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Rapid detection of thrombin and other protease activity directly in whole blood

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Rapid Detection of Thrombin and Other Protease Activity Directly in Whole Blood

A Thesis submitted in partial satisfaction of the requirements for the degree Master of Science

in

Bioengineering

by

Johnson Chung Sing Yu

Committee in charge:

Professor Michael J. Heller, Chair
Professor Xiaohua Huang
Professor Geert Schmid-Schönbein

2012
The Thesis of Johnson Chung Sing Yu is approved and it is acceptable in quality and form for publication on microfilm and electronically:

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Chair

University of California, San Diego

2012
DEDICATION

I want to dedicate this thesis to everyone that has provided me guidance, support, and insight in my academic journey thus far. This includes faculty members, classmates, friends, and most importantly, my family. I sincerely thank you all for being there every step of the way.
The following analogy helps to visualize nano-scale detection:

"It's not just finding a needle in a haystack, but finding a needle in the state of Iowa..."

Dr. Michael J Heller
TABLE OF CONTENTS

Signature Page.................................................................................................................. iii
Dedication........................................................................................................................ iv
Epigraph........................................................................................................................... v
Table of Contents.............................................................................................................vi
List of Figures.................................................................................................................. vii
List of Tables................................................................................................................... ix
List of Graphs.................................................................................................................. x
Acknowledgements......................................................................................................... xi
Abstract of the Thesis...................................................................................................... xiii

Chapter 1. Detection of Thrombin Activity Directly in Whole Blood ...................... 1
  Background on Thrombin.............................................................................................. 1
  Current platforms to detect Coagulation activity......................................................... 3
  Background on Electrophoresis...................................................................................... 6
  Background on Peptides............................................................................................... 6
  Proven Platform for Protease Detection ..................................................................... 7
  Thrombin Charge-changing substrate platform....................................................... 8
  Thrombin Specific Peptide Substrate Experiments................................................... 8
  Determining the detection limit of thrombin in human whole blood.................... 10
  Real time detection of thrombin in whole human blood......................................... 11
  Specificity of the thrombin substrate........................................................................ 11
  Thrombin Peptide Experiment Results..................................................................... 11
  Conclusion.................................................................................................................... 25

Chapter 2. Design and Testing of a POC Thrombin Detection Device .................. 26
  Horizontal Gel Format Device................................................................................... 28
  Horizontal Format Device Testing Results............................................................. 34
  Vertical Gel Format.................................................................................................... 45
Vertical Format Device Testing Results .................................................................48
Future Direction ........................................................................................................58
Chapter 3. Bacterial Protease Detection........................................................................60
   Expanding the Existing Platform for the Detection of Bacterial Protease ..............60
   Background on Omptin T Surface Protease ..........................................................61
   Designing a Charge-changing OmpT Peptide Substrate .....................................62
   Bacterial Protease Detection Procedure ..............................................................67
   Future Direction ......................................................................................................68
References ..................................................................................................................69
LIST OF FIGURES

Figure 1. The blood sample is combined with the substrate. ........................................... 13
Figure 2. Electrophoresis of Sample. .................................................................................. 13
Figure 3. Gel with various thrombin concentrations in substrate and 1x PBS. .................. 15
Figure 4. Gel with various concentrations of thrombin in citrated blood. ................... 15
Figure 5. Gel of Thrombin Activity Over Time for Donor #1. ....................................... 17
Figure 6. Gel of Thrombin Activity Over Time for Donor#2. ......................................... 18
Figure 7. Gel of Thrombin Activity Over Time for Aspirin User .................................. 20
Figure 8. Comparison of Gels Between Normal Donor and Aspirin User ...................... 21
Figure 9. Gel of Thrombin Activity Over Time for Heparinized Blood ....................... 23
Figure 10. Isometric View of the Device.......................................................................... 28
Figure 11. Top View of the Device. .................................................................................. 29
Figure 12. Side View of the Device. .................................................................................. 29
Figure 13. Exploded View of the Device. ........................................................................ 30
Figure 14. General Configuration of the Gel Medium..................................................... 31
Figure 15. Horizontal Format Device Setup. ................................................................... 33
Figure 16. Preliminary Device Running Cleaved Substrate. .......................................... 35
Figure 17. Redesigned Device Running Cleaved Substrate........................................... 36
Figure 18. Running cleaved substrate in 0.5x TBE and 15% Doped gel. ....................... 38
Figure 19. Running cleaved substrate and blood in 15% Doped Gel. ......................... 40
Figure 20. Running reacted substrate and blood in 15% doped gel. ............................. 41
Figure 21. Running reacted substrate and heparinized blood in 15% Doped gel. ........... 44
Figure 22. Vertical Gel Format Configuration................................................................. 46
Figure 23. General Setup for the Vertical Gel Format Device........................................ 47
Figure 24. Running negatively charged substrate through the 15% gel under reverse polarity. ........................................................................................................... 49
Figure 25. Running uncleaved substrate through the 15% gel under normal polarity. .... 50
Figure 26. Confirmation of cleaved thrombin substrate (T3) sample. ........................... 52
Figure 27. Running uncleaved substrate in a 6%/15% gel in reverse polarity. ................ 54
Figure 28. Running cleaved substrate in a 6%/15% gel in normal polarity..................... 56
Figure 29. Running substrate in fresh blood through a 6%/15% doped gel at higher voltage.

Figure 30. Crystal Structure of OmpT. Front and side views are shown.

Figure 31. CPK Space Fill Model of OmpT Substrate.

Figure 32. Cartoon Model of OmpT Substrate.

Figure 33. Ball and Stick Model of the OmpT Substrate.

Figure 34. OmpT Specific Charge-Changing Peptide Substrate Reacting with Sample Containing the OmpT Enzyme.
LIST OF TABLES

Table 1. Substrate Sequences............................................................................................................ 12
Table 2. Recipes for Different Gel Mediums.................................................................................... 31
Table 3. Kinetic parameters for the OmpT-catalyzed hydrolysis of peptides corresponding to sequences selected by bacteriophage display methods............................. 64
Table 4. OmpT Charge-changing Peptide .......................................................................................... 65
LIST OF GRAPHS

Graph 1. Thrombin in 1x PBS and Citrated Blood ................................................................. 15
Graph 2. Thrombin Activity Over Time for Donor #1 .............................................................. 17
Graph 3. Thrombin Activity Over Time for Donor #2 .............................................................. 18
Graph 4. Thrombin Activity Over Time for Donor #3 .............................................................. 19
Graph 5. Thrombin Activity Over Time for Aspirin User ......................................................... 20
Graph 6. Thrombin Activity of Normal Patients vs Aspirin Users ............................................ 21
Graph 7. Thrombin Activity Over Time for Heparinized Blood .................................................. 23
Graph 8. Thrombin Activity of All Groups Tested ...................................................................... 24
Graph 9. Fluorescence Detected in Reading Window of Cleaved Substrate in TBE. ........ 35
Graph 10. Fluorescence Detected in Reading Window of Cleaved Substrate in 15% Gel for New Channel .................................................................................................................. 37
Graph 11. Fluorescence Detected in Reading Window of Cleaved Substrate in 15% Doped Gel .............................................................................................................................................. 38
Graph 12. Fluorescence Detected in Reading Window of Cleaved Substrate in Citrated Blood at Various Time Points ............................................................................................................. 40
Graph 13. Fluorescence Detected in Reading Window of Substrate in Fresh Blood. ............... 42
Graph 14. Fluorescence Detected in Reading Window for Substrate in Heparinized Blood ....................................................................................................................................................... 44
Graph 15. Fluorescence Detected in the Vertical Gel Reading Window for Substrate Under Reverse Polarity .......................................................................................................................... 49
Graph 16. Fluorescence Detected in the Vertical Gel Reading Window for Substrate Under Normal Polarity ....................................................................................................................... 51
Graph 17. Fluorescent Signal of the Cleaved and Uncleaved Substrates ............................... 52
Graph 18. Fluorescent Signal Detected in Reading Window of Substrate in 6%/15% Gel, Reverse Polarity ...................................................................................................................................... 54
Graph 19. Fluorescent Signal Detected in Reading Window of Cleaved Substrate in 6%/15% Gel .............................................................................................................................................. 56
Graph 20. Fluorescent Signal Detected in Reading Window of Substrate and Fresh Blood in 6%/15% Gel .......................................................................................................................... 58
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Chapter 1, in part is currently being prepared for submission for publication of the material by Augusta Modestino and Johnson Yu. The content was jointly written by the authors and the paper was edited by Dr. Michael Heller.

