POLYURETHANE SURFACES BEARING IMMOBILIZED THROMBIN BINDING AGENTS: PREPARATION AND INTERACTIONS WITH PLASMA

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ABSTRACT

Novel polyurethanes bearing D-phe-pro-arg chloromethylketone (PPACK), sulphonate groups and polyethylene oxide were synthesized using block copolymerization and chain-grafting methods. The structure and the PPACK attachment mode of the PPACK-bearing polyurethane were studied by 2D NMR. The distribution of the functional groups on the corresponding biomaterial surfaces was studied by XPS. PPACK was grafted onto the polyurethane chains in such a way that its bioactivity (interactions with thrombin) was retained. The protein layers deposited from plasma onto the different polymer surfaces were studied by SDS-PAGE in conjunction with immunoblotting methods. The initiation and generation of active coagulation factors in plasma contacting different polymer surfaces were investigated by chromogenic substrate methods using specific oligopeptide substrates. The polyurethane bearing PPACK residues effectively inhibited the activation and generation of factor XIIa and thrombin in plasma contacting the surface, and prevented the clotting of the plasma. This much improved in vitro biocompatibility indicated the validity of using non-heparin antithrombin agents to prevent surface induced coagulation, and it showed that the PPACK-based polymers have potential as novel biomaterials to improve the biocompatibility of current medical devices such as small diameter vascular prostheses, hemodialyzers, stents, and catheters.

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ABBREVIATIONS

A or pNA	p-nitroaniline
AT III	antithrombin III
B	surface binding site
BDDS	4,4'-Diamino 2,2'biphenyl-disulphonic acid sodium salt
C3	complement factor 3
DADFAT	human citrated plasma depleted of ATIII and fibrinogen, and with added thrombin
DMF	N,N'-dimethyl formamide
DMSO	dimethyl sulphoxide
ESCA	electron spectroscopy for chemical analysis
GPC	gel permeation chromatography
h, hr	hour
HCP	human citrated plasma
HK	high molecular weight kininogen
HMWK	high molecular weight kininogen
HSA	human serum albumin
HT	human thrombin
IgG	immunoglobulin G
IIa	thrombin
IPN	interpenetrating network
Ks	equilibrium or affinity constant

kD	kilo Dalton
k_r	reaction rate constant for thrombin-substrate interaction
k_t	reaction rate constant for thrombin inhibition by surface binding sites
MDA	methylene dianiline
MDI	4,4'-diphenylmethane diisocyanate
min	minute
MW	molecular weight
NMR	nuclear magnetic resonance
OD	optical density
PEO	polyethylene oxide
PEOBDDS	a polyurethane made from BDDS, PPO, PEO and MDI
PEOMDA	a polyurethane made from MDA, PPO, PEO and MDI
pNA	p-nitroaniline, or p-nitroanilide (when it is bound to a tripeptide)
PPACK	D-phe-pro-arg chloromethylketone
PPACKPPO	PPACK-containing PPOBDDS
PPO	polypropylene oxide
PPOBDDS	a polyurethane made of BDDS, PPO and MDI
PPOMDA	a polyurethane made of MDA, PPO and MDI
Pst	polystyrene
RFGD	radio frequency glow discharge
RT	retention time
S	substrate
SDS	sodium dodecyl sulfate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis

SEM-EDAX	scanning electron microscope with energy dispersive analysis of X-rays
t	time
T	thrombin (concentration), temperature
TBS	Tris buffered saline
Tris	tris(hydroxymethyl)aminomethane
VN	vitronectin
XIa	activated factor XI
XII	factor XII
XIIa	activated factor XII
XPS	X-ray photoelectron spectroscopy
YPACK	N-p-tyr-pro-arg chloromethylketone
YPACKPPO	YPACK-containing PPOBDDS

POLYURETHANE SURFACES BEARING IMMOBILIZED THROMBIN BINDING AGENTS: PREPARATION AND INTERACTIONS WITH PLASMA

CHAPTER 1
INTRODUCTION

The development of blood-contacting biomaterials has been hindered by the inherent thrombogenicity of artificial surfaces. For example, the high mortality and morbidity associated with the use of vascular prostheses and implanted blood pumps are directly related to the poor blood compatibility of the synthetic materials used in their construction (Moritz et al., 1988; Ifudu et al., 1994; Iglehart, 1993). The interactions of a biomaterial surface with human blood stimulate platelet activation, coagulation and thrombus formation (Ryan and Worthington, 1992; Bevers et al., 1991; Elam and Nygren, 1992; Brash, 1988). The initial step is the adsorption of blood proteins on the surface (Brash, 1988). Platelets then adhere and become activated. Finally the coagulation cascade produces fibrin to consolidate the thrombus.

The plasma protein thrombin plays a central role in coagulation and thrombogenic processes (Fenton II, 1986). Thrombin converts fibrinogen to fibrin and initiates several cellular and vascular responses including platelet activation, endothelial cell changes, and activation of coagulation factors V, VIII, and XIII (Mustard and Packham, 1970; Carney and Cunningham, 1978; Gospodarowicz et al., 1978; Kitaguchi et al., 1979; Salem et al., 1984; Fenton

II et al., 1991). The activated factor V and factor VIII form a complex with factors IXa and Xa respectively and increase their activity several hundred times so that the production of thrombin is greatly accelerated. This feedback mechanism of thrombin is the key for production of physiologically effective concentrations of thrombin itself. Due to the various roles of thrombin in thrombotic processes, thrombin has become a primary target in strategies to prevent thrombosis (Scharfstein and Loscalzo, 1992; Hijikata-Okunomiya and Okamoto, 1992). It is widely believed that to understand the interactions of thrombin is to understand thrombosis, so that to control thrombin is to control thrombosis. Accordingly, we propose that a blood compatible surface should exhibit thrombin-inhibiting properties.

Among the different strategies for improving blood compatibility, surface heparinization to inhibit thrombin and prevent thrombosis has been one of the most favored. Polymeric solids bearing heparin are either controlled release (Kim et al., 1983; Albanese et al., 1994) or covalently "grafted" systems (Smith and Sefton, 1992, 1993; Larsson et al., 1987; Park et al., 1992). Such materials have been found to be less effective than desired, and their clinical application is often accompanied by supplementary systemic heparinization (von Segesser et al., 1992).

Upon careful examination of human blood-surface interactions, problems with surface-heparinization approaches become evident: (1) A biomaterial surface might directly participate primarily in activating the contact system while the production of "physiological" concentrations of thrombin is mainly on the phospholipid membrane of the platelets which are adsorbed and activated on the biomaterial surface. If this is true, then attention should be focused on inhibiting

the thrombin released into the blood from the platelet membrane as well as that adsorbed to the biomaterial surface. (2) The rapid adsorption of proteins onto a biomaterial surface is known to be the first event in blood-biomaterial interactions, with subsequent reactions controlled to a large extent by the nature of the adsorbed protein layer (Brash, 1991). van Delden et al. (1994) reported that preincubating a heparin-grafted polymer surface with plasma significantly decreased the activity of the heparin. Releasable heparin may be expected to behave differently from covalently grafted heparin by diffusing through the protein layer, thereby retaining its antithrombin activity. However, no advantage of such a heparin-releasing system over a heparin-grafted system has been demonstrated. (3) An important consideration is the recent finding that heparin-antithrombin III (ATIII) complex inhibits only free thrombin and has little effect on the fibrin- or matrix-bound thrombin which is a major contributor to thrombus growth (Weitz et al., 1990; Hogg and Jackson, 1989). The inactivation of fibrin-bound thrombin by the heparin-ATIII complex is most likely sterically hindered (Hubbell and McIntire, 1986). (4) Heparin has a number of undesirable side effects (Hirsh, 1991), particularly with regard to platelets; it may promote platelet adhesion and aggregation, possibly by direct interactions (Brace and Fareed, 1985). Kelton et al. (1988) reported that heparin binds to the Fab portion of IgG to form an immune complex which can initiate the platelet release reaction by binding to the platelet Fc receptors.

To inhibit the thrombosis process more effectively at the blood-biomaterial interface, ATIII-independent antithrombin agents should be considered, and these agents should be able to break through the "barrier" of the newly formed protein layer to ensure inhibition of platelet-dependent thrombosis. In addition,

since the feedback activation process of thrombin is very fast, the antithrombin agents on the biomaterial surface must be able to inhibit the initially produced thrombin and thus block the thrombin feedback mechanisms. A key consideration is to keep the correct balance of thrombin inhibition and activation. Therefore, a highly specific thrombin inhibitor is needed for the synthesis of new antithrombotic biomaterials, and the incorporation of the inhibitor into the polymer surface must not reduce its activity.

The tripeptide PPACK (D-phe-pro-arg-CH_2Cl) is an extremely effective inhibitor of thrombin. It is V-shaped with a positively charged "head" that binds to the aspartic acid at the center of thrombin's active site (Kettner and Shaw, 1979; Hijikata-Okunomiya and Okamoto, 1992). Given the relatively small size (MW 453) of this tripeptide, it seems unlikely that there will be significant conformational change when it is immobilized. Its efficacy in binding thrombin may thus remain intact in the surface-bound state. Therefore, in this research, PPACK was chosen for immobilization to polymer surfaces as an approach to the neutralization of thrombin activity.

Another reason for the slow progress in the development of blood compatible biomaterials is that the interactions between biomaterials and the many components of blood and tissue are extremely complicated and not well understood. The mechanisms of thrombosis on biomaterial surfaces have been studied for over two decades, but are still not clear (Ratner, 1993; Courtney et al., 1994).

Protein adsorption is believed to play a critical role in coagulation and platelet activation. The mechanism of protein deposition and the composition of the protein layer formed in blood-biomaterial contact has been the focus of much

research (Brash, 1991). In many cases, only a monolayer of proteins is adsorbed to the surface; therefore radio-labeling and other highly sensitive methods have been used to measure the trace amounts of proteins adsorbed onto the biomaterial surface. A drawback of the radio-labeling method is that only one or two proteins can be measured at a time. Recently, Mulzer and Brash (1989) used an immunoblotting method to directly detect more than 20 important plasma proteins adsorbed on hemodialyzer membranes. Valuable information on plasma-surface interactions was obtained. It may be anticipated that this approach will be further developed to study systematically the adsorption and activation of proteins on well defined model surfaces, and consequently to probe the mechanisms of protein-surface interaction in greater depth.

It is known that complicated multiple reactions occur simultaneously at the plasma-polymer or blood-polymer interface. Using factor-depleted plasmas (for example, fibrinogen-depleted plasma) information may be obtained on that particular protein (Brash, 1988; Adams et al., 1984). However, this is an indirect approach using modified plasmas. It is perhaps preferable to develop new methods to examine directly the interactions of individual components from normal plasma. In the case of enzymes, chromogenic substrates can be used for this purpose (Huseby and Smith, 1980). With certain highly specific chromogenic substrates, the activity of thrombin, factor XIIa, and other activated clotting factors can be directly measured in the plasma by measuring the colour released during the reaction.

The objective of the present research is to synthesize new antithrombogenic materials by incorporating the specific thrombin inhibitor PPACK and to combine immunoassay and chromogenic assay as an integrated

method to study the mechanisms of protein adsorption and clotting factor activation on the newly synthesized material surfaces.

The specific aims of this research are:

(1) To develop new biomaterials bearing the ATIII-independent thrombin-inhibitory peptide PPACK;

(2) To acquire a knowledge of the composition of the protein layer deposited on these biomaterial surfaces from plasma and its influence on the activity of the antithrombin agent;

(3) To acquire a knowledge of the kinetics and mechanisms of coagulation factor activation at the biomaterial surfaces;

(4) To devise new approaches for synthesizing blood-compatible biomaterials by incorporating highly specific antithrombin peptides with both surface grafted and controlled release characteristics, thus overcoming the "barrier" of the protein layer formed on the biomaterial surface.

CHAPTER 2
BACKGROUND AND LITERATURE REVIEW

The processes identified in blood-material interactions are summarized in Fig. 2.1, taken from Brash (1987). As shown in Fig. 2.1 (p. 30), the initial step is the adsorption of plasma proteins on blood-contacting surfaces, followed by platelet interaction and coagulation. The interactions in this Figure are briefly reviewed in this section. The central goal in this research is to develop thrombin-neutralizing biomaterials. Therefore, recent developments in anticoagulant therapy and anticoagulant biomaterials are addressed in this section. Finally biomaterial synthesis techniques will be briefly reviewed.

2.1 PROTEIN ADSORPTION

Upon contact with blood, a material is immediately covered by a layer of proteins. The composition of this layer and the conformation of the proteins determine the subsequent events which lead to thrombus formation.

Proteins are long chain poly(amino acids) and are able to form many different binding sites/domains to bind cell surface receptors or other extracellular matrix components (Yamada, 1983; Hynes, 1985; Culp et al., 1991; Lewandoska et al., 1992). On artificial surfaces protein binding may be non-specific as well as specific, and it is currently impossible to predict the adsorption behavior of proteins on blood-contacting biomaterial surfaces. The relative affinities of proteins for different surfaces has been studied by

competitive adsorption among several proteins, and the conformation and biological activities of adsorbed proteins have been investigated using a variety of physical and biological methods. Earlier work has been summarized by Andrade et al. (1985), although much additional data have been obtained in more recent work. For example, Fabrizius and Cooper (1991) studied the competitive adsorption of vitronectin (VN) with albumin, fibrinogen, and fibronectin in binary mixtures, and reported that VN has higher affinities for various polymer materials than the other proteins tested. However, no general interpretation has been given for these and other experimental observations. For example the energy change associated with protein conformational change on surfaces cannot be estimated and the binding constants cannot be predicted. The effects of surface composition on protein adsorption, except for a few generalities, are essentially unknown. Molecular modeling is able to predict the three dimensional conformation of proteins exposed to different environments and appears to be a useful approach (Fasman, 1989), but little work of this type has been done in the biomaterials area.

According to the in vitro work pioneered by Vroman et al. (Vroman and Adams, 1986), the proteins in blood interact with a contacting surface sequentially. The sequence begins with albumin, followed by IgG, fibrinogen, HMWK, and coagulation factors XII, and XI (Vroman and Adams, 1986; Elwing and Tengvall, 1991). The replacement sequence was found by others to be dependent on the protein concentration and surface affinity (Brash 1988). Rapoza and Horbett (1990) reported that postadsorptive transitions in fibrinogen are dependent on the adsorption time and the nature of the surface. Glassy, rigid polymers showed high fibrinogen adsorption, regardless of

whether the polymer was hydrophilic or hydrophobic. Binding strength (as measured by elutability) was much lower on hydrophilic polymers and oxygen-containing hydrophobic polymers. However, recent work by Tengvall et al. (1991) showed that, on a hydrophobic surface, the adsorption of albumin and fibrinogen is stable, and no displacement of one protein by another was observed.

As indicated it is important to be able to control protein adsorption since the behaviour of adsorbed proteins determines subsequent events. It has been found, for example, that surfaces precoated with fibrinogen, fibronectin, gamma-globulins, thrombospondin, and von Willebrand factor, among others, enhance thrombus formation. However, other proteins such as albumin and transferrin tend to passivate artificial surfaces against thrombosis (Packham, 1988).

2.2 PLATELET INTERACTIONS ON ARTIFICIAL SURFACES

Platelets play several important roles in thrombogenesis on biomaterial surfaces (Packham, 1988). Many studies have shown that platelet adhesion is related to the amount of fibrinogen adsorbed on the biomaterial surface (Vroman et al., 1980; Zucker and Vroman, 1969; Chaikof et al., 1990; Elam and Nygren 1992; Packham, 1988). It is believed that glycoprotein IIb/IIIa complexes (gpIIb/IIIa, fibrinogen receptors) on the platelet surface are involved in the process of fibrinogen-related platelet adhesion. There are approximately 50,000 gpIIb/IIIa complexes present on the surface of the resting platelet. It is currently thought that the activation of platelets by thrombin or the adhesion of platelets to surfaces will induce a conformational change in the gpIIb/IIIa complex which results in the expression of the binding sites of gpIIb/IIIa for

fibrinogen (Bennett, 1992; Beardsley, 1990; Plow et al., 1984). Therefore, one possible role of the adsorbed fibrinogen in the platelets' response to artificial surfaces is to facilitate aggregation and stabilization of the adsorbed platelets.

Other proteins such as fibronectin and von Willebrand factor (vWF) also induce or enhance platelet adhesion (Fressinaud et al., 1990). The fibronectin receptor gpIc/IIa is active in unstimulated platelets and thus is available to support directly the adhesion of platelets to surfaces (Bennett, 1992). Fibronectin is known to adsorb to artificial surfaces (Elam and Nygren, 1992) and thus it will contribute to the adhesion of intact platelets possibly leading to platelet activation. vWF is a multimeric glycoprotein composed of identical subunits (Sadler et al., 1991). All of the subunits can bind to the gpIb/IX receptor on the platelet surface thus enhancing the affinity of vWF to platelets and the ability of the vWF-bound platelets to resist high shear stress in flowing blood (Weiss, 1991). Ordinarily, vWF does not interact with platelets. It is possible that the adsorption of vWF to biomaterial surfaces results in a conformation change in vWF which renders it adhesive to platelets.

There are many other cell adhesion molecules: the immunoglobulin superfamily, the integrin family and the selectin family (Ryan and Worthington, 1992). Collagen, fibronectin, laminin, microfibrils of subendothelium, vitronectin, fibrinogen, thrombospondin, aggregates of IgG, vWF, etc., may all provide ligands for platelet adhesion. The interactions of these adhesion molecules with artificial surfaces may constitute important pathways for platelet adhesion and activation as well as activation of coagulation.

2.3 COAGULATION

The coagulation pathways are illustrated in Fig. 2.2 (p. 31). The important roles of platelet phospholipids in factor X activation and thrombin formation are emphasized (PL in Fig. 2.2). The known multipathways of initiation and feedback have made the picture quite complicated. Fig. 2.2 shows that all of the procoagulant reactions converge at thrombin. No bypass has been found.

The well accepted sequence of the intrinsic pathway (Kaplan and Silver, 1987) is as follows: Factor XII, a single chain glycoprotein, is adsorbed on negatively charged surfaces and undergoes a conformational change (Fair et al., 1977). The denatured XII is then activated by kallikrein and by other proteases (Griffin, 1978; Revak et al., 1977). The activated factor XII (XIIa) is able to activate factor XI and prekallikrein. High molecular weight kininogen (HK) is required for the adsorption of both prekallikrein and factor XI to the negatively charged surface and for the formation of complexes with factor XII (Griffin and Cochrane, 1979). Surface-bound factor XIa then activates factor IX (DiScipio et al., 1978).

There are still disagreements about the details and role of the contact phase. For example, deficiencies of factor XII, HK and prekallikrein are not associated with abnormal bleeding. Also, most artificial surfaces are not negatively charged and yet all of them activate blood coagulation to some extent. Naito and Fujikawa (1991) reported that factor XI can be autoactivated, or can be activated on negatively charged surfaces by thrombin. They proposed that factor XI is possibly the enzyme which initiates the intrinsic pathway of coagulation. Broze (1992) also considered that the activation of

factor XI is independent of other contact factors.

It may well be that platelets play the major role in the intrinsic pathway (Walsh, 1987). It has been found that platelets can release HK (Kerbiriou-Nabias et al., 1984; Schmaier et al., 1983) and factor XI (Lipscomb and Walsh, 1979; Tuszynski et al., 1982). Although the quantities of intrinsic clotting factors in platelets are small compared to the plasma content of these proteins, there may be enough to cause the initiation of the coagulation cascade locally.

Various steps of the coagulation cascade are influenced by platelets (Bennett 1992). The intrinsic pathway is accelerated by platelets (Walsh, 1987), and two essential steps of the coagulation cascade, the formation of factor Xa and the formation of thrombin, require the negatively charged phosphatidylserine of the platelet surface on which the activating complexes are assembled (Bevers et al., 1991). Kinetic studies have shown that the catalytic effect of phosphatidylserine on thrombin formation or fibrin formation is more than a million-fold (Mann et al., 1990; Tans and Rosing, 1986).

Thrombin plays a central role in the process of coagulation and thrombogenesis (Fenton II, 1986). Fibrin-bound thrombin can cleave circulating fibrinogen and convert it to fibrin, and can activate factors V, VIII and XI thereby resulting in the autocatalytic generation of a high concentration of free thrombin in the vicinity of the thrombus. Thrombin also causes platelet activation, endothelial cell changes, and factor XIII activation (Mustard and Packham, 1970; Carney and Cunningham, 1978; Gospodarowicz et al., 1978; Kitaguchi et al., 1979; Salem et al., 1984; Fenton II et al., 1991).

There are many ways in which the blood vessel wall may be attacked and injured. The repair mechanisms involving "thrombus" formation are

correspondingly versatile. Accordingly, thrombogenesis can be initiated by various stimuli, and it is thus expected to be very difficult if not impossible to design a simple artificial surface which inhibits all of the various prothrombogenic mechanisms. However the coagulation process can be blocked completely by inhibiting thrombin, as indicated in Fig. 2.2, although thrombin is probably not involved in the initial stages. For this reason, thrombin has become the primary target of antithrombotic treatments and therapies (Scharfstein and Loscalzo, 1992; Hijikata-Okunomiya and Okamoto, 1992).

2.4 PREVENTION AND INHIBITION OF THROMBOSIS

Since the enzymatic activity of thrombin is essential in thrombus formation, inhibition of thrombin is an attractive antithrombotic strategy. Indeed most of the known effective antithrombotic agents are thrombin inhibitors (Table 2.1). There are two groups of thrombin inhibitors. Direct inhibitors such as hirudin and indirect inhibitors such as heparin which either interfere with thrombin generation or require a cofactor to inhibit thrombin activity.

Table 2.1. Thrombin inhibitor (Scharfstein and Loscalzo, 1992)

Direct Inhibitors	Indirect Inhibitors
Hirudin	Heparin
Hirugen	Heparan sulfate
Hirulog	Antithrombin III
PPACK	Warfarin
Argatroban	Heparin cofactor II
	Activated protein C
	Thrombomodulin
	Factor Xa inhibitors

2.4.1 Heparin

Heparin (Fig. 2.3, p. 32) is an acidic mucopolysaccharide composed of D-glucuronic acid and D-glucosamine (Casu, 1989), having potent anticoagulant properties (Hirsh, 1991). Heparin is an indirect thrombin inhibitor which forms a complex with ATIII. ATIII then becomes an effective inhibitor of a number of coagulation enzymes, including thrombin, and activated factors X, XII, XI, and IX (Rosenberg, 1987). Only about one third of heparin molecules in a given population can bind to ATIII and these are responsible for most of the anticoagulant activities exhibited (Lam and Silbert, 1976). The mechanism of heparin inhibiting coagulation is not completely clear. There is evidence suggesting that its major function is the inhibition of the thrombin-induced activation of factors V and VIII (Ofosu et al., 1987; Ofosu et al., 1989; Beguin et al., 1988). It has also been found that the heparin-ATIII complex only inhibits free thrombin and has little effect on fibrin-bound thrombin which is considered to be a major contributor to thrombus growth (Hogg and Jackson, 1989; Weitz et al., 1990). The inactivation of fibrin-bound thrombin by heparin-ATIII complex is most likely sterically hindered by fibrin (Hubbell and McIntire, 1986). Electrostatic repulsion effects may also play a role.

Heparin has a number of undesirable side effects (Hirsh, 1991), particularly with regard to platelets; it may promote platelet adhesion and aggregation, possibly by direct interactions (Brace and Fareed, 1985). Kelton et al. (1988) reported that heparin binds to the Fab portion of IgG to form an immune complex which can initiate the platelet release reaction by binding to the platelet Fc receptors.

Heparin has been incorporated into biomaterial surfaces with the intention of rendering them antithrombogenic. The performance of these surfaces has not been satisfactory, and co-incorporation of antiplatelet agents into the material surface to counteract the side effects of heparin appears to be necessary (Kim et al., 1987). In addition, due to the inevitable conformational changes of heparin on immobilization, it is very difficult if not impossible to maximize its antithrombotic effects while limiting its adverse side effects (Nemets and Sevastianov, 1991; Barbucci et al., 1991).

2.4.2 Hirudin

Hirudin was originally isolated from the leech as a specific inhibitor of thrombin (Markwardt, 1970). It is a single polypeptide chain of 65 amino acids and is composed of an N-terminal core region held together by 3 disulfide bonds and a flexible C-terminal tail (Markwardt, 1989). The N-terminal binds to the active site of thrombin and the C-terminal binds to the positively charged anion-binding exosite of thrombin (Sturzecher and Walsmann, 1991), as shown in Fig. 2.4 (p. 33). The extremely high affinity ($K_d = 10^{-13}$-10^{-14} M) of hirudin for thrombin is achieved by 212 contacts of less than 4 Å across the full diameter of the thrombin molecule (Rydel et al., 1990). Hirudin inhibits not only the thrombin-catalyzed conversion of fibrinogen to fibrin but also thrombin-mediated cellular events, such as platelet aggregation and secretion (Fenton II, 1989; Hoffman and Markwardt, 1984; Glusa and Urban, 1988; Glusa, 1991). In contrast to heparin, hirudin reacts only with thrombin and not with other serine proteases, has no known side effects, and is a weak immunogen (Markwardt, 1989; Kaiser, 1991; Glusa, 1991).

2.4.3 Hirulogs

Hirulogs are synthetic peptides of about 10 amino acid residues based on what is believed to be the active portion of hirudin (Maraganore et al., 1990). The sequence D-phe-pro-arg is employed as the active site inhibitory moiety and a segment homologous to residues 53-64 of the HV2 hirudin variant is used to interact with the anion-binding exosite of thrombin (Bourdon et al., 1991). The affinity of hirulogs for thrombin is lower than that of hirudin because they do not interact with the apolar binding site of thrombin.

2.4.4 PPACK

Smaller peptides such as PPACK (D-phe-pro-arg-CH_2Cl, see Fig. 2.5, p. 34) and argatroban [(2R, 4R)-4-methyl-1(N^2-((3-methyl-1,2,3,4-tetrahydro-8-quinolinyl)sulfonyl)-l-arginyl))-2-piperidinecarboxylic acid] has been shown to bind to the active site of thrombin. PPACK binds to thrombin covalently, i.e. irreversibly, in a substrate mode. Argatroban binds to thrombin reversibly in an inhibitor binding mode (Banner and Hadvary, 1991). These molecules are usually V shaped with a positively charged "head" that interacts with the aspartic acid at the center of thrombin's catalytic site (Hijikata-Okunomiya and Okamoto, 1992). Since only the catalytic site of thrombin is inhibited, these inhibitors are less specific than hirulogs. PPACK-inhibited thrombin can still promote fibrin polymerization via its exosite activity (Kaminski et al., 1991).

2.4.5 Antiplatelet agents

The first step in platelet activation on surfaces is adhesion, but only

platelet aggregation is physiologically important in the production of thrombus. Antiplatelet agents are therefore focused on this aspect (Packham, 1988). There are many antiplatelet agents having different mechanisms of inhibition. Aspirin is the one most widely used and has been shown to be able to inhibit thrombin formation (Szczeklik et al., 1992). Since thrombin is the most potent activator of platelet aggregation, inhibition of thrombin may inhibit both coagulation and platelet activation. There have been only a few attempts to develop antithrombotic biomaterials based on immobilization of antiplatelet agents such as prostaglandins (Kim et al., 1987).

2.5. MATERIAL DEVELOPMENT FOR ANTITHROMBOSIS

As discussed in Section 2.2, protein adsorption is the first observed step in blood-material interactions leading to thrombogenesis on biomaterial surfaces. Accordingly, it has been proposed that surfaces excluding protein adsorption will be blood compatible (Courtney et al., 1993). As already indicated another important approach to antithrombogenic material synthesis is heparin grafting. We will review these two approaches since they are closely related to the present work. Other approaches such as endothelialization of surfaces will not be discussed.

2.5.1 Inert surfaces based on protein selectivity or protein resistance

Adsorbed albumin appears to passivate nearly all materials, minimizing platelet adhesion and thrombus formation (Courtney et al., 1993). However, Goodman et al. (1991) found only a small influence of adsorbed albumin on

platelet reactivity to material substrates and showed that the approach of covering a biomaterial surface with albumin may not be successful if the albumin is denatured.

Polyethylene oxide (PEO) has become a major focus of recent research in biomaterials, since surfaces grafted with PEO have shown remarkably low protein adsorption and platelet adhesion (Chaikof et al., 1990). The mechanism of these effects is still under debate. PEO is very water soluble, and perhaps there is a close match between the energy of interaction of PEO chains and water molecules. PEO chains also exhibit high flexibility (Bailey and Koleske, 1976; Merrill and Salzman, 1983; Kjellander and Florin, 1981; Andrade et al., 1987), a large excluded volume (Atha and Ingham, 1981; Hermans, 1982; Knoll and Hermans, 1983), and a low interfacial energy at the PEO-water interface. Thus the driving force for protein adsorption is low (Coleman et al., 1982). A high degree of flexibility gives PEO a high configurational entropy, and adsorption of proteins to PEO may result in a thermodynamically unfavorable loss of entropy.

2.5.2 Anticoagulant and antithrombogenic surfaces

Heparin has been incorporated into biomaterial surfaces to impart anticoagulant properties (Smith and Sefton, 1993; Ito, 1987; Jozefowicz and Jozefonvicz, 1984) and some degree of success has been achieved, especially for short term applications. As already indicated, there is some question as to the possible activation of platelets by heparin. Kim et al. (1987) addressed this question by the use of a conjugate of prostaglandin E1 (PGE1)-heparin on the basis that PGE1 would minimize the platelet response. On the other hand,

Larsson et al. (1987) reported that no platelets adhered to a heparin grafted surface. Thus, it seems clear that consensus does not exist on the platelet reactivity of heparin-bound surfaces.

A significant limitation of covalently bound heparin surfaces is their susceptibility to degradation by heparinases (Jozefowicz and Jozefonvicz, 1984). Anionic functional group incorporation into a biomaterial has been suggested as a way to mimic the anticoagulant properties of heparin while avoiding heparinase recognition (Jozefowicz and Jozefonvicz, 1984; Grasel and Cooper, 1989; Ito et al., 1991; Silver et al., 1992). Such surfaces are less specific with respect to inhibition of thrombin and show no tendency to prevent or minimize interactions with blood proteins and cells. Nevertheless good blood compatibility has been observed in some cases (Silver et al., 1992). Ito et al. (1991) reported that poly(sodium vinyl sulphonate) (PVS) was only 7% as effective as heparin in inducing the conformational change in ATIII which potentiates its interaction with thrombin.

Boisson-Vidal et al. (1991) reported that polystyrene surfaces modified with sulphonate groups appeared to promote the adsorption of plasminogen (a key protein in the fibrinolytic pathway) and its activation to a plasmin-like molecule. Okkema and Cooper (1991) reported that the incorporation of propyl sulphonate groups into polyurethanes dramatically reduced the number and activation of platelets adherent to the polymer surface, in spite of the fact that fibrinogen adsorption increased with increasing sulphonate content. Okkema et al. (1991) compared the effects of propyl sulphonate and ethyl carboxylate groups. They found that carboxylate-containing polymers had no effect on blood response, whereas a polymer containing 20 mol% propyl sulphonate

groups significantly reduced platelet deposition and activation while also exhibiting enhanced fibrinogen deposition.

Santerre et al. (1992) incorporated sulphonate groups into the hard segment of polyether polyurethanes. They found that fibrinogen adsorption from plasma increased as the sulphonate 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 sulphonate groups. To further enhance the antithrombotic activity of the sulphonated polymers, these authors derivatized the sulphonate groups with arginine methyl ester, aspartic acid and lysine (Santerre et al., 1992). They found that fibrinogen adsorption was reduced by derivatization. However, the thrombin times of plasma in contact with these materials were essentially the same for the derivatized and underivatized materials.

Ito et al. (1992) attached a thrombin inhibitor argatroban to acrylamide polymers and reported a deactivation of thrombin and suppression of platelet adhesion.

2.6 SURFACE MODIFICATION TECHNIQUES

A number of surface modification techniques have been used to improve the biocompatibility of biomaterials. Only the techniques relevant to the present work will be discussed in this section.

2.6.1 Radio frequency glow discharge

Radio frequency glow discharge (RFGD) is an important surface treatment technique which has found applications in polymer science generally

(Boenig, 1988; Ratner, 1992). The material to be deposited is introduced into a reactor containing appropriate gases and radio frequency energy is passed through the system. Radicals and ions are formed at the surface of the material and the ensuing reactions cause etching and activation of the surface. If the gas is polymerizable, a highly crosslinked layer of polymer is formed on the surface with elemental composition similar to that of the gas.

A major advantage of this technique is that the surface can be modified without changing the bulk properties of the material. Almost any polymeric material is suitable for RFGD treatment and the choice can be made based on the required mechanical properties. The disadvantage is that with the current state of the art it is impossible to introduce a specific functional group composition into the surface to achieve the high specificity required for control of protein binding. Control over the surface chemistry of the product surface is lacking.

RFGD treated surfaces are rich in radicals, and functional vinyl monomers can be incorporated into RFGD treated surfaces by radical polymerization. RFGD can thus be used to prepare a surface for subsequent chemical modification.

2.6.2 Attachment of bioactive ligands to surfaces

This is perhaps the most common method currently used to modify polymer surfaces for biological applications. In general a ligand which is expected to interact with the biological system (blood, tissue) in a beneficial way is incorporated. The disadvantages of this approach are: (1) The surface must have functional groups which can react under appropriate chemical conditions

with the ligand. (2) The possible reaction conditions do not necessarily give high yields. Therefore it is very difficult to achieve 100% conversion of all functional groups and the surface is only "partially" modified, leaving many sites which do not bear ligands. (3) The chemical bond between the surface and the ligand may not be sufficiently stable for long term "viability".

A number of binding methods have been used for ligand attachment: ionic bonding, hydrophobic interaction or simple physical adsorption. The material stability clearly depends on the method of attachment. Chaikof et al. (1990) and Arkles (1983) used the interpenetrating polymer network (IPN) method and reported high coverage in the case of silicone polymer with PEO as ligands. In principle, the IPN method does not have the disadvantages of the simple chemical bonding method mentioned above, but it requires that the bulk materials are crosslinked.

Polyurethanes have become important as biomaterials and their modification is therefore of interest to this discussion. Feng et al. (1985) used ceric salt initiation and a controlled oxidation method to graft hydrogel-forming polymers onto polyurethanes. Bamford et al. (1968; 1983) grafted vinyl monomers onto a chlorinated polyurethane by radical polymerization initiated by $Mo(CO)_6$. These techniques are versatile and in principle can be used to graft any vinyl monomer onto polyurethane surfaces.

Santerre and Brash (1990) used biphenyl diamino disulphonic sodium salt (BDDS) as a chain extender to incorporate sulphonate groups into the hard segment of polyurethane chains. Subsequently the sulphonate group was reacted with the amine group of amino acids using oxalyl chloride as an activator, thus giving materials with amino acid ligands in the hard segment.

2.6.3 Glass surface silanization

Silanization of glass is widely used in industry as a surface modification method. A proposed mechanism for the silanization of glass is shown in Fig. 2.6 (p. 35).

The silanol HO-SiR_3 is obtained via the reaction of RO-SiR_3 with water (R is an organic group such as -CH_2CH_3). Accordingly, many methodologies use wet solvent for silanization. A high concentration of water prevents the reversible silanization reaction, so the oxane formation is slow in the presence of water. Since a layer of water is usually present on the glass surface, silanization of glass can be carried out with dry solvents. For example, Robinson et al. (1971) used acetone, Grushka and Kikta (1974) and Phillips and Hercules (1986) used dry toluene.

Initial condensation of alkoxysilanes (or silanols) with glass or glass-like surfaces can be driven to near completion by heating or by the use of catalysts (Kaas and Kardos, 1976). A temperature of 100°C is considered sufficient to bind silanes to regular glass, while reaction with silica may require a temperature of 120-200°C (Hertl, 1968). It is possible that the metal ions in glass catalyze oxane bond formation at lower temperatures. Amines, titanate esters and tin compounds are catalysts for condensation of alkoxysilanes with mineral surfaces (Plueddemann, 1974).

Silica surfaces are strongly acidic, whereas commercial glasses are basic, because metal ions like Na and K are present. Since base catalyses the reversible silylation in both directions, it should be removed if a stable surface is to be realized. Therefore, metals in commercial glass are usually removed by treating with acid (HCl or HNO_3) for several hours (Phillips and Hercules,

1986).

The reaction of hydrophobic coupling agents with water-resistant minerals like quartz can produce composites that show essentially no deterioration after several days in boiling water. The coupling agent itself should not form a hydrophilic interface. When a hydrophilic coupling agent is needed, it should be used together with a second hydrophobic silane. For example, the diamine functional silane $H_2NCH_2CH_2NHCH_2CH_2CH_2Si(OCH_3)_3$ (AEAPT) provides a very good initial bonding of polyurethane to glass, but the bond is not very resistant to water due to the unreacted hydrophilic silane remaining at the interface. With addition of 90 to 98% of a vinyl silane $CH_2=CHSi(OCH_3)_3$, water resistance is considerably improved (Plueddemann, 1974). Scouten (1986) suggested a three-layer approach using a Teflon-like resistant inner layer to increase the stability of the silylated surface.

The primary amine group of AEAPT would be oriented toward the silica surface through an acid-base interaction. Because AEAPT exists in water as an internal zwitterion, electrokinetic effects may be important in controlling the orientation of this silane. Adjusting the pH value of the solution was shown to influence the orientation of AEAPT and significantly changed the mechanical properties of the silanized surface (Plueddemann, 1974).

2.7 POLYURETHANES

Because of their excellent mechanical properties and relatively good biocompatibility, polyurethanes have been used as blood-contacting materials for a number of years (Lelah and Cooper, 1986). In this work, polyurethane elastomers were used as substrates for surface attachment of antithrombin

agents. Therefore, polyurethane elastomers are now briefly introduced.

Polyurethane elastomers are alternating block copolymers composed of so-called soft and hard chain segments. The hard segments usually contain residues of aromatic diisocyanate and low molecular weight diol or diamine chain extenders. The molecular weights of the hard segments are in the range of 500 to 5000. This chain length range is preferred if the hard segments are to undergo micro phase separation into glassy or semicrystalline domains. Too short, too long or too high a concentration of hard segments may influence the proper distribution of the hard domains in the polymer matrix. The soft segments are usually polyesters or polyethers such as polypropylene oxide and polytetramethylene oxide with molecular weights in the range of 500 to 5000. They typically form an amorphous, elastic matrix in which the hard domains are dispersed in moderate concentration and act as physical crosslinking sites and reinforcing fillers. Thus segmented polyurethanes exhibit both thermoplastic and elastomeric behaviour.

A two step procedure is frequently used in the synthesis of polyurethanes. A polyol is reacted with a diisocyanate to give a short prepolymer with the desired molecular weight. The prepolymer and chain extender then react to form the segmented polyurethane. For example:

$$HO\text{-}(R'O\text{-})_nH + 2\ OCN\text{-}R\text{-}NCO \longrightarrow OCNRNHCOO\text{-}(R'O\text{-})_nCONHRNCO$$

$$OCNRNHCOO\text{-}(R'O\text{-})_nCONHRNCO + H_2N\text{-}R''\text{-}NH_2 \longrightarrow$$

$$\text{—}[CONHRNHCOO\text{-}(R'O\text{-})_nCONHRNHCONH\text{-}R''\text{-}NH]_m\text{—}$$

Microphase separation greatly influences the properties of the polyurethanes. The benefit of the two step polymerization compared with the alternative one-shot method where all three reactants are brought together at the same time is a narrower chain length distribution of the hard segments which results in a significant increase in tensile modulus (Harrell, 1969). The total molecular weight of the polyurethane chains has little effect on the physical properties when it is higher than 25,000 (Schollenberger and Dinbergs, 1973). The chemical structures of the polyols and isocyanates used also affect the micro phase separation. Aromatic structures in the hard segment promote phase separation (Lelah and Cooper, 1986), as does incorporation of sulphonate and other ions into the hard segment (Al-Salah et al., 1987).

2.8 ASSAYS FOR BLOOD COMPATIBILITY

The methods currently used to evaluate the blood interactions of biomaterials depend on prior knowledge of these interactions. The most direct method is the use of animal models. Such in vivo studies are slow and expensive, and the data obtained are of a "global" nature and difficult to interpret from a mechanistic view point. Therefore, simplified and well defined in vitro methods must be used to complement in vivo methods. In this work, several in vitro methods were used to evaluate the polymers that were developed and to study possible blood-polymer interactions. These methods are briefly discussed in this section.

2.8.1 Use of chromogenic substrate methods to measure clotting factor-material interactions

Oligopeptide chromogenic substrates have been used for many years to assay coagulation proteases (Huseby and Smith, 1980; Gallimore and Friberger, 1991; Claeson, 1994). The sensitivity and convenience of such assay procedures have led to their increasing use in preference to clotting assays.

Coagulation proteases exhibit strong subsite specificity and will cleave only one or a small number of peptide bonds in their natural substrates. On the other hand, some synthetic chromogenic substrates can be cleaved by more than one protease and the interpretation of the data obtained can thus be difficult. Therefore, the choice of chromogenic substrate for assaying a particular protease is of critical importance. Cho et al. (1984) reported that the best substrate for coagulation beta factor XIIa is Z-lys-phe-arg-pNA. Aurell et al. (1977) found that benzoyl-ile-glu-gly-arg-pNA is highly selective for factor Xa. Many pro-arg-pNA based tripeptides have been found to be highly selective for thrombin (Claeson, 1994). It is possible to assay factors XIIa, Xa and thrombin directly in plasma using these substrates, and while they may react with other coagulation proteases, the error thus introduced is usually negligible.

The clotting of plasma results in an increase in optical density due to the development of turbidity. Therefore, many chromogenic substrate assays are performed with highly diluted plasma. Although the data generally agree with clotting tests using undiluted plasma (Duncan et al., 1985), plasma dilution may

change the inhibitor/activator ratio and thus affect the results (Pixley et al., 1991). In using chromogenic substrate assays to evaluate plasma-polymer interactions experiments should therefore be done at both high and low concentrations to allow for the possible effect of plasma dilution on protein-surface interaction. Llanos and Sefton (1993) used a fluorogenic substrate to study thrombin generation in plasma, thus removing the problem of plasma turbidity, since the wavelengths of fluorescence and light scattering by the clot are different.

2.8.2 Protein adsorption

Radiolabeled proteins have been widely employed to study protein-surface interactions. The radiolabeling method is highly sensitive and is convenient for the study of single protein either in buffer, in plasma or in blood. The adsorption of proteins from plasma gives rise to a complex protein layer on biomaterial surfaces. The determination of the composition of this protein layer requires a sensitive, versatile method capable of detecting many proteins. Brash et al. (Mulzer and Brash, 1989; 1990; Cornelius and Brash, 1993) used immunoblotting methods to study the adsorption and activation of proteins from blood and plasma onto hemodialyzer membrane surfaces. More than 20 proteins were detected, and the possible activation or cleavage of certain proteins was found to correlate with the overall blood response of the materials. Other researchers (Elam and Nygren, 1992; Tengvall et al., 1992) have also used immunoassay methods to study protein adsorption from blood or plasma onto polymer surfaces.

The adsorption and activation mechanisms of certain proteins in relation

to coagulation has been studied in this work using radiolabeling and immunoassay methods.

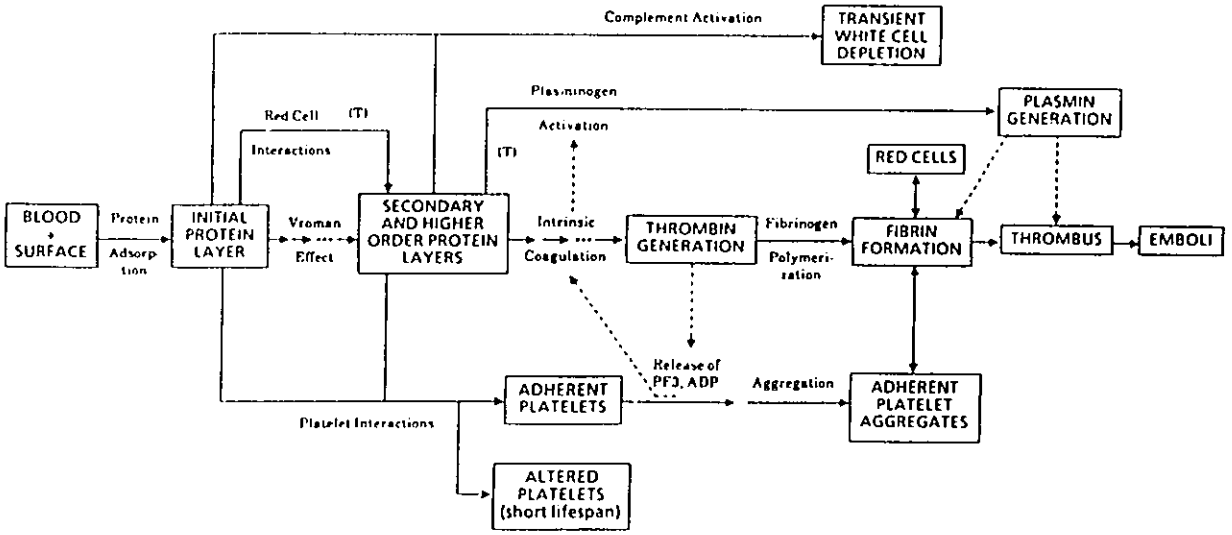
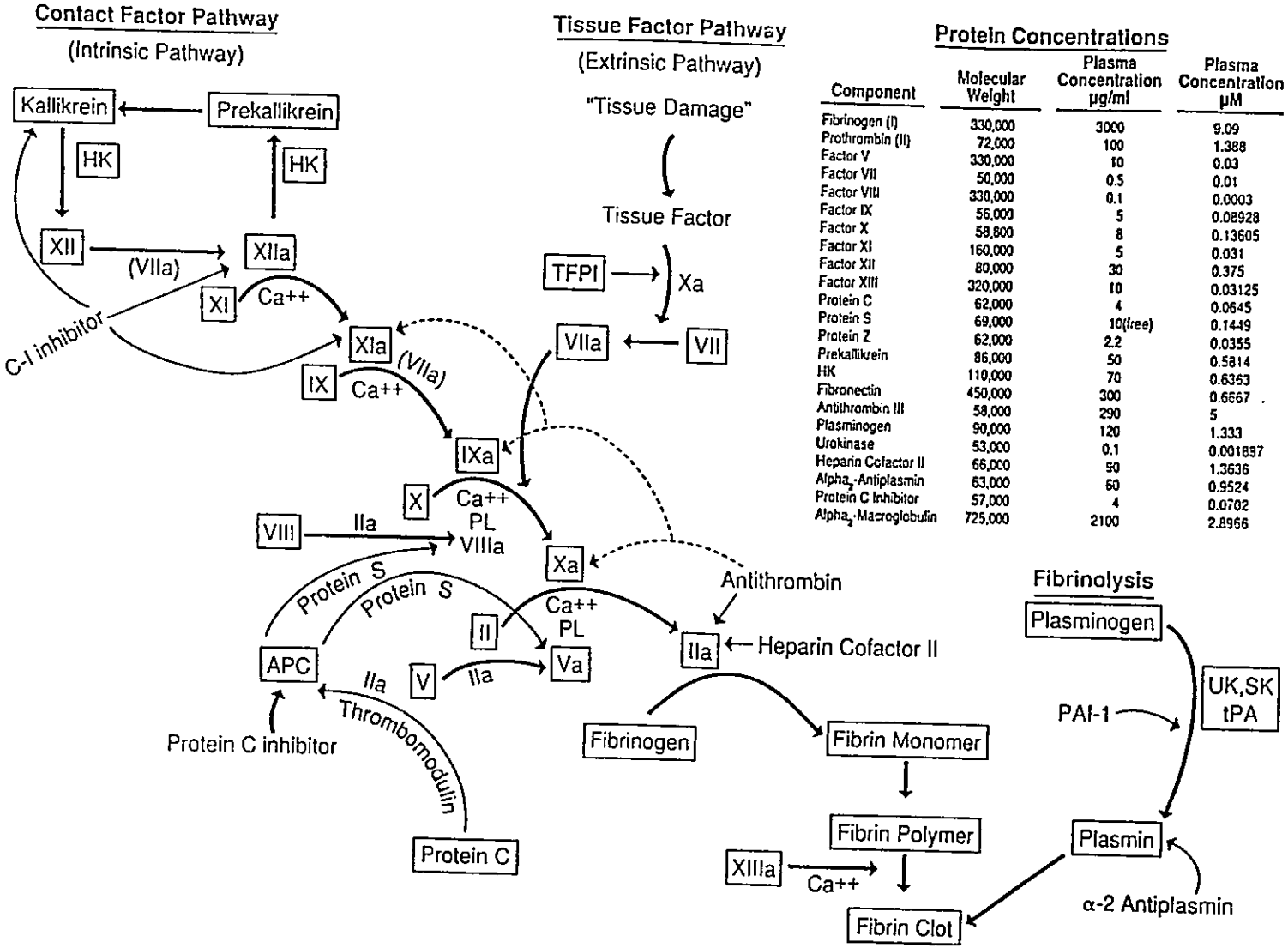


Fig. 2.i Blood-Material Interaction (Brash, 1987)



Protein Concentrations

Component	Molecular Weight	Plasma Concentration µg/ml	Plasma Concentration µM
Fibrinogen (I)	330,000	3000	9.09
Prothrombin (II)	72,000	100	1.388
Factor V	330,000	10	0.03
Factor VII	50,000	0.5	0.01
Factor VIII	330,000	0.1	0.0003
Factor IX	56,000	5	0.08928
Factor X	58,800	8	0.13605
Factor XI	160,000	5	0.031
Factor XII	80,000	30	0.375
Factor XIII	320,000	10	0.03125
Protein C	62,000	4	0.0645
Protein S	69,000	10(free)	0.1449
Protein Z	62,000	2.2	0.0355
Prekallikrein	86,000	50	0.5814
HK	110,000	70	0.6363
Fibronectin	450,000	300	0.6667
Antithrombin III	58,000	290	5
Plasminogen	90,000	120	1.333
Urokinase	53,000	0.1	0.001887
Heparin Cofactor II	66,000	90	1.3636
$Alpha_2$-Antiplasmin	63,000	60	0.9524
Protein C Inhibitor	57,000	4	0.0702
$Alpha_2$-Macroglobulin	725,000	2100	2.8966

Fig. 2.2 Coagulation Cascade (Enzyme Research Lab., Inc. Catalog, 1993)

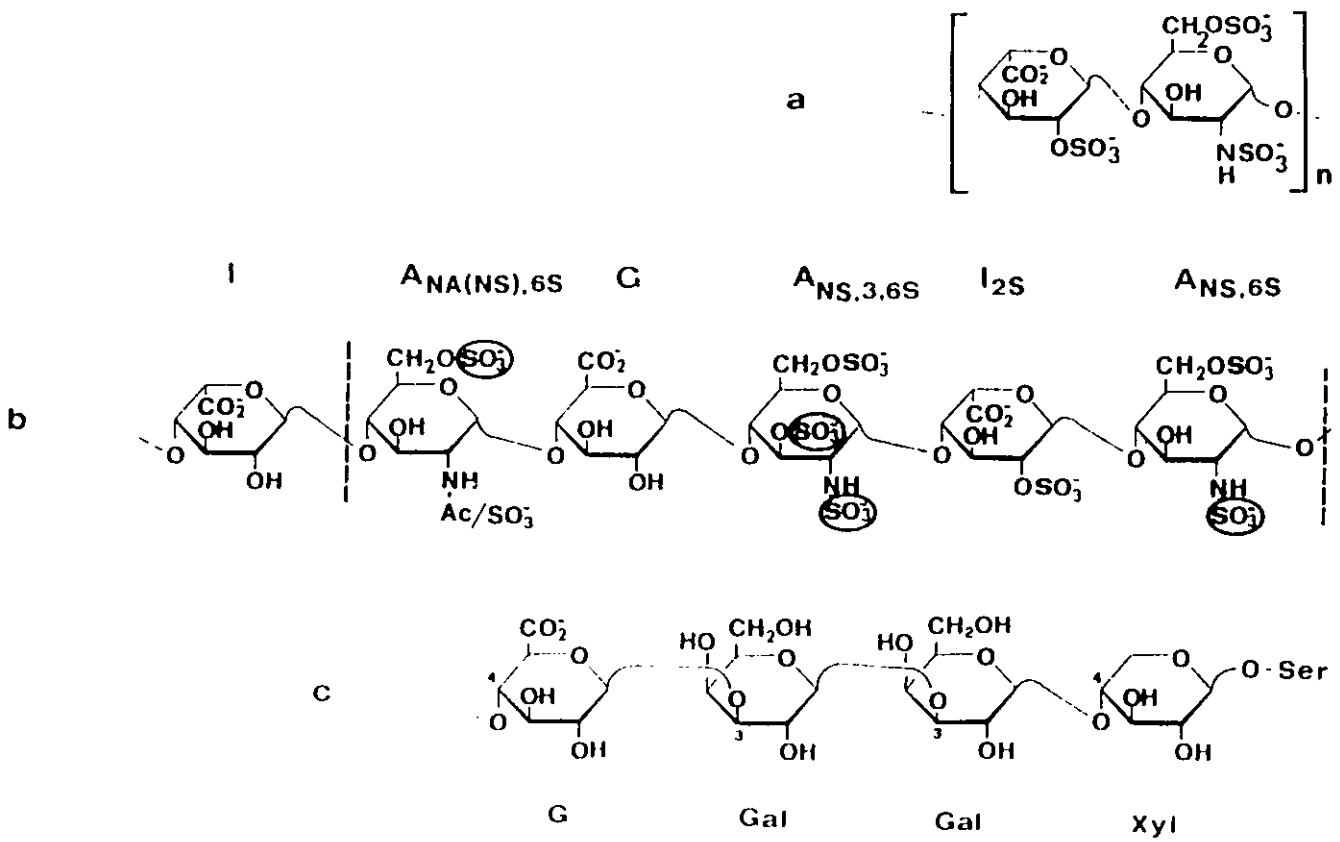


Fig. 2.3 Typical Heparin Sequences (Casu, 1988)

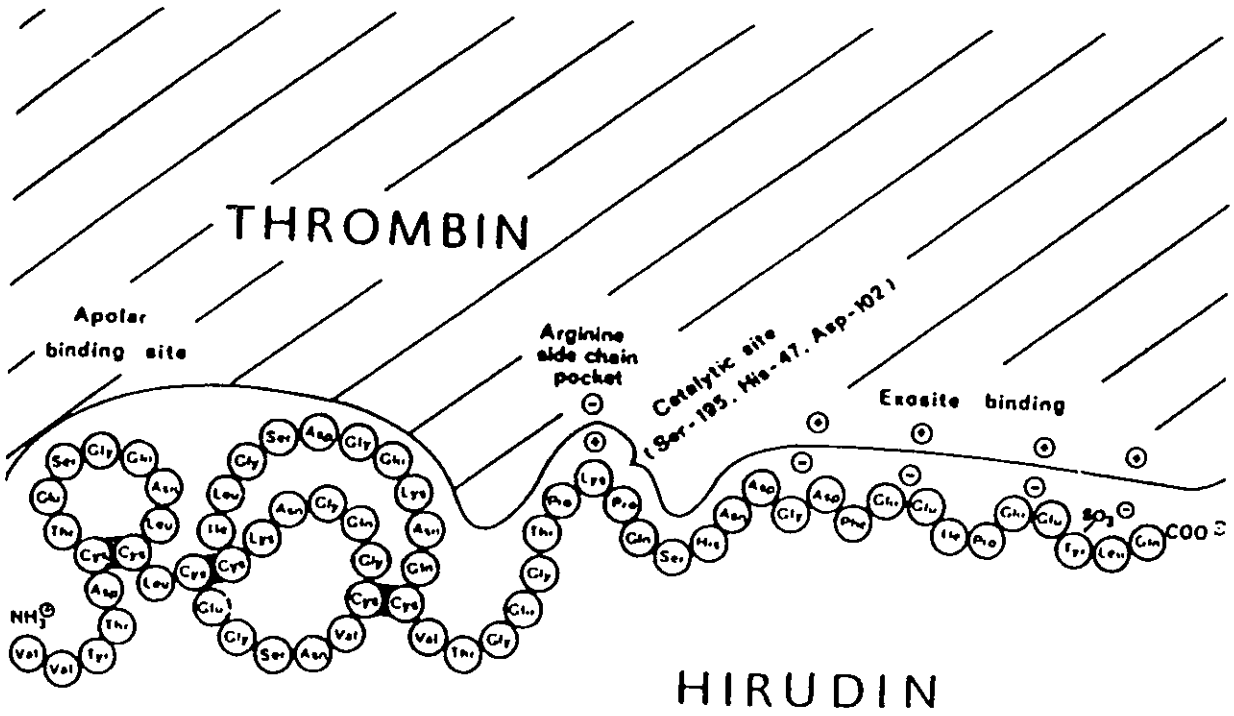


Fig. 2.4 Schematic of Thrombin-Hirudin Interaction (Markwardt, 1989)

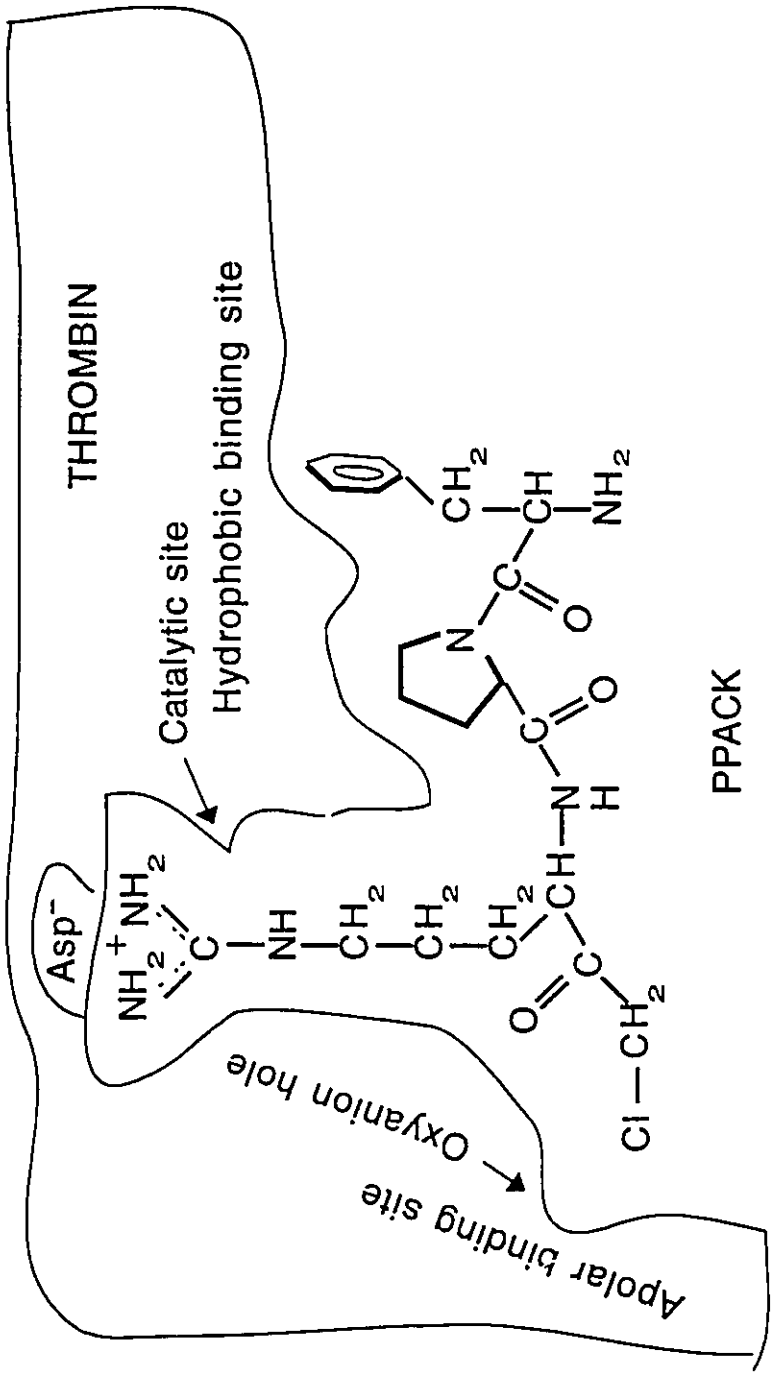


Fig. 2.5 PPACK-Thrombin Binding

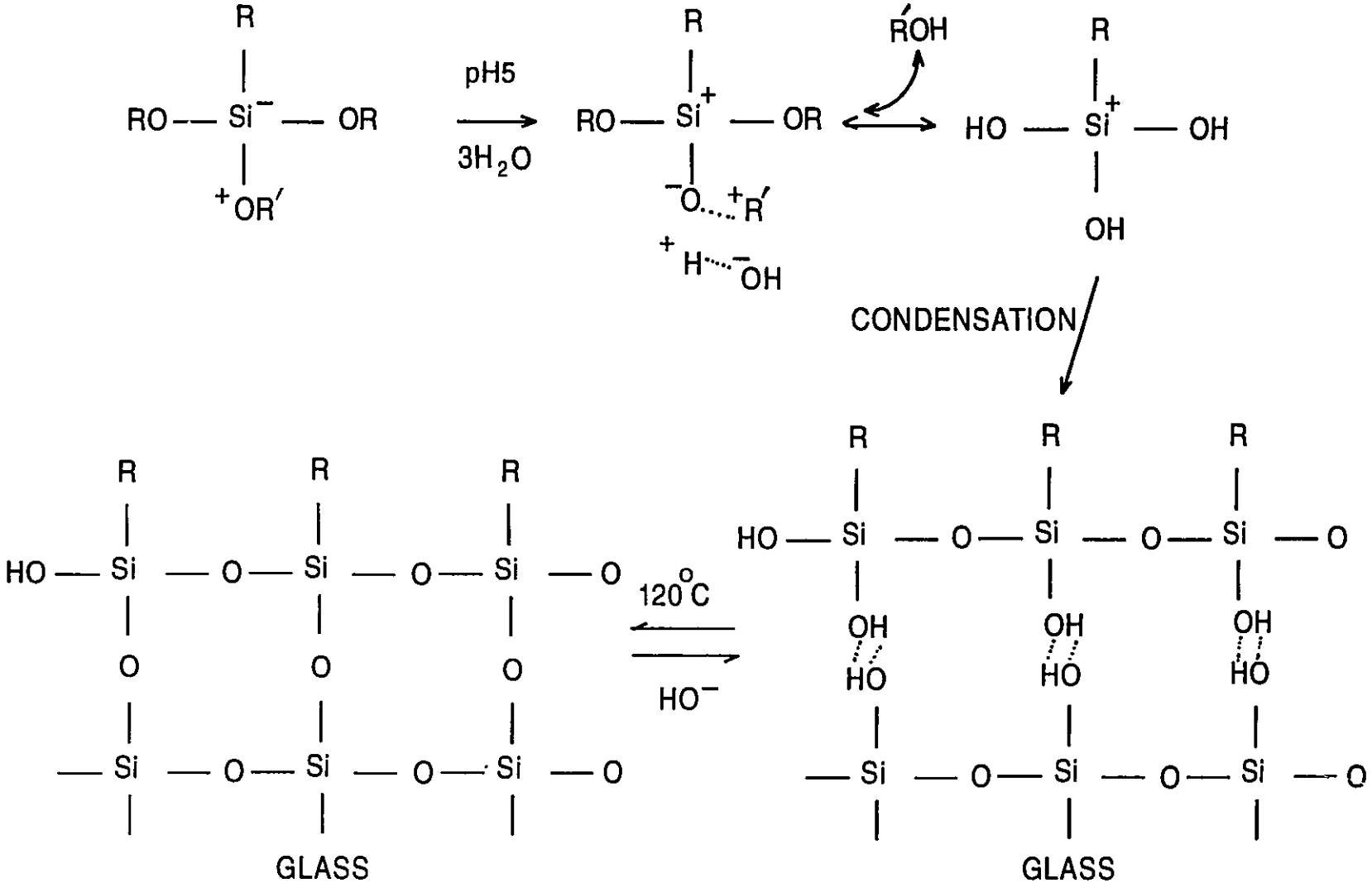


Fig. 2.6 Polysiloxane Formation and Silanization of Glass Surface (R is an organic group).

