IMPORTANCE OF PROTEIN ADSORPTION IN THE BLOOD COMPATIBILITY AND BIOSTABILITY EVALUATION OF POLY ETHERURETHANES (PEUs) CONTAINING SURFACE MODIFYING MACROMOLECULES (SMMs)

Alireza Jahangir

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Graduate Departments of Metallurgy and Material Science and Institute of Biomedical Engineering
University of Toronto

Importance of protein adsorption in the blood compatibility and biostability evaluation of poly etherurethanes (PEUs) containing surface modifying macromolecules (SMMs). Collaborative M.A.Sc., 1999, Alireza Jahangir, Depts. MMS and IBME, University of Toronto.

Abstract

Polyurethanes (PEUs) have been the materials of choice for the manufacturing of conventional blood-contacting devices. Nevertheless, biostability and blood compatibility, are still among the obstacles that limit their applications. Recent studies investigating the development of protective coatings for PEUs have indicated that biostability of the base polymer can significantly be improved by the use of different fluorinated surface modifying macromolecules (SMMs). It is therefore hypothesized that PEU surfaces modified by SMMs may also exhibit superior blood compatibility. To determine the blood compatibility of the PEU containing SMM, a series of *in vitro* experiment were designed to first study the pattern of protein adsorption from plasma, as well as to assess the nature of platelet adhesion and activation on each of the substrates. In the second aspect of this thesis, fibrinogen was used as a simple model to study the influence of plasma proteins on the biodegradation of polymers by a macrophage derived enzyme, cholestrol esterase (CE).

Total protein studies indicated that PPO212L and the PTMO212F were among the SMMs that adsorbed the least amount of proteins relative the other two SMMs (PTMO212I and PTMO322I) and non-modified base. More importantly, it was also observed that the three adhesive proteins (Fg, Vnc, Fnc), implicated in subsequent thrombogenic events, were adsorbed in lower amounts on these two SMM containing surfaces. Platelet adhesion and activation data further highlighted the differences among the various substrates. It was shown that the non-modified base had the highest number of adhered platelets relative to SMM modified surfaces. Among these substrates, PPO212L and PTMO212F had significantly lower platelet densities. Close morphological examination further revealed that platelets residing on PPO212L and PTMO212F were not appreciably activated. Hence, it is believed that the nature of protein/platelet interactions with the polymers was influenced by such factors as hydrophilicity, polymer chain mobility, SMMs' fluorine content, and volume exclusion phenomena.

The biodegradation analysis indicated that the pre-adsorption of Fg onto the modified and non-modified polymers provided a temporary delay in the hydrolytic degradation of the PEU by

cholestrol esterase (CE). This effect was temporal and was lost after seventy days of incubation in CE solution of phosphate buffer, pH. 7.0, at 37 °C.

In conclusion, it was shown that protein adsorption as well as platelet adhesion and enzyme induced hydrolytic degradation, were influenced by the presence of the SMMs at the interface of a PEU. Based on such data, it is believed that PPO212L and PTMO212F were shown the least thrombogenic substrates. Additionally PPO212L provided the most biostable SMM.

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1.0 INTRODUCTION

The field of science referred to as biomaterials dates back as far as two thousands years ago, when Persians, Roman and Chinese used gold in dentistry. However, it was not until the late 1960's that the field began to be recognized through the early symposium presentations on biomaterials such as those held at Clemson University. The first example of a polymeric biomaterial (polymethyl methacrylate) began in dentistry in 1937. After World War II, Voorhees experimented with parachute textile materials (Vinyon N) as a vascular prosthesis (Park 1992). In the late fifties', it was suggested to cardiac surgeon to pay a visit to their local draper's shop and purchase Dacron fabric that could be cut with pinking shears to fabricate arterial prostheses (Park 1992).

The use of biomaterials has evolved over the years and today they are being used for much more complex applications, such as: the growing of cells in culture, the apparatus for handling proteins in the laboratory, the devices used to regulate fertility in cattle, and in the near future they will be used in a cell-silicon "bio-chip" that is intended to be integrated into computers (Ratner 1997). A valid question at this point would be to ask: "how could we reconcile these diverse applications of materials into one field?" The common denominator is the interaction between biological systems and synthetic materials (Ratner 1997).

Despite the advances in materials development over past decades, several serious problems that limit the biomedical use of the biomaterials still persist. These could be referred to as biocompatibility issues. In a gathering of scientists in Chester UK in 1986, the following definitions for a biomaterial and the concept of biocompatibility were formulated: "A biomaterial is defined as a nonviable material used in the fabrication of a biomedical device, that interacts with biological systems"(Williams 1987).

Biocompatibility is defined as the ability of a material to perform with an appropriate host response in a specific application (Williams 1987). The host response is the keyword in the latter definition, since it is this response that determines the biocompatibility of a biomaterial; hence "host response is the reaction of a living system to the presence of a foreign material" (Williams 1987).

It is therefore believed that the biological performance will be determined by the interaction between the material and the living system.

The present thesis is an *in vitro* study which attempts to provide some further comprehension of certain preliminary steps involved in host response, initiated upon contact with a specific group of polyurethane surface active additives, namely surface modifying macromolecules (SMMs). SMMs are new polyurethane-based additives, that upon blending with a base polymer, the surface chemistry is altered, yielding a relatively more biostable and/or biocompatible polymer, while leaving the bulk relatively unchanged (Tang 1996).

1.1 Blood-Contacting Biomaterials

In the case of blood-contacting biomaterials, such as heart valves, vascular grafts, and hemodialysis membranes, an array of interactions between blood elements and the material surface must be confronted. Blood is a complex fluid, primarily made up of proteins and cells (Guyton, 1987). Therefore, any material placed in contact with it must not only meet all of the mechanical and processing criteria, but must also avoid any blood-based pathologic responses such as coagulation, thrombosis, and infection. The immediate problem concerning blood-contacting materials is surface induced thrombogenesis. The exposure of a foreign surface to blood invariably result in the thrombus formation and its potential detachment subsequently could lead to the formation of an emboli, which in turn can travel and lodge within blood vessels, leading to strokes and other atherosclerotic complications (Brash 1991). Although the phenomenon of material induced thrombosis has been an area of intensive research for many years, limited success has been achieved in circumventing the problem and the use of anticoagulants is still required (Hirsh, 1991). The latter mode of treatment often leads to bleeding complications with long term use.

The work reported here, deals primarily with the investigation of blood-material interactions from a standpoint of understanding the causative agents of thrombogenesis, namely plasma proteins and the blood platelets. Later on the effect of protein adsorption and its influence on biodegradation processes, induced by the hydrolytic enzyme, cholestrol esterase, will be studied.

1.2 Application of Polymers in Blood Contacting Devices

Polymeric biomaterials are often employed as blood-contacting biomaterials due to their versatile mechanical properties, the ability to chemically tailor these materials, and the relative ease to create a broad range of products (Lelah 1986). A number of polymeric materials are used in direct contact with blood in a variety of applications such as blood storage containers, catheters, drainage tubes, oxygenators, and various implants. Such materials include polyvinyl chloride, silicone rubber, polyethylene and polyurethanes. Of particular interest is the family of polyurethanes which, are block copolymers that have been found to exhibit relatively good blood compatibility (Lelah 1986). This unique class of polymers may be synthesized to form a variety of products that can display a range of both mechanical and chemical properties (Saunders 1967). It is the combination of versatility and biocompatibility demonstrated by these polymers that has led to their wide use as blood-contacting biomaterials. However, upon blood-polymer interaction, and due to the release of various enzymes and degradative elements from inflammatory cells, chemical biodegradation of the polymer can be followed. Specifically, the hydrolysis of ester bonds and oxygenation of other groups in polyurethanes are the prime mechanisms by which polyurethane can be degraded *in vivo*. Different approaches have been taken by different investigators to circumvent this problem, and to improve the biostability of the polymer (Ratner 1987). Recent investigations by Dr. Santerre's group have led to the synthesis of a series of surface modifying macromolecules (SMMs), that upon addition to base polymer significantly alter the surface chemistry of the segmented polyurethane (Tang 1995, Pham 1996, and Weiler 1997). It was shown that these fluorine containing SMMs migrate to the surface of the polymer mixture, yielding a new hydrophobic substrate (Tang 1996). Previous works have also shown that this new surface chemistry provided more biostable polyurethane by protecting the hydrolysable groups of the polyurethane (Tang 1995, Jahangir 1996, and Weiler 1997).

1.3. *In Vitro* Investigation of Blood-Material Interactions:

The study of blood-material interactions is complicated, both because of the array of biological reactions involved in the blood response to foreign materials and in the inherent difficulty in modeling the biological systems experimentally. To investigate a particular aspect of the blood-material response, some degree of simplification must be made. However, since the hemostatic

reactions are interdependent and often synergistic in nature, the validity of any simplified *in vitro* system is always in question (Guyton, 1987). Nonetheless, the impossibility of human *in vivo* examination requires that *in vitro* experimental procedures be developed which attempt to reflect, as far as possible, conditions of the *in vivo* situation. Examples of such systems have been described to simulate the *in vivo* situations both biologically and rheologically. (Baumgartner 1973, Feuerstein 1975, Sakariassen 1983, and Fukuyama 1989).

In addition, there is no single *in vitro* test for the assessment of material blood compatibility. A few examples of such tests include clotting time, protein adsorption, and platelet adhesion response. Brash *et al* (Brash 1986, Cornelius 1992, and Brash 1993) have been performing numerous studies to determine the influence of protein adsorption characteristics on various biomaterial surfaces and their subsequent interactions with other biological systems.

1.4 *In Vitro* Assessment of Blood-SMM Containing Materials

Due to the lack of sufficient information on the importance of blood derived elements on the blood compatibility and biostability of PEU containing SMMs, the following hypotheses were formulated:

1. The PEU surfaces modified by SMMs may alter the protein adsorption patterns at their interface.

2. As a direct result of changes in protein adsorption pattern, surface fluorination via SMMs will reduce platelet adhesion to the surface, rendering the surfaces less thrombogenic.

3. The adsorption of dominant plasma proteins (e.g.: fibrinogen) will inhibit the ability of cholestrol esterase to degrade the polyurethane surfaces.

The focus of the present work was to use *in vitro* biological tests to evaluate the polyurethane containing different SMMs. Based on above hypotheses the objectives were to assess plasma protein adsorption patterns onto these PEU containing SMMs, and to determine how this phenomena may influence subsequent biological processes such as, platelet adhesion and activation as well as enzyme catalyzed biodegradation of the underlying base polymer.

2.0. LITERATURE REVIEW

2.1 Polyurethanes

Biomedical applications of polyurethanes in blood contacting devices as well as tissue implants, began since 1930's (Lelah 1986). Polyurethanes have demonstrated mechanical and physical superiority, and more importantly possess acceptable blood compatibility (Lelah 1986). Their initial performance was poor and marked by the induction of thrombosis and inflammation upon blood contact. Over the years, however, it was found that these failures were partly contributed by the technical methods used to prepare the implants and more importantly due by poor understanding of their surface characteristic (Lelah 1986).

The segmented polyurethanes used in this research are a subgroup of the family of thermoplastic elastomers. They are composed of short, non-compatible, alternating blocks of soft and hard-segment units. The terminology for "soft" and "hard" segments drives from the morphological and the physical characteristics of each block provided. For example, the soft segment provides an amorphous, rubbery phase, and the hard segment provides a rigid crystalline phase (Lovell 1991).

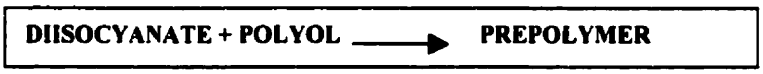
The chemical incompatibility between the hard and soft segments will lead to microphase separation, which is essentially formation of macroscopic domains consisting of pure homopolymer (Andrade 1985). Many of the thermal, mechanical, and biological properties can be explained in terms of these domain structures (Marchant 1987).

The hard segment normally consists of a highly reactive aromatic diisocyanate, and is polymerized with a low molecular weight diol or diamine, called a chain extender. The product of such a reaction is an oligomeric aromatic urethane or urethane-urea segment of molecular weight between 300-3000 (Lelah 1986). Two diisocyanates used in the present research are 2,4-toluene diisocyanate (TDI), and 1,6-hexane diisocyanate (HDI). Other aromatic diisocyanates include 2,6-toluene diisocyanate and methylene bis (p-phenylisocyanate) (MDI).

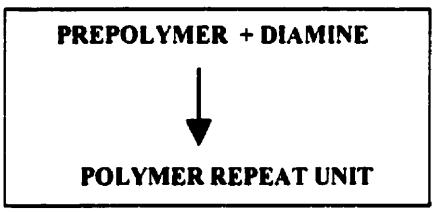
The soft segment is an oligomeric diol or a polyol. The oligomeric diols used in the current study are all polyether-based materials. Relative to polyester-based materials, the polyether based

urethane exhibit a relatively high resistance to hydrolytic cleavage and hence is more biostable. A commonly used polyether in the synthesis of polyurethanes is polytetramethylene oxide (PTMO). Polyurethanes prepared with this soft segment generally exhibit good mechanical strength and possess relatively good hydrolytic stability and water resistance (Lelah 1986).

Segmented polyurethanes can be synthesized using a two step solvent polymerization also known as the pre-polymer method. In this two step reaction procedure, an oligomeric pre-polymer is first formed by a reaction of a diisocyanate with the oligomeric diol, leading to the formation of a urethane linkage.



The second step, also known as the chain extension step, is the reaction of the prepolymer with the chain extender. This reaction may require several hours depending on the reactivity of the chain extenders toward the terminal isocyanate groups of the prepolymer.



Among the factors to consider during the polymer synthesis is the solvent. The choice of the solvent may affect both the rate of the reaction and the effectiveness of the catalyst. In general, solvents that readily complex with an active hydrogen compound or catalyst (for example, by hydrogen bonding or dipole moment interactions) will result in a slower reaction than with solvents that cannot readily associate with reactant or catalyst (Saunders 1962). Common solvents used in the synthesis include N, N-dimethylacetamide (DMAC), dimethylformamide (DMF), tetrahydrofuran (THF), and dimethylsulfoxide (DMSO). These are all polar solvents that can provide a nonreactive medium in which the diisocyanate, the oligomeric diols, the diol or diamine, and the synthesised polymers are all soluble (Lelah 1986).

2.1.1 Microphase Separation

The segmented block copolymers may be represented as $(AB)_n$, where A may be considered as hard block and B is the soft block. As previously mentioned, these two segments are thermodynamically incompatible, and once reacted they can form microdomains. Due to their low glass transition temperature (Tg), the soft domains can be rubbery and amorphous, while the hard domains may be rigid and crystalline, as result of their high melting temperature (Tm). In the solid specimen under tensile force, the soft domain will stretch while the hard domain provides physical cross-linking and dimensional stability. In fact, the elastomeric properties of segmented polyurethanes arise primarily from their domain microstructure (Bonart 1968).

The degree of phase separation is affected by several parameters, including: segment length, segment polarity, crystallisability, overall composition, mechanical and thermal history, and the possibility of hydrogen bonding between the urethane linkages as well as the carbonyl or ether functional group (Lelah 1986). In general, increased phase separation leads to improved mechanical properties, as the hard segment interaction is uninterrupted allowing for semi-crystalline domain formation (Lelah 1986).

At the same time, the biocompatibility of segmented polyurethanes is known to be influenced by polyurethane domain structure (soft and hard segments), particularly the relative amounts of these components at the surfaces of the materials (Lelah 1986, and Takahara 1991). Protein adsorption studies from plasma onto polyurethanes having both hydrophobic and hydrophilic characters, showed that fibrinogen exhibited the Vroman effect on these polymers (Brash 1986). This topic will be considered in the subsequent sections. Briefly, the Vroman effect is a displacement of the proteins in plasma or a multi-component solution that occurs in a competitive and a time dependent manner (Vroman 1969).

Similarly, cellular adhesion and specifically platelet reactivity on hydrophobic, segmented poly-ether-urethane-ureas was also affected by microphase separation (Takahara 1991). Other investigators have shown that polyurethanes with polypropylene oxide (PPO) soft segments supported a higher level of platelet adhesion than polyurethanes with a polyethylene oxide (PEO) soft segment (Whicher 1978). It is generally believed that a relatively good hemocompatibility is

associated with the type and surface concentration of the hard segment of polyurethane (Lelah 1986, and Grasel 1987).

2.1.2 Degradation of Polyurethanes

Despite the widespread interest in PEU as biomedical elastomers, little is known about their degradation behavior (Marchant 1987). There are various mechanisms by which polyurethanes can be degraded, including hydrolysis and oxidation. Oxidation can be initiated via heat, UV light or chemical oxidants (Young, 1991). Hydrolytic degradation of polyurethanes, on the other hand, takes place when water reacts with the ester, urea, amide or the urethane linkages of the polymer. When implanted these materials can undergo both oxidation and hydrolysis due to species existing within plasma and those released during the inflammatory or foreign body response (Lelah, 1986, and Boyes 1992).

Other factors that determine the material's biostability, include the physical nature of the polymer (i.e., degree of microphase separation, porous versus non-porous), the chemical nature of the polymer (i.e., aliphatic versus aromatic), mechanical stress, the size, the shape and the roughness of polyurethane implant (Lelah, 1986).

It is suggested that the initiation of polymer degradation occurs via free radical formation and peroxide oxidation (Szycher, 1991). Free radicals react with oxygen to form peroxy radicals. The peroxy radical reacts with unstable hydrogen to form another peroxy radical, which continues the cycle. Furthermore, this unstable product decomposes into an alkoxy radical and a hydroxyl radical which further amplifies the degradation cycle (Szycher, 1991). When this cycle is complete two radicals combine to form a stable product.

2.1.3 Enzymatic Degradation of Polyurethanes

In the early studies on biodegradation, it was shown that polyurethanes could be degraded *in vitro* by certain enzymes and under certain conditions (Lelah 1986). However, it was difficult to characterize the amount of polyurethane degradation, both *in vivo* and *in vitro*. The *in vitro* degradation studies were usually based on small changes in molecular weight and mechanical

properties, while *in vivo* animal studies were costly and the mechanisms were difficult to identify. In an effort to correlate enzymatic degradation with the degradation of polymers, polyamide, polyesters and polyurethanes were synthesized with the incorporation of a ^{14}C-label and were subsequently exposed to a variety of enzymes, *in vitro* (Williams 1986). Though the amount of degradation was low, they found that some of the polymers were more susceptible to surface degradation than others.

In other studies, polyurethanes were treated with three different enzymes: papain, chymotrypsin and leucine aminopeptidase and changes in molecular weights were measured (Ratner 1988). In contrast to conventional thought, they found that the polyester-urethane degraded the least. Other authors have employed techniques such as Fourier Transform Infrared spectrometry (FTIR), to study the polymer degradation. For example, the degradation of polyether-urethane incubated in trypsin for 5 months was noted, by changes in soft segment chemistry (Bouvier 1991).

Other investigators studied the process of papain mediated hydrolysis on a polyetherurethane urea, prepared with and without stabilizers (Marchant, 1989). Different methods of determining the extent of degradation were utilized, such as: fatigue testing, optical microscopy, electron spectroscopy chemical analysis (ESCA), FTIR and chromatographic methods (Marchant, 1989). It was noted that degradation occurred in both enzyme and water treated samples. However, degradation was limited to the surface of the material. It was found that the presence of stabilizers inhibited degradation in aqueous mediums but only reduced degradation in enzymatic mediums.

In an *in vivo* setting, polymers are degraded because body constituents (e.g.: enzymes, ions, acids, water, superoxide anions etc.) attack the biomaterials directly or indirectly. It is generally believed that following initial protein adsorption onto the implanted biomaterials, an acute inflammatory response is initiated characterized by the presence and adhesion of various cellular components (e.g., neutrophils and monocytes) of the blood (Coury 1988). For example it has been shown that activation of monocytes will lead to the release of a whole host of cytokines (e.g. Il-1, TNF α, and IL-6) and enzymes (Cholestrol Esterase) (Holmes 1993, and Labow 1998). It is suggested that enzymes initiate their different degradation mechanisms once they recognize and interact with a polymer or more accurately of polymer coated with specific serum proteins, *in vivo* (Pitt 1986). It is therefore believed that enzyme-polymer interaction is a specific phenomena and is determined by

the nature of the adsorbed protein layer. A few enzymes participating in such degradative phenomena include protease, esterase, lipases, and glycosidase. Each enzyme is named based on the molecular structure that they interact with (Coury 1996).

In more recent studies conducted by Santerre *et al* (Santerre 1993, 1994, and Labow 1998) polyurethanes having specific segments labeled with ^{14}C were investigated. These studies focused on the interaction of the enzymes with the chemistry of the polyurethane at the molecular level. They concluded that cholesterol esterase (CE), a hydrolytic enzyme, was an effective enzyme for the degradation of some polyurethanes (Santerre, 1994). In addition, they proposed that urethane/ester-linkages in the soft segments of the polyurethane were more susceptible to enzymatic attack by CE than the urea/urethane groups of the hard segment.

In other studies, Labow *et al* have used live cell culture systems to evaluate polyurethane susceptibility to biodegradation (Labow, 1995). They monitored the release of material from a variety of polyurethanes during exposure to neutrophils, isolated from human blood. Two of the identified enzymes were elastase (Labow 1995) and more recently catalase (Maslove, 1996) as possible contributors to enzymatic degradation of biomedical implants.

2.1.4 Polyurethane Surface Additives

To improve the biostability and to manipulate the blood compatibility, additives are incorporated into biomedical polyurethanes. Biocompatible polymeric surfaces can be classified into four categories based on the additives employed for regulate blood material interactions and the mechanisms by which they achieve this task.

The first approach is based on the physicochemical properties of the material. Highly hydrophobic and highly hydrophilic polymeric materials resist protein adsorption and therefore can reduce thrombus formation (Tsuruta, 1993). The second approach to regulate blood-material interactions is based on the specific manipulation of the interfacial chemistry. Blood compatibility can be enhanced by altering the surface chemistry to mimic the natural properties of the surrounding tissue wall. A third approach involves the addition of biologically active molecules to the material surface. These molecules might include anticoagulant molecules, heparin, prostaglandins or urokinase (Male, 1991).

The final approach involves the adsorption of plasma derived molecules to the biomaterial surface (Tsuruta, 1993). For example, engineered biomaterials which can selectively adsorb albumin have been shown to be relatively non-thrombogenic (Tsuruta, 1993). The review of literature in this section will focus only on few of the relevant approaches.

2.1.5 Physicochemical and Interfacial Additives

To produce a stable biomaterial a balance must be obtained between biostability, biocompatibility and the physical properties of the polyurethane. This balance can often be achieved with the use of additives. Many additives intended to improve the interfacial biostability or biocompatibility with the host environment are termed surface modifying additives (Ward 1984). The processing additives however are generally added to manipulate polymer properties (Carraher 1993). Table 2.1 summarizes some of the intended applications of polymeric additives.

plasticizers	**flame-retardants**
Lubricants	**viscosity modifiers**
Coloring agents	**filters**
Reinforcements	**stabilizers**
Antioxidants	**antimicrobial agents**
Impact modifiers	**antiblock agents**
Curing agents	**antistatic agents**
Foaming agents	**preservatives**

Table 2.1. Applications for Polymeric Additives, adapted from Carraher 1993.

2.1.6 Additives for Altering Biological Interaction

One method by which additives improve biostability is through the inhibition of crack formation. Microcracks cause the weakening of material as well as acting as sites for the nucleation of thrombus (fibrin network entrapping blood components predominantly platelets) and mineralization formation (Wu, 1991). Plasticizers have been used, in an effort to reduce the cracks. Typically, these materials lower the inter-polymer chain attraction, enabling the chains to slide past each other (Carraher, 1993). However, the universal use of plasticizers in various biomedical applications has raised some concerns. Low molecular weight plasticizers and additives can leach from the polymer into surrounding tissue or blood. The partitioning of the leachable is a function of the additive's inherent solubility in the polymer relative to the biological medium. Lipid soluble additives may

affect cell function by modifying cell membranes, although this may not be strictly detrimental (Frantantoni, 1992).

Another groups of polymer additives are anti-oxidants that are used to inhibit oxidative degradation. Although these additives can protect biomedical polyurethanes from oxidative stress cracking they can jeopardize biocompatibility when used in large quantities (Szycher, 1991). Free radical scavenger anti-oxidants inhibit oxidation via chain-propagating radicals. Their reactive -OH or -NH groups transfer hydrogen to free radicals or peroxy radicals, limiting further degradation.

Wesslen *et al* (1994) have shown that hydrophilic surfaces will adsorb less protein overall and therefore have a reduced thrombogenicity. On the other hand, other investigators have shown that a hydrophobic surface will passivate a substrate through the preferential adsorption of albumin (Munro 1981). It is believed that albumin does not contain specific amino acid epitope to which platelets can bind (Munro 1981). Therefore, preferential adsorption of albumin is believed to render the substrates non-thrombogenic. Hence, the specific surface chemistry, which dictates the hydrophobicity/hydrophilicity character of the polymer surface, is one of the contributing factors in the biocompatibility of the material.

2.1.7 Fluorinated Agents as Additives

As mentioned earlier the specific chemistry which dictates the surface energy character of the polymer surface plays an important role in the biocompatibility and biostability of the materials. In fact, it was observed that the rate of hydrolytic degradation of the polyurethane (PU) decreases in a highly hydrophobic system (< 2.0% water saturation) whereas highly hydrophilic polymers (e.g. hydrogels) perturb this effect (Coury 1996). As far as biocompatibility was concerned, it was observed that poorly wettable surfaces show very low coagulation activation level whereas those with highly wettable surfaces exhibited a high coagulation activity (Vogler 1995). In one particular study it was shown that the presence of fluoroalkyl groups as chain extenders in PU, would result in a polymer that is non-thrombogenic (Kashiwagi 1993). It is believed that due to fluorine group's low surface energy, they tend to be preferentially concentrated on the surface of the polymer and as such, inhibit protein adsorption as well as platelet adhesion and activation (Kashiwagi 1993).

In an effort to improve thromboresistance, Ward *et al* have reported the use of surface modifying additives (SMA) blended with biomedical polymers (Ward, 1984). Blending these, silicone based, amphipathic additives at low concentrations with a base polymer resulted in the spontaneous migration of the low energy SMA to the air-surface interface (Ward, 1984). The saturation of the SMA at the surface is thought to reduce thrombogenicity by minimizing protein denaturation or affecting protein adsorption. *In vivo* evaluation of materials containing SMAs were performed through the implantation of the Pierce-Donachy prosthetic ventricle with blood pumping sacs and cannulas fabricated with SMA blends (Ward, 1984). They found that the SMA copolymer blend was superior in thromboresistence character relative to the commercial polyurethane (Biomer®) without accompanied loss in mechanical or physical properties.

A number of new fluorinated surface modifying macromolecules (SMMs) with a broad range of molecular weights (1.6×10^4 to 5.5×10^4) were developed in Dr. Santerre's group, and their effect in inhibiting degradation of polyurethanes was studied (Tang 1995, Weiler 1997). These SMMs were linear polyurethanes with different polyol segments, end-capped with a fluorinated alcohol. It was observed, these SMMs were able to selectively migrate to the surface without affecting the bulk properties, while improving the biostability of the base (polyester-urethane). Contact angle measurements revealed that the fluorine rich surfaces reduced the surface energetic (Jahangir 1996) and it was suggested that presence of these fluorine moieties at the surface restricted the access of the enzymes to the susceptible groups in the base polymer (Tang 1997). In addition, this principle was further validated, when fibrinogen adsorption studies revealed that poly-ester-urethane containing SMMs significantly decreased fibrinogen adsorption which may indicate the non-thrombogenic character of this material (Tang, 1997).

2.2 Fundamentals of Protein Adsorption to Biomaterials

The adsorption of proteins is important in many interfacial phenomena, including blood/biomaterial contacting systems, cell culture on solid supports, bacterial adhesion to teeth and to implanted materials, soil bacterial growth, fouling of contact lenses in tear fluids, and the protein fouling phenomena that takes place during food processing (Brash 1994). In general proteins are surface active and tend to accumulate at interfaces (Norde 1991).

The chemical and physical characteristics of both the protein and the adsorbing substrate material influence the interactions. Proteins are natural polymers composed of "mer" units known as amino acid residues linked together via peptide (CO-NH) bonds. These molecules consist of one or more polypeptide segments, and range in length from approximately forty to over four thousand amino acid residues. Since the average mass of an amino acid residue is approximately 110 D, proteins have molecular masses that range from approximately 4 to over 440 kD (Voet1995). 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 its three dimensional arrangement; that is, the folding of its secondary elements, together with the spatial dispositions of its side chains. It results largely from unfavorable interaction between the amino acids. In solution the three-dimensional shape attained by the protein will be that which minimizes the Gibbs free energy (G) of the system including the interactions between the protein and the surrounding medium (Norde 1991). The major driving force for the folding of a polypeptide in solution is the dehydration of hydrophobic amino acid side chains (Norde 1991). Amino acids with hydrophobic side groups tend to be in the interior of the molecule, away from water. These charged or polar side chains tend to be predominantly on the periphery of the molecule (Norde 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 1991).

Enzymes are special class of proteins, which are responsible for many of the interactions, which occur when biomaterials are placed in blood. Enzymes are biological catalysts that carryout their biological functions via specific binding to a substrate and their activation are conformationally dependent. This binding is achieved via an "active site" which is characteristic of the enzyme. Factors that affect conformation such as temperature, pH, presence of other proteins, and adsorption to a surface may affect the enzymatic activity.

The surface properties of a material will influence its interactions with proteins and are likely to alter the protein conformation and enzymatic activity (Andrade 1985, and Norde 1991). In most of the protein adsorption studies, surfaces have frequently been classified as either hydrophobic or

hydrophilic. Protein adsorption is generally considered to be greater, onto hydrophobic surfaces than hydrophilic ones (Brash, 1992). However, others have shown that protein adsorption can not simply be correlated to surface hydrophilicity and/or hydrophobicity. For example, a maximal protein adsorption was obtained when both a hydrophilic and a hydrophobic content of the surfaces were present in intermediate amounts (Van Damme 1990). Other researchers have found stronger relationships between competitive and single protein adsorption studies and surface hydrophobicity (Brash 1979, Horbett 1981, Slack 1985, whitesides1991). Nevertheless, the exact relationship between hydrophobicity and protein adsorption from pure buffer and/or plasma has not clearly been established (Wojciechowski, 1992).

There is evidence to suggest that surface hydrophobicity/hydrophilicity influence the conformation of the protein when it is adsorbed. Conformational changes induced by protein/material interaction may result in the inactivation of enzymes. For example, it was shown that many enzymes were inactivated when adsorbed to a hydrophobic latex surface from solutions of low enzyme concentration (Sandwick 1987). It was also shown by other investigators, that complement factor 3 (C3), adsorbed to hydrophobic surfaces exposed antigenic determinants normally only accessible to antibody after denaturation or activation (Elwing 1987). Ito *et al* (1990) found that denaturation of proteins adsorbed to surfaces correlated with increased platelet adhesion and thrombogencity.

2.2.1 Single Protein Adsorption

The adsorption of protein is different and unique than other species, in that proteins are large and as mentioned earlier, chemically heterogeneous (i.e.: contain hydrophobic regions, electrically charged regions, and polar regions). This chemical heterogeneity is also present in surfaces of the solid substrates, which implies that a number of different protein-surface binding mechanisms are possible.

It is generally believed that protein adsorption, during the initial stages of the process (that is when fractional surface coverage is low, less than 10%), is rapid and the rate is therefore transport controlled. In a static system, the transport to the surface is by diffusion alone and the rate of adsorption equals the flux to the surface, given by:

$$d\Gamma/dt = C_o (D/\pi t)^{1/2} \quad [1]$$

so that

$$\Gamma = 2C_o (Dt/\pi)^{1/2} \quad [2]$$

Where,

Γ = Surface concentration

C_o = Solution concentration

D = Diffusivity

t = Time

Under the flow condition systems, both convective and diffusive mass transport occurs. In the later stages of adsorption, when surface coverage exceeds about 10%, the protein adsorption rate is controlled by such phenomena as protein-protein adsorption involving competitive displacement and other related mechanism (reaction control) (Brash 1992).

2.2.2. Protein Adsorption from Protein Mixture

As mentioned earlier, proteins are surface active, readily adsorbing to solid surfaces. In a protein mixture, one protein may preferably adsorb and its adsorption is 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. Due to the complexity of the blood and the plasma, simple mixtures of plasma proteins in buffer have been studied as models for both plasma and blood (Brash 1992).

The proteins most commonly investigated in competitive studies from buffer are immunoglobulin G (IgG), albumin (Alb), and fibrinogen (Fg) (Horbett, 1984, Pitt 1986, Sharma 1986, and Bale 1988). These have been selected for study because of their high plasma concentration (120-500 μM, 500-800 μM, and 9 μM respectively) and their availability.

It was shown that fibrinogen preferentially adsorbs from protein mixtures, particularly in the presence of IgG and albumin (Horbett, 1984, Vroman 1987, and Bale 1988). Using a three major plasma protein mixtures of IgG, fibrinogen, and albumin at representative physiological concentrations, Brash *et al* (1984) showed that the level of adsorption for each protein was the reverse of their relative concentrations in the bulk solution, e.g. albumin < IgG < fibrinogen. This was validated for a variety of surfaces. It was also found that Fg adsorption was preferred over albumin and IgG on several different surfaces including a segmented urethane-urea (Horbett 1987). Using a somewhat different protein mixture, it was shown that fibrinogen preferentially adsorbed on polystyrene latex (Bale 1988). It was estimated that the relative affinities of the proteins studied were in the order fibrinogen > fibronectin > IgG > albumin. However, evidence from simple competitive adsorption experiments has shown that hemoglobin adsorbs preferentially to polyethylene and the relative order for protein affinities to this material has been estimated as hemoglobin > fibrinogen > albumin ≈ IgG. In addition, other researchers have investigated less abundant plasma proteins, such as high molecular weight kininogen (HMWK) (Elwing 1987), and vitronectin (Fabrizius-Homan 1991). Results from these studies have indicated that fibrinogen may not always be preferentially adsorbed. In one study it was shown that vitronectin had a high surface affinity relative to human serum albumin, fibrinogen, and fibronectin when studied in both binary and competitive systems (Preissner 1991). In a study of fibrinogen adsorption onto polyethylene and glass using binary mixtures of either fibrinogen/albumin or fibrinogen/hemoglobin, fibrinogen adsorption showed a maximum surface concentration (~ 0.09 $\mu g/cm^2$) at 5% of normal plasma concentration (Slack 1987). It was shown that this maximum shifted to lower bulk fibrinogen concentrations when the ratio of competing protein to fibrinogen was increased. The same observation was seen in the plasma. In another experiment, it was found that a linear correlation exist 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 1986).

The data obtained from the extrapolation of competitive adsorption found in mixtures of proteins to plasma and whole blood data has not been always so clear. For example, there were 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 surfaces evaluated in plasma (Grainger 1990). Other investigators have found differences in the adsorption characteristics of a Fg

adsorbed from a mixture of proteins compared to its adsorption from plasma or whole blood (Horbett 1984, and Brash 1984). These studies suggest that, binary or ternary systems may not be an appropriate model for the plasma system or whole blood systems.

2.2.3 Protein Adsorption from Plasma and Blood

Protein adsorption from plasma appears to have very little to do with the relative concentration of a protein in plasma. For instance, albumin in general has been found to have very low adsorption levels from plasma onto surfaces (Brash 1979, Bale 1988). It was observed that other proteins, such as fibrinogen and albumin, undergo transient adsorption to surfaces manifested as a maximum in the adsorption data in a time or concentration dependent manners (Wojciechowski 1986, Brash 1989, and Andrade 1991). Such phenomena was first observed by Leo Vroman 1960's (Vroman 1969). Later other investigators studied the adsorption of fibrinogen, albumin, and IgG to three surfaces, and found that neither albumin nor IgG adsorption to glasses showed any dependence on plasma concentration (Brash 1984). In contrast, fibrinogen adsorption increased with plasma concentration and then decreased. Horbett obtained similar results for fibrinogen adsorption from plasma to polytetrafluoroethylene (PTFE), polyethylene and glass (Horbett 1984). It was later found that fibrinogen adsorption from plasma could not be correlated to surface chemistry alone (Wojciechowski 1991).

