The Development of an In Vitro, One-Pass, High-Throughput Model of Flow Dependent Thrombosis

by

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ABSTRACT

Thrombosis is an initiating response to a vascular injury, which aids in the repair and remodeling of the vessel wall. However, if this process remains unchecked, occlusion of the arterial lumen may quickly occur. The arterial vascular bed is a delicate and life-sustaining environment, in which a pathological thrombosis can bring about devastating conclusions such as acute vascular syndromes or post-interventional thrombosis. In order to explore these flow-dependent thrombotic reactions, it is essential to consider the physical environment present inside the vasculature. A novel in vitro, high-throughput method for creating one-pass blood flows has been developed to model the arterial environment. Flow is generated in a matrix of small glass tubes with varying inner diameters through the use of a constant pressure drop. Using this technique, a variety of flow rates are created in the numerous tubes, resulting in a variety of flow shear rates. In addition, this technique allows for the monitoring of sensitive, flow-dependent processes without the disturbances from pump action and circuit effects. A detailed discussion about the goals of the proposed systems is included, as well as the methodology employed to choose the optimal flow system, and the process by which the components of the system evolved in design. Finally, tests are formulated in order to explore the issues of biological feasibility, noise, precision, and accuracy related to the proposed system and make to make improvements on the design accordingly.

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CHAPTER 1
Background and Significance

1.1 Vascular Thrombosis

1.1.1 Significance
During states of health, all parts of the vascular system are lined with an endothelial barrier that separates and protects the highly reactive vessel wall from the blood flowing within. This biological environment is greatly affected by several cellular mechanisms such as gene expression patterns and surface receptor expression [1]. These mechanisms allow for the living endothelial surface to change according to the local physiological needs.

This protective endothelial surface is continually injured by environmental and genetic factors. Although these injuries are of very low severity, they cause the accumulation of subendothelial deposits of lipids and lipid-laden macrophages that initiate a complex cycle of chronic inflammation, smooth muscle cell proliferation and migration, extracellular matrix turnover, and the build up of cellular and necrotic debris [2-6]. The accumulation of these materials and processes develop into atherosclerotic disease and luminal stenosis. A healthy arterial lumen is compared against a lumen experiencing narrowing by atherosclerosis in Figure 1.

![Figure 1: A) Healthy arterial lumen B) Arterial lumen narrowed by atherosclerosis.](image)

These atherosclerotic plaques are characterized by a lipid laden, necrotic core that is covered by a fibrous cap region. This atherosclerotic process can continue over a lifetime and eventually lead to significant reduction in lumen area, on the level of >75% reduction. Regulatory mechanisms of the body can account for these progressively growing plaques by compensating with increased blood flow. Although this situation can be somewhat accounted for naturally and safely by the body, a plaque eruption or endothelial erosion can be unpredictable and treacherous [3-5, 7, 8]. One of these events can expose the highly reactive subendothelial layer and accelerate the progression of
disease, which can then bring about unstable angina, myocardial infarction, or ischemic sudden death.

1.1.2 Biological Components

Although great deals of factors are involved in vascular thrombosis, this study will focus on the blood and wall as the primary parts involved. Specifically, the element in blood that relates most to the vascular response to injury is the platelet. A platelet is a cell that circulates along with blood in concentrations ranging between $1.5 - 4.5 \times 10^8 \text{ cells/mL}$ [9]. Platelet reactions with walls are an incredibly important factor in thrombosis as is evidenced through the successful clinical use of anti-platelet drugs in order to minimize thrombotic risk [10]. Upon binding to a wall, these platelet-wall reactions occur in a series of steps. They first alter their expression of surface molecules and release substances which both attract and adhere to flowing cells, as well as promote the enzymatically driven coagulative response.

The adherence, or tethering, of a platelet to a vascular wall is the outcome of several biochemical processes actively occurring in the blood. The speed with which the bonds are created in these biochemical processes is a determining factor of the effectiveness of a platelet to tether to a surface under given flow conditions, and therefore the rate at which platelets collect at a site. Studies have shown that these tethering activities become increasingly important as blood shear rates increase above 300 s$^{-1}$, and are nearly the sole factor in platelet tethering at rates around 1500 s$^{-1}$ [11, 12]. After a platelet binds to the wall surface, it undergoes a shape change which is accompanied by the release of substances such as ADP and thromboxane. Platelets flowing freely in the blood can then be activated by the released molecules, which enhance their binding affinity to the activated receptors on the surface of the bound platelets. Platelets are necessary in the support of coagulative propagation, a process initiated by internal or external factors, but results with the conversion of monomeric fibrinogen to fibrin. The monomeric fibrin quickly polymerizes into a mesh network of filaments onto which platelets can adhere. This process strengthens the overall cohesiveness of this aggregating, hemostatic plug which helps to protect and close the wound.

In addition to the specific characteristics of blood that contribute to coagulation, the physiology of the arterial wall has great influence on the process of clotting. As explained before, the process begins with the damaging of the layer of the arterial inner wall which contacts blood. This layer is composed of endothelial cells which die when damaged. This damage can lead to sub endothelial layer exposure. The cells and proteins of this formerly protected layer can then react with the selectins or integrins found in blood. Specifically, the layer can react with collagen, fibronectin, and can absorb Von Willebrand Factor, which is present in all plasma. Also, the wall can react with tissue factor, the initiating factor in coagulation [2-6]. Initially, the test proposed in this document will focus on the effects of shear rates on collagen, later on tissue factor will be tested as well.
1.1.3 Physical Environment

Biological interactions occurring inside the vasculature are at least partly caused by the physiological environment, especially inside the hemodynamic setting of the body's vasculature. The flow of blood in this environment allows for the maintenance of diversity as well as the control of vascular responses. The flow conditions in this setting vary by both time and space, affected by the systolic and diastolic phases of a beating heart, as well as curving vessels, numerous branch points, and geometric pathologies [14-17]. The complex flow patterns created by these features must be taken into account when considering highly flow-sensitive events such as thrombosis.

1.1.3.1 Flow

Blood flow can affect the biological interactions occurring in a body, beginning at the molecular and microscopic scales, and up to the macroscopic scale. Some of these interactions include physical forces and mass transport, as well as sophisticated machinery developed by the body to adapt to changing surroundings.

1.1.3.2 Molecular Interactions

Blood flow can affect the biological interactions occurring in a body, beginning at the molecular and microscopic scales, and up to the macroscopic scale. Some of these interactions include physical forces and mass transport, as well as sophisticated machinery developed by the body to adapt to changing surroundings [18].

1.1.3.3 Microscopic Interactions

There are two important regulators of molecular interactions in the vascular environment: cellular transport and wall reactivity. Cellular components are transported to and from reactive surfaces by a special diffusion coefficient, specific to non-Newtonian fluids. This coefficient is dependent on the concentration of red blood cells in flow, which happen to migrate to the flow axis. This causes platelets to migrate to the flow periphery [13]. The reactivity of a vessel wall also matters with respect to the expression of surface adhesion molecules of endothelial cells [19]. These molecules are known as selectins, and they interact with respective ligands on free stream cells under high shear conditions. These connections are made quickly and are not strong, but initiate pathways of vascular cell activation, resulting in integrin receptor activation, and firm, shear-resistant adhesion to surface ligands.

1.1.3.4 Macroscopic Interactions

On the macroscopic scale, shear stresses caused by blood flows can impact the spread and development of a vascular disease. Atherosclerotic plaques are characterized by an atheromatous core region covered by a fibrous cap, which can cause the rupture of the plaque if removed [20-23]. As a thrombus increases in size by the aggregation of cells and molecules, it is subject to increasing fluid forces. These increased stresses can bring about the rupture of the plaque, which can eventually result in a dangerous embolism.
1.2 Methods of Studying Flow Dependent Thrombosis

A large variety of methods to study flow dependent processes and effects in the vasculature have been developed in the past. These tests have ranged from *in vivo*, to *ex vivo*, to *in vitro* technologies. New testing methods and devices have been devised to improve upon the performance limits previous models, as well as to include new information about the system gathered from recent studies. As a result of all these tests, each one has its own set of advantages and disadvantages, and therefore some are more suited to test certain variables and conditions than others. The use of all these experimental procedures has brought us to our current knowledge about the environment, physiological processes, and biochemical pathways that exist in the vascular setting.

