Development of a Microfluidic Platform for Trace Lipid Analysis

Andrew Paul Davic

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DEVELOPMENT OF A MICROFLUIDIC PLATFORM FOR TRACE LIPID ANALYSIS

By

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Approved March 14, 2016

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ABSTRACT

DEVELOPMENT OF A MICROFLUIDIC PLATFORM FOR TRACE LIPID ANALYSIS

By
Andrew Davic
May 2016

Dissertation supervised by Michael Cascio

The field of lipidomics encompasses the study of pathways, networks, and functionality of cellular lipids in biological systems. The lipid subclass, primary fatty acid amides, are crucial to nervous system signaling, receptor function, and numerous other physiological roles. Chapter 1 details these bioactive properties of several well-studied primary fatty acid amides as well as their biosynthesis, degradation, and most common analysis techniques. As these bioactive lipids are endogenously present in trace and ultra-trace abundancies, the field of microfluidics presents an attractive alternative analysis system to incorporate minimization of sample and reagent usage, analysis cost reduction, highly sensitive detection pairing, and decreased analysis time, all while limiting sample handling. Chapter 2 provides a microfluidics-based review of common device fabrication techniques, droplet microfluidics, and detection systems. Current primary fatty acid amide
analysis techniques have detection limits on the periphery of endogenous concentrations, presenting the need for a more sensitive detection system, such as fluorescence. Chapter 3 serves as the foundation in developing methodology to analyze these amides and their conjugate fluorescently-tagged primary amines. Chapter 4 focuses on the development of a microfluidic platform capable of efficient on-chip fluorescent tagging reactions and the coupling of a highly sensitive laser induced fluorescence detection system capable of detection limits several orders of magnitude lower than currently employed mass spectrometry techniques. In addition, the appendix details the method development for the quantitative analysis of the anti-inflammatory and anti-cancer drug, celecoxib, uptake into novel drug delivery vehicles.
DEDICATION

This thesis work is dedicated to the late Dr. Mitchell E. Johnson. I was first introduced to “Mitch” during the summer of 2007 through Duquesne University’s Research Experience for Undergraduates program. The valuable research experience, mentorship, and general welcoming that I felt from the Johnson Lab was enough to make me want to come back not only for a second summer research program, but also for the long journey of graduate school. I have Mitch to thank for finding and developing my interest and passion for scientific research, and helping to shape me into the scientist that I have become today. Mitch taught me to appreciate (and brew) a good beer, find the joys and humor in a terribly bad science movie, and to never, ever, cross the streams. I am forever grateful to have known him as close as I did and am proud to be his final Ph.D. student.
ACKNOWLEDGEMENT

Words cannot begin to describe the appreciation and gratitude that I have for my research advisor, Dr. Michael Cascio. Following the passing of my original research advisor, Dr. Mitch Johnson, Mike willingly took me under his wing and became my graduate mentor. Although the thesis work presented herein did not necessarily overlap with his research specialty, Mike was willing to do all that he could to not only learn about the work that I have been doing, but also to provide a helping hand whenever needed. I am thankful for the opportunity to have worked for you and alongside you over my graduate years and for the mentorship and friendship that you have continued to provide.

To my graduate committee members: Dr. Stephanie Wetzel, Dr. Partha Basu, and Dr. Dave Gallaher, thank you for all that you have done for me. Stephanie, I was lucky enough to have had the chance to be your TA for several years, and the experience gained and lessons learned have been invaluable. You have taught me to be proficient in using a plethora of analytical instrumentation and have really helped shape me into the analytical chemist that I am today. Partha, as a close friend of Mitch, I feel that you have been a person within the department that I knew I could always come and talk to, be it for scientific purposes or anything else. I am thankful for your continued guidance and for always pushing me to learn and achieve all that I can. Dave, as Mitch’s first graduate student, I have you to thank for largely pioneering the research that I have had the privilege of working on. You know the background of my work better than anyone else, and I am very much appreciative of the help and guidance that you have provided over the years.
To present and past members of the Johnson/Cascio Lab, thank you for making my graduate school experience one that I will always remember. I have developed deep friendships with many of you through the hardships and triumphs that we have all shared in. I thank you for welcoming me into the lab when I started doing undergraduate summer research, which ultimately led me to return for graduate school. I look forward to staying in touch with many of you and wish you all the best of luck in your future careers.

Lastly, none of this would have ever been a possibility without the overwhelming love and support from my friends and family. Mom, Dad, and Michael, you were always there for me growing up, providing unwavering support and encouragement for everything that I did in life. I could not ask for a better family as I am truly lucky to have all of you. Finally, to my amazing wife, Sarah, thank you for everything. It has been a long journey to say the least, and I cannot thank you enough for your patience and support. I look forward to experiencing what our future has in store with you by my side.

Andrew Davic
Duquesne University
2016
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<th>Description</th>
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<td>µPAD.</td>
<td>microfluidic paper-based analytical device</td>
<td></td>
</tr>
<tr>
<td>µTAS</td>
<td>micro total analysis system</td>
<td></td>
</tr>
<tr>
<td>5-HT</td>
<td>5-hydroxytryptamine</td>
<td></td>
</tr>
<tr>
<td>APCI</td>
<td>atmospheric pressure chemical ionization</td>
<td></td>
</tr>
<tr>
<td>APD</td>
<td>avalanche photodiode array detector</td>
<td></td>
</tr>
<tr>
<td>BPE</td>
<td>bipolar electrode</td>
<td></td>
</tr>
<tr>
<td>CBI</td>
<td>N-2-substituted-1-cyanobenz-[f]-isoindole</td>
<td></td>
</tr>
<tr>
<td>CI</td>
<td>chemical ionization</td>
<td></td>
</tr>
<tr>
<td>CID</td>
<td>collision induced dissociation</td>
<td></td>
</tr>
<tr>
<td>CoA</td>
<td>Coenzyme-A</td>
<td></td>
</tr>
<tr>
<td>CrEL</td>
<td>Cremophor EL</td>
<td></td>
</tr>
<tr>
<td>CSF</td>
<td>cerebral spinal fluid</td>
<td></td>
</tr>
<tr>
<td>DMF</td>
<td>digital microfluidics</td>
<td></td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
<td></td>
</tr>
<tr>
<td>DSC</td>
<td>Discovery</td>
<td></td>
</tr>
<tr>
<td>EI</td>
<td>electron impact</td>
<td></td>
</tr>
<tr>
<td>ESI</td>
<td>electrospray ionization</td>
<td></td>
</tr>
<tr>
<td>EUFS</td>
<td>energy units full scale</td>
<td></td>
</tr>
<tr>
<td>FAAH</td>
<td>fatty acid amide hydrolase</td>
<td></td>
</tr>
<tr>
<td>FITC</td>
<td>fluorescein isothiocyanate</td>
<td></td>
</tr>
<tr>
<td>FQCA</td>
<td>3-(2-furoyl)quinolone-2-carboxaldehyde</td>
<td></td>
</tr>
</tbody>
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FTIR. ................................................................. Fourier transform infrared spectroscopy
GABA. .................................................................. γ-aminobutyric acid
GC. ....................................................................... gas chromatography
HF. ...................................................................... hydrofluoric acid
HPLC ...................................................................... high performance liquid chromatography
ICP-RIE ................................................................. inductively coupled plasma reactive ion etching
KCN. ................................................................. potassium cyanide
LOD. ................................................................. limit of detection
MAG. ..................................................................... monoacylglycerol
MALDI. .................................................................. matrix assisted laser desorption ionization
MRI. ................................................................. magnetic resonance imaging
MRM. ................................................................. multiple reaction monitoring
MS. ...................................................................... mass spectrometry
MTBE. ............................................................... methyl-tert-butyl ether
NAE. .................................................................... N-acyl ethanolamine
NAG. ..................................................................... N-acyl glycine
NDA. ..................................................................... naphthalene-2,3-dicarboxaldehyde
NMR. ..................................................................... nuclear magnetic resonance
OPA. ...................................................................... o-phthalaldehyde
OTS. ..................................................................... octadecyltrichlorosilane
P105. ...................................................................... Pluronic P105
PAL. ...................................................................... peptidyl-α-hydroxyglycine α-amidating lysase
PAM. .................................................................... Peptidylglycine α-amidating monooxygenase
PCR. .............................................................. polymerase chain reaction
PDMS. ............................................................. polydimethyl siloxane
PEEK. ............................................................. polyether ether ketone
PFAM. ........................................................... primary fatty acid amide
PFC. ............................................................... perfluorocarbon
PIFA. ............................................................ [bis(trifluoroacetoxy)iodo]benzene
QqQ. .............................................................. triple quadrupole
LIF. ............................................................... laser induced fluorescence
QToF. .............................................................. quadrupole time of flight
R^2. ................................................................. coefficient of determination
Re. ................................................................. Reynolds number
S/N. ............................................................... signal to noise ratio
SEM. ............................................................. scanning electron microscope
SERS. ............................................................ surface-enhanced Raman spectroscopy
SIM. ............................................................. selected ion monitoring
SPE. ............................................................. solid phase extraction
TLC. ............................................................. thin layer chromatography
ToF. .............................................................. time of flight
UV. ............................................................... ultraviolet
UV-Vis. ........................................................ ultraviolet-visible
Chapter 1

Review of Primary Fatty Acid Amides and Lipidomics

1.1. BACKGROUND AND SIGNIFICANCE

Lipidomics is broadly defined as a comprehensive study of pathways, networks, and functionality of cellular lipids in biological systems. Lipids are relatively water-insoluble and are small, amphiphilic bioorganic molecules that have numerous biochemical functions and properties. They play important roles in cell makeup, energy storage, and signaling, while also acting as essential hormones. Lipids are divided into eight different categories, with each having several main classes and further subclasses. The broad range of molecular diversity is shown in Table 1-1 with each lipid category as well as a selected subclass depicted.\textsuperscript{1,2} The lipid category of fatty acyls, and more specifically its subclass, primary fatty acid amides (PFAMs), will be the focal point of this review.
<table>
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<th>Lipid Category (Subclass)</th>
<th>Common Example</th>
<th>Structure</th>
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<tr>
<td>Fatty Acyls (Fatty Amides)</td>
<td>Oleamide</td>
<td><img src="image1" alt="Structure" /></td>
</tr>
<tr>
<td>Glycerolipids (Monoalkylglycerols)</td>
<td>1-Oleylglycerol</td>
<td><img src="image2" alt="Structure" /></td>
</tr>
<tr>
<td>Glycerophospholipids (Diacylglycerophosphoserines)</td>
<td>PS</td>
<td><img src="image3" alt="Structure" /></td>
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<tr>
<td>Sphingolipids (Sphing-4-enines)</td>
<td>Sphingosine</td>
<td><img src="image4" alt="Structure" /></td>
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<tr>
<td>Sterol Lipids (Cholesterol and Derivatives)</td>
<td>Cholesterol</td>
<td><img src="image5" alt="Structure" /></td>
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<td>Prenol Lipids (Bactoprenols)</td>
<td>Decaprenol</td>
<td><img src="image6" alt="Structure" /></td>
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<tr>
<td>Saccharolipids (Pentaacyl-aminosugars)</td>
<td>Bordetella pertussis lipid A</td>
<td><img src="image7" alt="Structure" /></td>
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<tr>
<td>Polyketides (Polyenes)</td>
<td>Nystatin</td>
<td><img src="image8" alt="Structure" /></td>
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Table 1-1. Examples of the variety of lipid classes and example subclasses. A representative molecule was chosen from each lipid subclass, with structures shown to show diversity of classes.\(^1\) \(^2\)
1.2. PRIMARY FATTY ACID AMIDES

PFAMs, along with N-acyl amines, fatty acyl homoserine lactones, and N-acyl ethanolamines, are a subclass of fatty amides within the fatty acyl lipid category.\textsuperscript{1, 2} These bioactive lipids are important in nervous system signaling, receptor function, and numerous other physiological roles. Structurally, PFAMs contain a carboxamide head group and an acyl tail varying in both length and unsaturation. The high level of variation in bond length, unsaturation (both the number of unsaturated bonds as well as position), and the presence of isomers provide a great deal of biological and physiological diversity. Endogenous fatty acid amides within mammalian organisms are present only in even chain lengths, typically ranging from 16 to 24 carbons in the aliphatic region. Palmitamide (C16:0), palmitoleamide (C16:1\textsuperscript{9}), oleamide (C18:1\textsuperscript{9-cis}), elaidamide (C18:1\textsuperscript{9-trans}), and linoleamide (C18:2\textsuperscript{9,12}) were the first PFAMs to be identified in 1989 from luteal phase plasma.\textsuperscript{3}

1.2.1. Oleamide

Oleamide is one of the most widely studied PFAMs due to its vast array of physiological effects and functions. Oleamide was first characterized (along with erucamide [C22:1\textsuperscript{13}]) from the cerebrospinal fluid of sleep deprived cats.\textsuperscript{4-6} Interest in sleep induction research extends well back to the early 1900s with initial endogenous sleep inducers first realized in 1913 by Pieron et al.\textsuperscript{7} Cerebrospinal fluid (CSF) from sleep deprived dogs was transfused into control dogs, resulting in 6-8 hours of sleep induction. Conversely, control dogs receiving CSF from highly alert dogs remained awake.
Modern day sleep induction research conducted by Lerner and Cravatt analyzed CSF in search of novel compounds that accumulated in cats during sleep deprivation. Preparative high performance liquid chromatography (HPLC) along with mass spectrometry (MS) was able to identify a major component to be oleamide.\(^4\) Originally referred to as cerebriodien, oleamide was only present in trace amounts of the sleep deprived cats (0.1-5 pmol/100 µL) while not being detected in the control samples.\(^5\)

Unsaturated fatty acid amides have been of interest due to their potential ability to alter neuronal function and suppress neurotransmitter release through interactions with the cannabinoid receptor.\(^8-10\) Due to structural similarity to a prototypical endocannabinoid, N-acylethanolamine anandamide, as well as common enzymatic degradation by fatty acid amide hydrolase, oleamide has been studied as a potential cannabinoid receptor agonist. Although not technically classified as an endocannabinoid, as binding to endocannabinoid receptors only occurs at high concentrations, oleamide displays characteristic properties similar to that of anandamide. Because anandamide has vasorelaxant properties in the rat mesenteric arterial bed,\(^11\) oleamide was investigated for vasorelaxant properties in the same artery. Oleamide was found to exhibit potent vasorelaxation via multiple mechanisms of agonistic receptor protein binding involving the endothelium, vascular smooth muscle layer, and sensory nerves.\(^12\) Such dose-dependent vasorelaxation induction has been found to be mediated by endothelial cannabinoid receptors, endothelial independent transient receptor potential cation channel subfamily V member 1, as well as contribution from both nitric oxide and potassium channels.\(^13\) Additional physiological effects of oleamide similar to that of cannabinoids are also observed in food intake and sexual behavior.\(^14\) Systematic injections of oleamide in rats as compared with anandamide
injections resulted in overeating at all doses. Conversely, all doses of anandamide had
direct correlations to sexual behavior, whereas oleamide showed no effect, showing that
oleamide displays some cannabinoid-like properties, however it is not actually classified
as such. Oleamide induced hypnotic, analgesic, and anxiolytic effects with very low
dependence liability, contrasted by similar effects produced from exogenous cannabinoid
agonists that have a greater potential for inducing severe physical dependence syndromes.

While systematic administration of oleamide has been shown to be an effective
sleep inducer, decreases in both body temperature and locomotor activity have also been
observed. Dose-dependent hypothermic responses from oleamide administration has been
differentiated from the known relationship of the sleep-wake cycle and body temperature
(i.e., body temperature being at its highest during wakefulness and a gradual decrease
during sleep, with the lowest temperature typically observed during rapid eye movement
sleep), suggesting the mechanisms related to oleamide effects on body temperature
and sleep induction differ. Although the specific mechanism behind hypothermia
induction is unknown, a possibility is the ability of oleamide to potentiate both type-A \( \gamma \)-
aminobutyric acid receptors (GABA\(_A\)R) and subtype 1A 5-hydroxytryptamine (serotonin)
receptors (5-HT\(_{1A}\)). This hypothesis stems from findings that systematic activation of both
GABA\(_A\) and 5-HT\(_{1A}\) receptors induce hypothermia in rats. As a possible consequence
of oleamide’s hypothermic effects, intraperitoneal oleamide injections have also produced
low motility. The induction of sleep, hypothermia, and hypomotility caused by
administration of oleamide is not mediated by changes in blood pressure and/or heart
rate.
Oleamide is an endogenous brain lipid in mammals, inclusive of humans. It is highly selective in potentiating serotonin (5-HT$_{1A,2A,2C}$) and GABA$_A$ receptors, producing long-lasting inhibition of receptor function, and thereby affecting alertness, sleep, and mood.\textsuperscript{22-25} Although several naturally occurring fatty acids (e.g., oleamide, arachidonamide, anandamide, oleyl ethanolamide) have been shown to potentiate the 5-HT$_{2A}$ receptor, oleamide is the most effective, likely due to the presence, position, and stereochemistry of the $\Delta^9$-cis double bond (subtle structural variations reduce/eliminate activity).\textsuperscript{23} Oleamide also potentiates the metabotropic 5HT$_7$ receptor via an allosteric site. Potentiation of this G protein coupled receptor influences G protein signaling, thereby potentially influencing behavioral responses.\textsuperscript{23} It has also proven to be effective at blocking gap junction communication between rat glial cells, while not effecting intercellular calcium signaling.\textsuperscript{26,27}

In addition to its vast array of physiological properties, oleamide has recently been shown to provide potential environmental benefits, specifically as an algicide in controlling toxic cyanobacterial blooms.\textsuperscript{28-30} Amine derivatives of fatty acids, which are a main constituent of plasma membranes and thylakoids, display stronger antimicrobial effects than their corresponding free fatty acids, with oleamide reported to have inhibitory effects against pathogenic bacteria.\textsuperscript{28-30} Sewage effluent and agricultural run-off results in excessive nitrogen and phosphorus accumulation in water, resulting in hepatotoxic microcystins-producing cyanobacterial blooms, posing a high health risk to human populations.\textsuperscript{31} Oleamide has been determined to have an EC$_{50}$ of 8.60 ± 1.20 mg/L on the growth of \textit{Microcystis aeruginosa}, presenting an easily obtained and biodegradable solution for bloom-forming cyanobacterial control in water supplies.\textsuperscript{32}
1.2.2. Erucamide

Erucamide (C22:1<sup>13</sup>) was first isolated and purified in 1990 from bovine omentum. It was found to be the major bovine mesentery angiogenic lipid with activity assessed by chorioallantoic membrane assay with as little as two micrograms of lipid. Angiogenic activity was also found to occur with rat corneal micropocket and mouse dorsal air-sac assays. Mitchell et al. showed further dose-dependent angiogenic properties with implantation of microgram quantities of erucamide into injured skeletal muscle. This resulted in an increased amount of large diameter blood vessel formation, particularly around the implant site. Conversely, higher doses of erucamide have shown fewer large diameter blood vessel formations, potentially due to a toxic effect of erucamide at high doses. Erucamide also modulates water balance. Hamberger and co-workers reported the isolation of a previously unidentified inhibitor of induced diarrhea from big blood plasma to be erucamide. An administered dose of 50 pmol/kg body weight (approximately 50 pM) of erucamide gave maximal inhibition of cholera toxin-induced intestinal fluid hypersecretion, as compared with 8.6 nM erucamide found endogenously. Although this dose only represents a marginal increase in erucamide concentration once distributed throughout the body, initial concentrations may be very high in the blood. Erucamide has also been found in the brain, cerebral spinal fluid, lungs, liver, and intestines. The mechanisms of action for erucamide’s angiogenic and anti-diarrheal activities, however, remain to be determined. In addition to its physiological properties, erucamide is also used, along with oleamide and stearamide, as additives in plastics, textiles, rubber, and lubricant oils.
1.2.3. Linoleamide

Linoleamide (C18:2\textsuperscript{9,12}) is another endogenous PFAM that has been found to induce sleep in cats, rats, and humans\textsuperscript{4}. Also similar to oleamide, linoleamide was enzymatically synthesized in the brains of stimulated rats\textsuperscript{37}. Huang and co-workers were the first to examine the effects of linoleamide on isolated cells by measuring changes in calcium ion concentration in Madin-Darby canine kidney epithelial tubular cells. Linoleamide was reported to induce intracellular calcium ion concentration increases with an EC\textsubscript{50} of 20 µM. It is believed that this occurs by the release of internal Ca\textsuperscript{2+} from the endoplasmic reticulum\textsuperscript{38}. Linoleamide and its metabolites have been found in the urine of rats dosed with linoleamide, suggesting that it may also play a role in activating ion transport in renal cells\textsuperscript{39}. Prolonged elevations in Ca\textsuperscript{2+} concentrations have led to cell injury and apoptosis, suggesting the possibility that linoleamide may be nephrotoxic\textsuperscript{40}.

