Human Platelet Adhesion to Heparinized Silica Surfaces

by
Travis Samuel Sebastian Bolsinger

A THESIS

submitted to

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Travis Samuel Sebastian Bolsinger for the degree of Master of Science in Chemical Engineering presented on August 28, 2008.

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Joseph McGuire

Heparin was modified and immobilized to surface-activated silica surfaces using two different reaction schemes. End-aminated heparin was reacted with 2-iminothiolane to produce free thiol groups at the terminal ends of the heparin chains. The end-thiolated heparin was immobilized by reaction with a pyridyl disulfide activated poly[ethylene oxide]-poly[propylene oxide]-poly[ethylene oxide] triblock copolymer that was non-covalently adsorbed to hydrophobic silica. In addition, heparin was modified with adipic dihydrazide and covalently immobilized to silica treated with of 3-aminopropyl triethoxy-silane and succinic anhydride to favor a side-on-orientation of heparin at the surface. X-ray photoelectron spectroscopy (XPS) and contact angle analysis were performed at each stage of surface treatment to determine if successful immobilization had taken place. XPS results indicated that successful immobilization of adipic dihydrazide modified heparin had taken place. However, for end-thiolated heparin, XPS results were not entirely consistent with successful heparin immobilization and determination of surface-bound heparin was thus inconclusive. Platelet adhesion under dynamic conditions was investigated on each surface during the immobilization of heparin. Scanning electron microscopy (SEM) was used to characterize platelet adhesion from human platelet-rich plasma on each surface. The images recorded from SEM were used to determine the number and morphology of adherent platelets. For

both heparin immobilization methods, there was a significantly lower number of adherent platelets on heparinized surfaces in comparison to the non-heparinized surfaces. Also, the platelets that did adhere to the heparinized surfaces showed less aggregation and spreading in comparison to the non-heparinized surfaces, which is consistent with a lower extent of platelet activation on these surfaces.

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APPROVED:

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Major Professor, representing Chemical Engineering

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Chair of the School of Chemical, Biological, and Environmental Engineering

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Dean of the Graduate School

I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

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Travis Samuel Sebastian Bolsinger, Author

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CHAPTER 1
INTRODUCTION

Biomedical devices, such as catheters and stents, provide a valuable and important resource in modern medicine today. These types of implantable devices can be very effective tools in the treatment of patients; however, they can also cause very severe health problems. Improvements in biocompatibility have to be addressed to reduce and/or eliminate the instances of device failures upon implantation before these methods of treatment can become fully safe.

The initial event that occurs when foreign material comes into contact with the blood is a rapid and instantaneous adsorption of blood proteins (Vroman and Adams, 1969). When proteins adhere to the foreign material, they change their native conformation and begin to initiate further biological responses, such as platelet adhesion, platelet activation and aggregation, and the initiation of the clotting cascade (Brash and Horbett, 1987). These events will result in thrombus formation at the site of the implant, which can then break free from the material and cause severe adverse reactions, such as heart attack and stroke.

One method of treatment to combat site-induced thrombosis is the administration of anticoagulant drugs, such as heparin or coumarin derivatives, like warfarin. These treatment methods have a limited effectiveness and can be very problematic for long term use. Patients on anticoagulant therapies can experience difficulties with excessive bleeding during surgery or injuries; also, there can be adverse side effects in children and pregnant or nursing mothers. A significant number of patients undergoing prolonged heparin therapy have developed heparin-induced thrombocytopenia, which requires these patients to stop heparin therapy (Nowak et al., 1997). Because of the problems associated with anticoagulant therapies, alternate methods to reduce the instances of site-induced thrombosis are needed.

The current alternative to anticoagulant therapy, and the focus of much research, is to create surface coatings that can be imparted on the device that will make it more biologically inert. One method for accomplishing this is to create a

surface that can inhibit the initial adsorption of proteins and also incorporate an anticoagulant on the surface of the device so that the surface has a dual functionality. Heparinized surfaces are commonly used to achieve this objective; however, the long term effectiveness of such coatings is questionable (Olsson et al., 2000; Svenmarker et al., 2001). One of the main problems with immobilized heparin coatings is that the mode of action and interaction with proteins when heparin is covalently bound to the surface is not fully understood. Heparin can be oriented in different ways when it is bound to a surface, thus influencing its effectiveness. Also, because heparin is a very heterogeneous bioactive molecule, chemical modification and immobilization can affect its bioactivity.

The objective of this research was to chemically modify heparin through two different mechanisms and covalently attach it to functionalized silica surfaces followed by determination of the extent of human platelet adhesion to the heparinized surfaces. The two methods for heparin modification focused on alteration of internal carboxyl groups, which could be covalent bound to succinilated surfaces, giving heparin a side-on orientation. The second method involved thiolation of end-aminated heparin and covalently binding to hydrophobically adsorbed end-activated pluronics giving the surface-bound heparin an end-on orientation. Platelet adhesion was examined at each stage of immobilization to determine the amount of adhered platelets.

CHAPTER 2
LITERATURE REVIEW

2.1 Protein Adsorption and Biocompatibility

Adsorption of plasma proteins is the first event that occurs when a foreign material comes into contact with blood (Young, 1982). The adsorption of plasma proteins to the surface of foreign materials is a thermodynamically driven process. Protein adsorption is a very dynamic process that consists of non-covalent interactions such as hydrophobic/hydrophilic interactions, electrostatic forces, hydrogen bonding, and van der Waals forces (Andrade and Hlady, 1986). When proteins adsorb to the material, they change their conformation and can become activated. The ability of a material to inhibit or prevent these events from occurring is very important for its effectiveness for use in the body.

An extensive review on the past and present aspects of protein adsorption on device surfaces has been presented by Ratner and Bryant, 2004. Upon implantation, devices elicit nonspecific protein adsorption to the surface of the material. The proteins can then interchange with other plasma proteins and be replaced, desorb, and can change conformation on the surface. When proteins on the surface of a device change conformation from their native structure they can become activated and promote unwanted adhesion of monocytes, leukocytes, and platelets. This can lead to surface induced thrombosis and inflammation.

A strategy to prevent the initial adsorption of proteins is to alter the surface chemistry of the device in such a way as to prevent proteins from being able to adhere. Tanaka et al. (2000) studied the adsorption of plasma proteins, their secondary structure, and platelet adhesion on poly(2-methoxyethylacrylate)(PMEA), poly(2-hydroxyethylmethacrylate)(PHEMA) surfaces, and polyacrylate analogs. It was observed that very low, but similar amounts of plasma proteins adhered to both the PMEA and PHEMA surfaces. The secondary structure of adsorbed proteins was studied using circular dichroism (CD). It was found that even though similar amounts of proteins adhered to both the PMEA and PHEMA surfaces the proteins that adhered

to the PHEMA surface changed conformation significantly and the ones that adhered to the PMEA surface showed little conformational change from the native structure. It was also found that PMEA surfaces had the lowest adherence of platelets compared to the other polymer surfaces. This suggests that the amount of platelet adhesion is related to the degree of denaturation of surface adhered proteins.

Huang et al (2001) studied the analytical and structural properties of poly(L-lysine)-g-poly(ethylene glycol) (PLL-g-PEG) electrostatically attached to the surfaces of niobium oxide (Nb_2O_5), tantalum oxide (Ta_2O_5), and titanium oxide (TiO_2) through the use of reflection-adsorption infrared spectroscopy (RAIRS), angle-dependent X-ray photoelectron spectroscopy (XPS), and time-of-flight secondary ion mass spectroscopy (ToF-SIMS). They also examined the amount of human serum and fibrinogen that would adsorb to these PLL-g-PEG coated layers by the use of OWLS, an optical planar waveguide technique. It was found that the poly(L-lysine) would electrostatically attach to the surface of the metal oxides and orient parallel to the surface, where as the poly(ethylene oxide) chains would extend from the surface and orient perpendicular to the oxide layer. All metal oxide surfaces behaved similarly with respect to the amount of protein adsorption. The amount of human serum adsorption on each PLL-g-PEG was found to be below the detection limit (<1-2 ng/cm^2) for the technique used and that fibrinogen adsorption was reduce by 96-98% compared to the uncoated oxide surfaces.

One very common technique to inhibit protein adhesion to device surfaces is through the use of Pluronics™ which are a class of triblock copolymers. These molecules are surface active agents made up of a central hydrophobic poly(propylene oxide)(PPO) chain with a hydrophilic poly(ethylene oxide)(PEO) chain at each end. These molecules can be hydrophobically associated to surfaces and have shown protein repelling properties. Also, it is possible to modify the terminal end of the PEO chains for covalent attachment of other molecules. This provides the opportunity to make a surface with a dual functionality where one could attach a biologically active molecule to the PEO chain and create a surface that is able to repel proteins and have some sort of biological effect on the surface.