1.2.1 *In Vivo* Strategies

*In vivo* methods of studying flow dependent thrombosis are used routinely in human studies and clinical trials. During human studies, the primary goal is to apply technology and knowledge towards the immediate improvement of the patient’s condition. Animal studies, in comparison to human studies, are more controlled and cost less to administer, although the applicability to the human condition usually becomes a factor when results have been obtained. These types of investigations are valuable because they take into account complex biological interactions in the body, such as integrated, systemic, and long-term processes [9]. Advantages can also be found in disease characterization, patient risk stratification, and the efficacy of therapeutic options. On the other hand, because of the objectives of these tests, as well as the lack of ideal controls and the variations between individual patients, scientific progress during one of these endeavors is difficult. On top of that, the tests can also be expensive, take a great deal of time to complete, and the final conclusions may be unclear, and therefore not directly applicable to the towards progress in the research field.

1.2.2 *In Vitro* Strategies

*In vitro* testing procedures allow for parametric control over hemodynamic and complex biological environments. Generally during *in vitro* experiments of physiological flow dependent processes, tests fall into one of two categories: control is taken over the physical geometries, or control is taken over the flow conditions. When geometry is maintained constant the main advantage obtained is the possibility to maintain realistic vascular dimensions in the testing components. Several problematic issues are also associated with these types of testing, namely finding suitable methods for flow actuation and control. One pass and fluid loop systems have been devised to work out some problems, but new issues have risen. One pass systems generally contain a static holding volume for the blood to be tested, which greatly constrains available flow rates, experimental run times, and constant flow conditions [25]. Fluid loop systems allow for the use of small volumes of blood, but have problems associated with blood recirculation and air-fluid interfaces [26-28]. In order to better control flow activation and control, pumps may be implemented into a design, but it is difficult to find suitable pump whose drive train does not activate platelets [29-30]. The activation of platelets means that this
environment is too traumatic to appropriately represent the arterial environment in experiments.

The use of constant geometry in experiments required the use of high flow rates to generate arterial-like flows. Because of this, liberties have been taken with the architectural design of flow setups, in order to create small circuit geometries. Examples include parallel plate chambers that allow for high shear rates under low flow, high levels of control over the environment, and the ability to visualize in real time [26, 24, 31-33]. Another example is the cone-plate or annular ring device that creates wall shears through Cuvette-type flows [17, 34]. Through the use of non-dimensional parameters and specific conditions, a miniature model can be related to a physiological architecture, and therefore prove useful as a source of experimental information.

1.2.3 Rotational Systems
Among the *in vitro* constant flow experimental techniques a novel method to create flows and wall shears was developed by Kumaran Kolandaivelu [35]. The method developed involved the angular acceleration of fluid filled loops about their axis. Flow was generated by inducing wall motion relative to the contained fluid. The flow was predetermined and controlled in order to modulate the fluid motion in relation to the wall through the transmitted shear forces. In addition, a variety of pulsatile flow patterns could be developed and modulated in order to mimic the physiological hemodynamic setting. This system had the advantages that it was able to accurately monitor sensitive flow dependent processes, received no disturbances from a pump mechanism, and utilized low test volumes as well as generated low costs.
1.3 Motivation for High-Throughput Strategies / Proposed Flow System

Table 1 compares prior flow systems that have been utilized to study the hemodynamics of thrombosis with respect to the human condition.

Table 1: Comparison of flow system attributes. (Subjective scale that ranges from +++ → −−−, which is equal to Excellent → Poor.)

<table>
<thead>
<tr>
<th>Experimental Systems</th>
<th>System Performance Attributes</th>
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<tr>
<td></td>
<td>Complex Interaction</td>
</tr>
<tr>
<td><em>In Vivo</em> Clinical Trials</td>
<td>+++</td>
</tr>
<tr>
<td>Large Animal Studies</td>
<td>++</td>
</tr>
<tr>
<td>Small Animal Studies</td>
<td>+</td>
</tr>
<tr>
<td><em>In Vitro</em> Gravity Driven Systems</td>
<td>--</td>
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<tr>
<td>Peristaltic Systems</td>
<td>--</td>
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<tr>
<td>Parallel Plate Chambers</td>
<td>--</td>
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<tr>
<td>Cuvette Systems</td>
<td>--</td>
</tr>
<tr>
<td>Rotational Systems</td>
<td>--</td>
</tr>
<tr>
<td><em>Ex Vivo</em> Ex Vivo Circuits</td>
<td>-</td>
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</table>

The combination of all these types of tests has generated a great deal of the information we currently know about thrombosis and the intravascular setting. Some test types, such as *Ex Vivo* circuits have even bridged the gap between *In Vivo* and *In Vitro* studies. Still, problems remain with each of these tests, and therefore new methods must be developed in order to perform new studies and gather new types of information.

Newer testing methods, especially the apparatus that creates inertial flow caused by an accelerating loop, successfully tackled several issues related to previous testing methods. The inertial flow method focused on gaining more control over the biological environment, creating physiologically relevant flows, as well as maximizing the biological signal while reducing the background noise. While the system was successful in reaching these goals, it had several problems which were solved through the key criterion of our new mechanism design:

1. The ability to test a large variety of conditions concurrently
2. Utilize a small sample of blood to run all the tests
3. Allow for the operator of the machine to be able to output accurate and consistent data, while following simple instructions for use.
After considering several routes for improvement upon the spinning loop design, a high throughput, one-pass, pressure drop driven flow mechanism was developed. Briefly, blood flows through 384 glass capillaries, by the help of a pressure drop, and is experiences differing flow conditions in each tube. The construction of this one-pass system then allows for easy assaying of the sample and monitoring of the biological signal. This method allows for the use of small test volumes and cost, allowing for a large amount of parametric analyses to be carried out on the sample.
CHAPTER 2

High-Throughput Strategies

The new iteration on a flow system design, described at the end of Chapter 1, strives to serve as a high throughput blood flow testing mechanism, which operates on a relatively small sample of blood. More specifically, several constraints and goal parameters had to be met in order to consider this mechanism an improvement over other systems, especially over the successful accelerating loop design. The performance characteristics of several blood flow systems were compared in the Pugh chart found in Table 1. The new design of the system had to improve upon several system aspects, such as: range and number of flow conditions, minimum volume of blood per sample, time to run each phase of the test, and the ease of setting up the apparatus for each test phase.

2.1 Goal Parameters / Basic Constraints

One of the major problems associated with the accelerating loop design was the ability to vary the range and number of flow conditions. This system allowed for variations in flow conditions by manipulation of the program file that sent the input signal to the motor of the apparatus. If the correct mathematics were carried out, one could successfully output a variety of pulsatile flows, of varying frequencies and amplitudes. Unfortunately, due to the nature of this system, the number of flow conditions that could be tested in parallel was a function of the number of rotors built into the system. Because of the size of the incubator that held the apparatus, the maximum number of rotors that could be built into the system was about two to three. Although a number of experimental condition repeats could be mounted onto a single rotor, only about two different experimental conditions could be tested in the time allotted for a test. The new flow system needed to concurrently test a large number of one or more variables, and have repeats of each condition to ensure precision. Early in the design process it was determined that designing the tool for direct use with a 384 well plate would be an efficient way to ensure that a great deal of conditions may be tested. Therefore, three repeats of 128 condition variations could be potentially tested. This was already a large improvement in terms of testing volume from the rotating loop, which at maximum could handle six repeats of two testing conditions.