CHAPTER 3
SYNOPSIS OF RESEARCH PROGRAM

3.1 HYPOTHESIS AND OBJECTIVE

As indicated in Chapter 2 the main problem in the use of blood contacting biomaterials is thrombogenesis. A major difficulty is that there are several mechanisms by which platelets and coagulation are activated and it is unlikely that all of them can be controlled by a single approach. It was noted previously that all of the pathways involved in thrombosis appear to converge on and diverge from thrombin (Fig. 2.2). Thus, it appears reasonable that it may be possible to block the thrombotic process most effectively by inhibiting the thrombin which is generated by blood-material contact. The objective of this work is to investigate the hypothesis that properly attached antithrombin agents may block thrombotic processes on biomaterial surfaces and thus improve the blood compatibility of the material. This hypothesis has four components:

1) To control thrombin is to control thrombosis. It may be possible to block the thrombogenic process more effectively by inhibiting thrombin instead of by inhibiting the initiation of coagulation in the contact phase.

2) Surface-bound antithrombin agents may selectively bind and inhibit thrombin and thus block the coagulation process on a biomaterial surface.

3) Immobilized antithrombin agents may retain their biological specificity if they are properly bound to the surface.

4) Surface-bound antithrombin agents may give a surface which will adsorb proteins from plasma in a way that will not stimulate the initiation of the intrinsic pathway of coagulation and will not induce platelet adhesion and activation.

Three problems arise in relation to this hypothesis. 1) The surface-bound antithrombin agents may be masked by non-specifically adsorbed blood proteins and thus have no effect on thrombogenesis. However with sufficiently high thrombin-binding affinity and sufficient surface coverage of the antithrombin agents such nonspecific protein adsorption may be prevented. 2) The effect of surface-bound antithrombin agents may be limited to the surface and thus would have no effect on fibrin-bound thrombin or the thrombin which is located on the surface of platelet aggregates. However, because physiologically effective concentrations of thrombin are produced by autoproduction stimulated by "primordial" trace amounts of thrombin, it is hypothesized that the surface-bound agents will inhibit this "primordial" thrombin and thus inhibit the mechanisms of subsequent thrombin generation. 3) The affinity of antithrombin agents for thrombin may be high enough that the bound thrombin cannot be released and the immobilized agents will no longer be available to inhibit additional thrombin that may be generated.

Other questions that come to mind are: Will thrombin be changed and released by the surface-bound agents? Does the agent-bound thrombin have anticoagulant properties? Such is the case when thrombin binds to thrombomodulin and initiates the protein C anticoagulant mechanism (Walker and Fay, 1992). Investigations of these various questions constitute an

important part of the examination of the working hypothesis which forms the basis of this thesis.

In this project, polyurethane surfaces were modified to prepare suitable surfaces to which different functional ligands including PEO chains, sulphonate groups and PPACK were attached. The bioligand-attached surfaces were tested by contacting them with various protein solutions or plasma. The experimental data obtained using these surfaces were used to investigate the hypothesis stated above.

3.2 EXPERIMENTAL APPROACH

Five model polyurethane surfaces were synthesized (Chapter 4) which were anticipated to have various properties of interest. They are PEOMDA, an inert non-adhesive hydrophilic surface; PPOMDA, a hydrophobic surface; PPOBDDS, a sulphonated negatively charged surface that could be expected to have heparin-like properties; PEOBDDS, a negatively charged PEO-containing surface, and PPACKPPO, a surface with incorporated PPACK moieties as a potential thrombin scavenger/inhibitor. The chemical structures of the polymers were determined using NMR. The molecular weight, solubility, composition, and phase separation properties of the polymers were tailored to make them suitable as coating materials. The polymer surfaces were studied by SEM-EDAX and ESCA to determine the surface morphology and composition. Restructuring of the surfaces which occurred following aqueous contact was monitored using contact angle methods. Finally, the adsorption of thrombin and albumin were studied using radiolabeling methods.

In Chapter 5, the activity of thrombin adsorbed on the polymer surfaces, determined using a chromogenic substrate method is reported. The uptake of thrombin from solution by the functional groups on the polymer surfaces was monitored using an on-line method combining radiolabeling and chromogenic substrate assay. The PPACKPPO polyurethane surface was found to inhibit thrombin mainly by uptake thrombin with the surface-bound PPACK. It is interesting that the PPACKPPO surface also slowly release PPACK so that the surface may be more effective in inhibiting fibrin-bound thrombin.

In Chapter 6, the composition of protein layers deposited from plasma on the polymer surfaces is reported. The compositions of plasma and the protein layer formed on the polyurethane surfaces are in genral different. The contact phase was found to be activated on the PPOBDDS surface with a high concentration and activation of high molecular weight kininogen (HK) being adsorbed. Coagulation proteins that adsorbed to the PPACKPPO surfaces were in general in intact form. Albumin, IgG, fibronectin and vitronectin were found to be accumulated on the polyurethane surfaces, and factor V and C3 were adsorbed and activated. In fact, among the approximately 30 proteins studied in this work, most of the proteins were activated to some extent. Some proteins were found to be first adsorbed and then displaced. Other proteins such as HK and fibrinogen were found to be enriched on the surface at longer adsorption times.

In Chapter 7, contact phase activation studied using a chromogenic substrate method is reported. Factor XIIa was generated on the negatively charged PPOBDDS surface. PPACKPPO effectively inhibited the activation of the contact phase. Experiments to investigate the possible direct interaction of

PPACK and factor XIIa showed that PPACK is a potent inhibitor of XIIa. The PPOMDA and PEOMDA surfaces did not stimulate or inhibit factor XIIa generation. On these surfaces, factor XII appears to be activated eventually by other surface-independent mechanisms.

In Chapter 8, thrombin generation and the clotting of plasma as determined by chromogenic substrate methods are discussed. Thrombin generation was different on the different polymer surfaces. The data showed that polymer surfaces influence only the initiation of thrombin generation but not the subsequent thrombin-dependent propagation of coagulation. The incorporation of PPACK blocked the positive feedback of initially formed thrombin and thus prevented physiologically effective thrombin generation. In addition, the inhibition of factor XIIa by PPACK also contributed to the inhibition of coagulation on the PPACKPPO surface.

In summary, studies of the preparation, characterization, protein adsorption properties, plasma-derived protein layer composition, contact phase activation and clotting properties of five model polymer surfaces were carried out. The data suggest that the initiation of the contact phase and the production of the initial primordial amount of thrombin is the most important contributor to thrombogenesis on polymer surfaces. A passivating surface may delay coagulation but will not prevent it ultimately. The PPACKPPO surface inhibited both contact phase activation and the initially generated thrombin and thus effectively inhibited the clotting of plasma. Preliminary in vitro work showed that this surface might prevent thrombogenesis in vivo as well.

CHAPTER 4
POLYURETHANES BEARING THROMBIN INHIBITORS: SYNTHESIS, CHARACTERIZATION AND THROMBIN ADSORPTION

4.1 INTRODUCTION

The initial step in the process of thrombosis induced by artificial surfaces is the adsorption of proteins. The adsorption of the proteins of the contact system such as factor XII and kallikrein triggers the activation of coagulation, and the resultant thrombin activates platelets. Because formation of this protein layer influences blood-biomaterial interactions (Brash, 1988), researchers have focused on methods to control protein deposition by modifying the surface properties of biomaterials (Brash, 1981).

In this work, a number of polymers, some inert, some with heparin-like functions (sulphonate) and some with PPACK functions were synthesized. The objective was to examine and compare these surfaces to generate information about the interactions between blood proteins and artificial surfaces, with particular focus on the role of thrombin. Many questions arise related to the grafting of PPACK: Can the high specificity of PPACK for thrombin be retained when it is attached to polymers? When a PPACK-grafted polymer is in contact with blood, will the adsorption of plasma proteins and the formation of the protein layer reduce the antithrombin activity of PPACK? Will the PPACK

surface effectively inhibit coagulation? The synthesis, characterization and thrombin adsorption data on these polymers are reported in this chapter.

4.2 EXPERIMENTAL

Materials

Hydroxyl-terminated polypropylene oxide of molecular weight 1025 (PPO, Aldrich, Milwaukee, WI) and polyethylene oxide of molecular weight 1500 (PEO, Aldrich) were dried at 60°C in a vacuum oven for 24 h prior to use.

4,4'-Diphenylmethane diisocyanate (MDI, Eastman Kodak, Rochester, NY) was purified by vacuum distillation at 2 mmHg pressure. The resulting white solid was stored at -10°C for a maximum of one month prior to use.

4,4'-Diamino-2,2'-biphenyl-disulphonic acid (Eastman Kodak) was washed extensively with hot water and filtered until the filtrate was clear. The washed material was then titrated with 10% NaOH solution until the pH reached 7.0. At this pH it became soluble in water, indicating that the sodium salt (BDDS) had been formed. The BDDS was precipitated by the addition of acetone, and dried at 90°C for several days. It was dried again at 90°C for 24 h immediately prior to use.

Methylene dianiline (MDA) was purchased from Sargent Welch, Toronto, Ontario. Dimethyl sulphoxide (DMSO), dimethyl formamide (DMF) and oxalyl chloride were purchased from Aldrich. D-phe-pro-arg chloromethyl ketone (PPACK) was purchased from Calbiochem, La Jolla, CA.

Polymerization

All reactions were carried out under a nitrogen atmosphere using a two step condensation method developed in this laboratory (Santerre et al., 1992). The synthesis process and the product properties were monitored by proton NMR (nuclear magnetic resonance).

PPOMDA polyurethane: PPO (0.01 mole) was dissolved in 300 ml DMSO and the solution was maintained at 80°C. MDI (0.022 mol) dissolved in 300 ml DMSO was added, and the reaction was continued for 3 h at 80°C. The system was then cooled to 45°C, and 0.012 mol MDA in 300 ml DMSO was added dropwise and reacted for 12 h. The polymerization was stopped by slowly adding water to precipitate the polymer. The polymer was then washed extensively with water and dried at 60°C for 24 h.

PPOBDDS, PEOMDA and **PEOBDDS polyurethanes** were synthesized in similar fashion using the appropriate polyethers and chain extenders. PPOBDDS was identical to PPOMDA, with BDDS used as extender instead of MDA. For PEOMDA, the polyether was a mixture of PEO and PPO (1/4 mol/mol) and the chain extender was MDA. For PEOBDDS, the polyether was a mixture of PEO and PPO (1/4 mol/mol) and the chain extender was BDDS.

PPACKPPO polyurethane: PPOBDDS polyurethane (0.03 g) was dissolved in 1.0 g DMF and kept at 4°C in an ice/water bath. Oxalyl chloride (0.01 ml) was added and reacted for 60 min. PPACK (0.017 g dissolved in 0.2 g DMF) was then added and reacted at 20°C for 30 min, then at 4°C for 3 h. The resulting polymer was slowly precipitated by adding water to the solution, and

the polymer was then extensively washed with water. A total of six preparations of this polymer were carried out on different scales. The NMR spectra were identical in all cases and the yields were in the range of 75 to 81% (based on NMR data), indicating that the synthesis was reproducible. Typical data are shown in Table 4.1.

Table 4.1. PPACKPPO polyurethane. Yield of the PPACK-sulphonate reaction.

POLYURETHANE	Conversion of sulphonate to PPACK derivative (NMR analysis)	Conversion of sulphonate to PPACK derivative (*XPS analysis)
PPACKPPO2	81%	100%
PPACKPPO4	80%	**64%
PPACKPPO6	75%	N/A

* Measured at 10 degree take off angle. See page 45 for description of XPS method.

** From Chlorine peak. This value is lower than that from the NMR data due to the possible hydrolysis of chloromethyl ketone during the preparation of samples for XPS analysis.

Polymer surfaces

PPOMDA was used as a solid film cast from solution in DMF. The other polyurethanes were prepared as coatings using PPOMDA films as substrate. PPOMDA polyurethane films (0.58 x 0.75 x 0.1 cm, surface area 0.9 cm^2) were briefly dipped into solutions of the other polyurethanes in DMSO (1.0 %, w/w), dried at room temperature under vacuum, then washed with water extensively and stored at -40°C. Before use, the polymer films were washed again with

water and equilibrated with isotonic Tris buffer, pH 7.4, for 2 h.

Polymer Characterization

Elemental analysis was carried out by two methods. Sulfur, potassium, and sodium were determined by scanning electron microscopy combined with energy dispersive analysis of X-rays (SEM-EDAX). A sulphonated polyurethane of known composition was used as a standard. Nitrogen, sulfur, potassium, and sodium were also determined by conventional analytical techniques (Guelph Chemical Labs, Guelph, Ontario).

Proton NMR spectra were recorded on a Bruker 200 spectrometer using solutions of the polymers in DMSO-d_6 (2% w/w).

XPS (X-ray photoelectron spectroscopy) data were obtained at the Surface Analysis Facility of the Centre for Biomaterials, University of Toronto. Polymer films were dried at 45°C in a vacuum oven for 24 h before XPS measurements.

Molecular weights were measured by gel permeation chromatography (GPC) using DMF (with 0.1 wt% LiBr) at 80°C (Santerre, 1990). Polystyrene standards were used for calibration.

Contact angles were measured using a goniometer, by placing a small water drop on the dry polyurethane film surfaces, or by introducing a small air bubble to the water-polymer film interface (Wojciechowski, 1991). Each data point is the average of 10 to 20 measurements. The error is less than 4 degrees.

Protein adsorption

Human alpha-thrombin (3000 units/mg, Sigma) was labeled with $Na^{125}I$ (Amersham, Arlington Heights, IL) using the lactoperoxidase method (Enzymobead reagent, BioRad, Richmond, CA) as described previously (Santerre et al., 1992). After labeling, the Enzymobeads were separated by centrifugation, and the supernatant was dialyzed for 20 h at 4°C. The enzymatic activity of the radiolabeled thrombin was similar to that of the original thrombin as determined by a chromogenic assay method using N-p-tosyl-gly-pro-arg p-nitroanilide as substrate (Sigma).

Human serum albumin (HSA, Behring, Marburg, Germany) was radiolabeled using the same method as for thrombin except that free ^{125}I was removed using an ion exchange chromatography column (AG1 X4 anion exchange resin, BioRad, Richmond, CA).

The adsorption of protein to the polyurethanes was measured under static conditions at room temperature (22°C). Polymer films were placed in the wells of polystyrene microtitre plates and incubated with 3.0 ml isotonic Tris buffer, pH 7.4 for 2 h. The buffer was removed and the radiolabeled protein was added and incubated with the film for 2 h. The films were then rinsed three times with isotonic Tris buffer and the radioactivity was determined. For each polyurethane-protein system, three independent experiments were performed using freshly labeled protein each time, and within an experiment, 2 or 3 films were used for each data point. The data reported in this work are the averages of all the values obtained for all experiments under given conditions (6 to 9 replicates). The relative error of these data is estimated to be less than 20%. For a given material the data reported were obtained using polymer from one

batch. Similar data were obtained for other batches.

4.2 Results and Discussion

Polymer characterization

In this work, the polymerization process was followed and the polymer structures were investigated by proton NMR. In Fig. 4.1 (p. 69 - 71), the proton NMR spectra and the assignments of chemical shifts for the PPACKPPO polyurethane (Fig. 4.1a), and its two precursors, PPACK (Fig. 4.1b) and PPOBDDS polyurethane (Fig. 4.1c), are shown. The assignments are based on synthesized model molecules and two dimensional NMR techniques. The peaks of some protons are not well resolved and thus are not labeled in the figure.

The urethane groups (peak B7, Fig. 4.1a, p. 69) at 9.5 ppm are formed by reaction of the terminal hydroxyl groups of PPO and the isocyanate groups of MDI; therefore the intensity of B7 gives a measure of the amount of PPO incorporated into the polyurethane chains. Similarly, the urea groups at 8.55 ppm (peak B9, Fig. 4.1a) are formed by the reaction of the NH_2 groups of BDDS and the isocyanate groups of MDI, and the intensity of peak B9 gives a measure of the amount of BDDS incorporated into the polyurethane chains. The intensity ratio B7/B9 can be used to estimate the molar ratio of PPO and BDDS in the polyurethane. There are two methyl signals at 1.0 ppm (peak B3) and 1.16 ppm (peak B6), indicating that PPO has two kinds of methyl groups. Peak B6 represents the methyl group at the end of a PPO chain segment adjacent to the urethane group B7 which reduces the electron density of the B6 methyls and

causes a shift to the higher ppm position; B3 represents the methyl groups in the "interior" of the PPO chain segment and adjacent to the ether groups $-CH_2O-$. The intensity ratio B3/B6 thus provides information about the chain length of the PPO chain segment, and the intensity of peak B6 gives a measure of the PPO content of the polyurethane. The representative signal of PPACK (peak 23, Fig. 4.1a) is at 1.5 ppm and is assigned to the "central" methylene of the arginine side chain as shown in Fig. 4.1b (p. 70). From the intensity ratio of representative peaks of PPACK and BDDS, I(23)/I(B9), it was found that greater than 80% of the sulphonate groups of BDDS were converted to the PPACK derivative (Table 4.1).

Oxalyl chloride converts the sulphonate group of BDDS to sulphonyl chloride which is highly reactive with amine groups (Santerre et al., 1992). However, PPACK has an amine group on the phenylalanyl residue and a guanidino group on the arginyl residue, both of which could react with sulphonyl chloride. Since PPACK binds to thrombin through its arginine residue, it is important to protect the guanidino group on the arginine. The guanidino group is reactive only at high pH (~ pH 9), and it is protonated and unreactive at pH 7. Accordingly, in this work, PPACK was reacted with sulphonyl chloride in DMF solution where the guanidino group remains protonated.

Comparing Fig. 4.1a with Figs. 4.1b and 4.1c, it is clear that the NMR spectrum of PPACKPPO is not a simple summation of the PPOBDDS spectrum and PPACK spectrum. New peaks appear at 8.85 ppm (peak 9, reacted NH_2 of phenylalanyl residue) and 7.95 - 8.3 ppm (assigned as the benzene ring signal of the reacted BDDS), indicating that PPACKPPO is not a physical mixture of

PPOBDDS and PPACK and that the reaction between PPACK and BDDS results in new chemical structure in the PPACKPPO macromolecule. As can be seen in Fig. 4.1a, PPACKPPO shows a broad signal (peak 27) at ~6.5 - 7 ppm assigned to the guanidino group, the same as in the free PPACK molecule (Fig. 4.1b), suggesting that the guanidino group is intact in PPACK which has been attached to the polyurethane chains. In contrast, the NMR signal of the amine group on the phenylalanyl residue which appears in free PPACK at 8.5 ppm (Fig. 4.1b) is shifted to 8.85 ppm in the polymer (Fig. 4.1a), suggesting that a strong electron withdrawing group is attached to the amine group. These findings indicate that in the PPACKPPO polymer, PPACK is attached to the polyurethane chains via the amine group of the phenylalanyl residue of PPACK, and that the guanidino group remains free. The disappearance of the NMR signal of the amine group on the phenylalanyl residue at 8.5 ppm indicates that no detectable free PPACK remained in the sample.

Similar NMR analysis was done on the other polyurethanes which were synthesized as part of this work (the NMR spectrum of PPOBDDS polymer is shown in Fig. 4.1c). Based on NMR analysis, the molar ratios of the functional groups of the polymers were estimated and the data are summarized in Table 4.2. Also in Table 4.2, water contact angles and molecular weights of the polymers are given.

The molecular weight distributions of the polyurethanes were measured by GPC. The GPC curves were smooth and unimodal, and no "satellite" peaks were observed (data not shown), suggesting that residual monomers had been effectively removed. This is in agreement with the NMR results that PPACKPPO is not a physical mixture of PPOBDDS and PPACK. The GPC was calibrated

using narrowly distributed polystyrene standard samples; therefore, the molecular weight data should be considered as polystyrene equivalent values only.

Table 4.2. Composition (from NMR), molecular weight and water contact angles of polyurethanes

Polymer	PPOMDA	PPOBDDS	PEOMDA	PEOBDDS	PPACKPPO
Composition (mol/mol)	PPO/MDI 1.42/2.5	PPO/BDDS 0.81/0.77	PEO/PPO/MDI 0.83/4.16/9.3	PEO/PPO/BDDS 0.15/1/1.1	PPO/PPACK/BDDS 0.81/1.25/0.77
Expected values	(1.4/3.1)	(1/1.2)	(1/4/11)	(1/4/6)	(1/2.4/1.2)
Contact angle* (°) Advancing	73.8±4	68.9±3	69.6±2	78.5±2	67±2
Receding	42.1±5	27.7±5	32.2±5	32.0±5	24±4
Number average MW	140,000	81,000	36,000	37,000	86,000

* Polymer films for water contact angle measurement were dried in a 60°C oven for 24 hours. Mean ±SD (n>5).

SEM-EDAX and conventional elemental analysis were also carried out for the polymers. The results were essentially the same as from the NMR data.

Surface characterization

XPS spectra were recorded using polyurethane samples that had been dried at 45°C in a vacuum oven for 24 hours. The vacuum oven creates a hydrophobic environment that favors low-energy surfaces. As a result, low-energy components of the polymer (e.g., PPO and MDI groups) will tend to

migrate to the surface of the films. These components will therefore tend to be enriched, and high-energy components such as BDDS or PPACK will tend to be depleted in the surface. Thus the material composition right at the surface will be different from the bulk composition and a composition gradient will exist as a function of depth into the material. Since the X-ray induced photoelectrons in the XPS experiment can only travel a limited distance (about 100 Å) through the solid, the XPS signals obtained at different take-off angles (α) provide information about composition as a function of depth (100sinα Å, see Fig. 4.2a, p. 72). Using this method, the composition profile through the depth of the polymer film was measured.

Typical XPS survey spectra of the PPACKPPO, PPOMDA and PPOBDDS polyurethanes are shown in Figs. 4.2b - 4.2d (p. 73 - 75). The integration of the XPS peaks gives the intensity of the elemental signals which give a measure of the atomic composition. Typical data are shown in Table 4.3. For the PPACKPPO polyurethane, the nitrogen (N) and sulfur (S) contents increase as the take-off angle increases, indicating that BDDS residue which is rich in N and S and PPACK residue which is rich in N may be depleted in the outmost surface of the material, while PPO residues may be enriched at the surface.

As shown in Table 4.3, small amounts of silicon (Si) were found on the polyurethane surfaces. This was particularly noticeable on the PPOMDA surface. As can be seen the silicon content increases towards the surface so that it probably represents surface contamination. The silicon-containing contaminants are probably from silicone pump oils or silicone grease used as a lubricant in various procedures during polymer preparation and handling.

It is convenient to measure the representative elements of the functional

groups of the polymer, e.g. the chiorine of PPACK and the sulfur of BDDS. However, the chloromethyl ketone group of PPACK is sensitive to water and can be lost by hydrolysis during sample preparation (refer to Table 4.1). In addition, components such as PEO and MDI have no unique elements for identification purposes. On the other hand, each PPACK molecule contains six nitrogen atoms, so that the incorporation of PPACK into the polyurethane will significantly increase its nitrogen/carbon ratio as shown in Table 4.3. A calculation scheme based on this property was thus developed, as illustrated below.

Table 4.3 XPS data for polyurethanes at five take-off angles (data precision is ± 10%).

Polymer	Take-off angle (°)	Atom (%)				
		O	N	C	S	Si
PPACKPPO	10	21.4	1.7	72.9	0.4	3.6
	20	21.1	2.2	73.8	0.3	2.6
	30	20.3	3.7	73.0	0.6	2.4
	50	19.4	4.6	73.4	0.7	1.8
	90	19.7	5.5	73.1	0.7	1.0
PPOMDA	10	21.6	1.4	67.9	0.3	8.9
	20	21.0	1.7	70.9	0.1	6.2
	30	22.2	2.2	70.8	0.2	4.5
	50	20.8	3.3	72.7	0.1	3.1
	90	20.6	3.2	73.7	0.1	2.5
PPOBDDS	10	21.1	2.6	72.8	1.0	2.4
	20	19.2	3.1	74.8	0.8	1.5
	30	20.5	3.7	73.1	1.0	1.3
	50	21.3	4.0	72.3	1.2	0.6
	90	22.0	4.3	71.1	1.2	0.6

PPACKPPO polyurethane is composed of PPACK, PPO, MDI and BDDS residues. These components have the following formulas:

PPACK	$C_{21}H_{30}N_6O_2Cl$
PPO	C_3H_6O
MDI	$C_{15}H_{12}N_2O_2$
BDDS	$C_{12}H_8N_2O_6S_2Na_2$

Let K represent the moles of PPACK residues in the PPACKPPO polyurethane sample, P the moles of PPO residues ($-CH(CH_3)CH_2O-$), I the moles of MDI residues, and S the moles of BDDS residues. From the above formulas, each molecule of PPACK contains 6 nitrogen atoms, so K moles of PPACK will have 6K nitrogen atoms, similarly, I moles of MDI have 2I nitrogen atoms, S moles of BDDS have 2S nitrogen atoms, and so on. The total nitrogen atoms in the polyurethane sample is the sum (6K+2I+2S). Thus, the total atoms of different elements in the material can be written:

$Nn = 6K + 2I + 2S$	(nitrogen)
$Nc = 21K + 3P + 15I + 12S$	(carbon)
$No = 2K + P + 2I + 6S$	(oxygen)
$Ns = 2S$	(sulphur)

Nn, Nc, etc. can be measured directly by XPS at different take-off angles and used to calculate the composition (K, I, P, S) corresponding to different depths into the sample.

$S = 0.5Ns$

$$K = (3No + 4.6Nn - Nc - 7.5Ns)/12$$

$$I = (Nn - 6K - 2S)/2$$

$$P = No - Nn + 4K - 4S$$

Using these equations, the depth profiles of the components for the PPACKPPO polyurethane surface were obtained from XPS data, as shown in Fig. 4.3a, p. 76. Similar profiles for the other polymer surfaces were also obtained and are shown in Fig. 4.3b and Fig. 4.3c (p. 77 - 78).

Fig. 4.3a demonstrates that the MDI component of the PPACKPPO polymer is enriched in the surface while the PPACK is depleted, indicating that the polymer surface may undergo restructuring during the film preparation process. The sampling depth of the XPS experiment is about 100 Å or 10 nm. The length of a typical C-C bond or C-O bond is about 1.5 Å. Therefore, the length of a fully extended PPO segment (molecular weight 1020, degree of polymerization 17, length of repeat unit C-C-O- 5 Å) should be about 85 Å (5x17=85), similar to the sampling depth of the XPS signals. A hard segment of PPACKPPO polyurethane with degree of polymerization 5 will have about the same length. Since the glass temperature of the hard segment of the PPACKPPO polyurethane is above the film drying temperature of 45°C, phase mixing and long range molecular migration are unlikely, and cannot be used to explain the observed composition gradient and surface restructuring which is implied by it. Instead, the composition difference between 1 nm (10 Å) and 6 nm (60 Å) shown in Fig. 4.3a is more likely to reflect the short range orientation of side chains. For example, the hydrophobic environment in a 45°C air oven is expected to bring the hydrophobic benzene rings of the MDI residues to the

surface while causing orientation of the hydrophilic arginine residues and the chloromethyl ketone groups of PPACK towards the interior.

Fig. 4.3b demonstrates that some PEO segments of the PEOBDDS polyurethane remain in the outermost layers. Since PEO is hydrophilic, it is expected to be oriented towards the bulk material the same as for the arginine residues of the PPACKPPO polyurethane. It is possible that the relative flexibility of PEO allows it to adopt a wider range of conformations and orientations.

The surface composition of the polyurethanes is compared with their bulk composition in Fig. 4.3. The bulk composition was measured by elemental analysis, SEM-EDAX and NMR, yielding values for sulfur content of 2.5, 2.0 and 2.1 %w/w respectively for the PPOBDDS polyurethane. As shown in Fig. 4.3c, the composition of PPOBDDS measured by XPS agrees with the data from the other methods. In addition, the composition depth profile for the PPOBDDS surface indicates that MDI residues are enriched in the surface while PPO residues are depleted.

It should be noted that these calculations do not take into consideration the silicon contaminants. Therefore for the PPOMDA surface which has a relatively high silicon content, the use of the above composition relations is not justified. For the PPACKPPO and PPOBDDS surfaces, the silicon content is low and the calculated XPS data agree with the data measured by NMR, elemental analysis, and SEM-EDAX methods (arrows in Figs. 4.3a and 4.3c).

The XPS data suggest that the polyurethane surfaces have a compositional gradient at the surface due to processes of chain reorientation. Because the samples were prepared in a 45°C vacuum oven which is a

"hydrophobic" environment, the different segments of the polyurethane chains are redistributed at the surface to achieve a minimum energy state. Consequently, the low energy MDI residues tend to migrate/reorient to the hydrophobic air-polymer interface, while the higher energy PPACK and BDDS residues tend to migrate to the interior of the material.

Water contact angle

Table 4.2 shows advancing and receding water contact angle data for the polyurethanes. The advancing contact angles range from 78° to 67°, indicating that the materials are essentially hydrophobic. The receding contact angles are much lower, suggesting either significant roughness, chemical heterogeneity, or restructuring in contact with water (see below).

As shown in Fig. 4.3, the hydrophobic components of the polyurethanes were enriched at the outermost surface when the specimens were prepared at 45°C in a vacuum environment. In contrast, the hydrophilic components such as PPACK, PEO and sulphonate are expected to be enriched on the surface, when the materials are placed in a hydrophilic environment. In this work, polyurethane films were incubated in water and the hydrophobic-hydrophilic restructuring process was monitored by measuring water contact angle (air bubble method) as a function of time.

As shown in Fig. 4.4, p. 79, the water contact angles measured on the polymer surfaces decrease significantly as the hydration time increases, suggesting that the hydrophilic groups, "buried" in the bulk material under normal conditions, migrate/reorient to the interface between the water and the polymer. When equilibrated in water, the PPOMDA surface showed a final

contact angle of about 50°, a fairly high value, suggesting only limited restructuring, probably due to the lack of hydrophilic groups in this polymer. In contrast, the contact angles of the PPOBDDS and PPACKPPO surfaces decreased to about 20°, suggesting that the surfaces underwent significant restructuring to become highly hydrophilic. BDDS is presumably the primary hydrophilic component of the PPOBDDS polyurethane, and PPACK the primary hydrophilic group of the PPACKPPO polyurethane. The difference in final contact angle between PPOMDA (~50°) and PPOBDDS and PPACKPPO (~20°) suggests that the sulphonate and PPACK hydrophilic functional groups migrate to the surface as a result of restructuring.

Thrombin adsorption

Radiolabeled thrombin was used to study the adsorption of thrombin to the polyurethane surfaces. The data are shown in Fig. 4.5, p. 80. It should be noted that the thrombin concentrations studied are relatively low. In part this range of concentrations was chosen for reasons of availability of thrombin. It is clear from the data that saturation was not achieved and the slopes of the lines can be considered as reflecting the relative affinities of the surfaces for thrombin.

In a protein adsorption experiment, the adsorption of protein to the surface should not change the concentration of the solution significantly in order to maintain equilibrium. In this work, although low concentrations of thrombin were used, the concentration of the solution was not significantly reduced due to adsorption. For example, the highest adsorption shown in Fig. 4.5 on the PPOBDDS surface is about 0.14 $\mu g/cm^2$ and the total amount of adsorbed

thrombin is 0.126 μg (on 0.9 cm^2 surface), while the amount of thrombin in the solution is 1.62 μg (0.35 ml solution), i.e. about 12 times higher. Therefore, significant solution depletion of thrombin did not occur.

As shown in Fig. 4.5, different amounts of thrombin were adsorbed to the different polymers. Polymers containing free sulphonate groups adsorbed the highest amounts of thrombin. Thus PPOBDDS adsorbed about three-times more thrombin than PPOMDA and ten-times more than PEOMDA. Since the main difference between PPOBDDS and PPOMDA is that the former has sulphonate groups, these findings suggest that the sulphonate groups of PPOBDDS are responsible for the increased thrombin adsorption. The high protein binding capacity of sulphonated materials has been noted previously (Santerre et al, 1992; Wojciechowski, 1991).

The main difference between the PPOBDDS and PEOBDDS films is that the latter probably have PEO segments on the surface. As a result, there may be less BDDS on the PEOBDDS than on the PPOBDDS surface, thus leading to lower thrombin adsorption to the PEOBDDS material.

The PEOMDA polyurethane adsorbed only about 25% as much thrombin as the PPOMDA polyurethane. The chemical composition of these two polymers differ in that about 20% of the soft segment PPO units in PPOMDA are replaced by PEO in the PEOMDA polymer. Because PEO is more hydrophilic than PPO, the PEO segments of PEOMDA should be the dominant component in the surface, when completely hydrated. Neither PPO nor MDA is expected to be significantly exposed. If the PEO component is protein resistant, the PEOMDA polyurethane should therefore adsorb minimal amounts of thrombin.

Since all the components of the PPOMDA surface are hydrophobic, the

fact that greater amounts of thrombin are adsorbed onto this surface than onto PEOMDA suggests that hydrophobic interactions promote thrombin adsorption. However, in comparison with the surfaces of PPOBDDS and PPACKPPO, PPOMDA adsorbs relatively little thrombin, suggesting that hydrophobic groups provide relatively weak thrombin-binding sites compared to sulphonate and PPACK residues.

PPACKPPO adsorbs a relatively large amount of thrombin (Fig. 4.5), and since PPO and MDI components appear to adsorb small amounts of thrombin, this finding suggests that most of the thrombin adsorbed onto the PPACKPPO surface binds to the PPACK and residual sulphonate groups. The PPACK which is grafted to the polymer thus apparently retains its ability to bind thrombin. The conformation and activity of the thrombin on the PPACKPPO and PPOBDDS surfaces could, however, be different. PPACK may be expected to bind uniquely to the active site of thrombin so that the thrombin on the PPACKPPO surface would be deactivated, while the sulphonate groups of PPOBDDS could bind to thrombin nonspecifically. Thrombin on the PPOBDDS surface might thus still have active sites available. Interestingly, similar amounts of thrombin are seen to bind to the PPACKPPO and PPOBDDS surfaces, suggesting that the binding affinities, but not necessarily the binding mechanisms, are similar.

Albumin adsorption

Albumin is the most abundant protein in plasma, and since it is known to be relatively unreactive to platelets (Salzman et al., 1969; Lyman et al., 1979) it has been suggested that an albumin-covered surface might be compatible with

blood (Goodman et al., 1991).

In the present work albumin adsorption to the various polyurethane surfaces was measured for two reasons. First it was desirable to know whether the patterns of thrombin adsorption seen on the surfaces are specific to thrombin or whether they apply to plasma proteins in general. Albumin was chosen as a typical plasma protein for this purpose. Second, albumin adsorption data are required for the interpretation of the experiments reported below in which the ability of thrombin to displace preadsorbed albumin was evaluated.

As shown in Fig. 4.6, p. 81, the amounts of albumin adsorbed to the different polymers vary widely. Polymers containing BDDS adsorb the highest amounts of albumin. For example the PPOBDDS material adsorbs about four times as much as its unsulfonated analogue, PPOMDA. It thus appears that it is the sulphonate groups of PPOEDDS which are responsible for the increased albumin adsorption on this material.

In comparison with PPOMDA, PEOMDA adsorbs a relatively small amount of albumin (about 10% as much). The chemical composition of these two polymers is the same except for the PEO content of the PEOMDA soft segments. It seems likely that PEO residues dominate the PEOMDA surface and that these PEO chains are resistant to albumin adsorption. This property of PEO has been observed by others (Coleman et al., 1982; Chaikof et al., 1990), although usually when it is grafted to the polymer (or the surface) with one end free, as opposed to being part of the polymer backbone as in the present case. The small amount of albumin adsorbed by the PEOMDA surface might be attributed to the PPO and MDI components that are exposed on the surface.

As seen in Fig. 4.6, PPACKPPO also adsorbed a large amount of albumin. From the behavior of the PPOMDA material, we may infer that PPO, MDA and MDI residues adsorb only small amounts of albumin. Therefore it appears to be the grafted PPACK that adsorbs most of the albumin on the PPACKPPO surface. Even though the adsorbed amounts are similar, the albumin binding modes are likely different on the PPACKPPO and PPOBDDS surfaces. If so the ability of the adsorbed albumin to protect against platelet interactions may be different and might be of interest to explore further.

Although albumin and thrombin have very different chemical and biological properties, it appears that the binding of both proteins to polyurethane surfaces is influenced by the surface properties in a similar way since the order of amounts bound is the same for both. The charged surfaces, PPOBDDS, PEOBDDS and PPACKPPO adsorb more proteins (albumin and thrombin) than the hydrophobic surface PPOMDA, and the PEO surfaces adsorb the smallest amounts of proteins. This finding suggests that the chemical composition of the polyurethane surfaces is a major determinant of protein adsorption. Since electrical charge and hydrophobicity are global properties, most of the proteins adsorbed to the polyurethane surfaces are probably bound nonspecifically.

Adsorption of PPACK-thrombin complex

In order to examine the possibility of specific binding of thrombin to PPACKPPO, the adsorption of free thrombin and the adsorption of PPACK-thrombin complex to the PPACKPPO surface were compared. If the binding between polymer-grafted PPACK and free thrombin is only through the active

site of thrombin, it is expected that the affinity of PPACK-thrombin complex for binding to the PPACKPPO surface will be reduced compared to free thrombin, because its active site is pre-occupied by PPACK.

The data in Fig. 4.7 (p. 82) show that over the range of concentrations studied, adsorption is greater for free thrombin than for the PPACK-thrombin complex. Although the adsorptions are at least partly irreversible (see below), the initial slopes of the isotherms may give an indication of relative binding affinity, and on this basis the free thrombin binding affinity is greater than that of the complex. The data thus support the idea that at least some of the thrombin is bound specifically via the active site. The interpretation of the data is complicated by the fact that the complex differs from the free thrombin more than just in the sense that the active site is occupied. Certainly it does not seem useful to try to give any quantitative interpretation of the data in Fig. 4.7 in terms of fraction of free thrombin bound to the PPACKPPO surface by the active site.

It is interesting that adsorption shows saturation in these experiments, in which the concentration tends to higher values than in Fig. 4.5. The plateau values of surface concentration, which are of the order of 0.3 to 0.4 $\mu g/cm^2$, could well correspond to monolayers for a protein of molecular weight 35,000. Thus if we assume (Waugh et al., 1978) that thrombin is a prolate ellipsoid 101 Å long by 34 Å diameter then a side-on close packed monolayer would have a surface concentration of 0.2 $\mu g/cm^2$. Similarly an end-on oriented monolayer would contain 0.7 $\mu g/cm^2$. Since the actual values are intermediate between those for the close packed native molecule, it is likely that a layer of randomly oriented, variously conformed molecules is present.

The binding between thrombin and PPACK is reported to be irreversible

(Kettner and Shaw, 1979). If the binding between thrombin and the PPACKPPO polymer surface is through specific interactions between PPACK and the active site of thrombin, then thrombin should be covalently (i.e. irreversibly) bound to the polyurethane, and should not be desorbable into buffer. In contrast, the PPACK-thrombin complex adsorbed to the PPACKPPO surface should be more readily desorbed, since presumably it is not covalently bound. The data in Fig. 4.8 (p. 83) show that more free thrombin than PPACK-thrombin complex was desorbed from the PPACKPPO surface. However, the difference is not significant, and no firm conclusion can be drawn with the available data. It is well known that many protein-surface interactions are irreversible even if binding is not covalent (Chan and Brash, 1981) so that desorption may not occur or may occur very slowly. The interpretation of these experiments is therefore complex. As indicated, if thrombin is bound covalently to PPACKPPO, then desorption into buffer is not expected. It seems likely, therefore, that at least some of the thrombin, namely the desorbable fraction (with values up to 15%), is not bound to PPACK sites.

Adsorption of thrombin to surfaces pretreated with plasma or albumin.

To obtain further insight into the possible specificity of binding of thrombin by surface-bound PPACK, experiments were conducted in which the surfaces were preincubated with albumin or plasma. It was assumed that these pretreatments would result in occupation of the PPACK (and other) sites by albumin and other plasma proteins. The question to be addressed in the experiments in which these protein-adsorbed surfaces were subsequently

exposed to thrombin was whether the thrombin could displace the preemptive proteins and bind to the PPACK sites by virtue of high affinity specific binding. This is the anticipated mechanism of antithrombin action of the materials in blood contact situations following thrombin generation.

The results of these experiments are shown in Fig. 4.9 (p. 84) and Fig. 4.10 (p. 85). As shown in Fig. 4.9, the amount of thrombin adsorbed to the albumin-preadsorbed PPOBDDS surface is reduced by almost 50% compared to the untreated surface (Fig. 4.5), indicating that some of the thrombin-binding sites on the PPOBDDS surface interact strongly with albumin and are no longer able to bind thrombin, i.e. thrombin cannot displace albumin bound to these sites. Similarly, preadsorption of albumin to the PPACKPPO polymer reduced subsequent thrombin adsorption by more than 80%. In fact the PPACKPPO material adsorbed very little more thrombin than the PPOMDA material when preadsorbed with albumin. This suggests that much of the thrombin bound to this surface is not bound with an affinity greater than that of albumin. Of course it may be that thrombin is unable to displace albumin from the PPACK sites due to the fact that the albumin undergoes a conformational change or "relaxation" on the surface which effectively increases its affinity over time. In retrospect a better experiment to test the ability of thrombin to displace adsorbed albumin and other proteins from the surface would be to inject thrombin into the protein solution immediately after the initial exposure to albumin (or plasma).

The data in Fig. 4.5 and Fig. 4.9 show that the PPACK-grafted surface has no advantage over the PPOBDDS or PEOBDDS surfaces in binding thrombin following exposure to albumin. However, these simple adsorption or binding experiments do not provide information about the conformation and

activity of the adsorbed thrombin. In Chapter 5, experimental data on the activity of adsorbed thrombin are presented and show that in this respect there is indeed a major advantage of the PPACK-grafted surface.

Preexposure of the polymer surfaces to plasma results in deposition of a multicomponent protein layer (see Chapter 6) which may both promote and inhibit subsequent thrombin adsorption. Fibrinogen, for example, contains specific cleavage sites for thrombin and may attract thrombin to the surface on this account. Other proteins such as HK may also influence thrombin adsorption. Fig. 4.10 shows the amounts of thrombin which adsorbed to the surfaces after preexposure to plasma for two hours. The decrease in thrombin adsorption compared to the untreated surfaces is similar to that seen for the albumin preadsorbed surfaces, suggesting that the blocking effect of plasma is similar to that of albumin. This could be taken as an indication that the major blocking agent in plasma is albumin and that other proteins are not significantly involved.

Thrombin binding affinity

The adsorption data give, in principle, an indication of the affinity of binding between protein and surface, and many authors have obtained numerical estimates of affinity constants by application of the Langmuir isotherm (Andrade and Hlady, 1986). Unfortunately the Langmuir model is based on a number of assumptions which are clearly invalid in the present experiments. Among these are that adsorption is reversible, that there is only one type of binding site, and that adsorption is limited to a monolayer. The first two of these conditions are not met and the third is questionable in the present case.

Therefore it does not seem appropriate to obtain estimates of affinity constants by this method. As suggested previously, a semiquantitative indication of binding affinity may be obtained from the slopes of the isotherms at low solution concentration. Regardless of reversibility or the details of the binding sites, a surface which binds more thrombin at a given concentration presumably has a higher binding affinity. Clearly such an approach gives some kind of average affinity over the various site types. The data for experiments using the untreated surfaces, the surfaces preexposed to albumin, and the surfaces preexposed to plasma are given in Table 4.4.

Table 4.4 Relative "affinities" for thrombin binding to polyurethanes in Tris buffer, 22°C.*

	Slope of adsorption curve (10^{-3}cm)				
	PPOMDA	PPOBDDS	PEOMDA	PEOBDDS	PPACKPPO
Unmodified surfaces (Fig. 4.6)**	9.3	31.2	3.9	22.7	30.7
Surfaces pre-adsorbed with plasma*** (Fig. 4.10)	5.2	14.3	1.8	16.6	5.2
Surfaces pre-adsorbed with albumin**** (Fig. 4.9)	2.8	9.7	1.1	15.5	4.8

* Precision of affinities estimated at ±10%.

** Polymer surfaces were incubated with Tris buffer for 2 h, then with thrombin solution for 2 h.

*** Polymer surfaces were incubated with plasma for 2 h, rinsed with Tris buffer three times, then incubated with thrombin solution for 2 h.

**** Polymer surfaces were incubated with albumin solution (0.15 mg/ml) for 2 h, rinsed with Tris buffer three times, then incubated with thrombin solution for 2 h.

The data in Table 4.4 show that the PPACKPPO and PPOBDDS polyurethanes have the highest affinity for thrombin (~31 x 10^{-3} cm, second row of Table 4.4). The affinity of the hydrophobic PPOMDA polyurethane is only about one third (9.3 x 10^{-3} cm), and of the PEOMDA polyurethane about 12% (~4 x 10^{-3} cm) as great. These data indicate that surface chemistry has a significant influence on thrombin binding. Negative charges appear to promote thrombin adsorption, while hydrophilic components like PEO greatly reduce thrombin adsorption.

Interestingly, the very low affinity of the PEOMDA polyurethane for thrombin is further decreased by pre-exposing the surface to albumin or plasma (Table 4.4), with the relative affinity reduced fourfold from ~3.9 x 10^{-3} cm to ~1 x 10^{-3} cm. PEOMDA polyurethane without pretreatment has a low affinity for thrombin, presumably due to the presence of PEO in the surface. The further decrease in thrombin adsorption by the preadsorbed plasma proteins suggests that protein precoating is another effective technique to control thrombin adsorption. As shown in Table 4.4, the combined effects of PEO incorporation and protein precoating changed the thrombin affinity from the highest value ~31 x 10^{-3} cm to ~1 x 10^{-3} cm, a thirty-fold decrease.

SUMMARY

Five polyurethanes having a range of properties were synthesized. PPOMDA was intended as a conventional or "standard" hydrophobic polyurethane with no special chemical or other properties. PEOMDA, containing a polyethylene oxide soft segment, was intended as a potentially protein-resistant material. PPOBDDS and PEOBDDS are negatively charged

versions of PPOMDA and PEOMDA respectively, and PPACKPPO contains PPACK residues. It was thus expected that PPACKPPO would exhibit specific thrombin binding (scavenging) capacity. The chemical structure of the polymers was investigated by NMR, and the surface structure was studied by XPS and contact angle methods. The polymer surfaces prepared under hydrophobic conditions appeared to have the hydrophobic components present on the surface. Surface-restructuring in aqueous media brings the hydrophilic components and functional groups to the surface. The adsorption of radiolabeled thrombin and albumin to the polymer surfaces showed that these proteins have high affinity for the PPOBDDS and PPACKPPO surfaces. Preincubation of the surfaces with albumin or plasma significantly reduced the adsorption of thrombin to both PPOBDDS and PPACKPPO surfaces.

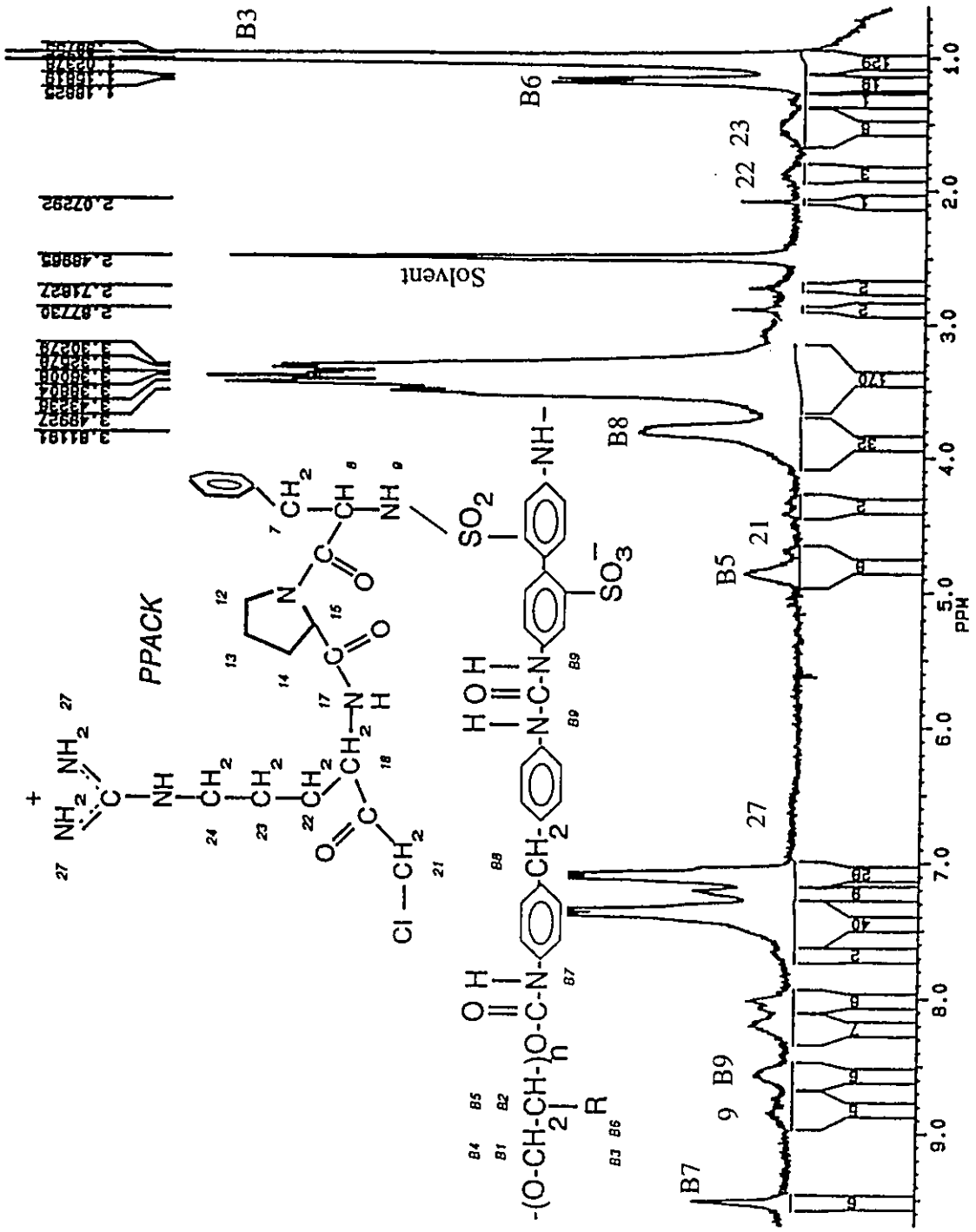


Fig. 4.1a Proton NMR spectrum (with assignments) of PPACKPPO polyurethane in DMSO.

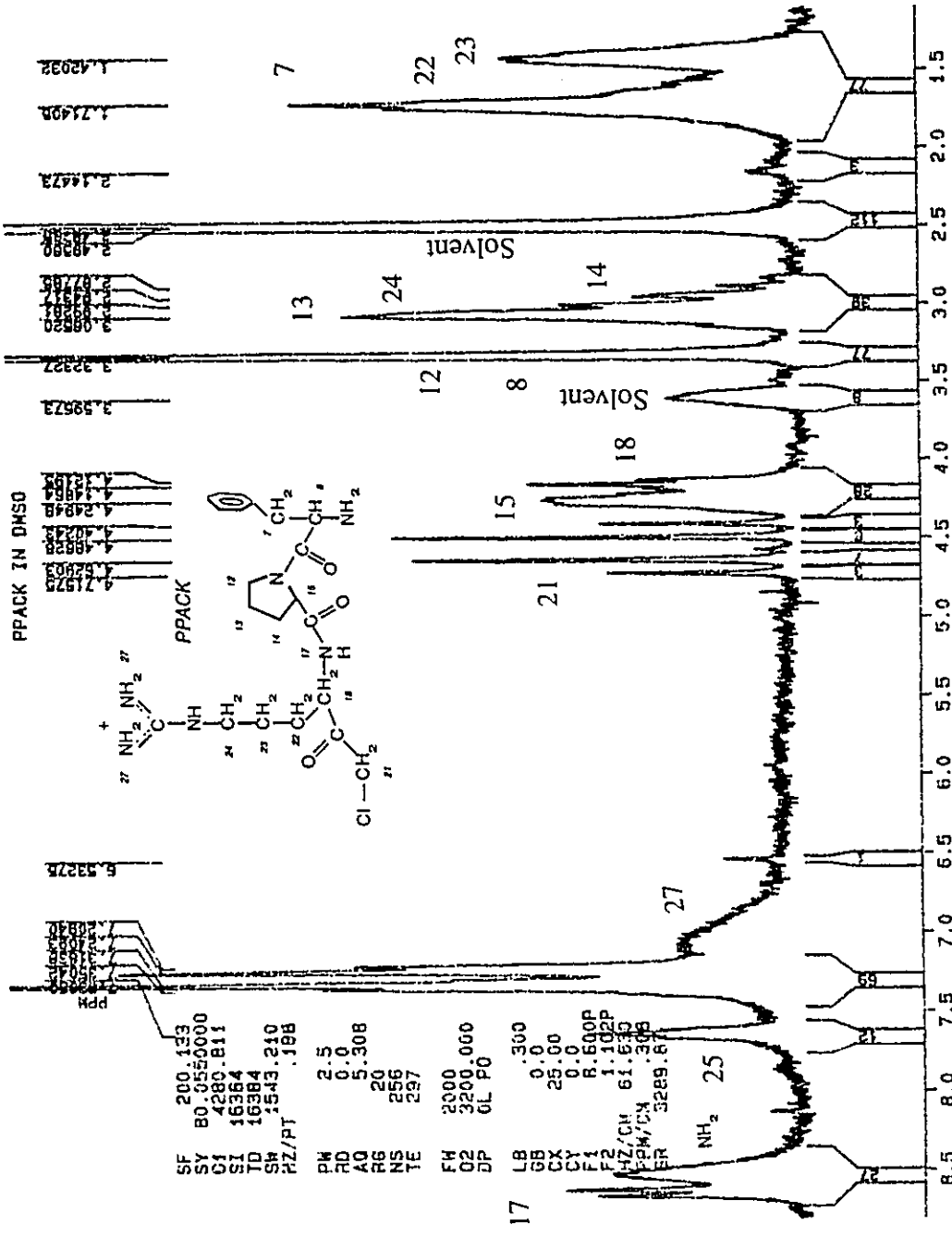


Fig. 4.1b Proton NMR spectrum (with assignments) of PPACK in DMSO.

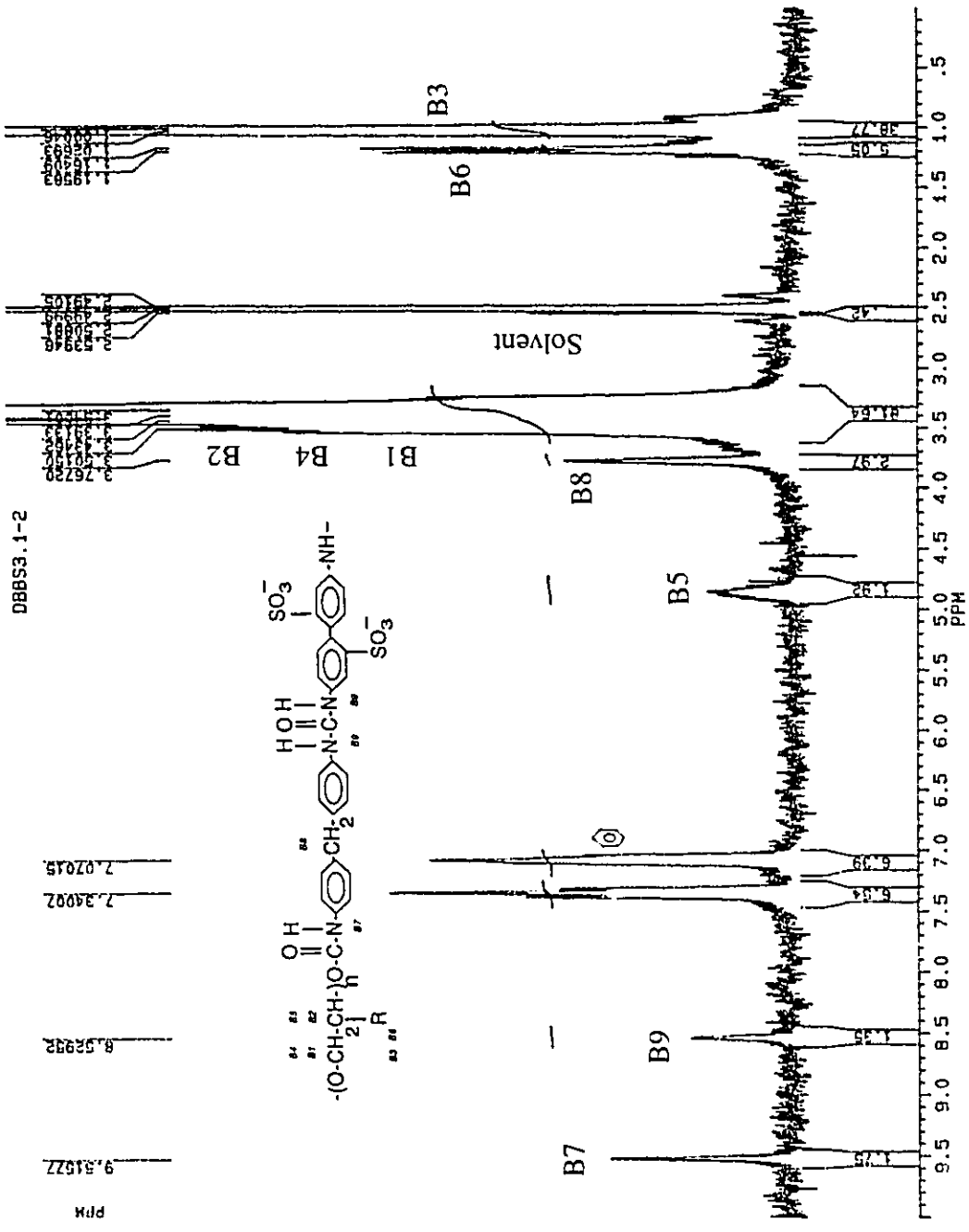


Fig. 4.1c Proton NMR spectrum (with assignments) of PPOBDDS polyurethane in DMSO.

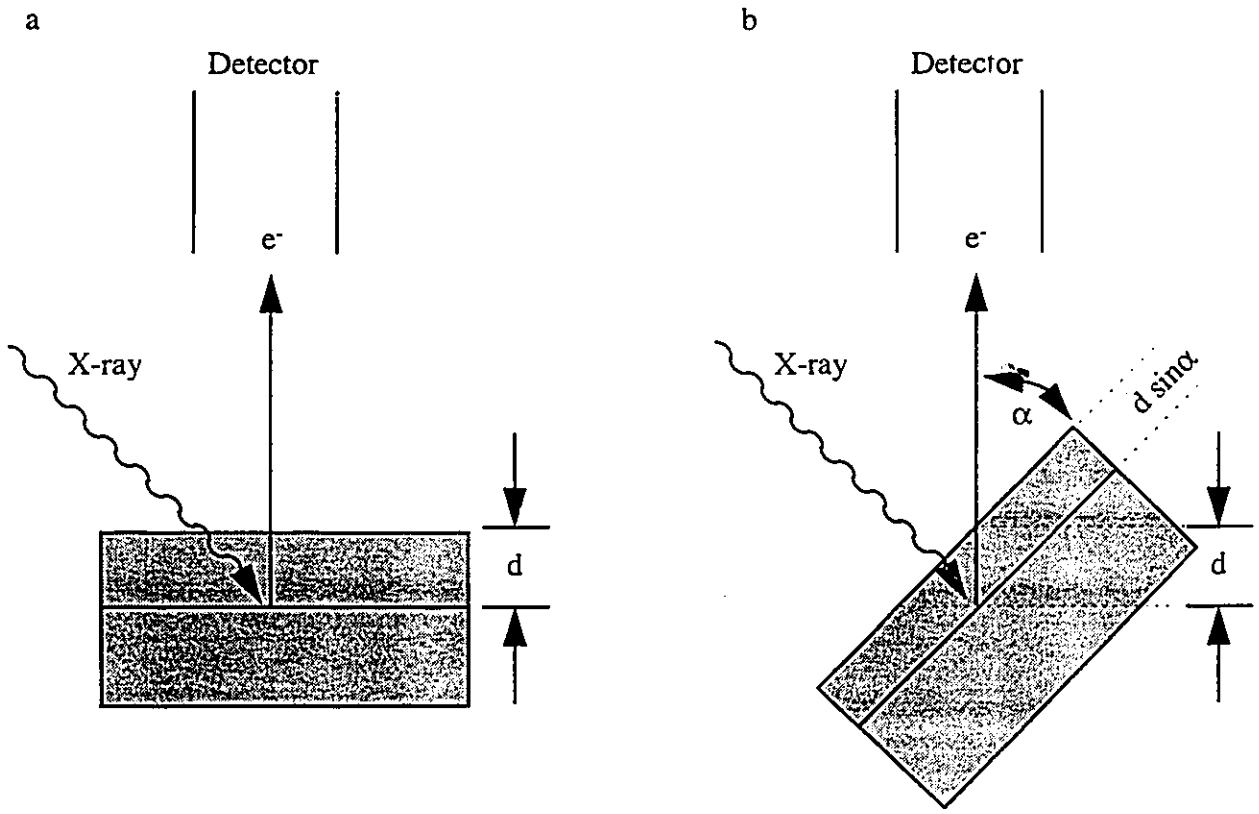


Fig. 4.2a (a) X-rays are directed towards the sample and electrons are emitted from a maximum depth, d (100Å). (b) Angle dependent XPS reduces the effective sampling depth (d sinα) when the take-off angle (α) is decreased.

