SURFACE MODIFICATION WITH AN ANTITHROMBIN-HEPARIN COMPLEX

ANTITHROMBOGENIC BIOMATERIALS: SURFACE MODIFICATION WITH AN ANTITHROMBIN-HEPARIN COVALENT COMPLEX

By

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ABSTRACT

Surface-induced thrombosis is a continuing issue in the development of biomaterials for blood contacting applications. Protein adsorption is a key factor in thrombosis since it occurs rapidly upon contact of a material with blood, initiating coagulation and other adverse reactions including platelet adhesion. The research presented in this thesis explores the use of a unique antithrombin-heparin covalent complex (ATH) for surface modification to provide antithrombogenicity. ATH was tethered to surfaces by various methods. Polyethylene oxide (PEO) was investigated as a linker-spacer molecule for surface attachment of ATH as well as for its antifouling properties.

In the first phase of the work gold was used as a model substrate. ATH was attached by three different methods: direct attachment, attachment via a short chain linker, and attachment via PEO. Analogous heparin-modified surfaces were prepared for comparison. Surfaces were characterized using contact angle measurements, x-ray photoelectron spectroscopy (XPS), ellipsometry and quartz-crystal microbalance (QCM). The data suggested that the heparin moiety of ATH was directed away from the surface, in an orientation allowing ready interaction with blood components. The ATH-modified surfaces showed greater antithrombin binding than the heparin-modified surfaces as measured by radioactive labelling and Western blotting analysis. Antithrombin binding was found to occur predominantly through the active pentasaccharide sequence of the heparin moiety of ATH, demonstrating the potential of the ATH for catalytic anticoagulant function. From measurements of the ratio of total heparin to active heparin

(anti-factor Xa assay), ATH-modified surfaces were shown to have greater bioactivity than heparin-modified surfaces. The adhesion of platelets to gold and modified gold surfaces was measured from flowing whole blood *in vitro* using a cone-and-plate device and was lower on all of the modified surfaces compared to bare gold. PEO-ATH surfaces were also shown to prolong plasma clotting times compared to control and heparinized surfaces.

In subsequent work, surface modification methods were developed for polyurethane (PU) substrates. Isocyanate groups were introduced into the PU surface for attachment of PEO and ATH was attached to the "distal" end of the PEO. Surfaces using PEO of varying molecular weight and end group were investigated to determine conditions for maximum anticoagulant activity and minimum non-specific protein adsorption. Surfaces were characterized using contact angle measurements and XPS, and protein interactions were studied using radiolabelling. The optimum balance of bioactivity and protein resistance was found to occur with PEO of low to mid range MW (ie. MW 300-600). These PU-PEO-ATH surfaces showed low fibrinogen adsorption and high selectivity for antithrombin. Consistent with results using gold substrates, platelet adhesion remained low when ATH was attached to polyurethane surfaces grafted with PEO. A hetero-bifunctional amino-carboxy-PEO (PEO-COOH surface) was compared with a "conventional" homo-bifunctional dihydroxy-PEO (PEO-OH surface) with respect to their effectiveness as linkers for attachment of ATH. The PEO-COOH-ATH surface was shown to bind slightly greater amounts of antithrombin, indicating higher catalytic anticoagulant activity. Thrombin binding was measured to determine whether the surfaces

could provide direct anticoagulant activity. The PEO-OH-ATH surface bound high amounts of thrombin, indicating potential for direct thrombin inhibition. It is hypothesized that the PEO properties (MW and functional end group) may have an effect on the orientation of ATH on the surface thus influencing its "preference" for catalytic vs. direct anticoagulant function.

This thesis provides new information regarding the interactions of proteins and platelets with ATH immobilized on biomaterials. ATH-modified surfaces were superior to analogous heparin-modified surfaces with respect to antithrombin binding and catalytic anticoagulant ability. Immobilized ATH was also shown to bind thrombin, suggesting potential for direct anticoagulant activity. It can thus be seen as a unique surface modifier with dual functioning anticoagulant activity. The modification of polyurethane with ATH using PEO as a protein resistant linker-spacer, may provide a material of improved antithrombogenicity for the construction of blood contacting devices.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF ABBREVIATIONS

ADP	Adenosine Diphosphate
AT	Antithrombin
ATH	Antithrombin-Heparin Covalent Complex
CBAS	Carmeda® BioActive Surface
COOH	Carboxylic Acid
DSP	Dithiobis(succinimidyl propionate)
FX	Factor X
FXa	Factor X (activated)
GIR-FTIR	Grazing Incidence Reflection Fourier Transform Infrared Spectroscopy
GP	Glycoprotein
HITS	High-intensity Transient Signals
HMWK	High Molecular Weight Kininogen
LDPE	Low Density Polyethylene
LMWH	Low Molecular Weight Heparin
MPC	2-Methacryloyloxyethyl Phosphorylcholine
MDI	Methylene-bis(phenyl-diisocyante)
NHS	N-hydroxysuccinimide
OH	Hydroxyl
PBS	Phosphate Buffered Saline
PEG	Polyethylene Glycol

PEO	Polyethylene Oxide
PET	Polyethylene Terephthalate
PF4	Platelet Factor 4
PMMA	Polymethylmethacrylate
PMP	Platelet Microparticles
PPACK	Phenylalanyl Prolyl Arginine Chloromethyl Ketone
PRT	Plasma Recalcification Time
PTMO	Poly(tetramethylene)oxide
PU	Polyurethane
QCM	Quartz Crystal Microbalance
RCL	Reactive Centre Loop
SAM	Self-assembled Monolayer
TAT	Thrombin-antithrombin
TEG	Thromboelastography
tPA	Tissue Plasminogen Activator
vWF	von Willebrand Factor
XPS	X-ray Photoelectron Spectroscopy

CHAPTER 1: INTRODUCTION

1.1. OVERVIEW

The research described in this thesis is aimed at gaining a better understanding of the interactions that occur at blood-material interfaces and improving the blood compatibility of biomaterials by reducing thrombosis. Biomaterials come in contact with blood in various medical device applications. This includes devices used for short periods of time such as peripheral catheters, oxygenators and hemodialysis membranes, as well as those implanted for long term use such as central venous catheters, stents, heart valves, vascular grafts, and artificial hearts. The prevalence of cardiovascular disease in Canada and across the world creates a significant need for many of these devices. For example in the year 2000, close to two million stents and valves were implanted worldwide, costing $75 billion US dollars, with growth rates increasing substantially (Lysaght and O'Loughlin 2000). The aging population also adds to the requirements for these and various other blood contacting devices.

Advances in science and technology have allowed the design and development of novel engineered devices for blood contact. However, the majority of these continue to suffer from complications, including thrombosis, infection, immunological reactions, improper healing and material degradation (Padera and Schoen 2004). Thrombotic complications remain the major issue affecting all materials and prevent some devices such as small diameter vascular grafts from being useful clinically (Ratner 2007). Due to the potential for thrombosis, continuous systemic anticoagulant therapy is also typically required in the use of blood contacting devices which can lead to additional risks.

Eliminating, or even reducing, the thrombogenicity of biomaterials would therefore have numerous benefits.

To improve blood compatibility, a targeted approach to designing biomaterials may elicit more desirable responses for specific applications. The optimal surface for contact with blood is the endothelial lining of healthy vessels. Therefore, an important aspect to consider in the design of improved materials is whether interactions occurring at the blood-material interface are similar to those at the blood-endothelial interface in the vasculature. This requires an in-depth understanding of the processes that take place when a material contacts blood, including protein adsorption, blood coagulation, platelet interactions and other cell interactions that can lead to thrombosis. This chapter will provide a review of all of these areas and will discuss the strategies that have been used for the design of biomaterials to reduce thrombogenicity.

Polymers are frequently used as biomaterials for blood contact due to their mechanical properties. However, synthetic polymers are “foreign” materials and elicit numerous adverse interfacial responses. Surface modification is an attractive strategy that allows conservation of the bulk mechanical properties, while permitting the surface to interact in a more favourable way with blood. Both “bioinert” and “bioactive” modifications have been used to improve blood-surface interactions. In this research, a combined bioinert-bioactive approach was investigated. Polyethylene oxide (PEO) was used for its protein and cell resistant properties, and a novel antithrombin-heparin (ATH) covalent complex for its anticoagulant activity. In initial work, gold was used as a model substrate to immobilize ATH through PEO and to study protein and platelet interactions

and anticoagulant function. An analogous PEO-heparin combination was also investigated. In later work, methods were developed for the attachment of PEO-ATH to polyurethane (PU) as a material more applicable for blood contacting devices.

1.2. LITERATURE REVIEW

1.2.1. Blood-Biomaterial Interactions

Interactions at the blood-material interface play a significant role in the compatibility of a device with the body and thus are essential to consider in the design of improved biomaterials. Blood consists of cells (red cells, white cells and platelets) and plasma, the liquid phase containing proteins and other dissolved substances. When exposed to blood, it is the surface of the material that interacts with these components. Blood-surface interactions are extremely complex involving an array of processes including protein adsorption, blood coagulation, fibrinolysis, complement activation, platelet interactions, and other cellular reactions (Courtney, Lamba et al. 1994) (Brash 1987).

An overview of the sequence of events occurring when blood contacts a foreign surface is shown in Figure 1.1. The initial and central role of protein adsorption and the importance of the protein layer composition is evident throughout the sequence. The various pathways are not independent of each other and many events are highly interrelated (Brash 1987). These reactions typically lead to significant adverse effects with thrombus generation as the end result. Thrombosis is detrimental at the site of the device if an obstruction occurs, and also if the thrombus embolizes into the systemic

circulation (Colman 1993). Clearly, the achievement of antithrombogenic materials requires a thorough understanding of these interactions and the ability to direct or control them in an appropriate way.

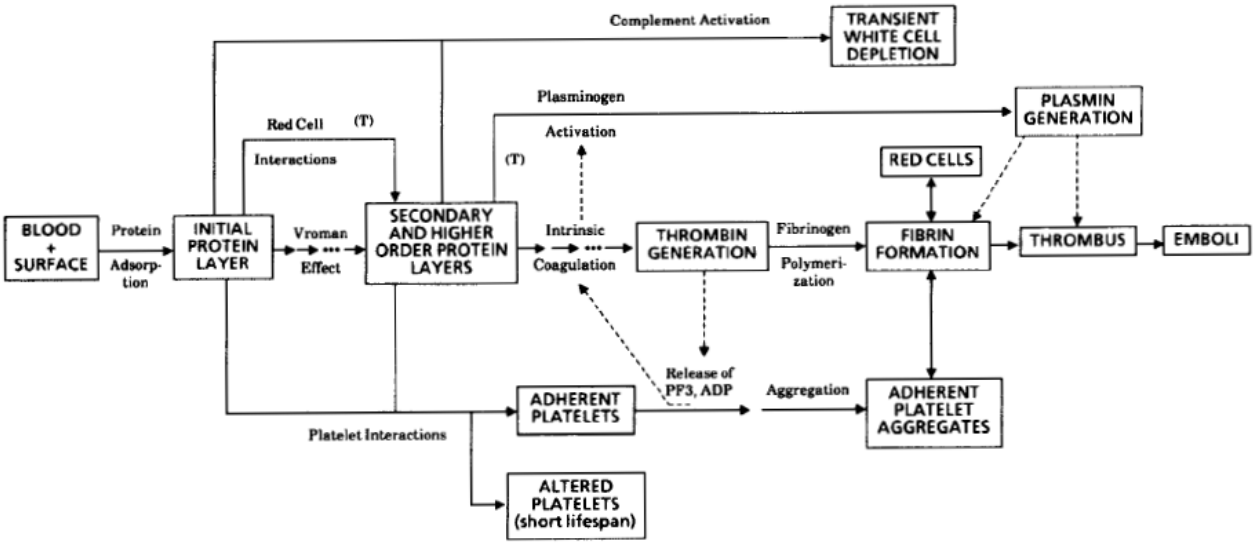

Figure 1.1. Sequence of events during blood-material interactions (Brash 1987). (With permission).

1.2.1.1. *Protein Adsorption*

The adsorption of proteins to surfaces is an important phenomenon in many applications including medical device implants, marine structures, biosensors and food processing. In blood contacting applications it is of particular importance due to the subsequent adverse reactions that protein adsorption can lead to. Many studies of proteins and their interactions at interfaces involve the use of blood and/or plasma proteins with a focus on coagulation proteins such as fibrinogen. Several in-depth reviews on protein adsorption have been published (Andrade 1985; Norde 1986; Brash 1991; Horbett 1993; Norde 2003).

Proteins are macromolecules composed of chains of amino acids linked together by peptide bonds to form polypeptides. Twenty standard amino acids are found in proteins, each containing a unique side chain. The side chains differ in chemical functionality, charge, polarity and reactivity (Hermanson 1996). The primary structure of a protein is determined by the composition and sequence of the amino acids. The conformation of the polypeptide backbone resulting in random, alpha helical and beta sheet sequences gives a protein its secondary structure, and folding and stabilization through electrostatic interactions, hydrogen bonding, hydrophobic interactions and disulfide bonds result in the tertiary structure which determines the 3-dimensional shape of the protein (Voet and Voet 2004). Larger proteins with multiple polypeptide chains can also be described by a quaternary structure based on the interactions between the protein subunits. Since some amino acids have negative charges and some positive charges, proteins have an isoelectric point (pI), defined as the pH at which the positive and negative charges are in balance, resulting in zero net electric charge. The wide variety of protein structures provides each protein with unique properties and functions.

When a foreign material contacts blood, it is well known that proteins adsorb within seconds (Baier and Dutton 1969; Brash and Lyman 1969). Many factors influence adsorption including protein structure, surface properties, and solution properties. Since proteins are amphiphilic, due to the varying polarity of amino acids, they have an intrinsically surface active character (Norde 1986). The effects of protein size, hydrophobicity-hydrophilicity and electric charge give rise to different mechanisms of protein-surface interaction. Protein-protein and protein-surface interactions both

determine the composition and quantity of protein on a surface. Once a protein is adsorbed, conformational and orientational changes can also take place, including spreading, unfolding and denaturation. Over time these structural transformations can result in an increase in the protein-surface bond strength (Rapoza and Horbett 1990).

1.2.1.1.1. Adsorption Kinetics

To be adsorbed a protein must be transported from the bulk solution to the interfacial region. Both diffusion and convection mechanisms may be involved. When a protein is far from the surface in the bulk and in its native state, any movement toward the surface is likely to be through convection (Ramsden 2003). In static conditions, or at low surface coverage when a protein reaches the near surface region, adsorption is controlled by diffusion and the surface concentration can be approximated as (Andrade 1985; Norde 1986):

$$\Gamma = 2C_o\left(\frac{Dt}{\pi}\right)^{1/2} \qquad \text{(Eq 1.1)}$$

where Γ is the surface concentration, C_o is the bulk protein concentration in solution, D is the diffusion coefficient, and t is time.

In flow conditions, an appropriate form of the convection-diffusion equation must be formulated and solved; various flow parameters are then involved. When the protein surface coverage is greater than about 10%, adsorption becomes reaction-controlled rather than transport-controlled and the rate is independent of flow conditions (Brash 1991). A study of the adsorption kinetics of various plasma proteins to common polymer

materials demonstrated the complexity of protein properties and their effect on adsorption (Young, Pitt et al. 1988). Computer simulation models have been used to describe protein adsorption kinetics from single-component systems, taking into consideration various physical effects (Wojciechowski and Brash 1990).

1.2.1.1.2. Thermodynamics

Protein adsorption occurs due to a decrease in the overall Gibbs free energy of a system leading to a more energetically favourable condition (Haynes and Norde 1994), described by:

$$\Delta G = \Delta H - T\Delta S < 0 \qquad \text{(Eq 1.2)}$$

where G is the Gibbs free energy, H is the enthalpy, T is the absolute temperature and S is the entropy. Enthalpic and entropic contributions from various interfacial interactions therefore determine the adsorption of proteins. The hydrophobic effect (interactions of hydrophobic species in aqueous systems) is important for protein adsorption; hydrophobic interactions result from more favourable contacts between water molecules versus contacts between non-polar groups or non-polar groups and water (Norde 1986). When proteins adsorb to surfaces, they may replace ordered water molecules leading to an increase in entropy and a decrease in free energy. Electrostatic interactions can be attractive or repulsive depending on the charges of the protein and surface; the solution pH and ionic strength have an effect on the electrostatic potential and charge (Tsapikouni and Missirlis 2008). Hydrogen bonding and van der Waals forces also play a role, but usually a less important one. Further decreases in Gibbs free energy can result from

structural changes such as protein folding and changes in conformation (Norde and Haynes 1995). Overall, the combination of these various mechanisms and the interplay between them drives the adsorption of proteins to surfaces.

1.2.1.1.3. Modelling Protein Adsorption

Various models have been used to describe protein adsorption. For single protein solutions, a simple monolayer model is frequently used where the data can be reasonably fit to either a Langmuir or Freundlich adsorption isotherm (Horbett 1993). The Langmuir model follows Equation 1.3:

$$\theta = \frac{\Gamma}{\Gamma_{max}} = \frac{KC_p}{(1 + KC_p)} \qquad \text{(Eq 1.3)}$$

where θ is the fraction of surface sites covered, Γ is the surface concentration (in mass or moles per unit area), Γ_{max} is the maximum surface concentration (typically a monolayer), K is the adsorption equilibrium constant, and C_p is the bulk protein concentration (in mass or moles per unit volume). Langmuir isotherms therefore increase monotonically with increasing protein concentrations until an asymptote or quasi plateau is reached, representing limiting coverage (Young, Pitt et al. 1988). When the protein concentration is low, adsorption increases linearly and the slope of the line can be used to calculate the adsorption equilibrium constant. At high protein concentration when all surface sites are occupied adsorption is at the monolayer level.

The Langmuir model assumes that: only one molecule is adsorbed per site, the surface is energetically homogeneous, lateral interactions between adsorbed molecules

are negligible, only one adsorbing species is present, the solution is dilute, and adsorption is reversible (Andrade and Hlady 1986). In the case of protein adsorption to solid surfaces, most of these assumptions are not satisfied. For example, on an ordinary time scale protein adsorption has been found to be essentially irreversible with respect to dilution (Chan and Brash 1981; Norde and Haynes 1995). Also, interactions between co-adsorbed proteins will certainly occur. Despite these apparent contradictions, a good deal of experimental data on protein adsorption has been found to fit well to the Langmuir equation (Brash and Lyman 1969; Brash and Davidson 1976; Horbett, Weathersby et al. 1977; Chan and Brash 1981; Young, Pitt et al. 1988).

The Freundlich model has also been found to be useful in describing protein adsorption (Equation 1.4):

$$\Gamma = aC_p^{1/n} \qquad \text{(Eq 1.4)}$$

where Γ is the surface concentration (in mass or moles per unit area), a is a measure of the adsorption capacity, C_p is the bulk protein concentration (in mass or moles per unit volume), and the exponent 1/n is a measure of the energy of adsorption (Young, Pitt et al. 1988). Both a and n are empirical constants. Adsorption increases much more slowly at high protein concentration than at low concentration and, in contrast to the Langmuir model, a well defined plateau is not usually reached. Many systems have been found to follow the Freundlich model, e.g. fibrinogen adsorption to electrically charged surfaces (Schmitt, Varoqui et al. 1983).

1.2.1.1.4. Surface Properties

Protein adsorption is strongly influenced by the specific properties of the adsorbing surface, e.g. its hydrophobicity/hydrophilicity, electrostatic charge and chemical reactivity. Also, surface topography and molecular motions can be important factors in determining adsorption behaviour (Horbett 1993). The composition of the surface is thought to play a greater role in low flow conditions, whereas the topography and molecular motions may be more important at higher flow (Hoffman 1987). In many cases hydrophobic surfaces have been found to adsorb greater quantities of protein and to bind the protein more strongly than hydrophilic surfaces (MacRitchie 1972; Elwing, Welin et al. 1987; Prime and Whitesides 1991). Electrically charged surfaces may influence protein adsorption via attractive interactions between oppositely charged proteins or repulsive interactions between similarly charged ones. For example on titanium films with increasing levels of negative charge, decreasing levels of fibrinogen adsorption were observed (Cai, Frant et al. 2006). However, in a study of polyelectrolyte surfaces of varying charge density, protein adsorption was found to be similar, in this case suggesting that hydrophobic interactions may have a greater effect than charge (Schmitt, Varoqui et al. 1983). The effect of surface topography has been a recent focus of research. In general adsorption has been found to increase with increasing surface roughness due to the increase in real surface area and to roughness-induced protein orientation (Rechendorff, Hovgaard et al. 2006).

1.2.1.1.5. Competitive Protein Adsorption

In complex, multi-protein systems such as blood, protein adsorption is known to be a selective and competitive process. Early studies by Vroman et al. on various surfaces showed that on contact with plasma, fibrinogen was present initially but rapidly became undetectable (Vroman and Adams 1969; Vroman, Adams et al. 1980). It was suggested that the adsorbed fibrinogen was "converted" to a form (perhaps a different conformation) that was no longer detectable by the immunochemical method used. Later work showed that the initially adsorbed fibrinogen was adsorbed and then displaced from the surface. This was termed the "Vroman effect" (Brash and Tenhove 1984; Horbett 1984) and is believed to reflect the competition among proteins of varying concentration and adsorption affinity. Proteins of higher concentration and lower affinity are preferentially adsorbed in the initial phase and are later displaced sequentially by proteins of lower concentration and higher affinity resulting in a dynamic protein layer that changes in composition with time.

Additional studies have investigated a range of surface types, surface chemistries, and protein types including coagulation factors and fibrinolytic system proteins (Wojciechowski, Tenhove et al. 1986; Brash, Scott et al. 1988; Slack and Horbett 1989; Brash and Tenhove 1993; Turbill, Beugeling et al. 1996). In general the timing and magnitude of fibrinogen adsorption and exchange has been found to vary with the surface: hydrophilic surfaces have been shown to be more "dynamic" than hydrophobic ones. More recent work has focused on the displacement of fibrinogen at the molecular level and the results suggest that the positively charged αC domains of the molecule play

a role in its adsorption and desorption from surfaces (Jung, Lim et al. 2003). The structural and conformational changes of proteins on surfaces are therefore also linked to adsorption dynamics.

1.2.1.2. *Blood Coagulation & Thrombosis*

Physiologically, blood coagulation is triggered in response to vascular damage so as to maintain vascular integrity. A balance among coagulation, anticoagulation and fibrinolysis regulates this process within the body (Spronk, Govers-Riemsiag et al. 2003). Coagulation is also initiated when a foreign object such as an implant contacts circulating blood. If coagulation is not controlled, a fibrin clot will be formed leading to thrombosis. The progression of blood coagulation is dependent on numerous complex interactions among plasma proteins, cells and other tissue components.

Traditionally, coagulation mechanisms have been described by "waterfall" or "cascade" models (Davie and Ratnoff 1964; Macfarlane 1964). The coagulation mechanism is generally understood as consisting of two pathways, the "intrinsic" pathway and "extrinsic" pathway, which converge to the "common" pathway (Figure 1.2) (Davie, Fujikawa et al. 1991). *In vivo*, fibrinolysis, as well as coagulation, is triggered by vessel wall damage so that the clot is dissolved when no longer needed.

A recent model of hemostasis with *in vivo* relevance highlights the importance of cellular components and involves overlapping stages termed initiation, amplification and propagation (Hoffman and Monroe 2001). The extrinsic pathway participates in the

initiation phase of this cell-based model and the intrinsic pathway plays a role in the propagation stage (Monroe and Hoffman 2006).

The coagulation cascade model continues to be seen as a functional model that adequately describes the interactions among the coagulation proteins (Figure 1.2). It consists of a series of enzymatic reactions involving coagulation factors and inhibitors along with cofactors. The reactions also depend on calcium ions, and some of them proceed by assembly of reactants on phospholipid surfaces (Furie and Furie 1988; Spronk, Govers-Riemsiag et al. 2003). The extrinsic pathway is initiated by release of tissue factor from damaged cells. Tissue factor (TF) binds to factor VII (FVII) on phospholipid, forming the TF/FVIIa enzymatic complex which activates both FIX and FX (Osterud and Rapaport 1977). The intrinsic (contact) pathway is triggered in response to a foreign surface and involves factors (proteins) that are contained in the blood. Contact activation is initiated by the adsorption of factor XII (FXII) to a material surface, and its conversion to activated FXII (FXIIa). FXIIa converts prekallikrein to kallikrein and factor XI to FXIa when bound to the cofactor high molecular weight kininogen (HMWK) (Colman 1984). The presence of HMWK also accelerates the conversion of FXII to FXIIa. FXIa activates FIX to FIXa and then FIXa, along with cofactor FVIIIa, calcium and phospholipid, converts FX to FXa (Colman 1993).

The common pathway begins with the conversion of prothrombin to thrombin. FXa, formed by either the intrinsic or extrinsic pathway, is required for this reaction. FVa, phospholipid and calcium, which combine with FXa to form the prothrombinase

complex, are also required (Davie, Fujikawa et al. 1991). The crucially important enzyme thrombin converts fibrinogen to fibrin, the material of the clot.

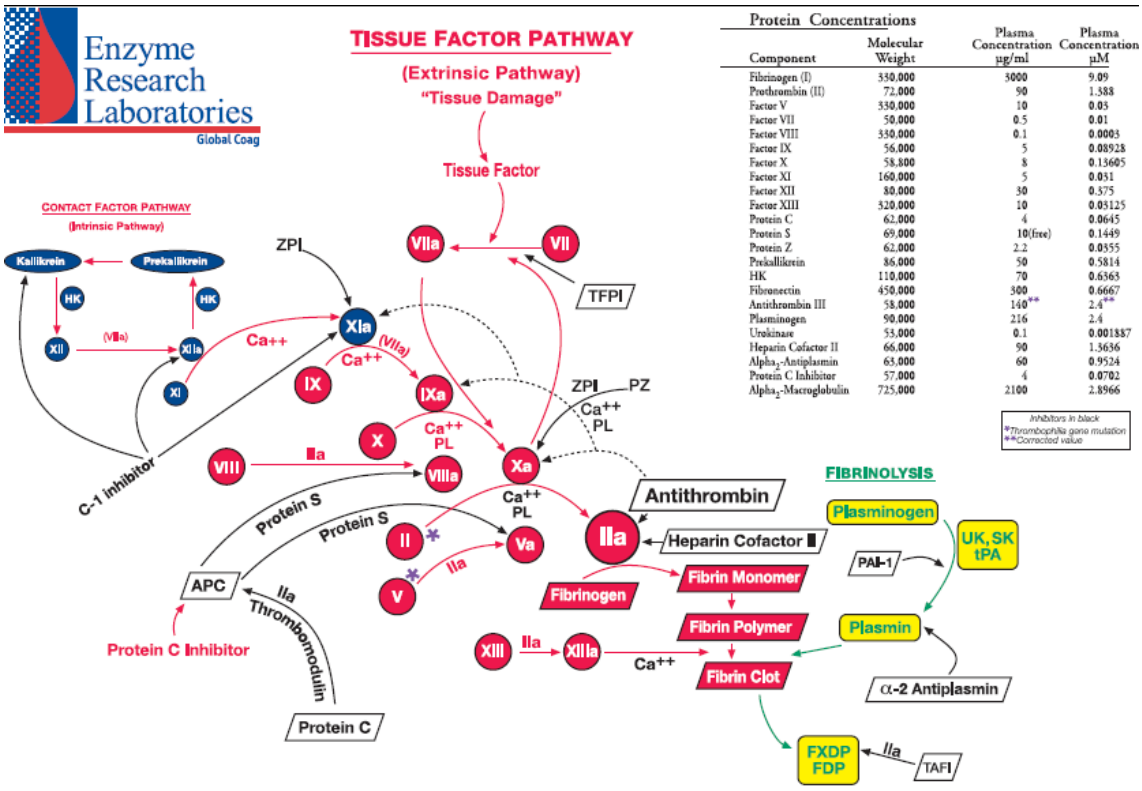

Figure 1.2. The blood coagulation cascade. (With permission from: http://www.enzymeresearch.com/CASCADES_2004/images/CASCADE_erl_2007.pdf)

1.2.1.2.1. Thrombin

Thrombin is recognized as the key enzyme in blood coagulation and thrombosis providing both positive and negative feedback activation. In addition to converting fibrinogen to fibrin, it has a number of other effects on coagulation and cellular reactions. Thrombin activates FV, FVIII and FIX. It also converts FXIII to FXIIIa, which crosslinks and stabilizes the fibrin clot. Thrombin is a powerful platelet activator and as such contributes greatly to platelet aggregation (Davie and Kulman 2006).

Although thrombin is typically viewed as procoagulant it can also act as an anticoagulant via the protein C pathway (Figure 1.2). Thrombin binds to thrombomodulin on endothelial cells to activate protein C. Activated protein C (with protein S as cofactor), in the presence of calcium and phospholipid, then inactivates FVa and FVIIIa (Kane, 1988). Thrombin also has biological functionality towards endothelial cells, smooth muscle cells and leukocytes (Tapparelli, Metternich et al. 1993). Physiologically thrombin is inactivated by α2-macroglobulin, heparin cofactor II, protease nexin I and its main inhibitor, antithrombin (Stubbs and Bode 1993).

1.2.1.2.2. Fibrinogen

Fibrinogen is important in coagulation and thrombosis due to its dual roles in fibrin formation and platelet adhesion. It is present in plasma at high concentration making up about 3-5% of the total protein content. Fibrinogen has an overall molecular weight of 340,000 Da, and is rod shaped with an approximate length of 450 Å and width of 60 Å (Bachmann, Schmitt-Fumain et al. 1975; Estis and Haschemeyer 1980). It is multidomained with two sets of three polypeptides, the α, β, and γ chains, that are connected by disulfide bonds (Doolittle 1984). In coagulation, fibrinogen is cleaved by thrombin to release fibrinopeptides A and B forming fibrin monomer (Blomback, Hessel et al. 1978). Fibrin monomer then polymerizes forming fibrin chains which are crosslinked by FXIIIa to form a fibrin network (Muszbek, Bagoly et al. 2008). A stable fibrin clot is produced that interacts with platelets and red cells to form thrombus. Specific fibrinogen and platelet reactions will be discussed in the next section.

The adsorption of fibrinogen to biomaterial surfaces has been investigated extensively because of its prominent roles in coagulation and in mediating platelet adhesion. Fibrinogen is adsorbed extensively to surfaces partly due to its high molecular weight and strong lateral interactions (Feng and Andrade 1995). Its high concentration in plasma leads to rapid initial adsorption and then displacement as discussed above with respect to competitive protein adsorption and the Vroman Effect. In general, fibrinogen adsorption is less affected by surface properties than other proteins and it has been found to adsorb to many surfaces including both hydrophobic and hydrophilic surfaces (Schmitt, Varoqui et al. 1983). In a study of fibrinogen adsorption to different polymeric materials from buffer or plasma, fibrinogen was found to adsorb to all materials, but the amounts varied significantly (Brash and Tenhove 1993). As discussed previously, the Vroman effect was originally observed via fibrinogen adsorption followed by desorption due to competition from other proteins for surface sites. The importance of fibrinogen-platelet interactions has been established in numerous studies that have shown a direct relation between fibrinogen adsorption and platelet adhesion on a range of different surfaces (Adams and Feuerstein 1981; Tsai, Grunkemeier et al. 1999; Tsai, Grunkemeier et al. 2002; Wu, Simonovsky et al. 2005). Fibrinogen adsorption to materials has also been shown to initiate acute inflammatory processes and the foreign body reaction (Tang and Eaton 1993; Tang and Eaton 1995; Hu, Eaton et al. 2001).

Following the formation of fibrin from fibrinogen, the fibrinolytic system is able to degrade fibrin clots, effectively reversing the process of thrombosis. The circulating plasma protein plasminogen is converted to the enzyme plasmin by activators such as

tissue plasminogen activator (tPA) or urokinase plasminogen activator (uPA) (Angles-Cano 1994). Plasmin cleaves fibrin resulting in the generation of soluble fibrin degradation products (Cesarman-Maus and Hajjar 2005). Rather than preventing coagulation on biomaterial surfaces, the idea of dissolving the inevitable clot by exploiting the fibrinolytic system is an alternative concept for materials of improved blood compatibility (Li, Chen et al. 2011).

1.2.1.2.3. Platelet Interactions

Platelets are non-nucleated, disc shaped cells, 2-5 μm in diameter, and are present in blood at an approximate concentration of 250,000 cells/μL. They originate from megakaryocytes and have a lifespan in the circulation of 7-10 days (White 2007). The platelet plasma membrane contains a number of glycoprotein receptors. Key platelet receptors are glycoprotein (GP) IIb/IIIa and GP Ib (Colman, Clowes et al. 2001).

Platelets play an important role in material-induced thrombosis due to their interactions with proteins and their contribution to the formation of a stable fibrin-platelet thrombus. Platelet adhesion, activation and aggregation on biomaterials are mediated by receptor binding to specific proteins present on the surface. Fibrinogen, fibronectin, vitronectin and von Willebrand Factor (vWF) are important proteins in platelet-surface interactions (Adams and Feuerstein 1981; Horbett 1993; Grunkemeier, Tsai et al. 2000). These proteins are all surface active and cell adhesive, and contain the specific RGD (Arg-Gly-Asp) sequence which acts as a ligand for platelet adhesion.