Li et al (1996) showed that poly(ethylene oxide)-poly(propylene oxide) triblock copolymers could be modified with the introduction of 2-pyridyl disulfide groups after coupling with 2-(2-pyridyldithio)ethylamine. This method allows for the covalent attachment of thiol-containing molecules to the triblock layer. Andersson et al (2006) showed that End-Group Activated Pluronic (EGAP) could be used to immobilize factor H to polystyrene surfaces. It was shown that covalently bound factor H onto EGAP coated polystyrene did not detach from the surface when incubated with human serum. It was also shown that bound factor H could significantly reduce complement activation. This showed that by attaching a biologically active molecule to pluronic surfaces it is possible to create a surface that is protein repelling, as well as biologically active.

2.2 Heparin Structure and Function

Heparin is a highly sulfated, polydisperse, linear polysaccharide made up of repeating units of 1→4 linked uronic acid and glucosamine residues. Because of the high amount of sulfate and carbonyl groups, heparin is the most negatively charged biomolecule known (Islam and Linhardt, 2003). Heparin is a member of the glycosaminoglycan (GAG) family of carbohydrates, which also includes heparan sulfate, hyaluronic acid, and chondrotin sulfate. Heparin is a very heterogeneous molecule and can vary widely between sources of origin. The molecular weight for unfractionated heparin can vary from 3000 – 40,000 Da with an average molecular weight of 12,500 Da (Tyan et al, 2002). Heparin has an average of 2.7 negative charges per disaccharide arising from the sulfate and carboxyl groups and, consequently, the prominent type of interactions between heparin and proteins are ionic (Capila and Linhardt, 2002).

Heparin is a very commonly used anticoagulant drug. Its anticoagulant activity arises mainly through its interaction with antithrombin (AT). Antithrombin is part of the serine proteinase inhibitor family of proteins and is the primary inhibitor of the blood coagulation proteinases, most notably thrombin and activated factor X. Antithrombin inhibits these blood clotting proteins by binding its reactive center loop

to the enzyme active site much like a substrate (Desai et al, 1998; Olson et al, 1981; Olson et al, 1992). This causes proteins, like thrombin, to become inactive and unable to form blood clots. This type of inhibition is normally very slow, but in the presence of heparin this process is increased by several thousand-fold (Gettins et al, 1996). There is a specific pentasaccharide sequence within heparin that is present in roughly one-third of all naturally occurring heparin chains and is responsible for heparin's ability to bind with antithrombin (Figure 1). In the case of thrombin inhibition, heparin must bind to both thrombin and antithrombin for inhibition to occur.

2.3 Immobilized Heparin

Because heparin has a very low bioavailability when given subcutaneously and no activity when given orally, it has to be administered intravenously. This means that heparin is allowed to spread throughout the body instead of being present only at the site of the implant where blood clotting is expected to occur. This results in an increased risk to patients because of the difficulty of forming clots following injuries and an increased surgical risk. Also, women who are pregnant or nursing are at increased risk and young children become at risk when undergoing heparin therapy. Because of the risks associated with systemic heparin therapy there is a need to create devices that can inhibit thrombosis at the surface to eliminate or reduce the need for intravenous heparin administration. One method for achieving this is by coating device surfaces with heparin so they are non-thrombogenic and only be active at the surface of the implant. Although studies have shown that immobilized heparin does improve biocompatibility (Svenmarker et al, 1997; Johnell et al, 2002), its exact mechanism and effectiveness has been questioned. For example, Blezer et al (1997) showed that because heparin is a very negatively charged molecule, immobilizing it on the surface can cause thrombosis to occur due to fact that negatively charged surfaces can result in the activation of the contact system. However, this is offset by the antithrombin activity of the surface bound heparin.

Heparin immobilization can be achieved by nonspecific, ionic, or covalent attachment (Goddard and Hotchkiss, 2007). The benefits of nonspecific and ionic type

coatings are that they are relatively quick and easily prepared. Nonspecific coatings can be prepared through the binding of heparin to other molecules, such as proteins, that can adsorb to the surface and thus create a heparinized surface. Mahoney et al (2004) showed that heparin could be immobilized onto the surfaces of microtiter wells through the use of heparin binding proteins. This method would permit non-covalent attachment of heparin to various substrates.

Because covalent attachment of heparin allows for more specific surface orientations of the heparin molecule and covalently immobilized heparin is bound more strongly (van Delden et al, 1996), it is considered the most ideal method for practical use. Marconi et al (1997) developed a method for immobilizing heparin onto the surface of ethylene-vinyl alcohol copolymer. Heparin was attached by both covalent and ionic methods. The amount and activity of the surface bound heparin were evaluated. It was found that covalent attachment of heparin to the surface provided a significantly greater hemocompatibility compared to that of the ionic attachment.

Joshi et al (2006) studied fibrinogen adsorption on covalently immobilized heparin to functionalized silica surfaces. Heparin was modified with adipic dihydrazide and covalently linked to surface-activated silica wafers. Surfaces were characterized through the use of X-ray photoelectron spectroscopy (XPS) and atomic force microscopy (AFM), which showed that successful heparin immobilization had occurred. Also, it was found that fibrinogen adsorbed to a much lesser extent on heparinized surfaces compared to unheparinized surfaces. In addition, the fibrinogen that did adsorb to the surface was more elutable, and thus less tightly bound, on heparinized silica versus unheparinized surfaces.

Chen et al (2005) studied the affinity adsorption of antithrombin (AT) and adsorption of fibrinogen on a silicone surface with heparin immobilized through a polyethylene glycol (PEG) spacer. The heparin surface density after immobilization to the PEG spacers was determined by a toluidine blue assay, and it was found that there was an immobilized mass of 0.68 $\mu g/cm^2$. Contact angles were taken during the immobilization process and it showed that there was a decrease in contact angle (i.e.,

increased hydrophilicity) upon PEG modification and a further decrease in contact angle upon heparin immobilization. The protein adsorption studies showed that there was a high specificity for ATIII binding when heparin was attached to the surface and that there was no significant ATIII adsorption when heparin was not present. It was also seen that fibrinogen did not adsorb in significant amounts on either the PEG or heparin grafted surfaces. Also, using a chromogenic thrombin generation assay, it was found that heparin modified surfaces showed greater thrombin resistance. This method of heparin immobilization shows that through the use of PEG linkers heparin can be successfully immobilized on the surface and show AT activity, as well as protein repelling behavior.

Immobilization of heparin has also been investigated for use in hemodialysis membranes (Lin et al, 2005). In this study the authors immobilized heparin onto the surface of polyacrylonitrile (PAN) membrane through two different techniques. One method involved the direct covalent immobilization of heparin onto a PAN membrane (PAN-H) using EDC as a crosslinking agent. The second method involved covalent attachment of heparin onto a chitosan-grafted PAN membrane (PAN-C-H) using glutaraldehyde. Protein adsorption, platelet adhesion, and thrombus formation were evaluated for each membrane. For both methods adsorption of the proteins human serum albumin (HSA) and Human plasma fibrinogen (HPF) were reduced on the heparin immobilized membranes, compared to the non-coated ones. Also, platelet adhesion and thrombus formation were significantly lower on the PAN-H and PAN-C-H membranes than the non-heparinzed ones. For all cases the PAN-C-H performed slightly better than the PAN-H membrane, but there was no significant difference. The anticoagulant activity of each membrane was determined by the activated partial thromboplastin time (APTT), prothrombin time (PT), fibrinogen time (FT), and the thrombin time (TT). The results showed that both the PAN-H and the PAN-C-H had significantly longer APTT and TT times compared to the control and unheparinzed membranes and the PAN-C-H showed the longest times out of all the membranes tested. However, the PAN-H membrane showed similar times to the controls for both the PT and FT tests where the PAN-C-H had much higher times for the PT tests. In

addition to the before mentioned tests the antibacterial activity was also measured on each membrane. It was found that the PAN-H and the PAN-C-H membranes were able to resist the growth of *Pseudomonas aeruginosa* for a period of up to 21 days. These results show that the immobilization of heparin onto PAN membranes using either of these heparin attachment methods creates a substantially more biocompatible surface than that observed with unheparinzed surfaces.

