PEO AND PEO-HEPARIN MODIFIED SURFACES
FOR BLOOD CONTACTING APPLICATIONS

by

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ABSTRACT

The conceptual design, synthesis and characterization of thiol-modified gold surfaces as well as studies of their behavior in contact with blood comprise the scope of this thesis. The attachment of different chemical and biological functionalities to surfaces with the aim of improving the blood compatibility of artificial materials is the main objective of this study.

The synthesis and characterization of various thiolated polyethylene oxide (PEO) polymers constitute a significant part of the work, from linear PEOs with different molecular weights, and different end groups, to star-type, multiarmed PEOs, to linear PEOs in which one end bears a thiol group and the other a bioactive group. Different reaction protocols were used for the synthesis of the different PEOs. Terminal thiol groups were attached to PEO by reaction with mercaptoacetic acid or cystamine. Conjugation methods were used to attach bioactive groups to the second terminus of thiolated PEO. Specifically for the chain-end heparinization of PEO, radicalization of the double bond in low molecular weight heparin (LMWH) was used to initiate reaction with a terminal thiol of PEO. NMR and IR spectroscopy, and GPC were used to confirm the reactions and characterize the products. The kinetics and mechanism of some of the reactions were investigated.

Thiolated PEOs were chemisorbed to gold films deposited on polyurethane or silicon wafer substrates. Combinations of PEO and chain-end functionalized PEO were used to passivate the gold films and impart specific bioactivity respectively. The modified
surfaces were characterized chemically and physically, by X-ray photoelectron spectroscopy (XPS), contact angle analysis, and graft density measurements.

From XPS, it was observed that the modified surfaces had decreased gold content compared to the unmodified surface. The sulfur content appeared to increase with decreasing molecular weight of the PEO, in agreement with the fact that proportionately more sulfur is present in low molecular weight PEOs.

A reaction protocol was developed to attach radioactive iodine to PEO, thus allowing the measurement of the graft density of PEO on the surface after chemisorption. The data showed a significant difference between surfaces treated with HS-PEO and PEO, reflecting the different mechanisms of adsorption.

The interactions of the modified surfaces with blood and blood proteins were investigated using radiolabeling, electrophoresis and immunoblotting methods. In measuring protein interactions with gold-based surfaces using radioiodine-labeling methods, it was found that the radioactive iodide ions \(^{125}\text{I}\) in the protein solution became bound to the surface along with the protein. To overcome this problem, two approaches were investigated: preexposure of the surface to "cold" (nonradioactive) iodide, and inclusion of cold iodide in the buffer in large excess compared to the radioactive iodide. Inclusion of cold iodide in the buffer was found to be effective in minimizing radioactive iodide binding during the protein adsorption experiments.

Data on the adsorption of fibrinogen and albumin from buffer and plasma onto surfaces modified with different PEOs showed that adsorption decreased with increasing PEO molecular weight, irrespective of the group on the "outer" end of the PEO (hydroxyl or methoxyl). However, protein adsorption on methoxy PEO was greater than on hydroxy
- PEO modified surfaces. Polypropylene oxide (PPO) modified surfaces were also found to suppress protein adsorption.

Surfaces were also prepared using PEO to which heparin was conjugated. Unfractionated heparin and low molecular weight heparin conjugated PEO were used to modify both gold and polyurethane surfaces. Both the passivation effect of PEO and the anticoagulant effect of heparin were found to be evident for these surfaces. A commercially available heparinized surface, the Carmeda Bioactive Surface (CBAS), was chosen to compare to the surfaces prepared in this project. Contact angle and XPS were used to evaluate the properties of the surfaces after modification. The data showed the effectiveness of the modification procedure.

Protein adsorption data from radiolabeling and SDS-PAGE/western blotting experiments showed complex protein binding patterns from plasma to the surfaces. The presence of heparin on the surface was indicated by AT adsorption. Anti-factor Xa assay showed that the surface bound heparin was active.
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<thead>
<tr>
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<th>Description</th>
</tr>
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<tr>
<td>μCi</td>
<td>microcurie(s)</td>
</tr>
<tr>
<td>μg</td>
<td>microgram</td>
</tr>
<tr>
<td>μL</td>
<td>microlitre(s)</td>
</tr>
<tr>
<td>μM</td>
<td>micromolar</td>
</tr>
<tr>
<td>°C</td>
<td>degrees Celsius</td>
</tr>
<tr>
<td>ADP</td>
<td>adenosine diphosphate</td>
</tr>
<tr>
<td>AFM</td>
<td>atomic force microscopy</td>
</tr>
<tr>
<td>AMP</td>
<td>adenosine monophosphate</td>
</tr>
<tr>
<td>APTT</td>
<td>activated partial thromboplastin time</td>
</tr>
<tr>
<td>Arg</td>
<td>arginine</td>
</tr>
<tr>
<td>Asp</td>
<td>aspartic acid</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>Au</td>
<td>gold</td>
</tr>
<tr>
<td>C3</td>
<td>complement factor 3</td>
</tr>
<tr>
<td>DMF</td>
<td>N,N'-dimethyl formamide</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulphoxide</td>
</tr>
<tr>
<td>EDRF</td>
<td>Endothelium derived relaxing factor, nitric oxide</td>
</tr>
<tr>
<td>FDP</td>
<td>fibrin degradation product</td>
</tr>
<tr>
<td>Fg</td>
<td>fibrinogen</td>
</tr>
<tr>
<td>FTIR</td>
<td>Furier transform infrared spectroscopy</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------------------------------------------</td>
</tr>
<tr>
<td>GPC</td>
<td>gel permeation chromatography</td>
</tr>
<tr>
<td>H</td>
<td>heparin</td>
</tr>
<tr>
<td>HAS</td>
<td>human serum albumin</td>
</tr>
<tr>
<td>HCII</td>
<td>heparin cofactor II</td>
</tr>
<tr>
<td>HMWK</td>
<td>high molecular weight kininogen</td>
</tr>
<tr>
<td>HS</td>
<td>heparan sulfate</td>
</tr>
<tr>
<td>IgG</td>
<td>immunoglobulin G</td>
</tr>
<tr>
<td>T, IIa</td>
<td>thrombin</td>
</tr>
<tr>
<td>KD, kDa</td>
<td>kilo Dalton</td>
</tr>
<tr>
<td>LMWH</td>
<td>low molecular weight heparin</td>
</tr>
<tr>
<td>MDA</td>
<td>methylene dianiline</td>
</tr>
<tr>
<td>pNA</td>
<td>p-nitroaniline</td>
</tr>
<tr>
<td>NHS</td>
<td>N-hydroxysuccinimide</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear magnetic spectroscopy</td>
</tr>
<tr>
<td>t-PA</td>
<td>tissue plasminogen activator</td>
</tr>
<tr>
<td>u-PA</td>
<td>urokinase-type plasminogen activator</td>
</tr>
<tr>
<td>PAI-1</td>
<td>Plasminogen activator inhibitor type-1</td>
</tr>
<tr>
<td>PAI-2</td>
<td>Plasminogen activator inhibitor type-2</td>
</tr>
<tr>
<td>PBMC</td>
<td>platelet adhesion and activation, and peripheral blood mononuclear cell</td>
</tr>
<tr>
<td>PBS</td>
<td>isotonic phosphate-buffered saline</td>
</tr>
<tr>
<td>PC</td>
<td>protein C</td>
</tr>
<tr>
<td>PEO</td>
<td>polyethylene oxide</td>
</tr>
</tbody>
</table>

XXXI
PGI₂ prostacyclin
PPACK D-phe-pro-arg chloromethylketone
PPO polypropylene oxide
PRT plasma recalcification time
PS protein S
PTMO polytetramethylene oxide
SAM self assembled monolayers
SDS sodium dodecyl sulfate
SDS-PAGE SDS-polyacrylamide gel electrophoresis
TBS Tris buffered saline
TFPI tissue factor pathway inhibitor
TM Thrombomodulin
UFH unfractionated heparin
VN vitronectin
XIa, Xa etc activated factor XI, X etc
XPS X-ray photoelectron spectroscopy
CHAPTER 1

INTRODUCTION

The work discussed in this thesis is directed towards improved understanding of blood-surface interactions and the development of blood compatible materials. In addition to discussing the research performed, it covers such issues as why blood compatible materials are important, what types of materials may be blood compatible, and which methods are appropriate to evaluate the blood compatibility of materials. Blood compatible materials hold the key to the alleviation of vascular disease via the use of devices that are implanted in the circulatory system, for example heart valve prostheses, vascular grafts, and intravascular stents.

When, in 1759, Hollowell united the edges of a severed brachial artery using a wooden peg and twisted thread (Wesolowski, 1963), he could not have foreseen future developments in the field now known as “biomaterials”. Biomaterials, being defined as “any substance other than a drug, or combination of substances, synthetic or natural in origin which can be used for any period of time as a whole or as a part of the system which treats, augments, or replaces any tissue, organ or function of the body” at a National Institutes of Health consensus development conference in 1987 (Williams, 1987), find their applications ranging from implants such as intraocular lenses, shoulder and finger joint replacements, skin grafts, prostheses, heart pacers and heart valves, to extracorporeal applications such as dialyzers and heart lung bypass systems. Many
materials, including naturally occurring materials, ceramics, polymers and metals have been used in these applications (Walivaara, 1996a; Walivaara, 1996b). Synthetic polymers developed in the last 40 to 50 years have played a major role in biomaterials development (Table 1.1). The need for biomaterials is ever increasing, due in part to the invention of new medical technologies that require new materials. For example, as recently as 1994 it was estimated that the market for blood-contacting devices was $6B US (Greco, 1994). However, the lack of a comprehensive theory or even simple guidelines for the development of blood compatible materials (or of biocompatible materials generally), and problems due to inadequate compatibility of existing biomaterials have limited the success of these devices and the potential application of biomaterials to other devices. Notwithstanding these difficulties, a considerable research effort over the past three decades has made progress in the understanding of protein and cell surface interactions, and in the development of methods to characterize and manipulate surface properties at the nanoscale level. These advances may accelerate the development of blood compatible materials.

Because blood usually contacts only the outer surface of a material, surface properties are critically important. The approach taken in this work is that the biomaterial will necessarily consist of a “bulk” material to provide appropriate mechanical properties, and that the surface can be modified to provide an interface which is blood compatible. Since blood-material interactions are complex and include both protein interactions (coagulation, complement and other plasma proteins) and cell interactions (red cells, white cells and platelets), considerable effort has been expended in developing completely inert materials or creating materials that will interact in specific ways with
blood. For example, modification of the surface of a biomaterial with poly (ethylene oxide), as will be discussed in this thesis, may provide benefits for blood compatibility.

The availability of truly blood compatible materials would permit the development of medical devices that currently are not feasible (e.g. small diameter vascular grafts). Other devices (e.g. heart valves currently requiring continuous systemic anticoagulation of the recipient) would also be greatly improved. In the next chapter, a detailed review of the literature on blood contacting materials and devices, the subject of the present thesis, will be given.

Table 1.1. Some typical biomaterials and their applications (adapted from (Greco, 1994))

<table>
<thead>
<tr>
<th>BIOMATERIAL</th>
<th>APPLICATIONS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cellulose</td>
<td>Sutures, hemodialysis membranes</td>
</tr>
<tr>
<td>Poly (ethylene glycol)</td>
<td>Tubings, shunts, syringes</td>
</tr>
<tr>
<td>Poly (propylene)</td>
<td>Sutures, prosthetic valves, plasmapheresis membranes, syringes</td>
</tr>
<tr>
<td>Poly (tetrafluoroethylene)</td>
<td>Vascular grafts, oxygenator membranes facial protheses, catheter coatings, stents</td>
</tr>
<tr>
<td>Poly (vinyl chloride)</td>
<td>Blood tubing, blood bags, plasmapheresis membranes</td>
</tr>
<tr>
<td>Polystyrene</td>
<td>Tissue culture dishes</td>
</tr>
<tr>
<td>Poly (methyl methacrylate)</td>
<td>Plasmapheresis membranes, dentures, bone cement, middle ear prostheses</td>
</tr>
<tr>
<td>Poly (hydroxyethyl methacrylate)</td>
<td>Membranes, drug release matrices, vascular prostheses coatings, catheter coatings, artificial organs</td>
</tr>
<tr>
<td>Material</td>
<td>Applications</td>
</tr>
<tr>
<td>----------------------------------</td>
<td>------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Poly (vinylpyrrolidone)</td>
<td>Blood plasma expanders</td>
</tr>
<tr>
<td>Poly (acrylonitrile)</td>
<td>Hemodialysis membranes</td>
</tr>
<tr>
<td>Polyamides</td>
<td>Sutures, hemodialysis membranes</td>
</tr>
<tr>
<td>Poly (ethylene terephthalate)</td>
<td>Vascular grafts, tissue patches, shunts</td>
</tr>
<tr>
<td>Poly (lactide-co-glycolide)</td>
<td>Biodegradable sutures, drug release matrices, tissue scaffolds</td>
</tr>
<tr>
<td>Polysulfone</td>
<td>Prosthetic valve and artificial heart structures, dialysis membranes</td>
</tr>
<tr>
<td>Polyvinylpyrrolidone</td>
<td>Catheter coatings</td>
</tr>
<tr>
<td>Poly (dimethylsiloxane)</td>
<td>Tubing, hydrocephalus shunts, oxygenator membranes, catheters, grafts, intra-aortic balloons, wound dressings, breast implants, reconstructive and orthopedic devices</td>
</tr>
<tr>
<td>Polyurethanes</td>
<td>Percutaneous leads, catheters, by-pass filters, tubing, intra-aortic balloons, wound dressings, artificial heart components, prosthesis cardiac valves</td>
</tr>
<tr>
<td>Metals and alloys</td>
<td>Fracture fixation devices, joint prostheses, heart valve components, stents</td>
</tr>
<tr>
<td>Ceramics</td>
<td>Bone substitutes</td>
</tr>
</tbody>
</table>
CHAPTER 2

GENERAL BACKGROUND

Implanted and extracorporeal blood contacting devices, such as catheters, heart valves and vascular prostheses require man-made biomaterials. It would be anticipated that the various systems of the body involved in the defensive responses to injury, or to the presence of foreign materials, would function in an integrated manner. These systems include the hemostatic, complement and kinin generation mechanisms.

2.1. CELLULAR AND MOLECULAR COMPONENTS IN HEMOSTASIS

Hemostasis refers to the arrest of bleeding following injury to the vessel wall and the ability of blood to prevent thrombosis inside the circulation. The haemostatic system consists of three parts: the coagulation system (Figure 2.1), the anticoagulation system (inhibition pathways, Figure 2.1) and the fibrinolytic system (Figure 2.2). Various components of the blood, composed of a fluid part, plasma, and cellular elements comprising red cells (erythrocytes), white cells (leukocytes) and platelets, and blood vessel walls, especially endothelial cells, are involved in the regulation of hemostasis.

Plasma contains proteins that are involved in the regulation of the blood coagulation, the anticoagulation and the fibrinolytic system, thus controlling the conversion of fibrinogen to fibrin, the material of the clot.
Figure 2.1. Schematic of haemostatic system pathways involving plasma proteins. Adapted from Furie et al, 1988 and Hoffman et al, 1995.
Figure 2.2. Scheme of the fibrinolytic system. Adapted from Furie et al, 1988 and Hoffman et al, 1996.

Blood coagulation is a host-defense mechanism that maintains the integrity of the circulatory system. As can be seen in Figure 2.1, a series of interdependent enzyme-mediated reactions translate the molecular signals that initiate blood coagulation into a biologic event, the formation of the fibrin clot (Courtney et al., 1994; Guyton, 1991; Hoffman et al., 1996). Clot formation involves the activation of factors XII, XI, IX, and VIII in the intrinsic pathway, or the activation of factor VII in the presence of tissue factor, in the extrinsic pathway (Furie et al., 1988). These pathways converge to the common coagulation pathway leading to activation of factor X to Xa, which then binds to the activated form of factor V
(Va). This complex (prothrombinase complex) converts prothrombin to thrombin. Thrombin acts on fibrinogen to generate fibrin monomer which polymerizes to form the fibrin clot (Furie et al., 1992).

To regulate coagulation, however, the inhibition pathways (Davie, 1995) may intervene (Figure 2.1). First, the serpin, antithrombin, the activity of which is greatly stimulated in the presence of heparin, inactivates thrombin as well as factors Xa, IXa and XIa (Kurachi et al., 1976). Heparin cofactor II (HCII), which is potentiated by dermatan sulfate and heparin, inactivates thrombin selectively. Second, tissue factor pathway inhibitor (TFPI) blocks the tissue factor pathway by forming complexes and inactivating VIIa bound to TF in a Xa-dependent reaction (Rao et al., 1987). Third, in the protein C (PC)/Protein S (PS) pathway, PC, following its activation by the thrombin-thrombomodulin complex, binds to PS and inactivates VIIIa or Va (Vehar et al., 1980). In addition, many synthetic or natural inhibitors, such as hirudin, have been used clinically as anticoagulants.

In the fibrinolytic system (Figure 2.2), an inactive pro-enzyme, plasminogen, is converted by plasminogen activators to the active enzyme, plasmin, which degrades fibrin (Verstraete, 1995). Two immunologically distinct plasminogen activators have been identified: tissue-type plasminogen activator (t-PA) and urokinase-type plasminogen activator (u-PA). The activators are inhibited by specific inhibitors such as types 1 and 2 plasminogen activation inhibitors (PAI-1 and PAI-2, respectively). Plasmin is inhibited by α-2-antiplasmin and α2-macroglobulin (Kruithof et al., 1986).

Platelets are disc shaped, non-nucleated granular cells with a volume of ~ 7 μm³ (Figure 2.3). Each cell contains a single peripheral microtubule coil, plasma membrane, a- granules, dense granules, mitochondrion and cytoplasm. The exterior coat is composed
of glycosaminoglycans, glycolipids, and glycoproteins responsible for the binding of agents inducing adhesion or aggregation of platelets. Platelets adhere readily to almost any surface other than normal endothelium, e.g. surfaces of damaged vessel walls, or artificial surfaces. Upon activation, platelets can release the contents of their various granules including various types of platelet factors, fibrinogen, albumin, ATP, ADP etc (Niewiarowski, 1981). These substances are generally involved in the propagation of hemostatic responses including platelet aggregation and plasma coagulation.

![Diagram of platelet organelles](image)

Figure 2.3. Schematic of platelet organelles (Andrews et al., 1997; Bentfeld-Baker et al., 1977). With permission.

Endothelial cells form a monolayer on the inner surface of the vessel, which normally contacts blood. Endothelium is not merely a passive barrier separating the blood from the interstitial matrix. Instead, it is an active participant in transportation, metabolic equilibrium and, most importantly, maintenance of the fluidity of the blood via its effects on the anticoagulant mechanisms (Figure 2.4). First, the endothelium contributes to the fibrinolytic pathway by releasing t-PA following damage to the vessel wall. Second, the endothelial cell surface contains heparin-like proteoglycans, heparan sulfate (HS), that
accelerate the inhibition of thrombin by AT and HC-II. Third, the endothelial cell surface

possesses a thrombin-binding protein, thrombomodulin (TM), which initiates the protein
C (PC) anticoagulant pathway and the ultimate inactivation of factors Va, VIIIa, and t-PA
inhibitor. Finally, following damage to the vessel wall, the endothelium releases both
prostacyclin (PGI₂) and endothelium-derived relaxing factor (EDRF), i.e. nitric oxide,
which inhibit platelet aggregation and cause vasodilatation (Ramamurthi et al., 2001;
Bohl et al., 2000; Bhatt, 1997). The conversion of adenosine triphosphate (ATP),
adenosine diphosphate (ADP), and adenosine monophosphate (AMP) to adenosine (A)
via an ecto-ADP-ase system also facilitates inhibition of platelet aggregation and
adhesion (Machovich, 1988). In addition, the release of a factor, designated vascular
relaxant factor (VRF), by endothelial cells inhibits the contraction of the smooth muscle
cells of the blood vessel wall. It is noteworthy, however, that once triggered, endothelial

Figure 2.4. Antithrombotic nature of the endothelium. Please refer to text for explanation.
Figure is from (Maruyama, 1998; Machovich, 1988).
cells can become procoagulant by generating various factors such as tissue factor and von Willebrand factor.

Ideally, blood-contacting biomaterials should behave in a manner similar to the normal vessel wall. However, on balance, unlike the endothelium, foreign surfaces promote thrombosis rather than anticoagulation or fibrinolysis. Thus, conceptually at least, development of improved surfaces should aim at tipping the balance towards the latter (Goldberg et al., 1980).

2.2. THE COMPLEMENT SYSTEM

The complement system, which is also activated by blood foreign surface contact, comprises a group of proteins which interact sequentially to cause irreversible damage of target cell membranes and, additionally, to produce a number of biologically active products. Activation of the complement system can take place through two major pathways, the classical pathway and the alternative (properdin) pathway (Figure 2.5).

In the classical pathway, activation of the first component of complement (C1), a complex of three proteins (C1q, C1r and C1s), is initiated by an antigen-antibody (IgM or IgG) complex. This leads to the formation of C3 convertase, a complex involving C1, C2 and C4, and thence to the formation of the membrane attack complex, C5b-9 (Ogston, 1983).

The alternative pathway is initiated by the interaction of serum components (factors B and D) with polysaccharides of cell walls, for example inulin and zymosan, lipopolysaccharides, and immune complexes containing antibodies of the IgA or IgD classes. This pathway bypasses C1, C4 and C2, and produces an alternative pathway C3 convertase, leading again to the assembly of the terminal lytic complex C5b-9 (Ogston,
Complement activation occurs to some extent on all foreign blood contacting surfaces and is believed to proceed by a mechanism akin to the alternative pathway (Maruyama, 1998; Kazatchkine et al., 1988).

Figure 2.5. The complement pathways. C1 – C9 are the complement components; C1a – C9a are the activated complement components; and C1a, C1b, ... etc, the subunits of the complement components. From Marieb (1995), with permission.
2.3. BLOOD COMPATIBILITY AND GENERAL STRATEGIES FOR SURFACE MODIFICATION

Within seconds following the exposure of a foreign surface to blood, adsorption of plasma proteins occurs, followed by adhesion and aggregation of platelets and leukocytes (Figure 2.6). This may initiate blood coagulation and complement pathways (Brash, 1991; Kim et al., 1998). Platelet aggregation and activation of coagulation results in thrombus formation at the biomaterial surface. Therefore, blood compatible materials which do not provoke these phenomena are required if blood contacting devices of improved performance are to be realized.

Blood compatibility of a material is hard to define due to the complexity of blood-material interactions. Some authors (Williams, 1992; Goldberg et al., 1980; Kim et al., 1998; Cahn, 1977; Lelah, 1986) have discussed blood compatibility in terms of incompatibilities.

![Blood response diagram](image-url)

Figure 2.6. Blood responses to a biomaterial initiated by protein adsorption (Courtney et al., 1994).
Thus, before going further into detail, it would seem useful to clarify the characteristics of a blood compatible material which form the background to the present research. In principle, the detailed mechanisms and factors that control the interactions between a biomaterial and blood should be known before effective modifications are possible (Brash, 2000; Duinhoven et al., 1995; Feng et al., 1995; Jacobs et al., 1988; Ikada, 1994). Unfortunately, other than the broad outlines indicated in the previous paragraphs, these mechanisms and factors are not well understood (Hoffman, 1982; Hench et al., 1981; Kim et al., 1992), a circumstance which has contributed to what has been characterized as the "blood compatibility catastrophe" (Ratner, 1993b). However, as indicated above, the rapid adsorption of proteins onto a biomaterial surface is regarded as the first major event following blood-biomaterial contact (Brash, 1991). These adsorbed proteins will, in turn, influence the interactions of cells which arrive later at the interface. For example, fibrinogen is known to promote the adhesion of platelets (Grunkemeier et al., 2000a), whereas albumin is nonreactive to cells (Jones et al., 2000; Amiji et al., 1993). The composition of the protein layer and the biologic status of the adsorbed proteins is therefore of great importance, and any rationale for achieving blood compatibility must recognize that these properties will be determined to a large extent by the chemical composition and structure of the material surface.

Therefore, based upon the author's understanding, a blood compatible biomaterial should be such that blood in contact with the material can fulfill both the general requirements as a fluid and the specific requirements as a bioactive medium. A blood compatible biomaterial should not initiate the blood coagulation pathways, activate complement, promote platelet adhesion and activation, or cause infection (Woodhouse et
al., 1992). Extensive studies can be found on anticoagulant surfaces, surfaces which are non-reactive to platelets, and surfaces which do not provoke infection (Brash, 2000; Grunkemeier et al., 2000a; Jones et al., 2000; Skarja et al., 1997; Groth et al., 1994; Kang et al., 1997; Seifert et al., 1997; Annich et al., 2000; Inoue H et al., 1997; Reynolds et al., 1993; Peckham et al., 1997; Baumann H et al., 2000).

In general, the following surface modification strategies may be proposed for blood compatibility: (1) passivation of the surface to minimize nonspecific protein adsorption; (2) incorporation of bioactive ligands into the surface to promote adsorption of specific targeted proteins (e.g. antithrombin, thrombin, plasminogen); and (3) a combination of (1) and (2). These strategies are now discussed in some detail.

2.3.1. PASSIVATION TO PREVENT NONSPECIFIC PROTEIN ADSORPTION

2.3.1.1. PASSIVATION MECHANISMS

Protein adsorption will depend on the properties of the surface, and of the proteins in question (Lee et al., 1996). The influence of hydrophobic interactions, van der Waals, electrostatic, and steric interactions can be illustrated in terms of their impact on the interaction potential, as shown in Figure 2.7 (Leckband et al., 1999b). By modifying a surface, we effectively change the net interaction potential. A commonly used strategy for surface modification is to make the surface "repellent" to the components of blood. As such, surfaces which repel proteins non-specifically are expected to minimize contact activation of coagulation and other adverse effects. Repulsion implies that the combined attractive interactions between surface and proteins, such as hydrogen bonding, van der Waals, electrostatic, and hydrophobic interactions, should be smaller than the repulsive
interactions including steric repulsion, giving the type of interaction potential shown in Figure 2.8.

Figure 2.7. Interaction potential between a protein and a surface for a system involving steric repulsion, van der Waals attraction, and electrostatic interaction. The net interaction potential (bold, solid line) is a superposition of the repulsive steric potential (long dashes), the attractive electrostatic potential (short dashes), and the attractive van der Waals potential (dots dashes). Adapted from Leckband et al., 1999b.
Figure 2.8. Hypothetical interaction potential between a protein and a passivated surface for a system involving steric repulsion, van der Waals attraction, and electrostatic interaction. The net interaction potential (bold, solid line) is a superposition of the repulsive, steric potential (long dashes), the attractive electrostatic potential (short dashes), and the attractive van der Waals potential (dots and dashes). Repulsion implies that the combined attractive interactions between surface and proteins, such as hydrogen bonding, van der Waals, electrostatic, and hydrophobic interactions, should be smaller than steric repulsion.

Surfaces which favor steric repulsion and minimize attractive interactions would be protein repellent. Steric repulsion, generally achieved by end attachment of polymer chains to the surface, has two contributing factors: the chain flexibility effect and the osmotic effect (Ryle, 1965; Hellsing, 1968). The chain flexibility effect results from loss of configurational entropy of the polymer chain caused by the reduction of available volume for polymer segments as the protein approaches the surface and compresses the
polymer chains (Nagaoka et al., 1983b; Mori et al., 1982). The osmotic effect results from the increase in effective polymer concentration caused by the mutual compression of the two macromolecules (Zalipsky et al., 1997b), causing osmotic transfer of solvent into the polymer coils and forcing the approaching protein away from the surface (Figure 2.9).

![Diagram](image)

Figure 2.9. Illustration of the steric repulsion of a surface with contributions from osmotic effect (a and b) and entropic effect (c). Adapted from (Shimada et al., 1998; Jeon et al., 1991b; Ikada, 1994).

The properties of the grafted polymer layer such as hydrophilicity, thickness (chain length), graft density, and end group all have the potential to influence steric repulsion and therefore protein adsorption (Duinhoven et al., 1995; Nagaoka et al., 1983a; Nagaoka et al., 1984; Nagaoka et al., 1983b; Nakao et al., 1987; Claesson, 1993; Andrade et al., 1993; Barentin et al., 1998; Beyer et al., 1997). These factors are now considered in more detail.

Surfaces based on immobilization of hydrophilic polymers have been shown to be protein repellent (Lee et al., 1995). Surfaces modified with hyaluronic acid, polyvinyl alcohol, PEO, and polysaccharide have been investigated (Defise et al., 1999;
Montdargent et al., 2000). Rabinow (1994) prepared polymer films using various surface-modifying polymers such as PEO, polyvinylacetate (PVA), and poly-N-vinylpyrrolidone (PNVP). When PVA of molecular weight 13 kDa was grafted to ethylene-vinylacetate copolymer, a sixfold reduction in IgG adsorption was obtained, suggesting the passivation effect of this hydrophilic modification layer. Cunliffe et al (1999), by grafting species of different hydrophilicity, hydrophobicity, chain length, and chemical functionality onto glass substrates, found that the hydrophilic uncharged surfaces modified by poly(ethylene glycol) showed the greatest resistance to protein adsorption.

Grafted polymers should be sufficiently flexible to provide the entropy contributions required for steric repulsion (Freij-Larsson et al., 1993; Ratner, 1993a; Zalipsky et al., 1992). In an aqueous fluid like blood, hydrophilic polymers should be able to adopt a range of conformations including fully extended ones, and thus should best satisfy the flexibility requirements for steric repulsion. Thus it is expected that polymers like PEO and certain polysaccharides will be effective. PEO in particular, given its inherent chain flexibility due to the low energy required for molecular rotations around the C-O bond, should be highly effective.

Polymer chains should be long enough so that the loss of entropy and osmotic repulsion upon chain compression are significant (Bergstrom et al., 1994). There are reports showed that protein adsorption decreases with increasing chain length of the modification layer (Jeon et al., 1991b; Leckband et al., 1999a; Archambault, 2000). PEO modified surfaces especially have attracted the attention of researchers worldwide (Szleifer, 1997b). For example, Cunliffe et al (1999), by changing the chain length of PEO on a glass surface, found that protein adsorption and bacterial adhesion decreased as
the molecular weight of PEO increased. Lee et al (1997), studied polyethylene surfaces modified with comb-like polyethylene oxide and found that plasma protein adsorption and platelet adhesion decreased with increasing PEO chain length and surface density. It has been found (Norde et al., 1994) that in many cases the steric repulsion model explains most of the experimental data on hydrophilic polymer modified surfaces. However, other authors (Harder et al., 1998; Wang et al., 1997) reported that short hydrophilic chains, with physically bound water, were also capable of producing a repelling surface. It has been shown (Harder et al., 1998; Wang et al., 1997; Palegrosdemange et al., 1991; Prime et al., 1993; Mrksich et al., 1997) that surfaces consisting of densely packed short PEO sequents (e.g. 2 units) are protein repellent, probably due to very tight water binding to PEO in the helical conformation.

Graft density is also an important parameter for protein repulsion. It is generally believed that to be optimally effective, the polymer should cover the surface completely so as to avoid "bare spots" (Bae et al., 1994). The influence of graft density on protein adsorption has been investigated by a number of groups (Malmsten et al., 1998b; Hommel et al., 1995; Afif et al., 1996; Desai et al., 1991b).

Szleifer considered protein adsorption on surfaces with end attached polymers from a theoretical standpoint (Szleifer, 1997a; 1997c). Assuming the grafted polymer-protein-solvent layer to be inhomogeneous in the direction perpendicular to the surface, he calculated the adsorption of the protein as a function of the surface coverage of grafted polymer, the bulk concentration of protein, and the type of solvent using single-chain mean-field theory. He concluded that the protein adsorption isotherms change
qualitatively for surfaces that have attractive interactions with the grafted polymer. The longer the polymer chain, the more effective the layer for protein repulsion.

Given a polymer in an athermal solvent, Halperin (1999) was able to show, by considering the force balance of the elastic and osmotic forces on the chains, that graft density depends on the number of repeating units and conformation of the chains.

The conformation of the polymer chains on the surface may have an influence on the effective graft density. Different conformations of end attached chains, e.g. so-called mushroom and brush structures (see Fig. 2.10) have been proposed (de Gennes, 1982) and their influence on protein adsorption studied.

