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THE INTERACTIONS OF PLASMINOGEN WITH MODEL SURFACES AND DERIVATIZED SEGMENTED POLYURETHANE UREAS

by

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ABSTRACT

The major complication limiting the usefulness of biomaterials in blood contacting situations is the activation of the coagulation pathways resulting in platelet activation and thrombus formation at the surface. These events are initiated by surface phenomena. The initial interaction is believed to be the adsorption of proteins onto the artificial surface followed by stimulation of cells and their subsequent adherence to the protein layer. The research presented in this thesis takes the approach of making the surface specifically interactive to achieve biocompatibility, and focuses on plasminogen, a plasma protein not previously studied in detail relative to blood interactions with artificial surfaces. The underlying working hypothesis of this research is that a potentially fibrinolytic, i.e. clot-lysing, surface may be formed via the specific and selective adsorption of plasminogen following blood contact.

Our approach was to create materials that would selectively adsorb plasminogen from blood by incorporating lysine residues (known to bind plasminogen) into the surface. Several different materials have been developed to facilitate this investigation: polyurethanes substituted with lysine derived from sulphonated precursors, classical polyurethanes, and model silica glass materials functionalized with sulphonic acid groups and subsequently derivatized with lysine.

The model surfaces (silica glass, silylated sulphonated silica glass, and lysinated silica glass) were used to develop a method for the lysine derivatization of materials which results in incorporation of lysine at the surface with a free ε-amine group. Chemical and physical characterization of the surfaces utilizing ninhydrin reagent, contact angle and X-ray photoelectron spectroscopy were done and molecular modelling of silylated glass surface was undertaken. The data obtained indicate both qualitatively and quantitatively that the silylation and lysine functionalization methods were successful.

Protein adsorption from buffer and from citrated human plasma was investigated on all materials. Generally, the plasminogen adsorption from buffer was Langmuirian. Surface concentrations corresponding to monolayer protein adsorption were observed at the isotherm plateaus. The sulphonated and lysine derivatized materials showed higher adsorption capacities relative to the other surfaces. Adsorption studies on the model materials from pure buffer, included both adsorption and desorption experiments in the presence of competing species, and

suggest that the binding mechanism of plasminogen is different on each of the surfaces. It seems likely that the lysine binding sites are involved at least to some extent in the adsorption of plasminogen to lysinated materials.

The adsorption studies of fibrinogen and plasminogen from plasma suggest that plasminogen may be a possible contributor to the fibrinogen Vroman effect. In addition these studies provide further data in support of the contention that sulphonate groups have a strong influence on the adsorption of proteins in general. The plasma studies also show that the ratio of plasminogen to fibrinogen is greater on the lysinated than on the control sulphonated material, again indicating a specific influence of the lysine residues.

Data on the enzymatic activity of plasminogen adsorbed on the lysinated silica glass and its sulphonated precursor are reported. These studies provide information on the effect of lysine derivatization on the very important question of plasmin activity of adsorbed plasminogen. It was found that the plasminogen is not "plasmin-like" when adsorbed to any of the surfaces studied in this system. However in the presence of plasminogen activator there is a highly significant difference in the enzymatic activity of plasminogen adsorbed to the lysinated material and to its sulphonated precursor. The lysinated material shows two-four times the plasmin activity on a mole basis compared to the sulphonated material. The implications of these findings for the development of fibrinolytic surfaces are discussed.

Preliminary investigations into polyurethanes derivatized using methods developed with the model materials are also reported.

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CHAPTER ONE
INTRODUCTION AND LITERATURE REVIEW

1.0 INTRODUCTION

According to Kambic et al, biomaterials are "substances or combinations of substances that can be used in a system that treats, augments, or replaces any tissue, organ, or function of the body" [Kambic et al, 1986]. Biomaterials include metals, polymers, glasses, ceramics and composites [Ratner et al, 1987] and are used in a wide variety of products such as sutures, artificial hearts, dental implants, vascular grafts, and prosthetic joints. A rapidly growing field for biomaterial use is controlled drug release [Langer, 1990].

With improvements in technology, treatment of diseases (particularly of the elderly), and longer life expectancies, the demand for biomaterials is increasing each year. In 1986, approximately 75,000 artificial heart valves were implanted worldwide, and 90,000 plasmapheresis procedures were performed in the United States alone [Kambic, 1986]. These numbers alone, which are by no means all encompassing, indicate the extent and importance of the field.

The material characteristics of devices that interact with the human body play a critical role in the biocompatibility of the device. For each application, the biological environment is different and demands different characteristics depending on the end use. For example, the environment of a hip replacement involving interaction with bone cells and other tissue components and high mechanical loading and stresses, requires very different properties compared to a vascular graft which is in continuous contact with flowing blood, or a contact lens in contact with the corneal epithelium.

This thesis deals specifically with blood contacting biomaterials. Thus background discussion will be limited to interactions at the blood-material interface.

Devices utilized in the vascular system include temporary devices such as left ventricular assists (LVAD), and intra-aortic balloons, as well as more permanent implants, for example prothetic heart valves and vascular grafts [Kambic et al, 1986; Lelah and Cooper, 1986]. Blood contacting biomaterials are also used in blood purification (plasmapheresis and haemodialysis), and heart-lung bypass systems.

The major complication limiting the usefulness of biomaterials in blood contacting situations is the activation of the coagulation pathways resulting in platelet activation and thrombus formation at the surface [Lelah and Cooper, 1986; Brash, 1987; Ratner et al, 1987; Packham, 1988]. In most applications, patients must be administered anticoagulants to inhibit clotting and thrombus formation for the duration of their treatment, not a desirable course of action for the long term. Uncontrolled clotting caused by blood contact with the prosthetic device can, in the case of a vascular graft, lead to complete occlusion. Clotting may also lead to emboli release from a device surface possibly giving rise to stroke.

Other serious problems caused by biomaterial-blood contact include activation of complement, and calcification. Complement activation during haemodialysis, for example, can lead to anaphylactic shock [Craddock et al, 1977] and has been related to the type of membrane used in the dialyser. Mulzer and Brash [1989] found the protein layers adsorbed from different dialyser membranes are complex and qualitatively different, but that the complement protein C3 is abundant on all the membranes. Calcification is a significant problem with bioprosthetic heart valves and severely limits their life. Although these and other problems can occur with blood-material contact, thrombotic events remain the major concern [Lelah and Cooper, 1986; Brash, 1987; Ratner et al, 1987; Packham, 1988].

These events are initiated by surface phenomena [Brash, 1987]. The initial interaction is believed to be the adsorption of proteins onto the artificial surface followed

by stimulation of cells and their subsequent adherence to the protein layer. Understanding and controlling the formation of this initial protein layer is important to the long term development of blood compatible materials. Researchers have investigated the surface properties of materials i.e. surface energy, hydrophobicity/hydrophilicity and attempted to correlate biological responses with these properties [Baier, 1972; Baier et al, 1984; Wojciechowski, 1992]. Clear relationships between any one surface characteristic and biocompatibility have still not been found.

Research directed to avoiding the thrombotic nature of blood contacting artificial materials has focused primarily on three strategies: making the surface inert, making the surface the same as the normal vascular wall, or making the surface specifically interactive with blood. The concept of making the surface inert was based on finding a single surface property which could be manipulated to improve the biocompatibility of biomaterials by minimizing the material interaction with blood components. This strategy has essentially been abandoned.

The second approach, i.e. simulation of the vascular endothelium, is based on developing a material which supports the growth and proliferation of endothelial cells. Vascular endothelium is highly specialized and has roles not only in haemostasis but in integration and induction of blood-borne signals, modulation of leucocyte interactions with tissues, synthesis and secretion of peptides, and regulation of inflammatory and immune reactions [Petty and Pearson, 1989; Pearson, 1991]. It is this dynamic regulatory interface that a biomaterial must replace when placed in the vasculature. The interactions of the endothelium are illustrated in Fig 1.1.

One might expect that over time, vascular grafts implanted in humans would eventually become covered in endothelial cells if clotting and other factors could be controlled. Although this is frequently the case with other species (e.g. dogs), vascular grafts do not generally endothelialize in humans [Burkel et al, 1987; Absolom et al, 1988]. Researchers in this field are attempting to modify materials to encourage endothelialization [Absolom et al, 1988]. One approach is to physically entrap endothelial

cells prior to implantation (seeding), and subsequently encourage their proliferation in vivo [Hunter et al, 1986; Burkel et al, 1987]; or to develop materials which act as a

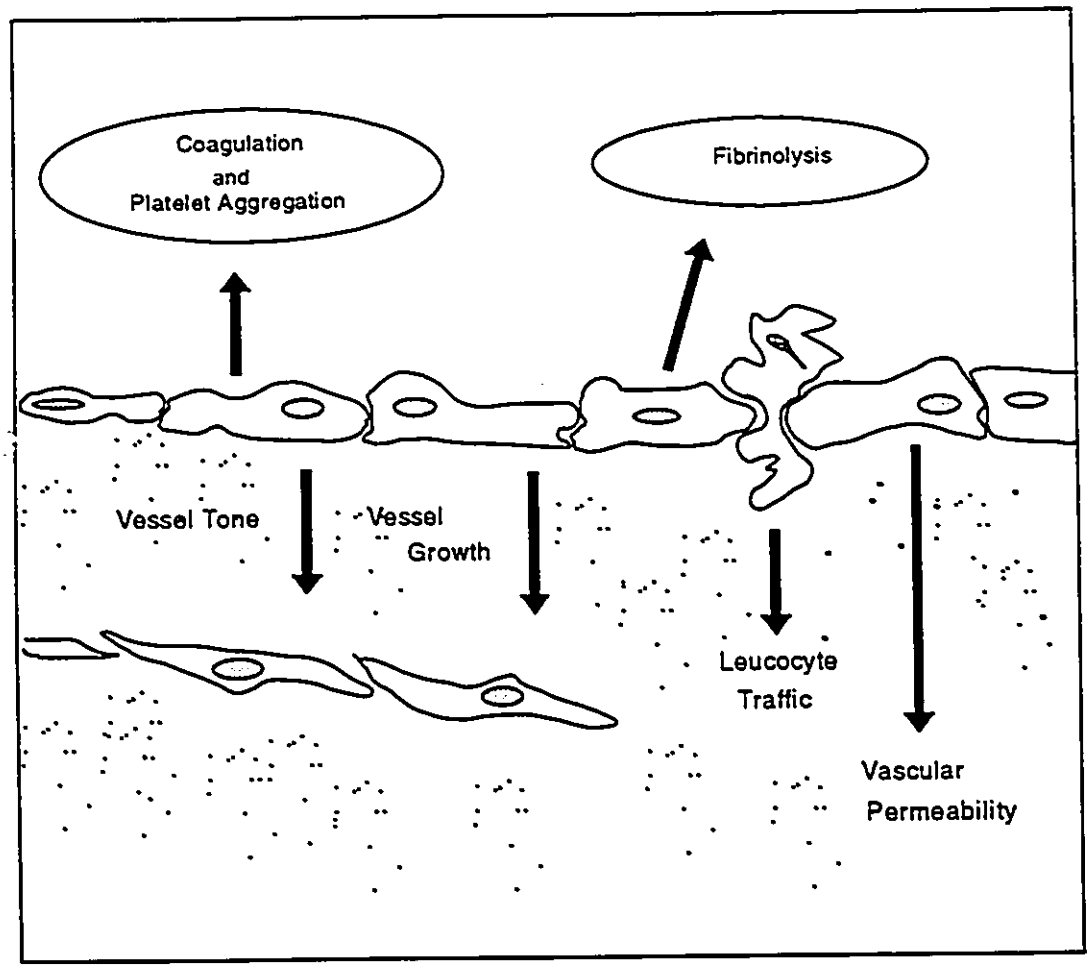


Fig 1.1: The endothelium and vascular homeostasis. Endothelial cells actively participate in the processes illustrated. This is generally achieved by the secretion or surface expression of bioactive molecules. Adapted from Pearson [1991]

substrate for the growth and proliferation of endothelial cells usually by surface modifications which result in cell attachment [Massia and Hubbell, 1991]. In the latter case, endothelial cells are grown in culture on these materials and are subsequently

implanted after cell growth and proliferation has occurred (sodding) [Williams et al. 1992). Although hampered by problems including slow cell growth, loss of cells from the material, and immunological barriers, this area is still the focus of a large research effort. Recent advances in cell microbiology and genetic engineering should advance this field of research considerably [Dichek et al. 1989].

The research presented in this thesis takes the last of the three approaches to achieving blood compatibility indicated above, namely making the surface specifically interactive, and focuses on plasminogen, a plasma protein not previously studied in detail relative to blood interactions with artificial surfaces. Plasminogen is the primary proenzyme of the fibrinolytic system, the function of which is to dissolve clots when they are no longer required. Plasminogen is composed of a single polypeptide chain containing 791 amino acids [Wallen and Wiman, 1972; Walker and Davidson, 1988]. It binds to fibrin, to the extracellular matrix and to cell surfaces via sites within the molecule which interact specifically with the amino acid lysine [Markus et al, 1978; Mangel et al, 1990; Mayer, 1990; Plow et al, 1991]. It is converted to its enzymatic form, plasmin, by cleavage of specific linkages by various activators [Mayer, 1990; Weitz, 1990]. Plasmin is a serine protease with a wide spectrum of activity including cleavage of both fibrinogen and fibrin into small fragments [Miyashita et al, 1988; Mayer, 1990].

The underlying working hypothesis of this research is that a potentially fibrinolytic, i.e. clot-lysing, surface may be formed via the specific and selective adsorption of plasminogen following blood contact. The long term objective of the research is to develop a clot-lysing or fibrinolytic material suitable for use as a blood contacting material.

The rest of this chapter presents background to this area of research. A general discussion of blood-material interactions is followed by descriptions of blood coagulation and fibrinolysis. Protein adsorption and segmented polyurethanes are major components of this thesis and are also reviewed.

1.1 BLOOD-MATERIAL INTERACTIONS

Blood-surface interactions are briefly summarized in Fig. 1.2. Blood contact with

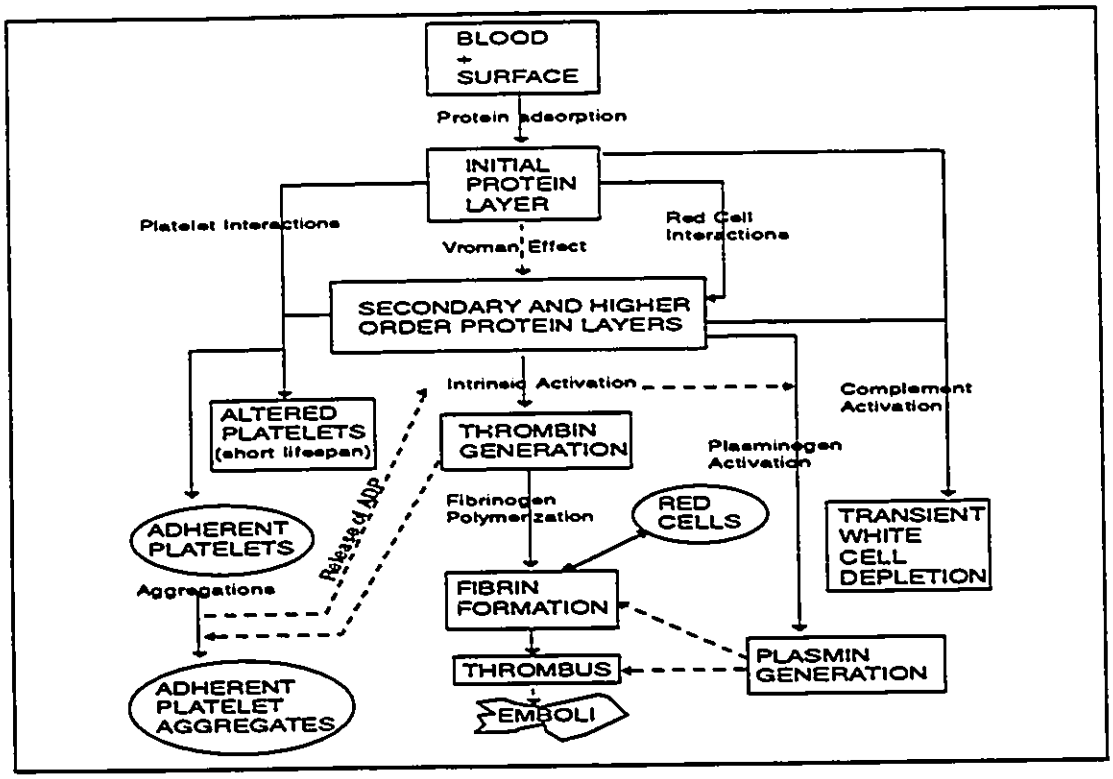


Figure 1.2: Blood-Material Interactions, Brash [1987]

a biomaterial results in the rapid adsorption of proteins to the material surface. This protein layer initiates coagulation via the intrinsic coagulation pathway (described in more detail later in this chapter) and results in the activation of platelets, principally through adsorption of fibrinogen. These early events trigger a series of cellular and protein interactions that can cause white cell and platelet depletion, immunological responses and clot formation. Clot formation may be followed subsequently by release of emboli at the clot surface. The fibrinolytic pathway is also stimulated by the protein layers. The consequences of contact activation depend on the balance achieved between coagulation

and fibrinolysis.

Coagulation, fibrinolysis, and protein adsorption are of prime importance for the present research and are now discussed in more detail.

1.2 COAGULATION

The coagulation pathway, which is activated by blood-material interactions is a complex cascade of enzymatically catalyzed reactions which culminates in the generation of thrombin (Thr), a serine protease which acts on the plasma protein fibrinogen (Fb) to form fibrin [Hirsh and Brain, 1983; Furie and Furie, 1988; Mann et al, 1988]. Fibrin is a polymer which constitutes the material of blood clots and is a stimulus of and anchor for the forming thrombus [Mann et al, 1988; Ribes et al, 1989].

Coagulation is a normal body response to blood vessel injury and results in the conversion of a developing platelet plug at the injury site to a fibrin clot. Clot formation is controlled by blood flow, inhibitors and fibrinolysis. When the clot is no longer required, fibrinolysis becomes dominant and the clot is dissolved.

There are two coagulation pathways, the extrinsic and the intrinsic. The latter pathway is dominant in blood-material interactions [Coleman et al, 1987]. An overview of the coagulation pathways is illustrated in Fig 1.3 and the intrinsic pathway is shown in more detail in Fig. 1.4.

Although the role of the surface is not totally understood, it is believed that in the intrinsic pathway the surface provides a site for conversion of the proenzyme factor XII to the active enzyme XIIa and similarly, factor XI to XIa by a series of interactions involving the plasma proteins prekallikrein, kallikrein and high molecular weight kininogen (HMWK)[Furie and Furie, 1988; Mann et al, 1988]. Activation of factors XII and XI is the initiation mechanism for subsequent stages of coagulation via the intrinsic pathway. The final step in this process, the action of thrombin on fibrinogen, results in the cleavage of fibrinopeptides A and B and the formation of fibrin monomers. These monomers then spontaneously polymerize and crosslink, forming the fibrin clot. The

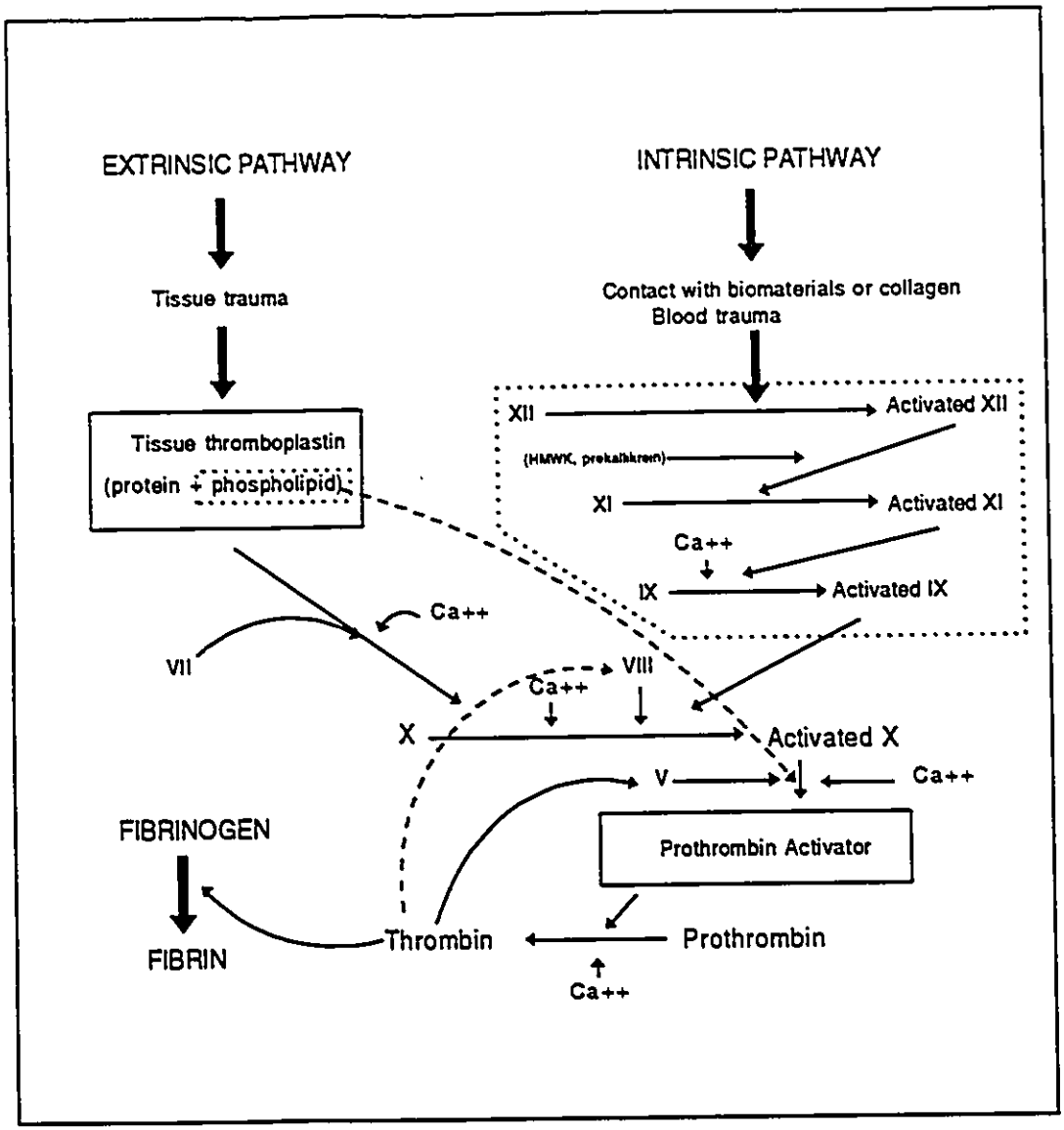


Figure 1.3: An overview of coagulation. The intrinsic and extrinsic pathways are shown. The extrinsic pathway is dominant under physiological conditions. Recent evidence suggests that activation occurs via Factor VIIa-tissue factor activation of Factor XI [Mann et al, 1988]. Many of the enzymes of the extrinsic pathway require cell membranes for activation. Adapted from Guyton [1986].

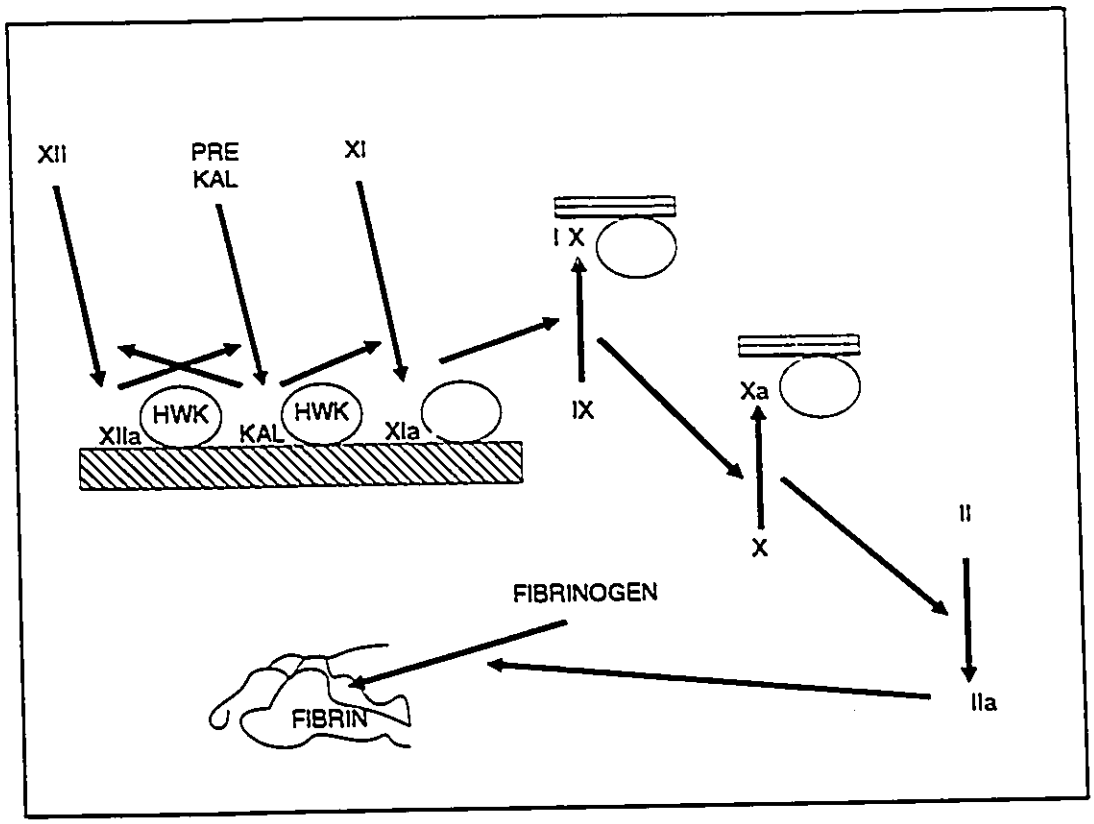


Figure 1.4: The intrinsic coagulation pathway. This pathway can be initiated by biomaterials, illustrated by the rectangle containing diagonal markings. The empty circles represent factors in the pathway which have not yet been defined. In this figure, KAL=Kallikrein, PRE KAL= prekallikrein, the precursor of kallikrein and HWK=high molecular weight kininogen. Adapted from Mann et al [1988].

stabilization of the monomers through crosslinking is achieved via the action of Factor XIIIa.

The characteristics of the proteins fibrinogen, fibrin and their interactions with the fibrinolytic pathway are important in this research. More detailed descriptions of each are provided below.

1.2.1 Fibrinogen and Fibrin

Fibrinogen is a protein occurring in relatively high concentrations (3-4 mg/mL)

in human plasma. It has been extensively studied in relation to blood-material interactions [Chan and Brash, 1981; Lelah et al, 1984; Wojciechowski et al, 1986; Lindon et al, 1986].

Fibrinogen is a large (340 kd), trinodal protein made up of three pairs of polypeptide chains, Aα, Bβ and γ [Doolittle, 1978; Nossel, 1981; Price, 1981; Wiesel et al, 1985; Dang and Bell, 1989]. The amino terminals of these polypeptide chains are linked together by disulphide bridges to form the central E domain, while their carboxyl terminals form the D domains [Doolittle, 1978; Nossel, 1981; Price et al, 1981; Wiesel et al, 1985; Dang and Bell, 1989]. This structure is illustrated in Fig 1.5.

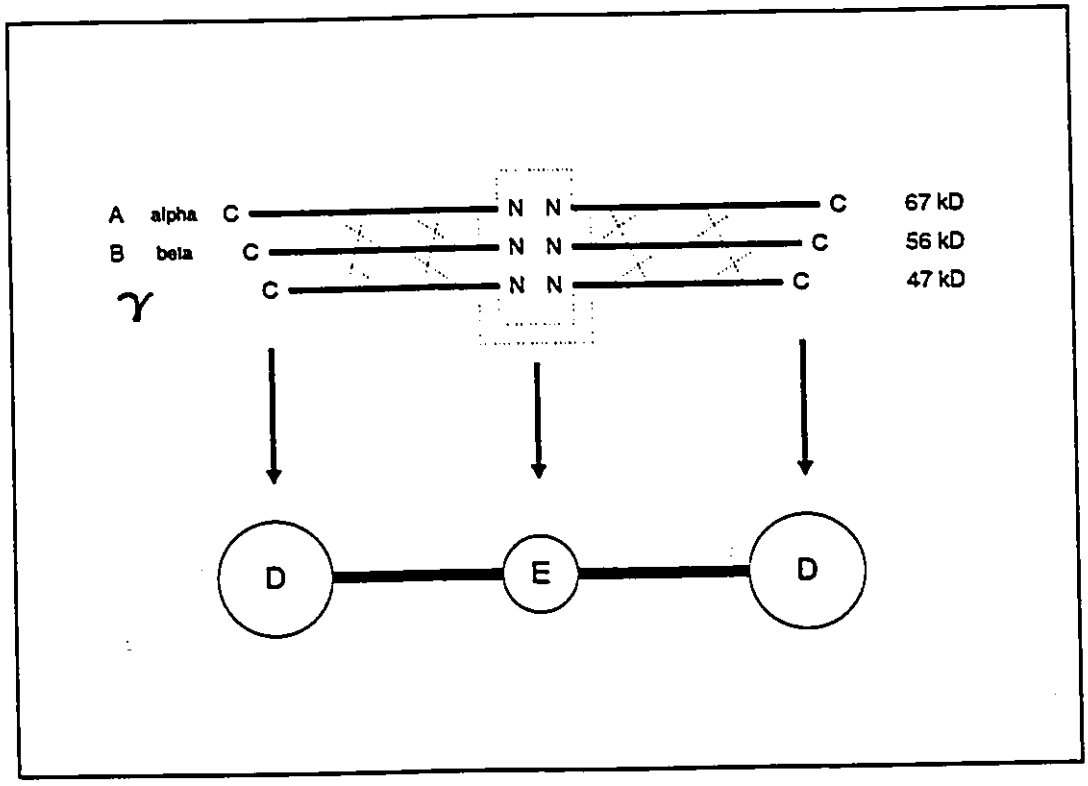


Figure 1.5: Fibrinogen structure. The fibrinogen molecule consists of pairs of Aα, Bβ and γ-chains linked together by disulphide bonds (**dotted**) at the amino-termini (N) that form the central E-domain. The bottom section of the figure shows the trinodular fibrinogen model. Adapted from Dang [1989].

Cleavage of fibrinogen by thrombin results in the release of fibrinopeptide A (FPA) and fibrinopeptide B (FPB) from the amino-terminal ends of the Aα and Bβ chains, respectively. Release of these peptides is believed to uncover polymerization sites within the E domain that participate in polymerization of what are now called fibrin I (release of FPA) and fibrin II (release of FPB) [Doolittle, 1978; Nossel, 1981; Wiesel et al, 1985; Dang and Bell, 1989]. Fibrin monomer polymerizes by interactions between the E domains of one fibrin molecule with the D domain of other fibrin monomers. FPB release enhances lateral association of the growing polymer chains and stabilizes the fibrin bundles. Factor XIIIa, a coagulation pathway protein, causes further stabilization of the bundles by crosslinking the γ and α chains. Polymerized fibrin provides substrate for thrombus growth and further participates in continuing clot formation [Doolittle, 1978; Nossel, 1981; Price et al, 1981; Wiesel et al, 1985].

1.3 FIBRINOLYSIS

Conversion of fibrinogen to fibrin is the culminating event in blood clot formation. However, during normal haemostasis the haemostatic plug or clot is temporary and is eventually dissolved. This dissolution is effected primarily by degradation of fibrin via the fibrinolytic pathway. Fibrinolysis is the degradation of fibrin by plasmin generated in the plasminogen/plasmin system, and the fibrinolytic system comprises elements which promote or inhibit this conversion [Walker and Davidson, 1988]. The fibrinolytic pathway is illustrated in Fig. 1.6.

1.3.1 Plasminogen

Plasminogen (Plg) is the primary proenzyme of the fibrinolytic system. Plasmin (Pli), the active enzymatic form of plasminogen, is a serine protease with a wide spectrum of activity. It is rendered specific for fibrin by the characteristics of the fibrin-plasmin-plasminogen interaction [Walker and Davidson, 1988], discussed in detail below.

Plasminogen is a beta globulin composed of a single peptide chain containing 791

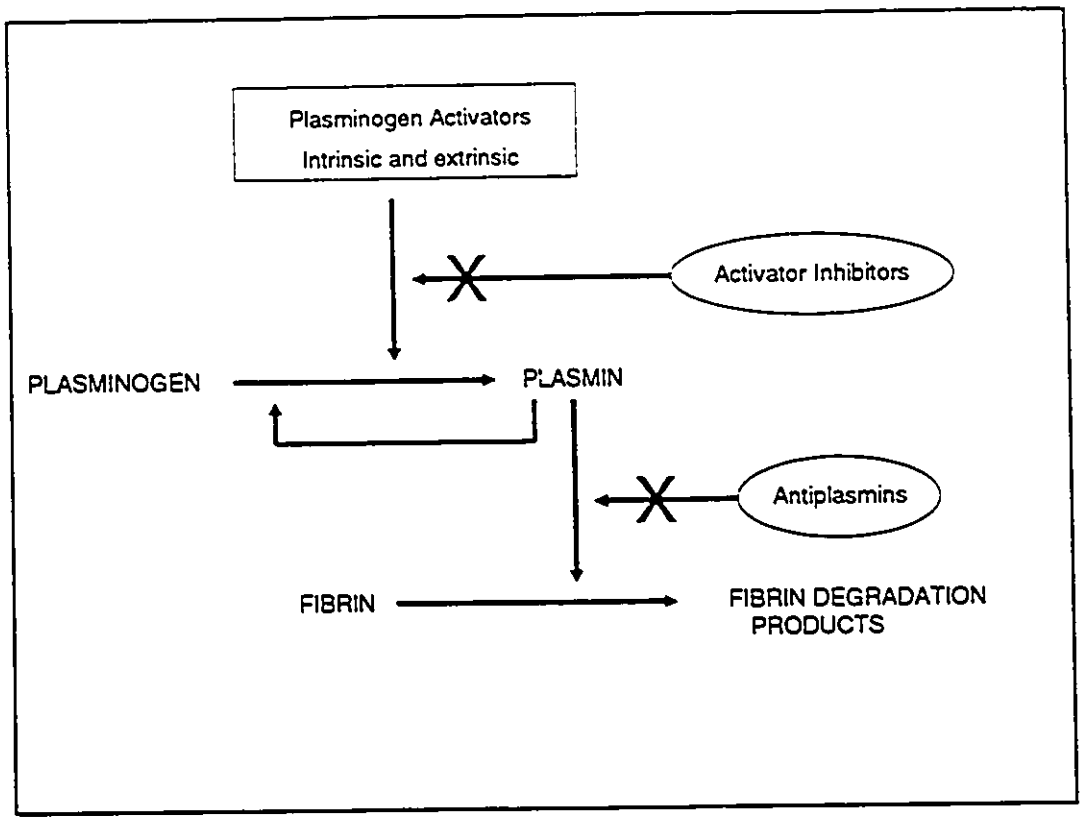


Figure 1.6: The fibrinolytic pathway, an overview. Adapted from Weitz [1990].

amino acids. It also contains approximately 2% carbohydrate which contributes to the electrophoretic heterogeneity of the molecule [Wallen and Wiman, 1972; Summaria et al, 1972; Summaria et al, 1973; Summaria et al, 1976; Walker and Davidson, 1988]. The protein is made up of several distinct regions [Walker and Davidson, 1988]: 1) the preactivation peptide (amino acid residues 1-76), a section of the molecule released in one of the activation pathways, 2) the (heavy) A region containing the five kringle structures associated with five weak and one strong lysine binding sites (LBS). These kringle areas are involved in the binding of plasminogen to fibrin and to α_2-antiplasmin. The A region contains amino acid residues 77-560, and 3) the light or B region, residues 561 to 791, containing the active site of the enzyme plasmin. The structure of plasminogen is

illustrated in Fig. 1.7 [Lijnen and Collen, 1982].

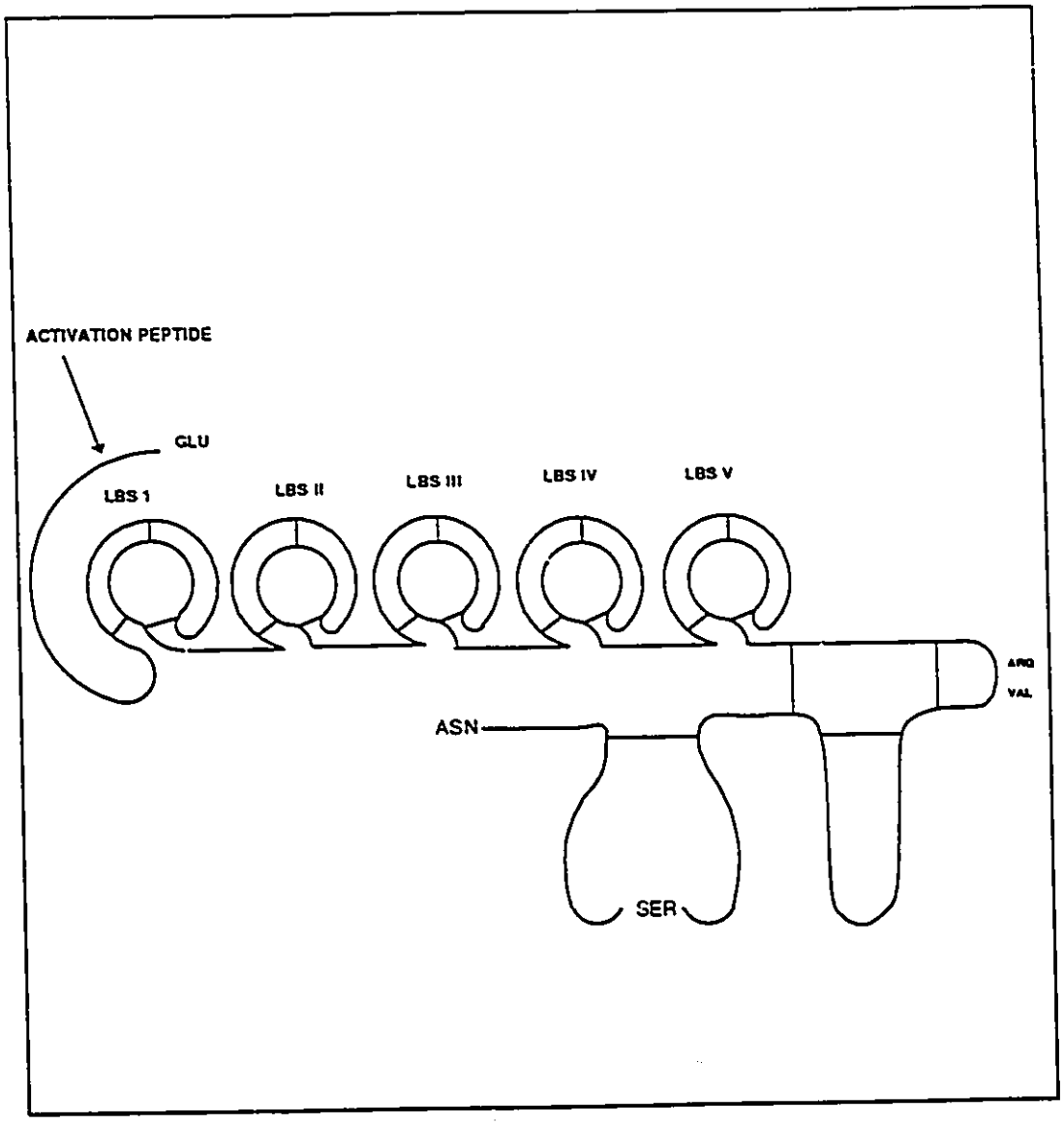


Figure 1.7: Schematic Representation of Plasminogen [Lijnen and Collen, 1982]. The kringle regions labelled LBS 1 to LBS 5 are associated with lysine binding sites.

The method used to isolate plasminogen from plasma affects the amino terminal amino acid residue on the preactivation peptide [Deutsch and Mertz, 1970; Wallen and Wiman, 1972; Summaria et al, 1972; Summaria et al, 1973; Hatton and Regoeczi, 1974;

Summaria et al, 1976]. The protein isolated may have either glutamic acid (Glu) or lysine (Lys) as the terminal amino acid. It is now believed that Glu-plasminogen is the native form of the proenzyme and that Lys-plasminogen exists as an intermediate in one of the two activation pathways which convert Glu-plasminogen to Lys-plasmin [Lijnen and Collen, 1972; Wallen and Wiman, 1973; Walker and Davidson, 1988]. These two forms of the protein differ in both chemical and functional properties [Summaria et al, 1972; Summaria et al 1973; Hatton and Regoeczi, 1974; Summaria et al, 1976; Springer, 1990].

Recently some aspects of the secondary and tertiary structure of plasminogen have been elucidated [Mangel et al, 1990]. Using small-angle x-ray scattering and vacuum ultraviolet circular dichroism(CD), Mangel et al [1990] found that plasminogen contains little α-helix or parallel β-sheet and consists predominately of anti-parallel β-sheet and β turns. Further when one of the weak lysine binding sites interacts with substrate, plasminogen undergoes a radical conformational change from a prolate ellipsoid structure (radius of gyration 39 Å) to a Debye random coil (radius of gyration 56 Å). The work of Mangel et al underscores the importance of the lysine binding sites to the functioning of plasminogen. Plasminogen binds to fibrin, to the extracelluar matrix and to cell surfaces via the strong lysine binding site in kringle 1 [Mangel et al, 1990]. In addition binding to this site results in protection from inhibition by α_2-antiplasmin. The weak binding sites are involved in activation [Markus et al, 1978].

1.3.1.1 Activation

Plasminogen does not degrade fibrin until it is converted to the enzyme plasmin. Therefore plasminogen activation (conversion to plasmin) is extremely important to the potentially fibrinolytic nature of a surface. In order for surface bound plasminogen to be profibrinolytic, the bound plasminogen should either have plasmin-like activity or should be activatable to plasmin. Several authors have documented plasmin or plasmin-like activity on adsorption of plasminogen to surfaces [Brash et al, 1985; Kichenin-Martin et

al, 1988; Shanker et al, 1987]. For example, Kichenin-Martin et al [1988], found that plasminogen adsorbed from buffer to either sulphonated polystyrene resins or polystyrene glutamic acid sulfamide resins was enzymatically active.

Plasminogen is sensitive to cleavage in the region of the amino terminal at the peptide bonds Arg(68)-Met(69), Lys(77)-Lys(78) and Lys(78)-Val(79) [Walker and Davidson, 1988; Fears, 1989; Mayer, 1990; Weitz, 1990]. Cleavage of these bonds releases the activation peptides causing conformational changes which facilitate the formation of plasmin [Walker and Davidson, 1988; Mayer, 1990]. Release of activation peptide converts Glu-plasminogen into Lys-plasminogen. This conversion is important since Lys-plasminogen has a higher affinity for fibrin and is more readily activated to plasmin than the precursor protein, Glu-plasminogen. Specific cleavage of the Arg(560)-Val(561) peptide bond results in the formation of the two-chain plasmin molecule. Kichenin-Martin et al [1988] attributed the plasmin activity of plasminogen adsorbed on polystyrene resins to an unusual activation mechanism which does not require cleavage of the activation peptide or the Arg(560)-Val(561) bond but exposes the active site, perhaps as a result of a conformational change which occurs upon adsorption.

Originally elucidated using urokinase as the activator, the proteolytic events resulting in the activation of plasminogen to plasmin are now believed to occur with many plasminogen activators [Walker and Davidson, 1988; Wun, 1988; Mayer, 1990]. However the sequence of the reactions and the exact mechanisms are not yet understood [Walker and Davidson, 1988; Wun, 1988]. The activation pathways, activators and inhibitors are important when assessing plasmin activity.

It is now generally agreed that there are two primary pathways of plasminogen activation in blood, the extrinsic and the intrinsic. Of the two, the extrinsic pathway is the more physiologically important. However the intrinsic pathway is likely the one that is activated by biomaterial-blood interactions [Walker and Davidson, 1988; Weitz, 1990].

The Extrinsic Pathway

The activators of the extrinsic pathway are classified into two types, tissue-type plasminogen activator (tPA) and urokinase-type plasminogen activator (uPA) [Wun, 1988; Haber et al, 1989; Weitz, 1990]. Tissue plasminogen activator is a 68 kd serine protease that binds fibrin and catalyses the conversion of plasminogen to plasmin on the surface of a fibrin clot [Hoylaerts et al, 1982]. It is stored and produced in the endothelial cells that line the vessel walls and is secreted into the blood [Beebe et al, 1989; Nachman and Hajjar, 1989; Wiman and Hamsten, 1990].

Tissue plasminogen activator is similar to other proteins of the coagulation and fibrinolytic systems in that it contains several structural domains. It is synthesized as a single chain [Barnathan et al, 1988; Wiman and Hamsten, 1990] form and is converted to the two-chain form via limited proteolysis by several different serine proteases [Wun, 1988, Beebe et al, 1989; Weitz, 1990]. tPA is unusual amongst serine proteases in that it is active in its single chain form [Wiman and Hamsten, 1990]

Tissue plasminogen activator has a high affinity for fibrin and is a relatively poor activator if fibrin is not present. Bound to fibrin, tPA shows changes in both its K_m and k_{cat} for plasminogen that result in a 1000-fold increase in its catalytic activity over its unbound form [Hoylaerts et al, 1982; Haber et al, 1989; Weitz, 1990]. Tissue plasminogen activator and plasminogen can interact on the surface of fibrin, resulting in the formation of a ternary complex [Hoylaerts et al, 1982]. Conformational changes in both proteins are suspected of promoting their interaction. It is interesting to note that the catalytic activity of tPA is enhanced not only by the presence of fibrin, but of fibrin(ogen) fragments, polylysine and heparin [Barnathan, 1988; Weitz et al, 1991].

The enzyme urokinase (u-PA) was originally isolated from urine [Ploug and Kjeldgaard, 1957] from which it derived its name. It is produced mainly in the kidneys as a single-chain molecule (scu-PA) with a molecular weight of approximately 54,000 [Wiman and Hamsten, 1990]. Whether or not scu-PA is active is still in dispute but its activation to the two chain molecule, u-PA by either plasmin or kallikrein results in

increased activity towards plasminogen [Loskutoff and Curriden, 1989; Wiman and Hamsten, 1990]. Urokinase plasminogen activator differs from tPA in that it does not require fibrin for its activity.

It should be noted that these serine proteases can cleave low molecular weight substrates, including chromogenic substrates, as well as plasminogen and fibrinogen [Weitz and Leslie, 1990, Weitz et al, 1991].

The Intrinsic Pathway

This pathway is shown schematically in Figure 1.8. Although this is not a dominant activation pathway in vivo, like the intrinsic coagulation pathway it may play a significant role in blood-material interactions.

The binding of factor XII to prekallikrein and high molecular weight kininogen activates not only the intrinsic coagulation pathway but factor XII-dependent fibrinolysis as well [Walker and Davidson, 1988; Weitz, 1990]. This process results in the conversion of prekallikrein to kallikrein and factor XII to XIIa by a reciprocal reaction. Kallikrein then activates plasminogen to plasmin via urokinase(see Fig 1.8).

1.3.1.2 Inhibition of Plasminogen Activators

Inhibitors of fibrinolysis can be divided into two types, antiactivators and antiplasmins. Of the two, the antiplasmins exert the greatest influence. Discussion of antiplasmins is delayed until plasmin has been introduced.

C1 inactivator is an α_2 globulin, having a molecular weight of approximately 104 kd, and is the main inhibitor of Hageman Factor in plasma and is therefore important in inhibiting intrinsic plasminogen activation [Walker and Davidson, 1988].

Plasminogen activator inhibitors, PAI-1 and PAI-2, are both members of the serpin (serine protease inhibitors) superfamily, and are difficult to detect and isolate [Andreasen et al, 1990; Mayer, 1990; Wiman and Hamsten, 1990]. PAI-1 was originally discovered in plasma, but is also found in several cell types including endothelial cells and in the α-

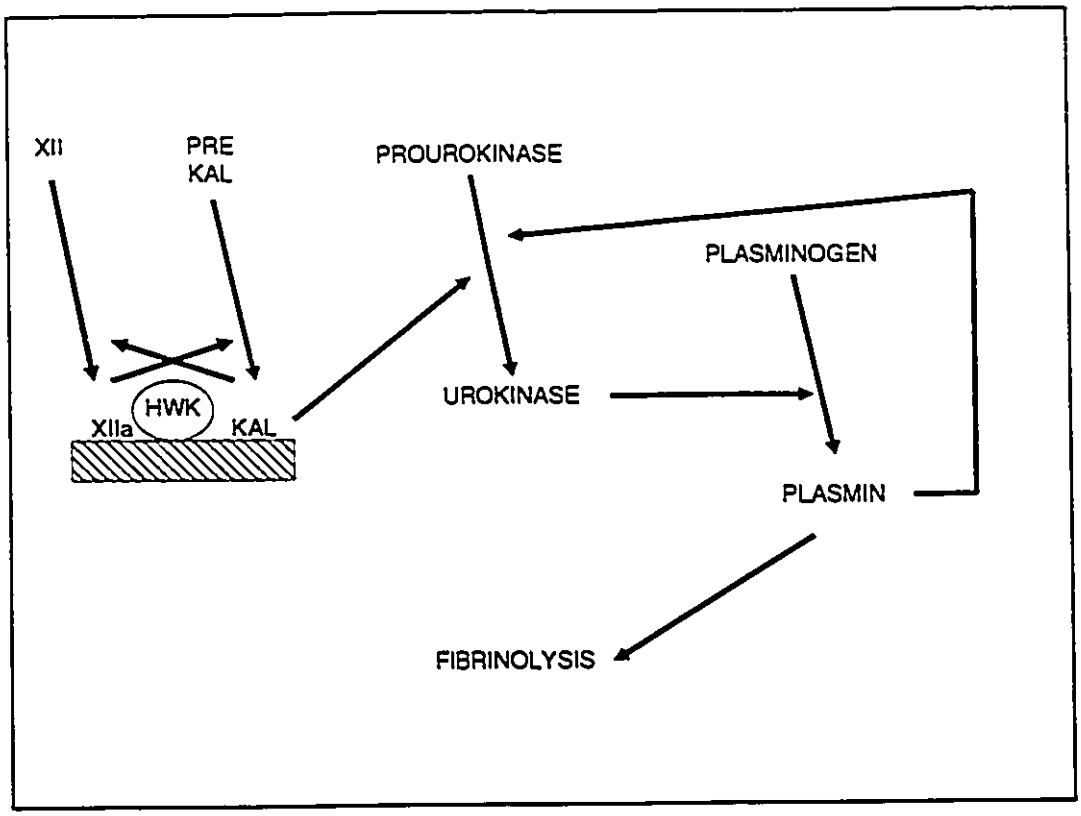


Figure 1.8: The Intrinsic Fibrinolytic Pathway [Weitz, 1990].

granules of platelets. It is released upon platelet activation [Andreasen et, 1990; Wiman and Hamsten, 1990]. Thought to be the principal inhibitor of tPA in plasma, it forms equimolar complexes with both tPA and uPA although it reacts more rapidly with tPA [Loskutoff and Curriden, 1989; Andreasen et al, 1990; Wiman and Hamsten, 1990]. Interestingly PAI-1 complexes with vitronectin, a strongly adhesive plasma protein which has been found to adsorb to biomaterials [Fabrizius-Homan and Cooper, 1991]. Vitronectin stabilizes PAI-1 in its active conformation. PAI-1 is rapidly inactivated under physiological conditions, with a half life of approximately two hours [Andreasan et al, 1990].

