EXAMINING THE ROLES OF MEMBRANE LIPIDS AND SECRETED BIOACTIVE

LIPIDS ON IMMUNE SYSTEM CELL FUNCTION: STUDIES ON MAST CELLS

AND PLATELETS

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Dedication

This thesis is dedicated to my spouse, Ben, and to my parents, Ann and Noel.

Abstract

The central theme of this dissertation is application of sensitive analytical chemistry tools to further the fundamental understanding of the role of both secreted lipids that act as short-range hormones and structural lipids that play an active role in cellular signaling in the function of immune system cells. Lipids, both as the cell-secreted mediators of inflammation and as important structural components of all mammalian cell membranes, have critically important roles in the functions of cells in the immune system. As such, this dissertation focuses on developing and applying ultra-performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS) methods to address the challenge of quantifying mast cell-secreted bioactive lipids in complex cell cultures as well as understanding the roles that membrane phospholipids play in platelet signaling.

With the exception of red blood cells, every mammalian cell uses phospholipids as enzyme substrates to generate and secrete bioactive lipid species that act as short-range hormones. Similarly, the phospholipids that compose the cellular membrane participate in cellular signaling and many physiological functions. Despite their prevalence, detection of secreted and structural lipids has traditionally presented a significant analytical challenge. The advent of UPLC-MS/MS, however, enables rapid, sensitive, and selective analysis of multiple structurally diverse lipids simultaneously, which enables us to study critical cellular functions and the effects of secreted bioactive lipids. In this dissertation, UPLC-MS/MS methods are developed (Chapter 2) for the rapid and simultaneous analysis of secreted phospholipids and eicosanoids to characterize mast cell function in complex cell

cultures (Chapters 2 and 3). UPLC-MS/MS methods are also applied to contribute to the fundamental understanding of exogenous phospholipids and their influence on platelet function (Chapters 4 and 5), both at the single platelet level and in the context of concerted activity between many platelets together in suspension. Both mast cells (Chapters 2 and 3) and platelets (Chapters 4 and 5) present interesting and challenging platforms for the application of new methods for studying secreted and structural lipid function.

Overall, the work in this dissertation contributes to the development and application of UPLC-MS/MS for simultaneous determination of structurally diverse lipids and exploits this method to achieve new insights into the fundamental role of phospholipids in mast cells and platelets.

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List of Abbreviations

5-HT, serotonin	5-hydroxytryptamine
A23187	calcimycin
AA	arachidonic acid
ACN	acetonitrile
ADP	adenosine diphosphate
ANOVA	analysis of variance
ATCC	American Type Culture Collection
ATP	adenosine triphosphate
BCA	bicinchoninic acid
BCS	bovine calf serum
CFMA	carbon-fiber microelectrode amperometry
CMFDA19	5-chloromethylfluorescein diacetate
DMEM	Dulbecco's Modified Eagle Medium
D-PS	phosphatidylserine containing a D-serine headgroup
ELISA	enzyme-linked immunoassay
ESI	electrospray ionization
ESI-MS/MS	electrospray ionization tandem mass spectrometry
FA	formic acid
FcεRI	high-affinity IgE receptor
HILIC	hydrophilic interaction liquid chromatography
HPLC	high performance liquid chromatography
IgE	immunoglobulin type E
IgG	immunoglobulin type G
IPA	isopropyl alcohol
LOD	limit of detection
LOQ	limit of quantitation
LPS	lipopolysaccharide
L-PS	phosphatidylserine containing a L-serine headgroup
LT	leukotriene
LTB$_4$	leukotriene-B$_4$
LTC$_4$	leukotriene-C$_4$

LTD$_4$	leukotriene-D$_4$
LTE$_4$	leukotriene-E$_4$
MeOH	methanol
MPMCs	murine peritoneal mast cells
MRM	multiple reaction monitoring
MS	mass spectrometry
ova	ovalbumin
PAF	platelet-activating factor
PAF-d$_4$	deuterated platelet-activating factor
PBS	phosphate-buffered saline
PC	phosphatidylcholine
PDMS	polydimethylsiloxane
PE	phosphatidylethanolamine
PF4	platelet factor-4
PG	prostaglandin
PGD$_2$	prostaglandin-D$_2$
PGE$_2$	prostaglandin-E$_2$
PLA$_2$	phospholipase A$_2$
PNAG	p-nitrophenyl acetyl-D-glucosaminide
PRP	platelet-rich plasma
PS	phosphatidylserine
RBL-2H3	rat basophilic leukemia cell line
RSD	relative standard deviation
SERT	serotonin specific reuptake transporter
SM	sphingomyelin
SPE	solid phase extraction
SRM	selected reaction monitoring
TNP	trinitrophenol
TQD	triple quadrupole mass spectrometry detector
UPLC	ultra-performance liquid chromatography
UPLC-MS/MS	ultra-performance liquid chromatography tandem mass spectrometry
β-hex	β-Hexosaminidase

Chapter 1: Introduction to the Determination of Mast Cell-Secreted Bioactive Lipids and the Understanding of Membrane-Bound Phospholipid Effects on Platelet Function

1.1 Cell-Cell Communication in Inflammation

The immune system is composed of a network of cells and tissues that interact with one another through complex and dynamic signaling cascades. It is well known that the immune system has critical roles in and valuable functions to human health, so an improved understanding of the cell-cell communication processes that underlie immune system function will benefit the studies of diseases that center on the malfunction of cell-cell communication in the immune system. The central theme of this dissertation is application of sensitive analytical chemistry tools to further the fundamental understanding of the role of both secreted lipids that act as short-range hormones and structural lipids that play an active role in cellular signaling in the function of immune system cells. In particular, this dissertation focuses on developing and applying methods to address the challenge of quantifying mast cell-secreted bioactive lipids as well as understanding the roles that membrane phospholipids play in platelet function.

The first half of the introduction to this thesis will cover the biological context of cell-secreted bioactive lipids, the challenges of bioactive lipid detection in complex biological matrices including a brief review of the liquid chromatography and mass spectrometry methods most relevant to the work that follows in the thesis. Additionally, the first half of the introduction explores the reasons for using mast cells as an interesting and physiologically relevant system for the development and application of new methods for secreted lipid detection. The second half of this introduction will cover the concept of membrane-bound phospholipids as active signaling molecules, the importance of

molecular asymmetry in the phospholipid membrane, and the compelling case for the use of platelets as an ideal platform for exploring the effects of exogenous phospholipids on cellular function.

1.2 Lipids: Ubiquitous Signaling and Structural Molecules

The term 'lipids' encompasses a broad and diverse class of molecules that are present in nearly all life forms on the planet. In conjunction with membrane-bound proteins, peptides, and cholesterol, phospholipids encase cellular contents in a continuous bilayer. The general structure of the phospholipid bilayer is well known: hydrophilic headgroups cluster together, while the fatty acid tails of the two layers interact to form a water-impermeable barrier. Far from passively shielding the cytosol from the extracellular milieu, however, cellular membranes have an active role in most cell-cell communication processes. With the exception of red blood cells, every mammalian cell uses phospholipids as enzyme substrates to generate and secrete bioactive lipid species that act as short-range hormones.[1,3] Similarly, the phospholipids that compose the cellular membrane participate in cellular signaling and many physiological functions.

1.3 Lipids as Mediators of Cell-Cell Communication

Secreted bioactive lipids are generally formed through one of two pathways: eicosanoid synthesis or platelet-activating factor (PAF) glycerophospholipid subclass synthesis. Eicosanoids are classified as fatty acyls formed from polyunsaturated fatty acids.[1] They generally contain a 20-carbon backbone, are synthesized and act with stereo

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selectivity, and are biologically active at concentrations in the nanomolar range. PAF-lipids are a family of approximately 20 soluble oxidized phospholipid mediators, and the structure for the most well-known PAF lipid (generally referred to as PAF) is shown in Fig. 1.1B.

Although the discovery of eicosanoids technically began with the 1930 discovery of essential fatty acids by George and Mildred Burr at the University of Minnesota, the structures of arachidonic acid-derived bioactive lipids were not elucidated until the 1970s.[2,3] By 1980, prostaglandin and leukotriene structures had been determined, and the inflammatory effects of these lipids had been evaluated.[3,4] From these early studies, several common features of eicosanoids emerged: cells manufacture them upon chemical or mechanical stimulus rather than store them, stereochemistry during formation affects their biological activity, and they are generally potent *in vivo* at nanomolar concentrations.[3] The pathway for eicosanoid synthesis involves the action of either phospholipase C or phospholipase A_2 on membrane-bound phospholipid precursors or diacylglycerol, which results in the formation of arachidonic acid (AA). AA is the precursor to nearly all of the eicosanoids formed in mammalian cells and certainly all of the eicosanoids examined in this thesis.[5]
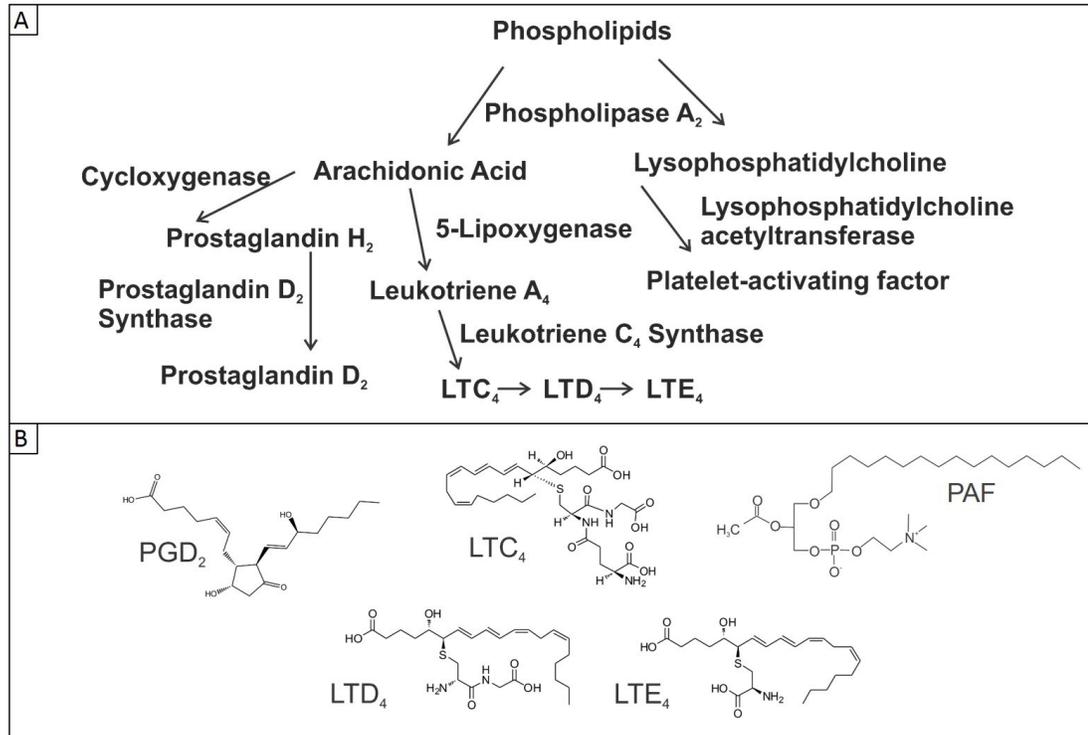
Figure 1.1. Simplified formation pathways (A) and structures of the mast cell-secreted bioactive lipids (B) examined in this dissertation.
Figure modified with permission from Reference 46.

PAF was first discovered in the early 1970s and was the first intact phospholipid metabolite shown to have paracrine signaling capabilities.[6-10] The work in this thesis focuses primarily on the PAF lipid shown in Fig. 1.1B, 1-O-alkyl-2-acetyl-sn-glycero-3-phosphocholine, with some focus on the 14-carbon chain version of this molecule, termed PAF-C14. PAF and other PAF lipids can be formed through two distinct enzymatic pathways. PAF formed during inflammatory response is generally thought to be the product of enzymatic replacement of the acyl functional group of 1-alkyl-2-acyl-sn-glycero-3phosphocholine with an acetate moiety.[11] Endogenous PAF is formed through a series of acetylation, dephosphorylation, and phosphocholination actions on lysophosphatidic acid.[12]

1.3.1 Lipid Determination in Biological Systems

Quantification of bioactive lipids in biological systems is challenging for multiple reasons. One primary challenge for lipid analysis is that bioactive lipids are difficult to extract from aqueous cell or tissue cultures because they contain both polar and non-polar functional groups. Most immune system cells simultaneously secrete a multitude of structurally diverse inflammatory compounds, and extraction methods that isolate one or several bioactive lipids of interest may not work for the isolation of other bioactive lipids.

As lipids are generally designed to be short-acting mediators of cell-cell communication, they have half-lives on the order of minutes to hours.[13-16] Lipids are also generally thermally unstable, susceptible to oxidation, and small variations in lipid structure can lead to large variations in biological activity. For example, while

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prostaglandin-D$_2$ (PGD$_2$) and prostaglandin-E$_2$ (PGE$_2$) are enantiomers, PGD$_2$ is a potent inducer of inflammation while PGE$_2$ has anti-inflammatory and sleep-regulatory functions. This dissertation attempts to address some of the challenges of lipid determination through the development and application of mass spectrometric methods for simultaneous determination of mast cell-secreted PGD$_2$, LTC$_4$, LTD$_4$, LTE$_4$, and PAF.

1.3.2 Liquid Chromatography-Tandem Mass Spectrometry

The detection of eicosanoids and secreted phospholipids such as PAF has traditionally been accomplished using radiolabelled isotopic immunoassays and enzyme-linked immunoassays. These assays have provided the foundation for the understanding of the biological roles of bioactive lipids, but they generally are time- and labor-intensive and suffer from high levels of cross-reactivity, which leads to overestimation of analyte concentration. With the advent of high performance liquid chromatography-tandem mass spectrometry (HPLC-MS or HPLC-MS/MS) and ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS or UPLC-MS/MS), however, the potential for rapid, sensitive, and selective analysis of bioactive lipids is much better than has been previously possible.

The application of HPLC-MS/MS to lipid research represents a significant advance in studies of bioactive lipids. In HPLC-MS/MS, bioactive lipids are separated by either analytical or small-bore chromatography columns prior to electrospray ionization (ESI), operated in either positive ion (yielding M+H$^+$ ions) or negative ion (yielding M+H$^-$ ion) modes.

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Eicosanoid analysis by HPLC has been explored extensively, which has created the foundation for the UPLC-MS/MS research presented herein. However, few published HPLC-MS/MS methods are capable of analyzing multiple classes of eicosanoids, due to the challenges associated with simultaneous extraction and efficient chromatographic separation of multiple structurally diverse eicosanoids. Previously, only one method has reported the analysis of multiple classes of eicosanoids in conjunction with phospholipid metabolites such as PAF, and it requires extensive SPE sample preparation and multiple analytical column separations.[17]

As with most small molecule analytes, quantitative mass spectrometric analysis of bioactive lipids following chromatographic separation is best performed with a triple quadrupole mass spectrometer (TQD) operated in multiple reaction monitoring (MRM) mode, as shown in Fig. 1.2.[1,5,18] A quadrupole mass spectrometer is composed of four metal cylinders onto which a radiofrequency voltage is applied in conjunction with a direct current voltage. Ions are separated by mass-to-charge ratio (m/z) based on the stability of their respective trajectories as they pass through the quadrupole under the influence of a particular range of voltages. As its name suggests, a triple quadrupole consists of a sequence of three quadrupoles: the first and third are operated as mass filters and an inert collision gas such as Ar is applied to the center quadrupole. In MRM studies, standards of analytes of interest are utilized to determine the molecular M+H$^+$ or M+H$^-$ ions with no collision gas. Then, the molecular ions are fragmented through the application of collision gas (i.e., in the 2nd quadrupole). Allowing the selected M+H$^+$ or M+H$^-$ ion through the first

quadrupole, followed by fragmentation in the collision cell and subsequent selective monitoring of known fragments in the third quadrupole is referred to as a transition. TQDs are capable of rapidly and constantly scanning pre-selected *m/z* ranges, with common dwell times on the order of 10 ms.

With HPLC, analyzing multiple classes of eicosanoids generally requires a minimum separation time of 25 min, and separations of 40-60 min are common.[1] Since bioactive lipids are generally unstable long-term in aqueous solutions, bioactive lipid detection utilizing much faster separations is preferred. With the advent of UPLC in the past decade, separations 5-10 times faster than HPLC are attainable. In UPLC, sub-2-μm-diameter particles in columns enable instrument pressures of 10,000-14,000 psi with flow rates of 0.4-0.8 mL/min. This dramatically increases resolution and decreases separation times, and when coupled to a rapidly scanning TQD, can increase sensitivity for improved lipid analysis.
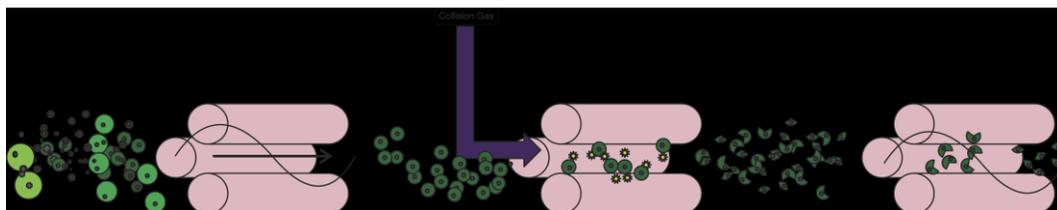
Figure 1.2. Multiple reaction monitoring scheme.
The three quadrupoles are represented by the purple cylinders. Green circles represent ions, black circles represent collision gas molecules, and green semi-circles represent fragmented ions.

In all LC/MS analyses of bioactive lipids, the use of internal standards is necessary. Minimally, due to different extraction efficiencies for different classes of eicosanoids, one deuterated internal standard per class of eicosanoid should be used, and ideally, co-eluting deuterated standards with equivalent transitions to each corresponding eicosanoid should be used for each target of interest.[5]

1.3.3 Sample Preparation for Studies of Bioactive Lipids

Bioactive lipids are found within complex matrices, necessitating an extraction step to permit detection by mass spectrometry. The extraction is complicated by the unstable nature of secreted lipid species that requires sample preparation methods devoid of pH extremes and high temperatures. Depending on the structural class of the lipid of interest, a number of liquid-liquid extraction techniques are available, including chloroform/methanol and hexane/isopropanol/acetic acid.[1,5] While these extraction methods work relatively well for non-polar analytes, polar lipids such as the prostaglandin sub-class of eicosanoids are incompletely extracted into the organic phase. Furthermore, different liquid-liquid extraction techniques are recommended for different eicosanoids, making liquid-liquid extraction challenging for simultaneous analysis of several structurally diverse eicosanoids.

Solid-phase extraction (SPE) is another widely used sample preparation method for lipid analysis. In SPE, biological samples are loaded onto cartridges containing a solid

phase for reverse phase, normal phase, or ion-exchange extraction. Bioactive lipids of interest are trapped on the solid phase, then extracted when the appropriate solvent is applied to the cartridge. SPE sample preparation is advantageous in that it can be automated and can concentrate analytes from relatively larger volumes of sample.[5] However, SPE is time- and labor-intensive and often prohibitively expensive.[1,5] Furthermore, SPE cartridges can suffer from batch-to-batch variability and the selection of the solid phase affects the recovery of bioactive lipid analytes.

1.3.4   Mast Cells as a Platform for Studies of Secreted Bioactive Lipids

In this thesis, mast cells are used as a model cell system for developing and implementing methods for determination of bioactive lipids in complex biological matrices. Mast cells were selected because they have a unique role in the immune system and present an interesting challenge in cell culture. Several immortal (cancer-based) cell lines exist for mast cell studies, but each demonstrates marked differences in receptor expression, morphology, or both from healthy mast cells.[19-21] Mast cells are tissue-bound cells, which makes isolation of large numbers of pure healthy mast cell populations difficult. The studies in the following two chapters of this thesis explore both bioactive lipid secretion from mast cells in an *in vivo*-like heterogeneous cell culture and from an immortal mast cell line. The method developed and the insights into cellular function in complex cell culture can be easily translated for use in other cell culture systems.

1.3.4.1 Mast Cells in the Immune System

Found in most connective tissues, including the skin, lungs, and peritoneum, mast cells are widely known for the primary role that they play in the production of the allergic response. Additionally, they have been shown to participate in the defense against both parasitic and bacterial infections as well as many other immune functions.[22-24] Fig. 1.3 illustrates the variety of immune processes that mast cells take part in, including the initiation of the intracellular signaling events characteristic of allergy (a function of the adaptive immune system) and response to bacterial pathogens (a function of the innate immune system). A series of complex interactions between multiple cell types give rise to the physiological symptoms that are characterized as allergic response. Briefly, when presented with antigens, macrophages or dendritic cells phagocytose (engulf) the allergen and present fragments of its proteins on their surfaces, and B cells produce antibodies against the protein fragments.[25] When exposed to bacterial or viral antigens, B cells generate immunoglobulin type G (IgG), which does not bind to mast cells. However, the antibodies produced against an allergen are immunoglobulin type E (IgE), which binds to the high-affinity IgE receptors (FcεRI) expressed on mast cells and basophils.[25] Subsequent contact with the same allergen results in the cross-linking of the receptor-bound IgE, which in turn initiates a cascade of events resulting in the physiological symptoms associated with allergies.[24,25]
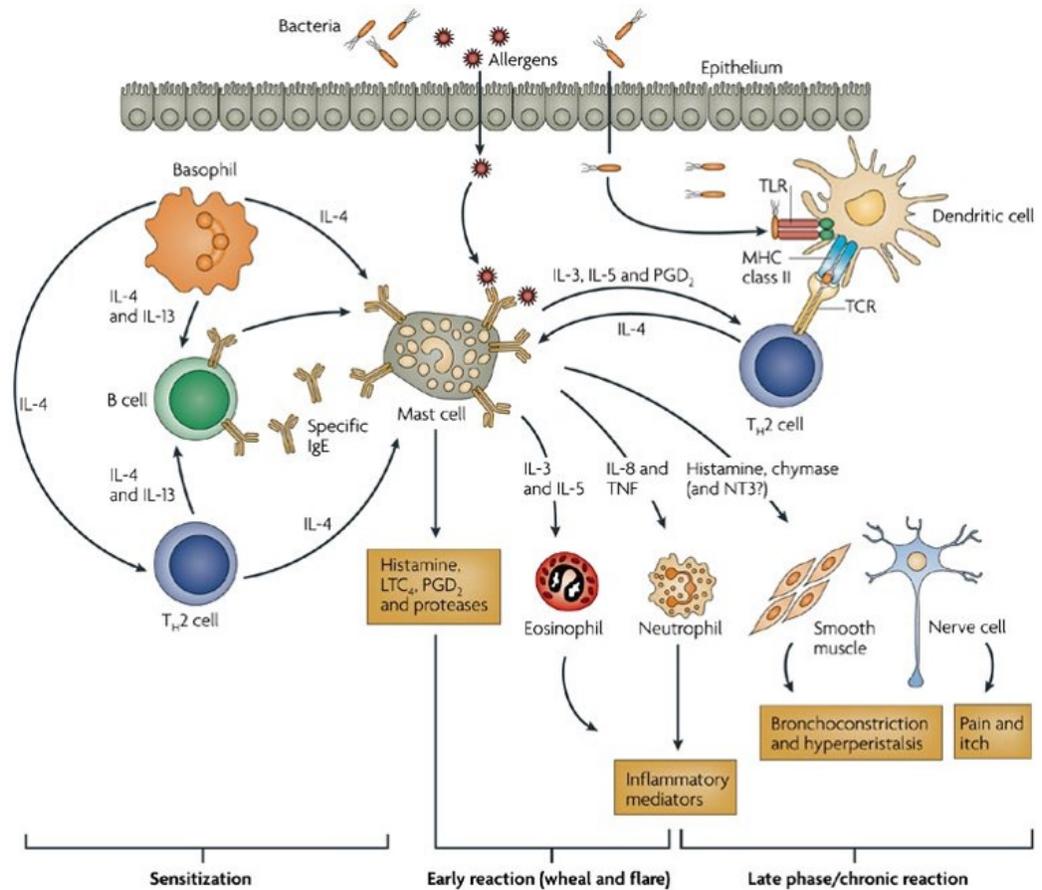
Figure 1.3. Schematic of the immune system with a focus on the interactions between mast cells and other immune system cell types.
Figure adapted with permission from Reference 22.

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Cross-linking between allergen and IgE bound to the mast cell surface results in the release of both granules and *de novo* synthesized chemical mediators from mast cells. The exocytosis of serotonin from the granules of murine peritoneal mast cells has been studied extensively.[26,27] While the granular contents of mast cells vary as a function of species and tissue type, the granules of most mast cells contain heparin, tryptase, chymase, histamine, other small molecules, and cytokines. However, the profile of mast cell-secreted cytokines can vary widely for mast cells responding to different conditions.[24] Fig. 1.4 shows an electron micrograph of a mast cell with the many dense-body secretory granules (vesicles) clearly shown.

Mast cells release mediators through multiple mechanisms. Exocytosis, in which mediator-containing granules fuse with the cellular membrane to allow the contents of the granules to diffuse into the extracellular space, has been studied more thoroughly than other mechanisms. As mentioned previously, the allergic response is initiated by mast cell exocytosis triggered by the cross-linking of surface-bound IgE by multivalent allergen. The subsequent intracellular signaling ultimately produces an increase in cytosolic Ca^{2+} concentration.[23] Ca^{2+}-activated granule fusion induces exocytosis via the interactions of vesicle membrane-associated proteins, such as synaptotagmin and synaptobrevin, and cell membrane-associated proteins, including SNAP-25 and syntaxin.[28] These and other

proteins coordinate to form SNARE complexes, which facilitate fusion of the vesicular and

plasma membranes and the release of the granular contents to the extracellular space.[28]
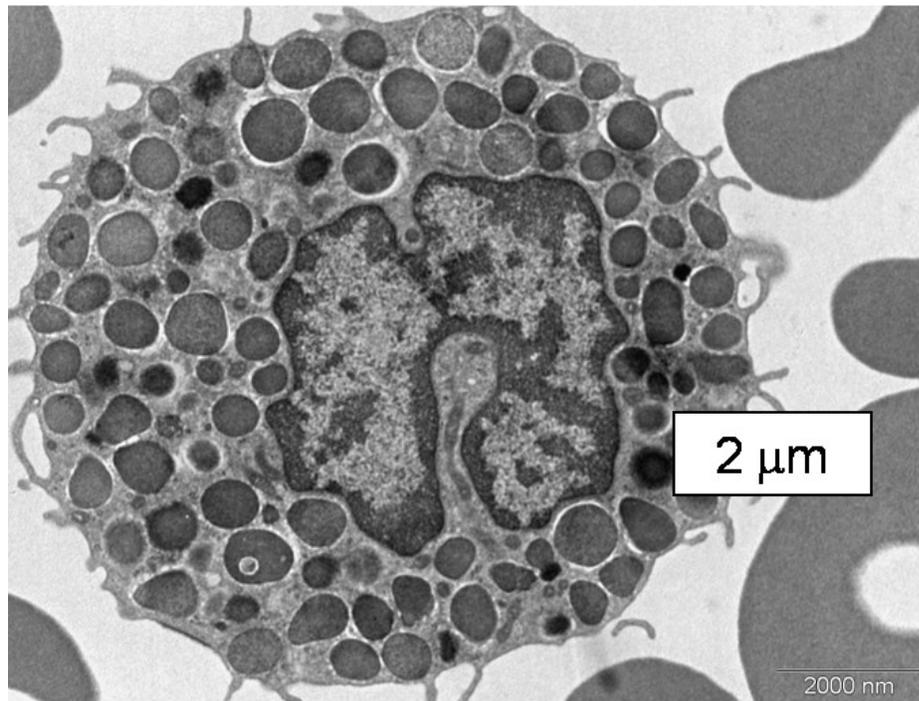
Figure 1.4. Transmission electron micrograph of a primary culture murine peritoneal mast cell.
Collected by Kathy L. Houle (nee Braun) and Dr. Bryce J. Marquis.

In addition to exocytosis of preformed mediators, mast cells also produce cellular signaling molecules via enzymatic action on the phospholipid molecules comprising the membrane, as described in section 1.3. Phospholipases are largely responsible for the production of AA, which is then subsequently transformed into the leukotriene and prostaglandin sub-classes of eicosanoids through enzymatic reactions of AA with hydrolases and synthases, as detailed in Fig. 1.1.[1,5,29] Mast cells also generate and secrete PAF; however, this biochemical pathway is much less understood.

1.3.4.2 Important Mast Cell-Secreted Lipid Mediators

There are many eicosanoid species with interesting physiological effects pertinent to a variety of cellular and disease processes, however, a full description of eicosanoid functions is beyond the scope of this thesis. Herein, this work will focus on two classes of eicosanoids: prostaglandins and leukotrienes. More specifically, this research involves PGD_2 and leukotrienes C_4, D_4, and E_4 (LTC_4, LTD_4, and LTE_4), as well as PAF, a non-eicosanoid inflammation-relevant soluble phospholipid, which is also synthesized via enzymatic transformations of phospholipids.

PGD_2 is formed as shown in Fig. 1.1. Once secreted, PGD_2 is rapidly dehydrated to form biologically active products, and it has been shown that the dehydration products of PGD_2, rather than PGD_2 itself, are responsible for many of the lipid's biological effects,

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including vasodilation and bronchoconstriction. PGD_2 is secreted by mast cells and has approximately 50x the bronchoconstriction potency of histamine.

LTC_4, LTD_4, and LTE_4 have historically been collectively referred to as the slow-reacting substance of anaphylaxis, as they were initially thought to be one substance. They are formed as shown in Fig. 1.1, with LTD_4 being a short-lived but highly potent bronchoconstriction intermediate between LTC_4 and LTE_4.

PAF is a potent mediator of anaphylaxis with biological activity at concentrations as low as 10^{-12} M. When secreted from mast cells, PAF activates other mast cells as well as surrounding cell types including platelets and macrophages.

1.4 Membrane-bound Phospholipids Have Critical Roles in Cell Signaling

Cell-secreted lipids are widely known to have incredible structural and functional diversity, and the phospholipid precursors from which they are generated also have important roles in influencing cellular function. Traditionally, the cellular membrane has been thought of primarily as a protective barrier separating and regulating the contents of the cell from the extracellular milieu, with membrane-bound phospholipids serving primarily a passive role as the structural foundation of this barrier.[30] Many recent studies have shown that, in fact, the cellular membrane plays a remarkably active role in many cellular functions, and membrane-bound phospholipids in particular are used as cellular signaling molecules.[30]

1.4.1 Molecular Asymmetry of Phospholipids in the Cellular Membrane

Within the cellular membrane, an enzymatically maintained asymmetry of the distribution of individual phospholipids is an important determinant of membrane function. The extracellular face, or outer leaflet, of the membrane is composed largely of phosphatidylcholine (PC) and sphingomyelin (SM), while the cytosolic side, or inner leaflet, is composed primarily of phosphatidylserine (PS) and phosphatidylethanolamine (PE). The asymmetry of phospholipids in cellular membranes is maintained through the actions of adenosine triphosphate (ATP)-dependent phospholipid translocases. In healthy unactivated cells, these enzymes selectively transport phospholipids to the appropriate leaflet whenever a concentration gradient in the lipids is detected.[31,32] When the asymmetry of the phospholipid membrane is disrupted, PS is exposed to the outer leaflet, which signals to surrounding cells that the cell exposing PS is apoptotic. Uncontrolled disruption of membrane asymmetry through defects in ATP-dependent phospholipid translocases is the cause of the bleeding disorder Scott syndrome, and is potentially responsible for other disorders related to cellular malfunction.[31]

1.4.2 Inner-Leaflet Phospholipids

The primary cell membrane inner leaflet phospholipids are the two aminophospholipids, PE and PS, whose structures are shown in Fig. 1.5.

PE is the second-most abundant phospholipid in mammalian cells, comprising 20-50% of the total membrane phospholipid content.[30] As with PS, PE is localized to the inner cellular membrane in resting cells and transported to the outer membrane upon cellular

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activation. Disruption of PE distribution within heart tissue has been implicated in the development of ischemia.[33] PE is the precursor for the synthesis of many secreted lipid metabolites and serves as a binding site for several surface-bound proteins with roles in cell signaling.[34]

PS is less abundant than PE, comprising approximately 2-10% of cellular phospholipid content.[30] Due to its role in the signaling of apoptosis, the maintenance of membrane asymmetry of PS has been studied extensively in the past decade.[30] PS is constrained to the inner leaflet via the action of an ATP-dependent aminotranslocase enzyme in resting cells. Upon cellular activation, aminotranslocase activity is suppressed, and Ca^{2+}-dependent lipid scramblase is activated, which results in exposure of PS to the outer leaflet, creating a more negatively charged cell surface. In platelets, this facilitates the binding of a variety of coagulation factors via ionic and stereoselective interactions.[35] PS facilitates the binding of vitamin K-dependent coagulation factors and allows conformational changes for maximum function of the coagulation proteins tenase and prothrombinase, leading to production of coagulation complexes.[35] There is evidence that membrane PS level and activity is regulated by other phospholipids, including PE.[36]
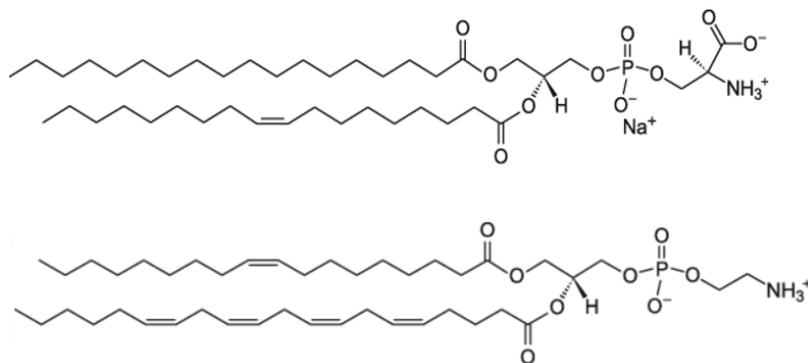
Figure 1.5. Structures of phosphatidylserine (top) and phosphatidylethanolamine (bottom).

1.4.3 Methods for Evaluating Membrane Phospholipid Content

Analysis of phospholipids and phospholipid metabolites such as PAF is traditionally performed using normal phase HPLC, hydrophilic interaction liquid chromatography (HILIC), or reversed phase HPLC. Studies employing normal phase HPLC or HILIC generally center on the separation of classes of phospholipids, i.e. phospholipids with a common headgroup and many variations of phospholipid tail length and unsaturation. Reversed phase HPLC enables separation of individual phospholipid species, but this can be problematic due to the incredible diversity of phospholipids with a common headgroup. The limitations of each type of chromatography present scientists with a choice in the study of phospholipids: analyze phospholipids by headgroup class or selectively analyze several specific phospholipid species. In either scenario, phospholipids must be extracted from cellular samples and separated within a reasonable timeframe, and phospholipid standards generally contain a predominant phospholipid species along with several other tail lengths. For this reason, phospholipid analysis is difficult, and few MRM methods are available because absolute quantitation of phospholipid species would require extensive purification of standards. Additionally, most LC/MS methods require at least 30 min separation times. In this dissertation, a compromise was made to evaluate platelet uptake of specific phospholipid species: UPLC-MS/MS was used for relative quantitation of the primary component of several phospholipid standards.

1.4.4 Platelets as a Platform for Understanding Phospholipid Function

Many studies regarding the function of phospholipids or the interaction of phospholipids with other membrane components have been performed using model membranes, which eliminate much of the biocomplexity of cellular membranes.[37-39] Studies on model membranes have established a foundation for understanding the interactions between phospholipids such as phosphatidylcholine, sphingomyelin, phosphatidylethanolamine, and phosphatidylserine with important membrane components such as cholesterol.[37] Exploring the effects of phospholipids on nucleated cells is challenging, as exposure to exogenous phospholipids can induce up- or down-regulation of proteins relevant to phospholipid conversion. In this dissertation, complications of protein effects are avoided while maintaining biocomplexity by studying anuclear platelets; these studies also facilitate critical insight about membrane uptake and the subsequent effects of phospholipids on this physiologically important system.
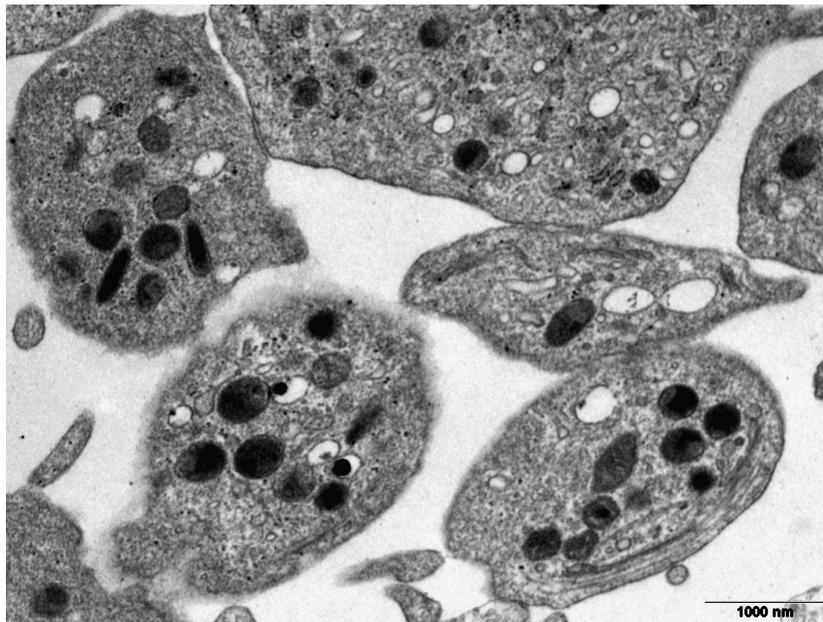
Figure 1.6. Transmission electron micrograph of murine platelets.
δ-granules are characterized by their dark electron-rich core within a larger granule, α-granules are the dark gray granules of heterogeneous color and shape, and lysosomes are light gray. Image courtesy of Drs. Secil Koseoglu and Melissa A. Maurer-Jones.

1.4.4.1 Overview of Platelets

Platelets are small (1-2 μm diameter) circulating cell-like bodies that were first discovered in the late 1800s through independent studies by Osler and Bizzozero on the blood-borne disc-like structures that were initially thought to be independent organisms in the blood.[40] A transmission electron micrograph of several murine platelets in Fig. 1.6 shows the anuclear nature and storage granule-rich cytosol of platelets. Anuclear platelets have a lifespan of 7-10 days, are generated from bone marrow-resident megakaryocytes, and are unique to mammals.[40,41] In circulation, platelets are discoid cell bodies that rapidly change their morphology in response to stimuli such as extracellular Ca^{2+}, glycoproteins, receptors on the surface of damaged blood vessels, tissue factor proteins, or fibrinogen. Upon activation, platelets form pseudopods before flattening on a damaged surface. While undergoing a change in shape, platelets secrete a wide variety of chemical messenger species that serve to activate other nearby platelets and assist in clot formation. Platelets have long been known for their role in clotting, and recent research has shown that platelets use surface receptors and other cell signaling molecules to promote wound repair, influence gene expression of white blood cells, and store/utilize mRNA, all despite the fact that they lack a nucleus.[42]
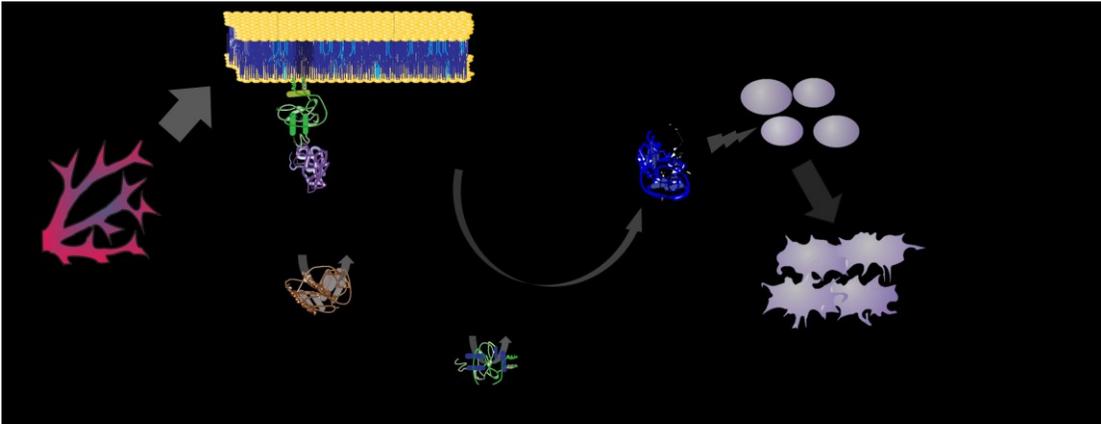
Figure 1.7. Simplified schematic of role of platelets the coagulation cascade.

1.4.4.2 Platelet Secretory and Adhesion Functions

Platelets contain three types of cytosolic granules that store and, upon initiation of platelet activation, secrete chemical messenger species while undergoing an irreversible shape change. Dense-body, or δ-granules, store serotonin, polyphosphate, ADP, and ATP. α-granules are heterogeneous and store a wide variety of proteins involved in coagulation, generally including platelet factor 4, P-selectin, and other soluble chemokines. Lysosomes store enzymes including β-Hexosaminidase that cleave protein products secreted from platelets. Platelets also secrete bioactive lipids, including eicosanoids and PAF, which contribute both to the amplification and resolution of the inflammatory response. When secreted by activated platelets, PAF acts through autocrine and paracrine mechanisms to further activate surrounding platelets.

Upon activation, platelets undergo a shape change that allows multiple platelets to adhere to one another, enabling clot formation.[43] Injury to blood vessel walls exposes the proteins collagen and von Willebrand factor, which activate platelets to secrete their chemical messenger cargo and expose PS and PE, which are binding sites for coagulation factor proteins, to their extracellular surface.[43-45] A cascade of reactions that cleaves the inactive circulating forms of clotting proteins to their active forms ultimately results in the formation of fibrin, which forms a mesh that operates in conjunction with activated platelets to repair damaged vessels.[43] Premature dissolution of clots is also prevented in part by platelet phospholipids, which prevent access of fibrinolysis enzymes to cleavage sites until the platelets incorporated into the clot lose their structural integrity.[44,45]

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1.4.4.3 The Platelet Plasma Membrane

As with most mammalian cells, the plasma membrane of platelets consists of membrane-bound proteins, cholesterol, and asymmetrically distributed phospholipids. Due to their anucleate nature, the plasma membrane of platelets plays a particularly important role in platelet signaling,[41] Platelets were selected for the studies in this dissertation in part because their anuclear character limits dramatic up- or down-regulation of protein production following exposure to exogenous phospholipids.

1.5 Conclusion

Lipids, both as the cell-secreted mediators of inflammation and as important structural components of all mammalian cell membranes, have critically important roles in the functions of cells in the immune system. The principal theme of this dissertation is developing and applying UPLC-MS/MS methods to improve fundamental understanding of both of mast cell function in complex cell cultures and of the role of phospholipids in platelet signaling.

The advent of UPLC-MS/MS enables rapid, sensitive, and selective analysis of multiple structurally diverse lipids simultaneously, which enables us to study critical cellular functions and the effects of secreted bioactive lipids. Both mast cells and platelets present interesting and challenging platforms for the application of new methods for studying secreted and structural lipid function.

Chapters 2-5 of this dissertation explore UPLC-MS/MS method development and application toward fundamental studies of mast cell and platelet function. Additionally,

this dissertation demonstrates the importance of the selection of cell culture model for understanding mast cell function. More specifically, Chapter 2 details the development of an UPLC-MS/MS method for simultaneous detection of five bioactive lipids and its application to lipid detection using a model inflammatory cell line and primary culture mast cells. Chapter 3 applies the method developed in Chapter 2 to explore the time-dependence of primary culture mast cell response to allergens and to inflammatory cytokines and explores the serotonin processing capacity of the heterogeneous cell culture.

Platelets are used as a platform to probe cellular uptake of exogenous phospholipids and the effects of phospholipid enrichment on secretory behavior, adhesion behavior, and platelet cholesterol content. Through this dissertation, the capacity of platelets to incorporate some, but not all, exogenous phospholipids into their membranes is revealed. Additionally, it is shown that platelets offer a compromise of biocomplexity between model membranes and nucleated cells that enables their use as an ideal platform for understanding phospholipid roles in cellular membranes. Chapter 4 details a study that determines whether platelets incorporate exogenous phospholipids into their membranes and how such incorporation affects their secretion and adhesion behaviors. Chapter 5 expands on the concept of phosphatidylserine effects on platelets by exploring concentration- and stereochemistry-dependent effects on platelet uptake of phosphatidylserine and explores how membrane enrichment of phosphatidylserine influences not only platelet behavior but also platelet cholesterol content.

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In Chapter 6 of this dissertation, the importance of scientific outreach activities is highlighted through the development and implementation of minimally guided inquiry-based mystery solving activity for middle school-age children. As described in the chapter, students are presented with a mystery and a kit of evidence, then guide themselves through analysis of the evidence by determining which of an available set of chemical tests to apply to the different pieces of evidence.

Overall, the work in this dissertation contributes the development and application of UPLC-MS/MS for simultaneous determination of structurally diverse lipids and exploits this method to achieve new insights into the fundamental role of phospholipids in mast cells and platelets.

Chapter 2: Isotope-dilution UPLC-MS/MS Determination of Cell-Secreted Bioactive Lipids

In part from: Audrey F. Meyer, John W. Thompson, Yiwen Wang, Secil Koseoglu, Joseph J. Dalluge, and Christy L. Haynes. Isotope-dilution UPLC-MS/MS determination of cell-secreted bioactive lipids. *Analyst*, 2013, 138, 5697-5705.

2.1 Overview

Secreted bioactive lipids play critical roles in cell-to-cell communication and have been implicated in inflammatory immune responses such as anaphylaxis, vasodilation, and bronchoconstriction. Analysis of secreted bioactive lipids can be challenging due to their relatively short lifetimes and structural diversity. Herein, a method has been developed using ultraperformance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) to quantify five cell-secreted, structurally and functionally diverse bioactive lipids (PGD_2, LTC_4, LTD_4, LTE_4, PAF) that play roles in inflammation. Sample analysis time is 5 min, and isotopically labeled internal standards are used for quantification. This method was applied to an immortal secretory cell line (RBL-2H3), a heterogeneous primary cell culture containing peritoneal mast cells, and murine platelets. In RBL cell supernatant samples, intrasample precisions ranged from 7.32–21.6%, averaging 17.0%, and spike recoveries in cell supernatant matrices ranged from 88.0–107%, averaging 97.0%. Calibration curves were linear from 10 ng/mL to 250 ng/mL, and limits of detection ranged from 0.0348 ng/mL to 0.803 ng/mL. This method was applied to the determination of lipid secretion from mast cells and platelets, demonstrating broad applicability for lipid measurement in primary culture biological systems.

2.2 Introduction

Bioactive lipids play critical roles in cell-to-cell communication and have been implicated in inflammatory immune responses such as anaphylaxis, vasodilation, and bronchoconstriction.[1,2] The prevalence of bioactive lipids in a wide array of physiological

systems necessitates rapid methods for detection, identification, and quantification of these molecules, particularly with regard to their role in inflammation. As such, examining bioactive lipid secretion from the RBL-2H3 cell line (RBL cells),[3] widely employed as a model for histamine-producing immune system cells, and from murine peritoneal mast cells (MPMCs) represent ideal platforms for proof-of-principle implementation of this new lipid analysis method. This work employs a newly developed UPLC-MS/MS method to study lipid secretion in a proof of principle study in RBL cells and subsequently applies the method to primary culture MPMCs to characterize the immune cell response to both allergen- and bacterial pathogen-based stimuli. The challenges in examining mast cells motivated the inclusion of both RBL cells and MPMCs: RBL cells are homogenous and prolific but like other 'mast cell' lines are not necessarily a good model of *in vivo* mast cell function, while it is difficult to obtain a homogenous sample of pure primary culture mast cells. The method demonstrated herein is applicable to lipid detection from both immortal (homogenous) and primary (heterogeneous) mast cells.

Immune system cells, including mast cells, release small molecule inflammatory mediators (e.g. histamine) in response to stimulants such as Ca^{2+} ionophore; in parallel, stimulated cells release bioactive lipids through enzymatic transformation of membrane phospholipids.[3, 4] During this process, phospholipases are largely responsible for the production of arachidonic acid, which is subsequently transformed into leukotriene (LT) and prostaglandin (PG) metabolites through hydrolase and synthase enzymatic reactions (Fig. 1.1).[5] The metabolites LTC_4, LTD_4, LTE_4, and PGD_2, especially, are of interest in

studies of inflammation as they have been implicated in a host of responses from bronchoconstriction in asthma to the wheal and flare reaction in hives.[4] PGD_2, LTC_4, LTD_4, and LTE_4 are all arachidonic acid metabolites with 20 carbon chain backbones.[6,7] Platelet-activating factor (PAF), an important lipid mediator not derived from arachidonic acid, plays a similar role in allergic/inflammatory response.[8] Moreover, PAF is both released from and an activator of immune system cells and has the ability to aggregate platelets.[7,9] Accordingly, these five species are the target analytes for the work presented herein. Although the method herein was validated for bioactive lipid secretion from RBL cells and a primary peritoneal cell culture containing mast cells, it could also be applied to quantitative analysis of lipid mediator secretion from other immune system cells such as macrophages, which also secrete LTC_4, LTD_4, LTE_4, and PAF.[10] Additionally, because this method allows simultaneous quantification of two classes of arachidonic acid-generated lipids as well as the phospholipid PAF, it could be easily adapted for quantitative analysis of other eicosanoids and PAF-like lipids.