1.3. PRIMARY FATTY ACID AMIDE BIOSYNTHESIS AND DEGRADATION

1.3.1. PAM Amidation

The biosynthesis, metabolism, and degradation properties of PFAMs have been a topic of interest to lipidomic researchers since the discovery of the physiological properties exhibited by endogenous oleamide. Divito and Cascio have compiled an extensive review outlining the progress that has been made in this field, while also highlighting PFAM physiology, receptor protein interactions, and current methods of analyses\textsuperscript{41}. The enzyme, peptidylglycine α-amidating monooxygenase (PAM), found in both the plasma and CSF
of mammals, is known to convert peptidyl glycines to their respective amide form and has a preference for hydrophobic peptide substrates.\textsuperscript{42-45}

The first reported naturally occurring non-peptide substrate for PAM was \textit{N}-myristoylglycine. PAM catalyzes the oxidative cleavage of \textit{N}-fatty acylglycines into PFAMs (and in this case, into myristamide), thus, proposing a catabolic pathway for the biosynthesis of PFAMS.\textsuperscript{46} Consistent with the finding that \textit{N}-myristoylglycine acts as a substrate for PAM is that the enzyme shows a preference in amidating glycine-extended peptide substrates having a hydrophobic amino acid at the penultimate position.\textsuperscript{42} This suggests that the majority of \textit{N}-fatty acylglycines will act as PAM substrates, and this has been proven with fatty glycines ranging from formylglycine through linoleoylglycine being amidated by purified recombinant type A rat PAM and verified by nuclear magnetic resonance (NMR) spectroscopy.\textsuperscript{47} These studies determined that the fatty glycines go through an intermediate form, identified as \textit{N}-acyl-\textit{α}-hydroxyglycine, prior to oxidative cleavage by peptidyl-\textit{α}-hydroxyglycine \textit{α}-amidating lysase (PAL).\textsuperscript{47}

Carpenter et al. analyzed PAM assays by HPLC showing the reactants, intermediates, and products for \textit{N}-octanoylglycine and \textit{N}-lauroylglycine (C8 and C12, respectively).\textsuperscript{48} HPLC chromatograms compared the relative abundance of all components during the PAM enzymatic conversion of \textit{N}-acylglycines to amides both in the presence and absence of Ac-Phe-pyruvate, a PAL inhibitor. Without the inhibitor added, the conjugate amides increased in abundance as both the fatty glycine and the intermediate \textit{α}-hydroxyglycine decreased relative to their abundance with the inhibitor added.\textsuperscript{48} Similarly, overexpression of PAM in N\textsubscript{18}TG\textsubscript{2} mouse neuroblastoma cells resulted in an increased flux of oleic acid to oleamide, while inhibition of PAM resulted in an accumulation of \textit{N}-
oleoylglycine, which was subsequently metabolized to oleamide.\textsuperscript{46, 47, 49} This was the first report of N-oleoylglycine from a biological system, and a three-step catabolic pathway was proposed for the biosynthesis of a PFAM beginning with the free fatty acid being converted into acyl-Coenzyme A (CoA), acyl-CoA converted into N-acylglycine, and finally N-acylglycine converted into a PFAM.\textsuperscript{46, 47, 49}

Cytochrome c has been discovered as an oleamide synthase from high percentage ammonium sulfate fractions of rat liver and kidney extract incubated with a radiolabeled acyl-CoA.\textsuperscript{50} Driscoll et al. were the first to report the ability of cytochrome c to catalyze the biosynthesis of oleamide from oleyl-CoA. The enzyme catalyzes the formation of acylglycine from acyl-CoA and glycine in the presence of ammonium ion and is significantly enhanced by hydrogen peroxide. The activity was found to be stable to both heat denaturation as well as limited exposure to proteolytic enzymes. The activity was found to display physiological temperature and pH, and was positively identified as cytochrome c by proteomic, biochemical, and immunologic analyses.\textsuperscript{50} These findings raise the possibility that PFAMs may be physiologically synthesized by a second or alternative mechanism of cytochrome c catabolism. In addition, it has been found that cytochrome c will catalyze formation of selected long chain fatty acyl amino acids. Mueller and Driscoll documented the successful catalytic formation of oleoylglycine from oleyl-CoA, glycine, and hydrogen peroxide as reactants.\textsuperscript{51} It appears likely that cytochrome c and PAM work together, with cytochrome c producing acylglycine from acyl-CoA, and PAM converting the acylglycine to the conjugate PFAM.

In addition to catalyzing the biosynthesis of PFAMs, cytochrome c exhibits the potential ability of generating a broad range of lipid signaling molecules and precursor
substrates for numerous other bioactive enzymes. The catalysis of bioactive messenger lipids by cytochrome c shows possible relevance to apoptosis as oleamide has been shown to inhibit gap junction communication.\textsuperscript{51} It is plausible that the failure in the biosynthesis of oleamide by cytochrome c may result in the spread of apoptosis via open gap junctions across healthy networks of cells, also known as bystander killing.\textsuperscript{26, 27} Immunoreactive cytochrome c has been found concentrated in secretory granules which contain PAM.\textsuperscript{52, 53} This localization suggests cytochrome c may play a role in intercellular communication, as the generation of oleoylglycine could be accomplished for the purpose of regulated secretion. Cytochrome c has also shown to catalyze the biosynthesis reaction with oleoyl-CoA using select amino acid substrates other than glycine as well as using other primary amines or long chain fatty acyl-CoA compounds as substrates.\textsuperscript{51, 54}

1.3.2. Primary Fatty Acid Amide Degradation

Fatty acid amide hydrolase (FAAH), a member of the amidase signature family, is a 65 kDa integral membrane enzyme consisting of a single N-terminal transmembrane domain. FAAH is one of the most highly characterized enzymes involved in the hydrolysis of bioactive lipids, inclusive of PFAMs. A prominent role of FAAH involves the termination of the signaling of endocannabinoids and oleamide in the central nervous system.\textsuperscript{55} FAAH was first reported in 1993 by Deutsch and Chin as anandamide amidase, which was capable of the hydrolysis of anandamide.\textsuperscript{56} This enzyme was found in several tissues, including liver and brain, and inhibited by phenylmethylsulfonyl fluoride, a serine protease inhibitor.\textsuperscript{56} Cloning of FAAH by Cravatt et al. in 1996 verified that FAAH exists as a single enzyme and that signal termination does not involve independent or combined
action of several different enzymes working together. Transfection into COS-7 cells showed high enzymatic activity in the membrane fraction of these cells. FAAH converted oleamide to oleic acid, but also converted anandamide to arachidonic acid, indicating that this enzyme can serve as a general inactivating enzyme for numerous PFAMs. FAAH differs from other members of the amidase signature family of enzymes in that it is an integral membrane enzyme, consisting of an N-terminal transmembrane domain that was not present in related familial enzymes, suggesting a straightforward mechanism for membrane binding. Deletion of the first 30 amino acids produced active membrane-bound enzyme, signifying the likelihood of multiple pathways for membrane integration.

1.4. ANALYSIS TECHNIQUES

1.4.1. Extraction and Isolation

Sample preparation of PFAMs often involves either extraction from biological tissue or isolation from similar fatty acyl lipid subclasses. The most well-known method of total lipid extraction from biological tissue involves the use of the Folch-Pi extraction. Originally used to prepare and purify brain lipids, the Folch-Pi extraction involves a two-step process. The first step requires tissue to be homogenized in 2:1 chloroform:methanol (v/v) at 20-fold the volume of tissue and the homogenate is filtered. Next, the total lipid-containing filtrate is added to 0.2-fold water, with the lower-phase containing the total pure lipid extract. Aqueous washing of the lipid-containing organic layer has been shown to remove essentially all of the non-lipid contaminants with minimal tissue-lipid loss. Following the Folch-Pi extraction, isolation of the desired fatty acyl lipid subclass can be
achieved using solid phase extraction (SPE).\textsuperscript{60,61} PFAMs were isolated from the total lipid extract from N\textsubscript{18}TG\textsubscript{2} mouse neuroblastoma cells using a Discovery (DSC)-Si SPE column. With the total lipid extract loaded onto the column, a series of increasingly polar elution steps followed, with the final fraction being found to contain PFAMs, monoacylglycerols (MAGs), N-acyl glycines (NAGs), and N-acyl ethanolamines (NAEs). This fraction was subjected to a second iteration of SPE using a DSC-NH\textsubscript{2} column. Another series of elution steps proceeded, with PFAMs being isolated to a fraction containing 3\% 2-propanol in chloroform. Percent recoveries of PFAMs by this SPE method were found to be greater than 80\% from 0.5 to 20 \( \mu \)g with 0.5 \( \mu \)g being the lowest possible mass to be reliably loaded and isolated.\textsuperscript{61} SPE lipid fractions can be quickly analyzed by thin-layer chromatography (TLC) with a three-stage development procedure. When separating lipid classes using TLC, visualization occurs by staining the plates with a primuline solution, which non-covalently binds to the lipids, and becomes visible under ultraviolet (UV) radiation.\textsuperscript{61}

1.4.2. High Performance Liquid Chromatography

Individual PFAMs can be chromatographically separated from one another via liquid and gas chromatography. When dealing with fully saturated PFAMs, a reverse phase HPLC column will follow an elution order relative to acyl chain length, with the shortest chain PFAM eluting first. PFAM separation usually requires the use of a C18 stationary phase column, however, slightly more polar C8 columns can also be used when the acyl chain is twelve or less carbons in length.\textsuperscript{48} Points of unsaturation in the PFAM (double bonds) result in an earlier elution than its corresponding fully saturated analog. Isocratic elution may be sufficient when separating a mixture of fully saturated PFAMs, however,
for more complex mixtures including PFAMs with one or more points of unsaturation, a
gradient elution profile must be developed with a mobile phase consisting of water and
either acetonitrile or methanol. The term “critical pair” results from a certain PFAM
containing one double bond having approximately the same retention as a fully saturated
PFAM containing an acyl chain of two fewer carbon atoms. Due to the highly nonpolar
acyl chain, PFAMs do not retain well using normal phase chromatography, however, this
technique can be applied to separating classes of polar neutral lipids from a total lipid
extract. Normal phase chromatography can successfully isolate fatty acyls,
triacylglycerols, diacylglycerols, MAGs, NAGs, PFAMs, and NAEs. Although a common
theme amongst polar neutral lipids are varying length acyl chains, the differences in head
group polarity allow for normal phase separation. This same principle has also been
applied to the separation of lipid classes using TLC.61

1.4.3. Gas Chromatography

Gas chromatography (GC) can also be used to separate either derivatized or
underivatized PFAMs. The most common technique of PFAM derivatization involved the
use of bis-trimethylsilyltrifluoroacetamide in toluene. This produced the highest signal to
noise ratios (S/N) when paired with ion trap mass spectrometry.62 Detection limits
approached the low picomol range with the use of selected ion monitoring (SIM). Further
work involved exploring derivatization techniques with a series of unsaturated PFAMs,
however, derivatization lacked adequate efficiency at low concentrations.61 Underivatized
PFAM analysis by GC/MS is successful in separating a series of saturated PFAMs as well
as PFAMs with varying points of unsaturation, however this technique falls short with its inability to distinguish by bond position due to the lack of column selectivity.\textsuperscript{61}

### 1.4.4. Detection Techniques

Detection of PFAMs is of particular importance as the technique must allow for sensitivity encompassing and surpassing the trace and ultra-trace abundance of these biological molecules. Arguably the most commonly used detection technique for HPLC is Ultraviolet-Visible (UV-Vis) spectroscopy. HPLC-UV-Vis detection at 210 nm (PFAMs) and 214 nm (NAGs) was used in developing a method to evaluate the PAM-mediated enzymatic conversion of NAGs to PFAMs by separating a series of PFAMs (C\textsubscript{2}-C\textsubscript{22}) and NAGs (C\textsubscript{2}-C\textsubscript{12}). UV-Vis detection resulted in detection limits estimated to be approximately 100 µM (1 nmol), which proved adequate for kinetic assays of PAM under physiological conditions.\textsuperscript{48}

The use of MS can complement both GC and HPLC techniques. PFAM separation using GC is typically detected using a mass selective detector. As previously mentioned, mass detection limits using an ion trap MS can approach low-picomole detection limits, however, derivatization techniques can be time and sample consuming and column selectivity can present separation problems.\textsuperscript{61-63} A soft-ionization mass spectrometry technique, atmospheric pressure chemical ionization (APCI), was used with HPLC to detect PFAMs in tallow with detection limits reaching the low femtomol range.\textsuperscript{64} Depending on the type of MS, SIM or multiple reaction monitoring (MRM) can be utilized to lower detection limits, allowing for trace analysis. Hard ionization sources such as electron impact (EI) ionization with GC allows for the nearly complete fragmentation of
the molecular ion. The resulting characteristic fragmentation patterns can be used to elucidate structural information or compared with fragmentation databases for component identification, when applicable. Tandem mass spectrometry (MS-MS) enabled the collection of collision induced dissociation (CID) patterns of saturated and unsaturated PFAMs using a quadrupole time of flight MS with electrospray ionization (ESI). CID resulted in characteristic spectra that were used to propose fragmentation mechanisms, which were able to differentiate saturated versus unsaturated PFAMs quickly and accurately. Additional tandem MS quantitation of NAGs in Sprague-Dawley rat brain showed quantitation limits in the picomole (6-895 pmol) range per gram of tissue using HPLC-MS-MS with a triple quadrupole (QqQ) detector in MRM mode.

As trace and ultra-trace detection methods are required for endogenous PFAM analysis and quantitation, fluorescence spectroscopy yields the potential of single-molecule detection limits when utilizing fluorophores with high quantum yields. Analytes such as PFAMs do not contain conjugated π-bonding systems capable of native fluorescence, therefore they must be derivatized, or fluorescently tagged, with an appropriate fluorophore, with the resulting fluorescence response directly proportional to analyte concentration. Furthermore, the growing trend of lower detection limits coinciding with “green” chemistry has popularized microfluidics with the capabilities of providing a total analysis system with “lab on a chip” technologies. The coupling of microfluidics with laser induced fluorescence (LIF) has the potential of being the most sensitive detection system to date for analysis of PFAMs and is further discussed in the subsequent chapters.
1.5. SUMMARY

Fatty acyls comprise a highly studied class of lipidomics focused largely on signaling. Of this specific class, a wide range of primary fatty acid amides have been found to be endogenously present and display a gamut of physiological properties. Oleamide is perhaps the most studied with initial research near the turn of the twentieth century linking it to sleep deprivation. Additional properties show similarities to the endocannabinoid, anandamide, resulting in the likelihood of oleamide acting as a cannabinoid receptor agonist. Oleamide also displays vasorelaxant, hypnotic, analgesic, and anxiolytic effects, stimulates overeating, and blocks gap junction signaling, amongst numerous others. Erucamide, like oleamide, has shown effects relating to sleep induction and angiogenesis as well as being used as additives in various synthetic materials. Erucamide has been found in several bodily organs and shown to modulate water balance. Linoleamide has been shown to increase calcium ion concentrations and activate ion transport in renal cells, albeit displaying potential nephrotoxicity.

The biosynthesis of PFAMs centers on PAM, the enzyme known to convert peptidyl glycines to their respective amide form via post-translational modification. Working harmoniously with PAM, cytochrome c catalyzes the synthesis pathway from acyl-CoA to the acyl-glycine form in the presence of ammonium ion, while PAM then converts the acyl-glycine to its conjugate PFAM form. The enzyme FAAH is responsible for the degradation of PFAMs by hydrolyzing the bioactive lipids. FAAH is also involved in the termination of the signaling of endocannabinoids and oleamide in the central nervous system.
PFAMs can be extracted from biological tissue using a Folch-Pi extraction technique where the analyte is solubilized in an appropriate solvent prior to a liquid-liquid extraction. Purification using solid phase extraction is followed prior to highly sensitive analysis and detection techniques. Both gas and liquid chromatography are often used to separate mixtures of PFAMs. Normal-phase liquid chromatography is suitable to separate out entire lipid classes, whereas reversed-phase liquid chromatography is used to separate out mixtures of lipids within a class. Direct detection of PFAMs can be accomplished using a mass selective detector, however, conversion to their conjugate amine form and subsequent fluorescent tagging provides the greatest sensitivity with a suitable fluorescence-based detector.
Chapter 2

REVIEW OF MICROFLUIDICS

2.1. BACKGROUND AND SIGNIFICANCE

The concept of microfluidics is broadly defined as a system that either processes or manipulates small volumes ($10^{-9}$ to $10^{-18}$ liters) of fluids within channels on the micrometer scale. Microfluidics presents a gamut of useful capabilities inclusive of the minimization of sample and reagent usage, analysis cost reduction, specificity of device design, high resolution chromatographic separations, highly sensitive detection pairing, and decreased analysis times. Several recently published reviews expand upon these capabilities and present new and innovative applications of this growing field. Various fabrication techniques exist, which can all be performed relatively inexpensively, as well as in-house. A few of the more common materials used in microfluidic device fabrication are polymer based with polydimethyl siloxane (PDMS), glass, thermoplastic, paper, and silicon. The actual technique of fabrication can vary widely based on materials used and application, however, the most common consist of soft lithography, wet etching, microcontact printing, and substrate bonding.