Another issue associated with the accelerating loop was the volume of blood per sample. Each tube loop required a 3 mL blood sample to provide data. While this is initially a feasible amount of blood to draw from a specimen, performing tests on a variety of conditions will soon require much greater amounts of blood. This would become a problem if the system were to ever enter into a population study where a large amount of samples and data would be necessary to arrive at any strong conclusions. One of the goal parameters of this new system would be to perform all 384 tests on 16 blood samples of 3 mL volume each. This system would have flow chambers of diameter smaller than physiological size in order to create high shear rate flows at low volumetric flow rates.

Finally, it was of great importance to count in the human factor of the design of this new device. The rotating loop apparatus had several problems associated with its ergonomics.
in conjunction with the time dependent characteristics of blood. Blood reacts with any surface it comes into contact, therefore minimizing the amount of surface the blood touches as well as the time in contact, is important in receiving a good biological signal from experimentation. Each test in the rotating loop took from three to five minutes to complete, but before running the test, each loop had to be filled with blood. The filling process could require up to one minute per loop, and if six were to be loaded onto a rotor before testing, the first loop would have sat five minutes longer than the last even before testing began. Considering the time involved in all these processes, a comparison between the signals received from each sample is flawed because of the large disparity in the times the blood samples spent in each loop. It was therefore necessary to find a method that would avoid blood loading issues by utilizing the fewest intermediate steps possible when loading the blood. Also it had to allow for all the blood to be loaded at the same time, or at least with a very small amount of time difference in storage waiting for testing. Specific methods to achieve these goals involved saving time during the experiment, by designing components of the apparatus that either served multiple purposes, or could be quickly swapped between different phases of the experiment.
2.2 Rationale for Rotational and Linear Flow Model Pursuit

Once the basic constraints and parameters of the new model were outlined, two high throughput flow models were proposed: a rotational and a linear flow model. The rotational model was the one most directly derived from the accelerating loop design. This model would utilize the same basic idea as the previous iteration, which was the application of a rotational acceleration to a fluid-filled container, in order to induce relative movement between the fluid and container wall. In this case, though, inspiration for the fluid containers came from a 384 well plate. The fluid containers, or wells, in this apparatus would each be very small and hold only 7.5 μL of blood. The plate that holds the wells would sit on a turntable which spins the plate about its center. Using programmed spinning combinations and varying the dimensions of each well, it would be possible to create prescribed shear rates inside the wells. This setup would be very simple to develop as it would use very few parts, and much of the components were the same as those utilized in the accelerating loop apparatus. The greatest obstacle in the developmental process of this system would be determining the mathematics associated with fluid mechanics in order to find the correct spinning parameters.

An alternative proposal to the rotational model involved a one-pass flow system. In this system a rectangular matrix of 384 glass capillaries would serve as blood flow chambers corresponding to each well of a 384-well plate. Each flow route would have a testing section that is the same for all samples, as well as a resistance section in which the inner diameter of the glass capillary varied from one sample to another. The combination of the resistance section of the tubing with a constant pressure drop across all the tubes allowed for each tube to test a different blood shear rate. The mathematics related to this system were much simpler than those for the rotational model but a greater amount of time was spent on the design of the various components to be used in the system, so that 384 blood samples may be handled safely and efficiently.

In order to initially determine the feasibility of either one of these systems, it was necessary to provide a theoretical model of the blood flows. In the case of the rotational model, it was of great importance to determine measurements of the well and the angular acceleration that must be imparted upon the plate in order to create the wall shear rates. It was necessary to figure out whether the accelerations and rotational speeds needed for this system could be reached by the motors and equipment provided in our lab. In addition, the feasibility of the system also rested on secondary effects of spinning, such as the centrifugation of cells, which would provide bad results. The one-pass flow system also needed to pass through feasibility testing by a theoretical model. This model would determine the amount of resistance that needed to be applied to the blood flow in order to reach our goal shear rates. Determining the amount of resistance directly informs us about component parameters such as the inner diameter and length of the resistance section tubing. These features must all be taken into consideration when designing the parts of the machine.
CHAPTER 3

High-Throughput Model Design

3.1 Rotational Flow Model (Model A)

3.1.1 Theory

3.1.1.1 Description of situation
The rotational flow model was a high throughput evolution of the flow model developed by Kolandaivelu [35]. A fluid filled container was accelerated about its axis of symmetry to create wall motion in relation to the fluid, whose angular speed lagged behind that of the wall because of its own inertia. The relation between the wall and fluid’s rotational velocity became the wall shear rate, which is of interest to us in our flow model. With the use of periodic accelerations and decelerations, one could ideally maintain a particular shear rate in a fluid filled container. In this particular model, the containers that held the fluid were the size of a well from a 96 well plate and were arranged on a circular disk that was spun from its center. The advantage of this setup was that it potentially allowed for the easy loading and testing of many samples of blood, as well as it would allow for an easier assay of the samples, and would allow us to obtain a great deal of data.

3.1.1.2 Rotating bucket example
The rotating bucket is a classic name given to the situation where a closed cylindrical fluid filled cavity is spun up from a standstill. The analysis of this type of system is much easier when assumptions such as the fluid being in rigid body rotation or an infinitely long cylinder, can be made. This approximation, though, cannot be applied to a finite length situation, as experimental results will vary greatly from the theoretical results. For a liquid of low viscosity, a relatively long time is required for the liquid to rotate as a solid body, and even through this transitional period, the fluid flow may become unstable [36]. The reason that an assumption that the cylinder is infinitely long cannot be made is because it had been found that the effects of cylinder ends on the fluid motion can actually dominate the flow, even in very slender cylinders. This fluid motion is affected by a secondary flow caused by the cylinder ends, in a region called the Ekman layer, which convects the fluid from the walls to the interior of the cylinder. As a result, the fluid attains rotational velocity much more quickly than without these secondary flows.

3.1.1.3 Equations and Solutions
In the particular case of rotationally symmetric flow, with small radial and axial secondary flow in the interior, Greenspan [37] explains that the azimuthal fluid spin-up is governed by:

\[
\frac{\partial v}{\partial t} + u \left( \frac{\partial v}{\partial r} + \frac{v}{r} \right) = \nu \left[ \frac{\partial^2 v}{\partial r^2} + \frac{1}{r} \frac{\partial v}{\partial r} \right],
\]

Eq. 1
where \( u \) is the radial velocity, \( v \) is the azimuthal velocity, and \( r \) is the well radius. This can then be solved by

\[
u = -K \left( \frac{v}{\Omega h^2} \right)^{1/2} (r\Omega - v), \tag{Eq. 2}\]

where \( \Omega \) is the angular velocity and \( K = 0.886 \). Greenspan explained that when \( K = 1 \), Equation 2 becomes identical to the corresponding equation for linear spin up. In addition, he made several substitutions into the resulting equation:

\[
\begin{align*}
R &= \frac{r}{a}, V = \frac{v}{a\Omega}, T = \frac{t(\nu\Omega)^{1/2}}{h}, A = \frac{a}{h}, E = \frac{v}{\Omega h^2}, \\
\frac{\partial v}{\partial t} + (V - R) \left( \frac{\partial V}{\partial R} + \frac{V}{R} \right) &= A^{-2} E^{1/2} \left[ \frac{2}{\partial R^2} + \frac{\partial}{\partial R} \left( \frac{V}{R} \right) \right]
\end{align*} \tag{Eq. 3}
\]

