MICROFLUIDIC PAPER ANALYTIC DEVICE FOR ASSESSMENT OF BLOOD COAGULATION

by

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Abstract

Monitoring blood coagulation while a patient is on cardiopulmonary bypass (CPB) is critical in preventing clots from arising in the bypass machine and consequently being sent into the patient’s bloodstream. Current methods used to monitor blood coagulation such as Activated Clotting Time (ACT) yield results that do not correlate coagulation time to heparin or protamine dosage and will typically take at least 400 s to yield a result that is safe to initiate bypass. Microfluidic paper-based analytical devices (μPAD) are advanced sensors based on a wide range of recently developed techniques for complex analytical methods. In this research, a point-of-care (POC) sensor was developed based on techniques adapted from lateral flow and μPAD. The effects of varied dosages of heparin and protamine were observed using this POC μPAD and an accompanying Raspberry pi-based monitoring device. Paper microfluidic channels were printed on nitrocellulose paper with a wax pattern. Human whole blood was added to an absorbent fiber glass sample pad preloaded with known amount of heparin or protamine. By having this absorbent pad on the inlet of the channel, the blood sample is able to travel through the channel via capillary flow. Significantly different (p < 0.05) rates of flow between blood samples with different doses of heparin and protamine show that the device can monitor the extent of coagulation and patient-specific responses to each drug. Thus a low-cost device was built that monitors the extent of blood coagulation and allows for individualized dosing of heparin and protamine in as little at 20 s and no more than 180 s.
Introduction

In cardiac surgery procedures requiring the use of cardiopulmonary bypass (CPB), maintaining blood fluidity in the circuit is vital. Monitoring the adequacy of anticoagulation plays a major role in maintaining sufficient CPB criterion. If inadequate anticoagulation occurs during surgery, major hemorrhagic and/or thrombotic events can arise\textsuperscript{1}. The current and most common method of assessing the adequateness of anticoagulation is the Activated Clotting Time (ACT) test\textsuperscript{2}.

In this age of precision medicine, it is vital that medical professionals managing complex cases and patients have the ability to frequently and accurately test for conditions that can lead to damaging clot formation or life-threatening excessive bleeding. The tools available to medical professionals are limited to those commercially available, which are often not designed for cardiac surgical needs. The specialized needs of cardiac surgical teams can be met by developing a POC \( \mu \)PAD that: is easy to use (to assist a multitasking perfusionist), provides rapid results, uses very small sample volumes (allows conservation of patients’ blood), and is extremely low cost (reduces hospital costs and barriers to commercialization). Successful application of this pilot device will lead to advanced capabilities able to test for more conditions such as other anticoagulants. Microfluidic systems are suitable for those developments since they can be designed to operate with small volumes of complex fluids with efficiency and speed but without the requirement for highly trained personnel.
Background

Cardiopulmonary bypass (CPB) was first utilized by Dr. John Gibbon on May 6, 1953. In 1813, a French physiologist, Le Gallois, suggested the concept of organ support through extracorporeal circulation. Le Gallois’ suggestion was followed up by von Frey and Gruber in 1885 who constructed an extracorporeal system that oxygenated blood. The discovery of heparin with its anticoagulant properties allowed the circulation of blood through a non-endothelium surface while avoiding the initiating of the coagulation cascade. In 1916, second year medical student, Jay McLean and physiologist William Howell studied the structures that controlled blood clotting. Howell isolated a fat soluble anticoagulant from canine liver tissue and formulated the term ‘heparin’ from the Greek prefix “hepar-” meaning liver. Despite the discovery almost a century ago, heparin is still the agent of choice for anticoagulation in cardiac procedures for many reasons; great quantities of anticoagulation can be quickly attained and the antidote, protamine, is readily accessible. A limiting factor of heparin is the requirement of cofactor, antithrombin (antithrombin III or ATIII) for its anticoagulation effect. Heparin forms a complex with ATIII which accelerates inhibition of coagulation factors IIa (thrombin), Xa, and inactivates IXa and XIa resulting in the halt of coagulation. Heparin is still used despite the development of newer anticoagulants and will be further discussed throughout this paper. The monitoring of anticoagulation and use of heparin is of vital importance - overdosing can result in life-threatening bleeding, while too low of a dose can cause thrombosis. If not monitored well, the CPB circuits can cause harmful thrombosis and/or the activation of the hemostatic inflammatory systems. Preventing intravascular thrombi and clot formation in CPB circuits are major goals in anticoagulation. Excessive bleeding following CPB is another major risk factor for effects after cardiac surgery.
Hemostasis

When there is vascular injury, normal hemostasis is the capability of the hemostatic system to control activation of clot formation and clot lysis in order to prevent hemorrhage without causing thrombosis.\textsuperscript{11}

The basic principles of normal hemostasis are\textsuperscript{11}:

- Two systems involved simultaneously in hemostasis: the procoagulant system and the fibrinolytic system
- The presence or absence of hemorrhage or thrombosis depends on a delicate balance between the procoagulant system and the fibrinolytic system
- Excess of procoagulants will result in thrombosis; too much activation of fibrinolysis will result in hemorrhage

Clotting factor biochemically interact with one another to strengthen the initial clot that is formed in the first part of hemostasis. The interactions result in the formation of fibrin clot which consists of loose mesh of strands that interlace with platelets to form a clot.\textsuperscript{12} Coagulation occurs via two different pathways: the extrinsic pathway and the intrinsic pathway. Whether the hemostasis system is activated by the intrinsic or extrinsic pathway or a combination, both the procoagulant and fibrinolytic systems are activated simultaneously, and the balance between the two systems will determine whether the patient will have normal hemostasis, bleeding, or develop thrombosis.\textsuperscript{13}

Extrinsic Pathway

The extrinsic pathway is faster (completed in a matter of seconds) and more direct than the intrinsic pathway. The extrinsic pathway is activated when damage occurs to the surrounding tissues, such as in a traumatic injury. Blood coagulation is believed to be predominantly initiated through the
extrinsic pathway through the presence of tissue factor (TF) together with factor VII. Damaged endothelial cells release tissue plasminogen activator (TPA), which activates plasminogen into plasmin and thus initiates the extrinsic fibrinolytic pathway.