Fibrinogen is considered to be the major mediator of platelet adhesion and aggregation. Platelet binding to fibrinogen occurs via the GP IIb/IIIa receptor and specific peptide sequences in the gamma and alpha chains of fibrinogen (Hawiger, Timmons et al. 1982). The conformation and orientation of fibrinogen in its adsorbed state has been shown to influence platelet adhesion (Lindon, McManama et al. 1986; Wu, Simonovsky et al. 2005). Studies have suggested that significant platelet adhesion will occur at fibrinogen surface concentrations greater than ~10 ng/cm^2 (Tsai, Grunkemeier et al. 1999; Zhang, Zhang et al. 2008).

At high shear rates vWF plays a prominent role in platelet adhesion and aggregation due to its increased affinity for GP Ib (Ruggeri 2001; Wu, Zhang et al. 2008). Binding of vWF to GP Ib activates GP IIb/IIIa and causes further interactions with fibrinogen, fibronectin and vitronectin. Blood flow dynamics are also important due to the interrelated effects on proteins, platelets, red blood cells and the material surface (Alkhamis, Beissinger et al. 1990).

Following adhesion to a surface, platelets become activated, change shape from discoid to spherical with pseudopod extrusions, and experience various spreading stages (Park, Mao et al. 1990). They also become more prone to platelet-platelet interactions and aggregation. Upon activation, platelets undergo secretion from dense granules and alpha granules. Released species include adenosine diphosphate (ADP), serotonin, platelet factor 4 (PF4), β- thromboglobulin, growth factors and clotting proteins (Blockmans, Deckmyn et al. 1995). The glycoprotein P-selectin is also released and expressed on platelet membranes after alpha granule secretion and can influence leukocyte adhesion

(Palabrica, Lobb et al. 1992; Gorbet and Sefton 2004). Activated platelets bind coagulation factors Va and Xa and contribute to the tenase complex and the prothrombinase complex that convert FX to FXa and prothrombin to thrombin, respectively. In addition, material-induced platelet activation has been found to lead to the formation of platelet microparticles (PMPs), which are procoagulant and support thrombin generation (Gemmell 2001).

Due to the complexity of platelet interactions that occur following contact of blood with a foreign surface, it is important to consider platelet adhesion, activation and aggregation along with other factors (Haycox and Ratner 1993). For example platelets undergo different degrees of spreading, they may become detached from a surface, and generate microparticles. Platelets can also be "consumed" and functionally altered by surface contact leading to a shortened lifespan in the circulation. Other interactions such as complement activation and leukocyte adhesion are also linked to platelet adhesion and thrombosis (Gorbet and Sefton 2004).

1.2.1.3. *Inhibition of Coagulation*

Since thrombin is central to the coagulation cascade and to platelet activation, its inhibition is key to controlling thrombosis. Various physiological and synthetic thrombin inhibitors are available including direct and indirect inhibitors. Hirudin is a powerful direct thrombin inhibitor that interacts with the active site of thrombin (Marki and Wallis 1990; Agnelli, Renga et al. 1992). It has advantages over other anticoagulants in that it is able to inactivate both circulating and clot-bound thrombin. Synthetic peptides, e.g.

phenylalanyl prolyl arginine chloromethyl ketone (PPACK), and other low molecular weight compounds have also been explored as direct thrombin inhibitors (Kettner and Shaw 1979; Tapparelli, Metternich et al. 1993). However, direct thrombin inhibitors with high binding affinity (irreversible binding) can inactivate only one thrombin and do not "regenerate"; thus, they have limited inhibitory capacity.

Heparin is a widely used anticoagulant that inhibits thrombin "indirectly" as discussed below (section 1.2.1.3.2.). Various "heparinoids" have also been explored as alternative anticoagulants. Low molecular weight heparins (LMWH), i.e. heparin fragments of average molecular weight ~5 kDa, have been shown to have advantages and disadvantages compared to standard heparin (Weitz 1997). Fondaparinux®, an even shorter heparin fragment consisting of the active pentasaccharide sequence, has recently been shown to be effective as an anticoagulant in some cases (Weitz 2011). Like heparin, these materials inhibit thrombin by an indirect catalytic mechanism, involving an intermediate antithrombin binding step. A novel antithrombin-heparin covalent complex (ATH) that can provide both rapid direct thrombin inhibition through AT and continuous catalytic inhibition through heparin has significant advantages over heparin and other anticoagulants. The use of ATH for modification of biomaterials is the focus of this research.

1.2.1.3.1. Antithrombin

Antithrombin (AT) is the main physiological thrombin inhibitor and regulator of blood coagulation. AT is a 58 kDa glycoprotein found in blood plasma at a concentration

of 0.125-0.150 mg/mL. It belongs to the family of serine protease inhibitors and its main functions are to inactivate thrombin and factor Xa, as well as factors IXa, XIa and XIIa (Pike, Buckle et al. 2005). AT forms a 1:1 complex with its target proteinase at its reactive centre loop (RCL) (Figure 1.3). AT circulates in two different isoforms, α-AT and β-AT. The α-AT isoform constitutes the majority of the plasma content of AT (90-95%), but the β-AT isoform has been found to bind more rapidly to the vessel wall (Frebelius, Isaksson et al. 1996). The isoforms vary structurally in their carbohydrate content and β-AT provides more rapid thrombin inhibition than α-AT (Peterson and Blackburn 1985). AT exists normally in a relatively inactive form, but upon binding to heparan sulfate or its derivative, heparin, the inhibitory activity of AT is increased significantly (Jin, Abrahams et al. 1997). The interaction between AT and heparin is an important intermediate step and is discussed in detail below.

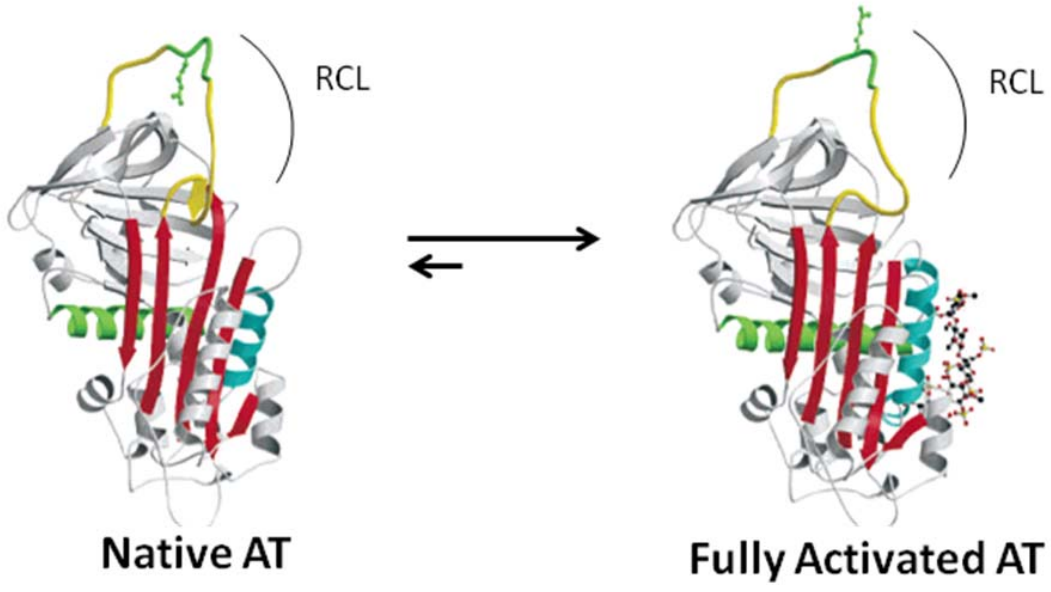

Figure 1.3. Antithrombin (AT) in its native and fully activated states. The reactive centre loop (RCL) is shown at the top of the antithrombin molecule. Adapted from: (Johnson and Huntington 2003). (With permission).

1.2.1.3.2. Heparin

Heparin is a highly sulphated heterogeneous polysaccharide. It is similar in structure to heparan sulfate, both being members of the glycosaminoglycan family; however, it is heparan sulphate that is expressed on the surface of cells, including vascular endothelial cells (Rabenstein 2002). Heparin on the other hand, is primarily synthesized by mast cells and can be found naturally in the mucosa of the intestine and lung. Unfractionated heparin has a broad molecular weight distribution ranging from 3 to 30 kDa, with an average molecular weight around 15 kDa. A unique pentasaccharide sequence is present in about one third of heparin molecules and this sequence binds specifically to AT (Figure 1.4) (Lam, Silbert et al. 1976; Olson, Bjork et al. 1992).

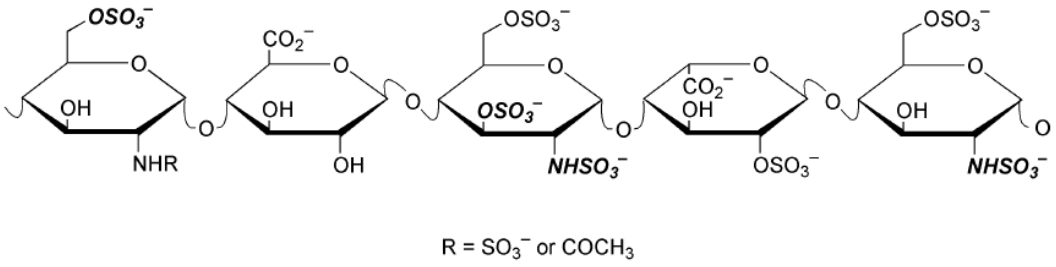

Figure 1.4. Unique antithrombin-binding pentasaccharide sequence in heparin (Rabenstein 2002).

Heparin binding to AT through its active sequence results in a conformational change in AT exposing its reactive centre loop (Carrell, Stein et al. 1994; Johnson and Huntington 2003). As a result, the rates of inhibition of thrombin and FXa by AT are enhanced on the order of 1000 fold. Thrombin inhibition is dependent on the heparin chain length since a bridging mechanism is required for the ternary interaction of heparin, antithrombin and

thrombin as shown in Figure 1.5 (Holmer, Lindahl et al. 1980; Olson, Bjork et al. 1992). The heparin catalysed inhibition of FXa is independent of heparin chain length; a conformational change in AT is induced without the formation of a ternary complex (Holmer, Lindahl et al. 1980; Weitz 1997). However, the "template effect" of heparin has also been found to be important for enhancing FXa inhibition in the presence of calcium (Rezaie 1998). For thrombin inhibition, heparin binds to antithrombin and a thrombin-antithrombin (TAT) complex is formed. Once thrombin is neutralized by antithrombin, heparin's affinity for the TAT complex decreases. The heparin is able to dissociate from the complex and repeat its catalytic function by interacting with another AT molecule; the cycle continues indefinitely (Carlstrom, Lieden et al. 1977; Byun, Jacobs et al. 1996).

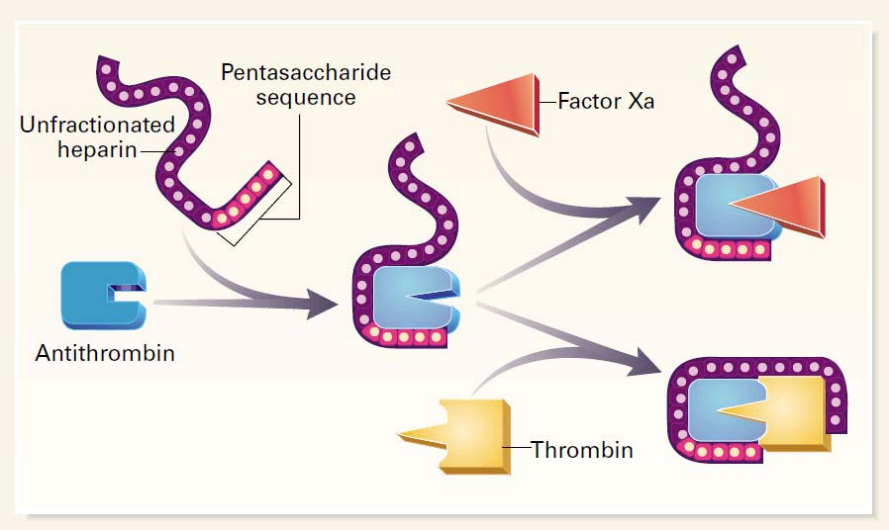

Figure 1.5. Catalysis of antithrombin-mediated inactivation of thrombin by heparin (Weitz 1997). (With permission).

Despite the extensive use of heparin as an anticoagulant, it has many limitations. The active pentasaccharide sequence required for AT binding is present in only about one third of the molecules in standard heparin (Lam, Silbert et al. 1976). This results in

relatively low and variable anticoagulant activity. Heparin is also confined to intravascular spaces and has a short half life in the circulation of about an hour (Kandrotas 1997). Its rapid removal is due partly to its propensity to bind to various proteins and cells. Many of the heparin-binding proteins such as vitronectin, fibronectin, von Willebrand factor (VWF), fibrin(ogen), tissue plasminogen activator (tPA), histidine-rich glycoprotein, platelet factor 4 (PF4) and thrombospondin are involved in hemostasis (Conrad 1998). The non-specific binding of heparin to these proteins not only limits its availability for AT binding, but also leads to variability in its anticoagulant effectiveness (Manson, Weitz et al. 1997). Heparin can also interact with platelets, macrophages and endothelial cells (Salzman, Rosenberg et al. 1980; Hirsh, Raschke et al. 1995). Another major limitation of heparin, is its inability to inhibit fibrin-bound thrombin and FXa in the prothrombinase complex (bound to FVa, phospholipid and calcium) (Hogg and Jackson 1989; Rezaie 2001). Further clinical concerns in the use of heparin include the risks of hemorrhage, heparin-induced thrombocytopenia and osteoporosis (Hirsh 1991).

1.2.1.3.3. Antithrombin-Heparin Covalent Complex (ATH)

Although heparin in the free state has limitations in the treatment of thrombosis, a covalent complex of heparin and antithrombin, its natural target, may provide improved function. This approach has been explored by a number of groups (Bjork, Larm et al. 1982; Ceustermans, Hoylaerts et al. 1982; Mitra and Jordon 1987; Chan, Berry et al. 1997). A primary advantage of an antithrombin-heparin complex (ATH) is that with heparin covalently attached to AT, the complex cannot dissociate and maintains its

activity. This may lead to increased inhibition of coagulation factors and other benefits. The relatively large complex should have a longer half life in the circulation, thus also giving a more predictable anticoagulant effect.

In initial work by Ceustermans *et al.* heparin was chemically modified by various means to introduce amino groups and was covalently attached to antithrombin using tolylene-2,4-diisocyanate as a coupling agent (Ceustermans, Hoylaerts et al. 1982). Although these complexes showed an increased half life compared to heparin alone, the inhibition of FXa and thrombin was not significantly different. Another approach involving modified heparin was reported by Bjork *et al.* where low molecular weight, high affinity heparin fragments were produced and reacted with AT via a covalent link between a heparin aldehyde group and a lysyl amino group on AT (Bjork, Larm et al. 1982). This complex showed considerable activity against FXa, but no thrombin inhibitory activity, likely due to the small size of the heparin component. A similar preparation of the complex using the method of Bjork *et al.* also showed no additional thrombin inhibitory activity (Dawes, James et al. 1994). Mitra and Jordan developed a covalently bound heparin-antithrombin complex, as described in their 1987 patent, using cyanogen bromide activated heparin (Mitra and Jordon 1987). All of the AT was found to be in the active state as demonstrated by full saturation of the heparin binding sites, and both FXa and thrombin showed high inhibitory capacities. However, no catalytic heparin activity was present and comparisons to AT and heparin non-covalent mixtures were not made.

The most recent ATH complex, developed by Chan *et al*., is the first to use standard heparin without chemical modification, along with native AT (Chan, Berry et al. 1997). The synthesis takes advantage of the natural conjugation of glycosaminoglycans and polypeptides via Schiff base-Amadori rearrangement mechanisms (Figure 1.6) (Berry, Chan et al. 1998).

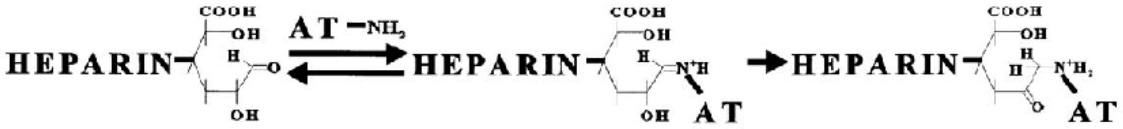

Figure 1.6. Synthesis of covalent antithrombin-heparin complex via Schiff base-Amadori rearrangement (Berry, Andrew et al. 2000).

Human AT is incubated with excess unfractionated heparin (molar ratio 1:200) in phosphate buffered saline (PBS) at 40°C for 14 days (Schiff base formation followed by Amadori rearrangement) (Chan, Berry et al. 1997). Any remaining Schiff base is reduced by $NaBH_3CN$. The ATH product is purified by hydrophobic chromatography on butyl agarose to remove unbound heparin, and subsequently by anion exchange chromatography on DEAE Sepharose to remove unbound AT. The resulting ATH complex possesses a stable keto-amine link between antithrombin lysyl □-amino groups and heparin aldehyde groups. ATH product yields under these conditions have been found to be consistently of the order of 50%, the highest among the various known preparation methods (Berry, Chan et al. 1998). The work reported in this thesis used the Chan et al. ATH preparation, and all subsequent discussion of ATH will refer to this preparation.

ATH has unique structural properties that contribute to its anticoagulant activity. The molar ratio of AT to heparin is typically very close to 1:1, and, inherent to the synthesis process, AT selects for heparin of larger average molecular weight than the starting heparin (18 kDa vs. 15 kDa) (Chan, Berry et al. 1997). During preparation, ATH also selects for heparin that has at least one pentasaccharide sequence (in some cases more than one) (Chan, Berry et al. 1997; Berry, Stafford et al. 1998). This is a significant advantage over uncomplexed, unfractionated heparin where only about one third of molecules contain the active sequence. As discussed above, AT is present in plasma in two different isoforms, varying in plasma concentration and binding affinity. The isoforms also vary in carbohydrate content and β-AT has a higher heparin affinity and inhibits thrombin more rapidly than α-AT (Peterson and Blackburn 1985). It was found that in ATH, the content of β-AT is greater than in the starting AT since heparin selects for this isoform during synthesis (Chan, Berry et al. 2003). This provides ATH with an AT component that is a more effective thrombin inhibitor.

As a systemic anticoagulant ATH has shown numerous advantages in various clinical applications. Initial work demonstrated its high anti-factor Xa activity and anti-thrombin activity compared to simple AT-heparin mixtures (Chan, Berry et al. 1997). The rate of reaction of ATH with thrombin was rapid and its intravenous half life was found to be greater than that of heparin. The permanently activated state of AT in the complex and its large size allowed ATH to be retained in the lung suggesting potential for prevention of thrombin-mediated fibrin deposition and for treatment for respiratory distress syndrome (Chan, Berry et al. 1997; Chan, Berry et al. 1998). ATH has been tested in both

venous and arterial thrombosis models. In a rabbit jugular vein model, ATH showed lower clot weight and fibrin accretion than heparin along with superior anticoagulant activity (Chan, Berry et al. 1998). In addition, the cumulative blood loss to anti-factor Xa activity ratio was higher for antithrombin-heparin mixtures than for ATH. In a rabbit arterial model, ATH showed a greater antithrombotic effect and lower blood loss than standard heparin (Chan, Rak et al. 2002). Recently, in a study using a pig cardiopulmonary bypass model, the ability of ATH to reduce high-intensity transient signals (HITS) and microemboli formation was assessed (Klement, Berry et al. 2010). It was found that ATH reduced the number of HITS in a dose dependent manner. Thromboemboli formation was also decreased by ATH.

Studies have demonstrated many other advantages of ATH due to its structure and mechanism of action. ATH is able to inhibit thrombin and factor Xa directly through its activated AT component (Berry, Stafford et al. 1998). The heparin moiety in ATH also provides catalytic inhibition of coagulation factors since exogenous AT can compete for the heparin pentasaccharide sequence (Paredes, Wang et al. 2003). The relative contributions of the direct and catalytic mechanisms of ATH anticoagulant activity have been studied using thromboelastography (TEG) (Atkinson, Mewhort-Buist et al. 2009). At high concentrations, ATH anticoagulant activity was found to be primarily through the AT-related direct mechanism and at low concentrations, through the catalytic mechanism. The ATH complex is able to inhibit fibrin clot-bound thrombin at a high rate, unlike uncomplexed heparin which forms a ternary fibrin-thrombin-heparin complex protecting thrombin from inhibition (Berry, Becker et al. 2002). Compared to uncomplexed,

unfractionated heparin, ATH showed less plasma protein binding and less binding to endothelial cells likely due to "shielding" by the AT portion of the ATH (Chan, Paredes et al. 2004).

ATH clearly has potential as a modifier of surfaces to be used for blood contact applications; this is the focus of the work reported in this thesis.

1.2.2. Materials for Surface Modification

Polymers are used extensively as biomaterials for medical device applications due mainly to their mechanical properties. However, the study of blood-material interactions (required in material development) is often difficult on polymeric materials due to their complex nature and to difficulties in the application of advanced surface characterization techniques. Instead of polymers, model materials such as gold can be used as substrates to evaluate surface modification methods. Well-defined surfaces can be prepared that are suitable for detailed chemical and physical characterization and for studying chemical and biological interactions. Data from work on gold can be used in the development of analogous polymeric materials, for example polyurethanes. Polyurethanes are widely used as blood contacting biomaterials and various surface modification methods are possible. In this thesis initial work was carried out using gold as a model substrate and the information generated was then applied to polyurethane.

1.2.2.1. Gold as a Model Substrate

Gold is an inert, stable and chemically simple material that can be used to create well-defined, characterizable surfaces. Under mild conditions, gold reacts strongly with sulfur containing moieties such as thiols or disulfides (Nuzzo and Allara 1983; Nuzzo, Zegarski et al. 1987). Chemisorption results in a surface with bound thiolate species (Ulman 1996):

$$R\text{-}S\text{-}H + Au^{o}_{n} \rightarrow R\text{-}S^{-}\ Au^{+} \cdot Au^{o}_{n} + \tfrac{1}{2}\ H_2$$

$$RS\text{-}SR + Au^{o}_{n} \rightarrow RS^{-}\ Au^{+} \cdot Au^{o}_{n}$$

Chemisorption to gold is carried out typically at room temperature in various solvents, over short periods of time. The procedures are thus relatively simple and rapid. Although gold is highly susceptible to surface contamination in laboratory air, it has been found that carbonaceous contaminants adsorbed to gold are readily displaced by chemisorbing thiols and disulfides (Troughton, Bain et al. 1988). Since the gold-thiol bond has been shown to oxidize over time resulting in desorption, materials must be used in a timely manner (Cerruti, Fissolo et al. 2008).

Self-assembled monolayers (SAMs) are formed on gold by sorption of long-chain alkanethiols. These are effectively structured organic layers. The chemistry, organization and structure of SAMs have been studied extensively. Binding to crystalline gold in the (111) orientation is thought to occur in the three fold hollows between gold atoms resulting in closely packed structures with sulfur atoms separated by a distance of 4.97 Å (Strong and Whitesides 1988; Whitesides and Laibinis 1990). This corresponds to a packing density of 21.4 $Å^2$ per thiolate and a maximum theoretical surface density of ~4.7

molecules per nm^2. The alkyl chains in close packed SAMs are in an ordered (stretched) conformation and are tilted approximately 30° to the surface normal, as shown in Figure 1.7 (Bain, Troughton et al. 1989). These well-ordered structures allow the study of various interfacial interactions.

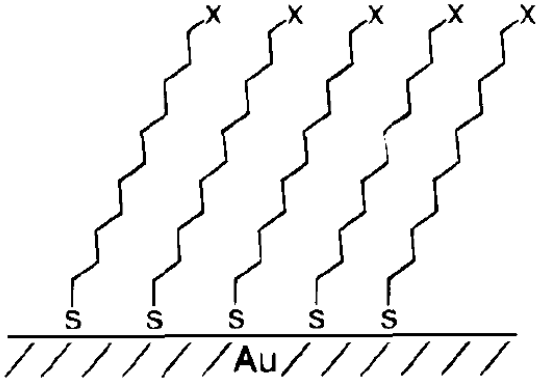

Figure 1.7. Model of an alkanethiolate SAM on gold with the alkyl chains tilted approximately 30° to the surface normal (Mrksich and Whitesides 1996).

SAMs are excellent model systems for the study of protein adsorption (Prime and Whitesides 1991). The properties of SAMs can be easily controlled by varying the terminal end group (X) to introduce a range of functional groups on the surface (Figure 1.7). Creation of mixed SAMs with two or more alkanethiols provides further controlled variable surface chemistry (Prime and Whitesides 1991). Lestelius et al. created SAMs of alkanethiolates on gold with five different end group functionalites, methyl, trifluromethyl ester, sulfate, carboxyl and hydroxyl, and investigated their effect on plasma protein adsorption (Lestelius, Liedberg et al. 1997). Due to the electrical conductivity and reflective properties of gold, a wide range of analytical techniques can

be used to study gold-based SAMs (Mrksich and Whitesides 1996; Ferretti, Paynter et al. 2000). This allows detailed characterization using state of the art methods.

SAMs based on short chain polyethylene oxide/glycol containing alkanethiols have been used to study the protein resistant nature of polyethylene oxide (PEO) (Prime and Whitesides 1993; Wang, Kreuzer et al. 1997; Benesch, Svedhem et al. 2001) (see section 1.2.3.1.1 for a detailed discussion).

Other thiol containing molecules have been attached to gold, including dithiobis(succinimidyl propionate) (DSP), a protein cross-linking reagent. DSP is chemisorbed to gold through its disulfide bond. Biomolecules can then be attached to the surface through the "distal" N-hydroxysuccinimide (NHS) ester end groups (Lomant and Fairbanks 1976; Katz and Solovev 1990). This technique has been used to attach various biomolecules and then to study protein interactions with the resulting surfaces (Storri, Santoni et al. 1998; Schmid, Stanca et al. 2006). Both PEO and DSP have been immobilized on model gold substrates in the research presented in this thesis.

1.2.2.2. Polyurethanes

Many blood contacting devices are made using polyurethanes due to the versatile physical and mechanical properties of these materials along with their "relatively good" biocompatibility compared to other polymers. Polyurethanes can be formulated with high tensile strength, flexibility, lubricity and abrasion resistance (Lamba, Woodhouse et al. 1998). Segmented polyurethanes, the most commonly used type, are block copolymers composed of alternating hard and soft segments. Rgid hard segment microdomains are

formed and are dispersed within a flexible soft segment matrix, thus generating a two phase microstructure (Bonart 1979). The thermodynamic incompatibility of the hard and soft segments provides the driving force for microphase separation (Lelah and Cooper 1986).

The ability to vary both the hard and soft segment types and their relative contents in the chains permits the synthesis of polymers with a range of different structures and properties (Zdrahala and Zdrahala 1999). Soft segments are usually polyesters, polyethers, polycarbonates or polydimethylsiloxanes of moderate molecular weight. The hard segment generally consists of an aromatic or aliphatic diisocyanate and a low molecular weight diol or diamine chain extender. The synthesis of polyurethanes relies on isocyanate chemistry and is usually carried out by a two step reaction process (Figure 1.8) (Lelah and Cooper 1986). In the first step, the diisocyanate and hydroxyl terminated polyether (or other soft segment component) react to form the prepolymer. The prepolymer is further reacted with the chain extender (diol or diamine) to produce the final block copolymer, a polyurethane (diol extender) or polyurethaneurea (diamine extender).

Polyurethanes are available from many different manufacturers under various trade names (Szycher 1999). The polyurethane work presented in this thesis used Tecothane®, a medical grade polyurethane from Thermedics® Polymer Products, as the base polymer. Tecothane is based on poly(tetramethylene)oxide (PTMO), methylene-bis(phenyl-diisocyante) (MDI) and butanediol.

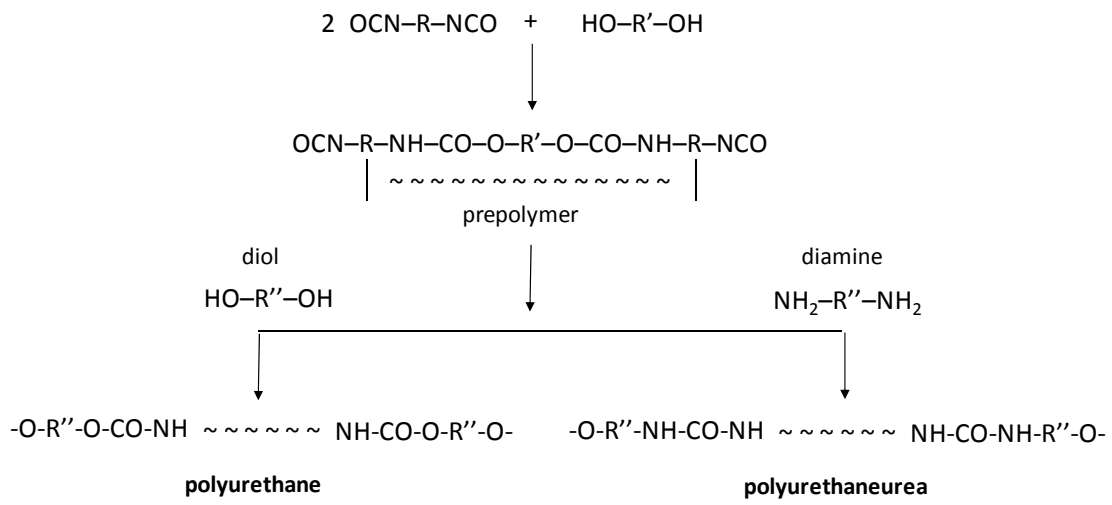

Figure 1.8. Prepolymer method of polyurethane synthesis. First step: diisocyanate and polyol form the prepolymer. Second step: Chain extension of prepolymer by diol or diamine.

Despite their versatility and extensive use, polyurethanes have limitations when used in medical devices. Like most materials, polyurethanes adsorb proteins and induce various biological responses when in contact with blood or tissue in both short and long term applications. Some polyurethanes have shown evidence of biodegradation during long term use, typically via hydrolytic, oxidative and enzymatic mechanisms (Griesser 1991; Santerre, Woodhouse et al. 2005). To improve their properties as biomaterials, polyurethanes can be "tailored" by bulk or surface modification methods. Bulk modification can be accomplished by variation of the soft segment, diisocyanate and chain extender used for synthesis. For example, sulfonated polyurethanes with improved biocompatibility, were prepared using a sulfonated diisocyanate (Santerre and Brash 1991; Silver, Hart et al. 1992; Skarja and Brash 1997) or a sulfonated soft segment (Silver, Hart et al. 1992). Polyurethanes have also been designed using a polycarbonate

soft segment with a view to improving their biostability (Pinchuk 1994; Stokes, McVenes et al. 1995). Surface modification can be achieved by blending with small or intermediate molecular weight materials that migrate to the solid aqueous interface (e.g. in contact with blood) (Freij-Larsson, Kober et al. 1993; Tang, Santerre et al. 1997; Tan and Brash 2008). Surface modification rather than bulk modification is often used to improve interfacial interactions with blood, tissue and other biological systems.

1.2.3. Surface Modification of Biomaterials

Since the surface properties of a biomaterial dictate biological interactions following blood (or other biosystem) contact, surface modification can be used to alter the material's response; at the same time the bulk properties are maintained. Surface modification can involve physical, chemical and biological methods. Various examples of physical and chemical modifications include physical coatings, chemical grafting, blending, etching and roughening, and gas plasma treatment (Hoffman 1987; Ikada 1994). Biological techniques may also involve physicochemical surface "priming" preliminary to incorporation of bioactive components. To provide protein and cell resistant properties, bioinert or anti-biofouling modification methods are required. In this work, bioinert and bioactive modifications were used in combination.

1.2.3.1. Modification for Bioinertness (Bioinert Surface)

Modification of biomaterials can be designed to create bioinert surfaces, i.e surfaces that eliminate or reduce protein adsorption and cell adhesion. This has been explored using various bioinert, usually hydrophilic polymers. The most extensively studied polymer in this connection is polyethylene oxide (PEO), also referred to as polyethylene glycol (PEG). Other hydrophilic polymers include dextran and tetraethylene glycol dimethyl ether (tetraglyme), the latter being PEO-like. The attachment of dextran to surfaces in particular conformations gave significant decreases in protein adsorption (Osterberg, Bergstrom et al. 1995; Martwiset, Koh et al. 2006). Various tetraglyme modified surfaces have shown reduced plasma protein adsorption, platelet adhesion, endothelial cell attachment and monocyte adhesion (Lopez, Ratner et al. 1992; Shen, Pan et al. 2001; Cao, Chang et al. 2007). Phosphorylcholine-containing polymers such as poly(2-methacryloyloxyethyl phosphorylcholine) (poly-MPC) have shown good protein resistance when attached to polymer materials (Ishihara, Tsuji et al. 1994; Feng, Zhu et al. 2005; Jin, Feng et al. 2010). Iwasaki and Ishihara (2005) have suggested that this behavior may be due to the resemblance of these surfaces to lipid bilayer (biomembrane) surfaces. Other zwitterionic polymers such as poly(sulfobetaine) and poly(carboxybetaine) have shown very low protein adsorption (Zhang, Chao et al. 2006; Zhang, Chen et al. 2006; Zhang, Chen et al. 2006; Zhang, Zhang et al. 2008). Jiang et al have proposed that these materials are protein resistant due to their zwitterionic nature with the positive and negative charges in exact balance (Chen, Yu et al. 2006).

