PEPTIDE MODIFIED GOLD COATED POLYURETHANE SURFACES AS THROMBIN SCAVENGERS

by

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ABSTRACT

Gold, as a chemically inert metal, does not form a stable oxide, but has strong specific interactions with sulfur functions. It has been found that thiols or disulfides can chemisorb to gold under mild conditions (room temperature), and form densely packed monolayers on the gold surface due to the high density of binding sites (gold atoms). Thus, it is possible to form closely packed and stable monolayers of thiolates containing desirable bioactive moieties. Thiol-gold chemistry may therefore be considered as a potentially important tool in the surface modification of materials for biomedical applications.

In order to develop surfaces with antithrombogenic properties, a number of thrombin inhibitors (heparin, hirudin and PPACK) have been bonded or immobilized to the material surfaces. In the present study, a series of short peptides, cys-pro-arg (CPR), cys-phe-pro-arg (CFPR) and cys-(D)-phe-pro-arg (C[D]FPR), analogues of PR and FPR respectively, were chosen as potential thrombin inhibitors to attach to gold-coated polyurethane surfaces via the cysteine residue. Their inhibitory activity against thrombin was verified by a chromogenic substrate assay. C(D)FPR, showed a relatively high level of inhibition activity.

The surfaces were characterized by water contact angle, XPS, AFM, SEM, ellipsometry, and infrared reflection-absorption spectroscopy, and the adsorption of thrombin from buffer and modified plasma was investigated. It was found that the

peptide modified gold surfaces adsorbed significantly more thrombin than the unmodified control surfaces. The C(D)FPR-modified gold surface showed the highest thrombin adsorption both from buffer and plasma. This results is in accord with previous studies showing that the D form of phenylalananine in the FPR peptide creates a favourable site geometry for binding to thrombin. The activity of thrombin adsorbed on these peptide modified gold surfaces was also investigated using a chromogenic substrate assay. Inhibition of adsorbed thrombin was demonstrated, and the C(D)FPR surface showed the strongest inhibitory activity.

The presence of thrombin on the peptide surfaces following exposure to modified plasma was verified by elution of proteins and identification of thrombin in the eluate. Probing of the eluates with antibodies to 25 plasma proteins showed that the peptide surfaces are relatively non-adsorptive, suggesting they have some degree of selectivity for thrombin binding.

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LIST OF ABBREVIATION

ABE	anion-binding exosite
AFM	Atomic Force Microscopy
APTT	activated partial thromboplastin time
Asp	Aspartate
ATIII	antithrombin III
BCIP	5-bromo-4-chloro-3-indolyl phosphate
C(L)FPR	Cysteine-(L)-Phelylalanine-Proline-Arginine
C(D)FPR	Cysteine-(D)-Phelylalaine-Proline-Arginine
CPR	Cysteine- Proline-Arginine
Cys	Cysteine
DMF	dimethylformamide
DMSO	dimethylsulfoxide
ED	ethylene diamine
gpIIb/IIIa	glycoprotein IIb/IIIa
gp Ic/IIa	glycoprotein Ic/IIa
GPC	gel permeation chromatography
His57	Histidine
HMWK	high molecular weight kininogen
Ile	Isoleucine
FT-IRAS	Fourier transform infrared reflection-absorption Spectroscopy
Leu	Leucine
MDI	4,4'-diphenylmethane diisocyanate
MPA	3-mercaptopropionic acid
NBT	nitroblue tetrazolium
PBS	phosphate buffer saline

PGI_2	prostaglandin I_2
Pro	Proline
Phe	Phelylalanine
PGE1	prostaglandin E_1
PEG	polyethylene glycol
PEO	polyethylene oxide
PMA	polymethacrylate
pNA	p-nitroaniline
PPACK	D-Phe-Pro-Arg chloromethyl ketone
PTMO	polytetra methylene oxide
PVC	Polyvinyl Chrolor
PVDF	polyvinylidene fluoride
SAMs	self-assembled monolayers
SDS-PAGE GEL	SDS-Polyacrylamide Gel Electrophoresis
SEM	Scanning electron microscopy
Ser	Serine
TCA	trichloroacetic acid
Trp	Tryptophan
vWF	von Willebrand factor
XPS	X-ray photoelectron spectroscopy

1. INTRODUCTION

As defined by Lelah and Cooper (1986), biomaterials are "artificial materials that are designed for or used in biomedical applications. Biomedical materials include metals, ceramics, natural and synthetic polymers, and combinations and composites of these materials. Biomaterials are used in contact with living tissue, resulting in an interface between tissue and biomaterial. The nature of the interactions at this interface will therefore determine the compatibility of the material with the biological environment in which it is placed (Klinkmann, 1983). The major properties of a biomaterial that need consideration are its material characteristics, mechanical behaviour, processability, and interactions with the biological environment.

1.1 Blood- material Interactions

As medical technology develops, biomaterials are being used more extensively in blood contacting devices including hemodialysers, access devices for dialysis, cardiopulmonary bypass systems, circulatory support devices, artificial valves, artificial hearts, artificial bypass grafts in large- and small-diameter vessels, apheresis equipment, and vascular catheters (Ratner, 1993). The response of blood to contact with an artificial surface has been subjected to numerous investigations, and the nature of these processes is beginning to be understood.

The response of blood to a biomaterial surface can be viewed (Figure 1.1) as beginning with protein adsorption, followed by the interaction of platelets, activation of intrinsic coagulation, participation of the fibrinolytic and complement systems, and the interactions of erythrocytes and leukocytes (Courtney, 1994). This simplified view provides a basis for the identification of features relevant to evaluating the influence of a biomaterial on blood.

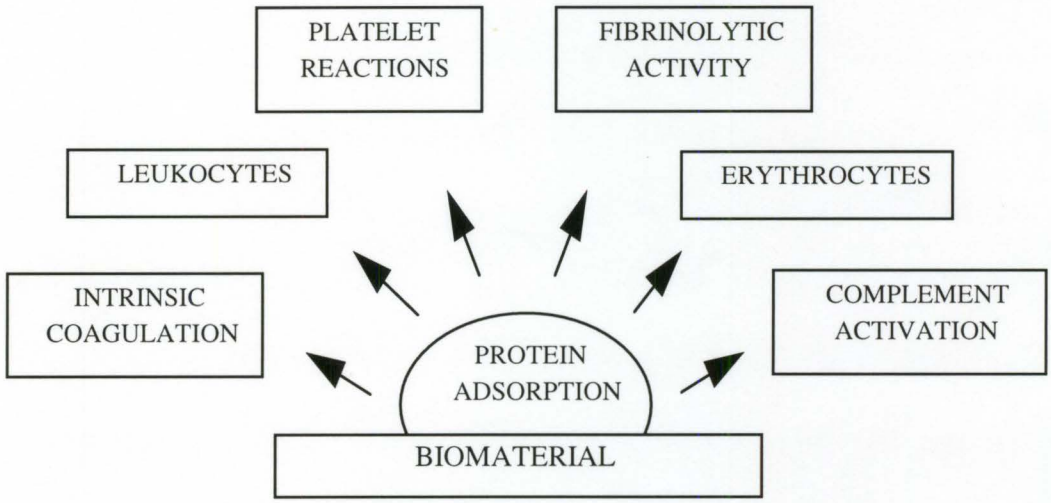


Figure 1.1 Response of blood to a biomaterial: general features. (Courtney, 1994).

The coagulation protein thrombin plays a central role in blood coagulation and thrombogenic processes (Fenton, 1986). Thrombin not only exhibits coagulant activities such as mediation of the fibrinogen-fibrin conversion, and activation of factors V, VIII, XI and XIII (Mustard and Packham, 1970; Hirsh et al, 1991), but also initiates the anticoagulant pathway via the activation of protein C (Esmon, 1987; Fenton, 1992). Furthermore, it initiates several cellular and vascular reactions including platelet

aggregation (Packham 1988; Bennett, 1992), endothelial cell proliferation (Coughlin 1994), and plasminogen activator release from the vessel wall (Coughlin 1994). Since the enzymatic activity of thrombin is essential in most of these processes, inhibition of thrombin has become a major concern in the development of blood compatible materials.

1.2 Antithrombogenic Agents Used in Biomaterials Development

The most widely used antithrombotic agent is the anticoagulant heparin. Heparin is a glycosaminoglycan composed of alternating glucosamine and uronic acid residues. In addition to inhibiting thrombin and thrombin-mediated activation of factors V and VIII, the heparin-ATIII complex inhibits other serine proteases in the clotting cascade including factors IXa, Xa, XIa, and XIIa (Scharfstein and Loscalzo, 1992). The antithrombotic effect of heparin is mainly mediated by antithrombin III (ATIII), and partially by heparin cofactor II. ATIII is a single chain glycoprotein which inactivates thrombin through the binding of its Arg-reactive site to the active site of thrombin, forming a 1:1 stoichiometric complex (Householder, 1991). However, this reaction is relatively slow in the absence of heparin. When heparin is present, it binds to the lysine-binding site on ATIII and causes a conformational change in ATIII which accelerates the ATIII-thrombin interaction (Householder, 1991).

Although heparin is an effective and widely used anticoagulant, several limitations and serious problems have been revealed during long term clinical applications. Heparin binds to several plasma proteins, such as fibronectin, vitronectin,

and von Willebrand factor (vWF). It is effectively neutralized by platelet factor 4 which is released by activated platelets (Johnson, 1994). The most serious limitation of heparin is that the heparin-ATIII complex inhibits only soluble thrombin, but has little effect on fibrin-bound thrombin, possibly due to the heparin binding site being blocked when the enzyme is bound to fibrin (Rubens et al, 1993; Weitz and Hirsh, 1993). Likewise, it has been shown that thrombin bound to the extracellular matrix is also protected from inactivation by heparin-ATIII complex. The ATIII-independent inhibitors, such as hirudin and PPACK have been found to be effective in inactivating clot-bound thrombin because their binding sites are not masked by thrombin binding to fibrin (Weitz, 1990).

Heparin has been used as an antithrombotic agent attached to material surfaces by different methods, and the antithrombogenic effects of these materials have been investigated in vivo and in vitro. Many research groups have demonstrated release of heparin from various polymer surfaces, including Teflon (PTFE; Plate et al, 1984), polyvinyl alcohol (Sefton, 1983), polystyrene (Sefton et al, 1978) and polyurethanes (Heyman et al, 1985). The surfaces showed potent antithrombotic activity as long as heparin was being released. Unfortunately, after a period of time, the heparin is exhausted; thus such biomaterials are suitable only for short-term applications. Heparin has also been covalently bound to suitable polymers. Some of these materials appeared to have antithrombogenic activity in vitro and some in vivo models (Sefton et al, 1981). However, the surface-bound heparin has also been shown to activate platelets (Larson et al, 1980). Various platelet adhesion and aggregation inhibitors (e.g. prostaglandin E_1

[PGE1]; Jacob, et al, 1986) or platelet-inert molecules conjugated with heparin (e.g. albumin, Hennick et al, 1983) have been investigated as surface modifiers. It has been found that heparin-albumin or heparin-PEG_1 conjugates retain the bioactivity of heparin while reducing platelet adhesion and aggregation on the surface. Other methods include immobilizing heparin to the surface using hydrophobic (e.g. C2 to C12 hydrocarbons; Ebert et al, 1982; Heyman et al, 1985), or hydrophilic (e.g. polyethylene glycol, PEG; Kim et al, 1988) spacer groups. Both types of model surface exhibited significantly prolonged clotting times in vitro.

Surface-bound heparin can be degraded under the action of heparinases when exposed to blood, and the use of such heparinized materials in long-term applications is thus limited. The incorporation of certain anionic functional groups into a biomaterial has been suggested as a way to mimic the anticoagulant properties of heparin while avoiding heparinase recognition (Jozefowicz and Jozefonvicz, 1984; Ito et al, 1991; Silver et al, 1992). Such surfaces are less potent with respect to inhibition of thrombin and have shown no tendency to prevent or reduce interactions with blood proteins and cells.

Due to the inadequacies of heparinized materials, direct thrombin inhibitors have been considered for use in place of heparin in surface modification applications. D-Phe-Pro-Arg chloromethyl ketone (PPACK) (Kettner and Shaw, 1979), an extremely powerful and highly specific thrombin inhibitor is one such material (Lumsden et al, 1993; Tapparelli et al, 1993a). PPACK binds to thrombin through the interaction of its

positively charged arginine residue with the aspartic acid residue in the thrombin active site. The chloromethyl ketone group of PPACK irreversibly alkylates the histidine residue in the active site of thrombin (Bode et al, 1989). A series of tripeptide aldehydes D-Phe-Pro-Arg-H (without the chloromethyl ketone group) have also been investigated as antithrombogenic agents in vivo and in vitro (Bajusz et al, 1990). These were found to have the same effect as PPACK on the inhibition of thrombin, but with reversible binding to thrombin. Thus it seems possible that when used as surface localized antithrombotic agents, these peptides could participate in a continuing cycle of binding-inhibition-release of thrombin with renewal of the surface function. If such a cycle could be realized a surface with a continuing antithrombotic effect would be obtained.

1.3 Surface Modification Strategies on Gold Coated Polyurethanes

Since blood is in contact with the material surface, surface modification reactions have been widely investigated as a means of improving blood compatibility. Most blood-contacting materials are polymeric in nature since polymers have elastomeric properties suitable for many devices and can be readily modified at the surface (Lelah and Cooper, 1986). Polymer surface modifications have involved reactions such as copolymerization, grafting, ionization, and simple physical coating (Jozefowicz and Jozefonvicz, 1982). Several modification techniques have been designed to achieve an increase in surface hydrophilicity such as grafting of polyethylene glycol (PEG), chemical modification including graft copolymerization (Brash, 1983), and cold plasma treatment

(Sefton, 1986). As discussed above the attachment of bioactive species such as antithrombotic agents (e.g. heparin, [Jozefowicz and Jozefonvicz, 1984]; hirudin [Markwardt, 1992]; and some platelet aggregation inhibitors, [Kim et al, 1993]), pretreatment of the surface with inert proteins (e.g. albumin, [Engbers et al, 1991]), fibrinolytic agents (e.g. urokinase, [Senatore et al, 1986]), and preparation of biomembrane-mimetic surfaces (Ishihara, 1993) are other approaches that have been tried. All these methods have been investigated in some depth. Difficulties in achieving sufficient coverage of the bioactive agent and in maintaining the agent on the surface in the case of physical attachment are frequently encountered.

A recent development in surface modification is the reaction of gold surfaces with thiol-containing compounds (Whitesides, 1990). These materials, particularly when self-assembled monolayers (SAMs) are formed, have well-defined, ordered surface structures (Ulman, 1993). Moreover the density of thiol coverage on the surface is potentially very high. In principle, therefore, such materials prepared with thiols that contain the desired bioactive moiety, may be useful as biomaterials. This approach has been used in the present work, and has been made more "practical" by modifying thin gold layers that are evaporated onto polyurethane substrates. As an introduction to the work reported in this thesis, some background on gold-thiol chemistry is now presented.

Gold metal is generally thought of as chemically inert in the sense that it does not form a stable oxide film, but it does interact strongly with sulfur-containing chemical functions. Thus thiol-containing compounds can be "grafted" to gold surfaces to form

tightly bound, dense monolayers. A well-known example is the self-assembled monolayers (SAMs) formed by alkane-thiols of chain length C_{11} to about C_{18} chemisorbed on gold surface (Bain et al, 1987). Studies have shown that alkanethiols and dialkyl disulfides chemisorb epitaxially on the Au (111) surface of crystalline gold with the sulfur atoms located in the "hollow" sites formed by three adjacent gold atoms (Figure 1.2). The intermolecular forces between extended hydrocarbon chains (van der Waals forces) act to establish quasi-crystalline order in the layer.

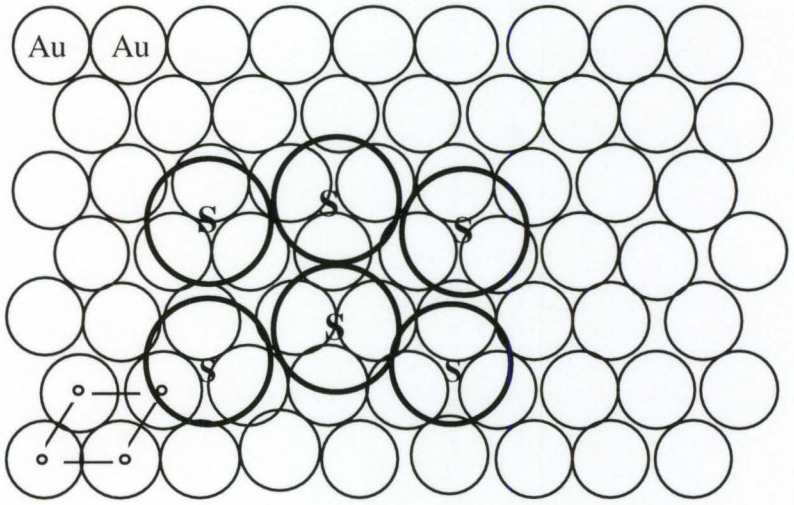


Figure 1.2 Arrangement of thiols adsorbed on Au (111) surface. Adapted from Whitesides, 1990.

Since there are sufficient binding sites on gold, the surface coverage of any gold-thiol layer is potentially high. Great versatility in the chemical properties of the grafted layers is possible through choice of thiol (or thiols) and in some cases a high degree of

structural order can be achieved by the processes of self assembly. In addition, the surfaces are easily prepared under essentially ambient conditions. The use of thiol-gold chemistry using thiolated bioactive agents for reaction with gold thus may be considered as a potential powerful tool in biomaterial surface modification.

The present work involves developing new antithrombogenic materials based on thiol-gold interactions. The three short peptides, Cys-Pro-Arg (CPR), Cys-L-Phe-Pro-Arg (C(L)FPR) and Cys-D-Phe-Pro-Arg (C(D)FPR) have been attached to gold coated polyurethane surfaces. These peptides are analogous to the thrombin inhibitory peptides discussed above except that an N-terminal cysteine residue is present to allow chemisorption to gold. The surfaces of these modified gold materials have been characterized chemically and physically, and their ability to bind and inhibit thrombin has been examined.

2.0 LITERATURE REVIEW

2.1 Blood-Biomaterial Interactions

Blood-material interactions are generally considered in terms of processes such as plasma protein adsorption, platelet activation, blood coagulation, and the control systems participating in thrombus inhibition and fibrinolysis. Without special modification, biomaterials cannot perform an active role in thromboresistance similar to that of the vascular endothelium, nor can they provide a non-interacting surface comparable to that of the endothelium.

2.1.1 Protein Adsorption

The rapid adsorption of proteins onto a biomaterial surface is regarded as the first major event following blood-biomaterial contact (Brash, 1987). Plasma proteins adsorb almost instantly and the composition of the protein layer is known to affect the subsequent interactions of blood cells, especially platelets (Zucker and Vroman, 1969; Packham, 1988; Elam and Nygren, 1992).

Proteins are macromolecules composed of several hundreds of thousands of amino acids linked by peptide bonds. Folding of the polypeptide chains to form the specific shape of the protein is driven by intermolecular forces, mainly hydrophobic interactions, i.e. dehydration of hydrophobic amino acid side chains in the proteins

(Norde and Lyklema, 1991). Amino acids with hydrophobic side groups tend to be in the interior of the molecule, away from water. The amino acids with charged or polar side chains tend to occupy the outer regions of the molecule. This folding can minimize the free energy of the system including the interactions between the proteins and surrounding medium (Macritchie, 1978; Norde and Lyklema, 1991).

Enzymes are a special class of proteins that catalyze many of the reactions in blood and other body fluids. They react through specific binding to a substrate involving interactions dependent on conformation. This binding is achieved either in cooperation with or independent of the "active site" which is characteristic of the enzyme. Factors such as temperature, pH, the presence of other proteins, and adsorption to a surface may affect enzyme activity.

The surface properties of a material will influence its interaction with proteins and are likely to affect the protein conformation and enzymatic activity (Andrade, 1985; Norde and Lyklema, 1991). In studies of protein adsorption, surfaces have frequently been classified as either hydrophobic or hydrophilic. Protein adsorption is generally considered to be greater on hydrophobic than on hydrophilic surfaces (Brash, 1992; Prime and Whitesides, 1991). However, Van Damme (1990) found that protein adsorption was maximal when both the hydrophobic and hydrophilic character of the surfaces were in balance. In the process of adsorption, some of the proteins may be denatured, their conformation may be altered or they may be replaced by other proteins with greater affinity for the surface (Andrade et al, 1984). Blood plasma contains over 200 different

proteins, but with respect to adsorption, only the most abundant types have been studied extensively. Indeed much of the previous work on protein adsorption has focused exclusively on fibrinogen and albumin.

The importance of fibrinogen adsorption comes from its central role in blood coagulation, and its interaction with platelets when adsorbed to a surface (Salzman et al, 1987; Lindon et al, 1986; Tomikawa et al, 1980). In addition, the replacement of initially adsorbed fibrinogen by high molecular weight kininogen (HMWK), a protein which participates in the activation of the intrinsic coagulation pathway (Brash, 1988), as well as a possible interaction between fibrinogen and leukocytes also underline the importance of this protein (Andrade et al, 1984). Numerous investigators have shown that initially adsorbed fibrinogen is displaced from the surface of many artificial materials, presumably by plasma proteins with a higher affinity for the surface (Vroman et al, 1980; Brash et al, 1988). Maxima in fibrinogen adsorption have been observed as a function of adsorption time (Horbett, 1986), plasma dilution (Slack et al, 1991), and fluid volume in narrow spaces (Vroman et al, 1982; Adams et al, 1984; Elwing et al, 1995). The term "Vroman effect" is now used to describe all these situations. However, the identity of the proteins displacing adsorbed fibrinogen is not clear, at least on some surfaces. The complexity of protein interactions with other proteins and with solid surfaces has hindered the development of a completely satisfactory description of this phenomenon.

The ability of adsorbed albumin to reduce platelet and leukocyte adhesion (Salzman et al, 1969; Lyman et al, 1979) and to inhibit thrombus formation (Engbers et

al, 1991) has encouraged the utilization of adsorbed albumin in the development of biomaterials with improved blood compatibility (Courtney et al, 1993). Materials that adsorb endogenous albumin preferentially from blood or that have been pre-adsorbed with albumin prior to blood contact, have been used for this purpose.

The adsorption of other proteins has been less thoroughly studied, although several platelet adhesion promoting proteins have been investigated. These include thrombospondin, which promotes platelet adhesion and release (Young, 1982), fibronectin which has a role in leukocyte adhesion (Adams, 1981; Grinnell et al, 1981; 1983; Vroman et al, 1983), and von Willebrand factor-factor VIII complex, which may have a role in increasing platelet adhesion (Horbett et al, 1984; Young et al, 1982). These proteins are discussed in more detail in Section 2.1.2.

2.1.2 Platelet Reactions

Platelet deposition usually occurs following the adsorption of plasma proteins onto artificial surfaces. This is followed by the release reaction in the adhering platelets by which constituents of internal granules (e.g. ADP) are secreted, provoking the formation of platelet aggregates (microthrombi) on the surface. Many studies have found that platelet adhesion mechanisms differ according to whether low or high shear conditions exist both on damaged endothelium and on the surface of blood contacting materials (Bennett, 1992). In vitro studies indicate that under static or low shear conditions, platelets adhere to surfaces coated with collagen, fibronectin, or laminin.

Platelets contain specific membrane receptors for these extracellular matrix proteins, which include glycoprotein Ic/IIa (gpIc/IIa) for fibronectin, α_6/IIa for laminin, and gpIa/IIa or gpVI for collagen. Under high-shear conditions as in arterioles, von Willebrand factor (vWF) is the major stimulus for platelet adhesion (Ruggieri, 1987). vWF is a multimeric glycoprotein composed of a number of identical subunits. All of the subunits can bind to the gpIb/IX receptor on the platelet surface thus enhancing the ability of the vWF-bound platelets to resist high shear stress in flowing blood (Fox et al, 1988).

Unlike platelet adhesion which is a passive process, platelet aggregation is active, requiring ongoing platelet metabolism and stimulation by one or more specific agonists. Of the various platelet agonists, thrombin is the most potent. Whether thrombin initiates platelet function by binding to and occupying a receptor or by cleaving a membrane protein has been unclear. A recent study showed that a platelet membrane protein exists whose cleavage by thrombin is clearly sufficient to stimulate the platelet hemostatic function (Bennett, 1992). Collagen stimulates platelet aggregation and also supports platelet adhesion. A number of proteins capable of interacting with collagen have been identified on the platelet surface (Brass et al, 1991). In addition to agonist stimulation, studies have shown that platelet aggregation requires the presence of a divalent cation such as calcium and an accessory molecule, usually fibrinogen. It is believed that glycoprotein IIb/IIIa (gpIIb/IIIa) complexes on the platelet surface is involved in the process of fibrinogen-related platelet aggregation (Phillip, et al, 1988). It is currently thought that the activation of platelets by thrombin and other agonists, or the adhesion of

platelets to surfaces induces a conformational change in the gpIIb/IIIa complex which results in the expression of the binding sites of gpIIb/IIIa for fibrinogen. Receptor-bound fibrinogen then cross-links adjacent platelets, resulting in the formation of platelet aggregates.

Besides those mentioned, there are many other cell adhesion molecules: e.g. the immunoglobulin superfamily, the integrin family and the selectin family. The interactions of these adhesion molecules with artificial surfaces may constitute important pathways for platelet adhesion and activation as well as activation of coagulation.

2.1.3 Blood Coagulation

As indicated, when artificial surfaces are placed in contact with blood, the activation of the coagulation process occurs through both the intrinsic and extrinsic pathways (Davie et al, 1991) (Figure 2.1). The initiation of the intrinsic pathway involves several "contact system" proteins, namely HMWK, prekallikrein, factor XII and factor XI. The extrinsic pathway is involved when damaged tissue containing tissue thromboplastin comes in contact with blood. In the presence of tissue thromboplastin (tissue factor) and calcium ions, factor VII is activated and this in turn activates factors IX and X. By either of the two pathways, the coagulation cascade leads to the formation of thrombin, the key enzyme of the coagulation process. Thrombin then catalytically cleaves fibrinogen into fibrin monomers which polymerize and are then crosslinked by activated factor XIII forming an insoluble fibrin clot (Hirsh et al, 1991).

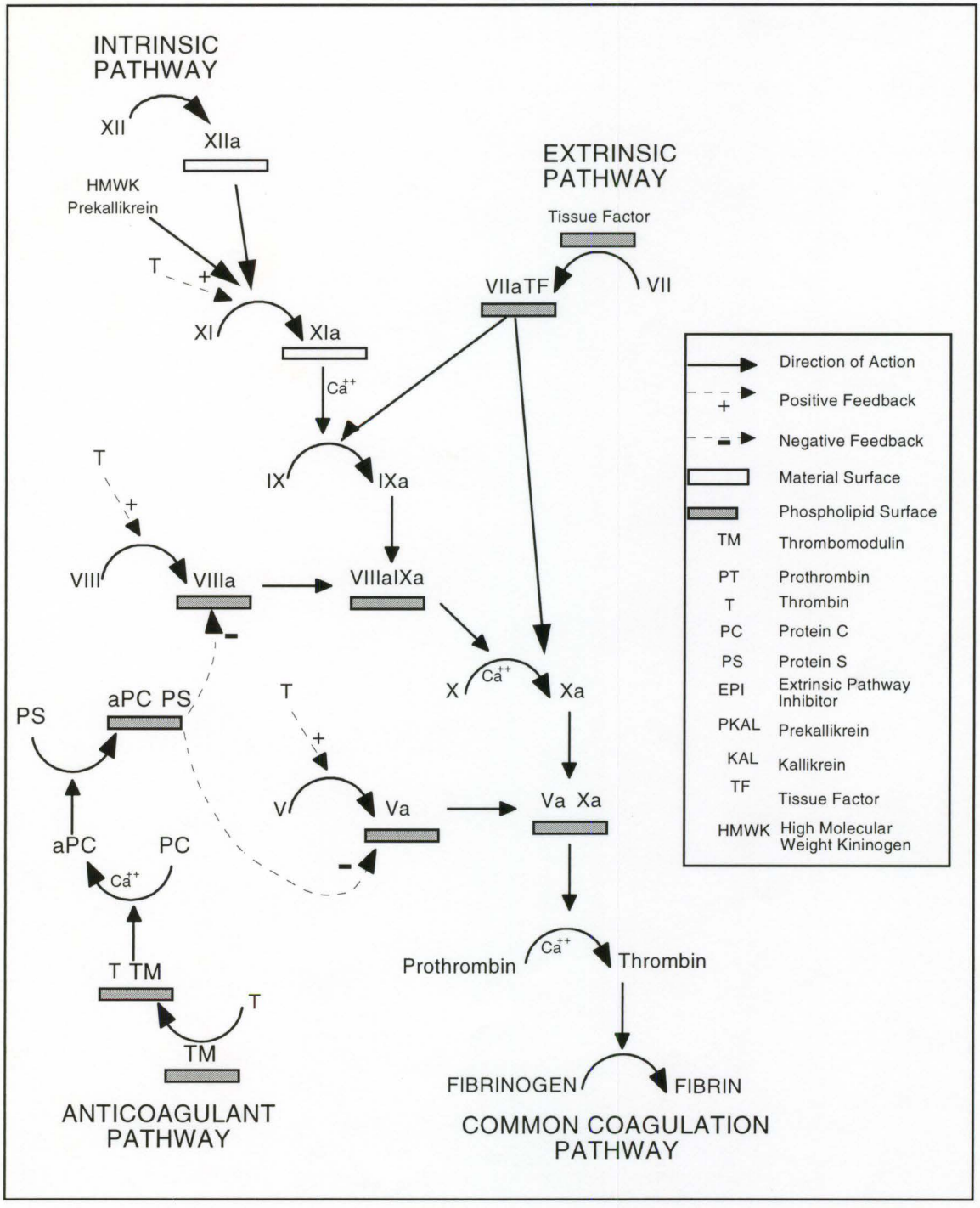


Figure 2.1 Coagulation Cascade. (Adapted from Enzyme Research Lab Inc. Catalog, 1993).