*Intrinsic Pathway*

The intrinsic pathway is much slower than the extrinsic pathway and is activated by a number of factors including platelets, the small amount of thrombin produced by the extrinsic pathway, exposed subendothelium, or damage to the inside of the vascular system. The intrinsic pathway reactions involve Factor VIII, Factor IX, and Factor XI. These clotting factors and their associated biochemical reactions are responsible for sustaining coagulation once the cascade has started to generate thrombin. Factor XII is activated by subendothelium collagen, which initiates the intrinsic pathway and activates plasminogen into plasmin and thus initiates the intrinsic fibrinolytic pathway.

Once the coagulation cascade is activated, whether through the intrinsic pathway, the extrinsic pathway, or a combination of both, thrombin is formed. Thrombin cleaves soluble fibrinogen into fibrin monomers, which spontaneously polymerize to form protofibril strands that undergo linear extension, branching, and lateral association leading to the formation of a three-dimensional network of fibrin fibers. Figure 1 shows the synthesis of fibrin in blood clots involving either the intrinsic pathway or extrinsic pathway, both of which lead to a common pathway.
Figure 1.
Fibrin clot synthesis Cascade
Analytic Tests

Viscoelastic Test

In 1948, Hellmut Hartert presented the first thromboelastography or viscoelastic test\(^{18}\) which was later used by MacFalone and Biggs to measure the thrombin generation in blood.\(^{19}\) In thromboelastography, clotting in fresh blood is initiated with an activator of the contact pathway and inserted into a cup. A rotating torsion wire is introduced into the mixing cup which spins continuously at a fixed angle. A clot will form gradually and the strength will increase, dampening the torsion wire rotation until it becomes almost fixed which resembles maximal clot stability. Thromboelastography also identifies the gradual resolve of a clot resulting from fibrinolysis. There is criticism that the viscoelastic method is highly sensitive to external vibrations and lacks the ability to detect single factor deficiencies.\(^{19}\) Improvements were made for automation, and the addition of trigger reagents showed more insight to the extrinsic pathway, the heparin effect (heparinase test), and resistance to lysis (aprotinin test)\(^{20}\). During the mid-1980s, the viscoelastic method began to be adopted as a point-of-care test (POC), monitoring hemostasis during cardiac surgery and liver transplantations.\(^{21}\) \(^{22}\)

Thromboelastometry (TEM)

Presently, there are two semi-automated thromboelastometry instruments; ROTEM-analyzer uses a fixed cup with a rotating pin and TEG-analyzer that uses a rotating cup, similar to the classical method.\(^{23}\) The advantage of these techniques is that they have the potential to measure the clotting process, starting with fibrin formation and continuing through to clot retraction and fibrinolysis at the bedside, with minimal delays.\(^{23}\) The intrinsic activated rotational TEM test (InTEM) activates the contact phase of hemostasis. The outcome is partial to platelets, coagulation factors, heparin and fibrinogen. Low molecular weight heparin (LMWH) is only detected at higher concentrations.
**Thrombin Generation Time (TGT)**

In 1953, MacFarlene and Biggs measured thrombin in whole blood. In thrombin generation tests, a trigger agent is used to imitate wall damage of a vessel.\textsuperscript{24} The clotting times of fibrinogen solutions estimated the concentration of thrombin after measurement against a thrombin standard is known as thromboplastin generation time (TGT).\textsuperscript{28} TGT can give more information on hemorrhagic diseases. Thrombin (factor IIA) is a serine protease that acts as a coagulation activator/inhibitor and cellular regulator. Thrombin plays an important role in the conversion of fibrinogen to fibrin by activating factors V, VIII, XI, and XIII. When bound to a cofactor found in the endothelial cells, thrombomodulin (TM), the thrombin-TM complex activates protein C (anticoagulant) and promotes the fibrinolytic cascade by releasing plasminogen activators. Prothrombin, the precursor to thrombin, is synthesized in the liver and secreted into the blood circulation. When a vascular injury occurs, prothrombin gets activated through the coagulation cascade\textsuperscript{25}. A major disadvantage for TGT is that it is not suitable for emergency cases given the duration of the test () and because the level of standardization is not for clinical use.\textsuperscript{27} Recent studies have shown that risk of frequent venous thromboembolism (VTE) is associated with high thrombin generation potential.

