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Development and Optimization of Microfluidic Devices for the Study of Free Fatty Acids

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DEVELOPMENT AND OPTIMIZATION OF MICROFLUIDIC DEVICES FOR THE STUDY

OF FREE FATTY ACIDS

By

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This dissertation is dedicated to my family for their encouragement and support.
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ABSTRACT

Free fatty acids (FFAs) are signaling molecules secreted by adipocytes, or fat cells. Individual FFAs differ in their ability to stimulate or attenuate insulin secretion depending on the chain length and degree of unsaturation of the FFA. Due to these varying effects of individual FFA on insulin release and the relationship of obesity and type 2 diabetes, it is important to temporally resolve the secretion of individual FFA from adipocytes. While the selective re-incorporation and secretion of FFAs have been extensively studied, few have monitored the temporal secretory profiles of individual FFAs to clarify their biological effects. One of the major obstacles in monitoring temporal secretory profiles of FFAs is the time- and labor-intensive sample preparation for analysis by gas chromatography-mass spectrometry (GC-MS). Conventional preparation procedures are often inefficient since multiple extraction steps are required followed by derivatization to fatty acid methyl esters (FAMEs). The total time can require up to 48 hours before samples can be quantified by a GC-MS method. These requirements hinder sample throughput and are often prohibitive to the detection of acute changes in FFA secretions. Microfluidics can address these drawbacks by reducing the amount of reagents consumed and automating the procedures by integrating sample preparation steps. This dissertation describes the development and optimization of an integrated microfluidic device and associated analytical methods that could be used to monitor individual FFAs to assess what role they might have in cellular communication between fat tissue and the pancreas.

The first module of the integrated device automated derivatization of FFA to FAME via acid catalyzed esterification using methanolic-HCl reagent. A statistical multivariate optimization protocol called Design of Experiment (DOE) was used to aid in the method development by determining the combination of derivatization time ($T_{der}$) and ratio of methanolic-HCl:FFA ($R_{der}$) that maximized the conversion of two model FFAs to their methyl ester forms. Optimal derivatization conditions for the two model FFAs were $T_{der} = 0.8$ min and $R_{der} = 4.9$ with a resulting total sample preparation time of 5 min. This combination of $T_{der}$ and $R_{der}$ was used to derivatize 12 FFAs producing a range of derivatization efficiencies from 18% to 93%. As compared to a conventional macroscale derivatization of FFA, the derivatization module decreased the volume of methanolic-HCl and FFA by 20- and 1300-fold, respectively.
The second module of the microfluidic device purified the derivatized sample by liquid-liquid extraction of FAME from the derivatization fluid into a hexane phase for GC-MS analysis. The derivatization module was coupled to the extraction module in a continuous-flow format. Phase contact with the hexane flow in stratified and segmented flow formats were qualitatively investigated as methods to extract FAME. The flow regimes in which both flow formats can be generated were explored and applied to generate parallel fluid flow or alternating aqueous and organic segments. Further work is needed to separate the two phases in both formats before the integrated device can be applied to study the temporal secretion of individual FFAs from adipocytes.

A second goal of this research was to design and numerically optimize a microfluidic perfusion chamber capable of housing a large number of pancreatic islets of Langerhans for the application of glucose concentration in sinusoidal waveforms. A DOE tool called response surface methodology (RSM) was used to determine the optimum perfusion chamber size and operating flow rate that would deliver homogeneous glucose waveforms to 40 islets of Langerhans with minimal shear stress. 3D computational models were used to calculate the homogeneity of glucose concentration and magnitude of shear rate within the chamber. The optimal perfusion chamber design with a chamber length of 1.84 mm and a flow rate of 10 µL min\(^{-1}\) was validated to show that the 40 islets experienced shear rate ≤ 50 s\(^{-1}\) and a 0.13% variability in the spatial distribution of glucose within the chamber. The optimized design will permit the sampling of secretory products in a format that can be easily incorporated in an online analysis to realize an integrated system for the quantification of secretory profiles from islets.

Separately, the devices described in this dissertation allow for the assessment of adipocytes and islet functions. When integrated in a single device, it will permit investigation of how temporal secretion patterns for individual FFAs from adipocytes might alter the signaling processes within the islets of Langerhans that lead to type 2 diabetes.
CHAPTER 1

INTRODUCTION

1.1 Background

1.1.1 Diabetes and obesity

Diabetes is a group of metabolic diseases characterized by high blood glucose levels due to defects in the body’s ability to produce and/or use insulin, a pancreas-derived hormone. Diabetes is classified as either type 1 or type 2, where type 1 is due to a lack of insulin production because of autoimmune destruction of insulin-producing cells. Approximately 90% to 95% of diagnosed cases of diabetes is type 2, where defective insulin production is combined with desensitization of the peripheral tissues to the hormone. The persistent elevation of blood glucose levels, hyperglycemia, and insulin dysregulation can both result in and may contribute to the development of type 2 diabetes. Hyperglycemia can lead to complications such as heart disease, hypertension, kidney disease, and blindness. The prevalence of diabetes is projected to increase from 14% in 2010 to 21% of the US population by 2050.

Parallel to the rapid rise in diagnosed cases of diabetes, the epidemic of obesity is also affecting an increasing percentage of U.S. population; an estimated 34% of adults aged 20 years or older are classified as obese. Obesity is a state of energy imbalance between energy intake and expenditure and may lead to the development of impaired lipolysis for adipose tissue. Obesity has been implicated as contributing to insulin dysregulation by the secretion of excess free fatty acids (FFAs) and cytokines leading to impaired insulin actions. The adipoinsular axis hypothesis states that the dysregulation of the bidirectional feedback loop that exists between adipose tissue and pancreatic cells may lead to development of obesity and insulin resistance. How FFAs might contribute to the adipoinsular axis is further discussed in section 1.1.3.

1.1.2 Islets of Langerhans and adipose tissue

Islets of Langerhans are clusters of at least five types of hormone-secreting cells located within the pancreas and their primary function is to help maintain glucose homeostasis. Chapter 4 describes the design and optimization of a perfusion chamber to be used for ~20 islets to monitor intracellular and extracellular responses of islets to glucose stimulation. The two
hormones most investigated are insulin and glucagon, which act in times of food consumption (insulin) and food deprivation (glucagon). When blood sugar levels are high, β-cells secrete insulin to induce glucose uptake in the peripheral tissues to lower blood levels. When blood sugar is low, α-cells secrete glucagon, signaling the liver to metabolize stored glycogen to glucose and also signaling the adipose tissue to break down triacylglycerides (TAG) to secrete FFA for use as fuel.

Figure 1.1. Interactions between an adipocyte and a β-cell. Glucose metabolism initiates the release of insulin from β-cells. In turn, adipocytes uptake glucose and convert it to glycerol-3-phosphate. FFAs are esterified with glycerol-3-phosphate to form triacylglycerides (TAG), which accumulate within lipid droplets. When the hormone sensitive lipase is activated, hydrolysis of TAG occurs and FFAs are released. FFAs cause an increased flux of calcium ions (Ca\(^{2+}\)) into the β-cell, affecting insulin secretion from the β-cell. Colored arrows are for processes that are inhibitory (red arrow) or stimulatory (blue arrow). Adapted from references 7 and 8.

Figure 1.1 shows a simplified depiction of some of the interactions that may occur between a β−cell in the pancreas and an adipocyte within adipose tissue. Adipose tissue is composed mainly of loose connective tissues and adipocytes, with nerve tissue, stromalvascular cells, immune cells, and preadipocytes constituting a minor fraction. The tissue acts as a complex metabolic and endocrine organ producing hormones, inflammatory cytokines, and adipokines to influence feeding behavior and the metabolism of glucose and lipids. The majority
of adipocyte cell volume is made up of intracellular lipid droplets that contain neutral lipids such as TAG. In a postprandial state, insulin promotes TAG storage in adipose tissue; in a fasting state, hormones such as glucagon stimulate hydrolysis of the stored TAG.

The molecular structure of a FFA is composed of a carboxylic acid group with an aliphatic tail that can be saturated or unsaturated. FFAs found in adipose tissue have an even number of carbon atoms ranging from 12 to 24 carbon atoms and the unsaturated forms have between one and six double bonds. The percent composition of individual FFAs differs slightly between visceral and subcutaneous adipose tissue deposits, but the most predominate are oleic acid, a monounsaturated 18-carbon long FFA, and palmitic acid, a saturated 16-carbon long FFA (Table 1.1). The accumulation of visceral fat is associated with increased risk for development of type 2 diabetes.

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>Visceral adipose tissue (%)</th>
<th>Subcutaneous adipose tissue (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lauric acid (12:0)</td>
<td>0.13 ± 0.03</td>
<td>0.14 ± 0.03</td>
</tr>
<tr>
<td>Myristic acid (14:0)</td>
<td>1.46 ± 0.06</td>
<td>1.43 ± 0.07</td>
</tr>
<tr>
<td>Myristoleic acid (14:1n9)</td>
<td>0.13 ± 0.02</td>
<td>0.12 ± 0.02</td>
</tr>
<tr>
<td>Palmitic acid (16:0)</td>
<td>19.55 ± 0.52</td>
<td>20.24 ± 0.37</td>
</tr>
<tr>
<td>(16:1 trans)</td>
<td>0.15 ± 0.01</td>
<td>0.15 ± 0.01</td>
</tr>
<tr>
<td>Palmitoleic acid (16:1n7)</td>
<td>3.53 ± 0.25</td>
<td>2.95 ± 0.15</td>
</tr>
<tr>
<td>(16:1n9)</td>
<td>0.64 ± 0.02</td>
<td>0.67 ± 0.03</td>
</tr>
<tr>
<td>Stearic acid (18:0)</td>
<td>3.70 ± 0.14</td>
<td>4.01 ± 0.18</td>
</tr>
<tr>
<td>Oleic acid (18:1n9)</td>
<td>45.71 ± 0.56</td>
<td>45.14 ± 0.66</td>
</tr>
<tr>
<td>(18:1 trans)</td>
<td>0.74 ± 0.11</td>
<td>0.90 ± 0.10</td>
</tr>
<tr>
<td>Vaccenic acid (18:1n7)</td>
<td>1.80 ± 0.07</td>
<td>1.93 ± 0.05</td>
</tr>
<tr>
<td>Linoleic acid (18:2n6)</td>
<td>16.23 ± 0.53</td>
<td>16.23 ± 0.53</td>
</tr>
<tr>
<td>(18:2 cis, trans)</td>
<td>0.07 ± 0.00</td>
<td>0.14 ± 0.00</td>
</tr>
<tr>
<td>α-linolenic acid (18:3n3)</td>
<td>0.68 ± 0.06</td>
<td>0.54 ± 0.05</td>
</tr>
<tr>
<td>γ-linolenic acid (18:3n6)</td>
<td>0.05 ± 0.01</td>
<td>0.06 ± 0.01</td>
</tr>
<tr>
<td>(18:4n3)</td>
<td>0.17 ± 0.01</td>
<td>0.16 ± 0.01</td>
</tr>
<tr>
<td>Eicosanoic acid (20:1n9)</td>
<td>0.55 ± 0.09</td>
<td>0.57 ± 0.05</td>
</tr>
</tbody>
</table>
Table 1.1. - continued

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>Visceral adipose tissue (%)</th>
<th>Subcutaneous adipose tissue (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(20:2n6)</td>
<td>0.39 ± 0.08</td>
<td>0.30 ± 0.02</td>
</tr>
<tr>
<td>Dihomo- δ-linolenic acid (20:3n6)</td>
<td>0.46 ± 0.05</td>
<td>0.52 ± 0.04</td>
</tr>
<tr>
<td>Arachidonic acid (20:4n6)</td>
<td>0.48 ± 0.06</td>
<td>0.57 ± 0.04</td>
</tr>
<tr>
<td>Eicosapentaenoic acid (20:5n3)</td>
<td>0.26 ± 0.07</td>
<td>0.14 ± 0.02</td>
</tr>
<tr>
<td>(22:5n3)</td>
<td>0.22 ± 0.03</td>
<td>0.31 ± 0.03</td>
</tr>
<tr>
<td>Docosahexenoic acid (22:6n3)</td>
<td>0.88 ± 0.24</td>
<td>0.49 ± 0.13</td>
</tr>
<tr>
<td>Lignoceric acid (24:0)</td>
<td>0.79 ± 0.05</td>
<td>0.85 ± 0.12</td>
</tr>
<tr>
<td>Nervonic acid (24:1n9)</td>
<td>0.09 ± 0.01</td>
<td>0.09 ± 0.01</td>
</tr>
</tbody>
</table>

Composition is presented as mean ± standard error of mean. Common name of FFA is used when available and in parenthesis is the omega nomenclature denotation of the fatty acid in the form C:DnX: C is the number of carbon atoms, D is the number of double bonds, n as a notation for unbranched fatty acid form, and X designated the carbon atom where the first double bond is located when counting from the terminal methyl carbon atom. Adapted from reference 10.

1.1.3 Adipoinsular axis

It is accepted that obesity and type 2 diabetes are connected, but the relationship is still uncertain. Approximately 1-30 fmol total FFAs cell\(^{-1}\) min\(^{-1}\) are secreted from perfused adipocytes in an oscillatory manner with an average period of approximately 5 min (Figure 1.2).\(^{12}\) Secretion of individual FFAs is dependent on chain length and degree of unsaturation; FFAs between 16- and 18-carbons and a high degree of unsaturation are secreted more readily from adipose tissue (Figure 1.3).\(^{13}\) The relative rate of FFA mobilization from the adipose tissue for a monounsaturated 20-carbon FFA is approximately 6-fold lower than a 20-carbon FFA with 5 degrees of unsaturation.\(^{14}\)
Figure 1.2. Oscillatory secretion of FFAs from perfused adipocytes. Secretion of FFAs from perfused adipocytes is oscillatory in presence of 2 mM glucose (open circle) as compared to flat secretory profile in absence of glucose (filled symbol). Modified from reference 12.

Figure 1.3. Selective secretion of FFAs from adipose tissue. A. Medium chain length FFAs, such as the 16- and 18-carbon long FFAs, are more readily secreted from adipose tissue (relative mobilization >1) compared to the amount stored within the tissues as TAG. B. FFAs with more than 3 double bonds within their chain length are more readily secreted than FFAs with less than 3 double bonds. From reference 13.

These individual FFAs have differing effects on glucose-stimulated insulin secretion (GSIS) from β-cells. Stearate, a saturated 18-carbon FFA, potentiates insulin secretion from
human islets to a greater extent than octanoate, an 8-carbon FFA (Figure 1.4). As the degree of unsaturation decreases, the effect of individual FFAs on insulin secretion increases dramatically. For example, stearate in the presence of 12.5 mM glucose potentiates insulin secretion 21-fold more compared to stimulation with glucose alone. The accumulation and long-term exposure of non-adipose tissues to FFAs result in lipotoxicity, which induces cellular dysfunction, apoptosis, and contributes to the development of type 2 diabetes. There are also hormones released from adipocytes, such as leptin, that are known to act directly and indirectly on β-cells. In addition, insulin is known to stimulate the production and secretion of several of these hormones from adipose tissue. One objective of this research was to develop analytical methods that could be used to monitor temporal secretory profiles of individual FFAs to assess what role they might have in cellular communication between adipose tissue and the pancreas.

![Graph](image_url)

**Figure 1.4.** Selected chain length and degree of unsaturation of FFAs effects on insulin secretion. At elevated level of glucose (black bars), saturated and medium chain length stearate potentiates insulin secretion to a larger extent than its unsaturated form (linolenate). Adapted from reference 17.

### 1.2 Multiphasic flow microfluidics

To monitor secretion profiles of individual FFAs (Table 1.1), the analytical methods must be both sensitive and selective to resolve changes in secreted FFAs of different chain-lengths and degrees of unsaturation. Most studies have focused on static incubations of adipocytes and often
examine the total amount of FFAs, while not quantifying individual FFAs. Such static incubation methods can have high temporal resolution if the sample collection time is short but can be disadvantageous because as many as $10^6$ adipocytes\textsuperscript{19} or 100 mg of adipose tissue\textsuperscript{14} must be used for the acute changes to be detected. An alternative to the static incubation method is to perfuse the media over the cells so that each collected fraction of the perfused media is a representation of the dynamic FFA secretion process.

Colorimetric assays\textsuperscript{20,21} have been used to quantify the total amount of FFAs, but when the relative rate of lipolysis is important, glycerol is quantified.\textsuperscript{22,23} However, with these methods, the molecular structure of FFAs can not be examined. To quantify individual FFAs, reversed phase liquid chromatography (RPLC) coupled to tandem MS/MS has been used, but the most commonly used method for compositional analysis of individual FFAs is gas chromatography-mass spectrometry (GC-MS).\textsuperscript{24,25} A typical procedure for analysis of FFAs by GC-MS calls for multiple extraction steps and derivatization to a more volatile form requiring as long as 48 hours before the samples can be quantified.\textsuperscript{26}

Microfluidic devices are a popular choice over the time- and labor-intensive sample preparation performed on the macroscale because they reduce the amount of reagents consumed, are easily automated, cost less, and permit faster analysis. There are several inherent advantages associated with the ability to perform chemistry on a micro-dimension that have driven the rapid growth of microfluidics. The concept of an integrated microfluidic system called micro total analysis system (µTAS) involves the integration of all aspects of analysis from sample preparation to separation and detection in an assembly with minimal footprint. Recently, a microfluidic device was developed for the quantification of total amount of FFAs secreted from perfused adipocytes with a temporal resolution of 5 min but no secretory profiles were built for the individual FFAs because a fluorescence-based enzymatic assay method was used.\textsuperscript{27}

In this dissertation, Chapters 2 and 3 are devoted to developing an integrated microfluidic device to automate derivatization of FFAs to a more volatile form, needed to analyze by GC-MS. To build temporal secretion profiles, fluid flow from the derivatization module was coupled to the extraction module to generate stable and robust multiphase flow. Multiphase flow systems are defined as having at least two fluids with different chemical compositions, such as liquid/liquid, or different physical states such as gas/liquid. Chapter 2 describes the development and optimization of a microfluidic module to rapidly mix two miscible flows within a heated
reaction channel to derivatize FFAs to fatty acid methyl esters (FAMEs). Chapter 3 describes the development of a microfluidic module used for the extraction of FAMEs into an immiscible hexane phase.

1.2.1 Miscible multiphase flow

Many applications of microfluidics in environmental chemistry, organic synthesis, and point-of-care diagnostics use miscible multiphase flows to take advantage of the reduction in amount of reagents consumed, enhanced mixing, and ease of automation associated with microfluidics. The synthesis of FAMEs from FFAs using microfluidic devices allows for high product yield due to enhanced heat and mass transfer. Esterification of FFAs is conventionally performed by the acid-catalyzed reaction of FFAs with borontrifluoride (BF$_3$/methanol), sulfuric acid (H$_2$SO$_4$/methanol), or hydrochloric acid (methanolic-HCl). Methanolic-HCl is a common reagent because it rapidly derivatizes FFAs with minimal side reactions. For example, 0.2% HCl in methanol has been used to quantitatively derivatize FFAs at 100 °C in 5 min.

The conversion of FFAs to FAMEs with methanolic-HCl is a one-step, acid-catalyzed, reversible esterification within a heated reaction channel. The amount of FAME product generated depends on factors such as acid concentration, temperature, volume ratio of FFA to derivatization reagent, and amount of water and alcohol present. To develop a microfluidic module capable of rapidly derivatizing FFAs, a statistical protocol called design of experiment (DOE) was used to perform multivariate optimization of several factors affecting the amount of FAMEs synthesized. DOE is discussed in detail in Section 1.3.