The fundamental insight that can be gained about cellular communication processes by examining bioactive lipid secretion is critical for elucidating mechanisms of cell-cell communication and subsequently identifying potential therapeutic targets in systems ranging from the spinal cord to endothelial cells and neutrophils.[11,12,13,14] Secretion of bioactive lipids is typically quantified using enzyme-linked or radiolabeled immunoassays, which lack the sensitivity of mass spectrometry and the ability to simultaneously examine secretion of multiple lipid species from a population of cells. Additionally, the short-lived

nature (half-lives of min to hours) of cell-secreted bioactive lipids[15,16,17,18] requires rapid, high-throughput analysis with the use of isotopically labelled internal standards for each analyte to accurately quantify these species. This is particularly true for PGD_2 that is rapidly dehydrated to physiologically relevant products with roles in fatty acid transport, and metabolism.[19] As such, use of isotopically labelled internal standards to assure that measurement of these products is correlative to initial PGD_2 concentrations (regardless of matrix) is imperative. Non-MS methods for lipid quantification such as flow cytometry,[20] enzyme-linked immunoassays,[21] or radioimmunoassays[22] are time-consuming, labor-intensive, and even when multiplexed, rely on antibodies, which can suffer from lack of specificity for lipid targets.[23] Previous studies using LC/MS-based methods to quantify lipid species require a column switching system,[23] chemical derivatization procedures,[24] or quantify only arachidonic acid metabolites.[25,26,27,28] In addition, although UPLC has been applied to eicosanoid determination, only one method is available for simultaneous determination of both PAF and arachidonic acid metabolites[23] and several do not use a comprehensive suite of internal standards, which is especially important to assure accurate quantitation of short lived cell-secreted bioactive lipids.[23,25,26] Due to limitations of previously reported methods, we have developed a method using ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) for the simultaneous quantification of the inflammatory cell-secreted lipids PGD_2, LTC_4, LTD_4, LTE_4, and PAF. The method reported here is in addition, significantly faster from the standpoint of total analysis time including sample preparation (180 samples, 19 h), than a previously reported

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method employing solid-phase extraction and column-switching LC/MS/MS for the same purpose (96 samples, 26 h).[23]

This work details the UPLC-MS/MS method development and its application to the targeted analysis of secreted lipid mediators from both a model inflammatory cell line, RBL cells, and a primary cell culture containing mast cells, exposed to lipopolysaccharides (LPS), a component of bacterial cell membranes. RBL cell response to LPS was examined in this study to demonstrate the method's feasibility and because recent studies indicate that mast cells play a critical role in the innate immune system in addition to their roles in inflammation. LPS, a component of bacterial cell membranes, has been found to induce release of cysteinyl leukotrienes in mast cells as well as to stimulate mast cell migration.[29,30] RBL cells were incubated with LPS and subsequently exposed to the Ca^{2+} ionophore calcimycin (A23187), which was used to induce RBL cell activation, enabling examination of RBL cell response under conditions modelling bacterial exposure during inflammatory response. Following development in the RBL cell line, this method was applied to examine bioactive lipid secretion from MPMCs. MPMCs are one component of a heterogeneous cell population that includes macrophages and fibroblasts. Examination of cell-cell interaction in a heterogeneous population is at once incredibly complex and necessary to understand the mechanisms by which immune cells communicate. To demonstrate the versatility of the method described here, it was also applied in a preliminary study of bioactive lipid secretion from platelets.

2.3 Experimental Approach

2.3.1 Reagents

LTC_4, LTD_4, LTE_4, PGD_2, and PAF and internal standards LTC_4-d_5, LTD_4-d_5, LTE_4-d_5, PGD_2-d_9, and PAF-d_4 were purchased from Cayman Chemical (Ann Arbor, MI) and used as received. J.T. Baker Ultra LC/MS-grade acetonitrile (ACN) and water were purchased from VWR International (Radnor, PA). Fisher Optima LC/MS-grade isopropyl alcohol (IPA) was purchased from Fisher Scientific (Pittsburgh, PA). Anti-TNP IgE was purchased from BD Biosciences (Bedford, MA), and LPS from *Escherichia coli* were purchased from Sigma Aldrich (St. Louis, MO) and used as purchased. Trizma-HCl, NaCl, KCl, *d*-glucose, $CaCl_2$, $MgCl_2$, and thrombin were purchased from Sigma Aldrich (St Louis, MO). Tris buffer was prepared with 12.5 mM Trizma-HCl, 150 mM NaCl, 4.2 mM KCl, 5.6 mM glucose, 1.5 mM $CaCl_2$, and 1.4 mM $MgCl_2$. High glucose Dulbecco's Modified Eagle Medium (DMEM) and Ca^{2+}, Mg^{2+}-free phosphate-buffered saline (PBS) were purchased from Invitrogen (Grand Island, NY), and a mixture of 100 units penicillin and 10,000 mg streptomycin mixture and bovine calf serum (BCS) were purchased from Fisher Scientific.

Table 2.1. Transitions monitored for UPLC-MS/MS detection of secreted lipids.

Compound	MRM Transition	Cone (V)	Collision Energy (eV)
LTC$_4$	626.4$\rightarrow$189.2	44	16
	626.4$\rightarrow$308.1	44	16
LTC$_4$-d$_5$	631.4$\rightarrow$194.2	45	17
	631.4$\rightarrow$308.2	45	17
LTD$_4$	497.3$\rightarrow$189.2	35	14
	497.3$\rightarrow$301.2	35	14
LTD$_4$-d$_5$	502.4$\rightarrow$194.3	33	11
	502.4$\rightarrow$306.3	33	11
LTE$_4$	440.3$\rightarrow$189.2	22	11
	440.3$\rightarrow$301.3	22	11
LTE$_4$-d$_5$	445.4$\rightarrow$194.2	31	10
	445.4$\rightarrow$306.3	31	10
PAF	524.4$\rightarrow$86.1	36	23
	524.4$\rightarrow$184.1	36	23
PAF-d$_4$	528.5$\rightarrow$86.2	50	22
	528.5$\rightarrow$184.1	50	22
PGD$_2$[a]	335.3$\rightarrow$233.2	29	10
	335.3$\rightarrow$299.2	29	10
PGD$_2$-d$_9$[a]	344.4$\rightarrow$242.3	30	12
	344.4$\rightarrow$308.3	30	12

[a] Transitions monitored for PGD$_2$ and PGD$_2$-d$_9$ correspond to a rapidly formed, physiologically relevant hydrolysis product of PGD$_2$.[19]

2.3.2 Sample Preparation and Ultraperformance Liquid Chromatography-Electrospray Ionization-Tandem Mass Spectrometry

Lipid extraction was necessary to isolate the polar lipids of interest from the Tris buffer, which contained non-volatile salts including NaCl, Trizma HCl, MgCl$_2$, CaCl$_2$, and KCl, all of which affect the sensitivity of the method and significantly foul the mass spectrometry ionization source over time. Several liquid-liquid extraction methods were explored, including extraction with 2/20/30 acetic acid/isopropanol/hexane,[31] sequential

extraction with acetone and hexane,[32] and the widely used Bligh & Dyer method of extraction with 1:2 $CHCl_3$:MeOH followed by extraction with $CHCl_3$ and H_2O.[33] Liquid-liquid extraction can induce error through user-to-user variability and sample loss through incomplete extraction of polar lipid analytes into the solvents used for extraction.[34] Additionally, liquid-liquid extraction techniques employed previously for prostaglandins are different from the extraction techniques required for cysteinyl leukotrienes and from platelet-activating factor.[35] The liquid-liquid extraction methods attempted were unsuccessful in simultaneous isolation of the five lipids of interest, as the analytes detected with this method have varying degrees of polarity. To avoid the pitfalls of liquid-liquid extraction, as well as time-consuming, labor-intensive solid-phase extraction procedures described in previously reported methods for lipid analysis[36], a sample preparation method was developed to rapidly remove the salts and proteins from cell supernatant samples while minimizing sample loss and recovery variation. By concentrating samples in a speedvac, it was possible to induce precipitation of salts in supernatants via addition of ice-cold ethanol and remove precipitated salts with centrifugation (see below).

A Waters Acquity UPLC coupled to a Waters triple quadrupole mass spectrometer (Acquity TQD) was used for separation and detection of mast cell-secreted lipids. A Waters BEH C_{18} 2.1 mm x 50 mm column (1.7 μm particles) at 45 °C was used during the following 5 min gradient separation with A: water containing 0.1% formic acid and B: 90/10 ACN/IPA containing 0.1% formic acid, at a flow rate of 0.6 mL/min: 30% B, 0 min to 0.5 min; 30% B to 50% B, 0.5 min to 1.0 min; 50% B 1.0 min to 2.0 min; 50% B to 90%

B, 2.0 min to 2.2 min; 90% B, 2.2 min to 3.2 min; 90% B to 30% B, 3.2 min to 3.5 min; and 30% B, 3.5 min to 5.0 min. By directly infusing each analyte and internal standard, cone voltages and collision energies for each selected reaction monitoring (SRM) transition were optimized. These SRM transitions are tabulated in Table 2.1. The transitions that produced the highest sensitivity for the determination of each analyte were selected for quantification: LTC_4: 626.4 to 189.2; LTD_4, 497.3 to 189.2; LTE_4 440.3 to 189.2; PAF, 528.5 to 184.1; PGD_2, 335.3 to 299.2. Dwell time for each transition was 0.01 s. For electrospray ionization tandem mass spectrometry (ESI-MS/MS) in positive ionization mode, parameters were as follows: capillary, 3.2 kV; extractor, 3 V; rf lens, 0.2 V; source temperature, 120 °C; desolvation temperature, 350 °C; desolvation flow, 800 L/h; cone gas flow, 20 L/h; low-mass resolution (Q1), 12 V; high-mass resolution (Q1), 12 V; ion energy (Q1), 0.3 V; entrance -5 V; exit, 1 V; varied collision energies listed in Table 2.1; low-mass resolution (Q2), 15 V; high-mass resolution (Q2), 15 V; ion energy (Q2) 3.5 V; multiplier, -542.8 V.

2.3.3 Method Validation

Spike recoveries with associated analytical precisions were assessed by quantification of each analyte in samples prepared by the addition of 25 ng/mL LTC_4, LTC_4-d_5, LTD_4, LTD_4-d_5, LTE_4, LTE_4-d_5, PAF, PAF-d_4, PGD_2, and PGD_2-d_9 to supernatants collected from a confluent RBL cell monolayer that had been incubated for three h with Tris buffer at 37 °C. To quantify spike recoveries, the concentrations for each lipid in the recovery samples were divided by the sum of 25 ng/mL and the average

concentration of the appropriate lipid in control samples. By quantifying spike recoveries in this way, it was possible to minimize interference from the endogenous levels of the bioactive lipids of interest. Cell-secreted lipids were quantified in samples prepared by the addition of 25 ng/mL LTC$_4$-d$_5$, LTD$_4$-d$_5$, LTE$_4$-d$_5$, PAF-d$_4$, and PGD$_2$-d$_9$ in 0.1% FA in 70/30 H$_2$O/90:10 ACN:IPA to supernatants immediately after collection from a confluent monolayer of RBL cells incubated appropriately for each experimental condition. Calibrants were prepared in 0.1% FA in 70/30 H$_2$O/90:10 ACN:IPA with 25 ng/mL LTC$_4$-d$_5$, LTD$_4$-d$_5$, LTE$_4$-d$_5$, PAF-d$_4$, and PGD$_2$-d$_9$. For standardization, 5 levels of calibration mixtures ranging from 0.5 ng/mL to 500 ng/mL were prepared for the five lipids and internal standards to achieve 5 different response ratios for each of the lipids in the mixtures, with the exception of PGD$_2$, for which 4 response ratios were used. These solutions were then analyzed by UPLC-MS/MS, and the data were subjected to a linear least squares analysis with the Waters Targetlynx™ software program. The peak area ratios of analyte:internal standard were then used in conjunction with the calibration curves to determine the concentration of individual lipids in the samples. Limits of detection and quantitation were calculated by determining the signal-to-noise values for samples spiked with 25 ppb analytes using the "Chromatograph" function of the Waters MassLynx™ software, then extrapolating the concentration at which the signal-to-noise value was 10 for LOQ or 3 for LOD.

2.3.4 Cell Culture and Stimulation

MPMCs were isolated from mice obtained from the University of Minnesota tissue sharing program. Briefly, 8-9 mL of Dulbecco's Modified Eagle Medium supplemented with 10% bovine calf serum and 1% penicillin and streptomycin were injected into the peritoneal cavity of each mouse immediately following euthanasia. Following a 30 second massage, approximately 6-7 mL per mouse were recovered. The collected cell suspension was pelleted at 450xg for 10 minutes and resuspended at 2×10^6 cells/mL in fresh media containing 0.5 µg/mL anti-TNP IgE (BD Biosciences). Cells were then plated on NIH-3t3 fibroblasts that had been plated 3 days prior to co-culture with the peritoneal cell suspension.

MPMCs incubated overnight with anti-TNP IgE were washed with Tris buffer to remove serum-containing media, then incubated for 2 hours with or without 2 µg/mL LPS. MPMCs with or without LPS exposure were then incubated for one hour in Tris buffer or Tris buffer containing 100 ng/mL TNP-ova or 10 µM A23187, a Ca^{2+} ionophore widely used to stimulate cell response through increasing intracellular Ca^{2+} concentration.[37]

RBL-2H3 cells purchased from American Type Culture Collection (Manassas, VA) were cultured in 75 cm^2 T-flasks and passaged using 0.1% trypsin. Passaged cells were plated in 12-well plates and cultured to a confluent monolayer. Cells were fed daily by replacement of half the volume of MEM/10% FBS/1% penicillin and streptomycin (a mixture collectively referred to as 'media') with fresh media. When a confluent monolayer had formed, cells were cultured overnight with 0.5 µg/mL anti-TNP IgE (BD Biosciences)

to sensitize the RBL cells. RBL cell stimulation was initiated through the addition of 20 μM A23187.[37]

Following overnight incubation with anti-TNP IgE, RBL cells were washed with Tris buffer to remove serum-containing media, then incubated for 2 h with or without 2 μg/mL LPS. RBLs with or without LPS exposure were then incubated for 1 h in Tris buffer or Tris buffer containing 20 μM A23187. RBL cells were exposed to A23187 after LPS to probe the role of cell response following bacterial pathogen exposure. Degranulation was terminated by incubating the cells on ice for 10 min. Supernatants were collected and spiked with 25 ng/mL of each internal standard, then concentrated to 50 μL. Salts and proteins were precipitated by the addition of 100 μL molecular biology grade EtOH containing 0.1% FA, followed by the addition of 300 μL 0.1% FA in 90/10 ACN/IPA. Precipitated salts were removed by centrifugation for 10 min at 2500 x g, and supernatants were concentrated to approximately 40 μL. Samples were then diluted to 100 μL with a 70/30 mix of LC mobile phase A/B.

2.3.5 Degranulation Assay

To assess degranulation of MPMCs and RBL cells, the secretion of the granule-stored enzyme β-Hexosaminidase (β-Hex) was quantified using a previously published protocol.[38] Briefly, from stimulated and unstimulated cell conditions, 25 μL of cell supernatant was incubated with 50 μL 0.1 M citrate buffer (pH 4.3) containing 1 mM p-nitrophenyl acetyl-D-glucosamine. To quench the enzymatic reaction, 150 μL of ice-cold glycine carbonate buffer at pH 9 was added. Absorbance values for six replicates of each

cell incubation and stimulation condition were read at 405 nm. As a background for intensity determination, absorbance at 630 nm was subtracted from absorbance at 405 nm. Secreted β-Hex for each RBL cell incubation and stimulation condition are reported as a percentage of secreted β-Hex content from unstimulated control cells.[38]

2.4 Results and Discussion

Determination of bioactive lipids in biologically relevant systems requires methods that are capable of purifying and detecting structurally diverse lipids, are sensitive, require minimal time for sample preparation and analysis, and are applicable to a wide range of cell or tissue culture platforms. The method presented in this paper is capable of purifying both eicosanoids (PGD_2, LTC_4, LTD_4, and LTE_4) and PAF, employs a 5 minute UPLC-MS/MS analysis time, and has been applied to an adherent model cell line, a primary culture of inflammatory cells, and a primary suspension culture platelets. Future applications of this method could include neutrophils, which would be an ideal platform for this method, as they play important roles in the immune system, can be easily isolated from human subjects, and are known to generate platelet-activating factor and eicosanoids. Primary culture macrophages would be another interesting system to which this method could be applied. Bioactive lipid generation in tissue or multi-cellular systems should also be explored, and applying this method to a diverse array of cellular and tissue models would require incorporation of additional tissue- or cell- appropriate lipids into the detection method.

2.4.1 Method Development

In this study, a method was developed to rapidly quantify secreted bioactive lipids from RBLs using UPLC-MS/MS. A variety of gradient chromatography methods were attempted to select the chromatographic separation that enabled rapid separation and detection of PGD_2, LTC_4, LTD_4, LTE_4, and PAF. Column selection, flow rate, and mobile phase composition were optimized to develop a 5 min method to simultaneously detect the lipids of interest.

Waters Acquity HSS-T3 C_{18} 2.1 mm x 100 mm, BEH Shield RB18 C_{18} 2.1 x 100 mm, BEH C_{18} 2.1 x 100 mm, and BEH C_{18} 2.1 x 50 mm columns were tested. The best separation was achieved using a Waters Acquity BEH C_{18} 2.1 x 50 mm column. Flow rates of 0.4 mL/min to 0.8 mL/min were tested, and 0.6 mL/min was found to be the optimal flow rate from the standpoint of chromatographic resolution combined with minimal analysis time. Organic mobile phases tested were 0.1% formic acid in 50:50 ACN:MeOH, 0.1 % formic acid in 50:50 ACN:IPA, and 0.1% FA in 90:10 ACN: IPA. Complete elution of all lipids within the desired timeframe without analytical carryover was achieved only when using 0.1% FA in 90:10 ACN:IPA. Using other organic mobile phases resulted in much longer retention times for PAF, whereas use of 90:10 ACN:IPA achieves separation of five structurally dissimilar lipids within 5 min. Representative SRM chromatograms for each bioactive lipid are shown in Fig. 2.1. Fig. 2.1 shows that LTC_4 and LTE_4 coelute. Cross-channel interference from these coeluting species or any of the analytes determined here has not been observed, and addition of isotopically labelled standards for each analyte

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compensates for any ionization suppression due to their co-elution. For RBL cell samples, all calibration curves were linear over the range of 10-250 ng/mL, and correlation coefficients averaged 0.989. For MPMC samples, all calibration curves were linear over the range of 1-250 ng/mL with the exception of PAF, which was linear over the range of 2.5-2500 ng/mL. Correlation coefficients averaged 0.994.

To evaluate the accuracy of the method, lipid analytes and internal standards were spiked into unstimulated RBL cell supernatants at a concentration of 25 ng/mL prior to sample processing. Samples were then processed with salt precipitation and concentrated as described for RBL cell secretion samples. UPLC-MS/MS was performed, and concentrations of lipid analytes were calculated from calibration curves for each analyte. The results of these spike/recovery experiments together with limits of detection, limits of quantitation, linear range, and precisions, for each lipid are shown in Table 2.2.

2.4.2 Application of the Developed Method to Assess Inflammatory Response of RBL Cells and MPMCs

To demonstrate that this method can be employed in the study of lipid based cell-cell communication, it was used to detect secreted lipids from RBL cells with and without exposure to LPS. RBL cells were exposed to Tris buffer as a control, A23187 to assess lipid secretion in allergic response, LPS to assess lipid secretion by mast cells in response to bacterial antigens, and a combination of LPS and A23187 to probe the effects of bacterial antigens on stimulated cell response. Table 2.3 illustrates the secreted quantities of LTC_4, LTD_4, LTE_4, PAF, and PGD_2 in response to each of these conditions. A similar study was

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performed using MPMCs stimulated by the IgE-mediated pathway in response to TNP-ova, with and without pre-exposure to LPS, and the secreted quantities of the bioactive lipids from MPMCs under these experimental conditions are shown in Table 2.4. Summaries of both studies follow.

2.4.2.1 RBL Cell Secretion of Leukotrienes

Control conditions and stimulation with A23187 without LPS pre-incubation did not produce detectable levels of LTC_4 or LTD_4; however, A23187 stimulation increased LTE_4 secretion. As Fig. 1.1 shows, LTC_4, LTD_4, and LTE_4 are generated sequentially when secreted from mast cells; as such, LTE_4 is expected to be the leukotriene highest in concentration. A marked increase, consistent with previously reported mast cell studies, in the amount of LTC_4, LTD_4, and LTE_4 secreted from RBL cells was observed after incubation with LPS followed by stimulation with A23187, (p =0.014 for LTE_4).[39] This increase in cysteinyl leukotriene secretion indicates that LPS incubation acts synergistically with A23187 stimulation, which could indicate that bacterial exposure has the potential to amplify RBL cell inflammatory response.

2.4.2.2 RBL Cell Secretion of Prostaglandins

As expected, PGD_2 was secreted upon stimulation with A23187. LPS incubation did not produce a significant difference in A23187-stimulated PGD_2 secretion from RBLs (p = 0.99). In contrast to enhanced leukotriene secretion by LPS-exposed RBL cells, the

lack of effect of LPS on RBL-secreted PGD_2 may indicate that bacterial exposure does not

promote RBL cell inflammation through pathways associated with PGD_2 production.
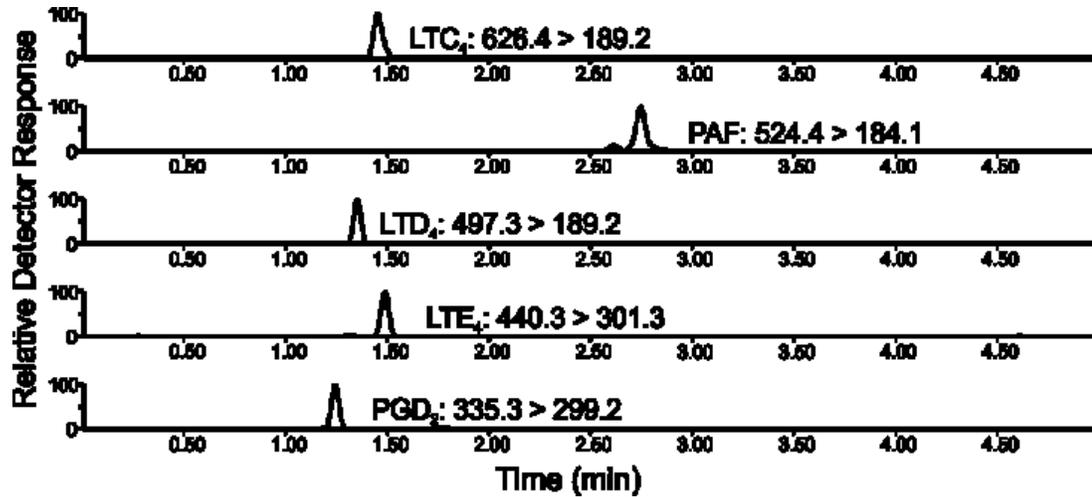
Figure 2.1. SRM transitions for five bioactive lipids secreted from RBL cells.

Table 2.2. Limits of detection, limits of quantification, linearities, precisions, and recoveries for lipid analytes.

Analyte	LTC_4	LTD_4	LTE_4	PAF	PGD_2
R^2 for Calibration	0.991	0.993	0.988	0.994	0.981
LOD (ng/mL)	0.283	0.540	0.0348	0.262	0.803
LOQ (ng/mL)	0.944	1.80	0.116	0.874	2.68
Recovery (% ± SD)	102 ± 15.8	98.2 ± 10.4	107 ± 9.5	88.0 ± 15.5	89.6 ± 8.0
Linear Range (ng/mL)	5-250	1-500	1-500	1-250	10-500
Instrumental Precision[a] (% RSD)	18.9	21.3	16.0	7.32	21.6
Biological Precision[b] (%RSD)	19.9	11.2	15.8	15.0	18.4

[a] Instrumental precision represents instrumental variability in RBL cell supernatant samples. In clean standards in 70/30 A/B with 0.1% FA, instrumental precision RSDs ranged from 6.65 to 20.4%, averaging 11.3%.

[b] These values represent biological variability, not analytical precision for n = 4 biological replicates. Three technical replicates were measured for each biological replicate.

Table 2.3. Bioactive lipids secreted from RBLs under conditions exploring LPS effects on A23187-stimulated cell function.[a]

Analyte	Control Incubation: Control Stimulation	Control Incubation: 20 µM A23187 Stimulation	2 µg/mL LPS Incubation: Control Stimulation	2 µg/mL LPS Incubation: 20 µM A23187 Stimulation
LTC_4 (ng/mL)	ND[b]	ND	ND	2.01 ± 0.48
LTD_4 (ng/mL)	ND	ND	ND	0.962 ± 0.11 Below LOQ for LTD_4
LTE_4 (ng/mL)	ND	1.68 ± 0.39	ND	2.65 ± 0.41
PAF (ng/mL)	6.54 ± 2.64	21.2 ± 0.4	2.63 ± 0.08	3.84 ± 0.47
PGD_2 (ng/mL)	ND	31.3 ± 8.8	ND	31.2 ± 6.6

[a] All concentrations are listed as ng/mL ± true biological standard deviation for n = 4 biological replicates for A23187-stimulated conditions and n = 3 biological replicates for control stimulation conditions. Three technical replicates were measured for each biological replicate.

[b] ND = not detected.

2.4.2.3 RBL Cell Secretion of Platelet Activating Factor

PAF secretion increased significantly upon stimulation with A23187 (p = 0.0007). LPS incubation without A23187 stimulation did not significantly increase PAF secretion over control levels (p = 0.1). The secreted level of PAF after LPS incubation plus A23187 stimulation was significantly higher than after LPS incubation alone (p = 0.03) and significantly lower than levels measured after A23187-stimulated PAF secretion without LPS incubation (p = 0.0001). This decrease, when considered in combination with the increase in secreted leukotrienes and lack of change in PGD_2 secretion indicates that LPS modulates RBL function, but has markedly different effects on the secretion of bioactive lipids formed through different enzymatic pathways.

Table 2.4. Bioactive lipids secreted from MPMCs under conditions exploring LPS effects on IgE-mediated and A23187-stimulated mast cell function.[a]

Analyte	Control Incubation: Control Stimulation	Control Incubation: 100 ng/mL TNP-ova Stimulation	Control Incubation: 20 µM A23187 Stimulation	2 µg/mL LPS Incubation: Control Stimulation	2 µg/mL LPS Incubation: 100 ng/mL TNP-ova Stimulation	2 µg/mL LPS Incubation: 20 µM A23187 Stimulation
LTC_4 (ng/mL)	ND	ND	16.1 ± 3.56	ND	ND	23.9 ± 2.0
LTD_4 (ng/mL)	ND	ND	ND	ND	ND	ND
LTE_4 (ng/mL)	ND	0.222 ± .077	2.83 ± 0.74	ND	0.385 ± 0.113	3.41 ± 0.87
PAF (ng/mL)	5.89 ± 1.16	5.67 ± 1.91	7.34 ± 3.78	3.91 ± 0.78	5.80 ± 2.61	7.13 ± 2.45
PGD_2 (ng/mL)	1.55 ± 0.91 Below LOQ	2.17 ± 1.07 Below LOQ	6.62 ± 2.77	1.94 ± 1.04 Below LOQ	4.38 ± 2.05 One biological replicate below LOQ	15.7 ± 5.2

[a] All concentrations are listed as ng/mL ± true biological standard deviation for n = 4 biological replicates. Three technical replicates were measured for each biological replicate.

[b] ND = not detected.

To further investigate the effect of LPS on RBL cell function, secretion of bioactive lipids detected using the UPLC-MS/MS method was correlated to RBL cell degranulation as assessed by the β-Hex assay, a standard method used in secretory cell analysis. β-Hex is a cytosolic granule-associated enzyme, and quantification of secreted β-Hex as a percentage of total β-Hex detected in lysed cells enables assessment of the effects of LPS on cell degranulation. As expected, stimulation with A23187 increased β-Hex secretion from control levels of 100% ± 19% to 282% ± 59% (note that reported uncertainties associated with measurement of β-Hex secretion represent absolute uncertainties, not RSDs, e.g., 100% ± 19% = 1.00 ± 0.19). LPS incubation did not significantly change β-Hex secretion (83.8% ± 12.3%, p = 0.1 versus control), and stimulation with A23187 of LPS-incubated cells with A23187 increased β-Hex secretion to 167% ± 36%, which is significantly lower than the percentage degranulation observed when RBL cells were stimulated with A23187 without LPS exposure (p = 0.002). When considering the effects of LPS exposure on A23187-induced RBL stimulation, it is evident that LPS incubation decreases the degree to which RBLs degranulate by 116% ± 38%. PAF and β-Hex secretion trends demonstrate LPS-induced inhibition of degranulation as well as decreased PAF secretion as shown in Fig. 2.2. It is evident that bioactive lipids are secreted from RBL cells through several mechanisms that can act synergistically with the well-characterized process of degranulation, as shown by the similarity in PAF and β-Hex secretion trends, but that lipid mediator secretion trends can deviate from exocytotic mediator secretion as

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well, as the trends in leukotriene and PGD_2 secretion are distinct from the bulk degranulation behavior.

2.4.2.4 MPMC Secretion of Leukotrienes

Under control conditions and exposure to TNP-ova, secreted LTC_4 was not detected from primary MPMCs. When exposed to A23187, LTC_4 secretion increased to 16.1 ± 3.6 ng/mL. When pre-incubated with LPS followed by exposure to A23187, LTC_4 significantly increased to 23.9 ± 2.0 ng/mL (p = 0.009). MPMC secretion of LTE_4 was observed under exposure to both TNP-ova and A23187. Upon exposure to TNP-ova, LTE_4 secretion increased to 0.222 ± 0.078 ng/mL. With pre-exposure to LPS, TNP-ova-induced LTE_4 secretion was 0.385 ± 0.113 ng/mL, which was not significantly different from LTE_4 secretion without LPS pre-exposure (p = 0.05). A23187-induced LTE_4 secretion with and without LPS pre-exposure was 2.83 ± 0.74 ng/mL and 3.14 ± 0.87 ng/mL, respectively, which were not significantly different (p = 0.6). LTD_4 was not detected in MPMC samples. Two separate experiments confirmed the trends observed in LTC_4 and LTE_4 secretion; in an additional experiment, increases in both LTC_4 and LTE_4 were observed under TNP-ova activation, and further increases were observed under A23187 activation. Pre-incubation with LPS enhanced the secretion of LTC_4 from MPMCs under A23187 activation but not TNP-ova activation, and it appears that LPS pre-incubation does not affect the secretion of LTE_4 from MPMCs.

2.4.2.5 MPMC Secretion of Prostaglandins

PGD_2 secretion from MPMCs was not consistently detected at levels above the limit of quantitation in control samples or control samples exposed to LPS. When exposed to TNP-ova, the increase in secretion of PGD_2 from MPMCs was not significant ($p = 0.4$). Likewise, exposing MPMCs to LPS followed by TNP-ova resulted in a non-significant increase in PGD_2 secretion ($p = 0.08$). When compared to control MPMCs, LPS pre-exposure followed by TNP-ova stimulation did significantly increase PGD_2 secretion ($p = 0.05$). However, A23187 induced a significant increase in PGD_2 secretion from both control (to 6.62 ± 2.77 ng/mL, $p = 0.013$) and LPS-exposed control MPMCs (to 15.7 ± 5.2 ng/mL, $p = 0.002$). A23187-induced PGD_2 secretion from MPMCs was also significantly greater from cells pre-incubated with LPS ($p = 0.02$), although LPS alone did not appear to induce PGD_2 secretion.

2.4.2.6 MPMC secretion of Platelet-activating Factor

Secretion of PAF from MPMCs was observed under all conditions, including from control samples. PAF secretion was significantly reduced by exposure to LPS in control samples from 5.89 ± 1.16 ng/mL to 3.91 ± 0.78 ng/mL, $p = 0.03$. Pre-exposure to LPS followed by A23187 significantly increased the secretion of PAF when compared to the non-activated LPS-exposed cells to 7.13 ± 2.45 ppb, $p = 0.05$. As MPMCs comprise a relatively small percentage of the heterogeneous cell population, and samples were incubated for a relatively long time with LPS and stimulants, it is possible that surrounding cell types transformed the secreted PAF prior to the collection of supernatants.

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The β-Hex assay was also applied to MPMC supernatants. Trends in β-Hex secretion from MPMCs matched those of RBL cells. TNP-ova stimulation was not tested in RBL cells, although it is interesting to note that in MPMCs, LPS exposure prior to stimulation reduced the extent to which mast cells degranulated when exposed to TNP-ova or to A23187 ($p = 0.004$ and 0.0001, respectively). Unlike RBL cells, the trend in the effects of LPS on MPMC degranulation did not match the trends in any of the lipids secreted. This is potentially due to the possibility that lipids secreted by other cell types present in the MPMC culture contribute to the detected lipid signal, particularly when cells are stimulated by A23187.

MPMC secretion of bioactive lipids in response to the effects of IgE-mediated antigen stimulation differed from that of RBL cells. This is likely due in part to the differences between a homogenous immortal cell line (RBL cells) and a heterogeneous primary cell culture (MPMCs). For example, LPS incubation increased secretion of PGD_2 secretion from MPMCs but did not affect PGD_2 secretion from RBL cells. While RBL cells increase PAF secretion in response to TNP-ova stimulation, curiously, MPMCs did not show a significant increase in PAF secretion. As previously described, this could be due to the heterogeneous cell culture of the MPMCs, which more closely models the *in vivo* environment of mast cells than the homogeneous immortal RBL cell line. Similarly, RBL cell leukotriene secretion in response to TNP-ova was much larger than that of MPMCs.

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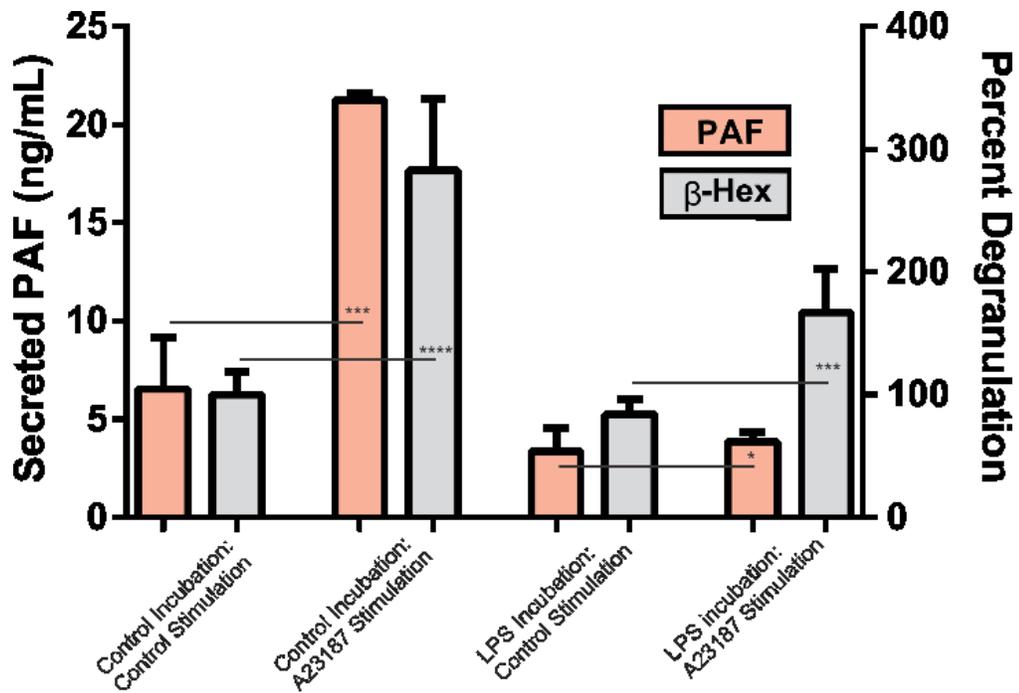
Figure 2.2. RBL cell secretion of PAF and β-Hex follows similar trends in response to LPS incubation and A23187 stimulation.
PAF secretion (red) is shown on the left-hand axis, and β-Hex secretion (gray) is quantified as a percentage of total RBL β-Hex content on the right-hand axis.
*p < 0.05; ***p<0.001, ****p<0.0001

To demonstrate the wide-ranging applicability of this method to detect lipid mediators in biological systems, it was also applied to the detection of PAF secretion from platelets. Platelets generate and secrete bioactive lipids, including PAF, upon thrombin activation. The accuracy of the UPLC-MS/MS method developed here for measurement of PAF in a platelet rich plasma matrix averaged 94.6% ± 17%, determined by spike/recovery experiments as described above (note that reported uncertainties associated with measurement of recovery represent absolute uncertainties, not RSDs, e.g., 94.6% ± 17.0% = 0.946 ± 0.17). To demonstrate measurement of PAF secretion in response to chemical stimulation, platelets were stimulated with thrombin, a physiological activator of platelets, which is critically important for platelet aggregation and thus, blood coagulation. Fig. 2.3 shows that, when stimulated by thrombin, platelet secretion of PAF increased significantly from 1.31 ng/mL ± 0.25 ng/mL to 1.93 ng/mL ± 0.44 ng/mL (p = 0.0018). This 47% increase in PAF secretion in response to thrombin stimulation is consistent with previously published data, although a paucity of information is available regarding platelet lipid secretion in the context of inflammation. LTC_4, LTD_4, LTE_4, and PGD_2 were not detected by this method as secreted from platelets. These preliminary findings suggest that the method developed herein can be used to quantitate bioactive lipid secretion from both immortal and primary culture adherent cells as well as murine platelets in suspension and should be widely applicable to monitor bioactive lipids in a variety of physiological systems.
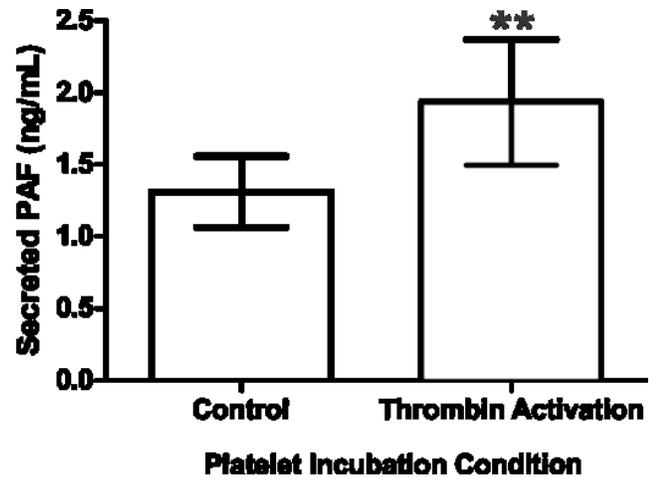
Figure 2.3. Platelet secretion of PAF increases significantly with thrombin activation, as expected.
 **p = 0.0018 vs. Control.

2.5 Conclusions

A method has been developed for the isolation and UPLC-MS/MS determination of five cell-secreted lipids with roles in inflammation. This five-minute method is useful for simultaneous quantification of structurally and functionally diverse lipids without liquid-liquid extraction, solid-phase extraction, or column switching. The lipids quantified with this method were leukotrienes C_4, D_4, and E_4, (LTC_4, LTD_4, and LTE_4) prostaglandin D_2 (PGD_2), as well as platelet activating factor (PAF). The method was applied to quantification of secreted lipids from a histamine-secreting immune cell line (RBL-2H3), murine primary culture mast cells, and murine platelets. In RBL cells, exposure to lipopolysaccharides, a component of bacterial cell membranes, modulated A23187-induced lipid secretion according to several different trends: LTE_4 secretion was significantly increased by pre-incubation with LPS, PAF secretion significantly decreased, and PGD_2 secretion was unaltered. In murine peritoneal mast cells co-cultured with NIH-3t3 fibroblasts, lipopolysaccharide exposure significantly increased the secretion of LTC_4 and PGD_2 when stimulated by A23187 but not when stimulated by IgE-mediated response to TNP-ova. LTE_4 secretion from MPMCs was stimulated by both TNP-ova and A23187, but was not significantly modulated by pre-incubation of the cells with lipopolysaccharides. Secretion of PAF from MPMCs was not observed to increase upon MPMC stimulation under the time-course of this study. Use of the method to monitor lipid secretion in platelets showed that secretion of PAF increased significantly upon activation

with thrombin. Future studies will use this method to gain a more comprehensive understanding of the function of a variety of cell types in inflammation.

Chapter 3: Time- and Concentration-Dependent Effects of Exogenous Serotonin and Inflammatory Cytokines on Mast Cell Function

In part from: Sarah M. Gruba[‡], Audrey F. Meyer[‡], Benjamin M. Manning, Yiwen Wang, John W. Thompson, Joseph J. Dalluge, and Christy L. Haynes. Time- and Concentration-Dependent Effects of Exogenous Serotonin and Inflammatory Cytokines on Mast Cell Function. *submitted*.

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3.1 Overview

Mast cells play a significant role in both the innate and adaptive immune response; however, the tissue-bound nature of mast cells presents an experimental roadblock to performing physiologically relevant mast cell experiments. In this chapter, a heterogeneous cell culture containing primary culture murine peritoneal mast cells (MPMCs) was studied to characterize the time-dependence of mast cell response to allergen stimulation, and the time- and concentration-dependence of the ability of the heterogeneous MPMC culture to uptake and degranulate exogenous serotonin using high performance liquid chromatography (HPLC) coupled to an electrochemical detector. Additionally, because mast cells play a central role in asthma, MPMCs were exposed to CXCL10 and CCL5, two important asthma-related inflammatory cytokines that have recently been shown to induce mast cell degranulation. MPMC response to both allergen exposure and cytokine exposure was evaluated for 5-HT secretion and bioactive lipid formation using the ultraperformance liquid chromatography coupled to an electrospray ionization triple quadrupole mass spectrometer (UPLC-MS/MS) method developed and described in Chapter 2. In this work, MPMC response was shown to be highly regulated and responsive to subtle alterations in a complex environment through time- and concentration-dependent degranulation and bioactive lipid formation. These results highlight the importance of selecting an appropriate mast cell model when studying mast cell involvement in allergic response and inflammation.

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3.2 Introduction

Mast cells are tissue-bound cells of hematopoietic origin widely known for their roles in inflammation and allergic response. They also have roles in innate immunity, host defense against parasitic and bacterial infection, wound healing, tissue homeostasis, and disease states such as vasculitis and fibrosis.[1-3] Mast cell function is often thought to be dominated by their cytosolic granules that contain inflammatory mediators, including enzymes such as tryptase and chymase, highly charged biopolymers such as heparin or chondroitin sulfate, and small molecule messengers such as serotonin (5-hydroxytryptamine, 5-HT) and histamine. In addition to granule-stored mediators, mast cells produce and secrete bioactive lipid compounds via enzymatic transformations of their phospholipid membranes. *In vivo* mast cell secretion of both granule-stored and *de novo* manufactured inflammatory mediators influence surrounding cell types, leading to symptoms commonly associated with allergic response, including mucus hypersecretion, bronchoconstriction, and vasodilation.[4,5] Based on their significant involvement in both innate and adaptive immune response, there are many research groups that aim to study mast cell behavior; however, there are several experimental roadblocks to performing physiologically relevant mast cell experiments.

Mature mast cells are tissue bound, and as such, it is difficult to isolate large numbers of pure populations for study; due to this challenge, several different *in vitro* strategies for mast cell culture are commonly used in experimental work. Isolation of pure populations of mast cells typically requires tissue homogenization followed by several

immunomagnetic separation steps, which can affect the activation state of the mast cells and gives low cell yields. To circumvent this challenge, studies of mast cells are often performed using immortal tumor-derived cell lines such as rat basophilic leukemia 2H3 (RBL) cells or the human mast cell lines HMC-1 or LAD2. Benefits of immortal cell lines include homogeneity and ease of culture; however, studies of adherence, receptor expression, and enzyme content have shown each of these mast cell-like cells lines to be only marginally representative of mature, tissue bound, non-transformed mast cells.[6-8] Another strategy for mast cell studies involves culturing mast cell-like cells from bone marrow or blood-derived immature precursors for 4-6 weeks with chemokines to drive mast cell maturation. Such cell cultures produce generally homogeneous mast cell-like cell populations, but they are not ideal due to length of culture time, expense of culture media, scarcity of precursor cells, and disparity between *in vivo* and *in vitro* culture conditions. Studies of primary culture mast cells employ the isolation of a cell suspension often from a mouse or rat peritoneal cavity. Cell suspensions generally contain a mixture of cells including mast cells and macrophages and are co-cultured with fibroblasts to maintain mast cell viability.[9] These heterogeneous cell cultures more closely model the *in vivo* environment of mast cells, which are in contact with macrophages and connective tissue cells but present challenges because mast cells make up less than 3% of the total cell population.

While important in many physiological processes, the time course of mast cell secretion of mediators in a heterogeneous cell culture, where mediators can stimulate other

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cell types or be cleared from the extracellular milieu, has not been evaluated. In this work, a heterogeneous cell culture containing primary culture murine peritoneal mast cells (MPMCs) is studied to characterize the time-dependence of mast cell response to allergen stimulation. The serotonin secretion behavior of MPMCs in a heterogeneous cell culture was compared to that from immortal RBL cells. When an unexpected 5-HT secretion trend was observed in the primary culture MPMCs, the time- and concentration-dependence of serotonin uptake in the heterogeneous MPMC culture was examined. Additionally, because mast cells play a central role in inflammatory diseases such as asthma, primary culture mast cells were exposed to CXCL10 and CCL5, two important asthma-related inflammatory cytokines that have recently been shown to induce mast cell degranulation at the single-cell level with different degranulation kinetics than IgE-mediated response to allergen.[10] To further understand these differences in time-dependent mast cell communication mechanisms, MPMC secretion of 5-HT and bioactive lipids was measured at various time points from 10 - 90 min following exogenous 5-HT or cytokine exposure. These experiments aim to reveal differences in mast cell biochemical pathways leading to exocytosis.

3.3 Experimental Approach

3.3.1 Reagents

All materials used for cell culture were obtained from HyClone (Logan, UT). Recombinant murine CXCL10 and CCL5 were purchased from Shenandoah Biotechnology Inc. (Warwick, PA) and stored as suggested. LTC_4, LTD_4, LTE_4, PGD_2,

and PAF and internal standards LTC$_4$-d$_5$, LTD$_4$-d$_5$, LTE$_4$-d$_5$, PGD$_2$-d$_9$, and PAF-d$_4$ were purchased from Cayman Chemical (Ann Arbor, MI) and used as received. LC/MS-grade acetonitrile (ACN) and water were purchased from JT Baker (Center Valley, PA). LC/MS grade isopropyl alcohol (IPA) and a penicillin/streptomycin mixture were purchased from Fisher Scientific (Pittsburgh, PA). Anti-trinitrophenol (TNP) IgE was purchased from BD Biosciences (Bedford, MA), while TNP-ovalbumin (TNP-ova) was purchased from Fisher Scientific (Hampton, NH).

3.3.2 Mast Cell Isolation and Exposure to Chemokines

MPMCs were harvested from 10 week old C57BL/6J mice purchased from The Jackson Laboratory (Bar Harbor, ME). Briefly, 8-9 mL of DMEM supplemented with 10% bovine calf serum and 1% penicillin and streptomycin (henceforth referred to as media) were injected into the peritoneal cavity of each mouse immediately following euthanasia (by CO$_2$ asphyxiation according to IACUC approved protocol #0807A40164). Following a 30 second abdominal massage, approximately 6-7 mL of peritoneal lavage fluid per mouse were recovered. The collected cell suspension was pelleted by centrifugation at 450xg for 10 minutes, resuspended at 2 x 10^6 cells/mL in fresh media, and plated over Swiss albino 3t3 fibroblasts (purchased from ATCC, Manassas, VA). Cells were then cultured overnight with additional 500 μL of fresh media containing anti-TNP IgE such that the concentration of IgE in each well was 0.5 μg/mL.

After overnight incubation, MPMCs were washed three times with Tris buffer (12.5 mM Tris, 150 mM NaCl, 4.2 mM KCl, 5.6 mM glucose, 1.5 mMCaCl$_2$, 1.4mM MgCl$_2$) at

37 °C to remove serum-containing media. To investigate 5-HT processing by the cell culture, MPMCs were incubated with 0.1-0.55 µM 5-HT or Tris buffer as a control, the supernatant was collected at specific time points and the cells were lysed with 0.5 µM $HClO_4$ for 30 min. The supernatant and lysate 5-HT content was assessed by HPLC at various timepoints. For experiments evaluating the effect of stimulation with TNP-ova, CXCL10, or CCL5 on MPMCs, cells were incubated for either 2 hours or particular time points of 10, 30, 60, and 90 minutes with Tris buffer or with Tris buffer containing 0.2-200 ng/mL CXCL10, 0.2-200 ng/mL CCL5, or 100 ng/mL TNP-ova. Following incubation, degranulation was terminated by incubating the cells on ice for 10 minutes, and supernatants were collected for analysis by UPLC-MS/MS or HPLC with electrochemical detection.

3.3.3 HPLC Analysis of Serotonin

MPMC-secreted serotonin was detected and quantified by HPLC coupled to an electrochemical detector using a modified version of a previously published protocol.[11] Briefly, following MPMC incubation, 250 µL of supernatant was collected and filtered using a 0.45µm filter plate from Millipore (Billerica, MA) at 3000xg for 5 min. 180 µL of the filtered supernatant was added to 20 µL of 5 µM dopamine (used as an internal standard) in 0.5M $HClO_4$ for final concentrations of 0.5 µM dopamine and 500 mM $HClO_4$. The samples were vortexed and injected by an autosampler into an Agilent 1200 HPLC (Santa Clara, CA). Separation was achieved using a 5µm, 4.6 x 150 mm Eclipse XDB C18 column attached to a Waters 2465 electrochemical detector with a glassy carbon-based

electrode. The working potential was set at 0.7 V vs. an *in situ* Ag/AgCl reference electrode with a current range of 50 nA. The HPLC flow rate was 2 mL/min using a mobile phase mixture consisting of 11.6 mg/L of the surfactant sodium octyl sulfate, 170 µL/L dibutylamine, 55.8 mg/L Na_2EDTA, 10% methanol, 203 mg/L sodium acetate anhydrous, 0.1M citric acid, and 120 mg/L sodium chloride.

A 5 point calibration curve was constructed by plotting the ratio of serotonin to dopamine (internal standard) peak areas in solutions containing 500 nM dopamine and 28-1000 nM serotonin in 0.5 M $HClO_4$. The line of best fit for an average calibration curve for several representative experiments was $y = 2.06 + 0.01$ with $R^2 = 0.9992$, where $y =$ peak area of 5-HT/peak area of internal standard and x = 5-HT concentration. Cell-secreted serotonin was quantified using the experimentally generated calibration curve.

3.3.4 UPLC-MS/MS Determination of Secreted Lipids

UPLC-MS/MS analysis was performed using a previously published method, which is described in detail in Chapter 2.[12] Briefly, supernatants from MPMCs were collected, spiked with 25 ppb of each of the internal standards LTC_4-d_5, LTD_4-d_5, LTE_4-d_5, PGD_2-d_9, and PAF-d_4 and concentrated in a centrifugal evaporator until the remaining volume was approximately 50 µL. After concentrating, salts were precipitated by the addition of 100 µL ice-cold ethanol containing 0.1% formic acid (FA) and 200 µL mobile phase B (90/10 ACN/IPA containing 0.1% FA). Precipitated salts were removed by centrifuging for 10 minutes at 12,000 RCF, and supernatants were again concentrated to approximately 20 µL. Samples were then resuspended in 100 µL 70/30 mobile phase

A/mobile phase B (with A: water containing 0.1% formic acid and B: 90/10 acetonitrile/isopropyl alcohol containing 0.1% formic acid). For UPLC-MS/MS analysis, a Waters (Milford, MA) Acquity UPLC coupled to a Waters triple quadrupole mass spectrometer (Acquity TQD) were used. Separation was performed on a Waters BEH C-18 2.1 mm x 50 mm column at 45 °C employing the following 5 min gradient separation at a flow rate of 0.6 mL per minute: 30% B, 0 min to 0.5 min; 30% B to 50% B, 0.5 min to 1.0 min; 50% B 1.0 min to 2.0 min; 50% B to 90% B, 2.0 min to 2.2 min; 90% B, 2.2 min to 3.2 min; 90% B to 30% B, 3.2 min to 3.5 min; and 30% B, 3.5 min to 5.0 min.