PAI-2 is found principally in the plasma of pregnant women and in placental

tissue [Andreasan et al, 1990; Wiman and Hamsten, 1990]. Its physiological function is still not fully understood but it is not believed to play a significant role in the inhibition of tPA.

1.3.2 Plasmin

The product of plasminogen activation, plasmin, exists in various forms, probably resulting from conversions of the different forms of the proenzyme. Plasmin is a two chain protein which cleaves lysyl and arginyl bonds. The heavy chain is referred to as A and the light chain as B. The light chain contains the active centre of the enzyme. Plasmin cleavage of crosslinked fibrin bundles produces D dimer (since the D domains are cross-linked) and E fragments and other fibrin degradation products (FDP)[Ferguson et al, 1975; Doolittle, 1978; Nossel, 1981].

Plasmin is a broad spectrum serine protease the activity of which is under tight control. In addition to cleaving fibrin, plasmin interacts with many other plasma proteins, including factors V and VIII [Mann et al, 1988]. It influences the coagulation, complement and kallikrein systems. Plasmin also causes platelet aggregation and release [Dang et al, 1989], breaks down the extracelluar matrix, and influences cell migration, tissue remodelling and tumour metastasis [Andreasan et al, 1990].

The cleavage of fibrinogen by plasmin is particularly important to this work. Thrombin and plasmin compete in the cleavage of fibrinogen and fibrin I [Nossel, 1981], but the products resulting from these cleavages are different. As discussed previously, thrombin cleavage of fibrinogen releases FPA and FPB, resulting in fibrin polymerization. Thrombin also activates factor XIII which then serves to crosslink the fibrin. On the other hand, plasmin degradation of fibrinogen and noncrosslinked fibrin I results in the release of the Bβ 1-42 peptide [Nossel, 1981; Liu et al, 1986; Weitz et al, 1986]. This produces an intermediate fragment, fragment X, which is subsequently degraded by plasmin through a series of intermediate steps to produce two unlinked D fragments and one E fragment from each fibrinogen or fibrin I molecule [Marder et al, 1969, 1972; Pizzo, S.V.

et al, 1972; Nossel, 1981; Liu et al, 1986; Weitz et al, 1986, Dang et al, 1989]. Degradation of fibrin II by plasmin releases the peptide β 15-42. These reactions are illustrated in Figure 1.9.

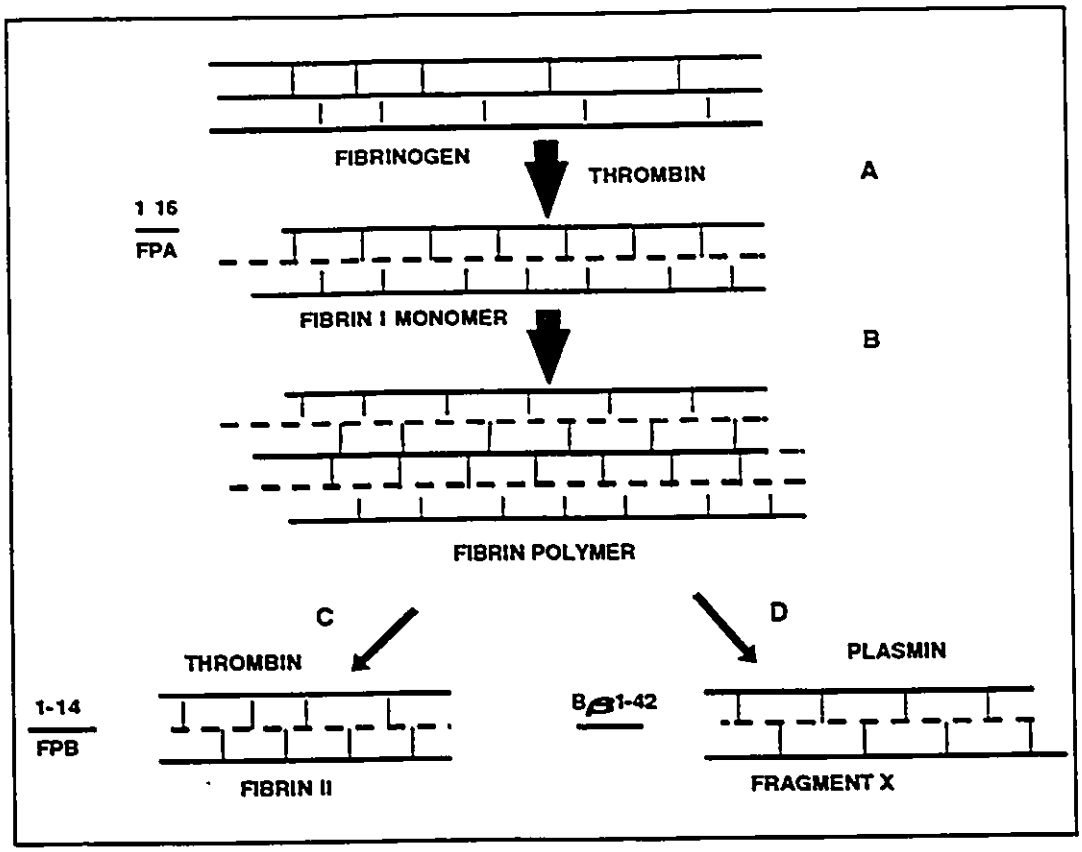


Figure 1.9 [Nossel, 1981]: Fibrinogen proteolysis in vivo. Reaction (A), thrombin cleaves the Aα chain of fibrinogen to produce free FPA and fibrin I monomer. Reaction (B), polymerization occurs. Reaction (C), thrombin cleaves fibrin I to produce free FPB and fibrin II. Reaction (D), plasmin cleaves fibrin I to produce Bβ1-42 and fragment X. Half fibrinogen molecules are shown.

1.3.2.1 Plasmin Inhibition

α_2-antiplasmin is an antiprotease which accounts for the major antiplasmin activity in plasma [Walker and Davidson, 1988]. Like PAI1 it is a member of the serpin

super family and is composed of a single chain polypeptide of approximately 70 kd [Mayer, 1990; Wiman and Hamsten, 1990]. α_2-antiplasmin forms a 1:1 complex with plasmin. This reaction is extremely rapid and takes place in several steps, one of which involves a lysine binding site [Mayer, 1990; Wiman and Hamsten, 1990]. Because of its affinity for the lysine binding sites, α_2-antiplasmin interferes with the binding of plasmin to fibrin. It reacts rapidly and irreversibly with circulating plasmin but more slowly with fibrin bound plasmin because in the latter case the lysine binding sites are occupied. It is a hypothesis of the present work that binding of plasmin(ogen) to a biomaterial surface functionalized with lysine may afford protection from the effects of this inhibitor by engaging the LBS.

α_2-macroglobulin is involved in the inhibition of plasmin by a relatively slow mechanism. The role of α_2-macroglobulin is to inactivate plasmin in excess of the inhibitory capacity of α_2-antiplasmin [Walker and Davidson, 1988]. This large glycoprotein (725 kd) is capable of binding several serine proteases including thrombin and elastase and is believed to inactivate these enzymes by steric hindrance of the active site [Walker and Davidson, 1988].

Other antiplasmins, including α_1-antitrypsin, have minimal influence in the presence of α_2-antiplasmin and α_2-macroglobulin.

1.3.3 The Fibrin Surface

The fibrin surface plays a central role in the localization and regulation of fibrinolysis [Fears, 1989; Mayer, 1989; Wiman and Hamsten, 1990]. Similarities have also been found between the binding of tissue plasminogen activator and plasminogen to fibrin and cell surfaces [Barnathan et al, 1988; Beebe et al, 1989; Nachman and Hajjar, 1989; Plow et al, 1991]. These similarities include specific binding sites [Barnathan et al, 1988; Beebe et al, 1989; Nachman and Hajjar, 1989; Plow et al, 1991], enhanced efficiency of activation of bound plasminogen, the role of lysine residues in binding to both types of surface, and the protection of bound plasmin from inhibition by α_2-antiplasmin [Plow et

al, 1991].

The fibrinolytic pathway is made specific for fibrin by the structural features found within the enzymes and proenzymes discussed in detail above [Fears, 1989; Mayer, 1990; Weitz, 1990; Plow et al, 1991]. Plasminogen, plasmin and tissue plasminogen activator all have " kringles" within their heavy chains which contain lysine binding sites (LBS). These sites mediate the specific interaction of the proteins with fibrin. It is the binding of these sites to fibrin/cell membranes that results in the conformational changes [Mangel et al, 1990], enhanced activation to plasmin by tPA [Barnathan et al, 1988; Beebe et al, 1989; Nachman and Hajjar, 1989; Plow et al, 1991], and protection from inhibition by α_2-antiplasmin, the principal inhibitor of plasmin in vivo, previously discussed [Beebe et al, 1989; Fears, 1989; Weitz, 1990; Plow et al, 1991].

In this work, it is hypothesized that mimicking the interactions of plasminogen with both fibrin and cell membranes utilizing a polymeric surface might lead to preferential adsorption of plasminogen from plasma to the surface. The nature of the adsorption via either the strong or weak lysine binding sites will have implications for the conformation, enzymatic activity, and susceptibility to inhibition of the adsorbed plasminogen.

1.4 BLOOD COMPATIBLE MATERIALS

As discussed in the introduction, there are primarily three strategies which have been employed to reduce the thrombogenicity of artificial surfaces: making the surface inert, making the surface specifically interactive or making the surface similar to the vascular wall. In keeping with the focus of this thesis, only interacting surfaces will be discussed in detail, and emphasis will be placed on heparinized and fibrinolytic materials.

1.4.1 Heparinized Surfaces

The best known examples of interacting surfaces are heparin-derivatized polymers or polymers with heparin-like activity.

Heparin is a linear sulphated polysaccharide and is highly negatively charged. It contains alternating 1,4 uronic acid and glucosamine residues, as illustrated in Figure

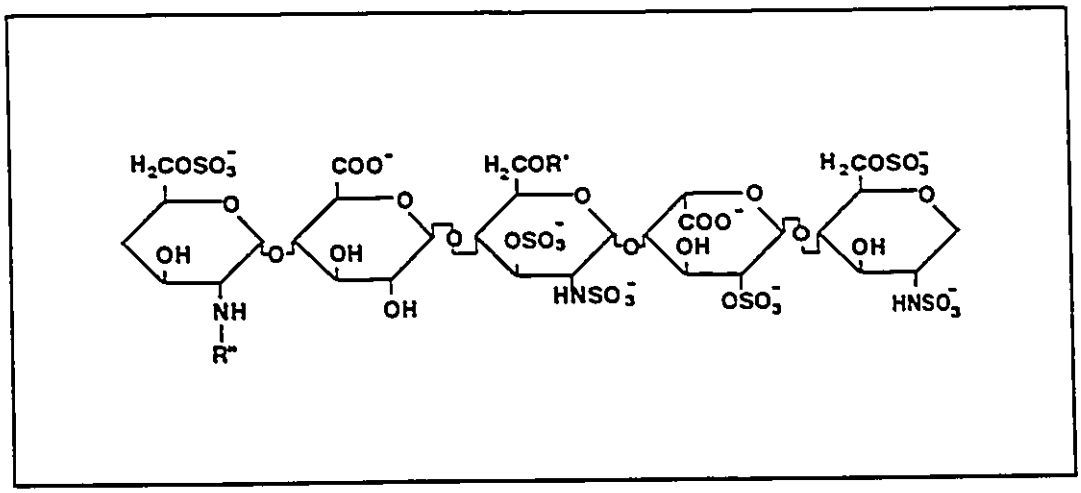


Figure 1.10: The structure of heparin. The pentasaccharide shown is the minimum sequence required for the high affinity binding of heparin with antithrombin III. The two rings on the right comprise a trisulphated disaccharide which is the active site for binding with antithrombin III [Casu, 1989].

1.10 [Hovingh et al, 1986; Lindahl et al,1986; Casu, 1989]. The major effect of heparin is to potentiate antithrombin III which then serves to inactivate thrombin, Factor Xa and other activated clotting factors. This results in an anticoagulation effect which can be utilized in the design of blood-compatible biomaterials. In addition, heparin can also influence cell proliferation, angiogenesis, modulation of the synthesis of collagen and increased synthesis of both fibronectin and thrombospondin by smooth muscle cells [Tan et al, 1989]. Heparin has a broad molecular weight distribution, ranging between 6000-25000 with approximately 2-3 sulphates per disaccharide unit [Alberts, 1983].

It was hypothesized very early that blood-contacting surfaces might be rendered anticoagulant, and thus non-thrombogenic, by the incorporation of heparin. One of the earliest approaches used a graphite-benzalkonium-heparin coating on metals and polymers

[Gott et al, 1964]. Subsequent work in this area concentrated on introducing cationic charges into the surface to which heparin could be ionically bound via its negative charges [Shibuta et al, 1986; Han et al, 1989a&b]. Several such materials showed good short-term anticoagulant properties. However when bound electrostatically, heparin is slowly released into contacting blood. The anticoagulant activity is thus of short duration [Goosen and Sefton, 1983; Shibuta et al, 1986; Larsson et al, 1987; Han et al, 1989b]. Other researchers immobilized heparin using glutaraldehyde as a coupling or crosslinking agent [Larsson et al, 1987; Han et al, 1989a].

Heparin has also been covalently attached to polymers and this approach has met with some success [Ito et al, 1986, Han et al, 1989a&b]. Investigators have reported anticoagulant and/or antithrombogenic activity using poly(dimethylsiloxane)-poly(ethylene oxide)-heparin block copolymer [Grainger et al, 1988; 1990], surface immobilized PGE_1 - heparin conjugate [Jacobs et al, 1989], and polyvinyl alcohol-heparin complex coated polyethylene tubes [Rollason and Sefton, 1989]. Recently, Mazid et al [1991] copolymerized heparin fragments with acrylamide and showed that such materials had antithrombotic properties.

Polyurethanes have been used extensively in the last several years as substrates for heparin. Polyaminoether urethaneureas containing tertiary amine groups were used by Shibuta et al [1986] to immobilize heparin to the polymer surface. Poly(amido amine) chains grafted to polyurethanes and glass were found to form stable complexes with heparin [Barbucci et al, 1987], and in a similar approach, polyurethanes containing poly-amidoamine blocks have been developed to enhance heparin adsorption [Tanzi and Levi, 1989]. Heparin has been immobilized onto segmented polyurethanes using hydrophilic PEO spacers to enhance its anticoagulant activity [Park et al, 1988] and Han et al utilized polyurethanes to immobilize heparin by several different methods [Han et al, 1989a].

Realization that the anticoagulant activity of heparin is due in some measure to the presence of sulphate and aminosulphate groups [Arge, 1956; Nelson et al, 1970; Hovingh, 1986] has led to the development of materials which incorporate these groups

and thus can be thought of as "heparin-like". This approach has been followed in particular by Jozefowicz and Jozefonvicz [Fougnot and Josefonvicz, 1979; Fougnot and Jozefowicz, 1979]. A comparison of heparin with a heparin-like material based on polystyrene is given in Fig 1.11. As early as 1956, sulphate esters of

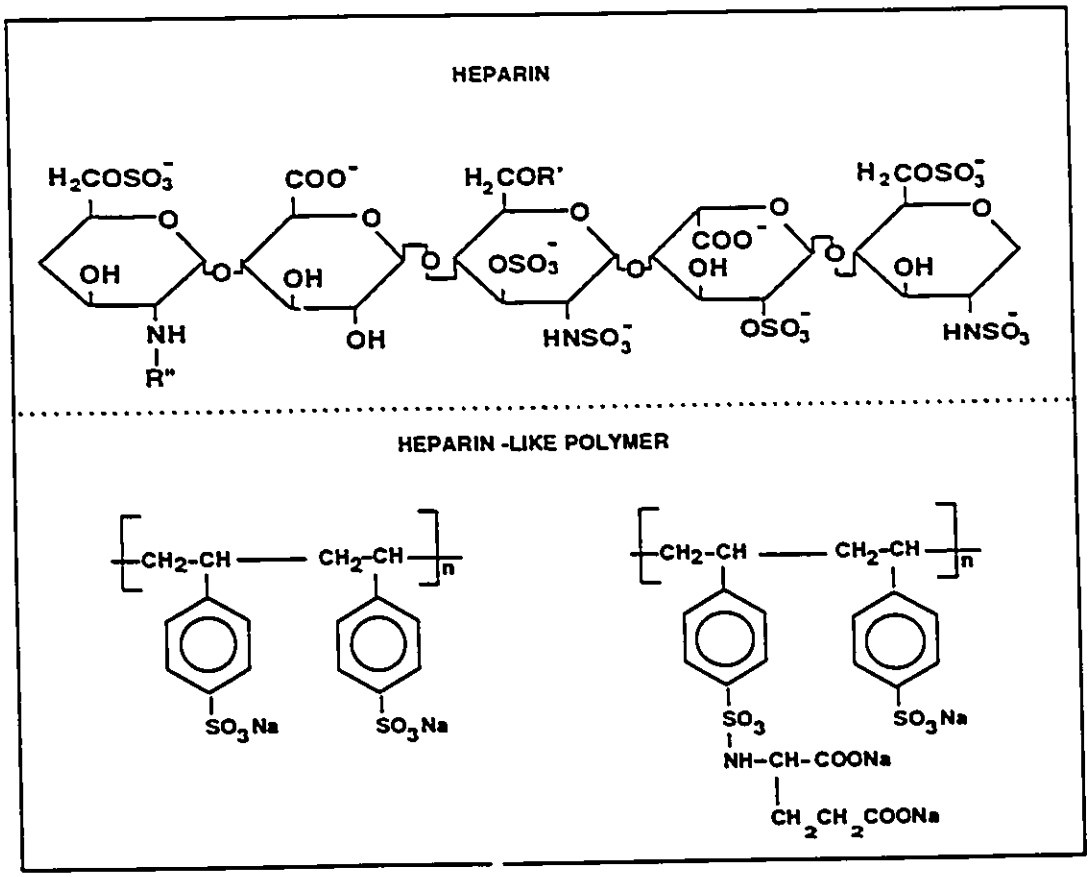


Figure 1.11: Comparison of heparin [Lindahl et al, 1986; Casu, 1989] and heparin-like polymers [Fougnot and Jozefowicz, 1979 a&b]

polysaccharides were found to have heparin-like activity [Arge, 1956]. Heparin-like activity has been achieved by Jozefowicz, Jozefonvicz, and coworkers through incorporation of sulphate and amino acid sulphamide groups into polystyrenes and dextrans [Fougnot and Jozefonvicz, 1979; Fougnot and Jozefowicz, 1979; Mauzac and

Jozefonvicz, 1984; Kanmangne et al, 1985; Douzon et al, 1987; Aubert et al, 1988; Migonney et al, 1988a&b]. They have shown that such materials possess anticoagulant and specifically, heparin-like characteristics.

Jozefowicz, Jozefonvicz and coworkers have also attached various amino acids to sulphonated polystyrenes in the expectation that specific adsorption of natural coagulation inhibitors, particularly antithrombin III, might occur [Fougnot and Jozefowicz, 1979; Fougnot et al, 1983]. Incorporation of aspartic and glutamic acids, was found to enhance antithrombogenic behaviour compared to sulphonated control polystyrenes [Fougnot and Jozefowicz, 1979; Fougnot et al, 1983]. Ito et al [1991a&b] have grafted polyetherurea film with poly(sodium vinyl sulphonate). The resulting materials showed enhanced binding of antithrombin III compared to unmodified polyetherurethane controls.

Heparinized or heparin-like materials, while showing some promise as antithrombogenic surfaces, are not without problems. As previously mentioned heparin activity is often short lived. In addition, heparinized surfaces have been associated with protein denaturation and platelet activation [Jacobs et al, 1989; Grainger et al, 1991; Ito et al, 1991a]. The interactions of surface bound heparin are not fully understood. For example in a study on heparinized polyurethanes, Ito et al [1986] compared the antithrombogenicity of anionic polymers with polyurethanes containing immobilized heparin. They found that a polyetherurethane with anionic charges on the surface selectively adsorbed albumin, did not appear to change the conformation of adsorbed proteins, suppressed platelet adhesion but not coagulation and showed what the authors termed moderate antithrombogenicity. The heparinized polyurethane, on the other hand, did not selectively adsorb albumin, altered the conformation of adsorbed proteins, activated platelets, deactivated the coagulation cascade, and showed excellent antithrombogenicity.

1.4.2 Fibrinolytic Surfaces

The attractiveness of the concept of a fibrinolytic surface, along with the paucity

of prior work based on this approach, provided the motivation for the work reported in this thesis. A fuller discussion of the rationale and objectives is given in Chapter 2.

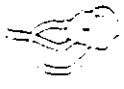
Work done by Kusserow in the early 1970s indicated that the attachment of urokinase to a surface modified clot formation [Kusserow et al, 1971]. More recently Foster and Bernath [1988] showed that urokinase immobilized on polytetrafluoroethylene vascular prostheses enhanced the fibrinolytic activity of these materials. In animal studies, Senatore et al [1986a] found increased patency for urokinase-bound Dacron-reinforced fibrocollagenous grafts versus controls without urokinase. Further work by Senatore and Bernath [1986b] on the same urokinase-modified grafts, indicated that the immobilized urokinase catalyzed the formation of fibrin degradation products at enhanced rates compared to controls.

Shankar et al [1987] immobilized plasmin on collagen beads and found fibrinolytic activity of the immobilized plasmin even in the presence of α_2-antiplasmin. This was in contrast to the results with soluble plasmin where the inhibitor significantly decreased the fibrinolytic activity of the sample.

Kichenin-Martin et al [1988] reported that plasminogen adsorbed from plasma to the sulphonated and amino acid derivatized polystyrenes of Jozefowicz et al[Kanmangne et al, 1985] and that via this adsorption plasmin-like fibrinolytic activity was generated, presumably due to a conformational change which exposed the active site. Brash et al have also found evidence for surface activation of plasminogen on glass resulting in degradation of adsorbed fibrinogen [Brash et al, 1985]. Recently, Yang and Henkin [1990] have refuted the findings of Kichenin-Martin et al [1988]. They found that rather than activating plasminogen upon adsorption, sulphonated or sulphonyl glutamate derivatized polystyrene resins selectively adsorb plasmin from solutions of plasminogen.

1.5 FUNDAMENTALS OF PROTEIN ADSORPTION TO BIOMATERIALS

As indicated in section 1.1, the adsorption of plasma proteins plays an important role in the interactions of a biomaterial with blood [Andrade, 1985; Brash, 1992]. This



is not a phenomenon specific to biomaterials. In general proteins are surface active and tend to accumulate at interfaces [Norde and Lyklema, 1991]. Thus protein adsorption is relevant to many other areas including food processing [Arnebrant and Nylander, 1986], purification of proteins by chromatography, waste water purification using membranes, and biosensors where protein-containing fluids are in contact with solid surfaces. Protein adsorption can result in the fouling of processing equipment and ultrafiltration membranes, decrease yields in chromatography, and support cell growth in bioreactors. Protein adsorption is also relevant to all biomedical implant situations, not just in blood contacting applications.

1.5.1 General Considerations

The chemical and physical characteristics of both the protein and the material influence their interactions. Proteins are macromolecules composed of amino acid residues linked one to another by peptide bonds. A protein may contain anywhere from fifty to over ten thousand residues with a specific chain sequence of the twenty or so amino acids commonly found. Proteins may assume a variety of conformations determined by the amino acid sequence (primary structure) which results in ordered secondary structures such as α-helices and β-sheets.

The tertiary structure of a protein refers to the way in which unordered and ordered secondary structures combine to give the protein its characteristic 3-dimensional shape. It results largely from unfavourable interactions between the amino acids. In solution the 3-dimensional shape attained by the protein will be that which minimizes the free energy of the system including the interactions between the protein and surrounding medium [Macritchie, 1986; Norde and Lyklema, 1991]. The major driving force for the folding of a polypeptide in solution is dehydration of hydrophobic amino acid side chains [Norde and Lyklema, 1991]. Amino acids with hydrophobic side groups tend to be in the interior of the molecule, away from water. The amino acids with charged or polar side chains tend to be predominantly on the periphery of the molecule [Norde and Lyklema,

1991]. The net result is a spontaneously folding protein with a hydrophobic core and a complex irregular exterior surface formed by the polar or hydrophilic side chains. A protein therefore has the potential of binding to a biomaterial through ionic, polar or hydrophobic interactions [Andrade, 1985; Norde and Lyklema, 1991].

Enzymes are a special class of proteins which are responsible for many of the interactions which occur when biomaterials are placed in blood. Enzymes are biological catalysts which effect their action through specific binding to a substrate involving interactions dependent on conformation. This binding is achieved via an 'active site' which is characteristic of the enzyme. Factors that affect conformation such as temperature, pH, the presence of other proteins, and adsorption to a surface may affect the enzymatic activity. Frequently enzymes are formed from precursors called proenzymes. Plasminogen, the focus of this thesis, is such a proenzyme. It must be converted to the enzyme plasmin for clot lysis to occur.

The surface properties of the material will influence its interactions with proteins and are likely to affect the protein conformation and enzymatic activity [Andrade, 1985; Norde and Lyklema, 1991]. Figure 1.12, adapted from Andrade [1985] illustrates the interactions generally considered to occur in protein adsorption.

In studies of protein adsorption, surfaces have frequently been classified as either hydrophobic or hydrophilic. Protein adsorption is generally considered to be greater to hydrophobic surfaces than hydrophilic surfaces [Prime and Whitesides, 1991; Brash, 1992]. Grainger et al [1989] found that surface hydrophobicity/hydrophilicity could not be correlated with protein adsorption onto phase separated block copolymers. However, Van Damme [1990], using silylated silica glass surfaces, showed that protein adsorption was maximal when both the hydrophilic and hydrophobic content of the surfaces were of intermediate values. Other researchers have found stronger relationships between competitive and single protein adsorption studies and surface hydrophobicity [Brash and Uniyal, 1979; Horbett, 1981; Slack et al, 1985; Elwing et al, 1987; Lee and Ruchenstein, 1988; Prime and Whitesides, 1991]. However, the exact relationship between

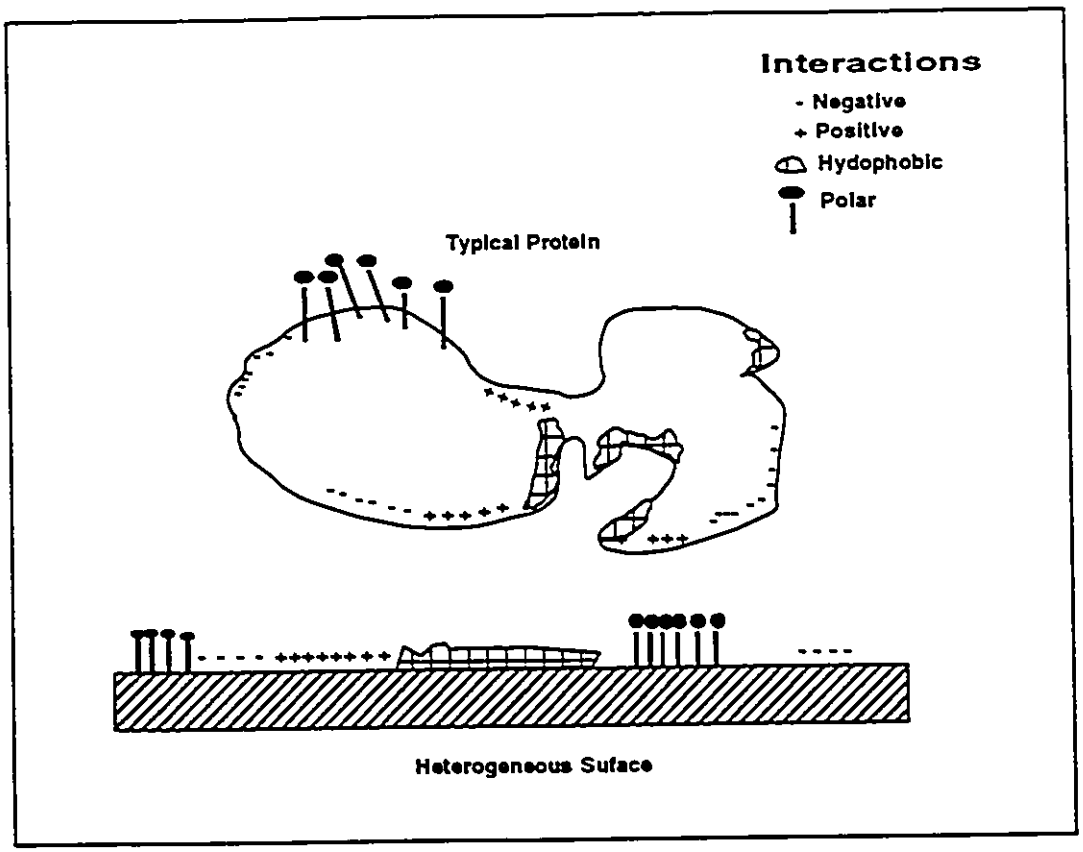


Figure 1.12: Protein/surface Interactions. Adapted from Andrade [1985]. The protein and the surface have a number of domains through which they may interact. These include hydrophobic or "greasy", polar (electron donor/acceptor interactions), and ionic domains.

hydrophobicity and protein adsorption from buffer and/or plasma has not been clearly established [Wojciechowski, 1992].

Investigators have also found that surface hydrophobicity/hydrophilicity affects the conformation of the protein when it is adsorbed. Conformational changes may result in inactivation of enzymes as discussed above, for example, Sandwick and Schray [1987] found that several enzymes were inactivated when adsorbed to a hydrophobic latex surface from solutions of low enzyme concentration. Elwing et al [1988] found that complement factor 3 (C3) adsorbed to hydrophobic surfaces exposed antigenic

determinants normally only accessible to antibody after denaturation or activation. Such effects were not observed on hydrophilic surfaces. Ito et al [1990] found that denaturation of proteins adsorbed to surfaces correlated with increased platelet adhesion and thrombogenicity. These results are particularly important for this work.

1.5.2 Protein Adsorption: Single Protein Systems

1.5.2.1 Kinetics

An important question in the study of interfacial phenomena is whether the rate is reaction or transport controlled. Protein adsorption is generally agreed to be transport limited when the fraction of the surface covered by the protein is low (less than 10% of monolayer coverage)[Andrade, 1985; Brash, 1991]. In this situation the flow conditions must be taken into account since convection as well as diffusion will be present.

The combined transport adsorption process may be modeled using the appropriate form of the convection-diffusion equation, equation 1.1, with appropriate boundary conditions [Lok et al, 1983].

$$\frac{\partial c}{\partial t} + v(y)\frac{\partial c}{\partial x} = D\frac{\partial^2 c}{\partial y^2} \qquad 1.1$$

where c is concentration, t is time, $v(y)$ is the velocity distribution across the flowpath, D is the protein diffusivity, x is the axial coordinate (direction of flow), and y is the radial coordinate perpendicular to the flow (e.g. in a tube).

An important boundary condition , equation 1.2, applies at the surface:

$$D\frac{\partial c}{\partial y} = R(c) \qquad 1.2$$

Equation 1.2 states that the flux at the surface is equal to the intrinsic (kinetic) rate of

adsorption R(c).

In general equation 1.1 must be solved numerically. However, analytical solutions are possible for simple cases such as the transport limited case in a nonflowing system (diffusion limited). In this case the flux at the surface, i.e. the adsorption rate is given by equation 1.3:

$$\frac{d\Gamma}{dt}=C_o\left(\frac{D}{\pi t}\right)^{1/2} \qquad 1.3$$

and the surface concentration is given by equation 1.4:

$$\Gamma=2C_o\left(\frac{Dt}{\pi}\right)^{\frac{1}{2}} \qquad 1.4$$

Where Γ=surface concentration; C_o= bulk concentration of the protein in solution; D=diffusivity; and t=time. Equation 1.4 indicates the $t^{1/2}$ dependence expected from a diffusion controlled process.

When adsorption is kinetically limited, whether in a flowing or nonflowing system, the rate is given by the intrinsic kinetics, of the general form of equation 1.5:

$$\frac{d\,\Gamma}{dt}=R(C_o) \qquad 1.5$$

As indicated, adsorption may initially be transport limited but as the surface sites become occupied, the intrinsic kinetics will determine the rate [Cheng et al, 1987].

1.5.2.2 Equilibria and Isotherms

Generally two kinds of model are used to describe single protein adsorption: namely theoretical and empirical [Silverberg, 1985]. Single protein adsorption isotherms

can be reasonably well explained by the Langmuir model (theoretical) and the Freundlich model (empirical) which were developed for gas adsorption.

The Langmuir model assumes that only one molecule adsorbs per site (monolayer assumption), that there is only one type of site present (energetically uniform surface, constant heat of adsorption), that the adsorption of one molecule does not affect the adsorption of another, and that the adsorption is reversible (i.e. equilibrium between the adsorbate on the solid surface and in solution is assumed) [Andrade, 1985; Silverberg, 1985; Brash, 1991]. Several investigators have found that protein adsorption is essentially irreversible on a reasonable time scale [Chan and Brash, 1981; Norde et al, 1986; Cheng et al, 1987]. Indeed the use of the terms "isotherm" and "equilibria" are questionable when applied to protein adsorption. However despite these difficulties, the Langmuir model has been used extensively in the literature and provides a reasonably good fit to many adsorption data [Young et al, 1988; Brash, 1992]. The success of the Langmuir model is surprising since the fundamental assumptions of this model are clearly not applicable to protein adsorption.

The Langmuir model can be derived using kinetic arguments [Moore, 1972]. Adsorption of protein P to surface site X may be described by:

$$P + X \underset{k_d}{\overset{k_a}{\rightleftharpoons}} PX \qquad 1.6$$

where k_a is the adsorption rate constant and k_d is desorption rate constant.

The rate of adsorption (R_{ads}) is then given by:

$$R_{ads} = k_a C_p (1-\theta) \qquad 1.7$$

where θ is the fraction of the surface area covered by adsorbed molecules at any time, C_p is the protein concentration.

The rate of desorption is given by:

$$R_{des} = k_d\theta \qquad 1.8$$

At equilibrium:

$$k_d\theta = k_a C_p(1-\theta) \qquad 1.9$$

Solving for θ:

$$\theta = \frac{KC_p}{1+KC_p} = \frac{\Gamma}{\Gamma_{max}} \qquad 1.10$$

Where Γ= adsorption, Γ_{max}= adsorption at the plateau (monolayer adsorption), and $K=k_a/k_d$ and is called the adsorption coefficient. The adsorption coefficient has the properties of an equilibrium constant.

A characteristic of this isotherm is that a plot of θ versus concentration yields a curve that rises monotonically with increasing concentration to a plateau of constant surface concentration (θ =1). This plateau level is assumed to represent complete coverage. Two limiting cases are generally considered when analysing adsorption data which fits a Langmuir model. In the first case when the concentration is very low equation 1.10 reduces to:

$$\theta \equiv KC_p \qquad 1.11$$

the curve approximates a straight line and the slope of this line can be used to determine the adsorption coefficient. In the second case, at high concentration with θ approximately equal to one, the plateau adsorption can be used to calculate the monolayer concentration

and the surface area occupied per molecule bound.

The other model frequently used to describe single protein adsorption is the Freundlich isotherm described by equation 1.12. This empirical model was originally used to express the relationship between pressure and surface coverage in gas adsorption [Thomson and Webb, 1968; Young et al, 1988]:

$$\theta = kC_p^{\frac{1}{n}} \qquad 1.12$$

In equation 1.12, θ is surface coverage, k and n are empirical constants characteristic of the system at a given temperature, and C_p is the bulk solution concentration of the protein.

If it is assumed that the heat of adsorption falls exponentially as adsorption proceeds and that the decrease in heat of adsorption is due to surface heterogeneity, the Freundlich isotherm may be derived theoretically [Thomson and Webb, 1968; Young et al, 1988].

Young, Pitt and Cooper [1988] investigated the adsorption isotherms of seven different proteins on four biomaterials and found that the data fit a Langmuir isotherm in the low region of the curve. However at higher adsorption levels the authors found that different models had to be applied to describe the adsorption isotherms. Moreno et al [1987] showed that protein adsorption could be fit to a Langmuir model but questioned the significance of the parameters obtained. These studies are but two examples of the extensive literature available on protein adsorption modelling and indicate the controversy surrounding this complex issue. The reader is referred to a series of excellent reviews by Brash [1991], MacRitchie [1978], Andrade [1985], and Norde [1991] for more detailed information.

1.5.3 Protein Adsorption from Protein Mixtures

As previously indicated, proteins are surface active, readily adsorbing to solid

surfaces. In a protein mixture, one protein may adsorb in preference to others, its adsorption influenced by the other proteins in the mixture and the surface characteristics of the solid material. The relative surface activity of a mixture of proteins will ultimately determine the composition of the protein layer on a solid surface in contact with that mixture. This initial layer has long term implications for biomaterials in contact with blood. Because of the complexity of blood and plasma, simple mixtures of plasma proteins in buffer have been studied as models for both plasma and blood.

The proteins most extensively investigated in competitive studies from buffer are IgG, albumin, and fibrinogen [Horbett, 1984; Pitt et al, 1986; Sharma and Joseph, 1986; Bale et al, 1987]. These have been chosen for study because of their high plasma concentration (120-500 μm, 500-800 μm, and 9 μm respectively) and ready availability.

Fibrinogen has frequently been shown to preferentially adsorb from protein mixtures, particularly in the presence of IgG and albumin [Horbett, 1984; Vroman, 1987; Brash and Davidson, 1976; Bale et al, 1988]. Using a protein mixture of IgG, fibrinogen and albumin present at physiologic concentrations, Brash et al [1984] showed that the level of adsorption of each protein in the mixture to a number of surfaces was the reverse of their relative concentrations in the bulk solution e.g. albumin had the lowest concentration on the surface. Horbett and Hoffmann also found that fibrinogen adsorption was preferred over albumin and IgG on several different surfaces including a segmented-urethane-urea. Using a somewhat different protein mixture, Bale et al [1987] found that fibrinogen preferentially adsorbed on polystyrene latex. They estimated the relative affinities of the proteins studied were in the order fibrinogen> fibronectin> IgG >albumin. However evidence from simple competitive adsorption experiments has shown the haemoglobin adsorbs preferentially to polyethylene and the relative order for protein affinities to this material has been estimated as haemoglobin > fibrinogen > albumin ≈ IgG. In addition, other researchers have investigated less abundant plasma proteins, such as high-molecular-weight kininogen [Elwing et al, 1987] and vitronectin [Fabrizius-Homan and Cooper, 1991] and these studies have also indicated that fibrinogen may not

always be preferentially adsorbed. Fabrizius and Cooper found that vitronectin has a high surface affinity relative to human serum albumin, fibrinogen, and fibronectin when studied in both binary and competitive systems.

One of the principal reasons for investigating protein mixtures is to extrapolate to what may happen in plasma and blood. For example, Slack et al [1987] studied fibrinogen adsorption from mixtures to polyethylene and glass using binary mixtures of either fibrinogen/albumin or fibrinogen/haemoglobin. They found that fibrinogen adsorption showed a maximum at intermediate protein concentrations and that this maximum shifted to lower bulk fibrinogen concentrations when the ratio of competing protein to fibrinogen was increased. This is similar to what has been found in plasma. Pitt et al found a linear correlation between the surface concentrations of albumin and fibrinogen on a polyvinyl chloride surface and platelet deposition when albumin adsorption was followed by fibrinogen in a sequential study [Pitt et al,1986].

Extrapolation from competitive adsorption data found in mixtures of proteins to plasma and whole blood data is not always so clear, however. Grainger et al found no correlation between competitive adsorption studies using a mixture of fibrinogen, albumin, and IgG in buffer to a series of polyethylene oxide polystyrene block copolymers and the same surfaces evaluated in plasma [Grainger et al, 1989], and other authors have also found differences in the adsorption characteristics of a protein adsorbed from a mixture of proteins compared to its adsorption from plasma or whole blood [Horbett, 1984; Brash and ten Hove, 1984, Wojciechowski et al, 1986]. These research groups found that fibrinogen adsorption from plasma to glass decreased at times longer than a few minutes while IgG and albumin adsorption increased. This is in exact contrast to the results obtained for this surface and the mixed protein systems. It is apparent that a binary or ternary system may not be an appropriate model for the plasma system or whole blood system.

1.5.4 Protein Adsorption from Plasma and Blood

Protein adsorption from plasma is frequently studied as an intermediate step between the simple protein mixtures discussed above and the more complex fluid, blood. Plasma is nonetheless relatively complex in nature, containing over two hundred proteins, and has the added advantages of being easier to store and work with than whole blood.

Protein adsorption from plasma appears to have very little to do with the relative concentration of a protein in plasma. Albumin for instance has been found by several investigators to have very low adsorption levels from plasma on surfaces in general [Brash and Davidson, 1976; Bale et al, 1988]. Many proteins, including fibrinogen and albumin, undergo transient adsorption to surfaces, manifested as a maximum in the adsorption data as a function of either time or plasma concentration [Brash and ten Hove, 1989; Andrade and Hlady, 1991; Scott, 1991] This phenomenon, first observed in the 1960's by L Vroman [1969], has been described by other researchers. Brash and ten Hove studied the adsorption of fibrinogen, albumin, and IgG to three surfaces and found that neither albumin nor IgG adsorption to glass showed any dependence on plasma concentration. In contrast, fibrinogen adsorption increased with plasma concentration and then decreased. The concentration at which the maximum occurred was surface dependent [Brash and ten Hove, 1984]. Horbett obtained similar results for fibrinogen adsorption from plasma to polytetrafluoroethylene, polyethylene, and glass [Horbett, 1984]. Wojciechowski et al [1986] found that the maximum in fibrinogen could also be measured as a function of time as well as plasma concentration. In the same study the authors found that none of the characteristics of the adsorption-concentration curves (i.e peak height) could be related to the water contact angle of the different surfaces. Using a series of silylated silica glass with different surface functionality, Wojciechowski found that fibrinogen adsorption from plasma could not be related to surface chemistry [Wojciechowski, 1992].

The Vroman effect reflects competitive adsorption and is believed to result from the displacement of initially adsorbed abundant proteins of low binding affinity by low

concentration proteins of high binding affinity. Vroman has suggested that high molecular weight kininogen (HMWK) is a major displacer on many surfaces and that its persistence in the adsorbed layer promotes surface-induced coagulation [Vroman et al, 1980; Andrade and Hlady, 1991; Scott, 1991]. However, the exact effect of HMWK on fibrinogen adsorption from plasma is not clear. Elwing et al [1987] found that on hydrophilic surfaces fibrinogen was displaced by HMWK but on hydrophobic surfaces there was no evidence of HMWK adsorption, despite the occurrence of the Vroman effect for fibrinogen. In a separate study using baboon fibrinogen, Slack et al [1987] found that there was no difference in fibrinogen adsorption from normal plasma and from kininogen deficient plasmas to silicone rubber or polyethylene.

It is clear that the exact mechanism of the Vroman effect is still not completely understood. It appears to be a surface and protein dependent phenomena with not all plasma proteins showing an adsorption maximum on all surfaces. Moreover its relationship to thrombotic events is not defined, although it is possible that an understanding of this mechanism may lead to a clearer understanding of thrombus formation at the material surface when placed in contact with blood. For this reason, detailed plasma adsorption studies using both fibrinogen and plasminogen are included in this thesis.

1.6 SEGMENTED POLYURETHANES

A component of the present project involves the modification of segmented polyurethanes to develop fibrinolytic surfaces. Therefore some background on polyurethanes is provided. Polyurethanes exhibit a wide range of physical and mechanical properties that allow their use in applications requiring hard plastics at one extreme to soft foams at the other. Because of this mechanical versatility and their relatively good biocompatibility, polyurethanes have been used in both blood contacting and tissue implants for a number of years. Examples of blood contacting devices include blood filters and oxygenators, prosthetic heart valves and cardiac assist devices [Lelah and

Cooper, 1986]. They are also used as coatings for cardiac pacemaker leads, in angiography catheters, and in intra-aortic balloons [Lelah and Cooper, 1986; Bruck, 1991]. A number of papers have described research on the used of polyurethanes to construct vascular grafts [Gogolewski, 1989; Brothers et al, 1990; Nojiri et al, 1990].

1.6.1 Chemistry

Polyurethanes are copolymers which contain the characteristic urethane linkage resulting from reaction between isocyanate and hydroxyl groups. The segmented polyurethanes used in this research are a subgroup of polyurethanes. They are block copolymers containing "hard" and "soft" chain segments which are thermodynamically incompatible i.e. their free energy of mixing is positive. Within the solid materials formed by these polymers, this incompatibility results in microphase separation with formation of "hard" and "soft" microdomains [Lelah and Cooper, 1986].

Isocyanates are highly reactive and their chemistry is central to the production of polyurethanes. In forming polyurethanes diisocyanates are reacted with either alcohols to produce urethane linkages, or with amines to give urea linkages. The isocyanate used in the present research is 4,4' diphenylmethane diisocyanate (MDI), an aromatic symmetrical diisocyanate (shown in Fig 1.13). Other diisocyanates commonly used include hexamethylene diisocyanate, cyclohexane diisocyanate, and toluene diisocyanate (TDI), which is asymmetric and therefore has isocyanate groups of unequal reactivity.

Chain extenders are bifunctional compounds used to link together the prepolymers via their terminal isocyanates, to form the high molecular weight polyurethane. The two general classes of chain extenders are diols and diamines. Diamines are generally more reactive than diols and were chosen for this research. Three chain extenders were used in this work. These are discussed in more detail in Chapter 3 of this thesis. Chain extension with diamines results in the urea linkages previously discussed and these polyurethanes are appropriately called polyurethane ureas [Lelah and Cooper, 1986].

Generally, segmented polyurethanes are synthesized in two steps (Figure 1.13):

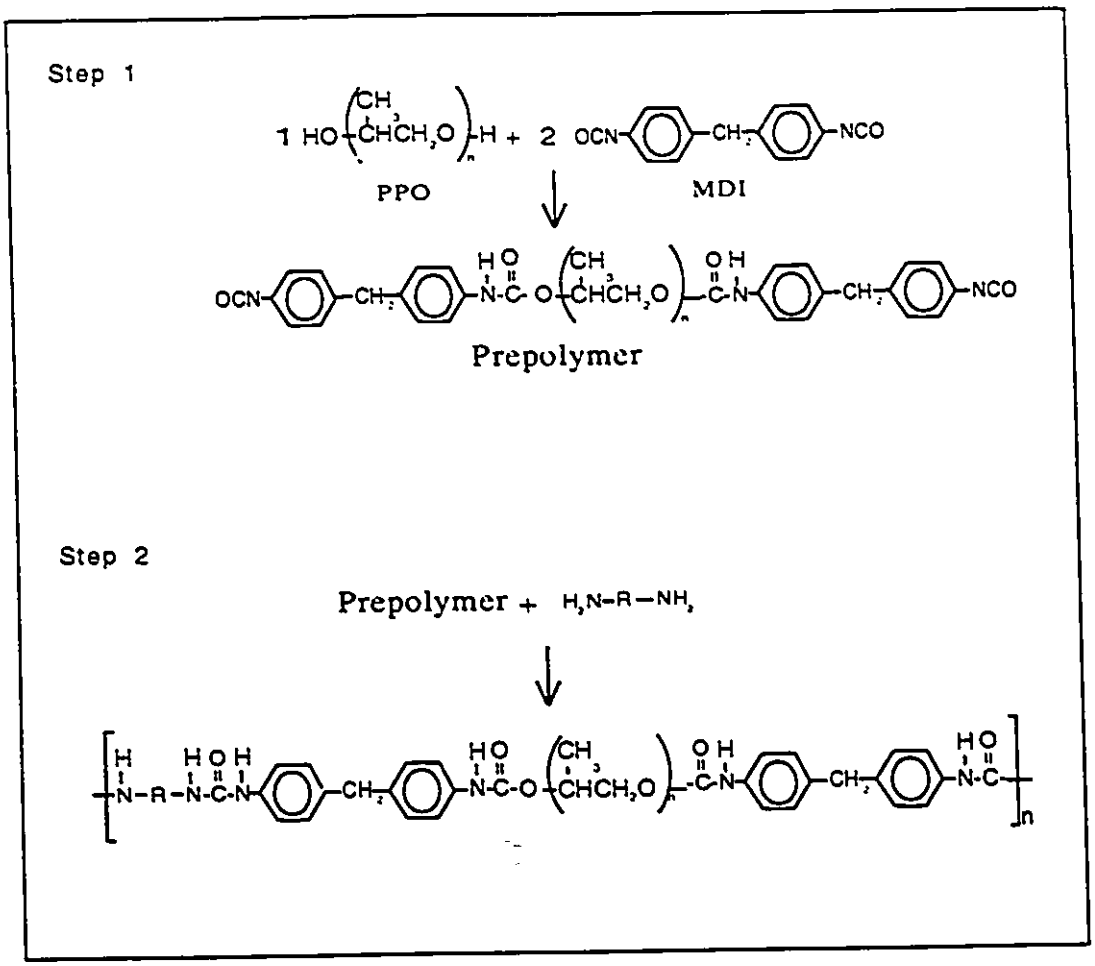


Figure 1.13: Two step synthesis of polyurethanes used in this work. Illustrated for polypropylene oxide (PPO), 4, 4' diphenylmethane diisocyanate (MDI), and a diamine chain extender.

i) A diisocyanate is reacted with a bifunctional hydroxyl-terminated polyester, polyether or other diol to produce an isocyanate terminated prepolymer which constitutes the soft segment. The most common soft segments are polyethers or polyesters with molecular weights in the range of 500 to 5000. In medical applications, the polyether-based materials, due to their relative hydrolytic stability, have proved to be more useful than the polyester based materials.

ii) The prepolymers are linked together or chain extended to form high molecular weight polymers using simple diamines or diols. The chain segments between soft segments consist of urethane and/or urea linked diisocyanates and diols or diamines and constitute the hard segments. The hard segments are relatively rigid and the corresponding microdomains are semicrystalline.