**Prothrombin Time and Partial Thromboplastin Time**

Prothrombin Time (PT) measures the integrity of the extrinsic system and the common components, and Partial Thromboplastin Time (PTT) measures the integrity of the intrinsic system and the common components. The prothrombin test specifically evaluates the presence of factors VII, V, and X, prothrombin, and fibrinogen while PTT measures the integrity of the intrinsic system (Factors XII, XI, VIII, IX) and common clotting pathways. A prothrombin time within the 11 -15 second range (depends on the source of thromboplastin used) indicates that the patient has
normal amounts of the above clotting factors. Prothrombin time (PT), and partial thromboplastin time (PTT), are elongated in the presence of fibrinolysis due to the presence of an excess of plasmin which biodegrades factors V, VIII, IX and XI, or due to an excess of fibrinogen degradation products (FDPs) that act as an anticoagulant. FDPs inhibit platelet aggregation and prevent the normal cross-linking of fibrin, which is necessary to render clots insoluble. The elongation of PT and PTT due to fibrinolysis are not distinguishable from the elongation that reflects a defect in the intrinsic or extrinsic pathways.

Activated Clotting Time (ACT)
Activated clotting time was first introduced in 1966 by Dr. Paul Hattersley where he studied three patients with potentially life threatening coagulopathic disorders. These disorders were overlooked during pre-operative history but were detected by ACT. In 1975, Brian S. Bull introduced the notion of ACT in the clinical setting of cardiac surgery. ACT is now established as the most common point-of-care (POC) method to determine the adequacy of anticoagulation during cardiac surgery. The ACT works with whole blood that is added to test tubes or cartridges that are exposed to coagulation activators such as celite, kaolin or glass. The interaction with coagulation activators triggers the intrinsic coagulation cascade. When a clot is formed in the ACT it is representative of the plasma coagulation, red blood cells, and platelets found in the whole blood clotting assay. ACT is measured in the number of seconds it takes for a blood clot to form. Many factors can affect the ACT, such as deficiencies in essential factors, fibrinogen levels, platelet function, temperature and interaction with activation inhibitors. With the use of different coagulation activators, prolonged ACT values can be seen.
POC Testing Devices

There are numerous ACT devices that are available commercially which vary in a number of ways such as: contact activators, sensitivity, method of clot detection, sample size, etc. Most ACT assays detect clot formation by mechanical methods including the use of a mechanical plunger (ACT Plus, Medtronic, Minneapolis, MN) or the use of displacement of a magnet (Hemochron, ITC, Edison, NJ). Individual manufacturers provide multiple tests using a single platform to address different levels of anticoagulation. The HEMOCHRON Signature Elite Whole Blood Microcoagulation is a battery operated handheld that have disposable single use cuvettes (HEMOCHRON Jr.) that contains reagents. The test shows linearity at heparin concentrations ranging from 1.0 to 6.0 units of heparin per mL of blood and is not affected by high dose aprotinin therapy. The HEMOCHRON Jr ACT-LR contains Celite as its activator, making it more sensitive to heparin. HEMOCHRON Jr ACT-LR shows linearity at heparin concentrations of up to 2.5 units of heparin per mL of blood. POC testing of ACT was never intended to assess low levels of heparin anticoagulation and there has been a reported poor correlation between ACT and PTT.

Medtronic Hepcon HMS Plus System

The Hepcon HMS provides the visibility into actual heparin levels that ACT alone cannot. By contrast to the standalone HEMOCHRON Jr, the Hepcon HMS specifically measures heparin concentration. The Hepcon HMS enables surgical teams to dose, manage and neutralize heparin precisely and appropriately for each individual, helping prevent fibrin formation and preserve patient clotting factors. The Hepcon HMS uses an individualized heparin dose-response curve (HDR) to calculate a patient-specific heparin bolus dose to achieve a targeted ACT. An individualized HDR is calculated by mixing the patient’s blood with 0-, 1.5-, and 2.5-U/mL doses of heparin. Unfortunately, no prospective, randomized studies have been conducted to determine
the superiority of the Hepcon HMS versus ACT monitoring. When compared to conventional ACT methods for a CABG or a single-valve case with a cardiopulmonary bypass run of <2 hours, the Hepcon HMS can cost an additional $65 per patient or $10,000 per year for institutions.\(^{38}\)

**Monitoring during Cardiopulmonary Bypass**

Heparin is administered before CPB to prevent coagulation while the patient is exposed to the extracorporeal circulation by maintaining ACT between 400 seconds and 480 seconds. Appropriate anticoagulation is a critical component of maintaining hemostasis within the CPB circuit. An ideal ACT value during CPB is definitively not established.\(^ {39}\) Brian S. Bull popularized a concept in the 1970s of a “safe zone” where ACT ranges from 300 to 600s.\(^ {40}\) In practice, clinicians typically target a minimum ACT value of 400–480 seconds.\(^ {41}\) ACT is known to require re-testing often, due to common failures such as sample loading errors, missing the narrow timeframe to load the sample, or inconsistent results. Once CPB surgery is complete, the heparin is reversed with protamine. Occasionally, the calculated dose of protamine given may not be sufficient to reverse the anticoagulation effects of heparin, since protamine dose is calculated based on the initial heparin dosage, not patient response.\(^ {42}\) When the patient comes off the bypass circuit, the circuit is scavenged for blood and this is returned to the patient—referred to as ‘pump blood’. The process of returning pump blood may result in additional heparin administration, resulting in alterations to the patient’s haemostasis. There is a routine check of ACT performed in ICU after the infusion of the pump blood with the aim being an ACT of less than 150 seconds; the expected ACT value range is 120–140 seconds.\(^ {43}\) ACT is currently the test of choice for examining anticoagulation during bypass due to its ease of use and exclusivity of being a POC test.
Problem Statement

Unfortunately, there is limited research into which test is more accurate as it is difficult to measure or perform crossover studies in the critical care environment. There appeared to be limited evidence related to various tests for ACT and their efficacy, efficiency and cost benefits, which complicates decisions related to hospital protocols for measuring ACT at the point of care. While commercial devices and cartridges are available for POC measurement of ACT, they are costly ($6K-27K) and are mostly indirect assays, thus do not predict the true extent of blood anticoagulation for varying doses of heparin or protamine except for the costly ($27K) multi-well, high sample volume Medtronic Hepcon HMS Plus system.