Chapter 1, in part is a reprint of the material as it appears in the poster "Rapid Detection of Thrombin and Other Protease Activity Directly in Whole Blood," by Augusta E. Modestino, Johnson C. Yu, Mrudul Bhide, Geert W. Schmid-Schönbein and Michael J. Heller. The poster was presented at the Jacobs School of Engineering Research Expo 2012.
ABSTRACT OF THE THESIS

Rapid Detection of Thrombin and Other Protease Activity Directly in Whole Blood

by

Johnson Chung Sing Yu

Master of Science in Bioengineering

University of California, San Diego, 2012

Professor Michael J. Heller, Chair

Thrombin is a serine protease that plays a key role in the clotting cascade to promote hemostasis following injury to the endothelium. From a clinical diagnostic perspective, in-vivo thrombin activity is linked to various blood clotting disorders, as well as cardiovascular disease (DVT, arteriosclerosis, etc). Thus, the ability to rapidly measure protease activity directly in whole blood will provide important new diagnostics, and clinical researchers with a powerful tool to further elucidate the relationship between circulating protease levels and disease. The ultimate goal is to design novel point of care (POC) diagnostic devices that are capable of monitoring protease activities directly in whole blood and biological sample.
A charge-changing substrate specific to the thrombin enzyme was engineered and its functionality was confirmed by a series of experiments. This led to the preliminary design, construction, and testing of two device platforms deemed fully functional for the electrophoretic separation and focusing of charged peptide fragments.

The concept of using the existing charge-changing substrate platform for bacterial protease detection was also investigated. Certain strains of E. coli are associated with severe symptoms such as abdominal cramps, bloody diarrhea, and vomiting. The OmpT protease is expressed on the outer membrane of E. coli and plays a role in the cleavage of antimicrobial peptides, the degradation of recombinant heterologous proteins, and the activation of plasminogen in the host. Thus, a synthetic peptide substrate specific to the OmpT protease was designed and modeled for the purpose of detecting E. coli in biological sample.
Chapter 1. Detection of Thrombin Activity Directly in Whole Blood

Background on Thrombin

Thrombin is a serine protease that plays a key role in the common pathway of the clotting cascade to promote hemostasis following injury to the endothelium. Serine proteases are prevalent in the intrinsic, extrinsic, and common pathways of the coagulation cascade. Prothrombin is activated by Factor Xa, Factor V, phospholipid, and calcium to form the thrombin enzyme, which in turn converts the soluble plasma glycoprotein, fibrinogen, to fibrin. Thrombin in the presence of calcium activates factor XIII, which promotes the cross linking of fibrin polymers. The cross linked fibrin then acts a hemostatic plug to stop blood loss.

From a clinical perspective, in-vivo thrombin activity is linked to various blood clotting disorders, which ties thrombin to a variety of disease conditions in the human body (cardiovascular disease, DVT, arteriosclerosis, cancer, etc).

The thrombin enzyme is a pivotal component in thrombus formation, which arises due to an imbalance between the activity of procoagulant factors and anticoagulant pathways. The thrombin protease is an activator of platelets, Factor V, and Factor VIII, which all contribute to the coagulation cascade. Thrombin generation is dramatically reduced in patients with bleeding tendencies, while it is increased in patients with risk of venous thromboembolism. Therefore, various groups utilize the thrombin enzyme as a biomarker for deep venous thrombosis. It has been shown that the administration of thrombin inhibitors can treat and prevent various complications such as cerebral venous thrombosis and stroke.

Thrombin and fibrin generating molecular machinery are also shown to regulate inflammation associated with atherosclerosis. Various experimental studies in mice
suggest a correlation between hypercoagulability and increased risk of atherosclerosis. The plaque phenotype relating to atherosclerosis appears to be modifiable through the administration of anticoagulants targeting thrombin formation in mice\textsuperscript{6}. Thrombin's role in inflammation includes the production of monocyte chemoattractant protein-1, tumor necrosis factor, and IL-1 or IL-6 in fibroblasts and endothelial cells. Its expression also enhances leukocyte migration\textsuperscript{7}.

HIV patients experience a higher risk of cardiovascular disease that is associated with elevated plasma D-dimer levels, which is a fibrin degradation product post coagulation. This implies that thrombin levels should be higher in HIV patients. However, one group measured the endogenous thrombin potential in HIV-infected individuals and found that thrombin generation decreased as a result of elevated levels of anti-thrombin activity\textsuperscript{6}. Thus, the medical research community is interested in clarifying the relationship between thrombin activity and HIV.

Thrombin directly contributes to thrombin activatable fibrinolysis inhibitor levels (TAFI), which down-regulates plasminogen activation\textsuperscript{9}. TAFI expression is believed to be involved in the pathogenesis and progression of inflammatory bowel disease (IBD)\textsuperscript{9}. Another study revealed the parameters of coagulation and fibrinolysis, which includes the thrombin enzyme, generally increased in patients with IBD\textsuperscript{10}.

One known symptom of sickle cell anemia is coagulation activation, which is dependent on tissue factor expression and elevated levels of thrombin activity\textsuperscript{11}. It is believed that abnormal coagulation contributes to increased levels of inflammation and vascular injury in patients with sickle cell anemia\textsuperscript{6}. As a result of hypercoagulation, patients also experience an increased risk of venous thromboembolism\textsuperscript{12}. Thus, multiple groups are interested in the relationship between procoagulant activity, such as that of thrombin, and sickle cell disease.
Thrombin is also involved with cancer biology as the enzyme indirectly stimulates receptors on tumor cells to communicate with the adjacent microenvironment, which has been shown to direct melanoma growth and metastasis. The thrombin enzyme also enhances VEGF secretion, which is elevated in cancer patients. Research has shown that the administration of thrombin inhibitor, argatroban, helps to suppress cancer metastasis. It is also been described in studies that procoagulant states, say thrombin, are activated in cancer patients, such as those with osteosarcoma. Hemostasis disorder is also prevalent in patients with prostate cancer.

Studies suggest that renal failure leads to enhanced coagulation activation as a result of increased thrombin levels. In fact, anticoagulation therapy consisting of warfarin, heparin, or direct thrombin inhibitor administration, is frequently used to treat or prevent thromboembolic disorders in those with chronic kidney disease. Hypercoagulation, a function of thrombin and other procoagulants, along with endothelial dysfunction increases the cardiovascular risk of patients with chronic kidney disease. Thus, the ability to measure thrombin activity in patients with renal complications is of interest to those performing research in this field.

Thrombin is undoubtedly a pivotal component in multiple disease conditions as the enzyme directly affects blood circulation. Therefore, the ability to monitor thrombin activity in whole blood and in real time will allow researchers to further elucidate the relationship between the thrombin protease and disease.

**Current platforms to detect Coagulation activity**

Current methods to assess thrombin levels and coagulation activity include prothrombin time (PT), activated partial thromboplastin time (PTT), thrombin clotting time
(TCT), bleeding time, platelet count, platelet function testing, aptamer-based assays, immunoassays, and others\textsuperscript{20–30}.

The prothrombin time assay strictly looks at the extrinsic and common pathways of coagulation. Blood is drawn into a vacuum tube loaded with citrate (1 part anticoagulant per 9 parts blood), which chelates the calcium ions in the sample. The blood is then mixed and centrifuged to separate out the red blood cells from the plasma. Excess calcium is dispensed into the plasma sample to enable coagulation. Tissue factor III is introduced into the mixture and the clotting time, usually within a normal range of 10-13 seconds, is then measured optically.

The activated partial thromboplastin time assay strictly evaluates the intrinsic and common pathways of coagulation. Again, the blood sample is drawn into a vacuum tube loaded with citrate (1 part anticoagulant per 9 parts blood) followed by intrinsic pathway activation by adding phospholipids, a silica activator, and calcium. Thrombus formation time is then measured. The presence of coagulation factors I, II, V, VIII, IX, X, XI, and XII will yield normal PTT times ranging from 25 to 39 seconds.

The thrombin time assay is the simplest to perform. Blood is drawn into a vacuum tube loaded with citrate (1 part anticoagulant per 9 parts blood) followed by centrifugation. The plasma sample is then mixed with bovine thrombin, which results in clot formation. The thrombin clotting time is measured optically and should fall into a normal range of 10 to 15 seconds or 5 seconds within the control. Platelet activity testing is commonly performed as well as thrombin expression promotes platelet activation.

Aptamers are nucleic acid ligands that can be synthesized against proteins and other biomolecules. Evidence indicates that aptamers can act as the biorecognition elements for various biosensors. The thrombin-binding aptamer is of great interest in
this field and has been taken as a model system by multiple research groups. Using the thrombin-binding aptamer, competitive assays have been developed by immobilizing the aptamer or protein on beads followed by electrochemical measurement\textsuperscript{20}. Thrombin specific aptamers can also be used as affinitiy probes in capillary electrophoresis\textsuperscript{21}. At this point, aptamer-based diagnostics remain uncompetitive compared to antibody-based testing as operating conditions have yet to be established or optimized. Current aptamer based methods also require extensive sample preparation.

Immunoassays also measure the presence of specific analytes in solution. Using various protein separation techniques, the thrombin enzyme can be isolated from the rest of the sample. Western blotting can then be performed to probe for the “immunoreactive” thrombin enzyme using various antigen-specific antibodies. A measurable signal is then detected and quantified to assess thrombin expression. Immunoassays also require extensive sample preparation and can be rather time consuming.