![Figure 2.10](image)

Figure 2.10. The conformation of PEO chains on a surface depends on the molecular weight and graft density. If molecular weight is low, extended conformations may form. If molecular weight is high, mushroom conformations may form. Mixtures of extended and mushroom conformation may coexist for some molecular weights and graft densities.

Malmsten (1998a) investigated the influence of electrostatic and hydrophobic effects on protein adsorption. Using surfaces of different hydrophilicity and charge, it was found that if the surface is hydrophilic and positively charged (diaminocyclohexane plasma surface), the charge on the protein determines the amount deposited with negatively charged proteins adsorbing in significant amounts. If the surface is hydrophobic and negatively charged (methylated silica), protein adsorption is more strongly dependent on the interaction between the surface and the protein. These results
stress the fact that both the surface and protein properties must be considered in designing surfaces for protein repulsion.

End groups are known to influence the conformation of polymer chains. For example, it was found that the glass transition temperature of polypropylene oxide (PPO) in its pure state varies significantly with the types of end group (Yoon et al., 1997). Chain end groups may have an influence on protein adsorption when the chains are grafted on a surface, both through compositional effects and through protein interactions with the chain ends.

Iwasaki et al (1996) observed protein adsorption from plasma to polymer-grafted surfaces where the polymer backbone, an acrylic polymer, is the same and the end groups are varied. Using PEO having phosphorylcholine (PC), hydroxyl (OH) and methoxyl (MeO) end groups, it was found that protein adsorption decreased in the order MeO>OH>PC.

Jeon and Andrade (1991a) investigated the effects of surface density of grafted PEO and of protein size on suppression of protein adsorption. They concluded that to achieve repulsion for small proteins (radius of spherical protein R ~ 20 Å) the distance, D, between the terminally attached PEO chains should be small (~ 10 Å), while for large proteins (R ~ 60 – 80 Å), D can be larger (~ 15 Å). Sofia et al (1998) and others (Arai et al., 1990a; 1990b) also studied the effect of protein size and other protein properties on adsorption to surfaces modified by PEO of linear and star types, as discussed in section 1.6 of this thesis.

Different types of surface can be modified using a variety of techniques (Gombotz et al., 1992; Llanos et al., 1993; Osterberg et al., 1995; Palegrodemange et al.,
1991; Santerre et al., 1991; Santerre et al., 1994; Gombotz et al., 1989; Han et al., 1993; Han et al., 1995; Nojiri et al., 1990a; Park et al., 1991; Llanos et al., 1991; Desai et al., 1991a; Noinville et al., 1995; Wesslen et al., 1993; Lea et al., 1994; Wang et al., 1993; Oehr et al., 1992; Mori et al., 1982; Bergstrom et al., 1992), and modifications can be of a chemical or biological nature. Physical immobilization, graft copolymerization and cold plasma treatment of polymer surfaces can be used for surface modification (Ikada, 1994).


2.3.1.2. PEO CONTAINING SURFACES: A MODEL FOR PROTEIN REPULSION

As discussed above, both steric repulsion and hydrophilicity are believed to be properties which reduce blood-surface interactions (Nakao et al., 1987; Lin et al., 1996). Hydrophilic polymers would seem to have a natural advantage, in that biomaterials are used in contact with aqueous media such as blood (Ikada, 1984). As may be noted in the previous discussion, many studies have been conducted on surfaces modified with poly (ethylene oxide) (PEO) (Osterberg et al., 1993; Han et al., 1989; Lin et al., 1991; Gombotz et al., 1991; Lee et al., 1988; Jeon et al., 1991b; Desai et al., 1992; Amiji et al., 1993; Tan et al., 1991; Nagaoka et al., 1983a; Park et al., 1992). PEO is a neutral, hydrophilic and highly flexible polymer (Hoffman, 1987). It is believed that relatively long flexible chains of PEO prevent adsorption by a combination of steric repulsion and high chain mobility (Malmsten et al., 1999; Nagaoka et al., 1984). When grafted to a surface, PEO is expected to give a protective effect against protein and cell interactions, and therefore is
chosen in this research as a surface modifying material. In addition to the influence of molecular weight and graft density of PEO on passivation, the end groups and architecture of PEO were also studied in relation to its protein repellency. In this section, therefore, the properties and applications of PEO are summarized to complement the discussions given in earlier paragraphs.

PEO is hydrophilic due to favorable interactions with water. The water solubility of PEO decreases with increasing temperature due to a decrease in hydrogen bonding interactions with bound water and a corresponding increase in hydrophobic interactions between polymer chains. Bjorling et al (1991) have shown that PEO adopts a helical conformation in polar solvents such as water and an extended conformation in non-polar solvents such as toluene. The helical conformation is better adapted for hydrogen bonding interactions (Harder et al., 1998; Wang et al., 1997).

PEO changes its conformation according to its molecular weight and chemical environment, either in solution or when grafted on a surface. In the latter case a relatively extended conformation is preferred when the molecular weight of PEO is low (Prime et al., 1993) and the surface density is high, thus providing brush-type layers. The random coil conformation-mushroom layer structure is expected to predominate when the molecular weight of PEO is high and the surface density is low. Mixtures of the two may coexist at intermediate molecular weights and densities (see Figure 2.10).

Star type PEO may adopt conformations that are unique and different from those of linear PEO of similar molecular weight. The synthesis and characterization of star type PEOs have been investigated by Merrill et al (1993). The relationship of molecular weight to molecular size was considered, and the dimensions of molecules in the dry and
wet state were investigated. It was found that when star-type PEO changes from the wet to the dry state it may either shrink uniformly, while maintaining its spherical shape, or it may change shape to a disk, the diameter of which is the same as that of the original wet sphere. It was also found that the graft density of PEO on a surface was dependent on the solution concentration (Sofia et al., 1998). The graft density of linear and star type PEOs on silicone surfaces could be varied by changing the concentration of the PEO solution.

The molecular weight of PEO has been shown to be influential for passivation against proteins. Jeon et al (1991b) modeled protein interactions on PEO-modified surfaces in water for the case of PEO chains interacting with a finite spherical, hydrophobic protein. They concluded that the PEO chains should be relatively long to produce the “optimal” layer thickness of PEO for a protein resistant surface. Mori et al (1982) studied the influence of PEO molecular weight on plasma protein adsorption and platelet adhesion to a methoxy-PEO grafted poly(vinyl chloride) surface. Both protein adsorption and platelet adhesion decreased with increasing chain length of the PEO grafts. Gombotz et al (1991) grafted PEO to polyethyleneterephthalate surfaces and found that protein adsorption decreased with increase in molecular weight of PEO. A dependence of protein adsorption on the molecular weight of PEO grafts has also been observed by Desai and Hubbell (1991b). In another study, albumin and fibrinogen adsorption were found to decrease with increasing molecular weight of PEO from 1,000 to 100,000 (Drumheller et al., 1995). Nagaoka et al (1987) showed a direct correlation between chain length and flexibility of PEO in aqueous solution and found that a lower limit on protein adsorption was reached at a degree of polymerization of 100 repeat units. Malmsten et al (1998b), using ellipsometry, studied the effect of PEO on serum protein adsorption. Efficient passivation was observed for PEO
of molecular weight 5000 irrespective of the grafting chemistry at high PEO graft
density. The protein size, relative to the average distance between PEO chains, was also
found to be important for protein rejecting ability.

Linear versus branched chains, and brush versus mushroom structures have been
shown to be important (Mcpherson et al., 1998; Claesson, 1993; Andrade et al., 1993;
Barentin et al., 1998; Beyer et al., 1997; Sofia et al., 1998). Palegrsosdemange et al (1991)
investigated the formation of self assembled monolayers (SAM) by chemisorption on
gold of thiolated alkane oligoethylene glycols (OEO) having the general structure
\( \text{HS(CH}_2\text{OCH}_2\text{CH}_2\text{)}\text{mOH} \). Protein adsorption was reduced on alkane-OEO compared to
alkane layers. The conformation of the oligo-PEO chains on the surface is also known to
be important. For example, it has also been shown (Harder et al., 1998; Wang et al.,
1997; Seigel et al., 1997) that when the OEO moieties form helical and amorphous
structures, the gold substrate is protein resistant. When the OEO moieties form a dense
“all-trans” structure, protein resistance is lost.

Linear PEO may provide more flexibility and higher coverage than branched PEO of
similar molecular weight. However highly branched PEO may allow for a higher
concentration of functional groups on the surface as required in some modification strategies.
The effects of branching and molecular weight of surface bound PEO on fibrinogen
adsorption were discussed by Bergstrom et al (1994). It was found that larger molecules
of both linear and branched PEOs were effective in preventing fibrinogen adsorption to
polystyrene surfaces. However, short-chain, small star-type PEOs were less effective than
their linear counter parts. It was suggested that star PEOs are restricted in their
conformational randomness and therefore have smaller exclusion volumes than the corresponding linear PEOs.

Irvine et al (1998) compared linear and star PEO modified silicon wafers with respect to their protein repelling effects. The effects of surface modification were found to be dependent on the solvent used, via effects on graft density. For linear PEO, high graft density was achieved in a poor solvent. For star PEO, on the other hand, essentially no dependence of graft density on solvent was observed. Protein adsorption was also found to depend on protein size. Albumin adsorption was inhibited by both linear and star PEO, while smaller proteins such as cytochrome-c were adsorbed on star PEO modified surfaces.

Using surfaces having gradients of PEO density, Lee et al (1997) measured the influence of graft density on protein adsorption, platelet adhesion, and platelet activation. All three phenomena decreased with increasing PEO chain length and surface density. Malmsten et al (1999) investigated the influence of chain length and interfacial density of various PEO-containing block copolymers (PEO/polytetramethylene oxide, PEO/polylactide, and PEO/polyethylene imine). Increasing the chain length and interfacial density of PEO improved the effective protein resistant character of these surfaces. Several methods for measuring the surface density of grafts have been discussed (Brash, 1991; Kim et al., 1998; Courtney et al., 1994; Mcpherson et al., 1998; Amiji et al., 1993; Karssen, 1995).

Proteins of different sizes were also used in adsorption experiments by Sofia et al (1998) using linear and star type PEOs. To achieve significant reduction in protein adsorption on linear PEO modified surfaces, it was found that the graft density has to be
high enough that a minimum half-overlapping of PEO chains occurs. To achieve such overlap, higher graft densities were needed for lower PEO molecular weights, indicating a dependence of protein adsorption on PEO molecular weight. Protein adsorption was thus considered to be dependent on the combination of graft density and molecular weight. In contrast to surfaces grafted with linear PEO, it was found that star PEO – grafted surfaces can prevent adsorption of larger proteins at a lower degree of overlap of PEO chains as long as the protein size is larger than the spaces between PEO chains. This is believed to be due to the fact that the polymer segment concentration is much higher than for linear chains of equivalent molecular weight (or equivalent size) which leads to greater steric repulsion forces. Accordingly, high concentrations of star PEO must be used to reach a graft density sufficiently high to passivate the surface for smaller proteins (Sofia et al., 1998).

2.3.2. Surface Modification with Bioactive Ligands.

The development of biomaterials which exhibit biorecognition and biospecificity is a desirable goal (Ratner, 1996). For example, coagulation inhibitors or fibrinolysis activators incorporated into biomaterials may selectively interact with blood components to prevent or reduce thrombosis (Ito et al., 1992). Antithrombin agents such as heparin, hirudin, and D-Phe-Pro-Arg-chloromethyl ketone (PPACK), a reagent that blocks the catalytic site of thrombin irreversibly, can be incorporated into a surface to inhibit coagulation by inactivating thrombin and other activated clotting factors. Enzymes and zymogens involved in fibrinolysis, such as urokinase, streptokinase, t-PA and plasminogen, can be used to promote the lysis of blood clots as they form.
2.3.2.1. COAGULATION INHIBITORS

Polymer surfaces can be modified with coagulation inhibitors including direct (e.g. hirudin) and indirect (e.g. heparin) thrombin inhibitors, and inhibitors of the protein C and protein S pathways such as thrombomodulin. TM initiates PC pathway by binding IIa and converting it into a potent activator of PC. Examples of anticoagulants include heparin, LMWHs, hirudin, hirulog, PPACK (Liu et al., 1994; Weitz, 1995; Mohler et al., 1986; Hanson et al., 1988), and a covalent complex of antithrombin and heparin, ATH (Chan et al., 1998; Berry et al., 1998). In favor of the direct (non-catalytic) inhibitors is that they react rapidly with thrombin, and are not limited by the requirement of a cofactor. On the other hand, indirect (catalytic) inhibitors are not limited to a single thrombin “knockout” but should, in principle, enable the continuing inhibition of thrombin, as long as cofactor is available (e.g. AT in the case of heparin).

A. HEPARIN AND HEPARINIZED MATERIALS

Heparin is a polydisperse mixture of highly sulfated polysaccharides, and has been used extensively as an anticoagulant. It is a strongly negatively charged glycosaminoglycan (GAG). The chain repeating units are disaccharides consisting of an uronic acid residue and a D-glucosamine residue. The uronic acid residue may either be D-glucuronic acid or L-iduronic acid, and the D-glucosamine residue may either be N-sulfated or N-acetylated. The disaccharides may further be O-sulfated at C6 and/or C3 of the D-glucosamine and at C2 of the uronic acid residue (Figure 2.11).

Heparin binds to several serpins such as antithrombin (AT) and heparin cofactor II (Rosenberg, 1987), and facilitates formation of covalent 1:1 complexes of serpins with their target protease (Olson, 1985), thus inhibiting the bioactivities of these proteins (Ragg
et al., 1990b; Ragg et al., 1990a). AT, for example, becomes highly activated when complexed to heparin (Rosenberg, 1975), and may inhibit coagulation by complexing with several of the blood coagulation factors, among them thrombin and factor Xa, two of the most important ones. The active binding sequence on heparin is a pentasaccharide unit which binds to a lysine-containing site on AT and produces a conformational change at the arginine reactive center of AT (Hirsh, 1991; Conrad, 1997). The molecular weight of heparin influences its ability to potentiate the anti-thrombin and anti-factor Xa activities of AT (Weitz, 1997; Bray et al., 1989). Because facilitation of AT inhibition of thrombin by heparin also requires thrombin binding to the polysaccharide, longer heparin molecules (MW > 12,000 Da) are preferred for tertiary complex formation. When the molecular weight of heparin is low (< 5,400 Da), the short chain length remaining after complexation with AT is insufficient to combine with thrombin. Thus, heparin in this molecular weight range only inactivates factor Xa.

![Chemical structure of heparin](image)

**Figure 2.11.** Chemical structure of heparin. The numbered residues constitute the pentasaccharide sequence which interacts specifically with AT.

The anticoagulant effect of heparin is modified by platelets, fibrin, vascular surfaces, and plasma proteins. Platelets inhibit the anticoagulant effect of heparin by secreting the heparin-neutralizing protein platelet factor 4 (Holt et al., 1985). Fibrin binds thrombin and protects it from inactivation by the heparin-antithrombin complex (Weitz et al., 1990). In plasma, approximately 20 times more heparin is needed to inactivate fibrin-bound thrombin.
than free thrombin, and this may be responsible for the higher concentrations of heparin required to prevent the extension of venous thrombi than their initial formation (Hirsh, 1991).

In addition to its role as an anticoagulant, heparin is also believed to possess many other biological activities, including the ability to modulate embryonic development, neural outgrowth, tissue homeostasis, wound healing, metastasis, cell differentiation, cell proliferation, and inflammation (Bazzoni et al., 1993; Nelson et al., 1993; Springer, 1994).

Heparinized surfaces have been investigated extensively in the context of blood compatibility. Heparin has been immobilized to many surfaces (Marconi et al., 1997; Bannan S et al., 1997; Bos et al., 1999), e.g. polyethylene (Pasche et al., 1987), polyethylene glycol (Llanos et al., 1992; Amiji et al., 1993; Nakayama et al., 1993; Piao et al., 1990), polyvinyl chloride (Zdanowski et al., 1997), polymethylmethacrylate (Lai et al., 1996), polystyrene (van Delden et al., 1995), polyacrylamide (Lindhout et al., 1995), polyurethane (Kang et al., 1997; Blezer et al., 1997; Park et al., 1988), polyvinyl alcohol (Rollason et al., 1989), polyethyleneterephthalate (Kim et al., 2000) and collagen (Bequemin et al., 1997). All of these surfaces have been found to exhibit anticoagulant properties. Surfaces based on both physical and chemical bonding have been developed and evaluated experimentally and in the form of devices used in patients (Kagisaki et al., 1997; Elgue et al., 1993; Svenmarker et al., 1997; Larsson et al., 1987).

Bae et al (1999) and Kang et al (1997) attached heparin to a PEO-modified surface via a two-step chemical reaction. A plasma modification method was first used to attach PEO to a polyurethane surface. Heparin was then attached to PEO using a coupling
reaction. Leaching experiments showed 3% of the bound heparin was lost in the first few hours, but very little subsequently. In vitro plasma recalcification time (PRT), activated partial thromboplastin time (APTT), platelet adhesion and activation, and peripheral blood mononuclear cell (PBMC) activation were used to evaluate the blood compatibility. It was found that PRT was prolonged on heparin-immobilized polyurethane (PU-Hep) when compared to a PU control. APTT was also significantly prolonged on PU-Hep, indicating that the immobilized heparin is capable of interacting with antithrombin. Platelet adhesion was significantly decreased by the immobilization of heparin on PU surfaces. Cells adhered less on heparin-immobilized PUs than other surfaces.

The effect of the configuration and manner of attachment of heparin on the surface has also been investigated. Elgue et al (1993) found that when heparin was attached via the chain end and presaturated with AT, both thrombin and factor Xa were inhibited. Sanchez et al (1997) reported that attachment of heparin via the chain end gives rise to a surface having strong anticoagulant activity. The Carmeda Company (Carmeda, 2000; Elgue et al., 1993; Riesenfeld et al., 1995) has reported that end-attached heparin is more effective as an anticoagulant than heparin in other configurations, presumably due to retention of the active pentasaccharide motif. Byun et al (1994) showed that the insertion of a “spacer” between heparin and the surface gave increased antithrombotic activity, suggesting that increased mobility increases the activity of the heparin.

Sanchez et al (1997) studied the relation between surface density of covalently bound heparin on a surface and inhibition of the plasma contact activation system. Six different heparin surfaces were prepared on polyethylene tubing and studied in contact
with human plasma. The surfaces expressing 4 pmol/cm² or more of specific antithrombin binding sites were found to generate potent anticoagulant activity. Below this level, the thromboresistant properties deteriorated as the density decreased.

It is worthwhile pointing out that a heparinized surface with sustained anticoagulant activity needs to show both effective catalysis of AT and the ability to maintain this activity over time. Nonspecific protein adsorption on a heparinized surface needs to be suppressed to a lower level for the heparin exposed to the blood to remain functional for long-term performance. Nishida et al (1999) investigated protein adsorption on cardiopulmonary bypass (CPB) circuits with both heparin-coated and control surfaces. Heparinized surfaces showed better blood compatibility. Although the protein adsorption patterns were not markedly different, fibrinogen appeared more abundant on the non-heparinized surface and conversion to fibrin was more pronounced. Van Delden et al (1996) investigated the effect of other plasma proteins on the inhibition of thrombin by heparin that was attached to a polycarbonate surface. They showed that only the surface immobilized heparin molecules, which bind AT in a reversible manner, contribute to the anticoagulant properties of the surface. Also, thrombin activity decreased as the AT concentration on the surface increased, and the activity of surface bound thrombin was not affected by the presence of other plasma proteins.

**B. LOW MOLECULAR WEIGHT HEPARIN (LMWH)**

LMWHs, varying from 1,800 to 12,000 Da, are obtained by partial depolymerization of unfractionated heparin (UFH). This has been carried out by treatment with HNO₂, heparinases, and base elimination following partial esterification of uronic acid carboxyls (Linhardt et al., 1999). Different preparations of LMWH exhibit
different anti thrombin activity, possibly due to varying contamination by higher molecular weight chains, and varying charge density. LMWH shows a significantly longer plasma half-life, probably due to reduction of non-specific protein binding by LMWH (Young et al., 1994; Huang et al., 1998). LMWH has great activity against factor Xa than against thrombin because at least half of the heparin chain are too short to bridge antithrombin to thrombin (Hirsh et al., 1998; Olson et al., 1992). LMWH exhibits reduced nonspecific binding to plasma proteins, platelets, endothelial cells and macrophages. Consequently, LMWH produces a more predictable anticoagulant response than heparin (Hirsh, 1999).

Although there have been numerous studies examining the utility of LMWH as a systemic anticoagulant (Hirsh et al., 1999; Marbet, 1999) as well as studies on the effect of LMWH on patients using different blood contacting devices (McMillan et al., 1997; Taylor et al., 1996; Alkhunaizi et al., 2000), research on surfaces modified with LMWH is limited. Byun et al (1994) carried out several studies on immobilized LMWH including investigation of the mechanism of thrombin inactivation, the effect of fibronectin on the binding of AT (Byun et al., 1996b), and the binding of AT and thrombin under flow conditions (Byun et al., 1996a). Also, there have been reports (Xiao et al., 1998) showing that LMWH on a surface reduces the adhesion of platelets, making it more attractive as an antithrombotic material. There is clearly a need for more thorough investigation of LMWH in the context of immobilization on biomaterials.

C. HIRUDIN, HIRULOG AND HIRUGEN

Hirudin, Hirulog® and Hirugen® are a family of thrombin inhibitors which interact directly with the active site or anion-binding exosite of thrombin. Hirudin, a 65
amino acid protein, binds both to the active site and the exosite, and thus is able to inhibit all thrombin-mediated reactions (both free and bound thrombin) with high potency (Rydel et al., 1990; Markwardt et al., 1992). Hirulog is a 20 amino acid analogue of hirudin and behaves similarly to hirudin (Maraganore et al., 1990). Hirugen is a synthetic N-acetylated dodecapeptide analogue of the carboxy-terminal of hirudin (Ac-Asn-Gly-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Glu-Tyr(SO3)-Leu). Hirugen interacts only with the exosite of thrombin and therefore is a less effective antithrombotic than hirudin (Kelly et al., 1992; Naski et al., 1990).

Hirudin has been immobilized on biomaterials by a number of methods (Phaneuf et al., 1998; Berceli et al., 1998; Seifert et al., 1997). Significant surface densities of hirudin have been achieved, giving surfaces that bind and inhibit thrombin. For example, Seifert et al (1997) studied hirudin-modified polylactide/polyglycolide surfaces and found that blood contacting properties were improved, e.g. platelet adhesion and activation were diminished. A fundamental disadvantage of hirudin as a surface modifier is that only one thrombin can be inactivated per hirudin molecule.

D. (D)FPR, (D)FPR-H AND PPACK

(D)FPR, (D)FPR-H and PPACK are forms of the tripeptide, D-phe-pro-arg. The tripeptides include the amino acids recognized by thrombin in its natural substrates such as platelet thrombin receptor and fibrinogen (Claesson, 1993; Tapparelli et al., 1993). The aldehyde group in DFPR-H and the chloromethyl ketone group in PPACK bind covalently to the histidine residue in the active site of thrombin, leading to a stronger inhibition effect than for the hirudin derivatives, or for the unmodified tri-peptide.
Polyurethane surfaces modified with PPACK have been prepared by Tian (1995) and were found to inhibit thrombin. Sun et al (2000) attached the cysteine derivative C(D)FPR, to gold surfaces via the thiol group of the cysteine residue and showed that, in contact with plasma, the peptide modified surface was able to bind and inhibit thrombin. These direct inhibitors are not expected to be regenerated after inactivating thrombin. Therefore a surface modified with such inhibitors would have limited thrombin inactivating capabilities.

E. COVALENT COMPLEX OF AT AND HEPARIN (ATH).

A new anticoagulant, ATH, has recently been proposed by Berry et al (2000). The preparation of ATH takes advantage of the fact that AT forms a natural complex with heparin. A Schiff base/Amadori rearrangement (Scheme 2.1) and the keto-amine linked conjugate of AT and heparin lead to the final covalent compound ATH after reduction (Berry et al., 2000).

The reaction of ATH with thrombin was shown to be faster than that of mixtures of heparin and AT because AT-heparin binding, the rate determining step in the heparin-catalyzed inhibition of thrombin by AT, is eliminated. Furthermore, only heparin molecules which have a pentasaccharide sequence were selected by AT to make ATH (only about one third of the heparins in commercial UFH have the pentasaccharide (Berry et al., 2000)).
Scheme 2-1. Schiff base formation and Amadori rearrangement lead to the final covalent compound ATH.

Since antithrombin has a greater variety of functional groups than heparin, there is a wider choice of chemistries available for surface attachment of the complex compared to heparin alone. Amino, carboxylate, guanidinium, imidazole, indole and phenolic groups on the protein allow for linkage of ATH to the surface through antithrombin (Berry et al., 2000). Since the surface already has permanently-attached antithrombin, it is less likely, in contrast to the heparin coated surface, that adsorption of other proteins in the blood will occur to any significant extent.

ATH has been attached covalently to the polycarbonate polyurethane Corethane™ (Corvita, Miami USA) (Corvita, 2000). Significant non-catalytic and
catalytic activities against thrombin were demonstrated (Chan et al., 1999; Klement et al., 2001). In vivo experiments using a rabbit model showed a 4 to 5 fold reduction in the weight of clot generated on ATH-treated compared to non-treated surfaces (Chan et al., 1999). In vitro and in vivo data showed also that a surface modified with ATH is superior to a hirudin-modified surface. The dual direct and indirect antithrombin activities of ATH may represent a new approach to the passivation of biomaterials for use in contact with blood.

F. THROMBOMODULIN (TM)

Thrombomodulin is an endothelial cell associated protein that functions as a potent natural anticoagulant by serving as a thrombin receptor. Once bound to thrombomodulin thrombin undergoes a conformational change that converts it from a procoagulant protease to an anticoagulant. Yagi et al (1989) immobilized bovine lung thrombomodulin on agarose gel (Sepharose 4B) and showed that this material inhibited the procoagulant activity of thrombin, and enhanced the thrombin-catalyzed activation of protein C. Vasilets et al (1997) grafted acrylic acid onto polytetrafluoroethylene and then used a CO₂ plasma-initiated vapor phase polymerization technique for immobilization of human thrombomodulin. The activity of immobilized human thrombomodulin was assessed by the protein C activation test. Kishida et al (1995) immobilized human thrombomodulin on regenerated cellulose films and hollow fibers. The activity of the immobilized TM was evaluated both in vivo and ex vivo. It was found that the immobilized TM retained its enzymatic activity for activation of the protein C anticoagulant pathway. The coagulation time of blood exposed to a TM-immobilized hemodialyzer was effectively prolonged.
2.3.2.2. PROMOTERS OF CLOT-LYSIS

Another strategy to develop a clot-free surface is to exploit the fibrinolytic or clot-dissolving system which removes clots after they are no longer needed (McClung et al., 2000). In fibrinolysis, plasminogen is converted to its active enzyme form plasmin which then degrades the clot (Collen et al., 1991; Baxmann, 1993; Fowers et al., 1997). Different authors (Woodhouse et al., 1994; Woodhouse et al., 1996; Fowers et al., 1997; McClung et al., 2000) have developed this approach. In general the surface is designed to bind endogenous plasminogen preferentially by incorporation of lysine residues.

Woodhouse et al. (1996) developed lysine-derivatized silica glass as a plasminogen-binding surface. They showed that, in the presence of t-PA, a preformed clot may be lysed by adsorbed plasminogen, and that clot lysis is significantly enhanced when the plasminogen is adsorbed via its lysine binding sites.

McClung et al. (2000) developed surfaces based on polyacrylamide to which lysine moieties were attached. Surfaces of varying lysine content in which the lysine was bound through the a-amino groups, leaving the epsilon-amino groups free, were investigated. These surfaces were able to adsorb large amounts of plasminogen, in the range expected for a compact monolayer. Evidence was presented showing that these surfaces adsorb plasminogen to the exclusion of the other plasma proteins.

2.3.3. SURFACES COMBINING GENERAL PASSIVATION AND BIOSPECIFICITY

Both of the modification strategies discussed above may be applied simultaneously to develop surfaces which prevent nonspecific interactions but promote specific interactions designed to give a desirable biological effect (Velichkova et al., 1998; Gok et al., 1994). For example, as has been discussed, end-attached hydrophilic
polymers should give a surface which is unreactive to proteins and cells. In addition, the incorporation of bioactive moieties may promote specific interactions with target proteins that will inhibit thrombosis (Ishihara et al., 1996; Doillon et al., 1994; Park et al., 1991). In our approach, a combination of PEO with heparin was selected to improve the blood compatibility of surfaces.

2.3.3.1 COMBINATION OF HEPARIN AND PEO

PEO may be modified by conjugation to heparin at the chain ends using a variety of methods. Several groups (Grainger et al., 1988; Bae et al., 1999; Llanos et al., 1992; Rollason et al., 1989; Park et al., 1992; Kim et al., 1991; Piao et al., 1992; Piao et al., 1990; Byun et al., 1996b; Byun et al., 1994) have modified various surfaces with both heparin and PEO, and found the blood compatibility to be greatly improved.

Nojiri et al (1990b) incorporated heparin into a segmented polyurethane urea (Biomer) surface via hydrophilic poly(ethylene oxide) spacers and block co-polymer PEO/poly(dimethylsiloxane) (PDMS) spacers. The long-term in vivo blood compatibility of vascular grafts tested in the abdominal aorta of dogs for up to three months was evaluated using Biomer and PEO grafted Biomer as controls. Protein adsorption (albumin, fibrinogen, and IgG) was studied by an immunogold staining method. Heparin immobilized surfaces showed a thin protein layer (300-600 Å) even after 3 months, with high concentrations of albumin and IgG and a relatively low concentration of fibrinogen. These surfaces showed high heparin bioactivity in vitro and excellent blood compatibility in in vitro-ex vivo experiments.

Piao et al (1990) attached heparin and PEO to poly(dimethylsiloxane) (PDMS). Diamino telechelic poly(dimethylsiloxane) (H₂N-PDMS-NH₂, MW 20,000) was first
reacted with toluene 2,4-diisocyanate. This modified PDMS was then coupled to
diamino-telechelic poly(ethylene oxide) (H₂N-PEO-NH₂ MW 2000, 4000, 6000) to create
BAB type block copolymers having terminal amino groups. The copolymers were finally
reacted with heparin containing terminal aldehyde groups to yield a bioactive block
copolymer. Surfaces prepared from such block copolymers showed heparin bioactivity in
vitro, and improved nonthrombogenic properties in *ex vivo* aorta-to-aorta shunt
experiments. Piao et al (1990; 1992) also developed a method to increase the
concentration of heparin immobilized on a polymer surface using PEO as a spacer.
Polyurethane-urea (PU) coated glass beads were first modified with diisocyanates
followed by treatment with polyfunctional polymers (PFP), including poly(vinyl alcohol),
poly(ethylenimine), and poly(allylamine), giving surfaces with -OH, -NH, and -NH₂
groups respectively. By reacting these PFP surface with diisocyanates and then α, ω-
diamino-terminated polyethylene oxide, multiple PEO chains were able to be introduced
to the surface. The amino groups on PEO were then allowed to react with heparin. A two-
fold increase in immobilized heparin content was obtained compared to surfaces where
heparin was immobilized directly. Bioactivity tests (including activated partial
thromboplastin time, thrombin time, and anti Xa activity) demonstrated increased activity
of heparin when immobilized through PFP-PEO compared with PFP and PU alone.

The same effect of PEO spacer has been reported by Tay et al (1989), in which
heparin was covalently bonded to poly(vinyl alcohol) (PVA) hydrogels and to
polyethylene oxide hydrogels activated by tresyl chloride. It was found that as the amount
of tresyl chloride used to activate PVA increased, the ability of the bound heparin to
inhibit thrombin decreased, suggesting that crowding of heparin units on a surface limits
their access to AT. PEO-heparin surfaces had almost ten-fold greater activity than PVA-heparin surfaces at comparable heparin concentrations, suggesting that the PEO spacer may give the heparin easier access to AT. Others (Park et al., 1992; Kim et al., 1991; Park et al., 1988; Park et al., 1991) succeeded in grafting heparin, through the use of PEO as a “spacer”, to a segmented polyurethane urea (SPUU) surface. The bioactivity of the immobilized heparin was enhanced by the incorporation of these spacers and the effect was dependent on the PEO chain length. The use of the PEO spacer reduced protein adsorption and subsequent platelet adhesion compared to Biomer controls. Ex vivo experiments showed the ability of the immobilized heparin to inhibit thrombosis.

