ELECTROCHEMICAL CYTOMETRY:
A NOVEL TECHNIQUE TO QUANTITATIVELY PROBE INDIVIDUAL NEUROSECRETORY AND ARTIFICIAL VESICLES

A Dissertation in
Chemistry
by
Donna M. Omiatek

© 2010 Donna M. Omiatek

Submitted in Partial Fulfillment of the Requirements for the Degree of
Doctor of Philosophy

May 2010
The dissertation of Donna M. Omiatek was reviewed and approved* by the following:

Andrew G. Ewing  
Professor of Chemistry  
Professor of Neural and Behavioral Chemistry  
J. Lloyd Huck Chair in Natural Sciences  
Dissertation Advisor  
Chair of Committee

Christine D. Keating  
Associate Professor of Chemistry

Scott T. Phillips  
Assistant Professor of Chemistry

Michael L. Heien  
Assistant Professor of Chemistry

Peter J. Butler  
Associate Professor of Bioengineering

Barbara J. Garrison  
Shapiro Professor of Chemistry  
Head of the Department of Chemistry

*Signatures are on file in the Graduate School
ABSTRACT

This thesis details the development of a novel analytical method, electrochemical cytometry, which has been used to probe the contents of individual biological vesicles extracted from the cell environment in a high-throughput format. This experimental approach is based on technology that I have developed to electrochemically interrogate individual vesicles using a hybrid capillary-microfluidic device. In this format, a vesicle suspension can be injected onto a fused-silica capillary and subsequently isolated to individual components in an applied electric field by capillary electrophoresis. The separation capillary terminates into a PDMS-based microfluidic device that continuously delivers surfactant from microchannels to the detection zone in a sheath-flow format. As individual intact vesicles exit the separation capillary, they are chemically lysed and their contents subsequently detected at a carbon-fiber microelectrode positioned at the outlet. Electrooxidation of vesicular contents by constant potential amperometry allows for the mole amount encapsulant to be quantified on a per vesicle basis.

The electrochemical cytometry method design and characterization studies are outlined in Chapter 2. Here, I investigated how sheath flow of the lysis buffer affected analyte dispersion in the detection zone of the hybrid capillary-microfluidic platform using confocal fluorescence microscopy, computational fluid dynamics simulations, and end-column electrochemical detection to monitor the eluent flow profile. Then a proof-of-concept study was performed to investigate artificial vesicles (nanoliposomes) both containing and lacking an electroactive analyte (dopamine) by electrochemical cytometry to highlight both the sensitivity and selectivity of the detection scheme. By modeling the amperometric peak characteristics with the theoretical flux of dopamine from of a lysed
vesicle at the detector, I was able to determine the coulometric efficiency of the electrochemical cytometry detection scheme to be 87% (which was increased to > 95% via less signal filtering for investigations in Chapters 3 and 4).

An interesting application of this novel technique is discussed in Chapter 3, where electrochemical data recorded from stimulus-coupled secretion experiments at single PC12 cells were compared to cell-free measurements of vesicular dopamine content using electrochemical cytometry. Although standard methods used to measure stimulus-coupled release from single cells have classically been thought to assess the entire content of vesicles, there is evidence in the literature that suggests the total transmitter stored in vesicles is not expelled during exocytosis. This hypothesis was directly interrogated using electrochemical cytometry which allows for the quantification of total vesicular neurotransmitter in a manner that circumvents biophysical release processes of the cell associated with exocytosis.

By comparing total dopamine content from electrochemical cytometry measurements at individual isolated vesicles with the vesicular amount released at single PC12 cells, it was determined that during full exocytosis only a fraction (~40%) of total transmitter load is released from a typical vesicle. The data from these experiments support the intriguing hypothesis that the average vesicle does not open all the way during exocytosis, resulting in incomplete distention of the neurotransmitter contents. The implications of these results to neuroscience are large; namely: even during full exocytosis vesicular neurotransmitter release is not necessarily all-or-none. This suggests that transmitter secretion can be regulated within a single exocytosis event, imparting a potential molecular basis for synaptic plasticity at the subcellular level. Upon
establishing that release in exocytotic processes proceeded in an incomplete manner, electrochemical data quantified from both single cell release experiments and electrochemical cytometry of vesicles were related to vesicular volume from electron microscopy measurements to investigate the location of intravesicular catecholamine stores (e.g., halo or dense core) in PC12 cells retained post-fusion.

In Chapter 4, the electrochemical cytometry method was expanded to investigate vesicular content from the midbrain neurons of a mammalian animal model. Vesicles were isolated from mice striatal tissue and individually probed for endogenous dopamine content in a manner that was independent of release. This brain region is of great interest in neuroscience research since dopamine pathways that terminate into the striatum have been directly linked to a variety of neurobiological phenomena including motor function, reward, addiction, cognition, and neurological dysfunction, including Parkinson’s and Huntington’s disease.

In addition, electrochemical cytometry was applied to monitor the effects of synaptic vesicle neurotransmitter loading and depletion from mice injected with various pharmacological agents. It was demonstrated that vesicular neurotransmitter levels were altered and variances observed from these treatments resolved from single synaptic vesicles in a high-throughput manner, thus providing an efficient methodology to screen for the effects of neurological therapeutics in the subcellular domain. Moreover, the effect of the psychostimulant, amphetamine was investigated and shown to significantly deplete dopamine in the average striatal synaptic vesicle.

In Chapter 5, the development and characterization of a reagentless modified carbon-fiber microelectrode sensor capable of monitoring pH in biological
microenvironments is presented. The voltammetric carbon-fiber sensor was modified using a simple and reproducible procedure that involved electrochemically grafting a commercially available diazonium salt (Fast Blue RR) onto the microelectrode surface. Fast-scan cyclic voltammetry was used to probe redox activity of a quinone-moiety on the surface bound diazonium. A quantifiable oxidative wave was observed to yield a linear pH-dependent voltammetric response by flow injection analyses. Then, the sensor was used to measure fluctuations of pH in vivo that were evoked by optogenetic stimulus-coupled secretion in the central nervous system of a mutant fruit fly.

The work in Chapter 6 outlines several future bioanalytical applications of electrochemical cytometry. The first few involve elements that apply to the technical aspects of the measurement to expand upon the separation and detection capabilities of the method. The latter describe specific applications in liposome research and neuroscience that can be investigated for the quantitative characterization of volume-limited submicron vesicles via electrochemical cytometry.
# TABLE OF CONTENTS

List of Figures .............................................................................................................. ix
List of Tables ............................................................................................................... xii
List of Schemes ............................................................................................................ xiii
Preface ........................................................................................................................ xiv
Acknowledgements .................................................................................................... xv

Chapter 1. Subcellular Analyses for Vesicular Content Quantification ............... 1
  Introduction ................................................................................................................ 1
  Imaging Exocytosis at Single Cells ........................................................................... 3
  Electrochemical Measurements of Vesicular Transmitter Content from Single Cells .................................................................................................................. 11
  Separation Techniques Used to Quantify Vesicular Transmitter Content .......... 18
  Scope of the Thesis ................................................................................................. 22
  References .............................................................................................................. 26

Chapter 2. Development and Characterization of a Hybrid Capillary-
  Microfluidic Device for the Electrochemical Cytometry of Vesicles ............... 31
  Introduction ............................................................................................................. 31
  Materials and Methods ......................................................................................... 34
  Results and Discussion ......................................................................................... 42
  Conclusions ........................................................................................................... 62
  References ............................................................................................................ 63

Chapter 3. Electrochemical Cytometry of PC12 Cell Vesicles Reveals
  That Only a Fraction of Quantal Content is Released During Exocytosis .......... 66
  Introduction ........................................................................................................... 66
  Materials and Methods ........................................................................................ 69
  Results and Discussion ......................................................................................... 77
  Conclusions ........................................................................................................... 95
  References ............................................................................................................ 96
Chapter 4. High-throughput Quantitative Analysis of Neurotransmitter Content from Individual Mouse Striatal Vesicles With Electrochemical Cytometry ................................................................. 99
  Introduction ................................................................................................................ 99
  Materials and Methods ............................................................................................... 105
  Results and Discussion .............................................................................................. 111
  Conclusions ................................................................................................................ 133
  References .................................................................................................................. 134

  Introduction ................................................................................................................ 138
  Materials and Methods ............................................................................................... 141
  Results and Discussion .............................................................................................. 146
  Conclusions ................................................................................................................ 164
  References .................................................................................................................. 165

Chapter 6. Electrochemical Cytometry: Conclusions and Future Bioanalytical Applications of a Novel Technology ................................................................. 168
  Introduction ................................................................................................................ 168
  Understanding the Mechanism of Separation by Electrochemical Cytometry ........ 168
  Expanding the Scope of Electrochemical Cytometric Detection ............................ 171
  Analytical Techniques to Improve Liposome Encapsulation and Characterization... 174
  Measuring in vivo pH Fluctuations in Drosophila with a Chemically-Modified Microelectrode Sensor ................................................................. 181
  Electrochemical Cytometry of Biological Vesicles and Future Applications in Neuroscience Research ................................................................. 181
  References .................................................................................................................. 184
LIST OF FIGURES

**Figure 1-1**: TEM micrographs demonstrate the effects of pharmacological manipulation on intravesicular dopamine levels in PC12 cell large dense-core vesicles......................................................................................................................... 4

**Figure 1-2**: Fluorescence methods used to probe secretory vesicles ...................... 6

**Figure 1-3**: Novel fluorescent probes used to mimic chemical messengers stored in vesicles.................................................................................................................................................. 10

**Figure 1-4**: Quantification of stimulus-coupled vesicular secretion using carbon-fiber amperometry at single cells.................................................................................................................. 13

**Figure 1-5**: Biophysical mechanisms of vesicular release can be elucidated from amperometric measurements at carbon-fiber microelectrodes................................................................. 15

**Figure 1-6**: New technologies developed to electrochemically map the location of active release sites on single cells.................................................................................................................. 17

**Figure 1-7**: Using CE to probe the contents of individual vesicles. ......................... 20

**Figure 1-8**: Electrochemical cytometry: a novel high-throughput methodology used to quantitatively probe individual vesicle transmitter content........................................................................ 23

**Figure 2-1**: Microfluidic device fabrication................................................................. 36

**Figure 2-2**: Hybrid capillary-microfluidic device for the end-column lysis and electrochemical detection of vesicles separated by capillary electrophoresis. ..................... 43

**Figure 2-3**: Bright field and confocal fluorescence images of the electrochemical cytometry device.................................................................................................................................................. 44

**Figure 2-4**: Effect of volumetric flow rate through lysis channels on the fluorescence detection of analyte.......................................................................................................................... 46

**Figure 2-5**: Effect of volumetric flow rate through lysis channels on end-column electrochemical detection...................................................................................................................... 48

**Figure 2-6**: Liposomes used as a model system for the lysis and detection of encapsulated molecules................................................................................................................................. 52

**Figure 2-7**: Representative electropherograms for the end-column lysis and detection of liposomes on the electrochemical cytometry platform......................................................... 53
Figure 2-8: Plot of the normalized frequency histogram for the apparent radius of individual dopamine vesicles measured by electrochemical cytometry versus DLS used to determine the hydrodynamic radii of particles in the same liposome suspension. ................................................................. 56

Figure 2-9: Plot of simulated data for the half-width and coulometric efficiency for the detection of dopamine following liposome lysis............................................... 60

Figure 3-1: Experimental approaches to measure vesicular transmitter content: single cell amperometry versus electrochemical cytometry ........................................ 78

Figure 3-2: Representative data for the amperometric measurement of vesicular catecholamine amounts in PC12 cells using stimulus-coupled secretion at single cells and electrochemical cytometry of isolated vesicles. .............................. 79

Figure 3-3: Normalized frequency histogram of the vesicular catecholamine amounts quantified from intact cells that underwent stimulated exocytosis versus isolated vesicles investigated on the microfluidic platform........................................ 81

Figure 3-4: Pharmacological treatment alters vesicular quantal size from both intact cells and individual vesicles isolated from PC12 cells. ........................................ 83

Figure 3-5: Western Blot of isolated PC12 cell vesicles ........................................ 88

Figure 3-6: Electron microscopy investigation of PC12 cell vesicular size and volume......................................................................................................................... 90

Figure 4-1: Electrochemical cytometry analysis of mouse synaptic vesicles isolated from primary striatal neurons. ................................................................. 112

Figure 4-2: Normalized frequency histograms of the vesicular transmitter from various secretory cell models shows versatility of electrochemical cytometry measurement.................................................................................................. 115

Figure 4-3: Example of electrochemical cytometry data for the pharmacological manipulation of striatal vesicle neurotransmitter content................................. 121

Figure 4-4: Normalized frequency histograms of vesicular neurotransmitter amounts quantified from isolated striatal vesicles for reserpine-treated (20 mg/kg i.p., 12-h), untreated, and L-DOPA-treated (50 mg/kg i.p., 2-h,) mice............. 123

Figure 4-5: The limits of detection on the electrochemical cytometry platform allow for the resolution of markedly small vesicle measurements................................. 124
Figure 4-6: Pharmacological treatment at two different time periods results in different vesicular neurotransmitter content in mouse synaptic vesicles as measured by electrochemical cytometry .......................................................... 126

Figure 4-7: Summary comparison of mouse striatal vesicles treated to alter neurotransmitter content measured by electrochemical cytometry .............................. 127

Figure 4-8: Electrochemical cytometry as a high-throughput approach to directly quantify neurotransmitter levels altered from exposure to drugs of abuse at the single vesicle level. ................................................................. 130

Figure 5-1: Cyclic voltammograms of a carbon-fiber microelectrode before and after FBRR attachment .................................................................................. 148

Figure 5-2: Electrochemical characterization of the FBRR microelectrode pH sensor in pH 7.5 AHL saline solution ........................................................................... 154

Figure 5-3: Cyclic voltammograms of a microelectrode modified with FBRR in AHL saline solutions of different pH measured with scan rate = 20 V/s ...................... 157

Figure 5-4: The anodic peak potential, $E_{pa}$, as a function of AHL saline solution pH for FBRR-modified electrodes .............................................................................. 159

Figure 5-5: Plot of $E_{pa}$ versus time during flow injection changes past the electrode of 0.2 pH units in AHL saline. The electrode is able to consistently measure either an acidic or a basic pH change ....................................................... 160

Figure 5-6: Physiological pH measurements in adult Drosophila CNS. ............... 162

Figure 6-1: Electrophoretic mobility-size relation of liposomes measured by electrochemical cytometry ......................................................................................... 170

Figure 6-2: Macromolecular crowding increases polymer and protein encapsulation in submicron lipid vesicles measured by fluorimetry ................................. 177

Figure 6-3: Preliminary data for the electrochemical cytometry of liposomes synthesized with and without the addition of a crowding agent ........................................ 180
LIST OF TABLES

Table 2-1: Effect of sheath-flow rate on the detection of 100-μM catechol solution eluting from separation capillary.................................................................50

Table 2-2: Liposome characterization from electrochemical cytometry and DLS analyses.....................................................................................................................58

Table 3-1: Vesicle size measurements from dynamic light scattering (DLS) and transmission electron microscopy (TEM). ......................................................................87

Table 3-2: Determining intravesicular catecholamine stores of PC12 cells using amperometric and TEM measurements. .................................................................93

Table 4-1: Average peak characteristics and quantified vesicular neurotransmitter amounts for the electrochemical cytometry of mouse striatal vesicles. ...............114

Table 4-2: Average peak characteristics and quantified vesicular monoamine amounts for the electrochemical cytometry of mouse striatal vesicles, PC12 cell vesicles, and mouse mast cell vesicles. .................................................116

Table 4-3: Electrochemical cytometry summary for the average vesicular peak characteristics and quantified amounts of neurotransmitter from mouse striata exposed to various drugs that alter dopamine levels in the brain. ...............................131

Table 5-1: Effect of varying voltammetric deposition parameters of the FBRR on the carbon-fiber surface .......................................................................................150

Table 5-2: Various reporter molecules investigated for the fabrication of a modified pH voltammetric carbon-fiber microelectrode sensor ........................................153
LIST OF SCHEMES

Scheme 5-1: Mechanism for the electrochemical reduction of the diazonium salt FBRR onto the carbon-fiber surface. .................................................. 147

Scheme 5-2: Proposed mechanism for the quinone moiety oxidation-reduction reaction on the surface bound FBRR-modified carbon-fiber electrode sensor .......... 151

The contents of Chapter 2 were reproduced in part from an article authored by DM Omiatek, MF Santillo, ML Heien, and AG Ewing, *Anal. Chem.*, 2009, 81, 2294-2303 (© 2009 American Chemical Society).


ACKNOWLEDGEMENTS

This work would have not been possible without the support of several key individuals. First, and foremost, I would like to thank my advisor Andy Ewing for taking me under his wing. Andy’s enthusiasm and his genuine desire to make a difference has inspired me over the past few years to grow as both a scientist and as a person. It has been an honor and a privilege to chase after a common goal with him, and an experience that I will always cherish.

I would also like to thank Michael Heien for playing an integral role in my graduate school experience. Like a true electrochemist, Michael has taught me that it is indeed cool to be coulometric. I would especially like to thank him for continually emphasizing the importance of fundamentals and for helping me to develop this new technology. There’s not enough PBR in the world to repay him for all that he has done.

I am thankful for the experiences that I have shared with fellow Ewing lab members over the past few years, as well. Of these individuals, one member stands out among the crowd, Monique Makos. From performing pH measurements in the fruit fly brain (Chapter 5) to concocting the perfect Bloody Mary (data not shown), Monique and I have worked together to accomplish our goals, and it has certainly made for an unparalleled experience. Along with this degree, I will take with me a friendship that I will forever hold near and dear to my heart. I wish her success and happiness in all that she does, for her future is surely bright and will most likely require some purple shades.

I have been given the opportunity to interact with many talented scientists through valuable collaborations that have contributed towards the work in this dissertation. I would like to thank Ann-Sofie Cans for supplementing part of the work in Chapter 1 and for taking the time to review several of my papers. Next, I would like to thank Chris Keating for sharing information about liposome encapsulation that helped to put meaning to the data in Chapter 2, and for including me in an exciting collaboration with her and Lisa Dominak to improve upon the synthesis and characterization of liposomes discussed in Chapter 6. I would also like to thank Michael Santillo for our many conversations about flow dynamics and for performing the simulations in Chapter 2. In addition, I
would like to acknowledge Yan Dong for performing the single cell amperometry experiments in Chapter 3, which were necessary for determining the fraction of vesicular transmitter released. I would also like to thank Anne Andrews and Amanda Bressler for providing me with the mice and striatal dissections for the work in Chapter 4, and Lauren Levine, for her friendship, and helpful tips about coupling chemistry that were used to develop the modified electrodes in Chapter 5. In addition, I would like to acknowledge Richard Cyr for granting me full access to his lab to perform vesicle isolations for experiments in Chapters 3 and 4.

Finally, it goes without saying, that I would like to thank my parents Tom and Carmella, and my brothers Tom and Matt to whom I am completely indebted (literally). This opportunity, and those that have preceded it, would have never been possible without the unconditional love, support, sacrifice, and understanding of my family, and for that, I will always be grateful. I would like to dedicate this work to my nephews TJ and Kyle and my nieces Celia and Kaitlyn.

“What lies behind us and what lies before us are tiny matters compared to what lies within us.” – Ralph Waldo Emerson
CHAPTER 1
Subcellular Analyses for Vesicular Content Quantification*

Introduction

The analysis of chemical messengers (e.g., neurotransmitters, neurohormones, and neuropeptides) transmitted throughout the nervous system has drawn a tremendous amount of attention throughout the last few decades, in both the physical and life sciences, to better understand the molecular basis for the physiological and behavioral aspects of an organism. Pioneering measurements of neurotransmission were performed by Katz in the 1950s using electrophysiology at the frog neuromuscular junction (1). These data formed the foundation for the quantal hypothesis, the principle theory of neurotransmission, which predicated that chemical messengers are secreted from the cell in fixed proportions (2). The vesicle has been identified as the primary intracellular storage unit of these messengers (3) and, therefore, has prompted the continuing analytical investigation of this organelle to determine its contributing factors in regulating neurotransmission.

Classical neurotransmitters (e.g., acetylcholine, GABA, glutamate, various monoamines, etc.) are synthesized intracellularly in the cytosol and packaged via specific transmembrane protein transporters into vesicles. The movement of these messengers from the intra- to extracellular domain occurs through exocytosis, a Ca$^{2+}$-dependent mechanism, where vesicles containing neurotransmitter migrate to the plasma membrane of a cell, fuse, and release their contents into the synapse. These messengers interact with various receptors on target cells to communicate, after which they are recaptured or metabolized. Exocytosis events occur on a sub-millisecond timescale with transmitter

*Part of this chapter was adapted with permission from a review article authored by DM Omiatek, AS Cans, ML Heien, and AG Ewing, Anal. Bioanal. Chem., in press. © 2010 Springer-Verlag.
release proportions varying from zepto- to femtomole amounts per vesicle (4), making them experimentally challenging to monitor.

Bioanalytical techniques have been developed to measure chemical messengers in the extracellular fluid following exocytosis from tissue \textit{in vivo} (5), and more recently to measure individual exocytosis events at single cells under \textit{in vitro} experimental conditions (4, 6-15). Herein, an overview of select imaging, electrochemical, and spectroscopic investigations of exocytosis that have supported our understanding of this complex mechanism to date is provided. Specifically, I will focus on the pre-synaptic regulation of transmission via the fundamental chemical messenger storage unit, the vesicle. Additionally, some emerging technologies are highlighted that have increased both the sensitivity and selectivity of traditional analytical measurements to better resolve the morphological, spatio-temporal, and quantal content characteristics of vesicular release.
**Imaging Exocytosis at Single Cells**

Imaging exocytosis has been a common goal in the neuroscience community since the first measurements of neurotransmission. Transmission electron microscopy (TEM) has historically been the reference standard for imaging subcellular components owing to its ability to measure submicron domains within the cell. This technique has been used to determine the size (typically 50- to 800-nm diameter) and morphology of secretory vesicles during exocytosis. Electron micrographs of the synapse have provided a static snapshot of omega-shaped vesicle fusion events at the plasma membrane upon exocytosis, offering insight into the mechanism of neurosecretion (16-18). The ability of TEM to effectively quantify vesicular size from cells subjected to pharmacological manipulation of transmitter content (19) has been demonstrated for vesicles from the dopaminergic cell line rat pheochromocytoma (PC12) cells as shown in Figure 1-1.

PC12 cells, along with related secretory cell models (e.g., chromaffin cells, mast cells, pancreatic beta cells, etc.), have been instrumental in the mechanistic study of exocytosis as they are relatively easy to culture and possess large dense-core vesicles (LDCVs) packaged with high levels of monoamine messengers. LDCVs are defined by the presence of a dense core, which is composed of a store of semi-crystalline monoamine and acidic proteins. The core is surrounded by a halo of solubilized monoamine, collectively bound from the cell cytoplasm by a limiting membrane. Colliver et al. demonstrated that pharmacological manipulation of transmitter levels with L-DOPA (a precursor to intracellular dopamine synthesis) and reserpine (an inhibitor of the vesicular monoamine transporter responsible for loading cytosolic dopamine into the vesicle) directly altered the size of vesicles, and therefore, the volume of the internal
Figure 1-1. TEM micrographs demonstrate the effects of pharmacological manipulation on intravesicular dopamine levels in PC12 cell large dense core vesicles (LDCVs). (A) Untreated cells. (B) Cells treated with 100-μM L-DOPA for 90 min. (C) Cells treated with 100-nM reserpine for 90 min. Scale bar = 500 nm. Adapted with permission from (19).
monoamine stores (19). Indeed, treatment with L-DOPA (Figure 1-1B) increases the size of the vesicular halo versus control (Figure 1-1A). Conversely, reserpine decreases the size of the vesicular halo (Figure 1-1C). Correlation of the calculated volume data with amperometric methods capable of measuring amounts of secreted species (vide infra) has provided a quantitative assessment of the concentration of transmitter stored in individual vesicles (20).

While static imaging methods, such as TEM, have provided information about the spatial aspects of secretion, and have aided in highlighting the vesicle as a potential pharmaceutical target, continuing efforts in the last two decades have been directed towards the development of novel fluorescence microscopy methods to visualize the dynamics of vesicular exocytosis in real time. Much of this work involves the use of fluorescent tracer molecules used to label various intracellular components involved in exocytotic release. Indeed, fluorophores can be integrated into lipid membranes (Figure 1-2A), attached to various synaptic proteins (Figure 1-2B), entrapped within acidic compartments such as the synaptic vesicle (pH ~5.5 (Figure 1-2C)), or used to stain the peptide vesicle cargo of dense-core granules (Figure 1-2D) (21-25). Fluorescent styryl dyes (e.g., FM1-43), bearing both lipophilic and divalent cation groups, have been widely used to label membranes for fluorescence microscopy investigations of vesicular exocytosis (21, 26). This is because styryl dyes can be integrated into vesicular membranes via endocytosis. During this process, the dye molecule partitions easily and reversibly into the vesicle membrane, which significantly increases its fluorescence intensity when compared to that in the bulk solution. Applications of styryl dye staining and destaining during endo- and exocytotic processes have been used to study the
Figure 1-2. Fluorescence methods used to probe secretory vesicles. (A) Styryl dye molecules placed on the outside of the cell membrane are encapsulated and partition to the inner leaflet of membrane vesicles during endocytosis. (B) Synaptic vesicular proteins have been labeled with pH-sensitive green fluorescent proteins, pHfluorins, that are fluorescently quenched when in the lower pH environment inside the vesicle and increase fluorescence when subjected to the higher pH of extracellular media. (C) Acidotropic dyes diffuse through membranes when neutral, but become charged in the low pH environment in a vesicle thereby concentrating and labeling the vesicle compartment. (D) GFP-labeled proteins in the dense core.
kinetics of vesicle cycling \((26, 27)\) and the prevalence of different exocytotic modes of release (e.g., full exocytosis versus kiss-and-run exocytosis, described in detail \textit{vide infra}) \((28, 29)\).

Monitoring fluctuations in pH associated with exocytosis has been another avenue pursued for measuring vesicular release in real time using optical microscopic methods. One way this has been accomplished is through the use of pH sensitive proteins, termed pHluorins, which are genetically engineered from enhanced green fluorescent protein (EGFP) \((24)\). By attaching a pHluorin to a synaptic protein, one can monitor changes in the fluorescence signal of EGFPs induced when the vesicle lumen (pH \(\sim 5.5\)) encounters extracellular media (pH \(\sim 7.4\)) during exocytosis. This owes to the fact that the fluorescence signal of the pHluorin is quenched at low pH present in the vesicle medium; however, when the pHluorin comes into contact with the extracellular medium containing higher pH levels, an increase in fluorescence intensity is observed. These pH sensitive proteins have been useful for the investigation of several aspects of exocytosis that involve the kinetics of synaptic vesicle cycling \((24, 30)\), the recycling dynamics of synaptic proteins after exocytosis \((31, 32)\), and the effects of changing vesicular pH on the kinetics of exocytosis \((25)\).

Emerging analytical technologies capable of imaging subcellular components have been used in conjunction with the development of novel biomimetic probes to effectively monitor the dynamics of neurosecretion. Two key measurement platforms in this area have been total internal reflection fluorescence (TIRF) and stimulated emission depletion (STED) microscopies, which have been used to monitor nanoscale subcellular components, including vesicles, that are otherwise difficult to resolve using conventional
light microscopies. The TIRF method uses an evanescent wave to excite and detect fluorophores at a distance within a few hundred nanometers of the imaging surface (33), providing a means to monitor single exocytosis events. This imaging method allows one to monitor vesicles stained with acidotropic dyes, molecules that accumulate inside acidic compartments of the cell (e.g., synaptic vesicles). These dyes possess a neutral charge at neutral pH and, in turn, become protonated at the low pH inside the vesicle lumen (Figure 1-2C). Hence, only the acidic stained organelle that localized to the plasma membrane will be visible by the TIRF imaging using this fluorophore. The TIRF microscopy approach has been used to monitor the location and movement of vesicles prior to and during fusion (34, 35), and to probe release mechanisms of the vesicle during exocytosis (22). An alternative imaging method, STED, uses an initial laser to excite and a second doughnut shaped laser to de-excite fluorophores, thus shrinking the excitation focal point down to subdiffraction limits (36). With an optical resolution of 66 nm STED has been used to effectively track the fate of synaptic vesicle-protein clusters on the pre-synaptic membrane (37) and, more recently single synaptic vesicles in live hippocampal neurons with an improved resolution of 62 nm (38).