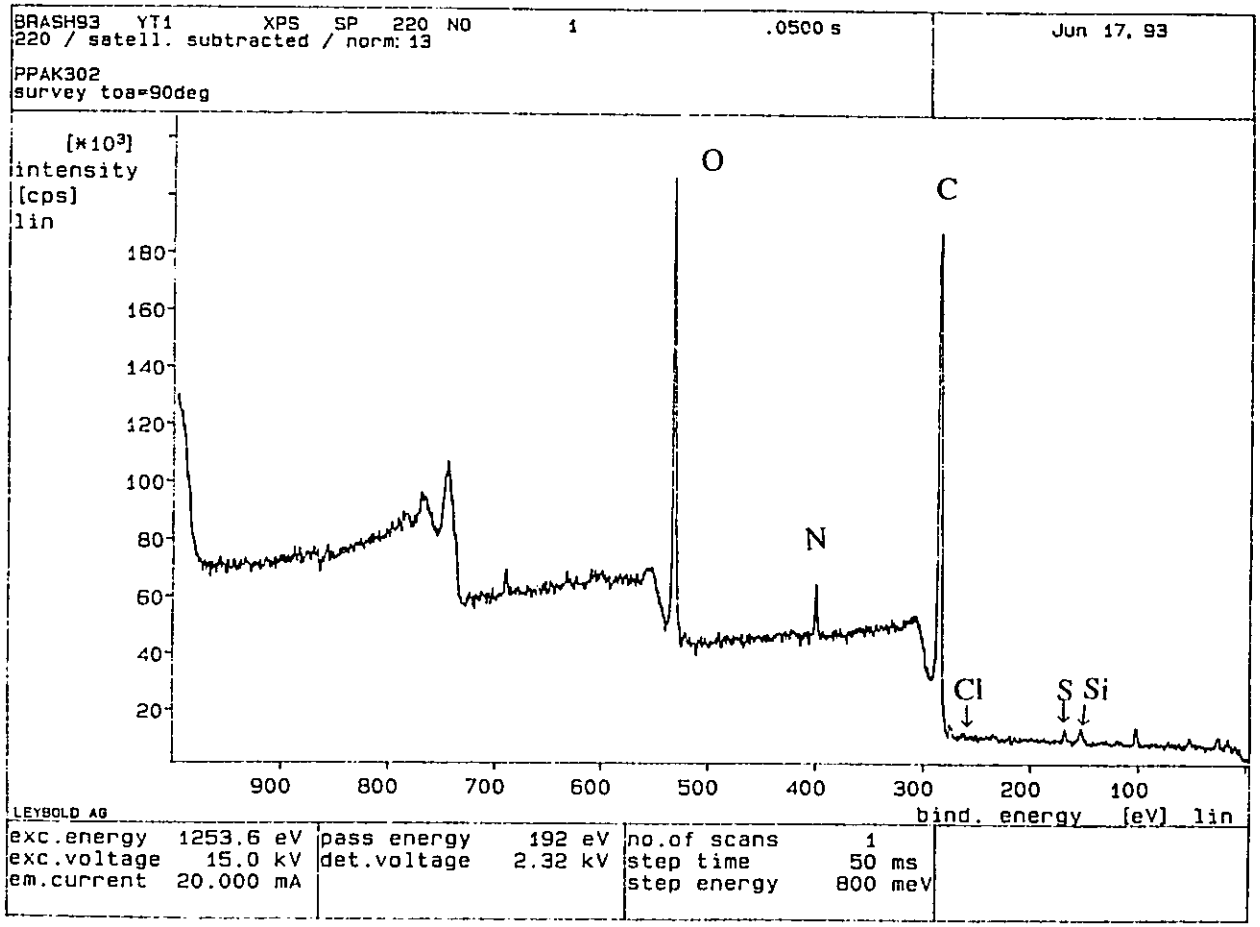


Fig. 4.2b XPS spectrum of PPACKPPO polyurethane film, take-off angle 90 degrees.

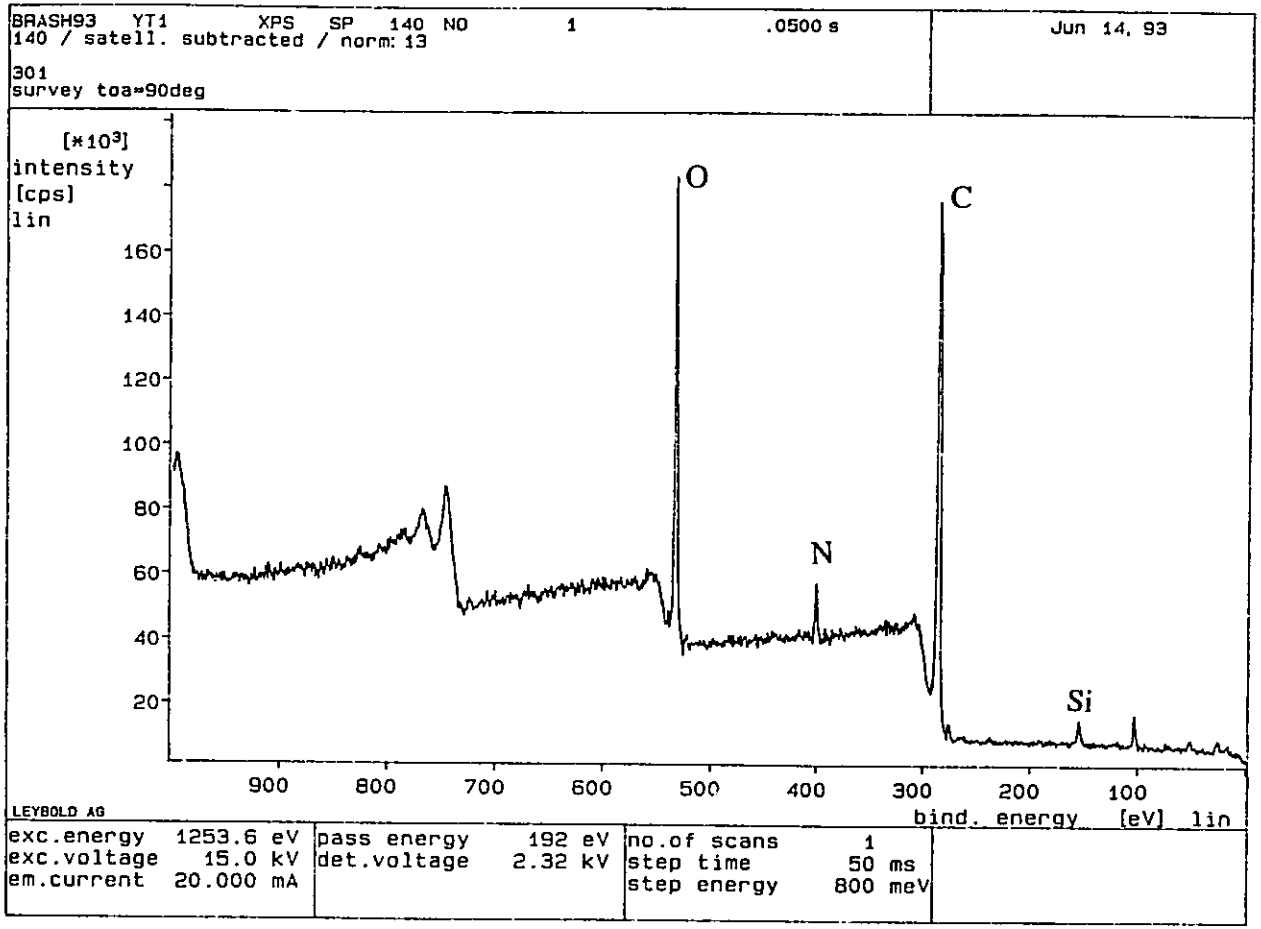


Fig. 4.2c XPS spectrum of PPOMDA polyurethane film, take-off angle 90 degrees.

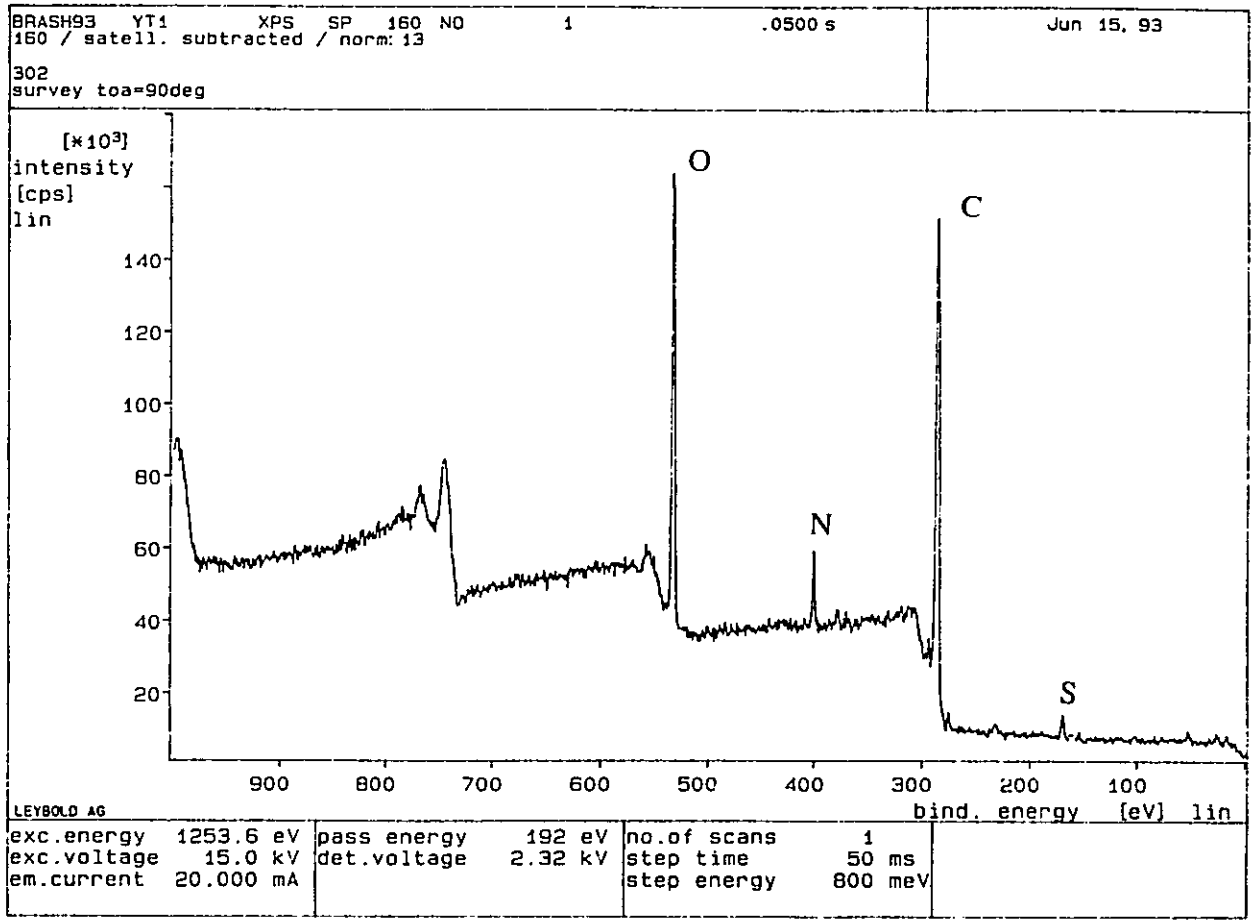


Fig. 4.2d XPS spectrum of PPOBDDS polyurethane film, take-off angle 90 degrees.

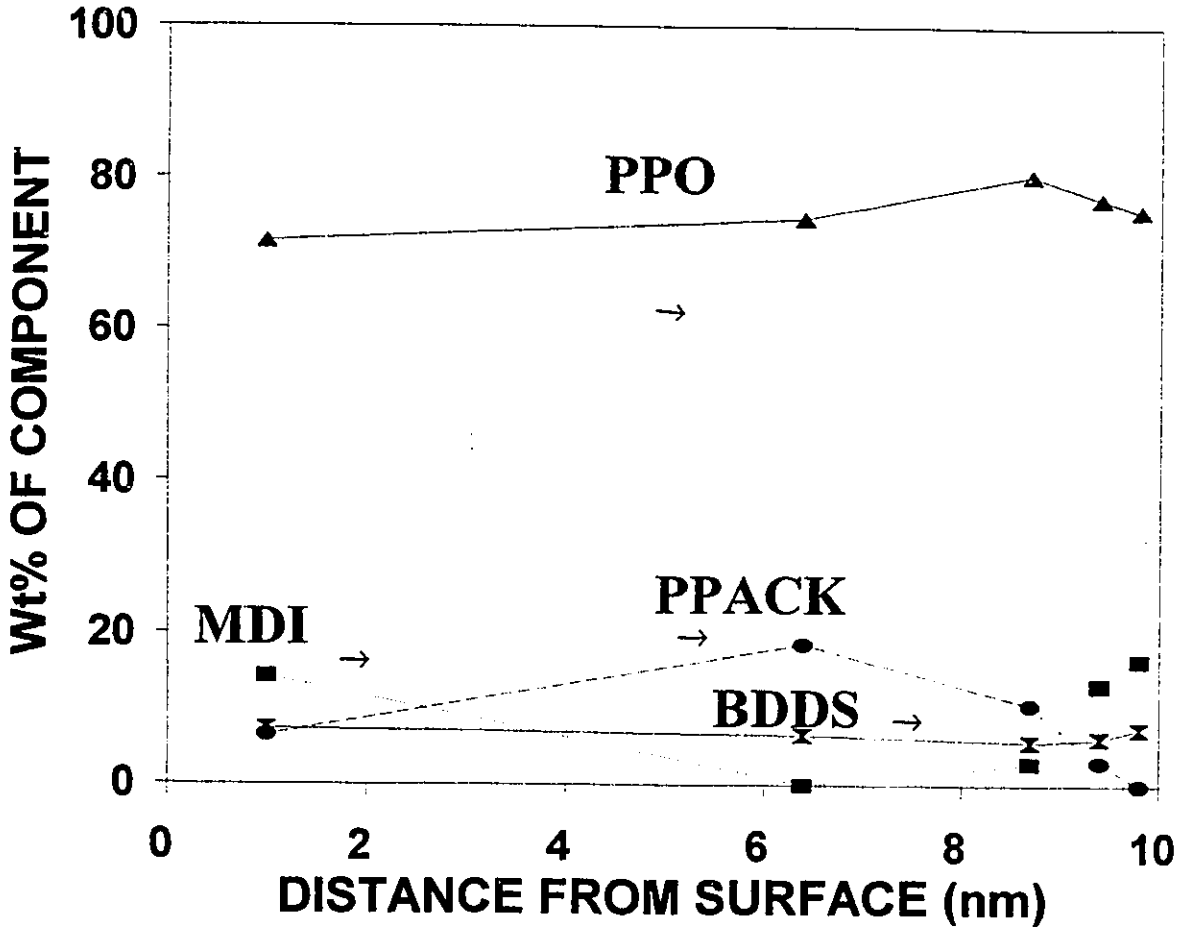


Fig. 4.3a Composition depth profile for PPACKPPO polyurethane surface measured by XPS at different take off angles (α), distance = 100 Å sinα. (→) Data from NMR and elemental analysis methods. Data precision is ±10%.

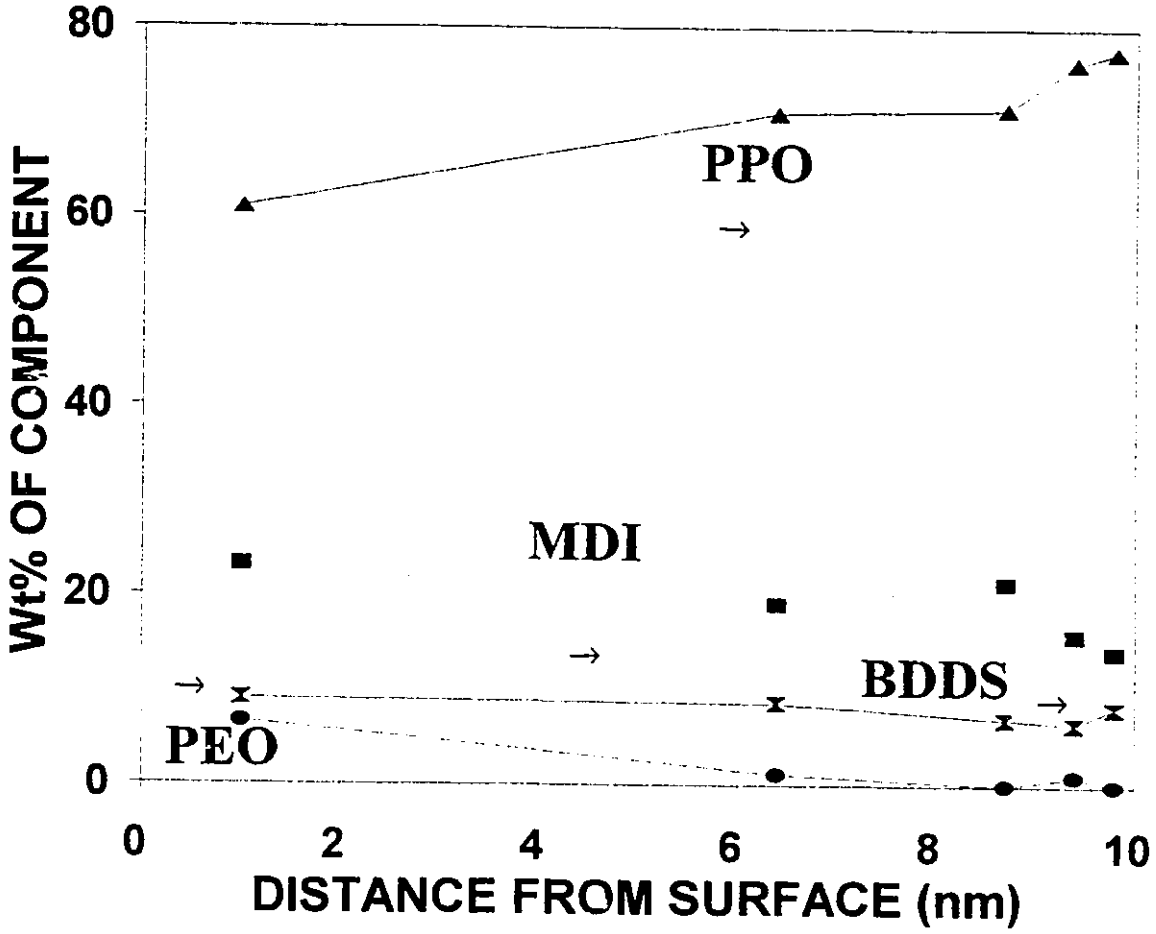


Fig. 4.3b Composition depth profile for PEOBDDS polyurethane surfaces measured by XPS at different take off angles (α), distance = 100 Å sinα. (→) Data from NMR and elemental analysis methods. Data precision is ±10%.

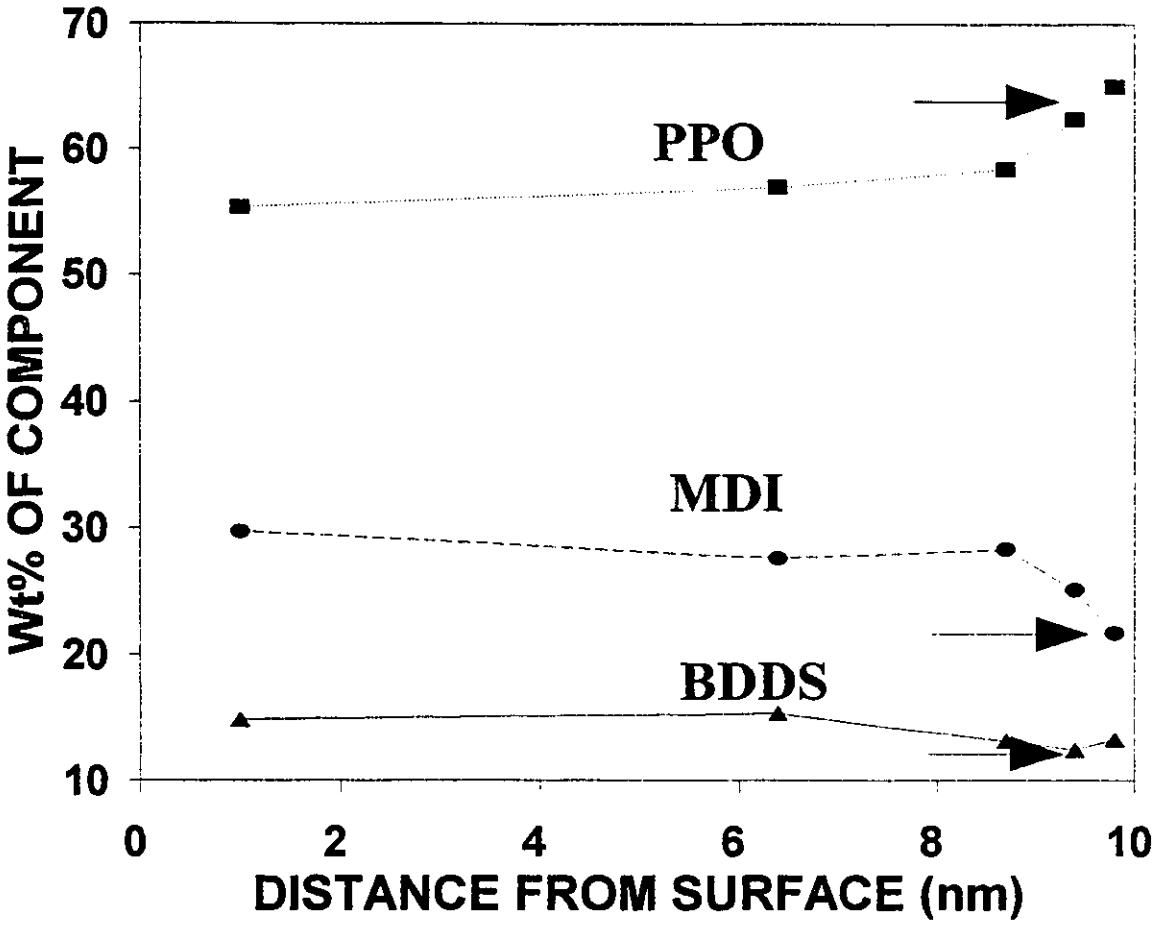


Fig. 4.3c Composition depth profile for PPOBDDS polyurethane surfaces measured by XPS at different take off angles (α), distance = 100 Å sinα. (→) Data from NMR and elemental analysis methods. Data precision is ±10%.

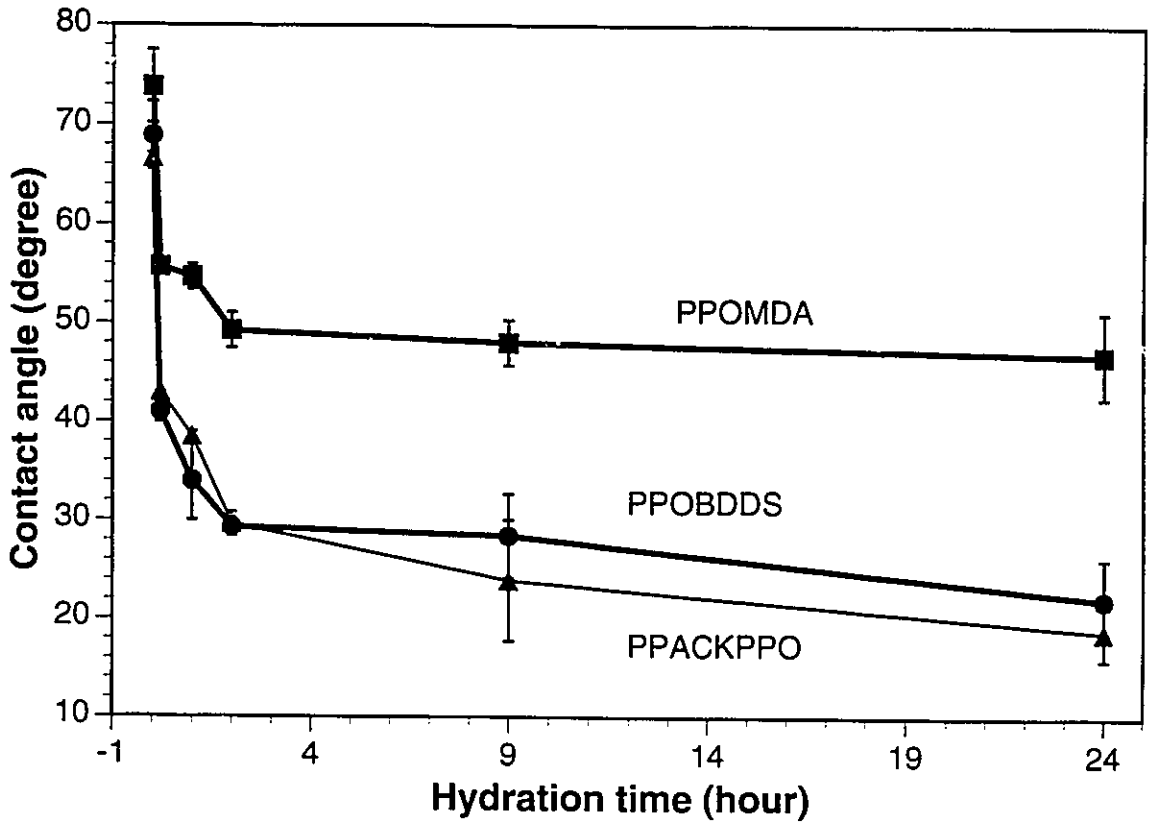


Fig. 4.4 Water contact angles of polyurethane surfaces as a function of hydration time. The films were immersed in water (22°C) and an air bubble introduced to the film-water intersurface. The contact angle made by the air bubble and the surface was measured. Error bars: ± SD, n > 5.

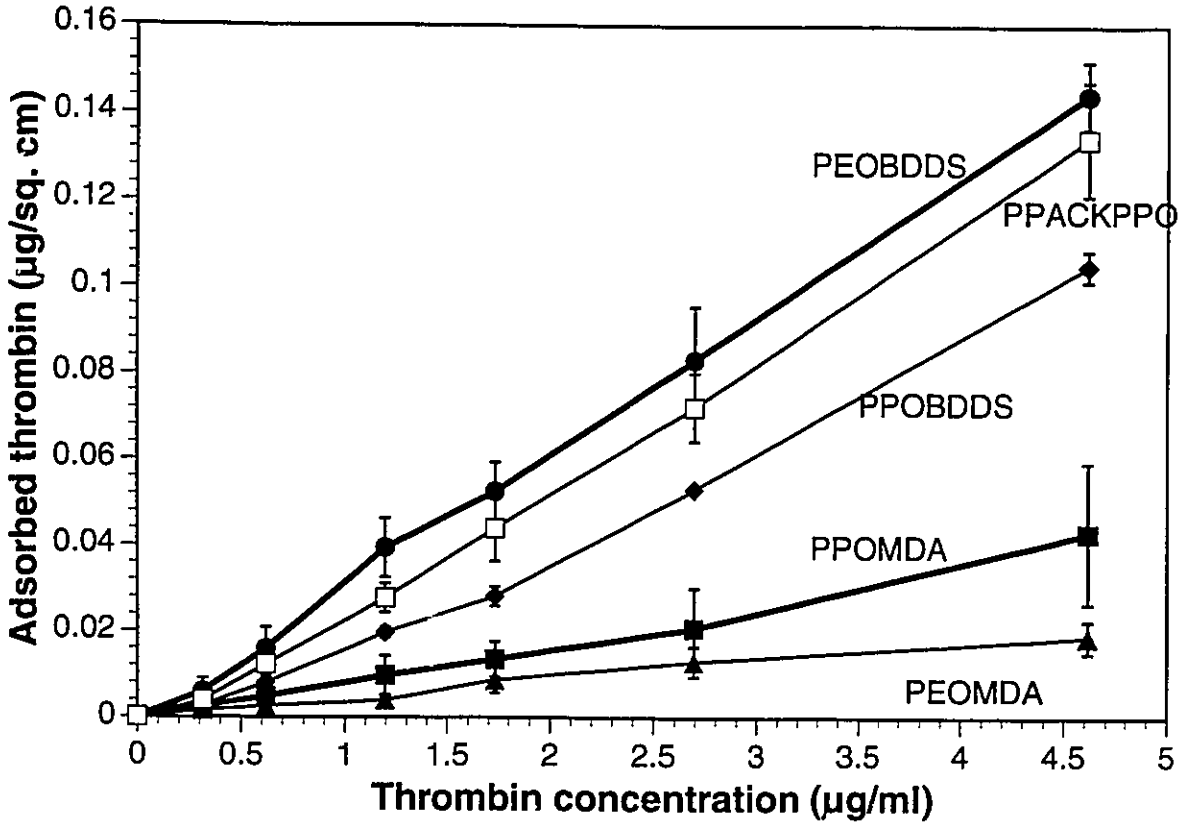


Fig. 4.5 Human thrombin (HT) adsorption to polyurethane surfaces. HT was labeled with ^{125}I. Adsorption was carried out in 96-well polystyrene microtitre plates, from isotonic Tris buffer, pH 7.4, at room temperature (22°C) under static conditions for two hours. Each point represents the average of 6 to 9 replicates. Error bars: ± SD, n > 5.

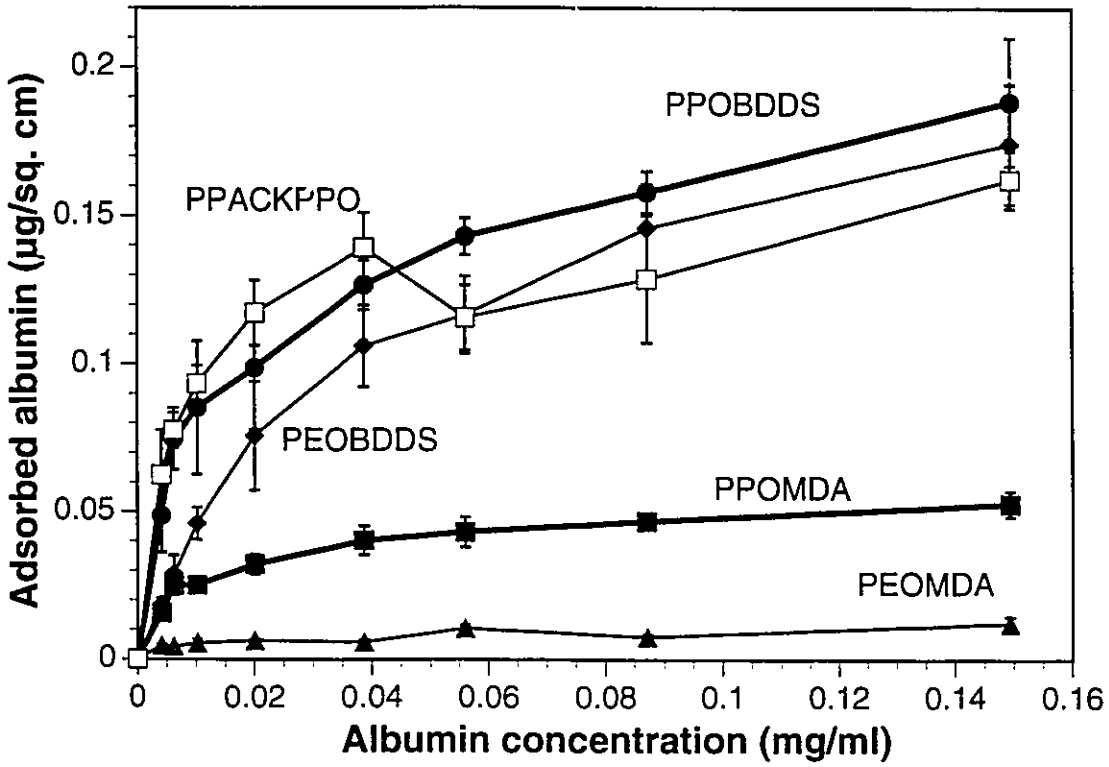


Fig. 4.6 Human serum albumin (HSA) adsorption to polyurethane surfaces. HSA was labeled with ^{125}I. Adsorption was carried out in 96 well polyurethane coated polystyrene microtitre plates from isotonic Tris buffer, pH 7.4, at 22°C under static conditions for two hours. Each point represents the average of 6 to 9 replicates. Error bars: ± SD, n > 5.

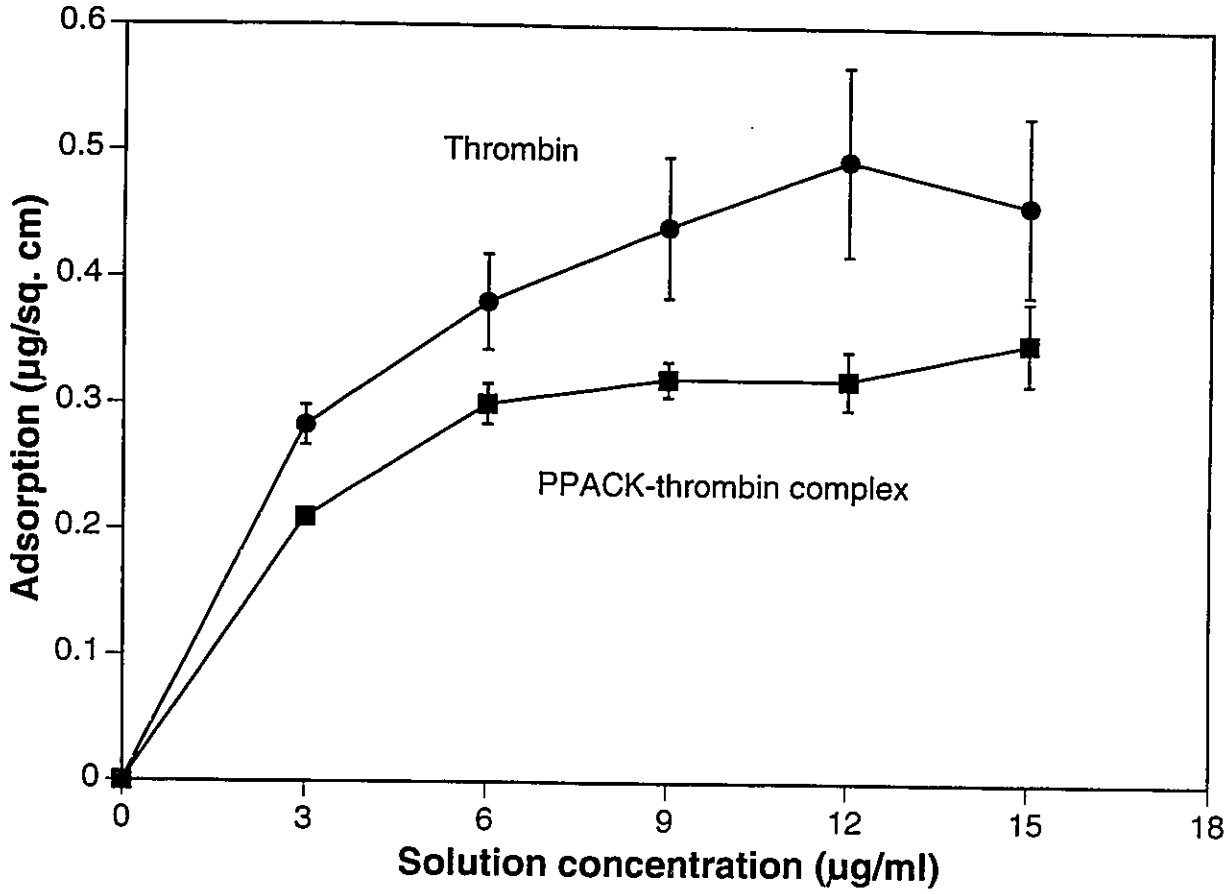


Fig. 4.7 Adsorption of human thrombin (HT) and HT-PPACK complex to the PPACKPPO polyurethane surface. HT was labeled with ^{125}I. Adsorption was carried out in 96-well PPACKPPO polyurethane coated polystyrene microtitre plates from isotonic Tris buffer, pH 7.4, at 22°C under static conditions for two hours. Each point represents the average of 6 to 9 replicates. Error bars: ± SD, n > 5.

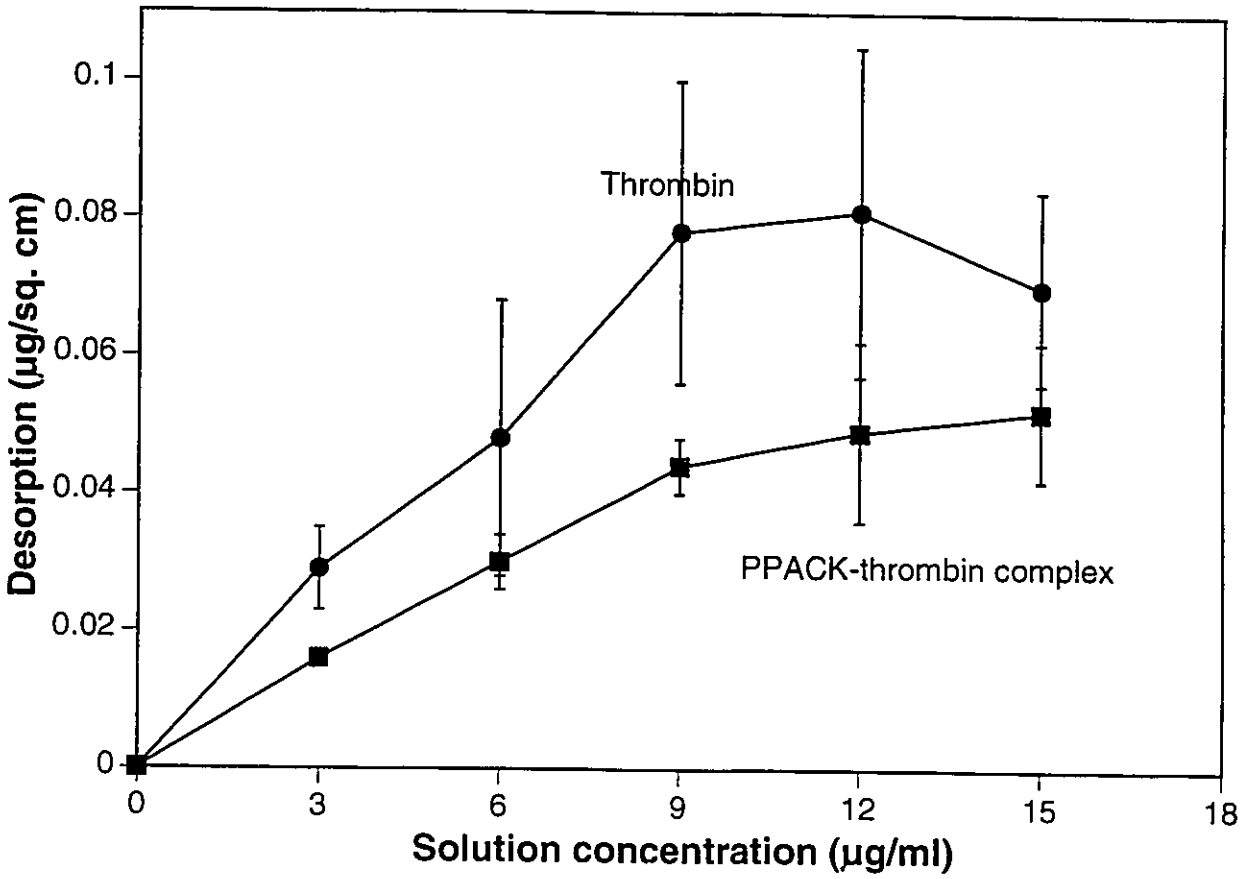


Fig. 4.8 Desorption of PPACKPPO surface-bound human thrombin into isotonic Tris buffer in 1 hour at room temperature. Thrombin was labeled with ^{125}I. Adsorption was carried out in 96-well PPACKPPO polyurethane coated polystyrene microtitre plates from isotonic Tris buffer, pH 7.4, at 22°C under static conditions for two hours. The wells were then rinsed with buffer 3 times, and incubated with buffer for 1 hour. The buffer solution was counted for radioactivity to determine the amount of thrombin desorbed into solution. Each point represents the average of 6 to 9 replicates. Error bars: ± SD, $n > 5$.

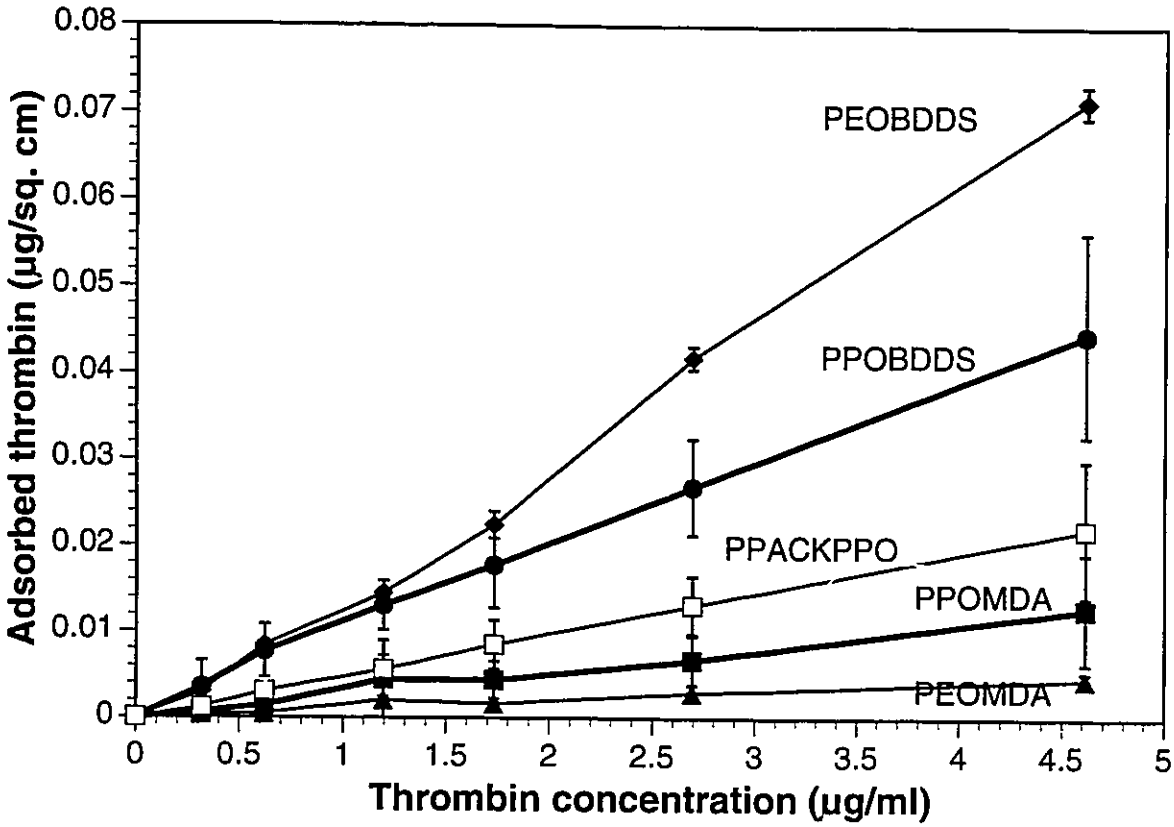


Fig. 4.9 Human thrombin (HT) adsorption to polyurethane surfaces preadsorbed with human albumin. Surfaces were incubated with human albumin solution (0.15 mg/ml) for two hours. Then the samples were rinsed three times with isotonic Tris buffer, pH 7.4. HT labeled with ^{125}I in Tris buffer was then added and the adsorption was carried out for 2 h at 20°C under static conditions. Each point represents the average of 6 to 9 replicates. Error bars: ± SD, n > 5.

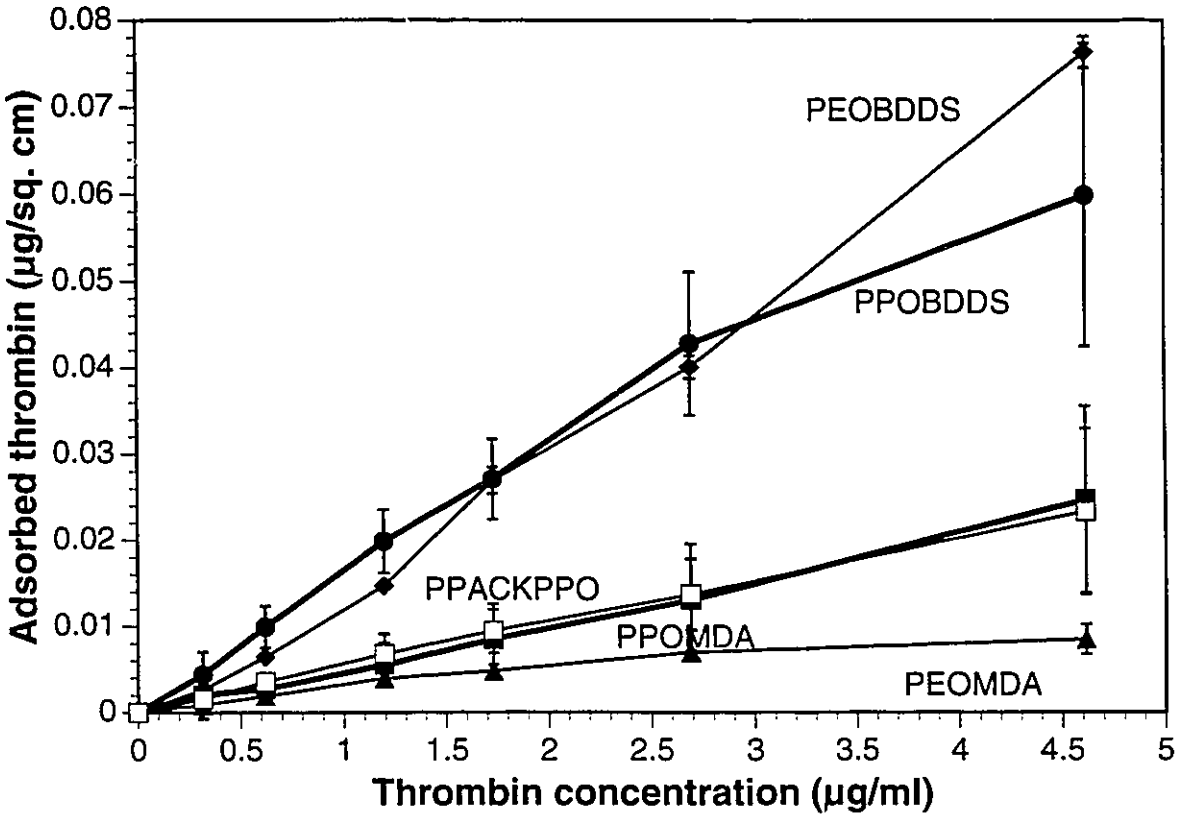


Fig. 4.10 Human thrombin (HT) adsorption to polyurethane surfaces preadsorbed with plasma proteins. Surfaces were incubated with pooled human plasma for two hours. Then the samples were rinsed three times with isotonic Tris buffer, pH 7.4. HT labeled with ^{125}I in Tris buffer was then added and the adsorption was carried out for 2 h at 20°C under static conditions. Each point represents the average of 6 to 9 replicates. Error bars: ± SD, n > 5.

CHAPTER 5
ACTIVITY OF THROMBIN ADSORBED ON POLYURETHANE SURFACES

5.1 INTRODUCTION

As discussed in Chapter 4, it was found that the incorporation of PPACK or sulphonate groups into polyurethanes significantly increased thrombin adsorption while polyethylene oxide (PEO) decreased thrombin adsorption. The preadsorption of plasma proteins reduced subsequent thrombin adsorption to the PPACKPPO surface, while the PPOBDDS surface was less influenced. One explanation for these observations is that certain of the plasma proteins bind tightly to PPACK and cannot be displaced by thrombin. Another possibility is that the surface-bound PPACK is chemically changed or is effectively "hidden" from any contacting fluid so that it can no longer bind thrombin with high specificity.

In addition to the quantity of protein adsorbed, the influence of the surface on the biological activity of the adsorbed protein is another important aspect of protein surface interaction. In this work it is of prime interest to develop knowledge of the ability of immobilized PPACK not only to bind thrombin, but to inhibit it as well. A number of methods have been developed to study the activity of adsorbed proteins. In the case of enzymes, methods using chromogenic peptide substrates are relatively simple and provide detailed and

valuable information on enzyme activity (Duncan et al., 1985; Rollason and Sefton, 1992). In this work, a chromogenic substrate method using the tripeptide substrate N-p-tosyl-gly-pro-arg-pNA was used to investigate: (1) the activity of surface-bound thrombin on different polyurethane surfaces; (2) the ability of the polyurethane surfaces to scavenge solution thrombin; and (3) the ability of surface-bound PPACK and released PPACK to inhibit thrombin.

5.2 EXPERIMENTAL

Materials

The synthesis of the polyurethanes was described in Chapter 4.

The chromogenic substrate for thrombin, N-p-tosyl-gly-pro-arg p-nitroanilide, and human alpha-thrombin (3000 units/mg) were purchased from Sigma (St Louis, MO) and used without further purification.

YPACK (N-p-tyr-pro-arg chloromethylketone) was purchased from Bachem, California.

YPACKPPO synthesis

PPOBDDS polyurethane was dissolved in DMF (3% w/w), and 0.15 ml of the solution was stirred at 90°C for 4 h. To this solution, 0.01 ml oxalyl chloride was added and reacted at 22°C for 3 h. A mixture of 1.41 mg YPACK and 3 μg ^{125}I-labeled YPACK in 0.2 ml dry DMF was added and the system reacted at 22°C for 2 h, and then at 5°C for 12 h. The polymerization was stopped by slowly adding water to precipitate the polymer. The polymer was then washed extensively with water and dried in a vacuum oven at 22°C for 24 h. The

polymer yield was 70 - 80 %.

Preparation of surfaces

Polymers were dissolved in DMSO (1% w/w), and 300 µl of the solution was added to the wells of 96-well polystyrene microtitre plates and then aspirated. The plates were dried in a vacuum oven at 22°C for 2 h, and the wells were then rinsed thoroughly with double distilled water and stored at -40°C. Before use, the wells were washed again with water three times and equilibrated with Tris buffered saline (TBS), pH 7.4, for 2 h.

Radio-labeling procedures

Radio iodination of YPACK: Into a vial containing 1 mCi ^{125}I (Amersham, Arlington Heights, IL) were added: 45 µl of 0.5 M $NaHPO_4$ buffer (pH 7.5), 5 µg YPACK, and 15 µl of a solution of chloramine-T (1.75 mg/ml, BDH, Toronto, Ontario). The mixture was incubated for 1 min, and 20 µl sodium metabisulfite solution (2.4 mg/ml, BDH, Toronto, Ontario) was then added and the mixture incubated for an additional 0.5 min. The reaction mixture was applied to a Sep Pak C_{18} cartridge (Waters, Mississauga, Ontario) which had been pre-equilibrated with aqueous 0.1% trifluoroacetic acid (TFA, Aldrich, Milwaukee, WI) solution. Free iodine was removed from the cartridge by washing with 0.1% TFA. The labeled YPACK was then eluted with 20% acetonitrile/80% aqueous TFA (0.1%). Fractions (1 ml) were collected and counted for radioactivity.

Radioiodination of human alpha-thrombin: Thrombin was labeled with

^{125}I using the lactoperoxidase method (Enzymobead reagent, BioRad, Richmond, CA) as described previously (Santerre et al., 1992). The Enzymobeads were separated from the reaction mixture by centrifugation, and the supernatant was dialyzed for 20 h at 4°C. The enzymatic activity of the radiolabeled thrombin was found to be similar to that of the original thrombin as determined by a chromogenic assay method using N-p-tosyl-gly-pro-arg p-nitroanilide as substrate.

Determination of thrombin activity in solution using chromogenic substrate

A typical experiment was as follows: after addition of 190 µl of thrombin solution (concentrations in the range 0.05 - 0.33 µg/ml) to the polyurethane-coated wells of microtitre plates, 10 µl of N-p-tosyl-gly-pro-arg p-nitroanilide solution (1.2 mg/ml) was added. The final concentration of the substrate was 0.06 mg/ml. The optical density (OD) of the p-nitroaniline (pNA) released from the substrate by thrombin was measured as a function of time at 405 nm and 22 or 37°C. The concentration of the substrate was chosen to ensure a maximum OD less than 2.0 so that the OD-concentration relationship is in the linear range.

Determination of the activity of surface-bound thrombin using chromogenic substrate.

A typical experiment was as follows: the polyurethane-coated wells of microtitre plates were incubated with 0.35 ml TBS, pH 7.4 for 2 h at 22°C. Immediately following the withdrawal of the TBS buffer, ^{125}I labeled thrombin (200 µl of a 2 µg/ml solution in TBS) was added to the wells and incubated for 2

h at 22°C. The wells were then rinsed 3 times with TBS buffer. The quantity of thrombin adsorbed to the wells was determined by measuring the radioactivity.

To measure the activity of the surface-bound thrombin obtained in the above experiment, 0.2 ml of 0.06 mg/ml N-p-tosyl-gly-pro-arg p-nitroanilide in TBS was added, and the optical density of the released pNA was measured at 405 nm using a plate reader. The amidolytic activity of surface-bound ^{125}I-labeled thrombin thus determined was compared with that of free radiolabeled thrombin. At the end of the incubation period, the supernatants were removed and counted for radioactivity as were the wells.

Release of PPACK from PPACKPPO polymer

Although the polyurethanes were extensively washed following synthesis, it seemed possible that some residual PPACK molecules would remain in the PPACKPPO polymer, and that some PPACKPPO macromolecules might be slightly water soluble. When the polymer was later placed in a solution containing thrombin, some free PPACK (PPACK molecule or soluble PPACKPPO) could diffuse into the solution and neutralize the thrombin.

The possible release of free PPACK was assessed as follows. After incubating PPACKPPO coated wells with 0.2 ml TBS for 1 h, half (0.1 ml) of the solution was transferred into an uncoated polystyrene well. Then various amounts of thrombin and substrate were added to measure the thrombin-inhibitory activity of any released PPACK. Similarly thrombin and substrate were added to the remaining 0.1 ml solution in the PPACKPPO-coated wells to measure the thrombin-inhibitory activity. In the polystyrene wells, the measured thrombin-inhibitory activity is from released PPACK only. In the PPACKPPO

coated wells, the measured thrombin-inhibitory activity is assumed to be from both released PPACK and surface-bound PPACK. Therefore higher thrombin-inhibitory activity is expected in the latter case.

The release of ^{125}I-YPACK from the YPACKPPO polymer was determined by measuring the radioactivity in TBS solution which had been incubated in YPACKPPO-coated wells for different times. Since the structure of the YPACKPPO polymer is similar to that of PPACKPPO, the data were used to provide an indication of the release of PPACK from the PPACKPPO polymer.

All assays were performed at least 3 times. The data shown are for typical, single assays. All data shown were obtained using polymers from a single batch. Again, similar data were obtained using replicate polymer batches.

5.3 RESULTS AND DISCUSSION

1. The activity of surface-bound thrombin

The amidolytic activity of adsorbed thrombin on different polyurethanes against the chromogenic substrate N-p-tosyl-gly-pro-arg p-nitroanilide is shown in Fig. 5.1 (p. 113).

Thrombin solution was incubated with the different polyurethane surfaces coated on the wells of a microtitre plate for two hours. The surfaces were then rinsed with buffer to remove any loosely bound thrombin. The remaining thrombin on the surfaces is considered adsorbed to the surface and its activity was determined using a chromogenic substrate assay. As shown in Fig. 5.1, no p-nitroaniline was released from the substrate by thrombin adsorbed on the

PPACKPPO surface, suggesting that all of the thrombin bound to the PPACKPPO surface was neutralized by its interaction with the surface. In contrast, variable amounts of the thrombin adsorbed on the negatively charged PEOBDDS and PPOBDDS surfaces remained active.

TABLE 5.1. The activity of surface-bound thrombin.*

Thrombin	PPACKPPO	PPOBDDS	Pst***	PPOMDA	PEOMDA	PEOBDDS
Total thrombin (ng/cm^2)	35	40	20	10	6	26
Active thrombin (ng/cm^2)	0	2.9	0.3	0.04	0.04	4.2
% active thrombin**	0	7.3	1.3	0.4	0.6	16

Note: Adsorption time, 2 h; thrombin concentration, 2 μg/ml (6 units/ml) in TBS; volume, 0.3 ml; polymer surface area, 0.95 cm^2; temp., 22°C.

* Data precision is ±5%.

** % active thrombin=active thrombin/total thrombin.

*** Thrombin concentration, 0.97 μg/ml; adsorption time 1 h; Pst surface area, 1.55 cm^2.

Using a kinetic model described below, the percentage of adsorbed thrombin which retained its activity against the substrate was estimated. These data are shown in Table 5.1. The total amount of thrombin adsorbed was measured using radiolabeled enzyme (in some cases the data reported in Chapter 4 were used, and in others additional data were obtained). The highest retention of thrombin activity (16%) was observed for the PEOBDDS surface. It is seen that BDDS-polyurethanes adsorb more thrombin with higher retention of activity than MDA-polyurethanes. It seems possible that on BDDS-polyurethanes, some of the thrombin binds to sulfonate groups via its anionic binding exosite thereby leaving the catalytic site "free" to interact with the

substrate. Thrombin bound to PPO-polyurethanes retains less activity than thrombin bound to PEO-polyurethanes, which may reflect the fact that the hydrophobic PPO segments bind to the hydrophobic pocket of thrombin that is adjacent to the catalytic site, thereby inhibiting the enzyme and leading to relatively low retention of activity on PPO-polyurethanes.

Thrombin adsorbed on polystyrene is also mostly inhibited. As shown in Table 5.1, this surface adsorbed a substantial amount of thrombin and only about 1.5% remained active against the chromogenic substrate. Since polystyrene is strongly hydrophobic and has no functional groups that are likely to interact specifically with thrombin, this finding suggests that the activity of thrombin can be neutralized by hydrophobic interactions.

The data in Fig 5.2 (p. 114) show that as the coverage of adsorbed thrombin on the PPOBDDS surface increases, there is a gradual increase in the percentage activity retained. As discussed in Chapter 4, monolayer coverage for thrombin is in the range of 0.2 to 0.7 $\mu g/cm^2$. The surface concentrations of thrombin adsorbed to PPOBDDS are within this range; therefore multilayer adsorption is not the main adsorption mode. However at the higher end of this range as the monolayer becomes more dense and there is less potential for deformation of the surface bound molecules by conformational change, there may be a greater tendency for the enzyme to remain active.

It should be noted in connection with the fact that the activity of the bound enzyme depends on coverage, that comparison among the different surfaces should strictly speaking be done at constant coverage. The activity data in Table 5.1 were taken at varying coverage and as a result may not be truly comparable.

Because of the nature of the enzyme activity experiment, there is some uncertainty as to whether all of the activity measured corresponds to adsorbed enzyme. This is due to the fact that some of the enzyme may be desorbed during the period of the assay, and this portion would be evaluated in solution where its activity may not be the same as on the surface. To investigate this possibility, the following experiment was carried out. Radiolabeled thrombin in TBS solution was incubated in PPOBDDS-coated wells for 2 h. The solution was removed and the wells were rinsed 3 times with TBS buffer. Only the tightly bound thrombin should then remain on the surface. To this surface-bound thrombin, chromogenic substrate solution was added and allowed to react for one hour. During this period, some of the thrombin may desorb into the solution. Accordingly, after the chromogenic assay, the solution was withdrawn from the well, and both the well and the solution were counted for radioactivity to determine the thrombin remaining on the well surface and the thrombin released into solution during the assay. The data on desorbed thrombin for the PPOBDDS surface are shown in Table 5.2. Comparison of the data in columns 3 and 4 shows clearly that the amount of thrombin released is less than the total active thrombin, thus confirming that a fraction of the surface-bound thrombin remains active on the PPOBDDS surface. It is not possible to estimate the relative contributions of released versus bound thrombin to the generation of pNA in the assay, but it could be that some of the activity measured is associated with the desorbed enzyme.

The overall conclusion from these experiments is that thrombin adsorbed on the PPACKPPO surface is completely inactive, whereas on the precursor PPOBDDS surface some of the enzyme remains active, suggesting that binding

of thrombin to PPACK sites results in inhibition.

Table 5.2. Interactions of thrombin on PPOBDDS polyurethane surface: adsorption, desorption, and enzyme activity.

Thrombin concentration µg/ml	adsorbed thrombin µg	desorbed thrombin µg	Active thrombin µg	Active thrombin %
3	0.42	0.057	0.069	16.6
6	0.51	0.040	0.088	17.3
9	0.52	0.064	0.103	19.8
12	0.58	0.053	0.116	20.1
15	0.60	0.045	0.131	21.8

Note: 0.2 ml ^{125}I labeled thrombin solution (concentration shown in the first column) was added to PPOBDDS-coated wells of microtitre plate (contacting surface area: 1.55 cm^2) and incubated for 2 h; temp: 22°C. After removing the thrombin solution and rinsing the surface with TBS 3 times, 0.2 ml fresh TBS (with 0.06 mg/ml tosyl-gly-pro-arg-pNA) was added to the wells and incubated 1 h to desorb surface-bound thrombin. Then, the radioactivity of the supernatant and the well was counted respectively to determine the amounts of the desorbed thrombin in the supernatant and the remaining thrombin on the well surface. Thrombin activity was measured by chromogenic substrate method during the incubation. Data precision is ±5%.

2. Thrombin scavenging/inhibition capability of polymer surfaces.

In the experiments just discussed, the activity of thrombin bound to the polyurethane surfaces is measured. In relation to the intended application of these materials, another important aspect is the ability of the surface to "scavenge" thrombin from solution and to inhibit its activity in the bound state. These are the two functions expected of the PPACKPPO material when used in contact with blood. Experiments designed to evaluate thrombin scavenging and neutralization simultaneously are discussed in this section.