3.3.5 Assay of Secreted β-Hexosaminidase

To determine if incubation with 5-HT induced MPMC degranulation, supernatant content of the granule-stored enzyme β-Hexosaminidase (β-Hex) was assessed as described previously and in Chapter 2.[12,13] Briefly, 50 μL supernatant samples were collected in a 96 well plate and stored overnight at -80°C. When thawed, supernatants were incubated for 60 min at 37°C with 100 μL 1 mM 4-nitrophenyl N-acetyl-β-D-glucosaminide in 0.1 M citrate buffer at pH 4.5. After 60 min, 150 μL ice cold 0.1 M carbonate buffer at pH 9 was added, and absorbance of each well was recorded at 405 nm with a background subtraction absorbance reading at 630 nm.

3.4 Results and Discussion

3.4.1 Time-dependent MPMC and RBL cell response to IgE-mediated stimulation

While MPMCs have been used for many studies of single mast cell secretion kinetics (usually tracking events on the ms to s timescale), the longer term (min-hrs) serotonin secretion of MPMCs in a heterogeneous cell culture has not been explored. To examine the time-dependence of mast cell response to IgE-mediated allergen stimulation, MPMCs were incubated overnight with anti-TNP IgE, washed three times, then exposed to 100 ng/mL TNP-ova. After exposure to TNP-ova, supernatants from the adherent cell culture were collected and analyzed for secreted mediators at discrete time points to probe the kinetics of long-term release of preformed granules. As previous research has indicated that exocytotic cells only partially secrete their granule contents upon direct stimulation,[14] it was hypothesized that supernatant 5-HT concentration would steadily increase over the course of 90 min exposure to allergen stimulation. However, Fig. 3.1A shows that over the course of 90 min, exposure to TNP-ova resulted in an increase followed by a decrease in secreted serotonin. Unsurprisingly, after 10 min of incubation with TNP-ova, secreted serotonin increased to $571 \pm 30\%$ of control serotonin. After 10 min, however, serotonin concentration in MPMC supernatants ($[5\text{-HT}]_{supernatant}$) steadily decreased to $351 \pm 30\%$ of 5-HT control cell concentration at 90 min (Fig. 3.1A). MPMCs are cultured in a heterogeneous cellular environment, and although this type of cell culture more closely models the *in vivo* cellular environment than homogeneous immortal cell lines, heterogeneity can obscure the fate of the secreted serotonin as it relates to mast cells.

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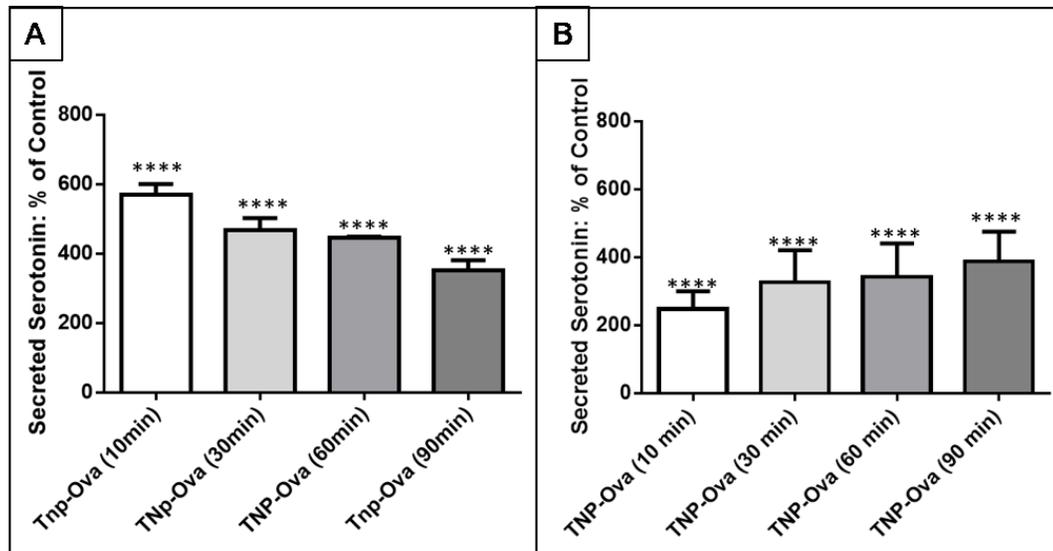
Figure 3.1. Time-dependent effects of serotonin secretion from A. MPMCs and B. RBL cells.
****$p < 0.0001$ vs. supernatant 5-HT concentration of control (unactivated) MPMCs or RBL cells. Control refers to the concentration of 5-HT in the supernatant of MPMCs for A RBL cells for B exposed to Tris buffer, normalized to 100%.

To probe the effect of the heterogeneous cell culture on secreted serotonin, MPMC 5-HT secretion was compared to that of the homogenous RBL cell line. RBL cells, while widely accepted as different from mast cells, share the common mast cell traits of adherence, FcεRI receptor expression, and degranulation in response to IgE-mediated stimulation.[8] Fig. 3.1 shows that the RBL cells continually release more serotonin over the course of 90 min, quite distinct from the serotonin secretion trend exhibited by MPMCs. When compared to the steadily increasing RBL cell [5-HT]$_{supernatant}$ concentration (Fig. 3.1B), the decreasing trend in MPMC [5-HT]$_{supernatant}$ (Fig. 3.1A) indicates a process of metabolism or reuptake by the cells in culture. This difference demonstrates the importance of the choice of cell culture when examining cell-to-cell communication behavior and interactions in response to changes in the environment.

3.4.2 Exogenous 5-HT effects on MPMC culture

Mast cell secretion of 5-HT (along with other granule stored and *de novo* manufactured mediators critical to cell-cell communication and inflammation) has several known downstream effects and potentially many other effects on mast cells themselves and surrounding cell types. Expression of the 5-HT specific reuptake transporter (SERT) has been detected on both mast cells and macrophages.[15,16] To explore the role that the cell culture microenvironment plays in MPMC 5-HT secretion, MPMCs were incubated with

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Tris buffer containing concentrations of 5-HT ranging from 0.1 μM to 0.55 μM. These incubations were intended to model the environment that un-activated (but IgE-sensitized) cells would experience when in contact with activated MPMCs. Previous experiments have shown that TNP-ova stimulated MPMCs generate supernatant 5-HT concentrations of 0.4 − 5 μM, and MPMCs exposed to inflammation-relevant cytokines generate 0.1 − 0.2 μM 5-HT in the cell culture supernatant. At 10, 30, and 60 min of serotonin incubation, the cell culture supernatants were collected and analyzed for 5-HT, and the adherent cells were lysed and analyzed for 5-HT content. In Fig. 3.2, the total system 5-HT is presented as a percentage of the sum of the control supernatant, control cell lysate, and incubation 5-HT concentrations. For this work, the total system 5-HT refers to the 5-HT in the cell supernatants and cell lysates. To account for the exogenous concentration of 5-HT in the cell cultures, Equation 3.1 was used to calculate the total system 5-HT.

Equation 3.1: Total System 5-HT

$$= \frac{[\text{5-HT}]_{\text{Lysate}} + [\text{5-HT}]_{\text{Supernatant}}}{\text{Control}[\text{5-HT}]_{\text{Lysate}} + \text{Control }[\text{5-HT}]_{\text{Supernatant}} + [\text{5-HT}]_{\text{Exogenous}}} \times 100\%$$

Surprisingly, the 5-HT content of the cell cultures exhibited a decreasing trend with increasing 5-HT incubation concentrations. The correlation between 5-HT as a percent of control and 5-HT incubation concentration was significant at 30 min ($p = 0.0009$) and 60 min ($p = 0.02$) but not at the earliest time point of 10 min ($p = 0.07$). This indicates that,

over the course of 30-60 min, the heterogeneous cell culture processes 5-HT. Exposing the cell culture to higher concentrations of exogenous 5-HT leads to a proportionally greater degree of cellular processing of 5-HT. To determine if supernatant-contained species were causing degranulation and/or caused the release of serotonin to be higher than that of our controls, a β-Hex assay was used to indicate extents of cell degranulation. When the β-Hex data were analyzed using a one-way ANOVA, the supernatants of MPMCs incubated with 0.4 μM and 0.55 μM 5-HT for 60 min released significantly more β-Hex than control MPMCs at the same timepoints ($p = 0.03$ and $p = 0.02$ for 0.4 μM 5-HT and 0.55 μM 5-HT incubation, respectively). No significant difference was detected between control β-Hex values and β-Hex values for the 0.1 – 0.55 μM 5-HT incubation at 10 min or 30 min or between controls and 0.1 – 0.3 μM 5-HT incubation at 60 min ($p > 0.05$). So, only the highest exogenous 5-HT concentrations and the longest incubation times induced significant increases in mast cell degranulation, meaning that degranulation alone does not account for the cells' 5-HT processing behavior (all β-Hex data can be found in Supplementary Information). Previous studies on the effects of exogenous 5-HT on mast cells using bone marrow-derived or blood progenitor-derived mast cell models determined that 5-HT affects mast cell adhesion but does not induce mast cell degranulation.[16] It is likely that mast cells in culture with a heterogeneous mix of other cells (as would be present *in vivo*) experience a different chemical microenvironment than that of mast cells derived from blood or bone marrow progenitor cells, and this may result in different autocrine and/or paracrine effects of exposure to cell-cell communication species, including 5-HT.

Changes in chemical microenvironment as a result of incubation with 5-HT after 60 minutes may induce release in mediators by surrounding cells, which stimulates mast cell degranulation of β-Hex and 5-HT.
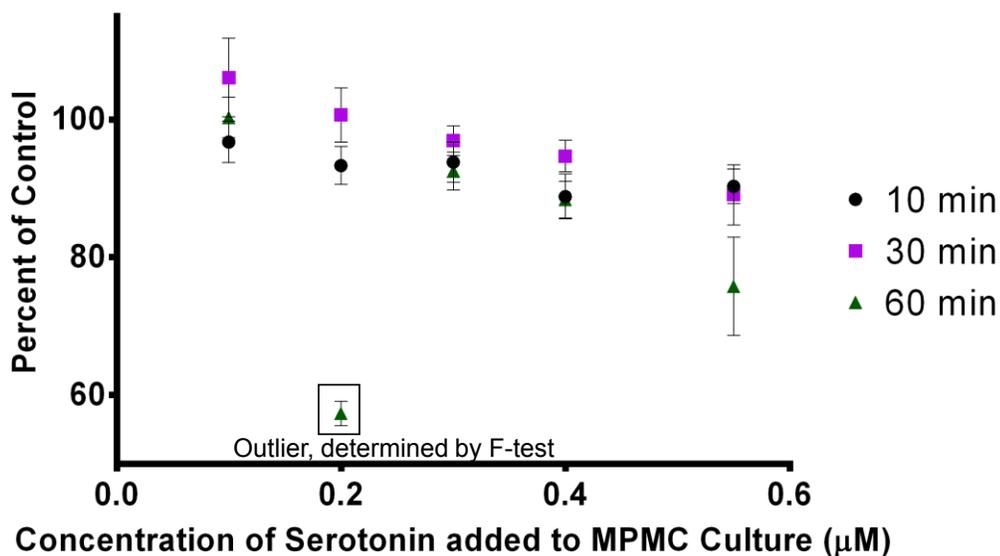
Figure 3.2. Time-dependent effects of incubating MPMCs with 0.1-0.55 uM 5-HT. Percents of control were calculated using Equation 1: each data point represents the total system serotonin (the sum of 5-HT concentration in supernatant as well as in lysed cells) as a percentage of the total system serotonin in control cells. The 0.2 μM incubation condition at 60 min was determined to be an outlier using the extra-sum-of-squares F tests ($p \leq 0.05$).

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To evaluate whether the trend in cellular processing of 5-HT shown in Fig. 3.2 was a result of supernatant 5-HT metabolism, lysate 5-HT metabolism (indicating intracellular MPMC 5-HT metabolism) or a combination of both, Fig. 3.3 shows the MPMC supernatant and lysate 5-HT trends for each 5-HT incubation condition separately. To compare the supernatant 5-HT concentrations of MPMCs incubated with exogenous 5-HT to control MPMC supernatants, the 5-HT values in the solid bars of Fig. 3.3 are represented as a percentage of the sum of the control (MPMC supernatant 5-HT + 5-HT concentration of the MPMC incubation). Trends in MPMC supernatant 5-HT content are shown in Table 3.1: after 10 min, the supernatant concentrations of 5-HT were significantly decreased in comparison to the 5-HT content of the supernatant of MPMCs exposed to no exogenous 5-HT ($p \leq 0.05$ using one-way ANOVA) at all exogenous 5-HT concentrations examined, when controlling for the concentration of 5-HT added to the cells. After 30 min of incubation with 5-HT, the supernatant 5-HT content did not change significantly from the 10 min timepoint supernatant 5-HT content at any of the exogenous 5-HT conditions ($p > 0.05$). After 60 min of incubation, however, the 5-HT content of MPMC supernatants exhibited a dependence on exogenous [5-HT]. The [5-HT] of supernatants of MPMCs incubated with lower concentrations of 5-HT, 0.1 and 0.3 µM, decreased significantly at 60 min vs. 10 min ($p < 0.0001$ for both). However, as the exogenous 5-HT concentration increased, the reverse effect was observed: at 0.4 µM and 0.55 µM exogenous 5-HT,

supernatant 5-HT content did not change significantly at 60 min incubation vs. at 10 min of incubation ($p > 0.05$). In summary, assessing supernatant β-Hex content indicated that at the longest incubation times and highest incubation concentrations, MPMC degranulation was induced, which would increase the 5-HT content of the supernatant. This may explain the trend of decreased supernatant 5-HT in MPMCs incubated with low concentrations of exogenous 5-HT and the opposite trend observed at higher concentrations of exogenous 5-HT. Cells exposed to the 0.4 -0.55 µM exogenous 5-HT for 60 min likely experience a chemical microenvironment similar to what they would experience when MPMCs are responding to allergen (as many mast cells degranulate). MPMC secretion of 5-HT must induce surrounding cells to secrete chemical species that in turn can induce further activation of mast cells. The difference between trends in supernatant 5-HT decrease at low (0.1 and 0.3 µM) and high (0.4 and 0.55 µM) 5-HT incubations indicate that 5-HT metabolism by the cell culture has both time and concentration dependence.
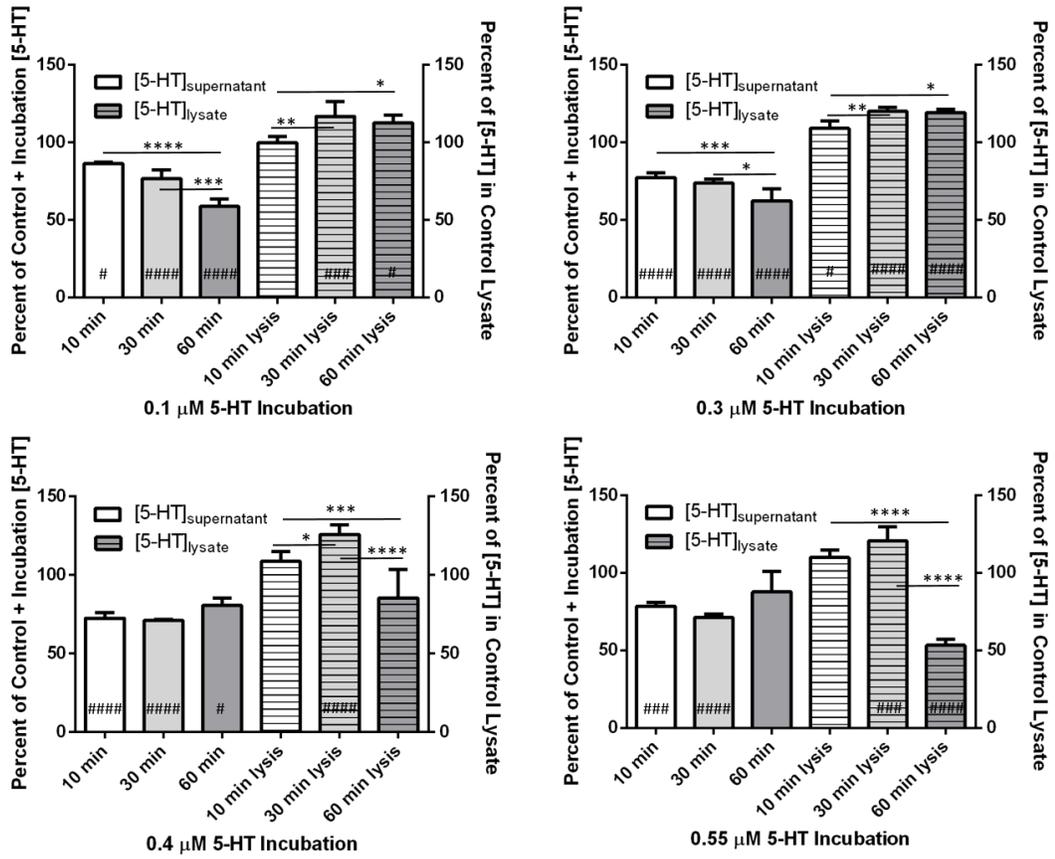
Figure 3.3. Supernatant and lysate 5-HT content of MPMCs incubated with 0.1 – 0.55 µM 5-HT for 10-60 min.
#p ≤ 0.05 vs. control; ###p < 0.001 vs. control; ####p < 0.0001 vs. control; *p ≤ 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001.

Several studies have reported on the mechanisms of clearance of 5-HT in the brain[17-19] and determined that while SERT has a high binding affinity for 5-HT ($K_d = 6.7$ nM[20]), there are also other mechanisms by which 5-HT is cleared, as SERT-knockout mice demonstrate the ability to clear 5-HT.[19] Although they have lower binding affinity for 5-HT than SERT, the extraneuronal monoamine transporter, plasmalemmal monoamine transporter, and the organic cation transporters 1-3 are several receptors with the ability to clear extracellular 5-HT efficiently.[19] While it is known that mast cells and macrophages (which compose the majority of the cells in the MPMC culture) express SERT, little is known about the non-SERT clearance of 5-HT by macrophages and mast cells. Additionally, as 5-HT clearance varies by local area within the brain,[21] it is highly likely that 5-HT clearance in non-neuronal tissue by SERT and non-SERT mechanisms will differ from brain synaptosomes. Clearly, further study on the clearance of extracellular 5-HT by peripheral tissues is warranted.

Analysis of the cell lysate 5-HT content revealed interesting trends in the uptake of 5-HT by the cell culture, which are detailed in Table 3.2. Of the 5-HT incubation concentrations examined, only 0.3 μM 5-HT produced a significant increase in cell-stored 5-HT concentration (compared to controls) after 10 min of incubation ($p = 0.05$). The trends in cell-stored 5-HT over the course of 10 to 60 min indicate significant increases at 5-HT incubation concentrations of 0.1 and 0.3 μM and significant decreases for 5-HT

incubation concentrations of 0.4 and 0.55 µM. The overall 60 min decrease at the higher

exogenous concentrations of 5-HT is likely due in part to the induction of MPMC

degranulation by the incubation itself.

Table 3.1. Trends in MPMC supernatant 5-HT content over the course of 60 min at varying exogenous concentrations of 5-HT.

Exogenous [5-HT] (µM)	10 min vs. Control MPMCs	10 min vs. 30 min	30 min vs. 60 min	10 min vs. 60 min
0.1	-13.7%	ns	-23.5%	-32.0
0.2	-16.9%	ns		
0.3	-22.9%	ns	-15.4%	-19.2%
0.4	-27.7%	ns	ns	ns
0.55	-21.5%	ns	+23.2%	ns

Where significant (p ≤ 0.05 using one-way ANOVA), the percent increase or decrease is indicated, and ns = not significant, p > 0.05 using one-way ANOVA

Table 3.2. Trends in MPMC lysate 5-HT content over the course of 60 min at varying exogenous concentrations of 5-HT.

Exogenous [5-HT] (µM)	10 min vs. Control MPMCs	10 min to 30 min	30 min to 60 min	10 min to 60 min
0.1	ns	+17.1%	ns	+13.0%
0.2	ns	+20.0%		
0.3	+9.01%	+9.97%	ns	+9.31%
0.4	ns	+15.8%	-21.4%	-21.4%
0.55	ns	ns	-55.8%	-51.5%

Where significant, (p ≤ 0.05 using one-way ANOVA), the percent increase or decrease is indicated, and ns = not significant, p > 0.05 using one-way ANOVA

3.4.3 Stimulation Effects: Inflammatory Cytokines CXCL10 and CCL5

The concentration and time-dependent trends in supernatant and lysate 5-HT concentration of MPMCs exposed to exogenous 5-HT have potential implications for *in vivo* effects of intercellular 5-HT. Mast cells are a critical cell type in the pathogenesis of inflammatory diseases such as asthma.[3] Their involvement in inflammatory diseases is influenced by cell-to-cell communication with surrounding cell types, which release chemokines recently shown to directly induce mast cell degranulation. Mast cells undergoing degranulation in response to IgE-mediated stimulation secrete more 5-HT than mast cells responding to inflammatory chemokines such as CXCL10 or CCL5.[10] The trends in Tables 3.1 and 3.2 indicate that the cells that surround MPMCs (and potentially mast cells localized to other tissues) will have different rates of uptake or metabolism of 5-HT, and this will likely affect the pathophysiology of surrounding cell response to MPMC secretory behavior following exposure to chemokines vs. allergen. To further explore MPMC response to stimulation over the course of min-hrs and environmental effects, the long term time-dependent response of MPMCs to inflammatory chemokines was compared to TNP-ova stimulation. Mast cells are most commonly known for their central roles in allergic response, and the TNP-ova incubation models that response. In this case, the MPMCs were incubated overnight with anti-TNP IgE, then exposure to TNP-ova induces IgE-mediated degranulation. Although response to allergen stimulation is the most widely known function of mast cells, these cells have diverse and complex roles in a number of

inflammatory diseases. By comparing the effects of inflammatory disease-relevant chemokines on MPMCs to the widely studied allergen response of MPMCs, this work attempts to probe the complex response of mast cells to subtle changes in chemical microenvironments. The inflammatory chemokines CXCL10 and CCL5 were selected because of their roles in mediating the cell-to-cell communication processes that give rise to asthma. CXCL10 in particular has a central role in the pathogenesis of asthma. IgE-sensitized MPMCs were exposed to 0.2-200 ng/mL CXCL10 or CCL5, and the 5-HT content of the cell supernatants were measured after 2 hrs of incubation. Direct stimulation of MPMCs was observed at a wide range of concentrations of both CXCL10 and CCL5 (results can be found in Supplementary Information). Based on the results after 2 hrs of exposure to a wide range of concentrations, two concentrations each of CXCL10 and CCL5 were selected for further time-dependent study based on the robustness of the MPMC response following exposure to them.
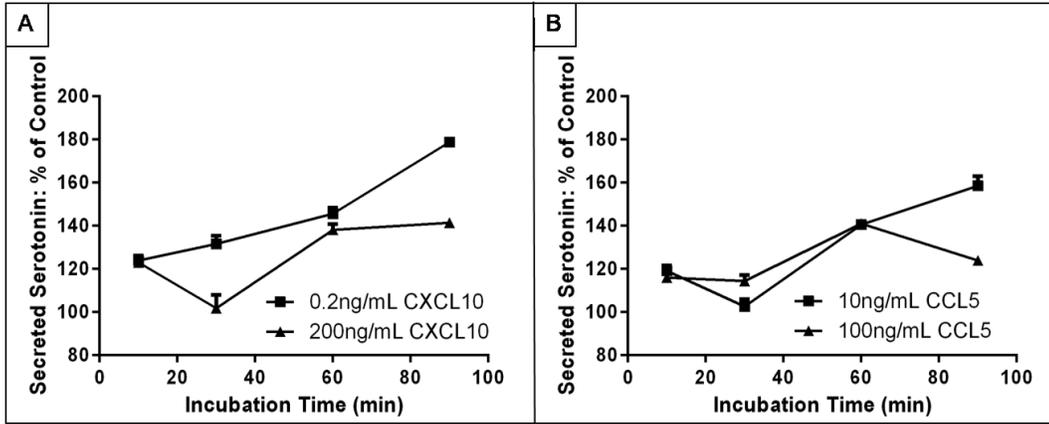
Figure 3.4. Supernatant 5-HT content as a percent of control MPMC supernatant 5-HT content after MPMC exposure to the inflammatory cytokines A. CXCL10 and B. CCL5.

MPMCs were exposed to CXCL10 at 0.2 and 200 ng/mL or CCL5 at 10 and 100 ng/mL, and supernatants were sampled at 10, 30, 60, and 90 min for 5-HT content measurement. Trends in 5-HT secretion in response to the cytokines are shown in Fig. 3.4. In contrast to the robust degranulation response that decreased over the course of 90 min following MPMC exposure to TNP-ova, exposing MPMCs to CXCL10 or CCL5 generated a smaller degranulation response with an overall increasing trend in secreted 5-HT over the course of 90 min. Stimulation with CXCL10 induced mast cell degranulation to a lesser extent than TNP-ova stimulation, which on average induced a 4.6 times greater secretion of 5-HT than CXCL10. The two stimulant concentrations of CXCL10 also induced different time-dependent trends in 5-HT secretion. Upon stimulation with 0.2 ng/mL CXCL10, supernatant 5-HT increased continuously over 90 min. MPMC exposure to 200 ng/mL CXCL10 resulted in an initial increase in supernatant 5-HT content at 10 min, followed by a return to control 5-HT levels at 30 min and another increase in 5-HT levels from 30-90 min. Interestingly, MPMC stimulation with CCL5 resulted in similar overall trends to stimulation with CXCL10, but the chemokine concentration dependence was reversed: 5-HT secretion following stimulation with 100 ng/mL CCL5 followed the same general trend as 5-HT secretion in response to 0.2 ng/mL CXCL10, and stimulation with 10 ng/mL CCL5 resulted in similar 5-HT concentration fluctuations as MPMC exposure to 200 ng/mL CXCL10. Both CXCL10 and CCL5 are mast cell chemoattractants that induce MPMC degranulation over a wide range of chemokine concentrations. The contrast in temporal response of MPMCs to low and high concentrations of these chemokines

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highlights the complex role of mast cells in inflammatory diseases. While *in vivo* mast cells are likely exposed simultaneously to multiple chemokines as they move toward the site of inflammation, examining the response of mast cells to individual mediators of inflammatory diseases can reveal information about MPMC response to different aspects of inflammation.

The response of surrounding cells to different concentrations of exogenous 5-HT is also informative of the diverse role of mast cells in inflammation. TNP-ova stimulation of MPMCs generates supernatant 5-HT concentrations of 0.4-0.55 µM, and stimulation with CXCL10 or CCL5 generates supernatant 5-HT concentrations of 0.1-0.2 µM. As the trends in Table 3.1 highlight, exposing MPMCs to 0.4-0.55 µM 5-HT results in an initial decrease in supernatant 5-HT concentration, likely due to MPMCs and macrophages taking up and/or processing the 5-HT, followed by an increase or no change in the supernatant 5-HT concentration. When the heterogeneous cultures were exposed to lower concentrations of 5-HT, the decrease in supernatant 5-HT concentration was more pronounced and continued for 60 min. When MPMCs are exposed to TNP-ova, the concentrations of secreted 5-HT are high enough that the surrounding cell culture does not process the secreted 5-HT as efficiently as the lower secreted 5-HT concentrations induced by the inflammatory chemokines CXCL10 and CCL5.
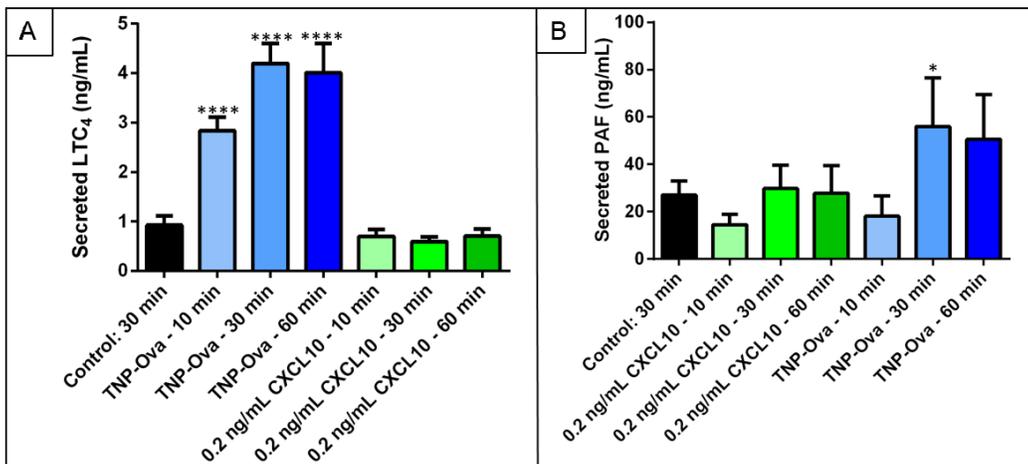
Figure 3.5. MPMC secretion of the bioactive lipids A. LTC4 and B. PAF in response to TNP-ova or 0.2 ng/mL CXCL10. *p = 0.02; ****p < 0.0001.

The secretion of 5-HT by mast cells is an important component of understanding mast cell function in both allergic response and inflammatory disease; however, mast cells secrete many other chemical species that contribute to their roles in physiological processes. In asthma, the effect of CXCL10 on mast cells is particularly important,[22,23] and previous studies have shown that CXCL10 potentially activates MPMCs through a different pathway than IgE-mediated allergen stimulation.[10] To further examine the effects of stimulation by response to allergen vs. inflammatory chemokines on MPMC function, the secretion of bioactive lipids from MPMCs exposed to 0.2 ng/mL CXCL10 or 100 ng/mL TNP-ova was also examined. MPMC supernatant concentrations of LTC_4 and PAF were determined using UPLC-MS/MS, and results are shown in Fig. 3.5. As expected, TNP-ova activation of MPMCs induced an increase (vs. unactivated MPMCs) of LTC_4 secretion and PAF secretion. CXCL10 exposure, however, did not significantly increase the secretion of LTC_4 or PAF ($p > 0.05$ vs. control MPMCs). In addition to the differences between CXCL10 and TNP-ova-induced degranulation of MPMCs, the mechanism by which CXCL10 activates mast cells likely does not induce bioactive lipid secretion. Further studies on the effects of cell-secreted cytokines are clearly needed to better understand the complex role that mast cell activation plays in inflammatory diseases.

3.5 Conclusions

In this work, MPMC response is shown to be highly regulated and responsive to subtle alterations in a complex environment through time and concentration dependent degranulation and bioactive lipid formation. These results highlight the importance of

selecting an appropriate mast cell model when studying mast cell involvement in allergic response and inflammation. This work probed both the effects of 5-HT concentration and time on surrounding cells in culture with MPMCs as well as mast cell response to different stimuli. The MPMC co-culture system actively responds to the presence of 5-HT, which *in vivo* may correspond to the ability of cells localized near mast cells to limit the concentration of inflammatory mediators in the extracellular microenvironment. The correlation between the processing of exogenous serotonin and time supports that heterogeneous cellular systems are able to efficiently respond to inflammation. When attempting to apply *in vitro* mast cell results to *in vivo* systems, considering the environment that the mast cell is experiencing is important for understanding mast cell contribution to inflammation.

MPMCs exhibited a controlled release dependence when exposed to different agents of mast cell activation. The relatively small degree of degranulation of MPMCs in response to the cytokines CXCL10 and CCL5 in contrast to the robust response of MPMCs to TNP-ova (allergen) are potentially due to the chemoattractant roles of the cytokines. Mast cell contact with chemoattractants *in vivo* generally occurs when mast cells are localized away from the site of inflammation. Mast cell secretion of small amounts of serotonin and other granule-associated mediators increases vascular dilation, which allows chemoattractants including CXCL10 and CCL5 to enable mast cells to move more efficiently to the site of inflammation.

Chapter 4: Exploring the Role of Phospholipids in Platelet Adhesion and Secretion

In part from: Secil Koseoglu[‡], Audrey F. Meyer[‡], Donghyuk Kim, Ben M. Meyer, Yiwen Wang, Joseph J. Dalluge, and Christy L. Haynes, Exploring the Role of Phospholipids in Platelet Adhesion and Secretion, *submitted,* 2013.

[‡]Authors contributed equally

4.1 Overview

In this chapter and the next, the focus of this dissertation will be on membrane-bound phospholipids rather than secreted bioactive lipids. The cellular phospholipid membrane plays an important role in influencing cell function and cell-cell communication, but its biocomplexity and dynamic nature presents a challenge for examining cellular uptake of phospholipids and the resultant effects on cell function. Platelets, small anuclear circulating cell bodies that influence a wide variety of physiological functions through their dynamic secretory and adhesion behavior, present an ideal platform exploring the effects of exogenous phospholipids on membrane phospholipid content and cell function. In this work, a broad range of platelet functions are quantitatively assessed by leveraging a variety of analytical chemistry techniques, including ultra-performance liquid chromatography-tandem electrospray ionization mass spectrometry (UPLC-MS/MS), vasculature-mimicking microfluidic analysis, and single cell carbon-fiber microelectrode amperometry (CFMA). The relative enrichments of phosphatidylserine (PS) and phosphatidylethanolamine (PE) (structures shown in Fig. 1.5) were characterized with UPLC-MS/MS, and the effects of the enrichment of these two phospholipids on both platelet secretory behavior and adhesion were examined. Results show that, in fact, both PS and PE influence platelet adhesion and secretion. PS was enriched dramatically and decreased platelet adhesion as well as secretion from δ-, α-, and lysosomal granules. PE enrichment was moderate and increased secretion from platelet lysosomes. These insights illuminate the critical connection between membrane

phospholipid character and platelet behavior, and both the methods and results presented herein are likely translatable to other mammalian cell systems.

4.2 Introduction

The perception of the cellular phospholipid membrane as an inactive barrier between the cytosol and the extracellular space has been challenged by many recent studies. In particular, membrane-bound phospholipids have been shown to have active roles in cellular signaling and receptor expression.[1,2] The membranes of mammalian cells contain phospholipids of numerous classes including phosphatidylserines, phosphatidylethanolamines, phosphatidylcholines, sphingomyelins, as well as cholesterol and many membrane-bound peptides and proteins.[3,4] Selectively examining the roles of individual membrane components is challenging because exposure to exogenous phospholipids can induce up- or down-regulation of any of the membrane components. Many studies employ model lipid bilayers, which eliminate nearly all of the biocomplexity of the cellular membrane and cytosol[3], and it is unclear if studies on such model lipid bilayers translate to physiologically relevant systems. In this study, primary blood platelets are used as a platform to examine whether cellular membranes can incorporate exogenous phospholipids and if so, what effects enrichment of membrane phospholipids have on cellular function. The anuclear nature of platelets makes them an ideal platform for studies of membrane phospholipids as they have minimal capacity to up- or down-regulate protein expression[5] in response to exposure to exogenous phospholipids. Additionally, platelets uniquely feature multiple types of secretory granules, each with a different type of stored

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cargo,[6] which enables the study of phospholipid effects on different classes of granules and chemical messenger cargo.

The asymmetric distribution of phospholipids within cellular membranes has important consequences in cell-cell communication.[1] Aminophospholipids, including phosphatidylserine (PS) and phosphatidylethanolamine (PE), are the second most abundant phospholipids in the plasma membrane, and they are localized to the inner leaflet of the plasma membrane.[7,8] Upon platelet activation, both PS and PE are exposed to the outer membrane surface. It has been shown that both the asymmetric distribution at rest and scrambling of the phospholipids upon activation are critical for cellular adhesion and the chemical messenger secretion process; in fact, disruption of the phospholipid asymmetry and redistribution is known to impair these functions.[9-11] When exposed to the outer leaflet of the platelet membrane, aminophospholipids serve as binding sites for circulating protein coagulation factors and also have catalytic activity in thrombotic reactions. In addition, fusion between the granular membrane and the plasma membrane is a critical step in exocytosis (the secretion of pre-formed granule-stored chemical messenger species), and the characteristics and actions of membrane lipid species are of innate importance in these events.[9,12]

Phospholipid content not only influences the fluidity and the curvature of the membrane but also promotes shape change and spreading of the platelets during exocytosis. In fact, it has been shown that incubation with exogenous phospholipids can mediate both the mechanism and the kinetics of exocytotic events in model exocytotic systems such as

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PC12 and chromaffin cells.[10,13] Due to their anuclear nature, platelet membranes are more stable and undergo minimal constitutive exocytosis, making it easier to draw conclusions about the direct effect of phospholipid substitution.

In addition to being an ideal model for studying the conserved process of exocytosis, platelets are also important players in various physiological processes, including hemostasis, inflammation, and angiogenesis.[6,14] As with other cells, an important regulatory component of the dynamic secretory and adhesion behavior of platelets is the phospholipid membrane, which plays an important role in influencing how platelets interact with their environment.[3] While clearly important, the exact role of membrane phospholipids in platelet activation and adhesion is not well characterized, mainly due to analytical limitations in characterizing cellular uptake of phospholipids. Herein, this work provides an improved fundamental understanding of how changes in the phospholipid membrane affect platelet behavior through the measurement of numerous platelet functions on both the single-cell and ensemble levels. The methods and results presented herein give general understanding about the role of the phospholipid bilayer in cell function and can be easily adapted for use with other cell types. Additionally, the physiological relevance of studies on platelets, specifically, will lead to enhanced therapeutic approaches related to thrombosis, inflammation, and angiogenesis, among others.

Due to their small size and short lifespan (generally 1 day *in vitro* or 1-5 days for clinical use), platelet secretory functions are typically studied using indirect methods. In fact, platelet secretion occurs from three distinct populations of storage granules (each

releasing unique messenger molecules) as well as on-demand manufacture of bioactive lipid species. To address the challenges associated with quantitative assessments of such a broad range of functions associated with a small and short-lived cell type, this work employs a variety of techniques to characterize the enrichments of PS and PE and the effects of PS and PE enrichment on both platelet secretory behavior and adhesion. In addition to measuring ensemble secretion of chemical messenger species from many platelets in suspension (PF_4 from α-granules, serotonin from δ-granules, and β-Hexosaminidase from lysosomes), which reveals information about the behavior of many platelets while they are in communication with one another, single cell carbon-fiber microelectrode amperometry (CFMA) measurements enable evaluation of the phospholipid content effects on quantal release and kinetics of individual δ-granule secretion. In parallel, the PS- or PE-enriched platelet adhesion behavior was assessed within a microfluidic platform where the feature size and endothelial cell coating mimic intravenous vasculature. Results show that, in fact, both PS and PE influence platelet adhesion and secretion. PS enrichment decreased platelet adhesion and decreased the secretion of δ-, α-, and lysosomal granules while PE enrichment increased secretion from platelet lysosomes. CFMA measurements showed that PS regulates granule recruitment and influences the frequency of secretion events while PS enrichment improves the stability of granule-cell membrane fusion events. Overall, this work reveals the importance of phospholipids in regulation of platelet behavior and highlights the utility of platelets as

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a platform for exploring the involvement of membrane phospholipids in the functions of mammalian cells.

4.3 Experimental Approach

4.3.1 Platelet Isolation and Phospholipid Incubation

All reagents used were analytical grade or higher quality. KCl, MgCl$_2$, N-2-hydroxyethylpiperazine-N'-2ethanesulfonic acid, NaHCO$_3$, and thrombin were purchased from Sigma Aldrich (St. Louis, MO). NaCl was purchased from BDH Chemicals (Radnor, PA). α-D-(+)-glucose was purchased from Acros Organics (Fair Lawn, NJ). LC/MS-grade H$_2$O and acetonitrile (ACN) were purchased from J.T. Baker (Center Valley, PA). LC/MS-grade isopropyl alcohol (IPA), Na$_2$CO$_3$, and citric acid were purchased from Fisher Scientific. Sodium bicarbonate and sodium citrate were purchased from Mallinckrodt (St. Louis, MO). Deuterated platelet activating factor (PAF-d$_4$) was purchased from Cayman Chemical (Ann Arbor, MI), and all phospholipids were purchased in chloroform from Avanti Polar Lipids (Alabaster, AL). Nine-week old C57BL/6J mice were purchased from The Jackson Laboratory (Bar Harbor, ME).

Blood was drawn via cardiac puncture from 10 week old mice euthanized via CO$_2$ asphyxiation (following IACUC-approved protocol #0806A37663) using 1 mL syringes pre-filled with 200 µL ACD, and platelet-rich plasma was isolated using the procedure described previously.[40] Briefly, whole blood was centrifuged at 130xg for 10 min without brake, and the upper plasma layer was transferred to new tubes. Platelets were pelleted

from the plasma by centrifugation at 500xg for 10 min and resuspended in fresh Tyrode's buffer.

200 µM PS, PC, PE, and SM solutions were prepared by drying appropriate volumes of phospholipids as received in chloroform in glass vials under a stream of nitrogen and sonicating in Tyrode's buffer for 2 hours, until all cloudiness had disappeared from the solutions. Phospholipid solutions were then filtered using 0.2 µm filters. Platelets were incubated with phospholipid or negative control solutions for 2h at room temperature.

4.3.2 Relative Quantification of Phospholipid Enrichment

Relative enrichments of PS, PC, PE, and SM in platelets were assessed using ultra-performance liquid chromatography coupled to tandem mass spectrometry (UPLC-MS/MS). Following incubation with PS, PC, PE, SM, or Tyrode's buffer (control), platelets were washed twice by centrifugation at 1000xg and resuspended in 6:1 Tyrode's buffer: ACD. After the final wash, platelets were resuspended in 100 µL Tyrode's buffer. Platelets were mixed with 400 µL $CHCl_3$/200 µL MeOH/10 µL 19.1 µM PAF-d_4 (internal standard) and sonicated for 20 min. 100 µL 0.1% acetic acid in 0.1 M NaCl was then added, and samples were sonicated for an additional 10 min. Samples were centrifuged for 5 min at 1500 RCF, and the upper aqueous layer was removed and discarded. The lower organic layer was dried under vacuum, and phospholipids were resuspended in 200 µL 0.1 % acetic acid in 40/60 A/B (A was 20 mM ammonium acetate in water, pH 5, and organic mobile phase; B was 0.1% acetic acid in 9:1 ACN:acetone) by 1 hour sonication. Samples were

centrifuged at 1500 RCF for 5 min and transferred to fresh tubes prior to UPLC-MS/MS analysis.

UPLC-MS/MS analysis was performed using a Waters Acquity Triple Quadrupole Mass Spectrometer using a modified version of the chromatography suggested by Rainville and Plumb with a Waters BEH C_8 2.1 x 100 mm column.[19] Deuterated platelet-activating factor (PAF-d_4) was used as an internal standard. Chromatography used a flow rate of 0.6 mL/min: 60% B, 0 min to 1.0 min; 60% B to 80% B, 1.0 min to 2.0 min; 80% B to 84% B, 2.0 min to 2.5 min; 84% B, 2.5 to 2.75 min; 84% B to 86% B, 2.75 to 3.0 min; 86% B, 3.0 min to 3.25 min; 86% B to 87.7% B, 3.25 to 3.5 min; 87.7% B, 3.5 to 3.75 min; 87.7% B to 95% B, 3.75 min to 4 min; 95% B, 4.0 min to 5.0 min; 95% B to 60% B, 5.0 min to 5.5 min; 60% B, 5.5 min to 7.0 min. Electrospray ionization tandem mass spectrometry (ESI-MS/MS) was operated in positive ionization mode using the following parameters: capillary, 3.8 kV; extractor, 3.00 V; rf lens, 0.30 V; source temperature, 120 °C; desolvation temperature, 400 °C; cone gas flow, 20 L/hr; desolvation gas flow, 800 L/hr; collision gas flow, 0.2 mL/min; low-mass resolution (Q1), 12.00; high-mass resolution (Q1), 12.00; ion energy (Q1), 0.30; and varied collision energies listed in Table 4.1.

Relative quantification of PS, PC, PE, and SM in each of control and phospholipid-incubated was accomplished using a calibration curve prepared exactly as the platelet samples were prepared. To account for variation in pelleting behavior of platelets induced by phospholipid incubation, phospholipid quantification results were normalized to

average protein values of control- or phospholipid-incubated non-activated platelet pellets, as determined using a Pierce bicinchoninic acid (BCA) assay.

4.3.3 Device Fabrication for Adhesion Measurements

A microfluidic device, employed to monitor platelet adhesion behavior with varied phospholipid content, was fabricated as was previously described.[41] Briefly, microfluidic channels were fabricated in polydimethylsiloxane (PDMS) using standard photo-/soft lithography techniques. The channel design was printed onto a transparent film (CAD/Art Services,), transferred onto a chrome mask plate (Nanofilm), and patterned onto a layer of SU-8 photoresist (SU-8 100, Microchem; Newton, MA) on a silicon wafer. The cell culture channel dimensions were 400 μm (width) x 100 μm (height) x 2500 μm (length). Once a master, patterned SU-8 on a silicon wafer, was complete, 10:1 resin:curing agent mixture of Sylgard 184 (Ellsworth Adhesives) was degassed in a vacuum chamber and then cast onto the master, followed by overnight curing at 80℃. Once cured, the PDMS layer was peeled off of the master, inlet and outlet holes were punched using a syringe needle, and the PDMS layer was washed to remove any remaining PDMS debris blocking the channels. Then, the PDMS layer was bonded to a clean glass substrate via oxygen plasma treatment to seal the channel. The device was brought into a bio-hood, and a 70 wt% ethanol solution was injected through the channel followed by a sterilized water rinse for sterilization purposes. This step was repeated three times, and after drying the channel, the device was exposed to UV light in the bio-hood for 5 minutes and then kept in the bio-hood until use.

4.3.4 Endothelial Cell Culture and Coating of the Microfluidic Device

An endothelial cell coating of the microfluidic channel is achieved as previously described[42] to simulate blood vessel architecture. The human endothelial cell line, Hy926, was purchased from American Type Culture Collection (ATCC, Manassas, VA). Dulbecco's Modified Eagle Medium (DMEM) with high glucose (formula: 4 mM L-glutamine, 4.5 g/L L-glucose, and 1.5 g/L sodium pyruvate (Gibco®, Carlsbad, CA)), supplemented with 10% fetal bovine serum and 1% penicillin and streptomycin (Sigma Aldrich, Milwaukee, WI) was used as the culture media, and cells were cultured in a T-flask with 5% CO_2 at 37ºC. Cells were fed every other day and split once a week. Endothelial cells were used only in passages three through twelve.

A day or two before the experiments, a microfluidic device was first incubated for 30 minutes with a 250 µg/mL human fibronectin solution in an incubator. Meanwhile, trypsinized endothelial cells from a T-flask were washed and re-suspended into the same culture media at a cell density of ~10^7 cells/mL. The cell suspension was injected through the device inlet into the cell culture channel and kept in an incubator for 3 hours. The fresh cell culture media was then injected through the channel to remove any non-adherent endothelial cells remaining in the channel, and the device was maintained in an incubator, feeding the cells every 12 hours. A monolayer of endothelial cells was achieved within 48 hours (80% of the devices), and devices that did not yield a complete, uniform monolayer of endothelial cells within 48 hours were discarded (20% of the devices).

4.3.5 Adhesion of Platelets Incubated with Phospholipids

Devices with a uniform monolayer of endothelial cells were selected for experiments, and the channel was washed with fresh Tyrode's buffer before exposure of endothelial cells to a stream of platelets. To facilitate visual distinction of platelets from endothelial cells, platelets were labeled with 5-chloromethylfluorescein diacetate (CMFDA19, Invitrogen) dye prior to phospholipid incubation. After phospholipid incubation, platelets were activated with 5 µM ADP and introduced into the microfluidic channel. ADP was used as it is a known platelet adhesion agonist that does not inducing platelet secretion. Flow control was accomplished using a syringe pump, and platelet suspensions were introduced onto the device through Teflon tubing. The endothelial cell-coated channel was exposed to a stream of platelets for 20 minutes at a constant flow rate of 30 µL/hr. This flow rate was chosen to avoid shear stress levels above 0.01N/m^2 (COMSOL simulation data not shown) to minimizes shear stress–induced platelet activation. After endothelial cell exposure to a stream of platelets, the cell culture channel was washed with fresh Tyrode's buffer and fluorescence images were obtained on an inverted microscope (Nikon, Melville, NY) equipped with a CCD camera (QuantEM, Photometrics, Tucson, AZ) using Metamorph Ver. 7.7.5 imaging software. Platelets adhered to endothelial cells were counted, and for each experimental condition, five images (450µm x 500µm each) were averaged; in each case, five biological replicates were measured.

4.3.6 Ensemble Platelet Secretion Measurements

Platelet δ-granule secretion was measured after incubation of the platelet-rich plasma (PRP) with phospholipids using a modified version of a previously published HPLC method.[43,44] For α-granule secretion, a PF_4 ELISA assay kit was purchased from R&D systems and used as directed. Lysosomal secretion was measured via a modified β-Hexosaminidase assay.[25] Briefly, following incubation of platelets with phospholipid solutions, platelets were exposed to Tyrode's buffer containing 10 U/mL thrombin (a physiological stimulant of platelet activation) or Tyrode's buffer (as control). Platelets were centrifuged at 500 RCF to pellet. Supernatant portions were used for the analysis of the secreted species (serotonin by HPLC, PF_4 with ELISA assay, and secreted Hexosaminidase with β-Hexosaminidase assay). Secreted platelet-activating factor (PAF) was measured using a previously published UPLC-MS/MS method as described in Chapter 2.[44] Briefly, a Waters BEH C18 2.1 x 50 mm column was used for separation, and a Waters Acquity triple quadrupole mass spectrometer was used for multiple reaction monitoring of secreted PAF with an internal standard of PAF-d_4. Selected reaction monitoring (SRM) transitions for PAF and PAF-d_4 were 524.4 > 184.1 and 528.4 > 184.1, respectively. Calibration curves were created by normalizing peak areas of PAF standards to peak areas for 25 ng/mL PAF-d_4 to create response curves over the concentration range of 0.5 – 500 ng/mL PAF. 25 ng/mL PAF-d_4 was spiked into supernatants from washed phospholipid-incubated platelets immediately after supernatant collection, and PAF secreted from platelets was quantified using response-based calibration curves. Due to low recovery of

PAF in Tyrode's buffer, PAF secretion was measured from platelets incubated in phosphate buffered saline (free of Ca^{2+} and Mg^{2+}, containing 1g/L glucose). A BCA protein assay was used for normalization of the results to prevent any differences in the data due to pelleting differences between control and phospholipid-incubated platelets. Ensemble platelet secretion measurements are reported as secreted species per μg pelleted protein.

4.3.7 Total Protein Quantitation

Total protein in pelleted platelets was quantified with a Pierce bicinchoninic acid (BCA) assay from Thermo Scientific, used as directed. Protein was extracted from platelet pellets with mammalian protein extraction reagent from Thermo Scientific, used as directed.