A recent trend in imaging exocytosis has involved the continuing development of novel fluorescent probes that directly mimic the molecular characteristics of chemical messengers, in terms of their chemical specificity and/or size, to resolve the spatio-temporal visualization of transmitter release (15, 39). Sulzer and Sames have collaborated on a report where they synthesized fluorescent artificial neurotransmitters and administered them to mouse chromaffin cells and primary neurons to monitor the dynamics of vesicular release using TIRF microscopy (39). The fluorophores used in this
work were engineered to be structurally similar to the monoamines native to the cells investigated and, therefore, were actively transported by vesicular transmembrane proteins to the interior lumen as shown in Figures 1-3A and 1-3B (39). As a result, it was possible to induce exocytosis, visualize neurosecretion from individual vesicles (Figure 1-3C), and ultimately determine that the exocytotic release was dependent on the frequency of stimulation. These findings provide evidence that suggests that an intracellular regulatory mechanism exists for pre-synaptic neurotransmission.

In a related microscopic investigation of vesicular secretion, Tsein and co-workers have studied the real-time dynamics of release modes associated with kiss-and-run exocytosis by loading quantum dots (15-nm diameter) into secretory vesicles of cultured hippocampal rat neurons (15). During kiss-and-run release it is thought that vesicles momentarily fuse with the plasma membrane, form a small pore through which transmitter can escape into the synapse, and subsequently close and retract prior to full distension and release of their chemical messenger contents. Tsein and coauthors hypothesized that the quantum dots were small enough to be transported into the vesicle (interior lumen space estimated as ~24-nm diameter), but would be spatially excluded from the narrow fusion pore (1- to 5-nm diameter) formed during kiss-and-run. Conversely, these particles could be expelled during full exocytotic collapse of the membrane, which cleverly allows direct interrogation of the mechanism of release (Figure 1-3D). They have compared the ratio of kiss-and-run events to full fusion events through a same-vesicle paradigm, by use of fluorescence video microscopy to track the consumed quantum dots throughout the synaptic vesicle cycle following exposure to an external stimulus. Their data suggest that the kiss-and-run mode of exocytosis dominates
Figure 1-3. Novel fluorescent probes used to mimic chemical messengers stored in vesicles. (A) FFN511 is a fluorescent false neurotransmitter that is structurally similar to monoamines packaged in mouse neuroendocrine and synaptic vesicles. (B) Fluorescent micrograph of a mouse chromaffin LDCV that has accumulated FFN511 in the vesicle lumen. Scale bar = 5 μm. (C) TIRF microscopy was used to monitor stimulus-coupled secretion of FFN511. The relative intensity of the fluorophore that is localized to the vesicular lumen decreases upon stimulation, which indicates secretion of the targeted analyte. Adapted with permission from (39). (D) Kiss-and-run (K&R) exocytosis was investigated from synaptic vesicles loaded with 15-nm nanoparticles. FCF refers to full-collapse fusion. Presented are stimuli consisting of a 0.1 Hz field stimulation for 2 min. Plot of K&R ratio for each stimulus (n = 8; Error is SEM). Adapted with permission from (15).
at the onset of stimulation, and that full exocytosis prevails following continuously repeated stimulation.

**Electrochemical Measurements of Vesicular Transmitter Content from Single Cells**

Electrochemical measurements of neurosecretion have been the most quantitative dynamic assessment of the chemical messengers released during exocytosis to date. The utility of electrochemical methods to investigate neurobiological phenomena is due to the fact that a select grouping of chemical messengers triggered during cell signaling processes in the nervous system are electroactive (e.g., dopamine, epinephrine, norepinephrine, serotonin, histamine, various neuropeptides, etc.), and therefore can be easily oxidized or reduced at a polarized surface (typically held at 0.2-1.0 V versus a Ag/AgCl reference electrode). The development of the voltammetric carbon fiber microelectrode sensor (40) has made it possible to measure these messengers in biological microenvironments. This methodology has served as a valuable tool for determining factors associated with the identity, amount, and time course of vesicular content secretion. The small double-layer capacitance of microelectrode sensors generates a rapid temporal response (microsecond) suitable for monitoring the dynamics of transmitter release in real time (41). Furthermore, the size of these sensors (typically ~5-μm tip diameter) permits spatial selectivity to screen for vesicular release in small regions of tissue or at single cells.

The first electrochemical measurements of individual vesicular release events at single cells were recorded by Wightman and co-workers on catecholamine-containing bovine adrenal chromaffin cells using constant potential amperometry at carbon-fiber
microelectrodes (13). This work revolutionized the analytical investigation of exocytosis by providing a controlled method to quantitatively monitor the stimulus-coupled secretion of vesicular transmitter. Representative current transients observed from the electrooxidation of vesicular catecholamine released from a secretory cell are shown in the amperometric trace in Figure 1-4A. Nearly each current transient on an amperometric trace can be attributed to a discrete vesicle fusion event. The measured charge ($Q$) from current transients on the amperometric trace can be related to the mole amount of transmitter ($N$) detected per vesicle using Faraday’s Law ($Q = nNF$), where $n$ is the number of electrons exchanged in the oxidation reaction (2e- for most monoamines) and $F$ is Faraday’s constant (96,485 C/mol).

The electrochemical method has since been applied to a number of secretory cell models for the investigation of vesicular release in real time to measure differences in the transmitter content of vesicles from brain (10-12) and peripheral cells (4, 6-8), as well as in a nerve cell of a living organism (14). Figure 1-4B shows a distribution for amperometrically measured amounts of transmitter detected from the induced exocytotic release of three commonly investigated LDCV-containing neurosecretory animal models (PC12 cells, human pancreatic beta cells, and rat mast cells) (4). Carbon-fiber amperometry at microelectrode sensors has also been widely used to probe variations in vesicular transmitter loading and release resulting from manipulations of protein expression (42-44) and pharmacology (45-48). An example is shown in Figure 1-4C, where representative current transients are plotted for vesicles pharmacologically treated to alter intravesicular dopamine stores in PC12 cells (49). As expected, $Q$ is increased with increasing vesicular dopamine for cells exposed to L-DOPA and, likewise,
Figure 1-4. Quantification of stimulus-coupled vesicular secretion using carbon-fiber amperometry at single cells. (A) Representative amperometric recording from the secretion of transmitter from LDCV-containing bovine adrenal chromaffin cells. Each spike identifies a single vesicle release event. Inset illustrates typical peak characteristics. Peak integration reveals the mole amount of transmitter released per vesicle. Adapted with permission from (13). (B) Normalized frequency histogram revealing the amount of transmitter released from typical LDCV-containing animal models measured using amperometry at carbon-fiber microelectrode sensors. $Q$ plotted as cubed root transform of amount to account for the spherical nature of the vesicle. Data are fit to Gaussian distributions to illustrate the heterogeneity of vesicular content from various secretory cells. Adapted with permission from (4). (C) Representative current transients observed from pharmacological augmentation vesicular of dopamine levels in PC12 cell vesicles. Same cell paradigm is used to monitor effect of depleting vesicular dopamine (with 100-nM reserpine, 90 min) and conversely increasing dopamine (with 100-μM L-DOPA, 90 min) versus control. In each case, the black trace marks the typical vesicular transmitter released from the cell prior to treatment and the gray trace marks the result after treatment. Adapted with permission from (49).
decreased for those treated with reserpine, which falls in line with volume measurements elucidated from TEM data discussed in the imaging section of this chapter (*vide supra*).

Carbon-fiber amperometry has also been used to unravel various intracellular biophysical processes that contribute to the mechanism of release. The extraordinary temporal characteristics of amperometric recordings at single cells have successfully resolved momentary features of exocytosis such as those related to the formation of the fusion pore observed as a pre-spike “foot” (*8, 9, 50*). This small current is thought to arise from oxidation of the transiently released transmitter that precedes the cumulative distension of vesicular contents as shown in Figure 1-5A (*51*). Amperometric recordings at single cells have also been used to identify partial vesicular release events that involve “flickering” fusion pores (*12*) and kiss-and-run exocytosis (Figure 1-5B and 1-5C) (*8, 9*). Figure 1-5B shows the amperometric signature for a flickering fusion pore, where each of the spikes on the complex transient are assigned to a unique flickering event in which the fusion pore connecting the vesicle lumen to the exterior is intermittently opened and closed (*12*). This is related to kiss-and-run exocytosis (Figure 1-5C (*52*)), a release mode discussed in the imaging section (*vide supra*). Mechanisms of partial release have been supported in the literature through hybrid experimental techniques that couple amperometry with capacitance measurements (e.g., patch-amperometry (*6, 8, 53*)) to measure changes in membrane area in tandem with secretion. Partial release models of exocytosis support the interesting contention that transmitter secretion can be regulated from single vesicles, which imparts a potential molecular basis for synaptic plasticity at the subcellular level and provides an explanation for the rapid membrane recycling necessary to efficiently to drive the synaptic vesicle cycle to completion (*54*).
Figure 1-5. Biophysical mechanisms of vesicular release can be elucidated from amperometric measurements at carbon-fiber microelectrodes. **(A)** Proposed mechanism and representative trace that depicts a pre-spike “foot”. It is proposed that the small rise in current that precedes the distention of vesicular contents is due to formation of the fusion pore. This is followed by a large spike, which has been associated with full collapse of the vesicle membrane and expulsion of the transmitter contents. Adapted with permission from (51). **(B)** Amperometric trace that displays a “flickering” fusion pore recorded from the stimulus-coupled secretion of dopaminergic mouse neurons. It is proposed upon fusion with the plasma membrane, the fusion pore intermittently opens and closes as indicated by the complex peak observed. Each label on the complex event is assigned to a unique flickering event. Adapted with permission from (12). **(C)** Cartoon depicting the mechanism of kiss-and-run exocytosis. This mechanism indicates partial release of a vesicle’s content and can be resolved using electrochemical measurements as depicted in the simulated traces. Adapted with permission from (52).
An emerging trend in neurosecretory analyses has revolved around developing technologies capable of electrochemically resolving the location of active release sites on secretory cells. Electrochemical methods to quantitatively map “hot spots” of vesicular release have included the use of dual microelectrode sensors (55), microelectrode arrays (MEAs) (56), and electrodes spatially located below and above the cell (57) to study exocytosis. In a recent report by Zhang et al., a multibarrel array of seven individually addressable carbon-fiber microelectrodes was fabricated and used to map the spatio-temporal vesicular release characteristics at stimulated PC12 cells (Figure 1-6A) (56). The individual electrodes (2.5-μm radius) were constructed in a multibarrel glass capillary, pulled to a fine tip to form an array, and polished to an angle for adequate placement on the target cell. This resulted in a MEA possessing a total diameter of about 20 μm, a dimension suitable to encompass the entire surface of a typical cell for somatic release recordings. The current traces in Figure 1-6A represent amperometric recordings from each of the individually addressed electrodes in the MEA array elicited via chemical stimulation of a PC12 cell. This approach allows the heterogeneity of release observed from various regions of the cell to be resolved by providing a quantitative assessment of measured transmitter.

Amatore and co-workers electrochemically investigated the frequency, kinetics, and content of vesicular release from both the apex and base of bovine adrenal chromaffin cells (57). This was accomplished by coupling amperometry with a carbon-fiber microelectrode placed above the cell and measurements at a thin film indium tin oxide electrode (ITO) placed below the cell. The experimental platform is depicted in Figure 1-6B, where adrenal chromaffin cells are cultured on the ITO electrode surface.
Figure 1-6. New technologies developed to electrochemically map the location of active release sites on single cells. (A) MEA composed of seven individually addressable carbon-fiber microelectrodes. Bright field image of the sensor on a PC12 cell is indicated by the arrow. To the left of this image is a micropipette containing elevated K⁺ solution to stimulate release. Shown below are representative amperometric traces recorded from each of the electrodes, demonstrating the heterogeneity of vesicular release across the cell surface. Adapted with permission from (56). (B) Electrochemical measurements of stimulated vesicular release from both the apex and base of chromaffin cells. Conventional carbon-fiber amperometry techniques are combined with amperometry measurements at a thin film ITO electrode upon which cells are cultured. Adapted with permission from (57).
and subsequently subjected to stimulus-coupled secretion. An overall higher amount of vesicular content released was observed from the basal pole when compared to the apical pole of the cell, possibly suggesting that sampling was achieved from distinct vesicle pools, although the different electrode material might have played a role as well.

**Separation Techniques Used to Quantify Vesicular Transmitter Content**

Analytical separations have been used to effectively isolate and identify a host of small molecules, peptides, and proteins involved in neurosecretory processes. Indeed, various gel separation techniques used in conjunction with mass spectrometric detection and bioinformatics have brought about a solution to the synaptic vesicle proteome (58). In the early 1970s, Adams and co-workers were among the first to couple liquid chromatography to electrochemical detection for the determination of catecholamine levels present in brain tissue homogenates, displaying a marked improvement over conventional bulk fluorometric and radiometric assays used to screen for transmitter content at the time (59). This paved the way for the quantitative assessment of neurosecretory products present in both the intra- and extracellular domains from a variety of organisms. Since this time, advancements have been made in chromatographic and electrophoretic technologies to improve the separation efficiencies, elution times, and detector sensitivities for neurosecretory investigations.

Capillary electrophoresis (CE) offers a number of analytical advantages for the investigation of subcellular constituents. With CE it is possible to sample small volumes (nano- to femtoliter), achieve high separation efficiencies (>10^6 theoretical plates), and the method can be coupled to sensitive detection platforms (e.g., UV, laser-induced fluorescence (LIF), mass spectrometric, electrochemical, etc. Basic detection principles
are reviewed in (60)). A recent trend in CE has revolved around developing methods to isolate and detect the contents of subcellular organelles. An example was reported by Chiu et al. using CE to investigate individual LDCVs originating from the atrial gland of the sea slug *Aplysia californica*. To accomplish this, they used a combination of techniques that included optical tweezing to isolate a single vesicle, CE to separate the components of the vesicle after lysis and online derivitization, and LIF to subsequently detect the fluorescently tagged vesicular components (61). Figure 1-7A depicts part of a microscopic video sequence from a trapped individual vesicle being electrokinetically injected onto a tapered separation capillary. Here, the vesicles were lysed and labeled with a fluorophore (NDA) that reacted with primary amines present in the vesicle lysate, to produce the NTP (NH$_2$- terminated peptide) peak observed in the resultant electropherogram from the separation of vesicular components in Figure 1-7A (bottom electropherogram). This was compared to a control experiment (Figure 1-7A top electropherogram) that lacked LDCVs to elucidate which peaks were attributed to vesicular constituents. This study demonstrated the concept of probing the contents of single biological vesicles extracted from the cell environment.

The Arriaga group has reported a high-throughput method using CE to efficiently separate a suspension of intact vesicles to individual components and subsequently detect them by LIF (Figure 1-7B, top electropherogram) (62). Artificial vesicles were encapsulated with a fluorescent dye and compared to a control suspension (Figure 1-7B, bottom trace) that lacked the target fluorophore, demonstrating the selectivity of the detection scheme. Those vesicles containing the fluorophore elicited a signal in the resultant electropherogram, whereas the control suspension was rendered transparent to
Figure 1-7. Using CE to probe the contents of individual vesicles. (A) LDCVs from the atrial gland of *Aplysia californica* are probed to identify molecules in vesicles. Present is an optical image of a single vesicle being optically trapped and subsequently injected onto a tapered separation capillary. Online derivitization of the vesicular components are accomplished for LIF detection. Peaks containing NTPs (NH$_2$-terminated peptides) are identified as constituents associated with vesicle content (bottom electropherogram). A control experiment excluding LDCVs is shown in the top electropherogram. Adapted with permission from (61). (B) High-throughput separation and quantification of artificial vesicles loaded with fluorescein. Each peak corresponds to the LIF-detection of a single vesicle. Top electropherogram corresponds to liposomes encapsulated with fluorophore. Bottom electropherogram represents a control suspension of liposomes that lack the target fluorescent analyte, demonstrating the selectivity of the detection scheme. Adapted with permission from (62).
the detection scheme. This group has had success in applying the CE-LIF methodology to investigate a number of subcellular components including cell nuclei (63), mitochondria (64), and various acidic organelles (65).
Scope of the Thesis

Central to this thesis has been the development of a new technology, termed electrochemical cytometry, to quantitatively probe the chemical messenger content of individual secretory and synaptic vesicles isolated from the cell environment. The cell-free assessment of quantal content provides a method to quantify endogenous vesicular transmitter in a manner that is independent of the biophysical release processes associated with exocytosis. This is important for the study of neurotransmission since the mechanism by which a vesicle releases its contents ultimately constitutes the measured quantal size, and can be influenced by extravesicular cues in exocytosis processes (e.g., temperature \(51\), osmolarity \(66, 67\), pH \(25\), \(\text{Ca}^{2+}\) levels \(68\), etc).

The electrochemical cytometry platform uses a hybrid capillary-microfluidic device to effectively separate, lyse, and measure the contents of individual vesicles. The development of electrochemical cytometry and its demonstration on artificial vesicles (liposomes) is discussed in Chapter 2 of the thesis. The electrochemical cytometry platform allows for vesicles to be injected onto a capillary and subsequently isolated to individual components by CE. The separation capillary terminates into a PDMS-based microfluidic device that continuously delivers surfactant to the detection zone in a sheath-flow format. As individual intact vesicles exit the separation capillary, they are chemically lysed and their contents quantified at a carbon-fiber microelectrode positioned at the outlet (Figure 1-8A). By modeling the amperometric peak characteristics with the theoretical flux of dopamine from of a lysed vesicle at the detector in a proof-of-concept experiment \(69\), the coulometric efficiency of the electrochemical cytometry detection
Figure 1-8. Electrochemical cytometry: a novel high-throughput methodology used to quantitatively probe individual vesicle transmitter content. (A) A schematic illustration of the device (not drawn to scale). (B) Sample amperometric data from the electrochemical cytometry of PC12 cell vesicles. A 1000-s portion of a representative electropherogram from the separation, lysis, and electrochemical detection of transmitter content from individual vesicles is shown. The inset contains an expanded axis to illustrate the typical peak characteristics.
scheme was determined to be 87% (which was subsequently increased to > 95% via less signal filtering (70, 71)).

The electrochemical cytometry method has been applied to quantify the content of individual vesicles extracted from the cell environment from various models including rat pheochromocytoma cells (PC12), normal mouse mast cells, and mouse primary neurons. A 1000-s portion of a representative electropherogram from the electrochemical cytometry of PC12 cell vesicles is shown in Figure 1-8B, as well as an expanded view of the axis in order to illustrate the typical peak characteristics. The resultant peaks can be integrated and subsequently related to the amount of transmitter per vesicle as described earlier in this chapter for amperometry at single cells.

Direct comparison of electrochemical data recorded from stimulus-coupled secretion experiments at single PC12 cells versus data obtained with electrochemical cytometry is discussed in Chapter 3 of the thesis. Use of this novel cell-free measurement reveals that, on average, each vesicle releases only a fraction (~40%) of its total transmitter load during exocytosis (70). The data from these experiments imply that average vesicle does not open all the way during exocytosis, resulting in incomplete distention of the neurotransmitter contents. This suggests that neurotransmitter secretion can be regulated within a single exocytosis event, imparting a potential molecular basis for synaptic plasticity at the subcellular level. More recently, studies have been performed on vesicles of primary midbrain neurons (71) as discussed in Chapter 4 of the thesis. These data are the first to quantify the levels of neurotransmitter in the vesicles versus what is released and suggest that the amount release per brain tissue is again a small part of the vesicular content. In both PC12 and mouse striatal vesicles,
electrochemical cytometry has been used to verify that vesicular dopamine levels can be altered with pharmacology and variances observed from these treatments resolved from single vesicles in a high-throughput manner, thus providing an efficient methodology to screen for the effects of psychoactive drugs and neurological therapeutics in the subcellular domain.

In Chapter 5, a novel pH sensor is discussed. pH is thought to play a significant role in neurosecretory processes. In a collaborative effort, a reagentless voltammetric carbon-fiber microelectrode has been developed to measure real-time physiological pH changes in biological microenvironments (72). The electrode was formed by via attachment of a diazonium modifier. Fast-scan cyclic voltammetry was used to probe redox activity of the chemical modifier as a function of pH. In vitro calibration of the sensor resulted in a linear pH-dependent anodic peak potential response. In addition, flow-injection analysis was used to characterize the modified microelectrode, revealing marked sensitivity to small pH fluctuations. Furthermore, the modified electrode was used to measure dynamic in vivo pH changes evoked during stimulus-coupled secretion in the central nervous system of a mutant fruit fly.

In the final chapter (Chapter 6), several potential applications of electrochemical cytometry are discussed. The first few applications involve expanding upon the separation and detection capabilities of the method, while the last few outline specific applications of electrochemical cytometry as a characterization method for volume-limited submicron vesicles.
References


secretion through the fusion pore in exocytic release from PC12 cells, *J. Neurosci.* 24, 303-309.


CHAPTER 2

Development and Characterization of a Hybrid Capillary-Microfluidic Device for the Electrochemical Cytometry of Vesicles*

Introduction

Although conventional stimulus-coupled secretion experiments measured using carbon-fiber amperometry at single cells have been thought to assess the entire quantal content of vesicles (1), the question still remains as to whether the total transmitter stored in vesicles corresponds to that quantified upon release. The formidable basis for this uncertainty revolves around the fact that the average transmitter quantified from single cell release experiments is dependent on a number of biochemical and biophysical processes that drive the exocytotic pathway, and thus, are capable of varying the quantal content characteristics of vesicular release (2-8). This chapter details the development and characterization of a novel technology using a hybrid capillary-microfluidic device to probe the contents of individual vesicles isolated from the cell environment, a process termed electrochemical cytometry. By doing so, a high throughput orthogonal analytical approach is provided to quantify total vesicular transmitter content in a manner that is independent of the physical release mechanisms of the cell.

Capillary electrophoresis (CE) has been shown to serve as a useful tool for the separation and isolation of individual micro- and submicron size particles, including subcellular components (9-14). The electrochemical cytometry platform discussed in this chapter takes advantage of the separation capabilities of CE to isolate submicron vesicles to individual entities in the presence of an applied electric field. In this format, a vesicle suspension can be injected onto a fused-silica capillary and electrophoretically separated.

*This chapter was reproduced in part from DM Omiatek, MF Santillo, ML Heien, and AG Ewing, Anal. Chem. 2009, 81, 2294-2302 with permission. © 2009 American Chemical Society.
to individual components. The separation capillary terminates into a PDMS-based microfluidic device that provides an interface for chemical lysis of the vesicle membrane and subsequent electrochemical detection of its contents. Lysis buffer is delivered to the detection zone in a sheath-flow format, with a carbon-fiber microelectrode positioned in the analyte flow stream, similar to previously reported electrochemical sheath-flow devices (15). The detection scheme presented here is well-suited for the analysis of transmitter housed in synaptic and secretory vesicle since the redox nature of various chemical messengers (e.g., dopamine, serotonin, epinephrine, norepinephrine, histamine, etc.) requires no external label and can be directly quantified as demonstrated with conventional single cell release experiments at carbon-fiber microelectrodes (1).

In this chapter, I will first describe how sheath flow of the lysis buffer affects the dispersion of a solution plug of analyte in the detection zone of the hybrid capillary-microfluidic platform using confocal fluorescence microscopy, computational fluid dynamics simulations, and end-column electrochemical detection. Then I will show how individual artificial vesicles (large unilamellar (LUVs), 200 nm diameter) loaded with dopamine (DA) were detected using electrochemical cytometry. LUVs are suitable a model for synaptic vesicles; they can be synthesized to be structurally similar to vesicles, contain similar membrane components, and can be selectively encapsulated with analyte such as dopamine, which possesses a well characterized electrochemical signature. Electrochemical quantification of the mole amount of vesicular dopamine was compared to an independent size measurement with dynamic light scattering to calculate the observed concentration of analyte heterogeneously encapsulated in the liposome suspension. Finally, by modeling the amperometric peak characteristics from the
dopamine-encapsulated liposomes with the theoretical flux of a lysed vesicle at the detector, the coulometric efficiency was determined to calibrate the detection scheme of the novel electrochemical cytometry platform.
Materials and Methods

Reagents. Catechol (CAT), 3-hydroxytyramine hydrochloride (dopamine, DA), N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES), sodium hydroxide, sodium dodecyl sulfate (SDS), fluorescein isothiocyanate (FITC), rhodamine B, and hydrofluoric acid (aq. 48%) were obtained from Sigma Aldrich (St. Louis, MO). All chemicals were used as received. The separation buffer consisted of 50 mM TES with 2% 1-propanol, and the lysis buffer was 50 mM TES with 5% (w/v) SDS. Buffers were adjusted to pH 7.4 using NaOH, filtered through 0.2 μm pore size filters (Nalgene, Rochester, NY), and purged with Ar (g) for 30 min prior to use to minimize oxidation of electroactive analyte. Standard solutions of catechol and dopamine were prepared as 10 mM stock solutions in separation buffer and diluted to the desired concentrations.

Liposome Preparation. Cholesterol (ovine wool) was obtained as a powder and 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) was obtained as a 10-mg/mL solution dissolved in chloroform (Avanti Polar Lipids, Inc., Alabaster, AL). Cholesterol powder was dissolved in chloroform to yield a 10-mg/mL stock solution. DPPC: cholesterol liposomes (80: 20 mole ratio) were prepared using a modified technique provided by the distributor to yield large unilamellar vesicles by extrusion (LUVET) (16). Briefly, aliquots of DPPC (154 μL) and cholesterol (16 μL) were combined with 100 μL of chloroform and evaporated for 4 h. Following this, 5 mL of 150 mM dopamine in rehydrating buffer (50 mM TES, pH 7.4) was added to yield a final lipid concentration of 0.34 mg/mL. Dopamine was excluded from the rehydrating solution for control liposome preparation. The lipid mixture was left to hydrate in the dopamine-containing buffer for 30 min. After this time period, the flask containing the mixture was
subjected to five freeze/thaw cycles to promote entrapment of water-soluble compounds into the liposomes (16, 17). This was accomplished by alternating the flask between a dry ice/acetone and warm water bath. The mixture was then extruded through a 0.2-μm polycarbonate membrane for a total of twenty passes (Mini Extruder Kit, Avanti Polar Lipids, Inc., Alabaster, AL) to yield a monodisperse suspension of unilamellar liposomes. Excess dopamine was removed from the liposome suspension by washing with a 10,000 MW dialysis cassette in DI water for 2 h (Slide-A-Lyzer, Thermo Scientific, Rockford, IL).

**Microfluidic Device Fabrication.** Microfluidic channels were fabricated using standard photo- and soft- lithography methods. A schematic for this process is shown in Figure 2-1. A photolithographic mask made from a high resolution laser photoplot (CAD/ Art Services, Inc., Bandon, OR) was printed with the device features. A master mold was developed by spin-coating 125 μm of SU-8 100 negative photoresist (MicroChem Corp., Newton, MA) on a 3-in. silicon wafer (Silicon Quest International, Inc., Santa Clara, CA). The mask was then placed over the wafer, exposed to ultraviolet light, and developed according to the resist manufacturer protocol.

Soft lithography was carried out using a Sylgard® 184 silicone elastomer kit (Dow Corning Corp., Midland, MI). A 10:1 ratio of poly(dimethylsiloxane) (PDMS) prepolymer base to curing agent was cast onto the master mold and cured at 70 °C for 2 h. The PDMS layer was then peeled from the master, revealing an impression of microfluidic channels. The center channel served to secure the separation capillary in the finished hybrid device. The other two channels, each 200-μm wide, are set at a 30° angle to the center channel and used to direct lysis buffer to the capillary outlet in a sheath-flow
Figure 2-1. Microfluidic device fabrication (not drawn to scale). A combination of photo- and soft lithographic processes are used to fabricate the PDMS-based microfluidic platform.
format. The three channels converge into a 650-μm wide channel where the electrode is placed for detection. A buffer reservoir for capillary electrophoresis was cut into a 2-mm layer of PDMS and plasma-bonded to a 75 x 50-mm glass micro slide (Corning Inc., Corning, NY). The layer containing imprinted microfluidic channels was then exposed to oxygen plasma and bonded onto the reservoir layer to form the three-layer hybrid device (100 W, 1 min.).

**Separations.** Fused-silica capillaries (15-μm i.d./150-μm o.d., Polymicro Technologies, Phoenix, AZ) were filled with separation buffer using a stainless steel reservoir with applied He pressure (400 psi). Electrokinetic injections were performed for 5 or 20 s at 5 kV and separations were carried out at 15 kV (333 V/cm) using a high voltage supply (Spellman, Hauppauge, NY). Capillaries were conditioned before each separation by rinsing at 15 kV with 1 M NaOH for 2 min, Ultratrol™ Dynamic Pre-Coat-HN (Target Discovery, Palo Alto, CA) for 5 min, and separation buffer for 10 min. Lysis solution was continuously flowed through the microchannels during separations at a volumetric rate of 0.5μL/min unless otherwise noted.

