FLOW CHAMBER FOR STUDYING CELL ATTACHMENT TO OPAQUE SUBSTRATES

By

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This document is dedicated to my parents, my brother, and “mi gordita Linda,” Paola.

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Endovascular stent-grafts for aneurysm repair have been appealing for several reasons, they are less invasive than conventional "open" operations, have a potential for regional anesthesia, and require a shortened hospital stay. These short-term benefits have been well documented, but procedures have been associated with long term complications such as endoleaks and late kinks or dislodgements of the grafts that can cause aneurysm rupture or graft occlusion. These complications suggest that the durability of the stent-grafts is unreliable and one contributing factor may be that endovascular grafts do not incorporate well enough.

The objective of our research is to develop a precise system that will allow us to measure the kinetics of endothelial cell adhesion on a variety of materials and substrates. Specifically, we are interested in determining the endothelial cell adhesion and detachment properties for 3 different polymer substrates on an opaque material like Dacron, which is a graft material that is widely used for stent graft treatments.

Endothelial cells will be cultured to grow on Dacron, Fibronectin, Matrigel, and Collagen IV, and their development and ability to reach confluence will be studied and documented. The attachment of cells will be qualitatively and quantitatively evaluated through shear testing in our system. The results should confirm the effectiveness of our device and indicate a good candidate or at least provide an effective protocol for determining an ideal stent-graft coating. In any case, the type of prosthetic polymer and substrate protein coating used to promote endothelial cell adherence are the two important factors which may determine the ultimate success of endothelial seeding in the operating room.

CHAPTER 1
INTRODUCTION

1.1 Objective

An abdominal aortic aneurysm (AAA), which is an area of localized widening (dilation) of the aorta, is a significant medical problem with a high mortality. The conventional treatment on the basis of surgical reconstruction is effective but associated with a significant morbidity and mortality rate (3% to 7%) [1]. Its alternative treatment, endovascular aneurysm repair (EVAR), has proven to be less invasive but several problems in the use of it have not been solved due to the deficient healing between the grafts and the blood vessels.

One of the major drawbacks of EVAR is endoleakage, which is defined as leakage at the proximal stent of the endovascular prosthesis or as a backflow into the aneurysm through one of the lumbar arteries [1]. Endoleakage leads to persisting pressure in the aneurysm, and has been reported as a cause of aortic rupture [1]. Additionally, EVAR is plagued by distal graft migration due to deficient graft healing that can lead to endoleakage.

It has been hypothesized that better healing leads to better graft fixation and a reduction in the incidence rate of endoleakage and distal migration of stent-grafts. Studies have been conducted to test the proliferation rates and shear resistance of cells on different substrates, but none have been able to give a real time analysis of cell detachment during a given flow rate. In fact, none have been able to give a real time analysis of cell detachment from an opaque material like Dacron. The objective of this

research is to develop protocols for determining cell adhesion and design and build a Parallel Plate Flow Chamber (PPFC) that can give us a better understanding of cell detachment from coated substrates or opaque substrates in real-time.

1.2 Specific Aims

- To design and fabricate a PPFC system for the real time study of cell attachment on opaque material like Dacron.
- To culture endothelial cells in a static fluidic environment on selected polymer substrates (Matrigel, Fibronectin, Collagen IV, and our control group Dacron).
- To quantify the cell proliferation and attachment of endothelial cells on each of the polymer substrates and control group.
- To analyze the cell attachment of endothelial cells on polymer substrates and the control group with our PPFC system in real-time.

1.3 Significance

The significance of our research is that via our PPFC, cell proliferation to stent-grafts can be analyzed on any given substrate in real time on virtually any conventional laboratory inverted microscope. Previous in vitro studies of cell adhesion to stent grafts have been conducted by analyzing the seeded substrates before and after large perfusion circuits that are susceptible to contamination and PPFC's that did not allow for real time analysis of cell adhesion [2-6]. This in vitro model has the ability to quantify not only the strength of endothelial adhesion but also the role of flow shear stress in cellular detachment.

Specifically, our PPFC system is designed so that cell properties on opaque surfaces can be observed under any conventional inverted microscope throughout the duration of fluid flow. Because the type of prosthetic polymer and the substrate protein coating used to promote fibroblast cell adherence are two important factors which may

determine the ultimate success of sent grafts in the operating room, it is important to be able to control and observe these variables under a physiologic shear stress.

CHAPTER 2
BACKGROUND

The most recent published data from the National Vital Statistics Report on Deaths from the year 2000 show that AAAs and aortic dissection composed the tenth leading cause of death in white men 65 to 74 years old and accounted for nearly 16,000 deaths overall [7]. Because the mortality rate associated with acute rupture of an abdominal aneurysm is high, treatment of the aneurysm before rupture is vital.

The aorta is the largest blood vessel in the body. It begins at the heart and passes backward to be located deep in the body just in front of the bony spine. When it passes through an opening in the diaphragm it becomes the abdominal aorta. As the aorta passes into the pelvis it splits into the common iliac arteries. The most important branches of the abdominal aorta feed the kidneys and bowel as shown in Figure 2-1. Frequencies of aneurysms in the abdominal aorta are related to abnormalities in blood flow as well as changes which occur to the blood vessel over time because of arteries hardening [8]. When an aneurysm does rupture, 50% of individuals do not survive long enough to receive medical attention and of those who make it to the hospital, 25%-30% die of postoperative complications.

Since the 1950s, the repair of aneurysms has involved invasive surgery. A typical procedure, lasting up to 6 hours, involves opening the aneurysm, removing any blood clots that may be present inside, suturing a graft in place of the aneurysm, and finally suturing the entire wound. Unfortunately, most of the patients that develop aneurysms are elder and have a variety of comorbidities. Thus a relatively high morbidity and

mortality rate is associated with invasive surgery [9]. So a modern solution to the treatment of aneurysms came in the form of endovascular aneurysm repair (EVAR), which is defined as the insertion of bypass conduits, usually fabric cover stents or stent-grafts, through a transfemoral or transiliac approach. This procedure bypasses the need of invasive surgery, does not require as much time to complete, and therefore may prove to be economically favorable.

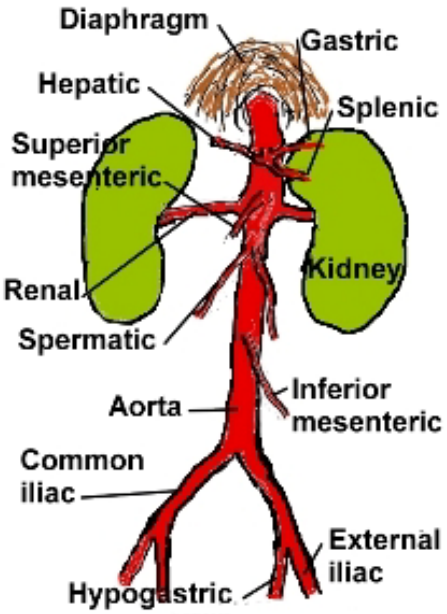


Figure 2-1. Anatomy showing the abdominal aorta and its branches. The abdominal aorta extends from the diaphragm to the iliac vessels. The branches feed the organs within the abdominal cavity [8].

But despite the feasibility of EVAR and the variety of innovative devices that have been designed, tested, and approved for clinical use, several problems in the use of EVAR have still not been solved [9]. One of the problems is endoleakage, which is defined as leakage at the proximal end of the stent-grafts or as a backflow into the aneurysm through one of the lumbar arteries. The prevalence rate of leaks with bifurcated endografts is 18%, and proximal and distal attachment leaks consist of 44%,

resulting in a total of 8% attachment site endoleaks [1]. Endoleaks have been classified into five types (Figure 2-2). Type I endoleak refers to leakage around the end of the stent at the stent-graft attachment site. Type II endoleak refers to retrograde filling of the aneurysms sack occurring from collateral vessels within the aneurized area. Type III endoleak occurs as a result of an insufficient seal at the graft joints or from a rupture in the graft material. Type IV endoleak refers to the leakage of fluid through a porous stent graft covering [1]. Type V leaks, "endotension," occur when pressure is transmitted to the sac without definable blood flow around the graft.

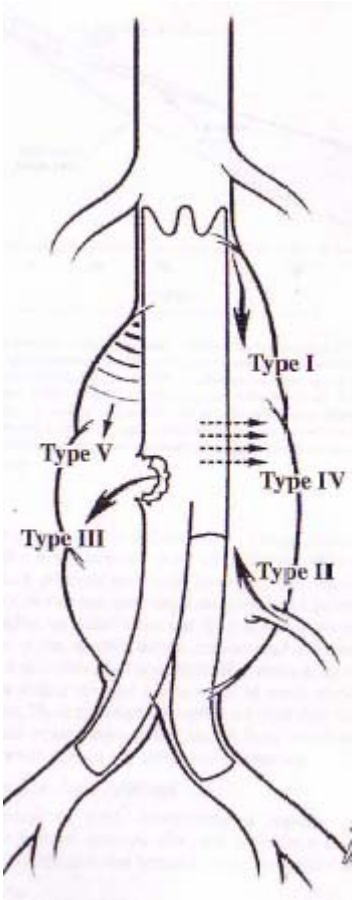


Figure 2-2. Five subdivisions of endoleaks [9].

One of the causes of endoleakage is imperfect sealing at the proximal landing zone. A second cause is distal migration during follow-up from deficient healing between the

aortic neck and the fabric of the stent-graft. Endoleakage leads to persisting pressure in the aneurysm, and rupture has been reported as a cause of persisting endoleak. A second problem of the deficient graft healing may be distal graft migration, which may also cause endoleakeage [1].

The belief is that better healing between the graft and the vessel will lead to a better graft fixation and will reduce the incidence rate of endoleakage and distal migration of stent-grafts. This improvement in the healing is believed to be achieved via mechanical fixation or with the coating of the graft material.

2.2 Mechanically Fixated Endovascular Stent-Grafts

The procedure for correcting aneurysms by use of a vascular stent-graft has proven to be an innovative and less invasive method to treating AAAs. Endovascular stent-grafts are made of an inner tube material, usually polyethylene terephtalate (PET or Dacron) or polytetrafluoroethylene (PTFE) and an outer mesh of nitinol or stainless steel. A number of systems have been developed to provide a durable fixation of the stent-graft to the aortic attachment areas. Three principles are applied to keep the stent-graft in position: outward radial forces of the stents, columnar strength of fully stented stent-grafts, and hooks penetrating the aortic wall as shown in Figure 2-4 [10]. The postoperative reduction of the aneurysm diameter indicates that stent grafting is a sound principle, but the long-term results remain uncertain [11, 12]. Specifically, this procedure has been associated with long term complications such as endoleak and migration of the stent-grafts [10, 13-15]. Reports of migration and endoleak are related to the lack of fixation between the stent-graft and the aortic wall.

