LEUKOCYTE RESPONSE TO ELASTIN-LIKE POLYPEPTIDE COATINGS

By

Meghan Kathleen Rooney

A thesis submitted to the Department Chemical Engineering

in conformity with the requirements for

the degree of Master of Applied Science

Queen's University

Kingston, Ontario, Canada

October, 2013

Copyright ©Meghan Kathleen Rooney, 2013

Abstract

Small diameter synthetic vascular grafts have yet to be clinically successful due to luminal narrowing from thrombosis and intimal hyperplasia. Current attempts to address this issue include the development of materials that support endothelialisation and protein modification to the material surfaces that reduce thrombosis. The extracellular matrix protein elastin has been found to be one of the least thrombogenic components of blood vessels, and its purified and recombinant forms have shown reduced thrombogenicity in both *in vitro* and *in vivo* models. Biomaterial coatings of elastin-like polypeptides (ELPs) recombinantly produced in the Woodhouse laboratory showed reduced fibrinogen adsorption, platelet adhesion, and platelet activity. However, the reason for their relative non-thrombogenicity is still not fully understood. In this work, the leukocyte response to ELP-coated materials was investigated. In particular, ELP1 and ELP4, which differ in molecular weight and sequence length, were physically adsorbed to a polyethylene terephthalate surface (MylarTM), yielding $0.22 \pm 0.13 \,\mu\text{g/cm}^2$ and $0.37 \pm 0.19 \,\mu\text{g/cm}^2$ surface coverage, respectively, as determined by the colorimetric assay, FastinTM Elastin. These surfaces were exposed to flowing citrated whole blood for surface and bulk evaluation of leukocyte activity using scanning electron microscopy and flow cytometry, respectively. Little leukocyte activation was observed on the surface of the controls, low-density polyethylene and uncoated MylarTM. In the bulk, tissue factor (TF) expression (monocytes: $ELP1 = 38.6 \pm 16.3$ %, $ELP4 = 33.9 \pm 18.1$ %) and platelet-leukocyte aggregates determined by CD61 (monocytes: ELP1 = 63.1 ± 17.1 %, ELP4 = 61.8 ± 16.8 %; granulocytes: $ELP1 = 62.7 \pm 17.0$ %, $ELP4 = 60.5 \pm 20.1$ %) were both decreased compared to uncoated MylarTM, while CD11b upregulation (monocytes: ELP1 = 18.7 ± 2.2 %, ELP4 = 19.7 ± 2.7 %; granulocytes: ELP = 21.4 ± 3.7 %, ELP4 = 22.0 ± 3.2 %) was increased. The statistical dependence of TF expression and platelet-monocyte aggregates was tested; however, no

correlation was found. Overall, platelet-leukocyte aggregate formation was reduced and there were conflicting results with regards to the reduction of leukocyte activation for the ELP coatings on $Mylar^{TM}$.

Acknowledgements

There are many people who have made this project possible and that the author would like acknowledge for their contributions. First, I would like to thank my supervisor Dr. Kimberly Woodhouse, whose guidance as a researcher and mentor has allowed me to grow and expand within my passion of Biomedical Engineering and as a person. Thanks for technical assistance goes out to Mr. Matt Gordon, for his expertise in flow cytometry, and to Mr. Charlie Cooney, for his knowledge and experience with scanning electron microscopy. I would also like to thank Dr. Yasuaki Shida and Dr. Alice van Velzen, for their phlebotomy skills, and the many volunteers for their generous time and blood. I am grateful for Dr. Brian Amsden and Dr. Lauren Flynn, and their labs, for the use of their equipment and guidance when necessary. Many thanks go to the funding agencies: the National Science Foundation Ireland, the Natural Sciences and Engineering Research Council of Canada and Queen's University. My fellow graduate students made this experience much more manageable and fun through the sharing of laboratory knowledge as well as friendship, and I thank them for this: Lindsay Hughes, for our technical discussions and companionship through the long days; Alix Murphy, for her elastin-like polypeptide production assistance and astute knowledge of our lab. I would also like to acknowledge my brother, Thomas Rooney, for his endless technical feedback, and my dad, James Rooney, for encouraging me to pursue this degree. Finally I would like to thank my family and friends who have supported me throughout these past years. Together you have pushed me when I needed pushing, given me outside perspective opinions when I was stuck, and had fun with me when I needed to take a break. For this, I thank you.

Abstract	. ii
Acknowledgements	iv
List of Figures	vii
List of Tables	ix
List of Abbreviations	. x
Chapter 1: Introduction	. 1
1.1 Objective	. 3
1.2 Specific Aims	. 3
Chapter 2: Literature Review	. 4
2.1 Blood-Material Interactions: Haemostasis and Thrombosis	. 4
2.1.1 Protein Adsorption	. 5
2.1.2 Coagulation	10
2.1.3 Platelets	13
2.1.4 Leukocytes	16
2.1.5 Complement Activation and Biomaterials	19
2.2 Anticoagulant	21
2.3 Effects of Shear on Thrombosis	22
2.3.1 Cone and Plate Device	26
2.4 Elastin	28
2.5 Elastin-Like Polypeptides	31
Chapter 3: Materials and Methods	34
3.1 Materials	34
3.2 Evaluation of Endotoxin Contamination	34
3.3 Elastin-Like Polypeptide Production and Adsorption	35
3.4 Fastin TM Elastin Assay	
3.5 Blood Collection	37
3.6 Cone and Plate Device	37
3.7 Random Sampling	38
3.8 Scanning Electron Microscopy	39
3.9 Flow Cytometry	40
3.10 Statistical Analysis	42

Table of Contents

Chapter 4: Results and Discussion	44
4.1 Endotoxin Testing	44
4.2 Elastin-Like Polypeptide Adsorption	44
4.3 Effects of Shear	47
4.4 Surface Evaluation – Scanning Electron Microscopy	47
4.5 Bulk Evaluation – Flow Cytometry	51
4.6 Inter-Donor Variability	59
4.7 Controls	60
Chapter 5: Conclusions and Recommendations	63
5.1 Summary of Findings	63
5.2 Conclusions	64
5.3 Recommendations for Future Work	65
References	68
Appendix: Research Ethics Board Approval	82

List of Figures

Figure 1: Langmuir's monolayer protein adsorption model
Figure 2: Freundlich's multilayer protein adsorption model
Figure 3: The coagulation cascade. Solid arrows indicate flow of activation, dotted arrows indicate amplification and T indicates coagulation inhibition
Figure 4: Fibrinolysis pathway
Figure 5: Stages of adherent platelet activation via scanning electron micrographs
Figure 6: Stages of adherent platelet aggregation via scanning electron micrographs 15
Figure 7: Platelet and leukocyte aggregate formation via fibrinogen and P-selectin
Figure 8: Adherent leukocytes with platelets on scanning electron micrographs
Figure 9: Complement cascade
Figure 10: Velocity profile model of laminar flow in a vessel
Figure 11: Cone and plate device profile
Figure 12: Typical blood vessel structure
Figure 13: Elastin-like polypeptide (ELP) gene structures for ELP1 and ELP4, and amino acid sequences
Figure 14: Schematic of cone and plate device used in this work
Figure 15: Typical scatter plot obtained from FlowJo vX flow cytometry analysis software 41
Figure 16: Histogram plots of the fluorescence intensity of monocytes from whole blood samples exposed to Mylar TM obtained from FlowJo vX flow cytometry analysis software
Figure 17: Typical Fastin TM Elastin standard curves for ELP1 and ELP4
Figure 18: Static surface adsorption concentrations of ELP1 and ELP4 on Mylar TM from a Fastin TM Elastin assay
Figure 19: Citrated whole blood with thrombin (1 mL/0.25 U), testing positive for ability to form a clot within seconds

Figure 20: Representative scanning electron micrographs of low density polyethylene (LDPE) and $Mylar^{TM}$ at shear rates of 100 s ⁻¹ , 200 s ⁻¹ and 300 s ⁻¹
Figure 21: Tissue factor expression on granulocytes and monocytes for ELP-coated Mylar TM compared to controls, at 300 s ⁻¹
Figure 22: CD11b upregulation on granulocytes and monocytes for ELP-coated Mylar TM compared to controls, at 300 s ⁻¹
Figure 23: CD61 expression on granulocytes and monocytes for ELP-coated Mylar TM compared to controls, at 300 s ⁻¹
Figure 24: Pearson correlations of the given surface between monocyte TF expression and platelet-monocyte aggregates
Figure 25: Inter-donor comparison of TF expression on monocytes
Figure 26: Scatter plots of positive (LPS) and negative (WB) static controls, shown left and right, respectively

List of Tables

Table 1: Common parameters for flow in vessels	
Table 2: Random sample configurations.	
Tuere 21 Tuminorin Sumpre Comingerations:	

List of Abbreviations

ANOVA	analysis of variance
AT	antithrombin
CD11b	integrin α_M
CD61	integrin β_3 , glycoprotein IIIa
ECM	extracellular matrix
ELP	elastin-like polypeptide
ELP1	elastin-like polypeptide 1 (20-(21-23-24) ¹)
ELP2	elastin-like polypeptide 2 (20-(21-23-24) ²)
ELP4	elastin-like polypeptide 4 (20-(21-23-24) ⁴)
ePTFE	expanded polytetrafluoroethylene
FDA	Food and Drug Administration
FITC	fluorescein isothiocyanate
FXII	factor XII
GPIIb/IIIa	glycoprotein IIb/IIIa, integrin $\alpha_{IIb}\beta_3$
HEPES	N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid
HMWK	high molecular weight kininogen
HTB	HEPES Tyrode's buffer
LAL	Limulus amebocyte lysate
LBS	lysine binding sites
LDPE	low density polyethylene
LPS	lipopolysaccharide, endotoxin
MAC	membrane attack complex
Mac-1	macrophage-1 antigen, integrin $\alpha_M \beta_2$
PAI	plasminogen activator inhibitor
PBS	phosphate buffered saline
PE	phycoerythrin
PEG	polyethylene glycol

PET	polyethylene terephthalate
PGVGVA	hexapeptide sequence: proline-glycine-valine-glycine-valine-alanine
RGD	arginine-glycine-aspartic acid
SD	standard deviation
SEM	scanning electron microscope
TF	tissue factor, CD142
TFPI	tissue factor pathway inhibitor
TPPS	5,10,15,20-tetrapenyl-21H,23H-porphine tetra-sulphonate
tPA	tissue-type plasminogen activator
uPA	urokinase-type plasminogen activator
WB	citrated whole blood

Chapter 1: Introduction

According to the World Health Organization, cardiovascular disease is the leading cause of death worldwide and is projected to continue to be so in the future [1]. Cardiovascular disease encompasses a range of heart and blood vessel disorders such as coronary artery disease and peripheral arterial disease, where coronary artery disease alone is responsible for 500,000 deaths per year in the United States [2]. The major issue with these diseases is the gradual atherosclerotic build-up of plaque in the vascular system that results in either an occlusion at the site of the build-up, or an embolism resulting in a blockage further down the narrowing vessel [3]. As of 2008, surgical procedures to treat these disease types, including bypass with small diameter autogenic vascular grafts, have risen to approximately 1.5 million procedures each year in the United States [3]. While some of these cases can be treated autogenically, there is a demand that has yet to be met for a synthetic small diameter vascular graft.

A vascular graft is a transplanted synthetic or non-synthetic blood vessel that replaces or bypasses a blood vessel that has been blocked or damaged by disease or trauma. Most grafts are produced synthetically, but autogenic and allogenic grafts have been used in clinical applications [4]. The ideal synthetic polymers for this application are required to closely match native tissue in both chemical and mechanical properties, as well as demonstrate resistance to degradation and thrombosis [5]. The most common materials used include polyethylene terephthalate (marketed as DacronTM), expanded polytetrafluoroethylene (ePTFE), and more recently, polyurethane [3]. DacronTM and ePTFE have been successful in large (inner diameter, ID > 10 mm) and medium (ID = 6-10 mm) diameter vascular grafts [6]. However, small diameter (ID < 6 mm) grafts of the same materials fail 5-10 years post implantation [5][7]due to luminal narrowing caused by thrombosis [8] and intimal hyperplasia [7]. Currently, autografts are used for small ID applications, but there are about 30% of cases for which this treatment cannot be administered due to previous surgery, disease or individual anatomy [4]. The motivation for further research is being driven by the need for small diameter vascular grafts that are highly resistant to both thrombosis and intimal hyperplasia.

The effectiveness of a blood-contacting biomaterial can be evaluated on the basis of its thrombogenicity, which encompasses several environment factors, some of which are better understood than others. Traditionally, platelets and clotting factors alone were thought to be responsible for coagulation. Recent research suggests that platelets, along with leukocytes and complement, interact in the process of thrombosis [9]. These three elements of thrombosis comprise the basis for assessing the biocompatibility of prospective small ID grafts.

Current strategies to overcome thrombosis and intimal hyperplasia include co-electrospinning polymer with elastin-like proteins [10], graft geometry alteration to redirect flow [11], and surface modification of existing vascular graft materials (i.e. cell seeding [12], bioactive molecule grafting [13], and coating [14]). Furthermore, clinical evidence indicates that a graft whose elastic properties (i.e. flexibility, compliance, elastic recoil) match those of the native blood vessel tissue is more successful against intimal hyperplasia [5]. The goal is therefore to mimic, as closely as possible, both the mechanical and chemical properties of the native tissue.

The extracellular matrix protein, elastin, has been found to be the least thrombogenic component of blood vessels and has been investigated for its potential as a biomaterial in purified and recombinant forms [15][16]. Elastin-like polypeptides (ELPs) recombinantly produced in the Woodhouse lab are based on the native elastin protein precursor, tropoelastin. These ELPs consist of alternating hydrophobic domains, 20 and 24, and cross-linking domains, 21 and 23, in the form of ELP20-(21-23-24)ⁿ, where n is the number of repeated 21-23-24 domains. The modification of a polyethylene terephthalate (PET) surface with these ELPs is the focus of this work. Previous work in our laboratory using an ELP as a coating on MylarTM, a semi-crystalline PET film,

2

indicated reduced fibrinogen adsorption [17][18], platelet adhesion [17][19], and platelet activation [18][19], as compared to uncoated MylarTM. The next step to more clearly understand the biocompatibility of elastin-like polypeptides is to evaluate the leukocyte response to ELP-coated materials under physiological shear.

1.1 Objective

To determine if elastin-like polypeptides ELP1 (ELP20-(21-23-24)¹) and ELP4 (ELP20-(21-23-24)⁴) developed in the Woodhouse lab physically adsorbed to the semi-crystalline polyethylene terephthalate film, MylarTM, impact the leukocyte response relative to controls.

1.2 Specific Aims

Using ELP1 and ELP4 the specific aims of this study are:

- To characterize surface leukocyte activation and platelet-leukocyte aggregates on ELPcoated MylarTM under shear conditions using scanning electron microscopy.
- To characterize bulk leukocyte activation and platelet-leukocyte aggregates of ELPcoated Mylar[™] under shear conditions. This specific aim will be evaluated by monitoring surface receptor proteins tissue factor and CD11b for leukocyte activation and CD61 expression on leukocytes for platelet-leukocyte aggregates with flow cytometry.

Chapter 2: Literature Review

2.1 Blood-Material Interactions: Haemostasis and Thrombosis

Haemostasis is the maintenance of normal blood flow in the body [20]. The system that controls it responds to any scenario that would disrupt normal blood flow, such as vascular injury. Coagulation, coagulation inhibition, and fibrinolysis are all processes called upon in this system to prevent blood loss by inducing clot formation, inhibition, and degradation to promote subsequent healing and return the vessel to its healthy state [20]. In normal native tissue, free of disease, these processes generally work very well for the desired outcome. However, when biomaterials are introduced into the body, normal haemostasis is disturbed and the response becomes much more complex [21]. This phenomenon is still not fully understood [21].

Biomaterials trigger a series of events including the adsorption of a plasma protein layer, the coagulation cascade, the activation and adhesion of both platelets and leukocytes, as well as the complement cascade [9]. These events provoke the formation of a thrombus, a cellular blood clot cross-linked with fibrin. Sometimes this thrombus can be dislodged, creating a thromboembolus, which has the ability to completely occlude downstream blood vessels. The thrombogenecity of a biomaterial is its ability to induce or promote coagulation or the formation of a thrombus [22].

There are four criteria that define non-thrombogenic biomaterials. Materials that meet some but not all criteria are still considered thrombogenic. The criteria are as follows:

- 1. Based on the first order autocatalytic model of thrombin (T) production, $d[T]/dt = k_p[T]$, the rate constant k_p is less than 0.0001 cm/s [23]
- 2. Low platelet activation and adhesion are present [21]
- 3. Low leukocyte activation is present [21]
- 4. Low complement activation is present [21]

Researchers have tried different strategies to overcome the issue of thrombogenecity; however, none of the strategies have met all of the criteria for a non-thrombogenic material. Some of these modification strategies include:

- a) heparin-coating surfaces [24],
- b) endothelialisation of biomaterials by adhesion promotion or pre-seeding [12],
- c) modification of the material surface with polyethylene glycol (PEG) grafting [13],
- d) alteration in mechanical structure to guide flow uniformly with a spiral guider[11] or helical shunt [25],
- e) biomaterial functionalization with peptides, such as RGD [14] or YIGSR [26],
- f) addition of lysine to promote fibrinolysis [27].

All of the mentioned methods have shown potential by meeting some criteria in a laboratory setting, and heparin coatings clinically [28], but research is ongoing to reduce the effect of thrombosis with biomaterials *in vivo*.

2.1.1 Protein Adsorption

The first phenomenon that happens to a biomaterial upon exposure to blood is protein adsorption [21]. This adhesion and accumulation at the blood-material interface occurs within seconds [29]. The plasma proteins involved in the initial adsorption are albumin, immunoglobulin, fibrinogen, fibronectin, factor XII (FXII) and high molecular weight kininogen (HMWK) [30]. This layer can induce coagulation, cell activation and adhesion, complement activation, and upon the conversion of fibrinogen to fibrin, the fibrinolytic response [9].

While plasma protein adsorption occurs on the surface of a biomaterial, particular proteins may not remain adsorbed over time. The Vroman effect describes the change in surface protein composition at blood-material interfaces, and other multicomponent systems, with time as proteins adsorb, desorb, and change shape [31]. This process is dependent on protein diffusivity and concentration, and the affinity for the surface of the plasma proteins [30]. It is initially driven by diffusion, where smaller proteins at high concentrations adsorb first, then larger proteins at lower concentrations adsorb later. Following this, affinity interactions take over where high concentration proteins with low surface activity are replaced by low concentration proteins with high surface activity [32]. The Vroman effect has been shown previously to occur with albumin, immunoglobulins and fibrinogen [29][33].

