Phenylketonuria (PKU) is a genetic inborn metabolic disorder which inhibits the functional production of the enzyme phenylalanine hydroxylase (PAH). It currently affects one in 15,000 Americans and is the most common amino acid metabolism error found in newborns. Individuals affected with PKU are incapable of metabolizing the amino acid, phenylalanine (Phe) and can face irreversible bodily damage, such as seizures or mental retardation, with elevated concentrations of Phe in the body. PKU is a lifelong disease that requires a regimented diet and total avoidance of foods containing Phe. Current methods for measuring Phe concentrations in blood require routine laboratory tests. It can take several days and even weeks for patients to get the results of these tests. This greatly limits the usefulness of measuring the patient Phe levels using current methods. The availability of these tests are limited to places with testing laboratories where patients can mail dried blood samples, which is generally only in developed nations. A point-of-care (POC) device capable of measuring blood Phe levels in low-resource settings, such as in homes or developing nations, could lower test cost and turn-around time and broaden the accessibility of Phe monitoring for affected individuals.
This report describes the conversion of a laboratory-based enzymatic/colorimetric assay (ECA) into a paper-based lateral flow test (LFT) that can detect relevant Phe concentrations in a whole blood sample. The proposed assay uses a well-known, two-step chemical reaction resulting in a colorimetric response that can visually be correlated to Phe concentration. An experiment was first developed to demonstrate assay capabilities with Phe-spiked human plasma in glass fiber and cellulose substrates. A plasma separation membrane was integrated that enabled the assay to accept and process whole blood samples. The assay successfully correlated blood Phe concentrations with the intensity of a visible colorimetric response from a Phe-spiked whole blood sample.
Development of a Paper-Based Whole Blood Phenylalanine Assay for PKU Diagnosis and Monitoring in Low Resource Settings

by

Robert Tyler Robinson

A THESIS

submitted to

Oregon State University

in partial fulfillment of the requirements for the degree of Master of Science

Presented June 3, 2016
Commencement June 2016
Master of Science thesis of Robert Tyler Robinson presented on June 3, 2016

APPROVED:

__________________________
Major Professor, representing Chemical Engineering

__________________________
Head of the School of Chemical, Biological and Environmental Engineering

__________________________
Dean of the Graduate School

I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

__________________________
Robert Tyler Robinson, Author
ACKNOWLEDGEMENTS

The author expresses sincere appreciation to Dr. Elain Fu. This work would not have been possible without her guidance, mentoring and encouragement. The author is extremely grateful for the continued love and support from his parents Steve and Michelle Robinson, brother Kyle Robinson, grandmother Susan Pons and late grandfather Robert L. Pons. They all played instrumental roles in building the foundation for the author’s professional development. The author would like to recognize his significant other, Rebecca Latiolait, for her loyalty and understanding during his pursuit of a graduate degree. Many thanks to Dr. Philip Harding, Dr. Karl Schilke and Dr. Matthew Johnston for being members of the author’s graduate committee. The author thanks his friends and peers Liam Wong, Corey Downs, Dylan Hinson, and Andrew Ferrara, for editing portions of this report. Special thanks to Keith Liebe and Alex Globus for help and guidance with photo editing software for making figures, Paige Molzahn for help with organizational tools. This research was sponsored by the National Phenylketonuria Alliance and Oregon State University.
<table>
<thead>
<tr>
<th>Section</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Introduction</td>
<td>1</td>
</tr>
<tr>
<td>1.1</td>
<td>Phenylketonuria</td>
<td>1</td>
</tr>
<tr>
<td>1.1.1</td>
<td>Discovery and History</td>
<td>1</td>
</tr>
<tr>
<td>1.1.2</td>
<td>Screening Systems and Incidence Rates</td>
<td>2</td>
</tr>
<tr>
<td>1.1.3</td>
<td>Diet Therapy and Monitoring</td>
<td>4</td>
</tr>
<tr>
<td>1.2</td>
<td>Paper-Based Microfluidic Devices</td>
<td>6</td>
</tr>
<tr>
<td>1.2.1</td>
<td>Use in Therapeutic Monitoring</td>
<td>6</td>
</tr>
<tr>
<td>1.2.2</td>
<td>Technical Microfluidic Background</td>
<td>8</td>
</tr>
<tr>
<td>1.3</td>
<td>Phenylalanine Monitoring Device</td>
<td>10</td>
</tr>
<tr>
<td>1.3.1</td>
<td>Current Laboratory Phe Measurement Methods</td>
<td>10</td>
</tr>
<tr>
<td>1.3.2</td>
<td>Alternative Phe Measurement Methods</td>
<td>11</td>
</tr>
<tr>
<td>1.3.3</td>
<td>Paper-Based Phe Assay Design Selection</td>
<td>12</td>
</tr>
<tr>
<td>2.</td>
<td>Phe Assay with Plasma Samples</td>
<td>14</td>
</tr>
<tr>
<td>2.1</td>
<td>Introduction</td>
<td>14</td>
</tr>
<tr>
<td>2.2</td>
<td>Materials and Methods</td>
<td>15</td>
</tr>
<tr>
<td>2.2.1</td>
<td>Card Fabrication and Image Acquisition Techniques</td>
<td>15</td>
</tr>
<tr>
<td>2.2.2</td>
<td>Enzymatic Reaction Sensitivity in Various PheDH conc.</td>
<td>16</td>
</tr>
<tr>
<td>2.2.3</td>
<td>Calibration Assay</td>
<td>17</td>
</tr>
<tr>
<td>2.3</td>
<td>Results and Discussion</td>
<td>19</td>
</tr>
<tr>
<td>2.4</td>
<td>Conclusion</td>
<td>24</td>
</tr>
<tr>
<td>3.</td>
<td>Phe Assay with Whole Blood Samples</td>
<td>26</td>
</tr>
</tbody>
</table>
# TABLE OF CONTENTS (Continued)

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.1 Introduction</td>
<td>26</td>
</tr>
<tr>
<td>3.2 Materials and Methods</td>
<td>28</td>
</tr>
<tr>
<td>3.2.1 Card Fabrication and Image Acquisition Techniques</td>
<td>28</td>
</tr>
<tr>
<td>3.2.2 Enzymatic Reaction Compatibility in Various Substrates</td>
<td>28</td>
</tr>
<tr>
<td>3.2.3 Colorimetric Reaction Compatibility in Various Substrates</td>
<td>29</td>
</tr>
<tr>
<td>3.2.4 Compatibility of PSM with Various Substrates</td>
<td>29</td>
</tr>
<tr>
<td>3.2.5 Calibration Assay</td>
<td>30</td>
</tr>
<tr>
<td>3.3 Results and Discussion</td>
<td>32</td>
</tr>
<tr>
<td>4.4 Conclusion</td>
<td>38</td>
</tr>
<tr>
<td>4. Conclusions and Next Steps</td>
<td>40</td>
</tr>
<tr>
<td>4.1 Summary of Conclusions</td>
<td>40</td>
</tr>
<tr>
<td>4.2 Next Steps</td>
<td>40</td>
</tr>
<tr>
<td>4.2.1 Automation</td>
<td>40</td>
</tr>
<tr>
<td>4.2.2 Calibration</td>
<td>42</td>
</tr>
<tr>
<td>4.2.3 Cell Phone Detection</td>
<td>43</td>
</tr>
<tr>
<td>Bibliography</td>
<td>45</td>
</tr>
</tbody>
</table>
## LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1</td>
<td>Paper-Based Phe Monitor Plasma Assay Card</td>
<td>15</td>
</tr>
<tr>
<td>2.2</td>
<td>Enzymatic Reaction Sensitivity for Various Concentrations of PheDH</td>
<td>20</td>
</tr>
<tr>
<td>2.3</td>
<td>Calibration Assay Results</td>
<td>23</td>
</tr>
<tr>
<td>3.1</td>
<td>Single Whole Blood Phe Assay</td>
<td>27</td>
</tr>
<tr>
<td>3.2</td>
<td>Enzymatic Reaction Compatibility in Various Substrates</td>
<td>33</td>
</tr>
<tr>
<td>3.3</td>
<td>Colorimetric Reaction Compatibility in Various Substrates</td>
<td>34</td>
</tr>
<tr>
<td>3.4</td>
<td>Plasma Separation Membrane Compatibility with Various Substrates</td>
<td>36</td>
</tr>
<tr>
<td>3.5</td>
<td>Calibration of Whole Blood Assay</td>
<td>37</td>
</tr>
<tr>
<td>4.1</td>
<td>Proposed Phe Assay with On-Board Calibration</td>
<td>42</td>
</tr>
</tbody>
</table>
# LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>Incidence of PKU in Various Countries</td>
</tr>
<tr>
<td>1.2</td>
<td>WHO Recommendations for an Ideal POC Device</td>
</tr>
<tr>
<td>1.3</td>
<td>Lab vs. Paper-Based Assay Comparisons</td>
</tr>
<tr>
<td>1.4</td>
<td>Assays Designed for Home or Low Resource Phe Detection</td>
</tr>
</tbody>
</table>
Chapter 1 - Introduction

Phenylketonuria

Discover and History

Phenylketonuria (PKU) was first discovered in 1934 by the organic chemist, Dr. Asbjørn Følling [1]. The discovery came after Følling was able to isolate phenylpyruvic acid in the urine of two severely mentally disabled brothers [1]. The disease has since been classified as an autosomal-recessive metabolic disorder caused by a deficiency of phenylalanine hydroxylase (PAH), the enzyme that converts phenylalanine (Phe) into tyrosine [2][3]. There have been more than 300 different mutations of the PAH gene recorded worldwide that can cause some form of PKU [4]. It is the most common inherited disease dealing with amino acid metabolism and there is currently no cure [5]. Similar, but less common, genetic inborn disorders involving amino acid metabolism include Maple Syrup Urine Disease and Urea Cycle Disorder [3].

