Shear Effect on Material Induced Blood Cell Activation

by

Xiaojian Chang

A thesis
presented to the University of Waterloo
in fulfilment of the
thesis requirement for the degree of
Master of Applied Science
in
Systems Design Engineering

Waterloo, Ontario, Canada, 2011

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

I understand that my thesis may be made electronically available to the public.

Abstract

Restenosis that occurs after stent implantation is a great threat to the quality of life of patients with coronary artery diseases. Studies on thrombosis have revealed that platelet is a primary component of blood clots, and leukocytes and other plasma proteins also contribute to thrombus formation. However, the mechanism of material-induced activation, especially with metallic materials, is currently not well understood and the effect of blood flow on material-induced blood cell activation has not been well characterized.

In vitro static and flow experiments were performed to assess the effect of flow on blood cell activation. Blood was taken from healthy donors. Two common stent metals, ST316L and TiAl6V4, were used as test materials. In static experiments, blood was incubated with metal disks at 37 ℃ for two hours. In flow experiments, blood was circulated in flow chambers preloaded with or without metal wires at shear rates of 100 s^{-1}, 500 s^{-1}, and 1500 s^{-1}. Platelet and leukocyte activation, leukocyte-platelet aggregation, and tissue factor expression on monocytes were measured by using a three-color FACSCalibur flow cytometer. Fibrin deposition and blood cell adhesion on metal surfaces were evaluated by scanning electron microscopy (SEM).

The results indicate that platelet and leukocyte activation under static condition was low. TiAl6V4 metal disk induced relative lower platelet and leukocyte activation, albeit no significant difference was found between control groups and the two metals tested. Shear stress significantly enhanced platelet activation as measured by an increase in platelet microparticle formation and platelet receptor CD61 expression. CD11b up-regulation, leukocyte-platelet aggregates, and tissue factor (TF) expression increased at 100 s^{-1}. However as shear rate increased, lower leukocyte activation was observed. TiAl6V4-induced leukocyte activation was generally lower than that of ST316L. Significant fibrin deposition and blood cell adhesion were observed on metal surfaces under static condition and with

shear rate of 500 s^{-1}. Adhesion significantly decreased with increasing shear rate to 1500 s^{-1}.

The flow cytometry analysis results and SEM images demonstrated that materials with different surface properties can induce different blood cell activation and adhesion after incubation with blood. While shear may have an effect on blood cell activation, the effect of biomaterials on cell activation under physiological shear conditions remains unclear. Further studies are required to determine if an increase in biomaterial surface area will provide some answers into the mechanisms of material-induced blood activation in the presence of flow.

Acknowledgements

It has been a wonderful experience at the University of Waterloo over the past two years and it is my pleasure to thank many people who made this thesis possible.

First of all, I would like to express sincere gratitude to my supervisor Dr. Maud Gorbet for giving me such a good opportunity to pursue my graduate studies in Systems Design Engineering, and sharing her extensive knowledge on biomaterials, flow models, statistics and much more.

I am especially grateful to Elizabeth Martell and Miriam Heynen for their great assistance in taking and donating blood. Many thanks also go to other blood donors. I could not have finished this study without your generous help.

I wish to thank my thesis readers Professor John Medley and Professor Jonathan Kofman for their valuable comments and suggestions. I also thank my fellow labmates in MIBS group: Tidi Gaamangwe, Laura Doody, Dan Cira, Sara Luck, and Sara Molladavoodi for the discussions and encouragement.

I would like to thank my entire family, especially my parents Chunhua Chang and Zhengyi Wang for their continued love and support throughout the years. Special thanks to my loving and supportive wife Zhao Ren.

This project is funded by NSERC Discovery grant.

Table of Contents

List of Tables

List of Figures

Abbreviations and Symbols

ADP	Adenosine diphosphate
ANOVA	Analysis of variance
BMS	Bare metal stent
CABG	Coronary artery bypass grafting
CAD	Coronary artery disease
CD	Cluster of differentiation
DES	Drug eluting stent
ESL-1	P-Selectin ligand-1
FDA	U.S. Food and drug administration
FITC	Fluorescein isothiocyanate
FSC	Forward scatter
g	Force of gravity
GP	Glycoprotein
HTB	HEPES-Tyrode's buffer
ICAM-1	Intracellular adhesion molecule-1
LDH	Lactate dehydrogenase
MFI	Mean fluorescence intensity

ml	Milliliter
mm	Millimeter
mM	Millimolar
μl	Microliter
μm	Micrometer
PBS	Phosphate buffered saline
PE	R-phycoerythrin
PGI2	Prostacyclin
PMA	Phorbol 12-myristate 13-acetate
PMP	Platelet microparticles
PRP	Platelet rich plasma
PSGL-1	P-Selectin glycoprotein ligand-1
CY5	Cytochrome 5
SD	Standard deviation
SEM	Scanning electron microscope
SSC	Side scatter
ST	Stainless steel
TF	Tissue factor
TXA2	Thromboxane A2
TNF	Tumor necrosis factor
VCAM-1	Vascular cell adhesion molecule-1
v/v	Volume/Volume
vWf	von Willebrand factor

Chapter 1

Introduction

1.1 Coronary Artery Disease and Stents

Coronary artery disease (CAD) is one of the leading causes of death worldwide. It occurs when the arteries that supply blood to the heart muscles become narrow. The inner walls of arteries are normally smooth and flexible, allowing blood to flow through them easily. However, fatty deposits or cholesterol plaques may build up on the inner walls of the arteries, and thus narrow the blood vessel [1]. As a consequence, blood supply to heart muscles reduces, resulting in the decrease of oxygen supply to the heart. About 90% Canadians have at least one risk factor for heart disease. Factors can be as common as smoking, drinking alcohol, physical inactivity, obesity, diabetes, high blood pressure, and high blood cholesterol. Although the rate of coronary artery disease has steadily declined over the past decade, it still accounts for 30% of death in Canada and costs tens of billions of dollars every year in physician services, hospital costs, and decreased productivity [2].

The selection of CAD treatment varies with different factors, such as the severity and

extent of CAD, overall heart function, and other medical conditions. For patients with early stage CAD that does not limit blood flow, medication and lifestyle changes can help to prevent progression. For patients with multiple areas of narrowed or blocked coronary arteries, coronary artery bypass grafting (CABG) is a common procedure. CABG creates a new path for blood to flow around the blockage in the stenotic arteries by grafting blood vessels from elsewhere in the body. In other cases, balloon angioplasty and stenting are applied. A balloon expands at the site of the stenotic artery to improve the blood flow, which is often followed by stent placement. Stents are small metal wire mesh tubes. They provide support to the artery wall and reduce the chance of blood vessel closing subsequently. The narrowing of a blood vessel after stenting is known as restenosis [1].

Bare metal stents (BMS) were first introduced in the mid 1980s. They gained wide clinical application throughout the 1990s. However, BMS were found to be a cause of stent restenosis [3]. This is because blood cells preferably adhere at the site of vascular injury induced during the stent placement, and smooth muscle cell proliferation results in thrombus formation in the stent. Drug-eluting stents (DES) preloaded with anti-proliferative or anti-inflammatory drugs were developed to solve the restenosis of BMS [4]. In 2007, cases of late thrombotic events and restenosis raised questions of the safety and efficacy of DES. Both BMS and DES are currently available on the market, but no significant differences in mortality rate at one year and beyond have been found [5]. Furthermore, the annual incidence of death, myocardial infarction, and repeated revascularization caused by stent implantation are more than 2% [6]. Late (between one month and one year) and very late (after one year) DES and BMS thrombotic complications remain a common issue.

1.2 Research Motivation and Objective

Researchers have noticed that some people get in-stent restenosis, while others receiving the same stent implantation do not. They have been trying to find out the roles of blood cells during contacting with stent materials and the mechanisms of blood cell activation, and to design new therapeutic approaches to prevent failures following stent implantation.

As the main component of thrombus, platelets are thought to be the primary factor that causes in-stent restenosis. However, blood-material contact actually involves the whole blood circulation system. As shown in Figure 1.1, thrombosis involves not only platelet activation, but also leukocyte activation, protein adsorption, and complement activation [7]. Moreover, shear conditions in blood vessels also take part in blood cell activation [8]. Each factor, but also their interactions between each other, can be important. Therefore, combined *in vitro* study of thrombogenic factors becomes necessary.

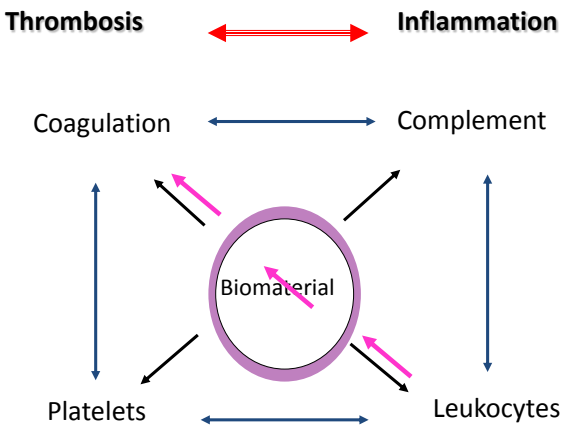


Figure 1.1: The interaction between blood cells and biomaterials [7].

Stent material also plays an important role in the blood cell activation which is generally characterized by events, such as an increase or decrease the number of membrane receptors, the secretion of microparticles, and the formation of cell aggregates. Titanium alloys and stainless steel (ST) alloys are widely used to fabricate stents because of their good mechanical properties and outstanding corrosion resistance [9]. Material surface finishing technologies can influence protein adsorption and cell-surface interaction [10]. Properties like surface roughness [10], chemical composition [11], and wettability [12] directly or indirectly affect the interaction between blood cells and materials.

Although there has been continued interests in material hemocompatibility, the mechanism of blood-material interaction is still not well understood, especially among metallic materials. In the present research, experiments have been conducted to understand the mechanism of blood cell activation induced by incubation with different stent metals. ST316L and titanium alloy TiAl6V4 have been selected and tested under both static and flow conditions. Flow cytometry was used to analyze blood cell activities. Protein adsorption and blood cell adhesion on the metal surfaces were assessed by scanning electron microscope (SEM). Hemocompatibility of the two stent metals was compared, and the shear effect on in-stent blood cell activation was studied.

1.3 Outline of Thesis

This thesis is organized as follows: Chapter 2 provides background on blood cells and current knowledge on their functions in thrombus formation. Chapter 3 summarizes the advantages and disadvantages of various flow models used in *in vitro* study. Chapter 4 describes the experimental methods and materials used in this work. Chapter 5 presents the experimental results. Discussion, conclusions and recommendations for future work

are presented in Chapter 6.

Chapter 2

Blood Cells and Thrombosis

Blood delivers oxygen and nutrient to every cell in the body. It transports CO_2 and other waste products out from these cells. The prevention of excessive blood loss is one of the important roles of blood vessels. The blood circulation system is able to rapidly detect blood vessel damage and generate an effective thrombus plug to stop bleeding. Nevertheless, increased circulating blood volume and pressure, as required in larger animals, make this function more significant and challenging.

The inner surface of blood vessel wall is the endothelium that acts as a selective barrier between blood and the rest of vessel wall. The endothelium consists of a thin layer of endothelial cells lining the entire circulatory system. They reduce the turbulence of blood flow, allowing blood to be pumped farther [13]. Under normal conditions, endothelial cells present a non-thrombogenic character by synthesizing and releasing two powerful soluble inhibitors against platelets activation, nitric oxide and prostacyclin (PGI2), and by expressing a thrombin inhibitor [14]. Their existence keeps blood vessel wall smooth and reduce the chances of firm attachment of cells and proteins.

Contact between blood and stent materials, and the trauma caused by stent placement can induce the blood coagulation through the intrinsic (material surface contact activation) and extrinsic (tissue factor) pathways respectively (Figure 2.1). The coagulation cascade is an important part of hemostasis, and involves a series of proteolytic reactions resulting in the formation of a fibrin clot which is one of the main components of blood clots [7,15,16]. Twelve factors (from factor I to XIII, except factor VI) are engaged in the process where an inactive factor is enzymatically activated following surface contact or after proteolytic cleavage by other enzymes; the newly activated factor then activates another inactive precursor factor [7]. Both pathways converge on common pathway of activating factor X, thrombin and fibrin.

Tissue factor (TF) is a key molecule during the activation of the extrinsic coagulation cascade, leading to the fibrin formation. With antibody against to tissue factor can inhibit fibrin deposition in thrombus [17]. Generally, tissue factor in normal blood is undetectable. Agents, such as inflammatory cytokines, virus infections, and thrombin, can induce TF synthesis [18]. Moreover, monocytes can synthesize and express tissue factor after stimulation by LPS, and P-selectin, or high shear stress [19], and may lead to leukocyte accumulation in areas of vascular injury associated with thrombosis and inflammation [20]. Blood contact with a material can induce TF expression on monocytes. A minimum of 60 min is needed for TF synthesis on monocytes [7]. In addition to monocytes, other sources of tissue factor include the neutrophils [21], endothelium and blood plasma [22].

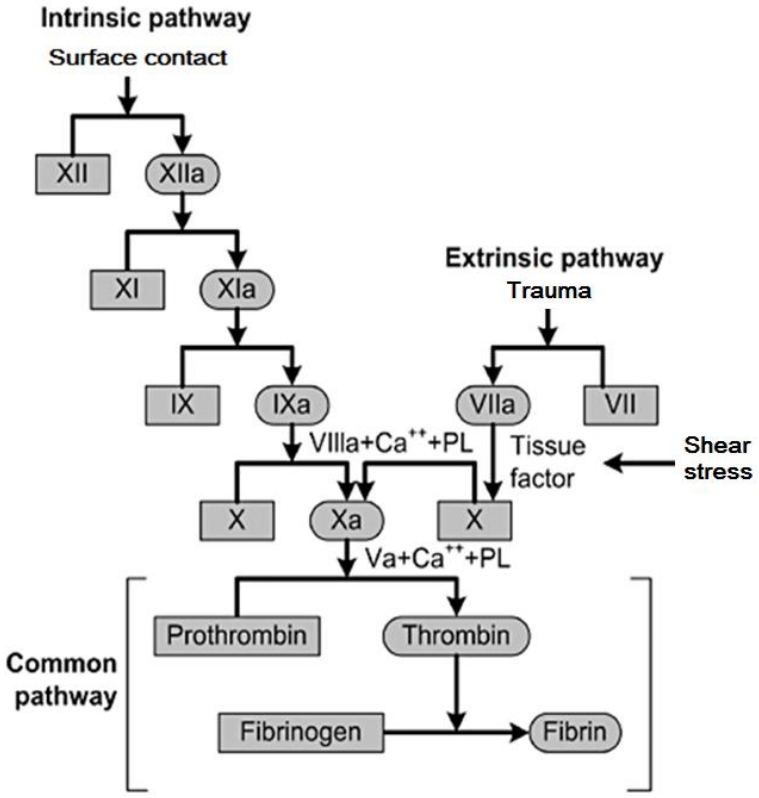


Figure 2.1: The intrinsic and extrinsic coagulation pathways. (Adapted from Adams,2009 [16])

2.1 Role of Platelets in Thrombus Formation

Platelets are disc-shaped anuclear cells with a diameter of 2-3 μm, and produced by megakaryocytes. They circulate at a concentration ranging from 200-500$\times 10^6$ cells/ml for 5 to 9 days after leaving the bone marrow. On an intact and healthy endothelium, platelets remain in their original and unactivated state. In most arteries, they appear to migrate toward the vessel wall in a flow- and hematocrit-dependent manner because of the

interaction between platelets and other blood cells. Platelet density near the vessel wall is 2 to 3 times higher than that in the center of the vessel [23].

Platelets play a crucial role in hemostasis and blood vessel repair through the formation of blood clots. They also participate in the development of coronary artery diseases through the pathological thrombus formation inside the blood vessel wall. Under normal conditions, soluble agonists, such as von Willebrand factor (vWf), collagen and fibrinogen in plasma, do not undergo significant interaction with platelets. However, platelets can rapidly adhere to exposed subendothelium and smooth muscle cells by binding to collagen and vWf at the site of blood vessel injury [7, 24], as shown in Figure 2.2. Tissue factor (TF, CD142) from exposed subendothelium promotes the formation of thrombin which is a potent platelet activator (See Figure 2.2). Thrombin mediates the conversion of fibrinogen to fibrin that also contributes to the formation of hemostatic plug and thrombus growth [24].

On activated endothelium or subendothelium, platelets roll, adhere, and form a monolayer of activated platelets [25]. With the presence of platelet activators or high shear conditions, platelets become activated. Platelet activation can elicit a series of physiologic responses, such as pseudopod formation and shape change, release of granules, biochemical membrane alterations, initiation of aggregation and the formation of procoagulant platelet microparticles (PMPs).

Once activation is initiated, platelets become more amorphous with projecting fingers, and transform into dendritic and spread shape [26]. They secrete dense granules containing ADP and α granules containing P-selecin, vWf and some other growth factors. Membrane enzyme phospholipase A2 is activated, leading to the formation of Thromboxane A2 (TXA2). Additional circulating platelets can thus be recruited to extend and stabilize the hemostatic plug by binding to the activating factors, like ADP, TXA2, and thrombin [7, 27, 28]. ADP acts via the G protein-coupled receptors, $P2Y_1$ and $P2Y_2$, to

reinforce the platelet aggregation. TXA2 binds to the corresponding receptor to stimulate the activation of new platelets and increases platelet aggregation (Figure 2.2). Moreover, PMPs contribute to further platelet activation and aggregation, and help with the hemostatic plug formation [29].

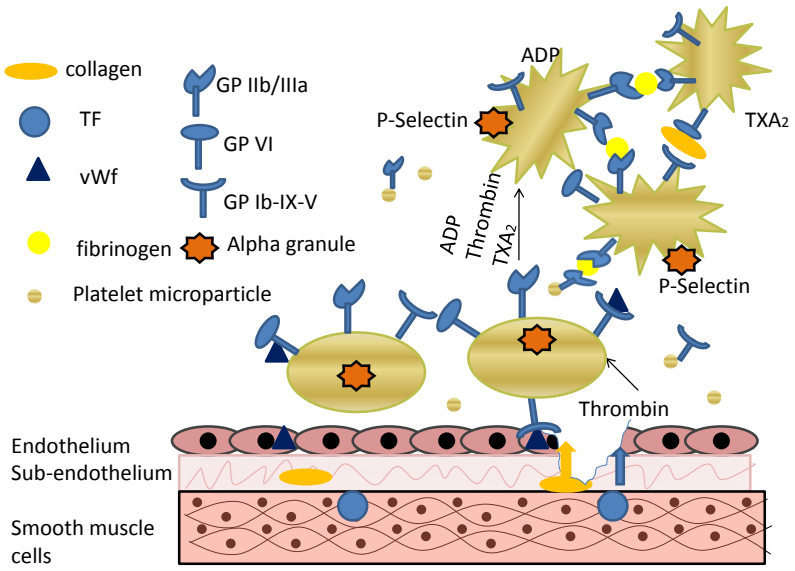


Figure 2.2: Platelet adhesion, activation and aggregation. Platelets adhere to the exposed TF and vWf, resulting in the conformational change, and P-selectin expression. Activated platelets release ADP, thrombin, and TXA2, leading to further platelets recruitment and aggregation at the site of injury.

2.1.1 Platelet receptors and agonists

An important platelet receptor is Glycoprotein Ib (GPIb), with a density of 25,000 copies per platelet. It is associated with GPIX and GPV at a 2:2:1 ratio [30]. Conformational changes of the GPIb molecule has been found to mediate the interaction between vWf and GPIb-V-IX complex [7, 30]. The initiation of platelet adhesion after vascular injury is primarily mediated by the interaction between GPIb-V-IX receptor complex to vWf [28].

Under static or low shear conditions, platelet receptor GPVI and GPIa/IIa can bind to collagen at the site of exposed subendothelium without the assistance of vWf. However, vWf is required to mediate platelet adhesion in arterial circulation [31]. Platelets adhere and decelerate until GPVI or integrin GPIIb/IIIa start to bind their respective ligands and form permanent adhesion, spreading and aggregation. It also binds to the neutrophil membrane receptor Mac-1 (also refer to as CD11b), P-selectin on activated platelets, and endothelial cells [32].

GPIa/IIa (CD49b/CD29) presents at the level of 1000 copies per platelet, and is required for platelet adhesion to collagen under static and flow conditions [33]. The interaction between GPVI and collagen can lead to shedding of membrane blebs (also referred to as microparticles) into circulation, which provides procoagulant surfaces [28].

Glycoprotein IIb/IIIa (GPIIb/IIIa also refer to as CD41/61), is also known as integrin $\alpha_{IIb}\beta_3$. GPIIb/IIIa is the most abundant adhesive receptor on the platelet surface at the density of about 50,000 on a resting platelet. It is also present in the α granules of platelets. Upon activation, α granules translocate to the platelet membrane and increase GPIIb/IIIa expression on platelets [33]. GPIIb/IIIa serves as a receptor for vWf, fibronectin, and vitronectin, and can be recognized by specific antibodies, such as PAC-1 and anti-CD41/CD61 [7, 33].

GPIIb/IIIa remains inactive on resting platelets, with a low affinity binding site for fibrinogen. Activated GP IIb/IIIa mediates platelet recruitment as well as platelet-platelet interactions. Conformational change after platelet stimulation leads to the expression of high affinity GPIIb/IIIa binding site for soluble fibrinogen. The initial platelet adhesion induced by GPIb-V-IX complex is reversible, while adhesion through GPIIb/IIIa can be strengthened through binding to fibrinogen and thus become irreversible [28, 34, 35]. Fibrinogen contains a second binding site to GPIIb/IIIa or Mac-1 on leukocytes, leading to the formation of platelet aggregates and platelet-leukocyte aggregates by cross-linking of receptors on different cells. GPIIb/IIIa inhibitors, such as c7E3, can prevent platelet aggregates formation [36]. Clinical studies have shown the efficacy of blocking GPIIb/IIIa to reduce ischemic complications on CAD patients [37].

P-selectin (CD62p) is a platelet membrane glycoprotein and a component of the α granules of resting platelets. It is only expressed on the platelet membrane surface after activation-dependent release from the α granule (Figure 2.2). The P-selectin exposure on platelet is partially regulated by vWf [38]. There are about 10,000 P-selectin molecules expressed on an activated platelet [39]. P-selectin mediates the rolling of platelets on endothelial cells [40] and the leukocyte-platelet aggregation through its leukocyte counter-receptor, P-selectin Glycoprotein Ligand-1 (PSGL-1). It can be identified by flow cytometry, and is used as an important marker of platelet activation both in research and clinical study [39, 41].

2.1.2 Platelets under shear condition

Blood flow conditions can be quantified by shear rate (unit s^{-1}). Shear rate refers to the gradient of the velocity between two adjacent laminar flows. It is zero at the center of the blood vessel, and maximum at the blood vessel wall (Figure 2.3) [42]. Sometimes,

shear stress τ is used to measure the blood flow, and it is proportional to shear rate with a proportionality factor blood viscosity μ. In the human blood circulation system, the physiological shear rate ranges from 20 to 2000 s^{-1}. Accordingly, shear stress varies from 1.4 to 60 dynes/cm^2. In certain pathological flow conditions, shear rate can reach 10^5 s^{-1} [23, 43]. Typical time average values for healthy blood vessels are presented in Table. 2.1.

High shear conditions, such as above 6000 s^{-1}, are able to induce platelet activation and aggregation in the absence of any other platelet activators. Platelets may experience different shear rates at the sites of abrupt reduction in blood vessel cross-sectional area [44]. It has been reported that significant increase of PMPs formation can be detected under pathological shear rate (10,500 s^{-1}), but no significant formation of PMP was observed at lower shear rates. This indicates that platelet activation proportionally correlates with the magnitude of shear rate [43]. Boreda *et al*. further proved that platelet activation is linearly dependent on the shear rate, and less dependent on the exposure time [45].

