Anti-thrombogenic Study of Ultrathin Monoethylene Glycol Adlayer on 316L Stainless Steel against Whole Human Blood

by

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A thesis submitted in conformity with the requirements for the degree of Master of Science

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Abstract

Implantable biomaterial made from austenitic 316L stainless steel have been widely used in a variety of clinical applications. However, exposure of foreign surfaces to blood can initiate platelet adhesion and trigger blood coagulation, leading to life-threatening consequences. In order to prevent undesirable surface platelet adhesion, we have developed an ultrathin anti-thrombogenic coating based on monoethylene glycol (MEG-OH) silane chemistry. Surface characterization of major surface modification steps are done by X-ray photoelectron spectroscopy and supplemented with contact angle goniometry. End stage anti-thrombogenicity assessment after 20 min blood exposure at 100 s\(^{-1}\), 300 s\(^{-1}\), 600 s\(^{-1}\), 750 s\(^{-1}\), and 900 s\(^{-1}\) shear rate has shown significant reduction (> 90%) of platelet adhesion, aggregation and thrombus formation on MEG-OH surface modified 316L stainless steel, compared with untreated ones. This finding is confirmed by real-time surface evaluation of 60 min blood exposure at 100 s\(^{-1}\), 600 s\(^{-1}\), and 900 s\(^{-1}\) shear rate.
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# Table of Contents

Abstract ........................................................................................................................................... ii
Acknowledgement ........................................................................................................................ iii
Table of Contents ........................................................................................................................... v
List of Tables .................................................................................................................................. vii
List of Figures ............................................................................................................................... viii
List of Appendices ......................................................................................................................... xii

1 Introduction ................................................................................................................................... 1
  1.1 Biomaterial .............................................................................................................................. 1
      1.1.1 Whole Human Blood Composition and Hemostasis ............................................... 1
      1.1.2 Biomaterial-blood Interaction ................................................................................. 4
      1.1.3 Plasma Protein Adsorption ..................................................................................... 5
      1.1.4 Platelet Adhesion and Subsequent Activation ....................................................... 7
      1.1.5 Hemodynamics and Platelet Adhesion .................................................................. 9
      1.1.6 316L Stainless Steel: the Most Common Used Biomedical Material ............. 11
      1.1.7 Hemocompatibility of 316L Stainless Steel Based Biomaterial ..................... 13
  1.2 Purpose of Research ................................................................................................................ 14
  1.3 Theory ..................................................................................................................................... 16
      1.3.1 Silane Chemistry ....................................................................................................... 16
      1.3.2 X-Ray Photoelectron Spectroscopy ..................................................................... 18
      1.3.3 Contact Angle Goniometry .................................................................................. 20
      1.3.4 Parallel Plate Flow Chamber and Confocal Microscopy ................................... 21

2 Materials and Methodology ......................................................................................................... 23
  2.1 General Remarks .................................................................................................................... 23
  2.2 Mechanical Polishing and Electrochemical Polishing .................................................... 24
  2.3 Surface Cleaning and Oxidation of 316L Stainless Steel ................................................ 25
  2.4 Water Vapor Physisorption unto Oxidized 316L Stainless Steel Surface, and
     Subsequent MEG-TFA Adlayer Formation ........................................................................ 26
  2.5 Surface MEG-TFA Solvolysis .............................................................................................. 28
  2.6 Parallel Plate Flow Chamber Preparation and Assembly ............................................ 29
  2.7 Blood Perfusion Experimental Setup ................................................................................ 32
  2.8 Contact Angle Goniometry .................................................................................................. 33
List of Tables

Table 1. Measured wt % composition of 316L stainless steel

Table 2. Calculated volumetric flow rate and required blood volume at 100 s\(^{-1}\), 300 s\(^{-1}\), 600 s\(^{-1}\), 750 s\(^{-1}\), and 900 s\(^{-1}\) shear rate

Table 3. Relevant literature C1s, O1s, Si2p, F1s and Cr2p XPS data

Table 4. XPS relative atomic percentage for untreated (bare); oxidized (activated); MEG-TFA modified (MEG-TFA); MEG-OH modified (MEG-OH)

Table 5. Calculated student’s t values, in comparison of mean platelet surface percentage coverage on bare and MEG-OH modified 316L stainless steel surfaces
List of Figures

Figure 1. Composition breakdown of whole human blood

Figure 2. Coagulation Cascade

Figure 3. Activation of coagulation cascade and complement system induced by a foreign surface

Figure 4. Surface platelet adhesion through interaction with various adhesion and aggregation factors.

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Figure 5. Fluid velocity profiles in a rigid cylindrical tube with low and high shear rates

Figure 6. Physiological blood shear rate in various vessels

Figure 7. MEG-OH surface modified quartz

Figure 8. Mechanism of organotrichlorosilane adlayer formation

Figure 9. The photoelectric effect, showing the photoexcitation of a 1s electron. Also shown an electron from an outer shell filling the vacancy (dotted line) and the ejection of an auger electron (in red).

Figure 10. Schematic diagram of an XPS instrumentation

Figure 11. Schematic diagram of contact angles, for hydrophilic and hydrophobic surfaces

Figure 12. Schematic diagram of custom-made parallel-plate flow chamber

Figure 13. Schematic diagram of a confocal microscope

Figure 14. 316L stainless steel surface cleaning and oxidation

Figure 15. Water physisorption onto oxidized 316L stainless steel surface

Figure 16. MEG-TFA adlayer formation onto 316L stainless steel surface

Figure 17. MEG-TFA adlayer solvolysis
Figure 18. Structure of DiOC<sub>6</sub>

Figure 19. Experimental setup used to record real-time platelet adhesion, aggregation and thrombus formation. Also shown a close-up view of the perfusion chamber under confocal microscope.

Figure 20. Relevant surface structures for XPS analysis: untreated (bare); oxidized (activated); MEG-TFA modified (MEG-TFA); MEG-OH modified (MEG-OH)

Figure 21. C 1s XPS narrow scan profiles at 20° and 90° take-off angles

Figure 22. O 1s XPS narrow scan profiles at 20° and 90° take-off angles

Figure 23. Si 2p XPS narrow scan profiles at 20° and 90° take-off angles

Figure 24. F 1s XPS narrow scan profiles at 20° and 90° take-off angles

Figure 25. Cr 2p XPS narrow scan profiles at 20° and 90° take-off angles

Figure 26. Measured static contact angles for untreated (bare); oxidized (activated); MEG-TFA modified (MEG-TFA); MEG-OH modified (MEG-OH); the error bars represent standard deviation, n = 5

Figure 27. XPS F/Cr and Si/Cr peak area ratios of MEG-TFA modified 316L stainless steel, analyzed at 20° take-off angle for 30 min, 60 min, 90 min, 120 min and 150 min silanization time

Figure 28. XPS CF<sub>3</sub>/Cr, F/Cr and Si/Cr peak area ratios of MEG-TFA modified 316L stainless steel, analyzed at 90° take-off angle for 30 min, 60 min, 90 min, 120 min and 150 min silanization time

Figure 29. Relevant surface structures for whole human blood surface thrombogenicity analysis: cleaned (bare); MEG-OH modified (MEG-OH)

Figure 30. Representative images after 20 min blood perfusion at 100 s<sup>-1</sup> shear rate, showing platelet adhesion, aggregation, and thrombus formation, or lack of, on bare (left) and MEG-OH modified 316L stainless steel (right).
**Figure 31.** Representative images after 20 min blood perfusion at 300 s\(^{-1}\) shear rate, showing platelet adhesion, aggregation, and thrombus formation, or lack of, on bare (left) and MEG-OH modified 316L stainless steel (right).

**Figure 32.** Representative images after 20 min blood perfusion at 600 s\(^{-1}\) shear rate, showing platelet adhesion, aggregation, and thrombus formation, or lack of, on bare (left) and MEG-OH modified 316L stainless steel (right).

**Figure 33.** Representative images after 20 min blood perfusion at 750 s\(^{-1}\) shear rate, showing platelet adhesion, aggregation, and thrombus formation, or lack of, on bare (left) and MEG-OH modified 316L stainless steel (right).

**Figure 34.** Representative images after 20 min blood perfusion at 900 s\(^{-1}\) shear rate, showing platelet adhesion, aggregation, and thrombus formation, or lack of, on bare (left) and MEG-OH modified 316L stainless steel (right).

**Figure 35.** Percentage of surface coverage due to platelet adhesion, aggregation and thrombus formation of bare and MEG-OH modified 316L stainless steel, after 20 min blood perfusion at 100 s\(^{-1}\), 300 s\(^{-1}\), 600 s\(^{-1}\), 750 s\(^{-1}\) and 900 s\(^{-1}\) shear rate; bars; the error bars represent standard deviation, n = 5

**Figure 36.** Percentage of platelet surface coverage reduction due to the MEG-OH adlayer on 316L stainless steel at 100 s\(^{-1}\), 300 s\(^{-1}\), 600 s\(^{-1}\), 750 s\(^{-1}\), and 900 s\(^{-1}\) shear rate; bars; the error bars represent standard deviation, n = 5

**Figure 37.** Representative frames captured every 3 min on bare 316L stainless steel surface during 60 min blood perfusion at 100 s\(^{-1}\) shear rate; the scale bars represent 20 µm

**Figure 38.** Representative frames captured every 3 min on MEG-OH modified 316L stainless steel surface during 60 min blood perfusion at 100 s\(^{-1}\) shear rate; the scale bars represent 20 µm

**Figure 39.** Representative frames captured every 3 min on bare 316L stainless steel surface during 60 min blood perfusion at 600 s\(^{-1}\) shear rate; the scale bars represent 20 µm
**Figure 40.** Representative frames captured every 3 min on MEG-OH modified 316L stainless steel surface during 60 min blood perfusion at 600 s⁻¹ shear rate; the scale bars represent 20 µm

**Figure 41.** Representative frames captured every 3 min on bare 316L stainless steel surface during 60 min blood perfusion at 900 s⁻¹ shear rate; the scale bars represent 20 µm

**Figure 42.** Representative frames captured every 3 min on MEG-OH modified 316L stainless steel surface during 60 min blood perfusion at 900 s⁻¹ shear rate; the scale bars represent 20 µm

**Figure 43.** Real-time platelet surface percentage coverage on bare and MEG-OH modified 316L stainless steel during 60 min blood perfusion at 100 s⁻¹ shear rate.

**Figure 44.** Real-time platelet surface percentage coverage on bare and MEG-OH modified 316L stainless steel during 60 min blood perfusion at 600 s⁻¹ shear rate.

**Figure 45.** Real-time platelet surface percentage coverage on bare and MEG-OH modified 316L stainless steel during 60 min blood perfusion at 900 s⁻¹ shear rate.
List of Appendices

Figure A. 1. XPS survey scans for bare (left) and oxidized (right) 316L stainless steel

Figure A. 2. XPS survey scans for MEG-TFA surface modified (left) and MEG-OH surface modified (right) 316L stainless steel
1 Introduction

1.1 Biomaterial

Over past fifty years, biomaterial have received a lot of attention with respect to potential adaptation for medical applications. In essence, biocompatibility refers to the ability of biomaterial to perform its desired function without eliciting any undesirable local or systemic effects in the recipient\(^1\). In today’s health care system, highly biocompatible material is urgently needed for medical diagnostic and treatment purposes, designed for direct exposure to patients’ biological tissue, such as blood. The contact between biomaterial and blood becomes unavoidable during procedures involving taking blood out of an individual's vascular system. A few examples include blood transfusion, cardiopulmonary bypass, blood dialysis and simply drawing blood through venipuncture for in vitro experiments. A variety of treatment methods involving disposable or implantable medical devices intended for short term and long term medical use, such as venous catheters, stents, vena cava filters, vascular grafts and prosthetic heart valves, also require biomaterial to be introduced into patient’s vascular system and brought into direct contact with blood. An ideal biomaterial should possess a combination of structural and surface characteristics, including excellent mechanical support, corrosion resistance, biocompatibility and thrombo-resistivity. In addition, other deployment advantages, such as superior vessel trackability, adequate radio compatibility, drug delivery capability as well as inexpensive manufacturing process should also be considered and incorporated into the biomaterial design\(^2\-^3\).