Factors that affect plasma protein adsorption on a biomaterial are both protein and surface related. In terms of the protein, concentration, structural stability and size all influence adsorption. Adsorption at lower bulk protein concentrations tends to result in higher surface interactions, as there is greater opportunity for conformational change through orientation and unfolding [32]. This can result in adsorption that is essentially irreversible for all practical purposes. Conversely, proteins adsorbed at higher bulk concentrations will maintain their conformation at the surface, which makes them more easily desorbed [32]. The conformational stability of a protein also influences the affinity for the surface. A highly stable protein allows for less conformational change, while an unstable protein can undergo more conformational change which will result in stronger surface attachment [32]. It has been shown that the conformationally stable protein lysozyme tends to adsorb to hydrophobic surfaces with minimal conformational change, while conformationally unstable proteins albumin and immunoglobulin adhere to all surfaces with a lot of conformational change [32]. A conformational change in a protein can alter its bioavailability by exposing or hiding binding sites. The availability of these binding sites can influence the processes following protein adsorption, and consequently, thrombosis.

Surface topography and chemical composition are surface specific factors that influence protein adsorption. Topographic structures and increased roughness promote more protein adsorption, as the increased surface area allows for more interaction [30]. The chemical composition of a

6

material determines its hydrophobicity and charge [30]. It is common for plasma proteins to have net negative charges, making them likely to adhere to positively charged surfaces [34]. They also tend to adhere more to non-polar, hydrophobic and charged surfaces compared to their counterparts [35]. Other factors that influence protein adsorption include the forces of attraction of van der Waals forces, covalent bonding and hydrogen bonding.

The plasma protein fibrinogen is of particular interest as it impacts thrombosis in the early stages. Fibrinogen has a high affinity for both hydrophilic and hydrophobic surfaces, although it tends to adhere more to hydrophobic surfaces [36]. Platelets adhere to fibrinogen on the material's surface, and over time, fibrinogen is replaced by HMWK on hydrophilic surfaces [37]. HMWK is a requirement for the intrinsic pathway of the coagulation cascade [37].

2.1.1.1 Protein Adsorption Kinetics

Protein adsorption undergoes three phases: initial fast diffusion-dependent adsorption, slower competitive adsorption in multi-component systems, and finally the desorption of loosely bound proteins. Diffusion-dependent adsorption is a case of unsteady state diffusion in a dilute solution that is driven by the concentration gradient in Fick's Second Law of diffusion (Equation 1). C is concentration, t is time, D is the diffusion coefficient of the protein, and *x* is position.

$$\frac{dC}{dt} = D \frac{d^2 C}{dx^2} \tag{1}$$

The diffusion coefficient, which is constant at low concentrations, is described by Equation 2,

$$D = \frac{k_B T}{6\pi\eta r} \tag{2}$$

where k_B is the Boltzman constant, T is temperature in degrees Kelvin, η is liquid's viscosity and r is the radius of the protein. Diffusivity for proteins is usually in the range of 10⁻⁷-10⁻⁶ cm²/s [38].

From Equation 1 and with appropriate boundary conditions, the protein mass adsorbed to the surface, m, can be expressed as Equation 3, where C_o is the initial bulk protein concentration and A is surface area.

$$m = 2C_o A \sqrt{\frac{Dt}{\pi}} \tag{3}$$

It can be seen in Equation 2 that the diffusivity depends on the size of the protein and from Equation 3 that a higher initial protein concentration, C_o , will result in greater mass transport to the surface. As mentioned in the previous section, concentration and protein size affect protein adsorption in the diffusion-dependent phase. To distinguish the extent of protein adsorption in this initial phase, there are two isotherms generally used to model the surface concentration of an adsorbed protein in relation to the bulk concentration: the Langmuir and Freundlich isotherms.

Langmuir's theoretical monolayer model, assumes a) only one protein can adsorb per site, b) the adsorption of one protein does not affect the adsorption of another, c) solution is dilute, and d) adsorption is reversible [38]. In terms of modelling, the rate of adsorption, R_{ads}, to a site is

$$R_{ads} = k_{ads}C(1-\theta) \tag{4}$$

And the rate of desorption, R_{des},:

$$R_{des} = k_{des}\theta\tag{5}$$

In Equation 5, θ is the fraction of surface area covered by adsorbed protein, k_{ads} and k_{des} are the rate constants for adsorption and desorption, respectively, and C is bulk protein concentration. At equilibrium, θ can be modelled as:

$$\theta = \frac{KC}{1+KC} \tag{6}$$

 $K = k_{ads}/k_{des}$ is the adsorption coefficient. This model, plotted in Figure 1, shows a steep increase in surface area coverage as the protein concentration increases, until it reaches a plateau at $\theta = 1$. This depicts the monolayer characteristic of the model. The initial steep increase can be used to calculate the adsorption coefficient, K, and the plateau can be used to determine the monolayer concentration.



Figure 1: Langmuir's monolayer protein adsorption model.

The adsorbed surface layer can also be modelled using the Freundlich isotherm, which is an empirical model that accounts for multilayer coverage, seen in Equation 7:

$$\theta = kC^{1/n} \tag{7}$$

 θ is surface area fraction of adsorbed protein, k is the adsorption capacity, C is the bulk protein concentration and 1/n is the adsorption intensity. Both k and 1/n are constants dependent on the system and temperature. Figure 2 shows that this model has an indefinite increase in surface coverage as protein concentration increases, suggesting multilayer protein adsorption.



Figure 2: Freundlich's multilayer protein adsorption model.

Most experimental data have been fit to the Langmuir model, however adsorption experiments have been reported to fit both models [33]. The elastin-like polypeptides used in this work are thought to exhibit monolayer surface coverage with multilayering at increased bulk concentrations, where the multilayering is thought to be the result of self aggregation of the proteins at the surface [39].

2.1.2 Coagulation

Coagulation follows a series of events in which the activation of one coagulation protein induces the activation of another coagulation protein in a continuous sequence until a cross-linked fibrin clot is formed. This is a process described as the coagulation cascade, seen in Figure 3. Within this cascade, there are two pathways: the intrinsic and extrinsic. Both share the conversion of prothrombin to thrombin, fibrinogen to fibrin and the cross-linking and stabilization of the fibrin polymer in the common pathway, where thrombin activates factor XIII to induce the crosslinking. Platelets, leukocytes, and erythrocytes can be caught in the fibrin network to form a thrombus.

10



Figure 3: The coagulation cascade. Solid arrows indicate flow of activation, dotted arrows indicate amplification, and T indicates coagulation inhibition. Reproduced from reference [40]. © Joe D / Wikimedia Commons / CC-BY-SA-3.0 License

The intrinsic pathway is initiated by activation of FXII, prekallikrein, and HMWK, which all require contact with a negatively charged surface [41]. Once adsorbed, FXII becomes activated (FXIIa) and transforms prekallikrein to kallikrein. The assembly of FXIIa, kallikrein, and HMWK converts FXI to FXIa.

Tissue factor (TF), a surface receptor expressed on damaged cells, initiates the extrinsic pathway. FVII binds with the TF on the cell surface to activate FVII to FVIIa. This forms the TF-FVIIa complex, which converts FX to its activated form.

The coagulation cascade is amplified with the production of thrombin, an enzyme that catalyzes the conversions of FXI, FVIII and FV to their activated forms [20]. Thrombin can also inhibit the coagulation cascade by activating protein C, which inactivates factors Va and VIIIa [9]. Other inhibitors of the coagulation cascade include tissue factor pathway inhibitor (TFPI), which blocks

the formation of the TF-FVIIa complex, and antithrombin, which inactivates FX and thrombin [9].

It was traditionally thought that biomaterial contact only initiated the intrinsic pathway [29]. However, recent research suggests that the extrinsic pathway, also referred to as the tissue factor dependent pathway, is also involved and more prominent in material-induced thrombosis than previously thought. Biomaterial studies with undiluted plasma showed only small amounts of thrombin generation, indicating that the absence of cells limited the extent of coagulation, further suggesting that the TF-dependent pathway is prominent [42][43]. Additionally, Burman *et al.* [44] found that a FXII-deficient patient was still able to produce levels of thrombin comparable to a healthy patient during cardiac surgery, indicating that FXII in the intrinsic pathway is not required for coagulation on a biomaterial. Further studies have suggested that after the TFdependent pathway has been initiated, FXa production from the intrinsic pathway propagates thrombin production [45]. Therefore material-induced coagulation is dependent on both proteins and cells.

Current research indicates that leukocytes must be present for the initiation of the coagulation cascade at the blood-material interface via the TF-dependent pathway [43]. The intrinsic pathway is initiated within seconds of protein adsorption, but the TF-dependent pathway takes at least 60 minutes for monocytes to express TF and generate thrombin [21]. FXIIa generation of the intrinsic pathway also depends on flow, which will vary in different vessels [46].

2.1.2.1 Fibrinolysis

In normal haemostasis, a cross-linked fibrin clot is temporary. During the healing, fibrin is degraded by plasmin in a process known as fibrinolysis, seen in Figure 4.

12



Figure 4: Fibrinolysis pathway. Black arrows indicate flow of activation, blue arrows indicate different activators and red arrows indicate inhibition. Reproduced from reference [47]. © Jfdwolff / Wikimedia Commons / CC-BY-SA-3.0 License

Plasminogen binds to fibrin, extracellular matrix (ECM) and cell surfaces where it specifically interacts with lysine [48]. There are five lysine binding sites (LBS) on plasminogen, to which fibrin and α_2 -antiplasmin can bind [48]. Plasminogen is converted to its activated form, plasmin, primarily by tissue-type plasminogen activator (tPA) and urokinase-type plasminogen activator (uPA) [49][50], where tPA requires the presence of fibrin to be effective [49]. Plasmin can cleave both fibrinogen and fibrin into degradation products [50].

To inactivate fibrinolysis, there are molecules that inactivate the plasminogen activators or specifically inactivate plasmin. Plasminogen activator inhibitors (PAIs) 1 and 2 inhibit tPA and uPA, where PAI-1 is more dominant and is released in the granules of activated platelets [50]. For direct plasmin inactivation, α_2 -antiplasmin is the primary regulatory molecule [50]. It binds to LBSs on plasmin, which interferes with its fibrin binding [50].

2.1.3 Platelets

Platelets were traditionally thought to be the major cellular participants in thrombosis because they were associated with thrombotic complications on cardiovascular devices [21]. As previously mentioned, platelets are important in the characterization of thrombosis, where adhesion [51] and activation [52] have been shown to lead to coagulation. Adhesion of platelets on to the initially adsorbed protein layer depends on their surface ligands and their state of activation [53]. The main protein mediating platelet adhesion is fibrinogen, with *in vitro* and *in vivo* results indicating that platelets bind to fibrinogen adsorbed to a material surface through the glycoprotein (GP) IIb/IIIa surface receptor [54]. It is noted that there need only be a very small amount of fibrinogen, less than 30 ng/cm², adsorbed on the surface to induce platelet adhesion [54]. Massa *et al.* [55] indicated that platelet adhesion was not directly related to the amount of fibrinogen adsorbed, and suggested that the conformational state of fibrinogen affects the extent of platelet adhesion. There are a pair of three GPIIb/IIIa binding sites on fibrinogen: two RGD motifs in the A α chain that binds GPIIIa [56], and a dodecapeptide sequence in the γ chain, H12 (HHLGGADQAGDV), which binds GPIIb [57]. Srokowski & Woodhouse [18] showed that the ELP coatings used in this study adsorbed fibrinogen with RGD sites partially (ELP1 and ELP2) and fully inhibited (ELP4), which could be the basis for reduced platelet adhesion.

A morphology change after adhesion from discoid, to round, to spread and spider-like is characteristic of platelet activation and is an indication of higher activation [21]. Figure 5 illustrates the change in platelet activation at a shear rate of approximately 100 s⁻¹, where the discoid shape adheres, rounds, presents filopodia extensions and spreads as activation increases.



Figure 5: Stages of adherent platelet activation via scanning electron micrographs. Lowest to highest activation at ~100 s⁻¹ shear rate: round (R), dendritic (D), spread-dendritic (SD), spreading (S), and fully spread (FS). Scale bar = 1 μm. Reprinted from [58] with permission from Elsevier.

Work was also done to investigate the steps of platelet aggregation in thrombus formation at a shear rate of 1800 s⁻¹ [59]. At shear rates above, but not below, 1000 s⁻¹, the activated platelets

present thin membrane tethers after adhesion in discoid shape, as seen in Figure 6 [59]. Generally, unstable aggregates are characterized by clustered discoid platelets with membrane tethers around a single, more activated platelet. The stabilized aggregate is distinguished by the spherical shape and long membrane filopodia, not membrane tethers, seen in the 90 second image in Figure 6.



Figure 6: Stages of adherent platelet aggregation via scanning electron micrographs. Shown at 1800 s⁻¹ shear rate, at 30, 60, and 90 seconds. Scale bar = 2 μm. Reprinted from [59] with permission from The American Society of Hematology.

Upon activation, platelet degranulation can occur, activating bulk platelets, leukocytes, promoting platelet-leukocyte aggregates (Figure 7) and supporting thrombin generation. Betathromboglobulin, thromboxane β_2 , ADP, and serotonin released in degranulation activate bulk platelets and released NAP-2 activates leukocytes [21]. Activated platelets also express P-selectin, an adhesion molecule that creates aggregates with monocytes and neutrophils via the P-selectin glycoprotein ligand-1 (PSGL-1) on the leukocytes [21][60]. Platelet-leukocyte aggregates can also be formed between GPIIb/IIIa (CD41/CD61) on platelets and Mac-1 (CD11b/CD18) on leukocytes, bridged by fibrinogen [60]. The concept of using platelet-leukocyte aggregates as an indication of thrombosis is relatively new [9].



Figure 7: Platelet and leukocyte aggregate formation via fibrinogen and P-selectin. Complement (discussed in more detail later) also interacts with platelets. Complement protein C1q has a receptor on platelets, and when bound, induces GPIIb/IIIa activation and P-selectin expression [61]. Complement protein C5b-9 has also been shown to induce thrombotic activity in platelets [62]. It is clear that thrombosis is a complex process involving plasma proteins, platelets, leukocytes, and complement proteins.

2.1.4 Leukocytes

There are five different kinds of leukocytes, distinguished by nuclear shape, size, and cytoplasmic inclusions. The two main groups are granulocytes, which have segmented nuclei and contain granules, and agranulocytes, which have a single nucleus. Neutrophils, eosinophils, and basophils are granulocytes, where neutrophils represent about 60% of leukocytes and eosinphils and basophils less than 5% together [63]. All granulocytes have a diameter of approximately 10-

12 μm. Agranulocytes include monocytes, which are larger leukocytes at a diameter of approximately18 μm and represent about 5% of the leukocyte population, and lymphocytes, which are the smallest leukocytes at a diameter of 6 μm, but represent about 30% of the leukocyte population [63]. Neutrophils and monocytes are considered to be the leukocytes involved in blood-material interactions. Neutrophils are the first leukocyte responders to biomaterials [63]. Neutrophils and monocytes will now be referred to in general as leukocytes.

Upon activation, leukocytes first exhibit a) a change in membrane receptor expression, followed by b) degranulation and release of inflammatory mediators by neturophils, c) an oxidative burst, and then finally, d) adhesion [21]. Membrane receptors on leukocytes that undergo change include the shedding of L-selectin and upregulation of CD11b, as seen in angioplasty [64][65], hemodialysis [66] and cardiopulmonary bypass [67][68]. TF, which may be specific to monocytes [69][70][71], is also expressed upon activation and partly depends on platelet activation and platelet-monocyte aggregates [72]. After changes with the membrane surface receptors, inflammatory mediators are released upon neutrophil degranulation. These include elastase [73] and lactoferrin [74], which are molecules that can neutralize anticoagulant proteins, but also activate platelets [9]. Other inflammatory mediators released are cytokines IL-1 and TNF- α , all of which can be chemoattractants for other leukocytes, further activate platelets and leukocytes, as well as promote endothelial cell adhesion [9]. After neutrophil degranulation and before adhesion, neutrophils and monocytes release oxidants like superoxide (O₂⁻²) and hydrogen peroxide (H₂O₂) which damage tissue and activate cells [75]. These reactive oxygen species have also been reported to degrade biomaterials [76][77].

The indicators of leukocyte activation directly involved in coagulation are CD11b upregulation and TF expression, where CD11b and TF are both transmembrane surface receptors. Figure 3 illustrates that TF directly participates in the coagulation cascade in the TF-dependent pathway to form a fibrin clot. It has been confirmed that upon material contact, monocyte TF expression results in a shorter clotting time *in vitro* [78]. TF expression is also dependent on both platelet and material presence [72]. CD11b indirectly affects the coagulation cascade by activating FX and converting fibrinogen to fibrin by surface binding, again showing pro-coagulant activity [21]. The degree of CD11b upregulation increases as surface area increases [78].

There will be an increase in leukocyte activation with an increase in surface area [78][79], and more adhesion with more activation [21]. Leukocyte adhesion depends on the surface and adherent proteins [80][81], as well as the presence of adherent platelets, discussed in a previous section [72][81]. This can be visualized in Figure 8, where the leukocytes are adhering to the surface with adherent platelets. The left portion of this figure shows a more activated leukocyte state, while the right portion shows a smooth morphology, characteristic of lower activation [82]. Leukocytes tend to be less activated and adherent to hydrophilic surfaces [83]. Leukocytes bind to fibrinogen [84] and adsorbed fibrinogen enhances leukocyte activation [80]. Complement is also thought to affect leukocyte adhesion, with *in vitro* studies showing that complement proteins C3a and C5a mediate leukocyte adhesion [85].



Figure 8: Adherent leukocytes with platelets on scanning electron micrographs. Shown at 150 s⁻¹ shear rate. Leukocytes are circled. Scale bars = 2 μm. Reprinted from [86] with permission from Elsevier.

Further research is still necessary concerning leukocyte activity in thrombosis. It is unknown how to distinguish leukocyte activation from adhesion, and the mechanism of leukocyte activation has

yet to be elucidated. Some suggestions for the activation mechanism include material contact, platelet activation, complement activation, and kallikrein [21]. However, it may be from a combination of factors like complement and platelet activation [21].

2.1.5 Complement Activation and Biomaterials

The complement system is part of the innate immune system, where it destroys pathogens by cell lysis via opsonisation or pore formation. The pathogens are then removed by inflammatory cells. Over 20 proteins are involved in this non-adaptive process (Figure 9) [9]. There are three different pathways, which follow a similar sequence, leading to the formation of a C3 and a C5 convertase. These three pathways are the classical, lectin, and alternative. The C3 convertase, which is C4b2b for the classical and lectin pathways and C3bBb for the alternative pathway, cleaves C3 into C3a and C3b. C3b coats the pathogen for disposal via opsonisation. The C5 convertase is formed with C3b, which is C4b2bC3b for the classical and lectin pathways and C3bBbC3b for the alternative pathway, and eventually forms the membrane attack complex (MAC) for cell lysis via pore formation. The cleaved C3a, C4a and C5a proteins are released cytokines used to attract immune cells, such as monocytes and neutrophils, and promote vasodilation, cell activation, and cell adhesion [87]. Biomaterials activate both the classical and alternative pathways [9].