Capillaries were prepared for electrochemical detection by removing 2 mm of the polyimide coating with a flame and subsequently etching the exposed fused-silica by purging He (250 psi) for 15 min in an HF bath. This resulted in an etch with a frustum geometry measuring approximately 40-μm wide at the base, which serves to both ease placement of the electrode at the capillary outlet, as well as to decouple the applied separation voltage from the electrochemical cell (18).

**Capillary Electrophoresis with Electrochemical Detection (CE-EC) on the Hybrid Capillary-Microfluidic Platform.** A CE system with end-column amperometric
detection was built in-house and used as previously described (19). Modifications were made to the system to integrate hydrodynamic flow of lysis buffer into the detection zone. Briefly, 45 cm of capillary was threaded into the center channel of the microfluidic device. A syringe pump (KD Scientific, Holliston, MA) was used to control volumetric flow of lysis buffer via 1-mL plastic syringes. Polyethylene tubing (0.86-mm i.d./1.52-mm o.d., Becton Dickinson, Franklin Lakes, NJ) was fed through inlet holes to the microchannels. Amperometric electrochemical detection was carried out with a two-electrode format. A 5 μm-diameter carbon fiber was sealed in a glass capillary as previously described (20). A cylindrical carbon-fiber electrode was cut to a length of approximately 500 μm from the glass seal. The electrode was held at 0.90 V versus a silver wire quasi-reference electrode (Ag QRE, 0.25-mm diameter, Alfa Aesar, Ward Hill, MA). The carbon-fiber electrode was positioned at the outlet of the capillary using an x,y,z- micromanipulator (Newport, Irvine, CA).

Current was measured using a Keithly model 427 (5 Hz bandpass) current amplifier (Cleveland, OH) and digitized at 100 Hz with a National Instruments PCI-6221 DAQ card using LabView 8.0 software (National Instruments, Austin, TX) written in-house. OriginLab 8.0 (Northampton, MA) was used to generate electropherograms and Mini Analysis (Synaptosoft Inc., Fort Lee, NJ) was used for analysis of the resultant peaks to determine amplitude, area, and half-width. Events were counted if the signal was greater than three times the RMS baseline noise and the half-width was less than 100 ms.

Confocal Fluorescence Microscopy. Fluorescence images were acquired using a laser-scanning confocal microscope (Leica TCS SP5, Mannheim, Germany). To
visualize fluid flow on the device, FITC solution (λ<sub>ex</sub> 488 nm, λ<sub>em</sub> 500-560 nm) was flushed through the microchannels and CE was carried out as described above. For the flow rate studies, rhodamine B (λ<sub>ex</sub> 543 nm, λ<sub>em</sub> 560-650 nm) in separation buffer was continuously injected into the capillary and run at 166 V/cm.

**Simulations.** Computational fluid dynamics (CFD) simulations were performed with Comsol Multiphysics 3.4 (Comsol, Inc., Burlington, MA), a finite element method solver (Performed by MF Santillo). A two-dimensional geometry was used to model a top view of the hybrid capillary-microfluidic system. The following constants were used: diffusion coefficient, \( D = 4.0 \times 10^{-6} \text{ cm}^2/\text{s} \); density, \( \rho = 1.0 \text{ g/cm}^3 \); dynamic viscosity, \( \eta = 1.0 \times 10^{-3} \text{ Pa s} \); initial bulk concentration, \( c_0 = 100 \text{ mM} \). Incompressible Navier-Stokes equations were initially solved, given by:

\[
\frac{\partial \mathbf{u}}{\partial t} + \rho \mathbf{u} \cdot \nabla \mathbf{u} = -\nabla p + \eta \nabla^2 \mathbf{u}
\]

\[\nabla \cdot \mathbf{u} = 0\]

where \( \mathbf{u} \) is velocity, \( t \) is time, \( \rho \) is density, \( p \) is pressure, and \( \eta \) is dynamic (absolute) viscosity. Subsequently, the convection/diffusion equation,

\[
\frac{\partial c}{\partial t} + \mathbf{u} \cdot \nabla c = D \nabla^2 c
\]

was solved, where \( c \) is concentration, \( t \) is time, \( \mathbf{u} \) is velocity, and \( D \) is the diffusion coefficient. The linear flow rate of solution exiting the capillary was fixed at 0.055 cm/s, and the velocity of sheath flow was varied, yielding unique concentration profiles corresponding to each sheath-flow rate.

To model the detection of dopamine in a liposome, a one-dimensional finite-difference simulation was used (Performed by ML Heien). The in-house simulation
program was written in LabView. Mass transport was considered diffusional and was described by Fick's second law, which for planar diffusion is:

\[
\frac{d[DA]}{dt} = D \frac{d^2[DA]}{dx^2}
\]

The dopamine concentration was initially set to zero, then the concentration in a liposome (assumed to be 150 mM) was introduced into spatial elements encompassing the experimentally measured diameter of a liposome (vide infra). The diffusion coefficient for dopamine \(D\) in this space was assumed to be \(6 \times 10^{-6} \text{ cm}^2/\text{s}\). Since amperometric measurements were performed, the concentration at the electrode surface was assumed to be zero as dopamine was oxidized to the ortho-quinone by the following reaction:

\[
DA \rightarrow DOQ + 2H^+ + 2e^-
\]

The determined flux is then converted to current by the following equation:

\[
i = -nFAJ
\]

Where \(n\) is the number of equivalents, \(F\) is Faraday’s constant, \(J\) is the flux, and \(A\) is the area. To yield accurate current amplitudes, the electrode area was set so the sum of the resultant volume elements containing a total amount of dopamine equal to that in a liposome. The resultant current versus time curve was digitally filtered (5 Hz low pass, one pole) to match the bandpass of the current amplifier, and a 100-ms time epoch was analyzed to match the time window for experimental peaks.

**Dynamic Light Scattering.** A Zetasizer Nano S (Malvern Instruments, Worcestershire, UK) was used to acquire light scattering data. Measurements were collected using a 4 mW HeNe laser operated at 633 nm. Liposome suspensions were
diluted 10-fold in 50 mM TES buffer. Data were collected in size mode at 25 °C and fit using instrument software.
Results and Discussion

Microfluidic Device Fabrication

A hybrid capillary-microfluidic device is presented in this study for the separation, chemical lysis, and electrochemical detection of vesicles (Figure 2-2). The device consists of two PDMS layers bonded onto a glass microscope slide. The top PDMS layer contains a microchannel to secure the separation capillary, which terminates into a buffer reservoir. Adjacent to it on either side are microchannels used to deliver solution in a sheath-flow format for the lysis of vesicles exiting the capillary. This top layer is bonded to a lower layer of PDMS containing a buffer reservoir where the electrical ground and reference electrode are placed. For the detection of redox active analyte, a cylindrical carbon-fiber microelectrode is positioned at the outlet of the separation capillary, parallel to the flow stream spanning 500 µm in length.

Bright field and confocal fluorescence micrographs of flow the assembled device are shown in Figure 2-3. These images illustrate both electrophoretic and hydrodynamic flow on the electrochemical cytometry platform. The capillary was placed approximately 500 µm beyond convergence of the central and lysis channels in order to ensure laminar flow was present in the detection zone. A solution of rhodamine B (red) was continuously injected onto the separation capillary at 133 V/cm, while FITC solution (green) was concurrently pumped through the lysis channels at a volumetric flow rate of 0.5 µL/min. The detection electrode is not pictured in these images.

Confocal Fluorescence Microscopy of Flow on the Device

Flow through the sheath microchannels affects analyte dispersion as it exits the separation capillary. An optimal flow rate would (i) confine the eluent to the diffusion
**Figure 2-2.** PDMS-microfluidic device for the end-column lysis and electrochemical detection of vesicles separated by capillary electrophoresis. Schematic of the hybrid capillary-microfluidic device (not drawn to scale). Three 200 μm x 125 μm channels converge into a 650 μm x 125 μm channel where contents exiting the capillary are lysed and detected at a carbon-fiber microelectrode.
Figure 2-3. Bright field and confocal fluorescence images of the electrochemical cytometry device. A continuous injection 100-μM rhodamine B solution (red) is flowed through the capillary using electroosmotic flow (166 V/cm) and 36-μM FITC solution (green) is flowed through the lysis channels using hydrodynamic flow controlled by a syringe pump (0.5 μL/min, scale bar = 200 μm).
layer of the detection electrode, and (ii) allow ample time for interaction of the eluent with the electrode. Analyte dispersion at the separation capillary outlet was investigated using confocal fluorescence microscopy and CFD simulations. Rhodamine B solution was continuously flowed through the separation capillary at a fixed rate (166 V/cm), while the rate of lysis buffer through the sheath channels was varied (0.1, 0.5, and 2 μL/min). The normalized concentration profile for the simulated diffusion of rhodamine B exiting the separation capillary is shown at different flow rates (Figure 2-4, black trace), and compared to confocal fluorescence microscopy data for the complementary experiment (Figure 2-4, red trace).

Confocal fluorescence micrographs and CFD simulations allow visualization of the relationship between flow rate and the eluent concentration profile. Line scans were taken 50 μm from the outlet perpendicular to the flow stream in both cases as indicated by the white arrows. As the flow rate is increased, the eluent was hydrodynamically focused, which minimized diffusional broadening in the detection zone. The experimental data matched that predicted by the CFD simulation. The correlation coefficient between the simulation and fluorescence data, \( r^2 \), was 0.99, 0.98, and 0.96 for the 0.1, 0.5 and 2.0 uL/min flow rates, respectively. This illustrates the importance of electrode position and flow rate selection for optimal detection on this platform. It can be estimated that an electrode with a radius of 3 μm has a diffusion layer thickness of approximately 20 μm (21). From examination of the simulation and fluorescence data in Figure 2-4, at 0.1 μL/min, the electrode diffusion layer does not encompass the eluent concentration profile. At 2.0 μL/min the diffusion layer encompasses the entire eluent concentration profile; however, this high flow rate limits the interaction time with the
Figure 2-4. Effect of volumetric flow rate through lysis channels on the fluorescence detection of analyte. A continuous injection of 100-μM rhodamine B solution was flowed through the capillary using electroosmotic flow (166 V/cm). Volumetric flow rates of buffer through the lysis channels were varied. Data are line scans taken perpendicular to the flow stream, 50 μm from the outlet as shown by the white arrows. Fluorescence measurements are indicated by the red trace and CFD measurements by the black trace. Confocal fluorescence microscopy images and CFD simulations for diffusion of separation eluent at 0.1, 0.5, and 2 μL/min are pictured to the right of the line scan traces (Scale bar = 50 μm). Fluorescence data was smoothed using a moving average of five points.
detector. Indeed, the calculated linear flow velocity in the channel is 0.082 cm/s, allowing only 0.6 s for the solution to interact with a 500-µm long electrode positioned at the capillary outlet. The intermediate flow rate investigated, 0.5 µL/min, allows approximately 2.4 seconds of interaction time and encompasses approximately 70% of the eluent concentration profile. Therefore this intermediate flow rate comes closer to satisfying the two requirements stated above, when compared to either extreme, and was used for further electrochemical cytometry experiments (*vide infra*).

**Electrochemical Detection on the Device**

Sheath-flow rate affected analyte dispersion and the interaction time with the detection electrode as determined from fluorescence experiments. In the next phase of the study, I quantitatively investigated how sheath flow influenced the amount of analyte detected and the separation efficiency using CE with electrochemical detection (CE-EC). A plug of 100-µM catechol solution was injected onto the capillary, and detected as the flow rate was varied. In these experiments, flow driven by the electric field (111 V/cm injection, 333 V/cm run potential) was fixed and the hydrodynamic flow rate of lysis buffer through the sheath microchannels was varied from 0 to 2.0 µL/min. It was determined that varying the flow rate of lysis solution affected the electrochemical detection of analyte, but not the elution time; elution times for catechol were uniform across the flow rates investigated. Figure 2-5A contains representative electropherograms without (black trace) and with sheath flow present at various flow rates (colored traces). When sheath flow was present, greater mole amounts of catechol were detected at lower flow rates, however, lower separation efficiencies were observed (Figure 2-5B). Lower flow rates allowed more time for the eluent to interact with the
Figure 2-5. Effect of volumetric flow rate through lysis channels on end-column electrochemical detection. A plug of 100-μM catechol solution was injected onto the capillary and run at 333 V/cm. Lysis buffer (50 mM TES/ 5%(w/v) SDS/ pH 7.4) was flowed through the microchannels at varying volumetric flow rates. (A) A series of electropherograms for the detection of catechol at varying flow rates from 0-2 μL/min. (B) Plot quantifying detection of catechol in terms of amount (Q) and separation efficiency (N) for the data in part A. Error bars omitted for clarity. SD listed in Table 2-1.
electrode surface, resulting in higher coulometric efficiency for the detection of catechol. In contrast, higher flow rates minimized the interaction time with the electrode surface, resulting in lower coulometric efficiency for the detection of catechol, but sharper peaks. Results for the detected analyte from this CE-EC experiment on the hybrid device are listed in Table 2-1.

In order to efficiently lyse vesicles and detect their contents, the velocity of the lysis solution must be slow enough to allow time for the surfactant to interact with the vesicle membrane, but fast enough to direct analyte to the electrode surface where they are subsequently detected. To determine the optimal flow rate, the mole amount of catechol and the total number of theoretical plates was plotted against flow rate for the solution studies (Figure 2-5B). The rate of 0.5 µL/min through the sheath channels was chosen because it yields the most efficient peak shape at high coulometric detection efficiency for the plug of catechol solution. This is in agreement with the fluorescence experiments, which predicted 0.5 µL/min as optimal for the detection criterion set.

**Separation and Detection of Liposomes**

Liposomes are often used as a model for the analysis of cellular and subcellular membrane-bound components (22, 23) as well as to characterize a number of analytical methods in the areas of separations (10, 24-27). Arriaga and co-workers have investigated liposomes loaded with a fluorescent dye by CE-LIF (10). They were able to determine the apparent radius of individual liposomes according to the signal that arose from the relative intensity of fluorophore inside. A similar approach is used in this chapter to characterize the electrochemical cytometry device; however, in this investigation the liposomes have been loaded with an electroactive molecule to directly
Table 2-1. Effect of sheath-flow rate on detection of 100-μM catechol solution eluting from separation capillary. $n = 3$. Error is SEM.

<table>
<thead>
<tr>
<th>Sheath-Flow Rate (μL/min)</th>
<th>Amplitude (pA)</th>
<th>Amount (fmol)</th>
<th>Coulometric Efficiency (%)</th>
<th>Theoretical Plates (N)</th>
<th>LOD (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>390 ± 15</td>
<td>9.5 ± 1.0</td>
<td>19 ± 2</td>
<td>26,000 ± 2,600</td>
<td>0.57 ± 0.02</td>
</tr>
<tr>
<td>0.2</td>
<td>430 ± 46</td>
<td>12.8 ± 6.0</td>
<td>26 ± 14</td>
<td>24,000 ± 1500</td>
<td>0.53 ± 0.05</td>
</tr>
<tr>
<td>0.5</td>
<td>480 ± 110</td>
<td>17.2 ± 6.1</td>
<td>36 ± 13</td>
<td>24,000 ± 18,000</td>
<td>0.50 ± 0.13</td>
</tr>
<tr>
<td>1.0</td>
<td>390 ± 170</td>
<td>7.8 ± 3.3</td>
<td>16 ± 6</td>
<td>37,000 ± 12,000</td>
<td>0.66 ± 0.27</td>
</tr>
<tr>
<td>2.0</td>
<td>250 ± 65</td>
<td>4.5 ± 1.0</td>
<td>9 ± 2</td>
<td>38,000 ± 13,000</td>
<td>0.95 ± 0.27</td>
</tr>
</tbody>
</table>
quantify the amount of analyte after membrane lysis by use of constant potential amperometry.

Bright field and confocal fluorescence micrographs of DPPC: cholesterol (80: 20) liposomes loaded with 100-μM rhodamine B solution are pictured in Figure 2-6. These images are indicative, but not a direct representation of those used for further analyses in this study by electrochemical cytometry and dynamic light scattering (DLS). The liposomes used for the further studies are smaller (~0.2 μm diameter) due to extrusion, and unilamellar according to the preparation protocol (16). The liposomes depicted in Figure 2-6 are larger (~3-20 μm diameter) for imaging purposes, and contain mixed lamellarities.

To calibrate the detection scheme of the novel microfluidic platform, I used the hybrid capillary-microfluidic device to separate and detect dopamine-loaded liposomes using carbon-fiber amperometry. Liposome suspensions were electrokinetically injected at 111 V/cm for 20 s and run at 333 V/cm. As the liposomes eluted from the separation capillary, they were lysed by surfactant, and the contents encapsulated were subsequently electrooxidated at an electrode positioned at the outlet of the separation capillary. A portion of a representative electropherogram for the separation and detection of these samples is in Figure 2-7A. Here, the electrochemical cytometry result for a suspension of liposomes loaded with 150 mM dopamine is shown. Each spike in the electropherogram corresponds to a single liposome. Using the frequency of detected events, there is a 0.5% probability that two liposomes would give rise to a single peak at a given time point in the electropherogram (based on Poisson statistics, 50 ms time bins, 0.09 events/bin).
Figure 2-6. Liposomes used as a model system for the lysis and detection of encapsulated molecules. Bright field and confocal fluorescence micrographs of liposomes loaded with 100-μM rhodamine B solution are pictured. Liposomes contain the same phospholipid composition as those used in further electrochemical experiments, but these were not extruded so that they could be imaged (Scale bar = 25 μm).
Figure 2-7. Representative electropherograms for the end-column lysis and detection of liposomes on the electrochemical cytometry platform. Liposomes injected for 20 s at 111 V/cm and run at 333 V/cm. Individual peaks are shown in the expanded view to the right. (A) Liposomes loaded with 150 mM dopamine. (B) A ten-fold dilution of the liposome suspension injected in part A. (C) Electropherogram of control liposomes that were loaded with buffer only (no dopamine).
Pictured to the right in Figure 2-7A is an expanded temporal axis of individual events to illustrate the peak characteristics associated with liposome lysis and detection.

The liposome suspension from Figure 2-7A was diluted ten-fold and injected again under the same parameters. The resultant electropherogram is shown in Figure 2-7B and an expanded view is to the right. As expected, a smaller number of peaks are detected upon dilution of the original suspension (140 vs. 2670 events). Figure 2-7C represents a control suspension of liposomes prepared using the same method as those in Figures 2-7A and 2-7B, but rehydrated in buffer that excluded dopamine. Few peaks were observed (4 peaks). The control suspension lacks the redox molecule required for electrochemical detection; therefore these liposomes do not yield a signal in the electrochemical cytometry experiments upon lysis, demonstrating the chemical selectivity of the detection scheme. The few events observed in the electropherogram are most likely due to minor mechanical perturbations of the electrode; upon close inspection the peaks do not possess characteristics observed in the traces obtained from liposomes loaded with dopamine (peak amplitude and half-width). This demonstrates that the amperometric peaks in the electropherograms of dopamine-loaded liposomes are due to electrooxidation of the dopamine at the electrode and are not an artifact of the measurement.

**Determination of Liposome Contents**

Quantification of dopamine detected from each liposome by electrochemical cytometry can be accomplished using Faraday’s Law (\[ Q = nNF \]). By integrating the area of each peak (\[ Q \]), the mole amount of dopamine (\[ N \]) detected per liposome can be determined (\[ n \] is the number of electrons transferred in the oxidation reaction (2 e- for dopamine).
dopamine) and \( F \) is Faraday’s constant (96,483 C/mol)). If a known concentration of analyte loaded into each liposome is used, the internalized volume of dopamine solution can be calculated from the mole amount detected and related to the equation of a sphere to elucidate the apparent radius as seen in Table 2-2 (\textit{vide infra}). However, this simplistic calculation makes two assumptions: 1) the encapsulation of dopamine is 100% efficient and 2) the entire contents of the vesicle are oxidized at the electrode after lysis. These assumptions are clearly imperfect; loading efficiencies are known to vary depending on liposome composition, size, lamellarity, and the solute investigated (17, 28-34). The liposome preparation in this study was designed to both maximize encapsulation efficiency and minimize leakage of dopamine out of the liposomes after synthesis. The phospholipid composition chosen used long-chain saturated fatty acid lipid (DPPC) to maximize encapsulation and cholesterol to minimize leakage (30). Despite this, the data clearly suggest that dopamine is not fully encapsulated or leaks out after formation of the liposomes.

The apparent radius distribution of individual dopamine-encapsulated liposomes detected by electrochemical cytometry is plotted next to an orthogonal measurement of vesicle size for the same liposomal suspension in Figure 2-8. DLS measures the diffusion of particles in a suspension over time and relates this measurement to the hydrodynamic radius of the particle by the Stokes-Einstein equation.

\[
 r(H) = \frac{kT}{6\pi\eta D}
\]

In this equation \( k \) is Boltzmann’s constant, \( T \) is the absolute temperature, \( \eta \) is the viscosity of the medium, and \( D \) is the translational diffusion coefficient of the particle. By correlating the measured diffusion coefficients for a number of particles in a given
Figure 2-8. Plot of the normalized frequency histogram describing (A) the apparent radius of individual dopamine vesicles calculated from the amount encapsulated measured by electrochemical cytometry on the hybrid capillary-microfluidic device platform and (B) DLS experiments used to determine the hydrodynamic radii of particles in the same liposome suspension. Liposomes were loaded with 150 mM dopamine and extruded to 200-nm diameter. Bin size for electrochemical cytometry data was 5 nm. Average amounts, radii, and standard deviation listed in Table 2-2.
time period, the hydrodynamic radius $r(H)$ of a population of particles is solved for and plotted as a frequency curve (Figure 2-8B). It was observed that the frequency curve in Figure 2-8B resembled the vesicle content distribution in Figure 2-8A, although the electrochemical cytometry data is shifted to smaller radii.

Table 2-2 lists the results tabulated for the electrochemical cytometry and DLS analyses of the liposome suspensions. The suspension was extruded through a 0.2-µm membrane, which should yield liposomes with a radius 100 nm or less. Electrochemical detection of the dopamine-loaded liposomes corresponded to zeptomole quantities of dopamine detected for each individual lysis event in the electropherogram. The calculated amounts translate to a mean radius of $80 \pm 18$ nm based on the measured internalized volume of the analyte for liposomes loaded with 150 mM dopamine, and extrapolate to a limit-of-detection corresponding to a liposome with a radius of 10 nm (based on peak amplitude, area, and loaded dopamine concentration). The hydrodynamic radius as measured by DLS for the same suspension was $115 \pm 36$ nm. There is a statistically significant difference between the two measurements (Mann-Whitney test, $p < 0.01$). The difference in size measurements further highlights the limitations in the assumptions regarding encapsulation efficiency and coulometric efficiency (vide supra). This difference could be due to two factors; 1) reported encapsulation efficiencies for LUVs in this size range vary from 15 – 60% (31) and 2) the detection may not be 100% efficient. Interestingly, the measured difference in size can be accounted for entirely by encapsulation efficiency, suggesting the detection scheme is highly efficient.
<table>
<thead>
<tr>
<th>Sample</th>
<th>Number of Peaks</th>
<th>Amplitude (pA)</th>
<th>Half-width (ms)</th>
<th>Amount of DA (zmol)</th>
<th>Apparent Radius Electrochemical Cytometry (nm)</th>
<th>Hydrodynamic Radius DLS (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>150 mM DA Liposomes</td>
<td>2670</td>
<td>1.4 ± 0.9</td>
<td>42.2 ± 17.4</td>
<td>367 ± 289</td>
<td>79.3 ± 18.4</td>
<td>115 ± 36</td>
</tr>
<tr>
<td>150 mM DA Liposomes</td>
<td>140</td>
<td>1.2 ± 0.7</td>
<td>27.2 ± 2.8</td>
<td>185 ± 108</td>
<td>64.5 ± 12.0</td>
<td>115 ± 36</td>
</tr>
<tr>
<td>Diluted</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control Liposomes</td>
<td>4</td>
<td>0.7 ± 0.3</td>
<td>62 ± 3.6</td>
<td>n/a</td>
<td>n/a</td>
<td>150 ± 48</td>
</tr>
</tbody>
</table>

**Table 2-2.** Liposome Characterization from Electrochemical Cytometry and DLS Analyses. No dopamine present in the control. Radius calculated from electrochemical cytometry assumes 150 mM dopamine concentration in the vesicle. Error is SD.
Modeling the Electrochemical Signal from Liposome Lysis

Although the difference between the expected amount of dopamine in a liposome and the experimentally determined amount can be accounted for with non-ideal encapsulation efficiencies, the coulometric efficiency of the device can also contribute to this discrepancy. Here coulometric efficiency is defined as the ratio of the amount of electroactive species detected to the amount present in a liposome. Unfortunately, there is no satisfactory reference standard to calibrate the device in order to determine coulometric efficiency of electroactive species released from membrane-bound components upon lysis (e.g., fluorescent microspheres are used to calibrate LIF detection). To approximate the coulometric efficiency of the device, the resultant electrochemical signal was modeled from contents measured upon liposome lysis and subsequent detection. In this explicit finite-difference simulation, liposomes were lysed a certain distance from the electrode, releasing their contents instantaneously, and the resultant flux of analyte at the electrode was determined as a function of time, converted to current, and filtered to allow direct comparison to experimental data. The shape of the resultant current versus time curves, particularly the half-width, was used to compare the simulation to experimental data.

The total flux at the electrode can be compared to the total amount of analyte originally loaded into the liposome to yield the coulometric efficiency. The distance away from the electrode where the lysis occurs was varied to yield curves detailing how half-width and coulometric efficiency change as a function of distance (Figure 2-9). As the distance from the electrode that a liposome lyses decreases, the coulometric efficiency increases. The experimental data in Table 2-2 show the half-width for the
Figure 2-9. Plot of simulated data for the half-width and coulometric efficiency for the detection of dopamine following liposome lysis. Inset: amperometric trace for a liposome filled with 150 mM dopamine, having a diameter of 200 nm, and lysing 0.25 µm away from the electrode. Distances are measured to the closest edge of the liposome in relation to the electrode.
measured liposomes as an average of 42.2 ± 17.4 ms, which in the simulated data, corresponds to a liposome lysing approximately 0.25 µm away from the electrode, yielding a coulometric efficiency of 87%. Using the temporal profile of the amperometric events, the model suggests the liposomes are lysing in a region close to the electrode (< 2 µm), corresponding to coulometric efficiencies ranging from 70 – 90%. Interestingly, liposomes at a distance of more than 5 µm away would not cross the signal-to-noise threshold for detection (~0.3 pA), resulting in a lower coulometric efficiency at 48%. Furthermore, liposomes at this distance have large half-widths (98 ms) not observed in the experimental data. If a coulometric efficiency of 87% is applied to experimental data for the quantification of individual dopamine liposomes in this study, the encapsulation efficiency is 41 ± 22 %, which is in agreement with the expected range for bulk analyses reported in the literature (31). Therefore, the electrochemical cytometry platform described in this chapter can be validated as a useful tool for the lysis and subsequent detection of individual submicron vesicles.
Conclusions

A hybrid capillary-microfluidic device has been fabricated for the separation, chemical lysis, and electrochemical detection of vesicular contents, a process termed electrochemical cytometry. Sheath flow of lysis buffer was shown to minimize the diffusional broadening of eluent in the detection zone as determined by confocal fluorescence microscopy, CFD simulations, and CE-EC. Submicron liposomes, a model for synaptic vesicles, were loaded with a known concentration of dopamine, followed by interrogation of the contents on the electrochemical cytometry platform. The electropherograms from diluted liposome suspensions and from the control liposomes possessed less peaks, confirming the selectivity of the detection scheme. Dopamine-encapsulated vesicle radii were plotted and compared to size distributions from DLS data. Correlation of these data demonstrated the heterogeneous non-ideal encapsulation efficiencies for individual liposomes in a biocolloidal suspension. Moreover, these data provided a means to characterize the sensitivity and selectivity of the electrochemical cytometry platform. Subsequent studies discussed in Chapter 3 and 4 use this novel device to investigate the amount of transmitter present in individual biological secretory and synaptic vesicles extracted from the cell environment.
References


CHAPTER 3

Electrochemical Cytometry of PC12 Cell Vesicles Reveals That Only a Fraction of Quantal Content is Released During Exocytosis*

**Introduction**

Neurons communicate through the release of chemical messengers (e.g., neurotransmitters, neurohormones, and neuropeptides), which are packaged intracellularity in submicron sized structures called secretory vesicles (1-3). During exocytosis, the fundamental method for neurotransmission, vesicles migrate to the plasma membrane of a cell, fuse, and release their contents into the extracellular space. These messengers can then bind to receptors on a target cell, thus inducing a cascade of signaling events in a complex network (3, 4). Researchers have focused on unraveling the elements that govern the exocytotic pathway, and direct measurements of chemical messengers released during exocytosis have allowed vesicle fusion dynamics and pre-synaptic regulatory processes of neurotransmission to be examined (5-10). Specific efforts have revolved around answering questions such as: how is vesicle fusion regulated, and how are chemical messengers extruded from vesicles into the extracellular space?

