COMPARISON OF NEUTROPHIL ATTACHMENT ACROSS 2D AND 3D IN VITRO MODEL PLATFORMS

A Thesis

by

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ABSTRACT

In vivo animal models provide the most realistic information on flow dynamics and drug delivery research. However, the high cost of animal models, the inherent complexity of biological systems, and ethical concerns can make research of specific variables challenging and can easily lead to data misrepresentation. Current in vitro models, such as parallel plate flow chambers and rectangular microfluidics channels, are commonly used to investigate vasculatures. However, these approaches may be inaccurate representation of in vivo conditions. Our group has developed a simple and low cost polydimethylsiloxane (PDMS) microchannel fabrication process to provide a realistic in vivo geometry testing platform. This platform will ultimately be used to study the effects of flow velocity and shear rate to the efficacy of vascular-targeted drug carriers. The main focus of this study is to compare neutrophil attachment on human umbilical vein endothelial cells (HUVECs) by using both existing 2D endothelial model in the parallel plate chamber and 3D cylindrical microchannel in vitro models. For microchannels, as shear rate was increased from 100 to 400 s^{-1}, there was a decreasing trend in neutrophil attachment. This trend is likely because the red blood cell – free layer reduces in width to the point where the layer thickness is smaller than neutrophil diameter. However, with the parallel plate flow chamber, neutrophil attachment increases with shear rate. This may be due to the red blood cell-free layer approaching a critical width, where the margination condition for neutrophils attachment is optimal. The attachment trends are opposed due to different channel geometry effecting the packing or distribution of red blood cell

differently. Overall, our findings suggest that circular microchannels may be a better platform for studying the functionality of vascular-targeted drug carriers due to the physiological relevant geometry.

DEDICATION

To my mother, Petrona Fuentes, whose love, support, and sacrifice allowed me to continue my education. Without you, nothing I have achieved would be possible.

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Contributors

This work was supervised by a thesis committee consisting of Professor Phapanin Charoenphol (Chair) and Professor Iman Borazjani of the Department of Mechanical Engineering and Professor Abhishek Jain of the Department of Biomedical Engineering.

The data gathered for flow experiments in Chapter 3 was partially assisted by Patrick Chai and Dr. Jorge Palma of the Department of Mechanical Engineering and Department of Biomedical Engineering, respectively. The statistical analyses depicted in Chapter 3 was conducted in part by Amir Akhlaghi of the Department of Mechanical Engineering.

All other work conducted for this thesis was completed by the student independently.

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NOMENCLATURE

ACD	Acetate – Citrate – Dextrose
CAD	Computer Aided Design
CFL	Cell Free Layer
CS	Cover Slip
EC	Endothelial Cells
FL	Free Layer
h	Gasket Height
HUVEC	Human Umbilical Vein Endothelial Cells
MC	Microchannel
MDS	Multistage-Drug Delivery System
NAC	Neutrophil Attachment Concentration
(NS)	Not Statically Significant
OFM	Optical Fiber Method
PDMS	Polydimethylsiloxane
Poly-prep	Polymorphprep
PPFC	Parallel Plate Flow Chamber
Q	Volumetric Flow Rate
r	Radius
RBC	Red Blood Cells
w	Gasket Width

$\dot{\gamma}$	Shear Rate
*	Statistically Significant

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1. INTRODUCTION

1.1. Background and Literature Review

In vitro models are commonly used to study cell behavior under exogenous conditions. A few examples include using these models to initialize the study of disease development and drug delivery methods [1, 2]. Although in vivo models provide realistic behavior on flow dynamics over the in vitro models, there are several disadvantages such as high cost of animal models, complexity of biological systems, regulations, and ethical concerns. Such factors can make research challenging and potentially lead to data misrepresentation. To date, several in vitro models such as a parallel plate flow chamber and rectangular microfluidic channels have been frequently used as a model of vasculatures. Unfortunately, these models tend to not well represent physiologically relevant conditions that are found in vivo [3]. There are several types of endothelial cells that can be used for in vitro modeling. Frequently, human umbilical vein endothelial cells (HUVECs) are used to coat channels or cover slips [4]. However, more recently, the idea of individualized treatment has been popularized with the ability to collect endothelial cells (ECs) from the patient's blood [5]. Blood collected ECs are then grown, harvested, and seeded onto a vascular, similarly as HUVECs [6].

Defining a vascular chip or organ-on-chip consists of a plane or conduit lined with ECs. Currently, there are several manufacturing methods to create the vascular chips. Three methods that will be discussed in this study include the use of 1) a parallel plate flow chamber equipped with a coverslip and gasket, 2) a rectangular/square microchannel

fabricated via photolithography on a silicon wafer, and 3) a circular microchannel made from optical fibers.

The first method utilizes a commercially available parallel plate flow chamber. This chamber has a rectangular cut out and can form a flow adhesion assay by attaching a cell-grown coverslip at the bottom of the flow gasket. Several methodologies exist to treat the cover slip surface, such as cross-linking, to facilitate the adhesion and growth of cell monolayer [7]. This assembled flow chamber has the cell monolayer formed only at the bottom planar, and thus is named a two-dimensional adhesion substrate (2D) in this study.

Photolithography, or more specifically, soft lithography, is used to create a protruding-stencil of a desired computer aided design (CAD) onto a silicon wafer. The modified wafer can then be used to imprint the desired channel path on cured polydimethylsiloxane (PDMS), a silicon-based bio-compatible material that cells adhere to [6]. This method allows for complex channel pathways, however; pathways cross-sections are fixed to rectangular or oval-like shapes [8]. Soft lithography requires unique equipment and environmental conditions to produce a positive-mold-wafer, making accessibility limiting [9].

The optical fiber method (OFM) is a “do-it-yourself” approach to making vascular chips. The required materials are inexpensive and off-the-shelf [3]. As the name implies, a segment of optical fiber is placed inside of a petri-dish and PDMS is poured in until the fiber is sufficiently covered. Once the PDMS cures, the extraction of fiber produces a circular conduit. This conduit shape is physiologically relevant [3, 10]. Though the OFM can produce circular ducts, a trained hand is needed to continuously produce useful

channels [3]. As a result, there is a need to develop a method for consistent circular channel production at various diameters.

Although there are several methodologies to create vascular chips, each procedure has limitations on achievable conduit-cross section silhouettes or path complexity. A few existing works have shown that the cross-sectional geometry of vascular chips could affect leukocytes cell margination behaviors [8]. In a recent study, the traffic of leukocytes were compared between rectangular and rounded-rectangular edge cross-sections on non-symmetric bifurcations [8]. The study indicates that migration of leukocytes is dependent on the cross-sectional geometry and when selecting the type of in vitro model, physiological geometry conditions must be considered. However, it is unclear how targeted cells attachment is affected by geometry. Therefore, for this research neutrophil attachment will be compared between a circular cross-section vascular chip and existing vascular chips with non-circular geometries at different flow conditions. This information will provide insights on similarities and differences in cell behaviors among in vitro vascular models. Additionally, the obtained in vitro data could potentially translate to the in vivo models.

1.2. Research Objectives

This research will investigate how the geometry of the testing platform plays a pivotal role on neutrophil attachment. The overarching goal of this research is to compare the adhesion of neutrophils to inflamed endothelium within existing non-circular micro-vessels to circular micro-vessel under various conditions. The effect of wall shear rate (100-400 s^{-1}), channel height (500-800 μm), and type of flow (laminar and pulsatile flows)

will be investigated. Additionally, beyond having alternate cross-sectional geometry, the plane in which the HUVECs is monolayer formed is different: along a bottom planar (2D for the non-circular microvessel) and along the channel wall (3D for the circular microvessel).