Figure 9: Complement cascade. Classical, lectin and alternative pathways are indicated. Reproduced with open access from reference [88].

The C1 complex consists of C1q, C1r, and C1s. The classical pathway is initiated when C1q binds to an antibody-antigen complex, aggregated immunoglobulins, or other substances. The lectin pathway is initiated when certain lectin binding proteins attach to carbohydrates on the surface of pathogens. A surface is required for the activation of factor B and the alternative pathway, which is activated when a non-self surface is recognized [9]. This surface could be a fungus, bacteria polysaccharide, lipopolysccharide, particle or a biomaterial [9]. Generation of the alternative pathway's C3 convertase, C3bBb, is amplified from C3b production in the classical and alternative pathways [9].

The complement reaction to biomaterials is not fully understood [9]. Although it was previously thought that it was solely the alternative pathway that was triggered by biomaterials, evidence of the classical pathway was shown in blood samples taken during cardiopulmonary bypass [89]. Investigations were also done with C4 deficient patients showing delayed MAC production [90], further supporting the connection to the classical pathway. Some biomaterials can generate high

levels of both C3b and C5b-9, and some high levels of C3b and not C5b-9 [21]. However, even low levels of C5b-9 were shown to activate leukocytes [91], and complement can also activate platelets [9], as previously discussed.

Proteins from the complement system interact with proteins in the coagulation system. From the coagulation system, FXIIa and kallikrein cleave C1s in the classical pathway [92]. Thrombin and kallikrein cleave C5 and factor B, while thrombin also cleaves C3 and C6 [9]. Antithrombin III from the coagulation system protects erythrocytes from MAC cell lysis [9]. Complement proteins Bb and C3bBb convert prothrombin to thrombin and the C1 inhibitor inactivates FXIIa and Kallikrein [9].

2.2 Anticoagulant

Anticoagulants are used in patients with diseases or prosthetics that require the elimination of blood clots either on a regular basis, or solely during a procedure. In terms of coagulation research, anticoagulants are used when collecting human blood from donors to reduce the coagulation events that may be initiated during the collection itself. Anticoagulants work in different ways to stop the formation of fibrin, either by inhibiting a coagulation protein or thrombin, or by chelating essential ions like calcium. Examples of coagulation protein inhibitors include heparin, PPACK, hirudin, TAP, and antistatin, all with varying ability to inactivate bound and unbound thrombin. Warfarin interferes with the vitamin K cycle and citrate is a calcium ion chelator. Depending on the nature of the research, the anticoagulant chosen must have minimal effect on the study of interest.

The most commonly used anticoagulant is heparin [9]. The mechanism by which it inhibits coagulation starts with the binding of antithrombin (AT). Once bound to heparin, AT undergoes a conformational change, inhibiting its ability to activate FIX, FX, FXI, and thrombin. It cannot inhibit surface- or fibrin-bound thrombin, like PPACK and hirudin, nor complexed-FXa like TAP

and antistatin [93]. Only about a third of administered heparin binds to AT, and the rest binds to other plasma proteins not involved in coagulation [94]. Heparin has also been shown to have some anti-complement activity [95].

Sodium citrate is also used, but it works as a buffer and calcium chelator. TF expression [96], formation of the TF-VIIa complex, activation of coagulation cascade proteins, IX and X, and conversion of prothrombin to thrombin require calcium for coagulation [9]. Platelet-leukocyte aggregates require calcium as well, and it has been shown that citrate reduces platelet-monocyte aggregates compared to use of hirudin, heparin, and PPACK, but not platelet-neutrophil aggregates [97]. The effect that citrate has on the complement system is also unclear [98].

2.3 Effects of Shear on Thrombosis

Flow and shear stresses play an important role in the formation of thrombi. Arterial and venous blood flow differ, and consequently, the associated cell and protein transport within the vessel also differ, impacting the thrombi produced from vessel to vessel. The expression of surface proteins on platelets and leukocytes is also influenced by flow, as vascular cells are very responsive to local mechanical forces [99][100].

A blood vessel can be modelled as a long, cylindrical tube exhibiting laminar, incompressible Newtonian fluid flow [101]. Velocity is only in the axial direction (v_z) , where the highest velocity is at the center (r = 0) and the lowest at the wall with a no slip condition [101]. A schematic of this can be seen in Figure 10:



Figure 10: Velocity profile model of laminar flow in a vessel. Blood is modelled as a Newtonian fluid with a no slip condition at the vessel wall. Radius (r), vessel radius (R), velocity in the z-direction (v_z) , and shear rate (γ) are labelled.

Flow is modelled using the following equation, where v is the mean fluid velocity, r is the radial position from the center and R is the vessel radius:

$$v_z = 2v \left[1 - \left(\frac{r}{R}\right)^2 \right] \tag{8}$$

In terms of shear, this model can be pictured as hypothetical fluid layers from the wall to the center. The layer directly touching the wall will have no velocity, and each subsequent layer will have a higher and higher velocity, reaching the maximum velocity layer at the center. Shear stress, τ , which is the tangential force over a unit of area, is proportional to the change in velocity across these layers described in Equation 9, where μ is the viscosity of blood.

$$\tau = \mu \frac{dv_z}{dr} \tag{9}$$

This change in velocity over the change in radial position is called shear rate, γ , commonly measured in inverse seconds (Equation 10).

$$\gamma = \frac{dv_z}{dr} = \frac{4vr}{R^2} \tag{10}$$

Contrary to velocity, it can be seen from Equation 10 that the shear rate will be highest at the wall and lowest at the center of the vessel. For studies of blood flow, the wall shear rate (γ_w) where r = R is of most concern. This is because the shear rate is the highest at the wall and will have the most impact on thrombus formation.

$$\gamma_w = \frac{4v}{R} \tag{11}$$

If v is expressed in terms of volumetric flow rate, Q,

$$v = \frac{Q}{\pi R^2} \tag{12}$$

then the shear rate at the wall becomes:

$$\gamma_w = \frac{4Q}{\pi R^3} \tag{13}$$

From this last equation, it can be seen that the shear rate at the vessel wall can vary largely, depending on different vessel diameters and volumetric flow rates. It is apparent that the major influence in this equation is vessel diameter, where shear rate will increase with decreasing diameter. This conclusion is supported by Table 1, which presents typical values of shear rate for a range of blood vessel diameters.

Vessel	Diameter (mm)	γ_{w} (s ⁻¹)
Arterioles	0.03	1900
Small arteries	0.3	1500
Left main coronary	4	460
Right coronary	3.4	440
Femoral artery	5	300
Common carotid	5.9	250
Internal carotid	6.1	220
Large veins	5-10	200
Inferior vena cava	20	40-60
Ascending aorta	23-45	50-300

Table 1: Common parameters for flow in vessels. Modified from [102].

Contrary to the flow model's assumption, blood does not actually act as a Newtonian fluid [103]. The plasma component of blood is a Newtonian fluid, however the cells, which are 99% by volume red blood cells [104], contribute to the non-Newtonian properties [105]. The viscosity of blood decreases with increased shear, due to the behavior of the red blood cells [103][106]. This is known as the Fahraeus-Lindqvist effect [107]. At low shear rates, the red blood cells tend to assemble in rouleaux, which are single cell stacks mediated by fibrinogen [103]. As the shear is increased, the rouleaux begin to break apart, decreasing the viscosity [106]. The increasing shear crowds red blood cells towards the center and tends to push other cells towards the vessel walls [108]. Increased shear increases protein transport to the vessel wall, and subsequent potential for coagulation at the wall, as reviewed by Eskin & McIntire [109]. Since both cells and proteins are forced closer to the wall at higher shear, there is a higher chance for thrombus formation in the smaller vessels.

Most of the research concerning shear and its effect on thrombosis has focused on platelets, although there has been some work investigating leukocytes and proteins. Higher shear can be the dominant initiator for platelet activation, and therefore for aggregation as well [110]. At higher shear, there is more platelet activation with less fibrin deposition, and at lower shear, lower
platelet activation with more fibrin deposition [46][111]. Results with leukocytes have been controversial, as they have indicated both increases and decreases with increased shear, which may be due to dependence of other cells and proteins present. Kuijper *et al.* [112] found that isolated leukocytes tended to adhere less to endothelium at a high shear rate, but when platelets were pre-adhered, the leukocytes adhered more at higher shear rates. Consequently, it appears that leukocyte deposition during shear is dependent on platelet adhesion [113]. Weber & Springer [84] found that leukocytes adhered less at higher shear rates, and Kuijper *et al.* [114] that more leukocytes adhered to fibrin at lower shear rates, but that the leukocytes adhered at higher shear rates were more clustered. Regarding coagulation, more FXa from the TF-dependent pathway was found to be generated with increased shear [115] and more thrombin produced from the intrinsic pathway with lower shear, but time to generation was shorter with increased shear [46]. These results indicate that at lower shear rates, the intrinsic pathway of coagulation appears to be dominant, and as shear increases, the TF-dependent pathway appears to be dominant.

2.3.1 Cone and Plate Device

The cone and plate device was originally designed to create constant shear rate and shear stress throughout the test fluid to measure viscosity. Like other rotational viscometers, known rotational speed and measured torque is used to calculate the viscosity of a Newtonian or non-Newtonian fluid. The cone and plate device was used to test starch pastes and gums in the textile industry to reduce measurement error compared to other methods [116] but has been expanded to include applications such as the viscosity measurement of whole blood and plasma in medical diagnostics [117]. In research, it can be used to create a constant shear environment.

The set-up of the cone and plate was optimized from the coaxial cylinder model, in which a cylinder rotated in a well of fluid [118]. Since the radius of the cylinder in the coaxial cylinder model was finite, the shear rate varied with radius [119]. To create a constant shear rate throughout the fluid of interest, the cylinder was modified to a cone in which the apex touched the

surface of the bottom of the well. This allowed for a thin layer of small sample volume, thus allowing more tests for a given volume, but which also enabled a rapid temperature stabilization with minimal fluctuation upon applied shear rate [119]. Note that temperature fluctuations that occur at cone angles higher than 4° can cause the shear rate to vary [119]. Like the coaxial cylinder, the cone and plate is a very simple design to set up and clean, where the constant shear rate can be easily adjusted. Shear rate, γ , can be calculated with the following equation when the cone angle is small, where Ω is cone rotation rate and θ is cone angle:

$$\gamma = \frac{a}{\theta} \tag{14}$$

Equation 14 applies when the cone angle and Reynolds number are small. Figure 11 schematically illustrates these parameters:



Figure 11: Cone and plate device profile. Cone angle, θ , rotation rate, Ω , and radius, r, are indicated. The particular cone and plate design used in this project is meant to simulate blood flow at constant shear to test the responses of different blood-contacting materials during shear. The bottom of the well consists of a test film or coating that allows for cellular response studies on the surface of the material. Like in straight sections of blood vessels, the flow in the cone and plate is

essentially laminar without secondary flow, as long as the test material is smooth [120]. However, the flow pattern spirals around the cone centre and the area at the centre is stagnant [121].

The cone and plate device has been used previously to test platelet response to blood-contacting biomaterials. During shear, it seems that the red blood cells are responsible for pushing the platelets to the surface due to the non-Newtonian properties of blood and the Fahreus-Lindqvist effect [107][122]. Experimentation was also done to show that the apparatus itself, the bystander material for example, was not responsible for platelet adhesion [120]. It was found that platelet activation is proportional to the shear rate from this device [123], consistent with findings previously described.

Other methods used to simulate blood flow, like the Chandler loop or flow chamber can require large volumes of blood or pumps that risk shearing blood cells. Since blood donors have high variability, using the same donor's blood from a single sample collection can help to reduce this variability. The cone and plate device is ideal, as it uses a small volume of blood (~1 mL), allowing for multiple samples to be run at the same time, and for there to be multiple runs per donor sample collection.

2.4 Elastin

Elastin is an extracellular matrix protein found in tissues requiring elastic recoil and resilience, such as blood vessels, lungs, and ligaments. In blood vessels, the dominant roles of elastin are cell signalling and mechanical properties. Elastin is made up of tropoelastin monomers, which are produced in vascular endothelial cells and in vascular smooth muscle cells. Once chaperoned to the cell surface by elastin binding protein, tropoelastin undergoes a process of self-assembly into elastin polymers. Tropoelastin is then crosslinked to form elastin fibres in a reaction catalyzed by lysyl oxidase [124]. Microfibrillar proteins are then incorporated and this forms mature elastic

28

fibres [125]. From this point on in the thesis, for simplicity, the word elastin will refer to the elastin protein.

There are three major layers to blood vessels, all of which contain elastin (Figure 12). In direct contact with the lumen, there is the tunica intima, followed by the tunica media and tunica adventitia on the outside. In elastic arteries, the adventitia is primarily comprised of collagen and elastin which are responsible for mechanical strength, elastic recoil, and resilience [126]. The media is made up of layers of alternating elastin-rich lamellae separated by smooth muscle cells which play an important role in the expansion and contraction of the vessel to maintain blood pressure during pulsatile flow [127]. The intima is simply a layer of endothelium on a basal membrane [126]. In between the intima and media, and media and adventitia, there is an internal elastic lamina and external elastic lamina, respectively, which are sheets of elastin fibres. These laminae, more so the internal elastic lamina, allow the vessel to contract after distention [126].



Figure 12: Typical blood vessel structure. Elastin-rich areas are identified with blue.

A canine study was done by Kabemba *et al.* [128] where vessels with removed endothelium were tested against vessels injured deeper than the internal elastic lamina. The vessels with absent

endothelium showed slower thrombus formation, thus indicating that the internal elastic lamina layer reduced thrombosis [128]. Elastin has been found to be the least thrombogenic component of blood vessels and has been investigated for its potential as a biomaterial in this regard [15][16].

Various forms of elastin have been used for synthetic vascular graft studies, including purified elastin from animal vessels and recombinantly produced elastin. Intimal hyperplasia was reduced and there was no inflammatory or thrombus response observed from a porcine common artery elastin-purified graft *in vivo* [129]. Simionescu *et al.* [130] compared both purified elastin and purified collagen porcine aorta grafts. Using platelet-rich plasma, the study showed that platelet adhesion and aggregation were both lower in the purified elastin graft *in vitro*, and significantly less platelet adhesion was observed *in vivo* [130]. Additionally, an elastin graft purified from porcine arterial elastin was compared to the clinically used ePTFE graft. The purified elastin graft remained patent longer, where patency refers to the state of being open or unblocked, and only showed signs of thrombosis at the suture line [131]. The ePTFE graft had signs of thrombosis throughout the graft.

Some groups have attempted to explain why elastin has non-thrombogenic characteristics, but as of yet, the reason for its relative non-thrombogenicity in comparison to collagen and other proteins is not fully understood [15]. Studies show that RGD sequences may be responsible for the binding between platelets and fibronectin and collagen, and suggest that elastin's lack of RGD sequence could be a reason for its low platelet adhesion characteristics [132]. Platelets also tend to bind to carbohydrates on glycosylated proteins [133], and like albumin, elastin lacks carbohydrate groups, which could also explain its low platelet adhesion [133].

It is noted that partially purified elastin fibres from vessels can cause calcification from the microfibrillar components [134]. Calcification is the hardening of tissue due to an accumulation of calcium, which can cause grafts to fail [134]. Contrary to partially purified elastin fibres,

30

purified tropoelastin, completely purified elastic fibres [135], and elastin-based peptides [136] inhibit calcification. Therefore, the materials based on these polymers are more likely to provide the best basis for tissue engineered scaffolds derived from elastin [15]. In addition, other investigations have shown that recombinant elastin-mimetic polymer coatings reduced fibrinogen [137], fibrin, and platelet [138] deposition relative to controls.

2.5 Elastin-Like Polypeptides

The Woodhouse laboratory, along with its collaborators, has been able to produce recombinant elastin-like polypeptides (ELPs) in *E. coli*, using genes based on the human tropoelastin gene [139]. The polypeptides produced contain the alternating hydrophobic and crosslinking domains characteristic of the elastin protein. Within the hydrophobic domains, there are non-polar amino acid residues, such as glycine, valine, proline, and leucine. In the crosslinking domains, alanine and lysine residues are prominent. These lysines are the sites for crosslinking in native elastin [140] and provide opportunities for crosslinking via different reagents *in vitro* [141]. The gene construct for the ELP peptides contains exons 20 and 24 alternated with exons 21 and 23 from the human tropoelastin gene. These exons code for the hydrophobic and crosslinking domains of the peptide, respectively. The structures of ELP20-24 (ELP1) and ELP20-24⁴ (ELP4) genes used in this work are shown in Figure 13. ELP20-24² (ELP2), not shown, has also been studied by others in the laboratory. Exon 24 is of particular interest because it codes for the amino acid sequence PGVGVA, which is repeated seven times. The hexa-peptide PGVGVA sequence is thought to reduce thrombogenecity [15] and has been implicated in the self-aggregation of elastin [139].



Human Exons:

Exon 20: PGFGVGVGGIPGVAGVPGVGGVPGVGGVPGVGISP Exon 21-23: EAQAAAAAKAAKYGVGTPAAAAAKAAAKAAQFG Exon 24: LVPGVGVAPGVGVAPGVGVAPGVGVAPGVGVAPGVGVAPGIGP

Figure 13: Elastin-like polypeptide (ELP) gene structures for ELP1 and ELP4, and amino acid sequences. Modified from [17] with permission from Taylor & Francis.

The elastin-like polypeptides have been shown to have physical and mechanical properties similar to native elastin [142]. After coacervation, Bellingham *et al.* [142] found that the ELP2 fibre formed was compact and ordered, which closely resembled that of tropoelastin. The formed ELP1 fibre had a lesser resemblance to the formed tropoelastin fibre. It was hypothesized that this difference between ELP fibres was because of the greater number of hydrophobic domains in ELP2 [142]. Crosslinked ELP2 and ELP4 materials were shown to be insoluble like native elastin and in terms of mechanical properties, were very similar to aortic porcine elastin [142]. However, the mechanical properties of ELP1 were not able to be evaluated because the protein was not able to form a material. It is suggested that a minimum of three hydrophobic domains, flanked with crosslinking domains, are required to attain self assembly and ultimately fibre formation. [142].

The thrombogenicity of physically adsorbed ELP2 has been tested on MylarTM (PET), TefzelTM (polytetrafluoroethylene/ethylene copolymer), and CorethaneTM (polyurethane) *in vitro*. All ELP-coated surfaces showed reduced P-selectin expression, platelet adhesion, and platelet-microparticle formation with the coating in comparison to uncoated controls [19]. The desorption level of the coatings from these materials indicated that the coatings were relatively stable, and all materials retained similar amounts of ELP2 [19].