Monoamines (e.g., dopamine, serotonin, histamine, epinephrine, and norepinephrine), are a redox-active family of chemical messengers that have been examined in both the CNS (9-11) and periphery (12-20) of various animal model systems. The release of monoamines at the single vesicle level can be probed on a millisecond timescale using electrochemical methods, making these molecules an

*This chapter was reproduced from DM Omiatek, Y Dong, ML Heien, and AG Ewing, ACS Chem. Neurosci. 2010, 1, 234-245 with permission. © 2010 American Chemical Society.
attractive target for quantitative mechanistic investigations of exocytosis. Constant potential amperometry performed at carbon-fiber microelectrodes has been used to monitor vesicular monoamine release from single cells (Figure 3-1A). In this measurement, Faraday’s Law \( Q = nNF \) is used to quantify the mole amount of released monoamine, \( N \), from the time integral of current transients \( Q \) on the amperometric trace, where \( n \) is the number of electrons exchanged in the oxidation reaction (2e\(^-\) for most monoamines) and \( F \) is Faraday’s constant (96,485 C/mol). Long-standing assumptions made in amperometric analyses of exocytosis at single cells are: (i) each current transient is attributed to a discrete vesicle fusion event and (ii) each vesicle releases its entire contents during fusion.

The transmission of chemical messengers by exocytosis has classically been thought to be an all-or-none process that is quantal in nature (21-24). However, the data in several reports suggest that full fusion does not result in complete expulsion of the transmitter in a vesicle, a concept that has not been widely accepted. To experimentally address this hypothesis, I used electrochemistry to investigate vesicle content from the neuroendocrine secretory cell model rat adrenal pheochromocytoma cells (PC12 cells). This immortalized mammalian cell line has been well characterized by the Ewing lab and others for quantification of neurosecretion (25). PC12 cells contain large dense-core vesicles that release catecholamines (dopamine and perhaps norepinephrine), a specific group of monoamine messengers implicated in physiological phenomena including neurological disease, learning and memory, and addiction.

In this study, direct measurements of vesicular catecholamine content are quantified using electrochemical cytometry. This novel experimental approach is based
on technology that I have developed to electrochemically interrogate individual vesicles using a microfluidic-based platform (26). In Chapter 2, the chemical selectivity of this detection scheme using artificial vesicles both containing and lacking an electroactive analyte was demonstrated (26). Here, the total catecholamine content of vesicles extracted from secretory cells is directly compared to the more conventional amperometric release experiments performed on intact single cells from matched populations. In doing so, it is shown that the entire catecholamine content of the vesicle is not released during fusion. This cell-free model simplifies the complexity of vesicular transmitter quantification measured from standard methods at single cells by eliminating elements of exocytosis that are governed by the cell machinery and membrane dynamics in fusion processes (27).
**Materials and Methods**

**Reagents.** Sodium chloride (NaCl), potassium chloride (KCl), magnesium chloride (MgCl₂), glucose, HEPES, and calcium chloride (CaCl₂), N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES), sodium hydroxide (NaOH), sodium dodecyl sulfate (SDS), reserpine, and 3,4-Dihydroxy-L-phenyl-alanine (L-DOPA), hydrodfluoric acid (aq. 48%), and the Synaptic Vesicles Isolation Kit were obtained from Sigma Aldrich (St. Louis, MO). All chemicals were used as received. Isotonic physiological saline used in single cell amperometry experiments was prepared with 150 mM NaCl, 5 mM KCl, 1.2 mM MgCl₂, 5 mM glucose, 10 mM HEPES, and 2 mM CaCl₂. For individual vesicle measurements on the microfluidic-based platform, the electrophoretic separation buffer consisted of 50 mM TES with 2% 1-propanol. The lysis buffer was 50 mM TES with 5% (w/v) SDS. All buffers were adjusted to pH 7.4 using NaOH and filtered through 0.2-μm pore size filters (Nalgene, Rochester, NY).

**Cell culture.** Rat pheochromocytoma cells (PC12) were purchased from the American Type Culture Collection (Manassas, VA, USA) and maintained as previously described (28). For stimulated exocytosis experiments at single cells, PC12 cells were grown on poly-D-lysine-coated culture dishes (Becton Dickinson, Bedford, MA, USA) in supplemented RPMI-1640 medium. For isolated vesicle experiments, cells were cultured on 75 cm³ poly-D-lysine-coated flasks. Cells were maintained in a 7% CO₂ atmosphere at 37 °C and used when confluency was reached.

**Vesicle Isolation.** A Synaptic Vesicle Isolation Kit (Sigma-Aldrich) was used to extract vesicles from the cells with a modified procedure provided by the manufacturer. All buffer constituents and isolation procedures are listed as proprietary knowledge of
Sigma Aldrich. Modifications were made to the protocol to ensure a sufficient amount of cells to suit the recommended procedures which have been tested on rat and rabbit brain tissue. To recreate a tissue-like model from this immortalized cell line, confluent cells from three 75 cm$^3$ flasks were forcefully released from the poly-D-lysine-coated surface and each of the three suspensions combined into one fraction. This fraction was then centrifuged at 1,500 x $g$ for 5 min to pellet the suspension. The supernatant containing growth media was then removed and discarded. According to the manufacturer-recommended protocol, cells were subjected to lysis in the presence of 10 mL of hypoosmotic buffer. This fraction was then centrifuged at 20,000 x $g$/4°C for 25 min. This allowed for removal of other organelles present in the cell, as they pelleted to the bottom of the centrifuge tube during this treatment. The supernatant, containing vesicles, was then recovered and subjected to ultracentrifugation at 70,000 x $g$/4°C for 1 h. Vesicles were recovered as a pellet in the bottom of the centrifuge tube. The supernatant was discarded and 2 mL of vesicle storage buffer was added to the pellet, resulting a crude suspension of vesicles for analysis. A Western Blot was performed to confirm successful vesicle isolation by targeting synaptophysin, a known integral membrane protein present on neuroendocrine vesicles (vide infra).

**Electrochemical recordings for Stimulated Exocytosis at Single Cells.** Carbon-fiber disk microelectrodes (5-μm diameter) were constructed as described previously (29) and back-filled with 3 M KCl. Electrode tips were polished at 45° angle on a diamond dust-embedded micropipette beveling wheel (Model BV-10; Sutter Instrument, Novato, CA, USA). Amperometric recordings were collected as described previously (13). Briefly, electrodes were held at + 0.65 V vs. a Ag/AgCl reference electrode (World
Precision Instruments, Inc., Sarasota, FL) using a commercially available patch-clamp instrument (Axopatch 200B; MDS Analytical Technologies, Sunnyvale, CA) configured as described previously (30). The output was filtered at 2 kHz using a four-pole low-pass Bessel filter and digitized at 5 kHz. Data were displayed in real time and stored in the computer with no further filtering. Exocytosis was monitored from 40 s intervals of measured current transients evoked with a 5-s, 20-psi pulse (Picospritzer II; General Valve, Fairfield, NJ, USA) of physiological saline containing elevated potassium (100 mM KC1). All experiments were performed at 37 ± 1°C. Exocytotic spikes were identified and the areas (fC) were determined using a multi-pass algorithm described previously (21). Mini Analysis (Synaptosoft Inc., Fort Lee, NJ) was used for analysis of the resultant peaks to determine area. Signals were designated as exocytotic events if their amplitude values were five times the RMS noise of the baseline when compared to a 1-s portion of stable baseline recorded before the first stimulation. All peaks identified by the program were inspected visually, and complex peaks were excluded manually from the datasets.

Microfluidic Device Fabrication for the Separation, Lysis, and Electrochemical Detection of Individual Vesicles. A hybrid capillary-microfluidic device was developed as previously described to investigate catecholamine amounts from individual vesicles isolated from the cell environment (26). Briefly, microfluidic channels were fabricated using standard photo- and soft- lithography methods. A master mold was developed by spin-coating 125 μm of SU-8 100 negative photoresist (MicroChem Corp., Newton, MA) on a 3-in silicon wafer (Silicon Quest International, Inc., Santa Clara, CA). A photolithographic mask containing imprinted device features was made from a high-
resolution laser photoplot (CAD/ Art Services, Inc., Bandon, OR). The mask was placed over the wafer, which was then exposed to ultraviolet light, and developed according to the resist manufacturer protocol.

Soft lithography was carried out using a Sylgard® 184 silicone elastomer kit (Dow Corning Corp., Midland, MI). A 10:1 ratio of poly(dimethylsiloxane) (PDMS) prepolymer base to curing agent was cast onto the master mold and cured at 70 °C for 2 h. The PDMS layer was then peeled from the master, revealing an impression of microfluidic channels. The center channel served to secure the separation capillary in the finished device. The other two channels, each 200-μm wide, are set at a 30 ° angle to the center channel and used to direct lysis buffer to the capillary outlet in a sheath-flow format. The three channels converge into a 2-mm wide channel where the electrode is placed for detection of catecholamine quantified from individual lysed vesicles. A buffer reservoir for capillary electrophoresis was cut into a 2-mm layer of PDMS and plasma-bonded to a glass micro slide (Corning Inc., Corning, NY). The layer containing imprinted microfluidic channels was then plasma-bonded onto the reservoir layer to form a three-layer device (100 W, 1 min.).

**Separation and Detection of Isolated Vesicles.** Fused-silica capillaries (45 cm in length, 15-μm i.d./150-μm o.d., Polymicro Technologies, Phoenix, AZ) were prepared for electrochemical detection by removing 2 mm of the polyimide coating with a flame and subsequently etching the exposed fused-silica by purging He (250 psi) for 15 min in an HF bath. This resulted in an etch with a frustum geometry measuring approximately 40-μm wide at the base, which serves to both ease placement of the electrode at the capillary
outlet, as well as to decouple the applied separation voltage from the electrochemical cell (31).

Electrokinetic injections of vesicles were performed for 5 s at 111 V/cm and separations were carried out at 333 V/cm using a high voltage supply (Spellman, Hauppauge, NY). Capillaries were conditioned before each separation to prevent non-specific binding of the vesicle membrane to the fused-silica by rinsing at 333 V/cm with 1 M NaOH for 2 min, Ultratrol™ Dynamic Pre-Coat-HN (Target Discovery, Palo Alto, CA) for 5 min, and separation buffer for 10 min. A syringe pump (KD Scientific, Holliston, MA) was used to control volumetric flow of lysis buffer via 1-mL plastic syringes continuously through the microchannels at a rate of 2 μL/min (0.05 cm/s).

Amperometric electrochemical detection was carried out with a two-electrode format. A 5-μm-diameter carbon fiber was sealed in a glass capillary and cut to a length of approximately 500 μm from the glass seal to fabricate a cylindrical microelectrode as previously described (32). The electrode was held at 0.90 V versus a silver wire quasi-reference electrode (Ag QRE, 0.25-mm diameter, Alfa Aesar, Ward Hill, MA) and the carbon-fiber electrode was positioned at the outlet of the capillary using an x,y,z-micromanipulator (Newport, Irvine, CA). Previous measurements and modeling indicated that this system yielded a coulometric efficiency of 87% for catecholamine in vesicles; however, with less filtering we have attained greater than 95% coulometric efficiency.

Current was measured using a Keithly model 427 (15 Hz bandpass) current amplifier (Cleveland, OH) and digitized at 500 Hz with a National Instruments PCI-6221 DAQ card using LabView 8.0 software (National Instruments, Austin, TX) written in-
OriginLab 8.0 (Northampton, MA) was used to generate electropherograms and Mini Analysis (Synaptosoft Inc., Fort Lee, NJ) was used for analysis of the resultant peaks to determine area (fC). Events were quantified if the signal was greater than five times the RMS baseline noise.

**Western Blot of Isolated Vesicles.** Vesicles isolated from the cell environment were investigated for the presence of synaptophysin, a known integral membrane protein present in synaptic and neuroendocrine vesicles. A 50-μL suspension of vesicles was combined with 50 μL of lysis solution containing 5% (w/v) SDS in 50 mM TES buffer. The mixture was vortexed vigorously for 1 min. Aliquots of this solution containing ~20 μg of protein were loaded into four lanes of a 12% SDS-PAGE gel. Separations were carried by applying 200 V for 2 h to the gel. A SeeBlue® Plus2 pre-stained standard protein kit (Invitrogen, Carlsbad, CA) was used to visualize the separation. Following this the gel was transferred onto nitrocellulose paper, followed by blocking and incubation with the primary antibody monoclonal anti-synaptophysin (2-h incubation at room temperature; 1:1000-dilution of antibody. Monoclonal Anti-Synaptophysin antibody was provided in the Synaptic Vesicle Isolation Kit (Sigma Aldrich)). The secondary antibody, HRP-conjugated anti-mouse IgG, was used for chemiluminescent detection of the targeted protein (ProteoQwest™ChemiluminescentWestern Blotting Kit, Sigma Aldrich). Results revealed a prominent band at 38 kDa, confirming the presence of synaptophysin in the vesicle lysate (Figure 3-5, *vide infra*).

**Dynamic Light Scattering of Isolated Vesicles.** A Zetasizer Nano S (Malvern Instruments, Worcestershire, UK) was used to acquire light scattering data. Measurements were collected using a 4 mW HeNe laser operated at 633 nm. Vesicle
suspensions were diluted 10-fold in buffer. Data were collected in size mode at 25°C and fit using instrument software.

**Transmission electron microscopy of Single Cells.** PC12 cells were rinsed with RPMI-1640 medium without serum and detached from the flasks. Single cell suspensions were transferred to Microfuge tubes and pelleted at 100 × g for 10 min. Cell pellets were fixed with an ice-cold fixative containing 2% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4 at room temperature for 1 h, and then incubated overnight at 4°C. The cells were post-fixed in 1% OsO₄ for 1 h and dehydrated by serial treatment in solutions of graded ethanol and embedded in Eponite 12. The areas of interest were selected under a dissecting microscope and 80-nm-thick sections were produced in an ultramicrotome (Reichart Microscopy, Depew, NY). Sections were contrast enhanced with uranyl acetate and lead citrate and examined with a JEOL JEM 1200 EXII transmission electron microscope (JEOL, Peabody, MA, USA) at 80 kV. Quantitative analysis of vesicle structures was performed using Image J 1.37v (Wayne Rasband, NIH, Bethesda, MD). Transmission electron microscopy images were imported into this software, and the limiting membrane of each vesicle as well as the perimeter of its dense core were traced. Once each object was inscribed, Image J determined its diameter (the average distance of the major and minor axis on the initial trace). Only vesicles in which a dense core could be clearly identified were measured. The diameters of the limiting membrane (vesicle) and dense-core diameter are measured, and converted to volume from the equation of a sphere \( V = \frac{4}{3}\pi r^3 \). Halo volume is the difference between the volumes calculated from the vesicle and dense core. Measured values were adjusted to account for thickness of the sample tissue slice (80 nm) as previously reported (33). For the correlation between
the amount of catecholamine detected (from amperometry measurements) and the volume in the halo (from TEM measurements) for measured release at single cells and isolated vesicles in Figure 3-6D, linear regression of release experiments on single cells yielded the equation $y = 38.97x + 74.76$ ($R^2 = 0.985$) and that for isolated vesicle experiments yielded $y = 124.7x + 153.2$ ($R^2 = 0.925$).
Results and Discussion

Schematic illustrations showing the experimental approaches used here for single cell amperometry and the electrochemical cytometry of vesicles are presented in Figure 3-1A and 3-1B, respectively. The electrochemical cytometry platform is based on methodology used to separate subcellular components by capillary electrophoresis (34, 35). First, vesicles from PC12 cells are isolated offline using differential centrifugation (Details provided in Materials and Methods). They are then injected onto a fused-silica capillary and separated by an applied electric field. The capillary terminates into a PDMS-based microfluidic device where individual vesicles undergo chemical lysis at the outlet. The vesicle contents are then quantitatively (>95% coulometric efficiency) detected using end-column amperometry at carbon-fiber microelectrodes.

Vesicular Release via Exocytosis is Not All-or-None

Measurements resulting from the amperometric electrooxidation of catecholamine in vesicles extracted from the cell were compared to measurements from single cell release experiments in which exocytosis was induced by chemical secretagogues (Figure 3-1B). Representative data for these measurements are shown in Figure 3-2A and 3-2B, where each current transient corresponds to the measurement of a single vesicle. Interestingly, as indicated by the amperometric traces for each of the two measurements, many more current transients are detected from the suspension of isolated vesicles (~5000 events per ~1 nL injection) when compared to that from stimulated exocytosis at single cells (~100 events with multiple cells and stimulations), demonstrating the electrochemical cytometry measurement as a high-throughput survey of vesicles within the cell.
Figure 3-1. Experimental approaches to measure vesicular transmitter content (not drawn to scale). (A) Stimulated exocytosis at single cells measured using amperometric detection at a carbon-fiber disk microelectrode. The carbon-fiber disk microelectrode is placed onto the cell and held at a potential above the formal oxidation potential of the monoamine investigated (typically ~ 0.7 V versus Ag/AgCl). A chemical secretagogue (e.g., elevated K⁺ solution) is puffed onto a single adherent cell using a micropipette to induce exocytosis. As vesicles undergo exocytosis, they fuse to the plasma membrane and release their contents into the extracellular space where they can be detected at the microelectrode sensor. (B) Isolated vesicle investigation on the microfluidic-based platform. Vesicles are isolated from cells offline using differential centrifugation. A suspension of vesicles is then electrokinetically injected onto a fused-silica capillary that terminates into a PDMS-based microfluidic platform. As individual vesicles exit the separation capillary, they are flushed with lysis solution from neighboring channels in a sheath-flow format. Their membranes are lysed when they interact with this solution and their contents subsequently detected using end-column amperometry at a cylindrical carbon-fiber microelectrode.
Figure 3-2. Amperometric quantification of catecholamine amounts in PC12 cells. (A) Representative amperometric trace resulting from exocytotic release at intact cells. Red arrows indicate elevated K⁺ application used to induce exocytosis. (B) Representative electropherogram for the end-column lysis and amperometric detection of individual vesicles. Vesicles were isolated offline by differential centrifugation from a matched population of PC12 cells investigated in panel A. Vesicles in panel B were injected at 111V/cm for 5 s and separated in an applied field of 333 V/cm. Individual events are shown in the expanded axes in panels A and B in order to illustrate typical peak characteristics observed for the analyses. The amperometric signals from single cell experiments were filtered at 2 kHz bandpass and at 15 Hz bandpass for the electrochemical cytometry experiments, providing different baselines for these different experimental approaches.
Using Faraday’s Law, the mole amount of catecholamine from individual current transients was quantified, binned, and plotted as a normalized frequency histogram of vesicle content (Figure 3-3). When the total vesicular catecholamine content (as measured in the cell-free model) is compared to the amount released (as measured from single cell amperometry experiments), the data reveal that, on average, approximately 40% of the catecholamine in a vesicle is released during exocytosis. Indeed, significantly less catecholamine was measured by stimulated exocytosis at single cells, 141 ± 3 zmol, versus 387 ± 2 zmol for isolated vesicles measured on the microfluidic platform (Mann-Whitney U-test, \( p < 0.0001 \); error is SEM). A Gaussian fit of the data reveals a shift in the distribution for the amount released from cells using stimulated exocytosis (red) compared to that from isolated vesicles investigated on the microfluidic platform (black). These data are plotted as the cube root transform of amount to account for the spherical geometry of the vesicle. Interestingly, if the data from the isolated vesicle experiments is fit to two Gaussian distributions (Figure 3-3, inset) the correlation coefficient of the fit increases from 0.90 to 0.99, which suggests two populations of vesicles. The mean of each distribution is greater than that measured from exocytosis at single cells (4.97 zmol\(^{1/3} \) for single cell experiments versus 5.5 and 7.7 zmol\(^{1/3} \) for isolated vesicles). This result challenges classical assumptions of quantal release for the stimulated exocytosis of single cells (\textit{vide supra}).

This hypothesis that exocytosis does not result in complete expulsion of the transmitter in a vesicle is supported in the literature. Vesicular content measurements have been shown to vary with experimental changes in stimulation (16, 36), the osmotic pressure differential between the vesicular and plasma membranes (30, 37), vesicular pH
**Figure 3-3.** Normalized frequency histogram of the vesicular catecholamine amounts quantified from intact cells that underwent stimulated exocytosis (red) versus isolated vesicles (black) investigated on the microfluidic platform. Exocytosis at single cells was induced by application of elevated K⁺ solution. Data plotted as the cube root transform to account for the spherical geometry of the vesicle. Bin size = 0.2 zmol¹/³. Fits were obtained from a Gaussian distribution of the data. Average amount of catecholamine measured from stimulated exocytosis of intact cell was 141 ± 2.67 zmol (n = 946 events), whereas that for isolated vesicles was 387 ± 1.87 zmol (n = 29,643 events). Inset: Two Gaussian distributions are fit to the data for isolated vesicles investigated with electrochemical cytometry. The mean of each distribution is greater than the Gaussian fit of the data from exocytosis experiments at single cells (4.97 zmol¹/³ for single cell experiments versus 5.5 and 7.7 zmol¹/³ for isolated vesicles). Correlation coefficient for fitting two distributions of these data is equal to 0.99 compared to 0.90 for fitting one distribution. Error is SEM.
manipulation (38), temperature gradients (39), and manipulation of second-messenger systems (40). It is possible that during exocytosis of large dense-core vesicles, the event does not result in full distention of the fused vesicle and thus rapid closure takes place prior to full release. This is consistent with studies where evidence suggests that in PC12 cells 97% of exocytosis events are followed by rapid endocytosis (41). In addition, if one compares the data from patch amperometry (where membrane area is measured in tandem with the amount released) to that from direct amperometry quantification of the former reveals three to five times the release than the latter (42). This is generally not addressed in the literature, but it is likely that the pressure applied during patch amperometry results in more complete distention, consistent with data in this chapter.

**Vesicular Content Is Altered with Pharmacology at the Single Vesicle Level**

The amount of catecholamine released was measured as a function of vesicle volume controlled by pharmacological manipulation. PC12 cells were treated with either L-DOPA (a synthetic precursor to dopamine) or reserpine (a potent inhibitor of the vesicular monoamine transporter (VMAT)), which are known to increase and decrease vesicular catecholamine levels, respectively (12, 13). Measurements of the amount released were made at single cells and compared to isolated vesicles as described above. Representative individual current transients observed from both treated and untreated cells are shown in Figure 3-4A for each measurement. When cells are treated with reserpine the charge, $Q$, is markedly smaller than that for untreated cells; conversely, the charge increases with L-DOPA treatment. In addition, the charge observed from experiments performed on isolated vesicles is greater than that from stimulated release experiments within each of the treatments.
Figure 3-4. Pharmacological treatment alters vesicular quantal size from both intact cells and individual vesicles isolated from cells. (A) Typical peak characteristics representing pharmacological manipulation of vesicular quantal size from both stimulated exocytosis at single PC12 cells and isolated vesicles. Data were collected from matched cell preparations. Cells were incubated with either 100 nM reserpine (a potent VMAT inhibitor) or 100 μM L-DOPA (a precursor in dopamine synthesis) for 90 min prior to stimulated exocytosis investigations and vesicle isolation. (B) Representative normalized frequency histograms describing the distributions of vesicular catecholamine amounts quantified from reserpine-treated (red), untreated (blue), and L-DOPA-treated (black) intact cells that underwent stimulated exocytosis. (C) Representative normalized frequency histograms of vesicular catecholamine amounts quantified from isolated vesicles from reserpine-treated (red), untreated (blue), and L-DOPA-treated (black) matched cell populations in panel B. Data plotted as cubed root amounts. Bin size = 0.2 zmol$^{1/3}$. Fits were obtained from a Gaussian distribution of the data. (D) Cumulative analysis for the average number of molecules of catecholamine quantified per vesicle from stimulated exocytosis of intact cells (striped) versus individual isolated vesicles (white) under pharmacological manipulation. The number of events measured for stimulated exocytosis at single cells was 312, 946, and 1,376 for reserpine-treated, untreated, and L-DOPA-treated cells, respectively. The number of events measured for the electrochemical cytometry of vesicles was 13,363, 29,643, and 22,270 for reserpine-treated, untreated, and L-DOPA-treated cells, respectively. Error in panel D is SEM.
Vesicular catecholamine amounts were measured and plotted as normalized frequency histograms for the amount released by amperometry from intact cells (Figure 3-4B). This is compared to electrochemical cytometry measurements of the amount in vesicles isolated from the cell environment (Figure 3-4C). Results from the reserpine-treated cells (red), untreated cells (blue), and L-DOPA-treated cells (black) are shown for each measurement. A cumulative analysis of average vesicular catecholamine content for matched cell populations is presented in Figure 3-4D. The amount of catecholamine detected following L-DOPA treatment significantly increased versus control (one way ANOVA, $p < 0.001$) whether observing release by amperometry or the total vesicle amount by electrochemical cytometry. Accordingly, the reserpine treatment significantly decreased the amount of catecholamine detected versus control for both release and vesicle content measurements (one way ANOVA, $p < 0.001$ for the amount released from intact cells and $p < 0.05$ for the amount in vesicles isolated from the cell environment).

In addition, the total vesicle content is significantly different than the amount detected in release experiments (one way ANOVA, $p < 0.001$) for control, L-DOPA, and reserpine treatment. Vesicular catecholamine amounts can be directly linked to the number of molecules detected using Avogadro’s number. For stimulated exocytosis at single cells, the release of $72,000 \pm 3,100$ molecules was detected for reserpine-treated cells, $85,000 \pm 1,600$ molecules for untreated cells, and $117,000 \pm 2,700$ molecules for L-DOPA-treated cells ($n = 312, 946, \text{ and } 1,376 \text{ events}; \text{ error is SEM}$). Vesicular amounts measured by electrochemical cytometry were $167,000 \pm 1,100$ molecules following reserpine treatment, $220,000 \pm 1,100$ molecules for untreated cells, and $320,000 \pm 1,600$ molecules following L-DOPA treatment ($n = 13,363, 29,643, \text{ and } 22,270 \text{ events}; \text{ error is SEM}$).
The average amount of catecholamine released following stimulated exocytosis at intact cells is significantly less than the total vesicular catecholamine amount. The fraction of average catecholamine amount released was 43 ± 2%, 39 ± 1%, and 37 ± 1% (error is SEM) for the reserpine-treated, untreated, and L-DOPA-treated cells, respectively, further indicating that the entire contents are not expelled from vesicles during the exocytosis processes. Moreover, the drug treatments yielded similar results for both changes in release and total vesicle content. The average vesicular release of catecholamine relative to control cells was reduced by 15 ± 4% with reserpine treatment and was increased by 27 ± 4% for L-DOPA treatment. This trend was maintained in the analysis of isolated vesicles by electrochemical cytometry; a 24 ± 1% decrease in average vesicular catecholamine content for reserpine treatment and a 31 ± 1% increase with L-DOPA treatment was observed when compared to control (error is SEM). Interestingly, the drug treatments demonstrated the utility of this novel cell-free method to serve as a simple and quantitative tool for performing high-throughput analyses to screen for direct physiological effects of pharmacological manipulation at the single vesicle level.

Size Measurements of Vesicles Validate the Electrochemical Cytometry Results

The results comparing vesicular catecholamine content by electrochemical cytometry to electrochemical measurements of release consistently show that the amount released upon stimulated exocytosis at single cells is approximately 40% of that measured from isolated vesicles. To assign this fraction to the hypothesis that the vesicle does not release its entire contents during exocytosis, several experimental parameters must be confirmed. These include: (i) that the methods are capable of quantifying similar-sized vesicles, (ii) the isolation procedures yield individual secretory vesicles for
analysis, and (iii) the isolated vesicles are representative of the vesicles sampled in single cell release experiments. The methods used are capable of quantifying similar-sized vesicles; the limits of detection (LOD) on the microfluidic-based platform are similar to those from release events in single cell experiments (LOD ~ 5,000 molecules for each method). Indeed, the smallest-sized vesicles measured at single cells are observed in isolated vesicle experiments, as indicated by the distributions in Figures 3-4B and 3-4C.