In the previous equation, \( a \) is the radial distance from the center of the well, \( A \) is the aspect ratio, \( E \) is the Ekman number, and the portion \( A^{-2}E^{1/2} \) can be substituted with \( \alpha_0 \). In their paper, Watkins and Hussey [38] explained that depending on the size of \( \alpha_0 \), the equation can be solved in different manners. A small \( \alpha_0 \) indicates that the system response to the rotation of the cylinder is very much dependent on the Ekman layer. On the other hand, if \( \alpha_0 \) is large, it indicates that the response of the fluid to the acceleration of its container is mostly to completely dependent on viscous effects from the cylinder wall. There is also the regime of \( \alpha_0 \) that falls in between both extremes and considers a combination of the two effects. Unfortunately, of the three regimes only the small \( \alpha_0 \) has been studied extensively, while only very rough solutions have been found for the rest. In this model we only want to deal with viscous effects, in order to avoid secondary flows in the well. In the case that \( \alpha_0 \) is large, its solution is found in the form:

\[
V = R + 2 \sum_{n=1}^{\infty} \frac{J_1(j_n R)}{j_n J_0(j_n)} \exp \left( \frac{-j_n^2 vt}{a^2} \right), \tag{Eq. 4}
\]

where \( J_1 \) is the Bessel function of the first kind of order 1 and \( j_n \) is the \( n^{th} \) zero of \( J_1 \). This equation was solved with the use of the program Excel for a variety of conditions. The extreme conditions, especially the maximum shear rates that we wanted to test, were focused on in order to determine the feasibility of this type of apparatus. While determining the parameters necessary to create the maximum shear rates, it was necessary to take note of the well radius, the maximum rotational speed reached by the spinning platform, \( \alpha_0 \), the centrifugal acceleration experienced by the fluid in the well, the volume of blood in the well, and the time for the fluid to reach a state of solid body rotation. The results of experimenting with the upper limit conditions revealed the results presented in Table 2.
The information in the table simply deals with the conditions necessary to produce the upper limit shear rate which was $4000 \text{ s}^{-1}$; as confirming whether the apparatus is capable of these limits determines the feasibility of the device. The largest difference between the two cases presented in the table is found in the radius of the well ($R_{\text{well}}$), where one well is about the size of a well in a 96 well plate, while the other is much larger to provide a good contrast. The advantages of a small well can be seen in this table. The small well requires a much smaller blood volume, allowing for nearly 60 experiments to be run from a normal sized blood sample, which is generally about 60mL. The larger volume would only allow around 4 tests to be taken. Also, the smaller well requires much less time for the fluid in the well to achieve a state of solid body rotation, because of viscous effects and its greater surface area to volume ratio. This figure is important when considering that the cylinder would have to cycle through acceleration and deceleration phases in order to continually create the shear rates required by the test. A $T_{\text{solid body rotation}}$ of 0.15s is much easier to work with than one of 10.0s, which would take up a great amount of time considering that each test would only run between 1-2 minutes. Advantages to using a larger well volume include the need to accelerate the cylinder to a considerably lower final rotation rate to achieve comparable shear rates, and therefore lower centrifugal accelerations are also expected. In theory, this system should reach its maximum rotational speed in less than 0.1s.

Outside of the advantages of one well size over another, the table provides information that indicates this testing method would not be feasible. In order to meet the goal shear rates of around $4000 \text{ s}^{-1}$, high rotation rates must be reached by the motors running the experiment. These rotation rates will then cause great centrifugal accelerations in each well, which quickly approach blood separation values as one increases the distance between the center of the plate and the well. As a rule of thumb, blood centrifuged at rates between 300-500 G's begins to separate between plasma and cells after only a few minutes. The generation of this effect would be very detrimental to the results of this experiment. In addition, the value of $a_0$ becomes very small at these rotational rates, which indicates that this system should actually be solved using the small $a_0$ approximations of Equation 3. Moreover, the fact that this system has a small $a_0$ denotes that the rotation of the fluid is very dependent on the Ekman layer, which in turn means that the fluid rotation is greatly influenced by secondary flows present. These secondary flows would be very detrimental in the simulation of the simplified physiological environment that this model is aiming to create, and needs to be avoided as much as possible.

Finally, during the research that was completed for this model, it was found that “spin down” or the deceleration of the fluid inside a sealed cylinder, was not symmetric to “spin up,” or the acceleration of the fluid [39]. Spin down can occur in an impulsive or
finite manner. During an impulsive spin down the fluid particles near the cylindrical wall will rapidly experience a centrifugal instability. This centrifugal instability can lead to the creation of “Taylor Görtler” vortices along the side wall [40]. As spin down proceeds, the smaller vortices coalesce into larger ones, generating a much more rapid spin down than what would be predicted by nonlinear theoretical arguments. This problem can be delayed by conducting spin down over a finite period of time. The problem may even be completely avoided in the slowest deceleration conditions, but the problem with this solution is clear in this test. The time needed to decelerate, and/or change the rotation direction of the fluid is of great importance, and a great deal of time cannot be applied towards deceleration of the fluid.

The combination of all of these technical difficulties led to the decision to explore the feasibility of a high throughput, one-pass linear flow device design.
3.2 Linear Flow Model (Model B)

3.2.1 Theory

3.2.1.1 Description of Situation
In order to create the desired flow profiles, and therefore, the desired wall shear rates, a fluid flow was generated down the length of the flow tube by a pressure drop. The difference between the pressures at the entrance and exit of the tube allowed for fluid to be moved from its initial container to a final collection reservoir. The wall shear rate in the tube was determined by the profile of the flow, which in turn was a factor of the tube dimensions as well as the fluid flow rate. Given a particular pressure drop and volumetric flow rate, the flow’s velocity was controlled by changing the cross-sectional area inside a tube. In the case of flow-dependent thrombotic reactions, the maintenance of velocity gradients or shear rates was the important flow factor.

Using this technique, we hope to initially create time-invariant flows, whose flow characteristics such as the dimensionless flow parameter Reynolds (Re) and wall-fluid shear rate were typical of physiological blood flows found in arteries. The theoretical model developed for this apparatus represents a straight pipe composed of three sections, as shown in Figure 2.

![Figure 2: Cross-sectional sketch of the theoretical straight pipe model.](image)

The three sections of the pipe are the test length, the contraction, and the resistance length. The test length section of the model will have constant parameters across all testing conditions. The contraction and resistance sections will vary with the shear rate conditions.

3.2.1.1 Equations
The most important parameter of the fluid flow in this model was the shear rate experienced between the fluid and the wall. This information could be found by solving only the axial (z) component of velocity of the cylindrical form of the Navier-Stokes equations (Equation 5)
\[
\rho \left( \frac{\partial v_z}{\partial t} + v_r \frac{\partial v_z}{\partial r} + v_\theta \frac{\partial v_z}{\partial \theta} + v_z \frac{\partial v_z}{\partial z} \right) = -\frac{\partial p}{\partial z} + \mu \left[ \frac{1}{r} \frac{\partial}{\partial r} \left( r \frac{\partial v_z}{\partial r} \right) + \frac{1}{r^2} \frac{\partial^2 v_z}{\partial \theta^2} + \frac{\partial^2 v_z}{\partial z^2} \right]
\]

Eq. 5

where \( V_z, V_r, \text{ and } V_\theta \) are the velocity components in the axial (z), radial (r), and tangential (\( \theta \)) directions, respectively. Also, \( t \) is time, \( \rho \) is the fluid density, and \( \delta P/\delta z \) is the axial pressure gradient. In the case of our specific model with its circumferential and axial symmetry, Equation 5 was simplified through the elimination of several dependencies. This was then be integrated to give

\[
V_z = \frac{1}{2\mu} \left( \frac{dP}{dz} \right)^2 + \frac{A_1}{\mu} r + \frac{A_2}{\mu}
\]

