Analyzing the Hemolytic Effects of a Microchannel-Based Blood Processing Device

by

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While hemodialysis is a unique therapy for treating chronic kidney failure, the application of extracorporeal blood processing presents the opportunity for treating a wider range of bloodborne diseases. The objective of the i-Blood research team is to develop a blood processing device that utilizes microscale-based technology as a platform for incorporating novel bioconjugation mechanisms to remove/degrade undesired solutes in the blood or administer drugs. The development of treatment mechanisms for hyperuricemia (high concentrations of uric acid in the blood) and iron overload (high concentrations of iron in the blood) have been the focus for other individuals on the research team. During preliminary development of the device, one concern that came up was the potential for mechanically induced hemolysis due to the foreign flow conditions imposed by the geometry of the device. While red blood cells (RBCs) are especially adept at modifying their shape to endure high pressures in arteries and small orifices in capillaries, the concern remains that the flow through the foreign materials of the device could inflict enough shear stress to break the cell membrane of RBCs. In order to provide a therapeutic benefit, it is essential that the team can identify flow conditions that minimize risks to the patient (i.e. minimized blood damage and coagulation) while simultaneously maintaining the intended functionality of the device for a given disease.

The primary objective of this project was to characterize the degree of blood damage in a novel microchannel-based blood processing device by quantifying the hemolytic effects of various flow parameters. A nuanced methodology of measuring the plasma-free hemoglobin concentrations via a hemoglobin detection assay and absorbance spectroscopy was developed. The effects of fluid velocity and number of passes through the device were investigated using a syringe

pump apparatus. Experimental results indicated that velocity had an insignificant effect on the hemolytic effects of the device for a single pass while increasing the number of passes at a constant flow rate caused an increase in hemolysis. No specific plate geometry recommendations can be made based on the current results, though recommended next steps and identified concerns for future investigation are discussed in detail. Ultimately, the research project was successful at developing a reliable methodology for quantifying blood damage which can be used throughout the design process.

Key Words: Microchannels, Extracorporeal Blood Processing, Plasma-Free Hemoglobin, Red Blood Cells, Hemolysis, Absorption Spectroscopy, Blood Damage, Hyperuricemia, Iron Overload, Chronic Kidney Disease

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Introduction

While over 468,000 Americans rely on hemodialysis to treat chronic kidney failure, the concept of removing bulk amounts of undesirable solutes from the blood via an extracorporeal procedure presents opportunities to treat a wide range of medical conditions [1]. This perceived versatility of extracorporeal blood treatment options has led to optimism for future development, although there are certain challenges that may limit the progression of expansion and ultimate effectiveness of the procedure for new applications. The foremost concern when removing blood from the body during hemodialysis is maintaining the condition of the blood throughout the process, especially when returned to the body after external treatment. Activation of coagulation is one clinical problem related to current hemodialysis treatments that may result from fluid stresses experienced within the dialysis machine. Returning blood into the body after the coagulation cascade has commenced raises the concern of thrombi developing in the patient's bloodstream [1]. This problem is combatted pharmacologically with the administration of anticoagulants such as heparin to disrupt the thrombotic process; however, for patients susceptible to bleeding, these blood thinners pose the risk of excess bleeding for patients [1]. While citrate has been recognized as a suitable alternative for reducing the risk of bleeding, studies have shown it can also increase the sensitivity of red blood cells to hemolysis [2] [3]. Additional complications that patients experience include hypotension, anemia, muscle weakness, and hyperkalemia as a function of the procedure itself which further complicates the body's ability to respond to the influx of blood with an inconsistent composition [4]. Previous research has suggested the enhanced inflammation and oxidative stresses imposed on blood during hemodialysis have an effect on atherosclerotic plaque formation and can increase cardiovascular risk for hemodialysis patients [5]. Unfortunately, limited research has been conducted to evaluate the dynamic effect these various changes to blood composition have on patients in the short and long-term [1] [5].

To improve the long-term quality of life for patients that have to routinely receive dialysis treatment, recent developments in hemodialysis have been focused on developing home hemodialysis (HHD) machines [6]. To ensure the effectiveness of home hemodialysis, updated dialysis models require design features that enable the patient and clinician to be confident in the machine's ability to limit medical complications without the need for frequent professional medical intervention [6]. Rather than relying on pharmacological solutions to address the myriad

of complications that can potentially develop from extracorporeal blood processing, developments for new treatment applications should prioritize preemptive measures in device design in order to limit the risk of coagulation and hemolysis. At Oregon State University, the i-Blood research team, led by Dr. Goran Jovanovic, is striving to develop a microchannel-based platform capable of employing bioconjugation mechanisms for treating a wide-range of hematological defects and imbalances not directly related to chronic kidney failure. The advantage of extracorporeal treatment is the ability to control conditions that may cause adverse biochemical complications internally. Developing extracorporeal treatment mechanisms could offer many patients that are currently relying on complicated drug mechanisms a safer, more convenient alternative; however, some of the complications that chronic renal patients experience as a consequence of dialysis could translate to these new applications as well. The two concerns guiding device development are the potential coagulative and hemolytic effects a microchannel-based platform may inflict on a patient's blood.

The focus of this study revolves around the desire to identify appropriate process flow conditions to mitigate the potential for undesired hemolysis occurring during treatment within the current plate geometry seen in *Figure 4.* While red blood cells (RBCs) are highly differentiated to survive constant fluctuations in flow conditions within the body's blood vessels, the foreign material of the microchannels may inflict increased shear stress causing elevated degrees of hemolysis and the activation of the coagulation cascade under certain flow conditions [7]. Quantifying the degree to which this foreign environment plays a role in increasing hemolysis is vital for understanding the effects the device has on blood before being reintroduced into the body. A series of experiments using bovine blood were performed with the latest version of the polycarbonate microchannel plate featuring an array of microscale posts. These experiments are intended to help establish reasonable flow parameters to inform the development of effective therapeutic mechanisms. A primary outcome of this research project was developing and validating a methodology for quantifying degrees of hemolysis for the investigation of blood damage in future versions of the device that incorporate these therapeutic components.

To quantify the degree of hemolysis caused by the device, the blood's plasma-free hemoglobin concentration was analyzed before and after passing through the device using absorption spectroscopy. It was assumed that since bovine and human blood have a comparable

composition and similar resiliency to shear, that results using bovine blood would model the expected results in human blood as well. One important difference between bovine and human blood is that the normal accepted average plasma-free hemoglobin in a healthy human is no greater than 5 mg/dL but the baseline concentration is much higher in bovine blood [8]. A hemoglobin assay kit from Sigma-Aldrich utilizing the Triton/NaOH method was used to convert plasma-free hemoglobin into the colorimetric complex product hematin, which is measurable at 400 nm using absorption spectroscopy [9]. This assay was originally intended for analyzing total hemoglobin concentrations as it features a high pH capable of causing the cell membrane of RBCs to burst [9]. Therefore, for the purposes of this study, the assay was performed only on plasma, which was separated using a centrifuge, such that the degree of hemolysis caused solely by the flow through the microchannel device could be determined via changes in only the *plasma-free* hemoglobin. The effect of flow rate (velocity) and number of passes through the device were explicitly evaluated during this project with the intent of developing a preliminary assessment of the device's tendency to cause mechanically induced hemolysis.

Background

Target Diseases for Therapeutic Extracorporeal Blood Processing

The following section details a brief background on the current application of extracorporeal blood processing (hemodialysis) and delves into the potential opportunities of expansion for new disease treatments that are currently being investigated by the i-Blood research team. Understanding and being aware of the potential requirements for developing a treatment specialized for each disease is advantageous for predicting how various therapeutic components may influence important flow parameters and the results of this study.

A. *Traditional Extracorporeal Dialysis Used to Treat Chronic Kidney Disease*

The majority of patients receiving dialysis treatment today do so to counteract the effects of prolonged chronic kidney disease (CKD) which eventually leads to the development of chronic renal failure (CRF) [10]. Loss of kidney function occurs in stages for CKD patients, but once total kidney failure occurs, they will rely on dialysis for the rest of their lives to perform the functions of an artificial kidney. Stages of CKD progression have been defined by the National Kidney Foundation based on estimated glomerular filtration rates (eGFR) which essentially characterizes the organ's ability to process blood [11]. End-stage renal disease is defined as an eGFR of less than 15 mL/min per 1.73 m^2 of renal tissue while a normal eGFR is defined as the ability to process at least 90 mL/min in that same area [11]. The kidneys play a crucial role in many metabolic, hormonal, and regulatory processes the body relies on to function properly. The main function of kidneys is to process blood by eliminating metabolic waste, minerals, and excess water via excretion in the form of urine; however, they perform other vital functions including the regulation of blood pressure, stimulation of red blood cell formation, and maintenance of vitamin D levels in the blood [12]. Many diseases can lead to renal impairment, but the two primary risk factors are diabetes (leading to diabetic nephropathy) and high blood pressure (leading to hypertensive renal damage) [12]. Patients with diabetes mellitus experience dangerous fluctuations in blood sugar levels causing damage to renal vasculature eventually leading to inhibited blood flow [13]. Alternatively, chronic hypertension causes arteries to weaken over time leading to the inability to deliver blood to kidney tissue for processing [12]. Regardless of the source of damage, the diagnosis of CKD is often associated with side-effects including fatigue, anemia, hematuria, muscle cramps, mineral concentration fluctuations, water retention (leading to edema), and

hormonal imbalances, if not successfully treated before advancing into later stages, nearing CRF [11].

Two dialysis options exist for patients in need of artificial blood filtration: peritoneal dialysis and hemodialysis. While both methods are effective for short-term treatment, patients enduring dialysis still experience a reduced life expectancy with large variations dependent on accompanying medical conditions or other complications associated with the treatment [11]. Both options operate using similar principles incorporating a semi-permeable membrane to promote simple diffusion of solutes down their concentration gradient to remove water, ionic solutes, urea, and other undesirable solutes from blood [14]. As seen in *Figure 1* below, peritoneal dialysis uses the peritoneum as a natural semi-permeable membrane to remove waste and excess water by filling the abdomen with fluid for a defined period of "dwell" time before the fluid, now containing increased concentrations of waste, is drained [15].

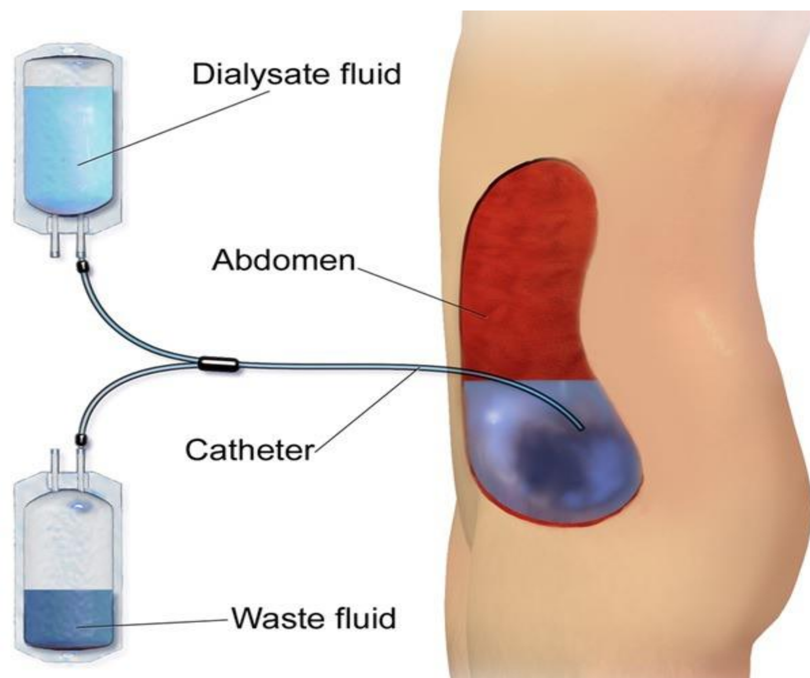

Figure 1 – Visual representation of peritoneal dialysis adapted from an article included in the Journal of Global Health [45]. The peritoneum acts as a natural semipermeable membrane for waste exchange from the surrounding vasculature into the fluid filled abdomen. The waste fluid is then removed through the same catheter, after sufficient exchange has occurred, now containing waste from the patient.