To confirm that the isolation procedures yield individual secretory vesicles for analysis, independent measurements of vesicle size from the treated and untreated PC12 cells investigated above were made using dynamic light scattering (DLS). As previously mentioned, pharmacology can be used to alter vesicular catecholamine levels in this cell model and these changes can be quantified directly from size measurements since vesicle volume is affected as a result of treatment (13, 14). DLS is a non-invasive measurement that relates the diffusion velocity of a suspension of vesicles in the cell-free model to the average hydrodynamic diameter using the Stokes-Einstein equation (26). The average diameters for reserpine-treated, untreated, and L-DOPA-treated PC12 cell vesicles are listed in Table 3-1. DLS measurements were used to provide a qualitative analysis of both treated and untreated vesicle suspensions. These measurements indicate that the vesicle isolation procedures used in the cell-free model are valid since the mean diameter is in agreement with independent measurements of PC12 cell vesicular size obtained in this study (vide infra) and in previous reports (12, 13). This was also verified by performing a Western Blot for synaptophysin, a known integral membrane protein present on neurosecretory vesicles (Figure 3-5).
<table>
<thead>
<tr>
<th>Treatment</th>
<th>Vesicle Diameter (nm)</th>
<th>Vesicle Diameter (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><strong>DLS</strong></td>
<td><strong>TEM</strong></td>
</tr>
<tr>
<td>Reserpine$^a$</td>
<td>174 ± 47</td>
<td>141 ± 2</td>
</tr>
<tr>
<td>Untreated$^b$</td>
<td>183 ± 24</td>
<td>153 ± 2</td>
</tr>
<tr>
<td>L-DOPA$^c$</td>
<td>194 ± 39</td>
<td>186 ± 4</td>
</tr>
</tbody>
</table>

**Table 3-1.** Vesicle Size Measurements from Dynamic Light Scattering (DLS) and Transmission Electron Microscopy (TEM). *Error = SD. **Error = SEM. For TEM data: $a = 206$ vesicles, $b = 217$ vesicles, and $c = 122$ vesicles.
Figure 3-5. Western Blot of isolated PC12 cell vesicles. Lysate from a crude suspension of PC12 vesicles was investigated for the presence of synaptophysin, a known membrane integral protein of both synaptic and neuroendocrine vesicles. Four repetitions for this experiment are depicted in each of the marked lanes. Results show the presence of synaptophysin in each of these samples as marked by the protein standard Alcohol Dehydrogenase with a molecular weight of 38 kDa.
Transmission electron microscopy (TEM) is a well-established quantitative method for measuring subcellular domains within the construct of the cell environment and, therefore, was employed for size analysis of vesicles. Representative electron micrographs for vesicle analyses in single cells are pictured in Figure 3-6A and measurements that quantify these data are listed in Table 3-1. To investigate whether vesicles from similar pools were being sampled in the single cell release and isolated vesicle experiments, I developed a set of criteria for two types of vesicles and compared their sizes as determined by TEM. The first group included vesicles close to, or already primed onto the plasma membrane (< 65 nm, less than the approximate average radius of the vesicles measured). These vesicles are considered to be in the readily releasable pool, and presumably the vesicles measured during stimulated release at single cells. I then defined a second group (> 65 nm from the membrane), designated as the reserve pool. In other secretory cell models, the reserve pool is thought to contain larger, more mature vesicles (39). It is important to note that the procedures employed for vesicle isolation sample from the entire cell, which should yield components from both the readily releasable and reserve pools.

The average diameter of vesicles in the readily releasable pool as measured by TEM was 148 ± 3 nm (n = 68; error is SEM). Notably, vesicles in the reserve pool, which are further from the plasma membrane possessed an average diameter of 153 ± 2 nm (n = 147; error is SEM). This difference is not statistically significant (Mann-Whitney U-test, \( p = 0.30 \)). The plot in Figure 3-6B illustrates this by showing that vesicles in these cells are the same size whether they are primed for exocytosis close to the plasma membrane or far from the membrane (measurements recorded for vesicles up
Figure 3-6. Electron microscopy investigation of PC12 cell vesicular size and volume. (A) Representative TEM images from treated and untreated PC12 cells. Scale bar = 400 nm. (B) Plot of average vesicle size versus distance from the plasma membrane determined by TEM for untreated PC12 cells. Error is SEM. Values marked ns are not statistically different, $p > 0.05$, Mann-Whitney U-test. (C) Plot describing average volume of dense-core vesicles and their constituents upon reserpine (white), untreated (black), and L-DOPA (striped) treatment measured by TEM. Values marked with *** in panel C are statistically different from untreated cells, with $p < 0.0001$, Mann-Whitney U-test. (D) Correlation between the amount of catecholamine detected in intact vesicles (cytometry; black boxes) and released from vesicles (amperometry; red circles) versus the volume in the vesicle halo (from TEM measurements). Data points for each of the measurements going from left to right are reserpine-treated, untreated, and L-DOPA-treated cells. Amperometric measured amounts of catecholamine are plotted against TEM measurements of halo volume. The slope is the concentration of catecholamine in the halo and the intercept is the amount of catecholamine in the dense core. Values listed in Table 3-2. Linear regression results available in Materials and Methods.
to 3 μm from the membrane). This validates that the isolated vesicles are representative of the vesicles sampled in single cell release experiments, and that measurements quantified from isolated vesicles are due to excess catecholamine retained during exocytotic release at single cells. In fact, it is likely that even less than 40% is released due to possible losses during the vesicle isolation procedure. Indeed, if catecholamine were to escape the vesicle after cell fractionation, this would cause an underestimation of the amount in each vesicle.

**Quantification of Intravesicular Catecholamine Stores: Halo vs. the Dense Core**

Once it was determined that the vesicle does not release its entire contents during exocytosis, I examined where the excess catecholamine remains after full fusion. TEM was used to map the size characteristics of intravesicular catecholamine stores. The TEM images in Figure 3-6A reveal the prime components of the large dense-core vesicles in this neurosecretory cell model; namely: a dark granule composed of a semi-crystalline matrix of acidic proteins and semi-soluble catecholamine (referred to as the dense core) encased within a membrane containing solubilized catecholamine that surrounds the dense core (referred to as the halo). The volumes of each intravesicular domain were calculated from TEM size measurements (Figure 3-6C). The data reveal that various constituents of the vesicle are altered upon pharmacological manipulation of the catecholamine stores as previously reported for this cell line (12), also demonstrating the existence of the halo as a genuine vesicular feature. A significant decrease in vesicle volume is observed when catecholamine levels are depleted with reserpine treatment, and conversely, significantly increased with L-DOPA treatment (Mann-Whitney U-test, \( p < 0.001 \)). The increase in halo volume with L-DOPA-treatment (Mann-Whitney U-test, \( p < \))
0.001) resulted in a much more pronounced effect than dense-core size, which was not statistically different from control (Mann-Whitney U-test, \( p > 0.05 \)). This phenomenon was conserved with reserpine treatment where a statistically significant amount of catecholamine was depleted from the halo when compared to control (Mann-Whitney U-test, \( p < 0.005 \)).

During the exocytotic full fusion processes it is thought that crystalline catecholamine present in the dense-core granule interacts with extracellular fluid, swells, and is solubilized as it is extruded through the fusion pore concomitantly with the halo contents (17). The results show a \(~60\%\) decrease in electrochemically measured catecholamine amounts between release at single cells and total content from the vesicles in the cell-free model. To account for this discrepancy, it is hypothesized that catecholamine constituents intercalated in both the halo and dense core are retained post-fusion. This was experimentally addressed by correlating the electrochemical measurements from each of the methods with volume data elucidated from TEM intravesicular size measurements as a function of pharmacological manipulation of catecholamine content. The result is shown in Figure 3-6D, where catecholamine amount data from release at single cells (red) and isolated vesicles (black) is plotted versus the volume of the halo, acquired from TEM data of treated and untreated PC12 cells. Linear regression analyses for the model systems investigated provide information about the average amount of catecholamine detected from the dense core (the intercept) and the concentration of catecholamine in the halo (the slope). The differences in the slopes of the linear regression for each method (Table 3-2) are not statistically significant (Student’s t-test, \( p = 0.15 \)). However, the intercept of these data reveals that single cells
<table>
<thead>
<tr>
<th></th>
<th>Amt. Detected (zmol)</th>
<th>Amt. in Dense Core (zmol)</th>
<th>[Halo] (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stimulated Exocytosis at Single Cells</td>
<td>141 ± 3</td>
<td>75 ± 10</td>
<td>39 ± 7</td>
</tr>
<tr>
<td>Isolated Vesicles</td>
<td>387 ± 2</td>
<td>153 ± 70</td>
<td>125 ± 35</td>
</tr>
</tbody>
</table>

**Table 3-2.** Determining Intravesicular Catecholamine Stores of PC12 Cell Vesicles using Amperometric and TEM Measurements. The average amounts of catecholamine were measured directly using amperometric detection of stimulated release at single cells and electrochemical cytometry of isolated vesicles. Error is SEM. Amount in the dense core is the intercept from Fig. 3-6D (error is SD). The concentration in the halo is the slope from Fig. 3-6D (error is SD).
expel an average of 50% of catecholamine associated with the dense core during exocytosis (Student’s t-test, $p < 0.05$). The notion of vesicular catecholamine stores being retained post-fusion is supported in the literature for partial release via processes such as “kiss and run” (14, 43) exocytosis and a “flickering fusion pore” (44). During these mechanisms, the vesicle is thought to momentarily fuse with the cell membrane forming a small pore through which transmitter can escape (44, 45). The pore then closes prior to full distention of vesicular contents. In single cell experiments, the amperometric signals for exocytosis are generally thought to represent complete vesicle distension. However, the electrochemical cytometry data reported here demonstrate incomplete expulsion of vesicular catecholamine. To explain this and the retention of the catecholamine in both the dense core and halo it is proposed that the average dense-core vesicle does not fully open during exocytosis, a process in between kiss and run and full membrane distension.
Conclusions

It has been demonstrated that individual vesicles isolated from the cell environment can be separated and probed to quantify transmitter content, a process termed electrochemical cytometry. These amounts can be directly altered with pharmacological treatment and variances due to the treatments can be resolved at the single vesicle level in a high-throughput manner. By correlating data from single cell release experiments with the cell-free model, it was demonstrated that vesicles release only 40% of their total catecholamine load during exocytosis, a premise that contradicts classical assumptions of complete quantal release. Furthermore, it has been shown that independent measurements of vesicular and sub-vesicular size both validate the novel method for monitoring transmitter content, and also contribute information with regards to which intravesicular domain excess catecholamine is stored. The implications of these results to neuroscience are large; namely: vesicular neurotransmitter and hormone release is not necessarily all-or-none. Therefore, exocytotic release might be regulated within a single event, which could have implications in neural disease states. Moreover, controlling the fraction of release from vesicles is a potential pharmaceutical target.
References


CHAPTER 4

High-Throughput Quantitative Analysis of Neurotransmitter Content from Individual Mouse Striatal Vesicles with Electrochemical Cytometry*

Introduction

The vesicle is the primary intracellular unit for the storage and release of chemical messengers in the nervous system and, therefore, is thought to play a determinate role in regulating neuronal cell signaling processes. In previous chapters, I have demonstrated that electrochemical cytometry can serve as a well-suited analytical methodology to assess total vesicular transmitter content. Here, I apply the general principles used to quantify artificial and secretory vesicle content in Chapters 2 and 3 towards the measurement of total neurotransmitter from synaptic vesicles isolated from primary midbrain neurons of the mouse. This investigation allows for a direct quantifiable measurement of neurotransmitter content from individual synaptic vesicles in a manner that is independent of the processes associated with exocytotic release.

The synaptic vesicles investigated were isolated from striatal tissue, a subcortical brain region, which is densely populated with dopaminergic innervations. Dopamine transmission pathways that terminate into the striatum have been an area of interest in neuroscience research as they have been directly linked to a variety of phenomena including motor function, reward, addiction, cognition, and neurological dysfunction, including Parkinson’s and Huntington’s disease (1). Gaining a fundamental understanding of the subcellular molecular components that dictate the functions of these neurons can provide information about how cell communication occurs in the

* A manuscript with the contents of this chapter authored by DM Omiatek, AJ Bressler, AM Andrews, ML Heien, and AG Ewing is in preparation for publication (Mar 2010).
mammalian brain. Moreover, it can open the possibility for new solutions in the treatment of non-ideal neuronal signaling associated with various disease pathologies.

Quantifying vesicular content from individual dopaminergic synapses in the central nervous system (CNS) has proven to be a challenging analytical measurement. Classical electrophysiology methods used to measure neurotransmission have been rendered ineffective at dopaminergic synapses due to the lack of post-synaptic currents associated with monoamine transmission (2). For example, during the neurotransmission of acetylcholine, the chemical signal rapidly diffuses across the synaptic cleft and binds to ionotropic receptors on a post-synaptic target, thus yielding an rapid point-to-point response that can be represented by mostly invariant currents attributed to vesicular exocytosis at the pre-synaptic terminal (3). Conversely, monoamines act on second messenger G protein-coupled receptors at the post-synaptic target and are known to elicit a more “social” response, where most of their actions are regulated extracellularly (e.g., via uptake, diffusion, or metabolism) (4-6). Therefore, recordings at the post-synaptic target of a dopaminergic cell can provide a poor representation of the neurotransmitter expelled presynaptically.

Midbrain primary neurons are populated mostly with electron lucent small synaptic vesicles (SSVs) which have been estimated by electron microscopy to be approximately 50-nm in diameter (7), although both large and small dense-core vesicles have been observed (8-10). These neurons are difficult to culture in vitro for single cell secretion experiments and have been reported to release very small amounts of vesicular monoamine messengers (1000-10,000 molecules on average) (4, 11-13). This is why most have turned to neurosecretory cell models (e.g., PC12 cells, chromaffin cells, mast
cells, etc.) to monitor the processes of exocytotic release. These cells are easier to maintain and contain vesicles with higher levels of monoamine messengers (~$10^4$-$10^6$ molecules) packaged in large dense-core vesicles (LDCVs) (14-17).

Conventional methodologies used to investigate exocytosis at LDCV-containing neurosecretory models often lack the spatio-temporal resolution necessary to quantify release at CNS terminals. Optical microscopy methods have been used to monitor modes of vesicular release (e.g., partial versus full exocytosis) at CNS neurons in culture (11, 18-20), but are difficult to quantify and relate to in vivo cell signaling scenarios since the actions of common fluorescent tracer molecules are temporally inferior to release at dopaminergic synapses (e.g., diffusion coefficient of FM1-43 $D = 1 \times 10^{-4}$ cm$^2$/s versus dopamine $D = 6 \times 10^{-6}$ cm$^2$/s) (18, 21). Likewise, capacitance measurements have been useful to measure changes in plasma membrane area upon exocytosis of LDCV granules (16, 17) and at large CNS synapses (e.g., the calyx of Held (22)), but do not possess the sensitivity to resolve these changes from small synapses that constitute the anatomical connections of midbrain primary neurons.

Electrochemical methods have been used to quantify dynamic measurements of chemical neurotransmission in the brain (23). The voltammetric microelectrode sensor has been a valuable tool for neurobiological investigations, especially for monitoring the extracellular presence of monoamines. Indeed, these sensors possess (i) a fast temporal response (sub-millisecond) capable of monitoring transient release, (ii) are micron-sized, allowing for measurements in spatially-confined regions of tissue or at single cells, and (iii) are selective for electroactive species (e.g., monoamine messengers) whose amounts and/or concentrations can be quantified from an electrochemical response at the detector.
Electrochemical methods have been used for \textit{in vivo} studies of neurotransmission at microelectrodes in the striatal tissue of rats\textsuperscript{(24, 25)} and mice\textsuperscript{(25)}, and micromolar levels of extracellular dopamine have been quantified following neural stimulation. In addition, quantitative electrochemical measurements have been employed to detect basal endogenous monoamine amounts from mammalian striata via direct sampling of extracellular fluid using microdialysis\textsuperscript{(26, 27)}, and at nerve endings (synaptosomes) purified from the brain milieu\textsuperscript{(28, 29)}. However, these approaches have looked at extracellular neurotransmitter and the focus of this study is relegated to the presynaptic investigation of neurotransmission via the fundamental chemical messenger storage unit, the vesicle.

Wightman demonstrated that constant potential amperometry at carbon-fiber microelectrodes can be used to quantitatively monitor the stimulus-coupled secretion of vesicular transmitter from LDCV-containing adrenal cells in culture\textsuperscript{(15)}. Although non-neuronal LDCV cells expel the same chemical messengers transmitted during neuronal signaling processes (e.g., monoamines), they lack analogous spatio-temporal release and/or quantal amounts that are characteristic to neurotransmission in the mammalian CNS (e.g., microsecond exocytosis events from functional neuronal processes). Studies have been reported on the electroanalytical quantitative investigation of synaptic vesicle content at single primary neurons\textsuperscript{(11, 12, 30-32)}, however, only a few have been able to effectively measure release from untreated midbrain dopaminergic neurons.

As mentioned earlier, the ability to successfully record release at primary neurons is experimentally challenging. For single cell electrochemical investigations of exocytosis at primary neurons, the neuron isolation, preparation, and measurement
procedures are intensive. To isolate dopaminergic neurons for these measurements, cells can be labeled with a fluorophore reactive to tyrosine hydroxylase (a cytosolic enzyme involved in intracellular dopamine synthesis) and dissected from the brain tissue. The cells then must be maintained outside the neuronal environment on culture dishes, which can compromise their viability and quantal release characteristics. These, among others, are reasons why there is limited information available regarding vesicular content from untreated midbrain dopaminergic SSVs via \textit{in vitro} amperometric investigations at single cells.

A mathematical model was developed to estimate the quantal content of dopaminergic vesicles from primary neurons and approximated 1000 molecules per vesicle (4). However, this value relies on a number of possible variables including the size of the vesicle, the length of the fusion pore, the thickness of the pre-synaptic membrane, the distance between the release site and detection electrode, and the extracellular transmitter concentration levels. Moreover, this model is rooted in the idea that all vesicles available for release at or near the synapse (as determine by microscopy data) undergo full exocytosis to contribute to the bulk measured extracellularly, which presents a generalized criterion for the average \textit{in vivo} exocytotic event. In Chapter 3, I provided direct evidence to show that dopaminergic secretory cells (PC12) do not expel their entire vesicular transmitter load during the average exocytosis event (33), and this concept of partial release that has been supported in the literature through electrochemical (31), capacitance (16, 17), and microscopic (34) investigations of exocytosis at both LDCVs and SSVs. Therefore, a significant underestimation of vesicular transmitter content on a per vesicle basis is plausible under such conditions.
Herein, I demonstrate the possibility of quantifying the total neurotransmitter content of vesicles from midbrain dopaminergic terminals through the use of electrochemical cytometry. These analyses were performed in a high-throughput format for thousands of individual vesicles per electrochemical cytometry experiment. Vesicles were isolated from mice striatal tissue and quantitatively investigated for endogenous vesicular dopamine content in a manner that circumvented the biochemical and biophysical processes of release. Next, it was shown that injecting a live mouse with pharmacological agents known to increase or decrease vesicular dopamine alters quantal content, and this change is quantified with electrochemical cytometry. In addition, electrochemical cytometry was used to measure a time dependent treatment effect at midbrain neurons, providing information about the period for certain drugs to act on the vesicular dopamine in the striatum. Finally, the effect of the psychostimulant, amphetamine (admin. 10 mg/kg i.p.), was investigated and shown to significantly deplete dopamine in striatal synaptic vesicles.
Materials and Methods

Reagents. N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES), sodium hydroxide (NaOH), sodium dodecyl sulfate (SDS), Trizma® hydrochloride (Tris-HCl), reserpine, and 3,4-Dihydroxy-L-phenyl-alanine (L-DOPA), benserazide hydrochloride, (+)-amphetamine, glacial acetic acid, hydrofluoric acid (HCl, aq. 48%), sodium chloride (NaCl), potassium chloride (KCl), glucose, calcium chloride (CaCl₂), magnesium chloride (MgCl₂), and the Synaptic Vesicles Isolation Kit were obtained from Sigma Aldrich (St. Louis, MO). All chemicals were used as received. All buffer constituents for isolation procedures are listed as proprietary knowledge of Sigma Aldrich (e.g., homogenization, lysis, and storage buffers). Mast cell isolation buffer consisted of 12.5 mM Tris-HCl, 150 mM NaCl, 4.2 mM KCl, 5.6 mM glucose, 1.5 mM CaCl₂, and 1.4 mM MgCl₂. The electrophoretic separation buffer for electrochemical cytometry experiments consisted of 50 mM TES with 2% 1-propanol. The lysis buffer for electrochemical cytometry was 50 mM TES with 5% (w/v) SDS. All buffers were made in ultrapure water, adjusted to pH 7.4 using NaOH, and filtered through 0.2-μm pore size filters (Nalgene, Rochester, NY).

Animal Care. Male and female Crl:CD-1(ICR)BR mice were obtained from Charles River Laboratories (Wilmington, MA) and were 2- to 5-months-old (average age 3 months) when they were used for these experiments. Animals were group housed by sex (2-4 mice per cage) in a temperature and humidity controlled room on an automatic 12-h light/dark cycle. Food and water were available ad libitum. Experimental protocols adhered to National Institutes of Health Animal Care guidelines and were approved by the Pennsylvania State University Institutional Animal Care and Use Committee.
Brain Dissection and Drug Treatments. Benserazide hydrochloride (10 mg/kg) was dissolved in distilled water and administered 30 min before treatment with L-DOPA. L-DOPA (50 mg/kg) was dissolved in 0.1 M HCl. Reserpine (20 mg/kg) was dissolved in 20 µL of glacial acetic acid and then diluted to volume with distilled water. (+)-Amphetamine (10 mg/kg) was dissolved in distilled water. Drug doses were calculated as the free base and were administered intraperitoneally (i.p.) in a 0.2-mL volume. Treatment time periods prior to animal sacrifice were either 0.5 h or 2 h for L-DOPA experiments, 6 or 12 h for reserpine experiments, and 1 h for amphetamine experiments. Following this time period, mice were sacrificed by cervical dislocation, decapitated, and their brains were removed rapidly and dissected over ice to isolate the striatum (Dissected by AJ Bressler). The striata from three mice were pooled for each vesicle isolation.

Peritoneal Normal Mouse Mast Cell Acquisition. CD-1 wild-type mice were used to isolate vesicles from peritoneal mast cells. The mice were euthanized by exposure to carbon dioxide vapor, then decapitated prior to cell isolation. Mast cell isolation buffer (5 mL) was injected into the peritoneal cavity of each mouse and its abdomen massaged for two minutes. The cavity was subsequently opened and the buffer containing the mast cells was collected. This procedure was performed on three mice and the cells pooled for vesicle isolation. The cell suspension was centrifuged at 400 x g for 5 min to pellet cells. The supernatant was discarded and the cells resuspended in homogenization buffer for further vesicle isolation (vide infra).

Vesicle Isolation. A Synaptic Vesicle Isolation Kit (Sigma Aldrich) was used to extract vesicles from the tissue by following a procedure provided by the manufacturer. To isolate vesicles (both synaptic and mast cell), tissue from three mice were
homogenized and centrifuged at 20,000 x g (4°C) for 25 min. The supernatant was discarded and the pellet of tissue in the bottom of the centrifuge tube was subjected to lysis in the presence of 10-mL hypoosmotic buffer. This fraction was then centrifuged at 20,000 x g (4°C) for 25 min. This removed other organelles present in the cell, as they pelleted to the bottom of the centrifuge tube during this treatment. The supernatant, containing vesicles, was then recovered and subjected to ultracentrifugation at 70,000 x g (4°C) for 1 h. Vesicles were recovered as a pellet in the bottom of the centrifuge tube. The supernatant was discarded and 2 mL of vesicle storage buffer was added to the pellet, resulting in a crude suspension of vesicles for analysis. This suspension was then homogenized and stored at 4°C prior to analysis.

Microfluidic Device Fabrication for the Electrochemical Cytometry of Individual Vesicles. A hybrid capillary-microfluidic device was developed as previously described in Chapters 2 and 3 to investigate transmitter amounts from individual isolated vesicles (33, 35). Briefly, microfluidic channels were fabricated using conventional photo- and soft- lithography methods. A master mold was developed by spin-coating 125 μm of SU-8 100 negative photoresist (MicroChem Corp., Newton, MA) on a 3-in. silicon wafer (Silicon Quest International, Inc., Santa Clara, CA). A photolithographic mask containing imprinted device features was placed over the wafer, exposed to ultraviolet light, and developed according to the resist manufacturer protocol.

Soft lithography was carried out using a Sylgard® 184 silicone elastomer kit (Dow Corning Corp., Midland, MI). A 10:1 ratio of poly(dimethylsiloxane) (PDMS) prepolymer base to curing agent was cast onto the master mold and cured at 70°C for 2 h. The PDMS layer was then peeled from the master, revealing an impression of
microfluidic channels. The center channel secured the separation capillary and the other two channels, each 200-μm wide, were set at a 30º angle to the center channel and used to direct lysis buffer to the capillary outlet in a sheath-flow format. The three channels converge into a 2-mm channel where the electrode was placed for detection of catecholamine quantified from individual lysed vesicles. A buffer reservoir for capillary electrophoresis was cut into a 2-mm layer of PDMS and plasma-bonded to a glass microslide (Corning Inc., Corning, NY). The layer containing imprinted microfluidic channels was then plasma-bonded onto the reservoir layer to assemble the device (100 W, 1 min.).

**Separation and Detection of Isolated Vesicles.** Fused-silica capillaries (45 cm in length, 15-μm i.d./150-μm o.d., Polymicro Technologies, Phoenix, AZ) were prepared for electrochemical detection by removing 2 mm of the polyimide coating with a flame and subsequently etching the exposed fused silica by purging He (250psi) for 15 min in an HF bath. This resulted in an etch with a frustum geometry measuring approximately 40-μm wide at the base, which served to both ease placement of the electrode at the capillary outlet, as well as to decouple the applied separation voltage from the electrochemical cell (36).

Electrokinetic injections of vesicles were performed for 5 s at 111 V/cm and separations were carried out at 333 V/cm using a high voltage supply (Spellman, Hauppauge, NY). Capillaries were conditioned before each separation to prevent non-specific binding of the vesicle membrane to the fused-silica by rinsing at 333 V/cm with 1 M NaOH for 2 min, Ultratrol™ Dynamic Pre-Coat-HN (Target Discovery, Palo Alto, CA) for 5 min, and separation buffer for 10 min. A syringe pump (KD Scientific,
Holliston, MA) was used to control volumetric flow of lysis buffer via 1-mL plastic syringes continuously through the microchannels at a rate of 2 μL/min (0.05 cm/s).

Amperometric electrochemical detection was carried out in a two-electrode format. A 5-μm-diameter carbon-fiber was sealed in a glass capillary and cut to a length of approximately 50 μm from the glass seal to fabricate a cylindrical microelectrode as previously described (37). A smaller detection electrode was used for synaptic vesicle analyses than those used in Chapters 2 and 3, which used a 500-μm carbon-fiber cylinder (500-μm electrode was used for mast cell vesicle experiments as well) to minimize the electrical noise as these amperometric peaks are very small and difficult to distinguish from the background. The electrode was held at 0.90 V (except for mast cell experiments where it was held at 1.5 V to oxidize histamine) versus a silver wire quasi-reference electrode (Ag QRE, 0.25-mm-diameter, Alfa Aesar, Ward Hill, MA) and the carbon-fiber electrode was positioned at the outlet of the capillary using a micromanipulator (Newport, Irvine, CA). Previous measurements and modeling indicate that this system provides a coulometric efficiency > 95% (33).