Figure 2-4. Hook/stent assembly of a stent-graft [10].

2.3 Coated Stent-Grafts

The lack of adhesion between the stent-grafts and the aortic wall is specifically related to the inability of the Dacron or PTFE to heal sufficiently and provide a firm fixation between the stent-graft and the vessel wall [13].

2.3.1 Dacron as a Graft Material

For our research, we are particularly interested in Dacron because of its superior mechanical handling properties during fabrication and implantation [16]. Three main types of Dacron prosthesis that are commercially available are: woven Dacron grafts, knitted grafts, and Dacron velour grafts [16]. For our research we will be using woven Dacron.

- Woven Dacron grafts (Figure 2-4) have adequate porosity to permit penetration and adhesion of fibrin. Types of applications include replacement of resected aortic aneurysms and vascular procedures for which bleeding is a primary concern.
- Knitted Dacron grafts (Figure 2-5) have porosity much higher than that of woven grafts. Before implantation, knitted grafts must be preclotted to minimize bleeding during operation and eliminate the risk of hemorrhage. Types of applications

include by-pass procedures, replacement in aneurismal and occlusive disease of the abdominal aorta, visceral arteries, and proximal peripheral arteries.

- Knitted velour Dacron grafts (Figure 2-6) have a high porosity and are either warp or weft knitted with loops extending either into the lumen or outside to provide for a more rapid and better adherence of the fibrinous lining. Knitted velour Dacron grafts, like knitted grafts, must be preclotted prior to implantation. Types of applications include implantation after resection of aneurysms of peripheral arteries or by-pass procedures for occlusive diseases or major peripheral and visceral arteries.

Figure 2-5 Woven Dacron graft at a magnification of x75 [16].

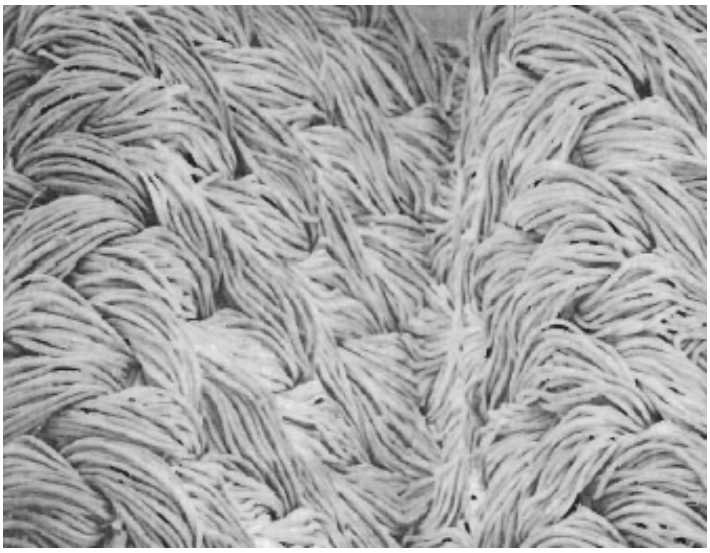


Figure 2-6 Knitted Dacron graft at a magnification of x70 [16].

Figure 2-7 Knitted velour Dacron at a magnification of x70 [16].

2.3.2 Graft Coatings

Since grafts do not achieve endothelial coverage in humans on their own, the idea is to endow them with endothelial cell growth factors and/or proteins to enhance the possibility of achieving endothelial coverage [6, 17]. A variety of protein substrates have been studied by investigators to improve endothelial cell adherence to stent-grafts, or more specifically, to the graft material. In fact it has been documented that the mere seeding of endothelial cells onto these grafts produce rapid endothelialization [15, 18]. But which of these substrates is ideal? The popular approach in choosing an ideal protein is to choose a protein that is already present in the extracellular matrix (ECM) of the blood vessel. In vivo endothelial cells are known to rest on the complex connective tissue structure of the basement membrane and ECM. These membranes are composed of several macromolecules such as collagens, glycosaminoglycans, and the two glycoproteins Fibronectin and laminin. For endothelial cell seeding, these substrates or a combination should result in effective cell adhesion.

The most prevalent of adhesive protein(glycoprotein) found in the ECM is Fibronectin, a large molecule containing about 5 percent carbohydrate and composed of two subunits with a combined molecular weight of about 500,000 dattons [19]. It is found as a dimmer in plasma and in multimeric form in the ECM and on cell surfaces. Its primary function is to bind cells to the ECM. In Dacron and in PTFE grafts, it has been found that Fibronectin increases significantly the percentage of endothelial cells retained after perfusion in in-vitro experiments [2, 3, 20].

Additionally, other studies have shown that Collagen IV substrates cause significant cell attachment too [20]. Collagen is the most prevalent component of the ECM. The characteristic feature of a typical collagen molecule is its long, stiff, triple-stranded helical structure, in which three collagen polypeptide chains are wound around one another in a ropelike super helix. The triple-stranded collagen molecules are packed together into a collagen fibril and the collagen fibrils are further packed together into collagen fibers. These collagen fibers are organized in many different ways to accommodate the function needed. Specifically, Col IV forms a meshwork of filaments localized in the basement membrane that helps anchor surrounding structures to the ECM [19].

Other naturally produced ECM substrates have also demonstrated excellent endothelial cell proliferation and resistance to shear stress on PTFE grafts [5].. But the major drawback that most of the adhesive and ECM proteins face is that they are also good substrates for platelet adhesion and thrombus formation, which is a concern for smaller diameter grafts[2, 5, 20].

Many groups are now either using combinations of proteins and growth factors as substrates for successful cell seeding or simply modifying existing ECM proteins to obtain better results. In 2002 a group in the Netherlands lead by J.M. Annemieke van der Bas M.D., showed that a Dacron graft impregnated with collagen, herparin, and bFGF (a fibroblast growth factor) is capable of inducing graft healing [1]. Currently, one group at the University of Florida is conducting studies on coating a stent-graft with poly(lactide-co-glycolide) (PLGA), which is a biodegradable polymer that promotes cellular reaction both in vitro and in vivo conditions [21]. Other groups have published significant cell attachement to PTFE grafts that were coated with preclot matrix and bFGF [20, 22].

2.3.3 Evaluation of Coatings

Research on coatings has been conducted via implantations, perfusion circuits (Figure 2-2), and parallel plate flow chambers (PPFC). But perhaps the major drawback of all these methods is that a clear picture of the process of cell detachment has not been researched. Specifically, a real-time analysis of the cell detachment from graft materials like Dacron has not been studied.

2.3.4 Background on Parallel Plate Flow Chambers

In-vivo studies aimed at understanding cellular responses to shear forces have the intrinsic problem that they cannot quantitatively define the exact features of the hemodynamic environment. Moreover, it is difficult to determine if the resultant response is due to shear stress or some other feature (i.e. eddy formation and altered mass transfer) associated with the complex in-vivo fluid dynamic environment [23]. In the past several years, engineering fundamentals have been used to design devices that simulate flow conditions encountered in the vasculature. These in-vitro experimental

systems are capable of generating controlled mechanical forces similar to those induced by hemodynamic forces in-vivo.

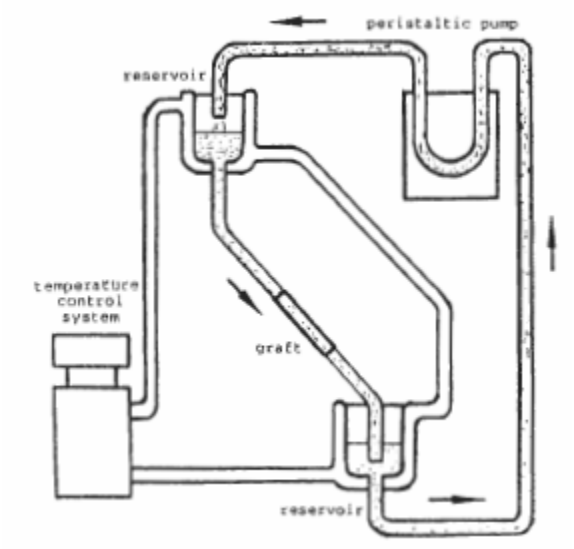


Figure 2-3. In-vitro perfusion loop. Arrows indicate the direction of culture medium flow. Transparent loop indicates circulating water of the temperature control system (37°C) (note the difficulty of analyzing cell adhesion and detachment in real time) [5].

The PPFC (Figure 2-7), frequently used by many researchers investigating cellular responses to shear forces [6, 24-27] provides a controlled and well-defined flow environment based on a fixed chamber geometry and a variable flow rate. Channel flow in a PPFC can be approximated as two-dimensional fully developed laminar flow with a simple parabolic velocity profile, since the channel height is a linear function of the volume flow rate through the channel.

Santhosh Kuman et al. actually used a PPFC to determine whether endothelial cell growth factor (ECGF) enmeshed with fibrin matrix enhances proliferation of endothelial cells [6]. But the major flaw of their research was that a control group (Dacron graft) was never established. Additionally, because of the way that their chamber was designed, the analyses of cell detachments were conducted before and after the substrates were

subjected to shear. Other groups have used PPFC to specifically monitor cellular responses to shear forces and have, additionally, successfully monitored the cells in real-time [26-28]. In fact we intend to modify a PPFC that was previously designed for other cell attachement studies in our Biorheology lab at the University of Florida [29]. Our modifications to the PPFC design will allow us to conduct real-time studies of cellular responses to shear forces on substrates and grafts (opaque samples) in relation to coated stent-grafts. Specifically, we want to satisfy the following criteria:

1. The chamber has to be small enough to be incorporated with conventional microscope setups.
2. The chamber design must be such that we can study cell adhesion to a coated opaque substrate.
3. The chamber has to be such that a fully developed laminar flow can be achieved to conduct accurate shear stress analysis with an upper limit of 60 dynes/cm^2.

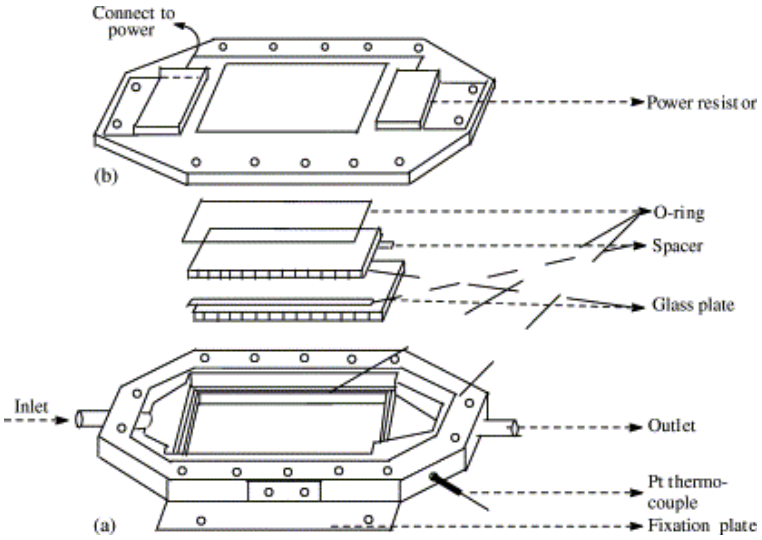


Figure 2-7. Sketch map of a typical PPFC [26].