1.1.1 Whole Human Blood Composition and Hemostasis

Plasma, making up roughly 55% of total blood volume, is mostly made up by water (up to 95% by volume), dissolved proteins (6-8% by volume, including serum albumins, globulins, fibrinogen and other clotting factors), and glucose, electrolytes and hormones (2% by volume). The buffy coat, making up less than 1% of total blood volume, mainly consists of leukocytes and platelets. The remaining 45% of blood volume is due to erythrocytes\(^4\). (Figure 1.1)
Blood, the primary component of human circulatory system, functions as a transport system supplying nutrients and oxygen to sustain living cells while simultaneously facilitating removal of metabolic wastes. In addition, blood borne cells and enzymes are also involved in initiating immune response against foreign microorganisms and substances. Since blood flows inside a close-loop pressurized vascular system, acute response mechanisms must be initiated to stop bleeding in case of blood vessel injury, in order to avoid excessive blood loss and maintain hemostasis.

The potent hemostasis regulatory mechanism is a highly efficient and complex system, keeping a delicate balance between bleeding and thrombosis\(^5\). In a healthy individual, the normal hemostatic response initiates when a blood vessel is ruptured or damaged. The primary stage of the hemostatic response involves adhesion of platelet to the damaged vessel wall. The activation of platelets attracts more platelet to the affected area, effectively forming a platelet plug, which stops the initial bleeding. The second stage of the hemostatic response involves the initiation of the coagulation cascade process\(^6\). (Figure 2)
In essence, the coagulation cascade is a complex network of step-by-step activation process between clotting factor zymogens and enzymes. The final product of this cascade response is formation of a soluble cross-linked fibrin clot. Coupled with activated platelets, the soluble cross-linked fibrin clot ultimately leads to a much more structurally stabilized fibrin network, which effectively seals the damaged area within a blood vessel until the vessel wall is fully repaired. When the blood vessel is restored, the clot is cleared by digestion of the fibrin network into smaller fragments through the fibrinolysis pathway, which is also characterized by a series of zymogen-enzyme activation and bears similarity to the coagulation cascade.
When regulatory mechanism of the potent hemostatic response fails or becomes too effective therefore no longer specific to vessel wall injuries, unwanted thrombosis or embolism, due to formation of blood clot within a blood vessel, could lead to vessel blockage and blood flow obstruction, ultimately resulting in severe physiological damage.

### 1.1.2 Biomaterial-blood Interaction

As previously mentioned, the normal hemostatic response is a highly efficient and complex system, which maintains the balance between unrestricted bleeding and thrombosis. The excellent hemocompatibility of the blood vessel is mediated by glycocalix of the endothelium cell lining covering the inner vascular surface\(^9\). However, when blood is exposed to surface of a foreign artificial object, which shares similar characteristics of an injured vascular vessel, this delicate hemostatic balance could be disrupted. The contact of blood to foreign surface could inadvertently activate leukocytes and platelets, as well as initiating the enzyme mediated coagulation cascade. (Figure 3) As a result, unwanted thrombus formation on the artificial object surface occurs.

For artificial vascular implants, formed surface thrombus could detach and form embolus, leading to life-threatening consequences. In order to prevent severe adverse physiological effects caused by artificial grafts and implants, a systemic antiplatelet and anticoagulation therapy is usually implemented during and after surgical procedures. Common examples include high dose of intravenous heparin treatment during and after cardiopulmonary bypass surgery, and continuous warfarin treatment for patients with prosthetic heart valve\(^{10}\). The current medical control measures for thrombus formation is clearly not ideal. Long period of antiplatelet and anticoagulant treatment could lead to many side effects, such as hemorrhage and thrombocytopenia\(^{11}\). Therefore, design of a biomaterial, offering improved if not perfect hemocompatibility, represents an urgent clinical need.
1.1.3 Plasma Protein Adsorption

The contact of blood with foreign surface could lead to a cascade of processes, which is initiated by surface plasma protein adhesion. The protein adsorption process is a rather rapid process. A thin layer (2-10 nm) of biologically active protein surface is generated within seconds after the contact of body foreign surfaces with blood\textsuperscript{12}. The most important protein species in this process include fibrinogen, vitronectin, immunoglobulins, Von Willebrand factor (vWF), HMW-kininogen, pre-kallikrein, Factor XI and Factor XII\textsuperscript{13-14}. The thin protein layer can subsequently interact with other blood borne mechanisms. Blood, as a complex biological fluid, is known to contain more than three hundred distinct protein with different characteristics and functions. Surface protein adsorption, an exothermic spontaneous process, is observed as the Gibbs free energy decreases:\[ \Delta G_{ads} = \Delta H_{ads} - T\Delta S_{ads} , \] where H, T and S stand for enthalpy, temperature and entropy. The observed decrease in the Gibbs free energy is driven by the increased system entropy due to a combination of factors, including the dehydration of proteins and adsorbent surface, interaction of protein and surface charged groups, and protein conformation change\textsuperscript{15}. The accumulation of protein on body foreign surface can result in up to 1000 fold surface protein concentration increase relative to the blood protein concentration\textsuperscript{16}.
The process of protein surface adsorption is also a dynamic process, depending on the chemical and physical properties of the surface and proteins involved. Surface adsorbed protein composition is observed to vary over time as one protein can be replaced by another protein minutes after the initial adsorption. This process, called Vroman effect, describes the dynamic nature of plasma protein-foreign surface interaction, in which the initial adsorbed highest mobility protein is replaced by later arrived proteins that have a higher affinity for the surface\textsuperscript{17-18}. The Vroman effect is observed to preferentially occur on hydrophilic, negatively charged surfaces\textsuperscript{19}. 

The surface wettability is also considered an important determinant for the protein adsorption process. It is widely accepted by the scientific community that hydrophobic surfaces tend to adsorb proteins more steadily than hydrophilic surfaces. The observed higher tendency of hydrophobic surfaces to adsorb protein is driven by the favorable surface interaction with hydrophobic domains and residues in the protein. The favorable process is further assisted by system entropy increase resulted from release of unfavorably organized water on the surface\textsuperscript{20}. A surface is generally considered hydrophilic when static or dynamic contact angle measurement with water is less than 65\textdegree\textsuperscript{21}. 

Other important factors, such as protein/surface charge and protein conformational change, can also contribute to the protein adsorption process. The maximal surface protein adsorption occurs when the blood pH is between the isoelectric points of the surface and protein, causing the protein and surface to possess opposite charges\textsuperscript{21}. Protein conformational change is also considered an important driving force for the surface adsorption process, especially under circumstances that no hydrophilic or hydrophobic protein-surface interaction is present. The dependence of protein surface adsorption on flow condition has also been analyzed. Studies have shown that there is no direct correlation between shear rate and the protein adsorption process, even under high shear rate of 2700 s\textsuperscript{-1}\textsuperscript{22}. 

1.1.4 Platelet Adhesion and Subsequent Activation

The natural, non-activated platelets, produced by megakaryocytes in the bone marrow (one megakaryocyte can produce up to 8000 platelets), are anuclear cellular fragments with 1-2 µm in length, 1 µm in thickness, and weigh ~10 pg\(^{24}\). Platelets circulate in the quiescent state for about 10 days throughout human body before cleared by macrophages and in liver and spleen. Most platelets are never activated in their lifespan, and their potential adhesiveness only become evident when the endothelium cell lining located at the inner blood vessel wall is damaged, or blood is exposed to foreign artificial material, such as cardiovascular implants.

Adsorbed protein on artificial surface can be recognized by platelet adhesion factors, which facilitates the process of adhesion, spreading and activation of platelets. The major platelet adhesion and aggregation mediators in natural human plasma include fibrinogen, fibronectin, vitronectin, immunoglobulins and vWF. It was conventionally thought that the surface adhesion of platelet was mediated through the mechanism of GPIIb/IIIa receptor binding to the Arg-Gly-Asp (RGD) like motif present on surface adsorbed fibrinogen. However, Latour et al.\(^{24}\) has recently shown that fibrinogen conformational change induced by surface adsorption leads to exposure of the binding motif and major modification in the protein’s biological activity. This finding explains why no platelets are activated and no clots are formed under physiological condition. The amount of adherent fibrinogen required to initiate platelet adhesion is very small, ~7 ng/cm\(^2\), and the initial platelet adhesion is completed through binding of platelet integrin \(\alpha\)IIb\(\beta\)3 receptor to surface adsorbed fibrinogen\(^{18}\). (Figure 4) The process of initial platelet surface adhesion is revealed to be kinetically rapid, requiring less than 5 s for hydrophobic surfaces and less than 30 s for hydrophilic surfaces\(^{25}\). The direct effect of surface wettability on platelet adhesion and activation has also been studied. The results show platelet adhesion occurs more frequently when the surface wettability is decreased\(^{26-27}\). This finding should not be particularly surprising as a greater extent of fibrinogen is expected to be adsorbed on hydrophobic surfaces than hydrophilic ones, indicated in the previously mentioned surface wettability and protein adsorption correlation study.
After initial platelet binding to surface adsorbed fibrinogen, a complex intra-cellular cascade is turned on to transduce the biochemical signal from the platelet plasma membrane to the cytoplasm, ultimately resulting in activation of platelet. The process of platelet activation is very fast, only requiring ~180 ms. The activated platelet, undergoing a change in shape due to rearrangement of cytoskeleton proteins, are then capable to release substances acting as vasoconstrictors in order to promote adhesion and aggregation of more neighboring platelets, facilitated by a variety of clotting factors and enzymes, such as fibrinogen, fibronectin and vWF. As the whole platelet adhesion and activation process is complex and highly dynamic, the number of each
membrane-bound receptors per platelet varies substantially, and most platelet receptors are not specific with respect to ligand/substrate.\textsuperscript{33} When sufficient number of platelets are activated near foreign surface, the intrinsic pathway is subsequently triggered through factor XII activation, ultimately leading to surface thrombus formation and stabilization by factor XIII-crosslinked fibrin.

1.1.5 Hemodynamics and Platelet Adhesion

Inside a blood vessel, the flowing blood stream leads to development of shear stress, which is applied by blood against the vessel wall. Shear stress is generated due to blood pressure gradient and transferred to all vessel layers. For a Newtonian fluid flowing in a planar surface, shear stress is determined by Newton’s law: \textsuperscript{34-35}

\[
\tau = \mu \cdot \frac{du}{dy},
\]

where \( \mu \) is the kinetic viscosity; \( u \) is the fluid velocity; \( y \) is the distance from the surface. Assuming blood behaves like a Newtonian fluid and blood flow maintains steady and laminar, shear stress due to blood flow inside an inelastic, cylindrical and straight vessel is defined by the Haagen-Poiseuille equation: \textsuperscript{36}

\[
\tau = \frac{32 \mu Q}{\pi d^3},
\]

where \( Q \) is the mean volumetric flow rate; \( u \) is the mean velocity; \( d \) is the vessel diameter. The determination of shear stress is based on the fundamental no-slip condition, which states that the fluid velocity upon surface is zero. Under this assumption, blood stream travel in layers parallel with the inner vascular endothelium surface, and velocity of each layer increases from zero at the vessel wall to the maximum value roughly at the center of blood vessel, leading to establishment of velocity gradient. (Figure 5) Shear rate, defined as the rate at which adjacent layers of fluid move with respect to each other and usually expressed in the unit of \( s^{-1} \), determines the shape of fluid velocity profile for a
If we consider blood vessel as a straight and cylindrical tube with rigid walls, the shear rate, or the velocity gradient, is given the following relationship, assuming blood is an ideal Newtonian fluid with constant viscosity and maintains steady and laminar flow:

\[
\dot{\gamma} = \frac{du}{dr} = \frac{32Q}{\pi d^3},
\]

where \( Q \) is the mean volumetric flow rate; \( u \) is the mean velocity; \( d \) is the vessel diameter. Under these condition, a parabolic velocity profile can be assumed.