A majority of these methods can only detect thrombin in serum or plasma as various components within whole blood cause strong background absorption, significant autofluorescence, and light scattering\textsuperscript{30}. Multiple methods can only detect thrombin indirectly by measuring early activation products. More advanced processes are either under-established for clinical use, require sample preparation, require complex equipment or cannot directly monitor thrombin activity in a point of care (POC) setting. Thus, it is necessary to develop a novel POC system to detect thrombin activity with minimal sample preparation and basic equipment.
Background on Electrophoresis

Electrophoresis is the movement of charged particles under the influence of an electric field. In biochemistry, gel electrophoresis is commonly performed to separate out proteins by charge and size, or DNA by length through molecular sieving mechanisms. A uniform electric field pulls positively charged particles toward the cathode and negatively charged particles toward the anode. The rate of travel depends on the mass of the molecule and the strength of the field. Larger molecules travel slower compared to smaller molecules, which results in the accumulation of identically sized particles in one band as the sample travels through a sieving medium. The gel usually consists of agarose, polyacrylamide, or starch. The gel composition and porosity is determined based on the target of interest. Small nucleic acids and proteins are usually separated in a polyacrylamide gel. Acrylamide before cross linkage is a potent neurotoxin. Polyacrylamide gels have a more uniform pore size distribution compared to agarose gels. Macromolecules are usually separated in agarose, which consist of long unbranched chains of uncharged carbohydrates. Electrophoresis takes place in buffer to minimize pH fluctuations as a result of the electric field. After electrophoresis, the gel is stained and the signal is quantified using a gel scanner.

Background on Peptides

Peptides are short polymers of amino acid monomers linked together by peptide bonds. Positively charged amino acid monomers include arginine, histidine, and lysine. Negatively charged amino acid monomers include aspartic acid and glutamic acid. A peptide bond (C(O)NH) is covalently formed between two molecules when the carboxyl group of one molecule reacts with the amino group of another. A water molecule is then released, which classifies this process as a condensation reaction. Peptide bonds can
be degraded by the addition of water, which causes amide hydrolysis, but the rate is very slow. The addition of enzymes specific to the peptide can significantly increase the cleavage rate.

Solid-phase peptide synthesis is a common way to construct peptides in the lab by linking together various amino acid monomers of interest. Small porous beads are first treated with linker molecules as a building platform for the peptide chain. The peptides are immobilized onto the beads. Thus, other liquid phase reagents and byproducts can be flushed away without removing the solid phase product, which is the peptide. The C-terminus of the first amino acid residue with a protected N-terminus is anchored to an insoluble support. Fmoc deprotection is then performed where the Fmoc protecting group is removed from the N-terminus by treating the sample with 20% piperidine in DMF. An excess of the second amino acid is introduced and the coupling reaction occurs. Excess reagents are removed by washing and the protecting group is removed from the N-terminus of the dipeptide prior to the addition of the third amino acid residue. The entire process is repeated until the desired peptide sequence is achieved. Trifluoroacetic acid is finally added to cleave the peptide from the resin and also remove any side-chain protecting groups. Mass spectrometry and Reversed phase high performance liquid chromatography (HPLC) are performed to verify peptide composition to illustrate peptide purity.

Proven Platform for Protease Detection

Lefkowitz, RB demonstrated the use of an electrophoretic platform for the detection of chymotrypsin, trypsin, elastase, MMP2, and MMP9 enzymes directly in whole blood. The platform consisted of a charge-changing fluorescent peptide substrate specific to the protease of interest and a polyanionic focusing gel. The
cleavage of the charge-changing fluorescent substrate by the target protease produced a positively charged fluorescent fragment, which can be separated from biological impurities and concentrated using electrophoresis. A clear signal was then captured and quantified. This platform only required a few microliters of blood with minimal sample preparation and the detection limit ranged from 100 to 200 ng/mL. Thus, this proven platform was adapted in a novel system to measure thrombin activity.

**Thrombin Charge-changing substrate platform**

A thrombin-specific charge changing peptide substrate was designed and synthesized according to sources investigating protease specificity. Negatively charged amino acids were positioned throughout the peptide sequence to engineer a substrate that will yield two oppositely charged fragments after thrombin cleavage between P1=Arginine and P1’=Glycine. The positively charged fraction was labeled with a fluorophore. The peptide sequence, acetyl-N-DD(Val)TPRGSAGAG-NH$_2$, was designed specifically for the thrombin enzyme. The contents of whole blood, which include red blood cells and other debris, are predominately negatively charged. Thus, both fractions can be separated and focused using electrophoresis. The engineered substrate in combination with an electrophoretic gel format established the foundation of the system.

**Thrombin Specific Peptide Substrate Experiments**

**Materials**

A thrombin-specific substrate of the following sequence, acetyl-N-DD(Val)TPRGSAGAG-NH$_2$, was synthesized by Aapptec (Louisville, KY, USA) and
Bodipy FL-SE (Invitrogen, Carlsbad, CA, USA) was then labeled onto the glycine residue’s amine group. The labeling was achieved by reacting equal volumes of the substrate (10mg/mL) in 100mM NaHCO₃ (pH 8.2), with 10mg/mL of the fluorophore in DMSO for 1 hour. The labeled product consists of the following sequence, acetyl-N-DD{Nle}TPRGSAGAG(e-amino-BodipyFL)-NH₂. Human plasma thrombin (2,955 units/mg), calcium-chloride dihydrate (C3881), and ethylenediaminetetraacetic acid were obtained from Sigma-Aldrich (St. Louis, MO, USA). Serum, sodium citrate (0.105M/3.2%), and sodium heparin (30 USP Units) vacuum tubes were obtained from BD Medical Supplies (Franklin Lakes, NJ, USA). Novex pre-cast 1-mm thick 20%T 2.6%C Polyacrylamide TBE gels and Novex gel cassettes (for casting custom 1-mm thick gels) were both obtained from Invitrogen. Whole blood was obtained from four healthy human donors.

Methods

Determining the detection limit of thrombin in 1 x PBS

The starting concentration of the thrombin-specific substrate was 5mg/mL. The stock solution of the substrate was then diluted in 1x PBS (pH 7.8) to achieve a final concentration of 0.45mg/mL. A stock solution of thrombin (100 units/mL) was prepared in 0.1% BSA (pH 6.5). Nine equal aliquots of the thrombin-specific substrate (0.45mg/mL) were prepared in 1 x PBS (pH 7.8) with 56mM CaCl₂ present as a co-factor for thrombin activity. Eight incremental volumes of the thrombin protease were dispensed into respective thrombin-specific substrate aliquots to achieve the following enzyme concentrations: 30, 50, 100, 200, 300, 400, 500, and 600 nM. 1x PBS (pH 7.8) was dispensed into the ninth substrate aliquot to serve as a negative control. After an incubation period of 30 minutes at room temperature, the 6μL samples were then loaded
onto the Novex Polyacrylamide gels submerged in 0.5x TBE running buffer (44.5 mM Tris-Borate, 1 mM EDTA, pH 8.0). The samples were electrophoresed at 500 V for 10 min and the gel was imaged using a BioDoc-It M-26 transilluminator (UVP, Upland, CA, USA) at excitation and emission wavelengths of 302 nm and 500–580 nm respectively. The gel was scanned in a Storm 840 workstation (Molecular Dynamics, Sunnyvale, CA, USA) loaded with the ImageQuant v5.2 software using the following settings: fluorescence mode, high sensitivity, 100 mm pixel size, 1000 V photomultiplier tube with a 450 nm excitation filter and a 520 nm long pass emission filter. The image was then quantified with ImageJ (NIH) and the fluorescent signal as a function of thrombin concentration was acquired.

**Determining the detection limit of thrombin in human whole blood**

The detection limit was confirmed by measuring the activity of known concentrations of the thrombin enzyme in whole blood. Endogenous level of thrombin activity was eliminated by drawing human blood samples directly into sodium citrate (0.105M/3.2%) vacuum tubes. Sodium citrate inhibits blood coagulation by chelating calcium, which is a cofactor for the conversion of prothrombin to thrombin. The substrate solution (0.45mg/mL) was reacted with incremental concentrations of the thrombin enzyme or the negative control (1x PBS, pH 7.8). The final substrate concentration for each reaction was 0.45 mg/mL and the final enzyme concentrations were 0, 20, 50, 100, 200, 300, 400, 500, and 600 nM. After a 30 min incubation period at room temperature, the 6μL aliquots were loaded onto the gels, electrophoresed, visualized, and quantified as described earlier.
**Real time detection of thrombin in whole human blood**

Thrombin activity was measured over time in human whole blood. This measurement required the inhibition of thrombin activity at different time points, which was achieved by the addition of EDTA. EDTA is a strong chelator that binds to the calcium in the sample, which is a co-factor for the thrombin activity to occur. Blood samples were collected from three normal donors and one donor who took aspirin on a daily basis. The substrate solution (0.45mg/mL) was reacted either with human whole blood samples drawn into serum tubes or the negative control (1x PBS, pH 7.8). The thrombin activity of each sample was then inhibited with EDTA at different time points from 0-36 min (3 min in between each time point). 6μL of each sample was then loaded onto the gel, electrophoresed, visualized, and quantified as described earlier.