Byun et al (1994) studied the effect of a PEO spacer on the binding kinetics of immobilized heparin to antithrombin and thrombin. The binding of AT and thrombin to control surfaces (no heparin), soluble heparin, heparin immobilized directly onto the surface, and heparin immobilized via spacer groups, were compared. It was found that soluble heparin bound both thrombin and AT, while heparin immobilized directly onto the surface bound only thrombin. Spacer-immobilized heparin bound both AT and thrombin, although to a lesser extent than soluble heparin. Thus, the enhanced bioactivity of spacer-immobilized compared to directly-immobilized heparin, was attributed to the retention of AT binding. Byun et al, (1992; 1996b; 1994) also attached PEO-heparin to a surface using toylene diisocyanate. Kinetic studies of the binding of AT and thrombin to this surface showed that the bioactivity of the immobilized heparin was reduced to about 16% that of the free heparin.

Kim et al (2000) developed an insulin and heparin co-immobilized polyethylene terephthalate (PET) material. This material (PET-I-H) was prepared by grafting PEO to
PET, followed by reaction first with insulin and then heparin. Plasma recalcification times and activated partial thromboplastin times were significantly prolonged for both heparin-immobilized PET (PET-H) and PET-I-H. Platelet adhesion was reduced by the introduction of PEO and insulin, and decreased further by the immobilization of heparin.

Llanos and Sefton (1992) immobilized a heparin-PEO conjugate to poly(vinyl alcohol) hydrogels through a terminal isocyanate. An increase in the in vitro whole blood clotting time of the modified gels was observed. Ex vivo assessment using a chronic canine A-V shunt in dogs showed that heparinization of the hydrogel had no effect on platelet activation.


Considerable research has already been done on surfaces modified with PEO or with heparin (including PEO-heparin conjugates). Heparin-PEO conjugates have been proposed by Kim et al and other groups from the standpoint of providing a spacer to improve the activity of the bound heparin. However, little work has been done where the two approaches are combined. Even less consideration is evident in previous work of the idea that nonspecific interactions of proteins other than AT should be prevented.

Our concept for a surface in which the two approaches are combined is shown in Figure 2.12. Some of the PEO molecules are conjugated to heparin at the “distal” chain end. Emphasis has been not only on identifying the functionality of heparin (both unfractionated heparin and LMWH were used), but on clarifying systematically the protein repellent properties of PEO as well.
Figure 2.12. Schematic representation of a gold surface modified with both PEO to prevent nonspecific protein adsorption, and PEO conjugated to a bioactive ligand A, e.g. heparin to promote adsorption of AT.

2.3.4. THIOL MODIFIED GOLD AS A MODEL SURFACE

Gold metal would potentially be suitable for biomaterial applications because of its inertness, and demonstrated nontoxicity (Eriksson et al., 1997). Gold surfaces can readily be modified via reaction with thiol containing compounds; such reactions occur at room temperature under generally mild conditions. In principle, thiol-terminated polymers can be “grafted” (chemisorbed) to a gold surface with high graft density corresponding to the density of thiol binding sites (Sarathy et al., 1997). On the (111) surface of crystalline gold these sites are located in the three-fold hollows between gold atoms, and due to the relatively large size of the sulfur atom, they are spaced about 5 Å apart (Ulman, 1991). In this work we have used gold-thiol chemistry to prepare a variety of model surfaces containing heparin and PEO of different chain length and architecture. These surfaces are models in the sense that the application to devices is not contemplated at this time. Rather the objective was to evaluate the effects of these modifications on protein interactions and blood coagulation.
The surface formed by chemisorption of thiol-terminated hydrocarbons on gold has been extensively investigated. When the chain length of the alkyl thiol compound is of an optimum value, densely packed, crystal-like assemblies are formed with fully extended zigzag carbon chains tilted at about 30° to the normal to maximize interactions between adjacent chains as shown in Figure 2.10 (Karssen, 1995). These structures belong to the class of materials known as self-assembled monolayers (SAMs); they result from noncovalent intermolecular interactions, including van der Waals interactions and hydrogen bonding (Nuzzo et al., 1987). Although these are weak interactions (0.1 to 5 kcal/mol) and of the same order of magnitude as thermal energies, SAMs are stabilized via the multiplicity of the interactions (Whitesides et al., 1991). Alkane thiols have been found to bind to (1, 1, 1) crystalline gold surface in a manner which reflects the structure of the surface. As indicated above the sulfur atoms are located in the threefold “hollow” sites of the gold surface (Whitesides et al., 1991). The nearest-neighbor distance between the sulfur atoms is about 0.5 nm, corresponding to a surface density of $4.6 \times 10^{14}$ molecules/cm$^2$ if all sites are occupied.

Surfaces having well-defined chemistry may be prepared using alkane thiols with different terminal functional groups (Whitesides et al., 1991). A number of factors, such as the method of preparation of the gold substrate (Buchers et al., 1994; Kuther et al., 1997; Schneegans et al., 1982; Creager et al., 1992; Lestelius et al., 1997; Tidwell et al., 1997; Zhang et al., 1997; Nuzzo et al., 1987; Kuther et al., 1997; Tao et al., 1997) and the solvent from which the thiol is chemisorbed (Bain et al., 1989; Kelemen, 1990) have been shown to influence the structure and quality of alkane thiol monolayers on gold.
Xu et al (1998) investigated structural changes during the growth of layers based on CH$_3$(CH$_2$)$_{17}$O(CH$_2$)$_{19}$-SH using time dependent AFM images. From the relationship of the change of thickness with time, kinetics of the adsorption process can be derived. It was concluded that the alkane thiols adsorb initially with their molecular axis parallel to the surface. As coverage increases toward saturation, a two-dimensional phase transition occurs to form clusters of molecules. Finally, the clusters grow and eventually merge to form a SAM.

![Figure 2.13](image)

Figure 2.13. Schematic representation of self-assembled monolayers of alkane thiols on a (1,1,1) gold surface. When the alkane thiol (structure shown explicitly for the rightmost chain) is long enough, densely packed, ordered assemblies with fully extended carbon chains tilted from the normal to the surface at about 30° are formed.

Although it is hard to know a priori whether HS-PEO chemisorbed on gold will form a self-assembled monolayer, it is probable that it can passivate the surface to nonspecific protein interactions. It is reported recently (Zhang et al., 2000) that PEO modified gold surface exhibit excellent passivation towards protein adsorption and platelet adhesion. Also, in principle, the density of PEO on these surfaces should be variable from very low to very high. Further, by attachment of appropriate bioactive species to the PEO it may be possible to prepare surfaces having a given biological response, e.g. heparin to inhibit blood coagulation, antiplatelet compounds to inhibit
platelet surface interactions. This is the approach on which the research presented in this thesis is based.

2.3.5. PEO- AND HEPARIN-MODIFIED GOLD SURFACES

From the discussion given above, it may be hypothesized that surfaces grafted with PEO at high grafting density should effectively protect surfaces from protein adsorption. The conjugation of heparin to PEO should additionally promote selective interactions with antithrombin thus giving the surface anticoagulant properties. When gold is used as a substrate, varying density (up to very high values) of the “grafted” species should be possible. In the limit, self assembled monolayers of well defined structure may be formed (Mrksich et al., 1997; Wang et al., 1997; Harder et al., 1998). Protein adsorption to thiol-modified gold surfaces can be used as an indication of the blood compatibility of surfaces (Orschel et al., 1998; Ishihara et al., 1995; Prime et al., 1991; Mrksich et al., 1995).

2.3.5.1. THIOLATION OF PEO

Considerable effort has been expended on the synthesis of functionalized PEOs, among them thiolated PEO as required for surface modification. Several methods have been described for the thiolation of PEO. Harris et al (1991) synthesized PEO thiols using tosylated PEO and the thiolation agent sodium hydrosulfide (Reaction 2.1). This reaction can be performed in water, but some disulfide may also be formed under these conditions (Reaction 2.2).

\[
\text{MPEG-OTs} + \text{NaSH} \xrightarrow{\text{H}_2\text{O}} \text{MPEG-SH} \quad (2.1)
\]

\[
\text{MPEG-SH} \xrightarrow{\text{H}_2\text{O}} \text{MPEG-S-S-PEGM} \quad (2.2)
\]
Herron et al (1994) used N-succinimidyl-3-(2-pyridyldithio)propionate (SPDP) to thiolate amino-terminated PEO (Reaction 2.3).

\[
\text{PEO}^\text{-NH}_2 + \text{N-SPP} \rightarrow \text{PEO}^\text{-NH}\text{C}\text{CH}_2\text{CH}_2\text{-SH}
\]  

(2.3)

The thiolation of poly(vinyl alcohol) (PVA), which, like PEO, is strongly hydrophilic, was investigated by Barbosa et al (1995). They used mercaptoacetic acid as a thiolation reagent. Because this reaction is simple and easy to perform, it has been adopted for use in the thiolation of PEO in the present research.

2.3.5.2. CONJUGATION OF PEPTIDES AND PROTEINS TO PEO

Although heparin was the only bioactive component investigated in this research, it was anticipated initially that proteins and peptides would also be of interest. Methods for the conjugation of these species to PEO were thus developed. The conjugation of amino acids, peptides and small proteins to PEO can be done using amino, carboxylic acid, thiol or other functional groups of the peptide. Zalipsky et al (1992) converted methoxy-polyethylene glycol of molecular weight 5000 to the reactive succinimidyl carbonate form (SC-PEG) (Reaction 2.4). SC-PEG gave high yields of protein conjugates under mild conditions, and was most reactive at pH 9.3. The commonly used succinimidyl succinate derivative of methoxypolyethylene glycol (SS-PEG) served as a reference standard to which the new reagent was compared. Scheme 2.1 lists various PEG-protein conjugation reactions found in the literature (Zalipsky et al., 1983; 1987; 1997 a, b).
Conjugation via amino groups in the protein or peptide is widely practiced since it has been found that biological activity in most peptides and proteins is thereby retained (Zalipsky et al., 1997b). The synthesis is usually carried out in two steps. The first step involves the activation of the PEO to form an electrophilic group at the chain end. The peptide can then be attached via the PEO chain end. For example one of the chlorines in trichloro-s-triazine, as illustrated in reaction (1) scheme 2.2, can be reacted with PEO.

\[
\text{PEO-OH} + \text{Cl-C-Cl} + \text{HO-} \xrightarrow{\text{Cl}} \text{PEO-O-C-O} \quad (2.4)
\]

Scheme 2-2. Conjugation of protein to MPEG (methoxy-PEO) via different reactions.

The other chlorines may then react with the amino groups of a protein or peptide (Brooks et al., 1988). This reaction provides an effective one-step activation of the polymer. However, the toxicity of the cyanuric chloride derivatives and the reactivity of these
compounds towards groups other than amines limit its application. Conjugation via succinic anhydride-modified PEO (Joppich et al., 1979), as shown in reaction 2, scheme 2.2, largely overcomes this difficulty.

In the present research, a protocol by which most amino acids, peptides or proteins may be attached to PEO was desirable. To maintain the structure and activity of proteins, the reaction should proceed under mild conditions (room temperature, normal pressure) and water should be used as the reaction medium. However, mild reaction conditions require that the reactivity of PEO with the targeted protein functional group should be high. At the same time reactivity with non-involved protein side groups should be low. The protocol using N-hydroxysuccinimide (NHS) was deemed to be a reasonable compromise. The complete reaction sequence is illustrated in Scheme 2.3.
CHAPTER 3

OBJECTIVES AND SCOPE OF THE PROJECT

The research described in this thesis aims at improving the blood compatibility of blood contacting materials and devices by surface modification. Previous research has used a number of different approaches. Some groups have focused on passivating the surface, i.e. inhibiting protein and cell interactions. Others have designed the surface to interact specifically with particular blood components to give anticoagulant or antithrombotic effects. There has been little work on designs in which both passivation and specific interactions are included.

As discussed in Chapter 2, there is considerable evidence that the attachment of PEO to a surface makes it protein repellent. Also heparin is well known as a potent antithrombotic agent, and because it inhibits thrombin by a cyclic catalytic mechanism it has the potential to be active over relatively long periods. Thus by the incorporation of heparin, the antithrombotic properties of a surface are expected to be improved. The incorporation of both PEO and heparin should provide protection from nonspecific protein adsorption, and active antithrombotic properties at the same time. This is the main hypothesis of the present research.

As discussed in chapter 2, the modification of gold surfaces with thiols is a simple process and should lead to surfaces that have reasonably well defined structures and controllable compositions. Moreover methods exist for the attachment of terminal
thiols to PEO. The objective of this research, therefore, was to investigate gold surfaces modified with PEO and heparin as model materials for blood contacting applications.

The work done includes: (1) the synthesis and characterization of HS-PEO and HS-PEO-A, where A is a bioactive moiety, with HS and A at opposite chain ends of the PEO chains. Both linear and star-type PEOs were used. (2) The modification of gold surfaces with the various thiolated PEOs. (3) The physical and chemical characterization of these surfaces. (4) The investigation of the blood interactions of these surfaces with emphasis on protein adsorption and anticoagulant effects.

Methods for the chain end thiolation of linear and star-type PEO and for the conjugation of amino acids, peptides proteins and other bioactive moieties to HS-PEO have been developed. Specifically PEO was synthesized with one end bearing a thiol group, and the other end a group such as methyl or hydroxyl, or heparin (expected to adsorb AT and inactivate thrombin).

Substrates consisting of thin gold layers deposited on polymer films or silicon wafers were used as substrates for chemisorption of HS-PEO and modified HS-PEO. Surfaces were prepared using linear PEO, star-type PEO, and heparin-PEO conjugates singly and in different combinations.

The structure and properties of the chemisorbed films were investigated using contact angle and X-ray photoelectron spectroscopy (XPS). A protocol was developed to attach a radioactive isotope to PEO as a means of estimating the “graft” density of PEO on the gold surface.

Biological interactions of the surfaces were investigated by measuring protein adsorption from single protein solutions and from plasma using radiolabeling methods.
Fibrinogen, albumin and IgG were studied in single protein experiments since they are the most abundant proteins in plasma and are important for blood compatibility. For the heparinized surfaces, the anti factor Xa activity of bound heparin was measured using a chromogenic substrate assay.

More comprehensive information on the proteins adsorbed from plasma to the different surfaces was obtained by gel electrophoresis and immunoblotting methods (Mulzer et al., 1989). The influence of PEO properties (MW and architecture) as well as those of heparin on the composition of the protein layer deposited from plasma onto the modified surfaces was investigated. These methods also provided information on the effect of the surfaces on various hematological responses, particularly the coagulation and complement pathways.
CHAPTER 4

SYNTHESIS OF MATERIALS FOR SURFACE MODIFICATION

4.1. INTRODUCTION

Thiol groups must be present at the PEO chain end in order for the PEO to be chemisorbed (effectively “grafted”) to gold surface. PEOs of different types, namely linear PEOs terminating in either OH or MeO, star-type PEOs, and heparinized PEOs, were used in this work and were thiolated using two methods. When it was required that one end only of the PEO chain be thiolated, simple esterification of the appropriate dihydroxy PEO with mercaptoacetic acid was carried out. The probability of monosubstitution and dissubstitution in a chain was considered as discussed below (section 4.3.1.2.). When it was required that one chain end be a thiol group and the other a bioactive ligand (e.g. heparin, proteins, peptides), the PEO was first activated with N-hydroxy succinimide at both chain ends and then reacted sequentially with the ligand followed by aminoethane thiol. This method appears to be applicable to most amino acids, peptides and proteins. In the present work, glycine, glycine methyl ester, lysine and N-BOC-lysine were conjugated to PEO. The major emphasis was on heparin as a bioactive ligand as discussed in chapter 7. Heparin was conjugated to PEO using the same coupling approach.
The products of these reactions were characterized by nuclear magnetic resonance spectroscopy (NMR), infrared spectroscopy (IR), and gel permeation chromatography (GPC).

4.2. EXPERIMENTAL

4.2.1. MATERIALS

Linear PEOs with a hydroxyl group at one end of the chain and a methoxyl group at the other (molecular weights 100, 350, 600, 1500, 2000, 3400, and 5000); linear PEOs with hydroxyl groups at both chain ends (molecular weights 100, 300, 600, 1500, 2000, 3400, and 5000); and star-type PEOs with hydroxyl groups at all chain ends (total molecular weights 2000 and 8000), were from Shearwater Polymers, Inc. (Huntsville, AL).

Tetrahydrofuran, N,N-dimethylaminopyridine, poly(propylene glycol) of molecular weight 1050, N-hydroxy succinimide, dicyclohexyl carbodiimide, poly(ethylene glycol) bis(carboxymethyl) ether (MW 600), methylene diphenyl diisocyanate (MDI), potassium carbonate, poly(tetramethylene oxide) (PTMO), mercaptoacetic acid, mercaptoethanol, isopropyl ether, dichloromethane, chloroform, ethylene diamine, methanol, ethanol, acetone, dimethyl sulfoxide, and dimethyl formamide were from Sigma-Aldrich (Oakville, ON). Cysteine, lysine, tyramine, heparin, Na$_2$HPO$_4$, NaH$_2$PO$_4$, H$_2$O, Alcian Blue, Tween 20 and NaCl were also from Sigma-Aldrich (Oakville, ON). Na$^{125}$I was purchased from ICN Biomedicals (ICN Biomedicals, Quebec, Canada). Iodogen was purchased from Pierce Chemical (Iodo-Gen Reagent, Pierce Chemical, Rockford, Illinois, USA).
4.2.1.1 INSTRUMENTATION FOR CHARACTERIZATION OF SURFACE MODIFYING MOLECULES

Proton and carbon-13 nuclear magnetic resonance (NMR) spectra were recorded using an AM-400 Bruker, 2D spectrometer and a DRX-500 Bruker spectrometer. Fourier transform infrared (FTIR) spectra were recorded using a Mattson Polaris FTIR spectrometer. Samples were in the form of KBr pellets. UV-visible spectra were recorded with a Beckman DU-65 spectrometer.

Gel permeation chromatography (GPC) (Waters Associates) for determination of the molecular weights of polyurethanes was carried out using four polystyrene (Styrage®) columns in series, a high-pressure pump and a differential refractive index detector. The polyurethane samples were dissolved at a concentration of about 0.2 mg/mL in a mobile phase containing 0.1 wt% LiBr in HPLC grade dimethylformamide. Samples of about 200 μL were injected and analyses carried out at 80°C and a flow rate of 1.0 mL/min. The calibration standards used were narrowly dispersed polystyrenes (Toyo Soda Manufacturing Co, Tokyo). Polystyrene equivalent molecular weights are reported.

4.2.1.2. SYNTHESIS OF POLYURETHANE

A polyether polyurethane was used as a substrate on which to evaporate gold. It was synthesized according to a two-step solution polymerization method described elsewhere (Brash et al., 1973). The procedure is illustrated in Scheme 4.1. In the first step, PTMO (MW 1000) was reacted with MDI (1:2 stoichiometry) in DMSO for 2 h at 90°C. This reaction gives a prepolymer of average composition MDI-PTMO-MDI. The prepolymer was then reacted with the chain extender ethylene diamine (1:1
stoichiometry) at 50°C for 1 h., to produce high molecular weight polymer. Both reactions were run in a nitrogen atmosphere to eliminate moisture.

\[
\begin{align*}
2n \text{O} &\equiv \text{C} = \text{N} - \text{C} = \text{O} + n \text{HO-PTMO-OH} \\
\text{HO-PTMO-OH} &\quad \text{NCO} \\
\text{H}_2\text{N-CH}_2-\text{CH}_2-\text{NH}_2 &
\end{align*}
\]

Scheme 4-1. Synthesis of the polyether polyurethane used as a substrate for gold.

4.2.2. THIOLATION OF PEO

A typical thiolation reaction was carried out as follows. Monomethoxy PEO of MW 350 (MPEO(350)-OH, 3.50 g, 10 mmol) was introduced into a 50 mL three-neck flask (Figure 4.1). The flask was equipped with a stirrer and a calibrated distillation trap to collect water produced by the reaction. The system was vacuum dried at 80°C for 30 min. Toluene (40 mL) was then introduced into the flask to dissolve the PEO. The flask was heated in an oil bath to 110°C. Mercaptoacetic acid (2.76 g, 30 mmol) and concentrated sulfuric acid (2 drops) were then added. The reaction was allowed to proceed for 2 h. The product was precipitated three times in ether. The final product (MPEO(350)-SH) was dried under vacuum at 40°C overnight. The volume of water produced was measured as a means
of monitoring the reaction kinetics. The procedures for the reaction of PEO-COOH with HO-PEO-OH were carried out in a similar fashion.

Figure 4.1. Reaction vessel for thiolation of PEO. A volume-calibrated distillation trap was used to collect water produced by the reaction. The quantity of water produced was used to monitor the reaction kinetics.

The thiolation of polypropylene oxide (PPO) of molecular weight 1025 was carried out in the similar manner.

The procedures for thiolation of star-type PEOs were similar; a molar ratio of 1:3, mercaptoacetic acid: hydroxyl groups (in PEO) were used in all cases.

4.2.3. **Conjugation of Bioactive Moieties to PEO**

The bioactive moieties discussed in this section are all amino acids. These were chosen as representatives of the broader class of amino acids, peptides and proteins.
Attention was focused on reaction between the terminal carboxyl group of the modified PEO and the terminal amino group of the bioactive component. Furthermore it should be noted that this part of the research was restricted to the synthesis of these conjugates, and no investigation of surfaces based on their chemisorption to gold was carried out. Studies of surfaces based on PEO-bioactive component combinations were restricted to PEO-heparin as discussed in chapter 7.

Conjugation of amino acids to PEO was carried out in three steps. In the first step, PEO-OH was converted to PEO-COOH by reaction with succinic anhydride (reaction a, Scheme 4.2). For example, monomethoxy PEO of molecular weight 350 (MPEO(350)-OH, 3.50g, 10 mmol) was vacuum dried at 80°C for 30 min in a round flask, and dissolved in tetrahydrofuran (THF, 40 mL). Succinic anhydride (SA, 2.00 g, 20 mmol) and N,N-dimethylaminopyridine (DMAP, 0.12 g, 1.0 mmol) were then added. The solution was held at room temperature for 8 h. The product was precipitated using isopropyl ether. The work up procedure was repeated two more times by dissolving the product in dichloromethane and precipitating in isopropyl ether (volume ratio 1:5). The final product was dried under vacuum at room temperature over night.

In the second step PEO-COOH was converted to PEO-NHS (reaction b, scheme 4.2). MPEO(350)-COOH obtained from the previous reaction (1.73 g, 4.0 mmol) was dissolved in THF (20 mL). N-hydroxy succinimide (NHS, 0.42 g, 4.0 mmol), and dicyclohexylcarbodiimide (DCC, 1.04 g, 5.0 mmol) were then added. The solution was held at room temperature for 4 h. After filtration to remove impurities, the solid product, NHS-PEO, was obtained by evaporating the solvent. It was then extracted with methylene chloride, CH₂Cl₂.
Scheme 4-2. Conjugation of amino acids to PEO. (a) Carboxylation of chain ends. (b) Conversion of carboxyls to NHS esters. (c) Conjugation of amino acids.

In the third step, the bioactive moiety (containing an amino group) was reacted with NHS-PEO to form an amide linkage (reaction c, Scheme 4.2). MPEO(350)-NHS from the previous reaction (1.04 g, 2.0 mmol) was dissolved in THF (20 mL). Glycine methyl ester, was used as model amino acid to evaluate the feasibility of the reaction. Amino acid (2.0 mmol) and potassium carbonate (0.15 g, 1.0 mmol) in 20 mL distilled
water were added to the MPEO(350)-NHS solution and the mixture allowed to react at room temperature for 1 h. The product, MPEO(350)-NHS, was precipitated using isopropyl ether. The work up procedure was repeated two more times by dissolving the product in dichloromethane and precipitating in isopropyl ether (volume ratio 1:5). The final product was dried under vacuum at room temperature over night.

For conjugates having a thiol group at one end of the PEO and a bioactive moiety at the other, NHS-PEO-NHS was used as the starting material. A mixture of aminoethane thiol and amino acid was used in the final step (Scheme 4.2). Because only a fraction of molecules was to be conjugated with bioactive moiety, a smaller amount of the latter was used compared to aminoethane thiol. Usually, a molar ratio of the amino acid to aminoethane thiol of 1:5 was used. To minimize the probability that both ends would have the same function, the amino acid was added to the reaction system 5 min prior to the aminoethane thiol.

4.2.4. REAGENTS FOR MEASUREMENT OF PEO GRAFT DENSITY

The surface coverage of PEO on gold (referred to as graft density) was measured by the coupling of radiiodinated tyramine to PEO. The reactions are indicated in Scheme 4.3.

Tyramine (0.137 g, 1.0 mmol) was dissolved in 2.0 mL distilled water. The pH was adjusted to 11.0 – 12.0 using potassium carbonate. Na$_{125}$I (5.0 μL) and Iodogen (0.13 g, 0.3 mmol) were then added and the solution was held at room temperature for about 15 min. D$_{2}$O was used as the reaction medium when NMR spectrometry of the products was carried out. The unreacted Na$_{125}$I was removed by addition of 50 μL AgNO$_{3}$ (1M) and centrifugation at 1000 rpm for 1 min. The supernatant was then treated with 50 μL NaCl
(1M) and centrifuged at 1000 rpm for 1 min. The supernatant was collected for PEO conjugation.

\[
\begin{align*}
\text{HO-} & \quad \text{CH}_2 \quad \text{CH}_2 \quad \text{NH}_2 + \quad \text{Na}^{125}\text{I} \\
\text{Iodogen} & \\
\text{HO-} & \quad \text{CH}_2 \quad \text{CH}_2 \quad \text{NH}_2 \\
\text{NHS-PEO-NHS} & \quad \text{Aminoethane thiol}
\end{align*}
\]

Scheme 4-3. Procedure for introducing radioactive iodine into PEO chains to measure graft density of PEO chemisorbed on gold.

For the conjugation of labeled tyramine to PEO, NHS-PEO-NHS (1.0 mmol, synthesized as described above) was dissolved in THF (2.0 mL). Aminoethane thiol (0.08 g, 1.0 mmol), radiolabeled tyramine (0.26 g, 1.0 mmol) and potassium carbonate (0.075 g, 0.05 mmol) dissolved in 5.0 mL distilled water were then added and the mixture allowed to react at room temperature for 1 h. To minimize the probability that both ends would have the same function, the amino acid was added to the reaction system 5 min prior to the aminoethane thiol. The product was purified on a Sephadex 40 column and characterized using NMR.
4.3. RESULTS AND DISCUSSION

4.3.1. THIOLATION OF PEO

Thiolation of linear monomethoxy PEO and dihydroxy PEO of various molecular weights was carried out by reaction with mercaptoacetic acid using sulfuric acid as catalyst (reaction 4-1).

\[
\begin{align*}
\text{HS-CH}_2\text{-COOH} + \text{HO-PEO-R} & \xrightleftharpoons{\text{H}_2\text{SO}_4} \text{HS-CH}_2\text{-COO-PEO-R} + \text{H}_2\text{O} \\
& \text{Toluene}
\end{align*}
\]  

To force the equilibrium to the product side, water-toluene azeotrope formed in the reaction was distilled as the reaction proceeded. Infrared and NMR spectroscopy were used to characterize the reaction products. Figure 4.2 shows a proton NMR spectrum of the product from MPEO(350)-OH. Peak (c) at 4.3 ppm is assigned to the methylene protons of PEO bonded directly to the carboxyl group of mercaptoacetic acid. The presence of peak (a) at about 2.1 ppm indicates that the SH group did not react further to form a disulfide linked dimer. The yield of this reaction calculated from NMR integration was essentially quantitative, as shown below.

Integration of the NMR spectra was used to estimate the yield of the reaction. The intensity of peak (d) at 3.67 ppm (I_{3.67}) indicates the number of methylene protons in the PEO repeating units excluding the methylene groups adjacent to the ester bond. The latter are represented by peak (c) at 4.3 ppm (I_{4.3}). The esterification yield is defined as:

\[
\text{yield} = \frac{\text{Number of end group protons reacted}}{\text{Number of end group protons initially}}
\]

In terms of the NMR data, this can be written as:
\[
Yield = \frac{I_{4.3}}{I_{3.67} + I_{4.3}} \frac{1}{N}
\]

where \(N\) is the chain length (degree of polymerization) of the PEO (estimated from the molecular weight data provided by the supplier). Table 4.1 lists typical esterification data. The peak integration data indicate that the esterification yield was in general greater than 90 percent.

Figure 4.2. Proton NMR spectrum of the product of reaction between MPEO(350)-OH and mercaptoacetic acid. Peak (c) at 4.3 ppm is assigned to the methylene protons of PEO directly bonded to the carboxyl group of mercaptoacetic acid. The presence of peak (a) at about 2.1 ppm indicates that the thiol group did not react further to give a disulfide-linked dimer.
Table 4.1. Thiolation of PEOs of different molecular weights and end groups by reaction with mercaptoacetic acid. Data are percent hydroxyl groups converted to \(-\text{OCOCH}_2\text{-SH}\), as measured by NMR.

<table>
<thead>
<tr>
<th>MW of PEO</th>
<th>114</th>
<th>300</th>
<th>600</th>
<th>750</th>
<th>1,025</th>
<th>1,500</th>
<th>3,400</th>
<th>4,600</th>
<th>5,000</th>
</tr>
</thead>
<tbody>
<tr>
<td>HO-PEO-OH → HS-PEO-OH</td>
<td>nd</td>
<td>53</td>
<td>50</td>
<td>nd</td>
<td>nd</td>
<td>47</td>
<td>nd</td>
<td>47</td>
<td>nd</td>
</tr>
<tr>
<td>HO-PEO-OCH(_3) → HS-PEO-OCH(_3)</td>
<td>~100</td>
<td>nd</td>
<td>nd</td>
<td>~100</td>
<td>nd</td>
<td>nd</td>
<td>~100</td>
<td>nd</td>
<td>~100</td>
</tr>
<tr>
<td>HO-PEO-OH → HS-PEO-SH</td>
<td>nd</td>
<td>~100</td>
<td>~100</td>
<td>nd</td>
<td>nd</td>
<td>~100</td>
<td>nd</td>
<td>~100</td>
<td>nd</td>
</tr>
<tr>
<td>HO-PPO-OH → HS-PPO-OH</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>~50</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
</tbody>
</table>

nd = not done

Infrared spectroscopic data also confirm the occurrence of the esterification reaction. Figure 4.3a and 4.3b show the FTIR spectra of MPEO(350)-SH and MPEO(3400)-SH. The presence of an ester group should give rise to a peak around 1750 cm\(^{-1}\) (C=O stretch). The peak at 1737 cm\(^{-1}\) in the spectra of Figure 4.3 suggests that esterification has occurred. Also, as expected, the intensity of this peak in MPEO(350)-SH is much higher than in MPEO(3400)-SH, in accordance with the amount of ester groups present in the samples.
Figure 4.3. IR spectra of thiolated PEO. The upper and lower panels show the spectra of thiolated PEO of molecular weight 350 and 3400. Both figures show a peak at 1737 cm\(^{-1}\) indicating the presence of an ester group. The intensity of this peak in the two spectra relative to an internal reference, for example the C-O-C vibration at about 1110 cm\(^{-1}\), indicates the relative amounts of ester bonds.
4.3.1.1. COMPETITION BETWEEN ESTERIFICATION AND SELF-CONDENSATION REACTIONS

It should be mentioned that by-products may be formed in this reaction by the self-condensation of mercaptoacetic acid as indicated in reaction 4-2. To investigate to what extent this reaction might be occurring, kinetic studies were carried out on the self-condensation and the conjugation reactions (reactions 4-2 and 4-1) by measuring the production of water using a volumetric method.

\[ 2 \text{HS-CH}_2\text{-COOH} \underset{\text{Toluene}}{\xrightarrow{\text{H}_2\text{SO}_4}} \text{HS-CH}_2\text{-C-S-CH}_2\text{-COOH} + \text{H}_2\text{O} \quad (4-2) \]

Figures 4.4 and 4.5 show the kinetics of the self-condensation reaction and of the reaction of PEO with mercaptoacetic acid respectively. The expectation from the simple mechanisms:

\[ \text{A} + \text{B} \rightarrow \text{C} + \text{D} \text{ and } 2\text{B} \rightarrow \text{E} + \text{D} \]

(where A,B,C,D, and E are PEO, mercaptoacetic acid, ester, water and mercaptoacetic acid dimer respectively) is that the reactions should follow second order kinetics. Accordingly plots of \(1/[A]\), in which \([A]\) was derived from \([A]_0-\text{[D]}\), were constructed to test the data. As can be seen, the plots are reasonably linear over the first 100 min for the self-condensation (Figure 4.4) and over the first 10 min for the esterification reaction (Figure 4.5). The rate constants were estimated from the slopes of the regression lines. The estimated rate constant for the self-condensation reaction \((1.4\times10^{-3} \text{ L mol}^{-1} \text{ min}^{-1})\) was found to be considerably smaller than that for the esterification reaction \((7.2\times10^{-2} \text{ L mol}^{-1} \text{ min}^{-1})\), indicating that the latter reaction is favored, thus making it unlikely that the self-
condensation polymerization of mercaptoacetic acid occurs to any significant extent under these conditions.