Woodhouse *et al.* [19] conducted an *in vivo* rabbit study on ELP2-coated polyurethane catheters in which the coated catheters remained patent for three times as long as the uncoated catheters. ELP1-, ELP2- and ELP4-coatings were further investigated on MylarTM *in vitro* in an attempt to explain this lowered thrombogenic response. Srokowski *et al.* [17], in reconstituted whole blood, and Srokowski & Woodhouse [18], in a fibrinogen solution and in whole blood, showed reduced fibrinogen adsorption, platelet adhesion and platelet activation with ELP-coated MylarTM under shear as compared to uncoated MylarTM. The biggest differences were noted with the ELP4 coating [17][18], which also showed an altered fibrinogen conformation as compared to the other ELP coatings [18]. In this work, the leukocyte response of ELP1- and ELP4-coated MylarTM was studied *in vitro* to further understand the possible mechanism for the relatively low thrombogenicity associated with these elastin-like polypeptides.

Chapter 3: Materials and Methods

3.1 Materials

All chemical reagents were purchased from Sigma-Aldrich Canada (Oakville, CA), unless specified otherwise. MylarTM (300 Å) sheets were purchased from Active Industries (Clifton Park, USA). It should be noted that MylarTM is the semi-crystalline film form of polyethylene terephthalate (PET), a commonly used biomaterial for vascular grafts [3]. Low density polyethylene (LDPE) (90 µm) sheets were purchased from KMac Plastics (Wyoming, USA). LDPE has been shown to be a negative control for platelet activation and thrombosis [143]. Circular MylarTM and LDPE surfaces (33 mm in diameter) were pre-treated with triplicate methanol rinses (Fisher Scientific, Whitby, CA) and equilibrated overnight at room temperature in 3 mL of plain Tyrode's buffer (0.14 M NaCl, 0.0025 M KCl, 0.012 M NaHCO₃, 0.0004 M Na₂HPO₄, pH 7.4), to ensure a clean initial surface.

3.2 Evaluation of Endotoxin Contamination

Endotoxin or lipopolysaccharide (LPS) is found in the outer cell membrane of gram-negative bacteria and becomes detrimental to humans and animals upon release after cell lysis [144]. It is the most significant pyrogen in parenteral drugs and medical devices [9], and therefore must be monitored closely when manufacturing devices that enter the body. The Food and Drug Administration (FDA) of the United States imposes a guideline with an acceptable LPS concentration of 0.5 EU/mL, where EU signifies endotoxin units [145]. On a cellular and molecular level, endotoxin is known to activate complement, the kinin system, leukocytes, platelets, and endothelial cells [144]. Some studies have indicated that LPS affects platelets at 1 μ g/mL (5000 EU/mL) and leukocytes at 0.01 ng/mL (0.05 EU/mL), as reviewed by Gorbet and Sefton [9]. However, these tests were done using purified LPS and their potency may be higher than that of environmental endotoxin and should be noted. It is very common to have LPS contamination *in vitro*, especially in the water source, as distillation and deionization do not remove it [9]. This makes it important to monitor and eliminate it in *in vitro* studies.

To reduce potential for endotoxin contamination and other microbes, all solutions were passed through 0.22 μ m sterile filters (Fisher Scientific, Whitby, CA) before use, although this may not remove all endotoxin. The water from the distillation and deionization system was tested using Pyrotell Gel Clot Formulation for endotoxin testing (Associates of Cape Cod, East Falmouth, USA). This is a *Limulus* amebocyte lysate (LAL) assay, with a sensitivity of 0.125 EU/mL. LAL assays are derived from horseshoe crab blood, which is very sensitive to coagulation. Endotoxin-free water (0.1 mL) was added to each tube to reconstitute the LAL powder and then 0.1 mL volumes of the distilled deionized water sample dilutions in endotoxin-free water were added corresponding to 0.125 EU/mL, 0.25 EU/mL and 0.5 EU/mL sensitivities to their respective tubes (0.1 mL, 0.05 mL and 0.025 mL distilled deionized water in 0.1 mL total samples of endotoxin-free water). Endotoxin-free water was used as a negative control and 50 EU/mL of reconstituted endotoxin was used as a positive control. All samples were thoroughly mixed before incubation in a water bath at 37 °C for 60 ± 2 minutes. A positive test was indicated by gel formation upon inversion immediately after the incubation time.

3.3 Elastin-Like Polypeptide Production and Adsorption

ELP1 and ELP4 were expressed, produced and purified using established methods [139]. Transfected BL21 *Escherichia coli* cells were cultured in a 10 L bioreactor for up to 5 hours, then induced with isopropyl- α -D-thiogalactopyranoside (IPTG), final concentration of 0.5 mM, and culture was continued for up to 3 more hours, or until cell culture growth reached a plateau. The cell pellet was retrieved by centrifugation, cells were lysed with formic acid and the ELP1 and ELP4 proteins were cleaved with cyanogen bromide before dialysis in distilled deionized water and in sodium acetate. Further purification was done using a SP sepharose cationic ion exchange column. The ELPs were then dialyzed in acetic acid before being lyophilized. A typical yield for ELP1 or ELP4 would be approximately 40 mg/L of cell culture.

Protein adsorption was carried out as previously described [17]. Pre-treated MylarTM films in 40 mm diameter disc form were affixed to the bases of the wells of the cone and plate device, where 32.5 mm film diameter was exposed in the well. ELP1 and ELP4 were solubilised overnight at 1 mg/mL in Dulbecco's phosphate buffered saline (PBS) (Fisher Scientific, Whitby, CA) and then were statically adsorbed (2.45 mL) to the pre-treated MylarTM surfaces for 2 hours at room temperature. Surfaces were rinsed with plain Tyrode's buffer before shear treatment.

3.4 FastinTM Elastin Assay

The colorimetric assay FastinTM Elastin (Biocolor Ltd., Carrickfergus, UK) was carried out in accordance with the manufacturer's instructions to determine the amount of each ELP adsorbed to the MylarTM surface by solution depletion. It uses a 5,10,15,20-tetrapenyl-21H,23H-porphine tetra-sulphonate (TPPS) dye to bind the basic and non-polar amino acid sequences in the ELP molecules and has a sensitivity range of 5-70 µg per test. After 2 hours of ELP adsorption (1 mg/mL in PBS), two 50 µL samples were taken from each well of the 6-well plate (33 mm diameter MylarTM film per well) and then precipitated in 50 µL of cold Elastin Precipitating Reagent and centrifuged in microcentrifuge tubes. After centrifugation, the pellet was redispersed in 1 mL of Dye Reagent and reacted for 90 minutes on a shaker plate to produce the elastin-dye complex. These elastin-dye complexes were centrifuged, then 250 µL of Dye Dissociation Reagent was added to the sample pellets and vortexed to release the dye into solution. TPPS samples were transferred to a 96-well plate and the absorbance of each sample was recorded on a PerkinElmer 2300 EnspireTM Multimode Plate Reader at a wavelength of 513 nm. Samples were compared to a standard curved generated from 0 µL, 12.5 µL, 25.0 µL, and 50 µL duplicate samples from the 1 mg/mL ELP solution run in parallel.

36

3.5 Blood Collection

Approximately 20 mL of human whole blood was collected into buffered 3.2% (0.109 M) sodium citrate vacutainers (BD Bioscience, Mississauga, CA) with a 21-gauge needle (BD Bioscience, Mississauga, CA) from healthy volunteers, free of medications for 10 days and in accordance with ethics approval from Queen's University (Queen's University ethics protocol #CHEM-003-11). The first collection tube was discarded (~2 mL) and the rest were stored at room temperature under constant gentle mixing. All samples were used and fixed within 8 hours of collection.

3.6 Cone and Plate Device

The cone and plate device used in this work has 4 wells of 32.5 mm diameter, with cone diameter of 31 mm and cone angle of 4°, which can be seen in Figure 14. Films can be placed in the plate to become the bottoms of the wells. Each run had a well of ELP1-coated MylarTM, ELP4-coated MylarTM, uncoated MylarTM and LDPE, the order of which was randomized per sample to mitigate the impact of confounding variables. LDPE was used as a negative control [143].



Figure 14: Schematic of cone and plate device used in this work. A is 1 of 4 cones, B is the rotating shaft of the cone, C is the motor housing, D is the lowering mechanism, E is the support structure, F is the motor, G is the plate housing, H is the 4-well plate and I is the base.

Citrated whole blood (0.8 mL) was added to each well, cones were lowered for the tip to barely touch the surface of the bottom of the well, and then the cones were rotated at a shear rate of 300 s^{-1} for 2 hours at 37 °C. This shear rate was chosen to represent that of small diameter vessels [102]. Shear rates of 100 s⁻¹ and 200 s⁻¹ were also tested for surface evaluation. During this 2 hour period, static controls of 1 mL resting citrated whole blood (WB) and 5 µg endotoxin in 1 mL of WB were carried out at 37 °C. A thrombin control of 0.25 U in 1 mL of citrated whole blood was used to qualitatively confirm that the blood demonstrated the ability to clot, in which all samples was positive.

3.7 Random Sampling

A random sample generator was used to calculate random configurations within the plate of the cone and plate device to reduce bias results within each well. The given output is seen in Table 2. Each plate set-up was changed sequentially and restarted at 'Run 1' once 'Run 20' was complete.

Run	Well 1	Well 2	Well 3	Well 4
1	LDPE	ELP1	ELP4	Mylar TM
2	Mylar TM	LDPE	ELP4	ELP1
3	ELP4	ELP1	LDPE	Mylar TM
4	ELP4	Mylar TM	ELP1	LDPE
5	Mylar TM	LDPE	ELP1	ELP4
6	ELP4	ELP1	Mylar TM	LDPE
7	ELP1	ELP4	LDPE	Mylar TM
8	LDPE	ELP1	ELP4	Mylar TM
9	LDPE	Mylar TM	ELP1	ELP4
10	Mylar TM	ELP1	LDPE	ELP4
11	ELP4	ELP1	Mylar TM	LDPE
12	Mylar TM	ELP1	LDPE	ELP4
13	ELP4	Mylar TM	LDPE	ELP1
14	ELP1	Mylar TM	ELP4	LDPE
15	ELP1	ELP4	Mylar TM	LDPE
16	LDPE	ELP1	ELP4	Mylar TM
17	ELP1	LDPE	ELP4	Mylar TM
18	LDPE	ELP4	ELP1	Mylar TM
19	LDPE	ELP1	Mylar TM	ELP4
20	ELP4	LDPE	ELP1	Mylar TM

Table 2: Random sample configurations. Each plate was set up differently to reduce bias within wells.

3.8 Scanning Electron Microscopy

Uncoated samples of MylarTM and LDPE from shear rates of 100 s⁻¹, 200 s⁻¹, and 300 s⁻¹ were used to evaluate the surface reaction of leukocytes. The sample preparation procedure for SEM was modified from Flynn [146]. After 2 hours of citrated whole blood exposure to shear, each surface was rinsed with plain Tyrode's buffer, and then fixed for 1 hour at room temperature and then overnight at 4°C with 2.5% gluteraldehyde. Samples were then rinsed again in plain Tyrode's buffer and were exposed to a series of ethanol dehydrations (70%, 90%, 95%, and 100%) before chemical drying with graduations of hexamethyldisilazane (HDMS) in ethanol (33%, 66%, and 100%). Samples were left overnight to completely dry before being cut into 8 mm discs with a biopsy punch (VWR, Mississauga, CA), gold sputter-coated and imaged on a JEOL JSM-840 scanning electron microscope (SEM) at an accelerating voltage of 10 kV.

3.9 Flow Cytometry

Flow cytometry is a precise analysis method that can simultaneously count cells and detect biomarkers, such as cell surface protein expression. Cells in suspension are stained with fluorescently-labelled antibodies before being injected into the flow cytometer. Once in the device, the cell suspension is encompassed in a sheath fluid that forces the cells and small membrane-derived vesicles (microparticles) in a single file. Lasers are shone on this fluid to excite fluorochromes and then the emitted light is detected by different fluorescence (FL) channels that can detect the number of cells that are fluorescent as well as the associated intensity. Light scattering can also be used to determine forward scatter and side scatter properties.

After shear exposure, 6-100 μ L samples of citrated whole blood were taken from each well and added to blocking solution (final concentration 5% BSA, 0.05% Tween-20 in PBS, pH 7.5) and incubated at room temperature for 15 minutes to reduce background staining. Each set of samples from the wells were single stained for 30 minutes in the dark at room temperature or at 4 °C with a) 10 μ L Alexafluor488-conjugated mouse anti-human tissue factor (R&D Systems, Minneapolis, USA), b) 10 μ L phycoerythrin (PE)-conjugated mouse anti-human CD11b (R&D Systems, Minneapolis, USA), b) 10 μ L phycoerythrin (PE)-conjugated mouse anti-human CD11b (R&D Systems, Minneapolis, USA), b) 10 μ L phycoerythrin (PE)-conjugated mouse anti-human CD11b (R&D Systems, Minneapolis, USA), c), 20 μ L FITC mouse anti-human CD61 (BD Bioscience, Mississauga, CA), and d) one sample per well was left unstained for gating purposes. Then, 2 mL of a proprietary erythrocyte lysing buffer, BD FACS lysing solution (BD Bioscience, Mississauga, CA), was added to each sample and incubated for 10 minutes. Samples were centrifuged twice for 5 minutes at 500xG, resuspended in 2 mL of HEPES Tyrode's buffer (HTB) (0.14 M NaCl, 0.0025 M KCl, 0.012 M NaHCO₃, 0.0004 M Na₂HPO₄, 0.001 M D-glucose, 0.35% BSA, 0.001 M MgCl₂, 0.01 M HEPES, pH 7.4) and then fixed with 0.5 mL of 1% paraformaldehyde for 30 minutes at room temperature. Samples were re-suspended in 1 mL of HTB for analysis on a Cytomics FC500 flow cytometer at the Cytometry and Imaging Facility at the Cancer Research

Institute at Queen's University. Unstained samples were used to determine fluorescence compensation settings.

Raw flow cytometry data obtained were analyzed using FlowJo vX flow cytometry analysis software from Tree Star Inc. The scatter plots were gated similarly to that in Figure 15. Since forward scatter indicates relative size and side scatter indicates relative complexity, the granulocytes were able to be distinguished from the monocytes. Figure 15 is a typical scatter plot, exhibiting an unstained citrated whole blood sample that was exposed to MylarTM.



Figure 15: Typical scatter plot obtained from FlowJo vX flow cytometry analysis software. An unstained citrated whole blood sample exposed to MylarTM is shown with gated lymphocyte, monocyte and granulocyte populations. Percentages are indicative of the fraction of total events for the given population.

With the populations identified, further gating was done to identify the TF, CD11b, and CD61 markers within the granulocyte and monocyte populations. Figure 16 shows typical histograms of monocytes from a citrated whole blood sample exposed to MylarTM. In a), the peaks stained for the platelet-leukocyte aggregate indicator CD61 (orange) and leukocyte activation indicator TF (blue) are compared to the unstained sample represented by the red peak. The area under the stained peaks beyond the area of the unstained peak is an indicator of the number of cells positive

for surface receptor expression, and this number can be used to compare between samples. There is a separate histogram shown in b), because unlike the markers in a), CD11b expression is generally present on leukocytes, regardless of activation state [147]. However, when activated, the number of CD11b surface receptor proteins on leukocytes increases [147]. To evaluate upregulation, the median fluorescence intensity of the CD11b marker is divided by the median fluorescence intensity of the unstained sample. In this analysis, the median of the positive events of the stained sample was divided by the median of the negative events of the unstained sample. This gives the fluorescence expression ratio and can be used to compare between samples.



Figure 16: Histogram plots of the fluorescence intensity of monocytes from whole blood samples exposed to MylarTM obtained from FlowJo vX flow cytometry analysis software. FL1 and FL2 indicate light channels for the 520 nm and 578 nm wavelengths, respectively. The red peaks represent an unstained sample, in a) the orange peak is from a single stained FITC mouse anti-human CD61 sample and the blue, Alexafluor488 mouse anti-human TF. In b), the blue peak represents a single stained PE mouse anti-human CD11b sample. Only positive events of the stained samples shown, as the negative events were removed for clarity.

3.10 Statistical Analysis

Statistical analysis was done using GraphPad Prism 6 statistical software. Surface comparisons were done using a repeated measures one-way analysis of variance (ANOVA), with Dunnett's multiple comparison post-hoc testing against uncoated MylarTM. Pearson's correlation test was used to test for dependencies between variables. Inter-donor variability was analyzed using a two-

way ANOVA with Tukey multiple comparisons post-hoc testing . All results are reported as mean ± 1 standard deviation (SD), with statistical significances reported based on p < 0.05.

Chapter 4: Results and Discussion

4.1 Endotoxin Testing

The LAL assay confirmed that there was less than 0.125 EU/mL endotoxin contamination in the distilled deionized water source as evidenced by the absence of gel formation for 0.125 EU/mL, 0.25 EU/mL, and 0.5 EU/mL thresholds. According to the FDA's guideline for maximum allowable endotoxin in biomedical devices of 0.5 EU/mL [145], the distilled deionized water used in this study is considered sterile and suitable for use in biomedical devices.

4.2 Elastin-Like Polypeptide Adsorption

After 2 hours of static adsorption in PBS, two samples from the supernatant of each ELP1 and ELP4 wells were tested using the FastinTM Elastin assay to determine the amount of ELP adsorbed to the MylarTM surface via solution depletion. There were a total of 10 and 9 tests taken for ELP1 and ELP4, respectively, over 4 independent experiments. Typical standard curves for 50 μ L samples of ELP-PBS solutions are shown in Figure 17. With known total solution volume and MylarTM surface area, it was determined that ELP1 adsorbed at a concentration of 0.22 \pm 0.13 μ g/cm² and ELP4 at 0.37 \pm 0.19 μ g/cm².



Figure 17: Typical FastinTM Elastin standard curves for ELP1 and ELP4. Data are shown for 50 µL samples of 0, 12.5, 25 and 50 µg of ELP in PBS.

The static adsorption quantification tests were used to confirm ELP adsorption to MylarTM, as well as to compare to the previously determined adsorption values of $0.76 \pm 0.48 \ \mu g/cm^2$ and $0.25 \pm 0.24 \,\mu\text{g/cm}^2$ for ELP1 and ELP4 [17], respectively, shown in Figure 18. ELP4 adsorption was not significantly different between current and previous work; however, ELP1 adsorption was significantly different. This could be due in part to differences in ambient conditions, where the temperature may be affecting adsorption. However, considering that all of the standard deviations are greater than 50% of their mean values for ELP adsorption, solution depletion may not be the most accurate method of adsorption detection. When using a solution depletion method, where the protein concentration left in solution is much higher than that adsorbed to the surface, the error associated with the concentration in solution would be on the same order of magnitude of the value of adsorbed protein. In other words, the magnitude of error is similar to that of the protein adsorption values, so that the low precision of the assay cannot detect small differences in the results. Therefore, for this work, the FastinTM Elastin assay is not precise, and other adsorption quantification methods, like protein radiolabelling [19], should be explored. Previous work with ELPs at a bulk concentration of 1 mg/mL adsorbed to MylarTM between 2-3 h had shown adsorption in the monolayer region of the Langmuir isotherm [39][148]. Thus, it is likely that ELP1 and ELP4 adsorption to MylarTM in this work is forming a monolayer consistent with the previous work.