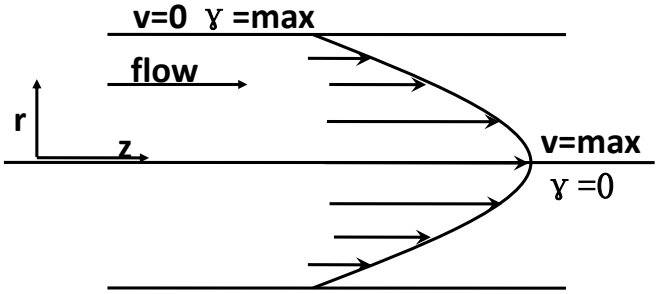


Figure 2.3: Shear rate definition and blood velocity profile within ideal blood vessel [42].

Table 2.1: Characteristic value of flow parameters within the human vasculature [23]

Vessel	Diameter (mm)	γ (s^{-1})	τ (dynes/cm^2)
Ascending aorta	23-45	50-300	2-10
Femoral artery	5.0	300	11
Common carotid	5.9	250	8.9
Internal carotid	6.1	220	8
Left coronary	4.0	460	16
Right coronary	3.4	440	15
Small artery	0.3	1500	53
Arterioles	0.03	1900	60
Large vein	5-10	200	7

Studies have also shown that platelet aggregation preferentially occurs at regions of flow disturbance where a shear acceleration zone is followed by a shear deceleration zone. This means that shear gradient or certain geometric blood vessel shape may be necessary for platelet aggregation formation. *In vitro* experiments have reported that under low shear rate, no platelet aggregation occurred, while under shear rates from 1000 to 10,000 s^{-1}, small transient aggregates formed through the development of membrane tethers. Large rolling aggregates can be found at pathological shear rates over 100,000 s^{-1} [46]. To

mimic shear condition around the stenosis, experiments were performed using a backward-facing step followed by an expansion zone [34]. Initial platelet recruitment occurred within the zone of peak shear stress, and was followed by large platelet aggregation within the shear deceleration zone. The result demonstrated that platelet aggregation appeared to be dependent on the magnitude of the shear gradient and GPIb, but not on the soluble platelet activators ADP, Thromboxane A2 and thrombin, soluble factors that would commonly be present in a recirculation zone [34]. While the effect of shear and shear gradient has been studied on platelet activation, most of these studies have not looked at the effect of biomaterials and have often been performed under short contact times and/or in the absence of red blood cells. To be able to design better biomaterials that will perform adequately under shear conditions, it is thus important to study *in vitro* flow models that could adequately assess material biocompatibility in blood.

2.2 Leukocytes and Thrombosis

There is increasing evidence indicating that leukocytes are involved in thrombosis [7, 19, 23, 47, 48]. Leukocytes consist of five different types of cells, which can be distinguished by the presence of granules. Polymorphonuclear (PMN) leukocytes contain more than one granule in their cytoplasm, and are composed of neutrophils, basophils and eosinophils. Mononuclear leukocytes are characterized by the absence of granules in cytoplasm, and include two subgroups, monocytes and lymphocytes.

Neutrophils, with a diameter of 8-15 μm, are the most abundant leukocytes (50-70% of all leukocytes). PMNs are the first line of defense as part of the innate immune response. They phagocytize foreign cells, toxins and viruses. Monocyte is the largest leukocyte with a diameter of 15-25 μm. Circulating monocytes account for 3-9% of all leukocytes. With

the assistance of fluorescent antibodies, they can be identified by the expression of large amounts of lipopolysaccharide (LPS) receptor component, CD14 [49].

2.2.1 Activation, adhesion and aggregates

Upon activation, at the membrane level, leukocyte will respond by modulating the expression of certain receptors (down-regulation through shedding or up-regulation) and will change in shape through pseudopod formation [50] (Figure 2.4). For example, L-selectin exists on most leukocytes and sheds from their surface following cell activation [7,51]. Inhibition of selectins can significantly reduce the leukocyte-platelet aggregation [52], leukocyte adhesion, and fibrin deposition [19].

CD11b (or Mac-1) is up-regulated within minutes of exposure to proinflammatory substances such as Tumor necrosis factor (TNF)-α, C5a, platelet-activating factor, and artificial material surfaces [7,53–56]. CD11b is one of the membrane protein subunits of integrin $\alpha_M\beta_2$, and can also bind to the inactive complement component iC3b. Leukocytes have been shown to adhere onto artificial surfaces through the iC3b/CD11b complex. The activation of CD11b integrin, which is partially dependent on the binding of P-selectin to PSGL-1, can firm leukocyte-platelet adhesion through fibrinogen [19, 57]. Similarly, it also mediates the firm attachment of leukocyte to the endothelial cell through its counter-receptor ICAM-1. Activated monocytes synthesize and express tissue factor which can initiate blood coagulation and mediates intracellular signaling events [21]. Studies on the effects of neutrophils and monocytes in material induced thrombus formation have been conducted as they are the main players defending against foreign materials [7].

Another marker for leukocyte activation is the formation of platelet-leukocyte aggregates which can be formed when P-selectin expressed on platelets binds to PSGL-1 on

leukocytes. Following activation, P-selectin is rapidly mobilized to the platelet and endothelial cell surface, and serves as a leukocyte adhesion receptor. E-selectin on activated endothelial cells involves the leukocyte rolling through its binding to PSGL-1 and E-selectin Ligand-1 (ESL-1).

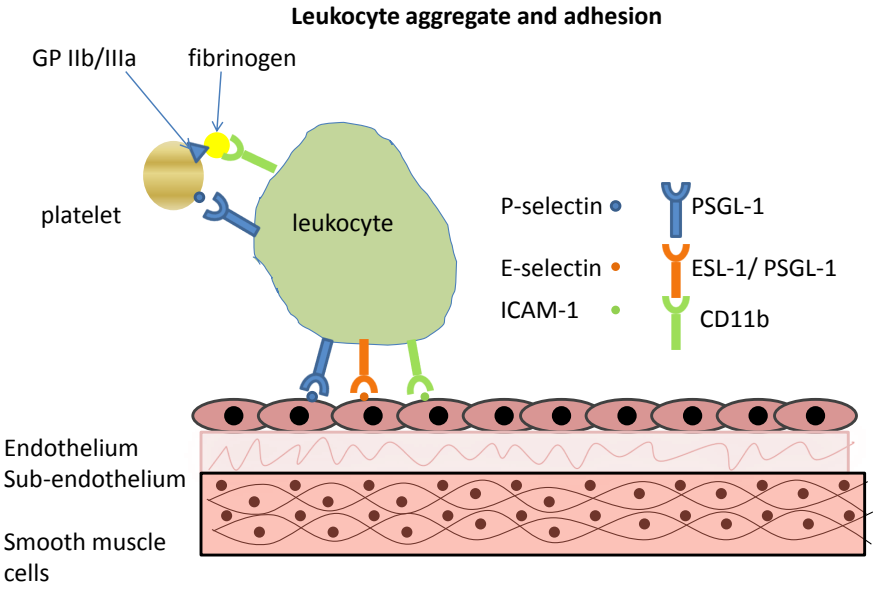


Figure 2.4: Leukocyte adhesion on endothelial cells and leukocyte-platelet aggregates formation.

As opposed to platelets, in the absence of leukocyte stimulus, physiological shear stress alone cannot induce pseudopod formation and β_2 integrin expression on leukocytes [58]. However, monocytes have been reported to adhere to the blood vessel wall under non-uniform shear stress, while no adhesion was observed under laminar shear stress.

2.3 Blood and Biomaterial Interaction

Hemocompatibility of biomaterials can be evaluated through blood clotting time, thrombin time, prethrombin time, and responses of platelet [59, 60]. The first event that occurs when a foreign material comes into contact with blood is the water and plasma protein adsorption onto its surface. The adsorbed protein layer is essential for the performance of biomaterials [61, 62].

Two plasma proteins, albumin and fibrinogen, have important roles in blood-biomaterial interactions. Albumin works as an inhibitory protein and inhibits platelet adhesion, while fibrinogen is an adhesive protein known to enhance the platelet adhesion and activation. The adsorption of albumin is generally faster than that of fibrinogen because albumin is much smaller than fibrinogen [61].

Initial reactions of platelets to foreign material, including platelet activation, adhesion and aggregation, are first in the cascade of events eventually leading to stent thrombosis [63]. Platelet response is a multiparametric process and is controlled by surface properties of biomaterials such as morphological characteristics [10], and chemical composition [61, 64], electrical properties [65], and wettability [66, 67]. Surface treatment techniques, such as mechanical polish, heat treatment, chemical treatment, have been shown to have a significant effect on the thrombogenicity of materials [68]. Increasing the complexity of the surface microtexture will enhance platelet adhesion and activation [10]. Platelet adhesion may be more important than platelet aggregation as it occurs before platelet aggregation. The shape change of platelets on biomaterial surfaces is strongly linked to the thrombogenicity of materials.

Interaction with stent materials and vascular injury caused by stent placement have been shown to induce leukocytes activation [55]. Leukocytes have also been found to be

involved in the early interaction with stent material, interacting and contributing to fibrin formation [12]. Moreover, it has been found that neutrophil adhesion is dependent on the wettability of the material surface. For example, hydrophobic materials (glass and polyethylene) induce a prominent adhesion of neutrophil, while the adhesive surface for neutrophil is low on hydrophilic material surfaces [12].

2.3.1 Stent metals

Metals possess different degrees of thrombogenicity. Stainless steel has a high electrochemical surface potential, allowing the transfer of electrons to proteins, such as fibrinogen. This promotes thrombus formation and neointimal hyperplasia. In contrast, titanium has low electrochemical surface potentials, and is biologically inert [69]. Platelet adhesion on ST316L and Cobalt-Chromium alloy was observed to be 2-fold higher than that on Magnesium alloy by SEM analysis [63].

Conventional metals are usually covered with surface oxide films which play an important role against protein adsorption and platelet adhesion [61]. A thin film of titanium oxide is formed shortly after TiAl6V4 contacted with air or blood, so that it is titanium oxide that blood actually contacts with during incubation. Titanium oxide is widely used in stent surface modification becaused of its excellent hemocompatibility [59]. Titanium oxide film composition and thickness of the oxide layer can influence platelet adhesion. Thicker titanium oxide layers displayed less platelet adhesion than thin oxide layers [70]. Heating, alkali, and H_2O_2 treated titanium substrate can decrease platelet adhesion and activation [71]. Compared with titanium alloys, the surface of Cr_2O_3 is expected to attract more proteins as the relative permittivity of Cr_2O_3 is lower than that of TiO_2. Taking the size of albumin and fibrinogen into account, the Cr_2O_3 surface adsorbs more albumin and thus has less platelet adhesion [61].

Nitinol is characterized as a shape memory alloy. Its biocompatibility and corrosion resistance are comparable or superior to that of ST316L [72]. Fibrinogen adsorption and platelet adhesion induced by nitinol have been shown *in vitro* to be significantly lower than ST316L. Furthermore, nitinol stents tend to exhibit less thrombus formation than ST316L stent [72].

2.3.2 Coating techniques

There is increasing interest in developing novel coatings to enhance the hemocompatibility of stent metals. Metal ions can be released from the BMS. For example, one of the concerns regarding the application of nitinol alloys in the vasculature is that released nickel may induce a toxic effect on the cells in the system or trigger thrombus formation [68]. Similarly, all of the elements (chromium, nickel, molybdenum, and manganese) of ST316L could be detected in the plasma samples after implantation. These metal ions may trigger platelet activation even at very low concentration [73]. With the use of coating techniques, ion release may be blocked.

Diamond-like carbon (DLC) coating was developed to enhance the hemocompatibility of metal implants. A comparative study showed that ST316L promoted a relatively high degree of platelet adhesion, spreading and aggregation, whereas only modest spreading and limited aggregation were observed on DLC surfaces. Platelet activation and adhesion were found to be higher on commercially pure titanium [74], and titanium alloy (TiN and TiC) [62] than those on DLC coated titanium samples. The index of platelet size on all of these materials was greater than that for the DLC coating indicating greater platelet spreading. A lower ratio of adsorbed albumin to fibrinogen is also believed to be responsible for the greater spreading of platelets observed on these surfaces [62].

Polymer coating on metal implants is a novel approach to improve hemocompatibility. Platelet adhesion can be reduced on stainless steel coated with different polymers [75, 76]. Compared with unmodified TiAl6V4 samples, platelet deposition on polyphosphorylcholine (PMPC)-grafted TiAl6V4 and platelet activation in blood were significantly reduced [77].

Adhesion and activation of the platelets on the titanium metal coated with apatite, albumin-apatite composite or laminin-apatite composite were definitely supressed as compared with those on uncoated titanium metal [11]. Coating of collagen and heparin on titanium surface improves the biocompatibility of titanium, because collagen is the major component of extracellular matrix, and heparin is a potent inhibitor of thrombin and factor Xa in cooperation with antithrombin III [78].

Although various metals and novel techniques have been developed, no real progress has been made in the development of truly blood compatible materials [79, 80]. Most materials and coatings remain at the laboratory stage. There is still a lack of understanding of the mechanisms involved in material-induced blood cell activation, especially under flow conditions. The gap in our knowledge can be illustrated by the fact that all cardiovascular implants still require anti-platelet and anticoagulant therapies and despite these, there are still events of thrombotic complications. Hence, there is a need to study the blood-material interaction and the effect of flow condition in blood cell activation so that better materials or coatings can be developed. This cannot come without first studying *in vitro* flow models using different biomaterials.

Chapter 3

Blood Activation in *In Vitro* Flow Models

The human body is carefully regulated to prevent unwanted or excessive thrombus formation so as to avoid occlusion both in the low shear environment in the venous system and high shear environment in the arterial system. Researchers believe that blood flow plays a critical role in thrombus formation. Under low shear rate, blood cells move relatively slowly to the vessel walls and have more opportunity to contact and interact with each other, because of the adhesion formed between the receptors on the surface of platelets and their ligands on the endothelium [23, 81]. As shear rate increases, the convective transport of platelets and plasma proteins adhesion to the vessel walls increase correspondingly. Studies have shown that thrombus formation preferentially occurs in regions where arteries undergo sharp fluid changes (Figure 3.1), indicating that thrombosis is affected by blood flow conditions [82].

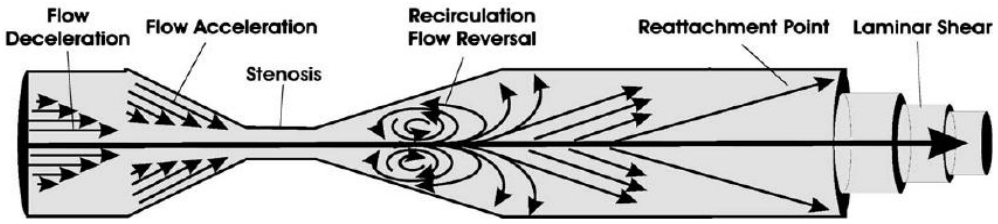


Figure 3.1: Blood flow profile within stenosed blood vessel [82].

Blood viscosity varies between individuals, and is dependent on the hematocrit and the viscosity of plasma. One should expect that blood is a non-Newtonian fluid, because the volume fraction of erythrocytes is about 40-45% in normal blood. However, blood viscosity is independent of shear rate under shear rates greater than 100 s^{-1}. Blood is assumed to have a constant viscosity in large vessels, as vessel diameter is large compared to that of single cell [83, 84]. Therefore, blood is always considered as a Newtonian fluid in the study of the coronary artery (diameter 3.2 to 4 mm).

Although *in vivo* studies on coronary artery diseases provide direct information on how cells react with stent materials in blood circulation systems, their applications are constrained by high costs and ethical issues. On the other hand, *in vitro* flow models have been greatly improved during the last decade. In this chapter, four major types of blood flow models used in the study of blood cell activation will be introduced. They are the cone and plate flow system, the chandler loop model, the microfluidic flow model, and the parallel plate flow chamber.

3.1 Cone and Platelet Flow System

The cone and plate flow system is a good option for investigating the effects of bulk shear stress on cellular functions [85,86]. In the cone and plate flow system (Figure 3.2), cells are either attached onto the stationary plate or suspended in the medium, the cone rotates at an angular speed ω, causing the fluid sample to move in the circumferential direction. At low shear rates, the flow is approximately uniform and laminar. In contrast, the fluid flow at high shear rates can begin to make a transition to three dimensional flow because of the centrifugal forces in the radial direction [43]. However, since the angle α between the cone and plate is small, the radial flow is small because the viscose and surface tension forces are much larger than the centrifugal forces, and can be neglected in thrombosis research. In the cone and plate flow system, shear rate is defined as

$$\gamma = \frac{\omega}{\alpha} \tag{3.1}$$

In biological experiments, shear rate produced in the cone and plate flow system ranges from 0 to 10,000 s^{-1}. Cone angle varies from 0.3 to 2 degrees. The shear rate is less than 2500 s^{-1} for neutrophil and endothelial cell studies, and it can reach 5000 s^{-1} in studies of shear-induced platelets activation [87, 88].

While the cone and plate flow system enables the study of the effect of various shear rates on cells within one experiment, hemolysis has been reported on several occasion [87, 88]. Thus, red blood cells or whole blood cannot be tested. Unfortunately, this significantly limits the validity of such flow experiments, as red blood cells play a significant role in cell transport.

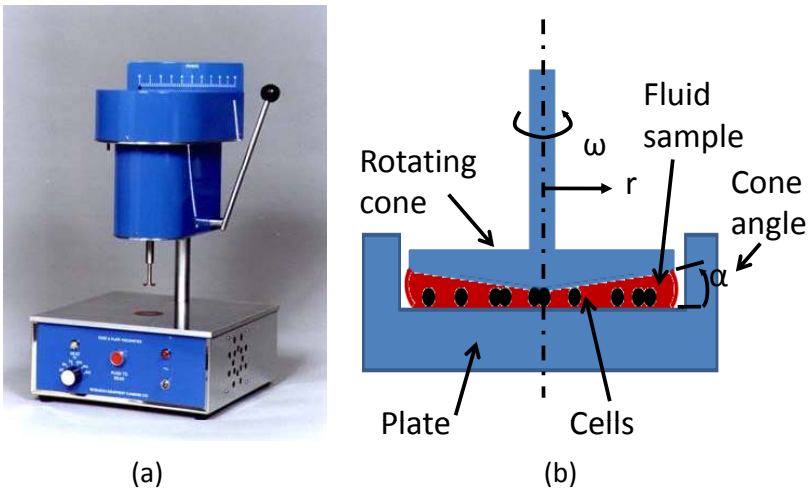
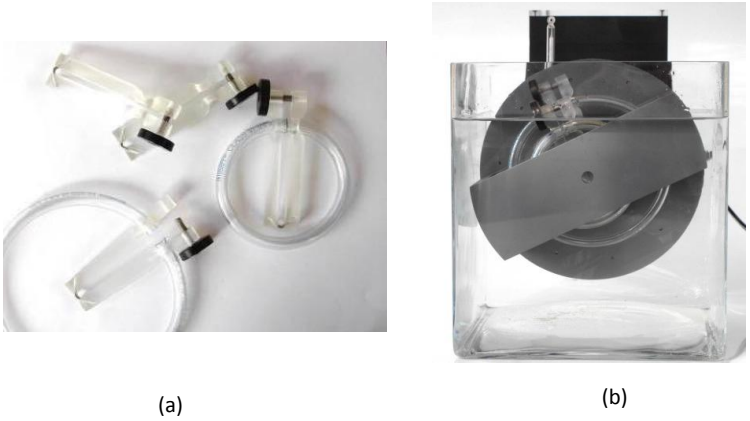


Figure 3.2: (a) Commercial product whose cone is made of titanium and plate is made of stainless steel (BS: 3900 A7, Research Equipment, UK), (b) The schematic of a typical cone-and-plate flow system.

3.2 Chandler Loop model

The chandler loop system was first created to form thrombi outside the human body in 1958. The system usually comprises a 50 cm long tube, with inner diameter of 4 to 6 mm [89,90]. The circular tubes are connected end-to-end after loading experiment samples (Figure 3.3(a)). The loop is then mounted on a rotating wheel. The chandler loop system is adapted to use in an incubator or water bath (Figure 3.3(b)). The rotation speed can be set at 20 to 60 rpm. The system is commonly used to mimic the blood flow condition in large vessels. However, one disadvantage of the sytem is the large volume of blood

consumption. Based on the tube mentioned above, 5 to 10 ml blood is needed per test sample. Hence, this design is not practical for either experiments with many test samples or experiments using blood from small animals. Moreover, it can only produce shear rates under 500 s^{-1}, and lacks the ability to produce high shear rates. The shear rate in the healthy human coronary artery is above 440 s^{-1} [23], and pathological shear rate is even much higher. Therefore, the chandler loop system is not a good candidate for studying the effect of pathological shear rate on blood cell activation.



(a) (b)

Figure 3.3: Chandler loop models. (a) Tube and connector, (b) Chandler loop system used in water bath (picture source: Chandler-Loop-System, Germany)

3.3 Novel Microfluidic Devices

Blood consumption is a major issue in blood experiments, and is limited by donors and the volume in each blood collection. As a result, various microfluidic flow systems have been developed, which can reduce the amount of blood volume for a single test sample [44, 91, 92]. Figure 3.4(b) is a typical example of microfluidic device. Photolithography

is first employed to fabricate the flow channels. It can minimize the channel width to a few hundred micrometers or even lower. This makes the cross sectional area very small, leaving the surface areas relatively large. This micro-dimension concept is especially practical for the cell adhesion experiment. Another beneficial component of such a system is the programmable pump, producing controllable flow rates. Multiple channels on the same device also make it possible to operate parallel experiments simultaneously under different shear conditions. Thus, shear force, flow rate, and temperature can be independently controlled in all wells allowing to potentially perform 24 experiments at once. Most devices are also made of silicone rubber polydimethylsiloxane (PDMS), an inexpensive but most importantly biocompatible and transparent. The material is inexpensive, and most importantly biocompatible and transparent. Thus, a microscope or a camera can also be added to the system to provide direct observation on cell adhesion and rolling.

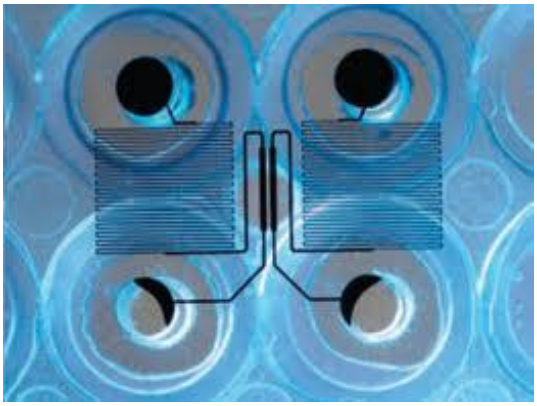


Figure 3.4: BioFlux^{TM} microfluidic well-platelet. The system is coupled to an SBS-standard 48-well plate. Each fluidic channel has a unique input and an output well. Up to 24 independent experiments can be run on a single BioFlux plate.

Despite the advantages mentioned above, microfluidic design also has some limitations.

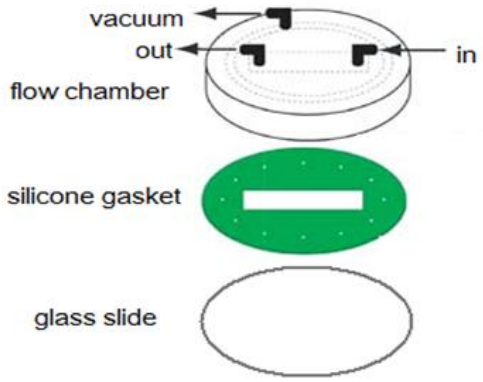
Due to its micro-dimensions, it can mimic the flow conditions in capillary blood vessels with a wide range of shear rates. However, it is not able to mimic the flow conditions in larger blood vessels, such as coronary arteries. Geometry differences between large blood vessels and capillaries result in different shear conditions, because blood viscosity decreases as the blood vessel diameter is reduced [93]. This phenomenon is named as Fahraeus-Lindqvist effect. Furthermore, blood in a micro-sized vessel is not a Newtonian fluid any more, because red blood cells move over the center of the vessel, leaving plasma flows near the vessel wall. This may result in shear rate calculation and measurement much more complex. Thus, it is not a suitable device for stent related thrombosis study.