In these experiments, the wells of microtitre plates were coated with the polyurethanes. Thrombin and chromogenic substrate solutions (TBS buffer, pH 7.4) were added simultaneously and the kinetics of release of pNA from the substrate at 37°C was measured. The experiment was carried out at different

initial thrombin concentrations.

Enzyme kinetics can be treated using Michaelis-Menten theory (Cornish-Bowden, 1976) involving the assumptions that the enzyme and substrate form a complex, and that the complex achieves a steady state. The theory has been used widely to calculate the initial reaction rates (when the substrate conversion is less than 5%) so as to estimate enzyme activity. However, a number of investigators have claimed that Michaelis-Menten behaviour for an enzyme is more the exception than the rule (Hill et al., 1977; Cornish-Bowden, 1976; Keleti, 1986; Wong, 1975).

For thrombin, many investigators (Lottenberg et al., 1981; Okamoto et al., 1981; Griffith et al., 1980; Lottenberg et al., 1983) have used the Michaelis-Menten equation to calculate the kinetic parameters K_m and k_{cat}, representing respectively the enzyme-substrate affinity and the catalytic activity of the enzyme. On the other hand, Izquierdo et al. (1987) found that the hydrolysis of several chromogenic substrates (including tosyl-gly-pro-arg-pNA) by human thrombin is best described by a steady-state equation of order 2:2 and concluded that "human thrombin is an enzyme that does not follow Michaelian kinetic behaviour." It is possible that thrombin can bind to chromogenic substrates with more than one binding site (Izquierdo et al., 1987), or that the release of pNA from the substrate is a relatively slow process. Thus, a consensus on the exact reaction mechanism of thrombin acting on chromogenic substrates has not been reached. In addition it is known that enzymes immobilized on surfaces show kinetic behaviour that is different from solution kinetic behaviour (Keleti, 1986).

In this work, we were interested in measuring the possible

adsorption/deactivation of thrombin on polymer surfaces in a period of 1 to 2 h (typical protein adsorption time). Under these conditions the substrate conversion is expected to be considerably greater than 5%. Simple conventional chemical kinetic approach was found to be adequate for the analysis of the experimental data.

We assume that the reaction between thrombin (T) and substrate (S) can be written as:

$$T + S \xrightarrow{k_r} A + T \tag{1}$$

where A is pNA and k_r is the rate constant. With S_0 and T_0 the initial concentrations of substrate and thrombin respectively, and $A = S_0 - S$, we can write:

$$\frac{dA}{dt} = k_r T_0 S \tag{2}$$

where t is reaction time. The solution to equation (2) is:

$$A = S_0\{1-\exp(-k_r T_0 t)\} \tag{3}$$

or

$$-\ln(1-A/S_0) = k_r T_0 t \tag{4}$$

Equations (2) and (3) assume that the total active thrombin concentration (T_0) is constant during the period of the assay. As discussed in the previous section, thrombin is mostly inactive when adsorbed to the polyurethane surfaces. Therefore comparison of the pNA release data obtained in the

scavenging experiments with equation (3) (derived on the assumption that thrombin activity remains constant, i.e. there is no scavenging) should provide an indication of whether any of the thrombin is adsorbed (i.e. scavenged) and inhibited by the surface before it can react with substrate.

Polystyrene

Fig. 5.3 (p. 115) shows the kinetics of release of pNA from the substrate at 22°C in thrombin solution contacting unmodified polystyrene surface in the wells of 96-well microtitre plates. The experiment was carried out at different initial thrombin concentrations. It is seen that the amount of pNA released reached a common plateau value at about 60 minutes except at the lowest thrombin concentration (0.1 μg/ml) where the release rate was slower.

The data were analyzed by plotting $-\ln(1-A/S_o)$ against time according to equation (4). As shown in Fig. 5.4a (p. 116) such plots are essentially linear up to about 10 min. From the data shown in Fig. 5.4a, the slopes (k_rT_0) were determined, and plotted against T_0 (initial thrombin concentration) to obtain an estimate of the rate constant k_r as shown in Fig. 5.4b (p. 117). A value of k_r = 0.24 $(\mu g/ml)^{-1}(min)^{-1}$ (22°C) was estimated. As shown in Fig. 5.3, fits of equation (3) to the data using this value of k_r are satisfactory only at short times. Fits at longer times are poor, and the experimental data are consistently below the model predictions, indicating that some of the thrombin may be adsorbed and deactivated by the polystyrene surface. In fact it appears that thrombin is adsorbed to the polystyrene surface rapidly (Fig. 5.5, p. 118). The adsorbed thrombin could lose activity (Table 5.1) via conformational change or orientation of the active site such that it is not accessible to the substrate.

PEOMDA polyurethane

Fig. 5.6 (p. 119) shows the kinetics of release of pNA from the substrate at 37°C using wells coated with PEOMDA. It is seen that the amount of pNA released reached a common plateau value at about 40 minutes except at the lowest thrombin concentration (0.05 μg/ml or 0.16 unit/ml) where the release rate was slower. A value of $k_r = 0.69$ $(\mu g/ml)^{-1}(min)^{-1}$ (at 37°C) was estimated for PEOMDA by the method indicated above for polystyrene. As shown in Fig. 5.6, fits of equation (3) to the data using this value of k_r are good, indicating that there is no significant adsorption of thrombin to this surface in the time frame of the experiment. This result is consistent with the thrombin adsorption experiments (Chapter 4), in which it was found that the PEOMDA surface adsorbed only small amounts of thrombin over a 2 h period (see also Table 5.1). Even when the concentration of thrombin used in the chromogenic substrate assay is high, for example 2 μg/ml, the greatest amount of thrombin that can be adsorbed by the PEOMDA surface during the experiment (usually less than 1 h) is less than 2%. Accordingly, the change in thrombin activity due to adsorption onto the PEOMDA surface is negligible.

PPOMDA polyurethane

Like polystyrene, PPOMDA polyurethane is also hydrophobic, but with a different surface chemical composition. As reported in Chapter 4 (Fig. 4.5), approximately 0.04 $\mu g/cm^2$ of thrombin could be adsorbed to the PPOMDA surface. In Fig. 5.7 (p. 120) low thrombin concentration (0.05 - 0.33 μg/ml) was used; therefore a high percentage of solution thrombin could be adsorbed and

deactivated and not available to react with the substrate. As shown in Fig. 5.7, at low thrombin concentration (for example 0.05 μg/ml), the pNA concentration versus time curve leveled off at a relatively low OD value, suggesting that some of the thrombin was adsorbed and deactivated by the polymer surface.

PEOBDDS polyurethane

Fig. 5.8 (p. 121) shows pNA release from the substrate in PEOBDDS-coated wells at different thrombin concentrations. Plateau values were reached at all thrombin concentrations in about 30 min, and the plateau values increased with increasing thrombin concentration. This is in contrast to the PEOMDA data which converged to a single plateau value independent of thrombin concentration (Fig. 5.6).

Comparing the pNA curves at 0.33 and 0.05 μg/ml thrombin concentration shown in Fig. 5.8, it is seen that a much higher level of pNA was produced in the 0.33 μg/ml solution. In both cases pNA production reached a plateau at about 20 min, suggesting that either thrombin or substrate was completely consumed at this time. If it is assumed that in the 0.33 μg/ml solution, all of the substrate was converted to pNA, then in the 0.05 μg/ml solution the much lower plateau value of pNA presumably indicates that the substrate conversion is less than 10% and that a high substrate concentration is still available for reaction. It may then be concluded that at 0.05 μg/ml all of the thrombin is completely inhibited at 20 min by interaction with the PEOBDDS surface. Similar behaviour was observed for the PPOBDDS surface (data not shown).

The conclusion that PEOBDDS interacts strongly with thrombin appears

to contradict the findings of other research showing that PEO containing surfaces are inert to proteins (Chaikof et al., 1990; Coleman et al., 1982). However from the XPS data shown in Chapter 4, it is clear that, even under vacuum, BDDS moieties are exposed on the outermost surface of the PEOBDDS polyurethane, indicating that PEO does not cover the entire surface. Sulfonate groups that are highly reactive to proteins (Santerre et al., 1992) are present at the interface between water and the PEOBDDS material. Therefore thrombin is rapidly adsorbed and inactivated by the sulphonate groups exposed on the PEOBDDS surface.

In contrast to PEOBDDS, the PEOMDA surface shows no deactivation of thrombin in this experiment. This difference is presumably attributable to the differences in hard domain composition and hydrophilicity resulting from the different chain extenders. MDA is hydrophobic so that when the PEOMDA polymer is in contact with the thrombin solution, the hydrophobic hard domains will be depleted and the PEO domains will be enriched at the interface. Conversely for the PEOBDDS polymer, the BDDS-based hard domains may be more hydrophilic than the PEO-based soft domains, so that the PEO domains may be depleted at the interface. If it is accepted that PEO is a passivating influence in relation to protein interactions then the PEOMDA polymer should be less active than PEOBDDS as is implied by the data in Figs. 5.6 and 5.8.

PPACKPPO polyurethane.

Fig. 5.9 (p. 122) shows the production of pNA in wells coated with the PPACKPPO polymer. No thrombin activity was observed at initial thrombin concentrations less than 0.54 μg/ml, indicating that the PPACKPPO surface

inhibits most of the added thrombin under these conditions.

At higher thrombin concentrations there is clearly significant thrombin activity, suggesting that the amount of thrombin in solution exceeds the scavenging/neutralizing capacity of the surface-bound PPACK. At 0.54 μg/ml thrombin, a very low rate of production of pNA is observed. If it is hypothesized that on this surface thrombin interacts by binding irreversibly to PPACK sites in 1:1 stoichiometry, then the number of PPACK sites on the 1.55 cm^2 of well surface is equivalent to 0.108 (0.54 x 0.2) μg of thrombin, i.e. a surface concentration of 0.072 $\mu g/cm^2$, which is close to the adsorption capacity of the PPACKPPO surface for thrombin (Chapter 4).

At the higher thrombin concentrations the data suggest that, the PPACKPPO surface rapidly adsorbs thrombin from solution in an amount corresponding to saturation of the PPACK sites on the surface (i.e. about 0.072 $\mu g/cm^2$). Then in a slower process the remaining thrombin is inhibited, resulting in a gradual reduction in slope of the pNA versus time curve. A possible interpretation is that this thrombin is inhibited by adsorption to non-PPACK sites on the surface. Alternatively the thrombin could be inhibited by PPACK which is slowly released from the surface.

The substrate N-p-tosyl-gly-pro-arg p-nitroanilide has a high affinity for thrombin, and as a small peptide it can diffuse rapidly in solution. Therefore it is expected to react rapidly with thrombin in solution. PPACK also has high thrombin binding affinity, but because it is immobilized on the surface, thrombin binding is expected to be diffusion controlled initially. The fact that thrombin appears to be inhibited suggests that the high affinity of PPACK for thrombin is retained by the surface-bound PPACK, enabling it to compete effectively with

the substrate in solution.

From the data presented in this section it appears that thrombin-surface interactions depend strongly on surface composition. For the surfaces studied, the scavenging/inhibition propensity follows the order: PEOMDA < PPOMDA < polystyrene << PEOBDDS << PPACKPPO.

3. Kinetics of thrombin/surface interaction

As the data presented in the above section show, the activity of thrombin in solution as measured by the chromogenic substrate assay in multiwell plates can be influenced by the nature of the well surface. In this section the data are reexamined using a model which includes enzyme surface interactions as well as enzyme substrate interactions in solution.

Assuming that in the experiments discussed in the above section thrombin in solution reacts with both the substrate and binding sites on the polymer surface, and also assuming that the binding of thrombin to the surface irreversibly deactivates the enzyme, we can write:

$$T + S \xrightarrow{k_r} A + T \qquad (1)$$

$$T + B \xrightarrow{k_t} T_d \qquad (5)$$

where B is a surface-binding site, T_d is bound and inactivated thrombin, and k_t is the rate constant for surface binding. If S_o, T_o, and B_o are the initial concentrations of substrate, thrombin, and surface-binding sites respectively,

we can write:

$$A = S_o - S \tag{6}$$

$$T_d = T_o - T \tag{7}$$

$$B = B_o - T_d \tag{8}$$

Under this scheme, thrombin is consumed only by surface binding, with rate:

$$-\frac{dT}{dt} = k_t B\ T \tag{9}$$

For pNA generation, we have:

$$\frac{dA}{dt} = k_r T\ S \tag{2}$$

The solution to these equations is given by equation (10).

$$A = S_0\{1-(\frac{\exp(k_t(T_0 - B_0)t)-B_0/T_0}{1-B_0/T_0})^{-k_r/k_t}\} \tag{10}$$

If the surface does not bind thrombin, then $B_o = 0$, and equation (10) reduces to equation (3):

$$A = S_0\{1-\exp(-k_r T_0 t)\} \tag{3}$$

Equation (10) was used to fit the experimental data obtained from the

chromogenic substrate assays for the surfaces where it was clear that some scavenging/deactivation was occurring. Fig 5.10 (p. 123) and Fig. 5.11 (p. 124) show the data and the fitted curves for PPOMDA and PEOBDDS respectively. For most of the conditions investigated the fit is good. The parameter estimates from the data fits are listed in Table 5.3 and show that the surface binding reaction is significant for PPOMDA and PEOBDDS.

It is clear from Fig 5.11 that the slow inhibition of thrombin by the PEOBDDS surface stopped the production of pNA at about 20 min, resulting in different plateau values for the pNA versus time curves. This behaviour is in contrast to the rapid inhibition of thrombin by the PPACKPPO polyurethane which stopped the production of pNA sooner (Fig. 5.9).

Table 5.3. Parameters used for fit of pNA production using equation (10), 37°C.

	PPOMDA	PEOBDDS
k_r $(\mu g/ml)^{-1}(min)^{-1}$	0.69	0.65
k_t $(\mu g/ml)^{-1}(min)^{-1}$	0.01	0.35
B_0 $(\mu g/ml)$	0.8	0.8

Note: Parameters were obtained by fitting equation (10) to the experimental data using Mathcad software. Parameter estimation was initiated using the parameters estimated using equation (4). For each polymer, a set of 600 experimental data points was used, and the sum of squared errors (SSE) was minimized. From 5 sets of experimental data, k_r (at 37°C) was found to be 0.65 $(\mu g/ml)^{-1}(min)^{-1}$ (sd=± 0.036, n=5).

The fit of equation (10) to the experiment data also gives calculated value of thrombin concentration. Using the value of k_t [0.35 $(\mu g/ml)^{-1}(min)^{-1}$] and B_0 (0.8 µg/ml) obtained from the data fit for PEOBDDS, the concentration T of

active thrombin can be calculated by the integrated form of equation (6). The concentration of deactivated thrombin (T_o-T) can then be estimated. Plots of T_o-T as a function of time are shown in Fig. 5.12 (p. 125). In Table 5.1, it is shown that most of the thrombin adsorbed on the polymer surfaces is deactivated except in the case of PEOBDDS. If we assume that during the chromogenic assay experiment, the adsorption of thrombin on the polymer surfaces is the only way that thrombin is deactivated, then deactivation as measured by the chromogenic substrate method can be considered as equivalent to adsorption.

In Fig. 5.13 (p. 126), the thrombin adsorption data obtained by radio-labeling (data points) are compared with the data (shown as lines) derived from the chromogenic substrate measurements as discussed above for PPOBDDS and polystyrene. At 5 min, thrombin adsorption on the polystyrene surface as measured by radio-labeling is a factor of four greater than thrombin deactivation as measured by the chromogenic substrate method. At longer times, adsorption and deactivation are similar.

On the PPOBDDS surface it is also seen that the amounts of adsorbed thrombin measured by the radio-labeling method are about 10 to 20% more than the amounts of deactivated thrombin measured by the chromogenic method. This finding is in agreement with the data in Table 5.1 showing that some of the adsorbed thrombin retains its activity on PPOBDDS.

It should be noted that the kinetic scheme [equations (1) - (10)] is a simple model of conventional chemical kinetics. Although the fit of equations (3) and (10) to the experiment data is good, it reflects only the macroscopic kinetic behaviour of thrombin, lacking detailed information about the molecular

processes such as complex formation and thrombin-surface binding. As pointed out by Izquierdo et al. (1987), much complicated reaction mechanism may be involved. Therefore, it is very desirable if a research can be done to develop an enzyme kinetic scheme to describe precisely thrombin reaction so that a solid and clear understanding of thrombin-polymer interaction can be obtained. Unfortunately, it is beyond the scope of this research.

4. Release of PPACK into solution from PPACKPPO surface

The primary objective of this project with respect to PPACK was to investigate the concept of a thrombin scavenging biomaterial. While originally it was assumed that the PPACK would remain attached to the surface, there was always the possibility that some of the immobilized PPACK might be released slowly from the surface and function as a thrombin scavenger in the fluid phase. Since PPACK is a potent inhibitor of thrombin, very small amounts (nano mol range) can have a significant effect on coagulation. With a load of 1 µmol PPACK in the polymer film and a release rate of 1 nmol per h, the polymer surface could maintain an effective PPACK concentration for 1000 h or more than one month. With this in mind the possible slow release of PPACK from the PPACKPPO polyurethane was investigated.

Release of ^{125}I YPACK from YPACKPPO polymer

Since it can be radio-iodinated, YPACKPPO polyurethane was used as a model to study the release of PPACK from PPACKPPO. Thus ^{125}I-labeled YPACK was used instead of PPACK to synthesize a labeled YPACKPPO polymer.

TBS buffer (0.2 ml) was added to ^{125}I-YPACKPPO-coated wells of microtitre plates, and aliquots of 10 µl were taken over time for measurement of radioactivity. The amount of YPACK released into solution was thus determined. The initial amount of YPACK on the 1.55 cm^2 of well surface was found to be $1.95X10^{-9}$ mol. This amount of YPACK is equivalent to $6.66X10^{-5}$ g thrombin and gives an indication of the potential of the surface for thrombin inhibition. As shown in Fig. 5.14 (p. 127), about 2% or $3.7X10^{-11}$ mol YPACK was released from the surface in 1 h. After 1 day (1500 min), as much as 8% of the YPACK was released.

The release rate of YPACK into albumin solution (1.3 µg/ml), thrombin solution (1.3 µg/ml), and plasma was also studied (data not shown). Identical data to those shown in Fig. 5.14 were found in all such experiments, suggesting that thrombin and other proteins binding to the YPACKPPO surface does not influence YPACK leaching. As shown in Fig. 5.14, the amount of YPACK released during the first few hours is linear with time. The initial release rate can be estimated at about $3.7X10^{-11}$ mol YPACK per h.

Inhibition of thrombin by released and surface-bound PPACK

As discussed above PPACKPPO-coated surfaces appear to contain both surface-grafted PPACK and free PPACK which can leach into the solution contacting the surface, and both the free and bound PPACK can inhibit thrombin effectively. It was of interest to distinguish the contributions of free and bound PPACK to the inhibition of thrombin by the PPACKPPO surface and the following experiments were designed for this purpose.

To the wells of a microtitre plate coated with the PPACKPPO polymer

(1.55 cm^2 of surface), 200 µl TBS buffer was added and incubated at room temperature for 1 h to allow the free PPACK to leach into the solution. This resulted in a system (free PPACK-PPACKPPO system) having both free PPACK in solution and surface-bound PPACK on the walls of the well. Half of the supernatant (100 µl) was then transferred to a polystyrene coated well to create a second system which had free, but not surface-bound PPACK (free PPACK-polystyrene system). In addition, to vary the amounts of released and surface-bound PPACK, different amounts of PPACKPPO polymer were coated onto the well surfaces by using different concentrations of the polymer solution.

To the free PPACK-polystyrene system, thrombin and thrombin substrate (N-p-tosyl-gly-pro-arg p-nitroanilide) were added [final concentration of thrombin, 1.33 µg/ml (0.037 µM); final concentration of substrate, 0.06 mg/ml] and the production of pNA by thrombin was measured. The apparent initial thrombin activity was estimated from the initial slope of the pNA versus time curve using equation (3) to fit the data.

Fig. 5.15 (p. 128) shows the data for three sets of measurements using the supernatant from wells coated using PPACKPPO polymer solutions of 0.46, 1.37, and 4.12 µg/ml respectively. For the 4.12 µg/ml coating, the "measured" thrombin concentration was reduced from the original 1.33 µg/ml to 0.03 µg/ml. It may be concluded that the PPACK released from the surface during 1 h incubation inhibited essentially all of the added thrombin (1.33 µg/ml). On an equimolar basis this corresponds to a free PPACK concentration of 16.4 ng/ml. For the 0.46 µg/ml coating, the measured thrombin concentration was reduced from the original 1.33 µg/ml to 1.2 µg/ml. Therefore it appears that the PPACK released from the surface during 1 h incubation inhibited only 0.13 µg/ml

thrombin, corresponding to a free PPACK concentration of 1.64 ng/ml.

The data for the analogous experiment in which the solution remained in the PPACKPPO coated wells (free PPACK-PPACKPPO system) are shown in Fig. 5.16 (p. 129). Comparing the data in Figs 5.16 and 5.15, it is clear that the system containing surface-bound as well as free PPACK has higher PPACK activity than the system containing free PPACK alone. For example, for the 0.46 μg/ml coating the measured thrombin concentration in the polystyrene wells is 1.2 μg/ml (Fig. 5.15), while in the PPACKPPO wells it is only 0.66 μg/ml (Fig. 5.16). This result suggests that the surface-bound PPACK contributes significantly to thrombin inhibition.

The data from these two experiments are shown in Table 5.4. Comparing columns 2 and 5 (0.46 μg/ml coating solution), it is seen that the PPACKPPO surface has total PPACK activity about 5 times that of the PPACK released into the solution in 60 min. Presumably the higher activity in the case of the PPACKPPO surface is associated with surface-bound PPACK. The data indicate that the active PPACK on the PPACKPPO surface has a thrombin inhibitory capability of 1.93 pmol or 0.069 $\mu g/cm^2$ (compare the data in Fig. 5.9, 0.072 $\mu g/cm^2$, p. 102). As the concentration of the polymer coating solution increases the amounts of released PPACK increase. When the concentration of released PPACK is high enough, all of the thrombin is inhibited in both free PPACK-polystyrene system and free PACK-PPACKPPO system. Thus the advantage of surface-bound thrombin is not observed and the measured amounts of "extra" PPACK appears to "decrease".

It should be noted that a thrombin molecule will occupy a relatively large surface area compared to PPACK, and one adsorbed thrombin molecule will

potentially cover many PPACK sites. The actual amounts of surface-bound PPACK are thus probably larger than suggested by the data in Table 5.4. In addition, a surface concentration of thrombin of 0.069 $\mu g/cm^2$ is close to the monolayer coverage (about 1 $\mu g/cm^2$ for side-on adsorption of thrombin) which is approximately the expected maximum thrombin scavenging capacity of the surface.

Table 5.4 The thrombin-inhibiting activity of free (released from surface) and bound PPACK (60 min incubation).

Well surface	Polystyrene			PPACKPPO		
Concentration of PPACKPPO coating solution (μg/ml)	0.46	1.37	4.12	0.46	1.37	4.12
Active thrombin (μg/ml)	1.2	0.57	0.03	0.66	0.18	0
Active PPACK (ng/ml)	1.64	9.56	16.4	8.43	14.1	16.7
"Extra" PPACK (ng/ml)	-	-	-	6.79	4.54	0.3

It should be noted from Figs 5.15 and 5.16 that equation (3) fits the experimental data only over the first 10 min or so of the reaction. The production of pNA gradually levels off, suggesting a slow release of PPACK into the solution and consequently a slow inhibition of thrombin, or a slow adsorption of thrombin to the polymer surfaces. This is similar to the deactivation of thrombin by the PEOBDDS surface shown in Fig. 5.8.

To summarize the results of these experiments, it appears that the PPACKPPO polyurethane contains both surface-bound PPACK and releasable PPACK. Both forms appear to retain thrombin-inhibitory activity over relatively

long times. It seems possible that the surface-bound PPACK may inhibit activation of coagulation by scavenging initially generated thrombin. Releasable PPACK may be able to inhibit additional thrombin.

Summary

It has been shown that PPACK grafted to and releasable from a polyurethane surface retains its ability to inhibit thrombin, and that it can effectively compete with the chromogenic substrate N-p-tosyl-gly-pro-arg p-nitroanilide which also has high specificity for thrombin binding. Thrombin adsorbed onto the PPACK polyurethane surface is almost completely inhibited. In contrast, thrombin adsorbed to other materials such as PEOBDDS and PPOBDDS shows significant retention of activity. It seems possible that when contacted with blood, the BDDS polymers will adsorb many different plasma proteins with the potential to initiate multiple biological responses. On the other hand, the PPACK polymer due to its specificity for thrombin binding, may show a more specific thrombin scavenging response. It may be possible also to improve the PPACK-type polyurethanes by using PEO instead of PPO as the soft segment in order to decrease non specific protein adsorption. An ideal thrombin scavenging surface should bind only thrombin while remaining inert to other plasma proteins.

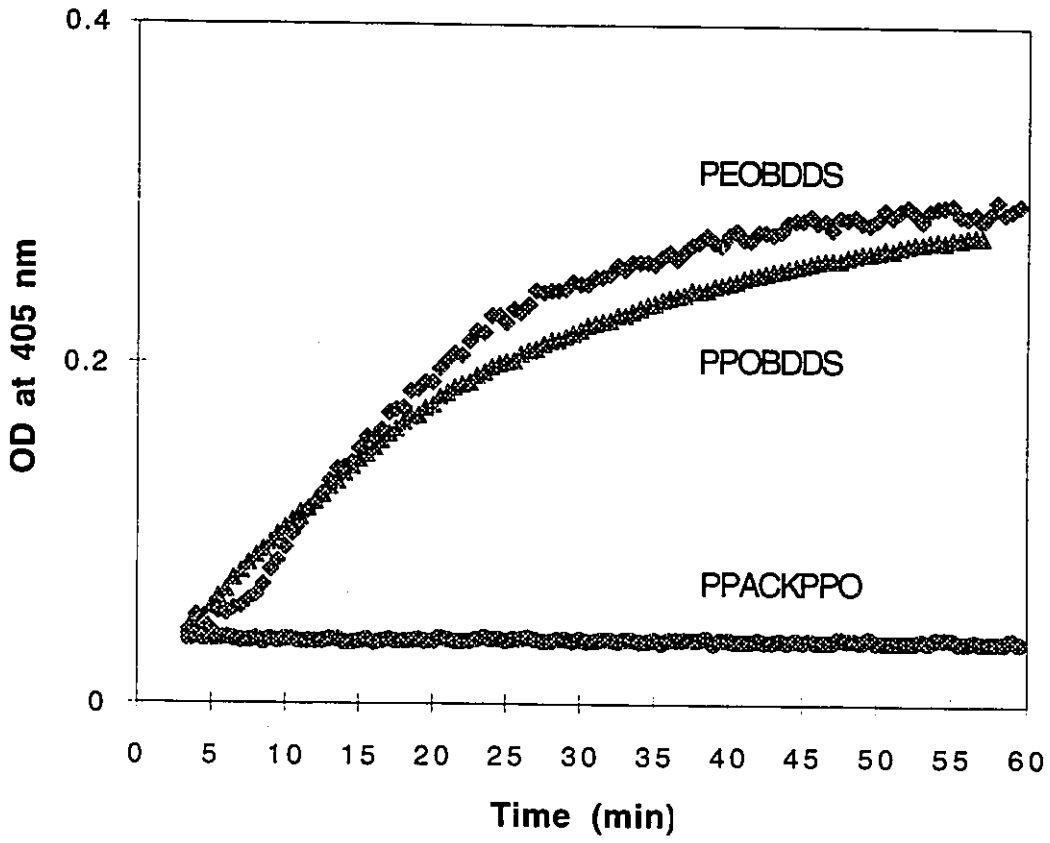


Fig. 5.1 p-Nitroaniline released from N-p-tosyl-gly-pro-arg p-nitroanilide by thrombin adsorbed on different polyurethane surfaces, measured by a plate reader at 405 nm and 37°C. The wells of the microtitre plate were coated with the polyurethanes. Thrombin solution was added to the wells and incubated for 2 h at room temperature. After rinsing with TBS, the thrombin remaining on the well surface was reacted with the substrate.

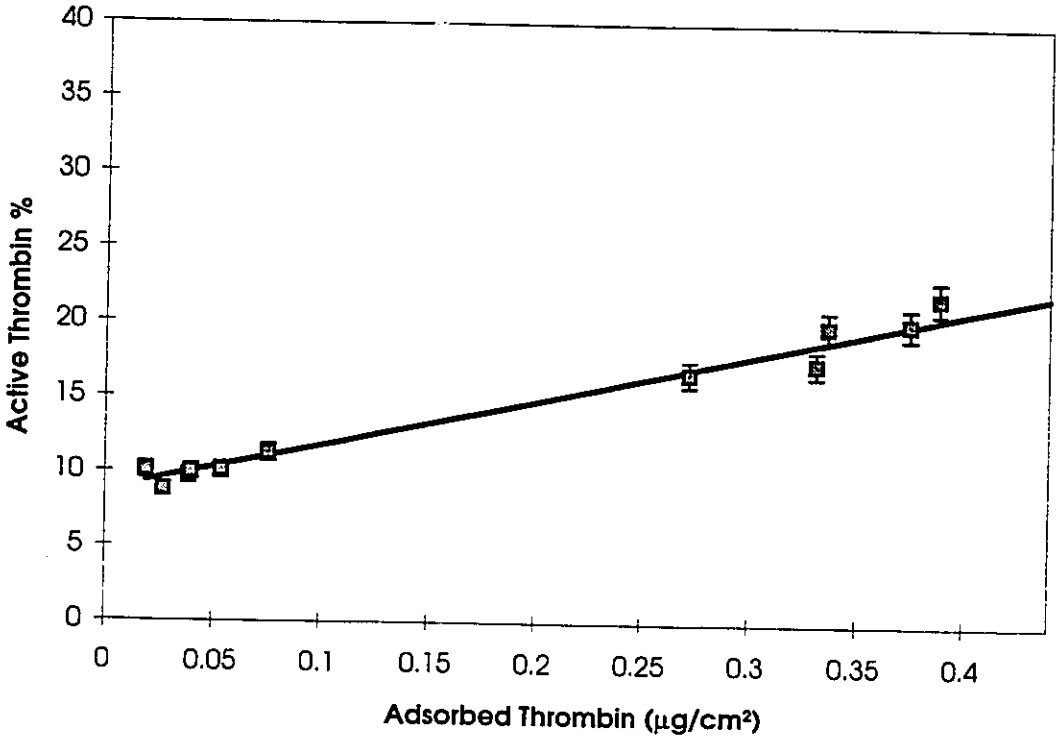


Fig. 5.2 The activity of thrombin adsorbed on PPOBDDS polyurethane surface versus surface concentration. Error bars: ±SD, n>5.

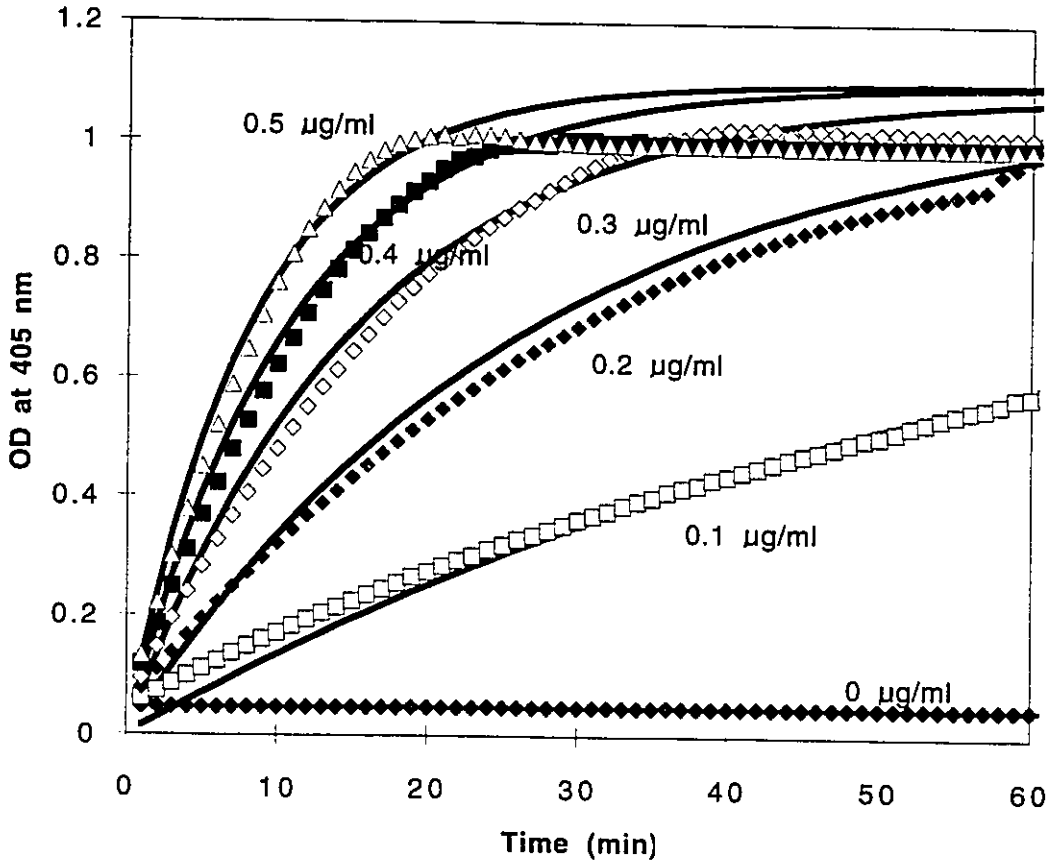


Fig. 5.3 p-Nitroaniline released from N-p-tosyl-gly-pro-arg-pNA by thrombin in TBS buffer, measured by plate reader at 405 nm at 22°C. The wells of the microtiter plate were of unmodified polystyrene. The concentration of thrombin is indicated on the curves. The solid lines are fits of the data to equation (3) with $k_f = 0.24\ (\mu g/ml)^{-1}min^{-1}$.

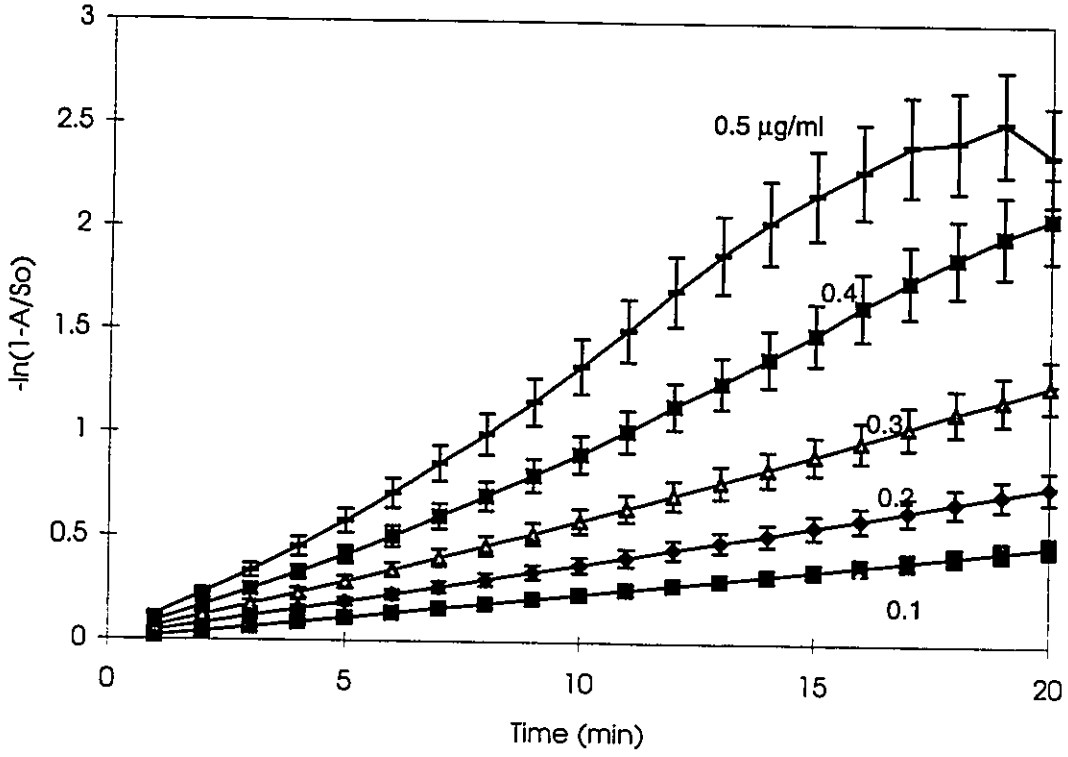


Fig. 5.4a - $\ln(1-A/S_0)$ versus time for polystyrene surface plotted with the data of Fig. 5.3. The concentration of thrombin is indicated on the curves. Error bars: ±SD, n>5.

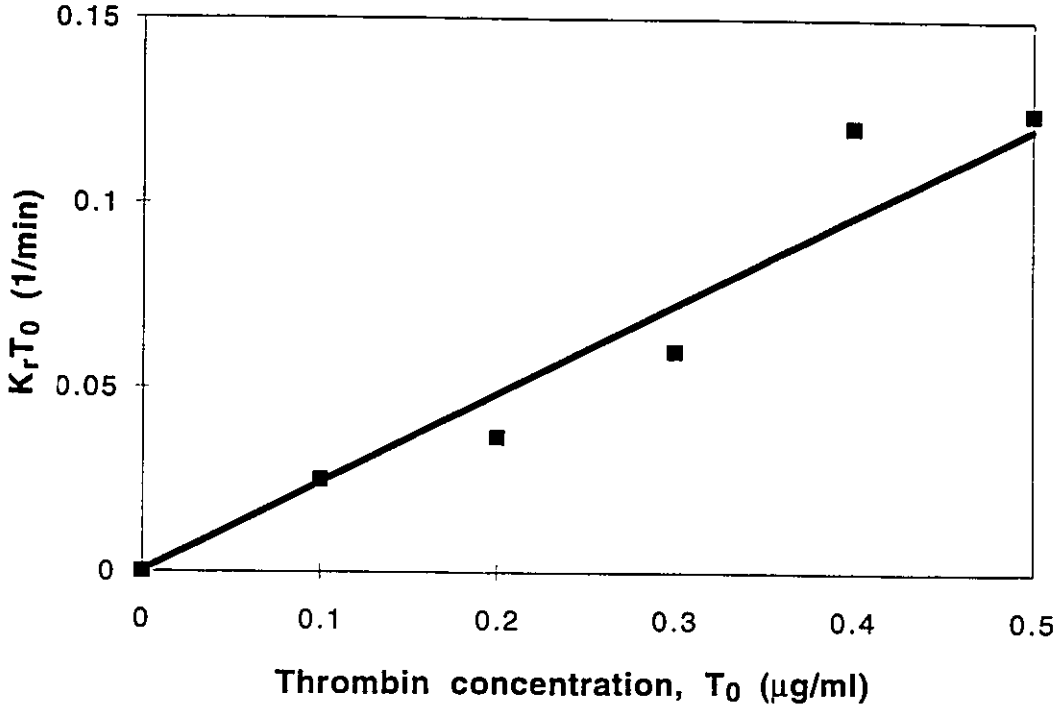


Fig. 5.4b k_rT_0 versus thrombin concentration (T_0) for polystyrene surface, 22°C.

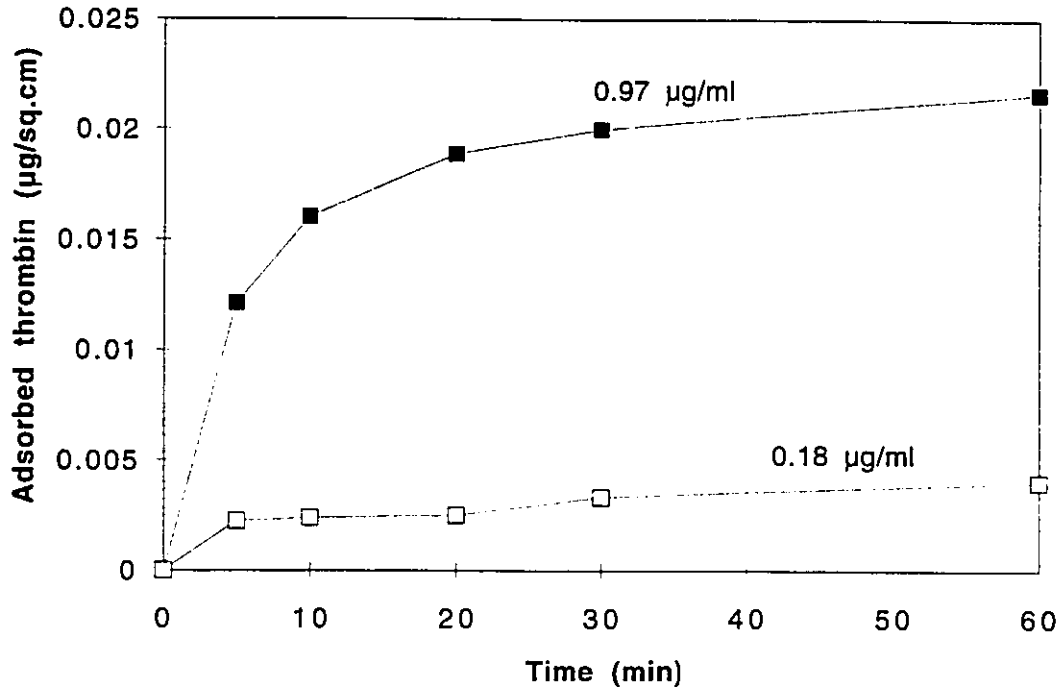


Fig. 5.5 Thrombin adsorption to polystyrene surface from TBS, 22°C, radio-labeling method. Total volume of thrombin solution is 0.2 ml, polystyrene surface area is 1.55 cm^2. The concentration of thrombin is indicated on the curves.

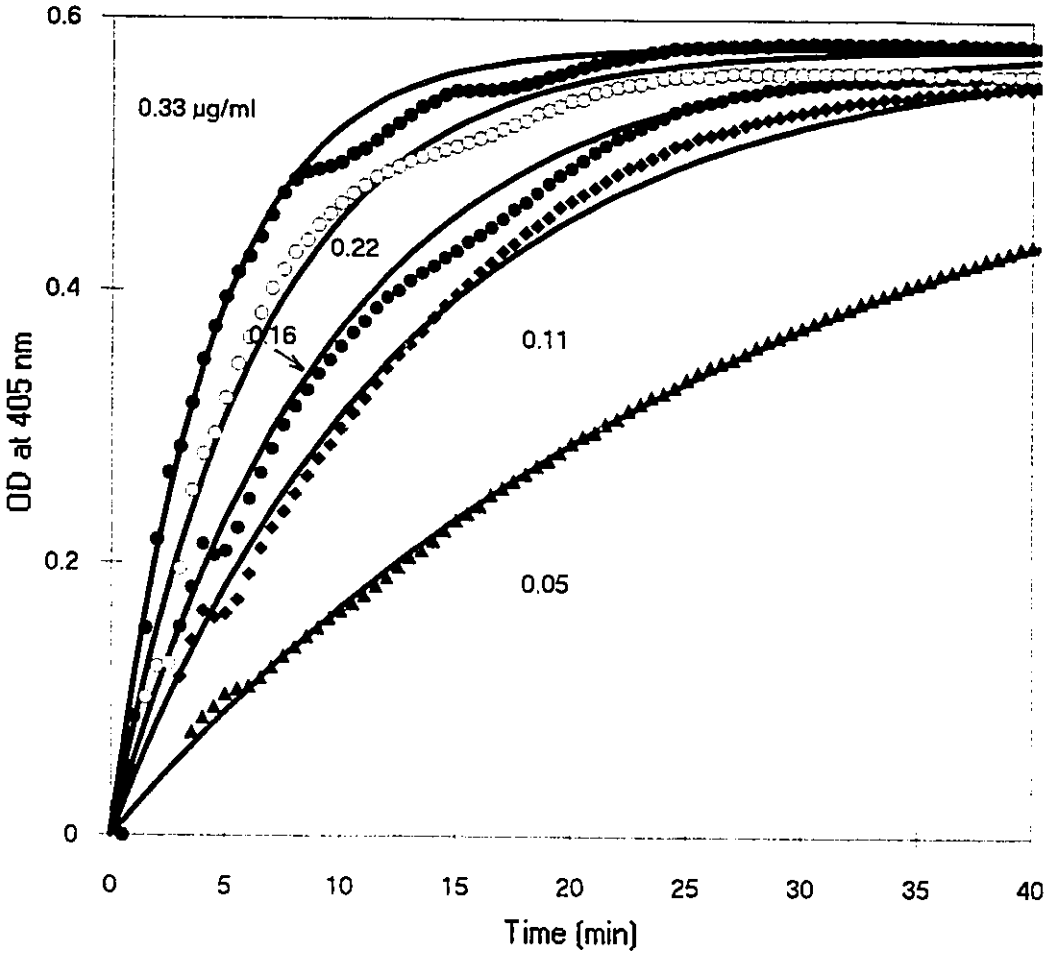


Fig. 5.6 p-Nitroaniline released from N-p-tosyl-gly-pro-arg p-nitroanilide by thrombin in TBS buffer, measured by plate reader at 405 nm and 37°C. The wells of the microtitre plate were coated with PEOMDA polyurethane. The concentration of thrombin is shown adjacent to the corresponding curves. The solid lines are fits of the data to equation (3) with $k_r = 0.69$ $(\mu g/ml)^{-1}min^{-1}$ (37°C).

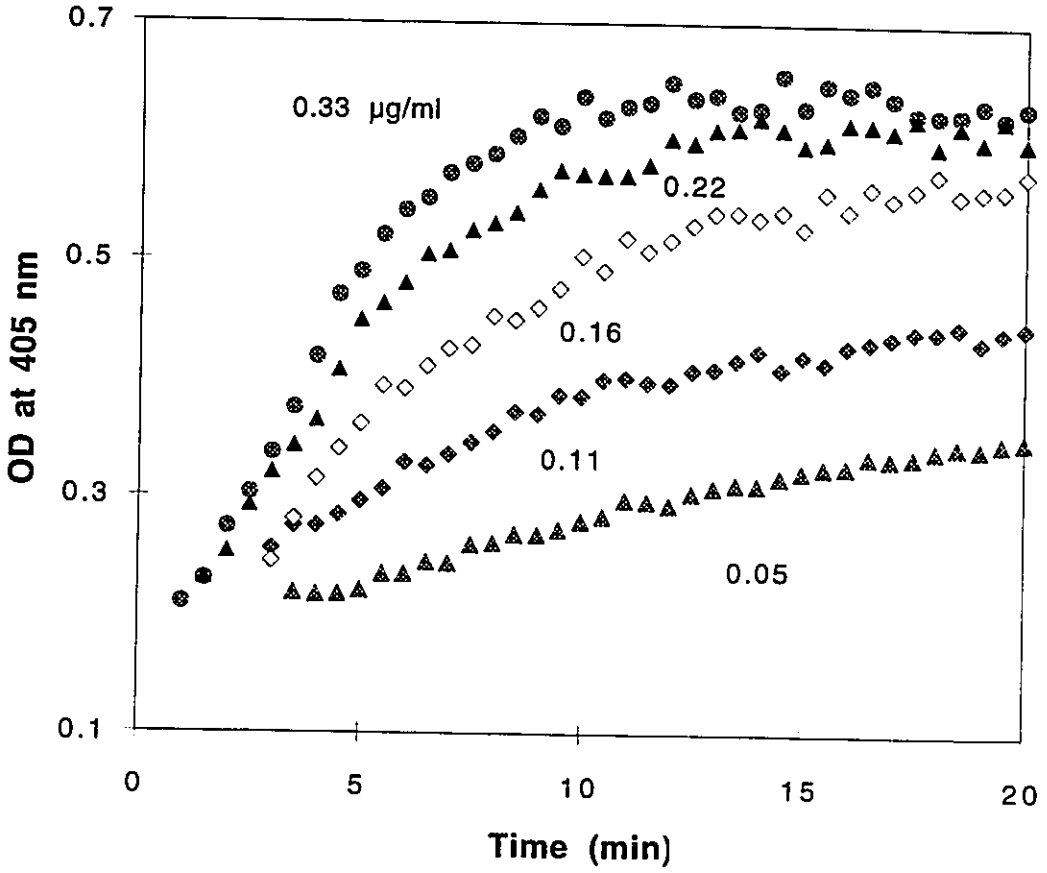


Fig. 5.7 p-Nitroaniline released from N-p-tosyl-gly-pro-arg p-nitroanilide by thrombin in TBS buffer, measured by plate reader at 405 nm and 37°C. The wells of the microtitre plate were coated with PPOMDA polyurethane. The concentration of thrombin is indicated on the curves.

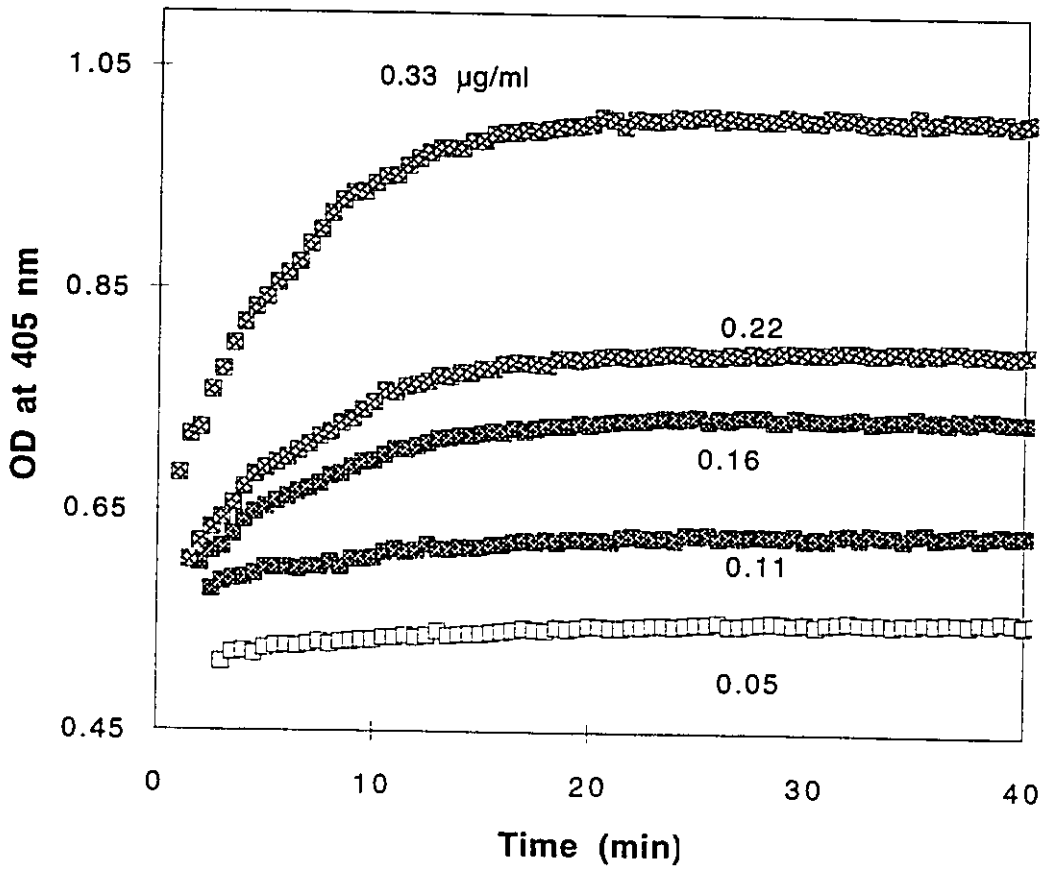


Fig. 5.8 p-Nitroaniline released from N-p-tosyl-gly-pro-arg p-nitroanilide by thrombin in TBS buffer, measured by plate reader at 405 nm and 37°C. The wells of the microtitre plate were coated with PEOBDDS polyurethane. The concentration of thrombin is indicated on the curves.

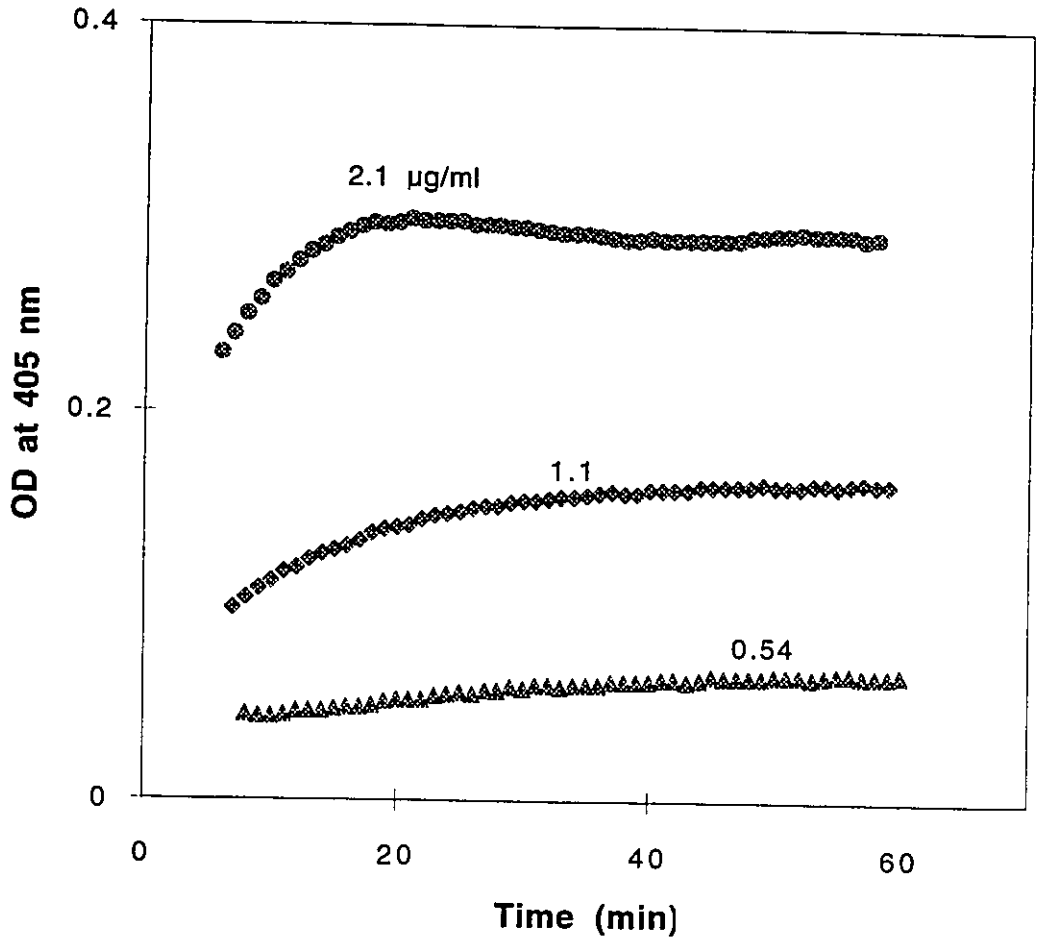


Fig. 5.9 p-Nitroaniline released from N-p-tosyl-gly-pro-arg p-nitroanilide by thrombin in TBS buffer, measured by plate reader at 405 nm and 37°C. The wells of the microtitre plate were coated with PPACKPPO polyurethane. The concentration of thrombin is indicated on the curves.

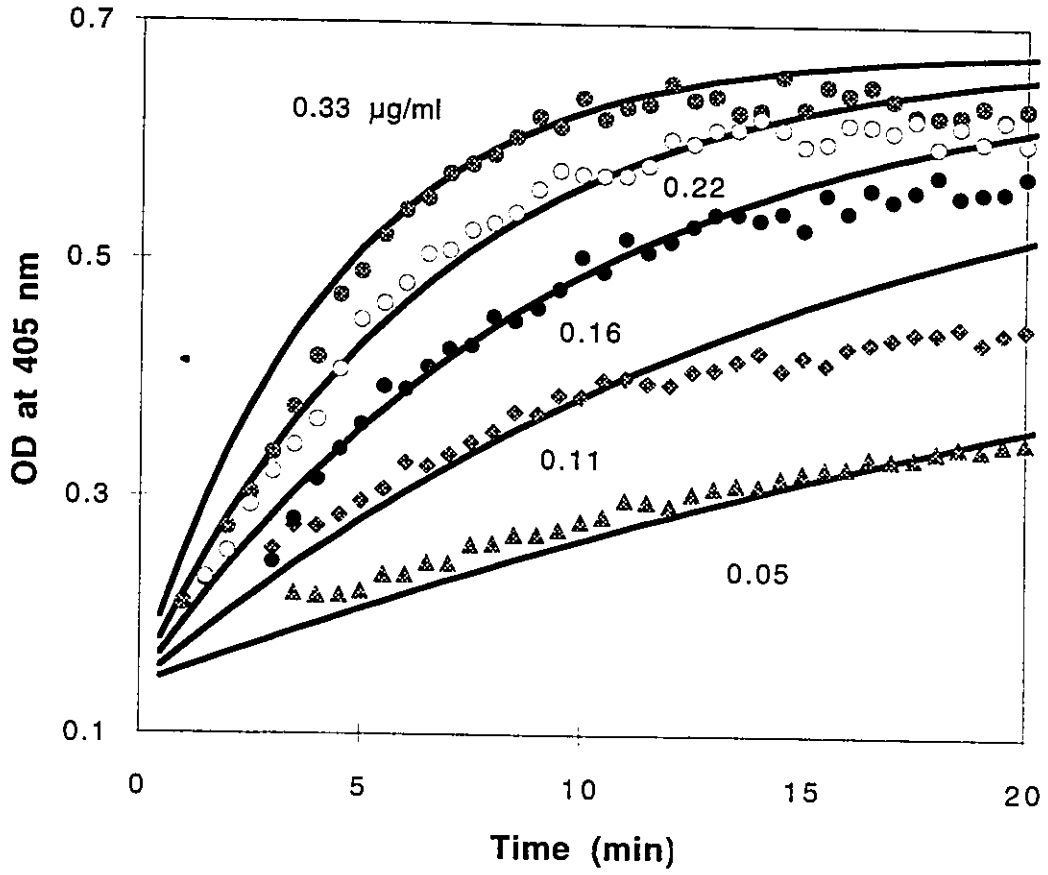


Fig. 5.10 p-Nitroaniline released from N-p-tosyl-gly-pro-arg p-nitroanilide by thrombin in TBS buffer, measured by plate reader at 405 nm and 37°C. The wells of the microtitre plate were coated with PPOMDA polyurethane. The concentration of thrombin is indicated on the curves. The solid lines are fits of the data to equation (10) with $k_r = 0.69\ (\mu g/ml)^{-1}min^{-1}$.

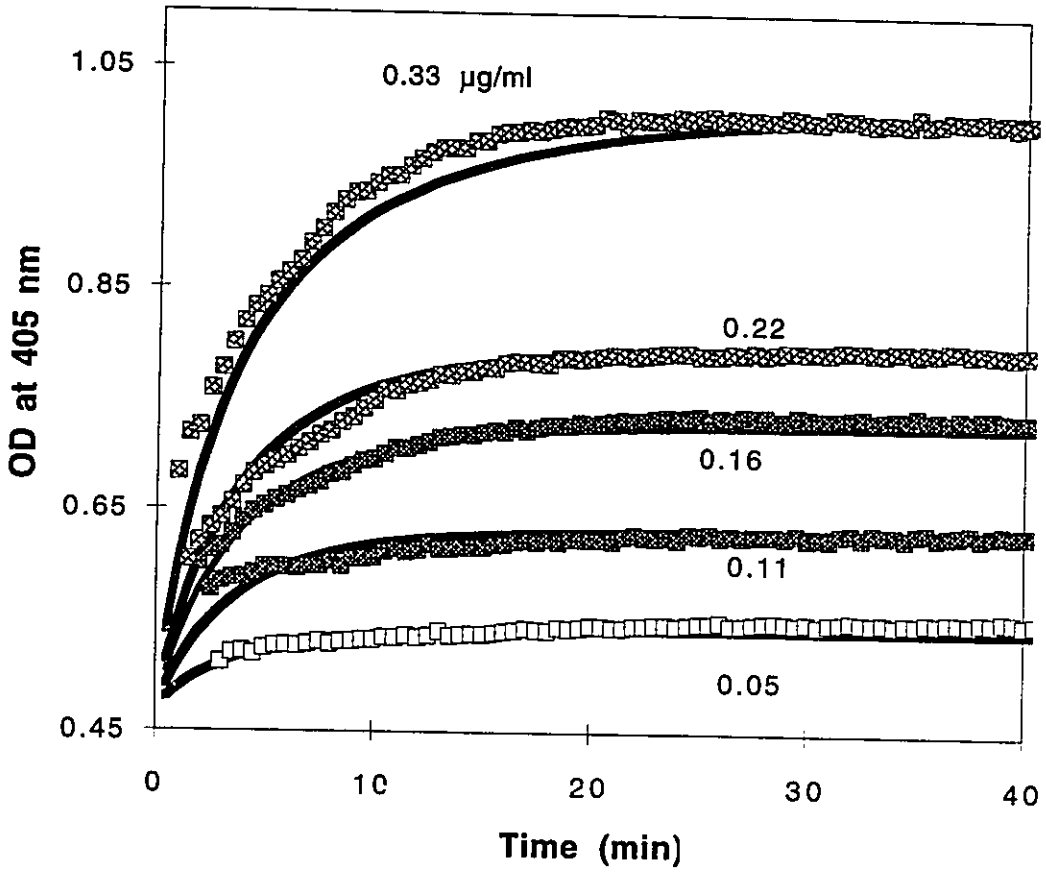


Fig. 5.11 p-Nitroaniline released from N-p-tosyl-gly-pro-arg p-nitroanilide by thrombin in TBS buffer, measured by plate reader at 405 nm and 37°C. The wells of the microtitre plate were coated with PEOBDDS polyurethane. The concentration of thrombin is indicated on the curves. The solid lines are fits of the data to equation (10) with $k_r = 0.65\ (\mu g/ml)^{-1}min^{-1}$.