1.2.3.1.1. Polyethylene Oxide (PEO)

Polyethylene oxide (PEO) is a non-ionic polymer that has widespread use in many different applications. The terms PEO and polyethylene glycol (PEG) are often used interchangeably although PEG usually refers to lower molecular weight species. In this thesis, the term PEO is used exclusively. As a surface modifier PEO is well known for its ability to reduce protein adsorption. The unique characteristics of PEO and its mechanism of resistance to protein adsorption are now discussed. A review of various PEO surface modification methods is also provided.

PEO is generally soluble in water and in some organic solvents. Its unique water solubility compared to other similar polyethers is thought to result from its good structural "fit" with water (Kjellander and Florin 1981). PEO chains are flexible and in an aqueous environment are well hydrated and highly mobile. As with many polymers, an excluded volume effect is experienced in PEO-protein systems (Hermans 1982). The free energy at an immobilized PEO interface is low, thus reducing the tendency for proteins to adsorb. However, this effect alone does not account for the unique behaviour of PEO since other polymers that exhibit low interfacial free energy do not reduce protein adsorption as effectively (Lee, Lee et al. 1995). It is likely that a combination of factors contributes to PEO's well known protein and cell resistant properties.

Two main "theories" or mechanisms have been advanced to explain the protein resistance of PEO (Morra 2000). These are the so-called "steric repulsion/exclusion" mechanism, and more recently, the "structured water barrier" mechanism. In relation to steric repulsion the surface density of PEO is important, particularly in surface grafted

materials (Figure 1.9). The "mushroom" regime occurs when the surface density is relatively low so that the chains are isolated and in the random coil conformation similar to PEO in solution. In contrast, the "brush" regime occurs as the coverage of polymer increases and the chains avoid overlapping by stretching away from the surface (Milner 1991). Specific factors have been investigated in this context including polymer chain length (molecular weight), graft density, conformation and protein properties.

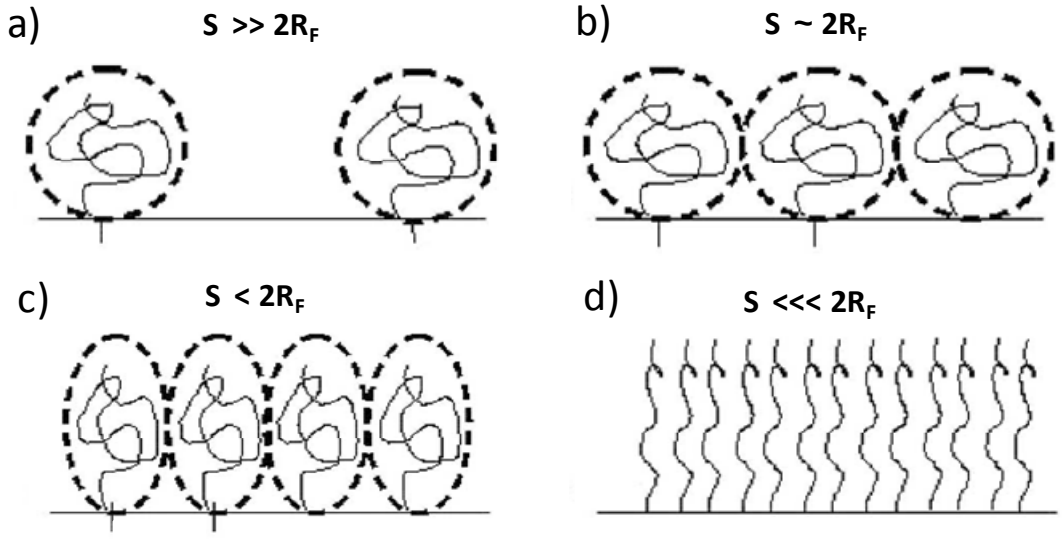

Figure 1.9. "Regimes" of surfaces based on tethered polymer chains: (a) nonoverlapping "mushroom" regime; (b) slightly overlapping "mushroom" regime; (c) dilute "brush" regime; (d) dense "brush" regime. S = distance between graft points. R_F = Flory radius Adapted from: (Unsworth, Tun et al. 2005). (With permission).

The steric repulsion mechanism was discussed in early theoretical studies by Jeon et al. Flexible PEO chains were assumed to be in the brush regime and terminally attached to a hydrophobic surface in water and proteins were assumed to be homogeneous and of infinite size (Jeon, Lee et al. 1991). They hypothesized that as a protein approaches the surface, the PEO chains are compressed with the generation of a repulsive interaction

from osmotic and elastic forces which overcomes the attractive van der Waals forces between the substrate and the protein. This model was extended in studies where the protein was assumed to have a spherical shape of finite size (Jeon and Andrade 1991). It was found that an optimum surface density of PEO is needed for protein resistance and that resistance increased indefinitely with PEO chain length. An explanation of steric repulsion has also been given in terms of a decrease in conformational entropy and increase in osmotic pressure as the PEO layer is compressed by the approaching protein (McPherson, Lee et al. 1995). Important shortcomings in the steric exclusion theory are the neglect of water interactions and its requirement for significant chain length of PEO.

An improved model was developed by Szleifer using single-chain mean-field (SCMF) theory. Various molecular interactions were taken into account including forces between the surface, the protein and the PEO (Szleifer 1997; Szleifer 1997). The grafted layer was assumed to be inhomogeneous such that the configurations of polymer chains transitioning from the mushroom to the brush regime were considered based on thermodynamic variables of the system. This theory is in better agreement with various experimental observations. For example, the use of self-assembled monolayers (SAMs) of PEO in work by Prime and Whitesides demonstrated protein resistance with short chain PEO of only a few units (Prime and Whitesides 1991; Prime and Whitesides 1993). The original steric repulsion model does not account for these observations (compression of a chain with only a few units is not significant). However, Szleifer's results using SCMF theory are in good agreement with the observations for short chains as well as for medium and long chains. This analysis was extended by Halperin by considering interaction

potentials along with brush layer thickness, graft density and protein size (Halperin 1999). These physically-based approaches still consider only forces resulting from compression and neglect water-PEO interactions.

The properties of these systems have more recently been considered taking into account hydration forces and chain conformation. The interaction of a surface with water results in both attractive and repulsive forces where hydrogen bonding in the hydration layer plays an important role (Besseling 1997). Grunze et al. have used SAMs of oligo(ethylene oxide) (OEO) to study the role of the interfacial water layer and molecular conformation on protein adsorption (Wang, Kreuzer et al. 1997; Harder, Grunze et al. 1998; Feldman, Hahner et al. 1999; Pertsin and Grunze 2000; Rosenhahn, Schilp et al. 2010). Protein adsorption was found to be lower on gold surfaces where the PEO chains were in a helical conformation than on silver surfaces where the chains were in the trans conformation. These results demonstrate the importance of molecular conformation and the stability of the interfacial water layer (Wang, Kreuzer et al. 1997; Harder, Grunze et al. 1998). The roles of electrostatic forces, hydration forces, hydrogen bonding and water penetration in the protein resistance of OEO have also been emphasized (Feldman, Hahner et al. 1999; Pertsin and Grunze 2000). The PEO-water layer has been shown to be highly organized (Heuberger, Drobek et al. 2004). The forces stemming from structured water around the PEO including hydrogen bonding provide a tightly bound water layer which is believed to provide an energy barrier to protein adsorption.

Modifications of various substrates with PEO have been carried out using a range of different strategies. Physical attachment of PEO has been performed by adsorption of

PEO-containing block copolymers on low density polyethylene (LDPE) and glass (Lee, Kopecek et al. 1989; Amiji and Park 1992). Materials based on blends of PEO-containing block copolymers with polyurethane, polyethylene terephthalate (PET) and polymethylmethacrylate (PMMA) have also been developed (Desai and Hubbell 1991). Covalent coupling (chemical grafting) of PEO to surfaces, resulting in more permanent attachment, has also been explored. PEO has been grafted to PET (Gombotz, Guanghui et al. 1991), polystyrene (Bergstrom, Osterberg et al. 1994) and poly(dimethylsiloxane) (PDMS) (Chen, Zhang et al. 2005); in all of these cases protein adsorption was reduced on the PEO modified surfaces compared to controls. Gombotz et al. attached PEO of different molecular weights (MWs) to PET and found that the adsorption of fibrinogen and albumin decreased with increasing MW up to a value of 3500 above which there was no further effect (Gombotz, Guanghui et al. 1991). Silicon surfaces were also grafted with PEO and showed decreasing protein adsorption with increasing grafting density (Malmsten, Emoto et al. 1998; Sofia, Premnath et al. 1998). As discussed previously, gold reacts with thiols and this chemistry can be used to attach PEO. Unsworth et al. chemisorbed chain-end thiolated PEO onto gold and demonstrated that the chain length, chain density and chain end group chemistry all influence protein adsorption (Unsworth, Sheardown et al. 2005; Unsworth, Tun et al. 2005).

Polyurethane surfaces have been modified with PEO to render them protein resistant. Block copolymers containing PEO segments were adsorbed to and blended with polyurethane to reduce protein adsorption (Freij-Larsson, Jannasch et al. 2000; Tan, McClung et al. 2008). In other work, PEO-containing block copolymers were blended

with polyurethane and then crosslinked to anchor them in the polyurethane matrix (Lee, Ju et al. 2000). Fujimoto et al. grafted PEO on polyurethane using a plasma technique and showed decreased protein and platelet adhesion on the modified surfaces (Fujimoto, Inoue et al. 1993). Many groups have grafted PEO to polyurethane surface by first reacting the surface with a diisocyanate, providing free isocyanate groups to react with a hydroxyl- or amine-terminated PEO (Han, Park et al. 1989; Freij-Larsson and Wesslen 1993; Wesslen, Kober et al. 1994; Kim, Han et al. 2003; Archambault and Brash 2004; Chen, Hu et al. 2008). Archambault and Brash modified polyurethane with PEO of varying MW and showed that protein resistance increased with increasing chain length, the effect levelling around PEO MW 2000 (Archambault and Brash 2004).

As well as being anti-fouling, PEO can act as a "spacer" for the attachment of biomolecules to surfaces. The direct attachment of a bioactive molecule to a surface can result in loss of activity and steric limitations on the access of target molecules in the biological medium. The interposition of a spacer such as a PEO chain positions the biomolecule away from the surface, thereby providing it with better mobility and enhancing its activity (Park, Okano et al. 1988; Goddard and Hotchkiss 2007). In addition, PEO can be synthesized with different chain end functional groups (Zalipsky 1995) which can be used for the attachment of the biomolecule to the surface. Du and Brash (2003) used thiol-terminated PEOs for chemisorption to gold surfaces (Du and Brash 2003). Hetero-bifunctional PEOs having two different groups on the chain ends, one end to bind selectively to the surface the other to react with the biomolecule, are useful in this regard (Zalipsky 1995; Won 2004). Examples of PEO-biomolecule surface

modification (e.g. PEO-heparin) will be presented in the section on heparinization (section 1.2.3.2.1.).

1.2.3.2. Modification for Anticoagulant Effect (Bioactive Surface)

The use of bioactive species for surface modification has been explored as a means to improve biomaterial interactions. Bioactive modification can involve the immobilization of molecules such as drugs, enzymes, antibodies and peptides. When introducing biological functionality, bioactive species may be attached by either non-covalent or covalent linkages.

Relevant to this work, a variety of approaches have been used to modify surfaces with bioactive moieties to provide anticoagulant function. The direct thrombin inhibitor hirudin has been attached to a number of substrates (Seifert, Romaniuk et al. 1997; Phaneuf, Szycher et al. 1998; Lahann, Klee et al. 2001; Alibeik, Zhu et al. 2010). Peptides that directly inhibit thrombin have also been immobilized to prevent surface-induced thrombosis (Sun, Sheardown et al. 2000). Attachment of heparin to biomaterials (surface heparinization) has been investigated extensively. In many cases bioactive modification is combined with modification for bioinertness, for example by conjugating bioactive agents to PEO, as discussed above.

1.2.3.2.1. Heparinization

The first investigation of immobilized heparin was performed by Gott et al. in 1963. Heparin was attached to a colloidal graphite surface through an intermediate

cationic surfactant benzalkonium chloride which interacted electrostatically with the sulfonate groups of heparin. This so-called GBH surface was shown to prolong clot formation in vitro (Gott, Whiffen et al. 1963). The technique was applied to various polymers and metals many of which showed decreased thrombogenicity (Gott, Whiffen et al. 1964; Leininger, Cooper et al. 1966). However, the ionically bonded heparin was subject to exchange with anions in the blood resulting in rapid loss of the heparin. In addition it was not clear that the active sequence of heparin was accessible for interaction with AT. Improvements to these initial approaches were attempted by covalently attaching heparin to surfaces (Hoffman, Schmer et al. 1972; Larm, Larsson et al. 1983; Olsson, Arnander et al. 1983). In particular, so-called "end-point" heparin attachment has proved to be a relatively effective method (Larm, Larsson et al. 1983; Larsson, Larm et al. 1987). This technique allows the active pentasaccharide sequence to extend away from the "immediate" surface, free to interact with antithrombin in the blood. Heparin immobilized by this method has been commercialized under the trade name Carmeda® BioActive Surface (CBAS®). The Carmeda surface has been used clinically for many years with application to devices such as extracorporeal circuits and blood oxygenators (Wendel and Ziemer 1999; Hussaini, Treanor et al. 2009). A number of other commercially available heparinized surfaces have also been compared in clinical studies (Noora, Lamy et al. 2003; van den Goor, van Oeveren et al. 2006; Zimmermann, Weber et al. 2007). These various heparin immobilization techniques have had some success, but mainly in short-term applications (Tanzi 2005).

Other methods of surface heparinization have been investigated at the research level. Michanetzis et al. investigated two methods of covalently binding heparin, one indirect method and one direct method, on four different polymers: silicone rubber, polyethylene, polypropylene and polyvinylchloride. The indirect method involved hydroxylation of the surface, introduction of free amino groups and immobilization of heparin using a carbodiimide coupling agent. The direct method used glutaraldehyde as a coupling agent to immobilize heparin. For both methods it was found that the heparin retained its biological functionality and both gave surfaces with decreased activation of coagulation (Michanetzis 2003). A number of studies have explored strategies for immobilizing heparin on polyurethanes (Ito 1987; Eloy, Belleville et al. 1988). In work by Barbucci et al. a polyurethane was crosslinked with poly(amido-amine) allowing attachment of heparin by electrostatic interactions. It was shown that, in contrast to control surfaces, the heparinized materials did not change the conformation of adsorbed plasma proteins (Barbucci and Magnani 1994). In work by Aksoy et al. heparin fractions of different molecular weight were covalently immobilized on polyurethane using gas plasma treatment and carbodiimide chemistry (Aksoy, Hasirci et al. 2008). Compared to controls the heparinized materials showed decreased protein adsorption and platelet adhesion at longer incubation times, but the effects varied with heparin molecular weight. Heparin was also attached to polyurethane through various spacer chains and the resulting surfaces showed decreased platelet interactions (Liu, Ito et al. 1991).

PEO has been used as a spacer to attach heparin to several polyurethane surfaces; the PEO serves a “bioinertness” as well as a spacer function. Park et al. modified

polyurethane with PEO using isocyanate chemistry and attached heparin to the PEO. They found that the PEO reduced protein adsorption and platelet adhesion and enhanced the activity of the heparin and that the effects were dependent on PEO chain length (Park, Okano et al. 1988). Bae et al attached heparin covalently to polyurethane following oxygen plasma treatment (Bae, Seo et al. 1999). They showed that plasma recalcification times (PRT) were prolonged on the heparinized surfaces (Kang, Seo et al. 2001). Platelet adhesion on polyurethane-PEO surfaces was similar to that on controls, but decreased on the surfaces that were additionally modified by heparin immobilization. Polyurethane containing side chain ester groups was modified by hydrolysis to give carboxylic acid groups. It was then treated with PEO followed by heparin (Kang, Baek et al. 1998; Wan, Baek et al. 2004). The PRT was found to be prolonged on the PEO-heparin surfaces, and platelet adhesion was reduced compared to controls (Wan, Baek et al. 2004). Byun et al. compared the mechanism of thrombin inhibition on surfaces where heparin was attached directly versus through a PEO spacer and found that with the spacer, the heparin was able to bind AT more effectively (Byun, Jacobs et al. 1996).

As mentioned previously, heparin has a number of limitations as an anticoagulant. Heparinized materials are correspondingly disadvantaged. The use of unfractionated heparin for surface modification implies that only a fraction of the heparin on the surface will have anticoagulant functionality since only about one third of the molecules contain the active pentasaccharide sequence. Since heparin, and in particular its inactive form, binds to many proteins and cells, these may accumulate on the surface in blood contact leading to variable efficacy and the potential promotion of procoagulant activity

(Rosenberg and Lam 1977; Cornelius, Sanchez et al. 2003). In general, both research and clinical studies have shown mixed results and it is evident that improved methods of inhibiting thrombosis on devices are needed.

1.2.3.2.2. Surface Modification with ATH

As discussed above ATH has been shown to have many advantages as a systemic anticoagulant, particularly compared to heparin alone. It also has great potential as an immobilized bioactive agent on biomaterials. The ability of ATH to provide both direct and catalytic anticoagulant activity, along with its high active pentasaccharide content, creates the potential for biomaterials with enhanced antithrombogenic properties. Structurally, ATH offers (1) more possibilities for surface attachment than heparin, (2) the potential for multiple covalent bonds to the surface through AT, and (3) since heparin is covalently bound to AT, hindrance to non-specific protein adsorption (Berry, Andrew et al. 2000; Berry and Chan 2008).

Initial work on surface modification with ATH involved direct attachment (no spacer) by covalent grafting; the substrate was a polycarbonate-urethane endoluminal graft (Klement, Du et al. 2002). Surfaces were modified with heparin and hirudin using the same method with graft densities six fold lower (heparin) and twenty-fold lower (hirudin) than that of ATH. The ATH surface was found to be stable and provided significant direct thrombin inhibitory activity. It also bound greater amounts of AT than the heparin surface indicating catalytic activity. In addition, *in vivo* experiments revealed

less clot formation in ATH-coated grafts than in control and hirudin-coated grafts (Klement, Du et al. 2002).

Surfaces were also prepared on which ATH was covalently linked to a polymer base coat that was applied to polyurethane catheters (Du, Klement et al. 2005; Klement, Du et al. 2006; Du, Brash et al. 2007; Klement, Berry et al. 2010; Du, Berry et al. 2011). The ATH in these materials was first linked to PEO. In an *in vivo* acute rabbit model, the ATH-modified catheters were shown to delay clotting compared to control and commercial heparinized catheters and were stable to sterilization, abrasion and protease attack (Du, Klement et al. 2005). The ATH-modified catheters also showed superiority in a chronic rabbit model where they remained patent over 106 days compared to the unmodified and heparin-modified catheters which occluded in 5-7 days (Klement, Du et al. 2006).

More recently, protein adsorption experiments were carried out on these ATH-modified catheters (Du, Brash et al. 2007). It was found that compared to commercial heparinized surfaces, ATH-modified surfaces bound greater quantities of antithrombin from plasma and smaller quantities of procoagulant proteins such as fibrinogen (Du, Brash et al. 2007).

Detailed chemical and physical characterization of the ATH coating were also carried out (Du, Berry et al. 2011). The modification reactions were verified, and the PEO-ATH was found to be strongly bound with a high graft density and high anticoagulant activity (Du, Berry et al. 2011).

In the work reported in this thesis ATH was attached to gold as a model substrate and on polyurethane as a more "practical" substrate using various conjugation procedures, with and without PEO. Analogous heparinized surfaces were prepared for comparison. The work provides new information on surface modification methods and on the interactions of blood with the heparin- and ATH-modified surfaces.

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CHAPTER 2: OBJECTIVES & CONTRIBUTIONS TO ARTICLES

2.1. OBJECTIVES

It is evident from the literature reviewed in Chapter 1 that many different methods have been explored to improve biomaterials by surface modification. Despite these efforts the biocompatibility problem has not been solved and, in particular, there remains a need for materials that do not provoke coagulation and thrombosis in contact with blood. In this regard, novel anticoagulants with advantages over standard heparin may have superior properties as surface modifiers. The combination of such bioactive modifiers with bioinert/non-fouling spacer molecules may provide further improvements.

The overall objective of this work was to investigate the use of an antithrombin-heparin covalent complex (ATH) for the surface modification of biomaterials to improve antithrombogenicity. There are two main parts to this research, (1) the immobilization of ATH on model gold substrates by various methods to better understand the mechanisms of interaction of blood components with "interfacial" ATH, and (2) the modification of polyurethane films by attachment of ATH for potential application in blood contacting medical devices.

In initial work with gold as the substrate, additional goals were (1) to create heparinized surfaces analogous to the ATH-modified surfaces, and (2) to determine conditions for maximal anticoagulant activity using ATH modification. Both ATH and heparin were attached to gold in three ways: directly, through a short linker molecule and through polyethylene oxide (PEO) as a linker/spacer that would provide resistance to non-specific protein adsorption. This approach allowed a direct comparison between ATH and

heparin as well as an assessment of PEO as an antifouling spacer. With gold as the substrate, characterization techniques including ellipsometry, quartz crystal microbalance (QCM) and grazing incidence reflection Fourier transform infrared spectroscopy (GIR-FTIR) were used to obtain graft density and functional group information. Additional information on the individual steps in the surface modification process was obtained from water contact angles and x-ray photoelectron spectroscopy (XPS). Protein interactions were studied by radioiodination and Western blotting analysis. Preliminary data on platelet adhesion and plasma clotting times were also obtained.

Based on the promising results from model gold studies, techniques were developed for the attachment of ATH to polyurethane surfaces. Polyurethane films were chemically activated to attach ATH directly and through PEO spacers. The molecular weight and functional end group of PEO was varied with the goal of developing an optimal surface that would balance the anticoagulant effect of ATH and the resistance to non-specific protein adsorption of PEO. Surface modifications were monitored by water contact angles, low and high resolution XPS and protein adsorption. Platelet adhesion and anticoagulant activity (both catalytic and direct) were measured on the ATH-modified surfaces. It is expected that surface modification using these methods could be used in the fabrication of blood contacting devices.

2.2. CONTRIBUTIONS TO ARTICLES

The research work reported in this thesis is presented as four journal articles in Chapters 3-6. I was the main contributing author to all of these. Professors John L. Brash and Anthony K.C. Chan provided the research focus and initial ideas for the direction of the work and I continued the development of these ideas. I performed all literature searches, experimental work and data analysis. I prepared the first draft of all papers and incorporated revisions from my supervisors and co-authors. I generated the initial responses to reviewers' comments and made required changes. Leslie R. Berry provided assistance with research ideas and paper revisions, particularly in regard to the antithrombin-heparin (ATH) complex. In Paper 1, Dr. Igor Zhitomirsky and Feng Sun assisted with quartz crystal microbalance (QCM) studies. In Paper 2, W. Glenn McClung assisted with the platelet experiments. In Papers 1 and 3, Dr. Rana Sodhi from Surface Interface Ontario at the University of Toronto provided assistance with x-ray photoelectron spectroscopy (XPS).

CHAPTER 3: PAPER ONE - Surface Modification with an Antithrombin-Heparin Complex for Anticoagulation: Studies on a Model Surface with Gold as Substrate

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Objectives:

The aim of this work was to immobilize ATH on model gold substrates using various modification techniques and compare these to analogous heparin modified surfaces. Surface characterization was used to confirm the modification steps. Protein interactions were studied with radiolabelling and western blotting analysis and provided information on the anticoagulant potential of ATH for biomaterial applications.

Main Contributions:

1. Using gold-thiol interactions, ATH was attached to the surface directly, with a small chain linker molecule and with polyethylene oxide. Analogous heparinized gold surfaces were prepared for comparison.

2. Surface modifications were confirmed and results suggested that when immobilized the heparin moiety of ATH is directed away from the surface in an optimal configuration for interaction with blood.

3. ATH-immobilized surfaces were found to bind significantly greater levels of antithrombin than heparinized surfaces from both buffer and plasma, demonstrating their enhanced anticoagulant potential.

Surface modification with an antithrombin-heparin complex for anticoagulation: Studies on a model surface with gold as substrate

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Abstract

Gold was used as a substrate for immobilization of an antithrombin-heparin (ATH) covalent complex to investigate ATH as a surface modifier to prevent blood coagulation. Three different surface modification methods were used to attach ATH to gold: (i) by direct chemisorption, (ii) using dithiobis(succinimidyl propionate) (DSP) as a linker molecule and (iii) using polyethylene oxide (PEO) as a linker/spacer. The ATH-modified surfaces were compared to analogous heparinized surfaces. Water contact angles and XPS confirmed the modifications and provided data on surface properties and possible orientation. Ellipsometry measurements showed that surface coverage of DSP and PEO was high. ATH and heparin densities were quantified using radioiodination and QCM, respectively. The surface density of ATH was greatest on the DSP surface (0.17 $\mu g/cm^2$) and lowest on the PEO (0.05 $\mu g\ cm^{-2}$). Low uptake on the PEO surface was likely due to the protein resistance of the PEO component. Using radioiodinated antithrombin (AT) it was shown that ATH-immobilized surfaces bound significantly greater amounts from both buffer and plasma than the analogous heparinized surfaces. Immunoblot analysis of proteins adsorbed from plasma demonstrated that surfaces chemisorbed with PEO, whether or not subsequently modified with ATH, inhibited non-specific adsorption. The immunoblot response for AT was stronger on the DSP-ATH than on the heparin surfaces, thus confirming the results from radiolabelling. The ATH surfaces again showed higher selectivity for AT binding than analogous heparin-modified surfaces, indicating the enhanced anticoagulant potential of ATH for biomaterial surface modification.

1. Introduction

Coagulation and thrombosis remains a major problem in the design of blood contacting medical devices despite many years of intensive research. A significant factor is the complex protein layer that rapidly forms on the material [1]. This layer initiates coagulation and other adverse reactions, ultimately leading to thrombosis. Efforts have therefore been directed to develop materials that either prevent non-specific protein adsorption [2, 3] and/or enhance the binding of specific proteins that promote a desirable biological function [4, 5]. Surface modification with hydrophilic polymers such as polyethylene oxide (PEO) has proved successful in minimizing the adsorption of proteins and the adhesion of cells on surfaces in a range of applications [6]. The ability of PEO to inhibit non-specific protein adsorption is influenced by its high chain flexibility and factors including chain length, conformation and surface density [7]. The attachment of bioactive molecules is also an effective way to control blood-material interactions, for example by promoting the inhibition of thrombin. The anticoagulant heparin is widely used for this purpose due to its ability to accelerate the thrombin-antithrombin (AT) reaction [8]. Heparin is a glycosaminoglycan that binds AT through an active pentasaccharide sequence, catalyzing the inhibition of thrombin and factor Xa [9]. However, as a systemic anticoagulant and as a surface modifier, heparin has a number of limitations. A major disadvantage is that only about one third of "standard" heparin molecules contain the pentasaccharide sequence needed to activate AT, leading to low and variable anticoagulant activity [10]. Also, heparin binds other plasma proteins [11,12]

and when this occurs on a biomaterial surface, it can lead to variable efficacy of the bound heparin and may even lead to procoagulant activity.

As an alternative to heparin, a covalently linked complex of antithrombin and heparin (ATH) has been developed. ATH has shown increased anticoagulant activity [13], probably because, unlike heparin, all ATH molecules have at least one pentasaccharide sequence, with some containing two or more [10]. Rates of thrombin inhibition are also faster since the rate limiting step in which AT binds to heparin is no longer required. The potential of ATH for both indirect anticoagulant activity through the catalytic effect of heparin, and direct activity through permanently activated antithrombin [10], gives the possibility of a biomaterial surface with improved antithrombogenic properties. Structurally, ATH offers more alternatives for surface attachment, the possibility of stabilization through multiple attachments, and because heparin is permanently bound to AT, restricted access for the binding of other unwanted proteins [14].

Preliminary studies have demonstrated the potential of ATH as a surface coating. ATH attached to an activated polycarbonate-urethane vascular graft displayed both non-catalytic and catalytic activity for thrombin inhibition [15]. *In vivo* studies in a rabbit model showed that ATH-modified catheters were stable and remained patent longer than commercial heparinized catheters [16,17].

The objective of the present work was to better define the mechanism of action of surface-immobilized ATH by investigation of its interactions with blood. For this purpose gold was used as a substrate on which well defined, well characterized ATH-modified

surfaces could be prepared. Surfaces modified with ATH and PEO separately and in combination were studied, and analogous surfaces modified with heparin were investigated for comparison with ATH.

2.1. Materials

Unfractionated heparin (UFH, sodium salt) from porcine intestinal mucosa, Grade I-A (187 U mg^{-1}) was from Sigma-Aldrich (Oakville, ON). Human antithrombin was from Affinity Biologicals Inc. (Ancaster, ON). ATH was prepared according to methods described previously [13]. Briefly, UFH and AT were incubated in PBS for 14 days at a molar ratio of 200:1 at 40°C, pH 7.3 to undergo Schiff base formation/Amadori rearrangement. Subsequently, $NaBH_3CN$ was added and allowed to react for 5 h at 40°C to remove remaining Schiff base. ATH was purified by hydrophobic chromatography with butyl-agarose to remove unreacted heparin, followed by anion exchange chromatography with DEAE Sepharose to remove unreacted AT. Fibrinogen was from Enzyme Research Laboratories (South Bend, IN). Dithiobis(succinimidyl propionate) (DSP) was from Sigma-Aldrich. Anhydrous dimethyl sulfoxide (DriSolv) was from EMD Chemicals (Gibbstown, NJ). N-hydroxysuccinimide (NHS)-terminated PEO ester disulfide (MW 1100) was from Polypure (Oslo, Norway). Standard electrophoresis reagents were from BioRad (Richmond, CA).

2.2. Surface preparation and modification

Gold substrates consisted of silicon wafers 1000 μm thick, polished on both sides, coated with a chromium adhesion layer followed by a layer of gold (approximately 1000 Å) and diced into 0.5 x 0.5 cm pieces (Thin Film Technology, Buellton, CA). The gold coated silicon was cleaned by immersion in a boiling solution of Milli-Q water, hydrogen peroxide and ammonium hydroxide (5:1:1 v/v) for 5 min followed by rinsing 3 times in Milli-Q water. For direct attachment of ATH to gold, surfaces were incubated in the appropriate solution of ATH or heparin. DSP modification was achieved by incubating gold in 20 mM DSP in DMSO for 2 h at room temperature to introduce NHS groups for specific attachment of ATH. Surface modification with PEO was carried out in a 1 mM solution of PEO-NHS ester disulfide in ethanol (room temperature, 2 h). Following functionalization with DSP or PEO, the surfaces were rinsed with solvent, dipped in PBS and transferred to the appropriate solution for attachment of ATH via reaction with the amino groups of lysine residues. A range of concentrations and times were tested to determine the minimum quantity of ATH needed to achieve maximum surface coverage. The optimal concentration was found to be 0.1 mg ml^{-1} with overnight incubation to achieve saturation. Parallel modifications were performed with unfractionated heparin at a concentration of 1.0 mg ml^{-1} and overnight incubation. Prior to surface characterization, samples were rinsed and dried in a stream of nitrogen.

2.3. Surface characterization

2.3.1. Water contact angle

Water contact angle measurements were performed using the sessile drop method with a Ramé-Hart NRL 100-00 goniometer (Mountain Lakes, NJ). Both advancing and receding angles were determined. Measurements were taken on both sides of the gold-coated surfaces at multiple points.

2.3.2. X-ray photoelectron spectroscopy (XPS)

XPS was performed on a ThermoFisher K-Alpha instrument with a monochromatic Al source and spot size from 30 to 400 μm. Survey scans were taken from 0 to 1000 eV and binding energies were referenced to the Au peak at 84.0 eV for calibration. Surfaces were analyzed at take off angles of 20º and 90º using low resolution for C, O, N, S and Au. Thermo Avantage Data Analysis Software was used to analyze the data.

2.3.3. Ellipsometry

An Exacta 2000 self-nulling ellipsometer (Waterloo Digital Electronics, Waterloo, ON) with a helium neon laser, wavelength 6328 Å, angle of incidence 70^{o}, was used to measure the thickness of deposited films. Gold surface, cleaned as described, was used as the substrate and the Exacta 2000 Software was used to calculate its refractive index and extinction coefficient. The refractive index of organic layers is typically about 1.5, and this value was used for DSP. The indices for PEO and heparin layers were taken as 1.47 [18] and 1.45 [19] respectively.