Figure 18: Static surface adsorption concentrations of ELP1 and ELP4 on MylarTM from a FastinTM Elastin assay. Previous (3 h adsorption) [17] and current (2 h adsorption) concentrations are compared, each with a minimum of 9 samples. Reproduced with permission from Taylor & Francis. Mean ± SD, * indicates significant differences at a p < 0.05.

Desorption has been previously investigated by Woodhouse *et al.* [19] who studied 21 h overnight static desorption of radio-labelled ELP2 (in PBS at 37 °C) on three surfaces: TefzelTM, CorethaneTM, and MylarTM. After 21 h, about 40% of ELP2 was desorbed from all surfaces as determined by radioactivity [19]. Srokowski & Woodhouse [148] also investigated desorption from MylarTM during shear of 300 s⁻¹ for 15 minutes at room temperature in PBS using goniometry, X-ray photoelectron spectroscopy (XPS), and a quartz crystal microbalance with dissipation (QCM-D) on ELP1, ELP2, and ELP4 [148]. Similar desorption results were found to the static overnight radio-labelled ELP2 desorption study at ~33% desorption [148]. This suggests that times greater than 15 minutes and shear do not affect desorption.

From the same studies by Srokowski & Woodhouse [148], ELP1 and ELP4 had ~52% and ~15% desorption, respectively [148], suggesting that the shorter ELPs have a higher tendency to desorb from the surface. This implies that ELP1 is a more conformationally stable protein as compared to ELP4, where ELP4 should be able to change conformation upon adsorption with a higher affinity for the surface. ELP1 was determined to have a more rigid and dehydrated layer than ELP2 and ELP4, and all ELP isotherm data showed a monolayer even after desorption from 30 minutes of rinsing in PBS [148]. For the purposes of this work, with shear exposure for a 2 hour

period, it is likely that ELP4 will maintain, at minimum, a monolayer and there is a chance that the ELP1 desorption may have exposed the underlying MylarTM surface.

4.3 Effects of Shear

All blood samples were tested for their ability to form a clot before being exposed to shear by subjecting them to thrombin (0.25 U in 1 mL citrated whole blood). All blood used was able to clot within seconds, as seen by the qualitative test in Figure 19. Static endotoxin in citrated whole blood (5 μ g/mL) against plain citrated whole blood samples (1 mL) were run in parallel with flow cytometry procedures to show that the cells had the ability to respond to stimulus.



Figure 19: Citrated whole blood with thrombin (1 mL/0.25 U), testing positive for ability to form a clot within seconds.

4.4 Surface Evaluation – Scanning Electron Microscopy

SEM was used to evaluate the surface leukocyte activation and platelet leukocyte aggregates. Lower shear rates of 100 s⁻¹ and 200 s⁻¹ in addition to the shear rate of interest, 300 s⁻¹, were used to determine if leukocytes had potential to adhere to the surfaces at various shears. Figure 20 illustrates a collage of scanning electron micrographs of LDPE and MylarTM samples exposed to shear rates of 100 s⁻¹, 200 s⁻¹ and 300 s⁻¹ with citrated whole blood at 37 °C for 2 hours. This test was run in triplicate. Generally the morphology of red blood cells is biconcave, but they can also appear crenated or spherical with spicules due to changes in osmotic pressure in the SEM preparation [149]. Leukocytes are distinguished by their larger size, $\sim 10 \ \mu\text{m}$ in diameter, and either smooth or rough surface morphology. The activated platelets can be recognized by their small size, a few microns in diameter, and especially their long spider-like extensions when activated. There appears to be fibrinogen or indications of fibrinogen present as well, identified as a film under the cells present, and sometimes completely covering the surface under the cells. This could be fibrinogen itself or the spreading of platelets on fibrinogen.





200s -1





Figure 20: Representative scanning electron micrographs of low density polyethylene (LDPE) and MylarTM at shear rates of 100 s⁻¹, 200 s⁻¹ and 300 s⁻¹. Red blood cells are indicated with arrows, and the leukocyte is circled. Scale bars are 20 µm.

At 100 s⁻¹ on the MylarTM, there appears to be both a leukocyte and activated platelets, indicating platelet-leukocyte aggregation. The 100 s⁻¹ sample of LDPE appears to have crenated red blood cells forming aggregates with the activated platelets. There is no evidence of any leukocytes adhering when the shear rate is increased to 200 s⁻¹ and 300 s⁻¹, although activated platelets are still present. These results are consistent with other studies that have shown that at lower shear rates, there is a tendency for leukocytes to adhere to surfaces because the cells have a greater chance of contacting the surface [84][114].

With respect to the platelets, they appear to be more activated at the lowest $(100s^{-1})$ and highest $(300s^{-1})$ shear rates as seen by their clustering or aggregation. Increases in platelet activation with shear rate have been reported by Turitto *et al.* [111]. The current results at 200 s⁻¹ and 300 s⁻¹ appear to be consistent with these findings; however, the increased platelet activation at 100 s⁻¹ on MylarTM could be due to the presence of leukocytes accelerating their aggregation [72]. Regardless of shear rate, the platelet activation appears to be higher on the MylarTM surface compared to the LDPE, indicated by the extent of platelet grouping and fibrinogen/spread platelet film. This finding supports the hypothesis that fibrinogen adsorption is required for platelet adhesion to take place [37], where platelets bind to fibrinogen's RGD sequence through their GPIIb/IIIa surface receptor [54], and is consistent with previous work [17].

Since leukocytes did not adhere to the uncoated MylarTM at the shear rate of interest of 300 s⁻¹, and previous work showed that there was a decrease in fibrinogen and platelet adsorption on ELP-coated MylarTM [17], it is unlikely that there would be leukocyte adsorption on the ELP-coated surfaces at this shear rate. Moreover, another group has shown that monocyte adhesion reflects some dependence on platelet adhesion [78], further indicating the unlikelihood of leukocyte adhesion on the ELP-coated surfaces. It was assumed that the ELP1 and ELP4 coatings would not likely promote leukocyte adhesion at 300 s⁻¹, and therefore bulk activation of the blood became the focus of the thesis from this point.

4.5 Bulk Evaluation – Flow Cytometry

The first sign of leukocyte activation is the changes in membrane receptors [21], including the expression of TF and upregulation of CD11b. Citrated whole blood exposed to the ELP1- and ELP4-coated MylarTM, uncoated MylarTM, and LDPE surfaces under 300 s⁻¹ shear rate for 2 h at 37 °C in the cone and plate device was tested for these membrane receptors. Percentages of cells in the monocyte and granulocyte populations expressing TF are summarized in Figure 21, and the degrees of CD11b upregulation of these populations in Figure 22. There were a total of 15 samples, where n = 1-4 samples per donor from N = 6 donors.



Figure 21: Tissue factor expression on granulocytes and monocytes for ELP-coated MylarTM compared to controls, at 300 s⁻¹. Static controls are LPS and WB. * indicates significant differences compared to MylarTM, p < 0.05. Mean \pm SD, 15 samples total (n = 1-4 samples per donor from N = 6 donors).

There were no significant differences in granulocyte TF expression for the ELP coatings as compared to the uncoated MylarTM. This may be because it is unknown whether granulocytes express TF [70] and these results may be an indication that they do not. Some research has shown that there is significant transfer of TF from monocytes to granulocytes [71], and others have detected little change to TF expression on leukocytes other than monocytes with respect to biomaterial exposure [72]. Therefore granulocyte TF expression may not be a good indicator of granulocyte activation and indirectly signifies monocyte activation.

In the monocytes, the TF expression was significantly lower for the ELP1 ($38.6 \pm 16.3\%$) and ELP4 ($33.9 \pm 18.1\%$) coatings as compared to the uncoated MylarTM ($62.0 \pm 21.5\%$) indicating that the ELP coatings are reducing monocyte activation. ELP1 and ELP4 seem to have a very similar response, which suggests that the sequence length does not largely influence the expression of TF by monocytes. TF expression requires calcium [96] and there is a possibility that the use of a citrate anticoagulant could impact these results. However, the data still shows TF expression differences between surfaces with the use of citrated anticoagulant. It is possible that the use of another anticoagulant, such as heparin, might indicate even greater differences between the surfaces. This is recommended for consideration in future work. Regarding static controls in Figure 21, it is unclear as to why the positive static control LPS was significantly lower than MylarTM and WB was not. This is discussed in depth later.

In Figure 22, both the granulocytes (ELP1 = 21.4 ± 3.7 %, ELP4 = 22.0 ± 3.2 %) and monocytes (ELP1 = 18.7 ± 2.2 %, ELP4 = 19.7 ± 2.7 %), ELP1- and ELP4-coated materials showed significantly higher CD11b upregulation as compared to uncoated MylarTM (granulocytes: 6.4 ± 2.5 %, monocytes: 9.6 ± 2.2 %). This result is intriguing because it appears to indicate that the ELP coatings are activating leukocytes relative to the uncoated MylarTM, which is contrary to the TF expression results. In contrast, other research has indicated that the TF expression and CD11b upregulation follow similar patterns upon leukocyte activation [72][150][151]. It is unclear as to why this is occurring with the ELP-coated MylarTM because the first indication of leukocyte activation is the change in surface receptors TF and CD11b [21]. This is discussed in more detail later. With respect to anticoagulant, the use of citrate has not been reported to likely have an effect on CD11b upregulation [152] and it has been suggested that heparin may falsely upregulate CD11b on leukocytes [153].



Figure 22: CD11b upregulation on granulocytes and monocytes for ELP-coated MylarTM compared to controls, at 300 s⁻¹. Static controls are LPS and WB. * indicates significant differences compared to MylarTM, p < 0.05. Mean ± SD, 15 samples total (n = 1-4 samples per donor from N = 6 donors).

Platelet-leukocyte aggregates were classified as positive CD61 expression, a platelet marker, in the granulocyte or monocyte populations. Reported percentages indicate the fraction of the cells positive for expression within the populations. Figure 23 shows that CD61 expression for the ELP coatings in both the granulocytes (ELP1 = $62.7 \pm 17.0 \%$, ELP4 = $60.5 \pm 20.1 \%$) and monocytes (ELP1 = $63.1 \pm 17.1 \%$, ELP4 = $61.8 \pm 16.8 \%$) is significantly lower compared to uncoated MylarTM (granulocytes: $80.7 \pm 16.5 \%$, monocytes: $75.0 \pm 14.1 \%$), which is consistent with previous findings for ELP4 at 300 s⁻¹ for 1 h [18]. This suggests that ELP1 and ELP4 coatings reduce platelet-leukocyte aggregates compared to uncoated MylarTM. While it is known that platelet-leukocyte aggregates require calcium [97], the use of a calcium chelating anticoagulant in this work has not prevented the ability to discern differences between surfaces. Platelet-monocyte aggregates in the static positive control LPS were lower than negative control WB and the reason for this is unclear. This is discussed in more detail later.



Figure 23: CD61 expression on granulocytes and monocytes for ELP-coated MylarTM compared to controls, at 300 s⁻¹. Static controls are LPS and WB. * indicates significant differences compared to MylarTM, p < 0.05. Mean ± SD, 16 samples total (n = 1-4 samples per donor from N = 6 donors).

Earlier discussion regarding ELP adsorption during the 2 hour exposure period to citrated whole blood in this work concluded that it was likely that the ELP4 coating on MylarTM would maintain a monolayer and that the ELP1 coating may undergo some desorption. ELP1 and ELP4 coatings showed similar monocyte TF expression (Figure 21), leukocyte CD11b (Figure 22), and platelet-leukocyte aggregate (Figure 23) responses. All of these responses were significantly different from the responses to uncoated MylarTM, suggesting that there was little desorption of the ELPs during the 2 hour time period. If there was some desorption, it does not appear to have affected the overall leukocyte response as compared to controls.

Figure 21 and Figure 23 show that both the TF expression on monocytes and the plateletleukocyte aggregates, respectively, have significantly decreased relative to the uncoated MylarTM, whereas Figure 22 shows that that CD11b upregulation has not. This suggests that there is a correlation between monocyte TF expression and platelet-leukocyte aggregates. Some studies have shown that TF expression is dependent on P-selectin expression, which is an indicator of platelet activation, and that TF mediates platelet-leukocyte aggregates [72][154]. Previous studies similar to the current project by Srokowski *et al.* [18] showed a significant reduction in the P-selectin expression for ELP2- and ELP4-coated MylarTM as compared to uncoated MylarTM,

54

which was reflected by their significant reduction in platelet-leukocyte aggregates in ELP2 and ELP4. This trend is consistent with the current findings, where the platelet-leukocyte aggregates were lower with the ELP coatings. It appears that the TF expression, platelet-leukocyte aggregates and P-selectin expression follow similar trends between results and is consistent with the literature. This further supports the idea that platelet activation, by P-selectin, affects leukocyte activation, through TF expression, which in turn mediates platelet-leukocyte aggregates.

The Pearson correlation was applied to assess the dependency between monocyte TF expression and platelet-monocyte aggregates for the ELP1- and ELP4-coated MylarTM, uncoated MylarTM, LDPE and static controls LPS and WB. An r value is calculated for each relationship. When r = 1, there is a perfect correlation, when r = 0, there is no correlation, and when r = -1 there is a perfect negative correlation. The results are illustrated in Figure 24 and show no significant correlations between TF and platelet-monocyte aggregates. This indicates that the parameters are not dependent, but, because previously discussed results and literature suggest that there is an association, an explanation in the differences might be the small sample size (15 data points). The association between TF expression and platelet-monocyte aggregates was not highly apparent in this work, but that may be due to the high variability between donors and within donors, discussed below. Additional runs could be performed to reaffirm this theory.



Figure 24: Pearson correlations of the given surface between monocyte TF expression and platelet-monocyte aggregates. r values are listed on plots, sample size is 15.

In this investigation, an increase in CD11b upregulation (Figure 22) was found when TF expression (Figure 21) decreased for the ELP-coated surfaces compared to controls. These findings show an interesting difference from previous studies. Research indicates that TF expression is dependent on the presence of platelets while platelets do not appear to influence CD11b upregulation [72][155]. This suggests that CD11b can be upregulated by a mechanism that differs from that of TF expression. Possible influential factors include time, degree of exposed biomaterial surface area and complement.

TF and CD11b are presented differently on the cell surface and as a result, the time to expression differs with these surface receptors. TF is internally synthesized before expression upon cell activation [156] and requires hours to be expressed [9]. Small amounts of CD11b are naturally present on the cell membrane, but upon cell activation, CD11b present in internal granules is translocated to the cell membrane to increase surface concentration [147]. CD11b upregulation requires less time than TF expression because transcription and translation do not need to occur upon leukocyte activation. The current experiments and previous *in vitro* and *in vivo* studies with recombinant ELPs had not been carried out past 4 hours [17][18][19][138]. The reduced thrombogenicity of these recombinant ELP coatings was based on fibrinogen and platelet activity. However, the increased CD11b upregulation of the current results suggests that perhaps leukocytes are in fact being activated and that TF expression may not be a good indicator of leukocyte activation in the short term.

Gourlay *et al.* [79] and Gorbet & Sefton [78] have found that the degree of exposed material surface area affects CD11b upregulation, but has little to do with TF expression [78]. As the material surface area to blood volume ratio increased by increasing number of polystyrene beads, Gorbet & Sefton [78] found that the amount of CD11b expression per bead increased, whereas the TF expression per bead remained the same. This suggests that perhaps the reason for the increased CD11b upregulation with the ELP-coated MylarTM in this work is due to an increase in

surface area after coating with ELP. There was work done using atomic force microscopy with reported 2D topographic images comparing uncoated to coated MylarTM with ELP1, ELP2 andELP4 [148]. However, the purpose for the experiment was directed towards surface composition and protein adsorption rather than surface roughness and surface area. With no roughness parameters listed, it is difficult to compare the surface roughness from the images to determine if in fact the ELP coatings have increased the surface area. This requires further investigation.

Complement may also be contributing to the increased CD11b upregulation. Complement proteins C3a and C5a attract leukocytes and C3b promotes leukocyte adhesion and activation [91][157]. It has been shown that leukocyte activation indicated by CD11b upregulation is highly influenced by complement activation, whereas TF expression is not [72]. Gorbet & Sefton [158] compared the CD11b upregulation on non-complement activating polystyrene beads and complement activating PEG-modified polystyrene beads to determine that CD11b upregulation via complement is material dependent. Additionally, complement can activate platelets with the complement protein C1q, causing them to express P-selectin and GPIIb/IIIa [61], which are known to induce platelet-leukocyte aggregates [72][154]. An indication as to why platelet-leukocyte aggregates are not increased with the ELP coatings in this work may be that it is the complement system's alternative pathway that is activated, which does not require the activation of C1q. This suggests that another factor to consider for increased CD11b upregulation and decreased TF expression on ELP-coated Mylar[™] is that these surfaces may be activating complement by the alternative pathway and requires further consideration.

The low thrombogenicity of these ELP coatings was shown in the initial *in vivo* studies where an ELP-coated polyurethane catheter remained patent for the duration of the experiment (4 hours), while an uncoated polyurethane catheter was completely occluded within 2 hours [19]. The mechanism for the non-thrombogenic nature of the ELPs was then further investigated *in vitro* on

58

Mylar[™] where fibrinogen adsorption [17][18], platelet adhesion [17][19], and platelet activation [18][19] were all reduced as compared to uncoated Mylar[™]. It is unexpected to see that leukocytes might possibly be activated by the coatings when there was low thrombogenicity overall in the *in vivo* model. This suggests that there is something in the *in vivo* model, such as endothelial cells, that may be reducing the possible leukocyte activation. TFPI, which inhibits TF [159], as well as CD46, CD55, and CD59, which inhibit complement via C3 convertase and MAC [160], are expressed on endothelial cells and may be impacting TF expression and CD11b upregulation *in vivo*. The impact of these molecules on ELP-coated Mylar[™] should be further investigated.

4.6 Inter-Donor Variability

A factor to take into consideration when evaluating the effects on thrombus formation when exposed to biomaterials is that blood composition may be different in each sample from donor to donor and also within the same donor. Studies have shown that peripheral blood gene expression varies with gender, age, time of day, and composition of the blood sample [161]. Preliminary inter-donor variability was executed in attempt to distinguish differences between subjects that could be contributing to variances in the results. There were 4 subjects tested with samples from 2 independent runs, totaling 2 samples per subject. A 2-way ANOVA was run with Tukey's multiple comparison post-hoc testing. Figure 25 is an example of these results, showing significant differences with some of the donors.



Figure 25: Inter-donor comparison of TF expression on monocytes. * indicates significant differences, p < 0.05. Mean \pm SD, n = 2 samples per donor from N = 4 donors.

While it can be seen that there are significant differences between subjects 2 and 4, the sample size is not adequate to detect representative differences between subjects. Within donor variability was unable to be analyzed due to the small sample size, but is something that should be further investigated.