3.4 Flow Chambers

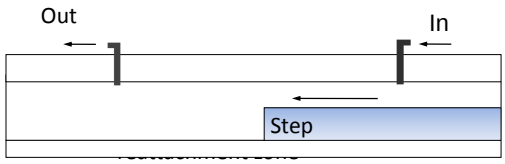
Flow chamber is another popular experimental device that has been widely used in blood-material interaction study. It was originally proposed by Lawrence *et al.* in 1958. After many years of development, various designs have been derived, such as parallel-plate flow chamber, rectangular flow chamber, and vertical step flow chamber. Figure 3.5 illustrates the design of these chambers. These flow chambers can produce not only constant shear rates [43], but also pulsatile flow and turbulent flow [94, 95]. Moreover, flow chambers can generate a wide range of shear rates, from 1 up to 10^4 s^{-1} with limited volume of blood as a syringe pump or roller pump can be used. It is also convenient to mount material wires or strips into the chamber, or coat the gasket with cells of interest [94]. Cell adhesion and movement in the chamber can be monitored or recorded by microscope or camera.

Taking blood vessel geometry, blood volume, and shear condition into account, the parallel-plate flow chamber appears to be the optimum device to study stent material-induced blood cell activation. The diameter of the tubing in the flow chamber system is

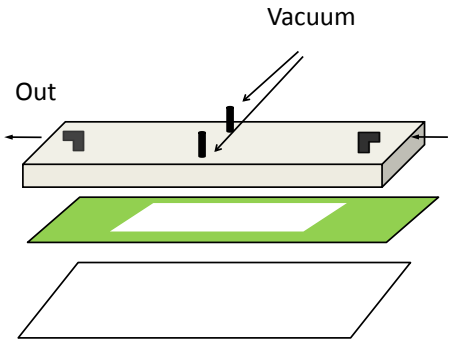
about 1.52 mm, which is close to the diameter of coronary arteries. Second, the blood volume needed for each sample can be reduced to less than 1.5 ml, so that more tests can be done with 5-10 ml of blood. For example, with 5 ml blood, which is acceptable for most blood donors, at least 3 tests can be operated at the same time. Third, a parallel-plate flow chamber can produce both physiological and pathological shear rates of coronary arteries, which simplifies the system design and operation.



(a)



(b)



(c)

Figure 3.5: Types of flow chamber designs. (a) Parallel-plate flow chamber, (b) Rectangular flow chamber, (c) Vertical step flow chamber.

Chapter 4

Materials and Methods

4.1 Materials

4.1.1 Reagents and monoclonal antibodies

LPS (*Escherichiacoli* serotype 0111:B4), endotoxin-free water, ethylenediaminetetraacetic acid (EDTA), thrombin receptor-activating peptide SFLLRN, 50% glutaraldehyde solution, and paraformaldehyde were purchased from Sigma-Aldrich Co(Oakville, ON, Canada). Phosphate-buffered saline (PBS) from Fisher Scientific Inc(Ottawa, ON, Canada) was used.

To quantify platelet activation, aggregates, and platelet microparticle formation, fluorescein isothiocyanate (FITC) conjugated mouse monoclonal antibody against human glycoprotein IIb/IIIa (CD61) (Cat: 555753) was used. R-phycoerythrin-cytochrome 5 (PE-Cy5) conjugated monoclonal antibody against CD45 (Cat: 555484) the pan leukocyte common antigen was used to label leukocytes. Leukocyte activation and tissue factor expression were detected through FITC conjugated mouse monoclonal antibody against

Mac-1 (CD11b) (Cat: 557701) and R-phycoerythrin (PE) monoclonal antibody against TF (Cat: 550312) respectively. All the monoclonal antibodies were purchased from Becton Dickinson Pharmingen (San Diego, CA, USA). FACS lysing solution, a red blood cell lysing buffer also containing paraformaldehyde, was obtained from Becton Dickinson (San Jose, CA). All other chemicals were of analytical reagent grade.

4.1.2 Metal samples and preparation

ST316L disks (thickness 0.5 mm, diameter 12mm) and TiAl6V4 disks (thickness 0.52 mm, diameter 12mm) were obtained from Goodfellow Co. (Oakdale, PA, USA). TiAl6V4 wire was purchased from Fort Wayne Metals (Fort Wayne, IN, USA), while ST316L wire was a generous gift from this company. To remove endotoxin on the surfaces and sterilize materials, both metal disks and wires were first cleaned twice in 70% ethanol and then rinsed in phosphate buffer (PBS). All washes took place in an ultrasonic water bath each time for 30 min. Rinsing samples in endotoxin-free water was performed after each ultrasonic clean cycle. Sterilized metals were then immersed in PBS and stored at 4 ℃ for 2 weeks maximum.

4.1.3 Blood collection

This study was approved by the research ethics committee of the University of Waterloo. Blood samples were taken by venipuncture with a 21 gauge needle from healthy donors (average age 30 years) who were medication-free for at least 3 days. Blood was then transferred to a polypropylene tube preloaded with 5U/ml anti-coagulant heparin (Sigma Aldrich Co), and inverted 2 to 3 times. Heparinized blood was kept at room temperature and processed within 30 min after collection.

4.2 Methods

4.2.1 Static experiment

To assess the effect of material on blood cell activation, static experiments were first performed by incubating material samples with heparinized whole blood or platelet rich plasma (PRP). PRP was prepared by centrifugation at 1000 g for 10 min at room temperature. PRP supernatant was then carefully collected and transferred into a small vial.

Sterilized ST316L and TiAl6V4 disks were fixed separately in one of the wells of a 24-well plate with a small Tygon^{TM} tubing ring (12.7 mm inner diameter, 15.9 mm outer diameter, and 10mm height; VWR, Mississauga, ON, Canada). The silicone tubing rings had also been cleaned in an ultrasonic cleaner as previously described above. A disk of sterilized silicone sheet (medical grade, thickness 0.015 inch; Specialty Manufacturing Inc, Saginaw, MI, USA) was placed at the bottom of a well and used as negative control, because of the good biocompatibility of silicone. Also, an empty polystyrene well was used as a positive control. Tygon^{TM} silicone tubing rings were also used in the control wells to ensure that blood was exposed to the same surface area with all samples. Samples were incubated with 300 μl heparinized whole blood or PRP at 37 ℃ for 2 hours. To prevent blood from settling, the 24-well plate was placed on an orbital plate shaker (Model type: Standard 3500, VWR) rotating at 300 rpm.

4.2.2 Flow experiment

The parallel-plate flow chamber was adopted because of the advantages mentioned previously in Chapter 3.4. Flow chamber kits were purchased from Glycotech (Gaithersburg, MD, USA). The flow chamber was comprised of a flow deck, a silicone gasket and a glass

side. The thickness of the gaskets was chosen to accommodate the laboratory metal wires that had a diameter of 0.005 inch. A smaller gasket chamber width was also preferred to reduce the blood volume needed for a single sample. Thus, the gasket with a thickness of 0.01 inch, a width of 2.5 mm and a chamber length of 19mm was selected.

For a healthy human, while also varying with age, gender, and lifestyles, the shear rate in coronary artery can be considered to be approximately 500 s^{-1}. For patients with CAD treated by stent placement, shear rate in their coronary arteries may also be considered to be close to the normal value in the short term, due to the stent support against blood vessel wall. Thus, 500 s^{-1} shear rate was chosen as the principal rate to be used in *in vitro* flow experiments. Meanwhile, shear rates of 100 s^{-1} and 1500 s^{-1} were also selected to study the effects of different flow conditions on blood cell activation.

A pump was needed to produce blood flow and mimic the flow condition in blood vessels. Shear rate in the parallel-plate flow chamber was calculated according the equation:

$$\gamma = \frac{6Q}{wh^2} \tag{4.1}$$

where γ is the shear rate, Q is the flow rate, w is the flow chamber width and h is the chamber height. Based on the chamber gasket parameters described above, flow rates of about 160, 800, and 2400 μl per minute were required to produce shear rates of 100, 500, and 1500 s^{-1}, respectively.

Peristaltic pump

A peristaltic pump (MasterFlex 77120-32, Cole Parmer, Montreal, QC, Canada) was used to circulate blood samples. Silastic^{TM} laboratory tubing (Dow Corning, Midland, MI, USA) was used to connect the whole system, as shown in the schematic diagram in Figure 4.1(a). Silastic^{TM} tube was secured in the retainers and occlusion bed (Figure 4.1(b)).

Blood sample in a 5 ml polypropylene tube first went through the peristaltic pump, and then entered into the circular flow chamber, and finally returned to the polypropylene tube. Continous flow could be produced by repeating this cycle. Since the pump could not display the actual output flow rate, the appropriate setting for the selected flow rate was first determined by measuring the time blood took to go through a 20 cm long silastic tube, as shown in Figure 4.1(c).

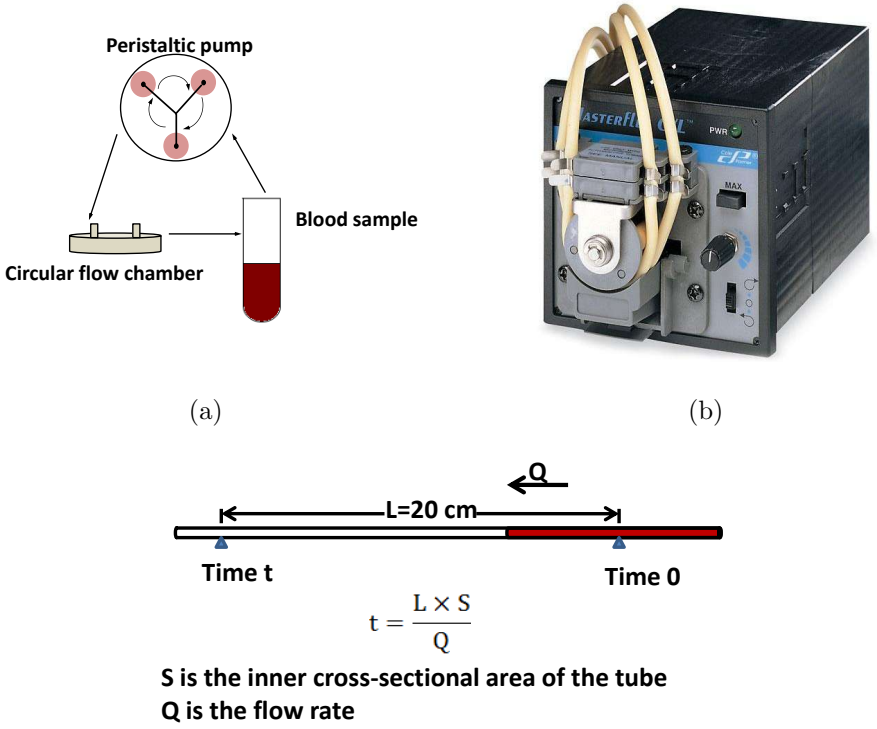


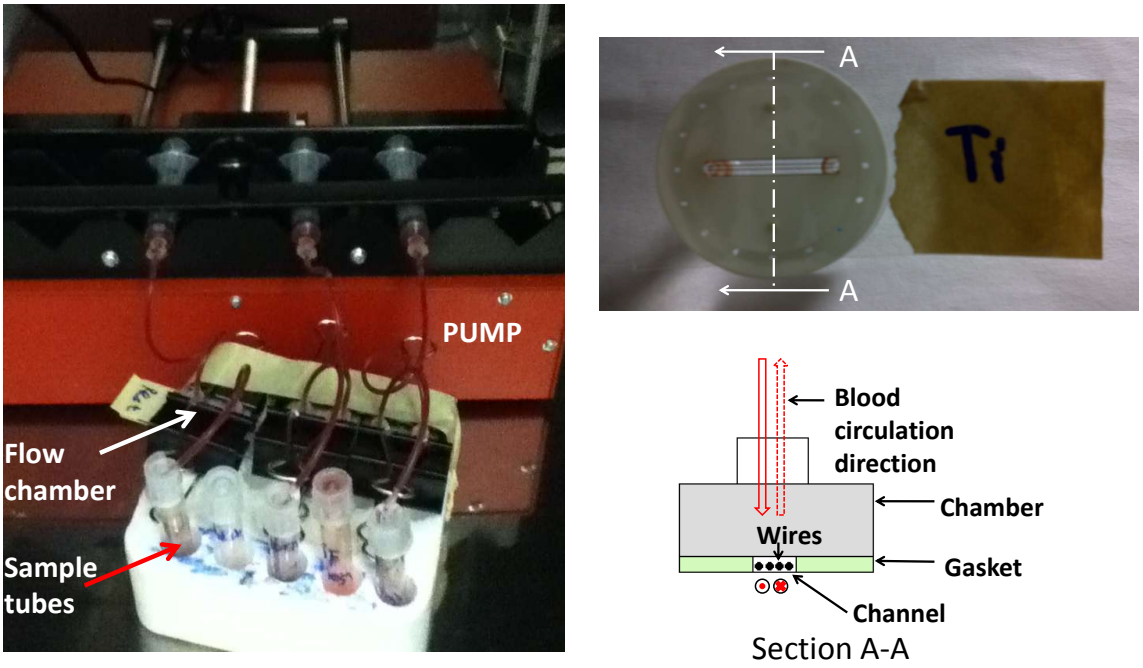
Figure 4.1: Peristaltic pump circulation models. (a) Schematic picture of blood circulation driven by peristaltic pump, (b) Peristaltic pump front view shows the tubing installation, (c) Flow rate was determined and then adjusted according to the time blood took to flow through a 200 mm long tube.

Syringe pump

A Cole-Parmer 75900-50 programmable 6-channel syringe pump was purchased to perform multiple sample tests simultaneously. Silastic tubing was used to connect the syringe, flow chamber, and blood sample tube (see Figure 4.2(a)). Blood was slowly withdrawn into the system. After the flow chamber and tubing were filled up with blood, the syringe was disconnected to expel the air bubbles and reconnected, and then loaded onto the syringe pump. To minimize further introduction of air bubble, standard BD 5 ml syringes were adopted.

According to the chamber width and length, 4 pieces of metal wires with a total length of 70 mm could be fixed into the chamber with minimum influence on the flow conditions. Figure 4.2(b) provides a bottom view of flow chamber preloaded with TiAl6V4 wires. A flow chamber configured without metal wire was used as negative control to obtain the baseline of shear effect. The glass slide surface was entirely covered by sterilized silicone sheet to minimize blood cell activation.

The total length of the tubing connecting the flow system was 340 mm, with a system dead volume of about 800 μl. This length was convenient for the experimental operation in the 37 ℃ incubator. A full circulation cycle was defined as blood sample being withdrawn into the syringe, then pumped back into the sample tube. Extra blood was needed to increase the circulation period. Taking the blood consumption and the times of blood flow direction change into account, a period of about 2 min was selected. Based on the above consideration, 0.95, 1.7, and 3.2 ml blood per sample was needed for the three different shear conditions.



(a) (b)

Figure 4.2: Blood circulation in syringe pump flow system. (a) Blood circulation in flow chambers was driven by the syringe pump, and the blood flow direction alternated once every cycle, (b) 70 mm metal wire was cut into 4 pieces to fit into the chamber (bottom view of the flow chamber and section view A-A).

Experimental controls

For all experiments, at the beginning of each experiment, a small aliquot of blood sample, -Rest0, was collected and directly processed for flow cytometry. Another 200 μl of heparinized whole blood, -Rest2h, was set aside in a microcentrifuge tube and incubated for 2 hours at 37 ℃ without flow or agitation. Blood samples added with LPS ($5\mu g$/ml) and EDTA (8mM final concentration) were also used as positive and negative control, respectively for the leukocyte study. Finally, as a positive control for platelet activation, thrombin peptide SFLLRN was added at the end of the 2-hour incubation to a small aliquot of Rest2h.

Platelet stimulation

To verify the performance of the *in vitro* flow model and assess how platelet activation, especially platelet microparticle formation, changed over time under flow condition, experiments with activated platelets were performed. Platelets were first pre-activated by adding 200 μl thrombin peptide SFLLRN into 3.5ml whole blood. After incubation at 37 ℃ for 30 min, flow-wholeblood sample was then circulated in flow chamber. The SFLLRN-activated blood was divided into 3 samples: (1) 1.7 ml of activated blood was directly circulated in flow chamber, flow-SF; (2) EDTA (85 μl, 8mM final concentration) was added to 1.7 ml of activated blood, and then circulated in flow chamber, flow-SF+EDTA; (3) 100 μl of activated blood was left "sitting" for 2 hours at 37 ℃, SF-rest. At the beginning of flow experiment, 200 μl of normal whole blood in a polypropylene tube was used as control Rest2h. Another 1.7 ml normal whole blood was also circulated in the flow chamber, flow-wholeblood. Small aliquots of blood were collected at 15 min, 60 min, 90 min, and 120 min to analyze the changes in platelet activation over time. To measure platelet activation

under pathological shear conditions, 3 ml blood sample or PRP were circulated at shear rate of 6200 s^{-1} for up to 45 min.

4.2.3 Flow cytometry

Flow cytometer

Flow cytometry is able to count and measure the properties of microscopic particles. After staining with fluorescent antibodies, samples are injected into a flow cytometer and enclosed by an outer sheath that contains faster flowing fluid, as shown in Figure 4.3. The sheath creates a single file of cells or microparticles which then passes through one or more laser beams. Light scattering or fluorescence emission provides information about the particle properties. Various dyes with difference absorbance and emission wavelength can be used to label cells, such as FITC, PE, Percp, and Alexa Fluor^{TM} series. The optical signal on cells is then selectively filtered by different lens and detected by separate fluorescence (FL) channels.

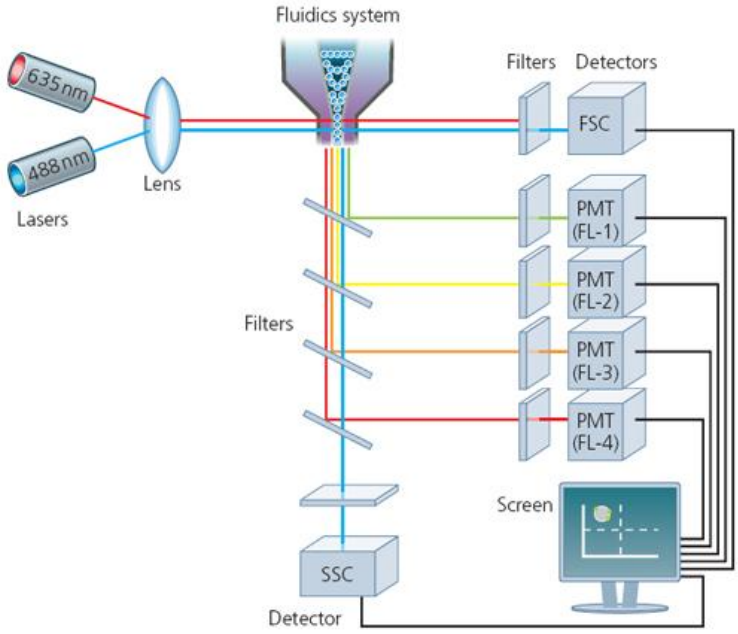


Figure 4.3: Schematic overview of a typical flow cytometer setup (figure source: Abdserotec Co.).

Fluorescent antibody labeling

In the present experiments, flow cytometry samples were processed before and after blood incubation with stent materials. Small aliquots of blood (5 and 30 μl) were diluted in 50 μl HEPES-Tyrode's buffer (HTB: 137mM NaCl, 2.7mM KCl, 5mM $MgCl_2$, 3.5mM HEPES, 1g/L Glucose, and 2g/L BSA, PH 7.4) containing saturating concentrations of monoclonal antibodies. Specifically, 8 μl anti-CD61 antibody was added to each platelet sample. For leukocyte study, 7 μl anti-CD45 antibody was added to all leukocyte samples. Leukocyte samples were divided into three groups: (1) group CD11b to study leukocyte activation, (2)

group CD61 to study leukocyte-platelet aggregates, and (3) group TF to study monocyte tissue factor expression. 5 μl anti-CD11b antibody was added to samples in group CD11b, 8 μl antibody anti-CD61 was added to samples in group CD61, 6 μl anti-TF antibody was added to samples in group TF respectively. All samples were incubated for 30 min at 4℃.

After incubation, samples for platelet study were diluted with 200 μl HTB and fixed with 200 μl 2% paraformaldehyde (final concentration 1%). For leukocyte samples, red blood cells were lysed with 700 μl FACS lysing solution, as they could hinder the detection of the target population. All leukocyte samples were incubated in the dark for 10 min. After that, leukocyte samples were washed at 1200 rpm for 6 min, and diluted with 150 μl HTB and fixed with 150 μl 2% paraformaldehyde.

Blood cell identification

In the present study, prepared blood samples were read on a three-color FACSCalibur flow cytometer (Becton Dickinson). Data acquisition and analysis were performed using CELL-Quest software (Becton Dickinson). Blood cells were normally detected by their forward scatter (FSC) and side scatter (SSC) profiles, corresponding to cell size and cell complexity (Figure 4.4). In the absence of a monoclonal antibody against a specific antigen, neither platelets nor leukocytes could be reliably identified in whole blood with FSC *vs* SSC characteristics.

Upon the addition of FITC labeled anti-CD61 antibody, platelets were identified by CD61-positive events in fluorescent channel-1 (FL-1). Platelet microparticles (PMPs) were defined as events being CD61 positive but smaller than intact platelets, based on forward light scatter, and were reported as a percentage of total platelet events. A FSC median value of platelets was first obtained from the initial blood sample Rest0, the median value is directly proportional to the average size of platelets in blood sample. The FSC upper limit

of PMPs was set to 35% of the median value of platelets, because PMPs are less than 1 μm and platelets are around 2-3 μm. Fluorescence intensities of CD61 and P-selectin on both platelets and platelets + PMPs were also recorded as a measure of platelet activation. The results are later reported either directly in arbitrary fluorescent units or as a percentage expression (up or down regulation) relative to one of the experimental controls.

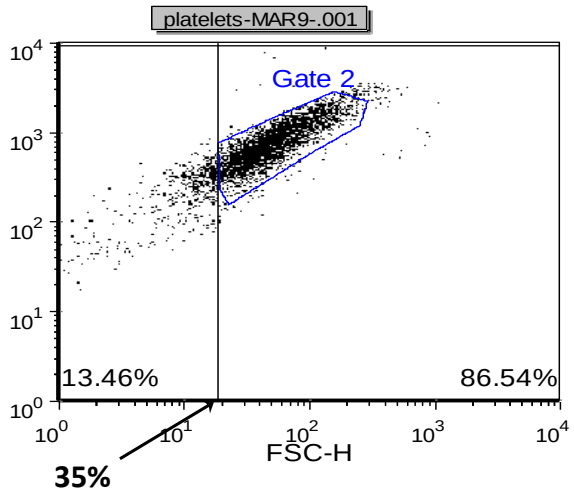


Figure 4.4: Flow cytometric analysis of platelets. Platelets and PMPs were analyzed by FSC *vs* SSC dot plot. Both FSC and SSC were set to logarithmic mode. Platelets were identified in Gate 2. Left quadrant represents the percentile of PMPs.