To control and fix the ambient temperature at a physiological condition (37 ºC) during flow adhesion experiments, an incubator is designed and constructed for the Nikon-Eclipse-Ts2R microscope. Beyond having to reach 37 ºC, the incubator must also be modular, spacious in order to store flow experiment equipment, and the user must have access to the microscope stage from various angles. Modularity for the incubator is required to access the inside panels for potential cleaning and to effortlessly place or extract the microscope.

2. MATERIALS AND METHODS

2.1. Blood Samples

All blood samples were collected from consenting healthy donors via venipuncture. Venous blood was obtained via a syringe containing an anticoagulant, acetate-citrate-dextrose (ACD), in a 9:1 whole blood to ACD ratio [11]. Drawn blood was then stored in a polypropylene-50-mL centrifuge tube and stored at 37 ºC before use. Blood samples were used within 4-hours of being drawn to avoid abnormalities. Total blood

All protocols were approved by the Texas A&M University Institutional Review Board (IRB ID: IRB2016-0450D) and in line with the standards set by the Helsinki Declaration.

2.2. Neutrophil Isolation

Polymorphprep (Poly-prep) solution is used to split whole blood into sectionalized components by centrifugation [12]. Specifically, the whole blood-ACD mixture was carefully layered on top of the poly-prep solution at a 1:1 volume ratio in a centrifuge tube. After the solutions were centrifuged for 30 minutes at 525 g with zero breaking, the whole blood components were separated into different layers. The neutrophil band was collected and washed with PBS (-/-). In the case where the neutrophil bands were overlapped with other components, the blood-Poly-prep samples were re-centrifuge at 500 g with zero breaking for 10 minutes. Lastly, upon running a flow experiment, neutrophils are diluted in PBS (+/+) to the desired cell concentration of 10^6 cells/mL.

2.3. Circular Microchannel Design and Fabrication

The fabrication of circular microchannels (MC) was adapted from Mannino's "do-it-yourself" approach [3]. In this study, a metal needle (at a manufacturable scale of 150-1000 μm) was used to generate a circular conduit instead of the optical fiber. A three-chamber mold and t-connectors needed for the channel fabrication, were all designed on Solidworks 2018, a CAD modeling software. Parts were printed on a stereolithography-Form 2 3D printer for tolerance and surface control. Each mold chamber has a glass coverslip inserted to ensure each micro-channel device has two clear-viewable surfaces.

Upon selecting the desired channel diameter, the corresponding needles were passed through t-connectors and pressed into the three-chamber mold. PDMS, which consists of a base and curing agent mixed in a 10:1 mass ratio, was then poured into the mold. The mold was then placed in a desiccator to remove any residual air from the PDMS. After the mold was baked at 80 ºC for a minimum of 12 hours in a furnace, the microchannels were then removed from the mold. Each microchannel was inspected for any surface scratches and underwent a trimming stage, where excess PDMS was cut to ensure channels are effortlessly imageable on both sides. Post inspection, channels were placed back into the furnace until the functionalization and endothelization phase, which will be discussed in the subsequent section.

With this design, there is no need to bio-punch inlets or outlets since the t-connector was used to mold a 3 mm opening for each device. Devices have a 13 mm channel length for cell seeding and an overall dimension of 27 x 20 x 5 mm. Dimensions can be altered based on use case.

2.4. Circular Microchannel Functionalization and Endothelization

The circular microchannels were washed with 70% 200 proof-ethanol followed by de-ionized water to remove any potential contaminant. The channels were then treated with oxygen plasma for 75 seconds on LOW-RF using the PDC-001 to render their surfaces hydrophilic. Subsequently, the channels were coated with 0.40 mg/mL of fibronectin for an hour and incubated in a 37 ºC and 5% CO_2 incubator.

Channels were then washed twice with PBS (-/-), followed by a wash of cell media. Channels were seeded with HUVECs which were harvested and strained, at the recommended cell concentration (6-8x10^6 cells/mL). After an hour of incubation, the channels were flipped and a fresh stock of HUVEC cells was seeded onto the device. Two hours after the second seeding, 200 μL of fresh media was perfused into the channels. The cell media was continuously replaced every 8 hours and the channels were being flipped to ensure channels had a confluent monolayer. Prior to the flow adhesion experiment, HUVECs in the channels and cover slip were activated with 800 and 2000 μL, respectfully, of 10 ng/mL of IL-1β to induce their inflammatory states.

To ensure the necessary substrate or fluid can enter the channel, a connector was designed alongside the microchannel. The quasi like L shaped connector was designed to fit the 3 mm microchannel cavity made by the t-connector when PDMS is baked over surface. The L-connector was designed around 10 -100 μL pipette tip, which are available is most wet labs. Furthermore, the connector has a small reservoir to hold media. Excess media is essential for cell growth and to avoid a dry channel caused by evaporation induced by the incubator.

2.5. Cover Slip Functionalization and Endothelization

Cover slips (CS) underwent an oxygen-plasma treatment on Low-RF for 75 seconds and were coated with the fibronectin substrate in the same manner as the micro-channel devices. Subsequently, the HUVECs were seeded on the surface treated cover slips and incubated for 15 minutes in 37 ºC and 5% CO_2. Following, 3 - 4 mL of media was added to the petri-dish. The media was replaced every 16 hours until the HUVECs cells on the coverslips were fully confluent. The confluent coverslips were then attached to a bottom of a parallel plate flow chamber to form a flow adhesion assay with a rectangular cutout.

2.6. Blood and Neutrophil Perfusion

Neutrophils at 10^6 cells/mL or whole blood were perfused through the confluent circular microchannel or the parallel plate flow chamber (PPFC) at a constant shear rate ($\dot{\gamma}$) : 100, 200, and 400 s^{-1}, for 5 minutes. The desired shear rate for microchannel and PPFC was controlled by adjusting the volumetric flow rate at a syringe pump as defined by Equation (1) and (2), respectively. In these equations, Q is the volumetric flow rate, r is the radius of the micro-channel, h is the height of the gasket, and w is the width of the gasket. The cryosection velocity profile for both the PPFC and microchannel will be representative of what is shown in Figure 1.

$$Q = \dot{\gamma}\frac{\pi r^3}{4} \tag{1}$$

$$Q = \dot{\gamma}\frac{h^2 w}{6} \tag{2}$$

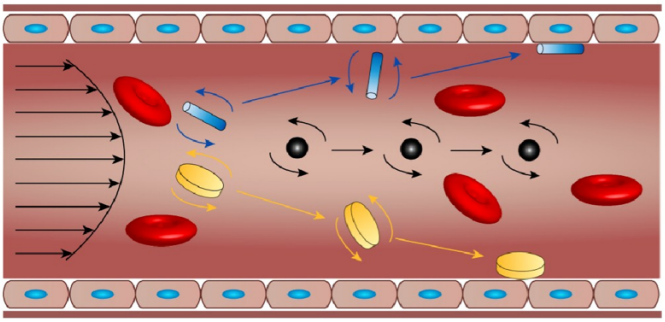

Figure 1 Effect of morphology and flow properties on migration of cells and particles, reprinted from [13].

2.7. Shear Flow Experiments

All flow experiments were conducted in a controlled environment of 37 ºC to mimic the homeostatic human condition. To achieve the controlled temperature, a custom incubator was designed for the Nikon-Eclipse-Ts2R and will be further discussed in section *3.3 Microscope Incubator Design.*

The experimental setup for microchannel adhesion flow experiments can be seen in Fig. 2. Tubing and l-connectors were used to connect the syringe pump, Legato 110 Syringe Pump, to the channel and then to the inlet reservoir syringe. Similarly, the parallel plate flow chamber has comparable setup to the micro-channel, but a vacuum pump was required to adhere the confluent cover slip and gasket to the parallel plate device, shown in Fig 3. Flow rates were alternated per device to the desired testing shear rate. Each shear rate tested consisted of 3 trials with 3 different healthy donors per trial. Blood donated was used within 4 hours of being drawn to avoid abnormal clumping. All data points from trial comparing the neutrophil binding within the PPFC and microchannel pairings were done in the same day with the same pool of blood, 30 minutes apart.