It is clear from the entire discussion above that strict control of the activity and the biological functions of thrombin is of crucial importance in regulating blood coagulation and platelet activity whether in relation to intravascular thrombosis or thrombosis on artificial surfaces. Since thrombin is a major focus of the work reported here, it is appropriate to give some additional details on its structure and function.

2.2 Thrombin Biochemistry and Physiology

Thrombin is a multifunctional enzyme, which can react specifically with a number of substrates (proteins) in the coagulation system, and a variety of cells (via receptors) as well as nonspecifically with surfaces (e.g., mucopolysaccharides). Its main functions are summarized in Figure 2.2.

2.2.1 Thrombin and Plasma Proteins

Besides its major role in cleaving fibrinogen to produce fibrin monomers, thrombin is also involved in the activation of factor XIII which catalyzes fibrin crosslinking (Hirsh et al, 1991). Thrombin stimulates its own production by activating factors V, VIII and XI, which are essential cofactors in the coagulation cascade. Thrombin is required for the activation of protein C (Figure 2.1) which results in the inactivation of factor Va and factor VIIIa. This action of thrombin can be regarded as a negative feedback control mechanism (Esmon, 1987; Fenton, 1993).

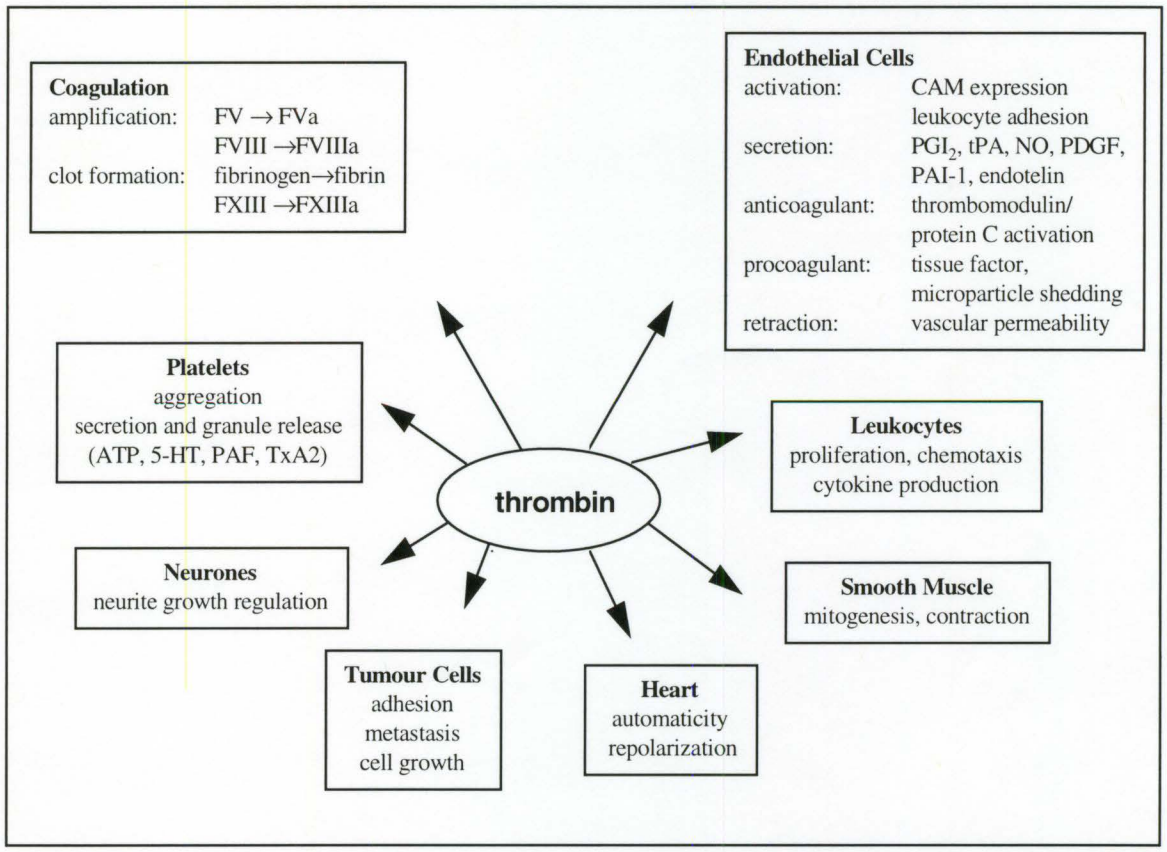


Figure 2.2 Scheme depicting the multiple enzymatic and cellular actions of thrombin. Abbreviations: FV, factor V; 5-HT, 5-hydroxy tryptamine; PAF, platelet activating factor; TxA_2, thromboxane A_2; PGI_2, prostaglandin I_2; tPA, tissue plasminogen activator; NO, nitric oxide; PDGF, platelet-derived growth factor; PAI-1, plasminogen activator inhibitor-1 (Tapparelli et al, 1993).

2.2.2 Interaction of Thrombin with Cells and Vessel Wall

In addition to interactions with the plasma proteins, thrombin also has various effects on cells in the vascular system (Coughlin, 1994). Thrombin-induced platelet aggregation contributes to the body of the clot, and the platelet provides a surface for the assembly of the protease-cofactor complexes that are involved in the coagulation cascade.

Thrombin also induces endothelial cells to secrete a number of agents, including prostaglandin I_2 (PGI_2), endothelin, nitric oxide, platelet-derived growth factor, vWF, tissue plasminogen activator, and plasminogen activator inhibitor-1. Moreover, thrombin stimulation of endothelial cells leads to an increase in vascular permeability and binding of mononuclear cells to the endothelial surface (Coughlin, 1994). Thrombin is a mitogenic stimulus for vascular smooth muscle cells, and it has been suggested that it may play a role in the development of proliferative lesions following vascular damage (Coughlin, 1994).

2.2.3 The Structure of the Thrombin

Thrombin, a member of the serine protease family, is made up of 295 amino acids that form two polypeptide chains joined by a single disulfide bridge. α-thrombin, the only form of human thrombin, has a molecular weight of 36,000; its A chain consists of only 36 amino acids, and its B chain, which possesses the serine protease domain, is 259 amino acids in length (Stubbs and Bode, 1995).

Like other members of the chymotrypsin family of serine proteases, thrombin is composed of two six stranded barrel domains, with the catalytic residues (His57, Asp 102, and Ser195) located at the junction between the two barrels (Figure 2.3). As in trypsin, residues of substrates of proteases are usually numbered from the cleaved bond: P_n-...-P_3-P_2-P_1-P_1'-P_2'-P_3'-P_n', where cleavage occurs at the P_1- P_1' bond; the

corresponding binding sites for these residues on the protease are designated as S or S' sites (Figure 2.3b).

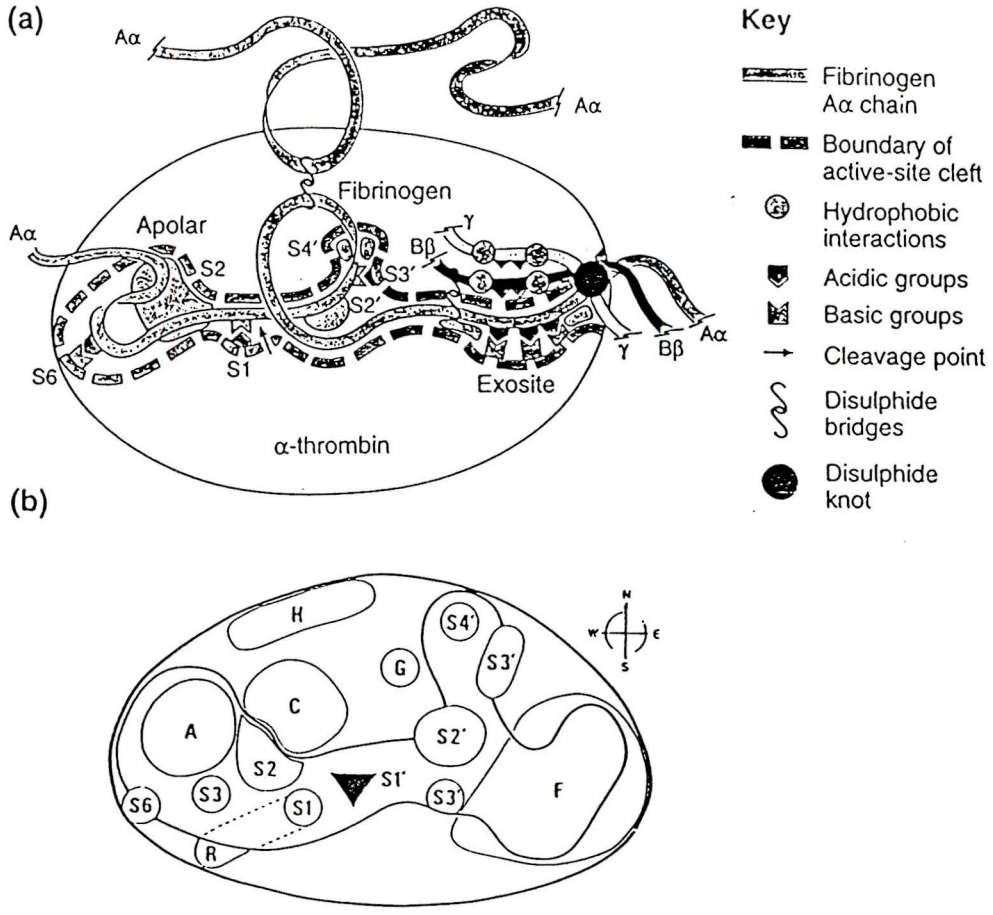


Figure 2.3 **(a).** Schematic diagram showing the major interactions of the fibrinogen Aα chain with thrombin. **(b)** Schematic diagram showing residues involved in the various subsites of thrombin.▼, active-site triad; S1-S6, specificity sites amino-terminal to cleavage; A, aryl-binding site ; F, fibrinogen-recognition exosite; H, heparin-binding site; G-glycosylation site; S1'-S3", putative specificity sites carboxyl-terminal to cleavage; R, RGD sequence. From Stubbs and Bode, 1995.

There is a primary specificity pocket or S_1 site on the thrombin surface, which consists of a deep channel with Asp189 located at the "bottom". This Asp residue serves as a salt bridge partner for positively charged residues (P_1) such as arginine or lysine which are present in the enzyme's natural substrates. However, thrombin has a more extensive substrate-recognition surface. The S_2 site of thrombin contains a loop shaped hydrophobic lid, restricting substrate P_2 residues mostly to those having small hydrophobic side chains such as valine or proline. It also opens to a large hydrophobic surface to the "west" that is ideally suited to receiving aromatic side chains ("aryl-binding site"). These two sites form the 'apolar' binding site. Particular also to thrombin is the presence of the 'exosite', which is a surface groove rich in basic amino acids that has been termed the anion-binding exosite (ABE). It can recognize elements far removed from the active site. These unique regions on the thrombin surface have been implicated in the interaction of thrombin with fibrinogen, heparin cofactor II, thrombin receptors, thrombomodulin and the inhibitor hirudin (Stubbs and Bode, 1993).

2.3 Inhibition of Thrombin

Inhibitors of thrombin may be characterized as direct or indirect. Direct inhibitors function by binding to one or more domains of thrombin, while indirect inhibitors require a cofactor for thrombin inhibition.

2.3.1 Indirect-thrombin Inhibitors

The major indirect thrombin inhibitors are the plasma serpins ATIII and heparin cofactor II. The inactivation of thrombin by these serpins is considerably enhanced by the action of heparin, which is widely used as an antithrombotic agent.

2.3.1.1 Heparin

Heparin is a glycosaminoglycan composed of alternating glucosamine and uronic acid residues with molecular weights in the range of 5,000 to 50,000. The antithrombotic effect of heparin is mediated by both ATIII and heparin cofactor II (Bjork, et al, 1982). As mentioned previously, ATIII inactivates thrombin through the binding of its Arg-reactive site to the active site of thrombin, forming a 1:1 stoichiometric complex (Householder, 1991). This inactive complex formation occurs normally at a relatively slow rate but is strongly catalyzed by heparin. Heparin binds to the lysine binding site on ATIII and causes a conformational change in ATIII which makes the original reactive site much more accessible for interaction with the active site of thrombin; the inactivation is accelerated at least 1,000 fold (Householder, 1991). In addition to inhibiting thrombin and the thrombin-mediated activation of factors V and VIII, the heparin-ATIII complex inhibits other serine proteases in the clotting cascade, including factors IX_a, X_a, XI_a, and XII_a. Heparin has been evaluated as adjunctive therapy in large thrombolytic therapy trials (Danielsson et al, 1986; Olson, 1988).

2.3.1.2 The Shortcomings of Heparin

As mentioned previously, heparin as an antithrombotic agent has a number of limitations and undesirable side effects. It can be inactivated by platelet factor 4 and heparinases, which are released from activated platelets (Weitz and Hirsh, 1993; Harker, 1994). It also binds to a number of plasma proteins, including fibronectin, vitronectin, and vWF which are acute-phase reactants, and may increase during illness. It is also known that surface-bound heparin may promote platelet adhesion and aggregation (Brace and Fareed, 1985). Kelton et al. (1988) reported that heparin binds to the F_{ab} portion of IgG to form an immune complex which can initiate the platelet release reaction by binding to the platelet F_c receptors. One of the most serious limitations of heparin is its inability to inactivate clot-bound thrombin which is known as a major contributor to thrombus growth (Hogg and Jackson, 1989; Weitz et al, 1990). The protection of clot-bound thrombin from heparin-ATIII complex may be due to steric hindrance by fibrin of the heparin binding site on thrombin (Hubbell and McIntire, 1986). In contrast to heparin, direct thrombin inhibitors can inactivate thrombin which is bound to fibrin as effectively as free thrombin (Weitz, 1990).

2.3.2 Direct Thrombin Inhibitors

Based on the binding domain where interaction occurs, direct-acting thrombin inhibitors can be further subclassified: (I) active-site inhibitors which interact with the thrombin active site; (II) exosite inhibitors which bind to the anion-binding exosite of

thrombin, and (III) bivalent inhibitors which bind to both the active site and anion-binding exosite (Winant et al, 1991).

The first class of inhibitors, mainly of the tripeptide type, contain an electron-deficient moiety for binding to the active site of thrombin. The second and third classes of inhibitors consist mainly of hirudin and its derivatives.

2.3.2.1 Hirudin and Its Analogues

Hirudin, isolated form the leech Hirudo medicinalis, is a 65-amino-acid protein with three disulfide bridges near its amino terminal and a highly acidic carboxy-terminal domain (Markwardt, 1992; Rydel, 1990). The N-terminal of hirudin binds to the apolar binding site of thrombin near the primary specificity pocket, whereas the C-terminal interacts with the anion-binding exosite of thrombin (Sturzbecher and Walsman, 1990). The Pro46-Lys47-Pro48 region of hirudin probably occupies the basic specificity pocket of the active site of thrombin (Figure 2.4). The direct interaction of hirudin with both the active site and ABE region account for its ability to inhibit all thrombin-mediated reactions, with equally high potency toward free and fibrin-bound thrombin (apparent, assay-dependent inhibitory constant, Ki = 10^{-14} M; Fitzgerald, 1994; Markwardt, 1989).

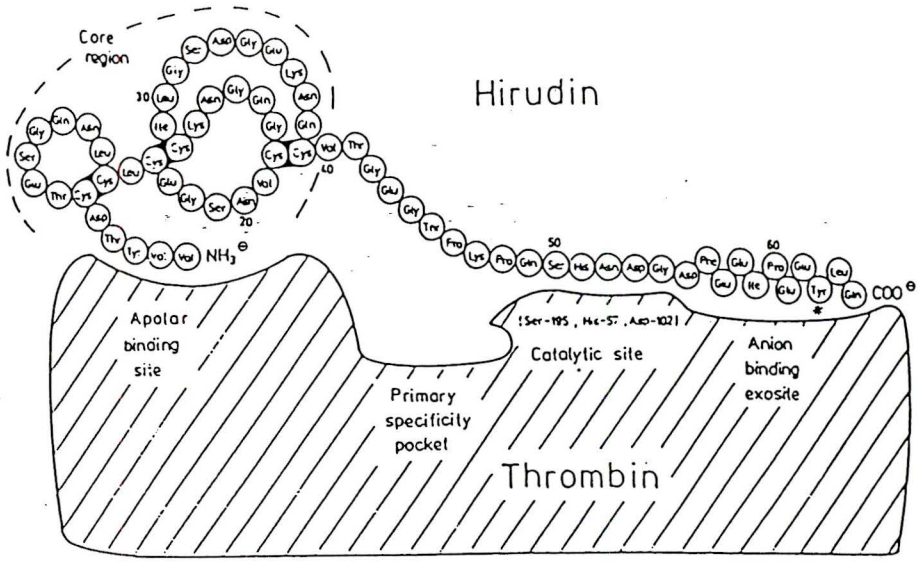


Figure 2.4 Schematic of the hirudin interaction with thrombin. From Markwardt, 1992.

Hirudin is also a potential inhibitor of thrombin-induced platelet activation, acting by interfering with the binding of the active site of thrombin to the receptor, and competing with the hirudin-like C-terminal receptor domain on platelets for the anion-binding exosite of thrombin (Fenton and Wilner, 1988; Hoffman and Markwardt, 1984; Glusa, 1991).

A number of analogues of hirudin have been developed as anticoagulants. Hirugen, is a 12-amino acid peptide derived from the C-terminal of hirudin, which inhibits thrombin's cleavage of fibrinogen by blocking its interaction with the ABE (Schmitz et al, 1991). It is less effective in preventing thrombosis in animal models than hirudin which binds to the active site as well as the exosite (Kelly et al, 1992). Hirulog is a 20-residue peptide consisting of an exosite binding sequence based on hirudin, and an "active site" specificity sequence (D-Phe-Pro-Arg-Pro) linked to a polyglycine as a spacer (Maraganore et al, 1990). Again, because of multiple sites of interaction with the

enzyme, hirulog appears to be highly specific for thrombin, in the same way as its parent molecule, hirudin. It also inhibits the thrombin-catalyzed clotting of fibrinogen, even though the Ki value of 10^{-9} M is relatively low (Bourdon et al, 1991).

2.3.2.2 PPACK

PPACK or D-phe-pro-arg-CH_2Cl, a chloromethyl ketone derivative, is a potent and selective inhibitor of thrombin. Its design (Kettner and Shaw, 1979) is based on the archetypal mechanism of thrombin substrate interactions (Figure 2.3b; Tapparelli et al, 1992).

The P_1 arginine of PPACK binds to the primary specificity pocket or S_1 site, which consists of a deep channel with Asp189 located at the bottom. Asp 189 forms a salt bridge with the P_1 arginine. The P_2 proline of PPACK fills the S_2 site of thrombin which is a hydrophobic pocket, well adapted for the binding of proline. The P_3 D-phenylalanine of PPACK binds to a site consisting of Leu99, Ile174, and Trp215 (the 'aryl-binding' site). This site is particularly suited to interaction with aromatic residues. The aromatic ring of the phenylalanine makes a favourable edge-to-face interaction with Trp215, and it is the D configuration of phenylalanine that is able to occupy the aryl-binding site. The side chain of an L-amino acid would extend away from the active site. The fit of the P1-P3 residues of PPACK to the S1-S3 sites on thrombin is almost optimal, and the chloromethyl ketone moiety irreversibly alkylates the active-site histidine, thus further re-inforcing the binding interactions.

The irreversible inactivation of thrombin by PPACK is a two-step process: (1) formation of a reversible active-site complex, and (2) rapid irreversible alkylation of the active centre histidine by the chloromethyl ketone group (equation 1; Collen, 1981):

$$E + I \xleftrightarrow{K_i} EI \xrightarrow{k_2} EI^* \qquad (1)$$

Kettner and Shaw (1979) reported the dissociation constant for the reversible complex (Ki) formed in the first step to be 3.7 nM, and the rate constant for formation of the enzyme-inhibitor complex (k_2) for the second step to be 0.49 s^{-1}. Table 2.1 lists the dissociation constants for several thrombin-inhibitor complexes. The data show very high affinity of these inhibitors for thrombin.

Table 2.1. Dissociation constants for various inhibitor-thrombin complexes (Kettner and Shaw,1979)

Inhibitors	Ki
PPACK	3.7 nM
Hirudin	20 fM
Hirugen	150 nM
Hirulog	2.3 nM
(D)FPR	75 nM
Boroarginines	4 pM
Argatroban	19 nM

The interaction of PPACK with the active site of thrombin results in the prevention of thrombin-mediated platelet activation. PPACK inhibits both free and clot-

bound thrombin with equal potency in vitro. A marked antithrombotic responsiveness of platelet-dependent thrombus formation to PPACK under conditions where heparin is ineffective has been demonstrated (Lottenberg, 1981). The PPACK-thrombin complex binds to high-affinity functional thrombin receptors on platelets without activation of the platelets, and then, saturated by this complex, subsequent thrombin activation is blocked (Harker, 1994). Consequently, not only does locally formed thrombin become inactivated by PPACK, but the resultant inactive complex, PPACK-thrombin, occupies the thrombin receptors on platelets to prevent subsequent activation by thrombin (Collen et al, 1982; Schaeffer et al, 1986). Since thrombin formation is generally a process associated with a cell surface, particularly the platelet membrane, irreversible inactivation by PPACK may occur after the activating cleavage and before thrombin is free from the platelet-bound prothrombin fragment 1.2 (Schaeffer et al, 1986; Harker, 1994). However, the high reactivity of the chloromethyl ketone moiety of PPACK can lead to alkylation of other proteins in plasma which may be nonspecifically modified by this reagent (Bady et al, 1993).

2.3.2.3 D-Phe-Pro-Arg Aldehydes (FPR-H) and Boronic Acid Derivatives

A novel class of direct-acting thrombin inhibitors of low molecular weight has been developed which incorporate the aldehyde form of the arginine residue, the aminoacid recognized by thrombin in its natural substrates, including fibrinogen and the platelet thrombin receptor (Tapparelli et al, 1993; Claeson, 1993; 1994).

Peptide aldehydes based on the D-Phe-Pro-Arg sequence are specific inhibitors of thrombin (Bajusz et al, 1990; Chiu et al, 1991; Chomiak et al, 1993; Shuman et al, 1995) and show antithrombotic effects in vivo (Bacher et al, 1993). These peptide aldehydes form a reversible complex with thrombin. D-Phe-Pro-Arg-H was shown to possess high and specific antithrombin activity (Bady et al, 1992; Ofosu, 1993; Shuman et al, 1995). Studies of the possible interactions of these inhibitors with plasma proteins and blood cells (especially red cells and platelets), shows that the tripeptide aldehydes specifically inhibit thrombin-induced platelet aggregation (Bady et al, 1992; Fitzgerald, 1994; Chiu et al, 1991). The antiplatelet effect of the tripeptide aldehydes is possibly due to a loose adsorption of the peptide on the platelet membrane, thus preventing the enzyme from binding to its high affinity platelet receptor site (Bady et al, 1992; Hauptmann and Markwardt, 1992).

Peptides containing a boronic acid derivative of arginine exhibit a similar spectrum of activities with Ki values in the picomolar range approaching that of recombinant hirudin (Table 2.1, Kettner et al, 1990). The tight binding of thrombin to FPR peptides in which the arginine residue is in the form of the boronic derivative is due largely to the interaction of boronate with the active-site serine. Consequently, it has been possible to modify other substituents of the inhibitor in order to achieve selectivity while maintaining reasonable potency. However, a potential disadvantage of this type of inhibitor is that it inhibits plasmin also, and this limits its use in thrombolytic therapy.

2.4 Development of Antithrombogenic Materials

The most common approach to developing blood-compatible materials is surface modification. This approach offers the possibility of controlling blood-material interactions (which occur at the surface), while maintaining other essential bulk properties of the biomaterial such as strength and elasticity. Surface modification techniques have been designed to achieve increases in hydrophilicity, to introduce chemical functions, to attach antithrombotic agents, to pre-coat with protein, and to prepare biomembrane-mimetic surfaces.

2.4.1 Surfaces Resistant to Protein Adsorption

Much effort has been directed toward the identification of biologically "inert" materials, i.e. materials that resist the adsorption of proteins and the attachment of cells. The most successful method of achieving resistance to protein adsorption is to coat the surface with poly(ethylene glycol) (PEG) (Gombotz, 1991). A variety of methods, including simple physical adsorption, chemical attachment, and radiation-initiated surface grafting, have been used to modify surfaces with PEG (Harris et al, 1992). In the late 1970s, Davis et al (1987) demonstrated that covalent attachment of PEG to plasma proteins causes minimal loss of biological activity and renders the protein nonimmunogenic and non antigenic, thus imparting greatly increased serum lifetime. Another application of PEGs is in synthesizing artificial enzymes by connecting binding sites and catalytic sites via PEG linkers. The PEG linker is a key aspect of this

application because of its length, hydrophilicity, and flexibility. It acts as a moisture preserver so that the normal environment around the protein is retained. Park and Kim (1988) have reported that attachment of bioactive heparin to the end of a PEG chain which is grafted to a polyurethane surface has a significant impact on antithrombogenic response. In this application, PEG acts to inhibit nonspecific protein adsorption on the surface. Additionally, the tethered molecules have been shown to be highly active, behaving essentially as molecules in free solution. This observation can be rationalized by assuming that the unusually long, well-hydrated PEG linker moves the active molecule well out into solution, some distance from the surface.

Surface-bound PEG has been used extensively for preventing protein adsorption. Studies by Andrade and co-workers (1991a; 1991b) showed that much less albumin, IgG, and poly-lysine are adsorbed to the PEG grafted surface compared to hydrophobic PVC or anionic polymethacrylate (PMA). Fibrinogen, the largest protein studied, gave slightly higher adsorbed amounts compared to albumin, IgG and poly-lysine. This may either be the result of interaction between domains on the protein and bare substrate sites not covered with PEG, or due to mere physical entanglement of PEG and protein segments. De Gennes and Andrade (1991) have reported that there is a significant decrease in adsorption with increasing PEG molecular weight up to 1500, but that above this value, only a slight decrease is observed. They proposed that surfaces modified with long PEG chains resist the adsorption of protein by "steric repulsion". In aqueous solution, the PEG chains are solvated and disordered. Adsorption of protein to the surface causes the chains

to compress, with concomitant desolvation. Both the energetic penalty of transferring water to the bulk and the entropic penalty incurred on compression of the layer serve to resist protein adsorption.

Polymers consisting of carbohydrate units also tend to passivate surfaces, but these materials are less stable and less effective than PEG (Liedberg et al, 1993; Wang et al, 1992). Another widely used strategy is to preadsorb a protein – usually bovine serum albumin — that resists the adsorption of other proteins (Bekos et al, 1995). However, this strategy suffers from problems associated with denaturation of the blocking protein over time or exchange of this protein with others in solution. A further limitation of this strategy is the inability to present other groups (e.g. ligands, antibodies) at the surface in controlled environments.

Surface-bound polymers exerting effective steric repulsion (e.g. PEO) must satisfy the following conditions: (1) tight anchoring to the surface; (2) complete surface coverage; and (3) flexibility of the grafted molecules. If polymer molecules are not tightly bound to the surface, they may be displaced by proteins or cells which have a greater affinity for the surface. Complete surface coverage is also important since proteins and cells can interact directly with any bare surface. Flexibility of the grafted molecules provides steric repulsion as discussed. In many investigations, the surface density of the grafted polymer molecules is unknown mainly due to the difficulty in measuring this property. Thus, if the grafting of hydrophilic polymers does not prevent protein adsorption or cell adhesion, it is possible that the surface concentration of the

grafted polymers is not high enough. Many attempts have been made to increase the surface density or coverage of grafted polymers, such as using cold plasma polymerization of monomers containing specific functional groups, or using gold as an intermediate between the polymer and the molecule to be grafted. The latter strategy is discussed in detail in section 2.5.