Despite all of the noted tests and devices discussed, there is still a clinical need for a rapid, inexpensive assay that gives a complete analysis of coagulation with the ability to provide a patient-specific, direct dose response to heparin and protamine. Although individually the tests and devices have their own benefits, there is not a device encompasses all of the needs. A combination of the different devices seen will create unnecessary costs, unrealistic blood volumes needed and unrealistic times.
Hypothesis and Specific Aims

Hypothesis

The purpose of this study was to develop a nitrocellulose microfluidic paper analytic device that was able to monitor the extent of coagulation through rate of flow with various amounts of heparin and protamine. It is hypothesized as heparin concentrations increase, there is a decreased degree of coagulation therefore an increased rate of flow. Also, as protamine concentrations increase, there is an increased degree of coagulation therefore a decreased rate of flow.

Specific Aims

i. Design and create a microfluidic paper analytic device (μPAD). Develop methods to preload channels with different combinations of clot activators, heparin and protamine.

ii. Optimize the pore size of nitrocellulose paper and the channel width and length. Perform characterization tests for different viscosities, temperatures and humidity conditions.
   - Reduce blood sample consumption and the assay time without compromising assay sensitivity and reproducibility

iii. Monitor the impact of specific and exact heparin doses on the coagulation of human whole blood.
   - Add exact, known, and clinically relevant dosages of heparin to blood drawn from volunteers
   - Immediately use this blood as a sample for the lateral flow assay designed and to monitor assay progress with a Raspberry Pi based camera to determine the extent of coagulation based on each specific heparin dose through this channel based on samples with controlled levels of coagulation
iv. Monitor the impact of specific and exact protamine doses on the coagulation of heparinized human whole blood.
   • Add exact, known, and clinically relevant dosages of protamine to blood drawn from volunteers after it has been heparinized

v. Optimize heparin and protamine concentrations to be tested
   • Clinical relevance and limit of detection

vi. Optimize assay times for heparin and protamine. Perform analytic statistical tests for different time points for each solution
Microfluidic Paper Based Analytical Devices

Microfluidic paper based analytical devices (µPAD) and lab on a chip (LOC) are advanced sensors based on a paper substrate. LOCs integrate several laboratory functions onto a small platform and are typically only millimeters or centimeters in size.44 A LOC is essentially a network of channels and wells that are etched onto silicon or polymer substrates to build miniature laboratories. LOCs normally involve the usage of small fluid volumes (usually on the µL or nL scale).45 Pressure or electrokinetic forces move small volumes of liquid in a controlled manner through the channels and introduces the division of microfluidics that deals with the control and manipulation of fluids at the µm scale. Therefore, LOCs are sometimes referred to as microfluidic devices or microfluidic chips. The LOC enables sample handling, mixing, dilution, electrophoresis and chromatographic separation, staining, and detection on a single, integrated system. The main advantages of the LOC are ease of use, speed of analysis, low sample and reagent consumption, and high reproducibility due to standardization and automation.46

µPADs have gained increasing attention for advantages including biodegradability, biocompatibility, availability, and price. Table 1 shows the comparisons of traditional materials used in these devices.47
<table>
<thead>
<tr>
<th>Property</th>
<th>Paper</th>
<th>PDMS</th>
<th>Glass</th>
<th>Silicon</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Structure</strong></td>
<td>Fibrous</td>
<td>Solid</td>
<td>Solid</td>
<td>Solid</td>
</tr>
<tr>
<td><strong>Fluid flow</strong></td>
<td>Capillary</td>
<td>Forced</td>
<td>Forced</td>
<td>Forced</td>
</tr>
<tr>
<td><strong>Flexibility</strong></td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><strong>Surface-to-volume ratio</strong></td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><strong>Biocompatibility</strong></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><strong>Biodegradability</strong></td>
<td>++</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><strong>High-throughput fabrication</strong></td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><strong>Sensitivity to moisture</strong></td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><strong>Functionalization</strong></td>
<td>++</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td><strong>Spatial resolution</strong></td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td><strong>Homogeneity of the material</strong></td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td><strong>Disposability</strong></td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><strong>Price</strong></td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><strong>Low initial investment</strong></td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 1.
Paper as sensor substrate in comparison with traditional materials

note: PDMS = polydimethylsiloxane

**Microfluidic Paper Analytic Device**

Microfluidic paper analytic device (μPAD) is a type of LOC where the substrate is substituted with paper. Chromatographic paper based test has typically been used in lateral flow immunochromatographic assays, or lateral flow assays (LFAs), also known as rapid kits. Li et al recently published their work demonstrating the use of a LFA to monitor blood coagulation. Their findings support the concept that RBC flow is a valid visualization of the coagulative
processes reflecting the gradual formation of fibrin threads during the movement of RBCs, ultimately halting it. Furthermore, their data provided evidence that paper based coagulation monitoring provided superior linear detection ranges compared to a traditional fibrin clot-based timed test.