The competitive nature of protein adsorption from plasma is explained by the Vroman effect, 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 1969). Vroman suggested that HMWK is a major displacer on many surfaces and that its presence in the adsorbed layer promotes surface induced coagulation (Vroman 1980, Andrade 1991, and Scott 1991). However, the exact effect of HMWK on fibrinogen adsorption from plasma has not been clarified. Other investigators have found that on hydrophilic surfaces fibrinogen was displaced by HMWK but the similar trend was not exhibited by hydrophobic substrates, despite the occurrence of the Vroman effect for fibrinogen (Elwing 1987). Slack *et al* have found that using baboon fibrinogen, there was

no difference in fibrinogen adsorption from normal plasma, and from plasma devoid of HMWK, to silicone rubber or polyethylene (Slack 1987).

Although, the details of the Vroman effect are still not completely understood, it appears that it is a surface related, as well as protein dependent phenomena. In addition, not all the proteins show the same adsorption behavior on all surfaces. Furthermore, the relevance of the Vroman effect to thrombotic events is not yet clear. It is believed more in depth understanding of this mechanism may lead us to identify the parameters that define the nature of biomaterial surfaces in contact with blood.

2.2.4 Effect of Polyethylene Oxide (PEO) on Protein Interactions

Incorporation of hydrophilic PEO chain as part of a polymer matrix has indicated that it will render the polymer non-reactive towards protein adsorption and cellular interactions (Lee 1997). It is believed that PEO's, minimum interfacial energy with water, hydrophilicity, high surface mobility, steric stabilization effect, and unique solution properties as well as molecular conformation in water, will endow polymer surfaces with more blood compatible character (Lee 1997).

2.3 Mechanisms of Hemostasis and Thrombosis

The term hemostasis means prevention of blood loss. In more general terms, humans have evolved an intricate hemostatic mechanism designed to maintain blood in a fluid state under physiologic conditions but primed to react to vascular injury in an "explosive manner to minimize the blood loss by sealing the defect in the vessel wall (Guyton 1989). This mechanism is composed of a complex series of interactions between blood proteins, cells and the vessel walls. Thrombosis may occur if the hemostatic stimulus is unregulated either due to impaired capacity of inhibitory pathways, or more commonly when the capacity of the natural anticoagulant mechanism is overwhelmed by the intensity of the stimulus.

In an *in vivo* setting blood vessels are normally covered by a single layer of cells known as endothelium, which maintain blood fluidity by inhibiting blood coagulation and platelet aggregation

and promote fibrinolysis. It also provides a protective barrier that separates blood cells and plasma factors from highly reactive elements in the deeper layers of the vessel wall. These hidden components include adhesive proteins such as collagen and von Willbrand factor (vWF), which promote platelet adhesion; as well as tissue factor, a membrane protein located in fibroblasts and macrophages that triggers blood coagulation. When vessels are damaged, they undergo vasoconstriction, hence diverting the blood from the site of injury. The blood however comes into contact with sub-endothelial structures, which stimulate the homeostatic plug formation by promoting platelet adhesion and aggregation, and by activating blood coagulation. When platelets are stimulated by sub-endothelial collagen, they expose and/or assemble membrane glycoproteins IIb and IIIa (GP IIb/IIIa), which can then bind to fibrinogen and vWF, which contain the specific amino acid sequence (RGD) required for platelet adhesion. Secretion of proteins from platelets' α granules is mediated by thromboxane A_2 synthesis, phosphorylation of specific proteins, and intracellular calcium translocation. Other protein co-factors accelerates factor X and prothrombin activation. This results in thrombin formation, which increases its own production many fold by converting Factors V and VIII into activated cofactors and stimulating platelet secretion (Guyton 1989).

The cellular and molecular reaction is modulated by endothelial cell elaboration of antithrombotic lipids (PGI_2), proteins (thrombomodulin), inorganic compounds (nitrous oxide), and several plasma protease inhibitors, most importantly anti thrombin III for Factors IXa, Xa, and thrombin. A major substrate for thrombin is fibrinogen, which after initial hydrolysis form fibrin monomers that then undergo spontaneous polymerization to form the fibrin clot (Cazenave 1990). Plasminogen, a plasma zymogen, is converted to plasmin by plasminogen activators released by endothelial cells. Plasmin does not normally act on fibrinogen in solution because of the presence of α_2-antiplasmin. However, plasmin on the surface of the clot is protected from the inhibitor, and fibrinolysis occurs with the formation of fibrin degradation products. Dissolution of the clot paves the way for the deposition of collagen, formation of fibrous tissue, and wound healing (Guyton 1989).

2.3.1 Platelet Physiology

Platelets ("little plates") are non-nucleated disk shaped cells having a diameter of 3-4 μm and an average volume of 10×10^{-9} mm^3. Platelets are produced by bone marrow, circulate at an average

concentration of about 250,000 cells per microliter and occupy about 0.3 % of the total blood volume. Platelets circulate for an average of ten days before being removed in the spleen and liver (Shulman 1987). Platelets perform the following functions in blood: 1. Initially arrest bleeding through formation of the platelet plugs. 2. Stabilize platelet plugs by catalyzing coagulation reactions, leading to the formation of fibrin (Hanson 1993).

Morphologically, platelets are composed of three basic regions: the peripheral zone containing the cell membrane, the sol-gel zone or cytoplasmic region, and the organelle zone consisting of various cell organelles. The platelet's peripheral zone consists of the outer membrane and closely associated structures, which provide the surface of the platelet including the walls of the surface-connected open canalicular system (OCS). This system enhances the surface area of the platelet membrane and provides a transport route for the uptake of plasma-borne substances and the secretion of granular products during the release reaction (White, 1987). At the same time, the OCS interacts with the dense tubular system (DTS) which is the site of calcium sequestration and enzymes utilized in prostaglandin synthesis. The external surface coat of the platelet contains membrane bound receptors (glycoproteins Ib and IIb/IIIa) that mediate the contact reactions of adhesion (platelet-surface), and aggregation (platelet-platelet). The adhesion response is closely related to cell activation whereby the activity of the membrane receptors triggers chemical messengers which act to cause physiological and chemical alterations in platelet function (Nishizuka, 1989).

The peripheral zone of the platelets acts both as a barrier between the cellular constituents and the flowing blood, as well as a communication pathway and selective transporter both into and out of the cell. Platelet's sol-gel zone is so named, since the cytoplasmic matrix of the cell resembles a gel. This area contains substantial amount of muscle protein (e.g., actin, myosin), which allow for shape change, pseudopodia formation, external contraction and secretion when necessary (White, 1987).

The organelles of the platelet are embedded in the cytoplasm. Platelets contain three types of cytoplasmic storage granules and two other constituents:

1. α-granules, which are numerous and contain the platelet –specific proteins platelet 4 (PF-4) and β-thromboglobulin (β-TG), and proteins found in plasma (fibrinogen, albumin, fibronectin, coagulation factors V and VIII);

2. Dense granules which contain adenosine diphosphate (ADP), calcium ions (Ca^{2+}) and serotonin;
3. Lysosomal granules containing enzymes (acid hydrolases);
4. Peroxisomes;
5. Mitochondria (Table 2.2).

Platelets can get activated upon minimal stimulation. This activation will cause a morphological change of the platelets; allowing them to become more "sticky" and alter their shape to irregular spheres with spiny pseudopodia, accompanied by internal contraction and release of storage granules into the extracellular environment (Hanson 1993).

Under normal physiologic conditions, platelets do not bind to the undisturbed endothelial lining of the vasculature. Platelets do however adhere to artificial surfaces, and injured blood vessels. Upon contact of platelets with such "abnormal" surfaces, and their subsequent activation, they respond by adhering to the surface and by releasing the molecules mentioned above, facilitating the subsequent coagulation mechanism (Niewiarowski 1987).

A platelet plug is created by the successive adherence of activated platelets upon each other and the platelet membrane provides a surface for the formation of coagulation complexes, thus increasing the rate of the coagulation reactions (Mann 1990). The concurrent initiation of the coagulation cascade results in the deposition of fibrin throughout the platelet mass stabilizing the plug and entrapping circulating red blood cells.

2.3.2 Platelet receptors

Platelet receptors are membrane proteins which, can interact with a specific agonist leading to a distinctive intracellular response which may be reversible. The importance of adhesive interactions in normal platelet function is reflected in the battery of receptors present on the cell membrane. These receptors include, integrins, selectins and leucine–rich glycoproteins (Kieffer 1990).
A degree of conformational similarity is necessary between ligand and platelet membrane receptors. Since the molecules affecting platelet function are diverse in structure, it can be expected that a different receptor must exist for most stimulating agents (Table 2.3).

Granule Type	Content
Dense body	Adenine nucleotides (ADP& AMP)
	Guanine nucleotides (GTP & GDP)
	Serotonin
	Calcium & Magnesium ions
	Inorganic phosphate
α Granules	Fibrinogen
	Von Willebrand factor
	Fibronectin
	Thrombospondin
	High molecular weight kininogen
	Albumin
	Platelet factor 4
	Coagulation factor V
	Platelet derived growth factor
	Transforming growth factor β
	α-2-antiplasmin
	plasminogen activator inhibitor
	Immunoglobulin G and A
Lysosomes	Heparitinase
	Cathepsin
	Acid phosphatase
	Aryl sulphatase
	β glucuronidase
Peroxisomes	Catalase

Table 2.2: Platelet granules and granule content, adapted from White 1987.

Receptors	***Ligands***	***Platelet function***
GP IIIb-IIIa	Fibrinogen	Aggregation
	Von Willbrand factor	
	Fibronectin	
	Thrombospondin	
	Vitronectin	
GP Ib-IX	Von Willbrand factor	Adhesion at high shear rates
GP Ia-Iia	Collagen	Adhesion
GP Ic-Iia	Fibronectin	Adhesion
GMP 140	Unknown	Platelet-lecocytes Interaction
GP Ic'-Iia	Laminin	Adhesion
Thrombin receptor	Thrombin	Activation
PECAM-11	Unknown	Unknown

Table 2.3. Platelet membrane receptors, ligands and the platelet function mediated by interaction, adapted from Kieffer 1990 and Coughlin, 1993.

Many receptors, however, contain common subunits and therefore recognize similar amino acid sequences present in adhesive proteins. Thus, it is possible to classify groups of receptors into gene families as described above. Most of the platelet glycoproteins have been cloned and sequenced permitting classification into known gene families.

Two main adhesion receptors expressed by platelets are the GP IIb-IIIa and GP IX complexes and, their respective ligands are vWF and fibrinogen (Ruggeri, 1993). On activated platelets, GP IIb-IIIa (member of integrin family) is a "promiscuous" receptor capable of interacting with several ligands (e.g., fibrinogen, vitronectin, fibronectin, and vWF). However, on non-activated platelets the receptor has been shown to interact only with immobilized fibrinogen (Savage 1991). The receptor complex requires free calcium ions in solution to achieve the correct conformation for adhesive interactions. Along with the fact that GPIIb-IIIa is the most abundant adhesive receptor on platelets (50,000 copies per platelet), it is also required for platelet spreading over the sub-endothelium with its variety of adhesive ligands (de Groot, 1990).

The GPIb-IX receptor (25,000 copies per platelet) is a member of the leucine-rich glycoprotein family of receptors and it is considered to be the principle receptor mediating arterial platelet adhesion (Roth, 1992). The interaction of GPIb-IX and vWF has been shown to be "shear-dependent". Under static conditions, the two molecules have no affinity for each other. However, when shear force is applied to the molecules, they develop a specific affinity for one another. Although the GP Ib-IX complex does not require platelet activation to interact with vWF, it appears to need the presence of wall shear rates in excess of 650 s^{-1} to facilitate vWF binding (Baumgartner, 1973). It is uncertain whether the presence of shear alters the vWF molecule or the receptor or both, thus enabling interaction (Roth, 1991).

2.3.3 Platelet Interaction with Thrombogenic Surfaces

Upon contact of platelets with a thrombogenic surface, such as the sub-endothelium or an artificial surface, platelet activation are triggered. The initial stimulus results in an increase in cytosolic calcium levels leading in turn to several activation responses such as receptor upregulation, shape

change, cytoskeletal organization, and centralization and release of granules. This response can be subdivided into three general phases: adhesion, aggregation, and secretion.

As mentioned earlier, it is unlikely that platelets ever contact the bare surface of an artificial material. Following the initial rapid protein adsorption, the adhesion of platelets to the vessel wall or other surfaces is one of the most crucial steps in the process of hemostasis or thrombosis.

Morphological alteration showed that platelets adhere to the sub-endothelium through following sequential stages: platelet-surface "contact", followed by activation and spreading of the individual contact platelets on the surface. Contacted platelets remain discoid in shape but are rigidly attached to the surface (Baumgartner 1973). This phase represents the first step of the adhesion process where a non-activated platelet stops and "sticks" at a reactive surface (Sarkariassen 1989). Following this initial contact, the platelet becomes activated and begins to spread over the surrounding surface. This shape change involves the extension of pseudopodia, up-regulating and redistribution of membrane receptors, and the organization of the platelet cytoskeleton microfilaments including granule centralization (Kieffer 1992). The characteristic fried egg, appearance of spread platelets results when viewed by electron microscopy (Barnhart 1972).

At the site of vessel injury, the adhesion process involves the interaction of platelet, GP Ib and connective tissue elements which become exposed (e.g., collagen) requiring plasma vWf factor as an essential cofactor. Platelet adhesion to artificial surfaces however is mediated through platelet GP IIb/IIIa, as well as through GP Ib-vWF interaction (Hanson 1997).

Following platelet adhesion, a monolayer of spread platelets adheres to the surface. Platelet aggregates begin to form by additional platelet adhesion to the monolayer of spread cells, attaching to each other. In fact, events such as the release of adenosine diphosphate (ADP), formation of small amount of thrombin and generation of thromboxane A_2, further encourage the recruitment of platelets into growing platelet aggregate. Furthermore, platelet stimulation by these agonists causes the expression on the platelet surface of activated GP IIb/IIIa, which then binds to specific sequence (Arg-Gly-Asp, or RGD) of certain plasma proteins (fibrinogen, vitronection, and fibronectin) that support platelet aggregation. In normal blood, fibrinogen, because of its relatively high concentration, is the most important protein supporting platelet aggregation. The platelet-platelet aggregation involves Ca^{2+}-dependent bridging of adjacent platelets by fibrinogen molecules. It was

shown that platelets would not aggregate in the absence of fibrinogen, GP IIb/IIIa or Ca^{2+} (Hanson 1993).

Platelets then begin to release their stored granules. This reaction can be induced by several agonist including thrombin, ADP, epinephrine and collagen as well as synthetic surfaces. Granule concentrating in the centre of the activated platelet is an immediate precursor to granule release. Most of these granules are extruded via OCS (White1987). This process is rapid and occurs within 20-120 seconds of stimulation depending on the strength of the stimulant. The contents of the different types of platelet granules are secreted at different rates and to different degrees by various agonists (Harrison 1993). For example, α-granule secretion may occur exclusively upon platelet stimulation by weak agonist whereas lysosomal secretion requires stronger stimulation (Holmsen 1982).

A platelet life span is completed, upon its granules extrusion. Non-viable platelets then serve as substrate for coagulation reactions and a physical impediment to flow until either embolization of thrombus dissolution occur. Platelet lifespan measured by radioisotope technique has been estimated to around 10 days. However, with the continuing thrombosis that may be produced by cardiovascular devices, platelets may be removed from circulating blood at a more rapid rate. Hence, a material's thrombogenicity can be determined by measuring the steady state elevation in the rate of platelet destruction as reflected by shortening of the platelets life span (Hanson 1980).

2.3.4 Platelet/Material Interactions

As mentioned earlier, platelet deposition onto the artificial surface follows the initial adsorption of plasma proteins and occurs on most of the artificial surfaces to varying degrees. Under normal blood flow conditions, platelets continually collide with the vessel surface. Once the platelet has reached the surface it may either "stick" or "bounce off" depending on activation state of the platelet as well as its surface binding affinity (Adams, 1985).

To withstand the shear forces exerted on adherent platelets by flowing blood, strong bonds are formed between the platelet and the surface. It is suggested that these bonds likely involve different

glycoproteins (GP IIb-IIIa) in the surfaces of platelet, since this receptor is able to bind to variety of adhesive ligands and is abundant in the platelet membrane (Ruggeri, 1993). Presumably the nature of the adsorbed protein layer dictates the strength of platelet adhesion.

The number of adherent platelets is the parameter most often used to characterize the platelet-contacting response of a material (Okkema 1991, and Takahara 1991). This type of data may be obtained either by radiolabeling platelets with ^{51}Cr (Donse 1976) or ^{111}In (Thakur 1976) isotopes or by microscope (SEM) evaluation. Other platelet parameters have been studied, including amount of granular contents released and degree of spreading of adherent platelets (Ito 1991, and Silver 1993). While the above measures of surface platelet reactivity can be obtained from *in vitro* studies, *in vivo* experiments, measuring platelet consumption tests are necessary performed *ex vivo* or *in vivo*. Consumption can indicate a type of platelet surface interaction that is not apparent in experiment which, measures adhesion at discrete time intervals (Ip 1991).

While simple studies of platelet adhesion are instructive in examining material thrombogenicity, the results obtained by *in vitro* and *ex vivo* methods are often different (Silver 1993). As well, there is no general agreement on what type of platelet response is most desirable. For example, a quickly formed spread monolayer of platelet may act to form a neointima which, is relatively unreactive but will yield high values of platelet adhesion by experimental observation. Conversely, a surface which stimulates platelet adhesion and subsequent release may appear to be unreactive towards platelets by standard measures such as adhesion number, but may result in high levels of platelet consumption and altered platelet function.

2.4 An Overview of Complement Cascade

The complement system consists of 20 proteins, and is one of the most important units of the immune system which mediate the host response by a series of biologic reactions, all of which serve in defense against foreign agent invasion (Robbins 1989). These biological responses include increased vascular permeability, chemotaxis, and opsonization prior to phagocytosis and lysis of target organisms (Robbins 1989).

The complement cascade is composed of activating and effector sequences. Activation happens quite fast and efficiently via the classical pathway initiated by an antigen (Ag)-antibody (Ab)

complex or more slowly via the alternative pathway, initiated by non-immunologic stimulation (Robbins 1989). Both pathways then converge into a common pathway of membrane attack complex (MAC) (Robbins 1989).

Proteins involved in classical pathway are C1, C2, C4, C1 inhibitor, and C4 binding proteins (C4bp) (Johnson 1996). The cascade begins when Ag-Ab binds to one of the subunit of the C1 (C1q), which self-activate and begin to cleave C4 and C2, the cleavage fragment form a complex better known as C3 convertase. C3 convertase is an important enzyme since it participates in further cleavage of the C3 into C3a and C3b (Robbins 1989). C3a is released into plasma; C3b forms a complex with C3 convertase to form C5 convertase, interacting with C5 to cleave C5a and C5b complex (Robbins 1989). Once again C5a is released into plasma and C5b combines with C6 and C7 to form C5b C67 complex, further binding of C5b67 with C8 and C9 to give rise to C5B-9 or better known as membrane attack complex (MAC).

The alternative pathway is more independent than the classical pathway and C3 is activated directly by stimulus such as complex polysaccharide, bacterial endotoxins, cobra venom and aggregated globulin (IgA). The C3 convertase complex of this pathway is formed by the help of factor B, D, and C3b in the presence of Mg^{2+} (Robbins 1989).

2.4.1 Blood-Membrane Interaction and Complement Activation

By far, complement activation through the alternative pathway is the hallmark of membrane-blood interaction, which further activates and influences other host responses (Colton 1994, Cornelius 1993, Holmes 1995).

In normal plasma, there is an ongoing process through which C3 is converted to C3b (C3 tickover); C3b possess a thiolester group available for binding to the foreign surfaces with nucleophilic sites; Factor B then recognizes the C3b, and gets cleaved by factor D, generating C3 convertase (Holmes 1995), leading to the amplification process, producing more C3b and releasing C3a into the fluid phase. As mentioned earlier, C3b binding on different dialysis membrane surfaces lead to the subsequent activation of the complement (Cornelius 1993). After the formation of the C3a, it will be

converted into C3a $_{\text{des arginine}}$ by serum carboxypeptidase. The additional C3b could then attach to the activating surface leading to additional C3 convertase sites (Holmes 1993). At some point, a C3b molecule binds to one of the C3 convertase sites to form C5 convertase, which cause the conversion of C5 to C5b and C5a (Holmes 1993).

C5a and its degradation product C5a $_{\text{des arg}}$ are important inflammatory mediators and are considered to be responsible for the host response of hemodialysis associated complement activation (Figure 5) (Holmes 1993). As far as C5b is concerned, it will bind to C6 to C9 to produce MAC in a similar fashion as the classical pathway (Holmes 1993).

For instance, during a chronic hemodialysis, the complement pathway is regulated primarily by factors H and I (Holmes 1993). Factor H speeds up the degradation activity of the C3 converatse, and more importantly, promotes the proteolytic degradation of the C3b by factor I to C3bi (Holmes 1993).

3.0 Experimental Methods

3.1. Material Synthesis and Characterization

A brief description of the base polymer as well as SMM synthesis will be given in this chapter. It should be mentioned that all the materials used in this experiment, were synthesized by Mr. C. McCloskey, and the details of synthesis as well as material characterization are found in his Master's thesis (M.A.Sc. thesis January 1999). These protocols are based on the work of Tang (Tang 1996), Pham (Pham 1996) and Weiler (Weiler 1997). The general chemical structure of the base polymer is defined in Figures 3.1. The non-radiolabeled base polymer with and without 5% by weight SMMs were used in the protein adsorption as well as platelet adhesion studies, while the radiolabeled analog of the base was used in the biodegradation experiment. The structure of the SMM is defined by the components found in Table 3.1 and Figure 3.2.

3.1.1 Base Polymer

A polyurethane base material (TDI/PTMO/ED) was synthesized using a conventional two-step prepolymer/chain extension reactions (Santerre 1994). The monomers included the following: 2,4-Toluene diisocyanate (TDI; obtained from Eastman Kodak, Rochester N.Y.); ^{14}C-labeled TDI (NEN, DuPont, Boston, Mass.) was used for the biodegradation experiment, while non-radiolabeled polymer was used for the rest of the experiments. Polytetramethylene oxide (PTMO; molecular weight = 1000, obtained from Dupont, Mississauga, Ont), and ethylene diamine (ED, obtained from Aldrich Chemical Company, Milwaukee, WI,).

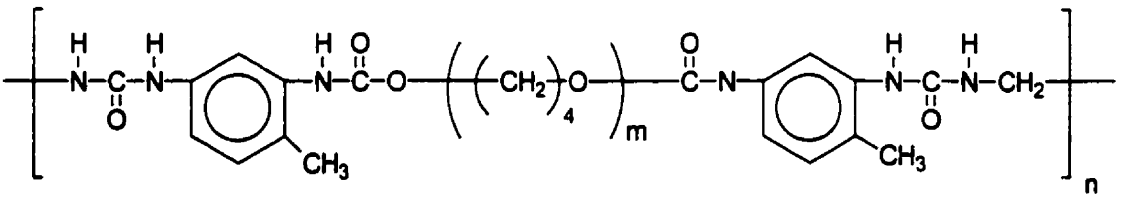
3.1.2 Preparation of Non-Radiolabeled and Radiolabeled Monomers

One batch of radiolabeled base polymer was synthesized. 2,4-Toluene diisocyanate (TDI), was labeled with ^{14}C in the phenyl ring (NEN, DuPont, Boston, Mass.), which was supplied in amber glass ampules each containing 0.24 mCi in 0.55 ml of anhydrous toluene. The non-radiolabeled TDI was vacuum distilled at 1.0 mm Hg and 25 °C. PTMO was degassed for 24 h at 0.5 mm Hg. ED was distilled under atmospheric pressure, to ensure the purity.

3.1.3 Poly-Ether-Urethane Synthesis

After the preliminary preparation of the monomers, the synthesis of both radiolabeled polyetherurethane (^{14}C-TDI-PTMO-ED) and non-radiolabeled polyurethane (TDI-PTMO-ED), with 2.1:1:1.2 stoichiometries of TDI, PTMO, and ED were conducted. Upon completion of the polymer synthesis, the materials were precipitated in distilled water, finely cut, and washed in 2.0 L of distilled water four additional times for periods of 12 h with vigorous stirring. These washing steps were done to ensure that the polymer was relatively free from unreacted monomers and low molecular weight radiolabeled materials, when ^{14}C base polymer was synthesized (Santerre 1994). The re-dissolution and re-precipitation were repeated an additional time to ensure the homogeneity of the polymer.

The molecular weight of the polymers was determined using gel permeation chromatography (GPC), and the specific radioactivity of the radiolabeled polymer was determined by Mr. C. McCloskey (M.A.Sc. thesis January 1999), using a scintillation counter (Beckman, Richmond Ca). The Chemical structure of the polyether-urethane is shown in Figure 3.1.



TDI **PTMO** **ED**

Figure 3.1 Chemical Structure of base Poly-Ether-Urethane.

3.1.4 Surface Modifying Macromolecules (SMMs)

Four SMMs were used in this experiment and their nomenclature is described in Table 3.1. the SMMs are fluorine-containing additives that possess two segments which differ in their compatibility with the base polymer (Tang 1996). It is speculated that the less compatible, fluorinated alcohol segment, of the SMM provides a driving force for the migration of the SMM toward the surface of the base, while the more compatible portion stabilizes the SMM within the

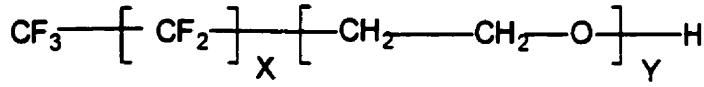
base polymer via non-covalent interactions and physical entanglement (Tang 1996). Detailed descriptions of SMM synthesis are given elsewhere (Pham 1994, Tang 1995, Tang 1996, Weiler 1997). A short general description is provided in the following section.

3.1.5 SMM Monomer Preparations

All the SMMs were synthesized using 1,6-hexanediisocyanate (HDI; obtained from Aldrich Chemical Company, Milwaukee, WI). Two polyols were used to synthesize the four SMMs: polypropylene oxide with molecular weight of 1000 (PPO; obtained from the Aldrich Chemical Company, Milwaukee, WI), and polytetramethylene oxide of molecular weight of 1000 (PTMO; Dupont). Two different mono-functional fluorinated alcohols (BA-L, and FSO-100 both obtained from Van Waters & Rodgers, Montréal, Que.) were used to carry out the end capping procedure. Similar to TDI, HDI was vacuum distilled at 70 °C and 0.025 mm Hg, while polyols (PPO and PTMO) were degassed at 40 °C and 0.1 mm Hg over night. Fluorinated alcohols (BA-L ,and FSO-100) were distilled into three fractions(Tang 1996, and Weiler 1997). The first fraction (distilled at 102 °C and atmospheric pressure) was designated as BA-L (Low; L). The second fraction (distilled between 70-80 °C under a vacuum of 0.01mm Hg) was called BA-L (Intermediate; I). Finally, the third fraction (distilled between 80-100 °C under vacuum at 0.01 mm Hg) was labeled as BA-L (High). Table 3.1 illustrates the SMM nomenclature for this thesis. The first three series of letters refers to the polyol segment used, the numerical sequence indicates the reagent stoichiometry, and the final letter refers to the fluoro-alcohol length contained in the SMM. The designation of F, is the only exception in PTMO212F. This defines the FSO-100 fraction (second fraction) used from the distillation of this fluoro-compound (Weiler 1997). The first distillation was conducted at 55 °C and 13.33 Pa and was colorless liquid. The second distillate was the residual of clear yellowish FSO-100 in the micro-distillation flask, and the yield was about 25% of the original stock, where as the first fraction was less than 2% of the original stock (Weiler 1997). The third fraction was a brown opaque solution which, remained in the initial vacuum distillation apparatus (Weiler 1997). A general structure of the fluoro alcohol is depicted on Figure 3.2.

Example: PTMO322I	
PTMO	Poly-tetramethylene-oxide (1000), is the polyether polyol segment used in this SMM. Other example includes PPO (1000).
322	Refers to the molar ratio of HDI : PTMO : Fluorinated Alcohol (BA-L or FSO-100).
I	Designate the length of the BA-L component of the SMM. Other choices include "H" or "L". "F" corresponds to FSO-100

Table 3.1 Example of SMM nomenclature, adapted from Tang 1996.



X = Odd number from 3-17

Y = 1 for PPO212L, PTMO212I, and PTMO322I

Y > 1 for PTMO 212F

Figure 3.2 Chemical structure of fluoro alcohol

3.1.6 SMM Synthesis

In the first step of the reaction, HDI was added to the appropriate polyol and the catalyst. After 2 hrs of reaction in a temperature range of 60-70 °C, the prepolymer mixture was cooled to 45 °C over a 15 minute period. In the next step, the appropriate fluorinated alcohol was added, and the mixture was allowed to react overnight. Upon completion of the reaction the polymer was precipitated in distilled water, and washed in another solvent to remove any residual fluorinated alcohol (Tang 1996).

3.1.7 Physical Characterization of the Materials

The SMMs were analyzed for their molecular weight, and elemental analysis (Table 3.2). The surface energetics of the SMM containing PEUs was analyzed using contact angle measurements

(Figure 3.3). All of these data were kindly provided by C. McCloskey (M.A.Sc. thesis, January 1999).

Base Modifier	*Wt % F*	*Wt % O*	*Wt % C*	*Wt % H*	*Wt % N*	**Mw (kDa)*	*Polydispersity*	*Synthesis Date*
PPO212L	18.0	22.3	50.18	7.25	2.30	19.2	1.20	10.12.96
PTMO212I	19.3	19.9	51.24	7.27	2.30	24.0	1.23	28.10.96
PTMO322I	11.3	21.5	55.49	8.72	3.30	35.1	1.32	04.10.96
PTMO212F	21.2	20.8	49.21	7.10	1.70	20.6	1.19	21.01.97

*Mw: Weight average molecular weight equivalent to polystyrene equivalent molecular weight

Table 3. 2 Physical characteristics of the SMMs

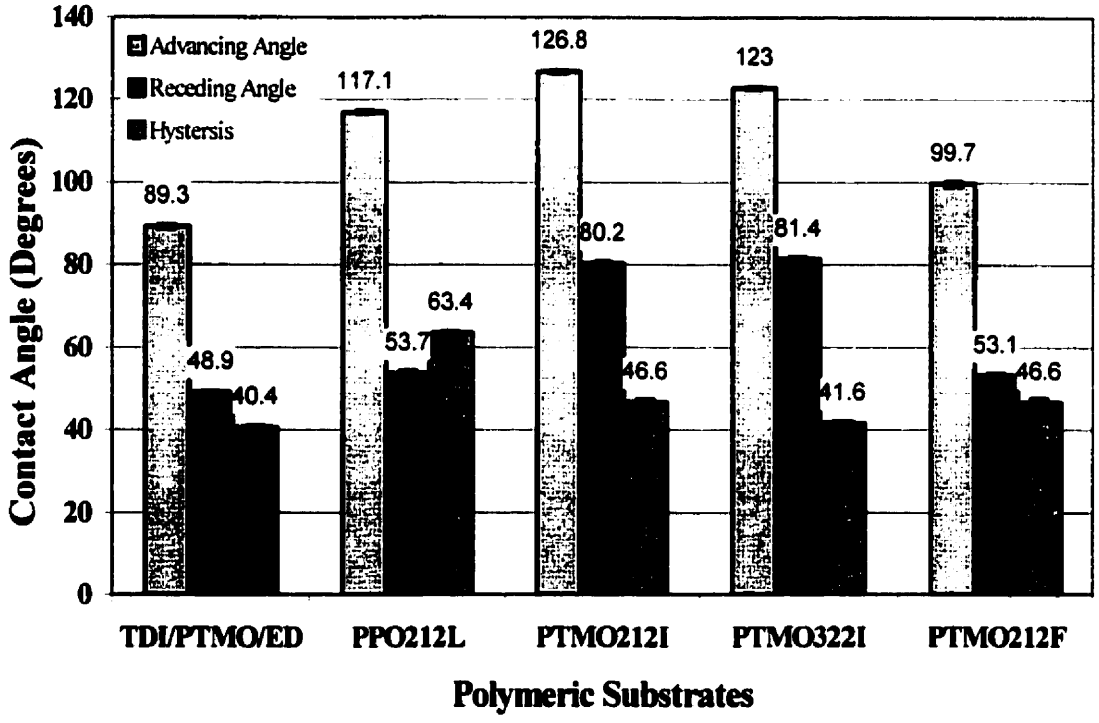


Figure 3.3 Contact angle measurements for PEU and PEU containing SMMs.

3.2 Total Protein Adsorption Studies by Western Blotting

It was desired to identify the presence of specific plasma proteins adsorbed to PEU and PEU containing the four different SMMs, using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting by western blotting. The methods followed were based on the work of Mrs. Rena Cornelius, in Dr. John Brash's laboratory, at Mc Master University, Hamilton, Ontario (Cornelius 1993).

3.2.1 Plasma Preparation

Human platelet poor plasma (PRP) was prepared from heparinized blood (3IU/ml) as described by the author (Cornelius 1993). The blood was obtained from normal healthy donor under no medication, and the plasma was obtained by serial centrifugation as follows 3 minutes at 2000g (x2), 15 minutes at 2000 g, at 20 °C.

3.2.2 Preparation of Polymer Coated Glass Tubes

Polymer samples were coated onto hollow glass tubes cut into 0.5 cm lengths (4 mm OD, 2 mm ID). Prior to coating, the tubes were cleaned in an ultrasonic bath for 30 minutes, rinsed thoroughly with deionized water and dried upright at 110 °C for at least 24 hours. Different polymer solutions were prepared by dissolving each polymer at a 5% (wt/vol.) concentration in N, N-dimethylacetamide (DMAC) (Aldrich, Milwaukee, WI). The SMM content in the base polymer was 5% wt/wt relative to the base polymer. Each tube was then dip casted using a pair of sterile tweezers, under the flow hood. Tubes were then blotted on a Kimwipe for a few minutes, and then transferred to a dry Teflon® plate to remove excess solution. The coated tubes were placed in an end up position and dried in a convection oven at 60 °C for 24 hours. The coating procedure was repeated three times, with the final coat dried in a vaccum oven at 60 °C, <100 KPa, for 24 hours. The resulting film thickness was approximately 10 μm. The day before the experiment the polymer coated glass tubes were pre-incubated in 0.05M PBS buffer, pH 7.0 at 4 °C. All the above procedures were conducted at the Faculty of Dentistry, University of Toronto.

3.2.3 Plasma Incubation with Polymeric Glasses

The equilibrated polymer coated glass tubes were removed from the vaccutainers and were placed in 96 well culture plates. Each polymeric group was run in triplicates and all the incubation experiments were conducted under sterile conditions, under the flow hood.

The frozen blood plasma was taken out of the –70°C freezer and thawed at 37 °C in a water bath. It was important to ensure that the plasma did not contain any ice residues or clumps. Then 250 μL of the plasma was loaded into each cell until the polymer coated glass sample was covered. These were then incubated at room temperature for three hours. Following the incubation period, each polymer coated glass tube was carefully rinsed three times with PBS buffer, pH 7.0. Following the last rinse, polymeric tubes were dried using the wick action of a Kimwipe.

Elution of the adsorbed protein onto polymeric surfaces was then carried out. The three tubes for each group were placed in one scintillation tube which, was then filled with 350 μL of 2% solution of SDS at 4 °C overnight. The eluents were then placed in the –20°C freezer until further use.