2.4 Platelet Adhesion

Platelet adhesion, aggregation, and activation are a crucial step in the body's response to injured blood vessels. When injury occurs within the body, platelets will adhere to the site via specific glycoprotein receptors that can bind to certain molecules, like collagen, that are exposed when injury occurs. After platelets adhere to the site of injury they will then aggregate by binding to other platelets that come into contact with them. They do this through the use of specific receptors on the surface of their membranes that can bind proteins, such as fibrinogen. This results in the formation of platelet plugs. For example, platelets use the glycoprotein receptor $\alpha_{IIb}\beta_{III}$ to bind to fibrinogen (Phillips, 1988). Once adhered, platelets will also secrete chemicals like ADP, collagen, epinephrine, and thrombin which will promote further aggregation of platelets. These events lead to the formation of a clot at the site of injury and the tissue will then be able to heal. When a foreign material comes into contact with the blood, these events will occur in a similar fashion on the surface of the material. The resulting platelet plug that is formed can detach from the surface of the material and lead to heart attack or stroke. The ability of a material to prevent these events is crucial for its viability to be used in a blood contacting device.

Uchida et al (2005) coated titanium metal with apatite (HA-Ti), albumin-apatite composite (AA-Ti), and laminin-apatite composite (LA-Ti) and studied platelet adhesion on each surface. The surfaces were prepared by immersion into sodium hydroxide solution followed by heat treatment. They were then exposed to a calcium phosphate solution containing either albumin or laminin depending on the substrate to be made. Platelet adhesion tests were carried out with a cone- and plate-type

viscometer using fluorescent labeled platelets. They found that there was a substantial reduction in the number of adherent platelets to the HA-Ti, AA-Ti, and LA-Ti samples compared to that of the untreated titanium surfaces. It was also noted that the platelets that adhered to the treated surfaces were more round in shape than what was seen on the untreated surfaces, indicating less activation of the platelets on the coated surfaces. Similar results were also found using apatite coated titanium surfaces under static platelet adhesion experiments (Muramatsu et al, 2003).

Yang and Lin (2003) studied platelet adhesion, clotting time, and protein adsorption on polysulfone (PSF) membranes treated with ozone then grafted with either acrylic acid or chitosan followed by the immobilization of heparin. They also varied the molecular weights of chitosan using 1170, 160,000, and 400,000 to see the effects of longer chain lengths on blood compatibility. Platelet adhesion was evaluated by exposing each surface to platelet rich plasma (PRP) under static conditions and then taking before and after platelet counts of the PRP to determine the number of platelets adhered. Membranes grafted with chitosan showed the highest amount of platelet adhesion, which was said to be due to the positive charge chitosan imparts to the surface. Heparin immobilized on chitosan showed the least amount of platelet adhesion and the heparin immobilized on the largest chitosan chains performed the best. Kang et al (1997) also found that platelet adhesion significantly decreased on surfaces with heparin immobilized on polyurethanes (PUs). Also, activated partial thromboplastin time (APTT) was prolonged on heparin coated PUs. The heparinized surfaces showed significantly longer clotting times than the controls and chitosan grafted membranes. Also, clotting times increased for heparin immobilized on chitosan surfaces as chitosan molecular weight increased. The same was found for the adsorption of HSA on each membrane, where the least amount of protein adsorption was found on the membranes with heparin immobilized on chitosan.

Higuchi et al (2003) also studied protein and platelet adsorption on polysulfone membranes coated with Pluronic® surfactants. They used Pluronic® with varying PEO chain lengths. It was found that increasing the coating concentration of the Pluronics® decreased the amount of protein that adhered to the membranes. Also, protein

adsorption decreased as the length of the PEO chain increased. Platelet adhesion to the membranes also decreased as the length of the PEO chain increased.

CHAPTER 3
MATERIALS AND METHODS

3.1 Synthesis of modified Heparin

3.1.1 ADH Heparin Synthesis

The method for adipic dihydrazide modification of heparin was adopted from Joshi et al. (2004). This technique for modification was based off of methods to modify hyaluronic acid (HA), which is another compound in the glycosaminoglycan family as described by Pouyani and Prestwich (1994), Pouyani et al. (1994), Vercruysse et al. (1997), and Luo et al. (2000). This method for chemical modification targets the carboxyl groups on the polysaccharide backbone of heparin through carboiimide compounds. Adipic dihydrazide introduces hydrazide groups extended from the heparin backbone that can be used to further couple to other compounds in mild aqueous environments.

Adipic dihydrazide modification was performed by first dissolving 200 mg heparin sodium (heparin, Celsus Laboratories, Inc., Cincinnati OH, Lot PH-40299) into 50 mL of double distilled water (DDW) for a final concentration of 4 mg/mL. To this mixture, 3.5 g adipic dihydrazide (ADH, Sigma, Lot A0638) was added. The pH of the solution was adjusted to 4.75 using 0.1 N HCl (HCl, Fischer Scientific, Fair Lawn, NJ), and maintained for 30 minutes. After 30 minutes, 382 mg of 1-ethyl-3-[3-(dimethyl-amino)propyl]carboiimide (EDCI, Sigma-Aldrich, Lot E6383) was added. The reaction solution was brought again to a pH of 4.75 by using 0.1 N HCl. The reaction was allowed to proceed for two hours or until no change in pH was observed. At this point the reaction was terminated by the addition of 1 N NaOH, bringing the pH of the solution to 7.0. Dialysis cassettes with a molecular weight cut off of 3,500 (Pierce, Rockford, IL) were then pretreated by soaking in DDW for at least 1 hour prior to use. The solution was then transferred to the pretreated dialysis cassettes and dialyzed against 100 mM NaCl (5.84 g/L) (Mallinckrodt Inc., Paris, KY) solution for 3 days in a 2 L vessel. The 100 mM NaCl dialysis solution was replaced every two

hours for the first six hours and then every 24 hours until the end of 3 days, when it was discarded. The reaction solution was then dialyzed against DDW for an additional 24 hours. The reaction solution was then removed from the dialysis cassettes and centrifuged at 21 °C, 2095Xg for 15 minutes. The supernatant was removed and placed into a -80 °C freezer until further processing; the pellet was discarded. The frozen supernatant was then lyophilized (Benchtop 5SL, VirTis Company, Inc., Gardiner, NY) for a minimum of 48 hours.

3.1.2 Thiolated heparin synthesis

Unfractionated heparin sodium amine (Hep-NH_2) was obtained from Celsus Laboratories (Cincinnati, OH). Modification of Hep-NH_2 to contain thiol groups was performed by reaction with 2-iminothiolane (Traut's reagent, Sigma-Aldrich, St. Louis, MO). An illustration of this reaction is given in Figure 2. First, 15 mg of Hep-NH_2 was dissolved in 1 mL of distilled water for a final concentration of 15% (w/v). A solution of 2-iminothiolane was prepared be dissolving 10 mg into 1 mL of 20 mM sodium borate buffer with 1 mM EDTA at pH 10. In a separate tube 770 µL of 20 mM sodium borate buffer with 1 mM EDTA at pH 10 was added in addition to 50 µL of the Hep-NH_2 solution and 180 µL of 2-iminothiolane solution for a total volume of 1 mL. Twenty tubes were prepared for a total combined volume of 20 mL. Each tube was then placed in a vortex mixer for 2 hours at 600 rpm at room temperature. To separate the Traut's reagent from the thiolated heparin after the two hour reaction time, 500 µL of the reaction solution from each tube was placed in a centrifugal filter device with a molecular weight cutoff of 3500 Da. They were then centrifuged at 13,400Xg for 90 minutes at room temperature to a stop volume of 10 µL. The filters were then taken out of the centrifugation tubes and placed inverted in new microcentrifugation tubes and centrifuged for 3 minutes at 100Xg to recover purified thiolated heparin. Next, 490 µL of 20 mM phosphate bufferd saline solution containing 1 mM EDTA and 150 mM NaCl at pH 7.5 was added to each 10 µL of purified, thiolated heparin solution bringing it up to a final volume of 500 µL. This

solution was then used to react with the end-group activated pluronic (EGAP) coated silica wafers.

3.2 Functionalizing Surfaces

3.2.1 Non-functionalized surface

The procedure for obtaining hydrophilic silica surfaces was previously reported by McGuire et al. (1995) and Joshi et al. (2004). Silicon (Si) wafers (crystal grade CZ, type P, boron doped, orientation 1-0-0, thickness 525 ± 18 μm, resistivity 0.01-0.02 ohm-cm) were purchased from Siltronic AG (San Jose, CA). Wafers were cut using a tungsten pen into 1 cm X 1 cm squares. Each square was then rinsed with acetone and placed in a 7 mL solution of NH_4OH:H_2O_2:H_2O (1:1:5 volume ratio). They were then immersed in an 80 °C water bath and left for 10 minutes. Then, they were taken out of the solution, rinsed with DDW, and each square was placed in a 7 mL solution of HCl:H_2O_2:H_2O (1:1:5 volume ratio). They were then placed in 80 °C water bath and allowed to sit for 10 minutes. Each slide was then removed from the solution, rinsed with large amounts of DDW, blown dry with nitrogen gas, and stored in a desiccator.