The major differences of μPADs from traditional LOCs are:

- Flow is spontaneous, via capillary flow through paper fibers, thus external pressure or electrokinetic force is not required
- Paper substrate can filter out larger molecules via the properties of the chromatographic filter paper.

Previously, μPADs had been fabricated using the same photolithographic methods used for conventional LOC fabrication. Recently, wax printing has been introduced as an easier fabrication method and is now widely being used. While wax printers were originally developed to generate high quality color images, they are equally strong at generating hydrophobic layers within the paper fibers to guide liquid flow through patterned channels and wells. μPADs are now considered an excellent alternative to both LOCs and LFAs, due to reduced sample and reagent consumption, lower power consumption (flow is spontaneous), low costs, user friendliness, and programmable assay (through microfluidic network of channels), etc.

**Paper Membrane**

The paper membrane is the most influential material used in a lateral flow assay (LFA). For LFA, the membrane must irreversibly bind capture reagents at the test or control lines. Chemical and physical characteristics of the membrane influence its capillary flow properties, which affects the reagent deposition, assay sensitivity, assay specificity, and consistency. The binding characteristics of the membrane are defined by its polymer composition. Commonly used polymers
and their binding characteristics are presented in Table 2. The electrostatic binding due to interactions between the strong dipoles of nitrate ester and those of peptide bonds (Fig. 2), means that nitrocellulose membranes are the most frequently used membranes.52

<table>
<thead>
<tr>
<th>Membrane Polymer</th>
<th>Primary Binding Mechanism</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nitrocellulose</td>
<td>Electrostatic</td>
</tr>
<tr>
<td>Poly(vinylidene fluoride)</td>
<td>Hydrophobic</td>
</tr>
<tr>
<td>Nylon (Charge-modified)</td>
<td>Ionic Electrostatic</td>
</tr>
<tr>
<td>Polyethersulfone</td>
<td>Hydrophobic</td>
</tr>
</tbody>
</table>

Table 2

Binding properties of different membrane polymers

Figure 2.
Structure of Nitrocellulose ester and protein dipoles52

Several qualities have resulted in nitrocellulose being the preferred substrate for lateral flow assays:53

- Nitrocellulose adsorbs protein at a high level
- Chemistries that compose the membrane wettable with aqueous solution do not significantly diminish protein adsorption
- Nitrocellulose membranes can be cast to have pores sufficiently large to allow lateral flow of fluid in a reasonable time
Sample Pad

The sample pad can have several functions, the most important of which is to evenly distribute the sample and transport the sample to other components of the LFA. The sample pad should be capable of transporting the sample in a smooth, continuous, and homogenous manner. The sample pad is usually permeated with buffer salts, proteins, surfactants, and other liquids to control the flow rate of the sample. Moreover, the pores of the sample pad can act as a filter in order to remove redundant materials, e.g. red blood cells.

Other characteristics that can be manipulated are:

- Increase sample viscosity to improve flow properties
- Enhance the ability of the sample to solubilize the detector reagent
- Prevent nonspecific binding of the conjugate and analyte to downstream materials
- Chemical modification of the sample to ensure immunocomplex formation at the test line

Commonly used materials for sample pads are cellulose and fiber glass. Sample application pads are sometimes designed to pretreat the sample before its transportation. Pretreatment may include separation of sample components, removal of interferences, adjustment of pH, etc.
Methods and Materials

Device Design

A Raspberry Pi-based device was designed to monitor the µPAD throughout the assay. Figure 3 shows the device, which consists of a Raspberry Pi 3, a touchscreen display, and a Raspberry Pi Camera housed in a 3D printed case. The Raspberry Pi is coded to capture images of the paper microfluidic chip at set time intervals (ex. 10 s, 20 s, 30 s, 40 s, 50 s, 60 s, 120 s, and 180 s). These images are automatically saved for analysis. The end goal is to have a stand-alone device that automatically analyzes the results, so the wax printed channels discussed later were designed with this purpose in mind.

Figure 3.
Assay monitoring device design and components

Evolution of Chip design

As noted earlier, the Li et al\textsuperscript{57} published paper in 2014 provided the preliminary design basis for this project, therefore similar channels was conceived here. The first chip design was a scalpel cut version and had dimensions of 3 mm x 50 mm in length with a sample pad size of 3 mm X 5 mm.
However, blood flowed up the edges of the strips because of the inconsistency in flow due to scalpel cut edges opening up the pores of the nitrocellulose paper, an alternative method of fabrication was needed. The development of the wax printed pattern was adopted because of its hydrophobic ability and the precision possible in channel fabrication, preventing problems with blood flow at the channel edges observed with previous methods. We were able to see a more consistent flow of blood within the channel. The wax printed channel, however, did not show a uniform red blood cell front apparently due to excess sample size of 14 μL. The final wax printed chip had optimized dimensions in order to minimize the blood sample volume needed to flow through the channel. With this final wax printed version, consistent separation of red blood cells and plasma was observed. While the distance traveled by the plasma front is our major parameter of interest, blood separation means that the nitrocellulose paper is in fact filtering the blood components as expected. Figure 4 depicts the evolution of the chip design as well as the respective blood flow on each chip.

