IS-T-1860

Capillary Electrophoretic Study of Individual Exocytotic Events
in Single Mast Cells

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MS Thesis submitted to Iowa State University

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Date Transmitted: February 12, 1999

PREPARED FOR THE U.S. DEPARTMENT OF ENERGY

UNDER CONTRACT NO. W-7405-Eng-82.

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TABLE OF CONTENTS

ABSTRACT	iv
CHAPTER 1. GENERAL INTRODUCTION	1
Thesis Organization	1
Single Cell Analysis	1
Capillary Electrophoresis with Laser-Induced Native Fluorescence	2
Cell Injection	3
Experimental Setup	5
Cell Isolation	6
Cell Release	7
Conclusion	8
References	8
* CHAPTER 2/ CAPILLARY ELECTROPHORECTIC STUDY OF INDIVIDUAL * EXOCYTOTIC EVENTS IN SINGLE MAST CELLS	11
Abstract	11
Introduction	11
Materials and Methods	12
Results and Discussion	14
Concluding Remarks	17
Acknowledgments	17
References	18
CHAPTER 3. GENERAL CONCLUSIONS	25
ACKNOWLEDGMENTS	26

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ABSTRACT

The peak profile of individual degranulation events from the on-column release of serotonin from single rat peritoneal mast cells (RPMCs) was monitored using capillary electrophoresis with laser-induced native fluorescence detection (CE-LINF). Serotonin, an important biogenic amine, is contained in granules (0.25 fL) within RPMCs and is extruded by a process termed exocytosis. The secretagogue, Polymyxin B sulfate, was used as the CE running buffer after injection of a single RPMC into the separation capillary to stimulate the release of the granules. Because the release process occurs on a ms time scale, monitoring individual exocytotic events is possible with the coupling of high-speed CE and LINF detection.

CHAPTER 1

GENERAL INTRODUCTION

Thesis Organization

Chapter 1 gives a general introduction on single cell analysis. Chapter 2 is a paper which has been accepted in a chemistry journal. Chapter 3 gives overall conclusions. Each chapter is followed by a reference list.

Single Cell Analysis

Single cell analysis has received much attention lately in the biological sciences due to the advances in the development of microscale analytical techniques. These techniques include immunoassay,¹ fluorescent imaging,² optical and electron microscopy,³ microscale ion-selective electrodes,^{4,6} and enzyme reactivity measurements.⁷ Some limitations associated with these methods include inadequate sensitivity, poor qualitative capabilities, and inability to determine multiple components. Microcolumn liquid chromatography and capillary electrophoresis coupled to electrochemical or laser-induced fluorescence (LIF) detection offer the best performance because of their ability to monitor chemical components and dynamics on a time scale appropriate for cellular processes and at extremely small sampling volumes.

The importance of determining the composition and dynamics of single cells will lead to the understanding of the biological functionality and roles of cells within their heterogeneous environments. By analyzing cells individually, one can identify abnormal cells that carry biological markers for certain diseases so that disease screening and understanding of the disease is possible. Because cells engage in numerous activities, such as chemical transport and reaction, self-regulation, and stimuli response, the ability to monitor such activities will provide a better understanding of how cells function, such as the effects of drugs and toxins on the chemical contents of cells. Such analyses can further provide information on cellular differentiation, intercellular communication, neurotransmitter uptake and release, signal

transduction pathways, and ion transport systems. In particular, there is great interest to investigate the link between secretion of chemical messengers and its biological reactions.

The secretion of neurotransmitters and hormones is central to many important biological functions and is a popular topic in single cell analysis. From the secretion of insulin from pancreatic β -cells in response to elevated glucose concentrations, to the secretion of neurotransmitters to control a variety of neuromodulatory functions of mammalian and nonmammalian species, the monitoring of chemical release from single cells is challenged by the extremely minute amount of chemical released, the small and complex working environment and the rapid time scale these processes occur within.

Capillary Electrophoresis with Laser-Induced Fluorescence (CE-LIF)

As mentioned above, there are many techniques currently available that have been used to analyze individual cells. To date, results from microcolumn HPLC and CE with electrochemical^{9, 10} and LIF detection^{11,12} offer excellent performance in terms of sensitivity and quantitative and multicomponent abilities.

Even though amperometry offers high sensitivity and voltammetry provides qualitative information, only a few biomolecules are intrinsically electroactive. Electrode-fouling due to contamination, restriction to the size of electrode and dilution due to diffusion are some challenges that are encountered with electrochemical detection.