2.4.2 Surfaces Modified with Antithrombotic Agents

It seems reasonable that the blood compatibility of biomaterials could be improved by the attachment of anticoagulants (Jozefowicz and Jozefonvicz, 1984), platelet aggregation inhibitors (Kim et al., 1987), or plasminogen activators (Woodhouse and Brash, 1994) and considerable effort has been devoted to this approach. The most frequently used agent is heparin. Heparin has been successfully incorporated into biomaterial surfaces by both ionic and covalent attachment (Kim et al., 1983). The strong anionic character of heparin can be used to bind it to cationic surfaces (Tanzawa et al. 1973, Kim et al., 1983). Since the early work of Gott et al. (1963), numerous studies have focused on ionically bound heparin. Polycationic polymers have been prepared from polystyrene and its derivatives, cellulose, silicone rubber, epoxy resins, and polyurethanes (Heymann, et al. 1985; Ebert et al, 1980; Kwon et al. 1994). The resulting materials have usually shown anticoagulant activity although it seems probable that many of them act by releasing the ionically bound heparin into the blood. Unfortunately, after a period of time, the heparin is exhausted; thus such biomaterials are suitable only for short

term applications. Heparin has also been covalently bound to suitable polymers (Sefton et al, 1978, 1981). Some of these surfaces have also been shown to be antithrombogenic in vitro and in some in vivo models. Nevertheless, some surfaces on which heparin is covalently bound have been shown to activate platelets when exposed to blood or platelet suspensions (Warkentin et al. 1992). However, preincubation of these surfaces with either ATIII solution or plasma appeared to passivate them with regard to platelet activation (Jozefowicz and Jozefonvicz, 1984). Certain platelet adhesion and aggregation inhibitors or platelet inert molecules have been incorporated into the surface along with heparin. Hennick et al. (1984) prepared heparin-human albumin complexes, and preadsorbed them onto polymer surfaces. They found that the heparin-albumin conjugate reduced both the amount of fibrin formation and the extent of platelet aggregation. Jacobs et al. (1986) prepared PGE_1-heparin conjugates and showed that when attached to a surface, the bioactivity of heparin was retained, while the PGE_1 reduced platelet adhesion and aggregation on the surface.

Synthetic heparin-like materials were also proposed to overcome the degradation problem of heparinized materials (Jozefowicz et al, 1979; 1981; 1982; 1983; 1984). Resins having a range of antithrombogenic activity were prepared by attaching sulfonate, carboxylic acid, amino acid sulfamide or amide groups to crosslinked polystyrene, polysaccharides, and polystyrene-polyethylene graft copolymers.

Ito et al (1992) attached a synthetic thrombin inhibitor MD-805 to polyurethane and polystyrene membranes by either blending the inhibitor with polymer resins or

chemically grafting it to the polymer surface. They found that the grafted membranes maintained antithrombogenic activity, in terms of suppression of platelet adhesion and thrombus formation, for a much longer time as compared to the blended materials. Santerre et al. (1992) incorporated sulfonate groups into the hard segment of polyether polyurethanes. However, it was found that fibrinogen adsorption from plasma increased as the sulfonate content increased. In addition displacement of fibrinogen by other plasma proteins via the Vroman effect was greatly reduced due to the tight binding of fibrinogen to the sulfonate groups. To further enhance the antithrombotic activity of the sulfonated polymers, these authors derivatized the sulfonate groups with arginine methyl ester, aspartic acid and lysine (Santerre et al, 1992).

Surface attachment of plasminogen activators has also been investigated as a means of producing materials which are capable of lysing small or incipient fibrin clots before they become problematic. Foster and Bernath (1986) immobilized urokinase (a plasminogen activator) on polytetrafluoroethylene vascular prostheses and found that the fibrinolytic activity of the surface was enhanced. Senatore and Bernath (1986) investigated the properties of urokinase-modified grafts both in vivo and in vitro, and showed enhanced fibrin degradation on the urokinase-modified surface. Shankar et al (1987) immobilized plasmin on collagen beads and found that the immobilized plasmin exhibited fibrinolytic activity even in the presence of α_2-antiplasmin. This was in contrast to results which indicated that the presence of inhibitor significantly decreases the fibrinolytic activity of plasmin. Brash et al (1985) have also found evidence for

surface activation of plasminogen on glass which results in the degradation of adsorbed fibrinogen. Kichenin-Martin et al (1988) reported that adsorption of plasminogen from plasma to sulfonated polystyrenes was capable of generating plasmin-like fibrinolytic activity on the surface, presumably due to a plasminogen conformational change. Somewhat in contrast, later work done by Yang and Henkin indicated that rather than activating plasminogen after adsorption, sulfonated or sulfonyl glutamate derivatized polystyrenes selectively adsorbed plasmin from solutions of plasminogen (1990). Woodhouse and Brash (1994) have reported that plasminogen on a lysinized surface is bound through a lysine binding site, thus potentiating its interaction with t-PA to form a ternary t-PA-plasminogen-fibrin complex. This resulted in more extensive clot lysis on this surface than on a control. This result is also in accord with the finding of Hoylaerts et al. (1982) that the activation of plasminogen bound to fibrin via a lysine binding site is a thousand times faster than for unbound plasminogen.

In general polymer surface modification is achieved through either chemical grafting or physical attachment (adsorption or deposition). Difficulties in achieving sufficient coverage of the bioactive agent or in maintaining the agent on the surface in the case of physical attachment are frequently encountered. The approach of using an intermediate gold film which can be derivatized at high surface density has been used in this work to overcome the problem of insufficient coverage.

2.5 Surface Modification Using Gold-thiol Chemistry

In 1983, Nuzzo and Allara showed that dialkyl disulfides (RS-SR) formed oriented monolayers on gold. Later, it was found that sulfur compounds coordinate strongly to gold, silver, copper, and platinum surfaces. However, most of the subsequent work has been done on gold surfaces, mainly because of the fact that gold as a chemically inert metal does not form a stable oxide, and thus it can be handled in ambient conditions.

In principle, any thiol- or disulfide-containing molecule can be "grafted" onto gold with a very high surface density. This approach is quickly emerging as the basis of a promising new methodology for surface modification, with the potential to attain well defined surface structures. Great versatility in the chemical properties of the grafted layers is possible and a high degree of structural order can be achieved by the processes of self assembly. In addition, the surfaces are easily prepared under essentially ambient conditions.

2.5.1 Self-Assembled Monolayers (SAMs) on Gold Substrates

SAMs on gold are generally formed by molecules containing hydrocarbon chains having a minimum of about 11 carbons. To prepare such a surface, a clean, hydrophilic gold substrate is usually immersed in a dilute solution (1mM) of the organosulfur compound in an organic solvent. Immersion times vary from several minutes to several hours for alkanethiols, while for sulfides and disulfides, immersion times of several days are needed. The result is a close-packed oriented monolayer (Figure 2.5), in which the

hydrocarbon chains are fully extended with a considerable degree of order in the layer structure. The effect of the sulfur function on the monolayer formation on gold was studied by Bain et al (1987). They examined a variety of gold-reactive groups and also conducted competition experiments in which mixtures of compounds having different reactive groups were used. They concluded that of all the groups studied, the thiol group gives the strongest interactions with gold surface.

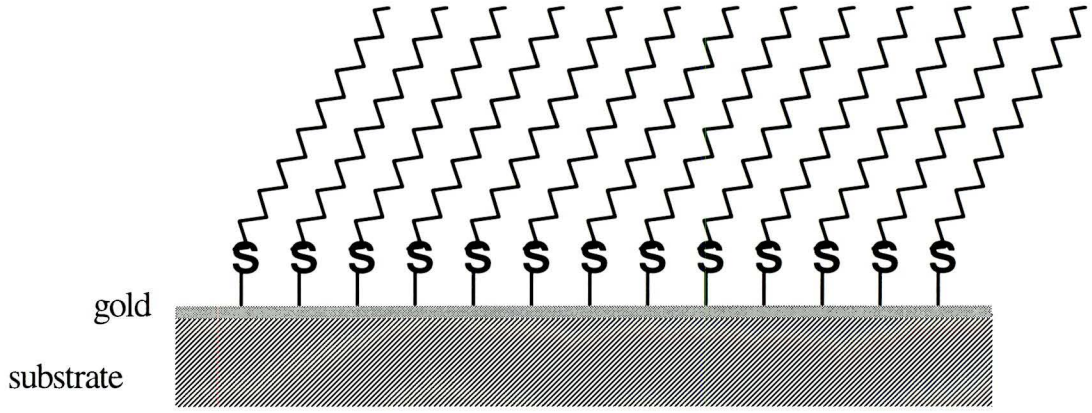


Figure 2.5 Schematic of self-assembled monolayer of hydrocarbon chains on modified gold surface. The chains are tilted at about 30° to maximize intermolecular interactions. Adapted from Ulman, 1990.

2.5.1.1 Kinetics of Formation of SAMs of Alkane Thiols on Gold

The kinetics of the formation of alkanethiol monolayers on gold was also studied by Bain et al (1987). In relatively dilute solutions (1 mM), they observed two distinct kinetic regimes: a very rapid initial adsorption (first few minutes) giving a layer with little or no order, followed by a slower process which lasts several hours, in which additional adsorption, displacement of contaminants, and most importantly, ordering of the

hydrocarbon chains to form a quasi crystalline structure (Figure 2.5) occurs. According to Nuzzo et al., the rate of formation of a SAM is influenced by many factors, some of which can be controlled relatively easily, such as temperature, solvent, concentration and chain length of the adsorbate, and cleanliness of the substrate. Other factors, such as the rate of reaction with the surface at a given temperature and the reversibility of adsorption of the components of the monolayer, are inherent to the system.

The effect of hydrocarbon chain length on the kinetics of formation of alkanethiol monolayers (SAMs) on gold was studied by Bain et al (1987) for C_{10} and C_{18} alkanethiols. It was found that the SAMs form faster for longer alkyl chains, due to van der Waals interactions which occur between adjacent chains on the surface, and provide a strong driving force for layer formation. Porter et al. (1991) studied the effect of chain length on the properties of monolayers for a series of n-alkanethiols, $CH_3(CH_2)_nSH$, measuring water and hexadecane contact angles and monolayer thickness. They found that SAMs of short alkanethiols ($n<9$) are liquid-like, and more sensitive to thermally induced disordering Thus, the properties of these monolayers are chain length dependent. When the chain length of the alkane thiols is long enough ($n>9$), the surface properties become independent of the chain length.

It has been noted that one may not be able to obtain a clean, uncontaminated gold substrate unless ultrahigh vacuum is used. This is because even with the most careful operation, there is always a film of contaminants on the gold surface, i.e. chemisorbed CO, O_2, H_2O, hydrocarbons, and physisorbed H_2O, hydrocarbons, and other organic

compounds that exist in the laboratory atmosphere. Such contaminants are always observed in x-ray photoelectron spectra of "clean" gold surfaces. Apparently, this contaminant film affects only the kinetics and not the extent of adsorption of thiols, and is displaced from the surface by the thiols in the process of monolayer formation.

The effect of solvent on the formation of monolayers has also been investigated by several research groups (Ulman, 1991; Bain, 1989a). Most of the work reported in the literature suggests the use of ethanol as the preferred solvent. From a practical point of view, the solubility properties of the alkanethiol being used dictate the choice of solvent. Bain et al. (1989b) showed that hexadecanethiol monolayers adsorbed from hexadecane have abnormally low water contact angles, presumably due to the incorporation of the solvent into the monolayers. Therefore, it is desirable to use a solvent that does not have a tendency to be incorporated. Ethanol, THF, and acetonitrile appear to be suitable in this respect.

2.5.1.2 Mechanism of the Thiol-Gold Interaction

Chemisorption of alkanethiols or dialkyldisulfides on gold (0) surfaces yields the gold (I) thiolate (RS^-) species. The mechanism of the reaction of alkanethiols with gold (0) is not completely understood. It is not clear whether the thiol hydrogen which is eliminated in the formation of RS^--Au^+ is released as H_2:

$$RS - H + Au_n^0 \Rightarrow RS^- - Au^+ + \frac{1}{2}H_2 + Au_{n-1}^0$$

or as H_2O via reaction with traces of oxidant:

$$RS-H+Au_n^0+oxidant \Rightarrow RS^- - Au^+ + \frac{1}{2}H_2O + Au_{n-1}^0$$

Strong and Whitesides (1988) studied monolayers of docosanethiol using electron diffraction. They found that the symmetry of sulfur atoms in a monolayer of docosanethiol on crystalline gold (111) surface is hexagonal with S··S spacing of 4.97 Å, and area per molecule of 21.4 $Å^2$. Later helium diffraction studies by Chidsey(1989) and Loiacono et al. (1990) suggested that alkanethiols and dialkyl disulfides chemisorb epitaxially on Au (111), with the sulfur atoms located in the three fold "hollow" sites of the gold surface (Figure 1.2). Porter et al. (1987) using scanning tunnelling microscopy confirmed the hexagonal symmetry in these systems.

Grazing-angle infared reflection absorption spectroscopy (IRAS) has been used extensively by several research groups (Nuzzo et. al, 1987; Porter et. al, 1987; Finklea and Melendrez, 1986; and Ulman, 1989) to study the orientation of the chains in long chain alkanethiol and disulfide monolayers on gold. It has been confirmed that alkanethiol monolayers are close-packed assemblies with a quasi-crystalline order, and that the extended chains have a tilt angle of 20^o - 35^o from the normal. This orientation is believed to maximize the van der Waals interactions between chains.

However, SAMs of alkanethiols exhibit a rich variety of packing and ordering phenomena, and can serve as excellent model systems for evaluating modern theories of wetting, spreading, adhesion, friction, molecular recognition and related phenomena (Ulman, 1993).

2.5.2 Application of Thiol-Gold Surfaces in Biomaterials Development

Since SAMs form dense and ordered layers on gold, they provide a means of preparing surfaces with well-defined chemistry which have been noted to be extremely useful for studying protein and other biomolecule adsorption (Whitesides, 1990). Mrksich and Whitesides (1995a) prepared SAMs from alkanethiols terminated in short oligomers of ethylene oxide. These SAMs showed resistance to the adsorption of several model proteins, as measured by both ellipsometry and surface plasmon resonance methods. It was observed that even SAMs containing as much as 50% methyl-terminated alkanethiolates, if mixed with oligo(ethylene oxide)-terminated alkanethiolates, resisted the adsorption of proteins. DeGennes and Andrade (1991) predicted that surfaces comprising densely packed, nearly crystalline chains of PEO might not resist the adsorption of protein, because there is sufficient free volume in the ethylene glycol layer of the SAMs to allow solvation by water, and on this basis, it is not expected that SAMs presenting densely packed tri(ethylene oxide) groups would resist the adsorption of protein, as was in fact found by Mrksich and Whitesides (1995b). Prime and Whitesides (1993) also used patterned SAMs on gold with elements ranging in size down to 1μm. Microcontact printing or photolithographic methods were used to produce these patterns and thus control the adsorption of protein to surfaces. The SAMs were patterned into regions terminated in different functional groups such as methyl, oligo(ethylene oxide) or negatively charged sulfonate groups (Lopez et al, 1993a). It was found that the

adsorption of a monolayer of protein occurred only on the methyl-terminated regions, and this pattern of protein could be imaged by SEM (Lopez, et al, 1993b). The same methods used to give patterned adsorption of proteins to surfaces have also been used to direct the attachment of cells to surfaces. Whitesides et al. again used microcontact printing to pattern SAMs into lines ranging from 10 to 100 mm in width. After incubating these surfaces with fibronectin, endothelial cells were confined to growth on the lines (Singhvi, et al. 1994, Spargo, et al, 1994). The ability to pattern the attachment of individual cells may be useful for single cell manipulation, toxicology and drug screening.

Delamarche et al. (1994) used a photoimmobilization method to attach antibodies to a SAM film with the goal of producing a homogeneous monolayer of IgG, in which the proteins maximize their activity in a solid phase immunoassay This method emphasizes the utility of SAMs in creating and controlling the properties of surfaces in a useful manner leading to improved understanding of macromolecular interactions at interfaces.

Besides alkane thiols (resulting in SAMs), short peptides and other molecules containing thiols have been adsorbed to gold surfaces and their interactions with plasma proteins and the immunologic system has been investigated. For example, Tengvall et al. (1994) have studied plasma protein interactions on bare gold and gold modified with L-cysteine (L-Cys) and 3-mercaptopropionic acid (MPA) using antibody probes as a method for detecting proteins.

SAMs of thiolates on gold provide the best system available with which to understand the interactions of proteins and cells with man-made surfaces. The ease with

which biologically relevant moieties can be presented in controlled environments, combined with simple methods that can pattern the SAMs in the plane of the monolayer, make these surfaces well suited for studies of fundamental aspects of biointerfacial science. Other advantages of these systems include the optical transparency of SAMs when supported on thin films of gold, the electrical conductivity of the underlying gold, the compatibility of these substrates with a range of analytical methodologies, their stability during storage and in contact with biological media, and the range of surfaces, including curved and nonplanar substrates, that can be used.

The use of thiol-gold chemistry to modify biomaterial surfaces for improving biocompatibility is an obvious extension of this technology and one which is being explored in this laboratory. Examples are attachment of PEO to provide a protein repellent surface, or of antithrombogenic agents to give surfaces of improved blood compatibility.

The work reported in this thesis falls into the latter category. The focus of the work is the use of peptide-modified gold coated polyurethane surfaces as potential antithrombogenic materials.

2.6 Hypothesis and Objectives of Present Research

As discussed previously, major problems in the application of blood contacting materials arise from protein deposition, platelet adhesion and aggregation, and triggering of the blood coagulation process. Thrombin plays a central role in blood coagulation and

platelet-related thrombogenic processes. Thrombin not only exhibits coagulant activities such as conversion of fibrinogen to fibrin, and activation of factors V, VIII, XI and XIII, but also initiates several cellular and vascular reactions. The inhibition of thrombin has therefore become a major concern in the development of blood compatible materials.

The objective of this work is based on the hypothesis that properly attached anti-thrombin agents may prevent the activation of platelets and of the coagulation process on a material surface and thus improve the blood compatibility of the material. The hypothesis includes four components:

(1) The surface-bound anti-thrombin agent should selectively bind thrombin and inhibit its activity thereby blocking the coagulation process on the biomaterial surface.

(2) The immobilized anti-thrombin agent should retain its biological specificity after it is appropriately bound to the surface.

(3) The surface coverage of antithrombin agent should be high, leaving no bare surface with which protein may interact.

(4) The surface-bound antithrombin agent should form a surface specific for thrombin but inert to other plasma proteins, specifically the contact phase proteins and platelet adhesive proteins, to prevent activation of coagulation, and platelet adhesion and aggregation on the surface.

Some work has previously been done in this laboratory concerning the first component of the hypothesis, involving the immobilization of PPACK to polyurethane surfaces through attachment to the polymer chains (Tian, 1995). These materials showed

some potential as thrombin inhibiting surfaces. However, due to the high reactivity of the chloromethyl ketone group, PPACK is relatively unstable both on a surface and in solution over long periods. The tripeptide (D)FPR without the chloromethyl ketone moiety also shows antithrombotic activity but is more stable than PPACK. (D)FPR also has low affinity for the binding of plasma proteins, and does not cause platelet activation (Bajusz et al, 1990), suggesting that it may be a specific and effective anti-thrombin agent when attached to a material surface.

Different methods have been used in this laboratory to graft anti-thrombin agents to surfaces including grafting to polymer chains, surface grafting, and cold plasma polymerization. In this work the attachment of anti-thrombin agents to surfaces using gold-thiol chemistry is explored. These agents are based on the FPR tripeptide to which an N-terminal cysteine is attached, i.e. CFPR.

Specifically, the objectives of this work are:

(1) To develop new antithrombogenic materials by attaching the peptides, CPR, C(L)FPR and C(D)FPR, potential thrombin inhibitors, to gold coated polyurethane surfaces through thiol-gold reactions.

(2) To measure the surface properties of these peptide-modified gold coated polyurethanes using contact angle measurements to investigate wetting and surface hydrophilicity; using ellipsometry and infrared reflection absorption spectoscopy (IRAS) to measure the peptide layer thickness and orientation; using XPS to investigate the

surface chemical composition; and using AFM and SEM to study surface morphology and topology.

(3) To study protein adsorption to these surfaces using radio-labelling techniques which can quantify the adsorption of single proteins either from buffer or plasma, particular emphasis to be given to thrombin adsorption.

(4) To investigate the ability of the peptide-modified gold surfaces to inhibit the enzymatic activity of adsorbed thrombin.

(5) To investigate the adsorption of plasma proteins more generally to the surfaces using gel electrophoresis and immunoblotting methods.

3. EXPERIMENTAL

3.1 Surface Modification

In this study, gold coated polyurethane film was used as the base material. Three potential thrombin inhibitors C(D)FPR, C(L)FPR and CPR were chemisorbed onto this gold surface. Cysteine modified gold and unmodified gold surfaces served as controls.

3.1.1 Materials

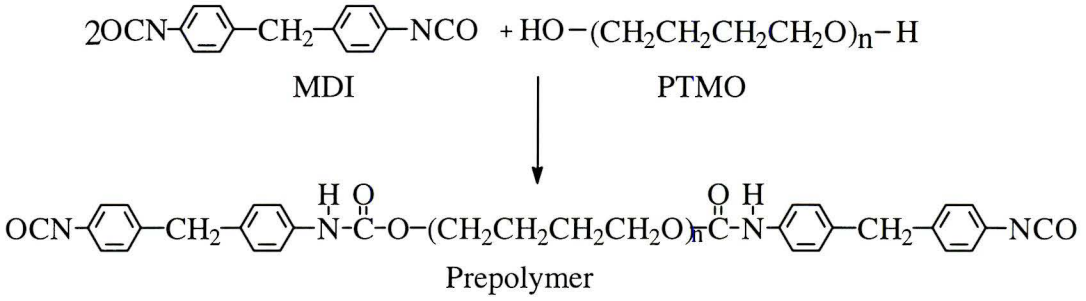
All chemical reagents, solvents and materials used in these experiments are listed in Appendix A along with the source and location of the supplier.

3.1.2 Polyurethane Synthesis

The base polyurethane used in these studies "ED980", was synthesized according to methods routinely used in this lab (Santerre, 1990). The polyol chosen for this synthesis was polytetramethylene oxide (PTMO) of molecular weight 980, since it is well known that PTMO-based polyurethanes show generally superior mechanical properties (Lelah and Cooper, 1986). Prior to use, PTMO was dried in a vacuum oven at 60°C for 24 h to eliminate any water contamination in the prepolymer reaction. The diisocyanate utilized in this synthesis, 4,4'-diphenylmethane diisocyanate (MDI), was purified by vacuum distillation at 2 mmHg pressure to remove any contamination by dimers and trimers of MDI. The purified MDI was stored in an airtight container at –10°C. The

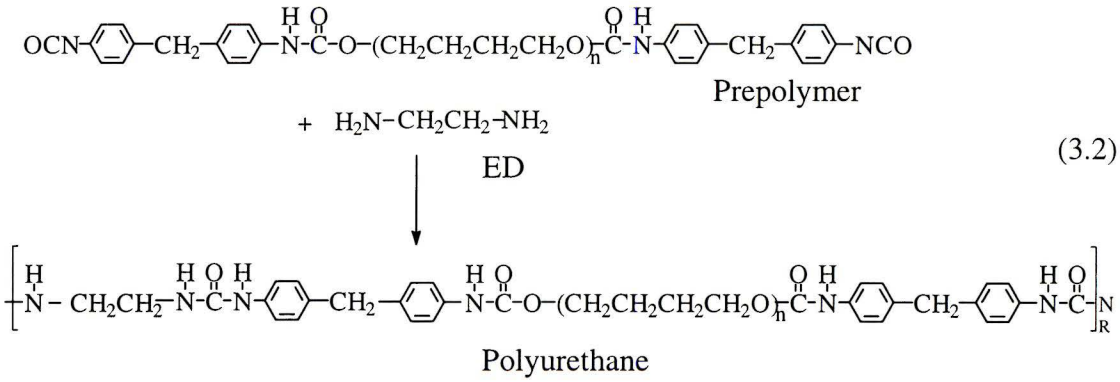
chain extender employed in this reaction, ethylene diamine (ED), was dried over 4Å molecular sieves for 24 h prior to use.

The polymerization reaction was carried out in dimethylsulfoxide (DMSO) using a two-step polymerization procedure (Santerre et al., 1992). In the first step, a prepolymer was synthesized by reacting MDI with PTMO in 2:1 stoichiometric ratio for 60 min at 90°C (Equation 3.1).



(3.1)

The prepolymer was then chain extended by reacting with ED in 1:1 stoichiometry for 30 min at 70 °C (Equation 3.2).



(3.2)

Reactions were carried out in a dry nitrogen atmosphere to prevent water contamination. The resulting polymer was precipitated and washed extensively in distilled water. The precipitate was then dried at 45°C for approximately two days in a conventional forced air drying oven. The dry polyurethane was stored in a brown glass bottle in the dark to prevent UV degradation until use.

3.1.3 Polyurethane Film Casting

Polyurethane in film form is required as a substrate for gold coating and surface modification. Dried ED980 was dissolved in dimethylformamide (DMF) at a concentration of 6 % (wt/vol). A series of 7 cm diameter glass Petri dishes (Pyrex) were placed level in a convection oven. Fifty milliliters of ED980 solution was poured into each dish. The dishes were covered with cardboard sheets to keep out dust and to prevent excessively fast solvent evaporation, which can result in surface irregularities. The films were held at 45°C for 2 - 3 days. After casting was complete, the films were placed in a vacuum oven at 45°C for at least 48 h. The thickness of the polyurethane films was about 0.5mm.

3.1.4 Gold Coating

The gold coating procedure was performed in the Department of Physics (Dr. M. Sayer) at Queen's University. Gold was deposited on the both sides of the ED980 polyurethane film surface in a standard vacuum evaporation system (EDWARDS AUTO 306 COATING SYSTEM). Polyurethane substrates were located at a distance of 12 cm

from the source in order to produce uniform film thickness. The system was evacuated to pressures of approximately 5 x 10^{-6} Torr before evaporation. The thickness of the gold coating was measured by use of a thickness monitor, and was typically of the order of 1000 Å. It has been found (Atre et al. 1995) that in general gold does not adhere well to polymer surfaces, particularly if they are smooth. Typically a very thin layer (~10 nm) of chromium or titanium is applied to the substrate prior to gold coating to improve the adhesion of the gold to the substrate (Tengvall, 1992; Liedberg, 1991; Nuzzo et al. 1986). Since the polyurethane surface cast in this lab was relatively rough, it was found that the gold adhered well without the need for chromium as a primer.

3.1.5 Chemisorption of Peptides to Gold Coated Polyurethane Surfaces

The peptides Cys-(D)-Phe-Pro-Arg (C(D)FPR), Cys-(L)Phe-Pro-Arg (C(L)FPR), and Cys-Pro-Arg (CPR) were obtained from Queen's University Peptide Synthesis Lab. The synthesized peptides were purified by HPLC, and the purity was determined by mass spectrometry. The resulting peptides were stored frozen at -10°C prior to use.

It is well known that gold is easily contaminated when exposed to lab air, and it is therefore important to clean the gold surface prior to the chemisorption process. Gold surfaces (6.5 mm diameter discs) were cleaned using a peroxide-ammonia solution (designated TL-1) made by combining one part (vol/vol) aqueous hydrogen peroxide (30 %), one part aqueous ammonium hydroxide (25 %), and five parts water. TL-1 solution is a strongly oxidizing mixture which can undergo explosion upon contact with organic materials and thus must be handled with care. The gold discs were immersed in TL-1

solution for 10 minutes at 80°C, then rinsed extensively with distilled water. The Petri dishes used for chemisorption were also cleaned in this manner in order to minimize contamination of gold during chemisorption. Clean, gold-coated polyurethane films were immersed respectively in solutions (2mM in water) of C(D)FPR, C(L)FPR, CPR and cysteine for 18 h at room temperature, then rinsed three times with distilled water. Some samples were immersed in pure water under the same conditions. These samples and the cysteine-chemisorbed materials served as controls.

3.2 Surface Characterization

All surfaces were characterized using several techniques: water contact angle, X-ray photoelectron spectroscopy (XPS), atomic force microscopy (AFM), scanning electron microscopy (SEM), and ellipsometry.

3.2.1 Water Contact Angle Measurements

Water contact angle data on the peptide modified gold surfaces were used to determine the relative hydrophilicity and hydrophobicity of the materials. Both sessile drop and captive air-bubble-in-water contact angles were measured with a goniometer (Rame-Hart, Bloomfield, N.J.). In the case of the sessile drop measurements, advancing and receding contact angle readings from both sides of each drop were measured on the modified gold surfaces of interest. The surfaces (polyurethane with gold and modified gold coatings) were mounted on glass microscope slides.

The advancing angle was obtained by introducing a small drop of water (approximately 10 μL) onto the test surface via a syringe. The drop was allowed to spread to an equilibrium position and the tangent at the contact line of the drop was measured. 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 receding and advancing angles is known as contact angle hysteresis. The captive bubble contact angle was measured by introducing a small (~ 2 mm) air bubble to the water-gold interface (Wojciechowski, 1991). Angles were again measured using a goniometer.

3.2.2 X-ray Photoelectron Spectroscopy (XPS)

XPS was employed to provide quantitative chemical information on surface composition. This technique requires exposing the material surface to an X-ray beam causing emission of photoelectrons from core levels. The energy of the emitted photoelectrons is indicative of the chemical elements and their bonding environment while the intensity of the emissions is related to the concentration of the chemical species (Turner, 1992). The method is surface sensitive since only electrons generated near the surface (within about 100Å) can escape to reach the detector.