Another advantage of microfluidics is the ability to achieve a “Lab on a Chip”, otherwise known as a micro total analysis system ($\mu$TAS). A true $\mu$TAS begins with the introduction of the fluidic sample or analyte, followed by reaction, separation, or isolation steps, and finally an applicable detection method. The introduction of sample or analyte into a microfluidic system is often accomplished by pressure driven flow. Once within microchannels, the fluidic flow is intrinsically laminar in nature, with the sole form of
mixing being simple diffusion between and within fluidic layers. In order to accomplish efficient mixing and reactions on-chip, some form of channel manipulation must be made to reorient the flow path. Reaction efficiency may also be increased by the production of droplets within the flow. Separation of analytes within a mixture can be achieved within a microfluidic device via the introduction of stationary phase packing material. The packing material can be incorporated into the device and held in place using frits or fritless weirs designed into the device fabrication procedure. Finally, several detection systems can be seamlessly implemented with the use of a microfluidic device such as spectroscopic, fluorescence, or mass selective techniques. In this chapter, we review common device substrates and fabrication techniques, droplet formation and manipulation, and microfluidic detection systems.

2.2. MICROFLUIDIC DEVICE FABRICATION TECHNIQUES

2.2.1. Polymer Based Devices

Of all the materials used for fabrication of microfluidic devices, the most commonly used are those consisting of polymers or elastomers. Elastomeric microfluidic devices share common advantages over more traditional substrates (glass- and silicon-based devices) such as reduced fabrication cost, simplified manufacturing procedures, and a wide range of available polymeric materials. Some of the various polymers used include PDMS, polystyrene, polypropylene, polyvinylcarbonate, polycarbonate, and polymethyl methacrylate. PDMS is the most commonly used polymer for microfluidic device fabrication due to many advantageous attributes, inclusive of its elastomeric properties,
biocompatibility, gas permeability, optical transparency, ease of molding sub-micron features, ease of self-bonding and glass-bonding, high chemical inertness, and low manufacturing costs.\textsuperscript{86, 87}

Initial fabrication of polymer based microfluidic devices is usually achieved via photolithography. Photolithographic fabrication of polymeric material is easier, more flexible, and more cost-effective than fabrication techniques used for devices comprised of glass or silicon. Also, the ease of polymeric device fabrication allows for usefulness in quick development of prototypical devices. The process of photolithographic fabrication of a PDMS microfluidic device is described in detail by McDonald and Whitesides.\textsuperscript{76} The first step in photolithographic fabrication requires the production of a master mold. This starts with the design of a photomask that contains the networks of channels and various spatial features required. The photomask can either be machine-fabricated with metallic coatings or designed and printed in-house with computer drawing software packages and a high resolution printer.\textsuperscript{78} Metallic photomasks, often consisting of chrome, can achieve spatial resolution as low as three micrometers, while lower-cost, high-resolution printed transparencies is often twenty micrometers or greater. A clean silicon wafer is coated with an epoxy-based negative photoresist to the desired thickness. The photoresist is important in defining channel depth profiles in the finished device. A pre-fabricated photomask is placed over the resist, and irradiated using a high power UV lamp, causing the exposed channel regions of the resist to crosslink. The exposed resist-coated wafer is developed in solvent that washes away the non-crosslinked region, leaving behind the master mold that can be used indefinitely.
PDMS is often supplied as two independent components, a base and a curing agent. When mixed at an optimal 10:1 (v/v) base:curing agent ratio, silicon hydride from the curing agent reacts with the vinyl groups present in the base to form a cross-linked, elastomeric solid. The liquid pre-polymer is poured over the master, cured, and then easily removed without damaging the master due to the low surface free energy and elasticity of the PDMS. PDMS has the ability to seal to either itself or to other surfaces, reversibly or irreversibly, without damaging or distorting channel features. For devices not designed to incorporate pressure-driven flow, PDMS provides a reversible seal by van der Waals contact that is watertight up to 5 psi. Additional pressure resistance can be added to reversibly-sealed devices with the use of adhesive tapes. Irreversible seals are often formed by exposure of the PDMS and the surface to which it will be sealed to air plasma, which serves multiple purposes. The plasma effectively cleans any organic contaminants on the surfaces and generates silanol groups by oxidation of methyl groups on the surface of PDMS. Plasma treatment allows for PDMS to seal to a multitude of components, such as glass, silicon, polystyrene, polyethylene, silicon nitride, or itself. Quick contact of the two surfaces must be made for a strong seal to occur as the oxidized PDMS surface will begin to reconstruct in the presence of air within a minute.

2.2.2. Paper Based Devices

Increasingly recognized as a low-cost, portable, disposable, and user-friendly microfluidic substrate, microfluidic paper-based analytical devices (µPADs) provide alternative technologies for diagnostic tools and point of care testing. These devices are often found to be useful with disease screening in developing countries with minimal
infrastructure and/or limited trained health care professionals. \textsuperscript{80} μPADs were first introduced in 2007 by Martinez et al. by patterning paper to create millimeter-sized channels.\textsuperscript{79} These devices can be used with miniaturized equipment to perform diverse functions, such as fluidic handling and quantitative analysis on the micro-scale, rendering them useful as a multiplexable point of care testing platform for use in pharmaceuticals and healthcare, as well as applications within environmental monitoring.\textsuperscript{104-106}

Depending on desired complexity and diagnostic applications, numerous two and three-dimensional fabrication techniques exist for producing paper-based devices. Two-dimensional fabrication techniques consist of direct printing (wax, inkjet, flexographic, screen, and wax-screen), photolithography, plasma and laser treatment, and wet etching. Many of these methods can also be similarly applied to fabrication of microfluidic devices consisting of various other substrates. These techniques primarily function by treatment of paper to convert hydrophilic sections of the paper to become hydrophobic, creating fluidic channels.\textsuperscript{80} The simplest, cheapest, and fastest two dimensional fabrication technique involves wax printing. Cai et al. describes the wax printing fabrication process, using only filter paper, a wax pencil, and an oven.\textsuperscript{107} Fluidic channels were drawn by hand with the wax pencil onto the filter paper using a template. The patterned paper was then briefly baked in an oven, causing the wax to melt and penetrate through the paper, forming hydrophobic walls, able to completely contain the liquid within. This device was able to perform a simple colorimetric assay capable of quantitatively analyzing amino acid content with a tea sample.\textsuperscript{107} The simplistic nature of wax-printed microfluidic devices also proves useful as a μPAD prototype for implementation of inexpensive bioassays.\textsuperscript{108} To fully automate the application of wax to the hydrophilic paper, a wax printer can be used.\textsuperscript{81}
Inkjet printing can be used to automate the hydrophobic fluid containment channels, resulting in higher precision microfluidic patterns. This can also be used as a precise deposition source of biomolecules and indicator reagents, creating complete high-volume biochemical sensing devices at low-cost. Photolithographic fabrication techniques pattern hydrophilic zones, or channels, bound by a hydrophobic polymer. Martinez et al. describes the photolithography process for fabricating a simple paper microfluidic device used for colorimetric glucose and protein assays in urine. Briefly, chromatography paper was soaked in an epoxy-based photoresist, spin coated, baked, and exposed to UV radiation through a photo-mask. Exposed portions of the photoresist were then cross-linked, and the unpolymerized region removed with propylene glycol monomethyl ether acetate before oxygen plasma cleaning to increase hydrophilicity. Clinically relevant ranges of glucose and bovine serum albumin were detected at comparable to or lower than detection limits for commercially-available colorimetric test strips.

Plasma treatment fabrication involves salinization of the paper using octadecyltrichlorosilane (OTS), or alkyl ketene dimer to render it hydrophobic, followed by plasma treatment using a mask containing channel features. The plasma degrades the hydrophobic OTS-cellulose fiber bond, rendering the exposed region hydrophilic. A caveat of plasma treatment, however, results from the long mean free paths in the energetic particles found in the plasma, causing over-etching of the substrate underneath the mask, thus resulting in the desired pattern to be larger than the mask. The degradation of OTS, and thereby degree of hydrophilicity and extent of over-etching, can be precisely controlled based on the length of exposure time. Plasma treated devices can be advantageous in that
functional elements such as switches, filters, and separators can be easily incorporated into the microfluidic system.\textsuperscript{110,111}

Laser-treatment fabrication uses polymerization of a photopolymer to produce desired fluidic channels or containment wells within the paper. This fabrication technique has produced leak-proof hydrophobic barriers as small as 120 µm and fluidic channels as small as 80 µm, which is the smallest reported paper-based fluidic pattern.\textsuperscript{112} The surface structure of devices using paper with a hydrophobic surface coating, such as wax paper, can be selectively modified and rendered hydrophilic using a CO\textsubscript{2} laser. The modified surface was shown to be highly porous, which helped to trap or localize chemical and biological aqueous reagents for analysis.\textsuperscript{113}

A novel, simple, and cost-effective approach for µPAD fabrication involves selective wet etching of hydrophobic filter paper by a paper mask. Ordinary hydrophilic filter paper was made highly hydrophobic using 2% trimethoxyoctadecylsilane. The paper mask, containing specific channel design features, was soaked in a sodium hydroxide solution and aligned onto the filter paper. The sodium hydroxide etching agent in the mask selectively etched the hydrophilic channel design into the filter paper, allowing for rapid fabrication within five minutes.\textsuperscript{114} Renault et al. demonstrated that simple and inexpensive carbon electrodes can be used as bipolar electrodes (BPEs) and screen printed onto standard µPADs.\textsuperscript{115} Electrode patterns were photolithographically created onto a mesh using a screen printing kit, and the carbon electrodes were strategically screen printed onto µPAD using the patterned mesh as a mask. Two driving electrodes connected to a power supply were sufficient to produce electrogenerated chemiluminescence on a µPAD containing an
array of 18 BPEs, showing the potential of conducting highly multiplexed electrochemical measurements on simple paper microfluidic devices.\textsuperscript{115}

\subsection*{2.2.3. Glass Based Devices}

As micro- and nanofluidic technology continues to evolve, the applications have expanded along with the vast array of materials and processes for fabrication of such devices. Glass based devices were of the earliest microfluidic devices developed with processes derived from semiconductor and microelectromechanical systems manufacturing.\textsuperscript{82-84} Although PDMS-type elastomers are primarily used as microfluidic device substrates due their ease, quick, and low-cost manufacturing processes, many applications exist where glass remains advantageous.\textsuperscript{85} Microfluidic applications that demand high temperature resilience, high aspect ratio structures, integration of electrodes or electronic circuits, or precise definition of nano-scale channels still routinely demand the use of silicon- or glass-based devices.\textsuperscript{85, 116, 117}

The most common form of glass microfluidic chip fabrication is bulk micromachining, where channel features are selectively removed from a silicon/glass wafer and channel encapsulation occurs by bonding the chip to a second wafer. Glass patterning is completed using either wet or dry etching. Wet etching occurs by a process initially similar to that of photolithography, where a glass wafer is spin-coated with a chrome layer followed by a photoresist later. The wafer is exposed to UV light through a photomask, and the exposed regions of the photoresist, followed by the exposed chrome, are sequentially removed by separate chemical treatments. The exposed glass is wet etched in an isotropic fashion typically using a hydrofluoric acid (HF) solution. Once the desired
degree of etching is achieved, the remaining photoresist and chrome layers are washed away.\textsuperscript{118} It is desirable to use glass with low content of oxides such as CaO, MgO, and Al\textsubscript{2}O\textsubscript{3} as these oxides result in insoluble products following the HF etching reaction.\textsuperscript{88}

The etch rate is largely dependent on the composition of the glass, but can be increased by raising solution temperature or incorporating ultrasonic agitation, and reduced by diluting the HF solution.\textsuperscript{119} Using a 49\% HF solution, Corning 7740 glass has an etch rate of 8 µm/min, while quartz has an etch rate of 1.3 µm/min under the same conditions.\textsuperscript{120} Dry etching of glass is a much more tedious and less reproducible process than that of wet etching, and thus is typically only used when an anisotropic etching profile is desired on a glass substrate. Common dry etching rates are approximately 0.5 µm/min and usually performed by a plasma etch in an inductively coupled plasma reactive ion etching reactor (ICP-RIE).\textsuperscript{85} High temperatures resulting from the large energy transfer can cause glass wafers to fracture, therefore, care must be taken to precisely control temperature gradients. Gas precursors used for plasma etching can be SF\textsubscript{6}, C\textsubscript{4}F\textsubscript{8}, CF\textsubscript{4}, or CHF\textsubscript{3} and a thick masking layer of electroplated Ni, silicon, or SU-8 photoresist is required due to the relatively low selectivity of the etching process.\textsuperscript{121, 122}

Direct bonding of glass microfluidic chips to another glass surface is required to provide enclosed fluidic channels within the device. Simple van der Waals force keeps two clean, flat glass surfaces in-tact with a bond strength of only 0.6 MPa.\textsuperscript{123} As glass begins to soften at high temperatures, fusion bonding of glass microchips can occur at a temperature of 650°C using an annealing furnace. Care must be taken to gradually cool the glass after annealing to prevent breakage.\textsuperscript{124} Fusion bonding can be combined with glass surface activation from a calcium solution or nitrogen radical activation by a
microwave reactor following an oxygen RIE treatment.\textsuperscript{125} Using the latter glass pretreatment followed by 600°C annealing temperature, bond strengths of up to 24 MPa were achieved.

\subsection*{2.3.1 DROPET-BASED MICROFLUIDICS}

The trend of miniaturization has enabled important technological advancement in recent decades in fields such as electronics, with transistors per integrated circuit roughly doubling every year since their invention in 1961, however, this trend in chemistry and biology has been pronouncedly less dramatic.\textsuperscript{126} Miniaturization of reaction volumes has been reduced only about 1,000 fold over this time, from milliliter volumes in test tubes to microliter volumes in microliter plates. Intrinsic problems in miniaturizing reaction volumes lie in evaporation rates and capillary action.\textsuperscript{127,128} The use of microfluidic devices have the potential to overcome these issues by droplet technology, which first came to realization in 2001 when mono-disperse droplets were formed within polyurethane microfluidic chips.\textsuperscript{129} Droplet-based microfluidics is a subcategory of microfluidics built around the premise of creating discrete volumes of fluid using immiscible phases. More traditional microfluidic systems incorporate continuous and laminar flow profiles, however, droplet microfluidics provide an alternative to continuous flow with a solution to overcome the challenges of laminar flow.\textsuperscript{130}

Droplets in microfluidics essentially serve as transport and reaction vessels, and are selectively introduced in pico- to nanoliter volumes at high throughput within encapsulated channels capable of mixing.\textsuperscript{131} Rapid reaction times are capable within droplets due to their high surface area to volume ratios, their efficient heat and mass transfer, and a small
Droplet manipulation can be automated or semi-automated by electrokinetic or hydrodynamic driven flow.\textsuperscript{132, 133} Droplet-based reactions are used for numerous chemical and biological applications, such as chemical synthesis, microfractionation, reaction kinetics monitoring, and particle formation, among others.\textsuperscript{134, 135}

\subsection*{2.3.2 Droplet Formation}

To prevent inter-droplet diffusion or droplet dilution by surrounding solvent flow, an immiscible carrier phase is often used. Fluorocarbon oils provide hydrophobic properties, which essentially eliminate diffusion of water-soluble compounds between droplets while causing less substrate swelling in PDMS-based microfluidic devices than hydrocarbon oils.\textsuperscript{136, 137} Droplets are most commonly generated via a T-junction geometry where a reagent channel intersects the carrier phase channel in an orthogonal arrangement, with droplet formation occurring at the intersection.\textsuperscript{92} Another method of droplet generation is flow focusing, where the carrier phase is supplied by symmetric channels surrounding the reagent-flowing channel. The carrier phase flow forces the reagent phase flow through a constricted channel feature leading into a larger channel. The pressure from the carrier phase pushes reagent through the constriction, and provides shear forces to separate the reagents into droplets.\textsuperscript{92} Both droplet-formation techniques (illustrated in Figure 2-1) can control droplet size, velocity, and frequency by adjusting flow rates, inlet pressures, or changing phase viscosities.
Figure 2-1. Illustration of two common methods of droplet formation within microfluidic devices. The use of a standard T-Junction (A) introduces the reagent fluid to the carrier fluid in an orthogonal manner. When the carrier fluid flow rate is greater than that of the sum of all of the reagent fluids, the reagent fluids will taper off into individual droplets. In a flow-focusing design (B), the carrier fluid forces the reagent fluid through a constricted feature in the channel. The increased pressure through the constricted feature causes sufficient shear force to form droplets from the reagent stream.

2.3.3 Droplet Manipulation

Analyses utilizing droplets can benefit from the ability to manipulate droplets, often by either division, fusion, or sorting. Division of droplets can be completed using an additional T-junction, where the droplet flows to the branch point, and is stretched out by the extension flow. Large enough droplets will become thin enough to split into two or more subsequent droplets. A flow-focusing technique can be used where the droplet flow is directed into a Y-junction, with carrier phase flow merging on both sides of the
droplet flow, causing droplet splitting along its length. The size and frequency of these daughter droplets can be controlled by changing flow rates of the additional carrier phase.\textsuperscript{139}

The ability to control fusion of droplets can be a critical step in chemical or biological on-chip reactions. For droplet fusion to occur, it is necessary to bring individual droplets into physical contact with one another in order to overcome the stabilizing effects provided by the carrier phase.\textsuperscript{140} Passive fusion of droplets may be achieved by geometrical features in the channel design. To get droplets in close enough proximity to one another, the downstream droplet flow must be slowed relative to the upstream droplet flow. This can be achieved by incorporating a channel feature where the dimensions are abruptly and briefly expanded so that trailing droplets can merge into those ahead as the droplet expands into the feature opening.\textsuperscript{139} Viscosity ratios of droplets and carrier fluid, as well as the presence of surfactant at their interface, play a significant role in droplet fusion. When the droplet has a lower viscosity, the interfacial film between fluids will easily rupture, resulting in droplet coalescence.\textsuperscript{141} In contrast, when the droplet viscosity is greater, the interface becomes less mobile, increasing the difficulty of coalescence. The presence of surfactant at the interface of the two phases will also inhibit fusion.\textsuperscript{89, 142}

Effective mixing within a droplet is of the upmost importance for rapid analysis and minimizing reaction times. Continuous flow microfluidics exhibits laminar flow due to low flow rates, as well as small channel dimensions, and mixing must rely on diffusion.\textsuperscript{89} Mixing efficiency is affected by the length of the fluid droplet or plug in which mixing is occurring, where longer droplet plugs are significantly less efficient in mixing than small, tight droplets. Mixing efficiency is also dependent on reactant dispersion within the
droplet, which is heavily dependent on the method of droplet formation. A process known as chaotic advection facilitates extremely rapid mixing of reagents within droplets through interaction of the droplet with all walls of a microchannel. The flow within droplets is continuously recirculated, resulting in shearing interactions of the droplets with channel walls. This shearing process is exacerbated by droplet flow through winding channels and is illustrated in Figure 2-2.