**Specificity of the thrombin substrate**

The specificity of the substrate was tested by drawing human whole blood into sodium citrate (0.105M/3.2%) and sodium heparin (30 USP units) tubes for analysis. Sodium citrate and heparin work as inhibitors for the thrombin enzyme. The blood samples were then allowed to react with the substrate (0.45 mg/mL) for 30 minutes. Upon completion of the reaction, EDTA was then added to all the samples to fully inhibit additional thrombin activity. 6μL of each sample was then loaded onto the gel, electrophoresed, visualized, and quantified as described earlier.

**Thrombin Peptide Experiment Results**

The substrate in Table 1 was developed for the detection of thrombin activity in whole blood. The substrate has a net charge of -1. Upon cleavage of the substrate at
the Arginine-Glycine bond by the thrombin enzyme, two peptide fragments are generated. One fragment was fluorescently-labeled with a net charge of +1 and the other fragment was not labeled with a net charge of -1. Utilizing a simple micro-electrophoretic format, the positively charged components were then separated from the negatively charged components of blood (red and white blood cells, plasma proteins, etc) and quantified using a fluorescent detector. Figure 1 shows a schematic of substrate cleavage and Figure 2 shows the separation of components by electrophoresis.

Table 1. Substrate Sequences

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence (N-terminal to C-terminal)</th>
<th>Net Charge</th>
</tr>
</thead>
<tbody>
<tr>
<td>Substrate</td>
<td>Acetyl-N-D’D’-{Nle}-TPR’/GSAGAGAG- diamino-ethyl- Bodipy-FL</td>
<td>-1</td>
</tr>
<tr>
<td>Cleaved</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fragments</td>
<td></td>
<td></td>
</tr>
<tr>
<td>N-terminal</td>
<td>Acetyl-N-D’D’-{Nle}-TPR’-O’</td>
<td>-1</td>
</tr>
<tr>
<td>C-terminal</td>
<td>H₃N’-GSAGAGAG- diamino-ethyl-Bodipy-FL-NH₂</td>
<td>+1</td>
</tr>
</tbody>
</table>
Figure 1. The blood sample is combined with the substrate. If the specific protease is present in the blood sample, cleavage of the substrate occurs. After cleavage, two fragments are generated; one positively charged fragment tagged with a fluorophore, and one negatively charged fragment. The substrate sequence in this figure above is specific to the thrombin enzyme.

Figure 2. Electrophoresis of Sample. The substrate and blood sample are allowed to react for defined period of time in a reaction tube. In some cases, EDTA is added into the sample to stop the reaction (EDTA is a strong Ca chelator, a cofactor of many proteases). After reaction, the samples are then loaded onto a polyacrylamide gel. Samples are then electrophoresed for 10 minutes at 500V. Separation of the charged components is observed. After 10 minutes the cleaved product can then be quantified using a fluorescent detector.
The fluorescent peptide substrate and the thrombin enzyme were first reacted in 1x PBS to verify the substrate was being cleaved by the protease of interest. Various concentrations of the thrombin enzyme were reacted with the substrate in 1 x PBS for an incubation period of 30 minutes. The simple environment ruled out any impurities that may affect reaction process. Aliquots of the reacted samples were electrophoresed in a NOVEX 20% polyacrylamide gel. The gel in Figure 3 shows the general trend in band density as a function of decreasing thrombin concentrations in 1x PBS. Graph 1 shows the fluorescence as a function of thrombin concentration in 1x PBS.

Various concentrations of thrombin enzyme were then reacted with the substrate in human whole blood treated with sodium-citrate (0.105M/3.2%) for an incubation period of 30 minutes. The process was more reflective of the intended use of the assay. Aliquots of the reacted solution were electrophoresed in a NOVEX 20% polyacrylamide gel. Figure 4 shows the typical results obtained for determining the detection of thrombin in citrated whole blood. Graph 1 also shows the relationship between fluorescence and increasing concentrations of thrombin in citrated blood.

The preliminary data showed that the substrate was recognized by the thrombin enzyme. The reaction time of all time points was approximately 30 minutes. As the thrombin concentration increased, so did the enzyme activity, which resulted in a higher fluorescence. From the data shown above, the lower detection limit was less than 50nM thrombin. The upper limit however, was never reached in this study, but it was greater than 600nM. The curve of the citrated blood was comparable to the curve of 1x PBS.

For the negative control, minimal cleaved substrate was detected and the uncleaved peptide substrate migrated to the anode since it had a net charge of -1. The fluorescent band obtained from cleaved product was distinguishable from the negative control where no enzyme was present.
Figure 3. Gel with various thrombin concentrations in substrate and 1x PBS.

Figure 4. Gel with various concentrations of thrombin in citrated blood. Signals were visibly different from one another.

**Thrombin Standard Curves**

Graph 1. Thrombin in 1x PBS and Citrated Blood
After assessing the detection limit of thrombin in 1x PBS and citrated whole blood, thrombin activity was measured as a function of reaction time. Blood samples were extracted from three different donors for this study. The blood was drawn and dispensed immediately into equal aliquots of substrate solution. Samples were allowed to react for increasing time intervals up to 36 minutes. As mentioned earlier, thrombin activity was inhibited at different time points by adding EDTA to the mixture. As the time increased, so did the signal as can be seen from left to right in Figures 5 and 6. All donors exhibited similar trends where fluorescence increases as the reaction time increases. Graphs 2, 3, and 4 shows the fluorescence as a function of increasing reaction time for blood samples collected from different donors. Cleavage rate can be assessed by calculating for the slope at the linear region of the graph. The signal eventually reached a maximum as all substrate molecules within the solution had been cleaved by the thrombin enzyme.

Blood was also drawn from a donor that takes aspirin on a daily basis and the thrombin detection process was performed on the sample. As reaction time increased, the signal also increased as shown in Figure 7. Graph 5 shows the fluorescence as a function of increasing reaction time for the aspirin blood sample.
Figure 5. Gel of Thrombin Activity Over Time for Donor #1. Samples of blood from Donor #1 reacting with the thrombin substrate for different time durations.

Fresh Blood Thrombin Assay with EDTA (Donor #1)

Graph 2. Thrombin Activity Over Time for Donor #1.
Figure 6. Gel of Thrombin Activity Over Time for Donor#2. Samples of blood from Donor #2 reacting with the thrombin substrate for different time durations.

Graph 3. Thrombin Activity Over Time for Donor #2.
Graph 4. Thrombin Activity Over Time for Donor #3
Figure 7. Gel of Thrombin Activity Over Time for Aspirin User. Samples of blood from a donor taking a daily dose of aspirin reacting with the thrombin substrate for different time durations.

Graph 5. Thrombin Activity Over Time for Aspirin User
Normal Blood

![Normal Blood Image]

Aspirin Blood

![Aspirin Blood Image]

Figure 8. Comparison of Gels Between Normal Donor and Aspirin User

Graph 6. Thrombin Activity of Normal Patients vs Aspirin Users.
Figure 8 visually compares the gels between normal donors and the aspirin user. As shown in the Graph 6 above, the red data points stand for the average fluorescence (n=3) at different time points of normal blood donors. The blue data points stand for the fluorescence at different time points of aspirin users. There was a clear delay in the thrombin activity of the aspirin user as the linear region of the curve experienced a shift to the right. The data demonstrated the sensitivity of this assay.

In addition to the studies performed earlier, thrombin substrate specificity was verified by performing the following. Blood was drawn from the donor and directly into a Heparin vacuum tube (1 part heparin, 9 parts whole blood). The blood was mixed by gently inverting the vacuum tube back and forth. Heparinized blood samples were immediately drawn from the tube and dispensed into the substrate aliquots. Samples were allowed to react for different time intervals. Once again, the samples were then electrophoresed and the signal was obtained. Heparin directly activated the enzyme inhibitor, antithrombin III, which then inactivated thrombin. Thus, any signal that surpassed the baseline signal indicated that the substrate was being cleaved by non-specific enzymes within the solution. Figure 9 shows the loaded gel duplicates after electrophoresis. No signal was visually detected in the first eleven wells from left to right. Graph 7 shows the fluorescence representing the cleavage of the thrombin substrate by the heparinized blood over time. Only a baseline signal was detected, which suggested the thrombin substrate did not interact with non-thrombin enzymes within the blood sample.
Figure 9. Gel of Thrombin Activity Over Time for Heparinized Blood. Heparinized blood samples reacting with the thrombin substrate for different time duration.

Graph 7. Thrombin Activity Over Time for Heparinized Blood. As reaction time increased, the fluorescence did not exceed the baseline of approximately 2.5 million, which indicated that the substrate was specific to the thrombin enzyme.
Results from the normal blood, aspirin blood, and heparin blood samples were combined in Graph 8. Thrombin activity started at approximately 2-3 million fluorescence at 0 minutes. Thrombin activity in heparinized blood remained constant for 0 to 30 minutes of reaction time. The linear region of the curve for the aspirin blood clearly shifted to the right. Both normal blood and aspirin blood reached a plateau in activity after all the substrate within solution have been cleaved by the thrombin enzyme.