The analysis was performed at the University of Toronto Surface Analysis Facility using a Leybold Max 200 X-ray photoelectron spectrometer. Following 18 h chemisorption, and extensive rinsing with distilled water, the CFPR-, CPR-, and cysteine-

modified gold surfaces were placed into the XPS vacuum chamber. Low resolution XPS spectra were recorded. In order to minimize surface contamination, the XPS spectra for the unmodified gold surface were taken immediately after washing in the TL-1 solution. The measurements were obtained using detector take-off angles ranging from 90^{o} to 10^{o} to obtain compositional depth profiles. The take-off angle determines the depth in the sample from which photoelectrons may reach the detector.

3.2.3 Atomic Force Microscopy (AFM)

AFM was used for examination of surface roughness and topography. The essential components of an AFM are as follows: a probe tip which interacts with the sample, a cantilever whose role is to translate the forces acting upon the tip into a measurable quantity. Either the sample or the cantilever assembly is controlled from a computer that enables raster scanning in the x (fast) and y (slow) directions. As a scan is made, the probe tip, and thus the cantilever, is deflected by its interaction with the surface. The AFM imaging process is shown schematically in Figure 3.1. Data on the deflection of the cantilever in the Z direction are collected as a raster scan over the surface is performed. The coordinates of points on the surface are collected and processed to produce a space-filling three-dimensional image (Goh, 1995).

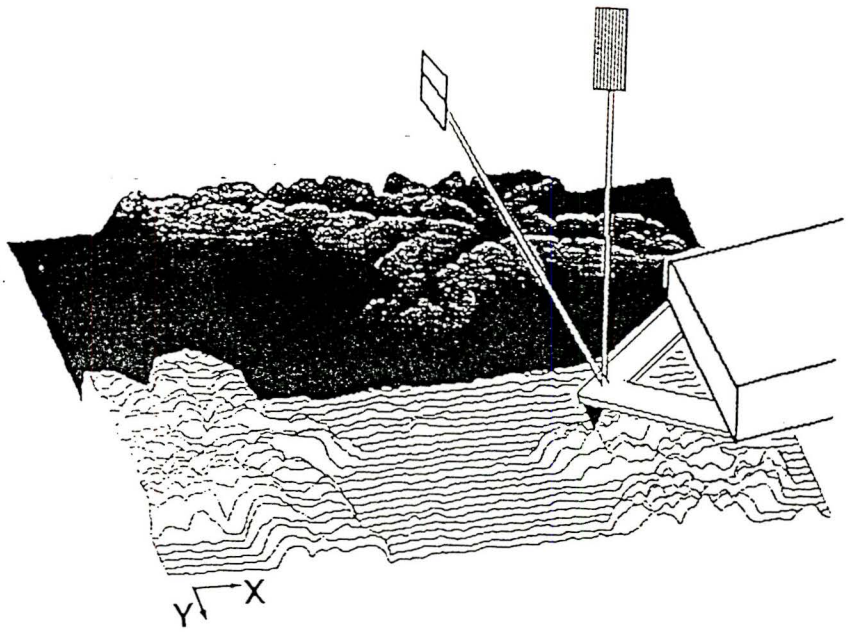


Figure 3.1 Illustration of the AFM imaging process. The fast (X) and slow (Y) directions of the raster scan are indicated. (From Goh, 1995.)

In this study, AFM was employed to obtain qualitative information on morphology differences between modified and unmodified gold surfaces, as well as on the morphology of adsorbed thrombin layers. The microscope used was a Nanoscope II (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 samples imaged in both the constant height and constant force mode. The constant force mode of imaging was used with an applied force of less than 8 x 10^{-9}N (Gale, et al, 1995). AFM was performed on the modified and unmodified cleaned gold surfaces. Images of the surface morphology were obtained, and the relative roughness of the surfaces was estimated. After thrombin adsorption to these modified surfaces, AFM images were taken again. Since the imaging process relies on

interactions between the sample and the probe tip, the probe tip is the crucial part of an AFM. Most problems concerning reproducibility in imaging may be attributed to uncertainties regarding the tip geometry (especially when materials from the surface are picked up), and the nature of tip interactions with the sample. In the AFM experiments on thrombin-adsorbed surfaces, it is possible that the protein may be "ploughed" or removed from the surface by lateral motion of the probe tip resulting in alteration of the protein layer.

3.2.4 Scanning Electron Microscopy (SEM)

SEM has been widely used for visualization of organic surfaces, especially in the study of surface morphology, domains, pinholes, other defects, and patterns (Janssen et al. 1980, Wasserman, et al. 1989). In this study, SEM was used for visualization of the gold and modified gold surface morphology and defects. Both gold-coated glass and gold-coated polyurethane samples were mounted on an aluminum stub, and grounded with silver paint. A Phillips 501-B SEM was used, mounted with a Polaroid camera to take polaroid images. The SEM was operated at 7 to 15 kv with a spot size between 200 and 500 nm.

3.2.5 Fourier Transform Infrared Reflection-Absorption Spectroscopy (FT-IRAS)

In order to evaluate the chemisorbed peptide layer in terms of coverage conformation and orientation, FT-IRAS spectroscopic measurements were made. This

work was done in Dr. Pentti Tengvall's laboratory in the Department of Applied Physics, Linkoping University, Linkoping, Sweden. Spectra were obtained using a BRUKER IFS 113 v Fourier Transform Spectrometer, equipped with a DTGS detector and a GIR (Grazing angle Infrared Reflection) accessory aligned at 83°. Information on the molecular structures of the formed monolayers was obtained from the absorption spectra by comparison with earlier measurements (Lestelius, 1994). All spectra were reported as -log (R/R_0) where R is the reflectivity of the peptide-on-gold interface and R_0 is the reflectivity of the bare gold reference surface.

3.2.6 Ellipsometry

Ellipsometry is an optical technique that can be used to determinate the thickness and refractive index of thin homogeneous films. When plane-polarized light impinges on a surface at some angle, the parallel and perpendicular components (s- and p-polarized, respectively) are reflected from the surface in different ways. The result is elliptically polarized light (Azzam and Bashara, 1977), the properties of which can be used to estimate the thickness of a transition region between the surface (Figure 3.3) and air by measuring the two quantities Ψ and Δ. Δ is the phase shift between the parallel and perpendicular components, and Ψ is the change in the ratio of the amplitudes of these two components. For a clean surface, Ψ and Δ are directly related to the complex index of refraction of the surface, which is related to the complex indices of both the film and the substrate, and to the film thickness (Birzer and Schulzer, 1986). An estimate of the

refractive index of the organic film is required in the calculation. Usually, a value of 1.50 is used as the refractive index for organic layers. This value is based on the assumption that the monolayer is crystalline, and similar to polyethylene. Its refractive index thus should be in the region 1.49 ~ 1.55 (Allara, 1985).

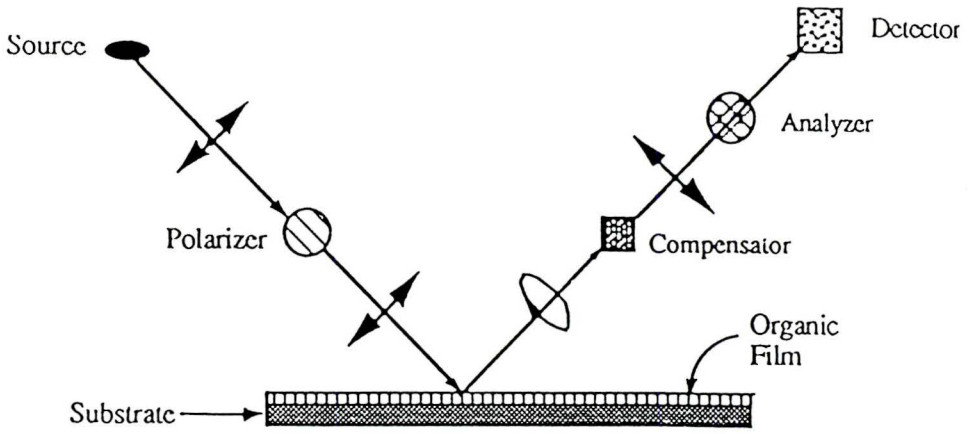


Figure 3.3 Schematic of an ellipsometer. (Adapted from Gaines, 1965.)

In this work, ellipsometry measurements were used to estimate the thickness of chemisorbed peptide layers. These measurements were carried out by Dr. P. Tengvall at Linkoping University in Sweden. A Rudolph Research AutoEL III ellipsometer equipped with a He-Ne laser (λ=632.8 nm) as light source was used to measure the analyzer and polarizer angles of the substrate at 632.8 nm. The measurement was repeated after chemisorption of the peptides, and the equivalent thickness of the peptide layers was calculated from the ellipsometric data using an isotropic three-phase (ambient/organic

film/gold substrate) model and a refractive index of 1.465±0.3 nm for the peptide layer (Lestelius & Tengvall, 1997).

3.3 Protein Adsorption

The first event of blood contact with artificial materials is the adsorption of plasma proteins to the material surface. The surface properties of the material will influence its interactions with proteins and are likely to affect the protein conformation and biological (enzymatic) activity (Andrade, 1985; Norde and Lyklema, 1991). In this study the peptides C(D)FPR, C(L)FPR and CPR, as potential thrombin inhibitors, were chemisorbed to gold surfaces. Such surfaces are expected to be selective for thrombin binding and to inhibit the activity of adsorbed thrombin. Adsorption of thrombin as a single protein from buffer and from plasma to these peptide modified gold surfaces was therefore studied in this work.

3.3.1 Plasma preparation

Human plasma was depleted of both antithrombin-III (ATIII) and fibrinogen for these studies. The objective in using this modified plasma is to enable investigation of thrombin-surface interactions during plasma contact. Depletion of antithrombin III is required so that formation of thrombin-ATIII complex can be avoided, and depletion of fibrinogen prevents plasma clotting. ATIII was removed from normal human plasma by affinity chromatography using a heparin-Sepharose gel (Miller-Andersson, 1974).

Sodium chloride was added to normal citrated plasma to achieve a high salt concentration (0.5 M). This plasma was then passed through a heparin-Sepharose column at room temperature at a flow rate of about 60 mL/h. The fractions with activated partial thromboplastin time (APTT) values identical to plasma were pooled and dialyzed twice at 4°C for two hours against 6 L of buffer containing 0.4 % tri-sodium citrate and 0.15 M sodium chloride, pH 7.35.

The ATIII-depleted plasma was then depleted of fibrinogen using the thrombin-like snake enzyme, Ancrod (Sigma, St. Louis, MO, USA). Ancrod solution (1 mg/mL in distilled water) was mixed with plasma (30 μL Ancrod per mL plasma), and the mixture was incubated in a water bath at 37°C for 15 min. The clotted plasma was then centrifuged for 4 min, and the clot was removed. The ATIII- and fibrinogen-depleted human plasma was stored at -70°C in 1.0 mL aliquots.

3.3.2 Thrombin Labelling with ^{125}I

For studies of thrombin adsorption from buffer and plasma to modified surfaces, the enzyme was radiolabelled with ^{125}I. In this work, 1,3,4,6- tetrachloro-3a,6a-diphenylglycoluril (Iodogen®) was used as a solid-phase reagent for iodination of thrombin (Knight, 1981; Millar and Smith, 1983).

$Na^{125}I$ was purchased from ICN Pharmaceuticals Inc. (Irvine, CA, USA), and Iodogen® from Pierce Chemical Company (Rockford, Illinois, USA). Human α-thrombin with activity of 3192 NIH U/mg was purchased from Enzyme Research Laboratories Inc.

(Southbend, Indiana, USA). The thrombin was diluted with phosphate buffered saline (pH 7.4) to 0.27 mg/mL, aliquoted in 1 mL volumes, and stored at -70°C.

For iodination, Iodogen® was adsorbed on the surface of a conical glass 3 mL vial by slowly evaporating a solution (100 μL, 0.1 mg/mL in chloroform) at room temperature in a dry nitrogen environment. The Iodogen-coated vials could be stored for up to 6 months at -20°C (Markwell and Fox, 1978).

$Na^{125}I$ (0.5 mCi, 5 μL) was added to the reaction vial, and the contents stirred for 1 min; 150 μL thrombin (0.27 mg/mL) was then added. After reacting for 15 min, the thrombin sample was removed from the vial to terminate the iodination reaction. The vessel was rinsed twice with 400 μL PBS buffer. In order to remove unreacted ^{125}I, the sample was injected into a SLIDALYZER® dialysis cassette (Pierce) and dialyzed three times at 4°C for 12 h against PBS (500 mL). The ^{125}I-radiolabelled thrombin was removed from the dialysis cassette, and the cassette was rinsed twice with 500 μL PBS to reduce protein loss.

3.3.3 Free-iodide Test

Since residual labelled iodide ion can bind to the gold-based surfaces, thereby invalidating the adsorption data (Cochran and Farrell, 1980, Sheardown et al, 1997), it is important to minimize the free or unbound radioiodide content of the labelled protein solutions. In this work, the trichloroacetic acid (TCA) precipitation method was used to

determine the amount of free iodide in a labelled protein sample. The method is as follows.

In each of six 1.5 mL micro-centrifuge tubes, 0.1 mL of a 1:10 dilution of labelled protein solution and 0.9 mL bovine serum albumin (1% w/v) were mixed. Three of these aliquots served as "A" solutions, and the radioactivity was determined. To the other three samples, 0.5 mL of 20 % trichloroacetic acid was added. The mixture was vortexed briefly and kept at room temperature for 10 min to precipitate the protein. It was then centrifuged for 2 min at 3000 RPM. Supernatant (0.5 mL) was added to 0.5 mL PBS and the radioactivity of these solutions (B) was determined. The free-iodide content (as a percentage of total radioactivity) was estimated using equation 3.3:

$$\text{Percent free } {}^{125}\text{I} = \frac{3(B_1 + B_2 + B_3)}{(A_1 + A_2 + A_3)} \text{x}100 \qquad (3.3)$$

Typically, the free iodide content of the thrombin solutions was between 2 and 5%.

3.3.4 Effect of Labelling on Protein Adsorption

To check the effect of labelling on thrombin adsorption, adsorption was measured using a series of labelled thrombin solutions having the same total protein concentration (10 U/mL) but varying proportions of labelled and unlabelled protein (1 to 100 % labelled protein). The adsorption experiments were run under the same conditions as described below (see section 3.3.5). As well, the effect of labelling on adsorbed thrombin activity was measured using a chromogenic substrate assay described in section 3.4.2.

3.3.5 Thrombin Adsorption from Buffer to Modified Gold Surfaces

All thrombin adsorption experiments were carried out in Linbro® "EIAII plus" multiwell microtitration plates (Flow Laboratories, Inc., McLean, Virginia, USA). Serial dilutions (concentrations ranging from 20 to 0.1 μg/mL) were made from an initial mixture of 20 % ^{125}I-labelled, and 80% unlabelled thrombin in PBS. Surfaces and unmodified gold surfaces were placed "upright" in the wells, and incubated with 250 μL of labelled thrombin solution for 3 h at room temperature. Following adsorption, the surfaces were rinsed three times in PBS, dried by blotting gently with filter paper, and the radioactivity determined. The adsorbed proteins were then eluted from the surfaces by incubating with 1 mL of 2 % SDS solution overnight. The eluted surfaces and the eluates were counted to determine the relative contributions of iodide ion and labelled protein to the total surface radioactivity, based on the assumption that the iodide ion cannot be removed by SDS while the protein is eluted (Sheardown et al, 1997). The surface concentration of thrombin was estimated from the radioactivity of the eluates using equation 3.4:

$$\text{Adsorbed thrombin} = \frac{Mass\ \ of\ \ Thrombin}{Surface\ \ Area} = \frac{CPM_2\ \ *\ \ C_{Th} * V_1}{\text{Surf. Area}\ \ *\ \ CPM_1} \qquad (3.4)$$

C_{Th}: Concentration of thrombin in original thrombin solution (μg/mL).
V_1: Volume of thrombin solution used for reference (mL).
CPM_1: Radioactivity of reference thrombin solution (counts per min, cpm).
CPM_2: Radioactivity of eluate (cpm).

Triplicate samples were run under given conditions (adsorption time, thrombin concentration, surface) within an experiment. Completed Experiments were done a minimum of three times.

3.3.6 Thrombin Adsorption from Plasma to Modified Gold Surfaces

Human plasma was depleted of both ATIII and fibrinogen as described in section 3.3.1. A mixture of 20 % ^{125}I-labelled and 80 % unlabelled thrombin was added to the plasma to attain a concentration of 10 U/mL (equivalent to 3.3 μg/mL, the average concentration in normal human plasma if all the prothrombin were converted to thrombin). This plasma was then serially diluted with PBS (pH 7.4) to make plasma solutions of 1 %, 10 %, 25 %, 50 % and 80 % strength. The modified and unmodified gold surfaces are incubated with this modified plasma immediately after I^{125}-labelled thrombin were added in to avoid the cross reaction between thrombin and Heparin cofactor II or α_2-microglucbin. The adsorption conditions and procedure were identical to those described in section 3.3.4.

3.3.7 Fibrinogen Adsorption from Plasma to Modified Gold Surfaces

Fibrinogen adsorption from plasma was studied for comparison to thrombin. The goal of these experiments was to provide a further test of thrombin specificity for the peptide-modified surfaces..

Normal human plasma was used in these experiments. Human fibrinogen (Calbiochem, La Jolla, California, USA) was labeled with ^{125}I using the ICl method as described by McFarlane (1958). ^{125}I-labelled fibrinogen was added to the plasma in amounts corresponding to 3 % of the endogenous fibrinogen concentration. The adsorption conditions and procedures were identical to those for thrombin adsorption from plasma described in Section 3.3.5.

3.4 Chromogenic Substrate Assay for Thrombin

In addition to the selective adsorption of thrombin from plasma, the ability of the CFPR- and CPR-peptide-modified surfaces to inhibit adsorbed thrombin is another important aspect of this study.

A number of methods have been developed to study the activity of adsorbed enzymes. In this work a chromogenic substrate assay was used as a simple and quick method to investigate: (1) the activity of free thrombin in solutions of the peptides as a measure of the inhibitory activity of the peptides; (2) the activity of adsorbed thrombin on the peptide-modified gold surfaces.

The thrombin substrate used in this work was the synthetic conjugated tripeptide N-p-tosyl-gly-pro-arg-p-nitroanilide (Sigma, St. Louis, MO, USA). It is rapidly cleaved at the arg-p-nitroanilide bond by thrombin to produce p-nitroaniline (pNA), a chromophore which can be measured colorimetrically at 405 nm. The change in absorbance per minute is related to the enzyme activity.

3.4.1 Activity of Thrombin in Solutions of Peptides.

As noted in the literature review, (D)FPR has been recognized as a powerful, direct thrombin inhibitor. The related peptides C(D)FPR, C(L)FPR, and CPR used in this work are expected also to be thrombin inhibitors. However, it was necessary before proceeding with the project to demonstrate that this was indeed the case. The activity of these peptides as thrombin inhibitors was therefore determined and compared to that of FPR-chloromethyl ketone (PPACK), which is well known as a powerful thrombin inhibitor of high specificity.

In order to establish optimum conditions for these experiments, thrombin concentrations of 0.03, 0.1, 0.33, 1.65 and 3.3 μg/mL were tested. Peptide concentrations were in the range of 2 to 20 mM, and the incubation time was varied from 5 to 15 min.

Using a 96-well polystyrene tissue culture plate, 200 μL of thrombin solution was added to each well. Solutions (50 μL) of C(D)FPR, C(L)FPR, CPR, PPACK and PBS (as a control) were added respectively to the thrombin solutions. After incubation, 50 μL of substrate (0.3 mg/mL in PBS, pH 7.4) was added. The ratio of substrate to enzyme was more than 100:1. The absorbance at 405 nm was measured immediately, and every 15s thereafter for 30 min in a UV plate reader. A series of assays was run at different thrombin concentrations to obtain a calibration curve in which the initial slope of the absorbance - time curve was plotted against mass of thrombin. The activity of thrombin in the inhibitor solutions was then estimated using the calibration curve.

3.4.2 Activity of Adsorbed Thrombin on Modified Gold Surfaces

Assays for activity of adsorbed thrombin on the modified surfaces were done as follows. Thrombin was adsorbed for 3 h from a pure thrombin solution in PBS, pH 7.4 at a concentration of 10 U/mL to the C(D)FPR, C(L)FPR, CPR, cysteine and unmodified gold surfaces as described in section 3.3.4. The surfaces were rinsed twice in fresh PBS (pH 7.4), and were then placed in the wells of a 96-well polystyrene tissue culture plate. Substrate solution (250 μL, 0.5 mg/mL in PBS) was then added to each well. The surfaces were incubated with the substrate and absorbance measurements taken at 1, 2, 3, 4, 5, 7, 10, 15, 20, 30, 40, 50 and 60 min (separate samples for each time), after stopping the reaction by adding 50 μL of acetic acid (1 mg/mL) to the well. The absorbance at 405 nm was measured after removing the surface specimen.

3.5 SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Immunoblotting

Gel electrophoresis is a convenient analytical technique for the separation of proteins in a mixture. When combined with the sensitivity and specificity of immunoassay, both qualitative and quantitative information on the separated proteins can be obtained.

3.5.1 SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

In SDS-PAGE an electric field is applied across a support of cross-linked polyacrylamide gel. The protein samples, treated with SDS, which confers the same negative charge density on all the protein molecules, are loaded onto the gel, and migrate in the applied field. Separation depends therefore on the molecular size (weight) of the proteins relative to the pores of the gel, and not on protein charge.

3.5.2 Western Blotting.

In this method specific antibodies are used to identify the proteins. After migration in an SDS-PAGE gel, the protein samples are transferred onto an Immobilon® polyvinylidene fluoride (PVDF) membrane by electrophoresis (Towbin et al, 1979; Gershoni and Palade, 1983). The membrane is incubated with primary antibodies specific to the proteins of interest, and then with a second antibody directed against the first and conjugated to alkaline phosphatase. A chromogenic substrate (5-bromo-4-chloro-3-indolyl phosphate [BCIP] and nitroblue tetrazolium [NBT], Bio-Rad, Richmond, CA, USA), is used to develop a color reaction with alkaline phosphatase, thus revealing the presence of the protein of interest.

3.5.3 Western Blot of Thrombin Adsorbed from Buffer and Plasma and Eluted

In this assay, a biotin-labelled sheep anti-human thrombin antibody was used as first antiboldy, and alkaline phosphatase-conjugated avidin for detection.

The detailed procedure for Western blots has been described in previous reports from this laboratory (Cornelius, 1993). All electrophoresis reagents were obtained from Bio-Rad, Richmond, CA. SDS-PAGE gel electrophoresis using a 12 % separating gel and a 4% stacking gel were run on a Mini-protean II® cell (Bio-Rad) at 200 V. After protein adsorption from buffer and from plasma, the proteins were eluted from the surfaces using very small amounts of 2% SDS solution (eight surfaces in 300 μL of 2% SDS). The protein-SDS eluates were separated by SDS-PAGE, and then transferred electrophoretically to a PVDF membrane. The blots were then cut into strips and, following blocking with 5 % nonfat milk in TBS buffer and rinsing three times with 0.1 % nonfat milk in TBS, the strips were incubated with a biotin-conjugated sheep antithrombin antibody in 1% nonfat milk with 0.05 % v/v Tween 20 in TBS buffer for 1 hour. After a further 3 rinses with 0.1 % nonfat milk, these strips were incubated with the appropriate alkaline phosphatase-conjugated avidin in 1% nonfat milk with 0.05 % v /v Tween 20, and rinsed again 3 times with 0.1 % nonfat milk. The color reaction was obtained by incubating these strips with the appropriate substrate, and stopping the reaction by rinsing with water. For thrombin, 10-column sample combs typically were used for one gel, and 10 μL of each thrombin-SDS eluate was loaded on each lane.

3.5.4 Westernblot Assay for Plasma Proteins

After exposure of surfaces to ATIII- and fibrinogen-depleted plasma (3h, room temperature) and elution with SDS, 100 μL of plasma-SDS eluate, or 5.0 μL of pure plasma, respectively, were loaded onto SDS-PAGE gels to separate the proteins. The

Western blotting procedure was similar to that for thrombin (Section 3.5.3). Typically, 100 μL SDS eluate was loaded on the gel for these experiments, and the blots were cut into 3 mm strips.

3.5.4.1 Primary Antibodies

Polyclonal antibodies to 25 different human plasma proteins were used in the immunoblots for protein identification. These primary antibodies were in the form of fractionated antisera (IgG fractions) developed in goat, sheep or rabbit, as listed in Table 3.1.

Table 3.1 Primary antibodies for Western blotting

Antibody to human protein	Raised in	Source
Factor XI	Goat	Nordic, Tilburg, Netherlands
Factor XII	Goat	Nordic, Tilburg, Netherlands
Prekallikrein	Sheep	Nordic, Tilburg, Netherlands
HMWK	Goat	Accurate, Westbury, NY
Fibrinogen	Goat	Sigma, St. Louis, MO
Plasminogen	Goat	Sigma, St. Louis, MO
Antithrombin III	Goat	Nordic, Tilburg, Netherlands
Complement C3	Goat	Sigma, St. Louis, MO
Transferrin	Goat	Sigma, St. Louis, MO
α1-antitrypsin	Goat	Enzyme Research Lab, Indiana
Fibronectin	Rabbit	Cappel Lab., Cochraneville,PA
Albumin	Goat	Sigma, St. Louis, MO
IgG	Goat	Sigma, St. Louis, MO
β-lipoprotein	Goat	Miles-YEDA Ltd, Israel
α2-macroglobulin	Goat	Sigma, St. Louis, MO
Vitronectin	Rabbit	Sigma, St. Louis, MO
IgM	Goat	Sigma, St. Louis, MO
Prothrombin	Sheep	Cedarlane Lab. Ltd, Hornby, ON
β2-microglobin	Rabbit	Sigma, St. Louis, MO
Hemoglobin	Rabbit	Sigma, St. Louis, MO

Haptoglobin	Rabbit	Sigma, St. Louis, MO
Complement C3c	Rabbit	Sigma, St. Louis, MO
Complement Factor B	Goat	Sigma, St. Louis, MO
Complement Factor H	Goat	Sigma, St. Louis, MO
Factor I	Goat	Sigma, St. Louis, MO

3.5.4.2 Enzyme-conjugated Second Antibodies:

The following affinity purified second antibodies used were: rabbit anti-goat IgG-alkaline phosphatase conjugate (Sigma, St. Louis, MO, USA); goat-anti-rabbit IgG-alkaline phosphatase conjugate (Bio-Rad, Richmond, CA, USA); rabbit anti-sheep IgG-alkaline phosphatase conjugate (Bio-Rad, Richmond, CA, USA).

In order to be able to compare the band intensities in different strips of the blots, the procedures were standardized, and all solutions and reagents were freshly prepared at the same concentrations. If we assume that all the proteins are eluted from the surfaces, then the intensities of the bands on the resulting blots can be used to compare the surfaces to one another; these intensities are related to the amount of protein adsorbed on each surface.

4. RESULTS AND DISCUSSION

4.1 Surface Modification

4.1.1 Polyurethane Synthesis

In this work, polyurethane ED980 was used as a substrate on which to deposit gold layers. It was synthesized using polytetramethylene oxide (PTMO) as soft segment, diphenylmethane diisocyanate (MDI), and ethylene diamine (ED) as chain extender following methods developed previously in this lab (Santerre, 1990).

4.1.2 Reaction Yield

The yield of the polymerization procedure was determined from the weight of the final dried polymer, and a value of 85 % (w/w of total reagents) was found. The polymer was precipitated in distilled water, and some losses may have occurred in this step.

4.1.3 Molecular Weight Determination

The molecular weight of polyurethane ED980 was determined by gel permeation chromatography (GPC). A typical GPC chromatogram is shown in Figure 4.1. The retention times were converted to number average molecular weights using a calibration curve constructed using polystyrene standards. The polymer peak is on the left of the

chromatogram (peak A), while the three narrow and small peaks B, C and D result from the presence of LiBr, water and air, respectively, in the system. Typical data for polymer ED980 gave 1.70 x 10^5 for $\overline{M_n}$, and 2.16 x 10^5 for $\overline{M_w}$, with a polydispersity, therefore, of 1.27. This is lower than the expected value of 2.0 for a step growth polymerization and the discrepancy may be due to losses in particular molecular weight ranges during the polymer work up process.