4.3.8 CFMA Measurements

CFMA experiments were performed as previously described.[28,45] After incubation with the phospholipid of interest, a drop of the platelet-rich plasma was added to an experimental chamber filled with Tyrode's buffer supplemented with the phospholipid of interest. A 7-μm-diameter carbon-fiber microelectrode (fabricated in house) was placed onto an individual platelet using piezoelectric micromanipulators, and then the platelet was activated by local application of Tyrode's buffer containing 10 U/mL thrombin solution. With an applied potential of 700 mV versus a Ag/AgCl reference at the carbon-fiber microelectrode, serotonin secretion from δ-granules was measured as current versus time for 90 s after the stimulation. Each phospholipid condition was compared with its own control measured from the same platelet preparation on the same day. Each current spike

was analyzed, and the aggregate data from at least 20 individual platelets was treated statistically as has been previously described.[43,46]

4.4 Overview of Experimental Results

4.4.1 Relative Quantitation of Phospholipids

Quantitation of lipid species presents an analytical chemistry challenge due to the limited solubility of phospholipids in solvents commonly used for liquid chromatography. Herein, relative phospholipid enrichment of platelet membranes was assessed using an ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) method. Mobile phase composition and column selection were based on a previously published work reported by Rainville and Plumb,[15] and chromatography was modified from the same report to a 7 min separation by adjusting the chromatographic conditions to optimize elution for each compound. An absolute quantification method was not possible because the phospholipid standards contained mixtures of fatty acid tails with a common headgroup, but this relative quantitation method can be adjusted for use with many phospholipids of interest. Fragmentation transitions for the primary component of each phospholipid were monitored, and lipids extracted from phospholipid-incubated platelets were compared to those extracted from control platelets. Phospholipid concentration values were then normalized to extracted protein values from platelet pellets incubated with phospholipids and washed under the same conditions as the platelets from which the phospholipids were extracted. Calibration curves were prepared to ensure a linear detector response over the relevant phospholipid concentration range. Selected reaction monitoring

(SRM) transitions that were used for phospholipid enrichment assessment are given in Table 4.1. All phospholipid transitions were monitored in all platelet samples. Enrichment results for PC and SM can be found in the SI. For PS, PE, and (to a much smaller extent) PC, enrichment was observed for the phospholipid in which the platelets had been incubated (Table 4.1). Thus, 2h incubation was enough time for platelets to take up exogenous phospholipid. This allowed a direct comparison of the effect of enrichment of each particular phospholipid on platelet secretion and adhesion behavior. The percent increase in phospholipid content was 808 ± 59 and 18.7 ± 7.5 for PS and PE conditions, respectively (Table 4.1). This study is the first to demonstrate that cells exposed to exogenous phospholipids can take them up and can do so selectively. The aforementioned errors in phospholipid enrichment are the standard deviations of the enrichment. Surprisingly, the % enrichment for PS incubation was drastically higher than the enrichment for PE.

In addition to PS and PE, effects of incubation with the phospholipids sphingomyelin (SM) and phosphatidylcholine (PC) were also examined. While these two phospholipids influenced platelet secretion and adhesion behavior, incubation with PC and SM did not consistently result in enrichment of the phospholipid with which the platelets were incubated. Effects of PC and SM on platelet behavior can be found in Supplementary Information.

Table 4.1. Summary of the UPLC-MS/MS analysis of each phospholipid

Phospholipid Incubation Condition	Control	PS	PE
Transition used for relative quantitation	n/a	812.5→627.4	718.3→577.3
Instrumental Precision (RSD)	n/a	5.91%	15.9%
Biological Precision (RSD)	n/a	23.3	13.3
Total protein in pelleted platelets (µg/mL ± SD)	117 ± 17	113 ± 26	118 ± 11
Percent increase in platelet phospholipid upon incubation (range in 4 replicates)	n/a	760 – 874	12.9 – 29.7
Average percent phospholipid increase (percent ± SD)	n/a	808 ± 59	18.7 ± 7.5

4.4.2 Phospholipids and Platelet Adhesion

The effect of plasma membrane phospholipid content on platelet adhesion was assessed on an endothelial cell-coated microfluidic platform to mimic *in vivo* vascular conditions. Consistent with other assessments in this manuscript, fluorescently labeled platelets were incubated with PE or PS prior to injection through the endothelial cell-coated microfluidic channel. In the microfluidic adhesion experiments, ADP was used to activate platelets; ADP as the stimulus makes it possible to decouple our investigation of adhesion from that of secretion because ADP is known to induce platelet adhesion without initiating

secretion.[20] While the adhesion of ADP-activated platelets from each condition was relatively consistent, the number of platelets adhered to endothelial cells varied from day to day; as such, comparisons of platelet adhesion to endothelial cells under each phospholipid condition were done with respect to the number of control platelets adhered to endothelial cells.

The ratios measured for each condition were tested to see if they were statistically different from the value 1 because a ratio of 1 indicates the same number of adhered platelet in the control and phospholipid-enriched platelets. As seen in Fig. 4.1, platelets enriched with PS showed reduced adherence to endothelial cells compared to the control condition (0.625 ± 0.075, $p = 0.004$). In contrast, platelets enriched with PE adhered to the endothelial layer in significantly higher numbers, with a ratio of 1.55 ± 0.02 ($p = 0.04$). Pre-incubation with either PC or SM also resulted in suppressed adhesion (data not shown).
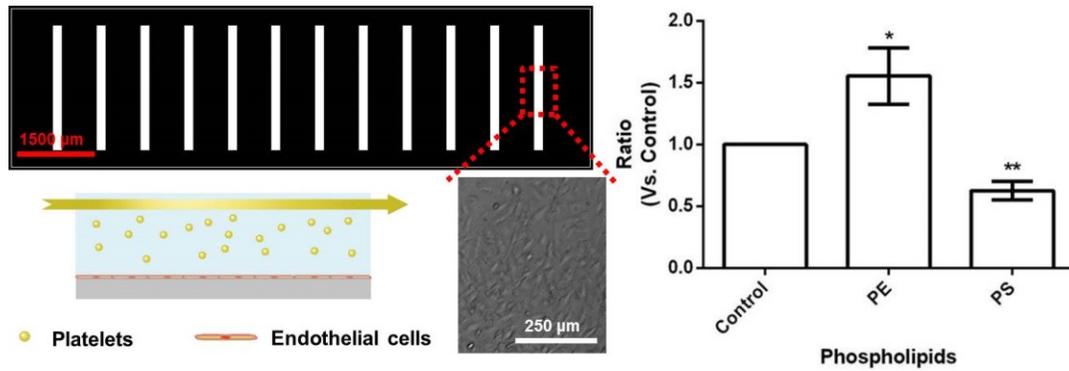
Figure 4.1. Platelet adhesion measurements.
(A) Schematic of microfluidic platelet adhesion experiment. (B) Ratio of the number of platelets adhered to the endothelial cell layer at each phospholipid enrichment condition compared to control. *denotes $p<0.05$, ** denotes $p< 0.01$).

4.4.3 Assessment of α-granule, δ-granule, and Lysosomal Release at Altered Phospholipid Levels with Ensemble Secretion Assays

Bulk secretion assays were performed under conditions with phospholipid enrichment. In all cases, the amount of secreted molecules from each type of granule was normalized according to platelet protein content. Platelet factor 4 (PF$_4$) was measured from α-granules, serotonin was measured from δ-granules, β-Hexosaminidase was measured from lysosomes, and platelet-activating factor (PAF) was measured as it was manufactured from platelet lipid membranes.

Each of the measured species was selected because of their important physiological functions, both in platelets and across other immune cell classes. The protein content of α-granules includes soluble proteins that are secreted to promote coagulation as well as proteins that are bound to the external platelet surface during activation.[21-23] PF$_4$, a soluble secreted protein, promotes blood clotting through binding to circulating anti-coagulation molecules such as heparin.[24] δ-granules influence coagulation through storage and secretion of small biogenic amines, polyphosphate, Ca^{2+}, ADP, and ATP. Due to its electroactive nature, serotonin can be detected at the single-platelet and bulk-suspension level, making it an ideal a marker of δ-granule secretion. Lysosomes contain acid hydrolases, which are a class of enzymes found in other secretory cells including mast cells and polymorphonuclear leukocytes.[25,26] While the secretion of δ-granules and α-granules from platelets has been studied extensively, relatively little is known about platelet lysosomal secretion. β-Hexosaminidase (β-Hex) is easily assayed using an absorbance

measurement and is widely used as a marker of acid hydrolase activity. As its name suggests, PAF is a secreted phospholipid that is widely regarded as a potent contributor to the platelet activation cascade. Additionally, PAF is secreted from and acts on many other cell types, including macrophages, mast cells, and polymorphonuclear leukocytes.[27]

Ensemble assay results show that an increase in the PS content impairs both α- and δ- granule secretion as well as lysosomal secretion. While control platelets secreted 0.043 ± 0.011 µmol serotonin/µg protein upon stimulation with thrombin, PS incubation led to a significant decrease in the average amount of δ-granule-localized serotonin secreted to 0.015 ± 0.010 µmol serotonin/µg protein (Fig. 4.2D; p=0.008). A more drastic effect was observed in α-granule secretion; upon PS enrichment, PF$_4$ secretion was completely attenuated, as PF$_4$ values were not significantly different (p = 0.2) from those of the unactivated platelets (Fig. 4.2B). PS also impairs lysosomal secretion and PAF secretion, though to a lesser extent than α- and δ- granule secretion. However, unlike α-and δ- granular release, lysosomal secretion and PAF secretion were unchanged by PC-, PE-, or SM-enrichment compared to control platelets.
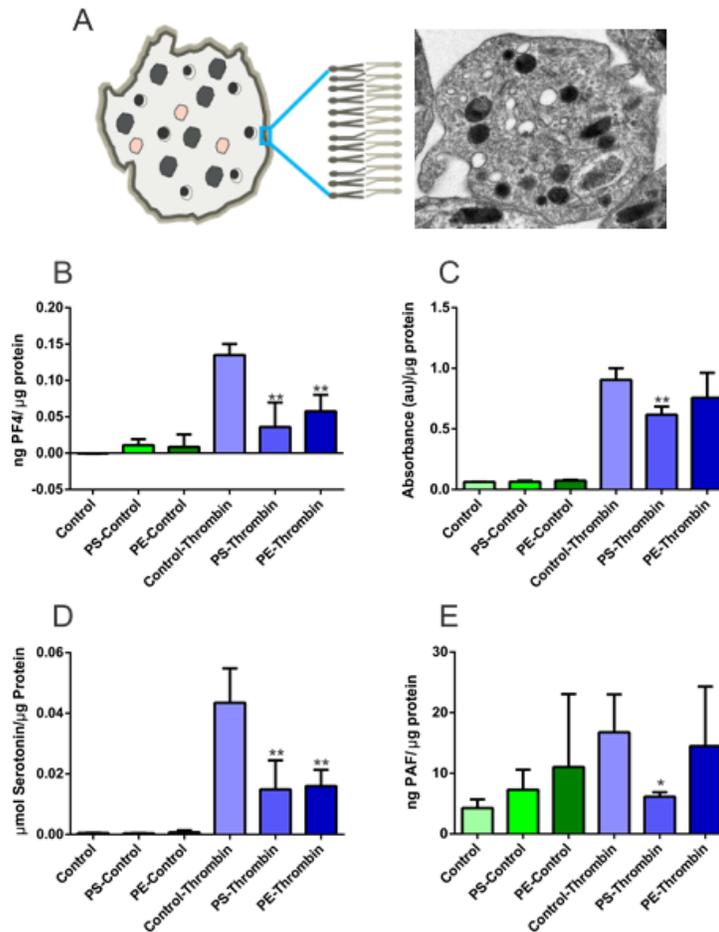
Figure 4.2. Assessment of phospholipid effects on ensemble platelet granule secretion.
(A) Transmission electron micrograph of a platelet and a representative figure illustrating three different platelet granule types; δ-granule, bull's eye shape; lysosome, pink shape; and α-granule, uniformly filled irregular shapes. Phospholipids are asymmetrically distributed in the platelet membrane. (B) PF_4 release from α-granules decreased with enrichment of each of the phospholipids studied. (C) Lysosomal release decreased with PS enrichment. (D) δ-granule secretion was also suppressed upon incubation with phospholipids. (E) Platelet-activating factor secretion was suppressed upon PS enrichment. *$p < 0.05$ and **$p < 0.01$ compared to thrombin-stimulated control platelets.

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4.4.4 Effect of Phospholipids on Single Platelet δ-Granule Secretion

While the ensemble assays clearly indicate that phospholipid content influences platelet secretion, mechanistic insight about why or how these changes occur would be helpful. In fact, because δ-granules contain electroactive serotonin, CFMA can be employed to achieve biophysical understanding of the phospholipid influence on platelet secretion. The sub-millisecond time resolution of the CFMA technique enables a detailed characterization and comparison of the δ-granule secretion in control and phospholipid-enriched platelets.[28] Parameters analyzed for each δ-granule, manifested in amperometric traces as individual current spikes, include the total secreted serotonin (Q), kinetics of secretion ($t_{1/2}$), total number of granule fusion events per platelet (N), and the percent of fusion events that exhibit a foot feature. In amperometric analysis of exocytosis, the presence of a foot feature (a small increase in measured current that is immediately adjacent to a large "full fusion" current spike) reveals information about the stability of the membrane fusion pore that forms prior to dilation of the pore for more complete chemical messenger secretion. The numbers of individual platelets analyzed using CFMA per phospholipid condition were: (control= 47, PE= 27); (control=20, PS=18); (control =20, PC= 21); and (control=58, SM=45).
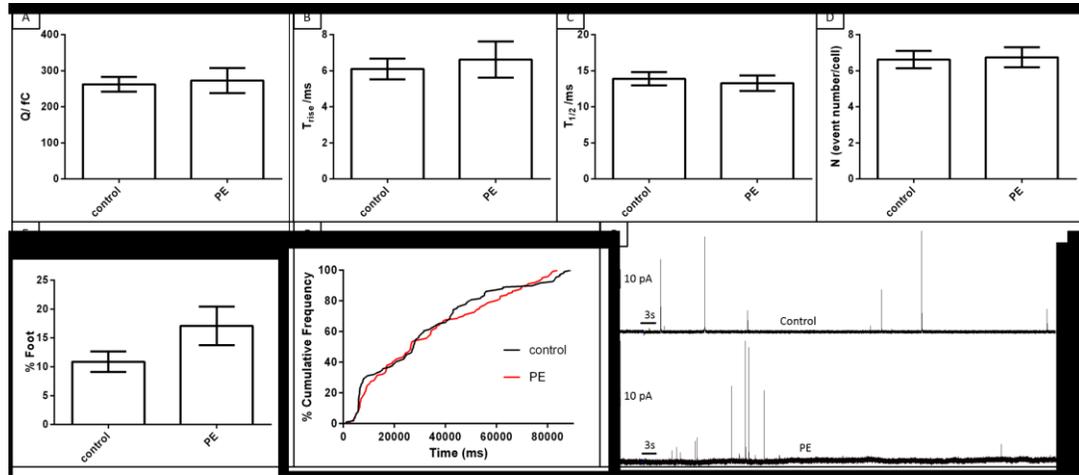
Figure 4.3. Effect of PE on δ-granule quantal release and release kinetics.
(A) PE did not influence the amount of serotonin released from single δ-granules. Kinetics of the release (B) and (C) were also unchanged. (D) Similar numbers of δ-granules were exocytosed from individual platelets with comparable fusion pore stability (E). Cumulative frequencies of the release are similar for each condition (F). (G) Representative amperometric traces from a control platelet (top) and PE-enriched platelet (bottom). Thrombin was applied for 3s, as indicated by the black bar on traces in (G).
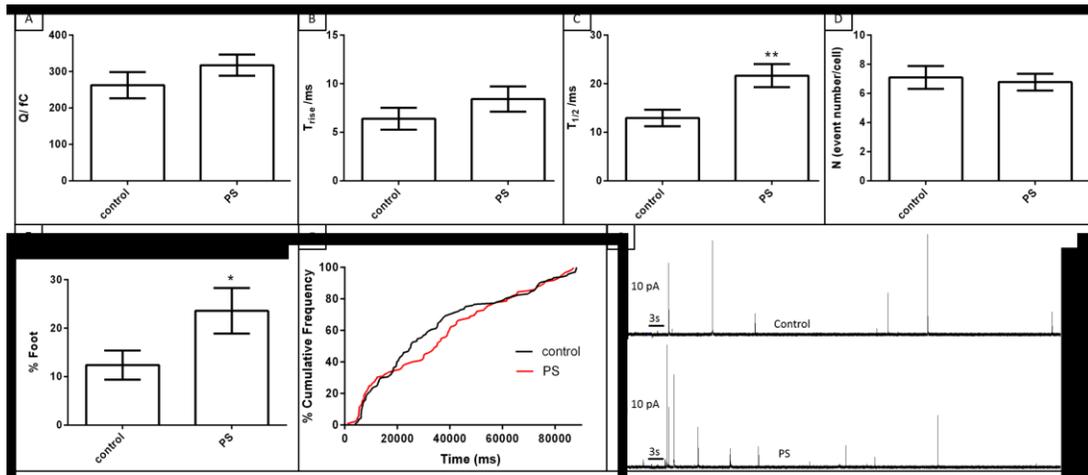
Figure 4.4. Effect of PS on δ-granule quantal release and release kinetics.
(A) PS did not influence the amount of serotonin released from single granules. Similar
T_{rise} (B) but higher $T_{1/2}$, **$p = 0.005$ (C) values were obtained, indicating slower
chemical messenger secretion with enriched PS. (D) The number of granules
exocytosed from single platelets did not change. (E) PS increased fusion pore stability,
*$p = 0.05$. (F) Cumulative frequency analysis shows initially fast but later slowed
granule trafficking in PS-enriched platelets. (G) Representative amperometric traces
from a control platelet (top) and PE-enriched platelet (bottom). Thrombin was applied
for 3s, as indicated by the black bar on traces in (G).

Although incubation of the platelets with PE resulted in enrichment of this phospholipid, PE-enriched platelets did not show a significant change in δ-granule secretion (Figures 4.4 and 4.5). The number of events with a foot feature was also not significantly changed with PE incubation compared to the control condition (p=0.08).

In contrast, PS, the most significantly enriched phospholipid of those considered, did induce platelet δ-granule secretion alterations. Upon PS enrichment, the amount of chemical messenger secreted per granule (the quantal secretion) did not change (Q= 262.70 ± 36.06 fC vs. 317.50 ± 29.10 fC for control and PS conditions, respectively; p=0.2). Since the average number of granules secreted from individual platelets also did not change between these conditions, it can be concluded that the total amount of serotonin secretion per platelet does not change with PS enrichment. This contradicts the bulk serotonin secretion measurement done using HPLC where enriched PS lowered the amount of secreted serotonin. Since single cell measurements are performed by measuring the action of one cell at a time in a relatively isolated space, it is unlikely that the activated secretion of one platelet can have a downstream effect of activation on another one. In a bulk suspension of platelets, however, activated platelets can secrete chemical messenger species that have the downstream effect of activating other platelets. By combining both single cell and ensemble measurements of platelet function, this study reveals insight into both the platelet behavior at the cellular level as well as the behavior of platelets in a suspension where they can influence one another. Ensemble measurements yield insight into the downstream effects of platelet activation and the feedback loops involved in

platelet function. The discrepancy between the single cell and ensemble measurement indicates that the downstream effect of activated platelets on the larger platelet population is important. However, without single cell measurements, it is not possible to observe the actual response to the stimulation, nor the heterogeneity in the response among a population of platelets.

Although CFMA reveals a similar amount of serotonin is extruded from platelet granules in both control and PS-enriched conditions, the secretion kinetics slow at higher membrane PS concentrations ($t_{1/2}$ values are 12.39±3.01 and 21.67±2.38 ms for control and PS conditions, respectively; p=0.005). While the number of granules secreted from individual platelets (N values) did not change, the platelets enriched with PS appear to have two different phases in terms of time evolution of exocytosis (Figure 4.4F). In the early phase, the granules that were docked before activation are released with a comparable frequency to the control condition. However, the frequency of the granular secretion decreases as the cell secretion continues, showing slower granule trafficking and docking compared to the control condition. Finally, fusion pore analysis showed that PS enrichment increases the stability of the fusion pore, with foot events occurring in 23.6±4.6% of the total secretion events, compared to 12.4±3.0% foot events observed for control.

4.5 Discussion of Experimental Results

Phospholipids are the major components of the plasma and granule membranes, and in addition to their structural importance as a cellular barrier that separates the intracellular and extracellular environment, they are also dynamically involved in and

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regulate many cellular processes. Platelets present an optimal platform for examining cellular uptake of phospholipids and the effects of phospholipid enrichment on cell-cell communication functions. Additionally in platelets, the phospholipid components are especially critical because it is likely that phospholipids are involved in platelet adhesion and secretion. However, little is known about how the different lipids regulate platelet behavior. In this work, common membrane phospholipids PS and PE were used to study the effect of phospholipid enrichment on platelet adhesion (using a microfluidic device to mimic the vascular environment), granular secretion (using CFMA and bulk secretion assays to evaluate the importance of phospholipids in platelet α- and δ- granule and lysosomal release), and bioactive lipid secretion (using UPLC-MS/MS to quantify secreted PAF).

Platelets were incubated with each of the phospholipids for 2h, long enough to enrich the platelets with the phospholipid of interest but short enough to minimize *de novo* regulation of other phospholipids. The drastically larger PS enrichment compared to PE enrichment is potentially due to a paucity of the particular PS species monitored in control platelets, which results in a much larger relative increase. Use of microfluidic channels coated with endothelial cells simulated blood vessels for evaluation of platelet adhesion. To separate platelet adhesion from secretion ADP, a strong agonist for platelet adhesion but not secretion, was used as the platelet stimulant.[20] 5 μM ADP provided a moderate platelet activation that could be monitored via fluorescent labeling of adherent platelets on the endothelial cell layer for each condition. Although PS is mostly known for its

procoagulant function, PS enrichment in fact diminished the platelet adhesion compared to control platelets. Besides providing a negatively charged surface to facilitate the binding of the coagulant factors, flipping PS from inner leaflet of the plasma membrane to the outer leaflet provides reorganization of the coagulation proteins in the plasma membrane. Upon PS incubation, it is likely that the PS concentration on the outer leaflet of the platelet membrane increased and impaired the translocation of the endogenous PS from inner leaflet to outer leaflet, thus diminishing the reorganization of the coagulation proteins that would facilitate platelet adhesion to endothelial cells. This observation is in agreement with previous findings in which the higher plasma level of PS decreased platelet coagulation and thrombus formation.[7,29] Unlike PS, PE enrichment boosted platelet adhesion by causing 50% more platelet adhesion than control platelets. Previous work published by Zieseniss et al showed that oxidized PE is one of the major components of low density lipoproteins that cause very strong thrombotic function.[30] A similar effect explains the observations of this work and reveals that PE synthesis and oxidation may be a good target for thrombosis prevention.

In addition to their role in platelet adhesion, phospholipids are major components of the highly conserved secretion process of exocytosis. During exocytosis, phospholipids not only serve as a matrix, accommodating essential proteins for membrane fusion but, by controlling the fluidity and curvature of the membrane, they influence the energy required for each step of exocytosis, especially the formation and stability of the granule-cell membrane fusion pore.[17,31-34] Ensemble secretion measurements were made on three

classes of platelet granules. Both α- and δ- granule secretion diminished greatly upon PS or PE enrichment. Lysosome secretion also decreased upon PS enrichment but not with PE enrichment. It is clear from literature precedent that phospholipids interact with the exocytotic machinery, and moreover, that there are specific sub-units of proteins involved in recruitment and secretion of exocytotic granules.[35,36] A similar scenario may be true for lysosomal release where specific interactions of PE are required. Although phospholipid regulation of lysosomal function has been reported in other cell types[37] there is not any precedent work either with evaluation of increased cellular phospholipid content or on platelets; thus, future work will explore these data further.

Platelets, like many other eukaryotic cell types, secrete bioactive lipids that are enzymatically synthesized from phospholipids, and it is interesting to consider that changes in the composition of the phospholipid bilayer may influence *de novo*-generated bioactive lipids. The interactions between phospholipids and circulating or membrane-bound enzymes are clearly necessary for bioactive lipid generation. Herein, PS enrichment suppressed PAF secretion, although similar effects were not observed with PE enrichment. This could be a result of the different degrees of enrichment of PS and PE or different action of the two species. For example, suppression of PAF secretion upon enrichment with PS could be a result of increased interactions between PS and the enzymes responsible for PAF synthesis (phospholipase A_2 and lyso-PAF acetyltransferase) that result in suppression of enzyme activity or suppression of enzyme interaction with phosphatidylcholine.

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Amperometric recordings of δ-granule secretion enabled detailed evaluation of the secretion at altered phospholipid levels. PE did not cause any change in the quantal release or the secretion kinetics. However, PS enrichment resulted in longer secretion events with a prolonged spike width (higher $t_{1/2}$) (Figure 4.4C). A more apparent two phase frequency of release was also observed with PS-enriched platelets, although the total number of granules released per cell were not significantly different. This implies a regulatory effect of PS on granular recruitment but not docking.

Although PE supports negative membrane curvature, PS packs better on positively curved membranes. The role of phospholipid curvature in exocytosis becomes more prominent during fusion pore formation.[17,31-34] Fusion pores are highly curved membrane structures that require phospholipids with positive curvature to pack in the outer leaflet and the phospholipids with negative curvature to pack on the inner leaflet for stabilization. To achieve a stable fusion pore, the phospholipid with appropriate curvature should be on the appropriate side of the membrane. Although PE incubation slightly increased the % of foot events, the changes were not significant (p=0.08). While it is known that the lipids with negative curvature stabilize the fusion pore better, PS (with positive curvature) provided more stable fusion pores compared to untreated platelets. This can be attributed to the exposed PS on the surface of the membrane upon activation which stabilizes the positive curvature of the fusion pore. Previous work has reported that the time of PS transport from the inner leaflet to the outer leaflet of the membrane is 3 min.[7] During 90 s of amperometric recordings, it is likely that more than half of the PS was exposed to the plasma outer

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surface. Moreover, previous work demonstrated that PC12 cells exposed to PS also displayed more stable fusion pore events; this was attributed to the interaction of PS with synaptotagmin, giving rise to a high negative curvature membrane. [17,31-34,38] Platelets have synaptotagmin-like protein 1, and its interaction with PS is not known but certainly may follow a similar behavior.[39]

4.6 Conclusions

Herein, this study has demonstrated the utility of platelets as a tool for understanding how different phospholipids can act on different aspects of platelet function. Platelets were exposed to two of the most common inner-leaflet phospholipids, PS and PE, and this study demonstrates that platelets can incorporate exogenous phospholipids into their membranes. Results indicated that enrichment of PS generally suppresses platelet function, and PE at the enriched level does suppresses platelet α- and δ-granule secretion, and it over-activates the thrombotic function of platelets. Moreover, this work highlights the possibility of interaction between phospholipids and important proteins involved in the secretion process, and the results and methods herein can be easily applied to other mammalian cell types using analogous cellular machinery. Elucidating the function of lipid-protein interactions will improve our current understanding of platelet secretion since it is obvious that the platelet secretion is regulated synergistically by both protein and lipid species.

Chapter 5: Stereochemistry- and Concentration-Dependent Effects of Phosphatidylserine Enrichment on Platelet Function

5.1 Overview

The results of the previous chapter clearly show that phosphatidylserine has important roles in platelet function. In this chapter, the work on phosphatidylserine and platelet function continues with an exploration of effects of both phospholipid headgroup stereochemistry and exogenous phospholipid concentration on platelets. Phosphatidylserine was selected for further study in part because of its importance in signaling apoptosis and because its binding is necessary for the function of enzymes such as protein kinase C. Additionally, recent studies have detected the presence of low levels of phosphatidylserine containing a d-serine headgroup, which 1) had not been previously known to exist in eukaryotic organisms and 2) has different physical and biological properties than the much more abundant phosphatidylserine containing a l-serine headgroup. Platelets are again used in this chapter as a platform for evaluating the phospholipid uptake and effects of phospholipid uptake on cellular function, with the inclusion of a study of the effects of phosphatidyl-d-serine and phosphatidyl-l-serine on platelet cholesterol content.

5.2 Introduction

Platelets are small (1-2 μm in diameter), circulating anuclear cell fragments that promote hemostasis by mediating the processes of clotting and angiogenesis. Although most widely known for their involvement in blood clot formation, they also play roles in inflammation and cancer malignancy. All of these actions are mediated by platelet secretion and adhesion, both of which are influenced by the platelet membrane

composition.[1] In addition to the importance of platelets in various physiological processes, the anuclear nature of platelets makes them an interesting platform for studying the role of membrane phospholipids in cell secretion and adhesion functions.

Phosphatidylserine is a particularly important component of cellular membranes due to its critical role in the cell signaling cascades, in particular, those that lead to clearance of apoptotic cells. In most cells, phosphatidylserine with an L-serine headgroup (L-PS) composes 2-10% of the total phospholipid content, and until recently, it was thought that all naturally occurring phosphatidylserine phospholipids were L-PS.[2] Recently, however, phosphatidylserines containing a D-serine headgroup (D-PS) have been detected at quantities of $0.05 – 0.9\%$ of the total phosphatidylserine content in rat tissues including cerebrum, heart, spleen, lung, testis, liver, and kidney,[2] and it is not clear if the biological roles of L-PS and D-PS are identical. When cells are unactivated, L-PS is localized to the inner leaflet of the membrane while the localization of D-PS is unknown. Upon activation, aminotranslocase, which maintains L-PS in the inner leaflet, is down-regulated, inducing activation of lipid scramblase. This activation, in conjunction with the rapid influx of Ca^{2+}, disrupts the phospholipid asymmetry, resulting in the exposure of L-PS to the outer leaflet of the membrane.[3-5] The reorientation of L-PS to the outer leaflet of the platelet is important as L-PS exposure to the extracellular environment signals apoptosis to surrounding phagocytes.[6-12] Several studies have also shown that the stereochemistry of the phosphatidylserine headgroup influences both the binding of cell signaling proteins and

the clearance of apoptotic cells by phagocytes including macrophages, which only recognize L-PS.[13]

Work presented in Chapter 4 has shown that platelets take up exogenous L-PS within 2 hrs of phospholipid incubation, and that the increased membrane L-PS suppresses platelet secretory and adhesion behavior.[14] In this study, L-PS, a particularly important membrane phospholipid, and its analog D-PS, are examined in depth for their relative effects on platelet function.

5.3 Experimental Approach

5.3.1 Reagents, Platelet Isolation, and Platelet Exposure to L-PS and D-PS

Unless otherwise specified, all reagents were purchased from Sigma-Aldrich (St. Louis, MO) with analytical quality or better. The α-D-(+)-glucose was purchased from Acros Organics (Fair Lawn, NJ), PAF and PAF-d_4 standards were purchased from Cayman Chemical (Ann Arbor, MI), and the L-α-phosphatidylserine (product #840032C) and 1,2-dioleoyl-sn-Glycero-3-Phospho-D-serine (product #79098) were purchased from Avanti Polar Lipids (Alabaster, AL). For UPLC-MS/MS, LC/MS-grade H_2O and acetonitrile (ACN) were purchased from J.T. Baker (Center Valley, PA). LC/MS-grade isopropyl alcohol (IPA), Na_2CO_3, and citric acid were purchased from Fisher Scientific.

Male C57BL/6J mice were purchased at 9 weeks old from The Jackson Laboratory (Bar Harbor, ME), euthanized via CO_2 asphyxiation (according to IACUC-approved protocol #0806A37663), and blood was drawn via cardiac puncture using syringes pre-filled with 200 μL ACD. For all experiments, platelets were isolated by centrifuging whole

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blood for 10 min at 130 RCF with no brake, transferring the plasma layer to fresh tubes, diluting the plasma layers 6:1 with ACD, and pelleting the platelets at 524 RCF.

Following isolation, platelets were incubated for 2 hrs with 10, 50, or 100 µM L-PS or D-PS prepared in Tyrode's buffer. To avoid solubility inconsistencies, 200 µM solutions of L-PS or D-PS stock solutions were prepared as described previously[15] and diluted as needed for incubations with different L-PS or D-PS concentrations. Briefly, solutions were prepared by evaporating the chloroform from appropriate volumes of the phospholipid solutions with a gentle stream of nitrogen, stirring overnight in Tyrode's buffer, then filtering with 0.2 µm filters.

All assessments of L-PS and D-PS enrichment, cholesterol content, and platelet secretory function were normalized to pelleted platelet protein content, which was quantified using a Pierce bicinchoninic acid (BCA) assay kit from Thermo Scientific (Rockford, IL). Where possible, measured values of secreted mediators were normalized to the pelleted protein content of individual replicates; when this was not possible, pelleted protein values represent the average of four biological replicates.

5.3.2 UPLC-MS/MS Assessment of Relative L-PS and D-PS Enrichment

Enrichment of L-PS and D-PS in platelet membranes was assessed using a Waters Acquity UPLC system in tandem with a Waters triple quadrupole mass spectrometer using a previously reported method[15] modified to include transitions for D-PS. Details of the UPLC method can be found in Chapter 4. Briefly, aqueous mobile phase A was 20 mM ammonium acetate at pH 5, organic mobile phase B was 90/10 ACN/IPA with 0.1% acetic

acid, and a Waters BEH C8 2.1 x 100 mm column at 60°C was used. Multiple reaction monitoring was used for mass spectrometric detection of the phospholipids: transitions monitored for L-PS, and D-PS can be found in Table 5.1. PAF-d4, with a transition from 524.4 > 184.1, was used as an internal standard. L-PS and D-PS were isolated from PRP after platelets incubated with the phospholipids were washed three times via centrifugation at 1000 RCF and resuspended in 4:1 Tyrode's buffer:ACD. After the final centrifugation step, platelets suspended in 100 µL Tyrode's buffer were sonicated with 400 µL chloroform and 200 µL methanol for 20 min, then sonicated for 10 more min following the addition of 100 µL 0.1% acetic acid in 0.1 M NaCl. Samples were centrifuged for 5 min at 1500 RCF, the upper aqueous layers were discarded, and the lower organic layers were dried under vacuum. Samples were resuspended by sonication in 40/60 A/B and analyzed by UPLC-MS/MS. Using a calibration curve prepared exactly as the platelet samples, relative L-PS and D-PS concentrations were acquired for each sample. To account for differences in platelet pelleting that could arise from increased phosphatidylserine content, L-PS and D-PS values were normalized to pelleted protein content for each platelet incubation condition. Statistical differences between experimental conditions were assessed using one-way ANOVA.

5.3.3 Cholesterol Content of Platelets Enriched with L-PS and D-PS

The cholesterol content of platelets was assessed using an Amplex® Red cholesterol assay kit from Life Technologies (Grand Island, NY) as directed. Statistical differences between experimental conditions were assessed using one-way ANOVA.

5.3.4 Ensemble Measurements of Platelet Granule Secretory Function

After incubation with either L-PS or D-PS, platelets were washed and incubated with either PBS buffer (control) or PBS containing 1.25 U/mL thrombin (activated). After 5 min of incubation, ACD was added for a final volume of 1:4 ACD: PBS and centrifuged at 1,000 RCF for 10 min to pellet the platelets. Supernatants were collected and analyzed for the presence of granule-specific secreted species and *de novo* manufactured bioactive lipids. Secretion of δ-granules in a bulk suspension of platelets was assessed using an Agilent HPLC coupled to a Waters electrochemical detector as described previously.[15,16] Supernatants analyzed for serotonin were collected both after the wash step to assess whether incubation with L-PS or D-PS induced platelet activation and after stimulation with either PBS or thrombin to assess the effects of L-PS and D-PS on δ-granule secretion. Dopamine served as an internal standard for serotonin detection. For all ensemble measurements, statistical differences between experimental conditions were assessed using one-way ANOVA.

Lysosome secretion from platelets was assessed using an absorbance assay for β-Hexosaminidase (β-Hex), a lysosome-stored enzyme as described previously.[14,15,17] Absorbances from L-PS- and D-PS-treated platelets were normalized to the absorbance of control platelets. Secretion of α-granules was assessed using an enzyme-linked immunoassay kit for platelet factor 4 (PF4) purchased from R&D Systems (Minneapolis, MN) and used as directed.

Platelet secretion of PAF, a *de novo* manufactured bioactive lipid, was assessed using a modified version of the previously described UPLC-MS/MS method described in Chapter 4.[14,15] Although previous studies have focused on the detection of PAF-C16, this work focuses on the detection of PAF-C14. Due to the lack of available internal standards for PAF-C14, PAF-C16-d4 was used as an internal standard.

5.3.5 Microfluidic Assessment of Platelet Adhesion Behavior

The microfluidic device used in this study is fabricated using standard soft lithography techniques, and the detailed procedure can be found in Chapter 4.[18] Briefly, a simple straight channel design is printed onto a transparent film (Cad/Art Service Inc., Bandon, OR) and transferred onto a 5" x 5" blank chrome mask plate (Nanofilm, Westlake Village, CA). For the master copy, a silicon wafer is spin-coated with SU-8 photoresist (Microchem, Newton, MA), and the device design was transferred onto the baked SU-8 silicon wafer with the previously completed mask. Once developed, a mixture of 10:1 PDMS monomer:curing agent (Ellsworth Adhesives, Germantown, WI) was cast onto the SU-8 master and cured overnight at 75 ℃. The cured PDMS layer is then peeled, cut, and punched for the inlet and outlet holes, and then bound to a clean glass substrate via oxygen plasma treatment. Once completed, the device is brought into a biosafety hood and the channels are washed three times with 70 % ethanol solution in sterilized MilliQ water (Millipore, Billerica, MA) to sterilize the device. Washed devices are then dried and kept in the biosafety hood until use. The channel dimensions were 450μm (width) x 100μm (height) x 2500μm (length).

A human endothelial cell line (Hy926, ATCC, Manassas, VA) was used to establish a confluent layer of endothelial cells in the microfluidic channels. The endothelial cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) with high glucose media (4mM L-glutamine, 4.5g/L L-glucose, and 1.5g/L sodium pyruvate (Gibco®, Carlsbad, CA)) supplemented with 10% fetal bovine serum and 1% penicillin and streptomycin (Sigma Aldrich, Milwaukee, WI). Conventional T-flask culture at 5% CO_2 and 37℃ was maintained with an every other day feeding schedule and a once a week splitting schedule. Herein, endothelial cells were only used between the fifth and eighth passages.

A 250 μg/mL human fibronectin solution was introduced into microfluidic channels for 30 minutes ahead of endothelial cells to facilitate endothelial cell adhesion onto the microfluidic channel surface. In parallel, endothelial cells in a T-flask were trypsinized, washed, and re-suspended into the same culture media at a cell density of $\sim10^7$ cells/mL. After exchanging the media in the microfluidic channel with endothelial cell culture media, 20 μL of the cell suspension was injected through the channel, kept in an incubator for an hour, and washed with the endothelial cell culture media. Finally, 20 μL of the cell suspension was injected through the channel, incubated for another hour, and washed with the culture media again. The devices were then kept in an incubator until use with a 12 hour feeding schedule. After 1-2 days, a confluent monolayer of endothelial cells was established; devices that did not establish uniform layers of endothelial cells were discarded after visual inspection.

After platelets had been incubated with L-PS, D-PS, or Tyrode's buffer, they were labeled with CMFDA dye to facilitate their visualization (2 μM, 20 minutes). The labeled platelets were then split into two populations - one of these populations was treated with 5μM ADP to activate them and the other was the same volume of Tyrode's buffer as an unactivated control. As ADP activation may cause significant platelet aggregation before introduction onto the microfluidic device, incubation was avoided and preparation of endothelial cell-coated microfluidic devices (washing channels with Tyrode's buffer) was done in parallel. Both platelet suspensions, activated by ADP or unactivated, were then introduced into the microfluidic channels at the flow rate of 30 μL/hour, where minimal shear-induced platelet and endothelial cell activation is expected, for 20 minutes. After exposure of platelet suspension to the endothelial cell layer, the microfluidic channels were washed with Tyrode's buffer and devices were brought to the microscope stage for fluorescence imaging (Microscope - \Nikon, Melville, NY, CCD camera – QuantEM, Photometrics, Tucson, AZ). Metamorph Ver. 7.7.5 was used as the imaging and analysis software. Statistical differences between experimental conditions were assessed using student's t-test with $p \leq 0.05$.

5.4 Results and Discussion

5.4.1 Enrichment of L-PS and D-PS in Platelet Membranes

Enrichment of L-PS and D-PS in platelet membranes was evaluated using UPLC-MS/MS after incubating platelets with 10, 50, or 100 μM solutions of each phospholipid, and results of the enrichment are shown in Fig. 5. 1 A and B and detailed in Table 5.1.

Using UPLC-MS/MS to determine relative enrichment offers several advantages to the analytical challenge of phospholipid detection. Selective reaction monitoring (SRM) enables the detection of specific mass transitions, which were set to the primary components of each L-PS and D-PS standard rather than just detecting all of the phosphatidylserine compounds in the membrane. A D-PS standard was selected that differed from the primary component of the L-PS standard by 2 mass units, or one unit of unsaturation, which enabled chromatographic separation of the two compounds, as shown in Fig. 5.1D. To evaluate platelet uptake of L-PS and D-PS, the concentration of L-PS in control platelets (not exposed to L-PS or D-PS) was normalized to 100%, and concentrations of L-PS and D-PS were calculated as the percent enrichment of the L-PS in control platelets. Comparing both phosphatidylserine species to the same levels of L-PS in control platelets enables a comparison of the abilities of the platelets to take up each of the phosphatidylserine species.

Platelets demonstrated significant uptake of both L-PS and D-PS at all incubation concentrations examined, and the uptake of D-PS and L-PS at each incubation concentration were not statistically different from one another ($p > 0.05$ using one-way ANOVA), i.e. incubation with 10 µM L-PS produced the same percent enrichment of total PS as the 10 µM D-PS. This indicates that platelets were enriched to a similar degree with either L-PS or D-PS, and if any differences are observed between the L-PS and D-PS at the same incubation concentration (10, 50, or 100 µM), they are likely due to the stereochemistry of the headgroup.

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Table 5.1. Relative enrichment of L-PS and D-PS in platelet membranes.

Incubation Condition	L-PS	D-PS
Transition used for relative quantitation	812.5 > 208	810.5 > 208
Instrumental Precision (RSD)	6.97	7.74
Biological Precision (RSD)	13.7	15.7
Percent increase upon 10 μM incubation for 2 hours Average percent increase ± SD[a]	345 ± 106	279 ± 91
Percent increase upon 50 μM incubation for 2 hours Average percent increase ± SD[a]	575 ± 68	638 ± 98
Percent increase upon 100 μM incubation for 2 hours Average percent increase ± SD[a]	730 ± 76	749 ± 94

[a]Percent increase is reported as the percent increase over the nmol PS/μg pelleted protein, as the amount of D-PS present in control platelets was much lower than the D-PS detected in platelets incubated with D-PS. The enrichment of D-PS as a percentage of D-PS content of control platelets was 2350 ± 767 for platelets incubated with 10 μM D-PS, 5370 ± 822 for platelets incubated with 50 μM D-PS, and 6300 ± 625 for platelets incubated with 100 μM D-PS.

5.4.2 Effects of L-PS and D-PS Enrichment on Platelet Cholesterol Content

Previous studies regarding the interaction between cholesterol and phosphatidylserine have generally been made using model membrane systems. The work of Wachtel and colleagues demonstrated that the solubility of cholesterol in membranes containing phosphatidylserine decreases as the molar ratio of phosphatidylserine increases.[19] As such, it was hypothesized that increasing the phosphatidylserine content of the platelet membrane would expel cholesterol, thus decreasing the cholesterol content of the pelleted platelets and increasing the cholesterol content of the supernatants, as compared to control platelets. This change in membrane cholesterol level may also have significant impact on critical cell functions such as exocytotic delivery of chemical messengers.

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Cholesterol is integral to cell membrane function, and the interaction between cholesterol and phosphatidylserine, examined in a variety of model membranes and monolayers, is clearly important in regulating the structure, fluidity, and function of the cellular membrane.[19] To determine whether L-PS or D-PS enrichment induces the expulsion of cholesterol from the platelet membrane, the cholesterol content of the both the pelleted platelets and the supernatants following incubation with L-PS and D-PS were evaluated. Incubation with either D-PS or L-PS induced an increase in platelet cholesterol content (Fig. 5.1C), but the cholesterol levels in the supernatant were not significantly different from the cholesterol per protein content of the incubation supernatants of control platelets. As Fig. 5.1C shows, the increase in platelet cholesterol content is higher following enrichment with 50 µM L-PS than 10 µM L-PS (p = 0.004) and with 50 µM D-PS than with 10 µM D-PS (p = 0.0001). Additionally, the increase in cholesterol content following phospholipid incubation was higher with L-PS than with D-PS (p = 0.0003 for 10 µM incubations and p < 0.0001 for 50 µM incubations).
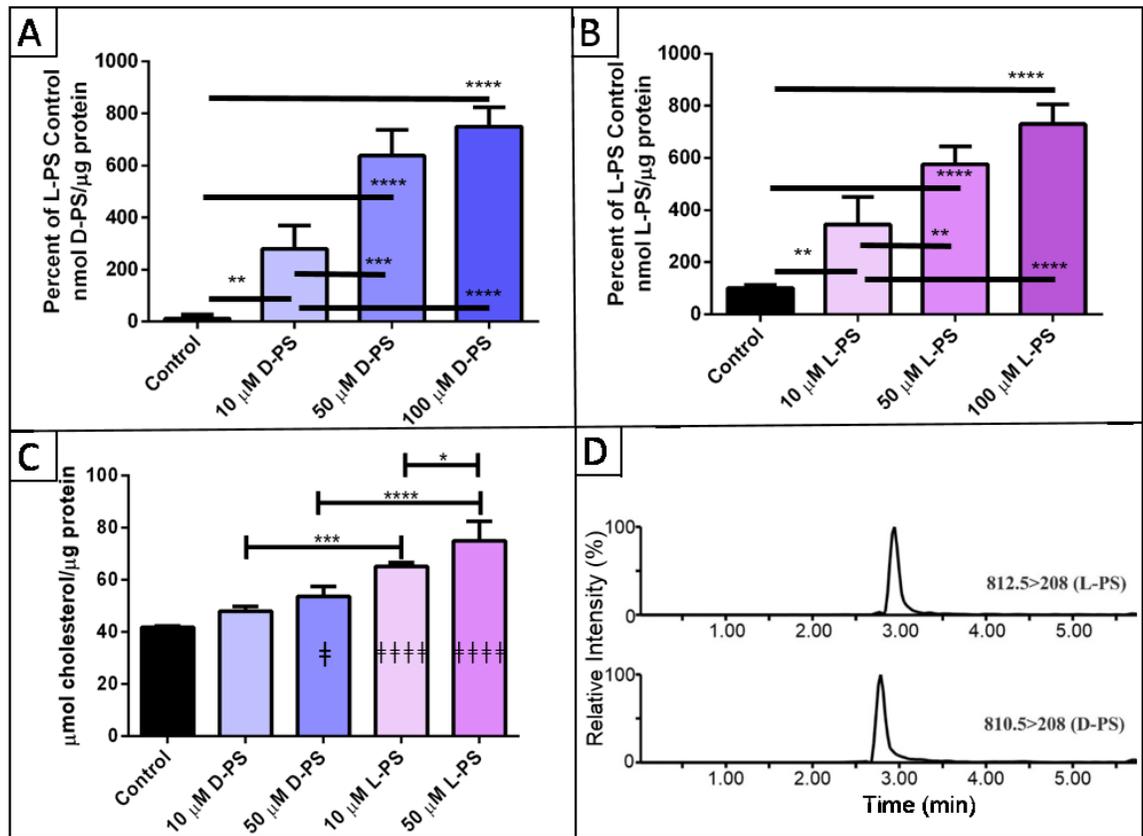
Figure 5.1. Percent enrichment of A. D-PS and B. L-PS, the cholesterol content of platelets, C, after incubation with either D-PS or L-PS, and D. Chromatograms of L-PS (top) and D-PS (bottom).
*$p \leq 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$ vs. indicated condition using one-way ANOVA. ‡$p \leq 0.05$, ‡‡‡‡$p < 0.0001$ vs. cholesterol control using one-way ANOVA. Error bars represent mean ± standard deviation.

The results of this study have demonstrated the opposite of the expected effect, indicating that either complexity of the platelet membrane affects the solubility of cholesterol in phosphatidylserine-containing membranes or cholesterol is expelled from the membrane but remains membrane-associated. Additionally, platelets were enriched to the same degree with either L-PS or D-PS, but the different forms of phosphatidylserine resulted in different levels of cholesterol content. There are several possible explanations for this effect. Within a phospholipid membrane, it has been shown that cholesterol, like phospholipids, is asymmetrically distributed in the membrane. Previous studies of cholesterol in phospholipid membranes support the theory that cholesterol exists within the membrane in two forms, both dissolved and crystalline. As cholesterol has been shown to have decreased solubility in phosphatidylserine, due to the increased hydrogen bonding around the negatively charged headgroup, it is possible that enriching platelets with phosphatidylserine results in the conversion of dissolved cholesterol to crystalline cholesterol. Another possibility is that the rigidity of phosphatidylserine decreases the solubility of cholesterol, which results in the conversion of cholesterol to its crystalline form.[19] When this occurs, the platelet would experience a shift in the equilibrium concentration of cholesterol and increase cholesterol production to compensate for the potentially decreased membrane stability induced by additional phosphatidylserine. The formation of cholesterol to a lesser degree in platelets incubated with D-PS may be a result of the extra unit of unsaturation; when in the membrane, a completely saturated tail will be relatively straight, allowing for tighter packing and interaction with the other tails, leading

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to extra rigidity. The extra unit of unsaturation in D-PS may cause the tail to deform, decreasing the extent to which the lipid tails can interact and pack, and, thus, increasing membrane fluidity. As shown in the work done by Wachtel and coworkers, this greater fluidity causes the solubility of lipid cholesterol to be increased, resulting in less crystallite formation and a smaller equilibrium shift in unactivated platelet cholesterol content.

5.4.3 L-PS vs. D-PS Effects on Ensemble Secretion of Chemical Species

To evaluate the effect of phosphatidylserine enrichment on platelet secretion, several ensemble measurements were used, including measurement of both stored and newly manufactured chemical messenger species. These measurements include *de novo* synthesis of PAF-C14, serotonin secretion from δ-granules, β-Hex secretion from lysosomes, and PF4 secretion from α-granules.

Effects of exposure to exogenous L-PS and D-PS on the secretion of PAF-C14 from platelets are shown in Fig. 5. 2. Exposure of control platelets to 1.25 U/mL thrombin for 30 min induced increased secretion of PAF-C14 ($p = 0.04$ vs. control unactivated platelets), while thrombin activation did not induce a significant increase in PAF-C14 secretion from platelets enriched with either D-PS or L-PS at 10 or 50 μM, when compared to their unactivated counterparts. For enrichment at 10 μM D-PS, this is almost certainly due to the significant increase in PAF-C14 secretion of unactivated platelets ($p = 0.003$ vs. control unactivated platelets). For platelets incubated with 50 μM D-PS and 10 μM L-PS, small but non-significant increases in the PAF-C14 secretion of unactivated platelets and small but non-significant decreases in the PAF-C14 secretion of thrombin-activated platelets

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likely contribute to the observation of no significant increases in PAF-C14 generation in response to thrombin. For platelets incubated with 50 μM L-PS, thrombin-activated secretion of PAF-C14 is completely attenuated (p = 0.04 vs. thrombin activated control platelets). This attenuation of PAF secretion is consistent with previous results for 100 μM L-PS incubation.