Genetic metabolic inborn errors are generally a result of an enzyme deficiency [3]. An absence of the PAH enzyme will lead to the accumulation of Phe, which can cause neurocognitive dysfunction if chronically left untreated [5]. Hyperphenylalaninemia (HPA) is defined as a clinical measurement of untreated patient blood Phe concentrations greater than 2 mg/dL. Further classification of HPA is categorized as either classical or non-classical PKU, which generally correspond to untreated blood Phe levels above or below 16.5 mg/dL, respectively [6].

Phe is a large neutral amino acid (LNAA) which directly competes with other LNAAAs in transport mechanisms across the blood brain barrier via the L-type amino acid
transport [7]. This disease can cause irreparable damage if the patient’s blood Phe levels are chronically high, making early detection a necessity [8]. High Phe levels dominate LNAA transport mechanisms, preventing other essential LNAAs from entering the brain. This lack of diversity in LNAA transport can cause mental retardation, behavioral disorders or seizures in patients with PKU [9]. Diagnosis and adequate treatment of infants with PKU within two weeks of life has proven to substantially improve health conditions and promotes normal growth and development [10]. Phe is found in high concentrations in many protein-rich foods, such as milk, eggs, nuts, beans and meats. Phe can also be found in processed consumables such as artificially sweetened beverages or gums and in some over-the-counter or prescribed medications [11,12]. A Phe avoidance diet therapy was first suggested and implemented in 1953, and by 1963, the success of patients towards normalization was realized [13].

**Screening Systems and Incidence Rates**

After the discovery of this disease, and the success of treating patients with a proper Phe-reduced diet, neonatal screening for elevated levels of Phe became a common occurrence in developed nations. A PKU screening assay was first introduced in the late 1960s with the bacterial inhibition assay [7]. As screening programs were showing success, industrialized countries started PKU screening programs in the 1980s, and within about a decade had greater than 99% screening rates [14]. Although most developed countries have the means to screen for PKU, many of them do not have complete coverage of newborns for reasons such as proximity to testing facilities capable of screening for PKU or home births [15].
PKU was the first inborn metabolic error disease to be identified through population-based screening, which pioneered a new era for diagnosis and treatment of genetic disorders [2]. There are many benefits to screening for this disease soon after a child is born. The U.S. Preventative Service Task Force (USPSTF) has a grade A recommendation for PKU screening, meaning there are substantial net-cost benefits to finding and treating this disease early [10]. All net-cost studies suggest a long term financial benefit to early detection and treatment of PKU when compared to the lifetime costs associated with caring for a person with untreated PKU [16]. Patients with early detection and chronic treatment of PKU generally have IQ levels within 5 points (although generally lower) of their unaffected siblings at the time of school entrance [17].

Despite overwhelming evidence that supports the benefits of neonatal screening, many countries do not have a screening program because they lack the infrastructure needed to test for this disease. In 2007, the number of newborns screened in China was 5 million, accounting for only 25% of the estimated 20 million births that year [18]. Many countries only diagnose PKU after it is already too late. This puts a larger strain on the population when an affected person undergoes stunted development and requires a caretaker for their entire life. Generally, in these cases, the main cause of late referral was the lack of newborn PKU testing [10].
Table 1.1 shows the incidence rate of PKU for various countries. There is a higher incidence rate in countries with large White and Native American populations, and a lower incidence in Black, Hispanic and Asian populations [16]. It is important to note that the incidence rate in many developing countries might be underestimated due to the lower screening rate [19]. It has been hypothesized that an explanation for such high incidence rates in countries such as Turkey or Tunisia could be due to higher instances of consanguinity. A screening in Tunisia found roughly 32% of marriages were consanguineous, and over 94% of the discovered PKU cases came from consanguineous parents [19, 20]. Countries like Japan and Finland are hypothesized to have much lower incidences due to a founders’ effect, where original settlers of those areas lacked the genetic mutation that hinders PAH generation [20].

**Diet Therapy and Monitoring**

Diet therapy is the gold standard treatment for many metabolic disorders. Diet therapy for PKU requires trained metabolic dietitians and a multidisciplinary team with members experienced with the disease to effectively treat individuals [3, 21]. Treatment with a low Phe diet has been successful in preventing brain damage among patients with PKU [17], but complications can arise from the lack of the metabolic products of Phe. Patients under a strict Phe avoidance diet are prone to low or deficient blood concentrations of tyrosine and tryptophan, which are precursors for dopamine or serotonin [22].

<table>
<thead>
<tr>
<th>Country</th>
<th>Incidence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Turkey</td>
<td>1:2,600</td>
</tr>
<tr>
<td>Lebanon</td>
<td>1:4,558</td>
</tr>
<tr>
<td>Tunisia</td>
<td>1:7,631</td>
</tr>
<tr>
<td>USA</td>
<td>1:15,000</td>
</tr>
<tr>
<td>Japan</td>
<td>1:125,000</td>
</tr>
<tr>
<td>Finland</td>
<td>1:200,000</td>
</tr>
</tbody>
</table>

Table 1.1 – Incidence of PKU in various countries.
The generally accepted Phe avoidance diet can require consuming a Phe-free metabolic formula for supplementing the lack of dietary protein [5,9,23]. Dietary restrictions and metabolic formulas are individualized and periodically need reevaluations to maintain proper amino acid levels during different stages of physical development [21]. The consequences of a person with PKU not following a low Phe diet can have irreversible adverse health effects, as mentioned previously. It is especially critical for young children and pregnant women to be under strict control of their blood Phe concentration, otherwise the developing child can suffer microcephaly or congenital heart failure [9]. It is recommended that blood Phe levels are measured at least weekly during infancy through age 4, every two weeks until the age of 10, and biweekly after that [17]. Long term, periodic monitoring is recommended to help clinicians and patients understand the needs of each individual and determine appropriate drug or diet therapies [17].

Some clinicians estimate at least 60% of adolescent and adult PKU patients do not follow a Phe-restricted diet [22]. Factors that inhibit therapy adherence are testing frequency, palatability of the metabolic formula, and the number of medical and nutritional personnel specialized in this field [17]. The frequency and access to blood Phe concentration measurements should be improved to allow patients to pinpoint exactly what foods do or don’t work with their individually tailored diet. Self-treatment within a closed loop system could allow a PKU patient better feedback to administer appropriate levels of therapeutic action, similar to the way diabetics have been successfully regulating their blood glucose levels for years [24].
One drug is currently available for the management of blood Phe levels. Sapropterin dihydrochloride (commercially known as Kuvan) is a synthetic form of an essential cofactor for the PAH enzyme, tetrahydrobiopterin. It is estimated that between 20 to 60% of PKU patients have a deficiency of this cofactor and could endogenously produce more PAH with this drug. Patients that are responsive to Kuvan have shown Phe levels lowered by 30% or more [5].

An alternative drug for managing blood Phe levels is in the third stages of FDA approval. Phenylalanine ammonia lyase (PAL) is an enzyme substitute for PAH that converts Phe into ammonia and trans-cinnamic acid. This enzyme is conjugated with polyethylene glycol (PEG) to reduce immunogenicity. This enzyme conjugated with PEG is commonly referred to as PEG-PAL. Patients participating in clinical trials have seen blood Phe levels decrease by about 50% when taking recommended doses [5].