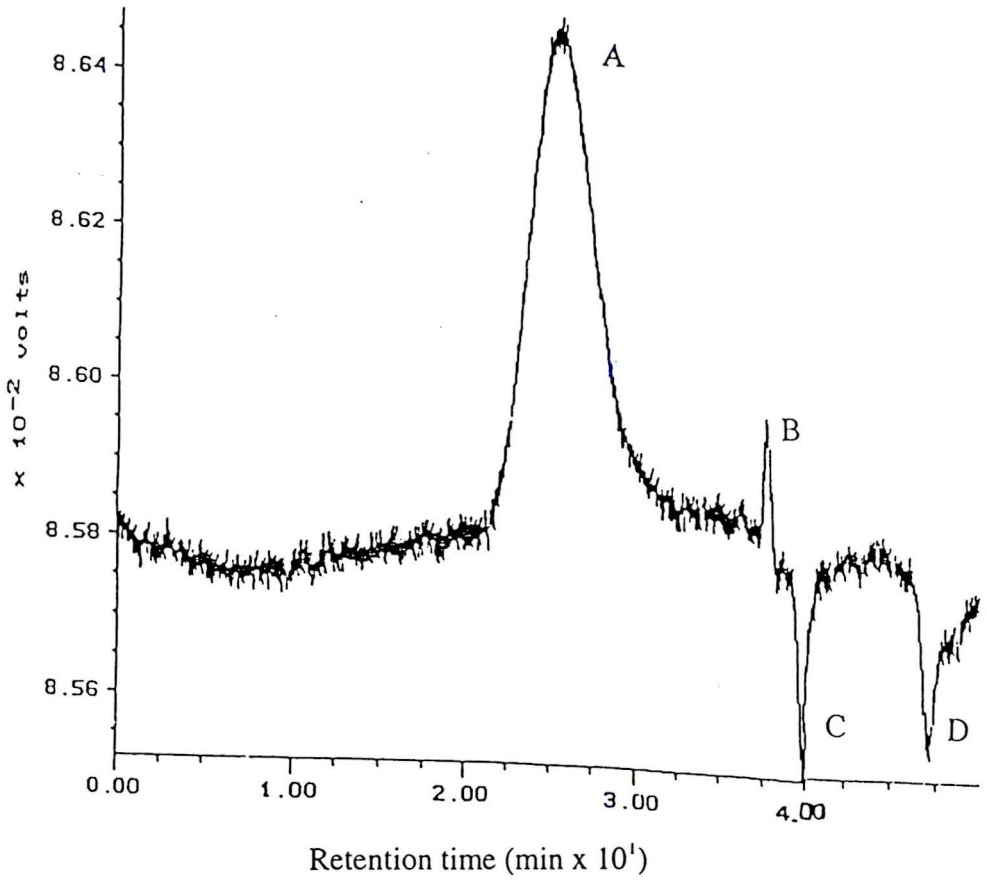


Figure 4.1 GPC chromatogram of ED980 polyurethane. The X-axis represents the retention time in minutes and the y-axis represents the relative intensity of the signal.

4.1.4 Film Casting

The quality of the polyurethane film formed in the glass casting dishes varied with drying temperatures and the position of the dish in the convection oven. Initially, the casting temperature was set at 50°C based on previous experience. However, a series of films formed in casting dishes located in the inside corner of the oven had high solvent evaporation rates which resulted in an irregular film, as determined by the rough nature of the surface. Upon reducing the casting temperature to 40 °C, the polymer films were formed more slowly (3 to 4 days). The final films under these condition were relatively smooth. During casting the polymer film has one side exposed to air and the other exposed to the smooth surface of the glass dish, and the roughness of these two surfaces appeared significantly different by visible inspection and by AFM. The roughness of the polyurethane surface affects, in turn, the roughness of the gold coating.

4.1.5 Gold coating

There are several gold coating techniques which are utilized in electronic applications, such as evaporation deposition, sputtering, and electrochemical deposition. The choice of method depends on the substrate material and geometry. Adhesion of gold to flat or smooth materials, such as glass or silicon wafers is poor(Atre et al. 1995). Typically a very thin layer (~10 nm) of chromium or titanium is applied to the substrate prior to gold coating. This serves to improve the adhesion of the gold to the substrate.

Since the polyurethane surface used in this work is relatively rough, it was found that using evaporation methods, the gold adhered fairly well without the need for chromium as an adhesive primer. This aspect is discussed in more detail in section 4.2.3.

4.1.6 Gold Surface Cleaning

Two cleaning methods were investigated: argon plasma cleaning and TL-1 washing (described in detail in Section 3.1.5). Following argon treatment, the water contact angle of the gold surface decreased from 65^{o} to 40^{o}, although the argon treatment did not remove all of the contaminants from the gold surface. The TL-1 washing procedure appeared to be more effective in this respect. The ammonia and hydrogen peroxide mixture is believed to oxidize the organic contaminants. A disadvantage of TL-1 washing was that some of the gold flaked off from the polyurethane during treatment. Inspection of the surfaces by SEM and AFM confirmed that some surface defects in the form of cracks and holes were present on the gold surface. This may have been caused by mechanical damage of the gold samples during boiling in the TL-1 solution. Thus, although TL-1 washing was an effective cleaning method, the gold surface was prone to damage if the treatment time was too long. Short treatments of 7 to 10 min were found to clean the gold surfaces adequately without causing significant damage.

4.1.7 Chemisorption of Peptides to the Gold Coated Polyurethane Surfaces

In order to optimize surface coverage of the peptides on gold, chemisorption times of 1 to 24 h were investigated. It was found that for cysteine, long periods of incubation gave surfaces from which the gold tended to flake off. This effect may be related to the cracks on gold produced by the TL-1 wash. It is possible that cysteine can gain access to the interface between the gold coating and polyurethane, causing gold to peel off from the polyurethane surface. For the peptides, C(D)FPR, CFPR and CPR, this access may be restricted. Even after a 24 h chemisorption time, the gold was not observed to peel off from these surfaces. Therefore the chemisorption time used for the majority of the experiments was 4 h for cysteine and 18 h for the peptides (C(D)FPR, CFPR, CPR).

4.2 Surface Characterization

The surface properties of the peptide-modified and unmodified gold coated polyurethanes were studied by several methods: wettability by water contact angle; surface composition and chemical structure by XPS analysis; morphology and topography by AFM and SEM; thickness of the peptide layers by ellipsometry; and peptide conformation and orientation by IRAS.

4.2.1 Contact Angle Measurements

Figure 4.2 shows water contact angle data for the modified and unmodified gold surfaces observed by both the sessile drop and the captive air-bubble-in-water techniques.

There is a clear decrease in the advancing and receding contact angles following chemisorption with cysteine, CPR, C(L)FPR, and C(D)FPR, indicating an increase in the hydrophilicity of the modified surfaces. Among all the surfaces examined, the cysteine-modified gold surface has the lowest advancing and receding water contact angles. The CPR-, C(L)FPR- and C(D)FPR-modified gold surfaces have similar advancing and receding angles, indicating similar surface wettability and hydrophilicity.

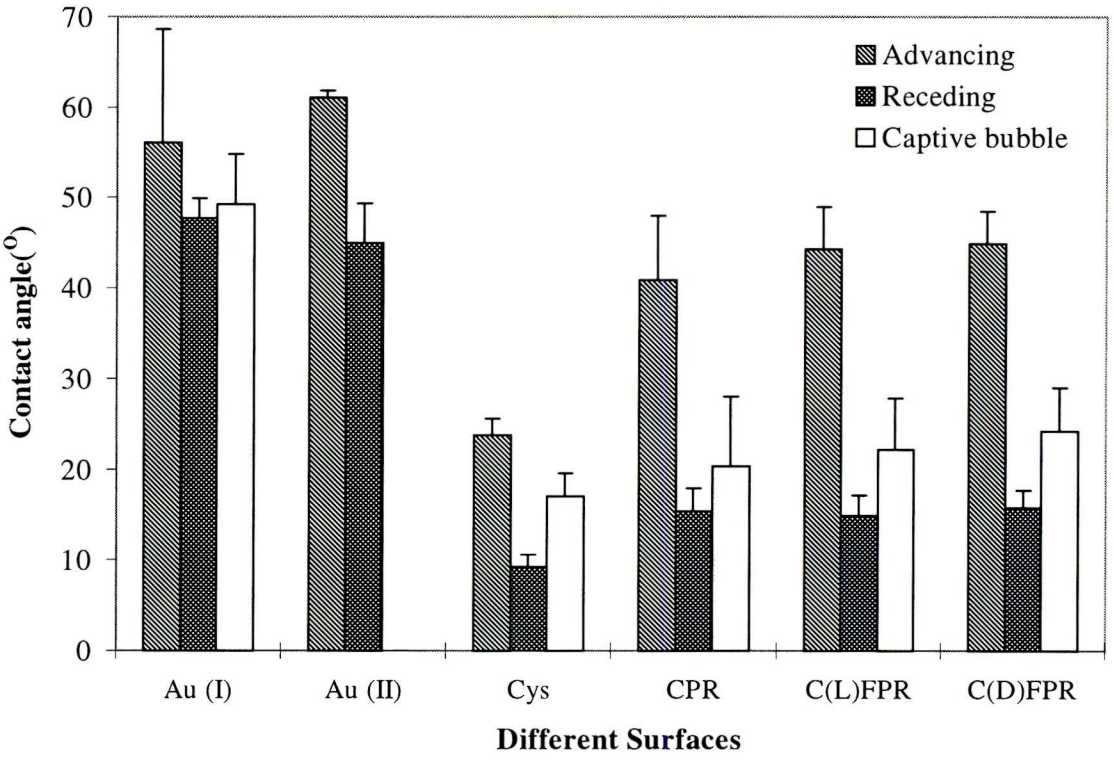


Figure 4.2. Water contact angles for the modified gold surfaces. Au (I), contact angle measured on gold after 18 hours incubation with water; Au (II), contact angle measured on gold immediately after TL-1 washing. Error bars are standard deviations (n = 10).

The gold surface itself, when measured immediately following the TL-1 wash, showed advancing and receding angles of 60° and 45° respectively. The contact angles did not change when the gold surface was incubated for 18h in water following a TL-1 wash. The unexpectedly high contact angles following cleaning probably result from rapid contamination by organic compounds which adsorb from the lab atmosphere to the gold surfaces. It is indicated in the literature (Bain et al. 1989) that contamination of gold occurs very rapidly, within minutes of exposure to air.

4.2.2 X-ray Photoelectron Spectroscopy (XPS)

The presence of chemisorbed C(D)FPR and CPR on the gold coated polyurethane surfaces was confirmed by XPS analysis. The low resolution spectra of the gold coated polyurethane as well as the C(D)FPR- and CPR-modified gold coated polyurethanes are shown in Figure 4.3, and demonstrate the presence of elements that are unique to the peptides, i.e. nitrogen and sulfur. Table 4.1 shows the atomic compositions derived from the low resolution spectra in Figure 4.3. The peaks for S (2p) and N (1s) were observed in the CFPR and CPR spectra but were absent from the unmodified gold spectrum, as expected. The ratio of nitrogen to sulfur was 5 : 1 for the CPR surface and 7 : 1 for the CFPR surface. These values are close to the theoretical nitrogen : sulphur ratios in these two peptides, namely 6 : 1 and 7 : 1 respectively. The XPS data thus confirm the presence of CPR and CFPR on the gold coated polyurethanes. The presence of the Au 4f signal in the CPR and CFPR surfaces may suggest that the peptides chemisorbed on gold

are not closely packed. The irregularities on the gold surface itself may be the cause of incomplete surface coverage. However, assuming a sampling depth of, say, 20 Å at 10° take off angle, the gold substrate may still be detected. High levels of carbon and oxygen were also observed on the unmodified gold surface indicating organic contamination. This is not unexpected since, as indicated, gold is quickly contaminated upon exposure to air. It appears that the gold-thiol reactions readily cause displacement of contaminants from the surface, although it may be concluded from the C and O content of the layer that some contamination remains. This also provides additional evidence that coverage of the peptides is incomplete.

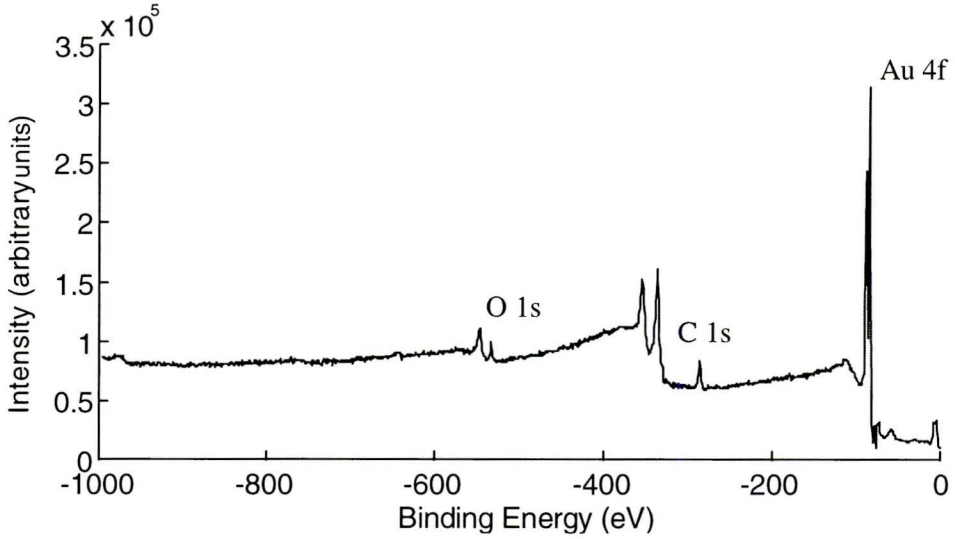


Figure 4.3 a XPS low resolution spectrum of gold coated polyurethane surface.

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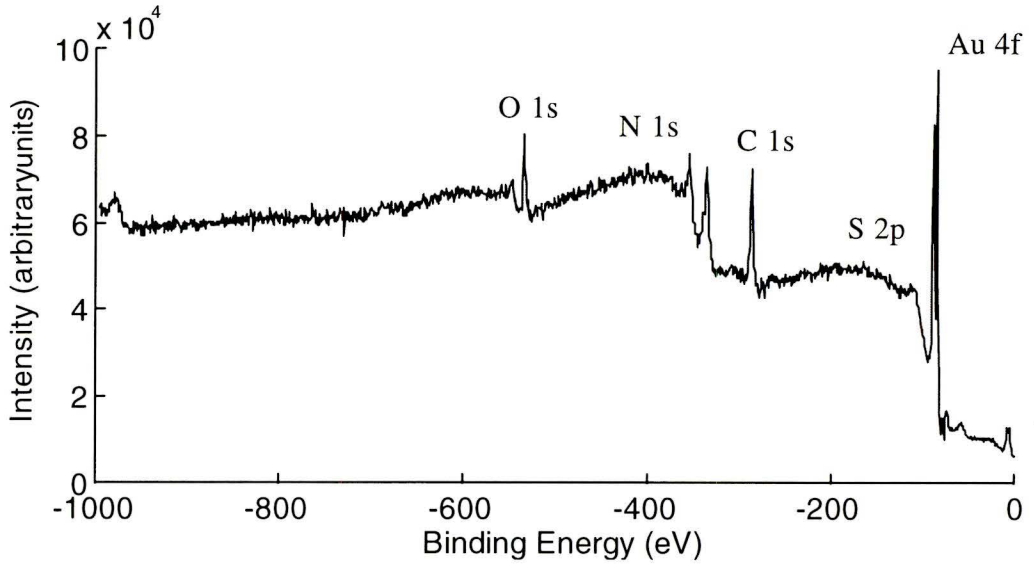

Figure 4.3 b XPS low resolution spectrum of CPR-modified gold coated polyurethane.

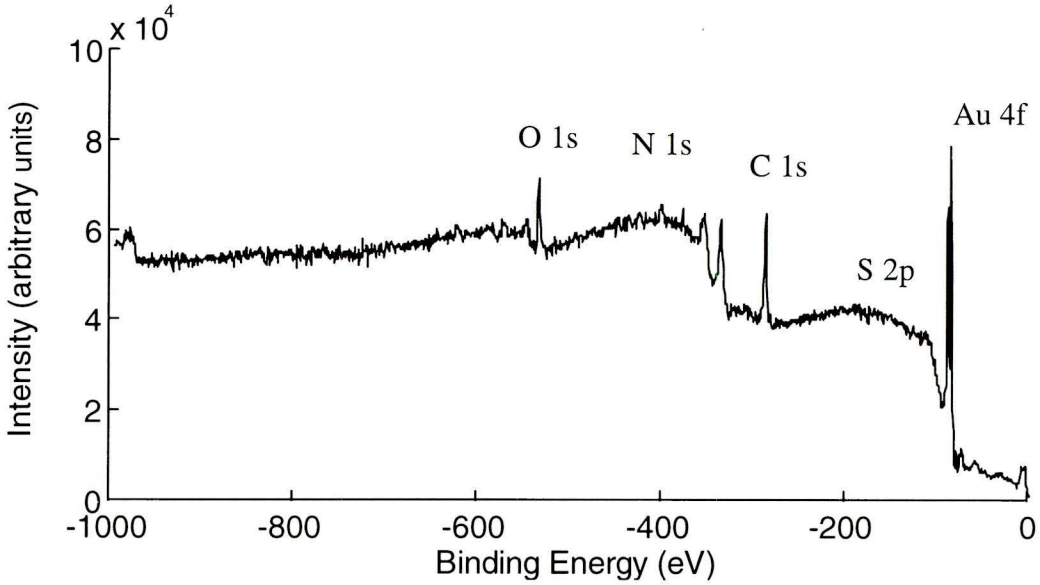

Figure 4.3 c XPS low resolution spectrum of C(D)FPR-modified gold coated polyurethane.

Table 4.1 Composition of peptide-modified gold coated polyurethanes from XPS data (atom %)[a].

Sample	C 1s	O 1s	N 1s	S 1s	Au 4f
Au [b]	53.3 [b]	10.3 [b]	-- [b]	-- [b]	36.4 [b]
CPR-Au[c, d]	72.6 [c] (56%) [d]	13.7 [c] (16%) [d]	3.6 [c] (24%) [d]	1.5 [c] (4%) [d]	8.6 [c] (--)[d]
CFPR-Au[c, d]	70.7 [c] (63.9 %) [d]	16.0 [c] (13.9%) [d]	4.6 [c] (19.4%) [d]	0.6 [c] (2.8%) [d]	8.1 [c] (--)[d]

a, Derived from XPS low resolution spectra at 10° take off angle (Figure 4.3). Data precision is ±10%
b, XPS taken immediately following TL-1 wash.
c, XPS taken following chemisorption at room temperature overnight.
d, Theoretical composition of peptide in brackets

4.2.3 Atomic Force Microscopy (AFM)

The surface morphology of the peptide modified gold surfaces and gold coated polyurethanes was investigated by AFM. Figure 4 shows AFM images of the surfaces used in this study. These pseudo 3-D images are generated in "false light" mode and give information on the overall appearance of the surface. The depth dimension in these images is exaggerated by a factor of 10. This imaging mode should be distinguished from more conventional AFM images in which depth is indicated by a range of colors or grey scale values. A number of clearly different surface features can be noted for the various materials. Upon gold coating, the rough and irregular appearance of the ED 980 surface (Figure 4.4a) changed to a somewhat more ordered morphology consisting of an array of gold "crystallites" (Figure 4.4b). Gold coated glass (Figure 4.4c) also showed a crystallite

morphology but was smoother and relatively homogenous compared to gold coated polyurethane. The irregularities on the gold coated polyurethane are believed to reflect the morphology of the polyurethane surface itself. After 18 h of chemisorption with CPR and CFPR peptides, the surfaces became smoother (Figure 4.4d). There were no obvious roughness or morphological differences among the CPR, C(L)FPR and C(D)FPR modified gold surfaces. After thrombin was adsorbed to the peptide modified gold, the surfaces again became rougher, but were less rough than the original gold coated materials. The nature of the structural elements seen in the thrombin-adsorbed surfaces is unknown. One possibility is that "macroscopic" aggregates of protein are formed on the surface.