Figure 4.4. Kinetics of self-condensation of mercaptoacetic acid catalyzed by sulfuric acid. Temperature: 113°C. The solid line is a linear regression of the data over the first 100 min. \([A] = \text{molar concentration of mercaptoacetic acid derived from } ([A]_0- [D]), \] in which \([A]_0\) is the concentration of \(A\) at time 0 and \([D]\) is the concentration of water at time \(t\).
Figure 4.5. Kinetics of the reaction of mercaptoacetic acid with MPEO(350) catalyzed by sulfuric acid. Temperature: 113°C. The solid line is a linear regression of the data over the first 10 min. \([A]\) = molar concentration of mercaptoacetic acid derived from \([A]_0 - [D]\), in which \([A]_0\) is the concentration of A at time 0 and \([D]\) is the concentration of water at time \(t\).

As indicated previously, linear PEOs of different molecular weights and having different groups at the chain ends were used in this work. Table 4.1 shows the products obtained in these reactions.
4.3.1.2. INVESTIGATION OF MONO- AND DI-SUBSTITUTION OF HO-PEO-OH IN THIOLATION REACTIONS

For the thiolation reaction of PEO where both chain ends have the same type of functional group, it is expected that a mixture of products will be obtained with one or both chain ends substituted (Reaction 4-3).

\[
\begin{align*}
\text{HO-PEO-OH} + \text{HS-CH}_2\text{-COOH} & \xrightarrow{\text{H}_2\text{SO}_4, \text{Toluene}} \text{HS-CH}_2\text{-COO-PEO-OOC-CH}_2\text{-SH} \\
& + \text{H}_2\text{O}
\end{align*}
\]

(4-3)

Monosubstitution products were required for most of the studies reported in this thesis. Theoretically, the product distribution should be dependent on the ratio of the reactants. As an example, in reaction 4-3, if “p” is the ratio of carboxyl to hydroxyl groups, and if the reaction of the hydroxyl at one end of the chain has no effect on the hydroxyl at the other end, then the probabilities that reaction will occur at both chain ends (product A), one end only (B), and not at all (C) will be \( p^2 \), \( 2p(1-p) \) and \( (1-p)^2 \), respectively. If \( p \) is 0.5, as is the case for most of the reactions studied in this research, these probabilities are then 0.25, 0.5 and 0.25, respectively.

A model system was used to investigate the distribution of the three products A, B and C: the reaction between dihydroxy PEO (MW 2000) and monomethoxy, monocarboxy PEO (MW 1000) was carried out (Scheme 4-4) using different stoichiometries. The products from the different reactions should have different molecular weights and should therefore be amenable to analysis by gel permeation chromatography (GPC). The molecular weights of the PEOs were chosen such that the
different products should be easily separated by the GPC system used in this research (see experimental section).

\[
\begin{align*}
\text{HO-PEO-OH} & + \text{MPEO-COOH} \xrightleftharpoons[H_2SO_4\text{Toluene}]{\text{H}_2\text{O}} \\
\text{MPEO-COO-PEO-OOC-PEO-M} & + \text{MPEO-COO-PEO-OH}\\
\end{align*}
\]

Scheme 4-4. Reaction of dihydroxy PEO with carboxy-methoxy PEO, showing expected products.

Figures 4.6 and 4.7 show typical chromatograms. Dihydroxy PEO of molecular weight 2000 (D) and monocarboxy, monomethoxy PEO of MW 1000 (M) were used. Figure 4.6 shows the GPC of the product of reaction at a molar ratio of 0.5, M:D. Accordingly, the functional group ratio (COOH:OH) would be 0.25. Three peaks are resolved and may be assigned to the bi-substituted product (31 min), the monosubstituted product (32 min), and unreacted PEO (35 min). It can be seen that considerable unreacted PEO of MW2000 remains, and that a significant amount of monosubstituted PEO is present. The amount of bi-substituted product, however, is small. Unreacted M-PEO-COOH was not detected, possibly because the lower separation bound of the column (MW = 600) was too close to the molecular weight of this species (1000). In effect the PEO of MW1000 would be included in the solvent peak. With a molar ratio M:D of 1.0 (functional group ratio 0.5) the amounts of unreacted PEO and bi-substituted products appear less than the amount of monosubstituted product.
Figure 4.6. GPC chromatogram of the product of reaction between dihydroxy PEO (MW 2000) and carboxy-methoxy PEO (MW 1000) with molar ratio 1:0.5. Three peaks are resolved and may be assigned to the bi-substituted product (31 min), the monosubstituted product (32 min), and unreacted PEO (monocarboxy, monomethoxy PEO of MW 2000, 35 min). The assignments of peaks are based on the calibration curve constructed from a series of monodispersed polystyrenes.
Figure 4.7. GPC chromatogram of the product of reaction between dihydroxy PEO (MW 2000) and carboxy-methoxy PEO (MW 1000) with molar ratio 1:1. Three peaks are resolved and may be assigned to the bi-substituted product (31 min), the monosubstituted product (32 min), and unreacted PEO (monocarboxy, monomethoxy PEO of MW 2000, 35 min). The assignments of peaks are based on the calibration curve constructed from a series of monodispersed polystyrenes.
All these observations are in accordance with the theoretical prediction discussed above. Quantitative data on the peak areas (normalized to the peak for monosubstituted product) are listed in Table 4.2 and compared to the calculated values. It can be seen from the table that the experimental and calculated values are in reasonably good agreement.

Table 4.2. Molecular weights and peak areas (given as ratios) of products from esterification of HO-PEO-OH (D) by MPEO-COOH (M) using different M:D molar ratios.

<table>
<thead>
<tr>
<th></th>
<th>Peak evaluation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2000</td>
</tr>
<tr>
<td>Elution time (min)</td>
<td>35</td>
</tr>
<tr>
<td>Peak areas (M:D=0.5, experiment)</td>
<td>1.67</td>
</tr>
<tr>
<td>Peak areas (M:D=0.5, theory)</td>
<td>1.5</td>
</tr>
<tr>
<td>Peak areas (M:D=1.0, experiment)</td>
<td>0.4</td>
</tr>
<tr>
<td>Peak areas (M:D=1.0, theory)</td>
<td>0.5</td>
</tr>
</tbody>
</table>

It should be mentioned, however, that for reaction 4-3, when such mixtures of products are used as reagents for chemisorption to gold, the mono and bi-substituted PEO can attach to the surface while the unreacted PEO cannot be chemisorbed and will be removed in the washing procedure. Clearly the bi-substituted PEO may chemisorb at either one or two chain ends. In the latter case PEO in a loop configuration would be present on the surface.

4.3.1.3. THIOLATION OF STAR-TYPE PEO

As well as linear PEOs, star-type PEOs were used in this research. In principle each "arm" of the star can be modified to have a bioactive moiety at the chain end.
Immobilization of these multi-functionalized star-type PEOs should provide surfaces having high densities of the bioactive groups. For the gold-thiol chemisorption method of surface attachment used in this work, at least one arm of the star-type PEO must have a thiol group.

Thiolation was again carried out by reaction with mercaptoacetic acid. As for the linear PEOs, the self-esterification of mercaptoacetic acid can occur in these reactions. Also a distribution of products having different numbers of hydroxyl groups converted to thiol is possible. However, for the same molar ratio of PEO to mercaptoacetic acid as in the linear PEO modification, the ratio OH:SH becomes much higher than in the case of linear PEO. Therefore, the effect of self-esterification and the probability of bi-, tri- etc substitution would be even smaller and probably negligible. As an example, estimates of the distribution of products for a star-type PEO can be made the same as for linear PEO. If “p” is the ratio COOH:OH, “n” is the number of arms on PEO, and m is the number of hydroxyls being esterified, the probability of a PEO having n arms with m arms reacted is:

$$P_m = \frac{n!}{m!(n-m)!}p^m(1-p)^{n-m}$$  \hspace{1cm} (4-2)

To compare with linear PEO, let us suppose the molar ratio of 8-arm PEO to mercapto acetic acid is 1:1, then “p”, the ratio COOH:OH, becomes 1:8. The substitution yield can be calculated with results as shown in Table 4.3. It can be seen that most of the products are monosubstituted (m=1), nonsubstituted (m=0) and bi-substituted (m=2) PEOs, leaving most of the hydroxyls in multiarmed PEOs unreacted. The yield of fully substituted PEO is essentially zero. In contrast, for linear PEO, it has been calculated that the ratio of unreacted, monosubstituted and bi-substituted PEO is 1.5:1.0 :0.17. In this
project, star PEOs of 4 arms (MW 2000, 10000) and 8 arms (MW 2000) were thiolated using a molar ratio of PEO to mercaptoacetic acid = 1:1.

Table 4.3. Calculation of thiolation degree of 8-arm PEOs with mercaptoacetic acid according to equation 4-2.

<table>
<thead>
<tr>
<th>m</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
</tr>
</thead>
<tbody>
<tr>
<td>n-m</td>
<td>8</td>
<td>7</td>
<td>6</td>
<td>5</td>
<td>4</td>
<td>3</td>
<td>2</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Pm</td>
<td>0.344</td>
<td>0.393</td>
<td>0.196</td>
<td>0.056</td>
<td>0.011</td>
<td>0.001</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Pm*</td>
<td>0.875</td>
<td>1.0</td>
<td>0.499</td>
<td>0.143</td>
<td>0.028</td>
<td>0.003</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Pm*: Pm normalized to monosubstituted (m=1) PEO

4.3.2. THIOLATION OF POLYPROPYLENE OXIDE (PPO)

Thiolation of linear PPO of molecular weight 1025 was also carried out by reaction with mercaptoacetic acid using sulfuric acid as catalyst (reaction 4-5).

\[
\text{HO–PPO–OH} + \text{HS–CH}_2\text{–COOH} \xrightleftharpoons[\text{Toluene}]{\text{H}_2\text{SO}_4} \text{HS–CH}_2\text{–COO–PPO–OH} + \text{H}_2\text{O} \quad (4-5)
\]

The reactants were present in 1:1 molar ratio, thus favoring the HS-PPO-OH product as discussed for PEO.

4.3.3. MODIFICATION OF PEO WITH BIOACTIVE LIGANDS

As detailed in the experimental section of this chapter (Scheme 4.2), the amino groups of amino acid residues in protein and peptide based bioactive ligands were used for coupling to the chain ends of PEO. Results relating to each reaction step in this process are discussed in this section.
4.3.3.1. CONVERSION OF PEO-OH TO PEO-COOH BY REACTION WITH SUCCINIC ANHYDRIDE (SA)

This reaction (reaction a, Scheme 4.2) was shown to be successful by proton NMR analysis of the products. Figure 4.8 shows the spectrum of the product of the reaction of SA with HO-PEO(1000)-OH. The singlet peak at 2.64 ppm (peak a) is assigned to the protons of SA after ring opening although it consists of signals from hydrogen bonded to different carbons. This assignment is consistent with the spectrum of the monomethyl ester of succinic acid found in the literature (Sadtler Research Laboratories, 1965b). The peaks in the vicinity of 4.3 ppm (b) are assigned to the methylene protons adjacent to the hydroxyl group of PEO after esterification by SA.

Figure 4.8. Proton NMR spectrum of the product of reaction between HO-PEO(1000)-OH and succinic anhydride using a 1:1 molar ratio. p-Dimethylaminopyridine was used as catalyst. Reaction was carried out at room temperature for 8 h. The peaks (a) at 2.64 ppm and (b) at 4.3 ppm verify the ring opening of succinic anhydride and the esterification reaction. Integration of the peaks indicates that the yield is essentially quantitative.
Calculation of the product yield based on integration of the spectral peaks indicates that the yield is essentially quantitative.

4.3.3.2. CONVERSION OF PEO-COOH TO THE N-HYDROXY SUCCINIMIDE (NHS) DERIVATIVE

Figure 4.9 shows the NMR spectrum of the product of reaction of PEO-COOH with NHS (see Scheme 4.2). To ensure that unreacted NHS was removed, extensive washing with dichloromethane, a good solvent for PEO and a nonsolvent for NHS, was carried out. The peaks at 2.83 ppm (a) indicate the presence of protons in NHS that has reacted. Also the singlet peak which appears at 2.64 ppm in Figure 4.8 (peak a) is now split into two triplets centered at 2.78 (c) and 2.92 ppm (d) respectively. This is because the chemical and magnetic environments of the protons of the two methylene groups in the succinic acid are different after reaction (one is adjacent to PEO (d) and the other to NHS (c)). Based on peak integration, the yields of this reaction were estimated to be greater than 90%.

4.3.3.3. CONJUGATION OF AMINO ACIDS TO NHS-ACTIVATED PEO

This reaction was performed using an interfacial reaction technique with water as one phase and methylene dichloride as the other. This approach has the advantage that it is suitable for many of the amino acids, which are soluble in water at room temperature. The reactions of NHS-PEO-NHS with glycine, glycine methyl ester, N(ε)-t-BOC-lysine, lysine methyl ester, and cysteine were carried out using this method. Also the reaction of NHS-PEO-NHS with both cysteine and lysine was attempted with the goal of synthesizing PEO derivatized with cysteine at one end, and lysine at the other. The ε-
amino group of lysine was protected, so that the α amino group would be used in the conjugation reaction, leaving the ε-amino group (after de-protection) free as in proteins.

Figure 4.9. Protein NMR spectrum of the product of reaction between PEO-COOH and NHS (II in scheme 4.2) at a 1:1 molar ratio. The reaction was carried out at room temperature. The peak at 2.83 ppm (a) is assigned to the protons of NHS after reaction (see text).
Figure 4.10 shows the NMR spectrum of the product of the reaction of glycine methyl ester with NHS-PEO. The new peaks at 3.78 ppm (g) and 4.04 ppm (a) indicate the presence, respectively, of methyl and methylene protons having chemical shifts in accordance with the structure of glycine methyl ester. Further, compared to Figure 4.9, the disappearance of the peaks at 2.83 ppm also indicates that the NHS end groups have been eliminated, thus confirming that the reaction has taken place.

Figure 4.10. Proton NMR spectrum of the product of reaction between NHS-PEO and glycine methyl ester. The new peaks at 3.78 ppm and 4.04 ppm indicate the presence of methyl and methylene protons similar to those of glycine methyl ester. The disappearance of the peaks around 2.8 ppm indicates the elimination of NHS groups.
Since the methylene proton signal at 2.64 ppm in the product of reaction (a) in Scheme 4.2 is a singlet ((a) in Figure 4.8), which indicates the presence of succinic acid, there was some doubt whether the triplets at 2.57 ppm and 2.71 ppm ((d) and (c) in Figure 4.10) are due to the protons of succinic acid. Spin decoupling at 4214.26 Hz (2.57 ppm in the 300 MHz machine) under a decoupling power 12L was therefore performed. Figure 4.11 shows the spectrum obtained from the decoupling experiment. Comparing this with Figure 4.10, it can be seen that the peak centered at 2.71 ppm (c) has partly collapsed to a singlet. This provides additional evidence that the glycine methyl ester has been linked to the succinic acid group of PEO because otherwise, the methylene protons in succinic acid should appear as in Figure 4.8.

![Spin decoupled proton NMR spectrum](image)

**Figure 4.11.** Spin decoupled proton NMR spectrum of the product of reaction between NHS-PEO and glycine methyl ester. The peak at 2.71 ppm is partly collapsed to a singlet, in contrast to the triplet which is seen before decoupling ((c) in Figure 4.10).
The NMR data indicate that the yield of this reaction is high, in fact somewhat higher than expected since interfacial reactions usually give low yields. One reason for the high yields may be that the reactivity of the NHS ester with amino groups is high. It should also be noted that interfacial reactions usually do not occur without surfactant. However, PEO is itself a powerful surfactant, and this may also contribute to the high yield of this reaction.

To prove directly the existence of a linkage between the terminal carboxyl residue of PEO and the amino group of the amino acid (see Scheme 4.2, reaction c), 2D NMR (heteronuclear multiple bond correlation, HMBC) was carried out. The coupling is hard to recognize by one-dimensional $^1$H NMR or $^{13}$C NMR, since the $^{13}$C signal at 178 ppm (carboxyl carbonyl), and the proton signal at 7.8 ppm (which includes the proton in the amide formed) are too weak. In 2D NMR, however, the signal becomes stronger when the interactions between the elements become strong. In this work COSY (coherent 2D spectroscopy) and HMBC (Heteronuclear Multiple Bond Correlation) spectra were used to investigate the structure of the substances before and after chemical reaction. Because these techniques are sensitive to changes several bonds away from the reaction site (multiple bond correlation), or more sensitive to subtle changes of chemical environment, they are more informative for the investigation of chemical reactions involving amino acids. Of these techniques HMBC appears to be more conclusive regarding the coupling reactions considered here, where the nearest neighbor elements after the reaction are N and O which cannot be analyzed by normal proton or carbon spectra. Figures 4.12 and 4.13 show, respectively, the $^1$H-$^{13}$C HMBC NMR spectra of a mixture of glycine methyl ester and PEO (molar ratio 1:2) and the reaction product of
glycine methyl ester with PEO. It can be seen from the expanded portion of the spectra that new peaks (178 ppm, 3.4 ppm) were present in the reaction product. This is the only change brought about by the reaction.

Figure 4.12. HMBC spectrum of a mixture of glycine methyl ester and PEO. Although the signals at 171 ppm, denoting the carbon of a carbonyl group, are weak, the intercorrelation peaks with the nearest protons (4.2 ppm), found in the region (4.2, 171) ppm, are nevertheless significant.
Figure 4.13. Expanded portion of HMBC spectrum for the region (4.2, 171) ppm. Top chart is for a mixture of glycine methyl ester and PEO. Lower chart is the reaction product of glycine methyl ester with PEO. The mixture lacks any interaction around (4.2, 171) whereas there is a significant interaction in the reaction product, indicating formation of an NH-CO bond.
Lysine was also investigated as a bioactive ligand for attachment to PEO because of its well-known affinity for plasminogen. It has been shown that surface-bound lysine is effective in “capturing” plasminogen from plasma (McClung et al., 2000). The adsorbed plasminogen may then impart fibrinolytic properties to the surface if it can be converted to plasmin. Figure 4.14 shows the proton NMR spectrum for the product of reaction of NHS-PEO-NHS with lysine and cysteine, the latter to provide a thiol group for chemisorption to gold. The molar ratio of lysine: cysteine: PEO was 0.2:1:1. In order to increase the likelihood that the PEO would be thiolated, cysteine was first added to the reactor, followed by lysine. Standard NMR spectra for L-lysine methyl ester and N-acetyl-L-cysteine are shown in Figures 4.15 and 4.16 respectively. It is seen that typical signals from lysine (peaks (a), (b) and (d) in Figure 4.15) and from cysteine (peaks (d), (e) and (h) in Figure 4.16) are also present in the spectrum of Figure 4.14. Because the purification process (dissolution in dichloromethane and reprecipitation into isopropylether) efficiently removed the unreacted lysine and cysteine, it is therefore evident that both lysine and cysteine are bound to PEO.

4.3.4 Radiolabeling of PEO for Graft Density Measurement

The PEO radiolabeling reactions (scheme 4.3) were monitored by NMR spectrometry. To investigate the reaction efficiency, a model reaction between tyramine and sodium iodide was carried out. It was found after a systematic study that under basic conditions (pH > 10), the reaction is more efficient at high sodium iodide concentration. Figures 4.17 and 4.18 show, respectively, the spectra of tyramine before and after the iodination reaction. The split peaks at 7.45, 6.85 and 6.45 ppm in the iodination product indicate the presence of three different types of proton in the aromatic ring (only two
types are present in the unreacted tyramine), and this suggests that iodination was successful.

Figure 4.14. Proton NMR spectrum of the product of reaction between lysine, cysteine, and NHS-PEO-NHS. It can be seen that all peaks expected for cysteine (3.10 and 4.03 ppm) and lysine (1.40, 3.02 and 3.88 ppm) are present, indicating that the reaction was successful.
Figure 4.15. Proton NMR spectrum of N\textsubscript{\alpha}-t-BOC-L-lysine (Sadtler Research Laboratories, 1965d). With permission.
Figure 4.16. Proton NMR spectrum of L-cysteine (Sadler Research Laboratories, 1965c). With permission.
Figure 4.17. Proton NMR spectrum of tyramine. The peaks at 6.92 and 6.62 ppm (c and d) are the two different types of protons in the aromatic ring (Sadler Research Laboratories, 1965a). With permission.
Figure 4.18. Proton NMR spectrum of tyramine after reaction with sodium iodide. The peaks at 7.45, 6.85 and 6.45 ppm are assigned the three different types of proton in the aromatic ring after iodination.

4.4. CONCLUSIONS

The synthesis and characterization of thiol-terminated PEOs and PPOs starting with linear PEOs of variable molecular weight, linear PPO and star-type PEOs, has been discussed.
Mercaptoacetic acid reacted with hydroxy-terminated PEO as shown by NMR spectroscopy. Kinetic studies suggested that the self-condensation of mercaptoacetic acid, a possible side reaction, did not occur to any significant extent. The distribution of products agreed with statistical predictions.

Synthetic methods for linear PEO, one end bearing a thiol group and the other a potentially bioactive group, were also investigated. In one approach, N-hydroxy succinimide activation was used to conjugate the amino acids glycine methyl ester, and lysine, to carboxyl terminated PEO.

A reaction protocol was developed to attach radioactive iodine to tyramine and then to conjugate the tyramine to PEO. This reagent was used to determine the density of PEO chemisorbed to gold as discussed below (Chapter 5)
CHAPTER 5

SURFACE MODIFICATION AND
CHARACTERIZATION

5.1. INTRODUCTION

The properties of the tissue-material interface are invariably central to biocompatibility. The composition and structure of the biomaterial surface, including the density and thickness of any surface modifier, and the structure and behavior of the adsorbed proteins and adherent cells, are of vital importance. In this work, contact angle, XPS, atomic force microscopy and graft density were used to investigate the properties of surfaces modified with the thiolated PEOs described in Chapter 4. The graft density of PEO achieved is expected to depend on both the molecular weight and the conformation adopted by the PEO on the gold surface, and provides quantitative information on the state of the polymer chains on the surface.

5.1.1 WATER CONTACT ANGLES

The hydrophilicity of a surface can be investigated via water contact angle measurements. Contact angle is related to wettability which can be defined as the extent to which a liquid makes intimate contact with a surface, quantified in terms of Young’s equation:

\[ \gamma_{SV} = \gamma_{SL} + \gamma_{LV} \cos \theta \]  

(5.1).
where $\gamma_{ij}$ are the interfacial free energies per unit area (or interfacial tensions) among the three phases solid (S), liquid (L) and vapor (V), and $\theta$ is the contact angle at equilibrium, as illustrated in Figure 5.1.

![Diagram showing interfacial tensions](image)

Figure 5.1. Contact angle, $\theta$, and the interfacial tensions for a solid-liquid-vapor system.

Modification of the solid surface (e.g. incorporation of interfacially active additives) changes its wettability by changing both $\gamma_{SV}$ and $\gamma_{SL}$. Apparent shifts from the equilibrium contact angle may also be observed as a result of surface roughness or surface heterogeneity. Either of these can lead to hysteresis in contact angle, which increases the apparent advancing contact angle and reduces the apparent receding angle (Marmur, 1998).

Ideally, equilibrium contact angles should be measured on a perfectly smooth and chemically homogeneous surface. Real surfaces are in general heterogeneous to some extent at the microscopic level and these effects are generally manifested as contact angle hysteresis, i.e. a difference between the advancing and receding angles. In practice measured contact angles are averages over a relatively large area and include the effects...
of roughness and heterogeneity. The contact angle of a liquid placed on a flat surface can most conveniently be measured by the sessile liquid drop or captive gas bubble methods.

As used in the present work, water contact angles provided information on the relative hydrophilicity of the various surfaces studied.

5.1.2 X-ray Photoelectron Spectroscopy.

In X-ray photoelectron spectroscopy (XPS), the surface is bombarded with X-rays which cause the emission of photoelectrons. The photoelectrons have a limited mean free path in a solid so that only those which originate near the surface can escape and be detected, thus making the method highly surface sensitive. The energy of the ejected electrons depends on the elements from which they derive (Turner et al., 1998). By measuring the abundance and kinetic energy of the electrons emitted from a sample following irradiation, the elements (except for hydrogen and helium) in the outermost layer to a depth of 10-100 Å can be quantified. Information on the chemical composition of the surface is thus obtained (Shard et al., 1997).

When the angle of the detector relative to the sample surface, i.e. the take-off angle, is changed, the escape depth, and thus the sampling depth is changed as shown in Figure 5.2. Varying the take off angle thus allows electrons emitted from different depths within the sample to be detected. A take-off angle of 90° (detector perpendicular to the surface) gives the greatest sampling depth, whereas lower take-off angles provide data for layers closer to the surface. Experiments using different take-off angles can be used to obtain chemical information as a function of depth into material.
Figure 5.2. Relationship of take-off angle ($\alpha$), X-ray pass ($d$) and sample depth ($d\sin\alpha$) in XPS experiment. The higher the contact angle, the deeper the sampling depth ($D=d\sin\alpha$).
5.1.3. ATOMIC FORCE MICROSCOPY (AFM)

AFM was used to obtain surface images (Xu et al 1998) of different materials such as protein bound on silanized silicon wafers before and after SDS elution (You et al, 1996). AFM generates images by recording the interaction force between the probe tip of the microscope and the sample. A cantilever transduces the forces acting on the tip into a measurable quantity. Either the sample or the cantilever assembly can be manipulated to enable raster scanning over the sample surface (Goh, 1995). Data on the deflection of the cantilever or the tip caused by the interaction are collected as the surface is scanned.

5.2. EXPERIMENTAL

5.2.1. MODIFICATION OF GOLD SURFACES WITH THIOLATED PEO

The polyurethane described in Chapter 4 was prepared as a thin film (~0.3 mm) by dissolving in DMF (6% w/v), pouring the solution into a glass Petri dish and evaporating the solvent slowly at 45 °C. A layer of gold, approximately 1000Å thick, was vacuum deposited onto both sides of the polyurethane surfaces. The gold coating operation was performed at Queen’s University, Physics Department, using a standard vacuum metal evaporation system (Edwards Auto 306 Coating System). In some experiments surfaces prepared by evaporation of gold onto silicon wafers (a kind gift from Dr. P. Tengvall, Linkoping University, Sweden) were used. Gold surfaces, as well as the glassware used in the modification procedures, were cleaned by treatment for 5 min in a mixture of boiling distilled water, ammonia (25% aq) and hydrogen peroxide (30% aq) in the volumetric ratio 5:1:1. After rinsing four times with distilled water, the surfaces were incubated in 5 mM aqueous solutions of the appropriate thiolated PEO for
4 h (Sheardown et al., 1998). The samples were washed three times in distilled water prior to characterization or adsorption experiments.

5.2.2. GRAFT DENSITY MEASUREMENT

As indicated in Chapter 4, PEO surface graft density was measured using a form of PEO radioiodinated by conjugation to tyramine. For example, thiolated PEO of MW 600 conjugated to radioiodinated tyramine (0.18 g, 20 μmol) was dissolved in 100 mL water in a 250 mL glass beaker with a magnetic stirring bar. Nonradioactive NaI (0.01 g) was added to eliminate the effects of possible unbound radioactive iodide (free iodide effect) as will be discussed in detail in Chapter 6. The gold surface samples of appropriate size (typically 0.7 cm²), after being washed with ammonia-peroxide, were then rinsed with the labeled PEO solution and incubated in the solution for 4 h at room temperature. After being washed three times with distilled water, the surfaces were placed in plastic vials for radioactivity counting. The graft density was estimated by comparing the surface radioactivity to the radioactivity of an aliquot of PEO-tyramine solution of known concentration.

5.2.3. CONTACT ANGLE, AFM AND XPS MEASUREMENTS

The sessile drop method was used to measure the advancing and the receding contact angles using distilled water and a Ramé-Hart NRL 100-00 goniometer (Mountain Lakes, NJ). The advancing angle was obtained by introducing a small drop of water (approximately 10 μL) onto the test surface using a syringe. The tangent at the contact line of the drop with the surface when the drop was spread to an equilibrium position was taken as the contact angle. The receding contact angle was observed by withdrawing
water from the drop, causing the contact line to recede across the surface to a new equilibrium position. The difference between the advancing and receding angles is the contact angle hysteresis.

AFM was performed at the Brockhouse Institute for Materials Research using a Nanoscope II microscope (Digital Instruments, Santa Barbara, CA) with square pyramidal silicon nitride tips (Nanoprobes, Digital Instruments) and a 0.58 N/m nominal spring constant. Scan rates were approximately 2 Hz, with all the samples imaged in both the constant height and constant force modes. In constant force mode the maximum applied force was 8 x 10^{-9} N.

XPS was used to monitor the gold-thiol chemisorption reactions by measuring the content and bonding environment of sulfur at the surface (Castner et al., 1996). Data were obtained at the University of Toronto Surface Analysis Facility using a Leybold Max spectrometer with a monochromatic Al source. Survey scans were run from 0 to 1000 eV. Binding energies were referenced to the carbon 1s peak for carbon bound to hydrogen with a binding energy of 284.6 eV. Surfaces were analyzed at low resolution for N, O, C, S, and Au at take-off angles of 20, 30 and 90 degrees. High-resolution scans were run on the carbon 1s and sulfur 2p peaks for all the tested surfaces. The high resolution peaks were fit using a Surface Science Instruments curve fitting package based on the minimum Chi squared calculation using 80% Gaussian criteria.
5.3. RESULTS AND DISCUSSION

5.3.1 CHARACTERIZATION OF GOLD SURFACE MODIFIED WITH LINEAR PEO

5.3.1.1 WATER CONTACT ANGLES

Contact angle data for surfaces modified with thiolated PEO of different molecular weights are shown in Figure 5.3. Compared to the unmodified gold, the PEO-modified surfaces have significantly lower contact angles. Pure, uncontaminated gold surface is very hydrophilic (Schneegans et al., 1982). Freshly cleaned gold surface, however, is rapidly (within seconds) contaminated with organic material when in contact with the normal laboratory atmosphere (Schneegans et al., 1982). A relatively high contact angle close to 55° is observed for the control gold surface, indicating contamination despite extensive cleaning with ammonia/peroxide. Because the contaminants are physisorbed, it is believed that they are easily displaced by thiols, with the heats of gold thiol reactions of the order of 30 kcal/mol. The high contact angle of the unmodified gold surface and the reduced contact angles of the PEO surfaces suggest that in fact the PEO-thiols did displace the physisorbed contaminants.