Droplet sorting is used to select specific droplets from a larger population, and their movement is influenced in either a serial or parallel fashion. Necessary forces for movement are often based on fluidic flow, acoustic waves, or coulombic attraction and repulsion. An electrically driven technique, such as dielectrophoresis, can quickly sort water droplets at a frequency of over 1.6 kHz by use of microelectrodes fabricated underneath a PDMS microfluidic device. Water droplets have also been manipulated using a microelectrode array and alternating current voltages across the electrodes at frequencies ranging from 50 Hz to 1 MHz.
Figure 2-2. Process of chaotic advection within droplets in a mixing region of a microfluidic device. Beginning with a droplet containing two discrete fluidic layers, the frictional forces induced on the droplet by the channel walls cause continuous refolding and recirculation of the droplet. As mixing within droplets is primarily based on inter-layer diffusion, the multiplication and subsequent thinning of fluidic layers results in highly efficient mixing within droplets.

2.4 Digital Microfluidics

Although similar to traditional microfluidics with respect to small volume analysis, digital microfluidics (DMF) approaches micro total analysis systems by manipulating individual droplets. These droplets move about on an open or closed chip via the application of electrical potentials from an array of electrodes. Droplets are deposited above an array of electrodes coated with a hydrophobic insulator. As an electrical potential is applied sequentially to adjacent electrodes, the droplets will move in the direction of applied current. During this process, known as electrowetting, the potential applied by the electrode charges the surface of the insulator, making it attractive for the fluid to wet the surface by decreasing the droplet-surface contact angle. This electrical manipulation process allowing for the automation of dispensing, transport, splitting, merging, and mixing of droplets. The advantage of DMF lies within its broad range of applications. Immunoassays were performed by the Sista group to detect insulin, interleukin-6, and cardiac troponin I by immobilizing the analytes using magnetic beads. On-chip glucose assays have been performed for concentration analysis in whole blood, serum,
plasma, urine, saliva, sweat, and tears.\textsuperscript{155-157} To avoid problems such as sample evaporation or air contamination, droplet-based bioluminescence assays of adenosine triphosphate using luciferase were conducted within an immiscible oil-phase.\textsuperscript{158} Other enzymatic applications of DMF have investigated pre-steady-state kinetics of tyrosine phosphatase phosphorylation ratios using matrix assisted laser desorption ionization (MALDI) – time of flight (ToF) - MS and the conversion of fluorescein diphosphate to fluorescein by mixing droplets with alkaline phosphatase.\textsuperscript{159, 160} Deoxyribonucleic acid (DNA) sample handling and purification were achieved via liquid-liquid extraction from a mixture of DNA and proteins.\textsuperscript{161} Damaged oligonucleotides, merged with a DNA repair enzyme, were repaired and this repair was subsequently verified using fluorescence microscopy.\textsuperscript{162}

By embedding a micro-heater to facilitate thermal cycling, polymerase chain reaction (PCR) has been conducted on-chip with DNA amplification comparable to benchtop methods with the added advantage of improved total analysis time and reduced sample consumption.\textsuperscript{153, 163, 164} The ability of DMF to individually control multiple reagents simultaneously makes it a beneficial system to use for proteomics. Macroscale protein purification requires multiple steps, relatively large reagent volumes, and time. Early DMF-proteomic applications demonstrated that six simultaneous peptide and/or protein samples can be purified from heterogeneous mixtures simply via sample drying and subsequent rinsing, followed by analysis using MALDI-ToF-MS.\textsuperscript{165-167}

For protein extraction and purification from more complex biological matrices, several important steps such as precipitation, rinsing, resolubilization, reduction, alkylation, and digestion have been incorporated on-chip.\textsuperscript{168-170} A form of “hybrid”
microfluidics integrates DMF with the more familiar channel microfluidics for in-line sample processing and separation. Following protein purification and digestion, the sample can be driven by DMF to the corresponding microchannel for separation using a packed stationary phase or capillary electrophoresis. Recently, dried blood droplets have been used in conjunction with DMF for the quantification of amino acids. The samples are processed on-chip, employing similar methods to standard fluid droplets; significantly, this technique negates the need for centrifugation or freezing of the sample, and allows for simplified transportation and cataloguing.

2.5 DETECTION TECHNIQUES

2.5.1 Spectroscopy

The use of spectroscopy, or the interaction of matter with electromagnetic radiation, is one of the most commonly used detection techniques with microfluidics as it can be sensitive and nondestructive. A simple absorbance detector using light-emitting diodes as the excitation source and optical fibers was developed for flexible integration at multiple device channel points for high-throughput droplet screening. Fourier transform infrared spectroscopy (FTIR) was used to image fast dynamic processes taking place within flowing droplets. Rapid scanning times of 50 ms allowed for active reaction monitoring within droplets. Additional spectroscopic techniques to monitor droplet-based reactions were Raman and surface-enhanced Raman spectroscopy (SERS). Kinetic studies of polymerization reactions within droplets were achieved using confocal Raman spectroscopy. SERS was used to provide high-speed detection for droplets containing
functionalized silver nanoparticle aggregates. Characterization of these nanoparticles was completed with sub-millisecond time resolution.\textsuperscript{175} Although spectroscopic techniques such as absorbance, FTIR, and Raman typically provide high-throughput, label-free detection with applications for biological assays and reaction monitoring, the need for more highly sensitive analysis and quantification should rely on either fluorescence or MS, or a combination of the two.

2.5.2 Fluorescence

Fluorescence detection can be used as either a highly sensitive and quantitative detection technique or a qualitative imaging detection mechanism. Microfluidic devices suit both techniques well due to their high optical transparency. Fluorescent microscopy often uses cameras to image fluorescing droplets and can measure single cell enzymatic activity. LIF incorporates a laser as an excitation source, optical filters to minimize extraneous noise, and photodetectors to quantitate orthogonal fluorescence photons.

The fluorogenic reagent, 4-methylumbeliferyl-\(\beta\)-\(\eta\)-glucuronide, was used to monitor enzyme activity by time-lapse fluorescence imaging of droplets.\textsuperscript{176} A custom-designed microfluidic device detected rare, aberrant cells in a population of wild-type human cells through rolling circle-enhanced enzyme activity single molecule fluorescence detection assay.\textsuperscript{177} The high sensitivity allowed for multiplexed detection of individual enzymatic events at the single cell level. A device known as “DropLab” was developed as a multifunctional droplet-based microfluidic platform that provides picoliter scale droplet manipulation with single-cell enzyme activity detection with a fluorescence microscope.\textsuperscript{178} A droplet microfluidic platform with fluorescence imaging setup was designed to meet
needs of high-throughput and high-dynamic range with multiple droplet processing schemes within a single chip. The chip design incorporated PCR amplification and fluorescence detection and generated over one million 50 pL droplets within 2-7 minutes capable of simultaneous visualization and analysis.\textsuperscript{179} In other studies, a microfluidic device coupled with LIF was used to detect glutathione concentrations within single erythrocytes collected from people in different age ranges and pathological states.\textsuperscript{94} Cells were injected, separated, and detected on-chip, with cell population and single cell data simultaneously collected from electropherograms.

2.5.3 Mass Spectrometry

While most optical detection techniques using droplet microfluidics require some form of chemical derivatization to analyze a reaction, MS presents the possibility of label-free or derivatization-free analysis. In addition to high sensitivity, MS can provide information about mass to charge ratios and/or structural information based on ion fragmentation patterns, commonly predicated by the combination of two or more mass analyzers. Due to the effluent from a microfluidic chip already being in liquid form, ESI is a common detection choice for microfluidic analysis. Much work has been reported on techniques of coupling droplet microfluidic systems to ESI-MS.\textsuperscript{96, 97, 180-182} The primary interfacial procedures involve either directly flowing the droplet stream into the ESI source or extracting the droplets into the source. Maintaining segmented flow into the source allows samples to remain encapsulated until fragmentation and detection, avoiding dilution and sample carry-over.
ESI-MS has been coupled to nanoliter scale flow from segmented flow microfluidics to screen inhibitors for cathepsin B. A series of T-junctions add enzyme, substrate, and quenchant to an array of nanoliter droplets containing test compounds prior to infusion into a metal-coated, fused silica ESI emitter for MS analysis. A direct segmented flow-ESI-MS analysis system was also applied for the online monitoring of a droplet-based micro-reaction for alkylation of peptides and to screen acetylcholinesterase catalyzed hydrolysis of acetylcholine. Segment flow was coupled to MS by capacitive charge transfer across the droplet-carrier phase boundary for monitoring single and biphasic reactions. A spyhole drilled on top of the microchip was used to sample passing droplets by applying high voltage pulses from an electrode placed below the chip. Electrostatic-spray ionization enabled MS analysis without dilution or an oil removal step, surfactants, or sheath-flow.

MALDI has been coupled with microfluidic technology to illustrate the feasibility of screening methods that consume minimal quantities of substrates per reaction. An optimized deacetylation reaction using only 20 µg of substrate was performed within segmented samples and deposited onto a plate for MALDI-MS analysis. A novel interface between droplet microfluidics and MALDI-MS enabled label-free analysis of up to 26,000 individual aqueous droplets on a microarray plate. A stage containing the plate was synchronized with the output of droplets from the device, preventing cross-contamination. The system monitored the formation of angiotensin II from angiotensin I over a several hour enzymatic reaction.
2.6 SUMMARY

Microfluidics has become a burgeoning field of chemical and biological sciences over the past two decades. The push toward a true µTAS platform that includes sample preparation and handling, separations, reactions, and analysis all within a single microfluidic device brings about nearly limitless potential. Increased sample throughput and reproducibility, reagent and sample minimization, improved reaction time and efficiency, and the capability of coupling highly sensitive and selective detection systems are what makes µTAS attractive to the modern day analyst. Fast, easy, and cost effective in-house device fabrication consisting of substrate materials such as PDMS, glass, or even paper can be used depending on the analysis requirements. The use of segmented flow, or droplet-based microfluidics allows for specialized reactions to be performed on-chip in a fraction of the time required on a larger scale. Integrated microchannel features can allow for precise manipulation of droplets via splitting, fusion, and sorting. While still technically droplet-based, digital microfluidics moves away from flow-based fluid handling and instead incorporates electrode-induced movement of individual droplets, providing an enhanced droplet manipulation ability. Finally, microfluidics can be tailored to numerous detection techniques, with the most commonly used being fluorescence and MS. Both techniques offer high levels of sensitivity, with fluorescence adding the ability of imaging-based detection and MS providing structural and mass related information.
3.1. ABSTRACT

The field of lipidomics is of increasing importance as the physiological properties of specific lipid classes become better understood. One such specific class of lipids, PFAMs, has been shown to display a myriad of signaling. A common difficulty in analysis of these biological PFAMs is their presence in trace concentrations, as they are often found at pmol/g quantities in tissue. Current analysis techniques, such as HPLC-MS, have detection limits that lie on the periphery of endogenous PFAM concentrations, making their quantitative analysis difficult. The need for a more sensitive detection system, such as fluorescence, exists in this field, and this chapter will serve as the foundation in developing methodology to analyze PFAMs and their conjugate amines.

Successful extraction of PFAMs from both complex lipid mixtures and biological tissue has been performed using either SPE or a Folch-Pi liquid-liquid extraction procedure. GC-MS analysis of PFAMs presents the ability for both their identification and quantitation, without the need for derivatization. To incorporate a highly sensitive fluorescence detection scheme into PFAM analysis, both an amide conversion and subsequent fluorescent tagging reaction must be employed, and is described herein. HPLC-fluorescence detection provides the most sensitive analysis-detection scheme, with 5 fmol detection limits being achieved.
3.2. INTRODUCTION

Lipids are small amphiphilic bioorganic molecules that play an important role in several different areas of mammalian biology. Lipids have important biochemical roles such as cell makeup, energy storage, essential hormones, signaling, and messenger functions. As described in Chapter 1 and recent reviews, PFAMs are one specific subgroup of the fatty acyls lipid class that are of growing interest due to their variety of physiological properties.\textsuperscript{47} PFAMs are contained in the CSF of humans, cats, mice, and also within bovine omentum.\textsuperscript{3, 5, 185} Oleamide (C18:1\textsuperscript{9}), a monounsaturated PFAM, displays several physiological effects such as sleep induction in cats, vasodilation, and potential anti-cancer qualities by suppressing spontaneous metastasis in the BL6 line of melanoma cells.\textsuperscript{27, 186-189} Anandamide (C20:5\textsuperscript{4,5,8,11,14}) also plays a potential role in mediating certain oleamide-like sleep induction effects.\textsuperscript{190-192} Erucamide (C22:1\textsuperscript{19}) stimulates angiogenesis, as well as regulates fluid imbalances.\textsuperscript{35} Quantitative studies of signaling lipids can be quite difficult as these are found in trace concentrations in biological systems, requiring extremely sensitive instrumentation. The need for increasingly lower detection limits becomes necessary to lipidomics researchers as concentrations within samples are often at trace, nano- to picomolar (10^{-9}-10^{-12}), or even ultra-trace, sub-femtomolar (10^{-15}), levels.

One method of PFAM detection utilizes fluorescence, which occurs when a molecule is excited at a given wavelength and then emits a photon of lower energy during relaxation back to the ground electronic state. This shift in energy (which corresponds directly to the inverse of the wavelength) from excitation to emission is called the Stokes shift.\textsuperscript{193, 194} In fluorescence, the emitted photon has a longer wavelength than the excitation photon.\textsuperscript{195} Optimal fluorophores display large Stokes shifts due to limited structural
rigidity, allowing for more energy loss in the vibrational relaxation step prior to emission of a photon. This increased energy loss, correlating to a large Stokes shift, results in an emission wavelength being significantly longer than the excitation wavelength, thus limiting systematic noise caused by an incident radiation source when proper optical filters are used in a fluorescence detection system.

Due to the delocalization of electron density caused by the carbonyl group, amide-reactive fluorophores are relatively non-existent, whereas amine-reactive fluorophores are plentiful. In order to incorporate a fluorescence detection system into PFAM analysis, the PFAM must first be converted to its conjugate amine form using a modified Hoffman rearrangement reaction. Since primary fatty amines do not have a conjugated π-bonding system required for strong fluorescence in the UV or visible range, these molecules must be derivatized with a fluorophore to allow potential fluorescent detection. The development of isoindole-forming derivatives such as o-phthalaldehyde (OPA) and naphthalene-2,3-dicarboxaldehyde (NDA) approximately three decades ago proved to be a critical advancement in the fluorescence analysis of amino acids and aliphatic amines. In the presence of a nucleophile (cyanide), NDA reacts with primary amines, forming highly fluorescent N-2-substituted-1-cyanobenz-[f]-isoindole (CBI) derivatives. These CBI derivatives have several advantages over similar OPA derivatives, such as improved chemical stability, visible excitation maxima, and enhanced quantum yield. Wang et al. recently showed the potential of NDA for trace analysis of short chain aliphatic amines (C1-C8) using HPLC with a fluorescence detector. Amines were extracted from an aqueous matrix by vortex-assisted liquid-liquid microextraction and subsequent studies provided limits of detection (LOD) between 10 pM and 40 pM. NDA was also used to
fluorescently tag a series of six neuroactive amines commonly found in brain dialysate. Offline derivatization with NDA showed LODs for arginine, citrulline, taurine, histamine, glutamine, and aspartate ranging from 50 nM to 250 nM; comparable to or exceeding the sensitivity required for detection of these analytes within the physiological concentration range.202-206

3.3 MATERIALS AND METHODS

3.3.1 Chemicals

Methanol (HPLC grade), acetonitrile (HPLC grade), hexane (ACS grade), glacial acetic acid, ammonium hydroxide, and fluorescein isothiocyanate (FITC) were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Oxalyl chloride, oleic acid, erucic acid, petroselaidic acid, anhydrous methylene chloride, decylamine, dodecylamine, octadecylamine, monooleoylglycerol, phosphatidyl-choline, N-oleoglycine, and N-oleylethanolamine were purchased from Sigma Aldrich (St. Louis, MO, USA). Heptadecanoic acid, eicosanoic acid, chloroform, and diethyl ether were purchased from Aldrich Chemical Company (Milwaukee, WI, USA). Lauric acid, myristic acid, palmitic acid, stearic acid, docosanoic acid, tetradecylamine, hexadecylamine, [bis(trifluoroacetoxy)iodo]benzene (PIFA), potassium cyanide (KCN), and acetone were purchased from Acros Organics (New Jersey, USA). Elaidic acid was purchased from MP Biomedical, Inc (Solon, OH, USA). 3-(2-furoyl)quinoline-2-carboxaldehyde (FQCA) and NDA were purchased from Molecular Probes (Eugene, OR, USA).
3.3.2 Synthesis of Primary Fatty Acid Amides

PFAMs were synthesized in-house from their corresponding fatty acids according to a modified synthesis procedure originally developed for low-aliphatic amides (three to six carbons in length).\textsuperscript{207} Fatty acids were dissolved in methylene chloride and reacted with oxalyl chloride to form the acid chloride in a sealed vial under an argon atmosphere and constant stirring. The reaction solvents were dried using a vacuum rotary evaporator at elevated temperature to reduce drying time, resulting in a colorless oily residue. A syringe filled with ammonium hydroxide was inserted into the vial containing the acid chloride and the ammonia was allowed to slowly outgas. The reaction was visibly monitored and deemed to be complete when the acid chloride turned to a white solid, characteristic of the PFAM form. The final reaction product was solubilized in chloroform and the liquid portion was removed, dried, and stored in a desiccator. Reaction verification and purity analysis were conducted using GC-MS and further detail is provided in Section 3.3.4.