In short, adhesive proteins such as fibronectin, collagen, whole ECM, and a combination of all these with growth factors have been investigated by research groups around the world for their ability to enhance attachment and proliferation of endothelial cells on vascular grafts [6]. Our goal is to develop a protocol for the analysis of

proliferation rates and adhesion properties of these substrates that will provide a better picture. Currently analyses of cell detachment are being conducted at several time intervals. After exposing cell seeded substrates to flow via a PPFC for a give rate and time, the substrates are washed and viewed immediately under a microscope to detect morphological alterations and cells loss. We propose a more in depth analysis of cell detachment by providing a real-time analysis of the entire flow exposure. We will attempt to verify results published by other groups on 2 different substrates (Matrigel and Collagen IV) and provide a better analysis of their work.

CHAPTER 3
MATERIALS AND METHODS

3.1 Cell Preparation

Porcine Vascular Endothelial Cells (PVEC) from the pulmonary artery were seeded and then cultured on T-75 flasks for our studies. All cells were derived from third or fourth passage. Cells in each of our experiments were derived from the same source. After cells reached confluence in 75 cm^2 tissue culture flasks, they were counted via a hemocytometer and then seeded onto the respective coated substrate. Other cell types that are frequently used in similar studies are smooth muscle cells and fibroblast cells.

3.1.1 Cell Growth Media Preparation

A Fibroblast Bulletkit (BioWhittaker, Inc., USA) that included a 500 ml bottle of Fibroblast Basal Media (FBM) and fibroblast growth supplements (hFGF-B, 0.5 ml; Insulin, 0.5 ml; FBS, 10 ml; GA-1000, 0.5 ml) was used to prepare the cell growth media needed for our studies. When ready for our experiments, the supplements were thawed in a water bath and the external surfaces were decontaminated for each supplement vial and the 500 ml basal media bottle with 70% ETOH before being placed inside of the hood. The contents of each supplement vial were added to the basal media with a pipette. The supplemented media, or just media, was then stored in a refrigerator after each use.

3.1.3 Cell Isolation and Culture

PVEC's were obtained from a pig's pulmonary artery as mentioned previously and then frozen at -70°C. When ready for conducting experiments, the cells were thawed and centrifuged in media at 800 rpm for about 3-5 minutes. Once the cells were ready to be

cultured, they were added to about 10 ml of media into a 75 ml polystyrene flask that has a 75 cm^2 culture area and then incubated under 5% CO_2 at 37°C. Cultures were fed with fresh media after every 48h. At confluence, the cells were harvested by treatment with tyrpsin solution for 15 min which was followed by immediate dilution and centrifugation in the complete medium. The cells were then distributed to their respected well-plates as explained later.

3.2 Preparation of Substrate Coatings

For our studies, glass microscope slide coverslips measuring 1.8 cm x 1.8 cm were coated with Fibronectin, Collagen IV, and Matrigel and used to conduct both cell proliferation and shear stress resistance studies. Our coated coverslips or coupons were compared to a control group made up of 1.8 cm x 1.8 cm woven Dacron (Boston Scientific, USA) squares.

3.2.1 Coverslip Sterilization

Before conducting any coating protocols, the coverslips were sterilized in a petri-dish under the hood. Specifically they were washed with ethanol under the hood and then rinsed with PBS and set to dry.

3.2.2 Fibronectin Coating Protocol

A 1 mg vial of Fibronectin (BD Biosciences, USA) was used to make a1 ml Fibronection solution with a concentration of 1mg/ml. Fibronectin is a broad range natural cell adhesion factor. As mentioned previously, it is found as a dimmer in plasma and in multimeric form in the ECM and on cell surfaces. When we were ready to conduct our Fibronectin coatings, 0.450 ml of the 1 ml solution was poured into 14.55 ml of phosphate-buffered saline solution (PBS, pH 7.3-7.4; Cellgro, USA), free of Ca^{2+} and Mg^{2+}, in order to make a 15 ml Fibronection solution with a concentration of 30 µg/ml.

Once our Fibronectin solution was made, our sterilized coverslips were added into the wells of 3 6-well-plates. In order for us to make sure that the entire surface of the well plate and coverslip was coated with Fibronectin, 0.962 ml of the 15 ml solution was added into each well. After incubating the wells for at least 1 h at room temperature, the well-plates were ready for cell seeding.

3.2.3 Mouse Collagen Type IV Coating Protocol

A 5 m1 vial of 0.49 mg/ml Mouse Collagen Type IV (BD Biosciences, USA) was used for our Collagen IV coatings. Recall that Collagen IV is found in relatively large basement membrane structures and complex organs. When we were ready to conduct our Collagen IV coatings, 0.918 ml of the 0.49 mg/ml Collagen IV solution was mixed with 14.08 ml of 0.05 M HCl to make a 15 ml solution with a Collagen IV concentration of 30 μg/ml.

Once our Fibronectin solution was ready, our sterilized coverslips were added into the wells of 3 6-well-plates. In order for us to make sure that the entire surface of the well plate and coverslip was coated with Collagen IV, 0.962 ml of the 15 ml solution was added into each well. After incubating the wells for at least 1 h at room temperature, the well-plates were ready for cell seeding.

3.2.4 Matrigel Basement Membrane Matrix Coating Protocol

A 5 ml vial with a concentration of 12.1 mg/ml of Matrigel Basement Membrane Matrix (BD Biosciences, USA) was used for our Matrigel coatings. Matrigel Basement Membrane Matrix is a solubilized basement membrane preparation extracted from the Engelbreth-Holm-Swarm (EHS) mouse sarcoma, a tumor rich in ECM proteins. Its major component is laminin, followed by Collagen IV, heparin sulfate proteoglycans, entactin, and nidogen. When we were ready to conduct our Matrigel coatings, 14.97 ml

of PBS was mixed with 0.0372 ml of Matrigel solution to attain a 15 ml solution with a Matrigel concentration of 30μg/ml.

Once our Matrigel solution was ready, our sterilized coverslips were added into the wells of 3 6-well-plates. In order for us to make sure that the entire surface of the well plate and coverslip was coated with Matrigel, 0.962 ml of the 15 ml solution was added into each well. After incubating the wells for at least 1 h at room temperature, the well-plates were ready for cell seeding.

3.3 Analysis of Cell Proliferation

The Proliferation of PVEC's on each coupon and Dacron coupon was analyzed using fourth passage cells. Once the Dacron and coverslip coupons were prepared, cells were harvested, via Trypsin, from their assigned flasks and then resuspended in complete medium. A total amount of 0.2 ml of the suspension was added into each well of a 6 well-plate. For our specific tests, we seeded 12 Fibronectin coupons, 12 Collagen IV coupons, 12 Matrigel coupons, and 12 Dacron coupons.

Following seeding, the cultures were then incubated at 37°C with 5% CO_2 and after 48 hrs the non-adherent cells were aspirated and the coupons placed in new well plates. The attached cells were again harvested by treatment with trypsin solution and then dyed with Trypan Blue so that they could be counted using a Hemocytometer. For a Hemocytometer, the number of cells per milliliter and total number of cells is found by the following calculations:

$$\text{cells/mL} = \left(\frac{\#\text{ of cells counted}}{5}\right)\left(10^4\right)\left(\text{dilution factor}\right) \qquad 3.1$$

$$\text{total cells} = \left(\text{cells/mL}\right)\left(\text{volume of original cell suspesion}\right) \qquad 3.2$$

Once the total number of cells per well was determined, those numbers were used to calculate the percent adhesion on PVEC's on each substrate. The calculation was as follows:

$$\% \text{ of cell adhesion} = \frac{(\text{coverslip cell count after 48hrs})}{(\text{initial cell count})} \qquad 3.3$$

Additionally, coupons representing each substrate were set and stained and then photographed via the image acquisition software AxioVision 3.1 (Carl Zeiss Microimaging, Inc., USA) and video camera that is hooked up to an AxioPlan2 IE Manual microscope (Carl Zeiss Microimaging, Inc., USA). The data was collectively organized and each coupon was compared to one another as well as with the Dacron coupon.

3.4 Shear Stress Resistance of Monolayer

Each Dacron coupon and each coupon coated with fibronectin, collagen IV, and matrigel was evaluated for shear stress resistance once a confluent monolayer of PVEC's was formed (48hrs later). On confluence, the monolayers were washed with serum-free medium and the coupons will then be fixed on the PPFC that will be discussed in further detail on the next chapter. The PVEC monolayer on each coupon and cover slip was exposed to a flow rate of 182 ml/min for 1hr using a roller pump (L/S Easy-Load II Pump, Cole-Parmer, USA). After exposure, each coupon was rinsed and then harvested with Trypsin-EDTA solution and counted using a Hemocytometer. The average percent of cell detachment for each substrate was then calculated by the following equation:

$$\text{Ave. \% of Cell Detachment} = \frac{(\text{Ave. cell count before shear})}{(\text{Ave. cell count after shear})} \qquad 3.4$$

Recorcdings of several coupons were also done via a CCD camera hooked up to microscope setup. The wall shear stress exerted will be calculated from the flow rate (cm^3/s), media viscosity, and height of the flow channel (refer to equation 2-1).

3.5 Experimental Set-Up

Figure 4-1 shows the data acquisition set-up for our shear stress experiments. Fluid was introduced into the PPFC chamber via its ports and a roller pump (L/S Easy-Load II Pump, Cole-Parmer, USA) that provided for a closed fluid circuit.

Images were acquired with an AxioPlan2 IE Manual microscope (Carl Zeiss Microimaging, Inc., USA) set-up for incident light transmission. The image acquisition software used was AxioVision 3.1 (Carl Zeiss Microimaging, Inc., USA).

Figure 3-1. Data acquisition set-up including roller-pump, microscope, and computer for image acquisition.

3.6 Statistical Analysis

Comparison between groups was performed with a one-way analysis of variance (ANOVA), and a Tukey-Kramer multiple comparison post test was then used to determine exactly which groups were different. Statistical significance was defined as $p \leq 0.05$. The results were expressed as mean ± standard error of the mean (SEM).