Figure 4.
Chip design progression
Channel Design

Proper cutting of the nitrocellulose channel was vital to the development process and the lateral flow test. Jagged cutting can lead to uneven edges and increased pore sizes along the cut edge, resulting in flow that is also irregular because flow will want to go through the path of least resistance. Limitations due to cutting the paper membrane were the first obstacles noted when beginning this project. Wax pattern printing was proposed to overcome these limitations. Optimization of the fluidic circuit was done with regards to reliability and repeatability of the fluid flow. Different fabrication methods were investigated for their impact on the flow as well. Many experiments were conducted in order to determine the variation of the wax spreading. We found that there was a standard deviation of about 2 pixels (in an approximately 8 pixel wide channel) when comparing flow through multiple channels under the same melting and time conditions. The concept of pretreating the sample pad with heparin/protamine is another factor considered but it could lead to variations of reagent concentration in the material itself.

Reliability and repeatability are important factors for analytical devices, particularly for the market admission of medical products\textsuperscript{59}. They can only be assessed with constant assay results and without defective products. In order to have consistent test results for paper based microfluidic analytical devices, constant flow characteristics over all fabricated devices are required. The optimization of the channel design has a great impact on the repeatability and reliability of the microfluidic circuit. Many small deviations have been explained by human error (i.e. sample pad misaligned) which can lead to poor/no flow or flow observed outside of the channel (leaking). A solution to this problem was the development of the 3D printed stamper. Having this stamper apply all 8 sample pads allowed for even force onto the inlet of the channels and consistent sample pad placement. It was also observed that channels that were not fully melted appeared to have very
leaky blood flow which impacted the assays therefore a minimum amount of 60 seconds of melting time was implemented.

**Chip Fabrication Preparation**

Fabrication of all chips in this study was done using Millipore HF075 nitrocellulose membrane as the analytical membrane in the test strips on which blood coagulation took place. Millipore HF180 nitrocellulose membrane was tested and found to have a less reproducibility of blood separation, therefore HF075 was the preferred membrane. The HF075 membrane used in this study has a capillary water flow rate of 0.5 mm s\(^{-1}\) (4 cm per 75 sec), which is within the range specified by the manufacturer.\(^5\) Using SolidWorks (Dassault Systemes®), the chips were designed with eight channels per chip to allow for multiple, simultaneous assays. For a computer to automatically separate blood from the printed channel, a contrast in color is required. The required absence of red color in the channel outline results in the use of channels with a teal color (teal component values = 0 red + 255 green + 255 blue). All chips were fabricated in batches of 10 chips (8 channels in each chip) per sheet. The channels were printed on the membrane paper using a solid ink printer with solid wax ink (Xerox ColorQube 8570). After cutting chips individually, all channels were introduced to a hotplate (Corning PC-420 Stirrer Hot-Plate) for 60 seconds at 65°C to allow for uniform melting of the pattern into the nitrocellulose membrane. Millipore G041 glass fiber conjugate pads were machine cut into 4 x 4 mm grids. G041 was chosen as the sample pad due to its high porosity permitting plasma and blood cells to flow easily. Different fluids were evaluated for their flow and separation behavior, in order to generate data to calculate the channel length. The chip design was optimized with regards to size minimization, complexity of fabrication, and minimal sample size. Figure 5 shows a sample of the chip design that is used during the assays.
Heparin Solutions

Heparin solutions were prepared utilizing dilution methods. Due to its similar characteristics to human plasma (its content of electrolytes, osmolality, and pH), plasma-lyte A (Baxter Healthcare Corporation) was used to dilute heparin sodium injection (Sagent). Due to similar concentrations used in clinical settings, the solutions were made into 0, 2, 4, and 6 USP/mL (for average 70 kg, this correlates to 0, 9000, 18000, 27000 USP, respectively) from the supplied 10,000 USP/mL heparin solution. Each solution was vortexed for at least 60 seconds in order to ensure homogenous distribution of the mixture.

Protamine Solutions

For a normal 70 kg patient, a table dose of heparin would be approximately 300 USP/kg, which equates to 21000 USP administered to the patient, in turn this will show approximately 3 USP/mL in the blood circulation. A concentration of 3 USP/mL is the same amount of heparin that is added to our vials once blood is drawn. The protamine solutions were diluted to 0, 2, 4, 6 mg/mL (once dried, 0.0, 0.016, 0.032, 0.048 mg on sample pads, respectively). For a normal 70 kg the standard to reverse heparin is approximately 1 mg/100USP, which equates to 210 mg needed to reverse the heparin. In turn, this calculates as approximately 0.038 mg/mL of protamine needed to reverse
heparin. Since the protamine solution is being absorbed into a sample pad, excess protamine is needed in order to ensure the blood is saturated with protamine. Protamine solutions were also prepared utilizing dilution methods. Similar to heparin solutions, plasma-lyte A was used to dilute protamine sulfate salt derived from salmon (Sigma). The solutions were made into 0, 2, 4, 6 mg/mL (once dried, 0.0, 0.016, 0.032, 0.048 mg on sample pads, respectively). Each solution was vortexed for at least 60 seconds in order to ensure homogenous distribution of the mixture.

**Blood samples**

*Purchased blood*

Sodium heparinized human whole blood (Innovative Research) containing 158 USP Heparin was originally purchased for the assays. The process to receive the blood was extensive and would take on average 1-2 weeks. Once the blood was received, there were visible clots and the integrity of the blood was questioned. When assays were performed, reproducibility and blood separation were not achieved. Due to inconsistency in delivery and product quality, heparinized human whole blood from a different vendor (Zen bio) was used. Amount of heparin in each blood vial was not provided, however, the amount of heparin affected the protamine assays in that made it difficult to perform assays.