Several undesirable side reactions can occur during the production of polyurethanes. They include reaction of the reactive hydrogen of urethane and urea groups with diisocyanate to produce allophanates and biurets respectively, formation of dimers or trimers of the diisocyanates, and reaction of isocyanate groups with water which produces carbon dioxide [Lelah and Cooper, 1986]. In the case of water, the formation of carbon dioxide is sometimes used to advantage in producing polyurethane foams. Allophanate and biuret formation can lead to chemical crosslinking within the polymer and generally undesirable mechanical and physical properties [Lelah and Cooper, 1986]. During polymerization exposure to water should be minimized. Solvent distillation and nitrogen blanketing of the reaction vessels are usually employed for this purpose.

1.6.2 Properties

As previously discussed, segmented polyurethane chains are composed of hard and soft segments, and these segments are thermodynamically incompatible. For this reason the segments separate into microdomains, the soft domains are rubbery and amorphous, the hard domains, rigid and semicrystalline. In a solid specimen under tension, the soft domains will stretch while the hard domains provide virtual crosslinking and stability. It is their domain microstructure that gives segmented polyurethanes their elastomeric properties and contributes to their value as biomaterials.

Driven by incompatibility between the segments, phase separation is influenced by the ability of the urethane segments to form interurethane bonds, the segmental length, the crystallisability of the segments, the chemical composition, and the potential for hydrogen bonding between segments [Lelah and Cooper, 1986]. Hydrogen bonding is

involved in the formation of domains and in polyurethanes involves the amide group as the donor and the carbonyl of either the ester or ether as the acceptor [Lelah and Cooper, 1986]. Hydrogen bonding is particularly important in the hard segment. Soft segments with higher molecular weight and a greater potential for hydrogen bonding yield polymers with less phase mixing [Lelah and Cooper, 1986].

The nature of the chain extender also affects the degree of microphase separation in segmented polyurethanes. Aliphatic extenders tend to give more phase mixing than aromatic because of their greater compatibility with the soft segments. Therefore polymers formed with aromatic extenders in general have better phase separation. Diamines also give better phase separation than diols, possibly because of increased hydrogen bonding within the hard segment domains [Lelah and Cooper, 1986]. As previously mentioned, both aromatic diisocyanates and diamines were used to synthesize the polyurethanes in this work. The resulting materials are therefore expected to be well phase separated.

Despite the relatively good blood compatibility of segmented polyurethanes, activation of the coagulation pathways still occurs when they are used in blood contacting situations. The biocompatibility of segmented polyurethanes is known to be influenced by the soft and hard segment components, particularly the relative amounts of these components at the surface of the material [Lelah et al 1986; Takahara et al, 1991]. Brash and Hudson [1986] studied protein adsorption from plasma to polyurethanes having both hydrophobic and hydrophilic character and found that fibrinogen exhibited the Vroman effect on these polymers. Takahara et al found that platelet reactivity on hydrophobic segmented poly(etherurethaneureas) was influenced by microphase separation. Whicher and Brash [1978] found that polyurethanes with a polypropylene oxide soft segment supported higher levels of platelet adhesion than polyurethanes with a polyethylene oxide soft segment. Other investigators [Grasel and Cooper, 1986;Lelah et al, 1986; Grasel et al, 1987] have found that biocompatibility is associated with the type and surface concentration of the hard segment of the polyurethane.

1.6.3 Ion-containing Polyurethanes

The segmented polyurethanes used in this work contain ions (sulphonate and quaternary ammonium). This has implications for the mechanical properties of the polymers as well as for their biocompatibility. Polyurethanes containing ionic groups (polyurethane ionomers) combine the microstructure of both polyurethanes and ionic polymers.

In the early seventies Eisenberg [1977] proposed that ion association in pairs, multiplets and clusters within the polymeric material should, theoretically, be energetically favoured. He defined ion clusters as aggregations of multiplets incorporating both ionic and nonionic materials and exhibiting the properties of a new phase. While it is now generally accepted that ion multiplets and clusters exist within ionic polymers, very little is actually known about the detailed morphology of these structures [Eisenberg and King, 1977; Tant, 1987; MacKnight, 1987]. It is also generally accepted that ion pairs form not only intramolecular but intermolecular associations which effectively constitute ionic "crosslinks". These structures are important in polyurethane ionomers.

1.6.3.1 Properties

Incorporation of ionic groups into segmented polyurethanes is generally in the hard segment and increases microphase separation by increasing the incompatibility of the hard and soft segments [Hwang et al, 1981; Miller et al, 1983; Yang et al, 1983; Speckhard et al, 1984; Al-Salah et al, 1987; Lee et al, 1988a&b]. This also promotes the formation and cohesion of the hard domains.

Cooper and co-workers have conducted extensive research on the effects of ion incorporation on the physical properties of polyurethanes. Early work by Hwang, Yang and Cooper [1981] on 4, 4'diphenylmethane diisocyanate (MDI)/methyl diethanolamine (MDEA)/polytetramethylene oxide (PTMO) polymers with sulphonate groups attached to the MDEA moiety indicated that increasing sulphonate content improved microphase separation. They observed lower and narrower soft domain glass transitions with

increasing ion concentration. Increasing ion content also caused increases in both the hard segment melt temperature and tensile strength for the materials studied by Hwang, Yang and Cooper. Other investigators obtained similar results for MDI\MDEA\PTMO and on MDI/MDEA/polypropylene oxide (PPO),polybutadiene (PBD), polyethylene oxide (PEO) and PTMO systems [Miller et al, 1983; Yang et al, 1983; Speckhard et al, 1984; Lee et al, 1988a&b].

Ion incorporation will also influence the properties of the polymers in solution as well as the solid state. The behaviour of the solids in contact with aqueous solutions is important because biomaterials are generally in contact with aqueous fluids. Longworth [1983] reviewed results on the absorption of water by thermoplastic ionic polymers and found the following common characteristics: water adsorption increases with increasing ion content and is dependent on the type of ion in the polymer. Hydration reduces or eliminates the small angle X-ray scattering (SAXS) peak probably indicating the break up of multiplets and clusters due to ion solvation. In addition solvation affects the relaxation characteristics and generally has a plasticising effect.

In their extensive study of polyurethane ionomers, Al-Salah et al [1987] investigated water absorption by anionomers and found that the type of counterion affected water uptake. Santerre [1990] found differences in water absorption with increasing ionic content and with the type of ion attached to the polyurethane. These findings are discussed more fully in Chapter 3 of this thesis.

In addition to affecting chemical and mechanical properties, ion incorporation into polyurethanes has also been found to influence blood compatibility. Lelah et al found that anionic and zwitterionic polyetherurethanes were less thrombogenic than the comparable material containing cations [Lelah et al, 1984].

Okkema et al [1991] found that polyurethanes chain extended with N,N-bis(2-hydroxylethyl)-2-aminoethane-sulfonic acid with polytetramethylene soft segments showed significantly reduced activation of adherent platelets compared to controls. Interestingly fibrinogen deposition was found to increase with increasing sulfonic acid content in this

study but was not correlated with platelet activation. The same polyurethanes with a polyethylene oxide soft segment showed both enhanced platelet activation and fibrinogen deposition.

Santerre [1990] found increasing fibrinogen adsorption levels with increasing sulphonate content of MDI/PPO polyurethanes chain extended with a sulphonated diamine (these polymers are discussed in more detail later in this thesis). These materials also showed a Vroman effect for fibrinogen adsorption from plasma but the maximum peak occurred at higher concentrations than the controls i.e. the Vroman effect was "delayed" [Santerre et al, 1992]. Santerre also found that sulphonated polyurethanes had longer thrombin times when compared to control polystyrene resins. Han et al [1991] found that sulphonate groups had a synergistic effect with polyethylene oxide grafted to polyurethanes, significantly delaying occlusion of grafts in rabbits when compared to controls.

The incorporation of ions into polyurethanes modifies the chemical properties, microphase separation, and microdomain formation. Interactions with blood and other biological systems are also correspondingly modified. The mechanical properties and the biocompatibility of polyurethane ionomers are the result of a balance between the effects of ions and counterions and the underlying polyurethane structure.

The following chapters will describe in detail studies on plasminogen adsorption to segmented polyurethanes, plasminogen interactions with model surfaces including information on the enzymatic activity, and finally initial work on lysine derivatized polyurethanes.

CHAPTER TWO
RESEARCH OBJECTIVES AND CONTRIBUTIONS

2.0 STATEMENT OF OBJECTIVES AND CONTRIBUTIONS

The objective of this work is to investigate the hypothesis that a surface which preferentially and selectively binds plasminogen from plasma may be profibrinolytic or clot lysing. This hypothesis has three components:

i) Surface fibrinolytic activity may be a useful approach to surface antithrombogenicity.

ii) Surface fibrinolytic activity may be conferred by preferential adsorption of plasminogen during blood contact followed by the acquisition of surface-induced plasmin-like activity.

iii) Preferential adsorption of plasminogen may be achieved by incorporating the amino acid lysine, which is known to have specific binding affinity for plasminogen, into a surface.

The contributions of this work are as follows:

1) Fundamental information about plasminogen adsorption and behaviour on surfaces. This protein has not previously been studied in relation to blood interactions with artificial surfaces.

2) A method for derivatizing silylated silica glass with lysine that leaves the ε-amine group free for interaction with plasminogen. The silylated silica glass materials serve as model surfaces for studying the plasminogen/lysine interaction on other, more complex solids. It is not anticipated that these materials will be used in the construction of blood-contacting devices. Rather, the ultimate long term goal of this research is the development of derivatized polymeric materials (e.g. polyurethanes) since these should provide useful materials with appropriate mechanical properties for fabrication as vascular

grafts and other blood contacting devices.

3) Information on the enzymatic activity of plasminogen nonspecifically adsorbed to control surfaces, i.e silica glass and sulphonated silylated glass, and specifically adsorbed via lysyl groups to lysinated silica glass.

4) Preliminary investigation into alternative methods of derivatizing sulphonated polyurethanes with lysine. This work builds on that of Santerre [Santerre, 1990] and should lead to methods that would allow high levels of lysine incorporation, and would leave the lysine in the appropriate configuration for interaction with plasminogen.

2.1 DISCUSSION

Research on "interactive" biomaterials for blood contacting applications has focused primarily on the development of materials which are actively anticoagulant in nature, the premise being that clot formation will be minimized or eliminated. Very little work has been conducted on the development of profibrinolytic surfaces which would lyse incipient clots before they can lead to adverse consequences.

The fibrinolytic pathway has characteristics which offer advantages for the development of a biomaterial. In vivo, the proteins of this pathway rely on binding to surfaces for a full expression of their activity. Fibrin and other surfaces potentiate plasminogen activation to plasmin, confer protection from inhibition of plasmin by α_2-antiplasmin, and ultimately localize and potentiate clot lysis. The protein structures which interact with surfaces, i.e. the lysine binding sites, and the nature of this interaction are reasonably well defined. It is reasonable then to hypothesize that surface fibrinolytic activity may be a useful (and relatively novel) approach to surface antithrombogenicity; that surface fibrinolytic activity may be conferred by preferential adsorption of plasminogen during blood contact followed by generation of plasmin activity (either by surface induced conformational changes or direct activation); and that preferential adsorption of plasminogen may be achieved by incorporating lysine into a surface.

More generally, the protein layer which is initially adsorbed to biomaterials placed

in contact with blood can have a significant influence on subsequent interactions between the material and its biological environment ultimately determining its long term viability. Plasma is a complex fluid containing a large number of proteins, all of which are likely to adsorb to the biomaterial. Some of these proteins have been investigated in detail (IgG, albumin, fibrinogen), others very little. Many of the proteins investigated are involved directly in the coagulation pathway, for example, thrombin and fibrinogen. Others have been studied principally because of their high concentration in plasma (albumin, IgG) and because they are known to provide ligands for platelet interactions, for example fibronectin and VWF. The fundamental studies on plasminogen adsorption reported in this thesis are to the author's knowledge, the first of their kind. These studies contribute new information to an important aspect of biomaterial interactions with blood.

The ultimate long term goal of this research is to produce a fibrinolytic material that is suitable for use as an implant. The silylated amorphous silica used as a model material is not suitable for the construction of vascular implants. This material serves two functions in this work. Silica glass and silylated silica glass have a fairly extensive history of use as model biomaterials [Arkels et al, 1986]. Silica glass is rigid, non-swelling, and can be readily functionalized using silanes via surface OH groups. Recently Massia and Hubbell used silylated glass as a substrate to couple adhesion peptides for endothelial cells, and used these materials to investigate the interaction of these cells with the different peptides [Massia and Hubbell, 1991]. Johsson et al [1987] investigated the adsorption of IgG to silica, and Wojciechowski utilized silylated silica glass surfaces to investigate the role of different chemical functionalities on protein adsorption [Wojciechowski, 1992].

Work on the model surfaces was expected to facilitate subsequent polymer development by clarifying the interaction of plasminogen adsorption to a lysinated surface during blood contact. These interactions are more difficult to determine on polymeric surfaces (e.g. the functionalized polyurethanes) due to the chemical and physical complexity of these materials. Clear silica glass is available in many different forms and

provides a surface which is relatively impenetrable to water and water-borne species. Polyurethanes are known to swell to varying extent in contact with aqueous fluids such as blood and may absorb as well as adsorb blood components. In addition, pure silica glass contains only silicon and oxygen whereas polyurethanes are chemically complex, containing a wide array of functionalities that could interact with proteins. Our studies with model materials are considered pivotal in providing the fundamental information on plasminogen adsorption discussed above. In addition, with the appropriate choice of the silane, the methods developed for attaching amino acids and other modifiers to the silica glass may be adapted for use with polyurethanes.

Polyurethanes are used extensively in medical applications. These polymers are versatile, with chemical and physical properties suited to producing a biomaterial for vascular applications. The development of polyurethanes as fibrinolytic materials is presented in two parts in this thesis. The first is a detailed study of plasminogen adsorption to polyurethanes derivatized with lysine utilizing methods previously used in this laboratory. The second presents preliminary investigations into and material characterization of polyurethanes synthesized using a method developed via the model surfaces. These results are reported in Appendix I.

Plasminogen adsorption studies are not sufficient to fully assess the potential of the materials as profibrinolytic, and are but the first step in a more detailed in vitro evaluation. As discussed in the introduction, plasminogen by itself does not lyse clots, but must be activated to the serine protease plasmin. Therefore evaluation of the plasmin activity associated with the surfaces is required and is reported in this work. Assessment of the enzymatic activity of the surfaces necessitated development of new methods, distinct from traditional techniques used to determine enzyme activity in solution.

CHAPTER THREE
ADSORPTION OF PLASMINOGEN TO SEGMENTED POLYURETHANES

3.0 INTRODUCTION AND SUMMARY

In this chapter, studies on the adsorption of plasminogen to segmented, derivatized polyurethane ureas are discussed. These materials were synthesized using methods developed previously by Santerre and Brash [1991].

As indicated these studies form part of a larger project, the objective of which is to develop a fibrinolytic surface for blood contacting applications. Our approach was to create a material that would selectively adsorb plasminogen from blood by incorporating lysine residues (known to bind plasminogen) into the surface. Lysine-containing polyurethanes were used for this purpose. The adsorption of plasminogen to these surfaces as well as to glass, "conventional" polyurethanes and precursor sulphonated polyurethanes (all serving as "controls" for the lysinated materials) was investigated. Adsorption from isotonic Tris buffer, pH 7.4, was measured under static conditions at room temperature using radioiodinated plasminogen. Adsorption on most of the surfaces except glass reached isotherm plateaux at about 0.1 mg/mL solution concentration. The sulphonated and lysine-derivatized polyurethanes showed higher adsorption capacities than any of the other surfaces but there appeared to be little difference in either capacity or apparent binding affinity between corresponding "lysinated" and sulphonated materials.

The adsorption data for glass and the two conventional polyurethanes were fit to an "irreversible" Langmuir model. These two polyurethanes differed by chain extender, one being based on methylene dianiline (MDA), the other on ethylene diamine (ED). A computer simulation was used to assess mass transfer effects in the cylindrical geometry of the experiment. Two parameters, Γ_{max}, the maximum monolayer adsorption, and k, the

rate constant for adsorption, were estimated for the three surfaces. Monolayer adsorption on glass appears to be about half that on MDA, while on ED it is intermediate between glass and MDA and the residual error is much larger. The Γ_{max} values suggest that plasminogen attains a more spread conformation on glass than on MDA assuming that both surfaces are equally smooth. The rate constant is also highest for the MDA surface, intermediate for the ED-based polyurethane and lowest for glass.

Adsorption from citrated human plasma diluted with isotonic Tris buffer, pH 7.4, under static conditions at room temperature using radioiodinated plasminogen was also investigated. The following trends were observed: 1) Adsorption increases monotonically with increasing plasma concentration and there is no suggestion of transient adsorption (Vroman effect) on any of the surfaces studied. 2) Sulphonate groups appear to have a strong effect on plasminogen adsorption as was found previously for adsorption from buffer. 3) The lysine derivatized material having the highest lysine content shows a slight increase in plasminogen binding affinity compared to its sulphonated precursor.

The synthesis, and some of physical and chemical characterization of the materials discussed in this chapter were done by Santerre during the course of his Ph.D. work at McMaster [Santerre, 1990]. Therefore detailed discussion of Santerre's methods is not provided here. The work in this chapter has resulted in two papers: Woodhouse, K.A., Wojciechowski, P.W., Santerre, J.P., and Brash, J.L., "Adsorption of plasminogen to glass and polyurethane surfaces", J. Colloid Interface Sci., 152, 60-69, (1992), and Woodhouse, K.A., and Brash, J.L., "Adsorption of plasminogen from plasma to lysine-derivatized polyurethane surfaces.", Biomaterials, in press. The author would like to acknowledge the assistance of P.W. Wojciechowski with the modelling section of this chapter.

3.1 EXPERIMENTAL

3.1.1 Polyurethane Synthesis

Eight segmented polyurethanes based on 4,4' diphenylmethane diisocyanate (MDI, Eastman Kodak, Rochester, NY) and hydroxyl-terminated polypropylene oxide of

molecular weight 1000 (PPO-1000, BDH Chemical, Toronto, Ontario) were used in this work. Various chain extenders were used to produce either "conventional" polymers or polymers containing sulphonate groups in the hard segment. Some polymers were then further modified by attaching the amino acid lysine to the sulphonate groups.

The two conventional polyurethanes were based respectively on ethylene diamine (ED, Aldrich, Milwaukee, WI) and methylene dianiline (MDA, Sargent Welch, Toronto, Ontario) as chain extenders. MDA was chosen because of its chemical similarity to the sulphonated chain extender, 4,4'-diamino 2,2'-biphenyl-disulphonic acid or biphenyl diamino disulphonic acid (BDDS, Eastman Kodak) used to synthesize the sulphonated polymers. Three polymers were made using BDDS as chain extender and thus contain sulphonate groups in the hard segment. These polymers differ by sulphur content in the final product and are referred to by the acronym BDDS followed by the weight percent sulphur, e.g. BDDS-1.4. The chemical structures of the three chain extenders are given in Figure 3.1.

The polyurethanes were synthesized using a two-step solution procedure [Brash et al, 1973] with DMSO (BDH Chemical, Toronto, Ontario) as solvent. In the first step a prepolymer was synthesized by reacting MDI with PPO-1000 in 2:1 stoichiometry for 3 h at 90°C. The prepolymer was then chain extended to high molecular weight by reacting with chain extender in 1:1 stoichiometry. The chain extension step was carried out at 25°C for 15-20 min (ED and MDA chain extenders) or at 25°C overnight (BDDS chain extender). Reactions were run in a nitrogen atmosphere to eliminate moisture.

Sulphonated polyurethanes derivatized with the amino acid lysine (Sigma, St.Louis, MO) were prepared using the method developed by Santerre and Brash [Santerre and Brash, 1991]. The lysine-derivatized polyurethanes were synthesized using BDDS-extended polymers. Sulphonate groups were converted to sulphonyl chloride by reaction with oxalyl chloride (Aldrich) in DMF (BDH Chemical). The sulphonyl chloride groups were subsequently converted to lysine sulphonamide by reaction with lysine. The reaction pathway for lysination is shown in Figure 3.2. From this figure it may be noted that no

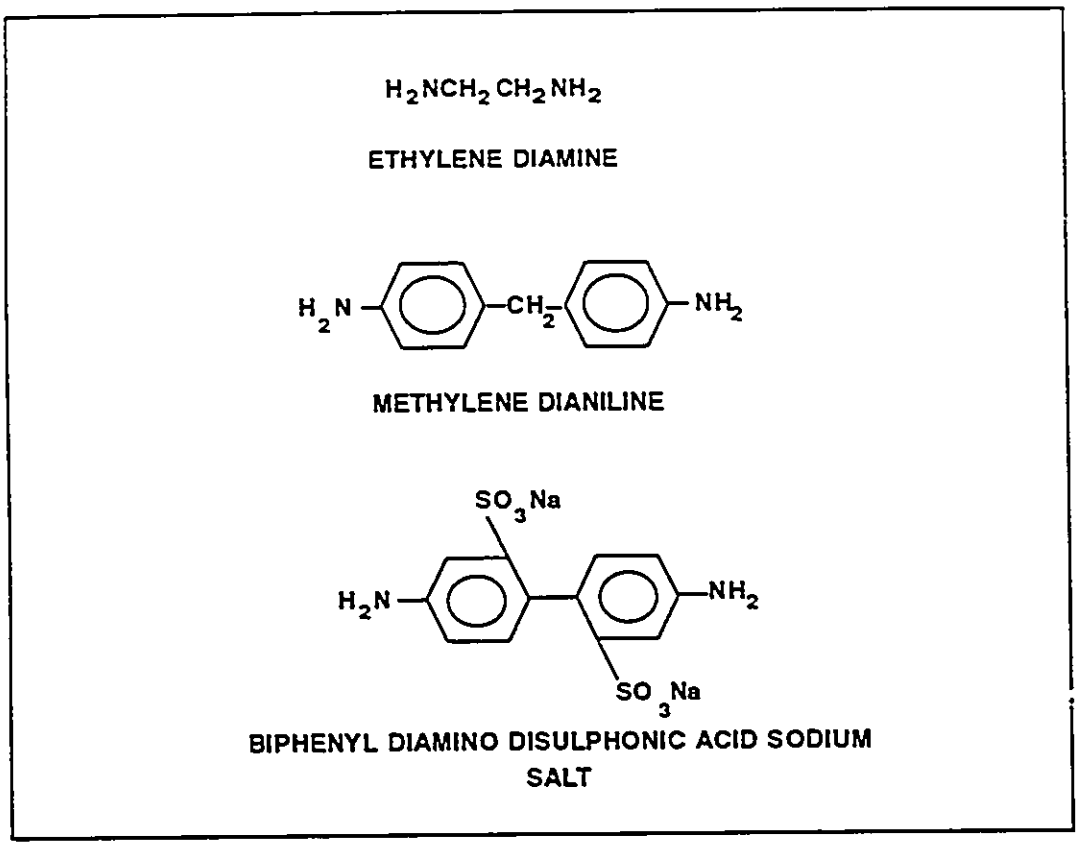


Figure 3.1: The chemical structures of the three chain extenders used to synthesize the polyurethanes.

attempt was made to control which of the two amine groups of lysine was involved in this reaction. It is therefore assumed that both α- and ε-amines participated in a random manner. The polymers were characterized extensively with respect to molecular weight and chemical composition as reported previously [Santerre and Brash, 1991].

The three lysine derivatized materials used in these studies are referred to as BDDS-1.4-LYS, BDDS-0.8-LYS and BDDS-0.2-LYS to indicate their association with the parent BDDS polymers. Derivatization of polyurethanes BDDS-0.8 and BDDS-1.4 resulted in 30% and 100% conversion respectively of the sulphonate groups to lysine sulphonamide.

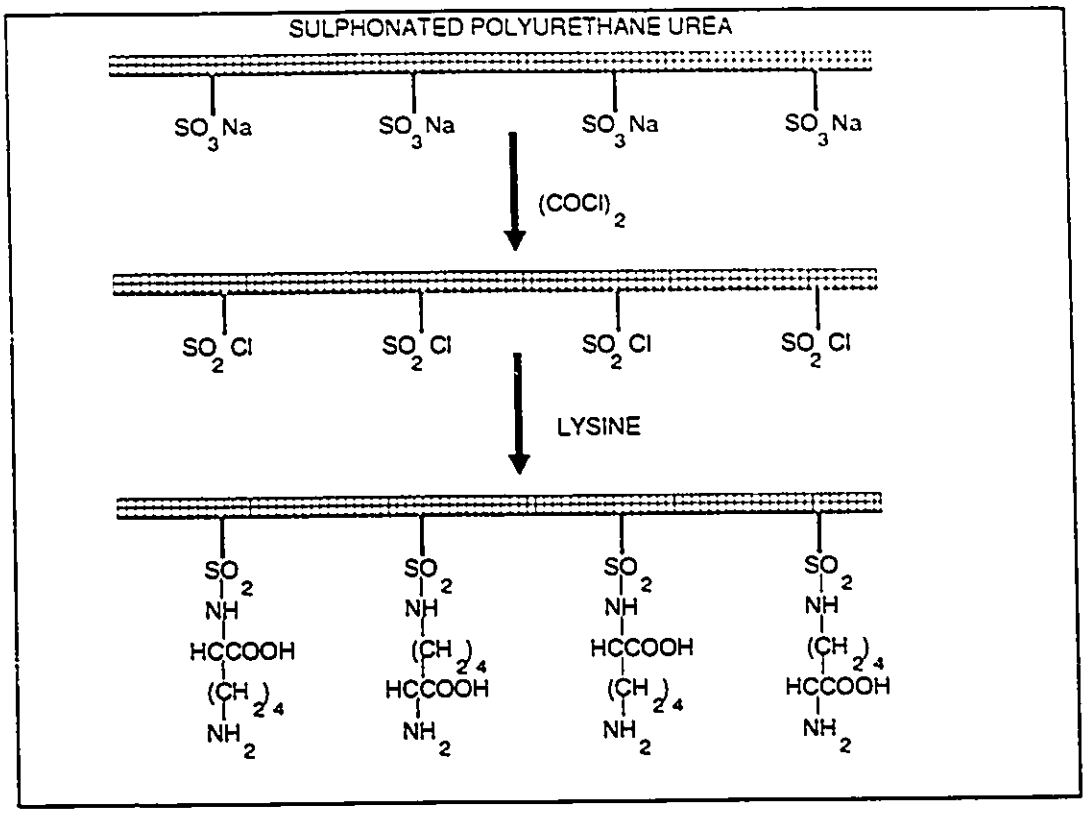


Figure 3.2: Lysine derivatization of sulphonated polyurethanes.

3.1.2 Physical Characterization of the Polyurethanes

3.1.2.1 Contact Angle Measurements

Water contact angles were measured with a goniometer (Rame-Hart, Bloomfield, N.J.) using glass slides coated with the polymer of interest. Both the sessile drop and captive air bubble methods were used [Andrade et al, 1985]. For the captive air bubble method, the surfaces were first equilibrated for 24 h in isotonic Tris buffer, pH 7.4 and then the contact angle was measured in Tris. The contact angle was assessed in this manner so that a measure of the wetted surface that the protein would "see" in the adsorption experiments could be obtained.

3.1.2.2 Gel Permeation Chromatography

Gel permeation chromatography, also referred to as size exclusion chromatography, is a separation technique which separates on the basis of molecular size. A solution of the polymer is applied to a column containing a bed of microporous particles. Diffusion into and out of the pores depends on the molecular size of the polymer, and thus the pore size distribution of the particles determines the molecular size range within which the separation occurs. An elution profile is obtained which can be converted to a molecular size (weight) distribution by a suitable calibration.

In this study four Ultrastyragel columns (Millipore Waters, Cdn) in series were used to determine the molecular weights of the polyurethanes. The nominal pores sizes of the column packings were 10^3Å, 10^4Å (2 columns), 10^5Å. A high pressure solvent delivery system (Waters M6000 A pump), and a differential refractive index detector (Waters R-401) were the other main components of the system.

The operating conditions for GPC are very specific for the polymer system under study. The polyurethane samples were dissolved at a concentration of 0.2 mg/mL in a mobile phase containing 0.1 wt% LiBr in dimethylformamide (HPLC grade). This mobile phase was filtered through a Millipore 0.5 µm glass frit filter before use. Santerre [1990] found that LiBr was required as an additive when DMF was used as the solvent. It was found that in the absence of LiBr, the polyurethanes form microgel particles which elute at shorter retention times and result in erroneous molecular weight data. LiBr disperses the microgel particles [Santerre, 1990]. Both the solvent and the LiBr can occasionally contain small particulate matter which will plug the high pressure pump of the GPC so the mobile phase must be filtered before use as indicated above.

Samples of approximately 200 µL were run through the columns at a flow of 1.0 mL/min and a temperature of 80°C. At this flow rate a typical retention time of 40 min was obtained [Santerre, 1990]. The column bank is kept at 80°C to lower the viscosity of the polyurethane sample. At lower temperatures the viscosity increases and results in unacceptably high operating pressures (>1500 psi) for the refractometer and the columns.

The calibration standards used were narrowly distributed polystyrenes from Toyo Soda Manufacturing Co., Ltd, Tokyo. Polystyrene equivalent molecular weights are reported. The chromatograms for the polyurethanes discussed in this chapter were reported by Santerre [1990].

3.1.3 Plasminogen Preparation Method

Plasminogen was prepared from citrated human plasma by affinity chromatography on Sepharose 4B-Lysine following a modification of the method of Deutsch and Mertz [Deutsch and Mertz, 1970] developed by Brockway and Castellino [Brockway and Castellino, 1972]. An additional modification was added to eliminate contamination by tissue plasminogen activator [Radcliffe and Heinze, 1978].

The method and associated techniques were modified and optimized over a period of several months. This was necessary because the amounts of plasminogen to be isolated were approximately ten times greater than those purified by typical laboratory scale procedures. The method thus had to be scaled up. Column size was a particular problem, as well as low yields and the long times required for sample concentration.

3.1.3.1 Preparation of Sepharose-Lysine

Sepharose 4B (Pharmacia,Dorval, Quebec) was used as the chromatographic matrix. This material was activated for lysine attachment using cyanogen bromide (CNBr). CNBr activation is believed to involve the reactions illustrated in Figure 3.3 [Joustra and Axen, 1975; Pharmacia, 1988]. The lysine coupling reaction is strongly influenced by the pH of the coupling buffer, the temperature and the ligand concentration.

A standard preparation required that 250 g (dry weight) of Sepharose 4B was weighed out and washed extensively on a coarse sintered funnel with distilled water (1-2 L). The cleaned resin was then mixed with an equal volume of distilled water.

Fresh CNBr (25 g) was dissolved in approximately 20 mL of distilled water with gentle heating. The CNBr solution was then added to the Sepharose resin with constant

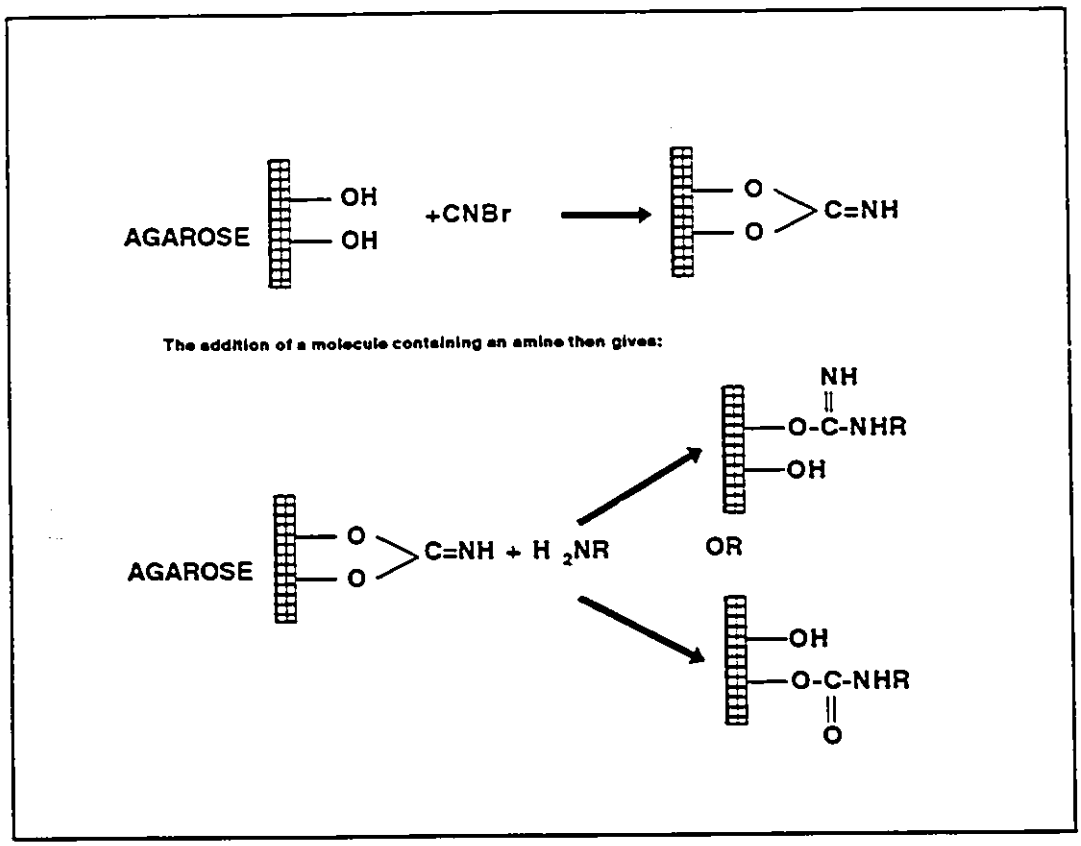


Figure 3.3: Cyanogen bromide activation of agarose resin [Pharmacia, 1988].

stirring. The pH was monitored continuously and the mixture titrated with 4N NaOH to a pH between 10.5 and 11.5, and maintained at this condition by addition of NaOH as required, until the pH no longer changed significantly. If the pH goes much below 10 or above 11.5 the reaction stops. The reaction time under these conditions was approximately 15 minutes.

The resin was then extensively but rapidly washed with 0.1M $NaHCO_3$, pH 9 (approximately 4 L), and then resuspended in the same buffer. Immediately following this step, 75 g of lysine in 0.1M $NaHCO_3$ (Sigma) was added to the suspension of resin and gently stirred at 4° C for 1 h. At this time the pH was checked and the resin

suspension titrated to a pH of 9.0. The resin was then incubated at 4° C overnight.

At pH 9.0 the binding of the α-amino group (pK=8.95) is favoured over that of the ε-amino group (pK=10.53). The ε-amino group of lysine is strongly basic, losing its proton only at very high pH. In developing affinity chromatography methods for plasminogen it has been shown that the ε-amino group is necessary for the binding of plasminogen. This has implications for work presented later in this thesis and is discussed in detail at that time.

Following activation and attachment of lysine, unreacted sites on the Sepharose must be blocked to minimize nonspecific binding of proteins to the matrix. This is achieved by treating the fresh Sepharose-lysine with 0.1M Tris, pH 8, for 1-2 h at room temperature. Uncoupled lysine is then washed out of the resin with several two step washing cycles as follows: 1) 0.1M acetate buffer (pH 4.0) containing 1M NaCl, 2) 0.1 M borate buffer (pH 8.0) containing 1M NaCl. The resin is stored at 4° C in 1.5M NaCl containing 0.1% sodium azide. If stored properly the resin retains its activity for periods up to a year.

3.1.3.2 Plasma Preparation

Sample preparation is very important in affinity chromatography. Removal of contaminants before loading the sample on the column facilitates the purification and extends the column life. The large quantity of "extraneous" proteins and the high lipid content of plasma causes columns to clog and necessitates frequent column washings and regeneration. Ammonium sulphate is often used as an initial treatment of plasma to precipitate unwanted proteins. In the present work Arvin or Ancrod, a snake venom derivative which cleaves fibrinogen and leads to fibrin clots was used [Hatton, 1974]. The clots were then removed from the sample. In this manner fibrinogen, the most serious potential contaminant, was removed from the plasma prior to chromatography.

Citrated human plasma obtained from the Canadian Red Cross, Ottawa, Ontario (1 volume citrate/dextrose/phosphate to 10 volumes of blood) or from volunteer donors

in the laboratory (1 volume acid citrate dextrose to 7 volumes blood) was treated with Arvin (donated by Dr. M.W. Hatton, McMaster University) or Ancrod (Sigma, St. Louis, MO.) for 2 h at 37°C following the method of Hatton [1974]. Fifteen units of the enzyme were added to approximately one litre of plasma which was then incubated at 37°C for 2 h. The fibrin clots thus formed were then removed and the serine protease inhibitor benzamidine (Sigma) added at a final concentration of 5mM to inhibit plasminogen conversion to plasmin. The plasma was aliquoted into 250 mL batches and stored at -40°C until use.

3.1.3.3 Elution

Two basic elution methods are commonly used in affinity chromatography: linear gradient elution and step elution. In linear gradient elution the concentration of the eluent increases linearly between two limits, for example 0 to 0.5M. The eluent is continuously applied to the column and the gradient is achieved via a mixing system. Linear gradients are often used when the exact eluent molarity required for elution of the product is unknown or when a series of products is desired. Step elution involves the application of eluent of a specific concentration to the column in separate stages. This type of elution requires knowledge of the optimum molarity of the eluent but results in a more concentrated product than does a linear gradient. Both techniques were used in the present work and are discussed below.

The final column used was a Pharmacia 25/26R (6 cm I.D.) reservoir modified to hold the Sepharose-lysine. In a typical procedure the column is packed with 250 g (dry weight) of Sepharose-lysine and then equilibrated overnight in 0.1M phosphate buffer, pH 7.4. Approximately 250 mL of plasma diluted 1:1 with 0.1 M sodium phosphate buffer, pH 7.4, is then loaded on the column at a rate of approximately 50 mL per h (gravity flow). The plasma is kept on ice during this period while the column is at room temperature. The column is then washed overnight with approximately 1 L of 0.2 M arginine in 0.1M phosphate buffer, pH 7.4 at a flow rate of about 100 mL per h (gravity

flow). The arginine wash is to remove tPA [Radcliffe and Heinz, 1978]. The plasminogen is then eluted from the column with either a 500 mL linear gradient of ε-amino caproic acid (EACA), 0 to 0.02 M in 0.1M phosphate buffer, pH 7.4, or a step gradient of 0.02 M EACA in phosphate buffer, pH 7.1. If separation of the protein into its affinity forms is desired then the gradient elution must be run.

The protein was concentrated using pressure dialysis followed by ultrafiltration. Approximately 50% of the preparation is pressure dialysed overnight against 0.1 M phosphate, pH 7.2 and 50% in isotonic Tris, pH 7.4 at 4°C. This provides for plasminogen for labelling (phosphate buffer) and the adsorption experiments (isotonic Tris). For the pressure dialysis approximately 125 mL of protein solution was placed into a high pressure dialysis membrane (1mm width, 14000 MW cutoff) sealed with dialysis clips at one end and affixed to a 250 mL glass pressure dialysis bulb. The bulb was pressurized using nitrogen to approximately 15 psig and clamped. The membrane and bulb were placed in 500 mL of 0.2M phosphate buffer, pH 7.2 or isotonic Tris, pH 7.4 and dialysed overnight. The dialysis buffer was changed three times over this period. Pressure dialysis is useful for concentration of large volumes of dilute protein and for obtaining the protein in the appropriate buffer for labelling and adsorption studies.

Pressure dialysis was followed by ultrafiltration using a 50 mL Amicon Diaflow Ultrafilter membrane system with a YM10 membrane (Amicon, W.R. Grace Co, Danvers MA) at 40 psi for approximately 20 min. A final protein concentration > 0.6 mg/mL was achieved. Finally, the protein solution was flash frozen and stored at -40° C.

SDS-polyacrylamide gel electrophoresis (7.5%) was run on each plasminogen preparation. Western blots using an antibody to plasminogen (Sigma) were run on a number of the preparations to confirm the identity of the plasminogen. These procedures are described later in this section.

3.1.3.4 Column Regeneration

After each plasminogen isolation, the packed column was washed with 1.5-2.0 M

NaCl with 0.1% sodium azide and stored at 4°C until the next run. After three preparations, the column was unpacked, and the resin washed extensively with 1.5-2.0 M NaCl in a coarse sintered glass funnel, and stored in 1.5M NaCl containing 0.1% sodium azide . After approximately six plasminogen isolations with a given batch of resin it was found that the plasminogen yield drops well below 50% due primarily to the irreversible nonspecific binding of lipid and proteins to the resin. This is a common problem in chromatography. Because the resin preparation is somewhat hazardous and since the materials are expensive the used resin was "regenerated" (i.e. nonspecifically bound materials were removed) by washing in succession with 1 L of 10.0 M urea (personal communication from M. Hatton), 10 L of distilled water, and approximately 5 L of 1.5-2.0M NaCl/0.1% sodium azide buffer. After regeneration the resin had a useful life of approximately three preparations. In all approximately fifty plasminogen isolations were run using six resin preparations. A total of about 3500 mg of plasminogen was obtained, representing between 45%-85% yields per batch.

3.1.4 SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Electrophoresis is a technique used to separate mixtures of proteins either in a solution or on a solid support. The method takes advantage of the fact that proteins have net negative or positive charges due to the charged amino acids on their surface and will migrate in an electric field. The migration rate and direction depends on the net charge on the protein, its shape and size [Andrews, 1981; Alberts, 1983].

In SDS-PAGE an electric field is applied across a support of cross-linked polyacrylamide gel. The protein samples, treated with SDS, are loaded onto the polyacrylamide gel and migrate in the field. Staining then reveals the final positions of the proteins after migration is complete.

Treatment of proteins with sodium dodecyl sulphate, a powerful, negatively charged detergent, does two things. Firstly it causes denaturation and solubilization. Secondly the detergent associates with the proteins thereby imparting a net negative

charge of approximately the same density to all the protein molecules. The proteins will then migrate in the applied field towards the positive electrode with the same electrophoretic mobility. Separation depends on the pore structure of the gel and is determined by size, the smaller proteins migrating through the gel more quickly [Andrew, 1981; Alberts, 1983]

Pre-treatment of proteins with addition of beta-mercaptoethanol, a reducing agent, breaks disulphide linkages thus yielding the polypeptide chains of multichain proteins to be separated by molecular weight [Alberts, 1983].

All electrophoresis reagents were obtained from Bio-Rad, Richmond, CA, or were made to the specifications in the Mini Protean Dual Slab Cell Instruction Manual, (BioRad). Low molecular weight markers were obtained from Pharmacia, Piscataway, NJ. SDS gel electrophoresis using 7.5% separating gels (7 cm x 8cm x 1mm) and 5% stacking gels was run on a Mini-Protean II Electrophoresis Cell (BioRad) at 200 V for 1 h on each plasminogen isolation [Mulzer and Brash, 1989]. The samples were reduced using β-mercaptoethanol 90°C for 5 min. Between 0.3 µg to 4 µg of plasminogen was added per lane of the gel. The gels were fixed for 1 h in 10% trichloracetic acid (Fischer Scientific, Fair Lawn, N.J.) and stained for protein overnight with Coomassie Blue. The gels were dried for 25 min at 80°C in a Bio-Rad 583 Gel Dryer.

3.1.5 Western Blots

SDS-PAGE allows the estimation of molecular weight but this is not sufficient to identify the protein completely. Western blots using an antibody to plasminogen (Sigma) were run on a number of the preparations to confirm the identity of the plasminogen. In immunoblotting protein bands from SDS-PAGE gels are transferred onto a sheet of solid material (e.g. a nitrocellulose membrane) by either electrophoresis or diffusion [Towbin et al, 1979; Gershoni and Palade, 1983]. The protein bands on the membrane are then incubated with antibodies specific to the proteins of interest. These are in turn detected by application of second antibodies containing either a radioactive or an enzyme

label [Gershoni and Palade, 1983; Johnson et al, 1984].

In the present work we used primary antibodies specific for plasminogen and fibrinogen. Goat antiserum to human fibrinogen was obtained from Miles Scientific, Rexdale,Ont, and rabbit antiserum to plasminogen from Behring Diagnostics, Montreal. The secondary antibodies, affinity purified goat anti-rabbit IgG-horseradish peroxidase and affinity purified rabbit anti-goat-alkaline phosphatase conjugate were obtained from Bio-Rad and Cedarlane Laboratories, Hornby, Ont, respectively.

For the samples that were to be blotted, SDS-PAGE was run as described above. However the gels were not fixed. Rather a small quantity of Pyronin-Y red dye in sample buffer was added to the wells just before the electrophoresis was completed. This marked the top of the separating gel before transfer. A Mini Transblot cell (Bio-Rad) was used for blotting and the following procedures were followed in sequence: after electrophoresis the gel was equilibrated in transfer buffer (25 mM Tris, 192 mM glycine, 15% HPLC grade methanol, pH 8.2-8.3) for 15-20 min; an Immobilon PVDF transfer membrane (Bio Rad) was cut to size, prewet with 100% methanol(1-3 sec) and distilled water (1-2 min), and soaked in transfer buffer. The membrane and gel were clamped together and blotted for 1 h at 100V (200 mA).

The marker lanes were stained separately from the rest of the blot. These lanes were stained with amido black, 0.1% (w/v) in 10%(v/v) methanol (HPLC grade) 7% acetone (HPLC grade), then destained first in 45% methanol 7% acetic acid, then in 90% methanol 7 % acetic acid, and dried.

The rest of the blot was wet with 100% methanol, rinsed in distilled water and incubated for 1 h with gentle agitation in a "blocking" solution of 5% w/v nonfat dry milk powder in Tris buffered saline (TBS). This blocking procedure limits the amount of nonspecific antibody binding to the membrane. After blocking, the blot was rinsed 3 times for 5 min in 0.1% (w/v) non-fat dry milk in TBS (solution A).

The blot was then incubated for 1 h in a solution of 1% (w/v) nonfat dry milk, 0.05% (v/v) Tween 20 in TBS (solution B) containing the primary antibody. The

fibrinogen antiserum was present at a 1/5000 dilution and the plasminogen antiserum at a 1/1000 dilution. The blot was then rinsed as stated above with solution A.

These steps were then followed by incubation with the enzyme-conjugated secondary antibody in solution B, again for 1 h. A 1/3000 dilution was used for the horseradish peroxidase conjugate and a 1/1000 dilution for the alkaline phosphatase conjugate. The blot was again washed with solution A.

Colour development was achieved by incubating the blot with the appropriate substrate. For the horseradish peroxidase conjugate an aqueous solution of 0.8 mM 3-amino-9-ethyl-carbazole, 11mM hydrogen peroxide, 29 mM sodium acetate, and 4mM succinic acid (Sigma) was used. The alkaline phosphatase substrate system consists of 5-bromo-4-chloro-3-indolyl phosphate, and nitroblue tetrazolium (Bio-Rad). This substrate was prepared as described by the supplier. The colour reactions with the substrate were stopped by rinsing the blot in distilled water.

3.1.6 Protein Labelling

Plasminogen was labelled with ^{125}I ($Na^{125}I$, Amersham, England) by the lactoperoxidase method using the reagent Enzymobeads (Bio-Rad, Richmond, CA) [Morrison et al, 1971]. This labelling method uses an enzymatic reaction which oxidizes radioactive sodium iodide to iodine using hydrogen peroxide and attaches the label to the protein via the tyrosine residues. It has been reported that this method involves a minimum of damage to the protein [Ardaillou and Larrieu, 1974; Grant et al, 1977].

The Enzymobead reagent is a blend of lactoperoxidase and glucose oxidase immobilized on polyacrylamide beads. When glucose is added to an Enzymobead/protein/^{125}I system, the glucose oxidase generates hydrogen peroxide. The lactoperoxidase then catalyses the peroxide oxidation of iodide to iodine, which reacts rapidly with the tyrosine residues in the protein.

For each labelling procedure, one 50 µL aliquot of Enzymobead reagent in distilled water was used. To this aliquot 0.1 to 1.0 mg of protein in 0.1M (or 0.2M) phosphate

buffer, pH 7.2 was added, followed by 1.0 mCi of $Na^{125}I$. 50 µL of 1% Beta-D-Glucose (or 2% Alpha-D-glucose allowed to mutarotate overnight) was then added and the reaction allowed to proceed for 30 min. The reaction was then stopped by centrifuging and separating the beads from the suspension. The ^{125}I-labelled protein solution was then passed through an anion exchange resin (AG 1-X4, Biorad) to remove unreacted free iodide. Finally the labelled protein was diluted with isotonic Tris buffer, pH 7.4, to the appropriate level for the adsorption experiments.

The amounts of protein and $Na^{125}I$ used in this procedure varied with the experimental requirements. However care was taken to keep the iodine to protein ratio less that unity to avoid damage to the protein [Ardaillou and Larrieu, 1974].

3.1.6.1 Free Iodine Test

After labelling, each protein preparation was assessed for free or unbound radioiodine. This is necessary because the free isotope may bind nonspecifically to the test surface, so that high levels of unbound isotope may result in erroneously high surface radioactivity and inaccurate determination of protein bound to the surface. The method for determining free radioiodine is based on precipitation of protein with trichloroacetic acid and determination of radioactivity in the supernatant.

To determine non-protein bound ^{125}I, 0.1 mL of radiolabelled protein solution is added to 0.9 mL of 1% bovine serum albumin in 0.2M phosphate buffer, pH 7.4. The radioactivity of this solution [A] is determined and then 0.5 mL of 20% trichloroacetic acid in distilled water is added. The mixture is kept at 0°C (on ice) for 10 min, to precipitate the protein. This solution is then centrifuged and 0.5 mL of the supernatant is added to 0.5 mL of 0.2M phosphate buffer (or isotonic Tris, pH 7.4). The radioactivity of this solution [B] is then determined. The fraction of free ^{125}I is calculated from the equation: $I = 3*B/A$

Typical values found in this work were between 1-4% free ^{125}I. Preparations with greater than 5% free isotope were rejected.