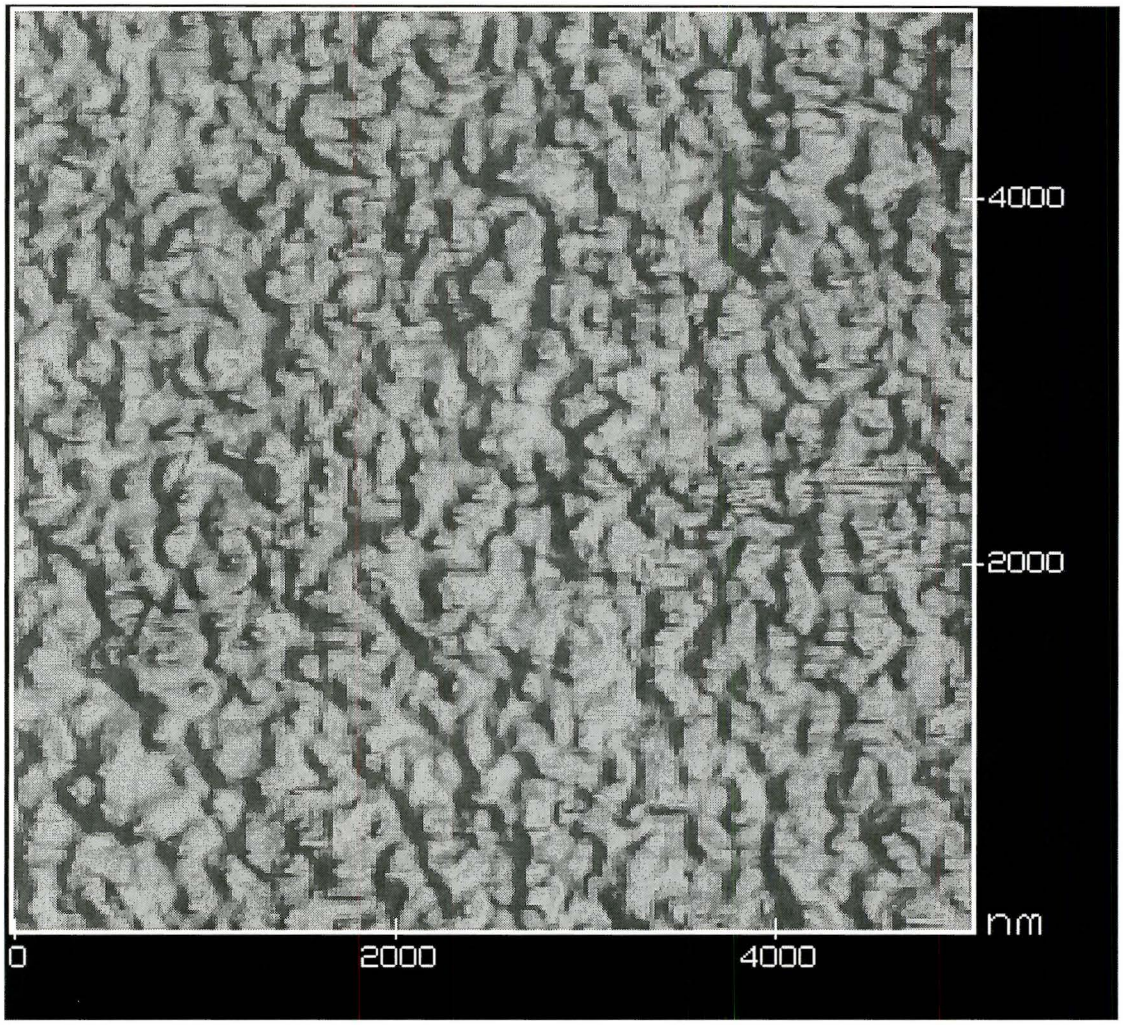

Figure 4.4a AFM image of ED 980 polyurethane surface

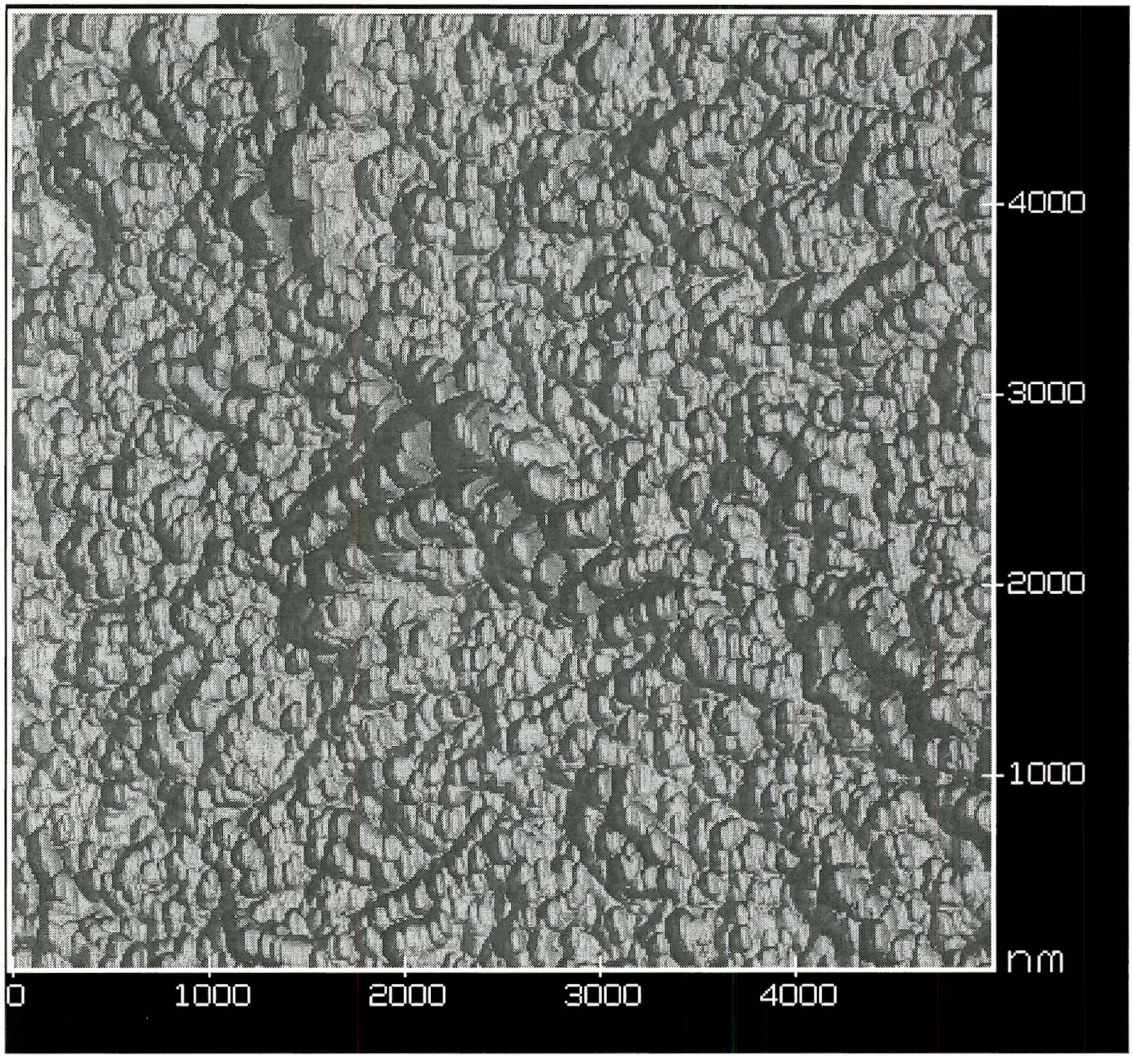

Figure 4.4b AFM image of gold coated polyurethane surface

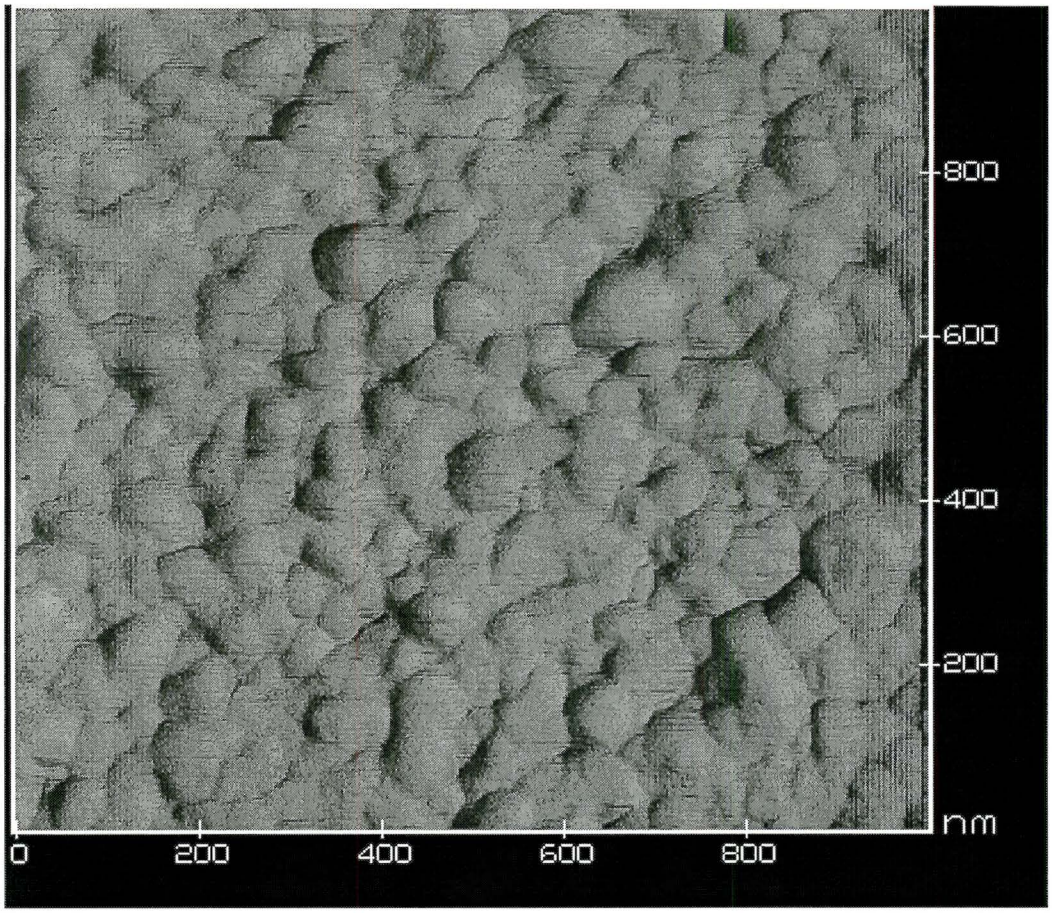

Figure 4.4c AFM image of gold coated glass surface

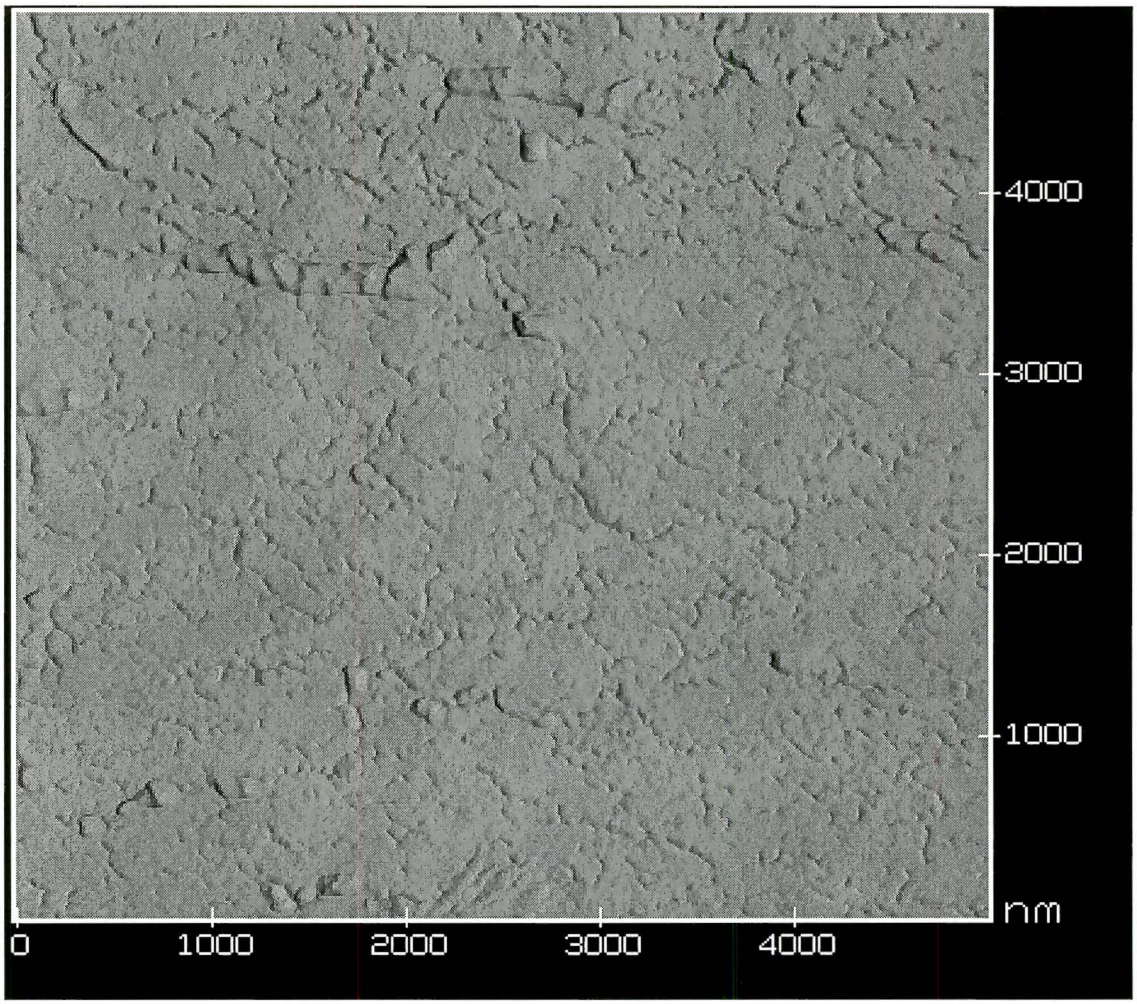

Figure 4.4d AFM image of C(D)FPR modified gold coated polyurethane surface

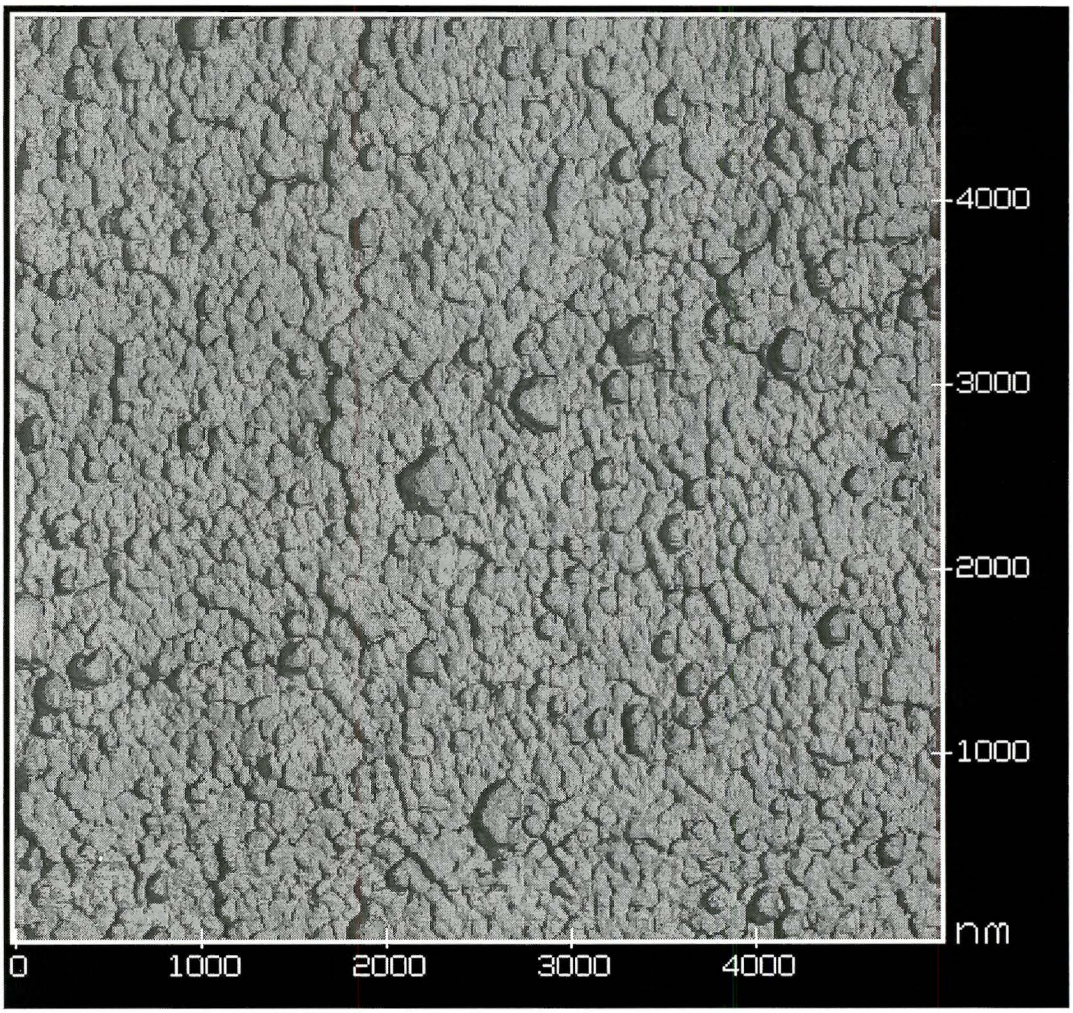

Figure 4.4e AFM of adsorbed thrombin on the C(D)FPR modified gold coated polyurethane surface

4.2.4 Scanning Electronic Microscopy (SEM)

An SEM image of gold coated polyurethane is shown in Figure 4.5. The surface is relatively rough with a "cracked mud" appearance. Following TL-1 treatment, the cracking becomes more pronounced and many more holes (approximately 0.5 μm) develop on the surface. The SEM image of gold coated on glass shows an entirely smooth, homogenous surface with no irregularities. Following TL-1 treatment, the surface remains smooth with the exception of a few small holes or imperfections (black areas on image). It is clear that topographical features are seen in more detail in the AFM than in the SEM images.

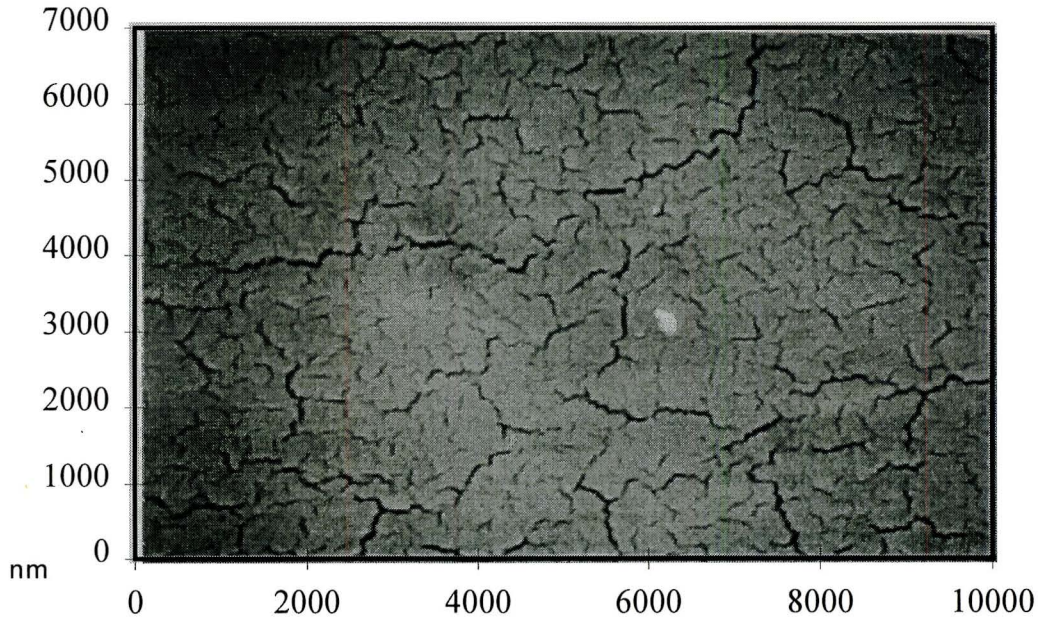

Figure 4.5 SEM of gold coated polyurethane surface.

4.2.5 Infrared Reflection-Absorption Spectroscopy (IRAS)

Both the transmission spectra (KBr pellets) of peptides CFPR and CPR and the reflection-adsorption spectra of the peptides chemisorbed to the gold surfaces were measured. The peak frequencies of the major absorption bands are listed in Table 4.2 together with their vibrational assignments.

Table 4.2 Peak assignments in the transmission spectra of peptides CFPR and CPR, and the R-A spectra of peptides CFPR and CPR chemisorbed on gold.

CPR			CFPR		
In KBr	**On Gold**	**Assignment**	**In KBr**	**On Gold**	**Assignment**
3425			~3678		
3383	~3463	}ν N-H	~3401	3365	}ν NH
3096		}ν CH(proline)	3089		}ν CH(proline)
2983			3070		
2962		ν_{as} CH_3	2954		ν_{as} CH_3
2930		ν_{as} CH_2	2930		ν_{as} CH_2
2888		ν_s CH_3	2879		ν_s CH_3
2569		ν H-S	2564		ν HS
1667	1666	Amide I		1731	ν C=O
1535	1547	Amide II	1671	1672	Amide I
1454		ν C-C	1534	1547	Amide II
1364	1235	ν CO_2^-	1453	1457	ν C-C(phenyl)
1202	1164	Amide III	1348	1342	ν CO_2^-
1134	1029	ν arginine	1202	1213	Amide III
518		ν S-S	1131	1036	ν-arginine

Note: ν N-H: Stretching vibrations of N-H at 3030 ~ 3070.
ν C-H: Stretching vibrations of C-H in proline at 3030 ~3500.
ν_{as}: Antisymmetric stretching vibrations of C-H in CH_2 and CH_3.
ν_s: Symmetric stretching vibrations of C-H in CH_2 and CH_3.

The peaks associated with CFPR and CPR, such as the amide I, II and III bonds, the three carbon-hydrogen ring bonds in proline, the C-C bonds in phenylalanine, and arginine specific vibrations appear in both the transmission and R-A spectra [1666 (amide I), 1547 (amide II), 1202 (amide III), 3089 (CH of proline), 1454 (C-C in phenylalanine), and 1134 cm^{-1} (arginine)]. The peak assigned to the H-S bond (~2600 cm^{-1}) appears in the transmission, but not in the R-A spectra. These data provide strong confirmation that the peptides CFPR and CPR are chemisorbed on gold, and that bonding occurs through the SH group.

4.2.6 Ellipsometry

The results of XPS analysis and the IRAS spectra provide evidence for the chemisorption of peptides to the gold coated polyurethanes. Additional evidence was obtained using ellipsometry to measure the thickness of the peptide layers on gold. Two independent experiments were run, and fresh surfaces were prepared for each experiment. For CPR, the ellipsometric thickness values were 24Å and 19Å, and for CFPR, the values were 17Å and 16Å. These data again confirm that the chemisorption reactions modified the surface. In addition, the thickness values are reasonable for monolayers of the peptides. The thickness of the CPR layer appears to be greater than that of CFPR, suggesting that on the CFPR modified surface the peptide is oriented relative to the surface at a lower angle or that the conformation is less extended than for CPR. It is not clear why this would be so

4.3 Protein Adsorption

It has been shown that protein adsorption to the biomaterial surface is the first significant event in blood-biomaterial interactions (Brash, 1987). Protein adsorption experiments have become a very valuable method to investigate blood compatibility. Since the surfaces studied in this work are modified gold coated polyurethanes which incorporate potential thrombin inhibitors, C(D)FPR, C(L)FPR and CPR, the selectivity of these modified gold surfaces for thrombin binding as well as their ability to inhibit the activity of bound thrombin are of interest. Therefore, studies of thrombin adsorption from buffer and from human plasma were conducted. The binding of a number of plasma proteins on the peptide-modified gold surfaces was also investigated.

4.3.1 Preparation of Plasma

As stated previously, it was necessary to deplete the plasma of antithrombin III (ATIII) and fibrinogen. The presence of ATIII in plasma would produce ATIII-thrombin complex, while fibrinogen had to be removed to prevent fibrin formation in the presence of thrombin. The ATIII-depleted plasma did not contain any detectable functional ATIII, although trace levels (5 μg/mL) of ATIII were detected immunologically. The normal plasma concentration of ATIII is of the order of 0.1 mg/mL. The levels of plasma clotting factors in the ATIII-depleted plasma were somewhat lower than in the control

plasma (see Table 4.3), but could still be considered to be within the normal range. After depletion of fibrinogen, there was no detectable fibrinogen in the plasma.

Table 4.3 Hemostatic function assay for ATIII-depleted plasma

Proteins	**Depleted plasma range** (U/mL)*
Prothrombin	0.84
Factor VIII	0.72
Factor V	0.84
Factor VII	0.94
Factor X	0.89
Factor XI	0.62
Factor XII	0.73
Factor IX	0.82

* Data from Clinical Haemotology Lab, McMaster University. Values for control plasma were 0.1 U/mL.

4.3.2 Protein Labelling

The adsorption experiments in this study used ^{125}I-labelled protein as a tracer. After radiolabelling the protein and then dialyzing against PBS to remove unbound radioactive iodide, the final protein concentration was estimated from absorbance at 280 nm.

It has been shown that iodide ion interacts strongly with gold (Cochran & Farrell, 1980). Therefore, the possibility arises that the radioactive free iodide present in the labelled protein solution will chemisorb to the gold surfaces. Clearly this would cause an

error in estimating protein adsorption, and thus it was important to monitor the concentration of free iodide in the final labelled protein solutions. After labelling, a known volume of the working solution of labelled thrombin was mixed with bovine serum albumin solution as carrier, and the protein was precipitated by trichloroacetic acid. The free iodide content of the working solution (as a percentage of total radioactivity) was estimated by the difference of radioactivity between the supernatant following precipitation and the original solution. It was found that the content of free iodide varies with the different proteins, and the scale of the labelling procedure. The free iodide in labelled thrombin solutions was consistently in the range of 2 to 5 % of the total radioactivity of the solution.

4.3.3 Effect of Free Iodide on Protein Adsorption

For the unmodified (bare) gold surface it was found that even very small concentrations of free iodide ion in the protein solution could result in extremely high levels of radioactivity on the surface, due to free iodide ion binding to gold. Indeed following treatment with SDS to remove the adsorbed protein (see below for more detailed discussion) up to 95 % of the radioactivity remained on the surface, indicating that iodide ion has much greater affinity than proteins for binding to gold. Several methods have been investigated in this lab to suppress free iodide ion interactions with gold surface, or to estimate the relative contribution of iodide ion and protein to the total surface radioactivity. It has been shown (Du et al, unpublished) that a small amount of

non-radioactive iodide ion added to the radiolabelled protein solution reduces the binding of radioactive free iodide to the gold surface, due presumably to the fact that the ratio of unlabelled to labelled iodide in the solution is high, and iodine interactions are predominantly with non-radioactive iodide.

It is also important to know whether the presence of free iodide ion or the presence of the label in thrombin affects its enzymatic activity. To investigate the effect of labelling on activity, two thrombin solutions of the same concentration, one of which contained 15% of the enzyme in labelled form, while the other one contained only unlabelled thrombin, were incubated with the gold surface. After adsorption, the activity of the thrombin in the supernatants was measured by chromogenic substrate assay. The data showed a higher concentration of active thrombin in the supernatant of the unlabelled protein (Figure 4.6). The difference in the slopes of the absorbance-time curves (related to the thrombin activity) is significant (Table 4.4), suggesting that either the presence of the label on thrombin or radiolabelled free iodide in the solution affects the interaction of thrombin with the gold.

The enzymatic activity of labelled and unlabelled thrombin solutions independent of surface contact was also measured (Figure 4.7). Although there is a difference between the labelled and unlabelled thrombin, it is considerably less than after contact with gold, suggesting that the interaction of the enzyme with gold has a greater effect on thrombin activity than the presence of the label.

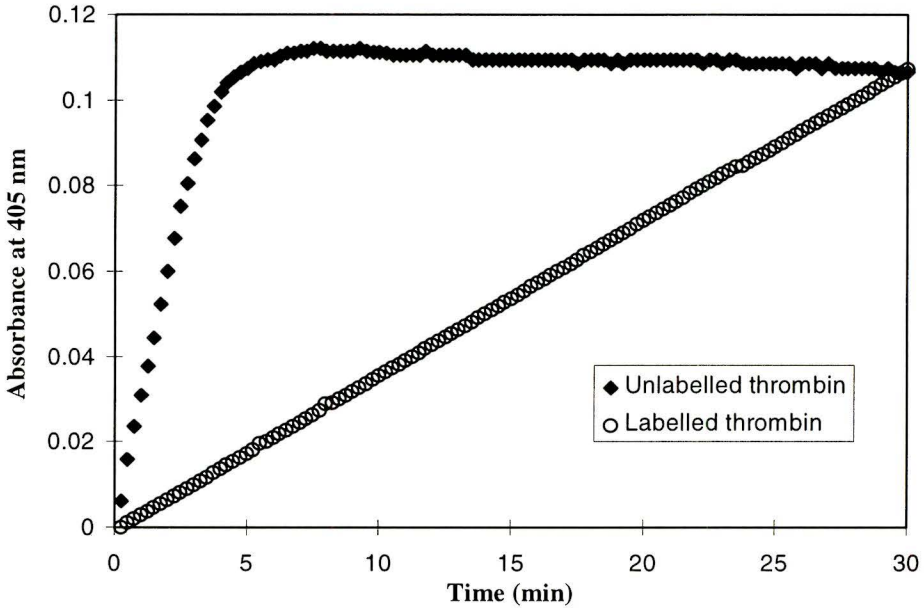

Figure 4.6 Enzymatic activity of labelled and unlabelled thrombin solution after exposure to gold surface.

Table 4.4 Effect of labelling on thrombin activity in the presence of gold coated polyurethane surfaces. (Slope of A_{405} vs time curve.)

Thrombin solution	Slope	Upper 95 % * confidence level	Lower 95 % * confidence level
100% Unlabelled	0.0192	0.0201	0.0184
15% Labelled	0.0035	0.0037	0.0033

*, C.I.'s determined using MS EXCEL™ regression macro.

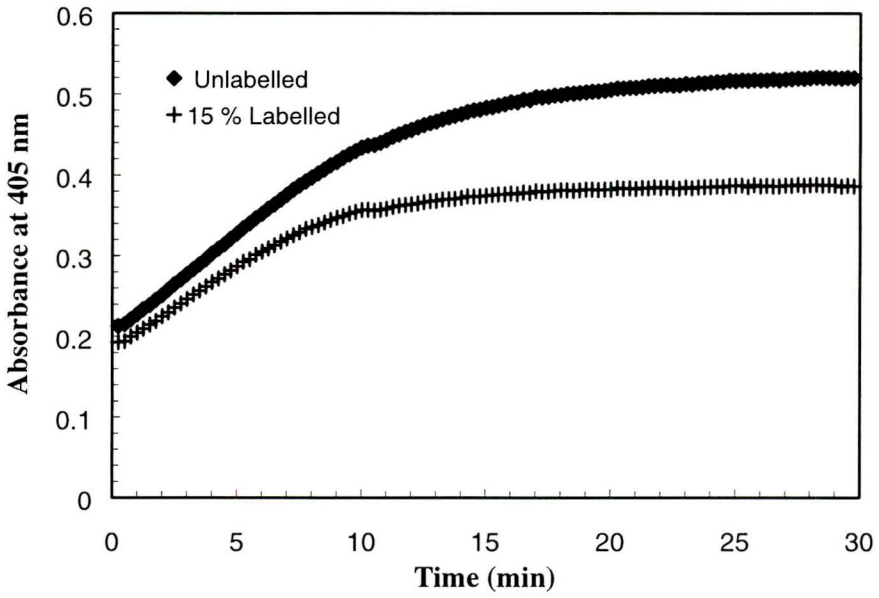

Figure 4.7 Enzymatic activity of labelled and unlabelled thrombin (no surface exposure).

4.3.4 Adsorption of Thrombin from Buffer to "Control" and Modified Gold Surfaces

The adsorption of thrombin from buffer was investigated to obtain a measure of the relative capacities and affinities of the surfaces for thrombin. All adsorption experiments were carried out for 2 h with thrombin concentrations ranging from 1 to 20 μg/mL. The isotherms (22°C) for the unmodified gold, the peptide- and cysteine-modified gold surfaces are shown in Figure 4.8. It is clear that the CFPR and CPR modified gold surfaces adsorbed more thrombin than the cysteine modified and unmodified gold. In particular, the CFPR surface showed the highest amount of adsorbed thrombin, up to 0.85 $\mu g/cm^2$, approaching the level of a close packed monolayer.

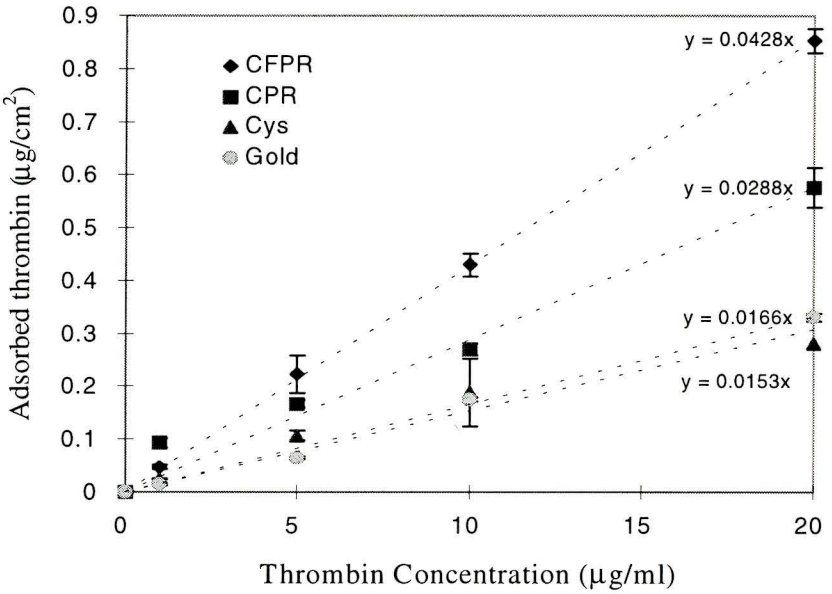

Figure 4.8 Adsorption of thrombin from buffer to the modified gold coated polyurethane surfaces. Three independent experiments were done for each surface, and 3 replicate samples were measured for each concentration in each experiment. The standard deviations, calculated by pooling all replicates from the three experiments, are represented by the error bars. Fits of the data by linear regression are also shown.

The difference in apparent affinity between CPR and CFPR may be related to the peptide structure. As was discussed in section 2.2.3, thrombin has very specific binding sites S_1-S_3 on its surface which are related to the P_1-P_3 sites on the thrombin substrates. S_1 is the active site containing Asp189, which can form a salt with arginine (P_1). The S_2 site of thrombin contains a loop shape of hydrophobic lid, well adapted for the binding of proline (P_2). The S_3 site of thrombin called the 'aryl-binding' site involves Leu99, Ile174, and Trp215, and is particularly suited to aromatic residues. The aromatic ring of the phenylalanine makes a favourable edge-to-face interaction with Trp215. It is the D

configuration of the phenylalanine that allows it to occupy to the aryl-binding site. The fit of the P1-P3 residues of (D)FPR to the S1-S3 sites on the thrombin surface is almost optimal (Bagdy et al, 1992, Fenton II et al, 1993). Due to the specific affinity of the tripeptide sequence D-Phe-Pro-Arg for thrombin, its derivative C(D)FPR may also be specific for thrombin binding.

In order to quantify the interactions between surfaces and thrombin, a protein adsorption model was used based on the Langmuir mechanism. The Langmuir mechanism assumes reversible adsorption of protein (P) to equivalent surface sites (S) with formation of a monolayer.

$$P + S \xleftrightarrow{K} PS$$

It is easily shown that at equilibrium:

$$\theta = \frac{KC}{1 + KC} \qquad 4.1$$

where K is an adsorption affinity constant, C is bulk protein concentration, and θ is fractional surface coverage given by:

$$\theta = \frac{\Gamma}{\Gamma_{max}} \qquad 4.2$$

where Γ is surface concentration. At low concentration, eq. 4.1 reduces to:

$$\theta = KC \qquad 4.3$$

$$\Gamma = \Gamma_{max} KC \qquad 4.4$$

The fits of the thrombin adsorption data to Eq. 4.4 are shown in figure 4.8 and the values of the parameter Γ_{max} K are listed in Table 4.6. The CFPR surface shows a higher value than the other three surfaces, suggesting a relatively high binding affinity for CFPR. This assumes that the Γ_{max} values are similar for all the surfaces. Clearly the isotherms (Figure 4.8) do not reach plateau levels at the low thrombin concentrations used, so the Γ_{max} values are unknown. In general protein adsorption plateaus are reached at concentrations of the order of 0.5 mg/mL, so it is perhaps not surprising that the thrombin isotherms would continue to increase at concentrations greater than 20 μg/mL.

Table 4.5 Thrombin binding affinity for the peptide-modified gold surfaces: comparison to cysteine and unmodified gold, from linear regression of the data in Fig. 4.8.

Surface	C(D)FPR	CPR	Cysteine	Gold
$\Gamma_{max}K$ **(mL/cm^2)**	0.0428	0.0288	0.0166	0.0153
R^2	0.9997	0.9843	0.9947	0.9636

4.3.5 Adsorption of Thrombin from Plasma to the Control and Modified Gold Surfaces.

The adsorption of thrombin from plasma to the peptide modified gold surfaces was measured in order to investigate the competition between thrombin and the other

proteins in plasma and thus to assess the potential of the surfaces to "scavenge" or adsorb thrombin preferentially from plasma. The adsorption isotherms for the C(D)FPR-, C(L)FPR-, CPR-, and cysteine-modified surfaces and for unmodified gold coated polyurethane are shown in Figure 4.9. The three peptide surfaces adsorb more thrombin than the control surfaces as was observed for thrombin adsorption from buffer. C(D)FPR adsorbs the greatest amounts of thrombin, followed by C(L)FPR, and CPR, again suggesting that the C(D)FPR surface has a relatively high binding affinity and therefore specificity for thrombin.