**Paper-Based Microfluidic Devices**

*Use in therapeutic monitoring*

Disease diagnosis and monitoring can be a large financial burden. There are many developing countries that lack the funds or laboratory equipment necessary to diagnose and treat individuals, which decreases the likelihood of administering treatment in a timely manner [25, 26]. This creates an even greater financial burden after irreversible neural damage has occurred when compared to a PKU patient that routinely receives treatment. It is highly recommended that a point-of-care (POC) device be developed for diagnosing and monitoring PKU [16, 17, 21].
The World Health Organization (WHO) created the ASSURED criterion, an acronym for characteristics that should be considered when developing an ideal POC device [22]. Table 1.2 describes in detail what each letter of the ASSURED acronym represents. It is also important to consider the disposal of the POC device. Waste removal in developing countries can be extremely primitive, often as simple as an outdoor incinerator or fire pit [26]. POC devices should be created on substrates that have a minimal environmental impact when incinerated or burned.

The field of microfluidics has gained traction over the last few decades as research has explored the miniaturization, automation and cost-reduction of gold standard laboratory assays and converted them into Lab-on-Chip (LOC) devices [7]. These devices are generally single-use, pre-packaged with all necessary assay reagents dried on-board, and have visually detectable results. The choice of materials used for the device can alleviate the use of pumps for fluidic transfer by capitalizing on capillary forces to transport fluid [27]. Devices that transport fluid laterally from a source material, through a material containing dried reagents and into a sink material are appropriately named lateral flow tests (LFT). Paper is of great interest as a rapid, inexpensive and easily disposable device for disease detection and monitoring in a LFT format [26, 28], and satisfies many of the ASSURED criteria. Paper-based assays are compared with traditional laboratory based assays in Table 1.3.

<table>
<thead>
<tr>
<th>Affordable – For screening and routine therapeutic monitoring</th>
<th>Sensitive – Little to no false-negatives</th>
<th>Specific – Little to no false-positives</th>
<th>User-friendly – Simple to use with little to no training</th>
<th>Rapid and Robust – Quick time to result and can run in various environments</th>
<th>Equipment-Free – Visual detection</th>
<th>Deliverable to End Users – No cold chain and preferably can be sent via mail</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 1.2 – WHO recommendations for an ideal POC device.
Paper-based LFTs are currently being used for applications such as home testing for pregnancy or illicit drug use, and disease detection in low-resource settings [29]. The Whitesides group sparked much interest in the paper-based microfluidics field in 2007 with their demonstration of a test that could simultaneously detect glucose and protein in urine on a cellulose matrix previously patterned with reagents [8]. Since then, significant improvements have been made in creating paper-based devices with self-calibration [30, 31], tunable valves [32-34], signal amplification [29, 33] and assay multiplexing [27, 32, 33] which significantly improves the likelihood of integrating more complex laboratory assays into paper-based devices.

**Technical Microfluidic Background**

Lateral flow tests require flow rates that are controlled and reproducible in order to provide useful information. Flow in a porous media can be classified in three states; imbibition, steady-state and drainage. The latter of the three states, drainage, is not of particular interest in this paper, due to assays generally being read before drainage occurs. It is assumed here that all of the paper strips presented have constant width.

<table>
<thead>
<tr>
<th>Laboratory Based Test</th>
<th>Paper Based Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Not rapid</td>
<td>Rapid (&lt; 20 min)</td>
</tr>
<tr>
<td>Need trained personnel</td>
<td>Easy to use</td>
</tr>
<tr>
<td>Expensive instruments</td>
<td>Equipment free</td>
</tr>
<tr>
<td>Needs electricity</td>
<td>No electricity</td>
</tr>
<tr>
<td>Cost variable</td>
<td>Low cost</td>
</tr>
</tbody>
</table>

Table 1.3 – Laboratory vs. Paper based assay comparisons
channels, unless otherwise stated, and flow is one dimensional.

The first fluid transport phenomena discussed, imbibition, is the initial wetting of the porous material. During this phase, it was assumed that flow is one-dimensional and coming from an infinite source. This flow is modeled with the Lucas-Washburn equation, \( L^2 = \frac{\gamma D t}{4\mu} \), where \( L \) is the distance the fluid front has traveled, \( \gamma \) is surface tension, \( D \) is the average pore diameter, \( t \) is time and \( \mu \) is the fluid viscosity [35]. If it is assumed that the surface tension, pore diameter or the fluid viscosity are not changing during an assay, then the distance the fluid front travels is proportional to the square root of time. In some LFTs, fluids are mixed with reagents and traveling from one media to another. This would make modeling fluid flow in the assay more complicated, but as long as surface tension, viscosity, and average pore diameter can be estimated, the equation could be treated as a piecewise function.

The second fluid flow phenomena discussed occurs at steady state, when fluid is traveling in a wetted material and has reached a constant flow rate. Flow under these conditions can be modeled with Darcy’s Law, \( Q = \frac{-\kappa A}{\mu L} \Delta P \), where \( Q \) is volumetric flow, \( \kappa \) is the porous material permeability, \( A \) is the surface area of the channel perpendicular to the flow direction, \( \mu \) is the fluid viscosity and \( \Delta P \) is the pressure drop occurring across a distance, \( L \) [35]. Similar to the Lucas-Washburn equation, many of these variables are constant in a LFT. Assuming the permeability, viscosity and change in pressure per length does not change, then the volumetric flow rate is dependent on the surface area of the channel perpendicular to flow. The flow rate of an assay can easily be decreased by
increasing the width of the strip, or vice versa.

**Phenylalanine Monitoring Device**

**Current Laboratory Phe Measurement Methods**

Routine blood Phe concentration measurements are essential to understanding how well a patient is responding to their diet therapy. Patients either need to go to a clinic where a blood sample can be drawn, or send blood samples through the mail to a center capable of measuring Phe concentrations. The downside to the current method is the slow response time when samples are sent via mail. Current turnaround times are a few days to two weeks, which is too slow to be practically useful in monitoring or optimizing a patient’s therapy [37]. It is believed that the usefulness of measuring blood Phe levels improves greatly the quicker the patient can receive the information [24].

Surveys are routinely administered to PKU patients, as well as caretakers and dietitians involved in treating PKU, to better understand how to treat the individuals with PKU. Many respondents believe that home monitoring would greatly improve PKU management by allowing more regular and timely feedback [21]. The rapid feedback would allow patients to understand and isolate various sources of Phe, as well as administer intermediate remedial action as needed. PKU camps, where patients are allowed to monitor their blood as frequently as they wish, have proven rapid monitoring can lower blood Phe levels by allowing patients to see exactly how certain foods affect them [24]. Measurement frequency could greatly improve if home monitoring, similar to home blood glucose testing, was available for measuring blood Phe levels [16, 17].

Phe is currently measured in the U.S. by taking a blood sample, followed by
laboratory analysis, and the patient receives results generally within a week or two [17, 21]. There are several methods for determining a patient’s Phe concentration. The gold standard for measuring Phe concentrations in a laboratory setting is tandem mass spectroscopy (MS/MS). The total estimated cost for a single Phe concentration measurement with MS/MS after sample collection, transportation, processing and interpretation, is approximately $15 [ZZ]. Some other methods used for measuring Phe concentrations are high performance liquid chromatography (HPLC), bacterial inhibition assay (BIA), an enzymatic-colorimetric assay (ECA) or amino acid analysis (AAA) [38].

Studies suggest there can be significant variation between results obtained with the previously mentioned methods. When Phe samples were measured in a clinically relevant concentration range, MS/MS results on average were 0.6 mg/dL, 2.3 mg/dL and 3.4 mg/dL lower than HPLC [9, 39], AAA [9, 38] and ECA [39], respectively. Many guidelines for recommended Phe cut-offs did not mention the method used to determine blood Phe concentrations, suggesting more than minimal inter-method differences when measuring Phe [38]. Differences across methods should compel clinicians to understand which method was used to measure blood Phe levels so they can suggest appropriate therapeutic actions [38].

*Alternative Phe Measurement Methods*

Alternative methods have been created with the goal of lower cost Phe detection and those are briefly outlined in Table 1.4. Although many of these methods offer low limits of detection, require small sample volumes and can detect a large range of Phe concentrations, they all require electricity and designated equipment which make them
poor candidates for low resource settings [8]. The cost, ease of use and portability of each one of these methods does not currently make any of them an ideal candidate for use as a home Phe monitor, but the translation of one of these methods into a paper-based version could offer a solution to these issues.