3.1.7 Adsorption Experiments: Buffer system

Pyrex glass tubes (11 cm long, 0.22-0.25 cm I.D.) were cleaned in chromic/sulfuric acid for 1 h at room temperature and then rinsed extensively with distilled water. Polyurethanes were coated from solution in DMF onto the inside surface of clean glass tubes. For this purpose the polyurethanes were dissolved in DMF (BDH) at a concentration of 5% (w/v). To provide good adhesion to the glass two coats of ethylene diamine chain-extended polymer were applied prior to the test material. Then two coats of the polyurethane to be studied were applied. Each coat was dried for 3 h at 70°C before the next coat was applied. The finished tubes were dried for 24 h at 70°C under vacuum.

The pyrex glass or polymer coated tubes were equilibrated overnight at room temperature in isotonic Tris buffer, pH 7.4. The tubes were fitted at each end with three-way valves to facilitate removal of air bubbles [Brash and ten Hove, 1989]. For a given experiment, a series of tubes was exposed under static conditions for 3 h to plasminogen solutions of different concentrations containing 3-5% ^{125}I-labelled protein. Data points at a number of concentrations were replicated. After adsorption the tubes were rinsed twice for 5 min with 10 mL isotonic Tris buffer, pH 7.4. This rinsing procedure was established after considerable experimentation and was considered optimum to prevent loss of loosely held protein on the one hand and to remove solution protein on the other. The tubes were then cut into 4 cm lengths for radioactivity counting.

3.1.8 Effect of Labelling on Adsorption

To check the effect of labelling on plasminogen adsorption, adsorption was measured (see above) using a series of labelled plasminogen solutions having the same total protein concentration (0.1 mg/mL) but varying proportions of labelled and unlabelled protein (1 to 100 % labelled protein). These experiments were carried out at 25°C for 3 h.

3.1.9 Adsorption Experiments: Plasma System

Pooled, citrated human plasma was obtained from the Canadian Red Cross, Ottawa, Ontario. The anticoagulant used was citrate dextrose phosphate (10 vol blood to 1 vol citrate). The plasma was aliquoted and frozen at -40°C until used.

Polymer coated tubes were prepared in exactly the same manner as for the buffer experiments. The pyrex glass or polymer coated tubes were equilibrated overnight at room temperature in isotonic Tris buffer, pH 7.4. The plasma was defrosted at room temperature for 10 min and then incubated at 37°C until melted. Care was taken to avoid cryoprecipitation. Plasma was manipulated in plastic equipment to minimize activation of the intrinsic coagulation pathway.

For adsorption experiments ^{125}I-labelled plasminogen was added to plasma in amounts corresponding to 5 to 10% of the normal plasma pool. For a given experiment, a series of tubes was exposed under static conditions for 3 h to plasma solutions of different concentration, obtained by diluting plasma with isotonic Tris buffer, pH 7.4. After adsorption the tubes were rinsed twice for 5 min with 10 mL isotonic Tris buffer, pH 7.4. The tubes were then cut into 4 cm lengths for radioactivity counting.

3.2 RESULTS AND DISCUSSION

The chemical and physical properties of the polyurethanes used in this work have been described in detail in previous publications [Santerre and Brash, 1991; Santerre et al, 1992]. The materials are named by chain extender (ED, MDA, or BDDS) followed by weight percent sulfur for the BDDS polymers, followed by LYS to indicate lysine derivatization as appropriate. Glass and the polyurethane ED were used as hydrophilic and hydrophobic control surfaces, respectively. The polyurethane series MDA, BDDS-"x", and BDDS-"x" -LYS was used to assess the effects of sulphonate and lysine on plasminogen adsorption.

3.2.1 Contact Angles

Table 3.1 shows contact angle data observed by both the sessile drop and the air-bubble-in-water techniques. The sessile drop data, which are advancing angles, show significantly higher values than the air bubble data indicating that significant hydration of these surfaces occurs when in contact with aqueous fluids. From both the bubble data and, though perhaps less clearly, the sessile drop data, it is seen that the conventional polyurethanes ED and MDA are more hydrophobic than the sulphonated and lysine-containing materials. This difference indicates that at least some of the sulphonate and lysine residues are present in the surface layers, and that they produce a pro-hydrophilic effect. Although not shown in Table 3.1, the sessile drop contact angle of water on glass is about 24° in our hands. Thus glass is more hydrophilic than any of the polyurethanes.

3.2.2 Physical and Chemical Characteristics of Segmented Polyurethanes

These data were taken with permission from the thesis of J.P. Santerre [Santerre, 1990]. They are presented here for completeness and are discussed only very briefly.

The polystyrene equivalent molecular weights of the polyurethanes are shown in Table 3.2. The weight average and number average molecular weights of the polyurethanes show the same trends. Santerre concluded that in the BDDS series of polyurethanes the molecular weight decreased as the prepolymer size decreased. The higher molecular weight for the ED polyurethane was attributed to the greater reactivity of ethylene diamine compared to either of the aromatic chain extenders, MDA or BDDS.

The analytical data, shown in Table 3.3, indicate that the sulphur content determined by elemental analysis is very close to the anticipated values of 0.2, 0.8 and 1.4 wt% sulphur. Santerre found that an average of approximately 50-54% of the sulphur was titratable as free sulphonate, in the BDDS-0.8 and BDDS-1.4 polyurethanes.

The change in the nitrogen content when the sulphonated polyurethane is compared to the same polymer after derivatization with lysine is very small. The exact amount of lysine attached to the polymer is thus difficult to determine. Santerre utilized

SURFACE	CONTACT ANGLE	
	SESSILE DROP (Degrees ± SD)	CAPTIVE BUBBLE (Degrees ± SD)
ED	74 ± 2.8	38 ± 2.5
MDA	76 ± 1.9	43 ± 2.7
BDDS-0.8	70 ± 1.8	29 ± 3.2
BDDS-1.4	67 ± 2.9	25 ± 2.0
BDDS-LYS-0.8	74 ± 2.6	28 ± 2.7
BDDS-LYS-1.4	68 ± 2.0	26 ± 2.9

Table 3.1: Sessile drop and captive bubble water contact angles for the polyurethane surfaces. The values are averages of 10 drops for each test.

several different methods including ^{14}C-labelled lysine in an attempt to quantify the amount of lysine incorporated into the polyurethanes. Data for the BDDS-0.2-LYS and BDDS-0.8-LYS materials were found to be unreliable. For the BDDS-1.4, 100 % of the sulphonate groups were found to be derivatized with lysine.

As discussed in Chapter 1, the presence of ions in the polyurethanes will result in changes in the mechanical properties of the polymers. Mechanical property data for MDI/BDDS/PPG (MW 1000) polymers are shown in Table 3.4 [Santerre, 1990] and show changes in the mechanical properties with increasing ion content. These changes in mechanical and physical properties impose an upper limit of about 4% on the sulphonate concentration [Santerre, 1990]. Above this level the ionomers become excessively brittle.

SURFACE	Mw ± 10%	Mn ± 10%	Mw/Mn
ED	270000	159000	1.7
MDA	82000	66000	1.2
BDDS-0.2	209000	135000	1.5
BDDS-0.8	87000	68000	1.3
BDDS-1.4	67000	51000	1.3

Table 3.2: Molecular weight data for the polyurethanes (polystyrene equivalents) [Santerre, 1990]. Mw is the weight average molecular weight, Mn the number average.

SURFACE	ELEMENT %NITROGEN ± 0.3%	% SULPHUR ± 0.2%
BDDS-0.2	2.9	0.1
BDDS-LYS-0.2	3.4	0.15
BDDS-0.8	3.6	0.8
BDDS-LYS-0.8	3.9	0.8
BDDS-1.4	4.3	1.4
BDDS-LYS-1.4	4.9	1.4

Table 3.3: Elemental analysis data for the substituted segmented polyurethanes [Santerre, 1990].

Polymer	Tensile Strength MPa (S.D. ± 10%)	Initial Modulus MPa (S.D. ± 15%)	% Elongation at Break (S.D. ± 12%)
ED	12.8	3.0	1340
MDA	10.1	33.6	365
BDDS-0.2	>0.8	6.0	>3900
BDDS-0.8	6.0	8.0	750
BDDS-1.4	28	60	380
BDDS-2.0	11	100	240
BDDS-2.6	Too brittle to test		
BDDS-0.2-LYS	>0.8	0.9	>1300
BDDS-0.8-LYS	21.8	4.0	1200
BDDS-1.4-LYS	37	32	380

Table 3.4: Tensile stress-strain properties of the segmented polyurethanes. The materials all have MDI and PPO as the diisocyanate and the soft segment respectively. The chain extenders are changed as described in the text.

Santerre also found that ion content affected the water uptake of the polyurethanes. Differences in water sorption with increasing ion content and with the type of ion were observed. The data are summarized in Table 3.5. It can be seen from this table that the polyurethanes of higher sulphonate content have a greater (but inconsistent) water uptake with increasing sulphonate content. Interestingly the lysinated materials do not show an effect until the materials are incubated with water for two days at 70°C, then the water uptake is also correlated with increasing ion content.

Polymer	% Water Uptake (w/w) 30 Days, 25°C	% Water Uptake (w/w) 2 Days, 70 °C
ED	40	63.5
MDA	5	9.5
BDDS-0.2	37	205
BDDS-0.8	38	374
BDDS-1.4	18	237
BDDS-2.0	410	4600
BDDS-2.6	180	Partially dissolved
BDDS-3.1	Dissolved after 2 days	-----
BDDS-0.2-LYS	51	359
BDDS-0.8-LYS	22	610
BDDS-1.4-LYS	22	705

Table 3.5: Water uptake for the substituted polyurethanes [Santerre, 1990]

3.2.3 Plasminogen Preparation

When plasminogen was first isolated in large quantities in the early 1970s it was found that the method of preparation affected the N-terminal amino acid residue on the preactivation peptide [Summaria et al, 1972;1973;1976; Mangel et al, 1990]. Depending on conditions, the protein isolated had either glutamic acid (Glu) or lysine (Lys) as the N-terminal amino acid. It is now believed that Glu-plasminogen is the native form of the proenzyme and that Lys-plasminogen exists as an intermediate formed by the loss of the preactivation peptide in one of the two activation pathways which convert Glu-plasminogen to Lys-plasmin [Wallen and Wiman, 1972, 1973; Walker and Davidson, 1988]. The two forms of plasminogen differ in both chemical and functional properties.

To further complicate matters, each of the forms of plasminogen, (Glu and Lys), is eluted from a Lysine-Sepharose affinity chromatography column in two different forms [Hatton, 1974; Summaria et al, 1976]. Summaria [1976] has named these various forms Glu-1-, Glu-2-, Lys-1-, and Lys-2- plasminogen. The relative amounts of both zymogens and affinity forms are dependent on the conditions of preparation, particularly the starting material, the inhibitors used, and, as Hatton [1974] has shown, the number of usages of the affinity chromatography column.

In comprehensive studies by Summaria et al [1972, 1973, 1976], it was determined that the various affinity forms differed in sialic acid content. The 1 forms have a much higher overall content of sialic acid than the 2 forms. These investigators also found that molecular weight decreased in the order Glu-1 > Glu-2 > Lys-1 > Lys-2 [Summaria, 1976]. Their results given in Table 3.6

PLASMINOGEN FORM	APPARENT Mw	SIALIC ACID (MOL/MOL)*
Glu-1	93,600 ± 625	2.25 ± 0.06
Glu-2	91,600 ± 825	1.40 ± 0.08
Lys-1	89,400 ± 1,125	2.44 ± 0.10
Lys-2	87,900 ± 1,375	1.37 ± 0.02

* Calculated on the basis of molecular weights of 88,000 for Glu-plasminogen and 82,400 for Lys-plasminogen.

Table 3.6: Molecular weight differences in plasminogen forms, adapted from Summaria, 1976.

Typical results for a plasminogen isolation run in the present work are illustrated in the chromatogram of Fig 3.4. In this figure the eluent was a linear gradient. This chromatogram shows two peaks corresponding to the two affinity forms, but they

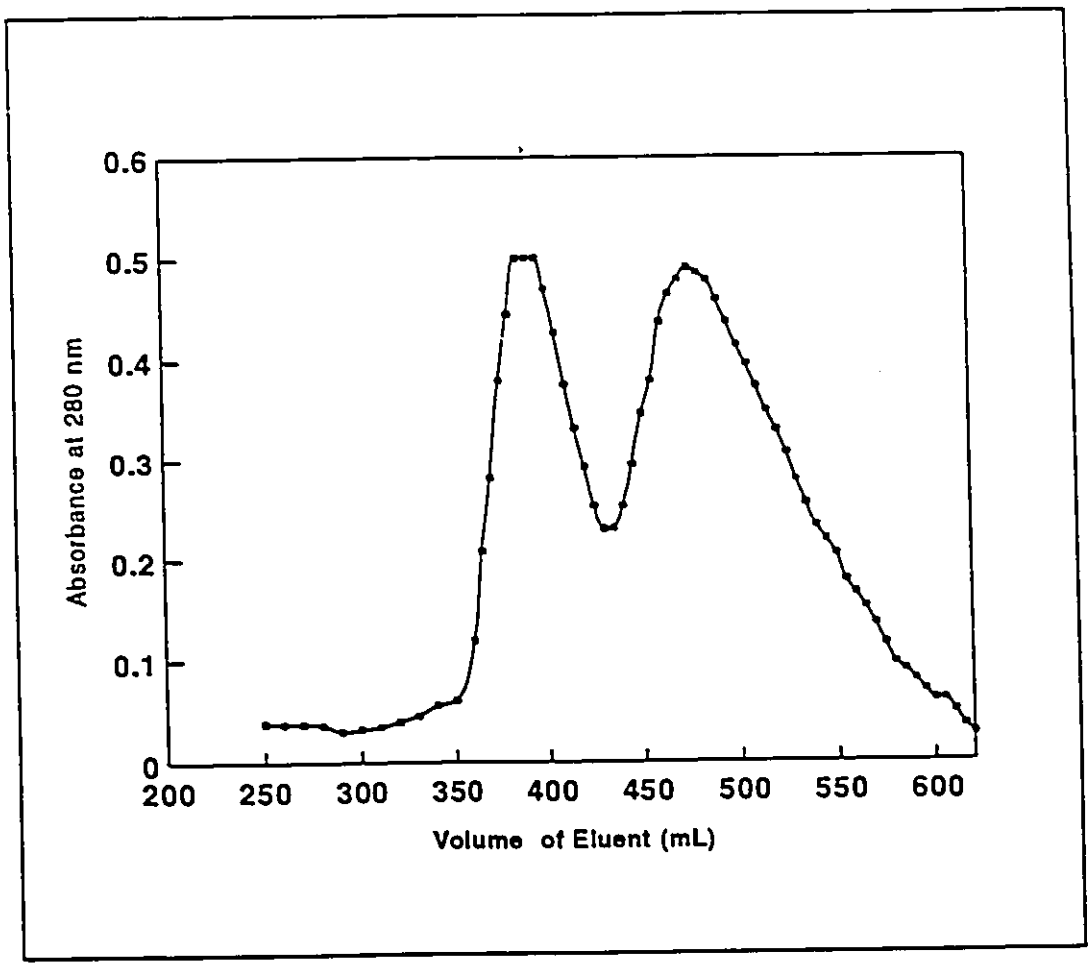


Figure 3.4: An example of a typical chromatogram for a linear gradient elution of plasminogen from a Sepharose lysine column.

are not completely resolved. The poor resolution is likely due to the large size and shape of the column, the high flowrate used and the method of elution. Optimization of the column performance around these parameters could be improved so that better peak separation would be achieved.

Hatton has found that the ratio of affinity forms changes with column usage. Similar behaviour was observed in the present work as illustrated in Figure 3.5. Because

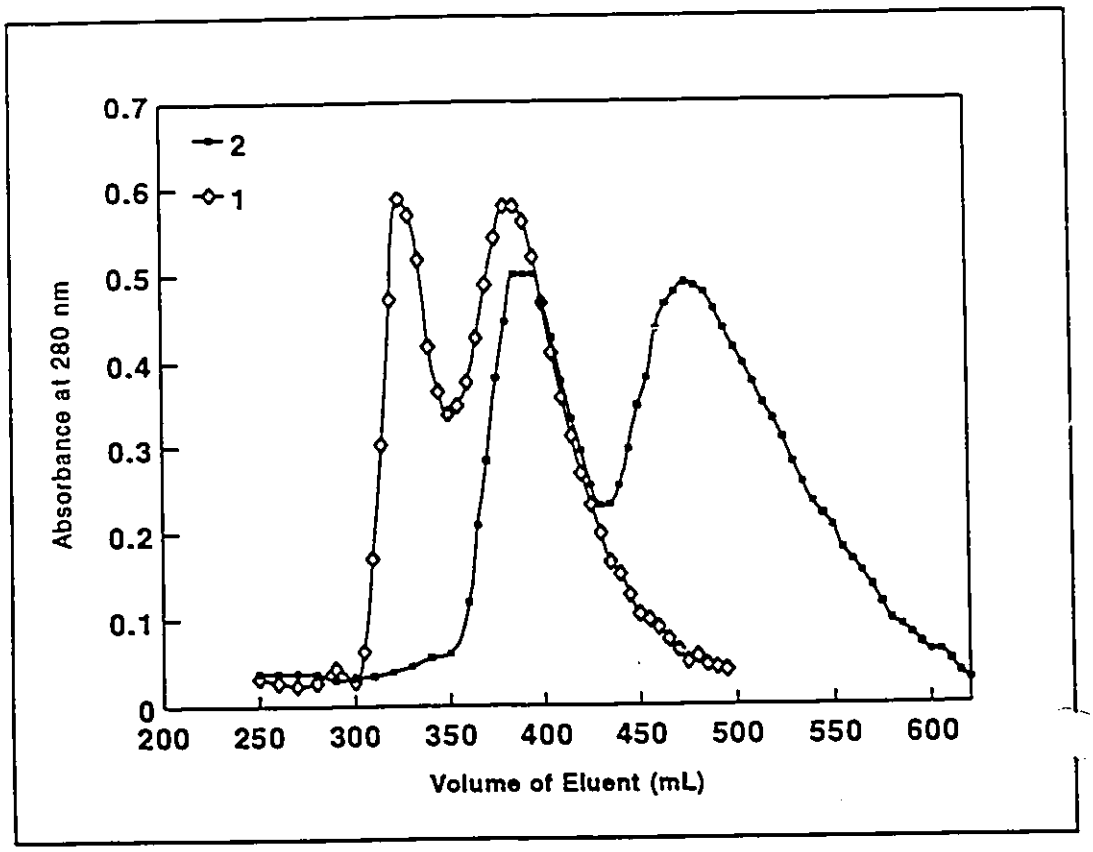


Figure 3.5: Chromatograms for two consecutive plasminogen preparations. Chromatogram 1 represents the first and chromatogram 2 the second run. Elution was by linear gradient,(0-0.2M EACA in phosphate buffer, pH 7.2).

of the potential for significant batch-to-batch variations in the plasminogen preparation a number of preparations were pooled for a given series of experiments. This was done by combining equal amounts of protein from different batches for each experiment.

As judged by polyacrylamide gel electrophoresis and protein immunoblotting criteria, the final preparations of plasminogen are homogeneous, and in particular are free of significant contamination by plasmin. Typical gel and protein blotting data are shown in Figure 3.6 and 3.7, respectively.

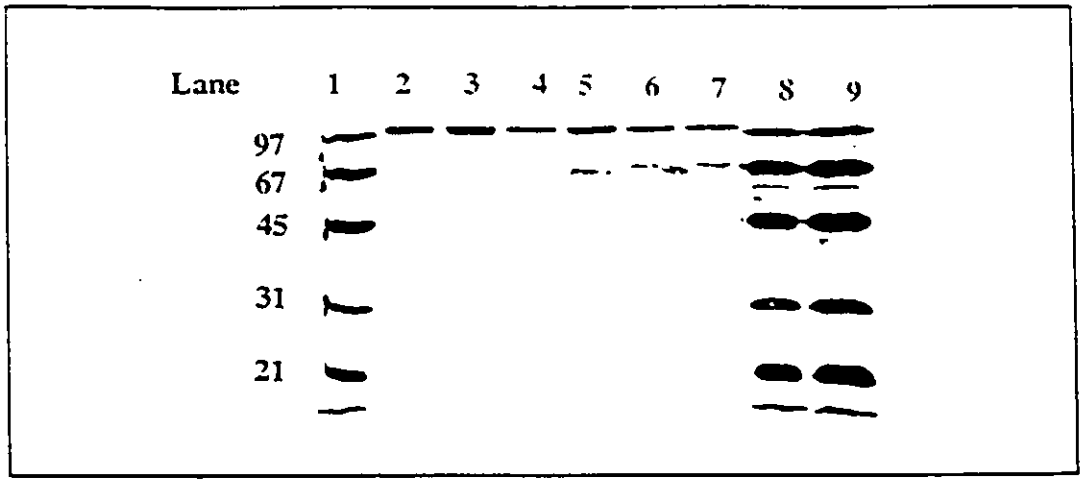


Figure 3.6: SDS-PAGE reduced gels of several different plasminogen preparations. Lanes 2&3 contain batch A loaded at 2.2 µg and 3.6 µ respectively. Lanes 4 & 5 contain batch B loaded at 0.7 µg and 1.5 µg respectively. Lanes 6 & 7 contain preparation C loaded at 0.5 µg and 0.8 µg respectively. Lanes 1, 8 & 9 contain the low molecular weight markers. The molecular weights are represented beside lane 8. Plasminogen is seen on these gels in a band very close to the 97000 molecular weight marker. Plasmin will be found near the 67000 molecular weight marker. These gels are typical of those found for most preparations.

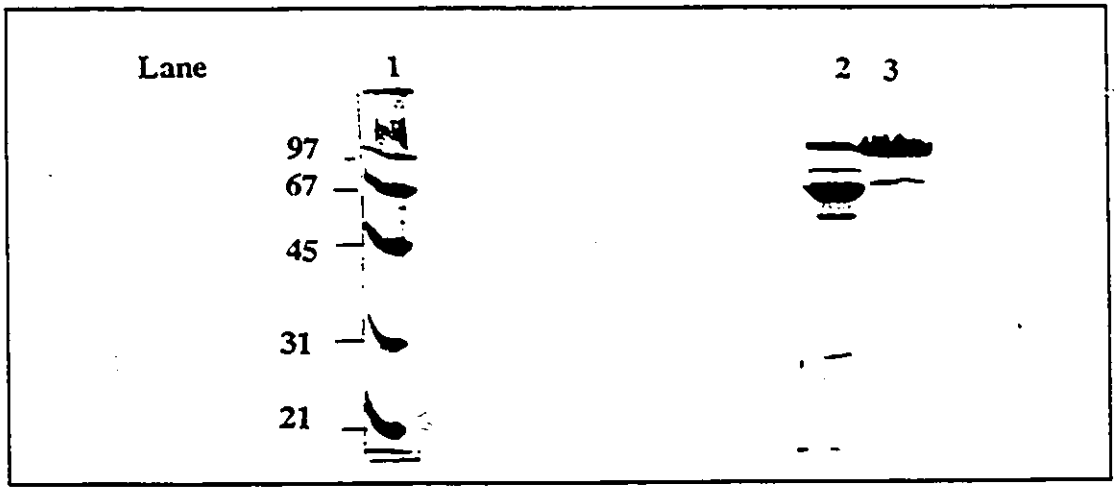


Figure 3.7:A Western blot of two plasminogen preparations using an antibody against plasminogen/plasmin. Lane 1: Low molecular weight markers. Lane 2: Preparation 20, provided here as an example of a preparation which was not used because of significant plasmin contamination shown at a molecular weight of 67000. Lane 3: Shows a typical plasminogen preparation with a heavy band at approximately 94000 and minimal plasmin contamination. Both lanes were loaded with 4 µg of protein. Details of the blotting conditions are given in the text.

3.2.4 Effect of Iodine Labelling of Plasminogen on Adsorption

Studies done on fibrinogen [Ardaillou and Larrieu, 1974] have indicated that iodine labelling with the lactoperoxidase method does not affect the its biological activity or adsorption if the number of iodine atoms per protein molecule is kept at or below an average of one. To our knowledge, no studies of this nature have been done on plasminogen. Therefore checks were performed to determine the effect, if any, of iodine labelling of plasminogen on its adsorption to the surfaces of interest.

Labelling studies were conducted on glass, the ED and MDA based polyurethanes and on one of each of the derivatized polyurethanes. The data are shown in Figure 3.8.

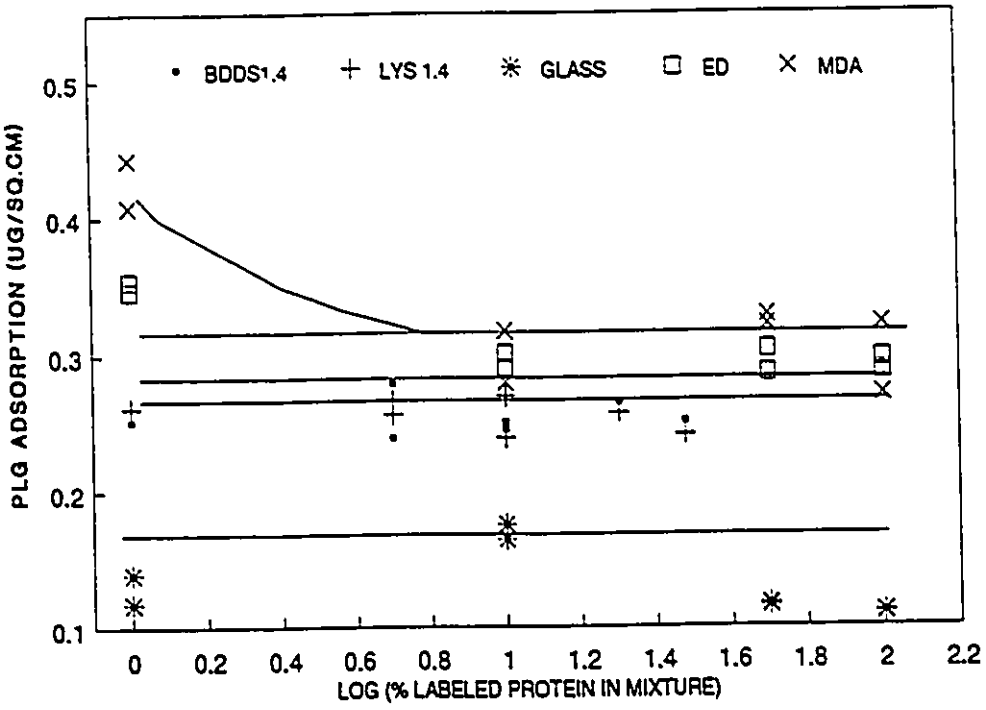


Figure 3.8: Effect of ^{125}I labelling on plasminogen adsorption. Total solution concentration was constant at 0.1 mg/mL. The solid lines do not represent models and are shown only to aid visual presentation of the data.

A variation in adsorption at the lowest labelled protein content is evident for the MDA chain extended polymer. The other surfaces show no effect of labelling on adsorption. It is not clear that the apparent labelling effect for the MDA polymer is significant and there is no obvious reason why this surface should behave differently from the others. The isotherm for this material is very steep in the vicinity of the bulk concentration used in these experiments (see Fig 3.9) so that slight discrepancies in solution concentration could shift adsorption values significantly. It is possible that the concentration of the solution at the lowest labelled protein content is in fact greater than the nominal value.

Based on these data, isotherm studies were carried out using solutions containing 3 to 5% labelled protein. This level minimized the quantities of labelled protein required, and allowed determination of the smaller adsorbed amounts with acceptable precision.

3.2.5 Adsorption of Plasminogen from Buffer

3.2.5.1 Glass and Conventional Polyurethane Surfaces

Figure 3.9 shows adsorption isotherms for glass and the polyurethanes ED and MDA. The data presented are from four replicate experiments for glass and MDA, and three replicate experiments for ED. It is clear that there is considerable scatter in the data and this aspect will be discussed in detail in the section on modeling.

The MDA isotherm (Fig 3.9C) suggests high affinity, Langmuir type adsorption, with a rising initial portion, characterized by a relatively steep slope, trailing to a quasi plateau. The glass (A) and ED (B) isotherms continue to rise and do not have well defined plateaux over the concentration range studied. This suggests that the maximum possible surface coverage has not been reached at the bulk concentrations studied. Plasminogen appears to have a lower affinity for the ED polyurethane and glass than for MDA, as indicated by the initial slopes of the isotherms.

The MDA isotherm plateau of approximately 0.2 $\mu g/cm^2$ is within the expected range for monolayer protein adsorption. Young et al [1988] have found monolayer surface concentrations of about 0.14 $\mu g/cm^2$ for albumin (MW 69,000) and 0.74 $\mu g/cm^2$

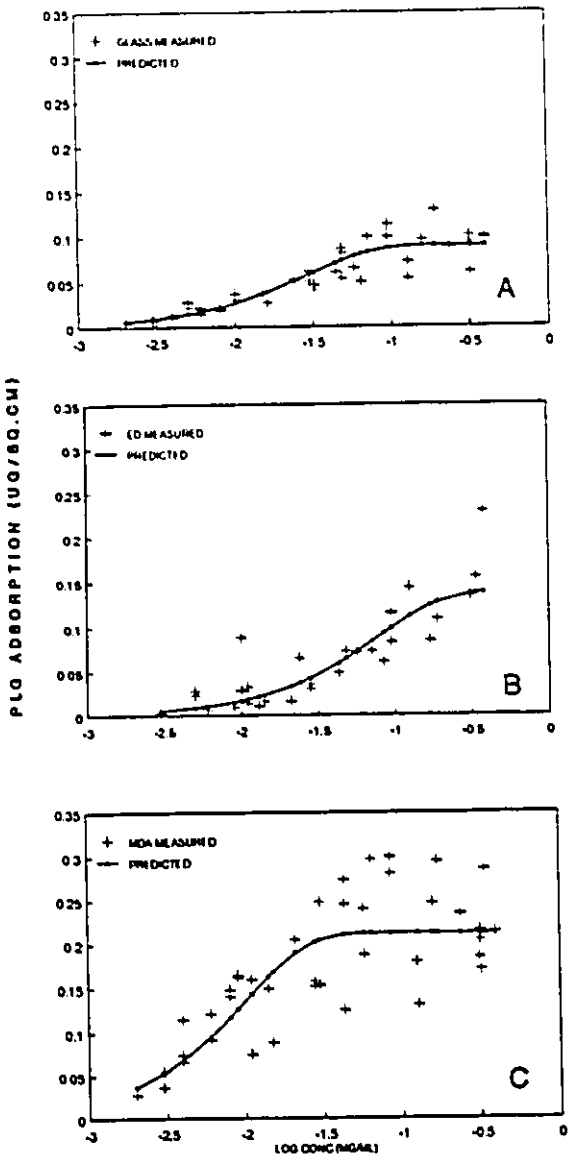


Figure 3.9: Plasminogen adsorption from Tris buffer, pH 7.4, onto glass (A), ED (B) and MDA (C) chain extended polyurethane surfaces: three hour isotherms at room temperature. The solid lines represent data fits to an irreversible Langmuir model. The model and results are discussed in detail in the text. The data shown are from four experiments for glass and MDA and from three experiments for the ED chain extended polyurethane.

for fibrinogen (MW 340,000), on various polymeric materials. It is therefore likely that the MDA polyurethane adsorbs approximately a monolayer of plasminogen (MW 94,000) at concentrations greater than about 0.1 mg/mL.

3.2.5.2 Sulphonated Polyurethanes (BDDS Polymers)

The data for these surfaces are shown in Figure 3.10. Total adsorption, as indicated by the quasi plateaux of the isotherms, increases with degree of sulphonation. However, although it is tempting to conclude that adsorption capacity increases with sulphonate concentration it could also be that the relatively high values for BDDS-0.8 and BDDS-1.4 reflect absorption as well as adsorption since it has been found that these sulphonated polymers swell in aqueous fluids [Santerre, 1990]. Slight differences in surface roughness could also influence adsorption capacities. The apparent affinities of plasminogen for the two sulphonated polyurethane surfaces in Fig 3.10, as indicated by the initial slopes of the isotherms, are about the same. The curves are virtually coincident in the low concentration region and the differences are not significant. Thus it cannot be concluded that sulphonate groups, at least at the concentrations present in these materials, show specific binding affinity for plasminogen.

Three independent replicate experiments were done for each of the BDDS surfaces. The general trends in all experiments were the same as shown in Fig 3.10 in which the values are averaged across all experiments. However there was considerable variability in the adsorption levels among the replicate experiments and this increased with increasing sulphonate content. A closer examination of the data showed that the variance among experiments was large, and correlated with the level of protein adsorption, whereas the variance within an experiment was small and independent of the amount of protein adsorbed.

There are several potential sources of data scatter among experiments including variation in rinsing technique, variable swelling of the polyurethane surfaces, coating differences, and polymer and plasminogen batch differences. Rinsing may have a large

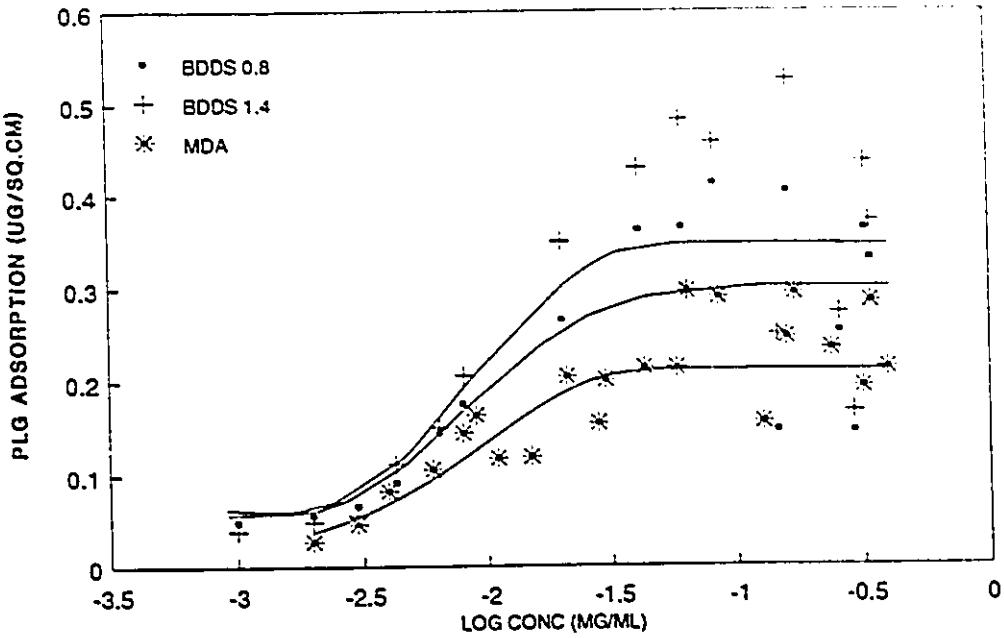


Figure 3.10: Plasminogen adsorption from Tris buffer, pH 7.4, onto MDA, BDDS-0.8 and BDDS-1.4 polyurethane surfaces: three hour isotherms at room temperature. The solid lines for the sulphonated polyurethanes do not represent models and are shown only to aid visual presentation of the data. For the MDA chain extender polyurethane the curve represents the fit to an irreversible Langmuir model. The data shown are for four experiments for MDA and for three experiments for the sulphonated polyurethanes. For given concentrations, averages of all replicates are shown.

effect on adsorption measurements particularly if multi or loosely bound layers of protein are involved. This effect may contribute to the correlation between variance and adsorption level.

Batch to batch variation in plasminogen is virtually inevitable since purified plasminogen is heterogeneous as previously discussed [Summaria et al, 1972]. The protein has several different forms with differing isoelectric points, carbohydrate content

and molecular weight [Summaria et al, 1972]. The composition of the preparation is affected by the inhibitors used, the column loading and number of uses [Hatton, 1974], and the starting plasma isolation technique. This inherent heterogeneity may also contribute to the variation in adsorption measurements.

3.2.5.3 Lysine Derivatized Polymers

The adsorption isotherms for these polymers are shown in Figures 3.11 and 3.12 along with data for the corresponding sulphonated polymers. Comparing the BDDS-0.8 and BDDS-0.8-LYS materials (Figure 3.11), a slight decrease in adsorption capacity (high concentration) is seen upon lysine derivatization. However there is little difference in the apparent affinity of plasminogen for the two surfaces (low concentration). The adsorption isotherms for the "1.4" polymers, shown in Figure 3.12, are similar to those for the "0.8" polymers although there is considerably more scatter in the data from experiment to experiment. In both Figure 3.11 and Figure 3.12 it appears that adsorption decreases at the highest concentrations. This behaviour is unusual in single protein systems and for the moment we have no convincing explanation for it. One possibility is that complexes of lower binding affinity are formed at the higher concentrations. The effect seems to be specific to the sulphonated and lysinated polyurethanes.

It was anticipated that introduction of lysine sites into a polyurethane surface would increase its binding affinity if not its binding capacity for plasminogen, but these expectations are not supported by the data. There appears to be no significant difference between the sulphonated and corresponding lysine-derivatized materials. Thus it cannot be concluded that the lysine-containing materials interact with plasminogen differently from their sulphonated precursors.

The similar behaviour of these materials is surprising since it is well known that plasminogen interacts specifically with lysine [Markus et al, 1978; Miyashita et al, 1988; Wun et al 1988; Mangel et al, 1990; Weitz, 1990]. Several possible explanations may

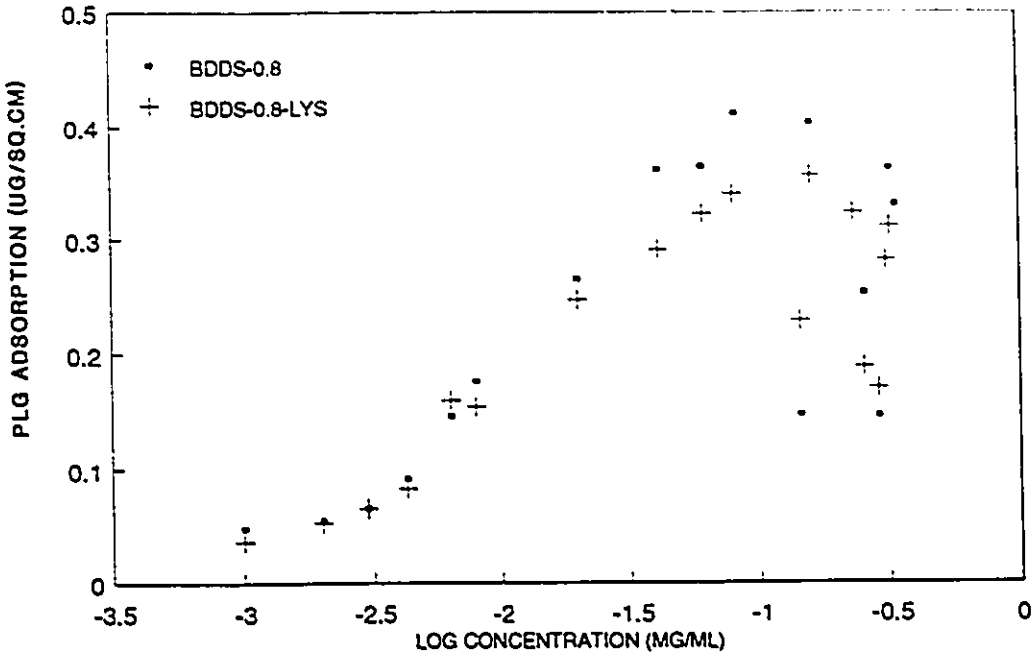


Figure 3.11 Plasminogen adsorption from Tris buffer, pH 7.4, onto BDDS-0.8 and BDDS-0.8-LYS polyurethanes: three hour isotherms at room temperature. Data from three independent experiments have been averaged and combined.

account for this apparent anomaly. First the sulphonate groups and the lysine residues that replace them may have similar binding affinities. To our knowledge, however, there is no report of any specific interaction between sulphonate groups and plasminogen. Second there may be only a few lysine residues per unit area of surface so that most of the binding sites in the precursor and lysine-derivatized materials are the same. Boisson et al [1987] found that the specificity of interactions of arginine-derivatized polystyrene resins with thrombin depended strongly on arginine content. A third explanation is that the lysine residues are inappropriately attached to the polyurethanes and cannot exhibit

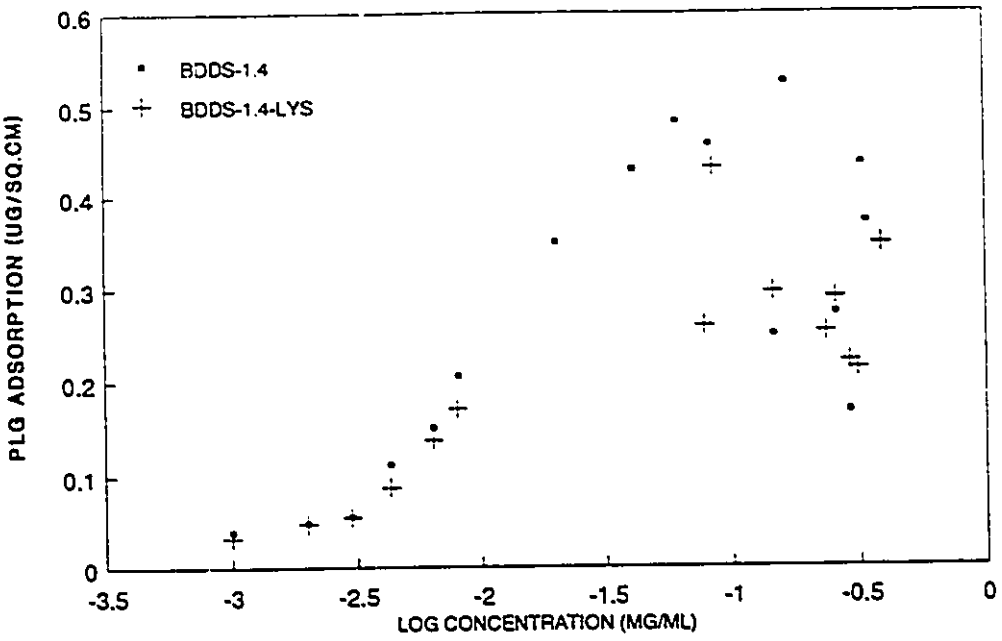


Figure 3.12: Plasminogen adsorption from Tris buffer, pH 7.4, onto BDDS-1.4 and BDDS-1.4-LYS polyurethanes: three hour isotherms at room temperature. Data from three independent experiments have been averaged and combined.

plasminogen binding. In developing Sepharose-lysine as an affinity chromatography support for plasminogen purification it has been shown that the ε-amine group of lysine is essential for interaction with plasminogen [Hatton and Regoeczi, 1974, 1975]. If this group is unavailable to the lysine binding site of plasminogen, adsorption will most likely occur in a non-specific manner. This situation could arise if the ε-amine group rather than the α-amine reacts with the sulphonate group of the polyurethane during derivatization. It is also possible that both amine groups of lysine react with sulphonate during the coupling reaction and that both are covalently bound to the surface and unavailable for plasminogen binding.

The inappropriate attachment of lysine may be anticipated to decrease plasminogen binding relative to the precursor sulphonate groups which were shown to increase binding. Thus the replacement of sulphonate groups with lysine residues in which the ε-amine groups are unavailable may actually reduce adsorption, as was found for the BDDS-0.8 materials.

3.2.6 Comparison of Adsorption Data to a Mechanistic Adsorption Model

The fitting of experimental protein adsorption data to adsorption models may be used to verify the mechanisms of adsorption and/or to quantify the interactions between surfaces and proteins via the numerical values of model parameters. Adsorption models used previously have incorporated such features as reversibility, exchange of surface and solution protein, and distributions of bound states involving differences in orientation, conformation, and surface-protein binding mechanisms. Lundstrom has written a comprehensive review of protein adsorption models [Lundstrom, 1985].

In the present work a previously developed simulation of the tubing adsorption experiment [Wojciechowski and Brash, 1990] was used to fit adsorption data to a simple model describing irreversible surface filling to a single adsorbed state. The use of more complex models did not seem justified since the data set is limited to a single adsorption time (3h). Rigorous model discrimination is more appropriately done using kinetic data [Wojciechowski and Brash, 1990]. Also it did not seem appropriate to apply such a simple model to the sulphonated and lysine derivatized polyurethanes, which as well as adsorbing plasminogen nonspecifically, have the possibility of interacting specifically via their functional groups. Therefore only the data for glass and polyurethanes ED and MDA were used in these investigations.

The model used is based on an irreversible Langmuir mechanism described by equation 3.1:

$$\frac{d\,\Gamma}{dt}=kC(1-\theta) \qquad 3.1$$

where Γ is the surface concentration of protein, t is time, k is a first order adsorption rate constant, C is bulk protein concentration and θ is fractional surface coverage given by:

$$\theta=\frac{\Gamma}{\Gamma_{max}} \qquad 3.2$$

where Γ_{max} is the maximum surface coverage. This mechanism involves simple surface filling with no desorption. Equation 3.1 was incorporated into a simulation of the adsorption experiment which accounts for diffusion under static conditions in cylindrical geometry [Wojciechowski and Brash, 1990]. The simulation is based on solving the diffusion equation, equation 3.3, using equation 3.4 (incorporating the model) as a boundary condition as described previously [Wojciechowski and Brash, 1990].

$$\frac{\partial C(r,t)}{\partial t}=\frac{D}{r}\frac{\partial}{\partial r}r\frac{\partial C(r,t)}{\partial r} \qquad 3.3$$

$$D\frac{\partial C(r,t)}{\partial r}=kC(r,t)(1-\theta) \quad at\ r=R \qquad 3.4$$

where r is radial position, R is the inner radius of the tube, and D is protein diffusivity assumed to be constant. Data were fit using the simulation and a nonlinear parameter estimation routine [Meeter and Wolf, 1965]. The procedure yields values of the parameters k and Γ_{max} for plasminogen adsorption on the various surfaces.

The model fits for plasminogen to glass, ED, and MDA are shown in Figure 3.9

Surface	Glass	MDA	ED
k (cm/s)*	(3.03 ± 0.45) X 10^{-7}	(2.49 ± 0.61) X 10^{-7}	(1.69 ± 0.52) X 10^{-7}
Γ_{max}* (ug/cm^2)	0.090 ± 0.013	0.212 ± 0.026	0.139 ± 0.075
n	42	41	28
Variance of residuals	0.077	0.081	0.340

* Data are given as ±95%confidence interval.

Table 3.7: Parameters from fit of data to an irreversible Langmuir Model (Eq. 3.1).

and the estimated parameters are listed in Table 3.7. With data for only a single time, the effects of diffusion and adsorption were difficult to decouple and thus an a priori estimate of plasminogen diffusivity (D) was required. The diffusivity of plasminogen in buffer was taken as 4.5 * 10^{-7} cm^2/s based on a molecular weight of 94,000 and the known diffusivities of other proteins [Andrade and Hlady, 1987].

The model fits shown in Figure 3.9 are adequate for the data. Models with greater numbers of parameters could not be justified statistically due to the scatter in the data. It is interesting to note that the data, especially in Figures 3.9A and 3.9C, are apparently distributed between distinct upper and lower limits. One may speculate that within this range the plasminogen is partially desorbable and thus the data are strongly affected by

the rinsing process.

Based on the assumption that the Γ_{max} values listed in Table 3.7 are in fact representative of monolayer adsorption we may draw some conclusions regarding the effect of the three surfaces on plasminogen. Monolayer adsorption on glass appears to be about half that on MDA, while on ED it is intermediate between glass and MDA and the residual error is much larger. In fact the 95% confidence interval for Γ_{max} on ED included the estimates for Γ_{max} on glass and MDA. It is thus difficult to draw any conclusions concerning the magnitude of Γ_{max} on ED. The Γ_{max} values suggest that plasminogen attains a more spread conformation on glass than on MDA assuming that both surfaces are equally smooth (and therefore the apparent areas are equal). The conformational changes induced by a surface may have a strong influence the biological activity of the adsorbed protein.

3.2.7 Adsorption from Plasma

3.2.7.1 Control Surfaces

The adsorption data for glass, the ED and the MDA polyurethanes are shown in Figure 3.13. Adsorption to these three surfaces increases monotonically and approximately linearly (except for ED) with increasing plasma concentration and shows no evidence of surface saturation at the higher plasma concentrations.

These data show significant differences from those obtained for plasminogen adsorption as a single protein from buffer, which showed Langmuirian adsorption with well-developed plateau levels between 0.1 and 0.3 $\mu g/cm^2$. Amongst the control surfaces, for the pure buffer system, the MDA chain extended polyurethane showed the highest adsorption plateau and binding affinity, and glass the lowest.

The curves in Figure 3.13 may be considered as adsorption isotherms and the slopes at low concentration, may be taken as indicative of relative binding affinity. On this basis the order of affinities in the complex, competitive adsorption environment of plasma appears to be glass > ED ~ MDA.

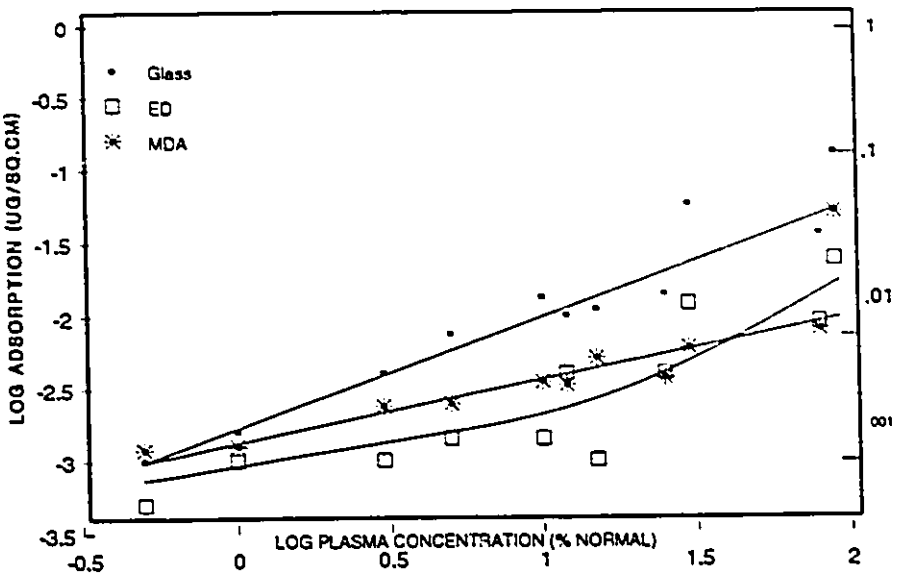


Figure 3.13: Plasminogen adsorption from citrated plasma onto glass, ED, and MDA chain extended polyurethane surfaces: three hour adsorptions at room temperature. The data shown are from two experiments. The average values are given. The curves are shown for clarity of presentation and do not represent model fitting.