Current was measured using a Keithly model 427 (15 Hz bandpass) current amplifier (Cleveland, OH) and digitized at 5 kHz with a National Instruments PCI-6221 DAQ card using LabView 8.0 software (National Instruments, Austin, TX) written in-house. OriginLab 8.0 (Northampton, MA) was used to generate electropherograms and Mini Analysis (Synaptosoft Inc., Fort Lee, NJ) was used for analysis of the resultant peaks to determine area (fC). Events were quantified if the signal exceeded a threshold greater than three times the RMS baseline noise.
**Western Blot of Isolated Vesicles.** Vesicles isolated from the cell environment were investigated for the presence of synaptophysin, a known integral membrane protein present in synaptic and neuroendocrine vesicles. A 50-μL suspension of vesicles was combined with 50 μL of lysis solution containing 5% (w/v) SDS in 50 mM TES buffer. The mixture was vortexed vigorously for 1 min. Aliquots of this solution containing ~20 μg of protein were loaded into four lanes of a 12% SDS-PAGE gel. Separations were carried by applying 200 V to the gel for 2 h. A SeeBlue® Plus2 pre-stained standard protein kit (Invitrogen, Carlsbad, CA) was used to visualize the separation. Following this, the gel was transferred onto nitrocellulose paper, followed by blocking and incubation with the primary antibody monoclonal anti-synaptophysin (2-h incubation at room temperature; 1:1000 dilution of antibody. Monoclonal Anti-Synaptophysin antibody was provided in the Synaptic Vesicle Isolation Kit). The secondary antibody, HRP-conjugated anti-mouse IgG, was used for chemiluminescent detection of the targeted protein (ProteoQwest™Chemiluminescent Western Blotting Kit, Sigma Aldrich). A prominent band at 38 kDa was observed, confirming the presence of synaptophysin in the vesicle lysate (data not shown).

**Dynamic Light Scattering of Isolated Vesicles.** A ZetasizerNano S (Malvern Instruments, Worcestershire, UK) was used to acquire light scattering data on vesicle preparations. Measurements were collected using a 4-mW HeNe laser operated at 633 nm. Vesicle suspensions were diluted 10-fold in vesicle storage buffer. Data were collected in size mode at 25 °C and fit using instrument software.
Results and Discussion

Electrochemical Cytometric Quantification of Total Synaptic Vesicular Neurotransmitter

To measure total content from synaptic vesicles, the striatum from three wild-type CD-1 mice were carefully dissected, pooled, and processed for synaptic vesicle isolation using similar procedures to those described in Chapter 3 (33). Approximately 1 nL of the synaptic vesicle suspension was injected onto a fused-silica capillary and separated by capillary electrophoresis (CE). As individual vesicles exited the separation capillary they were chemically lysed by surfactant delivered from neighboring channels in a sheath-flow format. Vesicular contents were subsequently detected at a cylindrical carbon-fiber microelectrode placed at the separation capillary outlet.

Figure 4-1A depicts a portion of a representative electropherogram for the electrochemical cytometry of synaptic vesicles isolated from the striatum, where each current spike corresponds to the detection of a single vesicle. The inset shows an expanded view of the axis in order to illustrate the typical peak characteristics. The species electrooxidated at the detector is most likely dopamine since it is the predominant electroactive species present in the brain region investigated, as confirmed by voltammetric release experiments (38, 39) and HPLC analyses (11) of striatal tissue. Integration of the peak currents and application of Faraday’s Law \( N = Q/nF \) yields an average mole amount of 55 ± 0.4 zmol/vesicle (n = 20,331 vesicles from 3 isolation experiments; Error is SEM). This mole amount pertains to an average of 33,000 ± 300 dopamine molecules per vesicle (Error is SEM). The cube root distribution of these data is plotted as a normalized frequency histogram in Figure 4-1B (mean = 3.7 zmol\(^{1/3}\), \( r^2 = 0.99 \) for single Gaussian fit). The electrochemical cytometry measurement of striatal
Figure 4-1. Electrochemical cytometry analysis of mouse synaptic vesicles isolated from primary striatal neurons. (A) A 1000-s portion of a representative electropherogram that demonstrates electrochemical detection of total neurotransmitter content in synaptic vesicles. The inset depicts an expanded axis to view the typical peak characteristics. (B) Normalized frequency histogram for the vesicular neurotransmitter amounts quantified from mouse striatal vesicles by electrochemical cytometry. Data are plotted as the cube root transform. Bin size = 0.2 zmol$^{1/3}$. Fit was obtained from a Gaussian distribution of the data. Correlation coefficient for a single Gaussian fit of these data is equal to 0.99. Distribution mean is 3.7 zmol$^{1/3}$. Average amount is 55 ± 0.4 zmol, which corresponds to 33,000 dopamine molecules ($n = 20,331$; Error is SEM).
vesicle neurotransmitter content was shown to be remarkably consistent among three separate isolations, as demonstrated in Table 4-1.

**Electrochemical Cytometry: Quantifying Vesicular Monoamine Content Across Cell Lines**

Vesicular neurotransmitter amounts have been reported to vary proportionally with the volume of the vesicle, whereas the concentration of vesicular transmitter has been shown to remain relatively constant \((40)\). This has been demonstrated by correlating electroanalytical data with electron microscopy data \((41)\). Here I use this approach to highlight the analytical versatility of the electrochemical cytometry platform by comparing results from dopaminergic midbrain synaptic vesicles, with PC12 cell vesicles and normal mouse mast cell vesicles.

Striatal synaptic vesicles are typically ~50-nm diameter and release primarily dopamine \((7)\), PC12 cell vesicles are ~150-nm diameter and also release dopamine \((33, 42)\), and normal mouse mast cell vesicles are ~800-nm diameter and release histamine and serotonin \((43)\). Electrochemical cytometric analyses yielded a mean vesicular content of 55 ± 0.4 zmol (33,000 molecules), 387 ± 2.0 zmol (220,000 molecules), and 700 ± 6.0 zmol (422,000 molecules, assuming 2 e- oxidation of contents) for the synaptic, PC12, and mast cell vesicles, respectively. A normalized frequency histogram these data collected by electrochemical cytometry is shown in Figure 4-2A. The cube root distributions for these data are plotted, and a single Gaussian fit resulted in distribution means of 3.7 zmol\(^{1/3}\) for synaptic vesicles (gray), 5.4 zmol\(^{1/3}\) for PC12 cell vesicles (red), and 8.4 zmol\(^{1/3}\) for mast cell vesicles (blue). Typical peak characteristics for these data and a summary of the electrochemical cytometry results are present in Table 4-2.
<table>
<thead>
<tr>
<th>Isolation</th>
<th>n</th>
<th>Halfwidth (ms)</th>
<th>Amplitude (pA)</th>
<th>Amount of Neurotransmitter (zmol)</th>
<th># Molecules of Neurotransmitter</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>7954</td>
<td>19 ± 0.04</td>
<td>0.5 ± 0.003</td>
<td>55 ± 0.4</td>
<td>33,000 ± 200</td>
</tr>
<tr>
<td>2</td>
<td>4221</td>
<td>21 ± 0.04</td>
<td>0.4 ± 0.002</td>
<td>56 ± 0.5</td>
<td>34,000 ± 300</td>
</tr>
<tr>
<td>3</td>
<td>8156</td>
<td>17 ± 0.03</td>
<td>0.6 ± 0.004</td>
<td>54 ± 0.4</td>
<td>33,000 ± 200</td>
</tr>
</tbody>
</table>

Table 4-1. Average peak characteristics and quantified vesicular neurotransmitter amounts for the electrochemical cytometry of mouse striatal vesicles. Error is SEM.
Figure 4-2. Normalized frequency histograms of vesicular transmitter from various secretory cell models shows the versatility of electrochemical cytometry measurement. (A) Distributions for vesicular monoamine amounts emanating from (i) mouse primary neuron synaptic vesicles (gray), (ii) PC12 cell vesicles (red), and (iii) mouse peritoneal mast cell vesicles (blue). Average amounts were 55 ± 0.4 zmol (33,000 molecules) for synaptic vesicles, 387 ± 2.0 zmol (220,000 molecules) for PC12 cell vesicles, and 700 ± 6.0 zmol (422,000 molecules) for mast cell vesicles. Bin size = 0.2 zmol^{1/3}. Fits were obtained from a Gaussian distribution of the data. The mean of each distribution was 3.7 zmol^{1/3}, 5.4 zmol^{1/3}, and 8.4 zmol^{1/3} for striatal, PC12, and mast cell vesicles, respectively. The single Gaussian distribution fits well to the synaptic vesicle data as shown in (B). However, the PC12 cell and mast cell data, which have correlation coefficients of 0.93 and 0.96 when fit to a single distribution, are better fit to multiple Gaussian distributions as shown in (C) and (D). For PC12 cell vesicles the mean of each distribution in panel C was 4.9 and 5.5 zmol^{1/3} with r^2 = 0.99. Likewise, mast cell vesicles yield distribution means of 5.9 and 8.8 zmol^{1/3} with r^2 = 0.99 in panel D. n = 20,331, 29,643, and 7209 vesicles for the synaptic, PC12, and mast cell vesicles, respectively; Error is SEM.
<table>
<thead>
<tr>
<th>Vesicle</th>
<th>n</th>
<th>Halfwidth (ms)</th>
<th>Amplitude (pA)</th>
<th>Vesicular Transmitter Amount (zmol)</th>
<th># Molecules of Transmitter</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse Striatum Synaptic Vesicles</td>
<td>20,331</td>
<td>19 ± 0.04</td>
<td>0.5 ± 0.003</td>
<td>55 ± 0.4</td>
<td>33,000 ± 300</td>
</tr>
<tr>
<td>PC12 Cell Vesicles</td>
<td>29,643</td>
<td>20 ± 0.09</td>
<td>3.2 ± 0.02</td>
<td>387 ± 2.0</td>
<td>220,000 ± 1000</td>
</tr>
<tr>
<td>Normal Mouse Mast Cell Vesicles</td>
<td>7209</td>
<td>17 ± 0.03</td>
<td>7.1 ± 0.04</td>
<td>700 ± 6.0</td>
<td>422,000 ± 4000</td>
</tr>
</tbody>
</table>

**Table 4-2.** Average peak characteristics and quantified vesicular monoamine amounts for the electrochemical cytometry of mouse striatal vesicles, PC12 cell vesicles, and mouse mast cell vesicles. Error is SEM.
Electrochemical cytometry can be used to unravel what kinds of vesicles are sampled during isolation (e.g., SSVs versus LDCVs). For example, amperometric measurements of serotonin release from the leech neuron resulted in multiple distributions leading Bruns and Jahn to suggest that the distributions represented SSVs and LDCVs (13). Figure 4-2B shows a single Gaussian fit of quantified vesicular neurotransmitter from the striatal vesicle experiments ($r^2 = 0.99$). The distribution of the data suggests that only one pool of vesicles is being sampled. This has been supported from microscopy data in the literature which has reported SSVs as the predominate vesicle type in this brain region (7). Moreover, these data have also been plotted as a non-cube root transform, and have yielded a distribution with a mean content of 42 zmol (~25,000 molecules) and $r^2 = 0.94$ (data not shown), resulting in few data points in a range outside of the mean (bin size = 0.5 zmol). Synaptic vesicles distributions can be compared to the distributions from LDCV-containing PC12 and mast cell vesicles in Figure 4-2C and Figure 4-2D. Here, the LDCV models are better fit to multiple Gaussians, inferring that multiple vesicle pools are sampled in the isolation procedures ($r^2$ increases from 0.93 to 0.99 by fitting PC12 cell vesicles to two Gaussians with mean content of 4.9 and 5.5 zmol$^{1/3}$ for the two distributions and from 0.96 to 0.99 when mast cell vesicles are fit to two Gaussians with mean content of 5.9 and 8.8 zmol$^{1/3}$). These data, in conjunction with the quantified vesicular amounts indicate that SSVs are sampled in the isolation procedures and quantified from electrochemical cytometric analyses.

Comparison of Electrochemical Cytometric Measurements of Total Synaptic Vesicular Transmitter to Measurements of Released Vesicular Transmitter

Electrochemical cytometry of synaptic vesicle content can be compared to in vitro electroanalytical studies of exocytosis performed at CNS dopaminergic neurons (11, 12,
Sulzer and co-workers have reported quantitative release measurements at single primary mouse midbrain neurons using carbon-fiber amperometry (11). They recorded an average of 3,000 vesicular dopamine molecules released from the chemical stimulation of cells in culture (n = 69 exocytosis events, 20 release sites) (11). This value is roughly 10% of the average content measured from the electrochemical cytometry of vesicles isolated from dopaminergic terminals in this study. Five larger events with an average of 48,900 ± 12,000 molecules were also observed from amperometric measurements of neurons in culture, but excluded from the data set. When the cultures were treated with GDNF, a neurotrophic factor reported to facilitate transmitter release as well as promote neuronal survival (11, 44, 45), they observed a nearly 4-fold increase in average vesicular quantal content and a 2-fold increase in exocytotic frequency (n = 109 events, 14 release sites). It is possible that untreated neurons in culture might not accurately represent the amount of vesicular dopamine packaged and released in vivo.

In a later study, Sulzer and co-workers demonstrated that the vesicular quantal size could be increased by overexpressing VMAT2 (vesicular monoamine transporter 2) on the vesicle membrane, a protein transporter responsible for loading monoamines from the cell cytosol into the vesicle lumen (12). Vesicular quantal size increased from an average of 7,800 molecules for control (n = 133 events) to 11,800 molecules at transfected cells, and the frequency of exocytosis increased 10-fold (n = 1,123 events). This demonstrated that the number of molecules reported from primary cultures can vary (260% increase in quantal size in the 2002 study versus the 1998 study), and these factors can be augmented from cues in the exocytotic machinery.
Chow and co-workers performed amperometric measurements of dopaminergic synaptic vesicle quantal size from rat substantia nigra slices, a midbrain region with interventions that project into the striatum \(32\). Exocytotic release was quantified from an environment that more closely represented an \textit{in vivo} experimental setting when compared to measurements at primary cultures, but one where it was not possible to easily measure from the synapse. Therefore, they measured release from the soma of these neurons and determined an average vesicle content of 14,000 dopamine molecules upon chemical stimulation.

Interestingly, when 14,000 dopamine molecules per vesicle from the work of Chow and co-workers is compared to the electrochemical cytometry average (\(\sim 33,000\) molecules), this results in \(\sim 40\%\) of neurotransmitter content, a value that falls directly in line with the fraction of transmitter released in PC12 cell experiments discussed in Chapter 3, thus supporting the notion of partial vesicular release in primary neurons \(33\).

Moreover, when primary midbrain neurons have been examined for complex vesicular exocytosis that involve “flickering” fusion pores, quantifiable amounts of neurotransmitter have been recorded for each flicker of the fusion pore \(31\). Sulzer and co-workers have shown that the average flickering fusion pore releases a certain fraction (\(\sim 11,000\) molecules for cells pre-treated to increase vesicular dopamine) with the first flicker, followed by an average \(\sim 70\%\) of this value with the second flicker, and \(\sim 55\%\) of the original vesicular dopamine amount with the third consecutive flicker at midbrain neurons in culture \(31\). If this is the general release mode for a synaptic vesicle, then the cumulative amount of transmitter from the flickers would more closely resemble the average amount of molecules previously measured via somatic release by Chow and co-
workers, and remain to be considerably less than that measured by electrochemical cytometry in this study.

**Pharmacological Manipulation of Striatal Vesicle Neurotransmitter Amount**

Conventional methods used to alter vesicular quantal content of cells for *in vitro* analyses typically involve immersion of the cultures in a bath of a pharmacological agent that directly acts on the cell. This methodology is less than ideal to investigate the neuronal response of a living organism to drug application, particularly in the mammalian CNS. In order to reach the site of action in the CNS, often a drug must travel through metabolic pathways in the periphery, cross the blood-brain barrier, and be processed intracellularly. This provides a number of obstacles to overcome before it reaches the targeted treatment site. These effects have been quantified using *in vivo* analyses for extracellular measurements of neurotransmission, but not for endogenous vesicular quanta at the subcellular level. Here, I have treated mice with pharmacological agents that act on vesicular dopamine levels, which allows for an *in vivo* treatment of the live animal with an *ex vivo* analysis of vesicular neurotransmitter through the use of electrochemical cytometry.

Striatal vesicles of mice acutely treated with agents to alter vesicular dopamine via intraperitoneal injections of the drugs were quantified with electrochemical cytometry. Figure 4-3 shows representative current spikes for dopaminergic quanta in striatal vesicles from mice treated with reserpine (an inhibitor of VMAT that blocks transport of dopamine into the vesicle, Figure 4-3A) for 12 h prior to sacrifice and L-DOPA (a precursor to intracellular dopamine synthesis, Figure 4-3C) for 2 h prior to sacrifice. The charge for striatal vesicles from mice treated with reserpine (3.8 fC,
Figure 4-3. Example of electrochemical cytometry data for the pharmacological manipulation of striatal vesicle neurotransmitter content. Mice were injected i.p. with reserpine (20 mg/kg) and L-DOPA (50 mg/kg) to alter vesicular neurotransmitter levels. (A) Average peak for reserpine-treated mice striatal vesicles. Striatal tissue dissected out the mouse brain after 12-h treatment with reserpine for vesicle isolation. Peak integral is equal to 3.8 fC (~12,000 molecules). (B) Average peak for untreated mice striatal vesicles. Peak integral is equal to 10.5 fC (~33,000 molecules). (C) Average peak for L-DOPA-treated mice. Striatal tissue dissected out of the mouse brain after 2-h treatment with L-DOPA for vesicle isolation. Peak integral is equal to 34.6 fC (~108,000 molecules). Data filtered offline using 50 Hz Lowpass filter (Butterworth).
~12,000 molecules) is markedly smaller than control (10.5 fC, ~33,000 molecules, Figure 4-3B). Likewise, that for L-DOPA (34.6 fC, ~110,000 molecules), which increases vesicular dopamine, is much larger than control.

The mole amounts of vesicular dopamine from these experiments are plotted as cube root transforms in Figure 4-4. The distribution for vesicles from reserpine-treated mice (green) is shifted to the left of control (blue), and L-DOPA (red) is shifted to the right on the amount axis. The average vesicular dopamine amounts for these vesicles were 20 ± 0.7, 55 ± 0.4, and 180 ± 1.1 zmol for the reserpine-treated, untreated, and L-DOPA-treated mice, respectively (Error is SEM). Results from these data are summarized in Table 4-3. The vesicle suspensions were also characterized using dynamic light scattering (DLS) to provide a qualitative analysis of the average size from vesicles in the isolation suspensions. The average diameters of striatal vesicles from mice were 31 ± 9 nm, 91 ± 15 nm, and 188 ± 20 nm when the mice were treated with reserpine, control, L-DOPA, respectively.

A noticeably smaller number of striatal vesicles were measured with the electrochemical cytometry of reserpine-treated mice versus the control and L-DOPA-treated groups (~1,000 events versus ~8,000 events for control and L-DOPA groups per 1-nL injection of vesicles). This could be due to the fact that a large number of these reserpine vesicles fell below the noise threshold of the baseline (3x RMS) to be counted in the dataset. In addition, it is possible that the smallest portion of the population of vesicles is lost in the isolation procedures. A current transient that is representative of the smaller subset of vesicles detected using electrochemical cytometry is presented in Figure 4-5. Integration of this peak yields 0.58 fC, which is equivalent to roughly 3 zmol or
**Figure 4-4.** Normalized frequency histograms of vesicular neurotransmitter amounts quantified from isolated striatal vesicles from reserpine-treated (20 mg/kg i.p., 12-h, green), untreated (blue), and L-DOPA-treated (50 mg/kg i.p., 2-h, red) mice. Mean number of dopamine molecules were 12,000 for reserpine-treated, 33,000 for untreated, and 109,000 molecules for L-DOPA-treated mice. Bin size = 0.2 zmol$^{1/3}$. Fits were obtained from a Gaussian distribution of the data. The mean of each distribution was 2.4 zmol$^{1/3}$, 3.7 zmol$^{1/3}$, and 5.5 zmol$^{1/3}$ for reserpine-treated, untreated, and L-DOPA-treated striatal vesicles, respectively. Average amounts and number of events for these data are listed in Table 4-2.
Figure 4-5. The limits of detection on the electrochemical cytometry platform allow for the resolution of markedly small vesicular content measurements. This event is representative of the smaller vesicles quantified on the electrochemical cytometry device. The peak integral is equal to 0.58fC (1,800 molecules, 3 zmol), and falls in line as one of the smaller vesicular neurotransmitter amounts quantified by amperometry (Others reports are between 1500-3000 molecules at cultured midbrain neurons undergoing stimulus coupled secretion (11)).
1,800 dopamine molecules. These are among some of the smaller amounts quantified for vesicular dopamine content at SSVs using electroanalytical methods (31).

**Measurements at Different Times Following Pharmacological Treatment**

After it was determined that changes in vesicular dopamine content could be measured, the effect of each drug at two different times following administration was investigated using electrochemical cytometry. These data are plotted as normalized frequency histograms in Figure 4-6. Figure 4-6A shows the distribution of vesicular dopamine following administration of reserpine to the mouse for 12 h (green) prior to sacrifice, and 6 h (purple) prior to sacrifice. At the 6-h treatment time period, the distribution is shifted to lower average amounts (50 ± 0.4 zmol) than control (55 ± 0.4 zmol), but higher amounts than the 12-h treatment (20 ± 0.7 zmol). Interestingly, the distribution for the 6-h treatment appears to be skewed to the left when compared to control, possibly indicating that one type of vesicle is preferentially depleted by reserpine at the shorter time scale. The average number of vesicular dopamine molecules quantified for the reserpine-treated mice was 12,000 ± 500 for the 12-h treatment and 30,000 ± 400 for the 6-h treatment. The treatments were statistically significant from each other and control (one-way ANOVA, $p < 0.001$; Error is SEM) as shown in Figure 4-7.

The administration of L-DOPA at two different times was also investigated and vesicular dopamine distributions are plotted in Figure 4-6C. Here, the mice treated for 0.5 h (gray) prior to sacrifice are compared to those treated for 2 h (red). The mean vesicular dopamine amount following 0.5 h of L-DOPA is less (84 ± 0.4 zmol) than that following 2-h treatment (180 ± 1.1 zmol), and both treatments are greater than control (55
Figure 4-6. Pharmacological treatment at two different time periods results in different vesicular neurotransmitter content in mouse synaptic vesicles as measured by electrochemical cytometry. (A) Normalized frequency histogram for reserpine-treated (20 mg/kg) striatal vesicles where mice were sacrificed 12 h (green) and 6 h (purple) after treatment. Mean number of dopamine molecules was 12,000 and 30,000 molecules for the 12- and 6-h reserpine treatments, respectively. The mean of each distribution was 2.4 zmol$^{1/3}$ and 3.5 zmol$^{1/3}$ for the 12- and 6-h treatments, respectively. (B) Normalized frequency histogram for untreated synaptic vesicles (average molecules = 33,000, distribution mean = 3.7 zmol$^{1/3}$). (C) Normalized frequency histograms for L-DOPA-treated (50 mg/kg) striatal vesicles where mice were sacrificed 0.5 h (gray) and 2 h (red) after treatment. Mean number of dopamine molecules was 51,000 and 109,000 molecules for the 0.5- and 2-h L-DOPA treatments, respectively. The mean of each distribution was 4.1 zmol$^{1/3}$ and 5.5 zmol$^{1/3}$ for the 0.5- and 2-h treatments, respectively. Data plotted as the cube root transform. Bin size = 0.2 zmol$^{1/3}$. Fits were obtained from a Gaussian distribution of the data. Average amounts, n values, and peak characteristics for these data are presented in Table 4-3.
Figure 4-7. Summary comparison of mouse striatal vesicles treated to alter neurotransmitter content measured by electrochemical cytometry. Statistical mean for average number of molecules of neurotransmitter per vesicle is plotted. Error is SEM. All treatment values are statistically different from each other (one-way ANOVA, $p = 0.001$). Average amounts, number of events, and peak characteristics for these data are presented in Table 4-3.
± 0.4 zmol). The L-DOPA treatments resulted in an average of 51,000 ± 200 molecules for the 0.5-h treatment and 109,000 ± 700 vesicular dopamine molecules for the 2-h treatment. The L-DOPA treatments were statistically significant from each other and control (one-way ANOVA, \( p < 0.001 \); Error is SEM), as shown in Figure 4-7. Results that include the average number of molecules, the distribution means, peak characteristics, and the number of events for these and the reserpine treatments are summarized in Table 4-3. Overall, these data demonstrate that the time dependent element of treatment for altering vesicular dopamine in the striatum can be monitored and quantified at single synaptic vesicles using electrochemical cytometry.

**Electrochemical Cytometry: Effect of the Psychostimulant Amphetamine on Vesicular Content in the Mouse Striatum**

Electrochemical cytometry can be used to resolve vesicular transmitter levels from synaptic vesicles of the mouse striatum and these values can be augmented by treating mice with pharmacology known to increase and decrease these levels. This can be expanded to determine the effect of drugs of abuse on vesicular dopamine in the striatum. Amphetamine is a psychostimulant that has been reported to both alter vesicular quantal size intracellularly and act on the cellular transmembrane dopamine transporter \( (46, 47) \). Jones and Wightman evaluated extracellular release from the striata of dopamine transporter knockout mice. They showed that extracellular dopamine was elevated upon treatment with amphetamine for wild-type mice, but not for mice lacking the transporter. In a separate study, Ewing, Sulzer, and coworkers reported on the mechanism of amphetamine by performing stimulated release experiments at PC12 cells and at the dopaminergic nerve of *Planorbis corneus* \( (46) \). Interestingly, amphetamine
was shown to cause an increase in extracellular dopamine from *Planorbis*, but a decrease in vesicular quantal size (to < 50%) from PC12 cells. These data, in combination with whole cell CE measuring cytosolic dopamine levels, supported the hypothesis that the actions of amphetamine cause the vesicle to redistribute dopamine to the cytosol.

To investigate the vesicular contribution to this hypothesis, I carried out electrochemical cytometry on striatal vesicles from mice that were injected (i.p.) with 10 mg/kg (+)-amphetamine for 1 h prior to sacrifice. Amphetamine-treated mice yielded an average vesicular dopamine content of 22 ± 0.3 zmol, which is equal to 13,000 ± 200 molecules per vesicle (Error is SEM). This amount is statistically significant from the average vesicular dopamine amount for control mice shown in Table 4-3 (one-way ANOVA, *p* < 0.001). The electrochemical cytometry results from amphetamine-treated mice striatal vesicles are plotted as a normalized frequency histogram of vesicular amounts (cube root) against control striatal vesicles in Figure 4-8. Amphetamine treatment was shown to reduce the average quantal content of striatal dopaminergic vesicles. Average amounts and peak characteristics for these data are listed in Table 4-3.