Many patients prefer hemodialysis which utilizes what is essentially an artificial kidney machine to remove blood from the body and filter it through a semipermeable membrane with a counterflow fluid promoting the transfer of waste before reentering the body [14]. The procedure of hemodialysis is depicted in *Figure 2* below.

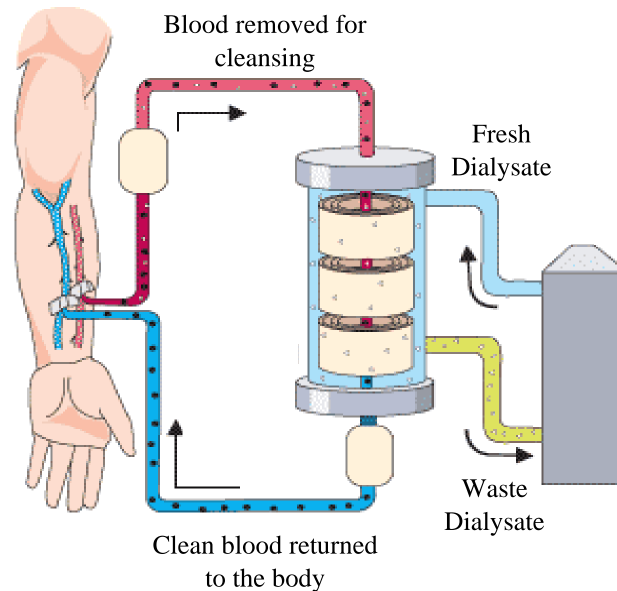

Figure 2 – Visual representation of the process of hemodialysis adapted from a review of dialysis waste disposal by Mike Kurz [46]. Fresh dialysate and blood needing cleansing are sent through the dialyzer device containing a semipermeable membrane to transfer solutes from the blood to the dialysate. The waste dialysate is disposed of and the filtered blood is returned to the patient.

The fundamental treatment model used in hemodialysis of processing blood outside of the body is the basis for the current development of more specialized blood processing mechanics. Although the i-Blood microchannel device in development is not designed for removing large volumes of water and does not incorporate membrane technology as in traditional dialysis, the platform is intended to support specialized enzymatic and biochemical features capable of targeting specific undesired solutes that are characteristic to a certain disease.

B. Sepsis

Sepsis is a systemic blood infection that results in a rapid immunological response causing several short-term effects including hypotension, hyperpyrexia, tachycardia, and dyspnea, but if untreated or mismanaged could lead to end-stage organ dysfunction and death [16]. Despite recent

developments in pathophysiological understanding of sepsis, the lack of advancement in hemodynamic monitoring tools for early detection and rapid resuscitation measures for late-stage action has led to 28-50% of the 750,000 cases per year of sepsis in the United States resulting in death [16] [17]. Dysregulated host response to an ordinary infection can develop into the medical emergency of septic shock due to endotoxins released by bacteria experiencing environmental stress, rapid growth, or cell wall disintegration [16]. Endotoxins are lipopolysaccharides found in the cell wall of Gram-negative bacteria that induce inflammation, severe hypotension, and blood vessel damage when introduced into the circulatory system of humans [17]. Current sepsis treatment approaches feature a protocol-based care plan featuring anti-inflammatory agents such as glucocorticoids, antibiotic administration, vasopressors to increase blood pressure, intravenous fluids, and immune system boosters [17]. Regardless of the treatment plan, the diagnosis of sepsis is an expensive ordeal accounting for 5.2% of the hospital cost in 2011 while also requiring extended hospital stays [16]. In an effort to expedite the recovery process, a recent OSU Honors College thesis discussed the possibility of using cationic amphiphilic peptides (CAPs) to selectively bind blood pathogens using a hemoperfusion process [18]. Further development related to this theory has led to a biocompatible design featuring polyethylene oxide (PEO) polymer chains terminating with the active CAP as a potential option for implementation into the microchannel blood processing device [19].

C. Hyperuricemia (Gout)

Hyperuricemia is characterized by abnormally high levels of serum urate (sUa) in the blood which can ultimately lead to gout, a common form of inflammatory arthritis characterized by the deposition of urate crystals found primarily in joints and the urinary tract [20]. The accumulation of serum urate develops due to an imbalance in the body's ability to metabolize uric acid [20]. The normal human metabolism produces uric acid via endogenous and exogenous purines catalyzed by the enzyme xanthine oxidase [20]. Although most mammals have the enzyme uricase to oxidize uric acid into a more soluble compound allantoin, humans lack this enzyme and instead rely entirely on the kidneys for uric acid disposal [21]. This enzyme deficiency leads to a relatively high normal uric acid concentration in humans, when compared to other mammals, of 0.5-2.0 mg/dL [20]. Hyperuricemia is commonly found with other comorbid diseases such as hypertension, cardiovascular disease, chronic kidney disease, and type 2 diabetes, though it is not

well understood if hyperuricemia is a factor in the development of these conditions or if it is a consequence of the disorders themselves [20].

Currently, there are several treatment options for patients including xanthine oxidase inhibitors to halt the production of uric acid, uricosurics for enhancing the kidney's ability to process uric acid, and intravenous administration of a functional recombinant uricase enzyme for degrading uric acid [22]. Pegloticase is one such recombinant mammalian uricase modified for treating refractory gout via the degradation of uric acid [23]. While effective, this option has a few limitations that have motivated further investigation into alternatives. The primary concern associated with the administration of pegloticase is the 20% chance that it will illicit an immune response in a patient when the common mPEG moiety is used [23]. Additionally, the initial treatment requires biweekly administration over the course of three months with the potential for monthly administration thereafter [23]. Research efforts have been made to use molecular engineering to enhance the enzymes efficiency, though an immune response remains a concern. If the uricase enzyme could be immobilized in a blood processing device, the amount of time the blood contacts the enzyme is greatly reduced, thus decreasing the potential for an immune response. Furthermore, the concentration of the enzyme can be adjusted to ensure maximum access to the active site, thus increasing efficiency. Although there may still be the potential for antibodies targeting the foreign uricase enzyme to develop after extended reliance on this procedure, utilizing an extracorporeal blood processing device offers a quicker and potentially safer treatment option. The i-Blood research team is currently investigating potential mechanisms for integrating uricase into the blood processing device for treating hyperuricemia.

D. Iron Overload

Iron is involved in many physiological functions including oxygen transport, DNA synthesis/repair, and the electron transport chain; however, most iron is safely bound to the glycoprotein transferrin in the body [24]. Non-Transferrin-Bound Iron (NTBI) is the fraction of ferric iron in the body not bound to transferrin or other iron transport proteins [25]. When ferric iron starts to exist in its loosely bound form within blood plasma, several physiological complications, including the potential for hydroxyl radical formation, become a potential threat to patients [25]. These radicals cause oxidative damage to the heart, liver, and other vital organs if left untreated [24]. There are several clinical conditions that can result in the development of iron

overload; two of the most notable are alpha/beta thalassemia and hemochromatosis [26]. Thalassemia is a genetic blood disorder that causes an abnormal form or inadequate supply of hemoglobin [25]. This lack of hemoglobin supply results in a large quantity of red blood cells to lyse causing anemia [25]. Additionally, since hemoglobin normally contains four iron atoms for oxygen transport, the lack of functional hemoglobin production causes the accumulation of iron as transferrin becomes saturated. Hemochromatosis is a disorder characterized by the over absorption of gastrointestinal iron [26]. Intrinsic iron metabolic mechanisms are unable to maintain homeostasis when these diseases impact a patient, resulting in iron overload [25].

Many treatments targeting high concentrations of loosely-bound iron utilize chelation therapy which neutralizes NTBI by forming a non-toxic complex with a chelator ligand that can be more easily excreted [26]. The most effective chelation therapies feature a continuous, non-toxic treatment with a long plasma circulation time and a strong selectivity for iron [26]. Administering these molecules as a drug have been an effective route for decreasing iron in the short-term, though most chelators used today have cytotoxicity concerns that ultimately limit the overall effectiveness of the treatment [26]. By immobilizing a specialized chelator molecule within a blood processing device, the iron can be bound and removed from the blood in one simple procedure rather than relying on the excretory for eventual removal. Furthermore, the concentration of these chelator molecules within the chelating film can be controlled to maximize chelation efficiency (percent of chelators that successfully bind iron).

Analysis of Hemolysis via Plasma-Free Hemoglobin

Blood contains four primary components including plasma, platelets, white blood cells (WBCs), and red blood cells (RBCs) [27]. Plasma compromises a large portion of human blood by weight containing water, enzymes, antibodies, and other proteins, making it vital for proper circulatory function [27]. Platelets are most often associated with coagulation as they form the basis of clots when damage to blood vessels occurs [27]. While platelet adsorption onto foreign surfaces is a functional concern of dialysis and blood processing in general, this project does not develop a methodology for analyzing the device’s tendency to trigger the clotting cascade. White blood cells come in a variety of forms including monocytes, eosinophils, basophils, lymphocytes, and neutrophils with each playing a crucial role in either the innate or adaptive immune system [27]. Selecting compatible materials of construction to reduce the potential for an undesired

immune response was a driving factor during preliminary design; thus, the initial design included a microchannel plate constructed from polycarbonate (using thermal embossing/etching), Tygon tubing, and a supportive structure composed of more polycarbonate and aluminum. All these materials have previously been used in FDA approved medical devices, promoting confidence that this combination of materials is capable of future approval [28] [29]. Avoiding leukopenia (shortage of white blood cells) is another obvious objective for extracorporeal blood processing since past research has suggested that WBC are potentially more susceptible to mechanical damage than RBCs [30]. Red blood cells are the component of blood responsible for oxygen transport throughout the body via the circulatory system and have multiple unique structural characteristics that enable its flexibility and resiliency to mechanical stresses [27]. This project's analysis of blood damage revolves around hemolysis (RBC membrane destruction) to simplify the complex interactions between the various blood components. By focusing on blood's most resilient component, the research team can develop a preliminary understanding of mechanical damage imposed by the device's geometry. If red blood cells are experiencing significant degrees of lysis under certain flow conditions, it can be deduced that white blood cells are also being damaged.

For the experiments conducted in this project, heparinized bovine blood was utilized due to safety regulations, availability, and similarities to human blood. Bottles of 500 mL of pre-heparinized bovine blood were purchased periodically from Hemostat Laboratories depending on the frequency of testing [31]. Previous research analyzing shear stress thresholds for RBC membranes compared human to bovine blood in their analysis, finding that bovine blood was slightly more resilient to mechanical stresses when compared to human blood [32] [33]. This is an important consideration when analyzing experimental results, though this relative mechanical fragility will not be used to extrapolate results directly due to the lack of control of shear stress when compared to the results of these previous studies. The serological similarities between the B antibody system for cattle and Rh antibody system for humans is another supporting factor for using bovine blood as a model for this study [34]. To reduce the occurrence of clot formation during storage and operation, the bovine blood was infused with the anticoagulant heparin. Despite this precaution, it was imperative that experiments used fresh blood to ensure minimal changes in the blood's natural characteristics.