The adsorption levels from plasma are significantly lower than from buffer. Even at 100 % plasma the surface concentrations are in the range from 0.01 to 0.1 $\mu g/cm^2$ whereas in buffer the isotherm plateaux were between 0.1 and 0.3 $\mu g/cm^2$. Clearly in plasma there are many other proteins besides plasminogen competing for surface sites. The complexity of the layer adsorbed from plasma has been observed in other studies [Young et al, 1988, Cuypers et al, 1987; Brash and ten Hove, 1989; Grasel and Cooper, 1989; Boisson et al, 1991].

It is important also to point out that there is no evidence that adsorption of plasminogen from plasma is transient as has been observed for fibrinogen and other proteins [Cuypers et al, 1987; Brash and ten Hove, 1989; Grasel and Cooper, 1989;

Andrade and Hlady, 1991; Scott, 1991]. The curves in Figure 3.13 increase monotonically with no indication of the adsorption maxima expected for transient adsorption [Cuypers et al, 1987; Brash and ten Hove, 1989; Grasel and Cooper, 1989; Andrade and Hlady, 1991, Scott, 1991]. The Vroman effect thus does not occur for plasminogen on these surfaces.

3.2.7.2 Sulphonated and Lysine Derivatized Polyurethanes

Plasminogen adsorption from plasma to the sulphonated and lysinated surfaces is shown in Figures 3.14 and 3.15. The data for the MDA chain extended polymer and glass are included for comparison. Figure 3.14 shows significantly greater adsorption to BDDS-0.8 and BDDS-0.8-LYS than to MDA, suggesting that the introduction of sulphonate or lysine-sulphonamide groups increases the plasminogen binding capacity of the polyurethanes. However there appears to be little effect of lysine since BDDS-0.8 and BDDS-0.8-LYS show similar adsorption.

Similarly Figure 3.15 shows that adsorption to BDDS-1.4 and BDDS-1.4-LYS is greater than to MDA. In this case adsorption to the lysine-containing polymer is somewhat greater than to the sulphonated precursor, though perhaps not significantly so. Binding affinities, as indicated by the slopes of the curves at low concentration, do not appear to be altered by sulphonate or lysine sulphonamide groups. Again there is no Vroman effect for plasminogen adsorption to these surfaces. The adsorption levels for the 0.8 and 1.4 series are similar.

Figures 3.14 and 3.15 also include a comparison of the sulphonated and lysinated materials with glass. The slopes of the glass curves are greater than for any of the other materials, and the adsorption levels are lower at the lower plasma concentrations and about equal at the higher plasma concentrations to those on the derivatized polyurethanes. This behaviour of glass is the reverse of that found in single protein adsorption where of all the surfaces studied, glass showed both the lowest apparent affinity and capacity for plasminogen. The reason for this behaviour is unclear.

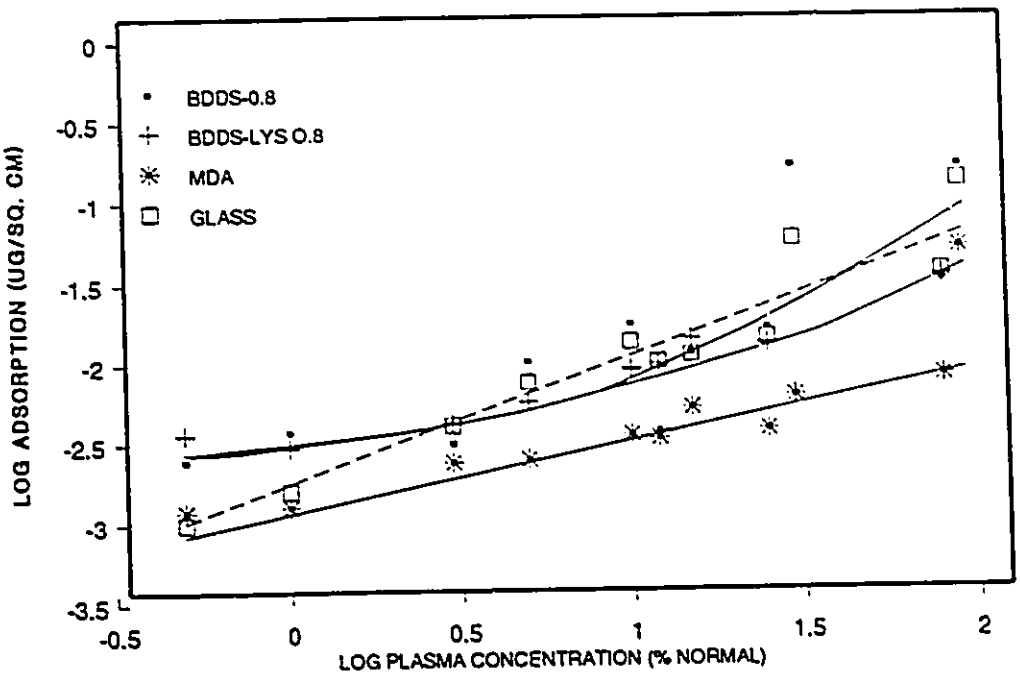


Figure 3.14: Plasminogen adsorption from citrated plasma onto glass, MDA, BDDS-0.8 and BDDS-0.8-LYS polyurethanes: three hour adsorption times at room temperature. The solid lines do not represent models and are shown only to aid visual presentation of the data.

These plasma adsorption data show several noteworthy trends: 1) Adsorption increases monotonically with increasing plasma concentration and there is no suggestion of transient adsorption (Vroman effect). 2) Sulphonate groups appear to have a strong effect on plasminogen adsorption as was found for adsorption from buffer. 3) The lysine

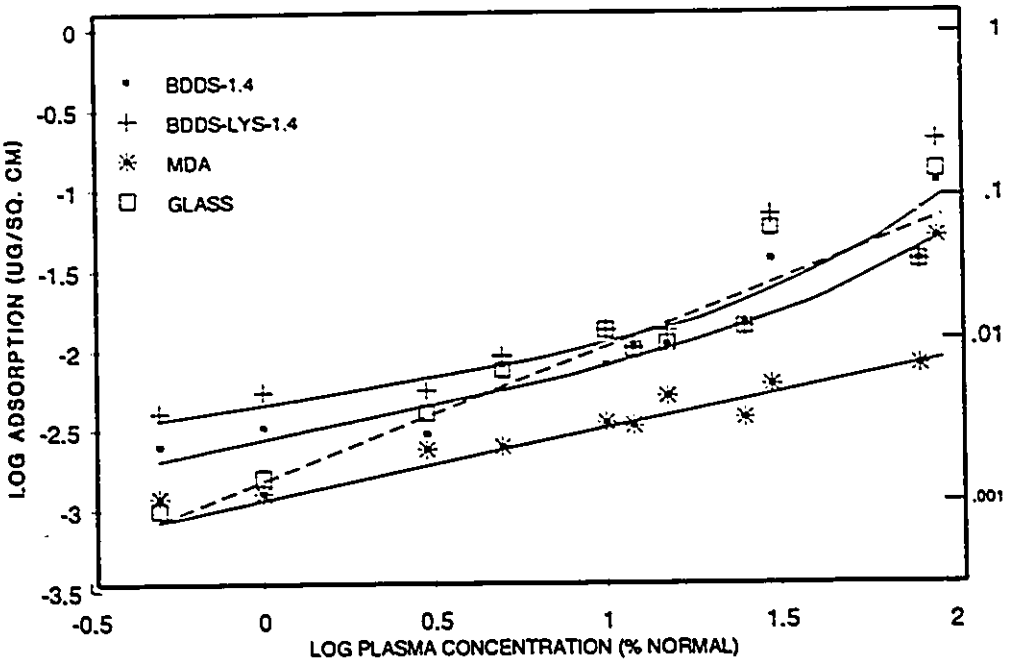


Figure 3.15: Plasminogen adsorption from citrated plasma onto glass, MDA, BDDS-1.4 and BDDS-1.4-LYS polyurethanes: three hour adsorptions at room temperature. The solid lines do not represent models and are shown only to aid visual presentation of the data.

derivatized polyurethane with the higher lysine content may show an increase inaffinity over its sulphonated precursor.

As discussed in Chapter 1 of this thesis many proteins, including fibrinogen and

IgG, undergo transient adsorption to surfaces, manifested as a maximum in adsorption as a function of either time or plasma concentration [Bale et al, 1988; Brash and ten Hove, 1989; Andrade and Hlady, 1991]. This phenomenon has been termed the Vroman effect. It is obvious that the Vroman effect does not occur for plasminogen adsorption on any of the surfaces studied. A comparison of plasminogen and fibrinogen adsorption from plasma is shown in Figure 3.16. The Vroman effect reflects competitive adsorption and

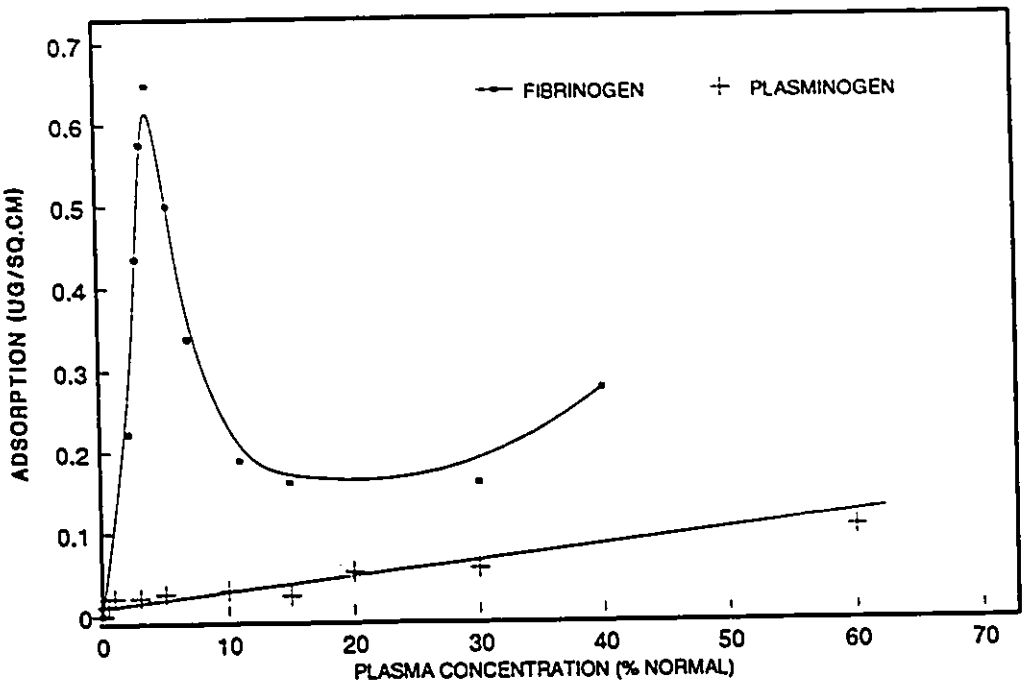


Figure 3.16: A comparison of plasminogen and fibrinogen adsorption from citrated human plasma to the MDA chain extended polyurethane. Adsorption was for three hours at room temperature. The fibrinogen curve illustrates the Vroman effect and contrasts with the monotonically increasing plasminogen curve.

is believed to result from the displacement of initially adsorbed high concentration

proteins of low binding affinity by low concentration proteins of high binding affinity. Thus it is possible that plasminogen (concentration about 0.2 mg/mL) may be a "displacer" protein in the early stages of plasma contact but may not itself be displaced due to its relatively high binding affinity. Vroman has suggested that high molecular weight kininogen (HMWK) is a major displacer on many surfaces and that its persistence in the adsorbed layer promotes surface-induced coagulation via the intrinsic pathway [Andrade and Hlady, 1991; Scott, 1991]. Plasminogen has not previously been considered in relation to the Vroman effect but the data presented here suggest that it may be involved. It is clear that the continued presence of plasminogen on a blood contacting surface is a positive finding in relation to the underlying theme of the present work, namely the development of a fibrinolytic surface by preferential adsorption of plasminogen. Displacement of plasminogen by competing proteins would clearly invalidate this approach. It is also worth noting that the presumed specificity of lysine binding sites is not required to prevent the displacement of adsorbed plasminogen since none of the surfaces investigated shows displacement.

The data presented in this chapter indicate that plasminogen adsorption is enhanced by the presence of sulphonate groups. The sulphonate group appears to have a strong influence on the adsorption of proteins generally [Grasel and Cooper, 1989; Boisson-Vidal et al, 1991; Okkema et al, 1991]. A number of investigators have shown enhanced protein adsorption to sulphonated materials, including fibrinogen [Grasel and Cooper, 1989; Boisson-Vidal et al, 1991; Okkema et al, 1991], and antithrombin III [Ito et al, 1991]. Recently Yang and Henkin [1990] found that polystyrene resins derivatized with sulphonate or sulphonyl-glutamate groups adsorbed plasmin preferentially from a plasmin-contaminated plasminogen solution, illustrating again the specific effect of the sulphonate group on protein surface interactions.

The mechanism of protein-sulphonate interactions has not been elucidated. As discussed, both sulphate and sulphonate groups are found in heparin [Casu, 1989], a glycosaminoglycan which binds antithrombin III. By analogy, the anticoagulant nature

of certain sulphonated materials has been attributed to these groups [Boisson-Vidal, 1991]. The glycosaminoglycans heparan sulphate and chondroitin sulphate also contain sulphate and sulphonate groups. Heparan sulphate exists as a cell membrane proteoglycan and has been implicated in cell-extracellular matrix interactions [Hovingh et al, 1986]. It is generally believed that the high negative charge on these molecules due to the sulphonate and sulphate groups contributes to their biological functions. The fact that molecules in the physiologic system associated with binding and adsorption phenomena contain significant amounts of sulphate and sulphonate may explain why synthetic materials containing similar groups show enhanced plasma protein adsorption.

It was anticipated that introduction of lysine sites into a surface would increase its binding affinity if not its binding capacity for plasminogen, but these expectations are not supported by the data for either pure protein or for plasma, although in the plasma studies a slight increase in affinity and capacity is observed for the lysine derivatized BDDS-1.4 polymer. As discussed in the pure protein section, lysine may simply not be present in high enough concentration at the surface to affect adsorption. It is also possible that the lysine, because of the manner in which it is attached to the polyurethanes, is not available to interact with plasminogen through the lysine binding sites. This would be true if the ε-amine groups of the lysine are not free.

3.3 CONCLUSIONS

The data presented in this chapter show that significant quantities of plasminogen are adsorbed from plasma and pure protein systems to various surfaces and that in plasma it is not displaced from the surface by other plasma constituents. These findings are encouraging for the concept of a fibrinolytic surface based on selective plasminogen adsorption. The lysine-containing surfaces do not show evidence of specific plasminogen binding but, as indicated, the surface concentration of lysine sites may not be sufficient for such specificity to be evident or the lysine may be bound in a manner which makes the ε-amine groups unavailable for interactions with plasminogen.

A number of questions remain to be answered before the concept of a plasminogen binding surface can be considered validated. In particular it must be demonstrated that the adsorbed plasminogen has plasmin or plasmin-like activity either via conformational changes induced by adsorption as found by Kichenin-Martin et al [1988] or by the binding of plasminogen activators to incipient fibrin clots. These questions are addressed in the chapters that follow.

CHAPTER FOUR

PLASMINOGEN ADSORPTION TO MODEL SILYLATED SILICA GLASS SURFACES

4.0 INTRODUCTION AND SYNOPSIS

The work discussed in Chapter 3, investigated the concept of a surface that would selectively adsorb plasminogen. The rationale was that surfaces which adsorb plasminogen may induce clot lysis, and that by utilizing binding characteristics of the physiologic system (the lysine binding sites), preferential adsorption of plasminogen might be attained. The initial approach described in Chapter 3 was to incorporate lysine residues into segmented polyurethanes, and although promising results were obtained, these materials presented a number of difficulties due to their physical and chemical complexity. It was thus decided to pursue the approach of lysine incorporation using "model" surfaces that would minimize these difficulties. The present chapter describes this work.

It was concluded in Chapter 3 that the plasminogen binding characteristics of the lysinated polyurethanes could be made more effective by assuring that the ε-amine of the lysine was free and available, and by increasing the lysine content of the polyurethanes by increasing the sulphonate content of the precursor polymer. It is largely the former issue that is addressed in this chapter.

Model surfaces have been utilized to develop a method of binding lysine to the surface which leaves the ε-amine free. Amorphous silica silylated with a sulphonate containing chlorosilane has been used as the precursor to the lysinated surface. The silane contains a sulphonyl chloride group which has been derivatized with ε-tBOC-lysine via the α-amine group to provide the lysinated material. The amorphous silica glass used as a substrate for these reactions affords a surface which is much better suited than the

polyurethanes for quantification of the chemical modifications and for method development. The model materials were characterized by contact angle analysis and two chemical methods to validate the silylation and lysine attachment methods. Molecular modelling of the silylated silica glass surfaces using the computer package PC Model [Serena Software, Bloomington, Ill.], was also undertaken to obtain an estimate of the density of sulphonate and lysine on the silica glass surfaces and whether or not these approximated a monolayer.

The model surfaces are also better adapted to the interpretation of adsorption data. It was difficult to determine the influence of the sulphonate and lysine groups on plasminogen adsorption to the polyurethanes because of their chemical complexity. As indicated, the amount of lysine incorporated and the availability of the ε-amine group available for interaction with plasminogen were difficult to assess. For these reasons the model materials were also used to study the interactions of plasminogen with both lysine and sulphonate groups in more detail.

It also became clear that a new method of evaluating protein adsorption to surfaces had to be designed and implemented. The method described in Chapter 3 used too much plasminogen and was simply not practical for the large number of experiments required for the work on the model surfaces. The development of this new method is also reported in this chapter.

Plasminogen adsorption from buffer to pure silica glass, sulphonated silica glass, and the corresponding lysinated silica glass was studied. The sulphonated materials were investigated for two main reasons: 1) Sulphonate groups have been associated with reduced thrombogenicity when they occur on a material surface. 2) Lysine was bound to the model surface by reaction with sulphonyl chloride groups, analogous to the approach used in the polyurethane modification [Santerre and Brash, 1991] described in Chapter 3. The sulphonated material is thus a precursor to the lysine form. The pure silica glass surface was included as a control material.

The single protein studies include adsorption isotherms, adsorption isotherms in

the presence of the lysine analogue ε-amino caproic acid (EACA) and desorption experiments against both buffer and buffer containing EACA. In total this series of single protein experiments comprises a detailed study of plasminogen adsorption to the model materials. The single protein adsorption studies from buffer were used to determine the plateau (monolayer) adsorption value and the relative affinities of plasminogen for the different surfaces. The Langmuir model was used to interpret the single protein adsorption data. The rational for the EACA experiments is given below.

As discussed previously, plasminogen, plasmin and tissue plasminogen activator all have "kringles" within their heavy chains which contain lysine binding sites (LBS). Lysine analogues can also bind to these lysine binding sites [Markus et al, 1978; Hatton and Regoeczi, 1974]. To further investigate the interaction between plasminogen and the model surfaces, adsorption studies were done in the presence of one such analogue, ε-amino caproic acid (EACA). Frequently used as an eluent for plasminogen in affinity chromatography, EACA has a high affinity for the lysine binding sites in unbound plasminogen [Markus et al, 1978]. The rationale for these experiments was that if the LBS were indeed involved in the interaction with the lysinated material, the presence of EACA in the buffer with plasminogen should result in a discernable inhibition of plasminogen adsorption to the lysinated surface compared to the sulphonated or control silica glass materials.

A series of experiments were run to determine the reversibility of plasminogen binding to the model surfaces. Similarly, desorption studies with EACA in the buffer were also performed. As well as being of intrinsic interest, data on desorption against buffer were required in designing the protocol for the enzymatic activity studies described in Chapter 5. It was necessary to evaluate the enzymatic activity associated with the bound plasminogen and therefore the experiments had to be run using surfaces which contained essentially no reversibly bound protein. This allowed assessment of adsorbed plasminogen only, and did not confound the results with the activity of unbound plasminogen.

Plasminogen and fibrinogen adsorption from plasma to the three model surfaces was also investigated. It was anticipated that the interactions of the two proteins would be different particularly on the lysinated and sulphonated materials. Plasminogen was expected to exhibit preferential adsorption from plasma to the lysine derivatized material compared to the sulphonated precursor. Comparing adsorption of both proteins to the surfaces would also provide data on the relative of affinity of the proteins for the materials. Sulphonated groups have previously been shown to strongly influence the adsorption characteristics of fibrinogen from both buffer and plasma [Fougnot et al, 1979a&b; Douzon et al, 1987; Grasel and Cooper, 1989].

In addition, fibrinogen adsorption from plasma is subject to the Vroman effect. From the results discussed in Chapter 3 of this thesis, plasminogen appears not to undergo the Vroman effect. Therefore the model materials were also used to further investigate the influence of sulphonate and lysine groups on the Vroman effect of the two proteins and whether or not plasminogen may contribute to this phenomenon for fibrinogen.

This chapter also includes a comparison of the protein adsorption studies for the model surfaces with the polyurethanes discussed in Chapter 3.

4.1 EXPERIMENTAL

4.1.1 Silylated Silica Glass

As discussed in the introduction, silica glass and silylated silica glass have a fairly extensive history of use as model materials [Arkels et al, 1986]. In referring to silica-based materials care must be taken to define clearly the material under discussion. The generic use of the word "quartz" which refers to a crystalline material composed of SiO_2 and having a specific crystal structure [Berezhnoi, 1960; Vogel, 1971] is inappropriate in the present work. The base material used in this work is correctly termed amorphous silica glass. The word glass refers to the method used to produce the materials from silica quartz [Vogel, 1971]: the silica quartz is heated to very high temperatures, essentially melted and then cooled, resulting in an amorphous structure [Vogel, 1971].

The term silica indicates that the material is composed of SiO_2. These glassy materials are often referred to as "fused silica quartz" by manufacturers.

The surface chemistry of amorphous silica glass is primarily dependent on the presence of hydroxyl at the surface [Hair, 1986]. It has been found that there are approximately 1.6 hydroxyl groups per nm^2 [Hair, 1986] at the surface of silica glass. These hydroxyl groups can be utilized to attach different functional groups to the glass surface via the use of silanes. Silanes may be regarded as the silicon analogs of alkanes and have the general structure, SiR_4 [Chvalovsky and Bellama, 1984]. Organosilanes have organic groups attached to the silicon atom via C-Si bonds [Voorhoeve, 1967]. Chlorinated silanes react readily with glass or silica and can be used to provide a surface containing different functional groups. This reaction, referred to as silylation, is the displacement of an active hydrogen, usually in a hydroxyl group, by an organosilane (see Fig 4.1).

4.1.1.1 Cleaning of the Silica Glass

Pure silica glass tubing (0.25 cm I.D.) (CFQ TO8 HLX tubing, Heraeus Amersil, Burford, GA, USA) was cut into 0.7 cm lengths and cleaned by soaking sequentially in 5 % NaOH for 1 h and in 100% HNO_3 for 1 h followed by alternate rinses in 18 Mohm water (Milli Q Water System, Millipore,), and HPLC grade methanol (BDH) (three rinses with each solvent ending with a methanol rinse). The base and acid washes were designed to clean the surface and expose the OH groups respectively [Phillips and Hercules, 1985; Van Damme, 1990; Wojciechowski, 1992]. Siloxane bonds are hydrophobic but treatment with acid results in the formation of the more reactive, hydrophilic silanol group at the surface. The silica glass was finally dried in a vacuum oven for 24 h at 90°C.

Samples to be used as controls were cleaned in exactly the same manner as described above. However before drying they were soaked in chloroform for 1 h and methanol for 1 h to remove hydrocarbons on the surface [Kallury, personal

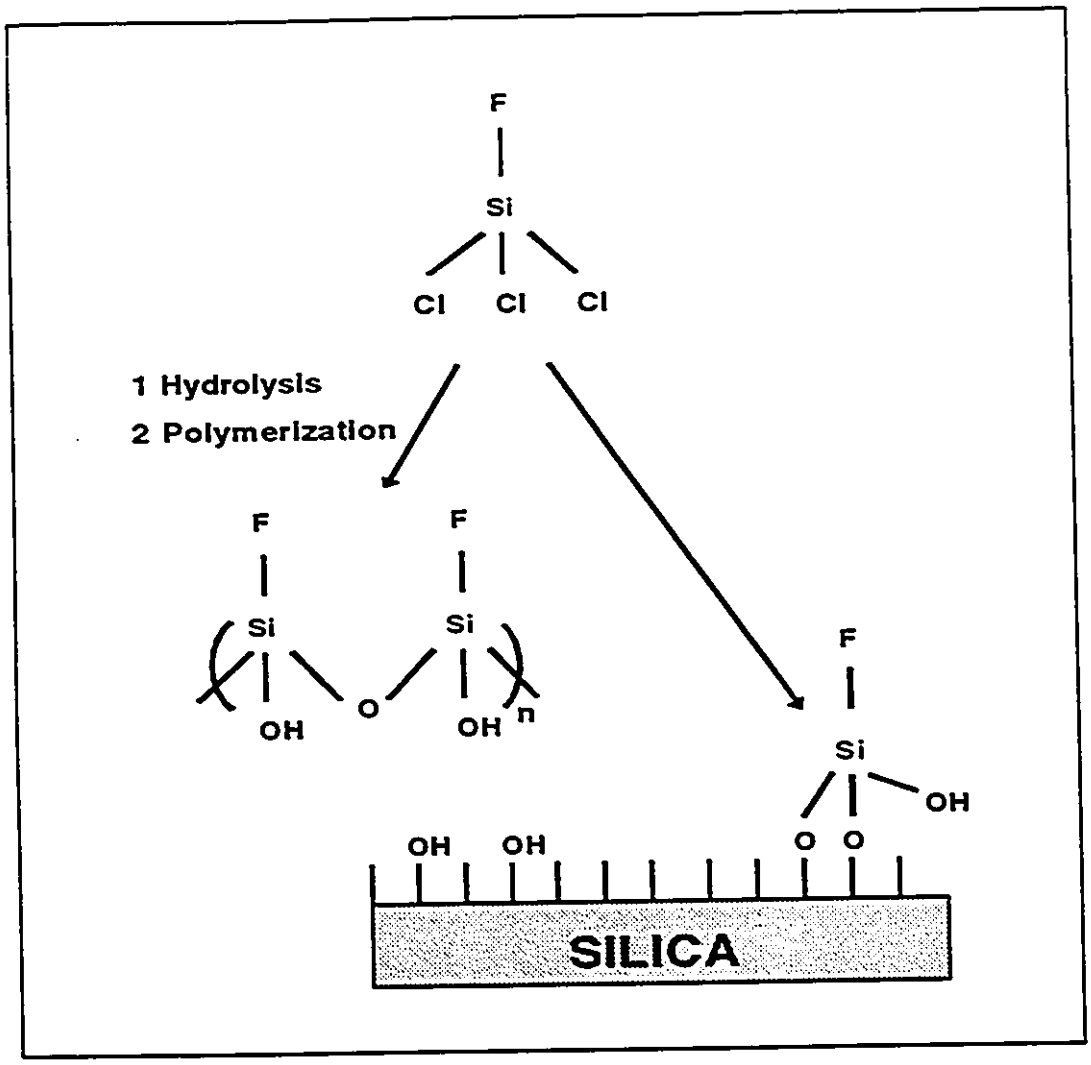


Figure 4.1: Silylation of silica glass. F represents a functional group (in this work F is SO_2Cl). A trichlorosilane is used in this work and the reaction with the surface is essentially the same as that shown here. The polymerization of the silane before reacting with the surface is also shown in this figure. The polymer may or may not subsequently react with the surface.

communication], and subsequently dried overnight at 90°C. The tubes were stored in clean capped vials and used within three weeks. X-ray photoelectron spectroscopy data indicate that the cleaned silica glass tubes were free from contamination by nitrogen,

chlorine, and sulphur.

4.1.1.2 Silylation

The methodology used to prepare silica glass surfaces covered with approximately a monolayer of a sulphonated silane is described in this section. Silylation of the cleaned silica glass tubes was done under nitrogen and using distilled toluene to minimize the amount of water present during the silylation reaction. Phillips and Hercules [1985] found that the level of water contamination in the system had a strong influence on the amount of silane attached to the surface. Minimizing the water impurity assists in limiting silane coverage to the monolayer level [Scouten, 1985]. Monolayer or submonolayer silane coverage is desired in this work because silane multilayers have been associated with leaching of the silane from the surface [Scouten, 1985].

Approximately 50 previously cleaned 0.7 cm silica glass tubes were placed in a nitrogen blanketed vessel. The pieces were then rinsed three times with distilled toluene. A 2% v/v solution of 2-(4-chlorosulfonylphenyl)ethyl trichlorosilane (CSPES) (Huls America, Piscataway, NJ, USA) in distilled toluene was then added to the silica glass tubes and allowed to react for 10 min at room temperature. These amounts and times were also chosen to minimize formation of unstable multilayers of the silane [Wojciechowski, 1992]. The surfaces were again rinsed three times with distilled toluene, and then reacted with 2% hexamethyldisilazane (HMDS: Huls America) in toluene for 30 min to block unreacted silanol groups [Wojceichowski, 1992]. They were then rinsed three times with toluene, washed for 1 h in chloroform to remove excess silane and 1 h in methanol before drying overnight at 90°C under vacuum. Treatment with HMDS raised some concern that the disilazane would react with the sulphonylchloride group of the sulphonated silane or affect the adsorption of the protein to this surface. To address this issue a comparison of protein adsorption to sulphonated silica glass with and without HMDS was run. No effect was found on the protein adsorption isotherms and the use of HMDS was discontinued.

4.1.1.3 Lysine Derivatization of the Chlorosulphonated Silica Glass

To produce a lysine-containing silica glass material the silylated silica glass surface was reacted with lysine. The silica glass was first treated with CSPES as described above, but omitting the HMDS blocking step. After silylation with the sulphonated silane, the tubes were immediately rinsed three times with DMF. A solution of 1 wt% Nε-tertbutoxycarbonyl(t-BOC)-lysine (Sigma, St Louis, MO) in DMF was added to the reaction vessel containing the sulphonated silica glass and reacted at room temperature for 30 min. The tubing segments were then rinsed extensively with fresh DMF and methanol and dried overnight at 90°C under vacuum. Removal of the protecting group, t-BOC was achieved by reaction of the dried silylated silica glass with 25% (v/v) trifluoroacetic acid (TFA) in dichloromethane(DCM) for 30 min. This was followed by extensive rinsing with DCM and methanol and drying overnight under vacuum at 90°C. These steps are illustrated in Figure 4.2.

The methods and materials used to attach lysine to the sulphonated silane via sulphonamide bond formation are similar to those utilized in solid phase peptide synthesis [McMurray, 1984; Stewart and Young, 1986]. Solid phase peptide synthesis was introduced in 1963 by Merrifield and its fundamental premise is that a peptide of desired amino acid sequence can be assembled from amino acids in solution while one end of the peptide is anchored to an insoluble support. This situation is very similar to the functionalization of silylated silica glass with lysine used in the present work.

In peptide synthesis, peptide bonds must be formed in a specific order and protection of functional groups that are not to be reacted is required. Carboxyl groups are frequently protected as their methyl or benzyl esters. The ε-amine side group of lysine has traditionally been protected using benzyloxycarbonyl (CBz) or substituted benzyloxycarbonyl groups [Bodansky et al, 1976; Bodansky and Bodansky, 1984; McMurray, 1984; Stewart and Young, 1986]. t-BOC is another protecting group frequently used to protect both α- and ε-amine groups. Both protecting groups have been used in this thesis, t-BOC for the model surfaces and CBz for the polyurethanes. The

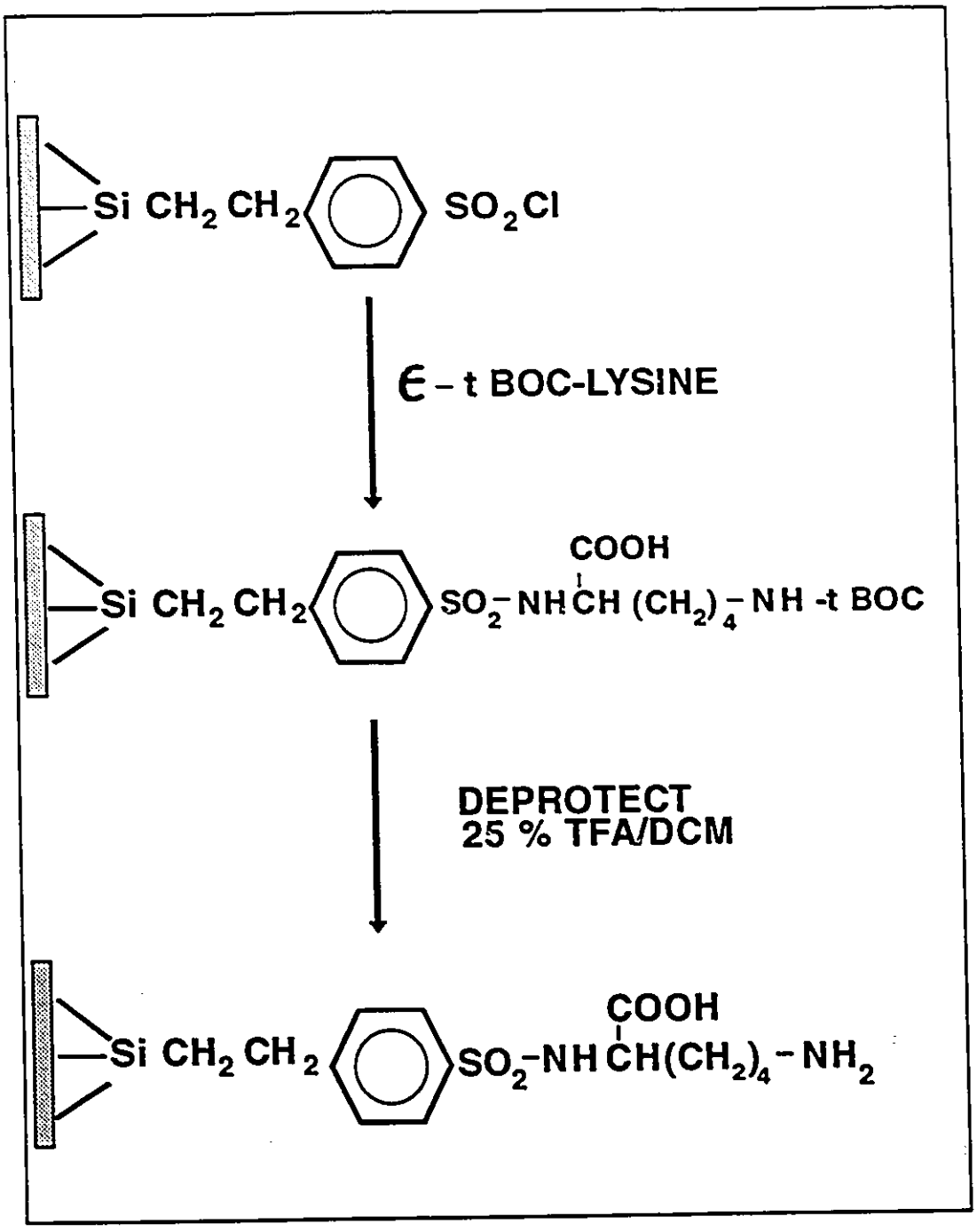


Figure 4.2: The functionalization of the chlorosulphonated silica glass surface with protected lysine. Deprotection is followed by extensive rinsing and drying overnight under vacuum at 90°C.

latter methodology is discussed in Appendix I.

t-BOC was used for the silylated materials because it was felt that the deprotecting reagent (25% TFA/DCM) would not affect the silane on the surface. This protecting group can be easily to removed under these relatively mild conditions [Bodanszky et al, 1976]. The results indicate that there is only a slight loss of silane after deprotection.

4.1.2 Physical and Chemical Characterization of the Model Silica Glass Surfaces

4.1.2.1 Contact Angles

Water contact angles were measured with a Rame-Hart goniometer (Gilmont Instruments GS-1200, Rame-Hart, USA) using the sessile drop method as described in Chapter 3 [Andrade et al, 1985]. Silica glass slides (Heraeus Amersil, CFQ TO8 commercial plate) were treated with CSPES and lysine as described above and used for the contact angle measurements. It was anticipated that changes in sessile drop contact angle would accompany and be indicative of silylation and lysine functionalization of the surfaces. Captive air-in-water bubble measurements were not taken because the model surfaces do not swell and it was anticipated that equilibrium contact angles would be rapidly achieved. The contact angle measurements were used to monitor the reaction protocol as well as to characterize the surfaces.

4.1.2.2 X-ray Photoelectron Spectroscopy

X-ray photoelectron spectroscopy (XPS) is a method that may be used to characterize the outermost 10 to 200Å of a surface. In XPS a sample is placed in a high vacuum (10^{-7} to 10^{-10} torr) and bombarded with monochromatic X-rays which will interact with the electrons in the sample. Through this interaction photoelectrons are emitted from the material and an analyzer determines the kinetic energy of the exiting electrons. The kinetic energies of the emitted photoelectrons can be related to chemical elements and their bonding environment. The intensity of the emission indicates the concentration of the chemical species.

Photoelectrons have a limited mean free path in a solid. Changing the angle of the detector relative to the sample surface allows electrons emitted from different depths within the sample to be detected. The take-off angle (θ) is defined as the angle of the sample from the perpendicular relative to the detector. Photoelectrons emitted from a depth equal to the cosine(θ) times the mean free path (or less) of the electrons will be detected. A take-off angle of 90° results in the lowest sampling depth and provides data for the outermost region of the surface. Thus using a series of take-off angles it is possible to obtain chemical information as a function of depth into the material.

Data were obtained for the silica glass tubing materials used in the adsorption studies. These tests were run at Surface Science Western, University of Western Ontario, London Ontario using a Surface Science Laboratories SSX-100 X probe spectrometer with a monochromatic aluminum source. The surfaces were analyzed for the presence of N,O,C,Si, and S at a take-off angle of 60°. Survey scans were run from 0 to 1000 eV. The binding energies were referenced to the carbon 1s peak for carbon bound to hydrogen with a binding energy of 284.6 eV. High resolution scans were run on carbon 1s and oxygen 1s for all surfaces, and on sulphur 2p and nitrogen 1s where appropriate. The high resolution scans were fit using a Surface Science Instruments curve fitting package based on the minimum Chi squared calculation using 80% Gaussian criteria.

4.1.2.3 Ninhydrin Analysis

A qualitative chemical method which could be used to monitor the lysine attachment and deprotecting reactions was desired. Ninhydrin (2,2 dihydroxy-1,3-indandione) is a pale yellow powder which gives a yellow aqueous solution. When ninhydrin reacts with primary amine groups it changes colour from yellow to purple (referred to as Ruhemann's purple after its discoverer), and is a commonly used analytic test for determining the presence of amino acids in solution [Stewart and Young, 1986]. With respect to lysine it should be noted that ninhydrin is known to react with both the α-amine and the ε-amine groups [Kemp, 1979].

Silica ground glass lenses of diameter 6 cm were silylated and derivatized with lysine using the protocol described above with the exception of the HMDS blocking treatment, which was omitted. Samples with a large surface area were used so that the colour development could be readily observed. Following the method of Kemp [1979], a 1% solution of ninhydrin reagent (Sigma) in ethanol was sprayed on the lenses which were then placed in an oven for 5min at 100°C. The samples were removed from the oven and colour development was observed.

4.1.2.4 Bolton-Hunter Analysis

Radiolabelled Bolton-Hunter reagent, 3-(4-hydroxyphenyl)propionic acid N-hydroxysuccinimide ester (BHR) has been used to label proteins [Bolton and Hunter, 1973], hormones [Thompson et al, 1987], and silanes containing amine groups on glass surfaces [Van Damme, 1990]. The ester acylates primary amine groups in the protein or other target molecules. This reaction is illustrated in Fig 4.3.

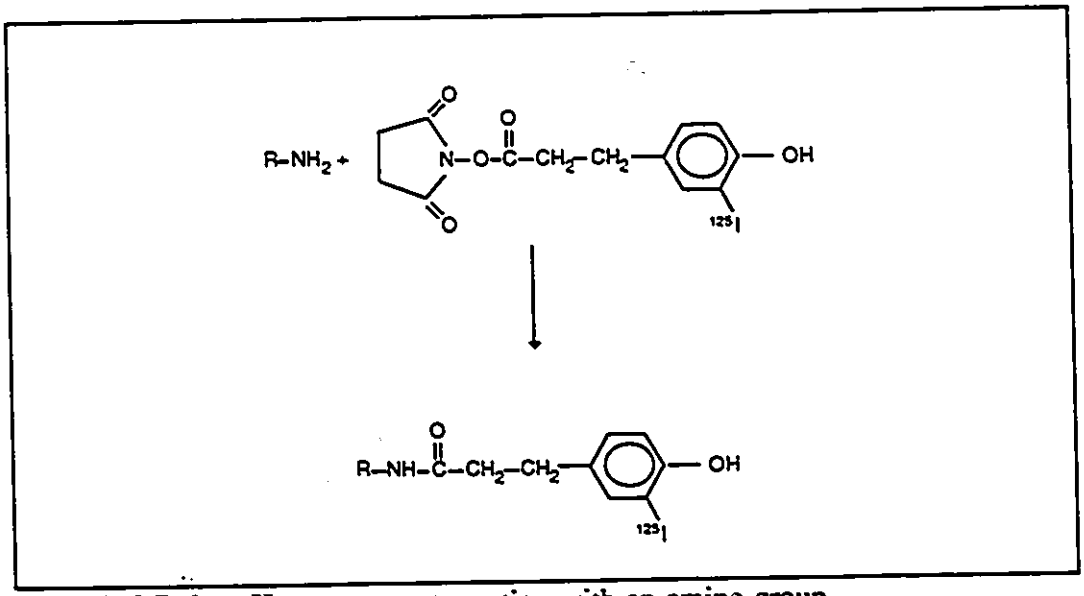


Figure 4.3:Bolton-Hunter reagent reaction with an amine group.

A water soluble form of BHR, sulfosuccinimidyl 3-(4-hydroxyphenyl) propionate (S-BHR)[Pierce Chemical Co. Rockford.Ill.) was used in the present work. S-BHR was radiolabelled following the method of Thompson et al [1987]. To iodinate S-BHR, 10 µL of a solution of S-BHR (1 mg/mL) in anhydrous dimethyl sulphoxide (DMSO) was placed in a 1.5 mL vial. To this solution the following reagents were added quickly and in sequence: 1 mCi $Na^{125}I$, 10 µL of a 5 mg/mL solution of chloramine T (Sigma) in 0.4 M sodium phosphate buffer, pH 7.4; 100 µL of hydroxyphenylacetic acid (1mg/mL in distilled water); and 10 µl of a solution of sodium metabisulphite (Sigma) (12 mg/mL in 0.05 sodium phosphate buffer, pH 7.5) [Thompson et al, 1987]. This solution was then added in is entirety to 5 mL of a 2 mg/mL solution of unlabelled S-BHR in 0.4M sodium phosphate buffer, pH 7.5 and mixed [Thompson et al, 1987; Van Damme, 1990].

The final mixture was added in 1 mL quantities to each of 5 vials containing sulphonated and lysinated silica glass tubes and allowed to react for 30 min. Each sample was then rinsed 3 times with 3 mL of 0.1M sodium phosphate buffer and counted for radioactivity. Duplicates of each test material were run simultaneously.

It is important to note that since both BHR and S-BHR are susceptible to hydrolysis, the labelling procedure should be completed in less than 8 min and the reagent should then be used immediately. In addition, solvents should be as free of water as possible.

4.1.3 Plasma Proteins and Plasma

The sources, purification methods and ^{125}I labelling methods for plasminogen were the same as described in Chapter 3. Plasma for adsorption experiments was again obtained from the Canadian Red Cross.

Human fibrinogen was purchased from Kabi (Stockholm, Sweden). The protein was dialysed overnight in either 0.1M phosphate buffer, pH 7.2 for labelling or isotonic Tris, pH 7.4. The fibrinogen was then aliquoted and stored frozen at -40° C until use.

4.1.4 Adsorption Experiments: Single protein system

The adsorption experiments described in Chapter 3 used segments of tubing 11 cm in length by 0.22-0.25 cm I.D. It was determined that these experiments were excessively consumptive of plasma and plasminogen, requiring approximately 12 mg of plasminogen per adsorption experiment. Therefore a procedure requiring much smaller protein volumes was developed using small fluid reservoirs (the wells of tissue culture plates) to contain the protein solution, and with smaller silica glass tubing segments immersed in the solution. The method was developed after extensive investigation into the rinsing methods, means of introducing the radiolabelled solution to the samples, and surface cleaning methods.

In the final procedure, 96-well polystyrene flat bottom tissue culture plates (Falcon 3072, Becton Dickinson, N.J., USA) were used. Immediately prior to the adsorption experiment, silica glass, sulphonated silica glass, and lysinated silica glass tubing segments, 0.25 cm I.D by 0.7 cm in length, described in section 4.1.1, were cleaned for 10 min in HPLC grade methanol (BDH) and rinsed extensively with distilled water. One tubing segment was then placed in each well and the samples were equilibrated for 30 min at room temperature in 280 μL of Tris buffer, pH 7.4, the medium used in the adsorption experiments.

Tubing segments of a given surface were exposed under static conditions for 3 h to plasminogen solutions of different concentration containing 3-5% ^{125}I-labelled protein. The protein solution was introduced into the well containing the test segment by first removing the equilibrating buffer with a pasteur pipette and then rapidly adding 280 μL of radioactive protein solution to the well. A fluid was thus maintained on the test surface at all times and the creation of a solid-solution-air interface, which can introduce complications such as Langmuir-Blodgett film transfer and protein denaturation, was avoided.

After adsorption the tube segments were rinsed twice by removing the sample from the radiolabelled protein solution using nylon tweezers and placing it successively

in two wells containing fresh Tris buffer for approximately 30 s. The sample was gently mixed in the solution during this time. This rinsing procedure was established after considerable experimentation and was considered optimum to prevent loss of loosely held protein on the one hand and to remove solution protein on the other. The silica glass segments were then counted for radioactivity in a gamma counter.

Data points at a number of concentrations were studied for all three surfaces (silica glass, sulphonated and lysinated silica glass) in each experiment. Replicates were also included in each experiment and five experiments were run in total. The plasminogen concentrations studied and the number of replicates at each concentration were randomized. The randomization was weighted so that the bulk solution concentrations studied emphasized the plateau and low concentration regions of the isotherm.

4.1.4.1 Adsorption Studies in the Presence of ε-Amino Caproic Acid

These experiments were carried out in exactly the same manner as just described, except that ε-amino caproic acid was present in the plasminogen/buffer solution at a concentration of 0.02M, and side-by-side comparison isotherms (pure plasminogen and plasminogen with EACA) were run simultaneously on each surface. The concentration of EACA is the same as that used as an eluent in affinity chromatography.

4.1.4.2 Desorption Experiments

The reversibility of protein adsorption is an important aspect of the study of biomaterial interactions with biological systems. One approach to determining the reversibility of protein adsorption is to attempt to displace the protein from the material surface after adsorption has occurred. This may be done by several methods including desorption against buffer or active displacement by competing species. Both of these methods were used in this work.

The desorption experiment against pure buffer were run using the 96-well plates in a manner similar to the adsorption experiments described above. Plasminogen

adsorption to 3 samples of each model surface was first carried out at a plasminogen concentration of 0.1 mg/mL in isotonic Tris, pH 7.4 for 2 h. Desorption was then initiated by placing samples in wells containing fresh Tris buffer, pH 7.4 for 5 min, rinsing once, and counting the surface radioactivity. The same sample was then placed in another well containing fresh isotonic Tris and desorption was continued. In this manner, each individual sample was sequentially exposed to desorbing buffer for contact times ranging from 5 min to 3 h. This experiment was repeated twice and thus 6 replicates were obtained for each surface. Additional desorption experiments were run in a similar manner with 0.02 M EACA in the buffer.

4.1.5 Adsorption Experiments: Plasma System

Plasminogen was labelled with ^{125}I by the lactoperoxidase method as described in Chapter 3. ^{125}I-labelled plasminogen was added to whole plasma in amounts corresponding to 5 to 10% of the normal plasma pool.

Adsorption experiments from plasma were conducted in a manner similar to that described for the single protein experiments. Tubing segments of pure silica glass, sulphonated silica glass and lysinated silica glass were cleaned as previously described and equilibrated in 96 well polystyrene tissue culture plates for 30 min at room temperature in isotonic Tris buffer, pH 7.4.

After equilibration, the buffer was rapidly removed from the well and replaced with a plasma solution. For a given experiment, the tubing segments were exposed under static conditions for 3 h to plasma of different concentrations obtained by diluting whole plasma with isotonic Tris buffer, pH 7.4. Following adsorption the samples were rinsed twice by immersing the sample in separate wells containing fresh isotonic Tris, pH 7.4 as previously described. The samples were then counted for radioactivity.

A total of twelve samples per surface were run in each experiment at plasma concentrations from 0.02% to 30% normal plasma. Replicates were run at several different plasma dilutions and three separate experiments were completed.

4.2 RESULTS AND DISCUSSION

All statistical methods in this chapter and in Chapter 5 used Minitab Statistical Software [Minitab, Release 7, Minitab Inc., State College, PA, USA] for the calculations unless otherwise stated.

4.2.1 Contact Angles

The contact angle data are given in Table 4.1. The silica glass and sulphonated

SURFACE	CONTACT ANGLE SESSILE DROP (Degrees ± SD)
Silica glass	46.6 ± 1.9
Sulph silica	44.4 ± 2.4
Lys silica	53.9 ± 2.6

Table 4.1: Contact angles of water on the model surfaces determined by sessile drop method. Sulph silica refers to the silica glass silylated with CSPES, Lys silica refers to lysine derivatized surface.

surfaces have effectively the same contact angle and are essentially hydrophilic. The lysinated surface shows a significantly greater contact angle and is therefore more hydrophobic. This is similar to the trend found for the polyurethanes discussed in Chapter 3. The unexpectedly high contact angles for the silica glass are likely due to the rapidly adsorbed hydrocarbon layer found on this high energy surface after even brief exposure to the air [Phillips and Hercules, 1985]. Phillips and Hercules [1985] found that acid leached silica glass slides contained carbon contamination from the leaching solution and the air. They also found that these carbon species were difficult to remove. After storage the surfaces also included organic carbon species and the extent of this organic contamination increased with time. This phenomenon is also found in the data presented here. It was found to be virtually impossible to prepare " totally clean" silica glass.