The data elucidated from the electrochemical cytometry measurements of vesicular content from amphetamine-treated mice may be supported by the the weak base theory (48-50), a hypothesis which contends that a number of psychoactive drugs (e.g., amphetamine, haloperidol, morphine, nicotine, tyramine, desipramine, etc.) are lipophilic weak bases that can disrupt the homeostasis of vesicular proton gradients activated in vesicular neurotransmitter loading (48). Indeed, most of these molecules have pK values between 8-11 at physiological pH (7.4), while the synaptic vesicle has a resting pH of ~5.5 (49). Therefore, it has been proposed weak bases in the cytosol (e.g., amphetamine)
Figure 4-8. Electrochemical cytometry as a high-throughput approach to directly quantify neurotransmitter levels altered from exposure to drugs of abuse at the single vesicle level. Mice were treated with (+)-amphetamine (10 mg/kg i.p.), sacrificed after 1 h, and their striata dissected for vesicle isolation. Plotted are normalized frequency histograms of vesicular quanta for amphetamine-treated mice (gray) versus untreated mice (blue). Mean amounts of dopamine molecules were 13,000 and 33,000 for the amphetamine-treated and untreated mice, respectively. The distribution means were 2.7 zmol^{1/3} and 3.7 zmol^{1/3}, for the amphetamine-treated and untreated mice, respectively. Data plotted as the cube root transform. Bin size = 0.2 zmol^{1/3}. Fits were obtained from a Gaussian distribution of the data. Average amounts, number of events, and peak characteristics for these data are presented in Table 4-3.
Table 4-3. Electrochemical cytometry summary for the average vesicular peak characteristics and quantified amounts of neurotransmitter from mouse striata exposed to various drugs that alter dopamine levels in the brain. Error is SEM. Distribution fit to a single Gaussian (All with $r^2 > 0.96$).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>n</th>
<th>Halfwidth (ms)</th>
<th>Amplitude (pA)</th>
<th>Vesicular Transmitter Amount (zmol)</th>
<th># Molecules of Transmitter</th>
<th>Distribution Mean (zmol$^{1/3}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>12 h Reserpine</td>
<td>1,011</td>
<td>18 ± 0.05</td>
<td>0.2 ± 0.001</td>
<td>20 ± 0.7</td>
<td>12,000 ± 500</td>
<td>2.4</td>
</tr>
<tr>
<td>6 h Reserpine</td>
<td>3,029</td>
<td>20 ± 0.03</td>
<td>0.2 ± 0.001</td>
<td>50 ± 0.4</td>
<td>30,000 ± 400</td>
<td>3.5</td>
</tr>
<tr>
<td>Untreated</td>
<td>20,331</td>
<td>19 ± 0.04</td>
<td>0.5 ± 0.003</td>
<td>55 ± 0.4</td>
<td>33,000 ± 300</td>
<td>3.7</td>
</tr>
<tr>
<td>0.5 h L-DOPA</td>
<td>23,700</td>
<td>20 ± 0.02</td>
<td>0.7 ± 0.002</td>
<td>84 ± 0.4</td>
<td>51,000 ± 200</td>
<td>4.1</td>
</tr>
<tr>
<td>2 h L-DOPA</td>
<td>8,712</td>
<td>21 ± 0.05</td>
<td>1.6 ± 0.009</td>
<td>180 ± 1.1</td>
<td>109,000 ± 700</td>
<td>5.5</td>
</tr>
<tr>
<td>1 h Amphetamine</td>
<td>2,939</td>
<td>16 ± 0.04</td>
<td>0.3 ± 0.001</td>
<td>22 ± 0.3</td>
<td>13,000 ± 200</td>
<td>2.7</td>
</tr>
</tbody>
</table>
can compete with endogenous neurotransmitter to accumulate into the vesicle lumen, buffer the intravesicular neurotransmitter charge, and cause the neutralized neurotransmitter to seep from the vesicle out to the cytosol (48). This results in an overall reduction of vesicular neurotransmitter quantal content and, therefore, an augmentation of the pre-synaptic chemical signal. In future studies, electrochemical cytometric methods can be used to further study the actions of amphetamine, as well as, alternative drugs of abuse to quantify their contribution to vesicular neurotransmitter loading and depletion.
**Conclusions**

Electrochemical cytometry has been used to quantify the total vesicular dopamine content from mouse midbrain SSVs in a high-throughput manner that is independent of the processes associated with exocytotic release in the CNS. Additionally, this methodology has been applied towards monitoring the effects of various drugs on synaptic vesicle transmitter loading and depletion. Mice have been injected with various pharmacological agents to alter vesicular neurotransmitter levels, and individual synaptic vesicles extracted from the striata of these mice have been quantified with the electrochemical cytometry platform. Finally, electrochemical cytometry has served to directly quantify vesicular dopamine depletion in the mouse striatum following administration of the psychostimulant amphetamine.
References


CHAPTER 5
Development and Characterization of a Modified Voltammetric Carbon-fiber Microelectrode Sensor for Monitoring pH Fluctuations Associated with in vivo Neurotransmission*

Introduction

Recently, there has been an interest in developing reagentless sensors to detect small pH changes in non-ideal environments (1). Carbon-based sensing materials have been attractive substrates for this application since they are intrinsically biocompatible, conductive, and apt for surface modification. Indeed, ion-selective reporter molecules can be tethered onto a carbon surface through a variety of methods including chemical oxidation of the surface with corrosive acidic cocktails and plasma treatment (2, 3), physical adsorption of organic precursors (4, 5), and electrochemically-assisted covalent attachment via the oxidation of amines (6-9) or the reduction of diazonium salts (10-16). Pioneered by Savéant and co-workers in the early 1990s, the electrochemical reduction of aryl diazonium salts onto carbon surfaces is a well-characterized method for the selective in situ attachment of organic moieties (13). The mechanism involves the electrochemical generation of a solution radical from the diazonium modifier and subsequent covalent linkage to the carbon surface, which has been shown to possess marked stability to external stimuli (14).

Electrochemical measurements in the central nervous system (CNS) can be used to quantify redox-active chemical messengers such as catecholamines and indolamines, which are thought to play a fundamental role in the physiological and behavioral aspects of an organism. In vivo voltammetry at carbon-fiber microelectrodes has been used since

*Part of this chapter was reproduced with permission from an article authored by MA Makos, DM Omiatek, AG Ewing, and ML Heien, Langmuir, in press. © 2010 American Chemical Society.
the early 1970s to monitor chemical neurotransmission of these species in the CNS of various mammalian animal models (17-19). Neurosecretory events are often accompanied by a flux of endogenous species (e.g., H⁺, ascorbate, etc.) which can interfere with the voltammetric signature of targeted electroactive chemical messengers (5, 19-26). Of particular interest are pH fluctuations in the surrounding matrix, which are thought to occur as a result of metabolic processes that follow stimulated neurotransmitter release (20, 27-29). Indeed, Wightman and co-workers have measured small acidic pH changes in rat brain slices subjected to electrically stimulated secretion with liquid membrane ion-selective microelectrodes (ISMs) (20). With the emergence of new volume-limited, CNS-containing animal models such as the fruit fly, Drosophila melanogaster, comes the need to develop microanalytical tools capable of measuring pH fluctuations associated with neurotransmission (30). Makos et al. have reported on a system for performing in vivo electrochemical measurements in the CNS of this exquisitely small animal model using fast-scan cyclic voltammetry (FSCV) (31, 32). Herein I describe the development and characterization of a reagentless, biocompatible modified voltammetric carbon-fiber microelectrode sensor for measuring dynamic physiological pH changes associated with in vivo neurotransmission in the Drosophila CNS.

Voltammetric pH-sensors measure changes in the redox-potential of a surface-bound electroactive species as a function of pH. This methodology for measuring pH has been demonstrated with quinone-based surface modification of various electrodes (33-36). In a recent study by Tommos and co-workers, the formal potential of a surface-bound quinone on a gold electrode shifted to more negative potentials with increasing
solvent basicity (33). Whereas a variety of quinone-modified electrodes have been reported to respond to pH, few have been developed on biocompatible materials that exhibit activity in a physiologically relevant pH range (1, 37). In this work I discuss a collaborative effort to electrochemically graft a variety of quinone-containing diazonium derivatives onto carbon-fiber microelectrode sensors to probe pH alterations. Modification of the carbon surface with Fast Blue RR (FBRR), a commercially available diazonium salt, resulted in a sensor capable of performing real-time, reagentless pH measurements in biological microenvironments. The redox response of the FBRR-functionalized electrode was characterized using FSCV in biological media set to a physiologically relevant pH range. Following in vitro calibration of the sensor, the FBRR-modified voltammetric microelectrode was used to measure small pH fluctuations in the CNS of Drosophila elicited from an optogenetic stimulus.
**Materials and Methods**

**Reagents.** 4-Benzoylamino-2,5-dimethoxybenzenediazonium chloride hemi(zinc chloride) salt (Fast Blue RR, FBRR, diazonium salt), 4-nitrobenzenediazonium tetrafluoroborate, gallic acid, 3,4-dihydroxyphenylacetic acid (DOPAC), homogentisic acid, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC, coupling agent) hydroxybenzotriazole (HOBT), diisopropylethylamine (DIPEA, 99.5%), tetraethylammonium tetrafluoroborate (TEABF₄), NaCl, KCl, CaCl₂, MgCl₂, NaHCO₃, NaH₂PO₄, sucrose, tris(hydroxymethyl)amino-methane (Trizma base®, and acetonitrile (ACN, anhydrous, 99.8%) were obtained from Sigma Aldrich (St. Louis, MO). Ferrocenecarboxylic acid and trehalose were obtained from Fluka BioChemika (Buchs, Switzerland). Fast Red AL salt was obtained from Acros Organics (Geel, Belgium). All chemicals were used as received. Adult hemolymph like (AHL) saline (108 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 8.2 mM MgCl₂, 4 mM NaHCO₃, 1 mM NaH₂PO₄, 5 mM trehalose, 10 mM sucrose, 20 mM Trizma base®, pH 7.5) was made using ultrapure (18 MΩ cm) water and filtered through a 0.2 µm filter (38). The pH of AHL solutions was adjusted with 0.5 M NaOH and HCl.

**Electrode Preparation.** Cylindrical carbon-fiber microelectrodes were fabricated as previously described (31). Briefly, a PAN-based 5 µm-diameter carbon fiber (T-40 12K, Amoco, Greenville, SC) was aspirated into a borosilicate glass capillary (1B100-4, World Precision Instruments, Inc., Sarasota, FL) and sealed using a regular glass capillary puller (P-97, Sutter Instruments, Novato, CA). The carbon fiber was trimmed to a length of 50 or 200 µm measured from the glass junction. Electrical contact was made by back-filling the capillary with a silver composition (4922N DuPont, Delta
Technologies Ltd., Stillwater, MN), followed by insertion of a tungsten wire, resulting in a 5 µm-diameter cylindrical carbon-fiber microelectrode. The 200-µm long cylindrical electrodes were used for all characterization experiments while the 50-µm long electrodes were used for the *in vivo* *Drosophila* applications.

**Chemical Modification of the Carbon-Fiber Microelectrode Surface.** For the development of Fast Blue RR and Fast Red AL electrodes, the commercially available diazonium salts were directly grafted onto the carbon-fiber microelectrode. Deposition of the diazonium onto the carbon-fiber microelectrodes was carried out using cyclic voltammetry on an Ensman Instruments EI400 microelectrode potentiostat (Bloomington, IN) operated in the two-electrode mode. A 2 mM solution of Fast Blue RR salt was prepared in acetonitrile containing 0.1 M TEABF₄. Solutions were purged with Ar (g) for 5 min prior to deposition in order to eliminate signal attributed to the reduction of O₂. Electrodes were electrochemically modified via reduction of the diazonium onto the carbon surface by scanning from +0.4 V to -0.8 V vs. Ag QRE (3 mm dia., Bioanalytical Systems, West Lafayette, IN) at 0.5 V/s. Data were collected and processed using LabView 8.0 software (National Instruments, Austin, TX) written in-house. Electrode surface coverage was calculated by subtracting the background current of a bare carbon-fiber microelectrode in 0.1 M TEABF₄/ACN from current attributed to the deposition of the diazonium. The typical surface coverage obtained using the experimental conditions listed above was ~20 nmol/cm².

For the fabrication of the ferrocenecarboxylic acid, DOPAC, homogentisic acid, and gallic acid modified sensors, 2 mM 4-nitrobenzenediazonium tetrafluoroborate was first electrochemically reduced onto a carbon-fiber surface by scanning from +0.4 V to
-0.8 V in 0.1 M TEABF₄/ACN at 0.5 V/s. The NO₂ moiety on the diazonium derivative was then electrochemically reduced to an NH₂ group by scanning from 0 V to -1.4 V in 0.1 M KCl dissolved in a 10% EtOH in H₂O. The reporter molecule was then linked to the NH₂ functionality through the formation of an amide bond using conventional coupling chemistry. The coupling chemistry cocktail was synthesized by reacting a carbodiimide coupling agent (e.g., EDC, 10 mM) with a carboxylic acid containing reporter molecule (e.g., DOPAC, 10 mM) in the presence of 10 mM hydroxybenzotriazole dissolved in 40 mM diisopropylethylamine (to activate the carboxylic acid-carbodiimide complex) at 0°C for 1 h. The NH₂-terminated diazonium electrode was then immersed in this solution and left to stir at room temperature for 24 hours. In this time period, the NH₂ group on the electrode is linked to the carboxylic acid on the reporter molecule through the formation of an amide bond, resulting in the various quinone-modified electrodes discussed later in the chapter.

**Electrochemical Measurements.** Voltammetric responses of the diazonium-modified electrodes as a function of pH were collected using either a Dagan Chem-Clamp potentiostat (Dagan Corporation, Minneapolis, MN) or a flow-injection analysis apparatus with a current amplifier (428, Keithley Instruments, Inc., Cleveland, OH). Both systems were run by the TH 1.0 CV program (ESA, Chelmsford, MA) (39) coupled with two data acquisition boards (PCI-6221, National Instruments). A Ag/AgCl electrode, which served as the reference in all experiments following the initial deposition of FBRR, was made by chloridizing a silver wire (0.25 mm diameter, 99.999% purity, Alfa Aesar, Ward Hill, MA). Electrodes were positioned using x,y,z-micromanipulators (421 series, Newport, Irvine, CA). All cyclic voltammograms were
obtained using a triangular waveform scanned from -0.7 to +0.8 V vs. Ag/AgCl at 20 V/s and repeated every 200 ms unless otherwise noted (FSCV data collected by MA Makos). Electrochemical responses were plotted and statistical analysis performed using Prism 5.0 (GraphPad Software, La Jolla, CA). Anodic peak potentials ($E_{pa}$) were determined using a fifth order polynomial fit from LabView 8.0 software written in-house. Cyclic voltammetry was used to estimate the heterogeneous electron-transfer rate constant, $k^0$, for this system via the method of Nicholson (40).

**In vivo Drosophila Preparation.** Female flies carrying Channelrhodopsin-2 (ChR2), a light activated ion channel, were crossed with male flies expressing tyrosine hydroxylase (TH) to produce mutant flies containing dopaminergic neurons that can be controlled through blue light stimulation (TH-GAL4/UAS:ChR2 genotype) (41). Male mutant flies, 3–7 days old, were maintained at 25 °C in the dark and fed yeast containing 10 mM all trans-Retinal (light sensitive chemical necessary for ChR2 function) for 2 days prior to experimentation. Blue light was applied through computer control of a 3 watt Luxeon Star LED with a peak intensity of ~470 nm (LXHL-LB3C, Newark, Chicago, IL). Flies were prepared as previously described for in vivo FSCV measurements (31). Briefly, ice was used to temporarily immobilize flies before they were mounted in a homemade collar (38.1 mm diameter concave plexiglass disk with 1.0 mm hole in center) with low melting agarose (Fisher Scientific, Pittsburgh, PA). Microsurgery was performed on a stereoscope (Olympus SZ60, Melville, NY) to remove the cuticle from the top portion of the head, thus exposing the brain region. The head was covered with 0.1% collagenase solution for 30 min to relax the extracellular matrix in the brain then
rinsed and bathed with AHL saline with the preparation maintaining its viability for 1.5 - 2.5 h (*Fly surgery performed by MA Makos*).
Results and Discussion

Cyclic Voltammetry Deposition of the Diazonium Salt onto a Carbon-fiber Microelectrode Surface

FBRR was electrochemically grafted onto a carbon-fiber surface using cyclic voltammetry by scanning from +0.4 V to -0.8 V vs. Ag QRE at a rate of 0.5 V/s in a 2 M FBRR/0.1 M TEABF₄ –supported ACN. The proposed mechanism for this reaction is presented in Scheme 5-1. A representative voltammogram of the diazonium salt reduction onto a cylindrical carbon-fiber microelectrode is shown in Figure 5-1A (blue trace). An irreversible reductive wave is observed around -0.5 V which was attributed to the solution radical formation of the diazonium derivative and its subsequent covalent linkage to the carbon-fiber surface, as reported for a similar molecule (12).

The charge \( Q \) of the diazonium deposited onto the surface is quantified from the current-time integral of the voltammetric trace. The small charge observed from the solvent background (black trace) has been subtracted from the charge due to diazonium deposition (blue trace). Faraday’s Law \( Q = nNF \) was used to convert \( Q \) to the corresponding number of moles of diazonium \( N \) deposited onto the carbon-fiber surface. In this equation the number of electrons exchanged in the reduction reaction, \( n \), is 1, and \( F \) is Faraday’s constant (96,485 C/mol). The surface coverage of diazonium on the electrode is calculated by dividing the number of moles of FBRR by the geometric area of the 200 μm cylinder \( (3.2 \times 10^{-5} \text{ cm}^2) \). This results in a typical coverage of 20 nmol/cm². As a point of reference, typical monolayer coverage for a surface-bound small organic molecule has been reported as 300 pmol/cm² (42). Therefore, this suggests a multilayer deposition of FBRR onto the sensor presented here, a result commonly observed for the reduction of aryl diazonium salts onto carbon surfaces (14). The amount
Scheme 5-1. Proposed mechanism for the electrochemical reduction of the diazonium salt FBRR onto the carbon-fiber surface.
Figure 5-1. Cyclic voltammograms of a carbon-fiber microelectrode before and after FBRR attachment. (A) Cyclic voltammogram of the electrochemical reduction and subsequent covalent attachment of FBRR onto the carbon-fiber surface. Background charge of the bare carbon-fiber electrode in solvent only (black trace). Reduction of FBRR at the carbon-fiber surface (blue trace). Diazonium concentration = 2 mM in 0.1 M TEABF$_4$/ACN. Potential window = +0.4 V to -0.8 V vs. Ag QRE. Scan rate = 0.5 V/s. (B) Cyclic voltammograms (average of 5 scans each) of a bare carbon-fiber microelectrode (dashed black line) and a carbon-fiber microelectrode modified with FBRR (solid blue line) in AHL saline. Potential window = -0.7 V to +0.8 V vs. Ag/AgCl. Scan rate = 20 V/s.
of FBRR deposited onto the carbon-fiber surface (Table 5-1) is dependent on both the scan rate and potential window of the voltammetric sweep. The voltammetric deposition of the diazonium is a time-dependent process; therefore, scanning at slower rates or to an extended negative waveform potential increases the amount of FBRR deposited onto the electrode surface. Interestingly, there is no significant effect in varying the concentration of diazonium in solution in increments from 0.5 to 5 mM on the resultant amount deposited onto the electrode (data not shown).

The presence of FBRR on the surface was confirmed using FSCV. In Figure 5-1B, cyclic voltammograms recorded at a bare carbon-fiber microelectrode (dashed black line) and the same microelectrode following modification with FBRR (solid blue line) show a clear indication of the presence of the electroactive diazonium salt on the electrode surface \( \nu = 20 \text{ V/s} \). The voltammogram of the FBRR redox system signifies quasireversible behavior with an apparent redox potential of -0.1 V in AHL saline at physiological pH \( \text{(pH 7.5)} \). Integration of the oxidative peak area from the redox-active molecule in Figure 5-1B results in an observed surface coverage of 40 pmol/cm\(^2\). This is approximately two orders of magnitude smaller than that calculated from the diazonium deposition in Figure 5-1A. A proposed mechanism for the oxidation-reduction reaction of the surface-bound quinone derivative is listed in Scheme 5-2. It is thought that voltammetric interrogation of this molecule initially induces a two-electron/two-proton oxidation to convert the \( p \)-methoxy moiety on the conjugated ring to its \( p \)-quinone analogue. The quinone is then chemically reduced in a two-electron exchange to form the hydroxy derivative of the molecule. Using the method of Nicholson, (40) the heterogeneous electron-transfer rate constant, \( k^o \), was determined to be 0.13 cm/s. This
Table 5-1. Effect of varying voltammetric deposition parameters of the FBRR on the carbon-fiber surface. [FBRR] = 2 mM in 0.1 M TEABF$_4$/ACN. $n = 3$ electrodes for each measurement. Error is SEM.

<table>
<thead>
<tr>
<th>Scan Rate (V/s)</th>
<th>Potential Window (V vs. Ag/AgCl)</th>
<th>$\Gamma$ (nmol/cm$^2$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.500</td>
<td>+0.4 $\rightarrow$ -0.2</td>
<td>2.7 $\pm$ 0.9</td>
</tr>
<tr>
<td>0.500</td>
<td>+0.4 $\rightarrow$ -0.4</td>
<td>9.9 $\pm$ 3.5</td>
</tr>
<tr>
<td>0.500</td>
<td>+0.4 $\rightarrow$ -0.6</td>
<td>14.5 $\pm$ 1.4</td>
</tr>
<tr>
<td>0.500</td>
<td>+0.4 $\rightarrow$ -0.8</td>
<td>21.5 $\pm$ 2.7</td>
</tr>
<tr>
<td>0.500</td>
<td>+0.4 $\rightarrow$ -1.0</td>
<td>25.0 $\pm$ 2.7</td>
</tr>
<tr>
<td>0.050</td>
<td>+0.4 $\rightarrow$ -0.8</td>
<td>29.4 $\pm$ 6.4</td>
</tr>
<tr>
<td>0.100</td>
<td>+0.4 $\rightarrow$ -0.8</td>
<td>24.4 $\pm$ 1.2</td>
</tr>
<tr>
<td>0.500</td>
<td>+0.4 $\rightarrow$ -0.8</td>
<td>20.0 $\pm$ 2.1</td>
</tr>
<tr>
<td>1.0</td>
<td>+0.4 $\rightarrow$ -0.8</td>
<td>19.7 $\pm$ 0.6</td>
</tr>
<tr>
<td>5.0</td>
<td>+0.4 $\rightarrow$ -0.8</td>
<td>9.9 $\pm$ 1.4</td>
</tr>
</tbody>
</table>
Scheme 5-2. Proposed mechanism for the quinone moiety oxidation-reduction reaction on the surface bound FBRR-modified carbon-fiber electrode sensor.
indicates that the FBRR undergoes outer-sphere electron transfer on the carbon-fiber surface, which is consistent with previous studies that have examined electron transfer kinetics over a wide insulating layer (43).

It has been demonstrated in Figure 5-1 that the FBRR-modified electrodes were well suited to operate under conditions applicable for measuring in vivo pH fluctuations in biological systems. Indeed, these sensors have displayed a stable and resolvable electrochemical signature in solutions of relevant physiological pH (AHL saline, pH 7.4) and have been shown to withstand fast scan rates ($\nu = 20 \text{ V/s}$) necessary to monitor dynamic changes associated with neurosecretion. To investigate whether these effects could be extrapolated to a number of surface bound formulations, a variety of reporter molecules were tethered to carbon fiber microelectrodes and probed for redox activity in solutions of varying pH as demonstrated in Table 5-2. Modification procedures are detailed in Materials and Methods. Although each of these modified electrodes displayed redox activity, none did so under conditions that would be relevant to measure dynamic pH changes of biological systems in vivo (e.g., displayed resolvable peaks at high scan rates and in physiological media). Therefore, the FBRR electrode was chosen for further characterization and eventual application for in vivo pH measurements in Drosophila (vide infra).

**Electrochemical Characterization of the FBRR Microelectrode pH Sensor**

The effect of scan rate on the electrochemistry of a FBRR-modified carbon-fiber microelectrode has been investigated using FSCV. Cyclic voltammograms of a FBRR microelectrode in pH 7.5 AHL saline solution at scan rates of 10, 20, and 50 V/s are plotted in Figure 5-2A. Because current is directly proportional to scan rate, the current
<table>
<thead>
<tr>
<th>Reporter Molecule</th>
<th>Structure</th>
<th>Representative Cyclic Voltammogram</th>
<th>Limitations as a Sensor for pH Measurements in the CNS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ferrocene Carboxylic Acid</td>
<td><img src="image" alt="Structure" /></td>
<td><img src="image" alt="Voltammogram" /></td>
<td>pH 7.4 $v = 0.05$ V/s Poor peak resolution at $v &gt; 0.1$ V/s.</td>
</tr>
<tr>
<td>DOPAC (3,4-dihydroxyphenyl acetic acid)</td>
<td><img src="image" alt="Structure" /></td>
<td><img src="image" alt="Voltammogram" /></td>
<td>pH 3 $v = 0.05$ V/s Poor peak resolution at pH &gt; 4 and $v &gt; 0.1$ V/s.</td>
</tr>
<tr>
<td>Homogentisic Acid</td>
<td><img src="image" alt="Structure" /></td>
<td><img src="image" alt="Voltammogram" /></td>
<td>pH 3 $v = 0.2$ V/s Poor peak resolution at pH &gt; 4 and $v &gt; 0.5$ V/s.</td>
</tr>
<tr>
<td>Gallic Acid</td>
<td><img src="image" alt="Structure" /></td>
<td><img src="image" alt="Voltammogram" /></td>
<td>pH 7 $v = 5$ V/s Poor peak resolution at $v &gt; 5$ V/s.</td>
</tr>
<tr>
<td>* Fast Red AL</td>
<td><img src="image" alt="Structure" /></td>
<td><img src="image" alt="Voltammogram" /></td>
<td>pH 11 $v = 0.05$ V/s Poor peak resolution at pH &lt; 9.</td>
</tr>
</tbody>
</table>

**Table 5-2.** Various reporter molecules investigated for the fabrication of a modified pH voltammetric carbon-fiber microelectrode sensor. The molecules investigated possess surface-bound quinone redox activity similar to the mechanism in Scheme 5-2 (with the exception of the ferrocene carboxylic acid). Experimental limitations are listed to explain why these molecules are non-ideal for measuring pH fluctuations associated with *in vivo* neurotransmission. The reporter molecules were linked to a surface bound diazonium through the formation of an amide bond using conventional coupling chemistry (details in Materials and Methods). *Fast Red AL was directly grafted onto the carbon-fiber surface and required no additional synthetic steps to probe redox activity (similar to FBRR).
Figure 5-2. Electrochemical characterization of the FBRR microelectrode pH sensor in pH 7.5 AHL saline solution. (A) Cyclic voltammograms (average of 5 scans each) of the FBRR redox couple at 3 scan rates. The current scale on the y-axis has been divided by scan rate so the peak positions at the different scan rates can easily be compared. In this scan rate range, neither the anodic peak potential ($E_{pa}$) nor the cathodic peak potential ($E_{pc}$) significantly shifts in value. (B) The effect of continuous cycling of the electrode on anodic peak current ($i_{pa}$). Error bars are SEM ($n = 3$).
scale on the y-axis has been divided by scan rate to allow for comparison of the peak positions at the different scan rates. Notably, neither the anodic peak potential ($E_{pa}$) nor the cathodic peak potential ($E_{pc}$) significantly shifts in value with varying scan rate in this range. At scan rates higher than 100 V/s (up to 350 V/s), the $E_{pa}$ becomes more difficult to identify due to a decrease in the ratio of the faradaic to the capacitive current. By inspection, the $E_{pa}$ is well resolved from the background current at 20 V/s, a scan rate that should suffice for monitoring rapidly occurring neurosecretory events during *in vivo* applications. Therefore, this scan rate has been chosen to monitor pH changes in the remainder of this study. Furthermore, the anodic peak current vs. $\nu$ (data not shown) is linearly dependent for scan rates 10 – 350 V/s ($r^2 > 0.99$). This confirms that the oxidation and reduction of FBRR is a surface-confined reaction, as expected, and provides evidence that the diazonium compound is sufficiently tethered to the carbon-fiber surface.

The long-term stability of the FBRR microelectrode pH sensor was studied by continuously cycling modified electrodes in pH 7.5 AHL saline solution for 2.5 h (-0.7 to +0.8 V vs. Ag/AgCl at 5 Hz with scan rate = 20 V/s). This corresponds to 45,000 voltammetric sweeps over the 2.5 h period. An 8% decrease in peak current is observed during the first 10 min of cycling (Figure 5-2B). During the remaining 2.5 h, the peak current remains fairly stable, decreasing an additional 13%. Therefore, the stability of the FBRR-modified microelectrode provides an ample time window for monitoring the pH in the CNS of *Drosophila* during *in vivo* electrochemistry measurements.

The selectivity of the sensor for H$^+$ has been investigated to determine if alternate ionic species present in the biological media interfere with the voltammetric response.
To accomplish this, FBRR microelectrodes \((n = 3)\) have been tested with FSCV in a series of AHL saline solutions that contained elevated concentrations of various inorganic cations. When the \(\text{Na}^+\) concentration in the first AHL saline solution is increased by 40\%, the \(E_{\text{pa}}\) remains unaltered. In addition, increasing the \(\text{Mg}^{2+}\) concentration by 45\%, \(\text{Ca}^{2+}\) concentration by 50\%, or \(\text{K}^+\) concentration by 60\% does not cause a shift in the \(E_{\text{pa}}\). These studies validate that changes in the concentration of these four cations do not contribute to the shift measured in the \(E_{\text{pa}}\), which suggests charged species, other than \(\text{H}^+\) ions, in the AHL saline solution are not affecting the voltammetric pH response of the FBRR microelectrode.

**pH Response of Microelectrode Sensor Deposited with FBRR**

To calibrate the voltammetric response of the sensor, the FBRR-modified carbon-fiber microelectrode has been investigated in AHL saline solutions of varying pH. The peak characteristics of cyclic voltammograms recorded over a pH range of 5.0 – 9.0 with a scan rate of 20 V/s have been examined. Figure 5-3 depicts representative voltammograms for the redox behavior of the FBRR microelectrode in three different pH solutions. The \(E_{\text{pa}}\) noticeably shifts to more negative potentials as pH is increased. The \(E_{\text{pc}}\) follows the same trend with pH as the \(E_{\text{pa}}\); however, the peak becomes difficult to distinguish from the background current in higher pH solutions (≥ pH 8), as reported previously for chemically modified electrodes in physiological media (5). Therefore, the \(E_{\text{pa}}\) was chosen as the identifier for the sensor calibration and subsequent in vivo studies instead of the \(E_{1/2}\).

