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Peptide Modified Gold Coated Polyurethanes as Biosynthetic Vascular Prostheses

By

© Robin McMillan

A thesis submitted in partial fulfillment of the requirements for the degree of
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Abstract

Cardiovascular disease is the leading cause of death worldwide. In an effort to combat the disease, surgeons are often required to perform vascular transplants. The optimal choice for replacement of the occluded vessel is an autologous vein. However, persons suffering from cardiovascular disease do not always have a suitable vein. This has led to the development of an artificial artery or vein for use in situations where an autologous transplant is not possible.

The use of large diameter artificial veins has been generally successful. However, when the diameter of the graft falls below approximately 5 mm, the patency rate of the graft drops off considerably. Failure of the graft is often due to occlusion of the vessel resulting from initiation of coagulation. Therefore there is a need for development of a small caliber vascular graft that does not initiate the coagulation cascade.

Healthy vasculature possesses a single layer of vascular endothelial cells on the interior lumen of the vessel. It is these cells that are responsible for the non-thrombogenicity of the natural blood vessel lumen. The generation of this layer on the surface of an artificial graft could provide the necessary non-thrombogenic properties to the artificial prosthesis.

In this work, synthetic cell adhesion peptides were designed, synthesized and chemisorbed onto the surface of a gold coated polyurethane. The polymer substrate was chosen in order to maintain good mechanical properties in the artificial vein or artery, while the gold coating served the purpose of permitting surface modification with the cell

adhesion peptides. The peptides were custom designed to specifically be active to cell surface receptors on vascular endothelial cells. The peptides also contained a thiol present in a cysteine amino acid which was necessary for interaction with the gold on the polymer surface.

The modifications to the surface were confirmed by various methods including water contact angles, and surface analysis techniques such as: x-ray photoelectron spectroscopy, atomic force microscopy and scanning electron microscopy. The surfaces were tested for their ability to promote the initial attachment of both vascular endothelial cells and mouse 3T3 fibroblasts. The modified surfaces were shown to be capable of increasing the initial attachment of the cells, with the peptides specifically designed to interact with the vascular endothelial cells showing the greatest amount of cell adhesion. The system shows considerable promise for the development of vascular endothelial cell lined vascular grafts for implantation purposes.

Acknowledgments

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Chapter 1: Introduction and Literature Review

1.1 Introduction

Cardiovascular disease is the leading cause of death worldwide. Nearly 30% of all deaths occurring in the united states in 1990 were attributable to heart attack or stroke (Wilmore and Costill 1994). While the patients' own vessels are generally the best choice, the prevalence of cardiovascular disease in our society has led to the development of artificial vascular prostheses for replacement of diseased or damaged blood vessels, especially in cases where an autologous transplant is not possible.

Attempts at constructing artificial vascular grafts with materials such as Dacron or expanded polytetrafluoroethylene (ePTFE) have been generally successful for large caliber (over 5 mm inside diameter) vascular prostheses. Problems however, have been encountered in the development of small caliber (under 5 mm inside diameter) vascular prostheses which are not significant in larger diameter prostheses. These problems have led to the failure of smaller diameter vascular prostheses in such applications.

Failure of the small caliber grafts is generally associated with the formation of a blood clot, or thrombus, on the surface of the vessel. When blood contacts any surface other than the naturally occurring endothelial cell layer on the lumen of the vessel, the

coagulation process is initiated. Thrombus formation can lead to graft failure by constricting the blood flow through the vessel, causing tissue death due to lack of oxygen.

Many different attempts have been made to circumvent the problem of thrombus formation when blood contacts artificial surfaces. Heparin is a naturally occurring thrombin inhibitor. Attempts have been made to attach heparin, heparin-like molecules and other thrombin inhibitors to artificial surfaces in an effort to inhibit thrombus formation (Charef *et al.* 1996, Smith and Sefton 1993). These efforts have shown some success in reducing coagulation but are complicated by the requirement that a cofactor be present for the heparin to be active.

Initiation of coagulation is dependent on the patterns of protein adsorption that occur at the artificial surface (Scott 1991). Some researchers have tried to prevent this initial protein adsorption through the use of polymers that do not adsorb significant amounts of protein. For example, modification of the surfaces with polyethylene oxide to prevent protein adsorption and subsequently thrombus formation has been attempted (Smith and Sefton 1993).

Further, the creation of a surface capable of degrading a thrombus as it forms has also been attempted. Several efforts have been made to promote the adsorption of the natural clot-lysing enzyme plasminogen through the attachment of the plasminogen activator lysine to the surfaces. Note that the action of plasminogen causes the degradation of fibrin, which forms the basis of the clot, and thus lyses the clot (Woodhouse *et al.* 1994 and 1996).

As a result of this research, the blood compatibility of artificial materials has been greatly improved. However, there is still no artificial surface which is comparable to that which occurs naturally in the vasculature - the endothelial cell layer. It is believed that the creation of a surface capable of supporting a confluent monolayer of endothelial cells would prevent blood from contacting the foreign material and therefore avoid the initiation of the coagulation response. As a consequence several attempts have been made to create surfaces which are capable of supporting the growth of endothelial cells (Massia and Hubbell 1990, Sipehia *et al.* 1993, Lin and Cooper 1994, Holland *et al.* 1996 and Walluscheck *et al.* 1996).

Great potential for improving the blood compatibility of artificial materials lies in constructing a surface amenable to the long term adhesion and growth of vascular endothelial cells. It is this approach that has been chosen in this thesis in an effort to improve the long term patency rates of small caliber vascular grafts. This study endeavors to create an artificial surface which is amenable to the adhesion and growth of human vascular endothelial cells. The attachment of the cells to the surface will be promoted through the use of synthetic peptides attached to the lumen of the artificial blood vessel. The peptides will be designed to bind integrins expressed by vascular endothelial cells. Attachment of the peptides to the lumen will rely on chemisorption of the peptides to a thin gold layer through the thiol moiety of the amino acid cysteine. This thin gold layer is evaporated onto the surface of a polymer which possesses the desired mechanical properties that will ultimately be necessary for the vascular graft.

1.2 Literature Review

1.2.1 Blood Composition

Blood has many functions that are essential to life. The blood is responsible for transporting oxygen to respiring tissues and removing carbon dioxide. Nutrients are delivered to the tissues and metabolic wastes removed, by the blood. The blood is also responsible for maintaining homeostasis in the body by transporting electrolytes, water, heat and hormones throughout the body. The transportation of immune and clotting system factors is also a function carried out by the blood.

The many functions of the blood require that there be various constituents forming its volume. Approximately 45% of the total blood volume is composed of different cells that fall under three main types. The erythrocytes, or red blood cells, are responsible for transporting oxygen and carbon dioxide in the blood. Thrombocytes, or platelets, are major players in the coagulation response. Platelets agglomerate to plug up small leaks in the vasculature and are essential for the formation of a stable fibrin polymer in the thrombus. The smallest group of cells are the leukocytes, or white blood cells. They are partially responsible for protecting the body from infection.

The remaining 55% of the blood volume containing no cells is referred to as the plasma. While the plasma is 93% water, the remaining 7% contains proteins, inorganic ions and organic metabolites. Many of the proteins, such as albumin and transferrin, are necessary for transporting insoluble materials around the body. Other proteins serve a

variety of other functions including the maintenance of hemostasis following injury or damage to a vessel.

1.2.2 Blood Coagulation on Artificial Surfaces - The Contact System

Blood coagulation is the natural physiologic response to vascular injury designed to arrest blood loss from the damaged blood vessel and allow for subsequent repair (Colman *et al.* 1994). A similar sequence of events which results in the same terminal steps can also be initiated when blood comes into contact with a "foreign" surface (Colman 1993, Scott 1991). This is referred to as contact activation of the coagulation cascade.

A foreign surface is any surface, natural or artificial, which is not the naturally occurring endothelial cell layer present on all inner luminal surfaces. This is of particular importance in the case of an artificial vascular implant. Coagulation, or thrombosis, on the surface of a vascular implant can potentially lead to a blockage of blood flow or occlusion of the vessel. If the clot or thrombus breaks apart as a result of the shear forces of the blood flowing through the vessel, the portion carried away is known as an embolus. An embolus can become lodged in a smaller vessel further along in the circulation, relative to where it was formed. Both of these scenarios ultimately result in occlusion of the blood vessel.

These results can have a catastrophic outcome for the patient. The presence of an occlusion limits the flow of blood through a vessel and results in the failure to supply an adequate amount of oxygen and nutrients to the tissues supplied by the blood vessel. If

the clot is not lysed by the naturally occurring mechanisms quickly enough, tissue death may occur due to lack of oxygen. If this takes place in one of the arteries feeding the heart muscle for example, the result is a myocardial infarction or heart attack. A cerebral vascular accident, or stroke, may result from an embolus becoming lodged in one of the vessels feeding the brain.

There are two independent pathways for the initiation of the coagulation cascade: the intrinsic and extrinsic pathways. The cascade is depicted schematically in Figure 1. The extrinsic pathway is initiated by factors derived from other tissues, and is usually the result of tissue damage (Colman *et al.* 1994). Physical or chemical trauma to the vasculature or surrounding tissue is capable of initiating coagulation through activation of the extrinsic mechanism of blood coagulation. The mechanism is of particular importance when a blood vessel becomes ruptured.

More important in the study of blood-material interactions is the initiation of coagulation through the intrinsic pathway. This mechanism is initiated when blood contacts any material which is not the naturally occurring intact vascular endothelial layer. It is this pathway which is of interest for the development of an artificial vascular implant as it is initiated by adsorption of contact factors onto a foreign surface (Colman 1993, Scott 1991). Both pathways are designed to arrest the flow of blood following injury and both converge into a single common pathway leading to the formation of fibrin threads which enmesh blood cells to form the actual clot. The composition of the clot is dependent on where in the circulation the clot is formed. Arterial clots are composed of

aggregated platelets joined by fibrin strands while venous clots consist mainly of erythrocytes trapped in fibrin threads (Colman 1993).

There are at least twelve coagulation factors responsible for contact activation that circulate continuously in the blood (Colman *et al.* 1994). The inactive factors, or zymogens, become enzymatically active following contact with a foreign surface or by proteolytic cleavage by other enzymes. Activation of each zymogen allows for activation of the next factor in the cascade. The activated factors are then capable of catalyzing reactions involved in the cascade.

As can be seen in Figure 1, the intrinsic system is initiated by the adsorption of factor XII and high molecular weight kininogen (HMWK) onto the foreign surface and the subsequent autoactivation of factor XII to the active coagulation factor XIIa. Once factor XII is converted to its active form (XIIa), the enzyme is capable of acting on its substrates, prekalicrine (Pka) and factor XI. These enzymes are then able to catalyze the next reaction in the cascade. The cascade continues, activating another coagulation factor at each step until prothrombin is converted into the active enzyme thrombin. Since each step results in the activation of an enzyme, and since an enzyme can act on many substrate molecules before becoming inactivated the reactions quickly amplify the amount of thrombin produced.

When thrombin is activated it causes the activation of circulating platelets, making them adhesive as well as the conversion of fibrinogen monomers into fibrin polymer which becomes the insoluble fibrin clot (Colman 1993, Colman *et al.*1994, Scott 1991). Thrombin also feeds back to factors XII and V to amplify its own production. Therefore,

only a small amount of factor XII needs to be activated to obtain significant thrombus formation.

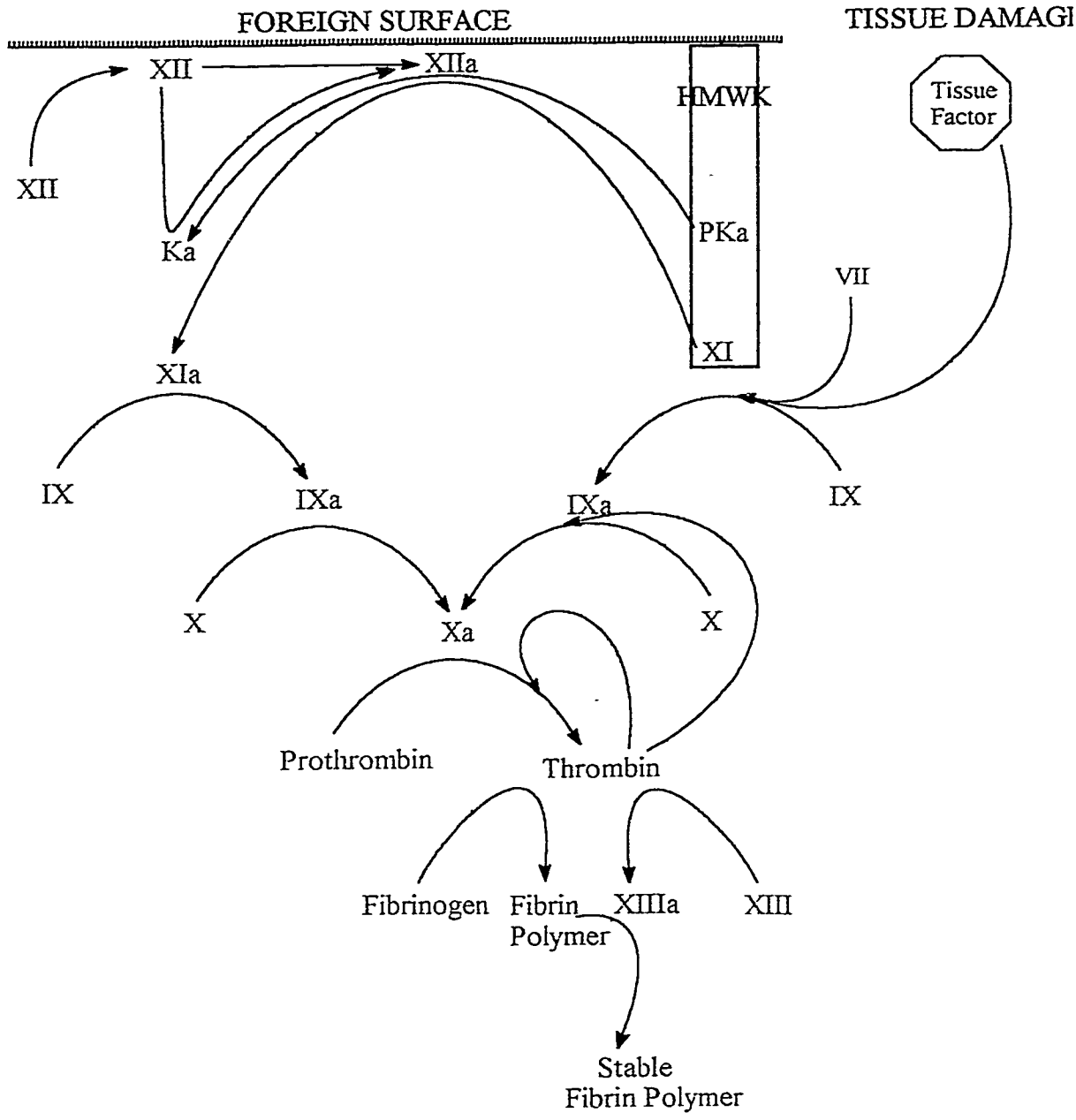

Figure 1: Schematic representation of the blood coagulation cascade. Roman numerals represent enzyme or zymogen factors in coagulation cascade. Lines indicate direct action of enzyme on zymogen or protein. HMWK represents the non-enzymatic cofactor high-molecular-weight kininogen.

Thrombin is one of the most important enzymes in the cascade. Thrombin is responsible for catalyzing the polymerization of the fibrinogen monomers into fibrin polymers, and because it can feed back to further activate increased amounts of factors V and XII, thrombin activation quickly results in the conversion of fibrinogen monomer to fibrin which is then polymerized. Fibrin is the main component of a blood clot and is responsible for forming the bulk of the clot and entrapping blood cells in the thrombus.

1.2.3 Physiology and Anatomy of the Arterio-Venous System

The vasculature is designed to transport blood throughout the human body, carrying blood and nutrients to the cells and removing carbon dioxide and waste products for excretion from the body. There are basically three layers in the blood vessels as depicted in Figure 2.

The outermost layer is composed primarily of connective tissue. This layer provides the physical structure to the vessel. The intermediary layer is composed of elastic fibers and smooth muscle cells. These cells are responsible for contraction and dilation of the vessel in response to blood flow requirements of the tissue. The innermost layer is composed of the vascular endothelium which is responsible for preventing blood coagulation in a normal intact blood vessel.

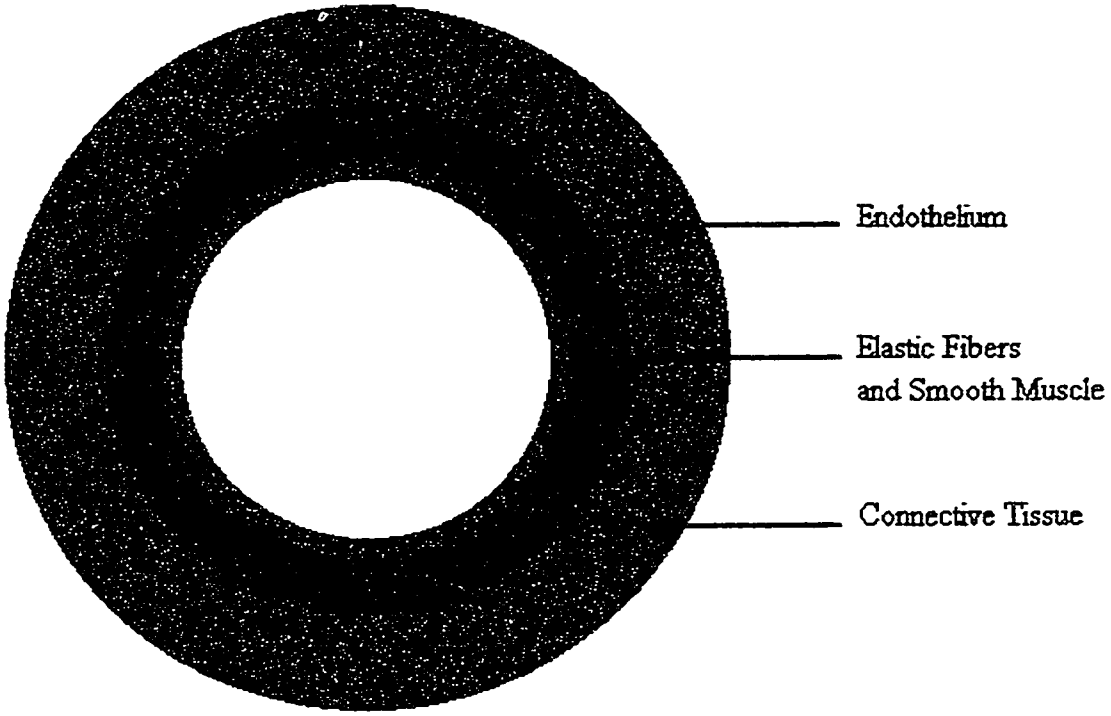

Figure 2: Schematic representation of a typical blood vessel in cross section. Blood flows through the inner lumen in contact with the single-celled endothelium.

1.2.4 Vascular Endothelium

The endothelium is composed of a single layer of specialized endothelial cells. These cells form a complete monolayer over the underlying material preventing it from coming into direct contact with the blood. It is the unique properties of the endothelial cells which give the natural vasculature its non-thrombogenic properties. Blood contacting any of the underlying tissue that is not endothelial in nature, as in the case of vessel damage, will result in coagulation. This is the natural mechanism which protects an individual, by stopping blood loss in the case of vessel injury (Colman *et al.* 1994).

While the most important function of the vascular endothelium is the maintenance of hemostasis in the vasculature, the endothelium can be responsible for the initiation of coagulation under certain circumstances. If the endothelium becomes damaged due to physical or chemical stress, activation of the extrinsic coagulation system can be initiated by the release of blood clotting factors such as tissue thromboplastin, a thrombin activator.

While the pro-coagulant capabilities of the endothelium are important in the human body, it is the anticoagulant properties that will be exploited in this work for the development of a suitable blood contacting material. There are several factors expressed by the vascular endothelial cells that are responsible for maintenance of hemostasis.

The vascular endothelial cells express a layer of glycocaylix, a mucopolysaccharide, on their cellular membranes. This layer repels the protein clotting factors and platelets naturally present in the blood from binding to the lumen of the vascular wall. This effect prohibits the platelets from becoming activated, a significant contributor to thrombus formation. As well, it prevents the initiation of the intrinsic mechanism of blood clotting by adsorption and subsequent activation of the blood coagulation factor XII (Guyton 1991). The blood vessel endothelial cells also express thrombomodulin on their cell membranes which helps prevent the formation of thrombi on the surface. Thrombomodulin can bind thrombin thereby removing it from the blood and inactivating it. The thrombin-thrombomodulin complex can also inactivate several cofactors in the coagulation cascade including factors V and VIII.

The body's primary natural anticoagulant, heparin, is also expressed by the vessel endothelial cells. Heparin, in concert with antithrombin III (ATIII), is also capable of

inhibiting the action of thrombin. By reducing the amount of active thrombin present, the coagulation cascade can be reduced significantly (Hanson *et al.* 1993).

1.2.5 Protein Adsorption

Proteins are large amphipathic molecules. Due to this fact, proteins are endowed with the ability to migrate and accumulate at the interface between phases. Protein interaction with surfaces is generally the first and fate determining step following contact of any surface with a protein-containing solution. These interactions between protein and surfaces are normally non-covalent. Proteins can be bound to surfaces by hydrogen bonding, electrostatic and hydrophobic interactions. Usually several different types of interactions are involved in the adsorption of a particular protein to a surface (Horbett and Brash 1995).

The adsorption of proteins to surfaces has been found to be generally irreversible (Lundstrom 1995). While the adsorption is irreversible, once adsorbed, the protein can undergo conformational changes to maximize interaction with the surface. Proteins have been found to be influenced more by nonionic or hydrophobic surfaces rather than by polar or hydrophilic surfaces (Horbett and Brash 1995 and Lundstrom 1995).

Most applications involving proteins occur in complex biological fluids that contain many different proteins. The adsorption of proteins from a mixture is surface specific. The properties of the surface and the composition of the protein mixture will ultimately determine the composition of the adsorbed protein layer.

The adsorption of proteins onto surfaces is central to many biological processes. Important to blood-material interactions is the effect of protein adsorption on complement and coagulation activation. Both of these processes can be initiated through adsorption of circulating protein factors in the blood. It is desirable in most material interactions with blood to avoid activation of these processes.

1.2.6 Development of Clot Resistant Surfaces

In vascular transplant surgery, such as in a heart bypass, the optimal choice for the transplant is the patient's own vein. Often the physician will use a portion of the patient's saphenous vein for the transplant. The use of an autologous vein is not always possible due to the fact that all of the available veins in the patient's body can be affected to some degree by their vascular disease, rendering the veins unsuitable for transplant. In cases such as these, the use of artificial vascular prostheses is ideal if not necessary.

The most commonly used artificial vascular prostheses are a synthetic expanded polytetrafluoroethylene (ePTFE) polymer and the human umbilical vein graft (Yoshida *et al.* 1996). However, even these systems are often "preclotted" prior to implantation in order to improve patency rates. While these products have been successful for large caliber transplants, they are still not viable for long term patency when used as small caliber grafts. One of the main causes of graft failure for small caliber prostheses is coagulation of the blood after contact with the "foreign" surface of the graft.

Coagulation occurs on these materials independent of internal diameter. For larger diameter (greater than 5 mm) vessels, the clot does not pose a significant problem. A

layer of clotted blood forms on the lumen of the graft, but does not interfere significantly with blood flow through the vessel as this initial clot represents a surface which permits the flow of blood without further clotting. For smaller diameter grafts, the clotting process has more severe consequences. The graft fails due to a layer of clotted blood that builds up on the luminal wall to the point where the flow of blood through the vessel is significantly reduced. Also, there is the potential for the formation of an embolus, or detached free floating clot, which can occlude the vessel down stream from the graft. This results in the same effect as an occlusion of the graft itself. In larger diameter vessels, embolus formation does not usually cause a problem because the natural fibrinolytic processes can lyse the clot before it becomes lodged in a smaller vessel.

Researchers have tried improving graft patency by functionalizing both the surface as well as the bulk polymer with heparin, heparin-like molecules or another thrombin inhibitor to reduce the clotting caused by the artificial surface (Charef *et al.* 1996, Smith and Sefton 1993). Other groups have developed surfaces which have low protein affinity in an attempt to reduce the initiation of coagulation that is initiated by adsorption of specific proteins to the surface (Smith and Sefton 1993). Heparin, in combination with antithrombin III (ATIII) is a powerful anticoagulant. The heparin acts in conjunction with ATIII to inactivate thrombin. When thrombin is inactivated the fibrinogen monomers cannot be polymerized into fibrin and a clot cannot be formed.

Other groups have tried to incorporate lysine and other plasminogen activating proteins on the surface in an attempt to break down any clots forming at the surface (Woodhouse *et al.* 1994 and 1996). Plasminogen is the principal zymogen in the

fibrinolytic, clot lysing, pathway. The lysination of the surfaces was shown in these studies to result in the activation of plasminogen to the active zymogen plasmin and the subsequent breakdown of the forming thrombi.

While the results of these studies are encouraging, several problems are routinely encountered which must be overcome in order to develop surfaces that are truly clot resistant. For example, the attachment of heparin to the polymer surface is complicated by the need for the cofactor ATIII in order to have an effective thrombin inhibiting surface. Problems with the attachment of a sufficient number of molecules whether they are thrombin inhibitors, small peptides or other polymers onto the surface of a polymer remains a difficulty to be overcome. Furthermore, with the use of these techniques, the complicated and still not completely understood mechanisms of protein adsorption are responsible for maintenance of hemostasis.

1.2.7 Endothelial Seeding of Vascular Grafts

Rather than manipulating the protein interactions that occur at the vascular graft surface, it has been suggested that the attachment of the non-thrombogenic surface present on the interior of the native vessels may reduce complications associated with implantation of small diameter vascular grafts. Therefore, another method that has been exploited in an effort to produce a blood compatible vascular prosthesis is to grow the naturally occurring endothelial cell layer on the lumen of the vascular graft (Massia and Hubbell 1990, Sipehia *et al.* 1993, Lin and Cooper 1994, Holland *et al.* 1996 and Walluscheck *et al.* 1996).