In fact, whole human blood does not behave like a Newtonian fluid. Due to the ability of erythrocytes to deform and aggregate, blood exhibits non-Newtonian behavior, especially at low shear rate (< 100 s\(^{-1}\)).\(^{38-39}\) Furthermore, blood viscosity is dependent on shear rate, and blood viscosity decreases as shear rate increases.\(^{40}\) Since shear stress is a function of blood viscosity, which is not constant and cannot be readily determined by any straightforward mathematical relationships, shear rate becomes a much easier-to-control experimental parameter than shear stress for most in vitro experiments. According to this equation, shear rate is primarily influenced by diameter of the blood vessel. High shear rate is present when the blood flow is fast and vessel diameter is small, and low shear rate is present when the blood flow is slow and vessel diameter is large. Based on previously published data,\(^{41-42}\) typical physiological blood shear rate in major vessels are outlined below. (Figure 6) The reported clinical values involve estimation of mean volumetric flow rate and vessel diameter, which can vary substantially from person to person.

Figure 5. Fluid velocity profiles in a rigid cylindrical tube with low and high shear rates.
Blood stream shear rate determines which protein platelets preferably adhere to and with which receptors. At high shear rate (> 5000 s\(^{-1}\)), platelets adhesion is found to be mediated specifically by vWF.\(^{43}\) At medium shear rate (1000-5000 s\(^{-1}\)), fibrinogen is the primary platelet adhesion meditator, and at low shear rate (< 1000 s\(^{-1}\)), platelets adhere to fibronectin, vitronectin, and fibrinogen.\(^{44}\) As shown in the figure, except arteriole and capillaries, which have relatively small diameter, most major blood vessels fall within the low shear rate regime (< 1000 s\(^{-1}\)).

1.1.6 316L Stainless Steel: the Most Common Used Biomedical Material

For the past twenty years, implantable biomaterial made from AISI type 316L stainless steel have been widely used in a variety of clinical applications, including metallic stents deployed during coronary angioplasty. For an optimal coronary stent, surface and bulk characteristics, metallic property as well as surface design and chemistry are all important factors to consider. The backbone material must be able to maintain excellent mechanical and chemical properties under stringent physical conditions. For example, the backbone material required for an expendable stent must have sufficient mechanical plasticity to fit the required diameter size within a blood vessel when deployed. In addition, self-expanding stents must be manufactured from material
that offers enough elasticity so that the stents can be compressed and expanded within
an affected vessel while retaining high radial hoop strength to prevent the blood vessel
from recoiling or collapsing once fully deployed.\textsuperscript{45} To date, most first generation
commercial coronary stents are manufactured from AISI 316L stainless steel,\textsuperscript{46} which
offers outstanding physical characteristics to address most mechanical requirements
mentioned above.

<table>
<thead>
<tr>
<th>Element</th>
<th>Measured wt. % composition</th>
<th>Element</th>
<th>Measured wt. % composition</th>
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<tbody>
<tr>
<td>Iron</td>
<td>68.04</td>
<td>Chromium</td>
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<tr>
<td>Sulfur</td>
<td>0.01</td>
<td>Aluminum</td>
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</tr>
</tbody>
</table>

Table 1. Measured wt % composition of 316L stainless steel

Chemical properties of the backbone material is also crucial, especially corrosion
resistance. Saline solution, such as blood, can quickly oxidize the implant surface and
cause corrosion particles to migrate to other parts of the body. It is commonly known that
surface formation of metal oxides film can effectively retard corrosion. In this regard, AISI
316L stainless steel provides exceptional anti-corrosion characteristics. The L
designation indicates low carbon content (< 0.03 wt. %). As outlined in the Table 1, the
alloy is composed of predominantly iron, chromium, nickel, molybdenum and manganese.
The high chromium content (~17 wt. %) provides the alloy superior corrosion resistance
as well as mechanical advantages in hardness and strength.\textsuperscript{47}
1.1.7 Hemocompatibility of 316L Stainless Steel Based Biomaterial

Despite excellent mechanical characteristics and corrosion resistance, clinical application of bare 316L stainless steel, like other material, is constrained by limited hemocompatibility and often plagued by surface thrombus formation when exposed to blood. So far, a variety of surface treatment has been attempted and shown to improve hemocompatibility of 316L stainless steel based biomedical implants. Sheth et al.\textsuperscript{48} has shown the effects of mechanical polishing to decrease surface irregularities on thromboses. Mechanically polished Palmaz-Schatz stents exhibit significant less thrombus formation compared with unpolished ones, in an ex-vivo porcine arteriovenous shunt model study. In addition, De Scheerder et al.\textsuperscript{49} demonstrate a drastic reduction in thrombus formation on electrochemically polished 316L stainless steel stents both in a rat arteriovenous shunt model and in a pig coronary model compared with untreated stents.

In the past twenty years, surface coatings have received a lot of attention in the scientific community, in order to combine distinctively favorable characteristics from different material. Many passive coatings as well as active, drug-eluting have been developed and tested under ex-vivo condition. Prior to year 2000, a variety of chemically inert passive coatings have been tested on 316L stainless steel. However, some are shown to be merely marginally effective against platelet adhesion and thrombus formation while some are reported to be capable of reducing platelet adhesion to a significant extent but cause increased neointimal formation in animal studies compared with untreated stainless steel. Some notable examples of such coatings include cellulose, poly-l-lactic acid, polynitrosated nitric oxide albumin, fibrin, silicone carbide, cross-linked phosphorylcholine, polyorganophosphazene, and polyurethane.\textsuperscript{50-56} In the last decade, newer generations of organic polymer coatings have shown promising results towards development of a surface inert to platelet adhesion and activation of coagulation. A few such coating examples are heparin,\textsuperscript{57} poly (ethylene glycol),\textsuperscript{58} which are prepared by surface covalent immobilization, and poly (vinylidene fluoride-co-hexafluoropropylene),\textsuperscript{59} which can prepared by either solvent casting or electrospinning. Recently, Qin Zhang et
al. has shown that SiCOH plasma nanocoating on 316L stainless steel stents can effectively reduce percentage of stenosis to 26.3±10.1% after 12 weeks.

1.2 Purpose of Research

Currently, the most commonly used surface modification coatings to enhance biomaterial’s resistance to protein and platelet adhesion are heparin and Poly (ethylene glycol) derivatives, and some biomedical implant manufacturers like GORE VIABAHN Endoprosthesis®, have already brought heparin-coated vascular stents to the market. Both coatings require surface covalent immobilization. But heparin and PEG coatings represent different approaches to minimize undesirable blood-material interactions. One approach is to avoid coagulation, and heparin is known to facilitate the elimination process of thrombin and other clotting factors such as factor Xa while suppressing activation of the complement system. However, this approach can lead to long term side effects such as surface protein clogging, accumulation of lipids and ultimately calcification. Another approach is to enhance the surface resistance to protein adhesion and subsequent platelet/cell adhesion, through immobilization of poly (ethylene glycol) molecules. The rationale behind this approach is that absence of adsorbed plasma protein layer prohibits platelets, clotting factors and cells in the blood stream from actively interacting with the foreign surface. It is speculated that substantially reduced protein-platelet interaction would lead to proportionally decreased blood coagulation, rendering the surface anti-thrombogenic.

Due to PEG’s susceptibility to oxidation, recent research efforts have been directed towards shorter chain oligo (ethylene glycol) OEG-based coatings. Recently, Sheikh et al. have reported a study involving in-serum adsorption dynamics of ultrathin monoethylene glycol-terminated (MEG-OH) adlayer on quartz, (Figure 7) monitored using electromagnetic piezoelectric acoustic wave sensor (EMPAS). Compared with bare quartz, ~17 fold frequency shift reduction is observed, and the frequency shift reduction is attributed to the antifouling property of the MEG-OH adlayer. In an effort to analyze the speculated anti-thrombogenic potential of MEG-OH adlayer on 316L stainless steel, the current stage of research consists of the following objectives:
1. Immobilize 2-(3-trichlorosilylpropyloxy) ethyl-trifluoroacetate (MEG-TFA) linker covalently onto freshly prepared surface oxidized 316L stainless steel samples, followed by surface solvolysis to generate MEG-OH adlayer, then characterize the said modified surface.

2. When the immobilization protocol is proven successful, a time trial experiment is used to determine the optimal immersion/deposition time, followed by characterization of all modified surfaces.

3. Scale up and apply the optimized immobilization protocol to larger dimension 316L stainless steel samples. Analyze and compare the anti-thrombogenicity of MEG-OH surface modified with untreated 316L stainless steel under specified shear rates against fluorescent-labeled whole human blood.

Mirror and electrochemically polished 316L stainless steel surface will be functionalized with hydroxyl groups by treatment with piranha solution (1:3 98% H₂SO₄ to 30% H₂O₂ by volume) after rigorous surface cleaning procedure involving successive sonication in multiple polar and nonpolar solvents. Following an overnight period of water
vapor physisorption inside a humidity chamber, the hydroxylated 316L stainless steel surface is subjected to immobilization treatment as mentioned above. Surfaces following each major step of surface treatment will be characterized with contact angle goniometry and X-ray photoelectron spectroscopy (XPS). Fresh whole human blood are drawn from healthy volunteers on the day of experiment and subsequently fluorescent-labeled with 10 µM 3,3'-dihexyloxacarbocyanine iodide (DiOC\textsubscript{6}, \lambda\text{exc}:\lambda\text{em} = 484/501 nm) in DMSO solution prior to use. Platelet adhesion and thrombus formation are observed in real-time under a confocal microscope, equipped with a digital recording camera.

1.3 **Theory**

1.3.1 **Silane Chemistry**

In order to ultimately form an MEG-OH terminated adlayer on a substrate, it is necessary to first immobilize 2-(3-trichlorosilylpropyloxy) ethyl-trifluoroacetate (MEG-TFA) linker onto the surface. Despite there are many types of organosilane, the typical organosilane molecular species used for adlayer formation have the following generic structure:

![Silane Structure]

where X stands for the head functional group of the organosilane molecule and is usually substituted with chlorine, in the case of trichlorosilane, or other reactive alkoxy groups.\textsuperscript{62} The organic bone of the molecule, the R group, is usually a long alkyl chain or oligo (ethylene glycol), and for our purposes, monoethylene glycol trifluoroacetate.
Figure 8. Mechanism of organotrichlorosilane adlayer formation

So far, the exact mechanism for organosilane adlayer formation, or simply the surface silanization process, is still under debate. However, it is currently in consensus that the silanization process occurs through a multi-step reaction mechanism. The whole process starts with diffusion of the organosilane to the substrate-solution interface, followed by hydrolysis of the tricholorsilane $-\text{SiCl}_3$ moiety by surface-adsorbed water molecule to generate trisilanol $-\text{Si(OH)}_3$ species. (Figure 8 step 1) The second step involves the chemisorption of the linker onto the substrate through hydrogen bonding interaction between the surface hydroxyl groups and the linker silanol groups, immediately followed by condensation reaction between the two groups. (Figure 8 step 2 and 3) The second step usually results in formation of islands (nucleating) over the surface. Subsequent reorganization and aggregation, driven by intermolecular Van der Waals forces between neighboring organic backbones, lead to adlayer formation. Ideally, cross-linkage between neighboring surface-bound silanol groups is known to occur to further strengthen the adlayer stability. However, such events may be limited due to steric effects between terminal groups.
There are several factors that can influence the eventual formation and structure of the adlayer, such as reaction temperature, linker concentration, solvent type, immersion/deposition time, and amount of surface-adsorbed water. In an effort to ensure maximal experimental reproducibility, all silanization reactions are carried out under inert (N$_2$) and anhydrous (P$_2$O$_5$) conditions. Solvent choice as well as the linker concentration are kept consistent throughout this study. In addition, time allowed for each substrate inside a humidity chamber is also closely monitored to ensure sufficient surface adsorbed water for complete adlayer formation and to prevent excess water deposition from causing premature linker polymerization, resulting in thick inhomogeneous adlayer. The optimal immersion/deposition time for the silanization reaction is later determined in a time trial study.