It is seen from Figure 5.3 that the contact angle decreases with increasing molecular weight of the PEO to a lower limit of about 30° for MW = 3400. This trend can be explained in terms of the changes of interfacial energy. Because PEO is a surfactant, it reduces the surface tension of water from roughly 72 dyn/cm to around 35 dyn/cm (Vagafit, 1983). In an analogous manner, when attached to a surface, PEO is expected to decrease the interfacial energy to an extent depending on the graft density.
and chain length of the PEO (Bae et al., 1994; Lee et al., 1989). When the graft density is low or the chain length is short, the ability of PEO to decrease the interfacial energy may be limited since the interactions between water and the gold surface will not be efficiently screened. Relatively high contact angles would be observed under these circumstances. As the molecular weight (and thus the surface coverage) of the PEO increases, it is expected that the contact angle should decrease and attain a minimum constant value, as seen in Figure 5.3. This would also be the case for increasing graft density (at fixed

Figure 5.3. Water contact angle data for gold deposited on polyurethane and modified with thiolated PEO. The gold surfaces were incubated for 4 h in 2mM aqueous solutions of MPEO(350)-SH, MPEO(3400)-SH, MPEO(5000)-SH and HO-PPO(1025)-SH. Control gold surface, Au-Ctrl, was subjected to the same treatment as the chemisorbed materials except that the incubation medium was distilled water. The two leftmost columns for each surface indicate the advancing and receding angles measured immediately after chemisorption. The two rightmost columns show the advancing and receding angles after incubation in saline (0.9% aqueous NaCl) for 3 days at room temperature. Data are mean ± S.D, n=8.
molecular weight). Similar trends for the dependence of contact angle on molecular weight of PEO have been reported by others (Silver et al., 1994). For example, Desai et al (1991a) showed that the surface properties of poly(ethylene terephthalate) modified with PEO are similarly dependent on the molecular weight of the PEO. Kim et al (2000) also found that reorganization of PEO at the surface resulted in decreasing contact angle and interfacial energy between the surface and water. Hancock et al have shown a large decrease in contact angle for a block copolymer of polysulfone and PEO (33°) compared to polysulfone itself (111°). Unfortunately there appear to be no data on contact angle for a pure PEO surface, although this would be expected to be very low (Hancock et al., 2000).

For comparison to PEO, polypropylene oxide (PPO) of MW 1025 was also chemisorbed onto gold. Because of its known hydrophobic properties (Bransdrup, 1999), PPO is expected to give surfaces of higher contact angle for similar surface coverage. As seen in Figure 5.3, contact angles in the mid sixties (advancing) and high forties (receding) were observed, i.e. higher than the bare (contaminated) gold. In comparison with the PEO modified surfaces, it is observed that the PPO surface shows greater hysteresis, possibly indicating more extensive rearrangement upon contact of the surface with water. It might have been expected that more extensive rearrangement would occur for PEO since it should interact more extensively than PPO with water. It may be that these interactions are rapid enough that they occur largely within the time required to make the advancing angle measurement. Thus the interactions would not be manifested in contact angle hysteresis.

To investigate the stability of the modified surfaces with respect to retention of the thiolated PEO, the materials were immersed in physiological saline for 3 days at room
temperature. As seen in Figure 5.3, little change in contact angle occurred as a result of saline incubation for the PEO modified surfaces, indicating good stability under these conditions. The unmodified gold surface showed a significant decrease in contact angle upon saline incubation, perhaps indicating removal of some of the hydrophobic contaminants which are normally present.

**5.3.1.2. XPS AND AFM STUDIES**

XPS was used to quantify the chemical elements in the outermost layer of the materials (10-100 Å), thus providing information on the chemical composition of the surface. Data were collected at low and high resolution and at different take-off angles to assess compositional change as a function of depth into the material. The low-resolution data allow determination of the elemental surface composition. High-resolution data for a particular electron (e.g. C1s) indicate the different bonding environments of the element from which it originated. For example, analysis of the sulfur S2p peak at high resolution gives information on the relative amounts of surface-bound and unbound thiol. This is of great value in monitoring the effectiveness of the gold-thiol chemisorption reaction.

Figures 5.4 and 5.5 show typical XPS survey spectra of unmodified gold and the gold surface modified with hydroxy-terminated PEO of molecular weight 2000 at the take-off angle of 90°. The relative intensity of the XPS signals (via peak integration) gives a measure of the atomic composition. However, the different electrons have different sensitivities for detection as shown in Table 5.1. From these data it is clear that the Au 4f peak as seen in the raw spectral data gives an inflated view of the surface gold content. The survey scans must be viewed in general with the different elemental sensitivities in mind. Compositional data presented below are based on the integrated peak areas and sensitivity factors. Compared to
the unmodified gold, the modified surface shows a weaker gold signal (Au 4f peak at 85.7 eV), presumably due to the presence of the PEO on the surface. Strong carbon 1s (286 eV) and oxygen 1s (532.7 eV) peaks are seen in the spectrum of the modified surface, again attesting to the presence of PEO.

![Graph](image)

Figure 5.4. XPS survey scan of unmodified gold on polyurethane. The surface was washed in ammonia-peroxide prior to analysis. Take off angle 90°.

Figures 5.5 and 5.6 compare the spectra of surfaces modified with PEO of the same molecular weight (2000) measured at take-off angles of 90 and 20° respectively. It can be seen that the gold peak is much stronger at 90 than at 20°. Since the data at 90° emphasize the deeper layers of the material, this result again suggests the presence of a layer of PEO on the gold as would be expected.
Figure 5.5. XPS survey scan of gold surface (polyurethane) modified with hydroxy-terminated PEO of molecular weight 2000. Take off angle 90°.

Table 5.1. Sensitivities of common elements in XPS (Leybold, 1994)

<table>
<thead>
<tr>
<th>Electron</th>
<th>Energy (eV)</th>
<th>Sensitivity factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>C 1s</td>
<td>284.5</td>
<td>0.3190</td>
</tr>
<tr>
<td>O 1s</td>
<td>531.7</td>
<td>0.750</td>
</tr>
<tr>
<td>N 1s</td>
<td>402.0</td>
<td>0.510</td>
</tr>
<tr>
<td>S 2p</td>
<td>165.3</td>
<td>0.760</td>
</tr>
<tr>
<td>Au 4f</td>
<td>85-89</td>
<td>6.700</td>
</tr>
<tr>
<td>F 1s</td>
<td>686.0</td>
<td>1.000</td>
</tr>
</tbody>
</table>
Figure 5.6. XPS survey scan of gold surface (polyurethane) modified with hydroxy-terminated PEO of molecular weight 2000. Take off angle 20°.

The elemental analyses of the various PEO-modified surfaces based on low resolution XPS data are listed in Table 5.2. The sulfur content is of particular interest. It shows a decrease with increasing molecular weight of the PEO, in agreement with the fact that the lower molecular weight HS-PEOs contain proportionately more sulfur. Further, for surfaces modified with PEO of given molecular weight, the sulfur content (as well as the gold content) decreases as the take off angle decreases, indicating that the sulfur is located at some depth into the materials. This is in accordance with the expectation that the sulfur is coupled directly to gold and thus should be found at the gold-PEO interface, i.e. at some depth into the modified material.

The ratios of carbon-to-sulfur based on the known compositions of the HS-PEO modifiers are listed in Table 5.2 and compared to the measured values. The measured ratios are seen to be consistently higher than the values for the HS-PEOs. It would be expected that
at the 20° take off angle especially, the layer “sampled” would be representative of the entire
HS-PEO component and thus the C:S ratios would be similar. The discrepancies may be due
to the fact that the sensitivity factor used for sulfur is inappropriate for these materials
(Leybold, 1994). Remaining surface contaminants containing carbon may also complicate
the situation. Possibly for the same reason, methoxy PEO and hydroxy PEO did not show
significant differences in composition using this method (data not shown).

Table 5.2. Surface composition of PEO modified gold surfaces (polyurethane)
determined by XPS. Data are atom percent*. Gold surfaces were formed by vacuum
deposition of gold onto polyurethane films.

<table>
<thead>
<tr>
<th></th>
<th>O</th>
<th>C</th>
<th>S</th>
<th>Au</th>
<th>C:S Measured</th>
<th>C:S in HS-PEO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unmodified gold</td>
<td>14.2</td>
<td>60.7</td>
<td>-</td>
<td>25.2</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>(90° take-off)</td>
<td></td>
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<tr>
<td>Unmodified gold</td>
<td>16.4</td>
<td>71.7</td>
<td>-</td>
<td>11.9</td>
<td>-</td>
<td>-</td>
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<td>(20° take-off)</td>
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<td></td>
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<tr>
<td>HSPEO(350)CH₃</td>
<td>14.9</td>
<td>58.6</td>
<td>2.0</td>
<td>24.5</td>
<td>30:1</td>
<td>15:1</td>
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<tr>
<td>HSPEO(350)CH₃</td>
<td>17.8</td>
<td>70.2</td>
<td>1.0</td>
<td>10.9</td>
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<tr>
<td>HSPEO(2000)CH₃</td>
<td>23.3</td>
<td>66.0</td>
<td>0.4</td>
<td>10.3</td>
<td>165:1</td>
<td>90:1</td>
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<tr>
<td>HSPEO(2000)CH₃</td>
<td>24.6</td>
<td>71.4</td>
<td>0.0</td>
<td>4.1</td>
<td>-</td>
<td>90:1</td>
</tr>
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<td>(30° take-off)</td>
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</tr>
<tr>
<td>HSPEO(2000)CH₃</td>
<td>23.9</td>
<td>73.5</td>
<td>0.0</td>
<td>2.6</td>
<td>-</td>
<td>90:1</td>
</tr>
<tr>
<td>(20° take-off)</td>
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<tr>
<td>HSPEO(5000)CH₃</td>
<td>21.1</td>
<td>65.5</td>
<td>0.1</td>
<td>13.3</td>
<td>650:1</td>
<td>226:1</td>
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<tr>
<td>(90° take-off)</td>
<td></td>
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<tr>
<td>HSPEO(5000)CH₃</td>
<td>22.1</td>
<td>72.6</td>
<td>0.1</td>
<td>5.2</td>
<td>726:1</td>
<td>226:1</td>
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<tr>
<td>(30° take-off)</td>
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</tr>
<tr>
<td>HSPEO(5000)CH₃</td>
<td>24.7</td>
<td>72.5</td>
<td>0.2</td>
<td>2.7</td>
<td>363:1</td>
<td>226:1</td>
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<tr>
<td>(20° take-off)</td>
<td></td>
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</table>

* Precision of data: ±5% of mean.

To investigate the type of sulfur-gold linkage on these surfaces, i.e. to verify the
occurrence of chemisorption, high resolution scans of the sulfur peak at 160 -166 eV
were performed (Castner et al., 1996). Figures 5.7 and 5.8 show typical spectra for
surfaces modified with thiolated PEO of molecular weight 350. The sample used for
Figure 5.7 was washed with ethanol and water after chemisorption. Following the
analysis of Castner et al (1996) the overall S2p envelope was fitted with four peaks:
162.3 eV is assigned to the S_{2p3/2} electrons in sulfur which is bound to the gold surface;
163.7 eV is assigned to $S_{2p3/2}$ sulfur not bound to gold; 163.9 and 164.7 eV are assigned to the $S_{2p1/2}$ electrons in sulfur bound and not bound, respectively, to gold. It can be seen that the peaks for bound gold are greater than those for unbound. Since the sample represented in Figure 5.7 was washed extensively after surface modification, it can be concluded that some of the thiols which are not attached to the surface cannot be easily washed off. Since the PEO thiolation reaction can produce both mono- and di- thiolated
PEO (see Chapter 4), it may be that the unbound thiol groups are due predominantly to dithiolated PEO which is bound to gold at one end only. Of course it is not possible to distinguish between these HS groups and those associated with unbound thiolated PEO.

![Graph showing sulfur 2p peaks](image)

**Figure 5.8.** High-resolution XPS of sulfur 2p peaks for gold surface (polyurethane) modified with thiolated PEO of molecular weight 350. The sample was not washed following the chemisorption procedure. Peak assignments same as in figure 5.7.

High resolution carbon 1s spectra (Figures 5.9 and 5.10) revealed some details about the nature of the carbon environments. All scans of carbon 1s peaks were fitted using the software provided with the instrument, with the lowest peak in the scans referenced to 285 eV. For a PEO (350) modified surface, it can be seen that different carbon environments, C-C (~285 eV), C-O (~288 eV) and C=O (~290 eV), are present,
and the proportion of C-O bonds is significant. These data confirm the presence of PEO.

In contrast, the data for the unmodified gold surface (Figure 5.10) showed that most of the carbon bonds were of C-C type, with very limited concentrations of C-O and C=O. This suggests that contamination on the unmodified surface is likely in the form of hydrocarbons, in agreement with the observation that the gold surface is hydrophobic even after cleaning.

Figure 5.9. High-resolution XPS of carbon 1s peak for gold surface (polyurethane) modified with thiolated PEO of molecular weight 350. Different carbon environments, C-C (~285 eV), C-O (~287 eV) and C=O (~290 eV), can be distinguished in this sample.
AFM was also used to compare the unmodified and modified gold surfaces. Figure 5.11 shows AFM images (height mode) of unmodified gold (top) and HS-PEO-OH (1500) modified gold (bottom) surfaces (silicon wafer base). The surface roughness is indicated by the color scheme, with dark indicating low areas, and light indicating high areas. The gold surface appears fairly smooth (typical peak-to-vally of about 3 nm). Surface morphology was not changed significantly by PEO modification at the resolution level of the AFM method. AFM observations for the other PEOs and for the polyurethane based materials were similar, although the modified surfaces showed differences from the unmodified when examined by XPS and contact angle. It was noticed that improper treatment of the gold on polyurethane surface by thiolated PEO solutions (too long incubation time or too concentrated thio solution) tended to cause removal of the gold layer from the surface, due possibly to the reaction of gold with the thiol.
Figure 5.11. AFM images (height mode) of unmodified gold (top) and HS-PEO-OH (1500) modified gold (bottom) surfaces (silicon wafer base). Surface morphology was not changed significantly by PEO modification at the resolution level of the AFM method. AFM observations for the other PEOs and for the polyurethane based materials were similar.
5.3.1.3. GRAFT DENSITY MEASUREMENTS

The parameters that are expected to affect the properties of the PEO-modified surfaces and their interactions with blood include the PEO graft density, the PEO chain length, and the presence of functional (biologically active) groups on the PEO chains. In general it is expected that graft density, chain length and functional group interactions will be interdependent, so that changing one of them may affect the others. For example, the graft density achievable under given conditions (temperature, time, thiol concentration) may depend on the molecular weight of the PEO and on the conformation the PEO adopts on the gold surface. Certainly it would not be valid to draw conclusions about the effects of chain length on surface properties and blood interactions without knowledge of graft density. In this work the investigation of graft density achieved with PEOs of varying molecular weight was undertaken using radiolabeled PEO derivatives.

Table 5.3 shows graft density data for surfaces treated with HS-PEO of MW 600 and 2000, and with unthiolated PEO of MW 600. Comparing the thiolated and unthiolated PEO 600 surfaces, it is seen that the graft densities measured when the surface is washed with water following chemisorption are similar and the same order of magnitudes the theoretical values, estimated on the assumption that all of the thiol binding sites on gold are occupied. However when ethanol is used as the wash fluid, the graft density measured on the HS-PEO surface is of the order of $0.2 \mu g/cm^2$ while on the unthiolated PEO surface it is essentially zero. Since unthiolated PEO cannot be chemisorbed to gold, it is expected that it can be washed off completely with a suitable solvent. It appears that ethanol is such a solvent but water is not. The residual HS-PEO
after ethanol washing is taken to be chemisorbed to the gold, while the HS-PEO removed by the ethanol wash is presumably physisorbed or merely deposited on the surface.

Table 5.3. Graft densities (μg/cm²) of PEO on gold surface (polyurethane). Chemisorptions were carried out at room temperature for 4 h and at HS-PEO concentrations of 2 mM.

<table>
<thead>
<tr>
<th></th>
<th>HS-PEO, MW600</th>
<th>PEO, MW600</th>
<th>HS-PEO, MW2,000</th>
</tr>
</thead>
<tbody>
<tr>
<td>Theoretical</td>
<td>0.46</td>
<td>-</td>
<td>1.54</td>
</tr>
<tr>
<td>Water wash</td>
<td>1.07±0.30</td>
<td>0.93±0.03</td>
<td>0.39±0.35</td>
</tr>
<tr>
<td>Ethanol wash</td>
<td>0.18±0.02</td>
<td>0.003±0.001</td>
<td>0.09±0.03</td>
</tr>
</tbody>
</table>

Data are mean ± SD, n= 8

The measured graft densities are compared to the maximum theoretical values for chemisorption to the (1,1,1) surface of crystalline gold. For (1,1,1) gold surface, the distance between gold atoms is approximately 2.88 Å (Hubbard, 1995). Because the gold-thiol binding sites are in the threefold hollows between gold atoms in the hexagonal array (see Figure 5.12), the maximum density of chemisorbed thiols can be estimated to be 4.6 x 10^{14} /cm² (Karssen, 1995). For a PEO molecular weight of 1000, the maximum surface density can be estimated at 1000 x 4.6 x 10^{14} / (6.023 x 10^{23}) = 0.77 μg PEO/ cm² (for PEO of MW 600 it is 0.46 μg / cm²). It is not clear how closely the gold surfaces used in this work resemble the (1,1,1) surface. It may be that the maximum possible density will be lower for these surfaces. Nonetheless the value calculated for the (1,1,1) surface provides a reference to which the measured values can be compared.

The measured graft density for HS-PEO 600, i.e. 0.18 μg/cm², is thus lower than the theoretical maximum coverage by a factor of about three. Clearly the maximum theoretical coverage was not reached, possibly due to steric effects by which access to vacant sites is hindered once adjacent sites are occupied. For example the chemisorbed
PEO could adopt conformations (e.g. random coil) which effectively cover adjacent unoccupied sites.

Figure 5.12. Arrangement of thiols adsorbed on Au (111) surface under ideal conditions. Adapted from Whitesides, 1990.

For the HS-PEO of molecular weight 2000, the graft density, at 0.09 μg/cm², is lower than for the 600 MW HS-PEO, and in fact is only one sixteenth of the theoretical maximum. One possibility to account for this discrepancy is that steric effects as described above are more severe for the higher molecular weight species. For example, the conformation of PEO is likely to be closer to random coil than fully stretched when the molecular weight is high. The maximum monolayer coverage is therefore probably not attainable.

For a random coil conformation for PEO of MW 2,000, the root mean square end-to-end distance is:

\[ \sqrt{r^2} = N^{1/2} \cdot a \]  
(5.2)
where \(a = 1.44\) nm is the average length of a repeat unit, \(N = 45\) is the number of repeat units on the PEO chain.

If we assume the area occupied by one such PEO is the area of a circle of radius \(\sqrt{r^2}\), then:

\[
\text{Area per PEO, } A = \pi \left(\sqrt{r^2}\right)^2 = 360\, \text{nm}^2.
\]

That is, one such PEO will occupy an estimated 360 nm\(^2\), corresponding to a graft density of 8.0 ng/cm\(^2\). The measured graft density is therefore higher than the random coil estimate and lower than the “fully stretched” estimate. It may be that a mixture of fully stretched and random coil conformation is present, as illustrated in Figure 2.10. The conformation of PEO chains is assumed to depend in part on the molecular weight of PEO and in part on coverage. If the molecular weight is high and the coverage low, then the mushroom regime is likely. If the molecular weight is low and the coverage high the brush regime would be favored.

Considerably more work would be required to elucidate thoroughly the structure and molecular properties of these chemisorbed PEO surfaces. Such investigations were beyond the scope of the present research.

5.3.2 **Characterization of Gold Surface Modified with Star-type PEO**

Figure 5.13 shows the water contact angle data for surfaces modified with star-type PEOs. As can be seen contact angles are in the range of 30 to 40°, similar to those for the linear PEOs (Figure 5.3). The surfaces are thus moderately hydrophilic. There is no clear effect of any of the structural variants (number of arms, length of arms). No
molecular weight dependence of contact angle was observed, as reflected by the identical contact angles of PEO-4 arm (2k) and PEO-4 arm (10k). Also, the contact angles were the same for star PEOs of the same total molecular weight (2000) and different numbers of arms (4 and 8). This result is contrary to expectation since for a greater number of arms the surface concentration of hydroxyls is expected to be higher, resulting in a more hydrophilic surface.

Figure 5.13. Water contact angle data for gold surfaces (polyurethane) modified with thiolated star-type PEOs. The gold surfaces were incubated for 4 h in 2 mM aqueous solutions of the PEOs for chemisorption. The control gold surface, Au Ctrl, was subjected to the same treatment as the chemisorbed materials except that the incubation medium was distilled water. The left and right columns for each sample are the advancing (A) and receding (R) angles respectively.

XPS was also performed on these star-type PEO modified surfaces. The survey spectra were very similar to those for the linear PEO modified surfaces, although there were some differences as well. As was the case for the linear PEOs, the star-type PEOs show
lower gold content than the unmodified surface (Table 5.4), reflecting the presence of the PEO layer. The decrease in intensity with decreasing take-off angle seen for the Au4f peak also indicates that the PEO is present as an overlayer on gold. Furthermore, the sulfur content decreases while the carbon and oxygen contents increase as the take-off angle decreases, again indicating the presence of PEO at the surface. The expected ratios of carbon-to-sulfur based on the known compositions of the HS-PEO modifiers are also listed in Table 5.4. The sulfur content decreases with increasing total molecular weight of the PEO. This is in line with the expectation that higher molecular weight PEOs should have lower sulfur content.

The dependence of the sulfur content of star-type PEO of molecular weight 2000 on take-off angle was not as a clear cut as for its linear analogue. The sulfur content of PEO (10 K) clearly decreased with decreasing take-off angle as expected if the sulfur is located at the gold-PEO interface (i.e. at some depth into the material).

Table 5.4. Atomic compositions of star-type PEO-modified gold surfaces (polyurethane) from XPS. Data are atom percent.

<table>
<thead>
<tr>
<th></th>
<th>O</th>
<th>C</th>
<th>S</th>
<th>Au</th>
<th>C:S measured</th>
<th>C:S expected</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unmodified gold (90° take-off)</td>
<td>14.2</td>
<td>60.7</td>
<td>-</td>
<td>25.2</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Unmodified gold (20° take-off)</td>
<td>16.4</td>
<td>71.7</td>
<td>-</td>
<td>11.9</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>HS-4armPEO(2k)-OH (90° take-off)</td>
<td>8.7</td>
<td>30.6</td>
<td>5.1</td>
<td>55.6</td>
<td>6.0:1</td>
<td>76:1</td>
</tr>
<tr>
<td>HS-4armPEO(2k)-OH (30° take-off)</td>
<td>14.6</td>
<td>46.4</td>
<td>5.8</td>
<td>33.1</td>
<td>8.0:1</td>
<td>76:1</td>
</tr>
<tr>
<td>HS-4armPEO(10k)-OH (20° take-off)</td>
<td>18.3</td>
<td>57.7</td>
<td>4.7</td>
<td>19.4</td>
<td>12.3:1</td>
<td>76:1</td>
</tr>
<tr>
<td>HS-4armPEO(10k)-OH (90° take-off)</td>
<td>9.1</td>
<td>40.0</td>
<td>0.7</td>
<td>50.2</td>
<td>57.1:1</td>
<td>380:1</td>
</tr>
<tr>
<td>HS-4armPEO(10k)-OH (30° take-off)</td>
<td>16.6</td>
<td>53.9</td>
<td>nd</td>
<td>29.5</td>
<td>-</td>
<td>380:1</td>
</tr>
<tr>
<td>HS-4armPEO(10k)-OH (20° take-off)</td>
<td>19.6</td>
<td>60.4</td>
<td>nd</td>
<td>20.0</td>
<td>-</td>
<td>380:1</td>
</tr>
</tbody>
</table>

Data precision is of the order of ± 5%
Nd = not detected.

It is interesting to note that the measured ratios of carbon to sulfur are consistently lower than the expected values, i.e. the opposite of what was observed for linear PEO modified surfaces. It seems unlikely that random error can account for this discrepancy.
Other factors such as the ‘real’ carbon-to-sulfur ratio in PEO and the orientation of PEO may be responsible. It is relevant to consider Table 4.3, where the statistically predicted products and thiolation levels are listed. The expected C:S ratio in Table 5.4 was calculated assuming that each PEO chain has one thiol group. However, from Table 4.3, it can be seen that the actual situation is probably different. Statistically, it is predicted that 34.4% of an 8-arm PEO will not be thiolated (31.6% for 4-arm PEO). As such, these molecules will not be able to chemisorb to gold. Excluding this unthiolated PEO, the PEO-thiol ratio for 4-arm PEO is \(1/(1-0.316)=1.46\). The expected C:S ratio then becomes \(76/1.46 = 52\), i.e. smaller than the expected ratio listed in Table 5.4.

In addition, some of the PEO chains on the gold surface will have more than one thiol group. If only one thiol is chemically bound to the surface there is a high probability that the other thiols will be located near the outermost surface of the material. As discussed previously, XPS is more sensitive to surface elements than elements deep down in the material. This may also contribute to a decrease in the C:S ratio.

5.4. CONCLUSIONS

Linear thiolated PEOs, and star-type thiolated PEOs were used to modify gold surfaces by chemisorption. Water contact angle, XPS, atomic force microscopy and graft density measurements provided surface property data on the modified surfaces. The linear PEO modified gold surfaces showed lower water contact angles than the unmodified surface. The modified surfaces were found to be moderately hydrophilic (water contact angles 30 to 40°). Also, the modified surfaces were stable on exposure to saline solution, reflecting the relatively strong attachment of the modification layer. From the XPS data, the modified surfaces were found to have lower gold content than the
unmodified surface. It was also deduced that the PEO is present as an overlayer on the gold, and that some of the sulfur atoms (and therefore the PEOs) are chemically bonded to gold. The physisorbed PEO is removed by washing with appropriate solvents. The graft density data indicated PEO coverage at about a third to a sixteenth (depending on the PEO molecular weight) of the maximum possible monolayer coverage based on the density of thiol binding sites on gold. The lower-than-maximum values are attributed to steric hindrance effects as the surface fills with the relatively large PEO molecules.
CHAPTER 6

THE INTERACTIONS OF BLOOD PROTEINS WITH PEO-MODIFIED SURFACES

6.1. INTRODUCTION

The study of protein adsorption can provide information regarding the affinity of specific proteins for a surface and the selectivity of the surface for different proteins in multicomponent solutions, such as blood. Such information can also give insights into the biocompatibility of the surface. A variety of techniques have been developed to investigate protein adsorption (Linden et al., 1999; Robers et al., 1999; Hook et al., 1998; Burgess et al., 1991; Leize et al., 1998; Regoeazi, 1987; Orschel et al., 1998). In this project, radiolabeling, and SDS-PAGE/immunoblotting techniques were used to study protein adsorption to the various surfaces developed.

The radiolabeling of proteins with iodine isotopes has been used extensively in this laboratory (Woodhouse et al., 1994; Santerre et al., 1992) and elsewhere (Ardaillou et al., 1974; Liu et al., 1998) as a method to determine the quantity of individual proteins adsorbed from buffer or plasma. The method is simple, sensitive and generally convenient and provides adsorption data of relatively high accuracy. Moreover it can be used to follow the behavior of individual proteins in complex mixtures.

In the context of gold-based surfaces, however, a difficulty arises for iodine labeling in that gold-iodide interactions are known to occur, forming complex ionic
species such as \( \text{AuI}_2^- \) and \( \text{AuI}_4^- \) (Sheardown et al., 1998; Letnikov et al., 1990). Previous work in this laboratory on protein adsorption to gold using radioiodinated proteins indicated that trace amounts of unbound or “free” radioactive iodide ion present in the protein solution can lead to serious overestimation of protein adsorption (Sheardown et al., 1998). The radioactivities of gold surfaces increased following their exposure to solutions of the same protein concentration but increasing amounts in free radioactive iodide. Typically 60 to 95% of the total radioactivity associated with the surface was found to be due to \( \text{I}^{125} \) (Sheardown et al., 1998).

An efficient way to suppress iodide uptake was necessary to obtain “clean” data. Incubation of gold with nonradioactive NaI was hypothesized to saturate the surface with iodide ions, thus pre-empting any subsequent binding of radioactive iodide to gold during protein adsorption experiments. To determine whether this treatment protocol was effective, the radioactivity associated with the surface following protein adsorption, with and without pre-exposure to NaI, was measured. Also, addition of nonradioactive iodide to the buffer was tried as a means of suppressing the adsorption of radioactive iodide. Following successful development of these methods, protein adsorption data were collected on the various modified surfaces discussed in previous chapters. Both albumin and fibrinogen were studied as single proteins in buffer. Adsorption of these proteins from plasma was also investigated.

To get information on a much wider range of proteins, SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was used to study the proteins eluted from surfaces after plasma contact. Because proteins have a number of positive and negative charges resulting in a net or global charge, either positive or negative depending on the pH, they
migrate when placed in an electric field. In SDS-PAGE, SDS treatment gives the components of the mixture the same negative charge density. Treatment with beta-mercaptoethanol, a reducing agent, breaks the disulfide linkages between subunits in the proteins (Alberts, 1983). With the application of an electric field, the protein-SDS complexes (or subunit-SDS complexes if reducing conditions are used) then migrate differentially (and thus are separated from one another) according to their molecular size. The smaller proteins migrate faster (thus farther in a given time) than the larger ones. The degree of separation is determined to some extent by the pore size of the gel (Andrews, 1981; Alberts, 1983).

Immunoblotting was used to identify proteins after separation by SDS-PAGE. Following separation, the proteins are transferred from the gel onto a solid membrane, e.g. polyvinylidene difluoride (PVDF), by electrophoresis. The membrane is incubated with primary antibodies directed against the proteins of interest and then with a secondary antibody-enzyme conjugate directed against the first antibody. Finally, using a chromogenic substrate to develop a color reaction with the enzyme, the presence of individual proteins separated by SDS-PAGE is revealed (Gershoni et al., 1983). The method is essentially qualitative or at best semi-quantitative as currently practised.

6.2. EXPERIMENTAL

6.2.1. SURFACES

The surfaces used in the protein adsorption experiments were the same as those described in previous chapters. The gold-coated polyurethane and silicon wafer samples were stored covered at room temperature prior to use. As discussed previously, the gold
surfaces used as controls and as substrates for modification were cleaned by immersion in a peroxide-ammonia solution at 85°C and washed in boiling water for 5 min. The surfaces were then rinsed extensively in distilled water and incubated in the different thiolated PEO solutions for modification as described in Chapter 4.

6.2.2. PROTEINS AND PROTEIN LABELING

Human albumin and fibrinogen were used in this study. Plasminogen-free human fibrinogen from Calbiochem (La Jolla, California, USA) was dialyzed against isotonic Tris buffer, pH 7.4, aliquoted, and stored at -70°C. Human albumin from Behringwerke A.G. (Marburg, Germany) was reconstituted with isotonic phosphate-buffered saline (PBS), pH 7.4, aliquoted, and stored at -70°C. Proteins were labeled with ¹²⁵I (ICN Biomedicals, Quebec, Canada) using the iodogen technique (Iodo-Gen Reagent, Pierce Chemical, Rockford, Illinois, USA) (Regoeczi, 1984; Regoeczi, 1987; Knight et al., 1981). A typical labeling procedure for fibrinogen was as follows. Protein solution (200 μL, 5 mg/mL) was placed in a glass tube precoated with iodogen. Radioactive Na¹²⁵I (1.0 mCi) was then added to the tube followed by stirring for 15 min with a magnetic stirrer. The labeled protein was mixed with 800 μL PBS buffer and dialyzed (MW cut-off limit 10000, Slide-a-lyzer system, Pierce) overnight against Tris buffered saline (TBS) (40.6 g Tris, 22 mL HCl adjusted to 2000 mL using distilled water, pH of 7.5-7.55) with three changes of dialysate to remove unbound radioactive iodide. Trace amounts of free radioactive iodide, still present in the resulting labeled protein solutions, were determined by counting the supernatant following trichloroacetic acid (TCA, 20% w/v in water) precipitation of the protein (Cornelius et al., 1997). The dialyzed product (1 mL) was mixed with nonradiolabeled fibrinogen (4.99 mL, 10.0 mg/mL). This solution was diluted
with TBS to different concentrations for fibrinogen adsorption studies. The concentration of fibrinogen solutions was determined by measuring absorbance at 280 nm (ε = 1.55).