Growing the naturally occurring layer of cells on the lumen would prevent the blood from contacting the artificial surface directly and thereby avoid the initiation of coagulation.

Different methods of promoting the growth of cells on artificial surfaces have been attempted. Surfaces have been coated with extracellular matrix proteins such as fibronectin and collagen to provide a surface similar to that which would be encountered by the cells *in vivo* (Massia and Hubbell 1990). Modification of synthetic polymers such as ePTFE with cell binding peptides has also been assayed in an attempt to improve the growth of endothelal cells on the artificial vascular graft (Walluscheck *et al.* 1996). While these attempts have demonstrated improvements the adherent cells may not achieve or sustain a confluent monolayer. Implantation if the artificial graft also requires that the adherent cells be capable of withstanding the shear forces caused by blood flow.

1.2.8 Integrins and Cell Binding Peptides

The adhesion of cells onto natural surfaces is mediated by the interaction of cell surface receptors with specific peptide sequences in the extracellular matrix (Drumheller and Hubbell 1994 and Lin *et al.* 1992). The cell surface receptors responsible for adhesion are membrane-spanning proteins known as integrins (Massia and Hubbell 1992a).

Integrins are heterodimeric proteins composed of α and β subunits. There are a variety of different α and β proteins that have been characterized. Each functional integrin is composed of one α and one β protein. Many combinations of α and β subunits have

been found to be expressed by different cells types. Each combination of the two subunits provides unique characteristics to the particular integrin. There are three main families of integrin receptors that have been distinguished based on the β subunits. The integrins that are associated with cell-extracellular matrix interactions for specific cell types are the β_3 integrins (Massia and Hubbell 1992b). As membrane spanning proteins, integrins are involved in many biological processes including: platelet aggregation, inflammation, wound healing, immune reactions, tumor metastasis, tissue remodeling in development and cell-extracellular matrix interactions (Healy *et al.* 1996).

The extracellular matrix is an intricate network of macromolecules which constitute the majority of the extracellular space. The matrix is composed of polysaccharides and proteins that are secreted by the local cells. The composition of the extracellular matrix can be very important in determining cell function. The two types of macromolecules that make up the majority of the extracellular matrix are glycosaminoglycans, such as heparin, and fibrous proteins, such as collagen, fibronectin, laminin and vitronectin.

The integrins on the cell surface recognize and bind specific amino acid sequences in extracellular matrix proteins such as fibrinogen, vitronectin and fibronectin. Binding of the receptors to the appropriate extracellular proteins serves to anchor the cells to the surface and promotes proper function of the attached cell (Lin *et al.* 1992). This binding and communication with the extracellular environment is essential in maintaining the appropriate cellular functions.

The amino acid sequences present in the binding region of the adhesion proteins are highly conserved. In many cases they are variants of the Arg-Gly-Asp-Ser (RGDS) sequence. Synthetic peptides based on this sequence and its variations have been shown to bind their respective integrin with nearly the same affinity as the entire protein (Hubbell *et al.* 1992). This finding has allowed the use of specific peptide sequences instead of whole proteins to promote the attachment and growth of specific cell types on artificial surfaces (refer to Figure 3 for a schematic representation of the action of integrins with the extracellular matrix).

Short synthetic peptides can be constructed easily. Use of these synthetic peptides in place of proteins avoids the task of isolating and purifying specific proteins from biological sources. Also, the binding regions are based on only a small portion of the entire protein chain. By using only the specific binding region, the majority of the protein need not be present. This could be important in avoiding any complications which may result from the interaction of other domains of the proteins with the surroundings. Furthermore, while the entire protein molecules tend to have significant immunological properties, these short chain peptides do not elicit any significant immune reaction. Finally, attachment of these peptides to surfaces is much more uniform as there exists only one or possibly two potential sites for binding compared with the hundreds of potential sites in proteins. Therefore, the conformation of these peptides on the surface is much more uniform.

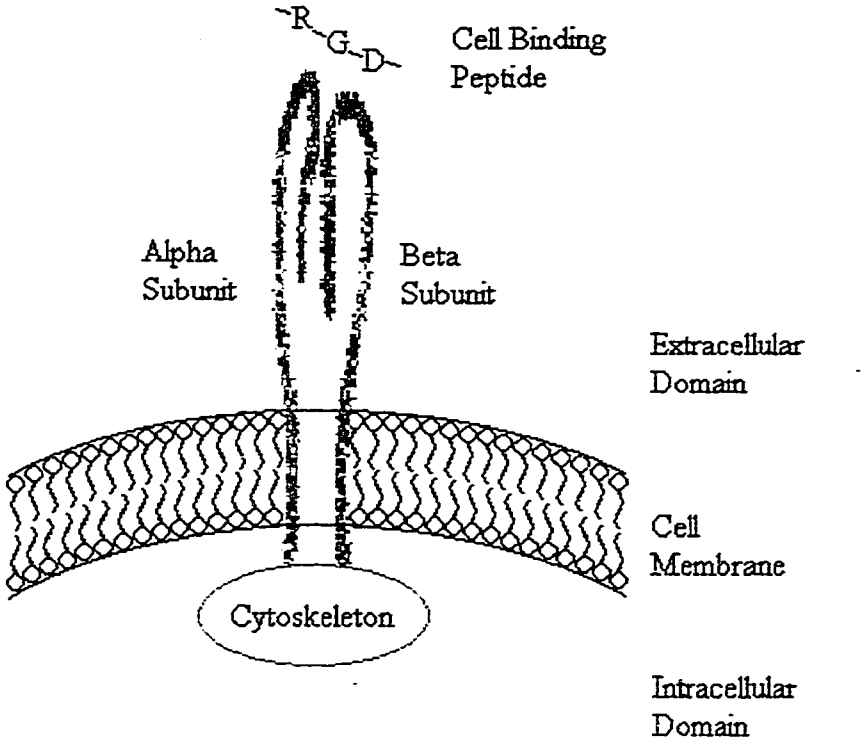

Figure 3: Schematic representation of an integrin binding a synthetic cell adhesion peptide. The α and β subunits are shown spanning the cellular membrane. The intracellular portion of the integrin binds elements of the cytoskeleton and the extracellular portion binds the synthetic peptide RGD.

The integrin on vascular endothelial cells that has been targeted in this study is the $\alpha_v\beta_3$ integrin. The ability of the synthetic adhesion peptide to selectively promote the adherence of vascular endothelial cells would reduce the possibility of graft failure due to platelet induced thrombosis or neointimal thickening, which results from ingrowth of smooth muscle cells into the graft, causing a constriction in blood flow through the vessel. Platelets and smooth muscle cells have not been found to express the $\alpha_v\beta_3$ integrin.

1.2.9 Gold-Thiol Chemistry

The study of sulphur-metal interactions began with the discovery that dialkyl disulfides formed oriented monolayers on gold. It was found that molecules containing thiol or disulfide groups could form dense and highly ordered monolayers on the surfaces of metals such as gold, silver, copper and platinum. If the attached molecules are of sufficient length, self assembly of the molecules on the surface occurs and hence these have been termed self-assembled monolayers (SAMs).

Much of the early work in this area was performed using alkane thiols. Alkane thiols were initially investigated to gain a better knowledge of the physics and chemistry of interfaces. The SAMs provided a means to create a very specific and consistent interface. The potential of SAMs in technological applications such as lubrication, microelectronics, photochemical, electrochemical and biological interactions has led to the study of many different thiol containing molecules (Whitesides *et al.* 1990, Nuzzo *et al.* 1987 and Bain *et al.* 1989).

Recently, this work has been adapted to study the chemisorption of sugars, DNA, enzymes and amino acids (Fritz *et al.* 1996, Hegner *et al.* 1993, Uvdal *et al.* 1992, Sawaguchi *et al.* 1992 and Ihs and Liedberg 1991). These studies have demonstrated that, in principle, any thiol- or disulfide-containing molecule can be chemisorbed to a gold surface. This has great potential for specifically tailoring surface chemistries and for studying biological interactions with the tailored surface.

A SAM can be prepared by immersing a clean gold surface into a dilute (1 mM) solution of the sulfur-containing compound. If the immersion time is sufficient, the result will be a closely packed monolayer on the surface as shown in Figure 4.

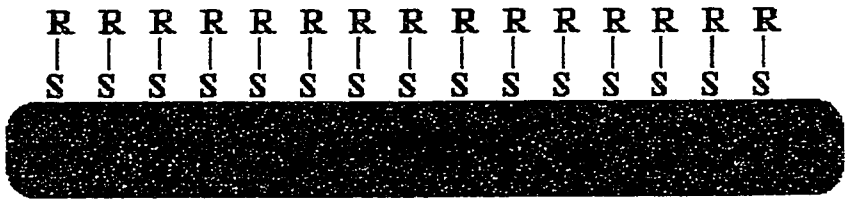

Figure 4: Schematic representation of thiol containing organic molecule chemisorbed on a gold surface. R represents the organic portion of the thiol containing molecule.

Chemisorption of thiol-containing molecules is capable of producing a surface with a very high density of the chemisorbed molecules on the surface. The regular 5Å spacing of gold atoms in colloidal gold allows for an extremely dense monolayer and hence a high concentration of the ligand of interest to be formed at the surface. Densities as high as 4.4×10^{14} molecules/cm^2, or 0.73 nmol/cm^2, have been found for chemisorption onto colloidal gold (Nuzzo *et al.* 1987). The use of thiol-containing molecules for chemisorption to gold has tremendous potential for specifically tailoring surface chemistries. By altering the R group it is theoretically possible to chemisorb any molecule to the gold surface. This provides unlimited potential in constructing surface chemistries.

1.2.10 Polyurethane Chemistry

Polyurethane block copolymers come from a subclass of the family of thermoplastic elastomers. The polyurethane elastomer consists of alternating rigid and flexible segments. The hard segment is diisocyanate and is chain extended with a low molecular weight diol or diamine to form a polyurethane urea. The soft segment is usually a polyol. The three building blocks of the polyurethane combine to form the polyurethane depicted in Figure 5.

HO~~~~~UGUGU~~~~~UGU~UGUGU~~~~~UGUGUGUGU~~~~~OH

Where: U is the diisocyanate hard segment
G is the diol chain extender
~ is the polyol soft segment

Figure 5: Schematic representation of macromolecular polyurethane structure. Lengths of hard and soft segments are variable.

The physical properties of the polyurethane can be manipulated by altering the molecular weights and molar ratios of the segments. Also changing the types of molecules for the three building blocks will alter the physical properties of the polyurethane. The soft segment used for elastomer synthesis is generally a polyol, polyether, polyester, polyalkyl or polydimethylsiloxane.

The two most commonly used isocyanates for polyurethane synthesis are toluene diisocyanate and methylenedi-p-phenyl diisocyanate (MDI). Many other alternatives are used for creating polymers with different physical properties.

The isocyanates react with the chain extenders to create the hard segment. Again, a wide variety of chain extenders can be used, each providing the polymer with different characteristics. Generally, aliphatic chain extenders yield softer polyurethanes than aromatic chain extenders.

Polymerization procedures can be carried out in a single or two step process. The two step process is more common. In the first step an intermediate oligomer, the prepolymer, is formed by reacting the diisocyanate and the polyol. This is depicted in Figure 6.

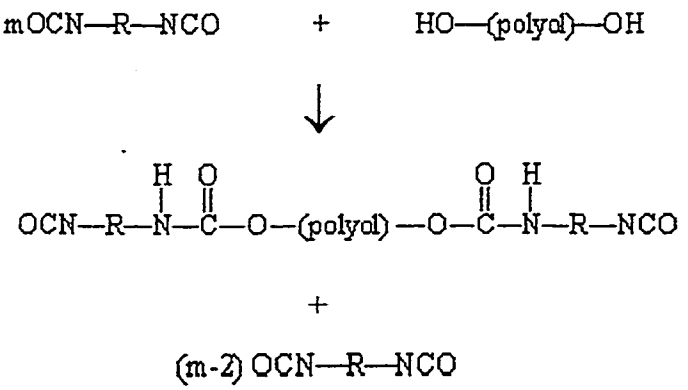

Figure 6: Polyurethane prepolymer synthesis. Two diisocyanates are reacted with one polyol to form the prepolymer.

After the prepolymer is formed the second step involves reacting the prepolymer with the chain extender. This is the chain extension stage. The reaction is shown in Figure 7.

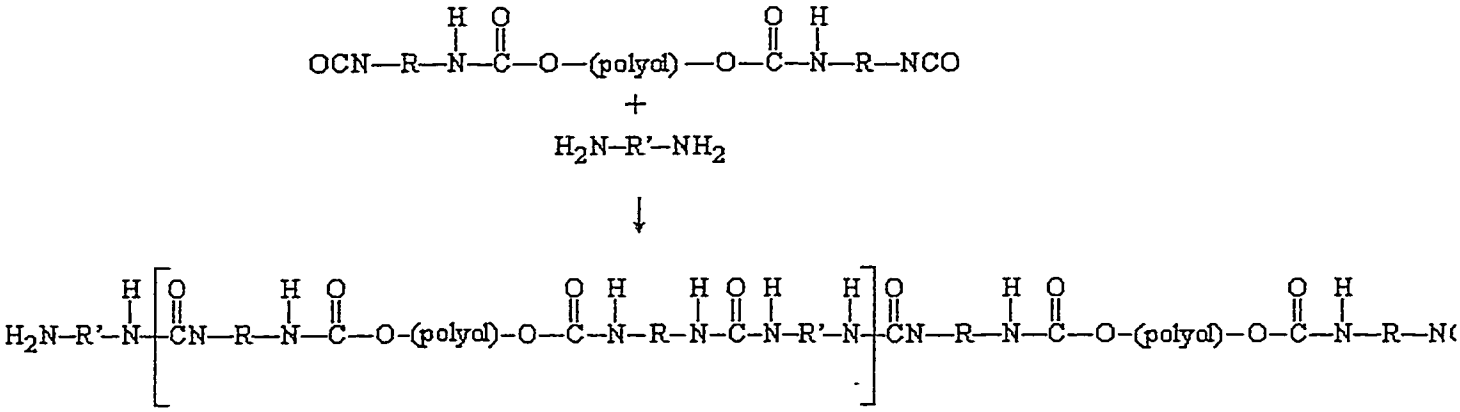

Figure 7: Polyurethane chain extension reaction. The second reaction produces the final polymer.

1.3 Experimental Analytical and Characterization Techniques

1.3.1 Water Contact Angles

Water contact angle measurements can be used to determine the relative hydrophilicity or hydrophobicity of a surface. When the surface in question is being altered in some way, a change in the water contact angle can indicate a change in the surface properties.

Water contact angle measurements can be conducted in several different ways. The principle of all of the methods is the same. The most straightforward measurements come from the sessile drop technique. A drop of water is introduced onto the surface and the tangent of the line where the water drop contacts the surface is considered the contact angle. Surfaces that are more hydrophobic will have a larger contact angle whereas surfaces that are more hydrophilic will have a greater degree of wetting and therefore a relatively smaller contact angle as shown in Figure 8. Information is qualitative rather than quantitative since surface chemistry is not specifically determined using this technique.

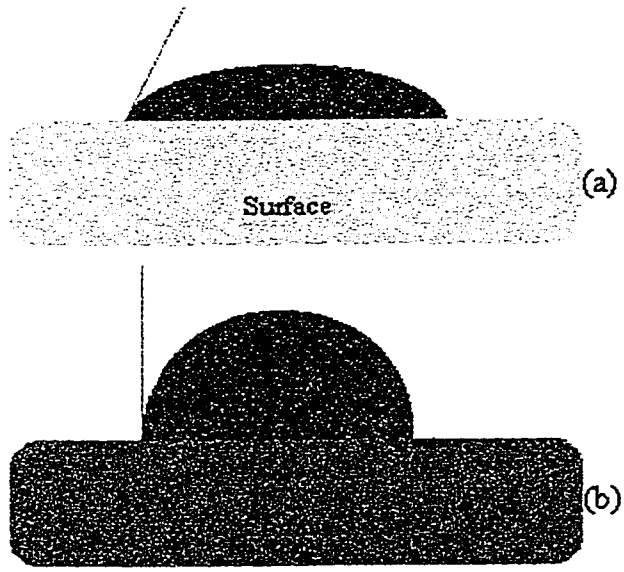

Figure 8: Schematic representation of surfaces with differing degrees of hydrophilicity. Surface (a) is more hydrophilic than surface (b) this is evident by the fact that the water tends to spread over and wet surface (a).

1.3.2 X-Ray Photoelectron Spectroscopy (XPS)

XPS can be used to obtain quantitative information about the surface chemistry. XPS functions by bombarding the surface with an X-Ray beam (refer to Figure 9 for a schematic representation).

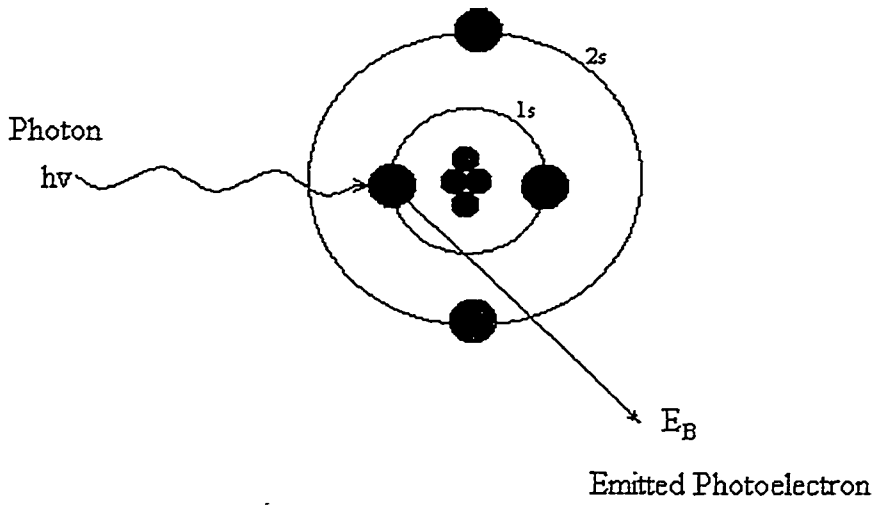

Figure 9: Schematic representation of the emission of a photoelectron from a core electron after bombardment with an x-ray.

The energy from this beam causes the emission of electrons from core atomic levels of atoms on the surface. The energy of the emitted photoelectron corresponds to the binding energy of the core electrons. By measuring the emissions from the surface, it is possible to determine what elements are present and the bond configurations of these elements. The method is highly surface sensitive since electrons emitted can only pass through a maximum of about 100 Å of material to reach the detector. A low resolution XPS scan will give quantitative information about the elements present on the surface. To

gain information on the bonding of particular elements on the surface a high resolution scan can be used. High resolution scans are focused on particular elements. Based on qualitative fits of the peak intensities, the bonding configurations of the elements present on the surface can be determined. By varying the angle at which the x-rays bombard the surface, or take off angle, more surface specific information may be obtained.

1.3.3 Scanning Electron Microscopy (SEM)

SEM is a technique that is used to gain information on the surface morphology of a sample (see figure 10 for a schematic representation of a scanning electron microscope).

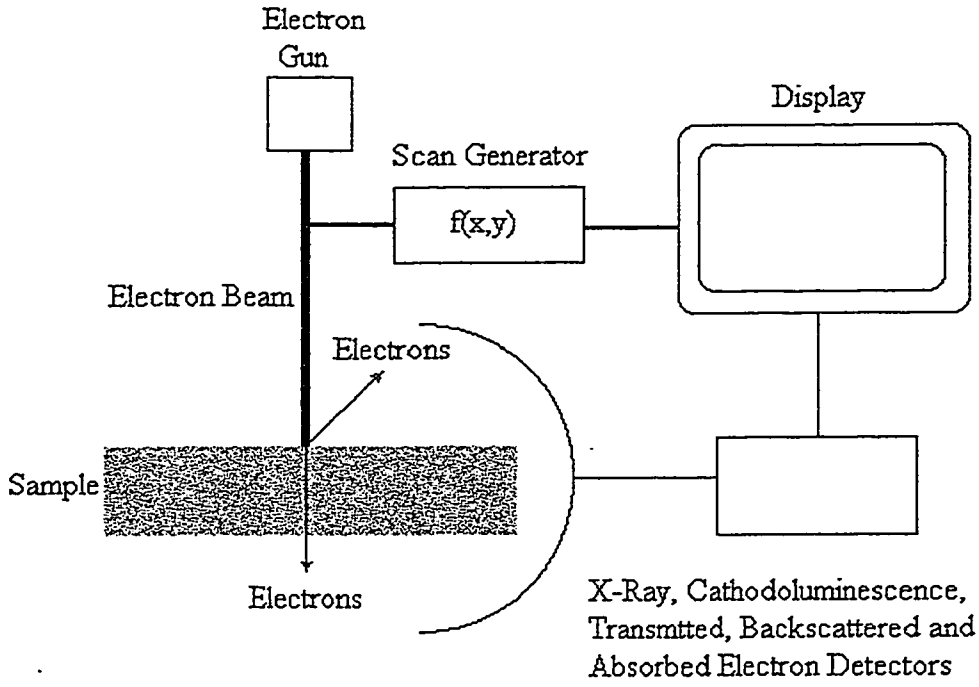

Figure 10: Schematic representation of a scanning electron microscope.

The SEM acts similarly to the light microscope except that by using an electron beam, which has a much smaller wavelength than visible light wavelengths, the resolution of the image can be enhanced significantly. The SEM functions by bombarding the surface with an electron beam. A secondary signal arising from the surface is recorded by a detector. A contrast on the recorded image will appear when the intensity of the signal changes as the surface is scanned. In this manner the light and shaded areas on the scanned image will appear similar to a conventional light microscope.

1.3.4 Atomic Force Microscopy (AFM)

AFM is a technique that provides information about the surface topography or surface roughness. In essence, this technique results in the generation of a "picture" of the surface topography (refer to Figure 11 for a schematic representation of the apparatus).

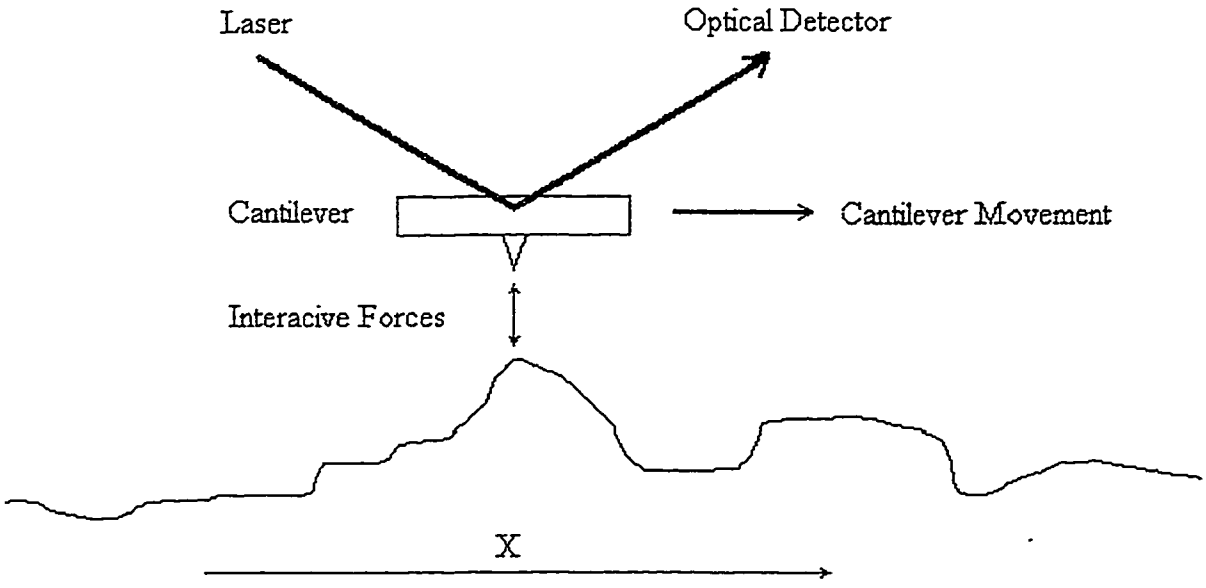

Figure 11: Schematic representation of atomic force microscopy.

In AFM, a sharp cantilever is brought into contact with the surface. The changes in elevation are sensed by the AFM by detection of the deflection of the cantilever as it passes over the surface. The deflection of the cantilever is detected by reflecting a laser off it. The changes in the force required by the laser is indicative of the changes in topography as the tip passes over the surface.

1.3.5 Sodium Dodecylsulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Gel electrophoresis is a common method of separating proteins in a mixture. Combining this technique with the use of sodium dodecylsulphate (SDS) allows for the separation of proteins based solely on molecular weight only and avoids the secondary effect of charge. SDS binds all proteins in such a manner as to confer the same charge density to all proteins. In this way, the charges on the individual proteins are masked and the molecular weight differences are the only means by which the proteins can be separated.

Following incubation with SDS the proteins are loaded onto a two phase polyacrylamide gel and subjected to an electric field. The electric field acts on the negative charges of the proteins conferred by the binding of SDS. The electric field causes the proteins to flow through the gel.

A two phase gel is used to improve the resolution of protein separation. The first phase, the stacking gel, is composed of large pores and serves to concentrate the proteins

into tight bands to provide sufficient differentiation after separation. The second phase of the gel is the separation gel, and is composed of smaller pores. The flow of larger proteins is retarded while the smaller proteins flow more easily through the gel.

The result is a gradient with low molecular weight proteins concentrated at the cathode and high molecular weight proteins concentrated at the anode. A low molecular weight tracer is added to the proteins and indicates that separation is complete when it reaches the cathode. Upon staining, bands corresponding to specific proteins can be seen and the proteins can be identified by molecular weight.