Single cell analysis by HPLC often involves the use of an internal standard to account for the variables associated with the sample preparation and injection.⁹ After isolation and transfer of a single cell by a micropipette into a microvial, the cell is homogenized and centrifuged. A sample of the supernatant is then injected into the LC microcolumn. A drawback of this method is the inability to analyze dynamic cellular processes, such as release and uptake.

Since many biomolecules will naturally fluoresce when excited with suitable laser wavelengths, laser-induced native fluorescence (LINF) is a favorable detection technique used

for single cell analysis. As a direct detection method, LINF avoids problem associated with the derivitization reaction, such as incomplete or slow reactions, dilution of reaction products and contamination due to sample handling. Even though the small quantities of material in a cell and the low fluorescence efficiency would not typically be considered to provide sensitive detection, the detection limits obtained by LINF are impressive.¹³ Many neurotransmitters and proteins excite at wavelengths that closely match the UV wavelengths of argon ion lasers.

Single cell analysis using CE has many advantages over HPLC. Because of the small dimensions of the capillary tubes, small sample volumes can be used. Cells typically have volumes in the femtoliter range. By using higher potential fields and shorter capillary lengths (migration distances), high separation efficiency and speed are obtained and axial diffusion is limited. A whole cell can be sampled by directly drawing it into the tip of the capillary by electrokinetic or vacuum injection. All the cellular contents are sampled giving better detection limits for low-level components. In this way, the capillary column can be used as the site of single cell manipulation, such as derivitization or on column release. Recently, CE has also been shown to provide a dual role in the separation and temporal monitoring of a biological event.¹⁴

Cell Injection

The vacuum injection method we used is diagrammed in Figure 1. An air-tight septum is placed over the outlet buffer vial of the CE system. The detection end of the capillary and a syringe are inserted into the septum. After placing the inlet end of the capillary into a drop of cell suspension solution on a microscope slide and looking under a light microscope (100X magnification), one can control the pressure of the syringe and visually confirm the cell injection and adhesion onto the capillary wall. Cell adhesion is important if cellular release is the process being studied. We have found that pretreating the capillary occasionally with NaOH between runs helps in cell adsorption to the capillary wall.¹⁴

Figure 1. Schematic of single cell injection into a capillary.

Experimental Setup

A typical setup used for single cell analysis using CE-LINF is diagrammed in Figure 2. The appropriate wavelength from a laser is used as the excitation source and is isolated with a prism. The light is directed into a safety interlock box where CE is performed. A quartz lens focuses the light onto the detection window of the capillary. The detection window is held securely with a capillary holder to prevent unnecessary movement. Fluorescence is collected at 90° with a 20X microscope objective and passed through appropriate filters and onto a photomultiplier tube (PMT). Electropherograms are recorded and stored on a computer.

Figure 2. Schematic of experimental setup.

Cell Isolation

There have been many cell types studied in single cell analysis. The measurement of native fluorescence from their cellular components include insulin from pancreatic β cells,¹⁵ serotonin from rat peritoneal cells,¹⁴ catecholamines in adrenal medullary cells,¹⁶ and hemoglobin variants from human erythrocytes.¹⁷

Depending on the cell type of interest there are many different procedures to isolate viable cells. In our study, we used rat peritoneal mast cells (RPMCs). Mast cells occur primarily in the loose connective tissue surrounding blood vessels, glandular ducts, and under epithelial, serous, and synovial membranes.¹⁸ It is very important to isolate mast cells free of peritoneal macrophages which make up the majority of peritoneal cells. Contamination with lysosomal enzymes from macrophages may digest surface components of perigranular and plasma membranes or the granule matrix itself.¹⁹ There have been many methods utilizing density gradient centrifugations.¹⁹⁻²² A simple, rapid, and reliable method to obtain high-yield and high-purity mast cells with preservation of morphological and biological integrity of the cells is a primary concern. Generally, the healthy mast cell is round and smooth with dense cytoplasmic granules occupying the cytoplasm. The average mast cell measures 12.6 μm in diameter, and each granule is approximately 0.2 to 0.4 μm in diameter.¹⁸ The procedure we followed was based on the method of Parpura and Fernandez.²⁰