1.2.2 Immiscible multiphase flow

While derivatization of FFA to FAME uses miscible flow, the subsequent step is the purification of the generated FAMEs by extracting them into an organic solvent for offline GC-MS analysis. The development of this extraction module uses immiscible multiphase flow consisting of methanolic-HCl derivatization phase and hexane phase. Systems that utilize miscible multiphase flow are less complex than immiscible multiphase systems. The complexities in multiphase flow originate from the downscaling, coexistence, and confinement of multiple phases in one system. For immiscible multiphase flow systems, the type of flow patterns obtained depends on several physical properties of the two fluids, but can be predicted
using dimensionless numbers that express the competing physical fluid dynamics present. For example, scaling from macro to micro dimensions allows viscous and interfacial forces to become dominant over gravitational and inertial forces. If the bond number ($Bo$) is less than 1, interfacial forces are dominant over gravitational force and immiscible liquids will exist as parallel flow streams. Reynolds number ($Re$) below $\sim 10^3$ characterizes the laminar flow regime where viscous forces are dominant over inertial forces and give constant fluid motions within parallel flow streams. Capillary number ($Ca$) is most often used to describe how multiphase flow phenomena, such as flow formation, coalescence, and splitting, can exist at the microscale level based on the competing viscous and interfacial forces. $Ca$ can vary between $10^{-6}$ and $10^{-1}$ and flow pattern maps (Figure 1.5) are used to depict the transitional regimes where different flow patterns can be observed at certain combinations of $Ca$ for the organic and aqueous phases. For the microfluidic devices described throughout Chapter 3, $Bo$ and $Re$ were $< 10^{-1}$ and $Ca$ varied to produce both stratified flow and segmented flow. In the following sections, the applications of fluid dynamics that dictate the generation and stabilization of stratified and segmented flows are presented.

![Image](image_url)

**Figure 1.5.** Flow patterns as a function of organic and water $Ca$ values. A. The variety of flow patterns (I-V) and distinct subregimes (i-iii) that can be generated within a single device as a function of the $Ca$ of the individual phases. B. Examples of flow patterns in the different $Ca$ regimes (I-V) corresponding to those outlined in A. Black is the organic phase and white is the water phase. From reference 43.
1.2.2.1 Stratified flow

Stratified flow consists of parallel laminar flow streams with a stable interface that exists along the contact length of the flow streams. This type of flow is accessible over a wide range of flow rates (Figure 1.5) and has been shown to be applicable for simultaneous contact of two-phase\(^36\) or even five-phase\(^37\) flows. This flow format decreases the time required for quantitative extraction by an order of magnitude compared to using a separatory funnel and mechanical shaking.\(^38\) The establishment of a stable interface between hexane and derivatization phase is key to successful application of stratified flow to the purification of FAMEs and is one focus of research performed in Chapter 3.

The location of the interface within an unmodified channel is dependent upon the pressure difference between the phases due to viscous flow (\(\Delta P_{\text{Flow}}\)) and on the Laplace pressure (\(\Delta P_{\text{Laplace}}\)) due to liquid-liquid interfacial tension.\(^39\) When two fluids with different viscosities come into contact, there is a \(\Delta P_{\text{Flow}}\) (Equation 1.1) between the organic phase (\(P_{\text{org}}\)) and aqueous phase (\(P_{\text{aq}}\)). The pressure of each phase in Equation 1.1 can be determined by estimating the pressure loss by each phase as it enters the channel through the inlet tubing (\(\Delta P_t\)), flows down the channel (\(\Delta P_c\)), and exits at the outlet at atmospheric pressure (\(P_{\text{atm}}\)).

\[
\Delta P_{\text{Flow}} = P_{\text{org}} - P_{\text{aq}}
\]

\[
P_{\text{org}} = P_{\text{atm}} + \Delta P_t + \Delta P_c
\]

Each of the factors within Equation 1.2 can be represented by the Poiseulle equation to yield the full form of Equation 1.1 as:

\[
\Delta P_{\text{Flow}} = \frac{32\eta_{\text{org}} v_{c,\text{org}} L_{c,\text{org}}}{D_{c,\text{org}}^2} + \frac{32\eta_{\text{org}} v_{t,\text{org}} L_{t,\text{org}}}{D_{t,\text{org}}^2} - \frac{32\eta_{\text{aq}} v_{c,\text{aq}} L_{c,\text{aq}}}{D_{c,\text{aq}}^2} - \frac{32\eta_{\text{aq}} v_{t,\text{aq}} L_{t,\text{aq}}}{D_{t,\text{aq}}^2}
\]

\(\Delta P_{\text{Flow}}\) is a function of viscosity (\(\eta\)), fluid velocity in the inlet tubing or channel (\(v_t\) or \(v_c\)), length of inlet tubing or channel (\(L_t\) or \(L_c\)), and hydraulic diameter of the outlet tubing or channel (\(D_t\) or \(D_c\)).

The Laplace pressure, (\(\Delta P_{\text{Laplace}}\)), is a result of the interfacial tension between two phases confined in a channel and is estimated based on the Young-Laplace equation:

\[
\frac{2\sigma \cos(\theta_{\text{ad}})}{d} < \Delta P_{\text{Laplace}} < \frac{2\sigma \cos(\theta_{\text{rec}})}{d}
\]
\( \Delta P_{\text{Laplace}} \) is dependent on the interfacial tension \( (\sigma) \), the curvature radius of the liquid-liquid interface \( (d) \), and the advancing and receding contact angle of the aqueous phase on the glass surface while in contact with an organic phase \( (\theta_{\text{ad}} \text{ and } \theta_{\text{rec}}) \). Since these are all constants, \( \Delta P_{\text{Laplace}} \) is restricted to values between the two sides of Equation 1.4.

The balance between \( \Delta P_{\text{Flow}} \) and \( \Delta P_{\text{Laplace}} \) determines the position of the interface within the channel and also affects phase separation at the outlet of the microfluidic device (Figure 1.6). Experimental parameters such as fluidic tubing dimensions and microfluidic geometry can increase or decrease \( \Delta P_{\text{Flow}} \) in comparison to \( \Delta P_{\text{Laplace}} \), and shift the organic phase toward (Figure 1.6C) or away (Figure 1.6E) from the aqueous phase. Ideally, experimental parameters are optimized to fix the interface to the middle of the channel (Figure 1.6A).

Figure 1.6. Pressure balance at liquid-liquid interface. A. The interface lies in the middle of the channel when the pressure difference in the flow \( (\Delta P_{\text{Flow}}) \) and Laplace pressure \( (\Delta P_{\text{Laplace}}) \) are balanced. B. Equal balanced flow in A allows for complete recovery of two separated phases at the outlets. C. \( \Delta P_{\text{Flow}} \geq \Delta P_{\text{Laplace}} \) causes the organic phase to shift towards the aqueous phase. D. The result of C is that the organic phase will partly exit out of the aqueous outlet. E. When \( \Delta P_{\text{Flow}} \leq \Delta P_{\text{Laplace}} \), the aqueous phase takes up more than half the width of the channel. F. In the case of E, some aqueous phase is recovered from the organic outlet. A, C and E are cross sectional profile views of main channel in B, D, and F, respectively. A, C, and E are from reference 39.

However the position of the interface within the channel would be easily affected by disturbances in the flow rates of syringe pumps and affect the downstream phases separation.
process (Figure 1.6D and F) hence methods to stabilize the interface are often utilized. Microfluidic features such as guide and pillar structures\textsuperscript{38,40,41} and methods such as the selective modification with octadecyltrichlorosilane (ODS)\textsuperscript{40} have been used to stabilize the interface for stratified flow. The combination of fabricated microfluidic features and modified surfaces act as a “fail-safe system”\textsuperscript{42} in case either one alone did not stabilize the interface. These interface stabilization techniques were tested in the development of FAMEs extraction module described in Chapter 3.

1.2.2.2 Segmented flow

When different phases are injected as adjacent streams into one channel, one will preferentially wet the channel walls and encapsulate the other if the interfacial forces dominate over the viscous forces. These two phases are known as the continuous phase and the dispersed phase, respectively. Modification of the channel walls can be performed to select which phase will be the dispersed phase, allowing for the generation of either aqueous phase segments or organic phase segments.\textsuperscript{43} The difference in the flow rates, and thus $Ca$, and the extent to which the continuous phase and dispersed phase interact with the channel walls yields complex flow patterns such as the segmented and stratified flows depicted in Figure 1.5.\textsuperscript{44}

The size of the encapsulated dispersed phase is used to sub-classify the flows as either segmented or droplet. A flow pattern is classified as droplet flow when the dimension of the dispersed phase is less than the width and depth of the channel. With segmented flow, the dispersed phase occupies the entire width of the channel and is investigated in Chapter 3. Segmented flow is ideal for continuous-flow extraction devices because of the high interfacial surface area, small molecular diffusion distance at the interface, fast thermal transfer, and minimal hydrodynamic dispersion. It has been demonstrated that extraction in this format gives 10-1000 fold enhancement of mass transfer compared to conventional extraction processes.\textsuperscript{45}
Common microfluidic geometries to produce segmented flow include T-junction and flow focusing (Figure 1.7). T-junction devices can easily couple the flow stream of one component to another component while allowing for droplet generation frequencies up to several hundred droplets per second. Unlike stratified flow, segmented flow occurs when the interfacial force is larger than the viscous force and can be obtained by tuning the geometry of the microfluidic device and surface chemistry as well as the fluid viscosity and flow velocity. Segment break up process have been observed to include three distinct flow transition regimes described as squeezing, dripping, and jetting. Because the majority of research has been in the dripping regime and that regime is the focus of our work, we will not go into details of the other regimes. Techniques to generate and coalesce derivatization phase segments were explored in Chapter 3.

1.3 Statistical Design of Experiment

The optimization of the FFA derivatization in Chapter 2 and a perfusion chamber design for islets of Langerhans in Chapter 4 can be labor and time intensive if an iterative and univariate approach is taken. Analytical method development and optimization processes have frequently utilized chemometric tools to reduce the number of experiments needed. Multivariate chemometric tools, such as DOE, vary multiple parameters and use mathematical models to assess the statistical significance of how each parameter affects the result. In the DOE method, the variables are termed “factors” and the result is the “response” which can be either qualitative or quantitative. Factors are typically incremented in “levels.” For example, in Chapter 2, DOE
was used to optimize the derivatization of FFAs by varying the derivatization time ($T_{der}$) and methanolic-HCl:FFA ($R_{der}$) ratio to maximize the response (the derivatization efficiency of the conversion of FFA to FAME). Whereas in Chapter 4, DOE was used determine the optimal perfusion chamber design and operating condition by varying the size of the chamber and flow rate to minimize flow-induced shear rate and maximize homogeneity of glucose.

To determine which factors have a significant effect on the response and to guide more extensive studies, an initial screening experiment may be performed. After identifying factors and responses, the minimum and maximum value for each factor define the experimental domain to be investigated. The combination of factors to be tested is determined based on the selected statistical model design. The most common designs are full factorial, central composite, Box-Behnken, Doehlert matrix, and mixture designs. Figure 1.8 depicts the design space of three factors with 3 levels, with each (●) denoting a combination of the factors to be performed in random order. A 3-level, 3-factor full factorial design has all possible combinations of the factors adding up to $3^3$ or 27 total experiments, increasing if replicates are performed. The central composite design (Figure 1.8A) decreases the minimum number of experiments to 15 while providing comparable statistical results. The Box-Behnken design is an alternative to central composite design where the minimum number of experiments is 13 (Figure 1.7B) and was used for the optimization of a perfusion chamber design in Chapter 4. Whereas in Chapter 2, the design was user-defined since it was a 3-level, 2-factor multivariate optimization.

Once all experiments are performed, analysis of variance (ANOVA) is performed to aid in the selection of the lowest-order polynomial model that adequately describes the factors that affect the response(s). The polynomial model can be represented graphically and is called a response surface which can be used to predict the response(s) at combination of factors the experimenter did not test. The response surface can have a linear, quadratic, or higher order polynomial relationship between the factors and their response. The more the factors interact with each other to affect the response, the greater the degree of curvature in the response surface.
1.4 Dissertation overview

To quantify the temporal secretion of individual FFAs based on their chain length and degree of unsaturation, a rapid and automated microfluidic device to derivatize FFAs into FAMEs and then extracted into hexane using segmented and stratified flows was developed. The second goal of this research was to design and optimize a perfusion chamber to contain ~20 islets of Langerhans for analysis of their responses upon stimulation with glucose waveform.

Chapter 2 describes the development of a microfluidic device to mix FFAs and a derivatization reagent within a heated reaction channel to yield FAMEs with a total sample preparation (derivatization and extraction) time of 5 min. In Chapter 3, stratified and segmented flow patterns were investigated as methods by which the extraction of FAMEs into hexane phase can be performed. In Chapter 4, DOE was used to numerically optimize a perfusion chamber design by assessing how flow rate and the chamber size affect the broadening of sinusoidal glucose waveform used to stimulate a group of islets. Chapter 5 summarizes the research and provides direction for future work.
CHAPTER 2

A MICROFLUIDIC DEVICE FOR THE AUTOMATED
DERIVATIZATION OF FREE FATTY ACIDS TO FATTY ACID
METHYL ESTERS

2.1 Introduction

Free fatty acids (FFAs) are major components of triacylglycerols and are ubiquitous in biological and environmental samples. In many cases, analysis of the FFA composition, as well as the total amount of fatty acids, is required. As an example, FFA compositions of soil\textsuperscript{52}, cellular secretions\textsuperscript{53}, and in foodstuffs\textsuperscript{54,55} have been performed. To underscore the importance of determining FFA composition, the relative amounts of the FFAs in dietary milk fats has been proposed as a potential determinant of childhood obesity.\textsuperscript{56}

Gas chromatography-mass spectrometry (GC-MS) is often used for both quantitative and qualitative analysis of FFA. Prior to GC-MS analysis, FFAs must be derivatized to a more volatile form, such as a fatty acid methyl ester, but the sample preparation procedure can be laborious and time-intensive. The derivatization process may require up to 24 hours of reaction time\textsuperscript{26}, high temperature,\textsuperscript{57,34} and/or large volumes of hazardous reagents and solvents. One way to decrease reagent consumption and increase sample preparation throughput would be to automate the derivatization process. Automation of the FAME preparation procedure using a robotic system with acetyl chloride derivatization reagent has been performed, but the overall derivatization reaction required up to 1 hour at elevated temperatures with multiple liquid-liquid extraction steps,\textsuperscript{58} making the system expensive and complicated.

Microfluidic devices, and more generally continuous-flow microreactors, are ideal for automation because they can integrate multiple sample preparation steps, while decreasing reagent and sample consumption.\textsuperscript{38} These devices have been used for organic synthesis\textsuperscript{31,59} and have increased product yield in a shorter amount of time than comparable macroscale reactions. Integrated systems also open the possibility for rapid on-line screening allowing highly functional devices.\textsuperscript{30} To date, there have been no reports in the development of a microfluidic device for derivatization of FFAs to FAMEs prior to GC-MS analysis.
In this report, we describe a microfluidic chip that can be used to derivatize FFA with methanolic-HCl to produce the corresponding FAME. Tridecanoic acid and stearic acid were used as representative short and long chain FFA, respectively, to optimize reaction conditions. Through initial screening, derivatization time ($T_{\text{der}}$) and the relative ratio of methanolic-HCl to FFA ($R_{\text{der}}$) were two variables that could be easily varied on the device and alter the amount of FAME generated. Since the method development would be time-consuming and inefficient if a univariate approach was taken, a Design of Experiment (DOE) protocol was used to screen 13 combinations of $T_{\text{der}}$ and $R_{\text{der}}$ to maximize the responses, in this case, derivatization efficiencies of tridecanoic acid and stearic acid. After performing these experiments, equations were fit to these responses and used to predict responses at experimental conditions that were not performed. A numerical optimization was performed to determine the combination of $T_{\text{der}}$ and $R_{\text{der}}$ which would optimize the derivatization efficiency of all FFAs at one condition. The predicted response at the optimal combination was validated and used to derivatize a mixture of 12 FFAs with varying chain lengths and degrees of saturation to their corresponding FAMEs.

### 2.2 Materials and Methods

#### 2.2.1 Reagents and materials

Heptadecanoic acid, gamma-linolenic acid, oleic acid, eicosadienoic acid, and gondoic acid were from Nu-Chek Prep, Inc. (Elysian, MN). All other FFAs, methanolic-HCl, and a 37-component FAME mixture were from Sigma-Aldrich (St. Louis, MO). HPLC grade methanol and hexane were from EMD Chemicals (Gibbstown, NJ). All PEEK tubings, microfluidic reservoirs, and coupling adapters were purchased from Upchurch Scientific (Oak Harbor, WA). Capillary was from Polymicro Technologies (Phoenix, AZ).

#### 2.2.2 Fabrication of microfluidic devices

The design of the microfluidic derivatization device had two inlet channels (1 cm length each) that intersected and formed a reaction channel 20 cm in length (29 µL volume). The devices were fabricated using conventional photolithography and wet etching with hydrofluoric acid as described with the following additional steps. After the exposed photoresist and chrome layer were removed, the glass piece was baked at 105 °C for 30 min to harden the unexposed photoresist. Channels were etched to 230 x 510 µm (depth x width). Fluidic access holes for the channel inlets were drilled with a 0.012” (Industrial Power Tools, North
Tonawanda, NY, USA) diamond drill bit, whereas the outlet access hole was made with a 0.04” size drill bit (Abrasive Technology, Inc., Lewis Center, OH). The different sized access holes were to minimize dead volume at the inlets and to decrease backpressure at the outlet. After the microfluidic device was thermally bonded, microfluidic reservoirs for 360 µm o.d. capillary (Upchurch Scientific) were attached to the device according to the manufacturer’s instructions.

2.2.3 Experimental system

A Rheodyne 6-port HPLC injector (IDEX Health & Science, Rohnert Park, CA) and two syringe pumps (KDScientific, Inc., Holliston, MA) for methanolic-HCl and water (18 MΩ·cm water, Millipore, Bedford, MA) were connected to the two inlets of the microfluidic device. A 7.4 µL loop made with a 250 µm i.d. capillary was used with the 6-port injector. The syringe pump pushing water was connected to the injector using 0.04” i.d. PEEK tubing. Connections from the 6-port injector to the device, and from the other syringe pump pushing methanolic-HCl to the device, were made with 100 µm i.d. capillaries. The outlet reservoir of the device was connected to a 50 µm i.d. x 14 cm length capillary. The temperature of the derivatization reaction channel was maintained at 55 °C by a Kapton flexible heater (Omega Engineering, Inc., Stamford, CT) and a temperature controller (Omega Engineering, Inc., Stamford, CT).

2.2.4 Off-chip preparation of internal standard

Methyl pentadecanoate solution was used as the IS and was made by reacting 6.0 mg of pentadecanoic acid in 2 mL of methanolic-HCl at 55 °C for 5 min. Then, 10 mL of hexane was added and mixed by a vortexer for 2 min. 9 mL of the hexane phase was then removed. A second extraction of the aqueous phase was performed by addition of 2 mL of hexane to the original methanolic-HCl solution and extracted as before. 1 mL of the hexane phase was removed and combined with the 9 mL from the first extraction and placed in a 50-mL volumetric flask. Hexane was added to obtain approximately 100 µg mL⁻¹ IS solution.