Eq. 6

Equation 6 provided the velocity profile of the flow. Inputting the boundary conditions of this system (at \( r = R_{\text{tube}} \), \( V_z = 0 \), and at \( r = 0 \), \( dV_z/dr = 0 \)) gave us

\[
V_z = \frac{1}{2\mu} \left( \frac{dP}{dz} \right)^2 - \frac{1}{2\mu} \left( \frac{dP}{dz} \right) R^2
\]

Eq. 7

Taking the derivative of this function with respect to radial distance yielded a function for the shear rate of the flow.

\[
\frac{\partial V_z}{\partial r} = \frac{1}{\mu} \left( \frac{dP}{dz} \right) r
\]

Eq. 8

Finally, inputting a radial distance of half the tube diameter into this formula gave us the fluid’s shear rate against the wall.

\[
\frac{\partial V_z}{\partial r} = \frac{1}{\mu} \left( \frac{dP}{dz} \right) R
\]

Eq. 9

In order to determine the flow profile and the wall shear rate, it was necessary to find out the pressure head loss across a certain piping distance (major head loss), which was found with the use of the Darcy-Weisbach equation:

\[
\Delta P = \frac{64}{Re} \left( \frac{L}{D} \right) \left( \frac{\rho V^2}{2} \right)
\]

Eq. 10

Equation 10 was used to determine the head loss across the testing and resistance lengths of the tubing. The head loss associated with the tube contraction could then be found with

\[
\Delta P = \frac{1}{4} \rho V^2 \left[ 1 - \frac{D_2}{D_1} \right]
\]

Eq. 11.
where $V$ is the linear velocity of the fluid, and $D_1$ and $D_2$ are the diameters of the tubing before and after the contraction, respectively.

Finally, the total head loss was equated to the sum of the losses incurred by each of the tubing sections. The length or diameter measurements of each resistance section were then modified until the head loss equaled the difference between the input and exit pressures of the tube. The difference between the input and exit pressures of the tube were set by using Equation 10 to determine the pressure drop necessary to move blood through a simplified model of the tubing at a shear rate of 4000 s$^{-1}$ (the maximum shear rate to be tested). The parameters for the tubing included a diameter equal to that of the test section and a total length equal to the test and resistance sections combined.

### 3.2.1.1 Solutions

Using the linear straight tube approximation to move blood at a shear rate of 4000 s$^{-1}$ it was found that the necessary pressure drop was equal to 56535 Pa. Using this figure, it was then possible to determine the dimensions of the tube sections. All the calculations involved the same dimensions for the test section of the tubing, which was chosen for the reason that we could meet our targeted volumetric flow rate goals while using enough blood for a good assay signal, which was 7.5 μL. Only the dimensions of the resistance section changed according to the different shear rates. The dimensions of the test section are found in Table 3.

#### Table 3: Dimensions of the test section of the tubing.

<table>
<thead>
<tr>
<th>Attribute</th>
<th>Measurement</th>
</tr>
</thead>
<tbody>
<tr>
<td>Length</td>
<td>10.00 cm</td>
</tr>
<tr>
<td>Internal Diameter</td>
<td>0.03 cm</td>
</tr>
<tr>
<td>Cross-sectional Area</td>
<td>$7.06 \times 10^{-4}$ cm$^2$</td>
</tr>
</tbody>
</table>

For the dimensions of the resistance section tubing, a choice had to be made about whether the lengths or the diameters of the sections should be varied. Variation of length was first investigated, but it was quickly found that the length of the longest tube would be over 80 times the length of the shortest tube which would bring about problems with the actual manufacture and design of the apparatus. Variation of the tube’s inner diameter would require variation between 90 microns and 300 microns, which are well within the capabilities of modern glass capillary tube manufacturing. The dimensions of the resistance section tubing are found in Table 4.
Table 4: Dimensions of the resistance section of the tubing.

<table>
<thead>
<tr>
<th>Rated Shear Rate [s(^{-1})]</th>
<th>50</th>
<th>100</th>
<th>200</th>
<th>500</th>
<th>1000</th>
<th>2000</th>
<th>4000</th>
</tr>
</thead>
<tbody>
<tr>
<td>Length [cm]</td>
<td>15</td>
<td>15</td>
<td>15</td>
<td>15</td>
<td>15</td>
<td>15</td>
<td>15</td>
</tr>
<tr>
<td>Internal Diameter [cm]</td>
<td>0.009</td>
<td>0.0105</td>
<td>0.0125</td>
<td>0.0160</td>
<td>0.0192</td>
<td>0.0235</td>
<td>0.0300</td>
</tr>
<tr>
<td>Cross-sectional Area [cm(^2)]</td>
<td>6.4 (x) 10(^{-5})</td>
<td>8.7 (x) 10(^{-5})</td>
<td>1.23 (x) 10(^{-4})</td>
<td>2.01 (x) 10(^{-4})</td>
<td>2.9 (x) 10(^{-4})</td>
<td>4.34 (x) 10(^{-4})</td>
<td>7.06 (x) 10(^{-4})</td>
</tr>
</tbody>
</table>

In addition, it was found that the head loss due to the tubing contraction (minor head loss) was very small in comparison to the major head loss, so that the manner in which the tubing contracts (gradual vs. sudden) matters little in this case. With the application of a specific pressure drop, we could then control the volumetric flow rate, and therefore, the wall shear rate in the tubing.
3.3 Overview of Testing Protocol Using Linear Flow Strategy

Once the basic method to create blood flows and shear rates was determined, it was necessary to continue designing according to the original high throughput demands of the new design. The high throughput requirement of this system extended further than just the core method used to create the shear rates. The entire system needed to be easy to use to minimize human effort and human error in the results. Therefore, the testing protocol had to be reviewed and revised several times in order to change the ergonomics of each component in the system. The protocol followed during each test was composed of four main steps: setup, the test run, passing of buffer through the system, and assaying the results. The following is an overview of the protocol to be followed during testing with the final apparatus. A description of all the apparatus components is provided in Section 3.4.

3.3.1 Setup

Prior to assembly of the testing mechanism, several of the components must be prepared. All the components, including the vacuum connection, resistance block, testing blocks, blood loading mechanism, and other blood containers, must be sterilized by autoclave. Also, before testing, the test and resistance tubes must be coated with their respective solutions. The resistance tubing must be coated with 1% albumin solution, while the test tubing can be coated with collagen, Von Willebrand Factor (vWF), tissue factor (tF), or other proteins of interest. The test tubing must be soaked in a solution of either water or phosphated buffered saline with about 2.1mg of the coating as solute, per 1mL of solvent, for about two hours. Once the components have been prepared, they can be assembled together. First, four rods are screwed into the holes at the corners of the vacuum connection. The resistance section can then be slid onto the rods and mated against the lip of the vacuum connection. Once this connection has been made, the additional rods for the individual test blocks can be inserted and screwed in. The test blocks can be slid into place one at a time once the three rods that correspond to it have been installed. The test block must be inserted slowly to ensure the safe mating of the male and female ends of the capillary tubes in the test and resistance blocks, respectfully. Each of the 16 test blocks is fed by its individual blood supply, which allows for the testing of blood from different persons or blood with different treatments, concurrently. If fewer than 16 blood supplies were to be tested, then a blank test block could be installed in the place of a regular test block. The blank test block would close off those vacuum routes, and nothing would flow through those resistance tubes.

Once all 16 of the test block spots have been filled a bent connecting rod is passed through both rows of blocks, and secured with a pair of washers and nuts. This feature helps to keep all the test blocks together for easier handling. The blood loading mechanism is placed below the test blocks, and mated to the stepped ends of the blocks. The stacked components are then placed on top of a raised platform that allows for tube access to the underside of the blood loading mechanism. The containers that hold the 3mL blood supply for each test block, called Vacutainers©, are then connected to their respective nozzles on the underside of the blood loading mechanism. Next, vacuum hoses
are connected to the two exits on the vacuum connection piece. The primary vacuum connection, which lies at the top of the apparatus, is monitored and regulated to ensure that a consistent pressure difference of about 56535 Pa exists throughout the entire test. Finally, the secondary vacuum exits, on the sides of the test blocks, are connected to another vacuum source.