Briefly, mast cells were isolated by peritoneal lavage from male Sprague-Dawley rats. The rats were anesthetized and then decapitated. Approximately 50 mL of the lavage saline consisting of CO_2 -independent medium containing 0.175% fatty acid-free bovine serum albumin was injected into the peritoneal cavity. The rat was inverted and massaged for ~5 minutes, an incision made into the cavity and the lavage saline withdrawn. The lavage was centrifuged for 10 minutes (700 rpm, 100g, 10°C) and the resulting pellet was resuspended in

1 mL of fresh lavage saline and layered over 2 mL of 0.22% metrizamide (dissolved in the lavage saline). After a 20 minutes (1400 rpm , 400g, 10°C) centrifugation, the supernatant was discarded. The remaining pellet was resuspended and centrifuged for 10 minutes (700 rpm, 100g, 10°C). Again, the supernatant was discarded and the cells were resuspended in the lavage saline until use.

Cellular Release

Methods used to release cellular contents include cell lysis, either by a lysing agent¹⁸ or a difference in osmotic pressure¹⁹ and exocytosis.¹⁴

Exocytosis is a common secretory mechanism. Secretory cells, such mast cells, chromaffin cells, and PC12 cells, contain large numbers of secretory granules that contain biologically active mediators. The discharge of the contents of a granule requires the granule membrane and the plasma membrane of the cell to come into contact and fuse, forming an opening through which the granular contents can be released. This process is termed exocytosis and is stimulated by proper secretagogues or releasing agents.

There are a number of compounds that cause noncytotoxic degranulation of secretory cells. Common agents for mast cells include concanavalin A (Con A), dextran, Compound 48/80, Polymyxin B sulfate (Pmx), and calcium ionophore.¹⁸ For chromaffin cells, nicotine,²⁴ Ba^{2+} ,²⁵ and digitonin,²⁶ are some secretagogues used to induce exocytosis of catecholamines. K^+ - and nicotine-solutions evoke release of catecholamine release from PC12 cells.²⁷

Specifically, mast cells synthesize, store and release serotonin, a neurotransmitter associated with psychiatric disorders within the brain and its presence in other peripheral tissues suggests its association with other disorders, and histamine, a neurohormone that triggers a variety of allergic reactions.

The sequential exocytosis of mast cell granules is depicted in Figure 3.²³ Membranes of the secretory granules in the cytoplasm first fuse with the plasma membrane, followed by

extrusion of the granules into the extracellular matrix. Mediators are then displaced from the granular core. Multiple fusion events may occur in which more than one granule fuse together before being released.²³

CONCLUSION

Because exocytotic events are thought to occur on a submillisecond time scale, providing temporal resolution to resolve individual events is necessary. The use of fast CE has been demonstrated before using high applied potentials. The following chapter describes the application of high-speed CE with LINF detection to resolve the peak profile of individual degranulation events released from RPMCs.

REFERENCES

1. Ishikawa, E.; Hashida, S.; Kohno, T.; Hirota, K. *Clin. Chim. Acta* **1990**, *194*, 51-72.
2. Tank, D.W.; Ugimori, M.; Connor, J.; Llinas, R.R. *Science* **1988**, *242*, 773-777.
3. Tan, W.; Shi, Z.-Y.; Smith, S.; Birnbaum, D.; Kopelman, R. *Science* **1992**, *258*, 778-781.
4. Jankowski, J.A.; Schroeder, T.J.; Holz, R.W.; Wightman, R.M. *J. Biol. Chem.* **1992**, *267*, 18329-18335.
5. Lau, Y.Y.; Takayuki, A.; Ewing, A.G. *Anal. Chem.* **1992**, *64*, 1702-1705.
6. Leszczysyn, D.J.; Jankowski, J.A.; Viveros, O.H.; Diliberto, E.J.; Near, J.A.; Wightman, R.M. *J. Neurochem.* **1991**, *56*, 1855-1865.
7. Giacobini, E. *Neurosci. Res.* **1968**, *1*, 1.
8. Hofstadler, S.A.; Swanek, F.D.; Gale, D.C.; Ewing, A.G.; Smith, R.D. *Anal. Chem.* **1995**, *67*, 1477-1480.
9. Kennedy, R.T.; Jorgenson, J.W. *Anal. Chem.* **1989**, *61*, 436-441.
10. Ewing, A.G.; Wallingford, R.A. *Anal. Chem.* **1988**, *60*, 258-263.
11. Egger, D.; Reisback, G.; Hultner, L. *J. Chromatogr. B* **1994**, *662*, 103-107.