SDS is a detergent that is often used in biochemical preparations, and binds quite tenaciously to proteins causing them to assume a rod shape. The large negative charges that the SDS impart, masks the protein's intrinsic charge so that SDS-treated proteins tend to have identical charge-to mass ratio and similar shapes. Hence, the electrophoresis of proteins in SDS-containing polyacrylamide gel separates them based on their molecular mass because of their different ability to migrate (Voet 1995).

3.2.4 SDS-Polyacrylamide Gel Electrophoresis (PAGE)

Gel electrophoresis is among the most effective and yet conveniently used methods of macromolecular separation. The polyacrylamide gels have pores of molecular sizes that can be specified. The molecular separations are based on the gel filtration as well as the electrophoretic mobility of the molecules being separated. The method takes advantage of the fact that proteins have negative or positive charges due to the charged amino acids on their surface and will migrate in an electric field. The rate of travelling and its direction depends on the net charge of the protein,

the shape and the size (Alberts 1983, and Voet 1993). Staining then determines the final positions of the protein after migration is finished.

The addition of the β-mercaptoethanol, a powerful reducing agent, can be used to break the proteins disulfide linkages, yielding the polypeptide chains of multi-chain proteins to be separated by molecular weight (Albert 1983).

The SDS-PAGE 10 well ready gel (12% resolving gel, 4% stacking) gel was purchased from Bio-RAD (Richmond, Ca). The gel was run on a Mini-Protean II Electrophoresis Cell (Bio-Rad, Richmond, Ca.) at 200 volts for 1 hour based on the protocol detailed by Mulzer (1989). 20μl of each eluent and 5 μL of Tracking dye was added per lane. With each gel a low range pre-stained SDS-PAGE standard (Bio-Rad, Richmond, Ca.), and a low range molecular weight standard (Bio-Rad, Richmond, Ca), were also run.

3.2.5 Western Blotting

Following the SDS-PAGE the protein bands in the gel were transferred electrophoretically (blotted) from the gel onto an Immobilon polyvinylidine difluoride (PVDF) membrane (Millipore Co, Bedford, MA). Just before the electrophoresis was completed a small quantity of Pyronin-Y red dye was added to the wells. This marked the top of the separating gel before transfer. A mini Transblot cell (Bio-Rad, Richmond, Ca.) was used for blotting and the following procedures as followed in sequence: (1) after electrophoresis, the gel was equilibrated in transfer buffer for 15-20 minutes; (2) an Immobilon PVDF transfer membrane was cut to size, pre-wet with 100% methanol (1-3 seconds) and distilled water (1-2 minutes), and finally soaked in transfer buffer; (3) The membrane and the gel were clamped together and blotted for 1 hour at 100 volts (200mA) at 4 °C.

The blots were then cut into 1.5 mm strips, were wet with 100% methanol, rinsed in distilled water and incubated for 1 hour in a special multi lane incubation vessel, with gentle agitation in a blocking solution of 5% nonfat dry milk powder (Quality, Oshawa, Ont.) 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 minutes each time in 0.1% (w/v) non-fat dry milk in TBS.

The blots were then incubated at room temperature for 1 hour in a solution of 1% (w/v) nonfat dry milk, 0.05% (v/v) Tween 20 in TBS (solution A), containing the primary antibodies to the different proteins, for 1 hour without shaking. A 0.1% solution is used for all first anti-sera. Each blot was then rinsed as stated above with solution A. The next step consisted of incubating the blots in solution B (the preparation method for all the above solutions are given in Appendix A), again for 1 hour. Blots were incubated with secondary antibodies present at a 1/1000 dilution of an alkaline phosphatase conjugate. Following last incubation the membranes was once again washed with solution A.

The final step consisted of color development, which was achieved by incubating the blots with appropriate substrate. Since all the secondary antibodies were conjugated with alkaline phosphatase, a standard Alkaline Phosphatase (AP) conjugate substrate kit was purchased from Bio-Rad (Richmond, Ca) for color development. The alkaline phosphatase substrate system consists of 5-bromo-4-chloro-3-indolyl phosphate (BCIP), and nitroblue terazolium (NBT). This substrate was prepared as described by the supplier. The blots were covered by this substrate while agitating in a shaker for 3 hours in a special incubation. Once the bands appeared on the PVDF membrane, the color reactions was then stopped by washing the blots with distilled water.

3.2.6 List of Primary and Secondary Antibodies

In this study, antibodies to 20 different human plasma proteins were used. These primary antibodies, as well as, their source are listed in Table 3.3.

Primary Antibody	*Source*
Factor XI[a]	**Nordic Immunology, Tilberg, The Netherlands**
Factor XII[a]	**Nordic Immunology, Tilberg, The Netherlands**
Prekallikrein[c]	**Nordic Immunology, Tilberg, The Netherlands**
HMWK[a]	**Nordic Immunology, Tilberg, The Netherlands**
Fibrinogen[a]	**Cappel Laboratories, Cochraneville, PA**
Plasminogen[a]	**Nordic Immunology, Tilberg, The Netherlands**
Thrombin III[a]	**Cappel Laboratories, Cochraneville, PA**
C3[a]	**Cappel Laboratories, Cochraneville, PA**
Fibronectin[b]	**Cappel Laboratories, Cochraneville, PA**
Albumin[a]	**Nordic Immunology, Tilberg, The Netherlands**
IgG[a]	**Miles Scientific, Rexdale, Ont.**
β-Lipoprotein[a]	**Miles Scientific, Rexdale, Ont.**
α-2-Macroglobulin[a]	**Sigma Chemical Co. St. Louis, MO**
Vitronectin[b]	**Calbiochem, Behring Diagnostic, La Jolla, CA**
Prothrombin[c]	**Nordic Immunology, Tilberg, The Netherlands**
β-2-Micoglobulin[b]	**Sigma Chemical Co. St. Louis, MO**
Hemoglobin[b]	**Sigma Chemical Co. St. Louis, MO**
C3c[b]	**Cappel Laboratories, Cochraneville, PA**
Factor B[a]	**Calbiochem, Behring Diagnostic, La Jolla, CA**
Factor H[a]	**Calbiochem, Behring Diagnostic, La Jolla, CA**

Table 3.3 List of Primary antibodies

The list of the enzyme conjugated secondary antibodies are listed in the Table 3.4.

Enzyme Conjugated Secondary Antibodies	*Source*
[a]Rabbit anti-goat IgG-alkaline phosphatase	**Sigma, St Louis, MO.**
[b]Goat anti-rabbit IgG-alkaline phosphatase	**Bio-Rad Richmond, CA**
[c]Goat anti-mouse IgG alkaline phosphatase	**Bio-Rad Richmond, CA**

Table 3.4 List of Secondary Antibodies

3.3. ^{125}I-Radiolabeled protein Adsorption Studies

Protein adsorption is one of the primary events that takes place when blood encounters an artificial surface such as polymeric materials (Brash 1985). It is believed that the subsequent cellular events with the material occur through a mediating layer of adsorbed proteins (Horbett 1986). Hence, recognizing the protein adsorption pattern of different surfaces provide us with crucial information relevant to the materials' biocompatibility.

The protein adsorption studies were conducted with the following main objective in mind: to identify the plasma proteins adsorbed to PEU and PEU containing SMM; then to compare their adsorption profile. The latter analysis will provide some measures of the potential for activation by the surfaces.

Protein adsorption data were obtained by undertaking single protein adsorption experiments, using two of the most abundant proteins in the plasma. Fibrinogen and albumin (Brash 1985), where ^{125}I-radiolabelled proteins were adsorbed onto the surfaces from single protein solutions and plasma (Brash 1984).

Two different radio-iodination methods were used to label proteins in these experiments. Radio-iodination involves the introduction of radioactive iodine into certain amino acids of the protein chains, usually Tyrosine. Two common labeling methods are the IODO-GEN® (Pierce, Rockford, IL), and iodine mono-chloride (ICl) methods. In both methods, radioactive ^{125}I are incorporated into proteins, via Na^{125}I chemical oxidation reaction. Fibrinogen was radiolabelled by the ICl method and albumin was radiolabelled by IODO-GEN iodination. The differences in methodology lie simply for the protein needed to be radiolabeled. All of the work was conducted by the candidate in Dr. John Brash's Laboratory, Mc Master University, Hamilton, On. Training was kindly provided by Mr. Glenn McClung.

3.3.1 Preparation of Polymer Coated Glass Tubes

Similar glass coating procedure as described in section 3.2.2 was also used here.

3.3.2 Albumin Labeling

As explained previously the radio-iodination of the protein takes place by iodination of the tyrosine residues on the protein. Lypholyzed human serum albumin (HSA) was purchased from Behringwerke (Marburg, Germany). The product was dissolved in distilled water and dialyzed overnight at 4°C against 0.05 M PBS, pH 7.0. The solution was clarified by centrifugation, and frozen in aliquots (1 ml), and stored in –70 °C until required.

Albumin was labeled with ^{125}I ($Na^{125}I$, Amersham, England) by the IODO-GEN method, described previously by Regoeczi (1984). Fraker *et al* (1978) was the one who first investigator to describe IODO-GEN® (Pierce, Rockford, Il.) as a reagent for the iodination of the proteins. In this method the oxidant is immobilized on the surface of a reaction vessel. Immobilization of the oxidants would result in a two-phase system, thus limiting direct contact of the oxidant with the protein. This results in a slower but more easily controlled reaction. Also immobilization of an oxidation reagent would allow for easy separation of the reagent from the reaction mixture. The reagent is virtually insoluble in aqueous media, and remains plated even after iodinated protein is removed (Regoeczi 1984).

Reaction vessels for IODO-GEN consisted of a scintillation vial, which was already prepared using the method described by Regoeczi (1984), by Mr. Glen McClung at Mc Master University.

The protocol used to prepare ^{125}I albumin with the IODO-GEN method begins by thawing 1 mL aliquot of 10 mg/mL of HSA working solution in a 37 °C water bath. This solution was further diluted to 1 mg/mL by adding additional PBS. The aliquots were subsequently mixed in a 1.5 mL eppendorf for 1 minute, and 0.1% of HSA was radio-iodinated by IODO-GEN technique.

In this method, 0.288 mL of albumin (1 mg/mL) was first added to the reaction vessel containing IODO-GEN reagent plated on the interior surface. The incubation time for this reaction was 15 minutes. Following this latter step, 5.0 μL of $Na^{125}I$ solution was quickly added to protein solution and the content was mixed for 15 seconds. The content of the vial was then removed by a syringe and was added to a dialysis cassette (Lyzer Membrane, Pierce, Rockford, Il) at 4 °C. The dialysis step was carried out for three hours by changing the dialysate solution with fresh buffer every hour. The purpose of dialysis was to remove any unbound radiolabeled iodine. It is important to mention

that due to the size of the reaction vessel, only 300 μg batches of the protein can be radiolabeled at one time.

The method of measuring the free iodide relies on the precipitation of protein with tri-cholro-acetic-acid (TCA) and determination of radioactivity in the supernatant. To determine non-protein bound with ^{125}I, a 1:10 dilution of ^{125}I-HSA (1 mg/mL) and PBS buffer was made. From this diluted ^{125}I-HSA solution, 100 μL was removed and added to 900 μL of 1 % bovine serum albumin in 0.2 M phosphate buffer, pH 7.0. This was labeled as solution [A]. A solution similar to solution [A] was prepared and 500 μL of 20% TCA was added to it. This was labeled solution [B]. The mixture was then centrifuged at 300 rpm for 1 minute. 500 μL of the supernatant was subsequently added to an equal volume of 0.2M PBS buffer (pH 7.0) in tube [C]. The radioactivity of vials A and C were then measured on Gamma Counter. The percentage of free iodide was then calculated from Equation 3.1. Preparations with greater values than 5% free isotope were discarded.

%Free I = 3 x Average radioactivity [C] ÷ Average radioactivity [A] x100

Equation 3.1 Free iodide calculation

3.3.3 Albumin Adsorption Studies

Prior to the single protein adsorption experiment the equilibrated polymer coated glass tubes, were removed from the buffer and blotted with a Kimwipe to remove residual buffer. Then, the polymer coated tubes (triplicates), were placed in a 96 well polystyrene cell culture plate. All the samples were exposed to a buffer solution which, contained 1.0 mg/mL of albumin of which 0.1% was ^{125}I-labeled albumin. The samples were incubated in this solution for 2 hours under static condition. After the incubation period, the tubes were blotted dry using the wick action of a Kimwipe and were immediately rinsed twice for 5 minutes with PBS buffer, pH 7.0. The rinsing procedure was considered optimum to prevent loss of loosely held protein on the one hand and to remove solution protein on the other. The polymeric tubes were then placed in scintillation vials and were counted overnight for radioactivity by Gamma Counter (Minaxi, Canberra Packard Canada) overnight. The

counts were then converted to μg albumin per cm^2 based on the radioactivity of a known concentration of stock solution and the known surface area of the glass tubes.

The methodology used to conduct the study with plasma was similar to the one described above. The only difference was the method of preparation of albumin in the plasma. Pooled, citrated human plasma (obtained from healthy donors) was prepared by Ms. Rena Cornelius at Dr. Brash's laboratory at Mc Master University, Hamilton, Ontario. The anticoagulant used was citrated dextrose phosphate (10 volume unit of blood to 1 volume unit of citrate). The plasma was aliquoted and frozen at –40 °C until further use. In this experiment, the polymeric glass tubes were incubated for 2 hours under static conditions with plasma solution containing 1.0 mg/mL of the albumin of which 0.1% was ^{125}I-labeled albumin.

3.3.4 Fibrinogen Labeling

Lypholyzed human plasma fibrinogen was purchased from Calbiochem (La Jolla, CA). The protein was dissolved in distilled water and dialyzed overnight at 4°C against 0.05 M PBS, pH 7.0. The solution was clarified by centrifugation, frozen in aliquots (1 mL), and stored in –70 °C until required.

Before radiolabeling, one of the Fg aliquots was removed from the freezer, thawed at 37 °C in a water bath for 5 minutes, and its concentration was determined by a photospectrometer (Biochrom, Cambridge, England). The values obtained were then substituted in the Equation 3.2.

$$[Fg] = (A_{280} \div \xi) \times \text{Dilution Factor}$$

[Fg] = Fg Concentration
A_{280}= Absorbance @ 280 nm
ξ = Extinction Coefficient for Fg (1.55)

Equation 3.2 Determination fibrinogen concentration adapted from Brash 1984.

Fibrinogen was then labeled with ^{125}I ($Na^{125}I$, Amersham, England) using the iodine monochloride (ICl) method, described previously by other investigators (McFarlene 1958, Horbett 1981, Uniyal 1982, and Brash 1984). The principle advantage of this system is that the number of potential

oxidation steps, in which ^{125}I is transferred to the protein is limited. At the time of addition to the reaction mixture, the iodine atoms are already in a reactive state. The number of reactions (substitution or oxidation) which can take place is equal to the number of active iodine atoms (Regoeczi 1984). Studies by Horbett have shown that the ICl-labeled proteins do not exhibit preferential adsorption relative to the unlabeled protein (Horbett, 1981). Finally, from a health hazard aspect, the ICl method can be carried out with a minimum of exposure to radiation (Regoeczi 1984).

Usually during the labeling of a protein 1-4 folds molar excess of ICl is used, due to protein loss. The protocol used to prepare ^{125}I fibrinogen with the ICl method began, by labeling two 1.5 mL eppendorfs as vial 1, and vial 2. To vial 1, 0.654 mg of the unlabeled Fg and 100 μL of 2M glycine pH 8.8, was added. Then, 2.3 μL of the ICl reagent, 40 μL of 2M glycine buffer and 10 μL of $Na^{125}I$ were added to vial 2. The content of this latter vial was mixed by a pipette for 1 minute. The content of vial 1 was then added to vial 2 and they were mixed for 2 minutes using a 3 cc. syringe. The protein mixture was then dialyzed, using a dialysis cassette (Pierce, Rockford, Il) at 4 °C. The purpose of dialysis was to remove the unbound radiolabeled iodine from the protein solution.

Following Fg labeling, proteins were assessed for free or unbound radio-iodine. This was necessary since the free isotope may bind non-specifically to the test surface, so that high levels of unbound isotope may yield very high radioactivity levels as well as inaccurate determination of protein bound to the surface.

To determine non-protein bound ^{125}I, 100 μL of radiolabeled protein solution was added to 900 μL of 1 % bovine serum albumin in 0.2 M phosphate buffer, pH 7.0. The radioactivity of this solution [A] was determined and then 500 μL of 20% trichloroacetic acid in distilled water was added to precipitate the protein. After 10 minutes, the mixture was centrifuged at 300 rpm for 1 minute. 500 μL of the supernatant was added to an equal volume of 0.2M PBS buffer (pH 7.0) in tube [B]. The radioactivity of this solution [B] was then determined. The fraction of free ^{125}I was calculated from the Equation 3.1, shown in section 3.3.2. Typical values of free ^{125}I, for Fg labeling were found to be between 1-4%. Preparations with greater values than 5% free isotope were discarded.

3.3.5 Fibrinogen Adsorption Studies

Prior to the single protein adsorption experiment, the equilibrated glass tubes, as described earlier, were removed from the buffer and blotted with a Kimwipe to remove residual buffer. Then, the polymeric tubes in triplicates were placed in 96 well polystyrene cell culture plates. All the samples were exposed to a buffer solution which, contained 0.5 mg/mL of fibrinogen, of which 2% was ^{125}I-labeled fibrinogen. The samples were incubated in this solution for 2 hours under static condition. After the incubation period, the tubes were dried using the wick action of a Kimwipe and were rinsed twice for 5 minutes with PBS buffer, pH 7.0. The rinsing procedure was previously optimized to prevent the loss of loosely held protein on the one hand and to remove solution protein on the other. The polymeric tubes were then placed in scintillation vials and were counted overnight for radioactivity by Gamma Counter (Minaxi, Canberra Packard, Miss., Ont.). The counts were then converted to μg fibrinogen per cm^2 based on the radioactivity of a known concentration of stock solution and the known surface area of the glass tubes.

The methodology used to conduct the study with plasma was exactly, the same above. The only difference was the method of preparation of fibrinogen in the plasma. Pooled, citrated human plasma was obtained and processed in the same manner as described. The polymer coated glass tubes were incubated for 2 hours under static conditions, with plasma solution containing 2.5 mg/mL of fibrinogen, of which 5% was ^{125}I-labeled fibrinogen.

3.4 Platelet Adhesion Studies

To assess the surfaces for their susceptibility to adhere and activate platelets, adhesion studies were carried out. The number density of adherent platelets, as well as extent of their spreading could be used as parameters to characterize the interaction of platelets with a material (Park 1990, Okkema 1991). It is believed that rotational motion of the red blood cells (RBC) influences the lateral platelet transport, and increases the lateral deposition of the platelets (Turitto 1979).The deposition of the platelets have been shown to increase under the high flow condition, since it is believed that the larger red blood cells are being concentrated in the centre of the stream and the smaller platelets are pushed toward the periphery of the flow (Aarts 1983). Hence in this study, the platelet-surface interactions were investigated using a cone and plate device, located in Dr. John Brash's laboratory at the Department of Pathology, McMaster University, Hamilton, Ontario.

Platelet adhesion data were obtained by radiolabeling of the cells with ^{51}Cr and by scanning electron microscopy evaluation. The cone and plate device consists of a cone, the tip of which touches a flat base plate covered with a film of the test material. The blood is placed in the gap between the cone and plate, where the cone is rotated at a fixed speed. This cone and plate geometry, under uniform laminar flow conditions, leads to a uniform shear rate in the fluid across the entire radius of the rotating cone provided the angle is small (Walters 1975). The hemodynamic considerations were quite imperative in this study, since it was suggested that disturbances in the flow could potentially lead to non-surface related (nonspecific) activation of the platelets (Skarja 1997). Equation 3.3 gives the equation for the fluid shear rate for small angles, where γ = Cone rotation rate (sec^{-1}), Ω = Cone angle (rad/sec), and θ = Fluid shear rate (rad).

$$\gamma = \Omega/\theta$$

Equation 3.3 Fluid shear rate

3.4.1 Cone and Plate Apparatus

The cone and plate device used in this experiment is illustrated in Figure 3.3. The device was constructed using four Plexiglas cones, 32 mm in diameter with cone angles of 7° (Skarja 1997).

Reynolds number generated by this angle can range from 0 to approximately 3000 when the rotational speed is varied from 0-350 rpm. Under such specification for the cone angles, as well as the Reynolds number ($Re = \rho\Omega R^2/\mu$, where ρ = fluid density, R cone radius, and μ = fluid viscosity), it was reported that there was no significant deviation in fluid shear rate (Skarja 1997).

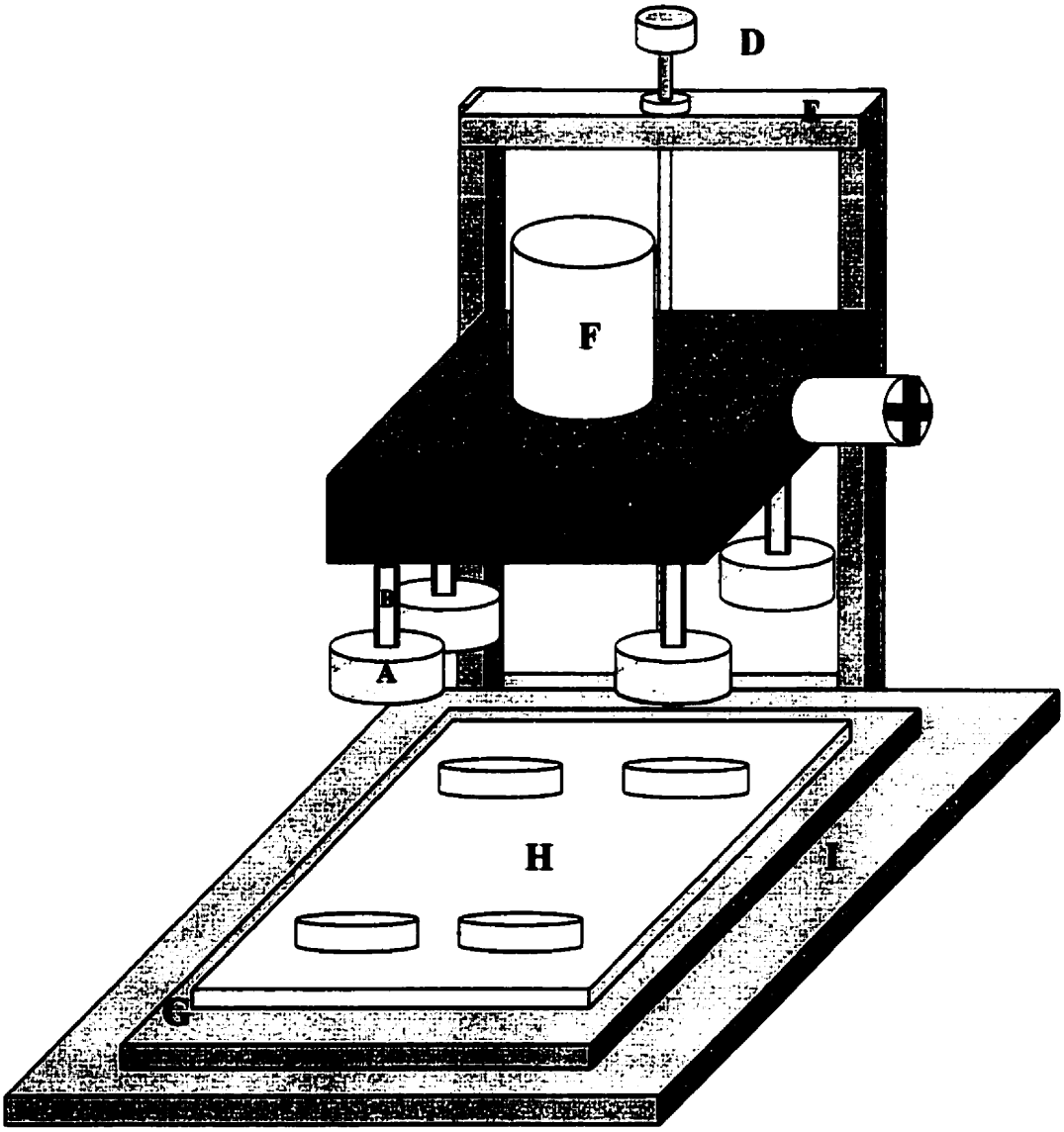


Figure 3.4. **A schematic representation of the cone and plate apparatus. *A.* Cone; *B.* Drive spindle, *C.* Cone-motor assembly; *D.* Positioning screw; *E.* Support frame; *F.* DC motor; *G.* Bracket for four-well plate; *H.* Four-well assembly; *I.* Base plate , adapted from Skarja 1997.**

3.4.2 Preparation of Human Platelet Suspension

The method used for isolating the human platelets in this experiment was described by Mustard (Mustard 1972). Tyrode buffer (preparation method is listed in Appendix B), which contains albumin and apyrase is used to inhibit adenosine di-phosphate (ADP) which may be present in the suspension. Platelets prepared by such a method proved to be healthy and viable for subsequent experiments (Rathbone 1977, and Cazenave 1979).

To begin the preparation, 100 mL of blood from healthy donors was collected by a sterile syringe, and was divided and added to two sterile 50 mL polyprolpylene (PP) tubes. Tubes were labeled as A and B. Tube A contained acid citrate dextrose (ACD) solution (ACD 1: 6 whole blood) and was used as the source of red blood cell (RBC) and platelets in subsequent stages. ACD is an anticoagulant agent used to prevent surface-induced coagulation during handling and preparation (Skarja 1994). Tube B which was used as the source of plasma, contained 50 mL of whole blood and 100 μL Low Molecular Weight Heparin (LMWH) (0.25 U/mL) (Rhône-Poulin Roren, Montreal, Qué.) as an anticoagulant agent.

Immediately upon collection, the both tubes A and B were centrifuged at 2500x g for 90 seconds twice. This was conducted to separate platelet rich plasma (PRP) from RBCs. The RBC from tube A was collected and the method of preparation is explained in section 3.4.4. The RBC from Tube B was discarded. The PRP from both tubes were then centrifuged at 2500x g, this time for 15 minutes at 37°C. This led to the formation of platelet poor plasma (PPP), and a platelet pellet (milky in appearance) at the bottom of the PP tube. At this time the PPP from Tube A was discarded and the pellet transferred into an initial washing solution (preparation method Appendix B) using a transfer pipette. This washing solution was contained in a 15 mL, polyethylene centrifuge tube, incubated in a 37 °C water bath, prior to platelet addition, and was used for further processing, as described in section 3.4.3.

The platelet pellet from Tube B was discarded, and the supernatant (LMWH-PPP), was kept at room temperature until final reconstitution.

3.4.3 Platelet Radiolabeling

To radiolabel the platelets with chromium-51, 500 μL of sodium ^{51}Cr-chromate (1 mCi/mL) was added to the 10 mL platelet suspension. This solution was incubated for 1 hour at 37 °C in the water bath. The time of incubation could vary between 45- 60 minutes depending on the level of labeling required in a particular experiment. After radiolabeling of the platelets, the suspension was centrifuged at 1200x g (2200 rpm) for 10 minutes, at 37 °C. The supernatant was then discarded and the platelets were transferred by a transfer pipette for washing into a second washing solution of platelets (preparation method Appendix B). The platelets were incubated for 20 minutes to allow for acclimation and recovery from the transfer step. The concentration of the apyrase (used to block the release of ADP from platelets) in this final suspension fluid was reduced to 1 μL/mL of suspension. The concentration of radiolabeled platelets was determined using a Coulter Counter (Coulter Electronics Canada). The counting solution was prepared by transferring a 5μL sample of the suspension was transferred to 15 ml of saline solution (Isoton II, Coulter Electronics Canada). Through this procedure a final platelet suspension concentration, of about 400,000 platelets/μL was achieved. It was shown previously by Kinlough-Rathbone (1977) that the platelets prepared by the above method, and kept at 37 °C remain responsive to ADP for many hours in the presence of fibrinogen.

3.4.4 Preparation of Red Cell Suspension

The preparation and washing of the red cells was done separately from the platelets. The collected red cells were washed three times using a washing solution containing the plain Tyrodes buffer, and 0.1% wt/volume glucose at pH 8.3 (Skarja 1994). The Tyrode buffer was added to the red cell solution in a 50 mL polyethylene centrifuge tube, mixing the suspension by inverting the tube several times, and then centrifuging the suspension at 2500 x g for 10 minutes. The supernatant was discarded at the end. This step was repeated three times to minimize the plasma carryover. The red cell concentrate was then suspended in the Dulbecco's Modified Eagle Medium (D-MEM) Base solution (GibcoBRL, Grand Island NY) containing glucose (0.1% wt/vol.) and bovine albumin (4% wt/vol.) at pH 8.3. The red cells were kept at room temperature in this solution until being used.

3.4.5 Preparation of Final Working Suspension

The final working suspension, also known as "pseudo whole blood" suspension used for the platelet adhesion experiment, had a volume of 3 mL and consisted of reconstituted radiolabeled platelets in LMWH-PPP (now called LMWH-PRP), along with washed red cells (approximately 1.62 mL of LMWH-PRP and 1.38 of RBC). The red cell suspension was centrifuged, immediately prior to adding to the platelet suspension. The "pseudo whole blood" contained LMWHP-PRP at a final platelet concentration of 250,000 platelets/μL and RBC at 40% hematocrit. This final suspension was stored at 37°C water bath until used in the platelet adhesion experiment.

3.4.6 Polymer Film Preparation

The polymer films prepared for the platelet adhesion experiment consisted of base poly-ether-urethane, and the base containing the 4 SMMs (PPO 212L, PTMO 212I, PTMO 322I, and PTMO 212F). These polymer films were made using 5% (wt/vol.) of base (non-labeled TDI/PTMO/ED) in N, N-dimenthylacetamide (DMAC) (Aldrich, Milwaukee, WI). The SMM content consisted of 5% of the weight for the base polymer. All of the polymer preparation was done in Faculty of Dentistry at University of Toronto.

A 5.0 cm^2 Teflon® mould was used to cast the polymers. Approximately 10 ml of each solution was cast in the mould, and the solvents were allowed to evaporate for 2 days in a 60 °C air-flow oven, followed by 3 days in vacuum oven at 60 °C and 100 KPa. The resulting polymer films were then removed from the mould by sterile tweezers, and they were placed in 15.0 mL of PBS buffer pH 7.0, at 4°C overnight. While at this temperature, the polymer films were then transferred to Mc Master University. The air cast polymer interface of the films was marked by a permanent marker and polymer discs having a diameter of 40 mm in diameter were punched out, and rinsed with methanol to remove any organic surface contamination. The polymer samples were then immersed in normal Tyrodes buffer (with no albumin) and allowed to equilibrate for at least 5 hours. These were then immediately removed from the buffer solution prior to the adhesion experiment and placed directly into the four well cone and plate device.

3.4.7 Platelet Adhesion Experiment

To avoid any platelet deterioration due to prolonged exposure to the ex-vivo environment, all the adhesion experiments were conducted the same day as the blood was taken from the donors.

One day prior to the experiment, the Plexiglass cones of the apparatus were cleaned by placing them in a detergent solution (4% Liquinox in distilled water). The cones were then rotated at approximately 200 rpm for 1 hour in this solution. This was followed by two rinsing procedures using double distilled water. The plates were also thoroughly washed with detergent and allowed to air dry overnight.

Different pre-wet polymeric materials were then placed between the base and the four-well plates. The plates were then tightly screwed together to avoid any leakage from the wells during the course of the experiments. The four-well plate was subsequently placed into the positioning bracket on the base of the cone and plate apparatus.

1.2 mL of the final suspension (platelet/red cell suspension), which was prepared earlier and kept at 37 °C in the water bath, was then added to each well using a syringe. The position of each cone was such that, the tip of the cone was touching the polymeric film. The point of contact was determined by observing the displacement of the test fluid from the center. In fact, a clear white point is observed at the center of each well when contact is made with the disc. While it is believed this is a practical way of determining the cone height, it was nevertheless not a quantitative one.

The experiment began by rotating the cones at 150 s^{-1}, for 15 minutes. The cone rotation was halted at 15 minutes and they were raised. After the plate was removed from the positioning bracket, the suspension was aspirated from each well using a Pasteur Pipette. The wells were rinsed out 3 times by pipetting 2 mL of Tyrodes buffer containing 0.01 M EDTA (ethylenediamine tetraacetic acid) into each well. The EDTA was used to disrupt any aggregate formation due to platelet-platelet interactions.

The four well plates were then disassembled and the polymeric discs were taken out. A 26 mm sample disc was originally punched out form each test surface. However, it was observed that the

flow pattern near the walls of the device differed significantly from that in the center (Hou 1991). As a result in order to obtain a more accurate evaluation of platelet adhesion and activation the size of the discs was deliberately chosen to be smaller in diameter (13 mm) (Figure 3.5), and were also taken from the central region of the plate where steady and laminar flow was expected to occur.

Three 13 mm discs were then placed into three separate 20 mL scintillation vials (Beckman). Also, 50 µl aliquots of the final working suspension and 950 µL of Tyrodes buffer were placed in the three scintillation vials and samples counted on a Gamma Counter (Minaxi, Canberra Packard Canada). The counts per minute (CPM) were then converted to platelet surface concentration by comparison to the CPM of the stock platelet suspension of known concentration.

3.4.8 Microscopic Evaluation of Platelet Adhesion

A morphological assessment of the platelets found on the test materials was carried out by scanning electron microscopy. Following the completion of the platelet adhesion experiment, the test surfaces (13 mm in diameter) were immediately immersed in a 2% glutaraldehyde solution containing, 0.1 M sodium cacodylate solution for fixation. A small rectangle (2-5 mm in width) was cut from the 13 mm diameter disc, fixed and prepared for mounting (Figure 3.5).

Polymeric semi discs were then dehydrated through graded ethanol and dried in a CO_2 critical point dryer (Polaron, CPD7501, Sussex, England). The samples were then mounted on aluminum specimen stubs, coated with gold, and examined on a Hitachi 2500 scanning electron microscope (Nagasaki, Japan). SEM data collection was carried out by of Mr. Robert Chernecky, Faculty of Dentistry, University of Toronto.

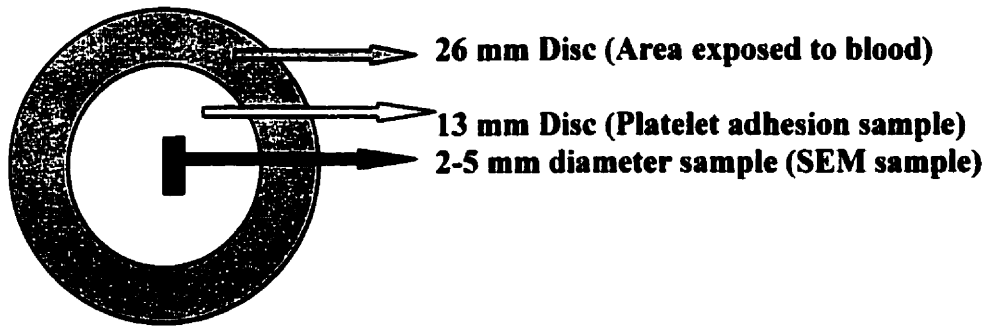


Figure 3.5. Schematic representation of test zones for the polymeric film.

3.5. Biodegradation Experiment

3.5.1 Preparation of Glass Tubes

The surface/volume ratio and sample preparation, are believed to be an important consideration for the interaction of hydrolytic enzymes, and protein in general with polymer surfaces. Thus surface conformity can be optimized using the coated tube method as described by Santerre *et al* (Santerre 1994).

Samples for biodegradation experiments consisted of polymer coated glass tubes. The polyurethane (^{14}C-TDI/PTMO/ED) with and without SMM was coated onto glass tubes using a 5% wt/wt polymer-DMAC solution. The SMM content was 5% wt/wt relative to the base polymer All experiments used the same batch of polyurethane to eliminate the possibility of the experimental results being influenced by batch to batch variations. The tubes had an inner diameter of 2 mm and an outer diameter of 3 mm. The total surface area was found to be approximately 36 cm^2.