1.4 Rationale / Scope of Thesis

In this work, the effect of using gold coated polyurethanes as substrates for cell growth was studied. The purpose of the polymer layer was to maintain the desired flexibility for use in such applications as vascular grafts. A thin layer of colloidal gold was evaporated onto the surface of these polymers. This surface could then be modified with various thiol containing peptides, including those specific for promoting the adhesion of vascular endothelial or other cell types. The close packed nature of the gold allows for the development of a relatively dense layer of peptide on the surface. It was hypothesized that gold coated polymer surfaces could be modified with appropriate peptides to obtain a high density of peptide on the surface. These modified surfaces would have significant potential to promote the adhesion of vascular endothelial cells.

Thiol containing peptides which were thought to be specific for promoting the adhesion of vascular endothelial cells were attached to the gold coated surfaces. These

surfaces were evaluated chemically as well as biologically. Surface analysis techniques such as water contact angles, x-ray photoelectron spectroscopy, atomic force microscopy and scanning electron microscopy were used to obtain surface-specific information. Protein adsorption studies and SDS-PAGE were used to obtain information about the protein adsorption on the modified surfaces. The ability of the various modified surfaces to promote the adhesion of mouse 3T3 fibroblasts and vascular endothelial cells was examined. This work was performed in effort to advance the development of a small caliber, implantable, bioartificial vascular prosthesis.

Chapter 2: Experimental Procedures

All chemical reagents, solvents and materials are listed in Appendix A. Reagent source of supply and location of supplier are also listed. All prepared solutions and their method of preparation are listed in Appendix B.

2.1 Surface Preparation and Modification

In this study, a polyurethane was used as the base material for supporting the gold surface. After synthesizing the polymer and casting to form a membrane, the polyurethane was coated with gold by vacuum evaporation. The gold surface was then modified by chemisorption of an amino acid or specific peptides.

2.1.1 Synthesis of Polyurethane

Polyurethane was prepared based on the method described by Santerre *et al.* (1992). Polypropylene glycol (PPG), average molecular weight 1200, was degassed under vacuum. Anhydrous dimethyl sulfoxide (DMSO), N,N-dimethyl formamide (DMF), methylene-di-p-phenyl diisocyanate (MDI), ethylenediamine (ED) and methanol were used as received.

A two step polymerization method was used to formulate the polymer. In the first step, prepolymers were formed by reacting MDI and PPG dissolved in DMSO in a 2:1 molar ratio at 90°C for 90 minutes. In the second step, ED dissolved in DMSO was added to the reaction flask such that the ratio of MDI:PPG:ED was 2:1:1. The reaction was allowed to proceed at 70°C for 60 minutes. The above reactions were performed in a 500 ml three necked round bottom flask under a dry nitrogen environment. A schematic representation of the apparatus is depicted in Figure 12.

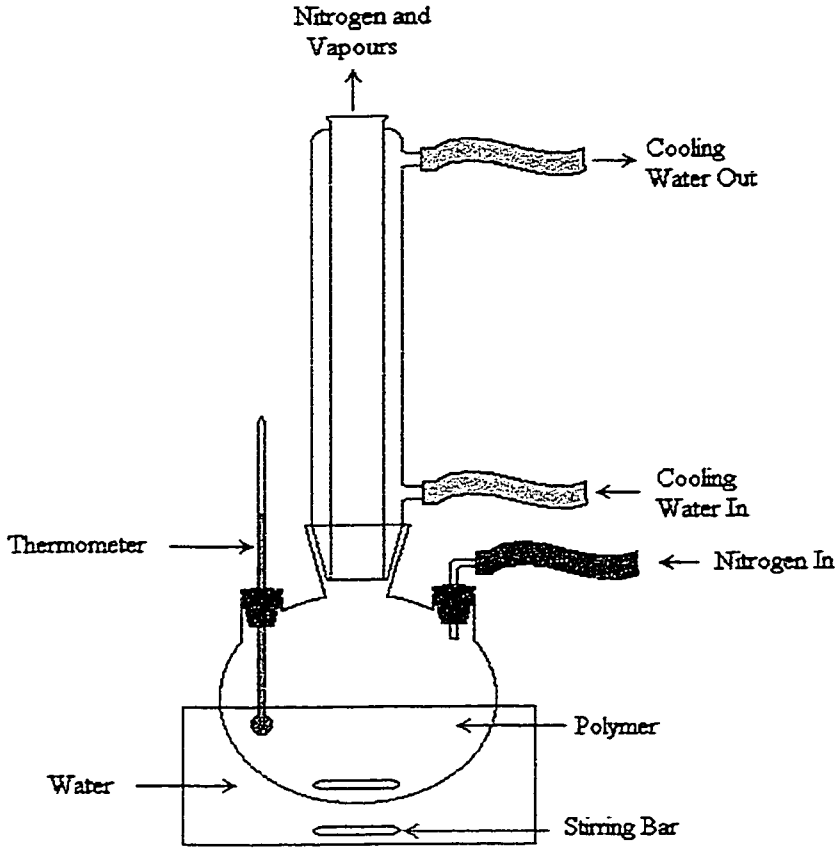

Figure 12: Schematic representation of polymerization apparatus. Nitrogen is passed into the reaction vessel to maintain an inert atmosphere.

2.1.2 Polyurethane Film Casting

A 12% (w/v) solution of the polyurethane in DMF was prepared. Fifty ml of the polyurethane solution was poured into 100 mm glass petri dishes, covered with cardboard and placed in an oven at 50°C for 5 days to allow the solvent to evaporate. The films were then removed from the dishes, rinsed with methanol, allowed to air dry and stored in plastic bags for future use.

2.1.3 Gold Coating of Polyurethane Films

The coating of the polyurethane surfaces with gold was performed by vacuum evaporation with an Edward's Auto 306 Coating System. This service was provided by Dr. Michael Sayer in the Department of Physics at Queen's University. The gold layer was deposited directly onto the surface of the polymer with no intermediate layer required to increase adhesion. The system was evacuated to a pressure of approximately $5x10^{-6}$ Torr with the polyurethane film positioned 12 cm from the evaporating source. Gold was then deposited on the film to a thickness of approximately 1000 Å. Gold was coated on either a single side or both sides of the films depending on the requirements of the particular experiment.

2.1.4 Chemisorption of Peptides onto Gold Coated Surfaces

Gold coated polyurethane disks of the desired diameter were cut with a brass punch. A cleaning solution consisting of 1 part hydrogen peroxide, 1 part ammonium hydroxide and 5 parts water was prepared. The gold coated surfaces, glassware required for the chemisorption and equipment contacting the surfaces were immersed in the cleaning solution which was subsequently heated to 80°C and maintained there for a period of 10 minutes. The surfaces and glassware were then removed from the cleaning solution and rinsed with distilled water.

Three synthetic peptides were used in these studies. These peptides are shown in Table 1. The peptides used for chemisorption were synthesized by the Core Facility for Protein/DNA Chemistry at Queens University in Kingston, ON. The synthesized peptides were purified by high performance liquid chromatography (HPLC) and their purity was determined by mass spectrometry. The chromatograms are included in Appendix C. The peptides were used as received.

Table 1: Synthetic cell adhesion peptide specificity for binding integrins. Amino acid sequences are shown with single letter abbreviations in parentheses.

Amino Acid Sequence	Binding Characteristics
Cys-Arg-Gly-Asp (CRGD)	Non-specific integrin binding
Cys-Arg-Glu-Asp-Val (CREDV)	Specific for binding $\alpha_4\beta_1$ integrin
Cys-Cys-Asp-Asp-Gly-Asp-Trp-Lys-Cys (CCRRGDWLC)	Specific for binding $\alpha_v\beta_3$ integrin

The RGD peptide was selected based on its relatively non-specific integrin binding capabilities. The RGD has been noted to be highly conserved in a variety of extracellular matrix proteins. The REDV peptide was chosen for its ability to specifically bind the $\alpha_4\beta_1$ integrin which is present on the surface of vascular endothelial cells.

Several studies have been conducted to test the ability of certain peptide sequences to bind specific integrins. One study in particular (Healy *et al.* 1996) randomly altered the two amino acids on either side of the RGD sequence through all possible combinations to find the optimal amino acid sequence capable of binding the $\alpha_v\beta_3$ integrin. The sequence RRGDWL was found to have a significantly higher binding ability over any other sequences.

Two studies performed on the binding ability of synthetic peptides to integrins have shown that steric hindrance of the peptide can significantly improve the binding affinity (Healey *et al.* 1996 and Zhang *et al.* 1996). Using these two findings, synthetic peptides targeted at binding the $\alpha_v\beta_3$ integrin were constructed. This peptide combined the optimal amino acid sequence for binding the $\alpha_v\beta_3$ integrin as well as the increased binding affinity resulting from cyclization through a disulfide bond between two cysteine residues. In all cases a cysteine residue was added to the N terminus such that a thiol group would be available for chemisorption to the gold surface.

Stock solutions of the peptides and the amino acid cysteine with a concentration of $1x10^{-2}$ M were prepared in distilled water. The stock solution was serially diluted down to a concentration of $1x10^{-6}$ M. Ten ml glass beakers were then filled with the desired

concentration of peptide and the gold coated surfaces were immersed in the peptide solution. Surfaces incubated in water served as controls.

The density of gold atoms on a smooth surface is calculated to be a maximum of approximately 10 $x10^{18}$ atoms/m^2. Six mm disks of the gold coated polyurethane were used for chemisorption. The disks would have a maximum of 2.8 $x10^{12}$ atoms on the surface which corresponds to 4.7 $x10^{-12}$ moles of gold on the disk. Therefore in a 10 ml volume any solution with a peptide concentration of approximately 5 $x10^{-10}$ M would have sufficient peptides to be in excess based on the fact that all of the sites on the surface are not expected to be occupied by gold. All concentrations used in this study were well above this minimum.

The surfaces were incubated at room temperature with mild agitation. Peptide concentrations at various times were examined in order to evaluate the effects of these parameters on the final surfaces. A summary of the different concentrations and incubation times are shown in Table 2. After incubation, the surfaces were removed from the incubation solution, rinsed three times with distilled water and stored in stoppered glass vials until use.

Table 2: Time and concentrations used in studying effects on chemisorption. The conditions marked with an X were not considered useful due to excessive delamination of the gold from the polymer.

	Time [hrs]			
Concentration [M]	1	5	25	125
0				
$5x10^{-5}$				
$5x10^{-4}$				
$5x10^{-3}$				X
$5x10^{-2}$			X	X
$5x10^{-1}$			X	X

2.2 Surface Characterization

The gold coated polyurethane used in this study was modified by chemisorption of the amino acid cysteine and custom synthesized peptides to the gold surface. The physical properties of the modified gold coated surfaces and the underlying polyurethane were analyzed using several different techniques.

2.2.1 Polystyrene Equivalent Average Molecular Weight

The molecular weight of the polyurethane used as the substrate for gold coating was determined by Gel Permeation Chromatography (GPC). The polyurethane was dissolved at a concentration of 0.1% (w/v) in DMF with 0.1% (w/v) lithium bromide (LiBr). The sample was run through a series of three Waters Styragel® HR4 GPC columns. The retention time of the polyurethane was determined using a Waters 410 Refractometer. This was compared to retention times of polystyrene standards. The polystyrene equivalent molecular weight was determined by the use of a calibration curve constructed by the use of the polystyrene standards with a range of molecular weights of 11 300 to 172 000 Da.

2.2.2 Water Contact Angle Measurements

The physical properties of the gold coated polyurethane surfaces after chemisorption of the peptides were studied by the use of advancing water contact angle

measurements. The contact angle measurements are depicted schematically in Figure 13 showing the variables involved in determining the angle.

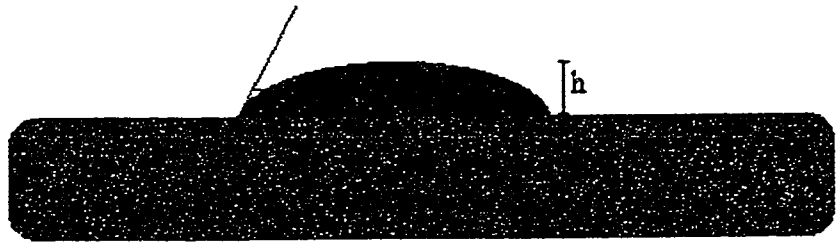

Figure 13: Schematic representation of contact angle measurement and variables used in calculating the angle θ. h and d are the height and diameter of the water droplet respectively.

The surfaces were allowed to air dry. A drop of distilled water (approximately 10 µl) was placed on the surface. The diameter of the drop in contact with the surface was measured with a vernier caliper, while the height of the drop was measured using a wetometer. The contact angle could then be determined from Equation 1.

$$\theta = \tan^{-1}\left[\frac{2h}{d}\right] \qquad (1)$$

Where: θ is the contact angle

h is the height of the water droplet

d is the diameter of the water drop

2.2.3 X-Ray Photoelectron Spectroscopy (XPS)

XPS was used to gain knowledge of the surface chemistry of the gold coated polyurethane and its peptide modified derivatives. The analysis was performed by the ICPET at the National Research Council of Canada. The analysis was performed using a Kratos AXIS HS X-Ray photoelectron spectrometer. Following chemisorption and washing as described above, the samples were placed in the vacuum chamber of the XPS. Low resolution spectra were obtained for all surfaces. High resolution spectra were recorded for gold, carbon, nitrogen and sulfur chemical species.

2.2.4 Scanning Electron Microscopy (SEM)

SEM was used in this study to visualize the gold coated polyurethane and the various peptide modified derivatives. The analysis was performed by the ICPET at the National Research Council of Canada without any further surface preparation. Image sizes ranged from 10 000 μm^2 to $6.25x10^4$ μm^2.

2.2.5 Atomic Force Microscopy (AFM)

AFM was used to examine the surface roughness and topography of the gold coated polyurethane and its peptide modified derivatives. The analysis was performed at the Brockhouse Institute for Materials Research at McMaster University without any further surface preparation. A Digital Instuments Nanoscope II was used in the analysis.

Scanning sizes of both $1x10^6$ nm^2 and $1x10^8$ nm^2 were performed on each sample. An estimate of the surface roughness was calculated from the scans using equation (2).

$$R_a = \frac{1}{L_x L_y} \int_0^{L_y} \int_0^{L_x} |f(x,y)| dxdy \quad (2)$$

2.3 Protein Adsorption

Since protein adsorption is the initial and fate-determining step following incubation of a surface with any protein-containing solution and since the composition of the adsorbed protein layer will have a significant effect on cell attachment via integrins on the cell surface, the effect of peptide modifications on protein adsorption from plasma was investigated in this study. The adsorption of albumin from plasma onto the modified surfaces was investigated. The native gold-coated surface and cysteine, CRGD, CREDV and CCRRGDWLC chemisorbed surfaces were compared.

2.3.1 Preparation of ^{125}I labeled Albumin in Plasma

Albumin was labeled with ^{125}I using the iodogen method prior to the adsorption studies. Briefly, albumin was diluted with phosphate buffered saline (PBS), pH 7.4, to a concentration of 1.0 mg/ml. A solution of 1,3,4,6-tetrachloro-3a,6a-diphenylcoluril (Iodogen®) in chloroform (0.1 mg/ml) was prepared. One hundred µl was plated in a conical glass 3 ml vessel. The chloroform was evaporated at room temperature under a

dry nitrogen environment. 5µl of radioactive sodium iodide ($Na^{125}I$, 0.5 mCi) was added to the glass vessel and stirred for approximately one minute. Following this, 100 µl of albumin (1.0 mg/ml) and 100 µl of PBS buffer were added to the reaction vessel and stirred for fifteen minutes. The albumin was then removed from the reaction vessel to terminate the iodination reaction.

Free iodide remaining in the albumin solution was removed by dialysis. The labeled albumin was injected into a SLIDALYZER® dialysis cassette and dialyzed against 500 ml of PBS buffer, at 4°C overnight with three changes of the dialysate. Following this, the ^{125}I labeled albumin was removed from the dialysis cassette and the cassette was rinsed twice with 500 µl of PBS to remove any remaining albumin.

A 0.25% labeled albumin solution was prepared by adding 0.9 ml of the labeled albumin solution to 5.04 ml PBS buffer. To obtain a plasma concentration of 10% (v/v), 0.66 ml of plasma was added to the albumin. The final albumin concentration was approximately 6000 µg/ml. This solution of 0.25% labeled albumin was used in all subsequent experiments involving albumin.

2.3.2 Plasma Preparation

Human pooled plasma was used for this application. Plasma from several different donors was combined and frozen in 1 ml volumes. Prior to use, the plasma was thawed to room temperature.

2.3.3 Determination of Free Iodide in ^{125}I Labeled Albumin

Free iodide in the labeled albumin can bind to the gold surfaces used in this study (Sheardown *et al.* 1997). The labeled albumin was tested to ensure that the level of free iodide was acceptably low.

To each of six 1.5 ml centrifuge tubes, 100 µl of 10% labeled plasma and 0.9 ml bovine serum albumin (BSA) (1% w/v) were added. Three of these aliquots served as "A" samples. To the other three tubes, which served as "B" samples, 0.5 ml of 20% (w/v) trichloroacetic acid (TCA) was also added to precipitate the protein. The tubes were then vortexed briefly to mix the reagents. After incubation for ten minutes at room temperature, the tubes were centrifuged at 3000 rpm for two minutes. A 0.5 ml aliquot of the supernatant, now free of protein, was removed and added to 0.5 ml of PBS. The radioactivity of these "B" solutions as well as the "A" solutions containing the ^{125}I labeled albumin were determined by a gamma counter and the free iodide in the solution determined using Equation 3. The free iodide content of the albumin used in these experiments was typically 0.5%

$$\%I = \frac{3(B_1 + B_2 + B_3)}{(A_1 + A_2 + A_3)} \times 100 \qquad (3)$$

Where: A is the protein containing solution

B is the protein free solution

2.3.4 Albumin Adsorption onto Modified Gold Surfaces From Plasma

Since any free iodide, even the trace amounts present, have an extremely strong affinity for gold and can therefore give erroneously high protein adsorption results, the surfaces were incubated overnight in NaI buffer prior to use in adsorption studies to reduce the amount of free iodide in the labeled plasma solution binding the gold surface. Serial dilutions of the labeled plasma solution to give 10%, 5%, 2%, 1% and 0.1% plasma in PBS were prepared. The surfaces onto which the albumin was to be adsorbed were placed in the wells of a 96 well microtitration plate. A volume of 250 µl of the albumin solutions were added to the wells. After incubating at room temperature for two hours, the surfaces were removed from solution and dip-rinsed three times in PBS buffer. The radioactivity of the surfaces were measured with a gamma counter and the counts compared to known standards.

The adsorbed albumin was estimated using Equation 4.

$$Adsorbed\ \mathrm{Pr}otein = \frac{CPM_1 \times C_{Alb} \times V}{Area \times CPM_2} \qquad (4)$$

Where: CPM_1 is the radioactivity of the surface [cpm]

C_{Alb} is the concentration of albumin in plasma solution [µg/ml]

V is volume of reference albumin solution [ml]

$Area$ is surface area of gold coated polyurethane disk [mm^2]

CPM_2 is the radioactivity of reference albumin solution [cpm]

2.3.5 Separation / Staining of Proteins Adsorbed to Modified Surfaces

In order to assess differences in protein adsorption patterns on the different modified surfaces, sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) was used. The resulting gels were then stained using a gold stain technique.

2.3.5.1 SDS Polyacrylamide Gel Electrophoresis

The separation technique required two different gel concentrations to achieve optimal separation. A 4% polyacrylamide gel was used for "stacking" or concentrating the proteins before separating to achieve a sharp band after separating. A 12% polyacrylamide gel was used for separating the proteins. See Appendix B for the formulation of gels used in these studies.

The 12% polyacrylamide gel solution was poured into a gel mould and the top was overlaid with water. After polymerizing for approximately one hour, the stacking gel was prepared. The water was removed from the top of the gel and the stacking gel was added. A ten well comb was then fitted into the top of the gel. The stacking gel was then allowed to polymerize for one hour.

To remove the proteins adsorbed to the gold and modified surfaces they were incubated overnight in a 2% SDS solution. The following day the samples and standard markers were loaded onto the gel. A volume of 0.5 µl of the standards and 25 µl of the eluted proteins were loaded into their respective wells at the top of the gel. The electrophoresis apparatus was assembled and 200 volts were applied across the gel for

approximately one hour (refer to Figure 14 for schematic representation of apparatus). When the proteins were separated on the gel, they were run with a tracking dye. The tracking dye provides a reference for timing the migration of the proteins. See Appendix B for formulation of tracking dye.

After electrophoretic separation, the proteins were transferred to an Immobilon® PVDF transfer membrane. The membrane was prewet with methanol and water then soaked in transfer buffer. The gel was applied to the membrane and 200 volts were applied for a period of one hour.

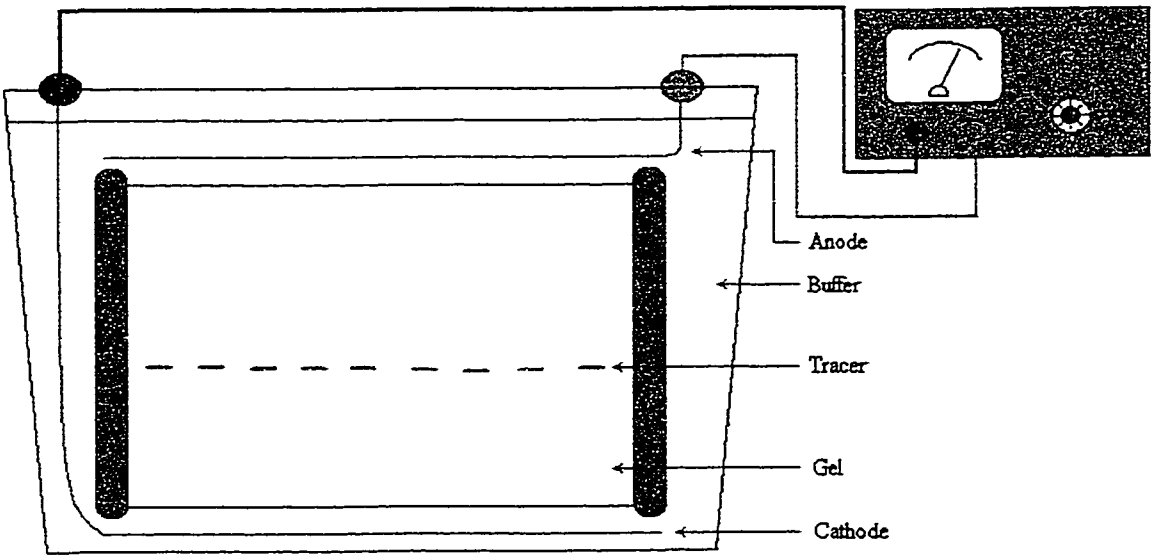

Figure 14: Schematic Representation of SDS-PAGE Apparatus. The polyacrylamide gel is shown submerged in buffer. The low molecular weight tracers are running down the gel from the anode to the cathode.

2.3.5.2 Gold Staining

After transfer of the proteins to the membrane, they were stained for visualization. The membrane was washed twice in PBS. The unbound membrane sites were then blocked by incubation in PBS containing 0.3% Tween 20 at room temperature for one hour. This was followed by washing the membranes three times with the same solution for five minutes each time. A wash with water for one minute was then repeated three times. The membranes were then incubated in 75 ml of Protogold solution overnight. After staining, the membranes were rinsed with water and allowed to air dry.

2.4 Cell Culture Studies

Two different cell types were used in this study to determine the effect of the peptide surface modifications on the attachment and growth on specific cell types. Mouse 3T3 fibroblasts were obtained from American Type Culture Collection. Human vascular endothelial cells were obtained from a cell line grown at the University of Ottawa Eye Institute and were fully characterized prior to use. All materials used in the cell culture, if not received sterile from the manufacturer, were sterilized with a solution of 70% (v/v) ethanol in water or by autoclaving with steam at 121°C. All work with sterile materials was performed in a sterile laminar flow tissue culture fume hood.

2.4.1 Cell-Type Specific Culture Reagents

The different cell types used in the cell culture studies had different requirements for growth media and culturing substrate. The substrate and media selected were to give optimal growth for the specific cell types under laboratory conditions.

2.4.1.1 Mouse 3T3 Fibroblast Cell Culture Reagents

The mouse fibroblasts were grown in supplemented Medium 199. Each 500 ml bottle of growth medium was supplemented with 50 ml fetal bovine serum and 0.1 ml gentamycin. The cells were cultured on 100 mm tissue culture plates.

2.4.1.2 Human Vascular Endothelial Cell Culture Reagents

The endothelial cells were grown in the BIOCOAT Endothelial Cell Growth Environment. The Endothelial Cell Growth Environment contained the growth medium and all reagents required to supplement the medium. The E-STIM growth medium was reconstituted and supplemented with Epidermal Growth Factor (EGF) and Endothelial Cell Growth Supplement (ECGF) as described by the supplier. A volume of 0.1 ml of gentamycin was also added to each 500 ml bottle of supplemented growth medium.

2.4.1.2.1 Preparation of Collagen Coated Tissue Culture Plates

The tissue culture plates used for culture of the endothelial cells were coated with collagen. A 1% (w/v) solution of type A porcine skin gelatin was prepared and sterilized by autoclaving at 121°C for 30 minutes. After cooling, 10 ml of this solution was spread on the tissue culture plates and allowed to stand for 10 minutes. The excess was then pipetted off and the plates were rinsed with 10 ml of sterile distilled water. This was followed by a rinse with 10 ml of growth medium. The plates were then used immediately for tissue culture.

2.4.2 Cell Recovery

Both cell types were received frozen. The frozen stock was rapidly thawed by placing in 37°C water bath temporarily. The thawed stock was then transferred to a 100 mm petri dish containing 10 ml of growth medium warmed to 37°C. The dish was covered and incubated at 37°C with a humidified, 5% CO_2 atmosphere overnight. The following day the cells were subcultured as described below.

2.4.3 Cell Maintenance

The growth media was replaced twice weekly. The used growth media was pipetted off of the surface of the tissue culture plate and discarded. Fresh growth media

was warmed to 37°C in a water bath. Ten ml was added to each 100 mm plate. The plates were then returned to the incubator.