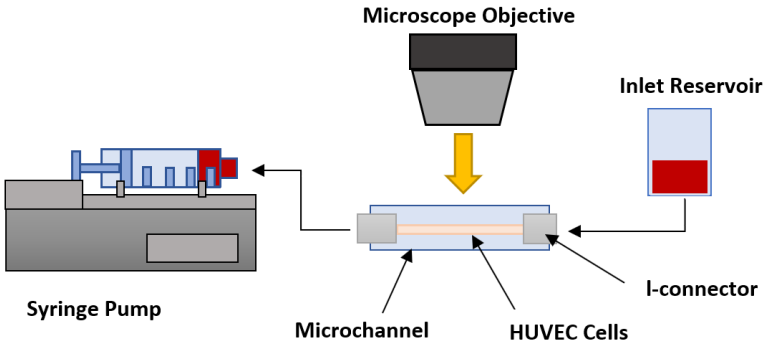

Figure 2 Microchannel adhesion experimental flow setup.

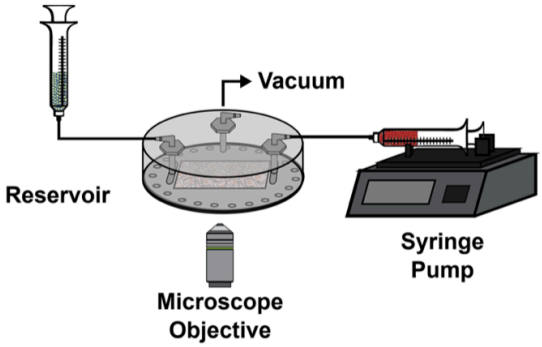

Figure 3 Parallel plate flow chamber experimental flow setup, reprinted from [14].

2.8. Assessment of Neutrophil Attachment

After the adhesion flow experiment and the devices become imageable, screen shots were taken per channels or cover slip. For micro-channels, neutrophils were counted on the focal plane and divided by the focal plane area; this is known as attachment concentration. A similar process is done with the PPFC; however, images are taken at or near the mid-flow streamline. Counting neutrophils at or near the entrance or exit of a

channel or cover slip was avoided to dismiss any discrepancies with the flow development region.

2.9. Statistical Analysis

All results are expressed as data mean with a standard error (n = 3). Statistical analysis of all quantitative data was performed either via Student's t-test or one-way ANOVA with Post-Hoc Tukey analysis using MiniTab. Statistical significance and not statistical significance are demonstrated as (*) = $p < 0.05$ and (NS) respectfully.

3. RESULTS

3.1. Research Aims

This research project is broken down into three aims: circular microchannel design and manufacturing, custom microscope incubator design and construction, and neutrophil adhesion experiments.

The first aim consists of developing a method to construct circular microchannels at various diameters. The developed method of manufacturing channels should be streamlined, consistent, and ensure component reusability. Additionally, cells inside channels must be viewable when bright-field imaging.

The second aim is to construct an incubator for the Nikon-Eclipse-Ts2R microscope. The custom incubator is designed to provide and sustain homeostatic temperatures during the flow adhesion experiments. In addition, the incubator casing must be modular, provide sufficient space to store flow experiment equipment, and, most importantly, supply the heat for the internal temperature to reach 37 ºC within a practical time frame (i.e. two hours).

Lastly, the third aim, which is the primary research goal, is to investigate and compare cell margination in a conventional non-circular flow assay and the circular microchannel. Specifically, the margination of neutrophils from human blood flow and their adhesion on inflamed endothelium, these in vitro models will be studied under various conditions. The flow conditions include various shear rates (100-400 s^{-1}), channel heights (500 and 800 μm), and flow types (laminar and pulsatile). Prior to conducting any

experiments, a common protocol for HUVECs cell seeding on the coverslip (used in the non-circular flow channel) and the microchannel must be developed since different seeding protocols are currently used. The results from this aim will explore the effect of channel geometry on neutrophil cell adhesion. This information is critical and should be known before using the flow adhesion assays for different applications, e.g. for testing the effectiveness of a drug delivery system.

3.2. Aim 1: Circular Microchannel Fabrication

The first aim of this research project is to create a method of manufacturing circular-cylindrical microchannels. Conventional methodologies for creating oval or rectangular shaped microchannels typically rely on a lithography technique [8]. This technique involves a process of etching a preselected design into a silicon wafer to generate a channel template. Then, PDMS is poured and allowed to cure over the design. A key disadvantage to soft lithography is the absence of circular channel cross-section, which is more physiologically accurate than rectangular or rounded edge channels [3]. Recent works have investigated an alternative method in creating a circular microchannel. Researchers have used a wire/fiber or glass capillary tubes as a channel template instead of the silicon wafer [3]. Though this method cannot construct complex channels and requires a trained hand to consistently produce useable channels, this method is overall much simpler and requires fewer materials.

In this study, 3D printer's nozzle needle cleaners were used over the wire and glass capillary tubes due to improved durability, tolerance control, smooth surface finish, minimal surface scratching, and manufacturability scale of 150 to 10000 μm diameters.

Using this method for the microchannel production allowed for consistent construction of circular microchannels at various diameters.

Several characteristics were considered for the mold and connector design. This included the thickness of a mold block, channel length, and the elimination of bio-punching the inlet and outlet of a device. The motivation behind eliminating the bio-punching was to minimize total manufacturing steps while also standardizing the cavity for connector usage. After several design iterations, the final mold and connector designs are shown in Fig. 4. A few modifications made to the mold included the addition of a reservoir for excess PDMS and filleted hole slots for t-connectors. The t-connector can be pressed into the mold and will create a 3 mm cavity on the MC if PDMS is baked. The use of fillets on t-connectors were essential for re-usability, otherwise connectors would break in 1-3 uses due to the high stress concentration caused by a 90-degree bend [15, 16].

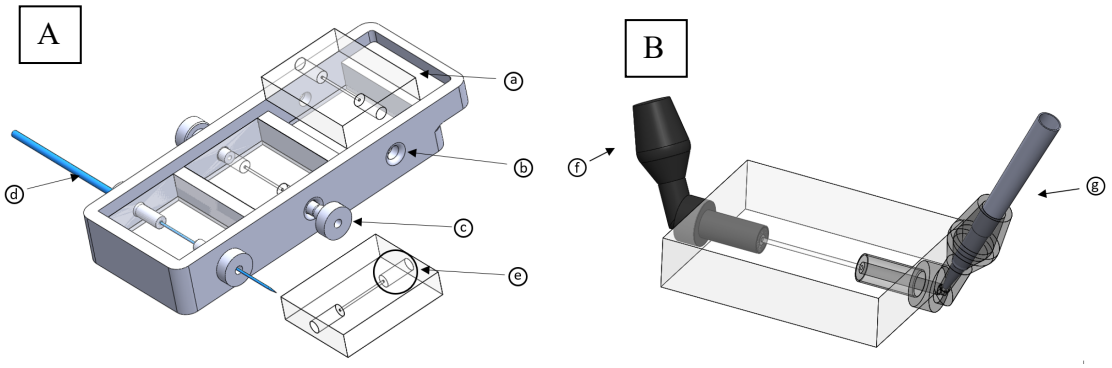

Figure 4 A) The three-chamber mold is designed to have a (a) reservoir for excess PDMS and (b) filleted hole slots for (c) t-connectors. Once PDMS is cured, (d) needles and t-connectors can be removed allowing the MC to be extracted. Additionally, t-connectors will leave a (e) cavity on MC device. B) The microchannel can be fitted with two (f) L-connectors; connectors are designed to be used with 10 – 100 µL pipet tips.

The circular microchannel is formed after the PDMS cures and the needle and t-connectors are removed. The L-connector, shown in Fig. 3B, was designed to be used alongside a 10-100 uL pipet tip to seed cells or replace media from the MC.