4.2.2 Ninhydrin

Only qualitative results were obtained from the ninhydrin analysis for the lysinated and sulphonated silica glass. The sulphonated surface gives no reaction with ninhydrin, i.e. no purple colour was observed. The lysinated material, when treated with ninhydrin, acquired an uneven pattern of purple areas with colour-free areas between. This pattern suggests partial conversion of the sulphonyl chloride groups with lysine. In addition it appears to indicate submonolayer coverage of the derivatized silane.

4.2.3 Bolton Hunter Analysis

Typical results for the sulphonated BHR experiments are shown in Table 4.2. As can be seen in this table, S-BHR is not specific for reaction with amine groups on the silylated surfaces. The reagent appears to also react with the silane possibly through the sulphonic acid. As can also be seen, there is some nonspecific adsorption of the reagent onto the pure silica glass material, although it is small compared to S-BHR binding to the sulphonated and lysinated silica glass. Van Damme [1990] used BHR, with success, to

SURFACE	SAMPLE	CPM [1]	MEAN CPM	MEAN CPM AFTER [2] WASHING	% CPM REMAINING
Sulph silica	1	64880	59512	39570	66.5
	2	54140			
Lys silica	1	51070	53500	40224	75.2
	2	46930			
	3	62500			
Silica glass	1	4130		670	14.0

1 CPM= counts per minute
2 The samples were exposed to Tris buffer, pH 7.4 for 72 h

Table 4.2: Typical data for the reaction of sulphonated Bolton-Hunter reagent with silica glass, lysinated silica glass and its precursor sulphonated surface.

determine the amount of an amine containing silane on the surface of glass. However the silanes used in his work did not contain either aromatic groups or sulphonic acid groups as is the case here. Numerous experiments were conducted with both the sulphonated and unsulphonated Bolton-Hunter reagents to determine reaction conditions which would give specificity toward the amine groups. No such conditions were found.

Table 4.2 also shows data after extensive exposure of the surfaces to buffer. In this portion of the experiment the samples were incubated in Tris buffer, pH 7.4. for 72 h after reaction with the sulphonated Bolton-Hunter reagent. This was done for two reasons: first to desorb nonspecifically bound radioactivity and thus obtain an estimate of

specifically bound S-BHR, and secondly to determine if the silanes were removed from the surface in aqueous buffer. Loss of the silanes in aqueous solutions has been reported [Scouten, 1985] and a significant amount of silane loss would be detrimental to the protein adsorption studies. The Bolton-Hunter reagent nonspecifically adsorbed to the pure silica glass surface is readily desorbable as shown in Table 4.2. If one assumes that the radioactivity is associated with the silane then the data in Table 4.2 indicate that there is likely to be minimal loss of silane from the silylated surfaces over the three hour time period used in the adsorption experiments. In addition, the amount of radioactivity associated with the samples was reasonably reproducible for each surface and across different sample preparations. This suggests that the silylation method used (again assuming that S-BHR reacts with the silane either through the sulphonic acid or the amine) gave a reproducible surface.

It is unfortunate that this method could not be used to quantify the lysine on the model lysinated surface. Other methods of quantification were considered, including the biotin-streptavidin reaction. The biotin-streptavidin interaction is the strongest known noncovalent interaction between a protein (avidin or streptavidin) and a ligand (biotin) [Green, 1975] and is frequently used as a detection and protein purification method [Bayer and Wilchek, 1980].

Avidin is a basic glycoprotein found in egg white. Streptavidin is similar in structure to avidin but contains no carbohydrate [Green, 1975]. Biotin is a vitamin found in tissue and blood. It interacts rapidly with either avidin or streptavidin and once formed the complex is unaffected by extremes in pH, or organic solvents [Green, 1975]. To accomplish detection of a protein using the biotin-streptavidin reaction, the protein is first biotinylated (biotin is inserted into the protein via reaction with an amine) followed by incubation with avidin or streptavidin. Streptavidin can be conjugated with markers including fluorochromes, horseradish peroxidase and ^{125}I, and the appropriate detection system used to determine the amount of amine present. For example, streptavidin has been radiolabelled and the biotin-^{125}I-streptavidin conjugate used to enhance tumour

images [Oehr, 1988].

Although it is possible that the biotin-avidin interaction could be adapted to the determination of lysine bound to silica glass, a number of difficulties would have to be overcome. For example, biotinylation of proteins requires an activated form of biotin such as hydrosuccinimide ester-biotin and the key reaction is between the succinimide ester and amine groups similar to the Bolton-Hunter reagent.

An alternate method of determining the lysine content at the surface would be to derivatize the sulphonated silane using a dipeptide of lysine and tyrosine. The tyrosine could then be radiolabelled with ^{125}I using one of several labelling methods. Use of the dipeptide in this manner would have to be controlled for its effect on the plasminogen adsorption and would likely require further modification of the derivatization method. This method could yield quantitative data if successful.

No attempt was made in the present work to use either the biotin-streptavidin reaction or to attach tyrosine to the lysine. The present work relies heavily on the XPS data to confirm the silylation and lysine derivatization methods.

4.2.4 X-ray Photoelectron Spectroscopy

The results for the model surfaces are shown in Table 4.3. The silica glass surface contains only oxygen, carbon and silicon in the atomic ratio 4.5:1:2.3. The oxygen to silicon ratio is approximately 2:1 as expected for SiO_2. The presence of carbon indicates some impurities on the material surface. Examination of the high resolution carbon 1s spectra (Figure 4.4, Table 4.4) reveals some details about the nature of these carbons. The predominant form of carbon bonding found in the contaminants is C-H at 281.27 eV and represents 55% of the C1s peak. The spectra also indicate the presence of C-OH (282.03 eV), C-O (283.10 eV) and carboxyl groups (283.62 eV). The peak at 285.85 eV is a satellite peak. Unfortunately the presence of these contaminants makes the C1s spectra of the silylated materials more difficult to interpret. The data also indicate that the silica glass surface is free of contamination from the cleaning process,

SURFACE	ELEMENT (ATOM %)				
	O	N	C	S	Si
Silica glass	53.20	—	12.80	—	29.00
Sulph Silica	39.90	0.80	33.40	2.00	23.90
Lys-tBoc Silica	50.10	1.30	21.40	1.20	26.00
Lys Silica	48.60	0.90	22.90	1.90	25.70

Table 4.3:XPS data for the model surfaces at a take off angle of 60°.

since N, Cl, and Na were not detected.

In the sulphur-containing surface (sulph-silica), the presence of sulphur is indicative of the silane. The presence of nitrogen indicates the hexamethyldisilazane used to block the unreacted OH groups at the surface. The silane and silazane contribute a large proportion of the carbon seen on this surface. The ratio of carbon to silicon has changed to 1:0.7 compared to a ratio of 1:2 found in the pure silica surface. Once again, the balance of the carbon content is likely due to contaminants. The high resolution C1s spectrum for this surface (Figure 4.4) shows an increase in the percentate of C-H bonding as would be expected with silylation. Carbon bonded to OH groups constitutes 25% of

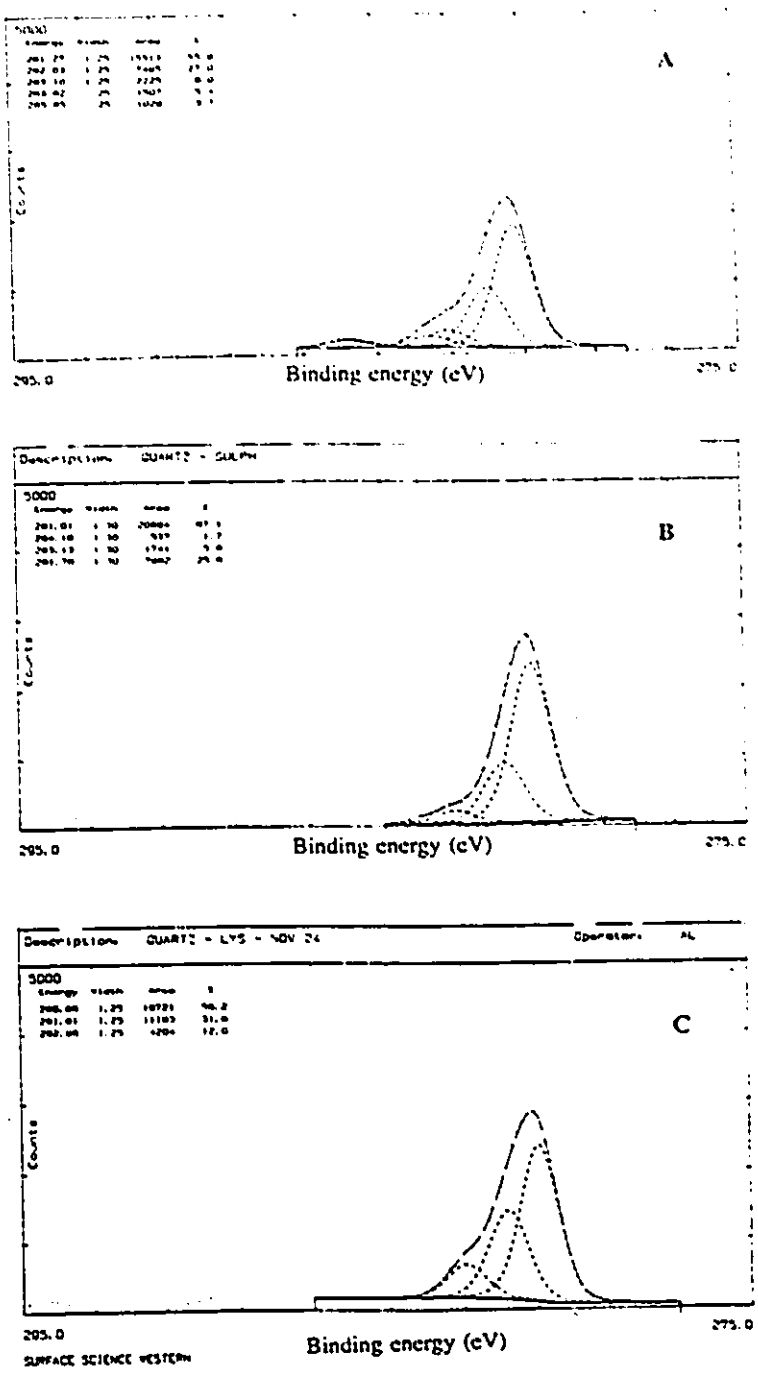


Figure 4.4: High resolution C1s XPS scans for: A) silica glass B) sulphonated silica glass C) lysinated silica glass.

SURFACE	CARBON BONDING (% OF C1s spectrum)			
	C-H	C-OH	CARBONYL	CARBOXYL
SILICA GLASS[1]	55.9	27.0	8.0	5.4
SULPH SILICA[1]	67.1	25.6	5.6	—
LYS SILCIA	56.2	31.9	12	—

[1] The unaccounted percent corresponds to noise.

Table 4.4: High resolution spectra for carbon 1s of the silica glass, sulphonated silica glass and lysinated silica glass.

the carbon spectrum while the carbonyl and carboxyl contributions are small.

In the lysinated surface before deprotecting (Lys-tBoc silica) there is approximately one nitrogen atom for every sulphur indicating that not all of the sulphonated silane available was derivatized, since a 2:1 ratio would then have been obtained. It would appear that approximately 25% derivatization was achieved. The oxygen to carbon ratio increased to 2.3:1 with derivatization, also indicating the presence of the protecting group. After deprotecting (lys-silica) the nitrogen content is reduced by approximately 30%. This is likely caused by loss of some lysine from treatment with trifluoroacetic acid.

A comparison of the carbon 1s spectra for the sulphonated and lysinated materials shows several significant trends (Figure 4.4 and Table 4.4). The most significant result is the lack of a carboxyl peak for the derivatized material. The presence of this peak was

anticipated with the addition of the lysine. The lysinated surface also shows an increase in the carbonyl carbon content compared to the sulph-silica, which may indicate the presence of residual protecting group. Incomplete deprotecting may also be indicated by the relatively similar oxygen:carbon ratios of the lysinated material before and after deprotection (2.3:1 versus 2:1). However the background contamination makes the data difficult to interpret. The increase in the carbonyl peak may also indicate reaction of the carboxyl group possibly with the sulphonyl chloride although this would appear unlikely because of the presence of the amine. Unfortunately high resolution scans for the lysinated material before deprotection, which would have allowed a more direct comparison to the lysinated material, could not be obtained because the samples were subject to charging during processing.

The question of whether the silylation method used to functionalize the silica glass resulted in multilayer, monolayer or submonolayer needs to be considered. Wojciechowski [1992] evaluated three silylated silica glass surfaces at different take-off angles in an attempt to determine the thickness of the silane layer. He found that the layer thickness was similar in size to the silane molecule and concluded that multilayering had been avoided in his experimental system. The silylation methods, silanes used and results obtained in this work are similar to those of Wojciechowski and it is reasonable to assume that monolayer to submonolayer coverage has been achieved. In addition, Phillips and Hercules [1985] in their extensive work on organosilane layers on silica glass, estimated that one sixth of the XPS signal was contributed by a silane monolayer. This approximation was obtained from models and the authors chose to use this assumption rather than attempting to deconvolute silicon lines from the silane and the bulk material. Using this assumption, the XPS data for the silylated silica glass indicates submonolayer coverage. This result was anticipated given the ninhydrin data for the lysinated surface.

The XPS data were also used to obtain the number of silane molecules present on the silica glass surface. This was done in anticipation of modelling the silane at the surface (described in detail later in this chapter) and is an attempt to obtain more

quantitative information about the silane layer. Some assumptions must be made about the underlying silica glass structure to account for its contributions to the XPS signal. The assumptions made are based on the prevailing theories of glass structure.

Two dominant theories of glass structure have been proposed over the last 50 years, the Zachariasen-Warren hypothesis and Lebedev's crystallite theory [Vogel, 1971; Goodman, 1987]. The former theory considers the SiO_4 tetrahedron to be the smallest building unit of glass but that these units are not symmetrically connected in a network as in the parent material, quartz. A comparison of the proposed glass structure according to this hypothesis with that of quartz is given in Figure 4.5. In the second theory, Lebedev considered glass to made of many small microcrystalline structures of SiO_2. Until recently, these two theories were considered to be at odds, however Goodman [1987] has proposed that a combination of the two may explain many of the characteristics of glass.

In attempting to define a model for glass and silica glass in particular, a good deal of physical information has been obtained about bonding in the material. The average interatomic distances have been found to be 1.6 ± 0.05 Å for the Si-O bond and 3.0 ± 0.05 Å for the Si-Si bond [Porai-Koshits, 1966]. To obtain an estimate of silane coverage on silica glass the surface has been modelled as a network of contiguous equilateral triangles with the length of the side equal to the Si-Si bond length. Equilateral triangles were used in order to facilitate the calculations. These are discussed in more detail later.

To estimate the amount of silane on the surface from the XPS data the following assumptions were made: 1) The sampling depth of the XPS experiment was 30Å [Tanuma et al, 1991]. 2) A pure silica surface contains 34 atomic % Si (carbon contamination discounted). 3) The average Si-Si bond distance in an amorphous silica glass structure is 3Å as discussed above. 4) The silica surface contains layers 3 Å deep [Porai-Koshits, 1966; Vogel, 1971] (Si-Si bonding) and these layers are stacked one on top of each other. These assumptions can be used to estimate the silica contribution to the signal from the

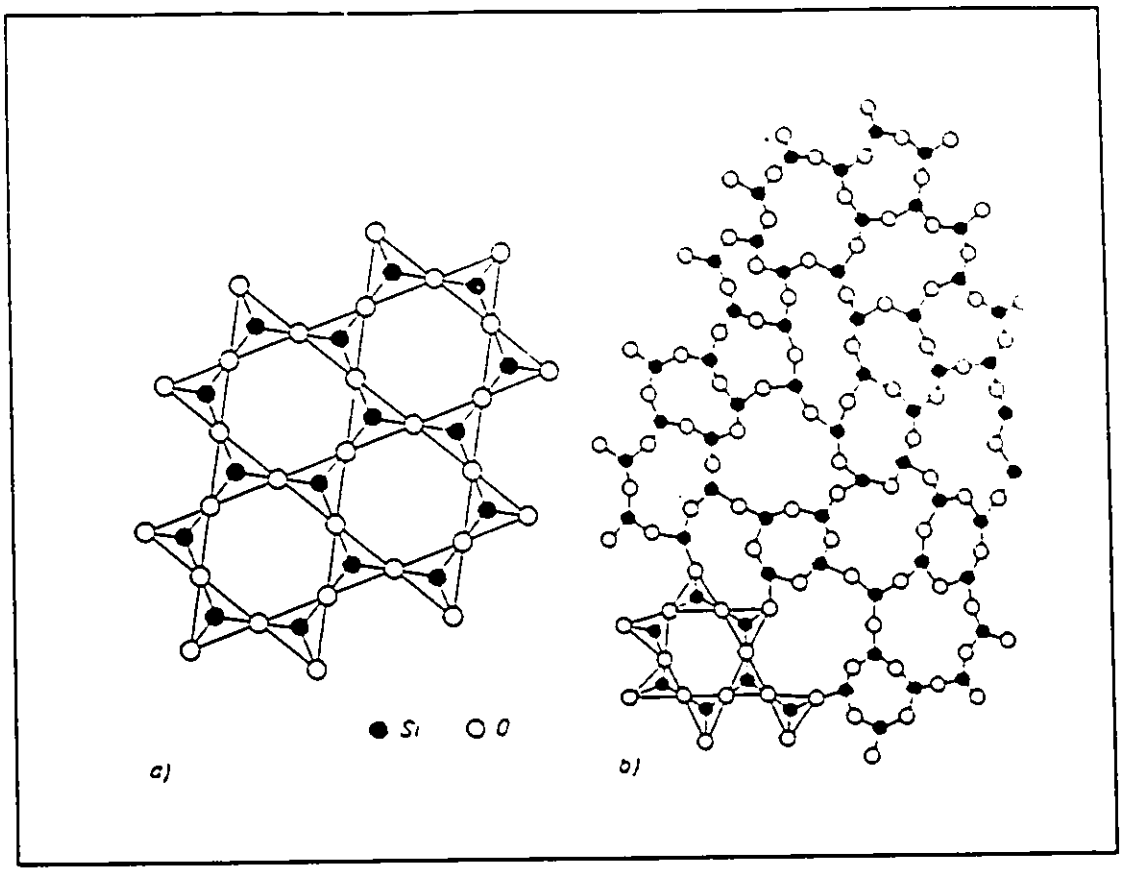


Figure 4.5: Diagrammatic representation of SiO_2 structures in a cross-section through the $[SiO_4]$ tetrahedra spatial networks described by the Zachariasen-Warren hypothesis. Three oxygen atoms are represented in each $[SiO_4]$ tetrahedron. The fourth oxygen atom is either above or below the plane of the diagram. All $[SiO_4]$ tetrahedra are cross-linked by oxygen atom bridges. A) Represents the regular ordered $[SiO_4]$ tetrahedra in crystallized silica., B) Represents the irregular 3-dimensional $[SiO_4]$ tetrahedra in amorphous silica glass according to Zachariasen and Warren.

bulk silica material.

The next step was to obtain the number of silane molecules sampled by the XPS spot used to generate the elemental analysis. It is here that the geometric characteristics of equilateral triangles are employed. The area evaluated by the XPS instrument can be approximated by a hexagon (suggested also because of the geometric arrangement of the

SiO_4 tetrahedra in the glass and quartz structure). The XPS spot area was therefore modelled as a hexagon with a "diameter" equal to the diameter of the circular spot area. Modelled in this manner the six vertices of the hexagon intersect the circle, and the hexagon can be divided into 6 equilateral triangle with sides equal in length to the radius of the circle, as illustrated in Figure 4.6.

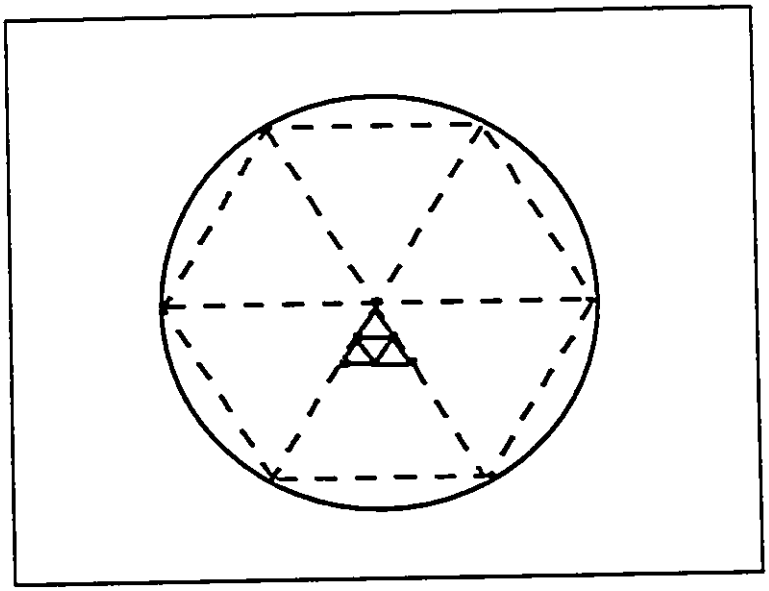


Figure 4.6: A representation of the method used to calculate the number of silane molecules on the surface of the silica glass. The spot area is modelled as a hexagon which contains 6 equilateral triangles with sides of length equal to the radius of the spot. The number of silanes is obtained by finding the number of equilateral triangles of side equal to the Si-Si bond length that can be fit into the larger equilateral triangles. One silane molecule is represented by a vertex of the smaller equilateral triangles.

It is a characteristic of equilateral triangles that a number of smaller equilateral triangles can be fit into a larger triangle. The number of smaller triangles in the larger triangle is proportional to the ratio, n, of the sides of the large and small triangles. If we assume that one silane molecule is located at each vertex of the smaller triangle network then by simple geometric considerations:

$$number\ of\ silanes\ in\ (XPS\ spot\ area) = 6[\frac{n(n+3)}{2}+1]$$

$$where\ n = \frac{radius\ of\ XPS\ spot}{silane\ separation\ distance}$$

This expression reduces to $3n^2$ when n is large.

Calculation of the number of silane molecules at the surface based on the XPS data, then proceeded in the following manner. Using the expression above, the number of silicon atoms in one "layer" of the silica glass substrate was obtained. The penetration depth was assumed to be 30Å and the number of "layers" in the sample was therefore 10. The single layer value was then multiplied by ten to obtain the total number of silicon atoms sampled in the experiment and this number was assumed to correspond to 34 atom % Si. The silicon content contributed by the silane was taken as the difference in the atomic % values between the XPS data and the theoretical 34% value. The contribution made by the silane was assumed to be equivalent to the atomic % sulphur. Using these assumptions the number of silane molecules on the sulphonated surface was calculated as 6.73×10^{10} for an XPS spot with a diameter of 1000 μm. This is equivalent to 8.5×10^4 silane molecules per μm^2. Assuming a derivatization of the sulphonated silane with lysine of approximately 25%, this would give approximately 2.1×10^4 lysine residues per μm^2 of silica glass surface.

These results were compared to the data of Massia and Hubbell [1991] for a comparable system. In a study using radiolabelled peptides attached to silylated glass substrates, they obtained a surface concentration of 7.3×10^4 per μm^2 and calculated a spacing between molecules of 4 nm. Using the method of calculation described above a separation distance of 3.6 nm is obtained for their data. The results from the two methods are thus in reasonable agreement and would indicate that the method used to calculate the silane contribution from the XPS data is appropriate.

4.2.5 Estimation of the Surface Concentration of Silane by Molecular Modelling

In the previous section, the amount of silane on the silica glass surface was estimated from the XPS data using several assumptions about the underlying silica glass structure. The number obtained appears to be in reasonable agreement with the results of other authors but the question still remains whether this number constitutes a monolayer of silane. To obtain an estimate of the maximum possible overall silane coverage on the silylated silica glass a chemical modelling approach was used.

It should be noted that this is in no way meant to be a detailed attempt at modelling the silane interactions with the surface but is simply a technique to give a rough estimate of the maximum number of silanes which could be contained in a monolayer, based on the chemical structure of the silane.

The modelling approach is based on molecular mechanics. In molecular mechanics a molecule is assumed to be a collection of atoms held together by forces which can be described by potential energy functions associated with structural features such as bond angles [Boyd and Lipkowitz, 1982]. The combination of these potential energy functions is a force field which describes the ideal state. The energy, E (often termed the steric energy), of a molecule in the force field results from deviations from "ideal" structural features and is approximated by a sum of energy sources. The contributing energy sources include bond stretching, van der Waals forces, rotational energy etc, and are additive. E is the difference in energy between the real molecule and a hypothetical molecule (estimated from experimental data) where all the structural values are ideal. E is therefore a measure of the intramolecular strain in the molecule relative to an "ideal" state and has no meaning by itself.

In a molecular mechanical modelling calculation using one of the available software packages, a force field is constructed by the computer using ideal values for the potential energy functions obtained from experiment. When a new chemical structure is then input, an initial E is calculated based on the new input and one of several gradient search methods is used to minimize E. Molecular mechanics can be used to predict

reaction pathways and chemical structures and is significantly faster than the quantum mechanical approach [Boyd and Lipkowitz, 1982]. Many computer programs are now available to do these calculations. PC Model by Serena Software [Bloomington, Ill. USA] has been used in this work.

In these calculations, E, the steric or overall energy, and the van der Waals energy contributions to E were used to estimate the minimum separation distance that two silane molecules on a plane surface could achieve before the repulsive interactions between the two prohibit closer contact. The minimum distance obtained was then translated into the number of silane molecules on the surface using a calculation analogous to that discussed in the previous section.

The modelling was done in two stages and is illustrated in Figures 4.7 to 4.11. In the first stage, the silica glass was modelled as a network of interconnecting cyclohexane rings. Cyclohexane was chosen to represent the silica glass because of the six-membered ring structure formed by the tetrahedron substructures in silica glass (illustrated in Figure 4.7) . The C-C bond angles in cyclohexane (120°) are reasonably close to the average Si-Si angles in silica glass (148°) [Porai-Koshits, 1966]. In addition, use of the cyclohexane ring structure simplified the computation and eliminated the need for using two separate computer programs. Before the second step of the modelling exercise was undertaken, the energy of this cyclohexane surface was minimized. This resulted in a rigid, flat structure.

In the second step, four sulphonated silane molecules were constructed and covalently "attached" to carbons in the "silica " surface. These silanes are represented by O, R, V, and P in Figure 4.7 and by the orange, red, green, and purple in Figures 4.8-4.11.

These four molecules were placed close together on the surface at distances that restrict their movement away from each other and force silanes R and V to remain relatively stationary. A fifth silane molecule (white in Figures 4.8-11) was then "walked" toward the group of four molecules by sequentially attaching it to different carbons in the

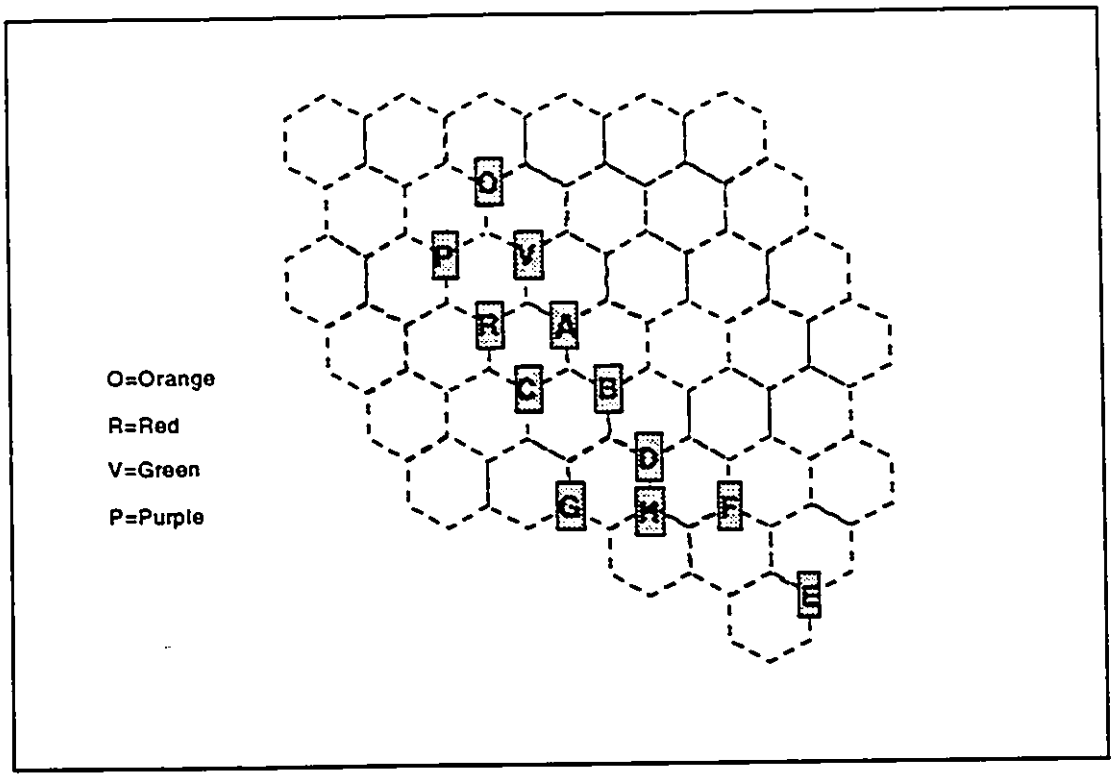


Figure 4.7 The surface and the points of attachment of the silanes to the surface are illustrated. The surface is represented by the hexagons. The first four silanes are in fixed positions. The letters A-H show the changing placement of the fifth silane. Energy calculations were done at each of these positions.

cyclohexane rings of the surface.

At each position, E, the steric energy and the van der Waals energy were calculated. These two energy functions were used because the they were felt to provide the most appropriate criterion by which to determine the effect of distance on the overall energy of interaction between the silanes. The van der Waals energy is an intermolecular term and is strongly influenced by the distance between molecules. The surface and the points of attachment of the silanes are illustrated in Fig 4.7.

At some point, as the "white" silane approached the other molecules, the energy E and the van der Waals energy rapidly increased. At this point the energy barrier was

considered too high and the limit of closest approach of the silanes to each other was considered to have been reached. Using this method the closest distance of approach was estimated to be about 5.3 Å for a close packed monolayer, and the greatest radius that a freely moving, unrestricted silane would sweep out would be 11 Å.

Figures 4.8 to 4.11 further illustrate the modelling process. These figures are in two parts. The first part gives a profile view and the second a plan view of the system. In Figures 4.8 and 4.9 the white silane is placed in a position corresponding to D in Figure 4.7. Figures 4.10 and 4.11 show the results for a silane placed in a position corresponding to location E in Figure 4.7.

The model predicts, purely from a chemical perspective, that a close packed monolayer with a minimum distance of 5.3 Å between each silane gives a coverage of 2.67×10^{12} silane molecules on the XPS spot. The model gives a result for unrestricted attachment of the silane to the surface and is not based on the availability of OH groups. Reaction of the silane with all the available OH groups would result in 1.56×10^{11} molecules on the silica surfaces [Hair,1986]. Therefore, the model predicts that using this silane, a reasonably close packed monolayer could be achieved by reaction with the OH groups and would not be sterically hindered. The model also indicates that the silane on the silica surface studied here is at submonolayer concentrations. At these concentrations the silane, from a fixed position on the surface can sweep out a circle of radius 11Å. The exact implication of this relatively unrestricted movement by the silane and its effect on the reaction of lysine is unclear.

Extensive modelling of the lysine derivatized silane was not undertaken. The closest separation distance for the lysine derivatized molecule is expected to be greater than 5.3Å and the unrestricted molecule would sweep out an circle of greater than 11 Å in radius. However given the relatively low concentration of lysine residues these dimensional considerations are not likely to constitute limitations on the reaction.

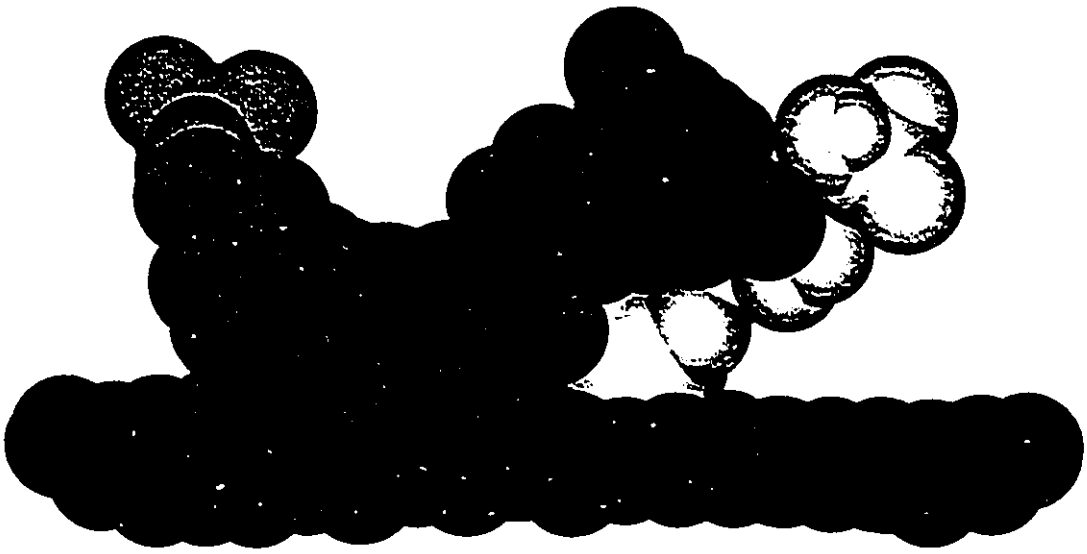


Figure 4.8: Profile view of the silanes on the silica glass surface with the white silane in position D shown in Figure 4.7.

Figure 4.9: Plan view of the silanes on the silica surface with the white silane in position D shown in Figure 4.7.

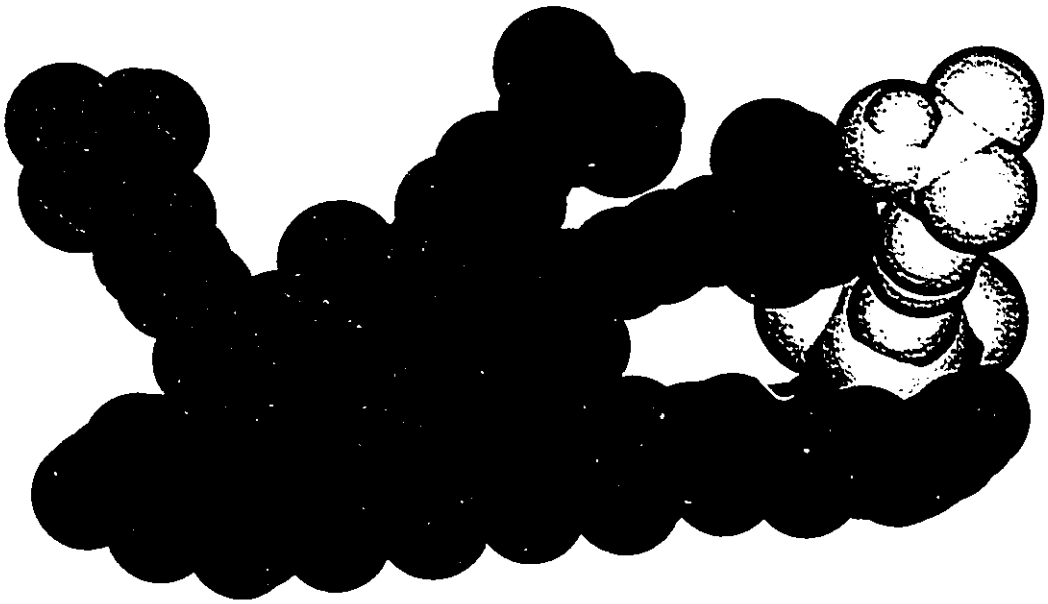


Figure 4.10: Profile view of the silanes on the silica glass surface with the white silane in position E shown in Figure 4.7.

Figure 4.11: Plan view of the silanes on the silica surface with the white silane in position E shown in Figure 4.7.

4.2.6 Plasminogen Adsorption from Buffer

Plasminogen adsorption from isotonic Tris buffer, pH 7.4 was studied on the pure silica glass, sulphonated silica glass and lysinated silica glass after deprotection. Five independent replicate experiments were done for each of the surfaces as described in section 4.1.4. The data for all three model materials are summarized in Figure 4.12. While

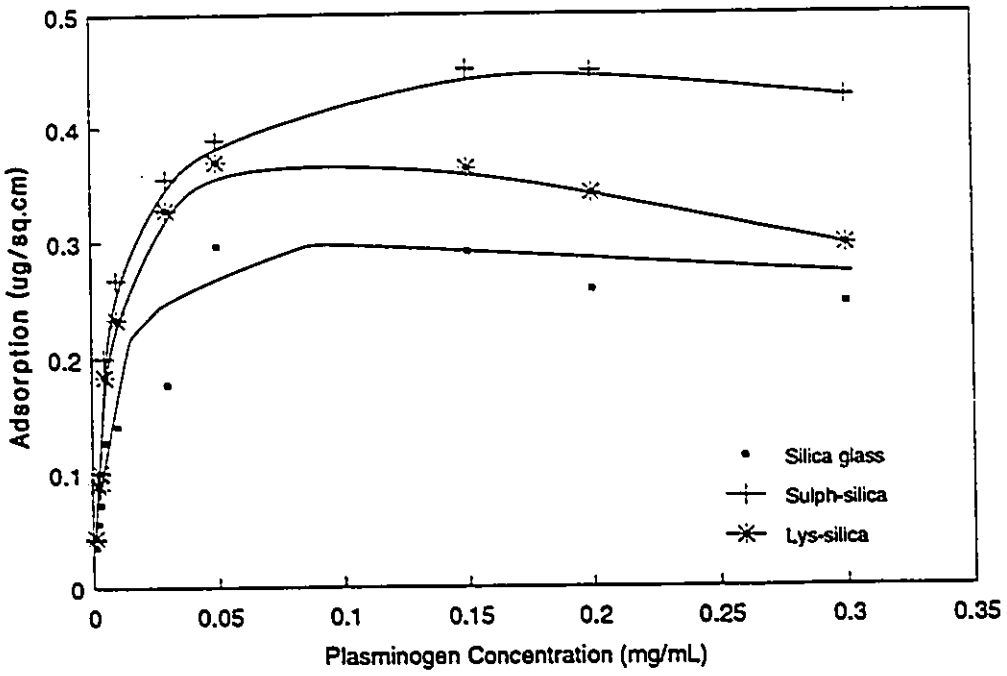


Figure 4.12 Plasminogen adsorption to silica glass, sulphonated, and lysinated silica glass model surfaces from isotonic Tris buffer, pH 7.4: three hour isotherms at room temperature. The solid lines do not represent models and are shown only to aid visual presentation of the data. The data shown are for five experiments. For given concentrations, averages of all replicates are shown.

the data are averaged for all five experiments, it should be emphasized that the trends for

each experiment taken separately were the same as those shown in this figure. The isotherms suggest high affinity, Langmuir type adsorption, with a rising initial portion, characterized by a relatively steep slope, trailing to a plateau. The isotherm plateau values are 0.443 $\mu g/cm^2$ for the sulphonated silica, 0.331 $\mu g/cm^2$ for the lysinated silica and 0.246 $\mu g/cm^2$ for the unmodified silica glass. These values are within the expected range for monolayer protein adsorption, as discussed in Chapter 3. The entire data sets for each surface are shown in Figure 4.13.

A two sided hypothesis test of the mean (μ) values of the isotherm plateaus was done at the 95% confidence level. The results are summarized in Table 4.6. The data in Table 4.6 confirm statistically what can be visualized in Figure 4.13. (1) The scatter in the data for the pure silica is greater than for the other two surfaces and the relatively large scatter in the data from different experiments is particularly obvious. This may be due to the variable hydrocarbon layer which rapidly forms on acid cleaned silica surfaces [Phillips and Hercules, 1985] previously discussed. (2) The plateau levels are highly significantly different at the 95% confidence level ($p<0.001$). The order of increasing adsorption is silica glass < lys silica < sulph silica.

In Figures 4.12 and 4.13 it appears that adsorption may be maximal at intermediate concentrations, decreasing again at the highest concentrations for the silica glass and lysinated silica glass. This behaviour is unusual in single protein systems and for the moment we have no convincing explanation for it. The same trend was noted for plasminogen adsorption to segmented polyurethanes (Chapter 3). One possibility is that plasminogen complexes of lower binding affinity are formed at the higher concentrations, as discussed in Chapter 3. Another possibility is that impurities in the plasminogen preparation may lead to lower adsorption at higher concentration via competitive adsorption.

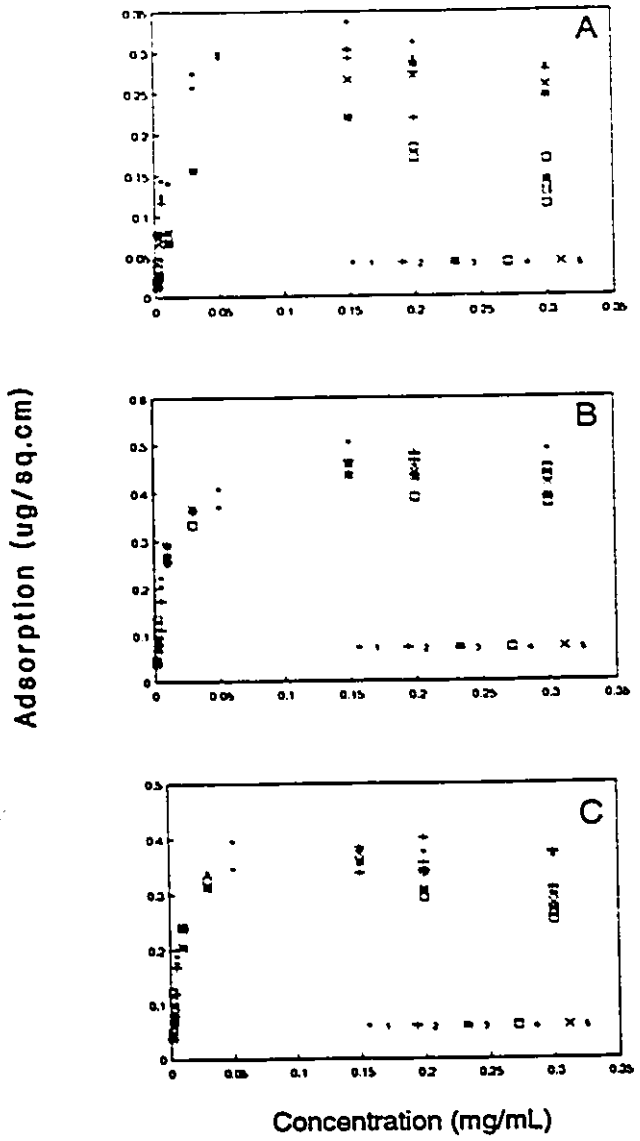


Figure 4.13: Plasminogen adsorption from Tris buffer, pH 7.4, onto silica glass (A), sulphonated silica glass (B), and lysinated silica glass (C): three hour isotherms at room temperature. The data are from five separate experiments, as indicated.

SURFACE	PLATEAU ADSORPTION DATA			
	MEAN (ug cm^{-2})	SD [1]	n [3]	95% C.I. [2] (ug cm^{-2})
Silica Glass	0.246	0.064	25	(0.221, 0.271)
Sulph Silica	0.443	0.034	28	(0.427,0.454)
Lys silica	0.331	0.045	27	(0.315, 0.348)

1 SD=standard deviation
2 C.I.= confidence interval
3 n=Number of data points

Table 4.5: The mean values of the plateau plasminogen adsorption for the silica, sulphonated and lysinated silica glass. 95% confidence interval is shown.

One possible contaminant, particularly for the effects associated with the lysinated surface, is lipoprotein(a). Lipoprotein(a) is a macromolecular complex that is assembled from low density lipoprotein (LDL) and a glycoprotein, apolipoprotein(a) [Utermann, 1989]. Apolipoprotein(a) is a high molecular weight glycoprotein which has homology with plasminogen. Upon disulphide reduction, lipoprotein(a) disassociates into LDL and apolipoprotein(a). Apparent molecular masses of apolipoprotein(a) range between 200 and 700 kd [Miles, 1989; Utermann, 1989]. It is therefore unlikely that the lipoprotein(a) or apolipoprotein(a) would be found with the gel protocol used in this work. Apolipoprotein(a) may contain 37 or more kringle structures which are 75-85% identical to the fourth kringle of plasminogen [Miles, 1989; Utermann, 1989]. The degree of homology of apolipoprotein(a) with plasminogen is so high that immunochemical studies show cross-reactivity with plasminogen [Utermann, 1989]. Lipoprotein(a) does compete

with plasminogen for lysine on cell surfaces [Hajjar et al, 1989; Miles et al, 1989]. Miles et al [1989] found that lipoprotein(a) and plasminogen compete for binding to monocyte-like U937 cells with equal affinity in a dose dependent manner.

From the above discussion it is possible that lipoprotein(a) may be present in trace quantities in the plasminogen preparations used in this work. The decrease in the adsorption values at high solution concentration for the lysinated material may be due to competitive adsorption of lipoprotein(a) which becomes significant at the higher concentrations.

4.2.6.1 Plasminogen Affinity for the Model Silica Glass Surfaces

As discussed in Chapter 1, the initial portion of a Langmuir isotherm approximates a straight line. The slope of this line can be used along with the plateau value to estimate a binding affinity of the protein-surface interaction. Figure 4.14 shows the lower portion of the isotherms for the three model surfaces. The initial slopes of the glass and sulphonated silica glass data are virtually coincident. The slope for the lysinated surface is greater, indicating that the affinity of plasminogen for this material is likely greater than for the silica glass or sulphonated silica glass. Estimates of affinity constants (K) were obtained by fitting the initial portion of the isotherm obtained from the adsorption experiments in buffer using linear regression. The slope of the line obtained was then used with the Langmuir equation ($\theta << 1.0$; $\theta = K C_p$) and an estimate of the affinities obtained. The data used in the calculations are given in Table 4.7. The affinities obtained are within the range previously found by other researchers for binding of proteins to solids [Boisson et al, 1988].

These estimates are approximate for a number of reasons. Moreover they should more appropriately be called "apparent" affinities. The "apparent" nature of K stems from the use of the Langmuir isotherm to model the data. As discussed, reversibility and

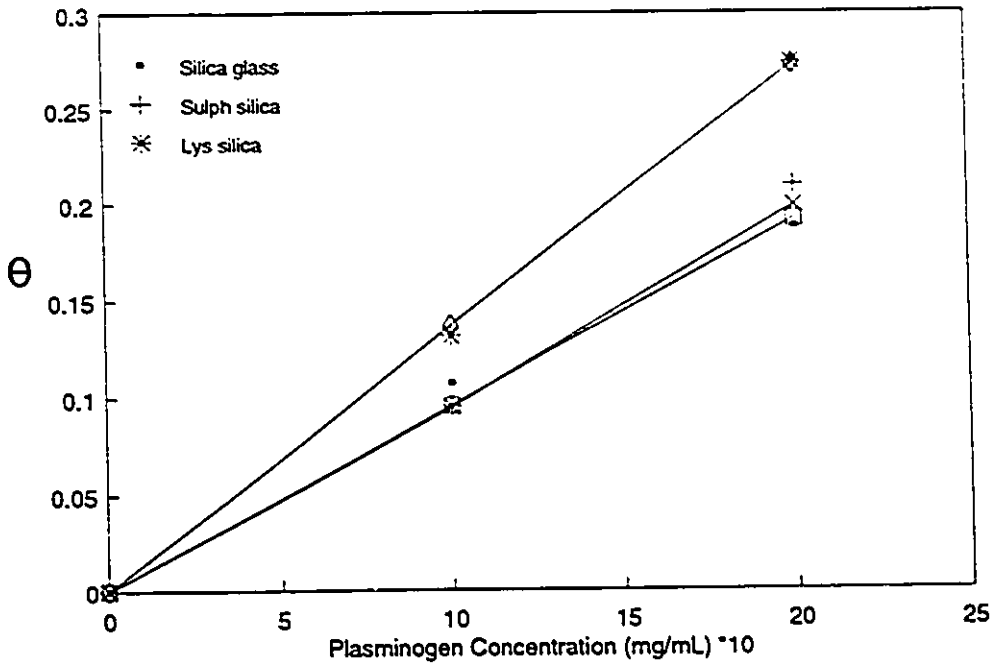


Figure 4.14: The lower region of the Langmuir isotherms for the model surfaces. θ plotted verses concentration. The slope of the curve is indicative of the "apparent" affinity of plasminogen for the surface, the greater the slope the greater the affinity. The values have been averaged from all experiments.

energetically homogeneous binding sites are two underlying assumptions of this model. It will be shown in the next section of this thesis that plasminogen adsorption is essentially irreversible for all practical purposes. In addition, the surfaces, particularly the sulphonated and lysinated silica glass, are heterogeneous. Thus the K values estimated from the Langmuir isotherm are "composite" affinities which would include specific and non-specific binding to different sites on the surface. However, given that the lysinated material is derived from the sulphonated silica glass a comparison of the

SURFACE	Γ_{max} (ug cm^{-2})	K (L/mol)	SD
Silica glass	0.246	9.20×10^{6}	1.40×10^{6}
Sulph silica	0.443	9.10×10^{6}	5.10×10^{5}
Lys silica	0.331	1.30×10^{7}	8.10×10^{5}

Table 4.6: Estimates of K from the isotherm data using a Langmuir model and linear regression.

relative values of K is appropriate. The difference in affinity values between these two materials, with the lysinated surfaces somewhat greater than the sulphonated, is presumably related to the presence of lysine.