Table 1.4 - Assays Designed for Home or Lower Resource Phe Detection

<table>
<thead>
<tr>
<th>Method</th>
<th>Sample Volume</th>
<th>Sample Fluid</th>
<th>LOD [mg/dL]</th>
<th>Range [mg/dL]</th>
<th>Equipment Free</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chemiluminescence on PDMS Chip[11]</td>
<td>Dipped ‡</td>
<td>Soft Drink</td>
<td>4.0e-6</td>
<td>2.5e-5 – 2.0e-3</td>
<td>No</td>
</tr>
<tr>
<td>Electrochemical Based[40],[41]</td>
<td>Dipped ‡</td>
<td>Artificial Urine</td>
<td>8.3</td>
<td>1.3e2 – 1.3e4</td>
<td>No</td>
</tr>
<tr>
<td>Enzymatic/Colorimetric[42]</td>
<td>25 µL</td>
<td>Serum</td>
<td>N/A</td>
<td>0 – 33</td>
<td>No</td>
</tr>
<tr>
<td>Fluorescent detection using UV LED[43]</td>
<td>Dipped ‡</td>
<td>Buffer</td>
<td>8.3e-2</td>
<td>0.17 - 170</td>
<td>No</td>
</tr>
<tr>
<td>DNA Hybridization on Compact Disc[44]</td>
<td>1.5 µL</td>
<td>Blood</td>
<td>7.0e-2</td>
<td>N/A</td>
<td>No</td>
</tr>
<tr>
<td>Spectrofluorometric in sol-gel[45]</td>
<td>5 µL</td>
<td>Artificial Serum</td>
<td>1.7e-2</td>
<td>0.83 - 33</td>
<td>No</td>
</tr>
</tbody>
</table>

‡ A portion of the assay is dipped into the Phe solution, therefore, a specific volume was not reported.

**Paper-Based Phe Assay Design Selection**

The Enzymatic-Colorimetric Assay (ECA) was chosen as the model laboratory test to integrate into an LFT because the reaction produces a visibly detectable colorimetric response, which offers promise for keeping the assay equipment free, and has a range spanning clinically relevant Phe concentrations. The reactions for this assay are well understood and are shown below.

\[
Phe + NAD^+ \xrightleftharpoons{\text{PheDH}} NADH + NH_4^+ + \text{Phenylpyruvate} \tag{1}
\]

\[
NADH + NBT \xrightarrow{\text{mPMS}} \text{Formazan Dye} + NAD^+ \tag{2}
\]
In Reaction 1, Phe reacts with the oxidized form of nicotinamide adenine dinucleotide (NAD$^+$) in the presence of phenylalanine dehydrogenase (PheDH) to form the reduced form of nicotinamide adenine dinucleotide (NADH), ammonium (NH$_4^+$) and phenylpyruvate. In Reaction 2, the NADH formed in Reaction 1 reacts with nitroblue tetrazolium (NBT) in the presence of the electron facilitator 1-methoxy-5-methylphenazinium methyl sulfate (mPMS) to form a visible, purple formazan dye and reduce the nicotinamide adenine dinucleotide back into NAD$^+$.

There is generally an excess of NAD$^+$ in the Reaction 1, making Phe the limiting reagent. This means that NADH formed in Reaction 1 is proportional to the original amount of Phe in the assay. The intensity of the colorimetric response formed in Reaction 2 is proportional to NADH concentration, and therefore also the Phe concentration originally present in the assay.

Previous work, with reagents in a model buffered solution, has shown that Reaction 1 is compatible when dried in glass fiber and reaction two is viable when the reagents are dried in cellulose [7]. Subsequent chapters here will follow the development of a paper-based ECA as significant milestones are explored and met. Chapter Two further describes an early version of the paper-based ECA, characterizes the enzymatic reaction performance with different reagent concentrations and shows a functioning assay capable of accepting a plasma sample. Chapter Three focuses on significant changes in materials needed for separation of a whole blood sample, as well as show a functioning assay that accepts whole blood. Chapter Four assesses current issues and next steps for the Phe home monitor development.
Chapter 2 – Phe Assay with Plasma Samples

Introduction

Diagnostic tests are often performed on blood (or derivatives of blood, such as plasma) because changes in its composition often indicate the current status of pathological processes throughout the body [46]. Previous work towards a home Phe monitor successfully converted a laboratory enzyme-based colorimetric assay performed in a well, into a paper-based assay that used a Phe sample in a model buffer solution. The result of that work provided a proof of concept for a paper-based enzymatic-colorimetric assay that could detect relevant levels of Phe in a low-resource setting [47]. The use of a model buffer solution does not accurately account for the chemical complexity of a sample of whole blood [48] which is the ultimate goal for sample fluid. A significant milestone towards the development of a whole blood home Phe assay is one that uses a sample of plasma as the Phe source.

Whole blood is roughly 55% plasma, although this number can vary as endogenous functions consume or replenish blood components [49]. A whole blood sample in a home setting is commonly obtained from pricking a finger with a lancet. The volume of blood obtained by this method depends on lancet size, as well as the depth the lancet penetrates into the skin. Assuming a whole blood volume of 40 µL is achievable from a finger prick, the current design of the home Phe assay was sized to accept a plasma volume of roughly 20 µL.
A schematic of the paper-based assay demonstrated in this chapter is presented in Figure 2.1. This design was used for calibrating the Phe assay for use with human plasma.

![Figure 2.1 Paper-based Phe monitor plasma assay card. (a) Glass fiber pads were impregnated and dried with NAD⁺ (1) and PheDH (2). Cellulose was impregnated with NBT (3) and mPMS (4). Samples of Phe spiked plasma pipetted in the glass fiber pads initiated the enzymatic reaction. A rectangular portion of the card was removed to allow the card to be folded after several minutes. (b) Plasma flows laterally through the cellulose and a colorimetric reaction developed downstream.](image)

**Materials and Methods**

*Card Fabrication and Image Acquisition Techniques*

Mylar (Tekra, New Berlin, WI, USA) folding cards and pieces of porous materials were designed with the software RetinaEngrave3D (Full Spectrum Laser, Las Vegas, NV, USA). A CO₂ laser cutter (H-Series, Full Spectrum Laser, Las Vegas, NV, USA) was used to cut substrates to the desired size. The cutting parameters (speed, power and number of passes) of the laser were adjusted to ensure the substrate was either cut entirely through, or partially etched on the surface. Porous materials used for the LFTs were cut to the desired dimensions and then placed onto an adhesive Mylar backing that housed the LFT. Porous materials were sized to hold specific fluidic capacities, which are roughly equal to void space. The Mylar backing allowed porous materials to
remain segregated on adjacent sides of a folding card until a user would fold a card and initiate fluidic contact.

A flat bed scanner (Perfection V700 Photo, Epson, Nagano, Japan) was used to image the assay. Images were saved as 16-bit TIFF files. A custom MATLAB (Natick, MA, USA) program was used to average the intensity values within a circular region of interest 100 pixels in diameter. Fiducial marks were etched onto the Mylar folding card to allow for spotting in a consistent location. A plate reader (Ultramark, Bio-Rad Laboratories, Hercules, CA, USA) measured absorbance at 340 nm.

*Enzymatic Reaction Sensitivity in Various PheDH Concentrations*

A stock solution of 64 mg/dL Phe (Sigma Aldrich, St. Loius, MO, USA) was created in deionized water. Three two-fold serial dilutions and deionized water were used to prepare a Phe concentration profile of 0, 4, 8, 16, 32, 64 mg/dL. A 10 µL volume of each concentration was pipetted into a 384 well plate. A 40 U/mL concentration of PheDH (Sigma Aldrich, St. Loius, MO, USA) was diluted to either 1.54, 6.15 or 12.3 U/mL in 220 mM bis-tris propane (Sigma Aldrich, St. Loius, MO, USA), pH 9.3, and 26 µL was added to each well containing a Phe solution. The reaction was initiated by the addition of 4 µL of 100 mM NAD$^+$ (Sigma Aldrich, St. Loius, MO, USA) in deionized water to each well simultaneously with a multi-channel pipette. A final volume of 40 µL in each well was used to calculate final concentrations. The final Phe concentration profile was 0, 1, 2, 4, 8 and 16 mg/dL. Final PheDH concentrations were either 1, 4 or 8 U/mL. The final NAD$^+$ concentration was 10 mM. Absorbance was measured within 15 seconds of reaction initiating. A total of 18 measurements were taken over the course of
10 minutes with six replicates for each sample. This experiment was designed and performed by Liam Wong.

**Calibration Assay**

A Mylar folding card was laser cut and etched to house the substrates, shown in Figure 2.1a. Fiducial marks were etched along the edges perpendicular to the direction of flow to allow for reproducible reagent hand-spotting placement, as well as reproducible image analysis.