As shown in Figure 4.5(a), leukocyte subpopulations were clearly identified by a dot plot of FSC *vs* SSC after red blood cells lysis. Neutrophils and monocytes were gated for further analysis. The activation of leukocytes was quantified by measuring CD11b expression. Formation of leukocyte-platelet aggregates was determined using the fluorescent intensities of CD61 on leukocytes as illustrated in Figure 4.5(c). Tissue factor expression on monocytes was determined by double gating the monocyte population based onon size characteristic

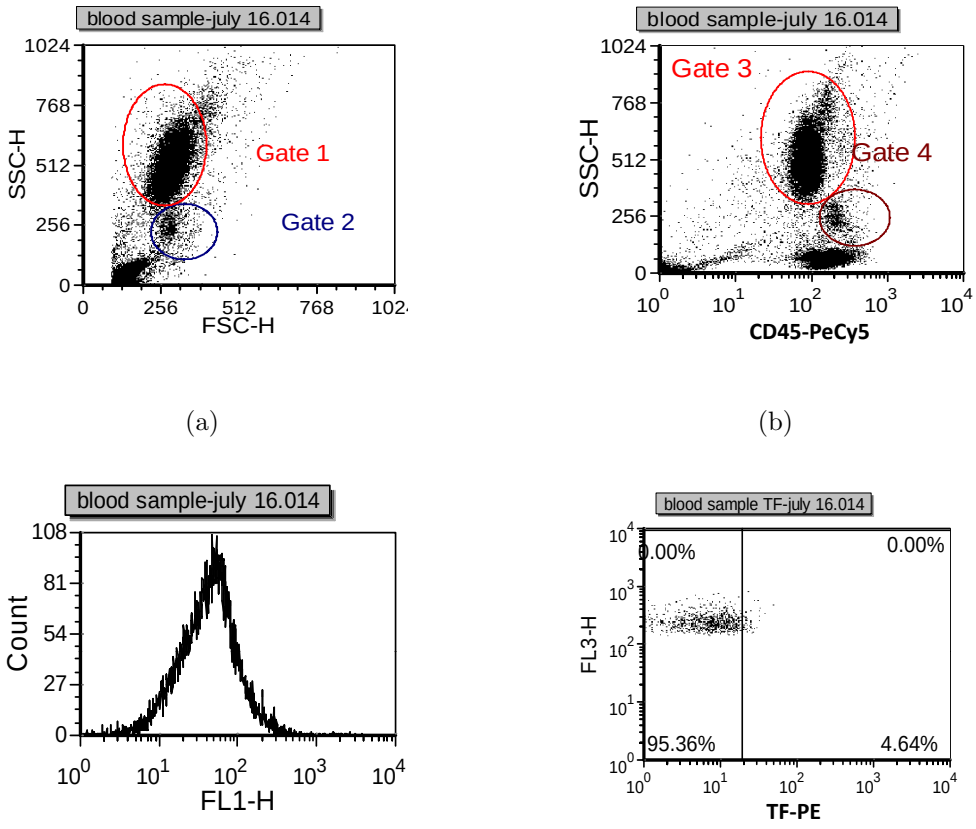


Figure 4.5: Flow cytometry analysis of leukocytes. (a) Leukocytes can be distinguished according to their FSC *vs* SSC characteristics; gate 1 identifies PMNs while gate 2 is for monocytes. (b) Leukocytes can also be identified in a dot plot of SSC versus PE-Cy5 conjugated anti-CD45 antibody (FL-3 channel); gate 3 identifies PMNs while gate 4 monocytes. (c) Channel FL-1 fluorescent histogram where FITC conjugated anti-CD11b and anti-CD61 antibodies can be observed. (d) Dot plot of monocyte population against fluorescent signal of PE-conjugated anti-TF antibody (channel FL-2).

and anti-CD45 positivity (Gate 2 and Gate 4). Both the fluorescent intensity and the percentage of monocytes staining positive for TF expression were recorded (Figure 4.5(d)). The leukocyte results are later reported as a percentage expression (up or down regulation) relative to one of the experimental controls.

4.2.4 Scanning Electron Microscopy analysis

For electron microscopy of adherent blood cells, ST316L and TiAl6V4 metal samples, and a piece of silicone sheet in flow chamber were rinsed with 0.2mM sodium phosphate buffer (v/v 36 ml Na_2HPO_4: 14 ml NaH_2PO_4 PH 7.2) after blood-material incubation, fixed with 2.5% glutaraldehyde and stored at 4 ℃ for a maximum of 30 days. The day before microscopic analysis, samples were dehydrated with a graded series of ethanol dilutions (25% to 100%) and dried overnight. The metal samples were coated with 8-10 nm thick gold by sputtering, and observed with a scanning electron microscope (LEO FESEM 1530) for cell adhesion and aggregation analysis.

4.2.5 Statistical analysis

All results are reported as the mean ± standard deviation (SD). To evaluate the effects of stent metals on platelet and leukocyte activation under the same blood flow condition, one-way ANOVA followed by Tukey HSD test was carried out using SAS 9.2 (Cary, NC, USA). A p value of less than 0.05 was required for statistical significance. The number of experiments was always equal to or greater than three.

Table 4.1: Flow cytometry experiment summary

Static condition	Heparinized whole blood was incubated with TiAl6V4 and ST316L disks, silicone sheet, and polystyrene for 2 hrs at 37 ℃. Platelt study: negative control-Rest0, positive control-SFLLRN. Leukocyte study: negative control-EDTA and Rest2h, positive control-LPS.
	Platelet rich plasma (PRP) was incubated with TiAl6V4 and ST316L disk for 2 hrs at 37 ℃. Negative control: Rest2h
Flow condition	Peristaltic pump: heparinized whole blood was circulated at 500 s^{-1} in flow chamber loaded with ST316L wires and no wires. Negative control: Rest2h
	Syringe pump: 1. Heparinized whole blood was circulated at 100 s^{-1}, 500 s^{-1}, and 1500 s^{-1} for 2 hrs at 37 ℃ in flow chambers loaded with TiAl6V4 wire, ST316L wire, and without wire. Platelt study: negative control-Rest0, positive control-SFLLRN. Leukocyte study: negative control-EDTA and Rest2h, positive control-LPS 2. Heparinized whole blood and PRP were circulated at 6200 s^{-1} for 30-45 min at 37 ℃. Flow cytometry results before and after circulation were compared. 3. Flow-sf, Flow-sf+edta, and Flow-wholeblood were circulated at 500 s^{-1} for 2 hrs at 37 ℃. Controls: SF-rest and Rest2h.

Chapter 5

Results

In this section, flow cytometry data from blood samples incubated under static and three different shear conditions are presented. The effects of material and shear stress on platelet and leukocyte activation, leukocyte-platelet aggregation, and tissue factor expression are compared. SEM images of cell adhesion and aggregation, and fibrin deposition on the surface of materials are presented.

5.1 Blood Cell Activation Under Static Condition

5.1.1 Platelet activation in platelet rich plasma

After TiAl6V4 and ST316L metal disks were incubated with 300 μl platelet rich plasma (PRP) at 37 ℃ for two hours, platelet activation was measured by flow cytometry with the PRP Rest2h being used as a negative control. Table 5.1 presents the data in terms of platelet microparticle (PMP) formation and platelet activation using the expression of

CD61 on intact platelets and all CD61 positive events (platelets and platelet microparticles). No significant difference in platelet microparticle formation in PRP was found between the Rest2h and metal samples. However, with PRP having contacted TiAl6V4, the CD61 signal intensity for all platelet events was significantly lower than ST316L (p=0.036) and Rest2h (p=0.025) samples.

Table 5.1: Platelet activation after metal disks incubated with PRP (n=3)

Marker	% PMPs	Platelet % CD61	Total % CD61
PRP TiAl6V4	8.8±2.8	93.8±7.7	92.7±3.9 *
PRP ST316L	8.9±4.5	101.8±8.6	100.7±2.6
PRP Rest2h	7.8±3.2	100±0	100±0

% PMPs represents the percentage of platelet microparticle formation in blood, as identified by sidescatter characteristics and CD61 positive events.
% CD61 signal expressed as a relative percent to the PRP Rest2h.
* TiAl6V4 significantly different from ST316L and Rest2h (p<0.04).

5.1.2 Platelet activation in whole blood

As expected, platelets were significantly activated with SFLLRN (positive control) with a platelet microparticle formation of about 60%, confirming that platelets functioned properly and thus would be able to respond adequately to a platelet stimulus. As can be seen in Table 5.2, no difference in platelet activation was found between the negative control

(Rest0) and all biomaterials that were incubated with blood for 2 hrs under static conditions. Compared with other biomaterials, TiAl6V4 had the lowest CD61 signal intensity, albeit not statistically significant.

Table 5.2: Platelet activation after incubation in blood under static conditions (n=3)

Marker	% PMPs	Platelet % CD61	Total % CD61
Rest0	6.9±2.1	100±0	100±0
SFLLRN	59.7±10.8†	94.5±5.8	60.9±4.4†
TiAl6V4	4.8±2.1	80.6±3.0^{*1}	82.2±1.9^{*2}
ST316L	5.0±2.4	94.6±7.0	96.3±5.8
Silicone	5.5±2.8	98.5±10.2	100.0±9.2
Polystyrene	6.1±3.8	97.6±0.7	98.6±2.2

% PMPs represents the percentage of platelet microparticle formation in blood, as identified by sidescatter characteristics and CD61 positive events.

% CD61 signal is expressed as a relative percent to the negative control Rest0.

^{*1} TiAl6V4 was significantly lower than Rest0, Silicone, and Polystyrene ($p<0.05$).

^{*2} TiAl6V4 sample was significantly lower than other samples ($p<0.05$).

† SFLLRN was significantly different from other samples ($p<0.002$).

5.1.3 Leukocyte activation in whole blood

Neutrophils and monocytes were identified using the anti-CD45 antibody. Antibodies against CD11b, CD61, and TF were employed to study leukocyte activation, leukocyte-platelet aggregation, and TF expression on monocytes. Leukocytes activation in negative control EDTA and positive control LPS are also reported to ensure the validity of experimental data and demonstrate the ability of blood leukocytes to be activated under normal conditions. Leukocyte activation induced by materials under static condition is reported in Table 5.3.

As expected, the neutrophil CD11b up-regulation in EDTA and Rest2h samples was low. Contact with metallic materials led to a higher expression of CD11b on neutrophils, and was in the order of Silicone≈ST316L<TiAl6V4≈Polystyrene although no statistically significant differences were observed with the Rest2h. With CD11b up-regulation on monocytes, high variations in expression were obtained between experiments making any comparison between sample conditions difficult.

With exposure to biomaterial under static conditions, formation of leukocyte-platelet aggregates appeared to be similar for all materials. Higher CD61 intensities were usually observed on monocytes, suggesting that an increased number of platelets bound to monocytes compared to neutrophils. Surprisingly, compared to all other conditions, leukocyte-platelet aggregates in the two-hour resting blood sample (Rest2h) was significantly higher. Minimal tissue factor expression on monocytes (both the expression and the population staining positive for TF) was observed in all test materials.

Table 5.3: Leukocyte activation after incubation in blood under static condition (n=3)

Marker	% CD11b		% CD61		% TF	
	neutros	monos	neutros	monos	monos	% monocytes express TF
TiAl6V4	47.0±11.4	58.1±35.5	25.0±3.3	44.4±13.7	96±14.8	2.8±1.7
ST316L	39.8±4.0	57.1±27.0	27.3±13.4	47.6±19.2	97.1±7.1	2.0±1.4
Silicone	39.6±5.2	57.9±22.4	23.9±6.0	44.3±8.3	95.1±16.6	3.3±2.4
Polystyrene	48.0±2.9	61.9±27.5	25.5±9.2	43.6±13.6	101.5±10.3	2.0±1.4
EDTA	22.7±13.4	44.2±32.0	32.8±26.4	66.1±17.4	89.2±11.2	2.4±1.9
LPS	100±0†	100±0†	41.6±23.6	75.6±9.1	100±0	53.3±15.9†
Rest2h	29.0±14.5	46.3±25.8	100±0‡	100±0	117±0.9	4.6±4.1

% CD11b and % TF expression on monos are expressed as a percent of fluorescent signal relative to the positive control LPS, while the % CD61 is based on Rest2h.

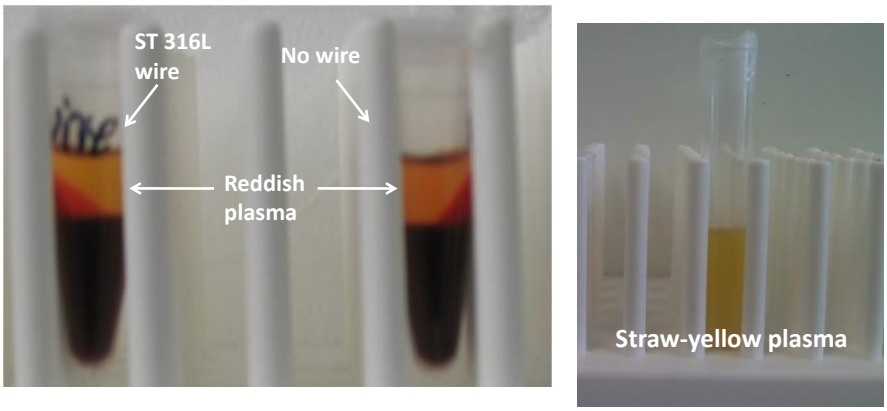
% monocytes express TF is represented as the percentage of monocytes staining positive for TF.

† LPS was significantly different from all other groups.

‡ Neutrophil-platelet aggregates were significantly different from all other groups.

5.2 Blood Cell Lysis in The Peristaltic Pump System

A blood flow experiment was first performed using the peristaltic pump. After circulating at a shear rate of 500 s^{-1} for two hours, 1 ml blood was collected from each sample and transferred into a small vial. After centrifuging at 1000 g for 10 min, reddish plasma was observed for both blood samples with and without metal wires (Figure 5.1(a)). The normal color of plasma being yellow (Figure 5.1(b)), it was obvious that red blood cell lysis had occurred during the recirculation with the peristaltic pump. The fact that lysis was observed in both samples, suggested that it was not the metal wires that were responsible for the lysing but the *in vitro* flow system. It is also likely that other types of blood cells were damaged in this *in vitro* blood flow experiment.



(a) (b)

Figure 5.1: Blood lysing with peristaltic pump. (a) Reddish plasma after circulation with and without metal wires, (b) Normal color of platelet rich plasma.

Table 5.4 reports the platelet and leukocyte activation after two-hour incubation. Compared with the control Rest2h, the lower CD61 values observed for both flow samples suggest potential platelet damage. On the other hand, both CD11b up-regulation and

leukocyte-platelet aggregates were higher in flow samples, but in the context of the red blood cell lysis observed under flow conditions, it is difficult to interpret these results as true material-induced activation. None of the blood samples had significant monocyte TF expression.

Table 5.4: Platelet and leukocyte activation after recirculation (n=1)

Marker	% PMPs	Platelet CD61	neutros CD11b	monos CD11b	MPA CD61	monos TF	% monos express TF
ST316L	27.2	150	453	403	1192	8.4	0.7
No wire	12.6	192	254	308	784	7.9	0.5
Rest2h	19	210	145	128	583	9.1	1.8

% PMPs represents the percentage of platelet microparticle formation in blood, as identified by sidescatter characteristics and CD61 positive events.
CD61, CD11b, MPA, and TF are presented as the signal in arbitrary fluorescent units of gated platelets, leukoyctes, monocyte-platelet aggregates, and monocytes.
% monos express TF shows the percent of TF positive monocytes.

5.3 Blood Cell Activation Under Shear Conditions

5.3.1 Platelet activation

Platelet activation under normal shear rates

As shown in Table. 5.5, the formation of platelet microparticles in blood was neither affected by material nor shear conditions. Expression of CD61 on platelets was comparable between materials and shear conditions, except for TiAl6V4 at 1500 s^{-1} where an increase was found. Results from P-selectin expression (CD62p) further confirmed this observation.

Table 5.5: Platelet activation under shear conditions

	% PMPs			Platelet % CD61			Total % CD61			% platelet CD62p+	
Shear	100 s^{-1}	500 s^{-1}	1500 s^{-1}	100 s^{-1}	500 s^{-1}	1500 s^{-1}	100 s^{-1}	500 s^{-1}	1500 s^{-1}	100 s^{-1}	1500 s^{-1}
Rest0	4.9±0.9	8.7±2.9	6.1±1.0	100±0	100±0	100±0	100±0	100±0	100±0	5.2±2.8	8.5±3.9
TiAl6V4	9.9±1.4	9.6±5.4	8.7±2.4	90.3±11.8	89.9±11.6	124±32.3	86.6±15.1	88.7±8.6	119±27.3	8.7±3.0	17.2±9.2
ST316L	9.0±3.6	9.8±4.9	9.7±3.4	88.8±9.1	95.5±11.9	104±7.0	85.7±13.9	94.9±9.4	99±3.4	9.1±4.7	12.4±5.5
No Wire	11.6±4.0	7.7±2.1	10.4±4.4	87.8±10.9	88.7±9.8	108±1.4	82.3±8.3	89.8±8.6	102±6.7	9.3±3.0	13.4±5.7

% PMPs represents the percentage of platelet microparticle formation in blood, as identified by sidescatter characteristics and CD61 positive events.

% CD61 signal is expressed as a relative percent to the negative control Rest0.

N=3 for 100 s^{-1} and 1500 s^{-1} groups. N=5 for 500 s^{-1}.

SFLLRN induced platelet activation at 500 s^{-1}

As increasing shear rate from 100 s^{-1} to 1500 s^{-1} did not appear to induce significant platelet microparticle formation, to ensure that platelet microparticles were not disappearing over time in the *in vitro* flow model, activated blood sample containing a significant population of platelet microparticles was circulated in the flow system for 2 hours at 500 s^{-1}. Blood was first activated by SFLLRN (as described earlier in the previous section) and the level of platelet microparticles was then monitored over 2 hours in the flow system.

After activation with SFLLRN, the initial platelet microparticle level in activated blood was 30±7%, and 6±0.9% PMPs were present in non-activated blood. As can be seen in Figure 5.2, an increase in microparticle formation was initially observed in SFLLRN activated blood samples under flow conditions. However, with increasing recirculation time, platelet microparticle presence in activated blood then decreased. Over the 2 hour recirculation at 500 s^{-1}, a small but steady increase in platelet microparticle formation was observed in normal blood. At 2 hours, 12±6% microparticles were present in normal blood circulated at 500 s^{-1}, which was in agreement with those previous results mentioned in Table 5.5.

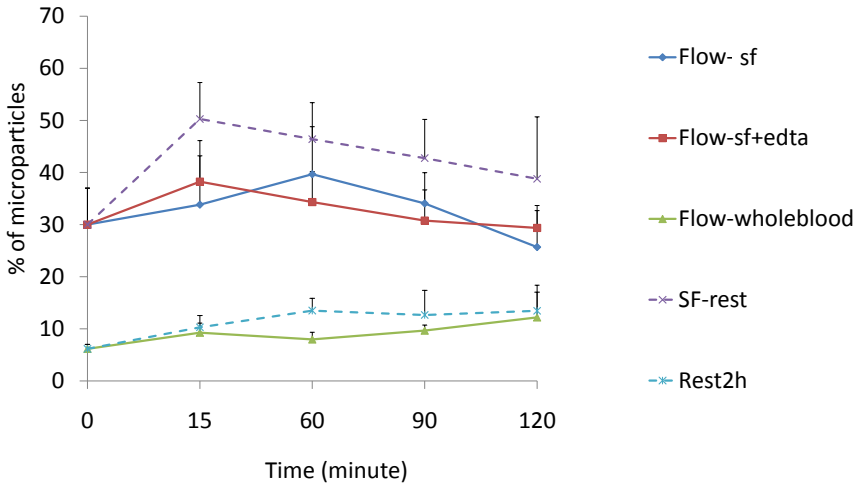


Figure 5.2: Platelet microparticles in blood exposed to 0 and 500 s^{-1} over 2 hours (n=3). All SF samples were from the same initial blood sample that had been activated with SFLLRN to generate platelet microparticles. EDTA was added to activated blood to prevent binding of platelets to leukocytes. Flow-sf: activated blood sample circulated at 500 s^{-1}; Flow-sf+edta: activated blood sample was inhibited with EDTA and then circulated at 500 s^{-1}; Flow-wholeblood: normal blood sample circulated at 500 s^{-1}; SF-rest: sitting activated blood sample; Rest2h: sitting normal blood sample.

Platelet activation under pathological shear condition

To further test the ability of the *in vitro* flow model to generate platelet microparticles, platelets were tested under higher shear conditions. Three milliliters of blood (n=2) and platelet rich plasma (PRP) (n=1) was circulated at 6200 s^{-1} for up to 45 min, and significant platelet activation was observed. Table 5.6 shows that PMP percent almost doubled in both whole blood and PRP sample after circulation. Correspondingly, the average size of platelets decreased, leading to the decrease of the total CD61 signal. CD61 fluorescent intensity of gated platelets in blood A and PRP sample decreased, while it slightly increased in blood B. Different circulation time (Blood A 30 min; Blood B: 45 min); the platelet activation status before circulation may have lead to the difference. Reddish plasma was obtained after centrifugation (Fig 5.3), suggesting blood cell lysed during recirculation.

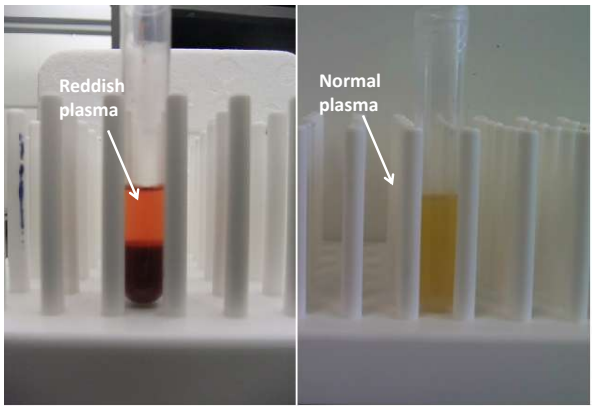


Figure 5.3: Blood cell lysis (left tube) after 30 min circulation at 6200 s^{-1}.

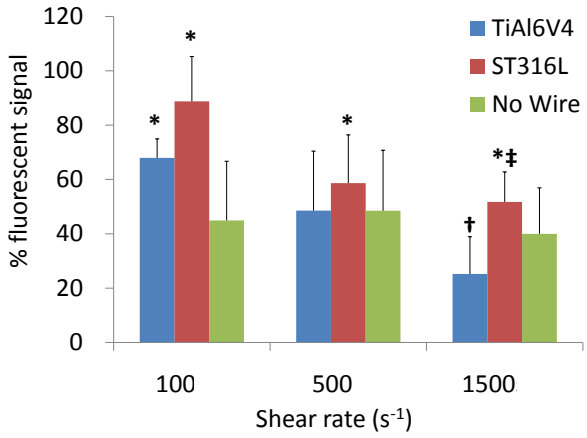
Table 5.6: Platelet activation at 6200 s^{-1}

	Shear	% PMP	Platelet CD61	Total CD61
Blood A	before	15.0	228	189
	after	26.7	154	112
Blood B	before	11.2	113	103
	after	27.4	117	97
PRP	before	5.4	202	188
	after	13.1	151	128

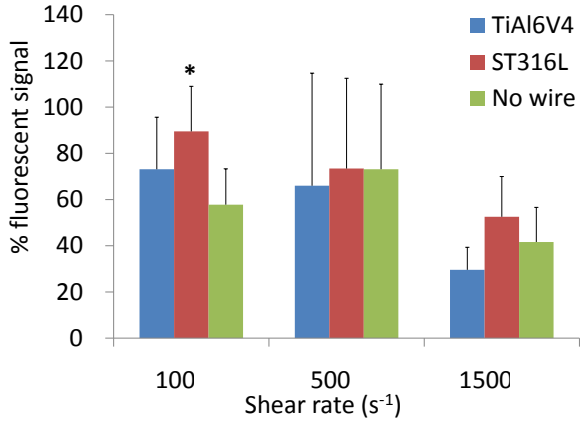
% PMPs represents the percentage of platelet microparticle formation in blood, as identified by sidescatter characteristics and CD61 positive events.
CD61 represents the absolute value of fluorescent intensity.