FSCV has been used to determine the response of the sensor to pH changes. In a pH range of physiological relevance (6.5 – 8.0), the \(E_{\text{pa}}\) varies linearly with pH (Figure 5-
Figure 5-3. Cyclic voltammograms of a microelectrode modified with FBRR in AHL saline solutions of different pH measured with scan rate = 20 V/s. (*) corresponds to the $E_{pa}$ for each voltammogram (average of 5 scans) with the dashed vertical line included for comparison. As the pH increases, the $E_{pa}$ visibly shifts to more negative potentials. (A) pH 6.5  (B) pH 7.5  (C) pH 8.0.
4, \( n = 9 \) electrodes). The calibration plot has a slope of 38 mV/pH unit which is less than the theoretical value of 59 mV/pH unit for a reversible, two-electron/two-proton redox reaction at room temperature (44). This deviation from the predicted Nernstian value suggests the attachment of the quinone couple to the carbon-fiber surface altered the electrochemistry of the FBRR. pH-sensitive, glassy carbon electrodes modified with alternative reporter molecules have been previously fabricated that exhibit expected Nernstian behavior, but practical limitations, such as high capacitive currents, larger diameters (millimeter), and lengthy time scales to obtain stable readings, have limited their biological usefulness (35,36). For example, Shiu et al. have reported the development of a glassy carbon electrode (3-mm diameter) modified by adsorption of an anthraquinonesulfonate film that possessed a near Nernstian slope of 56.4 mV/pH unit in aqueous pH buffers (35). However, it would not be feasible to use an electrode of this size to measure dynamic events associated with \textit{in vivo} neurosecretion in volume-limited model systems such as \textit{Drosophila}.

**Microelectrode Response Time to a pH Change**

Flow-injection analysis has been used to study the dynamic response of the sensor by introducing plugs of AHL saline solution of varying pH to a FBRR-modified microelectrode. Ideally, a fast electrode response time to a minor change in pH of the surrounding solution would produce a square-shaped \( E_{pa} \) vs. time trace. Figure 5-5 shows the \( E_{pa} \) response of the modified electrode sensor to 0.2 pH unit changes. After initial immersion in an AHL saline solution of pH 7.4, the electrode is exposed to a bolus of AHL saline solution of pH 7.2 (Figure 5-5A). Likewise, AHL solution of pH 7.6 is introduced to the electrode in pH 7.4 solution in Figure 5-5B. By inspection, the
Figure 5-4. The anodic peak potential, $E_{pa}$, as a function of AHL saline solution pH for FBRR-modified electrodes. The $E_{pa}$ varies linearly with pH in a physiological relevant pH range (6.5-8.0). Error bars are SEM ($n = 9$ electrodes).
Figure 5-5. Plot of $E_{pa}$ vs. time during flow injection changes past the electrode of 0.2 pH units in AHL saline. The electrode is able to consistently measure either an acidic or a basic pH change. (A) Initial AHL saline solution of pH 7.4 is decreased to pH 7.2. (B) Initial AHL saline solution of pH 7.4 is increased to pH 7.6.
modified electrode response to a 0.2 change in pH is square-like and consistent for measurement of either an acidic or a basic pH change. Indeed, flow injection calibration of the sensor revealed marked sensitivity for H^+, capable of detecting pH changes as small as 0.005 (based on S/N > 3) with a time response equal to 1.6 s (τ determined from exponential decay).

Measuring dynamic in vivo pH Changes in the Drosophila CNS

Makos et al. have developed a method for placing a carbon-fiber microelectrode into the CNS of *Drosophila* to electrochemically measure neurotransmitters *in vivo* with FSCV (31, 32). Here, the pH electrode described in this chapter was used to monitor a dynamic pH change associated with neurotransmitter release in the fruit fly brain. Blue light stimulation has been used on the mutant fly TH-GAL4/UAS:ChR2 to evoke dopamine neurosecretion as demonstrated previously in *Drosophila* larvae by Venton and co-workers (45). This mutant expresses blue light sensitive cation channels which are specific to dopaminergic neurons, allowing dopamine release to be controlled through timed blue light stimulations. Following microsurgery, a micromanipulator is used to insert the cylindrical FBRR-modified electrode into the CNS region of an adult mutant fly. Blue light stimulation was used to induce neurotransmitter release and a change in $E_{pa}$, was measured which corresponds to an ~0.034 acidic pH change in the mutant fly following a 5 s stimulation with blue light (Figure 5-6, solid red line). This value is in agreement with pH fluctuations observed as a result of stimulus-coupled secretion in rat brain slices (0.047 unit pH change in the cortex) reported by the Wightman lab using ISMs (20). To ensure the response is due to a biological change in the fly, the experiment has been repeated with the electrode in the surrounding solution outside of
Figure 5-6. Physiological pH measurements in adult *Drosophila* CNS. A representative trace of a dynamic, acidic pH change associated with neurotransmitter release is measured in a mutant fly CNS (solid red line) and plotted along with control stimulation of electrode in AHL saline solution only (solid black line). The black arrow corresponds to a 5-s stimulation with blue light.
the fly brain (solid black line). This experiment demonstrated the high temporal sensitivity of the FBRR sensor, highlighting its utility for real-time analyses of pH fluctuations associated with neurosecretion in emerging volume-limited biological microsystems.
**Conclusions**

A carbon-fiber microelectrode pH sensor was developed via the voltammetric reduction of the diazonium salt FBRR. The stability and sensitivity of the sensor for $H^+$ was characterized in biological media set to physiologically relevant pH ranges. FSCV was used to probe the surface-bound diazonium derivative as a function of pH. The peak corresponding to $E_{pa}$ for the FBRR-modified electrode was correlated to small changes in pH. Flow-injection analyses were used to characterize the temporal response of the sensor for solutions of varying pH, resulting in a limit of detection to 0.005 pH units. Furthermore, direct *in vivo* measurements of pH were carried out in the *Drosophila* CNS after stimulated neurotransmitter release, revealing an acidic change in a brain region dominated by dopaminergic nerve innervations. These data demonstrate the utility of this easily fabricated sensor for measuring dynamic changes in extracellular pH in this and other new emerging microanalytical animal models.
References


44. Laviron, E. (1983) Electrochemical reactions with protonations at equilibrium: Part VIII. The 2 e, 2H+ reaction (nine-member square scheme) for a surface or for a heterogeneous reaction in the absence of disproportionation and dimerization reactions, *J. Electroanal. Chem.* 146, 15-36.

CHAPTER 6

Electrochemical Cytometry: Conclusions and Future Bioanalytical Applications of a Novel Technology*

Introduction

The development of a new technology, electrochemical cytometry, has been discussed throughout much of this thesis for the high-throughput quantitative investigation of individual artificial, secretory cell, and neuronal synaptic vesicles. This novel method has been used for various applications including the characterization of a liposome suspension in Chapter 2, determination of the fraction of vesicular transmitter released during exocytosis in Chapter 3, and quantification of endogenous and pharmacologically manipulated vesicular dopamine from mouse primary neurons in Chapter 4. Here, conclusions from these studies and possible future applications for the electrochemical cytometry method are outlined. The first few applications involve elements that focus on the technical aspects of the measurement to expand upon separation and detection capabilities of electrochemical cytometry. The latter concepts involve specific applications to liposome research and neuroscience that can be investigated by electrochemical cytometry to quantitatively characterize of volume-limited submicron vesicles.

Understanding the Mechanism of Separation by Electrochemical Cytometry

In Chapter 2, I discussed how a single amperometric peak on an electrochemical cytometry electropherogram can be used to quantify vesicular content. Poisson statistics were used to determine that there is a 0.5% probability that two liposomes would give

*A manuscript with the contents from part of this chapter authored by LM Dominak, DM Omiatek, EL Gundermann, ML Heien, AG Ewing, and CD Keating is in preparation for publication (2010).
rise to a single peak at a given time point in the electropherogram (1), thus allowing for electrochemical interrogation of individual vesicles from a densely populated biocolloidal suspension. While it has been established that each amperometric peak is attributed to a single vesicle, the mechanism of separation for these and related reports involving liposome and subcellular organelle isolations by CE has not been determined (2, 3). Classical electrokinetic theory would predict that particles bearing the greatest positive charge would migrate to the cathode first, followed by neutrals, and then negatively charged particles. Likewise, if all particles possessed analogous surface charge, then vesicles with the largest surface area should elute first since they assume the greatest net charge.

Linear size-elution correlations have been observed for the separation of polymer microspheres which contain homogenous surface characteristics relative to their lipidic counterparts and, therefore, abide mostly by classical electrokinetic separation theory (4, 5). However, in agreement with related densely populated liposome separations by CE (3), no definitive size-elution relation was determined on the electrochemical cytometry platform as shown in Figure 6-1. Here the electrophoretic mobility, which describes the migration time of a particle by CE based on the length of the capillary and the magnitude of the applied electric field, is plotted against the observed radius for individual dopamine-encapsulated liposomes investigated in Chapter 2.

Elution times for liposomes of similar sizes can be influenced by several factors, including electrostatic repulsion between vesicles, hindered migration paths, and electric field perturbations that could be due in part to capillary wall adsorption (despite the pre-treatment conditioning of the capillary) (6, 7). These factors are amplified with
Figure 6-1. Electrophoretic mobility-size relation of liposomes measured by electrochemical cytometry. A density plot of individual liposome electrophoretic mobilities plotted against the apparent radius demonstrates no correlation of size to elution order in the separation of 100-nm radius liposomes ($r^2 = 0.04$). The mean apparent radius was 80 ± 18 nm measured by electrochemical cytometry. Liposomes (80:20 DPPC:cholesterol) were loaded with redox analyte and extruded to 200-nm diameter ($I$). The capillary length was 45 cm and the separation voltage was 333 V/cm. The apparent radius was based on the mole amount of redox molecule measured using amperometry. Amount was converted to volume from the concentration of analyte in the loading buffer. The apparent radii of individual liposomes were then determined from the equation of a sphere ($V = 4/3\pi r^3$). $n = 3362$; Error is SD.
increasing biological complexity, as various secretory and synaptic vesicles are known to contain a host of charged transmembrane proteins (8) which can contribute to elements that affect elution in the applied field. In future studies, efforts might be geared towards further characterization of liposome separations by electrochemical cytometry. By strategically altering factors such as liposome size, lipid composition, buffer pH, and ionic strength, one can unravel the terms that dictate the mechanism of separation. If these experiments are carried out in very dilute samples, the contribution of the biocolloidal electrostatic interactions should be nominal, and therefore can potentially yield a result that more closely follows classical electrokinetic theory. Providing an adequate calibration of these data opens up the possibility of a bilateral electrochemical cytometry detection scheme for synaptic vesicle applications, where both vesicular size and neurotransmitter content can be measured in concert with each other online.

**Expanding the Scope of Electrochemical Cytometry Detection**

As discussed in Chapter 1 and throughout this thesis, electroanalytical methods have been used extensively for the study of neurobiology since a select grouping of chemical messengers transmitted during signaling processes in the nervous system are electroactive, and therefore, require no external label for detection. The electrochemical cytometry measurement introduced in this thesis has employed the use of constant potential amperometry at carbon-fiber microelectrodes to effectively quantify the mole amount of vesicular transmitter from artificial (1), secretory (9), and synaptic (10) vesicles. By modeling the amperometric peak characteristics with the theoretical flux of dopamine from of a lysed vesicle at the detector in a proof-of-concept experiment in Chapter 2, I was able to determine the efficiency of the electrochemical cytometry
detection scheme to be 87% (1) (increased to > 95% via less signal filtering in Chapters 3 and 4).

In addition to amperometric detection, which quantifies the amount of species detected, it would be useful to determine the chemical identity of vesicular content. This can be accomplished by integrating end-column fast-scan cyclic voltammetry (FSCV) into the detection scheme. Cyclic voltammetry is a potential sweep technique that measures current as a function of electrochemical potential to yield voltammetric oxidation and reduction waves that are unique to the species being investigated. Therefore, the resultant voltammogram serves as a molecular “fingerprint” for various redox-active neurochemicals secreted during neurotransmission in the nervous system, as most oxidation and reduction waves have been well characterized for both in vivo and in vitro analytical investigations using FSCV (11-13). Moreover, cyclic voltammetry can provide information about the concentration of vesicular transmitter since it is proportional to the current measured in the oxidation-reduction reaction (14). FSCV has been used as a detection scheme for microchip separations in previous reports and, therefore, could feasibly be integrated for the electrochemical cytometry analyses of vesicular content (15, 16).

I demonstrated in Chapter 2 that the electrochemical cytometry signal measured by constant potential amperometry was specific to electroactive components since no amperometric peaks were observed when liposomes lacking the electroactive analyte were investigated (1). In addition, when the working electrode was held at 0 V versus Ag QRE for secretory vesicle experiments, a voltage insufficient for catecholamine electrooxidation, no signal was observed. It was assumed that the oxidative signal
observed in electrochemical cytometry experiments for the biological vesicles investigated was attributed to dopamine since orthogonal studies at PC12 cell large dense-core vesicles (LDCVs) (17) and mouse striatal small synaptic vesicles (SSVs) (18-20) have identified dopamine as the prominent electroactive component released upon exocytosis of these cells. It would be useful to directly confirm the identity of vesicular species by using electrochemical cytometry with FSCV detection in future work. In addition, FSCV can be used to determine the concentration of transmitter in these vesicles. In Chapter 3, catecholamine concentration was thought to remain constant upon varying the amount of transmitter in a vesicle, but this was based on independent measurements of vesicle size (9). Vesicular concentration measurements could add a useful detection element to the electrochemical cytometry platform for future applications in neuroscience research.

In addition to identifying the components of the vesicles described in this thesis, future work could use electrochemical cytometry with FSCV detection to directly monitor the coexistence of multiple transmitters in a single vesicle. Stimulus-coupled release experiments have been performed by the Wightman lab using FSCV on cultured chromaffin cells to differentiate between vesicular epinephrine and norepinephrine (21, 22) and on cultured mast cells to identify vesicular histamine and serotonin (23, 24). Electrochemical cytometry with FSCV could be used to investigate if the ratio of monoamine measured from these release experiments at single cells is in agreement with that measured after lysis and detection of total content from vesicles extracted from the cell environment. By comparing these ratios, one could determine whether certain
monoamines that coexist in vesicles are preferentially released during exocytosis whereas others are retained.

Analytical Techniques to Improve Liposome Encapsulation and Characterization

In Chapter 2, I demonstrated that electrochemical cytometry could be used to characterize nanoliposome suspensions via the quantitative measurement of encapsulated content. Although these vesicles were extruded to a fixed size (200-nm-diameter confirmed with light scattering), the data revealed heterogeneous non-ideal encapsulation efficiencies for individual liposomes in the biocolloidal suspension (1). Indeed, the average amount of dopamine encapsulated was shown to be $41 \pm 20\%$ of that expected (using light scattering data to estimate the radius) (1).

The result observed in Chapter 2 is common to lipid vesicle formation as poor solute encapsulation is often reported in the literature (25, 26), especially for nanometer-sized liposomes formed using passive hydration methods (27). This is problematic since nanoliposomes promise to play a pivotal role in emerging biomedical technologies, as they can be molecularly programmed for site-specific action and can encapsulate a host of bioactive agents, including genetic material and pharmaceutical therapeutics (28). It has been well established that the encapsulation efficiency of the vehicle in which a therapeutic agent is transported to the site of action is as important as the proposed efficacy of the encapsulant itself since it regulates the treatment dosage (29). Therefore, much work has focused on maximizing vesicular encapsulation in liposome syntheses (30). Some have turned to specific lipid-solute interactions for optimized formation (e.g.,
ion-pairing, complexing agents, etc.), which are useful for select applications but do not ameliorate the general inadequacies of nanoliposome encapsulation (30).

The Keating group has developed a method to increase the encapsulation of bulky high molecular weight polymers and biomolecules (e.g., > 500 kDa) into giant vesicles through the addition of a polymer co-solute, such as PEG 8 kDa (poly(ethylene glycol)), which serves to condense the encapsulant structure during the liposome formation (31, 32). This finding has offered a potential generic solution to increase encapsulation efficiency for many liposome-solute combinations formed by passive hydration. I have recently collaborated with this group to determine if the macromolecular crowding effects observed at giant vesicles could be extrapolated to their submicron counterparts (33). These smaller vesicles are more relevant for clinical applications, as vesicle size is known to play a determinant role in the ultimate efficacy of a therapeutic agent (34). Indeed, for liposomes used in a variety of immune virus and cancer therapies to act effectively in vivo, they must be small enough to avoid recognition by the MHC (major histocompatibility complex) pathway (~100-nm diameter), but not so small (< 10-nm diameter) that they are automatically cleared by the body (e.g., through the renal system) (34).

Submicron lipid vesicles (200-nm diameter, 80:20 DPPC:cholesterol) were formed in a manner similar to the procedures detailed in Chapter 2 (1). Gentle hydration of a lipid film was used to encapsulate the small molecule carboxyfluorescein (CF, 376 Da), the polymer dextran-conjugated fluorescein isothiocyanate (FITC-Dx 500,000 Da), and the protein thyroglobulin-conjugated AF488 (660,000 Da) into liposomes with and without the addition of 3% PEG 8 kDa (33). Unencapsulated fluorophore was removed
from the vesicle suspensions via dialysis prior to analysis to minimize non-specific signal. The vesicles were lysed with surfactant and measured by fluorimetry to quantify the intensity of expelled fluorescently tagged encapsulant in the lysate. The resultant signal was then converted to a concentration to calculate the bulk encapsulation efficiency (BEE) of the suspensions. Bulk encapsulation efficiency is defined as the ratio of the encapsulated solute concentration measured upon lysis to that in the initial loading buffer. These measurements were compared to matched vesicle suspensions that were formed in the presence of PEG to see if macromolecular crowding increased encapsulation as observed in the giant vesicle experiments. Results are plotted in Figure 6-2.

It was observed that the addition of PEG did not affect BEE of the small molecule carboxyfluorescein into the submicron liposomes (Figure 6-2, blue; Student’s t-test, $p = 0.63$), in agreement with previous reports that have investigated the crowding effects of small molecule encapsulation in giant vesicles (31, 32). However, crowding had a marked effect on the encapsulation of the large polymer FITC-Dx 500 kDa (Figure 6-2, green) and the biomolecule thyroglobulin (Figure 6-2, purple), which yielded BEEs that were statistically significant from control (Student’s t-test, $p = 0.03$ and 0.04 for FITC-Dx and thyroglobulin, respectively). Overall, this experiment demonstrated that a generic means of encapsulation could be applied to nanoliposome formation to improve the efficiencies by which biological species can be entrapped in vesicles. In addition to drug delivery, which typically encapsulates small molecules, these findings have the potential to impact a wide variety of applications that involve the use of liposomes as volume-
Figure 6-2. Macromolecular crowding increases polymer and protein encapsulation in submicron lipid vesicles measured by fluorimetry (33). BEE (bulk encapsulation efficiency) is the fraction of analyte from a lysed volume of vesicles divided by the initial concentration of analyte loaded into the vesicles. CF (carboxyfluorescein) is 376 Da, FITC-dextran (FITC-Dx) is 500 kDa, and thyroglobulin-AF488 is 660 kDa. These liposomes are represented by the solid bars. The striped bars represent the same respective encapsulants in the presence of 3% PEG 8 kDa to serve as a crowding agent. In all cases, liposomes were prepared by passive hydration of a lipid film, followed by temperature-controlled extrusion through polymer track-etch membranes with 200-nm pore diameters. Vesicle sizes were confirmed with dynamic light scattering. n = 3 for each data set; Error is SD. (*) designates $p < 0.05$. CF liposomes were not statistically significant from control with PEG addition (Student’s t-test, $p = 0.63$). FITC-Dx and thyroglobulin-AF488 encapsulated with PEG were statistically significant from control with $p = 0.04$ and 0.03 for FITC-Dx and thyroglobulin-AF488, respectively (Student’s t-test). Fluorimetry data was collected by LM Dominak.
limited “bioreactor” vessels to better understand mechanisms that regulate transcription (35), protein synthesis (36, 37), and enzymatic reactions (38) in cell biology.

Future work geared towards the investigation of nanoliposome crowding effects on encapsulation efficiency can be monitored using electrochemical cytometry to measure entrapped analyte in individual vesicles. The ability to perform measurements on a per vesicle basis would be compelling in liposome research technologies since no methods currently available are capable of quantitatively resolving content from individual submicron vesicles in a high-throughput format. Conventional optical microscopies and/or flow cytometry analyses cannot be used to monitor the fraction of solute encapsulated in a nanoliposome due to limitations associated with diffraction in light microscopy.

Most have relied on bulk scale characterization analyses, such as fluorimetry, to investigate encapsulation. These approaches are based on BEE calculations, as discussed above, and yield an average value representative of the cumulative encapsulant from a liposome lysate. Although single vesicle encapsulation measurements have been reported in the literature, they have required the use of advanced optical techniques (e.g., optical traps, optical tweezers, customized imaging platforms, etc.), which are notoriously low-throughput and yield information from merely a select grouping of liposomes in suspension (39, 40).

The electrochemical cytometry device provides a high-throughput method capable of resolving liposome encapsulants on a per vesicle basis. Preliminary sample data from a concept study using electrochemical cytometry to investigate 400-nm diameter liposomes (80:20 DPPC: cholesterol) loaded with tris-2,2'-bipyridyl ruthenium(II)
(Ru(bpy)$_3^{2+}$, a redox-active fluorophore, are shown in Figure 6-3A. This analyte is ideal to characterize the suspension since encapsulation efficiency can be measured both electrochemically on the device and offline using fluorimetry to calculate a BEE. Amperometric spikes on the electrochemical cytometry trace can be integrated and the coulometric charge related to Faraday’s Law ($N = Q/nF$) to yield the mole amount of Ru(bpy)$_3^{2+}$ per vesicle as demonstrated throughout this thesis.

Normalized frequency histograms of Ru(bpy)$_3^{2+}$-encapsulated vesicles are plotted as average radii for liposomes formed with (Figure 6-3C) without (Figure 6-3B) the addition of 3% PEG 8 kDa. These were determined by relating the amount detected by amperometry to a volume measurement, which was then converted to radius from the equation of a sphere as described in Chapter 2 (assuming 5 mM Ru(bpy)$_3^{2+}$ and 1 e- oxidation) (1). The average radii were measured as 188 ± 1.4 nm (n = 1467 events) for the vesicles formed in dilute solution (blue), and 197 ± 1.3 nm (n = 2713 events) for those formed with 3% PEG. This result is expected for the small Ru(bpy)$_3^{2+}$ molecule (MW = 750 Da), as demonstrated with CF in both the giant and submicron vesicle fluorescence analyses. The frequency histograms illustrate the heterogeneity of encapsulation, where multiple Gaussians provide the best fit to the data. From close inspection of the data, the liposomes formed in dilute solution appear to have a population of vesicles shifted to lower amounts that is not seen in the distribution from the liposomes containing PEG. However, this is preliminary sample data from one analysis and should be used only as a proof-of-concept template for future studies. Instead, it is more convenient to conclude that liposomes loaded with and without crowding agents can be detected on a per vesicle basis using electrochemical cytometry.
Figure 6-3. Preliminary data for the electrochemical cytometry of liposomes synthesized with and without the addition of a crowding agent. (A) Sample data for the electrochemical cytometry of 400-nm-diameter liposomes loaded with 5 mM Ru(bpy)$_3^{2+}$ in water (working electrode held at 1 V vs. Ag QRE). (B) Normalized frequency histogram quantifying the apparent radius of Ru(bpy)$_3^{2+}$ liposomes synthesized in water (blue). For calculation of radius see Figure 6-2. Data fit to two Gaussian distributions. Distribution means were 117 and 195 nm ($r^2 = 0.97$). Bin size = 5 nm. n = 1467 events, average radius = 188 ± 1.4 nm. (C) Normalized frequency histogram quantifying the apparent radius of Ru(bpy)$_3^{2+}$ liposomes synthesized with the crowding agent 3% PEG 8 kDa (orange). Data fit to two Gaussian distributions. Distribution means were 160 and 209 nm ($r^2 = 0.97$). Bin size = 5 nm. n = 2713 events, average radius = 197 ± 1.3 nm.
and future work can be geared towards optimizing this methodology for similar analyses where larger molecular weight species can be investigated, given the presence of a redox tag.

**Measuring in vivo pH Fluctuations in Drosophila with a Chemically-Modified Microelectrode Sensor**

In Chapter 5, I reported the development and characterization of a reagentless modified carbon-fiber microelectrode sensor capable of measuring dynamic *in vivo* pH fluctuations evoked during optogenetic stimulus-coupled secretion in the CNS of a mutant fruit fly (41). The voltammetric sensor was modified by a simple and reproducible procedure that involved electrochemically grafting a commercially available diazonium salt (Fast Blue RR) onto a carbon-fiber microelectrode sensor. FSCV was used to cycle the oxidation-reduction reaction of a quinone-moiety on the surface bound diazonium. A quantifiable oxidative wave was observed to shift to higher voltammetric potentials in acidic media and lower potentials in basic media. The next phase of this project will be to measure pH fluctuations in the CNS of a wild-type fly, but methods are still being optimized to elicit release from this microanalytical neuroscience model.

**Electrochemical Cytometry of Biological Vesicles and Future Applications in Neuroscience Research**

An interesting application of electrochemical cytometry has involved the study of vesicular content from the dopaminergic cell line PC12 cells in Chapter 3. In this work, the total transmitter content measured from PC12 cell vesicles extracted from the cell environment (measured by electrochemically cytometry) was compared to conventional *in vitro* stimulus-coupled secretion events at single cells to determine that the fraction of
transmitter released during exocytosis was less than unity (~40%) (9). The resultant cell-
free assessment of transmitter content from individual secretory vesicles provided a new
method to monitor vesicular transmitter content in manner that circumvented biophysical
processes of the cell associated with release. Moreover, this study provided direct
evidence to support the hypothesis that the mechanism of neurotransmission can be
regulated at the single vesicle level, which could play a role in synaptic plasticity and
potentiation.

In Chapter 4 it was demonstrated that individual synaptic vesicles from
mammalian midbrain dopaminergic neurons could be quantitatively probed in a high-
throughput format using electrochemical cytometry (10). This was an exciting finding
that has opened the possibility for a myriad of applications to study pre-synaptic
regulators of neurobiological phenomena from live animals that have been otherwise
difficult to investigate. Moreover, it was shown that the effects of neurotransmitter
loading and depletion as a result of pharmacological manipulation could be resolved from
single vesicles. This methodology was applied to investigate mechanisms that outline
subcellular signaling in the presence of the psychoactive drug amphetamine. Future
studies can be expanded to understand vesicular dopamine regulation as a function of
time course and dose for this and other drugs of abuse to provide molecular evidence for
subcellular actions that are thought to play a role in reward and addiction.

Future applications of the electrochemical cytometry can be geared towards the
investigation of vesicular monoamine from genetic mutant animal models. As discussed
in Chapter 4, it is useful to measure neurotransmitter in striatal vesicles since they come
from a brain region that is densely populated with anatomical dopamine inputs projected
from areas that span the mammalian brain. Neurological disorders such as Huntington’s disease have been characterized by the non-ideal signaling of dopaminergic neurons that are integrated into these pathways. Huntington’s is a grossly debilitating and, eventually, fatal disease that induces irregular cognitive and motor functions. The symptoms of Huntington’s disease are hypothesized to be attributed to impaired vesicle loading from VMAT dysfunction (42-44). An interesting application of electrochemical cytometry would be to study quantal vesicular dopamine content from R6/2 mice, a genetic mutant model for Huntington’s disease. Voltammetric measurements of extracellular catecholamine have been made from striatal brain slices of R6/2 mice, but these studies have not been able to resolve vesicular transmitter (43, 44). Since the implications of impairment are thought to arise at the vesicle, electrochemical cytometry could be used to directly confirm this hypothesis. Information gathered from these experiments could aid in better understanding the mechanism of the disease, as well as providing a useful high-throughput method to screen for potential therapeutics designed to reverse the effects of neuronal dysfunction at the subcellular level.
References


Vita - Donna M. Omiatek

EDUCATION
Ph.D. Chemistry, The Pennsylvania State University, University Park, PA (May 2010)
M.S. Chemistry, Villanova University, Villanova, PA (May 2010)
B.S. Chemistry (ACS Approved), Saint Joseph’s University, Philadelphia, PA (May 2003)

AWARDS
Norma Robinson Graduate Student Research Award (2009)
Pennsylvania State University Travel Award (2007)
Sigma Xi Research Award (2002)
Environmental Chemistry Research Award from the Philadelphia Coatings and Technology Society (2002)

SELECT PUBLICATIONS

SELECT PRESENTATIONS