2.4.4 Cell Subculture

When the cells became confluent and formed a complete monolayer, they were subcultured. Approximately every seven to ten days, the plates were examined under a microscope. The plates were considered to be confluent when a single cell layer could be observed to be covering the entire culture plate. At this point the plates would be subcultured.

2.4.4.1 Fibroblast Cell Subculture

The used culture medium was pipetted off the top of the plates and discarded. The plates were then rinsed with 10 ml of Ca^{2+} and Mg^{+} free phosphate buffered saline (PBS) solution. The PBS was subsequently pipetted off the surface and discarded. Two ml of Trypsin EDTA (Trypsin) was added to the plates to dislodge the cells from the culture surface. The plates were then returned to the incubator.

After incubation for five minutes, 8 ml of culture medium, which contained trypsin inhibitor, was added to the plate to inhibit the trypsin. The cells were then resuspended by pipetting up and down several times. Two ml of the cell suspension were then pipetted into fresh culture plates. The cells were thus divided into five new culture plates. Eight

ml of culture medium was then added to each plate to bring the total volume back up to 10 ml. The plates were then returned to the incubator and maintained as described above.

2.4.4.2 Endothelial Cell Subculture

The endothelial cells were subcultured as described above for the fibroblasts with several minor differences. The plates were rinsed and the cells trypsinized as described above. The culture medium for the endothelial cells did not contain trypsin inhibitor. This had to be added separately following trypsinization. After incubation for the trypsinization, 2 ml of trypsin inhibitor was added to each culture plate. This was followed by the addition of 6 ml of culture medium to bring the total volume up to 10 ml. The cells were subsequently resuspended and divided into five new tissue culture plates as described previously for the fibroblasts.

2.4.5 Cell Freezing

In order to continuously maintain an active cell line and reduce the number of cells to be maintained, when an excessive amount of cells were growing, a portion was frozen to keep for later use. In freezing the cultured cells, the same procedure described above for subculturing was followed up to the point where the volume is brought up to 10 ml with culture medium. At this point, the cell suspension was transferred to a polypropylene centrifuge tube. The cells were centrifuged at 1500 rpm in a Beckman J6-MC centrifuge.

After centrifuging for five minutes, the media was aspirated off the top. The pelleted cells were resuspended in 1 ml of culture medium containing 10 % (v/v) DMSO. The resuspended cells were then transferred to a 1.5 ml cryovial. The vial was placed in a -1°C/minute freezing container and the container placed in a freezer at -80°C for 24 hours. The following day the cryovials were transferred to a liquid nitrogen container.

2.4.6 Cell Adhesion and Growth Assay

The cell adhesion and growth studies were performed using the same techniques described above for maintenance and subculturing. The efficacy of the modified surfaces for supporting the attachment and growth of the cells was determined by introduction of the modified surfaces into the culture plates.

The gold coated polyurethane surfaces were modified as described above. These surfaces were sterilized by autoclaving at 121°C for 20 minutes. The sterile surfaces were placed into the bottom of a well in a 24-well tissue culture plate. The cells were subcultured and the volume of tissue culture medium was brought up to 10 ml with growth media as described above.

The cells were resuspended by pipetting up and down several times. A volume of 0.5 ml of the cell suspension was added to each well of the tissue culture plate. The wells were then topped off with 1.5 ml of warmed growth media. The culture plates were then placed in the incubator and maintained there for three days.

2.4.7 Evaluation of Adhesion and Growth on Modified Surfaces

After incubating for the desired period of time, the culture plates were removed from the incubator and the media was pipetted out of each well. The cells were then fixed with a 3% (v/v) solution of glutaraldehyde in distilled water, and the plates maintained at 4°C overnight.

The cells were stained for easier viewing under a microscope. A 0.01% (w/v) solution of Giemsa stain was prepared. The glutaraldehyde was pipetted out of the wells and 2 ml of the Giemsa stain was added. After ten minutes, the stain was pipetted out of the wells and the surfaces were rinsed with water three times.

The surfaces were then examined under a microscope. At a magnification of 450X, the number of cells attached to the surfaces were counted for ten randomly chosen fields of view. The average number of cells attached to the surfaces per unit area was then calculated from this data.

Chapter 3: Results and Discussion

3.1 Surface Preparation

3.1.1 Polyurethane Synthesis

A polyurethane based on a modification of the method proposed by Santerre *et al.* (1992) was used as the substrate for support of the gold layer in this study. The polyurethane was composed of a polypropyleneglycol (PPG) soft segment, a methylenedi-p-phenyl diisocyanate (MDI) hard segment and an ethylene diamine (ED) chain extender. The polyurethane was produced in a batch reaction. To produce sufficient quantities several batches were produced and combined to create a single polymer source for all films produced.

3.1.2 Reaction Yield

Each of the batch reactions was monitored to determine the yield of final polyurethane based on total reagent mass. The yield for all batch ranged between 89 and

97%. These yields are quite respectable give that it is likely that reduced recovery resulted from evaporative losses, polymer residues left on reaction vessel as well as losses incurred during the precipitation and filtration steps.

3.1.3 Polystyrene Equivalent Average Molecular Weight

The molecular weight of the polyurethane was determined through the use of gel permeation chromatography (GPC). The retention time of the polyurethane was converted to polystyrene equivalent weight and number average molecular weight by comparison to a standard curve constructed from known polystyrene standards (refer to Figure 15 for chromatogram from polyurethanes synthesized). The weight average molecular weight of the polyurethane was determined to be 76 300 daltons with a polydispersity of 1.256. This polydispersity is quite reasonable considering that the polymers were synthesized in a batch reaction and that the results came from a mixture of more than one batch of polymer. This suggests that the different batches of polymer were extremely uniform.

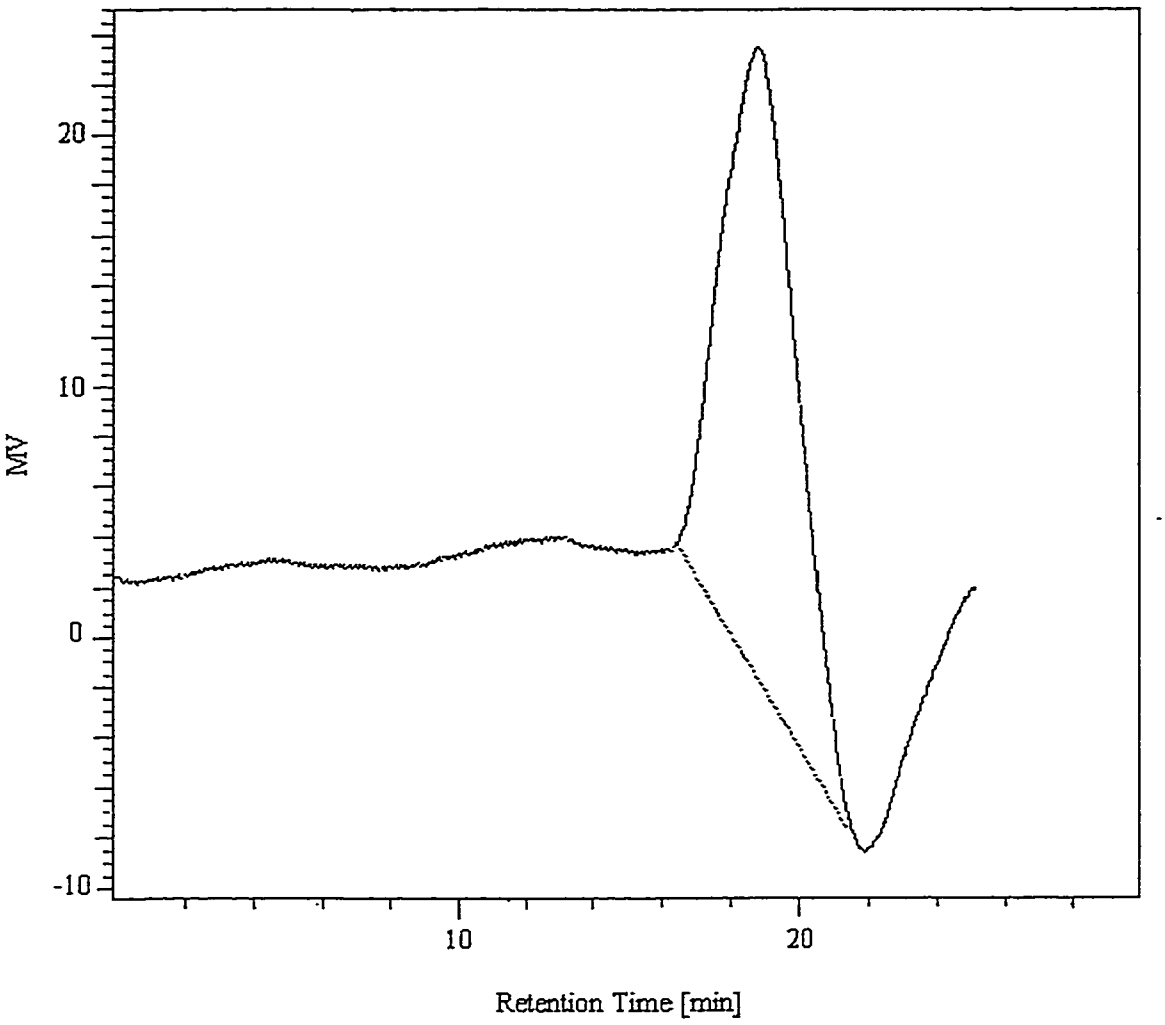

Figure 15: GPC chromatogram for polyurethane. The chromatogram shows a peak in the refractive index which is measured by the detector in mv.

3.1.4 Polyurethane Film Preparation

Thin films of polyurethane were prepared by dissolving the polymer in dimethylformamide (DMF), with subsequent evaporation of the solvent in an oven. The rate of solvent evaporation was found to affect the quality of the films. Exceedingly rapid

evaporation tended to result in a nonuniform film with inconsistent thickness and the presence of pockets on the polymer surface. By reducing the evaporation temperature to 50°C and placing a cardboard cover over the petri dish, the solvent evaporation rate was much slower. This resulted in films with uniform thickness and a relatively smooth surface.

3.1.5 Gold Deposition on Polyurethane

Often, the deposition of a gold layer onto a smooth surface is preceded by the coating of the surface with a thin layer of chromium or titanium. This intermediate layer improves the adhesion of the gold to the surface (Sun, X., M. Eng. thesis, McMaster University, 1998). It has been found that using evaporative methods of coating a polyurethane surface with gold does not necessarily require the intermediate layer to improve adhesion (Sayer, M. - personal communication). It is likely that the method of preparation of the polymer film results in a surface that is sufficiently rough to allow adhesion of the gold layer itself.

It was noted throughout the course of experiments that there was some loss of gold coverage on the polyurethane surface. Some of this loss could be attributed to mechanical destruction of the gold layer caused by manipulation of the surfaces. This however, resulted in only minor losses. There were also losses of the gold layer noted after chemisorption of the amino acid and peptides to the surface. A similar observation has been noted by other researchers (Brash, J. - personal communication). While loss of

the gold layer was noted, it was generally estimated to be less than 5% of the total surface coverage.

3.1.6 Cleaning Gold Coated Polyurethane

Contaminants present on the gold surface were removed by heating it in an ammonia/peroxide solution. There was no observable loss of gold from the polymer caused by the cleaning process. While there was no loss of gold, there was a change in the gold layer, observable to the naked eye. After the cleaning process, the surface was covered in cracks. Swelling in this polymer, while not significant (between 2 and 5% at room temperature (Meeks, B., B. A. Sc. thesis, University of Ottawa, 1998)) is thought the be the cause of this cracking. It is likely that an increase in size of the polymer film, resulting from expansion or swelling, caused the separation of the gold layer.

3.2 Surface Characterization

The gold-coated polyurethane surfaces chemisorbed with cysteine and the cell adhesion peptides were characterized by several methods and compared to the clean gold surface. The surfaces were characterized using water contact angles, SEM, AFM and XPS.

3.2.1 Water Contact Angle Measurements

Sessile drop water contact angle measurements were performed on clean gold surfaces as well as cysteine and peptide chemisorbed surfaces. The results are depicted in Figure 16.

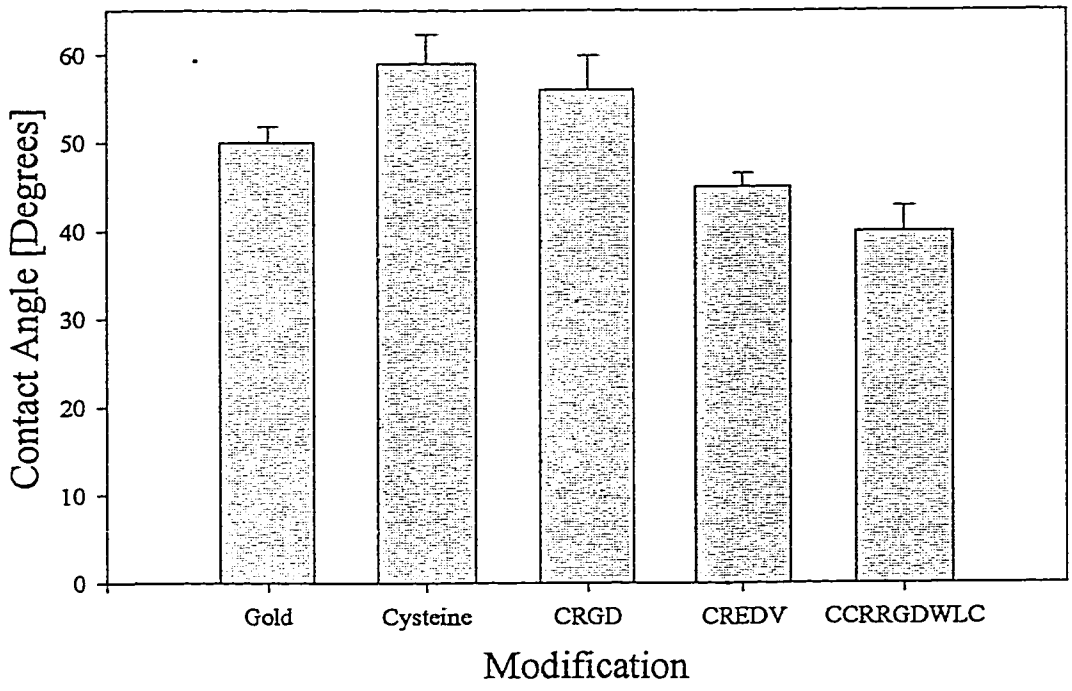

Figure 16: Contact angles for gold coated polyurethane and the peptide modified derivatives. The modified surfaces were incubated in 0.1 mM solutions of the chemisorption molecule for 25 hours at room temperature. The gold surface was incubated in distilled water. Error bars represent standard deviations.

The results show definite changes in water contact angle compared with the clean gold surface as expected. There is no obvious trend, with measured contact angles that are both greater and less than the control, depending on the chemisorption molecule. The

cysteine and CRGD modified surfaces displayed the highest water contact angles while the CREDV and CCRRGDWLC modified surfaces had lower contact angles than the clean gold surface. The differences in the contact angles are significant based on an analysis of variance ($\alpha<0.05$). While these differences are in no way indicative of the surface chemistry, they do demonstrate that changes to the surface properties of the gold coated polyurethane were made as a result of the chemisorption process and that these differences do depend on the peptide used for chemisorption. This suggests that the molecules are becoming fixed to the surface, thereby affecting the surface chemistry.

The contact angle of the clean gold surface is likely not indicative of the contact angle that would be observed with pure gold. As indicated by Bain *et al.*(1989), gold exposed to the atmosphere is immediately (times on the order of milliseconds) contaminated with organic molecules and it is this surface that is contacted by the water. Therefore, it is likely that there is significant contamination of the gold surface occurring even in the very short time between the surface cleaning procedure and immersion of the surfaces into the chemisorption solution.

3.2.2 Scanning Electron Microscopy (SEM)

SEM images of the gold surface after cleaning and after chemisorption with cysteine are shown in Figure 17. It should be noted that these images were taken using only the surfaces without any further evaporation of gold as is usually done for SEM.

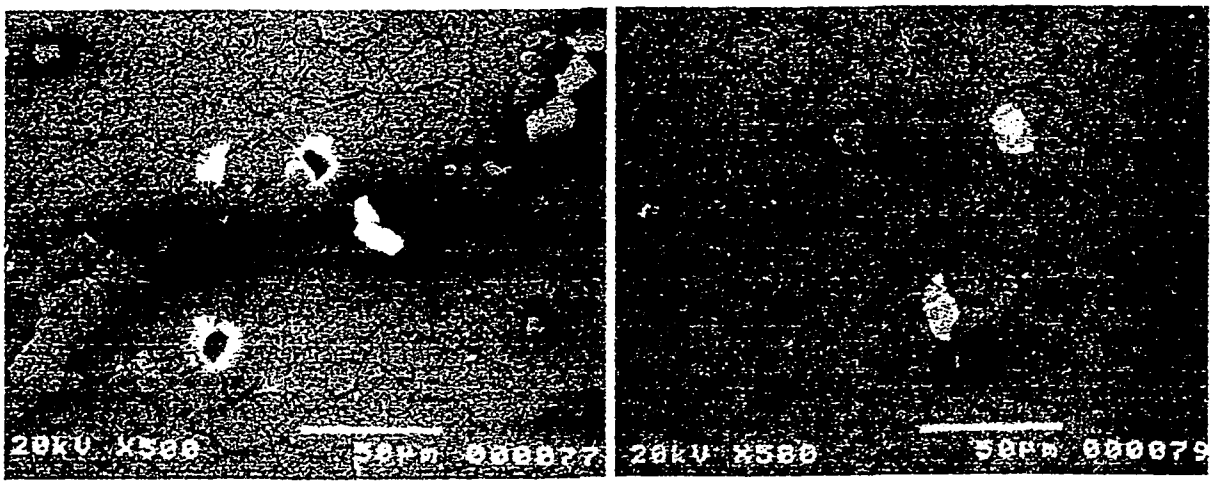

Figure 17: Scanning electron microscope images of clean (left) and cysteine chemisorbed (right) gold coated polyurethane surfaces. The cysteine modified surface was incubated for 25 hours at room temperature in a 0.1 mM cysteine solution. The clean gold surface was incubated in distilled water. Note the high degree of cracking evident on both surfaces.

The high degree of cracking of the gold layer is evident in both images. It can also be noted that several imperfections are present on the surface as indicated by the dark areas in both images. The SEM images reveal no observable difference between the two surfaces. It was not expected that differences would exist on the surfaces as a result of chemisorption since the peptides used in these studies had a size that was on the order of angstroms. Therefore, the peptides are of such a small size that their presence on the surface would not be seen with the SEM.

3.2.3 Atomic Force Microscopy (AFM)

AFM was used to study the surface morphology of the gold coated polyurethane and its peptide chemisorbed derivatives. An estimate of the surface roughness was also determined through the use of AFM. The AFM images are presented in Figures 18-21.

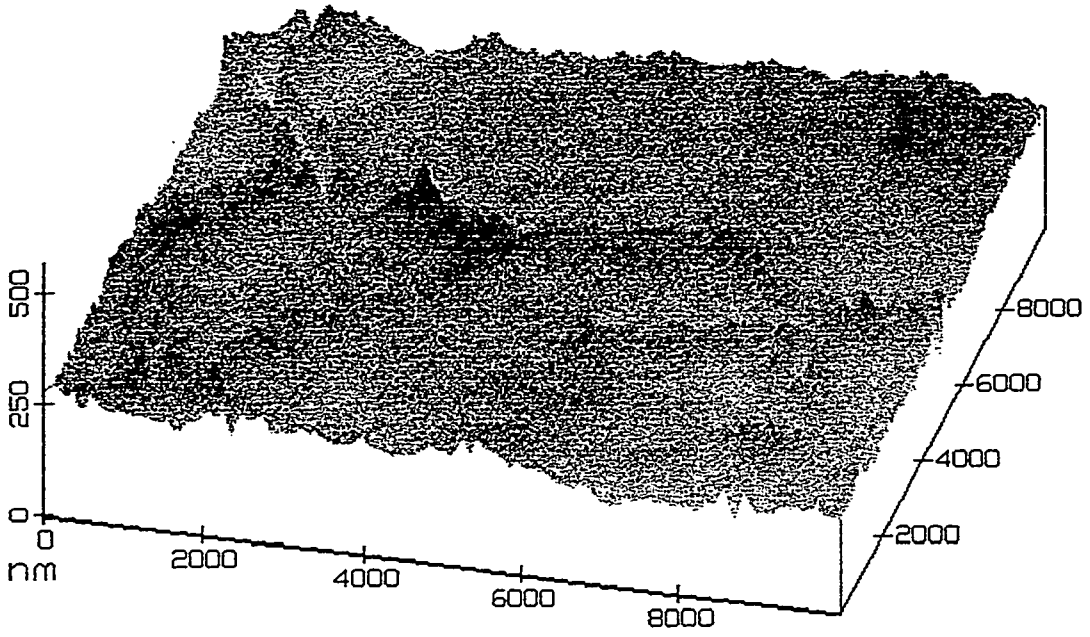

Figure 18: 100 μm^2 AFM image of gold coated polyurethane incubated in distilled water at room temperature for 25 hours. The X and Y axes represent distance in nanometers. The Z axis displays the elevation of the surface in nanometers. Note the presence of the sharp peaks and deep valley in the top left corner representative of a crack in the gold surface.

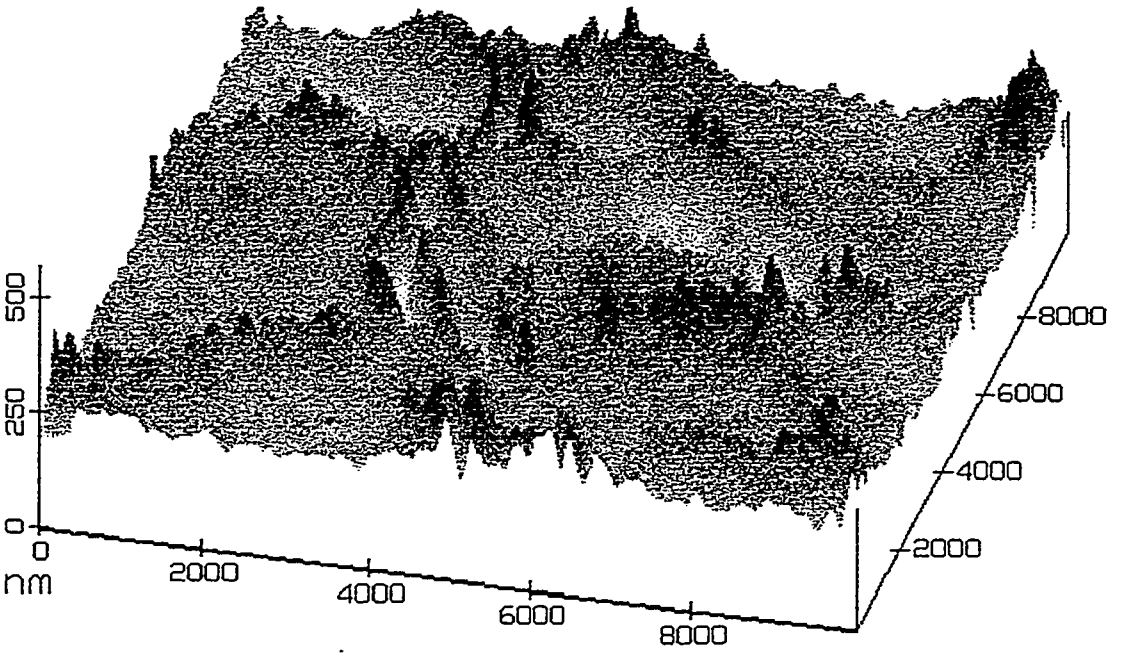

Figure 19: 100 μm^2 AFM image of cyclic cell adhesion peptide (CCRRGDWLC) modified gold coated polyurethane incubated in 0.1 mM cysteine at room temperature for 25 hours. The X and Y axes represent distance in nanometers. The Z axis displays the elevation of the surface in nanometers. Note the presence of the sharp peaks and deep valley in the center of the image representative of a crack in the gold surface.

The large area AFM scans (Figures 18 and 19) show deep valleys running across the surfaces in both images. The valleys represent the dramatic change in elevation of the surface where the cracks have opened up in the gold coating. The gold coating tends to rise significantly at the edge of the cracks. The valleys of the cracks and rising of the gold coating at the edges of the cracks would have an impact on the roughness of the surface. There are no discernible differences between the peptide chemisorbed surfaces and the

clean gold surfaces. Again this is not an unexpected result considering that the size of the peptides is much smaller than the size of the gold crystallites.

Much smaller scanning areas are depicted in Figures 20 and 21. These images give a much more detailed view of the topography of the surface.