2.2.5 On-chip derivatization

Volumetric flow rates and the sample collection and extraction time were calculated from the combination of $T_{der}$ and $R_{der}$ being tested. The reaction channel was 29 µL and at a given $T_{der}$, the total volumetric flow rate was calculated. Using this total flow rate at a given $R_{der}$, the flow rates of both the methanolic-HCl and FFA were calculated. For each on-chip derivatization
experiment, 5 µL of IS and 5 mL of hexane were added to a glass vial and stirred while held in an ice bath. The outlet capillary from the microfluidic device was placed into the hexane in this vial for a given time as described in the next paragraph.

Reaction conditions were optimized by using a mixture of 100 µg mL⁻¹ of tridecanoic acid and stearic acid in methanol. This solution filled the 7.4 µL injection loop and water was used to push this solution through the device. The start time for collecting the derivatized FAMEs was when the HPLC injector valve was actuated. The end of the collection time was twice the calculated time it took for the sample plug to flow through the sample loop, tubings, microfluidic chip, and outlet capillary at a given flow rate. After sample collection, the stirring in the collection vial was stopped and 1 min was allowed for phase separation. 4 mL of the hexane phase was transferred into another vial and evaporated using a stream of N₂. After evaporation, the sample was reconstituted in 100 µL hexane for GC-MS analysis. Between each derivatization, the device was flushed with 500 µL of methanolic-HCl and methanol.

2.2.6 GC-MS analysis

FAME detection was performed with an HP 6890 gas chromatograph with 5973N mass selective detector (Agilent Technologies, Inc., Santa Clara, CA). 1 µL splitless injection was performed onto the GC column (Restek Corp. RTX-5SIL MS column, 0.32 mm ID x 30 m length, 0.25 mm film thickness, Bellefonte, PA) with the injection port temperature held at 250 °C. The carrier phase was ultrahigh purity He at a flow rate of 1.0 mL min⁻¹. The chromatographic separation was t = 0 min/135 °C; t = 7.5 min/180 °C; t = 22 min/180 °C. For GC-MS analysis of the 37-component FAME mixture and 12 FFAs mixture, the temperature program was t = 0 min/135 °C; t = 7.5 min/180 °C; t = 22 min/180 °C; t = 23 min/208 °C; t = 34 min/230 °C; t = 44 min/230 °C. The temperature of the MS inlet and quadrupoles were 150 °C while the MS source was 230 °C. The m/z was scanned from 40-550 with a sampling rate of 1.47 scan sec⁻¹. On-chip derivatized peaks were identified by comparing their retention times with the 37-component FAME mixture and matching their mass spectra against a NIST reference mass spectra library (v 2.0d). Total ion count was used for integration of FAME peak areas. Calibration curves were made by diluting the 37-component FAME mixture 500, 167, 50 and 33-fold with hexane. These curves were used for calculating derivatization efficiency of the on-chip
reaction, expressed as a percent of the experimentally determined amount of FAME to the theoretical amount of FAME obtained if the derivatization efficiency was 100%.

2.2.7 Off-chip extraction efficiency

To test if the range of $T_{\text{der}}$ and $R_{\text{der}}$ used in the on-chip reactions affected off-chip extractions, four sets of samples at 1.2 min and 6.2 min extraction times at 0.4 and 5.0 $R_{\text{der}}$ were generated with a minimum of four replicates. Each sample contained identical volumes of methanolic-HCl, water and methanol, as well as identical concentrations of 100 µg mL$^{-1}$ methyl tridecanoate and methyl stearate mixture solution (prepared using the sample procedures as for IS solution), and IS. These volumes and concentrations were also identical to those samples derivatized in the microfluidic device. The samples were extracted and prepared for GC-MS analysis as described in GC-MS analysis section. A two-tailed $t$-test was used to compare the average FAME:IS peak areas.

2.2.8 Mixture of 12 FFAs

Individual stock solutions of 2 mg mL$^{-1}$ FFA in methanol were prepared for decanoic acid, lauric acid, tridecanoic acid, myristic acid, palmitoleic acid, palmitic acid, heptadecanoic acid, gamma-linolenic acid, oleic acid, stearic acid, eicosadienoic acid, and gondoic acid. A final mixture of the 12 FFAs was prepared with 300 µg mL$^{-1}$ decanoic acid and all other FFAs at 50 mg mL$^{-1}$.

2.2.9 Design of Experiment method

Design Expert® 7 software (StatEase, Minneapolis, MN) was used to guide the selection of $T_{\text{der}}$ and $R_{\text{der}}$ combinations to be tested. All statistical tests were deemed significant when $p < 0.05$. All values are given as the average peak area ± one standard deviation with the number of replicates given in the text.

2.3 Results and Discussion

To increase sample preparation throughput, a rapid method for converting FFAs to FAMEs prior to GC-MS analysis is needed. The microfluidic system shown in Figure 2.1 was developed for the rapid and simple derivatization of FFA to their corresponding FAME.
Figure 2.1. Experimental system. A syringe pump used water to drive a mixture of FFA from the sample loop of a 6-port injector into one arm of a microfluidic device. The conversion of FFAs to FAMEs occurred as the FFAs solution mixed with methanolic-HCl within the heated reaction channel. The flow rate ratio of the two syringe pumps ($R_{\text{der}}$) and the duration of time in the heated reaction channel ($T_{\text{der}}$) were varied using a DOE protocol to optimize the derivatization efficiency of two model FFAs.

Briefly, a 6-port HPLC injector was used to introduce a plug of FFA to one arm of a microfluidic device where it was mixed with methanolic-HCl derivatization reagent as it traversed a heated serpentine reaction channel. The FAME products then exited the device and were collected in a vial off-chip and extracted with hexane prior to injection onto a GC-MS system. Tridecanoic acid and stearic acid were used as model FFAs to be derivatized to methyl tridecanoate and methyl stearate, respectively.

To demonstrate the possibility of rapid on-chip derivatization of FFA, both $T_{\text{der}}$ and $R_{\text{der}}$ were optimized using a DOE protocol with the goal of the highest derivatization efficiency within a 6 min total sample preparation time. To accomplish this goal, $T_{\text{der}}$ was varied between 0.4 and 1.0 min, and $R_{\text{der}}$ varied between 0.4 and 5.0. $T_{\text{der}}$ was the time that the FFA had to react with methanolic-HCl in the heated reaction channel, while $R_{\text{der}}$ was the ratio of the volumetric flow rates of methanolic-HCl to FFA. The use of a DOE protocol allowed determination of
optimum $T_{der}$ and $R_{der}$ without the need to test a large experimental space. DOE protocols have been used to optimize reaction conditions, for example, to optimize the temperature, stoichiometry, and reaction temperature for removal of a protecting group from an amine in a continuous flow microreactor.\textsuperscript{49} In our optimization, 13 experiments with different combinations of $T_{der}$ and $R_{der}$ were performed with additional replicates added for assessment of precision, producing a total of 27 experiments over 3 days. Response surfaces were used to determine the condition where the derivatization efficiencies of tridecanoic acid and stearic acid were maximized.

2.3.1 Extraction variability

For each experiment, the combination of $T_{der}$ and $R_{der}$ being tested resulted in different extraction times and volume ratios of hexane:aqueous in the off-chip collection vial, which may have influenced the post-derivatization extractions of the FAMEs. To test if the different conditions affected the off-chip extraction, four samples with combinations of the smallest and largest sample collection time and $R_{der}$ used in the on-chip experiments, were extracted off-chip. We hypothesized that if the amount of FAME detected in these samples were not significantly different, the various extraction times and volume ratios of hexane:aqueous would not influence the amount of FAME extracted for all other samples.

Extraction with $R_{der}$ of 0.4 resulted in the same average FAME:IS peak area whether the extraction occurred for 1.2 min, $0.46 \pm 0.09$ for methyl tridecanoate and $0.54 \pm 0.04$ for methyl stearate ($n = 9$), or as long as 6.2 min, $0.50 \pm 0.11$ for methyl tridecanoate ($p = 0.38$) and $0.51 \pm 0.05$ ($n = 6, p = 0.19$). Similarly, when extracting with $R_{der}$ of 5.0, extraction times of 1.2 ($n = 8$) and 6.2 min ($n = 8$) resulted in the same FAME:IS ratio of $0.47 \pm 0.08$ versus $0.54 \pm 0.04$ for methyl tridecanoate ($p = 0.14$) and $0.55 \pm 0.04$ versus $0.56 \pm 0.02$ for methyl stearate ($p = 0.46$), respectively. The average FAME:IS peak area of these samples showed no statistical difference ($p > 0.05$) for both methyl tridecanoate and methyl stearate at any extraction time or $R_{der}$. The lack of statistical significance in these off-chip experiments implied that any differences in the FAME levels detected by the GC-MS were due to varying amounts of FAME produced on-chip because of differences in the $T_{der}$ and $R_{ders}$, not to processes outside the device.
2.3.2 On-chip derivatization

After demonstrating that the off-chip extraction procedure was not influencing the amount of FAME detected by the GC-MS, 27 on-chip derivatization experiments were performed with 13 different T_{der} and R_{der} combinations. After every two samples, a blank injection of hexane was made into the GC-MS and indicated no detectable carryover between runs. Blank runs were also performed through the device and showed no detectable FFA or FAME. In each of the 27 experiments, the same mass of FFA was subjected to derivatization. In most cases, unreacted FFA was not detected in the GC-MS chromatograms, except when T_{der} \leq 0.7 \text{ min}. To assess the inter-day precision, the percent relative standard deviation (%RSD) of the FAME:IS peak area ratio was calculated from replicates (n = 7) of the DOE centerpoint over 3 days. The DOE centerpoint was defined as the median values of the range of the two variables tested, T_{der} = 0.7 \text{ min} and R_{der} = 2.7. A 13% inter-day precision was obtained for tridecanoic acid and 15% for stearic acid. This poor precision may be due to incomplete derivatization for tridecanoic acid and stearic acid at these reaction conditions (67% and 86% derivatization efficiency, respectively). The DOE model assumed the same inter-day precision throughout all combinations of T_{der} and R_{der} tested. Intra-day precision (n = 3) was assessed as the %RSD of FAME:IS peak area ratio at one condition in one day and was found to be 14% for both tridecanoic acid and stearic acid.

2.3.3 ANOVA analysis of model designs

The model used to fit the derivatization response from the 27 samples was the quadratic model with the general form of:

\[ Y = \beta + A + B + (AB) + A^2 + B^2 + E \]  \hspace{1cm} (2.1)

where Y was the FFA derivatization efficiency, \( \beta \) was the model coefficient, A was T_{der}, B was R_{der}, and E the error term, while the terms in parentheses were interactions between the variables, and squared terms were the quadratic effects of the variables.\(^{51}\) Peak areas of methyl tridecanoate, methyl pentadecanoate, and methyl stearate from each on-chip derivatization sample were used to calculate the response expressed as the FFA derivatization efficiency as described in GC-MS analysis section. Analysis of variance (ANOVA) was performed to aid in the selection of the lowest-order polynomial model that adequately described the derivatization process. To perform ANOVA, an F-value for each variable was tabulated. In our experiments,
A $p$-value was calculated for each of the variables by comparing calculated $F$-values to a one-tailed $F$ distribution table. The $p$-value was the probability that the effect of the variable on the response was due to random error. Therefore, when $p < 0.05$, the effect was not due to random error and the variable had a significant effect on the response. A model reduction was done, which excluded the insignificant variables in equation 2.1 to the final reduced model form (equations 2.2 and 2.3), but still accounted for the variation of these terms in the $E$ term.

The final reduced model for the derivatization to methyl tridecanoate was linear with the form:

$$\text{Tridecanoic acid derivatization efficiency} = \beta + A + B + E \quad (2.2)$$

ANOVA results (Table 2.1) for the variables in Equation 2.2 indicated that $T_{\text{der}}$ was not significant ($p = 0.3086$), but was retained in the reduced model because $T_{\text{der}}$ was one of the tested variables. The model term estimated the factor effects and with $p = 0.0116$, $T_{\text{der}}$ and $R_{\text{der}}$ had significant effects on the derivatization of tridecanoic acid.

A reduced quadratic model was fit to the derivatization efficiency of stearic acid as described by equation 2.3:

$$\text{Stearic acid derivatization efficiency} = \beta + A + B + (AB) + B^2 + E \quad (2.3)$$

The ANOVA results for the variables in equation 2.3, as well as the excluded variables are shown in Table 2.1. Similar to the model for tridecanoic acid, the model term indicated these factors had significant effects ($p < 0.0001$) on the response.

### 2.3.4 Derivatization of tridecanoic acid

Equations 2.2 and 2.3 can be represented by multi-dimensional plots to visualize the derivatization efficiency response at a range of $T_{\text{der}}$ and $R_{\text{der}}$ values. The response surface plot for derivatization of tridecanoic acid to methyl tridecanoate is shown in Figure 2.2A. The shallow slope of the response over the range of $T_{\text{der}}$ demonstrates that this variable had less of an effect on the derivatization than $R_{\text{der}}$, which had a steeper slope. Using equation 2.2, the predicted vs. actual derivatization efficiency was plotted and showed a quality of fit ($R^2$) of 0.6042 (Figure 2.2B).
Figure 2.2. Model for derivatization efficiency of tridecanoic acid. A. Tridecanoic acid derivatization efficiencies were shown with respect to the two experimental variables, $R_{der}$ and $T_{der}$. B. Predicted vs. actual tridecanoic acid derivatization efficiency was linear with an $R^2$ of 0.6042.

One possibility for the low $R^2$ was that the model assumed the variability at all combinations of $T_{der}$ and $R_{der}$ were equal to the intra-day error of 13% found from replicates of a single combination of these variables, as mentioned in the on-chip derivatization section. If the variability in some responses were larger than this intra-day error, $R^2$ would be reduced. FFAs are known to adsorb onto silica in a chain-length dependent manner which may be pertinent in
our system. Regardless of the low $R^2$, as stated in ANOVA analysis discussion, the overall model was statistically significant (Table 2.1).

Table 2.1. ANOVA table for the response surface models.

<table>
<thead>
<tr>
<th>Source</th>
<th>Tridecanoic acid derivatization efficiency</th>
<th>Stearic acid derivatization efficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$df^a$</td>
<td>$F$ value</td>
</tr>
<tr>
<td>$A$</td>
<td>1</td>
<td>1.14</td>
</tr>
<tr>
<td>$B$</td>
<td>1</td>
<td>20.91</td>
</tr>
<tr>
<td>$AB$</td>
<td>1</td>
<td>0.41</td>
</tr>
<tr>
<td>$A^2$</td>
<td>1</td>
<td>0.53</td>
</tr>
<tr>
<td>$B^2$</td>
<td>1</td>
<td>1.20</td>
</tr>
<tr>
<td>Model</td>
<td>5</td>
<td>5.10</td>
</tr>
<tr>
<td>Error</td>
<td>11</td>
<td>1.70</td>
</tr>
</tbody>
</table>

$^a$Degrees of freedom for each source of variation
$^b$Not significant and moved to the Error term in the final models

Derivatization of tridecanoic acid had the lowest derivatization efficiency of 10% at the shortest $T_{der}$ and lowest $R_{der}$, and the highest conversion of 83% at the longest $T_{der}$ and highest $R_{der}$. While larger ranges of $T_{der}$ and $R_{der}$ could have been used in an attempt to obtain 100% derivatization efficiency, the ranges of $T_{der}$ and $R_{der}$ were limited intentionally to keep the total sample preparation time less than 6 min, which was the approximate time for macroscale sample preparation. There may be methods to increase tridecanoic acid derivatization efficiency without increasing total sample preparation, such as mixing the FFAs with the derivatization reagent in a faster manner, or by increasing the reaction temperature. Reproducibility of the tridecanoic acid derivatization varied from 13% - 28% in the range of $T_{der}$ and $R_{der}$ where no unreacted tridecanoic acid was detected.

**2.3.5 Derivatization of stearic acid**

Similar to tridecanoic acid, stearic acid derivatization efficiency (Figure 2.3A) was lowest at the shortest $T_{der}$ and lowest $R_{der}$, and was the highest at the longest $T_{der}$ and highest $R_{der}$. The derivatization efficiency of stearic acid was 100% when $T_{der} = 1.0$ min and $R_{der} = 5.0$. Curvature in the response near the longest $T_{der}$ and highest $R_{der}$ indicated that the response did not increase further and the optimum result was contained within the model. The $R^2$ value,
0.9235, of the reduced quadratic model for stearic acid derivatization (Figure 2.3B) showed the predictive power of the model. Reproducibility of stearic acid derivatization ranged from 15% - 23%.

Figure 2.3. Model for derivatization efficiency of stearic acid. A. The response surface for stearic acid derivatization is shown with maximum response at $T_{der} = 1$ min. and $R_{der} = 5.0$. B. The plot of predicted vs. actual stearic acid derivatization efficiency had an $R^2$ of 0.9235.
2.3.6 Numerical optimization

To determine the $T_{\text{der}}$ and $R_{\text{der}}$ that would generate the highest simultaneous derivatization of tridecanoic acid and stearic acid, a numerical optimization of the response surfaces was performed. Since unreacted tridecanoic acid was detected in most samples with $T_{\text{der}} \leq 0.7$ min at $2.7 R_{\text{der}}$, $T_{\text{der}}$ was restricted to 0.8 min. With this constraint on $T_{\text{der}}$, $R_{\text{der}}$ was set to be in the range of 4.9 – 4.95 to ensure that the total sample preparation time was less than 5 min. Tridecanoic acid derivatization efficiency was constrained to the range of 70% – 83%, the maximum experimentally obtained efficiency. Stearic acid derivatization efficiency was constrained to the range of 90% – 100%. Priority was given to tridecanoic acid derivatization being in the set range because derivatization of stearic acid was complete in this region. The solution meeting these criteria was $T_{\text{der}} = 0.8$ min and $R_{\text{der}} = 4.9$, which produced a 5 min sample collection time. On-chip derivatization of tridecanoic acid and stearic acid at these values of $T_{\text{der}}$ and $R_{\text{der}}$ was performed to validate the predicted solution. The derivatization efficiencies of $59 \pm 14\%$ and $94 \pm 4\%$ for tridecanoic acid and stearic acid, respectively, were statistically similar to the predicted solutions of $72 \pm 11\%$ and $94 \pm 8\%$, respectively ($p > 0.05$).

2.3.7 Derivatization of 12 FFAs

The microfluidic device was developed to increase sample preparation throughput by automating the derivatization of FFA to FAME prior to GC-MS analysis. To demonstrate the potential application for on-chip derivatization of a variety of FFAs, a mixture of 12 FFAs was derivatized to the corresponding FAMEs using the optimized combination of $T_{\text{der}}$ and $R_{\text{der}}$ (Figure 2.4).

All FFAs derivatized to their FAME forms with derivatization efficiencies ranging from 18% to 93% (Table 2.2). Decanoic acid had the lowest derivatization efficiency (18%) with a large percentage remaining as underivatized FFA, indicated by an asterisk in Figure 2.4. We believe the low derivatization efficiency of decanoic acid may be due to non-optimal reaction conditions for this FFA, as well as decanoic acid lost in pre-chip sample handling due to its high volatility. Derivatization efficiencies of tridecanoic acid and stearic acid correlated to the predicted solution described in the analysis of design models, indicating that derivatization efficiencies of the individual FFAs were not altered by inclusion in a more complex sample, making the applicability of this method for other samples feasible. One caveat of this approach
Figure 2.4. Validation of numerical optimization result. A mixture of 12 FFAs was derivatized using the optimal derivatization condition of $T_{\text{der}} = 0.8$ min. and $R_{\text{der}} = 4.9$ with the IS added. FAME peaks are (1) methyl decanoate, (2) methyl laurate, (3) methyl tridecanoate, (4) methyl myristate, (5) methyl palmitoleate, (6) methyl palmitate, (7) methyl heptadecanoate, (8) methyl gamma-linolenate, (9) methyl oleate, (10) methyl stearate, (11) methyl eicosadienoate, and (12) methyl gondoate. The asterisk (*) denotes underivatized decanoic acid.

was that FFAs longer than 20-carbons in chain length had limited solubility in methanol making their derivatization difficult with this procedure. However, in volume-limited samples, the method described here can be used for rapid derivatization of a number of short and medium chain length FFAs.