3.3.2 Test Run
Once all the blood container and vacuum connections have been made, the testing of the system can begin. The tubing system must first be primed with Hanks buffer (KCl 400mg/L, KH₂PO₄ 60 mg/L, NaHCO₃ 350 mg/L, NaCl 8000mg/L, NA₂HPO₄ 47.88mg/L). The vacuum suction can now be applied to the system, allowing blood to flow upward towards the vacuum connection piece, and out the side waste exit. The blood is allowed for flow for a period of 1 minute, or 60mL for all 384 tubes, at the prescribed pressure drop of about 56535 Pa. After this time is up the vacuum connection is removed and the system is allowed to come to rest at atmospheric pressure.

3.3.3 Buffer
After the system has achieved stable atmospheric pressure, the vacuum connection and resistance block pieces are lifted and removed from the system carefully. All of the rods are removed along with the vacuum connection and resistance block. The buffer hopper piece is installed on top of the test block pieces and Tyrodes buffer solution (MgCl₂ 0.75mM, CaCl₂ 1mM, KCl 2.7mM, NaCl 137mM, Glucose 5mM, NaHCO₃ 12mM, and HEPES 10mM) is poured into it. The secondary vacuum connections can now be opened, and the buffer is run in the reverse direction of the blood flow for 1-2 minutes at a shear rate of 1000 s⁻¹. After the buffer has been run through the tubing, the secondary vacuum connection is closed and the system is brought back to atmospheric pressure. Finally, the linked test blocks are removed from the blood loading mechanism and the buffer hopper, to enter the assay phase of the test.

3.3.4 Assaying of Results
Prior to performing the assay, a 384 well plate must be prepared. A quantity greater than 7.5μL of 1% Triton-X solution is placed into each of the 384 wells, since each test tube can contain exactly 7.5μL of fluid. Once the plate has been readied, the 384 well plate is mated to the bottom side of the 384 well plate connection piece, and a spacer is placed under the plate in order to fill in the gap below it. The connected testing blocks can then be mated to the other side of the well plate connecting piece, and the vacuum connection piece is then placed on top of the test blocks, allowing the lip to surround the blocks. Next, the vacuum suction can be applied to the system, and under hand control allow for the Triton-X in the wells to enter and fill all of the test tubes. The solution is suspended in place for about 30 minutes, after which the solution is expelled into a new 384 well plate. Cytotox 96® Cytotoxicity Assay (Promega) is used to measure the amount of LDH in the fluid that was expelled into the 384 well plate from the test tubes. LDH, or Lactate Dehydrogenase, is a chemical constitutively present in all cells. Therefore, the measure of
the amount of LDH in a solution gives a good indication to the amount of cells that were present when the solution was being created. Next, an amount cytotoxicity assay equal to the amount of Triton-X used in each test tube, is pipetted into each of the 384 wells of the plate. In this case, 7.5 µL of this assay would be placed in each well. This solution is allowed to rest for a period of one hour, after which a stop solution composed of diluted acetic acid is introduced into the mixture in an amount equal to the amount of cytotoxicity assay used in each well (7.5 µL). Finally, after the hour, the plate is placed in a plate-reading spectrophotometer, set to read at 490 nm wavelength. The instrument indicates how much chromagen is present in each well by determining how much of the 490 nm wavelength light is absorbed by the chromagen in the solution, which has peak absorbance at 490 nm. The outputted data from the instrument ranges numbers from 0 to 4, in proportion to the amount of 490 nm light absorbed by the solution, where 0 and 4 indicate that either none or all of the light has been absorbed, respectively.
3.4 Model Embodiment
The linear flow model was composed of six main components, namely the test blocks, resistance block, blood loading mechanism, vacuum connection, buffer hopper, and a connection to the 384 well plate. Each of these components went through individual evolutions, although the larger picture of the whole system had to be kept in mind the whole time. The main goals of the design of each part was to determine a combination of ease of use during operation and sanitizing, protection of fragile components, and accuracy that allowed for one to collect a great deal of data from properly run experiments. The design evolution of each of the six components is presented in the following sections, briefly describing the stages of the evolution.

3.4.1 Test Block
A schematic of a basic test block is shown in Figure 3 and three-dimensional drawing of the test block is shown in Figure 4. The test block was a specifically shaped block of silicone rubber cast around an 8 x 3 matrix of glass tube capillaries manufactured by Vitrocom. Each one of these capillaries had an outer diameter (OD) of 1mm and an inner diameter (ID) of 400μm. The length of each tube was also kept standard at 10cm. Each tube was spaced in the square tubing matrix according to the spacing between wells found in a 384 well plate. Therefore, there was a space of 4.5mm between two tube centers along either of the axes on the plate’s plane. At one end of the test block, the tubes extended past the face of the block in order to serve as a male end of the tubing to mate with the female end of the resistance tubing. The test block was designed to test eight different shear rate conditions, with three repetitions of each condition. In addition, the complete system will allow for the maximum use of 16 of these test blocks, which equaled to a total of 384 separate test runs. A 1mm step had been cast into the bottom face of the block in order to align the block into its respective position in the blood loading mechanism, which will be explained later.

As the rest of the testing process was thought out, the question about how buffer would be run through the testing section was brought up. The buffer would flow in the direction opposite to how the blood flowed and therefore a vacuum connection at the blood loading side of the test block was necessary. The bottom face of the test block, which faces down when the block sits on the blood loading mechanism has vacuum exit tubes built into it. These exit holes are connected to tubing which, in turn, exit out the narrow side of the testing block, and eventually to a vacuum source.

The first physical model of the test block is shown in Figure 5.
Figure 3: Schematic of the test block with dimensions included in mm. (B=4.00mm, C=2.00mm, D=1.00mm, E=2.00mm, F=2.50mm)

Figure 4: Three-dimensional drawing of the test block.
After creating the first physical model of the test block, several considerations were added to the block's design. First, it was found that the use of a transparent silicone would prove useful to simply view whether liquid was entering a particular tube, as well as inform the user about any defects or damages to the glass capillaries. The next observation that was made concerned the elasticity of the silicone material. The original silicone was found to be very elastic, and although that is good for the protection of the glass tubes, this characteristic can also be detrimental to the proper and accurate alignment of the male and female ends of the tubing. A material with different mechanical characteristics or a method of stiffening the structure would be necessary for the final design. Third, it was found that performing any operations around the protruding male ends of the tubing was potentially dangerous in that they could easily be broken. Therefore, any part in contact with this face of the block needed to have plenty of accuracy and clearance to account for these protrusions, to allow for any procedures involving this area to ensue without hitch. Finally, it was observed that as a product of the sheer number of connections between the testing and resistance blocks, it may be difficult to separate the connected blocks without possibly harming the tubes.

The first two issues were dealt with together by finding a suitable silicone rubber to use for the final application. The rubber needed to meet the particular demands of a long enough working time to pour into the mold, transparency in order to be able to see the tubing, and a stiff enough constitution to provide proper protection for the glass while allowing for relatively accurate positions of the glass tubing. The material also had to be suitable for biological experiments where it would come into contact with blood, and had to be able to withstand several autoclave cycles. After mixing and testing the properties of different brands and types of silicone rubbers, we found that the Silastic MDX4-4210 was the best choice.