3.3. Aim 2: Microscope Incubator Design

In order to simulate physiological conditions for the flow experiments, a custom incubator was designed to house the Nikon-Eclipse-Ts2R to maintain the 37 ºC ambient temperature (Fig. 5). Besides being temperature controlled, the incubator had to be ergonomic and spacious to store equipment needed for experiments. Modularity is achieved by having removable front and back panels to easily place or remove the microscope when needed. Following, the doorways allow for easy access to the microscope stage and the interior panels for cleaning. All CAD work for the custom casing was developed on Solidworks 2018.

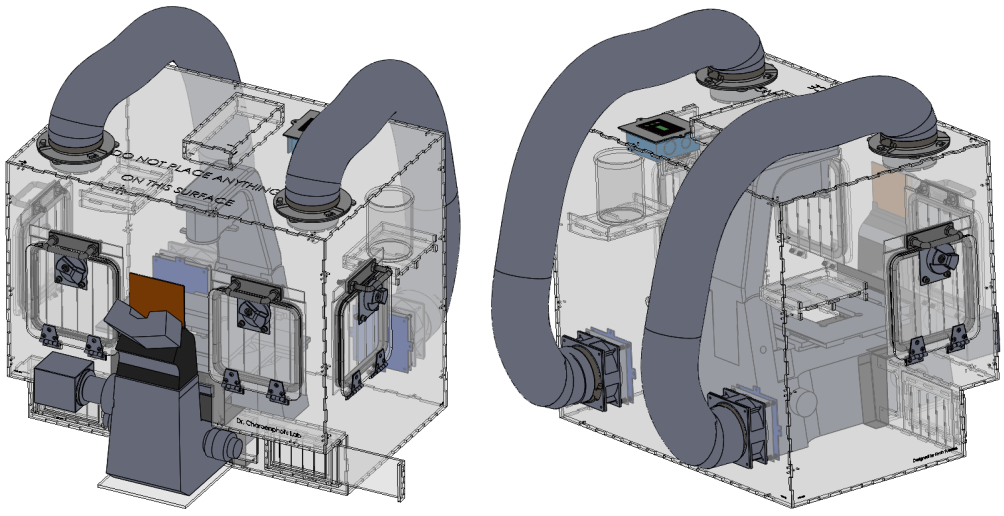

Figure 5 CAD model of a fully assembled incubator for the Nikon microscope.

The incubator is temperature controlled by two convective heating elements. Air within the incubator is continuously recirculated and heated to reduce the time and energy needed to reach the set point of 37 C. In order to minimize heat loss to the outside environment, several preventions have been put in place such as insulated ducts, rubber linings on doorways, and a black tarp to cover openings (Fig. 6). The puzzle design was used on most panels of the incubator to ensure a proper alignment during the assembly. An early rendition of the incubator design was built and can be viewed in Fig. 7. Using the prototype incubator for nearly 4-months revealed ergonomic and design issues. In response, counter measures were implemented on the 2.0 incubator design.

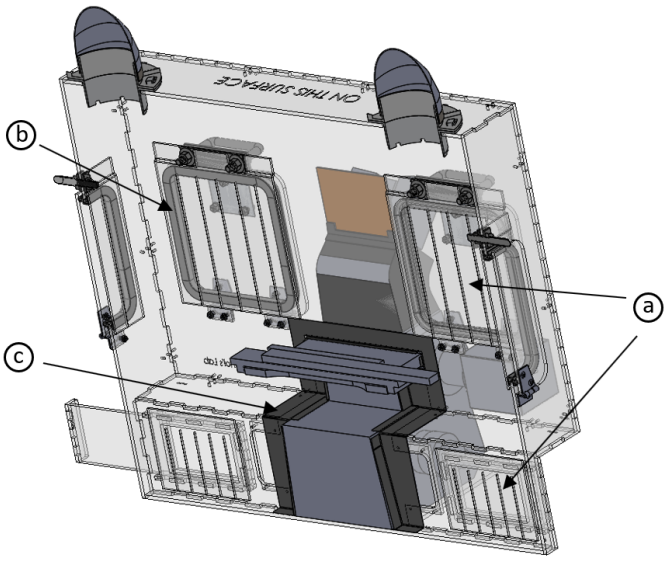

Figure 6 To minimize heat loss and to better stabilize the incubator temperature the use of (a) plastic flaps, (b) rubber linings on doorways, and (c) black tarp were implemented on the incubator design.

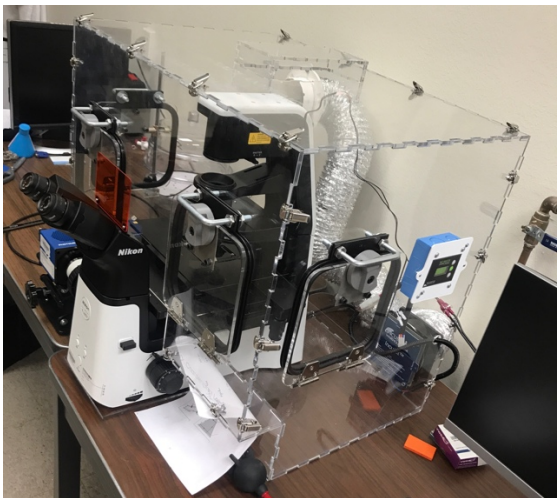

Figure 7 Physical build of the initial incubator design.

3.4. Aim 3: Comparing Cell Margination in a Conventional Non-circular Flow Assay and the Circular Microchannel

3.4.1. Seeding Protocol for Microchannels and Coverslips

Part of the third aim is to develop a seeding protocol for the microchannel, which can consistently achieve a confluent-monolayer of HUVECs along the channel wall. Our initial attempt was to determine a substrate that supports HUVECs growth and maintains the cell integrity during the flow experiment. Our results have shown that fibronectin and a combination of collagen with fibronectin were all found to be functional, shown in Fig. 8. All substrates allowed HUVECs cell to grow, form a monolayer and reach confluency throughout the entire channel wall within an expected time frame.

Since previous findings demonstrated that substrate can effect endothelial cells function, therefore, these microchannels were further tested for neutrophil attachment using different substrate variances [17]. Similar to the previous work, our results showed

that the level of neutrophil attachment on activated HUVECs is substrate-dependent (Fig. 9). The binding of neutrophils on HUVECs cells grown on 0.1 mg/mL of fibronectin was found to be significantly different that of 0.1 and 0.05 mg/mL of collagen and fibronectin. It should be noted for this particular investigation, isolated neutrophils were perfused through channels. For the remainder of the study, 0.4 mg/mL of fibronectin is used over the other two variances due to greater cell confluency success on the microchannels.