Serotonin, one species stored in δ-granules, was used as a marker of platelet δ-granule secretion. The effects of D-PS and L-PS enrichment on platelet δ-granule secretion are shown in Fig. 5. 3. At all enrichment levels of D-PS and L-PS, thrombin increased δ-granule secretion compared to their unactivated counterpart (p ≤ 0.05 for each thrombin-activated condition vs. unactivated platelets of the same enrichment condition). Unactivated platelets enriched with 10 or 50 μM D-PS or with 50 μM L-PS demonstrated increased δ-granule secretion vs. control unactivated platelets (p < 0.05). Meanwhile, thrombin-activated platelets enriched with 50 μM D-PS or 10 or 50 μM L-PS demonstrated decreased δ-granule secretion compared to thrombin activation of control platelets (p ≤ 0.05). The suppression of thrombin-stimulated δ-granule secretion was more pronounced with L-PS than with D-PS and more pronounced at 50 μM enrichment than 10 μM enrichment for both phosphatidylserine species. This effect is in contrast to the effects of D-PS and L-PS on δ-granule secretion of unactivated platelets, where both 10 and 50 μM D-PS enrichment induced δ-granule secretion, but only 50 μM L-PS did. Furthermore, 50 μM D-PS enrichment induced a greater degree of δ-granule secretion from unactivated platelets than enrichment with 50 μM L-PS (p = 0.04).
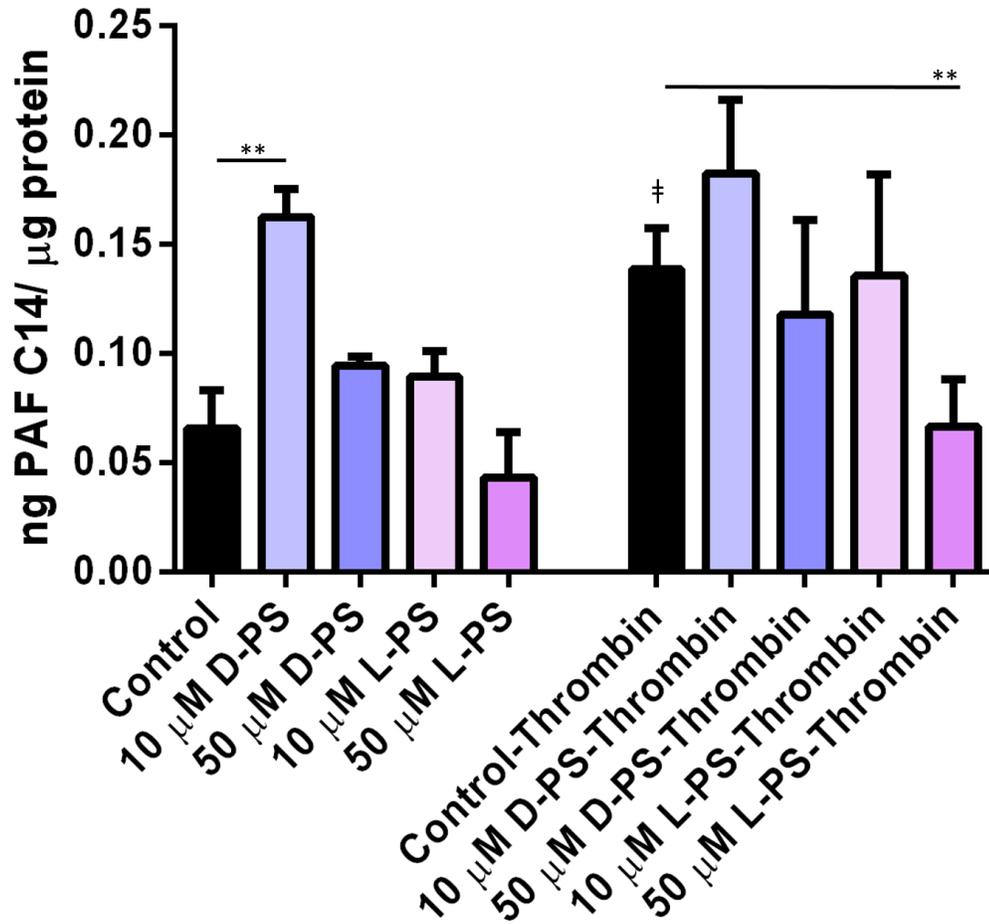
Figure 5.2. Secretion of PAF-C14 from platelets enriched with L-PS and D-PS.
 **p < 0.01 using one-way ANOVA. ‡p≤ 0.05 vs. control platelets.
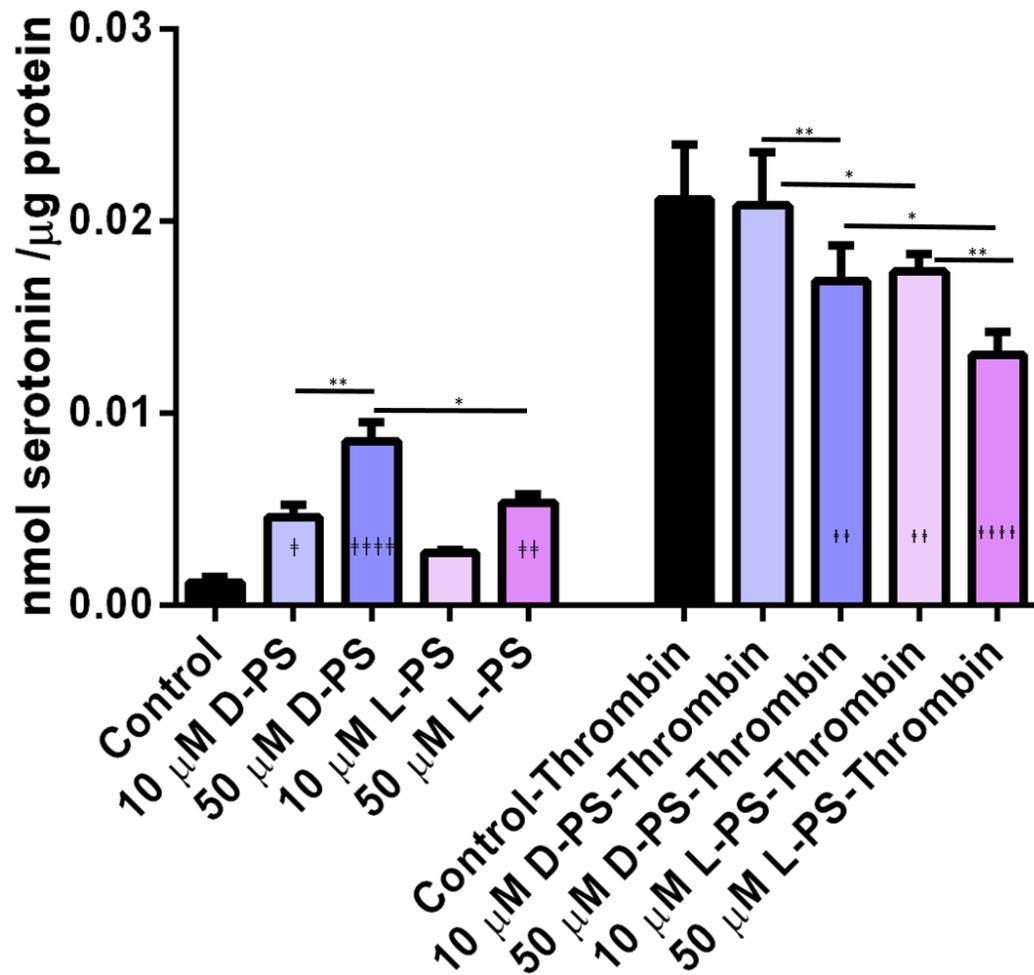
Figure 5.3. Effects of L-PS and D-PS enrichment on platelet δ-granule secretion.
*p ≤ 0.05, **p < 0.01 vs. condition as indicated using one-way ANOVA. ‡p ≤ 0.05, ‡
‡p< 0.01, ‡ ‡‡p< 0.0001 vs. Control (resting platelets) using one-way ANOVA. ‡‡p <
0.01, ‡‡‡‡p < 0.0001 vs. Control-Thrombin using one-way ANOVA.
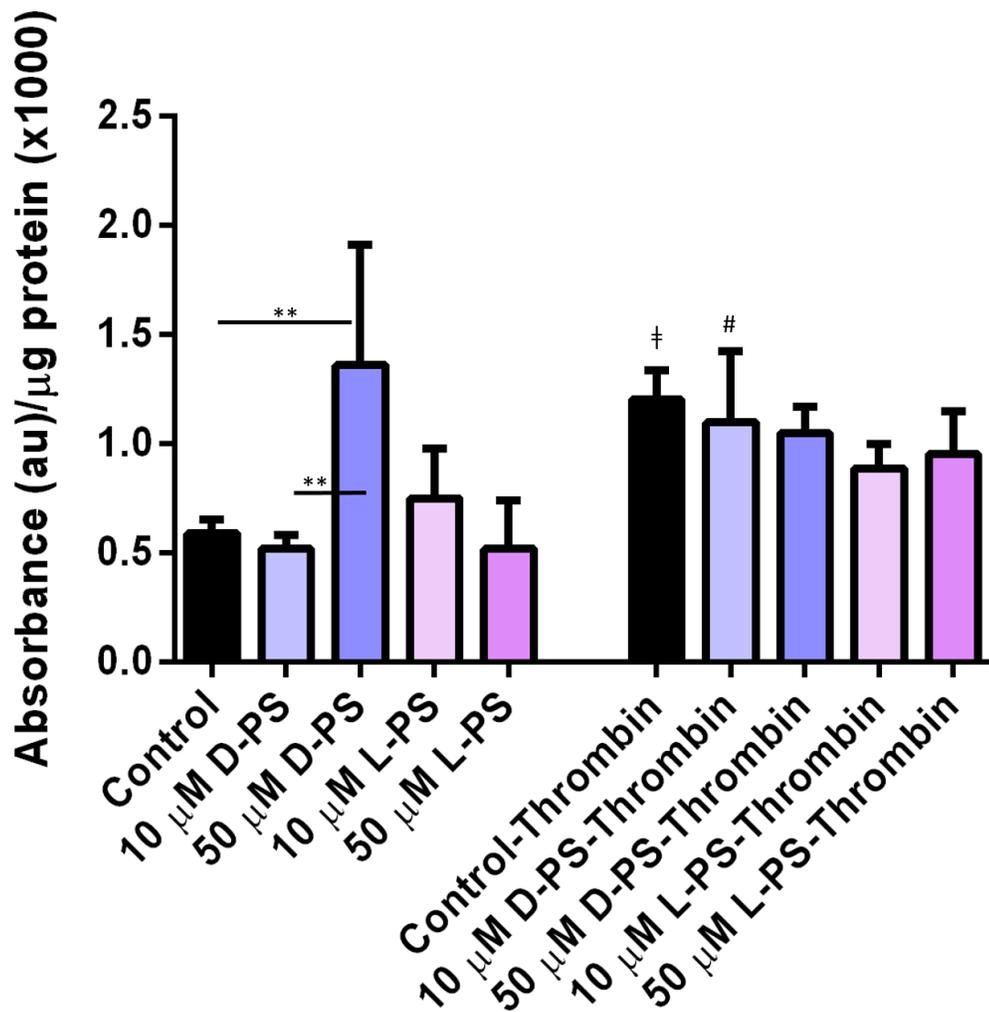
Figure 5.4. Lysosome secretion of platelets enriched with D-PS or L-PS.
**p < 0.01 vs. condition indicated; ‡p = 0.02 vs. Control resting platelets; #p = 0.04
vs. 10 µM D-PS enriched resting platelets using one-way ANOVA.
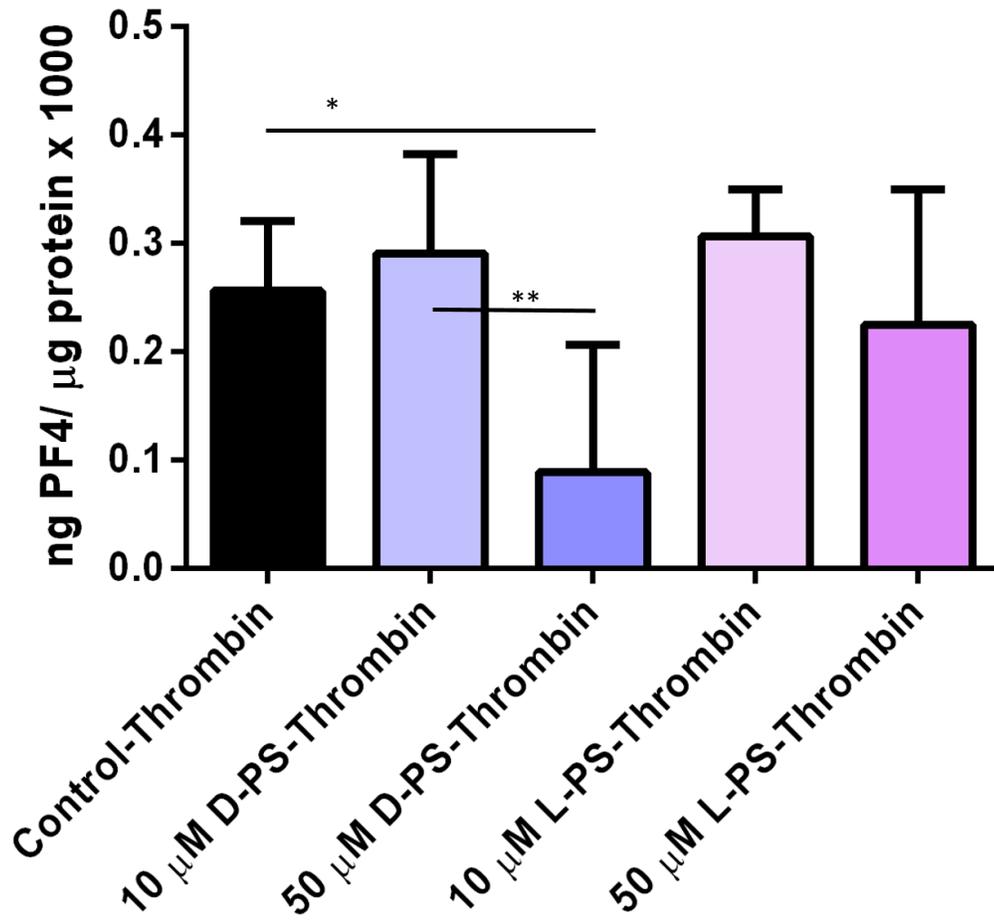
Figure 5.5. Thrombin-stimulated α-granule secretion of platelets enriched with D-PS or L-PS.
*p = 0.03; **p = 0.002, using one-way ANOVA. PF4 was not detected in resting platelet samples.

Lysosome secretion was assessed using an absorbance assay for β-Hex, one lysosome-secreted species, and the effects of L-PS and D-PS enrichment can be found in Fig. 5.4. When exposed to thrombin, control platelets and platelets enriched with 10 μM D-PS induced increased secretion of β-Hex. Unactivated platelets enriched with 50 μM D-PS increased lysosome secretion, but exposure to thrombin did not further increase the secretion of lysosomes from platelets.

The effects of L-PS and D-PS enrichment on thrombin-stimulated platelet secretion of α-granules are shown in Fig. 5. 5. PF4 was not detected in unactivated platelet samples. In thrombin-activated platelets, enrichment with 50 μM D-PS induced a decrease in the secretion of α-granules (p = 0.03 vs. control), but enrichment with L-PS or with 10 μM D-PS did not affect thrombin-stimulated platelet secretion of α-granules.

In general, enriching platelets with L-PS or D-PS suppresses the thrombin-stimulated secretion functions of platelets, while inducing granule secretion by unactivated platelets. In particular, in unactivated platelets, incubation with 10 μM D-PS induces the secretion of PAF-C14 and δ-granules, and incubation with 50 μM D-PS induces the secretion of δ-granules and lysosomes. Incubation with 10 μM L-PS did not affect the secretory behavior of unactivated platelets, and incubation with 50 μM L-PS induced the secretion of δ-granules, albeit to a smaller extent than with 50 μM D-PS. These results indicate that the incorporation of D-PS into the platelet membrane activates the protein machinery responsible for exocytosis, while L-PS incorporation into the platelet membrane does not. L-PS enrichment, unlike D-PS enrichment, had a greater effect on thrombin-

activated secretion of PAF-C14 and δ-granules release. While several studies have detected very small amounts of D-PS in brain and other tissues, clearly the effects of D-PS on platelets differ than from those of L-PS.

When considering the effects of L-PS or D-PS on platelet secretion function, it is important to also consider the increase in cholesterol with both L-PS and D-PS enrichment. The data in Fig. 5.1C indicate that the cholesterol remains associated with the platelets, although it is not known whether cholesterol excluded from the membrane is localized to the interior or exterior of the membrane.[19] One potential reason for the increased effect of L-PS on thrombin activation of platelets could be that cholesterol excluded from the platelet membrane prevents access of thrombin to the membrane-exposed phosphatidylserine. As cholesterol solubility in phosphatidylserine depends on the saturation of the lipid tail as well as the charge of the headgroup, the differences between the effects of L-PS and D-PS on platelet secretory function could arise from the solubility of cholesterol in L-PS vs. D-PS.
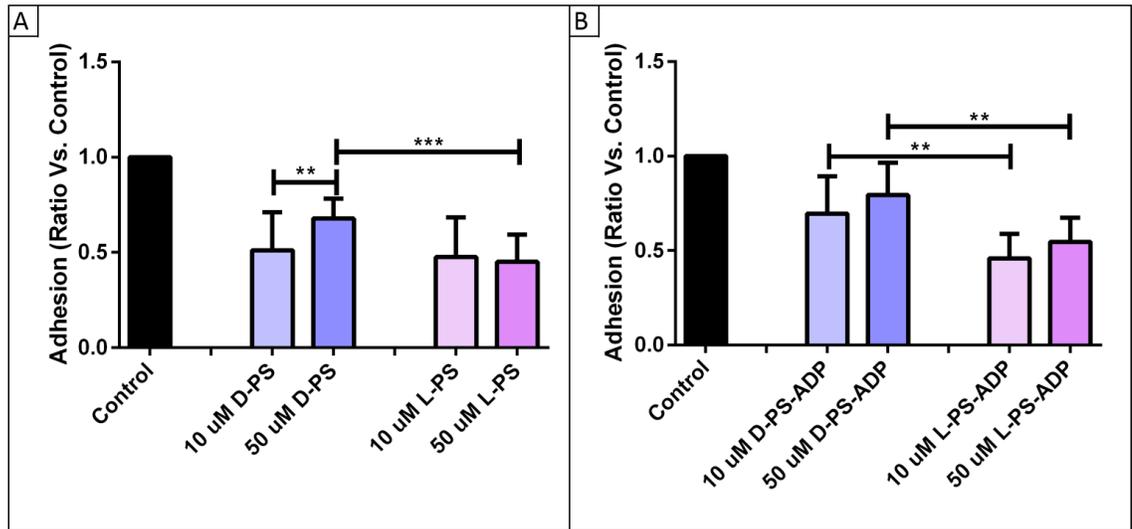
Figure 5.6. Microfluidic assessment of A. resting and B. ADP-activated platelet adhesion in platelets enriched with D-PS or L-PS.
$**p < 0.01$; $***p < 0.001$ using and unpaired t-test. In both A and B, all L-PS and D-PS enriched conditions are significantly different from the control platelet adhesion ($p < 0.0001$ using an unpaired t-test).

5.4.4 Microfluidic Assessment of Platelet Adhesion

With clear changes in the secretion behavior of platelets exposed to L-PS or D-PS, we wanted to explore another critical platelet function in this context – adhesion. The platelet adhesion ability, incubated with L-PS or D-PS, was evaluated on a layer of endothelial cells using a microfluidic platform.[20] Few studies are available regarding the relationship between membrane phospholipid content and platelet adhesion. However, in a recent study,[15] we found that L-PS enrichment in platelet plasma membranes causes reduced adhesion of platelets to endothelial cells, and now we can compare the relative effects of L-PS and D-PS. Experimental conditions herein are unactivated and ADP-activated platelets, and each has five sub-conditions of control (no pre-exposure to either L-PS or D-PS), pre-incubated with L-PS (10 μM or 50 μM), and pre-incubated with D-PS (10 μM or 50 μM). Platelet adhesion to the confluent endothelial cell layer in the microfluidic device was assessed simply by counting the number of fluorescent platelets present following introduction and rinsing.

As shown in Fig. 5.6A, unactivated platelets showed decreased adhesion to the layer of endothelial cells when they are pre-incubated with either phosphatidylserine species. Interestingly, while L-PS did not show concentration dependence, unactivated platelets pre-incubated with D-PS showed concentration-dependent adhesion; in fact, unactivated platelets pre-incubated with 50 μM D-PS showed higher adhesion than any other phosphatidylserine-pre-incubated platelets. In contrast, ADP activation of platelets incubated with 50 μM L-PS or 50 μM D-PS demonstrated that D-PS has less impact on

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activated platelet adhesion behavior. This concurs with the ensemble measurements made of thrombin-stimulated platelet chemical messenger secretion. Again, all PS-enriched platelets showed lower adhesion to endothelial cells than control platelets. These impacts of L-PS and D-PS on platelet adhesion could result from changes in the membrane curvature due to the added phosphatidylserine content in the platelet membrane.[15] However, the work in this paper also suggests the possibility that decreased adhesion is due to the increased PS membrane content reducing platelet secretion of chemical messengers. This possibility will be of significant interest for future study.

5.5 Conclusions

This work explores the effects of phosphatidylserine on platelet function by evaluating the effects of enriching platelets with either L-PS or D-PS. It was determined that incubation with either D-PS or L-PS results in the same degree of phosphatidylserine enrichment, and that the two stereochemical forms of phosphatidylserine have different effects on platelet secretory and adhesion function. Surprisingly, enrichment of both L-PS and D-PS increased the cholesterol content of the platelets, although the localization of the cholesterol in or around the platelet membrane is unknown. In general, platelet exposure to D-PS induces platelet activation in unactivated platelets while both L-PS and D-PS suppress thrombin-induced platelet secretory function, with a greater effect with L-PS than D-PS enrichment. Phosphatidylserine stereochemistry has implications in a broad range of biochemical processes, and this study demonstrates its effects on cellular function: while platelets take up L-PS and D-PS to the same degree, there are clear differences between

the effects of L-PS and D-PS on secretion and adhesion behavior of both unactivated and

activated platelets.

Chapter 6: A Chemistry "Whodunnit" to Explore the Scientific Method

6.1 Overview

The previous five chapters of this dissertation have focused on scientific advancements in the field of lipid research. In this chapter, the focus will shift to the responsibility of the scientific community to reach out to the public to increase scientific interest and proficiency among school-age children, as well as to increase the public visibility of scientists within their communities. The work in this chapter covers the design and implementation of a mystery-solving activity that was implemented in three consecutive years with three separate mysteries in a community center in St. Paul, MN.

In recent years, public interest in forensic science has increased greatly, due in part to television shows such as *CSI* and *Bones*, which feature scientists using analytical tools to solve crimes.[1,2] Although these shows contain many scientific and legal inaccuracies,[1] the increased public interest in forensic science presents an opportunity for chemists to engage students in the science behind chemical tests featured on television.[2] By leveraging student interest in the applied nature of forensic chemistry, educators also have an opportunity to use a practical, problem-solving approach to introduce students to the scientific method,[3] including formulating hypotheses, selecting an appropriate experiment from those available, performing positive and negative controls, and interpreting both conclusive and inconclusive results.

In this outreach activity, we engaged middle school students to select an assay, consider positive and negative controls, and use information gained from several assays to reach a conclusion and solve a mystery. Three mysteries were presented in consecutive

years to students participating in a summer program at the West 7th Community Center in St. Paul, MN. Mysteries included an environmental chemical spill, a jewel heist, and a case of sabotage at a solar cell factory. In the first and second years, students watched a video wherein an investigator described the mystery and interviewed a cadre of suspects. Students also viewed a short video describing the assays available to them. Working in groups of four, students were given a kit of evidence and challenged to solve the mystery. In the third year, a similar format was followed for the sabotage with the exception that the students did not watch a video of the demonstrations of the assays available. In the second and third years of the activity, students were asked multiple-choice pre- and post- questions about important scientific vocabulary. They used personal response devices, which have received generally positive student reviews,[4,5] to answer the questions, and student answers were analyzed to evaluate student learning during the course of the activity. Results of the student answers to pre- and post- experiment questions are included: in Year 2, student comprehension increased for two of the four questions asked from the pre – to the post-activity assessment, and in Year 3, increases in student comprehension were observed for all four of the questions from the pre- to the post-activity assessment.

6.2 Experimental Approach

6.2.1 Activity Format

This activity involves preparation beforehand of (1) a video (or other format presentation) detailing the events of the mystery to students, (2) evidence kits, and (3) solutions for several assays. The format of the activity as well as the assays made available

to students are described herein, and detailed transcripts of the 3 mystery videos as well as instructions for preparation of the evidence kits and assays can be found in Appendix II. Students watched a short video written, directed by, and starring University of Minnesota graduate and undergraduate students. Student participants were then split into groups of four and assigned to a graduate or undergraduate 'guide' who helped keep the students on-task during the activity. Students were given a box containing approximately 6 pieces of evidence that they were told had been collected from the suspects or at the crime scene and a list of stations where they could test their evidence. Graduate or undergraduate students were available at each of the assay stations to explain the chemistry concepts of the assay, how to perform the assay, and positive and negative controls where applicable.

Table 6.1. Overview of the evidence kit contents and available assays for the three mystery activities.

Activity	Contents of Evidence Kit[a]	Assays Available to Students
Chemical Spill	Contaminated river water sample Handwritten notes found in contaminated area Pens belonging to suspects Map with fingerprints and fingerprint samples from suspects Soil sample from contaminated area Homogenated fish sample from contaminated area	Acid/base detection of metal ions in solution Ink chromatography Flame test Iodine fingerprint developing or ninhydrin fingerprint developing Luminol test pH test
Jewel Heist	Fingerprint samples from each suspect Debris from crime scene with fingerprint present Glass from the display case Glass found in a suspect's car Powder found at crime scene Powder samples found on suspects Paint collected from security camera Paint from a suspect's hands Paint sample from a mural (suspect's alibi)	Acid/base detection of metal ions in solution Ink chromatography Iodine fingerprint developing or ninhydrin fingerprint developing Nanoparticle test for pesticides Iodine test for starch
Solar Cell Factory Sabotage	Piece of plastic left at crime scene Pieces of plastic from the workplaces of suspects Sample containing DNA from a cup at the crime scene DNA sequence pre-processed from each suspect Handwritten note found at the crime scene Pens belonging to suspects Metallic dust found at the crime scene Metallic dust found on shoes of suspects Fingerprint samples from suspects Piece of paper with fingerprint found at crime scene Sample of scent detected at crime scene Cologne or perfume from suspects	Plastic density comparison DNA extraction Metal ion flame test Ink chromatography Iodine fingerprint developing or ninhydrin fingerprint developing Silver plating test

[a]Positive and negative control samples should be provided to students at the assay stations.

For all three activities, evidence kit contents and assays available to the students are listed in Table 6.1. Detailed preparation instructions for the evidence kits, the assays, and positive and negative controls can be found in Appendix II. In scenario 1, "Chemical Spill on the Mississippi River," students watched a video that explained that a portion of the Mississippi River was mysteriously lacking in fish and wildlife and watched investigator interviews with the suspects that might be causing this environmental change. In scenario 2, "Jewel Heist at the Science Museum," students watched a video that explained that the Crown Jewels on display at the Science Museum had been stolen during a break-in, and they were shown interviews with suspects. In scenario 3, "Sabotage at a Solar Cell Factory," students watched a video depicting a crime of sabotage at the Solar Haynes Industries solar cell factory, including police investigator interviews with potential suspects. Students were presented with 4 suspects and 5-6 assays with which to test their evidence for each scenario. It is possible to change the guilty suspect by altering the evidence apparently collected from each suspect.

In addition to use of several well-known tests widely used in outreach activities (DNA extraction, starch detection with iodine, filter paper chromatography with felt-tip pens), we incorporated a series of less well-known tests that demonstrate chemical reactions. Some of these include an acid/base detection of metal salts in scenario 1 and a nanoparticle-based test for pesticide detection.

All mystery scenarios were tested with students ages 10-14 in a one-day event for socioeconomically disadvantaged students (average of 74% of students identify as racial

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minorities and 87% of students were from homes below 200% of the Federal Poverty Guidelines) participating in a summer program.[8] Although the mysteries were developed for use in an outreach event, all activities were translated and optimized by a certified Minnesota high school teacher for use in middle and high school classrooms as some tools and materials may be inaccessible to middle school classrooms. Changes made to the tests for middle school classrooms include preparing nanoparticles in advance for the silver nanoparticle aggregation test used in scenario 2 according to a previously published protocol[6] and inducing aggregation by the addition of a salt.[7] Also for scenario 2, paint samples were composed of mixtures of washable paint, and in post-activity optimization, it was determined that black tempera paint and black acrylic paint (readily available and inexpensive at craft stores) would be easier for implementation in a classroom. In scenario 3, a DNA isolation procedure was used that required the use of a centrifuge; however, as many middle and high school science classrooms may not have a centrifuge, an alternate DNA isolation procedure is included in Appendix II. A library of detailed descriptions of each scenario as well as teacher guides for implementation in a middle school classroom can be found in Appendix II.

6.2.2 Hazards and Safety Considerations

Safety hazard information and proper waste disposal procedures for the supplies used in each activity can be found in Appendix II. Gloves and goggles should be worn for all activities. Caution should be exercised when using an open flame. Iodine, silver nitrate, or ninhydrin can stain clothes and skin.

6.4 Results and Discussion

Students ages 10-14 participating in a summer program at a community center in St. Paul, MN participated in a forensic science mystery-solving activity. Three different mysteries were developed and implemented in three consecutive years, and student learning over the course of the activity was evaluated in the latter two years. Some students participated in the summer program for multiple years, leading to a small degree of overlap between students each year.

Students were asked several multiple-choice questions both before and after solving the mystery: (1) What is an observation, (2) What is a hypothesis, (3) What is a positive control, and (4) What is a negative control. They then watched a video in which an investigator described the mystery scenario, interviewed suspects, and collected evidence, followed by a short video with instructions for the assays available to them. Students were then divided into groups, where each group had a guide and was given a box containing samples of evidence matching the samples collected in the video, a set of worksheets to guide them, and access to 4-6 stations where they could perform chemical assays on the evidence in the kit. They were given no instructions regarding which assay to use on which piece of evidence. For each assay, students recorded the evidence tested and the results of the assay as well as the results of the positive and negative controls. Students were also asked to interpret their results in the context of the mystery. Once all students had completed the activity and made their conclusions about the potential suspects, they were asked Questions 1-4 again.

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During the Jewel Heist Mystery activity, all groups solved the mystery correctly, and the percent of students answering the pre-activity questions and post-activity questions correctly are listed in Table 6.2. Of the four questions asked, the percent of students answering correctly increased from the pre- to the post-activity assessment for Questions (3) and (4) (by 13% and 25%, respectively), did not change for Question (1) (0.0% change), and decreased for Question (2) (-8.3%). The increase in overall student scores from 60% before the activity to 68% after the activity was found to be non-significant using a two-tailed t-test (p=0.5). Based on the results in Table 6.2, it was clear that increased emphasis on the concepts of the scientific method (observations, hypotheses, and controls) was needed to increase student comprehension of the scientific method during the activity. As the goal of this activity was for students to learn through problem solving rather than direct instruction, preparations for the assay stations for the following year (Solar Cell Factory Sabotage) focused on improving emphases on observations, hypotheses, and controls. In doing so, students were exposed to these principles through the mini-experiments that they performed at each assay station.

During the Solar Cell Factory Sabotage Mystery activity in year 3, students were asked the same questions before and after the activity as for the Jewel Heist Mystery. Overall student scores increased significantly from 52% to 68% (p=0.03). The percent of students answering correctly increased from the pre- to the post-activity assessment for all four questions asked. The increase in students correctly answering was 3.7% for Question (1), 11% for Question (2), 22% for Question (3), and 26% for Question (4).

To assess the statistical significance between pre- and post-activity responses, the student responses to each question for both of the activities were pooled. Using McNemar's test,[9] the pooled responses showed a significant increase in comprehension for Questions 3 (p=0.01) and 4 (p = 0.01). Significant differences between the pre- and post-activity responses were not observed for Questions 1 (p=0.6) and 2 (p=0.6). These results indicate that an outreach activity with no direct instruction can lead to significant increases in student comprehension of concepts related to the scientific method and are promising for student learning when implemented in a middle school classroom.

Table 6.2. Student responses to pre- and post-activity questions.

	Percent of students answering correctly before and after the activity (before/after)	
Question	Jewel Heist	Solar Cell Factory Sabotage
Question 1: What is an observation?	79%/79%	70%/74%
Question 2: What is a hypothesis?	67%/58%	78%/89%
Question 3: What is a positive control?	50%/63%	30%/53%
Question 4: What is a negative control?	46%/71%	22%/48%
Total Percent Correct	60%/68%	52%/68%

While the focus of the investigations when implemented as an outreach activity was on engaging students in solving a mystery through problem solving using chemical assays, each investigation contains multiple activities that could be used to highlight a number of different chemical concepts, including solubility, acid-base chemistry, chemiluminescence, and atomic emission spectroscopy. Using a format with minimal direct instruction and instead allowing students to determine which chemical tests were appropriate for certain types of samples, student comprehension of concepts related to the scientific method increased when these concepts were emphasized during the explanation

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of the assay protocols. Results from the implementation of this activity at a community center were used to optimize the activities for use in middle and high school science classrooms, including detail regarding how these activities meet national science standards. The optimized versions of the activities are presented here, with input and perspectives from two high school teachers as to the choice of materials used and the questions presented on student worksheets during the activity. Appendix II contains activities as three separate and complete investigations including a teacher guide, student handouts, and assay protocols with each considering learning objectives such as making observations, organizing data, and drawing conclusions from data.

6.5 Classroom Science Standards

This activity includes components that address high school Next Generation Science Standards including Practice 1: Asking Questions and Defining Problems, Practice 3: Planning and Carrying Out Investigations, Practice 4: Analyzing and Interpreting Data, Practice 6: Constructing Explanations and Designing Solutions, and Practice 7: Engaging in Argument from Evidence.[10,11]

6.6 Conclusions

In the activity presented here, three separate investigations were developed where students use simple chemical assays to solve a mystery. In each investigation, students were given a kit of evidence, a list of assays, and minimal direct instruction. Although this activity was originally designed as an outreach event, the activities were modified and

formatted for implementation in a middle school classroom. Students work in groups, make decisions related to a course of action for solving a problem, formulate hypotheses, perform positive and negative controls, interpret data, and formulate conclusions based on results of multiple chemical assays. Instructors could also modify activities as needed for a greater focus on scientific concepts of interest to the class.

6.7 Additional Information for this Educational Activity

Detailed additional information for this activity includes Student and Teacher Materials for the Chemical Spill, Jewel Heist, and Solar Cell Factory Sabotage Mysteries, including detailed descriptions of evidence kit preparation, assays used in each activity, scripts used for the activities, worksheets for each piece of evidence, and teaching objectives met for the activities. This material is available in Appendix II.

Concluding Remarks

The field of lipid research has grown enormously over the last century, and the work in this dissertation demonstrates that many scientifically interesting and physiologically relevant avenues remain to be explored in the field of cell-cell communication through secreted bioactive lipids and phospholipids. The research in this dissertation uses UPLC-MS/MS to overcome the significant analytical challenges presented by lipid detection in complex biological systems and employs mast cells and platelets as platforms for examining bioactive lipid secretion and phospholipid signaling, respectively. The work in Chapters 2-5 provides a compelling case for 1) the utility of UPLC-MS/MS in quantitative determination of bioactive lipids and relative quantitation of phospholipids, 2) the importance of cell culture model when making measurements on immune system-relevant cells, and 3) the use of platelets as an ideal model system for studying the roles of phospholipids in cell signaling.

In Chapter 2, an UPLC-MS/MS method was successfully developed and implemented for simultaneous determination of three sub-classes of secreted lipids. This is important because cell-secreted bioactive lipids are ubiquitous, structurally diverse, and have a variety of roles in the cascade of cellular communication reactions that lead to inflammation and inflammatory diseases. In both Chapters 2 and 3, mast cells were selected as the cell type of interest because they have central roles in diseases such as asthma, and the work in Chapter 3 demonstrates that mast cell interactions with other cells in a complex heterogeneous culture are clearly important. Future studies in the area of lipid signaling

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should attempt to incorporate other lipids of interest into the UPLC-MS/MS method. Work on this front has begun with the incorporation of PAF-C14 in Chapter 5, and preliminary experiments exploring the incorporation of LTB$_4$, an important bioactive lipid in leukocyte functions, have begun.

In Chapters 4 and 5, the focus of the research shifts from secreted bioactive lipids to their phospholipid precursors. In particular, amino-phospholipids PS and PE were studied in Chapter 4, and Chapter 5 further explored the effects that concentration and stereochemistry of exogenous PS have on platelet function. The field of phospholipid research has benefitted greatly from the advent of LC-MS, and the application of UPLC to mass spectrometric studies of phospholipids has the potential for furthering studies of cellular uptake of phospholipids. For the work in this dissertation, platelets presented an ideal platform to develop and implement a method for determining whether cells can incorporate exogenous phospholipids into their membranes, which had not previously been demonstrated. However, future studies of phospholipid effects on cellular function would be equally exciting with endothelial cells or mucosal cells, as the surface chemistries of these cell classes have roles in many physiological processes. Overall, this work has demonstrated the utility of UPLC-MS/MS for both secreted bioactive lipid and phospholipid studies of interesting immune-relevant cell types and will enable further studies of lipid function in cell signaling.

Bibliography

Chapter 1

1. Ecker, J. Profiling eicosanoids and phospholipids using LC-MS/MS: Principles and recent applications. *Journal of Separation Science,* **2012**, *35*, 1227-1235.

2. Burr, G. O.; Burr, M. M. On the nature and the role of the fatty acids essential in nutrition. *Journal of Biological Chemistry,* **1930**, *86*, 587-613.

3. Funk, C. D. Prostaglandins and leukotrienes: Advances in eicosanoid biology. *Science,* **2001**, *294*, 1871-1875.

4. Samuelsson, B.; Goldyne, M.; Granstrom, E.; Hamberg, M.; Hammarstrom, S.; Malmsten, C. Prostaglandins and thromboxanes. *Annual Review of Biochemistry,* **1978**, *47*, 997-1029.

5. O'Donnell, V. B.; Maskrey, B.; Taylor, G. W. Eicosanoids: generation and detection in mammalian cells. *Methods in Molecular Biology (Clifton, N.J.),* **2009**, *462*, 5-23.

6. Prescott, S. M.; Zimmerman, G. A.; Stafforini, D. M.; McIntyre, T. M. Platelet-activating factor and related lipid mediators. *Annual Review of Biochemistry,* **2000**, *69*, 419-445.

7. Hanahan, D. J. Platelet-activating-factor - a biologically-active phosphoglyceride. *Annual Review of Biochemistry,* **1986**, *55*, 483-509.

8. Henson, P. M. Release of vasoactive amines from rabbit platelets induced by sensitized mononuclear leukocytes and antigen. *Journal of Experimental Medicine,* **1970**, *131*, 287-305.

9. Sirganian, R. P.; Osler, A. G. Destruction of rabbit platelets in the allergic response of sensitized leukocytes. *Journal of Immunology,* **1971**, *106*, 1244-1251.

10. Benveniste, J.; Henson, P. M.; Cochrane, C. G. Leukocyte-dependent histamine release from rabbit platelets: the role of IgE, basophils, and a platelet-activating factor. *Journal of Experimental Medicine,* **1972**, *136*, 1356-1377.

11. Snyder, F.; Lee, T. C.; Blank, M. L. Platelet-activating factor and related ether lipid mediators - biological-activities, metabolism, and regulation. *Annals of the New York Academy of Sciences,* **1989**, *568*.

12. Snyder, F. Platelet-activating-factor and related acetylated lipids as potent biologically-active cellular mediators. *American Journal of Physiology,* **1990**, *259*.

13. Schlondorff, D.; Neuwirth, R. Platelet-activating-factor and the kidney. *American Journal of Physiology,* **1986**, *251*, F1-F11.

14. Piomelli, D.; Feinmark, S. J.; Cannon, P. J. Leukotriene biosynthesis by canine and human coronary-arteries. *Journal of Pharmacology and Experimental Therapeutics,* **1987**, *241*, 763-770.

15. Giles, H.; Leff, P. The biology and pharmacology of PGD_2. *Prostaglandins,* **1988**, *35*, 277-300.

16. Haberl, C.; Hultner, L.; Flugel, A.; Falk, M.; Geuenich, S.; Wilmanns, W.; Denzlinger, C. Release of prostaglandin D_2 by murine mast cells: importance of metabolite formation for antiproliferative activity. *Mediators of Inflammation,* **1998**, *7*, 79-84.

17. Kita, Y.; Takahashi, T.; Uozumi, N.; Shimizu, T. A multiplex quantitation method for eicosanoids and platelet-activating factor using column-switching reversed-phase liquid chromatography-tandem mass spectrometry. *Analytical Biochemistry,* **2005**, *342*, 134-143.

18. Whitehead, S. N.; Hou, W. M.; Ethier, M.; Smith, J. C.; Bourgeois, A.; Denis, R.; Bennett, S. A. L.; Figeys, D. Identification and quantitation of changes in the platelet activating factor family of glycerophospholipids over the course of neuronal differentiation by high-performance liquid chromatography electrospray ionization tandem mass spectrometry. *Analytical Chemistry,* **2007**, *79*, 8539-8548.

19. Guhl, S.; Babina, M.; Neou, A.; Zuberbier, T.; Artuc, M. Mast cell lines HMC-1 and LAD2 in comparison with mature human skin mast cells - drastically reduced levels of tryptase and chymase in mast cell lines. *Experimental Dermatology,* **2010**, *19*, 845-847.

20. Passante, E.; Ehrhardt, C.; Sheridan, H.; Frankish, N. RBL-2H3 cells are an imprecise model for mast cell mediator release. *Inflammation Research,* **2009**, *58*, 611-618.

21. Passante, E.; Frankish, N. The RBL-2H3 cell line: its provenance and suitability as a model for the mast cell. *Inflammation Research,* **2009**, *58*, 737-745.

22. Bischoff, S. C. Role of mast cells in allergic and non-allergic immune responses: comparison of human and murine data. *Nature Reviews Immunology,* **2007**, *7*, 93-104.

23. Heib, V.; Becker, M.; Taub, C.; Stassen, M. Advances in the understanding of mast cell function. *British Journal of Haematology,* **2008**, *142*, 683-694.

24. Theoharides, T. C.; Kalogeromitros, D. The critical role of mast cells in allergy and inflammation. *Neuroendocrine and Immune Crosstalk,* **2006**, *1088*, 78-99.

25. Kindt, T. J.; Goldsby, R. A.; Osborne, B. a.; Kuby, J.: *Kuby Immunology*; W.H. Freeman and Company: New York, NY, 2007.

26. Okayama, Y.; Hagaman, D. D.; Metcalfe, D. D. A comparison of mediators released or generated by IFN-gamma-treated human mast cells following aggregation of Fc gamma RI or Fc epsilon RI. *Journal of Immunology,* **2001**, *166*, 4705-4712.

27. Ogawa, Y.; Grant, J. A. Mediators of anaphylaxis. *Immunology and Allergy Clinics of North America,* **2007**, *27*, 249-260.

28. Kim, D.; Koseoglu, S.; Manning, B. M.; Meyer, A. F.; Haynes, C. L. Electroanalytical eavesdropping on single cell communication. *Analytical Chemistry,* **2011**, *83*, 7242-7249.

29. Serhan, C. N.; Haeggstrom, J. Z.; Leslie, C. C. Lipid mediator networks in cell signaling: Update and impact of cytokines. *FASEB Journal,* **1996**, *10*, 1147-1158.

30. Vance, J. E. Thematic review series: Glycerolipids. Phosphatidylserine and phosphatidylethanolamine in mammalian cells: two metabolically related aminophospholipids. *Journal of Lipid Research,* **2008**, *49*, 1377-1387.

31. Zwaal, R. F. A.; Comfurius, P.; Bevers, E. M. Scott syndrome, a bleeding disorder caused by defective scrambling of membrane phospholipids. *Biochimica Et Biophysica Acta-Molecular and Cell Biology of Lipids,* **2004**, *1636*, 119-128.

32. Zwaal, R. F. A.; Schroit, A. J. Pathophysiologic implications of membrane phospholipid asymmetry in blood cells. *Blood,* **1997**, *89*, 1121-1132.

33. Powell, W. S. Extraction of eicosanoids from biological fluids, cells, and tissues. *Methods in Molecular Biology (Clifton, N.J.),* **1999**, *120*, 11-24.

34. Menon, A. K.; Stevens, V. L. Phosphatidylethanolamine is the donor of the ethanolamine residue linking a glycosylphosphatidylinositol anchor to protein. *Journal of Biological Chemistry*, **1992**, *267*, 15277-15280.

35. Zwaal, R.; Comfurius, P.; Bevers, E. Lipid-protein interactions in blood coagulation. *Biochimica Et Biophysica Acta-Reviews on Biomembranes,* **1998**, *1376*, 433-453.

36. Heemskerk, J.; Bevers, E.; Lindhout, T. Platelet activation and blood coagulation. *Thrombosis and Haemostasis,* **2002**, *88*, 186-193.

37. Bach, D.; Wachtel, E. Phospholipid/cholesterol model membranes: formation of cholesterol crystallites. *Biochimica Et Biophysica Acta-Biomembranes,* **2003**, *1610*, 187-197.

38. Epand, R. M.; Bach, D.; Epand, R. F.; Borochov, N.; Wachtel, E. A new high-temperature transition of crystalline cholesterol in mixtures with phosphatidylserine. *Biophysical Journal,* **2001**, *81*, 1511-1520.

39. Epand, R. M.; Bach, D.; Borochov, N.; Wachtel, E. Cholesterol crystalline polymorphism and the solubility of cholesterol in phosphatidylserine. *Biophysical Journal,* **2000**, *78*, 866-873.

40. Coller, B. S. A Brief History of Ideas about Platelets in Health and Disease Foreword. *Platelets, 2nd Edition,* **2007**, XXIII-XLII.

41. White, J. G. Platelet Structure. *Platelets, 2nd Edition,* **2007**, 45-73.

42. Rowley, J.; Oler, A.; Tolley, N.; Hunter, B.; Low, E.; Nix, D.; Yost, C.; Zimmerman, G.; Weyrich, A. Genome-wide RNA-seq analysis of human and mouse platelet transcriptomes. *Blood,* **2011**, *118*, E101-E111.

43. Bouchard, B. A.; Tracy, P. B. Platelets, leukocytes, and coagulation. *Current Opinion in Hematology,* **2001**, *8*, 263-269.

44. Fay, W. P.; Eitzman, D. T.; Shapiro, A. D.; Madison, E. L.; Ginsburg, D. Platelets inhibit fibrinolysis in-vitro by both plasminogen-activator inhibitor-1 dependent and inhibitor-1 independent mechanisms. *Blood,* **1994**, *83*, 351-356.

45. Varadi, B.; Kolev, K.; Tenekedjiev, K.; Meszaros, G.; Kovalszky, I.; Longstaff, C.; Machovich, R. Phospholipid barrier to fibrinolysis - Role for the anionic polar head charge and the gel phase crystalline structure. *Journal of Biological Chemistry,* **2004**, *279*, 39863-39871.

46. Meyer, A. F.; Thompson, J. T.; Wang, Y.; Koseoglu, S.; Haynes, C. L.; Dalluge, J. J. Isotope-Dilution UPLC-MS/MS determination of mast cell-secreted bioactive lipids. *Analyst,* **2013**, *138*, 5697-5705.

Chapter 2

1. Farooque, S., P.; Arm, J. P.; Lee, T. H.: Lipid Mediators: Leukotrienes, Prostanoids, Lipoxins, and Platelet-activating Factor. In *Allergy and Allergic Diseases*, *2nd Edition*; Kay, A. B., Kaplan, A. P., Bousquet, J., Holt, P. G., Eds.; Blackwell Publishing, **2008**; Vol. 1.

2. Murphy, R. C.; Gijon, M. A. Biosynthesis and metabolism of leukotrienes. *Biochemical Journal,* **2007**, *406*, 379-395.

3. Heib, V.; Becker, M.; Taub, C.; Stassen, M. Advances in the understanding of mast cell function. *British Journal of Haematology,* **2008**, *142*, 683-694.

4. Metcalfe, D. D.; Baram, D.; Mekori, Y. A. Mast cells. *Physiological Reviews,* **1997**, *77*, 1033-1079.

5. Serhan, C. N.; Haeggstrom, J. Z.; Leslie, C. C. Lipid mediator networks in cell signaling: Update and impact of cytokines. *FASEB Journal,* **1996**, *10*, 1147-1158.

6. Nijkamp, F. P.; Sitsen, J. M. A. Leukotrienes, allergy and inflammation. *Pharmaceutisch Weekblad-Scientific Edition,* **1982**, *4*, 165-171.

7. Karimi, K.; Kool, M.; Nijkamp, F. P.; Redegeld, F. A. Substance P can stimulate prostaglandin D_2 and leukotriene C_4 generation without granule exocytosis in murine mast cells. *European Journal of Pharmacology,* **2004**, *489*, 49-54.

8. Johnson, P. R. A.; Armour, C. L.; Black, J. L. The action of platelet activating factor and its antagonism by web-2086 on human isolated airways. *European Respiratory Journal,* **1990**, *3*, 55-60.

9. Ogawa, Y.; Grant, J. A. Mediators of Anaphylaxis. *Immunology and Allergy Clinics of North America,* **2007**, *27*, 249-260.

10. Kita, Y.; Takahashi, T.; Uozumi, N.; Nallan, L.; Gelb, M. H.; Shimizu, T. Pathway-oriented profiling of lipid mediators in macrophages. *Biochemical and Biophysical Research Communications,* **2005**, *330*, 898-906.

11. Wierzbicki, M.; Brzezinska-Blaszcyk, E. Diverse effects of bacterial cell wall components on mast cell degranulation, cysteinyl leukotriene generation and migration. *Microbiology and Immunology,* **2009**, *53*, 694-703.

12. Konopka, L.; Wierzbicki, M.; Brzezinska-Blaszcyk, E. Lipopolysaccharide from *Porphyromonas gingivalis* stimulates rat mast cells to cysteinyl leukotriene generation and upregulates Toll-like receptor -2 and -4 expression. *International Journal of Immunopathology and Pharmacology,* **2010**, *23*, 803-810.

13. Foreman, J. C.; Mongar, J. L.; Gomperts, B. D. Calcium ionophores and movement of calcium ions following the physiological stimulus to a secretory process. *Nature,* **1973**, *245*.