The third issue, which concerns the accuracy involved in lining up the components correctly in order to protect the fragile tube ends, also helped to solve the problem of the elasticity of the rubber. The design of the test block was adapted to allow for the passage of three guiding tubes along its length. These guiding tubes allow one to pass stiff metal rods through the body of the test block and then through the resistance block in order to more easily guide the male ends of the tubes in the test block into the female ends of the tubing in the resistance section. The extra stiffness imparted onto the test block structure
by the guiding tubes counteracted the excessive elasticity of the material. In addition, the organization of the three guiding tubes in each test block allowed for the intuitive orientation of the testing blocks when mating with the resistance block because of the fact that the blocks could only fit in one manner.

The final potential problem found through the physical model dealt with the ease of removing various test blocks from the large resistance block. In order to make this process easier, it was believed that if the various test blocks could be connected in some way to handle it as a single large block, this process would be much easier. Therefore, the design was once again modified to include the passage of two small holes that traverse the width of the block. When all the blocks were lined up, threaded rods could be passed through these holes in order to hold together the 16 blocks as a single unit.

3.4.2 Resistance Block
The resistance block was originally designed as a 13cm long block with the same footprint as the test block. Specifically, the dimensions of this design called for a 36mm x 13.5mm x 130mm block. This block sat upon the test block and provides the necessary amount of resistance to the blood flow through each tube in order to control the wall shear rate of the fluid. The original design for the resistance block called for 16 blocks to be arranged in an 8 x 2 manner in the apparatus, in order to line up with the wells of the 384 well plate. The glass tubes were again aligned in an 8 x 3 square matrix manner, with 4.5mm between the centers of each tube along both the length and width dimensions. The glass tubes used in the resistance block were of 3mm OD and an ID that varied between 0.09mm and 3mm, according to the results of the calculations from Section 3.2.1.1. A schematic of the resistance block is shown in Figure 6 and three-dimensional drawings are shown in Figure 7.
Figure 6: Schematic of the resistance block. With dimensions included in mm. (A=4.00mm, B=2.00mm, C=10.00mm)
In the case of the glass tubes used for the resistance block, it was initially decided that a wide entrance on the female end of the resistance tubing would be very helpful when trying to mate it with the male end of the test tubing. The female end of the resistance tubing was then designed to have a lead in which we would bore out in a shop. The female ends of the tubing would then sit flush against the surface of the resistance block that mates with the test block. The opposite face of the resistance block was designed to mate with a vacuum connection that would also allow for the exit of blood from the glass tubing. In order for blood to flow out of the tubing and out of the system without adding any resistance to the flow of blood behind it, it was determined that the tubing ends passing through this block face had to extend 2cm. This allowed the blood to run through the tubing and fall out into a basin that would remove the blood from the system in a sanitary manner.

After the creation of the first physical model of the test block, several improvements were made on the design of the resistance block. The initial design of the system included 16 resistance blocks to mate with 16 test blocks. It was found that there was no need for 16 individual resistance blocks, so a new single block was designed. This block utilized the same sized tubes, but they were arranged in a 24 x 16 matrix, still spaced at 4.5mm between the centers of the tubes. This design change would help create a better airtight connection with the vacuum connection, as well as ease the process of assembling the device, since aligning fewer parts makes the process quicker and safer.

Another observation made from the first physical model was the need for features that help to align the male and female ends of the tubes. This feature was already discussed in
the previous section. It involved the passing of guiding rods made of tungsten carbide through stainless steel tubes cast through the long dimension of the resistance block. The arrangement of the stainless steel guiding tubes was such that it matched exactly with the arrangement on the test blocks, and only allowed for the blocks to be mated in one manner, so as to not confuse the direction in which the blocks sat.

3.4.3 Blood Loading Mechanism
The blood loading mechanism (BLM) was designed as a stand for the whole apparatus, on which the test blocks would directly rest. This piece had 16 2mm deep wells arranged in an 8 x 2 configuration. These wells were dimensioned to give clearance to the 1mm step at the end of each test block, and align the blocks in relation to each other in order to match the tubes to the wells in the 384 well plate. At the bottom of each well in the BLM, there was a hole that passed through the thickness of the piece that allowed for a nozzle to connect the well to a blood supplying tube. A schematic including the dimensions of the device is shown in Figure 8, and a three-dimensional drawing of the piece is found in Figure 9.

![Figure 8: Schematic of the blood loading mechanism with dimensions included in mm. (A=3.18mm)]
The nature of this piece required it to be in contact with a great deal of blood, and therefore needed to be constructed of a material that was compatible with blood and did not react in its presence. The piece was designed to be made of Silastic® silicone rubber which is safe for use in medical devices and pharmaceutical applications, and is also safely sterilized in an autoclave.

3.4.4 Vacuum Connection

The vacuum connection (VC) was designed as the piece that would sit on top of the resistance block, which in turn sits on top of the test blocks. This connection would provide the system with the pressure drop necessary to drive the blood from its reservoir and through the test and resistance tubing. The VC has enough clearance to allow the resistance block to slip into the piece, with a 3.35mm deep lip surrounding the block, to ensure a tight vacuum seal. The piece also had enough clearance to allow the 2cm tube protrusions from the resistance block to fit safely. There was also another hole on the short side of the VC that allowed for blood that had flowed through the tubing and spilled out through the protruding end to escape the system. A nozzle was be fitted to this hole and another vacuum hose connected to the system to allow for the removal of the used blood. A schematic of this piece with dimensions is presented in Figure 10 and a three-dimensional drawing is presented in Figure 11.
Figure 10: Schematic of the VC with dimensions included in mm. (A=6.35mm, B=3.00mm, C=6.35mm)

Figure 11: Three-dimensional drawing of the VC.

As shown in Figure 11, the interior of the VC contained a set of tapped holes of 9.525mm (3/8") depth of a diameter equal to that of the guiding rods that passed through the test and resistance blocks. The VC will serve as a stable base off of which the guiding rods can be aligned, which in turn aligns the resistance and test blocks. In addition, the VC
could connect to the test blocks when they were organized in their 8 x 2 matrix, during the platelet assaying phase of the testing. During this phase, Triton X was sucked up into the test tubing, and held in place for about 30 minutes. A vacuum connection was necessary to introduce the Triton X into the test capillaries, and it was therefore convenient to be able to utilize the same vacuum connection for both phases of the testing. Finally, this piece will most likely be made of a material that is easy to machine but also strong enough to allow for the guiding rods to align parallel to each other. In this case, a material like aluminum may work best.

3.4.5 384 Well Plate Connection / Buffer Hopper

The buffer hopper (BH) was originally designed as a piece of this system that would fit on top of the test blocks and serve as a container that would simply hold a supply of buffer. This buffer was then sucked downward through the test tubing, into the blood hopper, and out through the secondary vacuum holes found on the long side of the test blocks. The design constraints of the BH were that it simply had to fit around and on top of the 16 test block matrix, create a water-tight seal against the rubber of the blocks, and be able to hold enough buffer solution to clear out the test tubes. The original design was composed of a sort of framing piece that sat around and above the test pieces with the use of a stepped lip, and had a tall wall that created an open top reservoir for the buffer with the use of the surface of the top of the test block.

The 384 well plate connection (WPC) was a piece designed very similarly to the BLM. The top of the WPC was designed and dimensioned exactly like the top of the BLM, with rectangular wells arranged in an 8 x 2 matrix. The bottom of the WPC was where the design differs from that of the BLM. The bottom had a section extruded from it that allowed for it to mate with the top of a 384 well plate. A 384 well plate had a particular rectangular shape with a cut off corner, which did not allow for confusion when mating the part to the WPC. A photograph of a 384 well plate is shown in Figure 12, and its dimensions are found in Figure 13.

Figure 12: A generic 384 well plate.
Since one side of the bottom mated directly onto the 384 well plate and the other side mated to test blocks, the piece lined up the test tubes to their corresponding wells on the plate. Another difference between the WPC and the BLM is that the wells in the WPB passed through the thickness of the material to create rectangular holes clear through the piece. The material left between the wells allowed the test pieces to align correctly against each other with the use of their stepped ends.