The glass tubes used for polymer coating were initially cut into 20.75 cm lengths using a carbide glasscutter. These lengths were notched at 2 cm, 4.55 cm, 6.55 cm, 9.1 cm, 11.65 cm, 14.2 cm, and 18.75 cm. The 20.75 cm tubes were then placed in a Branson 220 Ultrasonic bath filled with 10 mL of cleaning reagent (Contrad*70 provided by Baxter®). After about 30 minutes, the tubes were removed and rinsed several times with distilled deionized water. The tubes were then placed in a 400 mL glass beaker, covered with aluminum foil and placed in a 120°C oven to dry overnight.

3.5.2 Coating of Tubes:

Coating the glass tubes was performed under fumehood (Edge Guard Hood). Six vaccutainers were filled with solutions of the base polymer and the four polymer SMM blends. Using powder-less gloves, each tube was carefully held by a tweezer at the end and dipped into the solution so that the tubes were evenly coated on both the inside and the outside surfaces. Excess solution was then drained back into the vaccutainers by tapping the side of the cylinder, being careful not to damage the section of the tube that would be incubated in the biodegradation experiments. The tube was then blotted at the end on a Kimwipe® to further remove excess solution. The tubes were carefully

placed onto a drying racks ensuring that only one end of the tube was supported on the rack; a pasture pipette was then used to remove any solution that was collected at the bottom of the tube. Finally, the tubes were placed in a convection oven at 60°C, and allowed to dry overnight. This process was then repeated three times. After the last coating, the tubes were placed in a vacuum oven at 60°C for 48 hours, to further evaporate the residual solvent. The resultant polymer coating had an approximate thickness of 10 μm. This estimation was obtained by measuring the thickness of the polymer film under the light microscope. After the coating procedure, tubes were sectioned at pre-defined lengths to yield five 2.55 cm segments and 2 cm spacers. The spacers were used to manipulate tubing during dipping and were discarded. The five remaining sections were then used in the biodegradation experiments.

3.5.3 Polymer Tube Pre-Coating with Fibrinogen:

To study the effect of the fibrinogen on the biodegradation of the base poly-ether-urethane (PEU) as well as the four SMM blends, polymers were pre-coated with fibrinogen. Human plasma fibrinogen was provided by Calbiochem® (La Jolla, CA.) in lyophilized form. This product was reconstituted with 45 mL of MilliQ™ filtered water by injecting the water through the bottle's septum with a needle (gauge 20G) and a syringe (50 mL). The bottle was then placed in a 37°C-water bath for 10 minutes until the content completely dissolved. Throughout this process the bottle would be left undisturbed and unshaken, since excessive movement of the bottle would cause foaming and denaturation of the protein. Once thoroughly dissolved, fibrinogen was transferred into dialysis bags Spectrum Medical Industries, Inc. Cat. # 08-667B, Houston, TX.). The 3 sealed bags, each containing 15 mL of fibrinogen solution were placed in a beaker containing 4000 mL of autoclaved PBS buffer pH 7.0, at 4 °C, and were allowed to stir overnight.

The following morning, the content of each dialysis bag was carefully removed and dialysate was subsequently placed in 50 mL polypropylene tubes.

A Photospectrometer (Biochrome, Cambridge, England) was used to measure the concentration of the dialyzed fibrinogen solution. A diluted fibrinogen solution (1 vol. fibrinogen to 20 vol. PBS) was made. The spectrophotometer was turned on twenty minutes prior to the measurement; the selected wavelength was 280 nm, with Deuterium lamp turned ON. Three working solutions of

fibrinogen, in addition to a blank (pure PBS buffer) were prepared in specific Quartz Cell. Upon calibration the instrument with pure buffer solution, measurements were conducted, and the values were substituted into Equation 3.2 (section 3.3.3) to obtain the concentration of the fibrinogen.

The fibrinogen concentration of all the batches were found to be 7.53 mg/mL. The stock protein solutions were aliquoted into 1mL eppendorf tubes and stored in a –70°C freezer for further use. The concentration required for use in the biodegradation experiment was 1 mg/mL. This concentration of fibrinogen would provide a monolayer of protein on the polymeric surface. Hence, enough stock Fg solution was diluted to produce a final concentration of 1 mg/mL, at pH 7.0.

A fibrinogen coating was placed onto the polymer coated tubes. A 5 mL aliquot of the fibrinogen solution (1 mg/mL) was placed in 5 different vaccutainers, under a laminar flow hood. Tubes were subsequently incubated in the Fg solution for three hours at 37 °C. This was followed by washing three times in PBS, in order to remove non-adhered fibrinogen. These experiments were repeated in triplicate. The control groups consisted of polymer coated tubes incubated in PBS alone.

3.5.4 Preparation of the Phosphate Buffer Saline (PBS)

A liquid medium (1x) of Dulbecco's Phosphate Buffered Saline (GibcoBRL Cat. No. 21600-010) at pH 7.0 was prepared by dissolving 9.6 g of the powder in distilled water to a total volume of about 1 L. 1N NaOH was used to adjust the pH to 7.0.

Due to the length of the experiment (126 days), and the concern for possible contamination, a stock (100X) antibiotic-antimycotic solution (GibcoBRL) was used. The stock solution contained: 10,000 U/mL penicillin G sodium, 10,000 μg/mL streptomycin sulfate and 25 μg/mL amphotericin B as Fungizone®in 0.85% saline. This solution was then stored in a refrigerator at 4°C, until required.

The buffer incubation solution, where polymer coated tubes were kept, contained neutral pH 7.0 PBS buffer. A 2% vol/vol solution of Antibiotic-Antimycotic Solution stock solution was also added.

One unit (U) of CE activity was defined as the amount required to generate 1 nmol/min of p-nitrophenol from the hydrolysis of p-nitrophenyl acetate, at pH 7.0 and 25°C. An 80.0 U/mL solution was prepared form the PBS stock solution, and Genzyme CE (bovine pancreas #70-1081-01). This solution was added to an 8% solution of Antibiotic-Antimycotic Solution stock solution.

The amount of enzyme powder used was determined by the product specifications obtained from the supplier. The solutions were then filter-sterilized and immediately placed in liquid nitrogen to quickly freeze them. These stock solutions were stored in a freezer at -80°C until needed. The supplier, Genzyme, defined the unit of activity based upon the cholesterol acetate substrate at 37°C, pH 7.0.

3.5.5 Biodegradation Experiment Start-Up

All incubation experiments were prepared using sterile techniques. Sterile technique, in the ventilated fume hood, involved wiping the entire area of the experiment with 70% ethanol, prior and after each experiment. The solutions were then filter-sterilized using a 0.22μm filter. The experiment was done in triplicate, as follows (Table 3.5):

Polymer Name	*# of Vaccutainers*
^{14}C-TDI/PTMO/ED	3
(^{14}C-TDI/PTMO/ED & PPO-212L)	3
(^{14}C-TDI/PTMO/ED & PTMO-212I)	3
(^{14}C-TDI/PTMO/ED & PTMO-322 I)	3
(^{14}C-TDI/PTMO/ED & PTMO-212F)	3

SMM concentration is 5% of the weight of ^{14}C-TDI/PTMO/ED.

Table 3.5 Biodegradation experimental setup.

The coated glass tubes were broken in 2.55 cm segments and placed in labeled vaccutainers. 5 mL aliquots of the incubation solutions (PBS buffer pH.7.0) were added and the containers were tapped to remove adherent bubbles on the polymer surface. The vaccutainers were sealed with their rubber stoppers in the flow hood under sterile condition, and stored in a 37°C incubator for 24 hours.

3.5.6. Cholesterol Estease (CE) Addition and Sampling

The enzyme used in biodegradation studies was cholestrol esterase (CE), and is present in the intracellular granules of liver cells, aortic intima, and leukocytes (Labow 1983). Its main function is to catalyze the hydrolysis of fatty acid esters of cholestrol. However, it is also believed that during the chronic stage of inflammation, when adherent monocytes to substrates differentiate into macrophages, and release a whole host of degradative enzymes, including CE (Labow 1996). In a previous studies it was shown that CE could degrade ^{14}C-radiolabeled PEU and that degradation was dependent on the size of the hard segment domain (Santerre 1997).

Addition of CE was carried out throughout the experiment in order to maintain the activity levels of the enzyme in the incubated samples. In order to maintain volume levels in every tube following sampling. The aliquots of the enzyme solution was set at 100 μL delivered five days a week. All experiments were replenished under sterile condition, inside a laminar flow hood once a day and five days a week.

Aliquots of 1.0 mL were removed from the polymer incubation solution once every two weeks and added to 8 mLs of scintillation cocktail (Beckman, Fullerton, CA) solution in a plastic scintillation vial. The samples were then placed in the Liquid Scintillation Counter (Beckman, Fullerton, CA.) for counting. The amount of radiolabel released in the sample was counted for an initial five minutes and then repeated. The results were presented in the form of counts per minute (CPM). The amount of background radiation (≈25 ± 5 CPM) was subtracted from the final values.

4. Results

The rational for the current investigation was primarily to assess the nature of interaction between isolated components of the host response and a PTMO based PEU with and without the modification by specific SMMs. In addition, it was desired to evaluate the material surface stability in the presence of protein and a biodegradative enzyme. Thrombogenicity represents a multi-step phenomenon, which occurs upon blood-material exposure. This important component of the host response begins with the initial protein adsorption onto the substrates. This is followed by specific cellular (e.g.: platelet) interactions with the protein layer, which determines in part the successful outcome of the biomaterial (Brash 1995). Hence, it is believed that the thrombogenic nature of a surface is transmitted through a film of adsorbed protein (Baier 1969). A group of experiments was designed to determine the thrombogenic capability of a base PEU and four SMM containing PEU materials.

Protein adsorption results include: western blotting data, followed by single adsorption experiments for two proteins (fibrinogen and albumin) from plasma and buffer systems on the different polymeric surfaces. The next section will introduce the platelet adhesion data. These experiments were conducted to determine if the differences introduced by the SMMs had influenced protein adsorption to the extent that platelet adhesion and activation would be altered. Similarly, a final series of data describes long-term biodegradation experiments on polymeric surfaces by cholesterol esterase in the presence of pre-adsorbed fibrinogen.

4.1 Protein Adsorption Experiments

4.1.1 Western Blotting

The western blotting analysis was conducted using 20 antibodies on all 5 polymeric materials. Figures 4.1-4.5 show the immunoblot data for the 20 proteins eluted from all the modified and non-modified substrates. Data are provided for repeated experiments. A visual inspection of the immunoblots indicates that, different proteins are adhered on PEU and SMM modified analogs. For example, some fragments of both factor XI and factor XII are adsorbed onto the non-modified base (Figure 4.1) whereas these fragments are not apparent on the PPO212L surface (Figure 4.2).

Fibrinogen, as another example, presents itself on both of the polymers, although the distribution of bands appears to be different from base versus the PPO212L containing surface. It is based on such differences and similarities that one can qualitatively examine the substrate's protein profiles and attempt to characterize the surface as thrombogenic, contact system activator, or even passivating.

In this section, adsorption patterns for 9 of the 19 proteins will specifically be highlighted. The oligopeptide fragments associated with each of these proteins are identified and summarized in Tables 4.1-4.5. Figures 4.1- 4.5 exhibit the band patterns for all the substrates. It also should be noted that the duplicate blots are shown for each material. The number of each lane corresponding to specific proteins is given in the legend.

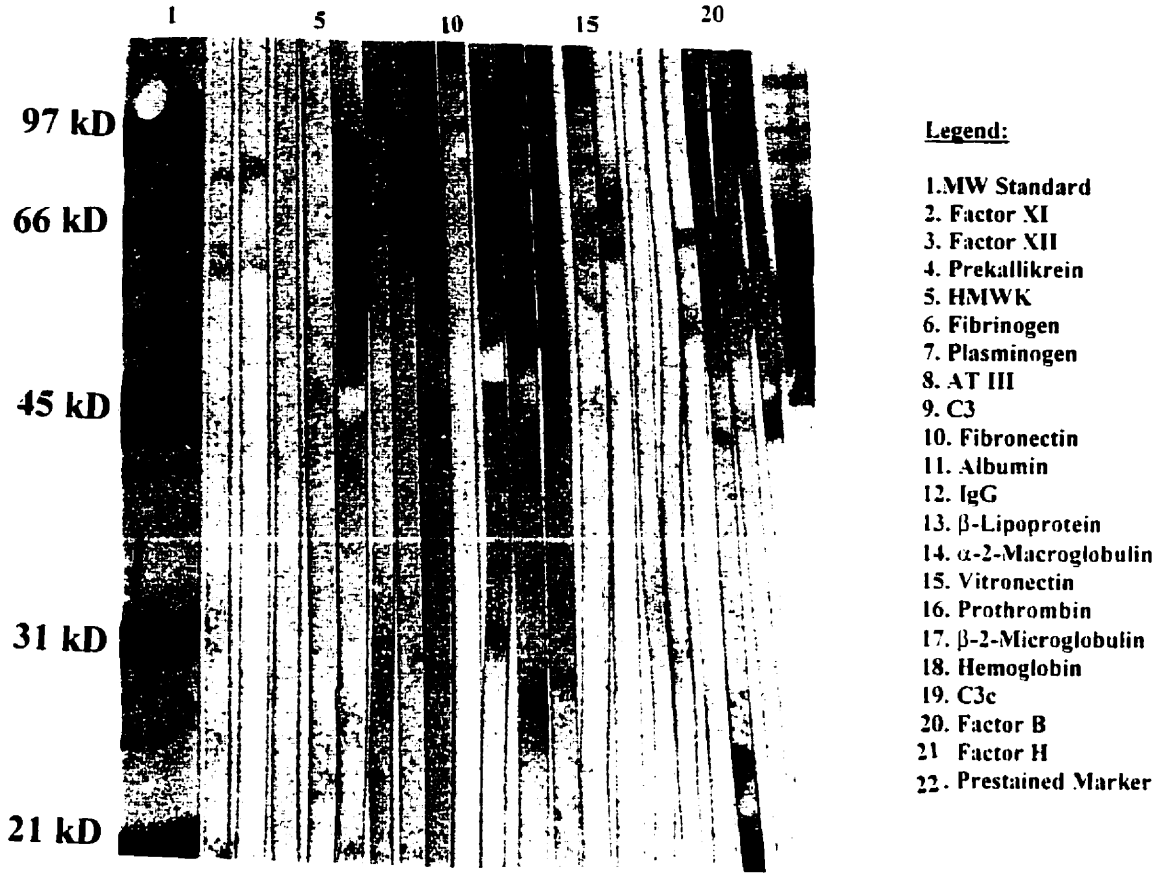


Figure 4.1.a. Immunoblot of proteins eluted from non-modified Base (TDI/PTMO/ED) (Gel 1).

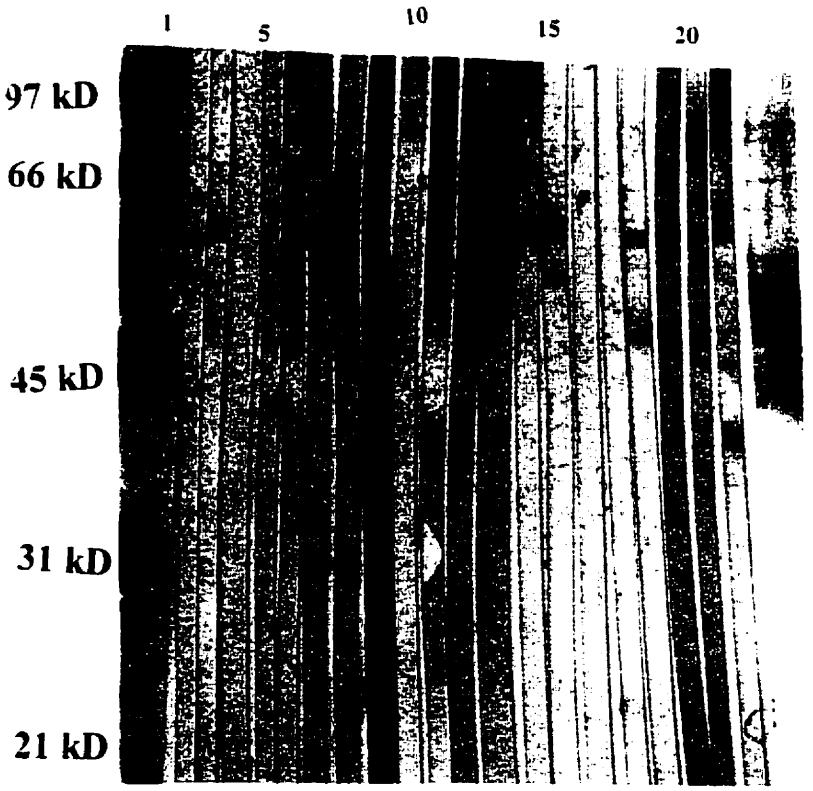
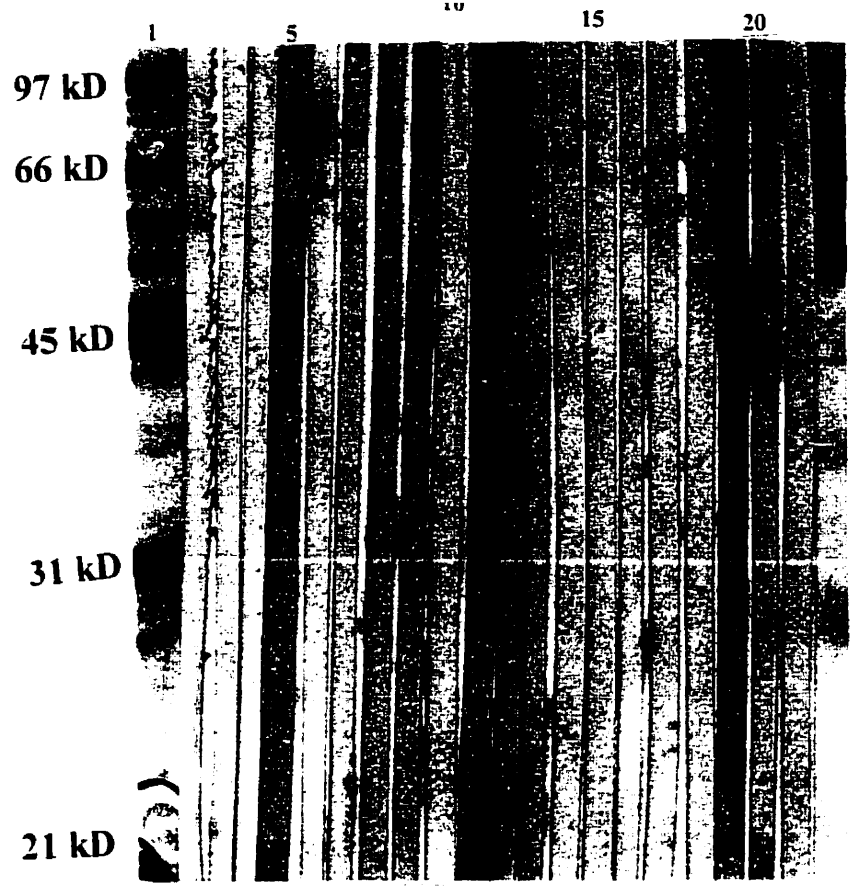


Figure 4.1.b. Immunoblot of proteins eluted from non-modified Base (TDI/PTMO/ED) (Gel 2).



Legend:

1. MW Standard
2. Factor XI
3. Factor XII
4. Prekallikrein
5. HMWK
6. Fibrinogen
7. Plasminogen
8. AT III
9. C3
10. Fibronectin
11. Albumin
12. IgG
13. β-Lipoprotein
14. α-2-Macroglobulin
15. Vitronectin
16. Prothrombin
17. β-2-Microglobulin
18. Hemoglobin
19. C3c
20. Factor B
21. Factor H
22. Prestained Marker

Figure 4.2.a. Immunoblot of proteins eluted from modified base with PPO212L (Gel 1)

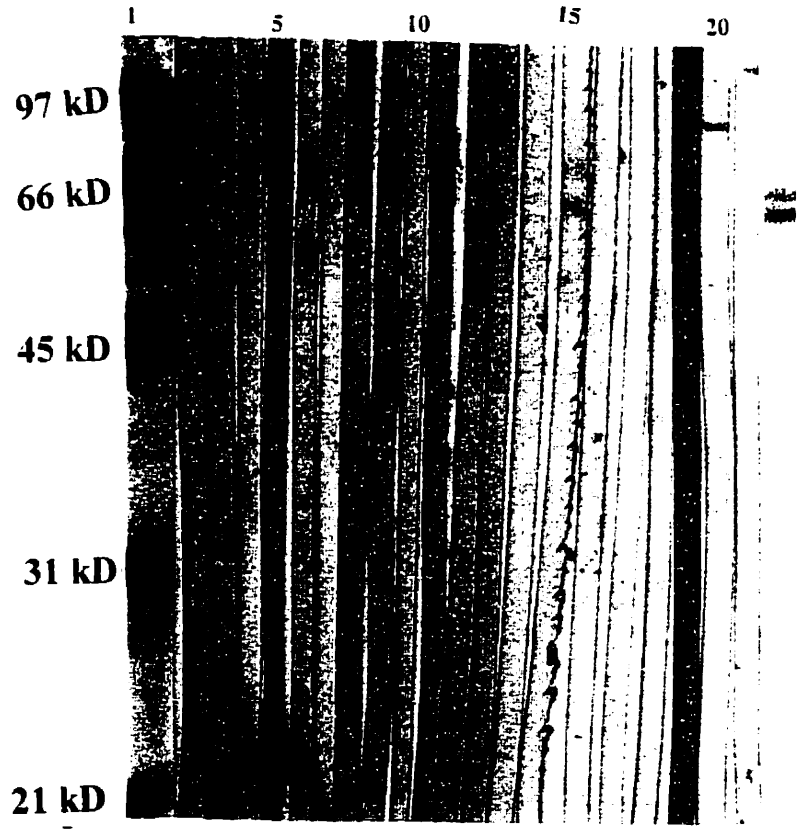
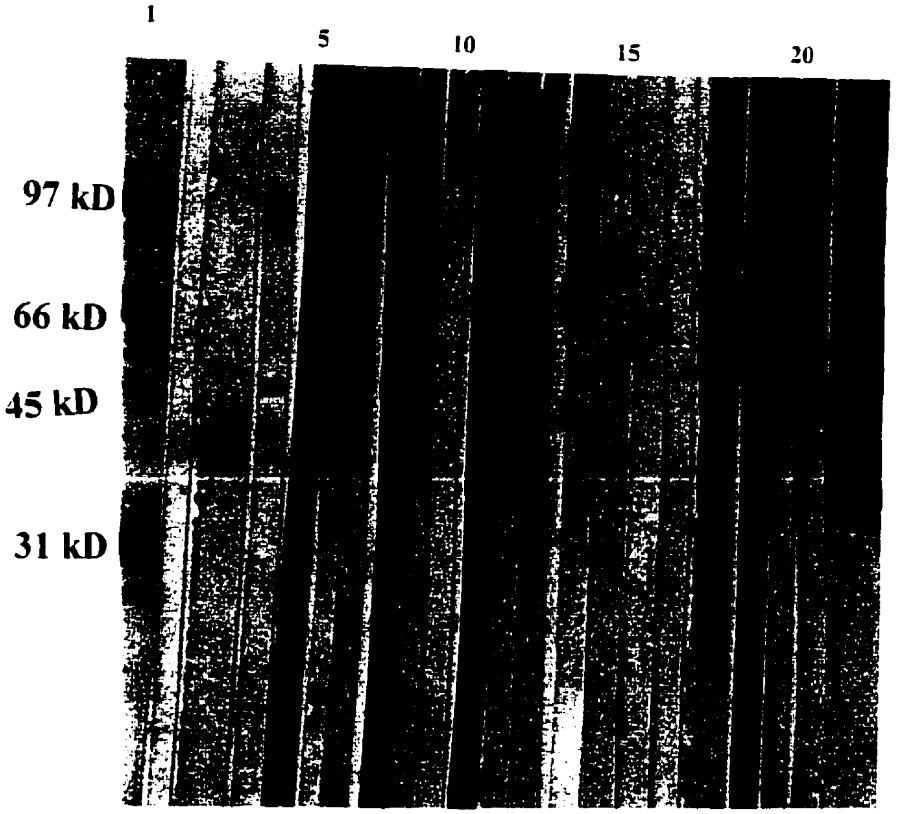


Figure 4.2.b. Immunoblot of proteins eluted from modified base with PPO212L (Gel 2).



Legend:

1. MW Standard
2. Factor XI
3. Factor XII
4. Prekallikrein
5. HMWK
6. Fibrinogen
7. Plasminogen
8. AT III
9. C3
10. Fibronectin
11. Albumin
12. IgG
13. β-Lipoprotein
14. α-2-Macroglobulin
15. Vitronectin
16. Prothrombin
17. β-2-Microglobulin
18. Hemoglobin
19. C3c
20. Factor B
21. Factor H
22. Prestained Marker

Figure 4.3.a. Immunoblot of proteins eluted from modified base with PTMO212I (Gel 1).

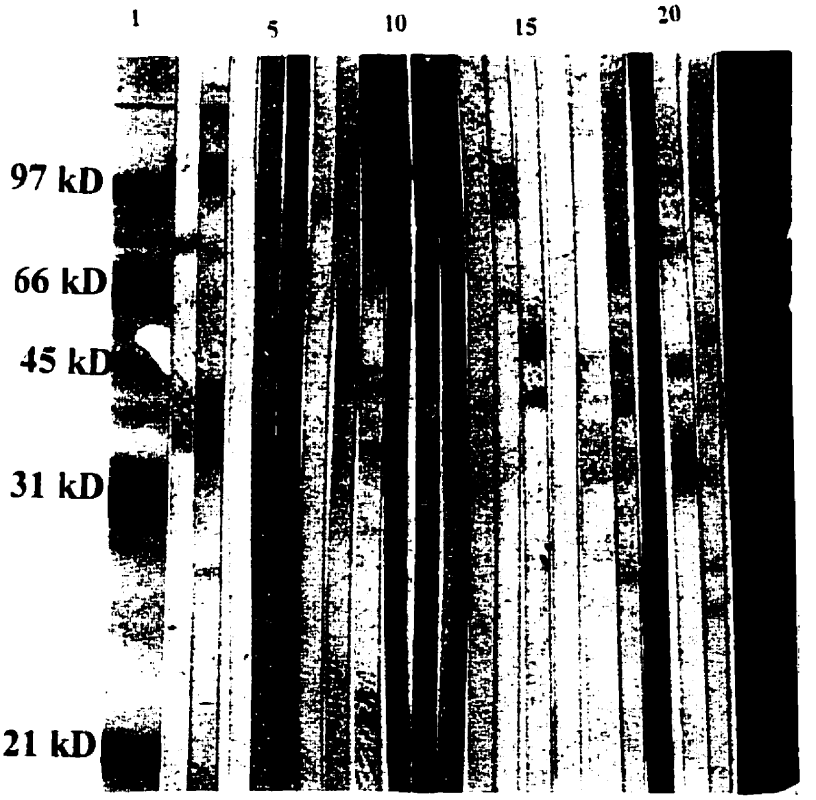


Figure 4.3.b. Immunoblot of proteins eluted from modified base with PTMO212I (Gel 2).

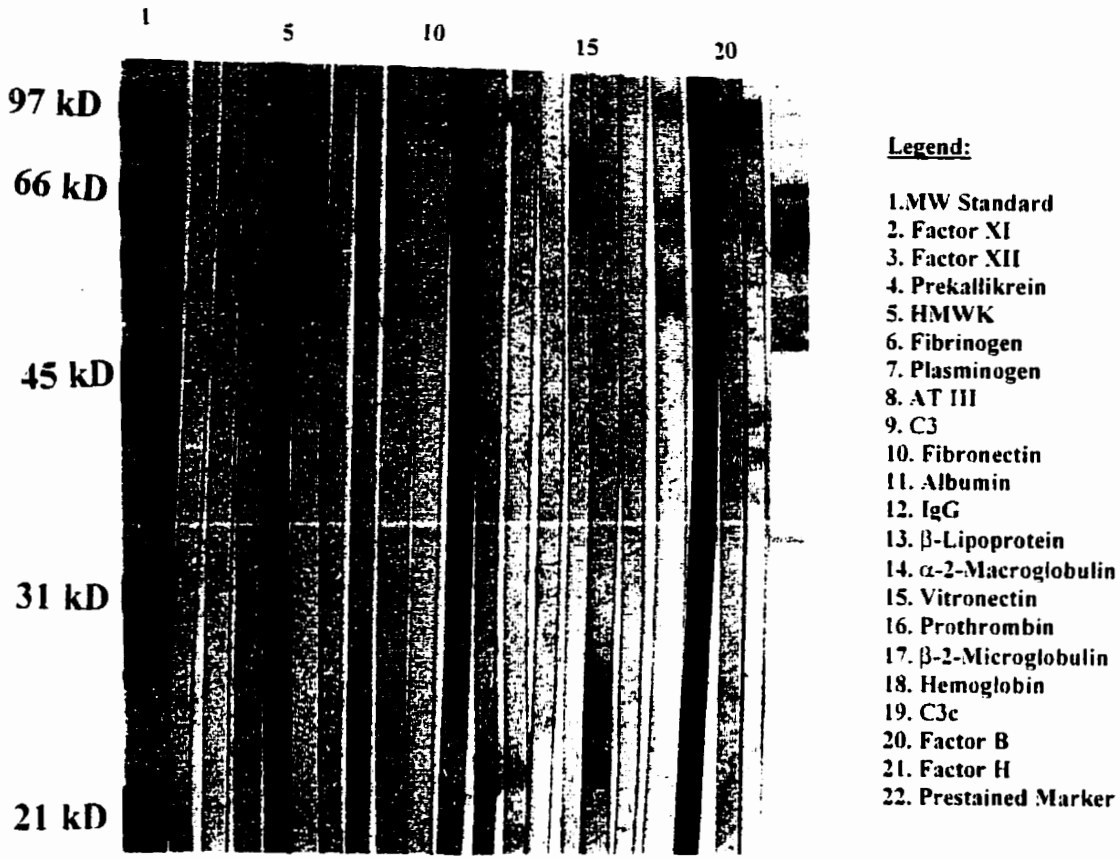


Figure 4.4.a. Immunoblot of proteins eluted from modified base with PTMO322I (Gel 1).

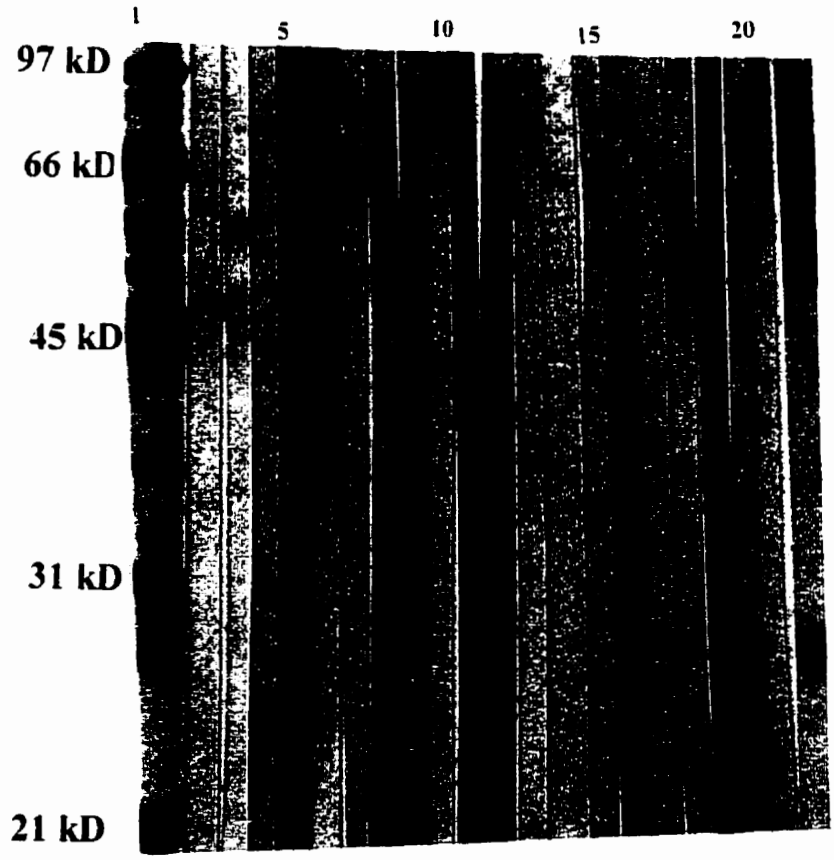


Figure 4.4.b. Immunoblot of proteins eluted from modified base with PTMO322I (Gel 2).

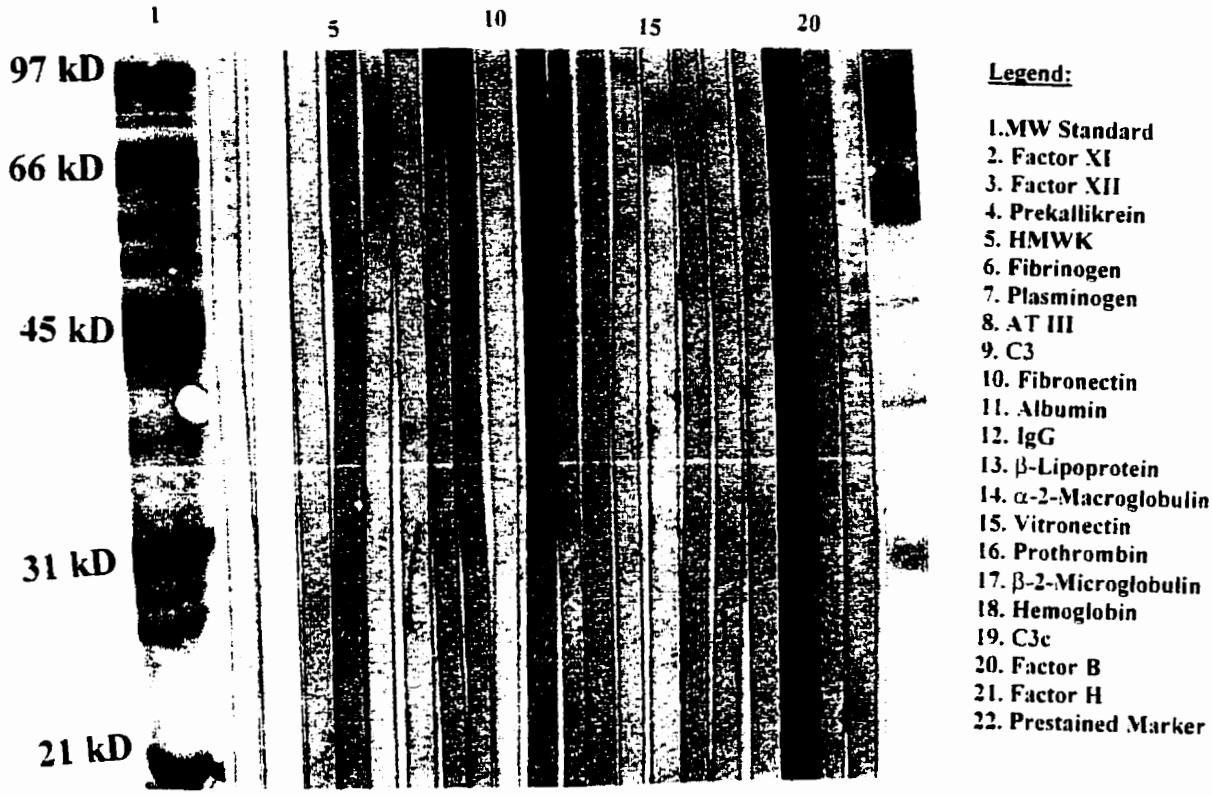


Figure 4.5.a. Immunoblot of proteins eluted from modified base with PTMO212F (Gel 1).