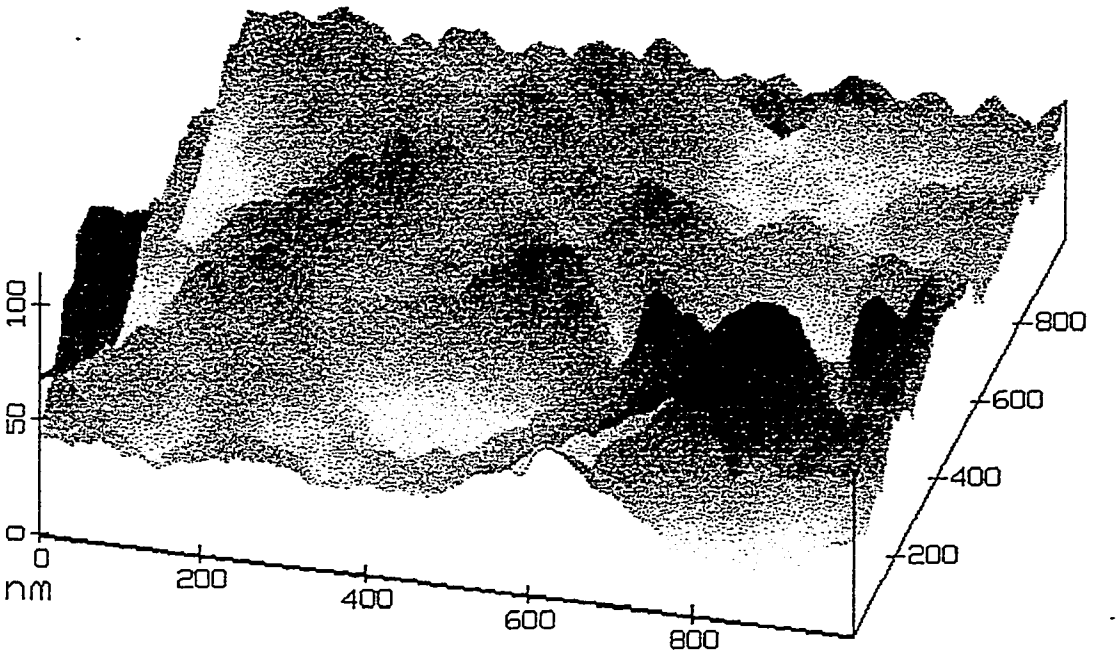

Figure 20: 1 μm^2 AFM image of gold coated polyurethane incubated in distilled water at room temperature for 25 hours. The X and Y axes represent distance in nanometers. The Z axis displays the elevation of the surface in nanometers. Note the spherical shaped entities that make up the surface. These are representative of the gold crystalloids that are evaporated onto the surface.

In the small area images, the gold crystalloids are clearly discernible as spherical shapes on the surface. The presence of cracks in the gold coating are also clearly presented. Chemisorption of cysteine and the peptides again can be seen to have no

obvious effect on the AFM images of the surface. The morphology was dominated by the deposition of the gold layer and its cracking.

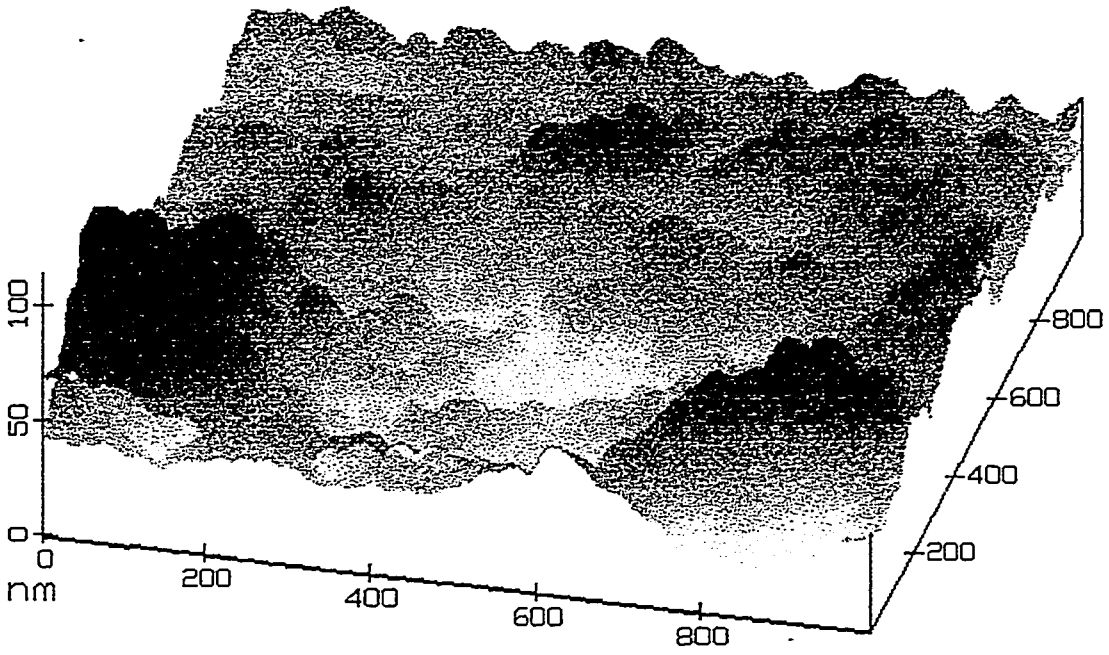

Figure 21: 1 μm^2 AFM image of cyclic cell adhesion peptide (CCRRGDWLC) modified gold coated polyurethane incubated in 0.1 mM cysteine at room temperature for 25 hours. The X and Y axes represent distance in nanometers. The Z axis displays the elevation of the surface in nanometers. Note the spherical shaped entities that make up the surface. These are representative of the gold crystalloids that are evaporated onto the surface.

The results of the roughness analysis are presented in Table 3. The roughness analysis was performed at two different scanning areas, 1 μm^2 and 100 μm^2. As can be seen from the table below, there does not seem to be a link between the surface modification and surface roughness, although the general trend is that the peptide modified

surfaces are much rougher than the unmodified surface. Furthermore, the chemisorption of the cyclic peptide on the surface resulted in a much smoother surface than did chemisorption of the other two peptides. This may be a function of the chemistry of the peptide or may suggest that self assembled monolayers are formed with this peptide, but do not occur with the others.

Table 3: Surface roughness of clean gold surface and the peptide chemisorbed derivatives. The roughness estimates of 100 μm^2 and 1 μm^2 areas are given.

Surface Modification	Roughness, 1 μm^2 [nm]	Roughness, 100 μm^2 [nm]
None	10.7	20.8
CRGD	18.0	93.7
CREDV	21.5	30.5
CCRRGDWLC	10.0	28.2

Again the presence of the peptides chemisorbed to the gold surface was not expected to have an effect on the surface morphology or roughness. The size of the peptides on the order of angstroms while the crystalloid sizes are on the order of approximately 50 nanometers. Hence the size difference in the peptides and gold crystalloids is approximately two orders of magnitude. Any effect on the surface morphology resulting from the chemisorption of the peptides to the gold is masked by the size discrepancy between the peptides and the crystalloids on the gold surface. This, combined with the even greater effects of the surface irregularities and cracking, make the effect of peptide chemisorption negligible on the surface morphology, and hence on roughness.

3.2.4 X-Ray Photoelectron Spectroscopy (XPS)

The presence of the chemisorbed molecules was confirmed through the use of XPS analysis. Both low and high resolution scans of the unmodified and modified gold coated polyurethane surfaces were performed. The surfaces were modified with the amino acid cysteine (refer to Figure 22 for the molecular structure of the molecule showing the presence of the thiol exploited in this work).

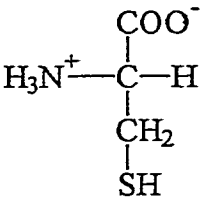

Figure 22: Molecular structure of cysteine. Note the presence of the amine (H_3N^+), carboxylic (COO^-) and the thiol groups (SH).

Low resolution scans of the clean gold surface and cysteine-chemisorbed surface are shown in Figures 23 and 24.

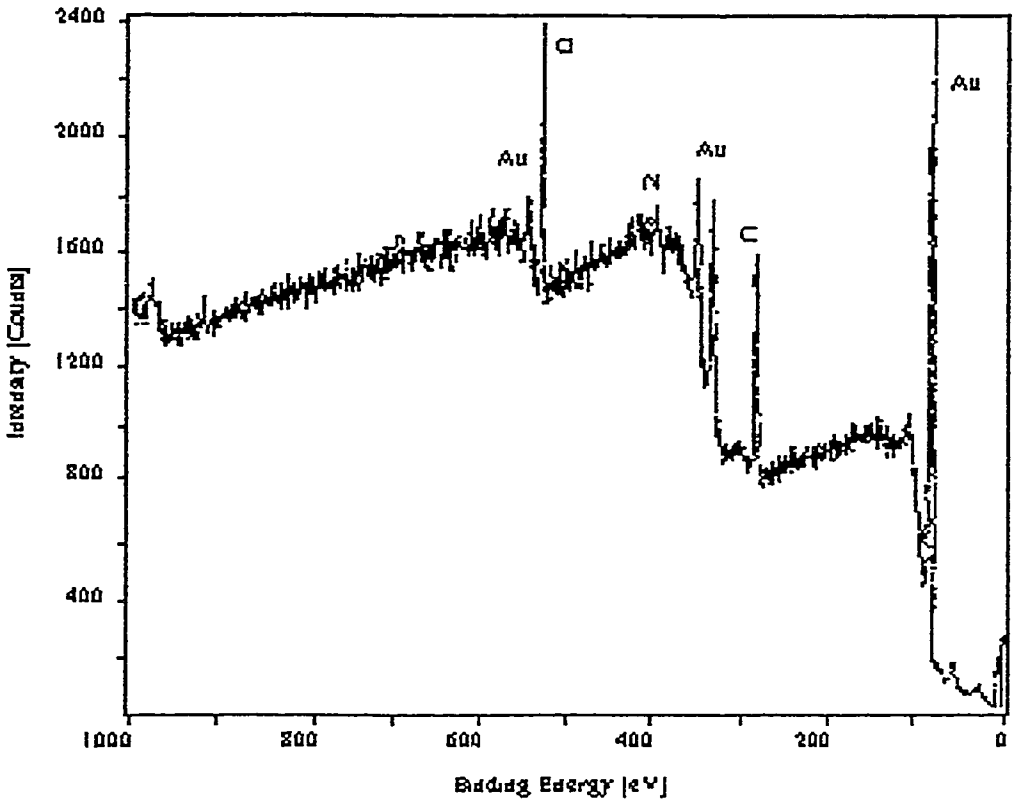

Figure 23: Low resolution XPS scan of gold coated polyurethane. The scan shows binding energies of emitted electrons representative of carbon (C), oxygen (O) nitrogen (N) and gold (Au) atoms on the surface. Contamination and cracking of the surface allow for C, N and O to be detected on the Au surface.

The low resolution scan of the clean gold surface, Figure 23, shows carbon (C), nitrogen (N) and oxygen (O) peaks. A perfectly clean surface devoid of any contaminating materials would ideally show only Au on the surface. This is not observed in this case. The surface composition on the clean gold was 58% carbon, 20% oxygen, 3% nitrogen and 19% gold. The fraction of gold on the surface of the polymer is quite low. Contamination of the gold surface with organic material from the atmosphere is likely responsible for some of the C and O found on the surface. The cracking and pitting of the gold coating on the surface is also likely making a significant contribution to the C, N and O peaks on the surface as well. The irregularities in the gold coverage would allow

some C, O and N to show through from the polymer and be detected by the XPS. A comparison of the carbon : oxygen : nitrogen ratio shows that while it differs somewhat from the theoretical ratio of the base polymer base polymer (77:3:20), the range of values is similar. Therefore, based on the observations and the chemistry, the polymer can be said to represent a significant fraction of the observed contamination.

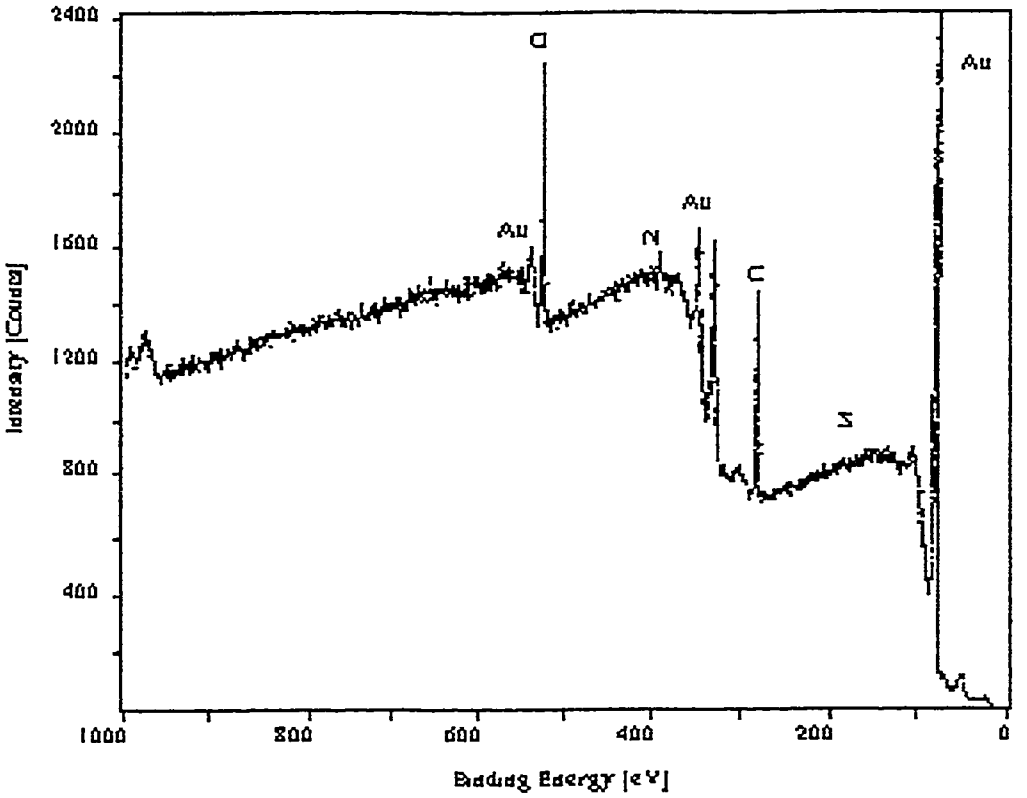

Figure 24: Low resolution XPS scan of cysteine modified gold coated polyurethane. The scan shows binding energies of emitted electrons representative of carbon (C), oxygen (O), nitrogen (N), sulphur (S) and gold (Au) atoms on the surface. S is representative of the presence of cysteine on the Au surface.

Investigation of Figure 24, showing the low resolution scan of the cysteine chemisorbed surface, shows some differences from the clean gold surface. Most notable in this scan is the presence of a small sulfur peak. While the amount of sulfur on the

surface is low, representing only 1% of the total atomic surface concentration, its presence does indicate chemisorption of the amino acid. This relatively low amount of cysteine and the significant C and O contamination of the surface tend to mask the effects on C and O detection of cysteine chemisorption on the gold surface under low resolution scans.

Variations in the time for chemisorption and the chemisorption concentration were performed in order to optimize the quantity of peptide on the gold surface. A summary of some of the low resolution XPS results including this data are shown in Table 4. Looking at the C, O, N and Au detected on the surface, it is difficult to see any consistent trend related to the effect of chemisorption time or concentration. It is possible that the differences in C, O, N and Au on the surface are a function of the surface cracking and contamination. However, there is a definite effect of time and concentration on the amount of sulfur on the surface.

The sulfur results for chemisorption time of 100 hours illustrates an effect of concentration on the chemisorption process. The higher concentration results in significantly more sulfur on the surface. Since the only source of sulfur is from the thiol moiety of the amino acid, the presence of sulfur is directly linked to that of the amino acid. The amount of cysteine on the surface, calculated based on the sulfur results is shown in the final column of Table 4. It should be noted however, that at high cysteine concentrations, the amount of delamination of the gold layer increased making it infeasible to increase the peptide concentration above a certain level.

Table 4: Summary of low resolution XPS results for clean gold surface and cysteine chemisorbed derivatives. Due to cracking and contamination of the surface, only sulphur indicates concentration and time effects on chemisorption process.

Chemisorption Cond.		Surface Chemistry					
Cysteine [mM]	Time [hrs]	C1s [%]	O1s [%]	N1s [%]	S2p [%]	Au4f [%]	Cysteine [nmol/cm^2]
0.05	100	63.6	23.7	3.1	0.7	3.9	0.012
0.50	100	66	21.1	3.6	1.2	3.1	0.020
0.05	25	63.7	23.4	4.0	0.5	6.5	0.008
0.50	25	63.6	22.7	2.7	1	10.0	0.017
0.50	5	63.3	26.0	3.9	0	6.4	0.000

The effect of chemisorption time can also be observed by comparing the results of the chemisorptions carried out with both the 0.05 and 0.50 mM solutions. After an incubation time of five hours at the higher concentration there is no detectable level of sulfur on the surface. However, as the time allowed for chemisorption increases the amount of sulfur detected on the surface increases. Delamination effects similar to those observed at high concentrations were also noted with long chemisorption times. It was noted that for high concentrations or incubation times, significant delamination of the gold surface was observed. This made the use of high concentrations and long incubation times unacceptable for use in the chemisorption process for these surfaces. It is possible that the high degree of cracking of the gold surface allowed the chemisorption molecules to infiltrate the area between the gold an polyurethane surfaces. Chemisorption of the molecules to the back of the gold film may have resulted in the delamination observed. Longer chemisorption times and higher concentrations are optimal but not feasible. The chemisorption conditions that were observed to give the best surface coverage with minimal losses of the gold layer were 0.1 mM for 25 hours. These conditions were used for all surfaces prepared for other experiments in this study.

The use of high resolution XPS scans allowed for better understanding of the changes to the chemistry of the surfaces taking place as a result of the chemisorption process. High resolution scans from the different elements on the different surfaces are depicted in Figures 25-28.

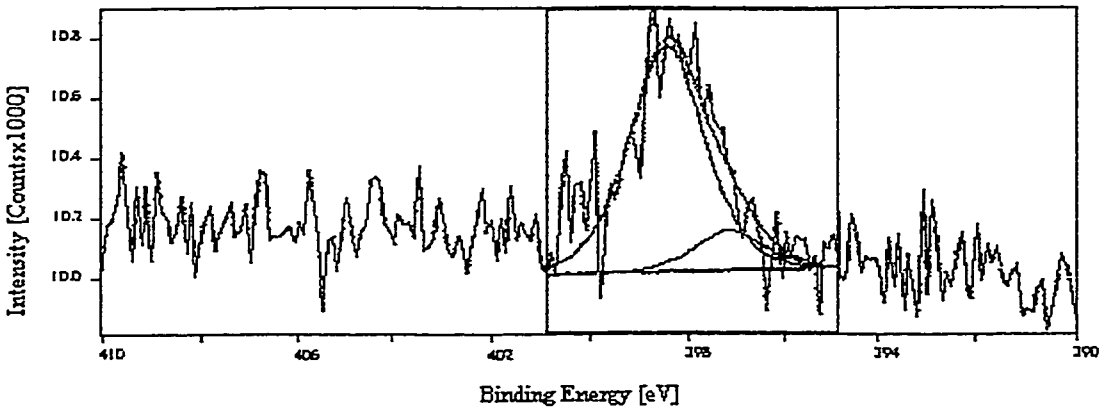

Figure 25: High resolution XPS scan of nitrogen on gold coated polyurethane. The scan shows that the nitrogen peak is composed of the atom in two different bonding conformations.

The high resolution scans showed definite changes in the surface chemistry of the gold coated polyurethanes resulting from incubation with the chemisorption molecules. The scan in Figure 25 shows the nitrogen 1s electrons on a clean gold surface. On this surface the N peak is composed of two peaks with binding energies of 400.4 eV and 399.1 eV which is consistent with the fact that there are two nitrogens present in the polyurethane structure. The nitrogen at 400.4 eV represents that present in the isocyanate and while that with a binding energy of 399.1 eV represents the nitrogen present in the ethylene diamine. The nitrogen present on the surface here is expected to be exclusively from the underlying polyurethane showing through cracks in the surface since nitrogen

compounds do not adsorb well to gold and are therefore not expected to contribute significantly to the N on the clean gold surface.

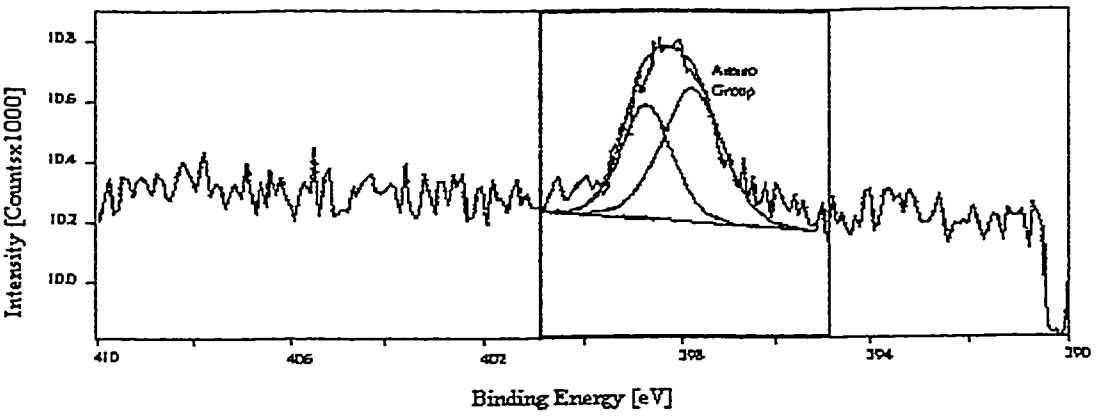

Figure 26: High resolution XPS scan of nitrogen on cysteine modified gold coated polyurethane. Gold coated polyurethane surface was incubated in 0.1 mM cysteine for 25 hours at room temperature. The scan shows that the nitrogen peak is composed of the atom in two different bonding conformations. The nitrogen peak has a much greater contribution from the peak at the lower binding energy. This peak is representative of an amino group which would indicate the presence of the amino acid on the surface.

After incubation of the surface in cysteine the contribution to the nitrogen peak is altered. This is evident in Figure 26. This scan shows that there is a much larger contribution to the N peak from the lower binding energy contributor, indicating the increased presence of an amine group which is indicative of cysteine chemisorption to the surface.

High resolution scans of the clean gold surface did not reveal any detectable level of sulfur on the surface as expected. A scan of the gold surfaces incubated with cysteine can be seen in Figure 27. As can be observed there is a slight but distinct peak corresponding to the 1s orbital of sulfur. This finding is also indicative of the presence of

cysteine on the surface with the presence of sulfur resulting from the thiol group on the amino acid.

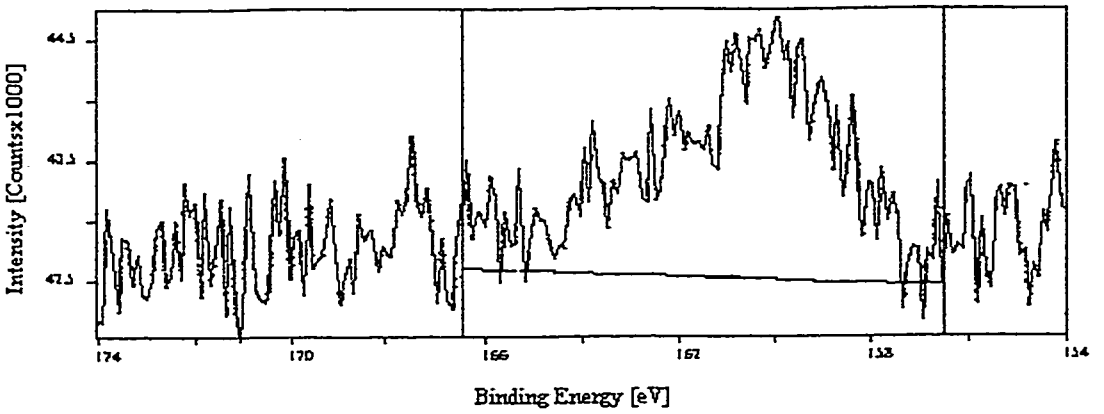

Figure 27: High resolution XPS scan of sulphur on cysteine modified gold coated polyurethane. Gold coated polyurethane surface was incubated in 0.1 mM cysteine for 25 hours at room temperature. The scan shows the presence of sulphur on the surface, indicating the presence of the amino acid.

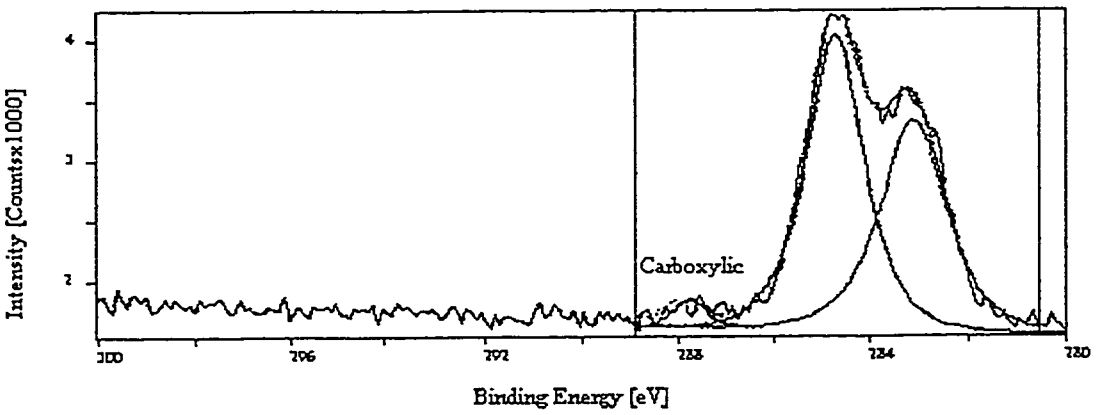

Figure 28: High resolution XPS scan of oxygen on cysteine modified gold coated polyurethane. Gold coated polyurethane surface was incubated in 0.1 mM cysteine for 25 hours at room temperature. The scan shows the presence of a carboxylic group on the surface, indicating the presence of the amino acid.

High resolution XPS scans of oxygen and carbon on the surface were also indicative of the presence of the amino acid after chemisorption. The XPS scan of the C1s orbital of a surface chemisorbed with cysteine is depicted in Figure 28. This figure indicates the presence of a small carboxylic peak. This peak should not be present as a result of other carbon contamination or the underlying polyurethane and is therefore further evidence of the presence of the amino acid on the surface.

3.3 Protein Adsorption

Protein adsorption to the artificial surface occurs virtually instantaneously upon blood-material contact. The adsorption of proteins onto the surface affects all subsequent cellular interactions with the surface. Therefore, these results could have a potentially significant impact on the cell adhesion and cell growth observed on the surfaces. The protein adsorptive properties of the peptide modified surfaces were compared to that of the clean gold surfaces. The adsorption of albumin from plasma was investigated. Albumin was selected as the model protein for these studies based on its relative abundance in plasma.