CHAPTER 4
PARALLEL PLATE FLOW CHAMBER DESIGN AND CHARACTERIZATION

To develop a PPFC that would benefit studies involving cell adhesion related to stent-grafts, we considered the following specifications:

- The chamber has to be small enough to be incorporated with conventional microscope setups.
- The chamber design must be such that we can study cell adhesion to a coated opaque substrate.
- The chamber has to be such that a fully developed laminar flow can be achieved to conduct accurate shear stress analysis with an upper limit of 60 dynes/cm^2.

For a standard laboratory inverted microscope (Figure 3-1) the stage and setup allows for a maximum working space of about 9''x 8''x 1.5''. So to fulfill our first specification, our chamber can not exceed these limits. Additionally, in order for us to be able to view our substrates in a conventional inverted microscope, we must have the surface in question face the objectives. Most importantly, the substrates must also be subjected to laminar flow in order to be able to conduct any valuable studies regarding shear stress.

Figure 4-1. Zeiss Axiovert 100.

Taking into consideration the space that we are constraining our chamber to (9''x 8''x 1.5''), we have to determine an appropriate equation that will describe the flow in our PPFC and also find an entrance length that will allow us to reach fully developed laminar flow. This is an important specification because our goal is to attempt to mimic physiological flow. From fluids mechanics, for the case of parallel plates, the following assumptions are made:

- Newtonian fluid: presence of cells is neglected
- Fully developed flow
- Incompressible fluid
- No-slip boundary condition
- 2-D flow

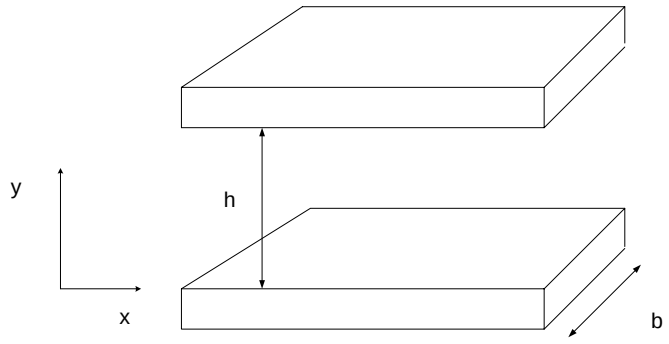


Figure 4-2. Labeled cross section for parallel plates

Under these conditions, the Navier Stokes equation in the direction of flow simplifies to equation 4-1 and is then integrated to get equation 4-2.

$$\frac{\partial^2 u}{\partial y^2} = \frac{1}{\mu}\frac{\partial p}{\partial x} \qquad 4\text{-}1$$

$$\frac{du}{dy} = \left[\frac{1}{\mu}\frac{\partial p}{\partial x}\right] y + C1 \qquad 4\text{-}2$$

By the definition of a Newtonian Fluid we know that equation 4-2 is proportional to shear stress on the walls of the parallel plate. So if we combine equations 4-2 and 4-3 and then apply the boundary conditions from Figure 4-2 we get equation4-5.

$$\tau = \mu \frac{du}{dy} \tag{4-3}$$

$$\tau = \mu \left(\frac{1}{\mu} \frac{dp}{dx} y + C1 \right) \tag{4-4}$$

$$\tau = h \left(\frac{dp}{dx} \right) \left[\frac{y}{h} - 2 \right] \tag{4-5}$$

Recalling that volumetric flow Q, we see that equation 4-5 can be rewritten as equation 4.7.

$$Q = b - \frac{1}{12\mu} \left(\frac{dp}{dx} \right) h^3 \tag{4-6}$$

$$\tau_{wall} = \frac{6Q\mu}{bh^2} \tag{4-7}$$

Note that b is the channel width, h is the height of the chamber, and μ the viscosity of the fluid flowing through the chamber. Thus from empirical data, we also know that this length or entrance length, L, is a function of the Reynolds number (Re) [30]:

$$\frac{L}{h} \simeq 0.06 \,\mathrm{Re} = 0.06 \frac{\rho \bar{V} h}{\mu} = 0.06 \frac{\rho Q}{b\mu} \tag{4-8}$$

where ρ is the density of the fluid, μ is the viscosity of the fluid, $\bar{V}$ is the average velocity of the flow, h is the distance between the top and bottom plates of a chamber, Q is the flow rate of the fluid flowing through the PPFC, and b is the channel width. The distance h between our plates is the thickness of our gasket, which is the area where our flow will be running through. So the thickness of this gasket will directly affect the entrance length

too. Since we are concerned with making the chamber as small as our specifications allow, we decided to make the gasket 0.58 mm in thickness. The gasket was made from a silastic T-2 silicone (Dow Corning, USA) using 2 offset glass plates. If we rearrange equation 4-7 and solve for the flow rate that we would need to attain a shear stress of $\tau_{wall} = 17.66\,\text{dynes/cm}^2$ knowing $h = 0.58$ mm, b = 20.62 mm, and $\mu = 6.85x10^{-4}$ N·s/m² for water at 37°C, we find the flow rate we will be using is Q = 182 ml/min. Thus from equation 4-1 we see that our entrance length is 0.09 cm, which means that we attained laminar flow. Note that the turbulent effects due to the geometry were not considered, which means that the entrance length is longer than calculated. However, the distance between the inlet and the beginning of the substrate pocket is 4.41 cm which is much greater than the entrance length calculated (L = 0.09 cm): it seems, then, reasonable to assume that the flow in through the pocket is fully developed. Figures 4-2 and 4-3 are computer assisted drawings (CADs) of the top and bottom plate of our PPFC.

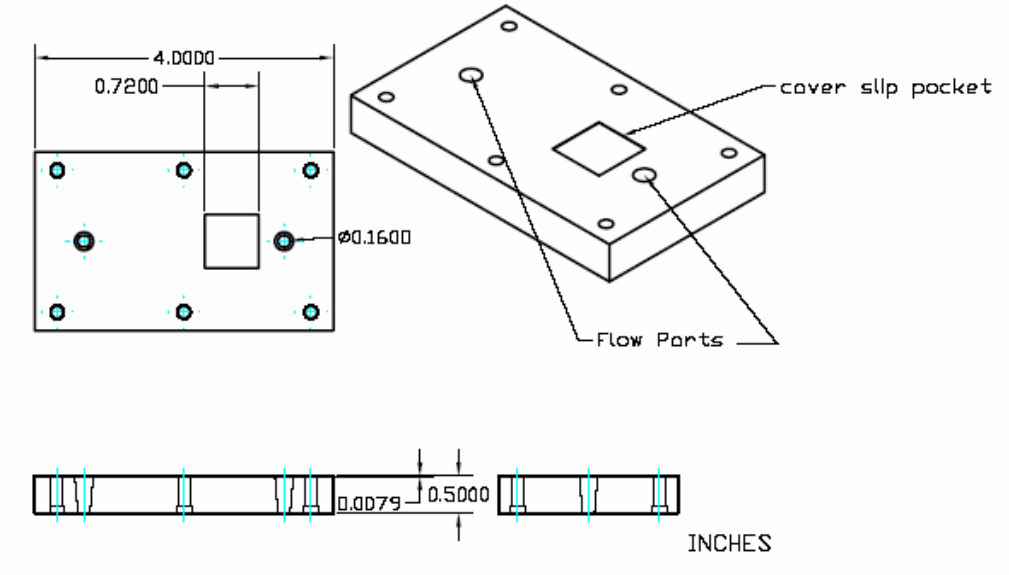


Figure 4-2. CAD of PPFC top plate (note the cover slip pocket and the flow ports).

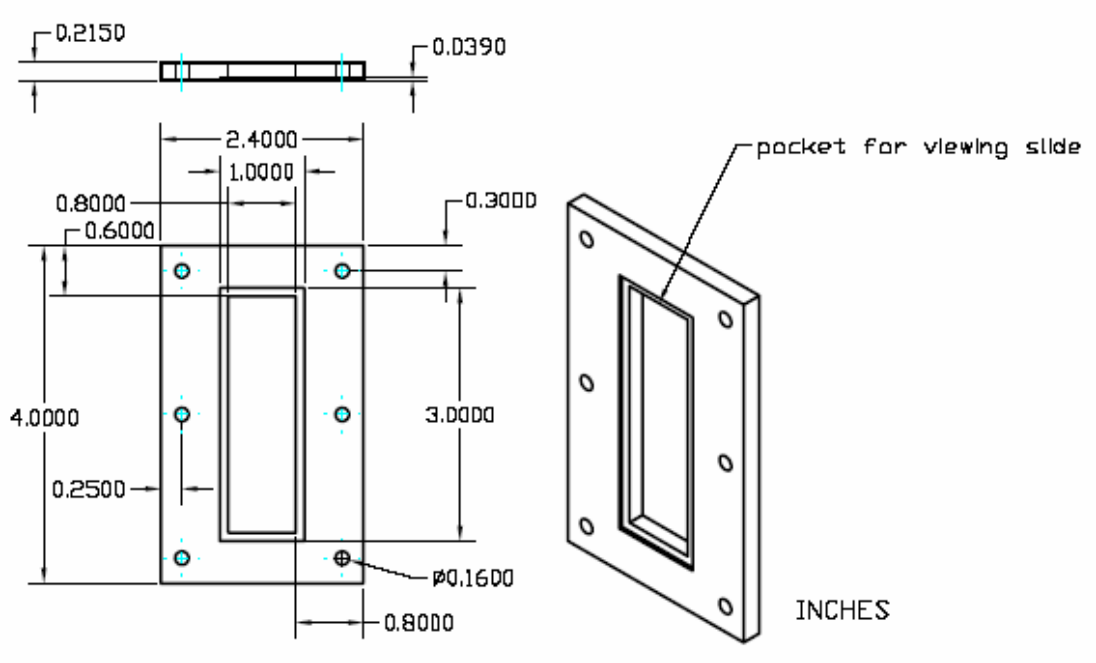


Figure 4-3. CAD of PPFC bottom plate (note pocket for viewing slide).

Figures 4-4 and 4-5 show the PPFC that we fabricated for our studies. The parts of this PPFC are top and bottom plates made out of polycarbonate material, specifically LEXAN (General Electric, USA), a glass microscope slide, a glass cover slip, and a silastic gasket.