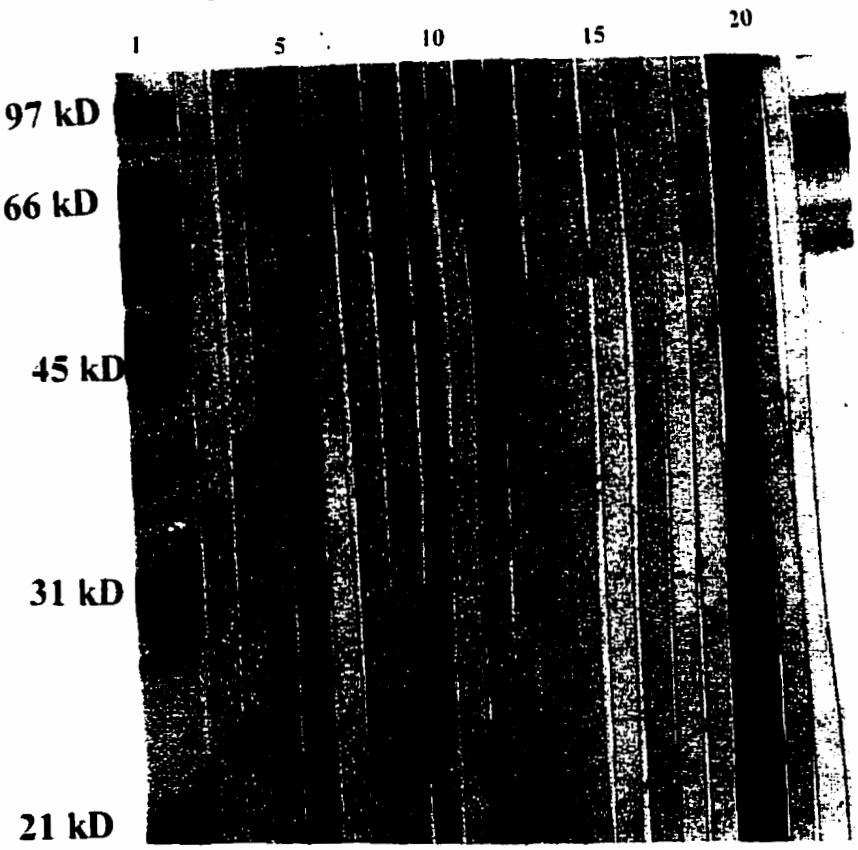


Figure 4.5.b. Immunoblot of proteins eluted from modified base with PTMO212F (Gel 2).

4.1.1.1. Adhesive Proteins (Fibrinogen and Vitronectin):

A: Fibrinogen:

Fibrinogen is one of the most abundant (2-3% of total plasma protein) and most important plasma proteins involved in the common pathway of the blood coagulation (Brash 1985). The intact form of this protein is composed of three pairs of non-identical but homologous polypeptide chains with molecular weight of 67 kD, 58 kD and 47 kD (Cornelius 1991).

Tables 4.1-4.5, provide a semi-quantitative comparison of band patterns for fibrinogen. The band corresponding to the 67 kD chain is present on all surfaces in the following order PPO212L < PTMO212F = PTMO212I < PTMO322I < TDI/PTMO/ED. The 58 kD is one of the intact Fg chains. It is present in a similar intensity to the 67 (Figures 4.1-4.5). In addition, some faint degradation fragments were found around 47 kD position. Such fragments are particularly observed in PPO212L, PTMO322I and PTMO212F. The reproducibility of the bands was acceptable in both blots.

B: Vitronectin (Vnc):

Vnc is an adhesive protein that promotes attachment and spreading of a variety of cells (Preissner 1991). The intact form of this molecule can be found as two subunits with molecular weights of 70 kD and 66 kD (Cornelius 1991).

The band patterns shown in Tables 4.1-4.5 indicate that both sub-units of the Vnc are adsorbed, on most surfaces. The amount of adsorption varies among TDI/PTMO/ED, PTMO212I and PTMO322I, having the highest bands and PPO212L, and PTMO212F showing lower amounts of protein fragments.

4.1.1.2. Complement System (C3, and Factor B):

A: C3:

C3 is one of the complement system proteins and is present in plasma at a concentration of about 1.5-1.7 mg/ml (Cornelius 1991). The intact form of the protein is composed of an α and β chains

with molecular weights of 119 and 73 kD respectively. C3 is believed to be activated (via alternative pathway) upon contact with negatively charged surfaces, producing the activated proteins $C3_a$ and $C3_b$ (Kazatchkine 1991). In previous work by Mulzer and Brash it was shown that the presence of a wide range of fragments was observed near 46-43 kD. These corresponded to the conversion of C3b to C3bi (Mulzer 1989). Another wide band was observed at 38-25 kD relating to C3c and possibly C3d (Mulzer 1989).

In the current study, most of the blots show the presence of both α and β chains of the C3 at 119 and 73 kD, as indicated in Tables 4.1-4.5. The intensity of each band signifies that various amounts of the proteins have been adsorbed onto different surfaces. For example, PTMO212I is the only polymer that has adsorbed more β chain than α chain. Another observation is that the TDI/PTMO/ED does not show the presence of some smaller fragments (30-38 kD). This could potentially indicate that no conversion of C3 to smaller fragments has taken place on this particular polymer. The smaller fragments are only present in the other SMM surfaces as shown in the Tables. The PTMO212I is the only SMM that indicated the presence of some amounts of smaller fragment of C3

B: Factor B:

Another protein component of the alternative pathway for the complement system is Factor B. This protein in its intact form has a molecular weight of 90 kD; also the degradation fragments have been seen between 59-47 kD (Cornelius 1991). Factor B binds to a surface via an activated C3b already on the surface.

Focusing on the band intensities recorded on Tables 4.1- 4.5, it appears that the intact form of the Factor B is present in Base, PPO212L, PTMO322I, PTMO212I and absent in PTMO212F. Interestingly, a similar trend is also repeated in the case of degradation product (47-59 kD). However, lack of reproducibility for the PTMO212I lanes does not allow us to make a definite statement about the presence or lack of the intact band.

4.1.1.3 Intrinsic Blood Coagulation Antibodies (Factor XII, HMWK, and PK)

A: Factor XII

Factor XII is a plasma glycoprotein that participates in the intrinsic pathway of blood coagulation. Factor XII is produced by the liver and is released as a zymogen, which is subsequently complexed with HMWK. Data from other investigations indicate that the intact form of this glycoprotein is composed of two identical 80 kD subunits held together by disulfide bonds (Cornelius 1991). It is believed that Factor XII is activated by binding to a negatively charged surface *in vitro*, through HMWK. The activated polypeptide fragments have molecular weights of 50 kD and 30 kD (Cornelius 1991).

The bands appearing on the immunoblots (Figures 4.1- 4.5) for Factor XII are generally very faint, indicating a very poor adsorption onto these surfaces. However, the intact form of the Factor XII is seen in the case of base polymer, PTMO322I, and PTMO212I. The PPO212L and PTMO212F do not appear to adsorb any intact form. Presence of some weak intensity bands at the lower end of the gel (50 kD and 30 kD) indicates the presence of the activated molecule.

B. High Molecular Weight Kininogen (HMWK)

HMWK is a protein of an apparent molecular weight of 110 kD and its estimated plasma concentration is about 70-90 ug/mL. Its cleavage by factor XI would result in two fragments (a heavy chain, 50 kD and a light chain, 46 kD) (Cornelius 1991). It is more likely that the cleaved form of the HMWK binds to greater extent to an activating surface, due to unmasking of its binding sites. The region responsible for surface binding has been hypothesized to be rich in histidine and glycine (Cornelius 1991).

Figures 4.1-4.5 illustrate the presence of different but weak bands. Such appearance indicates the poor nature of the adsorption. All of the surfaces except the base show various degrees of the higher molecular weight fragments adsorption. It appears that base substrate did not adsorb any amount of HMWK. The presence of heavy and light chain is clearly observed in PTMO212F and PTMO322I.

The poor quality of the blots and lack of bands reproducibility did not allow accurate interpretation of the results.

C: Prekallikrein (PK)

PK has a molecular weight of approximately 88 kD (Scott 1985). This single chain protein is also synthesized in the liver before its release into the plasma (estimated concentration in plasma 35 –50 ug/mL). PK is converted to its active form (Kallikrein) by the action of Factor XIIa on the surface augmented by HMWK or by Factor XIIa fragment in the fluid phase. The heavy and light chains are found at 52 kD and 28 kD respectively. The degradation band can be found at 36 kD (Cornelius 1991). It is believed that the ATIII and α2-Macroglobulin act to inhibit Kallikrein's activity (Cornelius 1991).

From the data in Figures 4.1-4.5, it is quite evident that none of the surfaces adsorbs any form of PK. The only exception was PTMO212I which adsorbed, although weakly, some intact and lower extent of the cleavage product.

4.1.1.4 Fibrinolytic System (Plasminogen)

Plasminogen is the zymogen form of the active fibrinolytic enzyme plasmin, which is synthesized in the liver, and its plasma concentration is about 210 mg/L (Cornelius 1991). This single-chain glycoprotein has a molecular weight of 98 kD in its intact form, and contains 791 amino acid residues with 24 disulfide bonds. Other intact states of the plasminogen can exist in two forms: glu-plasmin, 94 kD, and lys-plasminogen, 84 kD (not reported here) (Cornelius 1991). The active form of the plasminogen is plasmin which, has been implicated in the degradation of the Fg (Brash 1985).

As can be seen from Figures 4.1-4.5 as well as Tables 4.1-4.5, there are some distinct bands at 98 kD indicating the presence of the intact molecule in the base polymer and PPO 212L as well as PTMO212F. On the other hand the active plasmin fragment (63 kD) can be seen in PTMO322I and PTMO212F. This particular fragment is absent in TDI/PTMO/ED, PPO212L and PTMO212I. The

replication of the bands was not good for PTMO212I in the two immunoblots since one can detect some variability.

4.1.1.5 Other Proteins (Albumin)

This major plasma protein (35-45 mg/mL) is well known for its capability to passivate the surfaces upon its adsorption (Fabrizius 1991). The intact form of this protein has a molecular weight of about 66 kD, and degradation fragments could be observed around 47 kD (Cornelius 1991). The immunoblots of Figure 4.1-4.5 show the presence of the 66 kD band in all of the surfaces. Another band was seen on all of the surfaces with an extensive saturation from 33-47 kD. This lower banding pattern is an indication of the degradation of the protein on the surface (Cornelius 1991). Although some degradation products can be seen in different SMM, the base material does not seem to degrade the albumin (Tables 4.1-4.5). The poor quality of the blots in the Gel 2, make it impossible to interpret the reproducibility of the bands observed in the blots for Gel 1.

4.1.1.6 Summary Tables:

Protein	*Mw (Kda)*	*Significance*	*Gel 1*	*Gel 2*	*Reproducibility*
Factor XI	83	Intact	+	+	S
	47	Active Fragment	+	+	S
	33	Active Fragment	-	-	S
Factor XII	80	Intact	+	++	DI
	50	Active Fragment	-	-	S
	30	Active Fragment	-	-	S
Prekallikrein	88	Intact	-	-	S
	52	Heavy Chain	-	-	S
	36	Deg. Fragment	-	-	S
	28	Light Chain	-	-	S
HMWK	110	Intact	-	-	S
	50	Heavy Chain Deg.	-	-	S
	44	Light Chain Deg.	-	-	S
Fibrinogen	67	Intact	++	++	S
	58-47	Deg. Fragment	-	-	S
Plasminogen	98	Intact	++	++	S
AT III	58	Intact Heavy Chain	+	++	DI
	48	Deg. Fragment	++	++	S
	30	Light Chain	-	-	S
C3	119	C3 (α)	-	+	DS
	110	C3b (α')	++	++	S
	73	β	+++	+++	S
	50	C3f+iC3b	-	-	S
	46-42	C3b-C3bi	+	++	DI
	38-25	C3c	-	-	S
	30	C3d	-	-	S
Fibronectin	200	Intact	+++	+++	S
Albumin	66	Intact	+++	+++	S
	47	Deg. Fragment	-	-	S
IgG	150	Intact	+	+	S
	50	Heavy Chain	+++	+++	S
	30	Light Chain	-	-	S
β_2-Lipoprotein	71	Intact	++	++	S
	63-52	Deg. Fragment	-	-	S
α_2-Macroglobulin	100	Intact	-	-	S
Vitronectin	70	Intact	++	+	DI
	66	Intact	++	+	DI
Prothrombin	72		-	-	S
	34	Thrombin	-	-	S
β_2-Microglobulin	60	Intact	-	-	S
Hemoglobin	50	Intact Frgmt.	+	+	S
	66	Intact Frgmt.	+	+	S
C3c	70	β	++	++	S
	45	Intact	+	+	S
Factor B	90	Intact	++	++	S
	59-47	Deg. Fragment	+	+	S

+++: Very Dark Band, ++: Dark Band, +: Faint Band, -: Not Detectable, DI: Different Intensity, S: Similar, DS: Dissimilar

Table 4.1 Protein Adsorption pattern on non-modified PEU.

Protein	*Mw (kDa)*	*Significance*	*Gel 1*	*Gel 2*	*Reproducibility*
Factor XI	83	Intact	-	-	S
	47	Active Fragment	-	-	S
	33	Active Fragment	-	-	S
Factor XII	80	Intact	-	-	S
	50	Active Fragment	-	-	S
	30	Active Fragment	-	-	S
Prekallikrein	88	Intact	-	-	S
	52	Heavy Chain	-	-	S
	36	Deg. Fragment	-	-	S
	28	Light Chain	-	-	S
HMWK	110	Intact	-	-	S
	50	Heavy Chain Deg.	+	-	DS
	44	Light Chain Deg.	+	-	DS
Fibrinogen	67	Intact	+	+	S
	58-47	Deg. Fragment	+	+	S
Plasminogen	98	Intact	+	+	S
AT III	58	Intact Heavy Chain	+	+	S
	48	Deg. Fragment	++	++	S
	30	Light Chain	-	-	S
C3	119	C3 (α)	-	+	DS
	110	C3b (α')	-	-	S
	73	β	++	++	DS
	50	C3f+iC3b	-	-	S
	46-43	C3b-C3bi	+	+	S
	38-25	C3c	-	-	S
	30	C3d	-	-	S
Fibronectin	200	Intact	+	+	S
Albumin	66	Intact	+++	+++	S
	47	Deg. Fragment	+	-	DS
IgG	150	Intact	-	-	S
	50	Heavy Chain	++	+	DI
β_2-Lipoprotein	71	Intact	-	-	S
	63-52	Deg. Fragment	-	-	S
α_2-Macroglobulin	100	Intact	-	-	S
Vitronectin	70	Intact	+	+	S
	66	Intact	-	-	S
Prothrombin	72		-	-	S
	34	Thrombin	-	-	S
β_2-Microglobulin	60	Intact	-	-	S
Hemoglobin	50	Intact Frgmt.	-	-	S
	66	Intact Frgmt.	-	-	S
C3c	70	β	-	-	S
	45	Intact	-	-	S
Factor B	90	Intact	++	+	DI
	59-47	Deg. Fragment	+	-	DS

+++: Very Dark Band, ++: Dark Band, +: Faint Band, -: Not Detectable, DI: Different Intensity, S: Similar, DS: Dissimilar

Table 4.2 Protein Adsorption pattern on PEU surfaces with PPO212L.

Protein	*Mw (kDa)*	*Significance*	*Gel 1*	*Gel 2*	*Reproducibility*
Factor XI	83	Intact	-	+	DS
	47	Active Fragment	-	-	S
	33	Active Fragment	-	+	DS
Factor XII	80	Intact	-	-	S
	50	Active Fragment	-	+	DS
	30	Active Fragment	+	+	S
Prekallikrein	88	Intact	+	-	DS
	52	Heavy Chain	-	-	S
	36	Deg. Fragment	+	-	DS
	28	Light Chain	-	-	S
HMWK	110	Intact	+++	-	DS
	50	Heavy Chain Deg.	-	-	S
	44	Light Chain Deg.	+++	-	DS
Fibrinogen	67	Intact	++	+	DI
	58-47	Deg. Fragment	++	-	DS
Plasminogen	98	Intact	+++	-	DS
AT III	58	Intact Heavy Chain	++	+	DI
	48	Deg. Fragment	+++	+	DI
	30	Light Chain	-	+	DS
C3	119	C3 (α)	+	+	S
	110	C3b (α')	+	++	DI
	73	β	++	++	S
	50	C3f+iC3b	-	-	S
	42-46	C3b-C3bi	-	-	S
	38-25	C3c	++	-	DS
	30	C3d	-	-	S
Fibronectin	200	Intact	++	+	DI
Albumin	66	Intact		++	SATURATED
	47	Deg. Fragment		++	SATURATED
IgG	150	Intact	+	++	DI
	50	Heavy Chain	-	-	S
β_2-Lipoprotein	71	Intact	+	-	DS
	63-52	Deg. Fragment	-	-	S
α_2-Macroglobulin	100	Intact	+	+	S
Vitronectin	70	Intact	++	+	DI
	66	Intact	-	+	DS
Prothrombin	72		-	-	S
	34	Thrombin	-	-	S
β_2-Microglobulin	60	Intact	-	-	S
Hemoglobin	50	Intact Frgmt.	++	++	S
	66	Intact Frgmt.	++	++	S
C3c	70	β	+	++	DI
	45	Intact	+	++	DI
Factor B	90	Intact	+	-	DS
	59-47	Deg. Fragment	+	+	S

+++: Very Dark Band, ++: Dark Band, +: Faint Band, -: Not Detectable, DI: Different Intensity, S: Similar, DS: Dissimilar.

Table 4.3 Protein Adsorption pattern on PEU surfaces with PTMO212I.

Protein	*Mw (kDa)*	*Significance*	*Gel 1*	*Gel 2*	*Reproducibility*
Factor XI	83	Intact	-	-	S
	47	Active Fragment	-	+	DS
	33	Active Fragment	-	-	S
Factor XII	80	Intact	+	+	S
	50	Active Fragment	+	+	S
	30	Active Fragment	-	-	S
Prekallikrein	88	Intact	-	-	S
	52	Heavy Chain	-	-	S
	36	Deg. Fragment	-	-	S
	28	Light Chain	-	-	S
HMWK	110	Intact	-	-	S
	50	Heavy Chain Deg.	+	+	S
	44	Light Chain Deg.	+	+	S
Fibrinogen	67	Intact	+++	+++	S
	58-47	Deg. Fragment	++	++	S
Plasminogen	98	Intact	-	-	S
AT III	58	Intact Heavy Chain	-	-	S
	48	Deg. Fragment	-	++	DS
	30	Light Chain	-	-	S
C3	119	C3 (α)	-	-	S
	110	C3b (α')	-	-	S
	73	β	++	++	S
	50	C3f+iC3b	-	+	DS
	46-42	C3b-C3bi	-	++	DS
	38-25	C3c	-	-	S
	30	C3d	-	-	S
Fibronectin	200	Intact	++	++	S
Albumin	66	Intact	+++	+++	S
	47	Deg. Fragment	+	+	S
IgG	150	Intact	-	-	S
	50	Heavy Chain	-	++	DS
β_2-Lipoprotein	71	Intact	-	+	DS
	63-52	Deg. Fragment	-	-	S
α_2-Macroglobulin	100	Intact	-	-	S
Vitronectin	70	Intact	++	++	S
	66	Intact	++	++	S
Prothrombin	72		-	-	S
	34	Thrombin	-	+	DS
β_2-Microglobulin	60	Intact	-	-	S
Hemoglobin	50	Intact Frgmt.	+	+	S
	66	Intact Frgmt.	+	+	S
C3c	70	β	++	++	S
	45	Intact	-	-	S
Factor B	90	Intact	++	++	S
	59-47	Deg. Fragment	+	+	S

+++: Very Dark Band, ++: Dark Band, +: Faint Band, -: Not Detectable, DI: Different Intensity, S: Similar, DS: Dissimilar

Table 4.4 Protein Adsorption pattern on PEU surfaces with PTMO322I.

Protein	*Mw (kDa)*	*Significance*	*Gel 1*	*Gel 2*	*Reproducibility*
Factor XI	83	Intact	-	-	S
	47	Active Fragment	-	-	S
	33	Active Fragment	-	-	S
Factor XII	80	Intact	-	-	S
	50	Active Fragment	-	-	S
	30	Active Fragment	-	-	S
Prekallikrein	88	Intact	-	-	S
	52	Heavy Chain	-	-	S
	36	Deg. Fragment	-	-	S
	28	Light Chain	-	-	S
HMWK	110	Intact	-	-	S
	50	Heavy Chain Deg.	+	+	S
	44	Light Chain Deg.	+	+	S
Fibrinogen	67	Intact	++	++	S
	58-47	Deg. Fragment	+	+	S
Plasminogen	98	Intact	++	++	S
AT III	58	Intact Heavy Chain	+	+	S
	48	Deg. Fragment	+	+	S
	30	Light Chain	-	-	S
C3	119	C3 (α)	+	+	S
	110	C3b (α')	++	++	S
	70	β	++	++	S
	50	C3f+iC3b	-	-	S
	46-42	C3b-C3bi	+	+	S
	38-25	C3c	-	-	S
	30	C3d	-	-	S
Fibronectin	200	Intact	++	++	S
Albumin	66	Intact	++	++	S
	47	Deg. Fragment	+	+	S
IgG	150	Intact	-	-	S
	50	Heavy Chain	++	++	S
β_2-Lipoprotein	71	Intact	-	-	S
	63-52	Deg. Fragment	-	-	S
α_2-Macroglobulin	100	Intact	-	-	S
Vitronectin	70	Intact	+	+	S
	66	Intact	+	+	S
Prothrombin	72		-	-	S
	34	Thrombin	-	-	S
β_2-Microglobulin	60	Intact	-	-	S
Hemoglobin	50	Intact Frgmt.	+	+	S
	66	Intact Frgmt.	+	+	S
C3c	70	β	++	++	S
	45	Intact	+	+	S
Factor B	90	Intact	-	-	S
	59-47	Deg. Fragment	-	-	S

+++: Very Dark Band, ++: Dark Band, +: Faint Band, -: Not Detectable, DI: Different Intensity, S: Similar, DS: Dissimilar

Table 4.5 Protein Adsorption pattern on PEU surfaces with PTMO212F.

4.1.2. Single Protein Adsorption Analysis

In this series of experiments two separate radiolabelled proteins (^{125}I-Albumin and ^{125}I-Fibrinogen) were adsorbed onto PEU and PEU containing SMMs, from two media: Phosphate Buffer Solution (PBS) and blood plasma solution.

4.1.2.1. Albumin

Figure 4.6 shows the ^{125}I-HSA adsorption data from PBS, onto the five polymeric materials. The first bar in each group is the total amount of ^{125}I-HSA adsorbed onto the polymeric substrates. The second bar represents the amount of ^{125}I-HSA that could be eluted by 2% SDS from the surfaces and the third bar represents the amount of protein still adsorbed onto the surface after 2% SDS elution.

TDI/PTMO/ED and PTMO212F are among the polymers that are adsorbing the least amount of the albumin, however, this observation could not statistically be verified (ANOVA, Scheffe method with $p<0.05$). Note that the maximum amount of ^{125}I-HSA adsorbed from the buffer was around 1.6 $\mu g/cm^2$ and the minimum amount is about 0.8 $\mu g/cm^2$. In almost all cases, it is quite apparent that more than 90% of the proteins were removed from the surfaces, and only a small amount represented by the third bar in each group, was still bound to the surfaces (less than 10%).

Figure 4.7 represents the amount of the radiolabelled HSA adsorbed from plasma onto PEU and PEU containing SMMs. The first observation is that the pattern of adsorption is different in plasma and in buffer. In the plasma, it appears that PTMO212I and PTMO212F exhibit the lowest amount of adsorption. The other three surfaces (TDI/PTMO/ED, PPO212L, and PTMO322I) appear to have an equal amount of adsorption. Statistical analysis of variance (ANOVA) was conducted using "Scheffe " method with confidence interval of 95%. It was found that there were some significant statistical differences between the base and PTMO212F as well as PTMO212I. However, the differences between the other groups could not significantly be confirmed by ANOVA anlysis.

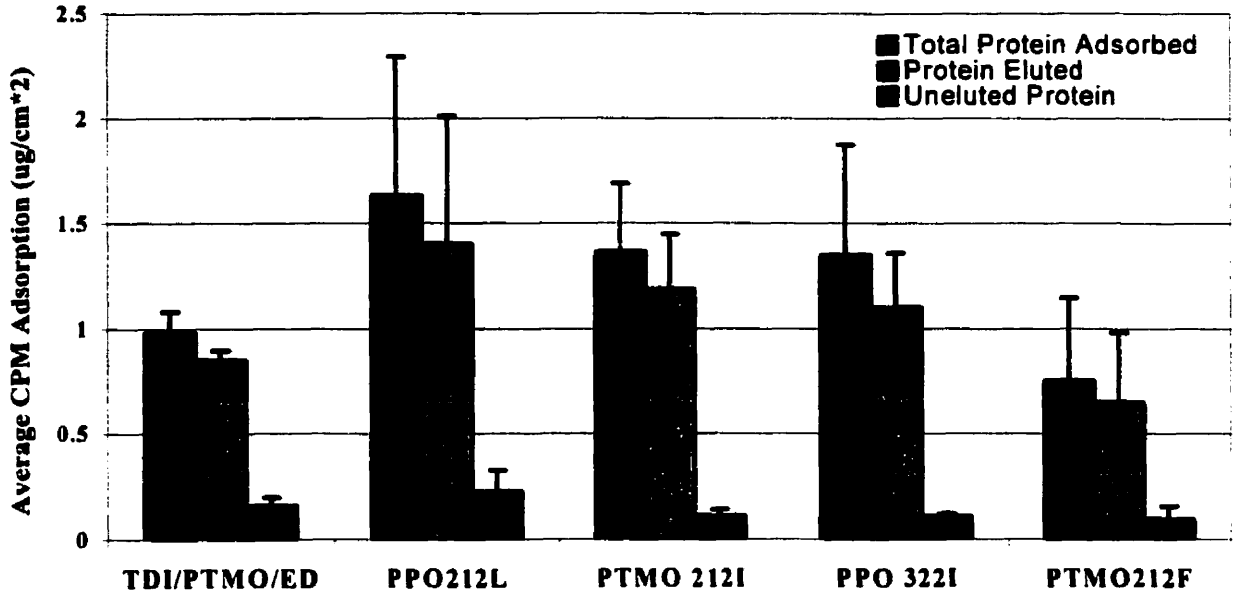


Figure 4.6. The adsorption profile of human serum albumin (HSA) from Phosphate Buffer Solution (PBS) pH 7.0, onto different polymeric surfaces, after three hours incubation at room temperature.

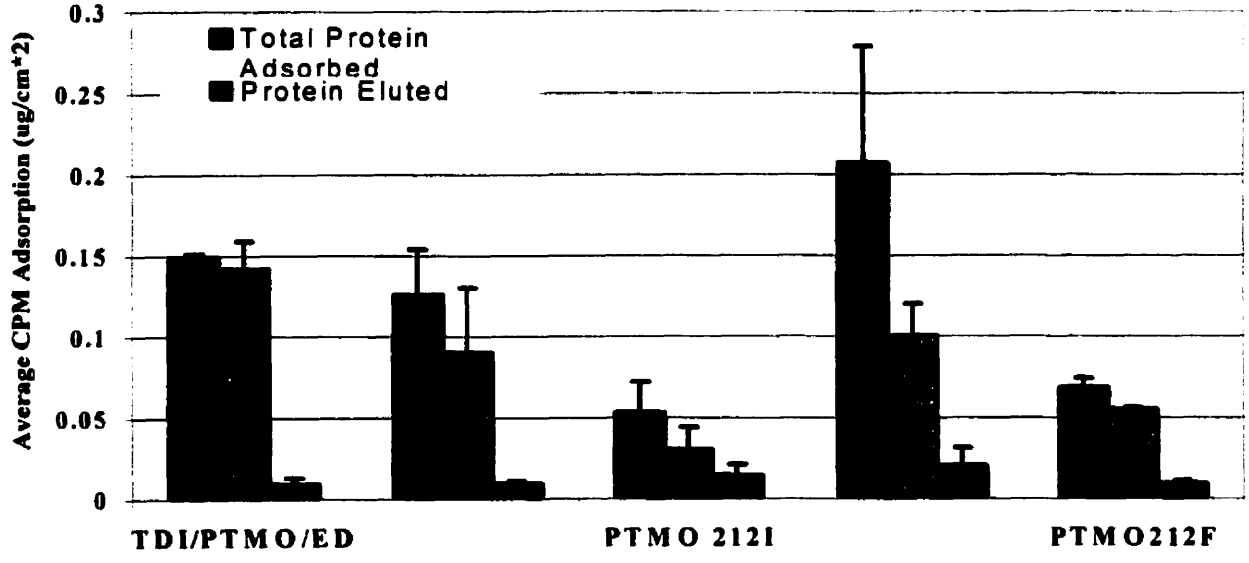


Figure 4.7. The adsorption profile of human serum albumin (HSA) from blood plasma, onto different polymeric surfaces, after three hours incubation at room temperature.

Figure 4.8 directly compares the amount of ^{125}I-HSA adsorbed from the plasma and from PBS. It is clear that HSA adsorption from pure buffer is almost 10 times higher than that of plasma, which

contains a multitude of other proteins competing for the surface (maximum adsorption 0.16 μg/cm^2 in plasma versus 1.6 μg/cm^2 in buffer).

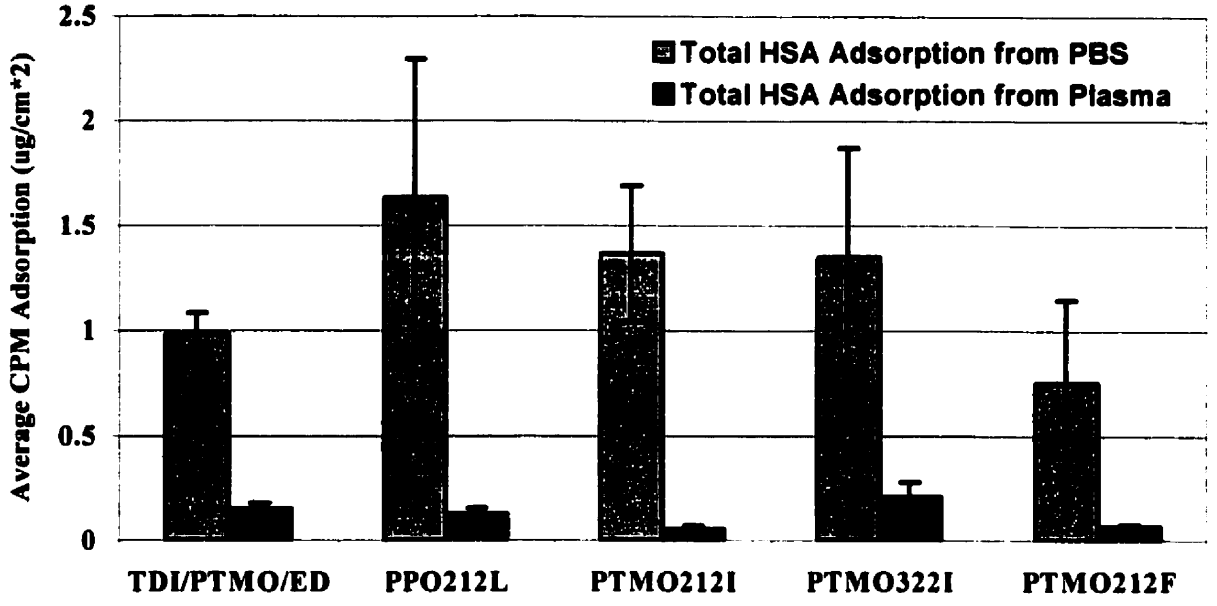


Figure 4.8 Comparison between amount of HSA adsorbed from PBS vs. blood plasma.

4.1.2.2 Fibrinogen

Figure 4.9 shows the ^{125}I-Fg adsorption data from PBS, onto the five polymeric materials. As was the previous case for albumin, the first bar in each material group is the total amount of adsorbed ^{125}I-Fg, while the second bar represents the amount of ^{125}I-Fg that was eluted by 2% SDS from the surfaces and finally the third bar represents the amount of the protein still adsorbed onto the surface after 2% SDS elution.

The total protein adsorption data shows that PTMO212I is the SMM that adsorbed the highest amount of Fg from PBS. In fact, after conducting ANOVA using "Scheffe" method with confidence interval of 95%, it was shown that Fg adsorption onto PTMO212I was significantly different from that of PTMO212F (p-value < 0.01). The amount of Fg adsorption onto TDI/PTMO/ED, PPO212L, and PTMO322I, were not statistically different based on ANOVA analysis (p-value < 0.05). Note that the maximum amount of ^{125}I-Fg adsorbed from the PBS is around 1.4 μg/cm^2 (PTMO212I)

and the minimum amount is about 0.6 μg/cm^2 (PTMO212F). In almost all cases, it was shown that more 90% of the proteins could be removed from the surfaces with the 2% SDS treatment.

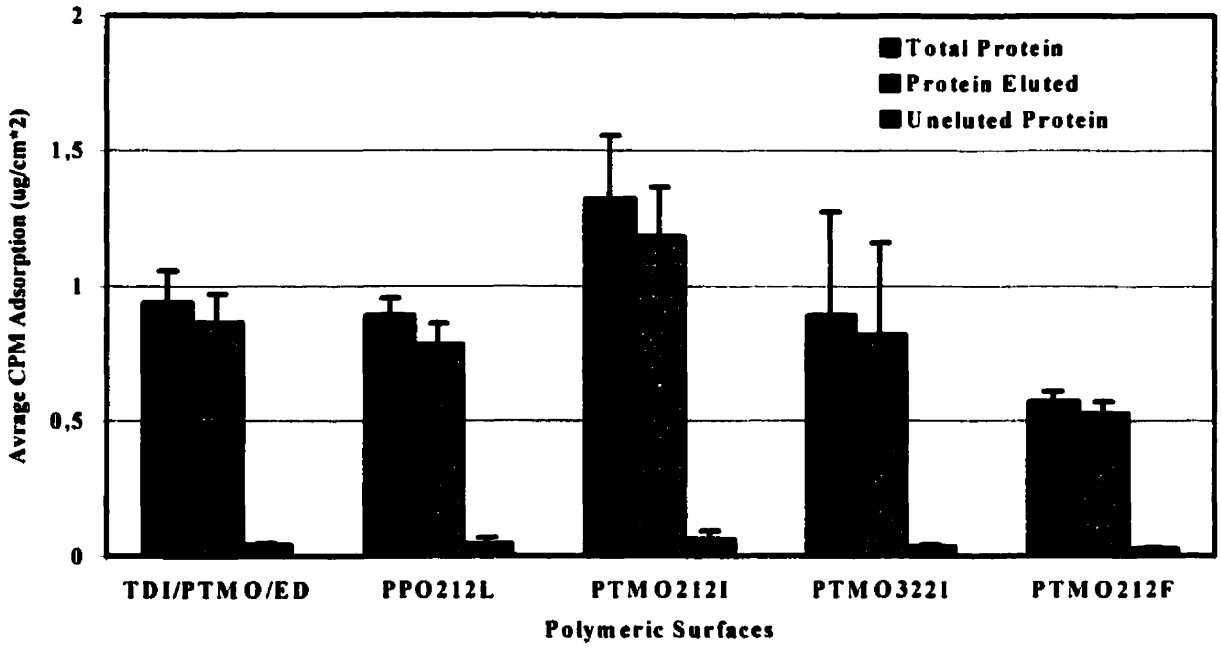
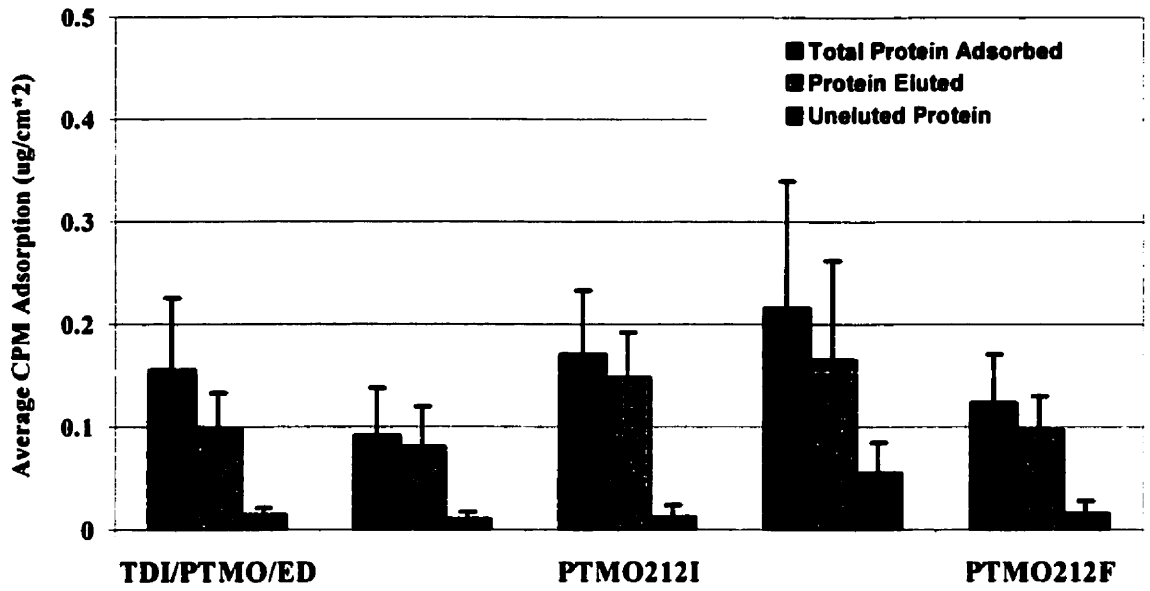


Figure 4.9. The adsorption profile of fibrinogen from Phosphate Buffer Solution (PBS) pH 7.0, onto different polymeric surfaces, after three hours incubation at room temperature.