12. Lee, T.L.; Yeung, E.S. *Anal. Chem.* **1992**, *64*, 3045-3051.
13. Yeung, E.S. *Adv. Chromatogr.*, **1995**, *35*, 1-51.
14. Lillard, S.J.; Yeung, E.S. *Anal. Chem.* **1996**, *68*, 2897-2904.
15. Tong, W.; Yeung, E.S. *J. Chromatogr. B* **1996**, *685*, 35-40.
16. Chang, H.T.; Yeung, E.S. *Anal. Chem.* **1995**, *67*, 1079-1083.
17. Lillard, S.J.; Yeung, E.S.; Lautamo, R.; Mao, D.T. *J. Chromatogr. A* **1995**, *718*, 397-404.
18. Metcalfe, D.D.; Kaliner, M.; Donlon, M.A. *CRC Critical Reviews in Immunology*, **1981**, 23-74.
19. Nemeth, A.; Rohlich, P. *European J. Cell Biol.* **1980**, *20*, 272-275.
20. Parpura, V.; Fernandez, J.M. *Biophys. J.* **1996**, *71*, 2356-2366.
21. Yurt, R.W.; Leid, W., Jr.; Austen, K.I.; Silbert, J.E. *J. Biol. Chem.* **1977**, *252*, 518-521.
22. Amende, L.M.; Donlon, M.A. *Biocimica et Biophysica Acta*, **1985**, *812*, 713-720.
23. Rohlich, P.; Anderson, P.; Uvnas, B. *J. Cell Biol.* **1971**, *51*, 480.
24. Leszczyszyn, D.J.; Jankowski, J.A.; Viveros, O.H.; Diliberto, E.J., Jr.; Near, J.A.; Wightman, R.M. *J. Biol. Chem.* **1990**, *265*, 14736-14737.
25. Schroeder, T.J.; Jankowski, J.A.; Senyshyn, J.; Holz, R.W.; Wightman, R.M. *J. Biol. Chem.* **1994**, *269*, 17215-17220.
26. Jankowski, J.A.; Schroeder, T.J.; Holz, R.W.; Wightman, R.M. *J. Biol. Chem.* **1992**, *267*, 18329-18335.
27. Chen, T.K.; Luo, G.; Ewing, A.G. *Anal. Chem.* **1994**, *66*, 3031-3035.

Figure 3. Diagram showing sequential exocytosis of mast cell granules. See text for explanation.

CHAPTER 3

GENERAL CONCLUSIONS

Answers to important questions in the field of cellular biochemistry can be provided by direct chemical analysis of single cells. Recently, many analytical techniques have been developed to provide methods to examine chemical and physiological events at the single cell level. Miniaturization of these techniques allows extremely minute samples to be detected in a rapid time scale.

CE-LINF is a highly effective and practical method due to the small sample requirements, high separation efficiency and speed, the ability to manipulate single cells on column, and sensitive detection. The ability to detect minute amounts of chemical species involved in cellular and neuronal functions, when combined with molecular biology techniques designed to determine structure-related proteins, will provide further understanding of the roles of single cells in complex biological environments.

ACKNOWLEDGMENTS

There are many special people to whom I am truly grateful for their valuable help during the past few years. First, I would like to thank Dr. Yeung for his encouragement and support as my major advisor. I would also like to acknowledge my committee members, Professors Donald Graves and Dennis Johnson, for their time and feedback this past semester. Finally, I would like to thank Professor Mike McCloskey for teaching me the cell isolation procedure and for allowing me to use his lab and rats.

To my family, I thank for encouraging my independence, enduring my various moods and providing support. And particularly to Evelyn, for understanding and supporting me throughout all the different challenges-personal and academic- that I've encountered these past few years.

I also appreciate the friendships I have developed within the lab. I'll always cherish my friendship with Eliza who continually hears and answers my many complaints and questions. To Sheri, Wei Tong, Smiley and Hui Su for their advice, friendship and helpful discussions. Outside of lab, I want to thank Jeana, Barb, Jill and Cathy, my running and tennis partners, for giving me something to look forward to on weekends and early mornings.

This work was performed at Ames Laboratory under Contract No. W-7405-Eng-82 with the U.S. Department of Energy. The United States government has assigned the DOE Report number IS-T 1860 to this thesis.