5.3.2 Leukocyte activation

Leukocyte activation as expressed by CD11b up-regulation is presented in Figure 5.4. Generally, leukocyte activation in positive control LPS was significantly higher than all other samples. As shear rate increased, the leukocyte activation in TiAl6V4 and ST316L samples decreased. The result shows that TiAl6V4 induced less leukocyte activation than ST316L under three shear conditions, whereas their difference at a specific shear rate was not significant. Neutrophil activation in ST316L was significantly higher than EDTA and Rest2h sample under all three shear rates. When shear rate increased from 100 s^{-1} to 1500 s^{-1}, neutrophil activation induced by TiAl6V4 ($P=0.0102$) and ST316L ($p<0.05$) wire decreased significantly. Shear effect on monocyte activation in both TiAl6V4 and ST316L sample were not significant.



(a)

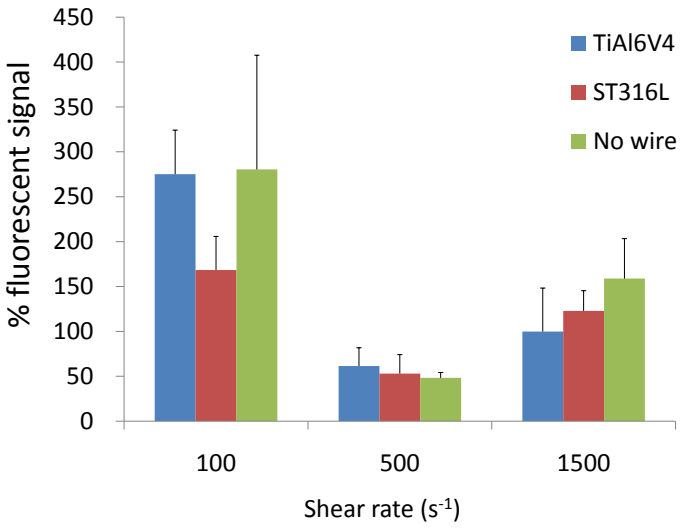


(b)

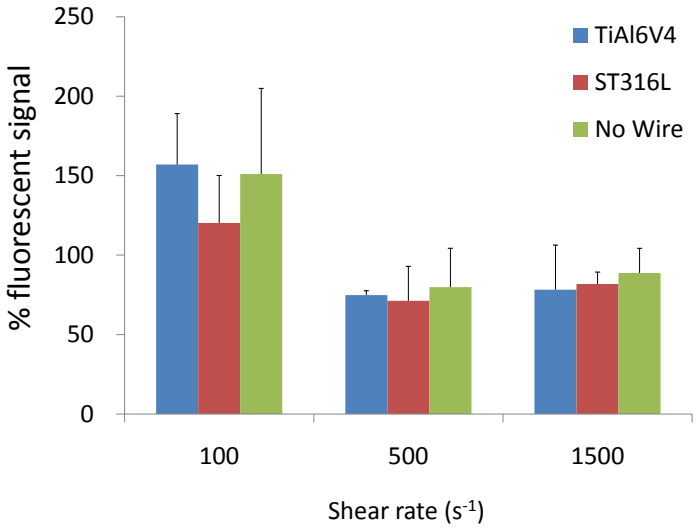
Figure 5.4: (a) Neutrophil and (b) monocyte activation under three shear rates. The values are expressed as a percent of CD11b fluorescent signal relative to positive control LPS. Leukocyte activation in LPS, EDTA, and Rest2h were consistent between experiments. The extent of activation in EDTA and Rest2h was similar, and was 17±6.7% for neutrophil activation and 34±13.9% for monocyte activation. ∗ Significantly higher than EDTA and Rest2h. † Significantly lower than TiAl6V4 at 100 s^{-1} and 500 s^{-1}. ‡ Significantly lower than ST316L at 100 s^{-1}. (n=3 to 5, mean±SD).

Under flow conditions, more leukocyte-platelet aggregates were formed in TiAl6V4 samples rather than in ST316L samples, except for the result in the 1500 s^{-1} group. The leukocyte-platelet aggregates in TiAl6V4 at 100 s^{-1} were significantly higher than those at 500 s^{-1} and 1500 s^{-1}. However, the Rest2h control at 500 s^{-1} was higher than the other two shear conditions, which might lead to relatively lower leukocyte-platelet aggregates.

Monocyte TF expression in TiAl6V4 sample was lower than in ST316L as can be seen from the fluorescent intensity of anti-TF antibody (Figure 5.6(a)). As shear rate increased, the monocyte tissue factor expression decreased, albeit not significantly. The maximal percent of TF positive events was obtained at 500 s^{-1}, but the variability between donor responses was high. The percent of TF positive cells in TiAl6V4 at 1500 s^{-1} was significantly less than that in ST316L ($p=0.02$).

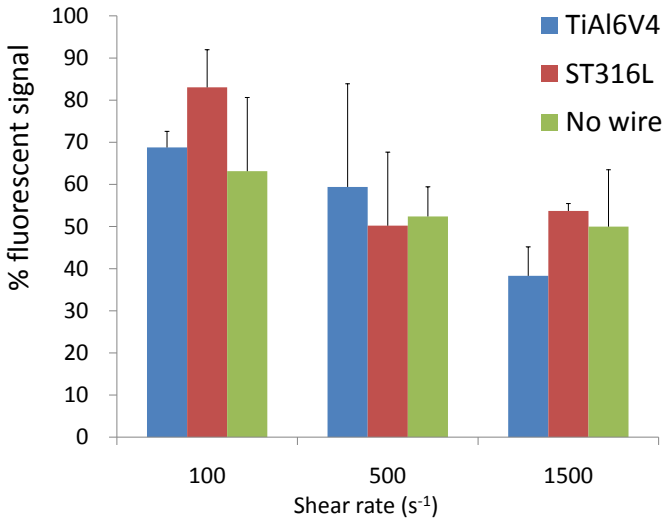


(a)

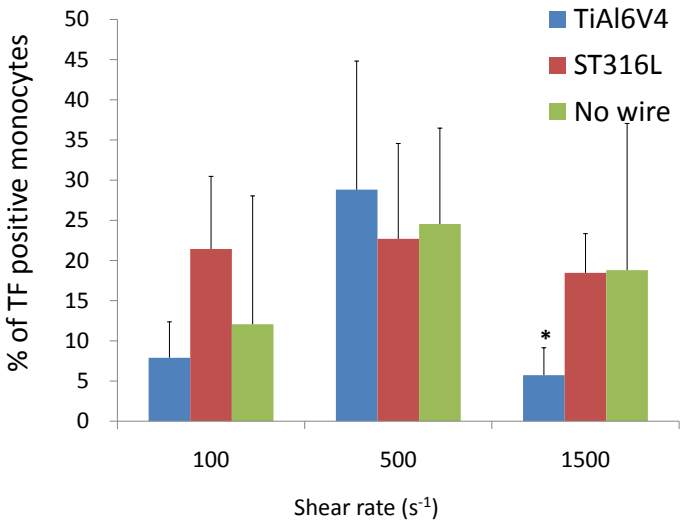


(b)

Figure 5.5: (a) Neutrophil-platelet aggregates, and (b) monocyte-platelet aggregates formation under three shear conditions. Fluorescent intensity of samples is expressed as a percent relative to Rest2h. (n=3 to 5, mean±SD).



(a)



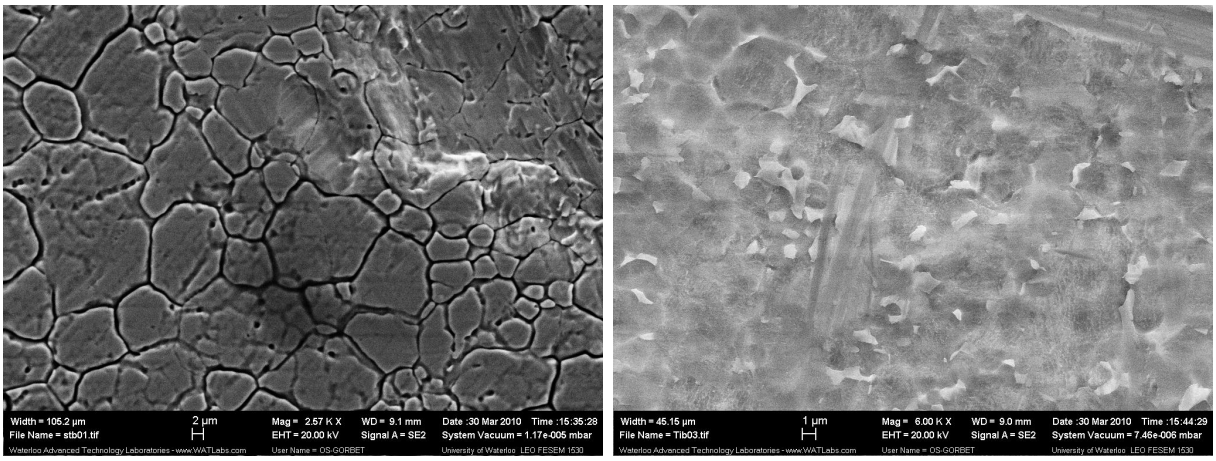
(b)

Figure 5.6: (a) TF expression on monocyte is expressed as a percent of fluorescent signal relative to LPS. Same extent of monocyte TF expression (42±5.8%) was obtained in EDTA and Rest2h. (b) Percent of monocytes staining positive for TF. * Significantly lower than other groups. (n=3 to 5, mean±SD).

5.4 Morphological Analysis of SEM Images

5.4.1 Bare metal disks

In order to explore the role of biomaterial-mediated cell adhesion and activation, SEM images of bare TiAl6V4 and ST316L disks were first obtained. Both disks were washed following the protocol described in Section 4.1.2. As can be seen from Figure 5.7, a "basin" was located at the top right corner, and smooth "islands" were separated by cracks on the ST316L disk surface. The TiAl6V4 disk featured some protrusive irregular shaped "grains" on its surface.



(a) (b)

Figure 5.7: SEM images of bare metal disk. (a) cracked ST316L surface, and (b) protrusive TiAl6V4 surfaces.

5.4.2 Metal disks incubated with blood under static condition

Red blood cell adhesion and aggregates, and large flake-like fibrin were frequently seen on the surface of ST316L disk as shown in Figure 5.8(a). The thickness of fibrin was more than 2-3 times the diameter of red blood cells. It appears that fibrin deposition may promote red blood cell adhesion and aggregation as red blood cell aggregates were often surrounded by fibrin, as shown in the magnified image in Figure 5.8(a). Platelets (and possibly some microparticles) were also attached to the fibrin. Figure 5.8(b) shows that highly activated platelets generated tethers and formed net-like platelet aggregates along fibrin. Some activated leukocytes were also observed on the metal surface (Figure 5.8(c)).

Short fibrin tethers but not large flake-like fibrin were always observed on the surface of TiAl6V4 disk (Figure 5.8(d)). Activated platelets with clear pseudopods, and small aggregates were presented in Figure 5.8(e). Red blood cells and their aggregates were also found on TiAl6V4 surface (Figure 5.8(f)). Comparing the two metal surfaces, there were less cell adhesion and aggregates on TiAl6V4 disk.

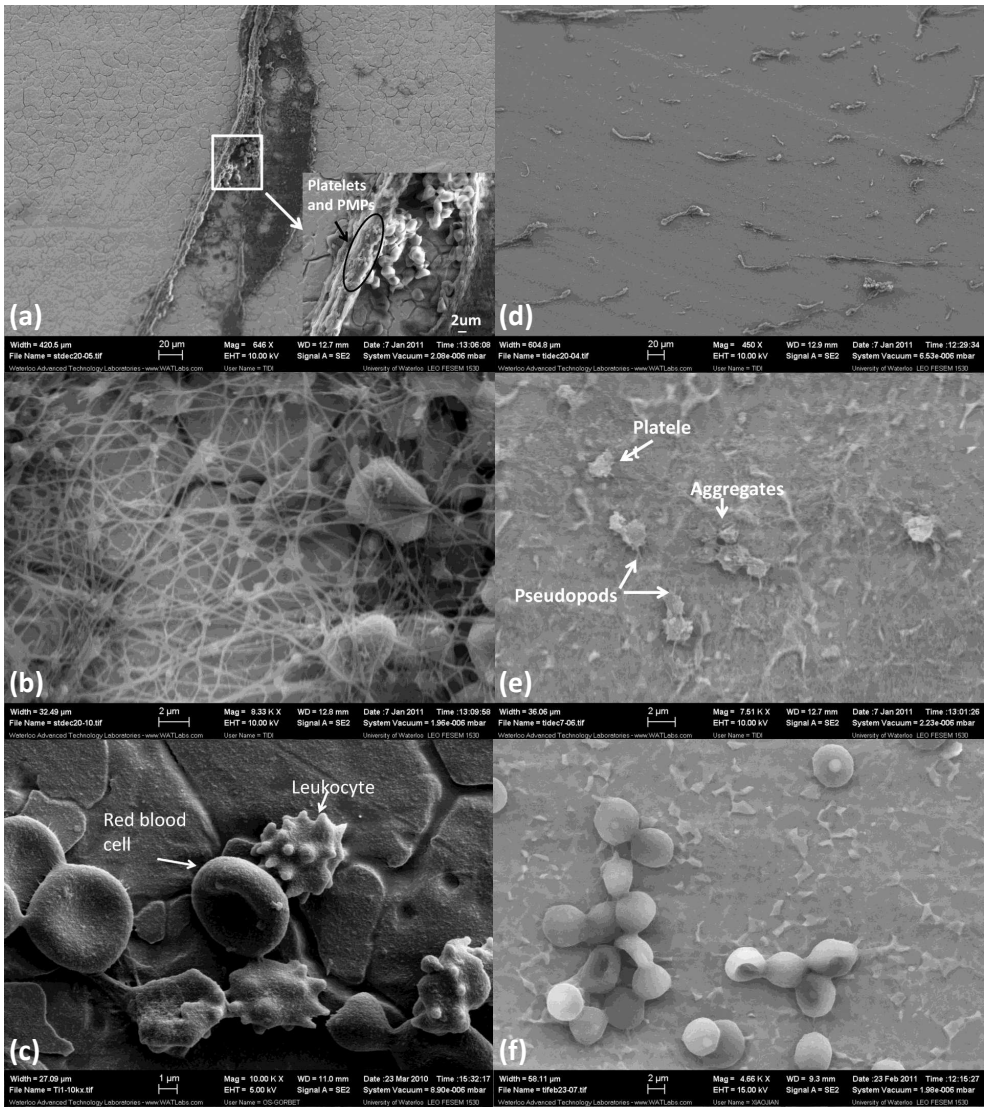
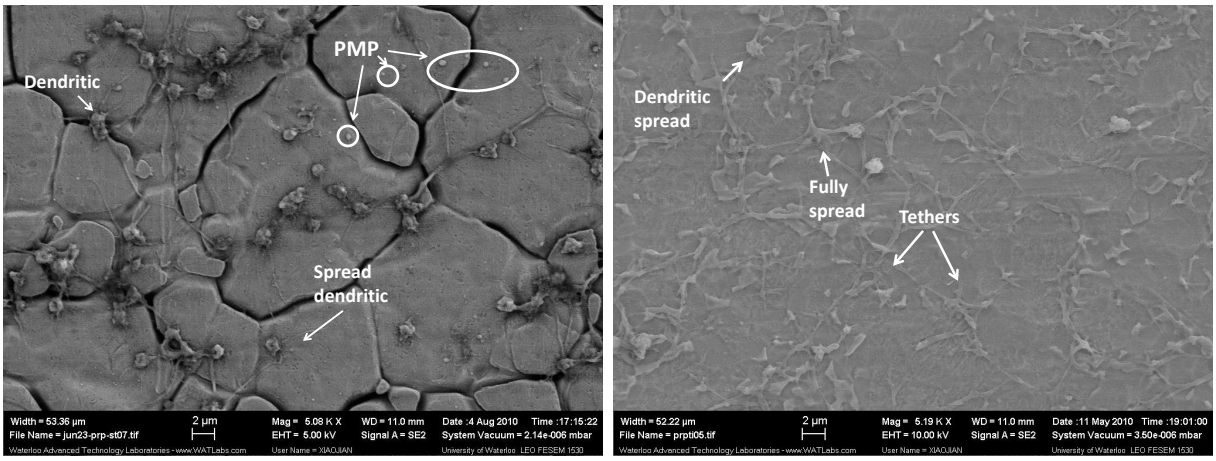


Figure 5.8: Blood cell adhesion and aggregation on metal disks under static condition. (a) to (c) display fibrin clot and cell adhesion on ST316L disk (magnification 646X, 8.33kX, and 10kX), while (d) to (f) illustrate TiAl6V4 disk surface after static incubation (magnification 450X, 7.51kX, and 4.65kX).

5.4.3 Metal disks incubated with platelet rich plasma

Fibrin tethers and large number of platelets and platelet aggregates were adhered on the surface of ST316L disk, as shown in the Figure 5.9(a). Two morphological forms of activated platelets can be identified: dendritic and spread dendritic, indicating different activation stages. Some small particles, most likely PMPs, were also present on the surface. Figure 5.9(b) shows only a few platelets were on TiAL6V4 surfaces. However, these platelets were highly activated and mainly in the spread dendritic and tethered morphological form.



(a) (b)

Figure 5.9: (a) ST316L disk and (b) TiAl6V4 disk incubated with platelet rich plasma.

5.4.4 Metal wires under flow conditions

Under shear rate of 100 s^{-1}

At 100 s^{-1}, small pieces of fibrin were spotted on the surface of the ST316L wire (see Figure 5.10(a)). Blood cell aggregates or PMPs covered by a thin film of fibrin were

observed in Figure 5.10(b). Fibrin clots were also present on the TiAl6V4 wire surfaces, while only a few platelets could be identified on the surface (Figure 5.10(c) and (d)).

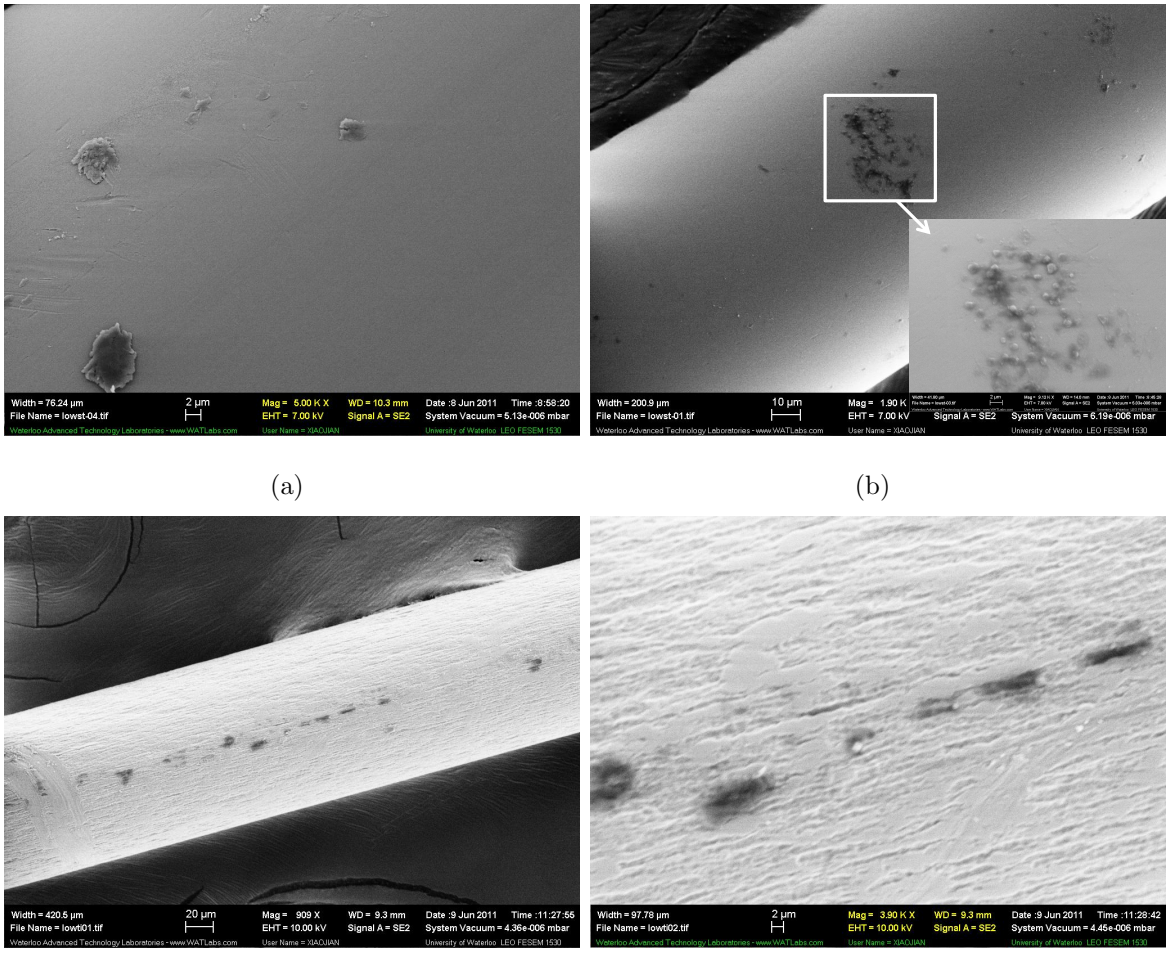


Figure 5.10: Cell adhesion and fibrin deposition on metal wires at 100 s^{-1}. (a) and (b) ST316L wire, (c) and (d) TiAl6V4 wire.

Under shear rate of 500 s^{-1}

Low magnification Figure 5.11(a) shows the detached fibrin tethers on ST316L wires. Leukocyte-platelet aggregates were assumed to be involved in the cell adhesion process, as activated platelets and leukocytes were observed in the black square of Figure 5.11(b). Moreover, a few leukocytes in Figure 5.11(c) were also found embedded or fully covered by a thin layer of fibrin. These findings suggest that leukocytes were also involved in the blood clot formation under flow conditions. Figure 5.11(d) shows the protein deposition, fibrin tethers, and possibly some PMPs on the ST316L wire surface.

Long protein fibers were present on the TiAl6V4 wires. The fiber in Figure 5.11(e) had a length of more than 1 mm. Higher magnification images show that the long fiber was composed of small fibrin fibers, red blood cells and platelets. Abundant platelets and PMPs were also found attached on or embedded in the fibrin fibers, as shown in Figure 5.11(f).