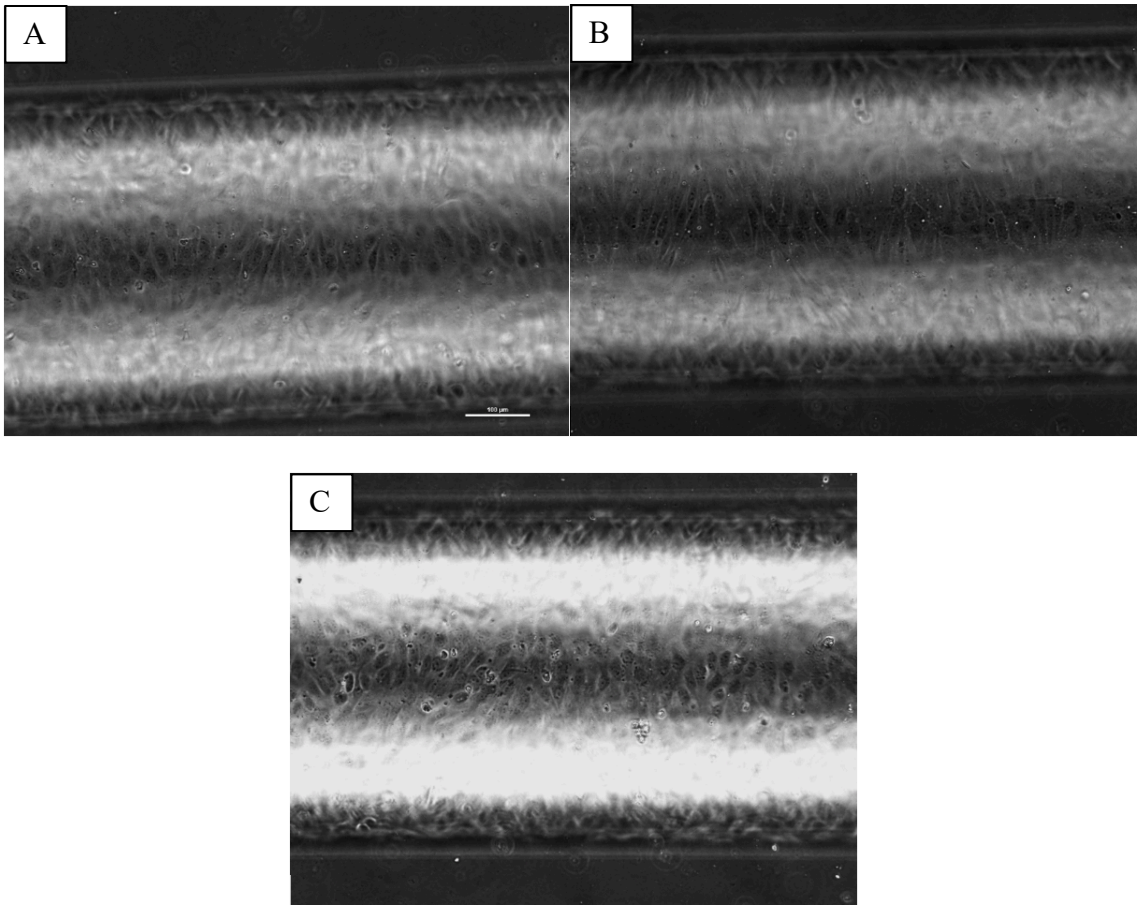

Figure 8: Confluent 500 μm microchannels with varying substrate. A) 0.1 mg/mL of fibronectin, B) 0.4 mg/mL of fibronectin, and C) 0.1 and 0.05 mg/mL of collagen and fibronectin.

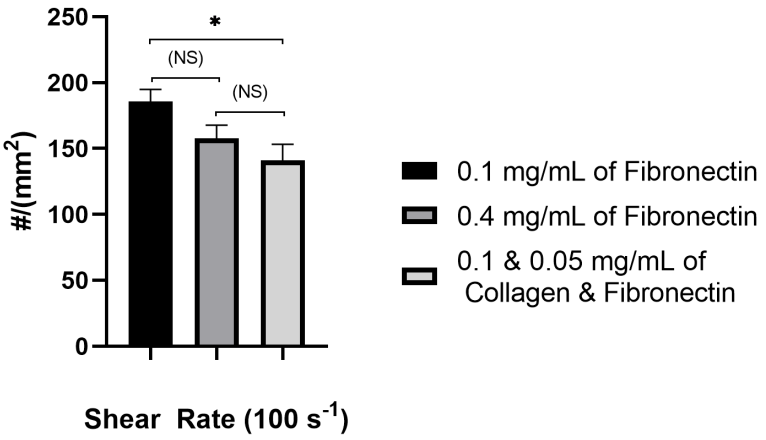

Figure 9 Discrepancies in neutrophil attachment concentration based on substrate variation.

Due to the discrepancies in neutrophil attachment on different concentrations and substrate combinations, there is a need to use a common seeding protocol for both microchannels and coverslips to avoid the substrate effect on the neutrophil adhesion. The seeding methodologies were previously described in the Methods and Materials, sections 2.4 and 2.5. The confluence of HUVECs within the microchannel or on the coverslip can be imaged using confocal, light (Fig. 10), and florescent microscopes. It is worth noting that as the diameter of the microchannel decreases, the imageable focal plane size also decreases.

Confocal imaging was used to confirm the confluency of HUVECs cells along the walls of the microchannel, as shown in Fig. 11. From both cross-sections shown, it is apparent that ECs are lining the channel contours. It must be noted that images are based on cells grown under static conditions.

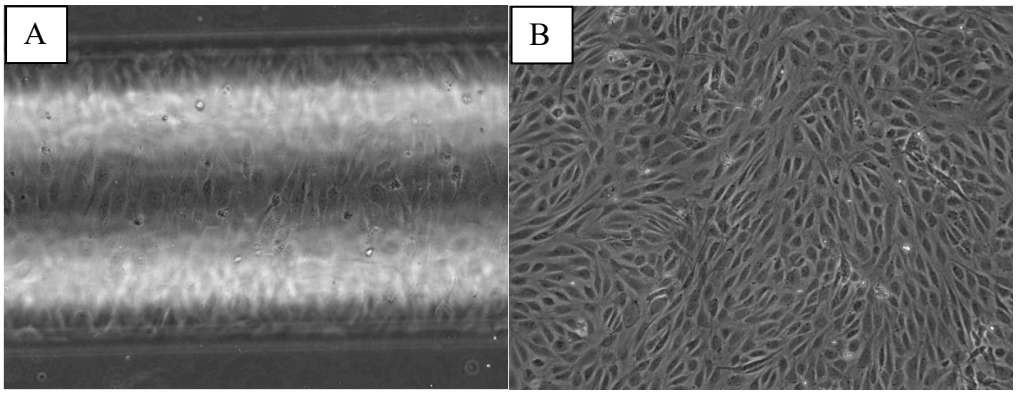

Figure 10 Optical microscope images of HUVEC cells within a 500 μm (A) MC and (B) CS on the PPFC.

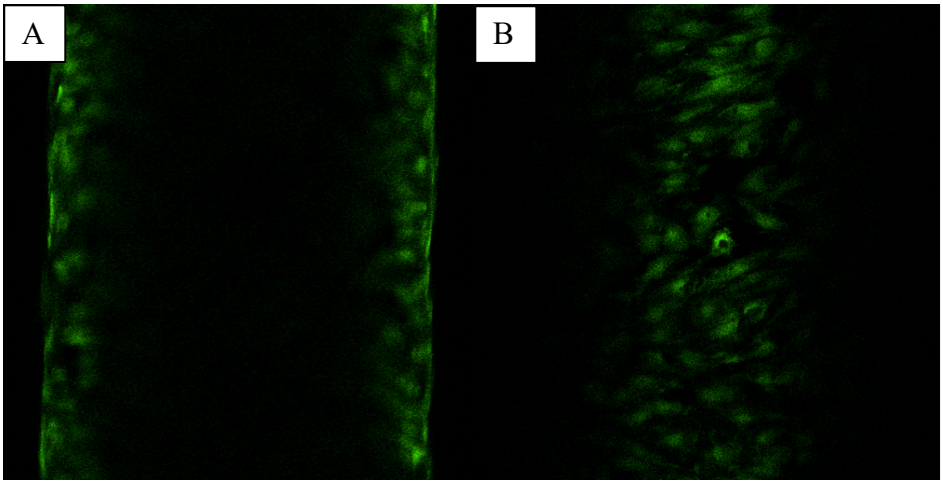

Figure 11 Confocal image of a 500 μm microchannel where A) is the cross-sectional profile and B) is the top view.

3.4.2. Flow Experiments

To determine the effect of channel geometry on cell adhesion, neutrophil attachment from human whole blood was observed in the circular microchannel and compared to the parallel plate flow chamber (PPFC) with different variables. In this

section margination of neutrophil will be compared by varying shear rate, channel height and type of flow.