*Volunteered blood*

In order to overcome the inadequacies of vendor supplied blood, whole blood was drawn from healthy volunteers according to the University of Arizona Institutional Review Board (IRB project no. 1612094853) guidelines. Blood draws were limited to one volunteer per week and were only drawn by a Certified Medical Laboratory Scientist. 10 mL of blood was drawn from the vein of the volunteer and divided evenly into one heparinized test tube and one unheminized test tube (Vacuette® Tube). Assays commenced immediately after a sample was taken.
**Heparin Assay Procedure**

The developed assay was prepared in a reproducible manner. Following the completion of preparations of the chips and solutions as noted previously, the chip was placed underneath the Raspberry Pi camera. Approximately 30 min before blood is drawn, 8 µL of each heparin concentration (0, 2, 4, 6 USP/mL) was preloaded onto a fiber glass sample pad housed on a 3D printed stamper and allowed to be fully dried. Once the blood was drawn into a test tube, the blood was pipetted into 8 wells of a 36 well plate. Once filled, an eight-tipped multi-pipette (ThermoFisher Scientific) was used to transfer the blood from the 36 well plate onto the sample pads. Once blood was introduced to the sample pad it was then incubated for 2 minute. The stamper was then flipped onto the inlet of the channels to begin the assay. To document the results of the experiments, a custom coded device (Raspberry pi) was initiated to capture and save a set number of images at set time intervals (ex. images at 10, 20, 30, 40, 50, 60, 120, and 180 s). Figure 6A depicts an outline version of heparin assay procedure and figure 6B depicts the use of the stamper as noted earlier.

**Protamine Assay Procedure**

The assay was prepared in a manner that was reproducible. Following the completion of preparations of the chips and solutions as noted previously, the chip is positioned. Approximately 30 min before blood is drawn, 8 µL of specified protamine concentration (0, 2, 4, 6 mg/mL) was preloaded onto the fiber glass sample pad housed on a 3D printed stamper and allowed to be fully dried. Once drawn into a test tube the blood is pipetted into 8 wells of a 36 well plate. Once filled, an eight-tipped multi-pipette (ThermoFisher Scientific) was used to transfer the blood from the 36 well plate onto the sample pads. Once blood was introduced to the sample pad it was then incubated for 1 minute. The stamper was then flipped onto the inlet of the channels and the assay begins. To
document the results of the experiments, a custom coded device (Raspberry pi) was then initiated to capture a set number of images at set time intervals (ex. Images at 10, 20, 30, 40, 50, 60, 120, and 180 s) and saved the images sequentially with one click. Figure 6A depicts an outline version of protamine assay procedure and figure 6B depicts the use of the stamper as noted earlier.

![Diagram of Heparin/Protamine Assay procedure](image1)

**Figure 6a. Diagram of Heparin/Protamine Assay procedure**

![Stamper flow chart](image2)

**Figure 6b. Stamper flow chart**
Results

Protamine Results

Figure 7 illustrates average distance normalized to 0 mg/mL at 50 s vs. concentration seen at 50 s for each concentration. Error bars represent standard error of the mean of a sample size of 3. Assays were analyzed with ANOVA for each time point from 10 s to 180 s and normalized to 0 mg/mL at 50 s (See Appendix 1 for complete analysis). ANOVA analysis of average normalized distance at 50 s showed a significance of \( p < 0.005 \) (See Appendix 1 for complete analysis). ANOVA relates to differences between experimental groups (various concentrations). Linear regressions analysis was performed and found a significance of \( p \leq 0.05 \). Linear regression analysis relates to if the data follows a trend (positive or negative slope). Figure 8A–D, depicts normalized protamine rate of flow vs. time. A linear trendline was fitted alongside the points and a slope and intercept was calculated. The intercept showed similarity to the average distance traveled at 50 seconds when blood had 0 mg/mL of protamine. The \( R^2 \) value (correlation coefficient) of Figure 7 is shown as 0.87494. Correlation coefficient is high as 0.9969 were observed in this assay (see Appendix 2 A-H). The \( R^2 \) value is a measure of how close the data is to the trendline.
Figure 7. Protamine average distance normalized
At 50 seconds for each concentration
Note: n = 3

Figure 8A - D. Protamine Rate of flow vs. time
Note: n = 3
**Heparin results**

Figure 9 illustrates average distance normalized to 0 USP/mL at 120 s vs. concentration seen at 120 s for each concentration. Error bars represent standard error of the mean and sample size of 3. Assays were analyzed with ANOVA for each time point from 10 s to 180 s and normalized to 0 USP/mL at 120 s (See Appendix 3 for complete analysis). ANOVA analysis of average normalized distance at 120 s showed a significance of $p \leq 0.05$ (See Appendix 3 for complete analysis). ANOVA relates to differences between experimental groups (various concentrations). Linear regressions analysis was performed and found a significance of $p \leq 0.05$. Linear regression analysis relates to if the data follows a trend (positive or negative slope). Figure 10A – 9D, depicts normalized heparin rate of flow vs. time. A linear trendline was fitted alongside the points and a slope and intercept was calculated. The intercept showed similarity to the average distance traveled at 120 seconds when blood had 0 USP/mL of heparin. The $R^2$ value (correlation coefficient) of Figure 9 is shown as 0.88486. Correlation coefficient as high as 0.9722 were observed in this assay (see Appendix 4 A-H). The $R^2$ value is a measure of how close the data is to the trendline.
Figure 9. Heparin Average Distance vs. Concentration
Note: n = 3

Figure 10A - D. Heparin Rate of flow vs. time
Note: n = 3
Discussion

Results

It was hypothesized that with the developed device we would see increased coagulation resulted in decreased rate of flow, due to the increased size and number of clots in the blood sample, and vice versa. Further, it was hypothesized that increased heparin concentration would result in decreased coagulation and therefore increased rate of flow. Protamine concentration was expected to result in increased coagulation and therefore decreased rate of flow until protamine was in excess of heparin in a blood sample, where protamine has been shown to act as a weak anticoagulant.