3.3.3 Solid Phase Extraction and Thin Layer Chromatography

A method of PFAM extraction from lipid mixtures containing N\textsubscript{18}TG\textsubscript{2} mouse neuroblastoma cells and bovine omentum and subsequent separation using TLC was originally developed by Sultana et al., and was reproduced in this work as a model for separation of lipid classes.\textsuperscript{61} A 3 mL polypropylene SPE tube was packed with approximately 500 mg of either DSC unbonded silica phase or DSC aminopropyl bonded silica phase between frits. A concentrated lipid mixture containing oleamide, oleic acid, monooleoylglycerol, phosphatidyl-choline, N-oleoglycine, and N-oleylethanolamine
were prepared and solubilized in hexane. A 100 µL aliquot of the lipid mixture was loaded onto the hexane-wetted unbonded silica phase column and eluted with 1 mL each of 99:1 (v:v) hexane:acetic acid, 90:10 hexane:ethyl acetate, 80:20 hexane:ethyl acetate, 70:30 hexane:ethyl acetate, 2:1 chloroform:isopropanol, and 2 mL of methanol. Fraction 6 (containing PFAM, MAG, NAG, and NAE classes) was subsequently dried and reconstituted in 200 µL of hexane. The reconstituted lipid mixture was loaded onto the hexane-wetted aminopropyl phase column and eluted with 2 mL each of hexane, chloroform, 1:50 isopropanol:chloroform, 1 mL of 1:50 isopropanol:chloroform, 3 mL of 1:33 isopropanol:chloroform, and 2 mL of methanol.

To verify SPE elution of the lipid classes, each fraction from both column phases was spotted on 10 cm × 10 cm TLC plates and separated using a three-stage development process. TLC plates were first developed to 5.5 cm in 95:5:1 chloroform:methanol:acetic acid and dried using a handheld dryer. The plates were re-developed to 8 cm in 60:40:5 hexane:diethyl ether:acetone and dried. The plates were re-developed to 9.5 cm in 97:3 hexane:diethyl ether and dried. A 0.05% primuline solution was evenly sprayed onto the plates and dried. The TLC plates were placed inside a UV box and viewed under broadband UV radiation to determine the presence of lipids in each fraction. Spots visible inside the UV box were circled to allow for future analysis as visibility of the primuline-stained spots fades with time.

3.3.4 Gas Chromatography-Mass Spectrometry

A Varian CP-3800 GC equipped with Varian Saturn 200 ion trap MS and Varian CP-8400 autosampler was used for analysis of PFAMs and to also verify PFAM synthesis
reaction completion and assess purity. GC separations were performed using a Varian Factor Four capillary column (VF-5ms). The column stationary phase consisted of 5% phenyl-methyl with dimensions of 30 m × 0.25 mm ID. Ultra-pure helium was used as the carrier gas with a flow rate of 1 mL/min. The inlet temperature was set to 250°C with a split ratio of 10:1. The temperature gradient began at 55°C and initially ramped to 150°C at 40°C/min with a 3.62 minute hold followed by a ramp to 275°C at 10°C/min with a 6.50 minute hold, resulting in a total run time of 25 minutes. PFAMs were ionized by either chemical ionization (CI) with methanol or EI ionization and analyzed using either total ion collection or SIM. Varian MS Workstation version 6.9 was used for all data analysis.

3.3.5 Primary Fatty Acid Amide to Amine Conversion

In order to conduct fluorescent labeling studies, PFAMs must first be converted to their conjugate amide form. PIFA was prepared to approximately 0.15 M in a 3:1 (v/v) acetonitrile:water solution. PIFA displays limited solubility, and must be extensively sonicated to aid in dissolution. Using a modified conversion method originally developed by Feng and Johnson, approximately 20 µmol of amide was added to an amber glass vial with 200 µL of the PIFA reagent and the solution was sonicated for at least 60 minutes. The reaction occurred overnight with the vials being kept in a dark environment. Samples not used immediately following a 24 hour reaction period were stored at -4°C until future use.
3.3.6 Fluorescent Labeling of Amines

For optimal fluorescent tagging of amines using FQCA, 10 mM solutions of both FQCA and amine were prepared in methanol, and a 10 mM solution of KCN was prepared in water. Optimal reaction conditions were determined to be 1:10:12 (v/v/v) equimolar amine:KCN:FQCA. The reactants were mixed in an amber glass vial and allowed to react for approximately 45 minutes at 40°C using a heating block. Fluorescent tagging of amines using NDA follows a similar procedure to that of FQCA. Each reactant stock solution was prepared at 10 mM with both NDA and amine in methanol and KCN in water. An equimolar 1:20:24 (v/v/v) solution of amine:KCN:NDA was required for maximum fluorescence intensity. The reactants were mixed in an amber glass vial and heated at 40°C for approximately 30 minutes for the reaction to proceed to completion.

3.3.7 Reversed Phase HPLC Separation of Primary Fatty Acid Amines

A Waters 1525 Binary HPLC Pump with Waters 2475 Multi Wavelength Fluorescence Detector and Waters Breeze version 3.30 software package was used for separation and fluorescence detection of amine mixtures. A Waters Xterra MS C18 with 5 um particle size, 3.9 mm × 150 mm column was used to separate saturated fluorescently tagged amine standards. A 5 uL injection loop was used for consistent injection volumes and the column was maintained at room temperature. An isocratic elution profile consisting of 100% methanol with a flow rate of 1.5 mL/min was used. For analysis of FQCA-tagged amines, an excitation wavelength of 488 nm and an emission wavelength of 590 nm was used with gain and energy units full scale (EUFS) settings at 100 and 1,000, respectively. For analysis of NDA-tagged amines, an excitation wavelength of 405 nm and
emission wavelength of 470 nm was used with gain and EUFS settings at 100 and 1,000, respectively.

3.4 RESULTS

3.4.1 Solid Phase Extraction

A concentrated lipid mixture containing pure oleamide, oleic acid, monooleoylglycerol, phosphatidyl-choline, N-oleoglycine, and N-oleylethanolamine standards were used to verify the SPE procedure designed to isolate lipids by class. After loading the concentrated lipid mixture onto the unbonded silica phase, the PFAM, MAG, NAG, and NAE classes were found to elute in fraction six (methanol). After drying, reconstitution, and application onto an aminopropyl phase column, PFAMs were isolated in fraction three (2 mL 1:50 isopropanol:chloroform) while the MAG, NAG, and NAE classes elute in fraction five (3 mL 1:33 isopropanol:chloroform). After application to and development on a TLC plate, a 0.05% primuline solution resulted in visible fluorescence of the lipids under UV light (data not shown).

3.4.2 Gas Chromatography

GC-MS was used to separate and identify PFAMs in a mixture of mid- to long-chain, as well as saturated and unsaturated PFAMs. A mixture sample containing myristamide (C14:0), palmitamide (C16:0), stearamide (C18:0), oleamide (C18:1⁹), and eicosanoamide (C20:0) were prepared at 200 µM per amide. A 1 µL sample of the PFAM mixture was injected with separation occurring using a VF-5ms capillary column. The GC
method conditions were sufficient for baseline resolution of all five PFAMs, inclusive of stearamide and oleamide, which only differ by one point of unsaturation in the aliphatic tail (Figure 3-1). CI with methanol was used as the ionization source. Fully saturated PFAMs with carbon chain lengths of 12, 13, 14, 16, 17, 18, 20, and 22 were individually run by GC-MS and their retention times were calculated versus carbon chain length. Consistent baseline resolution was found between peaks of fully saturated PFAMs with a coefficient of determination ($R^2$) of 0.99 for retention time versus the aliphatic chain length, showing a linear response between retention time and chain length.

GC-MS was also used to verify the completion of the PFAM synthesis reaction. Synthesized fatty acid amides, as well as precursor fatty acids, were solubilized in methanol, ionized by CI with methanol, and analyzed using SIM. The integrated peak areas of both the fatty acid precursor and the PFAM product were used to estimate overall purity, with the purity value presented as the peak area of the PFAM divided by the sum of the peak areas of the PFAM and residual fatty acid precursor. All PFAMs synthesized were found to have 98% or greater purity.
3.4.3 Primary Fatty Acid Amide to Amine Conversion

As amide-reactive fluorophores are relatively non-existent, PFAMs must be converted into their conjugate amine form prior to fluorescent tagging. The PIFA reaction occurs via a modified Hoffman rearrangement, or decarboxylation reaction, where the product amine contains one less aliphatic carbon than its precursor amide, and this reaction has been well characterized within our laboratory (Figure 3-2). A range of mid to long chain saturated PFAMs consisting of dodecylamide (C12:0), tetradecylamide (C14:0), hexadecylamide (C16:0), and octadecylamide (C18:0) were prepared to a concentration of 20 µM per amide individually, and equally mixed for a total amide concentration of 20 µM. Using a Waters Xterra MS C18 column (5 µm particle size, 3.9 mm × 150 mm), the resulting conjugate amines were separated and fluorescently detected (Figure 3-3). For preliminary PIFA conversion and verification by HPLC, FITC was used as the tagging
fluorophore with fluorescence detector excitation and emission wavelengths set to 495 nm and 520 nm, respectively.

Figure 3-2. Reaction scheme for the conversion of a PFAM to its conjugate amine form using PIFA. The reactants undergo a decarboxylation reaction that results in the conjugate amine having one less carbon than its precursor amide.

Figure 3-3. HPLC fluorescence chromatogram showing product primary amines resulting from precursor PFAMs following the PIFA conversion reaction. Converted PFAMs were injected independently (top four traces) and as a mixture (bottom trace). The fluorescence response was acquired at excitation and emission wavelengths of 495 nm and 520 nm, respectively.
3.4.4 Fluorescent Tagging of Primary Fatty Acid Amines

In order to utilize highly sensitive fluorescence detection with ultra-trace primary fatty acid amine analysis, amine-tagging reactions were optimized for response and efficiency. Preliminary fluorescent tagging experiments included the use of FQCA, however, extensive optimization of the fluorescent tagging of fatty amines focused on the fluorophore NDA, with further rational expanded upon in Section 3.5. The tagging reaction using NDA involves both the fluorophore and the fatty amine solubilized in methanol in the presence of excess cyanide ion. The basic reaction scheme is shown in Figure 3-4. To determine the optimal ratio of reactants to one another, decylamine (C10:0) was used as the target analyte. All reactants, inclusive of the amine, were prepared to 1 mM and reaction mixtures were incubated at 50°C for 45 minutes to ensure reaction completion. Following each reaction, the samples were diluted to an amine concentration of 1 µM and run in triplicate. The average peak area of NDA-tagged decylamine was determined by HPLC with fluorescence detection. Previous work using FQCA showed that optimal reaction conditions required an excess of NDA and KCN, therefore a reactant ratio of 1:10:12 (v/v/v) amine:KCN:NDA was used as the benchmark. Additional reactant mixtures used ratios of 1:5:6, 1:5:12, 1:10:6, 1:1:1, and 1:20:24 (v/v/v) amine:KCN:NDA, with the 1:20:24 reaction resulting in the largest average peak area (Figure 3-5).

For optimal fluorescence detection, the fluorescence emission maximum of the NDA-tagged amine was determined. Dodecylamine (C12:0) was tagged with NDA and diluted to an amine concentration of 1 µM and analyzed by HPLC with fluorescence detection. The excitation wavelength was held at 405 nm as per the fluorophore manufacturer’s recommendation and the emission wavelength varied from 450 nm to 500
nm to determine the emission maxima, with gain and EUFS settings held constant. Samples were run in triplicate at each emission wavelength, with the maximum fluorescence response, and therefore the emission maxima, was observed at 470 nm (Figure 3-6).

Each of the reactants were tested by HPLC with fluorescence detection to determine whether any native fluorescence may attribute to the overall fluorescence response of NDA-tagged amines. Each reactant was prepared to a concentration of 100 µM and mixtures of two or all three reactants followed a 1:10:12 (v/v/v) amine:KCN:NDA equimolar ratio. Each sample was also subjected to a 45 minute incubation time at 50°C. All three reactants (amine, NDA, and KCN) displayed negligible native fluorescence when injected independently. Dodecylamine reacted with NDA and dodecylamine reacted with KCN displayed no native fluorescence. NDA reacted with KCN did show a moderate fluorescence response, however, this response was dwarfed by that of the NDA tagged amine as shown in Figure 3-7.

Figure 3-4. Reaction scheme showing the fluorescent tagging of a primary fatty acid amine with NDA in the presence of a cyanide ion. Optimal reaction conditions were determined with a reaction mixture containing 1:20:24 (v/v/v) amine:KCN:NDA in equimolar concentrations. The reaction takes approximately 30 minutes at 45°C to reach completion, resulting in a maximal fluorescence emission of 470 nm.
Figure 3-5. Reaction mixtures displaying varying ratios of reactants (amine, KCN, and NDA). All solutions were prepared with reactants added in equimolar concentrations following the format of amine:KCN:NDA by volume. Average peak area of fluorescently tagged decylamine diluted to a concentration of 1 µM is shown for each reaction mixture, with standard deviations depicted (n=3).

Figure 3-6. Average peak areas are shown at varying intervals of fluorescence emission wavelength. Dodecylamine was fluorescently tagged with NDA and diluted to a concentration of 1 µM. Fluorescence emission wavelengths ranged from 450 to 500 nm with the excitation wavelength held at 405 nm. Standard deviations are shown (n=3).
Figure 3-7. HPLC chromatograms showing native fluorescence of reactants and varying reactant mixtures. All reactants were prepared to 100 µM and incubated at 50°C for 45 minutes. For mixtures, 1:10:12 (v/v/v) equimolar amine:KCN:NDA reaction conditions were used. A, B, and C. show fluorescence chromatograms of NDA, KCN, and dodecylamine, respectively. D. and E. show decylamine reacted with NDA and dodecylamine reacted with KCN, respectively. F. shows the fluorescence response of KCN and NDA reacted with one another. G. and H. show the full reaction of NDA-tagged dodecylamine and decylamine, respectively, for perspective with respect to retention time.
3.4.5 HPLC of Primary Fatty Acid Amines

HPLC with fluorescence detection was used to validate the amine-tagging reaction and for separation of individual primary fatty acid amines from mixtures. An NDA-tagged amine mixture containing decylamine, dodecylamine, tetradecylamine, and hexadecylamine was prepared at a concentration of 10 µM per amine using reaction conditions of 1:20:24 (v/v/v) amine:KCN:NDA. Isocratic elution profiles of 100% methanol, 90:10 (v/v) methanol:acetonitrile, and 80:20 methanol:acetonitrile as mobile phases at 1 mL/min flow rates were tested to determine optimal chromatographic resolution. While maintaining a 100% methanol isocratic elution profile, the flow rate was increased to 1.5 mL/min to determine the extent of resolution loss, which was apparent between decylamine and dodecylamine (Figure 3-8). The most retained component (hexadecylamine) eluted within four minutes for all mobile phase compositions regardless of the flow rate.

Detection limit studies were performed with an NDA-tagged amine mixture consisting of decylamine, dodecylamine, tetradecylamine, hexadecylamine, and octadecylamine. Each amine was reacted with NDA and KCN individually, with all reactants prepared to 1 mM. The reacted amines were mixed together and serially diluted to 1 nM per amine. In order to achieve maximum sensitivity, the gain and EUFS settings on the fluorescence detector were set to 1,000 and 1, respectively. A 5 µL injection loop was used, resulting in 5 fmol of each amine on-column (Figure 3-9).
Figure 3-8. HPLC fluorescence chromatograms showing NDA-tagged amines (decylamine, dodecylamine, tetradecylamine, and octadecylamine) at varying mobile phase compositions. All elution profiles were isocratic with flow rates of 1 mL/min. A. 100% methanol, B. 90:10 (v/v) methanol:acetonitrile, and D. 80:20 methanol:acetonitrile were used as mobile phases. 100% methanol was also analyzed with an increased flow rate of 1.5 mL/min (C.), which resulted in a loss of resolution between decylamine and dodecylamine.
Figure 3-9. An HPLC fluorescence chromatogram displaying instrumental detection limits. A mixture of pre-NDA-tagged decylamine, dodecylamine, tetradecylamine, hexadecylamine, and octadecylamine were serially diluted to a concentration of 1 nM per amine. A 5 µL injection volume resulted in the on-column load of each amine to be approximately 5 fmol.

3.5 DISCUSSION AND CONCLUSIONS

The need to develop fast, accurate, and highly sensitive determination of PFAMs for eventual analysis of samples with trace and ultra-trace concentrations of endogenous PFAMs has been addressed, and this chapter serves to develop the methodology for these future analyses. In order to analyze PFAMs with the sensitivity offered by fluorescence, the conjugate amine form must be used. This is due to the carbonyl group on the amide causing a delocalization of electron density, effectively prohibiting a fluorophore from binding to the amide head group. Several PFAMs were available for use as the fatty acid to PFAM synthesis was successfully developed and completed in-house. GC-MS proved to be helpful in verifying the successful reaction completion as well as determining the overall percent purity of the product PFAM aided by the specificity of SIM. GC-MS has the benefits of requiring no derivatization of PFAM samples and very sharp
chromatographic peaks due to its increased number of theoretical plates versus HPLC, however, detection limits range from the low µM to high nM range, with poor reproducibility near these concentrations. In addition to the chromatographic benefits that GC offers, detection by MS enables for mass identification using either CI or EI ionization. CI is a soft ionization source, resulting in little or no mass fragmentation. With analytes of known mass, individual peaks can be identified as the entire molecular ion will be intact in the mass spectrum. In addition, SIM adds increased selectivity as any extraneous noise or contamination peaks can be removed from a chromatogram when the analyte mass(es) is/are known. EI ionization is a much harder ionization source, with little to no molecular ion remaining, depending on the analyte. This is especially useful for identification of unknown materials as each molecule will display a characteristic fragmentation pattern that can be saved to a library. SIM may not be as useful with EI ionization unless a specific mass fragment is known (Figure 3-10).
Figure 3-10. GC/MS spectrum comparing CI versus EI ionization of stearamide. CI is a very soft ionization source (top), resulting in essentially all of the molecular ion mass remaining. This ionization mode can be especially useful for identification of analytes of known mass or for SIM. EI is a much harder ionization source (bottom), as shown by the significant extent of fragmentation resulting in little to no remaining molecular ion. This ionization mode is especially useful for identification of unknown components by characteristic fragmentation patterns that can be matched to a library database.