Table 2.2. On-chip derivatization efficiencies of 12 FFAs.

<table>
<thead>
<tr>
<th>Peak number</th>
<th>FAME common name</th>
<th>Derivatization efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Methyl decanoate</td>
<td>18</td>
</tr>
<tr>
<td>2</td>
<td>Methyl laurate</td>
<td>69</td>
</tr>
<tr>
<td>3</td>
<td>Methyl tridecanoate</td>
<td>61</td>
</tr>
<tr>
<td>4</td>
<td>Methyl myristate</td>
<td>78</td>
</tr>
<tr>
<td>5</td>
<td>Methyl palmitoleate</td>
<td>76</td>
</tr>
<tr>
<td>6</td>
<td>Methyl palmitate</td>
<td>93</td>
</tr>
<tr>
<td>7</td>
<td>Methyl heptadecanoate</td>
<td>71</td>
</tr>
<tr>
<td>8</td>
<td>Methyl gamma-linolenate</td>
<td>60</td>
</tr>
<tr>
<td>9</td>
<td>Methyl oleate</td>
<td>33</td>
</tr>
<tr>
<td>10</td>
<td>Methyl stearate</td>
<td>84</td>
</tr>
<tr>
<td>11</td>
<td>Methyl eicosadienoate</td>
<td>50</td>
</tr>
<tr>
<td>12</td>
<td>Methyl gondoate</td>
<td>45</td>
</tr>
</tbody>
</table>
2.4 Conclusions

DOE was used to optimize derivatization efficiencies of tridecanoic acid and stearic acid to their methyl ester forms. When compared to the typical sample preparation procedure listed in the product information sheet provided with methanolic-HCl, the optimized microfluidic derivatization resulted in a 20-fold decrease in the required methanolic-HCl and 1300-fold decrease in the amount of FFA required, all while maintaining a 5 min total sample preparation (derivatization and extraction) time. Future work will aim to integrate extraction of FAMEs with this derivatization module with coupling of the integrated microfluidic device to a GC-MS to realize continuous-flow preparation of FAMEs for an increased sample preparation throughput method.
CHAPTER 3

AN INTEGRATED MICROFLUIDIC DEVICE FOR THE DERIVATIZATION AND EXTRACTION OF FATTY ACID METHYL ESTERS

3.1 Introduction

FFAs are signaling molecules involved in cellular processes such as glucose metabolism in the muscle and liver. They regulate insulin production in pancreatic β-cells in a glucose-dependent manner and contribute to the insulin-leptin signaling pathway that exists between the pancreas and adipose tissue. GC-MS has been used to quantify FFA compositions in the intra- and extra-cellular environments of adipose tissue to examine the role of FFA in cell signaling. The majority of the sample preparation procedures of FFAs for GC-MS analysis are time- and labor-intensive since they call for multiple pre- and post-derivatization liquid-liquid extractions as well single- or multiple-step derivatization of FFA to a more volatile form of fatty acid methyl ester (FAME).

Microfluidics can address this lack of automation and inefficient preparation of FAME so that the temporal secretory profile of all individual FFAs, which other studies have not examine, can be built. Microfluidic devices, in a continuous-flow format, are ideal for automation because they can integrate multiple sample preparation steps, while decreasing reagent and sample consumption. Some modular microfluidic devices have been developed to perform simple sample preparation steps such as preconcentration, extraction, mixing, reaction, and purification. By integrating some of these discrete operation modular microfluidic devices into one device, multiple sample handling steps can be performed in an integrated continuous-flow device in an automated and efficient way.

The development and optimization of a microfluidic device for the derivatization of FFA to FAME has been described previously (Chapter 2 and in reference 68). The sample preparation procedure involved the derivatization of a mixture of 12 FFAs to FAMEs in a microfluidic device. The subsequent liquid-liquid extraction of the FAMEs into hexane was
performed offline prior to GC-MS analysis. To increase the automation and efficiency further, the development of a microfluidic module to extract FAMEs into hexane is needed.

In this chapter, extraction of FAME into hexane by both segmented and stratified multiphase flows were investigated. Since the extraction module was being developed concurrently with the derivatization module, some experiments were performed using different operating conditions than the previously described derivatization module. For segmented flow, the effects of the hexane flow rate and surface modification on the generation of segments were investigated. By varying the depth of the extraction channel, stratified flow was generated. Geometric features called guide structure were required to stabilize the interface in stratified flow. In both cases, the aqueous flow stream from the derivatization module was visualized by adding a colored dye. Visual confirmation of flow patterns was used as a qualitative method to assess the geometry and syringe pump conditions that would give stable and reproducible flows.

3.2 Materials and methods

3.2.1 Reagents and materials

Methanolic-HCl, anhydrous toluene, octadecyltrichlorosilane (ODS) were from Sigma-Aldrich (St. Louis, MO). Hexane was from EMD Chemicals (Gibbstown, NJ). Water (18 MΩ·cm, Millipore, Bedford, MA) and ethanol (Pharmco-aaper, Shelbyville, KY) were also used. All PEEK tubings, microfluidic reservoirs, and coupling adapters were purchased from Upchurch Scientific (Oak Harbor, WA). Capillary tubings were from Polymicro Technologies (Phoenix, AZ). Glass gas-tight syringes were from Hamilton Company (Reno, NV).

3.2.2 Fabrication of microfluidic devices

The design of the microfluidic derivatization device had three fluidic input channels (1 cm length each) for methanolic-HCl, water, and hexane. The derivatization reaction channel was 20 cm in length and the extraction channel length was varied from 1 cm to 20 cm. The devices were fabricated using conventional photolithography and wet etching with hydrofluoric acid (HF) as described previously with the following additional steps. Channels within the derivatization module were etched to 230 µm depth as described in Chapter 2. For certain geometry designs, HF-resistant tape (Semiconductor Equipment Corp., Moorpark, CA) was used to mask the areas where channel depths less than 230 µm were needed for the extraction module.
Other techniques used to fabricate guide structure and hydrophilic bridges were performed as described in Appendix B. Fluidic access holes for the channel inlets and outlets were drilled with a 0.012” (Industrial Power Tools, North Tonawanda, NY) diamond drill bit. After the microfluidic device was thermally bonded, microfluidic reservoirs for 360 µm o.d. capillary were attached to the device according to the manufacturer’s instructions.

3.2.3 Experimental

3.2.3.1 Experimental system

The system used was as described previously with the following additions for the development of the extraction module. KDScientific (Holliston, MA) or New Era syringe pumps were used to push hexane into the microfluidic device. Flow rates of derivatization fluids and hexane were varied as described in the results section.

3.2.3.2 Visualization of flow patterns

Prior to the observations of the flow pattern, the channels within the devices were wetted with ethanol to minimize the occurrence of air bubbles that would disrupt the flow downstream. Then methanolic-HCl, hexane, and water were flowed through the device for approximately 5 minutes to ensure the fluids properly wetted the walls of the channels. For visualization of flow patterns, green food coloring was added to the methanolic-HCl solution so that the fluidic flow from the derivatization module was distinguished from the colorless hexane flow. Videos were recorded for determination of flow characteristics using a microscope digital camera (MDC200, Hangzhou ScopeTek Opto-Electric Co., Zhejiang Province, China).

3.2.4 Stratified flow

Initial experiments were performed with a microfluidic device containing a serpentine extraction channel of various lengths and a depth of 30 µm. Then, devices with guide structure were fabricated using a photomask design containing two parallel channels separated by a variable distance. During HF etching, the distance between the two parallel channels was also etched and the etching process was stopped when the depths of the channels reached 230 µm. At the very bottom of the two now overlapping channels was a portion of unetched glass that formed a guide structure. Devices with guide structures of heights varying from 50 to 130 µm were fabricated. CARM design with two different depth channels was also tested for the generation of stable stratified flow. The CARM devices had the derivatization channel etched to
approximately 230 µm depth while the depth of the hexane channel depth was varied from 30 - 150 µm. For devices with hexane channel depths greater than 80 µm, the hexane channel was modified with a 1 wt% ODS solution in toluene using a combination of pressure driven flows from a syringe pump at the hexane inlet port and vacuum applied at hexane outlet port. Devices with hexane channel depths less than 80 µm were modified by adding 1-2 µL of 1 wt% ODS in toluene at hexane reservoir where capillary action then pulled the solution into the shallow channel.

3.2.5 Segmented flow

Serpentine channels with depths from 160 - 230 µm and lengths of 2 - 10 cm were used. To generate hexane segments in a derivatization fluid continuous phase, the extraction channel was bare glass. To generate derivatization fluid segment in hexane continuous phase, the extraction channel was modified with a 1 wt% ODS solution in toluene using a combination of pressure driven flow and vacuum. Coalescence of derivatization phase segment was attempted using a design with two parallel channels, one to generate and contain the segments (4 cm length) and the other to coalesce the segments (2 cm length) referred to as the extraction and external channel, respectively. A short, shallow channel of 6 µm depth acted as a fluidic contact region between the extraction and external channels. This approach is called a hydrophilic bridge because the main extraction channel was modified with 1 wt% ODS solution in toluene while the shallow channel and the external channel remained bare glass.

3.3. Results and discussion

Common geometries such as T-junction and flow focusing can be used to generate segments. T-junction devices are more appealing because the flow stream of one component can be easily coupled to the next component. With such ease of fabrication and operation, the T-junction configuration was used in the development of the extraction modules using stratified and segmented flow patterns. Strategies to form stable and robust stratified flow as well as methods to generate and coalesce segments in segmented flow were investigated.

3.3.1 Stratified flow

The influence of flow rate and geometry on the flow patterns generated within microfluidic devices with flow focusing geometry have been characterized. However, very
little is known about the factors affecting flow in multiphase systems with T-junction geometry using hexane and a mixture of methanolic-HCl and water. Therefore, initial screening experiments were performed without the derivatization module to determine the stratified flow regime. Upon integration of an extraction module to an optimized derivatization module, other microfluidic designs such as guide structures and CARM were attempted to stabilize the stratified flow. Factors such as the flow rate and backpressure dictated by the optimized derivatization module limit the operation conditions of the extraction module.

3.3.1.1 Shallow extraction channel

Based on published flow pattern maps depicting flow patterns observed at certain combinations of $Ca$ for the organic and aqueous phase, it was determined that both phases must be dominated by viscous forces at $Ca > 0.1$ to be in the stratified flow regime. The calculated depth of extraction channel needed to generate $Ca > 0.1$ was 20 $\mu$m for flow rate ratios of hexane:derivatization from 0.5:1 to 4:1 where the derivatization flow rate was 14.45 $\mu$L min$^{-1}$. The fabricated microfluidic device (Figure 3.1A) had three inlet channels for derivatization flow (Inlet$_{der}$) and hexane (Inlet$_{hexane}$) that met in a main channel and then two separate outlet channels, one for derivatization flow (Outlet$_{der}$) and another for hexane flow (Outlet$_{hexane}$). The depth of the extraction channel was 30 $\mu$m. Stratified flow in the form of parallel laminar flow streams was obtained in devices with extraction channel lengths of 1 cm to 20 cm at all tested flow rate ratios. The interface between two fluids did not remain steady as expected, but instead fluctuated back and forth with either phase occupying ~30 - 50% of the extraction channel width resulting in the derivatization fluid exiting from the Outlet$_{hexane}$. This fluctuation could be attributed to the differences in $\Delta P_{Flow}$ and $\Delta P_{Laplace}$ between the two phases. Therefore $\Delta P_{Laplace}$ was kept constant by maintaining a constant flow rate ratio and $\Delta P_{Flow}$ was altered in attempt to correct for this imbalance. Since $\Delta P_{Flow}$ is a function of the pressure drop in each channel and hence the inlet and outlet tubings dimensions (Equation 1.3), the fluid resistance of each channel can be varied by changing the length and i.d. of capillaries used for the inlets and outlets. Because the derivatization phase exited also from Outlet$_{hexane}$ then the $P_{aq}$ had exceeded $P_{org}$, thus $P_{org}$ must be increased so that hexane flow would push the derivatization flow away from Outlet$_{hexane}$. $P_{org}$ was increased 8-fold by using 20 cm length of 50 $\mu$m i.d. capillary at hexane outlet and the hexane flow pushed against the derivatization flow and occupied more than half the width of the extraction channel prior to the outlets. However the unsteady movement of the
fluids did not allow for conclusive observations to be made about how much the interface had shifted to prevent the undesirable derivatization flow from flowing into Outlet\textsubscript{hexane}. Unsteady flows might be due to the pulsatile performance of the New Era syringe pumps in these experiments, therefore less pulsatile KDScientific syringe pumps were used in the subsequent experiments.

![Diagram of extraction module](image)

Figure 3.1. Extraction module for the generation of stratified flow. A. Microfluidic device used for generation of stratified flow with the channels etched to a depth of 28 µm. B. Stratified flow observed throughout the entire 20-cm length of extraction channel for a wide range of volumetric flow rates ratio.

When the extraction module in Figure 3.1 was integrated with the derivatization module as in a single device, the backpressure generated by the shallow extraction channel prevented water and methanolic-HCl flows from entering the reaction channel within the derivatization module. Decreasing hexane flow rate or the extraction channel length did not alleviate the backpressure and the derivatization flow rate could not be changed because the derivatization reaction was already optimized. So the depth of the extraction channel was increased from 30 µm to 160 µm but segmented flow was obtained instead of the desired stratified flow. For that reason, methods to stabilize the interface of the parallel streams of laminar flow were investigated as described in section 3.3.1.2.

3.3.1.2 Guide structure

A guide structure feature was used to stabilize the stratified flow interface as the fluids traveled parallel to each other down one common extraction channel. A similar approach to the shallow extraction channel described previously was taken for the initial screening experiments.
of guide structures with microfluidic devices fabricated with a range of heights for the guide structure. For each device with a guide structure of a given height, the hexane:derivatization flow ratio was varied from 0.5:1 to 4:1 with the derivatization flow rate at 14.45 μL min⁻¹. In order to fabricate the ridge feature shown in Figure 3.2B called guide structure, the distance between the two channels was varied from 250 to 350 μm (Figure 3.2A) and both channels were etched to the same depth of 230 μm. The extraction channel’s length was 10 cm.

Unstable stratified flow was characterized by disruption of the interface between the two flow streams that changed the downstream flow into segmented flow. However as the guide structure’s height was increased from 50 μm to 115 μm, the length of extraction channel over which stable stratified flow was maintained increased from 1 mm to approximately 5 cm.

Using the insights gained in the screening experiments, microfluidic devices were fabricated to incorporate the derivatization module with extraction channel lengths of 3 cm or 5 cm and similar guide structure heights. The devices used for testing had guide structure heights of 130 μm with extraction channel lengths of 3 cm (Figure 3.3).
Figure 3.3. Stratified flow within guide structure microfluidic device with bare glass channels and ODS modified channels. A. When the channels were left as bare glass, stratified flow was established at the inlets region with the flow direction indicated by the white arrow. B. However, time elapsed snapshots (i-iii) of flow pattern at the outlets region showed a long slug of derivatization flow building up in hexane channel. C. Leakage of ODS coating into Inlet_{der} channel caused the hexane fluid to wet an area of the derivatization channel. D. Due to the ODS coating leakage at the inlets, the derivatization flow downstream at the outlets became unstable and resulted in a segment of derivatization fluid exiting from the Outlet_{hexane} channel.

The device with 5 cm length generated high backpressure and affected the flow in the derivatization module described as section 3.3.1.1. When the channels were uncoated, the derivatization flow wetted the hydrophilic walls of the Inlet_{hexane} channel, resulting in a thin green colored film (Figure 3.3A). Over time, the volume of the derivatization phase accumulated until the entire width of the Inlet_{hexane} was filled with a segment of the derivatization phase. Then the inflowing hexane phase pushed the segment downstream to give a segment of derivatization phase (Figure 3.3B) exiting Outlet_{hexane}. Several methods were attempted to strip
the ODS coating from the device, but ODS became covalently bonded to the surface silanol groups of the bare glass surfaces, therefore it was not possible to completely strip the ODS coating and regenerate the hydrophilic surfaces. Although the guide structure feature resulted in stratified flow, the surface wetting property of derivatization fluid in bare glass did not allow for stable stratified flow. Nevertheless, the ODS modification approach seems promising for generation of stratified flow.

### 3.3.1.3 Capillarity restricted modification

There are several techniques to control the interfacial tension to aid in the stabilization of the interface between two phases in stratified flow format. A microfluidic feature similar to guide structure and the ability to easily modify one channel was incorporated in CARM. \(^{40,41}\) CARM included two channels of different depths with the shallow channel modified with ODS to aid in the stabilization of the stratified flow’s interface. The asymmetrical cross section along with modified surface acts as a “fail safe” in case either one alone would not work. \(^{42}\)

![Diagram](image.png)

**Figure 3.4.** Capillary restricted modification (CARM). A. Photomask design used to fabricate the asymmetrical depths for the extraction module specified for CARM method. B. Profilometer scan depicting the cross sectional profile of the device with asymmetrical depths for hexane and derivatization channels.

CARM microfluidic devices were fabricated with the design shown in Figure 3.4A. The depth of the derivatization channel was kept constant at 230 µm while the hexane channel depths varied from 60 µm to 150 µm (Figure 3.4B) depending on the fabrication technique used. The tested volumetric flow rate ratio of hexane:derivatization was varied from 0.5:1 to 1:1 with the derivatization flow rate being 24.0 µL min\(^{-1}\). When the channels were bare hydrophilic glass,
derivatization fluid filled the shallow and narrow hexane channel in the area beyond the Inlet\textsubscript{hexane} (Figure 3.5). The derivatization flow wetted the hydrophilic wall of the Inlet\textsubscript{hexane} channel (red arrow in Figure 3.5A) and a thin film accumulated on the walls over time as described previously in section 3.3.1.2. As soon as the thin film volume spanned the entire cross-sectional area of the Inlet\textsubscript{hexane}, the inflowing hexane solution pushed the segment of derivatization phase fluid downstream. Then as the segment of derivatization fluid reached the derivatization channel, it merged with the flow stream and also caused instability in the hexane stream to generate a segment of hexane phase in the derivatization channel. As the result, the overall flow pattern observed was hexane segments separated by thin film of derivatization phase flowing downstream and exiting the Outlet\textsubscript{hexane} (Figure 3.5B).

![Figure 3.5](image)

**Figure 3.5.** Segmented flow in unmodified CARM microfluidic devices. A. The derivatization phase wetted the hydrophilic walls of Inlet\textsubscript{hexane} channel (red arrow) and generated hexane segments with the derivatization fluid as thin films in between. Depth of hexane channel was 140 \(\mu\)m and derivatization channel was 225 \(\mu\)m. B. Hexane segments and thin films of derivatization fluid exited Outlet\textsubscript{hexane} channel and mostly derivatization fluid exiting the Outlet\textsubscript{der} channel.

The ease in modifying the shallow channel in CARM devices had been attributed to the balance between the glass-liquid and air-liquid interfacial energies that aided in stopping the ODS solution at the boundary between the shallow and deep channel.\textsuperscript{41} The concept behind CARM was that water molecules in the derivatization fluid will adhere to the hydrophilic channel surface and prevent the liquid from flowing into the hydrophobic channel provided the pressure difference across the aqueous/organic interface does not exceed a critical value determined by the Young-Laplace equation.\textsuperscript{41} Stable and robust stratified flow should be
obtained for a wider range of volumetric flow rates compared to microfluidic devices that were not fabricated with the CARM feature.