Protamine Interpretation

Figure 7 depicts the average normalized blood flow with varied protamine concentrations. Due to a large standard deviation with the raw distance travelled, we normalized the data to 0 mg/mL at 50 s by dividing all data points by the average distance traveled at this point. We were able to optimize the normalized concentration and time after normalizing it at different points and seeing which showed the highest correlation, least amount of error, and significant differences between experimental groups. Looking at each time point and fitting a linear trendline, we were able to calculate the $R^2$ value (see Appendix 1 for complete analysis) in order to see which time point had followed the linear trend most closely. ANOVA analysis at each time point was calculated, after 10 s there was a significance of $p \leq 0.05$ which shows that there were distinct differences between each concentration. We propose that our assay has a minimum assay time as low as 20 s to determine patient specific protamine dose response. Our 20 s minimum assay time based on an 8 uL blood sample is significantly faster and lower volume than the typical Hepcon HMS predication of protamine titration (which takes at least 120 s and 3 mL of blood). Between the linear regression
analysis and the high correlation coefficient of the protamine assays a linear dose response was proven. Figure 10 A-D, shows the rate of flow at different time points. Figure 10 D shows a lower rate of flow in comparison to figure C which has a lower rate of flow in comparison for B, which has a lower rate of flow than A. Overall this is evidence that as there is a higher concentration of protamine, there is a lower rate of flow.

Heparin Interpretations

Figure 9 depicts the average normalized blood flow with varied heparin concentrations. Due to a large standard deviation with the raw distance travelled, we normalized the data to 0 mg/mL at 120 s by dividing all data points by the average distance traveled at this point. We were able to optimize the normalized concentration and time after normalizing it at different points and seeing which showed the highest correlation, least amount of error, and significant differences between experimental groups. Looking at each time point and fitting a linear trendline, we were able to calculate the $R^2$ value (see Appendix 4 A-H for complete analysis) in order to see which time point had followed the linear trend most closely. ANOVA analysis at each time point was calculated, after 120 s there was a significance of $p \leq 0.05$ which shows that there were distinct differences between each concentration. We propose that our assay has a minimum assay time as low as 120 s to determine patient specific heparin dose response. Our 120 s minimum assay time based on an 8 uL blood sample is a lower volume than the typical Hepcon HMS predication of heparin titration (3 mL of blood). It is known that heparin has a longer time to take effect in the patient due to its direct interaction with ATIII and amplification of the clotting cascade. Therefore, it would be expected that the detection of the impact of heparin would be longer than that of protamine. Between the linear regression analysis and the high correlation coefficient of the heparin assays a linear dose response was proven. Figure 10 A-D, shows the rate of flow at different time points.
Figure 10 D shows a higher rate of flow in comparison to figure C which has a higher rate of flow in comparison for B, which has a higher rate of flow than A. Overall this is evidence that as there is a higher concentration of heparin, there is a higher rate of flow.

**Clinical Translation**

While significant progress has been made toward the development of this device and assay, for full translation to a clinical setting improvements and refinements are necessary. Immediate steps would include optimizing exact heparin and protamine concentration for clinical relevance and optimizing the limit of detection of the assay. In order to optimize the concentrations of heparin and protamine preloaded onto the sample pads an analysis would need to be done on the interactions between the drugs and the sample pad (how much of the preloaded drug actively participates in the reaction with blood). To determine repeatability and reproducibility, increased sample sizes would need to be tested. Upon final development, the assay and device would have to undergo FDA approval and intense clinical trials.
Conclusion

We have successfully designed an assay and accompanying monitoring device which can monitor the extent of blood coagulation in as little as 20 seconds and no more than 180 seconds. Further, this device is able to monitor coagulation in an individualized response to varied dosages of heparin and protamine, which would allow for individualized dosing of these drugs. Around 30 – 40 seconds assay time, both heparin and protamine trends were established. With this, an even lower assay time may be possible. Successful application of this pilot device will lead to the development of advanced capabilities to test for more conditions (other anticoagulants) and versions that could be used by outpatients, similar to finger pricking in glucose strip/meter testing. Combined with the low cost of this assay and device, this could result in the broad use of these devices for diagnostic evaluation of individuals with both minor and acute coagulation conditions. The appealing cost and performance characteristics are of great advantage in drug monitoring activities, enabling single use of individual µPAD.

However, in order to make this approach a marketable reality several components will need to be pursued. Establishing reproducibility and automated data analysis will lead to a truly user-friendly device. Increasing the sample size will help with this aspect and lead to a better understanding in optimizing the device to its full potential.
Appendix

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1. ANOVA analysis for protamine concentration at each time point

2. A-H. Protamine blood flow with varied protamine concentration
ANOVA analysis for each time point for heparin concentrations

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3. ANOVA analysis for heparin concentration at each time point

4. A-H. Heparin blood flow with varied heparin concentration
References


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