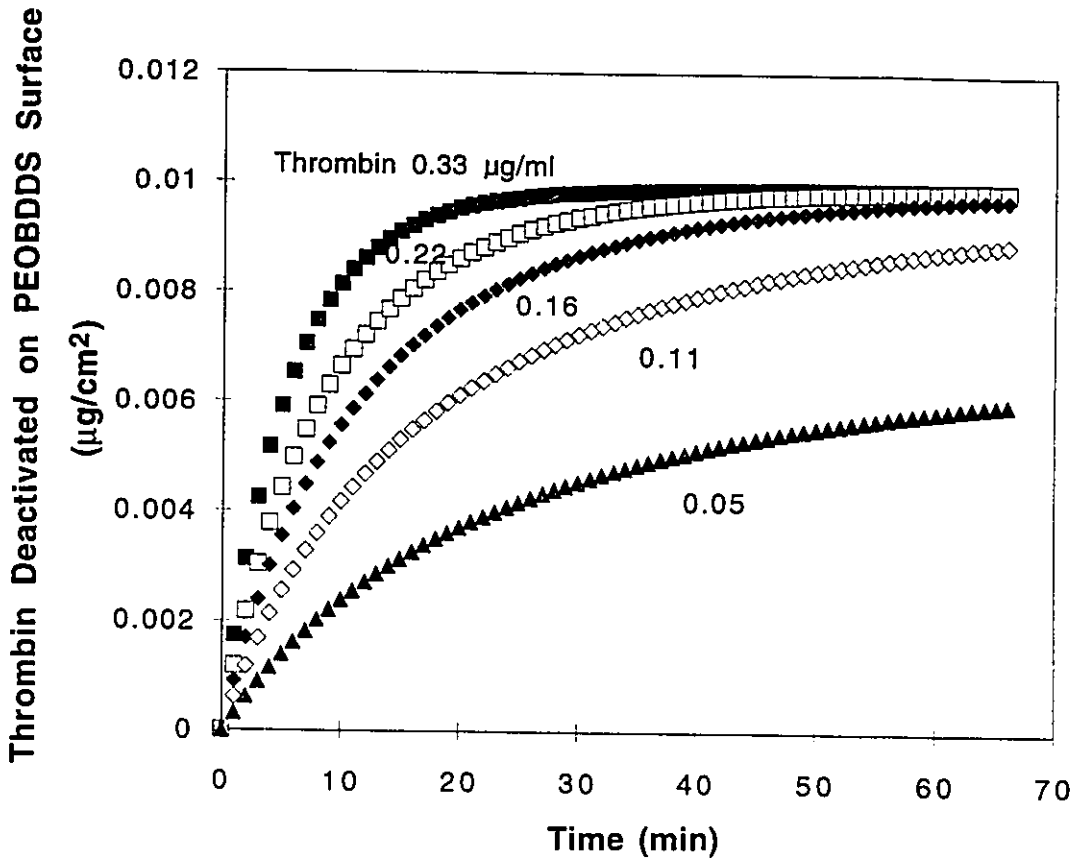


Fig. 5.12 Deactivation of thrombin on PEOBDDS polyurethane surface from TBS buffer, 37°C, measured by plate reader using a chromogenic substrate of thrombin. As discussed in the text, deactivation and adsorption may be considered equivalent for this surface.

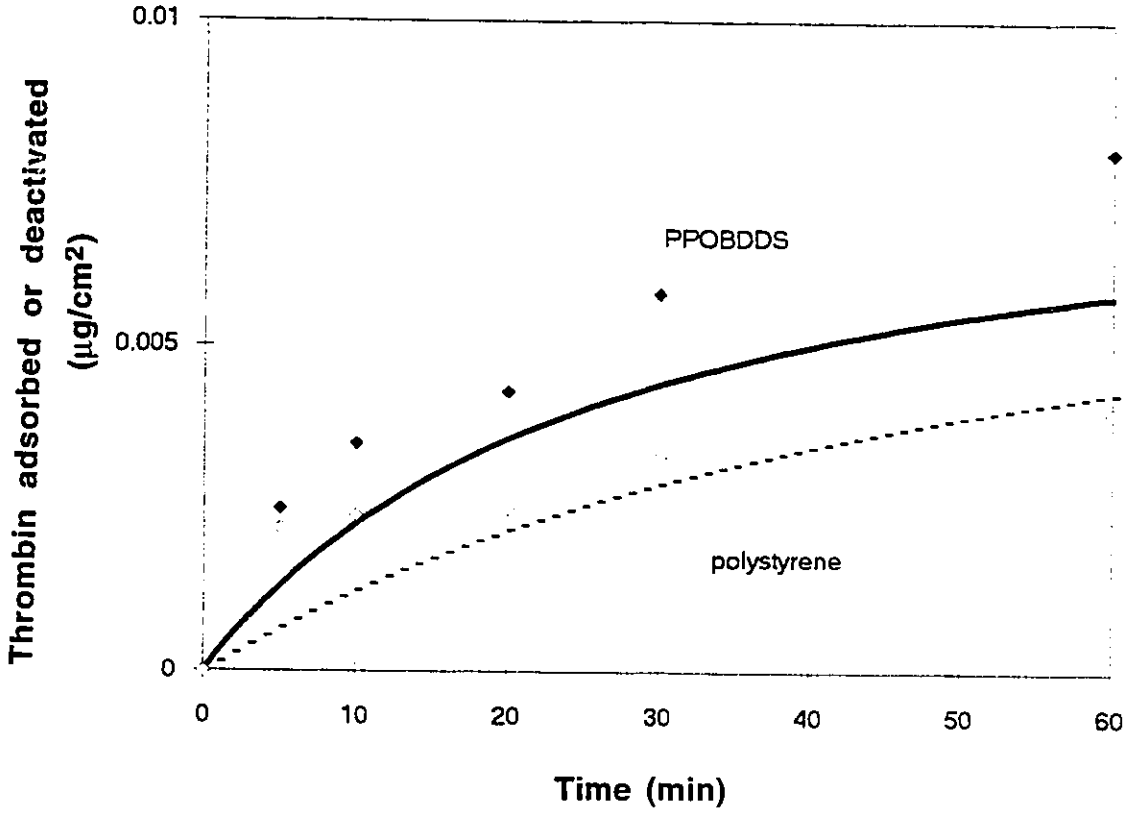


Fig. 5.13 Thrombin adsorption and deactivation on PPOBDDS and polystyrene. (____): Deactivation of thrombin as measured by the chromogenic substrate method on PPOBDDS; (.....): Deactivation of thrombin as measured by the chromogenic substrate method on polystyrene; (♦): Adsorption of thrombin on PPOBDDS surface measured by ^{125}I radio-labeling method; (◊) Adsorption of thrombin on polystyrene surface measured by ^{125}I radio-labeling method. Thrombin concentration in TBS buffer, 0.22 μg/ml; temperature, 22°C.

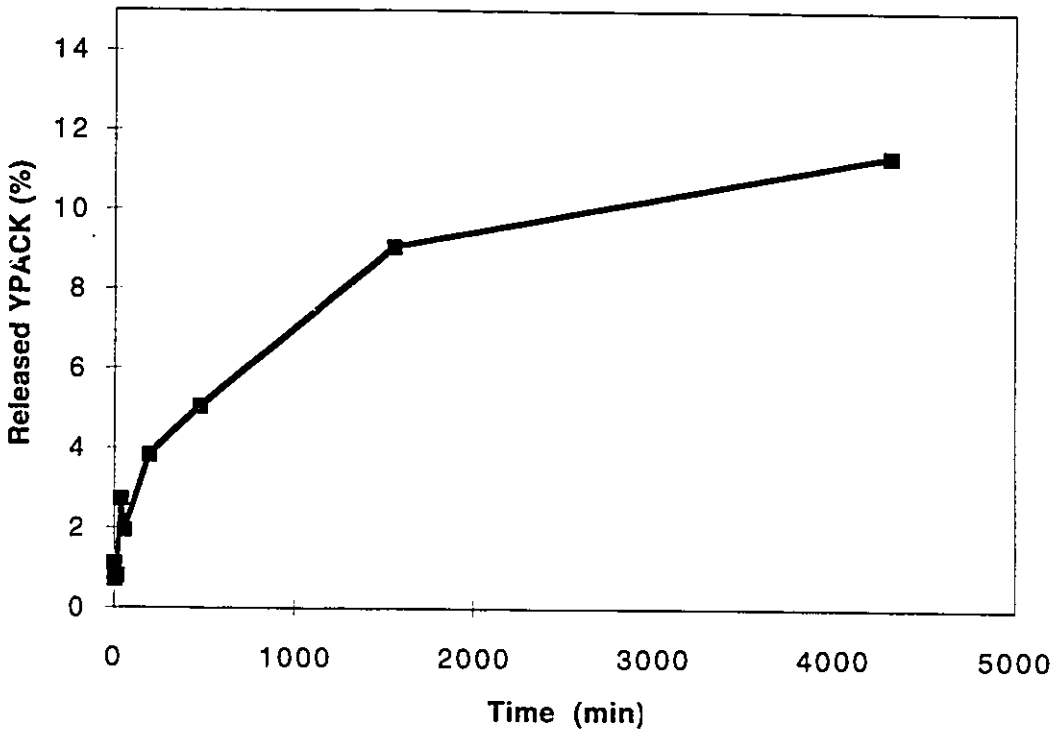


Fig. 5.14 YPACK released from YPACKPPO coated well into TBS buffer, measured by radio-labeling method, 22°C. Each data point shown is the average of 10 to 12 measurements, SD < 1.07, n > 9.

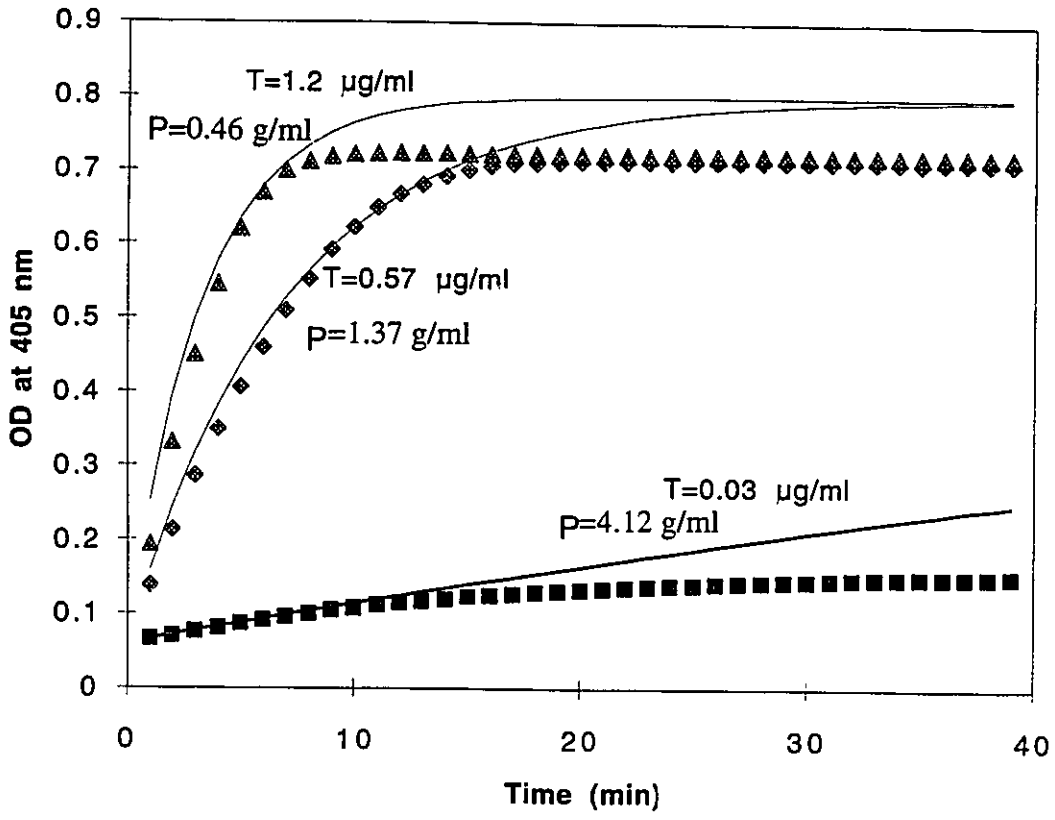


Fig. 5.15 Thrombin activity in TBS buffer which has been incubated in a PPACKPPO-coated microtitre well for 1 h, then transferred to a polystyrene-coated well. The concentration (P) of the PPACKPPO/DMSO solution used to coat the wells is indicated adjacent to the curves (g/ml). The solid lines are fits of the data to equation (3) with k_r = 0.24 $(\mu g/ml)^{-1}min^{-1}$ (22°C), and are used to estimate the initial concentration of thrombin (T), shown adjacent to the curves.

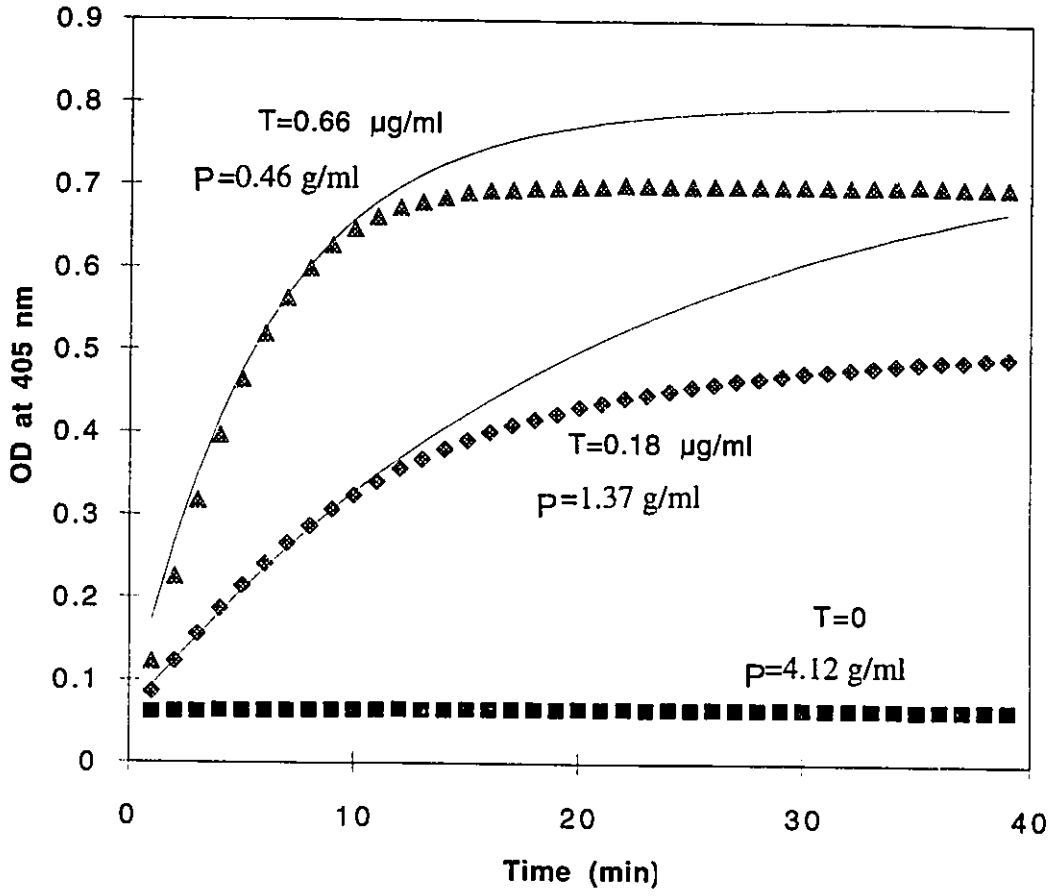


Fig. 5.16 Thrombin activity in TBS buffer which has been incubated in a PPACKPPO-coated microtitre well for 1 h, measured directly in the PPACKPPO coated well. The concentration (P) of the PPACKPPO/DMSO solution used to coat the wells is indicated adjacent to the curves (g/ml). The solid lines are fits of the data to equation (3) with k_r = 0.24 $(\mu g/ml)^{-1}min^{-1}$ (22°C), and are used to estimate the initial concentration of thrombin (T), shown adjacent to the curves.

CHAPTER 6
PROTEIN LAYER DEPOSITED ON POLYURETHANE SURFACES FROM PLASMA: ANALYSIS USING IMMUNOBLOTTING METHOD

6.1 INTRODUCTION

As discussed in Chapters 4 and 5, it was found that thrombin adsorption on polyurethane surfaces as well as the activity of the adsorbed thrombin are influenced by surface-bound PPACK, PEO and sulphonate groups. Immobilized PPACK shows thrombin scavenging activity unique in that it specifically adsorbs and inhibits thrombin. Incorporated PEO was found to reduce albumin and thrombin adsorption, while the immobilized sulphonate groups showed very high affinities for these proteins.

Another important aspect of protein-surface interaction is the formation of a protein layer on the polymer surface in contact with blood. The formation and the composition of this protein layer as well as the enzymatic activities of the proteins depend on the chemistry of the polyurethane surface. Analysis of this protein layer is therefore valuable for this work in order to study the influences of surface-bound functional groups PPACK, sulphonate and PEO on the composition of the protein layer, and subsequently to form a basis for understanding the protein activation mechanisms at the plasma-polymer interfaces (to be discussed in Chapters 7 and 8).

In Chapters 4 and 5, radiolabeling and chromogenic substrate methods were used to study protein-surface interactions in purified protein systems. For direct analysis of blood-surface interactions, electrophoresis and immunoblotting methods have been developed in this laboratory and valuable information on the composition of the protein layer deposited on polymer surfaces has been obtained (Mulzer and Brash, 1989; Mulzer and Brash, 1990; Cornelius and Brash, 1993). In this work, an immunoblotting method was used to examine the influences of surface-bound functional groups on the composition and activation of the protein layer deposited from plasma on polyurethanes. The immunoassay data are only semiquantitative due to the limitations of the methods for development of color and estimation of band intensities using a densitometer. The discussions presented below should be viewed in this light.

6.2 EXPERIMENTAL

Materials

The following polyurethanes were utilized in this study: PPOMDA, PPOBDDS and PPACKPPO. Their synthesis and molecular structure were discussed in Chapter 4. In this work, polyurethanes were coated onto polystyrene beads, and the beads were then used for protein adsorption studies. The coating method is as follows: In a polyethylene tube, polystyrene beads (200-400 mesh, 50 μm diameter, surface/weight 0.2 m^2/g, BioRad, Richmond, CA) were immersed in a polyurethane/DMSO solution (concentration 2% w/w) and placed in an ultrasonic bath for 5 min. Then the tube was centrifuged, the supernatant removed, and the beads washed with water 10 times. Finally, the polyurethane-coated beads were dried in a vacuum oven at room temperature

for 24 h. The beads were examined under a microscope before and after the coating; neither shape change nor particle aggregation was observed. The XPS spectra of the coated beads showed strong signals for O and N which are lacking in polystyrene, indicating that the bead surface was covered by the polyurethanes.

Pooled platelet poor human citrated plasma (**HCP**) was obtained from Red Cross, Hamilton, Ontario, prepared by drawing 9 volumes of blood (from more than 20 donors) into 1 volume of 3.8% trisodium citrate, and then centrifuging for 15 min, 2000 rpm and 10 min, 3000 rpm. The prothrombin time, the partial thromboplastin time and the concentration of coagulation factors were found to be in normal range. The plasma was aliquoted and stored at -70°C until use.

Fibrinogen-depleted, ATIII-depleted, thrombin-added human citrated plasma (**DADFAT**): To 1 ml pooled, citrated, and ATIII-depleted human plasma (Red Cross, Hamilton, Ontario), 25 μl Ancrod (Sigma) in isotonic Tris buffered solution (6 unit/ml) was added and reacted at 37°C for 15 min. The formed fibrin was removed by centrifuging the plasma for 3 min. Then, 10 μl thrombin (3000 unit/mg, Sigma) solution (500 unit/ml) was added. The final concentration of thrombin in the plasma was 5 unit/ml. This plasma was used to study the adsorption of thrombin, not normally present in plasma. Addition of thrombin to unmodified normal plasma (HCP) would form ATIII-thrombin complex and cause fibrin formation.

Primary antibodies

Polyclonal antibodies to 25 different human plasma proteins were screened and utilized in the immunoblotting experiments. These primary

antibodies were in the form of fractionated antisera (IgG fractions) developed in goat or rabbit, as listed in Table 6.1. The banding patterns of proteins measured with these antibodies were essentially identical to published data, and no crossreaction was found.

Table 6.1. Primary antibodies for Western blotting

Antibody to	Developed in	Source
Factor XI	Goat	Nordic Immunology, The Netherlands
Factor XII	Goat	Nordic Immunology, The Netherlands
Prekallikrein	Goat	Nordic Immunology, The Netherlands
HK	Goat	Nordic Immunology, The Netherlands
Plasminogen	Goat	Nordic Immunology, The Netherlands
Albumin	Goat	Nordic Immunology, The Netherlands
prothrombin	Goat	Nordic Immunology, The Netherlands
Fibrinogen	Rabbit	Cappel Lab. Cochraneville, PA, USA
Antithrombin III	Goat	Cappel Lab. Cochraneville, PA, USA
C3	Goat	Cappel Lab. Cochraneville, PA, USA
α_1-antitrypsin	Goat	Cappel Lab. Cochraneville, PA, USA
fibronectin	Goat	Cappel Lab. Cochraneville, PA, USA
α_2-macroglobulin	Goat	Sigma, St. Louis, MO, USA
Transferrin	Goat	Sigma, St. Louis, MO, USA
β_2-microglobulin	Rabbit	Sigma, St. Louis, MO, USA
Hemoglobin	Rabbit	Sigma, St. Louis, MO, USA
IgG	Goat	Miles Scientific, Rexdale, ON, Canada
ß-lipoprotein	Goat	Miles Scientific, Rexdale, ON, Canada
Vitronectin	Rabbit	Calbiochem, La Jolla, CA, USA
Protein C	Rabbit	Calbiochem, La Jolla, CA, USA
Factor V	Goat	Nordic immunology, The Netherlands
Factor IX	Rabbit	Red Cross, Hamilton, ON, Canada
Factor X	Rabbit	Red Cross, Hamilton, ON, Canada
Factor VII	Rabbit	Red Cross, Hamilton, ON, Canada
Factor VIII	Rabbit	Red Cross, Hamilton, ON, Canada

Enzyme conjugated second antibodies

Affinity purified second antibodies were as follows: rabbit anti-goat IgG-alkaline phosphatase conjugate (Sigma); goat anti-rabbit IgG-alkaline phosphatase conjugate (BioRad , Richmond, CA).

Protein adsorption

In a polyethylene vial, 280 µl HCP plasma or DADFAT plasma was incubated with 0.1 g (200 cm^2) polyurethane-coated beads at 37°C for 5 min or 2 h. The supernatant was then removed by centrifuging. To the plasma-incubated beads, 300 µl TBS buffer was added and the beads were shaken for 5 min to remove the loosely adsorbed proteins. The TBS and beads were then separated by centrifuging for 2 min. This washing process was repeated 6 times. After washing, 200 µl 20% SDS solution was added to the beads and the suspension was incubated at 4°C for 12 h. The eluted protein in SDS solution were subjected to immunoblotting analysis.

Polyacrylamide gel electrophoresis and immunoblotting

All electrophoresis reagents were obtained from BioRad, Richmond, CA. The polyacrylamide gel electrophoresis and immunoblotting procedures were performed in the manner that has been detailed in previous reports from this laboratory (Mulzer and Brash, 1989; Mulzer and Brash 1990; Cornelius and Brash, 1993). Briefly, for each experiment, 80.0 µl protein-SDS eluate, or 1.0 µl pure plasma or supernatant from plasma contacted with the polymer surfaces was treated by conventional SDS-PAGE to separate the proteins according to molecular weight. The proteins were then transferred electrophoretically

(blotted) from the SDS-PAGE gel onto an Immobilon PVDF membrane (Millipore Co., Bedford, MA). The blots were then cut into strips and, following blocking with 5% nonfat milk, the strips were incubated with the primary antibodies to the proteins listed in Table 6.1 and then with the appropriate alkaline phosphatase-conjugated second antibody. The substrate system used to develop a color reaction for alkaline phosphatase was 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitroblue tetrazolium (NBT) (both from BioRad), prepared as described by the supplier. For each experimental step, the same volume of freshly prepared reagents was used and the reaction time was controlled to ensure reproducibility and comparability. All assays were repeated at least twice, and the protein blotting patterns were essentially the same for the duplicate experiments. The data shown are for typical, single assays.

Several immunoblots for different surfaces are shown in Fig. 6.1 (p. 154 - 160). The blots were scanned using a densitometer (Howtek Scanmaster 3+) and integrated intensities were calculated using software supplied with the instrument (Bioimage Whole Band Analysis). These data are shown in Figs. 6.3 to 6.15 (p. 162 - 174).

In order to be able to compare the band intensities in different blots, the procedures were standardized as far as possible. All solutions and reagents were freshly prepared at the same concentrations and each step was timed exactly, so that the processes of antibody binding and color development in different experiments were essentially the same. The plasma volume and bead surface area used for plasma exposure were the same for all the polyurethane surfaces, and the same volume of eluates (80 µl) was taken for gel/blot

analysis. Therefore, the intensities of bands in the blots, which are related to the amounts of protein adsorbed, should be comparable from one surface to another.

6.3 RESULTS AND DISCUSSION

1. Immunoblotting

Immunoblot data for HCP and DADFAT plasmas which were not exposed to the polyurethane surfaces are shown in Figs. 6.1a and 6.1g. These blots serve as controls to compare to the blots of the eluates from the polyurethane surfaces, and give an indication of the reactivity of the antibodies with the proteins in normal plasma.

The immunoblot patterns of the protein eluates from the polyurethane surfaces are shown in Fig. 6.1b through Fig. 6.1f. The data show that the overall banding patterns vary with the polyurethane surfaces, and that the eluates from the polymer surfaces are markedly different in composition from the HCP control sample. The immunoblotting tests were positive for the majority of proteins tried. It thus appears that most of the plasma proteins were adsorbed to the surfaces but in relative amounts which vary from one surface to another.

2. Contact phase proteins

Factor XII

Factor XII (Hageman factor, HF, plasma concentration = 30 $\mu g/ml$) is the initiating protein for the activation of the contact phase of plasma coagulation. It

has been shown that adsorption of factor XII to negatively charged surfaces renders it several hundred times more susceptible than solution factor XII to proteolytic activation by kallikrein in the presence of high molecular weight kininogen (Griffin, 1978). The intact form of factor XII has a molecular weight of 80 kD. The activation cleavage of factor XII by activated factor XII (alpha factor XIIa or HFa) at site 1 and by kallikrein at sites 2 and 3 (Fig. 6.2, p. 161) forms a heavy chain (52 kD) and a light chain (28 kD) (Pixley and Colman, 1993).

The factor XII fragments in the protein layer deposited from HCP or DADFAT plasma on polyurethane surfaces were measured by the immunoblotting method (Fig. 6.3). The factor XII fragments in the starting plasmas HCP and DADFAT were also analyzed and are shown in Fig. 6.3 as controls (s1 and s10 in Fig. 6.3). As shown in the control HCP blot (s1 of Fig. 6.3), factor XII appears at 80 kD and 50 kD and is thus extensively cleaved, probably during the plasma preparation procedure.

Following a 5 min incubation of HCP with the PPOMDA and PPACKPPO surfaces a significant amount of factor XII was deposited (s2 and s3 in Fig. 6.3). In comparison, after a 2 h incubation smaller amounts of factor XII were eluted (s4, s5, s6 in Fig. 6.3), suggesting that factor XII was rapidly adsorbed to the polyurethane surfaces upon plasma-surface contact, and that other proteins of higher binding affinity slowly displaced it.

By comparing lane s6 (PPACKPPO) with lane s4 (PPOMDA) and lane s5 (PPOBDDS) in Fig. 6.3, it can be seen that more factor XII was adsorbed from HCP to the PPACKPPO surface. It seems likely that the PPACKPPO surface has a higher affinity for factor XII. As will be reported in Chapter 7, factor XII adsorbed to the PPOBDDS surface has strong amidolytic activity, while no

factor XIIa activity is observed on the PPACKPPO surface. Therefore, the adsorption of factor XII to the PPOBDDS surface renders it more susceptible to activation, while adsorption to PPACKPPO probably occurs through binding of the surface-bound PPACK and the active center of factor XIIa, so that factor XIIa is adsorbed and inactivated by PPACK. The possible inhibition of factor XIIa by PPACK is discussed further in Chapter 7.

DADFAT plasma is depleted of fibrinogen and antithrombin III and contains 5 unit/ml thrombin. The objective in using this 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. The immunoblot of DADFAT plasma using an antibody to factor XII is shown in lane s10 in Fig. 6.3 as a control. The deposition of factor XII from DADFAT on the polymer surfaces seems different from the deposition of factor XII from HCP (compare s11 - s14 with s4 - s6 in Fig. 6.3). Adsorption of factor XII from DADFAT onto PPOMDA (s12) and PPACKPPO (s14) surfaces was essentially zero. This interesting result suggests that thrombin does not promote the adsorption of factor XII to the polymer surfaces. However, thrombin should not prevent factor XII adsorption to surfaces, because it is not the dominating adhesive protein in plasma. Therefore, the decrease in factor XII adsorption to the polymers may be related to the absence of fibrinogen in the DADFAT plasma compared to the HCP. In other words, the adsorption of factor XII may require the preadsorption of fibrinogen on the polymer surfaces. This result is of great interest for further investigation since prevention of factor XII activation is critical for improving the blood compatibility of biomaterials.

High molecular weight kininogen

High molecular weight kininogen (HK, concentration in plasma, 60 μg/ml) is an important contact phase protein (Colman, 1984). It contains approximately one-fifth of the kinin (potent vasoactive peptide) content of plasma and exists as a single polypeptide chain of approximately 110 kD MW. HK can be cleaved by kallikrein to give a heavy chain of MW 62 kD and a light chain of MW 56 to 62 kD (Scott et al., 1984), and this light chain can be further cleaved to give a fragment of 45 to 47 kD. It has been demonstrated that the cleaved (activated) HK augments the adsorption of prekallikrein and factor XII to initiating surfaces and renders them thousands of times faster in activation (Scott et al., 1984). Therefore, the cleavage of HK is an indication of contact phase activation.

In this work, HCP and DADFAT were used to study HK adsorption on polyurethane surfaces. The immunoblot of HCP using an anti-HK antibody (s1 in Fig. 6.4) shows that most of the HK in this control sample is in intact form (110 kD). In contrast, the DADFAT sample (s10 in Fig. 6.4) has some cleaved HK (bands at 40 - 60 kD), indicating that the preparation process for DADFAT may have induced some activation of the contact phase.

The PPOMDA surface incubated with HCP for 5 min (s2 in Fig. 6.4) or 2 h (s4 in Fig. 6.4) adsorbed only a small amount of HK. DADFAT also deposited only a trace amount of HK on the PPOMDA surface (s12 in Fig. 6.4). PPOMDA is a neutral surface which has neither positive nor negative charges. Comparing the low adsorption of HK on the PPOMDA surface with the high adsorption of HK on negatively charged PPOBDDS (s5 and s13 in Fig. 6.4) and positively and negatively charged PPACKPPO (s3, s6, s14 in Fig. 6.4), it seems

likely that electrical charge plays an important role in HK adhesion. HK has been shown to contain an unusual domain with an amino acid sequence that is rich in positively charged histidine, lysine and glycine, and this domain has been shown to be essential for the binding of HK to negatively charged surfaces and for its cofactor activity (Kato et al., 1979; Sugo et al., 1980). Other experiments to be reported in Chapter 7 show that the contact phase is not directly activated by the PPOMDA surface, possibly due to the lack of HK adsorption on this material.

Incubation of either HCP or DADFAT with negatively charged PPOBDDS for 2 h (s5 and s13 in Fig. 6.4) deposited a significant amount of HK (40 - 50 kD) on the surface. Moreover in the eluates from this surface, the HK is essentially completely cleaved, whereas in the controls (s1 and s10) it is intact. The supernatant of the HCP sample that has been incubated with PPOBDDS shows only traces of HK (s8 in Fig. 6.4), providing further evidence of significant accumulation of HK on this surface. The activation and accumulation of HK suggested in Fig. 6.4 agree with the data which will be reported in Chapter 7 showing that the contact phase of coagulation is rapidly activated by the negatively charged BDDS-based polyurethanes.

It is interesting to note that significant amounts of HK were adsorbed but not activated on the PPACKPPO surfaces incubated with HCP or DADFAT for 2 h (s6 and s14 in Fig. 6.4). The intact bands of HK on PPACKPPO are in striking contrast to the activated bands of HK on PPOBDDS. It is possible that the positive charges on the PPACKPPO surface interact with HK in such a manner that the adsorbed HK is protected from cleavage by kallikrein. Another possible explanation is that the contact phase is inhibited by PPACK as discussed in

more detail in Chapter 7. This behavior of HK on the PPACKPPO surface suggests that adsorption of intact HK will not directly induce contact phase activation. Instead, its activity may be controlled by other mechanisms such as the inhibition of factor XIIa by surface-bound PPACK on the PPACKPPO surface (see Chapter 7).

The data in Fig. 6.4 show that thrombin does not influence HK adsorption. The adsorption and activation of HK on polymer surfaces are the same whether HCP or DADFAT is used (compare s4, s5, s6 with s12, s13, s14 in Fig. 6.4). It seems likely that while activation of the contact phase leads to thrombin generation, thrombin does not influence the contact phase (see Chapter 8 for further discussion).

Factor XI

Factor XI is a glycoprotein (concentration in plasma, 5 μg/ml) that contains two identical polypeptide chains of MW 80 kD. Each polypeptide chain can be cleaved by alpha factor XIIa to give disulfide-linked heavy (MW 50 kD) and light (MW 30 kD) chains (Cochrane and Griffin, 1982). As can be seen from the control blots (s1 and s10 in Fig. 6.5), factor XI appears at ~50 kD and is thus activated in HCP and DADFAT plasmas during the plasma preparation procedure. Adsorption of factor XI to the polymer surfaces in a 2 h incubation was essentially zero (s4 - s6, s11 - s14 in Fig. 6.5). However, in the 5 min eluates (s2, s3 in Fig. 6.5), small but significant amounts of factor XI were found. It seems likely that the initially adsorbed factor XI is displaced by other proteins. In Chapter 8, it is reported that thrombin can be slowly activated on the PPOMDA surface through a factor XII-independent mechanism. The small

amounts of factor XI adsorbed on PPOMDA in 5 min (s2 in Fig. 6.5) may contribute to the activation of thrombin on the surface.

Comparing HCP (s1 in Fig. 6.5) with DADFAT (s10 in Fig. 6.5), it is seen that factor XI was extensively activated by thrombin in DADFAT. However, essentially no factor XI was adsorbed from DADFAT to the polymer surfaces (s11-s14 in Fig. 6.5). It is possible that the depletion of fibrinogen from DADFAT prevented factor XI adsorption.

Prekallikrein

Prekallikrein (with a MW of 85 kD in its intact form, concentration in plasma 50 μg/ml) shows a relatively strong band at 50 kD in the 5 min eluates (s2, s3 in Fig. 6.6), while in the 2 h eluates less prekallikrein was found (s5, s6, s11-s14 in Fig. 6.6). Thus it appears that all of the contact phase factors: factor XII, prekallikrein and factor XI were adsorbed to the polymer surfaces in the early stages (5 min) of plasma-surface interactions, and were then either cleaved or displaced from the surface. On the other hand, for the contact phase cofactor HK, the amounts adsorbed on the polymer surfaces increased with time. These different adsorption properties of the contact phase enzymes and cofactor (HK) are of interest for further investigation.

3. Other coagulation proteins

Fibrinogen

The intact *alpha*, *beta* and *gamma* chains of fibrinogen have molecular weights of 67, 56, and 47 kD respectively (Dang et al., 1989). The control HCP

sample (s1 in Fig. 6.7) shows that most of the fibrinogen is in intact form. After a 2 h incubation with HCP, substantial amounts of fibrinogen were adsorbed to the polymer surfaces (s4-s6 in Fig. 6.7), and the intensity of the bands in the eluates is much higher than in the control plasma. Thus it appears that fibrinogen is a major component of the protein layer adsorbed to these polyurethanes from plasma.

The bands in Fig. 6.7 (s2-s6 in Fig. 6.7) in the region from 30 to 47 kD show that some fibrinogen was cleaved on the polyurethane surfaces. Fibrinogen cleavage products were particularly abundant on the PPOMDA and PPOBDDS surfaces (s4, s5 in Fig. 6.7), possibly indicating thrombin generation.

After 5 min incubation (s2, s3 in Fig. 6.7) smaller amounts of fibrinogen appeared to be adsorbed on the PPOMDA and PPACKPPO surfaces compared to 2 h (s4, s6 in Fig. 6.7). Therefore, fibrinogen adsorption on the polyurethane surfaces increased with time in contrast to other surfaces where it decreases via displacement (Brash, 1991). It seems possible that fibrinogen may be one of the proteins which displaces other initially adsorbed proteins such as the contact phase factors.

Prothrombin

Prothrombin, the precursor of thrombin, is a single chain glycoprotein of MW 72 kD (concentration in plasma, 100 µg/ml). It is cleaved by factor Xa into thrombin of MW 36 kD (Butkowski et al., 1977). The control HCP sample (s1 in Fig. 6.8) shows a strong band corresponding to the intact protein at 72 kD and also a strong band at 36 kD, indicating that some prothrombin was activated to thrombin.

After a 5 min incubation with HCP more prothrombin was adsorbed to the PPOMDA and PPACKPPO surfaces (s2, s3 in Fig. 6.8) than after 2 h (s4-s6 in Fig. 6.8). This is in contrast to fibrinogen adsorption discussed above. It appears that prothrombin has lower binding affinity for the polyurethane surfaces than fibrinogen and it can be slowly displaced at longer incubation times.

In the control HCP sample (s1 in Fig. 6.8), a strong band at 36 kD was found corresponding to cleaved/activated prothrombin. Surprisingly, no such strong band was found in the eluates from the polymer surfaces (s2-s6 in Fig. 6.8). The DADFAT plasma contained a high concentration of added thrombin, and the corresponding band at 36 kD is evident in the control blot (s10 in Fig. 6.8). However, no such bands were found in the eluates from the polymer surfaces (s11-s14 in Fig. 6.8). It is possible that thrombin binds to the polyurethane surface tightly so that it cannot be eluted easily.

Factor V

Factor V is a large plasma protein of MW 330 kD in its intact form (concentration in plasma, 10 µg/ml). When altered or activated by thrombin, it is converted to a form that binds to phospholipids and markedly enhances prothrombin activation by factor Xa (Mann et al., 1988). Thrombin cleaves the single chain factor V at arg 710 - ser 711 and arg 1545 - ser 1546 to release a large connecting chain (between terminal domains) of MW 170 kD, a heavy chain of 94 kD, and a light chain of MW 69 kD (Kane and Davie, 1988). The heavy chain and light chain are held together by calcium ions to form factor Va (Guinto and Esmon, 1982). Factor V was not significantly activated in HCP. As

shown in Fig. 6.9 lane s1, only small amounts of cleaved factor V at about 150, 70, and 25 kD were found. Adding thrombin to plasma (DADFAT) produced large amounts of factor V fragments at ~110 and 70 kD corresponding to the heavy chain and light chain of factor Va (s10 in Fig. 6.9).

On the PPACKPPO surface, HCP plasma deposited large amounts of factor V fragments after 5 min and 2 h incubation (s3 and s6 in Fig. 6.9). Surprisingly, the PPOMDA surface (s2, s4, s12 in Fig. 6.9) appeared to adsorb less factor V than the PPACKPPO surface. As will be discussed in Chapter 8, plasma clotting occurred on the PPOMDA surface but not on the PPACKPPO surface. Since factor V is important for thrombin generation, it might be supposed that more factor V would be adsorbed on the PPOMDA surface, i.e. in contrast to the data in Fig. 6.9. To interpret this finding, the effect of thrombin on factor V adsorption to the polyurethane surfaces was investigated. As shown in the control DADFAT sample (s10 in Fig. 6.9), thrombin did produce large amounts of factor Va in the plasma. However, the increase in factor Va concentration did not increase the amount of factor Va adsorbed to the polymer surfaces. Instead, the amount of factor Va adsorbed on the polymer surfaces from DADFAT (s11-s14 in Fig. 6.9) was significantly less than the amount adsorbed from HCP (s2-s6 in Fig. 6.9). Because DADFAT is depleted of fibrinogen, the reduced adsorption of factor V from DADFAT to the polymer surfaces indicates that fibrinogen may be important for factor V adsorption.

4. Adsorption of "antithrombogenic" proteins

Albumin

Albumin adsorbed on artificial surfaces is reported to have a passivating effect on blood-surface interactions, probably due to a reduction in platelet adhesion (Young et al., 1983; Chang, 1984). As seen in Fig. 6.10 significant amounts of intact albumin (66 kD) were adsorbed to the polymer surfaces from DADFAT plasma in 2 h incubation (s5-s8 in Fig. 6.10). After a 5 min incubation a small amount of albumin was adsorbed (s2, s3 in Fig. 6.10) from HCP plasma. The data suggest that the rate of albumin adsorption to the polymer surfaces is relatively slow, even though the concentration of albumin is high in plasma.

As seen from the data presented in this chapter many proteins are adsorbed on the polymer surfaces. Among them, fibrinogen and albumin appear to be the most abundant in the adsorbed protein layer (Figs. 6.7 and 6.10). However, in the initial stages, relatively little albumin was adsorbed. Thus, albumin appears not to be strongly adsorbed to the polyurethane surfaces, and it seems likely that it would never completely cover the surface in such a way as to prevent other proteins, especially fibrinogen, from adsorbing. Thus little in the way of a "spontaneous" protective effect of albumin is to be expected.

Plasminogen

Plasminogen (concentration in plasma, 120 μg/ml) exists in two forms of molecular weight 94 kD (glu-plasminogen) and 84 kD (lys-plasminogen). Activation to plasmin involves scission of the arg 560-val 561 peptide bond,

yielding a molecule having a heavy chain (60 kD) and a light chain of MW 24 kD linked by disulfide bonds (Lijnen and Collen, 1982). Therefore plasminogen may exhibit bands at 60, 24 and 10 kD in reduced gels depending on its state of activation. The control HCP and DADFAT blots (s1, s10 in Fig. 6.11) show strong bands at 94 and 85 kD, indicating that most of the plasminogen was present in the intact form in the control plasmas. The supernatants of the HCP plasma incubated with the PPOMDA and PPACKPPO polymer surfaces (s7, s9 in Fig. 6.11) show relatively strong bands at ~85 kD. Therefore, adsorbed plasminogen was mainly in the intact form and these two surfaces did not induce plasminogen activation. However, in the supernatant of the HCP plasma that was in contact with the PPOBDDS surface (s8 in Fig. 6.11), some cleavage products of plasminogen are seen at 30 - 50 kD, suggesting some activation of the fibrinolytic pathways.

Among the polymer surfaces investigated, PPACKPPO adsorbed more plasminogen than the others (s3, s6 and s14 in Fig. 6.11), and most of the plasminogen on the PPACKPPO surface was present in the intact form (~90 kD). Significantly less plasminogen was adsorbed to the PPOBDDS (s5 and s13 in Fig. 6.11), PPOMDA (s2, s4 and s12 in Fig. 6.11) and polystyrene surfaces (s11 in Fig. 6.11).

The adsorption of plasminogen does not appear to evolve significantly with time. Comparing 5 min adsorption on PPOMDA (s2 in Fig. 6.11) and PPACKPPO (s3 in Fig. 6.11) with 2 h adsorption on PPOMDA (s4 in Fig. 6.11) and PPACKPPO (s6 in Fig. 6.11), similar amounts of plasminogen are seen. Thus adsorption appears to be completed within 5 min.

Many cleavage products of plasminogen were found in the supernatant of the plasma that was incubated with the PPOBDDS surface (s8 in Fig. 6.11), indicating that activation had occurred. However, the same products were not evident on the surface itself (s5 in Fig. 6.11). Therefore, the activation of plasminogen may not be correctly indicated by surface-adsorbed plasminogen fragments.

Antithrombin III

Antithrombin III (ATIII) is the major plasma glycoprotein inhibitor of blood coagulation, and is composed of a single polypeptide chain of MW 65 kD (concentration in plasma, 290 µg/ml). It inhibits serine proteases of the coagulation pathway, mainly thrombin, by forming a stable 1:1 complex between an arginine residue of ATIII and the serine active site of thrombin (Rosenberg and Rosenberg, 1984). Heparin can bind to ATIII and change its conformation rendering the arginine residue at the active site of ATIII more readily available for binding to thrombin.

After a 5 min incubation with HCP more ATIII appeared to be deposited on the PPOMDA and PPACKPPO surfaces (s2, s3 in Fig. 6.12) than after 2 h (s4, s5, s6 in Fig. 6.12). This suggests that ATIII was among those proteins adsorbed to the polymer surfaces in the initial stages of plasma-surface interactions, and that it was displaced later by other proteins, possibly fibrinogen and albumin.

The supernatants of the plasma samples that were incubated with the PPOMDA and PPOBDDS surfaces for 2 h show complex patterns of strong bands in the 20 - 50 kD region (s7, s8 in Fig. 6.12), suggesting that ATIII was

extensively cleaved. In contrast, no such bands were found in the protein layer adsorbed on the corresponding polymer surfaces (s4, s5 in Fig. 6.12). The cleavage fragments of ATIII thus appear to be non-surface active, similar to the activation fragments of factor V and plasminogen.

PPOBDDS contains sulphonate groups which are among the major functional groups of heparin. Since heparin has a high affinity for ATIII, it might be expected that PPOBDDS would interact strongly with ATIII also. The ATIII band intensities on the PPOBDDS surface (s5 in Fig. 6.12) are even lower than on the PPOMDA surface (s4 in Fig. 6.12), so that PPOBDDS does not appear to have a high affinity for ATIII. This surprising result might be attributed to the extensive adsorption of fibrinogen, HK, and albumin on the PPOBDDS surface. These and other proteins may preempt the surface and prevent adsorption of ATIII. This explanation is consistent with the data reported in Chapter 8 showing that PPOBDDS does not effectively inhibit thrombin. Instead, it rapidly activates the contact phase of coagulation (Chapter 7), and the plasma in contact with PPOBDDS is clotted.

5. C3 component of complement

Complement activation is potentially a major problem during blood-biomaterial contact. Hemodialysis-induced complement activation and the associated clinical complications have been reviewed extensively (Johnson, 1994; Deppisch et al., 1994). The activation of complement can result in release of C3a and formation of C3b. C3a is an anaphylatoxin which can cause a severe allergic reaction.

In this work, the control plasma samples HCP (s1 in Fig. 6.13) and DADFAT (s10 in Fig. 6.13) show two major bands. The first appears at ~110 kD which corresponds to the intact 116 kD α-chain or to the 108 kD α'-chain of C3b. The second appears at ~75 kD corresponding to the β-chain (Sim et al., 1981). Only trace amounts of cleavage products were found in the 30 - 60 kD region which could be attributed to intermediate products in the conversion of C3b to C3bi (Sim et al., 1981; Davis and Harrison, 1982).

Similar amounts of subunits from intact C3 were deposited from HCP onto the PPOMDA and PPACKPPO surfaces after a 5 min incubation (s2, s3 in Fig. 6.13). After a 2 h incubation essentially the same amount of C3 was deposited on these two surfaces from HCP (s4 and s6 in Fig. 6.13) and from DADFAT (s12 and s14 in Fig. 6.13).

A common approach to assessing complement activation is to measure fluid phase C3 fragments (Cheung, 1994). As has been discussed, some proteins such as factor XII (Fig. 6.3), HK (Fig. 6.4) and factor V (Fig. 6.9) are strongly adsorbed to the polymer surfaces and thus their fluid phase concentrations were reduced. Similar results were found for C3. By comparing s4, s5, and s6 with s7, s8, and s9 in Fig. 6.13, it is clear that many C3 fragments which have MW below 60 kD were adsorbed on the polymer surfaces (s4, s5, s6 in Fig. 6.13), but not detected in the fluid phase (s7, s8, s9 in Fig. 6.13). This finding suggests that the fragments of C3 are strongly adhesive to polymer surfaces, and the more valid way of assessing them is to analyze the adsorbed protein layer, not the fluid phase.

6. Fibronectin and vitronectin

Fibronectin and vitronectin are adhesive proteins that promote the attachment and spreading of a variety of cells on surfaces (Sekiguchi and Hakomori, 1983; Hynes 1986; Ill and Ruoslahti, 1985). Fibronectin is a dimeric glycoprotein with subunit polypeptides of ~200 kD. The control plasma samples HCP and DADFAT (s1 and s10 in Fig. 6.14) show strong bands of fibronectin fragments of MW 40 - 180 kD, indicating that fibronectin may be degraded by enzymes (Sekiguchi and Hakomori, 1983; Mulzer and Brash, 1989) during the plasma preparation process. As can be seen in Fig. 6.14, substantial amounts of fibronectin fragments were adsorbed on the PPACKPPO surface after a 5 min incubation (s3 in Fig. 6.14). Some fibronectin fragments were also adsorbed to the PPOMDA surface after 5 min (s2 in Fig. 6.14). It is interesting that in 2 h only trace amounts of fibronectin were adsorbed on the surfaces (s4-s6 and s11-s14 in Fig. 6.14). This finding suggests that fibronectin is not strongly adsorbed to polyurethane surfaces and that initially adsorbed fibronectin is later displaced by other proteins. Large amounts of fibronectin fragments in the supernatants of the plasmas which contacted the polyurethane surfaces were found in the 40 - 140 kD region (s7 -s9 in Fig. 6.14), further indicating that fibronectin was extensively cleaved but the cleavage products did not adsorb to the surfaces.

Vitronectin is a major adhesive glycoprotein in plasma and certain extracellular locations. It can promote adhesion and spreading of anchorage-dependent cells (Hayman et al., 1982). Vitronectin is synthesized in the liver as a single chain of MW 75 kD or partly degraded two-chain (MW 65 kD and 10 kD) glycoprotein (Preissner and Jenne, 1991). Significant amounts of vitronectin were deposited from HCP onto different polymer surfaces after a 5

min incubation (s2 ~3 in Fig. 6.15), while the control plasma showed a smaller amount of vitronectin (s1 in Fig. 6.15). This finding indicates that vitronectin is strongly adsorbed to the polyurethane surfaces.

Summary

The composition of protein layers deposited from plasma on different polyurethane surfaces was directly analyzed using an immunoblotting method. The amount of protein adsorbed, the sequence of protein adsorption and the activation of proteins on the surfaces were found to be influenced by the properties of the surfaces. The PPACKPPO surface appears to adsorb proteins in such a way that the coagulation cascade is not activated. PPOBDDS on the other hand promotes the adsorption and activation of coagulation proteins.

Fibrinogen, HK, and albumin are the most abundant proteins in the protein layer deposited from plasma on the polymer surfaces. The adsorption of these proteins increases with time, suggesting that they may replace other initially adsorbed proteins on the polymer surfaces.

The contact phase factors XII, XI and kallikrein, as well as prothrombin, factor IX, ATIII and fibronectin were adsorbed on the polymer surfaces in the initial stages of plasma-surface interactions. They were apparently later displaced by other proteins, probably fibrinogen, HK, and albumin.

The adsorption of factors XII, XI, V and prothrombin on the polymer surfaces was significantly decreased when plasma depleted of fibrinogen was used, suggesting interactions between fibrinogen and these coagulation factors in normal plasma.

The compositions of plasma and the protein layer formed on the polymer surfaces are in general different. For example, large amounts of cleaved HK, C3, and factor V were found on the polymer surfaces, while the plasma supernatants were almost depleted of these proteins.

The adsorption of proteins on the polymer surfaces was found to be consistent with the findings from the chromogenic substrate assay studies reported in Chapters 7 and 8. The combination of immunoblotting and chromogenic substrate methods therefore represents a valuable approach for studying the mechanisms involved in plasma-surface interactions.

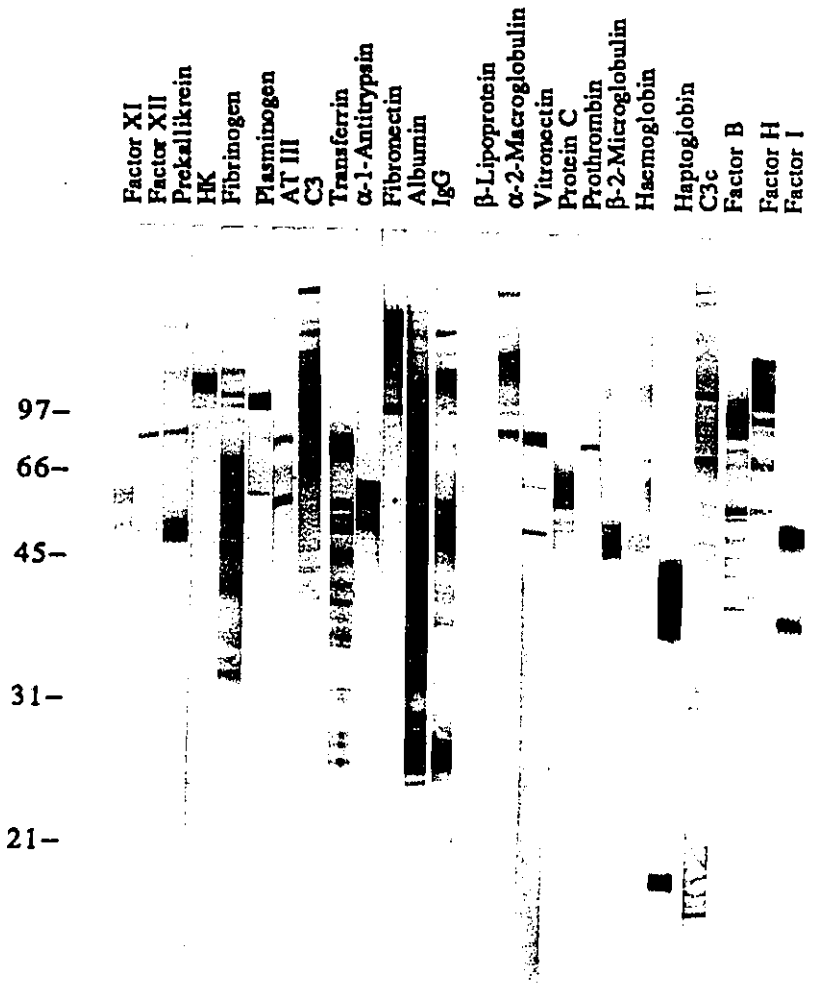


Fig. 6.1a Immunoblot from SDS-PAGE (reduced) of normal human citrated plasma. Immunoblotting patterns for specific antibodies are indicated. Molecular weight scale in kD.

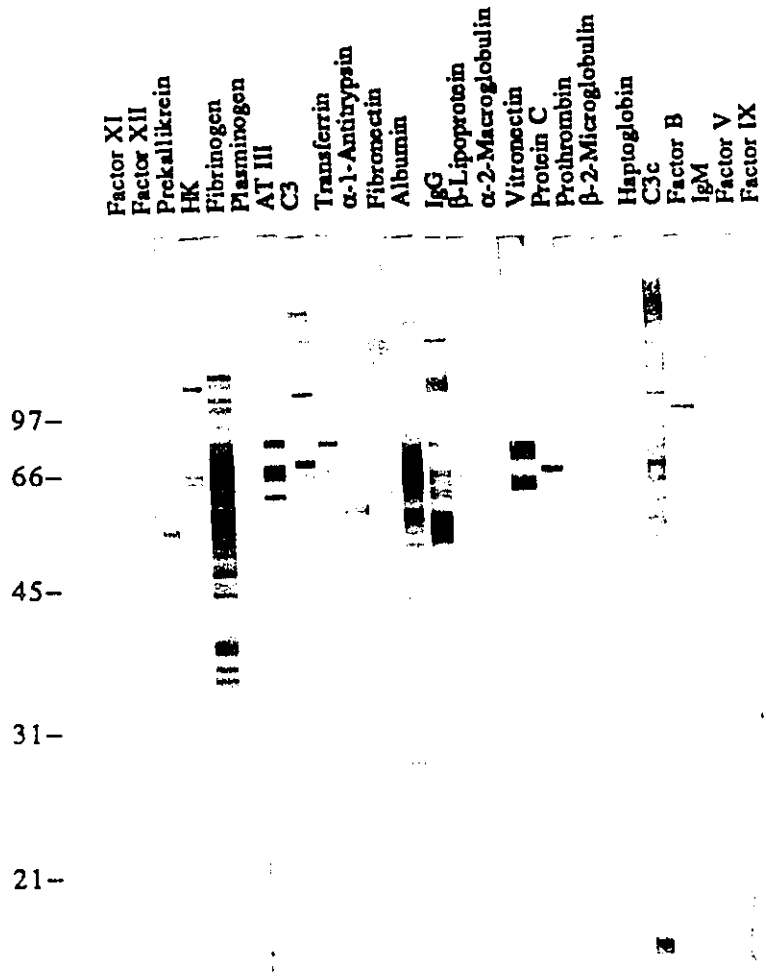


Fig. 6.1b Immunoblot from SDS-PAGE (reduced) of 2% SDS eluate from PPOMDA polyurethane surface after 5 min exposure to normal human citrated plasma. Immunoblotting patterns for specific antibodies are indicated. Molecular weight scale in kD.

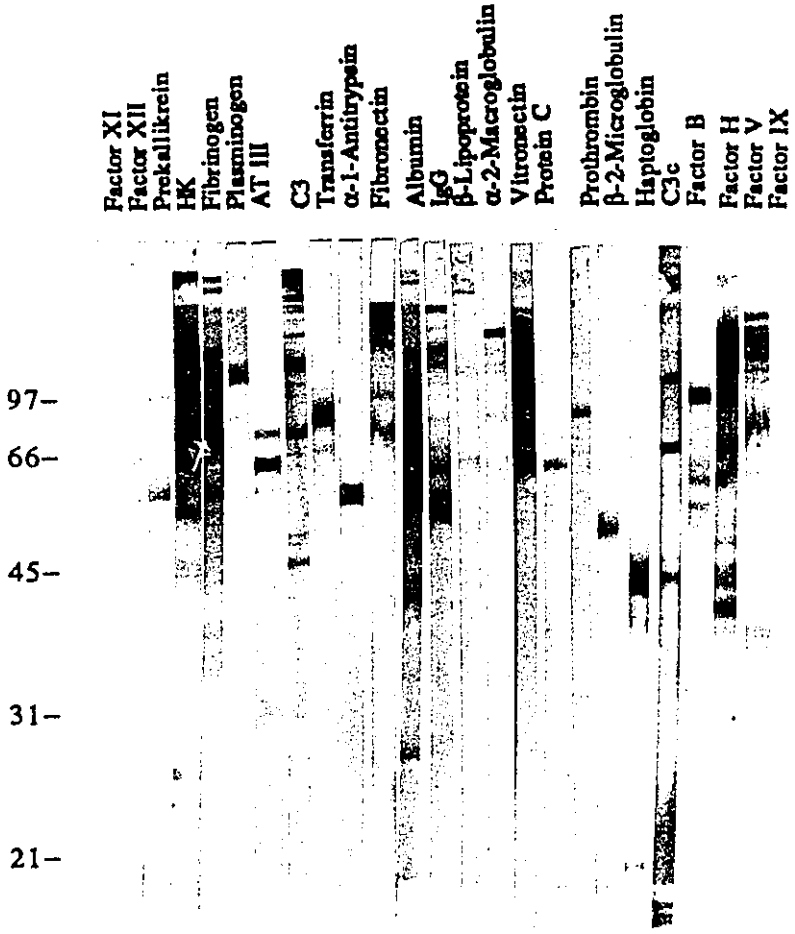


Fig. 6.1c Immunoblot from SDS-PAGE (reduced) of 2% SDS eluate from PPACKPPO polyurethane surface after 5 min exposure to normal human citrated plasma. Immunoblotting patterns for specific antibodies are indicated. Molecular weight scale in kD.

Fig. 6.1d Immunoblot from SDS-PAGE (reduced) of 2% SDS eluate from PPOMDA polyurethane surface after 2 h exposure to normal human citrated plasma. Immunoblotting patterns for specific antibodies are indicated. Molecular weight scale in kD.

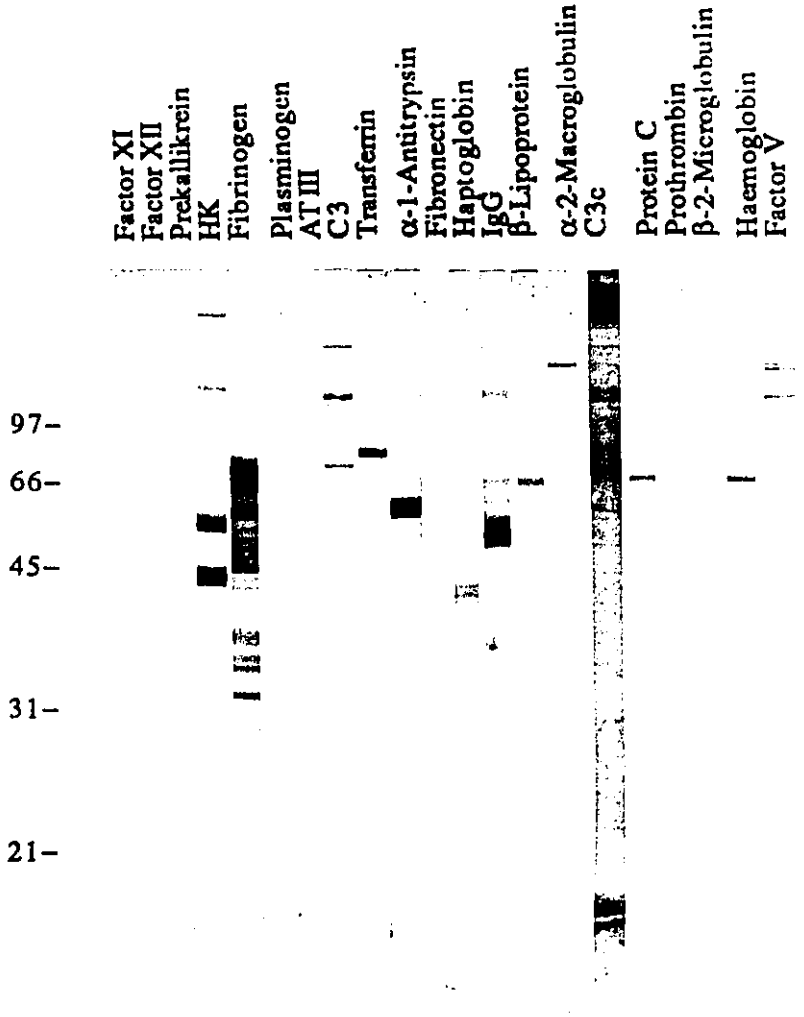


Fig. 6.1e Immunoblot from SDS-PAGE (reduced) of 2% SDS eluate from PPOBDDS polyurethane surface after 2 h exposure to normal human citrated plasma. Immunoblotting patterns for specific antibodies are indicated. Molecular weight scale in kD.