Glass fiber pads (8975, Ahlstrom, Helsinki, Finland) were laser cut into rectangles with a 10 µL capacity. Cellulose pads (CFSP22300, EMD Millipore, Darmstadt, Germany) were cut into comb shaped pieces to help with handling, placing and ensuring the correct direction once reagents were spotted. Each tooth in the comb shape was sized to hold 48 µL of fluid and were the same width as the glass fiber pads. Half of the glass fiber pads were filled to capacity with a 220 mM bis-tris propane buffer, pH 9.3, and the other half of the glass fiber pads were filled to capacity with 20 mM NAD$^+$ in deionized water solution. The cellulose pads were filled to capacity with a 220 mM bis-tris propane buffer, pH 6.3. The buffer was pipetted into the cellulose at the upstream edge, to wash reactive after-products from the cutting method downstream. The glass fiber and cellulose pads were placed into a desiccator (Secador, Bel-Art Products, Wayne, NJ, USA) and dried overnight.

Pads were removed from the desiccator and the glass fiber pad with 220 mM bis-tris propane buffer, pH 9.3, was placed onto the folding card on the location marked 2, as shown in Figure 2.1a. A 2 µL volume of 40 U/mL PheDH in 220 mM bis-tris propane, pH 9.3, was spotted into the center of the downstream edge of this glass fiber pad. The
A cellulose pad was placed onto the folding card and 2.5 µL of 2.4 mM NBT (Life Technologies, Eugene, OR, USA) and 2.5 µL of 0.1 mM mPMS (Dojindo Laboratories, Kumamoto, Japan) were pipetted into the cellulose in the locations marked 3 and 4 shown in Figure 2.1a, respectively. After the reagents were spotted into each pad, the card was placed back into the desiccator. The cards were removed after 2 hours and the glass fiber pad with NAD$^+$ was placed downstream of the glass fiber pad in the location marked 1 in Figure 2.1a so that there was 1.5 mm of contact between the pads.

Mock samples of various Phe concentrations were created in a solution of normal pooled human plasma. The highest Phe concentration was created by spiking 15 mg/dL of Phe into a solution of plasma. This solution was serially diluted twice to create samples of lower Phe concentrations. Subsequent measurements of Phe samples (Knight Diagnostic Laboratories, Oregon Health and Science University, OR, USA) confirmed that the Phe concentrations used in the assay were 0.9, 4.4, 7.9 and 15 mg/dL, from the normal sample to the highest spiked in concentration respectively.

A multi-channel pipette was used to dispense 20 µL of each Phe concentrations, as well as a PBS (Sigma Aldrich, St. Louis, MO, USA) solution, pH (7.4), into the upstream edge of the glass fiber pads to initiate the assay. The enzymatic reaction was allowed to incubate in the glass fiber pads for 6 minutes before the card was folded and the glass fiber pads came into contact with the cellulose pad. Plasma flowed laterally through the cellulose, where it was mixed with the NBT and eventually mPMS. The card was placed onto the flatbed scanner and imaged 10 minutes after the initial spotting of plasma. Four replicates were performed for each Phe concentration.
Results and Discussion

The paper-based home Phe sensor used the same two-step reaction as a laboratory-based enzymatic-colorimetric assay [42]. A plasma sample containing Phe was used to initiate the first reaction where NAD$^+$ was reduced in the presence of a phenylalanine dehydrogenase [50]. This enzymatic reaction produced NADH and other reaction products. An experiment was conducted with buffered solutions in a well to better understand the enzymatic reaction response to Phe when subject to various concentrations of PheDH. The goal of this experiment was to determine a suitable PheDH concentration for use in the paper-based assay. Absorbance data for the enzymatic reaction sensitivity experiment was analyzed after reagents were allowed to react for 8 minutes. Each replicate was averaged and the standard error was calculated.

Sensitivity here is the change in absorbance with respect to the change in Phe concentration. The sensitivity of the assay at clinically relevant Phe concentrations at three different PheDH concentrations are shown in Figure 2.2. The sensitivity for all PheDH concentrations were similar in a Phe concentration range between 0 and 4 mg/dL. However, the data indicates that the PheDH concentrations of 4 and 8 U/mL are similar and resulted in a higher sensitivity than 1 U/mL PheDH for Phe concentrations above 4 mg/dL.
Affordability is also an important metric when designing this assay. PheDH is a costly reagent, therefore, the lowest PheDH concentration that doesn’t sacrifice performance was desired. The PheDH concentration of 4 U/mL was used in the paper-based assay to reduce cost.

The enzymatic reaction took place in glass fiber, due to its relatively large fluid capacity (55 µL/cm²) and proven compatibility with this reaction [7, 47]. The PheDH and NAD⁺ were dried in the glass fiber before the assay began. A major concern when working with biological reagents is the environment in which they are stored. It has been found that NAD⁺ requires a slightly acidic pH, whereas the PheDH can maintain high activity in an alkaline environment. The recommended storage pH for PheDH was between 9 and 11 to maintain activity, maximize efficiency, and to reduce cross reactivity with other endogenous compounds found in blood [40, 42].

**Figure 2.2** – Enzymatic Reaction Sensitivity for Various Concentrations of PheDH. The plot shows the measured absorbance vs Phe concentration after 8 minutes for three concentrations of PheDH. Error bars represent the standard error between 8 replicates.
The enzymatic reaction occurs where the sample is pipetted, which was designed to be 20 µL when using plasma as the Phe sample. Due to the different storage requirements of NAD$^+$ and PheDH, it was decided to segregate the drying of these reagents into two glass fiber pads sized for 10 µL of fluid each and place them in contact once the glass fiber is dry. This made it possible to better control the environment the biological reagents were subject to when impregnating the pads with solutions. The NAD$^+$ in deionized water maintained the activity required when impregnated and dried in a glass fiber pad alone.

The PheDH required stringent control over the environment of the glass fiber pad it was pipetted into. Dilute enzymes often lose much of their activity when stored in dilute concentrations, as well as in environments dissimilar to their native environment [51]. The glass fiber pads needed for this reagent were previously impregnated and dried with a pH 9.3 bis-tris propane buffer. A smaller volume of a higher concentration of PheDH was then pipetted into the glass fiber pad to decrease the amount of activity that is lost when drying the reagent.

The colorimetric reaction utilizes the NADH created in the enzymatic reaction to facilitate a visible response. Tetrazolium salts are generally used for the colorimetric reaction due to their specificity for NADH and the generation of formazan dye. There are several types of tetrazolium salt and each one yields a unique formazan dye. This dye is created when electrons are transferred between the tetrazolium salt and NADH. Nitro blue tetrazolium (NBT) was chosen due to a low rate of auto production, the insolubility of the dark blue formazan dye product, and the stability at room temperature and an open atmosphere [7].
A potential downside to NBT was the slow rate of electron transferring between NADH and NBT to create the formazan dye. Previous work used the electron mediator 5-methylphenazinium methyl sulfate (PMS) to remedy this issue [7]. Concerns arise when using PMS because of the photosensitivity [52], which could affect assay performance in uncontrolled light. The assay presented here used 1-methoxy-5-methylphenazinium methyl sulfate (mPMS) because it is a photochemically stable and a versatile electron carrier [50].

The colorimetric reaction took place in cellulose. This material has a similar capacity (56 µL/cm²) to glass fiber, and has a larger capillary pressure, 2.64 kPa, compared to the glass fiber, 0.282 kPa [7]. The difference in capillary pressures will be the driving mechanism for fluid flow from the glass fiber and laterally through the cellulose when the two materials are placed into contact.

Previous work has shown that NBT exhibits appreciable amounts of auto-production when dried in cellulose [42]. Upon rehydration, it appeared that the auto-produced signal remained only at the location it was stored. The problem was avoided by segregating the storage of NBT and mPMS [7]. NBT was stored upstream of mPMS to allow for the signal to develop away from where auto-production was known to occur. An elevated pH has also been shown to be a significant source of auto-production [42], likely due to non-specific conversion of NAD⁺ to NADH. Maintaining a pH below 8 can significantly reduce the amount of auto-production, so it was decided to impregnate the cellulose with a slightly acidic pH buffer before the addition of the colorimetric reaction reagents to counteract the elevated pH required in the enzymatic reaction.
An experiment was conducted to calibrate the colorimetric response to the concentration of Phe spiked into a plasma sample. Several plasma samples were spiked with clinically relevant concentrations of Phe. This experiment was conducted in a paper-based lateral flow assay like that shown in Figure 2.1. A sample of Phe spiked plasma was pipetted into glass fiber pads containing reagents for the enzymatic reaction and allowed 6 minutes to react. The card was then folded and plasma flowed laterally through reagents needed for the colorimetric reaction, and a signal developed downstream. After 4 additional minutes, the region of interest (ROI) was scanned and analyzed, and the results are shown in Figure 2.3c.