To identify signs of disease in a diagnostic application, the complexities of blood often require an in-depth analysis to characterize any abnormalities or concentration fluctuations. Clinically, characterizing the relative health of all components found in a patient's blood is routinely performed via a complete blood count (CBC) analysis in tandem with other hematology tests to identify blood disorders and provide a holistic examination [4]. Applying this time-consuming test to evaluate the effect of adjusting multiple parameters related to an extracorporeal blood processing device was not considered a feasible option for this project. The decision to characterize blood damage via red blood cell lysis (more specifically the concentration of plasma-free hemoglobin) enabled a simpler methodology: a colorimetric hemoglobin detection assay. The Sigma-Aldrich assay employs the Triton/NaOH method for converting hemoglobin into hematin, a complex with Triton X-100, which absorbs light at a wavelength of 400 nm [9]. By measuring the degree of absorption at this wavelength with a spectrophotometer, the concentration of hemoglobin in a plasma sample can be determined [9]. The extreme alkalinity of the assay (pH of 13) would purposefully result in additional RBC lysis under normal conditions testing for total hemoglobin measaurements, blood samples were separated via a centrifuge to extract plasma samples treated using the assay. This extra step is performed to ensure that the changes in hemoglobin concentration occur only due to any mechanical damage inflicted by flowing through the microchannel device. Although hemoglobin is not normally present for long periods of time in plasma due to recycle mechanisms performed in the spleen, natural hemolysis causes the normal concentration in healthy humans to be as high as 5 mg/dL [8]. It should be noted that the plasma-free hemoglobin of the bovine blood was found to be considerably higher than this value. By comparing the concentration of plasma-free hemoglobin in experimental groups to the baseline concentration as a control, the relative degree of hemolysis can be deduced. Thus, significant changes in plasma-free hemoglobin will be easily detectable using the absorption spectroscopy procedure discussed in *Colorimetric Hemoglobin Detection Assays*. Other benefits of this methodology for estimating hemolysis include the relatively cheap cost of the assay and limited requirement for sample translocation.

A Closer Look at Red Blood Cells and Hemoglobin

Human blood is composed of approximately 45% hematocrit (RBCs) by volume which serves multiple physiological roles beyond its primary function of transporting oxygen and nutrients including interorgan communication and maintenance of sustainable blood rheology [35]. Due to their relative abundance, RBCs dominates blood viscosity with changes having a profound impact on flow biophysics due to their unique, elastic shape [36]. Healthy mammalian RBCs are biconcave discocytes (as seen in *Figure 3* below) that lack a nucleus creating more space for carrying hemoglobin, promoting a high surface-area-to-volume ratio, and enhancing flexibility to avoid deformation despite a constantly fluctuating circulatory system [36].

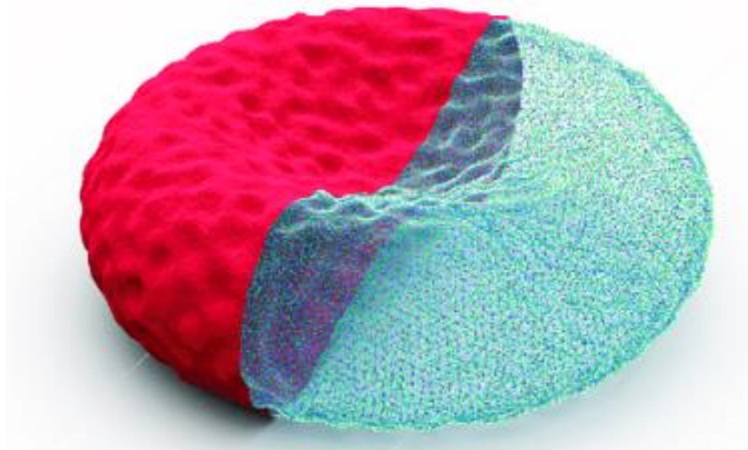

Figure 3 – Computer generated rendition of the biconcave geometry of a red blood cell modified from a simulation study from the Biophysical Journal [47].

In vivo, red blood cells experience a range of flow conditions and shear stresses from high flow in wide arteries to low flow in small capillaries, in some cases permitting only one cell at time [35]. A flexible membrane that contains more phospholipids and cholesterol than the average cell increases fluidity and allows red blood cells to alter their shape so long as their cytoskeleton integrity holds [35]. Hemolysis is a naturally occurring process in the body as a means of removing old red blood cells and releasing iron for recycling [37]. Elevated levels of RBC destruction most often occur due to hemolytic anemia in which an internal or external disturbance causes accelerated hemolysis resulting in an imbalance between this destruction process and the RBC replenishing capability of bone marrow [37]. Despite the flexibility and durability of RBCs, extracorporeal blood purification systems present foreign flow conditions creating enhanced mechanical stress

that is not encountered in healthy blood vessels. This increased mechanical stress could compromise the flexible cytoskeleton of the red blood cells passing through the microchannels, causing cell lysis. Targeting red blood cell lysis is thus an obvious choice to characterize blood damage because if even the most flexible component is susceptible to damage caused by flow through the microfluidic device, other components will also be at risk for deformation. Hemoglobin composes approximately a third of a red blood cell's total volume (270 million hemoglobin molecules per RBC) and plays a crucial role in its main oxygen delivery function [38]. Thus, when hemolysis occurs, millions of hemoglobin molecules are released into the plasma from a single RBC, which is the basis of this project's experimental approach to quantifying blood damage.

Hemoglobin is a metalloprotein found in red blood cells that binds oxygen for transport throughout the body [38]. The structure of hemoglobin itself consists of four polypeptide subunits (a tetramer) including two alpha chains and two beta chains used to stabilize a heme group which serves as the active site for oxygen binding to an iron cation containing ferrous group [38]. Hemoglobin denaturation via mechanical stress (flow through the device and centrifugation) or thermal conditions will be treated as negligible for the purposes of this study. While temperature and pH impact which conformation hemoglobin assumes, the assay remains functional regardless [9]. As previous blood damage research has suggested, operating at room temperature rather than heating the blood to physiological conditions won't have a profound impact on hemolytic effects and will eliminate the time-consuming process of safely heating the blood [39].

Materials and Methods

Schematic of the Microchannel-Based Blood Processing Device

Although new versions of the functional polycarbonate microchannel plate are in development, for all trials conducted in this project, the same design utilizing posts for altering flow was used. The main objective of these posts is to split up flow allowing for increased surface area for solute exchange with the active mechanisms ultimately integrated into the device. A schematic model of the device is provided in *Figure 4*.

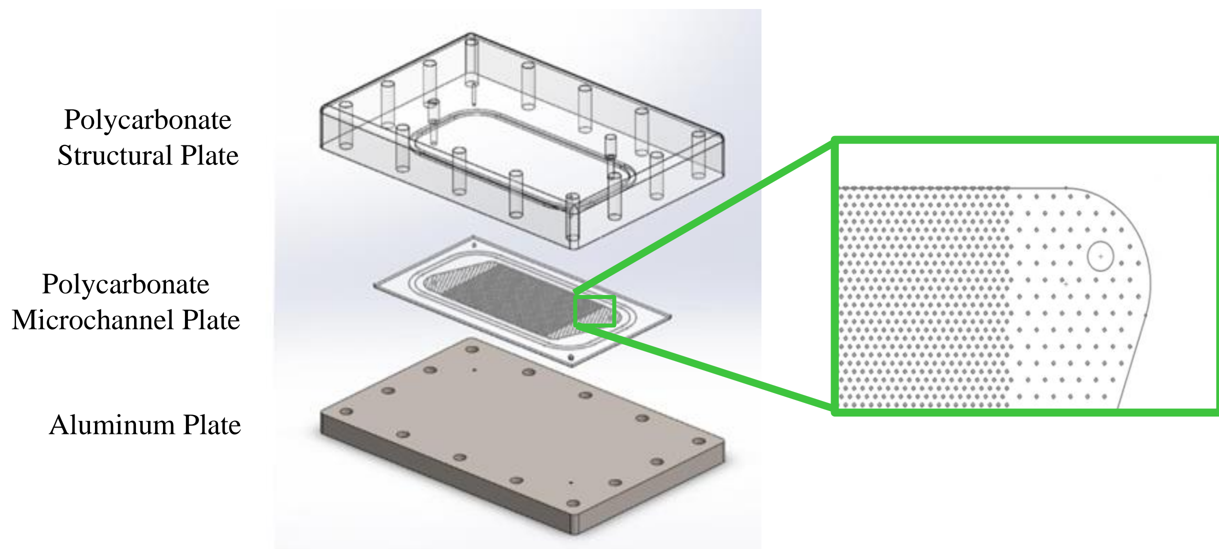

Figure 4 – This model is courtesy of Dr. Matt Coblyn, a contributor to the i-Blood project. Model of the sole plate design used throughout the blood damage project which consisted of an array of posts, designed to increase the surface area of blood available for contact with the integrated therapeutic mechanism. The average cross-sectional area of blood flow is between 3.47-4.47 mm^2. The average path length was approximately 10 cm and the width of the plate was approximately 5 cm.

Evaluated Flow Parameters

A. *Maximum Fluid Velocity*

The first parameter this project analyzed was the effect flow rate, and therefore fluid velocity, had on hemolysis rates observed in bovine blood traveling through the microchannel device. The main objective of the series of trials completed in this section of the project was to determine a reasonable range of velocities to inform future design and operating conditions. Ideally, a velocity and residence time to maximize the blood processing capabilities of the device without causing damage beyond an acceptable threshold can be identified by testing various flow rates with all other process parameters held constant; thus, all trials conducted in this section

featured a single pass through the device before a sample was taken for absorbance spectroscopy analysis. Three flow rates of 1 mL/min, 10 mL/min, and 30 mL/min were evaluated in the first set of experimental trials.

At elevated velocities, the flow will become increasingly turbulent, which is expected to cause increased mechanical stress on the system. Another concern turbulent flow introduces is the potential formation of dangerous air bubbles during operation that could ultimately develop into air emboli if returned into the patient's blood stream. The other extreme occurs if the bioconjugation mechanism being developed requires a velocity so low that coagulation becomes an increased threat. Although prospective patients may be administered an anticoagulant, clot formation is still an occasional complication that should be considered when developing a new blood processing device. Identifying realistic goals for internal average fluid velocity will thus greatly influence the development of these complicated therapeutic mechanisms.

Past research has analyzed this parameter of velocity with similar microfluidic dialysis devices and have successfully characterized an acceptable range of blood flow rates for their design, but there are expected to be significant differences when analyzing blood flow through the geometry of this particular blood processing device. This assumption is primarily due to the unique microchannels used as the active site for separations. For example, the first iteration of the microchannel plate uses a pillar configuration (see *Schematic of the Microchannel-Based Blood Processing Device*) for blood separation rather than straight channels that have been studied in previous work. This design was chosen to optimize surface area available for active coating but could cause excess turbulent flow resulting in elevated degrees of hemolysis.

To estimate the average fluid velocity through the microfluidic plate, the known flow rate set on the syringe pump is divided by an approximate cross-sectional area of the blood's path through the plate. The average cross-sectional area for the plate featuring pillars was determined to be between 3.47 mm^2 and 4.47 mm^2 with a total path length of approximately 10 cm. The smaller cross-sectional area estimate will require a velocity at the set flow rate. The recommended velocity range will attempt to minimize the amount of time the blood is within the microfluidic device while maximizing filtration efficiency and minimizing shear stress in this cross-sectional area of posts. The expected range of fluid velocities for the three flow rates tested is included in *Table 1*.

Table 1 – Summary of the expected velocity ranges for the three operating flow rates utilized in this study. For a detailed calculation example, see *Appendix II: Sample Calculations*.

Set Flow Rate $\left[\frac{mL}{min}\right]$	**Minimum Average Velocity** $\left[\frac{cm}{s}\right]$	**Maximum Average Velocity** $\left[\frac{cm}{s}\right]$
1	0.4	0.5
10	3.7	4.8
30	11.2	14.4

The estimated cross-sectional area and total straight path length of the microchannel plate were also utilized to calculate the total volume of the system to establish standards for cleaning procedures. Obviously, once the plate is being coated with a hydrogel or other material designed for solute extraction, the cross-sectional area will be reduced causing an increase in velocity; however, estimating an acceptable velocity range for operation will guide flow rate considerations in future designs.