1.3.2 X-Ray Photoelectron Spectroscopy

Despite Heinrich Hertz first discovered the photoelectric effect in 1887, it was Albert Einstein who first explained the photoelectric effect in 1905. After the WWII, Kai Siegbahn pioneered the development of XPS as an analytical technique after recording the first functional high-energy resolution XPS of cleaved sodium chloride. Ever since then, XPS has gradually become a standard surface probing analytical technique due to its high sensitivity with probing depth of 1-10 nm. A variety of information on a surface can be retrieved through XPS, including relative atomic composition (accurate to 0.01-0.3 %), electronic and chemical states of atoms and depth profile. The principle of XPS is based on the photoelectric effect, in which an X-ray photon interacts with a sample, causing excitation and subsequent ejection of a core-level photoelectron. (Figure 9) The phenomenon can be explained by the following relationship:

$$E_{binding} = E_{photon} - E_{kinetic} - \phi$$
where $E_{\text{binding}}$ stands for the binding energy of the atomic orbital from which the photoelectron is ejected (relative to Fermi level); $E_{\text{photon}}$ is the energy of the X-ray photon; $E_{\text{kinetic}}$ is the kinetic energy of the ejecting photoelectron; $\varphi$ is the work function term used for correction due to instrumental absorption.

A schematic diagram of an XPS instrumentation is displayed in the figure 10. For a Kα probe, an X-ray with radiation energy of 1486.6 eV or 1263.6 eV is generated from a twin anode made from either Mg or Al. After passing through a monochromator, the incidental X-ray hits the sample, situated in an ultra-high vacuum chamber. The sample is placed on a rotator platform (for ARXPS), which is capable to adjust the take-off angle by tilting the sample. The take-off angle is determined by the line of acceptance relative to the sample surface. ARXPS allows for varying emission angles by which the ejecting photoelectrons can be picked up by the analyzer, providing distributional information for depth profiling and elements in different surface regions. After the ejected photoelectrons pass through a series of lenses, they are directed towards the hemispherical electron energy analyzer, which is also an ultra-high vacuum environment. By adjusting the voltage applied to the hemisphere, electrons sorted based on energy levels will eventually reach the detector. When connected to a computer output, the detector records incoming electrons in counts/s as a function of binding energy, and the resulting graphs can be
used for quantitative analysis as the relative atomic percentage is determined from individual peak area. The survey scan summarizes and provides relative atomic percentage of all elemental species detected on the surface. The narrow scan, which outlines individual peak, is used to extract information from interested elemental species, such as F1s, Si2p, C1s, and O1s for our surface characterization purposes.

![Schematic diagram of an XPS instrumentation](image)

**Figure 10. Schematic diagram of an XPS instrumentation**

1.3.3 Contact Angle Goniometry

In our study, contact angle goniometry is primarily used to measure surface wettability and to provide supplementary information like surface tension prior to XPS analysis. Contact angle is defined as the angle made by the liquid-air interface and the solid-liquid interface, shown as $\Theta_c$ in the figure 11.
When measuring static contact angles, one drop of distilled water, or other liquid with known surface energy, is deposited onto the substrate surface by a syringe pointing vertically down. A high resolution camera captures the images, which are then analyzed by a image analysis software. The theory behind contact angle comes from the thermodynamic equilibrium of the three phases: solid (S), liquid (L) and gas (G). The interfacial tension for solid-liquid, liquid-gas, and solid-gas interfaces are designated as $\gamma_{SL}$, $\gamma_{LG}$, $\gamma_{SG}$, respectively, as shown in the figure. The relationship between contact angle and interfacial tensions can be expressed by Young’s equation: \[ 0 = \gamma_{SG} - \gamma_{SL} - \gamma_{LG} \cos \theta_c \]

In general, a surface with a high contact angle indicates low surface energy and wettability, and a low contact angle indicates high surface energy and wettability. By convention, a surface is considered hydrophilic if contact angle is less than 65°, and it is considered hydrophobic if contact angle is greater 65°. In this study, distilled water is used as the testing liquid consistently, and contact angle of every sample is checked after each major surface modification step.

1.3.4 Parallel Plate Flow Chamber and Confocal Microscopy

In order to analyze the anti-thrombogenic potential of MEG-OH adlayer, DiOC₆-fluorescent-labeled whole human blood is perfused over the stainless steel surface
through a custom-made parallel plate flow chamber. The flow chamber consists of a glass coverslip, channel slide, and substrate slide, which in our case, MEG-OH surface modified or untreated 316L stainless steel slides. (Figure 12) Assembly of the flow chamber is relatively simple and involves sandwiching the channel slide between the glass coverslip and substrate slide. Space between the two female Luer adapters is designed for fitting a microscope objective lens piece. Real-time platelet adhesion is observed under a confocal microscope, equipped with a digital camera. Confocal microscope offer several unique advantages over conventional light optical microscope, including ability to collect serial optical sections from thick specimen, elimination of out-of-focus fluorescence emission light ray, and shallow depth of field. The schematic diagram of a confocal microscope is shown in the figure 13. Real-time images on the substrate slide are recorded by the equipped digital camera within the perfusion channel while blood is flowing through the parallel-plate chamber.

Figure 12. Schematic diagram of custom-made parallel-plate flow chamber
2 Materials and Methodology

2.1 General Remarks

All glassware involved in silanization process, including 20 mL scintillation vials and 150 mm diameter, 65 mm deep pyrex petri dishes, was pre-treated with 5% (v/v) octadecyltrichlorosilane (OTS) in anhydrous toluene inside a glove box maintained under inert atmosphere. Silanized glassware was subsequently rinsed with large quantity of ACS grade toluene and acetone then dried under N$_2$ stream before use. 2-(3-trichlorosilylpropyloxy) ethyl-trifluoroacetate (MEG-TFA) linker used for stainless steel surface silanization was synthesized and provided by Dr. Christophe Blaszykowski. All silanization was performed inside an in-house Plexiglas glove box maintained under inert (N$_2$) and anhydrous (P$_2$O$_5$) atmosphere.

98% concentrated sulfuric acid was purchased from ACP Chemicals Incorporated. Anhydrous toluene, ACS grade toluene, ethanol, DMSO, OTS, and 98% 3,3'-
dihexyloxacarbocyanine iodide (DiOC$_6$) fluorescent dye were purchased from Sigma Aldrich. ACS grade Chloroform, n-pentane, acetone, and methanol were purchased from Fisher Scientific. Plain glass microscope slides (75.2 mm x 25.4 mm dimension, 1.0 mm thickness) was obtained from Ted Pella Incorporated. Threaded 1/16 inch, straight, male polyethylene tubing connectors was purchased from GlycoTech Corporation. Clear, impact-resistant, both sides smooth, 0.0508 mm thick polycarbonate film was purchased from McMaster-Carr Supply Company, along with 1/16 inch/1/8 inch (inside/outside) diameter moisture-resistant polyethylene vacuum tubing, Poly(methyl methacrylate) (PMMA) sheets, and Henkel Loctite Hysol® 0151 thixotropic epoxy paste adhesive.

For XPS surface analysis, annealed austenitic type 316L stainless steel foil, mirror polished on both sides, with thickness of 2.0 mm, was purchased from Goodfellow Ltd., Cambridge, UK. Due to restrictions on XPS sample size, the 316L stainless steel foil was cut into 10 mm x 10 mm square size by the University of Toronto Department of Chemistry Machine Shop. The 10 mm x 10 mm dimension with 2.0 mm thickness 316L stainless steel samples was subsequently sent to Electro-Kleen Alloy Polishing Ltd., Mississauga, Ontario, Canada for electrochemical polishing. 1.0 mm thick, Annealed, 2B mill polished on both sides, austenitic type 316L stainless steel plates, were obtained from Stainless Supply. Ltd., Monroe, NC, US. Stainless steel plates, shear cut into dimension of 50.8 mm x 25.4 mm, are used for the whole human blood surface thrombogenicity analysis

2.2 Mechanical Polishing and Electrochemical Polishing

No. 2B and buffed No. 8 (mirror polished) finish are both achieved through mechanical polishing. No. 2B finish is a bright, cold-rolled smooth finish. In order to polish a microscopically very porous and uneven stainless steel surface to No. 2B finish, the steel sheet must be first cold rolled under a hand sheet mills or continuous mills to a specified thickness, producing a dull finish. The steel surface then undergoes annealing and descaling, followed by a final light cold rolling pass under a polished roll.\textsuperscript{68} No. 2B finish is widely considered a general purpose surface polishing for most applications. Buffed No. 8, or mirror polishing gives the most reflective stainless steel surface that can be achieved through mechanical polishing. A buffed No. 8 surface finish can be prepared
by continuously polishing the surface with progressively finer abrasives, all the way up to 500-grit. The polishing procedure is then followed by extensive buffing with very fine white chrome bar buffing compounds.  

Electrochemical polishing of all 316L stainless steel samples was performed by Electro-Kleen Alloy Polishing Ltd., Mississauga, Ontario, Canada. Electrochemical polishing is known to be performed based on the following steps.  

The 316L stainless steel samples are submersed in an electrolytic solution, such as a mixture of H$_2$SO$_4$ and H$_3$PO$_4$. A direct electric current is then run through the electrolytic solution, making the stainless steel becoming the anode and nearby metallic conductor the cathode in an electrolytic reaction. As the polarized film covers the indents and protrusions on the steel surface, the rate of metallic dissolution is higher at protrusion than at indents because of higher polarized film thickness and resulting increased electric resistance at indents, ultimately leaving the steel surface microscopically featureless. 

2.3 Surface Cleaning and Oxidation of 316L Stainless Steel

![Figure 14. 316L stainless steel surface cleaning and oxidation](image)

All 316L steel samples were scratched “X” with a sharp tipped tweezers on one side prior to cleaning procedure, in order to differentiate the unscratched side for future analysis. Next, stainless steel samples were subjected to successive sonication in ACS grade n-pentane, ACS grade acetone, then 95% ethanol, for 20 minutes each. Afterwards, each 316L stainless steel sample was then carefully rinsed with a large quantity of 95% ethanol, followed by copious amount of distilled water. Cleaned stainless steel was subsequently dried under a steady stream of N$_2$ gas. All dried steel samples were then individually immersed and oxidized in piranha solution as follows: each 10 mm x 10 mm dimension samples was placed within a test tube, with the unscratched side facing
upward. 50.8 mm x 25.4 mm dimension samples were placed tilted against the glassware in a 120 mL beaker, which is capable of holding up to six samples. Each test tube or beaker was subsequently put in a hot water bath pre-heated to 90°C. Piranha solution was prepared by adding three equivalent of 98% concentrated sulfuric acid to one equivalent 30% hydrogen peroxide. (The reaction is very exothermic, and piranha solution is corrosive. Therefore, handle with caution.) Approximately 2 mL piranha solution was dispensed into each test tube, and about 100 mL piranha solution was poured into the 120 mL beaker to fully immerse all steel samples.

After 30 minutes, all oxidized 316L stainless steel samples were removed from piranha solution. Each steel sample was then rinsed with large amount of distilled water to wash off residual piranha solution on surface, followed by three times of ACS grade methanol rinse. Next, steel samples were individually transferred to a clean glassware and placed inside an oven maintained at 150°C. After two hours, steel samples were taken out of the oven and left to cool down to the room temperature for 15 minutes. Finally, each 316L stainless steel sample was plasma cleaned using an expanded tabletop plasma cleaner with a maximum power of 30 V for 5 min. One oxidized sample was stored in a clean scintillation vial under nitrogen awaiting XPS surface characterization.

2.4 Water Vapor Physisorption unto Oxidized 316L Stainless Steel Surface, and Subsequent MEG-TFA Adlayer Formation

![Figure 15. Water physisorption onto oxidized 316L stainless steel surface](image-url)
After plasma cleaning, all oxidized 316L stainless steel samples intended for surface modification were placed immediately inside a humidity chamber for 24 hours to facilitate water vapor physisorption onto the steel surface. The humidity chamber was maintained by an aqueous solution of magnesium nitrate hexahydrate (Mg(NO$_3$)$_2$·6H$_2$O) at relative humidity of 78% and 23°C. Next, 316L stainless steel samples were transferred inside a glove box, along with MEG-TFA trichlorosilane linker stock solution, micropipettes, silanized glassware and Parafilm®. 10 µL MEG-TFA linker was pipetted and diluted with 10 mL anhydrous toluene. 2.0 mL 1/1000 (v/v) MEG-TFA/anhydrous toluene solution was portioned into each silanized scintillation vials containing one 10 mm x 10 mm x 2.0 mm dimension stainless steel sample, with the unscratched side facing upward. For 50.8 mm x 25.4 mm x 1.0 mm size steel samples, a minimum of 20.0 mL 1/1000 (v/v) MEG-TFA/anhydrous toluene solution was required to fully immerse three samples of such dimension within a 150 mm diameter, 65 mm deep silanized Pyrex® petri dish. The scintillation vials containing steel samples immersed in MEG-TFA/anhydrous toluene solution were screw capped and subsequently sealed with Parafilm®. The petri dishes were placed in a desiccator before removing from the glove box. The scintillation vials and the desiccator were then placed

![MEG-TFA](image-url)
on a spinning plate for two hours since the stainless steel samples are initially immersed in MEG-TFA/anhydrous toluene solution. When silanization was completed, each stainless steel sample was rinsed three times with ACS grade toluene, followed by five minutes of sonication in ACS grade Toluene. Then the same rinsing and sonication process repeats with ACS grade chloroform on each steel sample, followed by drying under nitrogen stream. One MEG-TFA modified 316L stainless steel sample was stored under nitrogen in a clean scintillation vial, as previously discussed, for XPS surface analysis. The remaining samples then proceeded to the next step.