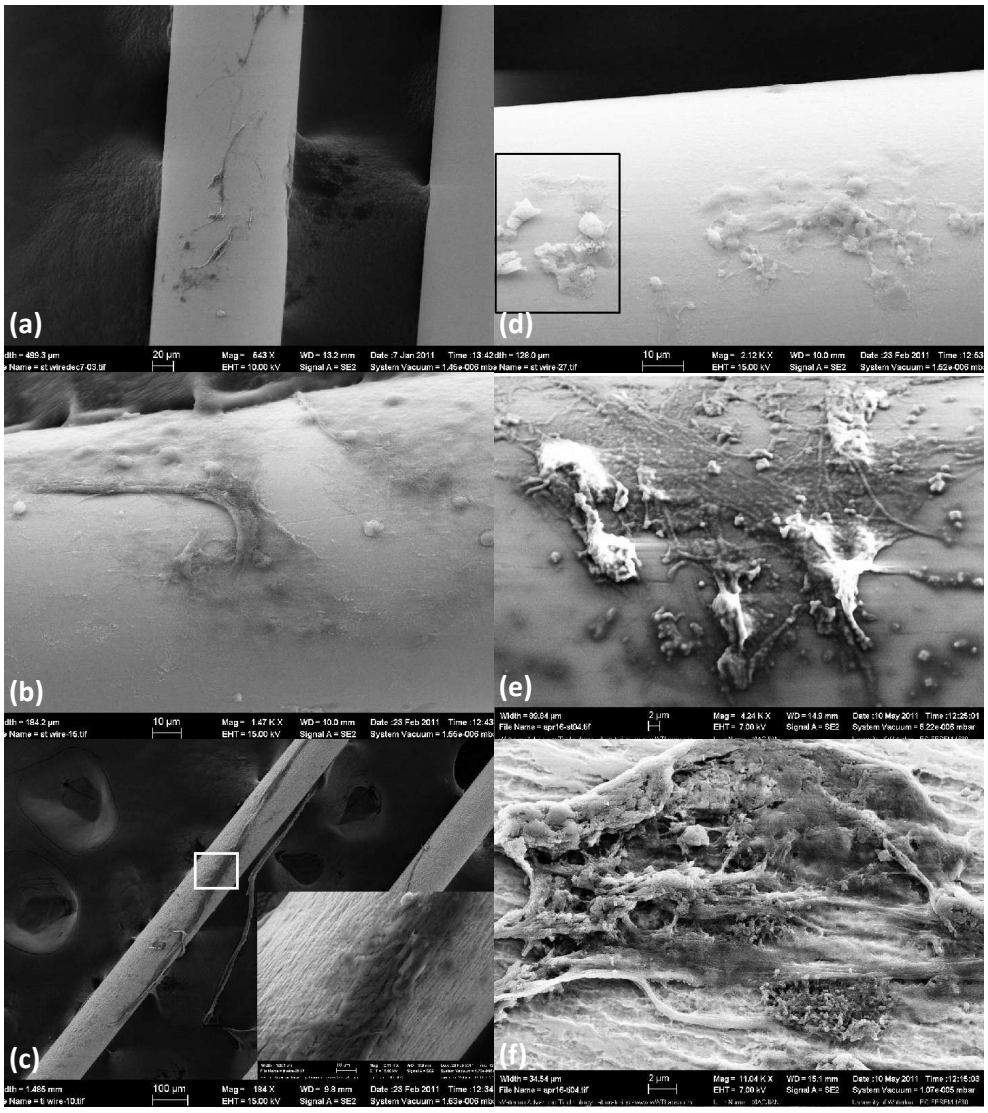
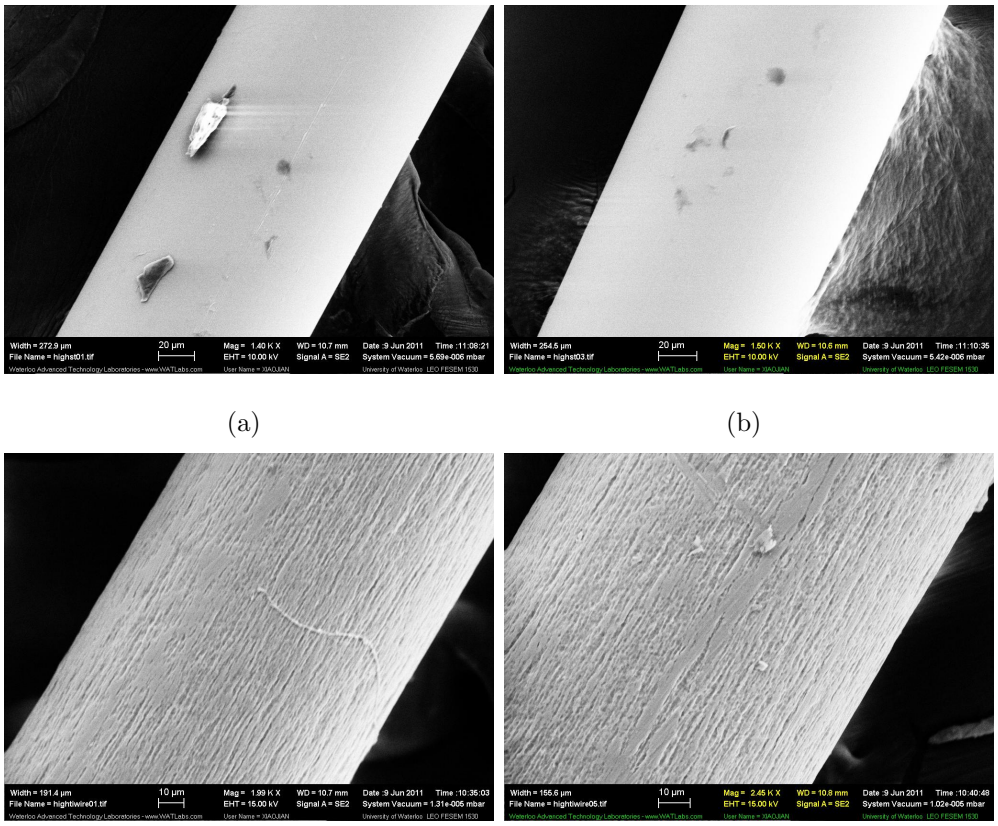


Figure 5.11: Blood cell adhesion on ST316L ((a) to (d)) and TiAl6V4 ((e) and (f)) wires at 500 s^{-1}, (a) Detached fibrin tethers, (b) Leukocyte-plaltelet aggregates adhesion, (c) Blood cells covered by protein, (d) Platelet and PMP adhesion on fibrin, (e) Blood cells embedded in long fibers, (f) Platelets and PMPs aggregates.

Under shear rate of 1500 s^{-1}

Generally, blood cell adhesion and protein adsorption at 1500 s^{-1} remained at a very low level on both metal wire surfaces. Only a few fibrin fibers were observed on ST316L wire surfaces (Figure 5.12(a) and (b)), with little blood cell adhesion. Small tethers were noticed on TiAl6V4 wire surfaces as shown in Figure 5.12(c), and a few platelets were found adhering to the surface (Figure 5.12(d)).



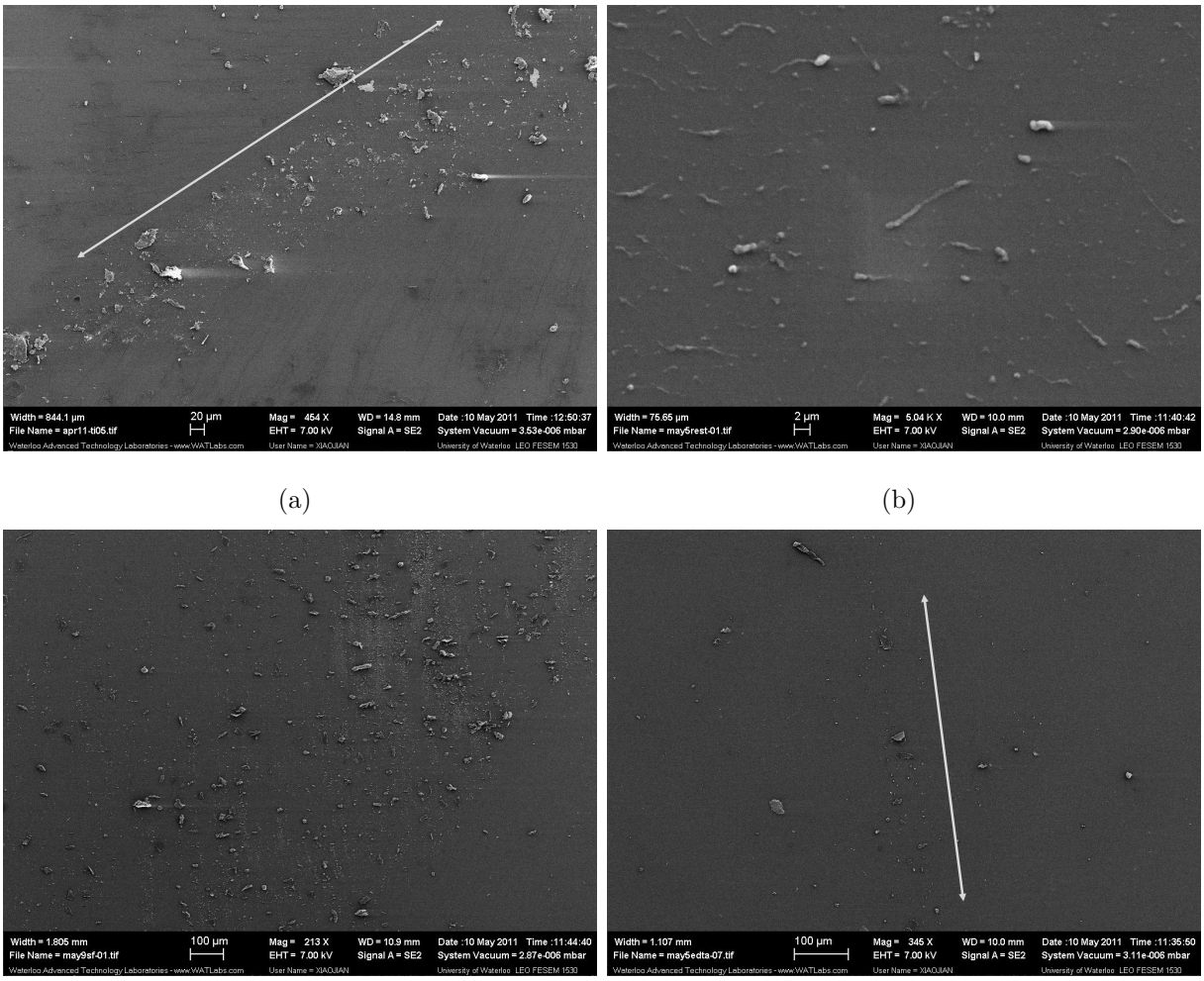
(a) (b)

(c) (d)

Figure 5.12: Cell adhesion and fibrin deposition on metal wires at 1500 s^{-1}. (a) and (b) ST316L wire, (c) and (d) TiAl6V4 wire.

5.4.5 Cell adhesion in flow system

Silicone sheets in the flow chamber were also collected and analyzed by SEM. The silicone sheets from different samples presented similar surface morphology. Fibrin and large amount of particles were attached. These particles had irregular shape and various size, and may be small pieces of fibrin, cell debris and their aggregates. Representative images of silicone sheets in the flow chambers are shown in Figure 5.13.



(a) (b)

(c) (d)

Figure 5.13: Surface morphology of silicone sheets in flow chambers after recirculation with (a) TiAl6V4 wire blood sample, (b) No wire blood sample, (c) SFLLRN activated blood sample, (d) EDTA inhibited blood sample. The arrow in the figure indicates direction of blood flow in the chamber.

Chapter 6

Discussion

6.1 Blood Circulation Model

In the study with the Master Flex peristaltic pump, red blood cell lysis occurred after two-hour recirculation. Clinical studies have reported that roller pumps can cause bleeding and hypotension from pump-induced platelet activation [96, 97]. Mechanical damage to blood cells was also noticed after *in vitro* blood circulation with the roller pump [98–102]. Rollers or impellers were suspected to crush blood cells and cause red blood cell lysis during blood circulation. It is thought that potential mechanical damage on platelets may have resulted in smaller average platelet size and less CD61 expression on each platelet. The latter was one of the observations made following the use of the peristaltic pump. Meanwhile, more PMPs might be shed during recirculation; these may bind to leukocytes and form aggregates further increasing the level of blood activation.

Hemoglobin is released into plasma either after red blood cell membrane rupture [103], or through the pores of a thinned membrane as a result of mechanical stress [100]. This

will lead to the red appearance of plasma. This was confirmed by a previous study in which researchers found a 2% hemolysis after two hour circulation with a roller pump [104].

Red blood cell fragments generated by the use of a roller pump has been reported previously. Linneweber *et al.* showed a tenfold fragments increase after a blood sample was circulated in a roller pump for two hours [101]. Blood viscosity may also increase due to the red blood cell aggregates and their fragments [105]. Moreover, the size distribution of red blood cell fragments is very close to that of intact platelets [99], and this could potentially affect the proper detection of platelets by flow cytometry.

Changes in roller pump parameters, such as impeller speed, flow rate, and tip gap, can affect the degree of hemolysis. It has been shown that blood damage increases with circulation time and impeller speed, as well as decreased flow rate [106]. Another potential drawback of the MasterFlex pump was that it only had two channels, and therefore did not meet the requirements of the simultaneous multiple-sample test for this study. The peristaltic pump circulation model was thus not adopted in later experiments.

6.2 Blood Cell Activation Under Static Conditions

6.2.1 Platelet activation

Blood-material contact can induce platelet activation and subsequent platelet microparticle shedding. In the static experiments, the platelet activation and adhesion of ST316L were higher than TiAl6V4, which may be primarily because of the difference of metal surface properties, including chemical composition and surface topography.

Platelet activation in both whole blood and PRP was also lower with TiAl6V4 disks than the negative controls (Rest0 and Rest2hr) and other test materials, as shown by the

reduced CD61 expression on platelets. This surprising result was in accordance with a previous study by Takami *et al.* [107], in which platelets in heparinized blood incubated with TiAl6V4 rod for three hours had a lower (although not statistically significant) P-selectin expression compared to their negative control (0 hour). It is known that procoagulant microparticles can mediate cell interaction through P-selectin [108] and bind to activated platelets and leukocytes, leading to both a decrease in PMPs in blood and reduced CD61 expression on platelets. The reduced CD61 expression on platelet microparticles with TiAl6V4 may thus be due to an increase in microparticle shedding, which then bind to leukocytes and are thus not sorted as PMPs. However, the presence of leukocyte-platelet aggregates did not increase with TiAl6V4, negating this hypothesis. The reduced CD61 expression may indicate that platelet activation in TiAl6V4 is suppressed because of higher albumin/fibrinogen ratio on TiAl6V4 surface. Albumin adsorption happens earlier than fibrinogen, and albumin adsorption being favored on the TiAl6V4 surface would then provide a more platelet-biocompatible surface and reduce platelet activation [109].

The static condition results showing higher platelet activation with ST316L compared to TiAl6V4 agree with published *in vitro* studies that indicat higher platelet activation with ST316L relative to other biomaterials [110, 111]. Increased platelet adhesion and aggregation were also found on ST316L compared to diamond like carbon (DLC) coated surface [112]. The chemical composition of ST316L is actually believed to play a key role in platelet activation. It has been reported that after blood contact with ST316L, GPIIb/IIIa receptors stored inside the platelet translocate to the platelet membrane and are more active than surface ones by displaying higher affinity towards anti-CD61 antibody and promoting higher levels of platelet adhesion and aggregation on biomaterials. Silicone and ST316L have also been shown to produce approximately the same levels of thrombin-antithrombin complex and complement C3a expression [113], which would agree with the

experimental data of this study whereby silicone and ST316L induced similar levels of platelet and leukocyte activation in the static experiments.

Blood incubation with polystyrene was used to create somewhat of a positive control material in the static test. However, bulk platelet activation was not significant after 2 hrs. Ye *et al.* had previously shown that protein adsorption and platelet adhesion on polystyrene was significantly higher than TiAl6V4 which may have suggested a higher platelet activation in the bulk, especially since platelet activation as measured by annexin V expression was also reported to be higher than TiAl6V4 [114]. Taken together, these studies and the current results from platelet activation and adhesion on the surfaces would tend to suggest that platelet activation in the bulk may not be observed despite significant platelet adhesion on a biomaterial. Such results further highlight the importance of studying what happens on the surface of a biomaterial and what remains in the flowing blood when testing material biocompatibility.

While these results have been discussed related to material-induced activation, it is also important to note that the blood collection protocol may also partially have contributed to the slight platelet activation in Rest0. In platelet activation studies, some researchers discard the first 1-3 ml blood, because it contains activated platelets caused by blood vessel injury and blood contacting with the syringe surface. In this study, blood was drawn without discarding the first milliliter. The anticoagulant heparin has also been reported to potentiate platelet aggregation and conformational change [115], which may explain the observation of higher values in the Rest0 sample. As mentioned previously, similar results at time 0 have also been noted by Takami *et al.* [107].

The overall PMP formation in the static experiments can be considered negligible. The inability to record changes in microparticle platelet formation may be due to how platelet microparticles were identified. Empirically gating [116, 117] (preferred method) and beads

calibration [118] are both used in identifying PMPs. However, the average platelet size is different between blood donors, likewise, the average size of their PMPs is different. In the current study, the average platelet size was first measured according to the median FSC value of gated platelets, then set the PMP limit to the 35% of the average platelet size, thus we may have not been able to record small change in platelet microparticle formation. It is also very likely that under the current experimental conditions (static), material-induced platelet activation is too low and thus platelets will not shed microparticles due to a lack of stimulation. Previous studies with metal discs have mostly focused on adhesion and thus there was little data available on measuring platelet activation in the bulk under static conditions. The low level of platelet microparticle formation compares well with those observed in Park *et al.*'s work using smooth disks [10]. However, it was surprising to see that increase contact time with the biomaterial did not increase microparticle formation: similar experimental conditions were used except incubation time was 30 min in their study while contact time was increased to 2hrs to observe the potential expression of TF.

6.2.2 Leukocyte activation

Under static conditions, CD11b upregulation is mainly dependent on the blood-material contact [53, 119]. While we were unable to detect platelet activation, leukocyte activation, as measured by CD11b upregulation, occurred with biomaterial samples. Material-induced leukocyte activation was most likely mediated by both soluble fibrinogen [56] and soluble complement factors C5a [119–121] and further experiments will be required to specifically determine the mechanisms of material-induced CD11b upregulation under static conditions.

While it was observed that some material-induced CD11b upregulation occurred, the difference was not statistically significant compared to the negative control (Rest2hr).

Smooth annealing treated Titanium surface has been shown to be more biocompatible than acid-roughed titanium surface [122] and this may explain the lack of statistical significance in the current results as only annealed titanium disks were tested. Acid-treatment of the titanium surface may help in further determining the sensitivity of the static model for leukocyte activation. It is also possible that more activated leukocytes were lost through adhesion on the material since besides upregulation, CD11b also undergoes a qualitative change [123] which further promotes cell aggregation and adhesion. There is also evidence from previous work with polymers that leukocyte activation is strongly dependent on surface area [53] and under the static experiment conditions, a similar surface area with polymer would also not have induced statistically significant leukocyte activation.

Monocyte-platelet aggregates can be viewed as a marker for both platelet and leukocyte activation. Similarly to the current results, higher CD61 intensities are commonly observed with monocytes since they are more sensitive to activated platelets and microparticles than neutrophils [47, 124]. Leukocyte-platelet aggregate formation is dependent on activated platelets but also on PMPs which serve as bridge between leukocytes [108]. PMPs have actually been shown to be more effective than platelets in bridging leukocytes [125]. Since both a low PMP level and low platelet activation were observed, it is not surprising that leukocyte-platelet aggregate formation remained low under static conditions.

A strong correlation has been found between platelet activation, monocyte-platelet aggregate formation and TF expression [126] and thus the low TF expression results are in accordance with the overall level of blood activation in the samples. It is also important to note that the absence of TF expression following biomaterial contact is indirect evidence that the biomaterials were endotoxin-free as any endotoxin contamination of the biomaterial would have resulted in significant TF expression on monocytes even in the absence of platelet activation.

6.2.3 Cell adhesion

Under static conditions, we were able to observe differences in blood cell adhesion between the two metal biomaterials tested. These results are in agreement with previous work by Takami *et al.* [107] and Tanaka *et al.* [61] who showed that the composition of the metal disk surfaces is one of the main factors determining platelet adhesion. Using up to seven different metals of similar surface roughness and either qualitative SEM observation or quantitative optical density measurements of CD61, they observed the effect of metallic material chemistry on platelet adhesion and fibrin formation.

Increased fibrinogen adsorption and blood cell adhesion was found on the surface of ST316L disk compared to the TiAL6V4 disks. While such a difference may be due to material chemistry, it is important to note that the surface of ST316L may also be rougher. A roughed material surface can increase surface area, which in turn increases plasma protein adsorption and subsequent platelet adhesion and activation [10]. Nygren *et al.* showed increased fibrinogen adsorption, platelet adhesion and P-selectin expression on the hydrofluoric acid-roughed surfaces compared to the smooth machined pure titanium [122]. Hong *et al.* observed more platelet adhesion on roughed TiN surfaces than smooth TiN surfaces [127]. Cracks with a depth of about 2 μm, were observed on the ST316L surface; this may have increased the overall surface area onto which proteins can adsorb but it may also have provided area where blood could be entrapped and create local sites of activation because of the localized high surface area to blood ratio in the cracks. Fibrinogen has the potential to promote leukocyte-platelet aggregates. However, considering low P-selectin expression, the leukocyte adhesion on ST316L disk was most likely mainly mediated by the interaction between CD11b and GPIIb/IIIa since both are expressed on resting cells. This was in accordance with a previous study that monocyte adhesion on ST316L was mediated by CD11b [128].

Hansi *et al.* [63] as well as Thierry *et al.* [72] found platelets strongly adhering to the surface of bare ST316L with the majority of the attached platelets displaying a dendritic shape with marked pseudopodia. However, during the incubation with platelet rich plasma (PRP), most platelets remained discoid or at a lower activation stage, such as dendritic or dendritic spread. This was in contrast to the blood experiments, where significant platelet activation and fibrin clots could be observed on the ST316L surface.

Compared to ST316L, low levels of platelets and platelet aggregates were observed on TiAl6V4 surface; it was also difficult to view if any PMPs were present due to their small size and the complex surface microtexture of TiAl6V4. Interestingly, despite low adhesion, the adherent platelets were at a higher activation stages and displayed spread or fully spread morphological shape compared to ST316L in PRP experiments. When comparing platelet adhesion on TiAl6V4 and ST316L, contradictory results have been obtained. Minimal platelet adhesion has been shown on TiAl6V4 compared with other biomaterials [107] while higher platelet adhesion level on TiAl6V4 than on ST316L was reported by Tanaka *et al.* [61]. Such differences in results may come from different experimental conditions (contact time, anticoagulant concentration, whole blood versus PRP) and material surface preparation (surface polishing versus none). In Tanaka *et al.* [61] experiment, shorter incubation time (20 min) and surface polishing were used. Within short incubation time, albumin is mainly responsible for blood cell adhesion, as it moves faster than other plasma proteins. The polishing step with SiC before incubation may also have altered their surface. In this regard, the titanium oxide film of the metal sample may be thicker and more stable, and thus lead to better hemocompatibility compared to their TiAl6V4 surface.

6.3 Blood Cell Activation Under Shear Conditions

6.3.1 Platelet activation

Compared to the static experiments, PMP formation almost doubled that found in the flow experiments. PMP formation also continued to increase at 6200 s^{-1} shear rate , further confirming previous results which showed that platelet activation depends on shear [43,129]. The increase in PMPs formation may come from the shear effect and/or contact with the *in vitro* flow system.

While we did observe an increase in PMP formation, there was no significant change between 100 s^{-1} , 500 s^{-1} and 1500 s^{-1} in the presence or absence of the wires. Thus physiological shear stress was too low to significantly activate platelets and the presence of biomaterial did not have an effect at these shear rates. Indeed, previous studies have shown that compared with the baseline at 10 s^{-1}, significant platelet activation was only observed at or above 6000 s^{-1} [87,130]. Performing short studies at 6200 s^{-1}, we further verified that the *in vitro* flow chamber model was able to agree with these published results and the lack of platelet activation was not due to an *in vitro* flow model that did not perform adequately but to a lack of material-induced activation. Based on a previous study showing PMP formation increasing with prolonged time under shear conditions [131], we had initially hypothesized that 2hrs recirculation with biomaterials would provide enough stimulation for platelet activation even under low shear conditions but the current results disproved the hypothesis. The fact that we did not observe any difference in CD61 expression and microparticle formation between the test materials may be explained by the small surface area/blood volume ratio. In flow experiments, metal wires with a total surface area of 0.3 cm^2 were circulated with more than 1 ml blood. The surface area/blood volume ratio decreased more than 10 times compared to the static *in vitro* model. Moreover, syringe,

sample tube, silicone tubing and sheet had a much larger surface area than the tested stent material itself. Thus any platelet activation induced by the metal wires was most likely buried in the background activation of the *in vitro* flow model. Furthermore, the result of SFLLRN flow experiments demonstrated that PMP percent gradually decreased during circulation, suggesting that they may bind to other blood cells, form aggregates and thus be sorted as cells or further decrease in size and become undetectable by flow cytometry. The PMP decrease may also be explained by them simply binding to the inner surface of the *in vitro* system. It is likely that a combination of these scenarios occurred over time.