6.2.3. PROTEIN ADSORPTION EXPERIMENTS USING RADIOLABELED PROTEINS

Surfaces (0.6 cm²) were exposed to 250 μL of protein solution in the wells of Falcon 96-well plates (Becton Dickinson, Oakville, ON) for 2 h at room temperature (Brash et al., 1993). Based on preliminary experiments it was found that adsorption was “complete” after about 1 h. Thus 2h was certainly sufficient time to achieve steady state. Following adsorption, samples were rinsed three times in PBS, wicked onto filter paper to remove residual adherent buffer, and the radioactivity determined. The quantity of protein adsorbed was determined by comparing the surface radioactivity to that of an aliquot of solution of known concentration. A minimum of four replicates was measured for each set of experimental conditions except when otherwise mentioned. All experiments were done in duplicate.

6.2.3.1. SUPPRESSION OF FREE IODIDE BINDING TO GOLD

As mentioned previously, gold reacts with iodide ion, which complicates the interpretation of adsorption experiments using radioiodinated proteins due to the presence of free radioactive iodide in the protein preparation.

Two methods were investigated to suppress free iodide on the surface. The term “radioactive iodide” or the symbol “¹²⁵I” are used to refer to radioactive iodide ion; the designations “nonradioactive iodide”, “iodide”, or “I” are used to refer to nonradioactive iodide ion.
A. PRE-EXPOSURE OF THE SURFACE TO A CONCENTRATED NONRADIOACTIVE NaI SOLUTION (METHOD A)

It was hypothesized that by pre-incubating the gold surface with concentrated NaI solution, the uptake of radioactive iodide on the gold during the adsorption experiment should be prevented. To investigate this hypothesis, experiments were carried out to measure the radioactivity of the surfaces with and without this pre-treatment.

B. ADDITION OF NONRADIOACTIVE IODIDE TO THE BUFFER USED FOR THE PROTEIN ADSORPTION EXPERIMENTS (METHOD B)

In this method, substitution of a small amount of the NaCl in the buffer with NaI was carried out. The concentration of nonradioactive iodide was of the order of 1 mol % of the NaCl concentration, and the total molar concentration of NaCl and NaI was the same as that of NaCl in PBS, pH 7.4. Thus 1L of PBS/NaI(0.01%) buffer contained 0.002 g NaI and 8.5g NaCl, while the other components were maintained unchanged. Osmolality data for some buffers are shown in Table 6.1. It should be noted that buffers typically used in protein adsorption studies have osmolalities in the range of 300 ± 20 mOsm/kgH₂O.

<table>
<thead>
<tr>
<th></th>
<th>PBS</th>
<th>PBS/NaI</th>
<th>PBS *</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH (adjusted)</td>
<td>7.38</td>
<td>7.40</td>
<td>7.45</td>
</tr>
<tr>
<td>Osmolality (mOsm/kgH₂O)</td>
<td>303 ± 5</td>
<td>293 ± 5</td>
<td>300±20</td>
</tr>
</tbody>
</table>

Table 6.1. pH and osmolality of PBS buffers.

PBS *: Normally accepted value for a typical PBS buffer

It was hypothesized that the presence of nonradioactive iodide during the adsorption experiment, at a concentration much higher than that of the adventitious radioactive iodide, would suppress the uptake of radioactive iodide to gold. To test this
hypothesis, the radioactivity associated with the surface following protein adsorption, with and without the addition of NaI to the buffer, was measured.

It was expected that both method A (pretreatment with NaI) and method B (addition of NaI to the buffer) would suppress free radioactive iodide uptake on the surface. To investigate the effectiveness of the two methods, surfaces were divided into four groups, designated 11, 12, 21, and 22, respectively, according to the principles of factorial experimental design (Montgomery et al., 1998). A typical experiment was as follows: two groups (11 and 12) of gold surfaces (0.6 cm²) were each pre-incubated in double distilled water for 4h; the other two groups (21 and 22) were incubated in aqueous NaI solution (0.5M) for 4 h in Falcon 96 well plates. The surfaces were rinsed by dipping three times into a well of 250 μL fresh distilled water. The surfaces in groups 12 and 22 were then incubated for 2 h in 250 μL radiolabeled albumin solution containing nonradioactive NaI. The surfaces in groups 11 and 21 were incubated in radiolabeled protein solution without NaI. Following adsorption, samples were rinsed three times in PBS, wicked onto filter paper to remove residual adherent buffer, and the radioactivity determined. The quantity of protein adsorbed was determined by comparing the surface radioactivity to that of an aliquot of solution of known concentration. Four replicates were run for each set of experimental conditions. Experiments were run in duplicate.

6.2.4. SDS-PAGE

All electrophoresis reagents were obtained from Bio-Rad, Richmond, CA, or were made to the specifications in the Mini-Protean Dual Slab Cell instruction manual (Bio-Rad). A 12% separating gel was prepared by mixing 3.35 mL distilled water, 2.5 mL 1.5 M Tris-HCl buffer (pH 8.8), 100 μL 10% (w/v) SDS solution with 4.0 mL 30%
acrylamide + 0.8% bisacrylamide solution and degassing for 15 min, mixing further with 5 μL tetramethylethylenediamine (TEMED) and 50 μL freshly made 10% ammonium persulfate. Soon after mixing, the gel solution was added into the SDS-PAGE apparatus. Distilled water was then injected above the separating gel, and the system was allowed to polymerize for 1 h. A 4% stacking gel was prepared in the same way using the following mixture: 3 mL distilled water, 1.2 mL 0.5 M Tris-HCl buffer (pH 8.8), 100 μL 10% (w/v) SDS solution, 0.65 mL monomer (30% acrylamide + 0.8% bisacrylamide) solution, 5 μL tetramethylethylenediamine (TEMED) and 25 μL freshly made 10% ammonium persulfate. The stacking gel solution was injected into the apparatus above the separating gel after the distilled water was withdrawn. Polymerization was allowed to continue for 1 h at room temperature.

Low molecular weight markers were obtained from Pharmacia, Piscataway, NJ. The protein samples were reduced by reaction with beta-mercaptoethanol at 90°C for 5 min. Samples containing approximately 5 μg of protein were loaded on each lane of the gel. Separations were run on a Mini-Protean II Dual Slab Electrophoresis Cell (Bio-Rad) at 200 V for 45 min. The gels were stained using “Protorgold” stabilized gold stain solution (British Biocell International, Cardiff, UK), and dried overnight at room temperature.

6.2.5. IMMUNOBLOTTING

For the samples that were to be investigated by immunoblotting, SDS-PAGE was run as described above. Before completion of the electrophoresis, a small quantity of Pyronin-Y red dye in sample buffer was added to the wells; this marked the top of the separating gel before being transferred to the blot membrane. A Mini Transblot cell (Bio-
Rad) was used for blotting and the following procedures were carried out in sequence. After electrophoresis the gel was equilibrated in transfer buffer (25 mM Tris, 192 mM glycine, 15% HPLC grade methanol, pH 8.2-8.3) for 20 min. An Immobilon® PVDF transfer membrane (Bio-Rad) was cut to size, pre-wet with methanol (3 sec) and distilled water (1 min) and soaked in the transfer buffer. The membrane and gel were clamped together and blotting was carried out for 1 h at 100 V (200 mA).

The marker lanes were stained separately from the rest of the blot. These lanes were stained with Gold Stain (Cedarlane), and then destained with distilled water.

The rest of the blot was cut into 3 mm-wide strips (usually 25 in total). The strips were prewet with 100% methanol, rinsed in distilled water and placed in the “blotting pan”. They were then incubated for 1 h with gentle agitation in a “blocking” solution of 5% (w/v) VIM instant skim milk powder (Ault Foods Limited, Etobicoke, ON) in TBS; this procedure limits nonspecific antibody binding to the membrane. After blocking, the strips were rinsed 3 times with 0.1% (w/v) nonfat dry milk in TBS for 5 min, and then incubated for 1 h in a solution containing a primary antibody (diluted 1000:1) directed against the protein of interest (Table 6.2), 1% (w/v) nonfat dry milk, and 0.05% (v/v) Tween 20 in TBS. After washing with 0.1% (w/v) nonfat dry milk in TBS three times to remove unreacted antibody, the strips were incubated with alkaline phosphatase-conjugated second antibody (diluted 1000:1) for 1 h. The strips were again washed 3 times with 0.1% (w/v) nonfat dry milk in TBS. Color was developed by incubating the strips with the appropriate enzyme substrate consisting of 5-bromo-4-chloro-3-indolyl phosphate (BCIP, 15 mg in 1000 µL DMF) and nitroblue tetrazolium (NBT, 30 mg in 300 µL water and 700 µL DMF). Substrate solution was prepared by adding the reagents
indicated to 100 mL of carbonate buffer (20 mg MgCl₂ 6H₂O, 840 mg NaHCO₃, ddH₂O to 100 mL, pH adjusted with NaOH to 9.8) just before use. The color reaction was stopped by adding distilled water.

Table 6.2. Antibodies used for immunoblotting. All primary antibodies were directed against human proteins

<table>
<thead>
<tr>
<th></th>
<th>Antigen</th>
<th>Host for first Ab</th>
<th>SUPPLIER</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Factor XI</td>
<td>goat</td>
<td>Cedarlane</td>
</tr>
<tr>
<td>2</td>
<td>Factor XII</td>
<td>goat</td>
<td>Cedarlane</td>
</tr>
<tr>
<td>3</td>
<td>Prekallikrein</td>
<td>sheep</td>
<td>Cedarlane</td>
</tr>
<tr>
<td>4</td>
<td>HMWK</td>
<td>goat</td>
<td>Cedarlane</td>
</tr>
<tr>
<td>5</td>
<td>Fibrinogen</td>
<td>goat</td>
<td>Cedarlane</td>
</tr>
<tr>
<td>6</td>
<td>Plasminogen</td>
<td>goat</td>
<td>Sigma</td>
</tr>
<tr>
<td>7</td>
<td>AT</td>
<td>sheep</td>
<td>Cedarlane</td>
</tr>
<tr>
<td>8</td>
<td>C3</td>
<td>goat</td>
<td>Calbiochem</td>
</tr>
<tr>
<td>9</td>
<td>Transferrin</td>
<td>goat</td>
<td>Sigma</td>
</tr>
<tr>
<td>10</td>
<td>α-1-antitrypsin</td>
<td>goat</td>
<td>Enzyme Res Labs</td>
</tr>
<tr>
<td>11</td>
<td>Fibronectin</td>
<td>rabbit</td>
<td>Sigma</td>
</tr>
<tr>
<td>12</td>
<td>Albumin</td>
<td>goat</td>
<td>Cedarlane</td>
</tr>
<tr>
<td>13</td>
<td>IgG</td>
<td>goat</td>
<td>Sigma</td>
</tr>
<tr>
<td>14</td>
<td>β-lipoprotein</td>
<td>goat</td>
<td>Sigma</td>
</tr>
<tr>
<td>15</td>
<td>α-2-macro</td>
<td>goat</td>
<td>Sigma</td>
</tr>
<tr>
<td>16</td>
<td>Vitronectin</td>
<td>goat</td>
<td>Cedarlane</td>
</tr>
<tr>
<td>17</td>
<td>Protein C</td>
<td>goat</td>
<td>Cedarlane</td>
</tr>
<tr>
<td>18</td>
<td>Prothrombin</td>
<td>sheep</td>
<td>Cedarlane</td>
</tr>
<tr>
<td>19</td>
<td>Haemoglobin</td>
<td>rabbit</td>
<td>Sigma</td>
</tr>
<tr>
<td>20</td>
<td>Haptoglobin</td>
<td>rabbit</td>
<td>Sigma</td>
</tr>
<tr>
<td>21</td>
<td>Factor B</td>
<td>goat</td>
<td>Calbiochem</td>
</tr>
<tr>
<td>22</td>
<td>Factor H</td>
<td>goat</td>
<td>Calbiochem</td>
</tr>
<tr>
<td>23</td>
<td>Factor I</td>
<td>goat</td>
<td>Calbiochem</td>
</tr>
<tr>
<td>24</td>
<td>Apolipoprotein</td>
<td>goat</td>
<td>Calbiochem</td>
</tr>
</tbody>
</table>

Rabbit Anti Goat IgG Alkaline Phosphatase Conjugate  | Sigma Chemical Co.
Rabbit Anti Sheep IgG Alkaline Phosphatase Conjugate  | Bethyl Laboratories Inc.
Goat Anti Rabbit IgG Alkaline Phosphatase Conjugate  | Bio-Rad Laboratories
6.3 RESULTS AND DISCUSSION

6.3.1. SUPPRESSION OF FREEIODIDE UPTAKE BY GOLD

The results of the free iodide suppression experiments (expressed in the form of an orthogonal experimental design) are illustrated in Figure 6.1. Following exposure to the protein, the surfaces were washed and their associated radioactivity determined. The data are listed in Table 6.3.

From these data the effect of addition of 1% NaI to the buffer (method B) and the effect of the presaturation of the surfaces by NaI (method A) can be estimated. The effect of the addition of 1% NaI to the buffer may be taken as the difference in radioactivity with and without NaI. The radioactivity without NaI can be estimated by averaging groups 11 and 21 while the radioactivity with NaI can be estimated by averaging groups 12 and 22. This calculation gives the effect of 1% NaI as $-263,600$ cpm. In a similar fashion, the effect of pre-saturating the surface with NaI can be estimated as $-44,400$ cpm. Thus it appears that addition of NaI to the buffer is more effective in suppressing free radioactive iodide uptake by gold surface than pre-treatment of the surface with NaI.

Using these results, the free radioactive iodide content of the non-suppressed surfaces can be estimated. Assuming that addition of NaI to the buffer eliminated all of the free iodide radioactivity, i.e. radioactivity on these surfaces is contributed solely by adsorbed protein, the percentage contribution of radioactivity from adsorbed protein on the non-suppressed surface can then be estimated by the ratio of radioactivity of surfaces with and without addition of NaI to the buffer. Both the ratio 22/21 and 12/11 give such a percentage contribution. In essence, the “mean” percentage contribution is calculated by taking the average of the two ratios:
Figure 6.1. Experimental design for the comparison of methods to suppress the uptake of free radioactive iodide by gold surface. Treatment groups are indicated above the columns. Typical radioactivity data are shown, for albumin adsorption (1.0 mg/mL, 2h).

Table 6.3. Comparison of methods to suppress the uptake of free radioactive iodide by gold surface (polyurethane). Adsorption of albumin, 1.0 mg/mL, 2h.

<table>
<thead>
<tr>
<th>Group</th>
<th>Pretreatment with NaI (A)</th>
<th>NaI present in buffer (B)</th>
<th>Average surface count (cpm)</th>
<th>S.D (N = 4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>11</td>
<td>no</td>
<td>no</td>
<td>340,200</td>
<td>28,450</td>
</tr>
<tr>
<td>12</td>
<td>no</td>
<td>yes</td>
<td>32,200</td>
<td>3,460</td>
</tr>
<tr>
<td>21</td>
<td>yes</td>
<td>no</td>
<td>247,750</td>
<td>32,990</td>
</tr>
<tr>
<td>22</td>
<td>yes</td>
<td>yes</td>
<td>28,560</td>
<td>3,970</td>
</tr>
</tbody>
</table>

\[
\frac{(32200/340200+28560/247750)/2}{2} = 10\%.
\]

The free iodide contribution is accordingly 90%. This number is in agreement
with the observation that the free iodide contribution to the radioactivity on gold surface in a typical protein adsorption experiment, without taking any precautions to suppress free iodide uptake, is between 60% and 90% (Sheardown et al., 1998).

6.3.1.1. VALIDATION OF METHOD B

It is important to point out that addition of NaI to the buffer may in itself change the properties of the buffer and may affect adsorption. The most important buffer properties are the pH and osmolality. To investigate the effects of NaI on the buffer, solutions having the same pH with and without NaI were prepared. The osmolalities were then measured. Table 6.4 shows data for PBS buffer in which 5 mol % of the normal NaCl concentration was replaced by NaI. The measured and expected values for the buffer without sodium iodide are also listed. It can be seen that the properties of the buffer with NaI are similar to those of “normal” PBS without NaI. Since buffers typically used in protein adsorption studies have osmolalities in the range of 300 ± 20 mOsm/kgH₂O, and since osmolality values within that range are acceptable in the present studies, it can be concluded that the addition of NaI at levels of 5% or less of the normal NaCl concentration does not lead to significant changes in osmolality.

Table 6.4. Composition, pH and osmolality of PBS and PBS + NaI (5%) buffers.

<table>
<thead>
<tr>
<th></th>
<th>PBS only</th>
<th>PBS+NaI (5%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Disodium hydrogen phosphate (g L⁻¹)</td>
<td>1.32</td>
<td>1.32</td>
</tr>
<tr>
<td>Sodium dihydrogen phosphate (g L⁻¹)</td>
<td>0.345</td>
<td>0.345</td>
</tr>
<tr>
<td>Sodium Chloride (g L⁻¹)</td>
<td>8.5</td>
<td>8.08</td>
</tr>
<tr>
<td>Sodium Iodide (g L⁻¹)</td>
<td>-</td>
<td>1.09</td>
</tr>
<tr>
<td>pH (adjusted)</td>
<td>7.38</td>
<td>7.40</td>
</tr>
<tr>
<td>Osmolality (mOsm/kgH₂O)</td>
<td>303 ± 5</td>
<td>293 ± 5</td>
</tr>
</tbody>
</table>
It also seemed possible that the presence of significant quantities of free iodide in the buffer might affect adsorption. To investigate this possibility, adsorption was measured using buffers with and without NaI. A surface (glass) that does not take up iodide ion was used in these studies. It should then be possible to conclude that any change in adsorption is due to the effect of iodide on the protein. To provide maximum information, two types of experiment were done: (1) using solutions of radiolabeled albumin (HSA$^{125}$I), presumably containing traces of $^{125}$I, and (2) using mixtures of unlabeled albumin and radioactive iodide (HSA + $^{125}$I). In both cases, experiments were done in the presence and in the absence of nonradioactive iodide in the buffer. The data are shown in Table 6.5.

Table 6.5. Effect of free iodide on albumin adsorption to glass.

<table>
<thead>
<tr>
<th>Expt</th>
<th>Protein Medium</th>
<th>NaI in Buffer</th>
<th>Radioactivity (cpm)</th>
<th>Radioactivity after SDS (cpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>HSA$^{+125}$I</td>
<td>no</td>
<td>34</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>HSA$^{+125}$I</td>
<td>5%</td>
<td>52</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>HSA$^{125}$I</td>
<td>no</td>
<td>17,670</td>
<td>1,500</td>
</tr>
<tr>
<td>4</td>
<td>HSA$^{125}$I</td>
<td>5%</td>
<td>15,670</td>
<td>1,130</td>
</tr>
<tr>
<td>5</td>
<td>HSA$^{125}$I</td>
<td>1%</td>
<td>19,150</td>
<td>-</td>
</tr>
</tbody>
</table>

From experiments 1 and 2 in table 6.5 it can be seen that as long as the protein is not radiolabeled (HSA$^{+125}$I), the glass surface does not acquire significant radioactivity, in agreement with the fact that free iodide is not taken up by the glass surface. Thus when radiolabeled protein is used, as in experiments 3-5, the entire increase in radioactivity must be due to protein adsorption. From experiments 4 and 5 it can be seen that the presence of NaI at the 1% level causes a small increase in adsorption while at 5% a slight decrease occurs. These data suggest that the effect of NaI on protein adsorption is
negligible. Further, the addition of a small amount of radioactive free iodide to the HSA$^{125}$I solution did not change the total radioactivity on the surface significantly.

From the data presented in this section, it is concluded that the addition of nonradioactive iodide to the buffer, while producing the desired suppression of radioactive iodide uptake on the gold surface, does not in itself cause changes in adsorption.

6.3.1.2. DEPENDENCE OF SUPPRESSION OF $^{125}$I UPTAKE ON SODIUM IODIDE CONCENTRATION

Suppression of $^{125}$I uptake on gold surface in the presence of nonradioactive iodide must depend on the ratio of nonradioactive to radioactive iodide. The ratio of NaI to Na$^{125}$I can be calculated starting from the half-life of $^{125}$I:

$$t_{1/2} = 60 \text{ days} = 60 \times 24 \times 60 \text{ min} = 86400 \text{ min}$$ (6.2)

Assuming 1 mol of $^{125}$I decays and that every disintegration is received by the gamma-counter, the disintegration rate $r_d$ can be estimated as:

$$r_d = \frac{6.02 \times 10^{23}}{t_{1/2}} \left( \frac{cpm}{mol} \right) = 3.484 \times 10^{18} \left( \frac{cpm}{mol} \right)$$ (6.3)

Given that the protein solution count is $1 \times 10^8$ cpm/100 μL, and that the free iodide content is 1%, the concentration of radioactive free iodide will be

$$m_i = \frac{1}{100} \times \frac{10^8 \text{ (cpm)} \times 10^6 \text{ (μL/L)}}{100 \text{ (μL)} \times r_d \text{ (cpm/mol)}} = 2.87 \times 10^{-9} \left( \frac{mol}{L} \right)$$ (6.4)

A liter of PBS buffer contains 8.5 g NaCl and if 1% of the NaCl is replaced by NaI, the concentration of NaI is:
\[ m_2 = \frac{1}{100} \frac{8.5 \text{ (g/L)}}{58.5 \text{ (g/mol)}} = 1.45 \times 10^{-3} \left( \frac{\text{mol}}{L} \right) \]  

(6.5)

The ratio of nonradioactive to radioactive free iodide is:

\[ m_2 / m_1 = \frac{1.45 \times 10^{-3}}{2.87 \times 10^{-9}} = 5.05 \times 10^5 \]  

(6.6)

From the above estimate it is seen that the ratio of NaI to Na\textsuperscript{125}I is high under typical experimental conditions, and uptake of radioactive iodide should therefore be suppressed. On the other hand, NaI may interact with the protein or with the surface to influence protein adsorption. Therefore only an amount of NaI sufficient to suppress free iodide uptake should be added to the buffer. The dependence of suppression on NaI content was therefore investigated.

Figure 6.2 shows data on the adsorption of albumin to glass and gold from solutions of varying NaI content. Expressed as mol % relative to NaCl, the content of NaI was set at 0%, 0.01%, 0.1%, 1% and 5%. For glass, the radioactivity on the surface following adsorption was essentially independent of NaI content. For gold, the surface radioactivity decreased dramatically with increasing content of NaI in the buffer, presumably reflecting suppression of \textsuperscript{125}I uptake. Even at 0.01% sodium iodide, the surface radioactivity was only about 50% of that in the complete absence of sodium iodide. Figure 6.2 suggests that a NaI content of about 1% is sufficient to suppress radioactive iodide uptake completely.

In summary, it can be concluded that of the two methods tried to correct for uptake of radioactive iodide by gold, method B involving the inclusion of nonradioactive NaI in the buffer, is the most effective. When NaI is present at a level of 1 mol % of the NaCl content of PBS buffer, the uptake of radioactive iodide is effectively suppressed. It
also appears that the inclusion of iodide at these levels has no effect on protein adsorption.

![Graph showing protein adsorption on glass and gold surfaces](image)

Figure 6.2. Albumin adsorption on glass and gold surfaces (polyurethane) as a function of NaI content in the buffer. Error bars are ±S.D (n=8)

### 6.3.2 Protein Adsorption to Modified Gold Surfaces

The adsorption of fibrinogen and albumin from buffer to surfaces modified with both linear and star PEO was investigated using the radiolabeling method.
6.3.2.1 ADSORPTION OF SINGLE PROTEINS FROM BUFFER TO GOLD SURFACES MODIFIED WITH LINEAR PEOs

Figure 6.3 shows typical data for the adsorption of fibrinogen to gold surfaces chemisorbed with methoxy-terminated PEOs of different molecular weights, ranging from 300 to 5000. Each point represents the average of three replicate samples. For any given surface, adsorption tends towards a plateau at higher protein concentration, indicating that saturation is reached. The plateau levels decrease with increasing molecular weight of PEO at least up to a MW of 5000, suggesting that the longer chains...
are more effective in repelling proteins from the surface. Because PEO is an electrically neutral polymer, it is unlikely that Coulomb interactions between PEO and protein are significant. As such the overall interaction energy decrease would come from either the decrease in long range interaction forces (most likely the electrostatic interactions) between protein and gold surface, or the increase in hydrophilicity due to PEO, as indicated by the decrease in water contact angle with increasing PEO molecular weight (Chapter 5).

PPO is a hydrophobic polyether and the chains are less flexible than PEO. Thus if the hypothesized mechanisms for repulsion of proteins by PEO are correct, PPO is not expected to be protein repellent. The PPO surfaces were thus used as controls having a similar chemical structure to PEO. It is seen in Figure 6.3 that the PPO surface adsorbs much less protein than the unmodified gold, and indeed shows adsorbed amounts similar to those on PEO. Thus, somewhat unexpectedly, chemisorbed PPO appears to have a protein repellent effect, the nature of which remains to be elucidated. Since PPO is hydrophobic, it does not dissolve to any extent in water. Therefore the conformation of the surface immobilized PPO in contact with water is probably a collapsed random coil that may lead to high effective coverage (Irvine et al., 1998) of PPO on the surface.

Although adsorption is considerably reduced on the PEO-modified surfaces, the levels, ranging from 0.1 to 0.2 \( \mu \text{g/cm}^2 \) are still significant and higher than those reported by others for PEO modified surfaces (Mao et al., 2000; Wesslen et al., 1993; Amiji et al., 1992; Bergstrom et al., 1992; Han et al., 1993; Maechling-Strasser et al., 1989). Certainly such levels of fibrinogen adsorption are more than enough to support platelet adhesion to blood contacting surfaces (Grunkemeier et al., 2000a; Grunkemeier et al., 2000b; Tsai et
al., 1999). A possible reason for the relatively high adsorption on these surfaces is that the methoxy (and/or thiol) end groups are involved in protein binding; if so, this would be expected to become more important at higher graft density. Therefore, to examine the possible effect of the end groups, surfaces modified with HS-PEO-OH instead of HS-PEO-OMe were also used in protein adsorption studies. Figure 6.4 shows fibrinogen adsorption data for such surfaces. As for the methoxy-terminated PEO, adsorption decreases with increasing molecular weight. Most strikingly, adsorption on these hydroxy-PEO surfaces is lower than on the corresponding methoxy-PEOs, with surface concentrations at the plateaus in the range of 0.04 to 0.12 μg/cm². Considering that the surface concentration of fibrinogen for a close packed a monolayer is in the range of 0.4 μg/cm² (side-on orientation) to 1.7 μg/cm² (end-on orientation) (Brynda et al., 1986), the data in Figure 6.4 show a very strong protein repulsion effect of PEO.

From Figures 6.3 and 6.4 it is clear that the terminal groups on PEO do have an effect on protein binding. This suggests that the terminal groups are present at a significant level at the outermost surface of the material when in contact with the protein solution, and thus that the chemisorbed PEO chains do not adopt a strictly random coil conformation, since in that case the end groups would tend to be “hidden” in the subsurface regions. This finding is, however, in contrast to those of others (Palegrosdemange et al., 1991; Prime et al., 1993; Mrksich et al., 1997) where the end groups on short PEO segments attached to C12 alkane thiol SAMs had no effect on protein adsorption. These contrasting observations are difficult to explain. If anything, one would expect the end group effects to be more important for a PEG-tipped SAM having a higher PEO surface density than the surfaces used here.
Figure 6.4. Fibrinogen adsorption to gold surfaces (polyurethane base) modified with hydroxy-terminated PEO of different molecular weights. Free iodide uptake was suppressed by addition of NaI to the buffer. Control is gold modified with hydroxy-terminated polypropylene oxide (HSPPO(1025)OH). Error bars are S. D. (n = 3).

For both the methoxy-PEO and hydroxy-PEO surfaces, fibrinogen adsorption is much lower than on the unmodified gold. The plateau adsorption level on unmodified gold surface is about 1.2 μg/cm², outside the range of the Figures.
Single protein experiments were also carried using albumin. Figure 6.5 shows data for adsorption to surfaces modified with methoxy-terminated PEO of different molecular weights. 

Figure 6.5. Albumin adsorption to gold surfaces (polyurethane base) modified with methoxy-terminated PEO of different molecular weights. Free iodide uptake was suppressed by addition of NaI to the buffer. Controls are unmodified gold, and gold modified with hydroxy-terminated polypropylene oxide (HSPPO(1025)OH). Error bars are S. D (n = 3).

molecular weights. Again it is seen that adsorption decreases with increasing molecular weight of the PEO. However in this case the PEO-350 surface adsorbs more than the unmodified gold. Again the PPO surface is protein repellent to a significant extent.
A similar dependence on PEO molecular weight for hydroxy-PEO surfaces is seen in Fig 6.6. It is clear that, as for fibrinogen, adsorption of albumin on the hydroxy-PEO surfaces is lower than on the corresponding methoxy-PEO surfaces, with plateau adsorptions in the range of 0.2 to 0.5 \( \mu g/cm^2 \) for methoxy, and 0.04 to 0.1 \( \mu g/cm^2 \) for hydroxy.

![Graph showing albumin adsorption vs. albumin concentration](image)

Figure 6.6. Albumin adsorption to gold surfaces (polyurethane base) modified with hydroxy-terminated PEO of different molecular weights. Free iodide uptake was suppressed by addition of NaI to the buffer. Control is gold modified with hydroxy-terminated polypropylene oxide (HSPPO(1025)OH). Error bars are S. D (n = 3).

Both fibrinogen adsorption and albumin adsorption showed dependency on the end groups of PEO. This may be due to either structural or functional differences between the two PEOs. Structurally, methoxy groups are more hydrophobic; they may
modify the conformation of the PEO chains in an aqueous environment, whereas the hydrophilic hydroxyl end groups may perturb the conformation less if at all. Functionally, methoxy groups are expected to interact with the hydrophobic portions of the protein while hydroxyl groups may interact more with the hydrophilic regions. Water binds to hydrophilic surfaces in preference to hydrophobic ones. Because the most abundant component of blood is water, it may be expected that proteins behave more normally facing a water-polymer layer than a polymer layer. As a result, hydroxy PEO shows less protein deposition than methoxy PEO.

As seen in figures 6.3 – 6.6, surfaces modified with polypropylene oxide (PPO), showed lower protein adsorption than unmodified gold. As discussed previously, PPO is hydrophobic, and does not dissolve to any noticeable extent in water. Therefore the conformation of the surface immobilized PPO in contact with water is probably a collapsed random coil that may lead to high effective coverage of PPO on the surface. It is unclear why PPO in such a conformation would have a protein repellent effect. Certainly there can be no question of a steric repulsion mechanism of the same type as has been postulated for PEO. In addition, it has generally been found that hydrophobic surfaces have relatively high affinity for proteins. Thus the repellent effect of PPO remains for the moment an unexplained observation. It may be that there is some kind of special property associated with polyethers. The effect of PPO would have to be confirmed using different types of PPO-modified surface (eg using polymeric substrates instead of gold).
6.3.2.2 ADSORPTION OF SINGLE PROTEINS FROM BUFFER TO GOLD SURFACES MODIFIED WITH STAR-TYPE PEO

It is the underlying hypothesis of this research that biocompatible surfaces will have two important properties, namely, resistance to nonspecific protein binding (passivation), and affinity for specific biological species (specific bioactivity). These requirements may be met using PEO to which appropriate bioactive components that will give the desired activity, are attached. Since the PEO chain end is the most convenient site for attachment, star-type or multiarmed PEOs, with a high number of chain ends per molecule, should have an advantage over linear PEOs in that surfaces with a greater density of biospecific groups should be possible. These ideas could be tested by chemisorbing to gold, star-type PEOs in which one chain end is thiolated and the others are conjugated to the bioactive species. In this work, as a preliminary step, star-type PEOs were thiolated at one chain end, leaving all the other ends with terminal hydroxyls. It was of interest to study protein interactions with gold surfaces to which these PEOs were chemisorbed, in particular to see whether the star-type PEO architecture would have an effect on protein resistance. Resistance could be affected by the density of terminal hydroxyls and by the flexibility of the PEO chains which could conceivably be different for star-type and linear PEO. With these considerations in mind, single protein adsorption to the star-type PEO modified surfaces was investigated.