4.2.7 Comparison of Single Protein Adsorption to the Model and Polyurethane Materials

Comparison of plasminogen adsorption from buffer to the polyurethane materials discussed in Chapter 3 and the model surfaces detailed in this chapter is of interest. Figure 4.15 shows the relevant data. It should be kept in mind that the experimental methods used to measure adsorption were different, and that the sulphonate contents of the two sets of materials were also different. The sulphur content of the polyurethanes in atom percent is as follows: BDDS-0.8 = 0.1; BDDS-1.4 = 0.3; BDDS-O.8-LYS = 0.2;

BDDS-1.4-LYS = 0.5. In contrast the sulphonated and lysinated silica glass have sulphur contents of approximately 2 atom percent. For the polyurethanes the relationship between bulk sulphur content and free sulphonate and lysine group content at the surface is unknown and the exact implications when comparing the data are thus not clear.

Several points emerge from Figure 4.15. First, the adsorption trends for the surfaces are the same for both types of material, consistently showing that the plateau levels are less on the lysinated materials than on the sulphonated materials. In addition, the model surfaces consistently show lower data scatter.

The plateau regions for the sulphonated and lysinated polyurethanes follow the same pattern as for the corresponding model surfaces. For the sulphonated silica glass, the plateau appears marginally more horizontal than for the polyurethanes. However the lysinated silica glass data trend downward, as is apparent in the polymers. The possible reasons for this decrease have already been discussed.

For the model materials, although the plateau levels are different in the lysinated and sulphonated materials with the former being lower, the "apparent" affinity of plasminogen for the lysinated surface is higher, accepting the limitations of the model used as discussed above. This result is different from those found in the polyurethane studies where there appeared to be no significant difference in the affinity of plasminogen for the sulphonated and corresponding lysine-derivatized polyurethanes. This difference between the two material types may be attributed to the heterogeneity of the polymer surfaces and their tendency to take up protein via swelling. Both of these effects tend to emphasize nonspecific binding of plasminogen, and make it difficult to assess specific binding to lysine sites.

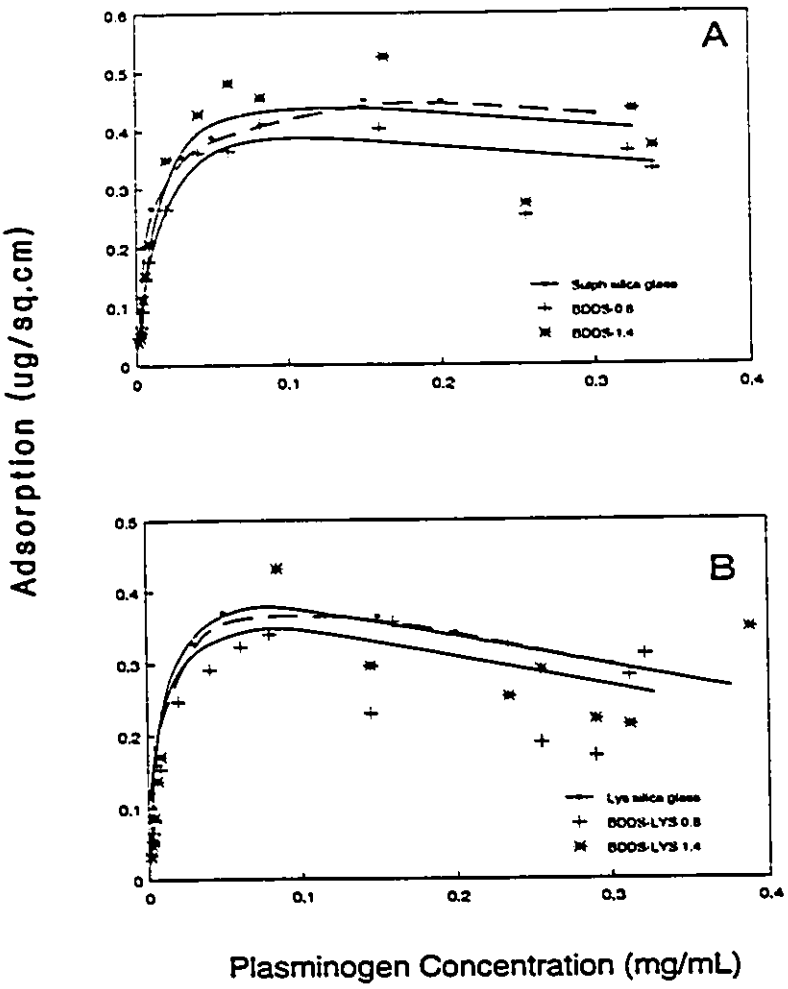


Figure 4.15: Comparison of plasminogen adsorption from Tris buffer, pH 7.4, to the sulphonated and lysinated model materials with adsorption to the sulphonated and lysinated polyurethanes discussed in Chapter 3. A) sulphonated materials, B) lysine derivatized materials. Three hour isotherms at room temperature. The solid lines do not represent models and are shown only to aid visual presentation of the data. The polyurethane data are for three experiments and the model surface data for five experiments. For a given concentration, averages of all replicates are shown.

4.2.8 Adsorption Studies in the Presence of ε-Amino Caproic Acid

ε-Amino-caproic acid binds to the lysine binding sites on plasminogen and thus could compete with lysine for these sites [Markus et al. 1978]. Experiments on the adsorption of plasminogen to the sulphonated and lysinated silica glass with EACA in the buffer were thus undertaken. It was anticipated that if plasminogen binds through the LBS to lysine sites on lysinated silica glass then EACA should inhibit this binding. On the other hand, binding of plasminogen to the sulphonated silica glass surface should not be influenced by EACA.

A statistical comparison (unpaired Student's one-sided t-test) of the plasminogen adsorption plateau values obtained for the sulphonated surface with and without EACA in the buffer (Figure 4.16 and Table 4.7) indicates that the plateau levels are the same at the 95% confidence level (p=NS). Therefore EACA appears to have no effect on plasminogen adsorption to the sulphonated material.

Comparing the plateau values for the lysinated surface in the same manner, the means are significantly different at the 95% confidence level ($p<0.05$). These data indicate that adsorption on the lysinated surface is lower in the presence of EACA as anticipated.

From Figure 4.16, there appears to be no difference in the slopes of the isotherms in the low concentration regions. This conclusion should be regarded as tentative because the data in this region are relatively sparse. It is therefore not possible make a definitive statement about the effect of EACA on the affinity of plasminogen for these surfaces.

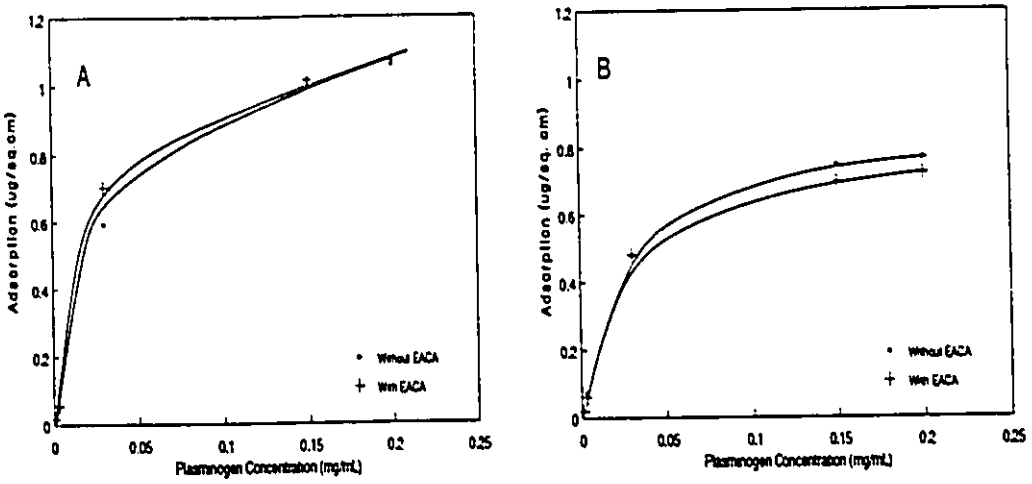


Figure 4.16: Comparison of plasminogen adsorption to the sulphonated and lysinated silica glass with and without EACA in the buffer. A) Sulphonated surface. B) Lysinated surface. Three hour isotherms at room temperature. The solid lines do not represent models and are shown only to aid visual representation. The data are for three experiments. For given concentrations, averages of all replicates are shown.

These studies suggest that the mechanism of adsorption of plasminogen to the lysinated surface is different from that for adsorption to the sulphonated material. It seems that some (but not by any means most) of the binding to the lysinated surface occurs through the lysine binding sites. The effect of EACA is small and this is probably

SURFACE	BUFFER[1]	MEAN	(n)	SD	p VALUE[2]
Sulph silica	Without	1.038	8	0.034	
	With	1.041	8	0.09	NS
Lys silica	Without	0.759	8	0.036	
	With	0.719	8	0.035	p<0.05

[1] With = 0.02 M EACA in the buffer

[2] p Value is for a one-sided t-test at the 95% confidence level.

Table 4.7: Comparison of plasminogen adsorption plateaus with and without EACA present in the buffer.

due to the relatively low density of lysine residues.

4.2.9 Reversibility of Adsorption: Desorption Studies

Reversibility is a controversial issue in protein-surface interactions [Baszkin et al, 1987; Evans et al, 1987]. Studies on the reversibility of plasminogen adsorption to the model surfaces were included in this project for two principal reasons, as discussed in the introduction; knowledge of reversibility is of intrinsic interest for modelling purposes, and is required to develop protocols to study the enzymatic activity associated with the adsorbed plasminogen (see Chapter 5).

There is often confusion about the exact definition of reversibility. Norde et al [1986] suggest that distinction should be made between reversibility towards 1) dilution;

2) changes in pH; 3) addition of low molecular-weight substances, including electrolytes; and 4) exchange against dissolved proteins. In this work reversibility of plasminogen adsorption towards dilution has been investigated. Data in the presence of EACA were also obtained. The data for the desorption studies are summarized in Figure 4.17 and Table 4.8.

Against pure Tris buffer, the order of increasing reversibility (fraction of reversibly bound protein) is sulph < lys < silica glass. For the lysinated and sulphonated surfaces approximately 70-80% of the protein appears to be irreversibly bound, with marginally higher reversibility on the lysinated silica glass than on its sulphonated precursor. At least part of this difference may be due to binding to the LBS.

The data from the experiments where desorption was measured against EACA-containing buffer (also shown in Table 4.8 and Figure 4.17), however, do not support this interpretation. It would appear that EACA causes increased desorption on both the sulphonated and lysinated surfaces and desorption is perhaps marginally higher on the sulphonated material although the data are inconsistent. This result is contrary to expectations.

The effect of EACA on desorption from glass is surprising. Desorption is actually less in the presence of EACA by almost a factor of two. The reason for this is unknown but may relate to changes in ionic interactions between the silica glass and plasminogen in the presence of the acid. The relatively high level of reversibility on the silica glass both in the absence and presence of EACA would suggest significantly different binding mechanisms for plasminogen to this surface compared to the silylated materials. It also appears that the binding mechanism is completely different from that involved in plasminogen-lysine interactions which are specifically susceptible to EACA.

In terms of the experimental protocol for the enzymatic activity assays, it should be noted that the rapid desorption of plasminogen from silica glass made it impossible to evaluate this surface in these assays. It would not have been possible to differentiate the activity associated with adsorbed plasminogen from that of unbound plasminogen which

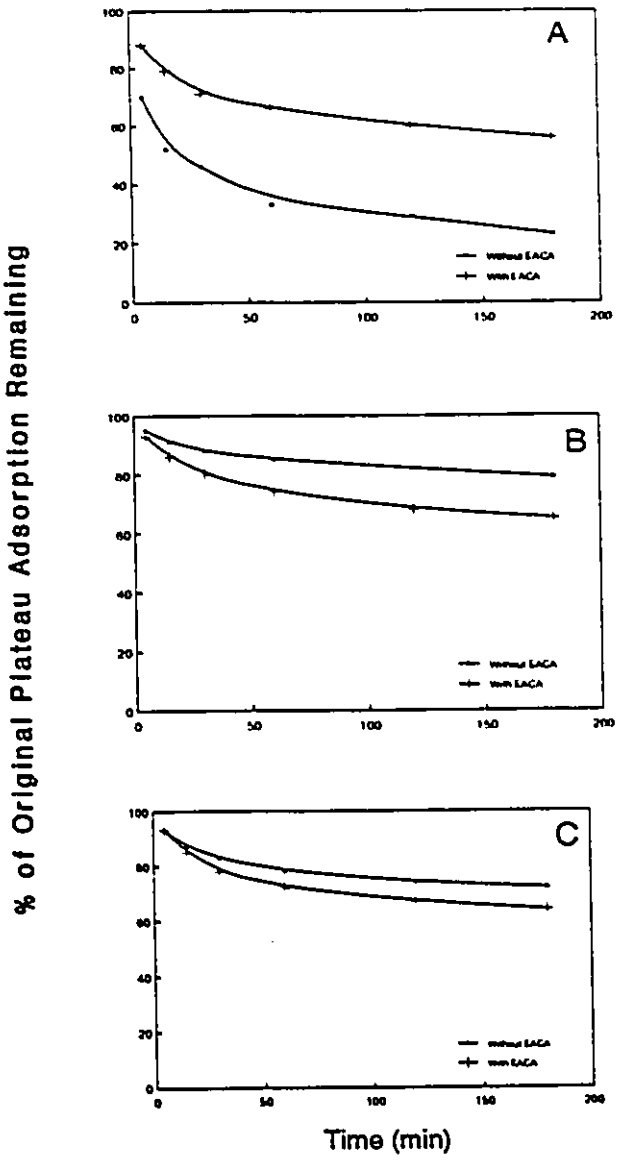


Figure 4.17: Plasminogen desorption from the model surfaces. The percent of adsorbed plasminogen remaining on the surface is plotted versus time. A) silica glass, B) sulphonated silica glass, C) lysinated silica glass.

SURFACE	TIME (min)	PERCENT REMAINING[1] WITHOUT EACA	WITH EACA	% DIFF (Without-With)
Silica glass	5	70	88	(25) [2]
	15	52	79	(51)
	30	46	71	(54)
	60	33	66	(100)
	120	29	60	(106)
	180	23	56	(143)
Sulph Silica	5	95	93	2
	15	91	86	5.3
	30	88	80	9.9
	60	85	74	12.8
	120	82	68	16.5
	180	79	65	18.7
Lys Silica	5	93	93	0
	15	87	85	1.6
	30	83	78	6.5
	60	78	72	8.2
	120	74	67	10.4
	180	72	64	11.2

[1] expressed as percent of the plateau adsorption level remaining.

[2] ( )=negative difference

Table 4.8: A summary of the plasminogen desorption data with and without EACA in the buffer. The % difference (without-with) between the two data sets is also given.

had desorbed from the surface. The silica glass surface was therefore not included in these experiments.

4.2.10 Plasminogen and Fibrinogen Adsorption from Plasma

The data for plasminogen and fibrinogen adsorption from plasma to silica glass,

sulphonated and lysinated silica glass are shown in Figure 4.18. Plasminogen adsorption to all three surfaces increases with increasing plasma concentration and shows no evidence of saturation at the higher plasma concentrations. Silica glass shows the highest adsorption capacity for plasminogen followed by the sulphonated and lysinated materials in that order. This result for silica glass could have been anticipated given the data for glass presented in Chapter 3. The reason for the higher adsorption on silica glass is not known, as previously discussed. The binding affinities, as indicated by the slopes at the low concentrations, are difficult to interpret from these data because the lysinated surface show a slightly negative slope in this region. It would appear that the silica glass has a higher affinity than either the sulphonated or lysinated material while the latter two materials appear to have similar affinities. The plasma behaviour of silica glass is in contrast to the single protein case where this surface showed the lowest adsorption of the three and in contrast to adsorption from buffer, the lysinated surface shows neither enhanced affinity nor enhanced capacity for plasminogen. These results are opposed to the notion (see Chapter 3) that the lysinated material should exhibit greater plasminogen affinity compare to the sulphonated material.

Fibrinogen adsorption from plasma to these surfaces illustrates the Vroman effect, a pattern typical for this protein. The fibrinogen curves vary markedly among the three surfaces with respect both to the peak height and peak position. The order of increasing peak height is :silica glass < lys silica < sulph silica. A high capacity for protein binding on sulphonated materials has been observed by others [Boisson et al, 1987; Santerre et al, 1992]. The plasma concentration at which the peak occurs increases in the same order. Again this probably reflects a relatively high binding affinity of fibrinogen on sulphonated materials.

It is clear that plasminogen and fibrinogen adsorption from plasma are very different. Plasminogen shows no Vroman effect on any of the three materials. This behaviour is similar to that observed with the derivatized polyurethanes discussed in Chapter 3.

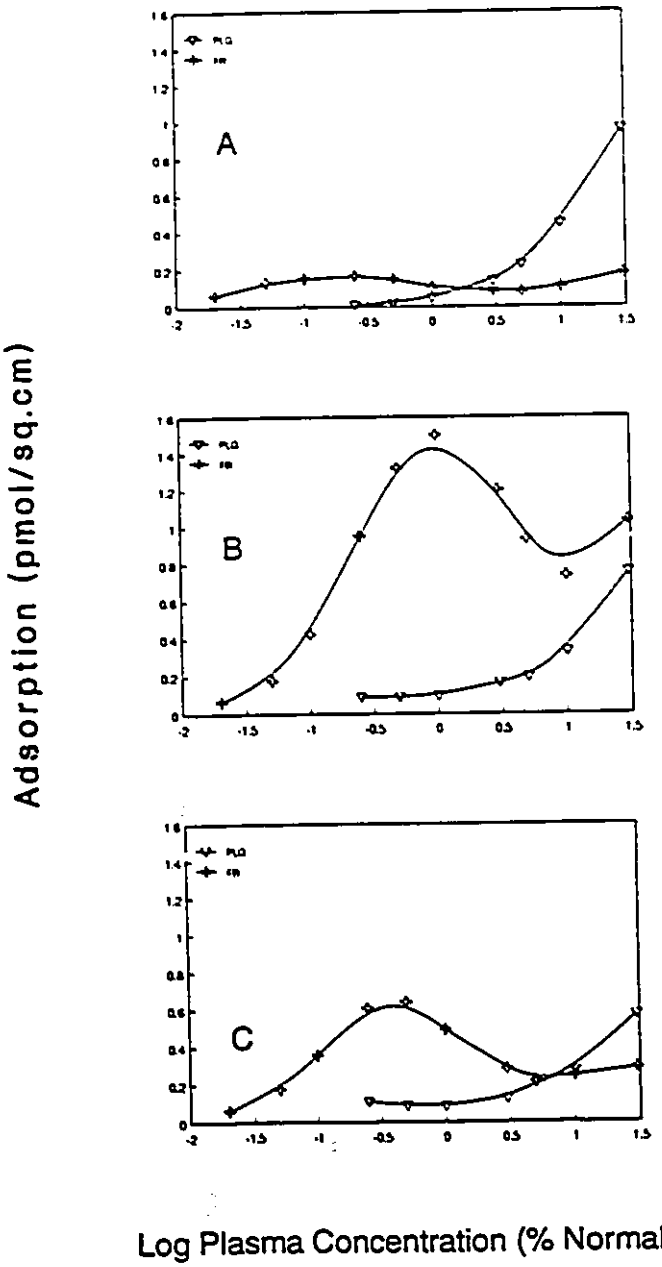


Figure 4.18: Plasminogen and fibrinogen adsorption from plasma to the model silica glass materials. A:silica glass, B:sulph-silica, C:lys-silica. Adsorption time was 3 h. Data are averages of three experiments. A minimum of three replicates were run at each concentration.

It is of interest to compare the levels of fibrinogen and plasminogen adsorption to the same surface. In the case of silica glass, (Figure 4.18 A), plasminogen adsorption is significantly greater than fibrinogen at concentrations beyond the Vroman peak. The same is true for the lysinated surface, illustrated in Figure 4.18 C. The sulphonated silica glass data show a different pattern. Plasminogen adsorption increases with increasing plasma concentration, but for this surface it only approaches the fibrinogen curve but does not cross it, indicating that the affinity of fibrinogen for the sulphonated material is relatively high so that it remains on the surface even at the higher plasma concentrations. Comparing the effects of lysine derivatization of the sulphonated surface on the two proteins, these data also indicate that the lysine residues cause enhancement of plasminogen adsorption and affinity relative to fibrinogen.

The adsorption of plasminogen relative to that of fibrinogen suggests that plasminogen may be involved in the Vroman effect, i.e. that it may be one of the fibrinogen displacing proteins, particularly when the surface has high affinity for plasminogen. Intuitively this makes a great deal of sense. Plasminogen is a protein which has been shown to have high affinity for physiologic surfaces, and its biologic activity is heightened when it is bound to a surface. One might even be tempted to suggest that displacement of coagulation proteins, particularly fibrinogen, which are involved in forming clots on surfaces, might offer a physiologic "advantage" to a protein whose principal function involves clot lysis.

The trends in plasminogen adsorption for the surfaces discussed in Chapter 3 were also observed in the model silica glass surfaces. The glass or control surfaces adsorbed the most plasminogen followed by the sulphonated and lysinated materials respectively. The model surfaces showed higher overall adsorption levels and less data scatter than the polyurethanes.

4.3 GENERAL DISCUSSION

The model surfaces discussed in this chapter, were used to aid in developing

methods for the lysine derivatization of materials such that lysine is present at the surface with the ε-amine group free. These model materials were also used to assess the effect of sulphonate groups and lysine residues on the adsorption of plasminogen to surfaces based on relatively simple substrate materials. Silica glass may be considered much less complex, both chemically and physically than the polyurethanes used in the initial work. The model materials appear to have served these two objectives reasonably well.

The chemical and physical analysis of the surfaces utilizing ninhydrin reagent, contact angle and XPS data indicate both qualitatively and quantitatively that the silylation and lysine functionalization methods were successful. Molecular modelling of the silylated surface is in general agreement with the other findings. The data also indicate that these materials have monolayer to submonolayer coverage of silane at the surface and that approximately 25% of the available sulphonated silane was derivatized with lysine.

The plasminogen adsorption studies in buffer, including both adsorption and desorption experiments, provide data suggesting that the binding mechanism of plasminogen is different on each of the three surfaces. It seems likely that the lysine binding sites are involved in some way in the adsorption of plasminogen to the lysinated material. Which of the LBS are involved and the exact mechanism are not clear. However the presence of lysine does affect the affinity of plasminogen for the surface as indicated both by the adsorption isotherms and by the fact that ε-amino caproic acid apparently competes with plasminogen for the binding sites on the lysinated surface. The specificity of the interaction of plasminogen with the lysinated surface will be further demonstrated in Chapter 5 of this thesis where the enzymatic activity associated with the bound protein is discussed.

The adsorption studies of fibrinogen and plasminogen from plasma implicate plasminogen as a possible contributor to the fibrinogen Vroman effect. In addition these studies provide further data in support of the contention that the sulphonated group has a strong influence on the adsorption of proteins in general. Sulphonated materials appear

to have high affinity for both plasminogen and fibrinogen. The plasma studies also show that the ratio of plasminogen to fibrinogen is greater on the lysinated than on the sulphonated material, again indicating a specific influence of the lysine residues.

Interestingly, with the exception of the affinity calculations, the trends in adsorption for the single protein systems on the model silica glass materials were the same as in the polyurethane studies discussed in Chapter 3. Extrapolating from the results of plasminogen adsorption experiments using the silica glass surfaces, it seems likely that the small amounts of sulphonate and lysine present at the surfaces of the polyurethanes exert considerable influence on the adsorption of plasminogen to these materials.

Use of the model materials also helped to clarify some of the earlier data on the polyurethanes. Of the three surfaces, plasminogen adsorption from plasma is greatest on the pure silica and lowest on the lysinated surface. The data presented in Chapter 3 suggested that the lysinated surface might have increased affinity for plasminogen in plasma systems. The data for the simpler, better defined model materials do not support this point of view. What they do suggest is that affinity of plasminogen relative to fibrinogen is greater for the lysinated materials than for the sulphonated precursor.

The work presented in this chapter indicates that lysine derivatization confers some degree of specificity for the adsorption of plasminogen to a surface. In Chapter 5, the results from a series of enzymatic assays on the lysinated material and its sulphonated precursor are reported. These studies provide information on the effect of lysine derivatization on the very important question of plasmin activity of adsorbed plasminogen.

CHAPTER FIVE

EVALUATION OF THE ENZYMATIC ACTIVITY OF PLASMINOGEN ADSORBED TO MODEL SILICA GLASS SURFACES

5.0 INTRODUCTION

The protein adsorption studies reported in Chapter 4 form only part of the work necessary to assess the fibrinolytic nature of a plasminogen-selective surface. The potential of the adsorbed plasminogen to exhibit enzymatic activity is an important additional aspect of the work presented in this thesis. To address this question, the plasmin or plasmin-like activity of plasminogen adsorbed to the model sulphonated and lysinated silica glass surfaces was investigated. The results of these studies are reported in this chapter.

As discussed in Chapter 1, plasminogen does not degrade fibrin until it is converted to its enzymatic form, plasmin [Weitz, 1990]. Therefore, plasminogen activation (conversion to plasmin) is extremely important to the fibrinolytic nature of a surface. In order for a plasminogen binding surface to be profibrinolytic, the bound plasminogen must exhibit either "plasmin-like" activity upon adsorption or must be readily activatable to plasmin. The enzymatic activity of plasminogen adsorbed to the model surfaces was assessed using two methods. In the first, plasmin activity was determined using a synthetic chromogenic substrate assay for plasmin. Cleavage of the substrate by the enzyme results in the release of a chromophore. The rate of colour production can then be monitored and related to the enzymatic activity. In the second, the amount of Bβ 1-42 fibrinopeptide produced when the surface is incubated with macromolecular fibrinogen was determined by radioimmunoassay. This method monitors fibrinogenolysis by plasmin. The use of these two methods to determine plasmin activity provides data using both a synthetic and a physiologic substrate for plasmin.

Both assays were performed in the absence and in the presence of tissue plasminogen activator. Studies in the absence of activator allowed quantification of the extent to which adsorbed plasminogen is inherently plasmin-like as suggested by Kichenin-Martin et al [1988], whereas with activator the rate and extent of activation of adsorbed plasminogen to plasmin could be determined. Finally, the experiments were repeated in the presence of both activator and the plasmin inhibitor, α_2-antiplasmin. These latter experiments were designed to determine whether plasmin bound to a lysine derivatized material is protected from inhibitors in a manner similar to plasmin bound to fibrin clots [Mayer, 1990; Weitz, 1990].

5.1 EXPERIMENTAL METHODS

Only methods that are new to this chapter are described in detail here. For all others the reader is referred to Chapters 3 and 4. Plasminogen used in these studies was prepared as described in Chapter 3.

5.1.1 Fibrinogen

The fibrinogen used in this work was prepared by the method described by Weitz and Leslie [1990] and donated by Dr. J.I. Weitz, McMaster University. Fibrinogen (Kabi, Grade L) used in the assays for enzymatic activity was made plasmin/plasminogen free by lysine-Sepharose 4B(Sigma) affinity chromatography in the presence of aprotinin (Sigma, 100 kallikrein inhibitory units/mL). The fibrinogen was then precipitated from solution with 25% ammonium sulphate and resuspended in 5 mM trisodium citrate (TSC), pH 7.4. This procedure was repeated three times and the protein then dialysed against 5 mM TSC for 48 h at 4°C. After dialysis, precipitate was removed from the preparation by centrifugation. The purified fibrinogen was then dialysed against 0.1 M NaCl in 0.05M TBS, pH 7.4 for 24 h at 4°C and stored at -70°C until use.

Prior to running SDS-PAGE the fibrinogen was incubated with streptokinase (3 mg/mL, Hoescht Celanese, Germany) for 48 h at 37°C. This should result in the

activation of any plasminogen present in the preparation to plasmin, and the resulting cleavage products should be detected by SDS-PAGE. SDS-PAGE (7.5% gels) under reducing and nonreducing conditions showed no significant degradation.

5.1.2 Evaluation of Enzymatic Activity

A brief background in enzyme kinetics is given to assist in the discussion of the chromogenic assay data. Enzyme kinetics is frequently described using the Michaelis-Menten equation. This equation is based on a two -step mechanism: The enzyme (E) first reacts with the substrate (S) to form a complex (ES) which then dissociates into free enzyme (E) and product (P):

$$E+S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES$$

$$ES \underset{k_4}{\overset{k_3}{\rightleftharpoons}} E+P$$

The second step is generally considered to be irreversible.

Based on this mechanism the Michaelis-Menten equation can be derived:

$$r=\frac{V_{max}[S]}{K_M+[S]} \qquad 5.1$$

where r = reaction rate, V_{max}= maximum rate, K_M is the Michaelis-Menten constant = k_2+k_3/k_1 and [S] = substrate concentration. An interesting characteristic of the Michaelis-Menten equation is that K_M is equal to the substrate concentration at which half V_{max} is attained. K_M has the dimensions of mol/litre and a low value of K_M indicates a high

affinity of the enzyme for its substrate.

In the chromogenic assays reported in this work, the V_{max} for plasmin cleavage of a synthetic substrate is obtained and used to estimate the plasmin activity present in the test sample.

5.1.2.1 Chromogenic Peptide Assay for Plasmin Activity

Chromogenic assays use a synthetic substrate to mimic the natural substrate for the enzyme. The chromogenic assay used to determine the plasmin activity of adsorbed plasminogen in this work used S2251 (Kabi, Sweden) as the synthetic substrate. S2251 is the tripeptide (D-Val)-Leu-Lys-p-nitroaniline and is rapidly cleaved at the Lys-p-nitroaniline bond by plasmin. This reaction is illustrated in Figure 5.1. p-Nitroaniline is

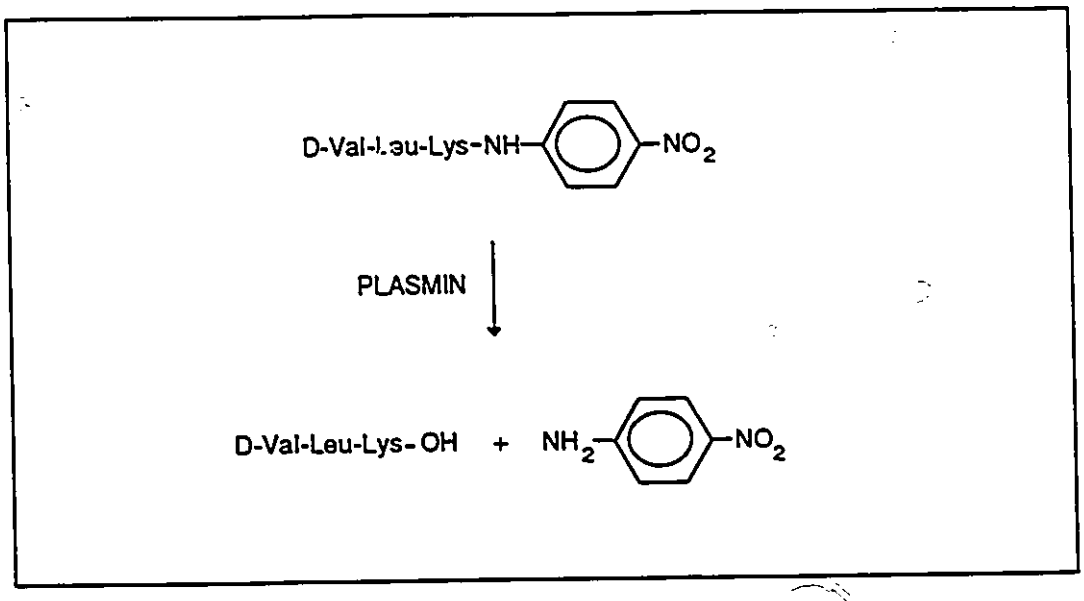


Figure 5.1: The cleavage of S2251 by plasmin to yield p-nitroaniline, a chromophore. The colour production over time can be monitored spectrophotometrically and used to estimate the activity of the enzyme.

a chromophore and its concentration can be measured spectrophotometrically at 405 nm.

The change in absorbance per min is related to the enzymatic activity. The cleavage of S2251 by plasmin has been shown to follow Michaelis-Menten kinetics [Friberger, 1982]. In addition product inhibition by either p-nitroaniline or the free peptide has not been observed [Friberger, 1982]. Friberger found that tPA also cleaves S2251, but at about half the rate of plasmin. For this reason the cleavage of S2251 by tPA was measured prior to running the adsorbed plasminogen assays and found to be negligible.

Assays were run under three separate conditions: with and without tissue plasminogen activator (tPA, Genentech), and with both activator and the plasmin inhibitor α_2-antiplasmin (Behring, Germany) present. The assays of the adsorbed plasminogen for plasmin activity using S2251 were done as follows. Plasminogen was adsorbed for 2 h from a pure plasminogen solution in isotonic Tris buffer, pH 7.4 at a concentration between 0.15 and 0.25 mg/mL to the sulphonated and lysinated silica glass surfaces as described in Chapter 4. This treatment resulted in a monolayer of plasminogen adsorbed to the surface as was determined in the pure protein studies.

The samples were rinsed twice in fresh buffer (isotonic Tris, pH 7.4) and then exposed to isotonic Tris, pH 7.4 for 2 h to desorb any loosely or reversibly bound plasminogen. This step was used to ensure that the enzymatic activity being evaluated was that of bound plasminogen. Observations on the pure protein system previously described, indicated that after 2 h in buffer the rate of desorption was negligible for the sulphonated and lysinated silica glass surfaces. However desorption from the unmodified silica glass continued over much longer periods. Therefore only the former two surfaces were used in the experiments discussed in this chapter.

After rinsing, the plasminogen-adsorbed silica glass samples, similar to those used in the adsorption studies, were placed in separate wells of a 96-well polystyrene tissue culture plate.

A total of 280 µL of solution was added to each well containing a tube segment. The solution contained tPA (Genentech, 2µL of a 100 µg/mL solution in Tris buffered saline (TBS), pH 7.4) where appropriate, α_2-antiplasmin (Behring, 10 µL of a 9 µmol/mL

solution in TBS, pH 7.4) where appropriate, substrate (30 µL of a 3.5 mmol/L solution in distilled water) and buffer (Tris buffered saline, pH 8.5) to make up the total. To evaluate unbound plasminogen the tube segment was substituted with approximately 5-10 pmol of plasminogen. This corresponded to the amount of plasminogen adsorbed to the silica glass tube segments. The average amounts of plasminogen adsorbed to the sulphonated and lysinated samples were 5.94 and 4.02 pmol respectively. The final tPA and α_2-antiplasmin concentrations were 1 µg/mL, and 0.3 µmol/mL, respectively. This α_2-antiplasmin concentration is in stoichiometric excess of both the unbound and surface bound plasminogen.

The reagents were added in the following order: plasminogen (or surface), buffer, tPA, α_2-antiplasmin, and substrate. In the inhibition experiments, the sample was incubated for 5 min prior to addition of the α_2-antiplasmin to allow some plasmin formation to occur. This method was found to give the most consistent results with α_2-antiplasmin.

The change in A405 (produced by cleavage of the substrate by plasmin) was read simultaneously for all samples and the data collected by a data collection program integrated with the incubator and spectrophotometer [Softmax, Molecular Devices Corp.]. The surfaces were incubated at 37°C and the change in absorbance versus time was followed for a 2 h period.

The quantities of chromogenic substrate (30 µL of a 3.5 mmol/L solution) added to each test well were in excess of the 6×10^{-4} mol/L value recommended by Kabi. The final volume of solution in each well (280 µL) resulted in complete coverage of the sample with solution but eliminated spillage in the incubator when the sample was mixed. The pH of the buffer is the recommended value that results in the greatest enzymatic activity under the conditions of this assay [Friberger et al, 1982]. A minimum of three replicates for each of the three experimental conditions were performed in each experiment.

5.1.2.2 Bβ 1-42 Radioimmunoassay

A radioimmunoassay is an assay based on the binding of an antibody to its antigen. A series of mixtures of radiolabelled and unlabelled antigen in different proportions is prepared. The same amount of antibody is added to each unlabelled/labelled antigen preparation and allowed to react. The antigen/antibody complexes formed are then precipitated and their radioactivity counted. The radioactivity will decrease in proportion to the amount of unlabelled antigen in the mixture because the antibody does not distinguish between labelled and unlabelled protein. In this manner a calibration curve can be constructed which relates the radioactivity of the precipitate to a known quantity of antigen. The amount of antigen in an unknown sample (with a radiolabelled tracer added) incubated with the same quantity of antibody, can then be obtained from the calibration curve [Riott,1991].

As discussed in Chapter 1, the Bβ 1-42 peptide is a specific cleavage product released by plasmin mediated proteolysis of the N-terminal region of fibrinogen or fibrin I. A radioimmunoassay specific for the Bβ 1-42 peptide (the antigen) developed by Weitz et al [1986] has been used in this work and is described in detail below.

The reagents for the radioimmunoassay were from the same sources as for the chromogenic assay. Prior to running the radioimmunoassay, sample tube segments of the sulphonated and lysinated silica glass were exposed to plasminogen in Tris buffer, pH 7.4 for 2 h as previously described for the single protein adsorption studies. The samples were then rinsed twice and incubated in isotonic Tris buffer, pH 7.4 for 2 h to remove loosely bound protein. Following exposure to buffer the samples were incubated in 1.5 mL vials containing either: 1) 300 µL of purified plasminogen-free fibrinogen (1 mg/mL) in 0.15 Tris buffered saline (TBS) [solution A] or, 2) Solution A containing tPA at a final concentration of 1 µg/mL [solution B], or 3) Solution B containing α_2-antiplasmin at a final concentration of 0.13 µmol/mL. The samples were incubated for 1 h at 37°C. These three experimental conditions are equivalent to those used in the chromogenic assay, i.e. without activator and inhibitor; with activator alone; and with both activator and inhibitor.

After incubation each sample was vortexed and 100 µL of the solution was removed and mixed with 300 µL of cold ethanol (BDH). This solution was in turn vortexed and allowed to sit at 4°C for a minimum of 30 min to precipitate unreacted fibrinogen before evaluation using the radioimmunoassay.

To assay for the Bβ 1-42 peptide, dilutions of the standard, and the test samples described above, and antiserum were made in 0.05mol/L Tris buffered saline, pH 8.5 containing 0.1% ovalbumin (Sigma) with 0.25% normal rabbit serum (Sigma)(Buffer 1). The standard is native Bβ1-42 peptide isolated by reverse-phase HPLC from plasmin digest of fibrinogen (donated by Dr. J.I. Weitz). A standard curve is prepared from a starting dilution of 1.25 pmol/mL of the peptide in Buffer 1. The test samples were prepared as described and included unbound plasminogen incubations as well as the supernatant for the incubated surfaces described above.

The antiserum to the Bβ1-42 (R142/3) was donated by Dr. J.I. Weitz, McMaster University. This rabbit antiserum does not cross-react with fibrinopeptide B or peptide Bβ 15-42 [Weitz, 1986]. In preparation for the assay, 100 µL of antiserum (1:4500 dilution) is aliquoted with 4.4 mL of buffer 1 with 0.25% normal rabbit serum and frozen at -70°C until use. Immediately before use the aliquots are thawed and 9.9 mL of buffer 1 with 0.25% normal rabbit serum, 5 µL of heparin [Sigma] at a concentration of 1000 U/mL, and one drop of food colouring are added. The aliquots are then kept at 0° until required.

Each assay tube contains 650 µL final volume consisting of 250 µL of buffer, 250 µL of either standard or test sample, 100 µL of Bβ1-42 antiserum, and 50 µL of tracer. The antiserum added is sufficient to bind between 25 to 35% of bindable counts under these experimental conditions. The tracer is ^{125}I labelled Bβ1-42 peptide (labelled using chloramine T) with a specific activity of 30-50 µCi/µg.

The samples are then incubated at 4°C for 24 h. After incubation, 750 µL of goat anti-rabbit IgG (Sigma, R3382) diluted 1/80 in a TBS (pH 8.5):2% PEG 8000 solution is added to each assay tube to precipitate tracer bound to Bβ1-42 antiserum. At this

dilution the goat anti-rabbit IgG is capable of binding all the rabbit IgG present in the assay. After the addition of this second antibody, the samples are vortexed and incubated at room temperature for 30 min. Following this incubation 2 mL of 0.9% NaCl is added to each tube and the samples are centrifuged at 2700Xg for 20 min at room temperature. The supernatant solution is aspirated and the pellet counted for radioactivity. The standard curve was then used to determine the amount of Bβ1-42 peptide generated in the test samples.

Within an individual experiment, a minimum of three replicates were run for each surface at each experimental condition (with tPA etc.). In addition unbound plasminogen was included as a control. Two experiments were run using tPA as the plasminogen activator and one using urokinase.

5.2 RESULTS

For both assay methods considerable differences were found in the absolute values of the measured variables (pmol of Bβ1-42 generated or casein units of plasmin activity). These differences are probably related to the two different plasminogen preparations used in the experiments. However the relative magnitude of the enzymatic activity associated with the lysinated and sulphonated surfaces, in the presence of plasminogen activator was constant. In addition the relationships between the data with and without activator, and with inhibitor and activator were also similar and consistent between experiments and assay methods.

5.2.1 Chromogenic Assay

A total of 12 or 13 replicates of the three experimental conditions were obtained from three separate experiments. Two different plasminogen preparations were used.

A print out of the raw data obtained from a typical assay is given in Figure 5.2.

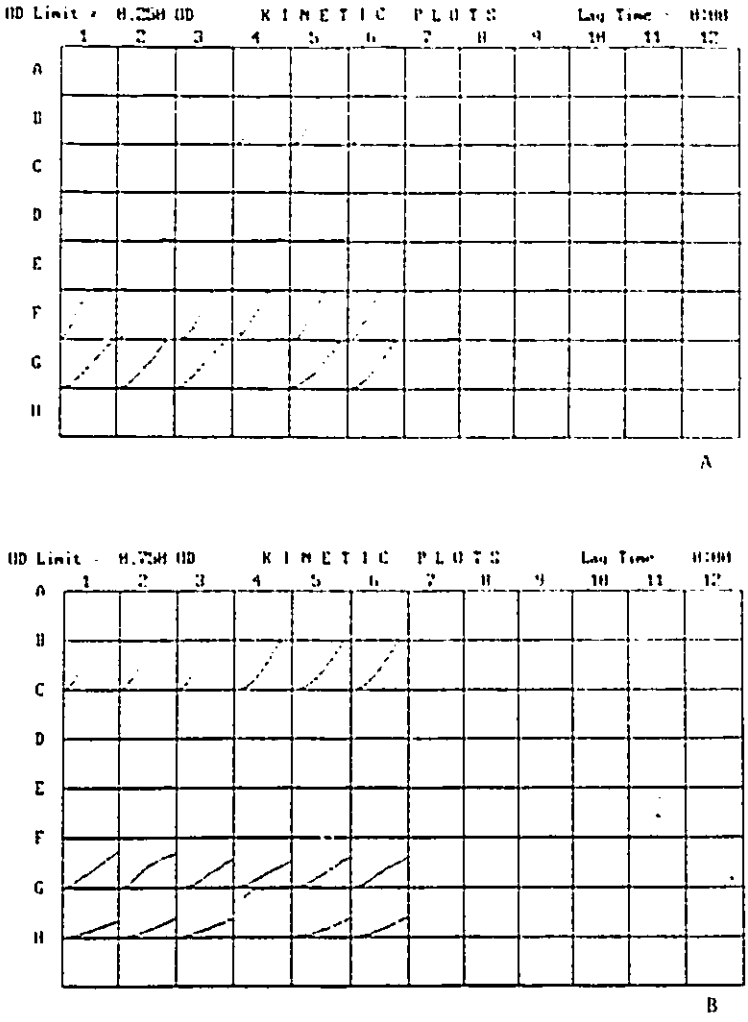


Figure 5.2: An example of the raw optical density data obtained from the Softmax data collection system for the chromogenic assay. Each square represents a well in the tissue culture plate. Optical density (OD) readings were obtained every 2 minutes for a two hour period and are represented as black dots. Well A1&A2 are controls (substrate and plasminogen), wells A3&A4 are unbound plasminogen without activator. Wells B1-B3 are unbound plasminogen with activator, wells B4-B6 are unbound plasminogen with activator and inhibitor. Wells D1-D6 are the surfaces without activator. D1-D3, sulphonated silica; D4-D6, lysinated silica Wells F1-F3 contain the sulphonated surface with activator and F4-F6 the lysinated surface with activator. Wells G1-G3 and G4-G6 are the sulphonated and the lysinated samples, respectively with activator and inhibitor. A) the data shown with an optical density upper limit of 250 mOD. B) the same data shown with an optical density upper limit of 750 mOD.

The maximum velocity of the enzymatic cleavage of the substrate, V_{max}, was obtained by taking the slope of the linear portion of the absorbance versus time curve and is reported in mOD/min. The slope was obtained using linear regression, and a correlation coefficient of 0.95 was the limit of acceptability for these data. The data were analyzed using both the Softmax Kinetics data package (Molecular Devices Corp) and Minitab linear regression (Minitab Statistical Software, Minitab Inc, PA; Regress). Both packages gave similar results. An example of a curve fit is shown in detail in Fig 5.3

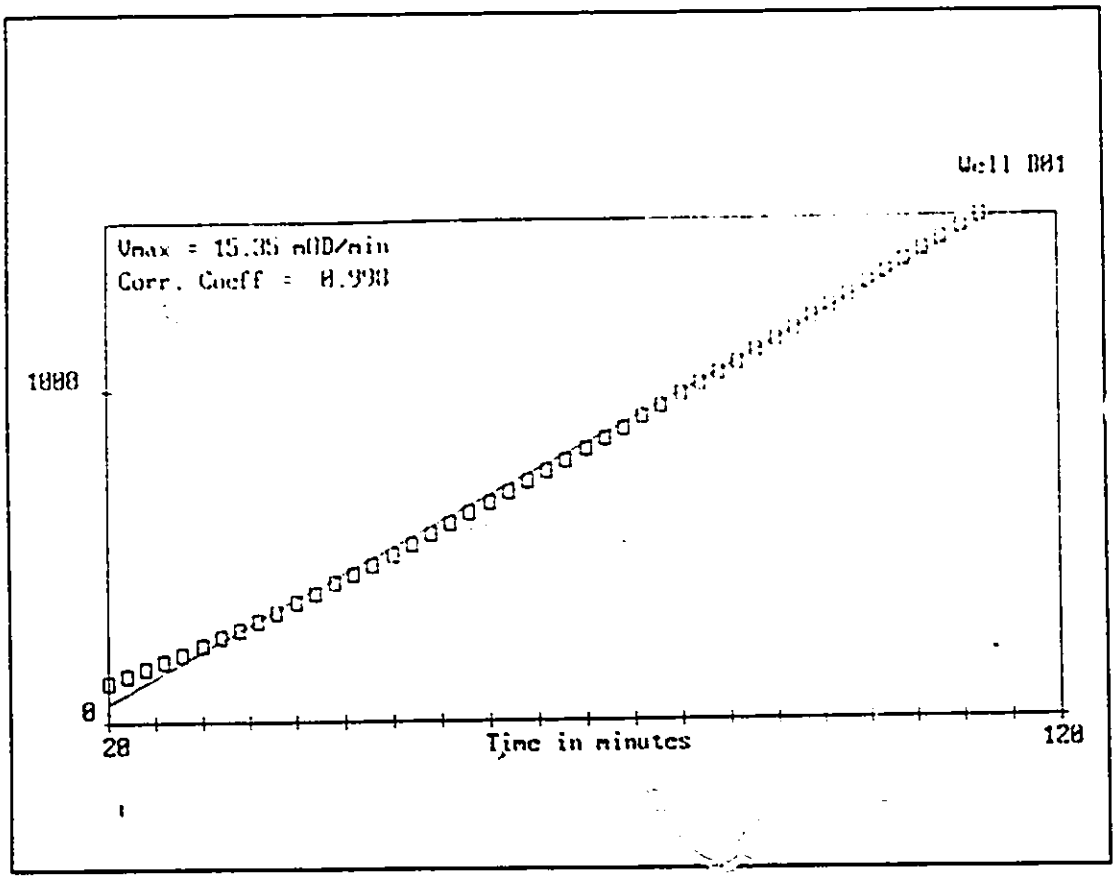


Figure 5.3: An example of linear regression of the mOD versus time data obtained from Softmax (Molecular Devices Corp. USA) for an unbound plasminogen sample with activator present. Minitab gave comparable results.

The V_{max} data are converted to enzymatic activity using information from the supplier of the chromogenic substrate. A change of absorbance of 50 mOD/min has been shown to be equivalent to 0.010 CU(Casein Units)/mL of human plasmin using S2251 [Kabi Diagnostica]. Activity may be calculated from the mOD/min data using the following equation:

$$A = \frac{0.01 * V * V_{max}}{50 * S} \qquad 5.2$$

where A = plasmin activity (CU mL^{-1}), V = volume of the well (0.28 mL), V_{max} = maximum velocity (mOD min^{-1}) and S = the amount of plasminogen in the well (pmol).

A summary of the data for the unbound plasminogen is given in Table 5.1. The raw V_{max} data can be deceptive because slightly different amounts of unbound plasminogen have been used in each assay. For this reason, the specific enzymatic activity per pmol of plasminogen present is provided along with the corresponding V_{max} data.

In the absence of activator, the enzymatic activity of the unbound plasminogen is not significantly above background indicating that the plasminogen preparations are free from any detectable plasmin contamination. However, with the addition of tPA, the enzymatic activity is greatly enhanced as anticipated. The average value for all the assays is 1.52×10^{-4} CU/pmol of plasminogen.

The data for the activity of the unbound plasminogen when both tPA and α_2 antiplasmin are present are more difficult to interpret. Averaged across all experiments, the enzymatic activity is 1.56×10^{-4} CU/pmol of plasminogen suggesting that no inhibition is occurring. This result may reflect the fact that the plasminogen was preincubated for 5 min with tPA, allowing the generation of plasmin and cleavage of substrate before α_2-antiplasmin was added. It may have been more appropriate to preincubate the sample with the inhibitor before adding the tPA.

ASSAY	PLG BATCH	ACTIVATOR	V max MEAN (mOD/min)	V max SD (mOD/min)	ACTIVITY ($(Cu/pmol) \times 10^{-4}$)
1	1	without	bk[1]	---	---
		with	5.86	1	1.61
		inhibitor	6.2	0.235	1.70
2	1	without	bk	---	---
		with	15.47	0.162	2.26
		inhibitor	12.24	0.560	1.79
3	1	without	bk	---	---
		with	9.16	0.465	1.35
		inhibitor	9.93	0.150	1.51
	2	without	bk	---	---
		with	7.31	0.205	1.11
		inhibitor	8.16	0.190	1.24
4	2	without	bk	---	---
		with	3.69	0.72	1.29
		inhibitor	4.57	0.59	1.60

1 bk=background

Table 5.1: Summary of the chromogenic assays for enzymatic activity of unbound plasminogen.