4.7 Controls

As seen from Figure 21, Figure 22 and Figure 23, the positive (LPS) and negative (WB) static controls are not always in agreement. Specifically, the monocyte TF expression and CD61 expression with LPS exposure are lower than those exposed to the negative control, WB, where LPS is known to strongly induce leukocyte activation [144]. Given this anomaly, a more in depth analysis of the flow cytometry results was undertaken.

It was found that the differences with the positive (LPS) and negative (WB) static controls may be attributed to the population gating in the flow cytometry analysis. A representative example of the LPS and WB scatter plots are shown in Figure 26 with granulocyte, monocyte and lymphocyte populations gated. It can be seen that the granulocyte populations are fairly distinct but that the monocyte populations are less so, particularly between the transition of lymphocyte and monocyte populations. Typically, in these scatter plots the monocyte population encompasses all mononuclear cells, including larger lymphocytes. This may impact the results presented in this work, which have assumed solely a monocyte population in the mononuclear gate. The lymphocyte population may be diluting the percentage of positive monocyte events. For example in the mononuclear gate, if the x number of monocytes expressing TF is consistent across samples and if the y number of lymphocytes is changing from sample to sample, the TF expression consistency may not be detectable.



Figure 26: Scatter plots of positive (LPS) and negative (WB) static controls, shown left and right, respectively. Granulocyte, monocyte, and lymphocyte population gates are indicated. Percentages are indicative of the fraction of total events for the given population.

Differences in CD11b upregulation for LPS and WB (Figure 22) were as expected, where the positive control (LPS) had higher upregulation than the negative control (WB). However, CD11b upregulation is determined by the median intensity of the fluorescent marker on each cell, not the percent of cells expressing the surface protein. Lymphocytes have been reported to express CD11b [162] but regardless of whether the lymphocytes have CD11b upregulation, the results presented for monocytes would still be true for the mononuclear population given that the
fluorescence intensity histogram follows a normal curve. For these reasons, the results concerning CD11b upregulation are thought to be reliable.

Surface markers that are measured by the percentage of cells with expression, such as TF and CD61, may be subject to the dilution effect of lymphocytes in the mononuclear population. This is especially the case if the lymphocytes do not express the surface receptor. It is thought that monocytes are the sole leukocytes expressing TF [70] and it has been shown that larger lymphocytes can bind to platelets to form aggregates [163], where the platelet-lymphocyte aggregates could appear in the monocyte CD61 expression results. Additionally, the monocyte gates for LPS and WB from Figure 26 are different in shape and vary with the forward scatter. This indicates that LPS has had an impact on the cells in this population regarding their size and potentially the ratio of monocytes to lymphocytes present. If there are more lymphocytes negative for expression present in this population with LPS than WB, the total mononuclear positive expression for LPS could falsely appear lower. This could be what is occurring for monocyte TF (Figure 21) and CD61 (Figure 23) expression for the static controls.

Contrary to the static controls, the mononuclear population gates in the scatter plots of blood exposed to shear are consistent across materials. This suggests that the mononuclear population is less likely to vary with lymphocyte dilution across the samples exposed to shear and results can be used as an indication of activation but not for quantitative analysis.

From these findings, we are still confident with the granulocyte results for differences with the surfaces. We are not as confident with the monocyte TF and CD61 expression results; however, they appear to follow a similar trend to those of the granulocyte CD61 expression. Further studies using the monocyte-specific marker, CD14, are warranted.

62

Chapter 5: Conclusions and Recommendations

5.1 Summary of Findings

Synthetic vascular grafts such as DacronTM and ePTFE have been clinically successful and are currently used in procedures requiring vessel inner diameters larger than 6 mm [6]. Contrarily, smaller grafts of the same material fail within 5-10 years of implantation due to thrombosis or intimal hyperplasia, and there is a need of over 1.5 million procedures each year that require small diameter synthetic vascular grafts [3]. Several approaches have been undertaken, with some success, to match the mechanical and chemical properties of a synthetic small diameter vascular graft to those of a native blood vessel, including endothelialisation, protein adsorption, and anticoagulant agent incorporation as well as fibrinolytic agent incorporation. Of particular interest is the prominent blood vessel protein, elastin, for its anti-thrombogenic properties. The basis of this work was to evaluate the effects two recombinant ELP coatings on MylarTM in terms of leukocyte response during shear with respect to thrombosis.

ELPs 1 and 4 were tested using the FastinTM Elastin assay to determine degree of surface adsorption to MylarTM and to ensure reproducibility of test materials from previous work [17]. Said studies are similar with a focus on platelet response in thrombosis, for the purposes of result comparison. ELP4 surface adsorption $(0.37 \pm 0.19 \ \mu\text{g/cm}^2)$ was not significantly different from the previous work, however ELP1 surface adsorption $(0.22 \pm 0.13 \ \mu\text{g/cm}^2)$ was significantly different and lower than previous work. This could be as a result of the different ambient conditions; however, it is likely the low precision of the FastinTM Elastin assay contributing to the differences. In terms of desorption during experimentation, it is thought that ELP4 coating would maintain at minimum a monolayer, and that ELP1 coating may desorb and expose the underlying MylarTM, which could increase the thrombogenicity.

63

Negative control, LDPE, and reference, uncoated MylarTM, were evaluated for surface adsorption of leukocytes. SEM results at 100 s⁻¹, 200 s⁻¹, and 300 s⁻¹ showed evidence of activated platelets, with some leukocyte activity at the lowest shear rate. Overall, higher platelet activation was noted on the MylarTM surface compared to LDPE. Since there was no evidence of leukocyte activation at the surface of the negative control and reference surface at the shear rate of interest, 300 s⁻¹, leukocyte surface activity for the ELP coatings was deemed unlikely and focus was turned to the bulk activation.

Membrane receptors TF and CD11b were evaluated for leukocyte activation, and CD61 for platelet-leukocyte aggregates in the bulk via flow cytometry. Both TF expression and plateletleukocyte aggregates were significantly different and lower for both ELP coatings on Mylar[™] as compared to uncoated Mylar[™]. Contrarily, CD11b upregulation was increased with the coatings, which was unexpected. It is thought that the TF expression and aggregates are related and consistent with literature; however, the sample size was inadequate to observe any correlation. The discrepancy with CD11b could be the result of the exposure time, material surface area, complement system, or as a result of a more complex mechanism. Donor variability analysis showed some significant differences between donors. However, the sample size was too small for both inter-donor and intra-donor variability analysis.

5.2 Conclusions

From the current and previous works, it is thought that ELP coatings may reduce the thrombogenicity of MylarTM. Bulk studies indicated reduced leukocyte activation in terms of tissue factor expression, as well as reduced platelet-leukocyte aggregates with the elastin-like polypeptide coatings. It was unexpected that CD11b upregulation was higher for both coatings, thus this finding requires further investigation. Both ELP1 and ELP4 coatings showed similar results in terms of leukocyte activation and platelet-leukocyte aggregation. However, previous

adsorption studies indicated that ELP1 is more likely to desorb. In conclusion, ELP1 and ELP4 may be promising surface coatings for blood contacting devices.

5.3 Recommendations for Future Work

The following are recommendations to further evaluate the effect of ELP coatings on leukocyte response and their practicality for use as a coating with blood contacting devices:

- The Fastin[™] Elastin solution depletion assay used in this work was deemed inappropriate to detect the quantity of ELP adsorbed to Mylar[™]. Should further investigation of the extent of ELP adsorption be of interest, it is recommended that another method be employed, such as radio-labelling ELP [19].
- 2. The anticoagulant used in this work, sodium citrate, is a mild calcium chelator. TF expression is known to require calcium [96] and CD11b upregulation is not affected by citrate [152] but may be falsely upregulated by heparin [153]. It would be interesting to compare this work's leukocyte response on ELP-coated MylarTM in citrate with the use of another anticoagulant, such as heparin, in similar experiments.
- Further investigation of the increased CD11b upregulation with the decreased TF expression on the ELP coatings is warranted. There are a few recommended approaches to this:
 - a. Exposure time. TF is internally synthesized upon leukocyte activation [156],
 requiring hours to be expressed on the surface [9], whereas CD11b is stored
 internally [147] and can expressed in a much shorter time frame. TF expression
 may not be an adequate indicator of leukocyte activation at shorter time periods,
 and longer experimental time periods are suggested.
 - b. Effect of surface area. Biomaterial surface area does not impact TF expression, but as surface area increases, the extent of CD11b upregulation increases [78].
 The ELP coatings may be changing the surface roughness of MylarTM, which

may be increasing the surface area and giving the result of increased CD11b upregulation. Atomic force microscopy can be used to quantify surface roughness.

- c. Complement activation. It has been shown that leukocyte activation indicated by CD11b upregulation is highly influenced by complement activation
 [72][85][158], whereas TF expression is not [72]. It would be of interest to investigate the effect of the ELP coatings on complement activation and associated CD11b upregulation with these conditions.
- 4. Endothelial TFPI [159], CD46, CD55, and CD59 [160] expression may be reducing leukocyte activation *in vivo*, while leukocytes may be activated in the current *in vitro* work. Investigation of the leukocyte response to ELP-coated MylarTM with these molecules is warranted.
- 5. Donor variability can have a high impact on results when using human blood samples. Gender, age, and time of day are just some factors that can influence the reactivity of the blood sample [161]. It is recommended that the blood of several donors undergo at least triplicate repeats of the study in order to better interpret the results.
- 6. Monocyte results in this work may have been subjected to a lymphocyte dilution effect in the analysis of the flow cytometry scatter plots. It is recommended that any further studies involving monocytes and flow cytometry use a monocyte-specific marker, such as CD14.
- Static adsorption of the ELPs was used here as a simple means of coating Mylar[™].
 However, this form of coating is subject to desorption and may not be a permanent solution. It is therefore recommended that other means of adsorption or protein incorporation be explored, such as covalent bonding [164].
- 8. It would be interesting to investigate ELP coating at various shear rates other than 300 s^{-1} to see how blood would respond to lower shear rates, like in recirculation areas, and to

higher shear rates, like at anatomical vessel bends. This would also allow for a better understanding of the response in various diameter vessels.

References

- [1] World Health Organization, "Cardiovascular Diseases (CVDs) Fact Sheet No317," 2012.
- [2] W. Rosamond, K. Flegal, K. Furie, A. Go, K. Greenlund, N. Haase, S. M. Hailpern, M. Ho, V. Howard, B. Kissela, B. Kissela, S. Kittner, D. Lloyd-Jones, M. McDermott, J. Meigs, C. Moy, G. Nichol, C. O'Donnell, V. Roger, P. Sorlie, J. Steinberger, T. Thom, M. Wilson, and Y. Hong, "Heart disease and stroke statistics--2008 update: a report from the American Heart Association Statistics Committee and Stroke Statistics Subcommittee.," *Circulation*, vol. 117, no. 4, pp. e25–146, Jan. 2008.
- [3] R. van Lith and G. A. Ameer, "Biohybrid strategies for vascular grafts," in in *Tissue Engineering*, N. Pallua and C. V Suscheck, Eds. Berlin, Heidelberg: Springer Berlin Heidelberg, 2011, pp. 279–314.
- [4] R. C. Darling and R. F. Linton, "Durability of Femoropopliteal Reconstructions," *Surgery*, pp. 472–479, 1971.
- [5] S. E. Greenwald and C. L. Berry, "Improving vascular grafts: the importance of mechanical and haemodynamic properties.," *The Journal of pathology*, vol. 190, no. 3, pp. 292–299, 2000.
- [6] B. H. Walpoth and G. L. Bowlin, "The daunting quest for a small diameter vascular graft," *Expert Review of Medical Devices*, vol. 2, no. 6, pp. 647–651, 2005.
- [7] B. W. Lytle, "Prolonging patency- choosing coronary bypass grafts," *The New England Journal of Medicine*, vol. 351, no. 22, pp. 2262–2264, Nov. 2004.
- [8] S. Ito, S. Ishimaru, and S. E. Wilson, "Effect of coacervated alpha-elastin on proliferation of vascular smooth muscle and endothelial cells.," *Angiology*, vol. 49, no. 4, pp. 289–297, 1998.
- [9] M. B. Gorbet and M. V Sefton, "Biomaterial-associated thrombosis: roles of coagulation factors, complement, platelets and leukocytes," *Biomaterials*, vol. 25, no. 26, pp. 5681– 5703, Nov. 2004.
- [10] S. G. Wise, M. J. Byrom, A. Waterhouse, P. G. Bannon, A. S. Weiss, and M. K. C. Ng, "A multilayered synthetic human elastin/polycaprolactone hybrid vascular graft with tailored mechanical properties," *Acta Biomaterialia*, vol. 7, no. 1, pp. 295–303, Jan. 2011.
- [11] Z. Zhang, Y. Fan, X. Deng, G. Wang, H. Zhang, and R. Guidoin, "Simulation of blood flow in a small-diameter vascular graft model with a swirl (spiral) flow guider.," *Science in China Series C: Life sciences*, vol. 51, no. 10, pp. 913–921, Oct. 2008.
- [12] O. F. Khan and M. V Sefton, "Endothelialized biomaterials for tissue engineering applications in vivo," *Trends in Biotechnology*, vol. 29, no. 8, pp. 379–387, Aug. 2011.

- [13] H.-S. Shin, K. Park, J. H. Kim, J.-J. Kim, and D. K. Han, "Biocompatible PEG grafting on DLC-coated nitinol alloy for vascular stents," *Journal of Bioactive and Compatible Polymers*, vol. 24, no. 4, pp. 316–328, Jul. 2009.
- [14] C. Chollet, C. Chanseau, M. Remy, A. Guignandon, R. Bareille, C. Labrugère, L. Bordenave, and M.-C. Durrieu, "The effect of RGD density on osteoblast and endothelial cell behavior on RGD-grafted polyethylene terephthalate surfaces," *Biomaterials*, vol. 30, pp. 711–720, Mar. 2009.
- [15] A. Waterhouse, S. G. Wise, M. K. C. Ng, and A. S. Weiss, "Elastin as a nonthrombogenic biomaterial," *Tissue Engineering: Part B*, vol. 17, no. 2, pp. 93–99, 2011.
- [16] S. W. Jordan and E. L. Chaikof, "Novel thromboresistant materials," *Journal of Vascular Surgery*, vol. 45, p. 104A–115A, Jun. 2007.
- [17] E. M. Srokowski, P. H. Blit, W. G. McClung, J. L. Brash, J. P. Santerre, and K. A. Woodhouse, "Platelet adhesion and fibrinogen accretion on a family of elastin-like polypeptides," *Journal of Biomaterials Science*, vol. 22, pp. 41–57, Jun. 2011.
- [18] E. M. Srokowski and K. A. Woodhouse, "Evaluation of the bulk platelet response and fibrinogen interaction to elastin-like polypeptide coatings.," *Journal of Biomedical Materials Research A*, p. Accepted Article, Mar. 2013.
- [19] K. A. Woodhouse, P. Klement, V. Chen, M. B. Gorbet, F. W. Keeley, R. Stahl, J. D. Fromstein, and C. M. Bellingham, "Investigation of recombinant human elastin polypeptides as non-thrombogenic coatings," *Biomaterials*, vol. 25, no. 19, pp. 4543– 4553, Aug. 2004.
- [20] R. W. Colman, A. W. Clowes, J. N. George, S. Z. Goldhaber, and V. J. Marder, "Chapter 1: Overview of hemostasis," in in *Hemostasis and Thrombosis*, 5th ed., R. W. Colman, A. W. Clowes, J. N. George, S. Z. Goldhaber, and V. J. Marder, Eds. Philadelphia: Lippincott Williams & Wilkins, 2006, pp. 3–16.
- [21] M. B. Gorbet and M. V Sefton, "Chapter 49: Biomaterial-associated thrombosis," in in *Hemostasis and Thrombosis*, 5th ed., R. Colman, A. Clowes, S. Goldhaber, V. Marder, and J. George, Eds. Philadelphia: Lippincott Williams & Wilkins, 2006, pp. 751–759.
- [22] Dictionary.com, "Thrombogenic," *The American Heritage Stedman's Medical Dictionary*, 2002. [Online]. Available: http://dictionary.reference.com/browse/thrombogenic. [Accessed: 11-Sep-2013].
- [23] G. Rollason and M. V Sefton, "Measurement of the rate of thrombin production in human plasma in contact with different materials," *Journal of Biomedical Materials Research*, vol. 26, no. 5, pp. 675–693, May 1992.
- [24] K. N. J. Stevens, S. Croes, R. S. Boersma, E. E. Stobberingh, C. van der Marel, F. H. van der Veen, M. L. W. Knetsch, and L. H. Koole, "Hydrophilic surface coatings with embedded biocidal silver nanoparticles and sodium heparin for central venous catheters," *Biomaterials*, vol. 32, no. 5, pp. 1264–1269, Feb. 2011.

- [25] C. G. Caro, N. J. Cheshire, and N. Watkins, "Preliminary comparative study of small amplitude helical and conventional ePTFE arteriovenous shunts in pigs," *Journal of the Royal Society Interface*, vol. 2, no. 3, pp. 261–266, Jun. 2005.
- [26] A. Andukuri, W. P. Minor, M. Kushwaha, J. M. Anderson, and H.-W. Jun, "Effect of endothelium mimicking self-assembled nanomatrices on cell adhesion and spreading of human endothelial cells and smooth muscle cells," *Nanomedicine: Nanotechnology, Biology, and Medicine*, vol. 6, pp. 289–297, Apr. 2010.
- [27] D. Li, H. Chen, and J. L. Brash, "Mimicking the fibrinolytic system on material surfaces," *Colloids and Surfaces B: Biointerfaces*, vol. 86, no. 1, pp. 1–6, Aug. 2011.
- [28] H. P. Wendel and G. Ziemer, "Coating-techniques to improve the hemocompatibility of artificial devices used for extracorporeal circulation," *European Journal of Cardio-Thoracic Surgery*, vol. 16, pp. 342–350, 1999.
- [29] J. L. Brash, "The fate of fibrinogen following adsorption at the blood-biomaterial interface," Annals of the New York Academy of Sciences, vol. 516, pp. 206–222, Jan. 1987.
- [30] D. R. Schmidt, H. Waldeck, and W. J. Kao, "Protein adsorption," in in *Biological Interactions on Materials Surfaces*, D. A. Puleo and R. Bizios, Eds. New York, NY: Springer US, 2009, pp. 1–18.
- [31] T. A. Horbett, "Mass action effects on competitive adsorption of fibrinogen from hemoglobin solutions and from plasma," *Thrombosis and Haemostasis*, vol. 51, pp. 174– 181, 1984.
- [32] N. Nath, J. Hyun, H. Ma, and A. Chilkoti, "Surface engineering strategies for control of protein and cell interactions," *Surface Science*, vol. 570, no. 1–2, pp. 98–110, Oct. 2004.
- [33] T. A. Horbett, "Principles underlying the role of adsorbed plasma proteins in blood interactions with foreign materials," *Cardiovascular Pathology*, vol. 2, no. 3, p. 137S– 148S, Jul. 1993.
- [34] D. L. Elbert and J. A. Hubbell, "Surface treatments of polymers for biocompatibility," *Annual Review of Materials Science*, vol. 26, pp. 365–394, 1996.
- [35] M. Rabe, D. Verdes, and S. Seeger, "Understanding protein adsorption phenomena at solid surfaces," *Advances in Colloid and Interface Science*, vol. 162, no. 1–2, pp. 87–106, Mar. 2011.
- [36] H. Nygren, M. Stenberg, and C. Karlsson, "Kinetics supramolecular structure and equilibrium properties of fibrinogen adsorption at liquid-solid interfaces," *Journal of Biomedical Materials and Research*, vol. 26, no. 1, pp. 77–91, Jan. 1992.
- [37] L. Vroman, A. L. Adams, G. C. Fischer, and P. C. Munoz, "Interaction of high molecular weight kininogen, factor XII, and fibrinogen in plasma at interfaces," *Blood*, vol. 55, pp. 156–159, 1980.