Converting a PFAM to its conjugate amine has been accomplished using PIFA, resulting in an amine removed of one carbon from its predecessor. This reaction has the potential to be a useful tool for enabling trace and ultra-trace detection of PFAMs via fluorescent tagging of their conjugate amines, as none of the side-products appear to have a fluorescent response. The PIFA reaction can fast become problematic, however, due to severe solubility issues. PIFA is highly soluble in aqueous solutions (or at least partially aqueous), whereas PFAMs display little to no solubility in water, worsening as the aliphatic chain increases in length. The usefulness of PIFA as a reactant to produce conjugate aliphatic amines of mid- to long-chain length does not appear to be realistic due to
solubility concerns. Future work must explore an alternative means to conduct PFAM to amine conversion reactions capable of handling solubility concerns of longer chain aliphatic amides.

When dealing with primary fatty amine standards, two modes of fluorescent tagging have been explored. Initial studies involved FQCA as an amine-tagging fluorophore, however, several drawbacks and the discovery of NDA changed the path of analysis. Comparatively, NDA displays a higher quantum yield, substantially longer fluorescence stability, and is significantly less expensive to purchase than FQCA. The NDA-amine tagging reaction was determined to produce optimal fluorescence response when reacted at 1:20:24 (v/v/v) equimolar ratios of amine:KCN:NDA. This reaction was stable for up to two weeks with minimal fluorescence loss when stored at 4°C (unpublished observation). Native reactant fluorescence studies showed that none of the reactants display native fluorescence alone, however a response is noticed when KCN and NDA are reacted with one another. This is likely due to dimerization/oligomerization of the NDA molecule, however, the response is approximately ten times less than that of NDA-tagged decylamine and is not well retained by reversed phase HPLC, eluting prior to the earliest eluting of any of the amines studied.

Reversed phase HPLC separation of fully saturated aliphatic amines can be relatively easily achieved in a relatively short amount of time (less than 4 minutes for octadecylamine). For ease of use, an isocratic elution of 100% methanol mobile phase can sufficiently separate saturated amines, however, resolution increases by slightly increasing the mobile phase polarity by adding 20% acetonitrile. A more extensive gradient elution profile was developed by Divito and Cascio for separation of mid- to long-chain aliphatic
amides both fully saturated and with varying levels of unsaturation. \(^\text{208}\) A sub 2 \(\mu\)m particle size column (Agilent RP C18, 2.1 mm × 50 mm, 1.8 \(\mu\)m particle size) was used with a 2\(\mu\)L injection volume and a gradient elution profile beginning with a 5 minute hold of 80:20 (v/v) methanol:water, ramp to 100 methanol over 5 minutes, and a four minute hold.

Fluorescence detection has the power of being a highly sensitive detection system for primary fatty acid amines, as evident by low fmol detection limits using an HPLC with fluorescence detector. Successful conversion of PFAMs to their conjugate amines using PIFA is key to progressing the developed methodology of fatty acid amine analysis, however, inherent problems develop as chain length increases, and solubility decreases. NDA has shown to be a highly fluorescent fluorophore selective to amine analysis. Successful optimization of this tagging reaction is evident in the extremely low detection limits by HPLC with fluorescence detection. As this research progresses into ultra-trace detection techniques, the methodology developed in this chapter serves as a strong foundation leading to microfluidic and LIF analysis of fluorescently tagged primary amines (Chapter 4).
Chapter 4

DEVELOPMENT OF A MICROFLUIDIC PLATFORM FOR ULTRA-TRACE ANALYSIS OF PRIMARY FATTY ACID AMINES

4.1 ABSTRACT

The inherent trace quantity of PFAMs found in biological systems presents challenges for analytical analysis and quantitation, requiring a highly sensitive detection system. The use of microfluidics provides a green sample preparation and analysis technique through small-volume fluidic flow through micron-sized channels embedded in a PDMS device. Microfluidics provides the potential of having a micro total analysis system where chromatographic separation, fluorescent tagging reactions, and detection could all be accomplished with no added sample handling. This chapter describes the development and optimization of a microfluidic-LIF analysis and detection system that can be used for the detection of ultra-trace levels of fluorescently tagged primary fatty acid amines. A PDMS microfluidic device was designed and fabricated to incorporate droplet-based flow. Droplet microfluidics have enabled on-chip fluorescent tagging reactions to be performed quickly and efficiently, with no additional sample handling. An optimized LIF optical detection system provided fluorescently tagged primary fatty acid amine detection approaching low amol LODs. The use of this LIF detection provides unparalleled sensitivity, with detection limits several orders of magnitude lower than currently employed LC-MS techniques.
4.2 INTRODUCTION

Initial discovery of PFAMs utilized NMR spectroscopy\textsuperscript{3}; however, current work more commonly involves coupling GC or HPLC for separation with MS for detection and structural identification of endogenous or derivatized analytes.\textsuperscript{3-6, 35, 48, 61, 63, 65, 185, 191, 209-212} MS detection, although widely employed and of great benefit, results in LODs on the periphery of endogenous PFAM concentrations (LOD of 10 – 400 nM depending on the species; Divito, unpublished), thus new methodologies for sensitive detection are required. The potential sensitivity of LIF makes this method an attractive alternative detection system.

The use of fluorescent labeling coupled with LIF detection presents the theoretical ability to approach single molecule detection using a fluorophore of high quantum yield.\textsuperscript{67-71} In the case of PFAMs, the amide must first be converted to its conjugate amine as the carbonyl of the amide causes delocalization of electron density, hindering the chemical derivatization required for fluorescent tagging at this chemical group.\textsuperscript{213} To achieve fast fluorescent labeling with maximal efficiency, a microfluidic platform can be utilized.

Microfluidics is the general label given to the technology of systems that process or manipulate small volumes ($10^{-9}$ to $10^{-18}$ L) of fluids by utilizing embedded channels with internal diameters on the micrometer scale and is reviewed in Chapter 2.\textsuperscript{214} Microchip fabrication can be performed quickly, safely, and economically via soft lithography using compounds such as PDMS.\textsuperscript{78} An inherent obstacle faced while performing microfluidic reactions on-chip is mixing efficiency, as laminar flow dominates the system at the micro-scale due to the low Reynolds number (Re < 2300). In laminar flow, the major form of mixing that occurs is diffusion between multiple streamlines, which is highly ineffective.
Segmented flow microfluidics presents the ability to conduct highly efficient reactions on-chip entirely within small-volume (nL) droplets. Droplet formation may be achieved by introducing at least two immiscible phases into the micro-channels via pressure-driven flow. One phase consists of a carrier fluid (perfluorodecalin, gas, etc.), which is continuous and encapsulates the second phase (analyte and all reaction-dependent solutions) into segmented droplets.\textsuperscript{215} Highly efficient mixing of nanoliter-sized droplets relies on the repeated stretching and folding of the intra-droplet fluidic layers until these layers become increasingly thin to the point where inter-layer diffusion becomes even more rapid and efficient.\textsuperscript{216, 217} The mixing process, known as chaotic advection, drastically reduces reaction time while improving reaction efficiency largely due to the significantly reduced mean free path as well as the absence of diffusion outside of the droplet, due to droplets being encapsulated by an immiscible phase.\textsuperscript{91} As the droplets flow through the micro-channels, frictional forces introduced via contact with the channel walls causes internal recirculation of the droplet, enhancing mixing. Implementing winding “S” shaped channels with several turns also enhances mixing by reorienting the droplet’s internal fluidic layers in the direction of droplet movement.\textsuperscript{91, 218, 219}

Herein, our efforts to develop a droplet-based microfluidic platform with LIF detection to enhance the fluorescent labeling of derivatized PFAMs and further reduce detection limits are described, which can be supplemented with previously developed MS methodology to aid in identification.\textsuperscript{208} The use of pre-chip or on-chip chromatographic separation, on-chip fluorescent tagging, and single photon counting detection has the potential to enable an in-line \( \mu \text{TAS} \) with detection limits sufficient for investigation of endogenous PFAMs.
4.3 MATERIALS AND METHODS

4.3.1. Chemicals

Methanol (HPLC grade) was purchased from Fisher Scientific (Fair Lawn, NJ, USA). Decylamine and dodecylamine were purchased from Sigma Aldrich (St. Louis, MO, USA). Tetradecylamine, hexadecylamine, chlorotrimethylsilane, isopropanol, and KCN were purchased from Acros Organics (New Jersey, USA). SU-8 2015 and SU-8 2075 epoxy based photoresists and SU-8 developing solution were purchased from MicroChem (Westboro, MA, USA). SlyGard 184 silicon elastomer kit containing base and curing agent was purchased from Dow Corning (Auburn, MI, USA). NanoStrip Piranha solution was purchased from Cyantek (Fremont, CA, USA). NDA was purchased from Molecular Probes (Eugene, OR, USA). Perfluorodecalin was purchased from Alfa Aesar (Ward Hill, MA, USA).

4.3.2 Microfluidic Device Fabrication

Microfluidic device fabrication is based on the principle of photolithography with PDMS as the device substrate. A 150 mm circular silicon wafer was cleaned in a NanoStrip Piranha solution (H$_2$SO$_4$ + H$_2$O$_2$) bath with constant agitation. The wafer was thoroughly rinsed with deionized water, and dried with nitrogen. A Laurell Technologies Corporation WS-650SZ-6NPP/Lite Spin Coater was used to evenly coat the wafer with either SU-8 2015 or 2075 epoxy based photoresist. The wafer was sealed to the spin coater rotor by vacuum and a constant flow of nitrogen was maintained within the chamber. Approximately 4-6 mL of the photoresist was applied in the center of the wafer,
and evenly coated to a thickness of approximately 100 µm by the following spin coating method: 100 rpm/s ramp to 500 rpm, hold for 5 seconds, 300 rpm/s ramp to 2000 rpm, hold for 30 seconds. The photoresist was semi-cured by heating from room temperature to 65°C for 5 minutes, cooled back to room temperature, heated to 95°C for 20 minutes, and cooled back to room temperature. A chrome-plated photomask designed in-house using Adobe Illustrator software package and manufactured by Fine Line Imaging was placed over the photoresist-coated wafer and irradiated by high intensity broadband UV light (350-400 nm) using a Newport 500 W mercury-arc lamp for approximately one minute (Figure 4-1). The photoresist was once again cured by heating from room temperature to 65°C for 5 minutes, cooled back to room temperature, heated to 95°C for 12 minutes, and cooled back to room temperature. The unexposed photoresist was selectively removed by washing the wafer in an SU-8 developing solution bath with constant agitation for 10 minutes. The wafer was then rinsed clean with developer, isopropanol, and finally deionized water and dried with nitrogen. A PDMS solution was prepared by thoroughly mixing SlyGard 184 silicon elastomer base and curing agent in a 10:1 (w/w) base:curing agent ratio, to a total mass of approximately 80 g. The PDMS mixture was degassed using a vacuum desiccator. The prepared silicon wafer was placed overtop a sheet of aluminum foil with the foil crimped vertically around the perimeter of the wafer, creating a boat to pour the PDMS mixture into. This was placed into an oven and baked at 100°C for at least two hours. Once cured, the polymeric PDMS is cut from the wafer using a razor and gently peeled away, taking care to not strip the photoresist from the wafer. A detailed schematic of the microchip fabrication process can be found in Figure 4-1.
Fluidic inlet and outlet holes were bored into the PDMS microchip using a needle punch of the appropriate gauge to match the outer diameter of the transfer tubing that will be attached. The PDMS microchip and a 25 mm × 75 mm glass microscope slide were thoroughly cleaned by rinsing with isopropanol and deionized water, and dried with nitrogen. The microscope slide and the microchip (feature side up) were placed a Harrick Plasma PDC-32G plasma cleaner inside an AirClean 600 laminar flow workstation. An oxygen-plasma was created within the plasma cleaner under vacuum pressure by allowing a slow and consistent stream of ambient air into the chamber. Verification of plasma formation was made by observing a pink-purple glow from within the chamber. The plasma cleaned microchip should be immediately placed (feature side down) onto the cleaned microscope slide and light pressure applied for several minutes to ensure a complete seal. Sealing the PDMS microchip to the glass allows for a closed fluidic channel system. To provide further PDMS-glass seal stability, a perimeter of quick-drying epoxy should be applied where the PDMS meets the glass. Polyether ether ketone (PEEK) inlet and outlet tubing was inserted into the pre-bored holes on the microchip and sealed into place with epoxy to prevent leaking.
Figure 4-1. Digital representation of a chrome-plated photomask used for PDMS microfluidic chip fabrication. The photomask is placed over the photoresist-coated wafer and irradiated under UV light. The channel features are transparent in the photomask, allowing for UV light to polymerize the photoresist underneath the mask. This photomask allows for 12 microfluidic chips to be produced in a single photolithographic fabrication process. Chip designs incorporate varying channel widths, which allows for differing flow rate capabilities. For size scale, the diameter of the circular photomask is approximately eight inches.
Figure 4-2. Schematic of PDMS microfluidic chip fabrication process using photolithography. An epoxy-based photoresist is spin-coated onto a clean silicon wafer (A). A chrome-plated photomask is placed on top of the wafer, and is irradiated under UV light (B) to polymerize the exposed photoresist (C). The wafer is washed with a developing solution to remove unexposed regions of photoresist (D), leaving behind a positive mold of the channel features. A PDMS mixture is poured over the mold (E) and cured at 100°C. The cured PDMS elastomer is peeled away from the positive mold, resulting in a microfluidic chip (F) that can be bonded to glass, forming a closed-channel system.
4.3.3 Scanning Electron Microscopy Analysis of Microfluidic Device

A Hitachi S-3400N scanning electron microscope (SEM) was used for analysis and verification of microfluidic device features. Prior to bonding to glass, a clean microfluidic chip was positioned feature side up on a 15 mm aluminum sample stub affixed to a copper stub holder. The high voltage was turned off and the SEM chamber was exposed to air prior to opening. Once opened, the stub holder, stub, and sample were placed onto the specimen stage and the total height was measured to ensure no contact is made with the metal optics guard. This height was inputted into the software program and the stage height was adjusted accordingly (sample should be approximately 1 mm from the optics guard). The chamber was closed and air was evacuated with a vacuum pump until internal pressure reached 50 Pa. With an accelerating voltage of 15.0 kV, the sample image brought into focus by adjusting magnification and focus knobs on the external controller. Brightness and contrast settings were also adjusted to provide the sharpest image. Both two and three dimensional images of the microfluidic device were acquired.

4.3.4 Development of a Laser Induced Fluorescence Detection System

For the development of a LIF detection system, all optical components were purchased from ThorLabs. The excitation source was a CrystaLaser Violet Blue solid state laser with a wavelength of 405 nm (± 5 nm), variable power source from 0-50 mW, and vertical polarization. The laser beam is reflected off of a series of mirrors and passes through a 405 nm bandpass excitation filter, a rotation-mounted linear polarizer set to 300°, and a pinhole filter. A longpass dichroic lens with cutoff wavelength of 425 nm reflected incident laser light vertically through a 40x microscopic objective lens, focusing the beam to the desired detection region of the microfluidic device. The resulting fluorescence
emission in the downward vertical plane passed back through the microscope objective and dichroic lens to a final mirror to reflect the fluorescence light horizontally to an optics tube. The optics tube contains a pinhole filter at the front end with a 472 nm emission bandpass filter and achromatic focusing lens within. The fluorescence emission beam is focused onto the 180 µm diameter circular active area of a single photon counting module equipped with a silicon avalanche photodiode array detector (APD). Individual photons generate transistor-transistor logic pulses, which are binned over 25 ms increments and transmitted to LabView data acquisition software. A schematic of the LIF detection system can be found in Figure 4-3.

Preliminary LIF detection system alignment was conducted by visually aligning the excitation laser beam downstream from component to component. Each optical component has alignment knobs to adjust both the horizontal and vertical angles of the component. In lieu of a fluorescing sample being placed on the horizontal sample stage above the focusing microscope objective, a mirror was used to reflect the laser beam back to the center of the dichroic lens. In order to align the optical components downstream from the second passing of the dichroic lens, a concentrated NDA-tagged dodecylamine solution was loaded into a capped, flat-sided quartz cuvette and placed on the sample stage. The resonant fluorescence beam was aligned through the dichroic lens, reflected by a mirror, and through the optics tube. A small, handheld dental mirror was used to ensure the fluorescence beam was properly focused from the optics tube onto the active area of the photon counting detector. Finally, a microfluidic chip undergoing segmented flow with NDA-tagged dodecylamine was placed on the sample stage and the stage aligned until the excitation laser beam was focused directly within a sample channel. Optical components
downstream from the sample stage as well as the sample stage horizontal and vertical alignment were adjusted until the detected fluorescence droplet response achieved the greatest S/N. All alignment procedures are completed in complete darkness to allow for maximum excitation and emission beam visibility.

Several optical components required optimization to achieve the greatest possible S/N of NDA-tagged amine. Signal was recorded while adjusting a pinhole filter from closed to its maximum diameter of approximately 2.6 mm, and precise diameters were measured using a caliper. The laser polarization was adjusted from vertical to horizontal alignment, as well as with the rotation-mounted polarizer removed from the beam path. Several emission filters inclusive of 495 nm and 472 nm bandpass filters and 470 nm and 450 nm longpass filters were tested. Using the variable power controller, the lasing power was tested between 5 mW and 50 mW. To determine variance caused by specific APDs, a Perkin Elmer APD was tested against two different models of EG&G APDs. On-chip fluorescent tagging efficiency was examined using two different channel designs; one design contained a droplet mixing region containing 10 turns before the linear detection channel and a second design contained 130 turns before the detection channel. A preliminary investigation of pre-chip chromatographic separation was conducted using a Dionex Ultimate 3000 HPLC Pump with a Waters Xterra MS C18 column (5µm, 3.9mm × 150mm). The flow rate was held constant at 4 µL/min and a 2 µL injection volume was used.
**Figure 4.3.** Schematic representation of the LIF detection system. A 405 nm excitation laser beam is emitted from a solid-state laser (A) and reflected off of two mirrors (B,C). The beam then passes through a 405 nm emission bandpass filter (D), rotationally-mounted polarizer (E), and pinhole filter (F). The beam reflected off of a 425 nm dichroic lens (G) and focused onto the sample (I) using a microscope objective (H). The resonant fluorescence beam then passes back through the objective and dichroic lens, is reflected off of another mirror (J), and into the optics tube (K) through a pinhole filter (L). Inside of the tube is a 472 nm emission bandpass filter (M) and achromatic focusing lenses (N). The focused beam is detected by a single photon counting APD (O).

### 4.3.5. Droplet Based Microfluidic Flow

The formation of droplet-based flow within the microfluidic device is crucial for conducting on-chip reactions. To optimize droplet formation flow rates, only two inlet ports were used. The inlet port with the channel that continues straight past the T-junction was used for the flow of perfluorodecalin, an immiscible oil-phase. One of the three inlet ports with channels entering the T-junction (perpendicular to the oil-phase channel) was used for the flow of NDA-tagged dodecylamine (prepared according to details provided in Chapter 3.3.6). Perfluorodecalin and the tagged-amine solution were loaded into separate
syringes and applied to the microchip using independent syringe pumps. Flow rates were adjusted until rapid and consistent droplets were formed on-chip. Droplet formation was detected visually due to differences in light refraction properties of perfluorodecalin and methanol, as well as by fluorescence detection, as described in Chapter 4.3.4.