2.5 Surface MEG-TFA Solvolysis

10 mm x 10 mm x 2 mm MEG-TFA surface modified stainless steel samples, placed in 20 mL clean scintillation vials, were individually immersed in 2.0 mL 1/1 (v/v) ethanol/distilled water on a spinning plate for overnight solvolysis under room temperature. Each 50.8 mm x 25.4 mm x 1.0 mm steel sample was placed in Falcon® 50 ml conical centrifuge tube, and volume of 1/1 (v/v) ethanol/distilled water used for solvolysis was increased to 40 mL. All stainless steel samples were subsequently dried under gentle
nitrogen stream. One MEG-OH 316L stainless steel sample was stored under nitrogen in a clean scintillation vial, as previously discussed, for XPS surface characterization, while others were stored for later blood perfusion experiments.

2.6 Parallel Plate Flow Chamber Preparation and Assembly

Commercially available flow chamber designs, such as GlycoTech®, were found incompatible with our experimental settings; therefore, not adapted due to various technical reasons. Most notably problems include relatively large volume of whole human blood required to maintain desired shear rate for extended period of time and inflexibility to accommodate nontransparent substrates slide or confocal microscope. Custom-made, single pass, parallel-plate blood perfusion chamber was consistently used for whole human blood surface thrombogenicity analysis. The transparent 50.8 mm x 25.4 mm x 1.0 mm dimension plain glass coverslip was adapted from commercial microscope slide. On each glass coverslip, two 1.0 mm diameter entrance and exit holes, both 10.2 mm and 12.7 mm away from each edge, and 30.4 mm apart from each other, were drilled perpendicularly to the glass surface. Two threaded female Luer adaptors made from PMMA were mounted and glued directly upon the inlet and outlet holes with Henkel Loctite Hysol® 0151 thixotropic epoxy paste adhesive. Two threaded 1/16 inch, straight, male polyethylene tubing connectors, each connected with 75.0 cm and 150.0 cm long 1/16 inch/1/8 inch (inside/outside) diameter moisture-resistant polyethylene vacuum tubing, were ultimately screwed into the glued female adaptors. All Luer connections and insertions were then sealed with Parafilm® extensively to prevent potential air or liquid leakage. The channel slide was made from clear, impact-resistant, both sides smooth, 0.0508 mm thick polycarbonate film, with up to 10% thickness tolerance. The polycarbonate film was cut into 50.8 mm x 25.4 mm size, and the flow channel was created by carving out a rectangular space with 4.0 mm width and 35.0 mm length.

The assembly of blood perfusion chamber proceeds as followed. Both the glass coverslip and channel slide were cleaned and sterilized with 95% ethanol. The channel slide was then gently applied onto the coverslip, making sure the entrance and exit holes were unobstructed and completely exposed within the rectangular flow channel.
Afterwards, a thin layer of distilled water was sprayed onto the channel slide to serve as lubricant and to prevent formation of air bubbles. The assembly of blood perfusion chamber was completed by sandwiching the channel slide with the unscratched side of 50.8 mm x 25.4 mm x 1.0 mm dimension bare or MEG-OH surface modified 316L stainless steel plate. In order to ensure the flow chamber was completely leak-tight under external pressure, each side of the chamber was extensively sealed with Parafilm® and further stabilized with small clamps.

2.7 Blood Flow Shear Rate and Regulation

Assuming whole human blood behaves like Newtonian fluid and follows a parabolic flow pattern, the parallel-plate wall shear rate due to blood flow can be calculated based on the following equation: \(^69\)

\[
\gamma = \frac{100Q}{wh^2},
\]

where \(\gamma\), \(Q\), \(w\) and \(h\) stand for wall shear rate (\(s^{-1}\)), volumetric blood flow rate (mL/min), channel width (mm), and channel height (mm), respectively. In this study, shear rates of 100 \(s^{-1}\), 300 \(s^{-1}\), 600 \(s^{-1}\), 750 \(s^{-1}\), and 900 \(s^{-1}\) were chosen for whole human blood thrombogenicity analysis on bare and MEG-OH surface modified 316L stainless steel plates. The corresponding volumetric flow rate and required blood volume at each chosen shear rate were calculated and displayed in the table 2, given that the channel width is 4.0 mm and channel height is 0.0508 mm:

<table>
<thead>
<tr>
<th>shear rate ((s^{-1}))</th>
<th>volumetric blood flow rate ((\mu L/min))</th>
<th>min. blood volume required (mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>20 min</td>
<td>60 min</td>
</tr>
<tr>
<td>100</td>
<td>10.3</td>
<td>0.206</td>
</tr>
<tr>
<td>300</td>
<td>31.0</td>
<td>0.619</td>
</tr>
<tr>
<td>600</td>
<td>61.3</td>
<td>1.24</td>
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<td></td>
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<td></td>
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<tr>
<td>-------</td>
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</tr>
<tr>
<td>750</td>
<td>77.4</td>
<td>1.55</td>
</tr>
<tr>
<td>900</td>
<td>92.9</td>
<td>1.86</td>
</tr>
</tbody>
</table>

Table 2. Calculated volumetric flow rate and required blood volume at 100 s\(^{-1}\), 300 s\(^{-1}\), 600 s\(^{-1}\), 750 s\(^{-1}\), and 900 s\(^{-1}\) shear rate

Harvard Apparatus® syringe pump was used throughout all whole human blood thrombogenicity analysis to enforce uniform blood flow rate at all times. The pulling mode was chosen over the pushing mode due to logistic concern and leakage precaution. The pulling mode eliminates the need to replace needles and to refill syringe with blood before each experiment, minimizing chances of manual contact with blood. The pulling mode also significantly decreases the possibility of blood leakage in case of unexpected channel or tubing clogging. In addition, when pulling mode is selected, blood can be pipetted into a reservoir, from which blood can then be drawn into the perfusion system as long as the setup is completely leak-tight.

2.8 Fluorescent Labelling of Platelets with DiOC\(_6\)

3,3'-dihexyloxacarbocyanine iodide (DiOC\(_6\), \(\lambda_{\text{exc}}\lambda_{\text{em}} = 484/501\) nm) fluorescent dye stock solution was prepared by dissolving 98% DiOC\(_6\) in ACS grade DMSO at 1 mM concentration. The working solution was diluted to 10 µM concentration and stored in an aluminum foil wrapped vial and kept in a refrigerator.

![Figure 18. Structure of DiOC\(_6\)](image)

All whole human blood samples are drawn from volunteers on the morning of the experiment day at Department of Hematology at St. Michael’s hospital, Toronto, ON,
Canada. Blood donors were told to maintain a regular diet and warned not to take any anticoagulant medicine one week prior to scheduled experiment date. Depending on specific experimental need, up to 18 mL of blood was drawn from a single blood donor’s median cubital vein each time. Collected whole human blood was stored in 6 mL lithium heparin BD plastic vacutainers. Whole human blood sample intended for 20 min surface thrombogenicity analysis was subsequently fluorescent-labeled with DiOC₆ working solution at 1/100 (v/v) ratio. For 60 min surface thrombogenicity analysis, DiOC₆ working solution was added to the whole human blood at 1/30 (v/v) ratio. The vacutainers containing fluorescent-labeled blood were then wrapped with aluminum foil and put on a rotator for at least 20 minutes before use.

2.7 Blood Perfusion Experimental Setup

![Experimental setup used to record real-time platelet adhesion, aggregation and thrombus formation. Also shown a close-up view of the perfusion chamber under confocal microscope.](image)

Pre-assembled flow chambers (five bare and five MEG-OH surface modified 316L stainless steel for each intended shear rate) were brought to Toronto Western Research Institute, Toronto, Ontario, Canada for surface thrombogenicity analysis against whole human blood under a BX61W1 (Olympus) confocal microscope, equipped with an EMC2 Q-imaging CCD camera (Rolera, QImaging). (Figure 18) The required exposure time was set to 300 ms without the camera gain. The excitation laser source used was an arc mercury lamp (X-cite 120 PC fluorescence illumination system, EXFO). A green
fluorescent filter cube set (FITC 3540B Semrock), which combines a 35 nm wide excitation bandpass filter centered at 482 nm and a 40 nm emission bandpass filter centered at 536 nm, was used to filter fluorescent signals. The dimension of the camera chip used was 1004 x 1002 with pixel size of 8 x 8 µm, and the objective lens used was a 40x Plan N (Olympus) with a numerical aperture of 0.65. This combined imaging setup would eventually give us a final pixel size of 0.2 µm. The microscope and camera setup allowed us to record live videos, from which we were able to extract specific frames. Extracted frames were then processed with imageJ® software to calculate surface coverage due to platelet adhesion, aggregation and thrombus formation on prepared substrates.

The outlet tubing of the blood perfusion chamber was connected to a 50 mL syringe mounted on a Harvard Apparatus syringe pump (not shown in the Figure), preset with pulling mode at a desired flow rate and placed far away from the testing platform to avoid disrupting image recording process. The inlet tubing was connected to an open 5.0 mL sterilized medical syringe, which will serve as the blood reservoir. Before starting blood perfusion, the parallel-plate flow chamber was stabilized on the testing platform and fitted under the microscope, followed by a 2.0 mL phosphate-buffered saline injection to purge and test leak-tightness. One to two drops of distilled water was added onto the coverslip for objective lens immersion purpose. Next, the syringe pump was turned on, and fluorescent labeled whole human blood was pipetted into the blood reservoir. The Image recording process did not start until blood reached the perfusion chamber.

2.8 Contact Angle Goniometry

Surface wettability of all 316L stainless steel samples after each major step of surface modification was measured using CAM 101 Attension optical tensiometer (contact angle meter), constructed by KSV Instruments Ltd. Distilled water was consistently used as the testing liquid.
2.9 XPS Surface Characterization

Selected bare, oxidized, MEG-TFA modified, and MEG-OH modified 316L stainless steel samples were submitted to Dr. Peter Brodersen at Surface Interface Ontario (Toronto, Ontario, Canada) for surface characterization by XPS. Angle-resolved XPS was analyzed using the Thermo Scientific Al Kα probe (Thermo Fisher Scientific, East Grinstead, UK). Take off angles of 20° and 90° relative to surface was chosen to be analyzed for each sample. Peak fitting and data analysis of all XPS spectra, including survey scans and elemental narrow scans of C1s, O1s, Si2p, F1s, Cr2p, Fe2p, Mn2p, Mo3d, Ni2p was processed using the accompanying Avantage® and commercial CasaXPS® software. Binding energy calibration was performed on all spectra by shifting C1s peak to 284.8 eV prior to peak fitting and data analysis.

3 Results and Discussion

3.1 MEG-OH Adlayer Formation and Characterization

3.1.1 X-ray Photoelectron Spectroscopy

Angle-resolved XPS with take-off angle of 20 and 90 degrees relative to the surface was performed on mirror and electrochemically polished 10 mm x 10 mm x 2 mm 316L stainless steel samples after every major surface modification step. For this purpose, the XPS characterization technique was used to both qualitatively and quantitatively monitor consequent surface compositional change at various stage.