3.2.2 Aminated surface

Bare hydrophilic Si wafers were functionalized with amine groups prior to being coupled with modified heparin. After Si squares were cleaned as previously described, they were then immersed in anhydrous acetone solution for at least two hours prior to use to ensure the Si wafers were clean. Acetone was made anhydrous by allowing it to sit in 4 Å molecular sieves. The Si squares were then placed in a 2 % (v/v) solution of 3-aminopropyl triethoxy-silane (3APTS, Aldrich) in anhydrous acetone at room temperature for 1 hour. Following incubation, the wafers were taken out and washed with anhydrous acetone followed by DDW, and blown dry with nitrogen. The wafers were stored in a desiccator until further use.

3.2.3 Carboxylated surface

It has been observed by Mason et al. (2000) that covalent attachment of HA is increased if the aminated sample is first treated with succinic anhydride. It was hypothesized that this is due to the extended chain from the substrate surface so that HA will experience less steric hindrance and, thus, will bind better to the surface.

Silicon wafers that had been treated previously with 3 APTES and stored in a desiccator were placed in 10 mM (1.0007 g/L) succinic anhydride (Aldrich Chemical Co. Inc., Milwaukee, WI) in anhydrous N, N-Dimethylformamide (DMF, EM Science, Gibbstown, NJ). The silica squares in the DMF solution were placed on a rotator and the reaction was carried out at room temperature for 10 hours. The wafers were then removed from the reaction solution and washed with DDW for 30 minutes, blown dry with nitrogen gas, and stored in desiccators until further use.

3.2.4 EGAP coated surface

Silica wafers were coated with end-group activated Pluronic® (EGAP) (gift from Allvivo Vascular, Lake Forest, CA) by hydrophobic association to be used as a linker for Hep-NH_2 immobilization. Silica wafers were cut and washed, as previously described, to create a clean hydrophilic surface. They were then made to be hydrophobic by treatment with PS200 (UCT Specialties, Bristol, PA). Treatment with PS200 was performed by immersing and agitating each wafer in a 5% (v/v) solution of PS200 in double distilled water for 20-25 sec. The wafers were then rinsed with water and dried in an oven for 1 hour at 100 °C. After the silica wafers were dried and allowed to cool, each wafer was immersed in a solution of 1% (w/v) EGAP in DDW. They were left on an orbital rotator overnight to allow sufficient adsorption time. Each wafer was then washed with double distilled water for at least 30 minutes, dried with nitrogen, and stored in a desiccator until further use.

3.3 Immobilization of heparin

3.3.1 Immobilization of ADH-heparin

The procedure for immobilization of ADH-heparin was obtained from the technique of HA attachment as described by Mason et al (2000) and modified for ADH-heparin. Si wafers that had been treated with succinic anhydride were each placed in a 7 mL solution of 1.2 g/L ADH-heparin, 50 mM (12.285 g/L) bis-tris HCl (Sigma), and 1 mM (0.192 g/L) EDCI (Sigma) for 24 hours. The reaction was performed at 4 ˚C at 90 rpm in an Aros 160 Adjustable Reciprocating Orbital shaker (Thermolyne), to prevent bacterial growth. Following incubation, the wafers were removed from solution, washed with large amounts of DDW to remove any physically adsorbed ADH-heparin that was not covalently attached to the surface, blown dry with nitrogen gas and stored in a desiccator until further use. An illustration of ADH-heparin attachment is given in Figure 3.

3.3.2 Immobilization of thiolated heparin

Thiolated heparin immobilization was performed by reaction with hydrophobically adsorbed EGAP on PS200 treated silica. The reaction between the thiol group on the terminal end of heparin and the pyridyl disulfide group from the PEO chain of the EGAP is a thermodynamically driven process (Figure 4). Immobilization was performed by immersing the EGAP-coated silica slides in 10 mL of the thiolated heparin solution previously described. The reaction was allowed to proceed overnight at room temperature on an orbital shaker. After the reaction was completed the slides were removed from the solution and washed in 10 mL of DDW for at least 30 min to remove any non-covalently attached heparin. The slides were then rinsed again with DDW and blown dry with nitrogen. The slides were stored in a desiccator until further use.

3.4 Surface characterization

3.4.1 Contact angle

Contact angle is a reflection of the forces that act when a liquid/vapor phase contacts a third solid phase. The contact angle is defined as the angle formed by the liquid at the three phase boundary where a liquid, gas, and solid interact. These measurements give an indication of a surface's hydrophilicity or hydrophobicity.

Contact angle measurements were obtained using a First Ten Angstroms 32 Video (FTA 32, First Ten Angstroms, Portsmouth, VA). A 1-2 μL drop was placed on the surface of a silica square and an image was taken and used to measure the contact angle of that sample. Contact angle measurements were taken and recorded from each surface during the immobilization of heparin.

3.4.2 X-ray Photoelectron Spectroscopy (XPS)

X-ray photoelectron spectroscopy is a quantitative spectroscopic method that is used to measure the elemental composition, empirical formula, chemical state, and electronic state of elements that exist in a substrate. It is performed by irradiation of a substance with x-rays and measuring the kinetic energy and number of electrons that are ejected from the surface.

XPS was used to investigate the elemental composition of each substrate during the immobilization of heparin. This was done to show the presence of heparin on the surface after immobilization.

The measurements were performed using a K-alpha XPS instrument (Thermoscientific, UK). The x-ray source was from monochromatized Al operated at 72 W. The spot size was taken at 400 μm and for the composition determination the pass energy was 75 eV.

3.5 Platelet Adhesion Studies

3.5.1 Buffer

Buffer used for platelet adhesion experiments was 10 mM sodium phosphate buffer with 100 mM NaCl, pH 7.4. Sodium phosphate dibasic heptahydrate (1.08 g/L, Mallinckrodt Inc., Paris, KY) and 0.53 g/L of sodium phosphate monobasic monohydrate (FischerBiotech, Fair Lawn, NJ) were mixed together in DDW with the addition of the appropriate amount of NaCl. The buffer was then filtered with a 0.2 μm filter and stored at 4 ˚C until needed.

3.5.2 Platelet Rich Plasma (PRP) collection

Platelet-rich plasma was collected to test the biocompatibility of surfaces. Human blood was taken from healthy adult donors who had not used any prescription or other medications, including aspirin, within 14 days of donation and had not consumed any alcohol at least 3 days prior to donation. One part sodium citrate (3.8 % v/v) was added to 9 parts whole blood after extraction to inhibit clotting. Platelet counts were recorded after whole blood was extracted and ranged from 175,000-200,000 platelets/μL. The whole blood was then centrifuged at 22 ˚C, 205xg for 20 minutes to separate blood cells from platelet-rich plasma. The top two thirds of the PRP supernatant were taken off and a platelet count was taken. The PRP was used within one hour of separation.

3.5.3 Parallel plate flow chamber and flow loop setup

A parallel plate flow chamber was constructed for platelet adhesion studies. A schematic of the device is given in Figure 5. The device was constructed using a two-part polyurethane mixture, which was cast into a frame containing the negative of the features. The flow chamber contains an inlet and outlet reservoir to reduce or eliminate entrance and exit effects on the flow of the fluid. The height of the channel

between the top and bottom plate is governed by a 0.01" gasket tape. There is also a 0.002" layer of adhesive so the total channel height is given as 0.012" (0.03048 cm). The width and length of the channel is 4.5 cm and 3.5 cm respectively. Platelet adhesion experiments are performed under flow conditions because it better simulates the environment under realistic physiological conditions. Cells and platelets under flow conditions compared to static conditions will undergo a different adhesion process, experience different binding strengths, and exhibit different morphologies. For these reasons this study uses flow conditions in place of static conditions.

A continuous flow loop apparatus was used to perform platelet adhesion experiments. Platelet-rich plasma was pumped through the parallel plate flow chamber using a peristaltic pump, and then was then passed to a 50 mL holding flask. The flask was set in a 37 ˚C water bath to keep the PRP at constant temperature. The PRP was then circulated back through the pump and into the flow chamber. After each use the tubing used to carry PRP was discarded and replaced with new tubing. Also, after each use the flow chamber was first washed with 2 % alconox soap solution and then immersed in 5% (v/v) bleach solution at 4 ˚C overnight to kill any bacteria that might have been present.