3.3.1 Protein Labeling

The adsorption experiments used in this study used radioactive iodine (^{125}I) labeled albumin. The protein was radiolabeled using Iodogen® and then dialyzed against phosphate buffered saline (PBS) to remove any unbound ^{125}I. It was necessary to remove

the free ^{125}I due to the high affinity of iodine for gold (Sheardown 1997). Excessive amounts of ^{125}I binding to the surfaces could result in erroneously high protein adsorption results.

After labeling, the albumin was mixed with bovine serum albumin (BSA) and precipitated with trichloroacetic acid (TCA). The proteins were separated from the supernatant via centrifugation and the amount of ^{125}I in the supernatant determined. The free ^{125}I in the albumin solution was calculated to be extremely low at 0.5% of the total radioactivity of the albumin solution.

3.3.2 Albumin Adsorption from Plasma

The adsorption of albumin from plasma at various dilutions was investigated to compare the adsorptive abilities of the clean gold surface to the peptide modified surfaces. The purpose of these experiments was to assess whether there were differences in the adsorption properties of the modified surfaces. The results of the adsorption experiments are shown in Figure 29.

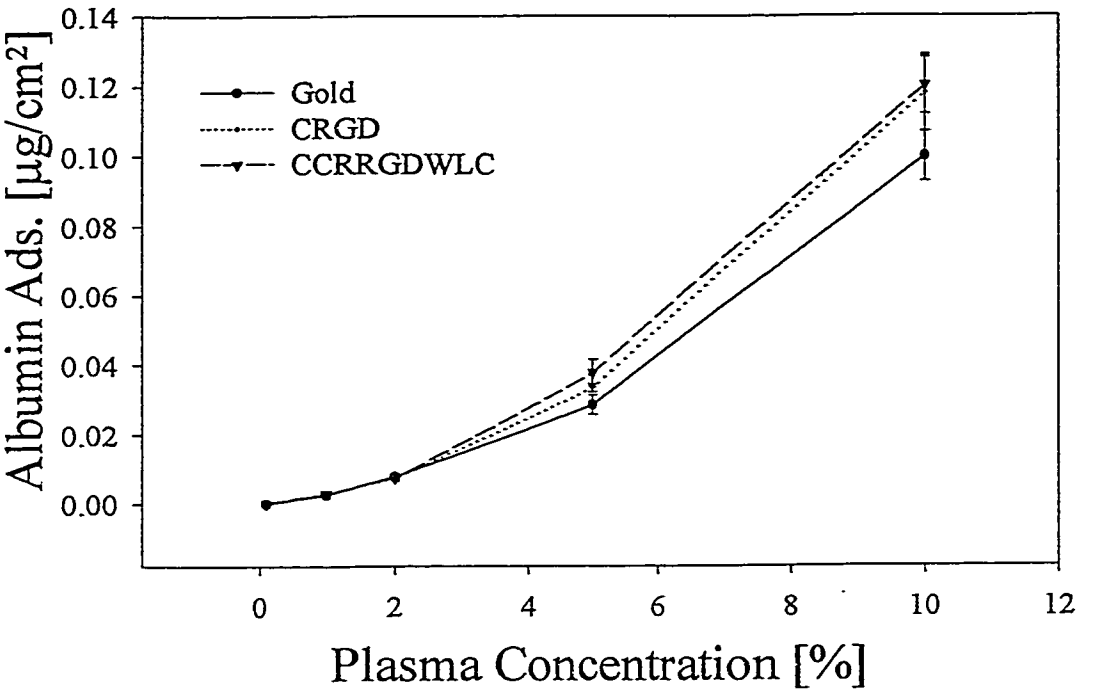

Figure 29: Albumin adsorption from plasma onto gold coated polyurethane and the peptide modified derivatives. The surfaces were incubated in human pooled plasma labeled with 125 I for a period of two hours at room temperature. Adsorption onto all surfaces increases with plasma concentration. Only significant differences occur at 10% plasma. Cysteine and CREDV modified surfaces were omitted to simplify the figure. However, similar results were obtained with these surfaces compared with the other peptide modified surfaces shown.

The figure shows, that in all cases, the peptide chemisorbed surfaces exhibit an increase in albumin binding affinity. The differences in albumin binding do not become significant until a plasma concentration of 10% is reached. The differences in protein adsorption at 10 % plasma are significant based on an analysis of variance ($\alpha<0.05$). This data indicates that the presence of the peptides on the surface is affecting the interaction between the surface and proteins in plasma. However differences between the different peptides were not apparent even at the highest plasma concentration. While differences

may be noted at plasma concentrations greater than 10%, these concentrations are generally not used in protein adsorption studies due to confounding effects that occur when high protein concentrations are present making it difficult to draw any conclusions about the adsorption process.

3.3.3 Polyacrylamide Gel Electrophoresis of Plasma Adsorbed Surfaces

The plasma protein adsorbed surfaces were washed with SDS to remove the adsorbed protein. The protein composition of the supernatants was then qualitatively determined by polyacrylamide gel electrophoresis. A typical SDS-PAGE gel of the proteins adsorbed from plasma onto a clean gold surface and the peptide modified surfaces is depicted in Figure 30. Duplicate runs are shown for comparison.

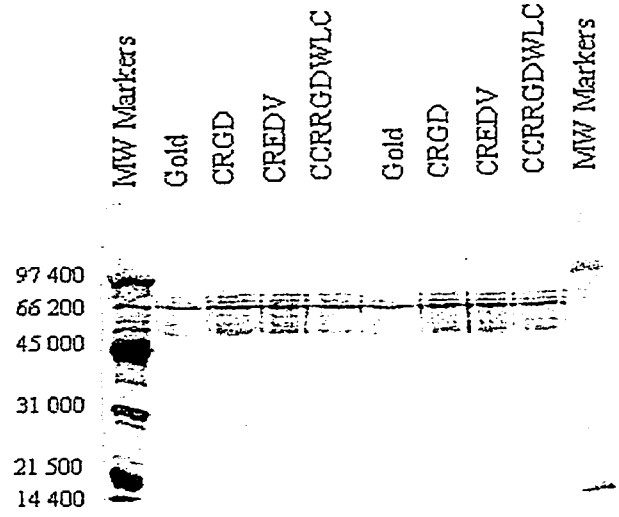

Figure 30: SDS-PAGE gel of proteins eluted off peptide modified gold coated polyurethanes. Gold coated polyurethane surface was incubated in 0.1 mM cysteine for 25 hours at room temperature. Duplicate runs are shown for

The two outside lanes show gels of molecular weight markers for determination of the molecular weights of the adsorbed proteins. The interior lanes, the "gold stained lanes" show the protein adsorption onto unmodified and peptide modified gold coated polyurethane. Each of these lanes shows a very strong band with a molecular weight of 66 200 which is representative of albumin. This band is repeated in all lanes for the peptide modified surfaces. All surfaces showed significant adsorption of albumin.

By investigating the intensity of each of the 66 200 bands the relative amount of albumin adsorbed to the surfaces can be determined. It can be noted that each of the peptide modified surfaces had an increased intensity for the albumin band. This is consistent with the radiolabeling finding that the modified surfaces had increased albumin binding.

All of the surfaces studied were capable of adsorbing proteins other than albumin as expected. This is demonstrated by the multitude of bands of differing molecular weights found in each lane. There were several differences between the gold and modified surfaces noted. Three proteins in particular, of molecular weight 42 000, 30 000 and 15 000, were found with varying intensity on the modified surfaces but not on the clean gold surface.

In general, the modified surfaces were found to act similarly in their ability to bind proteins from plasma. There were no significant differences observable from the electrophoresis results. All of the modified surfaces were capable of adsorbing more protein than the gold surface and in particular several proteins were found only on the modified surfaces.

3.4 Cell Adhesion and Growth

The main goal of this study was to create a surface which is amenable to the adhesion and growth of vascular endothelial cells. To this end, the effect of the peptide modified surfaces on the initial attachment of fibroblasts and endothelial cells was investigated. Once it can be established that the cells will attach to surface, future work will involve assessment of the various cell characteristics including expression of appropriate proteins. The cells, once attached, will create their own extracellular matrix and hopefully will proliferate to form a confluent monolayer on the surface of the graft. Two different cell types were investigated to ascertain whether differences in the surface peptide present resulted in enhanced binding of the cells and whether cell specific differences were encountered.

3.4.1 Mouse 3T3 Fibroblast Cells

The initial attachment of mouse 3T3 fibroblasts onto the polyurethane, gold and modified surfaces were investigated. Microscope photos of the surfaces after incubation with the cells are shown in Figures 31-36.

The base polyurethane, unmodified gold and cysteine modified gold surfaces served as controls. As can be seen in Figure 31, there are no cells present on the polyurethane. The polymer is apparently incapable of promoting the adhesion of the fibroblasts under the conditions studied.

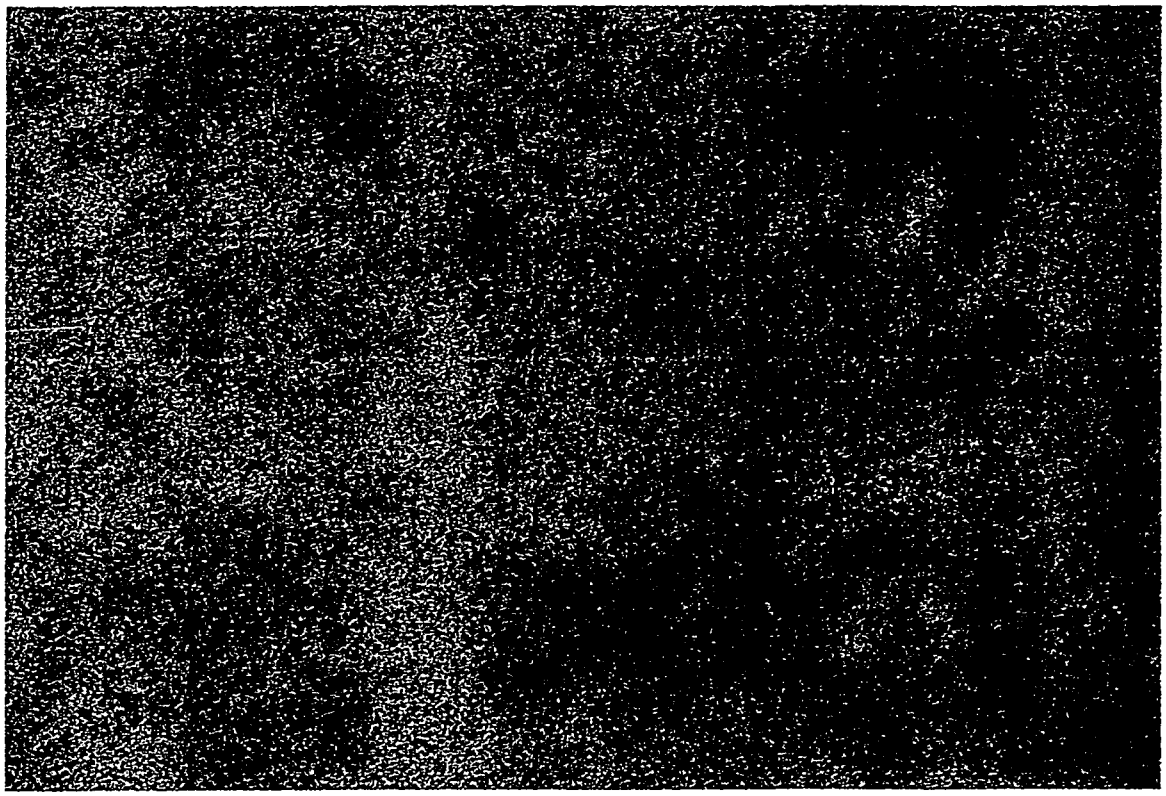

Figure 31: Photo of polyurethane surface incubated with mouse 3T3 fibroblasts for three days. There are no fibroblasts adherent to the polyurethane surface. Magnification 250X.

Figure 32 shows the adhesion of fibroblasts to the clean gold surface. In this image there is only a single cell present which is typical of the adhesion of the fibroblasts to the gold surface. There were very few cells found on the clean gold surface.

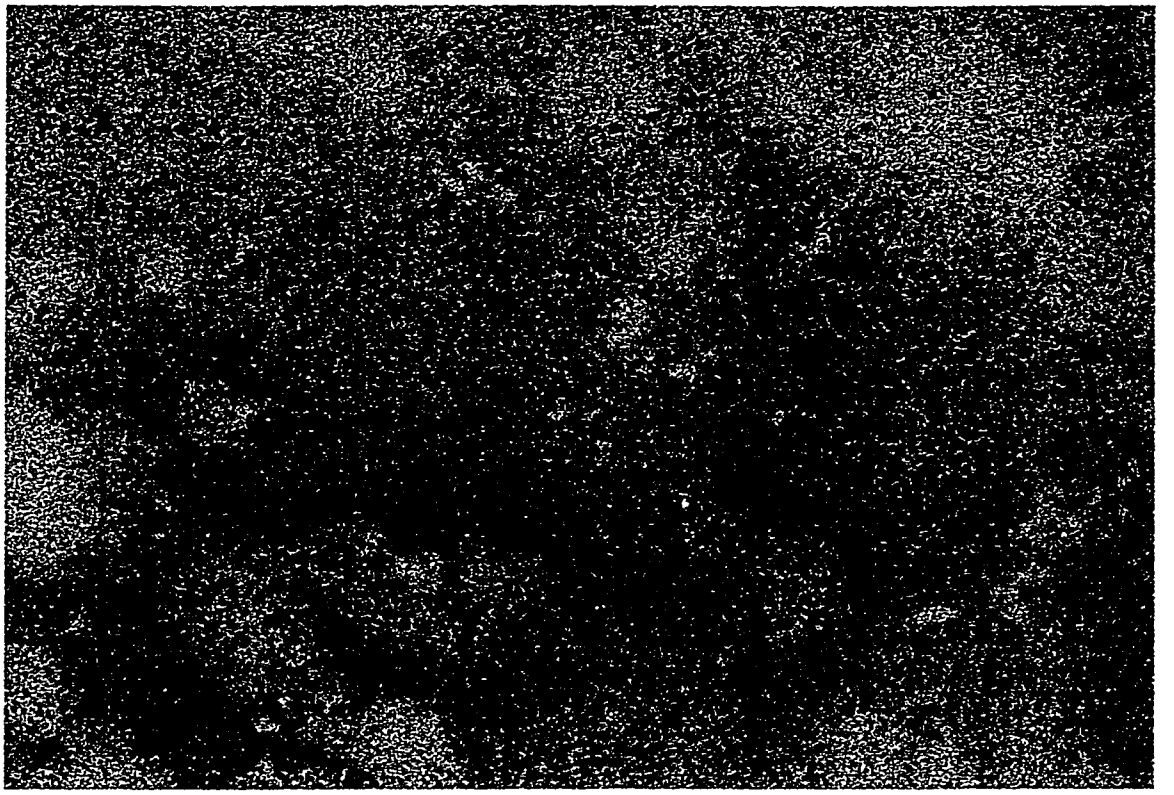

Figure 32: Photo of gold coated polyurethane surface incubated with mouse 3T3 fibroblasts for three days. There are no fibroblasts observed to be adherent to the modified surface. The cracking is evidenced by the lighter coloured lines. Some delamination of the gold is also evident by the lightly coloured areas. Magnification 250X.

A cysteine chemisorbed surface was also incubated with the fibroblasts. The cysteine chemisorbed surface did not show any improvement in cell adhesion as compared to the gold surface. This can be observed by referring to Figure 33.

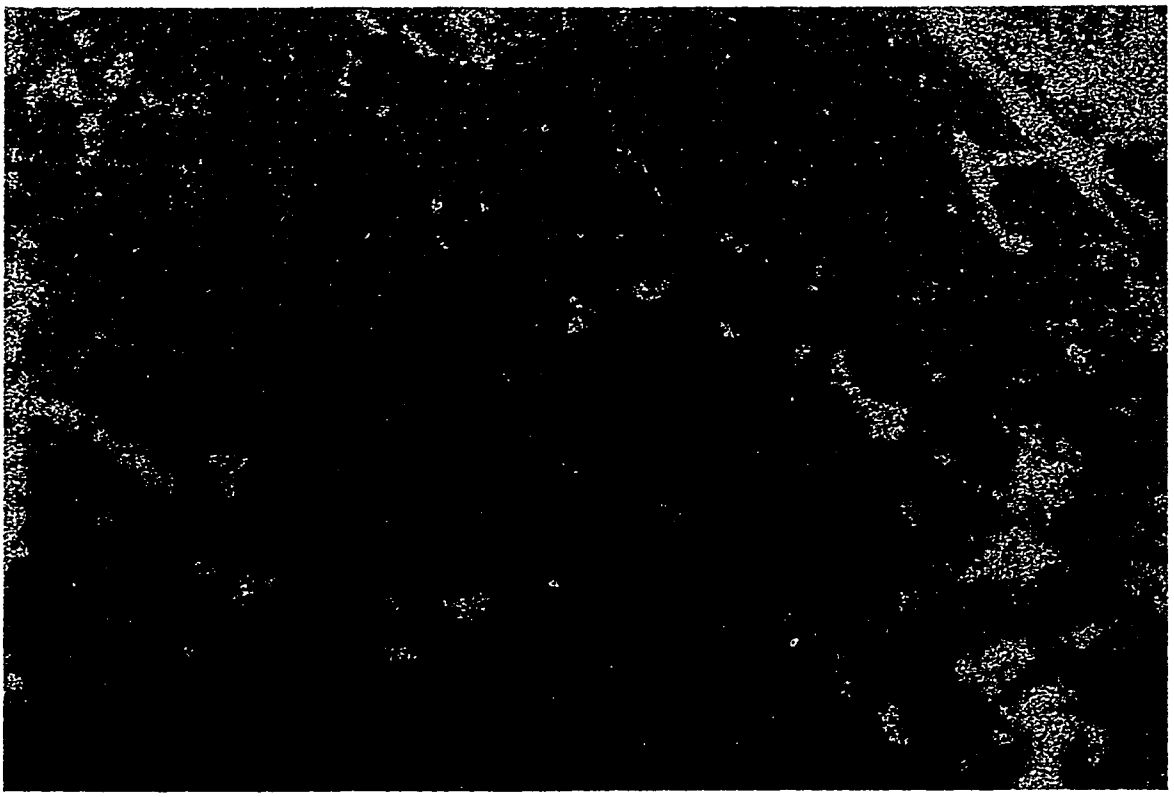

Figure 33: Photo of cysteine modified gold coated polyurethane surface incubated with mouse 3T3 fibroblasts for three days. There is a single fibroblast observed to be adherent to the modified surface in the upper right corner. The cracking is evidenced by the lighter coloured lines. Some delamination of the gold is also evident by the lightly coloured areas. The darkly stained areas are a Giemsa stain crystals. Magnification 250X.

The results of incubation of the peptide modified surfaces with fibroblasts are pictured in Figures 34-36. Each of these surfaces were capable of promoting the attachment of significantly more fibroblasts compared to the controls.

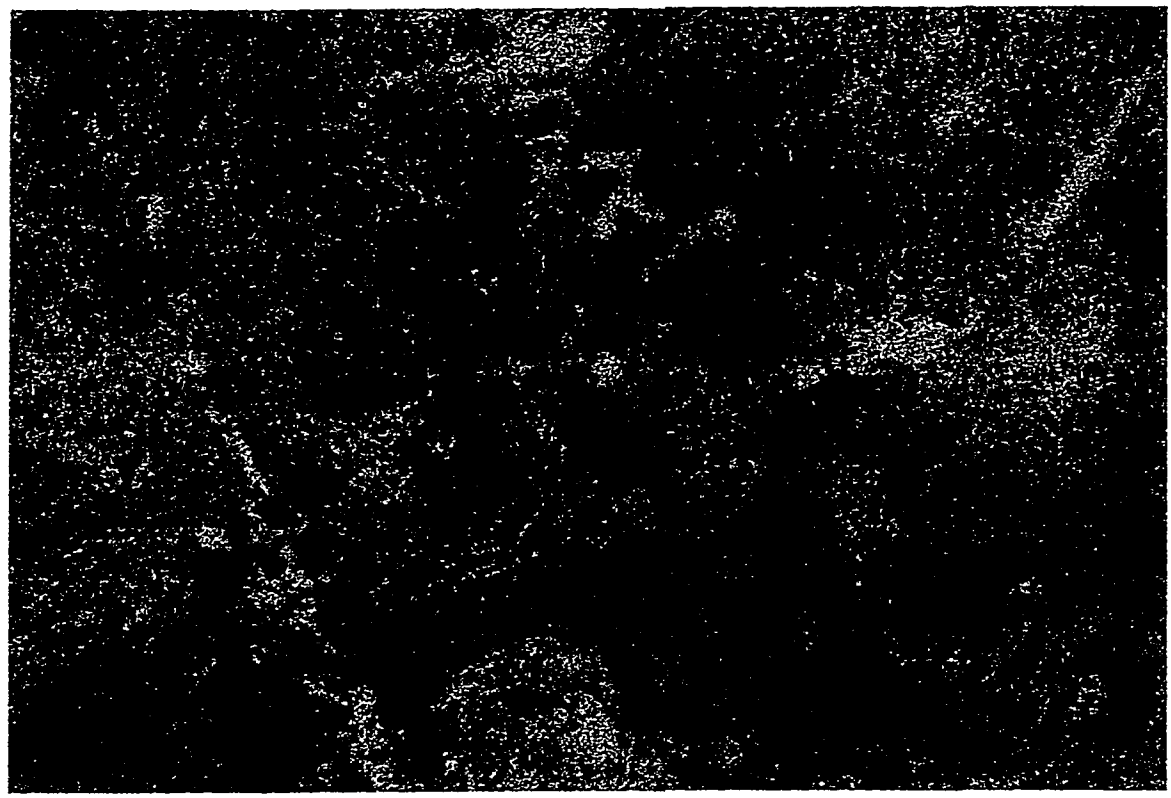

Figure 34: Photo of CRGD peptide modified gold coated polyurethane surface incubated with mouse 3T3 fibroblasts for three days. There are many fibroblasts observed to be adherent to the modified surface. The cells are well spread only over the areas where the gold surface is intact. Magnification 250X.

In particular, the greatest amount of adhesion was found to take place on the CREDV peptide chemisorbed surface. A summary of the adhesion results is depicted in Figure 37. A number of fields of view were selected and the cells contained within the field of view were counted to measure the ability of the surfaces to promote the initial adhesion of the cells. All measurements were taken at the same magnification.

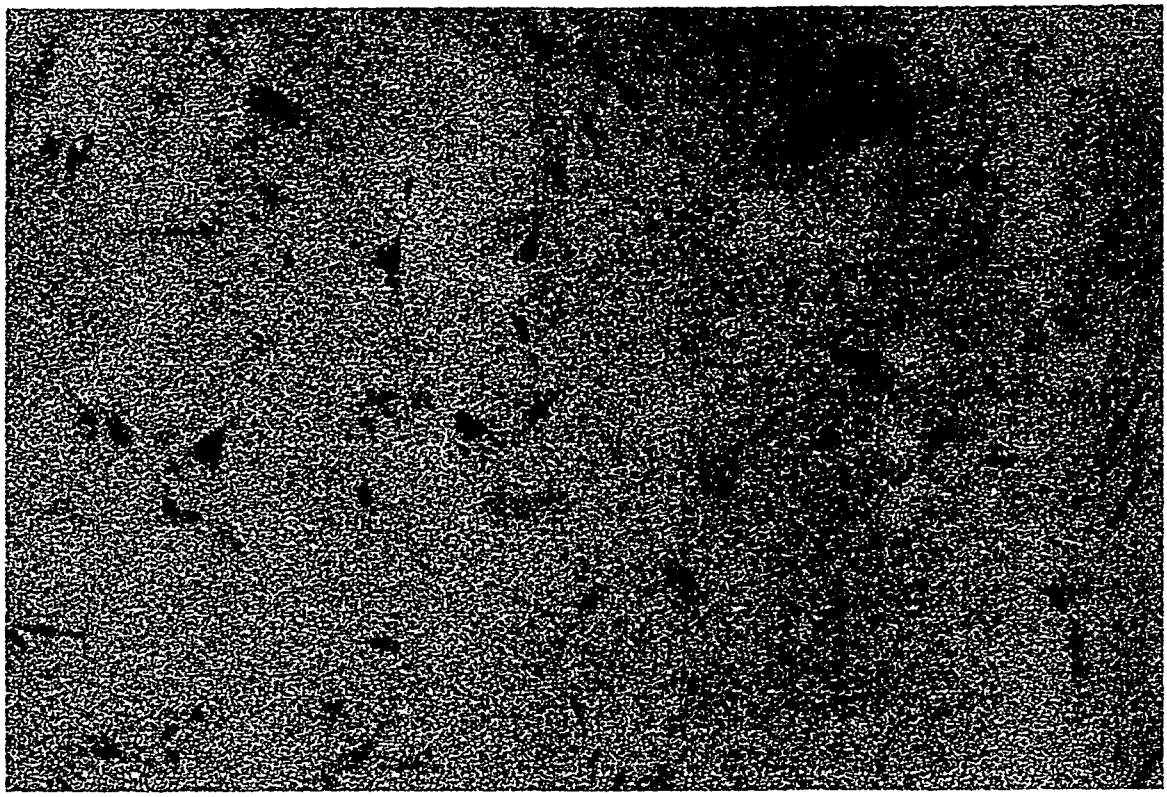

Figure 35: Photo of CREDV peptide modified gold coated polyurethane surface incubated with mouse 3T3 fibroblasts for three days. There are many fibroblasts observed to be adherent to the modified surface. The nuclei of the cells are stained darkly. Magnification 250X.