The objective of the first part of our investigation was to confirm the effectiveness of stainless steel surface cleaning and activation protocol as well as to prove the successful generation of MEG-TFA and subsequent formation of MEG-OH adlayer on 316L stainless steel surface. Due to the mechanism of tricholorosilane linker adlayer formation, the stainless steel surface intended for subsequent silanization process was required to be oxidized and functionalized by hydroxyl groups through treatment with piranha solution (3:1(v/v) 98% sulfuric acid to 30% hydrogen peroxide). Therefore, one bare, directly-from-manufacturer 316L stainless steel sample along with one surface
cleaned and activated 316L stainless steel sample were included in our XPS analysis to assess difference in respective surface elemental and structural composition. In addition, one MEG-TFA and one MEG-OH surface modified samples were also analyzed by angle-resolved XPS, the result of which would validate the adlayer formation and existence.

Each survey scan and elemental narrow scan spectra was processed using Shirley background. Subsequent elemental identification and modes characterized through deconvolution of carbon and oxygen narrow scan spectra were assigned based on the reported binding energy value from literature:

<table>
<thead>
<tr>
<th>Element (orbital)</th>
<th>Chemical state</th>
<th>Binding Energy (eV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C (1s)</td>
<td>C-H/C-C</td>
<td>284.8</td>
</tr>
<tr>
<td></td>
<td>C-O</td>
<td>~286</td>
</tr>
<tr>
<td></td>
<td>C=O</td>
<td>~289</td>
</tr>
<tr>
<td></td>
<td>CF₃</td>
<td>~294</td>
</tr>
<tr>
<td>O (1s)</td>
<td>Metal oxides (M-Oₓ)</td>
<td>529-530</td>
</tr>
<tr>
<td></td>
<td>Metal hydroxide (M(OH)ₓ)</td>
<td>530.9-531.7</td>
</tr>
<tr>
<td></td>
<td>Organic C-O</td>
<td>531.5-532</td>
</tr>
<tr>
<td></td>
<td>Organic C=O</td>
<td>~533</td>
</tr>
<tr>
<td>Si (2p)</td>
<td>Organic Si</td>
<td>~102</td>
</tr>
<tr>
<td>F (1s)</td>
<td>Organic F</td>
<td>688-689</td>
</tr>
</tbody>
</table>

Table 3. Relevant literature C1s, O1s, Si2p, F1s and Cr2p XPS data
Figure 20. Relevant surface structures for XPS analysis: untreated (bare); oxidized (activated); MEG-TFA modified (MEG-TFA); MEG-OH modified (MEG-OH)
C 1s, Take-off Angle = 20° (surface sensitive)

![Graph showing XPS narrow scan profiles at 20° take-off angles for Bare, Activated, MEG-TFA, and MEG-OH samples.]

C 1s, Take-off Angle = 90° (bulk sensitive)

![Graph showing XPS narrow scan profiles at 90° take-off angles for Bare, Activated, MEG-TFA, and MEG-OH samples.]

Figure 21. C 1s XPS narrow scan profiles at 20° and 90° take-off angles
Figure 22. O 1s XPS narrow scan profiles at 20° and 90° take-off angles
Si 2p, Take-off Angle = 20° (surface sensitive)

Si 2p, Take-off Angle = 90° (bulk sensitive)

Figure 23. Si 2p XPS narrow scan profiles at 20° and 90° take-off angles
**F 1s, Take-off Angle = 20° (surface sensitive)**

[Graph showing F 1s XPS narrow scan profiles at 20° take-off angle]

**F 1s, Take-off Angle = 90° (bulk sensitive)**

[Graph showing F 1s XPS narrow scan profiles at 90° take-off angle]

Figure 24. F 1s XPS narrow scan profiles at 20° and 90° take-off angles
Figure 25. Cr 2p XPS narrow scan profiles at 20° and 90° take-off angles
<table>
<thead>
<tr>
<th>Sample</th>
<th>TOA</th>
<th>C(1s)</th>
<th>O(1s)</th>
<th>Si(1s)</th>
<th>F(1s)</th>
<th>Cr(2p)</th>
<th>Fe(2p)</th>
<th>Ni(2p)</th>
<th>Mo(2p)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bare</td>
<td>20°</td>
<td>64.14</td>
<td>28.66</td>
<td>6.38</td>
<td>0</td>
<td>0.82</td>
<td>0</td>
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<tr>
<td></td>
<td>90°</td>
<td>37.81</td>
<td>42.56</td>
<td>12.53</td>
<td>0</td>
<td>3.46</td>
<td>3.54</td>
<td>0</td>
<td>0</td>
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<tr>
<td>Activated</td>
<td>20°</td>
<td>11.59</td>
<td>58.55</td>
<td>0</td>
<td>0</td>
<td>15.08</td>
<td>14.78</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>90°</td>
<td>9.66</td>
<td>59.27</td>
<td>0</td>
<td>0</td>
<td>10.14</td>
<td>8.15</td>
<td>6.43</td>
<td>6.35</td>
</tr>
<tr>
<td>MEG-TFA</td>
<td>20°</td>
<td>33.22</td>
<td>44.51</td>
<td>17.19</td>
<td>4.31</td>
<td>0.77</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>90°</td>
<td>27.32</td>
<td>45.25</td>
<td>13.26</td>
<td>12.6</td>
<td>1.58</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>MEG-OH</td>
<td>20°</td>
<td>35.70</td>
<td>47.95</td>
<td>15.17</td>
<td>0</td>
<td>1.17</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>90°</td>
<td>29.60</td>
<td>53.13</td>
<td>14.21</td>
<td>0</td>
<td>1.87</td>
<td>0.94</td>
<td>0</td>
<td>0.25</td>
</tr>
</tbody>
</table>

Table 4. XPS relative atomic percentage for untreated (bare); oxidized (activated); MEG-TFA modified (MEG-TFA); MEG-OH modified (MEG-OH)

The untreated bare 316L stainless steel sample surface was analyzed by XPS at 20 degrees (surface sensitive) and 90 degrees (bulk sensitive) relative to the surface. As expected, the bare 316L stainless steel sample surface has a thin layer of superficial contaminant deposition, despite the extensive mirror polishing and electrochemical polishing efforts. The presence of the contaminant deposition layer is highlighted by the staggeringlly high elemental C (1s) and Si (1s) relative atomic percentages at both 20 degrees and 90 degrees take-off angle, (Table 4) as both elements are considered impurities and cannot exist more than trace amount in the 316L stainless steel composition breakdown. (Table 1) The superficial contaminant layer on the bare 316L stainless steel sample is further evidenced by its deconvoluted C (1s) and O (1s) narrow scan spectra, showing significant C-C/C-H, C-O and C=O components. Through the deconvoluted O (1s) narrow scan spectrum of the bare 316L stainless steel sample, it can also be seen that the presence of abundant metal oxides and metal hydroxide species, primarily air-exposed Cr2O3 and Cr(OH)3, at respective 530.1 eV and 531.7 eV, on the untreated 316L stainless steel surface. This observation agrees with prior knowledge that the formation of chromium oxide layer on stainless steel surface blocks oxygen diffusion to the surface and contributes to the anti-corrosion property of stainless steel.
The activated 316L stainless steel sample was obtained after successive cleaning steps through sonication in various polar and nonpolar solvents, followed by surface oxidation carried out in piranha solution. Despite the fact that the XPS data from untreated sample revealed significant superficial metal oxide and metal hydroxide composition, we still decided to proceed the oxidation step to ensure extensive and uniform oxidation of the steel surface, which is required for the later MEG-TFA adlayer formation. Compared with the untreated bare sample, the activated 316L stainless steel surface exhibits distinctive surface composition. First of all, the relative atomic percentages of elemental C (1s) on the activated sample at both 20 degree and 90 degree take-off angles drastically decrease. A careful examination of the deconvoluted C (1s) narrow scan indicates significant reduction of the characterized C-C/C-H, C-O and C=O components’ peak height as well as integrated peak area. Due to presence of adventitious carbon, the elemental C (1s) relative atomic percentage does not decrease to zero, and deconvoluted peaks for each characterized carbon component, C-C/C-H and C-O in particular, are still observable; however, noticeably reduced in overall peak height and area. The effectiveness of the surface cleaning protocol is demonstrated by the zero Si (2p) relative atomic percentage and the complete disappearance of the relatively weak but broad peak due to Si (2p), spanning from 102-104 eV, previously seen on the untreated bare 316L stainless steel sample. In case of the deconvoluted O (1s) narrow scan, we also see metal oxides (M-Oₓ) and metal hydroxide (M(OH)ₓ) components increase substantially in terms of peak height and area at both 20 degrees and 90 degrees take-off angle, suggesting the activated sample is much more oxidized and has a higher surface hydroxyl group density than the untreated bare surface. This information confirms success of our surface oxidation protocol on 316L stainless steel. Furthermore, considerable peak height and area decrease of deconvoluted O (1s) components due to C-O and C=O also proves substantial reduction of surface contamination. As the superficial contaminant deposition layer is shown removed by our surface cleaning protocol, Cr (2p) relative atomic percentage and integrated peak area is observed to have a substantial increase. Other major 316L stainless steel elemental components besides Cr, including Fe (2p), Ni (2p), Mo (3d), which are not previously found on the untreated bare sample, are also become detectable by XPS at both take-off angles.
The MEG-TFA surface modified 316L stainless steel samples were prepared by immobilizing MEG-TFA trichlorosilane linker on the activated sample under inert and anhydrous environment, followed by rigorous rinse and sonication in toluene and chloroform. Success of this surface chemistry is most evidently demonstrated through the emergence of an intense and broad peak due to organic fluorine, located at ~689 eV on both 20 degrees and 90 degrees take-off angle F (1s) narrow scans of the MEG-TFA surface modified sample. As the bare and activated samples do not exhibit any fluorine content on either surface, the appearance of such an organic fluorine peak on F (1s) narrow scan would be a convincing evidence that at least the trifluoroacetyl group is present on the surface. An intense and broad peak due to organic silicon (2p), which is located at ~103 eV and not observed on activated sample surface, also appears on the Si (2p) narrow scan spectra at both take-off angles. In the case of the deconvoluted O (1s) narrow scan, the component peak due to metal hydroxide (M(OH)\textsubscript{x}) species completely disappear, and the metal oxide (M-O\textsubscript{x}) species component also decrease significantly in terms of peak area. Compared to the previous activated sample XPS result, the combination of observed intense Si (2p) peak as well as the disappearance and drastic peak area decrease of respective metal hydroxide and metal oxide species component on the modified surface strongly indicates the formation of the polysiloxane network, characteristic of the MEG-TFA adlayer formation. In addition, the formation of MEG-TFA adlayer on the substrate surface is further supported by the deconvoluted C (1s) narrow scan spectra at both take-off angles, from which we observe the appearance of the CF\textsubscript{3} component peak at ~294 eV and the C=O component peak at ~289 eV. Both the CF\textsubscript{3} and the C=O component peaks are characteristic of the trifluoroacetyl group. Furthermore, the peak height and area of the C-O and C-C/C-H component peaks, located at ~286 eV and 284.8 eV respectively, drastically increase at both 20 degrees and 90 degrees take-off angle compared with the activated sample surface, suggesting the incorporation of the monoethylene glycol backbone. The parallel effect for C-O and C=O is also observed from the deconvoluted O (1s) narrow scan spectra at both take-off angles. The presence of the polysiloxane network, monoethylene glycol backbone and trifluoroacetyl group confirms success of our surface silanization protocol, demonstrating successful MEG-TFA linker immobilization on the activated surface.
The MEG-OH surface modified 316L stainless steel samples was obtained by overnight solvolysis of the previously prepared MEG-TFA surface modified samples in 50:50 (v/v) 95% ethanol/distilled water. The purpose of the hydrolysis reaction is to hydrolyze the trifluoroacetyl group while leaving the monoethylene glycol backbone and the polysiloxane network intact. Compared with the MEG-TFA surface modified sample, the hydrolyzed sample does not exhibit any noticeable variation regarding the Si (2p) peak height, but the associated peak area is slightly decreased at both take-off angles, suggesting possible minor loss of the immobilized organosilane linker due to hydrolysis. The most significant difference between the two samples is the complete disappearance of the F (1s) peak observed from the hydrolyzed surface at both take-off angles. In addition, the losses of C (1s) component peaks due to CF₃ and C=O are also observed from the deconvoluted C (1s) narrow scan spectra of the hydrolyzed surface. The disappearances of peaks associated with organic fluorine, CF₃ and C=O on F (1s), C (1s) and O (1s) narrow scan spectra at both take-off angles indicate successful removal of the trifluoroacetyl group. A careful inspection of the deconvoluted C (1s) and O (1s) narrow scan spectra also reveals component peak height and area associated with C-O and C-C/C-H increase marginally, suggesting that the MEG backbone stayed intact and undamaged when TFA solvolysis was taking place. The loss of the labile TFA group and intact MEG silane linker backbone confirms our TFA solvolysis protocol and successful conversion of MEG-TFA to MEG-OH on 316L stainless steel surface.