Graph 8. Thrombin Activity of All Groups Tested

The sensitivity of the thrombin-specific charge-changing fluorescent peptide ranged between 50nM to greater than 600nM. Due to a limited supply of the thrombin enzyme, upper limit intensities of this assay were never acquire. However, a majority of biomolecular assays are geared towards the detection of minute levels of target. Thus, additional work was not pursued in determining the upper limits of the system.
The fluorescence of the calibration samples for all runs performed were comparable. Thus, various parameters of the system, which include electric field strength, temperature, gel quality, and buffer composition, remained consistent throughout each run.

The trends exhibited by the assay made sense as enzyme activity was limited by substrate availability. Thus, all time related assays eventually reached a plateau in fluorescence. However, the slope of the curves in the linear region varied from donor to donor. This was expected as coagulation activity does vary among individuals.

**Conclusion**

In this study we designed and tested a charge-changing substrate specific to the thrombin enzyme. We demonstrated that thrombin activity could be measured directly in whole blood. Since thrombin activity is correlated to the rate of coagulation, we were able to identify differences in the thrombin activity between blood from normal donors and aspirin users (delayed clotting). The thrombin assay required no sample preparation, used only a few micro liters of blood, and can sensitively detect low concentrations of the enzyme (~100nM).
Chapter 2. Design and Testing of a POC Thrombin Detection Device

We successfully demonstrated the detection of thrombin activity in whole blood using our thrombin-specific fluorogenic substrate platform. The detection limit, sensitivity, and assay specificity was deemed acceptable. We intend to integrate this platform in a point of care device that is acceptable in performance and intuitive to use.

We wanted to design and develop a device that can satisfy a specific set of design inputs. Prior to the establishing the concept for the device, we went ahead and answered the following questions.

1. What is the real need for the new device?
2. Where will the new device be used?
3. Who will use the new device?
4. How will the new device be used?
5. With what devices will the new device be used?
6. How long will the new device be used?

There is a real need for a novel system that is able to directly detect thrombin activity in whole blood in real time. The device will be used in the field, research laboratories, clinics, and hospitals of developed and developing nations. It will be catered to researchers, laboratory technicians, and clinicians. We want minimal sample preparation, where the user can just dispense or draw blood directly into the device that will then perform the separation process with the flip of a switch. The system will be used in conjunction with other common phlebotomy equipment, which includes vacuum tubes, needles, tubing sets and so forth. The system consists of a single-use disposable cartridge and a reusable base that contains the circuitry. Ideally, the process should
only take 45 minutes and the cartridge is replaced after each use. However, all electrical
components in the base will be reused until failure.

After answering those fundamental questions, we also assessed other design
requirements. The entire thrombin detection system should weigh less than 15 pounds,
which will allow the operator to simply carry it over to the site. As this device may be
operated in an environment without wall power, say out in the field of a developing
nation, the user should still be able to power the device using highly accessible battery
sources. Due to the intended operating environment of the device, sample preparation
must be minimized as laboratory resources may be limited. The operation of the device
should be intuitive as some of our users may not be trained scientists. We envision the
thrombin detection process to be as simple as the prick of a finger followed by the flip of
a switch. Total processing time should be short where the user can extract critical
information on the patient’s thrombin activity as a function of concentration, reaction rate,
and etc. As a point of care diagnostic tool, process performance is definitely critical. We
project the total processing time to be within 45 minutes of blood draw. The analysis of
the signal will require some computing power, which can be fulfilled by a simple laptop
loaded with basic imaging software.

With these basic design inputs and requirements in mind, we designed and
tested two potential platforms for this system. One relied on a horizontally positioned
polyacrylamide gel encased in an acrylic channel. The other relied on a vertically
positioned polyacrylamide gel that was encased in a glass pasteur pipette tip. The
design and test procedures for each format is presented.
**Horizontal Gel Format Device**

To assess the feasibility of various electrophoretic formats for the separation and concentration of the cleaved substrate fractions, we tested two configurations. The horizontal gel format consisted of an acrylic gasket (1x0.5x0.125cm) bonded onto a silica microscope slide (2.5x7.5cm) using a medical grade UV adhesive (Dymax 1181, Dymax, Torrington, CT, USA). The gasket was designed using CAD and material was removed using the LaserCAM. The electrode assembly consisted of an acrylic gasket solvent bonded onto two other acrylic plates with dichloromethane to create two grooves for the placement of two platinum wires. Two holes in opposing corners were cut out from the acrylic gasket and electrode assembly using the LaserCAM. One pin (0.25" long) was installed in each hole for the purpose of aligning the electrodes to the acrylic gasket. The assembly is shown in Figures 10, 11, 12, and 13.

![Isometric View of the Device](image)

**Figure 10. Isometric View of the Device.** Color selection of components does not reflect actual colors on device
Figure 11. Top View of the Device. All values are in mm.

Figure 12. Side View of the Device. All values are in mm.
The gel ingredients were mixed together in a 15mL conical tube, with the exception of TEMED. TEMED was then added into the mixture to start the polymerization process. 500uL of the mixture was then immediately transferred over to the rectangular reservoir within the acrylic gasket. The gel polymerized and conformed to the geometry of the acrylic gasket. The various gel mediums and respective ingredients are listed in Table 2. Using an X-Acto knife, sections of the gel were physically removed to achieve the configuration in Figure 14. The gel membrane shown
in Figure 14 helps to reduce the electrochemistry between the biological sample and the platinum electrode.

Table 2. Recipes for Different Gel Mediums

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Volume for 15% Gel (µL)</th>
<th>Volume for 15% Doped Gel (µL)</th>
<th>Volume for 6% Gel (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distilled H₂O</td>
<td>350</td>
<td>300</td>
<td>580</td>
</tr>
<tr>
<td>40% Acrylamide (1:29, Acrylamide:bis)</td>
<td>375</td>
<td>375</td>
<td>150</td>
</tr>
<tr>
<td>1.5 M Tris (pH 8.8)</td>
<td>250</td>
<td>250</td>
<td>250</td>
</tr>
<tr>
<td>10% SDS</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>10% Ammonium Persulfate</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>TEMED</td>
<td>5</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Poly-L-Glutamic Acid (5mg in 50µL H₂O)</td>
<td>0</td>
<td>50</td>
<td>0</td>
</tr>
<tr>
<td><strong>Total Volume</strong></td>
<td><strong>1000</strong></td>
<td><strong>1000</strong></td>
<td><strong>~1000</strong></td>
</tr>
</tbody>
</table>

Figure 14. General Configuration of the Gel Medium. The normal configuration usually consisted of the anode being on the same side as the sample well. The cathode was usually positioned in the buffer well, which opposes the sample well. The gel membrane was not present in earlier experiments, specifically for ones that did not involve the usage of blood.
Each well was filled with 0.5x TBE running buffer. The two platinum wire electrodes were each connected to either the positive or negative terminal of three 9V batteries in series as shown in Figure 15. The entire setup in Figure 13 was placed in a BioDoc-It M-26 transilluminator (UVP, Upland, CA, USA) with a camera positioned on top of the chamber facing downwards as depicted in Figure 15. The test sample (10μL) was then loaded into the sample well. Closing the switch completed the circuit, which led to the generation of an electric field that separated the charged fragments.
**Figure 15. Horizontal Format Device Setup.** The device and battery sat in the dark chamber of the BioDoc-It transluminator unit. The excitation wavelength was emitted by the light source, which then traveled through the horizontal format device. The fluorescent dye that was attached to the charge-changing substrate fragment was excited by the light source and emits the emission wavelength. The filter only allowed the emission wavelength to pass through and the signal was then detected by the camera. Images were then analyzed by the computer.
Horizontal Format Device Testing Results

The first experiment involved the usage of a pilot device. The components of this device were similar to that in Figure 13. However, the channel size was significantly wider at 1cm x 3cm x 0.3175cm. It was designed as such for simple manipulation of the gel medium. A 15% gel was casted and various regions were carefully removed using an x-acto knife to create two reservoirs. Each reservoir topped off with 0.5x TBE. The thrombin substrate was first cleaved according to the instructions provided by the Thrombin CleanCleave Kit (Sigma Aldrich). Approximately 10uL of the cleaved substrate was then added to the sample well. The power supply was set at 27V to replicate the low voltage potential of multiple 9V batteries in series. As presented in Figure 16, a concentrated amount of cleaved substrate was dispensed into the sample reservoir that was preloaded with 0.5x TBE at 0 minutes. All fluorescent data was collected through a stationary reading window that focuses on the gel medium as shown in Figure 16. The fluorescence of the reading window was measured and analyzed at 0, 9, 20, and 55 minute time points. According to Graph 9, the fluorescence of the reading window increased with electrophoresis duration, which meant the cleaved substrate traveled into the 15% gel. The feasibility of the platform was confirmed by this run.
Figure 16. Preliminary Device Running Cleaved Substrate. The red circle outlines the reading window of the 15% gel.