6.3.2 Leukocyte activation

Despite a rougher surface, CD11b expression induced by TiAl6V4 wire was generally lower than ST316L. Under flow conditions, previous studies have reported that leukocyte activation depended on material surface chemical composition and topography [132] and that CD11b up-regulation was also sensitive to material surface area [133]. The results would tend to disagree with such statements and suggest that material chemistry was the most important factor in material-induced leukocyte activation.

In parallel to increase in platelet activation with an upregulation of P-selectin induced by shear, leukocyte-platelet aggregates significantly increased compared to the static experiments. This is in agreement with recent studies showing that, under flow conditions, platelet and leukocyte interaction is mainly through the P-selectin-PSGL-1 rather than through the complex GPIIb/IIIa-fibrinogen-CD11b [81, 124, 134]. It is also possible that the *in vitro* model itself promoted the formation of platelet-leukocytes aggregates as they tend to form in regions where high shear stress converts to low shear stress [82, 135], or where pulsatile flow applies [136]. In the *in vitro* flow model, the inlet and outlet of the flow chamber were both connected to a silicone tube whose cross sectional area was big-

ger, resulting in the decrease of shear rate when blood flows out from the chamber; this configuration rather than shear itself may have contributed to aggregates formation in the flow experiments.

Leukocyte activation, as measured by CD11b upregulation and platelet-leukocyte aggregates, has been shown to increase with shear stress [81, 124]. However, in the flow experiments, both CD11b expression and leukocyte-platelet aggregates decreased with increasing shear rate, the latter even in the presence of an increase in platelet activation. In flow experiments, the blood volume used for each flow condition was 1 ml, 1.7 ml and 3.3 ml to keep the same cycle period and to ensure blood cells have the same number of passages in contact with the metal wires. With metal wire surface area unchanged but an increase in blood volume, the decrease of surface area/blood ratio likely may have led to a "dilution effect" for leukocyte activation. It is also possible that with increased shear rate, the cells have less chance to come into contact with the material as the flow rate is increased and this reduction in contact with the artificial surface may actually explain the reduced activation in 1500 s^{-1} compared to 100 s^{-1}.

TF is being recognized as an important player in thrombus formation and fibrin adsorption on artificial surfaces. Platelet activation has been shown to promote TF expression [17, 126]. In the current experiments, TF expression on monocytes and platelet activation correlated well at 100 s^{-1}. However as shear increased, despite an increase (albeit non significant) in platelet activation, TF expression decreased suggesting that under higher shear conditions platelet activation may not be able to contribute to TF expression due to the increase flow rate which may limit the ability of platelets to bind to monocytes.

6.3.3 Cell adhesion

Fibrin deposition and blood cell adhesion are dependent on material surface properties and shear stress magnitude [12, 50]. CD11b and TF have also been shown to mediate blood cell adhesion and fibrin deposition under flow conditions [17]. These results agree with these previous observations as higher CD11b upregulation and TF expression were obtained in ST316L blood samples, and correspondingly, more fibrin deposition and blood cell adhesion were observed on ST316L surfaces.

We were able to demonstrate from the SEM results with 500 s^{-1} and 1500 s^{-1} that, as shown in previous studies [12, 137, 138], fibrin deposition and cell adhesion on material surfaces decreased with increasing shear stress. However, significant adhesion on metal wires at 100 s^{-1} was not observed. This may be because the metal wires used for SEM analysis was only from one 100 s^{-1} flow experiment and may not have been a representative sample of the surfaces.

Cell debris and microparticle adhesion were also noticeable on the silicone sheet in the flow chamber, further indicating that material-induced adhesion occurred on the *in vitro* flow model making it difficult to assess the effect of the metal itself. The microparticle presence also provided further evidence that PMPs were consumed on the surface, providing some explanation to the slow disappearance of PMPs in the bulk of SFLLRN activated blood at 500 s^{-1}. We were unfortunately unable to clearly assess the surface of the silicone tubing that was used to pump blood in and out of the chamber as its small diameter made it impossible to properly observe under SEM.

Chapter 7

Conclusion and Future Work

7.1 Conclusion

Flow cytometry analysis was a reliable technique to assess both platelet and leukocyte activation in the bulk. SEM was also a valuable tool to qualitatively evaluate cell adhesion on material surface, but quantitative analysis of the number of platelets and their state of activation would provide important information on adherent cells and should be considered in the future.

Static experiments demonstrated that, with both blood and platelet rich plasma, little platelet activation occurred in the bulk and difference in platelet activation between materials could only be observed with platelet adhesion on the surfaces. While we did not always obtain significant differences between our samples, the *in vitro* flow model using the parallel plate flow chamber appeared to be a useful platform to study cell activation in blood under various shear conditions. The presence of shear resulted in increase platelet activation, but no difference between shear rate and materials was obtained under the

physiological shear rate conditions tested (100 to 1500 s^{-1}). On the other hand, significant differences were observed in material-induced leukocyte activation under shear conditions. At 100 s^{-1}, increase in CD11b upregulation, platelet-leukocyte aggregates and TF expression was detected. Interestingly as shear increased, material-induced leukocyte activation decreased either as a consequence of reduced contact time with the surface or because of a significant reduction in surface area to volume ratio. These results also demonstrate that TiAl6V4 is in general more blood compatible than ST316L under both static and flow conditions, and thus stent material should be well considered in new stent design to reduce the risk of in-stent restenosis. However while material had an effect on blood activation, it is important to note that there did not seem to be a synergistic or priming effect between the presence of a foreign material and shear in inducing blood activation. This may be due to experimental conditions such as the small material surface area tested and future work is needed.

7.2 Future Work

In the present research, blood was collected from healthy donors with an average age of 30. As coronary artery disease is less likely to happen in young people, comparing material-induced blood activation under shear conditions using blood from CAD patients, older or diabetic people with the blood from healthy donors would provide important information on how disease may affect material biocompatibility. The difference in blood, such as blood sugar, cholesterol, lipids and even in blood cells, together with other medical conditions may result in different blood cell activation under shear conditions. These differences may help explain the discrepancies that are currently observed in patients on anti-platelets and anticoagulant therapies.

The blood collection protocol also should be changed. In the current protocol, a tourniquet was applied at proper pressure when taking blood. Platelet activation in blood sample remains small as it usually takes less than 1 minute to collect the blood. However, as more blood volume is required to produce higher shear rates, and longer collection time is needed. This may lead to additional initial platelet activation and make it difficult to determine the effect of shear and materials on activation.

It was difficult to see any difference in material induced platelet activation under flow conditions and it is believed that this may be due to the small surface area/blood volume ratio and the large amount of silicone used in the *in vitro* flow system. Further experiments are needed to validate this hypothesis; this would also help elucidate our observations that leukocyte activation appeared to decrease with increased shear. To increase metal surface area, metal strips rather than wire can be used. For the *in vitro* model, the setup should use minimal amount of silicone tubing; one may also considered designing a new *in vitro* flow model to limit the use of silicone tubing.

For materials used in the experiments, material surface properties, such as roughness, chemical composition, and wettability should be characterized. This would provide valuable information on the materials and may help in identifying the important properties in material-induced activation under shear conditions. Treatments of the surface such as acid or laser treatment would also provide means to increase surface area.

SEM is limited in estimating the total number of adherent platelets on the surface. Other methods such as radiolabeling or LDH should be considered. It would also be very valuable to obtain a blood cell count at the end of each experiment and while there may be a significant cost involved, options should be evaluated.

APPENDICES

Appendix A: Ethical Approval Letter

Appendix B: Blood Donation Consent Form

UNIVERSITY OF WATERLOO

OFFICE OF RESEARCH ETHICS

Notification of Full Ethics Clearance of Application to Conduct Research with Human Participants

---

Principal/Co-Investigator: Maud Gorbet **Department:** Systems Design Engineering

ORE File #: 14242

Project Title: Cellular mechanisms involved in material-induced thrombosis

---

This certificate provides confirmation that the additional information/revised materials requested for the above project have been reviewed and are considered acceptable in accordance with the University of Waterloo's Guidelines for Research with Human Participants and the Tri-Council Policy Statement: Ethical Conduct for Research Involving Humans. Thus, the provisional ethics clearance status has been removed and the project now has received full ethics clearance. This clearance is valid for a period of ***four years*** *from the date shown below and is subject to an* ***annual ethics review process*** *(see Note 2). A new application must be submitted for on-going projects continuing beyond four years.*

Note 1*: This project must be conducted in accordance with the description in the application and revised materials for which full ethics clearance has been granted. All subsequent modifications to the application must be submitted for prior ethics review using ORE Form 104 and must not be intiated until notification of ethics clearance has been received.*

Note 2*: All ongoing research projects must undergo annual ethics review. ORE Form 105 is used for this purpose and must be submitted by the Faculty Investigator/Supervisor (FI/FS) when requested by the ORE. Researchers must submit a Form 105 at the conclusion of the project if it continues for less than a year.*

Note 3*: FIs and FSs also are reminded that they must immediately report to the ORE (using ORE Form 106) any events related to the procedures used that adversely affected the participants and the steps taken to deal with these.*

ADDITIONAL COMMENTS:

[✓] No additional comments

[ ] Additional comments emailed on date shown below.

Susan E. Sykes, Ph.D., C.Psych.
Director, Office of Research Ethics
OR
Susanne Santi, M. Math
Manager, Research Ethics

3/27/08
Date

University of Waterloo

March 17, 2008

Title of Project: ***Cellular Mechanisms involved in material-induced thrombosis***

Principal Investigator: **Maud Gorbet**
University of Waterloo
Department of Systems Design Engineering
519-884-4567 Ext. *32602*

Purpose of Study:

You have been asked to donate a blood sample for a research project aimed at evaluating how white blood cells interact with metallic materials used in cardiovascular devices (e.g.: stents and mechanical heart valves). The current study will use blood from healthy volunteers, who have not taken any anti-inflammatory or pain killers in the last 48 hours prior to the donation. This research project will contribute to a better understanding of how, upon contact with a foreign material, white blood cells may become activated and participate in the formation of blood clots. A better understanding of cell-material interaction will help in developing novel surface modifications and therapeutic approaches to reduce the incidence of clotting complications that can significantly impair the quality of life of many patients.

Procedures Involved in this Study:

We will require a small sample of blood (10 to 15 ml). A maximum of 15 minutes of your time will be required to fill out the consent form, the medical screening form and take the blood sample. Blood will be collected in a syringe from a vein in the arm by a certified phlebotomist (registered nurse, or a registered technologist trained in venipuncture). The blood will then be transferred immediately in a tube containing anticoagulants. If at any point during the blood donation you feel uncomfortable, we will remove the needle immediately. It is your choice if you want to be a regular donor for this study or if this is a one time only donation. If you choose to be a regular donor, for donor comfort, you will not be asked to donate more than twice a week and/or more than six times in a month. *The Red Cross policy for limiting blood donation is a volume of 450mL of blood once every two months.*

Personal Benefits of Participation:

You may not benefit personally from your participation in this study. However, the information obtained from this research may directly advance the development of better biomaterials and more appropriate targeted treatment strategies for reducing/preventing the occurrence of clotting complications with cardiovascular devices.

Risks associated with participation in the study:

This is considered a low risk study and minimal risks are anticipated. As with regular blood collection procedures for medical purposes, slight pain may be felt upon insertion of the needle in the

blood vessel. Small bruising may also occur. The donor may also feel faint when the blood is drawn. A fruit juice drink will be available after donating your blood.

Medical Screening Form:

This questionnaire asks some questions about your health status. This information is used to guide us with your entry into the study. Exclusions to participation in this study include any kidney problems or any cardiovascular diseases including bleeding disorders and anaemia.

Changing Your Mind about Participation:

You may withdraw from this study at any time without penalty. To do so, indicate this to the researcher or one of the research assistants by saying, "I no longer wish to donate blood for this study".

Confidentiality:

To ensure the confidentiality of individuals' data, each blood donor will be identified by a donor identification code known only to the principal investigator and his research assistants. Blood samples will be kept up to a week in the fridge. Serum or plasma derived from the blood samples will be kept up to six months in a -70oC freezer. All samples derived from the blood experiments will be used solely for the purpose of identifying inflammatory and thrombotic factors.

Contact Information:

If you have any questions about the study at any time, please contact Professor Maud Gorbet at her office 519-888-4567 ext. 32602, or by email at mgorbet@uwaterloo.ca.

Participant Feedback:

If you are interested in receiving publications derived from experiments performed with your blood donation, you can provide an email address below where we will send you our published studies.

Concerns about Your Participation

This study has been reviewed and received ethics clearance through the Office of Research Ethics. However, the final decision about participation is yours. If you have any comments or concerns resulting from your participation in this study, you may contact the Director, Office of Research Ethics at 519-888-4567 ext. 36005.

CONSENT FORM

I agree to take part in a research study being conducted by Dr. ***Maud Gorbet*** of the Department of ***Systems Design Engineering***, University of Waterloo.

I have made this decision based on the information I have read in the Information letter. All the procedures, any risks and benefits have been explained to me. I have had the opportunity to ask any questions and to receive any additional details I wanted about the study. If I have questions later about the study, I can ask the principal investigator ***Maud Gorbet (SYDE, 519-888-4567 x32602).***

[ ] I agree to be a one-time only donor for the study.

[ ] I agree to be a regular donor for the study. I will not be asked to donate more than twice a week and/or more than six times in a month.

I understand that I may withdraw from the study at any time without penalty by telling the researcher.

[ ] I wish to receive research publications on experiments that used my donated blood.

Email address: _____

This project has been reviewed by, and received ethics clearance through, the Office of Research Ethics at the University of Waterloo. I am aware that I may contact this office (519-888-4567, ext. 36005) if I have any concerns or questions resulting from my involvement in this study.

_____	_____
Printed Name of Participant	Signature of Participant
_____	_____
Dated at Waterloo, Ontario	Witnessed

HEALTH SCREENING FORM

STUDY: ***Cellular Mechanisms involved in material-induced thrombosis***

Name:
_____

Phone # (optional): _____ Year of Birth: _____

SELF REPORT CHECKLIST:

Known Health Problems (check appropriate):

[ ] Anaemia – Low red blood cell count

[ ] Rheumatic Fever

[ ] Epilepsy

[ ] Heart Murmur

[ ] Emphysema, Pneumonia,Bronchitis

[ ] High Blood Pressure

[ ] Disease of the Arteries

[ ] Congenital Heart Disease

[ ] High Cholesterol

[ ] Heart Attack

[ ] Heart Operation

[ ] Kidney and liver disease

[ ] Diabetes (diet or insulin)

[ ] Enteritis/Colitis/Diverticulitis

[ ] Ulcers

[ ] Bleeding from Intestinal Tract

[ ] Bleeding Disorders

_____
Signature of Participant

References

[1] Michaels A, Chatterjee K. Angioplasty versus bypass surgery for coronary artery disease. Circulation. 2002;106(23):E187–E190.

[2] Dai S, Bienek A, Walsh P, Stewart P, Wielgosz A. Tracking Heart Disease and Stroke in Canada 2009. Chronic Disease in Canada. 2009 Oct;29(4).

[3] Lemesle G, Slottow TLP, Waksman R. Very late stent thrombosis after bare-metal stent implantation: case reports and review of the literature. The Journal of invasive cardiology. 2009 Feb;21(2):E27–32.

[4] van der Hoeven BL, Pires NMM, Warda HM, Oemrawsingh PV, van Vlijmen BJM, Quax PHA, et al. Drug-eluting stents: results, promises and problems. International journal of cardiology, March 10. 2005;99(1):9–17.

[5] Kirtane AJ, Gupta A, Iyengar S, Moses JW, Leon MB, Applegate R, et al. Safety and efficacy of drug-eluting and bare metal stents: comprehensive meta-analysis of randomized trials and observational studies. Circulation. 2009 Jun 30;119(25):3198–3206.

[6] Holmes Jr DR, Kereiakes DJ, Garg S, Serruys PW, Dehmer GJ, Ellis SG, et al. Stent thrombosis. Journal of the American College of Cardiology. 2010 Oct 19;56(17):1357–1365.

[7] Gorbet MB, Sefton MV. Biomaterial-associated thrombosis: roles of coagulation factors, complement, platelets and leukocytes. Biomaterials. 2004 Nov;25(26):5681–5703.

[8] Messer RL, Mickalonis J, Lewis JB, Omata Y, Davis CM, Brown Y, et al. Interactions between stainless steel, shear stress, and monocytes. Journal of biomedical materials researchPart A. 2008 Oct;87(1):229–235.

[9] Mani G, Feldman MD, Patel D, Agrawal CM. Coronary stents: a materials perspective. Biomaterials. 2007 Mar;28(9):1689–1710.

[10] Park JY, Gemmell CH, Davies JE. Platelet interactions with titanium: modulation of platelet activity by surface topography. Biomaterials. 2001 Oct;22(19):2671–2682.

[11] Uchida M, Ito A, Furukawa KS, Nakamura K, Onimura Y, Oyane A, et al. Reduced platelet adhesion to titanium metal coated with apatite, albumin-apatite composite or laminin-apatite composite. Biomaterials. 2005 Dec;26(34):6924–6931.

[12] Otto M, Wahn B, Kirkpatrick CJ. Modification of human polymorphonuclear neutrophilic cell (PMN)-adhesion on biomaterial surfaces by protein preadsorption under static and flow conditions. Journal of materials scienceMaterials in medicine. 2003 Mar;14(3):263–270.

[13] Robert S Dieter RAD. Peripheral Arterial Disease. Columbus, Ohio: McGraw Hill; 2008.

[14] Watson SP. Platelet Activation by Extracellular Matrix Proteins in Haemostasis and Thrombosis. Current Pharmaceutical Design. 2009;15(12):1358–1372.

[15] Vine AK. Recent advances in haemostasis and thrombosis. Retina (Philadelphia, Pa). 2009 Jan;29(1):1–7.

[16] Adams RL, Bird RJ. Review article: Coagulation cascade and therapeutics update: relevance to nephrology. Part 1: Overview of coagulation, thrombophilias and history of anticoagulants. Nephrology (Carlton, Vic). 2009 Aug;14(5):462–470.

[17] Palmerini T, Coller BS, Cervi V, Tomasi L, Marzocchi A, Marrozzini C, et al. Monocyte-derived tissue factor contributes to stent thrombosis in an in vitro system. Journal of the American College of Cardiology. 2004 Oct 19;44(8):1570–1577.

[18] Camerer E, Kolsto AB, Prydz H. Cell biology of tissue factor, the principal initiator of blood coagulation. Thrombosis research. 1996 Jan 1;81(1):1–41.

[19] Maugeri N, Manfredi AA, Maseri A. Clinical and experimental evidences on the prothrombotic properties of neutrophils. Srpski arhiv za celokupno lekarstvo. 2010 Jan;138 Suppl 1:50–52.

[20] Furie B. P-Selectin and blood coagulation:its not only about inflammation any more. Arteriosclerosis, Thrombosis, and Vascular Biology. 2005 May;25(2):877–878.

[21] Maugeri N, Brambilla M, Camera M, Carbone A, Tremoli E, Donati MB, et al. Human polymorphonuclear leukocytes produce and express functional tissue factor upon stimulation. Journal of thrombosis and haemostasis : JTH. 2006 Jun;4(6):1323–1330.

[22] Osterud B, Bjorklid E. Sources of tissue factor. Seminars in thrombosis and hemostasis, February 01. 2006;32(1):11–23.

[23] Hathcock JJ. Flow effects on coagulation and thrombosis. Arteriosclerosis, Thrombosis, and Vascular Biology. 2006 Aug;26(8):1729–1737.

[24] Angiolillo DJ, Ueno M, Goto S. Basic principles of platelet biology and clinical implications. Circulation journal : official journal of the Japanese Circulation Society. 2010 Mar 25;74(4):597–607.

[25] Brass LF. Thrombin and platelet activation. Chest. 2003 Sep;124(3 Suppl):18S–25S.

[26] Goodman SL. Sheep, pig, and human platelet-material interactions with model cardiovascular biomaterials. Journal of Biomedical Materials Research. 1999 Jun 5;45(3):240–250.

[27] Davi G, Patrono C. Platelet activation and atherothrombosis. The New England journal of medicine. 2007 Dec 13;357(24):2482–2494.

[28] Jennings LK. Mechanisms of platelet activation: need for new strategies to protect against platelet-mediated atherothrombosis. Thrombosis and haemostasis. 2009 Aug;102(2):248–257.

[29] Sinauridze EI, Kireev DA, Popenko NY, Pichugin AV, Panteleev MA, Krymskaya OV, et al. Platelet microparticle membranes have 50- to 100-fold higher specific procoagulant activity than activated platelets. Thrombosis and haemostasis. 2007 Mar;97(3):425–434.

[30] Andrews RK, Berndt MC. Platelet physiology and thrombosis. Thrombosis research. 2004;114(5-6):447–453.

[31] Fuchs B, Budde U, Schulz A, Kessler CM, Fisseau C, Kannicht C. Flow-based measurements of von Willebrand factor (VWF) function: binding to collagen and platelet

adhesion under physiological shear rate. Thrombosis research. 2010 Mar;125(3):239–245.

[32] Andrews RK, Gardiner EE, Shen Y, Whisstock JC, Berndt MC. Molecules in focus Glycoprotein Ib-IX-V. The International Journal of Biochemistry and Cell Biology. 2003 Aug;35(8):1170–1174.

[33] Konstantopoulos K, Kukreti S, McIntire LV. Biomechanics of cell interactions in shear fields. Advanced Drug Delivery Reviews. 1998 Aug 3;33(1-2):141–164.

[34] Nesbitt WS, Westein E, Tovar-Lopez FJ, Tolouei E, Mitchell A, Fu J, et al. A shear gradient-dependent platelet aggregation mechanism drives thrombus formation. Nature medicine. 2009 Jun;15(6):665–673.

[35] Broberg M, Eriksson C, Nygeren H. GpIIb/IIIa is the main receptor for initial platelet adhesion to glass and titanium surfaces in contact with whole blood. Lab Clin Med. 2002 Mar;139(3):163–172.

[36] Matzdorff AC, Kuhnel G, Kemkes-Matthes B, Pralle H, Voss R, Fareed J. Effect of glycoprotein IIb/IIIa inhibitors on CD62p expression, platelet aggregates, and microparticles in vitro. The journal of laboratory and clinical medicine. 2000 Mar;135(3):247–255.

[37] Investigators TE. Use of a monoclonal antibody directed against the platelet glycoprotein IIb/IIIa receptor in high-risk coronary angioplasty. The New England journal of medicine. 1994 Apr 7;330(14):956–961.

[38] Broberg M, Nygren H. Von Willebrand factor, a key protein in the exposure of CD62P on platelets. Biomaterials. 2001 Sep;22(17):2403–2409.

[39] Merten M, Thiagarajan P. P-selectin in arterial thrombosis. Zeitschrift fr Kardiologie, November 01. 2004;93(11):855–863.

[40] Frenette PS, Johnson RC, Hynes RO, Wagner DD. Platelets roll on stimulated endothelium in vivo: an interaction mediated by endothelial P-selectin. Proceedings of the National Academy of Sciences of the United States of America. 1995 Aug 1;92(16):7450–7454.

[41] Murasaki K, Kawana M, Murasaki S, Tsurumi Y, Tanoue K, Hagiwara N, et al. High P-selectin expression and low CD36 occupancy on circulating platelets are strong predictors of restenosis after coronary stenting in patients with coronary artery disease. Heart and vessels. 2007 Jul;22(4):229–236.

[42] Colman R, Marder V, Clowes A, Georage J, Goldhaber S. Hemostasis and thrombosis : basic principles and clinical practice. Philadelphia: Philadelphia : Lippincott; 2006. Includes bibliographical references and index.; ID: vtug2229894.