Figures 6.7 and 6.8 show, respectively, data on fibrinogen and albumin adsorption to star-type PEO modified surfaces. It was anticipated that adsorption would be dependent on the total molecular weight of the PEO, on the number of arms, and on the length of the arms. As can be seen, for star-type PEOs having four arms, protein
adsorption was similar for arm molecular weights of 500 and 2500. For PEOs of total

![Graph showing Fibrinogen adsorption vs Fibrinogen Concentration](image)

Figure 6.7. Fibrinogen adsorption to gold surfaces (polyurethane base) modified with branched PEO of different structure, molecular weight. Free iodide uptake was suppressed by addition of NaI to the buffer. Controls are unmodified gold, and gold modified with hydroxy-terminated polypropylene oxide (HSPPO(1025)OH). Error bars are S. D (n = 3).

molecular weight 2000, adsorption was slightly lower for the 8-arm compared to the 4-arm polymer. However, neither the differences between PEOs of different number of arms (Fig 6.7 – 6.8) nor between linear PEOs and branched PEOs are significant (compare Fig 6.7, 6.8 to 6.4 and 6.6). The dependence of protein adsorption on molecular weight as well as on type of end group may be a factor in explaining these data. When the number of arms increases, the arm length decreases, and these effects may compensate each other to some extent. For example, PEO-(8 arm 2k)-OH has 8 arms with each arm having an average molecular weight of 250. For linear PEO of the same molecular
weight, the protein passivation effect is much less. Presumably the high density of terminal hydroxyl groups for the star-type PEO is responsible for its greater passivation. In fact protein adsorption on the PEO-(8 arm 2k)-OH modified surface is similar to that on the linear PEO(1,500)-OH.

![Graph showing albumin adsorption vs. albumin concentration](image)

Figure 6.8. Albumin adsorption to gold surfaces (polyurethane base) modified with star-type PEOs. Free iodide uptake was suppressed by addition of NaI to the buffer. Controls are unmodified gold, and gold modified with hydroxy-terminated polypropylene oxide (HSPPO(1025)OH). Error bars are S. D. (n = 3).

The observation that linear PEO and star PEOs give similar passivation effects is in contrast to findings of Sofia et al (1998) that for linear PEO, the surface was resistant to protein adsorption if the graft density was greater than a monolayer, while for star PEO-grafted surfaces, a protein passivation effect was observed only if the spaces between star PEOs were smaller than the effective size of the protein.
6.3.3. **Protein Adsorption from Plasma (Radiolabeling)**

While experiments on adsorption of single proteins from buffer provide data that can be relatively easily interpreted, it is obvious that the behavior of proteins in such a simple system may not necessarily be the same as in real biological fluids like plasma or whole blood. The classical example of differences between simple and complex systems is the Vroman effect in plasma, whereby proteins are adsorbed and then desorbed as a function of time (Vroman et al., 1969; Turbill et al., 1996; Brash et al., 1993; Scott, 1991; Leonard et al., 1991; Brash et al., 1988; Slack et al., 1992; Slack et al., 1995) due to differential diffusion rates and binding affinities. As discussed previously, the composition of the adsorbed protein layer determines the degree to which the cellular elements of the blood are bound to the surface and activated (Forbes et al., 1978; Salzman et al., 1987). Protein adsorption also leads directly to fibrin formation and complement activation (Rollason et al., 1992; Janatova et al., 1991). Therefore it was important to investigate the adsorption of proteins from plasma to the PEO-modified surfaces developed in this work, and in particular to determine whether the repellent effects seen for the simple buffer system would hold up in the more complex system.

Figures 6.9 and 6.10 show, respectively, albumin and fibrinogen adsorption to gold and PEO-modified gold surfaces from human plasma. The data were obtained in plasma diluted to different extents with buffer. Dilutions corresponding to 0.1, 2, and 20% of normal plasma concentration were investigated. Only hydroxy-terminated PEOs of different molecular weights, ranging from 300 to 5000 were used in this study, based
on the result that hydroxy-PEO is more efficient in suppressing protein adsorption than methoxy-PEO.

Figure 6.9. Albumin adsorption from plasma to gold surfaces (polyurethane base) modified with linear PEO of different molecular weights. Adsorption was measured in plasmas diluted to different extents with buffer. Plasma concentration is expressed as "percent normal plasma". Free iodide uptake was suppressed by addition of NaI to the buffer. Error bars are S. D. (n = 3).
Figure 6.10. Fibrinogen adsorption from plasma to gold surfaces (polyurethane base) modified with linear PEO of different molecular weights. Adsorption was measured in plasmas diluted to different extents with buffer. Plasma concentration is expressed as “percent normal plasma”. Free iodide uptake was suppressed by addition of NaI to the buffer. Error bars are S. D. (n = 3).

It appears that protein adsorption increases monotonically with increase in plasma concentration. The Vroman effect seen on many artificial surfaces (especially hydrophilic surfaces) is thus absent. It could be that PEO prevents the Vroman effect by reducing the interaction of displacing proteins, such as contact phase proteins, with the surface. Similar to single protein adsorption from buffer, for any given surface, adsorption tends towards a plateau at plasma concentrations of ~ 2%, indicating
saturation or pseudo equilibrium adsorption. Longer chains appear to be more effective for passivation in that plateau adsorption levels decrease with increasing molecular weight of PEO, similar to single protein adsorption. Thus albumin adsorption shows saturation levels of 0.05 and 0.02 μg/cm² for PEO of molecular weight 300 and 4600 respectively. The corresponding values for fibrinogen are 0.15 and 0.05 μg/cm².

Fibrinogen adsorption from plasma also shows similar trends to those observed for adsorption from buffer. In contrast to albumin, however, the PPO surface shows lower fibrinogen adsorption than the unmodified gold. Again this difference may refer to the difference in hydrophilicity of the two proteins.

6.3.4. PROTEIN ADSORPTION FROM PLASMA STUDIED BY SDS-PAGE AND IMMUNOBLOTTING OF ELUTED PROTEINS

As a complement to the measurement of adsorption from plasma using individual radiolabeled proteins, SDS-PAGE and immunoblotting were carried out on the proteins eluted from the surfaces after plasma contact. The data from these experiments can provide a comprehensive picture of the overall composition of the protein layer deposited on the surfaces. The influence of chemisorbed PEO of different molecular weights on the composition of the protein layer formed on gold was investigated.

It should be noted that for these experiments, identical surface areas were used for all the materials, identical volumes of SDS were used for elution of the proteins, and identical volumes of the eluates were loaded on the gels. Thus the intensity of gel and blot bands can be interpreted to a first approximation in terms of the relative amounts of the proteins eluted from the different surfaces.
It can be seen from Figure 6.11, showing gels of the plasma used for adsorption, and of the eluates from some of the surfaces, that the unmodified gold appears to adsorb the smallest amounts of the proteins. The HS-PEO(1500)-OH appears to adsorb the greatest amounts. This observation runs contrary to the expectation that PEO should passivate the surface. It is also in conflict with data from the experiments using radiolabeled proteins which showed that PEO is an effective passivator on these surfaces. It is believed that these discrepancies are due to the strong interactions of proteins with gold via complexation with thiol and disulfide groups present in the proteins. It seems likely that treatment with SDS will be ineffective in eluting proteins that are bound to the
gold via interactions with these groups. However when the gold is modified with PEO, these interactions will be reduced, and the adsorbed proteins will be more readily elutable. This problem does not arise for the radiolabeling experiments since in that case the proteins are measured directly on the surface.

The gels of the eluted proteins (Figure 6.11) show strong bands at about 66, 55, 48, and 27 kDa, probably indicating the presence of albumin, fibrinogen and IgG. Positive identification requires information from the immunoblots.

A Western blot of the plasma used in this study is shown in Figure 6.12. As expected, the abundant proteins, albumin, fibrinogen, C3, and IgG are clearly present. Most of the other proteins tested for also give positive responses. It is not clear why prothrombin (concentration 0.1 mg/mL) does not give a response. The β-2-microglobulin concentration is perhaps below the limit of detection. Blots of the proteins eluted from several surfaces by 2% SDS are shown in Figures 6.13 - 6.15. As mentioned in discussing the gels, specific interactions may occur between gold and adsorbed proteins, so that elution from the unmodified gold may be difficult. Thus a comparison of the blots of the gold and modified gold surfaces in terms of adsorption from plasma may be invalid. From an overall standpoint the blots of the three surfaces are qualitatively similar, although again the responses are somewhat stronger for the modified than for the unmodified surfaces, and stronger for the PEO1500 than the PEO4600.
Figure 6.12. Western blot of the plasma sample used for this study. The lane at the extreme left is the SDS-PAGE gel stained with “gold stain”.

Figure 6.13. Western blot of proteins eluted from unmodified gold surface (polyurethane base) after plasma contact.
Figure 6.14. Western blot of proteins eluted from HS-PEO (1,500)-OH modified gold surface (polyurethane base) after plasma contact.

Figure 6.15. Western blot of proteins eluted from HS-PEO (4600)-OH modified gold surface (polyurethane base) after plasma contact.
For individual proteins, the following observations can be made. The coagulation proteins, factor XI, factor XII, prekallikrein, and high molecular weight kininogen (HMWK) are of interest because they are involved in the contact phase of the intrinsic blood coagulation pathway (Davie et al., 1991; Colman, 1984; Cornelius et al., 1993). Factor XII is known to bind to negatively charged surfaces and to undergo a conformational change making it susceptible to cleavage by kallikrein. The factor XIIa generated activates more prekallikrein and the kallikrein generated activates more factor XII in a mutual amplification cycle. HMWK (120 kDa) is a cofactor of the contact phase and is known to form complexes with factor XI and prekallikrein. During coagulation it is degraded by factor Xla and kallikrein (Colman, 1984) to a 2-chain form with fragments of MW 56 and 46 kDa (Cornelius et al., 1999). It can be seen in Figures 6.13 - 6.15 that HMWK, was present in significant amount on all the surfaces tested largely in the 2-chain form. It also appears to be enriched on the surfaces compared to the plasma. The fragments were presumably produced by the action of factor Xla, reflecting activation of the contact phase of coagulation. The other contact phase proteins (factors XII, XI and prekallikrein), while detectable on all surfaces, were not present in great amounts and to be neither enriched nor depleted on the surfaces relative to the plasma.

Fibrinogen, with a molecular weight of 340 kDa, plays a critical role in the formation of fibrin clot. It is transformed to fibrin monomer by the activation of thrombin. The fibrin monomer then polymerizes to form fibrin. During clot dissolution, fibrin is degraded by plasmin forming degradation product (FDP). Intact fibrinogen as well as degradation products of fibrinogen can be seen in the eluates from the unmodified gold surface but are less evident in those from the PEO modified surfaces. This
observation is in agreement with data from experiments using radiolabeled fibrinogen (Fig. 6.10) which showed reduced adsorption on the PEO modified surfaces. It also suggests that fibrinogen does not interact with gold through thiol or disulfide interactions.

Complement C3, IgG, and complement factors B, H, I were also investigated in relation to the possible activation of the complement system. C3 is the most abundant protein of the complement system and it plays an important role in the complement pathways, which may be activated during the interaction of blood with foreign surfaces (Sim et al., 1981; Woffindin et al., 1988; Craddock et al., 1977). IgG (molecular weight 160 kDa) may be involved in the initiation of the classical complement pathway as an Ag-Ab complex, whereas factors B, H, and I, are involved in the alternative pathway which is believed to be more important for surface induced complement activation (Kazatchkine et al., 1988). C3 was present in the eluates from all three of the surfaces investigated. A strong band at about 45 kDa indicates degradation of the α-chain, and suggests that the complement system was activated in contact with the surfaces. It should be noted that in the plasma blot the α and β chains at 115 and 75 kDa respectively, are largely intact. IgG was more clearly evident on the PEO modified surfaces than on bare gold, again possibly due to difficulty of elution from the latter.

To summarize, all the surfaces studied appear to adsorb HMWK strongly and indicate contact system activation. Fibrinogen was less abundant on the PEO modified surfaces than on the unmodified gold surface. The C3 blot shows strong evidence of complement system activation. It is difficult to draw any definite conclusion regarding protein repulsion by the PEO modified surfaces, since proteins can interact specifically with gold via sulfur functions, thus making elution difficult.
6.4. CONCLUSIONS

Protein adsorption experiments from single protein solutions and plasma to gold and PEO modified gold surfaces were carried out using radiolabeling and SDS-PAGE/immunoblotting methods. The addition of nonradioactive NaI to the buffer used for protein adsorption was found to be effective in suppressing the uptake of radioactive free iodide onto gold surface. The data from the labeled protein work showed that PEO modification reduces the amounts of fibrinogen and albumin adsorbed onto the surface from both buffer and plasma media. In the single protein experiments adsorption decreased with increasing molecular weight of PEO in the range of 300 to 5000. PEOs with hydroxyl end groups were observed to adsorb much less protein than the corresponding methoxy terminated derivatives. Star-type PEO modified surfaces also showed reduced protein adsorption, and the extent of the reduction was similar to that seen for the higher molecular weight linear PEOs with terminal hydroxyls, providing further support for the idea that both molecular weight and end groups are important for protein rejection. Experiments on protein adsorption from plasma also showed a strong effect of PEO reducing the adsorption of albumin and fibrinogen.

Western blots of proteins eluted from surfaces after plasma contact showed evidence of contact phase activation and complement activation on all surfaces including those modified with PEO.
CHAPTER 7

SURFACES MODIFIED WITH PEO AND HEPARINIZED PEO

7.1. INTRODUCTION

In this chapter, the development of surfaces modified with PEO and heparinized PEO is discussed. The rationale for PEO modification was to reduce nonspecific protein binding whereas heparin was introduced to act as an anticoagulant. Heparin acts as an anticoagulant by binding antithrombin (AT), and inducing an allosteric modification in the reactive centre loop of antithrombin, thereby rendering it more accessible to serine proteases such as thrombin and factor Xa (Damus et al., 1973; Hirsh et al., 1999; David et al., 1989).

Low molecular weight heparin (LMWH) is widely used as a systemic anticoagulant in clinical applications (Hirsh et al., 1999). The use of LMWH as a surface modifier is also an attractive prospect. Most heparinized surfaces developed thus far have been based on unfractionated heparin rather than LMWH (Kang et al., 1997; Park et al., 1988; Park et al., 1991; Nakayama et al., 1993; Marconi et al., 1997; Nojiri et al., 1996; Alkhunaizi et al., 2000). In the present work, Enoxaparin® LMWH was conjugated to PEO for use in surface heparinization.
Both gold and polyurethane surfaces were used as substrates and different chemistries were employed for attachment of PEO and heparin-PEO conjugate. A commercial heparinized polyurethane material in which heparin is claimed to be end-point attached (Carmeda heparinized polyurethane surface) was evaluated for comparison. Unmodified PEO and heparinized PEO were attached simultaneously in an attempt to achieve both the passivation effect of PEO and the anticoagulant effect of heparin via specific interaction with AT. An anti-factor Xa assay was developed to measure the level of active heparin on the surfaces. To evaluate the passivation effect of PEO in the presence of heparin, fibrinogen and albumin adsorption from buffer were measured using radiolabeled proteins. The broad patterns of protein adsorption from plasma were investigated using Western blot analysis.

7.2 EXPERIMENTAL

7.2.1. MATERIALS

Unfractionated heparin (UFH) of MW ~18,000 was purchased from Sigma-Aldrich (Oakville, ON). A commercial low molecular weight heparin (LMWH), Enoxaparin®, was purchased from Rhone-Poulenc Rorer (RPR, Quebec, Canada). Carmeda heparinized polyurethane tubing, referred to as Carmeda bioactive surface (CBAS), was purchased from Solomon Scientific (Plymouth Meeting, PA, USA). Stachrom®, Hepanorm®, and Hepacontrol® reagents for determination of heparin activity were from Diagnostica Stago (Asnieres-sur-Seine, France). Acryloyl-poly(ethylene oxide)-NHS of molecular weight 3,400 was purchased from Shearwater Polymers Inc. (Huntsville, Alabama, USA). Azo isobutyronitrile (AIBN), ethylene glycol
dimethacrylate (EGDMA), poly(ethylene oxide) ether methacrylate, methyl methacrylate, alcian blue, acetic acid, ferrous ammonium sulfate, ammonium persulfate, tetrahydrofuran (THF), ammonia (25% aq) and hydrogen peroxide were purchased from Sigma-Aldrich. Diethylaminoethylene Sepharose (DEAE Sepharose™) was purchased from Amersham Pharmacia.

7.2.1.1. UNFRACTIONATED HEPARIN

Unfractionated heparin was conjugated to PEO of molecular weight 2000 by reaction with NHS-PEO-NHS as illustrated in Scheme 7-1. Briefly, NHS-PEO(2000)-NHS (2.0 g, 1.0 mmol) was dissolved in THF (10 mL). Heparin (1.0 g, ~0.3 mmol), 2-aminoethane thiol (0.07 g, 0.9 mmol) and potassium carbonate (0.045 g, 0.3 mmol) in 20 mL distilled water were added and the solution was allowed to react at room temperature for 1 h. The product was then precipitated in isopropyl ether, dissolved in dichloromethane, reprecipitated in isopropyl ether, and dried under vacuum.

For further purification, treatment with DEAE Sepharose was performed. DEAE Sepharose™ (20 mL) was centrifuged (~1000 rpm), washed and resuspended in 10 mL Tris buffer in a 50 mL centrifuge tube. The dried heparin-PEO product was dissolved in 10 mL distilled water and placed in the centrifuge tube. After mixing for 10 min, the beads were centrifuged, washed with 20 mL 0.1 M NaCl buffer, pH 7.4, centrifuged again and eluted with 2.5 M NaCl buffer. This procedure removes the HS-PEO-SH and PEO from the system. The product solution was stored at −20 °C.
Scheme 7-1. Conjugation of unfractionated heparin to PEO

SDS-PAGE of the purified product was run using the method described in chapter 6. The gel was washed with 40% methanol, 10% acetic acid solution for 30 min, equilibrated with 25% ethanol, 10% acetic acid solution for 30 min and stained in Alcian blue for 5 – 10 min. The gel was destained in 25% ethanol, 10% acetic acid solution three times for 5 min, 30 min and overnight respectively. The gel was then dried using a gel dryer (Model 583, Bio-Rad, Mississauga).
The molar ratio of PEO to heparin-PEO was adjusted to about 5:1 for surface modification. PEO was in excess to give a strong passivation effect. Fibrinogen and albumin adsorption were investigated to evaluate passivation. AT adsorption and antiXa activity were investigated to evaluate the anticoagulant properties of the heparinized surfaces.

7.2.1.2. LOW MOLECULAR WEIGHT HEPARIN (LMWH)

Enoxaparin® is a depolymerized heparin obtained by chemical hydrolysis of heparin benzyl ester from pork mucosa (Dietrich et al., 1999). As indicated in Fig 7.1, a

\[
\begin{array}{c}
\text{COO}^- \\
\text{CH}_2\text{OR}_1 \\
\text{OR}_1 \\
\text{OR}_1 \\
\text{NHR}_2 \\
\text{CH}_2\text{OR}_1 \\
\text{OR}_1 \\
\text{OR}_1 \\
\text{NHR}_2 \\
\end{array}
\]

\[R_1 = H \text{ or } \text{SO}_3\text{Na}, \quad R_2 = H \text{ or } \text{SO}_3\text{Na} \text{ or } \text{COCH}_3, \quad n = 0 \text{ to } 20\]

Figure 7.1. Structure of the LMWH, Enoxaparin®.

2-O-sulfo 4 – enopyranoseuronic acid group is present at the nonreducing end, and a 2 – N, 6 – O – disulfo – D glucosamine group is present at the reducing end.

The double bond in the terminal saccharide unit of the LMWH can be reacted with the thiol group of HS-PEO. The reaction used in the present research is illustrated in Scheme 7.2.
Scheme 7-2. Reaction of thiolated PEO with the terminal double bond of Enoxaparin\textsuperscript{®}. \( R_1 = H \) or \( \text{SO}_3\text{Na} \).

A typical reaction was carried out as follows. Aqueous LMWH (2 mL, 0.5 g, 0.1 mmol) was introduced into a 25 mL flask. PEO of molecular weight 600, thiolated at both ends (0.6 g, 1 mmol), ferrous ammonium sulfate (0.06 g, 0.15 mmol) and ammonium persulfate (0.04g, 0.17 mmol) were then added and the solution was allowed to react for 1 h at room temperature. The product was precipitated in isopropyl ether and extracted with dichloromethane.

Similar to UFH, the molar ratio of PEO to PEO-heparin used in preparing surfaces was adjusted to a high value to investigate both the passivation effect and the coagulation effects.

7.2.1.3. MODIFICATION PROCEDURE: GOLD

Modification of gold surfaces using mixtures of thiolated-PEO and thiolated PEO-Heparin was similar to thiolated PEO modification as described in chapter 6. The molar ratio to thiolated PEO over thiolated PEO-heparin was adjusted to 5:1 to emphasize the passivation effect of PEO. Gold evaporated onto silicon wafers, a kind gift of Dr. P. Tengvall (Linkoping University, Linkoping, Sweden), was used for this study. The surfaces, as well as the glassware to be used, were washed for 5 min in a mixture of boiling distilled water, ammonia (25\% aq) and hydrogen peroxide (30\% aq) in a
volumetric ratio of 5:1:1. After rinsing with distilled water 4 times, the samples were incubated in an aqueous solution containing the 1 mM heparin-PEO-SH (or LMWH-PEO-SH) and 4 mM PEO-SH for 4 hours. The samples were then washed three times in distilled water prior to surface characterization or adsorption experiments.

7.2.2. HEPARINIZATION OF POLYURETHANE (PU) SURFACES

Polyetherpolyurethane films were a gift of Jacques Archambault. The polymer used to make the films was prepared using the methods described in chapter 5. Films were cast from solution in dimethylformamide (3% w/v) in glass petri dishes and dried at 50 °C for ~3 days. The polyetherpolyurethane films were washed with dichloromethane. These films were then incubated in a dichloromethane solution containing NHS-poly(ethylene oxide) methacrylate (40% w/v, MW 3400, Scheme 7.3, A), ethylene glycol dimethacrylate, EGDMA (16% w/v, Scheme 7.3, B), poly(ethylene oxide) ether methacrylate (4% w/v, MW 2080, Scheme 7.3, C), methyl methacrylate (20% w/v, MW 100, Scheme 7.3, D) and azo-bis-isobutynitrile (AIBN) (0.01% w/v) at room temperature for 10 min. The films were then carefully removed to a flask and the solvent was evaporated at room temperature under nitrogen. They were then maintained at 80°C for 2h. The films were then immersed in aqueous heparin solution (5% w/v) for 30 min at room temperature, rinsed 3 times and stored at −20 °C.

Schemes 7.3 and 7.4 illustrate the putative reactions. As shown in Scheme 7.3, NHS-PEO-methacrylate and PEO-methacrylate are expected to copolymerize with EGDMA to give a cross-linked network polymer with pendant PEO chains having NHS residues at the “distal” chain ends. These are then expected to react with the free amino groups of heparin (Scheme 7.4) at room temperature to produce a heparinized acrylic
polymer network. The network formed in the presence of the polyurethane film is expected to be adherent to the film surface. The presence of heparin on the surface was confirmed by staining with Alcian Blue.

Scheme 7-4. Heparinization of NHS-PEO modified polyurethane film based on the reaction shown in Scheme 7.3

7.2.3. CARMEDA BIOACTIVE SURFACE (CBAS)

Carmeda’s “end point attachment” method is believed to bind heparin covalently to the surfaces of biomaterials while preserving its anticoagulant activity. The technology is based on a chemical modification of heparin that results in the formation of a reactive aldehyde group at one end of the molecule. This aldehyde group can then be reacted with a primary amino group introduced in advance on the surface of the material. The intermediate Schiff base is reduced to a stable covalent bond (Scheme 7.5). Because it is

Scheme 7-5. Illustration of Carmeda process for the end point attachment of heparin to an aminated surface.
linked by a single bond at one terminus ("end point"), the immobilized heparin molecule may, during blood contact, extend from the surface to interact with AT. In this way, bound heparin can express its local anticoagulant activity.

7.2.4. **Heparin Activity of Modified Surfaces**

The activity of surface immobilized heparin was measured using an anti-factor Xa assay as described by Teien et al (1976). The method was modified slightly from the Diagnostica Stago protocol using the Stachrom® heparin kit. The Stachrom kit is generally used to measure the activity of heparin in solution.

To measure heparin activity, the surface was incubated with an excess (known quantity) of AT for 4 min to ensure complete conversion of heparin to AT-heparin complex. An excess of factor Xa was then added and allowed to react for 5 min. The supernatant was removed and residual factor Xa measured by reaction with the chromogenic substrate CBS 31.39 (Stago) for 1 min. After the addition of glacial acetic acid to stop the reaction, released p-nitroaniline was determined by measuring absorbance at 405 nm. The amount of heparin present in the system was taken as inversely proportional to the absorbance at 405 nm. The heparin concentration was derived from a calibration curve generated using a series of solutions of known heparin concentration (unfractionated heparin) (Figure 7.2). The procedure is shown schematically in Figure 7.3.
Figure 7.2. Calibration curve for anti-factor Xa assay of heparin modified surface. The curve was generated using a series of heparin (UFH) solutions of known concentration. Curve generated by Excel regression program.

The anti-factor Xa activity of immobilized heparin also was measured in citrated plasma using a similar method. The heparinized surfaces were incubated in plasma for 2h and then rinsed three times with distilled water before incubating with excess AT.
Figure 7.3. Schematic representation of anti-factor Xa assay for surface-bound heparin. The surface is removed from the system after incubation for 5 min in excess factor Xa solution. The unreacted Xa is measured by reaction with chromogenic substrate and the absorbance at 405 nm is measured.
7.2.5. **Protein Adsorption on Modified Surfaces**

7.2.5.1. **Protein Adsorption using Radiolabeled Proteins.**

Proteins (albumin, fibrinogen and antithrombin) were radiolabeled as described in Chapter 6. The final protein solutions for measurement of adsorption consisted of 98% unlabeled and 2% radiolabeled protein.

For single protein studies, surfaces (0.6 cm²) were exposed (96-well plates) to 250 μL of protein solution (TBS, pH 7.4) for 2 h at room temperature, rinsed three times in PBS, wicked onto filter paper to remove residual adherent buffer, and the radioactivity determined. A minimum of 4 replicates was measured for each set of conditions in a given experiment. All experiments were done in duplicate.

For protein adsorption from plasma, the radiolabeling procedure was the same. Radiolabeled protein was added at the level of 2% of the normal plasma concentration of the protein under study (albumin, 45 mg/mL; fibrinogen, 3 mg/mL; AT, 0.29 mg/mL).

7.2.5.2. **SDS-PAGE**

SDS-PAGE analysis was performed to identify proteins bound to the surfaces following plasma exposure. The experimental protocol was similar to that described in Chapter 6. SDS-PAGE also was used to investigate the coupling reaction between PEO and heparin. The procedure to make the 4% stacking gel in this case slightly different. Thus, in preparing 10 mL of 4% stacking gel, 3 mL distilled water, 1.2 mL 0.5 M Tris-HCl buffer (pH 8.8), 100 μL 10% (w/v) SDS solution with 0.55 mL of (30% acrylamide + 0.8% bisacrylamide) solution, and 0.50 mL 5% PEO-heparin solution were mixed and degassed for 15 min. The solution was mixed further with 5 μL tetramethyl
ethylendiamine (TEMED) and 50 μL freshly made 10% ammonium persulfate. After mixing, the solution was injected into the stacking layer region in the electrophoresis apparatus. Polymerization was allowed to proceed for 1 h. SDS polyacrylamide gels were run on a Mini-Protean II Dual Slab Electrophoresis Cell (Bio-Rad) at 200 V for 45 min. Samples reduced by reaction with beta-mercaptoethanol at 90°C for 5 min containing approximately 5 μg of PEO-heparin conjugate were loaded on each lane of the gel. The gels were stained using Alcian blue (Sigma-Aldrich).

SDS-PAGE and Western blotting of proteins eluted from the surfaces after exposure to plasma were carried out as described in Chapter 6.

7.3. RESULTS AND DISCUSSION

In this study, only one PEO molecular weight (higher than 600) was chosen for each of the heparinization on the discovery that protein adsorption did not change significantly if molecular weight of PEO becomes higher than 600.

7.3.1. CONJUGATION OF UNFRACTIONATED HEPARIN TO PEO

For the reaction indicated in Scheme 7.1, proton NMR was used to confirm conjugation. The spectra show the presence of PEO protons in heparin-PEO (3.7 ppm, Figure 7.4) and in the unreacted PEO (Figure 4.8) but not in the unreacted heparin (Figure 7.5). Because the purification procedure removed unattached PEO, the spectra indicate the success of the coupling reaction. For detailed assignments of the heparin protons, it is convenient to use the conventional nomenclature of saccharide ring structure as illustrated in Figure 7.6. The left structure is a typical hexuronic acid (iduronic acid or glucuronic acid) ring and the right is a typical glucosamine ring. Also, because the
molecular weight of heparin is high, only the internal structures (repeating units) are shown. Table 7.1 lists the $^1$H-NMR assignments for the protons of the internal hexuronic acid and glucosamine residues (Horne et al., 1992a; 1992b). The protons of unreacted PEO appear at 3.67 ppm and 2.68 ppm.

![Proton NMR spectrum of the product of reaction between heparin and PEO. The presence of the strong peak at 3.7 ppm attributed to PEO, indicates the formation of the conjugate.](image)

Figure 7.4. Proton NMR spectrum of the product of reaction between heparin and PEO. The presence of the strong peak at 3.7 ppm attributed to PEO, indicates the formation of the conjugate.
Figure 7.5. Proton NMR spectrum of unreacted heparin. Refer to the text for the peak assignments.

\[ R_1 = \text{H or SO}_3\text{Na}, \quad R_2 = \text{H or SO}_3\text{Na or COCH}_3, \quad n = 0 \text{ to } 20 \]

Figure 7.6. Nomenclature of saccharide rings for proton assignment. The left structure is a typical hexuronic acid (iduronic acid or glucuronic acid) ring, and the right is a typical glucosamine ring.
Table 7.1. $^1$H-NMR assignments (ppm) for the protons of the internal hexuronic acid and glucosamine residues of heparin (Horne et al., 1992a).

<table>
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<th>H-1</th>
<th>H-2</th>
<th>H-3</th>
<th>H-4</th>
<th>H-5</th>
<th>H-6,6'</th>
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<td>4.32</td>
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<td>3.85</td>
<td>3.81</td>
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<tr>
<td>Glucosamine</td>
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<td>3.29</td>
<td>3.65</td>
<td>3.82</td>
<td>4.04</td>
<td>4.26, 4.39</td>
</tr>
</tbody>
</table>

7.3.2. **PRODUCT OF REACTION OF UNFRACTIONATED HEPARIN WITH NHS-PEO-METHACRYLATE**

To determine whether heparin had reacted with PEO, SDS-PAGE was performed for the heparin-PEO which would be present in both heparin-PEO conjugate (scheme 7.1) and the heparin-PEO-methacrylate reaction product (schemes 7.3 and 7.4).

As described in the experimental section, the reaction products were present during polymerization of the acrylamide in forming the stacking gel. In this situation the PEO-methacrylate will copolymerize with acrylamide through its double bond. If heparin is conjugated to the PEO-methacrylate, therefore, it will be retained in the stacking gel region because it is covalently bound. On the other hand the simple heparin-PEO conjugate will migrate in the gel.

Figure 7.7 shows an SDS-PAGE gel of the reaction product of heparin with NHS-PEO-methacrylate at room temperature for 2h. The left lane shows the gel for the methacrylate- PEO-heparin. The presence of Alcian blue staining material in the stacking gel indicates successful coupling of heparin to the PEO-methacrylate. The right lane shows the simple PEO-heparin conjugate. Most of the stain is at the bottom of the gel, reflecting the relatively low molecular weight of the heparin-PEO conjugate.
Figure 7.7. SDS-PAGE of heparin-PEO (Alcian blue staining). Heparin-PEO conjugate (right) and Heparin-PEO-methacrylate (left).