3.5.4 Platelet adhesion experiments

The protocol used for treating samples after exposure to PRP during platelet adhesion studies was adapted from Pan et al. (2007). Silicon wafer samples to be used were incubated at 37 ˚C under 10 mM PBS, 100 mM NaCl pH 7.4 buffer for at least 30 minutes prior to use to equilibrate surfaces. Also, PRP obtained as described above was placed into a holding flask, which was then set in a 37 ˚C water bath for at least 30 minutes prior to the experiment. Silicon wafers were placed into the parallel plate flow chamber and all tubing was attached. Platelet-rich plasma was then circulated through the system in a continuous flow loop for 1 hour at a volumetric flow rate of 0.262 mL/sec and a corresponding shear rate of about 480 sec^{-1}. After 1 hour the device and tubing were flushed with 20 mL PBS buffer to remove the PRP in the system. The samples were then taken out of the flow chamber and washed gently three

times with buffer to remove any non-adhered platelets. After washing the samples, they were first placed in a 2.5% glutaraldehyde (Sigma) and left for 30 minutes at room temperature and then placed in a 5% glutaraldehyde for 120 minutes to fix the adhered platelets. Once the adhered platelets were fixed, they were then washed once with buffer. Samples were then dehydrated using a series of ethanol-water solutions (50, 75, 90, and 100%, v/v) for 10 minutes in each solution and then allowed to dry in a desiccator at room temperature.

3.5.5 Scanning Electron Microscopy (SEM)

Scanning Electron Microscopy (SEM) is a type of microscopy that scans a surface with a high-energy beam of electrons. The electrons that reach the surface interact with the atomic structure of the surface, which then produces different signals that are emitted from the sample. The types of signals that are emitted from the surface are secondary electrons, back scattered electrons, characteristic x-rays, cathodoluminescence, specimen current, and transmitted electrons. Using SEM it is possible to obtain high resolution images of the topography of the sample surface. This permits clear visualization of the shape, orientation, and amount of platelets that adhere to each surface.

Scanning electron microscope images were taken of each surface after the platelet adhesion experiments were performed. Before SEM imaging each sample was sputter coated with gold for 25 sec at 40 mA using a Cressington 108A sputter coater. They were then imaged using an AmRay 3300 scanning electron microscope. The number of adhered platelets was determined by a manual count on at least three images from each sample using a viewing window of 81 X 71 µm.

CHAPTER 4
RESULTS AND DISCUSSION

4.1 Surface Analysis

4.1.1 Contact Angle

Table 1 gives the contact angles on each surface during the immobilization of heparin. The bare untreated silica surface had the lowest contact angle, most hydrophilic, compared to the other samples. Upon treatment with APTES and succinic anhydride the surface became much more hydrophobic, as indicated by the contact angle of 47.72^{o}. After the immobilization of ADH-Hep the contact dropped to 34.95^{o}, making the surface more hydrophilic. Because heparin is a highly hydrophilic molecule it is expected that the contact would lower upon immobilization. However, the contact angle is higher than what would be expected for a heparinized surface, this is most likely due to incomplete surface coverage with heparin. It was also observed that the contact angle decreased upon immobilization of Hep-SH onto the EGAP coated surface, from 59.9^{o} to 40.08^{o}. Again, this is higher than what we would expect, but is most likely explained by bare hydrophobic regions on the surface.

4.1.2 X-ray photoelectron spectroscopy

Table 2 gives the elemental composition of each surface during the immobilization of heparin. A graphical illustration is also presented in Figure 6, detailing the data given in Table 2. For the method of ADH-Hep attachment, it is shown that the percent of silicon slightly decreased after treatment with APTES and succinic anhydride and decreased significantly after immobilization of heparin. If we assume that there is a uniform coating on the surface, then this decreasing trend in silicon indicates a thicker layer present on the surface of the substrate. Also, the increasing amount of carbon, oxygen, and nitrogen indicates the presence of organic groups on the surface after each stage of activation. It is assumed that the carbon present on the bare silica surface is due to contamination of the substrate with air or

dust particles. Successful ADH-Hep immobilization is indicated by the presence of sulfur on the ADH-Hep surface. Heparin is the only group that contains sulfur so it should only be present on the heparinized surfaces, which is confirmed by the data. In the case of the EGAP and Hep-SH immobilized surfaces, there was no sulfur detected. Both heparin and EGAP contain sulfur, so the absence on the surface indicates that neither of these components is present. The elemental composition of the EGAP coated surface is very similar to the bare silica surface, suggesting that there is little to no EGAP present on the surface. However, after Hep-SH immobilization the percent silicon decreased by 18% and the percent carbon increased by about 25%. This would indicate that there is something present on the bare silica surface. Although great care was taken drying the EGAP and Hep-SH coated surfaces it is possible that some of the material was taken off during this step. Because EGAP is attached to the silica surface by hydrophobic association only, when the surfaces are dehydrated and taken out of a hydrophilic environment there is no driving force to keep the EGAP on the surface, therefore it would be possible for the EGAP to be removed. This would explain the inconsistent results during the Hep-SH immobilization.

4.2 Platelet Adhesion

Representative SEM images of adhered platelets on each type of surface are given in Figures 8 and 9 at a magnification of 1500 and 3000, respectively. From the images obtained, the amounts of adhered platelets were counted manually and are recorded in Table 3 and given in Figure 7. The platelet amounts are recorded as the total amount of adhered platelets per square millimeter area. The images were also used to compare the behavior of the platelets after adhering to each surface. From visual inspection it is possible to determine the relative degree of aggregation and activation of the platelets on each surface after exposure.

From Table 3 we can compare the amount of platelets that adhered to each surface during the immobilization of ADH-heparin. There is a significant decrease in the amount of platelets that were present on the bare silica surface compared to that of

the ADH-heparin immobilized surface ($p<0.05$). There is also significantly fewer platelets adhered to the ADH-heparin surface than to the APTES and succinic anhydride treated surface ($p<0.02$). Also, there was a slightly higher number of platelets observed on the APTES and succinic anhydride treated surface than what was found on the bare silica surface, although the difference was not significant. It has been well documented that immobilizing heparin onto a surface will show increased non-thrombogenic properties (Klement et al, (2002); Keuren et al, (2003); Tsai et al, (2001)). The results show that this method for heparin immobilization does inhibit the adhesion of platelets and is, thus, less thrombogenic than that of the untreated surface.

From Table 3 we can compare the amount of adhered platelets during the immobilization of thiolated heparin onto non-covalently immobilized EGAP. It was found that there were significantly fewer platelets were adhered on the EGAP coated surface compared to that of the bare untreated surface ($p<0.05$). There has been much research that shows that pluronic coated surfaces will inhibit the adsorption of proteins and will show a high resistance to platelet adhesion (Li and Caldwell, (1996); Neff et al, (1999)). Also, there was significantly fewer adhered platelets on the Hep-SH than that of the EGAP coated surface ($p<0.005$). This result might suggest that the lessened thrombogenic response from the Hep-SH immobilized surface is due to the heparin molecule interacting with the plasma proteins and inhibiting the contact system. It is known that negatively charged surfaces will promote the activation of the contact system (Colman, (1984); Kaplan and Silverberg, (1987)). Heparin is a very highly negatively charged molecule and because of this it should promote the activation of the contact system and platelet adhesion, which would cause there to be an increased amount of adhered platelets onto the surface. Because we do not see this and the heparinized surfaces show less affinity towards platelet adhesion than the EGAP coated surface it is a reasonable conclusion that the surface bound heparin shows some sort of bioactivity.

When we compare the number of adhered platelets on the ADH-Hep immobilized surface to that of the Hep-SH immobilized surface, there was no significant difference between the two. However, although to not a significant degree,

there was slightly fewer platelets observed on the Hep-SH immobilized silica compared to the ADH-Hep immobilized surface. This could be due to the fact that the EGAP coated surface showed much less platelet adherence compared to the APTES and succinic anhydride surface ($p<0.02$). If we assume that the surface coverage of heparin is equivalent for each surface and that there are areas where there is no heparin present, than the EGAP surface underneath would inhibit platelet adhesion more than the succinic anhydride surface.

From Figure 8 and Figure 9 we can compare the relative degree of aggregation and activation of the adhered platelets on each surface. It was observed that the platelets that adhered to the bare hydrophilic silica experienced much more aggregation compared to the other surface. During the immobilization of ADH-Hep it can be seen that the APTES and succinic anhydride treated surface showed similar degrees of platelet spreading compared to that on the bare silica surface. As stated before, negatively charged surfaces will promote the activation of the contact system so we would expect some degree of spreading for both the bare silica and succinic anhydride treated surface. When compared to the ADH-Hep immobilized surface it can be seen that the platelets that did adhere to the surface were more spherical in shape indicating less activation and there is no apparent aggregation. This indicates that the ADH-Hep coated surfaces show a higher level of biocompatibility than non treated surfaces.