Figure 4.10 represents the amount of the radiolabelled Fg adsorbed from plasma onto PEU and PEU containing SMMs. No significant differences could be seen among the different surfaces, following ANOVA analysis with 95% confidence interval. While it would appear the PPO212L adsorbs the lowest amount of the protein and PTMO322I the highest, the differences are not significant. Again, approximately 90% of the protein could be eluted from the surfaces. The presence of large error bars is rationalized by the complexity of plasma as test solution.



4.10. The adsorption profile of fibrinogen from blood plasma, onto different polymeric surfaces, after three hours incubation at room temperature.

Figure 4.11 directly compares the amount of ^{125}I-Fg adsorbed from the plasma and from PBS. The amount of ^{125}I-Fg adsorbed from plasma onto polymeric surfaces is again (similar to albumin studies Figure 4.8) about 10 times lower than the adsorption from the pure buffer. These data were verified statistically by ANOVA, and it was shown that Fg adsorption from plasma and buffer were significantly different for each substrate at p-value < 0.05.

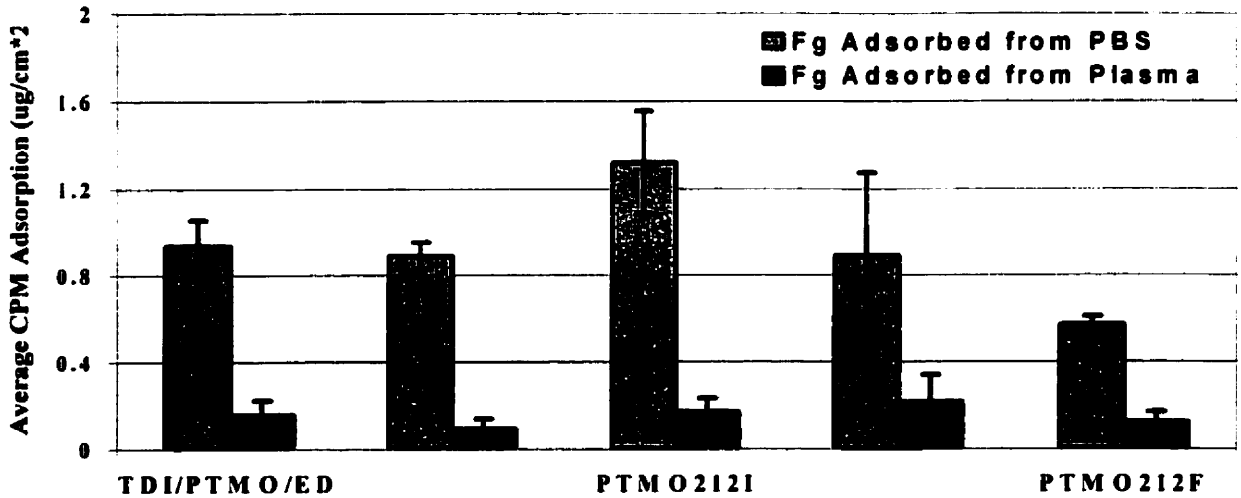


Figure 4.11 Comparison between amount of Fg adsorbed from PBS vs. blood plasma.

4.1.2.3 Comparative Data of the Adsorbed Proteins from PBS and Blood Plasma

Figure 4.12 and 4.13 show the graph of comparative adsorption analysis of Fg and HSA from PBS and plasma respectively. It appears that HSA and Fg are adsorbed equally from PBS as well as plasma onto different polymeric surfaces. Due to the presence of large error bars on the HSA group (especially in PPO212L), it is not possible to identify any differences between the two groups. Statistical analysis (ANOVA, Sheffe method, with confidence interval 95%) revealed that there were no significant differences among any of the groups. Interestingly, although both proteins are structurally and functionally different, their adsorption behavior onto each substrate seemed to be comparable.

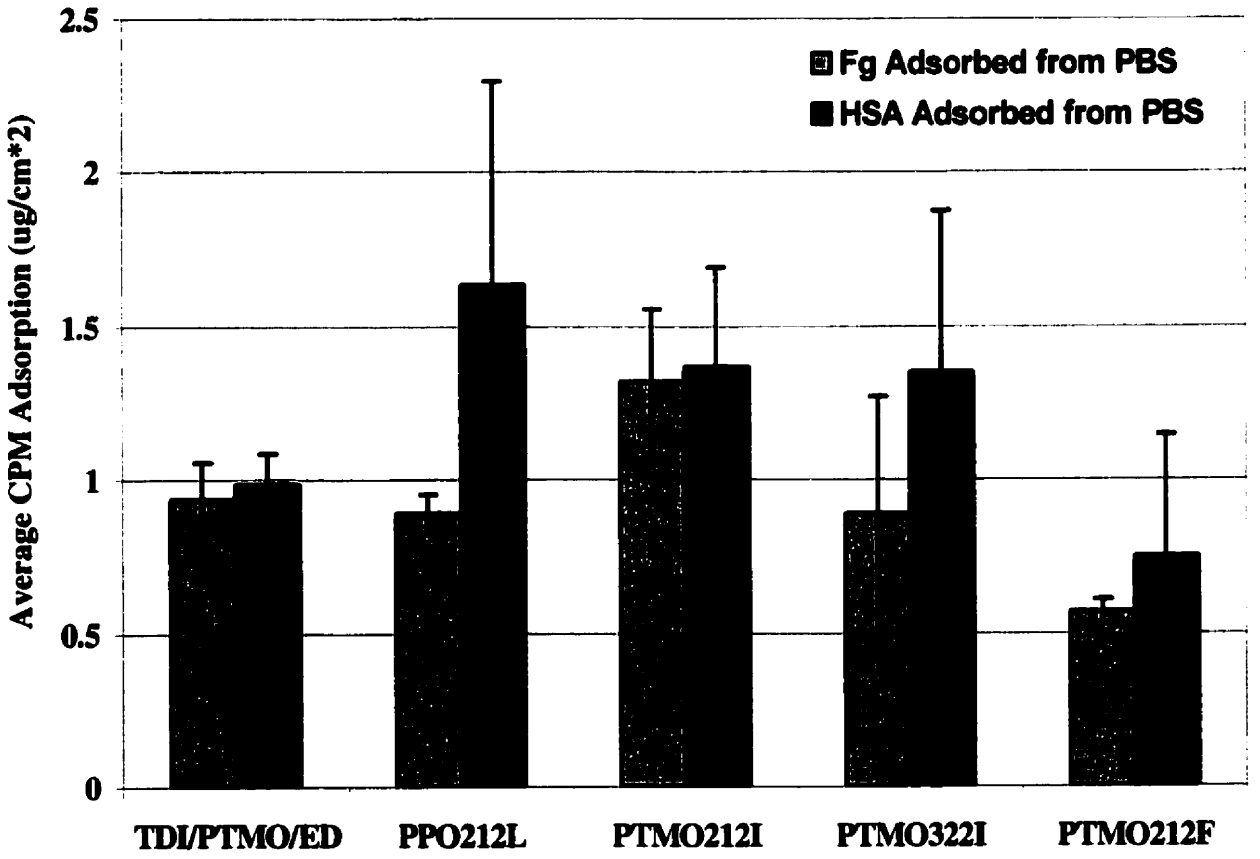


Figure 4.12. Comparative Studies on HSA and Fg adsorption from PBS.

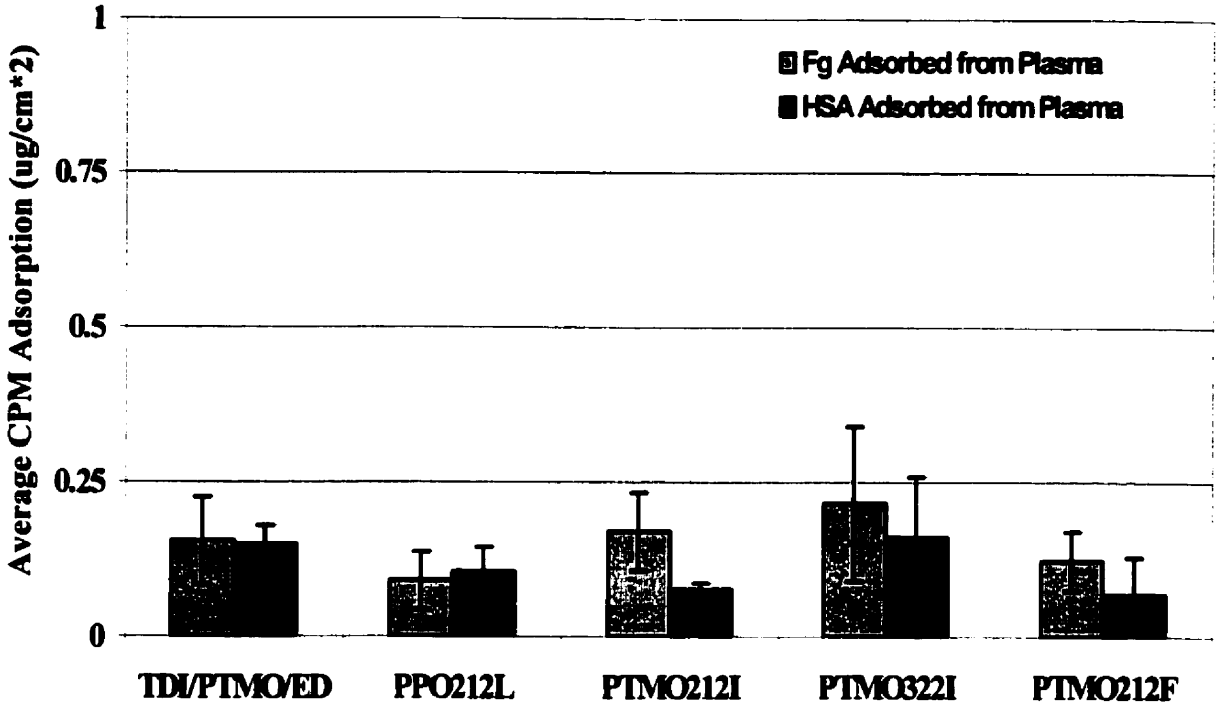


Figure 4.13 Comparative Studies on HSA and Fg adsorption from blood plasma.

4.2 Platelet Adhesion:

4.2.1 ^{51}Cr-Platelet Adhesion onto the Polymeric Materials

Adhesion of the radiolabeled (^{51}Cr) platelets from whole blood, onto different polymeric surfaces is shown in Figure 4.14. As can be seen, the platelet density on the TDI/PTMO/ED is very high in comparison to the other SMM containing PEUs. Among the SMMs, it is observed that PTMO212I and PTMO322I behave in a very similar manner as far as platelet deposition is concerned. PPO212L and PTMO212F have the least amount of platelets deposited onto their surfaces. Statistical analysis (ANOVA, Sheffe method with 95% confidence interval), revealed that all the PEU containing SMMs have significantly lower platelet density on their surfaces than the base polymer. This analysis also suggested that there were some statistical differences among the PEU containing SMMs at p-value < 0.05 (ANOVA). For example, the analysis revealed that PTMO212F and PTMO212I were significantly different (p-value , 0.05). There were no statistical significance between PPO212L and PTMO212F.

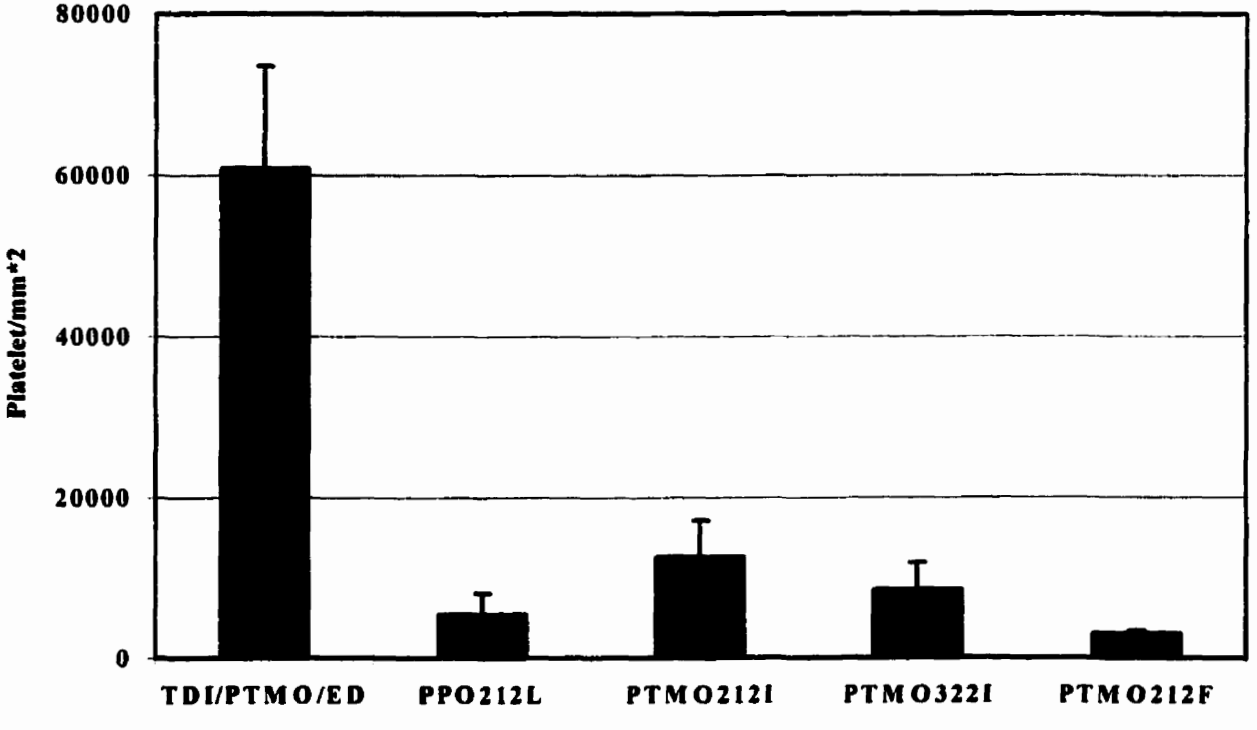


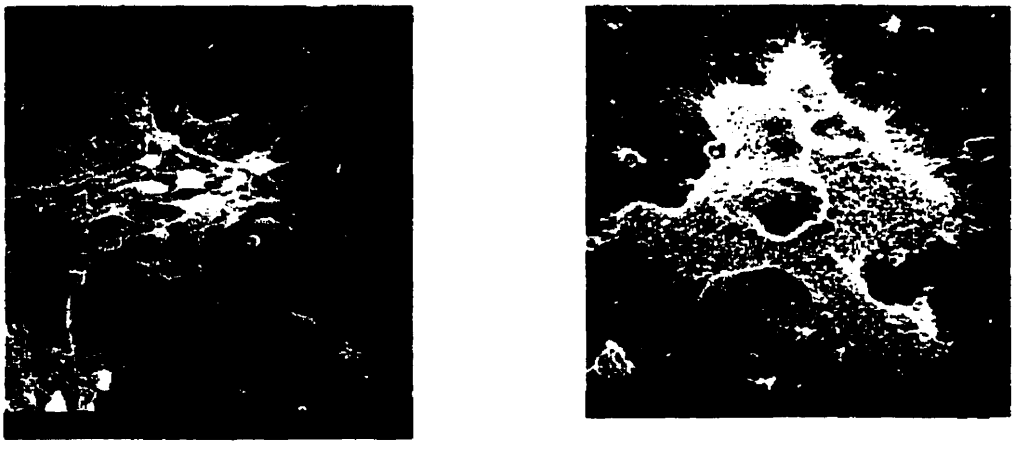
Figure 4.14. Platelet adhesion (normalized n=9) onto 5 different polymeric surfaces after 15 minutes of exposure to whole blood at 300 rpm (150 s^{-1}).

4.2.2 Activation Analysis of the Platelets by Scanning Electron Microscopy (SEM)

The morphology of the adherent platelets onto the five polymeric surfaces is indicative of the reactivity for the platelets when they encounter different surfaces. The differences observed in platelet morphology and spreading may be an indication of differences in thrombogenicity for the various materials. Different pictures obtained by scanning electron microscopy (SEM) of platelets on PEU and PEU containing SMMs revealed the activation state of each platelet in the early stages of contact and spreading.

4.2.2.1. TDI/PTMO/ED:

Figures 4.15. a and b show SEM pictures of the platelets on base polymer (TDI/PTMO/ED). The adherent platelets exhibited their activation by pseudopodia formation and an extensive degree of spreading. Clustering of the platelets on top of each other as well as the direct contact of the neighboring pseudopodia displayed the evidence of platelet-platelet interaction. Another sign of the platelet activation was that most the platelets had their central region elevated as an indication of activation (Goodman 1984), as shown in Figure 4.15 a. These pictures represented the later stages of the platelet activation (platelet-platelet interaction, white thrombus formation etc.).

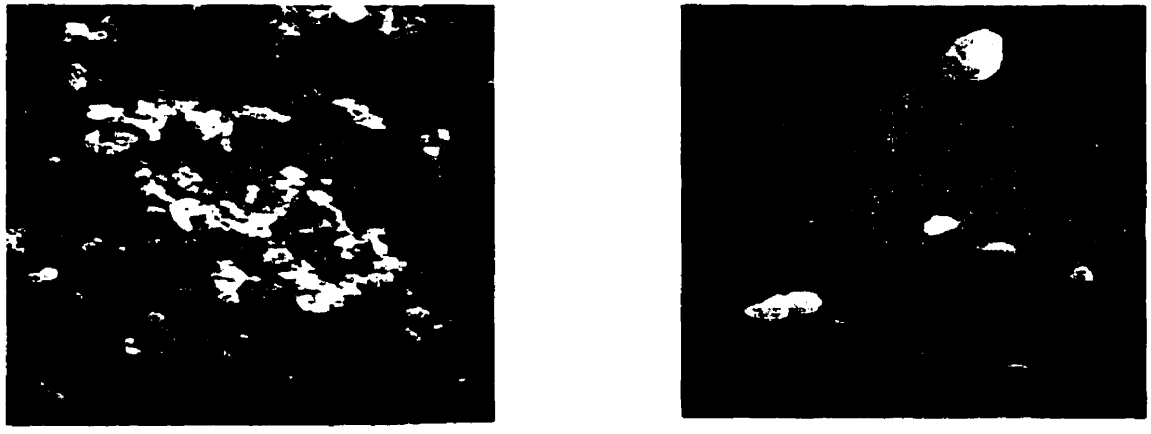


a b

Figure 4.15 Scanning electron micrograph of platelet adhesion onto TDI/PTMO/ED, after 15 minutes of exposure to whole blood at 300 rpm.(a. x 5.00 K, and b. x 1.00K original magnification).

4.2.2.2 PPO212L:

Figures 4.16 a and b show the morphology of the platelets present on the surface of PPO212L. As can be seen from the picture and based on the previous description of the activated platelets, these platelets were not present in their activated state. None of the platelets showed any amount of spreading, but rather a typical discoid shape. No evidence of pseudopodia formation was present. They were appeared to be dendritic and not in the spread form. Such morphology further suggested that these were aggregates of quiescent platelets, as opposed to activated cells, which should at least show some indication of spreading. These cells were present in single colony or present as aggregates of many cells. Had more intensive washing procedures been used, it is believed that these non-activated platelets could have been removed from the surface.



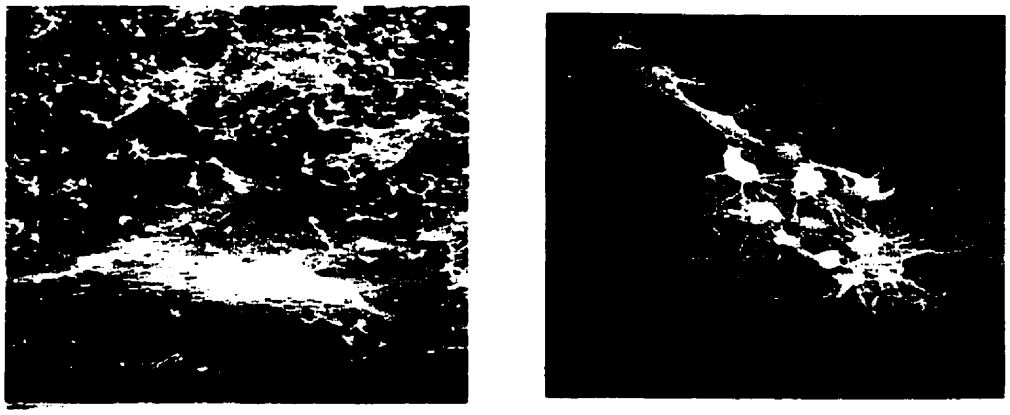
a b

Figure 4.16 Scanning electron micrograph of platelet adhesion onto PPO212L, after 15 minutes of exposure to whole blood at 300 rpm. (a. x 600 and b. x 5.00K original magnification).

4.2.2.3 PTMO212I

The morphology of adherent platelets on the surface of PTMO212I was a contrast to that of PPO212L. Figures 4.17 a and b, represented the morphology of the platelets on PTMO212I. These platelets showed a degree of circularity and spreading. They exhibited relatively long and thin pseudopodia, with the central region of the platelets being elevated. Figure 4.17 showed that some

platelet-platelet interaction were occurring with intermingling of their pseudopodia. The typical appearance of a platelet as "fried egg" was also observed in this micrograph. It could be noticed in Figure 17 b that the pseudopods werre strongly "anchored " onto the surface of the polymer.

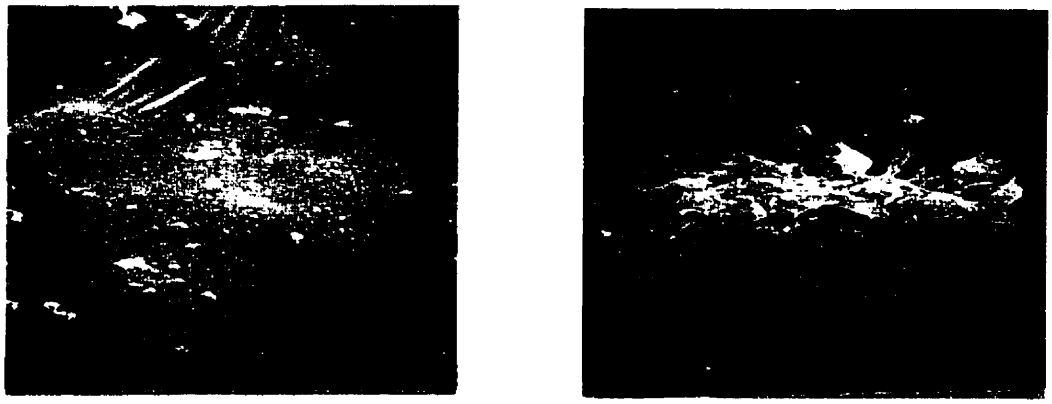


a b

Figure 4.17 Scanning electron micrograph of platelet adhesion onto PTMO212I, after 15 minutes of exposure to whole blood at 300 rpm. (a. x 600 and b. x 5.00K original magnification).

4.2.2.4. PTMO322I:

Platelets on PTMO322I exhibited very similar characteristics to those on the PTMO212I containing surface. Although a smaller number of platelets was attached to the surface, the adherent platelets exhibited pseudopodia formation and some spreading as indicated by their circular morphology (Figures 4.18 a and b). There was also evidence of platelet-platelet interactions. However, these activated platelets were scattered around the surface and were not concentrated throughout the surface as was found on the base (Figure 4.15).

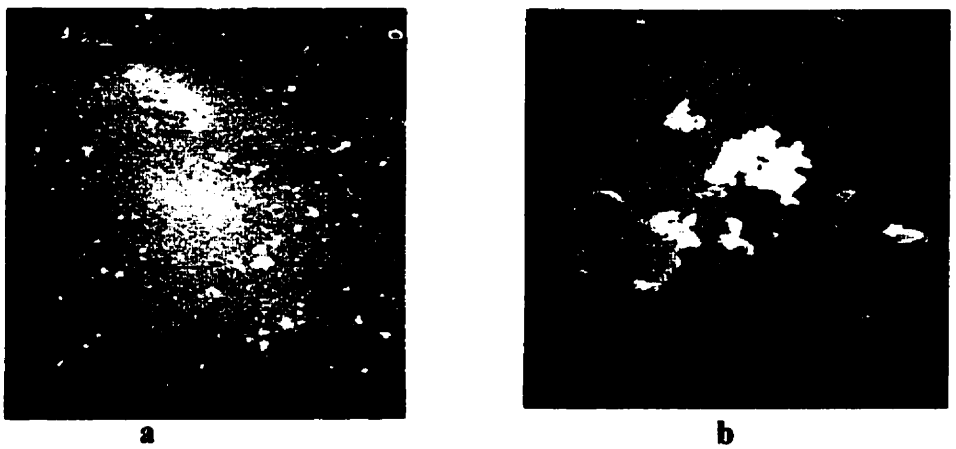


a b

Figure 4.18 Scanning electron micrograph of platelet adhesion onto PTMO322I, after 15 minutes of exposure to whole blood at 300 rpm. (a. x 600 and b. x 5.00K original magnification).

4.2.2.5 PTMO212F:

Figures 4.19 a and b showed relatively quiescent platelets on PTMO212F. Pseudopodia were absent, and no spreading or circularity of the platelets was observed. Platelets present on the surface consisted of aggregates that accumulated nonspecifically on top of each other or individually. These characteristics resembled those observed on PPO212L. As mentioned earlier, a more aggressive washing procedure might have removed these loosely held platelets from the surface of PTMO212F.



a b

Figure 4.19. Scanning electron micrograph of platelet adhesion onto PTMO212F after 15 minutes of exposure to whole blood at 300 rpm. (a. X 600 and b. X 5.00K original magnification).

4.3 Biodegradation Experiment

Previous data have indicated that PEU containing SMMs inhibited the biodegradation of the base PEU by cholesterol esterase (CE) (Jahangir 1995, and Tang 1996). Due to the importance of protein adsorption upon blood-material interactions, a series of experiments was conducted to study the influence of protein pre-adsorption on the biodegradation of the polymers with cholesterol esterase. This pre-adsorption model would be a good initial model for the *in-vivo* scenario, since it is believed that large amount of this protein adsorbs on the surface of the polymer shortly upon its exposure to blood. (Brash 1985, and Chinn 1992). These results were further confirmed earlier in this chapter (Figures 4.1-4.5).

Figure 4.20 showed the results of the long-term biodegradation experiment of PEU and PEU containing SMMs, by cholesterol esterase (80 units/mL). It should be mentioned that all the polymeric surfaces were pre-adsorbed with 1 mg/mL of fibrinogen, which should theoretically provide a uniform monolayer of protein on the surface of the polymer. As can be seen from the graph (Figure 4.20) the non-modified base polymer (TDI/PTMO/ED) showed the highest rate of the polymer degradation. TDI/PTMO/ED degradation was followed by PEU containing SMM: PTMO212F, PTMO212I and PTMO322I. The most resistant surface to the release of radiolabeled degradation product was PPO212L. All the polymers exhibited similar levels of product release until day 70. From this point onward, changes in the slopes were observed, indicating an increasing rate of radiolabel release of base polymer components. The amount of radiolabeled polymer degradation in the case of PPO212L always remained lowest of other three surface modified PEUs. The comparison of the final degradation levels revealed that PPO212L was able to inhibit most effectively 50% of the initial surface degradation, whereas other SMMs were not as effective (Table 4.6).

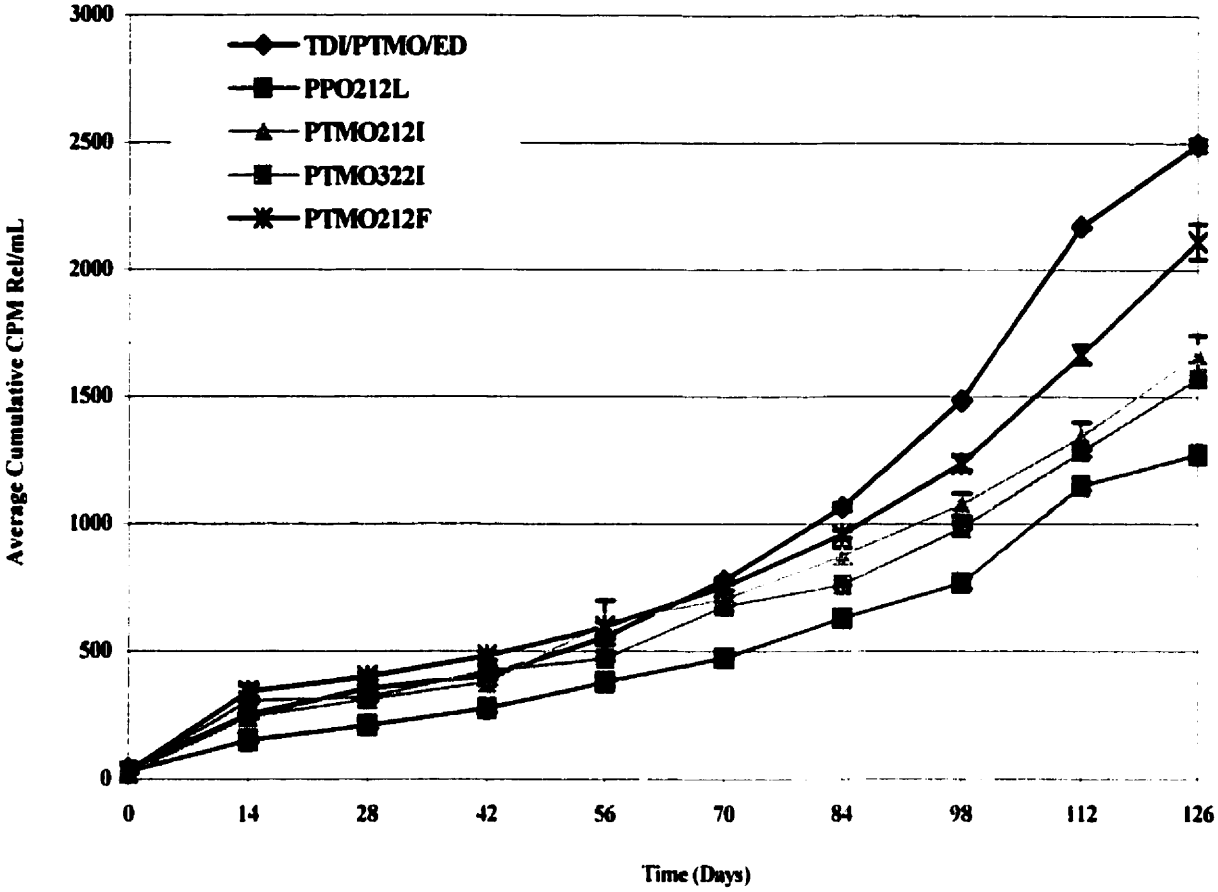


Figure 4.20. Long-term biodegradation of the fibrinogenated PEU and PEU containing SMM by CE (80 units/mL) at pH 7.0 (37 °C) for 126 days.

Surface Modifier	Fibrinogenated (Avg. CPM/mL)	% Degradation relative to base polymer
The Base: None (TDI/PTMO/ED)	2469 ± 24	100
The PPO: PPO212I	1275 ± 69	52
The PTMO: PTMO212I	1668 ± 75	68
The PTMO: PTMO322I	1572 ± 29	64
The PEO: PTMO212F	2114 ± 69	86

Table 4.6. The radiolabel release from the fibrinogenated PEU and PEU containing SMMs at day 126.

Figure 4.21 shows a comparative graph between fibrinogenated and non-fibrinogenated PPO212L containing surfaces. As can be seen from the graph, the rate of the degradation for the non-fibrinogenated polymer was initially a little higher than that of the fibrinogenated system. It is believed that the presence of Fg on the polymeric surface provides "some" delay of the biodegradative effect of CE (until the 70th day). However, this pseudo-protective influence progressively disappeared after this point. Both fibrinogenated and non-fibrinogenated groups had the same degradation rate by the 126th day.

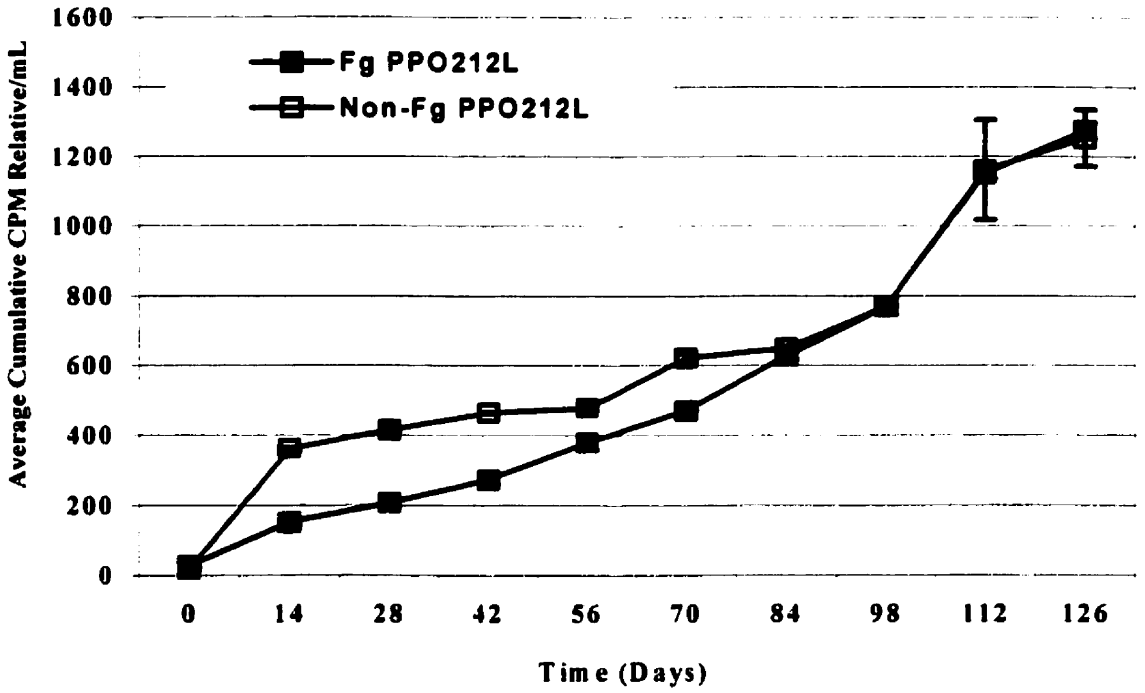


Figure 4.21. Radiolabel release from PPO212L incubated with CE (80 Units/ml) in 0.05 PBS, pH 7.0 at 37 °C.