2.3.4. Quartz crystal microbalance (QCM)

A QCA922 instrument (Princeton Applied Research, Oak Ridge, TN) was used along with WinEChem software for QCM measurements. AT cut quartz crystals of resonant frequency 9 MHz containing 0.2 cm^2 gold electrodes on each side with a 3000 Å thick sputtered layer of gold on a 100 Å foundation of titanium were used. The C_f proportionality constant used for the gold quartz crystals (Seiko EG&G, Matsudo, Japan) was 1.12 ng Hz^{-1} cm^2. The quartz crystal was used with a well type Teflon holder. The apparatus was modified to eliminate evaporation and pressure effects, and to allow the solution to be appropriately added to the well. Prior to use, the gold was cleaned by immersion in 1 M NaOH for 10 min, 1 M HCl for 5 min and concentrated HCl for 30 s, followed by multiple rinses with Milli-Q water [20].

2.4. Protein interactions

2.4.1. Protein radiolabelling

Antithrombin and ATH were labelled with either $Na^{125}I$ or $Na^{131}I$ using the iodogen method (Iodination Reagent, Pierce Biotechnology, Rockford, IL). The labelled proteins were dialyzed overnight using a Slide-A-Lyzer cassette (Pierce) with three changes of buffer to remove unbound radioactive iodide. Fibrinogen was labelled with $Na^{125}I$ using the iodine monochloride (ICl) technique. A column containing AG 1-X4 anion exchange resin was used to remove free iodide from the labelled protein solution. The working buffer used in these procedures was PBS (pH 7.4) containing nonradioactive NaI (PBS-NaI) to eliminate uptake of free radioiodide ion on the gold surface [21]. The

percentage of free iodide still present in the protein solutions after dialysis or ion exchange was measured by the trichloroacetic acid (TCA) protein precipitation method, and was below 2% in labelled AT and ATH solutions and below 1% in labelled Fg solutions. Protein solutions for adsorption experiments in buffer consisted of 98% unlabelled and 2% labelled protein and were diluted to the appropriate concentration with PBS-NaI. For plasma experiments, whole blood was collected from healthy volunteers into acid citrate dextrose (ACD). Platelet poor plasma was prepared from multiple donors, pooled, aliquoted and stored at -70^{o}C. Labelled protein was added to plasma at a concentration of approximately 10% of the endogenous protein level. Surfaces were incubated in the appropriate protein or plasma solution for 3 h at room temperature, rinsed three times in PBS-NaI (5 min each), wicked onto filter paper to remove fluid and placed in counting vials. Radioactivity was determined using a Wizard Automatic Gamma Counter (PerkinElmer, Waltham, MA). In certain experiments, to determine the quantities of protein on the surface not likely to be bound due to NHS-lysine reaction (i.e. covalently), the surfaces were treated with sodium dodecyl sulfate (SDS). This treatment is expected to elute physisorbed proteins. The protein-adsorbed surfaces were counted before and after overnight incubation in 2% SDS.

2.4.2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western Blotting

The procedures used have been described in previous publications [22, 23]. Surfaces were immersed in pooled human plasma for 3 h, rinsed and incubated overnight in 2% SDS solution to elute adsorbed proteins. To achieve a sufficient concentration of proteins in the eluate, surfaces were incubated in the smallest practical volume (0.20 mL). Reduced SDS-PAGE with 12% separating gels was used to analyze eluted proteins. The same volume of eluate was loaded for each sample. For molecular weight determination, gels were stained with stabilized gold sol (Protogold, Cedarlane Laboratories, Hornby, ON). After electrophoresis, the gels were electroblotted onto Immobilon polyvinylidene fluoride (PVDF) membranes (Millipore, Bedford, MA) and cut into strips. Unbound sites were first blocked with nonfat dry milk. Incubation in primary antibody solutions, then in alkaline phosphatase-conjugated secondary antibody, both at a dilution of 1:1000, was then carried out. Finally, the blots were treated with 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitroblue tetrazolium (NBT) substrate to develop the protein bands by colour reaction.

2.5. Statistical analysis

Analysis of variance (ANOVA) was used to determine significant differences between groups of data. When differences existed, student's t-test was used to compare sets of data. Differences were considered significant for $p < 0.05$. Data are reported as means ± standard deviation (SD).

3. Results and discussion

3.1. Surface preparation

Three approaches were used to create well-defined surfaces with ATH immobilized on gold (Fig. 1). The first approach, described as "direct attachment" (Fig. 1A), depends on physical adsorption with the potential also for reaction of gold with the sulfur functions of the protein. In the second approach, DSP was first

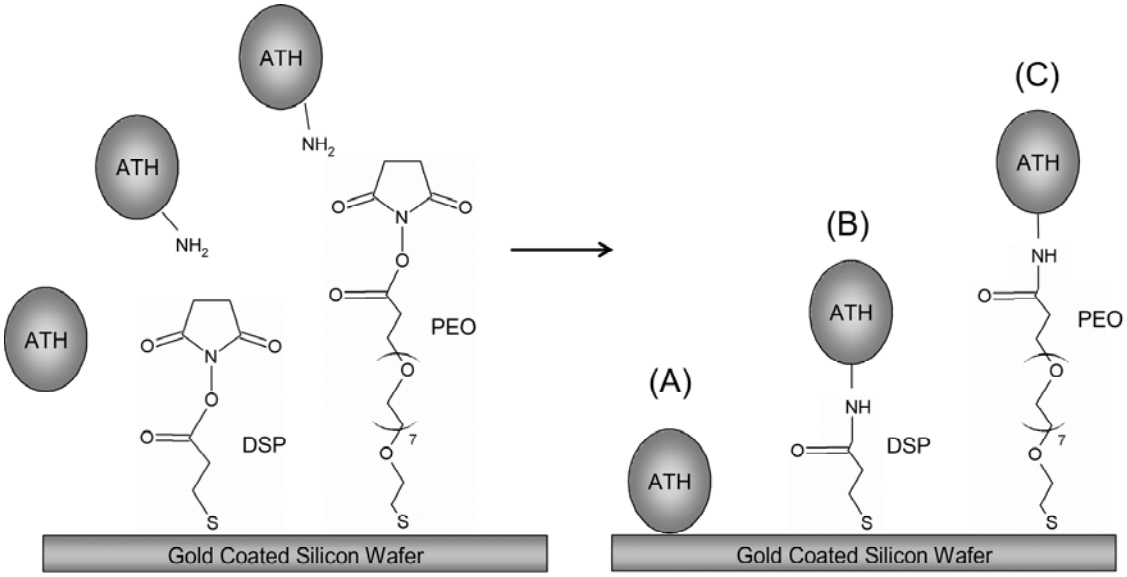

Fig. 1. Surface modification procedures. (A) Direct immobilization of ATH on gold. (B) Immobilization with dithiobis(succinimidyl propionate) (DSP) linker molecule. (C) Immobilization with polyethylene oxide (PEO) linker/spacer.

chemisorbed to gold to provide NHS functional groups that can react with amino groups in ATH or other target molecules, forming covalent bonds (Fig. 1B). Since the intermediate DSP molecule is quite small, steric hindrance still may limit the attachment of ATH to the surface. In the third approach (Fig. 1C), NHS–PEO–S–S–PEO–NHS was first chemisorbed to gold (via reaction with –S–S–) to again provide NHS groups for

reaction with amino groups in ATH. In this case, due to the PEO, the site for reaction with ATH is positioned further from the interface. This may result in greater reaction efficiency and greater availability of ATH to bind AT in contact with blood. In addition, PEO can potentially provide resistance to non-specific protein adsorption.

3.2. Surface characterization

3.2.1. Water contact angles

Water contact angle measurements were used to determine the relative hydrophilicity of the bare gold and modified gold surfaces. The data shown in Fig. 2 provide some evidence of the success of the modification procedures. Both the advancing

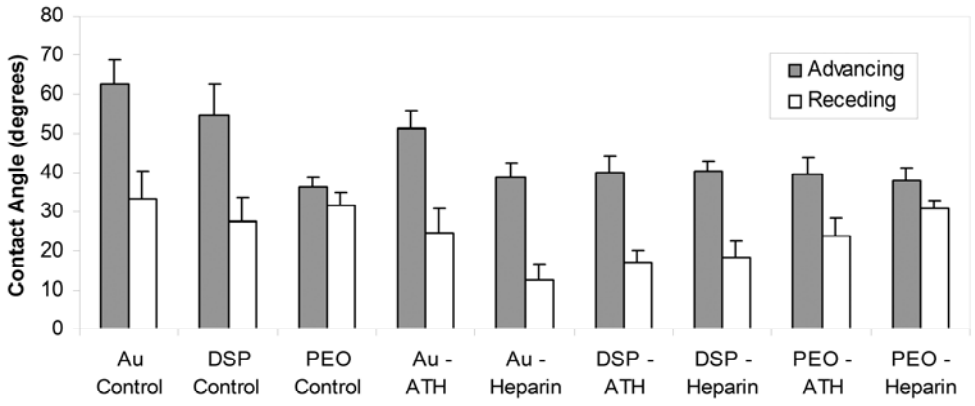

Fig. 2. Advancing and receding water contact angles. Data are means ± SD, n ≥ 12.

and receding angles were measured. Significant hysteresis was evident on most of the surfaces, and may be due to both roughness and chemical heterogeneity [24]. Although pure gold is hydrophilic and has low water contact angles, in air it is known to contaminate almost instantaneously, resulting in increased hydrophobicity and a relatively

high advancing angle value as observed in Fig. 2. The contamination layer on gold likely makes the surface rougher and more heterogeneous, thereby contributing to the observed hysteresis.

Following chemisorption of DSP, the contact angles did not change significantly. While this result cannot confirm that DSP was attached to the gold, the values are consistent with reports in the literature [25]. It appears that DSP binding to gold displaces contaminants with no change in wetting behaviour. In contrast, PEO chemisorption resulted in advancing contact angles that were significantly reduced, as would be expected. Notably, the PEO-modified surfaces showed very low hysteresis suggesting that the surfaces became more homogeneous and smoother.

Whereas the "direct" immobilization of ATH on gold did not cause a significant change in contact angles, the attachment of ATH via either DSP or PEO caused a decrease. The angles on the ATH-modified surfaces were similar to those on the heparin-modified surfaces. These data support the idea that on the surfaces modified with ATH, the heparin moiety is directed away from the surface and toward the solution. The ATH surfaces thus appear to have a twofold advantage over heparin alone: (i) optimal orientation and (ii) selection for 100% active heparin molecules. For the DSP-ATH and PEO-ATH surfaces, heparin orientation away from the surface is expected since the ATH should be attached via reaction of the terminal NHS groups on DSP or PEO with amino groups of AT leaving the heparin moieties relatively free. Furthermore, on the PEO-ATH and PEO-heparin surfaces, contact angle hysteresis is low suggesting more uniform coverage.

3.2.2. X-ray photoelectron spectroscopy (XPS)

Surface chemical composition data from XPS are summarized in Table 1. Following survey scans to establish elemental presence, low resolution scans were performed for carbon, oxygen, nitrogen, sulfur and gold at take off angles of 20º and 90º. The unmodified gold control was cleaned and stored in nitrogen prior to analysis, resulting in data showing high gold content and only slight contamination. The higher carbon and lower gold content at the lower, more surface sensitive sampling depth (20º) indicate that contaminants were mainly in the form of carbonaceous compounds from the atmosphere.

Table 1. Low resolution XPS data (at.%) at take-off angles of 90^o and 20^o.

Surface	90^o take-off angle					20^o take-off angle				
	C(1s)	O(1s)	N(1s)	S(2p)	Au(4f)	C(1s)	O(1s)	N(1s)	S(2p)	Au(4f)
Au	13.9	1.5	0.8	0.2	83.6	22.5	1.4	0.0	0.0	76.1
DSP	8.5	0.9	1.6	2.6	86.4	23.1	3.4	5.9	2.8	64.8
PEO	33.5	11.6	2.1	3.2	49.7	33.2	11.6	2.4	2.9	50.0
Au–ATH	19.2	2.9	3.2	1.6	73.1	28.7	4.5	6.1	2.7	58.0
DSP–ATH	18.1	4.6	2.1	3.6	71.7	22.7	3.0	8.2	7.0	59.2
PEO–ATH	31.0	10.1	1.1	3.9	54.0	38.3	13.5	4.1	4.9	39.2
Au–AT	34.5	8.8	6.4	0.9	49.4	54.4	14.7	9.1	1.2	20.7
DSP–AT	36.3	11.6	8.3	3.2	40.6	49.2	12.6	10.5	3.0	24.7
PEO–AT	42.3	15.2	2.9	2.7	36.9	51.0	17.1	6.0	2.6	23.2
Au–heparin	36.0	8.9	9.1	1.1	44.9	50.7	11.9	9.0	3.1	25.4
DSP–heparin	39.2	12.3	6.9	3.4	38.3	47.9	15.8	5.3	7.4	23.6
PEO–heparin	33.6	10.3	3.3	3.0	49.8	37.3	7.5	3.8	3.4	47.9

Data precision = ± 5%.

In general, surface modifications resulted in decreasing gold content. Chemisorption of DSP and PEO is expected to displace contaminants [26]. At 90º the DSP-modified surface showed lower carbon content, suggesting that contamination was decreased, and higher sulfur and nitrogen demonstrating the presence of DSP. At 20º the

sulfur and nitrogen contents were greater and the gold content was lower indicating that DSP was present at the outermost surface. Modification with the NHS-terminated PEO ester disulfide resulted in increases in oxygen, sulfur and nitrogen as expected. The observed C:O ratio was about 2.9:1, slightly greater than would be expected for pure PEO but similar to other reported values for PEO chemisorbed to gold [18].

With the attachment of protein to a surface, the presence of amino and amide groups should cause an increase in nitrogen levels. All of the ATH surfaces, i.e. direct, DSP and PEO, showed increased nitrogen content with the greatest increase on the DSP-ATH surface. Higher values were observed at the lower sampling depth. In comparison to the AT surfaces, the nitrogen increase for the ATH surfaces was smaller, showing that ATH results in different surface chemistry due to the presence of heparin. The sulfur contents of the ATH surfaces were similar to those of the heparin surfaces, and were again greater at the smaller sampling depth, suggesting that the heparin moiety of ATH is at the outermost surface as desired.

3.2.3.Ellipsometry

Ellipsometry data provided information on the thickness of single layer films, including DSP and PEO, on the gold surface. Thickness measurements can be converted to surface density using the following equation [27]

$$L = \frac{\rho_{dry} d N_A}{M}, \qquad (1)$$

where L is the estimated surface density (chains n^{-2}), ρ_{dry} is the density of the dry layer (assumed to be 1 g cm^{-3}), d is the thickness obtained from ellipsometry, N_A is Avogadro's

number and *M* is the molecular weight of the deposited species [27]. For crystalline gold in the Au (1 1 1) orientation it is believed that sulfur binding occurs at the threefold hollows between the gold atoms [28]. Based on the nearest distance between sulfur atoms of 4.97 Å and corresponding area per molecule of 21.4 $Å^2$, obtained from electron diffraction studies [29], the maximum theoretical surface density is estimated to be 0.78 nmol cm^{-2} or 4.7 molecules nm^{-2}. The ellipsometric data obtained for DSP and PEO layers gave surface densities of 0.57 ± 0.27 nmol cm^{-2} (3.5 ± 1.6 molecules nm^{-2}) and 0.49 ± 0.10 nmol cm^{-2} (2.9 ± 0.59 molecules nm^{-2}), respectively. These values are below the theoretical maximum for both molecules; this is as expected since 100% site occupancy is highly unlikely. When the PEO-NHS ester disulfide (MW 1100) chemisorbs to gold, the disulfide should dissociate to form a thiolate bond, resulting in "grafts" of MW ~ 550. Since the DSP molecule is smaller than PEO it is anticipated that a higher density would be possible for DSP. In a previous study investigating the chain density of PEO chemisorbed on gold, values of 0.40 to 0.70, 0.33 to 0.58 and 0.12 to 0.30 chains nm^{-2} were reported for PEO of MW 750, 2000 and 5000, respectively [18]. The values determined in this work are relatively high in comparison, but follow the same trend of increasing chain density with decreasing molecular weight. Higher density is expected for smaller molecules since steric hindrance to sorption as the layer fills should be reduced.

Ellipsometric measurements require knowledge of the refractive index of the substrate. When estimated using the ellipsometry software, this was found to fluctuate likely due to variation in the contamination layer that is present on gold. The substrate values used were those obtained immediately after cleaning. Measurements for modified

surfaces rely on the assumption that there is complete coverage of the gold and are based on an estimate for the layer refractive index. Although thiol binding is expected to replace contaminants, carbon and oxygen species may still contaminate uncovered areas of the surface (if coverage is not complete) potentially resulting in an overestimate of the graft density.

3.2.4. Surface density of ATH by radioiodination

The quantity of ATH immobilized on the gold and modified gold surfaces was determined using radiolabelled ATH. It was found that overnight incubation at a concentration of 0.1 mg ml^{-1} achieved saturation of the ATH-surface interaction: no increase in immobilization occurred at longer time or higher concentration. The quantities immobilized were in the order DSP > Au > PEO, with the PEO surface showing the smallest uptake, presumably due to the protein resistance of PEO. Thus it appears that protein resistance and protein specificity may be in conflict and that an optimum surface composition may exist with respect to these two properties. An estimate of 0.23 μg cm^{-2} can be made for the upper limit of ATH uptake based on monolayer coverage in the hydrated state, and assuming ATH has shape and Stokes radius similar to AT [30]. Uptake on the DSP modified surface was close to this value but was much lower on the PEO surface.

Susceptibility of the immobilized ATH to elution with SDS was used to distinguish between physically and more tightly (possibly covalently) bound ATH. It is

seen in Table 2 that on the DSP surface about 75% of the ATH was retained following SDS treatment whereas only 45% was retained on the gold and 50% on PEO.

Table 2. Surface densities of ATH and heparin on Au, Au-DSP and Au-PEO surfaces.[a]

Surface	Density (μg cm^{-2})	Density (pmol cm^{-2})	Remaining after SDS (μg cm^{-2})
Au–ATH	0.11 ± 0.01	1.40 ± 0.08	0.05
DSP–ATH	0.17 ± 0.02	2.16 ± 0.23	0.13
PEO–ATH	0.05 ± 0.01	0.68 ± 0.09	0.026
Au–heparin	0.14 ± 0.01	9.23 ± 0.86	-
DSP–heparin	0.19,0.20[b]	12.9,13.5[b]	-
PEO–heparin	0.25,0.28[b]	17.0,18.7[b]	-

[a]Surfaces were incubated overnight at concentrations of 0.1 mg ml^{-1} (ATH) and 1.0 mg ml^{-1} (heparin). Data are means ± SD, n ≥ 3.
[b]For DSP-heparin and PEO-heparin, data are from two independent experiments.

3.2.5. Surface density of heparin by ellipsometry and QCM

Ellipsometry was used to determine the density of heparin layers deposited directly on gold. As shown in Table 2, the density was found to be 0.14 ± 0.01 μg cm^{-2} (9.23 ± 0.86 pmol cm^{-2}) following incubation overnight in a 1 mg ml^{-1} solution, conditions that were expected to give maximum uptake. Since heparin has a broad molecular weight distribution, it is difficult to determine a unique value for the surface concentration of a monolayer, so the value of 0.14 μg cm^{-2} obtained cannot easily be related to coverage. This value is equivalent to ~9.2 pmol cm^{-2} and compares to a much lower value of 1.4 pmol cm^{-2} of heparin on the Au-ATH surface.

Heparin immobilization on Au-DSP and Au-PEO was determined by quartz crystal microbalance (QCM); ellipsometry could not be readily used since the multiple

layer models needed for data interpretation were not available. Representative QCM data are shown in Fig. 3 for the Au-DSP surface. Initially, PBS was injected into the cell

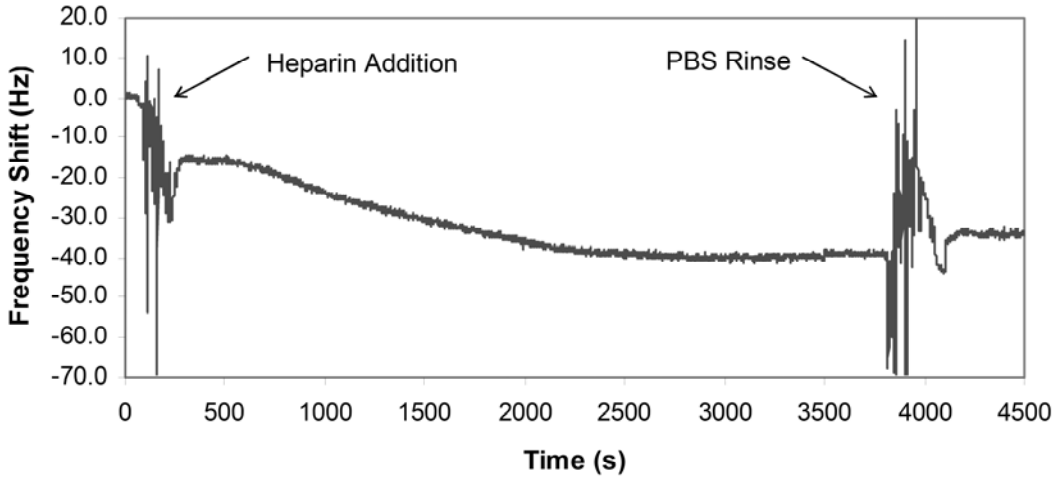

Fig. 3. Representative QCM curve showing frequency shift on addition of heparin (1 mg ml^{-1}) followed by PBS rinse: DSP-modified gold-coated quartz crystal.

containing the modified gold sample and frequency readings were taken over a short period while equilibrium was established. The resonant resistance was also monitored and was found to remain stable, indicating constant viscosity. When heparin (1 mg ml^{-1}) was added, the frequency immediately decreased and continued to do so for about 45 min after which it levelled off. The system was then flushed with PBS until a constant frequency was achieved. The net change in frequency due to heparin uptake was used to approximate the mass increase on the surface using the Sauerbrey equation

$$\Delta m = -C_f \Delta_F \quad (2)$$

where Δm is the mass change (ng), Δ_F is the change in frequency (Hz), and C_f is a proportionality constant [31]. The heparin density was found to be ~0.20 μg cm^{-2} (13.2 pmol cm^{-2}) as shown in Table 2. The primary amino group on the terminal serine residue

of heparin has the potential to react with the NHS on the DSP and PEO surfaces, and it is possible that some of the heparin was bound via this reaction. Following rinsing with PBS, only a slight increase in frequency was observed, indicating that most of the heparin was firmly bound.

The same procedure was followed for the PEO-modified surface. For the two experiments carried out, heparin densities of 0.25 and 0.28 ug cm^{-2} (17.0 and 18.7 pmol cm^{-2}) were obtained (Table 2). It is seen that on the PEO surface the uptake of heparin was much greater than that of ATH. Thus it appears that the PEO layer is much less resistant to interaction with heparin than with the AT-heparin complex, possibly due to the much smaller size of heparin. Also for the PEO surface, the PBS rinse did not alter the frequency, indicating that only a small amount of the heparin on the surface was loosely bound. Similar to the densities found in the present work, Chen et al [32] reported a density of 0.58 μg cm^{-2} for heparin immobilized on silicone, also by attachment through reaction with NHS functionalized PEO.

3.3. Plasma protein adsorption

3.3.1. Studies using radiolabelled proteins

AT adsorption from buffer was investigated to provide a measure of the specific interactions of immobilized heparin and ATH with their target protein, AT. Fig. 4 shows typical data for three groups of surfaces: AT "control" surfaces consisting of gold preadsorbed with AT, the three ATH-modified surfaces, and the three heparin-modified surfaces. It is evident that the ATH surfaces adsorbed much more AT than the

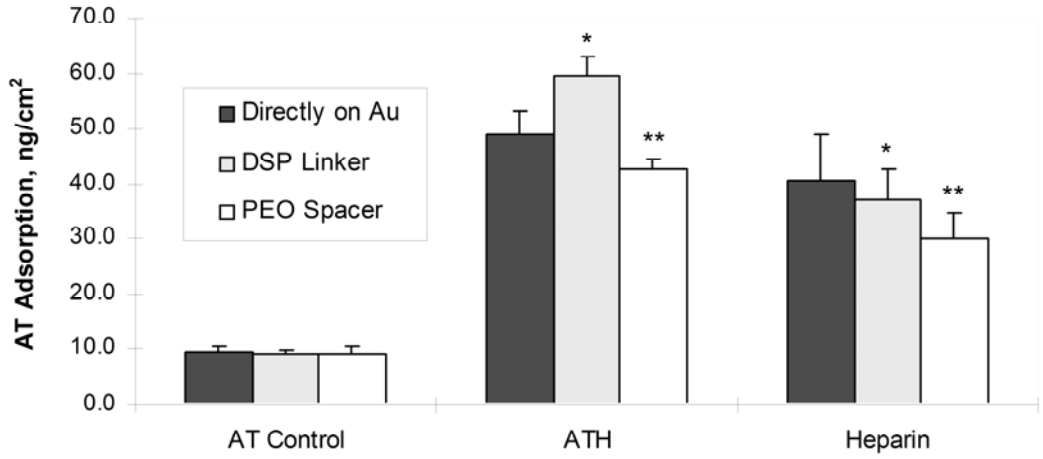

Fig. 4. AT adsorption from buffer to modified surfaces (0.15 mg ml^{-1}, 3 h). Data are means ± SD, n ≥ 3. Significant differences indicated by * for DSP-ATH vs. DSP-heparin and
** for PEO-ATH vs. PEO-heparin.

corresponding controls. The highest adsorption occurred on DSP-ATH (59.5 ng cm^{-2}) and Au-ATH (49.0 ng cm^{-2}) with slightly lower adsorption on PEO-ATH (42.7 ng cm^{-2}). These levels are; however, lower than would be expected for monolayers of AT (0.23 μg cm^{-2}) as discussed previously. Some of the AT adsorption on these surfaces may be through physical interactions rather than interactions with heparin. The heparin surfaces showed generally less adsorption than the corresponding ATH surfaces. In the case of the DSP and PEO surfaces, the differences are statistically significant ($p < 0.05$). A more valid comparison is on the basis of the AT adsorption normalized to the quantity of heparin on the surface. On this basis, since the amount of heparin on the "heparin alone" surfaces is greater than on the ATH surfaces, the latter showed much higher AT adsorption, indicating that the heparin moiety on ATH is functionally more active,

consistent with the fact that the heparin on ATH has a significantly higher proportion of molecules with active pentasaccharide sequences than standard heparin [10].

The adsorption of AT from plasma was also studied as a means of assessing the biospecificity of immobilized heparin and ATH in conditions much closer to blood contact. Fibrinogen adsorption was measured simultaneously to provide a comparison to AT and an evaluation of resistance to non-specific protein adsorption (Fig. 5). On the

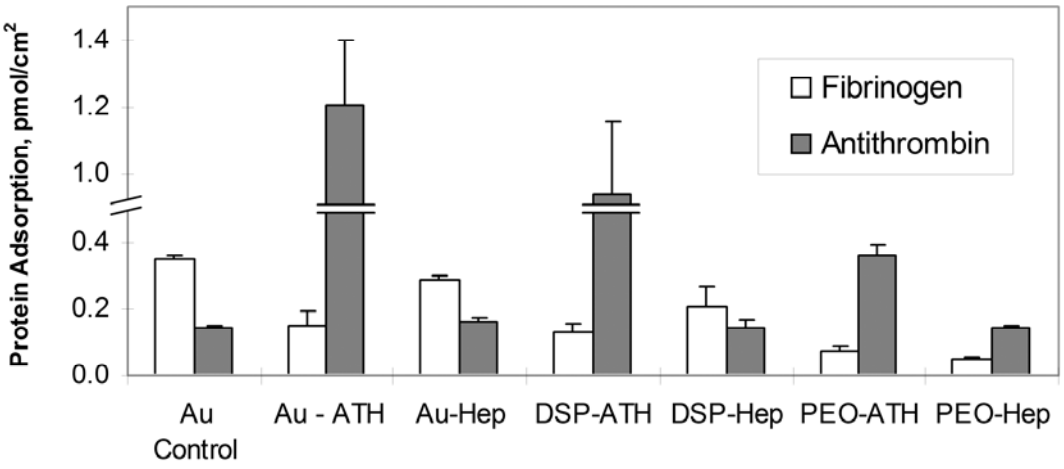

Fig. 5. Adsorption of fibrinogen and antithrombin from plasma (3 h) measured simultaneously by dual radiolabelling. Data are means ± SD, n =3.

gold control surface, fibrinogen adsorption was greater than AT as would be expected on the basis of the much higher concentration of fibrinogen in plasma. In addition, Au-heparin adsorbed significantly more fibrinogen than Au-ATH ($p < 0.05$), suggesting that the AT portion of the complex may contribute to protein resistance. On the PEO surfaces, fibrinogen adsorption was reduced by greater than 80% compared to the corresponding surfaces without PEO. These data demonstrate that PEO is able to resist the adsorption of fibrinogen even when the distal chain ends are derivatized with ATH and heparin.

In contact with plasma, the ATH surfaces showed very high specificity for AT. It is interesting to note that for Au-ATH and DSP-ATH, AT adsorption from plasma (~1 pmol cm^{-2}) was similar to adsorption from buffer (~55 ng cm^{-2} or ~0.95 pmol cm^{-2}). In contrast, the DSP-heparin and PEO-heparin surfaces showed relatively high adsorption of AT from buffer, but much lower adsorption from plasma. The PEO-ATH surface, although it adsorbed less AT than the Au-ATH and DSP-ATH, adsorbed substantially more than the Au control and the PEO-heparin. The former effect may be due to the lower density of ATH on the PEO-ATH surface (Table 2). In addition, the interaction of AT with the pentasaccharide sequence of heparin may be inhibited by the protein resistance of the PEO. Overall, these data show extensive uptake of the target protein antithrombin on the ATH surfaces, thus suggesting their potential anticoagulant activity.

It is expected that antithrombin and anticoagulant activity will correlate with AT binding on these surfaces, such that with greater binding there will be increased activity. Future work will focus on determining the catalytic and direct inhibitory activity of the surfaces in relation to coagulation enzymes including thrombin and factor Xa. Moreover conditions in whole blood and in vivo, where cells and vessel surfaces will play a role, may alter AT binding and activity compared to the simpler systems used here. It is nonetheless anticipated that the trends seen in these in vitro experiments will extend to the more complex systems.

Since during ATH synthesis, AT selects for heparin with at least one active pentasaccharide sequence [13], it is likely that the ability to catalyze antithrombin inhibition through heparin will be the major functional benefit of immobilized ATH over

unmodified heparin (in standard heparin only one molecule in three has the AT binding domain). It is thus necessary for the heparin moiety to be oriented away from the surface into the fluid phase to achieve enhanced AT binding as demonstrated. It should also be pointed out that there is potential for both catalytic and direct anticoagulant activity with the ATH complex [10]. Previous work has shown that upon attachment to a surface, the permanently activated AT of ATH possesses some direct activity; however this is small compared to ATH in the free state [15]. Thus, it is possible that direct AT functionality will be compromised upon attachment to the surface, depending again on the orientation of ATH. Despite this, as long as heparin is still free to interact, its active sites will provide increased biological activity.