Similar observations can also be made during the immobilization of Hep-SH to EGAP coated surfaces. The platelets that adhered to the EGAP coated surface compared to the bare silica surface were more isolated and showed no aggregation as was seen on the bare silica surface. It was seen, however, that there were more platelets and more platelet spreading than expected on the EGAP coated surface. This could be attributed to the fact that there could be bare regions on the EGAP coated surface due to either incomplete coating or from replacement of plasma proteins. It has been shown that non-covalently coated pluronic surfaces will show a certain degree of elutability when exposed to proteins. The method used in this study to expose the EGAP coated surface to platelet rich plasma was performed in a dynamic system

which will impart a shear stress at the surface. This will likely cause an increased amount of EGAP elution from the surface, which will create more bare hydrophobic regions and thus promote cell adhesion and spreading. It was also observed that the platelets that adhered to the Hep-SH surface were more isolated and showed less spreading than that of the bare silica and EGAP coated surfaces. This observation is consistent with surface bound heparin showing decreased thrombogenic response.

Upon comparison of the aggregation and activation of platelets on the ADH-Hep immobilized surface to the Hep-SH surface there is no observable difference between the two. Both surfaces show no platelet aggregation and little spreading of the platelets on the surface of the sample. From these results, it is inconclusive whether one method of heparin immobilization shows better biocompatibility compared to the other.

CHAPTER 5
CONCLUSION

Successful immobilization of adipic dihydrazide modified heparin was achieved as indicated by contact angle and XPS data. Based on the findings in this study, the extent of immobilization of Hep-SH onto end-group activated triblock copolymer-coated surfaces is inconclusive. The number of adherent platelets on the heparinized coated surfaces was significantly lower than that for the unheparinized surfaces. Also, the platelets that adhered to the heparin coated surfaces were more scattered from each other and showed less spreading on the surface than on non-heparinized coated surfaces. This is consistent with less platelet activation.

The results of this study indicate that attachment of heparin using both reaction techniques for heparin immobilization show a higher resistance to thrombus formation and, thus, are more biocompatible than unheparinized silica.

CHAPTER 6

LIST OF FIGURES

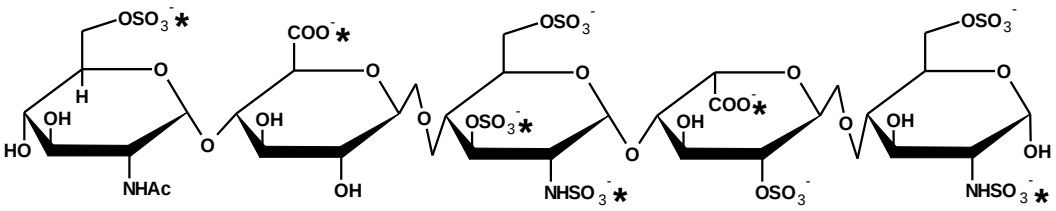


Figure 1: Pentasaccharide sequence of heparin responsible for binding to antithrombin III.

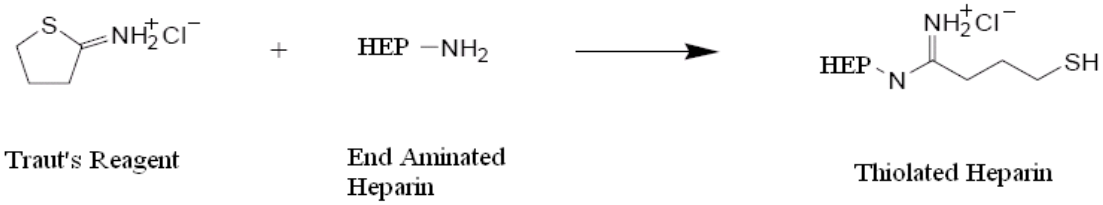


Figure 2: Reaction schematic of Traut's reagent with end aminated heparin.

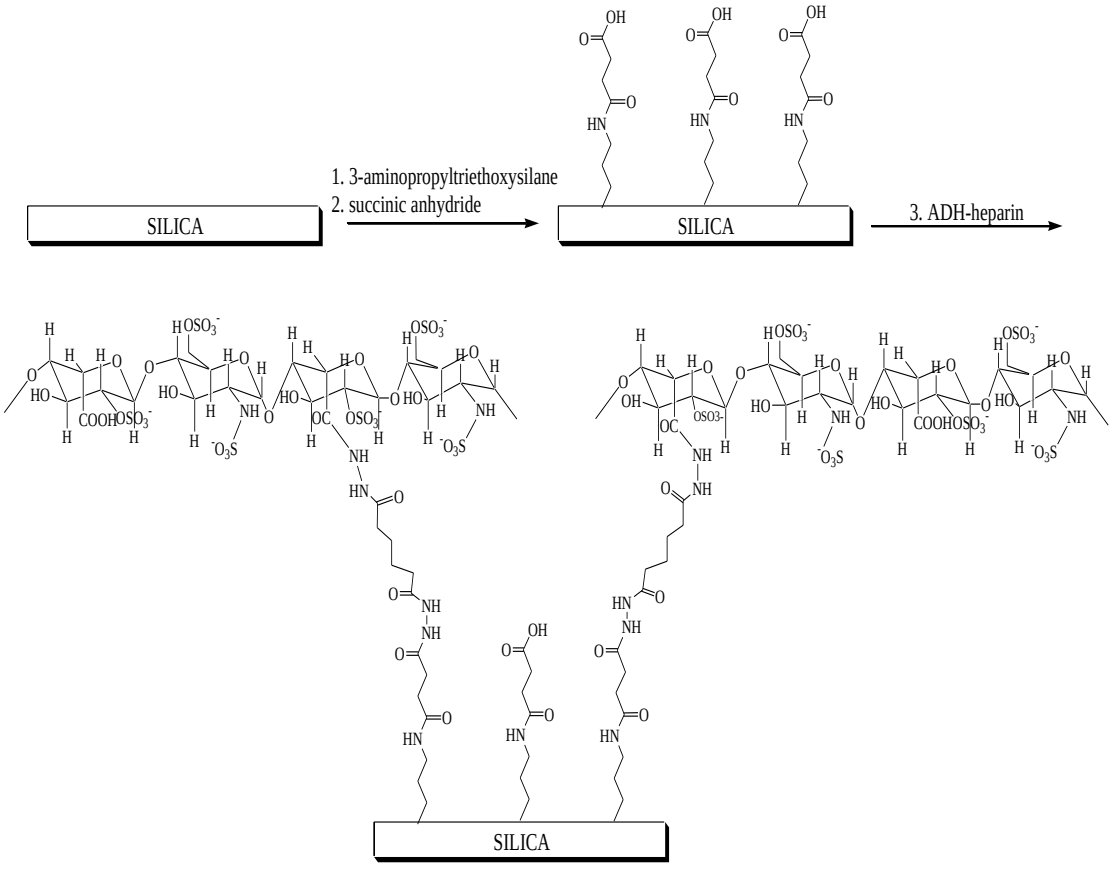


Figure 3: Immobilization of ADH-heparin on silica.

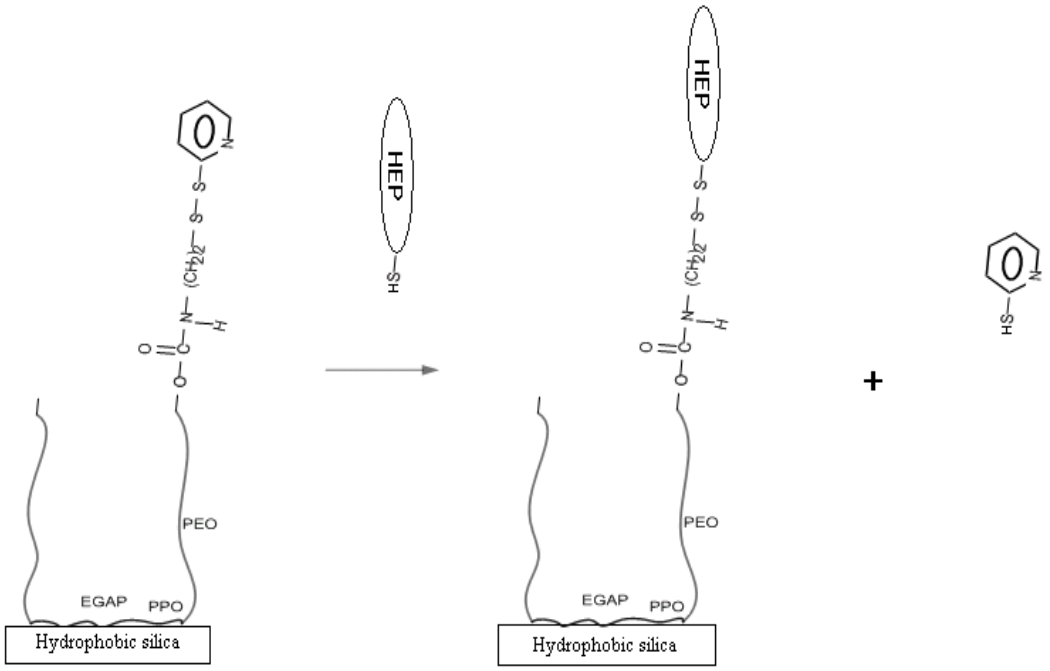


Figure 4: Immobilization of thiolated heparin with end-group activated pluronic.