3.4.2.1. Comparison Between 500 μm MC and PPFC with Laminar Flow

At a fixed height of 500 μm, shear rates were varied (100, 200, and 400 s^{-1}) for the MC and PPFC. Figure 12 shows neutrophil adhesion on HUVECs within the MC and PPFC after the 5-minute blood flow adhesion experiment at a shear rate of 100 s^{-1}.

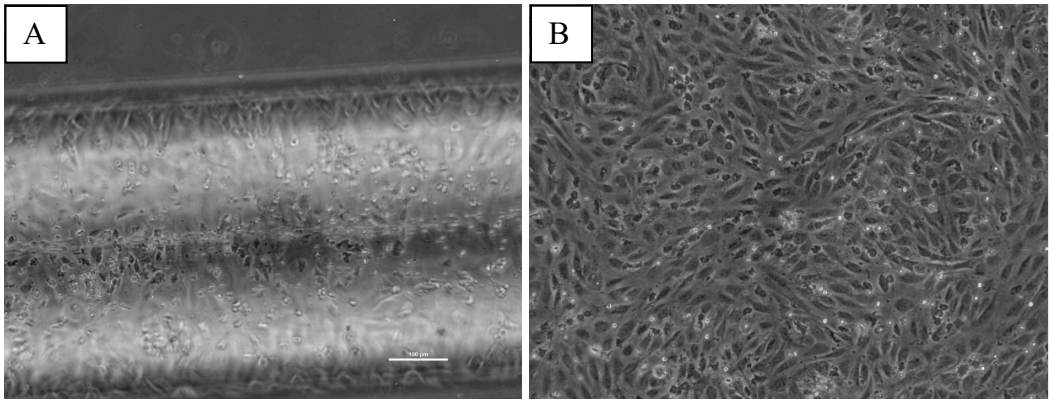

Figure 12 500 μm (A) microchannel and (B) CS on the PPFC post adhesion flow experiment, ran at 100 s^{-1}.

The plot, shown in Fig. 13, illustrates that as shear rate is increased from 100 to 400 s^{-1}, the level of neutrophil attachment decreases within the circular microchannels. However, the trend is opposite for the non-circular PPFC: the neutrophil attachment increases with the increase in shear rate, consistent with our previous work [18]. A one-way ANOVA statistical analysis was conducted for individual PPFC and MC groups. For PPFC, when comparing attachment at 100 and 200 s^{-1} shear rates, the level of neutrophil binding is not significantly different. However, for MC, the neutrophil binding at all shear

rates are significantly different from each other. Additionally, when doing a 2-sample t-test for all PPFC and MC shear rate pairings, results are significantly different. This data emphasizes that the level of neutrophil attachment is impacted by both channel geometry and shear rate.

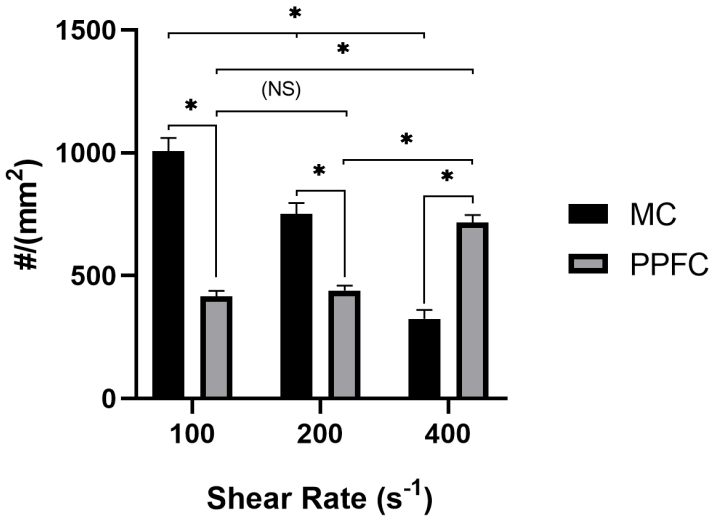

Figure 13 Neutrophil attachment concentration as it relates to shear rate at a fixed 500 μm height for MC and PPFC.

3.4.2.2. Comparison Between 800 μm MC and 750 μm PPFC with Laminar Flow

Similar to the 500 μm height condition, 800 μm MC and 750 μm PPFC were varied with different shear rates, 100 and 200 s^{-1}. Data for 400 s^{-1} was not collected due to the high volume of blood required per trial for the PPFC. Though PPFC and MC do not have equivalent heights, channels height values are justifiably similar where data trends can be compared. Figure 14 are sample images post the adhesion flow experiment for MC and PPFC at a shear rate of 200 s^{-1}.

Comparable to the 500 μm trend depicted in Fig. 13, Fig. 15 demonstrates as shear rate increases, neutrophil attachment decreases for MC. For the non-circular PPFC, neutrophil attachment increases with increased shear rate. A one-way ANOVA statistical analysis was conducted for individual PPFC and MC groups; neutrophil attachment was significantly different as shear rate varied for both respected groups. However, when conducting a 2-sample t-test for all MC and PPFC data pairings, neutrophil attachment is not significant at 100 s^{-1}, whereas at 200 s^{-1}, neutrophil attachment is statistically significant. This data set further emphasizes that neutrophil attachment is impacted by shear rate and geometry.

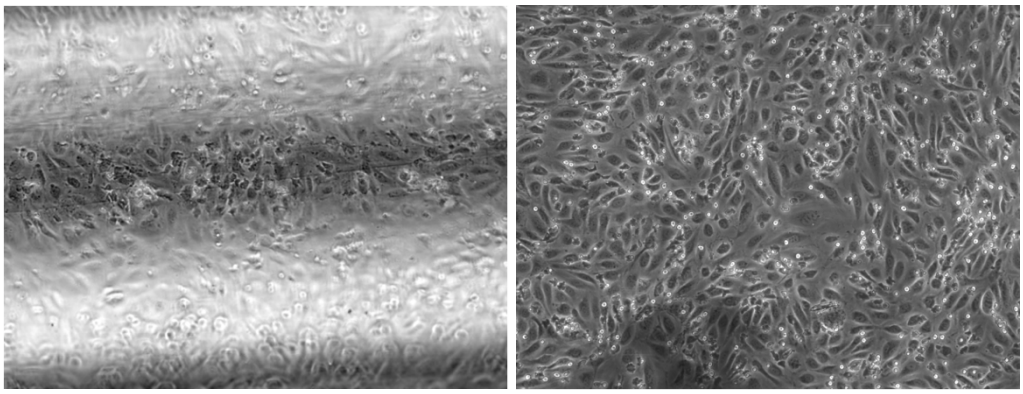

Figure 14 (A) 800 μm microchannel and (B) 750 μm CS on the PPFC post adhesion flow experiment, ran at 200 s^{-1}.

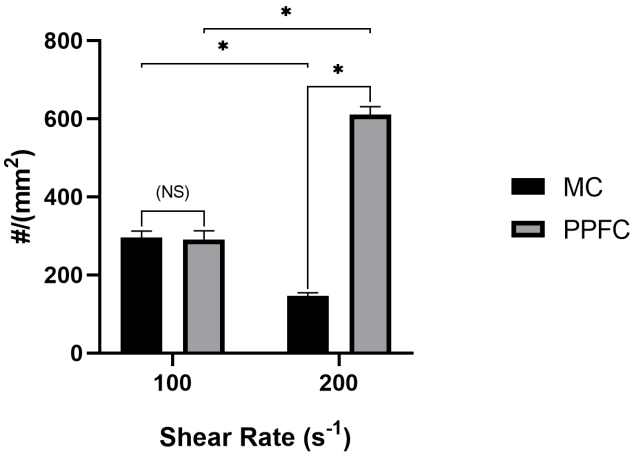

Figure 15 Neutrophil attachment concentration as it relates to shear rate at a fixed 800 μm height for MC and 750 μm PPFC.