B. Number of Passes Through the Device

The second parameter evaluated was the number of passes of the blood through the device using the syringe pump set-up and experimental methods. The purpose of evaluating this variable was to mimic actual blood processing operation and evaluate any "wear-and-tear" effects on the blood. For example, hemodialysis takes about four hours on average to slowly reduce the concentration of waste in the blood stream, which requires the cardiovascular system of the patient to pump blood through the dialyzer many times in a single sitting [40]. Potentially, successive passes through the device could cause wear on the RBCs resulting in elevated levels of hemolysis in later passes. Increased contact time with foreign surfaces could increase the potential for other concerns as well such as coagulation or an immune response. Due to the current experimental design using a syringe pump that must be reloaded with blood for each pass, the maximum number of passes through the device was limited five. Although the estimated number of passes required for the various therapies has not yet been quantified, it would be ideal to construct a continuous loop with a peristaltic pump such that total operation time for a given volume of blood could be tracked while other process variables are monitored. Such an integrated breadboard loop is

currently being developed by another member of the i-Blood research team but was not completed for the experiments in this project. For all multi-pass trials, the flow rate was held constant at 10 mL/min (the intermediate value of the three flow rates tested in the previous study).

Equipment Preparation

Before any equipment preparation was performed, it was ensured that all lab members wore the appropriate Bio Safety Level 2 PPE of safety glasses, a lab coat, and nitrile gloves. Before assembly, a Kim wipe and isopropyl alcohol (isopropanol or IPA) as needed were used to clean all device components with emphasis on surfaces that ultimately contact blood in the system. A rubber o-ring was placed into a groove in the top polycarbonate structural plate to seal the microchannel plate and prevent leakage during operation. On the aluminum structural plate, the microchannel plate was slid into place via small aluminum rods that linked the plate in notches bore into the aluminum plate. A total of 14 bolts, equally spaced on the edges of the two structural plates, were used to secure the device together using a torque wrench for tightening. Plastic adapter fittings for the inlet and outlet are threaded into the ports on the top of the polycarbonate plate for attaching the inlet and outlet Tygon tubing. Quick-twist connections were used to attach the 3/16" ID tubing to the fittings protruding from the top plate. *Figure 16* is an image of the equipment set-up during testing found in *Appendix I: Lab Photos.*

Collecting Samples

A. Flow Rate (Velocity) Trials

A syringe pump was used to adjust the fluid infusion flow rate such that consistent flow was maintained to sustain constant shear rates. The digitalized interface allows for simple user-input for altering the flow rate and starting/stopping flow during trials. The volume infused was monitored on the pump's display (and reset after each run) to ensure that the correct amount had been infused for each procedure. Prior to the introduction of blood, the entire system was sanitized using 15 mL (a standard of three times the total volume of the system for anytime a new fluid is introduced) of 95% purity isopropyl alcohol as a strong detergent/disinfectant. An additional 15 mL of deionized water was used to flush out the isopropanol because the pure alcohol causes rapid coagulation. Preliminary experiments showed that introducing blood immediately after the isopropanol was problematic due to excess coagulation in the microchannels. For each individual run, a total of 40 mL was infused into a sterile 60 mL plastic syringe. During operation, the first

15 mL of blood was pumped into the system at a standard flow rate of 10 mL/min with the outlet fluid being immediately disposed of. This step was performed to avoid potential variations in results for samples taken during start-up, and to push out any remaining water in the system from the cleaning procedure.

The syringe pump was readjusted to the desired flow rate of the trial and a minimum of 5 mL was ran through the system and collected in a waste beaker to avoid collection of blood that was infused at prep flow rate of 10 mL/min. The next 5 mL of blood infused through the system was collected in a fresh 20 mL beaker as the actual experimental blood sample. Once the syringe pump indicated 5 mL of blood had passed through the system into the fresh beaker, the syringe pump was manually stopped and the outlet tubing was taped to the top of the waste beaker without touching the waste within it to avoid contamination. From the 5 mL blood sample, three 1 mL samples were transferred into small 2 mL centrifuge tubes using a micropipette and stored in the centrifuge until all trials had been completed. For a single set of experimental results, each flow rate of 1 mL/min, 10 mL/min, and 30 mL/min produced three 1 mL samples for subsequent centrifugation. For a full schematic breakdown from the collected blood to the samples eventually loaded into the multi-well plate, see *Figure 6*.

B. Multi-Pass Trials

The same equipment set-up and pre-cleaning procedure was completed for the multi-pass trials as previously described. The sterile plastic syringe was filled to maximum capacity for these trials with the first 15 mL disposed of to introduce the blood into the system and clear out any remaining cleaning fluids (isopropanol or water). All of the remaining 45 mL of blood was infused into the system at a flow rate of 10 mL/min and collected in a fresh beaker. Three individual 1 mL samples were extracted and directly transferred into small 2 mL centrifuge tubes using a micropipette after each pass. The samples were loaded into the centrifuge and stored until all passes had been completed. The syringe was detached from the inlet and used to recover the remaining blood from the beaker before being reattached to the syringe pump. The beaker was recleaned before collecting the blood for the next pass. No additional cleaning of the plate was performed between passes. This process was repeated until a total of five passes through the device had occurred.

Separating Plasma via Centrifugation

For both the velocity and multi-pass trials, all samples were loaded evenly into a mini centrifuge to separate the plasma for further analysis. Evenly spaced tubes were essential for ensuring proper rotation of the device and a consistent separation. The samples were spun for 10 minutes at 3000 RPM at which point the tubes were removed from the centrifuge. It took multiple trials to perfect the centrifugation procedure in terms of RPM and duration, but the final separation achieved looked like *Figure 5*, below.

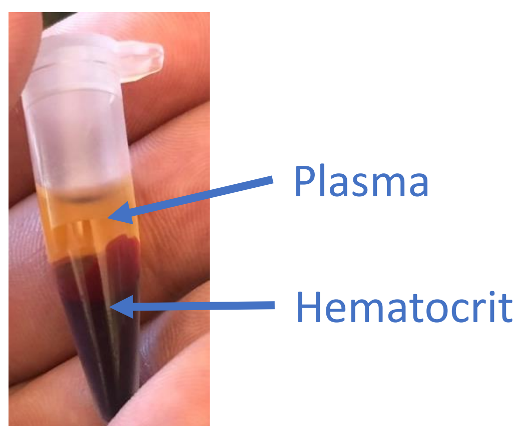

Figure 5 – Picture from the lab of a 1 mL sample after being centrifuged.

For each 1 mL sample that was spun in the centrifuge, three 5 µL samples were extracted from the plasma, avoiding the pellet of cells at the bottom of the tube. These small samples were injected directly into fresh tubes, awaiting dilution. The recommended dilution from Sigma-Aldrich was 100-fold with water, thus each sample was diluted with 500 µL of deionized water. Future experiments could benefit from a deviation from this dilution suggested by Sigma-Aldrich (since it was initially intended for bulk hemoglobin concentration determinations) because the absorbance values were on the low end of the spectrometer's range. At these low absorbance values, the sensitivity is more variable which could result in unreliable results. For a full schematic breakdown of the sample production, see *Figure 6* below.

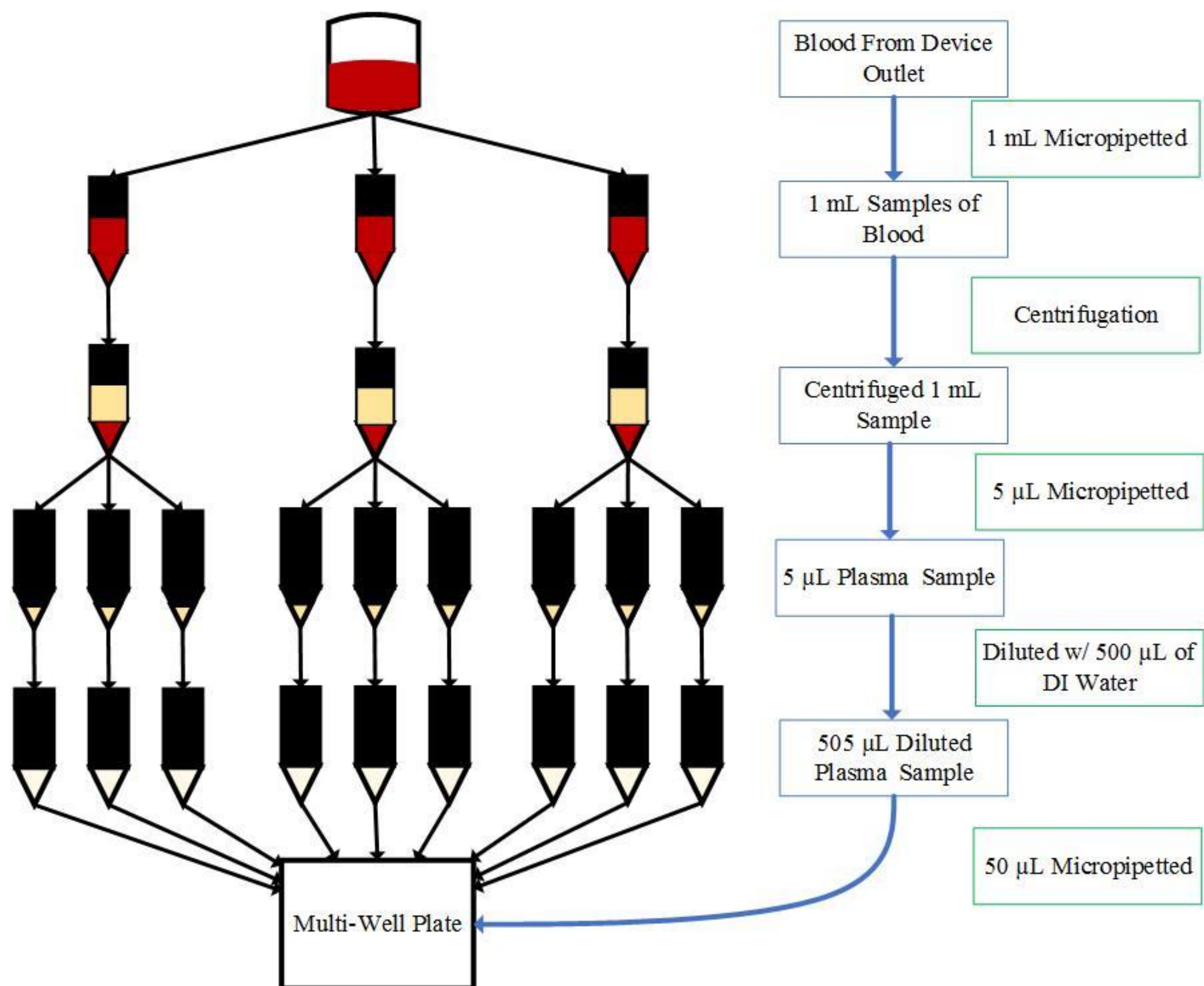

Figure 6 – Schematic breakdown of the sample production from the device outlet to the sample injected into the micro-well plate. This diagram represents the samples produced from a single pass through the device.

Colorimetric Hemoglobin Detection Assays

Sigma-Aldrich sells a total hemoglobin assay kit that can be bought in bulk with sufficient supplies for 250 colorimetric tests [9]. Past hemoglobin detection assays have used potassium cyanide and ferricyanide as an active ingredient to convert hemoglobin into cyanmethemoglobin, though the kit purchased from Sigma-Aldrich utilizes a Triton/NaOH reagent to minimize toxicity [9]. This reagent converts free hemoglobin into a different complex of hematin, a photoactive product that absorbs light at a wavelength of 400 nm [9]. The detectable concentration for this assay ranges from 0.9-200 mg/dL [9]. The average total concentration of hemoglobin ranges from 12-18 g/dL in normal human blood, thus careful dilution is required for detection [4]. To remain consistent with the suggested methodology provided with the kit, despite testing only plasma

samples, the same dilution procedure was conducted by diluting the samples 100-fold with deionized water.