3.3.2. SDS-PAGE and Western blotting

Information on adsorption from plasma for a wider range of proteins was obtained using SDS-PAGE and immunoblotting. Experimental conditions were identical for all surfaces (i.e. same plasma volume, surface area, incubation time, volume of buffer for elution, volume of eluate loaded on gels) allowing comparisons of gel and blot responses. Typical data are shown in Fig. 6. The blots for the gold surface showed strong responses for high molecular weight kininogen (HMWK), fibrinogen, albumin, vitronectin and apolipoprotein A-I, weaker responses for AT and C3, and no response for prothrombin. The patterns for HMWK and C3 suggest that both the contact coagulation and complement systems were activated by this surface. The adsorption of fibrinogen and vitronectin may be considered undesirable due to the role of fibrinogen in coagulation and

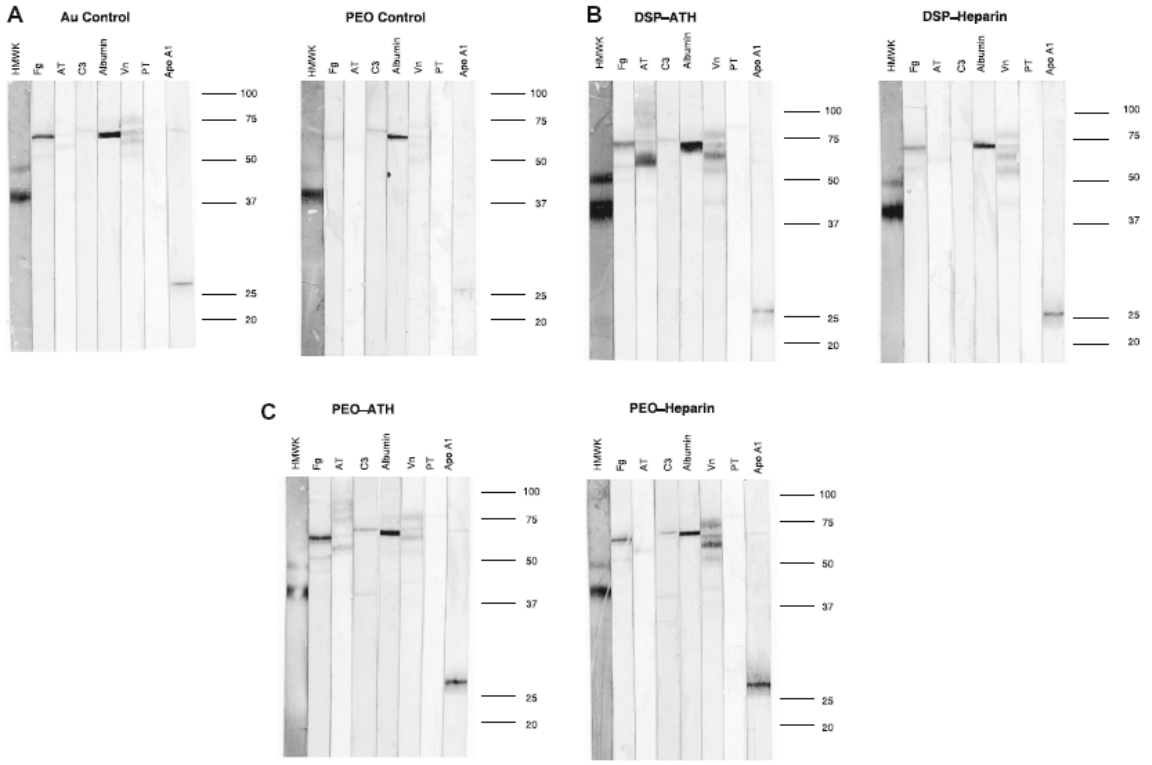

Fig. 6. Western blots of adsorbed proteins. (A) Au and PEO controls. (B) DSP-ATH and DSP-heparin. (C) PEO-ATH and PEO-heparin. Surfaces were incubated in plasma (3 h), and eluted with 2% SDS. Blots were probed with antibodies to HMWK, Fg, AT, C3, albumin, vitronectin (Vn), prothrombin (PT) and apolipoprotein A-I (Apo A1). Molecular weight scale in kD.

the cell adhesive properties of vitronectin. Conversely, since albumin is considered a passivating protein, its presence on the surface may be seen as beneficial. Apolipoprotein A-I has gained attention due to its adsorption from blood to a wide variety of biomaterial surfaces and it is suspected that it may play a significant, though as yet undefined, role in blood-material interactions [33].

Most of the proteins seen on gold were less evident on the PEO surface; the strongest response on this surface was for albumin. Thus the chemisorption of PEO

appears to reduce the adsorption of undesirable proteins, while allowing a potentially desirable one. There was, however, still a strong response for HMWK.

A notable feature of these data is the very strong AT response for the DSP-ATH surface. This is in contrast to the DSP-heparin surface which showed a very weak response for AT. These findings are in agreement with the results for AT adsorption using the radioiodinated protein (Fig. 5), and suggest that the ATH surface is potentially more effective than the heparin alone. The AT responses on the PEO-ATH and PEO-heparin surfaces were both relatively weak, with that for the PEO-ATH the stronger of the two. In general, compared to DSP, the PEO component appears to inhibit the adsorption of all proteins including AT. At the same time the attachment of ATH or heparin to the PEO clearly diminishes the protein resistance of the PEO. It may be that on the PEO linker/spacer surfaces a better balance between resistance to non-specific adsorption and specific adsorption of AT could be found, for example using PEO of lower molecular weight.

4. Conclusions

ATH was immobilized on gold surfaces using three methods. Analogous heparinized surfaces were also prepared. Physicochemical characterization confirmed surface modification and provided information about the possible orientation of immobilized ATH molecules, suggesting that the heparin moiety is directed away from the surface and is free to interact with contacting blood. ATH surfaces were able to bind significantly higher amounts of antithrombin from plasma than the analogous heparinized

surfaces, despite having lower surface density of the heparin moiety. The greater selectivity for the target protein AT indicates that surface immobilized ATH is likely to be more effective than heparin in preventing coagulation on blood contacting surfaces.

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CHAPTER 4: PAPER TWO - Immobilization of an Antithrombin-Heparin Complex on Gold: Anticoagulant Properties and Platelet Interactions

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Objectives:

ATH was attached to gold through active functional groups to study its anticoagulant activity in comparison to heparin. The contribution of the pentasaccharide sequence of the heparin moiety was assessed along with the bioactivity of immobilized ATH vs. heparin. Platelet adhesion was also measured from flowing whole blood to assess the modified surfaces in physiologically relevant conditions.

Main Contributions:

1. Antithrombin binding to ATH modified surfaces was found to be predominantly through the active pentasaccharide sequence of heparin and was significantly greater than AT binding on the corresponding heparinized surfaces.

2. The ratio of active heparin density, as determined by the inhibition of factor Xa, to total heparin density, was highest on PEO-ATH modified surfaces. All ATH surfaces showed significantly greater bioactivity compared to heparin modified surfaces.

3. Platelet adhesion measured from flowing whole blood was found to be lower on all modified surfaces compared to bare gold. This was the first investigation of platelet adhesion to ATH surfaces and results showed that the PEO-ATH had levels similar to PEO, a surface known to be platelet resistant.

4. The clotting time of ATH immobilized with PEO on gold was slightly prolonged in recalcified plasma, in contrast to heparin surfaces which showed similar clotting times to controls.

Immobilization of an antithrombin-heparin complex on gold: Anticoagulant properties and platelet interactions

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Abstract

The anticoagulant properties and platelet interactions of gold surfaces modified with an antithrombin-heparin (ATH) complex are reported. ATH was attached to gold through either a short disulfide (linker) or a thiol-terminated polyethylene oxide (PEO) (linker, spacer). Analogous surfaces were prepared with uncomplexed heparin. Antithrombin (AT) uptake was measured before and after selectively destroying the active pentasaccharide sequence of the heparin moiety, and was found to be predominantly through the active sequence on all of the surfaces. AT binding was higher on the ATH surfaces than on the corresponding heparin surfaces. Heparin activity was assessed by an anti-factor Xa assay. The ratio of active heparin density (from the anti-FXa assay) to total heparin density was taken as a measure of heparin bioactivity. The ratio was greater on the ATH- than on the heparin-modified surfaces: i.e. the PEO-ATH surfaces showed the greater proportion of active heparin. Platelet adhesion from flowing whole blood was found to be reduced on PEO- and ATH-modified surfaces compared to bare gold. The PEO-ATH modified surfaces, but not the heparinized surfaces, were shown to prolong the clotting time of recalcified plasma.

1. Introduction

When a foreign material is exposed to blood, rapid adsorption of plasma proteins takes place. The properties of the protein layer determine subsequent surface interactions including coagulation, complement activation, platelet adhesion and activation, and other blood cell responses [1, 2]. These phenomena are severely limiting on the use of blood contacting medical devices including stents, vascular grafts, oxygenators and catheters.

A popular strategy to reduce protein and platelet interactions on biomaterials is the incorporation of hydrophilic polymers such as polyethylene oxide (PEO) [3-5]. Due to its hydrophilic character PEO acts as a barrier to protein attachment. It can also function as a spacer for attaching bioactive molecules. For example, the anticoagulant heparin has been immobilized to surfaces through PEO spacers [6, 7]. The immobilization of heparin, on its own and in combination with other molecules such as PEO, has been extensively investigated [8-10] leading to a number of commercialized technologies [11]. Heparinized materials are, however, not without drawbacks. A significant disadvantage of heparin as a surface modifier, indeed for any application, is its variable activity and propensity to bind a large number of plasma proteins as well as antithrombin (AT) [12]. These limitations have led to the investigation of other anticoagulants including direct thrombin inhibitors such as hirudin, hirulog and PPACK [13-15], as well as a range of heparinoids.

Our lab has developed a novel covalent complex of antithrombin and heparin (ATH) with increased pentasaccharide content in the heparin moiety and high anticoagulant activity compared to standard heparin [16-17]. ATH has many advantages

over heparin including both catalytic and direct AT activity, reduced non-specific binding of plasma proteins, and the ability to inhibit surface-bound coagulation factors [18]. As a surface modifying agent ATH has an advantage over heparin in that attachment can take place through the AT moiety, leaving the heparin effectively "free". In addition the heparin is expected to be directed away from the surface and able to interact more efficiently with blood components such as thrombin.

In previous studies ATH was shown to have potential as a biomaterial surface modifier on polyurethane grafts and catheters [19-22]. The present work attempts to elucidate in more detail the interactions of surface-immobilized ATH using gold as a better defined substrate more amenable to precise surface characterization. Once optimized, the various chemistries studied on gold for attachment of ATH can be applied to more practical substrates for blood contacting materials, such as synthetic polymers. In a previous communication on gold-ATH [23], we described surface preparation to create well-defined surfaces through direct immobilization, using DSP, a short linker molecule, and with PEO as a linker/spacer. Detailed surface characterization and protein interaction studies were carried out and demonstrated that in comparison to analogous heparin-modified surfaces, ATH could bind greater amounts of AT from plasma. In the present work, the biological activity of these surfaces was further investigated. AT binding both biospecifically (through the pentasaccharide sequence of heparin) and non-specifically, inhibition of factor Xa, platelet adhesion from flowing whole blood, and plasma coagulation are reported.

2. Materials and methods

2.1. Materials

Human antithrombin (AT) was purchased from Affinity Biologicals Inc. (Ancaster, ON, Canada). Unfractionated heparin (UFH) was Grade I-A sodium salt from porcine intestinal mucosa (Sigma-Aldrich, Oakville, ON, Canada). ATH synthesis and purification was performed as described previously [16]. Briefly, AT and UFH were heated to 40ºC for 14 days followed by addition of $NaBH_3CN$ and further heating at 37ºC for 5 h. ATH was purified by butyl agarose hydrophobic chromatography to remove excess UFH followed by DEAE Sepharose anion exchange chromatography to remove unreacted AT. Dithiobis(succinimidyl propionate) (DSP) was from Sigma-Aldrich (Oakville, ON, Canada) and N-hydroxysuccinimide (NHS)-terminated PEO ester disulfide, MW 1100, was from Polypure (Oslo, Norway). $NaBH_4$ was from Caledon Laboratories (Georgetown, ON, Canada) and $NaIO_4$ was from Aldrich (St Louis, MO, USA).

2.2. Surface preparation

Gold surfaces were prepared as described previously [23]. Silicon wafers 0.5 mm thick and double side polished were coated with a titanium adhesion layer and 1000 Å of gold (Silicon Valley Microelectronics, Santa Clara, CA, USA). Four inch diameter gold coated wafers were diced into 0.5 x 0.5 cm squares for protein adsorption and activity studies or into four equal pieces for platelet studies. Gold was cleaned for 5 min in a boiling aqueous solution containing 1 part hydrogen peroxide, 1 part ammonium

hydroxide and 5 parts Milli-Q water, followed by extensive rinsing in Milli-Q water. Surface modification methods were as described elsewhere [23]. Briefly, gold surfaces were modified directly or functionalized by incubation with either DSP (20 mM in DMSO) or PEO NHS ester disulfide (1 mM in ethanol) for 2 h at room temperature, rinsed with solvent and dried in a stream of nitrogen. NHS functional groups on the distal ends of DSP and PEO were then able to react with amino groups of ATH or heparin. Surfaces were transferred to a solution of ATH in PBS (0.1 mg ml^{-1}) or, for parallel modification with heparin, a solution of heparin in PBS (1.0 mg ml^{-1}) and incubated overnight at room temperature. Surface modification schemes using these approaches are shown in [23].

2.3. Grazing incidence reflection Fourier transform infrared (GIR-FTIR) spectroscopy

GIR-FTIR spectra of the surfaces were obtained with a Nicolet 6700 FTIR spectrometer (Thermo Scientific) with Smart SAGA (specular apertured grazing angle) reflectance accessory. The instrument was equipped with a germanium polarizer and liquid nitrogen cooled, mercury cadmium telluride (MCT) detector. Measurements were made at 80º angle of incidence. The resolution was 4 cm^{-1} over a spectral range of 400 to 4000 cm^{-1}. The sampling area was a 5 mm diameter disc and 32 scans were taken. Bare gold was used as background. Samples were stored in nitrogen prior to measurements.

2.4. Antithrombin uptake (binding to heparin active site)

Some ATH- and heparin-modified surfaces were treated to selectively destroy the pentasaccharide sequence of the heparin moiety [20]. Surfaces were incubated in 0.1 M $NaIO_4$ at room temperature for 18 h in the dark and rinsed in Milli-Q water. They were then placed in 1.0 M $NaBH_4$ for 5 h at room temperature and rinsed again with Milli-Q water. Analogous untreated surfaces were used as controls.

AT was radiolabelled with $Na^{125}I$ using the Pierce Iodination Reagent (Thermo Scientific, Rockford, IL, USA). The labelled protein was dialyzed against PBS using a Slide-A-Lyzer dialysis cassette (Thermo Scientific) with three changes of buffer to remove unbound radioactive iodide. The free radioiodide content of the protein solution after dialysis was measured by the trichloroacetic acid (TCA) precipitation method and it was routinely found to be less than 2%. To avoid uptake of free radioactive iodide on the gold surfaces, PBS (pH 7.4) containing nonradioactive sodium iodide (PBS-NaI) was used as buffer [24].

Platelet poor plasma from multiple healthy donors was pooled, aliquoted and stored at -70ºC until needed. Radiolabelled AT was added to plasma at a concentration of approximately 10% of the normal endogenous level. Surfaces were incubated in plasma for 3 h at room temperature, rinsed 3 times, 5 min each time, with buffer and wicked onto filter paper. Samples were placed in counting vials and radioactivity was measured using a gamma counter.

2.5. Anti-factor Xa activity

The bioactivity of surfaces in terms of their ability to inhibit factor Xa (FXa) was assessed using a modified protocol with the Stachrom® Heparin kit from Diagnostica Stago (Asnières sur Seine, France). AT, FXa and the chromogenic substrate, CBS 31.39, were supplied with the Stachrom® kit. Surfaces were incubated in 96-well microtitre plates with 0.14 ml AT (1 mg ml^{-1}) for 10 min at 37ºC. FXa (70 µl, 7 mg ml^{-1}) was then added and allowed to react for 4 min. Samples (60 µl) were transferred to adjacent wells containing 60 µl chromogenic substrate. The reaction was stopped after 1 min by adding 60 µl 50% acetic acid. Residual FXa not inhibited by AT on the surface reacts with the chromogenic substrate to produce p-nitroaniline, determined via absorbance measurements at 405 nm. A standard curve was constructed using heparin solutions of known concentration.

2.6. Platelet adhesion

Platelet adhesion was measured in a whole blood preparation under controlled flow conditions. Platelets were isolated and labelled with ^{51}Cr for quantification. With the exception of the modifications outlined below, the method of Mustard et al. was used for isolation of platelets from human blood [25]. Briefly, human whole blood obtained from healthy drug-free volunteers was collected into centrifuge tubes containing either acid citrate dextrose (ACD) anticoagulant (6 parts ACD to 1 part blood) or low molecular weight heparin (Enoxaparine, Rhone-Poulenc, Montreal, QC, Canada) at a final concentration of 0.2 U ml^{-1}. Platelets were isolated from the ACD whole blood by multi-

step centrifugation at 2500×*g*, resuspended in Tyrodes albumin buffer with 2 mM Ca^{2+} and 1 mM Mg^{2+} and incubated for 45 min with 0.5 mCi ml^{-1} of $Na^{51}Cr$ (Perkin Elmer, Boston, MA, USA). Red cells isolated from ACD whole blood were washed three times with Eagle's MEM (Gibco, Burlington, ON, Canada) by centrifugation (2500×*g*). Platelet poor plasma (PPP) was separated by centrifugation of the low molecular weight (LMW)-heparinized plasma. After successive washes to remove unincorporated isotope, the ^{51}Cr-platelets were resuspended in LMW-heparinized plasma to give a final platelet concentration of 250,000 μl^{-1}. The washed red blood cells were added to the platelets suspended in heparinized plasma to give a reconstituted whole blood at 40 % hematocrit.

A cone-and-plate device was used to measure platelet adhesion [26]. Gold surfaces were assembled in the device and 1 ml of the whole blood preparation was introduced. The device was operated at a shear rate of 300 s^{-1} and exposures were for 15 min. Surfaces were rinsed with ethylenediamine tetraacetic acid (EDTA) buffer and placed in counting vials for radioactivity measurement.

2.7. Plasma clotting times

Human platelet poor plasma from healthy donors and anticoagulated with ACD was pooled and stored at -70ºC. Surfaces were incubated in 96 well microtitre plates (non-tissue culture treated) with 0.1 ml of plasma for 10 min at 37ºC. An equal volume of 0.025 M $CaCl_2$ was added to the wells and absorbance measurements at 405 nm were taken at 30 s intervals for 40 min. Clotting times are reported as time to half maximum (S-shaped absorbance-time curve).

2.8. Statistical analysis

Differences between data sets were assessed using analysis of variance (ANOVA). Student's t-test was used to compare sets when differences existed and differences were considered significant for $p < 0.05$. Data are reported as mean ± standard deviation (SD).

3. Results and discussion

3.1. Grazing incidence reflection Fourier transform infrared (GIR-FTIR) spectroscopy

Surface characterization confirmed the gold modification procedures as reported previously [23]. The functionality of the reactive groups on DSP and PEO was investigated by surface sensitive infrared spectroscopy. GIR-FTIR analysis (also known as infrared reflection absorption spectroscopy – IRAS) was used for this purpose. N-hydroxysuccinimide (NHS) esters are commonly used as coupling agents for attachment of molecules containing amino groups to surfaces. However, the reactivity of NHS groups can be reduced by surface immobilization; in addition they can readily undergo hydrolysis in wet conditions [27, 28]. By optimizing the pH and working under moisture free conditions these effects can be minimized. It is important, nonetheless, to verify the presence of functional NHS groups and this was done with information from GIR-FTIR. Following modification with DSP and PEO, samples were sealed in vials under nitrogen and spectra were taken immediately. Unmodified gold was used as a control. Surfaces modified with DSP and PEO showed similar characteristic bands. A representative spectrum for PEO-NHS is shown in Fig. 1. The band at 1748 cm^{-1} is indicative of the

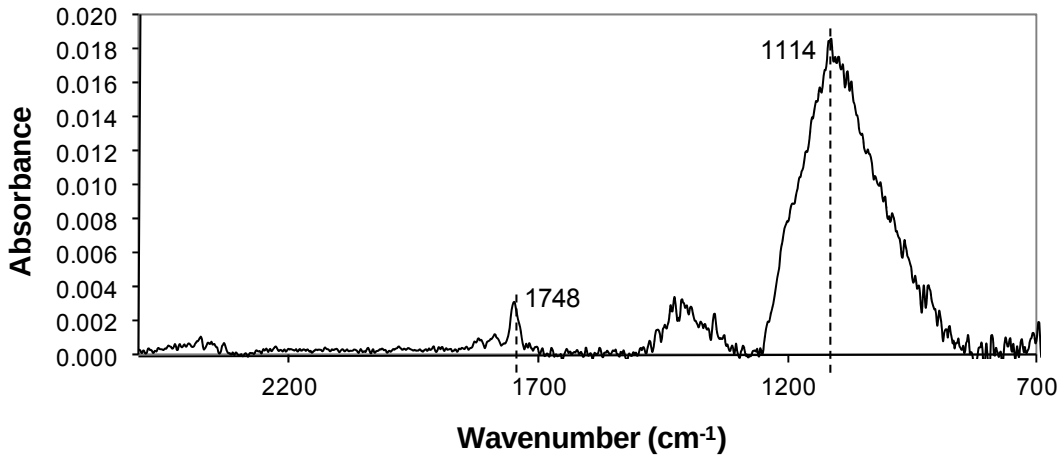

Fig. 1. GIR-FTIR spectrum of Au following modification with PEO-NHS showing characteristic stretching frequencies of NHS groups at 1748 cm^{-1} (C=O) and 1114 cm^{-1} (C-O).

succinimidyl carbonyl stretching vibration and the band at 1114 cm^{-1} of the ester C-O stretching vibration [27, 29]. Also, two smaller peaks are observed around 1820 and 1788 cm^{-1} corresponding to the ester carbonyl [30]. Dordi et al have shown that the succinimidyl carbonyl stretch disappears upon hydrolysis [27]. Wagner et al have also shown that this band disappears and that carboxylate carbonyl at 1696 cm^{-1} appears following hydrolysis [30]. The presence of the ester carbonyl stretching vibrations and the absence of carboxylate carbonyl in our samples suggest that hydrolysis has not occurred. Overall, the spectra obtained provide evidence of intact NHS ester groups and confirm that functionality was maintained following immobilization on gold.

3.2. Antithrombin binding to modified surfaces

AT adsorption from plasma provides an indication of the ability of the heparin moiety in ATH to bind the target protein AT. Some AT uptake may occur by physical adsorption rather than specific binding via the pentasaccharide sequence of heparin. To distinguish these two types of interaction, modified surfaces were treated with sodium periodate and sodium borohydride to selectively destroy the pentasaccharide sequence [20]. For all three ATH modified surfaces (direct, DSP and PEO), periodate treatment caused a decrease of greater than 85% in the uptake of AT from plasma (Fig. 2). This result suggests that the binding of AT to these surfaces is predominantly through the active pentasaccharide sequence of heparin rather than physical adsorption.

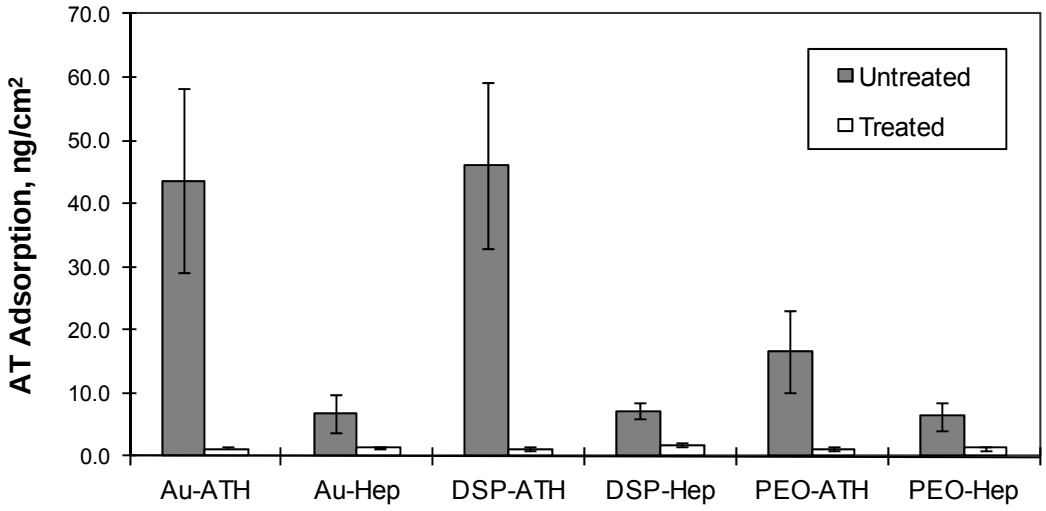

Fig. 2. AT adsorption from plasma to modified surfaces treated and not treated with $NaIO_4$ and $NaBH_4$, demonstrating the effect of destroying the active pentasaccharide sequence of the surface immobilized heparin. Data are mean ± SD, $n \geq 6$.

These data also demonstrate the increased binding of AT to ATH modified surfaces versus heparinized surfaces. Consistent with previous results [23], the Au-ATH and DSP-ATH surfaces showed the highest uptake of AT from plasma. Uptake on the PEO-ATH surface was somewhat less, probably due to the protein resistance of the PEO. In all cases AT uptake was greater on the ATH than on the corresponding heparinized surfaces suggesting enhanced anticoagulant activity of the ATH.

3.3. Anti-factor Xa assay

This assay was performed to determine the ability of surface immobilized heparin (as heparin alone or as ATH) to catalyze the inhibition of factor Xa. Using a chromogenic substrate, the activity remaining after incubation of a solution of FXa with the surfaces was measured and used to calculate the density of "active" heparin. As seen in Table 1,

Table 1. Active heparin densities (anti-FXa assay) and total heparin densities [23] on gold, DSP- and PEO-modified gold surfaces.

	Active heparin (pmol cm^{-2})	**Total heparin** (pmol cm^{-2})	**Bioactivity ratio** (%)
Au-ATH	0.17 ± 0.03	1.40 ± 0.08	12.1
Au-heparin	0.49 ± 0.17	9.23 ± 0.86	5.3
DSP-ATH	0.19 ± 0.04	2.16 ± 0.23	8.8
DSP-heparin	0.24 ± 0.06	12.9, 13.5[a]	1.8
PEO-ATH	0.20 ± 0.04	0.68 ± 0.09	29.4
PEO-heparin	0.34 ± 0.25	17.0, 18.7[a]	1.9

[a] For DSP-heparin and PEO-heparin, data are from two independent experiments.
The average was used to calculate the bioactivity ratio. All other data are mean ± SD, $n \geq 3$.

all three types of ATH surface showed similar anti-FXa activity with values close to 0.2 pmol cm^{-2}, demonstrating that when heparin is covalently linked to AT in ATH, it is still

able to bind exogenous AT and catalyze the inhibition of FXa. It was found previously, using Sepharose-AT chromatography and a similar anti-FXa assay, that binding of AT to ATH may be reduced due to complexation of FXa with ATH which causes steric hindrance and reduced access to the active pentasaccharide sequence [31]. This may explain at least in part why the Au-heparin surface showed significantly greater anti-FXa activity than the Au-ATH ($p < 0.05$). The DSP-heparin and PEO-heparin surfaces also showed slightly higher activity than the DSP-ATH and PEO-ATH surfaces; however the differences were not significant.

It must be kept in mind that this assay was carried out in buffer, i.e. no other proteins were present. For the heparinized surfaces, AT binding from plasma was much lower than from buffer, while for the ATH surfaces, binding remained relatively high in plasma [23]. Thus, in a blood contact situation the ability of a heparin-modified surface to interact with AT and catalyze the inhibition of FXa may be less than that of an ATH-modified surface.

The bioactivity ratio, which gives a measure of active heparin on the surfaces, was calculated from the total molar density of ATH or heparin determined previously [23] and the density from the anti-FXa activity assay (Table 1). The total densities were much greater on the heparin- than on the corresponding ATH-modified surfaces. The anti-Xa activity was also greater on the heparin-modified surfaces but the differences between ATH and heparin were smaller (Table 1). The ratios for the ATH-modified surfaces were much higher than for the heparinized surfaces, indicating that the heparin moiety in surface immobilized ATH has higher anticoagulant activity. The PEO-ATH surface, with

the lowest total density of ATH, had the highest bioactivity ratio (greatest proportion of immobilized heparin in active form). This may be due to the "spacer" effect of PEO whereby the heparin in ATH is more available to interact with AT than when attached directly or via DSP.

3.4. Platelet adhesion

Platelet adhesion to the surfaces following exposure to flowing whole blood was measured using ^{51}Cr labelled platelets [26]. It is important in such studies that red cells and flow, both of which strongly influence platelet surface interactions [32, 33], should be present. As shown in Fig. 3, platelet adhesion on gold was significantly higher than on the

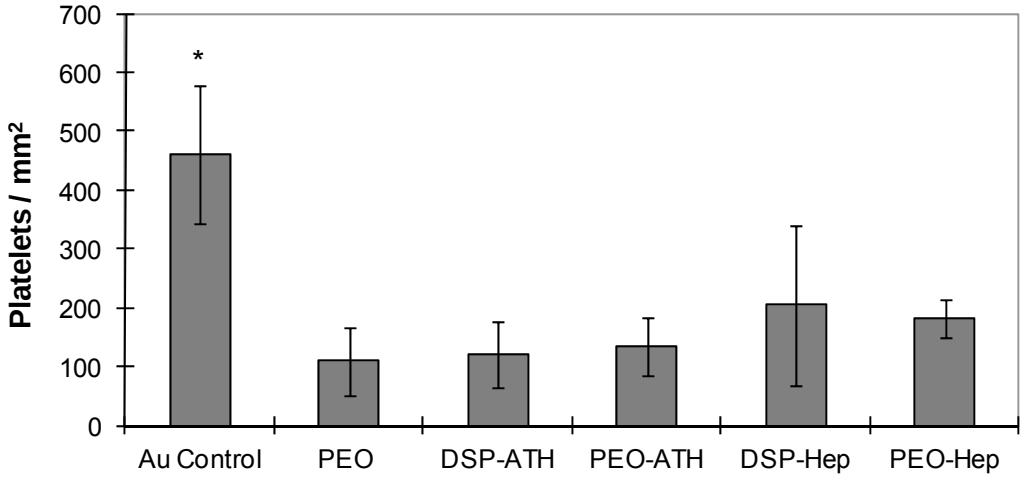

Fig. 3. Platelet adhesion to surfaces following exposure to flowing whole blood suspension for 15 min at 300 s^{-1}. Platelet concentration 250,000 platelets μL^{-1}, 40% hematocrit. Data are mean ± SD, $n \geq 4$. *Differences significant between Au Control and all other surfaces ($p < 0.05$).

various modified surfaces ($p < 0.05$). Low adhesion on the PEO-modified gold was expected based on previous reports [5, 34]. On both DSP-ATH and PEO-ATH modified gold, platelet adhesion was also low, and similar to the PEO-alone surface, indicating that ATH caused no additional platelet adhesion when tethered to PEO. Platelet adhesion is known to be mediated by various adsorbed plasma proteins, with fibrinogen playing a particularly important role [35]. In previous work, it was determined that fibrinogen adsorption was much lower on the PEO-ATH surface than on unmodified gold [23], thus correlating with the decreased platelet adhesion on these surfaces.

The DSP-heparin and PEO-heparin surfaces also showed reduced platelet adhesion relative to the gold. Adhesion was slightly greater on the heparin modified surfaces compared to the ATH; however the difference was not significant. The data for the DSP-heparin surface showed relatively large variability (Fig. 3). The reason for this is unclear. The effect of surface immobilized heparin on platelet adhesion and activation as reported in prior literature is conflicting, with some studies showing suppression of interactions and some showing enhancement [36-39]. The variability in heparin preparations and sources may account for these discrepancies. ATH has the advantage that the heparin component is homogeneous and consists of active heparin molecules. An increase in ATH density on the surface may result in a further decrease in platelet interactions. Other work has suggested that the formation of an antithrombin-heparin complex can reduce adverse platelet interactions [40]. Reduced platelet reactivity has been found on a range of different heparinized surfaces with high AT binding capacity [38]. Thus, due to the presence of AT in the ATH complex and to the increased capacity

of the heparin portion for AT binding from plasma, it is likely that access of platelet binding proteins to ATH immobilized surfaces would be restricted. Platelet adhesion, activation and aggregation should then be reduced.

This communication presents the first investigation of platelet interactions with ATH modified biomaterials. The data show that adhesion is lower ($p < 0.05$) than on unmodified gold. Adhesion was similar on all of the modified surfaces. The study was restricted to platelet adhesion; further work will be required to examine platelet activation, including aggregation, on these surfaces.

3.5. Plasma coagulation

The effect of ATH modified surfaces on clotting was assessed by a plasma coagulation test based on turbidity measurements. Surfaces were incubated in platelet poor, citrated plasma and absorbance at 405 nm was measured over time after the addition of $CaCl_2$. The time to reach half maximum absorbance was determined (Fig. 4), the maximum being taken as the attainment of a plateau. Clot formation was visually observable at that point. The gold surface reached half maximum between 200 and 300 seconds and most of the modified surfaces showed clotting times in this range, including the PEO surface. The sole exception was the PEO-ATH surface which showed a significantly longer clotting time compared to the gold control, PEO control and PEO-heparin surfaces ($p < 0.05$). Thus with PEO as linker, ATH showed a clear advantage over heparin. In contrast no differences were found between ATH and heparin surfaces prepared by direct attachment to gold or with DSP as linker. The PEO-ATH surface also

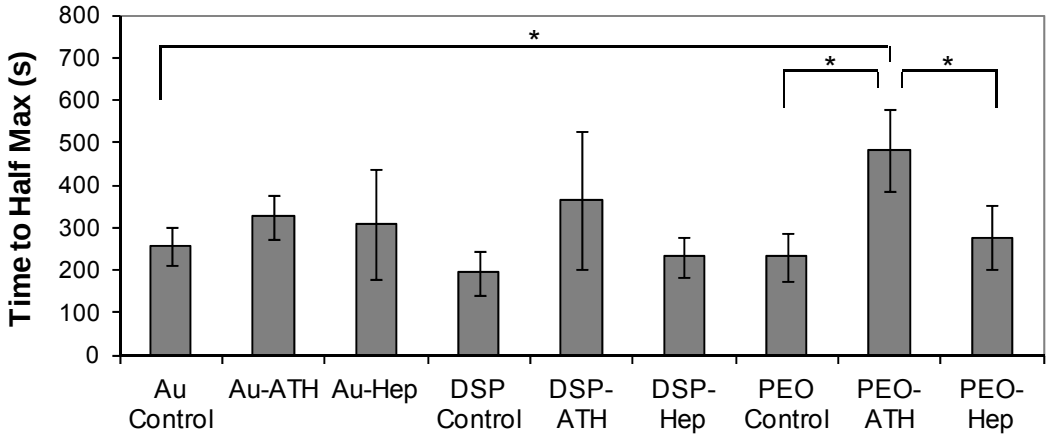

Fig. 4. Effect of surfaces on plasma coagulation. Time to reach half maximum absorbance at 405 nm following addition of 0.025 M $CaCl_2$ to citrated plasma. Data are mean ± SD, $n \geq 4$.
*Indicates significant differences ($p < 0.05$).

showed the highest bioactivity ratio (anti-factor Xa experiments) indicating the highest proportion of active heparin. In addition, protein adsorption and platelet adhesion were found to be low on this surface. All of these factors may contribute to the prolonged clotting time. It should be noted that the method used to assess clotting effects may be limited in that the amount of ATH on the surface is low, even for complete coverage. Our data suggest that there is potential for further prolongation of clotting time for PEO-ATH surfaces having an increased surface density of ATH. This is being explored in ongoing investigations with PEO-ATH attached to polymer substrates.