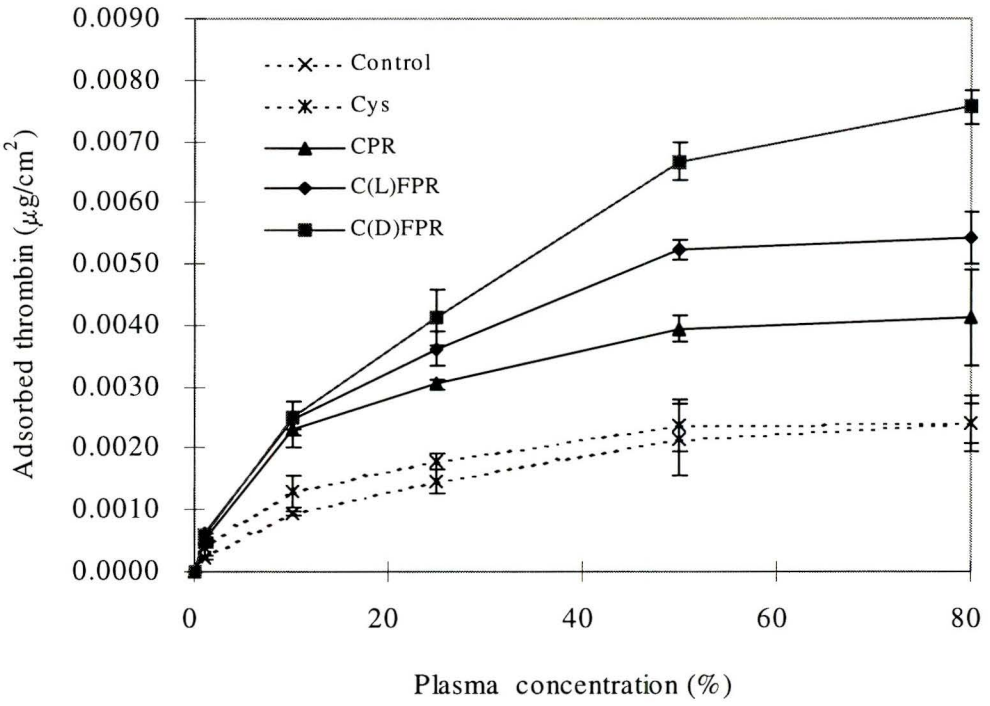

Figure 4.9 Adsorption of thrombin to modified gold coated polyurethane surfaces from plasma. The thrombin concentration in 100% (i.e. undiluted) plasma was 10 U/mL (3.3 μg/mL) with 20% labelled and 80% unlabelled. The error bars represent standard deviations (n=9).

Much less thrombin is adsorbed from plasma than from buffer. For instance, at a plasma concentration of 80 % (2.64 μg/mL thrombin), the amount of thrombin adsorbed to the C(D)FPR surface from plasma is 0.008 $\mu g/cm^2$ compared to about 0.12 $\mu g/cm^2$ from buffer at the same concentration. It is likely that the other proteins in the plasma, many of which have much higher concentrations, compete with thrombin for surface binding sites. Some of these sites will be non-specific as far as thrombin is concerned so that the binding of proteins of higher concentration will be favoured. This point is revisited in section 4.5.2 (Western blot data).

4.3.6 Adsorption of Fibrinogen from Plasma to Control and Modified Gold Surfaces

In order to investigate further whether the peptide modified gold surfaces are specific for thrombin binding, it was of interest to study the adsorption of another plasma protein. Fibrinogen was chosen for this purpose since it is a protein for which a large adsorption data base exists and which is not expected to interact specifically with the CPR and CFPR peptides. Normal human plasma (not depleted of fibrinogen or ATIII) was used for this study and ^{125}I-labelled fibrinogen was added at a level of 3 % of the endogenous fibrinogen concentration. The adsorption time, plasma concentrations and temperature were the same as for the thrombin adsorption experiments.

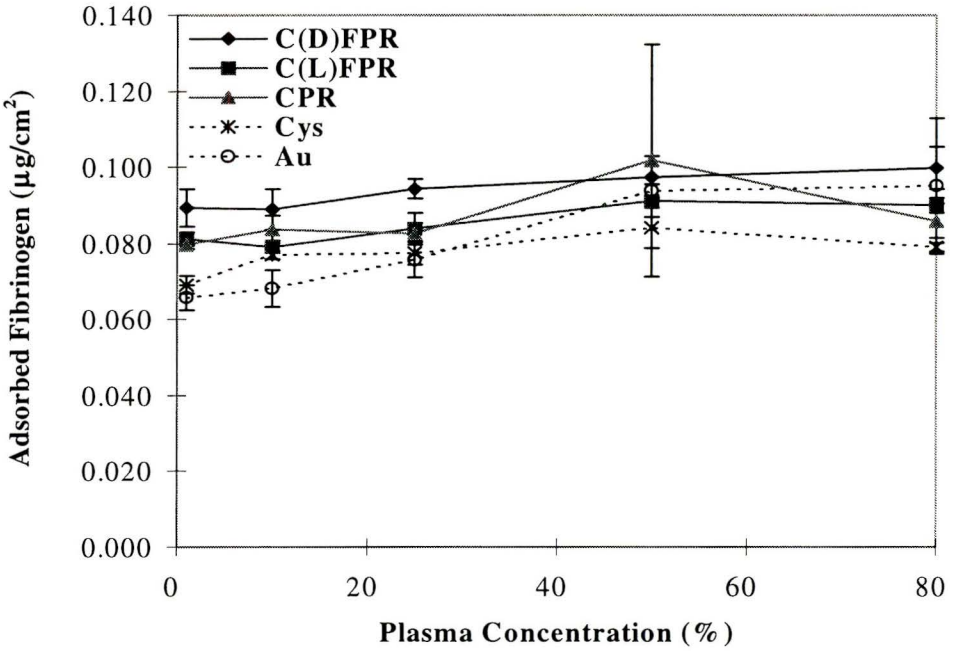

Figure 4.10 Adsorption of fibrinogen (as ^{125}I-fibrinogen) to modified gold coated polyurethane surfaces from plasma. The error bars are standard deviations (n=9).

The data (Figure 4.10) indicate that there is no difference in the quantities of fibrinogen adsorbed to the C(D)FPR-, C(L)FPR- and CPR-modified gold surfaces or to the control surfaces, thus providing added support for the conclusion that the peptides have some specificity for thrombin binding, but not for other proteins. It is interesting to note that on C(D)FPR and C(L)FPR surfaces, there is slightly more fibrinogen adsorbed from 1% plasma than that from 10 % plasma, suggesting the occurrence of a Vroman effect, i.e. displacement of initially adsorbed fibrinogen by other proteins (Vroman and Adams, 1969). More data collected at low plasma concentration would be of interest in order to demonstrate the presence or absence of the Vroman effect on these surfaces. The Vroman effect was not observed when investigating thrombin adsorption from plasma.

As indicated, the Vroman effect reflects competitive adsorption and is believed to result from the displacement of initially adsorbed high concentration proteins of low binding affinity by low concentration proteins of high binding affinity (Brash et al., 1988; Santerre et al., 1992; Vroman and Adams, 1980). Thus it is possible that thrombin may be a "displacer" protein in the early stages of plasma contact but may not itself be displaced due to its high binding affinity to these peptide surfaces (Brash et al. 1988; Slack and Horbett, 1995).

4.4 Thrombin Enzymatic Activity

Thrombin enzymatic activity was measured in two situations: (1) in solution with the various peptides present, and (2) when bound to the peptide modified gold surfaces. A synthetic peptide-conjugated nitroanilide, N-p-tosyl-gly-pro-arg-p-nitroanilide was used as a specific thrombin substrate (Abildgard et al. 1977). The cysteine-modified and unmodified gold surfaces were again included as controls.

4.4.1 Activity of Thrombin in Solution: Inhibition by Peptides

The activity of thrombin in the presence of the peptides was investigated to assess their inhibitory activity. The kinetics of release of p-nitroaniline (pNA) from the substrate by the action of thrombin at 37°C was studied by measurements of absorbance at 405 nm. Solutions of pure thrombin (no inhibitor) and of PPACK-incubated thrombin (strong inhibition) were included for comparison. The kinetic data were analyzed using

the Softmax 2000 kinetics data package (SLT- Labinstrument, Tecan). Typical kinetic data are shown in Figure 4.11.

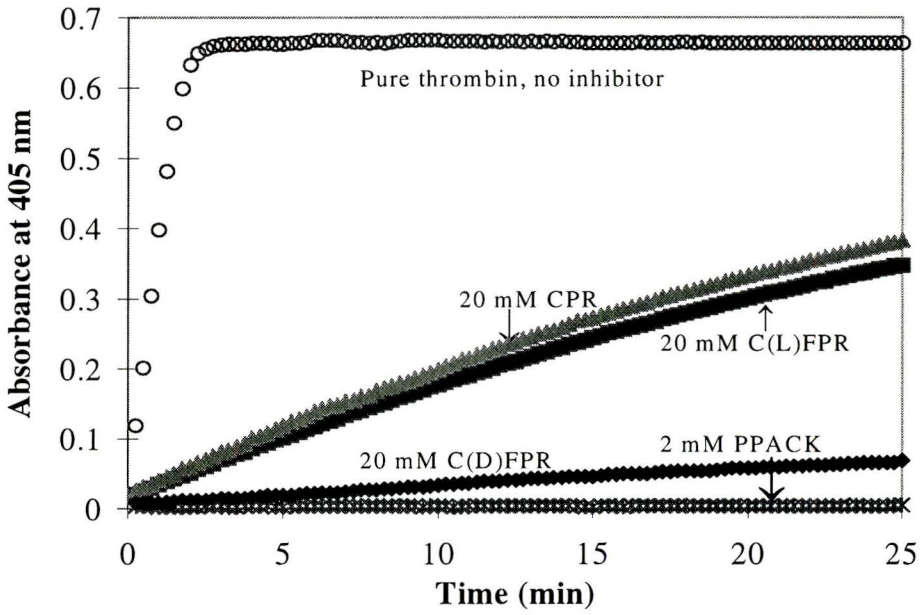

Figure 4.11 Chromogenic substrate assay of thrombin activity in the presence of peptides. Thrombin concentration, 0.1 μg/mL (0.3 U/mL, 2.8 μM); incubation time with inhibitor prior to beginning of assay, 15 min.

At a thrombin concentration of 0.1 μg/mL, the maximum absorbance value of pNA in pure thrombin solution was about 0.68, and the plateau was achieved within 3 min. However, when thrombin was incubated with 2 mM PPACK for 5 or 15 min, the absorbance did not rise above background indicating that thrombin was completely inhibited by PPACK. Compared to the pure thrombin and PPACK data, the release of pNA in the peptide-thrombin solutions was intermediate. For example, after incubation of thrombin with 20 mM C(D)FPR peptide, the absorbance was 0.05 after 25 min. Both

C(L)FPR and CPR peptides gave absorbance values of approximately 0.4. These data suggest that the C(D)FPR peptide is a relatively strong inhibitor, while C(L)FPR and CPR are weaker inhibitors of thrombin.

Chromogenic substrate assays were also run at different thrombin concentrations (0.03 to 1.65 μg/mL), peptide concentrations (2 and 20 mM) and pre-incubation times (5 and 15 min).

A set of standard thrombin activity kinetics curves were obtained using thrombin solutions of different activity. A calibration curve was then constructed using the initial slopes of the kinetics curves and the known amounts of thrombin (Figure 4.12).

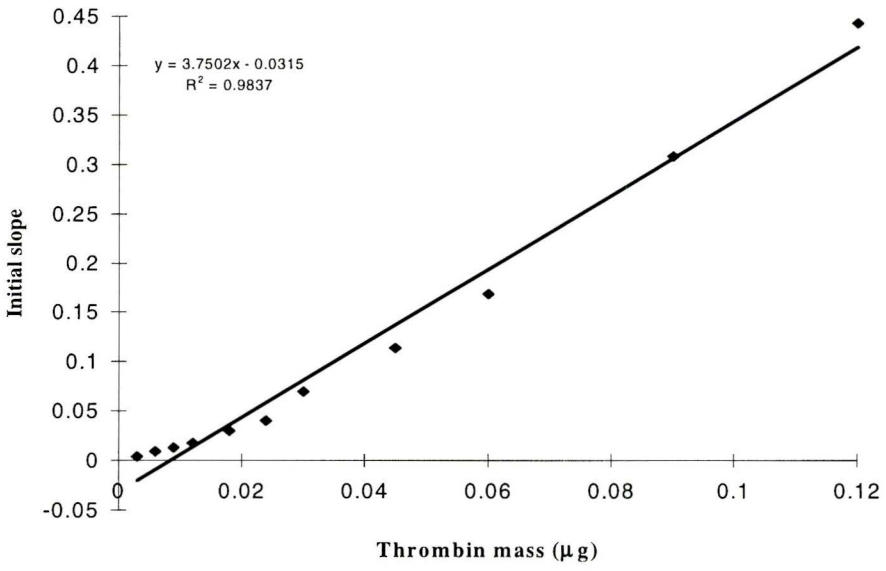

Figure 4.12 Calibration curve for thrombin activity measurements based on the initial slope of standard thrombin kinetics activity curves at different thrombin mass. The data were obtained by chromogenic substrate assay.

The mass of active thrombin in the peptide solutions could than be estimated from the calibration curve. The inhibitory activity of the peptides (as percentage thrombin inhibited) was determined from the activity of thrombin in solution following incubation for short times (5 to 15 minutes). The results are shown in Table 4.6.

Table 4.6 Inhibition of thrombin by peptides (PPACK 2mM, other peptides 20mM) at different thrombin concentrations. Incubation time prior to assay, 15 min. (Calculated from the initial slope of thrombin activity kinetics curves.)

Thrombin Concentration (μg/mL)	Inhibitor	% of thrombin Inhibited
0.03	PPACK	99.9
	C(D)FPR	99.9
	C(L)FPR	97.9
	CPR	93.4
	Control	0
0.33	PPACK	99.9
	C(D)FPR	98.7
	C(L)FPR	85.6
	CPR	45.8
	Control	0
1.65	PPACK	99.7
	C(D)FPR	88.7
	C(L)FPR	71.8
	CPR	37.3
	Control	0

PPACK shows the strongest inhibitory activity, and completely inhibits thrombin even at the highest thrombin concentration of 1.65 μg/mL, with a PPACK concentration of 2 mM and a 5 min incubation time. At low thrombin concentration (0.03 μg/mL), all peptides showed good inhibitory activity, with more than 90 % inhibition. At high

thrombin concentration (1.65 μg/mL), C(D)FPR showed relatively strong inhibitory activity with 89 % of the thrombin being inhibited, while C(L)FPR and CPR showed only 72% and 37% inhibition respectively. It is seen that the order of inhibitory activity remains as PPACK >> C(D)FPR > C(L)FPR > CPR regardless of conditions.

4.4.2 Activity of Thrombin Adsorbed on Peptide Modified Gold Surfaces

The enzymatic activity of thrombin adsorbed on the different modified gold surfaces was measured as follows. After thrombin adsorption for 3 h from PBS solution at a concentration of 10 μg/ml, samples of the modified and unmodified gold surfaces were incubated with a solution of the chromogenic substrate N-p-tosyl-gly-pro-arg p-nitroanilide. At specified times, the reaction was stopped by adding 1M acetic acid, and the surfaces were removed from the wells. The absorbance of the final reaction solutions was measured at 405 nm, and was plotted as a function of time. Since there are different amounts of thrombin adsorbed on the peptides and "control" surfaces, the Y axis is normalized to the quantity of thrombin present in the assay (Figure 4.13).

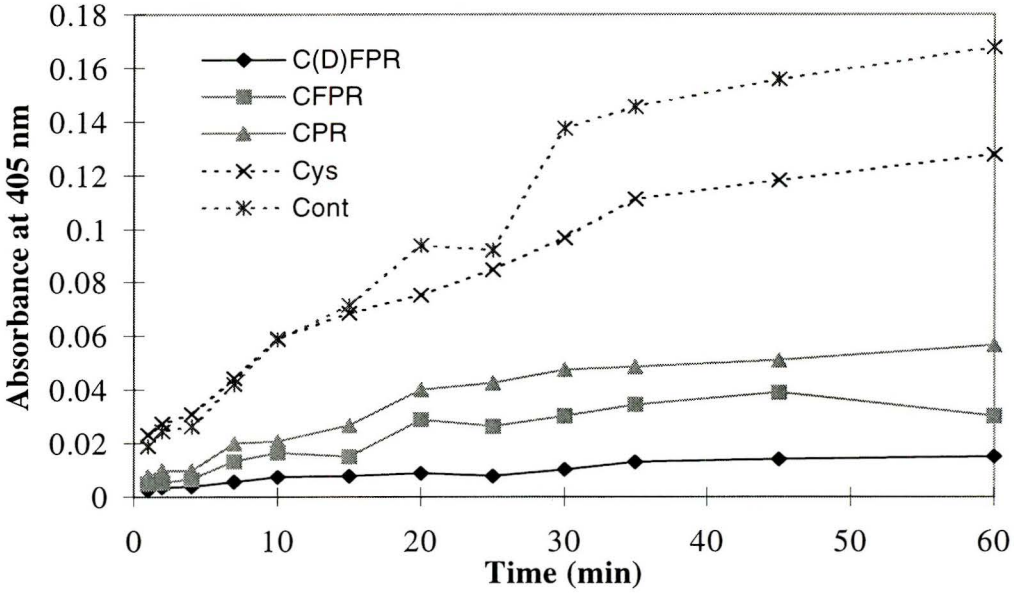

Figure 4.13 Thrombin activity on modified gold surfaces: absorbance (405nm) of pNA released from N-p-tosyl-gly-pro-arg-pNA by thrombin on surface. Data normalized to quantity of thrombin present.

The three peptide modified gold surfaces show low activity compared to the controls. The lowest thrombin activity was observed for the C(D)FPR modified gold surface with a maximum optical density of only 0.016. This is approximately one tenth of the thrombin activity observed on the unmodified gold control surface. These data suggest that the thrombin is bound to the C(D)FPR modified gold surface through its active site. In contrast, non-specific thrombin adsorption, where some of the thrombin active sites remain available, is presumably occurring on the control surfaces.

The data on thrombin activity both in peptide solutions and on peptide modified gold surfaces indicate that the C(D)FPR is the strongest thrombin inhibitor of the three

peptides investigated. Thus the C(D)FPR modified gold surface appears to have the greatest potential as a thrombin scavenging and thrombin inhibiting material.

4.5 Studies of Proteins Adsorbed from Buffer and Plasma using Imunoblotting Methods

Immunoblot assays were conducted to identify thrombin and other plasma proteins eluted from the modified and unmodified gold surfaces. The proteins were eluted with 2 % SDS solution following exposure of the surfaces to thrombin in buffer (PBS) or to ATIII- and fibrinogen-depleted plasma for 3 h. Thrombin was detected using a biotin-conjugated sheep anti-human thrombin antibody in conjunction with an avidin-conjugated colour reagent. This system provides a highly sensitive method for thrombin detection. Other plasma proteins were detected using specific polyclonal antibodies (Table 3.1), in conjunction with enzyme-conjugated second antibodies and a colour reaction detection system.

4.5.1 Western Blots of Thrombin Adsorbed from Buffer and Plasma

Typical immunoblots of thrombin eluted from peptide-modified, cysteine-modified and unmodified gold surfaces following exposure to thrombin-in-buffer or plasma is shown in Figure 4.14.

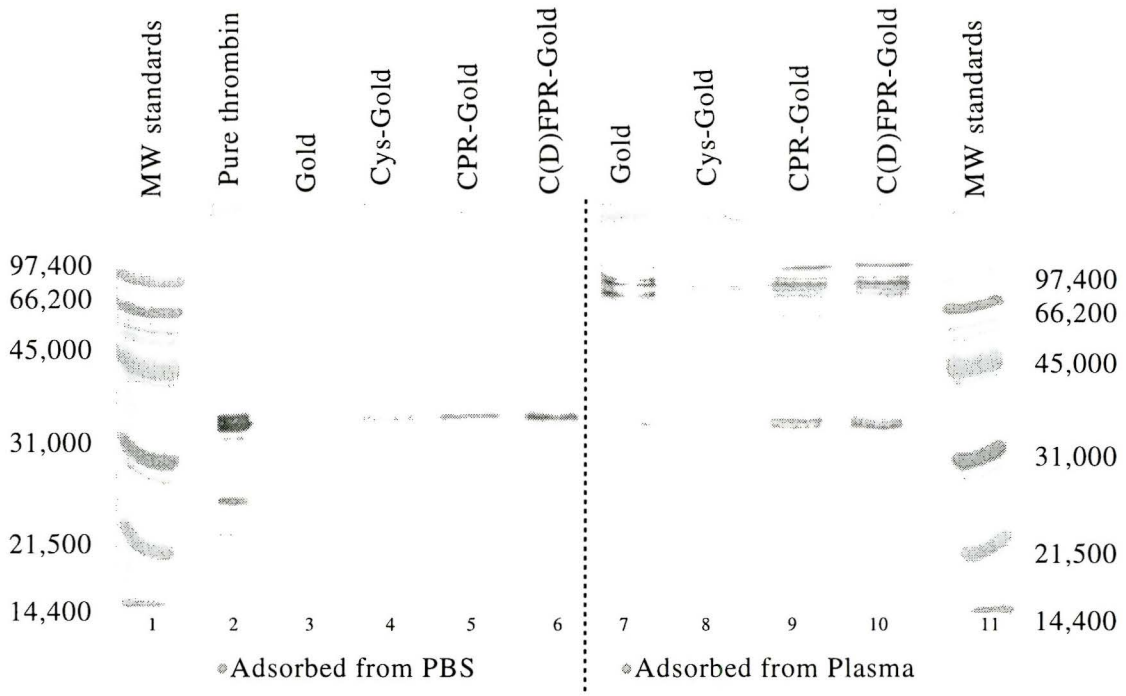

Figure 4.14 Immunoblot of thrombin adsorbed to the modified and unmodified gold surfaces. The blot was obtained from a 12 % reduced SDS-PAGE gel of the proteins eluted from the surfaces by 2 % SDS following adsorption from thrombin in PBS or from plasma.

Lanes 1 and 11 are gels of the molecular weight standards used. Lane 2 is an immunoblot of a pure thrombin solution which serves as a control to compare to the eluates from the modified and unmodified gold surfaces.

Lane 2 shows a very strong band at approximately 36 kD, and two fainter bands at about 25 and 22 kD corresponding to thrombin fragments. Lanes 3 to 6 show the blots of eluates from gold, cysteine, CPR and CFPR surfaces respectively, following exposure to thrombin in PBS. No bands are visible in lane 3 for the gold surface. A faint thrombin band is visible in lane 4 for the cysteine surface. The eluates from both the CPR and CFPR surfaces (lanes 5 and 6) show a relatively strong band at 36 kD, although no bands

corresponding to degraded thrombin are visible. From these blots for thrombin-in-buffer exposure, more thrombin appears to be present on the peptide modified surfaces than on the control surfaces, again suggesting a specific thrombin-peptide interaction.

Lanes 7 to 10 show typical immunoblots of thrombin following elution of proteins from the surfaces after 3 h contact with plasma. Again, there are clear bands visible at about 36 kD on the CPR and CFPR surfaces but only faint bands on the control surfaces, suggesting that the peptide surfaces have a higher affinity for thrombin than the controls. Bands are also visible at about 70 kD, possibly due to adsorbed prothrombin that is detected by the antithrombin antibody. A clear band appears at around 94 kD on the blots for the CPR and C(D)FPR surfaces, possibly due to ATIII-thrombin complex (57 kD ATIII, 36 kD thrombin) adsorbed by the peptides. This result is discussed in more detail in section 4.5.2. It is important to note that the amount of thrombin added to the plasma in the blotting experiments was 30 μg/mL. This is approximately 10 times the amount used in a typical thrombin-in-plasma adsorption experiment using radiolabelled thrombin.

4.5.2 Western Blots of Proteins Adsorbed from Plasma (depleted of ATIII and fibrinogen, with thrombin added)

The immunoblot patterns of the plasma eluates from the modified and unmodified gold surfaces are shown in Figures 4.15a to 4.15d. For comparison, the immunoblot for ATIII- and fibrinogen-depleted plasma which was not exposed to the gold surfaces is shown in Figure 4.15e.

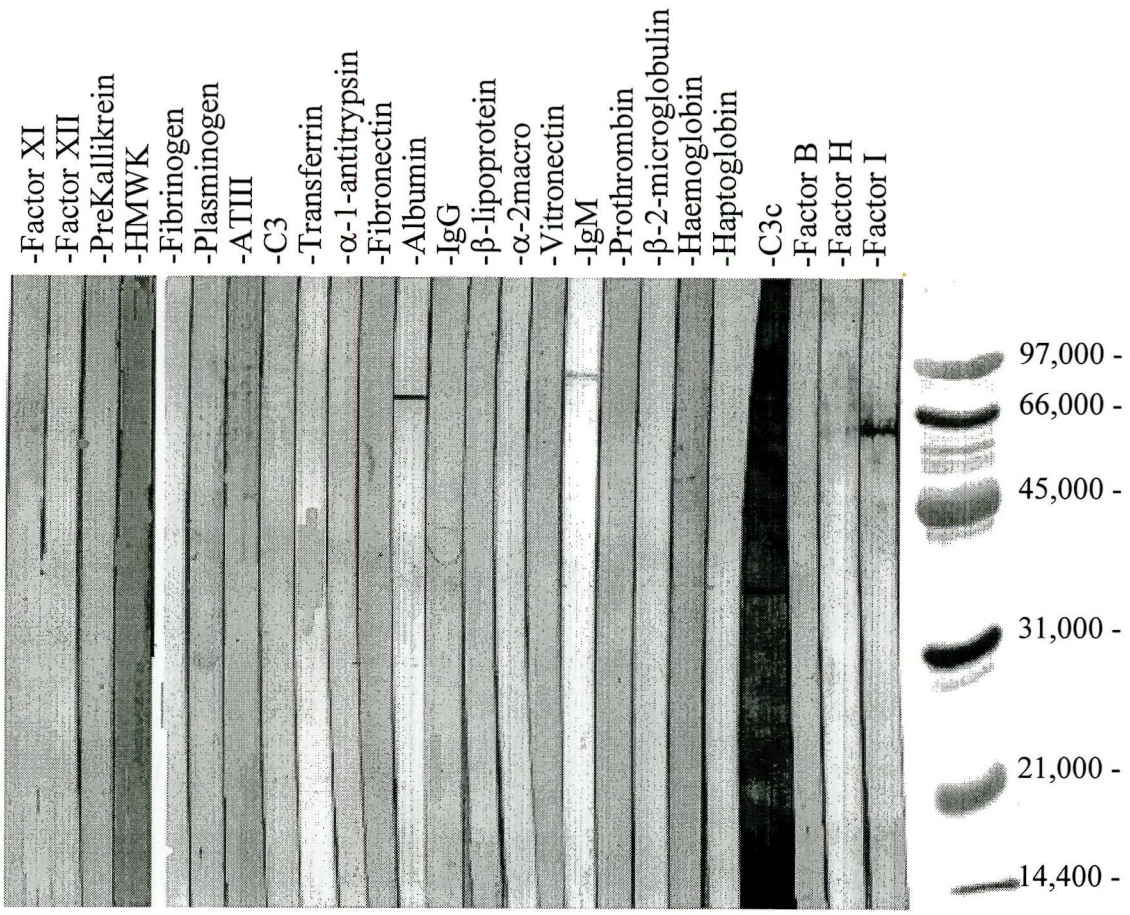

Figure 4.15 a Immunoblot from SDS-PAGE gel (reduced) of 2 % SDS eluate from unmodified gold surface after 3 h protein adsorption from plasma.

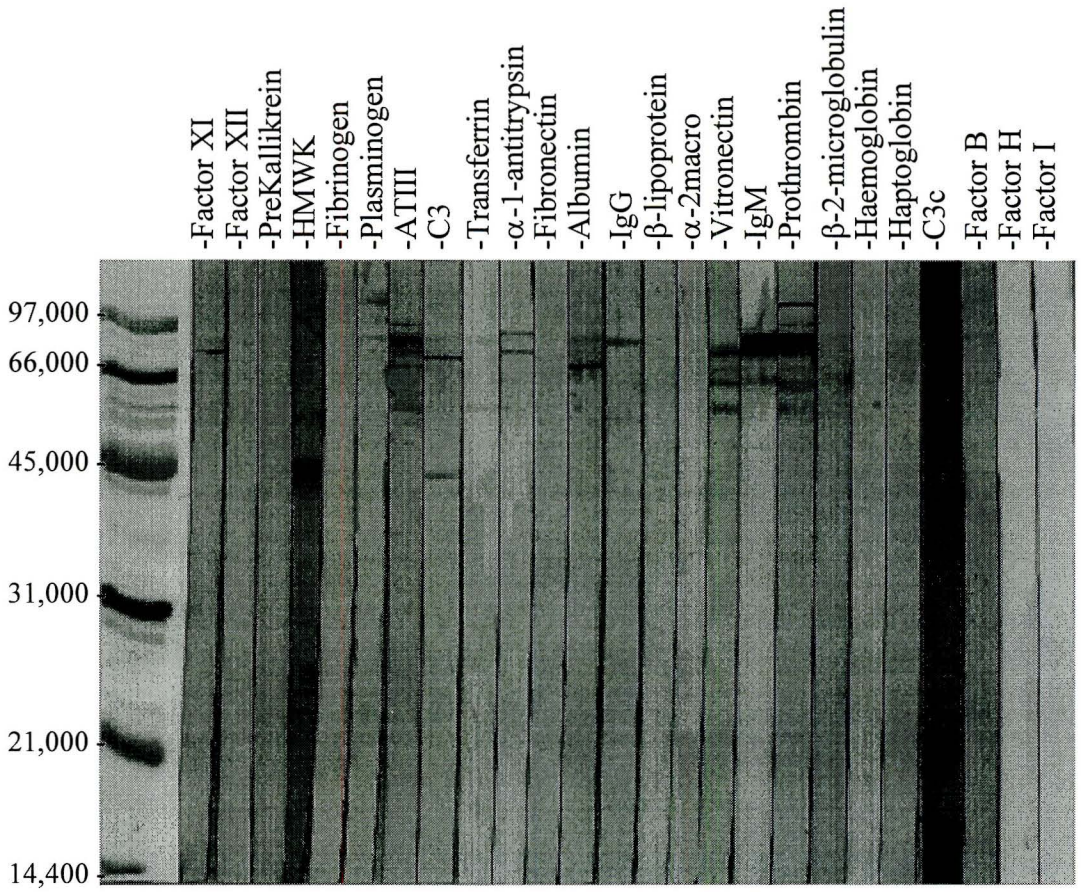

Figure 4.15 b Immunoblot from SDS-PAGE gel (reduced) of 2 % SDS eluate from cysteine modified gold surface after 3 h protein adsorption from plasma.