[43] Holme PA, Orvim U, Hamers MJ, Solum NO, Brosstad FR, Barstad RM, et al. Shear-induced platelet activation and platelet microparticle formation at blood flow conditions as in arteries with a severe stenosis. Arteriosclerosis, Thrombosis, and Vascular Biology. 1997 Apr;17(4):646–653.

[44] Tovar-Lopez FJ, Rosengarten G, Westein E, Khoshmanesh K, Jackson SP, Mitchell A, et al. A microfluidics device to monitor platelet aggregation dynamics in response to strain rate micro-gradients in flowing blood. Lab on a chip. 2010 Feb 7;10(3):291–302.

[45] Boreda R, Fatemi RS, Rittgers SE. Potential for platelet stimulation in critically stenosed carotid and coronary arteries. Vasc Invest. 1995 Jul;1:26–37.

[46] Ruggeri ZM, Orje JN, Habermann R, Federici AB, Reininger AJ. Activation-independent platelet adhesion and aggregation under elevated shear stress. Blood. 2006 Sep 15;108(6):1903–1910.

[47] Michelson AD, Barnard MR, Krueger LA, Valeri CR, Furman MI. Circulating monocyte-platelet aggregates are a more sensitive marker of in vivo platelet activation than platelet surface P-selectin: studies in baboons, human coronary intervention, and human acute myocardial infarction. Circulation. 2001 Sep 25;104(13):1533–1537.

[48] Freedman JE, Loscalzo J. Platelet-monocyte aggregates: bridging thrombosis and inflammation. Circulation. 2002 May 7;105(18):2130–2132.

[49] Thiriet M. Biology and mechanics of blood flows. New York, NY ;London : Springer, c2008; 2008.

[50] Makino A, Shin HY, Komai Y, Fukuda S, Coughlin M, Sugihara-Seki M, et al. Mechanotransduction in leukocyte activation: a review. Biorheology. 2007;44(4):221–249.

[51] Sperandio M, Pickard J, Unnikrishnan S, Acton ST, Ley K. Analysis of leukocyte rolling in vivo and in vitro. Methods in enzymology. 2006;416:346–371.

[52] Ritter LS, Stempel KM, Coull BM, McDonagh PF. Leukocyte-platelet aggregates in rat peripheral blood after ischemic stroke and reperfusion. Biological research fornursing, April 01. 2005;6(4):281–288.

[53] Gorbet MB, Sefton MV. Leukocyte activation and leukocyte procoagulant activities after blood contact with polystyrene and polyethylene glycol-immobilized polystyrene beads. The Journal of laboratory and clinical medicine. 2001 May;137(5):345–355.

[54] Tautenhahn J, Meyer F, Buerger T, Schmidt U, Lippert H, Koenig W, et al. Interactions of neutrophils with silver-coated vascular polyester grafts. Langenbeck's archives of surgery / Deutsche Gesellschaft fur Chirurgie. 2010 Feb;395(2):143–149.

[55] Inoue T, Uchida T, Yaguchi I, Sakai Y, Takayanagi K, Morooka S. Stent-induced expression and activation of the leukocyte integrin Mac-1 is associated with neointimal thickening and restenosis. Circulation. 2003 Apr 8;107(13):1757–1763.

[56] Rubel C, Fernandez GC, Dran G, Bompadre MB, Isturiz MA, Palermo MS. Fibrinogen promotes neutrophil activation and delays apoptosis. Journal of immunology (Baltimore, Md: 1950). 2001 Feb 1;166(3):2002–2010.

[57] Evangelista V, Manarini S, Coller BS, Smyth SS. Role of P-selectin, beta2-integrins, and Src tyrosine kinases in mouse neutrophil-platelet adhesion. Journal of thrombosis and haemostasis : JTH. 2003 May;1(5):1048–1054.

[58] Alcaide P, Auerbach S, Luscinskas FW. Neutrophil recruitment under shear flow: it's all about endothelial cell rings and gaps. Microcirculation (New York, NY: 1994). 2009 Jan;16(1):43–57.

[59] Huang N, Yang P, Leng YX, Chen JY, Sun H, Wang J, et al. Hemocompatibility of titanium oxide films. Biomaterials. 2003 Jun;24(13):2177–2187.

[60] Kim YJ, Kang IK, Huh MW, Yoon SC. Surface characterization and in vitro blood compatibility of poly(ethylene terephthalate) immobilized with insulin and/or heparin using plasma glow discharge. Biomaterials. 2000 Jan;21(2):121–130.

[61] Tanaka Y, Kurashima K, Saito H, Nagai A, Tsutsumi Y, Doi H, et al. In vitro short-term platelet adhesion on various metals. Journal of artificial organs : the official journal of the Japanese Society for Artificial Organs. 2009;12(3):182–186.

[62] Jones MI, McColl IR, Grant DM, Parker KG, Parker TL. Protein adsorption and platelet attachment and activation, on TiN, TiC, and DLC coatings on titanium for cardiovascular applications. Journal of Biomedical Materials Research. 2000 Nov;52(2):413–421.

[63] Hansi C, Arab A, Rzany A, Ahrens I, Bode C, Hehrlein C. Differences of platelet adhesion and thrombus activation on amorphous silicon carbide, magnesium alloy, stainless steel, and cobalt chromium stent surfaces. Catheterization and cardiovascular interventions : official journal of the Society for Cardiac Angiography and Interventions. 2009 Mar 1;73(4):488–496.

[64] Seyfert UT, Biehl V, Schenk J. In vitro hemocompatibility testing of biomaterials according to the ISO 10993-4. Biomolecular engineering. 2002 Aug;19(2-6):91–96.

[65] Tanaka Y, Matsuo Y, Komiya T, Tsutsumi Y, Doi H, Yoneyama T, et al. Characterization of the spatial immobilization manner of poly(ethylene glycol) to a titanium surface with immersion and electrodeposition and its effects on platelet adhesion. Journal of biomedical materials researchPart A. 2010 Jan;92(1):350–358.

[66] Karagkiozaki V, Logothetidis S, Kalfagiannis N, Lousinian S, Giannoglou G. Atomic force microscopy probing platelet activation behavior on titanium nitride nanocoatings for biomedical applications. Nanomedicine : nanotechnology, biology, and medicine. 2009 Mar;5(1):64–72.

[67] Chu CL, Wang RM, Hu T, Yin LH, Pu YP, Lin PH, et al. XPS and biocompatibility studies of titania film on anodized NiTi shape memory alloy. Journal of materials scienceMaterials in medicine. 2009 Jan;20(1):223–228.

[68] Armitage DA, Parker TL, Grant DM. Biocompatibility and hemocompatibility of surface-modified NiTi alloys. Journal of biomedical materials researchPart A. 2003 Jul 1;66(1):129–137.

[69] SG S. Titanium–the material of choice? Periodontology 2000. 1998 Jun;17(1):7–21.

[70] Takemoto S, Yamamoto T, Tsuru K, Hayakawa S, Osaka A, Takashima S. Platelet adhesion on titanium oxide gels: effect of surface oxidation. Biomaterials. 2004 Aug;25(17):3485–3492.

[71] Muramatsu K, Uchida M, Kim HM, Fujisawa A, Kokubo T. Thromboresistance of alkali- and heat-treated titanium metal formed with apatite. Journal of biomedical materials researchPart A. 2003 Jun 15;65(4):409–416.

[72] Thierry B, Merhi Y, Bilodeau L, Trepanier C, Tabrizian M. Nitinol versus stainless steel stents: acute thrombogenicity study in an ex vivo porcine model. Biomaterials. 2002 Jul;23(14):2997–3005.

[73] Klein C, N. The role of metal corrosion in inflammatory processes: induction of adhesion molecules by heavy metal ions. Journal of Materials Science: Materials in Medicine. 1994;5(11):798–807.

[74] Krishnan LK, Varghese N, Muraleedharan CV, Bhuvaneshwar GS, Derangere F, Sampeur Y, et al. Quantitation of platelet adhesion to Ti and DLC-coated Ti in vitro using (125)I-labeled platelets. Biomolecular engineering. 2002 Aug;19(2-6):251–253.

[75] Hietala EM, Maasilta P, Valimaa T, Harjula AL, Tormala P, Salminen US, et al. Platelet responses and coagulation activation on polylactide and heparin-polycaprolactone-L-lactide-coated polylactide stent struts. Journal of biomedical materials researchPart A. 2003 Dec 1;67(3):785–791.

[76] Vicario PP, Lu Z, Grigorian I, Wang Z, Schottman T. Cell adhesion and proliferation are reduced on stainless steel coated with polysaccharide-based polymeric formulations. Journal of biomedical materials researchPart B, Applied biomaterials. 2009 Apr;89(1):114–121.

[77] Ye SH, Johnson Jr CA, Woolley JR, Oh HI, Gamble LJ, Ishihara K, et al. Surface modification of a titanium alloy with a phospholipid polymer prepared by a plasma-induced grafting technique to improve surface thromboresistance. Colloids and surfacesB, Biointerfaces. 2009 Nov 1;74(1):96–102.

[78] Chen J, Chen C, Chen Z, Chen J, Li Q, Huang N. Collagen/heparin coating on titanium surface improves the biocompatibility of titanium applied as a blood-contacting biomaterial. Journal of biomedical materials researchPart A. 2010 Nov;95(2):341–349.

[79] Ratner BD. The catastrophe revisited: blood compatibility in the 21st Century. Biomaterials. 2007 Dec;28(34):5144–5147.

[80] Ratner BD. The blood compatibility catastrophe. Journal of Biomedical Materials Research. 1993 Mar;27(3):283–287.

[81] Hu H, Varon D, Hjemdahl P, Savion N, Schulman S, Li N. Platelet-leukocyte aggregation under shear stress: differential involvement of selectins and integrins. Thrombosis and haemostasis. 2003 Oct;90(4):679–687.

[82] Nesbitt WS, Mangin P, Salem HH, Jackson SP. The impact of blood rheology on the molecular and cellular events underlying arterial thrombosis. Journal of Molecular Medicine (Berlin, Germany). 2006 Dec;84(12):989–995.

[83] Ottesen JT, Olufsen MS, Larsen JK. Applied mathematical models in human physiology. Society for Industrial and Applied Mathematics; 2004.

[84] Eckmann DM, Bowers S, Stecker M, Cheung AT. Hematocrit, volume expander, temperature, and shear rate effects on blood viscosity. Anesthesia and Analgesia. 2000 Sep;91(3):539–545.

[85] Wu Y, Zhang M, Hauch KD, Horbett TA. Effect of adsorbed von Willebrand factor and fibrinogen on platelet interactions with synthetic materials under flow conditions. Journal of biomedical materials researchPart A. 2008 Jun 1;85(3):829–839.

[86] George NP, Konstantopoulos K, Ross JM. Differential kinetics and molecular recognition mechanisms involved in early versus late growth phase Staphylococcus aureus cell binding to platelet layers under physiological shear conditions. The Journal of infectious diseases. 2007 Aug 15;196(4):639–646.

[87] Reininger AJ, Heijnen HF, Schumann H, Specht HM, Schramm W, Ruggeri ZM. Mechanism of platelet adhesion to von Willebrand factor and microparticle formation under high shear stress. Blood. 2006 May 1;107(9):3537–3545.

[88] Shankaran H, Neelamegham S. Effect of secondary flow on biological experiments in the cone-plate viscometer: methods for estimating collision frequency, wall shear stress and inter-particle interactions in non-linear flow. Biorheology. 2001;38(4):275–304.

[89] Fink H, Hong J, Drotz K, Risberg B, Sanchez J, Sellborn A. An in vitro study of blood compatibility of vascular grafts made of bacterial cellulose in comparison with conventionally-used graft materials. Journal of biomedical materials researchPart A. 2011 Feb 9;.

[90] Johnell M, Larsson R, Siegbahn A. The influence of different heparin surface concentrations and antithrombin-binding capacity on inflammation and coagulation. Biomaterials. 2005 May;26(14):1731–1739.

[91] Sarvepalli DP, Schmidtke DW, Nollert MU. Design considerations for a microfluidic device to quantify the platelet adhesion to collagen at physiological shear rates. Annals of Biomedical Engineering. 2009 Jul;37(7):1331–1341.

[92] Gutierrez E, Petrich BG, Shattil SJ, Ginsberg MH, Groisman A, Kasirer-Friede A. Microfluidic devices for studies of shear-dependent platelet adhesion. Lab on a chip. 2008 Sep;8(9):1486–1495.

[93] Geddes JB, Carr RT, Wu F, Lao Y, Maher M. Blood flow in microvascular networks: a study in nonlinear biology. Chaos (Woodbury, NY). 2010 Dec;20(4):045123.

[94] Chiu JJ, Chen CN, Lee PL, Yang CT, Chuang HS, Chien S, et al. Analysis of the effect of disturbed flow on monocytic adhesion to endothelial cells. Journal of Biomechanics. 2003 Dec;36(12):1883–1895.

[95] Skilbeck CA, Walker PG, David T, Nash GB. Disturbed flow promotes deposition of leucocytes from flowing whole blood in a model of a damaged vessel wall. British journal of haematology. 2004 Aug;126(3):418–427.

[96] Borgdorff P, van den Bos G, Tangelder GJ. Extracorporeal circulation can induce hypotension by both blood-material contact and pump-induced platelet aggregation. The Journal of thoracic and cardiovascular surgery. 2000 Jul;120(1):12–19.

[97] Borgdorff P, Tangelder GJ. Pump-induced platelet aggregation with subsequent hypotension: its mechanism and prevention with clopidogrel. The Journal of thoracic and cardiovascular surgery. 2006 Apr;131(4):813–821.

[98] Polaschegg HD. Red blood cell damage from extracorporeal circulation in hemodialysis. Seminars in dialysis. 2009 Sep-Oct;22(5):524–531.

[99] Linneweber J, Chow TW, Kawamura M, Moake JL, Nose Y. In vitro comparison of blood pump induced platelet microaggregates between a centrifugal and roller pump during cardiopulmonary bypass. The International journal of artificial organs. 2002 Jun;25(6):549–555.

[100] Watanabe N, Sakota D, Ohuchi K, Takatani S. Deformability of red blood cells and its relation to blood trauma in rotary blood pumps. Artificial Organs. 2007 May;31(5):352–358.

[101] Linneweber J, Chow TW, Takano T, Maeda T, Nonaka K, Schulte-Eistrup S, et al. Direct detection of red blood cell fragments: a new flow cytometric method to evaluate hemolysis in blood pumps. ASAIO Journal (American Society for Artificial Internal Organs : 1992). 2001 Sep-Oct;47(5):533–536.

[102] Moen O, Fosse E, Dregelid E, Brockmeier V, Andersson C, Hogasen K, et al. Centrifugal pump and heparin coating improves cardiopulmonary bypass biocompatibility. The Annals of Thoracic Surgery. 1996 Oct;62(4):1134–1140.

[103] Sakota D, Sakamoto R, Sobajima H, Yokoyama N, Waguri S, Ohuchi K, et al. Mechanical damage of red blood cells by rotary blood pumps: selective destruction of aged red blood cells and subhemolytic trauma. Artificial Organs. 2008 Oct;32(10):785–791.

[104] Spiller D, Losi P, Briganti E, Sbrana S, Kull S, Martinelli I, et al. PDMS content affects in vitro hemocompatibility of synthetic vascular grafts. Journal of materials scienceMaterials in medicine. 2007 Jun;18(6):1097–1104.

[105] Undar A, Henderson N, Thurston GB, Masai T, Beyer EA, Frazier OH, et al. The effects of pulsatile versus nonpulsatile perfusion on blood viscoelasticity before and after deep hypothermic circulatory arrest in a neonatal piglet model. Artificial Organs. 1999 Aug;23(8):717–721.

[106] Kim NJ, Diao C, Ahn KH, Lee SJ, Kameneva MV, Antaki JF. Parametric study of blade tip clearance, flow rate, and impeller speed on blood damage in rotary blood pump. Artificial Organs. 2009 Jun;33(6):468–474.

[107] Takami Y, Yamane S, Makinouchi K, Niimi Y, Sueoka A, Nose Y. Evaluation of platelet adhesion and activation on materials for an implantable centrifugal blood pump. Artificial Organs. 1998 Sep;22(9):753–758.

[108] Forlow SB, McEver RP, Nollert MU. Leukocyte-leukocyte interactions mediated by platelet microparticles under flow. Blood. 2000 Feb 15;95(4):1317–1323.

[109] Dion I, Baquey C, Monties JR, Havlik P. Haemocompatibility of Ti6A14V alloy. Biomaterials. 1993;14(2):122–126.

[110] Tepe G, Wendel HP, Khorchidi S, Schmehl J, Wiskirchen J, Pusich B, et al. Thrombogenicity of Various Endovascular Stent Types: An In Vitro Evaluation. Journal of Vascular and Interventional Radiology. 2002;13(10, pp. 1029-1035):October.

[111] Schmehl JM, Harder C, Wendel HP, Claussen CD, Tepe G. Silicon carbide coating of nitinol stents to increase antithrombogenic properties and reduce nickel release. Cardiovascular Revascularization Medicine. 2008;9(4, pp. 255-262):October.

[112] Santin M, Mikhalovska L, Lloyd AW, Mikhalovsky S, Sigfrid L, Denyer SP, et al. In vitro host response assessment of biomaterials for cardiovascular stent manufacture. Journal of materials scienceMaterials in medicine. 2004 Apr;15(4):473–477.

[113] Muthusubramaniam L, Lowe R, Fissell WH, Li L, Marchant RE, Desai TA, et al. Hemocompatibility of silicon-based substrates for biomedical implant applications. Annals of Biomedical Engineering. 2011 Apr;39(4):1296–1305.

[114] Ye SH, Johnson Jr CA, Woolley JR, Snyder TA, Gamble LJ, Wagner WR. Covalent surface modification of a titanium alloy with a phosphorylcholine-containing copolymer for reduced thrombogenicity in cardiovascular devices. Journal of biomedical materials researchPart A. 2009 Oct;91(1):18–28.

[115] Gao C, Boylan B, Fang J, Wilcox DA, Newman DK, Newman PJ. Heparin promotes platelet responsiveness by potentiating alphaIIbbeta3-mediated outside-in signaling. Blood. 2011 May 5;117(18):4946–4952.

[116] Matzdorff AC, Kuhnel G, Kemkes-Matthes B, Pralle H. Quantitative assessment of platelets, platelet microparticles, and platelet aggregates with flow cytometry. The journal of laboratory and clinical medicine. 1998 Jun;131(6):507–517.

[117] Trappenburg MC, van Schilfgaarde M, Marchetti M, Spronk HM, ten Cate H, Leyte A, et al. Elevated procoagulant microparticles expressing endothelial and platelet markers in essential thrombocythemia. Haematologica. 2009 Jul;94(7):911–918.

[118] Daniel L, Fakhouri F, Joly D, Mouthon L, Nusbaum P, Grunfeld JP, et al. Increase of circulating neutrophil and platelet microparticles during acute vasculitis and hemodialysis. Kidney international. 2006 Apr;69(8):1416–1423.

[119] Gemmell CH, Black JP, Yeo EL, Sefton MV. Material-induced up-regulation of leukocyte CD11b during whole blood contact: material differences and a role for complement. Journal of Biomedical Materials Research. 1996 Sep;32(1):29–35.

[120] Sperling C, Maitz MF, Talkenberger S, Gouzy MF, Groth T, Werner C. In vitro blood reactivity to hydroxylated and non-hydroxylated polymer surfaces. Biomaterials. 2007 Sep;28(25):3617–3625.

[121] Bergseth G, Lambris JD, Mollnes TE, Lappegard KT. Artificial surface-induced inflammation relies on complement factor 5: proof from a deficient person. The Annals of Thoracic Surgery. 2011 Feb;91(2):527–533.

[122] Nygren H, Eriksson C, Lausmaa J. Adhesion and activation of platelets and polymorphonuclear granulocyte cells at TiO2 surfaces. The Journal of laboratory and clinical medicine. 1997 Jan;129(1):35–46.

[123] Diamond MS, Springer TA. A subpopulation of Mac-1 (CD11b/CD18) molecules mediates neutrophil adhesion to ICAM-1 and fibrinogen. The Journal of cell biology. 1993 Jan;120(2):545–556.

[124] Ahn KC, Jun AJ, Pawar P, Jadhav S, Napier S, McCarty OJ, et al. Preferential binding of platelets to monocytes over neutrophils under flow. Biochemical and biophysical research communications. 2005 Apr 1;329(1):345–355.

[125] Jy W, Mao WW, Horstman L, Tao J, Ahn YS. Platelet microparticles bind, activate and aggregate neutrophils in vitro. Blood cells, molecules and diseases. 1995;21(3):217–31.

[126] Gorbet MB, Sefton MV. Material-induced tissue factor expression but not CD11b upregulation depends on the presence of platelets. Journal of biomedical materials research. 2003 May;67(3):792–800.

[127] Hong J, Andersson J, Ekdahl KN, Elgue G, Axen N, Larsson R, et al. Titanium is a highly thrombogenic biomaterial: possible implications for osteogenesis. Thrombosis and haemostasis. 1999 Jul;82(1):58–64.

[128] Schuler P, Assefa D, Ylanne J, Basler N, Olschewski M, Ahrens I, et al. Adhesion of monocytes to medical steel as used for vascular stents is mediated by the integrin receptor Mac-1 (CD11b/CD18; alphaM beta2) and can be inhibited by semiconductor coating. Cell communication and adhesion. 2003 Jan-Feb;10(1):17–26.

[129] Nomura S, Tandon NN, Nakamura T, Cone J, Fukuhara S, Kambayashi J. High shear stress induced activation of platelets and microparticles enhances expression of cell adhesion molecules in THP-1 and endothelial cells. Atherosclerosis. 2001 Oct;158(2):277–287.

[130] Goto S, Ichikawa N, Lee M, Goto M, Sakai H, Kim JJ, et al. Platelet surface P-selectin molecules increased after exposing platelet to a high shear flow. International angiology : a journal of the International Union of Angiology. 2000 Jun;19(2):147–151.

[131] Miyazaki Y, Nomura S, Miyake T, Kagawa H, Kitada C, Taniguchi H, et al. High shear stress can initiate both platelet aggregation and shedding of procoagulant containing microparticles. Blood. 1996 Nov 1;88(9):3456–3464.

[132] Tarnok A, Mahnke A, Muller M, Zotz RJ. Rapid in vitro biocompatibility assay of endovascular stents by flow cytometry using platelet activation and platelet-leukocyte aggregation. Cytometry. 1999 Feb 15;38(1):30–39.

[133] Gourlay T, Stefanou DC, Asimakopoulos G, Taylor KM. The effect of circuit surface area on CD11b(mac-1) expression in a rat recirculation model. Artificial Organs. 2001 Jun;25(6):475–479.

[134] Evangelista V, Manarini S, Sideri R, Rotondo S, Martelli N, Piccoli A, et al. Platelet/polymorphonuclear leukocyte interaction: P-selectin triggers protein-tyrosine phosphorylation-dependent CD11b/CD18 adhesion: role of PSGL-1 as a signaling molecule. Blood. 1999 Feb 1;93(3):876–885.

[135] Zhang JN, Bergeron AL, Yu Q, Sun C, McIntire LV, Lopez JA, et al. Platelet aggregation and activation under complex patterns of shear stress. Thrombosis and haemostasis. 2002 Nov;88(5):817–821.

[136] Yang X. Pulsating flow and platelet aggregation. In: Sunderam VS, van Albada GD, Sloot PMA, Dongarra JJ, editors. Computational Science - ICCS 2005. 5th International Conference. Atlanta, GA, USA. Germany; 2005. p. 1048–1051.

[137] Kao WJ. Evaluation of leukocyte adhesion on polyurethanes: the effects of shear stress and blood proteins. Biomaterials. 2000 Nov;21(22):2295–2303.

[138] Dopheide SM, Maxwell MJ, Jackson SP. Shear-dependent tether formation during platelet translocation on von Willebrand factor. Blood. 2002 Jan 1;99(1):159–167.