![Calibration Assay Results](image)

**Figure 2.3** Calibration Assay Results. (a) Scanned images of ROIs from representative replicates. (b) A schematic of the plasma calibration card with developed ROI. (c) A plot showing average background subtracted intensity values vs Phe concentration. Error bars represent the standard error between 6 replicates.

The intensity values within the specified ROI on each strip were averaged. An image showing a set of ROIs after the colorimetric reaction can be seen in Figure 2.3a. The strips containing only PBS were used as to calculate background values for each card. The intensity of each sample contacting Phe was subtracted from the background intensity value calculated for the respective card. The average intensity value for each
Phe concentration is shown in Figure 2.3c. Error bars represent the standard error for each average.

The results show a promising monotonic relationship between the colorimetric intensity and Phe concentration. Statistically significant differences were observed between clinically relevant concentrations of Phe. The assay can distinguish concentrations between normal (~1 mg/dL), elevated (~4 mg/dL) and severely elevated (~15 mg/dL) levels of Phe. Further discussion with an experienced physician could isolate semi-quantitative Phe ranges that elicit a specific response. For example, Phe levels in the normal category would signify good Phe management and not require a response, Phe levels in the elevated category would suggest a stricter adherence to diet therapy is needed, and Phe levels in the severely elevated category would suggest that a patient should schedule an appointment with their physician for a reevaluation of their prescribed diet therapy, or an increase in their Phe reducing medication.

**Conclusion**

The enzymatic reaction used in the proposed paper-based ECA was investigated in this chapter to better understand how PheDH concentration effects the conversion of NAD$^+$ into NADH over a range of Phe concentrations. The reaction sensitivity with respect to Phe concentration was similar for all three PheDH concentrations investigated in the 0-4 mg/dL Phe concentrations range. A greater sensitivity was observed in reactions with 4 and 8 U/mL PheDH in the 4-16 mg/dL Phe concentration range, than in reactions with 1 U/mL PheDH. A 2 $\mu$L volume of 4 U/mL PheDH was chosen for use in the paper-based assay because it is the lowest concentration observed that performs similarly to elevated concentrations of PheDH.
The paper-based LFT developed in this chapter showed monotonicity between the intensity of a colorimetric response and Phe concentrations in human plasma samples. The assay is capable of semi-quantitatively determining Phe concentrations, which could be useful for determining a range of clinical actions. Assay results demonstrate that the materials and reagents used for the home Phe monitor are compatible with complex biological matrix such as plasma. This proof of concept is an instrumental milestone towards the development of an equipment-free device that can accept a finger prick of whole blood and routinely monitor blood Phe concentrations. Further device development will require an inexpensive and automated method for on-card plasma separation from a whole blood sample.
Chapter 3 – Phe Assay with Whole Blood Samples

Introduction

A paper-based Phe monitor that semi-quantitatively measured Phe concentrations in a sample of plasma was described and demonstrated in the previous chapter. To further assay utility, it must be capable of accepting and processing a whole blood sample. It is important to recognize that the color of red blood cells can interfere with colorimetric signals meant to be visually interpreted [8]. The previously designed buffer and plasma assays provided a relatively transparent background, however, a whole blood sample will add a significant visual interference if left unprocessed. A cheap, reliable and disposable blood separation technique will be required for further assay development.

Most biological assays require the separation of cells and plasma to reduce interference between the cells and the analytical device, which is currently one of the largest challenges for POC devices [47, 53]. Many microfluidic blood separation techniques have been shown to be effective at separating blood cells from plasma, such as electro-osmotic flow, bifurcation, geometric obstruction, acoustic standing wave forces, and cross-flow filtration. These filtration methods would not be suitable for this assay because they require additional equipment, such as syringe pumps, or high voltage power supplies [48].

Commercially available plasma separation filters are a paper-based solution that can be integrated upstream of the current Phe assay to process whole blood into plasma [36]. Plasma separation membranes (PSM) are capable of separating red and white cells from plasma by trapping the cell in an asymmetrically graded membrane and allowing
the plasma to flow downstream [48]. It was decided to place the PSM upstream of the assay. The blood sample will separate into plasma and perform similarly to the assay developed in Chapter 2.

Several challenges arose when integrating the PSM, which motivated several assay design changes. The substrates used to house the enzymatic and colorimetric reactions in the previous version of the assay did not allow for an adequate flow of plasma from the PSM, and thus an alternative glass fiber material (AEGF) was investigated for compatibility. The goal was to find a material that allowed efficient plasma transport and was compatible with the enzymatic and colorimetric reactions. The new card design with the incorporated PSM can be seen in Figure 3.1.

Figure 3.1 Single whole blood Phe assay. (a) A 40 µL sample of whole blood is deposited onto the card with a disposable glass capillary and plunger device (PTS Diagnostics). The card is folded at 6 minutes and a purple colorimetric response appears in the downstream region of the assay. (b) A schematic shows the substrates and location in which reagents are spotted (left). The sample is placed into the PSM (1). Plasma is separated downstream into two glass fiber pads, previously dried with PheDH (2) and NAD⁺ (3). The card is folded after six minutes and the reacted fluid comes into contact with the last glass fiber pad, which contains dried NBT and mPMS (4). Once the card is folded, the colorimetric reaction occurs (right).
Materials and Methods

Card Fabrication and Image Acquisition Techniques

Card designs were created in a 2D CAD software (Draftsight, Dassault Systémes, Velizy-Villacoublay, France). A CO$_2$ laser cutter (H-Series, Full Spectrum Laser, Las Vegas, NV, USA) was used to cut substrates to the desired size based on these designs. The laser cutting parameters were adjusted (power, speed, number of passes) until a setting was found for each substrate that fully cut or etched the substrates with minimal burning or melting of the substrate.

A flatbed scanner (Perfection V700 Photo, Epson, Nagano, Japan) was used to capture images of the card after a specified amount of time. The images were saved as either 8 or 16-bit TIFF files. The saved images were further processed with a custom MATLAB (Natick, MA, USA) program. The program used a predetermined fiducial mark on the image of the card to select and analyze a circular (8 mm diameter) region of interest (ROI). Absorbance was measured with a plate reader (Ultramark, Bio-Rad Laboratories, Hercules, CA, USA) at 340nm.

Enzymatic Reaction Compatibility in Various Substrates

The laser cutter was used to cut circles out of cellulose (CFSP22300, EMD Millipore, Darmstadt, Germany), AEGF (A/E, Pall Corporation, Port Washington, NY, USA) and GF (8975, Ahlstrom, Helsinki, Finland) sized to hold a fluid volume of 60 µL. A knife cutter (X-Acto 26642, Westerville, OH, USA) was used to cut cellulose squares with a similar fluid capacity (56 µL). The pads were filled to capacity with a solution of 10 mM NAD$^+$ (Sigma Aldrich, St. Louis, MO, USA) and either deionized water or 3.75 mg/dL Phe (Sigma Aldrich, St. Louis, MO, USA) in deionized water and placed in a
desiccator (Secador, Bel-Art Products, Wayne, NJ, USA) to dry overnight. The following day, pads were placed into modified pipette tips and were placed into 1.5 mL Eppendorf tubes. The pads were filled to capacity with 1 U/mL Phe in a 220 mM BTP (Sigma Aldrich, St. Louis, MO, USA) buffer (pH 9.3). After allowing three minutes for the reaction to take place, the Eppendorf tubes, containing the pipette tips and pads, were centrifuged at 16,000 g for 30 seconds. The pipette tips containing the pads were removed from the Eppendorf tube and 40 µL of solution was collected from each sample. A volume of 40 µL of each solution was pipetted into a 384 well plate. After a total of 13 minutes had elapsed, the plate was measured at 340 nm.

**Colorimetric Reaction Compatibility in Various Substrates**

Porous substrates were cut using the methods outlined for the enzymatic reaction. The pads were filled to capacity with either 0 or 3.75 mg/dL NADH (Sigma Aldrich, St. Louis, MO, USA) in 220 mM BTP (pH 6.3) and allowed to dry in a desiccator overnight. A Mylar (Tekra, New Berlin, WI, USA) folding card was fabricated to hold pads dried with NADH on one side, and hold virgin pads on the adjacent side. The virgin pads were filled to capacity with 1.2 mM NBT (Life Technologies, Eugene, OR, USA) and 0.1 mM mPMS (Dojindo Laboratories, Kumamoto, Japan) and the card was folded to bring both pads into contact. The card was imaged on a flatbed scanner after three minutes and the intensity was evaluated with the custom MATLAB program.