14. Blank, U.; Rivera, J.: Assays for Regulated Exocytosis of Mast Cells. In *Current Protocols in Cell Biology*; John Wiley & Sons, Inc., 2006.

Chapter 3

1. Page, S.; Ammit, A. J.; Black, J. L.; Armour, C. L. Human mast cell and airway smooth muscle cell interactions: implications for asthma. *American Journal of Physiology-Lung Cellular and Molecular Physiology,* **2001**, *281*, L1313-L1323.

2. Metcalfe, D. D.; Baram, D.; Mekori, Y. A. Mast cells. *Physiological Reviews,* **1997**, *77*, 1033-1079.

3. Bradding, P.; Walls, A. F.; Holgate, S. T. The role of the mast cell in the pathophysiology of asthma. *Journal of Allergy and Clinical Immunology,* **2006**, *117*, 1277-1284.

4. Theoharides, T. C.; Alysandratos, K. D.; Angelidou, A.; Delivanis, D. A.; Sismanopoulos, N.; Zhang, B. D.; Asadi, S.; Vasiadi, M.; Weng, Z. Y.; Miniati, A.; Kalogeromitros, D. Mast cells and inflammation. *Biochimica Et Biophysica Acta-Molecular Basis of Disease,* **2012**, *1822*, 21-33.

5. Theoharides, T. C.; Kalogeromitros, D. The critical role of mast cells in allergy and inflammation. *Neuroendocrine and Immune Crosstalk,* **2006**, *1088*, 78-99.

6. Guhl, S.; Babina, M.; Neou, A.; Zuberbier, T.; Artuc, M. Mast cell lines HMC-1 and LAD2 in comparison with mature human skin mast cells - drastically reduced levels of

tryptase and chymase in mast cell lines. *Experimental Dermatology,* **2010**, *19*, 845-847.

7. Passante, E.; Ehrhardt, C.; Sheridan, H.; Frankish, N. RBL-2H3 cells are an imprecise model for mast cell mediator release. *Inflammation Research,* **2009**, *58*, 611-618.

8. Passante, E.; Frankish, N. The RBL-2H3 cell line: its provenance and suitability as a model for the mast cell. *Inflammation Research,* **2009**, *58*, 737-745.

9. Marquis, B. J.; Haynes, C. L. The effects of co-culture of fibroblasts on mast cell exocytotic release characteristics as evaluated by carbon-fiber microelectrode amperometry. *Biophysical Chemistry,* **2008**, *137*, 63-69.

10. Manning, B. M.; Meyer, A. F.; Gruba, S. M.; Haynes, C. L.: Single cell analysis of mast cell exocytosis reveals direct induction of mast cell degranulation by the airway smooth muscle-associated chemokines CXCL10 and RANTES. **submitted**.

11. Koseoglu, S.; Gruba, S. M.; Maurer-Jones, M. A.; Haynes, C. L. Comparison of platelet delta-granule secretion from different species using single cell measurements. **submitted**.

12. Meyer, A. F.; Thompson, J. T.; Wang, Y.; Koseoglu, S.; Haynes, C. L.; Dalluge, J. J. Isotope-Dilution UPLC-MS/MS determination of mast cell-secreted bioactive lipids. *Analyst,* **2013**, *138*, 5697-5705.

13. Blank, U.; Rivera, J.: Assays for Regulated Exocytosis of Mast Cells. In *Current Protocols in Cell Biology*; John Wiley & Sons, Inc., 2006.

14. Omiatek, D. M.; Dong, Y.; Heien, M. L.; Ewing, A. G. Only a fraction of quantal content is released during exocytosis as revealed by electrochemical cytometry of secretory vesicles. *ACS Chemical Neuroscience,* **2010**, *1*, 234-245.

15. O'Connell, P. J.; Wang, X. B.; Leon-Ponte, M.; Griffiths, C.; Pingle, S. C.; Ahern, G. P. A novel form of immune signaling revealed by transmission of the inflammatory mediator serotonin between dendritic cells and T cells. *Blood,* **2006**, *107*, 1010-1017.

16. Rudd, M. L.; Nicolas, A. N.; Brown, B. L.; Fischer-Stenger, K.; Stewart, J. K. Peritoneal macrophages express the serotonin transporter. *Journal of Neuroimmunology,* **2005**, *159*, 113-118.

17. Blackburn, K. J.; French, P. C.; Merrills, R. J. 5-hydroxytryptamine uptake by rat brain *in vitro*. *Life Sciences,* **1967**, *6*, 1653-1663.

18. Blakely, R. D.; Berson, H. E.; Fremeau, R. T.; Caron, M. G.; Peek, M. M.; Prince, H. K.; Bradley, C. C. Cloning and expression of a functional serotonin transporter from rat-brain. *Nature,* **1991**, *354*, 66-70.

19. Hagan, C. E.; Schenk, J. O.; Neumaier, J. F. The Contribution of low-affinity transport mechanisms to serotonin clearance in synaptosomes. *Synapse,* **2011**, *65*, 1015-1023.

20. Neubauer, H. A.; Hansen, C. G.; Wiborg, O. Dissection of an allosteric mechanism on the serotonin transporter: A cross-species study. *Molecular Pharmacology,* **2006**, *69*, 1242-1250.

21. Daws, L. C.; Toney, G. M. High-speed chronoamperometry to study kinetics and mechanisms for serotonin clearance *in vivo*. *Electrochemical Methods for Neuroscience,* **2007**, *1*, 63-81.

22. Bradding, P. Asthma: eosinophil disease, mast cell disease, or both? *Allergy, Asthma, and Clinical Immunology,* **2008**, *4*, 84-90.

23. Brightling, C. E.; Ammit, A. J.; Kaur, D.; Black, J. L.; Wardlaw, A. J.; Hughs, J. M.; Bradding, P. The CXCL10/CXCR3 axis mediates human lung mast cell migration to asthmatic airway smooth muscle. *American Journal of Respiratory and Critical Care Medicine,* **2005**, *171*, 1103-1108.

Chapter 4

1. Zwaal, R. F. A.; Schroit, A. J. Pathophysiologic implications of membrane phospholipid asymmetry in blood cells. *Blood,* **1997**, *89*, 1121-1132.

2. Dykstra, M.; Cherukuri, A.; Sohn, H. W.; Tzeng, S. J.; Pierce, S. K. Location is everything: Lipid rafts and immune cell signaling. *Annual Review of Immunology,* **2003**, *21*, 457-481.

3. Bach, D.; Wachtel, E. Phospholipid/cholesterol model membranes: Formation of cholesterol crystallites. *Biochimica Et Biophysica Acta-Biomembranes,* **2003**, *1610*, 187-197.

4. Fadeel, B.; Xue, D. The ins and outs of phospholipid asymmetry in the plasma membrane: roles in health and disease. *Critical Reviews in Biochemistry and Molecular Biology,* **2009**, *44*, 264-277.

5. Schubert, P.; Devine, D. V. *De novo* protein synthesis in mature platelets: a consideration for transfusion medicine. *Vox Sanguinis,* **2010**, *99*, 112-122.

6. Ge, S.; White, J. G.; Haynes, C. L. Critical role of membrane cholesterol in exocytosis revealed by single platelet study. *ACS Chemical Biology,* **2010**, *5*, 819-828.

7. Heemskerk, J.; Bevers, E.; Lindhout, T. Platelet activation and blood coagulation. *Thrombosis and Haemostasis,* **2002**, *88*, 186-193.

8. Zwaal, R.; Comfurius, P.; Bevers, E. Lipid-protein interactions in blood coagulation. *Biochimica Et Biophysica Acta-Reviews on Biomembranes,* **1998**, *1376*, 433-453.

9. Zwaal, R. F.; Comfurius, P.; van Deenen, L. L. Membrane asymmetry and blood coagulation. *Nature,* **1977**, *268*, 358-360.

10. Chasserot-Golaz, S.; Coorssen, J. R.; Meunier, F. A.; Vitale, N. Lipid dynamics in exocytosis. *Cellular and Molecular Neurobiology,* **2010**, *30*, 1335-1342.

11. Uchiyama, Y.; Maxson, M. M.; Sawada, T.; Nakano, A.; Ewing, A. G. Phospholipid mediated plasticity in exocytosis observed in PC12 cells. *Brain Research,* **2007**, *1151*, 46-54.

12. Zhang, Z.; Hui, E.; Chapman, E. R.; Jackson, M. B. Phosphatidylserine regulation of Ca^{2+}-triggered exocytosis and fusion pores in PC12 cells. *Molecular Biology of the Cell,* **2009**, *20*, 5086-5095.

13. Amatore, C.; Arbault, S.; Bouret, Y.; Guille, M.; Lemaître, F.; Verchier, Y. Regulation of exocytosis in chromaffin cells by trans-insertion of lysophosphatidylcholine and arachidonic acid into the outer leaflet of the cell membrane. *Chembiochem,* **2006**, *7*, 1998-2003.

14. Kato, N.; Nakanishi, M.; Hirashima, N. Transbilayer asymmetry of phospholipids in the plasma membrane regulates exocytotic release in mast cells. *Biochemistry,* **2002**, *41*, 8068-8074.

15. Chernomordik, L. V.; Kozlov, M. M. Mechanics of membrane fusion. *Nature Structural and Molecular Biology,* **2008**, *15*, 675-683.

16. Chernomordik, L.; Kozlov, M. M.; Zimmerberg, J. Lipids in biological membrane fusion. *Journal of Membrane Biology,* **1995**, *146*, 1-14.

17. Zhang, Z.; Jackson, M. B. Membrane bending energy and fusion pore kinetics in Ca(2+)-triggered exocytosis. *Biophysical Journal,* **2010**, *98*, 2524-2534.

18. Flaumenhaft, R. Molecular basis of platelet granule secretion. *Arteriosclerosis, Thrombosis, and Vascular Biology,* **2003**, *23*, 1152-1160.

19. Rainville, P.; Plumb, R. *Separating Phospholipids with UPLC/MS*, Waters Application Note, Milford, MA, **2007**.

20. Platelets and megakaryocytes: Volume 1. Functional Assays. Gibbins, J. M., Mahaut-Smith, M. P., Eds.; in *Methods in Molecular Biology*; Humana Press, Inc.: Totowa, NJ, 2004; Vol. 1; pp 272.

21. Blair, P.; Flaumenhaft, R. Platelet alpha-granules: basic biology and clinical correlates. *Blood Reviews,* **2009**, *23*, 177-189.

22. Berger, G.; Caen, J. P.; Berndt, M. C.; Cramer, E. M. Ultrastructural demonstration of cd36 in the alpha-granule membrane of human platelets and megakaryocytes. *Blood,* **1993**, *82*, 3034-3044.

23. Suzuki, H.; Murasaki, K.; Kodama, K.; Takayama, H. Intracellular localization of glycoprotein VI in human platelets and its surface expression upon activation. *British Journal of Haematology,* **2003**, *121*, 904-912.

24. Briquet-Laugier, V.; Lavenu-Bombled, C.; Schmitt, A.; Leboeuf, M.; Uzan, G.; Dubart-Kupperschmitt, A.; Rosa, J. P. Probing platelet factor 4 alpha-granule targeting. *Journal of Thrombosis and Haemostasis,* **2004**, *2*, 2231-2240.

25. Blank, U.; Rivera, J.: Assays for regulated exocytosis of mast cells. In *Current Protocols in Cell Biology*; John Wiley & Sons, Inc., 2006.

26. Emiliani, C.; Ciferri, S.; Mencarelli, S.; Mezzasoma, A. M.; Momi, S.; Orlacchio, A.; Gresele, P. Defective platelet beta-N-acetyl hexosaminidase content and release in chronic myeloproliferative disorders. *Platelets,* **2006**, *17*, 20-29.

27. Prescott, S. M.; Zimmerman, G. A.; Stafforini, D. M.; McIntyre, T. M. Platelet-activating factor and related lipid mediators. *Annual Review of Biochemistry,* **2000**, *69*, 419-445.

28. Kim, D.; Koseoglu, S.; Manning, B. M.; Meyer, A. F.; Haynes, C. L. Electroanalytical eavesdropping on single cell communication. *Analytical Chemistry* **2011**, *83*, 7242-7249.

29. Silver, M. J. Role of calcium ions and phospholipids in platelet aggregation and plug formation. *American Journal of Physiology,* **1965**, *209*, 1128-1136.

30. Zieseniss, S.; Zahler, S.; Muller, I.; Hermetter, A.; Engelmann, B. Modified phosphatidylethanolamine as the active component of oxidized low density lipoprotein promoting platelet prothrombinase activity. *Journal of Biological Chemistry,* **2001**, *276*, 19828-19835.

31. Lai, A. L.; Tamm, L. K.; Ellena, J. F.; Cafiso, D. S. Synaptotagmin 1 modulates lipid acyl chain order in lipid bilayers by demixing phosphatidylserine. *Journal of Biological Chemistry,* **2011**, *286*, 25291-25300.

32. McMahon, H. T.; Kozlov, M. M.; Martens, S. Membrane curvature in synaptic vesicle fusion and beyond. *Cell,* **2010**, *140*, 601-605.

33. Churchward, M. A.; Rogasevskaia, T.; Brandman, D. M.; Khosravani, H.; Nava, P.; Atkinson, J. K.; Coorssen, J. R. Specific lipids supply critical negative spontaneous curvature--an essential component of native Ca^{2+}-triggered membrane fusion. *Biophysical Journal,* **2008**, *94*, 3976-3986.

34. Fuller, N.; Benatti, C. R.; Rand, R. P. Curvature and bending constants for phosphatidylserine-containing membranes. *Biophysical Journal,* **2003**, *85*, 1667-1674.

35. Reed, G. L.; Fitzgerald, M. L.; Polgár, J. Molecular mechanisms of platelet exocytosis: insights into the "secrete" life of thrombocytes. *Blood,* **2000**, *96*, 3334-3342.

36. Orita, S.; Naito, A.; Sakaguchi, G.; Maeda, M.; Igarashi, H.; Sasaki, T.; Takai, Y. Physical and functional interactions of Doc2 and Munc13 in Ca^{2+}-dependent exocytotic machinery. *Journal of Biological Chemistry,* **1997**, *272*, 16081-16084.

37. Clement, A. B.; Gamerdinger, M.; Tamboli, I. Y.; Lütjohann, D.; Walter, J.; Greeve, I.; Gimpl, G.; Behl, C. Adaptation of neuronal cells to chronic oxidative stress is associated with altered cholesterol and sphingolipid homeostasis and lysosomal function. *Journal of Neurochemistry,* **2009**, *111*, 669-682.

38. Richard, J. P.; Leikina, E.; Langen, R.; Henne, W. M.; Popova, M.; Balla, T.; McMahon, H. T.; Kozlov, M. M.; Chernomordik, L. V. Intracellular curvature-generating proteins in cell-to-cell fusion. *Biochemical Journal,* **2011**, *440*, 185-193.

39. Neumueller, O.; Hoffmeister, M.; Babica, J.; Prelle, C.; Gegenbauer, K.; Smolenski, A. P. Synaptotagmin-like protein 1 interacts with the GTPase-activating protein

Rap1GAP2 and regulates dense granule secretion in platelets. *Blood,* **2009**, *114*, 1396-1404.

40. Peters, C. G.; Michelson, A. D.; Flaumenhaft, R. Granule exocytosis is required for platelet spreading: differential sorting of α-granules expressing VAMP-7. *Blood,* **2012**.

41. Kim, D.; Haynes, C. L. Neutrophil chemotaxis within a competing gradient of chemoattractants. *Analytical Chemistry,* **2012**, *84*, 6070-6078.

42. Kim, D.; Lin, Y.-S.; Haynes, C. L. On-Chip Evaluation of shear stress effect on cytotoxicity of mesoporous silica nanoparticles. *Analytical Chemistry,* **2011**, *83*, 8377-8382.

43. Ge, S.; Woo, E.; Haynes, C. L. Quantal regulation and exocytosis of platelet dense-body granules. *Biophysical Journal,* **2011**, *101*, 2351-2359.

44. Meyer, A. F.; Thompson, J. T.; Wang, Y.; Koseoglu, S.; Haynes, C. L.; Dalluge, J. J. Isotope-Dilution UPLC-MS/MS Determination of mast cell-secreted bioactive lipids. *Analyst,* **2013**, *138*, 5697-5705.

45. Ge, S.; Woo, E.; White, J. G.; Haynes, C. L. Electrochemical measurement of endogenous serotonin release from human blood platelets. *Analyical Chemistry,* **2011**, *83*, 2598-2604.

46. Ge, S.; White, J. G.; Haynes, C. L. Quantal release of serotonin from platelets. *Analytical Chemistry,* **2009**, *81*, 2935-2943.

Chapter 5

1. Flaumenhaft, R. Molecular basis of platelet granule secretion. *Arteriosclerosis, Thrombosis, and Vascular Biology,* **2003**, *23*, 1152-1160.

2. Omori, T.; Mihara, H.; Kurihara, T.; Esaki, N. The distribution of phosphatidyl-d-serine in the rat. *Bioscience Biotechnology and Biochemistry,* **2010**, *74*, 1953-1955.

3. Heemskerk, J.; Bevers, E.; Lindhout, T. Platelet activation and blood coagulation. *Thrombosis and Haemostasis,* **2002**, *88*, 186-193.

4. Zwaal, R.; Comfurius, P.; Bevers, E. Lipid-protein interactions in blood coagulation. *Biochimica Et Biophysica Acta-Reviews on Biomembranes,* **1998**, *1376*, 433-453.

5. Zwaal, R. F.; Comfurius, P.; van Deenen, L. L. Membrane asymmetry and blood coagulation. *Nature,* **1977**, *268*, 358-360.

6. Fadok, V. A.; Voelker, D. R.; Campbell, P. A.; Cohen, J. J.; Bratton, D. L.; Henson, P. M. Exposure of phosphatidylserine on the surface of apoptotic lymphocytes triggers specific recognition and removal by macrophages. *Journal of Immunology,* **1992**, *148*, 2207-2216.

7. Martin, S. J.; Reutelingsperger, C. P. M.; McGahon, A. J.; Rader, J. A.; Vanschie, R.; Laface, D. M.; Green, D. R. Early redistribution of plasma-membrane phosphatidylserine is a general feature of apoptosis regardless of the initiating stimulus - inhibition by overexpression of bcl-2 and abl. *Journal of Experimental Medicine,* **1995**, *182*, 1545-1556.

8. Bennett, M. R.; Gibson, D. F.; Schwartz, S. M.; Tait, J. F. Binding and phagocytosis of apoptotic vascular smooth-muscle cells is mediated in part by exposure of phosphatidylserine. *Circulation Research,* **1995**, *77*, 1136-1142.

9. Casciolarosen, L. A.; Anhalt, G. J.; Rosen, A. DNA-dependent protein-kinase is one of a subset of autoantigens specifically cleaved early during apoptosis. *Journal of Experimental Medicine,* **1995**, *182*, 1625-1634.

10. Tanaka, Y.; Schroit, A. J. Insertion of fluorescent phosphatidylserine into the plasma-membrane of red-blood-cells - recognition by autologous macrophages. *Journal of Biological Chemistry,* **1983**, *258*, 1335-1343.

11. Schroit, A. J.; Madsen, J. W.; Tanaka, Y. *In vivo* recognition and clearance of red blood-cells containing phosphatidylserine in their plasma-membranes. *Journal of Biological Chemistry,* **1985**, *260*, 5131-5138.

12. Fadok, V. A.; Laszlo, D. J.; Noble, P. W.; Weinstein, L.; Riches, D. W. H.; Henson, P. M. Phagocytosis of digestible particles stimulates macrophages to recognize phosphatidylserine (ps) on apoptotic cells. *Molecular Biology of the Cell,* **1992**, *3*, A291-A291.

13. Fadok, V. A.; Laszlo, D. J.; Noble, P. W.; Weinstein, L.; Riches, D. W. H.; Henson, P. M. Particle digestibility is required for induction of the phosphatidylserine recognition mechanism used by murine macrophages to phagocytose apoptotic cells. *Journal of Immunology,* **1993**, *151*, 4274-4285.

14. Meyer, A. F.; Thompson, J. T.; Wang, Y.; Koseoglu, S.; Haynes, C. L.; Dalluge, J. J. Isotope-Dilution UPLC-MS/MS determination of mast cell-secreted bioactive lipids. *Analyst,* **2013**, *138*, 5697-5705.

15. Koseoglu, S.; Meyer, A. F.; Kim, D.; Wang, Y.; Meyer, B. M.; Dalluge, J. J.; Haynes, C. L. Phospholipid effects on platelet secretion and adhesion. **submitted**.

16. Koseoglu, S.; Gruba, S. M.; Maurer-Jones, M. A.; Haynes, C. L. Comparison of platelet delta-granule secretion from different species using single cell measurements. **submitted**.

17. Blank, U.; Rivera, J.: Assays for regulated exocytosis of mast cells. In *Current Protocols in Cell Biology*; John Wiley & Sons, Inc., 2006.

18. Kim, D.; Koseoglu, S.; Manning, B. M.; Meyer, A. F.; Haynes, C. L. Electroanalytical eavesdropping on single cell communication. *Analytical Chemistry,* **2011**, *83*, 7242-7249.

19. Bach, D.; Wachtel, E. Phospholipid/cholesterol model membranes: formation of cholesterol crystallites. *Biochimica Et Biophysica Acta-Biomembranes,* **2003**, *1610*, 187-197.

20. Kim, D.; Lin, Y.-S.; Haynes, C. L. On-chip evaluation of shear stress effect on cytotoxicity of mesoporous silica nanoparticles. *Analytical Chemistry,* **2011**, *83*, 8377-8382.

Chapter 6

1. Schweitzer, N. J.; Saks, M. J. The *CSI* effect: popular fiction about forensic science affects the public's expectations about real forensic science. *Jurimetrics,* **2007**, *47*, 357-364.

2. Milanick, M. A.; Prewitt, R. L. Fact or fiction? General chemistry helps students determine the legitimacy of television program situations. *Journal of Chemical Education,* **2013**, *90*, 904-906.

3. Kazilek, C.J.; Pearson, D. Using the scientific method to solve mysteries. http://askabiologist.asu.edu/teaching-scientific-method. (accessed Jul 2013).

4. Caldwell, J. E. Clickers in the large classroom: current research and best practice tips. *CBE Life Science Education,* **2007**, *6*, 9-20.

5. MacArthur, J. R.; Jones, L. L. A review of literature reports of clickers available to college chemistry classrooms. *Chemical Education Research Practices,* **2008**, *9*, 187-195.

6. Maurer-Jones, M. A.; Love, S. A.; Meierhofer, S.; Marquis, B. J.; Liu, Z.; Haynes, C. L. Toxicity of brine shrimp: an introduction to nanotoxicity and interdisciplinary science. *Journal of Chemical Education, 2013*, *90*, 475-478.

7. McFarland, A. D., Haynes, C. L., Mirkin, C. A., Van Duyne, R. P., and Godwin, H. A. Color my nanoworld. *Journal of Chemical Education,* **2004**, *81*, 544A.

8. Murphy, J. Personal Communication to C.L. Haynes, 2011-2013. Demographics of W. 7th Community Center.

9. Agresti, A.; Franklin, C. *Statistics: The Art and Science of Learning from Data*; 3rd ed.; Pearson: USA, 2012; pp 504.

10. Quinn, H.; Schweingruber, H.; Keller, T.: *A Framework for K-12 Science Education: Practices, Cross Cutting Concepts, and Core Ideas*; The National Academies Press: Washington, D.C., 2012.

11. Achieve, Inc. on behalf of the twenty-six states and partners that collaborated on the NGSS. *Next Generation Science Standards*, Appendix F; Achieve, Inc. on behalf of the twenty-six states and partners that collaborated on the NGSS, 2013.

Audrey Francis Meyer

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Education

2013 (expected)	Ph.D. University of Minnesota, Minneapolis, MN Advisor: Christy L. Haynes GPA: 3.7/4.0
2009	M.S., Chemistry, University of Minnesota, Minneapolis, MN GPA: 3.7/4.0
2008	B.A., Chemistry, Concordia College, Moorhead, MN GPA: 3.8/4.0

Research Experience

2008 - present	University of Minnesota, Department of Chemistry, Minneapolis, MN *Research Assistant* Advisor: Christy L. Haynes Developed and applied mass spectrometry methods for investigating mast cell and platelet-secreted lipids
2006 - 2008	North Dakota State University, Department of Coatings and Polymeric Materials, Fargo, ND Concordia College, Moorhead, MN, Department of Chemistry Advisors: Mark Jensen and Dennis Tallman (2007-2008)*Research Assistant* (2006) *Summer Undergraduate Research Experience Participant* Investigated surface preparation methods for electrodeposition of polypyrrole on Al alloys and examined the mechanism of mediated electrodeposition of polypyrrole on Al alloys

Awards and Recognition

2012	MN Division of ACS Travel Award
2012	University of Minnesota Doctoral Dissertation Fellowship
2011	FACSS 2011 Student Poster Award
2011	Graduate and Professional Student Association Travel Award
2009	Graduate Women in Science Agnes Hansen Travel Award
2009 - 2012	National Science Foundation Graduate Research Fellowship
2008 - 2012	3M Science and Technology Fellowship
2006	Centennial Scholars Research Scholarship
2006 - 2008	Porter and Lillian Erickson Scholarship for Excellence in Chemistry
2005 - 2008	Concordia College Faculty Scholarship
2005	Morrison County Farmers' Union Scholarship

Peer-Reviewed Publications

Stereochemistry- and concentration-dependent effects of phosphatidylserine enrichment on platelet function. **Meyer, A.F.***, Gruba, S.M.*, Kim, D., Meyer, B.M., Koseoglu, S., Dalluge, J.J., and Haynes, C.L. *in preparation.*

Time- and concentration-dependent effects of exogenous serotonin and inflammatory cytokines on mast cell function. Gruba, S.M.*, **Meyer, A.F.***, Mannng, B.M., Wang, Y., Thompson, J.W., Dalluge, J.J., and Haynes, C.L. *submitted.*
        *Authors contributed equally to this publication

Phospholipid effects on platelet function. Koseoglu, S.*, **Meyer, A.F.***, Kim, D., Wang, Y., Dalluge, J.J., Haynes, C.L. *submitted.*
        *Authors contributed equally to this publication

Single-cell analysis of mast cell exocytosis reveals direct induction of mast cell degranulation by the airway smooth muscle-associated mediators CXCL10 and RANTES. Manning, B.M., **Meyer, A. F.**, Gruba, S.M., and Haynes, C.L. *in revision.*

A chemistry "Whodunnit" to explore the scientific method. **Meyer, A.F.***, Knutson, C.M.*, Finkenstaedt-Quinn, S.A., Gruba, S.M., Meyer, B.M., Thompson, J.W., Halderman, S., Tillman, A.S., DeStefano, L., and Haynes, C.L. *submitted.*
        *Authors contributed equally to this publication

A workflow for preliminary identification of small molecules enriched in autophagosomes and activated mast cells using UPLC-MS^e, chemometrics, and mass spectral evaluation. Satori, C.P., Koopmeiers, J.S., **Meyer, A.F.**, Rodriguez-Navarro, J.A., Henderson, M.M., Taylor, T.H., Haynes, C.L., Arriaga, E.A., and Dalluge, J.J. *in revision.*

Isotope dilution UPLC-MS/MS determination of cell-secreted bioactive lipids. **Meyer, A.F.**, Thompson, J.W., Wang, Y., Koseoglu, S., Haynes, C.L., Dalluge, J.J. *Analyst*, 138, 5697-5705, (2013).

Scanning electrochemical microscopy and video microscopy investigations of tiron-mediated polypyrrole nucleation on AA2024-T3. Jensen, M.B., Karels, J.M., Cool, P.J., **Guerard, A.F**., Tallman, D.K., *Journal of Solid State Electrochemistry,* 16, 3363-3370 (2012).

Electroanalytical eavesdropping on single cell communication. Kim, D.*, Koseoglu, S.*, Manning, B.M.*, **Meyer, A.F.*** Haynes, C.L. *Analytical Chemistry*, 83 (19), 7242-7249, (2011).
        *Authors contributed equally to this publication.

Recent progress in SERS for biosensing. Bantz, K.C.*, **Meyer, A.F.***, Im, H., Lee, S.H., Wittenberg, N.J., Lindquist, N., Haynes, C.L., Oh, S.-H. *Physical Chemistry Chemical Physics*, 13, 11551-11567, (2011).
        *Authors contributed equally to this publication.

Studies of electrochemical transfer at aluminum alloy surfaces by scanning electrochemical microscopy. Jensen, M.J., **Guerard, A.F.**, Tallman, D.E., Bierwagon, G.P. *Journal of the Electrochemical Society*, 155, C324-C332, (2008).

Scientific Presentations

Contributed poster, "Phosphatidylserine Effects on Platelet Function Evaluated with UPLC-MS/MS," 62nd American Society for Mass Spectrometry Meeting on Mass Spectrometry and Allied Topics, Minneapolis, MN. June 2013.

Invited poster, "Phosphatidylserine Effects on Platelet Function," Doctoral Dissertation Poster Session, University of Minnesota, Minneapolis, MN. May 2013.

Invited seminar, "Phosphatidylserine Effects on Platelet Function," Physics and Chemistry Colloquium Series, Concordia College, Moorhead, MN. November 2012.

Invited oral presentation, "Phosphatidylserine Effects on Platelet Function," Doctoral Dissertation Fellowship Seminar, Minneapolis, MN. October 2012.
*supported by 2012 Doctoral Dissertation Fellowship

Contributed oral presentation, "Phosphatidylserine Effects on Platelet Function," ACS Fall Meeting 2012, Philadelphia, PA. August 2012.
*supported by 2012 MN Division of ACS Travel Award

Invited poster, "UPLC/MS/MS Examination of Mast Cell Mediators," 3M Graduate Fellowship Program Poster Session, Minneapolis, MN. October 2011.
*supported by 3M Science and Technology Fellowship

Contributed poster, "Rapid Detection of Ricin Using Aptamer-Modified Ag Colloids," FACSS 2011, Reno, NV. October 2011.
*supported by FACSS 2011 Student Poster Award

Contributed oral presentation, "Targeted UPLC/MS/MS Analysis of Mast Cell-Secreted Lipids," IUPAC Congress on Analytical Sciences, Kyoto, Japan. May 2011.
*supported by NSF Graduate Research Fellowship Program International Travel Allowance

Contributed oral presentation, "Mast Cell Function is Mediated by CXCL10: UPLC/MS/MS Analysis of Mast Cell-Secreted Lipids," 10th Annual University of Minnesota Graduate Research Symposium, Minneapolis, MN. May 2011.

Contributed oral presentation, "UPLC/MS/MS Analysis of Mast Cell Secreted Mediators," PittCon 2011, Atlanta, GA. March 2011.

Contributed poster, "UPLC/MS/MS Detection and Quantification of Mast Cell Secreted Mediators," FACSS 2010, Raleigh, NC. October 2010.

Contributed poster, "Surface-Enhanced Raman Spectroscopy for the Detection of Inflammatory and Vasoactive Mediators Produced during Allergic Response," PittCon 2010, Orlando, FL. March 2010.
*supported by Agnes Hansen Travel Award

Contributed poster, "Mechanistic Studies of the Mediated Electrodeposition of Polypyrrole on Aluminum and Aluminum Alloys," 3rd ASBMB/ACS Northwest Regional Undergraduate Affiliate Network Meeting, Moorhead, MN. October 2007

Contributed poster, "Surface Preparation for the Electrodeposition of Polypyrrole on Aluminum Alloy 2024-T3," Minnesota Private College Scholars at the Capitol, St. Paul, MN. February 2007

Professional and Community Service

2010 - 2013	*Mentorship at University of Minnesota*
	(2012-2013) Directed University of Minnesota undergraduate Yiwen Wang's research on phospholipid effects on platelet function
	(2012) Directed Creighton graduate Sarah Gruba's research on chemokine effects on mast cell function in asthma
	(2011-2012) Directed University of Minnesota undergraduate John Thompson's research on analysis of mast cell degranulation and lipid secretion in asthmatic inflammation and nanoparticle exposure
	(2010-2011) Directed University of Minnesota undergraduate Marsha Sintara's research on analysis of mast cell-secreted proteins

2009 - 2013	*Student Seminar Committee, University of Minnesota*
	2012-2013: Chair; 2011-2012: Vice Chair; 2009-2011: Member
	Invite student-selected scientists to give seminars and organize seminar speakers' schedules

2009 - 2013	*Outreach Activities at University of Minnesota*
	(2012 - 2013) Energy and U Volunteer, University of Minnesota
	(2009 - 2013) Cool Chem Day: developed and executed hands-on activities for a day of science for junior high school girls
	(2009 – 2013) West 7th Community Center Outreach, St. Paul, MN
	(2011-2012) Graduate School Workshops, Hamline University and St. Olaf College, St. Paul, MN and Northfield, MN
	(2011) North Minneapolis STEM Expo, Minneapolis, MN; University of Minnesota Math and Science Family Fun Fair; Poster Judge for Modern Instrumental Methods of Chemical Analysis
	(2010) Exploring Careers in Engineering, Physical Sciences, and Mathematics; CheckIT Out
	(2009) Centennial Elementary Chemistry Day, Circle Pines, MN

2005-2008	*Outreach at Concordia College, Moorhead, MN*
	(2005-2008) Chemistry Club: Served as president (2006-2008), assisted in organizing several days of hands-on activities per year for over 200 elementary school students, and organized major fund-raisers for the Chemistry Club Relay for Life team
	(2006-2007) Expanding Your Horizons: Organized and led a day of hands-on experiments for junior high school girls from rural Minnesota

Co-Authored Grant Applications

"Exploring the Role of Airway Smooth Muscle/Mast Cell Interaction in the Pathophysiology of Asthma," American Asthma Foundation, 2013
"The Roles of Membrane Proteins and Lipids in Platelet Secretion," National Institute of Health, 2012
"Exploring the Role of Airway Smooth Muscle/Mast Cell Interaction in the Pathophysiology of Asthma using Microfluidic Platforms," American Asthma Foundation, 2012

"Exploring the Role of Airway Smooth Muscle/Mast Cell Interaction in the Pathophysiology of Asthma," National Institutes of Health, 2011

"Exploring Airway Smooth Muscle/Mast Cell Interactions on Microfluidic Platforms," American Asthma Foundation, 2011

"Surface-Enhanced Raman Scattering Detection of Secreted Lipids," National Science Foundation, 2009

Teaching Experience

2008 – 2009	University of Minnesota, Department of Chemistry
	Teaching Assistant
	CHEM 1011: Introductory Chemistry Laboratory, Spring 2009
	CHEM 2111: Analytical Chemistry Laboratory, Fall 2008
	CHEM 2301: Organic Chemistry Laboratory, Summer 2008
	-Prepared pre-lab lectures and grading rubric, led chemistry experiments, graded lab reports, held tutor and office hours
2006 – 2008	Concordia College, Department of Chemistry
	Teaching Assistant, Laboratory Preparatory Assistant, and Course Grader

Professional Memberships

American Chemical Society
Coblentz Society
Society for Applied Spectroscopy

Appendix II: Supplementary Materials for Mystery Solving Activities

A1. Investigation of a Chemical Spill

A1.1. Teacher Guide

A1.1.1. Introduction

Recently public interest in forensic science has increased greatly, due in part to television shows such as *CSI* and *Bones*, which feature scientists using analytical tools to solve crimes. The increased public interest in forensic science presents an opportunity for science educators to engage students by leveraging student interest in the applied nature of forensic chemistry while practically introducing students to the scientific method. In this investigation students are presented with information about a crime scene and are asked to solve the crime using evidence gathered from the scene, evidence collected from the identified suspects, and a series of simple forensics type tests. Through this investigation students will become more familiar with making hypotheses, making observations, analyzing data, interpreting positive and negative results, and drawing conclusions.

In addition to giving students the background information relevant to the investigation, students will receive information about conducting each of the tests in the investigation before they are given the evidence kit. Each test is explained and its procedure detailed in the teacher guide section. You may consider demonstrating each of the tests to students in addition to explaining their purpose and relevant background information. There is a place for students to take notes on each of the tests in the student handout.

This investigation requires the preparation of an evidence kit for each group of students as well as preparation of materials for each of the tests. Preparation of the evidence kit and the tests are fully described in the teacher guide section. Amounts prepared may be adjusted to larger or smaller quantities depending on the number of students. While preparation may be initially intensive, most chemicals and solutions can be stored for later use, which greatly reduces preparation when repeating the activity. This investigation will likely take 3-4 one hour class periods, but may be extended or shortened if desired. One day will be required to introduce the investigation and the tests and 2-3 days will be required for students to complete the tests and draw final conclusions.

This investigation is centered around a fictitious chemical spill in the Mississippi River near St. Paul, MN (other important local body of water with fish may substitute). The complete story and dialogues are given in the student handout section. You may consider having students read the story and dialogues to preface the investigation or having students act out the story and dialogues in class. You may also consider creating a video with colleagues or students to present before the investigation begins based on the story and dialogues provided.

Tasks and Suggested Timeline for this Activity
Read teacher packet: 4 weeks prior to activity
Film dialog (optional): 3 weeks prior to activity
Prepare evidence kits: 2 weeks prior to activity
Prepare test stations: 1 week prior to activity
Demonstrate tests: day of activity

A1.1.2 The Evidence Kit and its Preparation

Clearly label all items with the bold text below. Do not include the non-bold text in the label. Place all pieces of evidence in a box to give to each group of students. You may consider including a slip of paper that lists the evidence available for testing in the box.

1. **Uncontaminated River Water Sample**
   a. Preparation: Obtain a sample of distilled water. Color with blue food coloring to match the contaminated river water sample, which will be blue from the addition of a copper II salt.
   b. Note: Using water with a pH of ~7 is essential in the preparation of materials for this investigation. If using water with a pH other than 7, preparation of some solutions may need adjustment.
   c. Note: Blue food coloring may be added to any of the prepared solutions in this investigation so that the solutions are not easily discerned from one another while conducting various tests.

2. **Contaminated River Water Sample** (sample contains 0.1 M copper II ions and is acidic)
   a. Preparation for 1 L of solution: Dissolve 20.0 grams of reagent grade copper II acetate $(Cu(C_2H_3O_2)_2 \cdot H_2O)$ in water to create 1 L of total solution, aliquot into 2-4 mL samples for evidence boxes.

3. **Handwritten Notes Found in the Area of Contamination**
   a. Preparation: Using a Vis-a-Vis wet erase marker, a Sharpie permanent marker, a Expo dry erase marker, or a Paper Mate felt-tip pen, write notes along the bottom of a strip of filter paper or a strip of a coffee filter.

4. **Pens Belonging to Pesticide Transporters, Corporate Executive, and DNR Agent**
   a. Preparation: Assign a Vis-a-Vis wet erase marker, a Sharpie permanent marker, an Expo dry erase marker, or a Paper Mate felt-tip pen to each of the above suspects, so that each suspect is assigned a different pen.
   b. Note: The marker or pen used for the handwritten notes above should match the pen assigned to the pesticide transporter.

5. **Fingerprint Samples from Possible Suspects**
   a. Preparation: Obtain ink fingerprint samples from seven people. Assign fingerprint samples to each of the suspects (Pesticide Transporter #1,

Pesticide Transporter #2, Poacher, Corporate Executive, DNR Agent, Teenager #1, and Teenager #2).

6. **Piece of Map Found Near Contaminated Area with Fingerprint**
   a. Preparation: Have one of the people from above press their fingerprint onto the edges of a piece of a piece of a paper map.
   b. Note: The fingerprint on the map should match the pesticide transporter's fingerprint sample.
   c. Note: Sweaty fingerprints are best for analysis. If possible, wrap the hand you are obtaining prints from in plastic wrap or wear a plastic glove for several minutes to produce extra sweat before printing. Fresh preparation of fingerprints is best. Protect fingerprints by inserting the paper in a plastic sheet.

7. **Soil Sample from the Uncontaminated Area**
   a. Preparation: Obtain a sample of soil.

8. **Soil Sample from the Contaminated Area**
   a. Preparation: Obtain a sample of soil.
   b. Note: The soil from the contaminated area does not contain any contamination.

9. **Homogenized Fish Sample from the Contaminated Area**
   a. Preparation: Obtain juice from a can of tuna for fish scent. Measure 0.5 L of the contaminated river water sample above and add the juice to it.

A1.1.3 The Tests and Their Procedures

Table A1. Summary of Tests for Chemical Spill Activity.

Test #	Test	Evidence to Test	Testing For...
#1	Detection of Lead or Copper in Solution using an Acid and a Base	Water and Fish Samples	Presence of Lead II or Copper II Ions
#2	Ink Chromatography	Handwritten Notes and Pens from Suspects	Identification of the Pen that was Used to Write the Notes
#3	Flame Test	Water and Fish Samples	Identification of Metal Ions in Solution
#4	Iodine Fingerprint Developing or Ninhydrin Fingerprint Developing	Piece of Map with Fingerprint	Identification of Fingerprints on the Piece of Map
#5	Luminol Test	Soil Samples	Presence of Blood
#6	pH Test	Water and Fish Samples	pH Level (Acidity or Basicity of a Solution)

Test #1: Detection of Lead or Copper in Solution using an Acid and a Base

Background:
By adding hydrochloric acid or sodium hydroxide, the presence of lead II or copper II ions in solution can be detected through a precipitation reaction. If lead II ions are present in the solution, addition of hydrochloric acid and sodium hydroxide will produce a solid precipitate in accordance with the following net ionic equations:

$$\text{With Addition of HCl: } Pb^{2+}_{(aq)} + 2Cl^-_{(aq)} \rightarrow PbCl_{2(s)}$$

$$\text{With Addition of NaOH: } Pb^{2+}_{(aq)} + 2OH^-_{(aq)} \rightarrow Pb(OH)_{2(s)}$$
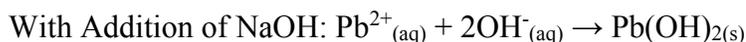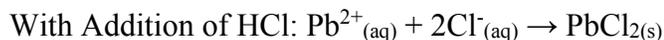
If copper II ions are present in a solution, addition of hydrochloric acid will not produce a reaction, but addition of sodium hydroxide will produce a solid precipitate in accordance with the following net ionic equations:

$$\text{With Addition of HCl: No Net Reaction}$$

$$\text{With Addition of NaOH: } Cu^{2+}_{(aq)} + 2OH^-_{(aq)} \rightarrow Cu(OH)_{2(s)}$$
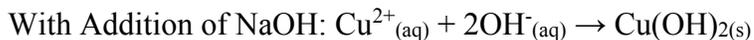
In this test, students will test a lead II salt solution (for a positive control), a copper II salt solution (for a positive control), and water (for a negative control) by the addition of hydrochloric acid and sodium hydroxide with the following anticipated results.

	Addition of HCl	**Addition of NaOH**	**Conclusions**
Lead II Salt	Precipitate (solid) forms	Precipitate (solid) forms	If a precipitate forms with both HCl and NaOH, then lead II is present
Copper II Salt	No precipitate forms	Precipitate (solid) forms	If a precipitate forms with NaOH, but not with HCl, then copper II is present
Water	No precipitate forms	No precipitate forms	If a precipitate does not form with HCl or NaOH, then neither lead II nor copper II are present

Students will then test the water and/or fish samples in their evidence kit against the above scheme to identify possible contaminants.

Materials:

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1. 1.0 M Hydrochloric Acid Solution
    a. Preparation of 1 L of 1.0 M hydrochloric acid solution from 12 M stock solution: Dilute 8.3 mL of 12 M reagent grade hydrochloric acid in water to create 1 L of total solution.
2. 1.0 M Sodium Hydroxide Solution
    a. Preparation of 1 L of 1.0 M sodium hydroxide solution. Dissolve 40.0 grams of sodium hydroxide in water to create 1 L of total solution.
3. 0.1 M Lead Salt Solution (suggested: lead II acetate)
    a. Preparation of 0.5 L of 0.1 M lead II acetate solution: Dissolve 19.0 grams of reagent grade lead II acetate ($Pb(C_2H_3O_2)_2 \cdot 3H_2O$) in water to create 0.5 L of total solution.
4. 0.1 M Copper Salt Solution (suggested: copper II chloride)
    a. Preparation of 0.5 L of 0.1 M copper II chloride solution: Dissolve 8.5 grams of reagent grade copper II chloride ($CuCl_2 \cdot 2H_2O$) in water to create 0.5 L of total solution.
5. Water
6. Water Samples from Evidence Kit
7. Homogenized Fish Sample from Evidence Kit
8. Droppers
9. Glass Test Tubes or Well Plate

Safety:
Hydrochloric acid is highly toxic by ingestion or inhalation and is severely corrosive to skin and eyes. Hazards for salts vary depending on the salt chosen. Sodium hydroxide is a corrosive solid and can cause skin burns. Heat evolves when adding sodium hydroxide to water. Sodium hydroxide is very dangerous to eyes and skin, so eye protection and gloves should be used when handling it. Lead II acetate is a probable carcinogen and an eye, skin, and respiratory irritant. Lead II acetate is slightly toxic. Copper II chloride is highly toxic by ingestion and inhalation.

Procedure for Positive Controls (Presence of Lead or Copper):
1. Place several drops of the lead II salt solution in a test tube or in one of the wells in a well plate. Add a dropperful of the hydrochloric acid solution. Record your observations.
2. Repeat with the sodium hydroxide. Record your observations.
3. Repeat steps 1-2 with the copper II salt solution.

Procedure for Negative Control (Absence of Lead or Copper):
1. Place several drops of water in a test tube or in one of the wells in a well plate. Add a dropperful of the hydrochloric acid solution. Record your observations.
2. Repeat with the sodium hydroxide. Record your observations.

Procedure for Unknowns:
1. Place several drops of an unknown in a test tube or in one of the wells in a well plate. Add a dropperful of the hydrochloric acid solution. Record your observations.
2. Repeat with the sodium hydroxide. Record your observations.

Waste Disposal:
Mixed chemicals may be collected together in waste container that is labeled with its contents. Unused solutions may be stored until later use.

Test #2: Ink Chromatography

Background:

Chromatography is a method for analyzing mixtures by separating them into their component chemicals. Even though a black marker or pen only writes in one color, the ink is actually made from a mixture of different colored pigments. To perform ink chromatography, a small dot of ink is placed at one end of a strip of filter paper. This end of the paper strip is then placed in a solvent. As the solvent travels up the paper strip, the mixture of chemicals that compose the ink are dissolved and pulled upward on the paper. The chemicals that dissolve best in the solvent will move up the paper strip further than chemicals that do not dissolve as well. Smaller molecules will also move up further than larger molecules. Forensic scientists are able to use ink chromatography to solve crimes by matching documents or stains found at a crime scene to a marker or pen that belongs to a suspect.

In this test, students will separate the components of three markers or pens that belong to some of the suspects and will then test the handwritten notes found near the contaminated area for comparison.

Materials:
1. Filter Paper of Coffee Filters (cut into uniform strips)
2. Different Black Markers or Pens (suggested: Vis-a-Vis wet erase marker, Sharpie permanent marker, Expo dry erase marker, or Paper Mate felt-tip pen)
3. Isopropanol (can substitute methanol, ethanol, acetone or similar)
4. Beaker or Cups
5. Handwritten Notes from Evidence Kit

Safety:

Isopropanol is a flammable liquid and thus a fire hazard. Isopropanol is slightly toxic by ingestion and inhalation.

Procedure:
1. Using each of the different black markers or pens, place a dot near the bottom of a paper strip. Use a different strip for each marker or pen.
2. Place about 10 mL of isopropanol in the bottom of a beaker or cup.
3. Hold one of the strips in the isopropanol using care to not dip the strip below the dot.
4. Allow the isopropanol to run up the filter paper, separating the pigments as it travels.
5. Remove the strip from the isopropanol when it has reached the top and allow it to dry.
6. Repeat with each of the remaining samples including the handwritten notes.

7. Compare each of the samples to the handwritten notes to determine which ink was used to write the notes.

Waste Disposal:
Excess used isopropanol should be placed in flammable organic waste. Unused isopropanol may be stored until later use. Used filter paper may be thrown away.

References:
Museum of Science+Industry Chicago.
http://www.msichicago.org/fileadmin/Education/learninglabs/lab_downloads/EvidenceLa b_ink_act.pdf (accessed July 2013).

Test #3: Flame Test

Background:

A flame test is a procedure used to detect the presence of metal ions based on each metal's characteristic emission spectrum. The test involves introducing a sample containing metal ions to a hot flame and observing the color that results.

In this test, students will conduct a flame test with a copper II salt solution, a sodium salt solution, a potassium salt solution, a barium salt solution, and water for observation of positive and negative controls with the following anticipated results:

Metal	Color Observed
Copper	Green
Sodium	Golden Yellow/Orange
Potassium	Lilac/Purple
Barium	Pale Yellow
Water (Blank)	None

Students will then test evidence in their kit against the above to identify possible contaminants.

Materials:
1. 0.1 M Copper II Chloride Solution
   a. Preparation: Use solution from detection of metal ions.
2. 0.1 M Sodium Chloride Solution
   a. Preparation: Use solution from detection of metal ions.
3. 0.1 M Potassium Chloride Solution
   a. Preparation: Use solution from detection of metal ions.
4. 0.1 M Barium Chloride Solution
   a. Preparation of 0.5 L of 0.1 M barium chloride: Dissolve 12.2 grams of reagent grade barium chloride ($BaCl_2 \cdot 2H_2O$) in water to create 0.5 L of total solution.
5. Water (for negative control)
6. Water Samples from Evidence Kit
7. Homogenized Fish Sample from Evidence Kit
8. Bunsen Burner
9. Wooden Stir Sticks

Safety:
Copper II chloride is highly toxic by ingestion and inhalation. Sodium chloride and potassium chloride are slightly toxic by ingestion. Barium chloride is highly toxic by ingestion.

Procedure for Positive Controls:
1. Dip a wooden stir stick in the copper II chloride solution.
2. Remove the stick from the solution and place over a Bunsen Burner flame. Record your observations.
3. Repeat steps 1-2 with the sodium chloride solution, the potassium chloride solution, and the barium chloride solution.

Procedure for Negative Control:
1. Dip a wooden stir stick in the water.
2. Remove the stick from the solution and place over a Bunsen Burner flame. Record your observations.

Procedure for Unknowns:
1. Dip a wooden stir stick in an unknown sample.
2. Remove the stick from the solution and place over a Bunsen Burner flame. Record your observations.
3. Compare the results from above to determine possible contaminants in the water.

Waste Disposal:
Used wooden stir sticks may be thrown away. Unused solutions may be stored until later use.

Test #4: Iodine Fuming Fingerprint Developing of Ninhydrin Fingerprint Developing

Background:

A fingerprint is an impression or mark made on a surface by a person's fingertip and can be used for identifying individuals based on its unique pattern. Fingerprinting is an important aspect of crime scene investigation and can be used forensically to identify individuals who were present.

There are three basic types of fingerprints: 1) plastic, 2) patent, and 3) latent. Plastic prints are prints made by impressions on a soft material, such as wax or clay. Patent prints are impressions made on colored materials or with colored materials that are on the fingertips. For example, a patent print might be one left in blood or one that is deposited because of blood on a fingertip. Both plastic and patent prints are visible without further processing. Latent prints are prints made by deposition of palmar sweat and natural oils to a surface. Latent prints require processing for visualization.