The data from the chromogenic assay experiments to evaluate plasminogen adsorbed to the lysinated and sulphonated silica glass are summarized in Table 5.2. Typical curves of mOD versus time are shown in Figure 5.4. It is clear from Table 5.2 that the data are strongly influenced by the plasminogen batch, and that there is considerable batch-to-batch variability. It is also clear that neither the plasminogen

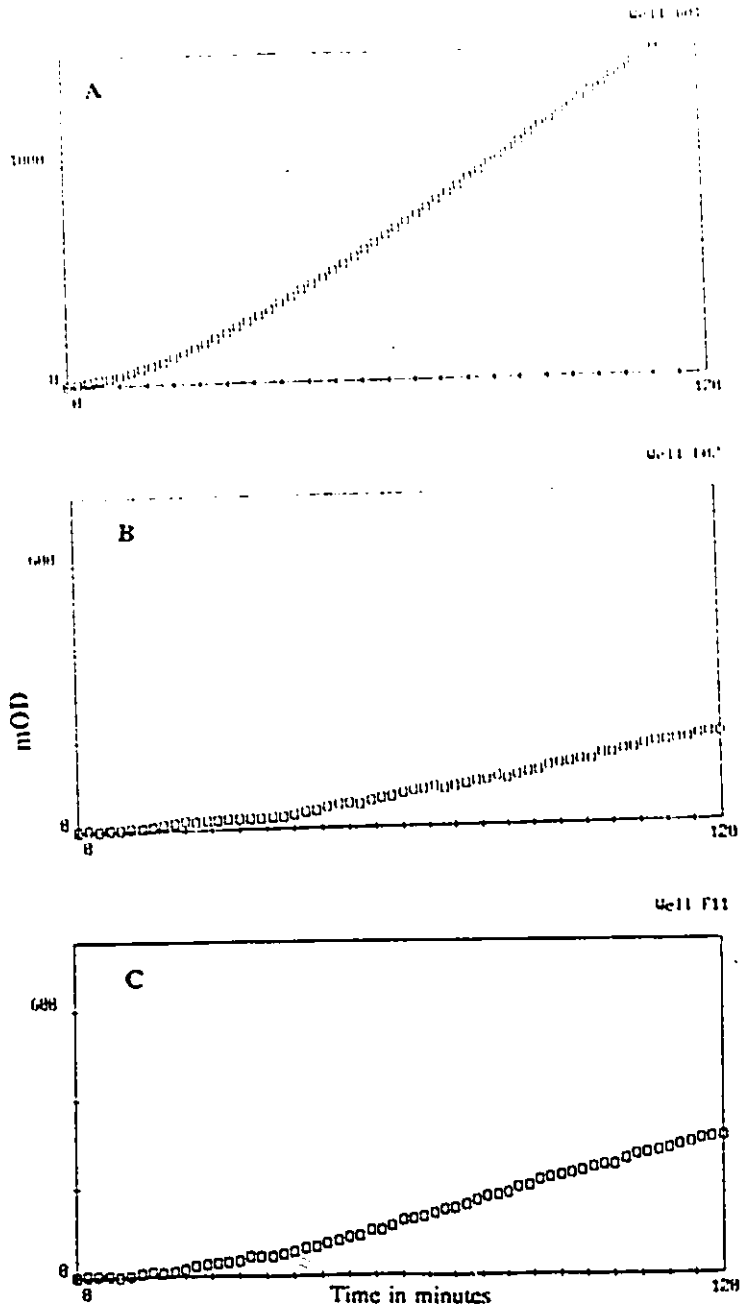


Figure 5.4: Comparison of the enzymatic activity associated with activated unbound plasminogen and plasminogen adsorbed to the lysinated and sulphonated silica glass. A) unbound plasminogen, B) plasminogen bound to the sulphonated surface, C) plasminogen bound to the lysinated surface.

PLG BATCH	ADDITIVES	SULPH SILICA V_{max} [2] (mOD/min)	SULPH SILICA ACTIVITY [3] (Cu/pmol)	LYS SILICA Vmax (mOD/min)	LYS SILICA ACTIVITY (Cu/pmol)
1&2	none [1]	—	—	—	—
1	tPA	3.66 ± 1.08	3.45×10^{-8}	4.88 ± 1.24	6.80×10^{-8}
	Inhibitor+tPA	2.82 ± 0.36	2.85×10^{-8}	3.01 ± 0.76	4.19×10^{-8}
2	tPA	5.05 ± 1.30	4.76×10^{-8}	9.25 ± 1.87	12.89×10^{-8}
	Inhibitor+tPA	3.60 ± 0.56	3.39×10^{-8}	3.02 ± 1.20	4.20×10^{-8}

[1] Background activity found for all assays without activator

[2] Mean value±SD

[3] Activity is expressed in Casein units per pmol of plasminogen

Table 5.2: Summary of the chromogenic assay data for the surface adsorbed plasminogen.

adsorbed to the sulphonated nor that adsorbed to the lysinated materials exhibits enzymatic activity in the absence of activator.

The data in Table 5.2 also indicate that there is a difference between the two surfaces in the enzymatic activity obtained after activation of the adsorbed plasminogen with tPA. This is confirmed by statistical analysis of the data using a two-sided hypothesis test at the 95% confidence level (Table 5.3) which shows that the enzymatic activity on the lysinated surface is highly significantly different from that on the sulphonated surface. The lysinated surface shows approximately two and half times the activity of its sulphonated precursor.

The data with inhibitor present are again not subject to clear interpretation. It appears that there is much more inhibition on the lys silica than on the sulph silica. Indeed there is very little inhibition by α_2-antiplasmin on the sulphonated surfaces.

SURFACE	ADDITIVES	ENZYMATIC ACTIVITY (CU/pmol) x10^{5} MEAN	(n)	SD	P value
Sulph silica	tPA	4.03	10	1.36	
Lys silica	tPA	10.67	13	3.80	p<0.001
Sulph silica	inhibitor + tPA	3.08	12	0.53	
Lys silica	inhibitor + tPA	4.20	12	1.46	p<0.02
Sulph silica	tPA	4.03	10	1.36	
Sulph silica	inhibitor + tPA	3.08	12	0.53	NA[1]
Lys silica	tPA	10.67	13	3.80	
Lys silica	inhibitor + tPA	4.20	12	1.46	p<0.001

[1] NA= not applicable, the sample size is not large enough to test.

Table 5.3: Comparison of the enzymatic activity associated with plasminogen adsorbed to the sulphonated and lysinated surfaces. (The mean values from the data in Table 5.2 are used).

However with α_2- antiplasmin present there is generally a much longer lag time and a more pronounced S shape in the kinetic data compared to the tPA-only data, perhaps indicating a difference in initial rate. It may be that a simple assessment of the data using V_{max} alone is inappropriate for this system which is likely to have complex kinetics. A more detailed examination of the initial rate period of the enzymatic activity will be necessary before any firm conclusions can be drawn from these data.

Finally a comparison of the average enzymatic activity associated with the unbound and surface adsorbed plasminogen is given in Table 5.4. The activity of the unbound protein (after activation with tPA) is approximately 1.5 times that of the

plasminogen adsorbed to the lysinated surface and 4 times that of the plasminogen adsorbed to the sulphonated surface. There is also a difference in the shape of the kinetics curves for the adsorbed and unbound plasminogen as in Figure 5.4. The maximum optical density achieved after 2 h is much lower for the adsorbed plasminogen compared to the unbound protein and the lag time is greater. This may be indicative of diffusion limitation effects on the interfacial reaction rates.

SURFACE	ADDITIVE	Vmax (mOD/min) MEAN	SD	ACTIVITY (Cu/pmol)
Unbound	tPA	8.30	4.0	15.2×10^{-5}
Sulph silica	tPA	4.28	1.37	4.04×10^{-5}
Lys silica	tPA	7.65	2.62	10.66×10^{-5}
Unbound	Inhibitor+tPA	8.40	2.7	15.7×10^{-5}
Sulph silica	Inhibitor+tPA	3.18	0.63	3.00×10^{-5}
Lys silica	Inhibitor+tPA	3.01	1.0	4.19×10^{-5}

Table 5.4: Comparison of the enzymatic activity associated with the unbound and adsorbed plasminogen as determined by chromogenic assay.

5.2.2 Bβ1-42 Radioimmunoassay

Three separate Bβ1-42 experiments were run, two with tPA as the activator and one with urokinase as the activator. A minimum of three replicates for each set of conditions were run in each experiment. Data for a typical Bβ 1-42 radioimmunoassay

using tPA are given in Table 5.5. The reproducibility of the data shown in Table 5.5 is similar to that found in the other experiments.

SURFACE	WITHOUT ACTIVATOR		WITH ACTIVATOR		WITH INHIBITOR	
SULPHONATED	0.51 1.01 1.82	1.11	1537.72 1821.94 1259.87 1308.66 1406.22 1410.47	1457.48	19.29 22.22 19.70 20.91 17.27 28.78	21.36
LYSINATED	1.21 1.11 1.31	1.21	3116.36 2719.73 3306.94 2642.46 3173.02 3574.79	3088.88	53.63 27.88 35.25 57.57 31.41 33.84	39.93

Table 5.5: Results for a typical Bβ1-42 radioimmunoassay. Data are given in pmol of Bβ 1-42 peptide produced per mL.

Table 5.6 summarizes the data for experiment three, and provides a direct comparison of the specific enzymatic activity associated with unbound plasminogen, and plasminogen adsorbed to both the lysinated and sulphonated surfaces in the presence of tPA and tPA and inhibitor.

Comparison of the data for the unbound versus adsorbed plasminogen (in both

SURFACE	ADDITIVES	ACTIVITY [1] (pmol peptide produced/ pmol plasminogen)
Sulph Silica	---	bk [2]
Lys Silica	---	bk
Unbound	---	bk
Sulph Silica	tPA	40.1
Lys Silica	tPA	171.6
Unbound	tPA	150.0
Sulph Silica	inhibitor+tPA	0.59
Lys Silica	inhibitor+tPA	1.75
Unbound	inhibitor+tPA	0.56

[1] All values taken from Assay 3 for a direct comparison.

[2] bk = background, found for all data without activator

Table 5.6: Summary of the enzymatic activity found in experiment 3 for unbound and adsorbed plasminogen. Data is presented for the assay with tPA alone, and with both tPA and inhibitor.

Tables 5.5 and 5.6) shows an interesting difference from the chromogenic assay. From the radioimmunoassay data, the enzymatic activity associated with plasminogen adsorbed to the lysinated silica glass with tPA present is comparable to that of the unbound plasminogen, indicating that the enzyme adsorbed to the lysinated material retains high activity against its physiologic substrate but not against a synthetic one. To assess possible desorption of the protein from the surface under the experimental conditions, labelled plasminogen adsorbed to both the sulphonated and lysinated silica glass was incubated with solutions containing either fibrinogen or fibrinogen and tPA. The extent

SURFACE	ACTIVATOR	ASSAY 1[a] Peptide (pmol/mL)	Activity	2 Peptide (pmol/mL)	Activity	3 Peptide (pmol/mL)	Activity
Sulph silica	without	bk[b]		bk		bk	
	with	1137.39 ± 133.5	31.3	393.29 ± 97.6	10.8	1457.46 ± 184	40.1
Lys silica	without	bk		bk		bk	
	with	1958.69 ± 337.0	108.8	880 ± 5.0	48.9	3088.88 ± 323	171.6
Control		893.24		14.95		48	

[a] Activator is urokinase at a concentration of 1ug/mL.

[b] bk=background levels

Table 5.7: Summary of enzymatic activity associated with adsorbed plasminogen as determined by Bβ1-42 R.I.A. The control is the amount of peptide generated when activator is incubated with fibrinogen.

of desorption was the same as observed previously for the incubation in buffer or EACA solutions (Chapter 4). Therefore it is unlikely that the higher enzymatic activity for plasminogen adsorbed to the lysinated surface, as assessed by RIA, is due to unbound plasminogen desorbed from the surface under the conditions of the assay.

Also as seen in Table 5.6, the data for unbound plasminogen in the presence of both tPA and inhibitor are clearer in the Bβ 1-42 assay than in the chromogenic assay. Almost complete inhibition of activity is found when α_2-antiplasmin is present in this assay system. Tables 5.5 and 5.6 also show the effect of α_2-antiplasmin on the enzymatic activity of surface adsorbed plasminogen. This is again clearer in the radioimmunoassay

than in the chromogenic assay. As was found with the unbound plasminogen discussed above, there is almost complete inhibition of the plasmin activity on both of the surfaces examined. There appears to be no difference between the two surfaces in this regard. This may indicate that binding to the lysinated surface does not confer protection from inhibition by α_2-antiplasmin. However this is not a kinetic experiment so no firm conclusions can be drawn about the relative effects on the plasminogen binding.

Table 5.7 summarizes all of the data obtained for the enzymatic activity associated with plasminogen adsorbed to both the sulphonated and lysinated materials. As previously mentioned, the values differ significantly from experiment to experiment. The difference between experiment 1 and experiments 2 and 3 is likely due to the use of urokinase as the activator. The difference between experiments 2 and 3 is likely due to the use of two different preparations of plasminogen (batches 1 and 2 discussed in the chromogenic assay section).

What does not change between experiments is the ratio of the specific enzymatic activity on the lysinated surface to that on the sulphonated material. The activity on the lysinated material is consistently about four times greater than that on the sulphonated precursor.

It appears from the experiment with urokinase that this activator has a direct effect on fibrinogen since the control sample (urokinase plus fibrinogen without plasminogen) shows significant peptide generation. Weitz et al [1990] have shown that incubation of fibrinogen with urokinase results in release of fibrinopeptide B (FPB) but not FPA. However they found no accompanying release of Bβ1-42 when purified urokinase was used. The urokinase used in this study may be contaminated with plasmin or plasminogen to a significant extent since no further purification was attempted. The later assays were therefore performed using tPA. If the background peptide generation in the control sample (urokinase and fibrinogen alone) is accounted for in the calculations, the trends in the assay using urokinase are the same as those in the experiments using tPA.

Finally, the data in all three tables indicate that, as was found with the

chromogenic substrate assay, minimal enzymatic activity is observed for the either the unbound plasminogen (Table 5.6) or plasminogen adsorbed to the surfaces in the absence of tPA but activation occurs readily in the presence of tPA (Tables 5.5-5.7).

5.3 DISCUSSION

From the bulk of the data presented in this chapter, the enzymatic activity of bound plasmin appears to be reduced in comparison to the unbound protein. (An exception is the lys silica surface as assessed by Bβ1-42 RIA.) This result is perhaps not surprising and similar effects have been found for other enzymes [Senatore and Bernath, 1986, Shanker et al, 1987].

Other authors have postulated that plasminogen adsorption to a surface may result in "plasmin-like" activity via conformational changes which occur upon adsorption [Brash et al, 1985; Kichenin-Martin et al, 1988]. From these considerations one would predict that either S2251 or fibrinogen would be cleaved by plasminogen adsorbed to the surface in the absence of any activator. However such cleavage was not observed using either assay method. The plasminogen-adsorbed surfaces incubated without activator show substrate cleavage only at background levels. Any enzymatic activity which is present may in fact be due to small amounts of plasmin adsorbed to the surface from the plasminogen preparation.

In both assays, however, the enzymatic activity associated with the bound plasminogen was significantly enhanced on both surfaces in the presence of activator. This indicates that the adsorbed plasminogen can be activated to plasmin which is capable of cleaving both a synthetic and physiologic substrate. This result implies that the active site of plasmin in the adsorbed state is available to interact with substrate.

It is a hypothesis of the present research that the lysine residues on the lys silica glass surface will result in a similar enhanced activation of bound plasminogen as is found on natural surfaces in the vascular system. In support of this hypothesis it was found that the specific enzymatic activity (i.e. activity per mole of plasminogen) of

plasmin on the lysinated material is approximately a factor of two greater than on the sulphonated material using the chromogenic assay, and a factor of four greater using the Bβ1-42 assay.

The difference in specific enzymatic activity of plasmin on these two surfaces may mean that plasminogen binding to the lysinated surfaces is more "physiologic", i.e. that the lysinated surface does indeed mimic fibrin, and that the lysine binding sites are utilized resulting in higher enzymatic activity on activation with tPA than on the sulphonated surface, where binding is presumably nonspecific.

Mangel et al [1990] found that in solution, plasminogen undergoes a radical conformational change from a prolate ellipsoid structure (radius of gyration 39Å) to a Debye random coil (radius of gyration 56Å) when one of its weak lysine binding sites interacts with substrate. These weak binding sites have been implicated in plasminogen activation, and when bound, the rate of activation of plasminogen by urokinase is increased relative to the unbound state [Mangel et al, 1990; Plow et al, 1991]. Mangel et al have proposed that the open Debye random coil may facilitate binding of the activator, and may be related to the enhanced activation. They have also suggested that this form would allow plasmin greater access to its substrate, fibrin, resulting in greater fibrinolytic activity. It seems possible that the open form of plasminogen could be present when the protein is adsorbed to the lysinated surface and not when it is adsorbed to the sulphonated material. The results presented here support both of the hypotheses of Mangel, and suggest that binding to the lysinated material could be via a weak binding site.

Another possible explanation of the difference in the enzymatic activity between the two surfaces, particularly with respect to the chromogenic assay is as follows. Kinetically, Lys-plasminogen is activated to plasmin at a rate 10-20 times faster than Glu-plasminogen [Hoylaerts, 1982; Walker and Davidson, 1988]. Hoylaerts et al found an intrinsic Michaelis-Menten constant for the activation of plasminogen bound to fibrin of 0.16 μM for Glu-plasminogen and 0.02 μM for Lys-plasminogen. In addition to being more rapidly activated when bound to fibrin, Lys-plasminogen also has a higher affinity

for fibrin. A close examination of many of the Western blots and SDS-PAGE gels for the plasminogen preparations used in this work showed the presence of both Lys- and Glu-plasminogen in the preparations. It is possible that the lysinated surface (mimicking fibrin) preferentially adsorbs Lys-plasminogen from the preparation resulting in a more rapid activation of plasminogen to plasmin compared to the sulphonated surface. In the chromogenic assay, V_{max} would be affected and enhanced activity found. Carrying this concept further, it seems likely that different batches of plasminogen would contain different ratios of Glu-and Lys-plasminogen (recall the elution profile of the plasminogen preparation in Chapter 3) and these differences may account for the variation in enzymatic activity for different plasminogen preparations. It would thus be of interest to study these two forms of plasminogen separately.

The data for the chromogenic assay in the presence of both tPA and α_2-antiplasmin are not conclusive. Although there appears to be a change in the shape of the kinetics curves and possibly a decrease in V_{max} when inhibitor is present, the data are not consistent. However more consistent information about the effects of α_2-antiplasmin on activation at the surfaces was obtained from the Bβ 1-42 assay. The addition of the inhibitor to the activated system virtually eliminated enzymatic activity on both the sulphonated and lysinated surfaces.

It is well known that the rate of inhibition of plasmin bound to fibrin is much slower than for unbound protein [Walker and Davidson, 1988]. While the Bβ1-42 data presented above indicate that total inhibition of bound plasmin activity is achieved, they do not provide information on the rate of inhibition. The chromogenic substrate assay data suggest that there may be an effect on the initial rate of substrate cleavage but further studies would be require to demonstrate this effect, convincingly.

In summary, plasminogen does not appear to be "plasmin-like" when adsorbed to the surfaces studied in this project. However in the presence of plasminogen activator, adsorbed plasmin is formed and there is significantly greater specific enzymatic activity

on the lysinated material than on its sulphonated precursor. This difference is not related to unbound protein. The data on susceptibility of bound plasmin activity to inhibition by α_2-antiplasmin are inconclusive, through it appears to be readily inhibited.

Although adsorption of plasminogen was greater on the sulphonated surface than on the lysinated surface in the buffer system, the factors indicative of biological response, i.e. enzymatic activity and selectivity in adsorption from plasma, were enhanced on the lysinated material.

Finally it is interesting to note that the enzymatic activity appears to be sensitive to what may be relatively small differences in the population of the bound molecules. On the lysinated surface it is likely that less than 25% of the sulphonated groups were modified with lysine, and thus that perhaps on the order of one quarter of the bound plasminogen is engaged by the lysine binding sites. Since a twofold to a fourfold increase in specific enzymatic activity is observed on the lysinated surface, this suggests that the molecules bound to lysine experienced a much higher increase, perhaps as much as ten fold. The investigations reported in this chapter indicate that continued efforts to produce polyurethanes with lysine residues appropriately bound are justified and would be of interest. Preliminary work in this direction is reported in Appendix 1 of this thesis.

CHAPTER SIX
CONCLUSIONS AND RECOMMENDATIONS

6.0 INTRODUCTION

The underlying working hypothesis of this research is that a potentially profibrinolytic surface may be formed via the specific and selective adsorption of plasminogen following blood contact. The anticipated contributions of this work were as follows: 1) Fundamental information about plasminogen adsorption and behaviour at the solid-solution and solid-plasma interfaces. This protein has not previously been studied in detail relative to blood interactions with artificial surfaces. 2) A method for derivatizing silanized silica glass with lysine that leaves the ε-amine group free for interaction with plasminogen. 3) Information on the enzymatic activity of plasminogen adsorbed to control surfaces, i.e silica glass and sulphonated silica glass, and to lysinated silica glass where the lysine binding sites of plasminogen are expected to be engaged. 4) Preliminary investigation into methods of derivatizing sulphonated polyurethanes with lysine so that high levels of lysine incorporation are achieved and the ε-amine group remains free.

6.1 CONCLUSIONS

1) Plasminogen adsorption from buffer and plasma to segmented polyurethanes modified with sulphonate groups and L-lysine was examined. Significant quantities of plasminogen are adsorbed from plasma and pure protein systems to the polyurethane surfaces. In plasma it is not displaced from the surface by other plasma constituents, i.e. it does not exhibit a Vroman effect. These findings are encouraging for the concept of a fibrinolytic surface based on selective plasminogen adsorption. The lysine-containing surfaces do not

show evidence of specific plasminogen binding but, as indicated, the surface concentration of lysine sites may not be sufficient for such specificity to be evident, and the lysine may be bound in a manner which makes the ε-amine groups unavailable for interactions with plasminogen.

2) Silica glass was modified by silylation with sulphonate groups and N-ε-tBOC-lysine to produce materials with, respectively sulphonic acid functionality and lysine attached via the α-amine group. Extensive characterization of the surfaces utilizing contact angles, ninhydrin, the Bolton-Hunter reagent, and XPS verified both qualitatively and quantitatively that the modification methods were successful.

3) XPS data were combined with a molecular modelling package (PC Model) to quantify the amount of silane on the silylated glass surface. The results suggest that silane coverage of the silica glass is less than a monolayer. In addition the model predicts that a more-or-less close packed monolayer could be obtained by reaction of the silane with all the available hydroxyl groups on the silica glass.

4) The silica glass materials were used to assess the effect of sulphonate and lysine groups on the adsorption of plasminogen to surfaces in a more direct way than was possible on the polyurethanes. Comprehensive studies of plasminogen adsorption and desorption in a single protein system provided data suggesting that the binding mechanism of plasminogen to the surfaces is different on each surface. The data from these studies also indicates that the lysine binding sites were involved in the adsorption of plasminogen to the lysinated material. The affinity of plasminogen appeared to be greatest on the lysinated surface in these single protein studies.

5) Data on the adsorption of fibrinogen and plasminogen from plasma to the model surfaces and to the segmented polyurethanes suggest that plasminogen may be a possible

contributor to the fibrinogen Vroman effect. In addition these studies provide further data in support of the contention that the sulphonate group has a strong influence on the adsorption of proteins from plasma in general. The plasma studies using the silica glass materials show that the lysinated surface is more selective of plasminogen than the precursor sulphonated surface.

6) The trends in plasminogen adsorption in both the single protein and plasma systems to both types of materials investigated in this thesis are the same. It would appear that even small amounts of sulphonate groups and lysine residues present on surfaces exert considerable influence on the adsorption characteristics of both plasminogen and fibrinogen.

7) Enzymatic activity associated with both unbound plasminogen and plasminogen adsorbed to lysinated and sulphonated silica glass was evaluated using two methods which provided data on enzymatic activity against a synthetic and a physiologic substrate, respectively. The enzymatic assays lead to the conclusion that adsorbed plasminogen is not inherently "plasmin-like". In the presence of plasminogen activators the enzymatic activity of associated with plasminogen adsorbed to the lysinated material was found to be significantly greater than on its sulphonated precursor. The data thus show that activation is potentiated on the lysinated surface as on the surface of a fibrin clot.

6.2 FUTURE WORK

1) The results from this work indicate that the binding of plasminogen to the sulphonated and lysinated surfaces is different. It would be of interest to further probe these differences. Monoclonal antibodies to specific epitopes on the plasminogen molecule could be used to determine specific regions of the molecule which are exposed in the adsorbed state. In this manner it might be possible to determine regions of the molecule

(e.g. strong and weak lysine binding sites) that are in close contact with the surface.

2) The model sulphonated and lysinated silica glass surfaces could be used to conduct a more extensive investigation into protein adsorption in both competitive and plasma protein systems. Systems including fibrinogen, plasminogen, HMWK and IgG could be studied. It would be interesting to determine plasminogen adsorption relative to these other materials.

3) The model materials could be used to investigate lipoprotein and lipid adsorption to biomaterials. This course of study is suggested by the possibility that lipoprotein(a) may be a contaminant in the plasminogen preparations. Lipid and lipoprotein adsorption has not been extensively investigated relative to biomaterials and may have implications for their biocompatibility.

4) The preferential adsorption of plasminogen to a surface may confer profibrinolytic potential to that surface. However adsorbed plasminogen does not inherently have plasmin activity. Future work should include the investigation of the potential for lysine derivatized materials to bind tPA from plasma. Materials which bind both plasminogen and tPA should have strong potential as fibrinolytic surfaces.

5) Determination of the enzymatic activity of adsorbed plasminogen is only the first step in the evaluation of the profibrinolytic or clot lysing activity of a surface. Future work in this area could include evaluation of the potential for adsorbed plasminogen to lyse clots. This could be done using radiolabelled fibrin clots. In addition, other importance blood responses particularly coagulation and platelet activation should be investigated.

6) There is clearly a need to develop a quantitative method to determine the amount of amino acid attached to both the polyurethanes and the silica glass. Lack of such a

method severely limits material development work. One possibility would be to use radioiodinated tyrosine, either alone or attached to the target amino acid as a dipeptide. This approach has been used by Massia and Hubbell [1991] to quantify peptide attachment.

7) It would be interesting to compare the enzymatic activity associated with lysine derivatized materials using ε-amine protected and unprotected lysine.

8) The methods of functionalizing the silane with a protected amino acid developed in this work open up the possibility of attaching more complicated peptides to surfaces. Future work in this area could include attachment of adhesion peptides for endothelial cells with the long term goal of developing an endothelializable polyurethane material.

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APPENDIX

INVESTIGATIONS INTO DERIVATIZED POLYURETHANES

INTRODUCTION

The model materials described in Chapter 4, although suitable for studying the specific interactions of lysine residues and sulphonated groups with plasminogen and for the development of surface derivatization methods, are not well suited for practical applications. One objective of the work on model surface was to develop methods that could be used to modify materials which would be useful for device construction, e.g. polyurethanes. In this appendix some preliminary work towards this objective is described. This work did not progress to the point that definite conclusions could be reached and will have to be pursued further to obtain information about plasminogen adsorption and enzymatic activity. This appendix describes the modified polyurethane synthesis and characterization and is included in the thesis primarily for documentation purposes.

One of the most difficult problems encountered with polyurethanes is to quantify chemical modifications. To this end, a model sulphonated polyurethane having a relatively high sulphonated content (corresponding to 6 wt%) was prepared which could be modified with lysine and other moieties as described previously. From a physical property stand point, this material is not well suited for fabrication and is not even capable of film formation. However the relatively high concentration of the modifier in this material makes it easier to characterise chemically. Nuclear magnetic resonance (NMR) spectroscopy was used in an attempt to quantify lysine derivatization and to define the appropriate protocol.

Four additional polyurethanes of lower sulphur content and capable of being cast in films were also prepared and characterized using ninhydrin, the Bolton-Hunter reagent,

XPS, and bulk chemical analysis. These were BDDS chain extended polymers containing 2.0 and 2.5% sulphur respectively (BDDS-2.0, BDDS-2.5), along with the corresponding lysine derivatized polymers (BDDS-2.0-LYS, BDDS-2.5-LYS). These polyurethanes provided materials containing higher weight percent sulphur and lysine than the materials discussed in Chapter 3. ε-Amine protected lysine was used thus leading to materials in which the ε-amine group remains intact.

EXPERIMENTAL

Polyurethane Synthesis

In total seven polyurethanes were synthesized all of which were based on polypropylene oxide soft segments. One polymer was extended with methylene dianiline (MDA). The others were chain extended with 4, 4'-diamino 2,2' biphenyl-disulphonic acid (BDDS) to produce materials containing 6, 2.5, and 2.0 weight percent sulphur respectively. These latter three polymers were each derivatized with ε-amine protected lysine. The polyurethanes having sulphur contents of 2 and 2.5 percent sulphur were synthesized using MDI, PPO 1000 and BDDS in molar ratios of 2.5:1.2:1.0 and 2.1:0.8:1.3 respectively, as described previously. The sulphonated material containing 6% sulphur was prepared using Jeffamine (Texaco), an amine-terminated PPO of molecular weight 1000 in place of the usual hydroxyl terminated PPO. This polymer was synthesized using a modification of the two step synthesis procedure previously reported. In the first step a prepolymer was synthesized by reacting MDI with BDDS in 3:2 stoichiometry for 2 h at 50°C. The prepolymer was then chain extended to high molecular weight by reacting with the Jeffamine in 1:1 stoichiometry. This step was carried out at 25°C overnight. Reactions were run in a nitrogen atmosphere to eliminate moisture.

Lysine derivatization was achieved using a modification of the method developed by Santerre and Brash [1991] and described in Chapter 3. This method uses unprotected lysine and presumably both the α-amine and ε-amine are involved in forming the

sulphonamide bond to the polyurethane. In the present work Santerre's method was modified to use ε-amine protected lysine in a manner similar the lysine derivatization of sulphonated silica glass as described in Chapter 4.

The lysine-derivatized polyurethanes were synthesized using the BDDS-extended polymers. Sulphonated groups were converted to sulphonyl chloride by reaction with oxalyl chloride in DMF. The sulphonyl chloride groups were subsequently converted to lysine sulphonamide by reaction with N-ε-benzyloxycarbonyl-lysine (CBz-lysine) as previously discussed in Chapter 3. The reaction pathway for lysination is shown in Figure 3.2. The resulting material was precipitated in methanol and dried for 24 h in a vacuum oven at 60°C.

Deprotection of the lysine on the polyurethane was achieved by hydrogenation of the material over a 10% Pd/charcoal (Sigma) catalyst. After drying, the N-ε-CBz-lysine derivatized polyurethane was dissolved in DMF at a concentration of 2% (w/v). In preparation for hydrogenation approximately 10.0 mg of catalyst was added to 20 mL of DMF in a modified 250 mL flask and maintained in suspension with a magnetic stirrer. The flask design allowed sample addition via a side arm and valve when the flask was in a hydrogen atmosphere.

The flask was evacuated and the air replaced with hydrogen. A solution (100 mL, 2 wt%) of protected derivatized polyurethane in DMF was then added to the catalyst. The mixture of polyurethane, catalyst and DMF was continuously stirred under hydrogen at atmospheric pressure for 1 h [Stewart and Young, 1986]. The resulting polymer was then filtered, precipitated, and extensively washed with distilled water and methanol (HPLC grade). Finally the sample was dried in a vacuum oven overnight at 60°C and stored in dark bottles at room temperature.

In these synthesis, CBz was used as the protecting group because it can be removed under relatively mild conditions by hydrogen [Bodansky and Bodansky, 1984; Stewart and Young, 1986] and it does not require the acid conditions necessary to remove t-BOC groups. It was assumed that the trifluoroacetic acid or other strong acids would

be detrimental to the polyurethane.

Elemental chemical analysis of the polyurethanes for carbon, nitrogen, and sulphur was done by Galbraith Laboratories Inc., Knoxville, Tenn.

Nuclear Magnetic Resonance (NMR) Evaluation of the 6.0 wt% Sulphur Polyurethanes

Proton NMR (Bruker AC200, McMaster University) was used to characterize the polyurethanes having the highest sulphur content. Samples for NMR were prepared by dissolving the polyurethane in deuterated DMSO (1-2 wt%) in 5mm diameter thin wall NMR tubes.

Film Preparation

Films of the materials were cast in glass petri dishes. The dishes were first cleaned for 1 h in chromic-sulphuric acid, rinsed extensively with distilled water and finally dried for 1 h at 90°C. Polymer solutions (50 mL of 5 to 8 wt% polymer in DMF) were poured into the bottom of a clean glass dish and covered with filter paper to protect from dust contamination. The glass dish was levelled and the solution was dried for 16 h in a convection oven at 60°C, and for a further 16 h in a vacuum oven at 60°C. After this two step drying process, the film was gently removed from the glass surface with tweezers and stored in sealed plastic bags in the dark until use. This method produced films of even thickness (about 0.5 mm).

A major concern was to remove the last traces of surface contaminants from the films without altering the polyurethane. Several methods were tried. Initially decontamination by solvent washing (ethanol, methanol, and acetone) was attempted. However all solvents used were found to alter the physical properties of the films so these cleaning methods were abandoned. The procedure of Horbett [1986] using ultrasonication in detergent was finally adopted as the cleaning method of choice. The polyurethane films were immersed in 1% Liquinox solution for 10 min in an ultrasonic bath (Branson

2200, Branson Ultrasonics Corp., Conn.) followed by 10 min in distilled water. The films were then dried overnight in a vacuum oven at 60°C.

RESULTS AND DISCUSSION

Nuclear Magnetic Resonance Spectra

NMR was used to monitor the lysine derivatization deprotection step. The proton NMR spectra for BDDS-6.0-LYS before and after deprotection of the lysine are shown in Figures 1 and 2. The peak assignments are shown in Table 1. As can be seen in

δ PPM	PROTON ASSIGNMENT
1-2	Methylene (PPO and Lysine)
2.5	DMSO
3-4	Aliphatic (PPO)
5	Water
7-7.5	Aromatic
>8	NH_2,NH

Table I: Peak assignments for proton NMR spectra of BDDS-6.0-LYS. Determined using Pecsok and Shields1 [968], standards,and personal commun. with Dr. A. Bain, McMaster.

Figures 1 and 2 there is a decrease in the relative intensity of the peak at 7.4 ppm suggesting loss of the protecting group however there does not appear to be a loss of

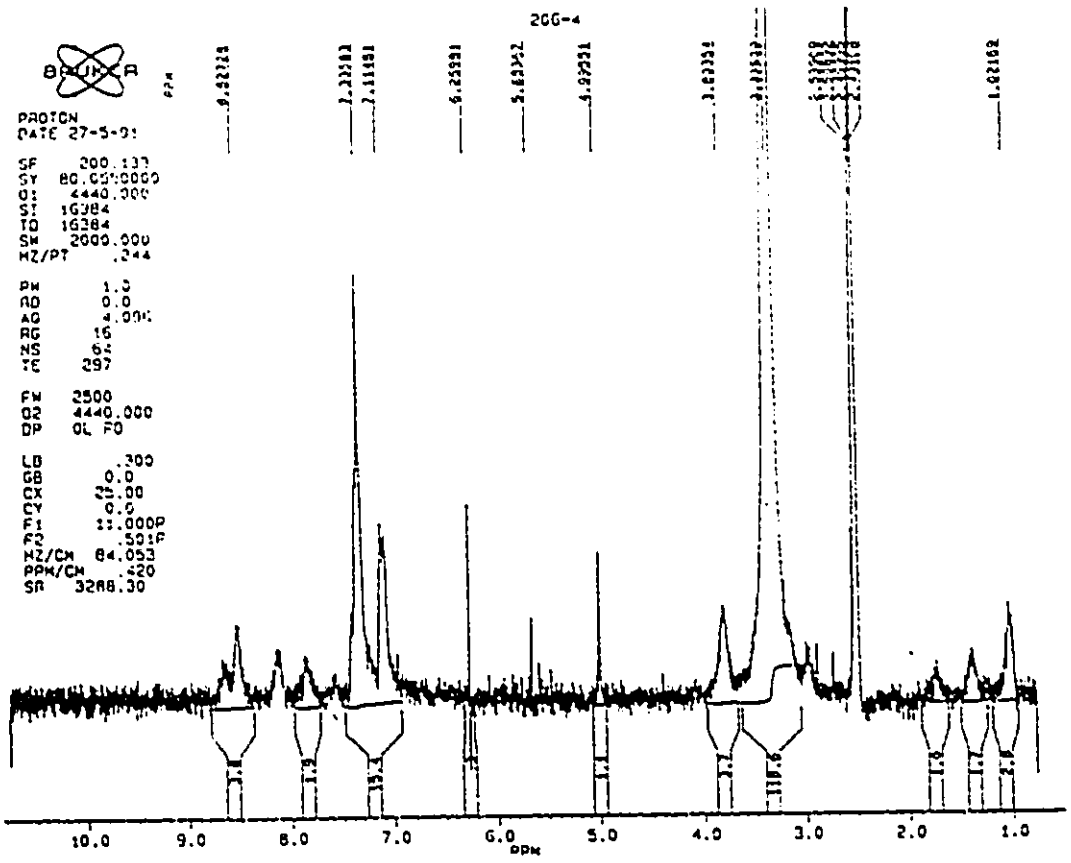

Figure 1: Proton NMR spectrum of the 6 wt % sulphur lysine derivatized polyurethane before deprotection.

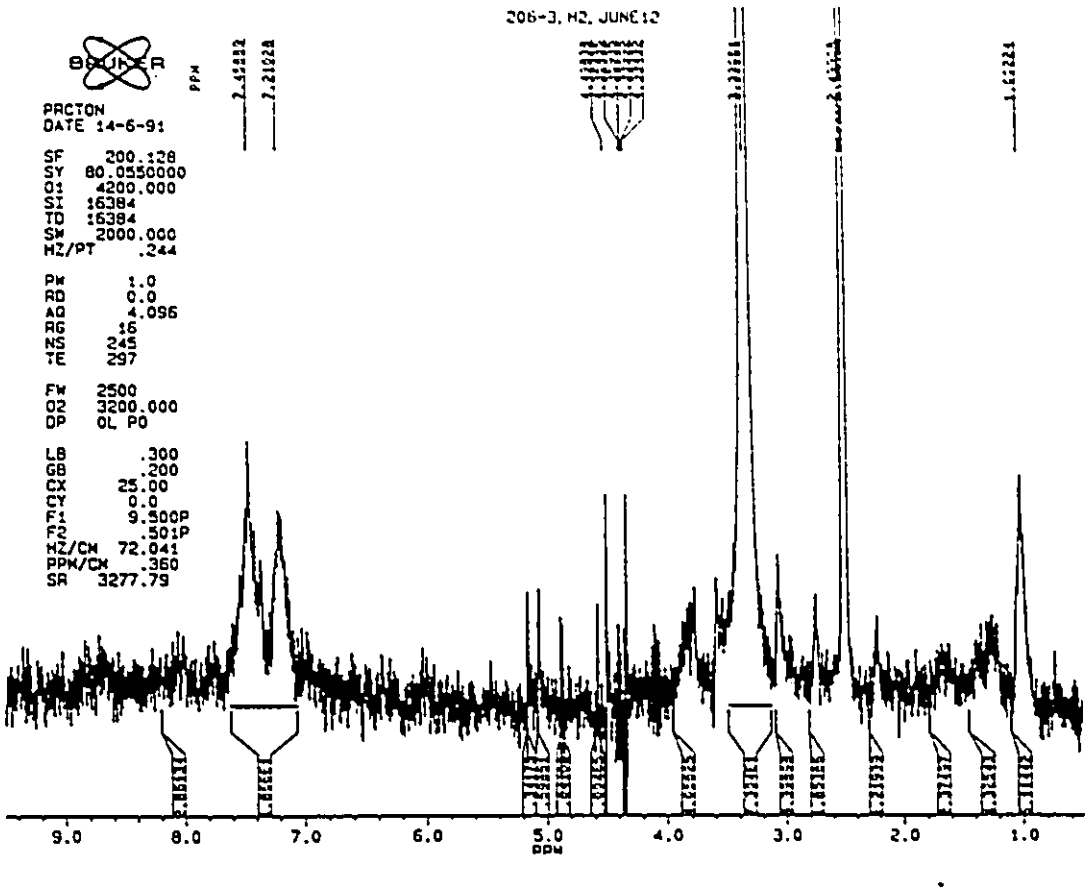

Figure:2 Proton NMR spectrum of the 6 wt% sulphur lysine derivatized polyurethane after deprotection with hydrogen using 10% Pd/activated charcoal catalyst.

peaks associated with the methylene protons in lysine, found between 1.0 and 2.0 ppm.

The changes in the NMR spectra after deprotection would appear to indicate that the hydrogenation method successfully removes the protecting groups but not a significant amount of the amino acid itself. It was assumed that the same would hold true for the polymers of lower sulphur content. An attempt was made to evaluate these materials directly using NMR but the critical peaks, particularly those representative of lysine, were too small to provide reliable indications.

Physical and Chemical Characterization of the Polyurethanes

Contact Angle

The data from the water contact angle measurements (sessile drop method) are shown in Table 2. The same trends are observed for the contact angles on these surfaces

SURFACE	CONTACT ANGLE SESSILE DROP (Degrees ± SD)
MDA	83.2 ± 2.5
BDDS-2.0	63.8 ± 4.6
BDDS-2.0-LYS	71.8 ± 3.5

Table 2: Water contact angle data for polyurethane cast films. The sessile drop method was used. The values given are averages of 12 measurements.

as were found for the polyurethanes discussed in Chapter 3. The MDA based polyurethane is the most hydrophobic followed by the lysinated and sulphonated materials in that order.

Gel Permeation Chromatography

The polystyrene equivalent molecular weights of the BDDS-2.0 and the BDDS-2.0-LYS polyurethanes were found to be 82,000 and 95,000 g/mol as determined by GPC. These molecular weights are approximately double those obtained previously [Santerre and Brash, 1991] for similar polyurethanes.

Elemental Analysis

The elemental analysis data are given in Table 3. These data show that the sulphur content was closer to the target of 2 wt% for the BDDS-2.0 material, and significantly higher than target for the BDDS-2.5 materials.

POLYMER	ELEMENT		
	CARBON	NITROGEN	SULPHUR
	(All data given as wt %)		
BDDS-2.0	62.7	3.6	1.78
BDDS-2.5	58.7	3.4	2.9
BDDS-2.0-LYS-CBz	62.4	4.5	1.17
BDDS-2.0-LYS	62.8	3.7	1.38
BDDS-2.5-LYS-CBz	59.8	3.6	3.10

SD are in the order of ± 0.05%

Table 3: Elemental analysis for the polyurethanes . CBz denotes a polymer before deblocking.

There is a significant difference between the nitrogen content of the BDDS-2.0 polyurethane and its lysine derivatized counterpart (BDDS-2.0-LYS-CBz), indicating that derivatization has indeed taken place. However, hydrogenation appears to cause some loss of lysine as well as deprotection because the nitrogen content is significantly less in the deprotected BDDS-2.0-LYS than in BDDS-2.0-LYS-CBz. Indeed the hydrogenated materials has a nitrogen content which is not significantly different from the parent sulphonated polyurethane. The ratio of carbons to nitrogen in BDDS-2.0-LYS-CBz is 14:1, 17:1 in the hydrogenated material, and 18:1 in the parent BDDS-2.0, perhaps indicating some loss of nitrogen with hydrogenation.

X-ray Photon Spectroscopy (XPS)

XPS was run directly on the cast films of polyurethane BDDS-2.0-LYS in its protected form, and on its sulphonated precursor, BDDS-2.0. (The protected form of BDDS-2.0-LYS was subject to charging during XPS analysis). The polyurethane BDDS-2.5 could not be dissolved to prepare films and no XPS data are available for this polymer.

The elemental analysis shown in Table 4, indicate that the sulphur content of the polyurethanes near the surface (take off angle of 60°) is very low in both the BDDS 2.0 and BDDS-2.0-LYS (compare data in Table 3). This may indicate that there is some soft segment enrichment at the surface of the material as has been found by others [Santerre, 1990]. There appears to be silicon contamination which may drive from the glass casting dish. There is, however, no evidence of contamination by palladium indicating that the filtration and precipitation steps following hydrogenation successfully remove the Pd catalyst.

High resolution carbon scans of the BDDS-2.0-LYS and its sulphonate precursor, compared in Figure 3, show very similar trends to those found with the model silica glass surfaces. There is a relative loss of the carboxyl peak (283 eV) and an increase in the carbonyl peak (285 eV) with derivatization.

SURFACE	ELEMENT				
	O	N	C	S	SI
	ATOM %				
BDDS 2.0	22.71	2.36	71.79	0.28	2.86
BDDS-2.0-LYS	22.40	2.51	71.22	0.45	3.42

Table 4: XPS results for the BDDS- 2.0 and BDDS-2.0-Lys polyurethanes. The take off angle was 60°.

Survey scans for elemental analysis were run on the cast films at different take-off angles in an attempt to determine if the composition of the polyurethanes changed with depth. Although there appeared to be some changes with depth particularly in sulphur and soft segment content, the data were inconsistent.

Determination of Lysine by Chemical Methods

Two chemical methods were used to evaluate the lysine derivatization of these polyurethanes, namely the ninhydrin and Bolton-Hunter reagents. The ninhydrin test was not able to distinguish between the lysine derivatized and non-derivatized polyurethanes. All materials gave a blue colour, indicating the presence of free amines.

Under conditions similar to those for the silylated surface, the Bolton-Hunter reagent reacted strongly with all the polyurethanes. Interestingly, the MDA based polymer gave the strongest reaction. It is likely that the reagent reacted with all amine

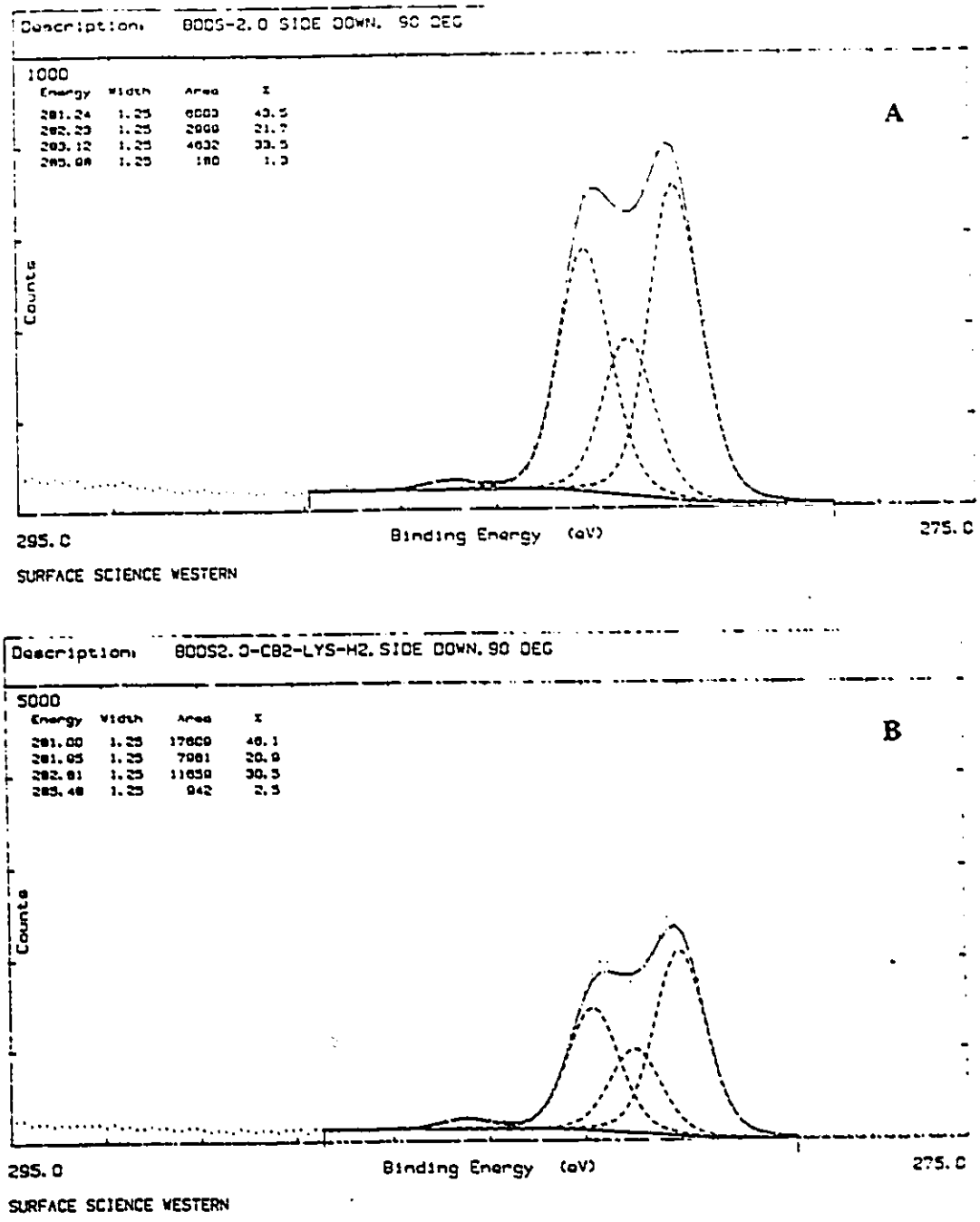

Figure 3: High resolution C 1s scans of the BDDS-2.0 and BDDS-2.0-LYS polyurethanes. A) spectra for BDDS-2.0, B) spectra for BDDS-2.0-LYS.

groups in the polymer and was not specific for the primary amines of lysine.

CONCLUSIONS

The work presented in this chapter points to two overriding conclusions: there is a strong need for control over the synthesis of the sulphonated polyurethanes, and for an analytical method that can reliably determine the amount of amino acid attached to the material. More work is therefore required to determine if lysine derivatization using the method presented in this appendix was successful.

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