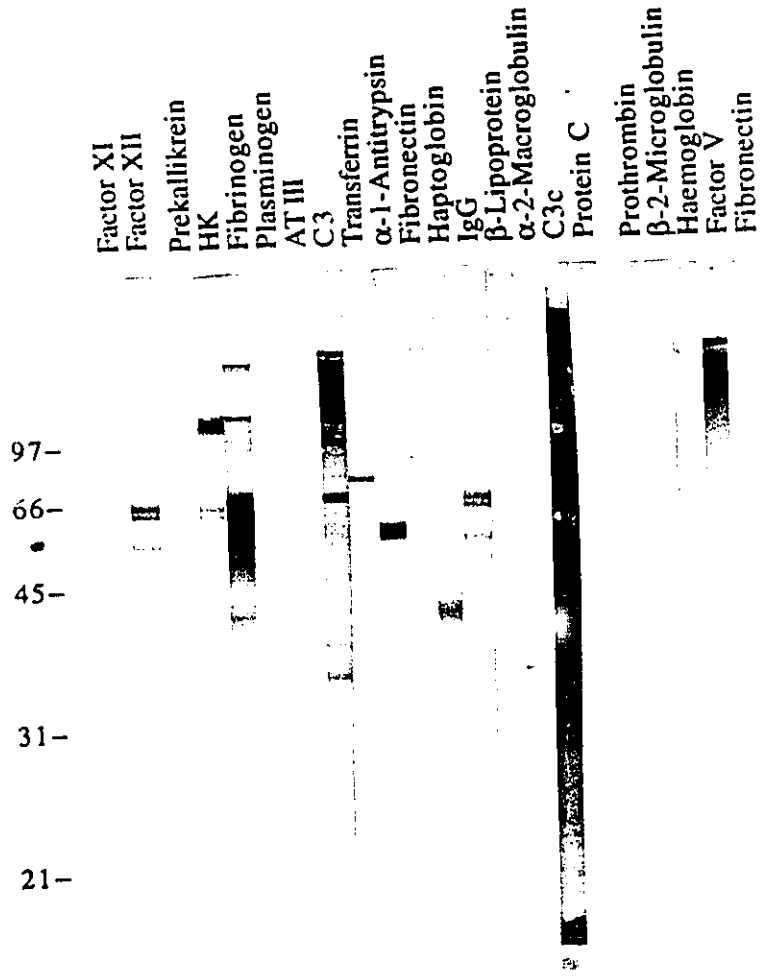


Fig. 6.1f Immunoblot from SDS-PAGE (reduced) of 2% SDS eluate from PPACKPPO polyurethane surface after 2 h exposure to normal human citrated plasma. Immunoblotting patterns for specific antibodies are indicated. Molecular weight scale in kD.

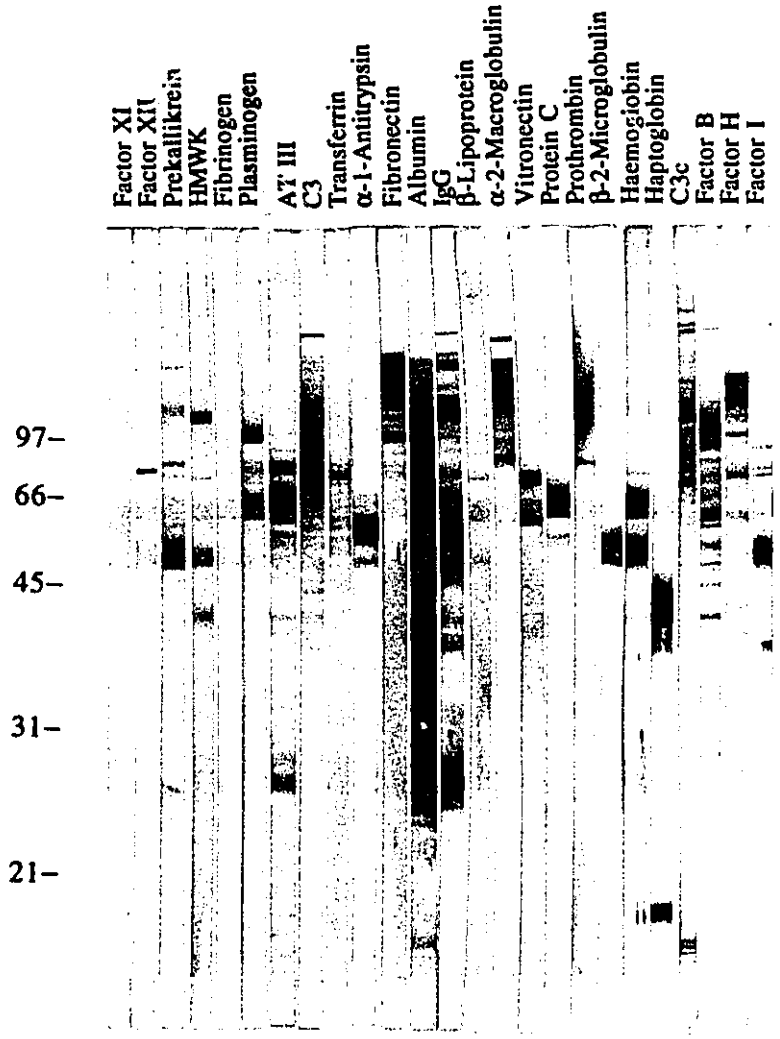


Fig. 6.1g Immunoblot from SDS-PAGE (reduced) of DADFAT plasma (human citrated plasma depleted of fibrinogen, depleted of ATIII, and with added thrombin (final thrombin concentration is 5 unit/ml)). Immunoblotting patterns for specific antibodies are indicated. Molecular weight scale in kD.

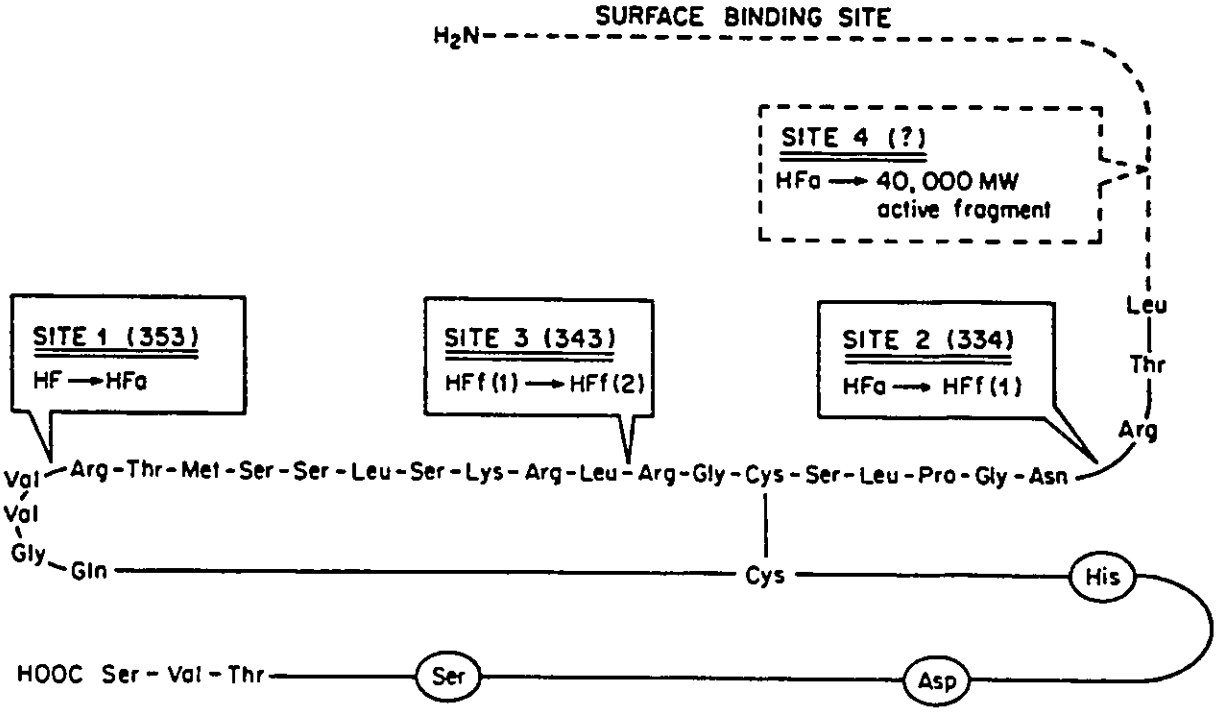


Fig. 6.2 Sites of cleavage of factor XII during contact activation. Site 1, within a disulfide bridge, converts factor XII to alpha factor XIIa. Cleavage external to the disulfide bond at site 2 and then 3 converts alpha factor XIIa to beta factor XIIa. The light chain of beta factor XIIa (residues 334 to 353 or 343 to 353) is derived from the C-terminal end of the heavy chain of alpha factor XIIa (Kaplan and Silverberg, 1987).

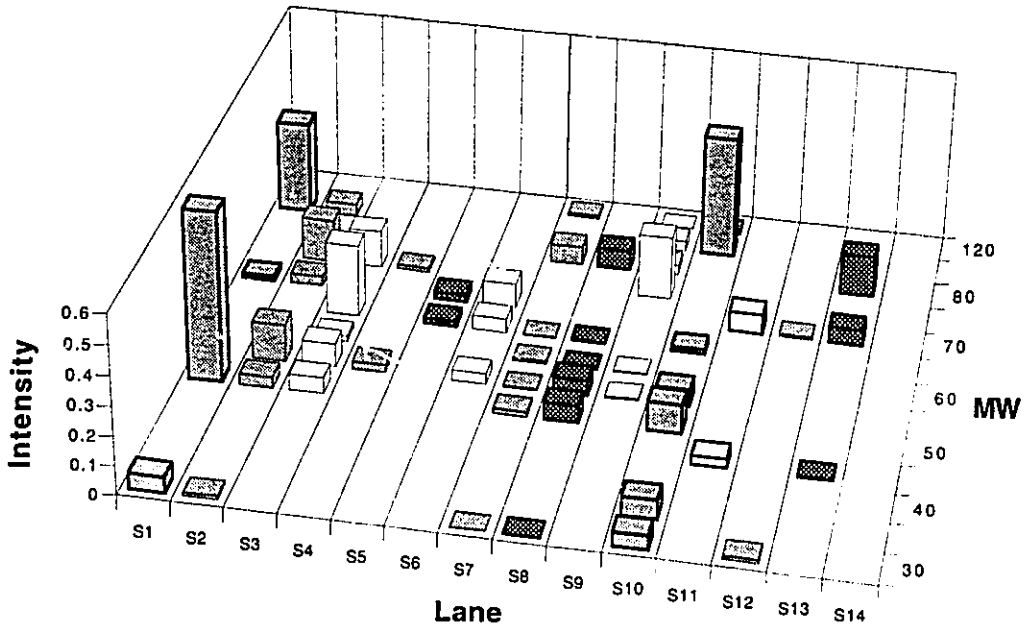


Fig. 6.3 Factor XII adsorbed from plasma to polyurethane surfaces, measured by immunoblotting method. Adsorption temperature 37°C.

S1, Human citrated plasma (HCP) sample used as a control;
S2, 5 min HCP adsorption on PPOMDA;
S3, 5 min HCP adsorption on PPACKPPO;
S4, 2 h HCP adsorption on PPOMDA;
S5, 2 h HCP adsorption on PPOBDDS;
S6, 2 h HCP adsorption on PPACKPPO;
S7, Supernatant of HCP contacted with PPOMDA for 2 h;
S8, Supernatant of HCP contacted with PPOBDDS for 2 h;
S9, Supernatant of HCP contacted with PPACKPPO for 2 h;
S10, DADFAT (human citrated plasma depleted of fibrinogen, depleted of ATIII and with added thrombin (final thrombin concentration is 5 unit/ml)) as a control;
S11, 2 h DADFAT adsorption on polystyrene;
S12, 2 h DADFAT adsorption on PPOMDA;
S13, 2 h DADFAT adsorption on PPOBDDS;
S14, 2 h DADFAT adsorption on PPACKPPO.

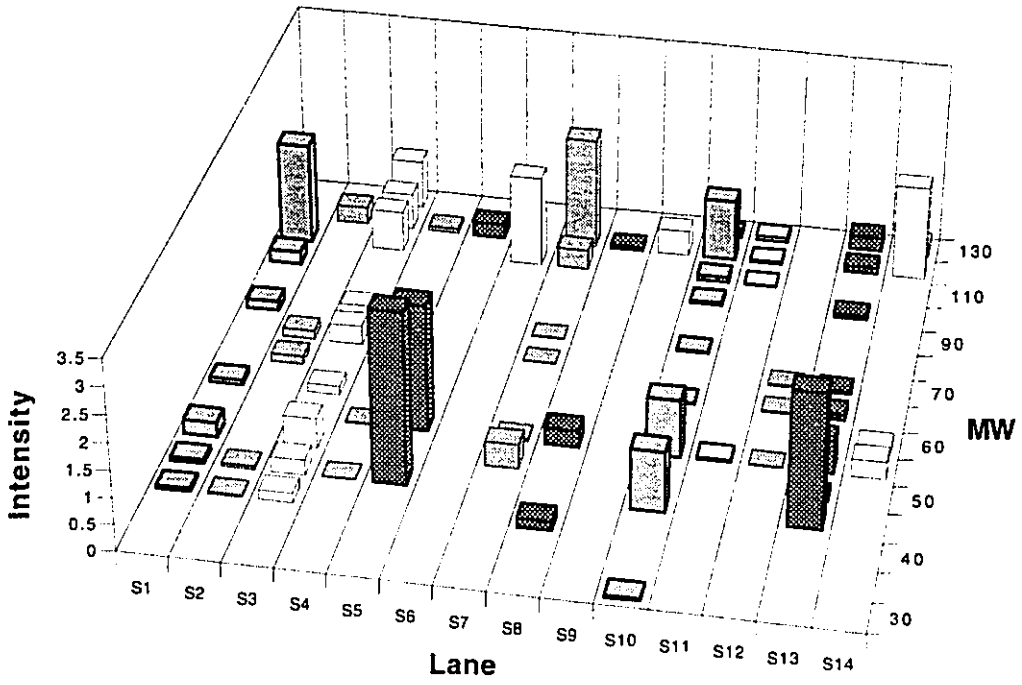


Fig. 6.4 High molecular weight kininogen adsorbed from plasma to polyurethane surfaces, measured by immunoblotting method. Adsorption temperature 37°C.

S1, Human citrated plasma (HCP) sample used as a control;
S2, 5 min HCP adsorption on PPOMDA;
S3, 5 min HCP adsorption on PPACKPPO;
S4, 2 h HCP adsorption on PPOMDA;
S5, 2 h HCP adsorption on PPOBDDS;
S6, 2 h HCP adsorption on PPACKPPO;
S7, Supernatant of HCP contacted with PPOMDA for 2 h;
S8, Supernatant of HCP contacted with PPOBDDS for 2 h;
S9, Supernatant of HCP contacted with PPACKPPO for 2 h;
S10, DADFAT (human citrated plasma depleted of fibrinogen, depleted of ATIII and with added thrombin (final thrombin concentration is 5 unit/ml)) as a control;
S11, 2 h DADFAT adsorption on polystyrene;
S12, 2 h DADFAT adsorption on PPOMDA;
S13, 2 h DADFAT adsorption on PPOBDDS;
S14, 2 h DADFAT adsorption on PPACKPPO.

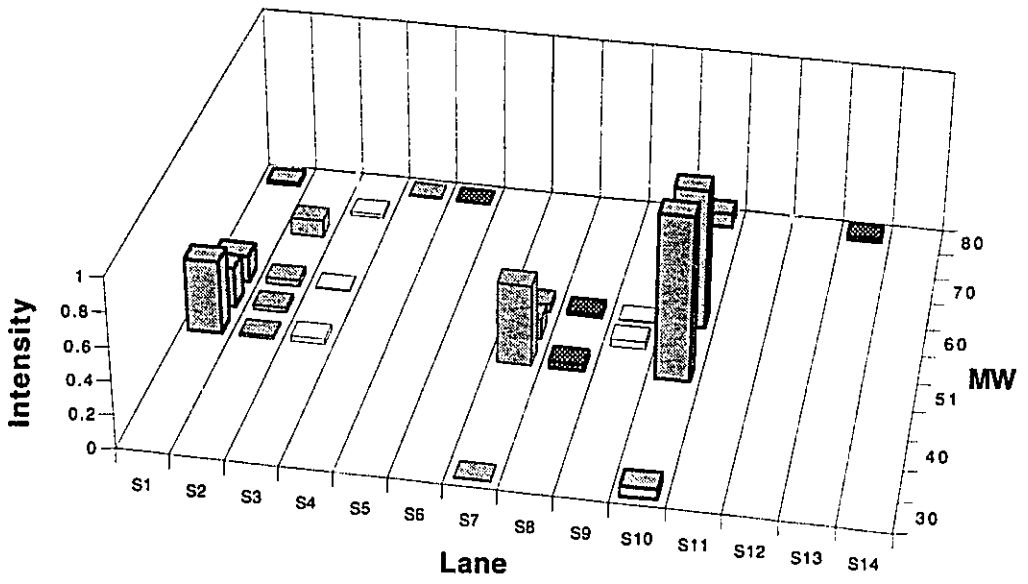


Fig. 6.5 Factor XI adsorbed from plasma to polyurethane surfaces, measured by immunoblotting method. Adsorption temperature 37°C.

S1, Human citrated plasma (HCP) sample used as a control;
S2, 5 min HCP adsorption on PPOMDA;
S3, 5 min HCP adsorption on PPACKPPO;
S4, 2 h HCP adsorption on PPOMDA;
S5, 2 h HCP adsorption on PPOBDDS;
S6, 2 h HCP adsorption on PPACKPPO;
S7, Supernatant of HCP contacted with PPOMDA for 2 h;
S8, Supernatant of HCP contacted with PPOBDDS for 2 h;
S9, Supernatant of HCP contacted with PPACKPPO for 2 h;
S10, DADFAT (human citrated plasma depleted of fibrinogen, depleted of ATIII and with added thrombin (final thrombin concentration is 5 unit/ml)) as a control;
S11, 2 h DADFAT adsorption on polystyrene;
S12, 2 h DADFAT adsorption on PPOMDA;
S13, 2 h DADFAT adsorption on PPOBDDS;
S14, 2 h DADFAT adsorption on PPACKPPO.

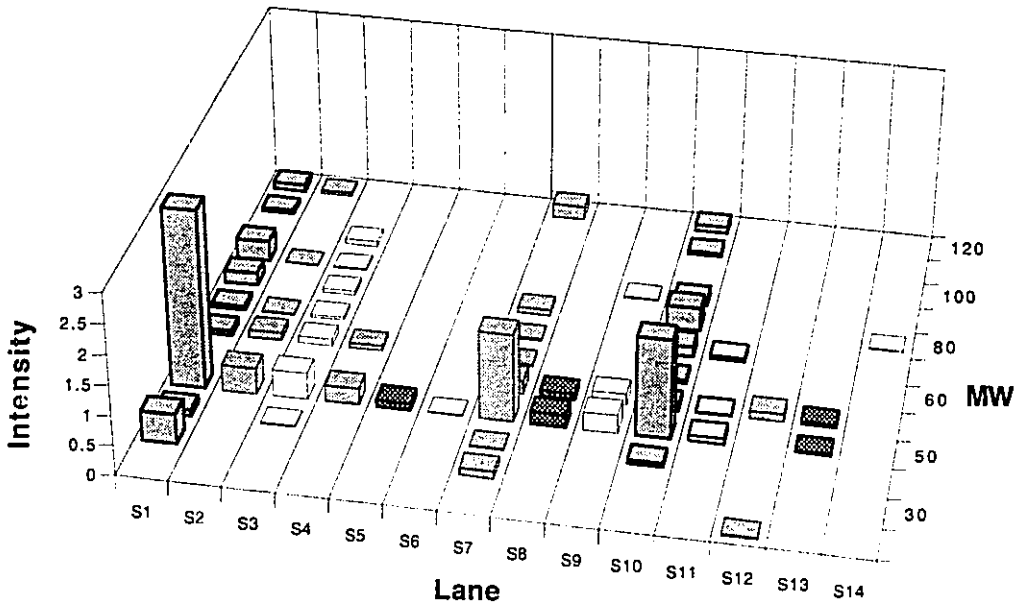


Fig. 6.6 Prekallikrein adsorbed from plasma to polyurethane surfaces, measured by immunoblotting method. Adsorption temperature 37°C.

S1, Human citrated plasma (HCP) sample used as a control;
S2, 5 min HCP adsorption on PPOMDA;
S3, 5 min HCP adsorption on PPACKPPO;
S4, 2 h HCP adsorption on PPOMDA;
S5, 2 h HCP adsorption on PPOBDDS;
S6, 2 h HCP adsorption on PPACKPPO;
S7, Supernatant of HCP contacted with PPOMDA for 2 h;
S8, Supernatant of HCP contacted with PPOBDDS for 2 h;
S9, Supernatant of HCP contacted with PPACKPPO for 2 h;
S10, DADFAT (human citrated plasma depleted of fibrinogen, depleted of ATIII and with added thrombin (final thrombin concentration is 5 unit/ml)) as a control;
S11, 2 h DADFAT adsorption on polystyrene;
S12, 2 h DADFAT adsorption on PPOMDA;
S13, 2 h DADFAT adsorption on PPOBDDS;
S14, 2 h DADFAT adsorption on PPACKPPO.

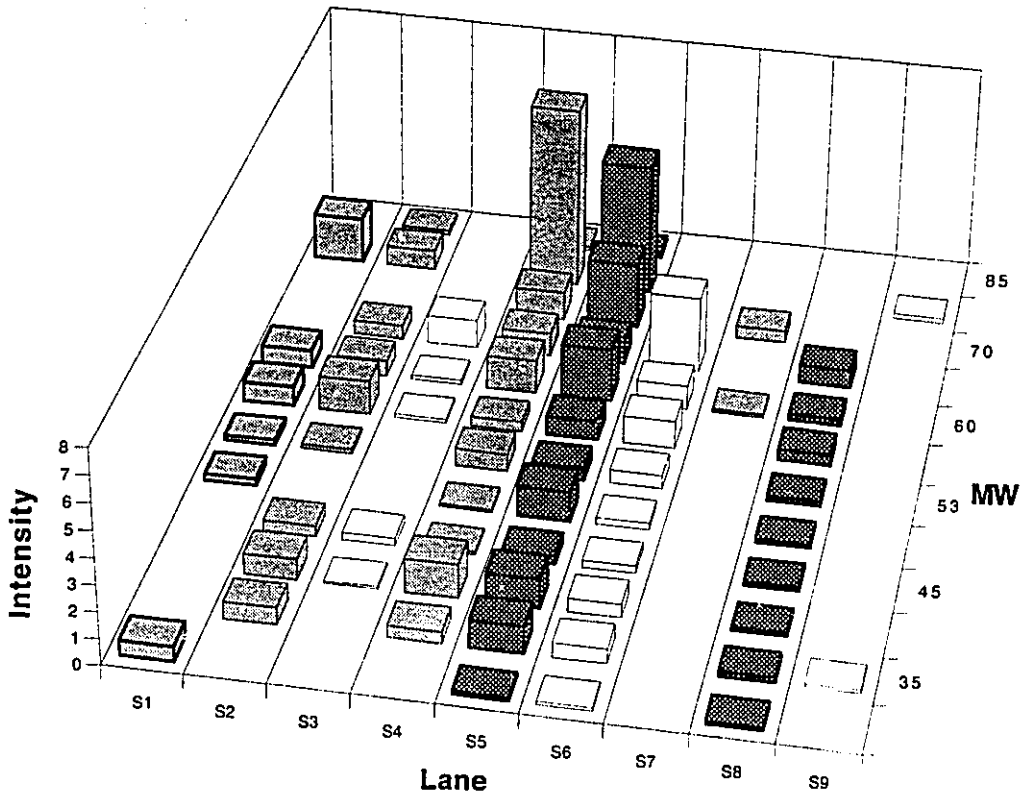


Fig. 6.7 Fibrinogen adsorbed from plasma to polyurethane surfaces, measured by immunoblotting method. Adsorption temperature 37°C.

S1, Human citrated plasma (HCP) sample used as a control;
S2, 5 min HCP adsorption on PPOMDA;
S3, 5 min HCP adsorption on PPACKPPO;
S4, 2 h HCP adsorption on PPOMDA;
S5, 2 h HCP adsorption on PPOBDDS;
S6, 2 h HCP adsorption on PPACKPPO;
S7, Supernatant of HCP contacted with PPOMDA for 2 h;
S8, Supernatant of HCP contacted with PPOBDDS for 2 h;
S9, Supernatant of HCP contacted with PPACKPPO for 2 h;

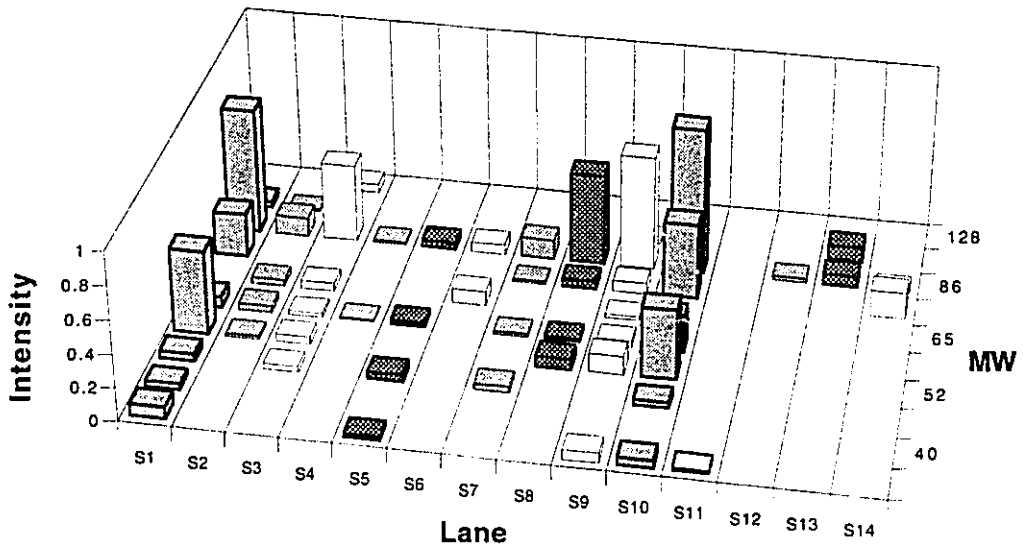


Fig. 6.8 Prothrombin adsorbed from plasma to polyurethane surfaces, measured by immunoblotting method. Adsorption temperature 37°C.

S1, Human citrated plasma (HCP) sample used as a control;
S2, 5 min HCP adsorption on PPOMDA;
S3, 5 min HCP adsorption on PPACKPPO;
S4, 2 h HCP adsorption on PPOMDA;
S5, 2 h HCP adsorption on PPOBDDS;
S6, 2 h HCP adsorption on PPACKPPO;
S7, Supernatant of HCP contacted with PPOMDA for 2 h;
S8, Supernatant of HCP contacted with PPOBDDS for 2 h;
S9, Supernatant of HCP contacted with PPACKPPO for 2 h;
S10, DADFAT (human citrated plasma depleted of fibrinogen, depleted of ATIII and with added thrombin (final thrombin concentration is 5 unit/ml)) as a control;
S11, 2 h DADFAT adsorption on polystyrene;
S12, 2 h DADFAT adsorption on PPOMDA;
S13, 2 h DADFAT adsorption on PPOBDDS;
S14, 2 h DADFAT adsorption on PPACKPPO.

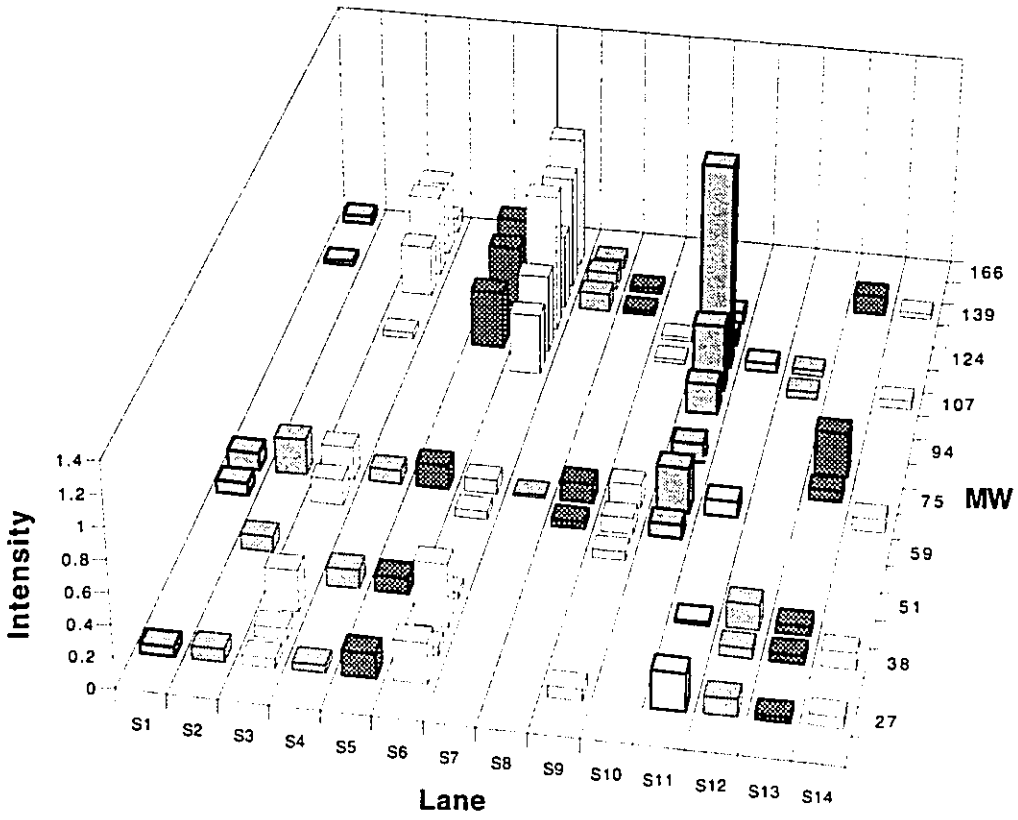


Fig. 6.9 Factor V adsorbed from plasma to polyurethane surfaces, measured by immunoblotting method. Adsorption temperature 37°C.

S1, Human citrated plasma (HCP) sample used as a control;
S2, 5 min HCP adsorption on PPOMDA;
S3, 5 min HCP adsorption on PPACKPPO;
S4, 2 h HCP adsorption on PPOMDA;
S5, 2 h HCP adsorption on PPOBDDS;
S6, 2 h HCP adsorption on PPACKPPO;
S7, Supernatant of HCP contacted with PPOMDA for 2 h;
S8, Supernatant of HCP contacted with PPOBDDS for 2 h;
S9, Supernatant of HCP contacted with PPACKPPO for 2 h;
S10, DADFAT (human citrated plasma depleted of fibrinogen, depleted of ATIII and with added thrombin (final thrombin concentration is 5 unit/ml)) as a control;
S11, 2 h DADFAT adsorption on polystyrene;
S12, 2 h DADFAT adsorption on PPOMDA;
S13, 2 h DADFAT adsorption on PPOBDDS;
S14, 2 h DADFAT adsorption on PPACKPPO.

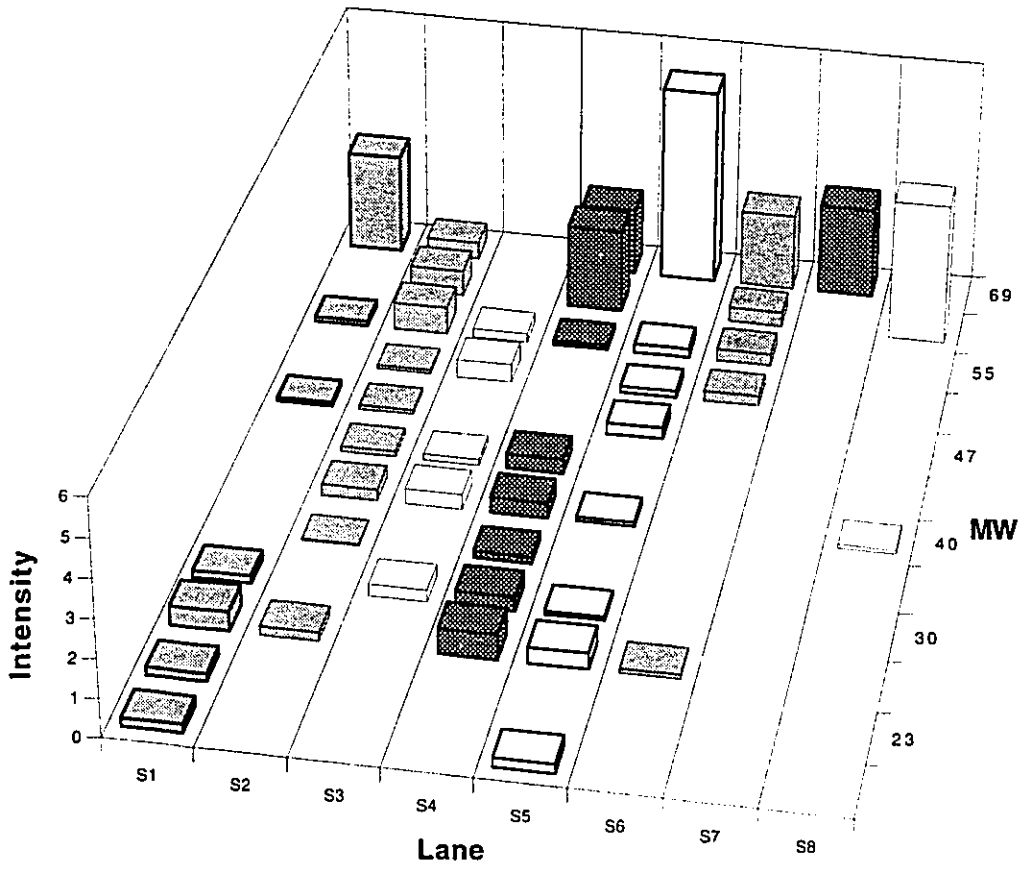


Fig. 6.10 Albumin adsorbed from plasma to polyurethane surfaces, measured by immunoblotting method. Adsorption temperature 37°C.

S1, Human citrated plasma (HCP) sample used as a control;
S2, 5 min HCP adsorption on PPOMDA;
S3, 5 min HCP adsorption on PPACKPPO;
S4, DADFAT (human citrated plasma depleted of fibrinogen, depleted of ATIII and with added thrombin (final thrombin concentration is 5 unit/ml)) as a control;
S5, 2 h DADFAT adsorption on polystyrene;
S6, 2 h DADFAT adsorption on PPOMDA;
S7 2 h DADFAT adsorption on PPOBDDS;
S8, 2 h DADFAT adsorption on PPACKPPO.

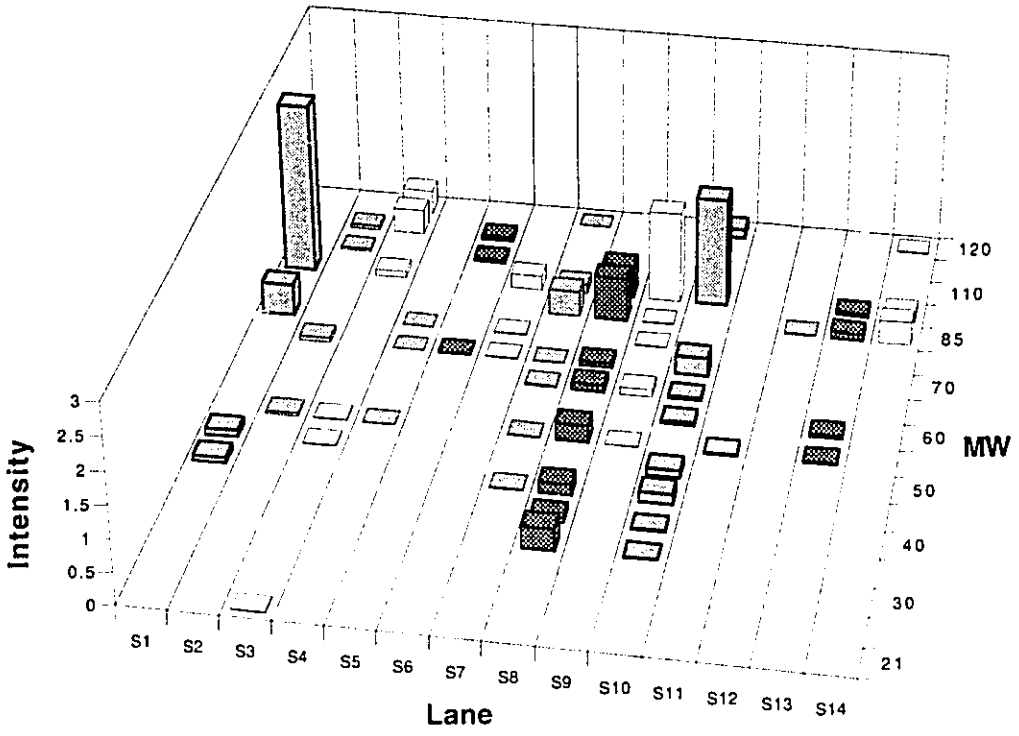


Fig. 6.11 Plasminogen adsorbed from plasma to polyurethane surfaces, measured by immunoblotting method. Adsorption temperature 37°C.

S1, Human citrated plasma (HCP) sample used as a control;
S2, 5 min HCP adsorption on PPOMDA;
S3, 5 min HCP adsorption on PPACKPPO;
S4, 2 h HCP adsorption on PPOMDA;
S5, 2 h HCP adsorption on PPOBDDS;
S6, 2 h HCP adsorption on PPACKPPO;
S7, Supernatant of HCP contacted with PPOMDA for 2 h;
S8, Supernatant of HCP contacted with PPOBDDS for 2 h;
S9, Supernatant of HCP contacted with PPACKPPO for 2 h;
S10, DADFAT (human citrated plasma depleted of fibrinogen, depleted of ATIII and with added thrombin (final thrombin concentration is 5 unit/ml)) as a control;
S11, 2 h DADFAT adsorption on polystyrene;
S12, 2 h DADFAT adsorption on PPOMDA;
S13, 2 h DADFAT adsorption on PPOBDDS;
S14, 2 h DADFAT adsorption on PPACKPPO.

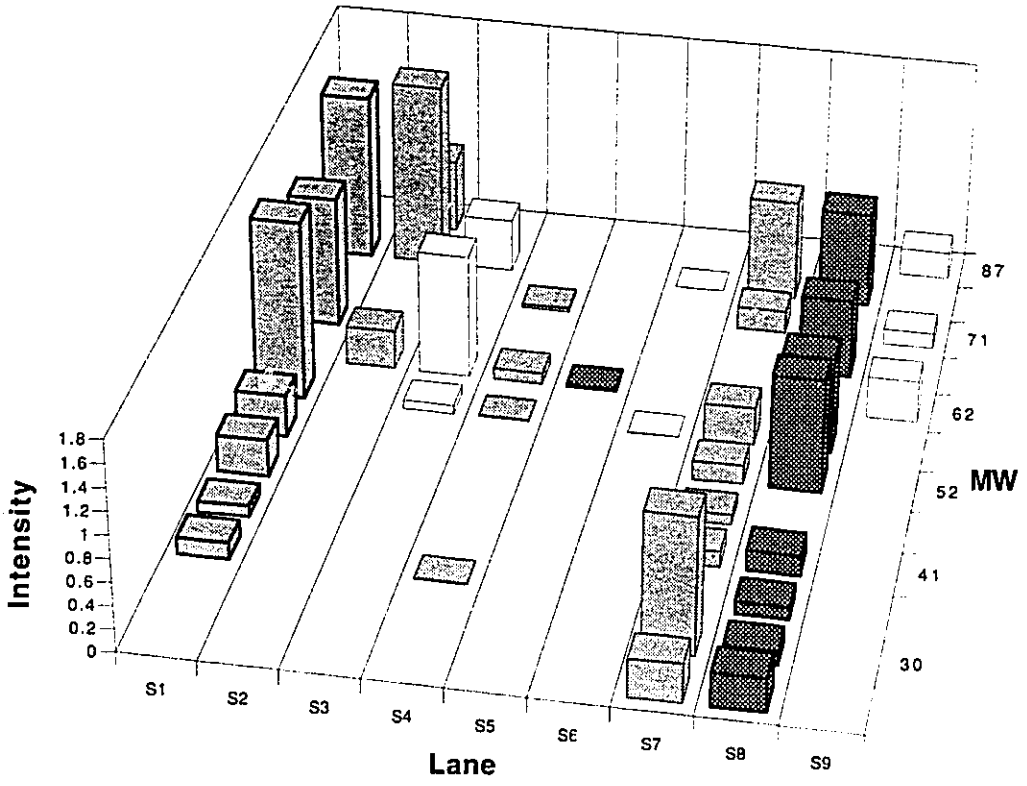


Fig. 6.12 ATIII adsorbed from plasma to polyurethane surfaces, measured by immunoblotting method. Adsorption temperature 37°C.

S1, Human citrated plasma (HCP) sample used as a control;
S2, 5 min HCP adsorption on PPOMDA;
S3, 5 min HCP adsorption on PPACKPPO;
S4, 2 h HCP adsorption on PPOMDA;
S5, 2 h HCP adsorption on PPOBDDS;
S6, 2 h HCP adsorption on PPACKPPO;
S7, Supernatant of HCP contacted with PPOMDA for 2 h;
S8, Supernatant of HCP contacted with PPOBDDS for 2 h;
S9, Supernatant of HCP contacted with PPACKPPO for 2 h.

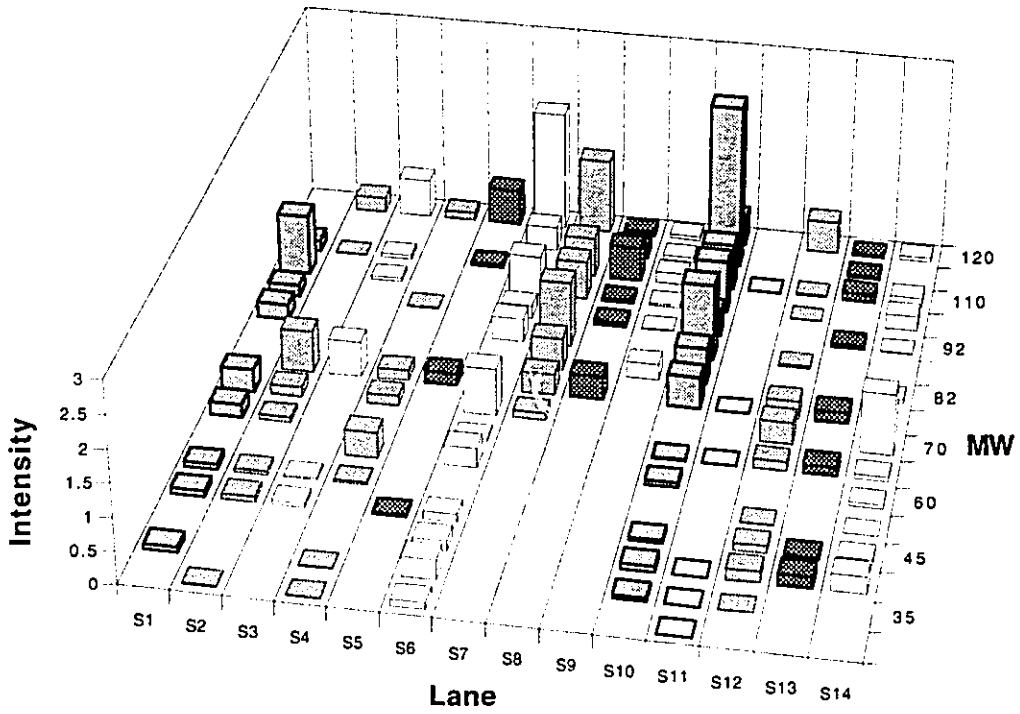


Fig. 6.13 C3 adsorbed from plasma to polyurethane surfaces, measured by immunoblotting method. Adsorption temperature 37°C.

S1, Human citrated plasma (HCP) sample used as a control;
S2, 5 min HCP adsorption on PPOMDA;
S3, 5 min HCP adsorption on PPACKPPO;
S4, 2 h HCP adsorption on PPOMDA;
S5, 2 h HCP adsorption on PPOBDDS;
S6, 2 h HCP adsorption on PPACKPPO;
S7, Supernatant of HCP contacted with PPOMDA for 2 h;
S8, Supernatant of HCP contacted with PPOBDDS for 2 h;
S9, Supernatant of HCP contacted with PPACKPPO for 2 h;
S10, DADFAT (human citrated plasma depleted of fibrinogen, depleted of ATIII and with added thrombin (final thrombin concentration is 5 unit/ml)) as a control;
S11, 2 h DADFAT adsorption on polystyrene;
S12, 2 h DADFAT adsorption on PPOMDA;
S13, 2 h DADFAT adsorption on PPOBDDS;
S14, 2 h DADFAT adsorption on PPACKPPO.

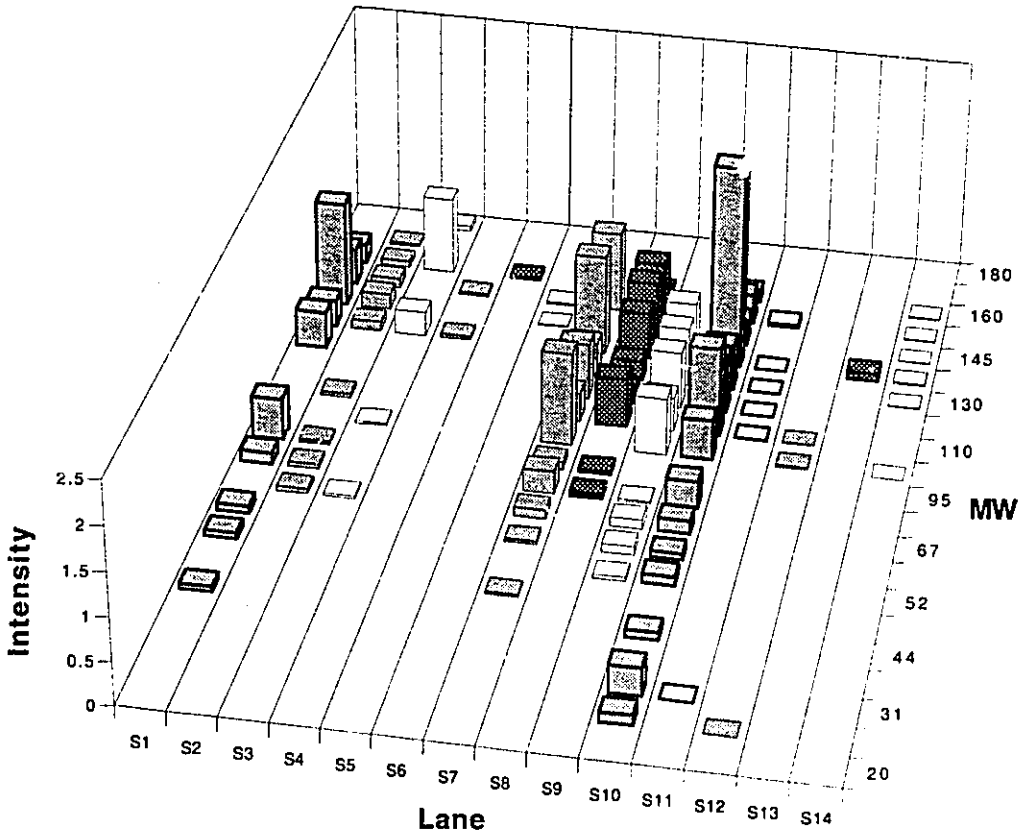


Fig. 6.14 Fibronectin adsorbed from plasma to polyurethane surfaces, measured by immunoblotting method. Adsorption temperature 37°C.

S1, Human citrated plasma (HCP) sample used as a control;
S2, 5 min HCP adsorption on PPOMDA;
S3, 5 min HCP adsorption on PPACKPPO;
S4, 2 h HCP adsorption on PPOMDA;
S5, 2 h HCP adsorption on PPOBDDS;
S6, 2 h HCP adsorption on PPACKPPO;
S7, Supernatant of HCP contacted with PPOMDA for 2 h;
S8, Supernatant of HCP contacted with PPOBDDS for 2 h;
S9, Supernatant of HCP contacted with PPACKPPO for 2 h;
S10, DADFAT (human citrated plasma depleted of fibrinogen, depleted of ATIII and with added thrombin (final thrombin concentration is 5 unit/ml)) as a control;
S11, 2 h DADFAT adsorption on polystyrene;
S12, 2 h DADFAT adsorption on PPOMDA;
S13, 2 h DADFAT adsorption on PPOBDDS;
S14, 2 h DADFAT adsorption on PPACKPPO.

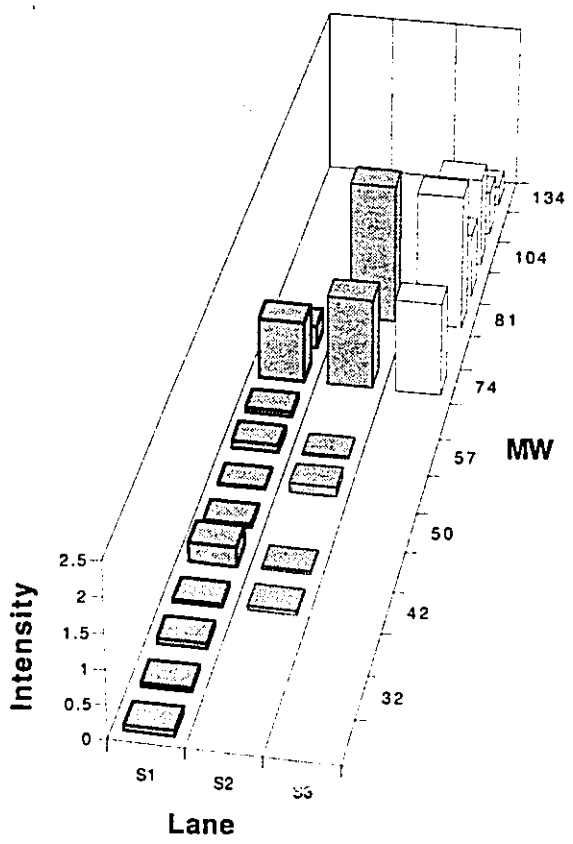


Fig. 6.15 Vitronectin adsorbed from plasma to polyurethane surfaces, measured by immunoblotting method. Adsorption temperature 37°C.

S1, Human citrated plasma (HCP) sample used as a control;
S2, 5 min HCP adsorption on PPOMDA;
S3, 5 min HCP adsorption on PPACKPPO.

CHAPTER 7

FACTOR XII ACTIVATION AT POLYURETHANE SURFACES

7.1 INTRODUCTION

The blood compatibility of biomaterials could, in principle, be improved by making the surface inert so that it would not activate platelets or the coagulation process. Some approaches along these lines have been based on incorporation of albumin or polyethylene oxide into polymer surfaces in order to prevent platelet adhesion (albumin) and protein adsorption (polyethylene oxide) (Young et al., 1983; Coleman et al., 1982; Chaikof et al., 1990). One particularly favored approach is to make the surface antithrombotic by heparinization (Smith and Sefton, 1992, 1993; Kim et al., 1983; Albanese et al., 1994; Larsson et al., 1987; Park et al., 1992).

Another logical and practical approach is to provide a surface which can inhibit the contact phase of plasma coagulation. The contact phase has been shown to be involved in the activation of the pathways of intrinsic coagulation, fibrinolysis, kallikrein-kinin generation and complement activation (Fuhrer et al., 1990), and these four systems are linked by a number of interactions. Therefore, the inhibition of the contact phase may have a significant impact on these various biological processes.

The mechanism of contact phase activation has been extensively studied (Cochrane and Griffin, 1982). Factor XII, prekallikrein, high-molecular-weight kininogen (HK) and factor XI are the major components of this system. As shown in Fig. 7.1 (p. 191), the initiating event is the activation of factor XII. Factor XII can either self-activate or be activated by negatively charged surfaces. Activated factor XII (XIIa) can then activate prekallikrein and factor XI. Both activated factor XI (XIa) and kallikrein "feed back" to activate more factor XII, thus forming a positive feedback cycle and resulting in an autoacceleration process. HK forms complexes with factor XIIa and kallikrein to promote these reactions (Kaplan and Silverberg, 1987).

The activation of the contact phase on negatively charged surfaces can be inhibited by proteins such as fibrinogen, IgG, and albumin (Cochrane and Griffin, 1982). The primary inhibitor of factor XIIa in plasma is C1-inhibitor. ATIII inhibits factor XIIa, factor XIa and kallikrein by forming a noncovalent complex (Stead et al., 1976; Beeler et al., 1985; Vennerod et al., 1976). The activity of factor XIIa also can be affected directly or indirectly by inhibitors of kallikrein such as *alpha-2*-macroglobulin and aprotinin.

The tripeptide D-phe-pro-arg CH_2Cl (PPACK) is an inhibitor of thrombin of high specificity. As reported in Chapter 4, PPACK has been grafted onto polyurethanes in an attempt to make the polymers antithrombogenic. Because the arginyl residue of PPACK is positively charged, the PPACKPPO surface may exhibit an inhibitory effect on the activation of the contact phase. As reported in Chapter 6, the PPACKPPO surface adsorbs proteins from plasma in such a way that coagulation is not activated. It is interesting that a significant amount of intact HK is adsorbed on the surface, indicating that the contact

phase is not activated in the plasma contacting the PPACKPPO surface. Due to the importance of the contact phase in initiating adverse blood-surface interactions, it was believed that the possibility that surface-bound PPACK might inhibit the contact phase should be investigated. In this chapter, studies of the generation of factor XIIa in plasma contacting polyurethane surfaces and the interaction of factor XIIa with the PPACKPPO polymer using a chromogenic substrate assay are reported.

7.2 EXPERIMENTAL

Materials

TBS buffer: 12.1 g Tris and 11.68 g NaCl were dissolved in 2 l of double-distilled water, and the buffer was adjusted to pH 7.4 with HCl ([Tris]=0.05 M, [NaCl]=0.1 M).

Factor XII substrate: 25 mg N-CBZ-lys-phe-arg p-nitroanilide (Nova Biochem, Switzerland) was dissolved in 50 ml TBS; the final concentration was 0.5 mg/ml.

TBS/Ca buffer: 1.11 g $CaCl_2$ was dissolved in 100 ml TBS ([Ca]=0.1 M).

TBS/Ca/Platelin buffer: 1.11 g $CaCl_2$ and 4 standard vials of Platelin®, a platelet factor 3 reagent (2.5 ml size, product No. 35501, Organon Teknika Corporation, Durham, NC) were dissolved in 10 ml double distilled water. The volume was then brought to 100 ml with TBS, ([Ca]=0.1 M; Platelin dilution, 1:10).

Aprotinin/TBS: 10 mg aprotinin (46 trypsin inhibitor units, Sigma, St. Louis, MO) was dissolved in 1 ml TBS; the final concentration was 10 mg/ml. Aprotinin was used as an inhibitor of kallikrein in this work.

Factor XIIa (Calbiochem, La Jolla, CA) was a 1 mg/ml solution in TBS.

Antithrombin III (ATIII, from human plasma, 300-400 units/mg, Sigma) was a 10 unit/ml solution in TBS.

Human citrated **plasma** was obtained from Red Cross, Hamilton, Ontario, prepared by drawing 9 volumes of blood (from more than 20 donors) into 1 volume of 3.8% trisodium citrate, and then centrifuging for 15 min, 2000 rpm and 10 min, 3000 rpm. The prothrombin time, the partial thromboplastin time and the concentration of coagulation factors were found to be in normal range. The plasma was aliquoted and stored at -70°C until use.

The synthesis of the polyurethanes PPOMDA, PPOBDDS, PEOMDA, PEOBDDS, and PPACKPPO was described in Chapter 4. The preparation of the polyurethane-coated surfaces was described in Chapter 5.

Determination of factor XIIa activity in solution using the chromogenic substrate

A typical experiment was as follows. After placing 210 µl TBS/Ca/Platelin buffer and 30 µl of N-CBZ-lys-phe-arg p-nitroanilide solution (0.5 mg/ml) into the polyurethane-coated wells of 96-well microtitre plates, 10 µl factor XIIa solution (1 mg/ml) was added. The final concentrations were: factor XII=0.04 mg/ml; [Tris]=0.042 M; [Ca]=0.08 M. The optical density (OD) of the p-nitroaniline (pNA) released from the substrate by factor XIIa was measured as a function of time at 37°C.

Determination of factor XIIa activity in plasma using the chromogenic substrate

A typical experiment was as follows: after adding 200 µl pooled human citrated plasma (diluted to be in the range of 5% to 100% of normal concentration), 20 µl TBS/Ca/Platelin buffer and 30 µl factor XII substrate were added to the polyurethane-coated wells of 96-well microtitre plates. The concentration of the pNA released by factor XIIa was monitored at 405 nm and 37°C. The plate was stirred for 5 seconds every min. The final concentrations were: plasma 1% to 80% of normal concentration; [Ca]=0.008 M; factor XII substrate=0.06 mg/ml; Platelin dilution, 1:125.

The other experiments have been described in the preceding chapters. In the work discussed here, all assays were performed at least three times. The data shown are for typical, single assays.

7.3 RESULTS AND DISCUSSION

Kinetic characteristics of factor XIIa generation in plasma

There are two forms (alpha and beta) of activated factor XII. Alpha factor XIIa is a surface-bound enzyme and it activates surface-bound factor XI and surface-bound prekallikrein (Kaplan and Silverberg, 1987). Beta factor XIIa lacks the binding site for surfaces and, once formed, is released into the fluid phase of plasma. Because factor XI activation is surface dependent (Revak et al., 1978), beta factor XIIa possesses only 2% to 5% of the coagulant activity of factor XIIa (Kaplan and Austen, 1971). On the other hand, beta factor XIIa is an

important prekallikrein activator (Revak et al., 1978; Kaplan and Austen, 1971; Wiggins et al., 1977). As reported in Chapter 6, the amounts of surface-bound prekallikrein are much smaller than surface-bound HK (compare Fig. 6.6 with Fig. 6.4) although they have similar concentrations in plasma (Fig. 2.2). Wiggins et al. (1977) reported that prekallikrein is not an adhesive protein and most of the surface-bound prekallikrein will be eluted into solution within 5 min. It was supposed that 80% of kallikrein is activated by beta factor XIIa in the fluid phase of plasma (Wiggins et al., 1977). Because kallikrein cleaves factor XII 3000 times faster than factor XII self-activation (Rosing et al., 1985), the activation of the contact phase is closely related to the generation of kallikrein by beta factor XIIa.

N-CBZ-lys-phe-arg p-nitroanilide is a highly specific substrate of beta factor XIIa (Cho et al., 1984). This substrate has two positively charged groups, lys and arg, which bind strongly to the active centre of beta factor XIIa resulting in the release of pNA. Because of the rapidity of the reaction between beta factor XIIa and N-CBZ-lys-phe-arg p-nitroanilide, the pNA formed when the substrate is present in plasma can be considered to be produced mainly by beta factor XIIa (Cho et al., 1984). Kallikrein has a potential to share substrates with factor XIIa (Kluft et al., 1983). It was found in the present work (Fig. 7.7) that inhibiting kallikrein with aprotinin does not influence significantly the release rate of pNA from N-CBZ-lys-phe-arg p-nitroanilide in plasma after the activation of the contact phase, indicating that the substrate has high specificity for factor XIIa versus kallikrein. Therefore in this work, N-CBZ-lys-phe-arg p-nitroanilide was used to measure the generation of beta factor XIIa in plasma contacting

different polyurethane surfaces. The data were used to describe the activity of factor XIIa as well as the activation of the contact phase in general.

pNA has a light yellow color which can be measured at 405 nm using a visible light spectrophotometer or plate reader. The concentration of pNA is proportional to the measured optical density (OD 405 nm) when the OD is in the range below 2.5. The slope of the OD versus time curve (or pNA concentration versus time curve) then gives a measure of the concentration of factor XIIa or the activity of factor XIIa when the concentration of the substrate is known. Plasma clotting results in an increase in plasma turbidity which contributes to the OD reading, especially at high plasma concentrations. In this work, a correction was made for turbidity, and only the contribution to OD from pNA is reported.

The OD 405 versus time curves measured in plasma contacting the PPOBDDS surfaces and the PPOMDA surfaces are shown in Fig. 7.2 (p. 192) and Fig. 7.3 (p. 193) respectively. The plasma concentrations were in the range of 1% to 80%, diluted with TBS containing 0.008 M Ca^{2+} and 1:125 diluted Platelin. Platelin is a preparation of the procoagulant phospholipids expressed on the activated platelet surface and is a reagent routinely used in plasma clotting experiments. In the normal procedure for the measurement of clotting time recommended by the supplier a 1:3 dilution of Platelin is used. In this work, a 1:125 dilution of Platelin was used, resulting in clotting times of plasma as long as 10 min. This longer clotting time or slower reaction rate provided a longer experimental time in which to examine the activity of factor XIIa in detail. As shown in Fig. 7.2 and Fig. 7.3, very different factor XIIa activity levels were found at different plasma concentrations.

The data shown in Fig. 7.2 and Fig. 7.3 indicate that there are three kinetic phases in the OD versus time curve or generation of factor XIIa activity. The first is the initiation or lag phase, where the slope of the OD versus time curve is zero for a certain time (initiation/inhibition processes in balance). Then the factor XIIa concentration (slope) increases rapidly over one or two min. For 10% plasma contacting the PPOBDDS surface (10% curve in Fig. 7.2), the initiation phase lasted about 2 min. For 10% plasma contacting the PPOMDA surface (10% curve in Fig. 7.3), it lasted about 10 min. The second phase is the propagation phase, where the slope of the pNA versus time curve (the factor XIIa activity) becomes constant for a certain time. For 10% plasma contacting the PPOBDDS surface, this phase lasted about 35 min, and in the 10% plasma contacting the PPOMDA surface, it lasted 5 min. The third phase is the termination phase, where the slope of the OD versus time curve starts to decrease and eventually reaches zero. In this phase, since the slope is proportional to both the factor XIIa concentration and the N-CBZ-lys-phe-arg p-nitroanilide concentration, a zero slope correlates to either a zero concentration of factor XIIa or a zero concentration of N-CBZ-lys-phe-arg p-nitroanilide remaining in the system.

As shown in Figs. 7.2 and 7.3, the concentration of plasma in the experiments influenced the shape of the OD versus time curves. However, all the curves show the three kinetic phases, suggesting that the activation mechanisms of factor XII at the plasma-polymer interface are the same for the different plasma concentrations.