4. Conclusions

Gold surfaces were modified with ATH and heparin using various modification methods as developed and characterized in previous work [23]. Preliminary modification with DSP and PEO was confirmed and gave functional NHS ester groups for attachment of ATH and heparin. Previously, ATH surfaces were found to bind significantly greater amounts of antithrombin than heparin surfaces [23]; in this work the binding of AT to immobilized ATH was shown to be predominantly through the active heparin pentasaccharide sequence. Based on the ratio of immobilized heparin able to inhibit FXa to total immobilized heparin, the heparin moiety in ATH-modified surfaces was found to be much more bioactive than that on heparin-modified surfaces and was greatest on PEO-ATH. Platelet adhesion was low on all ATH-modified surfaces and comparable to the gold-PEO- and heparin-modified surfaces. PEO-ATH-modified surfaces were able to prolong the clotting time of recalcified plasma compared to gold, gold-PEO and PEO-heparin. The combination of PEO and ATH for surface modification therefore shows promise as a strategy for improved thromboresistance.

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CHAPTER 5: PAPER THREE - Modification of Polyurethane Surface with an Antithrombin-Heparin Complex for Blood Contact: Influence of Molecular Weight of Polyethylene Oxide used as Linker/Spacer

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Objectives:

Polyurethane was activated with isocyanate groups in order to graft PEO of various molecular weights and study protein interactions on immobilized ATH. It was expected that a balance between protein repulsive effects and protein attraction would be needed to provide optimal anticoagulant function. Protein radiolabelling, Western blotting analysis and measurements of platelet adhesion were performed.

Main Contributions:

1. PEO was successfully modified with isocyanate groups to allow grafting of PEO 300, 600, 1000, 2000 and 4600, followed by NHS functionalization and attachment of ATH. Modifications were confirmed with contact angle, low and high resolution XPS.

2. Fibrinogen adsorption was significantly reduced on all PEO surfaces and decreased by close to 80% compared to PU for MWs 300-2000. The attachment of ATH to the distal end of functionalized PEO did not cause much change in adsorption except on the lowest MW PEO-300.

3. AT binding was greatest on PEO-ATH with lower MW PEO (300 and 600). Only immobilized ATH showed AT selectivity from plasma whereas heparinized surfaces showed very low binding.

4. Adsorption of various proteins and adhesion of platelets was minimized on PEO-ATH modified PU compared to bare PU.

5. PEO-600 was found to be the optimal MW for reducing non-specific protein adsorption, while permitting ATH attachment and highly selective AT binding from plasma.

Modification of Polyurethane Surface with an Antithrombin-Heparin Complex for Blood Contact: Influence of Molecular Weight of Polyethylene Oxide used as Linker/Spacer

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Abstract

Polyurethane (PU) was modified using isocyanate chemistry to graft polyethylene oxide (PEO) of various molecular weights (range 300-4600). An antithrombin-heparin (ATH) covalent complex was subsequently attached to the free PEO chain ends which had been functionalized with NHS groups. Surfaces were characterized by water contact angle and x-ray photoelectron spectroscopy (XPS) to confirm the modifications. Adsorption of fibrinogen from buffer was found to decrease by ~80% for the PEO-modified surfaces compared to the unmodified PU. The surfaces with ATH attached to the distal chain end of the grafted PEO were equally protein resistant, and when the data were normalized to the ATH surface density, PEO in the lower MW range showed greater protein resistance. Western blots of proteins eluted from the surfaces after plasma contact confirmed these trends. The uptake of ATH on the PEO-modified surfaces was greatest for the PEO of lower MW (300 and 600), and antithrombin binding from plasma (an indicator of heparin anticoagulant activity) was highest for these same surfaces. The PEO-ATH- and PEO-modified surfaces also showed low platelet adhesion from flowing whole blood. It is concluded that for the PEO-ATH surfaces, PEO in the low MW range, specifically MW 600, may be optimal for achieving an appropriate balance between resistance to non-specific protein adsorption and ability to take up ATH and bind antithrombin in subsequent blood contact.

INTRODUCTION

Polyurethanes are known for exhibiting a range of mechanical properties including abrasion resistance, toughness and tensile strength, and for their relatively good biocompatibility compared to other polymers.[1] For these reasons they are commonly used in the design of cardiovascular medical devices such as catheters, heart valves, extracorporeal circuits and artificial hearts. However, like most materials, polyurethanes are prone to thrombosis and other adverse surface-localized reactions. Protein adsorption is the initial source of these problems, occurring rapidly upon blood contact and leading to platelet adhesion and other cellular interactions.[2] It has been suggested that blood compatibility may be improved by directing interfacial interactions to simultaneously avoid non-specific protein adsorption while promoting the selective binding of proteins for a desired bioactive function.[3] These two strategies, however, may be antagonistic rather than synergistic due to competing protein repelling and protein attracting effects. An appropriate balance between the two may thus be necessary.

To reduce non-specific protein adsorption, various polymers such as polyethylene oxide (PEO), dextrans and poly(2-methacryloyloxyethyl phosphorylcholine) (polyMPC) have been investigated as surface modifiers.[4-6] PEO is well known for its protein and cell resistant properties, and different strategies have been used to modify surfaces with PEO including adsorption, blending and grafting.[7-12] Protein resistance has been found to increase with PEO molecular weight up to a few thousand Daltons and then levels off.[13,14] However, when modification with PEO and a bioactive agent to promote fibrinolysis were combined, the optimum PEO molecular weight was found to be in the lower range.[15]

Other approaches to selectively attract proteins of interest for blood contact include the use of heparin for antithrombin (AT) binding, C18 chains to adsorb albumin, and specific peptides to scavenge thrombin.[16-20]

The widely used anticoagulant heparin catalyzes the inhibition of thrombin, factor Xa and other coagulation factors by antithrombin.[21] However, in "standard" heparin only about one third of the molecules contain the active pentasaccharide sequence needed for antithrombin-heparin binding.[22] To overcome this and other limitations of heparin, we have developed a novel antithrombin-heparin covalent complex (ATH) that consists of human AT and heparin in a 1:1 ratio.[23] During synthesis, ATH selects for heparin that has at least one pentasaccharide sequence, leading to a heparin component with high catalytic activity.[24] The catalytic activity of heparin and the direct inhibitory activity of AT are combined in ATH to provide dual functions. For biomaterial applications ATH offers many advantages due to its unique structure and chemical properties. Along with its high anticoagulant activity, ATH may provide anti-inflammatory properties and is expected to have limited immune response. The presence of AT on the surface may inhibit non-specific adsorption of other proteins. It also provides a greater variety of functional groups for attachment to surfaces.[25]

Surface coatings containing ATH on polymeric materials have been investigated in several studies and have shown promise for use in blood contacting applications.[26-29] ATH has also been immobilized on gold as a model substrate to investigate protein interactions and anticoagulant properties compared to analogous heparinized surfaces.[30,31] It was shown that ATH on gold was more selective for AT adsorption from plasma and

had superior bioactivity compared to analogous heparin-on-gold surfaces. The protein resistance of PEO, however, limited the achievable surface density of ATH.

In the present work, a medical grade polyurethane, as a substrate appropriate for the development of blood contacting devices, was covalently grafted with PEO of various molecular weights. ATH was subsequently attached to the PEO chain ends. The main objective was to investigate the influence of PEO molecular weight on biointeractions of the surfaces.

EXPERIMENTAL DETAILS

Materials.

Tecothane® polyurethane (PU), TT-1095A, was from Thermedics® Polymer Products (Wilmington, MA). It was extracted with boiling methanol for 48 h (Soxhlet extractor). N,N-dimethylformamide (DMF), triethylamine (TEA, 99%), anhydrous toluene and anhydrous acetonitrile were from EMD Chemicals (Gibbstown, NJ) and were used as received. 4,4'-Methylene-bis (phenyl-diisocyanate) (MDI), dihydroxy polyethylene oxide (PEO) of MW 300, 600, 1000, 2000, 4600, N,N'-disuccinimidyl carbonate (DSC), and unfractionated heparin (Grade I-A sodium salt from porcine intestinal mucosa) were from Sigma Aldrich (Oakville, ON). Human antithrombin (AT) was from Affinity Biologicals (Ancaster, ON). ATH was prepared by incubating AT and heparin at 40ºC for 14 days, as described previously.[23] Human fibrinogen was from Enzyme Research Laboratories (Southbend, IN). Human plasma was obtained from multiple healthy donors following whole blood collection into acid citrate dextrose

(ACD) and separation of platelet poor plasma. This procedure has ethics approval from McMaster University (McMaster University ethics protocol #04-046). Plasma was pooled, aliquoted and stored at -70^{o}C. Standard electrophoresis reagents were from BioRad (Richmond, CA).

Surface Preparation.

Polyurethane films were made by casting from a 5% (w/v) solution of Tecothane in DMF on glass Petri dishes and drying in air at 75ºC for 48 h. PU discs 6 mm in diameter were punched from the films, rinsed with ethanol and dried under vacuum prior to use. Isocyanate (NCO) groups were introduced into the PU surface by reaction with MDI (7.5% in anhydrous toluene) at 50ºC under nitrogen flow with TEA (2.5%) as catalyst.[14,32] The reaction was carried out for 100 min and the surfaces were then rinsed with toluene. Dihydroxy PEOs (5%) of varying molecular weight (300-4600) were grafted to PU-NCO films by reaction in anhydrous toluene for 24 h at 40ºC. Films were rinsed with Milli-Q water to react any remaining NCO groups on the surface. The PU-PEO films were incubated for 6 h in a solution of DSC (1 mmol) in anhydrous acetonitrile in the presence of TEA (1 mmol) to convert distal hydroxyl groups to N-hydroxysuccinimide (NHS).[33] Samples were rinsed with acetonitrile and dipped in PBS (pH 7.4). ATH was attached via reaction of its amino groups with NHS (aqueous solution: ATH, 0.1 mg/ml; reaction time 16 h). Surfaces were incubated in 2M NaCl for 3 h to remove any loosely bound ATH and rinsed again with PBS. PU was also directly modified by adsorption of ATH from the same solutions (PU-ATH).

Surface Characterization.

Water contact angle measurements were carried out using the sessile drop method (Ramé-Hart NRL goniometer, Mountain Lakes, NJ). Advancing and receding angles were measured on both sides of the films.

XPS data were obtained using a Thermo Scientific Theta Probe instrument with a monochromatic Al K-alpha x-ray source and a spot diameter of 400 μm. Samples were run in angle resolved mode (60º angular acceptance) providing data at take-off angles of 30º, 50º and 70º relative to the surface normal. Two replicates of each sample at each take-off angle were measured (one spot on each) and averages are reported. Survey spectra (0 to 1000 eV) were taken and low resolution scans were acquired for C, O, N, S and Si at a pass energy of 200 eV and with binding energies referenced to C1s at 285.0 eV. High resolution C1s spectra were obtained at a pass energy of 30 eV. Thermo Avantage software was used for instrument operation and data processing.

ATH Surface Density, AT Adsorption and Fibrinogen Adsorption.

ATH and AT were labelled with ^{125}I using the Iodogen method (Pierce Iodination Reagent, Thermo Scientific, Rockford, IL). Free iodide was removed by dialyzing overnight against PBS, pH 7.4 with three changes of buffer. ATH solution (PBS, 2% labelled, 98% unlabelled) was prepared at a final concentration of 0.1 mg/ml (in terms of the AT moiety). For experiments to measure AT adsorption from plasma, labelled AT was added to the plasma at 5% of the normal endogenous level. Fibrinogen was

radiolabelled with ^{125}I using the iodine monochloride method. Free iodide was removed by ion exchange chromatography on an AG 1-X4 column.

Adsorption experiments were carried out in 96 well microtitre plates using 6 mm diameter disks. Following adsorption, surfaces were rinsed three times (5 min each time) with PBS and counted for radioactivity using a gamma counter. Surfaces were then incubated overnight in 2% sodium dodecyl sulfate (SDS) to elute loosely bound protein and counted again.

SDS-PAGE and Western Blotting.

Protein samples for immunoblotting analysis were obtained by incubating films of identical total surface areas in 100% plasma for 3 h at room temperature. Adsorbed proteins were eluted by incubation with 2% SDS solution (same volume for all surfaces). Polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting were carried out as described previously.[34,35] Briefly, equal volumes of each eluate were run on 12% gels under reducing conditions. Proteins were transferred from gels to polyvinylidene fluoride (PVDF) membranes, cut into strips and unbound sites blocked with nonfat dry milk. A panel of proteins of interest was selected and strips were incubated in the appropriate primary antibody solutions and then in alkaline phosphatase-conjugated secondary antibody.[36] Protein bands were developed by colour reaction of alkaline phosphatase with 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitroblue tetrazolium (NBT).

Platelet Adhesion.

Platelet adhesion from a whole blood preparation under flow was measured as described previously.[31] The platelet preparation method and the cone-and-plate instrumentation are based on work of Mustard et al.[37] and Skarja et al.[38], respectively. Briefly, washed platelets were isolated from citrate-anticoagulated human blood by centrifugation and labelled with ^{51}Cr. Packed red cells and platelet poor plasma (PPP) were recovered from the same blood sample. Labelled platelets and red cells were resuspended in the plasma. The final whole blood preparation contained platelets at a concentration of 250,000/μL and red cells at 40% hematocrit. Blood was placed in the wells of the cone-and-plate device in which the film samples formed the base of the wells. The device was operated at a wall shear rate of 300 s^{-1} and exposures were for 15 min. The surfaces were rinsed and counted for radioactivity to determine the surface density of adherent platelets.

Statistical Analysis.

Data are reported as mean ± standard deviation (SD). Significant differences within data sets were assessed using one-way analysis of variance (ANOVA). Student's t-test was used to compare sets when differences existed. Differences were considered significant for $p < 0.05$.

RESULTS AND DISCUSSION

Surface Preparation.

The sequential surface modification reactions carried out on polyurethane films are shown in Figure 1. The first step involves introducing isocyanate (NCO) functional groups into the surface by reaction with MDI. Attempts were made to eliminate traces of water in this step to avoid reaction with NCO groups, which converts MDI to methylene dianiline (MDA).[14] However, it was found that when rigorously water-free conditions were achieved, the reaction did not proceed as expected and PEO grafting was less efficient. The presence of trace amounts of water appears to be required, possibly due to the reaction of amino groups from MDA with surface NCO groups to form a urea linkage. The urea group would then be susceptible to further reaction with the NCO groups of MDI resulting in longer, branched polymer chains and enhancing the availability of NCO; this would be advantageous in the subsequent step where PEO is grafted to the surface by reaction of a chain end hydroxyl group with NCO. The influence of water in this reaction is an area for future study. PEO was used in excess to reduce loop formation (surface attachment of both hydroxyl end groups of PEO). Finally, the distal hydroxyl end of PEO was derivatized with NHS to allow coupling of ATH through its amino groups (Figure 1).

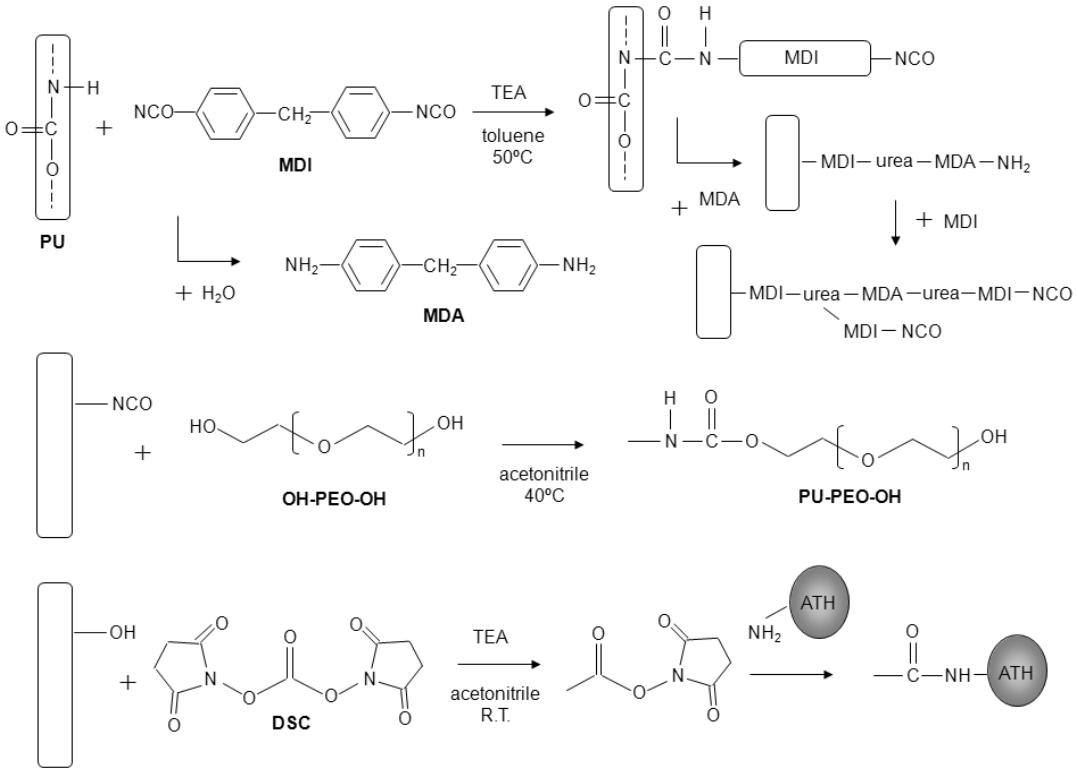

Figure 1. Polyurethane modification reactions.

Surface Characterization.

Water Contact Angles.

Water contact angle measurements were performed before and after surface modification to determine changes in wettability (Figure 2). The unmodified PU showed a relatively high advancing contact angle >90º indicating a hydrophobic surface. The receding angle was ~75º indicating moderate hysteresis. These values are consistent with data reported in the literature for thin films of Tecothane polyurethane.[39] Following modification with PEO the advancing contact angles decreased to 25-45º depending on PEO molecular weight. Given the hydrophilic character of PEO these lower values are

expected. No clear trend in contact angle as a function of PEO molecular weight was evident. Upon conjugation of ATH to the PEO, the contact angles increased slightly for PEO of MW 1000, but were still much reduced compared to the unmodified PU.

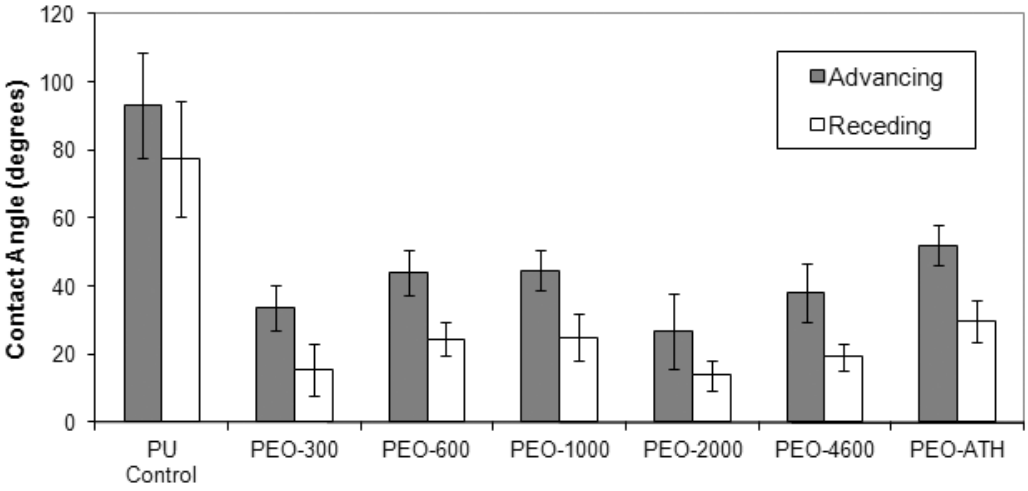

Figure 2. Water contact angles. PEO-ATH is a representative PEO-ATH surface with PEO MW=1000). Data are means ± SD, n ≥ 12.

X-ray Photoelectron Spectroscopy (XPS).

The chemical composition of the surfaces was determined by XPS at take-off angles of 30, 50 and 70º (Tables 1 and 2). The angles are relative to the surface normal, so that 70º samples closer to the surface and 30º closer to the bulk. The unmodified PU showed high carbon content which increased toward the surface (Table 1). The oxygen content was low but increased slightly toward the bulk. The C/O ratios varied from 11 to 17 depending on sampling depth; these values are much higher than would be expected for unmodified PU. Others have shown that extensive solvent extraction of medical grade polyurethanes can cause a decrease in the C/O ratio, probably due to the removal of processing additives.[40] In this work the PU was extracted in methanol prior to film

casting; however it seems that additives or other contaminants may still have been present at the surface. Silicon was also evident on the PU (and on the modified surfaces); this is due to contamination from silicones in laboratory air.[41] High resolution C1s scans for the PU indicated that most of the carbon was in the form of hydrocarbon, with some C-N and C-O (Table 2). There was also a small peak at 287.9 eV which may be due to amide groups from processing additives such as amide-containing lubricants.[40]

The first modification step, in which MDI was used to introduce NCO groups into the surface, resulted in a decrease in carbon content and increases in nitrogen and oxygen. NCO groups are highly reactive towards urethanes resulting in the formation of allophanate linkages. A peak at ~292 eV is evident only on the PU-NCO surface; this is likely a satellite peak attributable to the aromatic rings of the MDI. Grafting of PEO-600 and PEO-1000 resulted in a further decrease in carbon and a significant increase in oxygen content, as expected. High resolution C1s data showed a large increase in C-O content, further indicating the attachment of PEO. The attachment of ATH to PU modified with PEO of MW 600 and 1000 resulted in modest increases in nitrogen content. High resolution scans on the PU-(PEO-1000)-ATH surface showed a significant increase in the C-N peak, indicating the presence of the protein. Unexpectedly for the ATH surface, the sulfur content appeared to be at the trace level, possibly due to the lower sensitivity of the XPS for sulfur as well as the relatively low sulfur content of heparin in ATH.

Table 1. Low resolution XPS data (atom %) at take-off angles of 70, 50 and 30º (measured from surface normal)[a]

	Angle	C	O	N	S	Si	C/O	C/N
PU	70º	89.9	5.5	3.1	0.2	1.4	16.3	28.9
	50º	88.5	6.2	4.0	0.2	1.2	14.3	22.3
	30º	86.7	7.6	4.7	0.1	0.8	11.4	18.5
PU-NCO	70º	71.6	15.2	8.3	0.1	4.8	4.7	8.6
	50º	72.9	15.0	7.8	0.1	4.1	4.9	9.3
	30º	73.2	14.1	8.6	0.1	3.9	5.2	8.5
PU-PEO-OH	70º	64.6	26.4	2.5	0.3	6.2	2.4	26.2
(600)	50º	66.2	26.1	3.0	0.2	4.5	2.5	22.2
	30º	66.1	26.6	3.4	0.3	3.7	2.5	19.4
PU-PEO-OH	70º	64.3	26.0	2.4	0.2	7.2	2.5	26.8
(1000)	50º	66.2	25.1	3.0	0.2	5.4	2.6	22.1
	30º	66.0	25.9	3.7	0.2	4.1	2.5	17.6
PU-600-ATH	70º	72.5	21.2	4.2	0.2	1.8	3.4	17.2
	50º	72.8	20.9	5.0	0.1	1.2	3.5	14.4
	30º	71.7	21.5	5.3	0.2	1.3	3.3	13.5
PU-1000-ATH	70º	68.3	24.2	4.6	0.2	2.7	2.8	14.7
	50º	69.3	23.6	5.1	0.2	1.8	2.9	13.7
	30º	68.4	24.3	5.3	0.1	2.0	2.8	12.9

[a]Data are means of two replicates of each sample type. Data precision, ±5%

Table 2. High resolution carbon 1s XPS data (fraction of C1s signal, %) at take-off angles of 70, 50 and 30 º (measured from surface normal). Data are means of two replicates of each sample type.

		Fraction of C 1s Signal (%)						
		C-C/ C-H	C-O	C-N	O-C-N /C=O	urea/C-O-O/urethane		aromatic
	Angle	(285eV)	(285.8eV)	(286.7eV)	(287.9eV)	(288.8eV)	(289.4eV)	(291.5eV)
PU	70º	83.6	13.1	3.2	0.2	-	-	-
	50º	85.4	8.5	4.5	1.6	-	-	-
	30º	83.7	9.7	4.5	2.1	-	-	-
PU-NCO	70º	71.3	14.4	9.9	-	-	3.1	1.3
	50º	66.2	15.6	13.1	-	-	3.6	1.5
	30º	65.1	17.2	10.9	-	-	4.1	2.7
PU-PEO-	70º	51.1	12.0	33.4	-	-	3.4	-
OH-600	50º	45.5	12.7	38.0	-	-	3.8	-
	30º	42.1	12.6	40.9	-	-	4.4	-
PU-PEO-	70º	48.9	8.0	39.9	-	-	3.3	-
OH-1000	50º	45.4	12.4	39.2	-	-	3.0	-
	30º	43.5	12.1	39.9	-	-	4.5	-
PU-1000-	70º	36.7	25.5	34.1	-	2.2	1.4	-
ATH	50º	36.7	22.7	36.0	-	2.0	2.6	-
	30º	38.3	19.3	37.1	-	2.1	3.1	-

Data precision, ±5%

Protein Interactions

ATH Surface Density

ATH uptake on PU-PEO surfaces derivatized with NHS functional groups was quantified using the radiolabelled complex. In previous work using gold as substrate, it was found that grafted PEO (MW=1100) limited the uptake of ATH, presumably due to protein resistance of the PEO.[30] It was hoped that the present work on polyurethane substrate using a range of PEO MWs might indicate the optimum PEO MW for high protein resistance and high uptake of ATH (and subsequent high AT binding in blood contact). Figure 3 shows typical data. The unmodified PU showed high ATH uptake, close to that expected for monolayer coverage (~0.23 $\mu g/cm^2$), assuming a Stokes radius for ATH similar to that of AT.[46] However, upon treatment of the surface with SDS most of the ATH was removed, suggesting relatively weak physical adsorption. Of the NHS-activated PEO grafted surfaces, the highest uptake of ATH was on the PEO 300 surface; the density on this surface was close to that expected for a monolayer. Uptake of ATH was lower and essentially the same on the other PEO-NHS surfaces. In contrast to unmodified PU, only a small fraction of the bound ATH was removed from the PEO-NHS surfaces by SDS, suggesting that relatively strong (presumably covalent, NHS-amine reaction) binding was involved. The uptake of ATH on a representative PEO (MW 1000) surface, not NHS-activated, was similar to that on the PEO-NHS surfaces, but almost all of the ATH was eluted by SDS, providing further evidence that a stable amide bond is involved in attachment to the PEO-NHS surfaces.

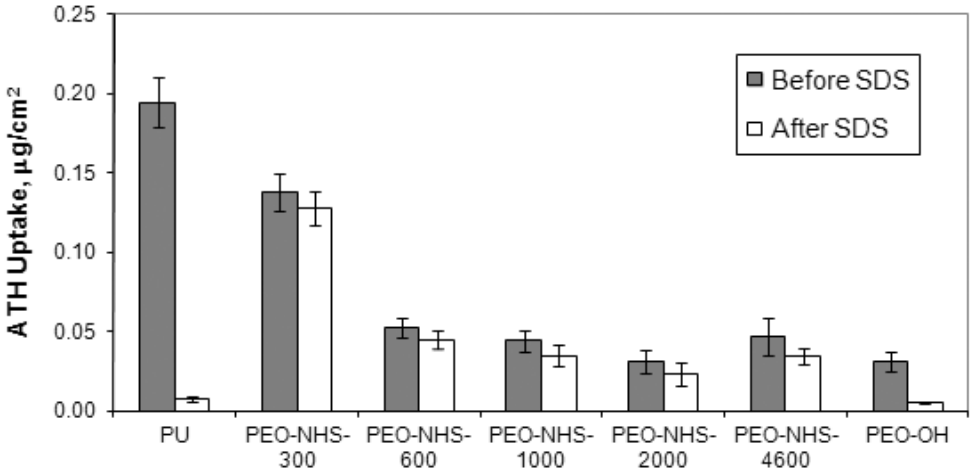

Figure 3. Density of ATH on surfaces after incubation in 0.1 mg/ml ATH. Surfaces were subsequently incubated in 2% SDS and the ATH density measured again. PEO-OH is a representative PEO-OH surface with PEO MW = 1000. Data are means ± SD, n = 3.

Fibrinogen Adsorption

To assess the ability of the surfaces to resist non-specific protein adsorption, fibrinogen adsorption from buffer was investigated. Fibrinogen is an important protein in blood-material interactions due to its role in coagulation and platelet adhesion. Since surface grafted PEO has been shown to inhibit fibrinogen adsorption on various surfaces,[42-44] adsorption data can also provide an indication of successful PEO attachment. Figure 4 shows typical data. Unmodified PU adsorbed ~0.6 µg/cm^{2}of fibrinogen. This quantity is close to that expected for monolayer coverage if fibrinogen of MW 340 kDa is considered to be rod-shaped with dimensions of 450 Å length and 60 Å width.[47] A closely packed monolayer of fibrinogen could then range from ~0.21 µg/cm^2 for side-on orientation to ~1.57 µg/cm^2 for end-on orientation. The PU-ATH adsorbed less than the PU, but still in the monolayer range. Following modification of PU with

PEO, adsorption decreased significantly. There was no clear trend with PEO MW in the 300-2000 range with reductions of ~80% for all MWs compared to PU. Others have shown that increasing PEO MW is correlated with increasing protein resistance up to a value of the order of a few thousand Daltons.[13,14,45] The advantage of attaching ATH to PU through PEO is apparent in comparison to direct attachment (PU-ATH surface). In general the presence of ATH on the distal chain end of the PEO did not cause a significant increase in fibrinogen adsorption. For the 300 MW PEO surface, attachment of ATH caused a slight increase, possibly due to the relatively high density of ATH on this surface (Figure 3). To take account of differences in ATH density, fibrinogen adsorption was normalized to the surface density of ATH (Table 3). The PEO-300, PEO-600 and PEO-1000 showed the lowest fibrinogen:ATH ratios, suggesting that low to mid range PEO MW may provide the optimum balance of protein resistance and ATH uptake.

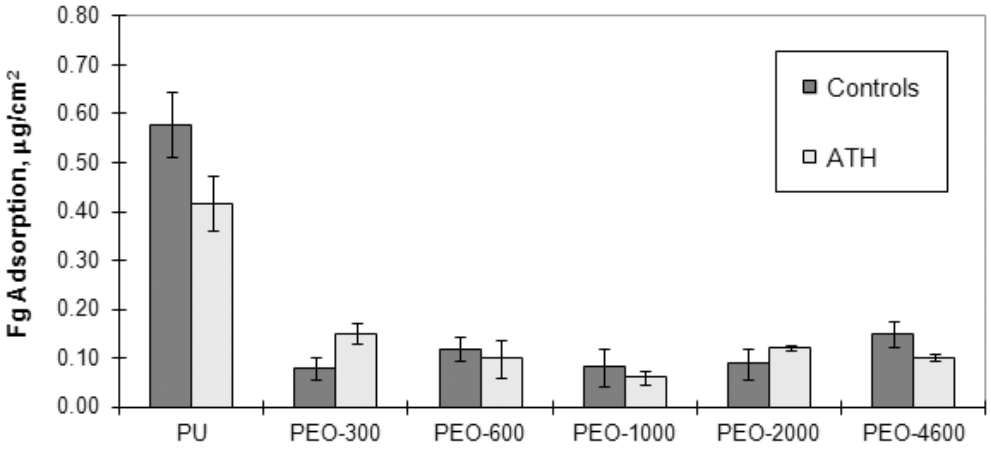

Figure 4. Fibrinogen adsorption from buffer (1 mg/ml, 3 h). Data are means ± SD, n = 3.

Table 3. Fibrinogen and antithrombin adsorption normalized to ATH surface density.