To conduct on-chip NDA-tagging reactions, a microchip was used with all four inlet ports accessible with PEEK tubing. The oil-phase inlet was maintained as previously described, with the other three inlets used for the three reactants in the NDA-tagging reaction. Amine, KCN, and NDA solutions were prepared at a concentration ratio of 1:20:24, respectively, and each was loaded into a syringe. The three reactant syringes were loaded onto a multi-syringe pump and all applied to the microchip simultaneously at the same flow rate. Flow rates were optimized for rapid and consistent droplet formation.

4.4 RESULTS

4.4.1 Microfluidic Chip Fabrication and SEM Verification.

The use of photoresists of varying viscosities, along with programmable spin coating, results in precise photoresist deposition, leading to eventual channel feature depth. SEM can be used to precisely measure channel features, providing validation of the fabrication process. Depending on the desired thickness of the photoresist substrate, either SU-8 2015 or SU-8 2075 epoxy-based photoresist was used. The SU-8 2015 photoresist has a viscosity of 1,250 cSt while the SU-8 2075 photoresist has a viscosity of 22,000 cSt. This viscosity difference has a direct correlation with the thickness of photoresist coating at a fixed spin speed. Spinning at 2,000 rpm results in a film thickness of approximately
25 µm and 100 µm for SU-8 2015 and SU-8 2075, respectively. Three dimensional imagery provided by SEM allowed for the verification of the channel dimensions for a microchip fabricated using the SU-8 2075 photoresist, with an average channel diameter and depth calculated to be 155 µm and 108 µm, respectively. Figure 4-4 shows electron micrographs of the inlet T-junction leading into the mixing region, a zoomed in view of the mixing channel, and a three dimensional image of a channel.

Figure 4-4. SEM imagery of channel features of a microfluidic device fabricated using SU-8 2075 photoresist. The four inlet channels, T-junction, and beginning of the mixing region is shown in a zoomed out image (A). A zoomed in image of the mixing channel shows consistent curving features which allow for on-chip mixing and reactions (B). The three dimensional image can be used to calculate distances between two points, enabling the determination of channel width and depth (C).
4.4.2 Optimization of Laser Induced Fluorescence Detection System

LIF is perhaps the most sensitive detection system known, providing sensitivity capabilities well below that which is necessary for the analysis of endogenous PFAMs. The LIF detection system required the optimization of nearly all of the adjustable optics to maximize S/N. Using 1 µM NDA-tagged dodecylamine, pinhole diameters were set to 0.762, 1.193, 1.752, and 2.641 mm and measured using a caliper. The fluorescence response from each pinhole setting was captured over the course of 7-10 droplets, and all fluorescence chronograms were overlaid (Figure 4-5). With the pinhole filter completely closed, no laser light is able to pass through, resulting in zero response. At 0.762 mm, the fluorescence response is approximately 4,000 counts, while the response is approximately 7,500 counts for each of the three larger diameter settings (Figure 4-6).

Using a 5 µM NDA-tagged hexadecylamine solution, the optimal laser polarization was determined. With the LIF system properly aligned and recording fluorescence response, the polarizer was slowly rotated a full 360° to determine the position that results in maximum response. At a vertical polarization of 300°, the response was at its maximum of approximately 15,000 counts and a S/N of nearly 380. By completely removing the polarizer from the beam path, the response increased to approximately 20,000 counts with a S/N of 430 (Figure 4-7).

Four emission filters with wavelengths at or near the emission maximum of NDA-tagged amines were used to record fluorescence response from a 5 µM NDA-tagged hexadecylamine sample. A 472x30 bandpass filter (472 nm ± 15 nm) provided the highest S/N at 430. The other bandpass filter tested, 495x40 (495 nm ± 20 nm) had the worst S/N.
at 139. Two longpass filters were tested with a 450 nm (≥ 450 nm) and 470 nm (≥ 470 nm) having S/N of 305 and 226, respectively (Figure 4-8).

The lasing power was analyzed using a variable power controller from 5 – 50 mW. A 1 µM NDA-tagged dodecylamine solution and 470 nm longpass emission filter were used as the S/N was calculated for each power increment. The S/N at 5, 10, 20, 30, 40, and 50 mW power was calculated at 55.5, 98.6, 58.6, 34.6, 20.0, and 16.1, respectively. Extrapolation of this data and fitting to a Gaussian distribution found the S/N_{max} to be at 12.7 mW of lasing power (Figure 4-9).

To determine detector-to-detector variance, three APDs were used with a 10 µM NDA-tagged dodecylamine solution, lasing power set to 20 mW, and both 495 nm bandpass and 450 nm longpass emission filters. All data recorded using the 495 nm bandpass filter showed a lower response than data collected using a 450 nm longpass filter. Both filter sets show the Perkin Elmer APD to have the highest fluorescence response, with the EG&G APD (large: Model SPCM-AQR-14) approximately 10,000 counts less than the Perkin Elmer APD (Model SPCM-AQR-14) and EG&G (small: Model SPCM-AQ-131) at nearly half the response of the Perkin Elmer APD using the 450 nm longpass filter (Figure 4-10).
Figure 4-5. Overlaid fluorescence chronograms showing the effect pinhole size on signal intensity. With the signal intensity being zero when the pinhole is closed, it is gradually increased as the pinhole size is increased, eventually reaching a maximum intensity at 1.193 mm in diameter.
Figure 4-6. Average fluorescence response shown at each pinhole diameter setting. At a diameter of 0.762 mm, the average response is nearly half of that at 1.193 mm. Further opening the pinhole filter past 1.193 mm does not significantly increase the average response, but, given the increase in the error, appears to increase the droplet-to-droplet variability (n = 7-10).
Figure 4-7. Fluorescence chronogram showing the effect of laser polarization using a rotation-mounted polarizer. The optimal laser polarization was found to be at 300° in the vertical position, which is also the native polarization of the laser. By removing the polarizer, the S/N was increased approximately 13%.
Figure 4-8. Four different emission filters were tested to determine maximum S/N of a 5 µM NDA-tagged hexadecylamine solution. The 472 nm bandpass filter showed the greatest S/N, with the 450 nm longpass, 470 nm longpass, and 495 nm bandpass filters showing progressive decreasing of the S/N.
Figure 4-9. Fluorescence chronogram showing the effect on response and S/N caused by lasing power. Using the variable power controller, the lasing power was set to 5, 10, 20, 30, 40, and 50 mW and fluorescence response was recorded at each increment. Of the individual power settings, 10 mW showed the highest S/N at 98.6, but extrapolation of the data fit to a Gaussian distribution showed the S/N_{mx} to be at 12.7 mW (inset).

Figure 4-10. Overlaid fluorescence chronograms showing the variable detector to detector response. In agreement with data from Figure 4-8, fluorescence responses obtained using the 450 nm longpass filter are greater than those using the 495 nm bandpass filter. The Perkin Elmer APD was found to give the greatest fluorescence response and was used in all subsequent studies.
4.4.3. Droplet Formation and On-Chip Fluorescent Tagging

Droplet-based microfluidics presents the ability to conduct highly efficient fluorescent tagging reactions on-chip, minimizing additional sample handling. For optimal rapid and consistent droplet formation, the flow rate of the immiscible oil phase must be greater than that of the reactant phase(s). It was found that a 4 µL/min flow rate of oil phase to 2 µL/min flow rate of the reactant phase(s) provided the consistent droplet size and frequency (Figure 4-11). It should be noted that when multiple reactants are used for fluorescent tagging on-chip, the summation of their individual flow rates should equal the 2 µL/min total flow rate. The acquisition software was set to produce a binned data point every 25 ms, which was sufficient to record a true maximum and minimum response for each droplet.

Detection limit studies were performed using optimized LIF detection system settings, a reactant flow rate of 1.5 µL/min, and NDA-tagged dodecylamine serially diluted to an amine concentration of 80.8 nM. With a droplet frequency of 4.6 droplets/second, each droplet was calculated to contain 5.4 nL of solution. At an amine concentration of 80.8 nM, the average droplet contained 436 amol of fluorescently tagged amine (Figure 4-12).

On-chip NDA-tagging efficiency was determined using two different channel designs. A 1:20:24 (M/M/M) amine:KCN:NDA reaction concentration ratio was maintained with all reactants independently administered to the microchip at a flow rate of 0.5 µL/min (1.5 µL/min total flow). The resulting concentration of hexadecylamine per droplet was calculated to be 3.33 µM. The initial chip design contained a mixing region with 10 turns in the channel, which resulted in incomplete fluorescent tagging as shown by
inconsistencies in maximum droplet response by the fluorescence chronogram (Figure 4-13). Transitioning to a new chip design containing 130 turns in the mixing region of the channel resulted in a completed fluorescent tagging reaction, as indicated by consistent maximum responses per droplet (Figure 4-14).

Figure 4-11. An example of a fluorescence chronogram showing consistent droplet formation. The lasing power is changed mid-acquisition with an immediate adjustment in response. Consistent droplet formation is determined by equal spacing between peaks (frequency) and equal peak widths (size).
Figure 4-12. Fluorescence chronogram showing NDA-tagged decylamine approaching the detection limit of the LIF detection system. The tagging reaction occurred pre-chip and the total amine concentration was serially diluted to 80.8 nM. The droplet frequency was determined to be 4.6 droplets/second and average droplet volume was 5.4 nL/droplet, resulting in approximately 436 amol of amine per droplet.
Figure 4-13.  Fluorescence chronogram showing NDA-tagging reaction efficiency performed on-chip. Reactants were maintained in a 1:20:24 amine:KCN:NDA molar ratio and applied to the chip independently at 0.5 µL/min. Using a chip design with 10 turns in the mixing region (inset) resulted in an incomplete reaction, indicative of inconsistent maximum peak responses.
Figure 4-14. Fluorescence chronogram showing NDA-tagging reaction efficiency performed on-chip. Reactants were maintained in a 1:20:24 amine:KCN:NDA molar ratio and applied to the chip independently at 0.5 µL/min. Using a new chip design incorporating 130 turns in the mixing region (inset) resulted in a completed tagging reaction, indicative of consistent maximum peak responses.

4.4.4. Pre-Chip Chromatographic Separation

The incorporation of chromatographic separation with the development of the microfluidic platform described in this work is the next step towards achieving a µTAS. Prior to pre-chip chromatographic separations, NDA-tagged dodecylamine was prepared as previously described in Chapter 4.4.3 and diluted to 1 µM. An HPLC pump was connected to the inlet port of the microchip, with an injection valve containing a 2 µL injection loop inserted into the flow path immediately preceding the chip. A 4 µL/min flow rate of methanol was maintained along with a 4 µL/min flow rate of oil phase on-chip. Once consistent droplet flow was achieved, an injection was made without using a column (Figure 4-15). NDA-tagged dodecylamine and hexadecylamine were independently prepared at 1 µM each for separation analysis. A column was inserted into the flow path
immediately following the injection valve and preceding the chip inlet and flow rate and injection conditions were maintained. Due to the poor chromatographic resolution, injections were made containing each tagged-amine separate from one another and the chromatograms were overlaid to enable easier identification of each component (Figure 4-16).

![Fluorescence chronogram showing unretained NDA-tagged dodecylamine. An HPLC flow rate of 4 µL/minute using 100% methanol was used and a 2 µL injection was made immediately preceding the chip inlet. Oil phase flow rate was held constant at 4 µL/min.](image)

**Figure 4-15.** Fluorescence chronogram showing unretained NDA-tagged dodecylamine. An HPLC flow rate of 4 µL/minute using 100% methanol was used and a 2 µL injection was made immediately preceding the chip inlet. Oil phase flow rate was held constant at 4 µL/min.
Figure 4-16. Overlaid HPLC fluorescence chronograms showing separation of NDA-tagged dodecylamine and hexadecylamine. An HPLC flow rate of 4 µL/minute using 100% methanol was used and a 2 µL injection was made with the injection valve and column immediately preceding the chip inlet. The oil phase flow rate was held constant at 4 µL/min. The dodecylamine and hexadecylamine are shown as red and black traces, respectively.

4.5. DISCUSSION AND CONCLUSIONS

The PDMS microfluidic device fabrication process using photolithography has numerous benefits inclusive of low cost per chip ($2 or less), relative safety (HF required with glass chip fabrication process), rapid fabrication (approximately 1 day), and overall ease of use. The key drawback to using PDMS is the relatively low pressure thresholds that induce the chip to unbond from the glass surface. When the PDMS is oxidized by air plasma inside of the chamber, hydrocarbons are etched leaving behind surface silanol groups, resulting in a hydrophilic surface. When placed in contact with an oxidized glass surface, bridged siloxane bonds are formed at the interface, creating the seal. This seal, however, has the tendency to fail with increasing pressures applied to the chip from external syringe or HPLC pumps. The application of an epoxy coating at the perimeter of
the chip and glass helps increase the pressure limits, however, there is still a tendency for relatively high pressures to force the fluid flowing through the channels to balloon outward, resulting in the chip failure and subsequent discarding.

During the photolithographic fabrication process, it is imperative to ensure a perfectly cleaned silicon wafer surface prior to spin coating. When conducting the soft bake process following UV exposure, the channel features will begin to faintly appear in the photoresist. This is caused by the exposed region underneath of the photomask to polymerize in the presence of UV light, resulting in this region being less soluble in the subsequent developing solution. Slight horizontal dispersion of the UV light through the photomask results in the channel width being slightly greater than that of the photomask. This is shown by the SEM micrograph having an average channel diameter of 155 µm, as opposed to the 150 µm width of the channels in the photomask.

The LIF detection system requires precise alignment of all optical components in order to achieve fluorescence detection, with minute adjustments required for optimal S/N. It was determined that a greater S/N was achieved by complete removal of the rotation-mounted polarizer. This was somewhat expected when results showed the greatest response to be in a vertically polarized position, which is the native polarization of the incident laser. The increase in response by removing the polarizer is due to the percent transmittance of the polarizer being less than 100%. In optimizing the pinhole filter diameter, the fluorescence response increased as the diameter of the pinhole filter was increased, up to 1.193 mm, at which point no further response gain was observed. As the filter was opened past 1.193 mm, a slight increase in noise was noticed while the signal remained constant. The goal of the pinhole filter is to block the lower, more variable outer
regions of the laser beam. A cross-sectional view of the laser beam would show a Gaussian distribution in laser intensity, with the center of the laser having the greatest intensity and the outer regions showing higher variability. The dichroic lens displays an important function in that it acts as an additional filter, preventing the incident laser wavelength from reaching the detector. At a 425 nm longpass cutoff, the dichroic lens reflects all light with a wavelength less than 425 nm (incident laser wavelength = 405 nm) and transmits all light with a wavelength greater than or equal to 425 nm (emission maxima = 470). Of the four emission filters tested, the 472 nm bandpass filter provided the highest S/N as it falls in line with the optimal emission maxima of NDA-tagged amines. A significant detector to detector response variability existed between the three detectors tested, with the Perkin Elmer APD displaying a significantly greater response as compared with the two EG&G APDs. The positioning of the microchip on the sample stage is crucial to achieving a maximum S/N. To prevent misalignment of the chip, it is held in place using tape, and the entire stage is carefully adjusted in the X, Y, and Z planes. Optimal positioning of the focused laser beam should be in the center of the detection region of the channel, and the bottom of the glass should be within 1 mm of the focusing microscope objective. Meticulous vertical alignment of the stage in relation to the microscope objective is required while monitoring real-time fluorescence response data until the greatest signal is achieved.

When optimizing flow rates for consistent droplet formation, it is necessary to have the immiscible oil phase be greater than that of the reactant(s) phase. The oil phase acts as a carrier fluid, with the reactants combining at the T-junction and tapering off into small volume droplets, which are carried down-channel by the immiscible oil. Due to the
extremely low flow rates and relatively long length of tubing, a significant amount of time is usually required for stable flow to occur, resulting in consistent droplet formation. The on-chip NDA-tagging reactions shown in Figure 4-13 and Figure 4-14 illustrate the importance of sufficient mixing. A linear channel exhibits laminar flow, with the only mixing occurring by simple inter-layer diffusion. By introducing the turns into the channel design, reorientation and folding of the fluidic layers within the individual droplets is induced. Each turn provides an additional fluidic layer interface, with subsequent turns rendering this fluidic layer infinitesimally thin to the point where simple diffusion becomes a fast and efficient form of mixing. Initial chip designs incorporating 10 turns into the mixing region proved insufficient, as shown by the resulting fluorescence chronogram. The varying peak widths found in Figure 4-13 are the result of unstable droplet formation, not that of incomplete reaction. The varying maximal response peak to peak shows that certain droplets exhibited greater reaction efficiency than others. Additionally, broad peaks (indicative of sample plugs rather than droplets) such as the one at approximately 2.75 minutes show a fluorescence response increasing within the individual droplet. This shows that the reaction efficiency at the front end of the droplet was worse than the efficiency at the tail end. Reaction efficiency was shown to be much improved using the modified chip design that incorporated 130 turns in the mixing region (Figure 4-14). The consistent maximal response for each droplet indicated that the reaction was complete.

The feasibility of conducting pre-chip HPLC separations was investigated in Chapter 4.4.4. Initial injections of tagged dodecylamine were made using the smallest injection loop readily available (2 µL) and without a column. In order to comply with on-chip flow rate restrictions, a 4 µL/min flow rate from the HPLC was used. By injecting a
tagged sample without a column, the degree of band broadening could be determined.

Extreme band broadening of approximately 100 seconds was shown in Figure 4-15. This is a result of a large longitudinal diffusion parameter of the van Deemter equation, which is exacerbated by the low flow rates and long tubing lengths between the injection valve and chip inlet. Figure 4-16 shows overlaid chromatograms resulting from the addition of a reversed phase column positioned between the injection valve and chip inlet port. Similar broadening issues exist, resulting in extremely poor resolution between a C12 and C16 amine.