Latent prints on non-absorbent surface can be developed with powder, while prints on soft or porous surfaces require other processing techniques. One technique used for fingerprint visualization is iodine fuming. Iodine can be heated to produce a violet vapor that is then absorbed by a fingerprint secretion to produce a yellow/brown pattern on the surface where the secretions were deposited. Such visualization is short-lived and needs to be chemically fixed by mixing with starch. Another technique is the use of a ninhydrin staining solution which turns purple upon heating in the presence of amino acids which are in palmar sweat.

In this test, students will develop fingerprints on the map from their evidence kit using one of two methods and will compare to the fingerprint samples of the suspects. While both methods work, the ninhydrin method generally results in more clearly defined prints.

Method #1: Iodine Fuming Fingerprint Developing

Materials:
1. Gentle Heat Source (oil candle, Bunsen burner, or similar)
2. Large Glass Test Tube (25 mm x 150 mm)
3. Rubber or Cork Test Tube Stopper
4. Test Tube Clamp
5. Forceps, Scoopula, or Spoon
6. Glass Rod
7. Iodine Crystals
8. 1% Starch Solution
   a. Preparation: Heat 25 mL of water to boiling. Add 1 gram of soluble starch to the hot water and stir. Dilute to 100 mL. Allow the starch solution to cool. The starch solution will not keep more than a few days. Add a small

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amount of salicylic acid to preserve the solution for a longer period of time.
9. Piece of Map with Fingerprint from Evidence Kit

Safety:
Iodine is toxic by ingestion or inhalation. Iodine reacts violently with reducing materials, sulfur, iron, alkali metals, metal powders, and phosphorus. Iodine irritates the skin and is corrosive to eyes and respiratory tract.

Procedure:
1. Using forceps transfer a single iodine crystal to a glass test tube.
2. Lightly stopper the test tube so the iodine vapor does not escape.
3. Place the test tube over a gentle heat source and allow the iodine crystal to sublime.
4. Place the fingerprinted material in the test tube and allow the fingerprint to absorb the iodine.
   a. Note: At this point the fingerprint should have a yellowish color and should be visible.
5. Permanently fix the fingerprint by dropping the starch solution on the fingerprint.
   a. Note: Fixing is required to keep the deposited iodine from evaporating and the fingerprint from disappearing.
   b. Note: At this point, the fingerprint should have a dark purple/black color.
6. Allow fixed fingerprint to dry.
7. Compare the fingerprint to the suspects' fingerprints for identification.

Waste Disposal:
Developed fingerprints may be thrown away. Unused iodine and starch may be stored until later use. If starch solution is stored it must be preserved.

Method #2: Ninhydrin Fingerprint Developing
Materials:
1. Fingerprint Staining Solution (ninhydrin, ethanol (acetone may be substituted), and acetic acid)
   a. Preparation: Dissolve 0.5 grams of ninhydrin in 125 mL of ethanol. Add 0.1 mL acetic acid to the solution.
   b. Note: Fresh preparation of solution is best for fingerprint developing.
2. Lysine Solution (for positive test)
   a. Preparation: Dissolve 0.25 grams of lysine in 50 mL of water and 50 mL of ethanol.
3. 1:1 Water:Ethanol Mixture (for negative test)
   a. Preparation: Mix 50 mL of water and 50 mL of ethanol.
4. Droppers or Spray Bottles

5. Paper
6. Heat Gun or Blow Dryer
7. Piece of Map with Fingerprint from Evidence Kit

Safety:
Ninhydrin is a body tissue irritant. Ethanol is a flammable liquid and thus a fire hazard. Ethanol is slightly toxic by ingestion and inhalation. Acetic acid is corrosive to skin and tissue and is moderately toxic by ingestion and inhalation

Procedure for Positive and Negative Controls:
1. On a piece of paper place a drop of the lysine solution on one side and a drop of ethanol on the other. Briefly allow to dry.
2. Drop or spray the fingerprint staining solution on the lysine solution and the 1:1 water:ethanol on the paper. Briefly allow to dry.
3. Using a heat gun or a blow dryer to apply heat to the paper.
   a. Note: A purple color indicating a positive test will develop where the lysine was dropped, and will not, indicating a negative test, where the ethanol was dropped.

Procedure for Unknown:
1. Drop or spray the fingerprint staining solution on the piece of paper with the fingerprint, essentially covering it. Briefly allow to dry. Using a heat gun or blow dryer to apply heat the paper.
   a. Note: If a fingerprint is present it should develop in the same purple color as observed in the positive test with lysine above.

Waste Disposal:
Paper may be thrown away. Unused chemicals may be stored until later use.

References:
Scientific Working Group on Friction Ridge Analysis, Study, and Technology.

http://www.swgfast.org/ (accessed July 2013).

Test #5: Luminol Test for Blood

Background:
The luminol chemiluminescence reaction is used by forensic scientists use to detect traces of blood at crime scenes. In this test, luminol powder ($C_8H_7O_3N_3$) is mixed with hydrogen peroxide (H_2O_2) and a hydroxide (such as KOH) in a spray bottle. The luminol solution is sprayed where blood might be found. The iron in the hemoglobin in the blood catalyzes the chemiluminescence reaction and producing a blue glow from the luminol. The blue glow fades quickly, but forensic scientists can take photographs for later analysis. In addition to blood, iron compounds, copper compounds, bleach, and other chemicals can activate luminol.

In this test, students will spray the luminol solution on the soil samples to test for the presence of blood.

Materials:
1. Luminol Stock Solution
    a. Preparation: Mix 2 g luminol, 15 g potassium hydroxide, and 250 mL water.
    b. Note: Fresh preparation of solution is best.
2. 3% Hydrogen Peroxide Solution (common over-the-counter concentration)
3. Soil Sample with Bleach Mixed In (labeled soil with animal blood; for positive control)
4. Soil Sample (labeled soil without animal blood; for negative control)
5. Soil Samples from Evidence Kit
6. Test Tubes or Cups
7. Scoopula or Spoon

Safety:
Potassium hydroxide causes severe blisters with skin contact. Potassium hydroxide is strongly corrosive as a solid and as a solution. Potassium hydroxide is very harmful if swallowed and is extremely dangerous to eyes. Many substances will cause hydrogen peroxide to decompose into water and oxygen gas. Hydrogen peroxide is severely corrosive to skin, eyes, and respiratory tract. Hydrogen peroxide is a very strong oxidant and presents a dangerous fire and explosion risk. Do not heat hydrogen peroxide. While a 3% solution of hydrogen peroxide is very weak, it is still an oxidizer and a skin and eye irritant.

Procedure for Positive Control:
1. In a test tube or cup, mix 10 mL of the luminol solution and 10 mL of the hydrogen peroxide solution.
2. Add a scoopula full of the soil sample with "animal blood."
    a. Note: A glow should appear indicating a positive test. A dark environment is best for viewing the glow.

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Procedure for Negative Control:
1. In a test tube or cup, mix 10 mL of the luminol solution and 10 mL of the hydrogen peroxide solution.
2. Add a scoopula full of the soil sample without "animal blood."
   a. Note: A glow will not appear indicating a negative test.

Procedure for Unknowns:
1. In a test tube or cup, mix 10 mL of the luminol solution and 10 mL of the hydrogen peroxide solution.
2. Add a scoopula full of a soil sample.
3. Observe the sample for glowing.

Waste Disposal:
Mixed chemicals may be collected together in waste container that is labeled with its contents. Unused solutions may be stored until later use.

References:
1. Helmenstine, A. About Chemistry.
   http://chemistry.about.com/od/glowinthedarkprojects/a/luminolblood.htm
   (accessed July 2013).

Test #6: pH Test

Background:
The pH scale is a measurement of the acidity or basicity of a solution. The pH scale generally measures from 0-14. The pH of pure water at room temperature is about 7. Solutions with a pH of less than 7 are said to be acidic and solutions with a pH greater than 7 are said to be basic. Chemical dyes, called indicators, change colors at various levels of pH and can be used to identify the pH of a solution.

In this test students will test various solutions to determine if they are acidic or basic and will approximate their pH level using pH paper. Students will then test evidence against solutions to identify possible contaminants.

Materials:
1. Solution with a basic pH (Label as Solution Containing Potassium Cyanide)
    a. Preparation: Add 1 M sodium hydroxide solution dropwise to 100 mL of water until a basic pH is achieved. Test pH with pH paper.
2. Solution with a neutral pH (Label as Solution Containing Potassium Nitrate)
    a. Preparation: Obtain a sample of water.
3. Solution with an acidic pH (Label as Solution Containing Lead)
    a. Preparation: Add 1 M hydrochloric acid solution dropwise to 100 mL of water until an acidic pH is achieved. Test pH with pH paper.
4. Solution with an acidic pH (Label as Solution Containing Barium Chloride)
    a. Preparation: Use solution from above.
5. Solution with an acidic pH (Label as Solution Containing Copper II Cyanide)
    a. Preparation: Use solution from above.
6. Solution with an acidic pH (Label as Solution Containing Ammonium Nitrate)
    a. Preparation: Use solution from above.
7. Water Samples from Evidence Kit
8. Homogenized Fish Sample from Evidence Kit
10. pH Paper (capable of determining a range of pH values)
    a. Note: Hydrion Insta-Chek 0-13 pH Test Paper or similar is suggested.

Safety:
Hydrochloric acid is highly toxic by ingestion or inhalation and is severely corrosive to skin and eyes. Sodium hydroxide is a corrosive solid and can cause skin burns. Heat evolves when adding sodium hydroxide to water. Sodium hydroxide is very dangerous to eyes and skin and eye protection and gloves should be used when handling it.

Procedure:
1. Dip a small strip of pH paper into each of the solutions.
2. Identify the approximate pH of each solution.

    a. Note: Allow the pH paper to dry for better analysis.
3. Compare the river water and fish samples and identify possible contaminants.

Waste Disposal:
Used pH strips may be thrown away. Unused chemicals may be stored until later use.

A1.2. Student Materials

Name: _____Class: _____

A1.2.1. Script for Investigation of a Chemical Spill

Some mysterious things have been happening near the Mississippi River in St. Paul, MN. Normally, the river is full of fish, which provide a plentiful food source for animals in the area. Recently, the fish have been dying and a number of animals that rely on the river for food have been found dead as well. As a forensic scientist, you have been called in to determine the cause of the mysterious deaths of the fish and the wildlife. Samples have been collected from the dead fish, the river, and the soil. A police detective has interviewed some of the many people who use the river for various purposes and has gathered evidence from the area of the river that is contaminated.

When you arrive to the scene, you are briefed by the police detective with the following information:

Police Detective: "We have narrowed our search to several suspects; some pesticide transporters, a poacher, a corporate executive of a car battery factory, a DNR agent, and some teenagers. Each are involved with toxins that could be causing the problem."

"The pesticide transporters denied that they have ever spilled any chemicals, but explained that some of their tanks are not up to standard. We confiscated one of their pens. They primarily transport copper cyanide and barium chloride."

"The poacher does not admit to poaching any fish or wildlife in the area, but reluctantly allowed that if she were to do such a thing there are two poisons she would use in addition to various traps. She mentioned barium chloride and potassium cyanide."

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"The corporate executive talked up her factory and explained how much she cares about the environment, heavily emphasizing how clean her factories are. We confiscated her favorite pen. Her factory makes car batteries which contain lead."

"The DNR agent admitted that he uses different chemicals for his purposes, but doesn't think they would cause any damage. We confiscated his pen. He uses potassium nitrate for starting controlled burns and ammonium nitrate as a fertilizer."

"Some teenagers stole a bunch of gasoline from a nearby factory recently. One refused to talk and denied everything. The other was caught with gasoline by his mother and admitted to everything but swore they never dumped any gasoline into the river."

"A worker from the battery factory stated that she is concerned that lead might be leaking out of the factories pipes into the river."

"I interviewed each of the suspects. The interviews have been transcribed for you to read over. We collected fingerprints from each of the suspects as well as some notes found near the contaminated area, a map that may have fingerprints, and some river and soil samples. I really hope you can help us to solve this. The river, the fish, and the wildlife are an important resource to our community."

Interview with the Pesticide Transporters

Police Detective: Good morning, I'm a police detective; do you mind if I ask you a few questions?

Pesticide Transporter #1: How long is this going to take? We have a lot of orders to deliver today.

Police Detective: Not long, but we have a very serious problem on our hands. It looks like there has been some kind of chemical spill in the Mississippi river—the fish and wildlife essentially disappeared in this area.

Pesticide Transporter #2: That's terrible; we will do whatever we can to help.

Pesticide Transporter #1: You don't think it's our fault do you? We have never had any accidents in the history of our company and we take great care in transporting chemicals.

Police Detective: I am not sure what's going on; we are doing our best to figure it out. What kinds of chemicals do you usually transport?

Pesticide Transporter #1: Usually copper cyanide and barium chloride.

Police Detective: Alright, and were you transporting anything the morning of July 7th?

Pesticide Transporter #2: Yes, both copper cyanide and barium chloride. I remember because we had quite a number of orders to fill that day.

Police Detective: And you're sure that nothing went wrong?

Pesticide Transporter #2: Yes, absolutely.

Police Detective: Alright, well that is pretty much everything, thank you for your time.

Pesticide Transporter #2: Please let us know what you find.

Police Detective: I will be sure to contact you once we have a better idea of what is going on. Oh, (to Pesticide Transporter #2) do you mind if I take that pen?

Pesticide Transporter #2: Um, sure.

Police Detective: Ok, thanks again, take it easy now.

Interview with the Poacher

Police Detective: Good morning, I'm a police detective; do you mind if I ask you a few questions?

Poacher: I do actually, what is this about? I have all my permits in order.

Police Detective: Actually, it is about what we think is a chemical spill—the fish and wildlife in this area have disappeared.

Poacher: Oh, I am sorry to hear that. In that case, ask away.

Police Detective: Look, I know you have been apprehended twice for poaching rather excessive numbers of fish and wildlife. Please, could you let me know how you normally poach these animals? I promise I am not here to bring you in for poaching again.

Poacher: I have absolutely no idea what you are talking about; I stopped all that stuff years ago…

Police Detective: Fine... Could you at least tell me what you *would* use if you *were* poaching fish or wildlife?

Poacher: Well I would never do this, but I have heard of people using potassium cyanide as well as barium chloride as a poison in addition to basic traps of course.

Police Detective: Alright, thank you for your time, and please always make sure to get a permit—they are not that expensive.

Poacher: Will do, you have a good day now.

Interview with the Corporate Executive from the Battery Factory

Police Detective: Hello, I'm a police detective; I spoke with your secretary about meeting with you and she said I would find you down here.

Corporate Executive: Oh, yes, come in. What is it you wanted to speak with me about?

Police Detective: There have been some terrible problems just upstream of your new car battery factory— the fish and wildlife are dying in record numbers.

Corporate Executive: Well, I am sorry to hear that, but I hope you are not implying that my factory has anything to do with this.

Police Detective: Not necessarily, but all this has happened since your factory has started operations so you can see why I am suspicious. Would you mind telling me the process you go through in dealing with hazardous waste?

Corporate Executive: I assure you that my factory meets and exceeds all environmental regulations. Our company is devoted to not only coexisting with the environment but helping to ensure that it remains pristine for generations to come. We have generously funded several efforts to clean up the very river you imply we are polluting in addition to

donating thousands of acres of wildlife habit to the Minnesota Valley National Wildlife Refuge.

Police Detective: Although it sounds like you are quite committed to helping the environment, you still have not answered my question: How do you deal with the large amounts of hazardous waste, especially lead, I know your factory produces?

Corporate Executive: I'll show you... Here is where we collect and package all of our hazardous materials for safe transport to a facility where they are recycled or disposed of properly.

Police Detective: Alright, well thank you for your time then, I am sorry to have bothered you.

Corporate Executive: I am always happy to clear up any concerns.

Police Detective: Oh, one more thing... Would you might letting me have your pen? We would like to run some tests with it.

Corporate Executive: That is no problem at all.

Police Detective: Thanks, have a nice day.

Interview with the DNR Agent

Police Detective: Hello, I'm a police detective from the investigative division over in St. Paul; this area here is your jurisdiction right?

DNR Agent: Good morning, yes, what brings you out here this early?

Police Detective: I am sure you have noticed the disappearing fish and wildlife in this area.

DNR Agent: I sure have. I don't think I have seen a single fish or animal in the past week. What do you think is causing all of this?

Police Detective: Well, we think it is some kind of chemical spill, and that is why I am wondering if you happen to use any chemicals routinely, especially ones that may be toxic to animals or fish.

DNR Agent: Well I start a lot of controlled burns using potassium nitrate, but most of it burns up.

Police Detective: Anything else?

DNR Agent: Oh, I sometimes use ammonium nitrate as a fertilizer to re-grow grass trampled by tourists, but again, I highly doubt I use enough of this to cause any sort of environmental damage.

Police Detective: Alright, well thanks for your cooperation, I will let you know what we find out.

DNR Agent: Please do so I can watch out for stuff like this in the future—I do not want to be contributing to the killing the fish and the wildlife in my efforts to help them.

Police Detective: I sure will. Oh, one more thing... Do you have a pen that you regularly use?

DNR Agent: Yes, in fact I have it with me right here.

Police Detective: Do you mind if I borrow it to run some tests?

DNR Agent: No, that would be no trouble at all.

Police Detective: Okay, thank you, have a good day now.

DNR Agent: Alright, I'll do my best, good luck finding out what or who is responsible for this and let me know if you have anymore questions.

A1.2.2. Student Worksheets with Anticipated Student Responses

Name: _____ Class: _____

Identify the suspects and the toxin(s) they are involved with that may be responsible for the river contamination.

Suspect	Possible Toxin Contaminating the River Water
Pesticide Transporters	Copper II Cyanide or Barium Chloride
Poacher	Barium Chloride or Potassium Cyanide
Corporate Executive	Lead
DNR Agent	Potassium Nitrate or Ammonium Nitrate
Teenagers	Gasoline

Question: Make your hypothesis... Who do you think contaminated the river?
Answers will vary.

Question: Why do you think it was the suspect(s) you identified?
Answers will vary.

Question: How will you determine if you are correct?
Testing the evidence will be needed to find out who contaminated the river water.

Test #1: Detection of Lead or Copper in Solution using an Acid and a Base

Notes about the experiment:

Question: What evidence will you test? Why?

The uncontaminated river water, contaminated river water, and fish samples will be tested because this test can identify if they contain lead or copper.

Complete the following chart:

Solution	Observation After Addition of HCl	Observation After Addition of NaOH	Conclusion
Solution Containing Lead II Ions	Precipitate (solid) forms	Precipitate (solid) forms	If a solution contains lead then... a precipitate will form with the addition of HCl and NaOH.
Solution Containing Copper II Ions	No change	Precipitate forms	If a solution contains copper then... a precipitate will form with the addition of NaOH, but not with the addition of HCl.
Water	No change	No change	If a solution does not contain lead or copper then... no precipitates will form.

Data Collection: Create a chart to document your observations of the evidence you decide to test.

Evidence	Observation After Addition of HCl	Observation After Addition of NaOH

Uncontaminated River Water	No change	No change
Contaminated River Water	No change	Precipitate (solid) forms
Fish Sample	No change	Precipitate (solid) forms

Question: What conclusions can you draw based on your observations from this test?
The contaminated river water and the Homogenized fish samples have copper II ions present. This indicates that the water may contain pesticides (copper II cyanide). This means the pesticide transporters may be responsible for the river contamination.

Test #2: Ink Chromatography:

Notes about the experiment:

Question: What evidence will you test? Why?

The handwritten notes and pens will be tested because this test will allow identification of the pen used to write the notes.

Complete the following chart:

Chromatograph from Pen #1 Suspect: Pesticide Transporter	Chromatograph from Pen #2 Suspect: Corporate Executive	Chromatograph from Pen #3 Suspect: DNR Agent
Varies depending on marker/pen assigned.	Varies depending on marker/pen assigned.	Varies depending on marker/pen assigned.

Data Collection: Document your observations of the evidence you decide to test.

Chromatograph from Handwritten Notes
Varies depending on marker/pen assigned.

Question: Why do the colors separate on the filter paper?

The inks are made of different pigments (colors) which separate on the filter paper based on their size and attraction to the solvent.

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Question: What conclusions can you draw based on your observations from this test?:
The pen used to write the notes belong to the pesticide transporters.

Test #3: Flame Test

Notes about the experiment:

Question: What evidence will you test? Why?

The uncontaminated river water, contaminated river water, and fish samples will be tested because this test can identify if they contain metals.

Complete the following chart:

Solution	Observations	Conclusions
Solution Containing Copper II Ions	Green Color	If a solution contains copper II ions then... a green color will be observed in a flame test.
Solution Containing Sodium Ions	Yellow/Orange Color	If a solution contains sodium ions then... a yellow/orange color will be observed in a flame test.
Solution Containing Potassium Ions	Lilac Color	If a solution contains potassium ions then... a lilac color will be observed in a flame test.
Solution Containing Barium Ions	Yellow Color	If a solution contains barium ions then... a yellow color will be observed in a flame test.
Water	None	If a solution does not contain metal ions then... no color change will be observed in a flame test.

Data Collection: Create a chart to document your observations of the evidence you decide to test.

Sample	Observations
Uncontaminated Water	No color produced from flame test
Contaminated Water	Green color produced from flame test
Homogenized Fish	Green color produced from flame test

Question: What distinguishes each sample?
Different metals give different colors.

Question: What conclusions can you draw based on your observations from this test?
The contaminated river water and the Homogenized fish samples have copper II ions present. This indicates that the water may contain pesticides (copper II cyanide).

Test #4: Fingerprint Developing

Notes about the experiment:

Question: What evidence will you test? Why?
The piece of map will be tested because this test can develop fingerprints left behind.

Data Collection: Draw a sketch of the fingerprint or glue/tape in your developed fingerprint.
This will vary based on the fingerprints used for analysis.

Question: What distinguishing patterns do you see on the fingerprint?
This will vary based on the fingerprints used for analysis.

Question: Which of the suspects do you think the fingerprint belongs to?
The pesticide transporter.

Question: What conclusions can you draw based on your observations from this test? The fingerprint matches that of the pesticide transporter indicating that they were present at the area of contamination.

Test #5: Luminol Test

Notes about the experiment:

Question: What evidence will you test? Why?
The contaminated and uncontaminated soil samples will be tested because this test can determine if they contain blood.

Complete the following chart.

	Observation	Conclusion
Dirt with Animal Blood	Glowing	If a sample of dirt contains blood then... the sample will glow with the addition of the luminol solution.
Dirt without Animal Blood	No Glowing	If a sample of dirt does not contain blood then... the sample will not glow with the addition of the luminol solution.

Data Collection: Create a chart to document your observations of the evidence you decide to test.

Sample	Observation After Luminol
Uncontaminated Soil	No Glowing
Contaminated Soil	No Glowing

Question: What component of blood generates the intense glowing?
Iron in the blood generates the glowing.

Question: Do you think human and animal blood would both be detected using this method? What further analysis could you do to determine the source of the blood?
Both human and animal blood would contain iron and could be detected using this method. DNA testing could be used to further analyze a blood sample to determine its source.

Question: What conclusions can you draw based on your observations from this test?
There is no blood in the soil indicating the fish and animals that are dead were not poached.

Test #6: pH Test

Notes about the experiment:

Question: What evidence will you test? Why?
The uncontaminated river water, contaminated river water, and fish samples will be tested because this test can identify if they are acidic or basic.

Complete the following chart:

Solution	pH (Tape pH paper below and Estimate pH value)
Potassium Cyanide	pH above 7 (Basic)
Potassium Nitrate	pH ~ 7 (Neutral)
Lead Containing	pH below 7 (Acidic)
Barium Chloride	pH below 7 (Acidic)
Copper II Cyanide	pH below 7 (Acidic)
Ammonium Nitrate	pH below 7 (Acidic)

Data Collection: Create a chart to document your observations of the evidence you decide to test.

Sample	pH
Uncontaminated Water	pH ~ 7 (Neutral)
Contaminated Water	pH below 7 (Acidic)
Homogenized Fish	pH below 7 (Acidic)

Question: Why is it important to test the pH of river and lake water?
Fish and other aquatic life cannot survive at extreme values of pH.

Question: What conclusions can you draw based on your observations from this test?
The contaminated river water and homgenated fish samples could have lead, barium chloride, copper II cyanide, or ammonium nitrate.

After completing each test, check the boxes where the suspect's sample matches the evidence found at the crime scene.

Test	Pesticide Transporters	Poacher	Corporate Executive	DNR Agent	Teenagers
#1: Detection of Lead II and Copper II Ions	x				
#2: Ink Chromatography	x				
#3: Flame Test	x				
#4: Fingerprint Developing	x				
#5: Luminol Test					
#6: pH Test	x	x	x	x	

Now that you have tested all of the evidence with the available tests, who do you believe is responsible for contaminating the river? Explain based on your observations obtained during each test why you believe this to be true and why you can rule out the other suspects. Was your hypothesis of who contaminated the river correct?
The pesticide transporters are responsible for contaminating the river... (Students should identify the pesticide transporters as the suspects responsible for contaminating the river. Students should support this with evidence from each test.)

A2. Investigation of a Jewel Heist

A2.1. Teacher Guide

A2.1.1. Introduction

Recently public interest in forensic science has increased greatly, due in part to television shows such as *CSI* and *Bones*, which feature scientists using analytical tools to solve crimes. The increased public interest in forensic science presents an opportunity for science educators to engage students by leveraging student interest in the applied nature of forensic chemistry while practically introducing students to the scientific method. In this investigation students are presented with information about a crime scene and are asked to solve the crime using evidence gathered from the scene, evidence collected from the identified suspects, and a series of simple forensics type tests. Through this investigation students will become more familiar with making hypotheses, making observations, analyzing data, interpreting positive and negative results, and drawing conclusions.

In addition to giving students the background information relevant to the investigation, students will receive information about conducting each of the tests in the investigation before they are given the evidence kit. Each test is explained and its procedure detailed in the teacher guide section. You may consider demonstrating each of the tests to students in addition to explaining their purpose and relevant background information. There is a place for students to take notes about each of the tests in the student handout.

This investigation requires the preparation of an evidence kit for each group of students as well as preparation of materials for each of the tests. Preparation of the evidence kit and the tests are fully described in the teacher guide section. Amounts prepared may be adjusted to larger or smaller quantities depending on the number of students. While preparation may be initially intensive, most chemicals and solutions can be stored for later use, which greatly reduces preparation when repeating the activity. This investigation will likely take 3-4 one hour class periods, but may be extended or shortened if desired. One day will be required to introduce the investigation and the tests and 2-3 days will be required for students to complete the tests and draw final conclusions.

This investigation is centered around a fictitious jewel heist that took place while the crown jewels were on display at the Science Museum in St. Paul, Minnesota (exact location may be modified to suit other local museum). The complete story and dialogues are given in the student handout section of this guide. You may consider having students read the story and dialogues to preface the investigation or you may consider having students act out the story and dialogues in class. You may also consider creating a video with colleagues or students to present before the investigation begins based on the story and dialogues provided.

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Tasks and Suggested Timeline for this Activity
Read teacher packet: 4 weeks prior to activity
Film dialog (optional): 3 weeks prior to activity
Prepare evidence kits: 2 weeks prior to activity
Prepare test stations: 1 week prior to activity
Demonstrate tests: day of activity

A2.1.2. The Evidence Kit and its Preparation

Clearly label all items with the bold text below. Do not include the non-bold text in the label. Place all pieces of evidence in a box to give to each group of students. You may consider including a slip of paper that lists the evidence available for testing in the box.

1. **Fingerprint Samples from Each Suspect**
    a. Preparation: Obtain ink fingerprint samples from 3 people. Assign fingerprint samples to each of the suspects (Security Guard, Former Executive, and Former Art Thief).

2. **Debris from Crime Scene with Fingerprint**
    a. Preparation: Have a different person from the ones above press their fingerprint onto the edges of a piece of a piece of crumpled paper.
    b. Note: The fingerprint will not be a match for any of the suspects.
    c. Note: Sweaty fingerprints are best for analysis. If possible, wrap the hand you are obtaining prints from in plastic wrap or wear a plastic glove for several minutes to produce extra sweat before printing. Fresh preparation of fingerprints is best. Protect fingerprints by inserting the paper in a plastic sheet.

3. **Glass from the Display Case from the Crime Scene** (sample is powdered, in solution, and contains 0.1 M lead II ions)
    a. Preparation of 0.5 L of solution: Dissolve 19.0 grams of reagent grade lead II acetate ($Pb(C_2H_3O_2)_2 \cdot 3H_2O$) in water to create 0.5 L of total solution.

4. **Glass Found in Former Executive's Car** (sample is powdered, in solution, and contains 0.1 M lead II ions)
    a. Preparation: Use solution from above.

5. **Powder Found at Crime Scene** ("pesticide")
    a. Preparation: Obtain a small sample of sodium nitrate.

6. **Powder Found on Security Guard's Arm** (flour)
    a. Preparation: Obtain a small sample of flour.

7. **Powder Found on Former Executive's Jacket** ("pesticide")
    a. Preparation: Obtain a small sample of sodium nitrate.

8. **Spray Paint Collected from Security Camera** (black acrylic paint)

a. Preparation: Dot black acrylic paint on the bottom of a strip of filter paper or a strip of a coffee filter.

9. **Paint from Former Art Thief's Hands** (black tempera paint)
    a. Preparation: Dot black tempera paint on the bottom of a strip of filter paper or a strip of a coffee filter.

10. **Paint from Art Mural** (black tempera paint)
    a. Preparation: Dot black tempera paint on the bottom of a strip of filter paper or a strip of a coffee filter.

A2.1.3 The Tests and Their Procedures

Table A2. Summary of Tests for Jewel Heist Activity

Test #	Test	Evidence to Test	Testing For
#1	Detection of Lead in Solution using an Acid or a Base	Glass Samples	Presence of Lead
#2	Paint Chromatography	Paint Samples	Identification of Paint Samples
#3	Iodine Fingerprint Developing or Ninhydrin Fingerprint Developing	Debris with Fingerprint	Identification of Fingerprints on the Debris
#4	Pesticide Test	Powder Samples	Presence of a Pesticide
#5	Starch Test	Powder Samples	Presence of a Starch

Test #1: Detection of Lead in Solution using an Acid or a Base

Background:

By adding hydrochloric acid or sodium hydroxide, the presence of lead II ions in solution can be detected through a precipitation reaction. If lead II ions are present in the solution, addition of hydrochloric acid and sodium hydroxide will produce a solid precipitate in accordance with the following net ionic equations:

$$\text{With Addition of HCl: } Pb^{2+}_{(aq)} + 2Cl^{-}_{(aq)} \rightarrow PbCl_{2(s)}$$

$$\text{With Addition of NaOH: } Pb^{2+}_{(aq)} + 2OH^{-}_{(aq)} \rightarrow Pb(OH)_{2(s)}$$
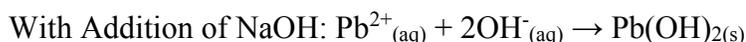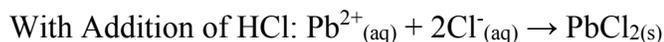
In this test, students will test a lead II salt solution (for a positive control) and water (for a negative control) by the addition of hydrochloric acid or sodium hydroxide with the following anticipated results.

	Addition of HCl	**Addition of NaOH**	**Conclusion Based on +/- Control Results**
Lead II Salt	Precipitate (solid) forms	Precipitate (solid) forms	If a precipitate forms with either HCl and NaOH, then lead II is present
Water	No precipitate forms	No precipitate forms	If a precipitate does not form with either HCl or NaOH, then lead II is not present

Students will then test the glass samples in their evidence kit to analyze for the presence of lead.

Materials:
1. 1.0 M Hydrochloric Acid Solution
    a. Preparation of 1 L of 1.0 M hydrochloric acid solution from 12 M stock solution: Dilute 8.3 mL of 12 M reagent grade hydrochloric acid in water to create 1 L of total solution.
            OR
   1.0 M Sodium Hydroxide Solution
    b. Preparation of 1 L of 1.0 M sodium hydroxide solution. Dissolve 40.0 grams of sodium hydroxide in water to create 1 L of total solution.
2. 0.1 M Lead Salt Solution (suggested: lead II acetate)

a. Preparation of 0.5 L of 0.1 M lead II acetate solution: Dissolve 19.0 grams of reagent grade lead II acetate ($Pb(C_2H_3O_2)_2 \cdot 3H_2O$) in water to create 0.5 L of total solution.
3. Water
4. Glass Samples from Evidence Kit
5. Droppers
6. Glass Test Tubes or Well Plate

Safety:
Hydrochloric acid is highly toxic by ingestion or inhalation and is severely corrosive to skin and eyes. Hazards for salts vary depending on the salt chosen. Sodium hydroxide is a corrosive solid and can cause skin burns. Heat evolves when adding sodium hydroxide to water. Sodium hydroxide is very dangerous to eyes and skin and eye protection and gloves should be used when handling it. Lead II acetate is a probable carcinogen and an eye, skin, and respiratory irritant. Lead II acetate is slightly toxic.

Procedure for Positive Control (Presence of Lead):
1. Place several drops of the lead II salt solution in a test tube or in one of the wells of a well plate. Add a dropperful of the hydrochloric acid or sodium hydroxide solution. Record your observations.

Procedure for Negative Control (Absence of Lead):
1. Place several drops of water in a test tube or in one of the wells in a well plate. Add a dropperful of the hydrochloric acid or sodium hydroxide solution. Record your observations.

Procedure for Unknowns:
1. Place several drops of an unknown in a test tube or in one of the wells in a well plate. Add a dropperful of the hydrochloric acid or sodium hydroxide solution. Record your observations.

Waste Disposal:
Mixed chemicals may be collected together in waste container that is labeled with its contents. Unused solutions may be stored until later use.

Test #2: Paint Chromatography

Background:
Chromatography is a method for analyzing mixtures by separating them into their component chemicals. Even though black paint only appears as one color, the paint is actually made from a mixture of different colored pigments. To perform paint chromatography, a small dot of paint is placed at one end of a strip of filter paper. This end of the paper strip is then placed in a solvent. As the solvent travels upward on the paper strip, the mixture of chemicals that compose the paint are dissolved and pulled upward on the paper. The chemicals that dissolve best in the solvent will move further up the paper strip than chemicals that do not dissolve as well. Smaller molecules will also move up further than larger molecules.

In this test, students will separate the pigments of paints for comparison.

Materials:
1. Paint Samples from Evidence Kit
2. Acetone (can substitute isopropanol, methanol, ethanol, or similar)
3. Beaker or Cups

Safety:
Acetone is a flammable liquid and thus a fire hazard. Isopropyl alcohol is slightly toxic by ingestion and inhalation.

Procedure:
1. Place about 10 mL of acetone in the bottom of a beaker or cup.
2. Hold one of the strips containing a paint sample in the acetone using care to not dip the strip below the dot.
3. Allow the acetone to run up the filter paper, separating the pigments as it travels.
4. Remove the strip from the acetone when it has reached the top and allow it to dry.
5. Repeat with each of the remaining samples
6. Compare each of the samples to determine which paint samples match.

Waste Disposal:
Excess used acetone should be placed in flammable organic waste. Unused acetone may be stored until later use. Used filter paper may be thrown away.

Test #4: Iodine Fuming Fingerprint Developing of Ninhydrin Fingerprint Developing

Background:

A fingerprint is an impression or mark made on a surface by a person's fingertip and can be used for identifying individuals from its unique pattern. Fingerprinting is an important aspect of crime scene investigation and can be used forensically to identify individuals who were present.

There are three basic types of fingerprints: 1) plastic, 2) patent, and 3) latent. Plastic prints are prints made by impressions on a soft material, such as wax or clay. Patent prints are impressions made on colored materials or with colored materials that are on the fingertips. For example, a patent print might be one left in blood or one that is deposited because of blood on a fingertip. Both plastic and patent prints are visible without further processing. Latent prints are prints made by deposition of palmar sweat and natural oils to a surface. Latent prints require processing for visualization.

Latent prints on non-absorbent surface can be developed with powder, while the prints on soft or porous surfaces require other processing techniques. One technique used for fingerprint visualization is iodine fuming. Iodine can be heated to produce a violet vapor that is then absorbed by a fingerprint secretion to produce a yellow/brown pattern on the surface where the secretions were deposited. Such visualization is short lived and needs to be chemically fixed by mixing with starch. Another technique is the use of a ninhydrin staining solution which turns purple upon heating in the presence of amino acids which are in palmar sweat.

In this test, students will develop fingerprints on a piece of debris from their evidence kit using one of two methods and will compare to the fingerprint samples of the suspects. While both methods work, the ninhydrin method generally results in more clearly defined prints.

Method #1: Iodine Fuming Fingerprint Developing

Materials:

1. Gentle Heat Source (oil candle, Bunsen burner, or similar)
2. Large Glass Test Tube (25 mm x 150 mm)
3. Rubber or Cork Test Tube Stopper
4. Test Tube Clamp
5. Forceps, Scoopula, or Spoon
6. Glass Rod
7. Iodine Crystals
8. 1% Starch Solution

     a. Preparation: Heat 25 mL of water to boiling. Add 1 gram of soluble starch to the hot water and stir. Dilute to 100 mL. Allow the starch solution to cool. The starch solution will not keep more than a few days. Add a small amount of salicylic acid to preserve the solution for a longer period of time.
9. Piece of Paper with Fingerprint from Evidence Kit

Safety:
Iodine is toxic by ingestion or inhalation. Iodine reacts violently with reducing materials, sulfur, iron, alkali metals, metal powders, and phosphorus. Iodine irritates the skin and is corrosive to eyes and respiratory tract.

Procedure:
1. Using forceps transfer a single iodine crystal to a glass test tube.
2. Lightly stopper the test tube so the iodine vapor does not escape.
3. Place the test tube over a gentle heat source and allow the iodine crystal to sublimate.
4. Place the fingerprinted material in the test tube and allow the fingerprint to absorb the iodine.
     a. Note: At this point the fingerprint should have a yellowish color and should be visible.
5. Permanently fix the fingerprint by dropping the starch solution on the fingerprint.
     a. Note: Fixing is required to keep the deposited iodine from evaporating and the fingerprint disappearing.
     b. Note: At this point the fingerprint should have a dark purple/black color.
6. Allow fixed fingerprint to dry.
7. Compare the fingerprint to the suspects' fingerprints for identification.

Waste Disposal:
Developed fingerprints may be thrown away. Unused iodine and starch may be stored until later use. If starch solution is stored it must be preserved.

Method #2: Ninhydrin Fingerprint Developing
Materials:
1. Fingerprint Staining Solution (Ninhydrin, Ethanol (acetone may be substituted), and Acetic Acid
     a. Preparation: Dissolve 0.5 grams of ninhydrin in 125 mL of ethanol. Add 0.1 mL acetic acid to the solution.
     b. Note: Fresh preparation of solution is best for fingerprint developing.
2. Lysine Solution (for positive test)
     a. Preparation: Dissolve 0.25 grams of lysine in 50 mL of water and 50 mL of ethanol.

3. 1:1 Water:Ethanol Mixture (for negative test)
    a. Preparation: Mix 50 mL of water and 50 mL of ethanol.
4. Droppers or Spray Bottles
5. Paper
6. Heat Gun or Blow Dryer
7. Piece of Debris with Fingerprint from Evidence Kit

Safety:
Ninhydrin is a body tissue irritant. Ethanol is a flammable liquid and thus a fire hazard. Ethanol is slightly toxic by ingestion and inhalation. Acetic acid is corrosive to skin and tissue and is moderately toxic by ingestion and inhalation

Procedure for Positive and Negative Controls:
1. On a piece of paper place a drop of the lysine solution on one side and a drop of ethanol on the other. Briefly allow to dry.
2. Drop or spray the fingerprint staining solution on the lysine solution and the 1:1 water:ethanol on the paper. Briefly allow to dry.
3. Use a heat gun or a blow dryer to apply heat to the paper.
    a. Note: A purple color indicating a positive test will develop where the lysine was dropped, and will not indicating a negative test where the ethanol was dropped.

Procedure for Unknown:
1. Drop or spray the fingerprint staining solution on the piece of paper with the fingerprint, essentially covering it. Briefly allow to dry. Use a heat gun or blow dryer to apply heat the paper.
    a. Note: If a fingerprint is present it should develop in the same purple color as observed in the positive test with lysine above.

Waste Disposal:
Paper may be thrown away. Unused chemicals may be stored until later use.

References:
Scientific Working Group on Friction Ridge Analysis, Study, and Technology.

http://www.swgfast.org/ (accessed July 2013).

Test #4: Pesticide Test

Background:

Nanoparticles are defined as particles with at least one dimension of 1 to 100 nanometers. The size of these particles is smaller than the wavelength of light, which gives rise to interesting optical properties in the bulk materials. One example of this is that nanoparticle solutions are often colored, and the color depends on the nanoparticle size. Due to this property, when nanoparticles aggregate, or begin to stick together, the color of the solution changes. Nanoparticle aggregation can be initiated by changes in surface charge, pH, or salinity of solutions. In addition, sulfur containing molecules bind to noble metal nanoparticles and can cause aggregation. Scientists have taken advantage of these and other properties of nanoparticles to create various nanoparticle-based sensors, including sensors for pesticide detection.

In this test, students will synthesize silver nanoparticles they can then use to test for the presence of a 'pesticide'. The various powder samples can be added to the nanoparticle solutions and monitored for the color changes indicative of aggregation.

Materials:

1. Silver Nanoparticles
   a. Preparation (for 1 test): Rinse all glassware 3-4 times with distilled water. Measure 13 mL of distilled water in a graduated cylinder and place in an Erlenmeyer flask. On a hot plate, heat the water to a boil. While heating, constantly stir. Add 1 mL of 7.5 mM silver nitrate (dissolve 0.13 grams of silver nitrate, $AgNO_3$ in 100 mL of water). Add 1 mL of 7.5 mM sodium citrate (dissolve 0.22 grams of lab grade sodium citrate, $Na_3C_6H_5O_7 \cdot 2H_2O$, in 100 mL of water). Continue stirring the solution. Add 2 drops of 10 mM sodium borohydride (dissolve 0.38 grams of sodium borohydride, $NaBH_4$, in 100 mL of water). At this point, the solution should be yellow in color, indicating the formation of silver nanoparticles. Continue stirring the solution for 3-5 minutes. Remove from heat and use immediately for pesticide test.
   b. Note: If the solution begins turning green or grey after addition of the 10 mM sodium borohydride before 3-5 minutes remove from heat immediately.
   c. Note: Even if solution is not yellow, the aggregation assay should still work.

     d. Note: Silver nitrate is sensitive to light and must be stored in a dark bottle or in aluminum foil.

     e. Note: Sodium borohydride must be made fresh for nanoparticle preparation.

     f. Note: Prepare extra nanoparticles prior to class in case students have trouble.

2. Sodium Nitrate (label as pesticide; for positive control)
3. Splenda (label as non-pesticide powder; for negative control)
4. Powder Samples from Evidence Kit
5. Test Tubes or Well Plate
6. Dropper
7. Scoopula

Procedure for Positive Control:

1. Place 1 dropperful of silver nanoparticles in a test tube or a well plate.
2. Add a small amount of sodium nitrate.
3. Observe the solution and record observations.
     a. Note: A change in color from yellow to a dark grey or black should be observed indicating a positive test.

Procedure for Negative Control:

1. Place 1 dropperful of silver nanoparticles in a test tube or a well plate.
2. Add a small amount of Splenda.
3. Observe the solution and record observations.
     a. Note: No color change will occur, indicating a negative result.

Procedure for Unknowns:

1. Place 1 dropperful of silver nanoparticles in a test tube or a well plate.
2. Add a small amount of unknown powder.
3. Observe the solution and record observations.

Safety:

Silver nitrate is a corrosive solid and causes burns. Silver nitrate will stain skin and clothing. Protective eyewear, gloves, and clothing should be worn when handling silver nitrate. Sodium borohydride is a corrosive solid and causes burns. Sodium borohydride is toxic by ingestion and is irritating to the skin and eyes.

Waste Disposal:

Mixed chemicals may be collected together in waste container that is labeled with its contents. Excess sodium borohydride may be placed in basic waste. Unused solutions may be stored until later use.

References:

McFarland, A. D., Haynes, C. L., Mirkin, C. A., Van Duyne, R. P., and Godwin, H. A. Color My Nanoworld. *Journal of Chemical Education* **2004**, *81*, 544A.

Maurer-Jones, M.; Love, S.; Meierhofer, S.; Marquis, B.; Liu, Z.; Haynes, C. Toxicity of Nanoparticles to Brine Shrimp: An Introduction to Nanotoxicity and Interdisciplinary Science. *Journal of Chemical Education* **2013**, *90*, 475-478.

Test #5: Starch Test

Background:

A starch is a polysaccharide (a long carbohydrate molecule) consisting of a large number of glucose units joined by glycosidic bonds. Iodine solutions are often used to test for the presence of starch. In the presence of starch, iodine solutions have a dark blue color because the iodine and the starch form an intensely colored chemical complex.

In this test, students use an iodine-potassium iodide solution to test for the presence of starch. When dropped onto a sample containing starch, the yellow-brown IKI solution changes to dark blue.

Materials:

1. Iodine Potassium Iodide (IKI) Solution
    a. Preparation: Dissolve 5 grams potassium iodide in 50 mL distilled water. Add 2.5 grams iodine to this solution. Store in an amber glass bottle or protect from light.
    b. Note: Fresh preparation is best.
2. Corn Starch (label as starch; for positive test)
3. Salt or Sugar (label as non-starch powder; for negative test)
4. Powder Samples from Evidence Kit

Procedure for Positive Control:

1. Place 1 dropperful of IKI in a test tube or a well plate.
2. Add a small amount of corn starch.
3. Observe the solution and record observations.
    a. Note: A change in color should be observed, indicating a positive test.

Procedure for Negative Control:

1. Place 1 dropperful of IKI in a test tube or a well plate.
2. Add a small amount of salt or sugar.

3. Observe the solution and record observations.
    a. Note: A change in color should be observed indicating a positive test.

Procedure for Unknowns:

1. Place 1 dropperful of IKI in a test tube or a well plate.
2. Add a small amount of unknown powder.
3. Observe the solution and record observations.

Safety:

IKI stains clothes and hands, so use caution when performing this assay.

Waste Disposal:

Mixed chemicals may be collected together in waste container that is labeled with its contents. Unused solutions may be stored until later use.

A2.2. Student Materials

A2.2.1. Script for Investigation of a Jewel Heist

Name: _____Class: _____

Investigation of a Jewel Heist

A break-in occurred at the Science Museum of Minnesota where the crown jewels were being displayed. The alarm system was deactivated, the security camera was painted over, the glass case holding the jewels was smashed, and the jewels were taken. Samples have been collected from the crime scene. A police detective has interviewed the suspects and has gathered evidence from them.

When you arrive to the scene, you are briefed by the police detective with the following information:

Police Detective: "We have narrowed our search to several suspects who each have motive for the break in: a security guard, a former executive, and a former art thief."

"I interviewed each of the suspects. The interviews have been transcribed for you to read over. We collected a fingerprint from the crime scene, fingerprint samples from the suspects, glass from the broken display case, broken glass from one of the suspect's car, powder from the crime scene, powder from two of the suspects' clothing, spray paint from the security camera, and paint from two of the suspects. I really hope that, with this evidence, you can help us to solve this crime."

Interview with the Security Guard

Investigator: Hello. I heard you were working during the time of the heist.
Security Guard: Yes. What do you want? I don't have much time, I've got another job I have to get to.
Investigator: How do you explain that during the heist you didn't hear or see a thing? Weren't you doing your rounds?
Security Guard: I had checked the jewel room earlier during the night. We only check each room once at the beginning of the night and then stay in the security room the rest of the night.
Investigator: Wouldn't you still have noticed when the thief painted over the cameras?
Security Guard: I've had this bug the last few days and haven't been getting much sleep. Nothing ever happens around here at night, so I kinda fell asleep. I overslept past my work time and had to hurry out. As I said, I've got another job. Speaking of which, I really need to get going.

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Investigator: Just a few more questions before you leave. How do you explain the alarms not going off when the jewel case was smashed?

Security Guard: I don't know. Someone must have turned them off. The guards aren't the only ones with access to the alarm system. All the executives have access, too.

Investigator: When we were looking through your things, we found a bag containing a set of jewels. How do you explain these being found in your possession?

Security Guard: They must have been planted there! Check if they're even real. It must have been that fired executive. He holds a grudge against me. I was the one who caught him giving private tours. That's what got him fired.

Investigator: Okay. Let's not throw too many accusations around here. We're checking all the leads. Last line of investigation - we found white powder both at the scene of the crime and on your security jacket. How do you explain this?

Security Guard: I told you! I've got another job. It's at a bakery, I work with flour there all the time.

Investigator: Alright. Thanks for your cooperation. We may contact you with more questions.

<div align="center">

Interview with the Former Executive

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Investigator: Hello sir. I understand you recently ended your employment at the Science Museum.

Former Executive: Yes, I did. To what does this regard?

Investigator: There has been a theft recently at the museum, and we need to ask you a few questions.

Former Executive: I guess I have time for a couple of questions, but I don't know how much help I will be. As you can see, I have traded the life of a powerful Science Museum executive to work on this farm and become at peace with myself and the land.

Investigator: We have reports that your employment at the Science Museum ended badly and that you were giving private tours. Care to elaborate on this?

Former Executive: No. But I will say that there is a very sneaky security guard who still works there. He often looked tired. Maybe he was up late planning something.

Investigator: Leave the investigative work to us, please. We've heard that you coordinated the loan and the security for the Crown Jewels before you were fired. We'll need to take a look around at your vehicle and your clothes from last night. Where were you last night, by the way?

Former Executive: I was here, working on the farm. Here is my jacket. The rest of my clothes are in the wash.

Investigator: We'll test this powder residue to see if it matches anything from the crime scene... Is this your vehicle?

Former Executive: Yes, but I share it with several other people who work here.

Investigator: This vehicle looks pretty clean... but we're going to take these pieces of glass out of the backseat. The glass case with the jewels at the Science Museum was shattered.

Former Executive: That glass could be from anything. Like I said, several people use this vehicle.

Investigator: Well, we'll go ahead and test it. The glass in that display case was a specialized material that contained lead and silver.

Former Executive: There's a lot of things on this farm that could have lead or silver in them. I wouldn't read too much into your test results.

Investigator: Uh huh.

Former Executive: I hear that that notorious art thief is living in Minneapolis. Instead of hassling hard working farmers like me, you should look into the activities of somebody with a history of heists and attempted heists.

Interview with the Art Thief

Investigator: Hello. I understand that you have a past as an art thief, but you claim that in recent years you have mended your ways. The Crown Jewels were stolen last night though, and you are Minneapolis' most notorious art thief. Perhaps this would have been too tempting an opportunity to pass up?

Art Thief: Hello investigator. I have already completed my sentence for allegedly stealing artworks. I now am committed to my new career as an artist for the public.

Investigator: Ah, but the evidence against you is compelling. Just confess, and the prosecutor will give you an easy sentence.

Art Thief: I will never confess! Any evidence you have is planted. I hear that there is rampant corruption in the ranks of the investigators.