Influence of surface type on factor XII activation in plasma

Fig. 7.4 (p. 194) compares the effects of the different polyurethane surfaces on factor XII activation. On the PPOBDDS and PEOBDDS surfaces, factor XIIa activity was observed immediately and the generation of factor XIIa was so fast that the acceleration period (slope increase of the OD versus time curve) was probably completed in seconds. This rapid generation of factor XIIa is typical of an autoacceleration process and supports the theory of positive feedback activation of factor XII by kallikrein shown in Fig. 7.1. As discussed in Chapter 6, factor XII in plasma appears to be activated to some extent during the plasma preparation. However, small amounts of factor XIIa usually are not able to induce factor XII autoacceleration because they are inhibited immediately by plasma inhibitors such as C1-inhibitor, ATIII and *alpha*-2-macroglobulin. A significant amidolytic activity of factor XIIa can be observed only when the balance of factor XII activation/inhibition is disrupted and the autoacceleration of factor XIIa generation begins (Kaplan and Silverberg, 1987). Either high levels of foreign activating factors or low concentrations of factor XIIa inhibitors will result in the autoacceleration of factor XIIa generation once the activation rate exceeds the inhibition rate. Therefore, the strong amidolytic activity of factor XIIa in the plasma contacting the PPOBDDS surface at the beginning of the reaction (Fig. 7.4, p. 181) suggests that the negatively charged sulphonate groups on the PPOBDDS surface are able to activate factor XII rapidly. This is in agreement with the published results of others (Cochrane and Griffin, 1982).

For the PPOMDA and PEOMDA surfaces, the pNA versus time curves are significantly different from those of the BDDS surfaces (Fig. 7.4). The initiation

phase of the OD versus time curve lasted as long as 15 min on the PPOMDA surface and 30 min on the PEOMDA surface. Neither of the surfaces has any negatively charged groups. On the contrary, the urethane and urea groups may be slightly positively charged, and these positive charges may contribute to the delay in factor XII activation (Cochrane and Griffin, 1982).

The generation rates of pNA (the slopes of the OD versus time curves) in the propagation phase on the PPOMDA and PEOMDA surfaces were also much slower than on the BDDS surfaces. This much slower generation of pNA suggests that not only is the initiation of the contact phase dependent on the surface, but the concentration of factor XIIa generated in the plasma during the propagation phase also depends on the contacting surface. This finding suggests that plasma needs a foreign surface as a cofactor to propagate the generation/autoacceleration cycle for factor XIIa, in agreement with the conclusion (Kaplan and Silverberg, 1987) that beta factor XIIa is produced by the cleavage of surface-bound alpha factor XIIa by kallikrein.

No factor XIIa activity at all was found on the PPACKPPO surface (Fig. 7.4). The positive charges on this surface may inhibit factor XIIa (Cochrane and Griffin, 1982), or the surface may be inert and so prevent factor XII activation. The second hypothesis seems less plausible than the first, because factor XII is activated in plasma contacting the PEOMDA and PPOMDA surfaces which may be considered to be relatively inert, probably by other activating mechanisms such as autoactivation, activation by the proteins adsorbed on the surfaces, or activation by the air-plasma interface. Because such activating mechanisms also potentially exist in the PPACKPPO system, factor XII should be activated in the plasma contacting the PPACKPPO surface. Therefore the finding that no

factor XIIa activity is detectable in plasma contacting the PPACKPPO surface suggests that factor XIIa is rapidly inhibited by the surface, so that the balance of initiation/inhibition is maintained as in normal blood.

The question still remains as to the mechanism by which the PPACKPPO surface inhibits factor XIIa. There are two possible mechanisms which could account for this inhibitory activity: (1) the positive charges on the surface may inhibit factor XIIa; (2) factor XIIa may bind to PPACK forming a complex which has no factor XIIa activity. Since the interactions of positively charged groups are non specific, their ability to inhibit factor XIIa may be limited. It may be that the strong inhibitory activity shown by the PPACKPPO surface is due to a specific interaction between factor XIIa and the surface-bound PPACK.

To investigate whether PPACK can inhibit factor XIIa, free PPACK was added to a purified factor XIIa solution in buffer. The reaction was repeated in different polyurethane (PPOMDA, PPOBDDS, PEOMDA, and PEOBDDS) coated wells of 96-well microtitre plate, and essentially the same results were found. As shown in Fig. 7.5 (p. 195) on the PPOBDDS surface, in the absence of PPACK (control curve), the OD 405 increased rapidly and reached a plateau at about 15 min. The high colour generation rate suggests that the substrate was very quickly consumed by factor XIIa. On the other hand, in the presence of PPACK (PPACK curve in Fig. 7.5), almost no pNA was produced. These data clearly show that PPACK is able to inhibit factor XIIa under these conditions. The inactivation of factor XIIa in plasma contacting the PPACKPPO surface may thus be attributable to the surface-bound PPACK. It is also possible that free PPACK leached from the surface may contribute to the inhibition of factor XIIa, especially considering that beta factor XIIa is not surface-bound. However, as

discussed in Chapters 5 and 8, surface-bound PPACK appears to play the major role.

Influence of inhibitors on factor XII activation

PPACK is known to be an inhibitor of thrombin of high specificity. It may also inhibit factor XIIa, but due to the complicated interactions between thrombin and other coagulation proteins, it is not clear at this time whether PPACK in plasma inhibits factor XIIa directly or indirectly through its effects on factor XIa, thrombin, and kallikrein. To investigate further the initiation and control mechanism of the contact phase, the behavior of the thrombin inhibitors ATIII and PPACK, and the kallikrein inhibitor aprotinin may be examined and compared.

ATIII is a major endogenous inhibitor of thrombin and factor Xa. Factor XI is known to be activated by thrombin, so that if the activation of factor XII in plasma is mainly due to feedback activation by factor XIa, the inhibition of thrombin by ATIII should block the feedback activation of factor XI by thrombin and thus inhibit factor XII activation. As shown in Fig. 7.6 (p. 196), for plasma containing 0.2 unit/ml ATIII, factor XIIa activity was observed at the very beginning of the reaction although the autoacceleration was slower and the propagation phase of the OD versus time curve was slightly delayed in comparison with the control. Since ATIII itself can slowly inhibit factor XIIa (Cochrane and Griffin, 1982), the slight delay of factor XII activation in ATIII-containing plasma should not be attributed to the inhibition of thrombin by ATIII. Thus the data suggest that the inhibition of factor XIIa, factor XIa and thrombin by ATIII has a very limited influence on the activation of the contact phase.

Aprotinin is a potent inhibitor of kallikrein (Trautschold et al., 1967), so that the addition of aprotinin to plasma will inhibit kallikrein, thereby blocking the acceleration cycle of the contact phase system. Thus, there are two possible mechanisms to account for the activation of factor XII in plasma containing aprotinin: (1) autoactivation of factor XII, (2) activation by factor XIa. As shown in Fig. 7.6, the activation of factor XII was influenced by aprotinin in two ways: first, factor XII activation was delayed by about 20 min; second, the amidolytic activity of factor XIIa was reduced (decrease in slope of the pNA versus time curve, Fig. 7.6). This finding confirms that kallikrein is an important part of the autoacceleration cycle of the contact phase, and that both the initiation and the later course of the generation of factor XIIa depend on kallikrein. The data shown in Fig. 7.6 also suggest that when the autoacceleration cycle is blocked by aprotinin, the activation of factor XII by factor XIa and factor XIIa itself becomes a slow process which lacks acceleration characteristics. This finding is in agreement with the result of the ATIII inhibition experiment discussed above, showing that factor XIa and thrombin are not important for the activation of factor XII. In other words, factor XIIa, HK and factor XIa without kallikrein cannot form an effective autoacceleration cycle for the contact phase.

To investigate further the mechanism of initiation of factor XIIa generation by kallikrein, aprotinin was added to plasma contacting the PPOBDDS surface at different times following contact (Fig. 7.7, p. 197). The data show that the influence of aprotinin on factor XIIa activity differed during the course of contact activation. The addition of aprotinin to plasma at 10 or 20 min produced a negligible effect on factor XIIa activity, while the same amount of aprotinin added at the beginning of the reaction greatly delayed and reduced factor XII

activation (Fig. 7.6). This result indicates that aprotinin can prevent factor XII activation but does not directly inhibit activated factor XII once formed. More importantly, the data in Fig. 7.7 suggest that aprotinin does not influence factor XIIa activity after its formation, neither inhibiting factor XIIa nor reducing factor XIIa generation during the propagation phase. In other words, kallikrein is involved only in the autoacceleration/initiation process of the contact phase. It may be that the propagation phase of the contact system involves factor XIIa only, and if so kallikrein would have no influence on it.

The data in Fig. 7.7 also show that inhibiting kallikrein by adding aprotinin to plasma did not reduce significantly the release rate of pNA from N-CBZ-lys-phe-arg-pNA, indicating that pNA is not produced by kallikrein. This result confirms that CBZ-lys-phe-arg-pNA is a highly specific substrate of factor XIIa.

In contrast to ATIII and aprotinin, PPACK completely inhibited factor XII activation in plasma (Fig. 7.6). As discussed above, PPACK probably influences factor XII activation by direct inhibition of factor XIIa, and not by other indirect mechanisms such as the inhibition of thrombin or kallikrein. In comparison with ATIII and aprotinin, PPACK appears to be a much more powerful inhibitor of contact phase activation. It seems likely that factor XIIa is the key enzyme of the contact phase, and inhibiting factor XIIa is therefore the most effective way to prevent contact phase activation.

Calcium ions and Platelin (similar to the phospholipids on activated platelets) are important cofactors for thrombin activation. The effect of thrombin on factor XII activation can thus be further investigated by varying the calcium ion and Platelin concentrations in plasma. As shown in Fig. 7.8 (p. 198), factor

XIIa activity was slightly reduced by adding Ca and Platelin to plasma. Since the addition of Ca and Platelin should strongly promote thrombin activity (Chapter 8), the decreased factor XIIa activity in the presence of increased thrombin activity shown in Fig. 7.8 agrees with the ATIII experiment (Fig. 7.6) and suggests again that thrombin does not influence factor XII activation, although factor XIIa will influence thrombin activation. This finding is very interesting because it contradicts the expectation that thrombin will feed back to activate factor XII.

Influence of plasma concentration and ionic strength on factor XIIa activity

To distinguish the effect of ionic strength from the effects of other factors such as surface charge, plasma concentration, and C1 inhibitor concentration, the activation of factor XII in diluted plasma on the negatively charged surface PPOBDDS and the "inert" surface PPOMDA were re-examined. The ionic strength of the plasmas contacting both polymers was the same.

As shown in Fig. 7.2, at the highest plasma concentration (80%), generation of factor XIIa started at 10 min, suggesting that the plasma inhibitors of factor XIIa overpowered the activating groups of the surface for 10 min. In the diluted plasmas (for example 10% plasma), the concentration of plasma inhibitors of factor XIIa such as C1 inhibitor is reduced while the concentration of surface activating groups is the same. In addition, the ionic strength was reduced by dilution. Therefore, factor XII was activated almost immediately due to decreased capacity for inhibition which presumably disrupted the balance of initiation/inhibition in the system.

Comparing the 10% curve in Fig. 7.3 with the 10% curve in Fig. 7.2, it is clear that pNA production on the PPOMDA surface was further delayed for more than 10 min in comparison with the PPOBDDS surface. Since the ionic strengths in the two plasma solutions were the same, the difference must be due only to the surfaces. The data thus indicate that surface is of major importance for factor XII activation, although ionic strength also plays a role.

Summary

From the experiments described in this chapter, it was found that surface-bound PPACK and free PPACK effectively inhibited factor XIIa and contact phase activation. Therefore PPACKPPO may be a material that can inhibit all of the pathways that are contact-phase dependent, including intrinsic coagulation.

From the data reported above, the following conclusions are drawn:

1. Factor XIIa generation is greatly accelerated by kallikrein. Indeed, kallikrein is a major activator of factor XII, with other activation mechanisms making a smaller contribution.

2. On plasma-contacting surfaces, activating agents such as negatively charged sulphonate groups have a strong influence on factor XII activation.

3. Soluble inhibitors such as aprotinin may compete with surface activation during the initial stages of contact phase activation. Only when the surface is able to overpower the inhibitors can the autoacceleration of factor XIIa production occur.

4. Although the contact phase has a significant influence on the activation of thrombin, thrombin does not appear to influence the contact phase.

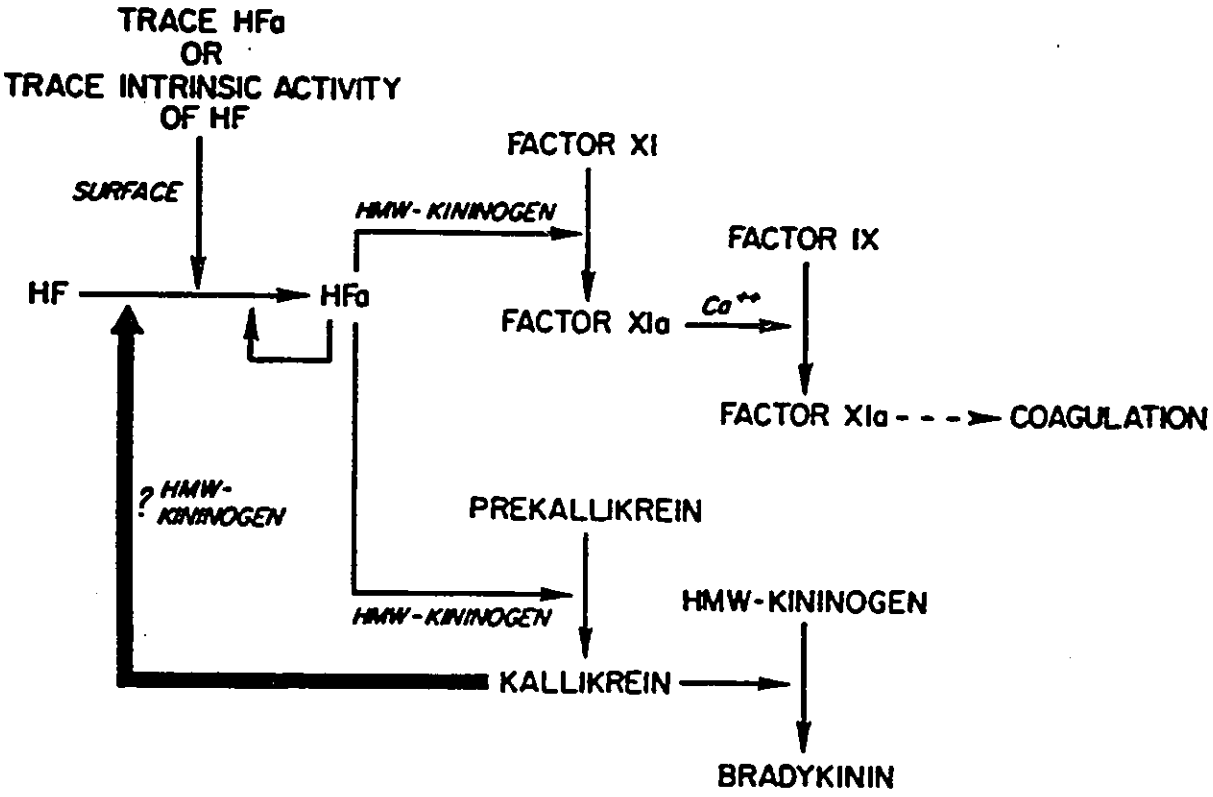


Fig. 7.1 Schematic diagram of the contact activation pathway depicting factor XII (HF) autoactivation, activation of kallikrein and factor XI, and the generation of bradykinin. The major cofactor role of HMW kininogen (HK) is on activation of HF substrates. Its direct role in HF activation by kallikrein is less clear and varies with the surface used (Kaplan and Silverberg, 1987).

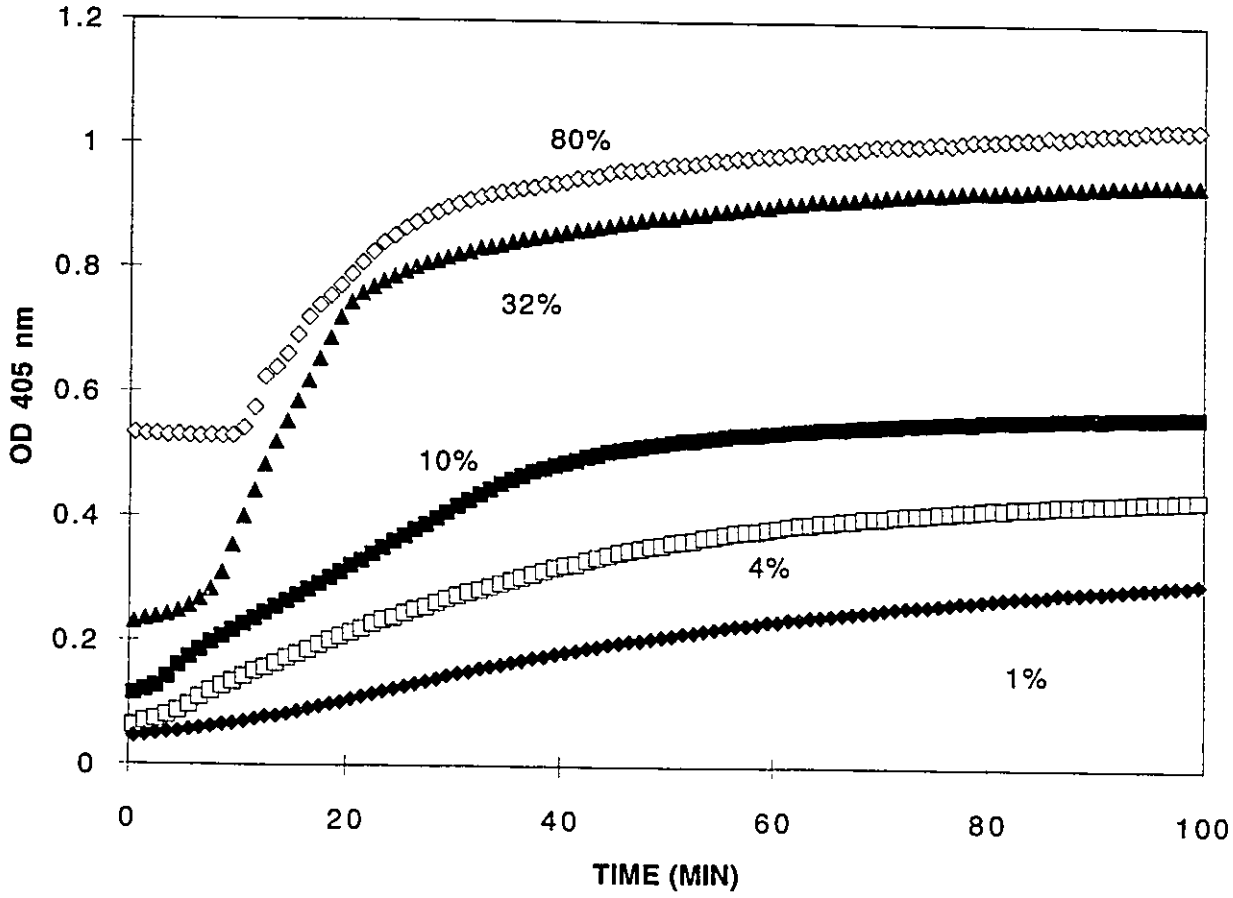


Fig. 7.2 Factor XIIa activity in plasma contacting a PPOBDDS polyurethane surface (37°C). Optical density (OD) of pNA released from Z-lys-phe-arg pNA by factor XIIa was measured as a function of time. The concentration of plasma is shown adjacent to the curves. TBS/Ca/Platelin buffer was used for dilution.

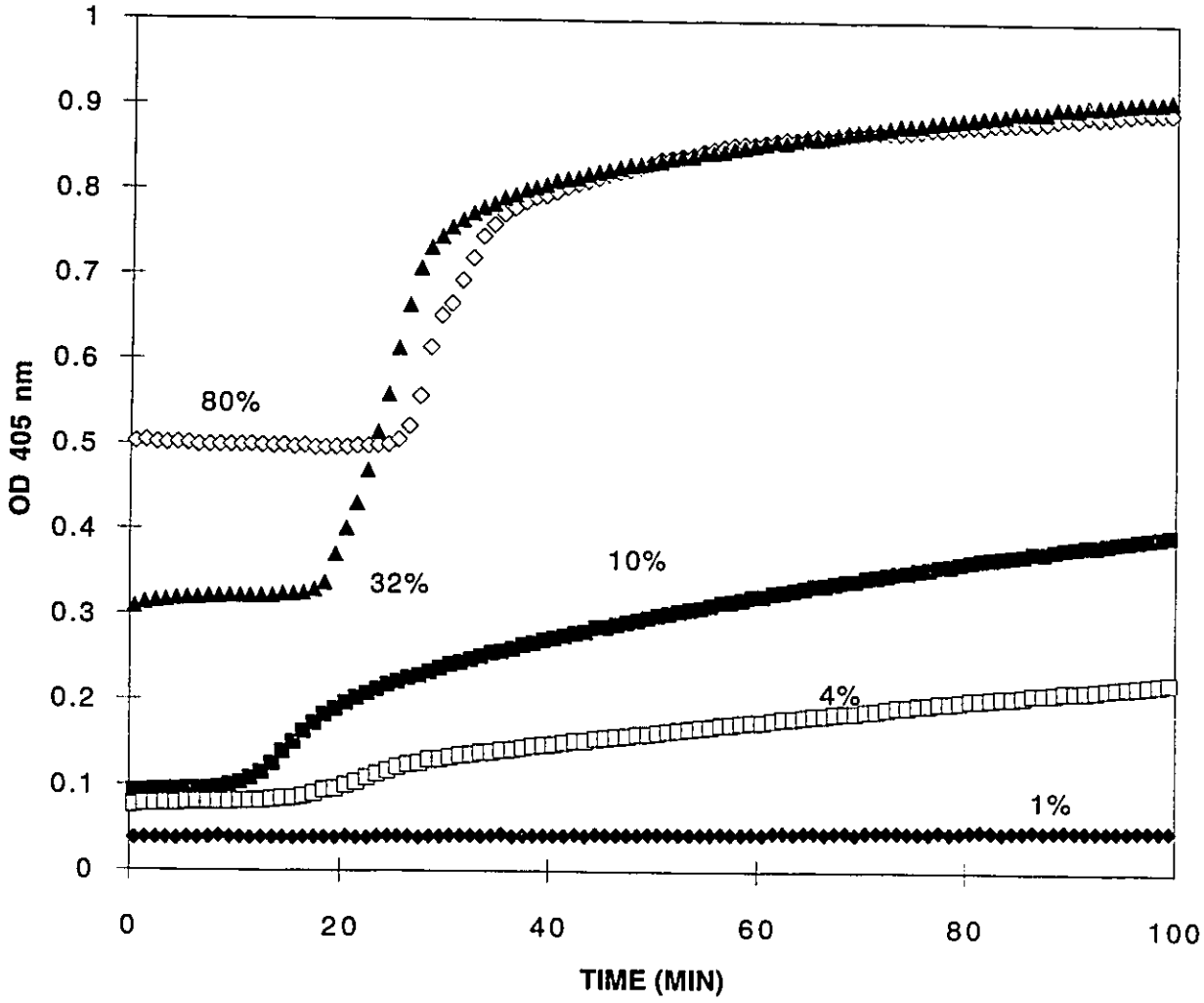


Fig. 7.3 Factor XIIa activity in plasma contacting a PPOMDA polyurethane surface (37°C). Optical density (OD) of pNA released from Z-lys-phe-arg pNA by factor XIIa was measured as a function of time. The concentration of plasma is shown adjacent to the curves. TBS/Ca/Platelin buffer was used for dilution.

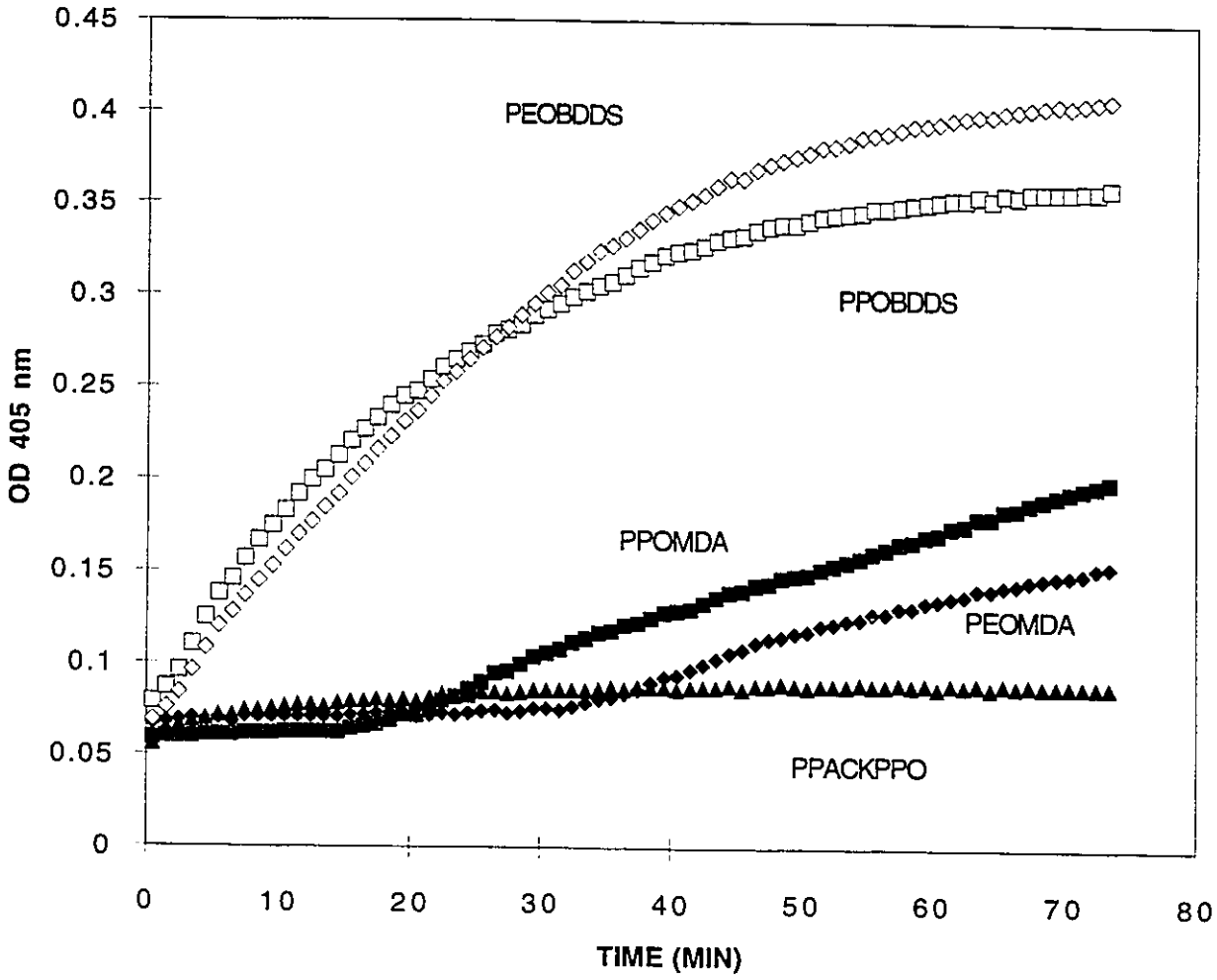


Fig. 7.4 Factor XIIa activity in 4% plasma contacting polyurethane surfaces (37°C). Optical density (OD) of pNA released from Z-lys-phe-arg pNA by factor XIIa was measured as a function of time. TBS/Ca/Platelin buffer was used for plasma dilution.

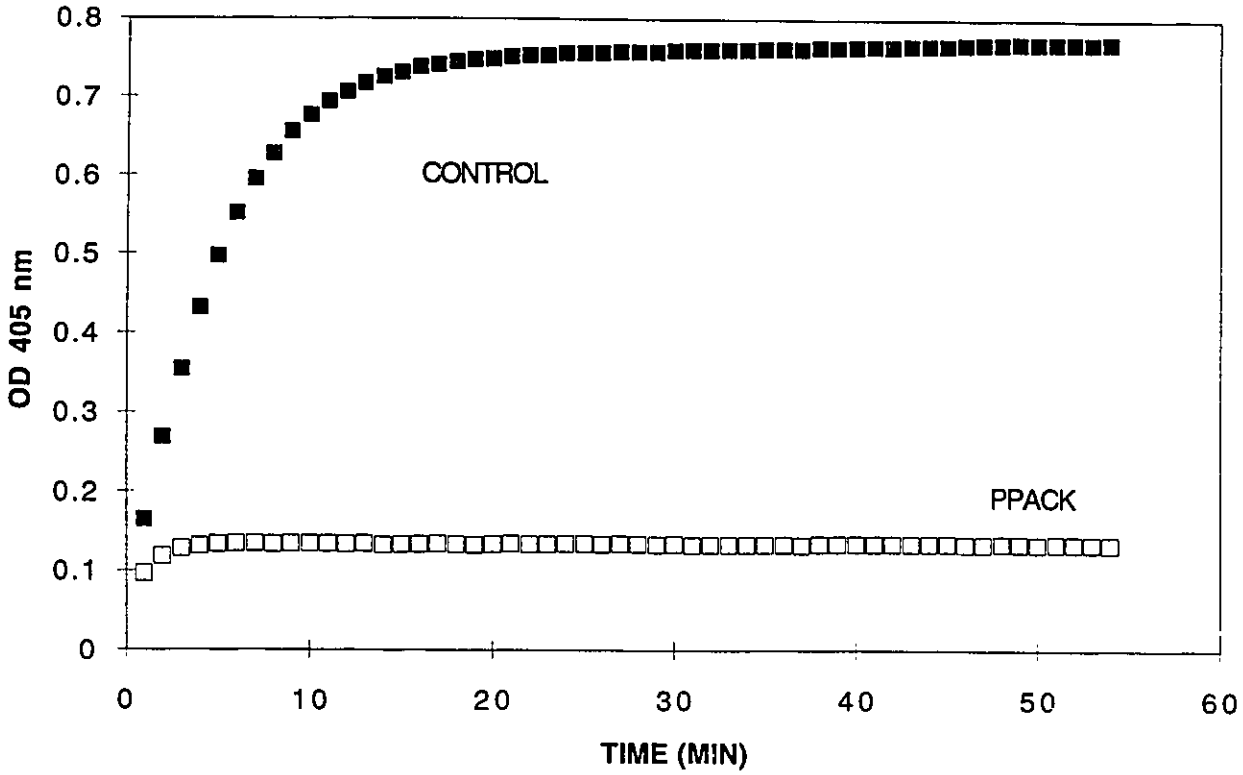


Fig. 7.5 Optical density (OD) of pNA released from Z-lys-phe-arg pNA by purified factor XIIa measured as a function of time on the PPOBDDS polyurethane surface at 37°C. Reaction medium: TBS/Ca/Platelin; PPACK concentration: 0.05 mg/ml; XIIa concentration: 0.036 mg/ml.

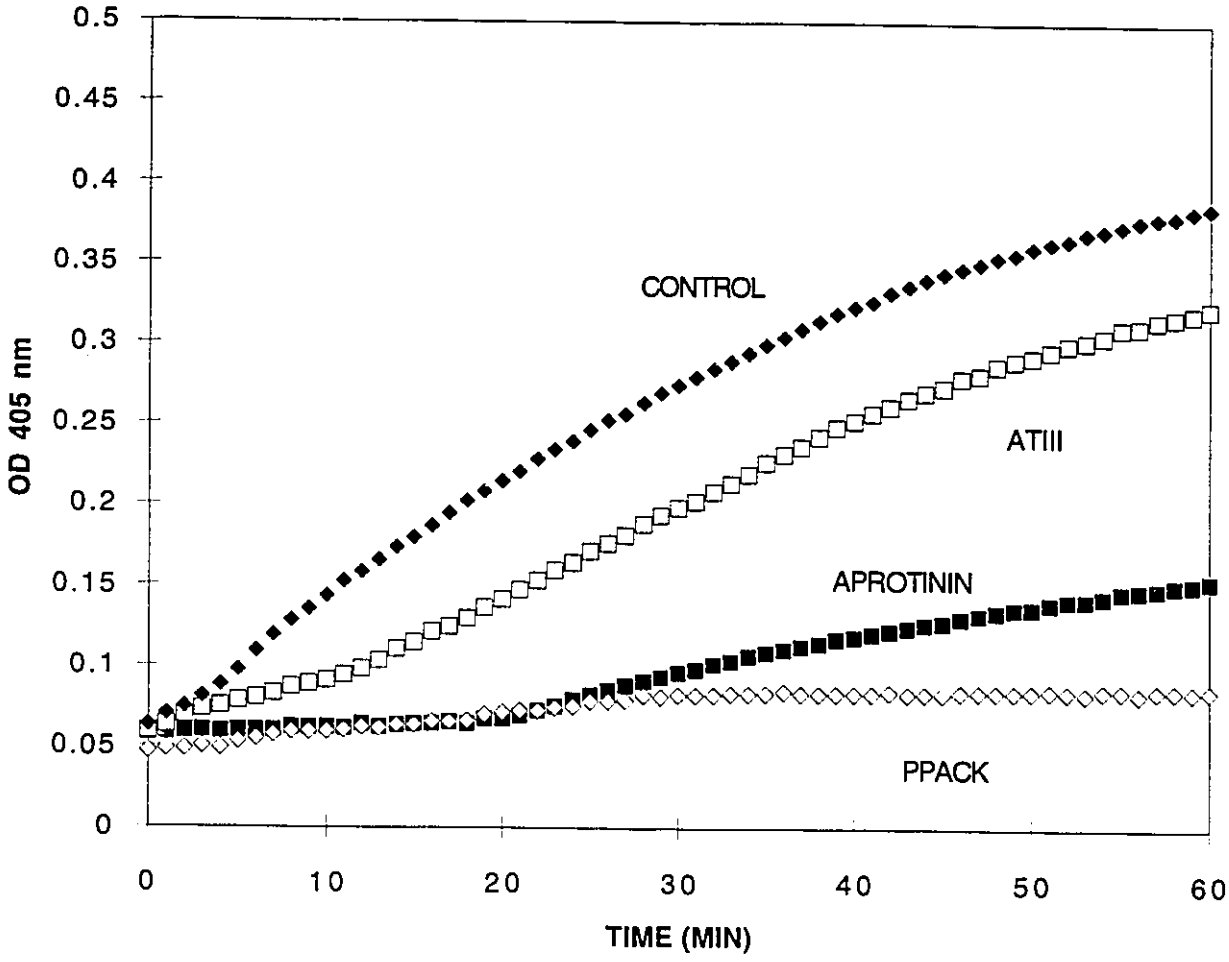


Fig. 7.6 Factor XIIa activity in 4% plasma diluted with TBS/Ca/Platelin buffer contacting an activating surface PPOBDDS polyurethane at 37°C. Optical density (OD) of pNA released from Z-lys-phe-arg pNA by factor XIIa was measured as a function of time. PPACK: 0.05 mg/ml, aprotinin: 0.2 mg/ml (0.92 unit/ml), and ATIII: 0.2 unit/ml were added to examine the influence of these inhibitors on the activity of factor XIIa.

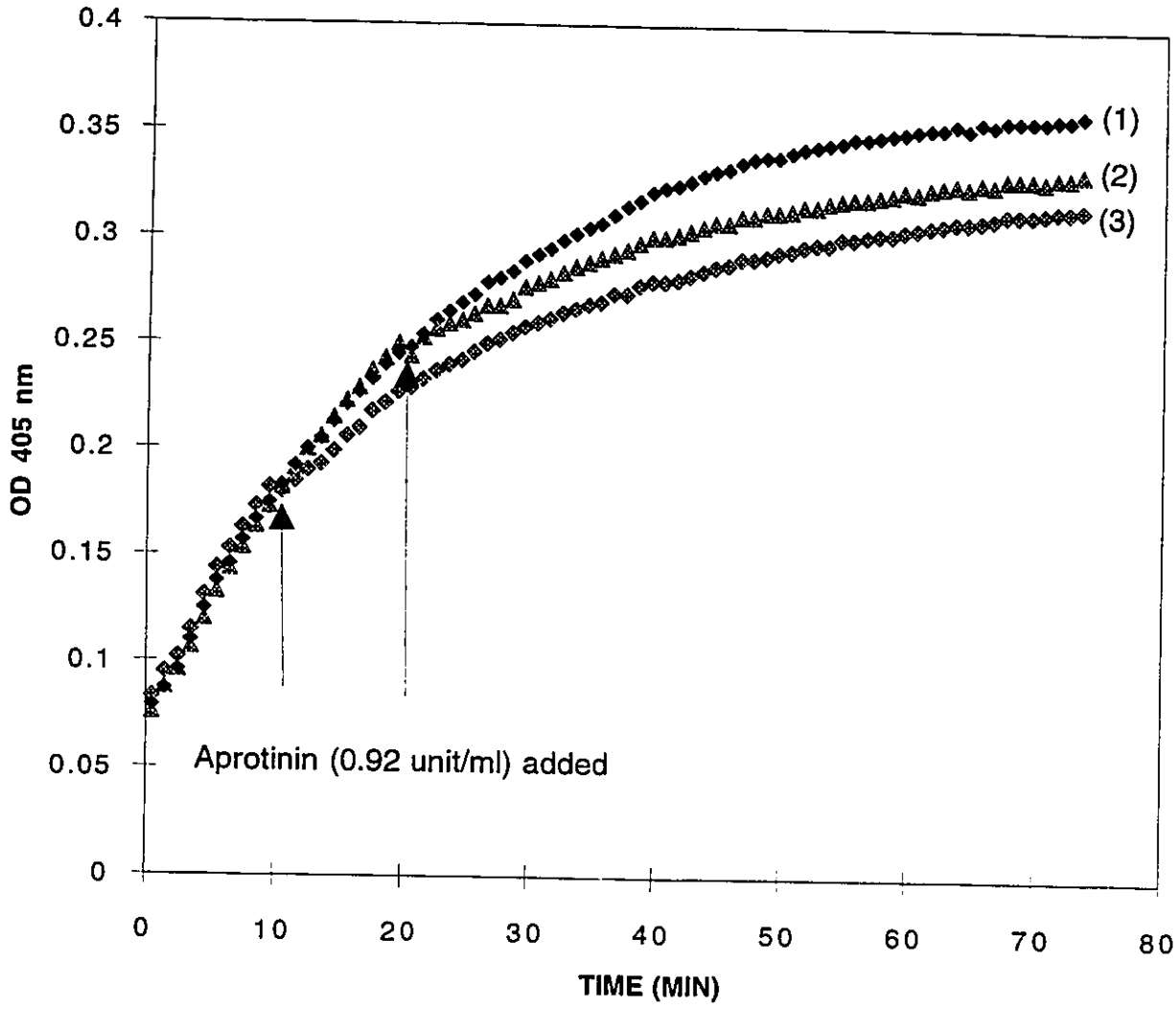


Fig. 7.7 Factor XIIa activity in 4% plasma diluted with TBS/Ca/Platelin buffer contacting an activating surface PPOBDDS polyurethane at 37°C. Optical density (OD) of pNA released from Z-lys-phe-arg pNA by factor XIIa was measured as a function of time. Aprotinin 0.2 mg/ml (0.92 unit/ml) was added at 10 min and 20 min respectively to examine its influence on the activity of factor XIIa. (1) No aprotinin added; (2) Aprotinin added at 20 min; (3) Aprotinin added at 10 min.

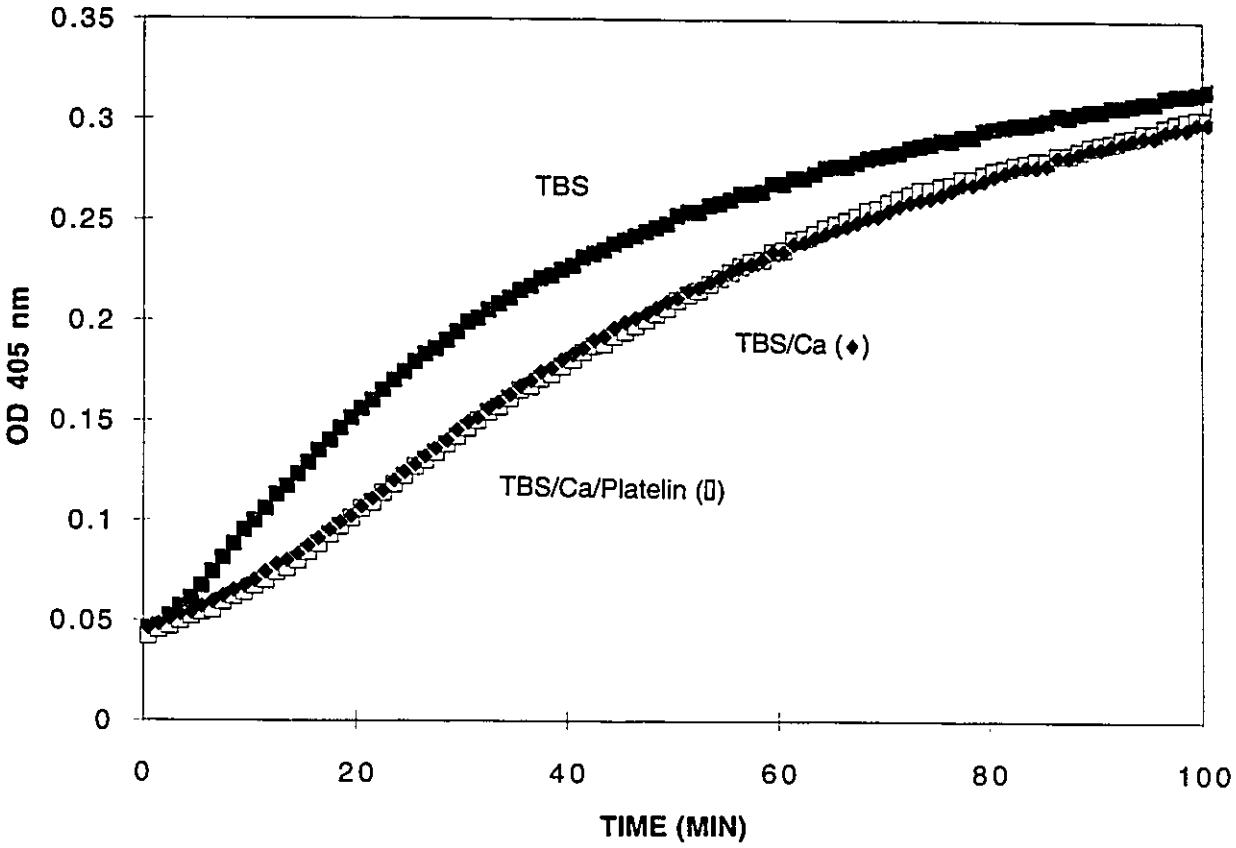


Fig. 7.8 Factor XIIa activity in 1% plasma contacting an activating surface PPOBDDS polyurethane at 37°C. Optical density (OD) of pNA released from Z-lys-phe-arg pNA by factor XIIa was measured as a function of time. Plasma was diluted with TBS, TBS/Ca, and TBS/Ca/Platelin buffers to examine the possible influence of Platelin on the generation of factor XIIa.

CHAPTER 8
THROMBIN GENERATION IN PLASMA CONTACTING POLYURETHANE SURFACES

8.1 INTRODUCTION

In the preceding chapters, thrombin adsorption (Chapter 4), the activity of adsorbed thrombin (Chapter 5), and the composition of the protein layer deposited from plasma (Chapter 6) on the various polyurethane model surfaces synthesized in this work were discussed. These studies provided a basis for understanding some of the interactions occurring at the plasma-polymer interface. With this foundation, a chromogenic substrate method was developed to examine the mechanism of factor XII-polyurethane surface interactions (Chapter 7).

The objective of the work reported in this chapter was to study an important practical question related to blood biocompatibility, namely the influence of biomaterial surfaces on the production of physiologically significant concentrations of thrombin during biomaterial plasma contact. This question has to do with the propagation of coagulation as opposed to its initiation discussed in preceding chapters. A chromogenic substrate method was used to study the coagulation behavior of plasma reconstituted in different ways in contact with the various polyurethane model surfaces. It was found that a negatively charged sulphonate-containing surface (PPOBDDS) promotes the initiation of the contact phase but has little effect on the subsequent production

of thrombin. An "inert" surface (PEOMDA) was found to delay but not prevent clotting. It is also concluded that for significantly improved blood compatibility, a practical approach might be to use materials containing PPACK both grafted to the surface and free for controlled release into the blood so as to inhibit completely both the contact phase and the later stages of coagulation which are thrombin-dependent.

8.2 EXPERIMENTAL

N-benzoyl-ile-glu-gly-arg p-nitroanilide, a chromogenic substrate of factor Xa, **N-benzoyl-pro-phe-arg p-nitroanilide**, a chromogenic substrate of kallikrein, and **N-p-tosyl-gly-pro-arg p-nitroanilide**, a chromogenic substrate of thrombin, were purchased from Sigma, St. Louis, MO. **Factor Xa** was purchased from Calbiochem, La Jolla, CA. Other materials were described in the preceding chapters.

Determination of thrombin activity in plasma using chromogenic substrate

A typical experiment was as follows: Pooled human citrated plasma (200 µl, concentration in the range 1.25% to 100%, diluted with TBS), 20 µl TBS/Ca/Platelin buffer and 30 µl thrombin substrate N-p-tosyl-gly-pro-arg p-nitroanilide (0.5 mg/ml) were placed sequentially in the polyurethane-coated wells of 96-well microtitre plates. The release of pNA by thrombin was measured at 405 nm and 37°C. The plate was shaken for 5 sec every min during the measurement. The final concentrations were: plasma, 1% to 80%; $[Ca^{2+}]$=0.008 M; Platelin dilution 1:125; concentration of thrombin substrate

0.06 mg/ml. The activities of factor XIIa, factor Xa and kallikrein in the plasma were measured in the same way as for thrombin using the appropriate chromogenic substrates (concentrations 0.06 mg/ml).

All assays were performed at least three times. The data were essentially the same for the replicate experiments. The data shown are for typical, single assays.

Other experiments have been described in the preceding chapters.

8.3 RESULTS AND DISCUSSION

Kinetic characteristics of thrombin activity

In this work, the formation of thrombin was investigated by assaying the amidolytic activity of the thrombin generated in plasma contacting polyurethane surfaces. N-p-tosyl-gly-pro-arg p-nitroanilide was used as a specific substrate of thrombin. It has the same amino acid sequence, gly-pro-arg, as the cleavage site of fibrinogen and is used routinely for thrombin assay (Huseby and Smith, 1980). When plasma reconstituted with calcium ions and Platelin was incubated with different polyurethane surfaces, differences in thrombin generation were observed as indicated by the release of pNA from the substrate (Fig. 8.1, p. 217 - 218).

It is interesting that the generation of amidolytic activity shown in Fig. 8.1 has two main kinetic phases: (1) the initiation phase from time zero to the onset of amidolytic activity; this phase is influenced by the polymer surface; (2) the propagation phase from the onset of amidolytic activity until the OD versus time curve reaches a plateau. The slope of the OD versus time curve gives a

measure of the concentration of thrombin in the plasma. In the propagation phase, the OD versus time curve is essentially concave up, indicating a continuously increasing concentration of thrombin.

While the slope of the OD versus time curve for factor XIIa activity (shown in Fig. 7.1) slowly decreases after the propagation phase, the corresponding slope for thrombin activity (shown in Fig. 8.1) decreases to zero in a very short time. In addition, all the curves for the different polymer surfaces reach a similar plateau value which represents the complete conversion of the chromogenic substrate. These data indicate that the thrombin concentration in plasma is maintained at a high level during the entire propagation phase until all the substrate in the system is consumed.

Activation of thrombin in plasma contacting polyurethane surfaces

As shown in Fig. 8.1a, thrombin activity is observed in 80% plasma contacting the PPOBDDS and PEOBDDS surfaces at about 6 min. The slope of the OD versus time curve increases sharply for several min, then becomes almost constant from 10 min until the curve reaches a plateau at 18 min. Similar OD versus time curves are found for the PPOMDA and PEOMDA surfaces, but the activation time (at about 13 min) is prolonged by about 8 min. For the PPACKPPO surface, however, no thrombin activity is observed up to 10 h (not shown). Similar data were obtained for 10% plasma (Fig. 8.1b).

The finding that the PPACKPPO surface completely inhibits the generation of thrombin (Fig. 8.1a) suggests that the incorporated PPACK is highly effective in inhibiting coagulation. It is well known that the contact phase and the extrinsic pathway produce only an initial trace amount of thrombin in

plasma, which can be inhibited via slow inactivation by ATIII (Saito, 1991). However, if the initial rate of thrombin generation exceeds the rate of inhibition by ATIII, the thrombin concentration then quickly reaches a level which can activate platelets and factors V and VIII which in turn act as cofactors for factors IXa and Xa, thus dramatically increasing the activity of these enzymes and leading to production of large amounts of thrombin. This positive thrombin feedback step is crucial for the production of a physiologically significant concentration of thrombin in plasma. Therefore a critical requirement for improving the blood compatibility of biomaterials may be seen as the inhibition of initial thrombin formation rapidly enough to block the thrombin feedback process. As reported in Chapter 7, PPACK was found to inhibit effectively the activation of factor XII in plasma. Thus it may be expected that the PPACKPPO surface will inhibit the initiation of coagulation by inhibiting the contact phase. Moreover, as shown in Fig. 8.1, the PPACKPPO surface also effectively inhibits thrombin generation. Therefore, the PPACK incorporated in the PPACKPPO material appears to retain its capacity to inhibit factor XIIa and thrombin. This dual inhibition of the contact phase and the thrombin dependent coagulation processes is presumably responsible for the effectiveness of the PPACKPPO surface in preventing plasma clotting.

Other polyurethane surfaces behaved very differently from the PPACKPPO surface. The sulphonate groups on the BDDS polyurethane surfaces appear to promote the activation of thrombin (Fig. 8.1a and Fig. 8.1 b). As reported in Chapter 7, the negatively charged sulphonate groups are expected to activate the contact phase with subsequent production of thrombin.

The PPOMDA and PEOMDA surfaces have no negatively charged groups to activate the contact phase (Chapter 7), and the generation of thrombin is delayed. However, the delay was only 7 min longer than for the BDDS surface, suggesting that, in addition to the rapid activation of the contact phase by negative charges, there are other mechanisms which generate thrombin more slowly, for example, the activation of coagulation by the autoactivation of factor XI or by interactions at the plasma-air interface (see below for further discussion).

It is interesting to note that the slopes of the OD versus time curves on all the polymer surfaces are similar in the propagation phase of coagulation (Fig. 8.1a). This result suggests that the production of thrombin was not influenced significantly by the nature of the polymer surface, although the surface did influence the initial phase of thrombin generation.

Influence of polyurethane surfaces on the propagation phase of coagulation

If the nature of the contacting polymer surface is not important for the generation of thrombin, then one may ask what the controlling factor is. Clearly, identification of the controlling factor or factors for thrombin generation following blood-material contact is of considerable importance for this area of research. To this end, the generation of thrombin in plasma contacting the sulphonated PPOBDDS surface was studied using different plasma media.

The removal of calcium ions and platelet phospholipid to inhibit the thrombin feedback process and the use of PPOBDDS to provide a negatively charged surface for contact phase activation should permit estimation of the

contribution of the contact phase to thrombin generation while diminishing the influence of the thrombin feedback mechanism. As shown in Fig. 8.2 (p. 219), the concentration of thrombin is reduced by about 90% (decrease in the slope of the "TBS" curve in Fig. 8.2) when calcium and Platelin are not present. Since the thrombin feedback mechanism is blocked, the generation of thrombin in the calcium-depleted plasma may be seen as representing the initial production of thrombin by factor IXa and factor Xa which are generated by the polymer surface through contact phase activation. The very low concentration of thrombin in the calcium-depleted plasma suggests that the contact phase is not directly responsible for the production of a physiologically effective concentration of thrombin in plasma, but is important only for coagulation initiation.

Adding calcium back to the plasma significantly increases the slope of the OD versus time curve ("TBS/Ca" curve in Fig. 8.2), indicating that calcium concentration is an important factor for the propagation phase of coagulation. Because calcium is needed for the binding of factor IXa and factor Xa to the platelet surface, it seems likely that the platelet surface, not the polymer surface, is the major production site of thrombin.

Platelin is a preparation which contains the phospholipids on the activated platelet surface. The addition of Platelin to plasma will lead to formation of liposome-like particles which can mimic the functioning of the activated platelet surface in blood. As expected, by adding Platelin (which may be considered "equivalent" to adding platelets back to plasma) the slope of the OD versus time curve is further increased ("TBS/Ca/Platelin" curve in Fig. 8.2). This finding suggests that the Platelin surface is the major site for factor IXa and

factor Xa assembly and therefore is the site for thrombin production in this experiment.

As shown in Fig. 8.2, in all the plasma samples (with or without calcium and Platelin) the initial phase of thrombin production was of the same duration (about 4 min), indicating that the mechanism of initiation of coagulation by the surface is the same. However, the propagation phase depends strongly on calcium and phospholipid. This can be considered a further indication that the initiation of coagulation depends on the contacting surface, while the propagation phase or steady production of thrombin depends only on interactions in the plasma itself.

It is known that factor IXa and factor Xa bind to the surface of activated platelets and thereby increase thrombin production by a factor of more than 10^6 in comparison with free factors IXa and Xa (Mann et al., 1990). Therefore, the physiological site for thrombin production in vivo is the platelet surface. The data in Fig. 8.2 show that in the in vitro plasma-polymer system, the major site for production of thrombin is also the "platelet" surface, not the polymer surface. This suggests that in blood-surface interactions, coagulation is initiated at the surface. The initially formed trace amount of thrombin then activates platelets, and the production of thrombin is transferred from the polymer surfaces to the highly efficient platelet surface shortly after the initiation phase. This understanding of the coagulation process at the biomaterial surface should be important for the design of blood-compatible materials.

The effect of a contact phase inhibitor on initial thrombin generation

In the above section, the influence of various polymer surfaces on the initial phase of coagulation was attributed to the contact system. To obtain additional support for this hypothesis it was useful to study directly the relationship between the contact system and thrombin generation. In this section, the discussion is focused on the effect of aprotinin, an inhibitor of kallikrein, on thrombin generation. Since kallikrein is involved in the activation of factors XI and XII, inhibiting kallikrein should block the autoacceleration cycle of the contact phase (Chapter 7) while allowing the direct generation of factor XIIa by the polymer surface to continue. The working hypothesis is that if kallikrein is completely inhibited by aprotinin in a plasma-polyurethane system, factor XI will then have three relatively minor possible activation mechanisms: (1) activation by factor XIIa which is generated on the polyurethane surface; (2) autoactivation (Naito and Fujikawa, 1991); (3) activation by the thrombin feedback mechanism (Naito and Fujikawa, 1991). If the activation of factor XI by factor XIIa in the absence of kallikrein and the autoactivation of factor XI are negligible in comparison with activation by the thrombin feedback mechanism, initiation of coagulation in aprotinin-containing plasma will be the same on all the polyurethane surfaces, because the thrombin feedback mechanism depends on platelets, not on the polymer surface (discussed in preceding section). The data in Fig. 8.3 (p. 220 - 223) show that in aprotinin-containing plasma, thrombin was generated after 40 min on the hydrophobic PPOMDA surface (Fig. 8.3a), 10 min on the negatively charged PPOBDDS surface (Fig. 8.3b), 45 min on the hydrophilic PEOMDA surface (Fig. 8.3c) and 12 min on the

PEO-containing negatively charged PEOBDDS surface (Fig. 8.3d). The coagulation process in aprotinin-containing plasma thus appears to be surface dependent. Therefore it seems likely that factor XIa was mainly generated from factor XI itself or from factor XIIa.

Another issue is to define the contribution of factor XIIa and factor XIa to the activation of factor XI in the absence of kallikrein. The data in Fig. 8.3 show that the generation of measurable thrombin ("II/aprotinin" curve) occurs about 10 min earlier than factor XII ("XII/aprotinin" curve) in aprotinin-containing plasma on all the polymer surfaces. Therefore it appears that the generation of thrombin in aprotinin-containing plasma in the early stages of the propagation phase is not due to factor XIIa. This finding suggests that factor XI is autoactivated in aprotinin-containing plasma, independent of factor XIIa.

The adsorption of factor XI on the polyurethane surfaces could conceivably cause a conformational change in factor XI making it more easily activated; thus the different polyurethane surfaces probably influence factor XI autoactivation through their different protein-binding properties (Chapter 6).

Comparing Fig. 8.3 with Fig. 8.1, it is noted that the effect of addition of aprotinin on the shifting of the OD versus time curve to a longer "initiation" time is similar to the effect of changing the surface from PPOBDDS to PPOMDA. The data show that the delayed thrombin generation in aprotinin-containing plasma and in plasma contacting the PPOMDA surface is due to the decreased activity of the contact phase. Therefore, the contact phase appears to be an important activation mechanism for thrombin generation. Although factor XI autoactivation can induce thrombin activation in aprotinin-containing plasma, the much-increased initiation time (45 min) in comparison with normal plasma (10 min) as

shown in Fig. 8.3, indicates that factor XI autoactivation-induced coagulation is a much slower and less important process than contact phase-induced coagulation.

Duration of thrombin activity

The fate of thrombin after its generation is another important issue for the understanding of blood-surface interactions. In vivo, small amounts of thrombin that may be generated can be neutralized at least in part by the thrombomodulin on the endothelial surface. This mechanism is unavailable in vitro in a blood-biomaterial contact situation.

Experiments were performed to investigate the duration of thrombin activity in our experimental system. In the experiment shown in Fig. 8.4 (p. 224), thrombin generation was measured in the manner reported previously (0 - 120 min) using the chromogenic substrate in plasma contacting the polyurethane surfaces. No further color was produced after about 70 min. Additional thrombin substrate was then added at 120 min, and a significant further increase in the production of pNA was observed, suggesting that thrombin was still active in the system. On both the PPOBDDS and PEOMDA surfaces, essentially the same increase in OD was found, indicating that there is no effect of the surface type on the activity of the remaining thrombin.

One explanation for the persistence of thrombin activity in this experiment is that the thrombin inhibitors in plasma were exhausted after a certain time, so that newly generated thrombin was not inhibited. If thrombin generation continued, the thrombin concentration could rise as high as the prothrombin concentration in the plasma (1.39 μM). In Fig. 8.4, by comparing the slope of

the OD versus time curve during the propagation phase (at 20 min) with the slope of the OD versus time curve after adding additional substrate (at 120 min), it is found that the slope at 120 min is smaller. Therefore the thrombin concentration at 120 min was not as high as at 20 min.

To study this phenomenon further, the "residual" concentrations of other coagulation proteins were investigated in a similar way. As shown in Fig. 8.5 (p. 225), very little residual amidolytic activity was found for factors Xa, XIIa and kallikrein upon adding additional substrates. These data suggest that virtually no factor XIIa, factor Xa or kallikrein remained in the system. The generation process of factor XIa by factor XIIa and the generation process of thrombin by factor X were stopped. Therefore, the residual thrombin at 120 min is produced probably in the early stages of plasma-surface interactions.

Influence of plasma concentration

Very different thrombin generation curves were observed for different plasma concentrations (Fig. 8.6, p. 226). The starting time for thrombin generation increased from ~5 min in 1% plasma to ~12 min in 80% plasma. The slope of the OD versus time curves also changed as the concentration of plasma increased. The data in Fig. 8.6 show that 32% plasma developed the highest thrombin activity during the clotting process, suggesting that an optimum plasma concentration exists with respect to thrombin generation.

The starting time of thrombin generation and the concentration of thrombin generated in plasma should depend on the balance of initiating factors and inhibiting factors. In the experiments shown in Fig. 8.6, the same volume of plasma was added to the same polymer surface area for all the

plasma concentrations. Therefore, for the same surface-to-volume ratio, it appears that the concentration of thrombin inhibitors in plasma decreased as the plasma concentration decreased. It is thus to be expected that with a relatively low inhibitor concentration (1% plasma) the time for initial thrombin generation is short (5 min), whereas at high inhibitor concentration (80% plasma) it is longer (12 min). The level of factor XIIa generated in a system low of inhibitor/surface ratio is also expected to exceed rapidly the level of C1-inhibitor, leading to rapid initiation of clotting via the intrinsic pathway.

All the curves in Fig. 8.6 show the same kinetic features. The differences in activation time and thrombin concentration can be explained in terms of the inhibitor/activator ratio and the surface/volume ratio. The data suggest that changing the plasma concentration will change the kinetics, but not the mechanism, of coagulation.

Influence of free and surface-bound PPACK on thrombin generation

Following the chemical process of grafting PPACK onto polyurethane chains (synthesis of PPACKPPO), it is possible that some unreacted free PPACK remains in the polymer, and that this free PPACK may be released when the material is in contact with plasma. Since PPACK is a powerful inhibitor of thrombin, a trace amount of free PPACK could have a significant influence on thrombin generation. While this free PPACK may increase the ability of the surface to inhibit thrombin, it may also cause side effects by inhibiting other proteins such as factor Xa, factor XIIa, tissue-type plasminogen activator, etc., if a significant systemic concentration is realized. On the other hand, surface-bound PPACK has the advantage of being localized. In any event the effect of the PPACK surface in preventing coagulation could be due to free PPACK as

well as surface-bound PPACK. Therefore, there is a need to compare the effect of surface-bound PPACK with the effect of free PPACK.

The rate of leaching of free YPACK into plasma from the YPACKPPO surface has been discussed in Chapter 5. YPACK was labeled with ^{125}I, so that the amount of free YPACK in plasma could be determined by measuring the radioactivity. The high sensitivity of the radiolabeling method makes it possible to measure concentrations of YPACK in the nanomolar range. Because of the similar molecular structures of YPACK and PPACK, YPACK was used as a model to study the leaching behavior of PPACK in this work.

To measure the activity of surface-bound YPACK, thrombin solution and plasma were added to YPACKPPO-coated wells of 96-well microtitre plate and the clotting process in the plasma was monitored (increase in turbidity measured at 405 nm). Because only a negligible amount of YPACK is leached in the first few min (Chapter 5), the measured antithrombin activity in this time frame can be attributed to surface-bound YPACK. To measure the activity of leached free YPACK, the plasma was incubated with the YPACKPPO surface for longer times (15, 30 and 60 min) to allow the leaching of measurable amounts to occur. The plasma containing the leached free YPACK was then removed from the YPACKPPO well into a polystyrene well (YPACK/Pst system), thrombin was added to clot the plasma, and the leached YPACK concentration was measured by radioactivity.