**Compatibility of Plasma Separation Membrane with Various Substrates**

The laser cutter was used to cut rectangles out of cellulose, AEGF and GF. The rectangles had a fixed width of 5 mm and a length chosen to ensure a 30 µL capacity. The plasma separation membrane (Pall Corporation, Port Washington, NY, USA) was
sized to hold 40 µL of fluid and had a width equal to the substrates with which it would be used downstream. A Mylar backing was fabricated to house the strips, as well as provide an on-card ruler to determine wicking distance along each substrate. Spacers of various heights were cut out of Mylar to border the lateral flow test in order to provide consistent contact between each substrate. Each substrate was placed onto the Mylar, had a PSM placed upstream of the substrate with 1.5 mm of overlap, and was covered by placing another piece of Mylar on top of the spacer. A 40 µL volume of whole blood was pipetted into the PSM and allowed to separate into the substrate downstream. The card was imaged after 6 minutes and the wicking distance plasma traveled in each strip was recorded.

**Calibration Assay**

Whole blood was collected from two healthy donors (about 10 mL each) and pooled in accordance with the IRB-approved study protocol. The pooled mixture was stored in a refrigerator when not in use, then equilibrated at room temperature for about an hour before experimentation. A 1500 mg/dL solution of Phe was made in deionized water and used to create 2 mL of a blood solution with 15 mg/dL more than endogenously present in blood. The solution with an added 15 mg/dL Phe was used to make three two-fold serial dilutions, with normal whole blood as the diluent. A 100 µL sample of blood at each concentration was spotted onto filter paper, allowed to dry and the Phe concentration was measured by mass spectroscopy (Wisconsin State Laboratory of Hygiene). The measured values of Phe from the mass spectroscopy were 1.1, 2.4, 4.0, 6.3 and 11 mg/dL. Mass spectroscopy has been shown to report lower values for Phe than other analysis techniques (see Chapter One), with the error generally increasing as
the Phe concentration increases. The normal Phe concentration of 1.1 mg/dL was assumed to be correct. The other Phe values were calculated using the spiked-in Phe concentration and the normal Phe as the diluent when making serial dilutions. The assumed concentrations of Phe in each sample were thus 1.1, 3.0, 4.9, 8.6 and 16 mg/dL. It should be noted that this is a more conservative estimate when calculating sensitivity.

The laser cutter was used to fabricate AEGF pads with 10 µL capacities, PSMs with 40 µL capacities and a Mylar backing to hold the cards and allow folding steps. A third of the AEGF pads were impregnated to capacity with 20 mM NAD$^+$ in deionized water. Another third of the AEGF pads were impregnated with 220 mM BTP, pH 9.3. The final third of AEGF pads were impregnated with 220 mM BTP, pH 6.3. The AEGF pads were placed in a desiccator and allowed to dry overnight. The following day, the cards were assembled into a folding card device, like that shown in Figure 3.1b. The pads with the 220 mM BTP, pH 9.3, were placed onto the card first (location 2 in Figure 3.1b), followed by the pads containing 220 mM BTP, pH 6.3 (location 4 in Figure 3.1b). A 2 µL volume of 40 U/mL PheDH was deposited into the center of the downstream edge of the pad that had been dried with 220 mM BTP, pH 9.3. A 3 µL volume of 1.2 mM NBT / 0.1 mM mPMS was spotted into the center of the upstream edge of the AEGF pad dried with 220 mM BTP, pH 6.3. The card with the drying AEGF pads was placed into a light shielded desiccator and allowed 2 hours for the PheDH and NBT/mPMS to dry, after which the pads were removed from the desiccator and the PSM (location 1 in Figure 3.1b) and AEGF pad dried with NAD$^+$ were placed on the card (location 3 in Figure 3.1b). The first flap was folded on top of the spacer to ensure good contact between the PSM, the AEGF pad containing PheDH and the pad containing NAD$^+$. 

A card was fabricated to hold five individual assays (Figure 3.5b) to allow simultaneously running all five concentrations of Phe in blood. A multi-channel pipette was used to add a 40 µL sample of each Phe concentration to the upstream region of the PSMs at the same time. After 6 minutes, the card was folded to allow contact between pads on adjacent sides of the card. Fluid flow between pads was assisted by gently compressing the pads together with two fingers. The cards were imaged two minutes after folding and analyzed with a custom MATLAB program. Six replicates for each concentration were performed.

Results and Discussion

The inclusion of the plasma separation membrane required a significant redesign of the substrates used to house the enzymatic and colorimetric reaction. Experiments were developed that measured both the sensitivity of each reaction and the efficiency of plasma separation for each substrate. An experiment was designed to measure the absorbance of NADH after undergoing the enzymatic reaction in various substrates. This experiment measured the change in absorbance at 340 nm due to NADH production from the enzymatic reaction. Experimental results are shown in Figure 3.2. Error bars represent the standard error calculated between the eight replicates.
Sensitivity used in the context of this assay refers to the change in absorbance per change in Phe concentration. It was desired to find a substrate with the greatest sensitivity. The results of the enzymatic reaction compatibility experiments seen in Figure 3.2 suggest that most substrates have a similar sensitivity as the reaction solely performed in a well, except for the reaction in laser cut cellulose. This significantly elevated signal in laser cut cellulose was the motivation to perform the reaction in knife cut cellulose, which performed similarly to the other laser cut substrates. It is hypothesized that the laser cutter was burning the edges of the cellulose and creating by-products that caused a non-specific reaction to occur with the enzymatic reaction, which would significantly elevate the signal of both concentrations of Phe.

Another experiment was designed to measure the intensity of the colorimetric reaction in candidate substrates where colorimetric reagents reacted in various substrates. Reacted cards were imaged and then analyzed using a custom MATLAB code. The results of the colorimetric reaction compatibility are shown in Figure 3.3. A
representative image from each of the six replicates is displayed in Figure 3.3a. The grayscale intensities for each replicate were averaged, and the intensity of the pads with 0 and 3.75 mg/dL NADH were subtracted from the averaged intensity of the corresponding substrate that was impregnated only with buffer. The averaged grayscale intensity results are shown in Figure 3.3b. Error bars represent the standard error between each replicate.

Figure 3.3 Colorimetric reaction compatibility in various substrates. (a) Representative images of each substrate. Pads that had either 0 or 3.75 mg/dL NADH were also impregnated with NBT/ mPMS. Virgin pads were wetted with only buffer to get a background value for each substrate. (b) Average intensity values from pads subject to either 0 or 3.75 mg/dL NADH were subtracted from the background intensity for the respective substrate and plotted. Error bars show the standard error between six replicates.

In order to accurately compare each substrate, pads wetted with only buffer were used as backgrounds for subtracting the intensities of corresponding substrates. The graph in Figure 3.3b shows how each substrate distinguished between 0 and 3.75 mg/dL NADH. Sensitivities were compared between candidate substrates for the colorimetric reaction. The laser cut cellulose showed a greater zero concentration response compared to other substrates, suggesting a non-specific reaction is occurring. The AEGF and GF both had relatively large sensitivities compared to the laser cut and knife cut cellulose.
The final metric for deciding between candidate substrates was how well each substrate wicked plasma out of a PSM. Substrates were placed onto an adhesive Mylar card with a PSM placed upstream. The materials had a 1.5 mm overlap, which allowed fluidic contact when plasma was separated from the PSM. A representative image from each substrate is shown in Figure 3.4a. Five replicates were performed for each substrate. Substrates were sized to hold the same volume, 30 µL. The farthest distance that fluid traveled downstream was assumed to be the fluid front. The length the fluid front traveled was divided by the total length of substrate to yield a fraction of distance that plasma penetrated into the substrate. The fluid front of plasma is likely not filling the substrate to capacity, so fractional filling in this context could be an overestimate if used to calculate plasma volume filling each substrate. A figure comparing the fractional filling for each substrate is shown in Figure 3.4b. It can clearly be seen that AEGF allowed for the greatest transport of plasma from the PSM. AEGF was chosen as the most suitable substrate to house the enzymatic and colorimetric reactions because it had both superior sensitivities in the reaction compatibility experiments and superior performance to other candidate substrates in the PSM compatibility experiment.
A new card was developed that incorporated the PSM and used AEGF to house the enzymatic and colorimetric reactions. A diagram of this new design can be seen in Figure 3.1b, and a depiction of a five-assay calibration card is shown in Figure 3.5b.

There were two significant differences between the plasma and whole blood assays. The integration of the PSM affected the enzymatic reaction time because the separation requires some time at the beginning of the assay. Although the time of 6 minutes was used for this version of the assay, a longer time might be useful in a later design to account for the time needed to separate plasma.