- [38] J. D. Andrade and V. Hlady, "Protein adsorption and materials biocompatibility : A tutorial review and suggested hypotheses," *Advances in Polymer Science*, vol. 79, 1986.
- [39] V. Chen, "Human elastin peptide coated synthetic materials in blood contacting appliations," 1999.
- [40] J. D, "Coagulation," *Wikimedia Commons, CC-BY-SA-3.0 License*. [Online]. Available: http://commons.wikimedia.org/wiki/File:Coagulation_full.svg. [Accessed: 28-Aug-2013].
- [41] A. H. Schmaier, "Contact activation: a revision.," *Thrombosis and Haemostasis*, vol. 78, no. 1, pp. 101–107, 1997.
- [42] K. W. H. J. van der Kamp, K. D. Hauch, J. Feijen, and T. A. Horbett, "Contact activation during incubation of five different polyurethanes or glass in plasma," *Journal of Biomedical Materials Research*, vol. 29, pp. 1303–1306, 1995.
- [43] J. Hong, K. Nilsson Ekdahl, H. Reynolds, R. Larsson, and B. Nilsson, "A new in vitro model to study interaction between whole blood and biomaterials. Studies of platelet and coagulation activation and the effect of aspirin," *Biomaterials*, vol. 20, no. 7, pp. 603–611, Apr. 1999.
- [44] J. F. Burman, H. I. Chung, D. A. Lane, H. Philippou, A. Adami, and J. C. R. Lincoln, "Role of factor XII in thrombin generation and fibrinolysis during cardiopulmonary bypass," *Lancet*, vol. 344, pp. 1192–11933, Oct. 1994.
- [45] M. D. Rand, J. B. Lock, C. van't Veer, D. P. Gaffney, and K. G. Mann, "Blood clotting in minimally altered whole blood," *Blood*, vol. 88, no. 9, pp. 3432–3445, Nov. 1996.
- [46] D. Basmadjian, M. V Sefton, and S. A. Baldwin, "Coagulation on biomaterials in flowing blood: some theoretical considerations," *Biomaterials*, vol. 18, pp. 1511–1522, Dec. 1997.
- [47] Jfdwolff, "Fibrinolysis," *Wikimedia Commons, CC-BY-SA-3.0 License*. [Online]. Available: http://en.wikipedia.org/wiki/File:Fibrinolysis.png. [Accessed: 28-Aug-2013].
- [48] W. F. Mangel, B. Lin, and V. Ramakrishan, "Characterization of an extremely large, ligand-induced conformational change in plasminogen," *Science*, vol. 248, pp. 69–73, 1990.
- [49] E. Haber, T. Quertermous, G. R. Matsueda, and M. S. Runge, "Innovative approaches to plasminogen activator therapy," *Science*, vol. 243, pp. 51–56, Jan. 1989.
- [50] M. Mayer, "Biochemical and biological aspects of the plasminogen activation system.," *Clinical biochemistry*, vol. 23, no. 3, pp. 197–211, Jun. 1990.
- [51] J. M. Grunkemeier, W. B. Tsai, and T. A. Horbett, "Hemocompatibility of treated polystyrene substrates: contact activation, platelet adhesion, and procoagulant activity of adherent platelets," *Journal of Biomedical Materials Research*, vol. 41, no. 4, pp. 657–70, Sep. 1998.

- [52] R. Nieuwland, R. J. Berckmans, R. C. Rotteveel-Eijkman, K. N. Maquelin, K. J. Roozendaal, P. G. M. Jansen, K. ten Have, L. Eijsman, C. E. Hack, and A. Sturk, "Cellderived microparticles generated in patients during cardiopulmonary bypass are highly procoagulant," *Circulation*, vol. 96, no. 10, pp. 3534–3541, 1997.
- [53] J. I. Sheppard, W. G. Mcclung, and I. A. Feuerstein, "Adherent platelet morpholgy on adsorbed fibrinogen: effects of protein in cubation time and albumin addition," *Journal of Biomedical Materials Research*, vol. 28, pp. 1175–1186, 1994.
- [54] W.-B. Tsai, J. M. Grunkemeier, C. D. Mcfarland, and T. A. Horbett, "Platelet adhesion to polystyrene-based surfaces preadsorbed with plasmas selectively depleted in fibrinogen, fibronectin, vitronectin, or von Willebrand's factor," *Journal of Biomedical Materials Research A*, vol. 60, pp. 348–359, 2002.
- [55] T. M. Massa, W. G. Mcclung, M. L. Yang, J. Y. C. Ho, J. L. Brash, and J. P. Santerre, "Fibrinogen adsorption and platelet lysis characterization of fluorinated surface-modified polyetherurethanes," *Journal of Biomedical Materials Research Part A*, vol. 81A, no. 1, pp. 178–185, 2006.
- [56] S. E. D'Souza, M. H. Ginsberg, T. A. Burke, S. C. Lam, and E. F. Plow, "Localization of an Arg-Gly-Asp recognition site within an integrin adhesion receptor.," *Science*, vol. 242, no. 4875, pp. 91–3, Oct. 1988.
- [57] S. E. D'Souza, M. H. Ginsberg, T. A. Burke, and E. F. Plow, "The ligand binding site of the platelet integrin receptor GPIIb-IIIa is proximal to the second calcium binding domain of its α subunit," *Journal of Biological Chemistry*, vol. 265, pp. 3440–3446, 1990.
- [58] S. N. Rodrigues, I. C. Gonçalves, M. C. L. Martins, M. A. Barbosa, and B. D. Ratner, "Fibrinogen adsorption, platelet adhesion and activation on mixed hydroxyl-/methylterminated self-assembled monolayers," *Biomaterials*, vol. 27, no. 31, pp. 5357–5367, Nov. 2006.
- [59] M. J. Maxwell, E. Westein, W. S. Nesbitt, S. Giuliano, S. M. Dopheide, and S. P. Jackson, "Identification of a 2-stage platelet aggregation process mediating shear-dependent thrombus formation," *Blood*, vol. 109, no. 2, pp. 566–576, Jan. 2007.
- [60] C. Eriksson and H. Nygren, "Polymorphonuclear leukocytes in coagulating whole blood recognize hydrophilic and hydrophobic titanium surfaces by different adhesion receptors and show different patterns of receptor expression," *The Journal of Laboratory and Clinical Medicine*, vol. 137, no. 4, pp. 296–302, Apr. 2001.
- [61] E. I. Peerschke and B. Ghebrehiwet, "Platelet receptors for the complement component C1q: implications for hemostasis and thrombosis," *Immunobiology*, vol. 199, no. 2, pp. 239–249, Aug. 1998.
- [62] P. J. Sims and T. Wiedmer, "Induction of cellular procoagulant activity by the membrane attack complex of complement," *Seminars in Cell Biology*, vol. 6, no. 5, pp. 275–282, Oct. 1995.

- [63] M. H. Ross and P. Wojciech, "Chapter 10: Blood," in in *Histology*, 5th ed., Philadelphia: Lippincott Williams & Wilkins, 2006, pp. 247–279.
- [64] A. J. Takala, I. T. Jousela, O. S. Takkunen, S.-E. R. Jansson, K. T. Kyosola, K. T. Olkkola, M. Leirisalo-Repo, and H. Repo, "Time of of β2-integrin CD11b/CD18 (Mac-1, αMβ2) upregulation on neutrophils and monocytes after coronary artery bypass grafting," *Scandinavian Journal of Cardiovascular Surgery*, vol. 30, pp. 141–148, 1996.
- [65] T. Inoue, Y. Sakai, S. Morooka, T. Hayashi, K. Takayanagi, and Y. Takabatake, "Expression of polymorphonuclear leukocyte adhesion molecules and its clinical significance in patients treated with percutaneous transluminal coronary angioplasty," *Journal of the American College of Cardiology*, vol. 28, no. 5, pp. 1127–1133, Nov. 1996.
- [66] K. von Appen, C. Goolsby, P. Mehl, R. Goewert, and P. Ivanovich, "Leukocyte adhesion molecules as biocompatibility markers for hemodialysis membranes," *American Society for Artificial Internal Organs*, vol. 40, pp. M609–M615, 1994.
- [67] D. Cameron, "Initiation of white cell activation during cardiopulmonary bypass: cytokines and receptors," *Journal of Cardiovascular Pharmacology*, vol. 27, pp. 1–5, 1996.
- [68] G. Asimakopoulos and K. M. Taylor, "Effects of cardiopulmonary bypass on leukocyte and endothelial adhesion molecules," *The Annals of Thoracic Surgery*, vol. 66, pp. 2135–2144, Dec. 1998.
- [69] C. R. Wilhelm, J. Ristich, R. L. Kormos, and W. R. Wagner, "Monocyte tissue factor expression and ongoing complement generation in ventricular assist device patients," *The Annals of Thoracic Surgery*, vol. 65, no. 4, pp. 1071–1076, Apr. 1998.
- [70] K.-E. Eilertsen and B. Østerud, "Tissue factor: (patho)physiology and cellular biology," Blood Coagulation & Fibrinolysis, vol. 15, no. 7, pp. 521–38, Oct. 2004.
- [71] E. M. Egorina, M. A. Sovershaev, J. O. Olsen, and B. Østerud, "Granulocytes do not express but acquire monocyte-derived tissue factor in whole blood: evidence for a direct transfer," *Blood*, vol. 111, no. 3, pp. 1208–1216, Feb. 2008.
- [72] M. B. Gorbet and M. V Sefton, "Material-induced tissue factor expression but not CD11b upregulation depends on the presence of platelets," *Journal of Biomedical Materials Research A*, vol. 67A, no. 3, pp. 792–800, Dec. 2003.
- [73] M. Haag-Weber, P. Schollmeyer, and W. H. Hörl, "β-2-microglobulin and main granulocyte components in hemodialysis patients," *Artificial Organs*, vol. 13, no. 2, pp. 92–96, Apr. 1989.
- [74] B. G. Stegmayr, K. Esbensen, A. Gutierrez, L. Lundberg, B. Nielsen, C. E. Stroemsaeter, and B. Wehle, "Granulocyte elastase, β-thromboglobulin, and C3d during acetate or bicarbonate hemodialysis with Hemophan compared to a cellulose acetate membrane," *The international journal of Artificial Organs*, vol. 15, no. 1, pp. 10–18, 1992.

- [75] J. A. Smith, "Neutrophils, host defense, and inflammation: a double-edged sword," *Journal of Leukocyte Biology*, vol. 56, pp. 672–686, 1994.
- [76] K. Sutherland, J. R. Mahoney, A. J. Coury, and J. W. Eaton, "Degradation of biomaterials by phagocyte-derived oxidants," *The Journal of Clinical Investigation*, vol. 92, no. 5, pp. 2360–2367, Nov. 1993.
- [77] S. A. M. Ali, S.-P. Zhong, P. J. Doherty, and D. F. Williams, "Mechanisms of polymer degradation in implantable devices," *Biomaterials*, vol. 14, no. 9, pp. 648–656, 1993.
- [78] M. B. Gorbet and M. V Sefton, "Leukocyte activation and leukocyte procoagulant activities after blood contact with polystyrene and polyethylene glycol-immobilized polystyrene beads," *The Journal of Laboratory and Clinical Medicine*, vol. 137, no. 5, pp. 345–355, May 2001.
- [79] T. Gourlay, D. C. Stefanou, G. Asimakopoulos, and K. M. Taylor, "The effect of circuit surface area on CD11b(mac-1) expression in a rat recirculation model," *Artificial Organs*, vol. 25, no. 6, pp. 475–479, Jun. 2001.
- [80] M. B. Gorbet, E. L. Yeo, and M. V Sefton, "Flow cytometric study of in vitro neutrophil activation by biomaterials," *Journal of Biomedical Materials Research*, vol. 44, no. 3, pp. 289–297, Mar. 1999.
- [81] G. Nimeri, L. Ohman, H. Elwing, J. Wetterö, and T. Bengtsson, "The influence of plasma proteins and platelets on oxygen radical production and F-actin distribution in neutrophils adhering to polymer surfaces," *Biomaterials*, vol. 23, no. 8, pp. 1785–1795, Apr. 2002.
- [82] S. Jaisson, S. Lorimier, S. Ricard-Blum, G. D. Sockalingum, C. Delevallée-Forte, G. Kegelaer, M. Manfait, R. Garnotel, and P. Gillery, "Impact of carbamylation on type I collagen conformational structure and its ability to activate human polymorphonuclear neutrophils," *Chemistry & Biology*, vol. 13, no. 2, pp. 149–159, Feb. 2006.
- [83] W. G. Brodbeck, G. Voskerician, N. P. Ziats, Y. Nakayama, T. Matsuda, and J. M. Anderson, "In vivo leukocyte cytokine mRNA responses to biomaterials are dependent on surface chemistry," *Journal of Biomedical Materials Research A*, vol. 64, no. 2, pp. 320– 9, Feb. 2003.
- [84] C. Weber and T. A. Springer, "Neutrophil accumulation on activated, surface-adherent platelets in flow is mediated by interaction of Mac-1 with fibrinogen bound to αIIbβ3 and stimulated by platelet-activating factor," *The Journal of Clinical Investigation*, vol. 100, no. 8, pp. 2085–2093, 1997.
- [85] W. J. Kao, "Evaluation of leukocyte adhesion on polyurethanes: the effects of shear stress and blood proteins," *Biomaterials*, vol. 21, pp. 2295–2303, 2000.
- [86] N. A. Riedel, B. S. Smith, J. D. Williams, and K. C. Popat, "Improved thrombogenicity on oxygen etched Ti6Al4V surfaces," *Materials Science and Engineering C*, vol. 32, no. 5, pp. 1196–1203, Jul. 2012.

- [87] A. Remes and D. F. Williams, "Immune response in biocompatibility," *Biomaterials*, vol. 13, no. 11, pp. 731–743, Jan. 1992.
- [88] M. Józsi, "Anti-complement autoantibodies in membranoproliferative glomerulonephritis and dense deposit disease," in in An Update on Glomerulopathies - Etiology and Pathogenesis, S. Prabhakar, Ed. 2011, pp. 31–46.
- [89] M. Tulunay, S. Demiralp, S. Tastan, H. Akalin, U. Ozyurda, T. Corapcioglu, and E. S. Akarsu, "Complement (C3, C4) and C-reactive protein responses to cardiopulmonary bypass and protamine administration," *Anaesthesia and Intensive Care*, vol. 21, no. 1, pp. 50–55, 1993.
- [90] K. Lhotta, R. Würzner, F. Kronenberg, M. Oppermann, and P. König, "Rapid activation of the complement system by cuprophane depends on complement component C4," *Kidney International*, vol. 53, no. 4, pp. 1044–1051, Apr. 1998.
- [91] M. Kazatchkine and M. Carreno, "Activation of the complement system at the interface between blood and artificial surfaces," *Biomaterials*, vol. 9, pp. 30–35, 1988.
- [92] P. M. Jansen, B. Eisele, I. W. de Jong, A. Chang, U. Delvos, F. B. Taylor, and C. E. Hack, "Effect of C1 inhibitor on inflammatory and physiologic response patterns in primates suffering from lethal septic shock," *Journal of iImunology*, vol. 160, no. 1, pp. 475–484, Jan. 1998.
- [93] J. Hirsch and J. I. Weitz, "Antithrombin therapy," in in *Acute Myocardial infarction and Other Acute Ischemic Syndromes*, R. Califf, Ed. St. Louis: Mosby, 1996.
- [94] L. Lam, J. E. Silber, and R. D. Rosendberg, "The separation of active and inactive forms of heparin," *Biochemical and Biophysical Research Communications*, vol. 69, no. 2, 1976.
- [95] R. E. Edens, R. J. Linhardt, C. S. Bell, and J. M. Weiler, "Heparin and derivatized heparin inhibit zymosan and cobra venom factor activation of complement in serum," *Immunopharmacology*, vol. 27, no. 2, pp. 145–53, 1994.
- [96] R. Bach and D. B. Rifkin, "Expression of tissue factor procoagulant activity: regulation by cytosolic calcium," *Proceedings of the National Academy of Sciences of the United States* of America, vol. 87, pp. 6995–6999, Sep. 1990.
- [97] S. Bournazos, J. Rennie, S. P. Hart, and I. Dransfield, "Choice of anticoagulant critically affects measurement of circulating platelet-leukocyte complexes," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 28, pp. e2–e3, Jan. 2008.
- [98] H. M. O. Straaten, J. A. Kellum, and R. Bellomo, "Clinical review : Anticoagulation for continuous renal replacement therapy - heparin or citrate ?," *Critical Care Medicine*, pp. 1–9, 2011.
- [99] S. M. McCormick, S. G. Eskin, L. V McIntire, C. L. Teng, C. M. Lu, C. G. Russell, and K. K. Chittur, "DNA microarray reveals changes in gene expression of shear stressed

human umbilical vein endothelial cells," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 98, no. 16, pp. 8955–8960, Jul. 2001.