	Molar Ratio Fg to ATH	Molar Ratio AT to ATH
PU	13.4	8.88
PEO-300	0.266	0.379
PEO-600	0.504	1.08
PEO-1000	0.400	0.646
PEO-2000	1.20	1.25
PEO-4600	0.668	0.354

Antithrombin Adsorption

The adsorption of radiolabelled AT from plasma to the ATH-modified surfaces was measured to determine the ability of the heparin moieties to select and bind AT (as occurs in the heparin-catalyzed inhibition of thrombin) in the presence of other plasma proteins. As seen in Figure 5, the PU and PU-PEO-OH surfaces, independent of PEO MW, showed very little adsorption of AT from plasma. The corresponding ATH-modified surfaces bound much higher amounts. PU-ATH showed high adsorption of AT corresponding to the high ATH density on this surface (Figure 3). However adsorption of fibrinogen was also high. In addition, ATH adsorbed to this surface was easily removed (Figure 3). It is evident that on PEO-ATH surfaces, the highest AT selectivity was achieved with PEO of lower MW (PEO 300 and PEO 600). Interestingly, although the PEO-600-ATH surface had a lower density of ATH than the PEO-300-ATH (Figure 3), it bound a similar amount of AT. This is further emphasized by comparing AT binding normalized to ATH density as shown in Table 3. The PEO-300 surface showed a low AT:ATH ratio. Although the highest ratio was seen for PEO-2000, this surface also had

the highest fibrinogen:ATH ratio. The PEO-600 showed a high AT:ATH ratio combined with high resistance to fibrinogen adsorption. It is possible that ATH attached distally to PEO of MW 600 (as opposed to higher or lower MW) may be optimally oriented/configured for AT binding. Previous work with gold as substrate showed that AT binding from plasma to analogous heparin-modified surfaces was lower than on ATH-modified ones.[30] This result is likely due to the competitive effect of other plasma proteins and shows the superiority of ATH for AT selectivity. The ability of immobilized ATH to interact with AT through its heparin moiety suggests that the heparin catalytic function is intact. Increased anticoagulant activity by heparin inhibition of thrombin and FXa may thus be expected.

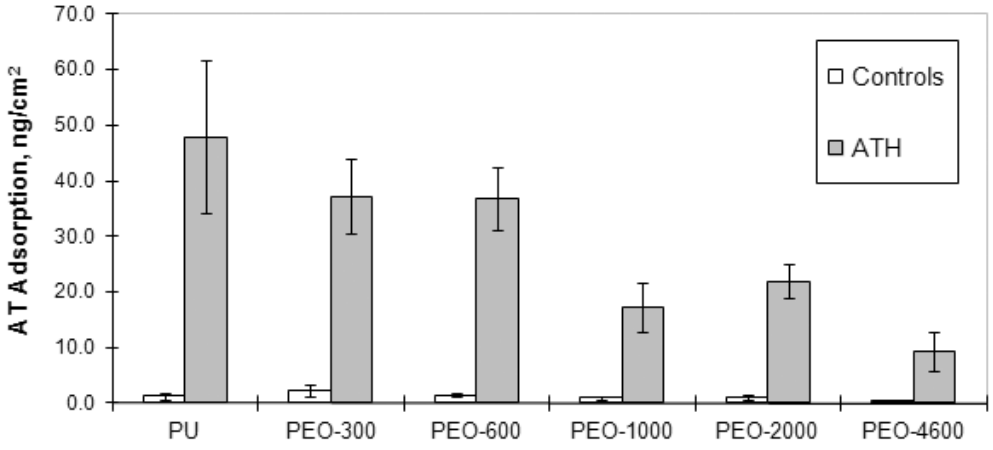

Figure 5. Antithrombin adsorption from plasma (3h). Data are means ± SD, n = 3.

SDS-PAGE and Western Blotting

SDS-PAGE and Western blots of proteins eluted from surfaces exposed to plasma were used to provide further information on protein-surface interactions. For each surface the areas, plasma volumes, adsorption times and volumes of eluate loaded on the gels were the same, thus allowing valid comparisons among immunoblot responses for a given protein on the different surfaces. Typical blots for unmodified PU, PU-PEO and PU-PEO-ATH surfaces are shown in Figure 6.

The unmodified PU surface adsorbed significant amounts of almost all of the proteins probed for. Fibrinogen and vitronectin, both well known as cell-adhesion promoting proteins, showed strong blot responses. The adsorption of these proteins is likely to facilitate platelet adhesion. Complement C3 (bands ~70 and 45 kDa) showed a weak response. The fragment at ~45 kDa is likely a degradation product of C3b and indicates that complement was activated.[48] Apolipoprotein AI, a protein that has been found on many surfaces on blood or plasma contact,[49,50] appeared as an intense band at 28 kDa. Albumin, the major protein component of plasma, was also strongly evident on the PU surface.

On both the PEO-600 and PEO-1000 surfaces the blot responses were weaker than on the PU. The PEO-1000 surface showed generally weaker responses than PEO-600, especially for fibrinogen, C3 and apo AI. The intensity of the vitronectin band was considerably lower than on PU for both the PEO-600 and PEO-1000. The albumin response was strong on both PEO surfaces, possibly due to the putative affinity of albumin for PEO,[51] or simply due to the high concentration of albumin in plasma.

The PEO-600-ATH and PEO-1000-ATH surfaces also showed weaker responses than the PU, in fact similar to those of the precursor PEO surfaces. The fibrinogen band was slightly stronger for the PEO-1000-ATH surface than for the PEO-1000, but not the PEO-600-ATH compared to the PEO-600. On both ATH surfaces the C3 response was weaker than on the PEO precursors, indicating that some "shielding" of PEO by ATH might have been occurring in this case. AT was not visible on the blot for PEO-1000-ATH, but appeared as a very faint band on the PEO-600-ATH. This is an unexpected result in view of the data showing significant quantities of AT on these surfaces as measured by radiolabelling (Figure 5). The blot data are, however, coherent with the radiolabelling experiments in that adsorption was greater on PEO-600-ATH than on PEO-1000-ATH in both cases. Adsorption on PEO-1000-ATH in the labelling experiments was significantly greater than on its precursor, but AT was not detected on the blot. Thus, the blot immunological sensitivity for AT was apparently insufficient to allow detection of the small amount adsorbed. Blot responses for the other proteins investigated were as weak as on the PEO precursor surfaces indicating that the PEO retained its protein resistance even with ATH attached distally.

The blot data again suggest that selective AT adsorption and general protein resistance on these dual functioning surfaces may be optimum for PEO MW in the low range.

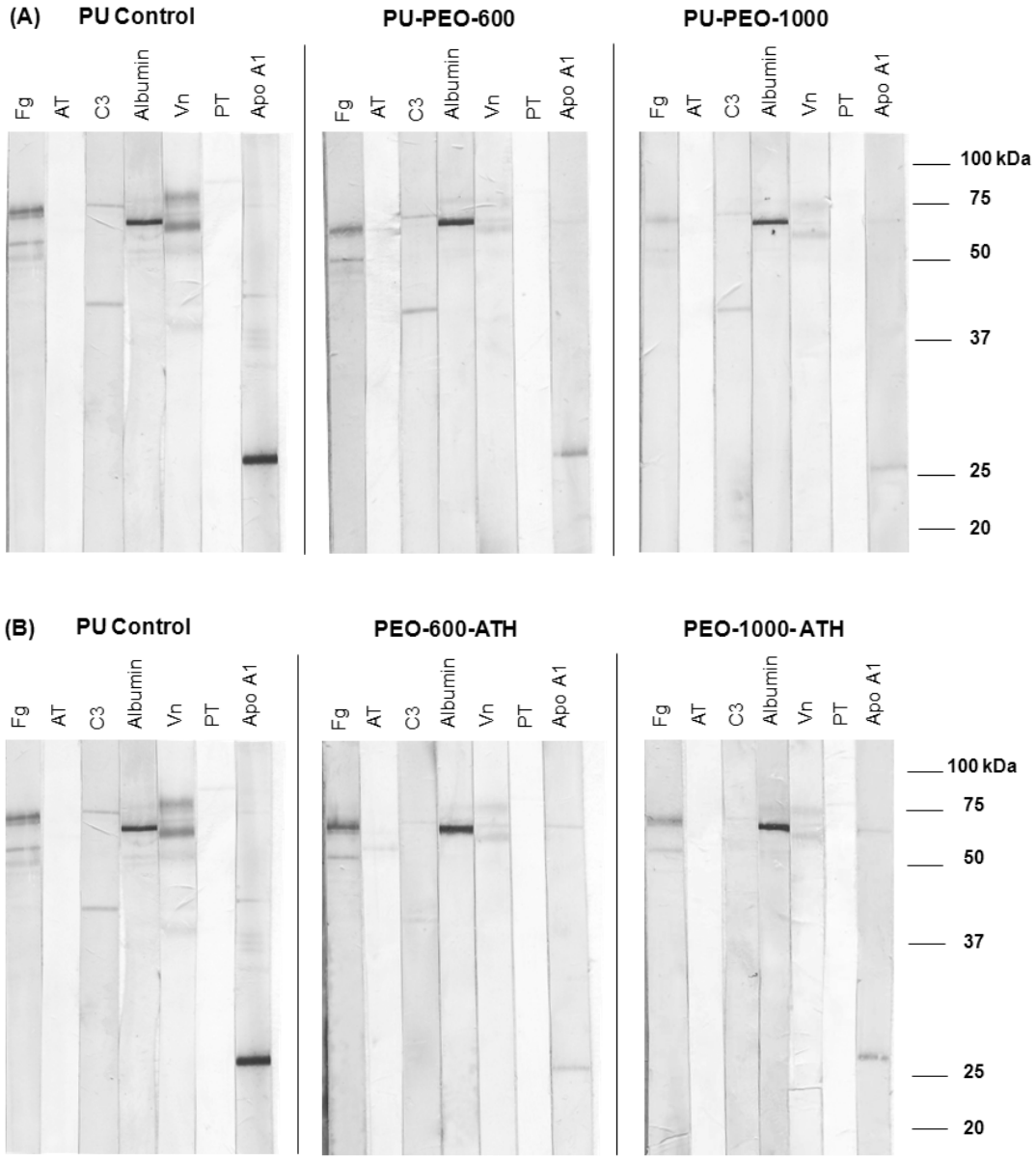

Figure 6. Western blots of proteins eluted (SDS) from surfaces following incubation in plasma (3h). (A) PU, PEO-600, PEO-1000. (B) PU, PEO-600-ATH, PEO-1000-ATH. The blots were probed with antibodies directed against fibrinogen (Fg), complement C3 (C3), antithrombin (AT), albumin, vitronectin (Vn), prothrombin (PT) and apolipoprotein A1 (Apo A1).

Platelet Adhesion

Platelet adhesion from flowing whole blood was measured using a cone-and-plate device (Figure 7). Unmodified PU was compared to surfaces modified with PEO of MW 1000 due to its high resistance to protein adsorption (Figures 3 and 6). The unmodified PU showed significantly more platelet adhesion than any of the modified surfaces. The PEO (MW 1000) grafted PU showed the greatest decrease, followed by the PU-PEO-1000-ATH and PU-ATH; platelet density was similar on all three modified surfaces. The decreases seen on PU-PEO and PU-PEO-ATH are consistent with the low levels of the platelet adhesive proteins, fibrinogen and vitronectin, adsorbed on these surfaces from plasma (Figure 6). In previous work on surfaces with gold as substrate, platelet adhesion was found to be as low on a PEO-ATH surface as on its PEO precursor, again indicating that the distal ATH did not eliminate the protective effect of the PEO to any significant extent.[31] Adhesion was also low on the PU-ATH surface prepared by directly attaching PU to ATH. This suggests that the ATH itself may be relatively inert to platelets, possibly via the favourable composition of the adsorbed protein layer which is expected to have a high AT content. These results are limited in that modifications were only performed with PEO of MW 1000. It would be of interest to also investigate the PEO-600-ATH surface due to its high protein resistance and AT binding. However, due to the large sample size needed for cone-and-plate studies and the limited availability of ATH, only one MW was investigated.

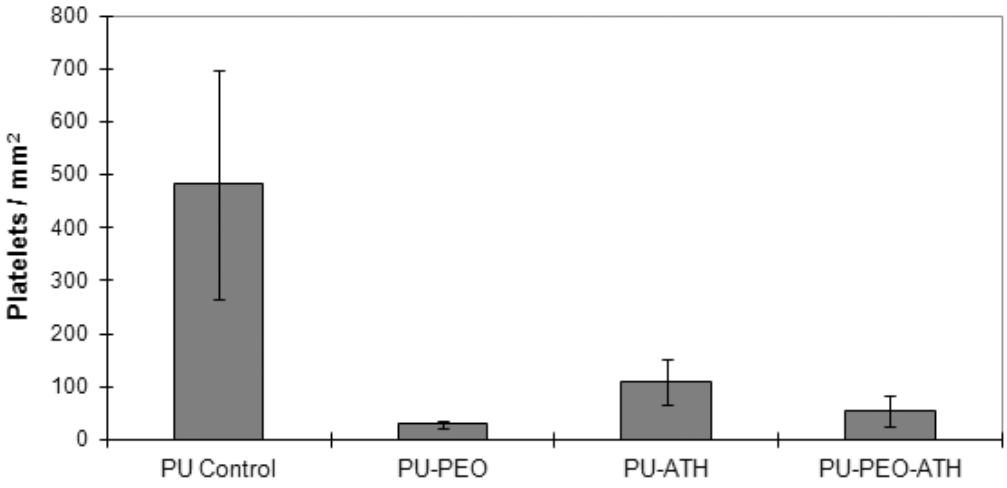

Figure 7. Platelet adhesion from human whole blood under flow (300 s^{-1}, 15 min). PEO MW is 1000. Data are means ± SD, n ≥ 3.

Conclusions

A biomedical grade segmented polyurethane was modified using isocyanate chemistry to enable grafting of PEOs of varying molecular weight, 300 to 4600. The hydroxyl end groups of grafted PEO were converted to NHS to facilitate attachment of the anticoagulant ATH. Water contact angles, XPS data and fibrinogen adsorption data indicated that the modification reactions were successful. Of the PEO modified surfaces (PU-PEO) uptake of ATH was highest on the surface with the PEO of lowest MW. The surfaces with ATH conjugated to PEO showed greater selectivity for antithrombin uptake from plasma, compared to controls. The PEO-ATH surfaces with PEO of MW 300 and 600 adsorbed the highest quantities of AT from plasma. Protein adsorption from plasma was investigated by Western blot analysis of eluted proteins and platelet adhesion was measured from whole blood. Both protein adsorption and platelet adhesion were low on the PEO and PEO-ATH modified polyurethane.

It is concluded from the data as a whole that a low to mid range PEO MW, e.g. MW 600, may be optimal for minimizing non-specific (and undesirable) protein adsorption, while allowing uptake of ATH and high subsequent AT binding for anticoagulant effect.

Acknowledgement

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CHAPTER 6: PAPER FOUR - Polyurethane Modified with an Antithrombin-Heparin Complex (ATH) via Polyethylene Oxide (PEO) Linker/Spacers: Influence of PEO Molecular Weight and PEO-ATH Bond on Catalytic and Direct Anticoagulant Functions

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Objectives:

The influence of PEO molecular weight and end group was investigated to determine an optimal method of attachment of ATH to polyurethane for anticoagulant effect. The hydrophilicity and protein resistance of PEO-grafted surfaces were measured to confirm modifications. Antithrombin and thrombin adsorption studies were performed to provide an indication of the catalytic and direct antithrombin potential of these surfaces.

Main Contributions:

1. ATH was immobilized on PU through grafting of homo-bifunctional dihydroxy-PEO and hetero-bifunctional amino-carboxy-PEO for comparison.

2. All functionalized surfaces showed significant uptake of ATH. The PEO-ATH surfaces had low adsorption of fibrinogen and high selectivity for AT demonstrating their catalytic antithrombin capacity.

3. Thrombin adsorption was found to be significant on PEO-ATH surfaces, with the hydroxyl-terminated PEO of lower MW showing the highest binding. These results suggest that ATH immobilization can also provide direct thrombin inhibition through AT.

4. The properties of the PEO spacer arm (length and end functional groups) may affect the orientation of ATH on the surface, in turn influencing the catalytic and/or direct anticoagulant activity

Polyurethane modified with an antithrombin-heparin complex (ATH) via polyethylene oxide (PEO) linker/spacers: Influence of PEO molecular weight and PEO-ATH bond on catalytic and direct anticoagulant functions

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Abstract

A segmented polyurethane (PU) was modified with polyethylene oxides (PEO) of varying molecular weight and end group. The PEO served as linker/spacers to immobilize an antithrombin-heparin (ATH) anticoagulant complex on the PU. Isocyanate groups were introduced into the PU to enable attachment of either "conventional" homo-bifunctional dihydroxy-PEO (PEO-OH surface) or a hetero-bifunctional amino-carboxy-PEO (PEO-COOH surface). The PEO surfaces were functionalized with N-hydroxysuccinimide (NHS) groups using appropriate chemistries, and ATH was attached to the distal NHS end of the PEO (PEO-OH-ATH and PEO-COOH-ATH surfaces). Water contact angle and fibrinogen adsorption measurements showed increased hydrophilicity and reduced fibrinogen adsorption from buffer on all PEO surfaces compared to unmodified PU. ATH uptake on NHS-functionalized PEO was quantified by radiolabelling. Despite the different PEO molecular weights and end groups, and NHS-functionalization chemistries, the surface densities of ATH were similar. The adsorption of fibrinogen and antithrombin (AT) from plasma was measured in a single experiment using dual radiolabelling. On PEO-ATH surfaces fibrinogen adsorption was minimal while AT adsorption was high showing the selectivity of the heparin moiety of ATH for AT. The PEO-COOH-ATH surfaces showed slightly greater AT adsorption than the PEO-OH-ATH surfaces. Thrombin adsorption on all of the PEO-ATH surfaces was greater than on the corresponding PEO surfaces without ATH, and was highest on the PEO-OH-ATH, suggesting potential anticoagulant properties for this surface via direct thrombin inhibition by the AT portion of ATH.

Introduction

Surface-induced thrombosis is an unresolved problem for blood contacting devices and generally leads to device failure. The adsorption of plasma proteins is the initiating event in thrombus formation. Consequently, various methods of surface modification using hydrophilic polymers have been developed to render the surface protein resistant.[1-4] Modification with polyethylene oxide (PEO) is by far the most popular method for this purpose. PEO is also effective as a spacer/linker agent for subsequent attachment of biomolecules.[5] Some conjugation methods use homo-bifunctional PEO derivatives (e.g. dihydroxy PEO), but the use of hetero-bifunctional PEO provides a more targeted approach.[6] With an appropriately designed hetero-bifunctional PEO, one chain end function can be chosen to react specifically with groups on the surface and the other to react with bioactive agents such as anticoagulants.

Thrombin is a key enzyme in the blood coagulation pathways, participating in several of the reactions in the cascade including the conversion of fibrinogen to fibrin. It is also a potent platelet activator. Inhibition of thrombin is therefore essential to prevent or reduce surface-induced thrombosis. Several direct thrombin inhibitors, i.e. agents that block the active site of thrombin, have been explored as anticoagulants. These include hirudin, PPACK and other peptides.[7-9] Hirudin is a strong inhibitor of both free and clot-bound thrombin and has been used to modify various biomaterials to prevent surface-induced thrombosis.[10-12] However, such direct thrombin inhibitors have the disadvantage that their capacity to inhibit thrombin is limited to a single interaction, generally involving formation of a “tight” complex.

Heparin is the most widely used thrombin inhibitor, both systemically and as a surface modifier. Heparin inhibits thrombin indirectly. It binds specifically, via an active pentasaccharide sequence, to endogenous antithrombin (AT) in the circulating blood. The AT then undergoes a conformational change that allows it to bind and inhibit thrombin at a much more rapid rate than in the absence of heparin, i.e heparin acts as a powerful catalyst for the thrombin-antithrombin interaction.[13] The thrombin-antithrombin (TAT) complex dissociates from the heparin, thus allowing the heparin to bind AT again and repeat the cycle.[14] Heparin immobilization on surfaces to reduce thrombosis has been investigated extensively resulting in commercially available heparin-based coatings for medical devices.[15-18] Yet the use of heparin, both systemically and as a surface modifier, has numerous limitations. These include the inability to inactivate thrombin bound to fibrin clots,[19] the low and variable efficacy of standard heparin products[20,21], and the fact that heparin binds many other plasma proteins besides antithrombin[22]. Strategies have been explored to attach heparin to various substrates through a PEO spacer to reduce protein adsorption and cell adhesion, and to enhance heparin bioactivity.[23-25] However clinical studies on heparinized surfaces in general have produced mixed results.[26-29]

In the present work, a covalent antithrombin-heparin complex (ATH) designed to address the limitations associated with heparin,[30] was used to modify the surface of a commercially available polyurethane (PU). The combination of antithrombin and heparin in ATH provides both recycling catalytic activity via the heparin component, and direct thrombin inhibition via the antithrombin. It also offers many other advantages including the ability to inhibit clot-bound thrombin, restricted access for plasma protein binding and

high anticoagulant activity.[31] We have previously reported on the surface modification of PU with ATH using "conventional" dihydroxy-PEO having a range of molecular weights as a spacer/linker.[32] Protein adsorption studies demonstrated that PEO of low to mid range was more effective at balancing protein repulsion effects with ATH binding and subsequent attraction of AT.

The present work extends these previous studies. The objectives were (1) to investigate a hetero-bifunctional PEO as the ATH linker/spacer for comparison to homo-bifunctional dihydroxy-PEO, and (2) to determine the relative contributions of the heparin (catalytic) and AT (direct) components of immobilized ATH to its anticoagulant properties. The PEO end group chemistry was varied and modification techniques were evaluated based on resistance to protein adsorption and ATH uptake. The catalytic activity of the surfaces was estimated by measurements of AT adsorption from plasma. Direct thrombin inhibition was assessed by measuring thrombin binding.

Materials and Methods

Materials

A biomedical grade PU, Tecothane® (TT-1095A), was from Thermedics® Polymer Products (Wilmington, MA). N,N-dimethylformamide (DMF), DriSolv toluene (anhydrous), DriSolv acetonitrile (anhydrous) and triethylamine (TEA) were from EMD Chemicals (Gibbstown, NJ). Dihydroxy-PEO of molecular weight (MW) 600 and 1000, 4,4'-methylene-bis (phenyl-diisocyanate) (MDI), N,N'-disuccinimidyl carbonate (DSC), and heparin (sodium salt, from porcine intestinal mucosa), were from Sigma Aldrich

(Oakville, ON). α,ω Amino-carboxy-PEO of similar molecular weight (600 and 1000) was from Polymer Source (Dorval, QC). 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide (EDC) and N-hydroxysuccinimide (NHS) were from Thermo Scientific (Rockford, IL). $Na^{125}I$ was from the McMaster Nuclear Reactor (Hamilton, ON). Human fibrinogen was from Enzyme Research Laboratories (Southbend, IN) and human antithrombin (AT) was from Affinity Biologicals (Ancaster, ON). Platelet poor plasma was prepared from citrated blood, pooled from multiple healthy human donors (McMaster University ethics protocol #04-046) and stored at -70°C. The preparation of ATH has been described in detail previously.[30] Briefly, AT and heparin, at a molar ratio of 1:200, were incubated for 14 days at 40°C. Subsequently, $NaBH_3CN$ was added and incubation continued for 5 h at 37°C. ATH was purified by butyl agarose and DEAE Sepharose chromatography in a two-step procedure.

Surface Preparation

The surface preparation methods have been described previously.[32] Briefly, films of the PU were cast from a 5% (w/v) solution in DMF and the solvent was slowly evaporated at 70ºC for 48 h. Discs of 6 mm diameter were punched from the PU films, rinsed in ethanol and dried at 60ºC in a vacuum oven. The first step of the PU modification procedure involved reacting PU with MDI (anhydrous toluene, 7.5%) at 50ºC as described previously.[32,33] TEA (2.5%) was used as catalyst. The reaction was carried out for 100 min and the films were then rinsed three times in toluene to yield PU-NCO films. In some cases, the PU-NCO films, after drying under vacuum, were modified

directly with ATH. For dihydroxy-PEO attachment, PU-NCO films were incubated with PEO (1%, toluene) of MW 600 or 1000 for 24 h at 40ºC, to give PU-PEO-OH surfaces. Hetero-bifunctional amino-carboxy-PEO was attached to the PU-NCO films after drying under vacuum by incubation with PEO (1%, acetonitrile) for 24 h at 40ºC, giving PU-PEO-COOH surfaces. Following the PEO attachment reactions, films were rinsed in Milli-Q water, dried and stored until needed. To convert "distal" OH groups to N-hydroxysuccinimide (NHS) on PU-PEO-OH, films were incubated with DSC (1 mmol, acetonitrile) and TEA (1 mmol) for 6 h at room temperature.[34] PU-PEO-COOH films were incubated with EDC (5 mmol, acetonitrile) and NHS (5 mmol) for 6 h at room temperature. For ATH attachment, PEO films were rinsed in acetonitrile, dried and dipped in PBS prior to overnight incubation in 0.1 mg/ml ATH.

Surface Characterization

The relative hydrophilicity of unmodified and modified surfaces was compared by measuring water contact angles using the sessile drop method (Ramé-Hart NRL goniometer, Mountain Lakes, NJ) and angles were recorded on both sides of films.

Adsorption of fibrinogen from buffer was used to assess resistance to non-specific protein adsorption. Fibrinogen was radiolabelled with ^{125}I using the iodine monochloride method and passed through an AG 1-X4 resin column to remove free iodide. Remaining free iodide was determined by trichloroacetic acid (TCA) precipitation and was always below 1%. Fibrinogen (5 % labelled, 95% unlabelled) dissolved in PBS, pH 7.4, at a final concentration of 1 mg/mL was used for adsorption experiments. Films were incubated in

the fibrinogen solution for 2 h at room temperature, rinsed in PBS and counted for radioactivity.

Surface Density of ATH

The density of ATH on the various modified films was determined using radio-iodinated ATH. ^{125}I labelling was performed using the Iodogen method (Pierce Iodination Reagent, Thermo Scientific, Rockford, IL). Unreacted iodide was removed by dialysis against PBS, pH 7.4, with three changes of buffer. Using the TCA method, the remaining free iodide was found to be below 2%. ATH (2% labelled, 98% unlabelled) was dissolved in PBS at a concentration of 0.1 mg/mL and incubated with films overnight at 4ºC. Films were then rinsed in PBS and their radioactivity counted. They were incubated overnight in 2% aqueous sodium dodecyl sulphate (SDS) and the radioactivity counted again.

Fibrinogen and AT Adsorption from Plasma

The adsorption of fibrinogen and AT from plasma was measured simultaneously using double labelling: fibrinogen was labelled with ^{125}I and AT with ^{131}I. The labelled proteins were present in the plasma in amounts corresponding to 5% of their average endogenous concentrations. Films were incubated in plasma for 2 h at room temperature. Radioactivity was counted at the same time for both isotopes.

Thrombin Binding

To determine the quantity of thrombin able to bind to ATH-modified PU, thrombin was radiolabelled with ^{125}I using the Iodogen method. Free iodide was removed by dialysis against PBS, pH 7.4. Thrombin (10% labelled, 90% unlabelled) was added to PBS to give a final protein concentration of 0.1 mg/mL. The films were incubated in the thrombin solution for 2 h, rinsed and assessed for radioactivity. They were then incubated overnight in 2% aqueous SDS to remove physically adsorbed protein and radioactivity was determined again.

Statistical Analysis

Data are reported as means ± standard deviation (SD). Significant differences within data sets were assessed using analysis of variance (ANOVA). Student's t-test was used to compare sets when differences existed. Differences were considered significant for $p < 0.05$.

Results and Discussion

Surface Preparation

The surface preparation and modification reactions using the dihydroxy-PEO were as described previously,[32] except that the concentration of dihydroxy-PEO was decreased to match that used in the alternative amino-carboxy-PEO procedure, thus allowing comparison between the two methods. The steps in PU surface modification are shown in Figure 1 for both PEO types. PU films were reacted with MDI to introduce isocyanate

(NCO) groups. One hydroxyl end group of dihydroxy-PEO or the amino end group of amino-carboxy-PEO was reacted with the PU-NCO surface. Since the dihydroxy-PEO is susceptible to loop formation if both end groups attach to the surface, it was used in excess. This possibility is also minimized by the use of the hetero-bifunctional PEO. In addition, it is expected that the amino groups will be more reactive than the hydroxyl groups.[35] The PEO distal ends were then functionalized with NHS groups by reaction with either DSC (PEO-OH) or EDC/NHS (PEO-COOH). Finally ATH was attached to the resulting PU-PEO-NHS by reaction of NHS groups with amino groups of ATH. Modification with dihydroxy-PEO was confirmed by low and high resolution x-ray photoelectron spectroscopy (XPS) in previous work[32].

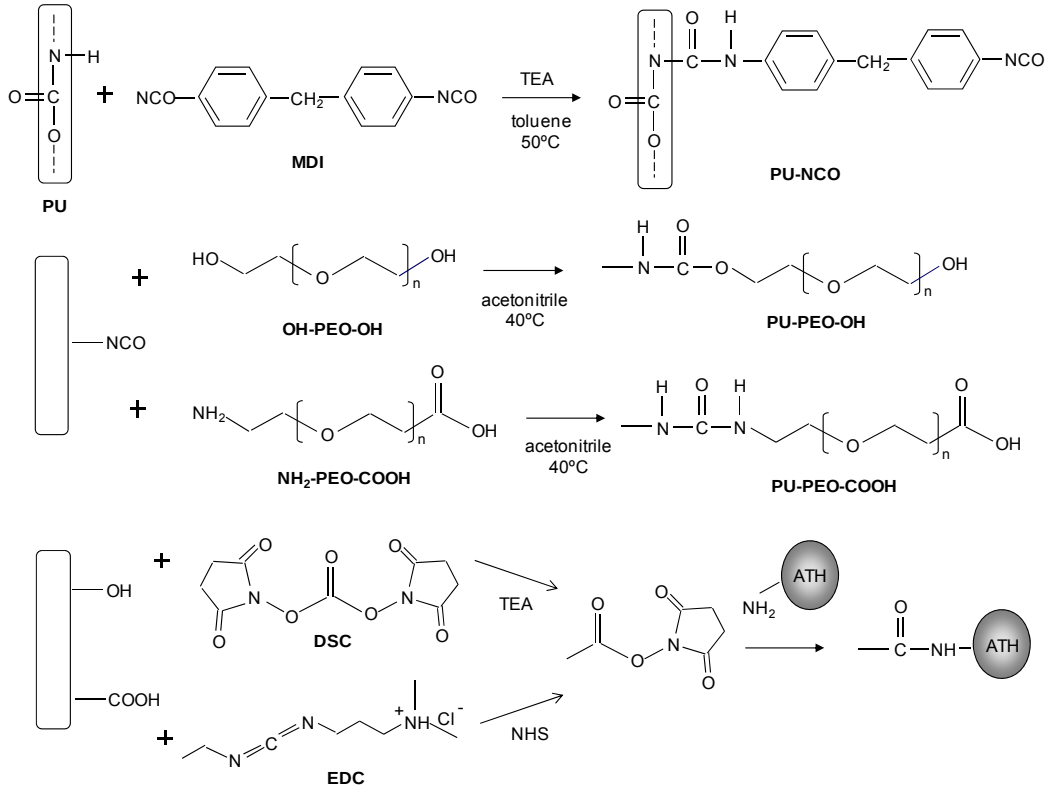

Figure 1: Polyurethane (PU) surface modifications using conventional polyethylene oxide (PEO) and hetero-bifunctional PEO.

Surface Characterization

Water contact angle data are shown in Figure 2. For the unmodified PU, the advancing angle was high (>100º); this was the most hydrophobic of the surfaces investigated. The receding angle was only slightly lower at ~90º, indicating mild hysteresis. After modification with dihydroxy-PEO (PEO-OH), the angles decreased significantly to ~50º (advancing) and ~30º (receding), for both MW 600 and 1000 PEO. This result is in agreement with previous work using these modification methods, and suggests that attachment of dihydroxy-PEO was successful.[32] Modification with the amino-carboxy-PEO (PEO-COOH) resulted in advancing and receding angles slightly greater than those of the PEO-OH surfaces for both MWs, but still significantly lower than the PU control. These differences may reflect the different effects of the two

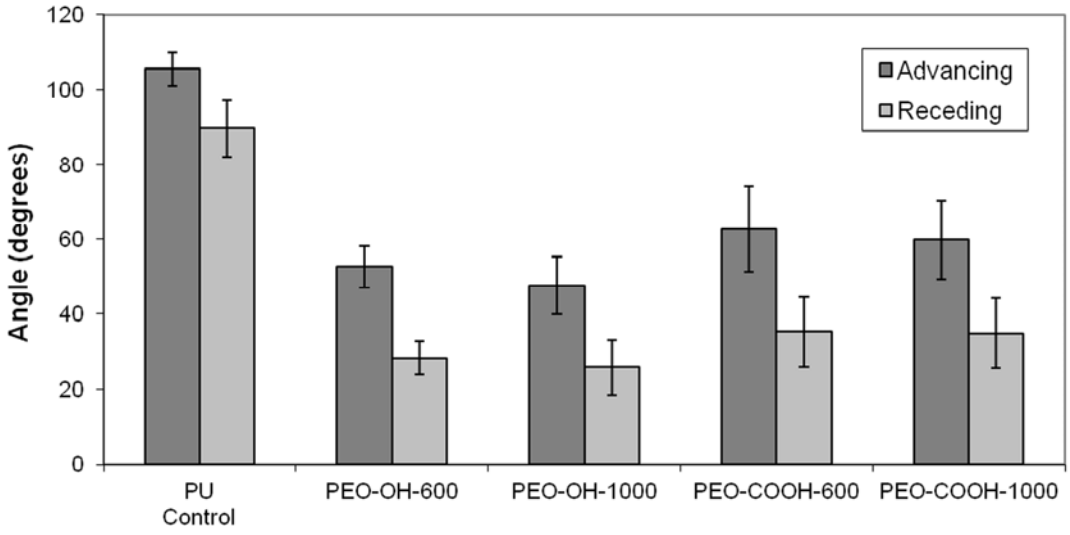

Figure 2: Water contact angles. Data are means ± SD, n ≥ 9.

end groups OH and COOH, and/or different surface coverage of the PEO. In a study on self-assembled monolayers with varying chain end functions, a COOH surface showed greater contact angles than OH.[36] Overall, the decreases in water contact angles following PEO modification indicate successful attachment.