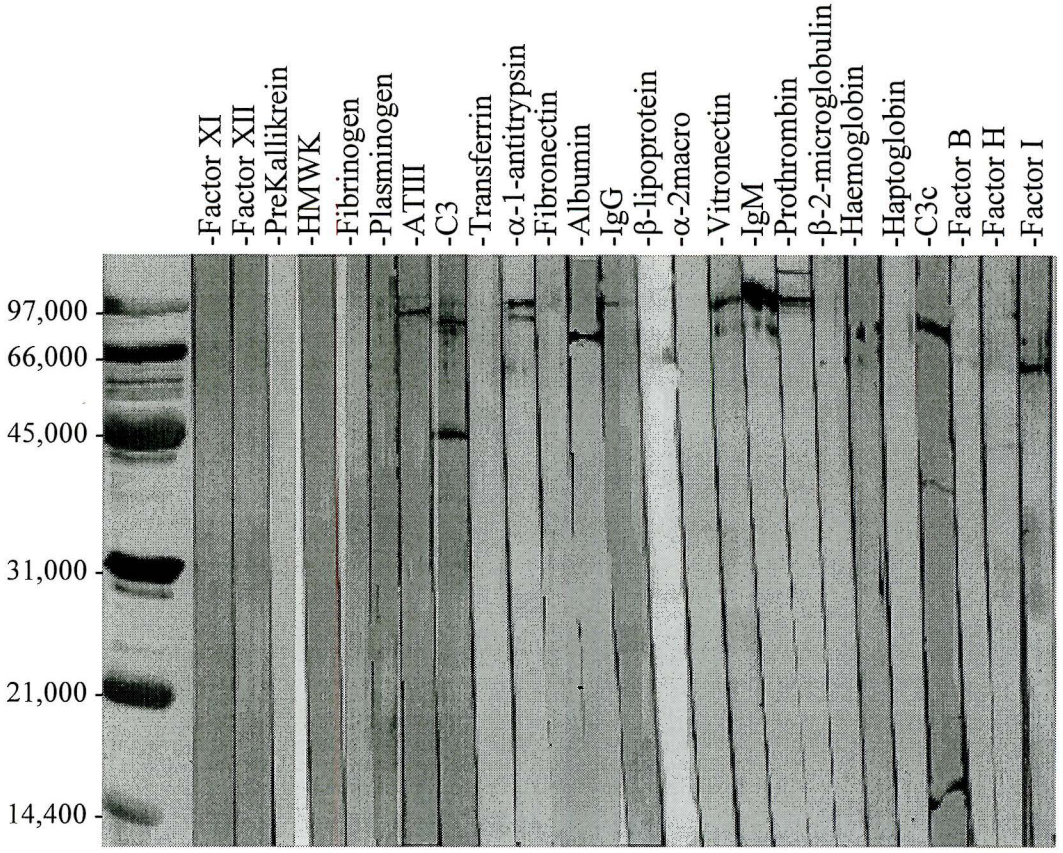

Figure 4.15 c Immunoblot from SDS-PAGE gel (reduced) of 2 % SDS eluate from CPR modified gold surface after 3 h protein adsorption from plasma.

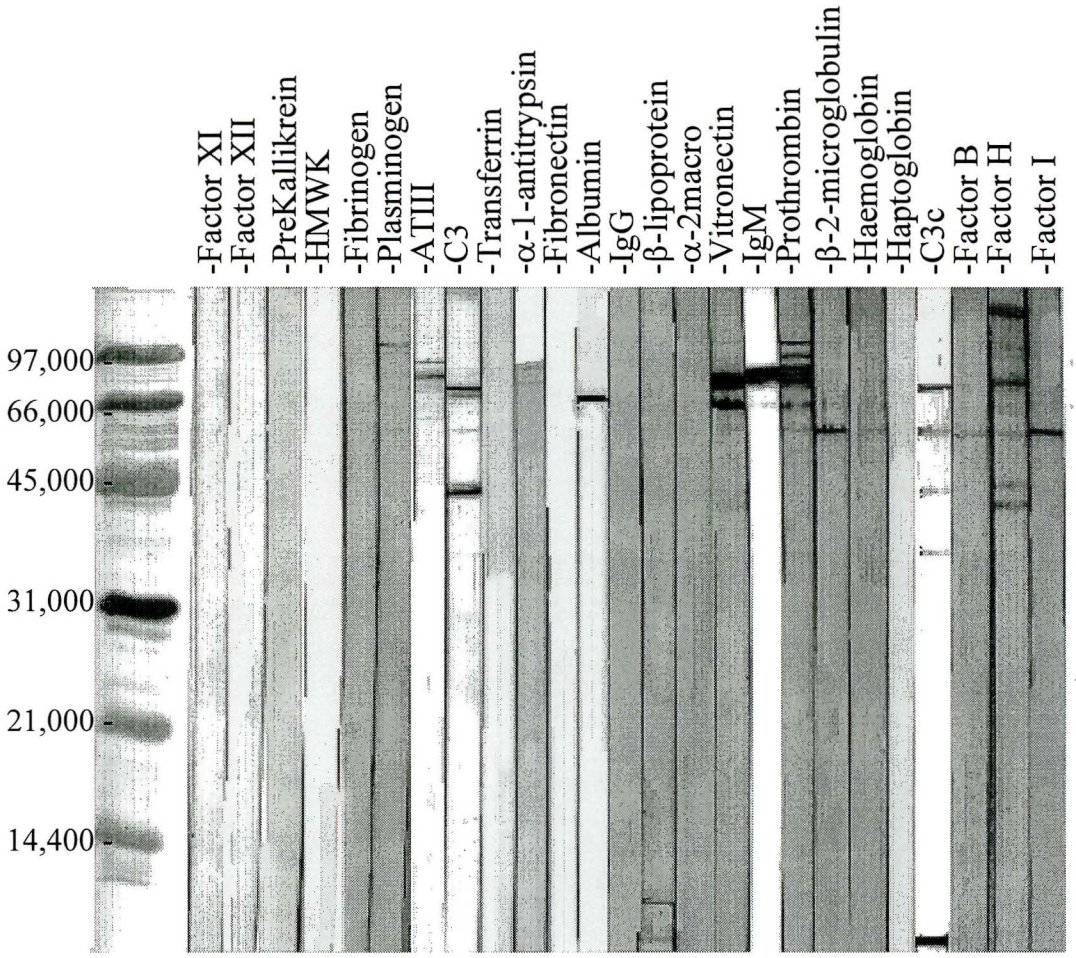

Figure 4.15d Immunoblot from SDS-PAGE gel (reduced) of 2 % SDS eluate from CFPR modified gold surface after 3 h protein adsorption from plasma.

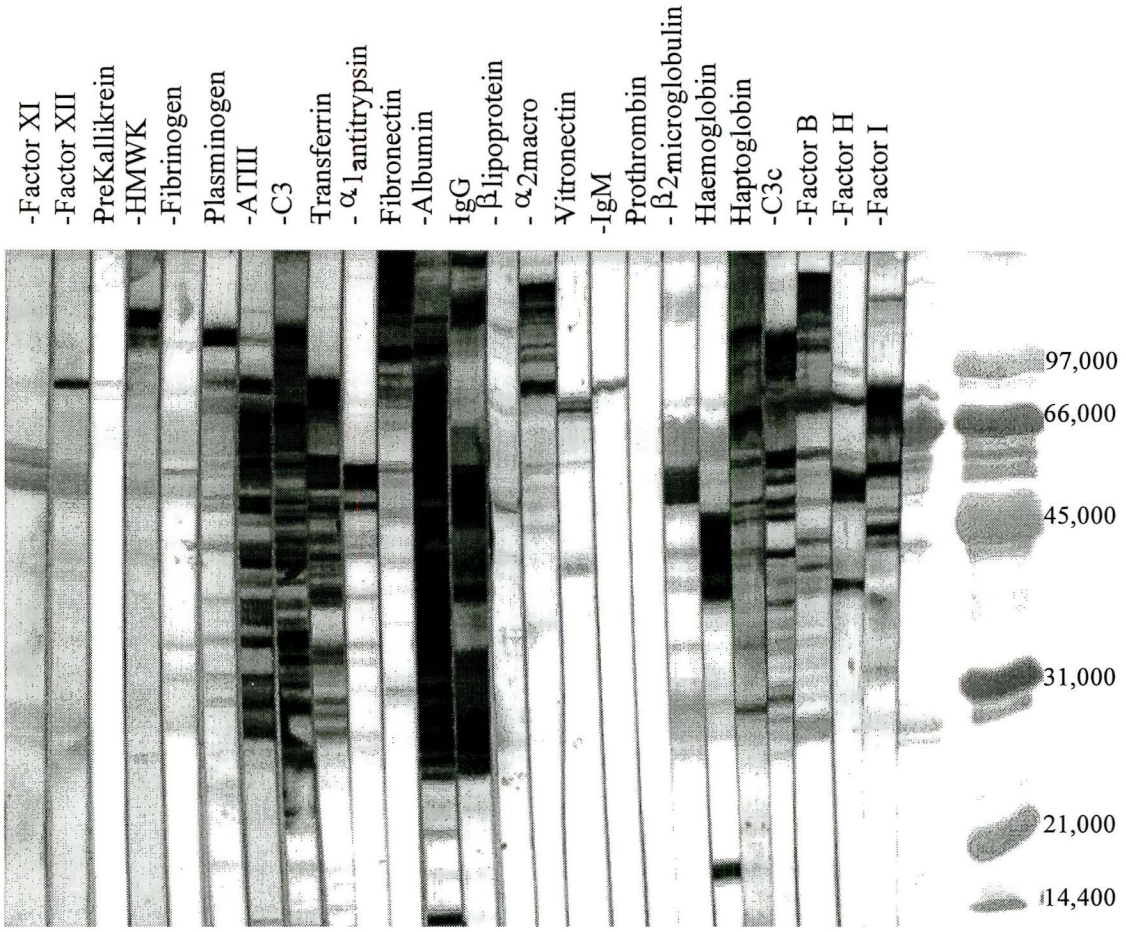

Figure 4.15e Immunoblot from SDS-PAGE gel (reduced) of ATIII and Fibrinogen-depleted human plasma.

The data show that while the overall banding patterns vary from surface to surface, the blots for the surfaces are much less complex than for the plasma itself. Therefore, it can be said that the surfaces show some protein selectivity in their interactions with plasma. The blot for the unmodified gold surface (Fig. 4.15a) shows virtually no proteins except for trace amounts of albumin, C3c, and complement factor I. A possible explanation for this result is that upon exposing the gold surface to plasma, the -S-S- and -SH groups in the proteins may react chemically with the gold. SDS may not be able to elute these "chemisorbed" proteins. This explanation is suggested by studies of Tengvall et al on the adsorption of plasma proteins to gold surfaces chemisorbed with 3-mercaptopropionic acid, L-cysteine and glutathione (Tengvall et al, 1992, 1994).

The blots of the protein eluted from the cysteine-, CPR- and C(D)FPR-modified gold surfaces (Fig. 4.15 b,c,d) show more proteins present than on the unmodified gold, but fewer than in the plasma control (Figure 4.5e). Assuming that proteins adsorbed to bare gold patches that may be present on the modified surfaces are not eluted by SDS, then the proteins seen in the blots must be associated with the peptide or cysteine sites. Albumin is seen at about 66 kD on all the modified gold surfaces. It has been reported that albumin adsorbed on artificial surfaces can reduce platelet adhesion, and thus may be considered to be a passivate ing protein with respect to blood-surface interactions (Young et al., 1983). Plasminogen, the major component of the fibrinolytic system, is present in trace amounts on the C(D)FPR and cysteine blots (94 kD), but not on the CPR blot. On the plasma control blot, plasminogen appears as a very strong band at 94 kD, the

value expected for the intact plasminogen molecule. When the fibrinolytic system is activated, plasminogen is converted to plasmin (the active fibrinolytic enzyme), a disulfide-linked 2 chain molecule with a heavy chain of 60 kD and a light chain of 24 kD. Therefore activated plasminogen is expected to show bands at 60 and 24 kD in a reduced gel. It appears that no conversion of plasminogen to plasmin occurs on the C(D)FPR and cysteine surfaces.

The contact phase coagulation factors, factor XII, high molecular weight kininogen (HMWK), factor XI, and prekallikrein are not detectable on the CPR and C(D)FPR blots, and only factor XI and HMWK bands are visible on the cysteine blot. These results suggest that the peptide- and cysteine-modified gold surfaces do not interact with the contact system proteins. If so, these surfaces may also be inert with respect to the intrinsic coagulation pathway.

An interesting point to note is the presence of prothrombin in the eluate from the modified gold surfaces (band at about 72 kD). Prothrombin is the precursor of thrombin, and when activated, is converted to thrombin (molecular weight 36 kD). A very faint band at 36 kD is visible on the cysteine blot, but not on the CPR and C(D)FPR blot. This suggests that prothrombin is adsorbed on the modified gold surfaces, but is not converted to thrombin.

Another point of interest is the ATIII-thrombin complex with a mass of 94 kD. A band in this position is seen in the ATIII blots for the three modified surfaces. This result indicates that some immuno detectable ATIII still remains in the ATIII- and fibrinogen-

depleted plasma. ATIII will bind to any thrombin present in plasma to produce an ATIII-thrombin complex which can be detected by ATIII antibody. The mechanism of binding and the binding sites on thrombin for ATIII are different from those for CPR and C(D)FPR as direct thrombin inhibitors. However, the ATIII-bound thrombin has no procoagulant activity, so the complex either in solution or adsorbed on a surface would not cleave thrombin substrate, either synthetic or natural.

Trace amounts of several complement proteins and IgG can be seen in the eluates from the modified gold surfaces. As well as C3 itself, C3c, factor I and IgM are seen in the blots for all three modified surfaces. IgG appears only in the cysteine blot, and only the heavy chain is detected

Adhesion proteins such as fibronectin and vitronectin (molecular 200 kD and 75 kD respectively) were also investigated. These proteins contribute to the attachment and spreading of blood cells (particularly platelets) on surfaces. Although no fibronectin was detected in the eluates from any of the modified gold surfaces, vitronectin is clearly present on all of them. The vitronectin band is particularly strong on the CFPR surface.

The immunoblot data suggest in general that the peptide- and cysteine-modified surfaces adsorb relatively small amounts of plasma proteins. At the same time, we have seen that the peptide-modified surfaces absorb more thrombin from plasma than the cysteine and gold control surfaces.

In summary, the peptide modified gold coated polyurethane surfaces showed significant thrombin adsorption from plasma in both radiolabelling and Western blot

assay experiments. In addition, very few other plasma proteins appear to be adsorbed on the peptide modified gold surfaces. The results of this study therefore suggest that the peptide modified gold surfaces have high affinity and specificity for thrombin binding.

The high amounts of thrombin on the peptide modified gold surfaces, and the very low activity of the adsorbed thrombin suggests that the peptide surfaces are capable of scavenging thrombin from plasma, and of inhibiting the adsorbed thrombin activity. They may therefore have potential as antithrombogenic surfaces.

5. SUMMARY, CONCLUSIONS AND RECOMMENDATIONS

5.1 Summary of Research

The hypothesis on which this work is based is that blood compatible surfaces can be prepared by the adsorption of thiols containing antithrombogenic functional groups to gold films coated on suitable substrates. The peptides CPR, C(L)FPR and C(D)FPR, which are analogues of a known powerful direct thrombin inhibitor (D)FPR chloromethyl ketone (PPACK), have been chemisorbed to gold coated polyurethane surfaces.

The chemical and physical characterization of these peptide modified gold coated polyurethanes were conducted using a number of techniques, such as contact angle measurements; XPS; AFM and SEM, as well as ellipsometry and IRAS.

The specificity of these peptide modified gold surfaces for thrombin binding was investigated by protein adsorption experiments using ^{125}I-labelled thrombin as a tracer. Thrombin adsorption from both single protein solutions and plasma to these peptide modified gold surfaces was measured by elution of the adsorbed protein from the surfaces using SDS. The validity of this procedure is based on the assumption that SDS can efficiently elute proteins from the surface, without removing iodide ion. Cysteine-modified and unmodified gold coated polyurethanes were tested simultaneously for comparison to the peptide-modified surface. Fibrinogen adsorption to these surfaces was

also measured in order to investigate the affinity of these peptides for plasma proteins other than thrombin.

The activity of thrombin adsorbed on the peptide modified gold surface was also investigated using a chromogenic substrate assay. Again the activity of thrombin on these peptide-modified surfaces was compared to that on the cysteine-modified and bare gold surfaces.

In order to positively identify thrombin adsorbed on the peptide modified gold surfaces, immunoblotting was conducted on eluted proteins using a biotin-conjugated anti-human thrombin antibody in conjunction with an avidin-conjugated alkylated colour reagent. Immunoblotting for 20 plasma proteins was also conducted to identify other proteins which had adsorbed to these peptide surfaces in contact with plasma.

5.2 Conclusions

1). The peptides CPR, C(L)FPR and C(D)FPR, were considered as potential thrombin inhibitors in this research. Their ability to inhibit thrombin was confirmed by a chromogenic substrate assay. All three peptides showed a significant level of of thrombin inhibition. The C(D)FPR peptide showed the greatest inhibitory activity, and C(L)FPR and CPR had weaker inhibitory effects.

2). The peptide modified gold surfaces were characterized by a number of techniques. Contact angle measurements showed an increased hydrophilicity for all the modified gold surfaces compared to the bare gold itself. The relatively high contact angle

on the gold surface is caused by contamination with organic compounds as revealed by XPS data. The sulfur content obtained from XPS agreed with the elemental analysis. The results of IRAS analysis confirmed the thiol-gold reaction, and showed the presence of the peptides on the gold surface. The thickness and surface density were estimated from the ellipsometry data, and the data suggested that monolayers of the peptides were formed on the gold.

3). The interactions of the peptide modified gold surfaces with blood were investigated by measuring thrombin adsorption from both single protein solutions and human plasma. Increased levels of thrombin were adsorbed on the peptide modified gold surfaces compared to the two control surfaces (the cysteine modified and unmodified gold surfaces). Furthermore, the C(D)FPR modified gold surface had the highest thrombin adsorption of all the surfaces studied. Fibrinogen adsorption from plasma was also measured and it was found that there was no difference in adsorption levels on the peptide modified gold surfaces and control surfaces. These data suggest that the peptide surfaces are specific to some degree for thrombin adsorption from plasma.

4). The inhibition of thrombin adsorbed on the peptide modified gold surfaces was investigated by a chromogenic substrate assay. The thrombin adsorbed on the peptide modified gold surfaces showed lower activity than that on the gold control surface. The C(D)FPR surfaces had the lowest levels of thrombin activity.

5). The thrombin adsorbed on these modified gold surfaces was identified by immunoblot assays. Clear bands were observed at 36 kD in the blots of eluates from

peptide modified gold surfaces. Only faint bands were observed on the blots for the cysteine and gold surfaces. Twenty-five plasma proteins were also tested for in the eluates from the surfaces following plasma contact. Few plasma proteins other than albumin and complement system proteins were visible on the blots of the modified gold surfaces, and no contact phase coagulation proteins were detected. Prothrombin was visible on the blots from the peptide surfaces, suggesting that the peptides have a high binding affinity for prothrombin as well as thrombin. Almost no protein was detectable in the eluates from the gold surface itself. This result is in accord with previous studies showing that proteins are chemisorbed to gold via thiol or disulfide groups, and following this, cannot be removed by SDS elution.

It is concluded that thiol-gold chemistry is a potentially powerful tool for surface modification to improve blood compatibility. The peptide-modified, gold-coated polyurethanes developed in this work have been shown to selectively adsorb thrombin when exposed to human plasma, and to inhibit the activity of bound thrombin. The C(D)FPR modified gold surface showed the strongest specificity for thrombin and the highest inhibitory activity of the three peptide surfaces studied. This approach may provide the basis for a powerful thrombin scavenging surface.

5.3 Recommendations for future study

1) In vitro studies of the peptide modified gold surfaces exposed to human plasma under flow conditions would be of interest.

2) In addition to the chromogenic substrate assay, the inhibitory activities of the peptide modified gold surfaces on thrombin should be measured using a clotting assay, e.g. thrombin time, prothrombin time or activated partial thromboplastin time (APTT). These assays would provide data more directly relevant to blood contact and would be a more severe test of anticoagulant activity.

3) The stability of these peptide-modified gold surfaces with respect to heat, pressure or solvents should be investigated.

4) Other than the peptides CPR, CFPR and C(D)FPR, more powerful and efficient thrombin inhibitors such as PPACK, hirudin or hirulog could be introduced to the gold surfaces by chemically attaching analogues of these compounds carrying a terminal cysteine.

5). Other surface moieties such as PEO or albumin as passivating agents, could be chemisorbed to the gold surface to prevent the nonspecific adsorption of other plasma proteins to the surface. As well, anti platelet aggregation agents such as PGI2 could also be chemically attached to the gold surfaces, although the attachment of these species in a way that retains their biological activity would present a considerable synthetic challenge. Combinations of all the ligands mentioned (thrombin scavengers, PEO, antiplatelet agents) would also be of interest.

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APPENDIX A

REAGENTS AND SOLVENTS

Solvents:

Dimethylsulfoxide (DMSO)	Sigma-Aldrich, Toronto, ON
Dimethylformamide (DMF)	Sigma-Aldrich, Toronto, ON

Polyurethane Reagents:

Polytetramethylene oxide 980 (PTMO)	BDH Chemical, Toronto, ON
4,4-Diphenylmethane diisocyanate (MDI)	Eastman Kodak, Rochester, NY
Ethylene diamine (ED)	Aldrich, Milwaukee, WI

Gold

Gold coated polyurethane	Dept. of Physics, Queen's University, Kingston, ON
Gold coated glass	Dept. of Applied Physics, University of Linkoping, Linkoping, Sweden.

Biological Materials:

Thrombin	3000 NIH/mg	Enzyme Research Laboratory Inc. Southbend, IN
Fibrinogen		Calbiochem La Jolla, CA
Ancrod		Sigma St. Louis, MO
Albumin		Sigma St. Louis, MO
N-Tosyl-Pro-Arg-N-p-Nitroanilide		Sigma St. Louis, MO

PPACK	Sigma	St. Louis, MO
CPR, C(L)FPR, C(D)FPR	Peptide synthesis Lab, Biochemistry, Queens University, Kingston, ON	
Cysteine	Sigma	St. Louis, MO
ATIII-depleted human plasma	4N44 of HSC, Red Cross Canada, McMaster University, Hamilton, ON	

Others

Isotope I^{125}	ICN Pharmaceuticals, Irvine. CA	
Iodogen® (1,3,4,6-tetrachloro-3a,6a-diphenylglycoluril)	Pierce	Rockford, IL
SLIDALYZER®	Pierce	Rockford, IL
Linbro® (EIAII plus microtitration) plates	Flow Lab Inc.	McLean, VA

APPENDIX B

WESTERN BLOT PROCEDURE

1. Prepare Gels

Separating Gel (12% gel):

Distilled water	3.35 mL
1.5 M Tris-HCl, pH 8.8	2.5 mL
10% (w/v) SDS stock	100 μL
Acrylamide/Bis (30 % stock)	4.0 mL

Degas above 4 reagents for 15 minutes at room temperature.

Add:	10 % ammonium persulfate	50 μL
	TEMED	5 μL

Fill gel plates with polymer solution, leaving enough space to add stacking gel later. Let it polymerize for approximately 2 min, then layer a small amount of water over gel (around 400mL each side or until the top line becoming smooth). Allow the separating gel to continue to polymerize for 1 h before adding stacking gel.

Stacking Gel (4 % gel):

Distilled water	3 mL
0.5 M Tris-HCl, pH 6.8	1.2 mL
10 % (w/v) SDS stock	100 μL
Acrylamide/Bis (30 % stock)	0.65 mL

Degas above 4 reagents for 15 minutes at room temperature.

Add:	10 % ammonium persulfate	25 μL
	TEMED	5 μL

Fill remainder of gel plates with stacking polyer solution, add comb (avoid bubbles). Allow polymerize for 1 h before use.

2. Prepare Samples

Sample buffer:

Distilled water	4 mL
0.5 M Tris-HCl, pH 6.8	1.2 mL
Glycerol	0.8 mL
10 % (w/v) SDS stock	1.6 mL

Mix the above four reagents, and aliquote into 225 μL volumes, and store in fridge until needed. Add below to sample buffer:

2-Bmercaptoethanol	30 mL
0.05% (w/v) Bromphenol Blue	30mL

Typical amount for a western blot using concentrated samples

(a) 1mL standard Mw marker, 10 mL TD

(b) 80 ~ 150 mL sample, 20 mL TD

(c) 7.5 mL prestained markers

If for SDS-PAGE gel, the samples are placed in 95°C water for 7.5 min.

3. Electrophoresis

Electrophoresis Buffer:

Tris base	15 g
Glycine	72g

SDS	5 g

Fill to 1 L with distilled water, check pH, do not adjust it.

Transfer Buffer:

Tris	3.03 g
Glycine	14.4 g
Methanol	200 mL

Fill to 1 L with distilled water pH should be 8.3, do not adjust. Keep it cold (4°C).

Electrophoretic Transfer:

Cut the immobilon PVDF transfer membrane to size, prewet with 100% methanol, water and soak in transfer buffer. Blot for 1 h at 100V (200 mA).

4. Molecular Weight Determination

Remove the standard molecule weight marker lanes. Wet with 100% methanol and water first, then stain using amido black 0.1% (w/v) in 10% (v/v) methanol, 7% acetic acid until the marker shows out. Destain using 45% methanol, 7% acetic acid followed by 90% methanol, 7% acetic acid. Air dry.

5. Block Unbound Membrane Sites

Cut remainder of blot into 3 mm wide strips. Wet with 100% methanol, rinse with distilled water, place into culture tubes. Incubate the strips for 1 h, with gentle

agitation in 5% w/v non-fat dry milk in TBS, pH 7.4. This procedure blocks the areas of the membrane devoid of bound proteins so that non-specific binding of antibodies to these areas will not occur. Wash strips 3 times for 5 min in 0.1 % (w/v) non-fat dry milk in TBS.

TBS Buffer:

Tris	6.057 g
NaCl	8.75 g

Fill to 1 L with distilled water. Adjust pH to 7.4.

PBS Buffer:

Na_2HPO_4	1.32 g
$NaH_2PO_4 \cdot H_2O$	0.34 g
NaCl	8.5 g

Fill to 1L with distilled water. pH adjust to 7.4.

6. Incubate with Primary Antibody

Incubate for 1 h in 1 mL 1 % w/v non-fat milk, 0.05% w/v Tween 20 in TBS containing the first antibody to the protein of interest.

I. 1:1000 is used for all first antisera. (3mL buffer + 3 μL of antibody)

II. 1:5000 is used for biotin-labelled first antisera. (3mL + 0.6 μL of biotin-labelled antibody)

III. If incubate the whole blot for 1 protein interest, 20 mL volume is needed.

Wash strips as before, in 0.1% (w/v) non-fat dry milk in TBS.

7. Incubate with second antibody

Incubate strips for 1 h with enzyme-conjugated second antibody in 1% w/v non-fat dry milk, 0.05% v/v Tween 20 in TBS.

I. 1:1000 dilution of alkaline phosphatase

II. 1:5000 dilution of strepavidin alkaline phosphatase conjugate.

Wash away excess and non-specific probe

I. Normal antibody washing 3 times of 5 min

II. Strepavidin washing 6~7 times of 5 min.

8. Detection

Incubate with the appropriate substrate to develop the colour reaction. Stop the reaction by rinsing with distilled water.

Substrate: 5-bromo-4-chloro-3-indolyl phosphate (BCIP)

Nitroblue tetrazolium (NBT)

PBS Buffer:

Na_2HPO_4	1.32 g
$NaH_2PO_4 . H_2O$	0.34g
NaCl	8.5g

Fill to 1 L with distilled water. pH is adjust to 7.4.

Carbonate Buffer:

$MgCl_2 \cdot 6H_2O$	20 mg
$NaHCO_3$	840 mg

Fill to 100 mL with distilled water. Adjust pH with NaOH to 9.8.

Reagents:

Immobilon® PVDF membrane	Millipore, Bedford, MA
BCIP (5-bromo-4-chloro-3-indolyl phosphate)	Bio-Rad, Richmond, CA
NBT (nitroblue tetrazolium)	Bio-Rad, Richmond, CA
Mini-protein II® cell	Bio-Rad, Richmond, CA
Tween 20 (polyoxyethylene sorbitan monolaurate)	Bio-Rad Lab, Hercules, CA