This temporary protective influence of Fg layer was also observed in the non-modified surface of base polymer (TDI/PTMO/ED) (Figure 4.22). The interesting point about this polymer was that the difference between the degradation of the fibrinogenated and non-fibrinogenated group is much larger than that observed for PPO212L. This might be explained by the fact that the presence of PPO212L on the surface was still the main mechanism for inhibiting the degradation by CE, for the SMM modified material. This point was further accentuated by the observation that the base polymer had a much higher rate of degradation than PPO212L, and even the other SMMs (see the slope in Figure 4.20).

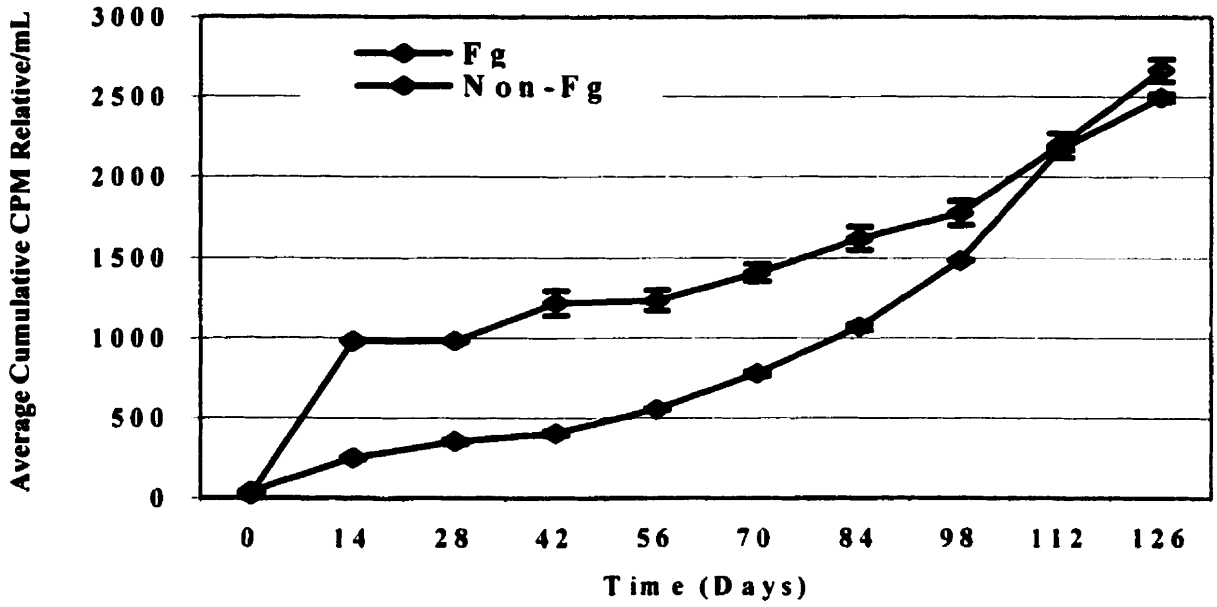


Figure 4. 22 Radiolabel release from TDI/PTMO/ED incubated with CE (80 Units/ml) in 0.05 PBS, pH 7.0 at 37 °C.

The cumulative radiolabel release data for day 126 are given in Table 4.6, for the base and the four SMMs modified materials. In all cases (with the exception of PTMO322I) the effect of fibrinogen adsorption in protecting the surfaces from the degradative effect of CE, had almost disappeared and in fact, for PTMO212F the degradation on the fibrinogenated surface was greater than that of the non-fibrinogenated surface (student T-test, with p-value < 0.01).

Surface Modifier	Fibrinogenated (Avg. CPM/mL)	Non-Fibrinogenated (Avg. CPM/mL)
The Base: None (TDI/PTMO/ED)	2469 ± 24	2668 ± 69
The PPO: PPO212I	1275 ± 69	1253 ± 82
The PTMO: PTMO212I	1668 ± 75	1693 ± 30
The PTMO: PTMO322I	1572 ± 29	1915 ± 53
The PEO: PTMO212F	2114 ± 69	1976 ± 9

Table 4.7. Comparative table of the radiolabel release from PEU and PEU containing SMMs incubated with CE (80 units/mL) at pH 7.0 (37 °C) for 126 days.

4.4 Summary of Results

In summary, the results presented indicate that SMM containing PEU demonstrate different interfacial behavior than the non-modified PEU. These trends suggested that almost all of the SMMs have adsorbed less protein, adhered and activated less platelet. However, variation also existed among the SMMs. For example, it was shown that PPO212L initiates the least amount of host response measured by adsorbing the least amount of proteins, as well as having low platelet adhesion and activation. The similar trend was seen in the case of PTMO212F. Interestingly, although the two SMMs had quite different chemical structures, they exhibited comparable blood compatibility character. The two other SMMs (PTMO212I and PTMO322I) behaved in a less blood compatible manner than the former two SMMs.

5.0 DISCUSSION

The objective of current study was to gain some insight into different aspects of polymer-blood interactions and specifically in regards to the contact of proteins and platelets with SMM modified surfaces. This was done with the intent of engineering surfaces which, may minimize the host response and remain relatively stable in the presence of deleterious environment.

The host response has been defined "as the reaction of a living system to the presence of a material" (Williams 1986). This response includes protein adsorption, platelet adhesion and the subsequent coagulation of blood at the surfaces of materials and inflammatory associated events (Brash 1985, and Lamba 1997). The experimental data obtained will be discussed under the following subject-matters: 1. The data from the adsorption of different plasma proteins onto surfaces, 2. The adsorption of two single proteins (fibrinogen and albumin) from plasma and buffer; 3. The adhesion of platelets onto different substrates and the assessment of their activation states, 4. The biodegradation of the PEU containing SMMs, by CE (a macrophage associated enzyme), when pre-adsorbed with fibrinogen. The latter topic may be considered a material response, since it is the response of the polymer to the degradative effect of CE that is being studied, hence a material response versus a host response.

5.1 Material Surface Properties

It is believed that polymeric surface properties have an important impact on the rate, the extent, and the mechanism of protein adsorption as well as cellular adhesive events (Andrade 1987). Hence, in order to better understand, the data acquired during the course of this study it would be essential to first discuss the surface chemistry and its dynamics for each of the PEU and SMM containing substrates.

According to classical surface chemistry, solid surfaces are assumed to be rigid, immobile and at equilibrium. Although such assumptions may apply to truly rigid solids, they are generally inappropriate for polymers (Williams 1971). Polymers are, in general, time and temperature dependant (Williams 1971). In fact, it is believed that most polymers rarely achieve true equilibrium due to their relatively high molecular weights (Andrade 1987). It could then be concluded that the

polymers are, in general, an inherently non-equilibrium structure, and as such, exhibit a range of relaxation times and properties under normal conditions and in response to a changing environment (Andrade 1987). According to North *et al,* polymer relaxation is defined as "A time dependent return to equilibrium of the system which has recently experienced a change in the constraints acting upon it" (Andrade 1987). The relaxation phenomenon is more pronounced in block copolymers that may have large domains of different surface properties. One of the most practical methods of studying surface phenomena is achieved by measuring the substrate's contact angle. In this method surface relaxation and motion is measured by preparing the surface under one set of conditions and examining it as a function of time and temperature in another environment (Andrade 1985). The tendency is for the polymer to minimize its surface energy, and therefore undergo an appropriate change in order to achieve such a state.

The contact angle data for each of the material surfaces studied in this work was presented in Figure 3.3 and has been kindly provided by C. McCloskey (M.A.Sc. thesis January 1999). It was generally accepted that for a water droplet in a water/air/surface system, the advancing contact angle was associated with the low surface energy domain determined by the polymer's hydrophobic groups (Andrade 1985). On the other hand, the receding contact angle measurements were used to assess the expression of the surface's hydrophilic component. The difference between advancing and receding angles, is contact angle hysteresis. The contact angle hysteresis has been shown to occur upon exposure of a water droplet at an air/polymer interface. Preferential reorientation of the polar groups from the polymer has been reported to occur in order to reduce the interfacial free energy of the system (Okkema 1991). Polymer hysteresis behavior may also be affected by surface roughness, by the incomplete removal of solvent from the surface, by polymer swelling and water adsorption during the advancing contact angle measurement (Wu 1982). In the case of the data presented in Table 3.1, it is reported that the observed hysteresis was primarily attributed to the surface heterogeneity of the polymer chains and not surface roughness since the coated glass slides were reported to be smooth upon visual inspection (McCloskey 1999). It should also be mentioned that the polymer coated slides, from which the contact angle measurements were conducted contained 4% weight SMMs, as opposed to 5% that were used for all the experiments in this thesis. It was reported by Tang that PEUs' surface saturation by SMM occurs at 2-3%, and therefore the difference between the two weight percents should be insignificant (Tang 1995). Hence, it is

appropriate to discuss the findings of this thesis in the context of the contact angle data, obtained from McCloskey (1999).

The physiochemical characteristics of a biomaterial at the interface, such as, its hydrophilicity, its smoothness, its degree of heterogeneity and distribution of different reactive chemical groups, not only contribute to the different surface dynamic phenomena but also to the selectivity of the adsorbed proteins, which may subsequently influence platelet adhesion with this layer (Cazenave 1990). Nevertheless, establishing specific correlation between plasma protein adsorption and these parameters have been difficult (Wojciechowski 1986)

The general trend observed in the contact angle data Figure 3.3 indicated that all of the PEU surfaces modified by SMMs had a higher advancing contact angle as compared to the non-modified PEU. This suggested that the presence of the fluorine component at the surface generated a low surface energy layer, a reflection of the increase in hydrophobic character. Tang *et al* confirmed the presence of the SMM molecules at the surface using X-ray photoelectron spectroscopy (XPS) (Tang 1996).

It should also be pointed out that there are some differences in advancing contact angle values among the SMMs. Although it is tempting to attribute these differences to the fluorine content of the SMM (Table 3.2), it was found that this relation did not exist. Tang *et al* showed that although fluorine contributes to a higher advancing contact angle, there did not seem to be a correlation between the higher fluorine content and higher advancing contact angle, in the SMMs (Tang 1995). This may be explained by the fact that surface saturation of the increased hydrophobic effect is achieved at about 2% SMM content (Tang 1995). Between the PTMO212I and PTMO322I, the former has one of the highest fluorine content (19.3%) and the latter has the lowest fluorine content (11.3%) (Table 3.2), yet they both have very similar hydrophobic character (Figure 3.3). It is interesting to note that both of these SMMs have a similar soft segment and diisocyanate component, however they vary in their respective ratios. Hence, this suggests that other structural and chemical characteristics of the SMMs contribute to the hydrophobic behavior at the interface. For SMMs containing more than 1-2% by weight of additive (i.e. above the saturation point) these differences are thought to be related to the specific chemical nature of the mid chain component for the individual SMMs themselves.

It was interesting to note that the two SMMs (PPO212L and PTMO212F), which contain different soft segments components than that of the base polyurethane (i.e. PPO and PTMO/PEO respectively), exhibited a difference in their advancing contact angle values. This was despite the fact that their fluorine contents were similar to that of PTMO212I. This further emphasizes the importance of the central segment of the SMM. The surface containing PTMO212F had the lowest advancing contact angle of the different modified surfaces and contained a poly-ethylene-oxide (PEO) spacer between its fluorinated alcohol and the diisocyanate of the SMM. The presence of the PEO group was attributed to generate SMMs with increased flexibility of the terminal fluorine chain, and elevated exclusion volumes. These properties may allow repulsive interactions to dominate over van der Waals attraction, when the SMM is positioned at the surface (Brash 1992).

Hence, these observations suggested that the hydrophobic nature of the polymer blends were not solely determined by the presence of the fluorine on the SMM. Rather, such features as difference in soft segments, length of fluoro-alcohol (I: intermediate, L: long, and F: FSO), and the polarity, as well as possible interactions between these parameters may contribute to their interfacial behavior. These characteristics could also be considered, to explain the differences in receding angles. For example, PPO212L and PTMO212F had comparable receding angles. Interestingly, the non-modified base polymer had a similar receding angle to that of the former SMMs. Since the values of receding angles were a reflection of the hydrophilic surface components, these three polymer substrates would be considered as having exhibited similar hydrophilicity despite their structural differences. It has been demonstrated by Takahara that the higher the hydrophilic nature of the polymer the lower its contact angle (Takahara 1991).

Contact angle hysteresis did not seem to follow the same trends as the actual measured contact angle. It was observed that the PPO212L had the highest hysteresis value among the SMMs. This might be due to the (-CH_3-) group present on the PPO soft segment of this SMM. Zisman *et al* have shown that the presence of the relatively short alkyl groups, reduced the van der Waals interaction among the polymer chains, yielding a more mobile "liquid like" character, with a lower packing density and coverage (Bernett 1971). The presence of such segments on the surface of the polymer was believed to have allowed for relatively facile restructuring of the soft segment on the surface upon a change of media, which in turn explains the large hysteresis value.

Interestingly, all of the SMMs containing PTMO groups and the non-modified PTMO containing base polyurethane exhibited similar hysteresis behavior. It was suggested that the length of the PTMO in the SMMs and the bulk polymer, as well as the absence of any alkyl group, contributed to stronger van der Waals interactions between the polymer chains, less mobility and hence reduced hysteresis. This was strongly supported by the knowledge that the PTMO segments in PEU were known to undergo crystallization (Lelah 1986).

In conclusion, from the contact angle data, it can be deduced that modification of the poly-ether-urethane by SMMs has effectively changed the surface chemistry, in such a manner that has rendered the substrates more hydrophobic, (as indicated by an average advancing contact angle values). This low surface energy characteristics varied from one SMM to another depending on the chemistry, length of the fluoro tail and nature of the central chains and their mobility. In addition, the hysteresis phenomena as indicated above, could be modified by such factors as chain mobility, polarity and the presence of small alkyl side chain. Consequently, the contact angle data provided a relative measure of the hydrophilic and hydrophobic elements expressed at the surface in the following manner:

Least Hydrophobic	Base < PTMO212F < PPO212L < PTMO322I ≤ PTMO212I	**Most Hydrophobic**
Most Hydrophilic	Base ≤ PPO212L = PTMO212F <PTMO212I = PTMO322I	**Least Hydrophilic**

5.2 Western Blot Analysis

In this section of the thesis, the banding patterns of proteins observed on the different substrates are presented in relation to their anticipated physiological importance, at least within the context of this thesis. As well, the patterns of binding will be discussed in light of the specific surface energetics of the materials.

A general comment should be made on the protein adsorption data presented in Figures 4.6, 4.7, 4.9, and 4.10, with regards to eluted and non-eluted proteins. The data showed that more than 95 % of the protein was effectively eluted into the eluent, and hence the "total protein eluted" bars in all cases represent a reasonable reflection of how much protein was originally adsorbed onto each of the substrates. This provides some level of confidence in the qualitative analysis that can be made from the Western blot data.

5.2.1 Adhesive Proteins (Fg, Fnc, Vnc):

Data in Tables 4.1-4.5 highlighted the protein adsorption from the plasma onto the five materials. It was noted that all the principal adhesive proteins (fibrinogen, fibronectin, and vitronectin) that are responsible for protein-protein as well as protein-cell interactions were present on these substrates.

A. Fibrinogen:

Fibrinogen has been implicated in platelet adhesion, by providing the required peptide (RGD) epitope for platelets' glycoprotein receptors (Fabrizius 1991). The contact angle data in Figure 3.3 indicated that, the non-modified base polymer did not have similar hydrophobic character to the PEU materials modified with SMMs. However, PPO212L and PTMO212F exhibited similar hydrophilicity to that of base PEU. It has been reported by other investigators that Fg adsorbs preferentially onto the surfaces with more hydrophobic character, and that its binding strength increased with hydrophobicity (Uniyal 1982, and Brash 1985). Based on the intensity of the Fg bands obtained in this study, it appeared that PTMO322I and the base (Table 4.4) had adsorbed the highest Fg levels, yet their hydrophobic character is quite contrasting (Figure 3.3). Hence it is believed, other factors must be at play. Kashiwagi *et al* have demonstrated that the presence of low surface energy fluoro-alkyl groups on the surface of PEUs, could reduce the level of adsorbed protein (Kashiwagi 1993). Interestingly, the lowest amount of Fg was indeed observed on two of the PEU containing SMMs: PPO212L and PTMO212F (Table 5.2). This low adsorption trend was also observed for PPO212L in the Fg adsorption studies from plasma (although only at p-value of < 0.9) (Figure 4.11). Further examination of the contact angle data, suggested that the PPO212L demonstrated the highest amount of hysteresis of all the SMM containing surfaces and the base polymer. It was suggested that due to the high molecular reorientation in response to change at the

liquid/solid interface, Fg had more difficulty to establish good binding. Similarly, PTMO212F had a comparable effect resulting from the presence of the PEO group

Examining the blots in more detail revealed another fact; all the SMM containing PEUs exhibited the presence of some low molecular weight Fg fragments (below 50 kD). Interestingly, non-modified base did not exhibit any such fragments. As Brash *et al* have also shown, these bands may correspond to the degradation fragments of the Fg caused by plasminogen (Brash 1986, and Cornelius 1992). This suggested that most of the SMM containing PEUs, in addition to their fibrinogen binding character might contain fibrinolytic properties as well. Interestingly enough, the band corresponding to plasminogen (98 kD) was observed in all (except PTMO322I) of the modified and non-modified surfaces (section 5.2.4) (Tables 4.1-4.5).

B. Vitronectin (Vnc) and Fibronectin (Fnc)

These two proteins were among the major adhesive glycoproteins in plasma (Preissner 1991). Like Fg, they also contain an RGD sequence, which was believed to mediate their binding to integrin receptors (i.e.: GPIIb/IIIa) in platelets. It was however, observed that Vitronectin appears to be superior to fibronectin with respect to the level of adsorption from plasma onto polymeric substrates (Preissner 1991). In other reports, it has been shown that for competitive experiments between Vnc and Fnc, that Vnc adsorbed before Fnc reached the surface (Fabrizius-Homan 1991). In another competitive experiment, it was indicated that Vnc might even arrive at the surface earlier than Fg because of its larger diffusivity and higher molar concentration (Fabrizius-Homan 1991).

The results obtained indicated that the level of protein adsorption was as follows: Fg > Vnc > Fnc, and the order of materials in terms of these three proteins, appeared to be the following: Base >PTMO212I = PTMO322I > PTMO212F > PPO212L. The low protein affinity of the PPO212L for Vnc and Fnc may again be attributed to the chemical structure of the PPO, and the elevated hysteresis exhibited by the PEU containing this additive.

5.2.2 Complement Proteins (C3 and factor B)

The involvement of the complement system proteins in regulating cellular, inflammatory and immune responses has been well-documented (Kazatchkine 1987, Frank 1991). It has been observed that upon contact of these proteins with "activating" surfaces, complement activation began through either the classical or the alternative pathways (Kazatchkine 1991). Complement proteins comprise at least 19 components; however, for the purpose of current studies and their relative importance, only three of these proteins have been studied. These include: factor H, factor B and C3. Special attention was given to C3, due to its importance, as the most abundant complement protein as well as its significance in the complement pathway (Sim 1981).

A. C3

Among the activation products the C3b-iC3b (MW range: 46-43) were present in base and PTMO212F. It has been suggested by Kazatchkine, that upon cleavage of C3b, a transient binding site could be expressed which allows for the covalent binding of the protein to surfaces. This specific binding took place via a trans-esterification reaction between the glutamyl group from an exposed internal thioester bond in the C3b molecule and OH^- or NH_2 groups on the substrate (Kazatchkine 1987). Based on such information, it was hypothesized that the C3b might bind more tightly to the surfaces which, was more polar and exhibited less hydrophobic nature. Interestingly enough, it was observed that the non-modified base and the PTMO212F containing surfaces, exhibited the least hydrophobic character (advancing contact angle data in Figure 3.1), and the highest amount of C3b adsorption. The poor quality of some of the blots, and lack of reproducibility did not allow for more accurate interpretation of the results.

B. Factor B:

It was believed that the difference between complement "activating" and "non-activating" substrates depended on the extent of the adsorption and the competition between factor B and H for the surface. It is thought that the C3, binding to factor H renders the surface non-activating and its binding to factor B renders it more activating to complement's alternative pathway. (Kazacnkine 1991).

Examination of the blots (Figures 4.1-4.5) revealed that Factor B was adsorbed on most the surfaces. The intensity of bands indicated heavy presence on non-modified PEU and PTMO322I (Tables 4.1, and 4.4). A more detailed inspection of the PTMO212F membrane exhibited complete absence of factor B. In light of such results, it was recommended that further experiments be conducted in order to assess the nature of complement activation since these surfaces all could potentially be strong complement activators.

5.2.3 Coagulation factors (Factor XII, Prekallikrein, and HMWK)

These proteins in addition to being involved in the contact phase of the intrinsic coagulation pathway also play an important role in the kallikrein/kinin system (Scott 1985). It was hypothesized by Cornelius that upon contact of these proteins with certain polymer membranes, some of them would exhibit cleavage. It was believed that negatively charged surfaces are strong contact activators of the plasma coagulation cascade and that positively, charged surfaces were not (Vroman 1972). In fact, Vogler *et al* have shown that polymer surfaces terminated with $-CO_2H$ exhibited very high procoagulant activities, whereas surfaces with $-NH_3^{\oplus}$ show very low pro-coagulant activities. They have also demonstrated that surfaces coated with $(CH_2)_2(CF_2)_7CF_3$ groups exhibit minimal pro-coagulant activity (Vogler 1995). The immunoblots for Factor XII indicate that it was the only band appearing on the surfaces (non-modified base and PTMO322I). Almost none of the other proteins were present in any of the surfaces. It was tempting to hypothesize that the reason for the low adsorption level of the coagulation proteins is directly related to the chemical structure of the SMMs and the base. This trend suggested that the combination of the fluorinated chemistry in the SMMs and the presence of specific amino groups within the urethane and urea groups of the base and SMMs might explain the low uptake of these proteins.

5.2.4 Other Proteins (Albumin)

Among the most abundant proteins in plasma were the "thrombo-resistant protein" albumin and IgG (Fabrizius 1991, Brash 1985). It has been recognized that the adsorption of albumin onto different polymeric substrates would passivate the surface, since it would decrease platelet adhesion and activation (Roohk 1976).

Albumin was strongly attached to all non-modified and SMM containing PEUs. It has been suggested by others that albumin had an affinity for surfaces that exhibit more water retaining capacity and were more hydrophilic (Brash 1985). However, the intact form of the albumin was adsorbed equally to all surfaces. In spite of their relatively hydrophobic character, upon contact of albumin with some of the PTMO containing SMMs, protein was extensively degraded and the degradation fragments were detected at the lower end of the blots. The amount of the degradation in PPO212L was significantly lower. Hence, due to such apparent contradiction a closer examination of albumin adsorption is required. However, ^{125}I-albumin studies did not provide any further insight, since the data indicated that PTMO212I and PTMO212F had the least amount of the adsorbed albumin from plasma (Figure 4.7).

In conclusion, many investigators have shown that the extent of protein adsorption and subsequent desorption from a surface may indeed be determined by a particular surface chemistry (Ishihara 1996), ratio of hard/soft segment (Grasel 1988), hydrophilicity (Brash 1984), to name a few. However, as far as the PEU containing SMMs are concerned, it was shown that the presence of fluorine containing SMMs at the interface of the base material, effectively changed the surface energetics (as indicated by contact angle measurements) (Figure 3.3), and this was translated to a change in the layer of the proteins adsorbed at the surface of each polymer. As was seen from the Western blot analysis, PPO212L and PTMO212F were the two SMM containing PEU that showed, important differences in their protein adsorption behavior (Tables 4.1-4.5). As far as the chemical of the polymers are concerned, the PPO212L had a soft segment different from the rest of the SMMs, and it had the second highest fluorine content of the SMMs. The PTMO212F on the other hand had a PEO spacer linked to its main chain that should supposedly resulted in more mobility of the terminal fluorine chain groups and hence provided steric hindrance effect (Tan 1993). Its fluorine content was, incidentally, very close to that of PPO212L. Hence, although contact angle data indicated that the presence of fluorine contributes to a change in surface chemistry and dynamics, clearly other factors played a decisive and determining role in the interaction of the polymer and the plasma since there were some important chemical differences these two SMMs.

5.3. Single Protein Adsorption Studies:

The main thrust behind these experiments was to further understand the adsorption behavior of the two proteins (albumin and fibrinogen) that were implicated frequently in different studies to explain thrombogenic phenomena (Brash 1985). The potential of a surface to exhibit thrombogenic character was believed to be transmitted through the film of adsorbed protein, which deposited on the surface prior to platelet deposition (Baier 1969 and Ihlenfeld 1978). The adsorption patterns of albumin and fibrinogen have widely been studied by Brash *et al* (Uniyal 1982, Brash 1985, Brash 1992). As far as thrombogenicity was concerned, the above two proteins were at the opposite ends of the spectrum. The adsorption of fibrinogen onto various polymeric surfaces was believed to encourage *in vitro* platelet adhesion (Salzman 1977). While the adsorption of the albumin, on the other hand, was believed to "passivate" the surface and inhibited platelet adhesion (Salzman 1977).

As mentioned earlier, albumin had a molecular weight of 66 kD and consists of a single peptide chain, and was negatively charged at physiological conditions with no carbohydrate. The molecule had, however, a high degree of α-helicity and high disulfide cross-link contents (Brynda 1978). Fibrinogen, on the other hand, was a much larger protein with a molecular weight of 360 kD, and was built up of two equivalent parts held by disulfide bridges and consists of three linear covalently bonded polypeptide chains. Fibrinogen was an asymmetric molecule, low in carbohydrate and conformationaly flexible (Andrade 1987). This conformational flexibility resulted in its high spreading and adaptability to a surface (Baszkin 1994).

5.3.1 Albumin Adsorption

Figure 4.7 represented the amount of HSA adsorbed from the plasma. An important observation that should be made at the outset is that HSA adsorption from plasma was significantly lower (0.21 $\mu g/cm^2$) than the average amount of HSA adsorbed from PBS ($1.21 \mu g/cm^2$). There were approximately six fold reductions in the plasma. This phenomenon has also been observed by Brash *et al* (Brash 1992). As mentioned earlier, this was due to the presence of many other proteins in the plasma and the competition among them to occupy the sites on the substrates. In addition, Warkentin *et al* have shown that protein-protein interactions can affect protein binding on the surface (Warkentin 1994). A statistically significant difference (p-value < 0.05) was observed

between the base material and PTMO212F as well as PTMO212I with the other three surfaces. Presently, there are no clear parameters that can define such trends. However, since the single protein binding experiment suggested that these surfaces all had a similar ability to adsorb the same level of protein (Figure 4.8), these observed differences in the plasma systems might arise from the preferential adsorption of other plasma proteins to these substrates.

The data presented in Table 3.2 showed that both PTMO212I and PTMO212F had very similar fluorine content, as well as similar molecular weights. The SMMs were different in their fluorinated alcohol (BA-L for PTMO212I and FSO-100 for PTMO212F), which were believed to explain the difference in contact angle measurements. Nevertheless, the similarity in protein adsorption indicated that both SMMs exposed the same sites and the same chemical groups appealing to albumin adsorption from plasma. It should be reemphasized that different structural features of two different SMMs inter-played to elicit similar level of adsorbed albumin. It is important to avoid generalization about one or two structural features to explain a given host behavior, even though the explanation will be true in one case it might be completely contradictory in another situation. The issue of polymer interaction with blood or plasma protein is a multi-factorial event that involves the interplay amongst different elements. One thing that is certainly true, is that the presence of the SMM at the interface has altered the polymer's ability to interact with the plasma.

5.3.2 Fibrinogen Adsorption

Brash *et al* have undertaken extensive studies where the adsorption of the Fg onto many substrates has been discussed (Uniyal 1982, Wojciechowski 1985, and Brash 1992). As mentioned earlier, due to the structural flexibility, the fibrinogen molecule can spread and adapt itself to different interfaces (Baszkin 1994). Considering the broad generalization in regards to surface properties, i.e. the "hydrophobic rule", it was stated, that the more hydrophobic the surface the greater the extent of protein adsorption (Brash 1994). It was shown that fibrinogen has a tendency to follow the above generalization. In fact, it was demonstrated that the "net" adsorption (adsorption - displacement) of fibrinogen on hydrophilic surfaces was rapid and extensive, whereas such a Vroman effect was much slower and less extensive on hydrophobic surfaces (Brash 1993).

Figures 4.9 and 4.10 illustrated the radiolabeled fibrinogen adsorption data onto the PEU and SMM containing PEUs, from buffer and plasma solutions respectively. The data indicated that PTMO212I showed the highest Fg adsorption levels from PBS (Figure 4.9). However, there was no significant difference between any of the materials, although PEU containing PTMO212F, showed some reduction relative to the base PEU, this trend could be verified statistically (ANOVA, scheffe method, 95% confidence interval). Such lower value trend could possibly be attributed to the ethylene oxide segment in this SMM (Figure 3.2), since polyethylene oxide used as a coating and as a spacer has been attributed with altering protein adsorption (Horne 1985). All of the surfaces showed that the level of adsorbed protein is in the range of a monolayer (1 mg/mL) (Brash 1979).

Data in Figure 4.10 confirmed that Fg was adsorbed to the surfaces of the materials even in the presence of other plasma proteins. The high variability and lower levels of fibrinogen adsorption were believed to reflect the competitive nature of protein adsorption in the more complex plasma solution. As shown in Figure 4.11, the level of fibrinogen adsorption from plasma was approximately 10 fold lower than that of PBS. Such a trend was also observed in the case of albumin adsorption. Two SMM containing surfaces, PPO 212L and PTMO 212F, showed lower adsorption levels than the other materials. Nevertheless due to high variability and lack of statistical significance (ANOVA, scheffe method with 95% confidence interval), these results could not be confirmed.

The competitive nature between Fg and Alb could not be verified from these studies. However Figures 4.12 and 4.13, compared the protein adsorption data from the PBS and plasma respectively. These data suggested that there was no preferential adsorption of one protein versus the other in the PBS as well as in the plasma, and that all the surfaces adsorbed protein within a similar order magnitude. Hence, it was rather difficult to narrow down the reason for relatively higher or lower protein adsorption levels to one or two factors. There exist a complex interrelationship between surface properties, such as surface chemistry, surface charge, domain morphology, water content, and surface molecular motion (Barenberg 1983).

It is generally accepted that Fg is adsorbed onto the hydrophobic surfaces (Brash 1994). The hydrophobicity of each SMM was determined by measuring the advancing contact angle (McCloskey 1998). The base polymer was excluded from this analysis since the expression of the

most hydrophobic element, i.e.: the fluorine containing SMMs were the target of this analysis. As stated earlier (section 5.3.2), hydrophobic surfaces were expected to show a greater extent of protein adsorption (Brash 1994). In this passage, it was attempted to establish a relationship between Fg adsorption from plasma and the advancing contact angle of SMMs. Calculating the correlation coefficient (r) would allow one to notice the strength of the linear association between the two variables (Moore 1993). The r value was found to be 0.54. This number indicated that there was a poor correlation between the Fg adsorption and the interfacial hydrophobic groups of the SMM (Figure 5.1). One explanation for such poor association could be related to the complex nature of the plasma protein pool from which Fg adsorption was conducted. As mentioned in the previous section, the presence of hundreds of other proteins and the competition among them for binding onto the SMM containing substrates could influence the Fg adsorption. Hence, it was suggested that although the hydrophobicity of each SMM may contribute to Fg adsorption, it was certainly not the only one factor involved. Interestingly, it was found that there was moderately good correlation coefficient (r = 0.92) between, receding contact angle and Fg adsorption (Figure 5.2). The correlation value further indicated that the adsorption of the Fg from plasma onto SMM containing PEU involved other factors than simply hydrophobicity of the surface.

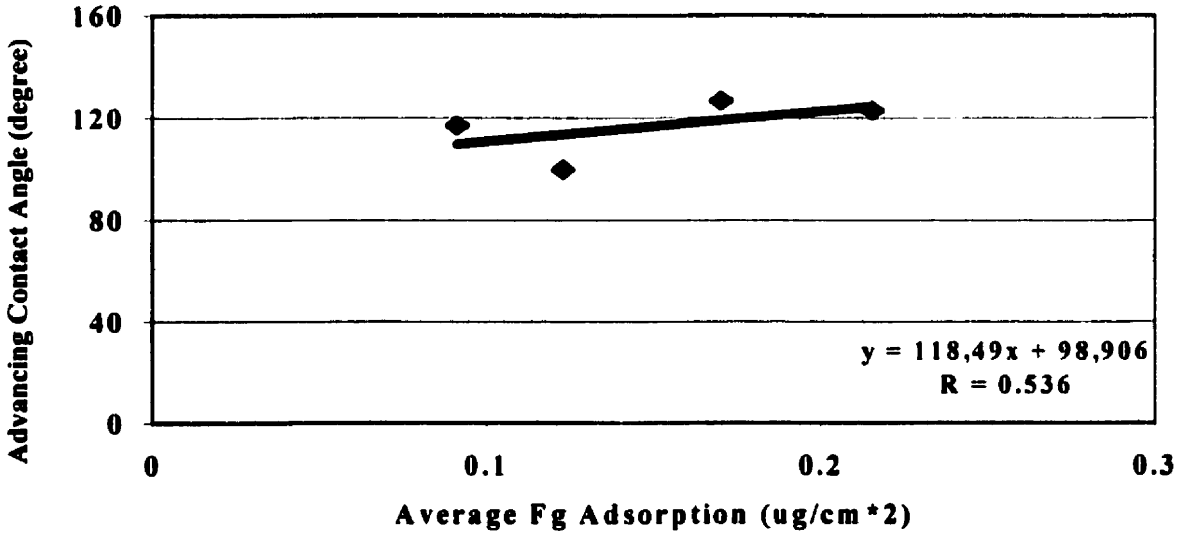


Figure 5.1 **Correlation between Fg adsorption from plasma and advancing contact angle of SMM containing PEU**

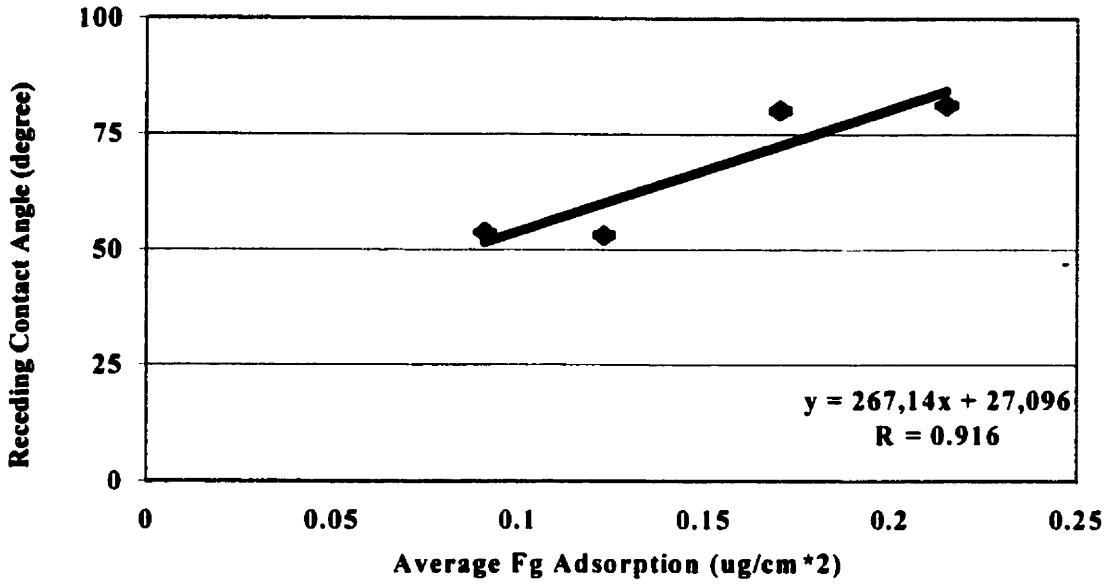


Figure. 5.2. Correlation between Fg adsorption from plasma and receding contact angle of SMM containing PEU

5.4. Platelet Adhesion Analysis

It is widely accepted that thrombosis in vascular implants is usually triggered by platelet adhesion onto the polymeric surfaces, following contact with flowing blood (Cazenave 1986). In fact, the sequence of events leading to the formation of the thrombotic mass can be categorized into the following steps: First the protein coated polymeric surfaces are recognized by platelets which upon their contact or adherence, becomes activated and changes its morphological shape from a discoid or round structure into a sphere. Secondly, this activation is followed by secretion of certain granular contents, and the expression of adhesive membrane receptors. This would lead to platelet spreading on the surface and inter-platelet binding with the formation of the surface bound aggregates. Thirdly, thrombin generation causes further platelet activation and transformation of Fg into polymeric fibrin, and hence coagulates the platelets into a secondary hemostatic plug (Mulhivill 1993).