After proper dilution, the tubes were well-mixed to ensure a homogenous mixture. From each tube, 50 µL of the diluted sample was transferred into individual wells in the 96 multi-well plate. Adding 200 µL of the reagent commences the reaction, converting hemoglobin to its photoactive complex of hematin with Triton X-100. For comparison with the experimental results, a calibration ladder (ranging from 1X to 100X dilutions) wass prepared following the procedure outlined by Sigma-Aldrich using the calibrator included with the assay kit [9]. According to the procedure, the calibration ladder diluted 50/50 with water will yield absorbance results indicative of a blood sample composed of 100 mg/dL of hemoglobin [9]. After preparation of the various diluted calibration samples, 50 µL of each is transferred into individual wells in a 96 multi-well plate using a micropipette. The same 200 µL of reagent was added to each well containing the calibration ladder. After five minutes of incubation, the microwell plate can be loaded into the spectrometer for absorbance testing. The plasma-free hemoglobin concentration of the fresh blood was analyzed for comparison with experimental trials to determine the change in concentration. The spectrometer produces absorbance values for each well tested which was converted into the concentration of hemoglobin via *Eqn. 1* found in the assay instructions [9]. To calculate the concentration of hemoglobin ($C_{Hb,sample}$) the equation requires absorbance measurements for the sample ($A_{400,sample}$), calibrator ($A_{400,cali.}$) at a known equivalent concentration ($C_{Hb,cali}$), and blank ($A_{400,blank}$). Calibrator absorbance values for an equivalent concentration of 100 mg/dL (1X dilution with water) will be used for all calculations to remain consistent and eliminate the concern for decreased confidence with successive dilutions. The sample's dilution factor (dF), reflects the ratio between the concentrated sample and the water added for dilution, which was maintained as 100 for all experimental and control samples.

$$C_{Hb,sample}\left[\frac{mg}{dL}\right] = \frac{(A_{400,sample}) - (A_{400,blank})}{(A_{400,cali.}) - (A_{400,blank})} * C_{Hb,cali.}\left[\frac{mg}{dL}\right] * dF \qquad \text{Eqn. 1}$$

Ultimately, it was determined that since this equation relied on only two data points, from the blank (theoretical concentration of 0 mg/dL) to one calibrator data point (equivalent

concentration of 100 mg/dL), a calibration curve would provide a more accurate prediction for the concentration of the sample. As previously discussed, a calibration ladder was prepared for each set of trials using the calibrator solution provided by Sigma-Aldrich with a total of seven dilutions such that a linear calibration curve could be applied to determine the concentration of a sample. Calibration curves were generated for each set of trials following the procedure outlined by Sigma-Aldrich. The curve was used to determine the concentration of the diluted sample, and then the dilution factor was used to determine the concentration of the sample prior to dilution as seen in *Appendix II: Sample Calculations.* An example of one such calibration curves can be found below in *Figure 7*.

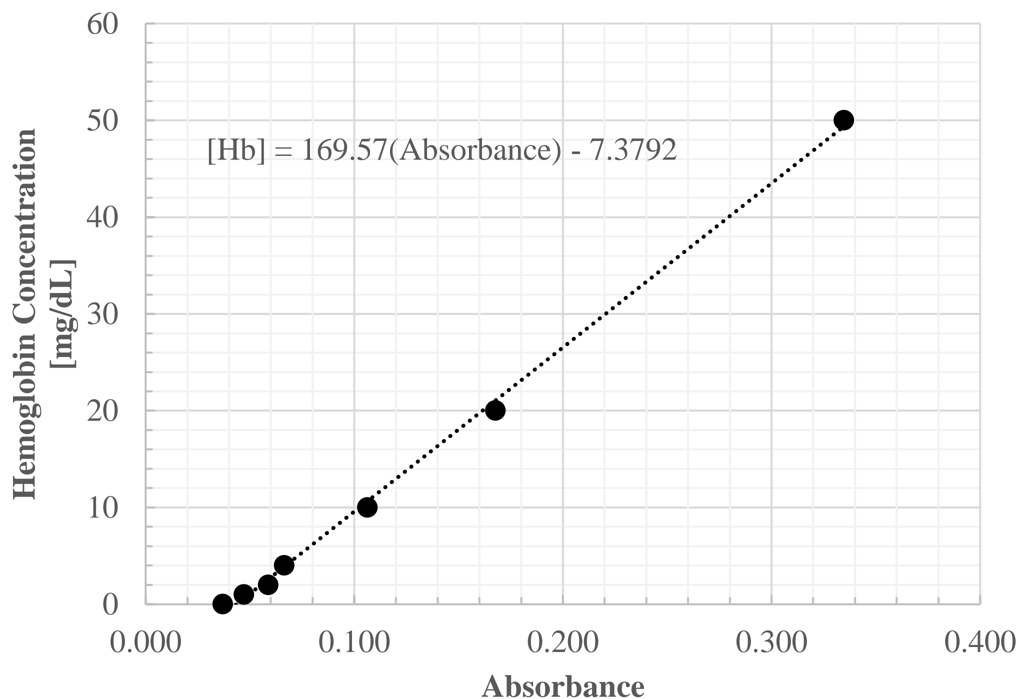

Figure 7 – Example calibration curve from one of the velocity set of trials. Note that the data point for the equivalent concentration of 100 mg/dL is excluded since all experimental data points were beneath an absorbance value of 0.100.

Thermally Induced Hemolysis Control

Since all procedures for determining the plasma-free hemoglobin concentration were novel approaches, it was imperative that the methodology was capable of indicating various degrees of hemolysis. Initially, a mechanically induced hemolysis control was included by flowing blood through the device at an elevated flow rate of 50 mL/min, but similar results to the experimental

trials led the research team to switch to a different approach. The primary concern was that the centrifugation process resulted in plasma samples that were an inaccurate representation of the true plasma-free hemoglobin due to improper separation or the tendency of hemoglobin to remain in the pellet. Even if the actual concentration values were skewed, it was essential to establish the ability for a relative comparison of damage.

Upon literature review, multiple options for inducing hemolysis thermally were found including exposure of blood to rapid temperature increases or an elevated temperature for an extended time. Due to simplicity of implementation, the latter option was favored. Although RBCs are resilient at low temperatures (enabling refrigerated storage for research and transfusion purposes), when exposed to a temperature of 45 ^{o}C, the typical unilamellar state of the membrane is converted to a multibilayer exposing lipid-free areas and effectively overcoming the activation energy of RBC membrane deterioration, resulting in rapid hemolysis [41]. A hot water bath was prepared using a 1000 mL beaker half-filled with water and a hot plate. Approximately 10 mL of fresh blood was poured into an Eppendorf tube and submerged in the water. A thermocouple was used to monitor the temperature of the blood directly and once a blood temperature of 45 ^{o}C was achieved, the temperature was maintained for 15 minutes before removing the Eppendorf tube. The sample was labeled and treated using the same procedure as all other samples by pipetting one mL of blood into a smaller tube for centrifugation. The separated plasma was prepared in the microwell plate using the same dilution and spectroscopy procedure as discussed previously in *Colorimetric Hemoglobin Detection Assays.*

Disposal of Waste

Pure bovine blood waste was collected in a waste beaker and could be disposed of directly down the sink drain according the Environmental Health and Safety (EH&S) department at Oregon State University [42]. This form of waste disposal was permitted because the blood used was bovine. A hazardous waste container was stored under the fume hood in the lab for disposing of waste containing high concentrations of isopropanol mixed with water and/or blood. After trials, EH&S was notified that the container was ready for pick-up so that they came to collect and dispose of the hazardous waste [42].

Results & Discussion

Absorbance measurements of the fresh blood yielded reasonable initial plasma-free hemoglobin concentrations with an average throughout all trials of 156 mg/dL. Note that this is significantly higher than the plasma-free hemoglobin concentration in human blood of 5 mg/dL, but the fresh blood control results were very consistent with a standard deviation of 40 mg/dL. This enabled a reliable baseline to be established using the fresh bovine blood as a control. Additionally, the thermally induced hemolysis control was successful at confirming the ability to indicate variations in the concentrations of hemoglobin in plasma samples by modifying the pre-existing methodology outlined by Sigma-Aldrich for determining the total hemoglobin concentration in blood. The average plasma-free hemoglobin in the blood that was submerged in a water bath at 45 ^{o}C for 15 minutes was ~2400 mg/dL, more than 15 times the concentration found in fresh bovine blood. While the sensitivity for determining differences in hemoglobin concentration was not confirmed, this control verified the desired ability to at least be able to compare relatively the degree of damage various flow conditions cause. One important consideration when evaluating the validity of the blood damage results is the utilization of plastic syringes and a syringe pump for repeated transfers of blood. A third control was developed for the final batch of velocity trials that indicated the procedure of using the syringe pump flowing through tubing on its own caused a 4-8% increase in plasma-free hemoglobin. All samples were treated the same way such that a relative comparison could be performed when interpreting results. Recommended modifications and areas for future development are discussed in *Recommendations for Future Work.*

The first parameter evaluated was the effect fluid velocity had on the amount of blood damage observed in the device. Based on the shear rate graph in *Figure 8* generated via a COMSOL Multiphysics model for a velocity of 4.8 cm/s (based on the velocity through the current geometry at a flow rate of 10 mL/min), the primary areas for concern are concentrated at the inlet, outlet, and on the edges of the posts. Note that there are some differences between the model used in COMSOL and the actual prototype used for collecting experimental data such as the boundary conditions near the inlet and outlet; however, the array of posts mimics the pattern used in the actual microchannel plate in terms of geometric size and spacing.

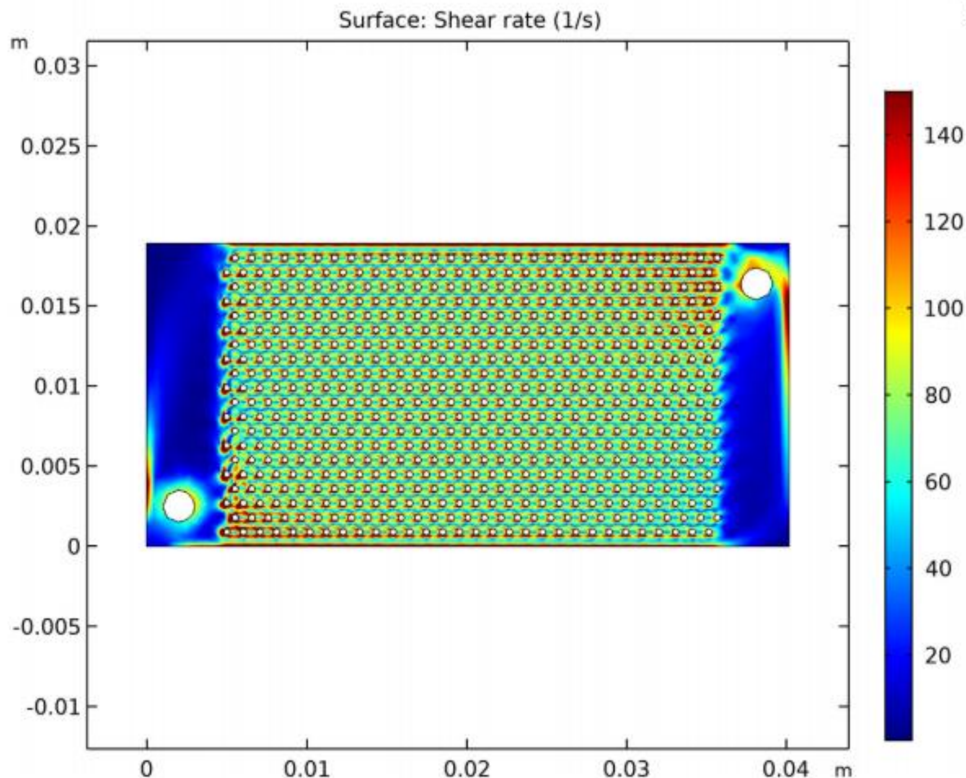

Figure 8 - COMSOL Multiphysics model displaying estimated shear rates for flow through a microfluidic device using a thin channel approximation for flow at a velocity of 4.8 cm/s. This velocity is the high-end estimate for the average fluid velocity of blood at a flow rate of 10 mL/min through the estimated cross-sectional area of 4.47 mm^2. The inlet is the large circle on the bottom left and the outlet is the large circle on the upper right. The properties of water were used as an approximation for blood in this model.

Exact shear rates could not be controlled or measured experimentally, although if elevated degrees of damage are observed for a given set of flow parameters, this flow model could be utilized for investigation of potential modifications to the device for future improvements. The experimental results of the plasma-free hemoglobin concentration as a function of the flow rate, displayed in *Figure 9*, indicate that for a single pass, velocity does not significantly affect the degree of damage inflicted on the red blood cells.