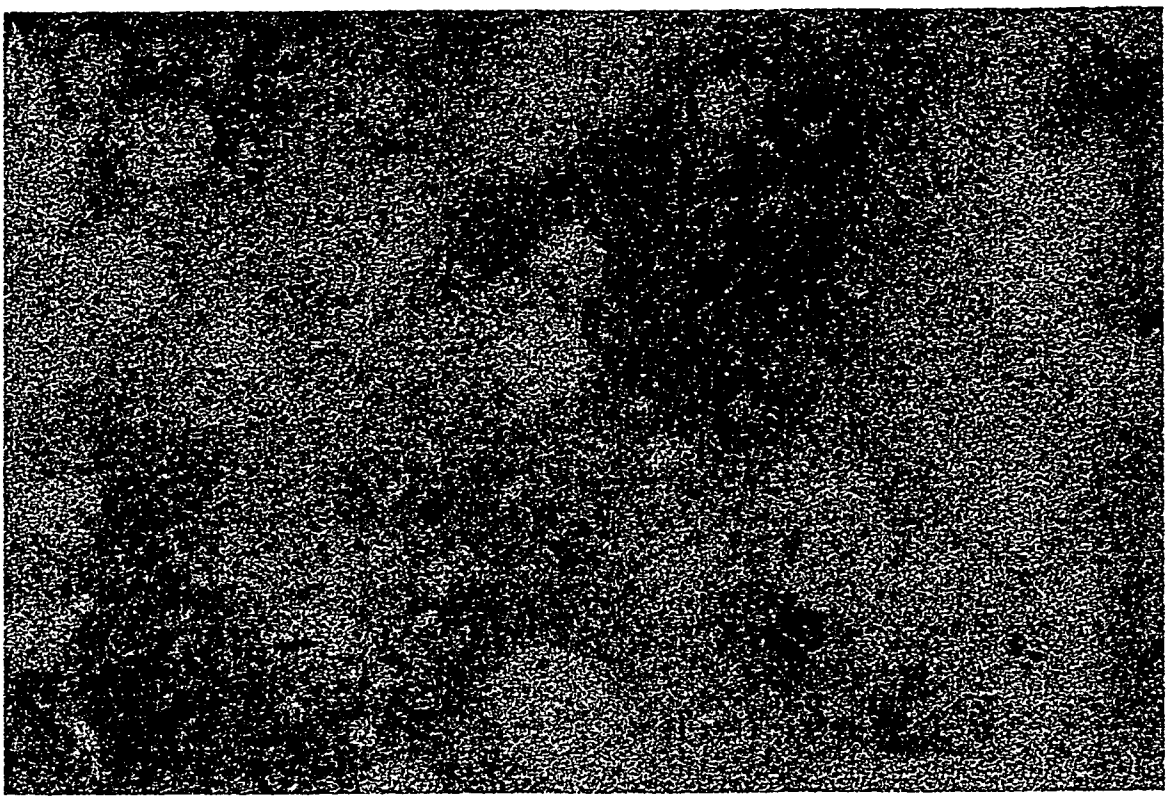

Figure 36: Photo of cyclic peptide modified gold coated polyurethane surface incubated with mouse 3T3 fibroblasts for three days. There are many fibroblasts observed to be adherent to the modified surface. The dark areas are Giemsa stain crystals. Magnification 250X.

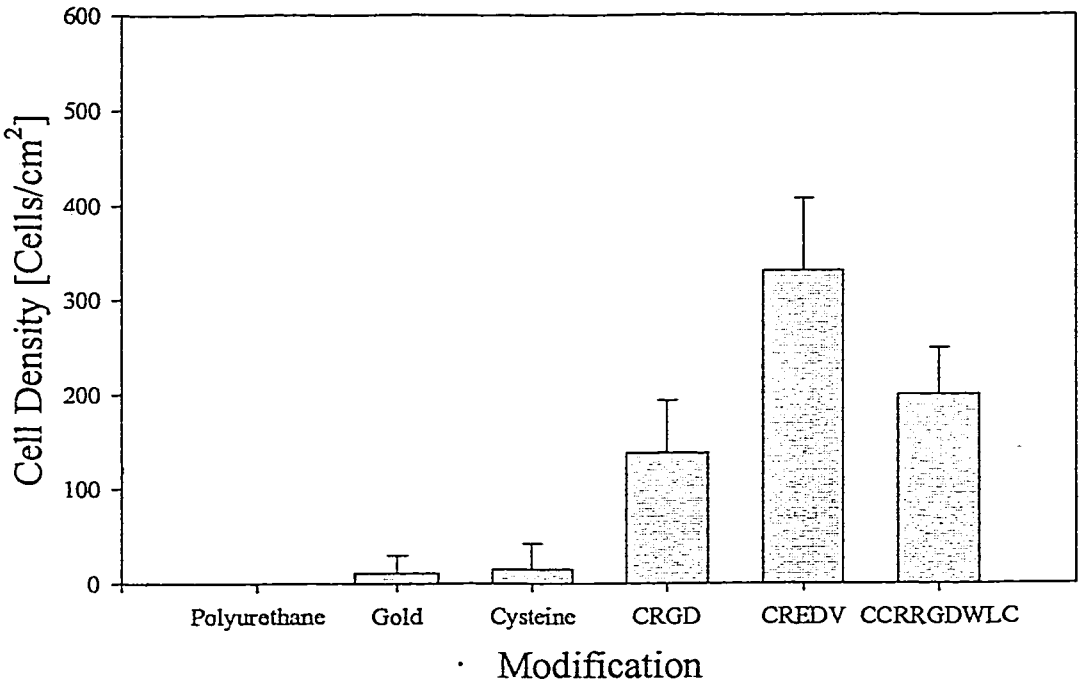

Figure 37: Adhesion densities of mouse 3T3 fibroblasts to peptide modified gold coated polyurethane. Surfaces were incubated for three days before adherent cells were fixed and counted. The polyurethane, gold and cysteine controls showed little adhesion. All peptide modified surfaces had significantly more adhesion.

Figure 37 shows that there was no detectable level of fibroblast adhesion on the polyurethane surface and a negligible amount on the gold and cysteine chemisorbed surfaces. All peptide modified surfaces were capable of greatly increasing the attachment of fibroblasts to the gold coated polyurethane. The differences in the cell adhesion are significant based on an analysis of variance ($\alpha<0.01$). The CREDV peptide chemisorbed surface was demonstrated to be the most proficient at encouraging the initial attachment of the fibroblasts.

It was not expected to see any significant growth on the polyurethane, unmodified gold or cysteine modified surfaces. These surfaces do not posses any integrin binding

sequences. Any adhesion to these surfaces was expected to arise from interaction of the cells with the adsorbed proteins from the culture medium. The peptide modified surfaces however, were expected to be capable of cellular adhesion due to the presence of the integrin binding peptides on the surface. This was observed for all three peptide modified surfaces tested.

3.4.2 Human Vascular Endothelial Cells

The effect of peptide modification of the gold coated polyurethane on the initial attachment of human vascular endothelial cells was also investigated. Figures 38-43 represent photographs of the various surfaces after incubation with the vascular endothelial cells.

As with the fibroblasts, there was no detectable adhesion seen on the polyurethane surface as shown in Figure 38.

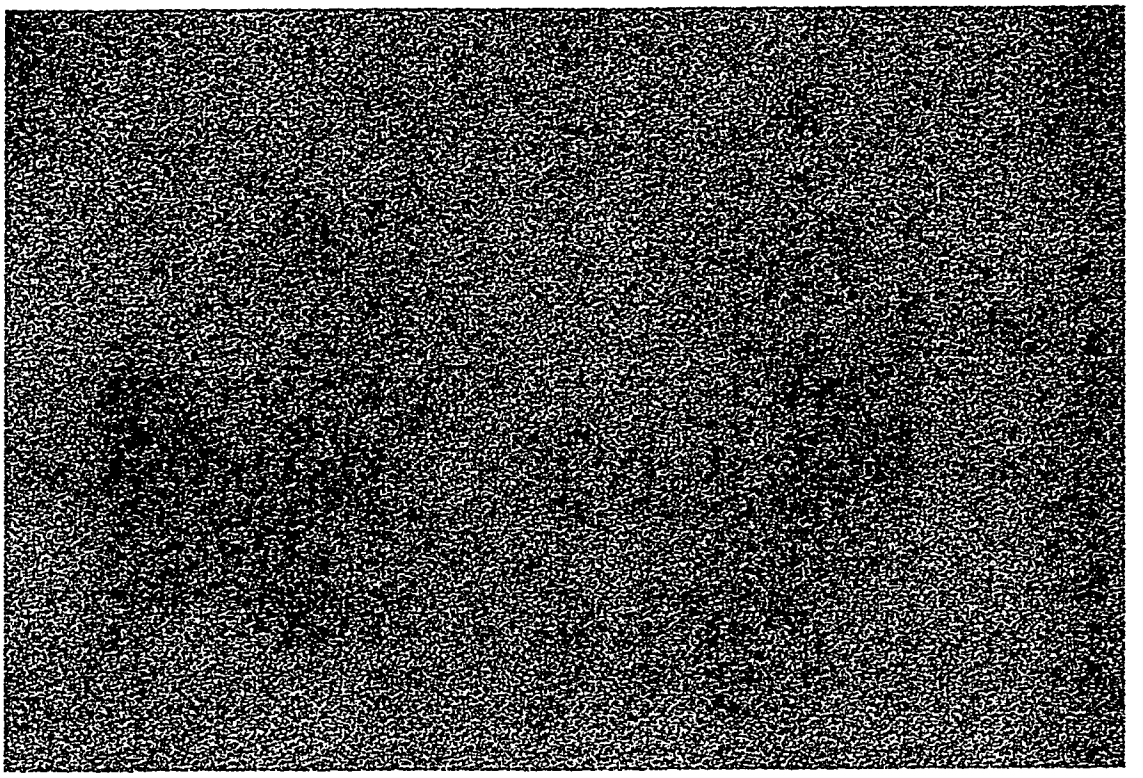

Figure 38: Photo of polyurethane surface incubated with human vascular endothelial cells for three days. There are no cells observed to be adherent to the modified surface. Magnification 250X.

Figure 39 depicts the adhesion of the endothelial cells to the clean gold surface. Surprisingly there were a significant number of cells adherent to the gold surface. This result was unexpected as the gold surfaces were not thought to support the adhesion of these cells.

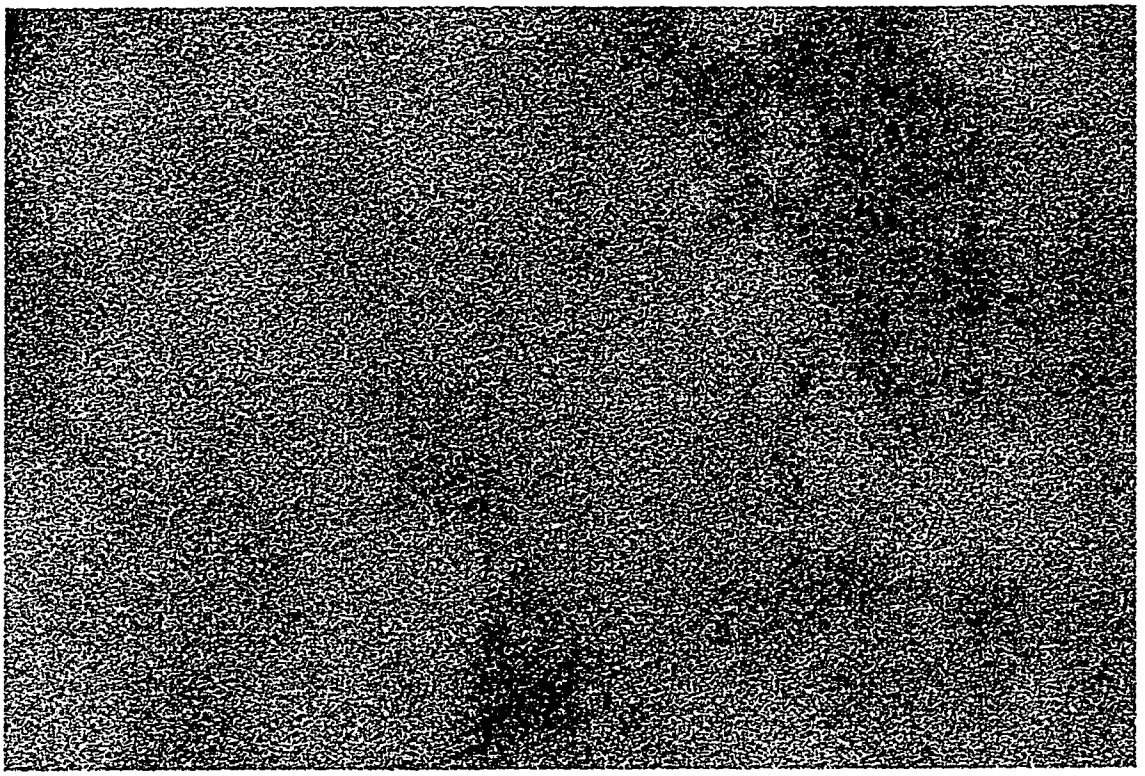

Figure 39: Photo of gold coated polyurethane surface incubated with human vascular endothelial cells for three days. There are several cells observed to be adherent to the modified surface. Magnification 250X.

There were also a large number of cells on the cysteine, CRGD and CREDV peptide chemisorbed surfaces. This is evident in Figures 40-42.

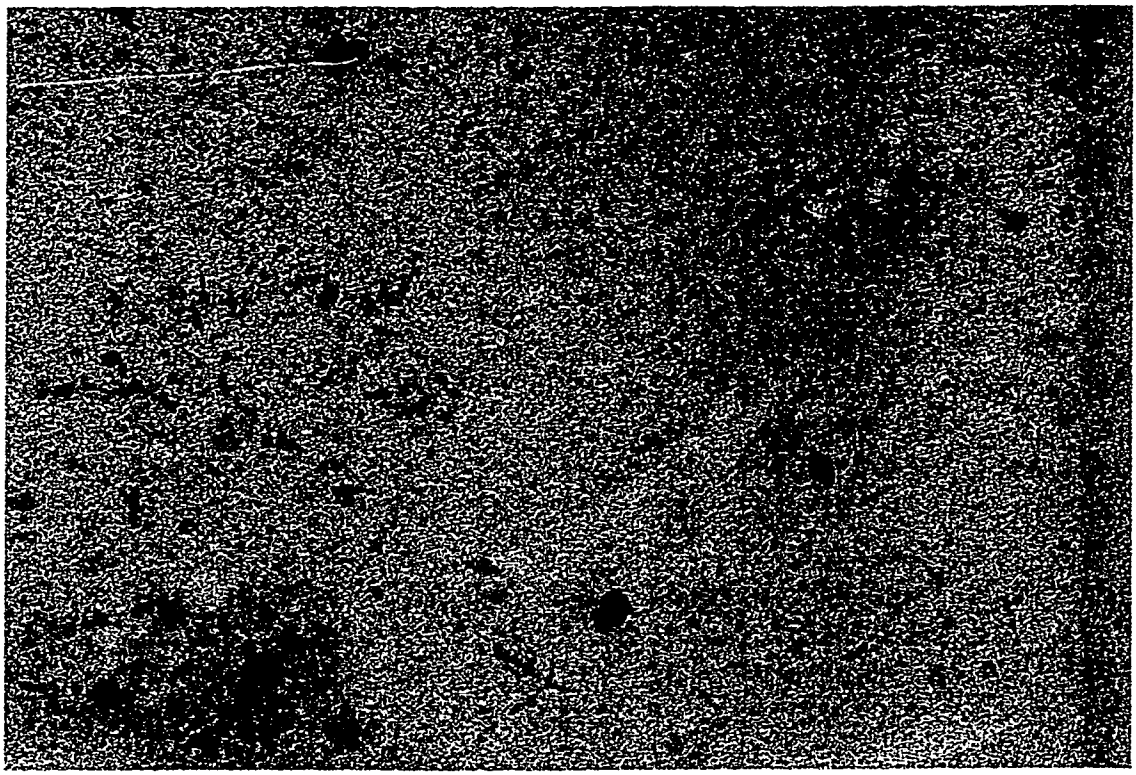

Figure 40: Photo of cysteine modified gold coated polyurethane surface incubated with human vascular endothelial cells for three days. There are several cells observed to be adherent to the modified surface. Magnification 250X.

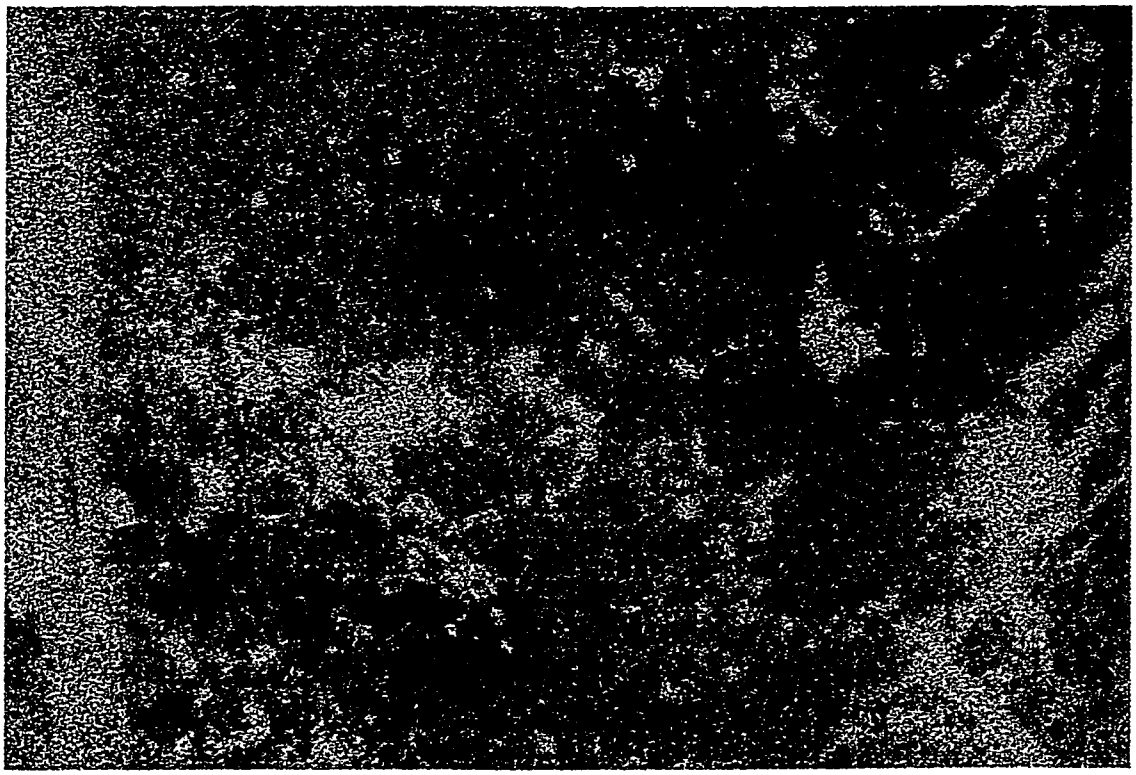

Figure 41: Photo of CRGD peptide modified gold coated polyurethane surface incubated with human vascular endothelial cells for three days. There are many cells observed to be adherent to the modified surface. The light coloured area represent delamination of the gold coating. Note the cells are only adherent to the portion of the surface covered with gold. Magnification 250X.

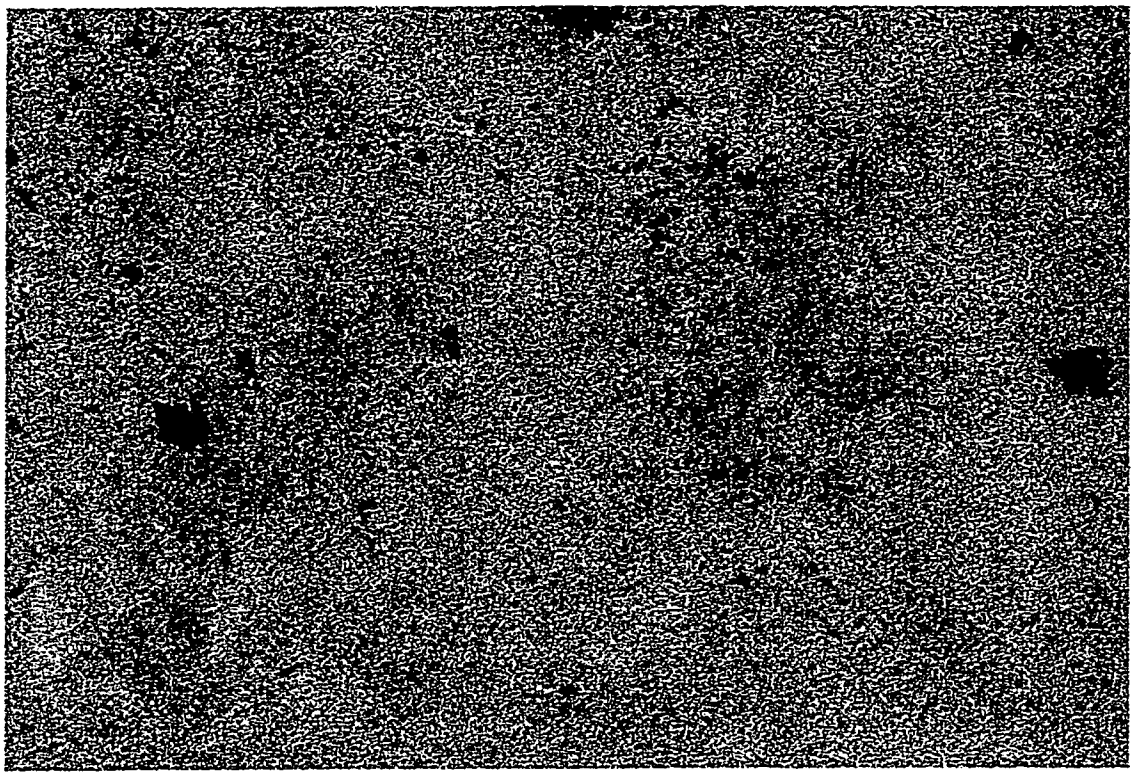

Figure 42: Photo of CREDV peptide modified gold coated polyurethane surface incubated with human vascular endothelial cells for three days. There are a number of cells observed to be adherent to the modified surface. The dark areas are Giemsa stain crystals. Magnification 250X.

Figure 43 shows the large number of endothelial cells which were present on the cyclic peptide chemisorbed surfaces.

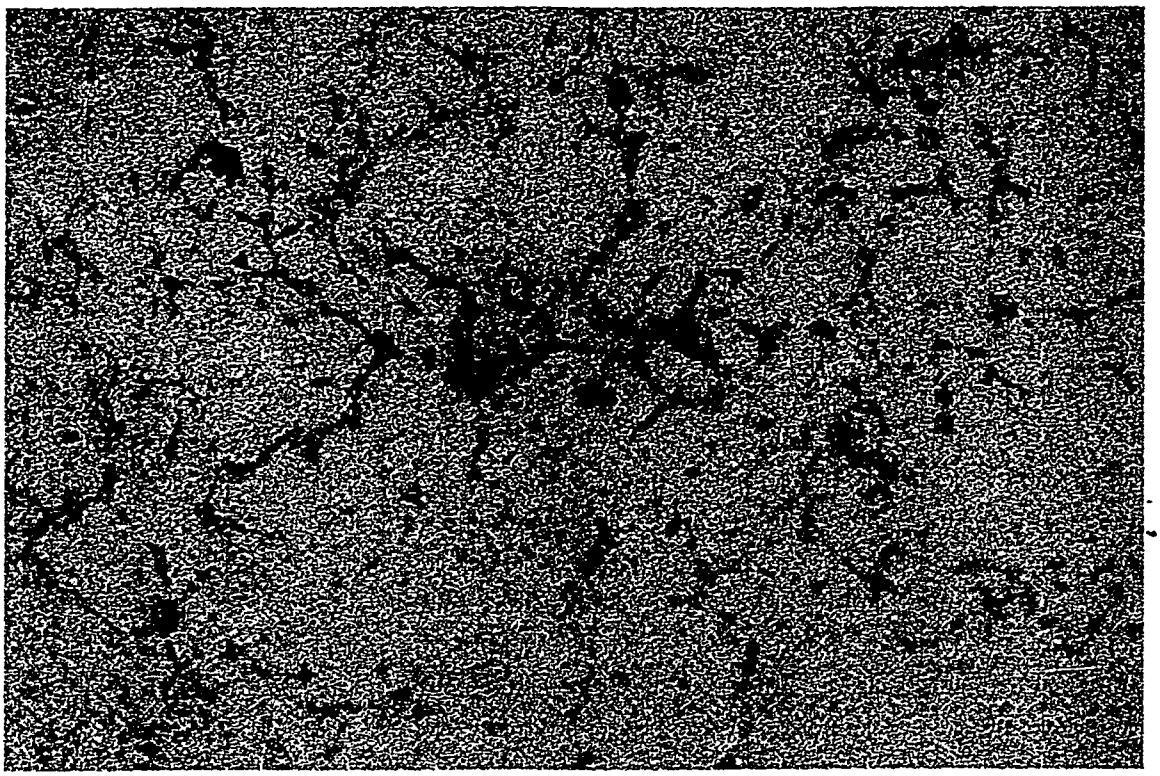

Figure 43: Photo of CCRRGDWLC (cyclic) peptide modified gold coated polyurethane surface incubated with human vascular endothelial cells for three days. There are a large number of cells observed to be adherent to the modified surface. The dark areas are Giemsa stain crystals. Magnification 250X.

The results for the initial endothelial cell adhesion to the various surfaces are summarized in Figure 44. The differences in the adhesion results are significant based on an analysis of variance ($\alpha<0.01$). In particular, differences between the CCRRGDWLC (cyclic) peptide and the other two peptides were noted. The adhesion of the cells to the clean gold surface was not expected.

To improve the growth of endothelial cells in culture, a collagen layer on the surface of the tissue culture dish is often used. Without this layer, the growth of endothelial cells in culture is difficult. Therefore plating these cells onto the gold surface was expected to result in little adhesion of the endothelial cells. This was not the case. It is possible that the proteins adsorbed onto the surface from the culture medium were

responsible for the adhesion of the cells to the gold. However, based on the protein adsorption results which demonstrated that the adsorption of proteins from plasma occurred to a lesser degree on the unmodified surfaces, this result would not have been predicted. It is possible that collagens are preferentially adsorbed from the tissue culture medium and that this was the reason for the initial adhesion noted. Western blots of the surfaces probing the supernatants for collagens may provide an explanation for the results noted.