Investigator: You have paint all over your hands. Paint that will no doubt prove to be the same paint as that spray painted over the security cameras at the Science Museum.

Art Thief: There is no way that I could have committed this heist. I was painting a mural for public enjoyment on the Greenway last night.

Investigator: Well, we will test the paint on your hands and see if you are telling the truth. Don't leave town for the next few days. We may have more questions for you.

A2.2.2. Student Worksheets with Anticipated Answers

Name: _____ Class: _____

Identify the suspects and their possible motives.

Suspect	Possible Motive
Security Guard	Answers may vary.
Former Executive	Answers may vary.
Former Art Thief	Answers may vary.

Question: Make your hypothesis... Who do you think stole the Jewels?
Answers will vary.

Question: Why do you think it was the suspect(s) you identified?
Answers will vary.

Question: How will you determine if you are correct?

Answers will vary.

Test #1: Detection of Lead in Solution using an Acid or a Base
Notes about the experiment:

Question: What evidence will you test? Why?
The glass samples will be tested because this test will determine if they contain lead.

Complete the following chart:

Solution	Observation After Addition of HCl	Observation After Addition of NaOH	Conclusion
Solution Containing Lead II Ions	Precipitate (solid) forms	Precipitate (solid) forms	If a solution contains lead then... a precipitate will form with the addition of HCl or NaOH.
Water	No precipitate forms	No precipitate forms	If a solution does not contain lead then... no precipitates will form.

Data Collection: Create a chart to document your observations of the evidence you decide to test.

Evidence	Observation After Addition of HCl	Observation After Addition of NaOH
Glass from Crime Scene	Precipitate (solid) forms	Precipitate (solid) forms
Glass from Former Executive's Car	Precipitate (solid) forms	Precipitate (solid) forms

Question: What conclusions can you draw based on your observations from this test? The glass found in the former executive's car matches the glass from the crime scene.

Test #2: Paint Chromatography:

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Notes about the experiment:

Question: What evidence will you test? Why?
The paint samples will be tested because this test will allow for comparison of the paints.

Complete the following chart with your observations:

Paint Sample from: the security camera	Paint Sample from: art thief's hands	Paint Sample from: mural
Varies depending on paint used.	Varies depending on paint used.	Varies depending on paint used.

Question: Why do the colors separate on the filter paper?
The paints are made of different pigments (colors) which separate on the filter paper based on their size and attraction to the solvent.

Question: What conclusions can you draw based on your observations from this test?

The paint on the art thief matches the paint from the mural and does not match the paint from the crime scene.

Test #3: Fingerprint Developing

Notes about the experiment:

Question: What evidence will you test? Why?
The piece of debris will be tested because this test can develop any fingerprints left behind.

Data Collection: Draw a sketch of the fingerprint or glue/tape in your developed fingerprint.
This will vary based on the fingerprints used for analysis.

Question: What distinguishing patterns do you see on the fingerprint?
This will vary based on the fingerprints used for analysis.

Question: Which of the suspects do you think the fingerprint belongs to?
The fingerprint does not belong to any of the suspects.

Question: What conclusions can you draw based on your observations from this test?

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This piece of evidence is not useful in solving the crime.

Test #4: Pesticide Test

Notes about the experiment:

Question: What evidence will you test? Why?
The powder samples will be tested because this test can determine if they are a pesticide.

Complete the following chart:

Sample	Observations	Conclusions
With Pesticide	The solution turns dark grey/black	If a pesticide is added to a solution containing silver nanoparticles then... the solution will turn dark grey/black.
Without Pesticide	The solution does not change	If something other than a pesticide is added to a solution containing silver nanoparticles then... the solution will not change.

Data Collection: Create a chart to document your observations of the evidence you decide to test.

Sample	Observations
Powder from Crime Scene	The solution turns dark grey/black
Powder from Former Executive	The solution turns dark grey/black
Powder from Security Guard	The solution does not change

Question: Why does the color of the solution change in the presence of a pesticide?
The pesticide causes the silver nanoparticles to aggregate (group together).

Question: What conclusions can you draw based on your observations from this test? The powder from the crime scene matches the powder from the former executive.

Test #5: Starch Test

Notes about the experiment:

Question: What evidence will you test? Why?
The powder samples will be tested because this test can determine if they are a starch.

Complete the following chart:

Sample	Observations	Conclusions
With Starch	The solution turns blue	If a starch is added to a solution containing iodine then... the solution will turn blue.
Without Starch	The solution does not change	If a something other than a starch is added to a solution containing iodine then... the solution will not change.

Data Collection: Create a chart to document your observations of the evidence you decide to test.

Sample	Observations
Powder from Crime Scene	The solution does not change
Powder from Former Executive	The solution does not change
Powder from Security Guard	The solution turns blue

Question: Why does the color of the solution change in the presence of a pesticide?
Starch forms a deep blue complex in the presence of iodide.

Question: What conclusions can you draw based on your observations from this test?
The powder from the crime scene does not match the powder from the security guard.

After completing each test, check the boxes where the suspect's sample matches the evidence found at the crime scene.

Test	Security Guard	Former Executive	Former Art Thief
#1: Detection of Lead in Solution		x	
#2: Paint Chromatography			
#3: Fingerprint Developing			
#4: Pesticide Test		x	
#5: Starch Test			

Now that you have tested all of the evidence with the available tests, who do you believe is responsible for stealing the Crown Jewels. Explain based on your observations obtained during each test why you believe this to be true and why you can rule out the other suspects. Was your hypothesis of who stole the jewels correct?

The former executive is responsible for the jewel heist... (Students should identify the former executive as the suspect responsible for stealing the jewels. Students should support this with evidence from each test.)

A3. Investigation of a Solar Cell Sabotage

A3.1. Teacher Guide

A3.1.1. Introduction

Recently public interest in forensic science has increased greatly, due in part to television shows such as *CSI* and *Bones*, which feature scientists using analytical tools to solve crimes. The increased public interest in forensic science presents an opportunity for science educators to engage students by leveraging student interest in the applied nature of forensic chemistry while practically introducing students to the scientific method. In this investigation students are presented with information about a crime scene and are asked to solve the crime using evidence gathered from the scene, evidence collected from the identified suspects, and a series of simple forensics-type tests. Through this investigation students will become more familiar with making hypotheses, making observations, analyzing data, interpreting positive and negative results, and drawing conclusions.

In addition to giving students the background information relevant to the investigation, students will receive information about conducting each of the tests in the investigation before they are given the evidence kit. Each test is explained and its procedure detailed in the teacher guide section. You may consider demonstrating each of the tests to students in addition to explaining their purpose and relevant background information. There is a place in the student handout for students to take notes on each of the tests.

This investigation requires the preparation of an evidence kit for each group of students as well as preparation of materials for each of the tests. Preparation of the evidence kit and the tests are fully described in the teacher guide section. Amounts prepared may be adjusted to larger or smaller quantities depending on the number of students. While preparation may be initially intensive, most chemicals and solutions can be stored for later use, which greatly reduces preparation when repeating the activity. This investigation will likely take 3-4 one hour class periods, but may be extended or shortened if desired. One day will be required to introduce the investigation and the tests and 2-3 days will be required for students to complete the tests and draw final conclusions.

This investigation is centered around a fictitious break in at a solar cell company, Solar Haynes Industries (alternative company names can obviously be used). During the break-in, newly developed solar cells were destroyed in an attempt to sabotage the company. The

complete story and dialogues are given in the student handout section. You may consider having students read the story and dialogues to preface the investigation or having students act out the story and dialogues in class. You may also consider creating a video with colleagues or students to present before the investigation begins based on the story and dialogues provided.

Tasks and Suggested Timeline for this Activity
Read teacher packet: 4 weeks prior to activity
Film dialog (optional): 3 weeks prior to activity
Prepare evidence kits: 2 weeks prior to activity
Prepare test stations: 1 week prior to activity
Demonstrate tests: day of activity

A3.1.2. The Evidence Kit and its Preparation

Clearly label all items with the bold text below. Do not include the non-bold text in the label. Place all pieces of evidence in a box to give to each group of students. You may consider including a slip of paper that lists the evidence available for testing in the box.

1. **Piece of Plastic Left at Crime Scene**
   a. Preparation: Obtain a piece (approx.. 2 cm x 2 cm) of polyethylene terephthalate plastic (PET or PETE, SPI Code #1, i.e. a soda bottle)

2. **Piece of Plastic from the Workspace of the Solar Haynes Employee, Murphy Sun Products Vice President, and Local Housing Developer**
   a. Preparation for Sample from Solar Haynes Employee: Obtain a piece of solid (not foam) polystyrene plastic (PS, SPI Code #6)
   b. Preparation for Sample from Murphy Sun Products Vice President: Obtain a piece of polyethylene terephthalate plastic (PET or PETE, SPI Code #1)
   c. Preparation for Sample from Local Housing Developer: Obtain a piece of polypropylene plastic (PP, SPI Code #5, such as a yogurt container or other food storage container)
   d. Preparation for Sample from Dino Coal Executive: No sample needed.
   e. Note: Many consumer products are made from various types of plastic. The Society of the Plastics Industry (SPI) established a classification system to allow consumers and recyclers to properly recycle and dispose of different types of plastic. Manufacturers follow a coding system and place an SPI code, or number, on each plastic product, which is usually molded into the bottom. The SPI codes are listed above to make finding the various types of plastic easier.

3. **Handwritten Note Found at Crime Scene**
   a. Preparation: Using a Vis-a-Vis wet erase marker, a Sharpie permanent marker, a Expo dry erase marker, or a Paper Mate felt-tip pen, write a threatening note along the bottom of a strip of filter paper or a strip of a coffee filter.

4. **Pen Belonging to Each Suspect**
   a. Preparation: Assign a Vis-a-Vis wet erase marker, a Sharpie permanent marker, an Expo dry erase marker, or a Paper Mate felt-tip pen to each of the above suspects, so that each suspect is assigned a different pen.
   b. Note: The marker or pen used for the handwritten note above should match the pen assigned to the Solar Haynes Employee.

5. **Sample Containing DNA from Cup at Crime Scene**
   a. Preparation: Create a 1% w/w salt water solution by dissolving 1 gram of sodium chloride in 100 mL of water. Vigorously swish 30 mL of the 1% salt water solution in your mouth for 30 seconds without swallowing, making sure to rub your tongue along your cheeks. Carefully spit the salt water/cheek cell mixture into a container.
   b. Note: Students will use the above sample to extract DNA. They will need to be given the "processed" DNA results below after extraction.
   c. Note: for younger students, instructors may want to use the "Shorter DNA Sequences" given below. If that is done, use the shorter DNA sequences here for the evidence kit.

The DNA you extracted was processed and two unique sequences of DNA were isolated.

Sequence 1:

TAAGAAAGTGGAGGGAGACATGTATGAG

Sequence 2:

AGCCTGTGGATGCCGTCAAGCTGAACCTCCCTGATTACTAT

Shorter DNA Sequences:

The DNA you extracted was processed and two unique sequences of DNA were isolated.

Sequence 1:

GCAGCTC

Sequence 2:

CCTCCAA

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6. **DNA Sequence Already Processed from Each Suspect**
   a. Preparation: Print the following.
   b. Note: For younger students, instructors may want to modify the sequences to be shorter. If this is done, the shorter sets of sequences below and in Evidence 3 'Sample Containing DNA from Cup at Crime Scene' should be used. Shorter DNA sequences will also need to be substituted into the Test #2 Instructions (p. 9) and Test #2 Student Handout (p.22).

DNA samples were collected and processed for each of the suspects. The following are each suspects' unique DNA sequences.

DNA Sequence from Solar Haynes Employee:

TCCAGACCCTGCAGCTCTGAAAGATCGCCGCATGGAGAACCTGGTTGCCTATGCTAAGAAAGTGGAGGG
AGACATGTATGAGTCTGCTAATAGCAGGGATGAATACTATCATTTATTAGCAGAGAAAATCTATAAAAT

DNA Sequence from Murphy Sun Products Vice President:

TACTTCCAATCCATGAACCCCCCGCCCCCAGAGACCTCCAACCCTAACAAGCCCAAGAGGCAGACCAAC
CAACTGCAATACCTGCTCAGAGTGGTGCTCAAGACACTATGGAAACACCAGTTTGCATGGCCTTTCCAGC
AGCCTGTGGATGCCGTCAAGCTGAACCTCCCTGATTACTATAAG

DNA Sequence from Local Housing Developer:

CTTTTATTTTATATTATAACCACTACGAGTACACGGGCGAGAAGGCATTAAAGTATAGTAACATAATATA
AAATATACTATGGTATTTATACAAAAAATTTTGTCATAACAAATATTTGTTCGGTATAAA

DNA Sequence from Dino Coal Executive:

TCACAAGTTTTAGTTTTTTGTGCGCGCAACTTCTCAGAAATTTTGTTTATAAAATGCTTCTTTTTGCCGATT
TGTCATTCCACGGAAATTTGTTTGTTCAGTGCAGCCAGCTTTTACGCGGAGAAAATAAATTTTA

Shorter DNA Sequences:

DNA samples were collected and processed for each of the suspects. The following are each suspects' unique DNA sequences.

DNA Sequence from Solar Haynes Employee:

TCCAGACCCTGCAGCTCTGAAAGATCGCCGCAT

DNA Sequence from Murphy Sun Products Vice President:

TACTTCCAATCCATGAACCCCCCGCCCCCAGAGACCTCCAAC

DNA Sequence from Local Housing Developer:

CTTTTATTTTATATTATAACCACTACGAGTACA

DNA Sequence from Dino Coal Executive:

TCACAAGTTTTAGTTTTTTGTGCGCGCAACTTCTCAGAAATTTT

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7. **Metallic Dust from Crime Scene** (in solution and contains 0.1 M copper II ions)
   a. Preparation of 0.5 L of solution: Dissolve 8.5 grams of reagent grade copper II chloride ($CuCl_2 \cdot 2H_2O$) in water to create 0.5 L of total solution.

8. **Metallic Dust from the the Workspace/Clothing of the Solar Haynes Employee, Murphy Sun Products Vice President, and Local Housing Developer** (in solution)
   a. Preparation for Sample from Solar Haynes Employee (contains 0.1 M copper II ions): Use solution from above.
   b. Preparation for Sample from Murphy Sun Products Vice President (two metals were isolated thus two solutions will be provided: one containing 0.1 M copper II ions and one containing 0.1 M calcium ions): For copper II solution, use solution from above. For the calcium solution dissolve 7.4 grams of reagent grade calcium chloride ($CaCl_2 \cdot 2H_2O$) in water to create 0.5 L of total solution.
   c. Preparation for Sample from Local Housing Developer: Obtain a sample of distilled water.
   d. Preparation for Sample from Dino Coal Executive: Obtain a sample of distilled water.

9. **Fingerprint Samples from Each Suspect**
   a. Preparation: Obtain ink fingerprint samples from 5 people. Assign fingerprint samples to each of the suspects.

10. **Piece of Paper with Fingerprint from Crime Scene**
    a. Preparation: Have one of the people from above press their fingerprint onto the edges of a piece of a piece of paper.
    b. Note: The fingerprint on the map should match the Solar Haynes employee's fingerprint sample.
    c. Note: Sweaty fingerprints are best for analysis. If possible, wrap the hand you are obtaining prints from in plastic wrap or wear a plastic glove for several minutes to produce extra sweat before printing. Fresh preparation of fingerprints is best. Protect fingerprints by inserting the paper in a plastic sheet.

11. **Trace Scent Found at Crime Scene** (in solution and contains 5% w/w glucose)
    a. Preparation: Dissolve 5 g of glucose in 95 mL of water.
    b. Note: Fresh preparation is best.

12. **Cologne or Perfume from each Suspect** (in solution)

    a. Preparation for Sample from Solar Haynes Employee: Obtain a sample of distilled water.
    b. Preparation for Sample from Murphy Sun Products Vice President (5% w/w glucose solution): Use solution from above (step #11).
    c. Preparation for Sample from Local Housing Developer (5% w/w glucose solution): Use solution from above.
    d. Preparation for Sample from Dino Coal Executive: Obtain a sample of distilled water.

A3.1.3. The Tests and Their Procedures

Table A3. Summary of Tests for Investigation of a Solar Cell Sabotage.

Test #	Test	Evidence to Test	Testing For
#1	Density Comparison	Plastic Samples	Densities of Plastics for Identification of Unknown Sample
#2	DNA Extraction	DNA	Extraction of DNA for Identification of Unknown Sample
#3	Flame Test	Metallic Dust Samples	Identification of Metal Ions in Solution
#4	Ink Chromatography	Handwritten Note and Pens from Suspects	Identification of the Pen that was used to Write the Notes
#5	Iodine Fingerprint Developing or Ninhydrin Fingerprint Developing	Fingerprint on Piece of Paper	Identification of Fingerprints on the Piece of Paper
#6	Silver Plating Test	Scent Samples	Presence of an Aldehyde

Test #1: Density Comparison

Background:

Density is a substance's mass per unit volume. Density can be used for identification purposes by comparing to known densities.

In this test, students will test the density of the pieces of plastic in the evidence kit relative to water and glycerol. If the plastic is more dense than the liquid it is placed in, it will sink. If the plastic is less dense than the liquid it is placed in it will float. Students will compare the densities of the pieces of plastic to determine who the piece of plastic left at the crime scene belongs to. Below are the anticipated results.

Sample	Water, D = 1.00 g/mL	Glycerin, D = 1.26 g/mL
Solar Haynes Employee Polystyrene, D = 1.05 g/mL	Sinks (More Dense)	Floats (Less Dense)
Murphy Sun Products VP Polyethylene terephthalate, D = 1.31 g/mL	Sinks (More Dense)	Sinks (More Dense)
Local Housing Developer Polypropylene, D = 0.86 g/mL	Floats (Less Dense)	Floats (Less Dense)
Unknown Sample	Sinks (More Dense)	Sinks (More Dense)

Materials:
1. Water
2. Glycerin
3. Beakers or Cups
4. Glass Stir Rod
5. Pieces of Plastic from Evidence Kit

Safety:
Some people are allergic to glycerin and may experience irritation to their skin and eyes.

Procedure:
1. Place water in a beaker.
2. Place a piece of plastic in the beaker. Poke the plastic beneath the surface of the water with the glass stir rod to break the surface tension. Record your observations.

3. Repeat steps 1-2 for each piece of plastic.
4. Repeat steps 1-3 with glycerin in place of water.
5. Compare the densities of the pieces of plastic for identification of the unknown piece of plastic.

Waste Disposal:
Glycerin can be placed in a container, labeled as used, and stored for repeating this activity.

Test #2: DNA Extraction

Background:

DNA, or deoxyribonucleic acid, is the hereditary material in humans. The information in DNA is stored as a code made up of four chemical bases: adenine (A), guanine (G), cytosine (C), and thymine (T). The order, or sequence, of these bases determines the information available for building and maintaining an organism. Each person's DNA is unique, thus it can be used for identification purposes.

In this test, students will extract DNA from a sample in the evidence kit. Students will then be given a "processed" DNA report and will use this to compare to the suspects' DNA sequences. The report shows that two DNA sequences were found in the sample. Students should match these sequences to the Solar Haynes Employee and the Murphy Sun Products Vice President.

Sequence 1:

TAAGAAAGTGGAGGGAGACATGTATGAG

Sequence 2:

AGCCTGTGGATGCCGTCAAGCTGAACCTCCCTGATTACTAT

DNA Sequence from Solar Haynes Employee:

TCCAGACCCTGCAGCTCTGAAAGATCGCCGCATGGAGAACCTGGTTGCCTAT
GCTAAGAAAGTGGAGGGAGACATGTATGAGTCTGCTAATAGCAGGGATGAA
TACTATCATTTATTAGCAGAGAAAATCTATAAAAT

DNA Sequence from Murphy Sun Products Vice President:

TACTTCCAATCCATGAACCCCCCGCCCCCAGAGACCTCCAACCCTAACAAGC
CCAAGAGGCAGACCAACCAACTGCAATACCTGCTCAGAGTGGTGCTCAAGAC
ACTATGGAAACACCAGTTTGCATGGCCTTTCCAGCAGCCTGTGGATGCCGTCA
AGCTGAACCTCCCTGATTACTATAAG

DNA Sequence from Local Housing Developer:

CTTTTATTTTATATTATAACCACTACGAGTACACGGGCGAGAAGGCATTAAAG
TATAGTAACATAATATAAAATATACTATGGTATTTATACAAAAAATTTTGTCA
TAACAAATATTTGTTCGGTATAAA

DNA Sequence from Dino Coal Executive:

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TCACAAGTTTTAGTTTTTTGTGCGCGCAACTTCTCAGAAATTTTGTTTATAAAA
TGCTTCTTTTTGCCGATTTGTCATTCCACGGAAATTTGTTTGTTCAGTGCAGCC
AGCTTTTACGCGGAGAAAATAAATTTTA

Materials:
1. Salt Water/Cheek Cell Mixture from Evidence Kit
2. 25% Soap Solution
    a. Preparation: Dissolve a light colored soap (e.g. yellow Dawn) in water in a 1:4 ratio.
3. 30 mL Cold Ethanol or Isopropanol
    a. Note: Addition of food coloring will be helpful in observing the water/alcohol layers, but is not essential.
4. 50 mL Centrifuge Tube of Test Tube
5. Pipet
6. Eppendorf tube
7. Centrifuge

Safety:
Ethanol and isopropanol are flammable liquids and thus a fire hazard. Ethanol is slightly toxic by ingestion and inhalation. Isopropanol is toxic by ingestion and inhalation.

Procedure:
1. Add 3-4 mL of the soap solution to a centrifuge tube or test tube.
2. Pour the salt water/cheek cell mixture into the tube until it is about half full.
    a. Note: It may be helpful to show students how the cheek cells were obtained by having obtain a sample of their own cheek cells. They can isolate their DNA with the following procedure along side the sample from the evidence kit for increased engagement.
3. Place a cap on the tube and gently rock it back and forth for 1-2 minutes.
4. Add enough cold ethanol to almost fill the tube.
    a. Note: Do not tip, shake, or mix, the tube or DNA may not be observed. At this point a line of separation between the water and alcohol layers should be observed. Bubbles attached with tiny hair like strings rising through the alcohol should be observed. The strings are DNA strands. This may take several minutes to observe.
5. Use a pipet to extract the alcohol/DNA mixture near the interface of the layers and transfer into an eppendorf tube.
6. Spin down the solution for 1 minute to pellet out the DNA.
    a. Note: Extraction and spinning is not required. The essential piece is for students to observe the DNA. At this point they will need to be given the "processed" DNA report for comparison to the suspects' DNA.

Waste Disposal:
Used ethanol should be placed in flammable organic waste. Excess soapy water and salt water may be discarded down the drain. Unused chemicals may be stored until later use.

References:
Harris, W. How Stuff Works. http://science.howstuffworks.com/life/genetic/dna-evidence.htm (accessed July 2013).
Saint Francis Preparatory School. http://www.sfponline.org/uploads/dnaextractlab.pdf (accessed July 2013).
Rothschild, A. PBS. http://www.pbs.org/wgbh/nova/body/extract-your-dna.html (accessed July 2013).

Test #3: Flame Test

Background:

The flame test is a procedure used to detect the presence of metal ions based on each metal's characteristic emission spectrum. The test involves introducing a sample containing metal ions to a hot flame and observing the color that results.

In this test, students will conduct a flame test with a copper II salt solution, a sodium salt solution, a potassium salt solution, a barium salt solution, a calcium salt solution, and water for observation of positive and negative controls with the following anticipated results:

Metal Ion	Color Observed
Copper II	Green
Sodium	Golden Yellow/Orange
Potassium	Lilac/Purple
Barium	Pale Yellow
Calcium	Red
Water (Blank)	None

Students will then compare the evidence in their kit to the above.

Materials:
1. 0.1 M Copper Salt Solution (suggested: copper II chloride)
    a. Preparation of 0.5 L of 0.1 M copper II chloride solution: Dissolve 8.5 grams of reagent grade copper II chloride ($CuCl_2 \cdot 2H_2O$) in water to create 0.5 L of total solution.
2. 0.1 M Sodium Salt Solution (suggested: sodium chloride)
    a. Preparation of 0.5 L of 0.1 M sodium chloride solution: Dissolve 2.9 grams of sodium chloride in water to create 0.5 L of total solution.
3. 0.1 M Potassium Salt Solution (suggested: potassium chloride)
    a. Preparation of 0.5 L of 0.1 M potassium chloride: Dissolve 3.7 grams of potassium chloride in water to create 0.5 L of total solution.
4. 0.1 M Barium Salt Solution (suggested: barium chloride)
    a. Preparation of 0.5 L of 0.1 M barium chloride: Dissolve 12.2 grams of reagent grade barium chloride ($BaCl_2 \cdot 2H_2O$) in water to create 0.5 L of total solution.
5. 0.1 M Calcium Salt Solution (suggested: calcium chloride)

      a. Preparation of 0.5 L of 0.1 M calcium chloride: Dissolve 7.4 grams of reagent grade calcium chloride (CaCl$_2$•2H$_2$O) in water to create 0.5 L of total solution.
6. Water (for negative control)
7. Bunsen Burner
8. Wooden Stir Sticks

Safety:
Copper II chloride is highly toxic by ingestion and inhalation. Sodium chloride, potassium chloride, and calcium chloride are slightly toxic by ingestion. Barium chloride is highly toxic by ingestion.

Procedure for Positive Controls:
1. Dip a wooden stir stick in the copper II chloride solution.
2. Remove the stick from the solution and place over a Bunsen Burner flame. Record your observations.
3. Repeat steps 1-2 with the sodium chloride solution, the potassium chloride solution, the barium chloride solution, and the calcium chloride solution.

Procedure for Negative Control:
1. Dip a wooden stir stick in the water.
2. Remove the stick from the solution and place over a Bunsen Burner flame. Record your observations.

Procedure for Unknowns:
1. Dip a wooden stir stick in an unknown sample.
2. Remove the stick from the solution and place over a Bunsen Burner flame. Record your observations.
3. Compare the results from above to the results for comparison of metals at the crime scene and metals present on the suspects.

Waste Disposal:
Used wooden stir sticks may be thrown away. Unused solutions may be stored for later use.

Test #4: Ink Chromatography

Background:

Chromatography is a method for analyzing mixtures by separating them into their component chemicals. Even though a black marker or pen only writes in one color, the ink is actually made from a mixture of different colored pigments. To perform ink chromatography, a small dot of ink is placed at one end of a strip of filter paper. This end of the paper strip is then placed in a solvent. As the solvent travels upward on the paper strip, the mixture of chemicals that compose the ink are dissolved and pulled upward on the paper. The chemicals that dissolve best in the solvent will move up the paper strip further than chemicals that do not dissolve as well. Smaller molecules will also move up further than larger molecules. Forensic scientists are able to use ink chromatography to solve crimes by matching documents or stains found at a crime scene to a marker or pen that belongs to a suspect.

In this test students will separate the components of markers or pens that belong to the suspects and will then test the handwritten notes for comparison.

Materials:
1. Filter Paper of Coffee Filters (cut into uniform strips)
2. Different Black Markers or Pens (suggested: Vis-a-Vis wet erase marker, Sharpie permanent marker, Expo dry erase marker, or Paper Mate felt-tip pen)
3. Isopropanol (can substitute methanol, ethanol, acetone or similar)
4. Beaker or Cups
5. Handwritten Note from Evidence Kit

Safety:

Isopropyl alcohol is a flammable liquid and thus a fire hazard. Isopropyl alcohol is slightly toxic by ingestion and inhalation.

Procedure:
1. Using each of the different black markers or pens place a dot near the bottom of a paper strip. Use a different strip for each marker or pen.
2. Place about 10 mL of isopropyl alcohol (i.e., isopropanol) in the bottom of a beaker or cup.
3. Hold one of the strips in the isopropyl alcohol using care to not dip the strip below the dot.
4. Allow the isopropyl alcohol to run up the filter paper, separating the pigments as it travels.
5. Remove the strip from the isopropyl alcohol when it has reached the top and allow it to dry.
6. Repeat with each of the remaining samples including the handwritten note.

7. Compare each of the samples to the handwritten notes to determine which ink was used to write the note.

Waste Disposal:
Excess used alcohol should be placed in flammable organic waste. Unused alcohol may be stored until later use. Used filter paper may be thrown away.

References:
Museum of Science+Industry Chicago. http://www.msichicago.org/fileadmin/Education/learninglabs/lab_downloads/EvidenceLab_ink_act.pdf (accessed July 2013).

Test #5: Iodine Fuming Fingerprint Developing or Ninhydrin Fingerprint Developing

Background:

A fingerprint is an impression or mark made on a surface by a person's fingertip and can be used for identifying individuals from its unique pattern. Fingerprinting is an important aspect of crime scene investigation and can be used forensically to identify individuals who were present.

There are three basic types of fingerprints: 1) plastic, 2) patent, and 3) latent. Plastic prints are prints made by impressions on a soft material, such as wax or clay. Patent prints are impressions made on colored materials or with colored materials that are on the fingertips. For example, a patent print might be one left in blood or one that is deposited because of blood on a fingertip. Both plastic and patent prints are visible without further processing. Latent prints are prints made by deposition of palmar sweat and natural oils to a surface. Latent prints require processing for visualization.

Latent prints on non-absorbent surface can be developed with powder, while the prints on soft or porous surfaces require other processing techniques. One technique used for fingerprint visualization is iodine fuming. Iodine can be heated to produce a violet vapor that is then absorbed by a fingerprint secretion to produce a yellow/brown pattern on the surface where the secretions were deposited. Such visualization is short lived and needs to be chemically fixed by mixing with starch. Another technique is the use of a ninhydrin staining solution which turns purple upon heating in the presence of amino acids which are in palmar sweat.

In this test, students will develop fingerprints on the piece of paper in their evidence kit using one of two methods and will compare to the fingerprint samples of the suspects. While both methods work, the ninhydrin method generally results in more clearly defined prints.

Method #1: Iodine Fuming Fingerprint Developing

Materials:

1. Gentle Heat Source (oil candle, Bunsen burner, or similar)
2. Large Glass Test Tube (25 mm x 150 mm)
3. Rubber or Cork Test Tube Stopper
4. Test Tube Clamp
5. Forceps, Scoopula, or Spoon
6. Glass Rod
7. Iodine Crystals
8. 1% Starch Solution

a. Preparation: Heat 25 mL of water to boiling. Add 1 gram of soluble starch to the hot water and stir. Dilute to 100 mL. Allow the starch solution to cool. The starch solution will not keep more than a few days. Add a small amount of salicylic acid to preserve the solution for a longer period of time.

9. Piece of paper with Fingerprint from Evidence Kit

Safety:

Iodine is toxic by ingestion or inhalation. Iodine reacts violently with reducing materials, sulfur, iron, alkali metals, metal powders, and phosphorus. Iodine irritates the skin and is corrosive to eyes and respiratory tract.

Procedure:

1. Using forceps transfer a single iodine crystal to a glass test tube.
2. Lightly stopper the test tube so the iodine vapor does not escape.
3. Place the test tube over a gentle heat source and allow the iodine crystal to sublimate.
4. Place the fingerprinted material in the test tube and allow the fingerprint to absorb the iodine.
   a. Note: At this point the fingerprint should have a yellowish color and should be visible.
5. Permanently fix the fingerprint by dropping the starch solution on the fingerprint.
   a. Note: Fixing is required to keep the deposited iodine from evaporating and the fingerprint disappearing.
   b. Note: At this point the fingerprint should have a dark purple/black color.
6. Allow fixed fingerprint to dry.
7. Compare the fingerprint to the suspects' fingerprints for identification.

Waste Disposal:

Developed fingerprints may be thrown away. Unused iodine and starch may be stored until later use. If starch solution is stored it must be preserved.

Method #2: Ninhydrin Fingerprint Developing

Materials:

1. Fingerprint Staining Solution (Ninhydrin, Ethanol (acetone may be substituted), and Acetic Acid
   a. Preparation: Dissolve 0.5 grams of ninhydrin in 125 mL of ethanol. Add 0.1 mL acetic acid to the solution.
   b. Note: Fresh preparation of solution is best for fingerprint developing.
2. Lysine Solution (for positive test)
   a. Preparation: Dissolve 0.25 grams of lysine in 50 mL of water and 50 mL of ethanol.

3. 1:1 Water:Ethanol Mixture (for negative test)
   a. Preparation: Mix 50 mL of water and 50 mL of ethanol.
4. Droppers or Spray Bottles
5. Paper
6. Heat Gun or Blow Dryer
7. Piece of Paper with Fingerprint from Evidence Kit

Safety:
Ninhydrin is a body tissue irritant. Ethanol is a flammable liquid and thus a fire hazard. Ethanol is slightly toxic by ingestion and inhalation. Acetic acid is corrosive to skin and tissue and is moderately toxic by ingestion and inhalation

Procedure for Positive and Negative Controls:
1. On a piece of paper place a drop of the lysine solution on one side and a drop of ethanol on the other. Briefly allow to dry.
2. Drop or spray the fingerprint staining solution on the lysine solution and the 1:1 water:ethanol on the paper. Briefly allow to dry.
3. Using a heat gun or a blow dryer to apply heat to the paper.
   a. Note: A purple color indicating a positive test will develop where the lysine was dropped, and will not indicating a negative test where the ethanol was dropped.

Procedure for Unknown:
1. Drop or spray the fingerprint staining solution on the piece of paper with the fingerprint, essentially covering it. Briefly allow to dry. Using a heat gun or blow dryer to apply heat the paper.
   a. Note: If a fingerprint is present it should develop in the same purple color as observed in the positive test with lysine above.

Waste Disposal:
Paper may be thrown away. Unused chemicals may be stored until later use.

References:
Scientific Working Group on Friction Ridge Analysis, Study, and Technology. http://www.swgfast.org/ (accessed July 2013).

Test #6: Silver Plating Test

Background:

This test, also known as a Tollen's test, determines if an aldehyde is present in a solution. If an aldehyde is present, the reaction will cause silver to plate (coat) the inside of a glass container. An aldehyde is an organic compound that consists of a carbon atom double bonded to an oxygen atom, singled bonded to a hydrogen atom, and singled bonded an alkyl chain (R). Its general structure is pictured below. Aldehydes are commonly used in fragrances. For example, the compound vanillin, which gives vanilla its scent and flavor, is an aldehyde.
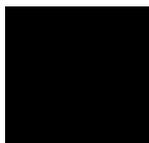
Aldehyde

In this test students will test the scents from their evidence kit to identify if an aldehyde is or is not present.

Materials:

1. 5% w/w Glucose Solution (for positive test)
   a. Preparation: Dissolve 5 grams of glucose in 95 mL of water.
   b. Note: Fresh preparation is best.
2. Water (for negative test)
3. 8% w/w Silver Nitrate Solution
   a. Preparation: Dissolve 8 grams of silver nitrate in 92 mL of water.
   b. Note: Fresh preparation is best. Silver nitrate solution must be stored in a dark bottle or container.
4. 12% w/w Ammonium Nitrate Solution
   a. Preparation: Dissolve 12 grams of ammonium nitrate in 88 mL of water.
5. 10% w/w Sodium Hydroxide Solution
   a. Preparation: Dissolve 10 grams of sodium hydroxide in 90 mL of water.
6. Medicine Droppers
7. Small Glass Vials (5 mL) with Caps
8. Wash Bottle
9. Scents from Crime Scene and Suspects

Safety:

Silver nitrate is a corrosive solid and causes burns. Silver nitrate will stain skin and clothing. Protective eyewear, gloves, and clothing should be worn when handling silver nitrate. Ammonium nitrate is a strong oxidizer and may explode if heated under confinement or at temperatures greater than 210 ºC. Ammonium nitrate explodes more readily if contaminated with combustible material. Ammonium nitrate is slightly toxic by

ingestion and is a body tissue irritant. Sodium hydroxide is a corrosive solid and can cause skin burns. Heat evolves when adding sodium hydroxide to water. Sodium hydroxide is very dangerous to eyes and skin and eye protection and gloves should be used when handling it.

Procedure for Positive Control (Presence of an Aldehyde):
1. Add 20 drops of glucose solution to a glass vial.
2. Add 10 drops of silver nitrate solution.
3. Add 10 drops of ammonium nitrate solution.
4. Cap the vial and mix the solutions for 10 seconds.
5. Add two dropperfuls of sodium hydroxide.
6. Cap the vial and shake the solutions for 60 seconds.
    a. Note: After about 60 seconds a silver plate should be observed on the walls of the glass vial.
7. Dump the excess solution in a waste container with its contents labeled.
8. Rinse the inside of vial with water and dump in the waste container.

Procedure for Negative Control (Absence of an Aldehyde):

1. Add 20 drops of water to a glass vial.
2. Add 10 drops of silver nitrate solution.
3. Add 10 drops of ammonium nitrate solution.
4. Cap the vial and mix the solutions for 10 seconds.
5. Add two dropperfuls of sodium hydroxide.
6. Cap the vial and shake the solutions for 60 seconds.
    a. Note: A silver plate will not form on the walls of the glass vial.
7. Dump the excess solution in a waste container with its contents labeled.
8. Rinse the inside of vial with water and dump in the waste container.

Procedure for Unknowns:

1. Add 20 drops of each sample to a different glass vial.
2. Add 10 drops of silver nitrate solution to each vial.
3. Add 10 drops of ammonium nitrate solution to each.
4. Cap each vial and mix the solutions for 10 seconds.
5. Add two dropperfuls of sodium hydroxide to each vial.
6. Cap each vial and shake the solutions for 60 seconds.

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7. Observe each vial for a positive or negative test.
8. Dump the excess solution in a waste container with its contents labeled.
9. Rinse the inside of each vial with water and dump in the waste container.

Waste Disposal:
Mixed chemicals may be collected together in waste container that is labeled with its contents. Unused solutions may be stored until later use.

A3.2. Student Materials

A3.2.1. Script for Investigation of a Solar Cell Sabotage

Name: _____ Class:_____

Investigation of a Solar Cell Sabotage

Late last Saturday night a break in occurred at Solar Haynes Industries, a solar cell factory. The culprit who broke in, damaged newly developed solar cell panels that Solar Haynes Industries has spent months working on. As a forensic scientist, you have been called in to determine who sabotaged Solar Haynes Industries. Samples have been collected from the crime scene. A police detective has interviewed the suspects and has gathered evidence from them.

When you arrive to the scene, you are briefed by the police detective with the following information:

Police Detective: "We have narrowed our search to several suspects who each have motive for the break in; a local housing developer who wants to get rid of Solar Haynes to grow their development and increase their profits, a Dino Coal executive planning to grow the coal industry to push their solar competitors out of business, Murphy Sun Products vice president who is in direct competition with Solar Haynes in the solar cell market, and a Solar Haynes employee who is upset by the way the higher ups are treating the factory workers."

"I interviewed each of the suspects. The interviews have been transcribed for you to read over. We collected a piece of plastic that we believe was dropped by the culprit, a threatening note, a cup that the culprit drank from, paper that we believe may contain fingerprints, a trace scent, and metallic dust from the crime scene. From the suspects we collected plastic from their workspaces, pens, DNA samples, fingerprint samples, cologne or perfume samples, and metallic dust from their clothing or workspaces. I really hope, with this evidence, that you can help us to solve this crime."

Interview with Solar Haynes Industries Employee
Police Detective: I'm sure you're aware the Solar Haynes was broken into and some solar cell panels were damaged. I've heard that you've had some trouble with the administration in the past. Do you care to comment?
Employee: Sure, I've had some run-ins with the higher ups, but they don't get us down here in manufacturing. We get the short end of the stick... long hours, low pay... I'm frustrated, but I wouldn't damage my own products. How would getting fired help me?

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Police Detective: Where were you last night around 2 am?

Employee: My business is my business.

Police Detective: Well I am sure if you aren't guilty you won't mind me collecting some evidence?

Employee: If you want evidence you should come back with a warrant. Unless you have a warrant, we are done here.

Interview with Murphy Sun Products Vice President

Police Detective: Hello! Can I get just a moment of your time to ask a few questions?

Vice President: Only a moment. I've got things to do! We're working on a new product line of cheap, lightweight plastic for use in solar cell manufacturing! It's going to be revolutionary, but we don't have time to waste if we are going to beat out our competitors.

Police Detective: Speaking of your competitors... Did you hear about the break in at Solar Haynes last night?

Vice President: I don't have time to keep up with those types of things. There are more important things that demand my attention. I really need to get going. You can see my assistant for anything else you need.

Police Detective: Alright. I will contact your assistant to collect evidence.

Interview with Local Housing Developer

Police Detective: Good afternoon, do you mind if I ask you a few questions?

Housing Developer: Are you from the law office? I've been waiting for a response for days now! When are we going to tear into that Solar Haynes company? They're infringing on our space and lowering property values.

Police Detective: Actually, I'm from the Police Department, here to talk to you about a break-in that happened at Solar Haynes Industries last night. It sounds like you've had some problems with them recently?

Housing Developer: Well, I mean… not exactly problems, per se. It's just that having a huge manufacturing plant right next to my new housing development is lowering my profit margin. I'd prefer if they moved their business elsewhere, but I wouldn't do anything to hurt them.

Police Detective: Where were you last night around 2 am?

Housing Developer: Are you kidding!? I was in bed. I wake up at the crack of dawn every day.

Police Detective: Alright, well if you weren't near Solar Haynes at the time of the break in then you won't mind us collecting some evidence to corroborate your story.

Housing Developer: No, go right ahead. Collect whatever you need.

Interview with Dino Coal Executive

Police Detective: Excuse me... Do you mind if I come in to ask a few questions?

Executive: No, no problem, come on in.

Police Detective: I've heard the coal industry has been having some hard times financially. How is Dino Coal handling it?

Executive: Well, it's hard with all these 'green' companies coming out with these 'green' products, but I've got a secret plan.

Police Detective: Really? Care to give some details?

Executive: Well it isn't a secret if I am telling everyone about it, but I'll let you know a little piece of it. We're changing our marketing strategy... we are going to have dinosaurs everywhere, all the time! Our employees are even going to have costumes! People won't be able to look any direction without being reminded of Dino Coal! We are going push our solar and 'green' competitors out of business with this strategy!

Police Detective: I am sure you heard about the break in at Solar Haynes. Is this part of your "marketing strategy?"

Executive: Naw, we would never do such a thing. We don't need to play cheap to leave the solar companies like Solar Haynes in the dust. Solar is a fad anyway, coal is for life!

Police Detective: Alright. I'll just collect a few samples and be on my way.

A3.2.2. Student Worksheets with Anticipated Responses

Name: _____ Class: _____

Identify the suspects and their motives.

Suspect	Motive
Solar Haynes Employee	Answers will vary.
Murphy Sun Products Vice President	Answers will vary.
Local Housing Developer	Answers will vary.
Dino Coal Executive	Answers will vary.

Question: Make your hypothesis... Who do you think sabotaged the solar cells?
Answers will vary.

Question: Why do you think it was the suspect(s) you identified?
Answers will vary.

Question: How will you determine if you are correct?
Answers will vary.

Test #1: Density Comparison

Notes about the experiment:

Question: What other evidence will you test? Why?

The pieces of plastic will be tested because this test can compare their densities.

Complete the following chart by identifying if the plastic is more or less dense than the substance it is placed in.

Evidence	In Water D = 1.00 g/mL	In Glycerol D = 1.26 g/mL	Conclusion
Plastic from Solar Haynes Employee	More	Less	The unknown plastic will match the employee's plastic if... it sinks in water, but floats in glycerol.
Plastic from Murphy Sun Products Vice President	More	More	The unknown plastic will match the vice president's plastic if... it sinks in both water and glycerol.
Plastic from Local Housing Developer	Less	Less	The unknown plastic will match the housing developer's plastic if... it floats in both water and glycerol.

Data Collection: Create a chart to document your observations of the evidence you decide to test.

	In Water	In Glycerol
Unknown Plastic	Sinks	Floats

Question: What two factors does density take into account?
Density takes into account an object or substance's mass and volume.

Question: If an object is put in a liquid and it floats, is the object more or less dense than the liquid?
The object is less dense than the liquid.

Question: Based on your observations, what can you conclude from this test?
The piece of plastic belongs to the Murphy Sun Product vice president.

Test #2: DNA Extraction

Notes about the experiment:

Question: What evidence will you test? Why?
The sample containing DNA will be tested because this test can extract DNA for analysis.

DNA from Suspects:

Suspect	DNA Sequence from DNA Sample
Solar Haynes Employee	TCCAGACCCTGCAGCTCTGAAAGATCGCCGCATGGAGAACCTGGTTGCCTATGCTA AGAAAGTGGAGGGAGACATGTATGAGTCTGCTAATAGCAGGGATGAATACTATCA TTTATTAGCAGAGAAAATCTATAAAAT
Murphy Sun Products VP	TACTTCCAATCCATGAACCCCCCGCCCCCAGAGACCTCCAACCCTAACAAGCCCAA GAGGCAGACCAACCAACTGCAATACCTGCTCAGAGTGGTGCTCAAGACACTATGG AAACACCAGTTTGCATGGCCTTTCCAGCAGCCTGTGGATGCCGTCAAGCTGAACCT CCCTGATTACTATAAG
Local Housing Developer	CTTTTATTTTATATTATAACCACTACGAGTACACGGGCGAGAAGGCATTAAAGTAT AGTAACATAATATAAAATATACTATGGTATTTATACAAAAAATTTTGTCATAACAA ATATTTGTTCGGTATAAA
Dino Coal Executive	TCACAAGTTTTAGTTTTTTGTGCGCGCAACTTCTCAGAAATTTTGTTTATAAAATGC TTCTTTTTGCCGATTTGTCATTCCACGGAAATTTGTTTGTTCAGTGCAGCCAGCTTTT ACGCGGAGAAAATAAATTTTA

Data Collection: Document your observations of the evidence you decide to test.

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Question: Do the DNA sequences from the unknown sample have any distinguishing characteristics?

There are unique combinations of letters in each sequence.

Question: Do the DNA sequences from the unknown sample match any of the suspects sequences?

Yes, the unknown DNA is a match to the Solar Haynes employee and the Murphy Sun Products vice president.

Question: Based on your observations, what can you conclude from this test?

The unknown DNA is a match to the Solar Haynes employee and the Murphy Sun Products

vice president indicating that they were present at the crime scene.

Test #3: Flame Test

Notes about the experiment:

Question: What evidence will you test? Why?

The metallic dust samples will be tested because this test can identify what metals are present.

Complete the following chart:

Solution	Observations	Conclusions
Solution Containing Copper II Ions	Green Color	If a solution contains copper II ions then... a green color will be observed in a flame test.
Solution Containing Sodium Ions	Yellow/Orange Color	If a solution contains sodium ions then... a yellow/orange color will be observed in a flame test.
Solution Containing Potassium Ions	Lilac Color	If a solution contains potassium ions then... a lilac color will be observed in a flame test.
Solution Containing Barium Ions	Yellow Color	If a solution contains barium ions then... a yellow color will be observed in a flame test.
Solution Containing Calcium Ions	Red Color	If a solution contains barium ions then... a red color will be observed in a flame test.
Water	None	If a solution does not contain metal ions then... no color change will be observed in a flame test.

Data Collection: Create a chart to document your observations of the evidence you decide to test.

Sample	Observations
Metallic Dust from Crime Scene	Green color produced from flame test
Metallic Dust from Solar Haynes Employee	Green color produced from flame test

Metallic Dust from Murphy Sun Products VP (#1)	Green color produced from flame test
Metallic Dust from Murphy Sun Products VP (#2)	Red color produced from flame test
Metallic Dust from Local Housing Developer	No color produced
Metallic Dust from Dino Coal VP	No color produced

Question: What distinguishes each sample?
Different metals give different colors.

Question: What conclusions can you draw based on your observations from this test?
The metallic dust from the crime scene matches the metallic dust found on the Solar Haynes

employee and the Murphy Sun Products vice president.

Test #4: Ink Chromatography

Notes about the experiment:

Question: What evidence will you test? Why?

The handwritten notes and pens will be tested because this test will allow identification of the pen used to write the notes.

Complete the following chart:

Chromatogram from Pen #1 Suspect: Solar Haynes Employee	Chromatogram from Pen #2 Suspect: Murphy Sun Products VP	Chromatogram from Pen #3 Suspect: Local Housing Developer	Chromatogram from Pen #4 Suspect: Dino Coal Executive
Varies depending on marker/pen assigned.	Varies depending on marker/pen assigned.	Varies depending on marker/pen assigned.	Varies depending on marker/pen assigned.

Data Collection: Document your observations of the evidence you decide to test.

Chromatograph from Handwritten Notes
Varies depending on marker/pen assigned.

Question: Why do the colors separate on the filter paper?

The inks are made of different pigments (colors) which separate on the filter paper based on their size and attraction to the solvent.

Question: What conclusions can you draw based on your observations from this test? The pen used to write the notes belong to the Solar Haynes employee.

Test #4: Fingerprint Developing

Notes about the experiment:

Question: What evidence will you test? Why?
The note will be tested because this test can reveal any fingerprints left behind.

Data Collection: Draw a sketch of the fingerprint or glue/tape in your developed fingerprint.
This will vary based on the fingerprints used for analysis.

Question: What distinguishing patterns do you see on the fingerprint?
This will vary based on the fingerprints used for analysis.

Question: Which of the suspects do you think the fingerprint belongs to?
Murphy Sun Products vice president.

Question: What conclusions can you draw based on your observations from this test? The fingerprint matches that of the Murphy Sun Products vice president indicating that they were present at the crime scene.

Test #6: Silver Plating Test

Notes about the experiment:

Question: What evidence will you test? Why?
The scents will be tested because this test can identify if they have an aldehyde present.

Complete the following chart:

	Observation	Conclusion
Aldehyde	Silver Plate Forms	If a sample contains an aldehyde then.. a silver plate will form when this test is conducted..
Water	No Change	If a sample does not contain an aldehyde then... a silver plate will not form when this test is conducted.

Data Collection: Create a chart to document your observations of the evidence you decide to test.

Sample	Observations
Scent from Crime Scene	Silver Plate Forms
Scent from Solar Haynes Employee	No Change
Scent from Murphy Sun Products Vice President	Silver Plate Forms
Scent from Local Housing Developer	Silver Plate Forms
Scent from Dino Coal Executive	No Change

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Question: What conclusions can you draw based on your observations from this test? The scent from the crime scene, the scent from the Murphy Sun Products vice president, and the scent from the local housing developer contain an aldehyde, thus the scent could be a match to one of those two suspects.

After completing each test, check the boxes where the suspect's sample matches the evidence found at the crime scene.

Test	Solar Haynes Employee	Murphy Sun Products VP	Local Housing Developer	Dino Coal Executive
#1: Density Comparison		x		
#2: DNA Extraction	x	x		
#3: Flame Test	x	x		
#4: Ink Chromatography	x			
#5: Fingerprint Developing		x		
#6 Silver Plating		x	x	

Now that you have tested all of the evidence with the available tests, who do you believe is responsible for the sabotage. Explain based on your observations obtained during each test why you believe this to be true and why you can rule out the other suspects. Was your hypothesis of who committed the crime correct?

The Murphy Sun Products vice president is responsible for the sabotage... (Students should identify the Murphy Sun Products vice president as the suspect responsible for the sabotage. Students should support this with evidence from each test.)