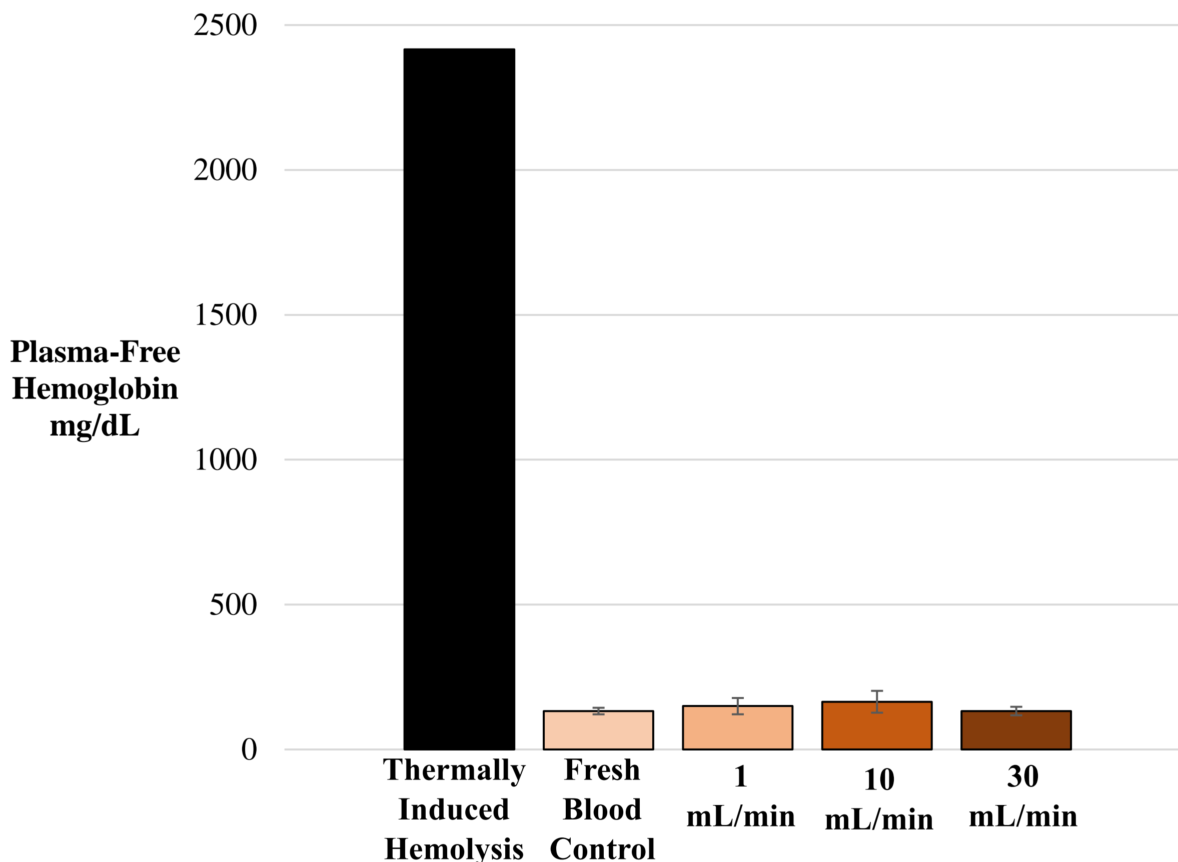

Figure 9 - Experimental results of the plasma-free hemoglobin content of bovine blood after passing through the microchannel device at various flow rate settings. The fresh blood control is based on blood directly taken from the bovine blood received from Hemostat. For comparison, the thermally induced hemolysis control is included. Error bars are included for the velocity results while the average value alone is displayed for the two controls.

Despite significantly different flow rates, the amount of plasma-free hemoglobin had an indeterminate relationship with the blood's flow rate through the device. The velocity trials indicated that for a single pass, the difference between 1 mL/min and 30 mL/min was essentially non-existent. In fact, the low and medium flow rates caused a greater increase of plasma-free hemoglobin from the fresh blood on average (17 mg/dL and 32 mg/dL respectively) when compared to the high flow rate which caused no damage at all on average. The most obvious explanation for this trend is systematic error because the effects of a single pass may not yield consistent results. Another potential explanation is inertial focusing, a phenomenon where denser components in a mixture flow closer to the center of a channel. According to this theory, at increased velocities the flow of the relatively large red blood cells may be concentrated toward the center of the channels resulting in a safer environment with lower shear rates once steady state is

achieved. Conversely, smaller components, such as platelets may be exposed to excess damage near the perimeter of the flow channel. Regardless of the correct reasoning, the results were consistently similar across all velocities.

The largest average increase in plasma-free hemoglobin was approximately 24%, which occurred at the intermediate velocity of 4.8 cm/s (high estimate for a flow rate of 10 mL/min). A third control was performed during the last batch of velocity trials to determine that a single transfer using the syringe pump procedure increased the plasma-free hemoglobin by 4-8%. When including the effects of the syringe procedure, the results suggest that the device itself increases the plasma -free hemoglobin only by 16-20% for this maximum change. Note that the average change in concentration for this percent change was only a 32 mg/dL delta between the experimental sample and control group due to the low concentrations of hemoglobin in plasma. Upon further analysis, it was found that there were two significantly higher concentration samples in this data set that, when eliminated as outliers, caused the percent change in plasma-free hemoglobin to fall to only 10%, with a much tighter standard deviation as seen in *Figure 9.*

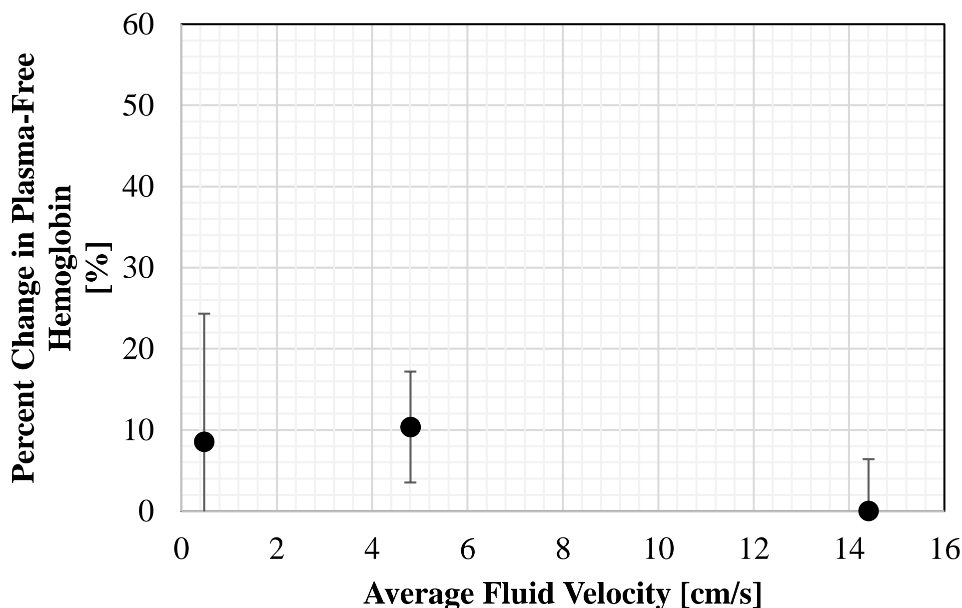

Figure 10 - Experimental results for the change in plasma-free hemoglobin as a function of the flow rate/velocity of operation ranging from 1-10 mL/min (0.5-14.5 cm/s). Error bars for each velocity are included based on the standard deviation of the results.

The wide error bars indicate a lack of confidence in the expected percent change in plasma-free hemoglobin; although, this may be improved in future experiments by performing less intense dilutions such that the absorbance values are in the middle of the required range. The trend suggests that within the operating limits for the therapeutic mechanisms, the velocity will not be a determining factor, since no mechanisms are expected to operate at velocities higher than 14 cm/s; however, it is recommended to repeat the experiments with the therapeutic mechanisms incorporated before confirming this trend.

Despite the inconclusive results of the velocity trials, the research team was optimistic that multi-pass trials would yield the expected trend of increased plasma-free hemoglobin with each pass through the device. During this study, only the intermediate flow rate of 10 mL/min was evaluated for multi-pass trials. The same microchannel plate with an array of posts used in the velocity trials was utilized for the analysis of multiple passes. The experimental results are summarized in *Figure 11* below. The maximum number of passes performed was limited to five for this study.

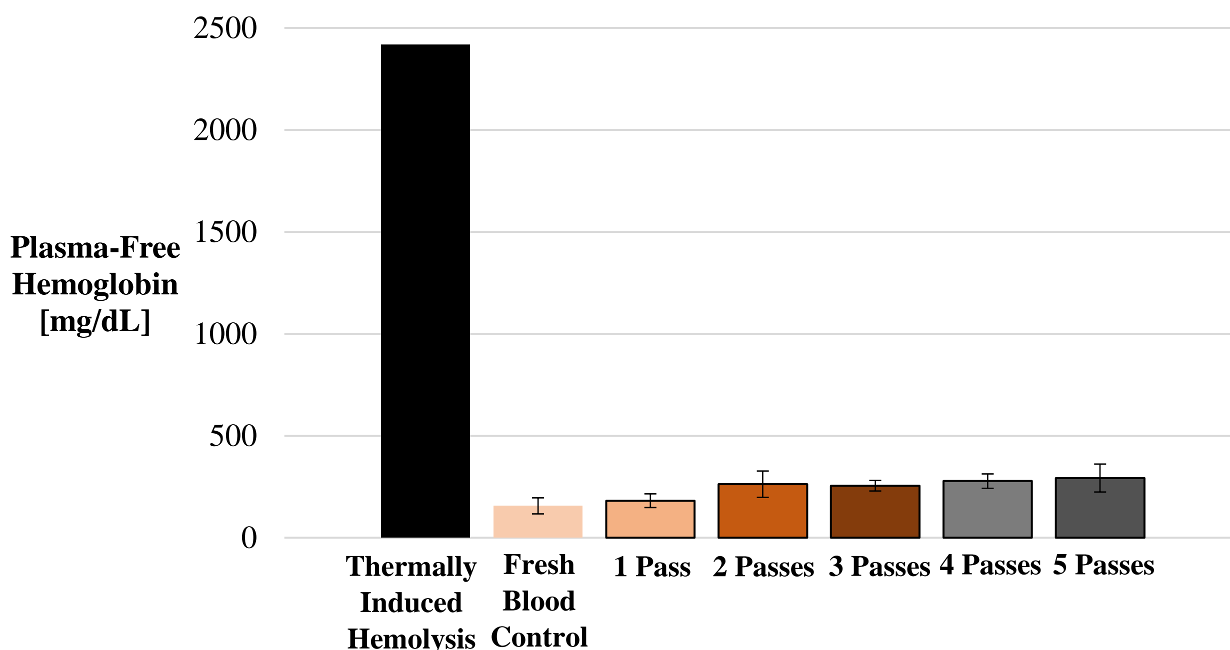

Figure 11 – Experimental results of the plasama-free hemoglobin content of bovine blood as a function of the number of passes through the blood processing device at a standard flow rate of 10 mL/min. The fresh blood control is based on blood taken directly from the bovine blood received from Hemostat. For comparison, the thermally induced hemolysis control is included. Error bars are included for the multi-pass experimental groups while the average value alone is displayed for the two controls.

The results indicate a consistent increase in plasma-free hemoglobin as a function of the number of passes through the device, showing a positive slope. Although the hemoglobin concentration increases more sharply in the first and second passes (increases of 22% and 45% respectively), the final three passes show a minimal increase relative to the last pass. This could indicate that there is internal diversity amongst the red blood cells and that those with less membrane stability are lysed early during operation while the RBCs with more stable membranes are resilient in successive passes. Another explanation may be that after multiple passes, the microchannels for flow are fully developed within the device, allowing for less contact with the posts resulting in lower overall shear. The primary takeaway from these results is that start-up procedures must be refined to ensure that the operation itself is not the cause for the steep hemoglobin increase in the first two passes. A linear regression was performed to develop a potentially useful relationship for projecting the plasma-free hemoglobin concentration as a function of the number of passes in *Figure 12*.