Figure 3.6. Stratified flow in ODS modified CARM microfluidic devices. A. When just the hexane channel was modified with ODS, stratified flow was observed at the inlet channels region. B. However, at the outlets region (i) a segment of derivatization fluid (red arrow in ii) slowly flowed into the Outlet\textsubscript{hexane} channel and then retracted back into the derivatization fluid stream (red arrow in iii).

When the hexane channel of the device depicted in Figure 3.5 was modified with ODS, a stratified flow pattern was observed as shown in Figure 3.6. Each fluid stream remained confined in its respective channel at the inlets with no sign of the derivatization fluid wetting the walls of the Inlet\textsubscript{hexane} (Figure 3.6A). However, in the outlet channels region a growing plug of derivatization fluid (red arrow in Figure 3.6ii) migrated down the hexane channel. Before this plug exited the channel, it retracted back into the derivatization flow stream (red arrow in Figure 3.6iii). Occasionally the derivatization plug would exit the hexane outlet, but usually the plug would move into the hexane channel before retracting backward into the derivatization channel. The growing-retracting behavior of the derivatization plug might be a result of the uneven ODS coating at this particular area of the hexane channel. If ODS did not properly coat the entire channel then any area that remained as hydrophilic bare glass would be wetted by the derivatization fluid. The retraction of the derivatization fluid plug from the hexane channel occurred because the cross flow stream of hexane pushed the plug, preventing the plug from breaking off from the derivatization flow.

The ODS modification procedure for the hexane channel was performed again and stratified flow was obtained throughout the channel in the same device. However, fluctuation in
the flow streams resulted in a segment of derivatization fluid being pushed out of the Outlet\_hexane as well as segment of hexane being pushed out of Outlet\_der. This fluctuation could be attributed to the pressure differences in the Inlet\_der and Inlet\_hexane that could be equalized if the two inlets were of equal length and depths or to vary the flow resistance of each channel until a pressure balance was achieved.\textsuperscript{39} Given that the Inlet\_der was the optimized derivatization module with a 20-cm serpentine channel and the Inlet\_hexane was 1-cm long, the inlet pressures would be different. In many experiments, different length and i.d. capillaries were used at the Inlet\_hexane, Outlet\_der, and Outlet\_hexane to induce higher or lower pressure. However, these experiments failed to establish parallel flow streams along the entire length of the extraction region. This application of balanced pressures concept might not be applicable in the microfluidic devices that had been modified with ODS and/or had guide structures since the concept was proposed for two-phase flow streams in unmodified microfluidic channel. The two different inlet channel lengths might contribute minimally to the establishment of stratified flow since the instability in the flow pattern was observed at the outlet channels region. The major source of the fluctuation in the flow streams that prevent the formation of stable and robust stratified flow might be due to pulsatile characteristic of the syringe.\textsuperscript{69} Other devices with hexane channel depths of 62 \( \mu \)m and 75 \( \mu \)m were also tested, but fluctuation in the flow streams at the interface resulted in a thin film of derivatization fluid flowing into the Outlet\_hexane. Instead of driving flows with syringe pumps, a different method using a stainless steel pressure bomb to generate a more stable gas-pressure driven flow might aid in obtaining a stable stratified flow pattern.\textsuperscript{70}

### 3.3.2 Segmented flow

Segmented flow does require strict control of the system needed for the generation, breakup, and merging of segments. The mechanisms of segment breakup within the T-junction configuration\textsuperscript{71,72} have recently been characterized, but even the systems were not similar to the flow rates and channel dimensions described in these experiments for hexane and a mixture of methanolic-HCl and water. Segmented flow can be obtained by tuning the microfluidic device geometry and surface chemistry as well as the fluid viscosity and flow velocity. Parameters such as flow rates, viscosities, wettability of the channel walls, interfacial tension, presence of surfactants, and channel dimensions have been observed to affect segment breakup. Therefore, the effect of extraction channel depth on the ability to generate segmented flow was surveyed
and techniques such as surface modification and varying flow rate ratio of hexane:derivatization were explored. Since the purpose of this integrated device is the preparation of FFAs for GC-MS analysis, recovery of the hexane phase from the device involves the complete separation of hexane phase from derivatization phase. Selective ODS modification and different device geometries were used to separate the hexane phase from the derivatization phase.

### 3.3.2.1 Generation of segments

Based on published flow pattern maps, it was determined that both phases must be in the interfacial tension dominated regime, $Ca < 0.1$, to be in the segmented flow regime. Therefore, microfluidic devices with an extraction channel depth greater than 160 µm operating at flow rate ratio of hexane:derivatization 0.5:1 to 2:1 would generate $Ca \sim 10^{-2}$ for both phases. Microfluidic devices were then fabricated using the design shown in Figure 3.7A with part of Inlet$_{der}$, the Inlet$_{hexane}$, extraction channel, and both Outlet$_{hexane}$ and Outlet$_{der}$ depths as 160, 200, or 225 µm, and an extraction channel length of 10 cm. For the device with an extraction channel depth of 160 µm, high backpressure from the extraction module caused the hexane flow to migrate up the Inlet$_{der}$ and pushed the methanolic-HCl flow up the water inlet channel, as previously described. However, when a device with an extraction channel depth of 200 µm was tested at low hexane:derivatization flow rate ratio of 0.5:1 ($Ca\sim 10^{-3}$ for both phases), hexane segments were generated with the derivatization fluid as the continuous phase (Figure 3.7B). The two immiscible phases formed an interface at the junction of the Inlet$_{der}$ and Inlet$_{hexane}$ channels. As the stream of hexane flow moved pass the junction, the derivatization flow penetrated into the extraction channel and then the hexane segment was pinched off by the expanding derivatization flow until it spanned the width of the extraction channel. The hexane segment flowed downstream in the main channel while the tip of the derivatization flow retracted slightly into the Inlet$_{der}$ channel. The described pattern was not applicable above flow rate ratio of 0.5:1 because high backpressure from the extraction module prevented the derivatization module flow from entering the extraction channel. Also the derivatization phase wetted the wall.
Figure 3.7. Microfluidic devices to generate segmented flow. A. The length and depth of serpentine extraction channels were varied in this design to explore flow regime in which segmented flow can be generated. B. When the channels were left as bare glass, hexane segments were generated with the derivatization fluid as the continuous phase. C. However the build up of derivatization fluid at the hydrophilic surface of Inlet_\text{hexane} channel lead to an attempt to modify only the hexane inlet channel with ODS. Unsuccessful ODS modification of just the Inlet_\text{hexane} channel resulted in regions of the extraction channel containing stratified flow instead of segmented flow.

of the Inlet_\text{hexane} channel and occasionally the accumulated derivatization phase segment was pushed downstream. To prevent the build up of the derivatization phase, ODS solution was used to modify the short Inlet_\text{hexane} channel and not pass the T-junction. However, the ODS solution traveled down into the extraction channel. This unsuccessful ODS modification caused a combination of stratified and segmented flows in the device (Figure 3.7B).

To alleviate the high backpressure caused by the long extraction channel length, a microfluidic device with a shorter extraction length of 7.5 cm and deeper extraction channel depth of 230 µm was used. As the previous attempt to modify only the Inlet_\text{hexane} with ODS was unsuccessful, the entire length of the Inlet_\text{hexane}, extraction channel, and Outlet_\text{hexane} was modified with ODS. For this device with an extraction channel length of 7.5 cm and channel depth similar to the derivatization module, no observable derivatization phase accumulated on the wall of the Inlet_\text{hexane} channel and reproducible derivatization phase segments were successfully generated.
3.3.2.2 Segment coalescence

The extraction of FAMEs from the derivatization phase into hexane segments also required a way to separate the two phases to recover the hexane phase from the device. Passive and active methods to control segment fusion could be used however, active methods would increase complexity in fabrication and device operations so passive methods were explored. Passive methods included the use of various channel geometries or fabricated pillars to trap or slow the movement of the leading segment long enough for the subsequent segment to merge.\textsuperscript{73,74} Modification of the surface that comes into contact with the fluids can also be used to induce coalescence of segments.

Figure 3.8. The exclusion of derivatization fluid from hexane outlet channel. As alternating segment of derivatization and hexane fluids migrated toward the outlets region, the green derivatization fluid was excluded from Outlet\textsubscript{der} channel and the hexane flow was split between Outlet\textsubscript{der} and Outlet\textsubscript{hexane} channels.

Using the surface modification approach, the short Outlet\textsubscript{hexane} channel was coated with ODS in order to utilize the preferential wettability of the phase for hydrophobic walls. In the microfluidic device shown in the time-elapsed snapshots in Figure 3.8, as the long derivatization phase segment approached the outlet channels, all the green colored derivatization phase migrated into the hydrophilic Outlet\textsubscript{der} and was being excluded from the hydrophobic Outlet\textsubscript{hexane} channel. Conversely, the hexane flow was split evenly between Outlet\textsubscript{der} and Outlet\textsubscript{hexane}
channels as shown in panel (vi) of Figure 3.8. For this selective modification method to work, the volume of hexane phase loss due to the hexane phase flow exiting via the Outlet\textsubscript{der} channel must be minimal. Devices with Outlet\textsubscript{der} channel cross-sectional area smaller than that of the Outlet\textsubscript{hexane} might minimize the volume of hexane phase loss. With this design, as the derivatization phase segment approached the outlet channels, the segments would flow out the hydrophilic Outlet\textsubscript{der} while the majority of the subsequent hexane continuous phase would exit the hydrophobic Outlet\textsubscript{hexane} because of its smaller hydraulic resistance to flow.\textsuperscript{73,75} Using this approach, the separation of derivatization fluid from hexane with minimal loss of extracted FAME can be realized.

Figure 3.9. Segment coalescence using hydrophilic bridge method. A. Design of microfluidic device used to fabricate the hydrophilic bridge. B. Picture of the hydrophilic bridge used to connect the fluids flow in the extraction and external channels. C. Segmented flow migrated down the extraction channel and as it reached the 6 μm deep hydrophilic bridge, the difference in the hydrophobic surfaces of the extraction channel and the hydrophilic bridge would coalesce the segment and exit via the hydrophilic external channel.

Another method used to coalesce the segments and to separate the two immiscible phases was a microfluidic design called a hydrophilic bridge.\textsuperscript{76} The design in Figure 3.9A has the derivatization module joined in a T-junction configuration to a straight extraction channel with the Inlet\textsubscript{hexane} and Outlet\textsubscript{der}. A 6 μm-depth channel called the hydrophilic bridge was fabricated on a second glass piece. The bridge provided fluidic contact between the extraction channel and
external channel, where the merged segment can be pushed out (Figures 3.9B and C). All channels, except the hydrophilic bridge, were etched to 230 μm depth. The main extraction channel was modified with 1 wt% ODS solution in toluene as previously mentioned while the shallow channel and the external channel remained bare glass. When the extraction module’s channels were unmodified, hexane segmented flow was generated (Figure 3.10A) and the derivatization fluid in between each pair of hexane segments partially migrated into the external channel via the hydrophilic bridge (Figure 3.10B).

Figure 3.10. Unmodified channels and hydrophilic bridge. A. Hexane segments in derivatization fluid continuous phase occurred in unmodified channels. B. As the hexane segments traveled downstream in the extraction channel (i), the derivatization fluid in between the segment emptied partially into the external channel (ii-iv).

An inversion of the phases was obtained when the extraction channel was modified with ODS solution. Similar to segmented flow discussed in section 3.3.2.1, as derivatization fluid from the Inlet_{der} channel entered the hydrophobic extraction channel, the derivatization phase segment grew while the hexane flow slowly pinched off the segment. Derivatization phase segment break up occurred when the hexane fluid reached the entire cross sectional area of the channel. The segment then was pushed downstream by the hexane flow until the derivatization flow penetrated into the extraction channel to generate another segment (Figure 3.11A). As the hexane segments travelled down the extraction channel, their length elongated (Figure 3.11B) and this had been attributed syringe pump pressure fluctuation. When the derivatization phase
segments reached the hydrophilic bridge region, only a portion of the segment wetted the hydrophilic bridge and migrated into the external channel. The leakage of the ODS modification into the external channel (Figure 3.11B) occurred since there was an irregularly shaped region of the external channel where the green derivatization phase resided with the rest of the region transparent due to the hexane phase wetting these surfaces.

Figure 3.11. ODS modified channel for segment coalescence using hydrophilic bridge. A. Derivatization phase segments were generated by the shear stress from hexane flow (i and ii) and the segment elongated as it traveled down the extraction channel (iii and iv). B. As derivatization phase segment traveled downstream, both hexane and part of the derivatization fluid migrated into the external channel (i-iii) but left a long segment of derivatization phase in the extraction channel (iv).

The factor limiting successful coalescence of derivatization phase segments in the hydrophilic bridge devices had been the leakage of the ODS solution into the hydrophilic bridge and external channel. To prevent the leakage of ODS solution in unwanted areas, the extraction channel could be modified with ODS in patches to create an emulsion inversion within the extraction channel. The Inlet\textsubscript{hexane} and a short length of the extraction channel, upstream of the hydrophilic bridge, can be modified with ODS so that derivatization phase segments in hexane continuous phase can be generated as in Figure 3.11A. As the derivatization phase segment in hexane flow arrive at the interface between the ODS modified channel and the bare glass hydrophilic channel, a phase rearrangement or inversion would occur to give hexane phase segments in derivatization phase flow. Coalescence of hexane phase segments within
unmodified channels had shown promise in Figure 3.10 as a single hydrophilic bridge-external channel design. Therefore, a series of hydrophilic bridge-external channel features in combination with partial ODS modification of the extraction channel might lead to a functional continuous-flow device where the extraction of FAMEs via segment generation and coalescence can be realized.

3.4 Conclusions

The majority of research in diabetes has focused on islets of Langerhans while few have examined the mechanism and effects of FFA signaling on islets. GC-MS was used to investigate the secretion of selected FFAs from adipose tissue. However, few studies have attempted to build the secretory profile of the individual FFAs to understand why individual FFAs have different acute glucose-dependent affect on islets insulin secretion depending upon the chain length and degree of unsaturation of FFAs. One of the major obstacles in mapping the secretory profiles of individual FFAs is the time- and labor-intensive sample preparation of FFAs for analysis by GC-MS.

To automate and streamline the preparation of FFAs, an integrated continuous-flow microfluidic device capable of rapid derivatization of FFAs to FAMEs and subsequent extraction of FAMEs into hexane phase was developed. The rapid derivatization of FFAs to FAMEs has been previously demonstrated; within this chapter, strategies to form stable and robust stratified flow as well as methods to generate and coalesce segments in segmented flow were investigated. Promising qualitative results were obtained to meet these goals. CARM feature allowed for the generation of stratified flow but an alternative method to generate non-pulsatile flow is needed to obtain stable interface between the two phases so that hexane phase without trace amounts of the derivatization phase can be recovered. Also, extraction of FAMEs using segmented flow was successful in that the generation of derivatization phase segment in hexane continuous phase and its emulsion inversion can be generated. Further work is needed to develop a method to separate the phases in segmented flow so FAMEs can be analyzed on an offline GC-MS. Selective ODS modification and geometry designs had been proposed for further work in segment coalescence. Once a robust and quantitative extraction method is realized, the integrated device can be applied to mapping the secretory profile of individual FFAs from adipose tissue to elucidate potential connections between diseases like obesity and diabetes.
CHAPTER 4

NUMERICAL OPTIMIZATION OF A MICROFLUIDIC PERFUSION CHAMBER DESIGN USING RESPONSE SURFACE METHODOLOGY

4.1 Introduction

Pancreatic islets of Langerhans are micro-organs that play an important role in the regulation of blood sugar levels. Persistent elevated levels of blood glucose can both result in, and contribute to, the dysregulation of glucose homeostasis-regulating hormones leading to the development of diabetes.\textsuperscript{78,79} Perfusion studies are routinely used to assess islet function as they allow dynamic measurement of intracellular and extracellular metabolic products.\textsuperscript{80-82}

Since many signaling processes are produced by temporally varying changes in the extracellular environment \textit{in vivo}, devices that can reproduce these complex patterns and allow measurement of intra- and extra-cellular factors are required. Several advantages make microfluidic devices an attractive tool to precisely control the cellular environment to mimic \textit{in vivo} processes in both spatial and temporal manner, such as reduced consumption of reagents, fast molecular diffusion, and ease of automation. The characterization of dispersion and diffusion of analytes\textsuperscript{83,84} within microfluidic channels to produce complex temporal gradients has been studied; however, the attenuation of the waveforms due to the perfusion chamber geometry that may be at least 50-fold larger in volume than the channel remains largely unexplored.

Pulsatile secretion of pancreatic hormones \textit{in vivo} have lead to the use of large number of islets to explore islets synchronized responses to glucose profiles to approximate global response of islets within pancreas \textit{in vivo}.\textsuperscript{85-87} Microfluidic devices used to mimick these profiles in waveform patterns are generated by mixing two flow streams with varying flow rates ratio and lateral mixing of the flow streams results in an axial concentration gradient, a gradient that varies only along the length of the channel. As the waveform enters the perfusion chamber, diffusion and dispersion can attenuate the waveform and change the spatial-temporal distribution of glucose within the chamber. Heterogeneity in the glucose concentration affected the glucose-
dependent responses of islets located in different area of the chamber differently and thus can yield inaccurate results about their global responses within our study.

Our research group has developed a perfusion system to study the synchronized intracellular responses of approximately 20 islets to sinusoidal glucose concentrations. However, these islets were situated in an open chamber that made it challenging to integrate downstream analytical methods for online analysis of secreted hormones. A closed chamber would be more ideal as it would allow analysis of the entire perfusate. These types of chambers have been used for islet experiments, with most utilizing a shallow channel to trap the islets while allowing perfusate to flow over. Changes in the design of the perfusion chamber have necessitated the optimization of device geometry and operating conditions to ensure the 20 islets were exposed to homogeneous concentration of glucose while temporally varying sinusoidal concentration waveforms were applied. Microfluidic pressure filters and the manipulation of channel dimensions have been developed to minimize pressure and concentration variations in fluid flow that attenuate waveforms. We took a different approach to minimizing attenuation by examining the effect the geometric dimensions of a perfusion chamber would have on the waveforms during the design development stage. We hypothesize that attenuation of glucose waveform could be minimized by controlling the flow rate of glucose so that there is adequate fluid exchange time in the chamber while ensuring that the glucose concentration within the entire chamber is uniform. This report describes the numerical optimization of a microfluidic perfusion chamber designed to hold ~40 islets. The optimization was performed using a statistical multivariate optimization approach called response surface methodology (RSM). The goal of the optimization was to maximize homogeneity of glucose concentration while minimizing flow-induced shear on islets within the perfusion chamber. The optimal chamber design was numerically validated for 20 and 40 islets.

4.2 Methods

4.2.1 Comsol modeling

3D finite element simulations were performed using COMSOL Multiphysics 4.2 (COMSOL Inc., Burlington, MA). The 3D geometry of the microfluidic device consisted of an inlet channel, a fan shaped chamber, and a shallow channel that kept the islets contained within the chamber (Figure 4.1). The inlet channel had a width and height of 350 µm, and the shallow
channel height was kept constant at 20 µm. A fan shaped chamber was drawn as a 90° sector of a circle with a radius whose length was varied from 1.2 to 3.6 mm. Chamber height was varied between 360 µm and 621 µm, and a volumetric flow rate of 2 - 50 µL min\(^{-1}\) was used. The inlet’s glucose concentration waveform was modeled as a sinusoidal with a median of 11 mM and an amplitude of 1 mM at 0.0033 Hz (300 s period).