3.1.2 Contact Angle Goniometry

Besides angle-resolved XPS, the outcomes of each major experimental stage, including surface cleaning/oxidation, MEG-TFA immobilization and subsequent MEG-OH adlayer formation were also monitored by static contact angles using distilled water as the testing liquid. All contact angles were measured within 30 seconds after the substrate surface came to contact with the water droplet to avoid significant surface modification. Multiple contact angles were also recorded for each test substrate to ensure data reproducibility.
As illustrated in the figure 25, the untreated bare 316L stainless steel presents a rather hydrophobic surface with contact angles (CA) of $86.6 \pm 2.8^\circ$ ($n = 5$). After rigorous surface cleaning procedure and treatment with piranha solution, the activated substrate surface wettability significantly increases, as CA drops to $48.9 \pm 2.7^\circ$ ($n = 5$), suggesting the surface has become hydrophilic and is fully saturated with hydroxyl group. The contact angle of MEG-TFA modified surface resumed to roughly same level of the bare sample: $CA = 84.3 \pm 4.6^\circ$ due to presence of the hydrophobic TFA groups. After removal of the TFA group through solvlysis, one hydroxy end of the MEG backbone is exposed, resulting in substantial CA decrease to $19.7 \pm 2.5^\circ$. Overall, the supplementary contact angle data matches the conclusion from the previous XPS surface characterization study.

3.2 Optimization of MEG-TFA Silane Surface Immobilization

Demonstrated by the previous XPS study, we have shown successful immobilization and adlayer formation of MEG-TFA silane linker on the activated 316L stainless steel surface. However, we arbitrarily chose the time allowed for the MEG-TFA silane linker surface immobilization process in the previous study to be 60 minutes and we performed the experiments accordingly. We realized that the time period chosen for
MEG-TFA surface immobilization may not result in an optimal linker surface packing order and coverage density. Therefore, the objective in this study was to determine the optimal silanization time required to yield the highest MEG-TFA and subsequent MEG-OH adlayer surface density.

In this silanization time trial study, a total of five MEG-TFA surface modified 316L stainless steel samples were prepared. All experimental parameters were kept consistent for all MEG-TFA modified samples as in the previous study, except the time interval allowed between the initial immersion of the prepared samples in 1/1000 (v/v) MEG-TFA/anhydrous toluene solution and exposure to open atmosphere, followed by rigorous toluene rinse. Each activated 316L stainless steel sample was subjected under 30 min, 60 min, 90 min, 120 min and 150 min of silanization time respectively. All prepared samples were submitted for angle-resolved XPS at take-off angles of 20 and 90 degrees relative to the surface. XPS spectra were calibrated to the C1s signal at 284.8 eV and processed using Shirley background.

![Figure 27. XPS F/Cr and Si/Cr peak area ratios of MEG-TFA modified 316L stainless steel, analyzed at 20° take-off angle for 30 min, 60 min, 90 min, 120 min and 150 min silanization time](image)

Integrated peak area of organic F (1s) and Si (2p), which are characteristic of MEG-TFA adlayer formation, was calculated and corrected with reference to that of Cr (2p), the most significant metallic component on 316L stainless steel surface. The peak area ratios
for each silanization time at take-off angle of 20 degrees were plotted in the figure 26, and the corresponding data of 90 degrees take-off angle, with addition of CF$_3$/Cr ratio, were shown in the Figure 27.

At 20 degrees take-off angle, F/Cr and Si/Cr peak area ratio trend noticeably resemble each other. Similar trend resemblance is also seen between CF$_3$/Cr, F/Cr and Si/F peak area ratios at 90 degrees take-off angle. However, the trend observed at the two take-off angles obviously do not completely coincide, particularly at 60 min and 90 min silanization time, possibly due to the reversibility of the polysiloxane formation as well as variation in the MEG-TFA molecular surface orientation. Nonetheless, the absolute peak area ratio maxima are consistently observed for F/Cr, CF$_3$/Cr and Si/Cr at both take-off angles at 120 min silanization time, suggesting the MEG-TFA and subsequent MEG-OH adlayer surface density should be optimized at 120 min silanization time for later whole human blood surface thrombogenicity study.

Figure 28. XPS CF$_3$/Cr, F/Cr and Si/Cr peak area ratios of MEG-TFA modified 316L stainless steel, analyzed at 90° take-off angle for 30 min, 60 min, 90 min, 120 min and 150 min silanization time
3.3 Whole Human Blood Surface Thrombogenicity Study

3.3.1 End-stage Measurement of Surface Platelet Adhesion

The next stage of our investigation involved assessing the speculated antithrombogenic property of the MEG-OH adlayer on 316L stainless steel. For all MEG-OH modified samples used for surface thrombogenicity analysis, the optimal silanization time of 120 min was consistently applied.

The objective of this study was to analyze and compare in vitro surface platelet adhesion, aggregation and thrombus formation on bare and MEG-OH surface modified 316L stainless steel after 20 minutes of whole human blood perfusion at 100 s\(^{-1}\), 300 s\(^{-1}\), 600 s\(^{-1}\), 750 s\(^{-1}\) and 900 s\(^{-1}\) wall shear rate, which were specifically chosen to simulate physiological hemodynamics of a healthy individual's venous and arterial blood flow. While syringes and needles were disposable after each experiment, the blood contaminated perfusion chambers were not reused until extensive rinse with 5% H\(_2\)O\(_2\), 5% NaClO and filtered water was completed. To ensure data reproducibility, five replicates of cleaned and MEG-OH surface modified 316L stainless steel plate were each analyzed as the control and testing group for every selected shear rate. The control groups were cleaned following the cleaning procedure used in the previous XPS surface characterization study.

![Figure 29. Relevant surface structures for whole human blood surface thrombogenicity analysis: cleaned (bare); MEG-OH modified (MEG-OH)](image)
Representative images after 20 min blood perfusion at 100 s\(^{-1}\) shear rate

Figure 30. Representative images after 20 min blood perfusion at 100 s\(^{-1}\) shear rate, showing platelet adhesion, aggregation, and thrombus formation, or lack of, on bare (left) and MEG-OH modified 316L stainless steel (right).

Representative images after 20 min blood perfusion at 300 s\(^{-1}\) shear rate

Figure 31. Representative images after 20 min blood perfusion at 300 s\(^{-1}\) shear rate, showing platelet adhesion, aggregation, and thrombus formation, or lack of, on bare (left) and MEG-OH modified 316L stainless steel (right).
Representative images after 20 min blood perfusion at 600 s\(^{-1}\) shear rate

Figure 32. Representative images after 20 min blood perfusion at 600 s\(^{-1}\) shear rate, showing platelet adhesion, aggregation, and thrombus formation, or lack of, on bare (left) and MEG-OH modified 316L stainless steel (right).

Representative images after 20 min blood perfusion at 750 s\(^{-1}\) shear rate

Figure 33. Representative images after 20 min blood perfusion at 750 s\(^{-1}\) shear rate, showing platelet adhesion, aggregation, and thrombus formation, or lack of, on bare (left) and MEG-OH modified 316L stainless steel (right).
Representative images after 20 min blood perfusion at 900 s\(^{-1}\) shear rate

Figure 34. Representative images after 20 min blood perfusion at 900 s\(^{-1}\) shear rate, showing platelet adhesion, aggregation, and thrombus formation, or lack of, on bare (left) and MEG-OH modified 316L stainless steel (right).

The speculated antithrombogenic potential of the MEG-OH adlayer on 316L stainless steel was assessed in real-time against whole human blood, fluorescent-labeled with 3,3’-dihexyloxacarbocyanine iodide (DiOC\(_6\), \(\lambda_{\text{exc}}\backslash\lambda_{\text{em}} = 484/501\) nm) under a confocal microscope, equipped with a CCD camera and a laser excitation source. DiOC\(_6\) is a commonly used cell-permeant, lipophilic, green fluorescent dye primarily used for staining membrane bound cellular organelles, such as mitochondria and endoplasmic reticulum.\(^73\) Despite DiOC\(_6\) was not specifically designed to fluorescent-label platelets, this fluorescent dye was chosen for several distinct advantages. First, DiOC\(_6\) does not cause platelets activation, even with extended period of excitation time. Second, in presence of red blood cells, only platelets and leukocytes appear fluorescent-labeled because hemoglobins within RBCs can effectively quench fluorescence.\(^73\) Due to the substantial size difference between leukocytes and platelets, respectively with \(~12-17\) \(\mu\)m and \(~1-2\) \(\mu\)m diameter on average, we can easily distinguish platelets from leukocytes under a high resolution confocal microscope. Third, platelet loading with DiOC\(_6\) is very straightforward, only requiring a minimum of 10 minutes of incubation at room temperature. For the purpose of our analysis, representative frames of the bare and MEG-OH modified surfaces were
extracted after 20 minutes of blood perfusion at selected shear rates and displayed in the Figures 29-33.

In a healthy human, blood flow is remarkably free of any turbulence. As a part of our objective to simulate physiologically relevant hemodynamic environment, the blood perfusion process must be maintained under constant laminar flow within our custom-made flow chamber. Reynolds number (Re) can be calculated to model the blood flow for each intended shear rate within our experimental setup. The dimensionless Reynolds number relationship can be written as:

\[ Re = \frac{\rho Q D_h}{\mu w h} \]

in which \( \rho \), \( Q \), \( D_h \), \( \mu \), \( w \), \( h \) respectively stand for blood density, blood flow rate, hydraulic diameter, blood viscosity, width and height of the perfusion channel. The hydraulic diameter is defined as \( D_h = \frac{4wh}{2(w+h)} = 0.100 \) mm, given that the channel width is 4.0 mm and channel height is 0.0508 mm. The corresponding Reynolds number for each chosen shear rate is calculated and displayed in the Table 4, provided \( \rho = 1.06 \) g/cm\(^3\); \( \mu = 3.5 \) gcm\(^{-1}\)s\(^{-1}\) for whole human blood.

<table>
<thead>
<tr>
<th>shear rate (s(^{-1}))</th>
<th>Q ((\mu)L/min)</th>
<th>Re</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>10.3</td>
<td>0.026</td>
</tr>
<tr>
<td>300</td>
<td>31.0</td>
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</tr>
<tr>
<td>600</td>
<td>61.3</td>
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<tr>
<td>750</td>
<td>77.4</td>
<td>0.192</td>
</tr>
<tr>
<td>900</td>
<td>92.9</td>
<td>0.230</td>
</tr>
</tbody>
</table>

Table 4. Calculated Reynolds number values for each intended shear rate within the experimental setup.

Based on the definition of Reynolds number, laminar flow occurs when \( Re < 2100 \). All of the calculated Reynolds number values corresponding to each shear rate selected for surface thrombogenicity analysis are much lower than 2100. Therefore, blood
flow generated in our custom-made flow chamber meets the criterion for laminar flow under all experimental conditions.

After every real-time recording under the confocal microscope, multiple frames were extracted around 20 min blood perfusion time for each analyzed surface. Extracted frames are first converted to gray-scale then binarized. The binarized images are subsequently imported to an image processing software, such as ImageJ®, in which image thresholding can be performed. The number of black (representing the background) and white (representing the fluorescent platelet) pixels in each selected frame can be calculated. The percentage of white pixels to the total pixel number in a thresholded frame, which can be calculated by the “area fraction” function in the ImageJ® software, represents the platelet surface coverage area.