It is believed that, the blood shear rate is one of the important factors that determine platelet adhesion (Mulhivill 1993), and that generally platelet adhesion enhances with an increasing shear rate. Using a cone and plate device, similar to the one used in this experiment, it was found that an optimum amount of platelet adhesion could be achieved with shear rate of 150 s^{-1} for 15 minutes (Skarja 1997). The same conditions were used by the current author in order to assay the platelet adhesion onto the SMM containing substrates.

In the first part of this section, the radiolabeled platelet adhesion data will be discussed and in the second part, comments on the nature of the activated platelets will be presented. Parallel to these analyses, it will be attempted to correlate the pattern of platelet adhesion data to the relevant adhesive proteins (Fg, Fnc, Vnc) that had adsorbed to the surfaces and the unique chemical nature of the SMMs.

5.4.1 Total Platelet Adhesion Assessment:

Figure 4.14 showed data for the platelet adhesion onto non-modified and SMM modified PEUs. As was seen, the non-modified base had the highest amount of platelets adhered onto its surface. This phenomenon could clearly related to the absence of the SMM and its fluoro-groups at the interface.

Protein adsorption data indicated that all the RGD (Arg-Gly-Asp) containing adhesive proteins adsorbed quite strongly onto this substrate.

It was therefore, quite clear that the presence of the fluorine containing SMMs and their unknown manner of interaction with the base polymer gave rise to surfaces that yielded significantly lower levels of adhered platelets relative to the non-modified surface (ANOVA, sheffe method with p-value < 0.05). Other investigators have also shown that the presence of components in PEU affects the surface chemistry of the bulk polymer and lowered the protein as well as platelet adhesion (Kashiwagi 1992). They attributed such observations to the water repelling and low surface free energy properties of the fluoroalkyl group (Kashiwagi 1993)

Among the SMMs, PTMO212F and PPO212L displayed the lowest amount of platelet adhesion (ANOVA, sheffe method with p-value < 0.05). Interestingly PTMO212I and PMO322I, despite their structural differences exhibited a very similar pattern of platelet adhesion. It was tempting to correlate the current data to the adhesion pattern of the adhesive proteins shown in sections 4.1, and 4.2.1.2. Earlier it was shown that there was a potential correlation between the receding contact angle of these four SMMs as well as Fg adsorption from plasma. By using the data for Fg adsorption from plasma onto different SMM containing substrates, and the current platelet adhesion data it would be possible to assess if there was any associations between these two variables. In doing so, the calculated correlation coefficient, was found to be around 0.61, which can be interpreted as a poor association between the two variables. As mentioned in section 5.2, Fg is only one of the proteins that contain the RGD epitope required for specific binding by platelets. It is believed, based on the banding pattern in the Western blot studies (Figures 4.1-4.5), that other RGD containing adhesive proteins (Vnc and Fnc) are present on all of the SMM surfaces and platelets might equally be associated with these proteins. It is suggested that similar experiments be conducted, using Vnc and Fnc, in order to obtain a more complete picture of the binding correlation that may exist between bound adhesive proteins and platelet adhesion.

It is believed that differences in platelet adhesion among the SMMs themselves could to some extent, be explained in terms of the structural characteristic of each SMM. As mentioned earlier PTMO212I and PTMO322I were similar in many respects and different in others. Their similarities arose from the common diisocyanates and soft segments they share. However, the ratio of each

segment varied (PTMO 2:1 and PTMO3:2) in each SMM. Their differences were rooted in their molecular weight (PTMO212I: 24.0 kDa, and PTMO322I 35.1 kDa), as well as differences in their respective fluorine content (PTMO212I: 19.3 % and PTMO322I 11.3 %) (Table 3.2). It was therefore believed that the similarity and the common soft segment might have led to the similar platelet adhesion pattern. The importance of the similarity was also highlighted in the adsorption pattern of Fg from plasma (Figure 4.10). Therefore, it is hypothesized that the same chemical groups that were expressed by the SMMs to give rise to a particular pattern of protein adsorption (Figure 4.10, and Tables 4.1-4.5), have also led to the particular adhesion trends in platelets among the SMM containing surfaces. These phenomena could only be explained when multiple factors (hydrophobicity, chain mobility, fluorine content, etc.) are considered together and their influences are being studied collectively and not individually. Since these factors were all encompassed within the contact angle measurements, these values were an ideal starting point for establishing relations between the materials and their platelet adhesion patterns.

PPO212L was one of the two SMMs that showed a quite low level of platelet adhesion. Grasel *et al* had suggested that in general, a PU with PTMO as its soft segment, exhibited more thrombogenic behavior than PPO based PEUs (Grasel 1987). It is hypothesized that specific characteristics of the PPO soft segment and the manner by which it interacted with the other polymeric chains of the system contributed to its unique behavior in platelet adhesion. By referring to the elemental analysis data (Table 4.1), it was quite clear that the PTMO212I and PPO212L share common traits with regards to their elemental content (F, C, N, and H), and molecular weights. They differ primarily in the nature of the soft segment and therefore such features need to be investigated in the future works.

The lowest amount of platelet adhesion was seen in PTMO212F. The reason for such trend, as was shown by other investigators, was believed to be related to the presence of a PEO spacer, which significantly reduced the platelet adhesion as well as protein adsorption of the polymer (Kishwagi 1993). As mentioned before, this characteristic of the PEO was attributed to the highly mobile and hydrophilic nature of this group at the interface (Grasel 1986).

In order to further identify a relationship between the surface character and trends of platelet adhesion, the correlation between advancing and receding contact angle with platelet adhesion was

investigated. The correlation coefficient (r) between the platelet adhesion and the advancing contact angle of SMMs was found to be 0.89, which implied a moderately good correlation between the platelet adhesion and its binding dependence on the nature of the hydrophobic groups at the surface (Figure 5.3). Interestingly, the r value for the receding contact angle was also moderately good (0.86), which implied the importance of the hydrophilic components at the surface. Hence, surface energetics contributed significantly to platelet adhesion, since this association was also shown to exhibit same level of correlation with Fg adsorption and it would indicate a role for the surface chemistry's ability to modulate subtle changes in protein adsorption and hence changes in platelet adhesion (Figure 5.4).

Also, a closer look at the protein adsorption pattern of the three adhesive proteins (Fg, Vnc, and Fnc) seems to be associated to the platelet adhesion data. Table 5.1 presents the adsorption patterns of three adhesive proteins (Fg, Fnc, and Vnc) (data taken from Tables 4.1- 4.5). Incidentally, the platelet density numbers (Figure 4.14) corresponded well with the intensity of protein bands in Western blotting (Table 5.1). As can be seen, all three RGD containing adhesive proteins, also appeared weakly on PPO212L and PTMO212F substrates.

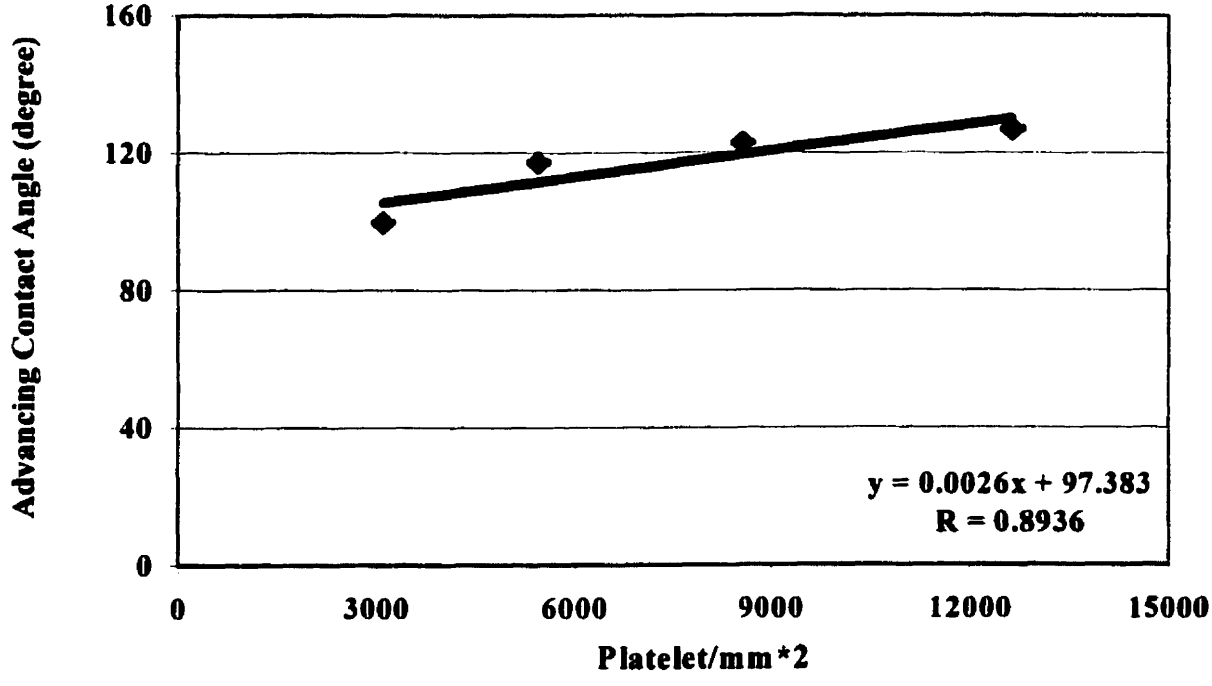


Figure 5.3. Correlation coefficient between platelet adhesion and advancing contact angle onto SMM surfaces.

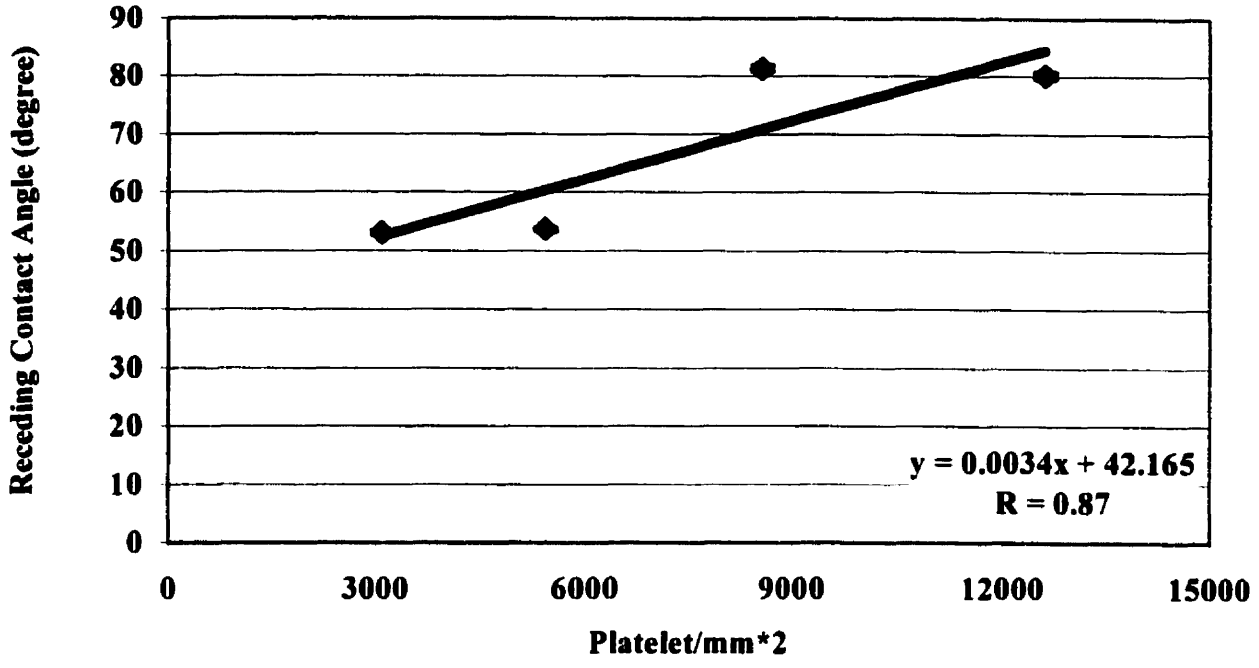


Figure 5.4. Correlation coefficient between platelet adhesion and receding contact angle onto SMM surfaces.

Adhesive Proteins	MW (kD)	Base 1	Base 2	PPO 212L 1	PPO 212L 2	PTMO 2121 1	PTMO 2121 2	PTMO 3221 1	PTMO 3221 2	PTMO 212F 1	PTMO 212F 2
Fg*	67	++	++	+	+	++	++	+++	+++	++	++
Fnc	200	+++	+++	+	+	++	++	++	++	+	+
Vnc	70	++	+	+	+	++	+	++	++	+	+
	66	++	+	-	-	-	+	++	++	+	+

* Fibrinogen degradation products were not shown in this table.

Table 5.1 Adhesive Proteins Adsorption Patterns

5.4.2 Morphological Analysis of the Platelets:

The SEM study was undertaken to assess the activation states of the platelets on the different substrates. It is believed that there was a direct correlation between the degree of platelet spreading on a polymeric surface *in vitro*, and the thrombogenicity of that material evaluated *ex vivo* (Silver

1993). Goodman *et al* have categorized platelet adhesion and activation into five different stages, characterizing them based on different morphological changes (Goodman 1984). These steps include, inactivated platelets which have predominantly round or discoid shapes, with an approximate diameter of 2 μm. As platelets become more activated, they begin to extend their pseudopodia, and the membrane between them extends as it is pushed out by the hyaloplasm until they reach their fully spread state. In the final state of activation, the platelets typically have a diameter of 7-10 μm. These criteria are used to assess the activation state of the platelets on the SMM containing PEUs.

Figures 4.15 a and b, exhibited the morphological state of platelets on the non-modified PEU. After exposure of the surfaces with blood, platelets were strongly attached, pseudopodia were formed and hyaloplasm could clearly be visualized among the pseudopodia. The surface was strongly activating the platelets. This was further proven by the size of the platelets which was approximately 7-9 μm, indicating the final step of the platelet activation. Figure 4.15. b, showed the picture of a white thrombus on the surface of the non-modified SMM, representing highly activated platelets (Park 1986).

This data could be related to the earlier Western blots of adhesive proteins which showed the presence of bands associated with Fg, Vnc, and Fnc (Table 5.1). These proteins contain the amino acid sequence (RGD, or Arg-Gly-Asp) which combines with GPIIb/IIIa receptor on the surface of platelets (Gaebel 1990, Sheppard 1994). In particular, two RGD sites have been found in Fg (E domain Aα 95-97 and E domain γ 572-575) (Rubens 1991). It was suggested that the conformational state of the protein, i.e. whether they were in compacted or expanded conformers, was crucial for the expression of these binding sites and the subsequent contact with the GPIIb/IIIa with the RGD sequence (Ugarova 1995). In fact, it was shown that post-adsorptive conformational changes of the proteins might prevented or inhibited the platelet adhesion, reduced binding of polyclonal antibody and reduce SDS elutibility (Grunkemeier 1996). These alterations were believed to be surface dependent phenomena (Rubens 1991). The non-modified base polymer surface chemistry, therefore not only allowed for the adhesion of the platelets on the surface, but also, its surface characteristics likely allowed the binding of the proteins in such a manner that they specifically exposed their RGD sequence to the platelets through an active adhesion process (Ito 1998).

Figures 4.16 a and b, represented the micrograph of platelet adhesion onto the PPO212L. It was observed that although platelets were present on the surface, they appear to be passively bound, i.e. not activated. It was believed that a more rigorous washing procedure in the post experimental stages might have removed these platelets from the surface. Their morphological shape was characterized by their discoid shape with complete lack of pseudopodium (Figure 4.16 b). The size of the platelet further reaffirmed their non-activated state ($\approx$ 2 μm) (Goodman 1984). This result was also in accordance with the Western blotting data presented earlier, in which the PPO212L blot exhibited very faint bands for the three adhesive proteins (Figure 4.3). Although these proteins were adsorbed, the nature of their conformational states is unknown. It was hypothesized that due to the particular chemical nature (diisocyanates:soft segment ratio, mobility of the side chains, hydrophilicity, surface charges, etc) of the SMMs at the interface, proteins might have either become denatured or were positioning themselves in such manner that "hid" their RGD sequence from the platelets, and hence a low platelet density and absence of activation was observed.

Due to the already established similarities between PTMO212I and PTMO322I, these SMMs were analyzed together. As the micro-graphs of Figures 4.17 and 4.18 exhibit, PTMO322I contains less platelet on its surface than PTMO212I. Nevertheless, closer morphological assessment revealed that platelets in both groups were activated to the same extent (Figures 4.17b and 4.18b). The former observation was further confirmed by referring to Figure 4.14, which shows fewer radiolabeled platelets on PTMO322I versus PTMO212I. It is therefore suggested that platelets recognized some distinctive chemical characteristics between PTMO212I and PTMO322I, in such a way that different adhesion levels were observed, yet the similar activation behavior was elicited from both SMMs. Platelets were however, less activated than the base, but more activated than for the PPO212L containing surfaces. The pseudopodia are beginning to bud out, the hyloplasms were not extending as entirely as they did in the base substrate.

Figure 4.19 contained the SEM micro-graphs of the adhered platelets onto PTMO212F. There were very few platelets adhered to the substrate, and those which were adhered passively, were quite scattered. As in the case of PPO212L, it was believed that a more aggressive washing technique might have removed these platelets from the surface. There was no indication of the presence of pseudopodia. These results again were in accordance with the platelet adhesion data presented in

Figure 4.14. Although Western blotting data indicated the evidence of moderate amounts of the adhesive proteins on this surface, it was suggested that the presence of the PEO group, among other factors, were rendering this particular SMM more hydrophilic, and more mobile allowing more mobility between the fluoro-tail and central component of the SMM. These factors might be contributing to post-adsorptive protein conformational changes, which were influencing platelet adhesion.

It was concluded that platelet adhesion and activation onto non-modified PEU was significantly different from the SMM containing PEUs. These differences were such that, the non-modified surfaces were more thrombogenic, than the SMM containing PEUs. It was suggested that the nature of protein/platelet interaction with polymer interface was influenced by such factors as: the hydrophilicity, chain mobility, fluorine content, diisocyanate-to-soft domain ratio, as well as other unique morphological features, yet undefined, induced by the SMMs.

5.5 Biodegradation Analysis:

The protective nature and the effectiveness of the different SMMs in shielding the underlying base polymer to degradation by enzymes have already been established by the previous studies in Dr. Santerre's group (Jahangir 1995, and Tang 1996). The main objective of the current work was to determine the effect that fibrinogen pre-adsorption could have on modified and non-modified PEU and to study its influence on the biodegradative effect of cholestrol esterase (CE).

Protein adsorption is one of the initial events that occur upon the blood-material interaction (Brash 1985). In addition, the results of protein adsorption experiments obtained so far indicated that Fg adsorbs quite avidly onto the surfaces whether they were or were not modified with SMMs. The role of Fg as an important thrombogenic protein as well as the most abundant protein in the plasma has also been established (Brash 1985).

As far as the stability of the Fg on the polymer surfaces was concerned, it was suggested that upon an extended period of Fg adsorption, the adsorbed protein can undergo significant conformational changes in such a manner that allows the protein to bind more tightly to the surfaces (Chinn 1992, and Slack 1992). In the presence of other proteins, however, despite such strong binding, fibrinogen has been shown to be competitively displaced by other proteins (Vroman effect) or be displaced due to enzymatic attack (Uniyal 1982).

Figure 4.20 exhibited the data for the long term biodegradation of fibrinogenated PEU and SMM containing PEUs. It was observed that most of the fibrinogenated surfaces, whether or not they were modified by SMMs, exhibited very similar degradation rate in the initial phase of the experiment. The PPO212L containing material was the only SMM substrate that showed significantly lower amounts of degradation than the base (student t-test, with p-value <0.01). It could then be hypothesized that Fg was effectively masking the hydrolysable groups, within the first 70 days, and hence, Fg was in fact, retarding the degradation of the underlying base polymer. Interestingly, from the 70^{th} day onward the gap between the SMMs and non-modified polymer began to appear, indicating that the enzyme was actually gaining access to the susceptible hydrolyzable bonds in the bulk polymer. In other words, the effect of Fg protection was believed to disappear by the 70^{th} day

of the experiment. Consequently, it could be said that the anticipated protective effect of each SMM is beginning to act from day 70.

A close inspection of the data in Figure 4.21 revealed that although there were initially significant differences (student t-test, with p-value < 0.01) between the fibrinogenated and non-fibrinogenated PPO212L, it only lasted until day 70. This implied that although Fg was acting, as a retarding barrier to degradation, did not significantly alter the SMM behavior. This trend seemed to be repeated in most of the SMMs (Table 4.7).

From these observations, the following hypotheses, with regards to the role Fg and the biodegradative phenomena have been put forward to explain the observations. A loss of the protective influence of the Fg in the biodegradation process after the 70^{th} day, may be due to the following:

1. As observed by many investigators, Fg could be replaced in a competitive manner via a process known as the Vroman effect (Brash 1985). It was conceivable that the Fg can indeed be replaced by CE. A possible explanation for such time dependent phenomena was that the presence of a certain threshold concentration of enzyme was necessary to enable the CE to successfully replace the Fg. It was only after such a competitive replacement that CE could begin its hydrolytic activities.

   To prove such a hypothesis, a simple competitive experiment between Fg and CE should be designed. The two proteins should be radiolabeled by two different isotopes and the displacement of one protein by the other should be monitored in a time dependant manner. In fact, the Fg should first be adsorbed onto the surface and later CE should be introduced. The logic behind the protein pre-adsorption would be to replicate the *in vivo* situation, where materials would be coated with a layer of proteins (Brash 1985). This phenomenon was usually followed with cellular interaction, which in turn, would release soluble factors and various enzymes (Brash 1992), e.g. macrophages releasing CE (Labow 1998).

2. It was believed that CE is a hydrolytic enzyme responsible for hydrolysing the ester bond of molecules (Labow 1983). Fg as a protein might contain some hydrolysable bonds or groups

prone to CE attack, which may lead to destruction of Fg. The enzymatic degradation of the Fg, and its subsequent removal from the surface was speculated by Uniyal *et al* (Uniyal 1982). Whether CE could degrade the Fg was still unknown. However, if this hypothesis was true, it was believed that this degradative process was a very slow phenomenon, and would take around seventy days for it to exhibit itself. It was only when enough Fg degraded, that CE could start its degradative action on the polymer.

To test the above hypothesis, the Fg should be exposed to CE in different concentrations. If CE is capable of degrading the Fg, then the degradation fragments of the Fg should be present in the buffer system. To detect the fibrinogen's degradation, a Western blot analysis should be conducted. Through such methodology, different banding patterns could correspond to different fragments of the Fg.

3. Thirdly, although it was believed, under idealistic conditions, that 1 mg/mL concentration of the Fg was capable of generating a confluent monolayer of protein at the surface, there might exist some unoccupied sites on the polymer surface not covered by Fg. These so-called "windows of opportunity" or "free access zones" could be penetrated by CE, leading to the subsequent degradation of the polymer. However, the reason for a delay of 70 days would lie in the fact that, although CE was capable of penetrating and degrading the polymer, the radiolabelled degradation products were incapable of being released, since a mesh-work of the Fg traps them underneath the adsorbed protein layer. The only time these fragments were released was when enough polymers were degraded and destabilized the Fg itself. At this time, the Fg along with these degradation products was released together into the buffer.

To test this hypothesis one should first gain some insight into nature of Fg present on the surface. This could be accomplished by Atomic Force Microscopy (AFM) analysis, to visualize a confluent layer of the protein on the surface (Ratner 1993). The Fg should also be radiolabeled as well as the polymer. In a time dependent experiments the rate of release of the fibrinogen, as well as the polymer should be monitored. A sudden release of the protein as well as polymer would create a peak in the radiolabel reading that should confirm the above hypothesis.

6. CONCLUSIONS

The contact angle data indicated that the surface chemistry of all PEU containing SMMs have been effectively altered in such a manner that yielded more hydrophobic surfaces (indicated by advancing contact angle), relative to non-modified surfaces. However, the variability in surface energetic was clearly exhibited among the four PEU containing SMMs. Such different behavior is believed to be dependent on the SMM's chemistry, length of the fluoro group as well as central chains, and mobility of the SMM chains and their components. It is believed that these specific differences have altered the manner by which proteins and ultimately platelets and other enzymes interact with the surfaces of SMM containing materials.

1. The total protein studies conducted by western blotting techniques provided us with a general profile of various plasma proteins adsorbed onto modified and non-modified PEUs. These data indicated that PPO212L and the PTMO212F were the surfaces that in general adsorbed the least amount of proteins. The other two SMMs, (PTMO212I and PTMO322I) behaved in a very similar manner to each other. The non-modified base adsorbed the highest amount of the proteins.

2. More importantly, it was also observed that the three adhesive proteins (Fg, Vnc, and Fnc) that were mostly implicated in subsequent thrombogenic mechanisms were adsorbed onto all the SMMs in various degrees. However, again, PPO212I and PTMO212F containing surfaces showed less fibrinogen and vitronectin adsorbed to their surfaces.

3. The single protein studies with albumin and fibrinogen from buffer as well as plasma protein pool revealed that despite lack of statistical differences (as verified by ANOVA), there were differences in adsorption behavior of both proteins from each protein pool. Both systems have shown that adsorption of albumin and fibrinogen from plasma was, however significantly lower than from PBS buffer. The higher adsorption level from the single protein pool contributed to the lack of other proteins competing for binding sites.

4. Despite the lack of strong statistical significance in the results, differences in Fg adsorption were observed between the non-modified substrates and modified substrates. Furthermore,

there was a moderately good correlation coefficient (r = 0.92) between the Fg adsorption from plasma and the receding contact angle (indication of hydrophilicity) of SMMs. This indicated that other factors than the hydrophobic nature of the SMMs modulated the protein adsorption. Hence, the protein adsorption phenomenon was believed to be dependent on many factors, and was influenced by several elements defining the surface energetics of a material. PPO212L and PTMO212F showed the lowest levels of fibrinogen adsorption from plasma and this confirmed the results from the Western blot data.

5. The platelet adhesion data clearly highlighted differences among the four modified PEU and the non-modified PEU. These results were statistically verified (ANOVA, with 95% confidence interval). The platelet density was shown to be significantly lower for the PEU containing SMMs as compared to the non-modified base.

6. Among the SMMs, the PPO212L and PTMO212F exhibited the lowest amount of platelet adhesion and PTMO212I and PTMO322I had adhered similar amounts. These similarities and differences among the SMMs were believed to be related to the specific chemical structure of each SMM, and the manner by which these different components interacted with polymer chain as well as the incubation medium at interface.

7. The morphological assessment of the platelets revealed that they were mostly activated on the base polymer surface and even the presence of the white thrombi was seen. PEU containing SMMs, on the other hand, had relatively lower amounts of platelet activation. Nevertheless some level of activation was seen on the PTMO212I and PTMO322I containing materials. The PPO212L, as well PTMO212F containing materials exhibited, minimal platelet activation.

8. The correlation coefficient between platelet adhesion and Fg adsorption was quite poor (r = 0,61). This led one to conclude that Fg could not be the only protein to which platelets were specifically attached. There was a moderately good correlation between the advancing/receding contact angle and platelet adhesion. This confirmed the importance of the hydrophilic/hydrophobic character in modulating adhesive protein adsorption and subsequent platelet interactions.

9. The biodegradation analysis indicated that the pre-adsorption of the Fg onto the modified and non-modified surfaces provided some temporary protection against the hydrolytic function of CE. However, this effect disappeared after day 70, and both fibrinogenated and non-fibrinogenated groups had the same degradation rate by the 126th day. It was observed that the initial gap between the fibrinogenated and non-fibrinogenated groups in the base polymer was significant, and that this difference was much smaller for the PPO212L containing material as compared to the other polymer surfaces.

10. The pre-adsorption of Fg did not appear to alter the SMMs' behavior in providing a more biostable surface.

11. Based on protein adsorption data as well as platelet adhesion and activation, it was believed that PPO212L and PTMO212F were the least thrombogenic substrates. Additionally PPO212L provided the most biostable SMM.

7.0 RECOMMENDATIONS

1. It would be valuable to conduct the western blot studies from whole blood rather than only plasma, since evidence suggests differences between the two systems may exist (Brash 1992).

2. Several more repeats of the single adsorption experiments are required in order to enhance the possibility of gaining statistical significance between the observed differences.

3. Protein adsorption studies (single and plasma) should be conducted with other adhesive proteins (Vnc and Fnc), and the correlation coefficient between these data should be calculated. This would potentially shed more light on the dependency of the adhesive proteins with the surface energetics and platelet adhesion for the SMM systems.

4. Adsorption experiments should be conducted where the three adhesive proteins (Fg, Vnc, and Fnc), where each protein is pre-adsorbed onto substrates and then is subsequently exposed to platelets. This experiment may provide some information on the exact nature of platelet adhesion with specific proteins.

5. The conformational state of each of the adhesive proteins should also be studied. This is important since it is believed that surface chemistry of each PEU containing SMM, could cause either denaturation of the protein, hence making protein adsorb stronger onto the surface, or it can prompt them to fold, and therefore, hiding their RGD sequence from the platelets. These experiments can be conducted by using monoclonal antibodies to specific amino acid sequence. Works similar to this have been reported by Chinn *et al* (1992).

6. It was mentioned in the discussion that a more aggressive washing procedure should have been used to remove non-adhered platelets. It is suggested that instead of three washes, more vigorous procedure be used to ensure the removal of the aggregates.

7. Experiments should be designed to understand the mechanism of Fg and CE interactions that led to the degradation of the polymers. A series of recommendations were noted in section 5.5

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9. Appendices

Appendix A. Solutions in SDS-PAGE and Western Blotting Experiment

A: SDS-PAGE:

1. 5x Electrophoresis Buffer:

Tris	15.0	g
Glycine	72.0	g
10% (w/v) SDS	5.00	g

Direction:

Add up to 1.0 Litre of deionized, distilled water (dd H_2O); adjust the pH to 8.3. Store at 4 oC until use. Working solution is diluted to 1x solution

2. Transfer Buffer:

Tris	3.03	g
Glycine	14.4	g
Methanol	200.0	mL

Direction:

Add up to 1.0 Litre of deionized, distilled water (dd H_2O). pH 8.3, Do not adjust the buffer. Prepare Fresh before each experiment

3. Tracking Dye:

Sample Buffer	225	µl

Sample Buffer Preparation:

0.5 Tris-HCl, pH 6.8	1.0	mL
Glycerol	0.8	mL
10% (w/v) SDS	1.6	mL

Direction:

Mix the above reagents, and aliquote into 225 µl 4 oC.

2-β-mercaptoethanol	30	µl
0.05% (w/v) Bromophenol Bleu	30	µl

4. Pyronin Dye:

Sample Buffer	225	µl
Pyronin	a drop	

B: Western Blotting Solution for Band Development:

1. Tris-Base Solution (TBS) Buffer:

50 mM Tris	6.05	g
150 mM NaCl	8.76	g

Direction:

Add up to 1.0 Litre of deionized, distilled water (dd H_2O); adjust the pH to 7.4.

2. 5% Blocking Solution:

Non-fat powdered milk	5.0	g
TBS	100	mL

3. 0.1% Washing Solution:

Non-fat powdered milk	0.5	g
TBS	500	mL

4. Solution A (1.0 % 1° Antibody solution):

Non-fat powdered milk	1.0	g
TBS	100	mL
Tween 20	50	µl
1° Antibodies	1/1000 diluted	

5. Solution B (0.1 % 2° Antibody solution):

Non-fat powdered milk	1.0	g
TBS	100	mL
Tween 20	50	µl
2° Antibodies	1/1000 diluted	

Appendix B. Solutions in Platelet Adhesion Experiment

1. Tyrodes Buffer Preparation:

Stock Solution 1

NaCl	160 g
KCl	4.0 g
$NaHCO_3$	20.0 g
NaH_2PO_4	1.0 g

Direction:

Add up to 1.0 Litre of deionized, distilled water (dd H_2O)

Stock Solution 2

0.1 M	$MgCl_2.6\ H_2O$	M.W.= 203.3

Direction:

20.33 g of the solid must be dissolved in 900 mL dd H_2O, and final volume must be brought to total volume of 1.0 Litre.

Stock Solution 3

0.1 M	$CaCl_2.6\ H_2O$	M.W. = 219.1

Direction:

21.91 g of the solid must be dissolved in 900 mL dd H_2O, and final volume must be brought to total volume of 1.0 Litre.

2. Plain Tyrode Solution Buffer (PTSB):

To 50.0 mL of the Stock Solution 1, add 1.0 litre of dd H_2O. Adjust the pH to 7.0.

3. Tyrode Albumin Buffer (TAS):

To 50.0 mL of Stock Solution 1, add 750.0 mL of dd H_2O, 10.0 mL of Stock Solution 2 and 20.0 mL of Stock solution 3. Dissolve 3.5 g of Bovine Albumin (supplied by Boehringer Mannheim-fatty acid free) and 1.0 g of detxrose. Diluted to 1.0 Litre and adjust the pH to 7.0.

4. Acid Citrate Dextrose (ACD) Preparation:

Trisodium Citrate	25.0 g
Citric Acid	14.0 g
Dextrose	20.0 g

Direction:
Solution was made by dissolving the above in 1.0 Litre of dd H_2O, and pH was adjusted to 4.5. Then 1 part ACD was used with 6 parts of whole blood.

5. Washing Solutions

5.1 First Platelet Washing Solution:

10 mL Tyrode buffer (plain) with 0.35% albumin was added (2 mM calcium, 1 mM magnesium, 0.1% glucose).
200 µL potato apyrase (20 µL/ mL),
100 µl heparin (Sigma) (10 µL/mL),
Adjust pH to 7.0.

5.2 Second Platelet Washing Solution:

10 mL Tyrode buffer (plain) with 0.35% albumin was added (2 mM calcium, 1 mM magnesium, 0.1% glucose).

100 μL potato apyrase (20 μL/ mL),

Adjust pH to 7.0.