Figure 4.1. 3D geometry of the perfusion chamber. (a) Top view of geometry used in simulation experiments with the flow rate from the inlet, chamber radius length, and height of chamber were varied. (b) Side view of the inlet channel, perfusion chamber, and a shallow channel with a constant depth of 20 µm.

Models were used to study the spatial-temporal distribution of glucose within a fan shaped chamber as its concentration was varied at physiological temperature of 37 °C. The chamber mesh size was varied between 150 - 200 µm to give approximately 20-30,000 elements. Non-transient Navier-Stokes equation (equation 4.1) for Newtonian flow (constant viscosity) of an incompressible fluid was used to calculate the velocity profile and shear rate:

\[
\rho \frac{\partial \mathbf{u}}{\partial t} - \eta \nabla^2 \mathbf{u} + \rho (\mathbf{u} \cdot \nabla) \mathbf{u} + \nabla p = \mathbf{F} \tag{4.1}
\]

\[
\nabla \cdot \mathbf{u} = 0 \tag{4.2}
\]
\[ \nabla \equiv i \frac{\partial}{\partial x} + j \frac{\partial}{\partial y} + k \frac{\partial}{\partial z} \quad (4.3) \]

where \( \rho \) was density of water, \( \eta \) was the dynamic viscosity of water, \( \mathbf{u} \) was the velocity field, \( p \) as pressure, \( \mathbf{F} \) as volume force, and \( \nabla \) as the del operator. Equation 4.2 is the equation of continuity for incompressible fluids. The velocity field solution was coupled to the time-dependent general diffusion equation (equation 4.4) for species transport by convection and diffusion to compute mass transport within the chamber where \( c \) was the concentration of glucose, \( D \) was glucose diffusion coefficient, and \( R \) was the reaction rate expression for glucose.

\[ \frac{\partial c}{\partial t} + \mathbf{u} \cdot \nabla c = \nabla \cdot (D \nabla c) + R \quad (4.4) \]

A 0.5 s time step was imposed on the solver to ensure the rapid changes in sinusoidal concentrations were not missed. All parameters used in the models were as given in Table 1. See Appendix D for more details for Comsol modeling.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Symbol</th>
<th>Nominal value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose diffusion coefficient</td>
<td>( D )</td>
<td>( 9 \times 10^{-10} \text{ m}^2 \text{s}^{-1} )</td>
</tr>
<tr>
<td>Density of water</td>
<td>( \rho )</td>
<td>( 994.27 \text{ kg m}^{-3} )</td>
</tr>
<tr>
<td>Dynamic viscosity of water</td>
<td>( \eta )</td>
<td>( 6.89 \times 10^{-4} \text{ Pa s} )</td>
</tr>
<tr>
<td>Glucose reaction rate(^a)</td>
<td>( R )</td>
<td>( 8 \times 10^{-3} \text{ mol m}^{-3} \text{s}^{-1} )</td>
</tr>
<tr>
<td>Intra-islet glucose diffusion coeff(^a)</td>
<td>( D_i )</td>
<td>( 3 \times 10^{-10} \text{ m}^2 \text{s}^{-1} )</td>
</tr>
</tbody>
</table>

\(^a\)From reference 93

### 4.2.2 Islets within perfusion chamber

Islets with a diameter of 150 ± 75 µm (mean ± standard deviation, \( n = 20 \)) were used. The consumption of glucose by islets was incorporated into the mass transport equation to identify how it affected the glucose profile. The islets were placed toward the back of the chamber, as close to the shallow channel as possible, to reflect how the islets would settle when
the flow stream entered from the inlet channel and exited through the shallow channel. These arbitrarily arranged 46 µm - 327 µm diameter islets were similar to the distribution of islet size in wild-type mice.\(^\text{94}\)

**4.2.3 Data analysis**

Glucose concentration and shear rate values at 150 µm from the bottom of the chamber were exported from Comsol and used for the calculations of the DOE responses, homogeneity and %shear rate. Homogeneity was calculated by taking the ratio of the average concentration of glucose in the chamber \((\overline{C}_{\text{chamber}})\) to the concentration at the inlet of the chamber \((C_{\text{inlet}})\) and dividing by the standard deviation in glucose concentration within the chamber \((\sigma_{\text{chamber}})\).

\[
\text{Homogeneity} = \frac{\overline{C}_{\text{chamber}}}{C_{\text{inlet}}} \div \sigma_{\text{chamber}} \quad (4.5)
\]

The number of the chamber’s \(xyz\) coordinates with shear rate values \(\geq 51\ \text{s}^{-1}\) \((x_{\text{shear} \geq 51 \text{s}^{-1}})\) were counted and was used to calculate %shear rate where \(n\) represented the total number of data points (equation 4.6).

\[
\%\text{shear rate} = 100 \times \frac{x_{\text{shear} \geq 51 \text{s}^{-1}}}{n} \quad (4.6)
\]

**4.2.4 Response surface methodology**

Box-Behnken model design was used to select the combinations of flow rate, chamber radius length and chamber height tested. Design Expert 7 software (StatEase, Minneapolis, MN) was used to analyze the results with \(p < 0.05\) deemed as significant.

**4.3 Results and discussion**

A multivariate optimization approach was taken in the design of a microfluidic perfusion chamber. Based on initial screening experiments, flow rate and chamber size were the two variables that had the largest effect on homogeneity. For large volume chamber, fast flow rate ensure high homogeneity within the chamber but changing flow rate meant flow-induced shear exerted on islets must be examined because the cellular imaging method used requires the islets to remain stationary. Shear rate above 100 s\(^{-1}\) may detach cells from the substrate and make the analysis difficult.\(^\text{95}\) We therefore set out to maximize homogeneity while minimizing shear rate.
A minimum of 13 experiments was required to build the response surface models. Fluid and mass transport variables such as shear rate and homogeneity in the chamber would be challenging to quantify within a 3D perfusion chamber so computer models were used. Therefore, each computer simulation represented different combinations of flow rate, chamber width, and/or chamber height. Shear rate magnitudes, or the velocity gradient, and glucose concentrations within the chamber were used to calculate %shear rate and homogeneity. Shear rate magnitudes \( \geq 51 \text{ s}^{-1} \) were expressed as %shear rate to reflect the percent of the chamber’s surface area where the magnitude of shear rate might cause the islets to detach from the bottom of the chamber. The extent to which attenuation of glucose waveforms affected the spatial distribution of glucose within the chamber was calculated as homogeneity. Data were analyzed using RSM to formulate equations that were used to predict %shear rate and homogeneity at experimental conditions of flow rate and perfusion chamber dimensions not examined with the 15 experiments performed. A desirability optimization was then performed to determine the optimal perfusion chamber design and flow rate that would minimize shear on islets while maximizing the homogeneity in the chamber.

### 4.3.1 Design model for RSM

Computer simulations were performed to estimate the flow induced shear rate on islets and to assess the effect device geometry and flow rate had on the spatial distribution of glucose within the chamber. The Box-Benhken design requires a minimum of 13 experiments and contained no replicates because the results would be identical due to the deterministic computer simulations. In actual experiments, however, the order in which the islets are loaded and their arrangement within the chamber would not be as drawn as in Figure 4.1A. Therefore, to induce changes in shear rate and homogeneity, replicates were added for the centerpoint combination by randomizing the arrangement of the islets for a total of three different configurations. The response surface models obtained upon the addition of two replicates for a total of 15 simulations did not statistically differ compared to when 13 combinations were performed.

A general second-order polynomial (equation 4.7) was used to model the predicted response:

\[
Y = \beta + A + B + C + (AB) + (BC) + A^2 + B^2 + C^2 + E \quad (4.7)
\]
where $Y$ is the %shear rate or homogeneity, $\beta$ is the model coefficient, $A$ is chamber height, $B$ is flow rate, $C$ is radius length, and $E$ the error term, while the terms in parentheses are interactions between the variables, and squared terms are the quadratic effects of the variables. For each simulation, %shear rate and homogeneity were calculated at 150 µm from the bottom of the chamber, on level with the average sized islets. The statistical significance effect of each variable on the response was interpreted using the $F$-test in an analysis of variance (ANOVA) and backward regression analysis was performed to exclude the insignificant variables in Equation 4.7 to the final reduced model form (equations 4.8 and 4.9). These reduced models were used to predict %shear rate and homogeneity for any given geometry design and operating flow rate.

4.3.2 Shear rate in chamber

Cell survival and responses in microfluidic devices are regulated by shear stress and mass transport of nutrients. High shear rate can induce cellular stress responses, including apoptosis. In our analysis of islet intracellular response to simulated glucose oscillations, the islets must remain stationary for the duration of the perfusion experiment because the movement of islets due to flow-inducing shear could make imaging analysis difficult. Islets have withstood blood flow induced physiological shear rates of ~30-200 s$^{-1}$ and depending on cell to substrate interaction and duration of exposure, shear rate above 100 s$^{-1}$ could detach some cells from the substrate. Therefore, we limit our desired parameter to shear rate values below 50 s$^{-1}$ and express the percent of shear rate magnitude above 50 s$^{-1}$ as %shear rate in this study.

The linear predictive model for the square root transformed %shear rate within the chamber was:

$$\left(\%\text{shear rate}\right)^{1/2} = 4.38 + 0.096B - 1.79C$$  \hspace{1cm} (4.8)

A square root transformation of the response was performed to normalize the large distribution of %shear rate values and to improve the fit of the model to the data. This statistically significant model ($p < 0.0001$) expresses %shear rate as a function of flow rate and the chamber radius length ($p = 0.0001$ and 0.0002, respectively) but not chamber height ($p = 0.7536$). As the chamber size increases due to the increase in the radius length and height, the percent of the chamber surface area with shear rate magnitude that can detach islets decreases whereas the opposite was observed for increasing flow rate (Figure 4.2A). The predicted quality of fit
(predicted $R^2$) of Eqn. 7 to experimental data was 0.6575. A low predicted $R^2$ might be due to the model assumption that the variability at all combination of chamber radius length, chamber height, and flow were equal to the variability of 17% due to the three random islet arrangements. If the variability in %shear rate was larger due to effects of variables than islets arrangement then the predicted $R^2$ would be reduced.$^5$1

![Response surface model for square root transformed %shear rate.](image)

(a) A predictive 3D response surface plot for %shear rate as a function of chamber radius length and flow rate. (b) 2D plot of the spatial distribution of shear rate magnitudes giving the lowest %shear rate at 150 µm from the bottom of the chamber. (c) The spatial distribution of shear rate magnitudes within this 2D plot resulted in the highest %shear rate of 48. White lines represent the velocity flow streams as flow entered from the inlet into the chamber and exited from the shallow channel. White cross-patterned areas are islets larger than 150 µm situated within the chamber.
As the glucose flow stream \((Re < 1)\) entered the chamber, the parabolic flow stream fanned out until it reached the islets (white lines in Figure 4.2), while the decrease in height from the chamber to the shallow channel caused the flow stream to rapidly increase in velocity as it exits through the shallow channel. Shear rate magnitudes up to 685 \(s^{-1}\) were observed with the maximum shear rate magnitude localized in the area where the large chamber height transitioned into the shallow channel (Figure 4.2C). Islets located in this area experienced maximum flow induced shear on the side facing the shallow exit channel. The height of the shallow channel might be a factor worth investigating in future studies to minimize shear rate on islets and could result in a higher predicted \(R^2\) value. Since all areas within the chamber had drastically different shear rate magnitude, \%shear rate was chosen to reflect the percent of the chamber surface area where shear rate is \(\geq 51\ s^{-1}\). A range from 0 to 48 \%shear rate was obtained. Experiments with flow rate of 2 \(\mu\)L \(min^{-1}\) generated shear rate values of less than 50 \(s^{-1}\) throughout the entire chamber (Figure 4.2B). A maximum \%shear rate of 48 was obtained from an experiment with a flow rate of 50 \(\mu\)L \(min^{-1}\), a chamber height of 491 \(\mu\)m, and a chamber radius length of 1.2 mm (Figure 4.2C).

### 4.3.3 Mass transport

A reduced model for the square root transformation of homogeneity was:

\[
\text{(Homogeneity)}^{1/2} = 7.50 + 0.266B - 2.17C \tag{4.9}
\]

Chamber height \((p = 0.5315)\) and the interactions between flow rate and radius length of the chamber had no significant effect on homogeneity. Only flow rate and radius length of the chamber had statistically significant effects on homogeneity \((p < 0.0001\) and \(p = 0.0172\), respectively). The predicted \(R^2\) value for the reduced linear response surface for homogeneity was 0.7035. Flow rate had a larger effect on homogeneity than the chamber radius length’s effect on homogeneity, as indicated by steeper slope of change for homogeneity as a function of flow rate (Figure 4.3A). Flow rate and radius length had opposite effects on the homogeneity as compared to trends observed for \%shear rate. High flow rate in combination with small chamber size yielded a more homogeneous distribution (Figure 4.3B) than low flow rate and large sized chamber (Figure 4.3C). This opposite trend in homogeneity and \%shear rate demonstrates the need for optimization of perfusion chamber design.
Homogeneity of 1.6 – 414 was obtained where higher homogeneity values meant less variability in the glucose concentration in the chamber. The lowest value of 1.6 was obtained for the maximum 5.4 % variability in glucose concentration (Figure 4.3C) for the experiment using a flow rate of 2 µL min\(^{-1}\), chamber radius length of 3.6 mm, and height of 491 µm. Homogeneity of 414 (0.02 % variability in concentration, Figure 4.3B) was obtained for the chamber radius...
length of 2.4 mm and 360 µm height at a flow rate of 50 µL min⁻¹. The large disparity in the range of homogeneity values can be attributed to the temporal delay of the waveform in large sized chambers. The combination of the lowest flow rate, 2 µL min⁻¹, and increasing chamber volume (0.4 – 6 µL), caused a temporal delay of up to 1.7 min in the glucose waveform. Due to the temporal delay of the waveforms, attenuation was different for the left and right sides of the chamber as compared to the center of the chamber, in direct flow path of the inlet channel. In most experiments, the attenuation was a 1% difference between the inlet glucose concentration and the concentration within the chamber. For a flow rate of 2 µL min⁻¹, a chamber height of 491 µm, and a radius length of 3.6 mm a 13% attenuation resulted in a low homogeneity within the chamber. Low homogeneity is detrimental to our investigation of glucose waveforms as a global signal synchronizing groups of islet since any changes in the applied waveform could skew our analysis of synchronization events.

4.3.4 Desirability optimization

From the response surface models (equations 4.8 and 4.9), the chamber design and flow rate that minimized %shear rate and maximized homogeneity within the chamber was determined by using a desirability function. The desirability function involves the mathematical transformation of multiple response surfaces into a single one so that the scale ranges from 0 (undesirable) to 1 (desirable). This optimization method is ideal because the chamber dimensions and operating flow rate have opposite trends in their effect on %shear rate and homogeneity.

An optimal design was defined as the combination of chamber dimensions and flow rate that would operate up to 10 µL min⁻¹, yield a %shear rate ≤ 10, and a homogeneity ≥ 20. To maximize the duration of our perfusion experiment for analysis of islets, we limited the flow rate to the lower range of flow rates tested within our experimental space. A minimum homogeneity value of 20 and maximum value of 10 for %shear rate ensured that a group of islets would be perfused with the same concentration no matter their location within the chamber and flow induced shear rate would not detach the islets from the bottom of the perfusion chamber.
Figure 4.4. Optimal perfusion chamber design. (a) The result of a multivariate optimization using RSM was a perfusion chamber with a radius length of 1.84 mm, 360 µm height, and flow rate of 10 µL min\(^{-1}\). (b) Attenuation minimally altered the sinusoidal glucose waveform as it entered the chamber (black line) and dispersed throughout the chamber (green, red, and blue lines). The optimal combination of perfusion chamber size and flow rate minimize temporal delay of the waveform for areas not in direct flow path of the fluid (green and blue lines). Colored dots within the chamber correspond to the data used to graph the colored lines in the waveform plot.

A solution meeting all the defined criteria in the desirability optimization method was a chamber with radius length of 1.84 mm and an operating flow rate of 10 µL min\(^{-1}\). A simulation was performed of this perfusion chamber containing 20 islets and yielded %shear rate value of 0 and homogeneity of 118 with 0.07% variability in the concentration of glucose in the chamber. These %shear rate and homogeneity were within the predicted values for the response surface models. When the same chamber design was filled with 40 islets (Figure 4.4A), the maximum shear rate obtained was 40 s\(^{-1}\) and homogeneity of 62 due to the 0.13% variability in glucose concentration in the chamber. This demonstrated the capability of the optimal perfusion chamber design to house more than 20 islets within a homogeneous environment where there is minimal attenuation of stimulatory glucose waveform (Figure 4.4B) and minimal shear.

4.4 Conclusion

By examining the influence of flow rate and perfusion chamber dimensions on dispersion of glucose waveform, a numerically optimized, closed microfluidic chamber design for analysis of up to 40 islets of Langerhans was found. Cellular stress due to flow-induced shear rate was minimal and the groups of islets in the chamber were exposed to homogeneous temporal gradients of glucose thus allowing for accurate assessment of the global response. The closed chamber design will permit the sampling of secretory products in a format that can be easily
incorporated in an online analysis to realize an integrated system for multi-analyte quantification of glucose homeostasis-regulating hormones important to the pathophysiological study of diabetes.
CHAPTER 5

SUMMARY AND FUTURE STUDIES

5.1 Summary

Microfluidic devices were developed and optimized for the preparation of FFAs needed to build temporal secretory profiles and to study islets of Langerhans intra- and extra-cellular responses to glucose waveforms. DOE was used to optimize a microfluidic derivatization module for the maximal conversion of FFAs to FAMEs in a rapid manner. Co-current to the development of this module was the development of an extraction module where segmented and stratified flow patterns were generated in order to extract the FAMEs into hexane phase. Computer simulation experiments and RSM were used to numerically optimize a perfusion chamber design to study islets responses to glucose waveforms. Separately, these devices allowed for the assessment of adipocytes and islets functions; when integrated in a single device, it will permit investigation of how temporal secretion patterns for individual FFAs from adipocytes might alter the signaling processes within the islets of Langerhans that lead to type 2 diabetes and obesity. Future work includes methods to recover the extracted FAMEs in hexane phase for segmented flow and an alternative method for the rapid MS quantification of selected underivatized FFAs.

5.2 Future studies

5.2.1 Automated fraction-collection with off-chip segment coalescence

As described within Chapter 3, the most promising result obtained in the development of the extraction module was when the hexane outlet channel was modified with ODS and the derivatization outlet channel was left as bare glass. A recent publication described a similar approach but was used to coalesce organic phase segments using a split tee connector to make fluidic contact to a short length of 5% diphenyl/95% dimethylpolysiloxane coated capillary and a hydrophilic flow restrictor. Using this off-chip segment coalescence approach, a programmable small-volume fraction collector can be used to collect the derivatized and extracted FAMEs from the end of the coated capillary and place the samples into the GC-MS.
autosampler. Complete automation of the sample preparation of FFA to GC-MS analysis would be complete using the described approach.