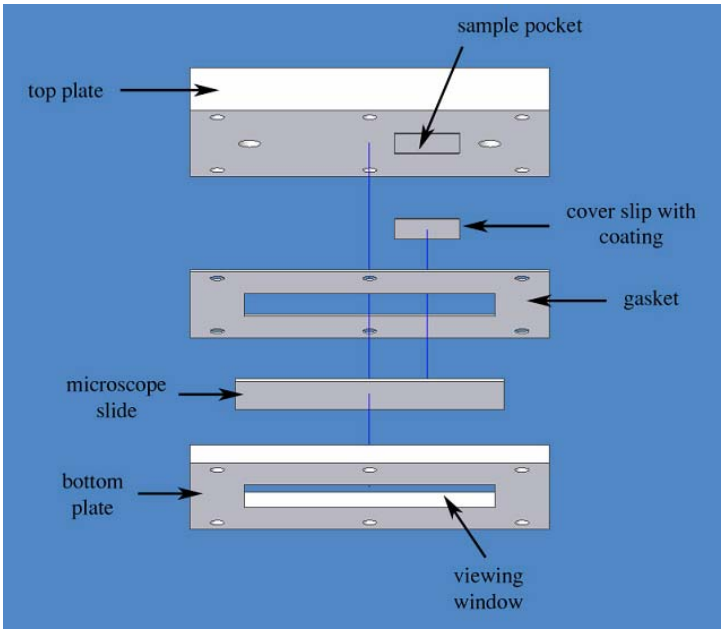


Figure 4-4. Exploded CAD view of PPFC

Figure 4-5. PPFC

The bottom plate is a LEXAN rectangle that is 4” x 2.4” x 0.215” thick with a 3” x 1” x 0.0390” thick counter sunk widow and a 2.8” x 0.8” x 0.215 rectangular hole. It is on this counter sunk window that a 3” x 1” x 0.0390” thick glass microscope slide perfectly sits to provide a viewing window. To create input and output ports in the 4” x 2.4” x 0.5” thick LEXAN top plate, diffusers were machined such that they start and end in the space provided by the 4” x 2.4” x 0.0197” thick silastic gasket with a 2.8” x 0.8” x 0.215 rectangular hole. Additionally, the 0.72” x 0.72” x 0.0079” thick counter sunk pocket on the top plate is for a 0.72” x 0.72” x 0.0079” thick glass cover slip to sit on.

Once the parts of the PPFC are put together and screwed, a sealed fluid circuit is created. After the seal is created, a 0.62 in inner diameter silastic laboratory tube (GlycoTech, USA) is connected to the fluid ports in the top plate by luer locks (ArkPlas Products, USA). The PPFC will then be ready for the microscope stage.

CHAPTER 5
RESULTS

5.1 Cell Proliferation

The retention of cells on tested substrates is expressed as a percentage of initially seeded cells (Figure 5-1). Making sure that the data for each substrate appeared to come from a normal distribution and performing a one-way analysis of variance (ANOVA) and Tukey-Kramer multiple comparison post test of the data sets, the PVEC adhesion to Collagen IV coated coupons was found significantly different to that of the Dacron and Matrigel coupons as shown in Figure 5-1. Note that if Fibronectin was included in this analysis, no significant difference will be found due to its large variability. Part of the reason for this is probably due to the size of our sample populations (n = 4). After 48h of seeding, the average amount of cells found on the Collagen IV coated substrate was about 18% of the initially seeded cells with a standard error of ±2%. On the other hand, about 6% of the initially seeded cells of the Matrigel substrate attached with a standard error of ±2%, which is about the same amount of cells that attached to Dacron coupons. Pictures of cells attached to Dacron and the other surfaces are shown in Figures 5-2 thru 5-6.

5.2 Resistance to Shear Stress

The effects of flow, for a flow rate of 182 ml/min and a calculated shear stress of ~18 dynes/cm^2, on the PVEC adherence to the studied substrates were found to be significant for Collagen IV when compared to Dacron. Physiological levels of venous and arterial shear stresses are 1-5 and 6-40 dynes/cm^2, respectively [31]. The cell detachment on the Collagen IV substrate was about 31% with a standard error of ±24%.

Figures 5-7 through 5-9 show pictures and a video of PVEC's attached to a Matrigel substrate resisting shear.

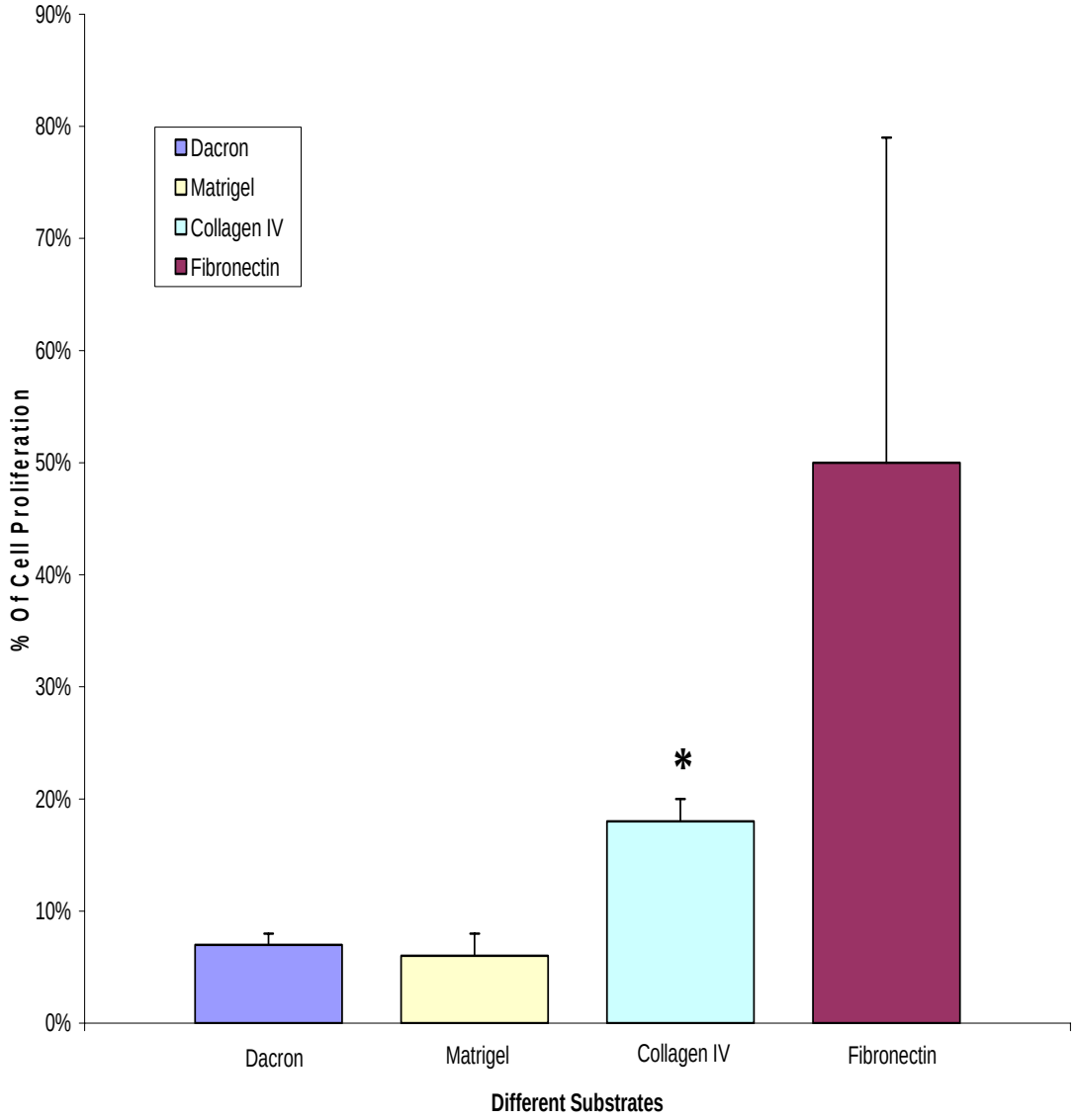


Figure 5-1. Comparison of the percentage of PVEC proliferation on different substrates. Dacron, Matrigel, Collagen IV, and Fibronectin, as detailed in methods were seeded with PVEC cells from the fourth passage. Each substrate has a concentration of 30µg/cm^2. Forty eight hours after seeding, unattached cells were washed and percentage of attached cells with respect to the total number of cells seeded was calculated. $^*p < 0.01$ compared to Dacron. The experiment was performed four times for each substrate and each point represents the mean ± SEM of the three substrates.

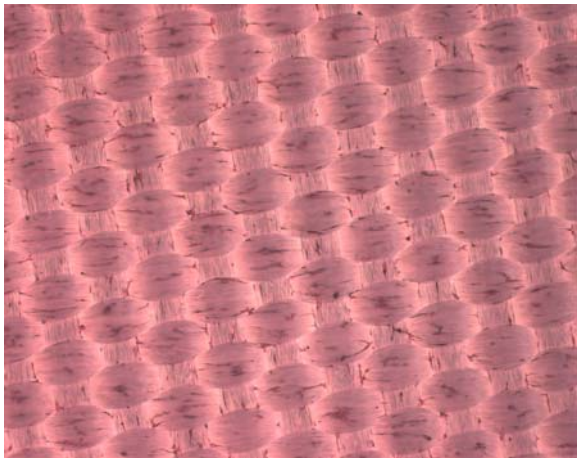


Figure 5-2. Cell spreading on Dacron at 100x.

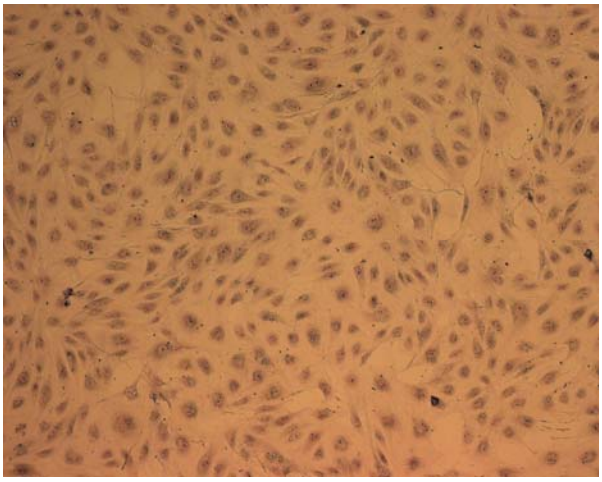


Figure 5-3. Cell spreading on Fibronectin substrate at 100x.