Graph 9. Fluorescence Detected in Reading Window of Cleaved Substrate in TBE.
The next experiment implemented the use of the setup described in Figure 15. The channel width had been reduced from 1 cm to 0.5 cm for the purpose of reducing sample well volume. At 0 minutes, approximately 10uL of cleaved substrate sample was dispensed into the well. The device was powered by three 9V batteries in series to show that it can be done in a POC setting without wall power. According to Figure 17 and Graph 10, there was some movement of the cleaved substrate into the 15% gel at 20 minutes as reflected by a slight increase in fluorescence. Due to high background resulting from the UV adhesive used to bond the acrylic gasket to the glass slide, the gel was excised at 30 minutes from the device and visualized independently. According to the Figure 17 and Graph 10, there was definitely an increase in fluorescence at the reading window, which implied the positively charged fractions did migrate into the gel medium. This experiment demonstrated the feasibility of the setup, but the signal can be improved by performing additional modifications to the gel medium.

**Figure 17.** Redesigned Device Running Cleaved Substrate. The red circle outlines the reading window of the gel.
Graph 10. Fluorescence Detected in Reading Window of Cleaved Substrate in 15% Gel for New Channel.

In order to increase the fluorescent intensity of the band, the gel medium was modified or doped with 0.5% w/v poly-L-glutamic acid. The glutamic acid is negatively charged. Thus, its addition to the 15% polyacrylamide gel should increase the affinity for positively charged substrate fragments, which should improve the focusing ability of the gel. The poly-L-glutamic acid came in a 25mg aliquot from Sigma Aldrich. The 25mg of glutamic acid was resuspended in 500uL of distilled H$_2$O and equally divided into ten 50uL aliquots. One aliquot of glutamic acid was then added to the 15% gel mixture to achieve a total volume of 500uL for gel casting. In this particular run, a gel membrane, shown in Figure 14, separated the sample well from the platinum electrode.

As shown in Figure 18 and Graph 11, the cleaved substrate concentrated itself in the gel medium as reflected by a 5 fold increase in fluorescence between the 0 and 30 minute time points. It can be concluded that the system worked in a setting without any biological impurities.
Figure 18. Running cleaved substrate in 0.5x TBE and 15% Doped gel.

Graph 11. Fluorescence Detected in Reading Window of Cleaved Substrate in 15% Doped Gel.
The next experiment assessed the functionality of the device that was more reflective of its intended use. 10uL of whole blood was added to the 5uL of cleaved substrate to create a more complex biological mixture. Again, the gel membrane was present to reduce the electrochemistry between the blood content and the positive electrode. At 0 minutes, 10uL of the sample mixture was dispensed into the sample well as shown in Figure 19. The process was again powered by three 9V batteries in series to supply 27V total. At 15 minutes, the charged fragments began to accumulate in the gel medium near the gel/buffer interface as there was a 2-fold increase in reading window fluorescence as shown in Graph 12. At 30 minutes, even more cleaved substrate migrated into the 15% doped gel. All the negatively charged molecules, which include red blood cells and uncleaved fragments, traveled towards the positive electrode. The gel membrane prevented the negatively charged particles from reacting with the positive electrode. The increase in reading window fluorescence with increased separation time, as shown in Graph 12, proved the system was functional even if there were biological contaminants in the sample.
Figure 19. Running cleaved substrate and blood in 15% Doped Gel. The red circle outlines the reading window for the gel.

Graph 12. Fluorescence Detected in Reading Window of Cleaved Substrate in Citrated Blood at Various Time Points.
The next run mimicked the intended use of this device. Blood was extracted from a healthy donor and 10uL of blood was reacted with 5uL of thrombin-specific substrate. The samples were incubated at room temperature for a total of 45 minutes. After that, 10uL of the reacted mixture was loaded into the 0.5x TBE-filled sample well. At 0 minutes, the red blood cells blocked out the fluorescent signal in the sample well. Again, three 9V batteries in series supplied a total of 27V to the system. According to Figure 20 and Graph 13, the reading window fluorescence peaked early on at 5 minutes, which reflected the accumulation of fragments within the gel medium. By 15 minutes, the signal reached a maximum. The focused band at the edge of the 15% doped gel was visually distinguishable from its surroundings. Thus, device functionality was confirmed by this experiment.

Figure 20. Running reacted substrate and blood in 15% doped gel. The red circle outlines the reading window for the gel.
Graph 13. Fluorescence Detected in Reading Window of Substrate in Fresh Blood.
System functionality was proven in the last run. Thus, a control study was also performed. Figure 21 and Graph 14 presents the results of the following study. 10uL of heparinized blood was reacted with 5uL of the thrombin-substrate and the mixture was incubated at room temperature for 45 minutes. Again, heparin inactivated the thrombin, which meant that thrombin activity should not exist in the mixture. Thus, we expected to see all the substrate travel to the positively charged electrode. 10uL of the mixture was then loaded into the 0.5x TBE-filled sample well. The system was powered by three 9V batteries in series for a total of 27V. At 0, 17, and 27 minutes, the device was masked meaning only the 15% doped gel section was exposed to the fluorescent source. At 27 minutes, the masking was removed and a photo was taken. At 0 minutes, the mixture was added to the sample well. The reading window fluorescence remained rather constant throughout the entire electrophoresis process as shown in Graph 14. The photo of the unmasked device at 27 minutes of separation showed a very clear and bright band of uncleaved substrate that migrated into the gel membrane. Thus, this negative control further demonstrated the specificity of the thrombin substrate. It also verified the device was able to separate out cleaved and non-cleaved fractions in a predictable manner as shown in Figure 21.
Figure 21. Running reacted substrate and heparinized blood in 15% Doped gel. The red circle outlines the reading window for the gel. The yellow circle outlines the collection of negatively charged uncleaved substrate in the gel membrane.

Graph 14. Fluorescence Detected in Reading Window for Substrate in Heparinized Blood.
**Vertical Gel Format**

The vertical gel format consisted of a glass Pasteur pipette tip as the cartridge. The overall setup and fabrication process was much simpler in comparison to the horizontal gel format. First, the upper section of the glass Pasteur pipette was cut using a glass cutter. Sharp edges were then melted and smoothed out using a flame from a propane torch.

The vertical gel format enabled the casting of a low density gel directly on top of another higher density gel medium. First, the gel ingredients were dispensed into a 1mL centrifuge tube with the exception of TEMED. The TEMED was then added into the mixture to start the polymerization process and 10uL of the gel mixture was immediately transferred over to a 50uL centrifuge tube. The glass pipette tip was then dipped into the gel mixture and the solution was transferred into the lumen through capillary action. Using a gel loading pipette tip, a layer of distilled water was dispensed directly on top of the gel to create a flat uniform surface. After polymerization, the water layer was carefully removed.

The stacking gel ingredients were added into a 1mL centrifuge tube with the exception of TEMED. TEMED was then added and 2uL of that mixture was loaded directly on top of the running gel medium. 2uL of distilled water was carefully dispensed on top of the stacking gel. After polymerization, 0.5x TBE was added into the upper well to keep the gel hydrated. Refer to Figure # for the configuration.

The gel loaded glass pipette tip was placed vertically on a stand. The bottom end of the pipette tip was submerged into a buffer well loaded with 0.5x TBE, which acted as the conductive medium. A platinum electrode was fixed onto the floor of the buffer well. A platinum electrode was also submerged into the upper reservoir of the pipette tip, which was topped off with 0.5x TBE. The upper electrode was connected to
the anode of the power supply, while the lower electrode was connected to the cathode as shown in Figure 22 below. The power settings were adjusted in each experiment.

The metal stand, gel-filled glass pipette tip, platinum electrodes, and buffer reservoir were placed in a BioDoc-It M-26 transilluminator (UVP, Upland, CA, USA) that was tipped over sideways with the camera positioned on the right side of the chamber facing the setup as depicted in Figure 23. A test sample of less than 3uL was dispensed into the upper reservoir. Activation of the power supply generated an electric field that pulled positively charged particles into the gel and negatively charged particles away from it.

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**Figure 22. Vertical Gel Format Configuration**
Figure 23. General Setup for the Vertical Gel Format Device. The dark chamber or the BioDoc-It transluminator, was tipped over sideways. The UV lamp emitted an absorption/excitation wavelength of 302 nm. As a result, the fluorophore emitted a wavelength of 500-580nm. The filter only allowed emission wavelengths to pass through and the signal was captured by the camera that was connected to a computer. Under normal operating conditions, the positively charged substrate fragments should travel into the gel, while negatively charged substrate fragments remain suspended in the upper reservoir.
Vertical Format Device Testing Results

The first run was performed to test the initial concept of the device and to ensure a signal can be acquired by the system. A 15% gel was casted in a glass pipette using capillary action. The upper reservoir was topped off with 0.5x TBE. The tip of the glass pipette was submerged into the lower buffer reservoir. A total of 3uL of uncleaved substrate was dispensed carefully into the upper reservoir. The results of this experiment are shown in Figure 24. At 0 minutes, the substrate hovered over the top of the 15% gel. The power supply provided 40V to the system. At 15 minutes, the sample reached the detection window and generated a fluorescent signal presented in Graph 15. Due to high residual levels of free floating fluorophore in the sample, the upper well had a detectable amount of background. The fluorophore had a neutral charge and remained unaffected by the electric field. However, the electrophoresis system did manage to focus the charged fractions within the gel, as demonstrated by the 1-fold increase in the fluorescence at 15 minutes in comparison to the control at 0 minutes.
Figure 24. Running negatively charged substrate through the 15% gel under reverse polarity. The red circle outlines the reading window of the gel.