5.2.2 Online ESI-MS detection of FFAs secretion from perfused adipocytes

Chapters 2 and 3 described an integrated microfluidic device for the preparation of FFAs for GC-MS analysis, however there are other methods in which FFAs can be quantify without the need for derivatization. Liquid chromatography-mass spectrometry (LC-MS) has been used to separate mixtures of secreted FFAs with MS detection in selected ion monitoring (SIM) mode. By taking advantage of the ease of coupling a microfluidic device for electrospray ionization (ESI) technique, the secretory profile of several major FFAs can be quantified near real-time. The proposed design for the microfluidic devices is as shown in Figure 5.1.

![Figure 5.1](image)

Figure 5.1. Perfusion of adipocytes and online extraction of FFAs for ESI-MS detection. A. Side view of perfusion chamber containing primary adipocytes with perfusion channel underneath the cells. B. FFAs from the perfusion chamber are purified in stratified flow pattern prior to ESI-MS for detection of FFAs.
Primary adipocytes can be loaded using the side channels and then closed off for perfusion experiment from the perpendicular channel. Perfusate containing secreted FFAs can be extracted using Bligh-Dyer method (chloroform: methanol mixture) and electrospray into MS interface. ESI-MS of chloroform:methanol mixture has been demonstrated previously\textsuperscript{99} thus the majority of the development and optimization procedures would focus on developing the microfluidic device.
APPENDIX A

QUANTIFICATION OF FAMES USING GC-MS

FAME detection was performed with an HP 6890 gas chromatograph with 5973N mass selective detector. There are several parameters for the GC-MS method that are crucial to the operation of the instrument and can affect the analysis of FAMEs but not mentioned within Chapter 2. Throughout the course of method development, other than the optimized temperature program needed to separate mixtures of FAME, it was found that parameters such as injection needle rinses and injection port programmed flows were as equally important for the quantitative analysis of FAMEs.

The pre- and post-injection rinses for the injection needle ensured that the syringe’s plunger movement was smooth and not resistant or “sticky” due to the use of hexane solvent. The automatic liquid sampler of the 6890 GC system allowed for pre- and post-injection injection syringe rinses with up to two solvents. Initial sample handling involved one post-injection syringe rinse with hexane but over time the FAME peak areas varied as much as 50%. Rigorous syringe rinses program consisting of one pre-injection rinse with methanol and two with hexane increased the reproducibility of FAME peak areas. Each rinse cycle pulled up 8 µL of the solvent. The addition of two post-injection rinses with methanol ensured that the plunger movement was smooth and it also pro-long the lifetime of the syringe.

After a 1 µL splitless injection was made, the gas flows within the injection port must be timed properly to minimize amount of sample loss. During a splitless injection, the valve (V) leading to inlet vent was turned off so there was no flow out of the split vent (Figure A.1). He gas flow at 4 mL min⁻¹ came in from the left of Figure A1 and split two-way with a default 3 mL min⁻¹ swept out as septum purge flow and the set 1 mL min⁻¹ going into the column. This configuration was maintained for 1 min to allow for the vaporized sample to be sweep into the column. After 1 min, V opened and total flow increased to 64 mL min⁻¹ with 60 mL min⁻¹ going to the inlet vent, 3 mL min⁻¹ out as septum purge flow, and 1 mL min⁻¹ into the column to purge the remaining vapors. A time of longer than 1 min for this step meant more samples swept into the column but larger variability in FAME peak areas were obtained. This purge flow configuration was held for 2 min and at time = 3 min, the inlet reverted to a gas saver mode to
conserv[e]e gas between injections by decreasing total flow to 24 mL min\(^{-1}\) with 20 mL min\(^{-1}\) exiting out the inlet vent, 3 mL min\(^{-1}\) out as septum purge flow, and 1 mL min\(^{-1}\) into the column.

![Diagram](image)

Figure A.1. Electronic pneumatic control inlet for splitless injection mode. GC inlet configuration to sweep the vaporized sample into the column. From reference 100.

FAME limit of detections (LOD) detected in (Table A.1) were calculated using Chemstation data analysis software (Agilent Technologies, Inc., Santa Clara, CA) based on three injections of a 1670-fold dilution of 37-component FAME reference standard mixture (Table A.1). LOD was defined as the amount of FAME detected that would give a peak height three times the peak-to-peak noise. First, a background-subtracted spectrum was obtained by using the time range of ~45-50 min within the hexane spectrum as the background signal to be subtracted from the sample’s spectrum. The time range spanning the width of the FAME peak was selected for the software to calculate the corrected FAME peak height. The time range for noise was selected as 1-2 min before the FAME peak. Chemstation performed a linear regression analysis to account for drift in the baseline for the data points within the selected time range. Then, the linear regression line was subtracted from the selected data points to yield drift-corrected noise. Peak-to-peak noise was calculated by subtracting the lowest intensity peak from the highest intensity peak in the drift-corrected noise range.
Table A.1. LOD of selected FAMEs.

<table>
<thead>
<tr>
<th>FFA</th>
<th>Average LOD (ng)</th>
<th>Standard deviation (ng)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10:0</td>
<td>0.82</td>
<td>0.15</td>
</tr>
<tr>
<td>12:0</td>
<td>0.13</td>
<td>0.01</td>
</tr>
<tr>
<td>13:0</td>
<td>0.10</td>
<td>0.01</td>
</tr>
<tr>
<td>14:0</td>
<td>0.08</td>
<td>0.01</td>
</tr>
<tr>
<td>15:0</td>
<td>0.08</td>
<td>0.02</td>
</tr>
<tr>
<td>16:0</td>
<td>0.07</td>
<td>&lt; 0.00</td>
</tr>
<tr>
<td>17:0</td>
<td>0.16</td>
<td>0.04</td>
</tr>
<tr>
<td>18:0</td>
<td>0.22</td>
<td>0.01</td>
</tr>
<tr>
<td>18:1n9</td>
<td>0.20</td>
<td>0.02</td>
</tr>
<tr>
<td>18:2n6</td>
<td>0.30</td>
<td>0.02</td>
</tr>
<tr>
<td>20:0</td>
<td>0.16</td>
<td>0.02</td>
</tr>
<tr>
<td>21:0</td>
<td>0.18</td>
<td>0.02</td>
</tr>
<tr>
<td>22:0</td>
<td>0.24</td>
<td>0.06</td>
</tr>
<tr>
<td>24:0</td>
<td>0.50</td>
<td>0.02</td>
</tr>
</tbody>
</table>
APPENDIX B

FABRICATION OF GLASS MICROFLUIDIC DEVICES

For each strategy implemented to obtain stable and reproducible segmented or stratified flow in Chapter 3, fabrication methods used to fabricate the glass microfluidic devices were also developed. Microfluidic devices were fabricated using conventional photolithography and wet etching with HF (Figure B.1). The steps from UV exposure to HF etching were used to fabricate devices used in Chapter 3. The borosilicate glass used was 5” x 5” so for each fabrication using a piece of glass yielded 4 – 8 microfluidic devices.

Figure B.1. Fabrication of glass microfluidic devices. A. A negative photomask contained the design for the channels. Upon exposure to UV light, these channels were areas where the photoresist layer became more solvent-soluble. B. After removing the photoresist and chrome layers, bare glass was exposed. C. HF etching of glass surfaces to obtain rounded channels. D. Remaining photoresist and chrome were removed, access holes were drilled, and chemical cleaning of the glass pieces were performed. E. A blank glass piece was used to close off the open channels. F. Thermal bonding of the glass pieces at 640 °C for 8 hr created a glass microfluidic device.
To fabricate the extraction module devices and integrated devices tested in section 3.3.1.2, a photomask containing two serpentine parallel channels separated by a certain distance was exposed to UV light. During HF etching, the distance between the two parallel channels was etched and the etching process was stopped when the depths reached a target depth (Table B.1). At the very bottom of the two now overlapping channels was a portion of unetched glass that formed a ridge or guide structure. Depending on fabrication conditions such as the quality of the coated borofloat glass pieces as well as concentration of the HF solution, variable guide structure’s heights of 54 to 124 µm were obtained.

Table B.1. Fabrication of guide structure feature.

<table>
<thead>
<tr>
<th>Fabrication method</th>
<th>Distance between parallel line (µm)</th>
<th>Height of guide structure (µm)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Extraction module (target depth = 250 µm)</td>
<td>350</td>
<td>54 ± 2 (n = 2)</td>
</tr>
<tr>
<td></td>
<td>375</td>
<td>64 ± 3 (n = 3)</td>
</tr>
<tr>
<td></td>
<td>400</td>
<td>76</td>
</tr>
<tr>
<td></td>
<td>450</td>
<td>116 ± 4 (n = 2)</td>
</tr>
<tr>
<td>Integrated device containing derivatization and extraction modules (target depth = 230 µm)</td>
<td>400</td>
<td>124 ± 7 (n = 3)</td>
</tr>
</tbody>
</table>

*Average ± standard deviation and n as number of devices where more than one device was fabricated

There were two methods used to fabricate devices with CARM feature, two asymmetrical depth channels, tested in section 3.3.1.3 (Table B.2). A photomask with a 400 µm distance between two parallel channels was exposed to UV light and etched with a target derivatization channel depth of 230 µm according to Figure B.1 and in Table B.1. However, fabrication of CARM feature using this method was not reproducible after two devices with hexane channel of 78 µm were fabricated. Therefore two methods were used to obtain hexane channel of depths varied from 60 µm to 150 µm while the depth of the derivatization channel was kept constant at 230 µm.
To fabricate devices with hexane channel depth $\geq 90 \, \mu m$, a photomask was exposed and the duration of the first HF etching step corresponded to the difference between the derivatization channel depth of 230 $\mu m$ to a target depth for the extraction channel. HF-resistant tape was used to protect the shallow hexane channel from over-etching while the derivatization channel was etched to 230 $\mu m$. To fabricate hexane channel depth $\leq 80 \, \mu m$, a photomask containing only the derivatization channel was first exposed and then etched until the derivatization channel depth correspond to 230 $\mu m$ minus the target depth for the extraction channel depth. Then, scratch-protective sheets of aluminum foil were carefully taped over the etched channels except for areas needed to perform the next step. A second photomask containing the design for only hexane channel was carefully aligned on the etched glass piece using a stereoscope. The etched channels were lit by the understage light of the stereoscope so the negative photomask containing only the extraction channel was moved until the two channels were overlapped. A second UV exposure was performed and the entire glass piece was further etched until the target depth for the hexane channel was obtained. This second UV exposure steps was performed separately for each microfluidic device contained on the 5” x 5” borosilicate glass piece to maximize number of devices meeting set specifications.

<table>
<thead>
<tr>
<th>Fabrication method</th>
<th>Distance between parallel line ($\mu m$)</th>
<th>Depth of derivatization channel ($\mu m$)</th>
<th>Depth of hexane channel ($\mu m$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>One UV exposure</td>
<td>400</td>
<td>230</td>
<td>$78 \pm 4 (n = 2)^*$</td>
</tr>
<tr>
<td>One UV exposure, HF etch while HF-resistant tape used to protect the shallow hexane channel</td>
<td>350</td>
<td>230</td>
<td>91, 137, 147**</td>
</tr>
<tr>
<td>Two separate UV exposures</td>
<td>280</td>
<td>214</td>
<td>76</td>
</tr>
<tr>
<td></td>
<td>300</td>
<td>215</td>
<td>62</td>
</tr>
</tbody>
</table>

*Average $\pm$ standard deviation and n as number of microfluidic devices fabricated

**Control depth of hexane channel by varying etching time of microfluidic device
A crucial technique in the fabrication of devices containing hydrophilic bridge for coalescence of segments described in section 3.3.2.3 was the alignment of two glass pieces with etched channels. After HF etching of the glass pieces, they were rinse thoroughly with deionized water and then dried with compressed N\textsubscript{2}. A drop of water was added between the two pieces of glass to hold the pieces together as the top piece was shifted to align the hydrophilic bridge between the extraction channel and external channel (see Figure 3.9B). Sharpie marker was used to mark the location of the access holes. After chemical cleaning, the two pieces were carefully alignment again under the microscope and let sit at room temperature for 30 min. This step let one correct the alignment of features if needed prior to the permanent thermal bonding step.
APPENDIX C

DETERMINATION OF CHANNEL CROSS SECTIONAL AREA

Fabrication of microfluidic devices using the HF solutions resulted in isotropic etching where the glass surface is etched equidistance in all direction and the cross sectional area \( A \) can be approximated as:

\[
A = ld + \frac{1}{2}(\pi d^2)
\]

where \( l \) was the width of the line drawn in the photomask and \( d \) was the etched depth of the channel (Figure C.1A). The ideal width \( W \) of the channel after HF etching and removal of the protective layers should be the sum of \( l \) and two times \( d \); however, for devices fabricated in Chapter 3, \( W \) was 50 – 250 µm larger than the ideal value (Figure C.1B).

![Figure C.1. Cross-section view of channel in glass devices. A. Ideal cross-section of channel upon isotropic etching of glass using HF with \( l \) as width of design on photomask, \( d \) as etched depth, and \( W \) as channel width. B. Cross-section of channel for a fabricated device with \( d \) of 217 µm and \( W \) of 544 µm when expected \( W \) to be 480 µm.](image)

Also, this method of fabrication had poor repeatability in the channel’s final dimensions since the process was dependent on many factors such as room temperature, HF concentration, glass quality, and quality of the protective layers. Accurate calculation of \( A \) was needed to establish \( R_{der} \) and flow rate ratios of derivatization and hexane phases for Chapters 2 and 3. These flow rate ratios were constant from one microfluidic device to another but the volumetric flow rates were different because they were dictated by each channel’s \( A \). A method was developed to quantitatively determine \( A \) that integrated profilometer measurement of the channel. ASCII data
file were exported from the profilometer and imported into Excel. The first column of data corresponded to data point number and was used to calculate the horizontal scan line in unit of µm (equation C.2).

\[
\text{scan\_line}(\mu m) = \text{data\_point\_number} \times \frac{1}{\text{scan\_frequency}} \times \frac{1}{\text{scan\_rate}}
\]

(C.2)

The column labeled as “Normal” was used to convert the channel’s height in Å to unit of µm. These two columns were saved in a text file and imported into a Mac OS graphing shareware called Plot (http://plot.micw.edu). Plot was used to integrate the line graph to obtain \( A \).
APPENDIX D

MODELING WITH COMSOL

COMSOL is a finite element analysis, solver, and simulation software that finds approximate solutions to partial differential equations (PDEs) and integral equations to model phenomena such as fluid dynamics, or chemical reaction kinetics. Similar to statistical tool such as DOE, tools like COMSOL can aid in making the approaches taken in development of perfusion microfluidic devices more efficient. Within this dissertation, the physic models called creeping flow and transport of diluted species were used extensively in order to couple and simultaneously model fluid flow, mass transport, and chemical reactions involving glucose and islets within the microfluidic device. The basic steps to using COMSOL to model any physical phenomena consist of (1) drawing the geometry, (2) specifying the physics of boundaries and walls, (3) meshing, (4) determining how the solver solves for the physics, and (5) post-processing data analysis. The design of the microfluidic device can be drawn in 2D or 3D. The walls and boundaries that make up the geometry of the device are then selected to assign variables and expressions to define the physics of the model. Then in the process called meshing, the geometry is partitioned into small units of simple shape called mesh elements so that the PDE is solved for each element. The steps taken by the solver to compute the PDEs are depended on whether the model is a steady state or time-dependent problem. Finally, in the post-process data analysis step, COMSOL approximate the response of the whole geometry by assembling solutions for the collection of all elements. The following details are step-by-step instructions on how to set up, solve, and export data for a model used in Chapter 4. Also included are step-by-step instructions on how to run the time-intensive models in batch processing mode using PC DOS command codes.
Figure D.1. Tree diagram for a Comsol model. All the basic steps to setting up a Comsol model.

**Model Wizard**

1. Go to Model Wizard window.
2. Click Next.
4. Click Add Selected.
5. In the Add physics tree, select Chemical Species Transport>Transport of Diluted Species (chds).
6. Click Add Selected.
7. Click Next.
8. Find the Studies subsection. In the tree, select Preset Studies for Selected Physics>Stationary.
9. Click Finish.

**Global Definitions**

**Parameters**

1. In the Model Builder window, right-click Global Definitions and choose Parameters.
2. Go to the Settings window for Parameters.
3. In the Parameters table, enter “depth” for name, “361[μm]” as expression to set the depth of the chamber. In the second row, enter “v0” for name, “0.000272[m/s]” as expression to set the inlet velocity corresponding to 2 μL min⁻¹.
**Waveform 1 (wv1)**

1. In the Model Builder window, right-click Global Definitions and choose Functions>Waveform.
2. Go to the Settings window for Waveforms.
3. In the Parameters section, set the Type to Sine, Angular Frequency to 0.0209333, Phase to 0, and Amplitude to 1.

![Model Builder screenshot](image)

- Chamber geometry.
- Inlet channel geometry.
- Shallow channel geometry.
- Adding depth in z-axis to each part of the device.
- Islets placed in chamber in a tightly packed configuration.

Figure D.2. Drawing 3D perfusion chamber and 20 islets. Basic geometric shapes were used to draw the fan-shaped perfusion chambers and 20 spheres to represent islets.

The following steps listed in bold or italic correspond to the tree branch of the same name in Figure D2.
Geometry 1
1. In the Model Builder window, right-click Model 1>Geometry 1.
2. Go to the Settings window for Geometry.
3. Set the Units>Length unit list to µm and Angular unit to Degrees.
4. Right-click Model 1>Geometry 1 and choose Work Plane.

Work Plane 1 (wp1)
1. In the Settings window for Work Plane 1, set the Plane Type as Quick, Plane as xy-plane, and z-coordinate as 0 µm.

Circle 1 (c1)
1. Right-click Work Plane 1>Geometry and add Circle.
2. In Circle Settings, select Solid as Object Type.
3. For Size and Shape section, set the Radius as 1200 µm and Sector Angle as 90 deg.
4. In Rotation Angle section, specify Rotation to be 225 deg.
5. Right-click Work Plane 1>Geometry and add a Bezier Polygon.

Bezier Polygon 1 (b1)
1. In the Bezier Polygon 1 Settings window, Get the General>Type as Solid.
2. Click on Add Linear and for Segment 1(linear) enter (x = 0, y =0) for the first coordinate and (x = -175, y = -175) for the second coordinate.
3. Add another linear segment and for Segment 2 (linear) enter (x = -175, y = -175) for the first coordinate and (x = 175, y = -175) for the second coordinate.
4. Add a third linear segment and for Segment 3 (linear) enter (x = 175, y = -175) for the first coordinate and (x = 0, y = 0) for the second coordinate.

Difference 1 (dif1)
1. In the Settings window for Difference, select c1 for Objects to Add and b1 for Objects to Subtract.
2. Deselect the Keep Input Objects and Keep Interior Boundaries boxes.
3. Right-click Geometry 1 and add Work Plane.

Work Plane 2 (wp2)
1. In Work Plane Settings window, set the Plane Type as Quick, Plane as xy-plane, and x-coordinate as 0.
**Rectangle 1 (r1)**
1. Right-click Work Plane 2>Geometry and add Rectangle.
2. In Rectangle Settings, select Solid as Object Type.
3. For Size section, set the Width as 350 μm and Height as 100 μm.
4. In Position section, specify x and y to be -175 μm.
5. In Rotation Angle, set the Rotation to be 0 deg.
6. Right-click Geometry 1 and add Work Plane.

**Work Plane 3 (wp3)**
1. In Work Plane Settings window, set the Plane Type as Quick, Plane as xy-plane, and x-coordinate as 0.

**Circle 1 (c1)**
1. Right-click Work Plane 3>Geometry and add Circle.
2. In Circle Settings, select Solid as Object Type.
3. For Size and Shape section, set the Radius as 1300 μm and Sector Angle as 90 deg.
4. In Rotation Angle section, specify Rotation to be 225 deg.
5. Right-click Work Plane 3>Geometry and add Circle.