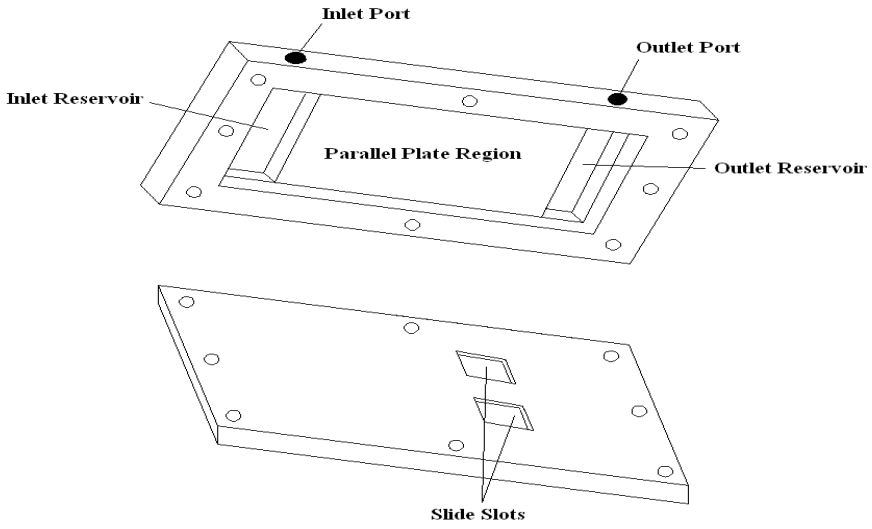


Figure 5: Parallel plate flow chamber used for platelet adhesion experiments.

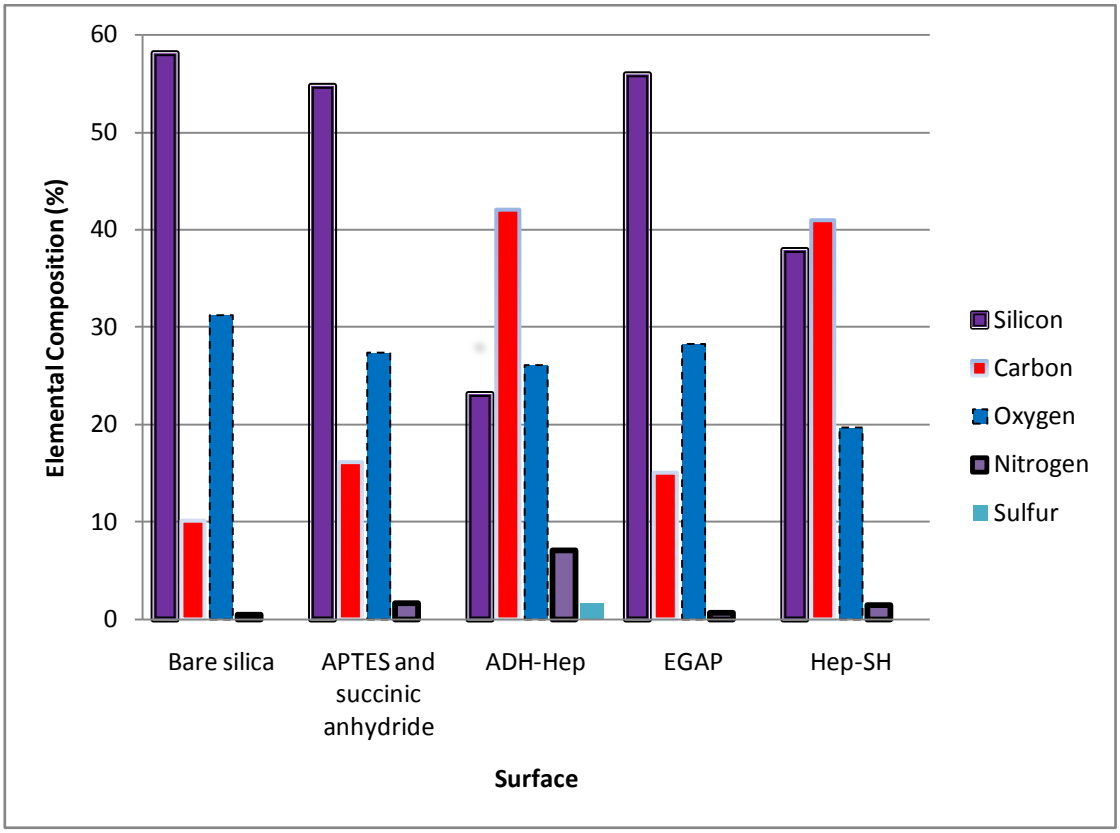


Figure 6: XPS results giving the elemental composition (%) on surfaces at each stage of heparin immobilization.

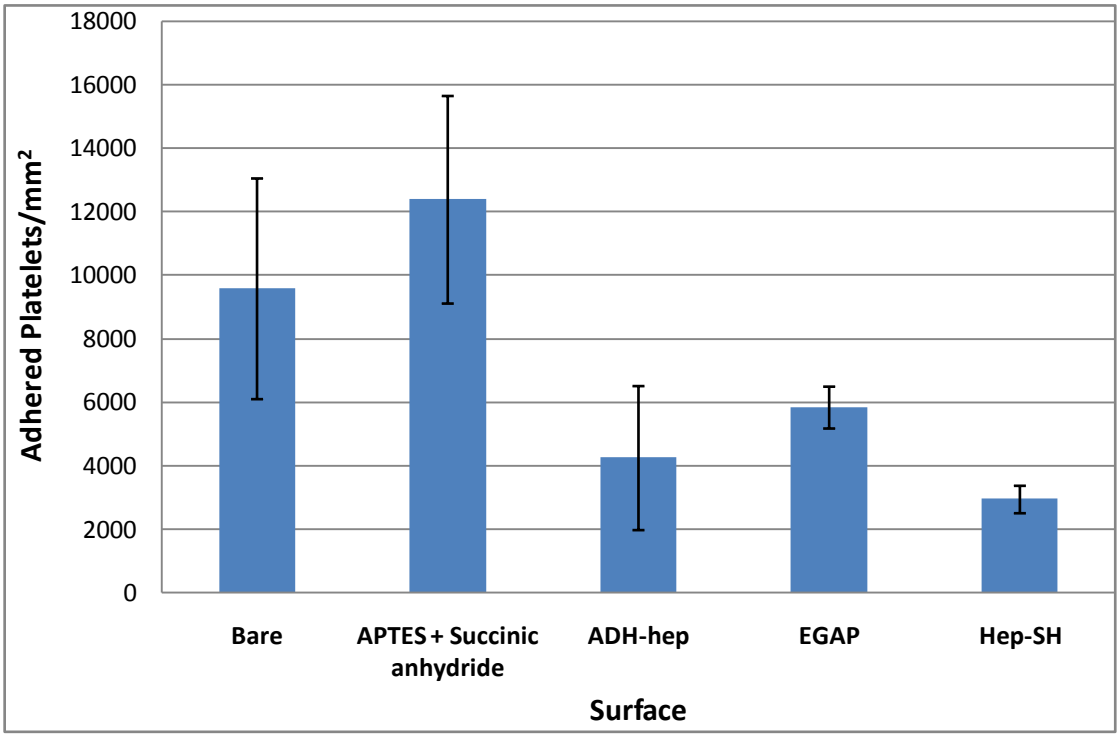
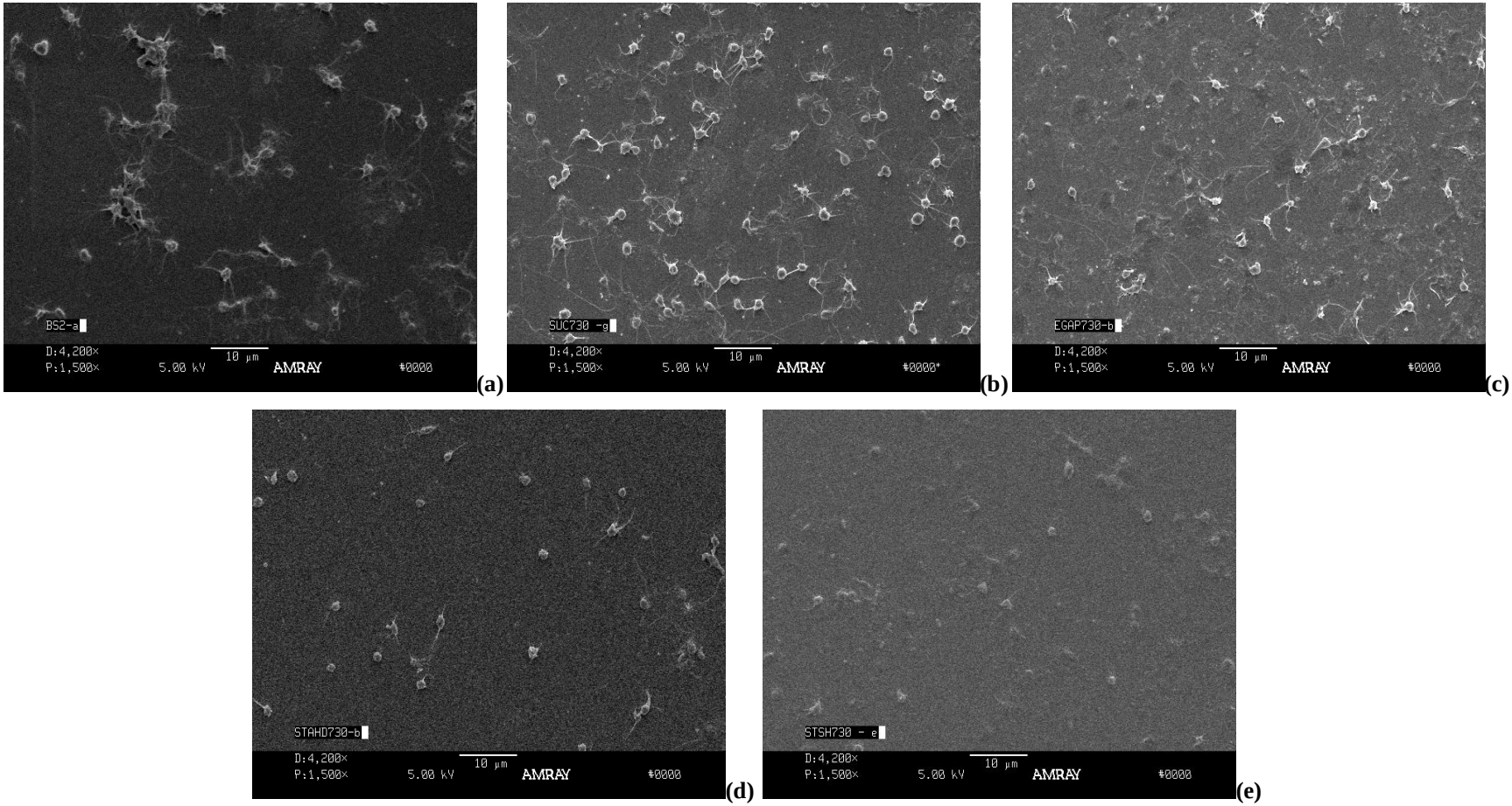