3.4.2.3. Comparison of Margination Between Flow Types

Lastly, by fixing the channel height, neutrophil margination can be compared between laminar and pulse type flow, shown in Fig. 16. For pulse flow, whole blood was perfused at a shear rate 200 s^{-1} for 5 seconds, flow was stopped for 5 seconds, and the cycle is repeated until the adhesion flow experiment is completed. Since the average shear rate of pulse type flow is 100 s^{-1}, results are comparable to laminar flow at the same shear rate. A one-way ANOVA was done for each common geometry set, resulting in statistically different attachment for flow type. Analogous to previous results, when conducting a 2-sample t-test, for all MC and PPFC data pairings, neutrophil attachment is significantly different. This data set emphasizes that flow type has an effect on neutrophil adhesion.

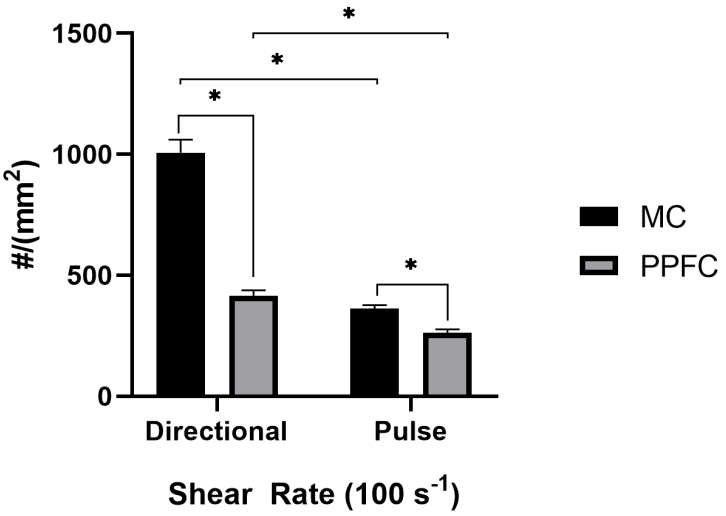

Figure 16 Neutrophil attachment comparison between 500 μm MC and PPFC with different flow types. The average shear rate is 100 s^{-1}.

4. DISCUSSION

4.1. Impact of Shear Rate and Channel Geometry on Red Blood Cell Free Layer

There are many variables that can alter neutrophil adhesion, a few being endothelial cell alignment, donor's blood, and geometry of conduit. Endothelial cell alignment is important for cell functionality; while geometry and shear rate determine the red blood cell – free layer (RBC – FL) thickness [19-21]. The RBC – FL is an RBC – excluded region, forming close to the blood vessel wall when whole blood experiences shear stress. This RBC – FL enables neutrophil or particle interaction with ECs lining the conduit wall, which facilitates their attachments [19].

To compare RBC – FL between the cylindrical microchannel and the parallel plate flow chamber, velocity profiles should be related. For circular channels, the velocity distribution is a symmetric-3D parabola, shown in Fig.17. The 3D velocity symmetry caused by the curvature geometry effects the close packing of RBCs, resulting in a thinner RBC – FL compared to a 2D geometry at the same shear rate and fixed channel height [19]. On the other hand, the PPFC has a uniform parabolic array velocity profile along the width of the flow chamber, shown in Fig. 18. The 2D packing of the RBC ultimately effects the thickness of the RBC – FL.

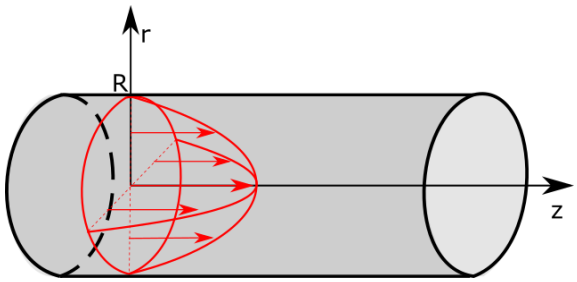

Figure 17 3D velocity profile for a circular channel, reprinted from [22].

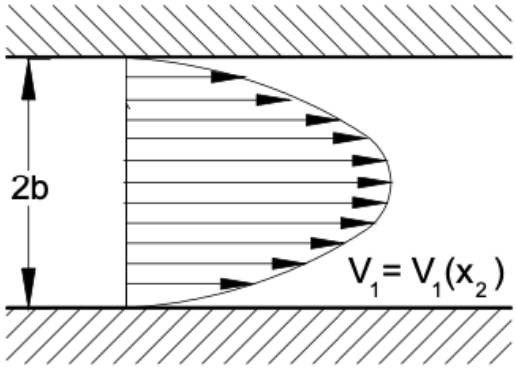

Figure 18 2D velocity profile for the parallel plate flow chamber, reprinted from [23].

Beyond being affected by geometry, the size of RBC – FL is dependent on shear rate and Fig. 19 depicts how neutrophil attachment can alter with the change of RBC – FL size. At low shear rates, RBC distribution is typically uniform, resulting in minimal formation of RBC – FL and negatable margination. As shear rate is increased, the RBC – FL is formed and surges in size, causing amplified margination [19]. However, if the RBC – FL becomes too large (above RBC – FL max), the margination probability decreases. Once the maximum width of the RBC – FL is reached, the RBC – FL decreases as shear rate increases, resulting in increased margination [19, 24]. By continuously increasing shear rate, the RBC – FL may decrease below a critical width (RBC – FL critical), in

which the RBC – FL becomes smaller than a particle or a neutrophil diameter. In this case, RBC can collide with the bound particles or neutrophils and decrease their attachment levels.

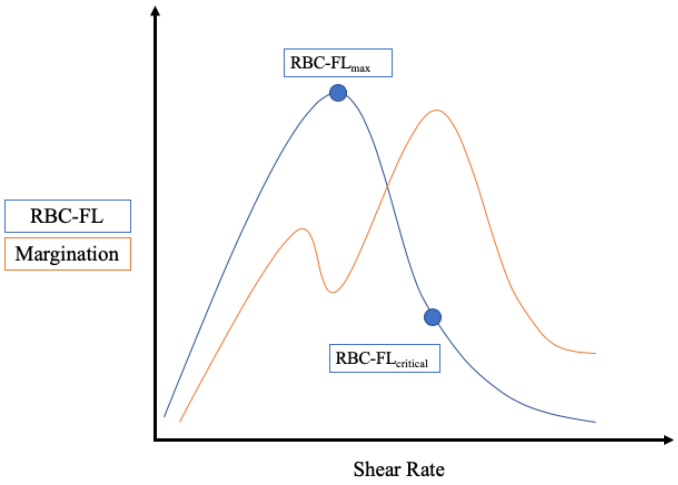

Figure 19 RBC – FL width is dependent on shear rate, which effects margination probability.

Using the description of RBC – FL as it relates to shear rate, along with Fig. 19, data trends can be described for Fig. 13 and 15. For MCs, as shear rate is increased, the margination decreases; this behavior is best described by the reduction of RBC – FL to the point where the RBC – FL width is smaller than the white blood cell diameter. In contrast, for the PPFC, the margination increases with shear rate. Fig. 19 suggests that the RBC – FL is reducing in size and approaching the RBC – FL critical width, which resulted in increased margination.

Looking at Fig. 13, comparing the data pairing for MC and PPFC at a shear rate of 100 s^{-1}, MC has significantly higher attachment than the PPFC. This is likely because the

RBC – FL within the microchannel is in a closer proximity to the RBC – FL critical value compared to the PPFC at 100 s^{-1} as shown in Fig. 19. It is worth noting that the RBC – FL thickness is a function of both geometry and shear rate. Thus, it is unclear whether the RBC – FL critical value will be the same thickness for MC and PPFC and whether the PPFC will have similar neutrophil margination compared to MC at a similar RBC – FL thickness. These research questions can be explored in future work.