Future work towards mitigating this band broadening issue must be addressed in order for the microfluidic device to provide a true µTAS. One such method of improvement would be to incorporate a flow-splitting device in the flow path immediately following the column and before the chip inlet port. This would enable higher, more traditional HPLC flow rates to be used, and necessary flow restrictions as dictated by the chip limitations would be incorporated by the flow-splitter. In theory, this would minimize band broadening until the flow-splitter, however, preliminary experimentation using this setup continued to show extensive band broadening occurring due to the low flow rates in the tubing connecting the flow-splitter to the chip inlet. Another option, which would significantly limit band broadening, would be to perform chromatographic separations on-chip. The modified chip design shown in the inset of Figure 4-14 shows an elongated inlet port, which contains a fritless weir prior to the junction point. This weir is a horizontally constricted region of the channel where the width decreases from approximately 150 µm to 15 µm. A slurry of reversed phase packing material with a 3 µm particle size can be prepared and injected into this inlet port. As the particles enter the front of the weir, the
density dramatically increases causing an aggregation of particles that prevents particles from flowing through the weir, therefore eliminating the need for a frit. The packing length can be adjusted to fill as little or as much of this inlet port as needed for optimal resolution. This fritless weir system of channel packing has been described in both PDMS as well as fused-silica capillaries. \(^{221-224}\) Preliminary work on packing chromatographic inlet channels using 3 \(\mu\)m particle size reversed phase packing proved difficult due to pressure restrictions caused by the PDMS-glass bond. As the length of the packed column increases, additional back pressure is required to maintain consistent flow rates. The fritless weir system was successful in stopping the flow of packing material through the column, but as the column length grew, the PDMS-glass bond broke, resulting in the solvent and packing material ballooning outward from the channel. A more robust device substrate such as glass may be required to meet the pressure demands caused by flow through a packed column.

The use of microfluidics with LIF provides the potential to detect ultra-trace quantities of bioactive lipids via fluorescence detection. Detection limits using the previously described analysis system have shown detection capabilities at 436 amol per droplet (Figure 4-12). We estimate that under these conditions, we could accurately detect concentrations down to \(10^{-17}\) moles per droplet, which provides an improvement of up to three orders of magnitude over that of the currently existing HPLC-MS methodology (20 fmol LOD). The ability to conduct fluorescent tagging reactions and the incorporation of chromatographic separations on-chip enables the possibility of a true \(\mu\)TAS system of analysis, dramatically reducing any errors caused by sample handling. Microfluidics also presents the opportunity to move towards a green analysis system, with a dramatic reduction of chemical and sample usage due to the extremely low flow rates and volumes
on-chip. As LIF is a non-destructive detection technique, microfluidic device fabrication can be further modified to incorporate seamless integration with MS detection. Using this proposed analysis system design, LIF can be used for highly sensitive quantification, while MS can provide accurate identification potential. Further work towards optimizing on-chip chromatographic separation efficiency by addressing the inherent PDMS robustness issues will be required prior to the investigation of using a microfluidic system for the analysis and highly sensitive detection of biologically endogenous PFAMs.
Appendix 1.

CELECOXIB DRUG UPTAKE INTO FLUOROCARBON NANOEMULSIONS

A.1. ABSTRACT

The use of theranostic agents and nanotechnology has the capabilities of providing new platforms for disease treatments and monitoring. Typical drug delivery vehicles require conjugation of a fluorescent dye to the surfactant in order to image biodistribution. A theranostic nanoreagent containing a perfluorocarbon (PFC) component acts as both a biologically inert drug delivery vehicle as well as a magnetic resonance imaging (MRI) tracer capable of in vivo monitoring. The combination of vehicle and tracer allows for unambiguous detection of drug delivery and its accumulation in target tissue. The development of a novel theranostic nanoreagents can potentially improve the therapeutic function of anti-inflammatory and anti-cancer drugs. This chapter provides insight to the use of HPLC-MS-MS to identify and quantitate a COX-2 inhibitor both in vitro and in vivo for use within theranostic nanoemulsions.

A.2. INTRODUCTION

Theranostics, or personalized medicine, is yet another biochemical field that has shown marked improvements with the advancement of micro- and nano-technology. The development of different systems and strategies for drug targeting to pathological sites, visualizing physiological processes, and the encompassing quantification has increased exponentially in recent years.225 The field of nanomedicine encompasses sub-micrometer
apparatuses used for diagnostics and treatments of disease, understanding pathophysiology of disease states, and improving patient quality of life. In the case of cancer, traditional treatment regiments prove incredibly difficult due to molecular heterogeneity between cancers of the same type, between tumors and their metastatic foci, and between cells within an individual tumor. Theranostic nanomedicine, in regard to cancer treatment, presents the ability to diagnose, deliver targeted therapy, and monitor therapeutical effects. Nanomedicine, as a whole, enable drugs to overcome chemical, anatomical, clinical, and physiological barriers by balancing localization in diseased-state versus healthy tissues and increasing accumulation at pathological sites. This, in turn, increases therapeutic efficacy and reduces both the number of and intensity of side effects, resulting in improved patient acceptance, compliance, and prognosis.

Specific drug targeting can come in either a passive or active form. Passive drug targeting often occurs in cancer treatment, as cancerous tumors often present with leaky and inflamed blood vessels, allowing for extravasation of drugs and nanomaterials into the pathological site. The enhanced permeability and retention effect allows for highly effective and selective passive drug targeting. In addition to having enlarged and leaking blood vessels, solid tumors lack functional lymphatics, resulting in the limited removal of nanomaterials from the drug target site. Conversely, localization and target cell uptake in active drug targeting often relies on antibodies, peptides, or sugar moieties, which are incorporated into the nanomaterial formulation.

A true theranostic nanomedicine contains both therapeutics and imaging agents within a single nanomaterial. The integration of diagnostics as well as therapy within a single nanomaterial has the potential of enabling both visualization of the material’s
biodistribution as well as treatment response.\textsuperscript{232, 233} A PFC nanoemulsion has been designed to incorporate a selective COX-2 inhibitor, celecoxib, which serves as a multimodal biological probe capable of studying COX-2 in macrophage tumor interaction.\textsuperscript{233} Additionally, the PFC nanoemulsion is equipped with dual-mode near-infrared fluorescence and \textsuperscript{19}F MRI imaging capabilities. The anti-inflammatory drug, celecoxib, has been reported to reduce cancer risk while suppressing tumor growth.\textsuperscript{234, 235} Additionally, celecoxib can induce COX-2 independent apoptosis, inhibit prostaglandin E\textsubscript{2} mediated anti-apoptotic proteins, and inhibit angiogenesis.\textsuperscript{236} Celecoxib is classified as a class II drug and has numerous adverse properties such as poor aqueous solubility (7 \textmu g/mL), requiring oral administration at high doses (200-400 mg, 2x/day) which can lead to severe cardiovascular side effects over a several month treatment regimen.\textsuperscript{237, 238} The rapid elimination from plasma experienced by celecoxib lowers the actual drug levels that reach the pathological site, resulting in the high required dosages.\textsuperscript{239, 240} Recent work with celecoxib containing nanoparticles has been reported for colon cancer treatment in a human xenograft mouse model.\textsuperscript{240} These adverse properties displayed, and potential benefits, have led celecoxib to be a promising drug for use within PFC nanoemulsions. Targeting circulating macrophages in and around pathological tissue sites, PFC nanoemulsions containing celecoxib can potentially suppress COX-2 activity while simultaneously allowing for the imaging of macrophage tumor infiltration dynamics.\textsuperscript{232, 233} The use of HPLC-MS-MS described herein provides a powerful analysis, identification, and quantification tool to further understand celecoxib uptake into both theranostic nanoemulsions as well as targeting efficiency into biological tissue.
A.3. MATERIALS AND METHODS

A.3.1. Chemicals

Methanol, optima grade, and formic acid, optima grade, were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Methyl tert-butyl ether was purchased from Sigma Aldrich (St. Louis, MO). PFC nanoemulsions, celecoxib standards, and surfactant mixtures were provided by Dr. Jelena Janjic (Department of Pharmacy, Duquesne University, Pittsburgh, PA).

A.3.2. Extraction of Celecoxib from Emulsion, Surfactant, and Cell Culture Matrices

Celecoxib was extracted from both PFC nanoemulsions and surfactant solutions for subsequent quantification by HPLC-QqQ-MS. The nanoemulsions were prepared with 1.38% Cremophor EL (CrEL) and 0.92% Pluronic P105 (P105) (w/v) and 0.188 mg/mL celecoxib. The surfactant solution was prepared with 3% CrEL, 2% P105, and 1% dimethylsulfoxide (w/v) and 0.188 mg/mL celecoxib. A 100 µL solution of celecoxib-doped nanoemulsion or surfactant solution was diluted to 4.93 mL with methyl tert-butyl ether (MTBE). The samples were sonicated for 3 × 10 seconds using a probe tip sonicator, followed by immersion in a sonicator bath for 30 minutes. A 25 µL aliquot of the MTBE dilution was further diluted to 1 mL with methanol modified with 0.3% formic acid. Both the nanoemulsions and surfactant solutions were diluted to celecoxib concentrations of 200, 100, and 50 nM prior to extraction to determine percent recovery. To eliminate any sample preparation or analysis bias, additional blind testing of nanoemulsions was conducted. Four nanoemulsions were submitted for analysis with one containing an
expected celecoxib concentration of 367 µM, another containing 445 µM, and two acting as control samples.

The extraction procedure of celecoxib from macrophage cell pellets was derived from previous work of Vioxx extraction from cell lysate. Four cell pellet samples (A-D) were submitted with pellet A containing $3.72 \times 10^6$ cells exposed to celecoxib containing nanoemulsion, pellet B containing $2.28 \times 10^6$ cells exposed to celecoxib containing nanoemulsion, pellet C containing $1.8 \times 10^6$ cells exposed to nanoemulsion in absence of celecoxib, and pellet D as the control containing $3.08 \times 10^6$ cells without exposure to nanoemulsion or celecoxib. 2 mL of deionized water was added to approximately 20 µL of each cell pellet and mixed thoroughly with a micropipette to break apart the pellet. The solution was placed in a sonicator for approximately 30 minutes, probe-tip sonicated for $3 \times 15$ seconds, and placed back in a sonicator for an additional 30 minutes to ensure complete cell lysing. Approximately 5 mL of MTBE was added to each sample and sonicated for 30 minutes. The organic layer was pipetted off, transferred to a test tube, and dried under nitrogen gas. The sample was resuspended in methanol modified with 0.3% formic acid. Celecoxib standards were prepared and a calibration curve was created to allow for quantitative analysis of celecoxib extracts.

A.3.3. Mass Spectrometry Analysis of Celecoxib

An Agilent 1200 series HPLC equipped with an Agilent 6460 QqQ-MS with APCI source was used for sensitive detection of celecoxib extracted from a variety of sample matrices. A 6 minute isocratic elution profile was used consisting of 80% methanol and 20% deionized water, both modified with 0.3% formic acid. A Phenomenex C18 column
(4.6 × 100 mm, 2.6 μm particle size) was used with an injection volume of 15 μL and flow rate of 0.5 mL/min. The MS instrumental parameters are as follows: fragmentor voltages of 135 V; dwell time of 500 ms; positive polarity; gas and vaporizer temperatures of 325 and 350°C, respectively; gas flow of 4 L/min; nebulizer pressure of 20 psi; capillary voltages of 4,500 V (+) and 4,500 V (-); and corona current of 4 μA (+) and 10 μA (-). MRM was optimized to monitor elution of the precursor ion at 382 m/z [M+H]^+ and the main product ion at 362 m/z with a collision energy of 30 V.

A.4. RESULTS

Celecoxib, a COX-2 inhibitor, is of great interest in theranostic medicine for its anti-inflammatory and anti-cancer properties. The HPLC-QqQ-MS methodology of detecting and quantifying celecoxib uptake into theranostic PFC nanoemulsions was developed to aid in the understanding of biological tissue targeting and therapeutic potential. A standard mixture of celecoxib was prepared at varying dilution levels from 1 – 500 nM and each sample run in triplicate to determine both linearity and reproducibility. Figure A-1 shows a calibration curve from the celecoxib standard data, with an R^2 value of 0.9989. Three nanoemulsion samples and three surfactant solution samples, each of which containing 50, 100, and 200 nM of celecoxib, were extracted and prepared according to method details in Appendix 3.2, and their celecoxib concentration was determined using the standard curve. Table A-1 summarizes the extraction results of celecoxib contained in both the nanoemulsions as well as the surfactant solutions. A 73%, 63%, and 61% recovery was obtained for the 50, 100, and 200 nM nanoemulsions, respectively. A 68%, 57%, and 59% recovery was obtained for the 50, 100, and 200 nM surfactant solutions, respectively.
The data points are plotted against the standard curve in Figure A-1 to show average peak area at the expected concentration of each extract as compared with the corresponding standard.

A blind study consisting of two nanoemulsion standards containing 367 µM and 445 µM and two control nanoemulsions without the presence of celecoxib were prepared and analyzed according to A.3.2. A new celecoxib calibration curve was prepared with concentrations ranging from 1 – 500 nM for quantitation of the nanoemulsion extracts. No celecoxib was detected in the two control samples. The 367 µM emulsion was found to contain 235 µM in the extract, representative of a 64% recovery. The 445 µM emulsion was found to contain 256 µM in the extract, representative of a 58% recovery.

Celecoxib concentrations were determined from the extract of four macrophage cell pellets, two of which were exposed to celecoxib containing nanoemulsion and two of which acted as controls. A freshly prepared calibration standard was used to create a standard curve used for quantitation. Pellets A and B were both exposed to celecoxib containing nanoemulsions and were found to contain 74.2 ng (973 nM) and 0.6 ng (8 nM) of celecoxib, respectively. Pellet C was exposed to nanoemulsion without celecoxib and contained 5.5 ng (76 nM) of celecoxib. Pellet D was not exposed to any nanoemulsion and was found to contain 8.5 ng (111 nM) celecoxib.
Figure A-1. Standard curve showing linearity and reproducibility of celecoxib standards run using HPLC-QqQ-MS. The standards were prepared in a concentration range from 1 – 500 nM. The data points for each of the three nanoemulsion and surfactant solution extracts are shown with the concentration plotted at their expected values. All samples were run in triplicate.

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>Expected Celecoxib Concentration (nM)</th>
<th>Actual Celecoxib Concentration (nM)</th>
<th>Celecoxib Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>E_{50}</td>
<td>50</td>
<td>36.6 ± 0.4</td>
<td>73%</td>
</tr>
<tr>
<td>E_{100}</td>
<td>100</td>
<td>63.4 ± 1.4</td>
<td>63%</td>
</tr>
<tr>
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<td>200</td>
<td>121.5 ± 2.0</td>
<td>61%</td>
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<td>S_{50}</td>
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<td>34.0 ± 2.1</td>
<td>68%</td>
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<td>100</td>
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<td>57%</td>
</tr>
<tr>
<td>S_{200}</td>
<td>200</td>
<td>118.0 ± 3.4</td>
<td>59%</td>
</tr>
</tbody>
</table>

Table A-1. Summary of celecoxib extraction from nanoemulsions and surfactant solutions. The expected celecoxib concentration reflects the sample preparation using known concentrations of celecoxib. The actual celecoxib concentration reflects the calculated concentration of celecoxib extracted from each sample according to the standard curve. The emulsions containing expected celecoxib concentrations of 50, 100, and 200 nM resulted in a percent recovery of 73%, 63%, and 61%, respectively. The surfactant solutions containing expected celecoxib concentrations of 50, 100, and 200 nM resulted in percent recoveries of 68%, 57%, and 59% respectively. All samples were run in triplicate.
A.5. DISCUSSION AND CONCLUSIONS

Instrumental parameters for highly sensitive detection of celecoxib using HPLC-QqQ-MS were successfully developed. The use of MRM detection allows for high selectivity towards the target analyte, eliminating any extraneous matrix effects. Fragmentation analysis from CID showed a quantitative product ion at 362 m/z, which was used for analysis. A linear range of 1 – 500 nM was determined and displayed a high $R^2$ value of 0.9989. The LOD for celecoxib was determined to be 15 femtomoles (1 nM). Celecoxib was shown to be successfully extracted from both PFC nanoemulsions as well as surfactant solutions, however, the measured concentration was always less than the expected concentration based on the amount of celecoxib added to each sample. Several explanations for the rational of less than 100% extraction efficiency may exist, such as errors in initial nanoemulsion and surfactant solution uptake of celecoxib, surfactant strength effects on micelle formation, or even analyte degradation over the course of sample preparation, storage, and extraction. The precise reasoning for the reported extraction efficiency, however, was not further pursued. The percent recovery of celecoxib did appear to decrease as the expected concentration in both the nanoemulsions and surfactant solutions increased, potentially suggesting that the actual amount of celecoxib uptake into the respective samples was not as great as anticipated. Average percent recoveries between nanoemulsions and surfactant solutions at the same expected concentration were comparable to one another. A blind extraction study of nanoemulsions both containing and in absence of celecoxib showed expected results, with the two control samples containing no detectable amounts of celecoxib, and the two samples with drug having comparable percent recoveries to initial nanoemulsion extractions. Inconsistent
results were noticed for the extraction of celecoxib from cell pellets. One of the two pellets that was exposed to drug showed a significant amount of celecoxib (74.2 ng) in the 20 µL cell pellet extract, where the other pellet exposed to drug only contained 0.6 ng of celecoxib in the extract. When analyzing the two control samples, the pellet exposed to nanoemulsions without drug contained 5.5 ng of celecoxib in the extract and the pellet exposed to neither nanoemulsions nor drug contained 8.5 ng. With the second pellet exposed to drug displaying such a low amount of celecoxib, it can be deduced that the sample was either mislabeled or incorrectly prepared for the drug uptake procedure. The two control samples found to contain low amounts of celecoxib show that sample contamination is likely of concern. By analyzing samples using MRM, it can be confirmed that these two control samples did in fact contain drug, and clean blanks injected between each sample set show that there was no sample carry-over issues present. Information pertaining to the amount of celecoxib that was added to each cell solution was not made available to the analyst, therefore the extraction efficiency was unknown.

In summary, the anti-inflammatory drug, celecoxib, is used to treat pain and inflammation relating to such diseases as osteoarthritis, rheumatoid arthritis, and acute pain, as well as show potential for reducing cancer risk by suppressing tumor growth. The prospect of targeted drug delivery using nanoemulsions allows for the drug to selectively accumulate at the pathological site, thus increasing therapeutic efficacy and reducing both the number of and intensity of side effects. PFC nanoemulsions present the prospect of not only a drug delivery vehicle, but also a fluorescent tracing probe that can be used to verify successful biodistribution using appropriate imaging techniques. Here, we have described a highly sensitive and selective method of detecting celecoxib in a variety of sample
matrices, inclusive of surfactant solutions, nanoemulsions, and macrophage cells. Future work of *in vitro* extraction and quantitation of celecoxib from pathological tissues, such as nerve cells, will be a concluding step in understanding and optimizing the uptake and delivery of chronic pain and anti-cancer treatments, with highly selective imaging techniques being used as verification.
REFERENCES