Graph 15. Fluorescence Detected in the Vertical Gel Reading Window for Substrate Under Reverse Polarity.
Another experiment was performed to ensure that fluorescent particles in general do not passively diffuse into the gel. In fact, all negatively charged uncleaved fragments should travel away from the gel. A total of 3µL of uncleaved substrate was carefully dispensed into the upper reservoir. At 0 minutes, the substrate settled directly on top of the 15% gel. Samples were then electrophoresed at 40V for 15 minutes. At 15 minutes, the substrate did not enter the gel. The fluorescence detected at the reading window remained constant from 0 to 15 minutes as shown in Graph 16. The loose fluorophore remained in suspension on top of the 15% gel. As hypothesized, the system was able to manipulate the flow of charged particles in a controlled manner.

![Image of the gel showing the flow of charged particles](image)

**Figure 25.** Running uncleaved substrate through the 15% gel under normal polarity. The red circle outlines the reading window of the gel.
The thrombin substrate was cleaved according to the instructions provided with the Thrombin CleanCleave Kit (Sigma Aldrich). 5uL of cleaved and uncleaved thrombin substrates were loaded onto a NOVEX 20% polyacrylamide gel. The samples were then electrophoresed at 500V for 10 minutes. A clear signal was detected in the lane loaded with the cleaved substrate sample as shown in Figure 26 and Graph 17. The lane with the uncleaved substrate sample served as a negative control. Thus, we confirmed cleavage of the thrombin substrate in the stock solution, which was reserved for other downstream experiments.
Figure 26. Confirmation of cleaved thrombin substrate (T3) sample.

Graph 17. Fluorescent Signal of the Cleaved and Uncleaved Substrates.
In an effort to improve the focusing of the fluorescent band for a cleaner read, a thin layer of 6% gel medium was added on top of the 15% gel with a gel loader tip. Theoretically, the substrate fragments should sift through the lower density gel quickly and line the surface of the 15% gel to create a tighter band. At 0 minutes, 3uL of thrombin substrate was dispensed carefully into the upper reservoir. A total of 40V was applied to the system and substrate began to travel into the 6% gel by 10 minutes (not shown here). By 45 minutes, a distinct band accumulated within the 15% gel, which resulted in strong signal shown in Figure 27 and Graph 18. However, the band remained scattered and neutral fragments lingered in the upper reservoir.
Figure 27. Running uncleaved substrate in a 6%/15% gel in reverse polarity.

Graph 18. Fluorescent Signal Detected in Reading Window of Substrate in 6%/15% Gel, Reverse Polarity.
The previous experiments demonstrated the ability of this system to manipulate the unidirectional migration of charged particles. Cleaved substrate, composed of positively and negatively charged fragments, in 0.5x TBE was then used to test the bi-directional separation of particles, which was more reflective of the system's intended use. Again, a thin 6% stacking gel (1.5uL) was dispensed directly on top of the 15% gel with a gel loader tip. The following results are shown in Figure 28. At 0 minutes, 3uL of the cleaved substrate sample was loaded into the upper reservoir. The power supply was set to 40V. At 30 minutes, the cleaved substrate scattered out, but traveled very slowly into the 6% gel. Due to the low migration speed, the power supply was ramped up to 90V at the 35 minute time point to increase the overall electric field strength. Electrophoresis was stopped at 45 minutes. Due to high background, it was hard to distinguish whether or not the charged fractions were in the gel. Thus, the sample in the upper well was extracted and 10uL of 0.5x TBE was added back into the well. Figure 28 and Graph 19 shows that fluorophore was detected in the reading window at 45 minutes after rinsing. The combination of positive and negative fractions in the same solution resulted in some charge confusion. Thus, the separation and migration speeds significantly decreased. One mitigation was to increase the electric field strength by ramping up the voltage powering the system.
Figure 28. Running cleaved substrate in a 6%/15% gel in normal polarity.

Graph 19. Fluorescent Signal Detected in Reading Window of Cleaved Substrate in 6%/15% Gel.
As evidenced by the previous experiments, the vertical platform device functioned for simple samples. Fresh blood was used in this next run to mimic the intended usage conditions of the device. Blood was collected from a healthy donor into a vacuum tube. 3.5uL of blood was then immediately dispensed into 4.2uL of thrombin substrate. The samples were allow to incubate at room temperature for 45 minutes. This time, a thin 6% stacking gel (1.5uL) was dispensed directly on top of the 15% polyacrylamide gel doped with poly-L-glutamic acid (0.5% w/v). The following results are shown Figure 29. At 0 minutes, 2uL of the mixture was carefully loaded into upper reservoir that was pre-filled with 0.5x TBE. After running the gel at a high voltage of 400V for 45 minutes, positively charged substrate fractions migrated into the 15% doped gel to yield a signal at the reading window shown in Figure 29. Graph 20 shows a 3-fold increase in reading window fluorescence at 45 minutes compared to 0 minutes.

Figure 29. Running substrate in fresh blood through a 6%/15% doped gel at higher voltage.
Future Direction

The horizontal gel format prototype device demonstrated the potential for using the current system for POC applications. The simplicity of the design allowed us to assess the overall concept of the system. We were able to power the device using three simple 9V batteries, which reflected the potential mobility of the setup. Thus, we can further develop this system to not only measure the coagulation characteristics of fresh blood, but also to use it as an IVD tool for other disease conditions.

Further development must be performed on the device to increase sensitivity, viability, and overall portability. Other electrode materials can be identified for the next generation device to reduce the overall cost of the setup. In an effort to reduce background, the next generation device will only expose a small region of the reading
window to the excitation wavelengths. More advanced fabrication techniques can be implemented in the near future to reduce the channel dimensions and complexity of the overall assembly by integrating the electrodes onto the channel itself using photolithography. We can also design and fabricate a more compact electronics foundation, where the camera will be replaced by a CMOS sensor and the current light source replaced by a small laser or hand held UV lamp. Thus, the dimensions of the dark chamber can be significantly reduced. The next generation device must also go through an optimization process to determine any relevant electrical, geometrical, and biological parameters or settings.

On the other hand, the vertical gel format deviated away from meeting the design inputs deemed necessary for a POC device. Longer processing times and the inability to effectively separate charged particles using a portable battery source limited the potential of this format. However, additional work can be performed in the near future to thoroughly assess and maybe overcome these limitations.

We will also design additional substrates for other proteases in the coagulation cascade. We intend to implement this system for various clinical research experiments to further elucidate the relationship between protease activity and disease.
Chapter 3. Bacterial Protease Detection

Expanding the Existing Platform for the Detection of Bacterial Protease

Various pathogenic outbreaks occur as a result of consuming contaminated food or water, which leads to serious food borne illnesses. The Centers for Disease Control and Prevention estimates that about 48 million food poisoning cases occur annually in the United States where at least 128,000 Americans are hospitalized and 3,000 actually die from the experience. The number of people affected worldwide is even greater. Food poisoning usually occurs as a result of poor handling, preparation, or storage of food and water. Common food borne bacteria include Salmonella, Escherichia coli, Campylobacter, Shigella and E. histolytica, which are all associated with acute bloody diarrhea\textsuperscript{3,4}. Therefore, it is necessary to develop a system that can rapidly detect the presence of such pathogens in food and water sources.

Cell surface proteases have been discovered on a variety of enterobacteria, such as Ompt in E. coli, PgtE in S. enteric, SopA in Shigella, and Pla in Y. pestis\textsuperscript{38–40}. Thus, the existing platform used to rapidly detect the serine protease, thrombin, within whole blood can be expanded for the detection of bacterial proteases as well. This theoretical system can be implemented in the food industry, clinical setting, or defense against bioterrorism. As there are various bacterial proteases of interest, we decided to first focus on the Omptin T surface protease, which is expressed on the surface of E coli. The gram-negative bacteria is widely used in biological research and certain strains, such as E. coli O157:H7, can cause severe abdominal cramps, bloody diarrhea, and vomiting\textsuperscript{44}.
Background on Omptin T Surface Protease

OmpT is a 33.5 kDa endoprotease present in E coli. The Omptin T (OmpT) surface protease of gram-negative bacteria was found to cleave antimicrobial peptides, degrade recombinant heterologous proteins, and activate human plasminogen. The role of this protease in bacterial virulence exceeds that of plasminogen activation, where the protease is related to the cleavage of various peptides that promote antibiotic activity.