**Circle 2 (c2)**
1. In Circle Settings, select Solid as Object Type.
2. For Size and Shape section, set the Radius as 1200 μm and Sector Angle as 90 deg.
3. In Rotation Angle section, specify Rotation to be 225 deg.

**Difference 1 (dif1)**
1. In the Settings window for Difference, select c1 for Objects to Add and c2 for Objects to Subtract.
2. Deselect the Keep Input Objects and Keep Interior Boundaries boxes.
3. Right-click Geometry 1 and select Extrude.

**Extrude 1 (ext1)**
1. In Extrude 1 Settings window under General section, select wp1 for Work Plane and check Keep cross-sectional faces box.
2. In Distances from Work Plane section, type in “depth” for Distances (μm).
3. Right-click Geometry 1 and select Extrude.

**Extrude 2 (ext2)**

1. In Extrude 2 Settings window under General section, select wp2 for Work Plane and check Keep cross-sectional faces box.
2. In Distances from Work Plane section, type in 350 for Distances (µm).
3. Right-click Geometry 1 and select Extrude.

**Extrude 3 (ext3)**

1. In Extrude 3 Settings window under General section, select wp3 for Work Plane and check Keep cross-sectional faces box.
2. In Distances from Work Plane section, type in 20 for Distances (µm).
3. Right-click Geometry 1 and select Sphere.

**Sphere 1 (sph1)**

1. In Sphere Settings, select Solid as Object Type.
2. For Size and Shape section, set the Radius as 101.5.
3. In Position section, set the settings as (x = -670, y = -840, z = 101.5).
4. Repeat steps 1-3 to add 19 more spheres with Radius and Position settings as in Table D1.

**Materials**

1. In the Model Builder window, right-click Model 1>Materials and choose Open Material Browser.
2. Go to the Material Browser window.
3. In the Materials tree, go to Built-In>Water, liquid.
4. Right-click and choose Add Material to Model from the menu.

*Water*

The first material added in this section applies to all domains by default so do not need to change unless needed.
Table D.1. 3D coordinates for 19 islets placement within the perfusion chamber.

<table>
<thead>
<tr>
<th>Name</th>
<th>Radius</th>
<th>x</th>
<th>y</th>
<th>z</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sphere 2 (sph2)</td>
<td>71</td>
<td>-530</td>
<td>-970</td>
<td>71</td>
</tr>
<tr>
<td>Sphere 3 (sph3)</td>
<td>59</td>
<td>-410</td>
<td>-1050</td>
<td>59</td>
</tr>
<tr>
<td>Sphere 4 (sph4)</td>
<td>25</td>
<td>-330</td>
<td>-1110</td>
<td>25</td>
</tr>
<tr>
<td>Sphere 5 (sph5)</td>
<td>84</td>
<td>-200</td>
<td>-1080</td>
<td>84</td>
</tr>
<tr>
<td>Sphere 6 (sph6)</td>
<td>50.5</td>
<td>-60</td>
<td>-1125</td>
<td>50.5</td>
</tr>
<tr>
<td>Sphere 7 (sph7)</td>
<td>23</td>
<td>20</td>
<td>-1155</td>
<td>23</td>
</tr>
<tr>
<td>Sphere 8 (sph8)</td>
<td>63.5</td>
<td>120</td>
<td>-110</td>
<td>63.5</td>
</tr>
<tr>
<td>Sphere 9 (sph9)</td>
<td>134</td>
<td>300</td>
<td>-990</td>
<td>134</td>
</tr>
<tr>
<td>Sphere 10 (sph10)</td>
<td>61.5</td>
<td>510</td>
<td>-1000</td>
<td>61.5</td>
</tr>
<tr>
<td>Sphere 11 (sph11)</td>
<td>126</td>
<td>620</td>
<td>-825</td>
<td>126</td>
</tr>
<tr>
<td>Sphere 12 (sph12)</td>
<td>41.5</td>
<td>-620</td>
<td>-700</td>
<td>41.5</td>
</tr>
<tr>
<td>Sphere 13 (sph13)</td>
<td>163.5</td>
<td>-400</td>
<td>-752</td>
<td>163.5</td>
</tr>
<tr>
<td>Sphere 14 (sph14)</td>
<td>82.5</td>
<td>-200</td>
<td>-900</td>
<td>82.5</td>
</tr>
<tr>
<td>Sphere 15 (sph15)</td>
<td>25</td>
<td>-90</td>
<td>-1050</td>
<td>25</td>
</tr>
<tr>
<td>Sphere 16 (sph16)</td>
<td>72.5</td>
<td>10</td>
<td>-1000</td>
<td>72.5</td>
</tr>
<tr>
<td>Sphere 17 (sph17)</td>
<td>65.6</td>
<td>115</td>
<td>-900</td>
<td>65.5</td>
</tr>
<tr>
<td>Sphere 18 (sph18)</td>
<td>105</td>
<td>210</td>
<td>-750</td>
<td>105</td>
</tr>
<tr>
<td>Sphere 19 (sph19)</td>
<td>90</td>
<td>405</td>
<td>-790</td>
<td>90</td>
</tr>
<tr>
<td>Sphere 20 (sph20)</td>
<td>52</td>
<td>-450</td>
<td>-535</td>
<td>52</td>
</tr>
</tbody>
</table>

**Creeping Flow (spf)**
1. In Creeping Flow Settings window, select only domains 1, 2, and 12.
2. Under Physical Model section, change the Compressibility to Incompressible flow.

**Fluid Properties 1**
1. In Model Inputs, set the temperature to 310.15[K].

**Initial Values 1**
1. Go to Settings window for Initial Values.
2. Specify Pressure to 0 Pa.
3. Right-click Creeping Flow and choose Inlet.
Figure D.3. Setting up the physics, solver, and post-processing steps. Solving the stationary fluid velocity profile and time-dependent mass transport within the perfusion chamber.

**Inlet 1**
1. Select Boundary 87 only.
2. Set the Boundary Condition as Velocity.
3. In Velocity section, select Normal Inflow Velocity and type \( v_0 \) in \( U_0 \).
4. Right-click Creeping Flow and choose Outlet.

**Outlet 1**
1. Select Boundary 2 only.
2. Set the Boundary Condition as Pressure, no viscous stress.
3. Type 0 in for Pressure: \( P_0 \).
Transport of Diluted Species (chds)
1. In Settings window, select All Domains under Domain Selection section.
2. Check the Convection box within Transport Mechanisms section.
3. Right-click on Transport of Diluted Species and add Inflow.

Inflow 1
1. Select Boundary 87 only.
2. Within the Concentration section, for \(c_0, c\) type \(11 + wv1(t[1/s])\)
3. Right-click on Transport of Diluted Species and add Outflow.

Outflow 1
1. Select Boundary 2 only.
2. Right-click on Transport of Diluted Species and add Convection and Diffusion.

Convection and Diffusion: chip
1. In Settings window, add domains 1, 2, and 12 to Domain Selection section.
2. Under Model Inputs section, select Velocity field (spf/fp1) from the drop down menu.
3. Under Diffusion section, set Bulk Material as Water.
4. Define Diffusion Coefficient by setting \(D_c\) as User Defined and type in \(0.9e-9[m^2/s]\).
5. Right-click on Transport of Diluted Species and add Convection and Diffusion.

Convection and Diffusion: islets
1. In Settings window, add all domains to Domain Selection section and then manually delete domains 1, 2, and 12.
2. Under Model Inputs section, select User Defined from the drop down menu.
3. Under Diffusion section, set Bulk Material as Water.
4. Define Diffusion Coefficient by setting \(D_c\) as User Defined and type in \(0.3e-9[m^2/s]\).
5. Right-click on Transport of Diluted Species and add Reactions.

Reactions 1
1. In Settings window, add all domains to Domain Selection section and then manually delete domains 1, 2, and 12.
2. For Reactions section, type in -8e-3 for \(R_c\).
3. Right-click on Transport of Diluted Species and add Initial Values.

Initial Values: chip
1. In Settings window, add domains 1, 2, 12 to Domain Selection.
2. Set the Initial Values>Concentration>c to be 11.
3. Right-click on Transport of Diluted Species and add Initial Values.

*Initial Values: islets*

1. In Settings window, add all domains to Domain Selection section and then manually delete domains 1, 2, and 12.
2. Set the Initial Values>Concentration>c to be 0.

*Mesh*

1. Right-click on Mesh and select Free Tetrahedral.
2. Select Entire Geometry from the drop down menu in Domain Selection>Geometric Entity Level.
3. Right-click on Free Tetrahedral1 and select Size.

*Size 1*

1. Select Entire Geometry from the drop down menu in Domain Selection>Geometric Entity Level.
2. In Element Size>Calibrate For select General Physics and set Prefined to Normal.

*Study 1*

**Step 1: Stationary**

1. Under Mesh Selection, Geometry 1 and Mesh 2 are the defaults since those are the only geometry and mesh built.
2. Under Physics Selection, deselect Transport of Diluted Species (chds) by clicking on the check sign under the Use column so that it changes to a cross.
3. Right-click on Study 1 and go to Study Steps>Time-dependent.

**Step 2: Time Dependent**

1. In Time Dependent window under Study Settings section, click on the right icon that has a green and red line.
2. A Range pop-up menu will come up. Set the parameters within this menu as “0” for start, “650” for stop, and “0.5” for step.
3. Click Add.
4. Under Mesh Selection, Geometry 1 and Mesh 2 are the defaults since those are the only geometry and mesh built.

5. Under Physics Selection, select Transport of Diluted Species (chds) by making sure there are check symbols under the Use column.

6. Right-click on Study 1 and select Show Default Solver.

**Solver Configurations**

1. Expand the Solver Configurations tree to locate the section titled Time-Dependent Solver 1.
2. Expand Time Stepping section.
3. Set the Method to BDF and Steps Taken by Solver to Intermediate.

**Results**

1. Right-click Results and select 3D Plot Group.

**3D Plot Group 1**

1. Right-click 3D Plot Group 1 and select Slice.
2. In Slice Settings window in Data section, select Solution 1 as Data Set and 375 as Time.
3. Under Expression section click on the icon on the right that has a green and orange arrow.
4. Select Transport of Diluted Species>Species c>Concentration.
5. In Plane Data section, set the settings to “xy-plane” as plane type, “coordinates” as entry method, and “range(150,1,150)” as z-coordinate.

**3D Plot Group 2**

1. Right-click 3D Plot Group 1 and select Slice.
2. In Slice Settings window in Data section, select Solution 1 as Data Set and 375 as Time.
3. Under Expression section click on the icon on the right that has a green and orange arrow.
4. Select Creeping Flow>Shear rate.
5. In Plane Data section, set the settings to “xy-plane” as plane type, “coordinates” as entry method, and “range(150,1,150)” as z-coordinate.

**Export**

**Plot 1**

1. Right-click on 3D Plot Group 1>Slice and select Add Plot Data to Export.
2. Right-click on 3D Plot Group 2>Slice and select Add Plot Data to Export.
Comsol Command File

To compute the Comsol simulations without a GUI in batch mode, a command file was created using DOS command script language so that it can be executed using Windows Terminal program.

Figure D.4. Windows DOS command for batch processing of Comsol models. Each line solved and saved a Comsol model while also generating a log file to record any error that might occur.

The first line of code “@echo off” turned off the display setting within the Terminal window so that the progress log can be saved in a separate log file. The second line titled the command script file and the third line of code tells the Terminal program to display “Starting Comsol simulation Runs.” For each Comsol model to be computed in a batch the following line of code was inserted:

```
<<comsolbatch –inputfile “filename.mph” –outputfile “filenamedone.mph” –batchlog “filenamedone.log”>>
```

“Comsolbatch” is a command for batch mode progressing. The codes -inputfile “filename.mph” and –outputfile “filenamedone.mph” specified the Comsol model to solve and the filename to save the results in. The code –batchlog “filenamedone.log” saved the steps Comsol software take to solve the model in a text file for review.

Creating a Command file

1. Open the Notepad program.
2. Type in the lines of codes shown in the above snapshot with the input filenames, output filenames, and batchlog filenames set as desired.
3. Save the Notepad file as a cmd.exe file type by selecting File and Save As.
4. Select the drop down cursor on Save as Type and select All files.
5. Type in the name of file and add “.cmd” designation at the end.

    For example:
    In the above snapshot, the file was named as “comsol_run_file” and it was saved by
typing the following “comsol_run_file.cmd”, without the quotations.

**Environmental Variables**

Specify the path to which “comsolbatch” exe file is located as well as the directory where the
comsol command file used to run the Comsol models without the GUI.
1. Right-click My Computer, and then click Properties.
2. Click the Advanced tab.
3. Click Environment variables.
4. Scroll down the System Variables window to locate the variable named Path.
   Click on Path and click Edit.
5. Type in the directory paths for comsolbatch.exe file and where command file and Comsol
   models were stored.

    For example:
    “C:\COMSOL42\bin\win32\” was inserted without the quotations to indicate the directory
    in which the “comsolbatch.exe” file is located
    “C:\Users\Comsol\” was inserted without the quotations to indicate the subdirectory in
    which the Comsol models and command file are located.

**Running Comsol command file**

To compute the Comsol models without the GUI:
1. Click on Start.
2. Type in “cmd” without the quotations into Search Programs and Files box to open Windows
   Terminal program as in Figure D5.
Figure D.5. Screen capture of batch processing of Comsol models. Windows DOS command scripts were executed to solve Comsol models without a graphic user interface (GUI).

3. Type in the command script “cd” and the folder name that contained the command file located within C:\Users\COMSOL\ subdirectory. Then press Enter.

   For example:
   
   “cd testbatch” was typed in the Figure D5 to direct the Windows Terminal program to go into the folder “testbatch” located with the user named COMSOL. The fourth line showed the new subdirectory the Windows Terminal program is now in.

4. Then type in the filename of the command file used to compute Comsol models without the GUI. Press Enter.

   For example:
   
   “comsol_run_file” was typed and the following line 5-6 automatically popped up to indicate that the file named “comsol_run_file” has started and batch processing of Comsol simulation runs have started.

5. For each line of script, the Terminal window would compute the Comsol model and the Terminal window would update as shown in Figure D5.

6. To check the progress of the Comsol computation, the log file with the designated filename can be opened.
APPENDIX E

POROUS MEMBRANE-PDMS PERFUSION CHAMBER DESIGN

The perfusion chamber design described within Chapter 4 allows for study of 40 islets to assess their global responses to glucose waveform. However, it can be challenging to load the islets into the perfusion chamber using the inlet channel and the capability of the design to house more than 40 islets is limited. The global response of the islets can more reflective of the pancreas response to changes in blood glucose levels if a large population of islets, ~100, is used. 3D computer simulations were performed using Comsol and the preliminary results for a design of a perfusion chamber that consisted of parallel channels, porous membrane, and a 2.5 mm diameter circular perfusion chamber were promising (Figure E.1 and Table E.1).

Table E.1. Parameters used in Comsol modeling of hybrid perfusion chamber.

<table>
<thead>
<tr>
<th>Design feature</th>
<th>Nominal value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Perfusion channels</td>
<td>200 µm width x 50 µm depth spaced 200 µm apart</td>
</tr>
<tr>
<td>Porous membrane</td>
<td>Polycarbonate 14 µm pore size 9 µm thickness 7.7 % porosity 1120 kg m⁻³ bulk density 3.05 x 10⁻¹⁴ m² permeability</td>
</tr>
<tr>
<td>Chamber</td>
<td>2.5 mm diameter 300 µm height (6 µL)</td>
</tr>
<tr>
<td>1-input design</td>
<td>2 µL min⁻¹ for each channel</td>
</tr>
<tr>
<td>2-input design</td>
<td>3 µL min⁻¹ for each channel</td>
</tr>
<tr>
<td>6-input design</td>
<td>0.5 – 1.5 µL min⁻¹ depending on channel lengths (5.0 mm diameter chamber)</td>
</tr>
</tbody>
</table>
Figure E.1. Porous membrane-PDMS hybrid perfusion chamber. A. Side view of the 3-layer design for the hybrid design. B. Homogeneity of glucose concentration within the 2.5 mm diameter chamber for the 1-input channels design. C. 2-input channels design for improved homogeneity of glucose concentration in the chamber. D. A 6-input design generated undesirable heterogeneity in glucose concentration for 5.0 mm diameter chamber. B and C are glucose concentrations at 75 μm from the bottom of the chamber.

Sinusoidal glucose waveform of 300 s period, median of 11 mM, and amplitude of 1 mM was used to test the performance of Figure E.1B and C designs. A step change from 0 to 1 mM glucose was used to test Figure E.1D design. Glucose solutions were flowed in the parallel perfusion channel and were delivered to the islets housed within the chamber by diffusion through a porous membrane. The benefit to this design is that the islets would not be in direct flow path of the fluid flow thus flow-induced shear stress would not be a major factor that limited how fast the fluid can be delivered to ensure homogeneous spatial distribution of glucose...
concentration within the chamber. Rapid prototyping of the channels and chamber using polydimethylsiloxane (PDMS) made fabrication simpler and more controllable than the wet etching fabrication method using glass.

When inflowing solutions were in the same direction in a 1-input design, the glucose concentration within the chamber was parabolic-shaped, as expected for pressure-driven flow. Glucose concentration being applied at the time point depicted in Figure E.1B was 12 mM and the concentration within the chamber was 11.84 ± 0.12 (1% variability). To ensure that all islets resided in the chamber were challenged with the same glucose concentration, the perfusion channels design was changed to a 2-input design and a faster flow rate was applied. This design resulted in an average glucose concentration of 11.88 ± 0.11 (0.9% variability) in the chamber. There was an improvement in the spatial-temporal distribution of glucose concentration in the center of the chamber as the temporal delay of the waveform for the 1-input design decreased to 0.75 min for the 2-input design.

A larger perfusion chamber of 5 mm diameter was used to house more than 200 islets but the 1-input and 2-input designs did not generate adequate homogeneity of glucose in the chamber so the number of inlets underneath the chamber was increased to enhance the homogeneity. However, it was found that if the fluidic inlets were located too close to the fluidic outlets the homogeneity of glucose concentration in the chamber would decrease because glucose exited out the outlets faster than the rate of diffusion across the porous membrane and into the chamber. Figure E.1D depicts a 6-input design where each group of 3 channels colored red were fluidic inlets located next to fluidic outlets. Comsol modeling for this snapshot solved the time-dependent mass transport of glucose for time = 1000 s (16.7 min) and results shown indicated that glucose concentration in the middle of the chamber had not equilibrated to 1 mM because glucose exited out the outlets faster than it diffuse into the chamber. Nevertheless, results for 2.5 mm perfusion chamber demonstrated the proof-of-concept of this design and the concepts might be worth exploring for the development and optimization of larger microfluidic perfusion chamber designs.
REFERENCES


68. Duong, C. T.; Roper, M. G., A microfluidic device for the automated derivatization of free fatty acids to fatty acid methyl esters. *Analyst* 2012, 137, 840-846.


BIOGRAPHICAL SKETCH

Cindy Duong received her Bachelor of Science degree in Chemistry in December 2005 from University of North Carolina at Chapel Hill (UNC-CH). After graduating from UNC-CH, she worked for a contract research organization in Durham, NC. In the Fall of 2007, she enrolled in the Department of Chemistry and Biochemistry at Florida State University. In the Spring of 2008, she joined Dr. Roper group. Her research on the development of microfluidic devices to study the cell signaling role of fatty acids has been presented at several regional and national conferences. She is expected to receive her Ph.D. in Analytical Chemistry in Fall 2012.