- [100] Y. Feng, J.-H. Yang, H. Huang, S. P. Kennedy, T. G. Turi, J. F. Thompson, P. Libby, and R. T. Lee, "Transcriptional profile of mechanically induced genes in human vascular smooth muscle cells," *Circulation Research*, vol. 85, no. 12, pp. 1118–1123, Dec. 1999.
- [101] L. Waite and J. Fine, "Review of basic fluid mechanics concepts," in in Applied Biofluid Mechanics, McGraw-Hill, 2007, pp. 1–33.
- [102] J. J. Hathcock, "Flow effects on coagulation and thrombosis," *Arteriosclerosis, Thrombosis, and Vascular bBology*, vol. 26, no. 8, pp. 1729–1737, Aug. 2006.
- [103] E. W. Merrill, "Rheology of blood," *Physiological Reviews*, vol. 49, no. 4, pp. 863–888, 1969.
- [104] H. Gray, "Chapter 5: Blood, lymphoid tissues and haemopoiesis," in in *Gray's Anatomy: The Anatomical Basis of Clinical Practice*, 39th ed., S. Standring, Ed. Elsevier Science Health Science Division, 2005, pp. 69–233.
- [105] E. W. Merrill, G. C. Cokelet, A. Britten, and R. E. Wells, "Non-newtonian rheology of human blood- effect of fibrinogen deduced by 'subtraction'," *Circulation Research*, vol. 13, no. 1, pp. 48–55, Jul. 1963.
- [106] H. Schmid-Schönbein, P. Gaehtgens, and H. Hirsch, "On the shear rate dependence of red cell aggregation in vitro," *The Journal of Clinical Investigation*, vol. 47, no. 6, pp. 1447– 1454, Jun. 1968.
- [107] R. Fahraeus and T. Lindqvist, "The viscosity of the blood in narrow capillary tubes," *American Journal of Physiology*, vol. 96, no. 3, pp. 562–568, 1931.
- [108] W. S. Uijttewaal, E.-J. Nijhof, P. Bronkhorst, E. Den Hartog, and R. M. Heethaar, "Nearwall excess of platelets induced by lateral migration of erythrocytes in flowing blood," *American Journal of Physiology*, vol. 1, no. 15, pp. H1239–H1244, 1993.
- [109] S. Eskin and L. McIntire, "Chapter 48: Rheology of thrombosis," in in *Hemostasis and Thrombosis*, 5th ed., R. Colman, A. Clowes, S. Goldhaber, V. Marder, and J. George, Eds. Philadelphia: Lippincott Williams & Wilkins, 2006, pp. 737–750.
- [110] J. R. O'Brien, "Shear-induced platelet aggregation," *The Lancet*, vol. 335, pp. 711–713, 1990.
- [111] V. T. Turitto, H. J. Weiss, and H. R. Baumgartner, "The effect of shear rate on platelet interaction with subendothelium exposed to citrated human blood," *Microvascular Research*, vol. 19, no. 3, pp. 352–65, May 1980.
- [112] P. H. M. Kuijper, H. I. Gallardo Torres, J. A. M. van der Linden, J. W. Lammers, J. J. Sixma, L. Koenderman, and J. J. Zwaginga, "Platelet-dependent primary hemostasis

promotes selectin- and integrin- mediated neutrophil adhesion to damaged endothelium under flow conditions," *Blood*, vol. 87, pp. 3271–3281, 1996.

- [113] E. L. Yeo, J.-A. I. Sheppard, and I. A. Feuerstein, "Role of P-selectin and leukocyte activation in polymorphonuclear cell adhesion to surface adherent activated platelets under physiologic shear conditions (an injury vessel wall model)," *Blood*, vol. 83, no. 9, pp. 2498–2507, 1994.
- [114] P. H. M. Kuijper, H. I. Gallardo Torres, J. A. M. van der Linden, J.-W. J. Lammers, J. J. Sixma, J. J. Zwaginga, and L. Koenderman, "Neutrophil adhesion to fibrinogen and fibrin under flow conditions is diminished by activation and L-selectin shedding," *Blood*, vol. 89, pp. 2131–2138, 1997.
- [115] C. H. Gemmell, Y. Nemerson, and V. Turito, "The effects of shear rate on the enzymatic activity tissue factor-factor Vlla Complex," *Microvascular Research*, vol. 40, pp. 327– 340, 1990.
- [116] R. S. Higginbotham, "A cone and plate viscometer," *Journal of Scientific Instruments*, vol. 27, no. 5, pp. 139–141, May 1950.
- [117] X. Wang, F. L. Liao, and J. F. Stoltz, "A new simple cone-plate viscometer for hemorheology," *Clinical Hemorheology and Microcirculation*, vol. 19, no. 1, pp. 25–31, Sep. 1998.
- [118] M. Mooney and R. H. Ewart, "The conicylindrical viscometer," *Physics*, vol. 5, no. 11, pp. 350–354, 1934.
- [119] R. Mckennell, "Cone-Plate Viscometer," Analytical Chemistry, vol. 28, no. 11, pp. 1710– 1714, 1956.
- [120] G. A. Skarja, R. L. Kinlough-Rathbone, D. W. Perry, F. D. Rubens, and J. L. Brash, "A cone-and-plate device for the investigation of platelet biomaterial interactions," *Journal of Biomedical Materials Research*, vol. 34, no. 4, pp. 427–438, Mar. 1997.
- [121] D. Cox, "Radial flow in the cone-plate viscometer," Nature, vol. 193, p. 670, 1962.
- [122] E. F. Grabowski, L. I. Friedman, and E. F. Leonard, "Effects of shear rate on the diffusion and adhesion of blood platelets to a foreign surface," *Industrial & Engineering Chemistry Fundamentals*, vol. 11, no. 2, pp. 224–232, May 1972.
- [123] P. A. Holme, U. Orvim, M. J. A. G. Hamers, N. O. Solum, F. R. Brosstad, R. M. Barstad, and K. S. Sakariassen, "Shear-induced platelet activation and platelet microparticle formation at blood flow conditions as in arteries with a severe stenosis," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 17, no. 4, pp. 646–653, Apr. 1997.
- [124] H. M. Kagan and K. A. Sullivan, "Lysyl oxidase: preparation and role in elastin biosynthesis," *Methods in Enzymology*, vol. 82, pp. 637–650, 1982.

- [125] S. G. Wise and A. S. Weiss, "Tropoelastin," *The International Journal of Biochemistry & Cell Biology*, vol. 41, no. 3, pp. 494–497, Mar. 2009.
- [126] Gray, "Chapter 7: Smooth muscle and the cardiovascular and lymphatic systems," in in *Gray's Anatomy: The Anatomical Basis of Clinical Practice*, 39th ed., S. Standring, Ed. Elsevier Science Health Science Division, 2005, pp. 137–155.
- [127] J. E. Wagenseil and R. P. Mecham, "Vascular extracellular matrix and arterial mechanics," *Physiological Reviews*, vol. 89, pp. 957–989, 2009.
- [128] J. M. Kabemba, J. E. Mayer, and G. L. Hamond, "Experimental arterial thrombosis formation in vivo by proteolytic enzyme perfusion and the role of elastin layer," *Surgery*, vol. 73, no. 3, 1973.
- [129] S. K. Karnik, B. S. Brooke, A. Bayes-Genis, L. Sorenson, J. D. Wythe, R. S. Schwartz, M. T. Keating, and D. Y. Li, "A critical role for elastin signaling in vascular morphogenesis and disease," *Development*, vol. 130, no. 2, pp. 411–423, Jan. 2003.
- [130] D. T. Simionescu, Q. Lu, Y. Song, J. S. Lee, T. N. Rosenbalm, C. Kelley, and N. R. Vyavahare, "Biocompatibility and remodeling potential of pure arterial elastin and collagen scaffolds," *Biomaterials*, vol. 27, no. 5, pp. 702–713, Feb. 2006.
- [131] M. T. Hinds, R. C. Rowe, Z. Ren, J. Teach, P.-C. Wu, S. J. Kirkpatrick, K. D. Breneman, K. W. Gregory, and D. W. Courtman, "Development of a reinforced porcine elastin composite vascular scaffold," *Journal of Biomedical Materials and Research A*, vol. 77A, no. 3, pp. 458–469, 2006.
- [132] D. Bax, U. R. Rodgers, M. M. M. Bilek, and A. S. Weiss, "Cell adhesion to tropoelastin is mediated via the C-terminal GRKRK motif and integrin αVβ3," *The Journal of Biological Chemistry*, vol. 284, no. 42, pp. 28616–28623, Oct. 2009.
- [133] R. G. Lee and S. W. Kim, "The role of carbohydrate in platelet adhesion to foreign surfaces," *Journal of Biomedical Materials Research*, vol. 8, no. 6, pp. 393–398, Nov. 1974.
- [134] W. F. Daamen, J. H. Veerkamp, J. C. M. van Hest, and T. H. van Kuppevelt, "Elastin as a biomaterial for tissue engineering," *Biomaterials*, vol. 28, pp. 4378–4398, Oct. 2007.
- [135] Y. Seyama and H. Wachi, "Atherosclerosis and matrix dystrophy," *Journal of Atherosclerosis and Thrombosis*, vol. 11, no. 5, pp. 236–245, 2004.
- [136] R. E. Sallach, W. Cui, F. Balderrama, A. W. Martinez, J. Wen, C. A. Haller, J. V Taylor, E. R. Wright, R. C. Long, and E. L. Chaikof, "Long-term biostability of self-assembling protein polymers in the absence of covalent crosslinking," *Biomaterials*, vol. 31, no. 4, pp. 779–91, Feb. 2010.
- [137] K. M. Defife, K. M. Hagen, D. L. Clapper, and J. M. Anderson, "Photochemically immobilized polymer coatings: effects on protein adsorption, cell adhesion, and leukocyte

activation," *Journal of Biomaterial Science - Polymer Edition*, vol. 10, no. 10, pp. 1063–1074, 1999.

- [138] S. W. Jordan, C. A. Haller, R. E. Sallach, R. P. Apkarian, S. R. Hanson, and E. L. Chaikof, "The effect of a recombinant elastin-mimetic coating of an ePTFE prosthesis on acute thrombogenicity in a baboon arteriovenous shunt," *Biomaterials*, vol. 28, no. 6, pp. 1191–1197, Feb. 2007.
- [139] C. M. Bellingham, K. A. Woodhouse, P. Robson, S. J. Rothstein, and F. W. Keeley, "Selfaggregation characteristics of recombinantly expressed human elastin polypeptides," *Biochimica et Biophysica Acta*, vol. 1550, pp. 6–19, Nov. 2001.
- [140] P. D. Yurchenco, D. E. Birk, and R. P. Mecham, Eds., *Extracellular Matrix Assembly and Structure*. 1994, pp. 281–314.
- [141] C. M. Bellingham and F. W. Keeley, "Self-ordered polymerization of elastin-based biomaterials," *Current Opinion in Solid State and Materials Science*, vol. 8, no. 2, pp. 135–139, Mar. 2004.
- [142] C. M. Bellingham, M. A. Lillie, J. M. Gosline, G. M. Wright, B. C. Starcher, A. J. Bailey, K. A. Woodhouse, and F. W. Keeley, "Recombinant human elastin polypeptides selfassemble into biomaterials with elastin-like properties," *Biopolymers*, vol. 70, pp. 445– 455, 2003.
- [143] M.-C. Belanger and Y. Marois, "Hemocompatibility, biocompatibility, inflammatory and in vivo studies of primanry reference materials low-density polyethylene and polydiemthylsiloxane: a review," *Journal of Biomedical Materials and Research*, vol. 58, pp. 467–477, 2001.
- [144] W. A. Lynn and D. T. Golenbock, "Lipopolysaccharide antagonists," *Immunology Today*, vol. 13, no. 7, pp. 271–276, Jul. 1992.
- [145] FDA, "Guideline on validation of the limulus amebocyte lysate test as an end-product endotoxin test for human and animal parental drugs, biological products and medical devices," 1987. .
- [146] L. E. Flynn, "The use of decellularized adipose tissue to provide an inductive microenvironment for the adipogenic differentiation of human adipose-derived stem cells," *Biomaterials*, vol. 31, no. 17, pp. 4715–24, Jun. 2010.
- [147] D. F. Bainton, L. J. Miller, T. K. Kishimoto, and T. A. Springer, "Leukocyte adhesion receptors are stored in peroxidase-negative granules of human neutrophils," *Journal of Experimental Medicine*, vol. 166, pp. 1641–1653, 1987.
- [148] E. M. Srokowski and K. A. Woodhouse, "Surface and adsorption characteristics of three elastin-like polypeptide coatings with varying sequence lengths," *Journal of Materials Science: Materials in Medicine*, vol. 24, no. 1, pp. 71–84, Jan. 2013.

- [149] D. Zhong, Y. Jiao, Y. Zhang, W. Zhang, N. Li, Q. Zuo, Q. Wang, W. Xue, and Z. Liu, "Effects of the gene carrier polyethyleneimines on structure and function of blood components," *Biomaterials*, vol. 34, no. 1, pp. 294–305, Jan. 2013.
- [150] X. Chang and M. Gorbet, "The effect of shear on in vitro platelet and leukocyte materialinduced activation," *Journal of Biomaterials Applications*, vol. 0, no. 0, pp. 1–9, Jul. 2012.
- [151] S. T. Fan and T. S. Edgington, "Coupling of the adhesive receptor CD11b/CD18 to functional enhancement of effector macrophage tissue factor response," *The Journal of Clinical Investigation*, vol. 87, no. 1, pp. 50–57, Jan. 1991.
- [152] H. Repo, S.-E. Jansson, and M. Leirisalo-Repo, "Anticoagulant selection influences flow cytometric determination of CD1 lb upregulation in vivo and ex vivo," *Journal of Immunological Methods*, vol. 185, pp. 65–79, 1995.
- [153] M. H. El Habbal, L. Smith, M. J. Elliot, and S. Strobel, "Effect of heparin anticoagulation on neutrophil adhesion molecules and release of IL8: C3 is not essential," *Cardiovascular Research*, vol. 30, pp. 676–681, 1995.
- [154] A. Celi, G. Pellegrini, R. Lorenzet, A. De Blasi, N. Ready, B. C. Furie, and B. Furie, "P-selectin induces the expression of tissue factor on monocytes," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 91, no. 19, pp. 8767–8771, Sep. 1994.
- [155] D. E. Lorant, M. K. Topham, R. E. Whatley, R. P. McEver, T. M. McIntyre, S. M. Prescott, and G. A. Zimmerman, "Inflammatory roles of P-selectin," *The Journal of Clinical Investigation*, vol. 92, no. 2, pp. 559–570, Aug. 1993.
- [156] N. Semeraro, A. Biondi, R. Lorenzet, D. Locati, A. Mantovani, and M. B. Donati, "Direct induction of tissue factor synthesis by endotoxin in human macrophages from diverse anatomical sites," *Immunology*, vol. 50, pp. 529–535, Dec. 1983.
- [157] B. P. Morgan, "Clinical complementology: recent progress and future trends," *European Journal of Clinical Investigation*, vol. 24, no. 4, pp. 219–228, Apr. 1994.
- [158] M. B. Gorbet and M. V Sefton, "Complement inhibition reduces material-induced leukocyte activation with PEG modified polystyrene beads (Tentagel) but not polystyrene beads," *Journal of Biomedical Materials Research A*, vol. 74A, no. 4, pp. 511–522, Sep. 2005.
- [159] B. A. Lwaleed and P. S. Bass, "Tissue factor pathway inhibitor: structure, biology and involvement in disease," *The Journal of Pathology*, vol. 208, no. 3, pp. 327–339, Feb. 2006.
- [160] F. Tedesco, F. Fischetti, M. Pausa, A. Dobrina, R. B. Sim, and M. R. Daha, "Complementendothelial cell interactions: pathophysiological implications," *Molecular Immunology*, vol. 36, pp. 26–268, Jan. 1999.

- [161] A. R. Whitney, M. Diehn, S. J. Popper, A. A. Alizadeh, J. C. Boldrick, D. A. Relman, and P. O. Brown, "Individuality and variation in gene expression patterns in human blood," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 100, no. 4, pp. 1896–901, Feb. 2003.
- [162] S. Muto, V. Větvicka, and G. D. Ross, "CR3 (CD11b/CD18) expressed by cytotoxic T cells and natural killer cells is upregulated in a manner similar to neutrophil CR3 following stimulation with various activating agents," *Journal of Clinical Immunology*, vol. 13, no. 3, pp. 175–184, May 1993.
- [163] N. Li, Q. Ji, and P. Hjemdahl, "Platelet-lymphocyte conjugation differs between lymphocyte subpopulations," *Journal of Thrombosis and Haemostasis*, vol. 4, no. 4, pp. 874–881, Apr. 2006.
- [164] A. Waterhouse, Y. Yin, S. G. Wise, D. V Bax, D. R. McKenzie, M. M. M. Bilek, A. S. Weiss, and M. K. C. Ng, "The immobilization of recombinant human tropoelastin on metals using a plasma-activated coating to improve the biocompatibility of coronary stents," *Biomaterials*, vol. 31, no. 32, pp. 8332–8340, Nov. 2010.

Appendix: Research Ethics Board Approval



QUEEN'S UNIVERSITY HEALTH SCIENCES AND AFFILIATED TEACHING HOSPITALS ANNUAL RENEWAL

Queen's University, in accordance with the "Tri-Council Policy Statement, 1998" prepared by the Medical Research Council, Natural Sciences and Engineering Research Council of Canada and Social Sciences and Humanities Research Council of Canada requires that research projects involving human subjects be reviewed annually to determine their acceptability on ethical grounds.

A Research Ethics Board composed of:

Dr. A.F. Clark, Emeritus Professor, Department of Biochemistry, Faculty of Health Sciences, Queen's University (Chair) Dr. H. Abdollah, Professor, Department of Medicine, Queen's University Dr. C. Cline, Assistant Professor, Department of Medicine, Director, Office of Bioethics, Queen's University, Clinical Ethicist, Kingston General Hospital Dr. R. Brison, Professor, Department of Emergency Medicine, Queen's University Dr. M. Evans, Community Member Dr. S. Horgan, Manager, Program Evaluation & Health Services Development, Geriatric Psychiatry Service, Providence Care, Mental Health Services Assistant Professor, Department of Psychiatry Ms. J. Hudacin, Community Member Dr. J. MacKenzie, Pediatric Geneticist, Department of Paediatrics, Queen's University Mr. D. McNaughton, Community Member Ms. P. Newman, Pharmacist, Clinical Care Specialist and Clinical Lead, Quality and Safety, Pharmacy Services, Kingston General Hospital Ms. S. Rohland, Privacy Officer, ICES-Queen's Health Services Research Facility, Research Associate, Division of Cancer Care and Epidemiology, Queen's Cancer Research Institute Dr. B. Simchison, Assistant Professor, Department of Anaesthesiology and Perioperative Medicine, Queen's University Dr. A. Singh, Professor, Department of Psychiatry, Queen's University Dr. J. Tang, Medical Resident, Department of Emergency Medicine, Queen's University Ms. K. Weisbaum, LL.B. and Adjunct Instructor, Department of Family Medicine (Bioethics) has reviewed the request for renewal of Research Ethics Board approval for the project Elastin Like

Polypeptide Biomimetic Surfaces for Blood Contacting Devices as proposed by Dr. Kimberly Ann Woodhouse of the Department of Chemical Engineering, at Queen's University. The approval is renewed for one year, effective January 05, 2013. If there are any further amendments or changes to the protocol affecting the participants in this study, it is the responsibility of the principal investigator to notify the Research Ethics Board. Any unexpected serious adverse event occurring locally must be reported within 2 working days or earlier if required by the study sponsor. All other adverse events must be reported within 15 days after becoming aware of the information.

albert J. Clark.

Date: January 09, 2013 Chair, Research Ethics Board Renewal 1[] Renewal 2 [X] Extension [] Code# CHEM-003-11 Romeo file# 6005689