The OmpT structure has been investigated by various groups. The protein is folded in a conserved 10-stranded β-barrel structure with extracellular loops that protrude out of the lipid bilayer. The active site of the enzyme is exposed on the surface of the bacteria, which allows it to interact with various substrates. The crystal structure is shown in Figure 30.

Figure 30. Crystal Structure of OmpT. Front and side views are shown. Figure was adapted from reference 42.
The OmpT enzyme employs a unique proteolytic mechanism that involves histidine and aspartate. In order to have any proteolytic activity, OmpT must be associated with lipopolysaccharide molecules, which are found in the outer membrane of gram-negative bacteria. Such a mechanism ensures the protein is only activated with it is transported from the bacterial cytoplasm to the outer membrane\textsuperscript{42}.

The OmpT protease has a narrowly-defined cleavage specificity. Its main substrate includes proteamine P1, which is a 50 amino acids long peptide where 24 of those amino acids are arginines. According to the Schechter and Berger nomenclature, P3-P2-P1-P1'-P2'-P3', cleavage occurs between P1 and P1'. It was found that OmpT requires a basic amino acid at the P1 position, whereas P1' can vary. The OmpT specificity was analyzed using various detection methods. Again, the arginine was confirmed to be a requirement in the P1 position. Lysine, glycine, or valine were confirmed in the P1' position. Valine or alanine was common for the P2' position and the P3 and P4 positions had a preference for Trp or Arg\textsuperscript{41}.

**Designing a Charge-changing OmpT Peptide Substrate**

In order to expand the current protease detection platform to probe for OmpT activity in biological samples, a new charge-changing peptide substrate must be designed and synthesized specifically for this enzyme. The substrate must meet several key requirements, which include specificity for OmpT, high sensitivity for OmpT as bacterial samples are less concentrated compared to thrombin in whole blood, and the entire substrate must have a negative net charge. The labeled fragment after substrate cleavage at P1-P1' must be positively charged. Ideally, the unlabeled fragment should be negatively charged so it can migrate away from the gel medium during
electrophoresis. The overall length of the peptide should be as short as possible to reduce any structural complications.

The first task was to identify an OmpT specific peptide, whether it be a synthetic or natural sequence, that is either negatively charged or close to neutral. Additional amino acid residues can be added to the N-terminus (amine group) and C-terminus (carboxyl group) to achieve the right charge configuration. The net charge of the entire substrate must be net negative. The N-terminus or the C-terminus must have the proper chemistry for labeling with a fluorescent dye. The fluorophore will be labeled onto the positively charged fraction terminal.

After going through multiple sources, the peptide configuration was narrowed down to the following sequences in Table 3. McCarter's group tested many synthetic and natural substrate sequences in their library using in situ cleavage of phage that display protease-susceptible peptides by E coli expressing OmpT and in vitro cleavage of phage-displayed peptides using purified enzyme. The peptide sequence was chosen based on the charge configuration and enzyme efficiency measured by \( \frac{K_{cat}}{K_m} \). \( K_{cat} \) measures the number of substrate molecules turned over per enzyme molecule per second. \( K_m \) measures the substrate concentration that is required for effective catalysis. The sequence of interest is highlighted in Table 3. Notice the \( \frac{K_{cat}}{K_m} \) for the synthetic peptide is significantly higher than that of the peptide sequence corresponding to the cleavage site of human plasminogen.
Table 3. Kinetic parameters for the OmpT-catalyzed hydrolysis of peptides corresponding to sequences selected by bacteriophage display methods. Material borrowed from reference 41.

<table>
<thead>
<tr>
<th>Phage clone and peptide no.</th>
<th>Amino acid sequence of peptide substrate</th>
<th>$k_{cat}$ (s⁻¹)</th>
<th>$K_m$ (µM)</th>
<th>$k_{cat}/K_m$ (M⁻¹ s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-3, 1</td>
<td>Ac-WGGRKR / RGGTk-NH₂</td>
<td>1.100 ± 0.70</td>
<td>150 ± 16</td>
<td>7.3 ± 0.2 x 10⁻⁶</td>
</tr>
<tr>
<td>5-4, 2</td>
<td>Ac-WGGRKR / RGGTk-NH₂</td>
<td>1.90 ± 0.19</td>
<td>37 ± 8</td>
<td>5.1 ± 0.1 x 10⁻⁶</td>
</tr>
<tr>
<td>4-12, 3</td>
<td>Ac-WGGRKR / RGGTk-NH₂</td>
<td>1.00 ± 0.14</td>
<td>6.8 ± 16</td>
<td>1.5 ± 0.5 x 10⁻⁶</td>
</tr>
<tr>
<td>4-20, 4</td>
<td>Ac-WGGRKR / RGGTk-NH₂</td>
<td>2.0 ± 0.14</td>
<td>28 ± 16</td>
<td>8.2 ± 0.4 x 10⁻⁶</td>
</tr>
<tr>
<td>4-16, 5</td>
<td>Ac-WGGR / RGGtk-NH₂</td>
<td>1.1 ± 0.1</td>
<td>25 ± 9</td>
<td>4.2 ± 1.5 x 10⁻⁶</td>
</tr>
<tr>
<td>4-28, 6</td>
<td>Ac-WGGR / RGGtk-NH₂</td>
<td>—</td>
<td>—</td>
<td>1.7 ± 0.1 x 10⁻⁶</td>
</tr>
<tr>
<td>Pseudogene cleavage site, 7</td>
<td>Ac-WGGR / RGGtk-NH₂</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

* Primary cleavage sites determined by ESIMS are indicated by the gaps in sequences (—). For substrates 4 and 7, conjugation could not be observed within the range of concentrations used and therefore $k_{cat}$ and $K_m$ values could not be calculated (—). The dimeric consensus residues are shown in bold; additional consensus residues are underlined.

The highlighted peptide with the sequence Ac-WGGK(+)YR(+) / R(+)AWGTR-NH₂ has a net charge of +3. Thus, 4 negatively charged residues must be added to the sequence to achieve a net charge of -1. Again, the peptide must be kept as short as possible to avoid complications in structure.

We suggest the following peptide sequence in Table 4 for the detection of OmpT activity in a biological sample. Aspartic acid, which is negatively charged, was chosen for the construction of the peptide as it is less bulky in comparison to glutamic acid. A majority of the negatively charged residues were added to the n-terminus since that will be the unlabeled end of the peptide. Such a configuration will yield a more positively charged labeled fraction and more negatively charged unlabeled fraction after proteolytic cleavage, which is optimal for electrophoresis separation.
Table 4. OmpT Charge-changing Peptide

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence (N-terminal to C-terminal)</th>
<th>Net Charge</th>
</tr>
</thead>
<tbody>
<tr>
<td>Substrate</td>
<td>Acetyl- D'D'D'WGGK'YR'/ R'AWGTID'-diamino-ethyl-Bodipy-FL</td>
<td>-1</td>
</tr>
<tr>
<td>Cleaved</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fragments</td>
<td></td>
<td></td>
</tr>
<tr>
<td>N-terminal</td>
<td>Acetyl- D'D'D'WGGK'YR'/O</td>
<td>-2</td>
</tr>
<tr>
<td>C-terminal</td>
<td>H3N'-R'AWGTID'-diamino-ethyl-Bodipy-FL-NH2</td>
<td>+1</td>
</tr>
</tbody>
</table>

In an effort to clarify the structure of the synthetic peptide substrate, modeling was performed using the RaptorX software developed by the Xu group. RaptorX is a protein structure prediction program that can also perform the alignment of distantly-related proteins with sparse sequence profile and that of a single target to multiple templates. The target peptide sequence was inputted into the RaptorX program and the computer generated various 3-dimensional structural models as presented in Figures 31, 32, and 33. The cleavage site consist of two arginine residues and is positioned at the center of the β-barrel. These models demonstrate that the peptide sequence will yield a substrate that is simple in structure with a highly accessible cleavage site.
Figure 31. CPK Space Fill Model of OmpT Substrate.

Figure 32. Cartoon Model of OmpT Substrate.
Figure 33. Ball and Stick Model of the OmpT Substrate.

Bacterial Protease Detection Procedure

The detection method for the OmpT protease will be similar to that used for thrombin detection in Chapters 1 and 2. The charge-changing substrate specific to OmpT will be dispensed into a biological sample, which can be derived from food and water sources, human waste, and even whole blood as shown in Figure 34. The samples will incubate at room temperature for a defined period of time. Samples are then loaded onto a gel device and positive and negative fragments can then be separated using electrophoresis. The resulting fluorescent signal can then be visualized and quantified to determine the bacterial protease activity. The corresponding enzyme concentration value can then be derived using a standard curve. The entire detection process should take no more than 1 hour and both detection platforms presented in Chapter 2 are applicable to this substrate as well.
Future Direction

Due to limited time and resources, the OmpT charge-changing substrate must be synthesized and tested at a later time. Various experiments must be designed and performed to confirm the specificity of the enzyme, to determine the consistency of the new assay, and to determine if the POC platform can detect bacterial protease levels directly in crude samples.
References