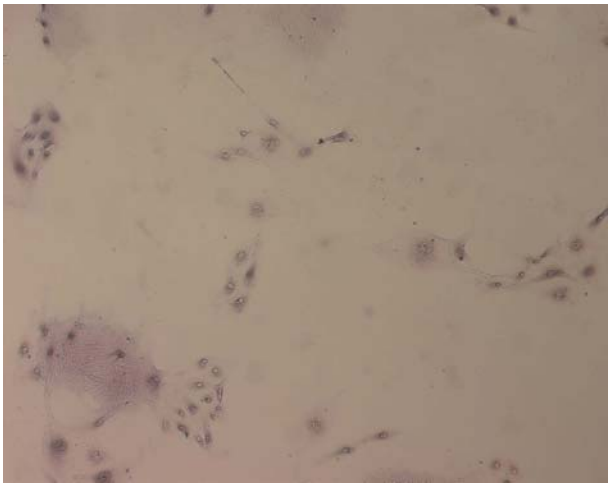


Figure 5-4. Cell spreading on Matrigel substrate at 100x.

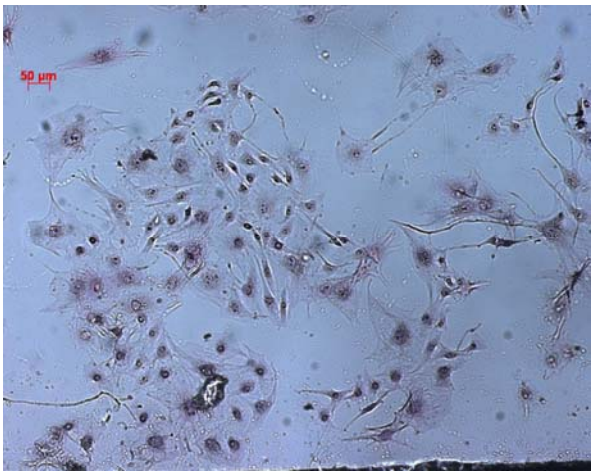


Figure 5-5. Cell spreading on Collagen IV substrate at 100x.

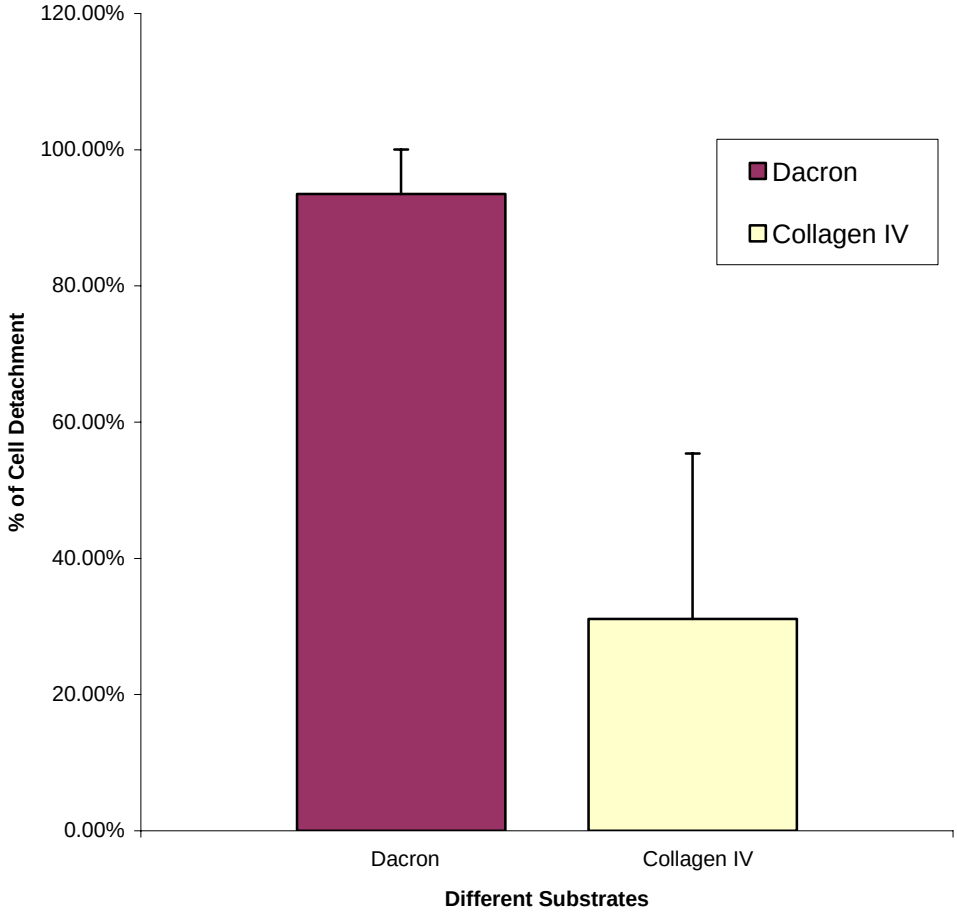


Figure 5-6. Comparison of the percentage of PVEC's detached from Dacron and Collagen IV substrates. The substrates were exposed to a shear stress of ~18 dynes/cm^2 for 1h.

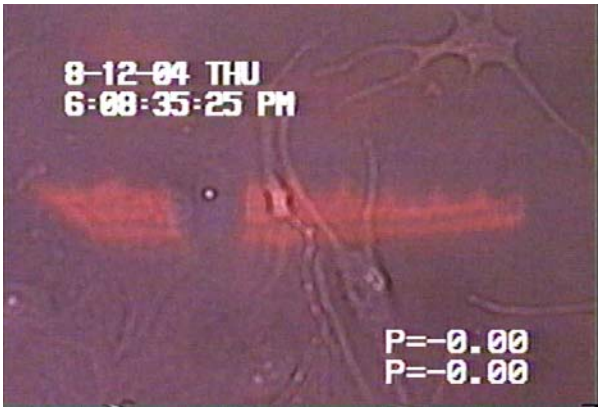


Figure 5-7. Still picture of PVEC attached to Matrigel substrate and resisting ~18 dynes/cm^2 at 40x.

Figure 5-8. Still pictures of PVEC moving from Matrigel substrate due to shear.

CHAPTER 6
SUMMARY AND CONCLUSIONS

The objective of this study was to develop a Parallel Plate Flow Chamber (PPFC) for the analysis of cell detachment on opaque materials in real-time. Further more, the viability of our chamber was tested by developing protocols that allowed us to quantitatively measure cell adhesion and detachment from polymer substrates and untreated woven Dacron.

6.1 Summary of Results

The experimental results presented in our studies suggest that it is possible to modify the surface of biomaterials used for vascular implants to increase their ability of cell adhesion which supports the viability of our PPFC for the study of cell adhesion. Specifically, our results show that Collagen IV increases the ability of a biomaterial, like Dacron, to have endothelial cells attach to it, which confirms what other groups have found [20]. In the case of Matrigel, results have been mixed regarding the enhancement in the attachment and proliferation of endothelial cells on vascular biomaterials [6], in our studies, a significant difference in cell adhesion when compared to Dacron was not found. For our cell detachment studies, Collagen IV on average had significantly fewer cells detach from it when compared to Dacron.

The main objective of our research was to show that our PPFC design allowed for the real-time analysis of cell detachment with special attention to opaque materials. Specifically were able to follow the detachment of cells when exposed to flow and were able to do the same for an opaque material like Dacron. Our results matched those

concluded by others and our new design allows for a more in depth analysis of cell detachment.

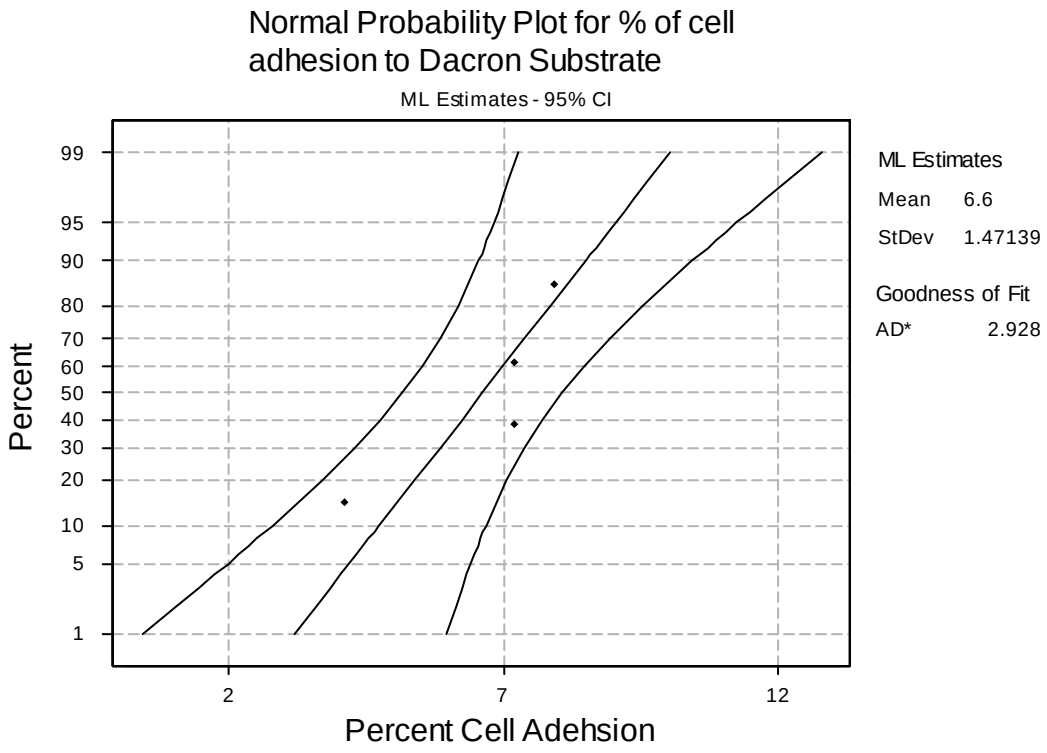
6.2 Discussion and Future Work

The results that were obtained in this study provide evidence of future success in using these protocols and PPFC design for evaluating the ultimate success of stent-graft coatings. Our in-vitro set-up allows for real time analysis of cell detachment, which can serve as a value tool when testing substrate adhesion to opaque materials like Dacron. However, there are a couple of suggestions to consider prior to future testing. The most important of these suggestions is slight modification in our PPFC design. When designing our PPFC, one of our major concerns was to be able to reach laminar flow. This was important because we wanted to simulate physiologic flow. In doing this, not much attention was focused an exit effects of the chamber, and because of this we feel that we might have machined our substrate pocket too close to the exit port. Future modifications would include increasing the length of our PPFC or moving the location of our substrate pocket to more of a central position. Additionally we feel that the placement of our ports on the top of the PPFC was perhaps not the best location. We feel that we can get more versatility from our PPFC if we were to machine the ports on the sides of the chamber. This way the ports will never be a factor when attempting to provide a light source to our PPFC and will allow for better video quality.