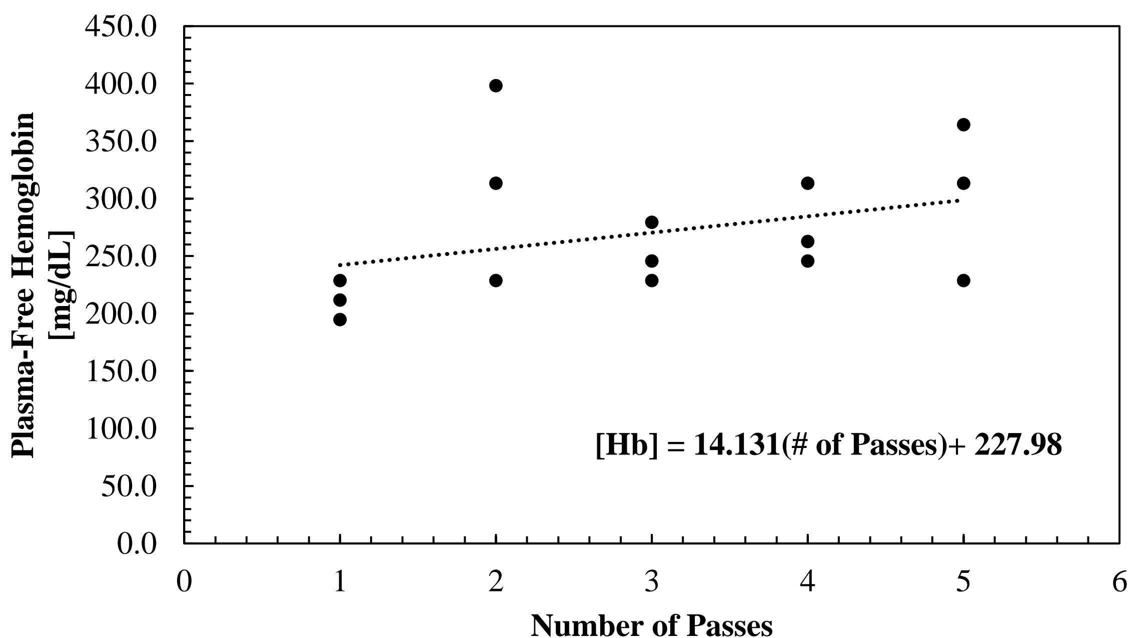

Figure 12 – Statistical analysis of the experimental results for the multi-pass trials using a linear regression trendline to determine the magnitude of the positive slope. Experimental data is based on one set of multi-pass trials with individual data points displayed.

The slope describing the dependence the plasma-fee hemoglobin content has on the number of passes through the device was determined to be approximately 14.1 for the experimental results obtained at a flow rate of 10 mL/min. Due to a small sample size and high degrees of variance throughout the experimental results, there is no confidence in this trend for extrapolating outward to more passes. As expected, the average plasma-free hemoglobin increased with the number of passes. Due to the observations discussed earlier about the first two passes inflicting more damage relative to others, a linear relationship may not be an appropriate representation. This argument is strengthened by *Figure 13*, which displays the percent increase in plasma -free hemoglobin relative to the previous pass.

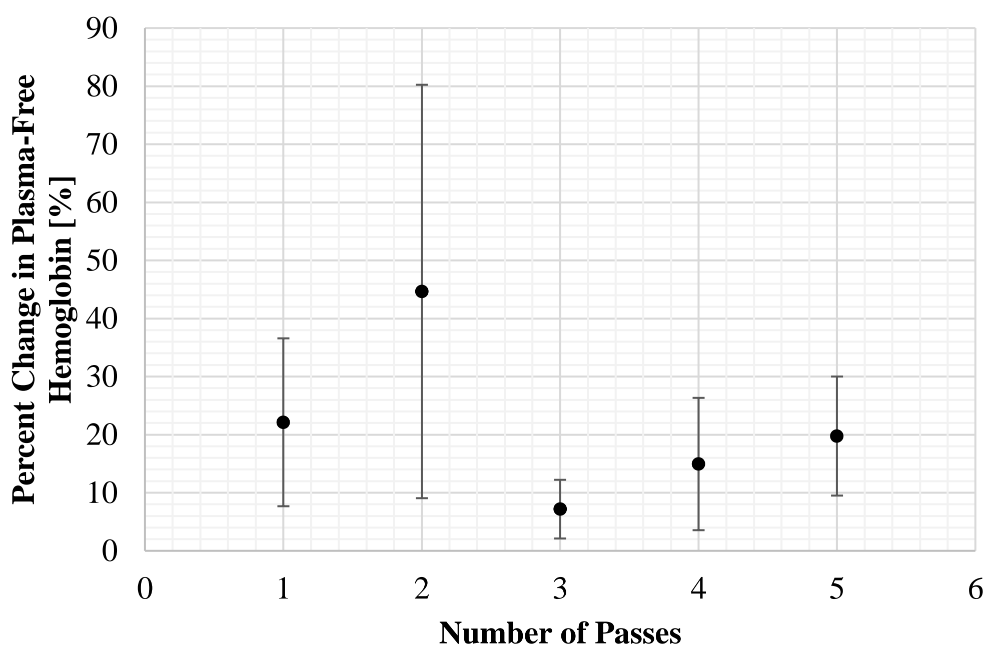

Figure 13 – Experimental results for the change in plasma-free hemoglobin between the number of passes. The concentration for each pass is compared to the pass immediately preceding it with the first pass being compared to the fresh blood control. Error bars are included and based on the standard deviation for the percent change between a single data point of that pass and the average of the previous pass.

Overall, the biggest takeaway from the multi-pass trials was that the start-up procedure needs to be investigated to ensure quick progression into steady state operation while minimizing damage. Additional data is required to further analyze the effect multiple passes has on the hemolysis rates observed such that a reasonable number of passes can be determined for a given flow rate. Further investigation using human blood, which has a much lower initial plasma-free hemoglobin, is expected to yield similar results based on literature review indicating similar resilience to shear As discussed in the results of the velocity trials, the current syringe pump methodology remains a limitation for obtaining genuine data representing the damage caused solely by flow through the device. During the multi-pass investigation, another concern related to this methodology arose when excess coagulation was observed when cleaning the device after all passes had been completed. Unfortunately, since the multi-pass trials required discontinuous flow allowing blood to sit stagnant while reloading the syringe, the cause of this coagulation seen in *Figure 14* could not be verified.

Figure 14 – Image taken in the lab during disassembly of the device following multi-pass trials. The inlet and outlet are labeled.

If the syringe procedure was the root cause for the coagulation, it was likely due to the stagnation of blood sitting between passes; however, if this were the sole contribution to coagulation, an equal distribution of clotted blood across the entire plate would be expected. As seen in *Figure 14*, the coagulation is concentrated near the outlet of the device. A potential explanation for this observation is that the stagnation caused even coagulation initially, and then the flow conditions exacerbated the problem toward the outlet where increased shear rates are experienced as seen in *Figure 8.* A suitable explanation for the coagulation not being concentrated at the inlet is the higher pressure near the inlet causing more clots to be knocked free and rejoin flow. Additionally, the coagulation causes increased pressure drop across the plate such that the decrease in pressure is enhanced.

If the coagulation occurred due to the repeated flow of blood through the device, the places with excess shear could initialize coagulation and result in clots forming between the flow microchannels, behind the posts. The velocity profile in *Figure 15* displays this phenomenon where velocity “dead” zones exist in these areas of significantly lower velocity than observed in the microchannels where blood flow is streamlined. The shear around the posts may cause local hemolysis which triggers the coagulation cascade that more readily adsorbs in the areas with decreased flow. Replacing the discontinuous procedure using the syringe pump with a continuous alternative could help future research into the effects of these “dead” zones.

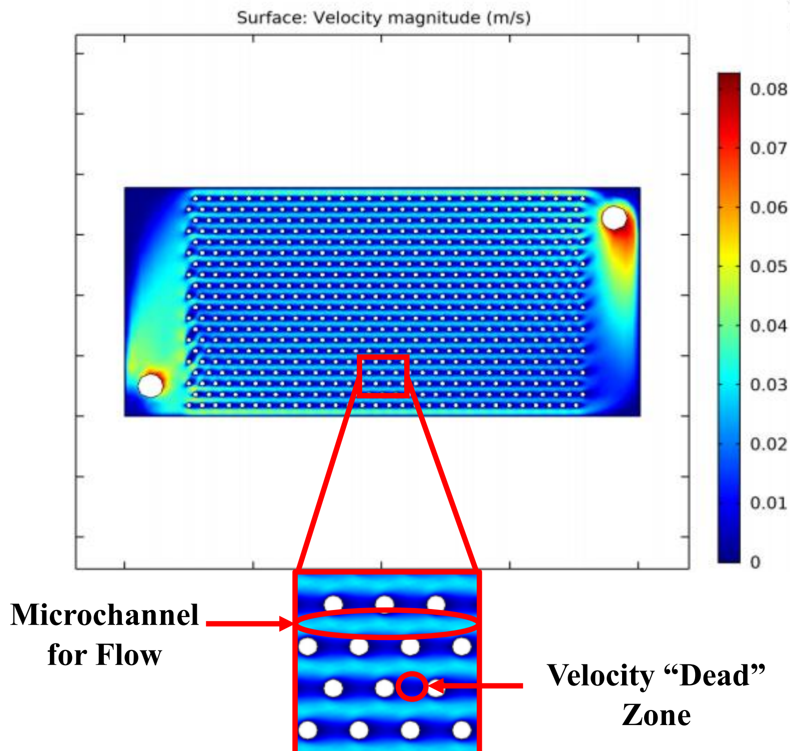

Figure 15 - COMSOL Multiphysics model displaying estimated velocity for flow through a microfluidic device using a thin channel approximation for flow at an entrance velocity of 4.8 cm/s. The inlet is the large circle on the bottom left and the outlet is the large circle on the upper right. The properties of water were used as an approximation for blood in this model.

Conclusion

Ultimately, this research project was successful at confirming the effectiveness of using a hemoglobin detection assay for determining the plasma-free hemoglobin concentration of various bovine plasma samples. The thermally induced hemolysis control verified the retention of hemoglobin in plasma following centrifugation and the ability to quantify differences in hemoglobin concentrations using absorbance spectroscopy. The refined procedure for comparing various plasma samples relative to one another is a reliable platform for future work investigating the hemolytic effects of various flow conditions within the blood processing device. One major flaw of the current procedure is the use of a syringe pump for flow, which was found to cause a 4-8% increase in plasma-free hemoglobin. Future studies should investigate potential alternatives where the source of blood damage can be isolated with more precision to the flow of the blood through the device itself.

Hemolysis rates were relatively constant as a function of velocity such that the relationship between the average fluid velocity of flow and subsequent hemolysis is inconclusive with current results. Due to the decreased sensitivity at the low-end of the assay's range, confidence in these results remain minimal until further control groups are developed for evaluating the effectiveness of the assay at low concentrations. Determining whether inertial focusing plays a role in the shear experienced by the red blood cells, and therefore the blood damage, would support the current data and explain the trend of decreased damage at higher flow rates.

The multi-pass trials revealed several potential concerns for further investigation with the first related to the start-up procedure as larger degrees of blood damage were observed in the first two passes through the device. Implementing a continuous process could eliminate this concern and speed up the time it takes to reach steady state within the device. With few exceptions, the general trend of the multi-pass trials suggested that with increasing number of passes, the plasma-free hemoglobin concentration increases as well; however, the inconsistent rate of increase is reason for further investigation. The multi-pass trials further exposed the limitations of the syringe pump procedure as excess coagulation was observed during testing, though the source could not be pinpointed.