7.3.3 CONJUGATION OF LMWH TO PEO

LMWH was conjugated to thiolated PEO by reaction at the terminal double bond (Scheme 7.2). NMR spectra of the reaction products (Figure 7.8) show signals from both heparin and PEO. It can be concluded that LMWH was successfully conjugated to PEO. It is possible, however, that some of the thiolated PEO may have reacted with LMWH at both ends. This possibility was not investigated further.
7.3.4. Properties of Heparinized Surfaces

7.3.4.1 Water Contact Angles

Figure 7.9 shows the contact angle data for gold surfaces (Si wafer substrate) before and after PEO and heparin modification. The PEO, UFH, and LMWH surfaces were found to have lower receding angles than the unmodified gold, reflecting the slightly more hydrophilic nature of the modified surfaces. Perhaps surprisingly, the heparinized surfaces appear to be somewhat more hydrophilic than the PEO surface. The
advancing angles are similar for all the surfaces, though slightly lower for the modified ones. The strong hysteresis seen for the heparinized surfaces suggests that these surfaces may undergo significant rearrangement on contact with water, perhaps via changes in heparin orientation or conformation.

![Bar chart](chart.png)

**Figure 7.9.** Water contact angle data for gold surfaces (Si wafer substrate) modified with heparin-PEO. The gold surfaces were incubated for 4 h in 5mM aqueous solutions of HS-PEO(600)-Heparin or HS-PEO(600)-LMWH. The control gold surface, AuCTRL, was subjected to the same treatment as the chemisorbed materials except that the incubation medium was distilled water. The left and right columns for each sample are the advancing and receding angles respectively. Error bars are S. D. (n=8).

Figure 7.10 shows contact angle data for the polyurethane based surfaces. The heparinized surface shows advancing and receding angles of 47° and 18° respectively, whereas the values for the PEO surface are 45° and 26°. The low contact angles on both the heparin and PEO surfaces indicate that both modifiers increase the surface
hydrophilicity. It is not appropriate to compare the PEO and PEO-heparin data since the
graft densities are not known. It appears that heparin is at least as effective as PEO in
increasing the surface hydrophilicity.

![Graph showing contact angle data for different materials.](image)

Figure 7.10. Water contact angles of modified polyurethane surfaces. Left and right
columns of each sample are the advancing and receding angles respectively. Error bars
are S. D. (n=8).

The greater contact angle hysteresis seen for PEO and PEO-heparin modified
polyurethanes indicates that upon exposure to water, the surface rearranges so that the PEO
and PEO-heparin grafts are more exposed and the polyurethane substrate is masked. In
contrast, the unmodified polyurethane substrate shows lower hysteresis, presumably because
there is less opportunity for surface rearrangement. For polyurethane, it is known that if there
is microphase separation based on hard and soft segments, the microdomains can rearrange on the surface to give hysteresis effects (Kajiyama et al., 1991).

Similar effects have been reported for poly(dimethylsiloxane)-poly(ethylene oxide)-heparin block copolymers by Grainger et al (1990).

7.3.4.2. XPS

Elemental composition data from XPS measurements are shown in Table 7.2.

Because the ratio of elements for heparin is approximately O:C:N:S = 14:12:2:1, any increase of sulfur and oxygen on the surface indicates the presence of heparin. For the gold based surfaces, sulfur was seen on the heparin-modified material. However, the oxygen content is much lower than in heparin itself. For the heparinized polyurethane surfaces the oxygen and nitrogen contents are higher than those of the substrate polyurethane, indicating the presence of significant amounts of heparin and PEO. The presence of sulfur also indicates that heparin is present on surface. The Carmeda heparinized surface shows a very high oxygen content, in fact higher than expected for a surface consisting only of heparin. The nitrogen content is also

<table>
<thead>
<tr>
<th></th>
<th>O</th>
<th>C</th>
<th>N</th>
<th>S</th>
<th>Si</th>
<th>Au</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unmodified gold</td>
<td>14.2</td>
<td>60.7</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>25.2</td>
</tr>
<tr>
<td>Au-PEO(2000)CH₃</td>
<td>23.3</td>
<td>66.0</td>
<td>-</td>
<td>0.4</td>
<td>-</td>
<td>10.3</td>
</tr>
<tr>
<td>Au-PEO-heparin**</td>
<td>14.0</td>
<td>47.1</td>
<td>-</td>
<td>4.0</td>
<td>-</td>
<td>34.9</td>
</tr>
<tr>
<td>Polyurethane</td>
<td>14.9</td>
<td>78.6</td>
<td>5.4</td>
<td>-</td>
<td>1.1</td>
<td>-</td>
</tr>
<tr>
<td>PU-PEO-heparin**</td>
<td>20.1</td>
<td>74.6</td>
<td>4.0</td>
<td>1.3</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CBAS</td>
<td>11.1</td>
<td>0.9</td>
<td>87.2</td>
<td>0.9</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Precision of data: ±5%
*CBAS = Carmeda heparinized polyurethane
**Mixed PEO, PEO-heparin surfaces
unexpectedly high. This may indicate that the Carmeda surface, although putatively polyurethane based, may be covered with a modification layer of higher nitrogen content, such as polyalkylamine (Wendel et al., 1999).

7.3.5. ANTI-FACTOR XA ACTIVITY OF HEPARINIZED SURFACES

Data on heparin activity associated with the surfaces are shown in Table 7.3. The various surfaces exhibit different activities, presumably reflecting the different immobilization chemistries and the type of heparin used (unfractionated or LMWH). For gold surfaces modified with LMWH, the heparin activity was estimated to be 0.008 IU/cm², corresponding to a graft density of ~ 58 ng/cm² assuming 140 IU/mg LMWH (Berry et al., 2000). This is much lower than the density expected if there were complete occupancy of all thiol binding sites on gold (0.76 nmol/cm² or 15.3 µg/cm² for gold (111)). The gold surface modified with UFH showed heparin activity of 0.012 IU/cm², similar to that of the LMWH modified surface.

Table 7.3. Surface heparin activity and graft density on heparinized surfaces

<table>
<thead>
<tr>
<th>Surface</th>
<th>Heparin activity (anti-factor Xa IU/cm²)</th>
<th>Graft density (µg/cm²) *</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Surface as prepared</td>
<td>Surface after plasma incubation</td>
</tr>
<tr>
<td>Gold-PEO-LMWH</td>
<td>0.008 ± 0.005</td>
<td>0.010 ± 0.008</td>
</tr>
<tr>
<td>Gold-PEO-UFH</td>
<td>0.012 ± 0.003</td>
<td>0.010 ± 0.006</td>
</tr>
<tr>
<td>PU-PEO-UFH</td>
<td>0.041 ± 0.007</td>
<td>0.036 ± 0.013</td>
</tr>
<tr>
<td>CBAS</td>
<td>0.095 ± 0.011</td>
<td>0.082 ± 0.015</td>
</tr>
</tbody>
</table>

*Assuming 140IU/mg for LMWH and 180 IU/mg for UFH. Data are given as mean ± S.D (n=4)

Because the heparin–PEO molecule is big relative to the thiol binding site, it is highly likely that it effectively “occupies” more than one such site. Furthermore, the
PEO conformation in PEO-heparin may be such that the reaction of the terminal thiol group of PEO with the binding site on gold is sterically hindered, resulting in a relatively low heparin graft density.

For the PU based surface, the heparin activity was 0.04 IU/cm², i.e. significantly higher than for the gold surface, suggesting that the heparin modification of PU may be more efficient. For comparison, the PU based Carmeda bioactive surface (CBAS) was also investigated. This material showed significantly higher heparin activity than either the gold or polyurethane materials developed in this work, corresponding to a graft density of 30 pmol of heparin/cm². This is in agreement with heparin densities in the range of ~ 20 to 100 pmol/cm² reported for these materials (Sanchez et al., 1997).

Interestingly, the heparin activity on these surfaces did not decrease significantly after 2 h incubation at room temperature in plasma, indicating that under these conditions, heparin activity was retained. Considering that heparinized surfaces have been shown to lose their activity rapidly when implanted in the circulatory system, due either to heparin leaching or adsorption of proteins which mask the heparin (Conrad, 1997), this result is noteworthy. As will be shown below, the heparinized surfaces developed in this work certainly absorb considerable amounts of protein when exposed to plasma, thus suggesting that the heparin can exert its effect in the presence of this protein layer.

7.3.6. **Protein Adsorption**

To investigate whether the PEO-heparin modified surfaces are able to reduce nonspecific protein adsorption, adsorption of fibrinogen and albumin from buffer were measured. Figures 7.11 and 7.12 show typical data for the heparinized gold surfaces.
Increases in adsorption for both fibrinogen and albumin were observed for the heparin versus the PEO surface. However adsorption on both the PEO and PEO-heparin surfaces was lower than on the unmodified gold (see Chapter 6).

Figures 7.13 and 7.14 show typical fibrinogen and albumin adsorption data for the heparinized PU surfaces. As for the gold based surfaces, the presence of heparin increases the adsorption of both fibrinogen and albumin relative to the PEO surface. Heparin is a highly negatively charged species and may enhance the adsorption of a variety of proteins by electrostatic interactions. It is not expected to be protein repellent. Consequently, protein adsorption on these surfaces is expected to depend on the ratio of PEO to heparin. Possibly

\[ \text{Figure 7.11. Fibrinogen adsorption to Au-PEO/Au-PEO-heparin and Au-PEO surfaces (silicon wafer base). Adsorption was from fibrinogen in TBS buffer at room temperature for a time of 2h. Error bars are SD (n=3).} \]
because the heparin densities are relatively low (molar ratio of PEO to PEO-heparin 5:1, Figure 7.2), the heparin appears to have little effect on protein adsorption. The main effect presumably comes from the PEO. Protein adsorption on surfaces having different heparin contents may be required for further understanding of the mechanisms of interaction of proteins with these surfaces.

Figure 7.12. Albumin adsorption to Au-PEO/Au-PEO-hep and Au-PEO surfaces (silicon wafer base). Adsorption was from albumin in TBS buffer at room temperature for a time of 2h. Error bars are SD (n=3).
Figure 7.13. Fibrinogen adsorption to polyurethane modified with heparin and PEO. Adsorption was from fibrinogen in TBS buffer at room temperature for a time of 2h. Error bars are SD (n=3).

Figure 7.14. Albumin adsorption to polyurethane modified with heparin and PEO. Adsorption was from albumin solutions in TBS buffer at room temperature for a time of 2h. Error bars are SD (n=3).
The quantities of protein adsorbed at the highest protein concentrations used are summarized in Table 7.4. These data correspond more or less to saturation of the surface based on the concentration dependence as seen in Figures 7.11 – 7.14. Clearly, the heparinized surface shows lower protein adsorption than the corresponding control surface but higher than the PEO modified surface.

Table 7.4. Protein adsorption to different surfaces: saturation levels (μg/cm²).

<table>
<thead>
<tr>
<th></th>
<th>Au</th>
<th>Au-PEO</th>
<th>Au-PEO-heparin</th>
<th>PU</th>
<th>PU-PEO</th>
<th>PU-PEO-heparin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fibrinogen (buffer)</td>
<td>0.32</td>
<td>0.06</td>
<td>0.14</td>
<td>0.67</td>
<td>0.16</td>
<td>0.30</td>
</tr>
<tr>
<td>Albumin (buffer)</td>
<td>0.43</td>
<td>0.06</td>
<td>0.10</td>
<td>0.31</td>
<td>0.07</td>
<td>0.10</td>
</tr>
</tbody>
</table>

7.3.6.1. ADSORPTION OF ANTITHROMBIN

If heparin is able to catalyze the inactivation of thrombin by antithrombin, antithrombin must bind to heparin. To be active on a surface, the heparin molecules must be in a conformation that favors their interaction with AT (Jackson, 1990; Eriksson et al., 1967; Gott et al., 1989; Grode et al., 1969; Labarre et al., 1977; Miyama et al., 1977). Therefore, it was of interest to investigate antithrombin adsorption to the heparinized surfaces.

Figure 7.15 shows antithrombin adsorption to the different surfaces from plasma. The CBAS surface shows the highest adsorption. The PU-heparin and PU-PEO surfaces developed in this work show much lower adsorption with values not significantly different from each other. The small amount adsorbed on the PU-PEO surface is presumably bound nonspecifically, suggesting that on the PU-heparin surface, it is the difference between PU-PEO and PU heparin that is bound via interaction with AT. This amounts to about 0.005 μg/cm². It is more difficult to correct for nonspecific adsorption
in the case of the CBAS surface since we do not have data for the control surface without heparin. If we assume that all the heparin is bound to CBAS via AT interactions then the graft density of heparin can be estimated at about 1 pmol/cm². By comparison with the antifactor Xa assay, which gave a functional heparin graft density of 10 pmol/cm², it appears that only one out of 10 heparins on the surface that has anti-factor Xa activity is able to bind AT. Similarly, for the PU-heparin surface the graft density from the antifactor Xa assay is about 0.4 pmol/cm² and from the AT adsorption about 0.05 pmol/cm² again suggesting that adsorption of AT from plasma does not provide an accurate measure of heparin activity.

Figure 7.15. Antithrombin adsorption on heparinized polyurethane based surfaces from plasma. Adsorption time 2h. Error bars are S.D. (n=3).
7.3.7. Western Blot Analysis of Proteins Adsorbed after Plasma Contact

Western blot analysis of proteins eluted from different surfaces after exposure to plasma was performed. The data are shown in Figures 7.16 to 7.18. In general, the protein deposition patterns are complex. Most of the proteins tested for on the different surfaces were found to be present. However, there were differences in the relative amounts of the proteins adsorbed on the different surfaces. In some cases both intact proteins and fragments were found.

![Western blot image](image-url)

Figure 7.16. Western blot of proteins eluted from gold surface (silicon wafer base) modified with HS-PEO (MW 600) and HS-PEO-heparin (PEO MW 600) after plasma contact.
Generally, the protein adsorption patterns on the CBAS and heparinized PU surfaces are similar, indicating that the same proteins are adsorbed on both types of surface. This is not unreasonable since both are heparinized surfaces. Because experiments were carried out using the same volume of SDS solution to elute surfaces of the same areas, the stain intensities reflect the relative amounts for a given protein.

Figure 7.17. Western blot of proteins eluted from PU surface modified with HS-PEO (MW 600) and HS-PEO-heparin (PEO MW 600) after plasma contact. The lane at the extreme left is the gold stained SDS-PAGE gel.
Figure 7.18. Western blot of proteins eluted from the Carmeda heparinized polyurethane surface (CBAS) after plasma contact. The lane at the extreme left is the gold-stained SDS-PAGE gel.

In general fewer proteins gave positive responses on the heparinized gold than on the PU-based surface. This may reflect the fact that elution from this surface is more difficult as discussed in chapter 6. Also, all three surfaces adsorbed significant amounts of AT, but CBAS adsorbed particularly large amounts. This result is in agreement with the $^{125}$I-AT experiments (Fig 7.15).

Small amounts of the intact contact factors prekallikrein, factor XI and factor XII were found on the CBAS surface. On the heparinized polyurethane surface, only
prekallikrein was found and on the heparinized gold surface only factor XII was present. It would be expected, however, that if heparin coverage is extensive, there would be little difference between these two surfaces. Therefore the blot data provide further evidence of differences in heparin density on these surfaces. Assuming that the contact system is activated, fragments of the contact factors should be present. The absence of some of these factors in the eluates may result from the fact that the factors and their activation fragments are adsorbed on the surface and then released. Alternatively they may be strongly bound and not eluted by SDS. A possible way to address this question would be to examine the surfaces immunohistologically, that is, probe the surface directly with antibodies directed against the protein of interest and then to a second fluorescent antibody.

Antithrombin is of strong interest for these heparinized surfaces since the anticoagulant activity of heparin is mediated by this protein. Antithrombin is a serine protease inhibitor that inhibits thrombin and factor Xa and, to a lesser extent, factors IXa, XIa, XIIa, tPA, urokinase, trypsin, plasmin, and kallikrein (Menache, 1991; Lahiri et al., 1976). It has a molecular weight of approximately 58 kDa and is normally present in the plasma at concentration of 140 to 300 µg/mL (Rosenberg et al., 1986). As already mentioned, large amounts of AT are eluted from the CBAS surface, reflecting the ability of heparin on that surface to bind antithrombin. Two AT bands are apparent, and these are presumably the alpha and beta forms of antithrombin. The heparinized polyurethane and gold surfaces show smaller amounts of antithrombin, reflecting the lower concentrations of heparin on these surfaces.
Fibronectin and vitronectin (Ginsberg et al., 1988; Ill et al., 1985) are adhesive proteins for cell-cell or cell-solid surface interactions. Along with fibrinogen, they are known to mediate platelet adhesion to blood contacting surfaces (Gawaz et al., 1997). Fibronectin is a dimeric glycoprotein, with subunit polypeptides of 200 kDa. Both intact and fragmented fibronectin were found on the CBAS surface. Less intact fibronectin and no fragmented fibronectin were found on the heparinized polyurethane surface. No fibronectin related bands were present on the heparinized gold surface. Vitronectin, with a molecular weight of ~70 kDa (Preissner et al., 1986), was found on CBAS and the heparin-modified polyurethane surface, but not on the heparin-modified gold surface.

Adsorption of albumin to artificial surfaces is known to have a passivating effect on blood response, probably due to a reduction in platelet adhesion. All three heparinized surfaces show strong bands at 66 kDa representative of the intact molecule while CBAS and the heparinized polyurethane surface show several faint lower molecular weight bands indicating cleavage products.

IgG, and C3 are present on all three of the surfaces investigated, with the heparinized gold surface showing the weakest C3 response. The C3 blots for all three surfaces suggest some activation of the complement system since the activation fragment at ~ 45 kDa is present. The effect is particularly noticeable on the CBAS surface. Factors B, H and I antibodies also gave stronger responses on the CBAS and the heparinized polyurethane surfaces than on the heparinized gold surface.

7.4. SUMMARY AND CONCLUSIONS

Methods for conjugating heparin and low molecular weight heparin to PEO have been developed, and gold and polyurethane surfaces have been modified with PEO-
heparin conjugates. To attach low molecular weight heparin to PEO, Enoxaparin® was used because it contains a double bond in the terminal saccharide unit. A free radical initiator was used to initiate the reaction of the thiol terminus of HS-PEO with the heparin double bond. PEOs were functionalized with N-hydroxysuccinimide (NHS) at the chain ends and reacted with unfractionated heparin. These conjugates were used for gold surface modification. A three-step procedure was used to coat polyetherpolyurethane films with unfractionated heparin.

The modified surfaces were characterized by water contact angle measurements. The advancing angles for the heparinized gold surfaces were essentially the same as those of the PEO modified gold. The heparinized gold showed strong contact angle hysteresis, suggesting that heparin on the surface may undergo changes in conformation and orientation on contact with water. For the polyurethane-based materials, the heparinized surface showed a lower advancing angle than the PEO surface. The receding angle for the heparinized surface was lower than that for the PEO-PU, indicating that heparin, as a polysaccharide with substantial negative charge, causes an increase in surface hydrophilicity.

XPS measurements showed that the procedures used for heparinization of gold were successful. However the coverage appeared low based on the sulfur content. Similar conclusions apply to the polyurethane-heparin surfaces.

Anti-factor Xa assays were used to evaluate the functional activity of immobilized heparin on the different substrates. On the gold substrate the data showed, in agreement with the XPS, that the heparin density was low. It is suggested that HS-PEO-heparin chemisorption is limited by steric effects and the relatively large size of the PEO-heparin
conjugate. The anti factor Xa activity of the polyurethane-bound surface was significantly greater than that of the heparinized gold, suggesting that the reactions used for the heparin modification of PU may be more effective. The heparin activity on these surfaces did not decrease significantly after 2 h incubation at room temperature in plasma, indicating that heparin activity was retained despite the fact that a layer of protein was adsorbed to the surface.

Investigation of the adsorption of fibrinogen and albumin from single protein solution in buffer showed that the heparinized surfaces, with PEO present as a "passivating" agent, adsorbed less protein than the unmodified surfaces (gold or polyurethane). These results suggest that nonspecific protein adsorption on these surfaces when in contact with blood would be relatively low. The heparinized surfaces adsorbed slightly more protein than the corresponding PEO surfaces without heparin.

Experiments were also conducted to measure the adsorption of antithrombin from buffer and plasma. A commercially available heparinized polyurethane surface (CBAS, Carmeda) as well as the heparinized surfaces developed in this work was examined. However from plasma, where AT adsorption presumably reflects specific interaction with heparin, the adsorbed amounts were considerably lower. The Carmeda surface adsorbed more than the heparinized gold or heparinized polyurethane, indicating a higher heparin content.

Western blot analysis of proteins adsorbed from plasma to the various surfaces was also performed. In general, the protein deposition patterns were complex. Most of the proteins tested for on the different surfaces were found to be present. However, there were some differences in the compositions of the proteins on the different surfaces.
Antithrombin was seen on all the heparinized surfaces. The Carmeda surface showed a particularly strong AT response, in agreement with the experiments using radiolabeled AT. Indications of complement activation were also evident on the Western blots, with the appearance of a band at ~ 45 kDa in the C3 blot. This effect was stronger on the CBAS surface than on the heparinized gold and polyurethane surfaces.
CHAPTER 8

SUMMARY AND CONCLUSIONS

Polyethylene oxide on a surface has been shown by several research groups to have a protective effect against protein adsorption. The effect is nonspecific and in general proteins are believed to be more or less equally repelled from the surface. In principle, therefore, a PEO-modified surface would be expected to prevent the adsorption of desirable and undesirable proteins alike. This difficulty can be overcome by purposely introducing functional groups or ligands into PEO that attract or stimulate the functions of the desirable proteins. The work described in this thesis is intended to investigate the protein adsorption characteristics of PEO or PEO-ligand modified surfaces. Heparin was chosen as the ligand, and gold and polyurethane as substrate surfaces for attachment of PEO. Gold can be viewed as a model surface to which HS-PEO can be attached by chemisorption, while polyurethane can be regarded as a practical biomaterial with excellent mechanical properties and a considerable history of use in blood contacting devices. Polymer synthesis, surface modification and characterization, protein adsorption, and functional assays are included in this study, along with method development and evaluation wherever necessary.

Passivation and functionalization of gold surfaces with thiolated PEOs and derivatives were exploited as a strategy for blood compatible biomaterials. Methods for the synthesis and characterization of thiolated PEO or PEOs with a thiol group at one end and a bioactive group at the other end were developed. Modification of gold surfaces
with the synthesized PEOs and characterization of the modified surfaces were also carried out. Evaluation of the surfaces for blood compatibility was based on determining protein interactions using radiolabelled proteins and gel electrophoresis-Western blotting methods.

Methods for chain-end thiolation of linear and star-type PEOs having different molecular weights and end groups, and methods for chain end (opposite) conjugation of amino acids and peptides to HS-PEO were developed. For thiolation of PEO, esterification of HO-PEO with mercaptoacetic acid was found to be suitable. NMR and IR spectroscopy confirmed the occurrence of these reactions. A kinetic study suggested that the self-condensation of mercaptoacetic acid, a possible side reaction, did not occur to any significant extent. When \( \alpha, \omega \) dihydroxy PEO and star-type PEO were subjected to thiolation using mercaptoacetic acid, the distribution of thiol groups on the PEOs were in agreement with theoretical values based on statistical considerations. Activation of HS-PEO with N-hydroxysuccinimide was used as a preliminary to the conjugation of amino acids and proteins. Glycine methyl ester, lysine, t-BOC-lysine and unfractionated heparin were successfully conjugated to HS-PEO using this method. The reactions were confirmed by NMR and GPC.

Linear and star-type PEOs of different molecular weights, thiolated in this study, were used to modify gold surfaces by chemisorption. Water contact angle, XPS, and PEO surface density measurements, as well as atomic force microscopy, were used to investigate the properties of the modified gold. The PEO modified gold surfaces were shown to have lower contact angles and to be more stable when incubated in saline solution than the unmodified surfaces, presumably due to the hydrophilicity and the
inertness of PEO respectively. XPS revealed that the thiol of HS-PEO was bonded to the gold surface, although AFM images did not show any detectable morphological difference between modified and unmodified samples. A method for measuring the surface density of PEO using radiolabelled PEO was developed. Data obtained using this method indicated that coverage of the gold surfaces with PEO was typically rather sparse when compared to the theoretical density based on complete occupancy of thiol binding sites. Coverage decreased with increasing molecular weight of the PEO, suggesting steric hindrance to the approach of PEO molecules to the surface as the sites become occupied.

Protein adsorption from solutions of single proteins (albumin and fibrinogen) in buffer or from plasma to control and PEO modified surfaces was studied using radiolabelled proteins. Inclusion of excess cold iodide ion in the buffer was developed as a method to suppress the uptake of free radioactive iodide (which otherwise introduces massive error into the measurement). Adsorption was found to decrease with increasing molecular weight of PEO. PEOs with hydroxyl end groups adsorbed much less protein than methoxy terminated PEOs. Star-type PEOs did not show significant differences from their linear counterparts, suggesting that the architecture of PEO did not influence the adsorption of the proteins studied. It is therefore believed that PEO is effective in passivating surfaces and protein adsorption can likely be adjusted by “tailoring” the PEO chain length and outer surface properties. It was found unexpectedly that PPO modified surfaces showed low protein adsorption levels, similar to PEO modified surfaces.

Adsorption from plasma was also studied by adding radiolabelled proteins to the plasma as tracers. The effects of PEO and PPO on fibrinogen and albumin adsorption
from plasma were found to be similar to those seen in buffer systems. Fibrinogen adsorption from plasma was reduced on the modified surfaces and the Vroman effect was eliminated. Studies of adsorption from plasma using Western blotting methods showed very little uptake on the PEO modified gold surfaces. However, for the contact phase proteins of the coagulation system, the molecular weight distributions apparent in the immunoblots suggested that activation of the coagulation pathways occurs on many of the surfaces studied.

Methods for chain end conjugation of heparin to thiolated PEOs, and modification of gold surfaces with the resulting HS-PEO-heparin were also developed. Both low molecular weight heparin and unfractionated heparin were used. For the conjugation of low molecular weight heparin (Enoxaparin™) to PEO, a radical reaction of thiol groups with the double bond of Enoxaparin was used. For unfractionated heparin, PEO was activated with N-hydroxysuccinimide (NHS) prior to use in the conjugation reaction between the activated PEO and heparin in the presence of cystamine. The advancing water contact angles of the heparinized gold surfaces were not significantly different from those of the precursor PEO modified gold surfaces. Significant contact angle hysteresis was observed, suggesting that heparin on the surface may undergo a major re-organization upon contact with water such that the surface becomes more hydrophilic. XPS data for the heparinized gold surface, showed, as expected, that the gold was overlaid with PEO and heparin. However, the nitrogen and sulfur contents were relatively low, suggesting a relatively low content of heparin on the gold surface. Anti-factor Xa assays, providing a measure of the content of active heparin, were also performed on these surfaces. The data confirmed the difficulty of achieving high heparin
surface density using the HS-PEO-heparin chemisorption approach. This result may indicate that the complex, which is large relative to the PEO used in this study, effectively “occupies” multiple thiol binding sites or that steric effects are even more severe than for the HS-PEO.

Passivation and functionalization of polyurethane (PU) with heparinized PEO was also investigated as a strategy for blood compatible biomaterials. As for the gold surface, HS-PEO-heparin synthesis, surface characterization, protein adsorption and functional assays were carried out. PU films were incubated in a mixture of monomers including NHS-poly(ethylene oxide) methacrylate and heated to 70°C to initiate polymerization. The surfaces were then immersed in aqueous heparin. SDS-PAGE gels and NMR spectra indicated the success of the syntheses. It was found that the heparinized surface had a lower advancing water contact angle than a PU surface; more significantly, the receding angles of the heparinized surfaces were not only lower than PU surface, but also lower than those of the PEO-PU, indicating that immobilization of heparin, a polysaccharide chain with negative charges, leads to strongly hydrophilic surfaces.

The heparinized PU showed greater anti-factor Xa activity than the heparinized gold surface, suggesting that the reactions for heparin modification of PU may be more efficient. Also, the heparin activity did not decrease significantly after a 2 h incubation at room temperature in plasma, indicating that heparin activity was retained despite the presumed adsorption of a layer of protein. It is possible that AT is a major component of the layer, and that this accounts for the retention of activity.

Single protein adsorption (fibrinogen, albumin) revealed that the heparinized surfaces, with PEO present as a co-passivating agent, appear not only to promote AT
adsorption, but also to reduce nonspecific protein adsorption in contrast to the unmodified PU surface.

A commercial heparinized surface from Carmeda (CBAS) was used for comparison to the experimental surfaces developed in this work. The Carmeda surface has no PEO or other passivator. Non-specific adsorption of proteins on this surface was found to be extensive, caused most likely by the cationic nature of the substrate and no passivation effect took place. In contrast, CBAS has strong anticoagulant properties as indicated by extensive AT adsorption from plasma and high antifactor Xa activity.

Western blot analysis of proteins adsorbed from plasma onto the different heparinized surfaces showed that in general the protein deposition patterns are complex. Most of the proteins tested for on the different surfaces were found to be present. However, there were differences in the relative amounts of the proteins on the different surfaces. As expected for heparin containing surfaces, AT was clearly adsorbed, but was strongest on the CBAS surface, presumably due to the relatively high surface density of heparin, and to the fact that so-called end-point attachment preserves the AT binding pentasaccharide sequence on this surface. With regard to complement activation (assessed by the appearance of a fragment at MW 45 kDa in the blot of C3) the data are inconclusive, although activation appears to be more extensive on the CBAS surface than on the other heparinized surfaces prepared in this work.

In conclusion, this research suggests the advantages of both passivation and functionalization of surfaces to improve bloodcompatibility.
Suggestions for Future Work

The measurements of PEO density on the gold surfaces indicated that coverage is far from 100% of the total thiol binding sites, and it was found that coverage decreased with increasing PEO molecular weight, suggesting steric hindrance in the chemisorption reaction. More comprehensive studies of PEO coverage may be helpful in understanding the configuration of PEO on these surfaces, and may provide valuable information in revealing the relationship between surface density of PEO and protein repellency.

Western blotting provides important information on proteins eluted from a blood-contacting surface. However, because some proteins are not elutable from the surface using SDS, this approach probably does not provide complete information on protein adsorption profiles. In addition, the spatial distribution of proteins on the surface cannot be obtained by this method. Such information is crucial to understand the performance of medical devices, especially those with complicated geometry. A method with the capability of determining the quantity and location of proteins on a surface after contact with blood should be of great benefit in future studies. One possibility is to combine ELISA (enzyme linked immunosorbent assay) and histotechnology. By providing antibodies with fluorescence, peroxidase, phosphatase or other activity, and by applying proper fixing, embedding and staining methods, proteins, cell populations and cell product distributions (protein and cytokines) could be characterized.

In studying the sorption of thiolated PEO on gold surface, the variability of graft density and its dependence on the molecular weight of PEO casts some doubt on the commonly cited strength of the thiol-gold bond. Although it is claimed that the bond strength is around 30 kcal/mol, it is not clear that this value is applicable in the case of
HS-PEO. It might be possible to increase the effective bond strength by introducing multiple thiol groups as well as hydrophobic anchors (e.g. amphiphilic triblock copolymers).

A biomaterial is not blood compatible without the ability to prevent platelet adherence and activation. Thus, it is obvious that studies of platelet interactions with the surfaces developed here should be undertaken. From then on, testing of these surfaces in plasma and whole blood and in animal models may allow us to understand more about factors in materials and blood that affect blood compatibility, as well as lead to improved surface properties.

As discussed in Chapter 2, ATH is superior to heparin in several respects. Attachment of ATH to an artificial surface may improve its blood compatibility significantly. Especially, the presence of antithrombin increases the type of possible linkages for grafting ATH onto the surface while leaving the heparin portion unmodified. For example, the method developed in this thesis for attaching heparin to polyurethane surface would be more easily applied to the ATH complex because amino groups are more accessible and numerous. CBAS surface may serve as an excellent control surface to evaluate the significance of introducing ATH into artificial surfaces.

Considerable potential exists to exploit the conjugates of structure HS-PEO-peptide (and HS-PEO-amino acid) developed here. Cell adhesion peptides, direct anti thrombin inhibitors (eg PPACK) and thrombolytic agents are examples that might prove to be useful in improving blood compatibility.
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