The other significant difference between the plasma and whole blood assays was the format of the colorimetric reaction. In the previous test, NBT and mPMS were stored spatially separated in cellulose to reduce the amount of auto-production. It was found that this auto-production was reduced when using AEGF for drying the two reagents. The colorimetric compatibility assay discussed earlier in this chapter suggests the reason behind the auto-production in earlier designs may have simply been because laser cut cellulose was used and the slightly burnt edges were creating a non-specific conversion.

Figure 3.4 Plasma separation membrane compatibility with various substrates. (a) Representative images of plasma wicking into each substrate. The bars to the left of each substrate shows how far the fluid front traveled in each material. (b) A bar graph of the fraction each strip was filled with plasma. The standard errors between the five replicates were too small to represent in the bar graph.
of NBT into the purple formazan signal. The AEGF is thinner than cellulose, which made imaging the colorimetric reaction from the back side of the card possible. Instead of fluid moving the products from the enzymatic reaction laterally through the assay, fluid was flowed vertically into the pad with the colorimetric reaction reagents. Fluid wicked more rapidly between the enzymatic and colorimetric reaction, decreasing the overall assay time, and provided better control over the location where the colorimetric reaction developed.

Representative images of the assay after the colorimetric reaction are shown in Figure 3.5a. A background value was calculated by imaging plasma in AEGF without any dried reagents, and averaging the intensity between five samples. The graph in Figure 3.5c shows the measured intensity values after being subtracted from the background value.

![Figure 3.5](image)

**Figure 3.5** Calibration of whole blood assay. (a) Representative images of the colorimetric response for each concentration of Phe spiked whole blood sample. (b) A schematic of the five card calibration assay after the folding step and colorimetric response. (c) A plot of the average grayscale intensity for each whole blood sample at various Phe concentrations. Error bars represent the standard error between six replicates.

The newly designed card was used to calibrate Phe concentration from a whole
blood sample with a colorimetric response. The card was successfully able to accept
whole blood, process it into plasma and perform the enzymatic and colorimetric reaction,
with limited user interaction. An alternative glass fiber material, AEGF, was selected to
house the enzymatic and colorimetric reaction because it compatible with both reactions
and could separate plasma better than previously used substrates. The results show
promise for monitoring clinically relevant levels of Phe from a whole blood sample.
Similar to the calibration assay performed with plasma samples, a monotonic relationship
was seen between the colorimetric intensity and Phe concentration. This whole blood
assay can semi-quantitatively distinguish several ranges of Phe concentrations that could
help users determine a clinical action that should be taken to better control their Phe
levels. The assay meets another significant milestone towards the development of a home
Phe monitor. Future work should investigate better colorimetric reagent concentrations to
improve the response sensitivity and the colorimetric intensity development.

Conclusion

Development of a home Phe monitor included the incorporation of a plasma
separation membrane that enables processing of whole blood samples. Significant design
changes were needed in order to utilize the PSM. The substrate in which the enzymatic
and colorimetric reactions take place was an alternative glass fiber (AEGF) that is
compatible with each reaction and efficiently wicks plasma out of the PSM. The novel
whole blood home Phe monitor was used to correlate a colorimetric response with an
initial Phe concentration in a 40 µL sample. The assay shows a monotonic response for
Phe concentrations in a clinically relevant range. Semi-quantitative Phe ranges can
quickly be determined, which could be useful for users to determine an appropriate clinical action required to maintain healthy blood Phe levels.
Chapter 4 – Conclusions and Next Steps

Summary of Conclusions

The previous chapters have covered the motivations, background and work towards a home Phe monitor for people with PKU. An assay was developed based on a laboratory enzymatic/ colorimetric assay. It was first integrated into a paper-based LFT capable of receiving a Phe sample in plasma, and eventually into an LFT that accepted and processed whole blood samples. The Phe monitor described in this report shows significant progress towards creating a rapid, robust, equipment-free home Phe assay. There are still several issues regarding this assay that could be addressed, but are outside of the scope of this report. This chapter will describe possible next steps for improving device automation, calibration and detection.

Next Steps

Automation

Two issues with the current Phe home monitor could be improved with device automation. The time-dependent user folding step between the enzymatic and colorimetric reactions is an issue that requires the user’s attention and a reliable timing method in order to provide accurate results. The other issue with this device is uncontrolled metering of plasma volumes wicking out of the PSM. The volume of available plasma from a whole blood sample can vary significantly due to hematocrit variations between users [49]. This could alter the concentrations of rehydrated reagents in the assay and affect the result of the colorimetric result. Paper-based valve systems have been useful in other assays where controlling volumetric flow rates and volumetric
metering are needed [34] and should be considered when progressing the development of this assay.

There are three categories of paper-based valves used for controlling fluid transport in porous media. Geometry-based valves rely on changes in channel width to increase or decrease the volumetric flow rate based on the Lucas-Washburn relationship (mentioned in Chapter 1). Chemical-based valves have reagents dried onto the LFT that momentarily delay fluid flow and then resumes flow once the material has been fully dissolved. This valve is often made with a dried sugar that does not interfere with the chemistry of the assay reactions. The delay time can be tuned by varying the dried reagent concentration on the LFT. Physical-based valves rely on breaking fluidic contact to halt the flow. Discontinuous segments of a LFT connected by a solid and porous reagent bridge (similar to the reagents used for chemical-based valves) will allow fluid to flow between the two segments until the bridge is fully dissolved, and the fluidic contact is lost [32, 34].

A chemical-based valve used in the home Phe monitor could delay fluid flow between the enzymatic and colorimetric reaction for several minutes while the enzymatic reaction takes place. This solution would alleviate the need for the time-dependent user folding step that initiates fluidic contact between the enzymatic and colorimetric reactions and does not require any user intervention after the sample is placed on the card. A physical-based valve could be used in the home Phe assay to regulate the plasma volume out of the PSM and available for downstream reactions. This would control the concentration of rehydrated reagents and would not require the user to precisely monitor the sample volume placed on the card.
Calibration

Several environmental factors affect the performance of a LFT. Variations in the temperature, humidity, pressure or light exposure during storage and operation, as well as variations from user to user, could significantly alter the colorimetric response of the assay. An on-board calibration method is recommended for this assay to provide the user with a range of colorimetric standards to compare the sample colorimetric response.

Previous work used tree-shaped structures to calibrate glucose and protein assays. Each branch of the assay was subjected to the exact same environmental and timing conditions, so the calibration was unique to every assay [30, 31]. A similar tree-shaped design could be used for the home Phe monitor, like that shown in Figure 4.1. Additional branches would be added that have colorimetric responses representing a low and high Phe concentration. The pad labeled 1 in Figure 4.1 would be spotted with enzymatic reagents like in the previous chapter. Pads labeled 2 and 3 in Figure 4.1 would be spotted with low and high concentrations of NADH, respectively. The pads labeled 4 in Figure 4.1 would be spotted with colorimetric reagents like in the previous chapter. Folding the card will initiate the colorimetric reaction in all three pads.

The “low” pad concentration could correspond to normal, endogenous levels of

![Figure 4.1: Proposed Phe card with on-board calibration.](image)
Phe (~1.1 mg/dL). The user will be able to determine if the assay is falsely negative in the colorimetric response if the sample pad develops lighter than the response in the “low” pad. The “high” pad concentration could correspond to a Phe level that requires clinical intervention (~15 mg/dL). A similar colorimetric response between the “low” and “high” pads would signify a decreased sensitivity for the whole card, possibly due to poor storage or fabrication methods. These conditions could inform the users when assay results should be discarded to decrease the likelihood of gaining misinformation.

**Cell Phone Detection**

Having on-board calibration would add to the complexity of the device and increase the difficulty of user interpretation. An optional handheld reader could help users interpret and understand the assay results. Improvements in cell phone camera technology has sparked an interest in using them for extracting quantitative data from LFTs [33]. Several devices have already been developed that attach to the camera portion of a cell phone. Some of these assays have been used to detect malaria, tuberculosis and HIV using either an iPhone or Android-based smartphone [54]. Images can either be sent electronically to trained personnel for interpretation [8], or a custom cell phone app can be created that images and analyzes the LFT results [54].

A cell phone reader could be designed as an additional readout method for this device. The ability to visually read the assay would remain, but users that can afford a cell phone reader could have a less biased form of assay interpretation. A cell phone app could also track Phe levels and record information such as diet and time of sampling. Physicians with access to this information could suggest more tailored therapy regimens and better understand the individual needs of a patient with PKU.
Bibliography