The most current design for the 384 well plate connection combined its design with that of the buffer hopper. The overall design of the WPC remained mostly the same. The only difference was found in the greater thickness of the material, and the increased depth of the extrusion in the material that allows for the 384 well plate to mate with the WPC. The WPC can be utilized as a buffer hopper by turning it over and mating one side against the open end of the test blocks during the buffer step of testing. The other side of the WPC, which was capable of mating with a 384 well plate, could now double as a buffer hopper that sat above the test blocks. A schematic and a three-dimensional drawing of this piece are shown in Figures 14 and 15 respectively.

Figure 13: Dimensioned schematic of a 384 well plate with dimensions included in mm and inches.
Figure 14: Schematic of the 384 well plate connector / buffer hopper with dimensions included in mm.

Figure 15: Figure __: Three-dimensional drawing of the 384 well plate connector / buffer hopper. The top side, which mates to the bottoms of the test blocks to connect to the 384 well plate, is shown on the left. The bottom side, which is the buffer hopper, is on the right.

Since the depth of the extrusion that mates with the 384 well plate was increased, a spacer now has to be placed under the plate in order to raise it off of a table and butt it up against the inside lip at the end of the extrusion. The piece will be fabricated from delrin because compatibility with blood is not required in this piece, and the material is easy to machine. The combination of two functions into one part made the whole setup of the apparatus easier and it was simply one piece less to manufacture.
4.1 Purpose of Biological Validation
The final type of bench top testing that was performed was a biological validation. The efficacy of the actual testing method needed to be examined before the components were created. These tests were basically trying to answer the question about whether a significant biological signal could be read from the 10cm long, 300 micron I.D tubes. These tests would also indicate other problems that may arise once the components of the machine were created and assembled, such as the formation of blood clots in the tubing, the effects of reversing the flow when passing buffer through the tubes, and the effect of priming the apparatus before running it.

4.2 Test Method Employed During Biological Validation
The test method used was basically the core steps of the method outlined earlier for the use of the final testing apparatus. Six capillary tubes were introduced into the ends of pipette tips, which were, in turn, connected to rubber tubing that connected all the pipette tips in series. This rubber tubing was then connected to the end of a syringe pump. This setup is shown in Figure 15.

The syringe pump was filled with about 3mL of blood, turned on, and left at a setting of 108 mL/h. The blood from the syringe is expelled through the capillary tubing and out to a waste container with bleach. After the 1-2 minutes of running the blood through the tubes, the tubes were removed from the pipette tips and connected to a new rubber tubing/pipette tips setup. This tubing system was initially primed with Tyrodes buffer, and then the open ends of the capillary tubes were placed into a bath of more buffer and the pump was set at 108 mL/h. The buffer ran in the opposite direction as the blood, to ensure that no more blood passed through the tubing and changed the results of the initial blood run. The buffer solution ran through the tubing for about 2 minutes, after which, the capillary tubes were removed from the
rubber tubing/pipette tip setup, and installed at the end of new pipette tips on a multipipetter. At this point, 7.5 μL of prepared 1% Triton X solution were sucked into each of the six tubes, and the fluid was suspended in the tubes for a period of 20 minutes. Finally, the solution in the tubes was expelled into 6 wells of a 384 well plate. Another 7.5 μL of Cytotox 96 was placed into each of the wells, and the plate was placed into a plate-reading spectrophotometer set at 490 nm wavelength. Finally, the plate reader outputted its light readings in the form of a print out with relative light intensity measurements on it.

4.3 Results and Conclusions

The biological validation testing results indicated that the capillary tube provided biological signals on the same level as the rotating tube tests [35]. The more important observations derived from these tests dealt with the fragility of the glass tubes, the difficulty in lining them up, and the ease with which blood could clot inside the tubes. Because of the fragility of the tubes and the amount of times that they needed to be removed from one set of rubber tubing and attached to another set, a connector was made to hold all of the glass tubes together. This connector decreased the amount of broken tubes and the amount of time needed to make and break all the connections. The decreased amount of time between test phases also helped with the clotting of blood, which was found to be a considerable problem at times. The longer blood stayed stagnant in the pipette tips or in the capillary tubing, the higher the chance of a blood clot rose. The emergence of a blood clot would block nearly, if not all the blood to one of the tubes, which changes the flow rates through all the other tubes and provides no useful results from the blocked tube. At times, a blood clot would also be difficult to detect during a test, so that a possibly fixable problem would go unnoticed and unresolved. It was finally determined that this sort of problem would be much less prevalent in the proposed apparatus because of the easy and swift manner in which one can pass from one phase of a test to another. In addition, the apparatus will have very little volume devoted to loading the blood into the tubes, where blood could either become stagnant or completely clot a path.
CHAPTER 5

Conclusions and Future Directions

In review, this document described the process taken in choosing the one-pass, linear flow, high-throughput system as opposed to the rotational high-throughput system. The testing system was chosen on a theoretical fluid mechanics basis. The multiple components for this system were then designed individually, and the designs evolved for usability of the machine as a whole. Solid models of the evolved components were created as a "rapid prototyping" mechanism, in order to determine potential problems during the use of the machine. In order to test some of the important mechanical and biological aspects of the apparatus, bench top tests had been formulated. A preliminary model of the testing block was created and handled in order to find ergonomics and safety problems that could not be determined from solid models on a computer screen. Currently, biological bench top testing of the blood flow mechanism is being conducted, to finalize the details concerning blood shear rates, flow of buffer through the tubes, and assaying and reading of the biological signal.

The most immediate next steps in this project involve the design of mold pieces to quickly and easily cast the test and resistance blocks. After that, the mold pieces and other parts of the machine will be machined and assembled. After the flow system machine has been created, a series of test may be applied to establish the machines accuracy when, for example, different concentrations of chemicals are used to treat blood samples or coat the tube surfaces. Also, tests would be formulated to determine the precision of the system and the useful number of experimental repeats to ensure the dependability of the results.

Pre-clinical studies should follow these accuracy and precision tests. These pre-clinical studies include testing on: "normal" populations, animal studies, and validation studies. Testing on a "normal" population means that the tests will utilize the blood of relatively healthy persons. These tests would allow for the establishment of the range of biological signals derived from healthy persons, which in turn, begins the calibration of this instrument as a possible future clinical tool. The next step, animal studies, will allow for the creation of perturbations in the animal's blood and cardiovascular system. These perturbations by coagulants and anticoagulants will create controlled variations in animals, resulting in specific biological signal readings to add to the calibration of the device.

The final type of testing in the pre-clinical studies is the validation study, where a population with a known disease would be chosen and tested upon. These results could be compared to those from the animal testing and can add to the results used toward the calibration of the device. The patients utilized in this study fall into two categories: low-coagulant states and pro-coagulant states. Persons in low-coagulant states may exhibit, for example, vWF disease, Thrombophilia A or B, Factor 8 or 9 deficiencies, Bernard Soulier syndrome, or Gladzmann's thrombocytopenia. Persons in pro-coagulant states may exhibit Factor 5 ligand - hypercoagulable state, high homosystein, lupus anticoagulant, antithrombin 3 deficiency, or protein c or s deficiency.
Following the pre-clinical studies, the last step is the clinical studies. By this phase, several machines will have been produced and placed into clinical settings. In the case of clinical thrombosis, patients are usually given antithrombotics or anticoagulants. At this point, the goal would be to have this machine used as a tool to determine if and how much anticoagulant should be used on a particular patient. In conclusion, the ideal case to test out the capabilities of this system would include application on a patient or population where the decision to use antithrombotics or anticoagulants in the treatment of thrombosis is not obvious or easy to make. This sort of future testing would test out the last set of capabilities and performance parameters, which could potentially open the door to widespread use of this new useful and efficient clinical and research tool.
References


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