4.2. Neutrophil Attachment Across Different Channel Heights

Beyond researching the effects of neutrophil attachment on different cross-sectional geometries, a few studies were conducted to determine how neutrophil attachment changes with altered channel heights, shown in Fig. 20. For the MC, the neutrophil attachment concentration within the 500 μm channel was statistically greater than the 800 μm channel at two different shear rates. For the PPFC, the attachment was greater for the smaller channel at 100 s^{-1}, though results were not statistically significant. At 200 s^{-1}, the attachment was statistically greater for the larger channel. Though not definitive with all results, data can be best described by: RBC – FL thickness reduces as channel height decreases, resulting in increased margination [19]. However, this deduction is not so black and white, RBC – FL is a function of shear rate, meaning this conjecture can alternate.

The behavior of the RBC – FL, as depicted in Fig. 19, will be similar regardless of channel height. In contrast, the margination trend of common geometries at different heights will skew from one another as illustrated in Fig. 21. Again, this is because the RBC – FL changes thickness when channel height is altered at a fixed shear rate.

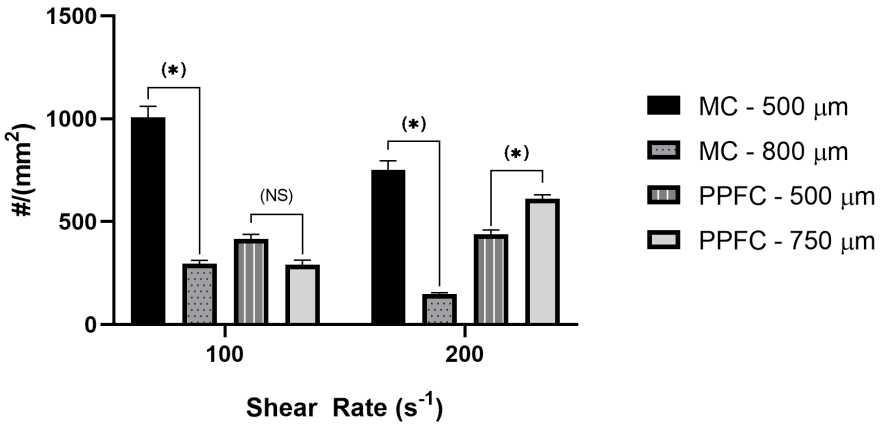

Figure 20 Neutrophil attachment across the MC and PPFC at different shear rates and heights.

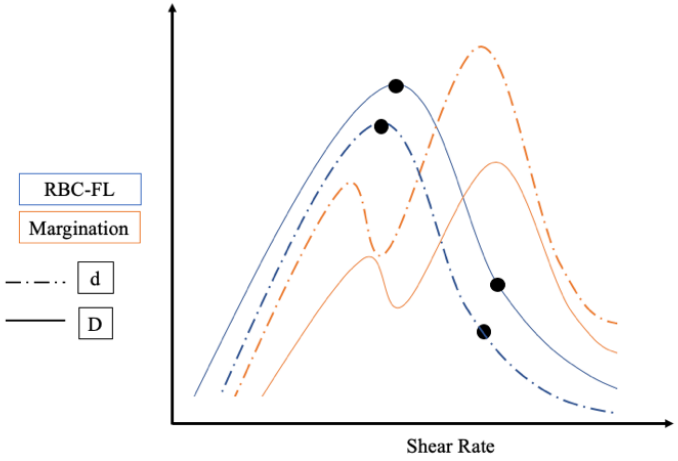

Figure 21 Margination comparison between similar cross-sectional geometries, but with different heights (d, D), where d < D.

4.3. Margination Across Different Flow Types

Lastly, margination was compared across two different flow types, laminar and pulse, at a fixed 500 μm height across the MC and the PPFC. Comparing attachment across

equivalent geometries, neutrophil attachment was statistically lower for pulse flow. The low attachment in pulse flow compared to laminar flow can be related to the RBC – FL. Since in the pulse flow, whole blood was running at a shear rate of 200 s^{-1} for 5 seconds, stopped for an additional 5 seconds and repeated until the experiment ended. The RBC – FL fluctuates in thickness, which in turn effects margination probability.

The RBC – FL fluctuation is different for the PPFC compared to MC on pulse type flow. However, the results are the same: pulse flow has decreased neutrophil attachment compared to laminar. The decreased attachment trend for the MC and PPFC is best explained by the RBC-FL alternating between two states: existing and not existing. At 200 s^{-1}, the RBC-FL exists, and facilitates neutrophil attachment onto ECs. At the static condition, the RBC – FL is not formed, causing neutrophil attachment to be minimal. Ultimately, margination should decrease compared to laminar flow.

These experiments only used one of many possible pulse conditions. Nevertheless, there was a statistically significant difference in attachment across laminar and pulse flow types using two different geometries. While comparing laminar and pulse flow using a similar average shear rate, RBC-FL fluctuation determines neutrophil margination success. That said, it is important to test new drugs or delivery systems using physiologically relevant geometry, shear rates, and flow types because all variables affect the CFL, which alters margination probability.

5. CONCLUSIONS AND FUTURE WORK

5.1. Conclusion

From this research, a streamlined methodology and equipment were constructed to manufacture circular micro-channels at various diameters. Though complexity of channel design is limiting, device use-case-ability is not. The in vitro model can provide initial insight on drug effectiveness by using physiologically relevant geometry and shear rates. Adding onto, this research showed how margination of white blood cells was affected by substrate coating, geometry, shear rate, and flow type. Initial findings determined that neutrophil binding can be substrate dependent. Therefore, it is important to use a common seeding protocol for HUVECs within and across different testing platforms to compare migration results.

For the testing shear rates, 100 – 400 s^{-1}, attachment decreased for MCs as flow rate was increased. However, the trend was opposite for the PPFC, attachment increased with increased flow rate. This trend is best described by the RBC – FL altering with geometry and shear rate. Ultimately, conduit geometry determines how the RBC are packed, which correlates with RBC – FL thickness.

Though not definitive for all gathered data points, as conduit size decreases, margination efficiency increases. This can be described by RBC – FL reducing in size as channel height follows. However, this deduction is not absolute, since the RBC – FL is also a function of shear rate. Additionally, it is important to understand how varying RBC – FL effects margination. Although only one pulse flow condition was tested, margination

values were statistically different when compared to laminar flow at the same average shear rate. With all this said, when testing a new drug or delivery system, it is important to use physiological relevant conditions such as channel geometry, flow type, and shear rates to name a few, because all variables can affect margination probability.

5.2. Future Work

From previous studies, it has been reported that ECs alignment effects cell functionality [20, 21]. A series of experiments can be done to determine how cell alignment impacts the white blood cell attachment trends at various shear rates. Cell alignment can be varied by continuously perfusing media through the channel using a syringe pump. Additionally, similar experiments can be done to compare the effect of cell alignment between alternate channel heights and flow types. These experiments can further our understanding on the relevancy of using physiological conditions by showing statistically significant differences in attachment trends. Separately, additional data points for alternate channel heights, beyond existing data, will give a better understanding on how channel height plays a role on neutrophil attachment.

Beyond cell alignment, comparing margination at a common RBC – FL thickness could give additional insight on how hemodynamics/shear rate plays a role in attachment on different and similar geometries. Computational simulation can be used to calculate shear rate for equivalent RBC – FL with alternate height and geometry [19]. Results will be interesting due to different shear forces acting on the wall.

Penultimately, channel path complexity must be adjusted. Having a linear channel is useful for several applications, however, design is limiting. Future channels should

implement bifurcation, stenoses, and other physiological relevant contours [10]. The development of the circular micro-channels was aimed to test the effectiveness of a multistage-drug delivery system (MDS) developed by our research group. The goal is to investigate whether MDS outperforms the nanoparticles in terms of EC attachment, since nanoparticles are commonly used as drug carriers in various applications [25].

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