With regards to our attachment experiments, a question we are interested in further investigating is whether the cells detached from the substrate proteins or if the substrate protein detached from the glass coverslip. One of our objectives was to determine if the substrates we chose would increase the cell attachment of Dacron. In other words, the end goal would be to find a protein that increases cell attachement and then to precoat a

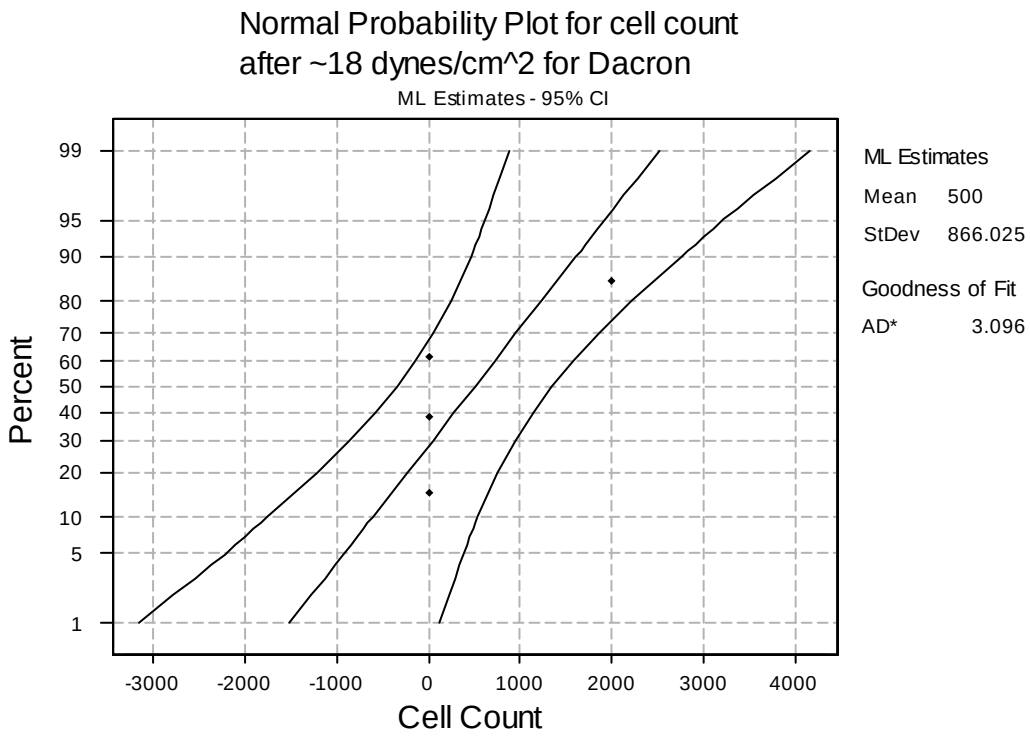
Dacron graft with this protein before treatment insertion. But because we coated glass coverslips with our substrates, we cannot say with absolute certainty that it was not our sustrate that was detaching from our coverslips. So future experiments should use Dacron or Mylar "coverslips." Mylar is simply just Dacron in film form.

APPENDIX A
DACRON SUBSTRATE STATISTICS



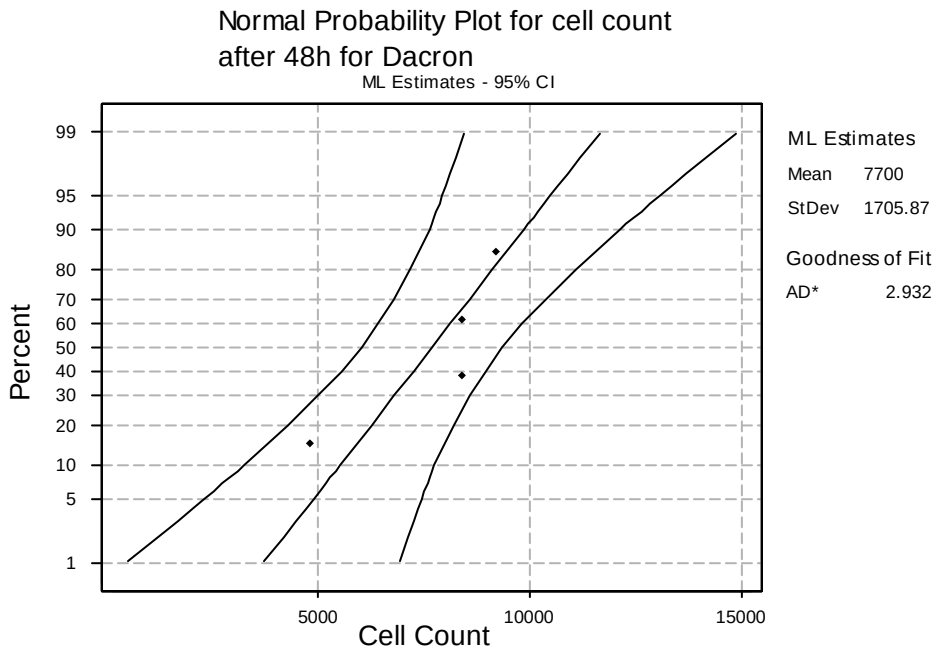
One-Sample T: % of cell adhesion (dacron)

Variable	N	Mean	StDev	SE Mean	95.0% CI
% of cell ad	4	6.600	1.699	0.850	(3.896, 9.304)



One-Sample T: cell count after ~18 dynes/cm^2 (Dacron)

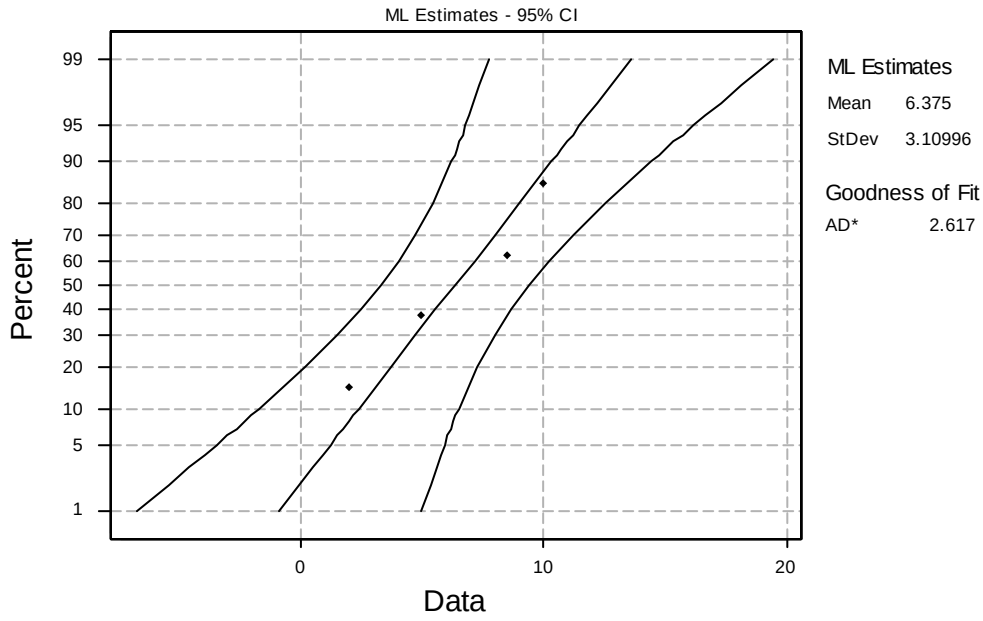
Variable	N	Mean	StDev	SE Mean	95.0% CI
cell count a	4	500	1000	500	(-1091, 2091)



One-Sample T: cell count after 48h (dacron)

Variable	N	Mean	StDev	SE Mean	95.0% CI
cell count a	4	7700	1970	985	(4566, 10834)

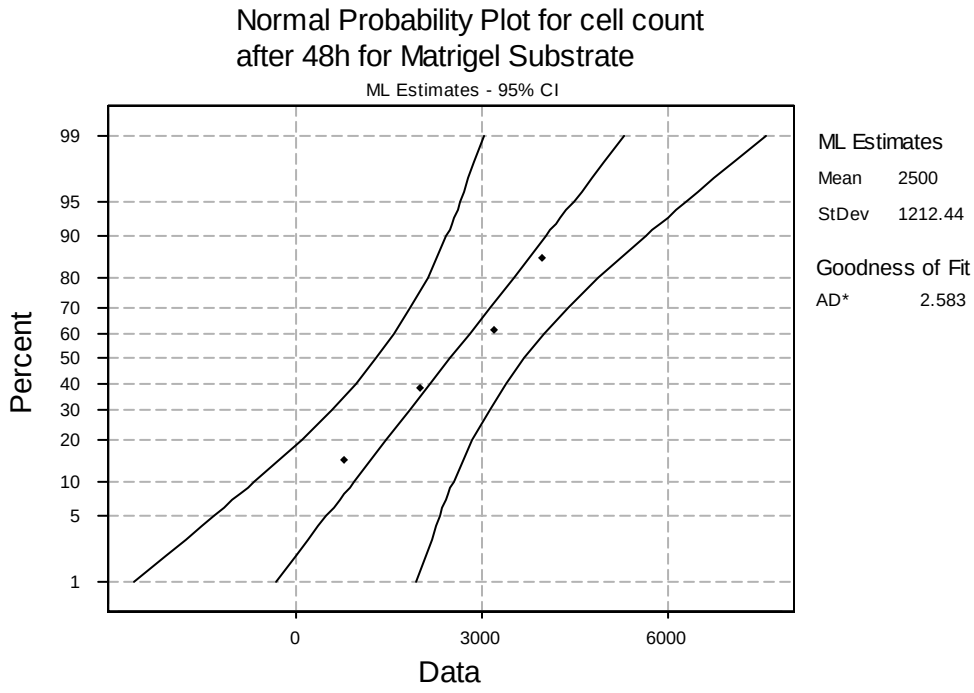
APPENDIX B
MATRIGEL SUBSTRATE STATISTICS



Where the data represents % of cell adhesion

One-Sample T: % of cell adhesion (Matrigel)