Figure 7: Number of adherent platelets on each surface during the immobilization of heparin after exposure to platelet rich plasma (error represents 95% confidence interval, n=3).



(a) **(b)** **(c)** **(d)** **(e)**

Figure 8: SEM images (1500X magnification) of platelets adhered to test samples: (a) bare, untreated silica; (b) APTES and succinic anhydride treated silica; (c) non-covalently immobilized EGAP onto PS200 treated silica; (d) ADH-Hep immobilized onto APTES and succinic anhydride treated silica; (e) Hep-SH immobilized onto EGAP.

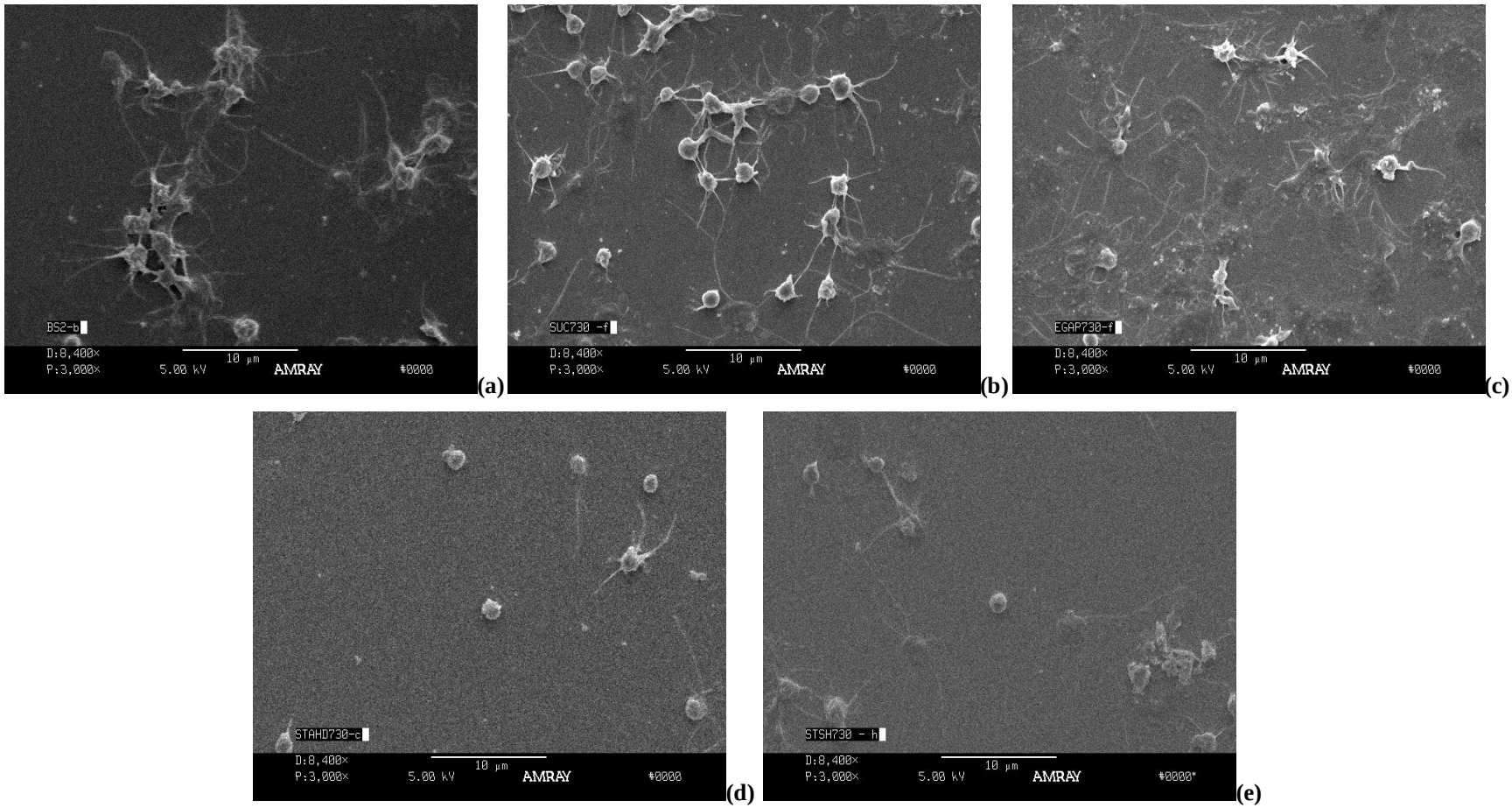


Figure 9: SEM images (3000X magnification) of platelets adhered to test samples: (a) bare, untreated silica; (b) APTES and succinic anhydride treated silica; (c) non-covalently immobilized EGAP onto PS200 treated silica; (d) ADH-Hep immobilized onto APTES and succinic anhydride treated silica; (e) Hep-SH immobilized onto EGAP

CHAPTER 7
LIST OF TABLES

Table 1: Contact angles of each surface during the immobilization of heparin.

Surface	Contact angle (^{o})
Bare silica	12.86 ± 3.65
APTES and succinic anhydride	47.72 ± 1.20
ADH-Hep	34.95 ± 1.05
EGAP	59.9 ± 5.23
Hep-SH	40.08 ± 1.07

Table 2: Elemental composition (%) of surfaces during immobilization of heparin.

Sample	Si	C	O	N	S
Bare silica	58.13	10.18	31.23	0.46	nd
APTES and succinic anhydride	54.73	16.16	27.42	1.69	nd
ADH-Hep	23.15	42.01	26.12	7.08	1.64
EGAP	55.91	15.1	28.28	0.71	nd
Hep-SH	37.91	40.95	19369	1.45	nd

nd : not detected

Table 3: Number of adhered platelets on each surface during immobilization of heparin.

Surface	Average number of platelets per mm^2	Standard deviation
Bare Silica	9588	1399
APTES and succinic anhydride	12392	1315
ADH-Hep	4257	913
EGAP	5848	265
Hep-SH	2953	174

CHAPTER 8

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