Figure 35. Percentage of surface coverage due to platelet adhesion, aggregation and thrombus formation of bare and MEG-OH modified 316L stainless steel, after 20 min blood perfusion at 100 s⁻¹, 300 s⁻¹, 600 s⁻¹, 750 s⁻¹ and 900 s⁻¹ shear rate; the error bars represent standard deviation, n = 5
As shown in the Figure 34, the cleaned bare 316L stainless steel surfaces, which were analyzed as the control group in this study, triggered significant amount of platelet adhesion, aggregation and thrombus formation after 20 minutes of exposure to fluorescent-labeled whole human blood. The surface platelet percentage coverage on the control groups were calculated at 99% confidence interval: 7.72±0.38% (n=5), 5.79±1.39% (n=5), 4.98±0.60% (n=5), 1.08±0.08% (n=5) and 0.94±0.11% (n=5), for respective 100 s\(^{-1}\), 300 s\(^{-1}\), 600 s\(^{-1}\), 750 s\(^{-1}\), and 900 s\(^{-1}\) shear rate. In the contrast, the MEG-OH modified 316L stainless steel surfaces, demonstrated remarkably superior resistance to thrombus formation after 20 minutes of whole human blood exposure. (Figure 35) As illustrated in the Figure 29-33, platelet adhesion on MEG-OH modified surfaces, highlighted by the red arrows, were barely observable at most. The calculated platelet percentage coverage on the MEG-OH modified 316L stainless steel surfaces were reported at 99% confidence interval: 0.67±0.06% (n=5) at 100 s\(^{-1}\) shear rate; 0.29±0.10% (n=5) at 300 s\(^{-1}\) shear rate; 0.37±0.04% (n=5) at 600 s\(^{-1}\) shear rate; 0.10±0.02% (n=5) at 750 s\(^{-1}\) shear rate and 0.08±0.01% at 900 s\(^{-1}\) shear rate. Student’s t tests, assuming unequal sample variances, were performed to evaluate whether the calculated mean platelet surface percentage coverage on the bare and MEG-OH modified 316L stainless
steel surfaces is equal. The evaluated t values for each blood shear rate is shown in the Table 5:

<table>
<thead>
<tr>
<th>Shear rate (s⁻¹)</th>
<th>100</th>
<th>300</th>
<th>600</th>
<th>750</th>
<th>900</th>
</tr>
</thead>
<tbody>
<tr>
<td>Student’s t value, at 99% confidence</td>
<td>52.70</td>
<td>23.01</td>
<td>20.92</td>
<td>33.49</td>
<td>26.12</td>
</tr>
</tbody>
</table>

Table 5. Calculated student’s t values, in comparison of mean platelet surface percentage coverage on bare and MEG-OH modified 316L stainless steel surfaces

The calculated student’s t values at each tested shear rate is much greater than the tabulated student’s t critical values at degrees of freedom = 4, which is reported as 4.604. Therefore, there is a highly statistically significant difference of platelet surface coverage on bare and MEG-OH modified 316L stainless steel.

3.3.2 Real-time Measurement of Surface Platelet Adhesion

This study was essentially an extension of the previous end-stage surface thrombogenicity experiment. The objective of this study was to assess the real-time anti-thrombogenic profile of MEG-OH modified 316L stainless steel surfaces compared to the bare ones for extended period of time. Without major changes made to the experimental protocol, a few parameters were modified. In this study, the time allowed for fluorescent-labeled whole human blood perfusion was increased to 60 min, three times longer than the previous study. Therefore, a significantly larger volume of whole human blood was required for each perfusion. Much higher blood concentration of DiOC₆ fluorescent dye also became mandatory in order to avoid complete photobleaching of labeled platelets already adhered to the substrate surface, as photobleached platelets would become unobservable under confocal microscope. In addition, the intensity of the excitation laser were tuned down, to ~50% of the intensity set for the previous experiment. Prior to each experiment, all perfusion chambers involved were thoroughly checked for air and liquid leakage to ensure blood can maintain laminar flow under constant shear rate for at least 60 min. One of each bare and MEG-OH modified 316L stainless steel surface was tested under 100 s⁻¹, 600 s⁻¹ and 900 s⁻¹ shear rate.
Bare 316L SS surface, 60 min blood perfusion at 100 s⁻¹ shear rate

Figure 37. Representative frames captured every 3 min on bare 316L stainless steel surface during 60 min blood perfusion at 100 s⁻¹ shear rate; the scale bars represent 20 μm
MEG-OH modified 316L SS surface, 60 min blood perfusion at 100 s\(^{-1}\) shear rate

![Figure 38. Representative frames captured every 3 min on MEG-OH modified 316L stainless steel surface during 60 min blood perfusion at 100 s\(^{-1}\) shear rate; the scale bars represent 20 \(\mu\)m]
Bare 316L SS surface, 60 min blood perfusion at 600 s\(^{-1}\) shear rate

Figure 39. Representative frames captured every 3 min on bare 316L stainless steel surface during 60 min blood perfusion at 600 s\(^{-1}\) shear rate; the scale bars represent 20 µm
MEG-OH modified 316L SS surface, 60 min blood perfusion at 600 s\(^{-1}\) shear rate

Figure 40. Representative frames captured every 3 min on MEG-OH modified 316L stainless steel surface during 60 min blood perfusion at 600 s\(^{-1}\) shear rate; the scale bars represent 20 µm
Bare 316L SS surface, 60 min blood perfusion at 900 s$^{-1}$ shear rate

Figure 41. Representative frames captured every 3 min on bare 316L stainless steel surface during 60 min blood perfusion at 900 s$^{-1}$ shear rate; the scale bars represent 20 µm
MEG-OH modified 316L SS surface, 60 min blood perfusion at 900 s\(^{-1}\) shear rate

Figure 42. Representative frames captured every 3 min on MEG-OH modified 316L stainless steel surface during 60 min blood perfusion at 900 s\(^{-1}\) shear rate; the scale bars represent 20 µm
In this real-time surface thrombogenicity study, image on each prepared surface was recorded at 120 ms time interval between each frames, while the exposure time allowed for individual frame recording by the camera is set to 100 ms. After 60 min of blood perfusion at a specific shear rate over one surface was completed, individual frames captured at every 3 min, were extracted from the image stack and processed using same procedure as in the previous experiment. The compiled twenty frames for every surface recorded at each shear rate, starting from 3 min to 60 min perfusion time and with 3 min interval between each frame, are presented in the form of montages shown in the Figure 36-41.

Figure 43. Real-time platelet surface percentage coverage on bare and MEG-OH modified 316L stainless steel during 60 min blood perfusion at 100 s⁻¹ shear rate.

Figure 44. Real-time platelet surface percentage coverage on bare and MEG-OH modified 316L stainless steel during 60 min blood perfusion at 600 s⁻¹ shear rate.
Both the bare and MEG-OH modified 316L stainless steel surface appeared to trigger platelet activation immediately after the exposure to the fluorescent-labeled whole human blood, as both samples started to have surface platelet adhesion within 3 minutes of blood perfusion under 100 s\(^{-1}\), 600 s\(^{-1}\) and 900 s\(^{-1}\) shear rate. However, the entire process of surface platelet adhesion, aggregation and thrombus formation is observed to occur at a much lower pace on the MEG-OH modified 316L stainless steel surface than the bare one. Illustrated in the Figure 42, the percentage coverage due to platelet adhesion and eventual thrombus formation on bare 316L stainless steel follows a near exponential increase pattern under 100 s\(^{-1}\) shear rate, reaching an alarming ~53% surface coverage after 60 minutes of blood perfusion. The blood stream flow path becomes visibly obstructed by massive surface thrombus formation under 100 s\(^{-1}\) shear rate after 60 minutes, shown in the Figure 36. As shear rate increase to 600 s\(^{-1}\) and 900 s\(^{-1}\), platelet surface coverage on the bare 316L stainless steel correspondingly decrease at each recorded time period; however, the previously observed high platelet adhesion and aggregation rate does not appear to slow down. Evidenced by the Figure 43 and 44, the platelet surface coverage on bare 316L stainless steel nearly triple from 20 minutes to 60 minutes, eventually reaching 20.2% and 10.7%, respectively. In the stark contrast, MEG-OH modified 316L stainless steel surfaces show remarkably high degree of resistance to surface plate adhesion, reducing the surface platelet coverage by over 90% under all tested shear rate after 60 minutes blood perfusion, compared with the bare 316L stainless
steel. In addition, the surface platelet micro-aggregate formation on MEG-OH surface modified 316L stainless steel is observed to maintain a minimal and steady rate, particularly under 600 s\(^{-1}\) and 900 s\(^{-1}\) shear rate. In this real-time study, The MEG-OH modified 316L stainless steel surfaces demonstrate a significantly higher anti-thrombogenic profile at 100 s\(^{-1}\), 600 s\(^{-1}\) and 900 s\(^{-1}\) shear rate relative to bare 316L stainless steel during the entire 60 min blood perfusion process.

4 Conclusion

In summary, we have been able to translate and apply our proprietary MEG-OH terminated organotrichlorosilane linkers to 316L stainless steel, a widely used backbone material for many commercially available biomedical implants. In addition, we have taken the proven antifouling characteristics of MEG-OH adlayer one step further and shown the anti-thrombogenic potential of the said adlayer. We first demonstrated the feasibility of immobilizing the MEG-TFA terminated organotrichlorosilane linker onto 316L stainless steel. The MEG-TFA surface modified 316L stainless steel samples were subjected to careful surface characterization of XPS and contact angle measurements. Compared with untreated bare stainless steel, emergence of an intense and broad peak due to organic fluorine and organic silicon indicated successful surface linker immobilization, which was also backed up by appearance of the CF\(_3\) component peak on C1s narrow scan at 90 degrees take-off angle as well as drastically increased peak height and area of the C-O and C-C/C-H component peaks at both 20 and 90 degrees take-off angles. After solvolysis, complete disappearance of organic fluorine peak as well as the significantly decreased contact angle confirmed successful conversion of MEG-TFA to MEG-OH.

Collectively, the surface thrombogenicity assays involving fluorescent-labeled whole human blood has shown outstanding resistance to surface platelet adhesion and thrombus formation due to the MEG-OH adlayer under all experimented shear rates. Statistical test has indicated that there is a highly statistically significant difference of platelet surface coverage on bare and MEG-OH modified 316L stainless steel. MEG-OH adlayer has shown to reduce surface platelet coverage by at least 90% on average under all tested shear rates in our 20-minute end stage blood perfusion study. 60 min real-time
surface thrombogeicity study has also indicated similarly promising anti-thrombogenic behavior of the MEG-OH adlayer, particularly under 600 s\(^{-1}\) and 900 s\(^{-1}\) shear rates.

5 Future work

Several additional studies involving the MEG-OH adlayer on 316L stainless steel could be very beneficial to further support the findings discussed in the present study. This should include further testing on more replicates under a wider range of physiologically relevant shear rate. Due to technical constraints in the present study, only five replicates for untreated control and MEG-OH surface modified test group were tested under each selected shear rate, which all fall under the low shear rate regime (< 1000 s\(^{-1}\)). If more replicates as well as higher shear rates can be included in the follow-on study, data reproducibility and reliability would be greatly improved.

In the present study, whole human blood used for testing purposes was drawn from three volunteers. Certain parameters of blood, such as platelet and other blood cell counts, can vary significantly from person to person. Blood with high red blood cell count would certainly behave more like a non-Newtonian fluid compared with blood with low blood cell count, especially under low shear rate due to red blood cell aggregation and deformation.\(^{38-39}\) In addition, high platelet concentrate can also cause blood to be more sensitive to coagulation triggers. In order to avoid potential systemic biases and errors, it would be very beneficial to diversify blood sources in the future study.

Lastly, an investigation of stability of MEG-OH adlayer on 316L stainless steel would be interesting. For future clinical application of MEG-OH surface modified 316L stainless steel based biomaterial, long term adlayer stability can be crucial. Certain organosilane adlayers are known to be susceptible to cleavage by hydrolysis when immersed in aqueous or buffer environment for extended period of time.\(^{74}\) In case of a similar long term instability issue with the MEG-OH adlayer, new immobilization methodologies other than the existing trichlorosilane chemistry could be explored.
References


71. AV Shchukarev and DV Korolkov, *CEJC* 2(2) 2004 347-362


Appendices

Figure A. 1. XPS survey scans for bare (left) and oxidized (right) 316L stainless steel.

Figure A. 2. XPS survey scans for MEG-TFA surface modified (left) and MEG-OH surface modified (right) 316L stainless steel.
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