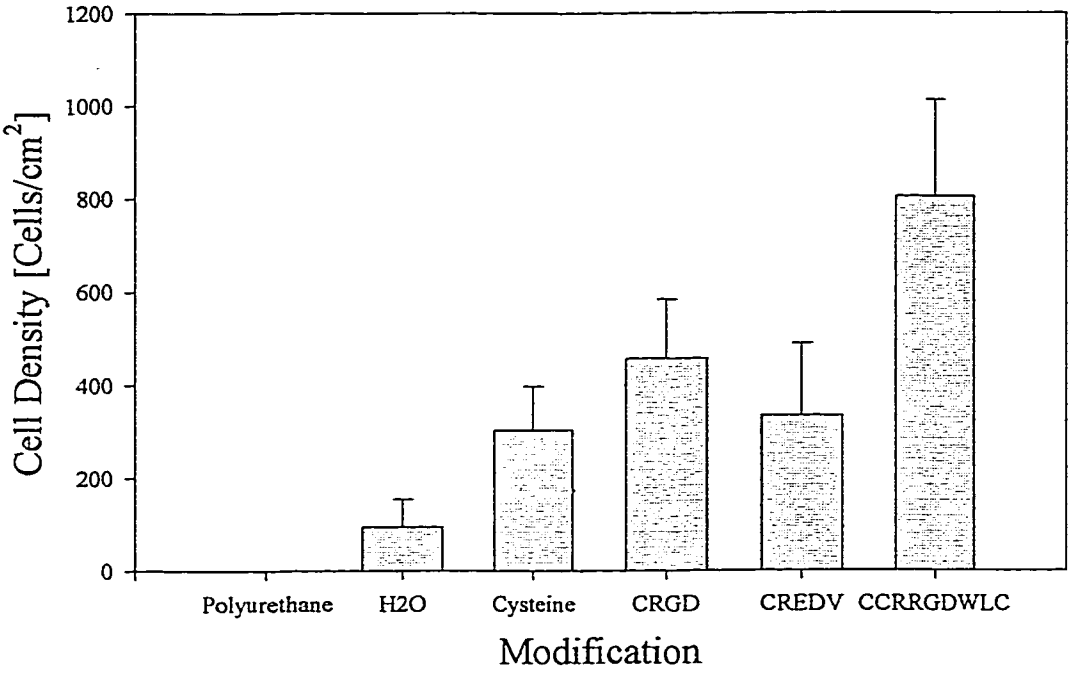

Figure 44: Adhesion densities of human vascular endothelial cells to peptide modified gold coated polyurethane. Surfaces were incubated for three days before adherent cells were fixed and counted. The showed no detectable adhesion. All other surfaces demonstrated some degree of adhesion. The greatest number of adherent cells were found on the CCRRGDWLC (cyclic) peptide modified surface.

The cysteine modified control surface was capable of supporting many more attached cells than the clean gold surface. Again, adhesion to this surface was not expected to be significant as the cell surface integrins were not expected to interact with the cysteine. However, the presence of the amino acid was shown to increase the binding of proteins from plasma. As with the gold surface, the adsorption of proteins, specifically cell adhesion proteins, onto the surface may have contributed to the adhesion of the endothelial cells. Cysteine was shown to cause greater adsorption of plasma proteins. Therefore, if a greater amount of the proteins responsible for the adhesion of the cells was present on the cysteine modified surface it could be expected that the ability of this surface to promote cell adhesion would be greater than the clean gold surface. Further analysis using Western blots is warranted.

The CRGD and CREDV peptide modified surfaces have approximately the same effect on cell adhesion as the cysteine modified surface. These surfaces were shown to adsorb approximately the same amount of protein from plasma. It is possible that the effects of these peptides are due in part to their ability to enhance protein adsorption. However, the peptide sequences were designed specifically to interact with the integrins on the cell surface and it is therefore expected that this also contributes to the effects noted.

The cyclic peptide modification was shown to promote the adhesion of the greatest number of cells. This surface was capable of promoting approximately 40% more endothelial cells as compared to the next best surface, the CRGD peptide modified

surface. The protein adsorption experiments demonstrated that all the peptide modified surfaces adsorbed similar amounts of proteins. Therefore it is expected that the difference between the cyclic peptide and the other peptides in promoting adhesion results primarily from the presence of the peptide. The cyclic peptide was designed to produce the maximum response from the endothelial cells and this is what was observed. Further studies of the effects of the different peptides and surface modification on the adsorption of different proteins from plasma may provide a more conclusive answer to the causes for the effects noted in this work.

Chapter 4: Conclusions and Recommendations

4.1 Peptide Chemisorption

Three different cell adhesion peptides were designed and synthesized for determination of their effect on fibroblast and endothelial cell adhesion. These peptides were chemisorbed to a gold coated polyurethane substrate. The presence of theses peptides on the surface was confirmed through XPS analysis. Peptide surface concentrations were on the order of 0.01 nmol/cm^2 which was consistent with the literature. Chemisorption of the peptides from solution onto the gold surfaces was shown to have definite time and concentration effects. However, high peptide concentrations and long chemisorption, which were shown to result in the greatest surface concentrations of the peptide, also resulted in the significant delamination of the gold from the surface, making the use of these concentrations and times infeasible in this work.

4.2 Surface Characterization

The modified surfaces used in this study were characterized using a variety of surface analysis techniques. Water contact angles showed significant changes in the

hydrophilicity of the surfaces following chemisorption. XPS analysis revealed the presence of elemental sulfur, amino and carboxylic groups on the surface after chemisorption indicating the presence of cysteine.

The surface morphology of the modified surfaces was not different when the peptide chemisorbed and the clean gold surfaces were compared. This is not unexpected given that the peptide size is much smaller than that of the gold crystalloids deposited on the surface during the evaporation process. Scanning electron microscopy and atomic force microscopy demonstrated the presence of microcracks in the gold coating. There was an observable difference in the surfaces following cleaning suggesting that this morphology change may have been brought about by the swelling of the polymer during the actual cleaning process.

4.3 Protein Adsorption

The interaction of the surfaces with plasma proteins was investigated. It was found that all modified surfaces had increased ability to adsorb albumin and other proteins from plasma. Also, several proteins were found to adsorb only to the modified gold surfaces.

4.4 Cell Adhesion

The ability of the synthetic cell adhesion peptides to affect the adhesion of fibroblasts and vascular endothelial cells was studied. All of the peptides were found to

greatly enhance the adhesion of fibroblasts to the gold surfaces. A similar result was also found for the endothelial cells. While a clear effect of the CRGD and CREDV peptide modified surfaces compared to the controls was not evident however, the cyclic peptide did show a dramatic effect on adhesion to the surface. This result was as expected based on the design of the peptides.

The results of the experiments performed in this work have shown that synthetic cell adhesion peptides are capable of increasing initial cell attachment to artificial surfaces. Furthermore, the results demonstrate the modification of gold coated polymers represents a significant tool for the evaluation of various surface groups. While this finding in itself could be very useful in any work concerned with cell adhesion, it may be of particular importance for the artificial vascular graft.

4.5 Recommendations for Future Study

Several recommendations for future studies have been brought about by the results of this work. While the results clearly demonstrate that the initial adhesion of the cells to the surfaces is enhanced by chemisorption of the peptides to the surface, it would be important to determine whether a confluent monolayer of vascular endothelial cells could be grown on an artificial surface for a prolonged period of time. If a monolayer of cells could be grown, the resistance of the cells to detachment due to the shear forces of the blood flowing through the vascular graft would need to be investigated. As well, the ability of the cells to maintain their native physiology under such conditions should also be investigated.

It was shown that there is clearly a chemisorption time and solution concentration effect on the quantity of peptide that was present on the modified surfaces. However, these surfaces were not optimized in any way in order to promote the attachment of the cells. It would therefore be very worthwhile to determine the effects of different peptide surface concentrations on the initial attachment of the cells as well as on their long term growth and physiology. In this way, the surfaces could be tailored to optimize the potential for attachment and growth.

The effects of time and concentration were evaluated. However, additional factors may also contribute to the chemisorption process. It would therefore be very valuable to assess the effects of these other factors on the chemisorption process.

Finally, the blood compatibility of the adherent vascular endothelial cells would need to be studied. While the vascular endothelial cells are non-thrombogenic in their natural environment, it is possible that they could lose this characteristic when adhering to an artificial surface.

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Appendix A: Reagents, Solvents and Materials

Reagent	Acronym	Supplier	Location	
1,3,4,6-tetrachloro-3a,6a-diphenylcoluril	Iodogen®	Pierce Chemical Company	Rockford	IL
2-βmercaptoethanol		Sigma Chemical Company	St. Louis	MO
Amonium hydroxide		Fisher Scientific Ltd.	Ottawa	ON
Ammonium Persulfate	AP	Sigma Chemical Company	St. Louis	MO
BIOCOAT endothelial cell growth env.		Becton Dickinson Labware	Bedford	MA
Bovine serum albumin	BSA	Sigma Chemical Company	St. Louis	MO
Bromophenol Blue		Sigma Chemical Company	St. Louis	MO
Ca^{++}, Mg^{+} free phosphate buffered saline	PBS	Gibco BRL	Grand Is.	NY
Chloroform		Sigma Chemical Company	St. Louis	MO
Cys-Arg-Gly-Asp	CRGD	Queen's University	Kingston	ON
Cys-Arg-Glu-Asp-Val	CREDV	Queen's University	Kingston	ON
Cys-Cys-Arg-Arg-Gly-Asp-Trp-Leu-Cys	CCRRGDWLC	Queen's University	Kingston	ON
Cysteine		Sigma Chemical Company	St. Louis	MO
Dimethyl sulfoxide	DMSO	BDH	Toronto	ON
Disodium Hydrogen Phosphate	Na_2HPO_4	Sigma Chemical Company	St. Louis	MO
Endothelial Cell Growth Factor	ECGF	Becton Dickinson Labware	Bedford	MA
Epidermal Growth Factor	EGF	Becton Dickinson Labware	Bedford	MA
E-STIM Growth Medium	E-STIM	Becton Dickinson Labware	Bedford	MA
Ethylenediamine	ED	Acros Organics	Pittsburgh	PA
Fetal bovine serum		Gibco BRL	Grand Is.	NY
Gentamycin		Gibco BRL	Grand Is.	NY
Giemsa Stain	Giemsa	Sigma Chemical Company	St. Louis	MO
Glutaraldehyde		Sigma Chemical Company	St. Louis	MO
Glycerol		Sigma Chemical Company	St. Louis	MO
Human Albumin	Albumin	Behringwerke	Marburgh	GY
Human Vascular endothelial cells	Endos	Ottawa Eye Institute	Ottawa	ON
Hydrogen peroxide		BDH	Toronto	ON
Lithium bromide	LiBr	Sigma Chemical Company	St. Louis	MO
Medium 199		Gibco BRL	Grand Is.	NY

Reagent	Acronym	Supplier	Location	
Methanol	MeOH	BDH	Toronto	ON
Methylenedi-p-phenyl diisocyanate	MDI	Acros Organics	Pittsburgh	PA
Mouse 3T3 fibroblasts	Fibros	American Type Culture Collection	Rockville	MD
Polypropylene glycol	PPG	Matheson Coleman & Bell	Norwood	OH
Porcine skin gelatin	Gelatin	Sigma Chemical Company	St. Louis	MO
Radioactive Sodium iodide	$Na^{125}I$	ICN Pharmaceuticals	Irvine	CA
Sodium Chloride	NaCl	Sigma Chemical Company	St. Louis	MO
Sodium Dihydrogen Phosphate	NaH_2PO_4	Sigma Chemical Company	St. Louis	MO
Sodiumdodecylsulfate	SDS	Sigma Chemical Company	St. Louis	MO
Sodium Iodide	NaI	Sigma Chemical Company	St. Louis	MO
Trichloroacetic acid	TCA	Sigma Chemical Company	St. Louis	MO
Trypsin EDTA	Trypsin	Gibco BRL	Grand Is.	NY
Trypsin inhibitor		Gibco BRL	Grand Is.	NY

Appendix B: Prepared Solutions and Method of Preparation

NaI PBS (pH 7.4)

Table 5: Reagents Required for Phosphate Buffered Saline Solution

Reagent	Acronym	Mass (g)
Disodium Hydrogen Phosphate	Na_2HPO_4	1.32
Sodium Dihydrogen Phosphate	NaH_2PO_2	0.345
Sodium Iodide	NaI	6.5
Sodium Chloride	NaCl	2

The above reagents were combined and brought up to one liter with distilled water. The pH was subsequently adjusted with NaOH or HCl to 7.4.

PBS (pH 7.4)

Table 6: Reagents Required for Phosphate Buffered Saline Solution

Reagent	Acronym	Mass (g)
Disodium Hydrogen Phosphate	Na_2HPO_4	1.32
Sodium Dihydrogen Phosphate	NaH_2PO_2	0.345
Sodium Chloride	NaCl	8.5

The above reagents were combined and brought up to one liter with distilled water. The pH was subsequently adjusted with NaOH or HCl to 7.4.

Separating Gel

The separating gel was prepared by combining the first four reagents listed in the table below.

Table 7: Reagents required for Fabrication of Polyacrylamide Gel

Reagent	Acronym	Separating Gel Volume (ml)	Stacking Gel Volume (ml)
Distilled Water	DW	3.35	3.0
	Tris-HCl	2.5	1.2
	SDS	0.1	0.1
	Acrylamide/Bis	4.0	0.65
Ammonium Persulfate	AP	0.05	0.025
	TEMED	0.005	0.005

After degassing these reagents for fifteen minutes at room temperature the ammonium persulfate and TEMED were added.

Stacking Gel

The stacking gel was prepared in the same manner as the separating gel by combining the first four reagents, degassing for fifteen minutes then adding the last two reagents to begin the polymerization.

Tracking Dye

Table 8: Reagents Required for Preparation of Tracking Dye

Reagent	Acronym	Volume (ml)
Distilled Water	DW	4.0
	Tris HCl	1.0
Glycerol		0.8
Sodium Dodecylsulphate	SDS	1.6
2-â mercaptoethanol		0.03
Bromophenol Blue		0.03

The first four reagents were combined and a 225 µl aliquot was subsequently used. The last two reagents were added to this aliquot.

Appendix C: SIMS chromatograms for synthetic peptides

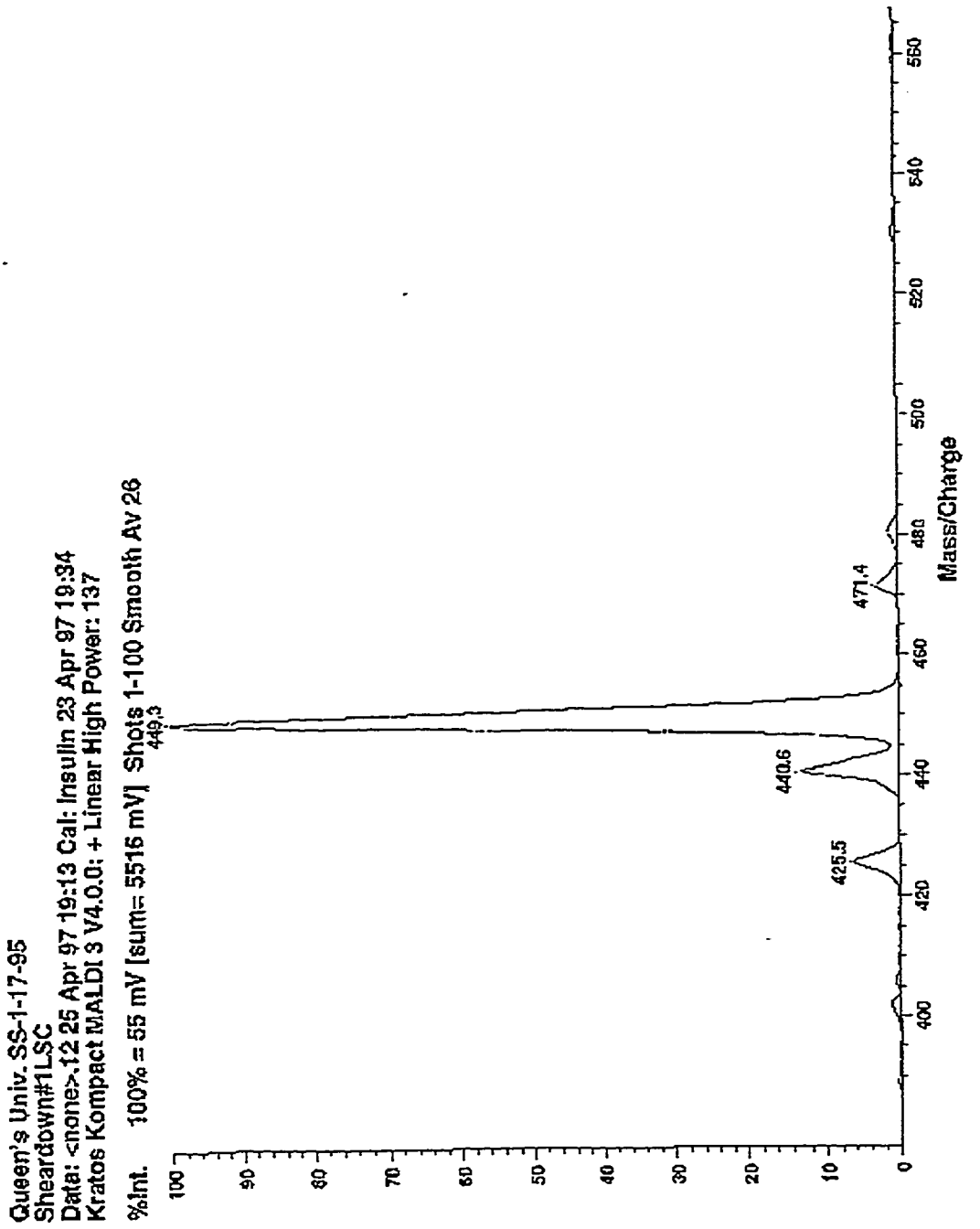

Figure 45: SIMS chromatogram for synthetic CRGD peptide

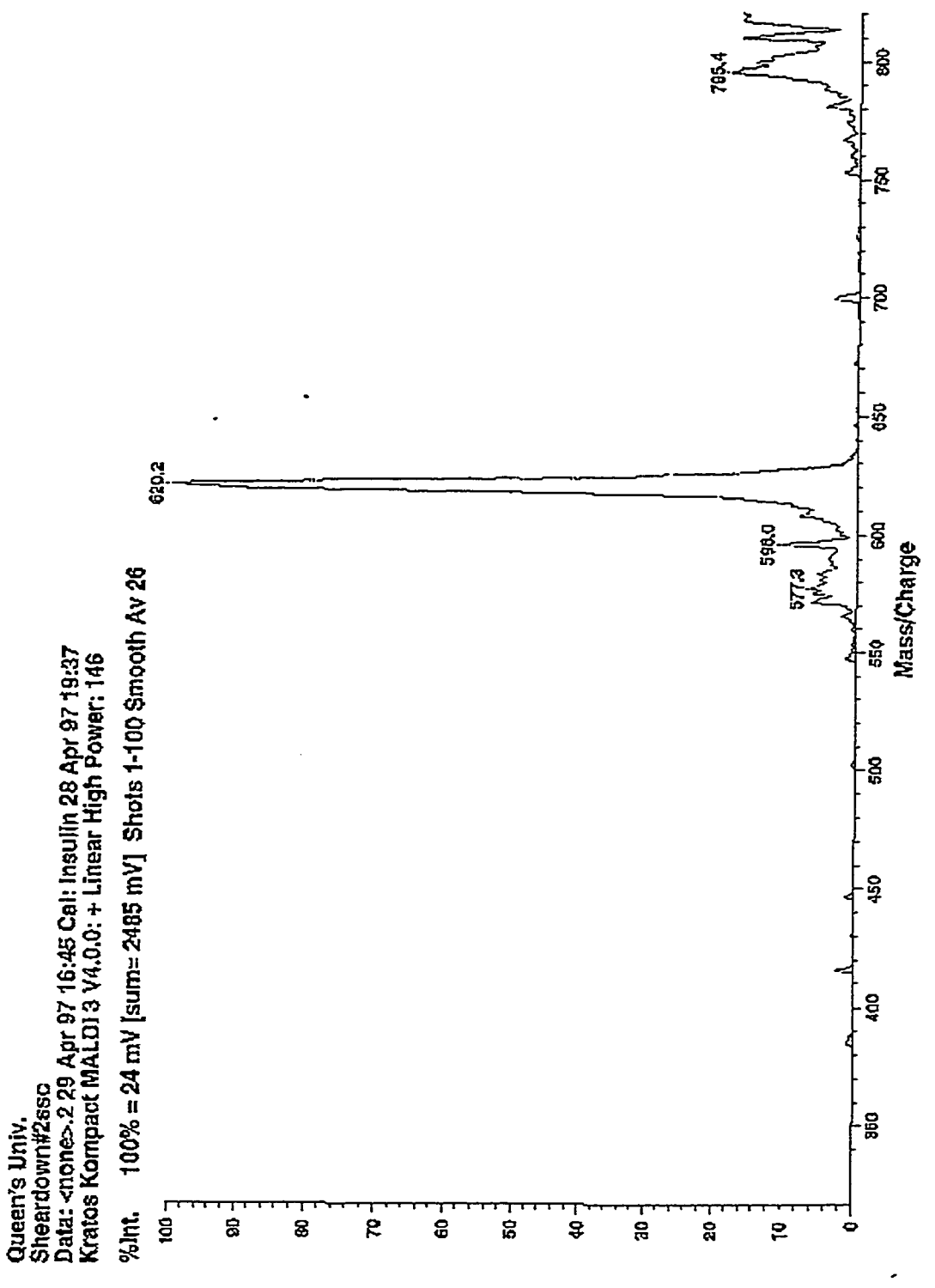

Figure 46: SIMS chromatogram for synthetic CREDV peptide

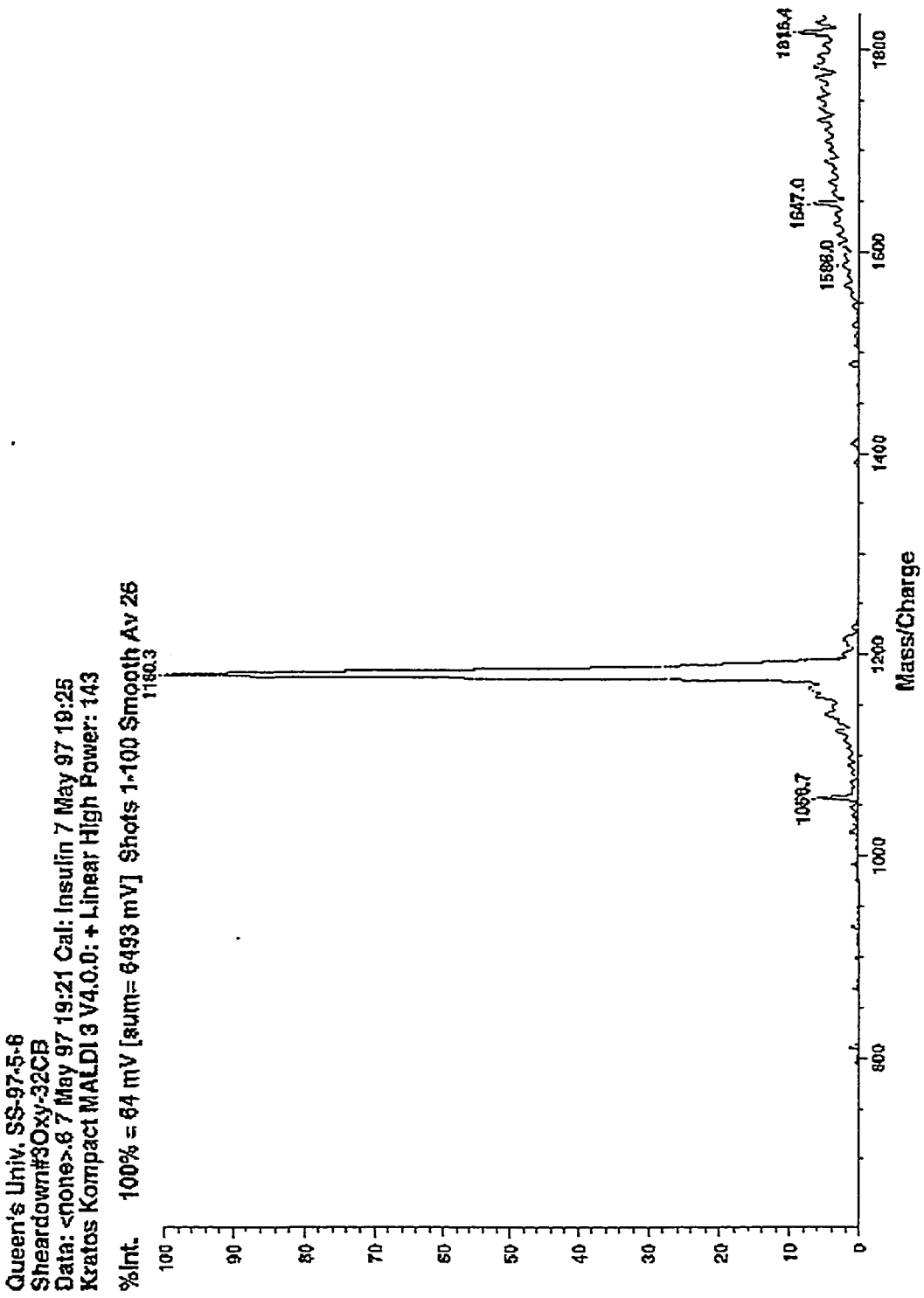

Figure 47: SIMS chromatogram for synthetic CCRRGDWLC (cyclic) peptide

IMAGE EVALUATION TEST TARGET (QA-3)

150mm

6"