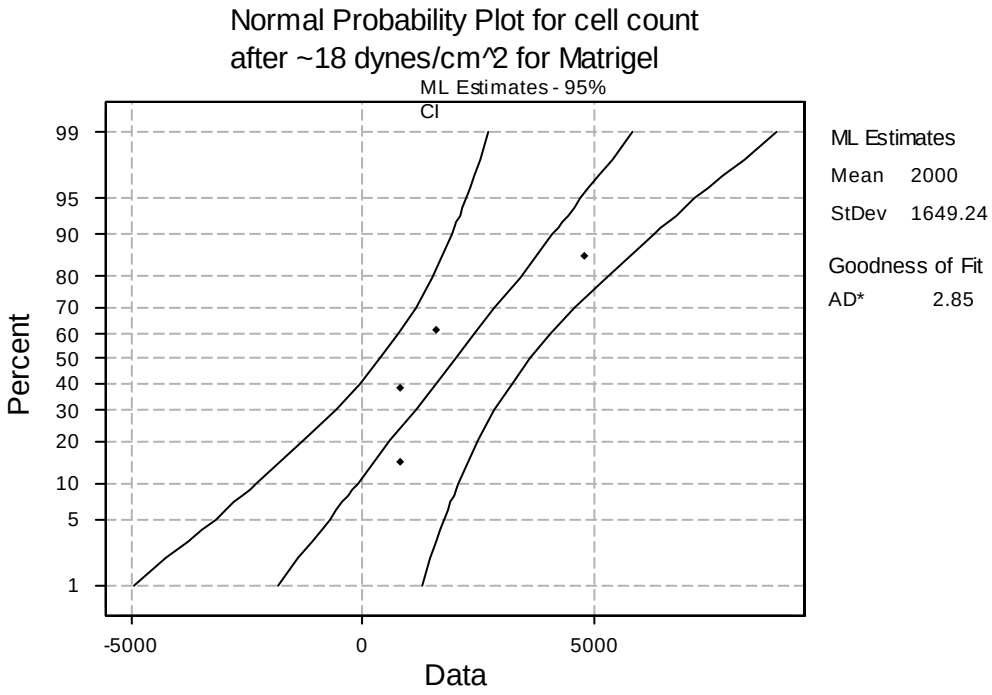
Variable	N	Mean	StDev	SE Mean	95.0% CI
% of cell ad	4	6.38	3.59	1.80	(0.66, 12.09)



Where the data represents cell count

One-Sample T: cell count after 48h (matrigel)

Variable	N	Mean	StDev	SE Mean	95.0% CI
cell count a	4	2500	1400	700	(272, 4728)

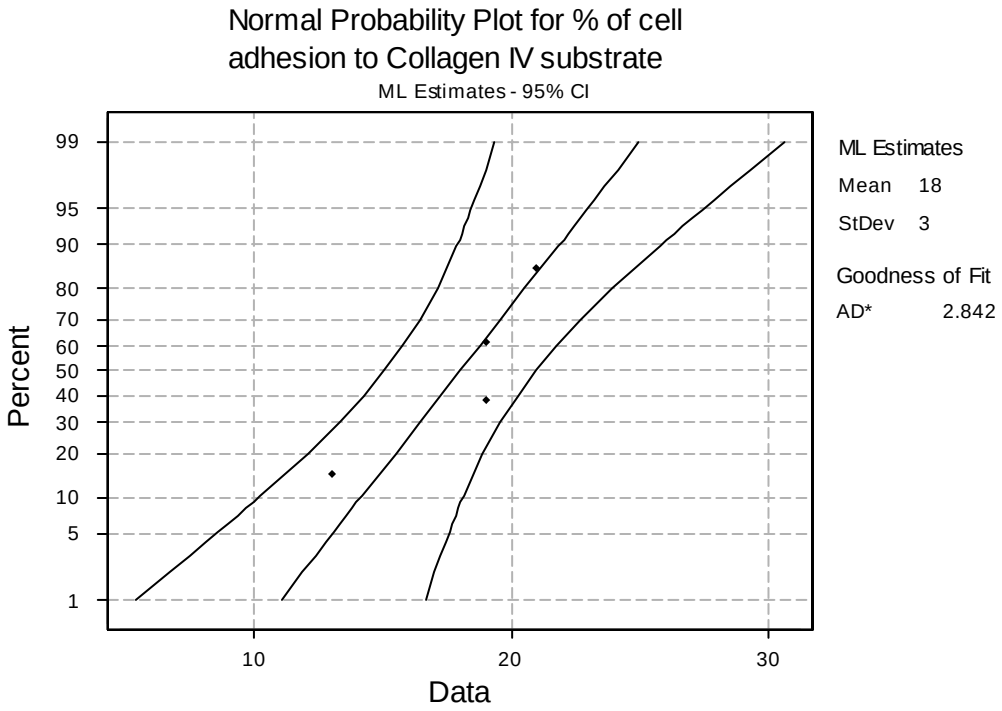


Where the data represents cell count

One-Sample T: cell count after shear 1 (matr)

Variable	N	Mean	StDev	SE Mean	95.0% CI
cell count a	4	2000	1904	952	(-1030, 5030)

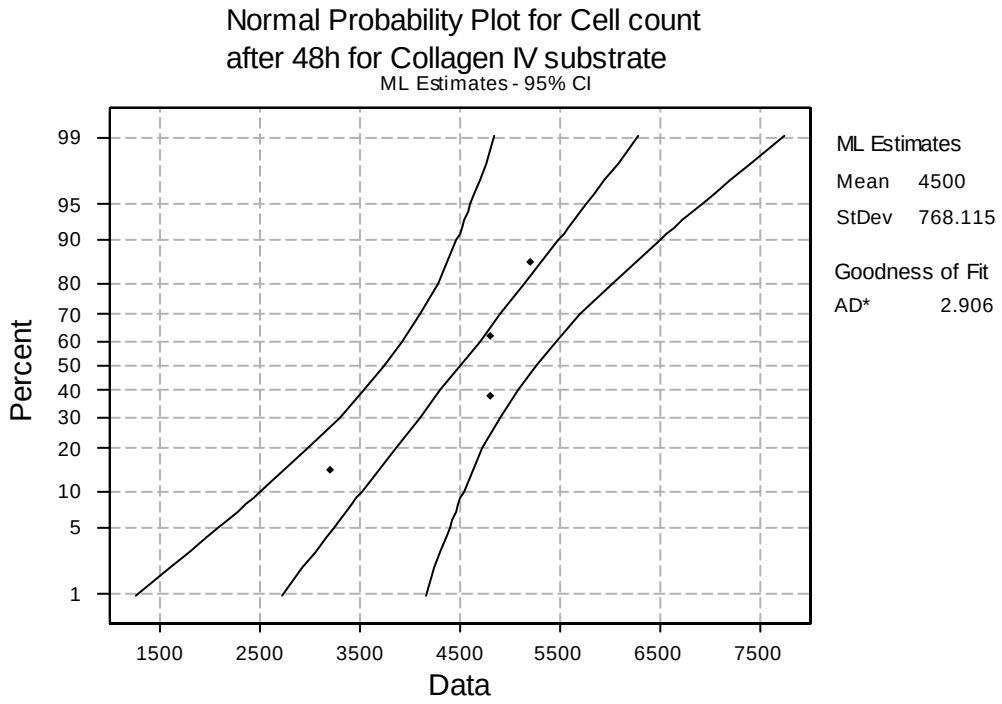
APPENDIX C
COLLAGEN IV STATISTICS



Where the data represents % of cell adhesion

One-Sample T: % of cell adhesion (Collagen IV)

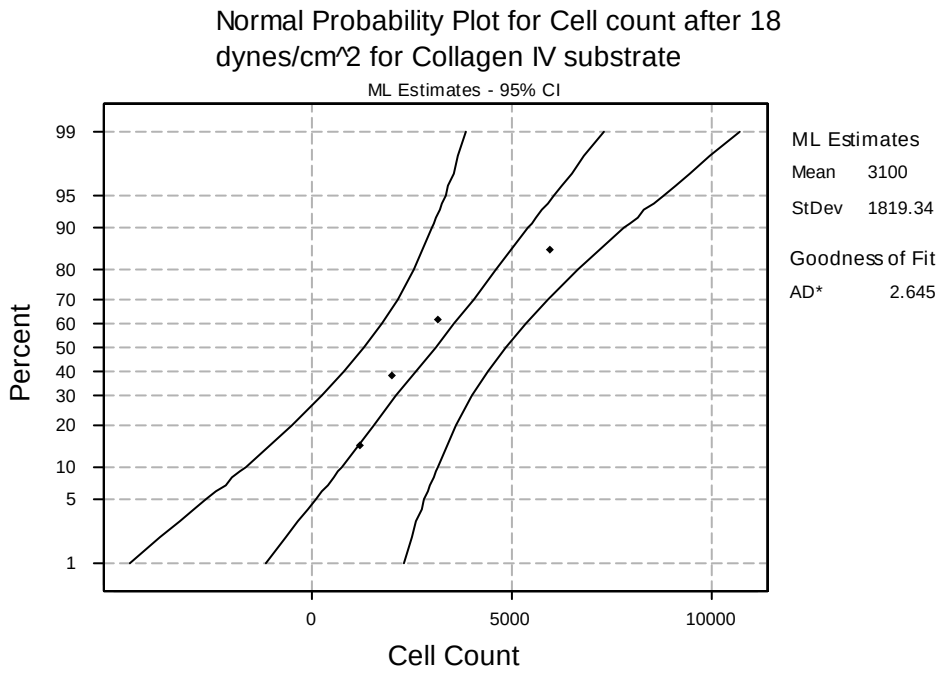
Variable	N	Mean	StDev	SE Mean	95.0% CI
% of cell ad	4	18.00	3.46	1.73	(12.49, 23.51)



Where the data represents cell count

One-Sample T: Cell count after 48h (colIV)

Variable	N	Mean	StDev	SE Mean	95.0% CI
Cell count a	4	4500	887	443	(3089, 5911)



One-Sample T: Cell count after Shear 1(colIV)

Variable	N	Mean	StDev	SE Mean	95.0% CI
Cell count a	4	3100	2101	1050	(-243, 6443)

APPENDIX D
SUBSTRATES STATISTICAL COMPARISONS

One-way ANOVA: Substrate Comparisons

```
Analysis of Variance for C11
Source     DF        SS        MS        F        P
C12         2    353.54    176.77    19.09    0.001
Error       9     83.35      9.26
Total      11    436.88
                                    Individual 95% CIs For Mean
                                    Based on Pooled StDev
Level       N      Mean     StDev  ------+---------+---------+---------+
C           4    18.000     3.464                      (-----*-----)
D           4     6.600     1.699   (-----*-----)
M           4     6.375     3.591   (-----*----)
                                    ------+---------+---------+---------+
Pooled StDev =    3.043               6.0      12.0      18.0      24.0

Tukey's pairwise comparisons

    Family error rate = 0.0500
Individual error rate = 0.0209

Critical value = 3.95

Intervals for (column level mean) - (row level mean)

                C           D

       D       5.390
              17.410

       M       5.615      -5.785
              17.635       6.235



Fisher's pairwise comparisons

    Family error rate = 0.113
Individual error rate = 0.0500

Critical value = 2.262

Intervals for (column level mean) - (row level mean)

                  C           D

       D       6.533
              16.267

       M       6.758      -4.642
              16.492       5.092
```

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BIOGRAPHICAL SKETCH

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