The clotting behavior of the plasma in the YPACK/Pst system was compared with that of fresh plasma in a YPACKPPO-coated well. The data are shown in Fig. 8.7 (p. 227), where the incubation time and the leached YPACK concentration are also indicated. The plasma clotted within 2 min in all the YPACK/Pst systems. The clotting of plasma in the YPACKPPO-coated well

("YPACKPPO" curve in Fig. 8.7) was faster than that of plasma containing 0.09 μg/ml free YPACK ("0.09 μg/ml YPACK/Pst" curve in Fig. 8.7), but slower than that of plasma containing 0.055 μg/ml YPACK ("0.055 μg/ml YPACK/Pst" curve in Fig. 8.7). This result suggests that the surface-bound YPACK in the YPACKPPO system is equivalent to more than 0.055 μg/ml of free YPACK in solution, corresponding to the concentration following 30 min of leaching. This result is in agreement with our previous finding (Chapter 5) that the thrombin-inhibitory activity of the YPACKPPO surface is mainly attributable to the surface-bound YPACK.

The surface-bound YPACK is immediately available for inhibition of thrombin, while there is a significant time delay before solution YPACK becomes effective. The experiment with ^{125}I-YPACK shows that 30 min is required for the YPACKPPO surface to leach 0.055 μg/ml YPACK into plasma. The data therefore suggest that to inhibit thrombin rapidly and effectively, surface-bound YPACK is needed. A polymer surface that has no surface-bound YPACK and can only leach YPACK at a rate of about 0.002 μg/ml per min (Chapter 5) is likely to clot immediately in the presence of as little as 1.1 μg/ml thrombin (see later).

Of course YPACK has different thrombin inhibition properties from PPACK, presumably due to the replacement of phenylalanine by tyrosine (Pauber et al., 1988). The capacity of YPACK to inhibit thrombin is less than that of PPACK and the binding affinity is lower. However, the functional groups on both inhibitors are the same. The mechanisms of grafting YPACK and PPACK onto a polyurethane chain and the leaching of YPACK and PPACK from the polymer surfaces into plasma are also the same. Therefore, it may be concluded that YPACKPPO can be used as a model to mimic the functioning of PPACKPPO. As shown in Fig. 8.7, the PPACKPPO surface prevented plasma

clotting upon adding 1.1 µg/ml thrombin. It seems likely that the thrombin-inhibiting action of the PPACKPPO surface is due both to the surface-bound PPACK and the PPACK which is released into the plasma.

Influence of protein adsorption on PPACK activity

To verify further that surface-bound PPACK or YPACK makes a significant contribution to the inhibition of plasma clotting on the peptide grafted surfaces, a surface which has "impaired" surface-bound YPACK but could leach free YPACK is needed. For this purpose, a YPACKPPO surface was incubated with plasma for one hour and was then rinsed with buffer to remove the loosely bound proteins. The surface thus becomes a "plasma-coated" YPACKPPO surface. Fresh plasma with 0.1 µg/ml factor IXa was then added and the clotting behavior was studied.

On the plasma-coated YPACKPPO surface, a protein layer is present which could mask or at least impair the activity of the surface-bound YPACK. As reported in Chapter 4 (page 64 and Figs. 4.9 and 4.10), coating the PPACKPPO surface with albumin or plasma significantly reduced the ability of the surface-bound PPACK to bind thrombin. On the other hand, the leaching of YPACK was found not to be influenced by protein adsorption (see Chapter 5, page 108 and Fig. 5.14). This surface can therefore be used to study the effect of YPACK leaching on plasma clotting. As shown in Fig. 8.8 (p. 228), the plasma clotting time was reduced from 10 min on the YPACKPPO surface to 5 min on the plasma-coated YPACKPPO surface. This clotting time is similar to the clotting times on the PPOBDDS surface and "0.035 µg/ml YPACK, Pst" surface, indicating that the ability of the plasma coated YPACKPPO surface to prevent clotting is reduced. Because both the YPACKPPO surface and the plasma-

coated YPACKPPO surface can leach free YPACK into plasma, and the protein layer on the plasma-coated YPACKPPO surface is not likely to reduce significantly the leaching rate of free YPACK (see Chapter 5), it is reasonable to assume that both surfaces will leach free YPACK into plasma at a similar rate. Therefore, the different clotting time on the two surfaces may be attributed to the different availability of the surface-bound YPACK. On the plasma-coated YPACKPPO surface, some of the surface-bound YPACK was presumably unavailable to inhibit thrombin due to the adsorbed protein barrier, resulting in a shorter clotting time. This result indicates that the surface-bound YPACK is important in inhibiting plasma clotting. Similar results were observed on plasma-coated PPACKPPO surfaces (data not shown).

The faster clotting of plasma on the plasma coated YPACKPPO surface suggests that protein adsorption at a longer plasma-surface contact time may eventually bury and mask the surface-bound YPACK or PPACK. Therefore, the leaching or sustained release of PPACK from the polymer surface might be needed for inhibition of thrombin at longer plasma-surface contact times. The concept of a PPACK sustained release material should be further investigated.

Summary

The blood-contacting surface has an important influence on the initiation of coagulation. Negatively charged sulphonate groups appear to initiate the contact phase rapidly. An inert surface such as PEOMDA polyurethane seems to have no direct effect on the activation of the contact phase. However, thrombin generation can be initiated by several other mechanisms, for example factor XI autoactivation. Once a trace amount of thrombin is generated, a physiologically effective concentration of thrombin is achieved on the platelet

surface (or Platelin liposome surface) by the thrombin feedback mechanism, and the polymer surface no longer has any significant effect. Therefore, in developing blood-compatible biomaterials, the surface should be designed to inhibit the initiation of the contact phase and at the same time scavenge trace amounts of thrombin produced by all possible generation mechanisms.

Thrombin activity was not observed in plasma contacting the PPACKPPO surface (Fig. 8.1). Activities of factors XIIa, Xa, and kallikrein were also not observed on the PPACKPPO surface (data not shown). As reported in Chapter 7, surface-bound PPACK can effectively inhibit factor XIIa generation in plasma. Because factor XIIa is inhibited, kallikrein activation by factor XIIa is therefore blocked. In addition, the activation of factor Xa in plasma depends on the activation of factors V and VIII by thrombin, and because thrombin is inhibited by the PPACKPPO surface (Fig. 8.1), the activation of factors V and VIII is blocked. Therefore factor X cannot be activated at the PPACKPPO surface. Since the PPACKPPO surface will effectively inhibit both factor XIIa and thrombin to prevent the activation of most other coagulation proteins, this surface shows a superior ability in preventing clotting.

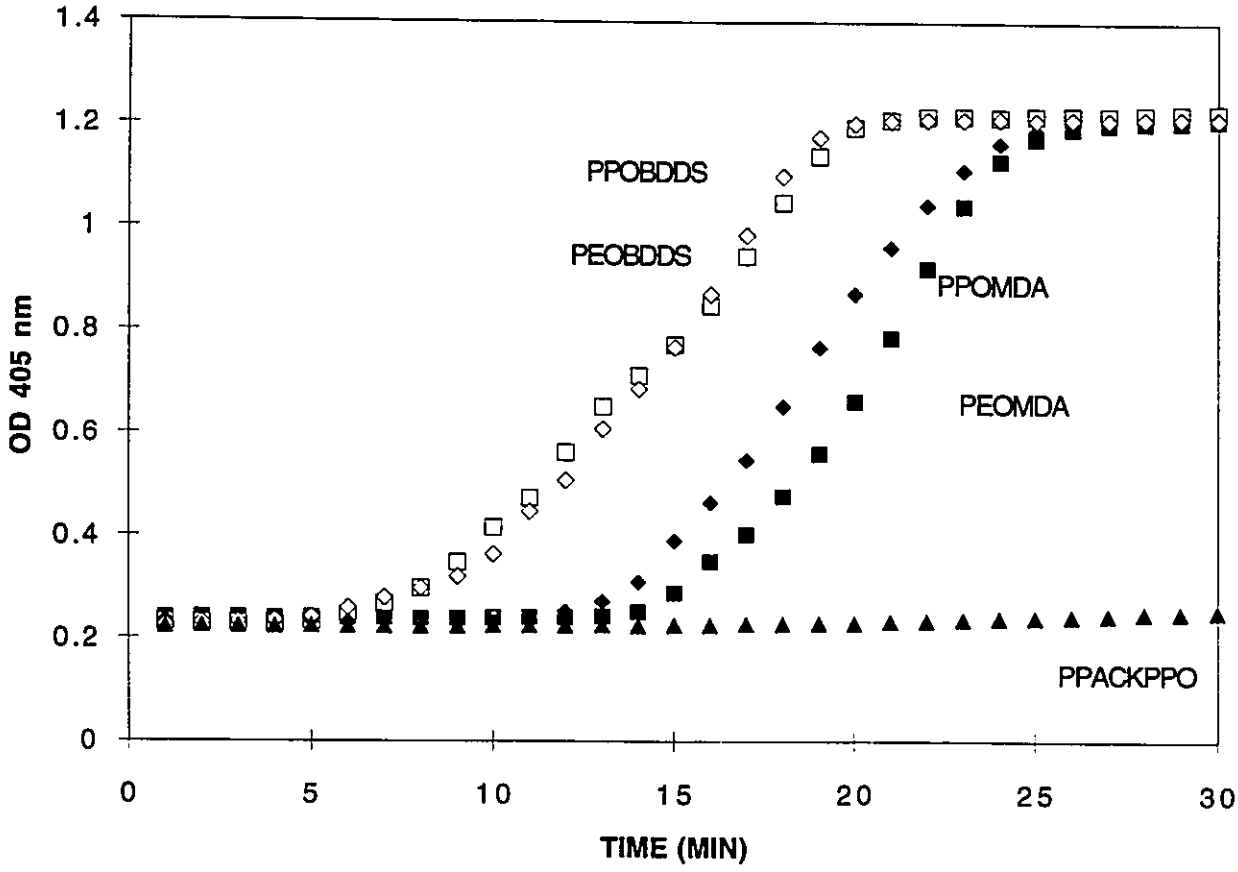


Fig. 8.1a Thrombin activity in plasma contacting polyurethane surfaces at 37°C. Optical density (OD) of pNA released from N-p-tosyl-gly-pro-arg pNA by thrombin in 80% plasma diluted with TBS/Ca/Platelin buffer was measured as a function of time. [Ca]=0.08 M, Platelin dilution 1:125. (◊) PPOBDDS; (□) PEOBDDS; (♦) PPOMDA; (■) PEOMDA; (Δ) PPACKPPO.

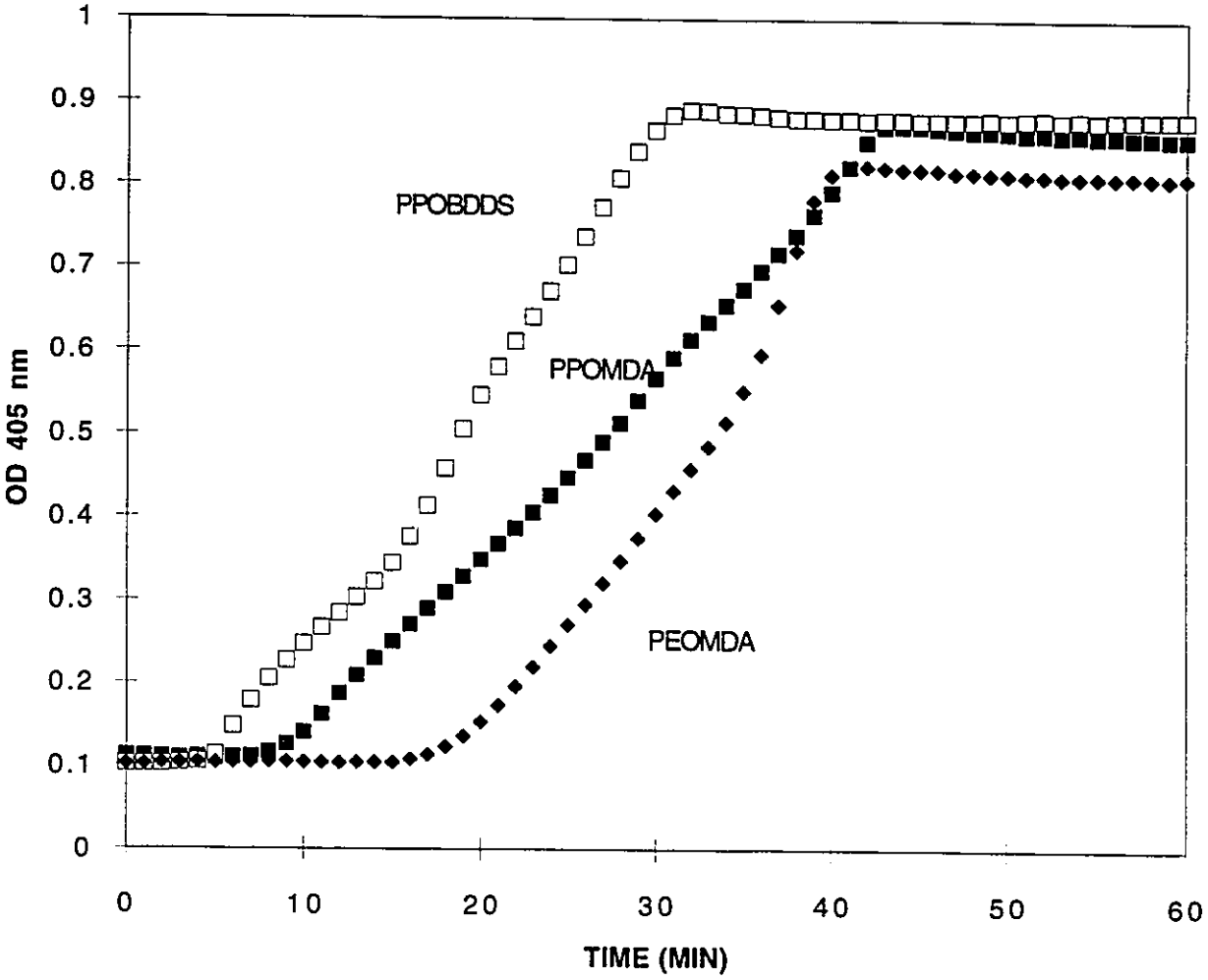


Fig. 8.1b Thrombin activity in plasma contacting polyurethane surfaces at 37°C. Optical density (OD) of pNA released from N-p-tosyl-gly-pro-arg pNA by thrombin in 10% plasma diluted with TBS/Ca/Platelin buffer was measured as a function of time. [Ca]=0.08 M, Platelin dilution 1:125. (□) PPOBDDS; (■) PPOMDA; (♦) PEOMDA.

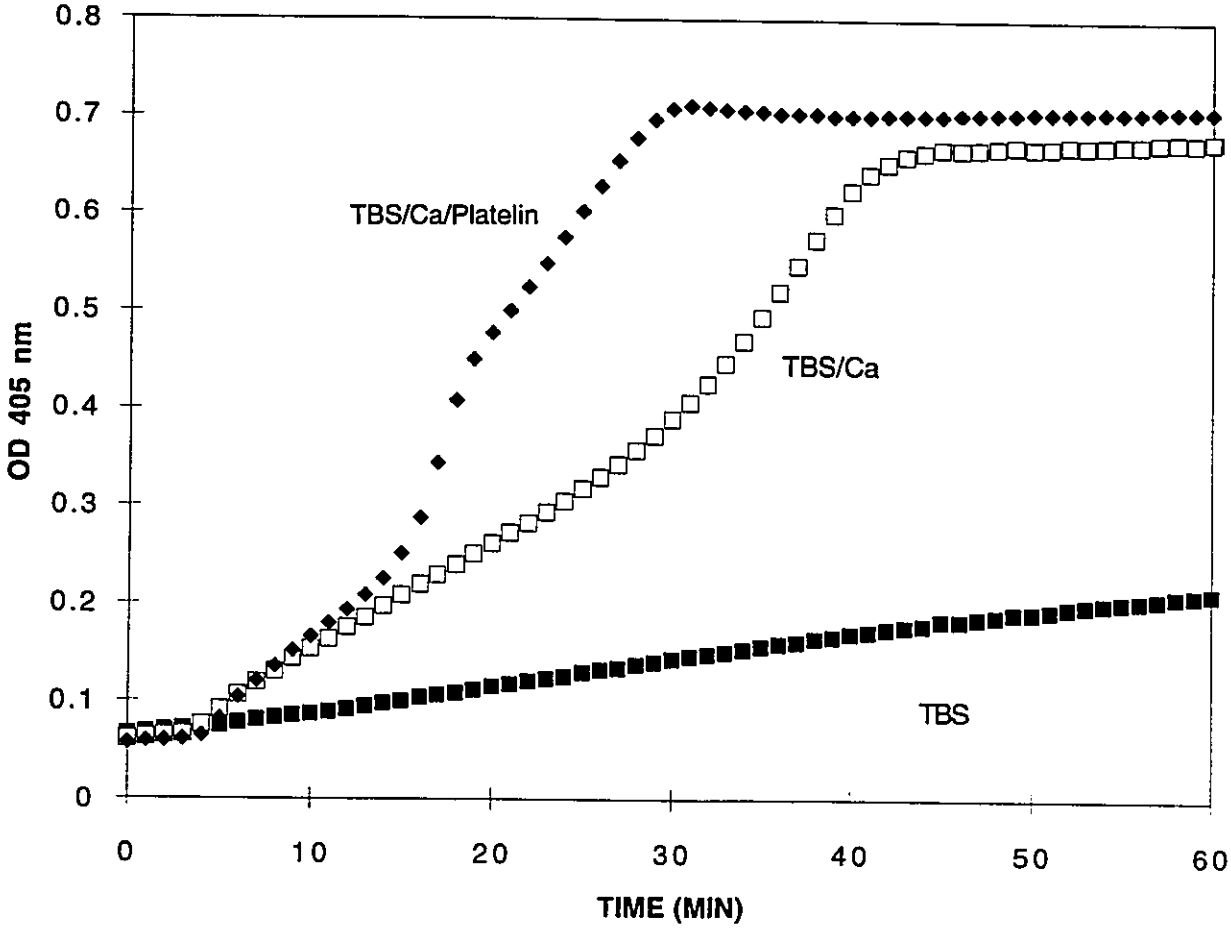


Fig. 8.2 Thrombin activity in plasma contacting polyurethane surfaces. Optical density (OD) of pNA released from N-p-tosyl-gly-pro-arg pNA by thrombin in 4% plasma measured as a function of time at 37°C. Different diluting buffers were used to examine their effects on thrombin generation: TBS: 0.05 M Tris, 0.1 M NaCl, pH 7.4; TBS/Ca: 0.1 M $CaCl_2$ in TBS; TBS/Ca/Platelin: 1 volume Platelin solution diluted in 9 volumes TBS/Ca, Platelin dilution 1:125.

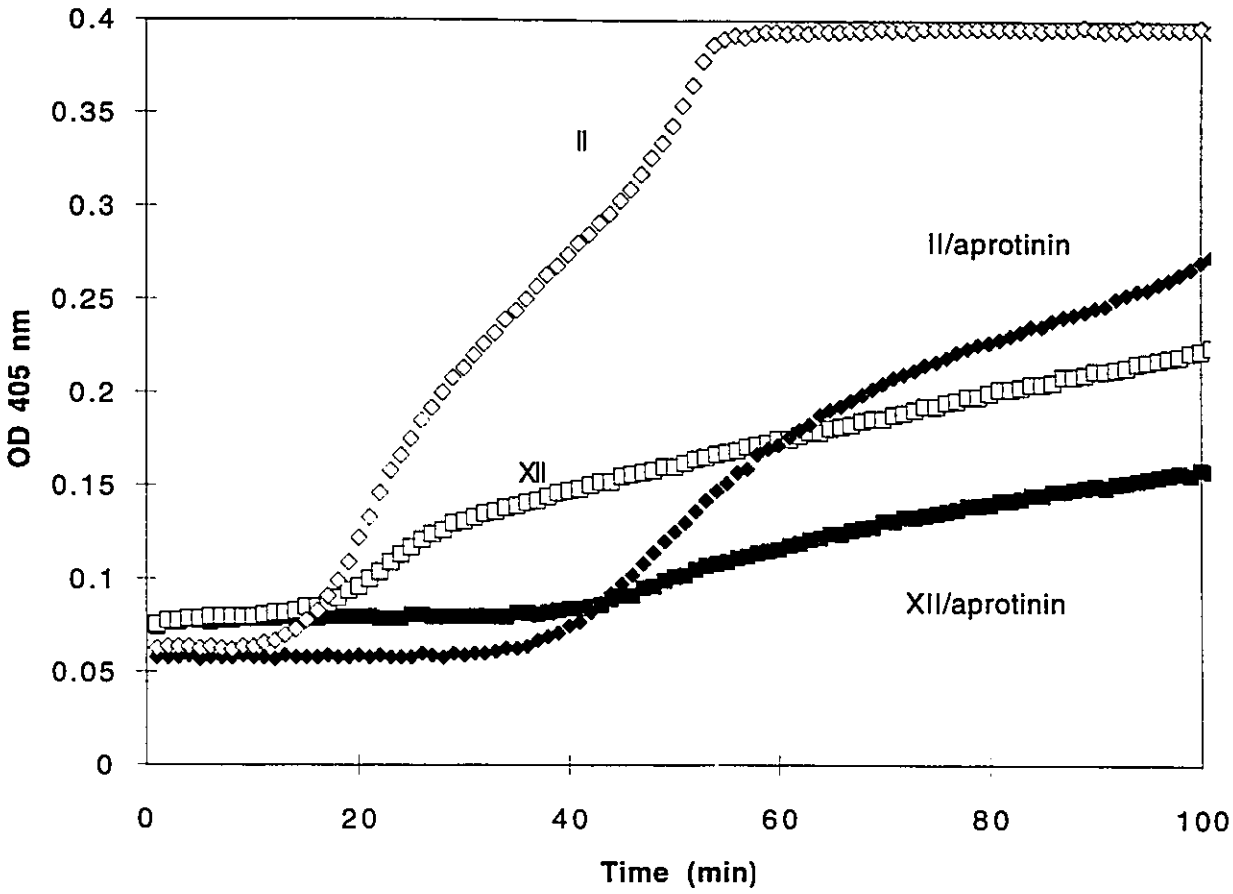


Fig. 8.3a Thrombin and factor XIIa activity in plasma contacting the PPOMDA polyurethane surface. Optical density (OD) of pNA released from N-p-tosyl-gly-pro-arg pNA by thrombin (curve "II") and released from Z-lys-phe-arg pNA by factor XIIa (curve "XII") in 4% plasma diluted with TBS/Ca/Platelin buffer measured as a function of time. [Ca]=0.08 M, Platelin dilution 1:125, 37°C. Aprotinin was added to examine the influence of the contact phase on thrombin and factor XIIa generation ("II/aprotinin" and "XII/aprotinin"); aprotinin concentration 0.2 mg/ml (0.92 unit/ml).

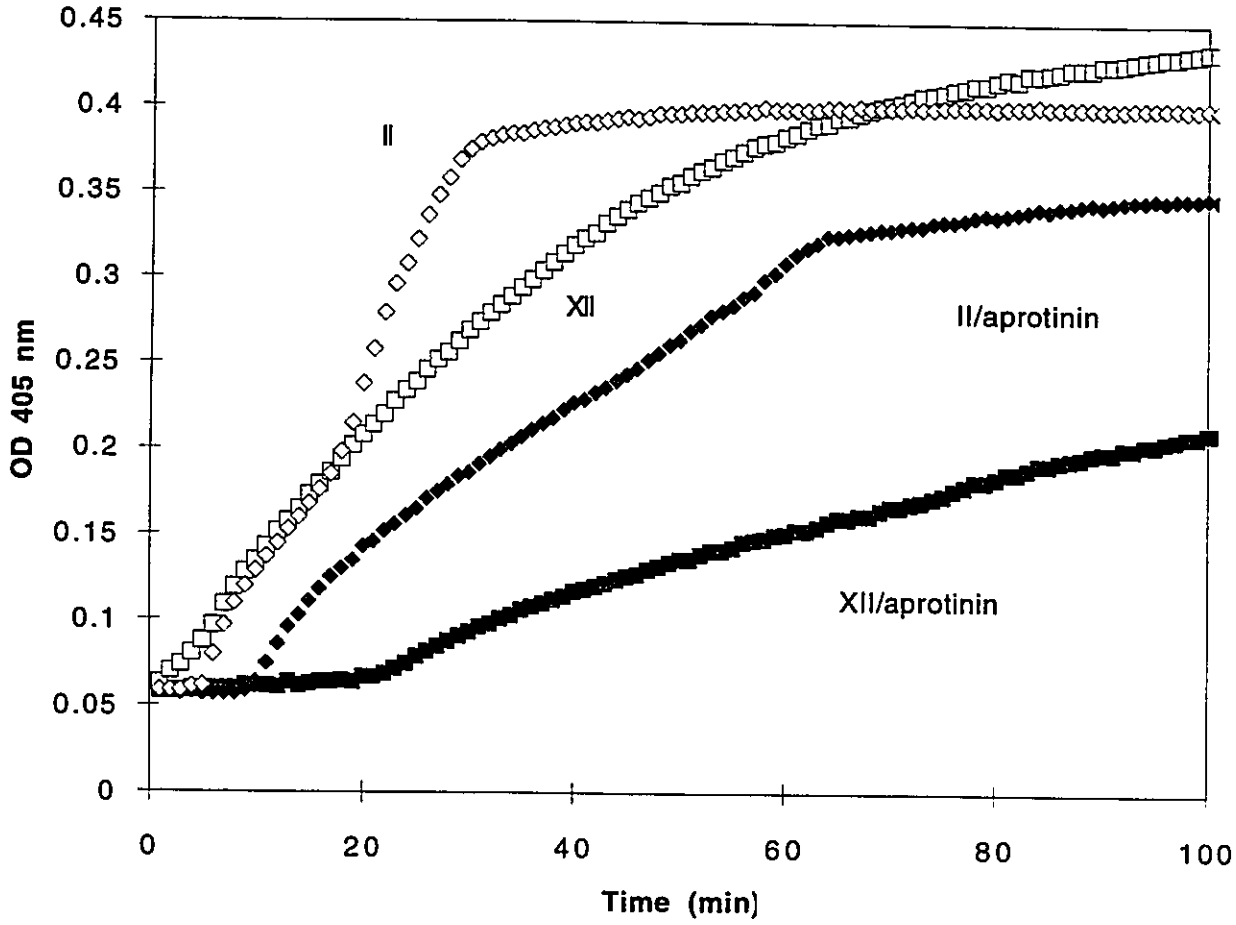


Fig. 8.3b Thrombin and factor XIIa activity in plasma contacting the PPOBDDS polyurethane surface. Optical density (OD) of pNA released from N-p-tosyl-gly-pro-arg pNA by thrombin (curve "II") and released from Z-lys-phe-arg pNA by factor XIIa (curve "XII") in 4% plasma diluted with TBS/Ca/Platelin buffer measured as a function of time. [Ca]=0.08 M, Platelin dilution 1:125, 37°C. Aprotinin was added to examine the influence of the contact phase on thrombin and factor XIIa generation ("II/aprotinin" and "XII/aprotinin"); aprotinin concentration 0.2 mg/ml (0.92 unit/ml).

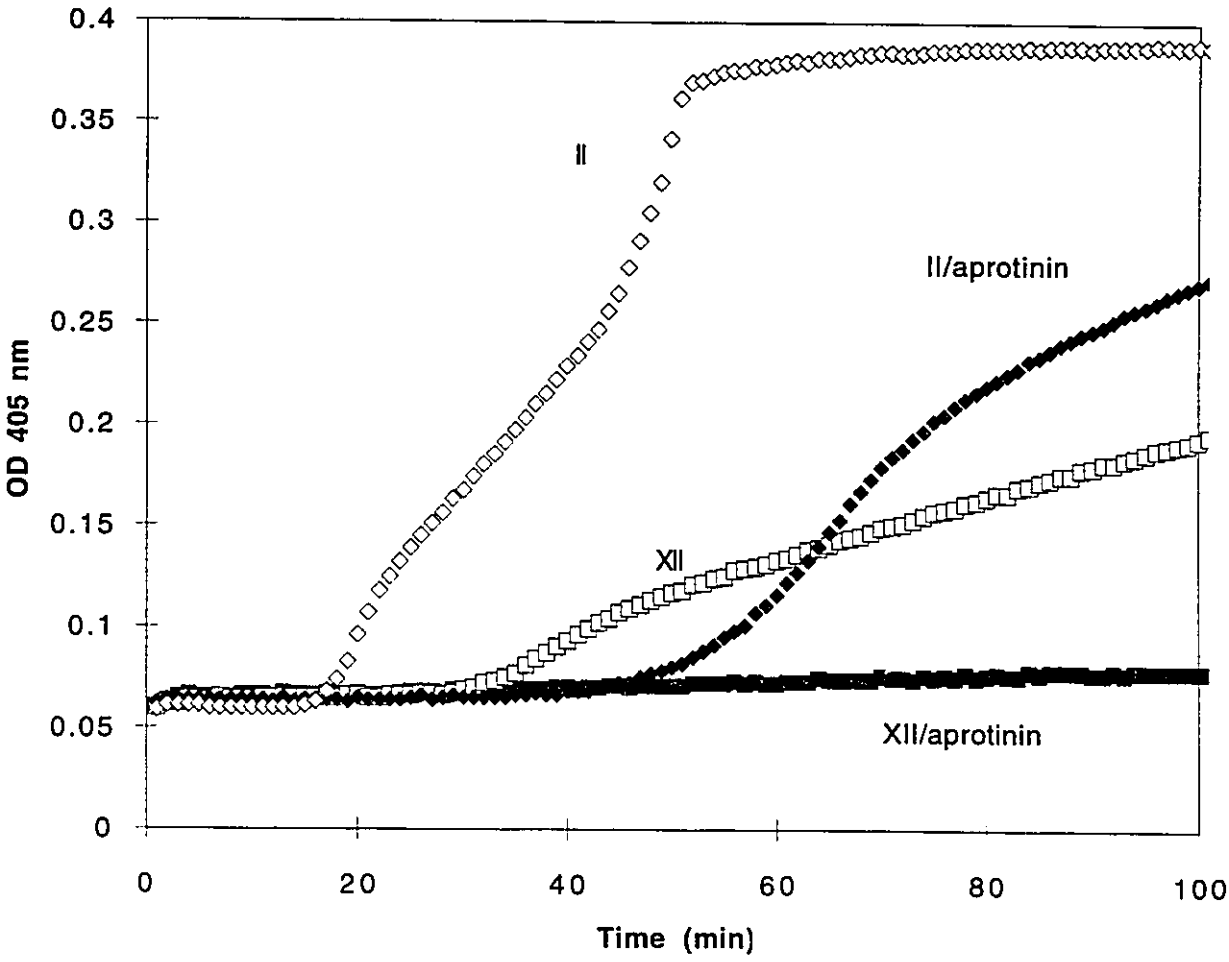


Fig. 8.3c Thrombin and factor XIIa activity in plasma contacting the PEOMDA polyurethane surface. Optical density (OD) of pNA released from N-p-tosyl-gly-pro-arg pNA by thrombin (curve "II") and released from Z-lys-phe-arg pNA by factor XIIa (curve "XII") in 4% plasma diluted with TBS/Ca/Platelin buffer measured as a function of time. [Ca]=0.08 M, Platelin dilution 1:125, 37°C. Aprotinin was added to examine the influence of the contact phase on thrombin and factor XIIa generation ("II/aprotinin" and "XII/aprotinin"); aprotinin concentration 0.2 mg/ml (0.92 unit/ml).

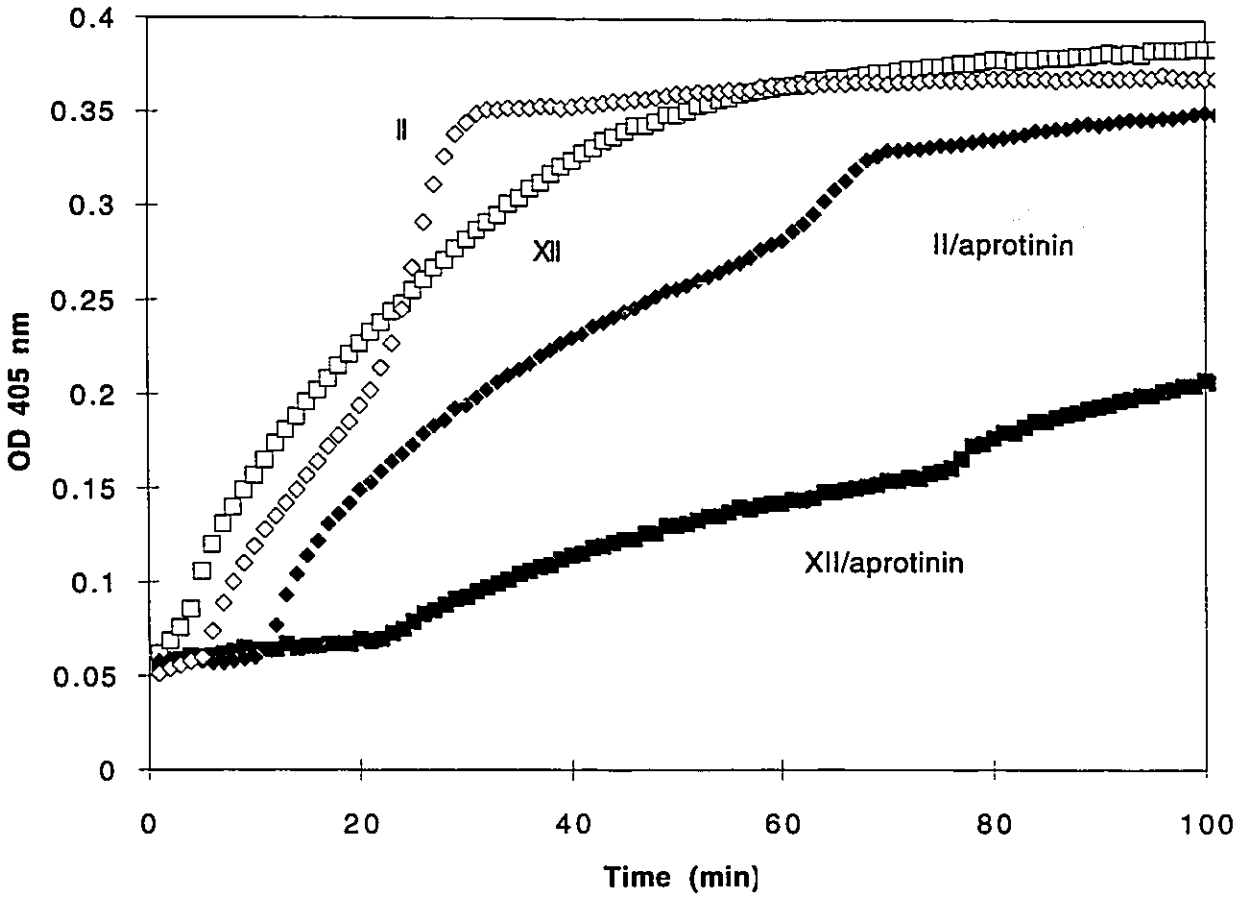


Fig. 8.3d Thrombin and factor XIIa activity in plasma contacting the PEOBDDS polyurethane surface. Optical density (OD) of pNA released from N-p-tosyl-gly-pro-arg pNA by thrombin (curve "II") and released from Z-lys-phe-arg pNA by factor XIIa (curve "XII") in 4% plasma diluted with TBS/Ca/Platelin buffer measured as a function of time. [Ca]=0.08 M, Platelin dilution 1:125, 37°C. Aprotinin was added to examine the influence of the contact phase on thrombin and factor XIIa generation ("II/aprotinin" and "XII/aprotinin"); aprotinin concentration 0.2 mg/ml (0.92 unit/ml).

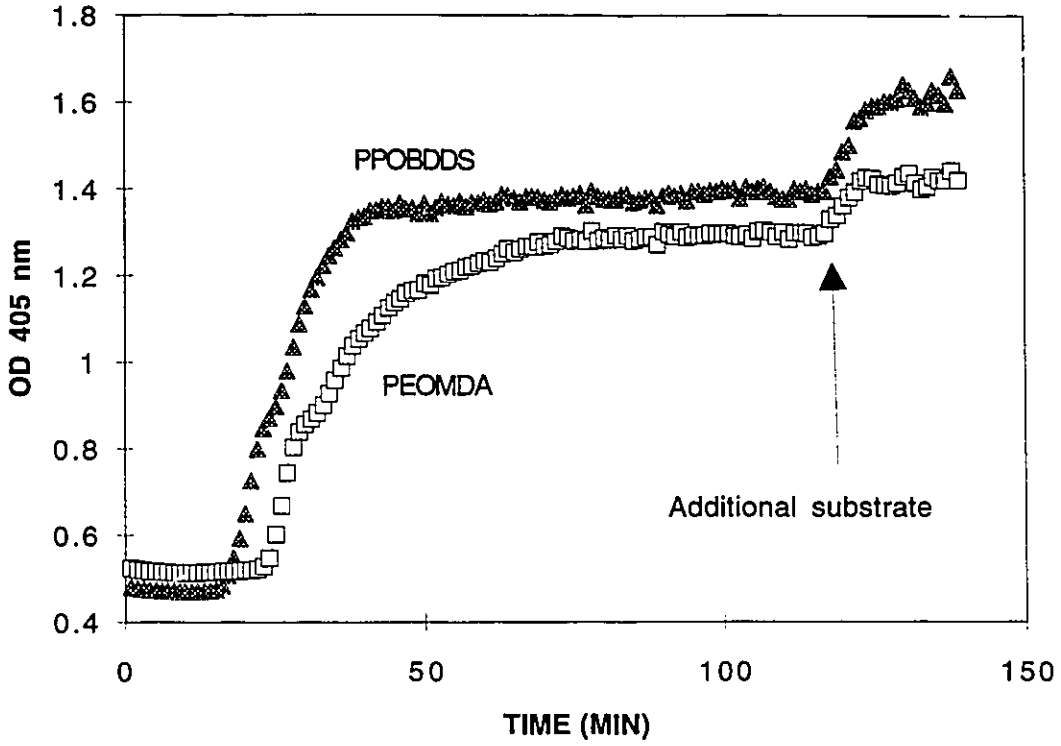


Fig. 8.4 Thrombin activity in plasma contacting the polyurethane surfaces PPOBDDS and PEOMDA. Optical density (OD) of pNA released from N-p-tosyl-gly-pro-arg pNA (0.06 mg/ml) by thrombin in 80% plasma diluted with TBS/Ca/Platelin buffer measured as a function of time. [Ca]=0.08 M, Platelin dilution 1:125, 37°C. Additional substrate (final concentration 0.12 mg/ml) was added at 120 min to examine the activity of the remaining thrombin (arrow).

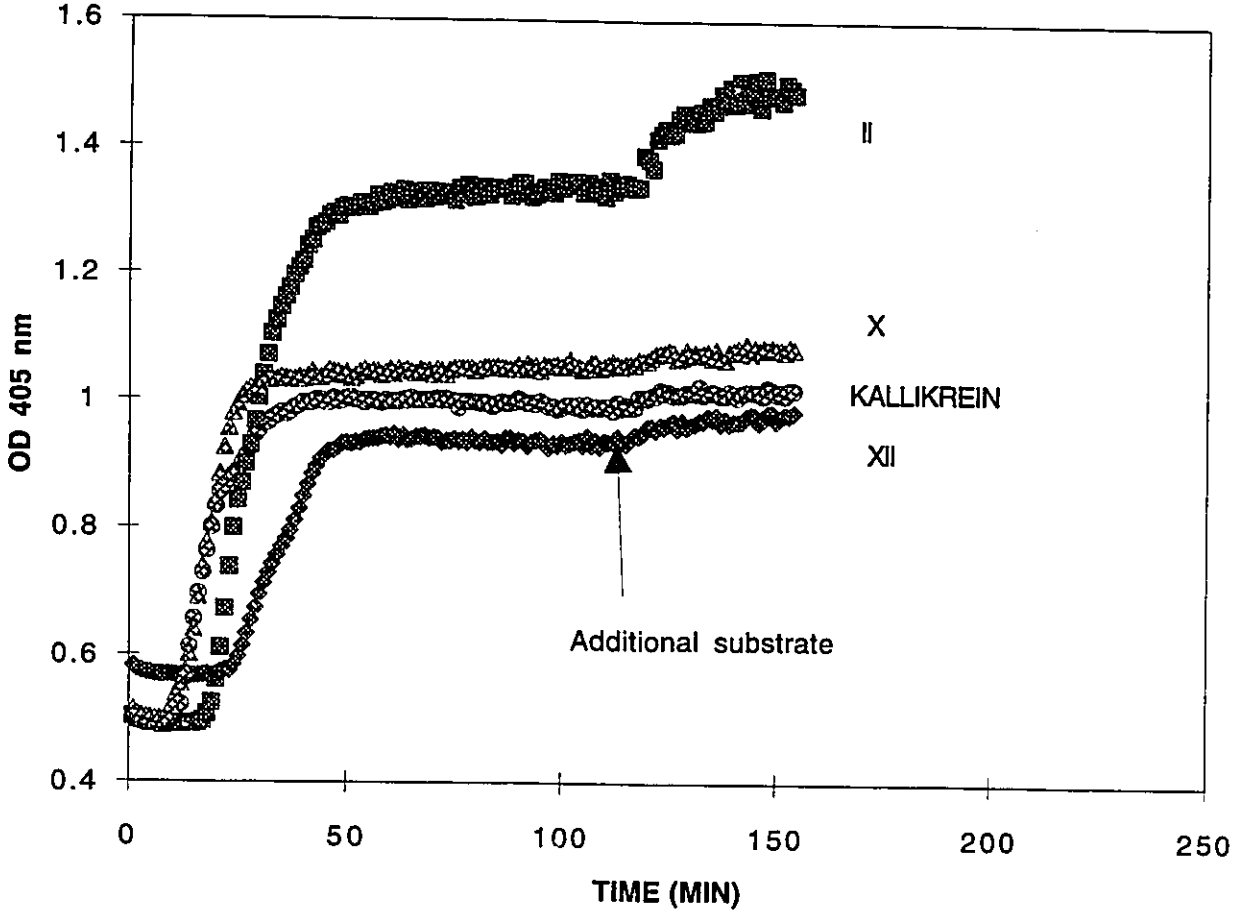


Fig. 8.5 Optical density (405 nm) of pNA released from N-p-tosyl-gly-pro-arg pNA (0.06 mg/ml) by thrombin, from Z-lys-phe-arg pNA (0.06 mg/ml) by factor XIIa, from N-benzoyl-ile-glu-gly-arg pNA (0.06 mg/ml) by factor Xa, and from N-benzoyl-pro-phe-arg pNA (0.06 mg/ml) by kallikrein in 80% plasma diluted with TBS/Ca/Platelin buffer contacting the PPOBDDS polyurethane surface. [Ca]=0.08 M, Platelin dilution 1:125, 37°C. Additional substrate (final concentration 0.12 mg/ml) was added at 120 min to examine the activity of the remaining enzymes (arrow).

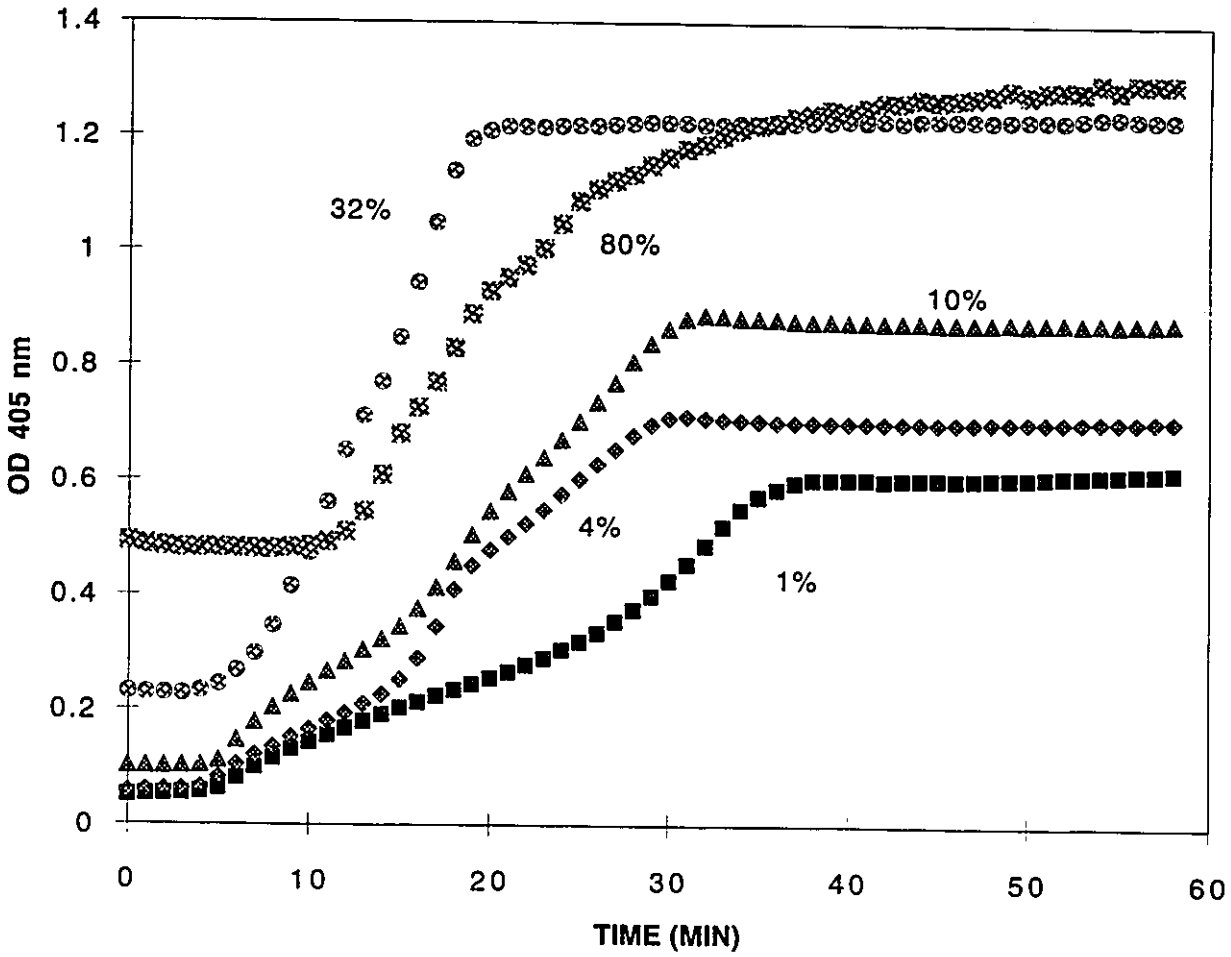


Fig. 8.6 Thrombin activity in plasma contacting the PPOBDDS polyurethane surface. Optical density (OD) of pNA released from N-p-tosyl-gly-pro-arg pNA. [Ca]=0.08 M, Platelin dilution 1:125, 37°C. Different plasma concentrations (plasma diluted with TBS/Ca/Platelin buffer) were used to examine the effect of dilution on thrombin generation.

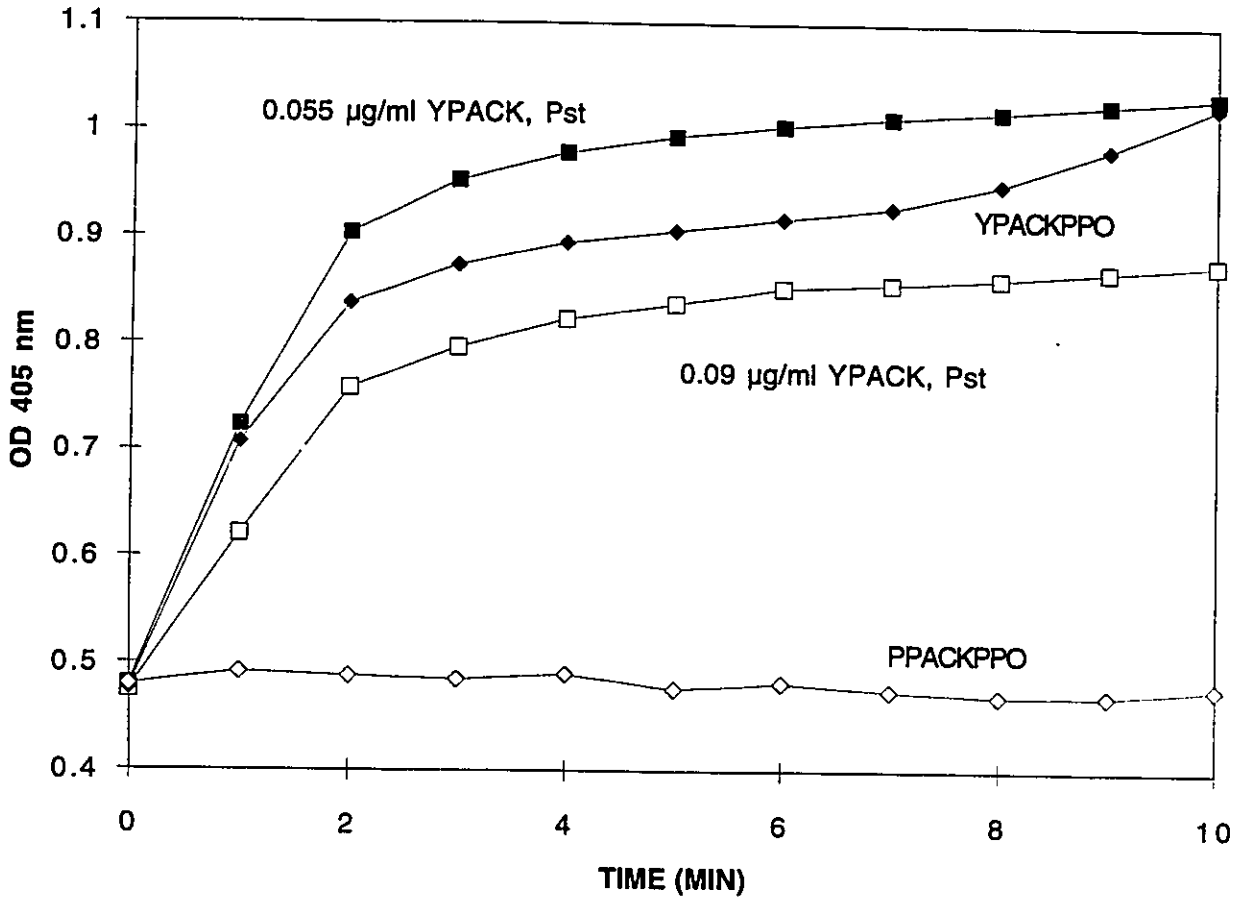


Fig. 8.7 Clotting of plasma on addition of thrombin (final concentration 1.1 µg/ml), plasma concentration 80% (diluted with TBS/Ca/Platelin), Platelin dilution 1:125, [Ca]=0.008 M, 22°C, on different polymer surfaces. The turbidity of the plasma at 405 nm was used as a measure of clotting.

Curve (**0.055 µg/ml YPACK, Pst**): plasma clotting on polystyrene surface with 0.055 µg/ml YPACK (equivalent to 30 min leaching).

Curve (**0.09 µg/ml YPACK, Pst**): plasma clotting on polystyrene surface with 0.09 µg/ml YPACK (equivalent to 60 min leaching).

Curve **YPACKPPO**: plasma clotting on the YPACKPPO-coated surface.

Curve **PPACKPPO**: plasma clotting on the PPACKPPO-coated surface.

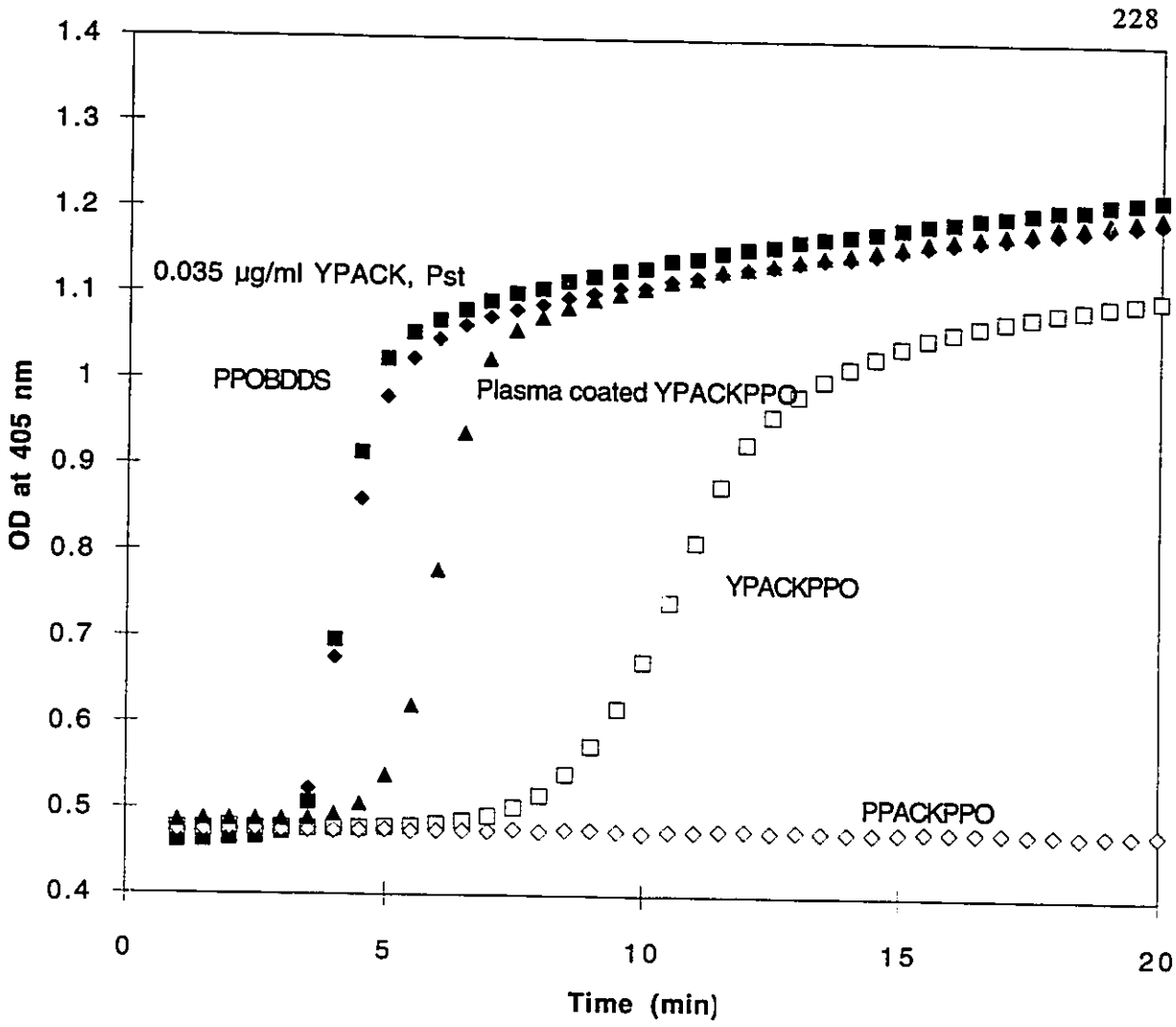


Fig. 8.8 Clotting of plasma on different polymer surfaces on addition of factor Xa (final concentration 0.1 µg/ml), plasma concentration 80% (diluted with TBS/Ca/Platelin), Platelin dilution 1:125, [Ca]=0.008 M, 22°C. The turbidity of the plasma at 405 nm was used as a measure of clotting.

Curve **PPACKPPO**: clotting of plasma on the PPACKPPO-coated surface.

Curve **YPACKPPO**: clotting of plasma on the YPACKPPO-coated surface.

Curve **Plasma coated YPACKPPO:** clotting of plasma on the YPACKPPO surface which has been coated with a protein layer deposited from plasma.

Curve **PPOBDDS**: clotting of plasma on the PPOBDDS-coated surface.

Curve **0.035 µg/ml YPACKPPO, Pst**: clotting of plasma on polystyrene surface with 0.035 µg/ml YPACK (equivalent to 15 min leaching).

CHAPTER 9

SUMMARY

In this work, five polyurethanes having a range of properties were synthesized. PPOMDA was intended as a conventional or "standard" hydrophobic polyurethane with no special chemical or other properties. PEOMDA, containing a polyethylene oxide soft segment, was intended as a potentially protein-resistant "inert" material. PPOBDDS and PEOBDDS are negatively charged versions of PPOMDA and PEOMDA respectively, and PPACKPPO contains PPACK residues. The chemical structure of the polymers was investigated by NMR using model compounds and 2D techniques. The surface structure including the functional group distribution on the surface were analyzed by XPS. The polymer surfaces prepared under hydrophobic conditions appeared to have the hydrophobic chain components present on the surface. Surface-restructuring in aqueous media appeared to bring the hydrophilic components and functional groups to the surface, and this restructuring process was monitored by following the water contact angle changes.

The adsorption of radiolabeled thrombin and albumin to the polyurethane surfaces showed that these proteins have high affinity for the PPOBDDS and PPACKPPO surfaces. The surface concentration of thrombin ranged from 0.005 $\mu g/cm^2$ on the PEOMDA surface to 0.5 $\mu g/cm^2$ on the

PPACKPPO surface. Thus protein adsorption is to a great extent influenced by surface chemistry. Preincubation of the surfaces with albumin or plasma significantly reduced the adsorption of thrombin to both PPOBDDS (50% decrease) and PPACKPPO (80% decrease) surfaces. Since these preadsorbed proteins are essentially not displaceable, it seems likely that they change conformation and become irreversibly bound.

It has been shown that PPACK grafted to and releasable from a polyurethane surface retains its ability to inhibit thrombin, and that it can effectively compete with the chromogenic substrate N-p-tosyl-gly-pro-arg p-nitroanilide which also has high specificity for thrombin binding. Thrombin adsorbed onto the PPACKPPO surface is almost completely inhibited. In contrast, thrombin adsorbed to other materials such as PEOBDDS and PPOBDDS shows significant retention of activity. It is likely that when contacted with blood, the BDDS polymers adsorb many different plasma proteins with the potential to initiate multiple biological responses. On the other hand, the PPACK polymer due to its specificity for thrombin binding, may show a more specific thrombin scavenging response.

The interaction between thrombin adsorbed on the polyurethane surfaces and the chromogenic tripeptide substrate can be described by a conventional first order kinetic scheme. It seems likely that surface-bound PPACK is able to scavenge solution thrombin effectively.

PPACK was found to be released slowly from the PPACKPPO surface into solution, thus contributing to the inhibition of thrombin especially when the surface-bound PPACK is masked by adsorbed proteins.

It may be possible also to improve the PPACK-type polyurethanes by using PEO instead of PPO as the soft segment in order to decrease non specific protein adsorption. An ideal thrombin scavenging surface should bind only thrombin while remaining inert to other plasma proteins.

The composition of protein layers deposited from plasma on different polyurethane surfaces was investigated using an immunoblotting method. The amount of protein adsorbed, the sequence of protein adsorption and the activation of proteins on the surfaces were influenced by surface properties. The PPACKPPO surface appears to adsorb proteins in such a way that the coagulation cascade is not activated. PPOBDDS on the other hand promotes the adsorption and activation of coagulation proteins.

Fibrinogen, HK, and albumin are the most abundant proteins in the protein layer deposited from plasma on the polymer surfaces. The adsorption of these proteins on the surface increases with time, suggesting that they are possibly among the proteins which replace other initially adsorbed proteins on the polymer surfaces.

The contact phase factors XII, XI and prekallikrein, as well as prothrombin, factor IX, ATIII and fibronectin were also shown to be adsorbed on the polymer surfaces in the initial stages of plasma-surface interactions. They were later displaced by other proteins such as fibrinogen, HK, and albumin.

Depleting the plasma of fibrinogen changes significantly the composition of the adsorbed protein layer. The adsorption of factors XII, XI, V and prothrombin on the polymer surfaces was significantly decreased.

The adsorption and activation of proteins on the polymer surfaces as determined by immunoblotting was found to be important for interpreting the

findings from the chromogenic substrate assay studies reported in Chapters 7 and 8. The combination of immunoblotting and chromogenic substrate methods therefore represents a valuable approach for studying the mechanisms involved in plasma-surface interactions.

From the experiments described in Chapter 7, it was found that surface-bound PPACK and free PPACK effectively inhibited factor XIIa and contact phase activation. Therefore PPACKPPO may be a material that can inhibit all of the pathways that are contact-phase dependent, including intrinsic coagulation.

On the polyurethane surfaces, the contact phase shows the following characteristics:

1. Factor XIIa generation is greatly accelerated by kallikrein. Indeed, kallikrein is a major activator of factor XII, with other activation mechanisms making a smaller contribution.

2. Activating agents such as negatively charged sulphonate groups have a strong accelerating effect on factor XII activation.

3. Soluble inhibitors such as aprotinin may compete with surface activation during the initial stages of contact phase activation. Only when the surface is able to overpower the inhibitors can the autoacceleration of factor XIIa production occur.

4. Although the contact phase has a significant influence on the activation of thrombin, thrombin does not appear to influence activation of the contact phase.

It was found (Chapter 8) that the blood-contacting surface has an important influence on the initiation of coagulation. Negatively charged sulphonate groups appear to initiate the contact phase rapidly. An inert surface

such as PEOMDA polyurethane seems to have no direct effect on the activation of the contact phase. However, thrombin generation can be initiated by several other mechanisms, for example factor XI autoactivation. Once a trace amount of thrombin is generated, a physiologically effective concentration of thrombin is achieved on the platelet surface (or Platelin liposome surface) by the thrombin feedback mechanism, and the polymer surface no longer has any significant role. Therefore, in developing blood-compatible biomaterials, the surface should be designed to inhibit the initiation of the contact phase and at the same time scavenge trace amounts of thrombin produced by all possible generation mechanisms.

Thrombin activity was not observed in plasma contacting the PPACKPPO surface. Activities of factors XIIa, Xa, and kallikrein were also not observed. Since the PPACKPPO surface will effectively inhibit both factor XIIa and thrombin to prevent the activation of most other coagulation proteins, this surface shows a superior ability in preventing clotting.

Two interesting findings (Chapter 8) are: (1) thrombin generation is initiated on the polymer surface and then shifts to the platelet surface; (2) protein adsorption masks the surface-bound thrombin. Thus, it appears that surface-bound PPACK should not be very effective in inhibiting thrombin generation in plasma. Interestingly, this work showed that surface-bound PPACK contributes significantly to the prevention of clotting on the surface, while released PPACK plays a minor role. It is possible that surface-bound PPACK exists in a very high effective concentration on the surface.

The materials synthesized in this work may be of value for use in blood-contacting medical devices. In vivo evaluation will be required before preceding to this stage.

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