Fibrinogen Adsorption

The effectiveness of PU modification with PEO of different MWs, end groups, and attachment chemistries in terms of resistance to protein adsorption was assessed by measuring fibrinogen adsorption from buffer. Fibrinogen is a key protein in blood coagulation and is converted to fibrin by thrombin in the final step of fibrin formation. Platelets also interact with adsorbed fibrinogen leading to platelet adhesion, activation and aggregation.[37] Data on fibrinogen adsorption to control and modified surfaces are shown in Figure 3. The unmodified PU adsorbed 0.82 ± 0.20 μg/cm^2. Based on approximate dimensions of fibrinogen, as a rod with of length 450 Å and diameter 60 Å, [38] this quantity is within the range that would be expected for a close packed end-on-oriented monolayer.[39]

Adsorption was also measured after reaction with MDI (PU-NCO). This surface adsorbed an even greater quantity of fibrinogen, 1.08 ± 0.24 μg/cm^2. In other work on a polyurethane urea (PUU) modified similarly with MDI, fibrinogen adsorption was also found to be higher than on the unmodified PUU.[33] Since isocyanates can readily react with amino groups to form covalent urea linkages, it is likely that the PU-NCO surface bound at least some fibrinogen by reaction with amino groups in the protein.

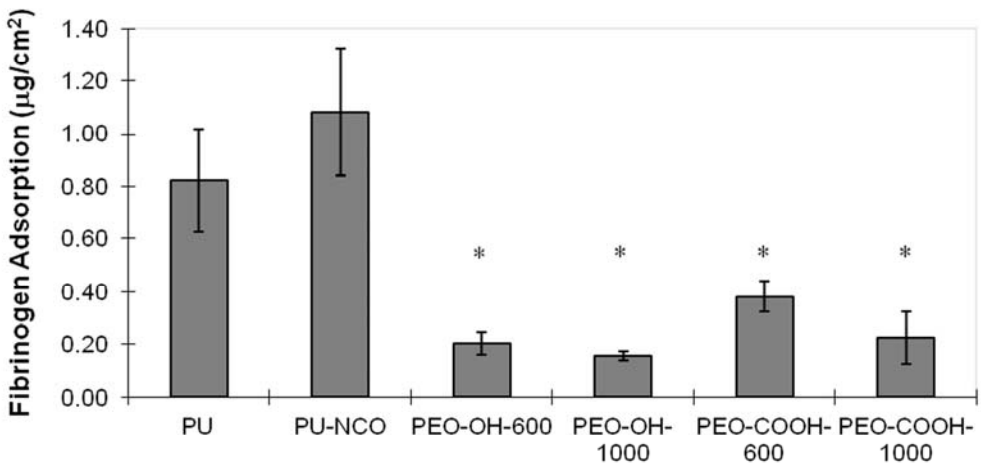

Figure 3: Fibrinogen adsorption from PBS (1 mg/mL, 2 h) on PEO-modified PUs. Data are means ± SD, n = 3. *All PEOs were significantly lower than PU and PU-NCO ($p < 0.05$).

After attachment of dihydroxy-PEO of MW 600 and 1000, fibrinogen adsorption decreased by approximately 75% and 81%, respectively. This is similar to the decrease found for PU-PEO-OH previously,[32] despite the lower concentration of PEO in the present work. The amino-carboxy-PEO surfaces also showed decreased fibrinogen adsorption. The decrease was slightly less than for the dihydroxy-PEO, particularly in the case of the 600 MW polymer. Fibrinogen on these surfaces is likely physi-sorbed, although some chemisorption may occur through any NCO groups that did not react with PEO.

MW and end group chemistry are known to influence protein adsorption to PEO-modified surfaces. For example adsorption has been found to decrease with increasing MW. The effect "plateaus" at MW in the range of a few thousand, beyond which no further decrease is seen.[33,40] Previous work with dihydroxy-PEO and a range of MWs

demonstrated that PEO of MW from 300 to 2000 gave similar levels of fibrinogen adsorption with only small decreases as the MW increased further to 4600.[32] The chain end group of PEO has been shown to have an effect on fibrinogen adsorption, with hydroxy- and carboxy-terminated PEO both demonstrating reduced levels compared to methyl- or methoxy-terminated PEO.[36,41] The present work shows that both PEO-OH and PEO-COOH end groups give protein resistant surfaces with PEO-OH the more effective of the two. These adsorption data confirm the modification reactions with the various PEOs. While the data are limited to fibrinogen in a buffer environment, they also suggest that the surfaces may be generally resistant to the non-specific adsorption of proteins in contact with plasma.

ATH Surface Density

To confirm ATH attachment and to determine its surface density on the PEO modified films, uptake was measured using radiolabelled ATH. Figure 4 shows uptake both before and after treatment with SDS. The unmodified PU adsorbed high quantities of ATH of ~0.33 ± 0.12 μg/cm^2. SDS treatment removed the majority of the ATH suggesting that it was only loosely attached. The PU-NCO adsorbed a greater amount of ATH, and in contrast to the PU, most of the ATH remained after SDS treatment, suggesting that attachment to the PU-NCO surface was much stronger and that ATH may be bound covalently through its amino groups.

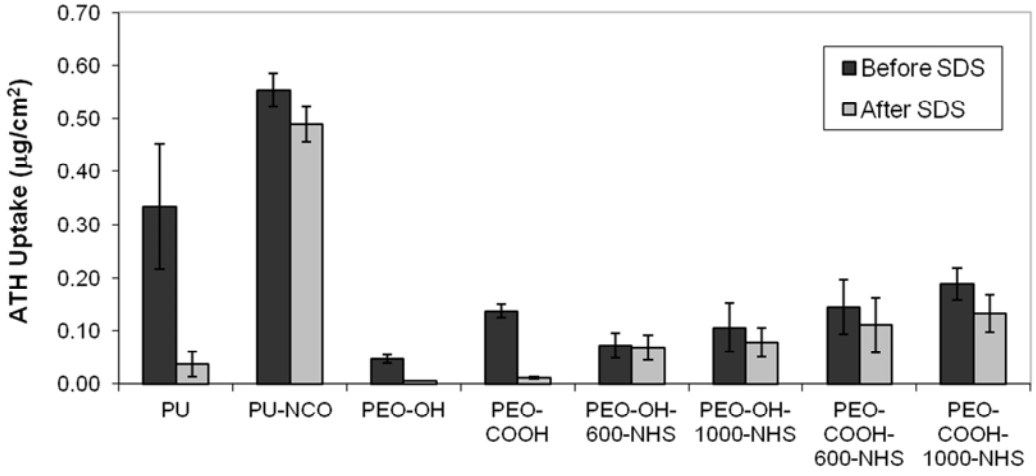

Figure 4: ATH uptake from PBS solution (0.1 mg/mL). Quantities remaining after SDS treatment are also shown. Surfaces are representative PEO-OH and PEO-COOH with MW = 1000.
Data are means ± SD, n = 3.

Uptake of ATH was also measured on PU-PEO surfaces before and after conversion of the end groups to NHS. On both the PEO-OH and PEO-COOH surfaces (MW = 1000) some ATH was adsorbed, but was almost completely removed on SDS treatment. After conversion to PEO-NHS (either PEO-OH-NHS or PEO-COOH-NHS) for both MW 600 and 1000, similar levels of ATH uptake were evident. The PEO-COOH-NHS surfaces showed somewhat greater uptake than the PEO-OH-NHS, but the differences were not significant. In contrast to the precursor PEO surfaces, the PEO-NHS surfaces retained most of the adsorbed ATH after treatment with SDS, suggesting covalent reaction between NHS groups and amino groups in ATH.

The data in Figure 4 show that the hetero-bifunctional PEO did not in general provide a significant increase in the density of ATH compared to the homo-bifunctional dihydroxy-PEO. Factors other than density, including the orientation of ATH on the surface, may provide more optimal conditions for AT binding and thrombin inhibition.

Fibrinogen and Antithrombin Adsorption from Plasma

Simultaneous measurement of fibrinogen and AT adsorption from plasma was performed using dual radiolabelling (Figure 5). On the PU surface, fibrinogen adsorption was less than from a single protein solution, as expected due to competition from other plasma components. The time frame of this experiment was long enough that any displacement of initially absorbed fibrinogen by proteins of higher affinity (Vroman effect) should have occurred.[42] Fibrinogen adsorption on the PU-NCO surface was high and close to that from buffer. Since fibrinogen is an abundant protein in plasma and has high surface activity, it is expected to adsorb rapidly. The high adsorbed quantity observed after 2 h contact suggests that attachment to PU-NCO is strong and probably occurs through covalent bond formation as discussed. Displacement of initially adsorbed fibrinogen by a Vroman effect thus appears unlikely. Both types of PEO-modified PU, i.e. dihydroxy and amino-carboxy, adsorbed slightly less fibrinogen than unmodified PU.

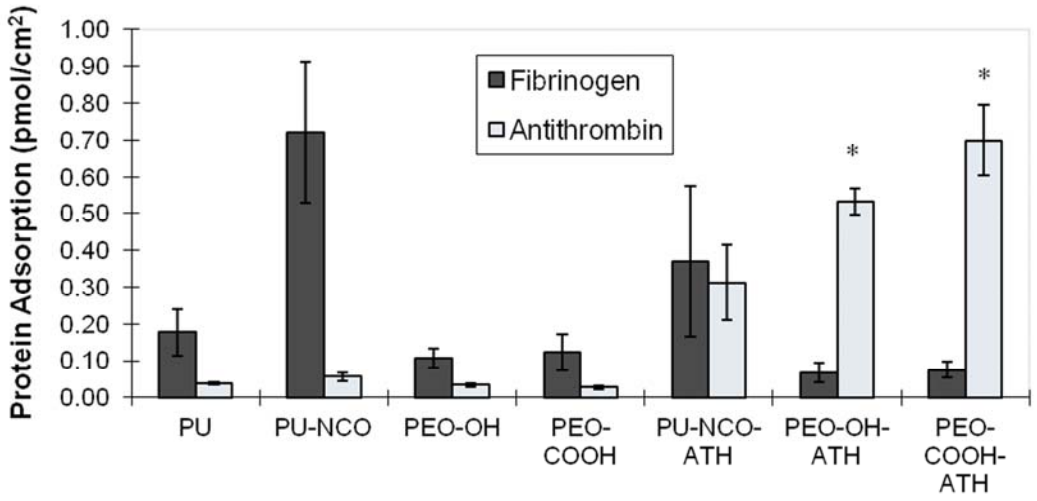

Figure 5: Fibrinogen and antithrombin (AT) adsorption from plasma (2 h). PEO MW = 1000. Data are means ± SD, n = 4. *AT adsorption on PEO-OH-ATH and PEO-COOH-ATH is significantly greater than all other samples ($p < 0.05$).

Antithrombin adsorption on the PU, PU-NCO, PEO-OH and PEO-COOH surfaces was lower than fibrinogen adsorption, presumably due to the lower concentration of AT in plasma (~0.15 μg/mL vs. ~3.0 μg/mL). None of these surfaces has specificity for AT. On the PU-NCO-ATH surface (ATH attached directly) AT adsorption was higher than on its PU-NCO precursor; this is in accord with the high ATH density on this surface (Figure 4). However, fibrinogen adsorption was also relatively high, showing the inability of this surface to resist non-specific adsorption. On the PEO-OH-ATH and PEO-COOH-ATH surfaces, AT adsorption was much higher on the precursor surfaces and fibrinogen adsorption was lower. These results demonstrate the effect of the PEO spacer in "amplifying" the specific AT-heparin interaction, resulting in greater adsorption of AT and reduced (nonspecific) adsorption of fibrinogen. It is expected that these high levels of AT should translate to anticoagulant properties for these surfaces.

In previous work on gold substrates it was found that AT binding to ATH-modified surfaces was predominantly through the active pentasaccharide sequence of the heparin moiety in ATH.[43] It is likely that the selective AT binding observed in the present work is also due to binding through the active sequence of the heparin portion of ATH.

Overall the results of these experiments show that the PEO-OH-ATH and PEO-COOH-ATH surfaces have a strong preference for AT over fibrinogen, suggesting that the ATH on the surface is able to express its specificity for AT and the PEO its resistance to non-specific adsorption.

Thrombin Binding

The catalytic function of immobilized ATH through its heparin moiety has been demonstrated here and in other studies.[32,43-45] ATH also has the potential to inhibit thrombin directly by interacting with the covalently bound AT portion.[30,46] To investigate whether thrombin is able to interact with AT in immobilized ATH, surfaces were incubated with solutions of radiolabelled thrombin in buffer and then with SDS. Thrombin adsorption was then measured (Figure 6).

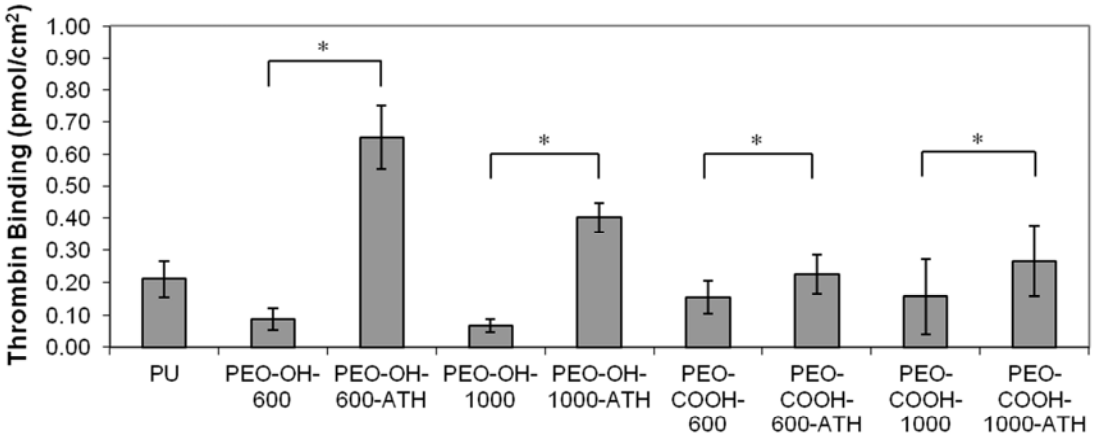

Figure 6: Thrombin adsorption from PBS (0.1 mg/mL, 2 h). Surfaces were treated with SDS prior to measurement. Data are means ± SD, n = 4. * Indicates significant difference between pairs of data ($p < 0.05$).

SDS treatment should remove non-specifically bound thrombin, leaving thrombin that is attached to the AT portion via specific interactions (TAT complex). Thrombin bound in this way should be inhibited. On the PU surface, thrombin adsorption was relatively low; a portion of this may be due to physically attached thrombin that was not removed by SDS. On PEO-OH (600) and PEO-OH (1000) thrombin adsorption was even lower than on PU, thus showing again the resistance of these surfaces to non-specific protein

adsorption. The PEO-COOH surfaces adsorbed greater quantities similar to those seen on the PU, again indicating the role of the PEO chain-end group in the protein interactions of grafted PEO.

The PEO-OH (600)-ATH surface adsorbed and retained approximately 0.65 pmol/cm^2 thrombin, the highest among the ATH-modified PEO surfaces and much higher than any of the controls (no ATH). The PEO-OH-(1000)-ATH adsorbed approximately 0.40 pmol/cm^2 thrombin, less than the MW 600 analogue, but also significantly higher than any of the controls. Some of the thrombin may be physically adsorbed, as on the PU and PEO controls. However, it appears that most of the retained thrombin is bound by specific interaction with ATH and this is expected to be translated to equivalent thrombin inhibition. For the PEO-COOH-ATH surfaces (both MW 600 and 1000), thrombin binding was lower than on the PEO-OH-ATH surfaces, and although not significantly higher than on the PU surface, it was significantly higher than on the precursor PEO-COOH surfaces. It seemed possible that conjugation methods using a hetero-bifunctional PEO might increase ATH binding and consequently allow increased AT binding and inhibition of thrombin. However although AT binding from plasma was slightly greater on the PEO-COOH-ATH surfaces, thrombin adsorption was not. Thus ATH on the PEO-OH-ATH surface was apparently in a more favorable state for the catalytic function of ATH, but not for the direct thrombin inhibition function. These differences may be due to different orientations or conformations on the different surfaces. Additional studies investigating the thrombin inhibitory effects of immobilized ATH and more direct measurements of its anticoagulant activity will be of interest.

Conclusions

ATH was immobilized on polyurethane via PEO spacer/linkers: either dihydroxy- or hydroxy-carboxy-terminated PEO. Both PEO types showed increased surface hydrophilicity, as measured by water contact angles, and decreased fibrinogen adsorption from buffer compared to unmodified polyurethane. The ATH surface density was similar for both PEO types, and the ATH was not removed from the surface by SDS, indicating strong ATH-PEO interactions. In contact with plasma, immobilized ATH adsorbed AT in preference to fibrinogen, indicating strong biospecificity and intact catalytic function for the heparin portion. All PU-PEO-ATH surfaces showed greater thrombin binding capacity than the precursor PEO surfaces, demonstrating their potential to provide direct anticoagulant activity. The surfaces based on hydroxy-terminated PEO of lower molecular weight showed the highest thrombin binding. The results of this work demonstrate the unique ability of immobilized ATH to provide surfaces with "dual" anticoagulant mechanisms, i.e. the catalytic mechanism due to heparin and the direct mechanism due to antithrombin. Different PEO molecular weights and different methods of attachment of ATH to the surface-bound PEO, may give different orientations of ATH with variable catalytic and/or direct antithrombin activity.

Acknowledgements

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CHAPTER 7: SUMMARY & RECOMMENDATIONS FOR FUTURE WORK

7.1. SUMMARY

Thrombotic complications remain a major problem for blood contacting biomaterials. Many different methods of surface modification have been applied to biomaterials to provide them with bioinert and/or appropriate bioactive properties. In this work an antithrombin-heparin covalent complex (ATH) was studied for its ability to reduce thrombosis on biomaterials. Modification with ATH was compared to that with heparin on model gold surfaces with and without the use of a bioinert PEO linker/spacer molecule. ATH was also immobilized on polyurethane to determine conditions for maximal anticoagulant activity and as a potential material for the construction of blood contacting devices.

ATH was immobilized on gold using gold-thiol chemistry. Three methods of attachment were developed: (i) direct chemisorption to gold, (ii) attachment via dithiobis(succinimidyl propionate) (DSP) as a short chain linker molecule, and (iii) attachment via polyethylene oxide (PEO) as a protein resistant spacer molecule. Analogous heparinized surfaces were also prepared using these same immobilization techniques. Various methods of characterization confirmed the modifications and provided information on surface densities of the modifiers. The density of ATH was highest when attached through the DSP linker and lowest with the PEO linker/spacer. This difference is thought to be due to the protein repellent nature of PEO and demonstrates that an optimum ratio of ATH:PEO may be needed to give a balance of anticoagulant effect and protein resistance. The results also suggested that when ATH is

attached via gold thiol chemistry, its heparin moiety is directed away from the surface leaving the active sequence readily available to interact with contacting blood. Studies in both buffer and plasma showed that ATH-modified surfaces were able to bind greater quantities of antithrombin (AT) per heparin residue than the analogous heparin-modified surfaces.

The gold surfaces modified with ATH were investigated for their anticoagulant properties and platelet interactions. The functionality of the DSP and PEO reactive end groups for ATH attachment was confirmed. The binding of antithrombin was shown to be mainly through the active sequence of the heparin moiety on ATH. Based on the ratio of total heparin to active heparin, measured by anti-factor Xa activity, the ATH-modified surfaces had greater bioactivity than the heparinized surfaces. Platelet adhesion measured from whole blood under flow conditions was significantly reduced for all modified surfaces compared to bare gold. The clotting time of recalcified, citrated plasma was prolonged when ATH was immobilized through the PEO linker/spacer in contrast to controls and heparinized surfaces.

In the second phase of work, polyurethane was used as substrate to immobilize ATH. Reactive isocyanate groups were first introduced into the polyurethane surface. Dihydroxy PEOs of molecular weight from 300-4600 Da were then attached. The free hydroxyl end groups of PEO were functionalized for attachment of ATH. Physicochemical surface analysis confirmed that the modification steps were successful. The ATH surface density was highest on the surfaces with PEO of lower MW, and stable (presumably covalent) attachment was evident. The adsorption of fibrinogen decreased

significantly on PEO-modified polyurethane (all MWs) and with subsequent ATH attachment the adsorption levels remained low. AT binding from plasma was also found to be the highest when ATH was attached through PEO in the low to mid MW range (300 and 600). The surface with ATH attached via PEO of MW 600 showed the best balance of protein resistance and bioactivity (low fibrinogen adsorption, high AT adsorption). Western blot analysis of proteins adsorbed after exposure to plasma further indicated that the ATH-modified surfaces adsorbed low amounts of other proteins. In addition, platelet adhesion from flowing whole blood was low on PEO-modified and PEO-ATH-modified polyurethane surfaces.

Polyurethane grafted with the conventional homo-bifunctional dihydroxy-PEO (PEO-OH) was compared to a hetero-bifunctional PEO, one chain end of which was an amino group for reaction with isocyanate activated polyurethane and the other a carboxylic acid group for further functionalization (PEO-COOH). Following conversion of the end groups to N-hydroxysuccinimide (NHS), ATH was attached. The PEO-modified surfaces were more hydrophilic than the unmodified polyurethane, and the PEO-OH surfaces were more hydrophilic than the PEO-COOH. Fibrinogen adsorption was low on all of the PEO-ATH surfaces while AT selectivity was high. The PEO-COOH-ATH surfaces showed slightly greater AT adsorption via the catalytic effect of the heparin moiety. All of the PEO-ATH surfaces showed greater thrombin binding capacity than controls, demonstrating their potential for direct inhibition. The PEO-OH-ATH surfaces of lower PEO MW showed higher thrombin adsorption. Thus, the PEO spacer influenced protein adsorption as well as anticoagulant activity.

From an overall standpoint this work provides new insight into the interactions of blood components with ATH-immobilized biomaterials and leads to the conclusion that surface modification with ATH is a promising method for improving blood contacting medical devices.

7.2. RECOMMENDATIONS FOR FUTURE WORK

This work has demonstrated the advantages of combining ATH and PEO for improving the blood compatibility of biomaterials. Various aspects of the work could be explored in more detail. New avenues are also suggested by the results generated thus far.

While PEO graft densities could be determined on the gold surfaces, the densities on polyurethane surfaces could not be obtained. A method of quantifying PEO on polymer surfaces is needed. This would provide a more precise indication of the contribution of protein resistance and might permit improved attachment of ATH and further optimization of the ATH-PEO balance. Other types of PEO could also be studied including PEO with different functional groups, branched PEO, and multi-arm or star PEO. Grafting methods such as surface initiated atom transfer radical polymerization (ATRP) could also be explored as a means to achieve higher densities of PEO and ATH (Jin, Feng et al. 2009).

Since questions have been raised about the long-term stability of PEO (Harris and Zalipsky 1997), the stability of PEO-ATH surfaces should be investigated. In addition,

alternatives to PEO as the protein resistant component, e.g. poly(methacryoyloxyethyl phosphoryl choline) (polyMPC) (Iwasaki and Ishihara 2005) should be investigated.

Preliminary studies were carried out to measure platelet interactions with the surfaces developed in this work. The measurements were limited to determining numbers of adherent cells under a single set of conditions (flow, temperature). Adherent platelets generally exhibit secondary responses including shape change, spreading and aggregation. Scanning electron microscopy (SEM) can be used to observe the morphology of adherent platelets and qualitatively assess their degree of activation. This would be done in experiments where the platelets are not radiolabelled. Furthermore, bulk platelet activation and microparticle formation can be studied using flow cytometry (Gemmell 2001). Platelet interactions may also be linked to other cellular interactions such as leukocyte adhesion. Since leukocyte adhesion results in inflammatory responses and the foreign body reaction (Anderson, Rodriguez et al. 2008), this is also an area for future studies of ATH-modified surfaces.

Clotting times of recalcified plasma were found to be prolonged on ATH-modified gold substrates, but reliable data were not obtained for modified polyurethanes. Several limitations exist with the optical technique used in this work including low sensitivity, the effects of "bystander" surfaces (microtitre plate wells, air interface), and maintaining the position of the film samples in the wells during measurements. Suggestions for improvement include increasing the surface area of the polymer samples, use of longer incubation times, pre-coating the wells with nonfouling materials, and improvements in the plasma mixing method. Alternative methods of assessing clot

formation could also be explored, e.g. thromboelastography (TEG) (Peng 2010). This technique may eliminate some of the problems indicated and can be carried out in either plasma or whole blood.

ATH has shown advantages in various applications both systemically and when attached to surfaces. One of the early studies demonstrated its potential for treating neonatal respiratory distress syndrome (Chan, Berry et al. 1997). ATH may also provide benefits when immobilized on biomaterials used specifically for pediatric devices. However, infant plasma and adult plasma differ in protein content and protein adsorption to polymeric materials has also been found to differ (Cornelius, Archambault et al. 2002). It would thus be of interest to compare the interactions of proteins from infant vs. adult plasma to ATH-modified materials.

This work has focused on the use of gold and polyurethane to immobilize ATH. Many other polymers could also be used as materials to attach ATH, using strategies similar to those presented in this thesis. Work is also needed to "translate" the methods developed here for flat surfaces and films to more complex geometries such as are found in devices. For example, techniques for modification of microfluidic devices with PEO have been developed (Wu, Sask et al. 2012). It would be of interest to extend the modifications to include ATH as well.

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APPENDIX A

A.1. PUBLICATIONS AND AWARDS

A.1.1. Journal Publications (Peer Reviewed)

Sask KN, Berry LR, Chan AKC, Brash JL. Polyurethane modified with an antithrombin-heparin complex (ATH) via polyethylene oxide (PEO) linker/spacers: Influence of PEO molecular weight and PEO-ATH bond on catalytic and direct anticoagulant functions. *Journal of Biomedical Materials Research Part A*. 2012. DOI: 10.1002/jbm.a 34218.

Wu W-I, **Sask KN**, Selvaganapathy PR, Brash JL. Polyurethane-based microfluidic devices for blood contacting applications. *Lab on a Chip*. 12:960-970, 2012.

Sask KN, Berry LR, Chan AKC, Brash JL. Modification of polyurethane surface with an antithrombin-heparin complex for blood contact: Influence of molecular weight of polyethylene oxide used as linker/spacer. *Langmuir*. 28:2099-2106, 2012.

Sask KN, McClung WG, Berry LR, Chan AKC, Brash JL. Immobilization of an antithrombin-heparin complex on gold: Anticoagulant properties and platelet interactions. *Acta Biomaterialia*. 7:2029-2034, 2011.

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Nielsen DR, **Sask KN**, McLellan J, Daugulis AJ. Benzene vapor treatment using a two-phase partitioning bioscrubber: an improved steady-state protocol to enhance long-term operation. *Bioprocess and Biosystems Engineering.* 29:229-240, 2006.

A.1.2. Abstracts (Peer Reviewed)

Sask KN, Berry LR, Chan AKC, Brash JL. Polyurethane Immobilized with a Covalent Antithrombin-Heparin Complex for Reducing Thrombosis on Blood Contacting Devices. XXIII Congress - *International Society on Thrombosis and Haemostasis, 2011, Kyoto, Japan.*

Wu W-I, **Sask KN**, Selvaganapathy PR, Brash JL. Fabrication of Polyurethane Microfluidic Channels and Their Surface Modification for Blood Contacting Applications. *MicroTAS: The 15th International Conference on Miniaturized Systems for Chemistry and Life Sciences, 2011, Seattle, WA, USA.*

Sask KN, Berry LR, Chan AKC, Brash JL. Polyurethane Modified with an Antithrombin-Heparin Complex (ATH) via PEO: Effect of PEO End Group on ATH Immobilization and Subsequent Antithrombin and Fibrinogen Binding. *Society for Biomaterials Annual Meeting, 2011, Orlando, FL, USA.*

Sask KN, Berry LR, Chan AKC, Brash JL. Influence of Polyethylene Oxide Spacer on Anticoagulant Properties of Immobilized Antithrombin-Heparin Complex. *28th Canadian Biomaterials Society Meeting, 2010, Kingston, ON, Canada.*

Wu Z, **Sask KN**, Alibeik S, Chen H, Brash JL. Tissue Plasminogen Activator Containing Polyurethane Surfaces for Fibrinolytic Activity. *28th Canadian Biomaterials Society Meeting, 2010, Kingston, ON, Canada.*

Sask KN, Berry LR, Chan AKC, Brash JL. Influence of Polyethylene Oxide Spacer on Anticoagulant Properties of Immobilized Antithrombin-Heparin Complex. *Society for Biomaterials Annual Meeting, 2010, Seattle, WA, USA.*

Grant D, Müller M, Tominac P, Guan L, **Sask K**, Brash J, Davies J. Significantly Reduced Burst Release by Sequestration of Protein Delivery Vehicle Within Unique Three-Phase Composite Scaffold for Bone Tissue Engineering and Regenerative Medicine. *Abstracts of the IBMS Davos Workshops: Bone Biology & Therapeutics, 2010, Davos Switzerland.*

Sask KN, McClung WG, Berry LR, Chan AKC, Brash JL. Biomaterial Surface Modification with an Antithrombin-Heparin Covalent Complex for Improved Intravascular Devices. XXII Congress - *International Society on Thrombosis and Haemostasis, 2009, Boston, MA, USA.*

Sask KN, Berry LR, Chan AKC, Igor Zhitomirsky I, Brash JL. Surface Modification of Model Gold Substrates with an Antithrombin-Heparin Anticoagulant Complex. *27th Canadian Biomaterials Society Meeting, 2009, Quebec City, QC, Canada.*

Sask KN, Berry LR, Chan AKC, Igor Zhitomirsky I, Brash JL. Surface Modification of Model Gold Substrates with an Antithrombin-Heparin Anticoagulant Complex. *Society for Biomaterials Annual Meeting, 2009, San Antonio, TX, USA.*

Sask KN, McClung WG, Zhitomirsky I, Berry LR, Chan AKC, Brash JL. Surface Modification with an Antithrombin-Heparin Covalent Complex. *8th World Biomaterials Congress, 2008, Amsterdam, Netherlands.*

A.1.3. Abstracts (Non-Peer Reviewed)

Sask KN, Berry LR, Chan AKC, Brash JL. Influence of Polyethylene Oxide on Polyurethane Modification with an Antithrombin-Heparin Anticoagulant Complex. *PolyMAC Conference, McMaster University, 2011, Hamilton, ON, Canada.*

Sask KN, McClung WG, Berry LR, Chan AKC, Brash JL. Surface Modification with an Antithrombin-Heparin Covalent Complex. *The WISE Initiative 2008 International Women's Day Research Conference, 2008, Hamilton, ON, Canada.*

Sask KN, McClung WG, Berry LR, Chan AKC, Brash JL. Antithrombogenic Biomaterials: Surface Modification with an Antithrombin-Heparin Complex. *2nd Annual Health Research in the City Conference, 2008, Hamilton, ON, Canada.*

A.1.4. Awards and Scholarships

• Oral Presentation Award - PolyMAC Conference, McMaster University: *2011*

• Dave Williams Graduate Scholarship in Biomedical Engineering ($1000): *2010*

• Oral Presentation Award - 28th Meeting, Canadian Biomaterials Society ($250): *2010*

• Graduate Student Association Travel Awards - awarded 3 times ($500/yr): *2009-2011*

• NSERC Postgraduate Scholarship (PGS) ($21000/yr - for 3 years): *2009-2012*

• Ontario Graduate Fellowship - McMaster Prestige Award ($12000/yr): *2007-2009*

• B.E.C. Joyce Memorial Award (outstanding graduate in Chemical Engineering): *2006*

• Dean's Scholar recognition and Dean's Award ($500): *2005-2006*

• Charles Allan Thompson Summer Research Award ($4500): *2005*

• Queen's University Entrance Award & Honours with Merit ($4500): *2002*

• Aiming For the Top Tuition Scholarship ($2450): *2002*