The project was successful at developing a proven option for quantifying various degrees of hemolysis via quantification of the plasma-free hemoglobin concentration using a Sigma-Aldrich assay and absorbance spectroscopy. Specific conclusions related to recommendations for specific flow conditions are thus far inconclusive, though observations during this study can effectively inform future efforts for more controlled, reliable results. Preliminary findings suggest that the current microchannel-based blood processing device is capable of accomplishing the desired flow pattern without causing significant damage, though the bounds of operation require further investigation.

Recommendations for Future Work

Integration of a Continuous Flow Loop

A primary cause for many of the inconclusive results of this project were due to the use of a syringe pump for discontinuous flow through the device during testing. While useful for controlling the flow rate though the device, the syringe pump itself caused significant damage resulting in elevated plasma-free hemoglobin concentrations. Ideally, the apparatus used for flow would minimize damage such that increases in plasma-free hemoglobin could be isolated to the mechanical damage caused by flow through the microchannel device. Current extracorporeal blood processing applications, such as dialysis, use peristaltic pumps which feature low shear operation while maintaining the sterility of the blood [43]. Incorporating a peristaltic pump into a loop that integrates the blood processing device would not only eliminate some of the concerns related to research purposes but would also more closely imitate the actual therapeutic procedure.

Recently, another undergraduate chemical engineering student on the i-Blood research team has developed an integrated breadboard system that can be used in future experiments evaluating blood damage. This system utilizes a peristaltic pump and has the capability of monitoring temperature and pressure with the potential for integrating other measurement features. The vision for this breadboard device is the ability to monitor and record various conditions such that the safety and effectiveness of the eventual application can be maximized. A primary advantage of this system would be the ability to evaluate the effect time of operation has on hemolysis rates. Although the number of passes through the device could not directly be determined, the difference in plasma-free hemoglobin at different time points during operation could be evaluated. Additionally, with integrated measurement systems, other parameters could be evaluated such as pressure drop and temperature dependence. The original goal of this project was to repeat the experiments conducted using the syringe pump with this updated system, but lack of access to the lab during the COVID-19 pandemic inhibited such testing. It is recommended that all future in vitro blood damage studies incorporate this device for enhanced control, continuous operation, and more reliable results.

Quantifying Degrees of Coagulation

Although blood damage results indicate more testing would benefit the device development, another primary takeaway from experimental testing was the potential for coagulation build-up as seen in *Figure 14*. The source of the coagulation could not be isolated due to the limitations of the current instrumentation but regardless of the source, if significant degrees of coagulation persist in future trials, the i-Blood research team must address this issue. This issue could be resolved by incorporating the integrated breadboard loop system if the bulk of the observed coagulation occurred due to the discontinuous nature of the syringe pump method. Regardless, the device development could benefit from a study confirming that coagulation will not be a limiting factor moving forward in the design process. One potential option would be to develop a similar methodology as described in this study except focusing on quantifying coagulation rates rather than hemolysis rates at different flow conditions. This proposed study could reveal that while the resilient nature of red blood cells allows for operation at elevated velocities without significant damage, the increased shear rates could result in increased rates of coagulation cascade activation.

Blood coagulation testing for diagnostic purposes is commonly conducted in a clinical setting for identifying risks of hemorrhage and monitoring the effects of anticoagulant therapy [44]. The motivation for such tests is determining the health of coagulative functions in patients rather than quantifying the amount of coagulation directly. Conceivably, these tests, such as a clot-based assay, could be used to determine the change in the blood's ability to form clots after flowing through the blood processing device, but such a test would not aide in determining how much coagulation the device directly causes [44]. There are several assays that can determine the concentration of various clotting factors and natural anticoagulants that could be used to indicate the effects flow through the blood processing device has on coagulative mechanisms. For example, evaluating the prothrombin time could indicate whether there has been an upregulation in clotting factors such that clots form at quicker rates than usual. Regardless of the methods used, the development of the device would benefit from a formal investigation of the coagulative effects of the microchannel-based blood processing device.

Integrating Therapeutic Features

Once the previous recommendations of adjusting the blood damage analysis methodology and developing a coagulation quantification procedure are addressed, these tests can be performed with the therapeutic components integrated into the device. Immobilizing a therapeutic molecule or enzyme in a hydrogel or other bioconjugation mechanism will decrease the cross-sectional area for flow. This will benefit the efficiency of the treatment, but potentially result in flow complications since the decreased cross-sectional area will result in larger fluid velocities for a given flow rate. The flow rate will need to be reduced to maintain reasonable shear rates, though this may increase the concern for coagulation. Luckily, the micro-channel device is designed such that the flow channels are maintained even under low flow conditions. These issues will inevitably be considered and addressed in future versions of the plate design since this could extend procedure times for a therapy and inform therapeutic mechanism development. Note that some other therapeutic options in development are on a microscale utilizing a thin coating only microns thick onto the functional plate. This project has successfully developed a procedure for evaluating the hemolytic effects of the blood processing device, which will be useful as therapeutic applications are more refined.

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Appendices

Appendix I: Lab Photos

1. Equipment Set-Up

Figure 16 – Syringe pump set-up during a cleaning procedure before actual flow of blood using isopropanol and water.

2. Loaded Microwell Plate

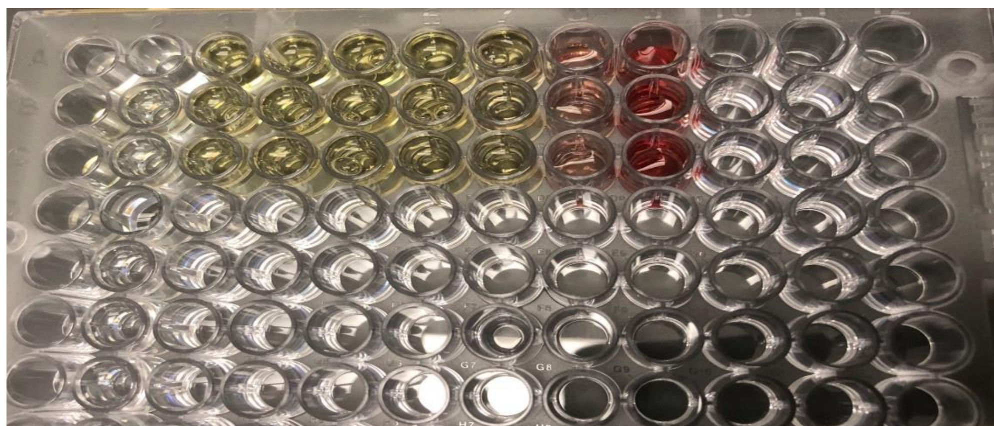

Figure 17 – Image of a partially loaded micro-well plate containing whole blood samples during the preliminary trial run with the assay and absorbance spectroscopy. The yellow wells (first five rows) have already been treated with the Triton/NaOH reagent and the reaction has gone to completion, the light pink row (sixth row) is undergoing the reaction after the addition of the reagent, and the far right row (seventh row) has been diluted but not yet treated with the reagent. Note that plasma sampled had much less vibrant colors due to the decreased hemoglobin concentration.

3. Air Bubbles During Operation

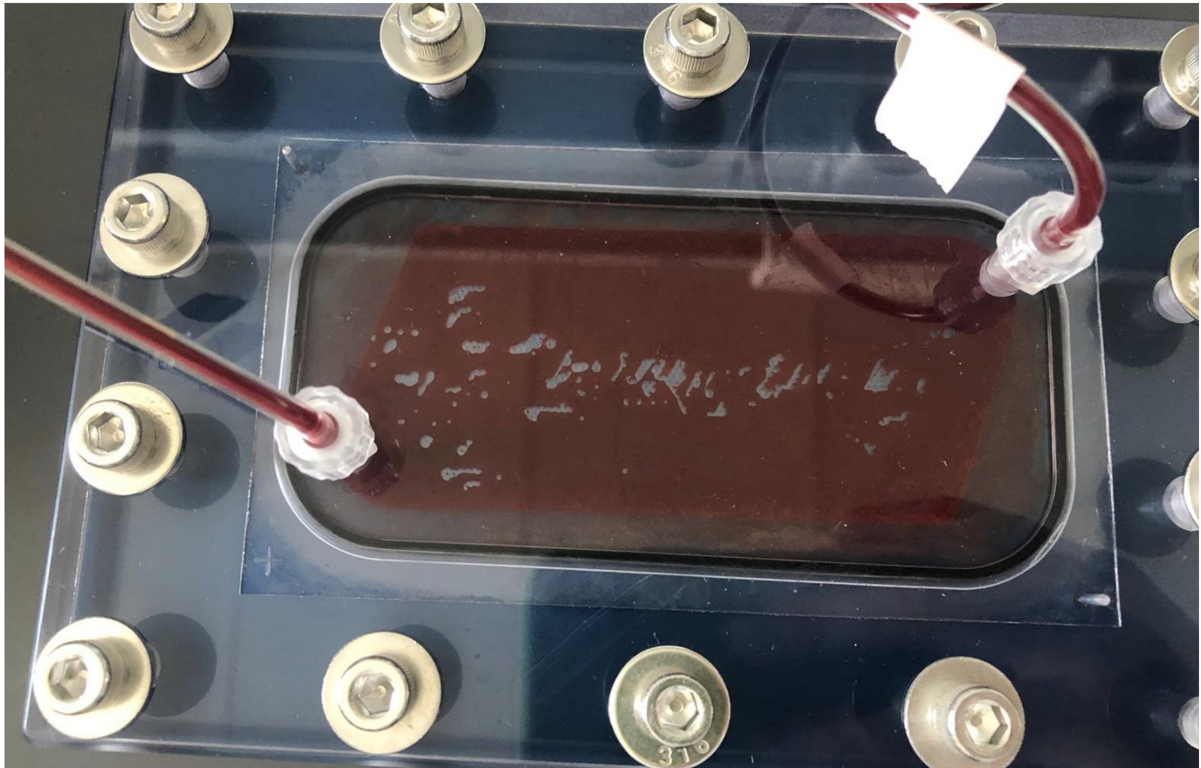

Figure 18 – Image exemplifying the air bubbles that develop due to the lack of control of the syringe pump set-up prior to the establishment of steady state operation. When switching from cleaning fluids to blood, air inadvertently enters the system.

Appendix II: Sample Calculations

1. Average Fluid Velocity

The known flow rate set by the syringe pump was used to determine the average fluid velocity through the device based on the estimated cross-sectional area. The high estimate for the cross-sectional area was used for the following calculation.

$$v\left[\frac{cm}{s}\right] = \frac{F\left[\frac{cm^3}{s}\right]}{A\,[cm^2]}$$

Eqn. 2

$$v\left[\frac{cm}{s}\right] = \frac{1\left[\frac{mL}{min}\right]\left[\frac{cm^3}{mL}\right]\left[\frac{1\ min}{60\ s}\right]}{4.47\,[mm^2]\left[\frac{1\ cm}{10\ mm}\right]^2} = 0.373\left[\frac{cm}{s}\right]$$

Eqn. 3

2. Plasma-Free Hemoglobin Concentration

Option 1: Sigma-Aldrich Equation

The following equation from Sigma-Aldrich uses one data point from the calibration ladder to determine the plasma-free hemoglobin concentration from an absorbance measurement.

$$C_{Hb}\left[\frac{mg}{dL}\right] = \frac{(A_{400,sample}) - (A_{400,blank})}{(A_{400,cali.}) - (A_{400,blank})} * C_{Hb,cali.}\left[\frac{mg}{dL}\right] * dF$$

Eqn. 4

$$C_{Hb}\left[\frac{mg}{dL}\right] = \frac{(0.054) - (0.037)}{(0.498) - (0.037)} * 100\left[\frac{mg}{dL}\right] * 100 = 369\left[\frac{mg}{dL}\right]$$

Eqn. 5

Option 2: Calibration Ladder

To determine the plasma-free hemoglobin concentration for a given sample, its absorbance value was used in comparison with the calibration ladder.

$$C_{Hb}\ \left[\frac{mg}{dL}\right] = (169.57(Absorbance) - 7.3792) * df$$ *Eqn. 6*

$$C_{Hb}\ \left[\frac{mg}{dL}\right] = (169.57(0.054) - 7.3792) * 100 = 178\ \left[\frac{mg}{dL}\right]$$ *Eqn. 7*