Hemocompatible polymer thin films fabricated by Electrostatic-Self Assembly (ESA)

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Abstract

Stent is one of the coronary angioplasty techniques that expands the narrowed coronary arteries due to the accumulation of fat, cholesterol and other substances in the lumen of the arteries. The major complication of stent is restenosis. Current development of drug-eluting stents shows successfully reduce the occurrence of restenosis. Other than using drugs, electrostatic self assembled (ESAd) thin films may be the potential candidates to prevent restenosis.

ESA is a process to fabricate thin films bases on the electrostatic attraction between two oppositely charges. We used this technique to fabricate four PVP films and four PEI films. All films were exanimated by XPS and AFM. XPS data showed our coatings were successfully fabricated on substrates. AFM images revealed PVP coating was uniform, but PEI coatings had different morphologies due to diffusion and pH during the process.

Three preliminary hemocompatibility testes were performed to evaluate the hemocompatibility of the coatings. Platelet adhesion study showed the thin films inhibited platelet adhesion. All thin films were able to inhibit coagulation and were less cytotoxic. The studies suggested the ESA films were potentially hemocompatible.

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Chapter 1: Introduction

Coronary heart disease (CHD) is a disease of the heart's blood vessels whereby arteries become narrowed because of the accumulation of fat, cholesterol and other substances in the lumen. A number of risk factors are associated with CHD such as diabetes, high blood pressure and obesity. Coronary angioplasty is a technique to treat CHD by inflating a balloon or a stent in the blockage site to relieve coronary narrowing. Nevertheless, through a natural process termed restenosis, the artery becomes narrowed after the procedure is performed. Restenosis can cause occlusion within days or up to six months after angioplasty is performed. A stent is a deformable wire mesh tube that is implanted into the narrowed coronary artery to reopen the blockage. The use of different materials for stents and their design may alter the cellular responses that initiate the development of restenosis, and platelets are believed to be involved in this process. To overcome this problem, drug-eluting stents have been developed and proven successful in recent clinical trails. The success of drug-eluting stents has encouraged the development of hemocompatible coatings on stents that inhibit restenosis. Here, electrostatically self-assembled (ESAd) thin films seem to be the optimal potential candidates to be the hemocompatible coatings due to the ability to control multiple properties in the coating. ESA is a technique based on the alternate adsorption of oppositely charged materials on a substrate surface to create a multilayer thin film. We believe using this technique and incorporating polymers will enable us to create a number of hemocompatible thin films. The design, synthesis and characterization of such films are discussed in this thesis. In this chapter, we discuss coronary heart disease, the use of

coronary angioplasty, the mechanisms of restenosis, the functions of platelets, and the theory of ESA.

1.1 Coronary Heart Diseases and Coronary Angioplasty

Cardiovascular diseases (CVD) have become the number one cause of death in the United States since 1900 and accounted for one out of every 2.6 deaths in 2001. Within CVD, coronary heart disease leads to 54% of all deaths. It is generally believed that the primary cause of coronary heart disease (CHD) is the formation of plaque, which is the accumulation of fat, cholesterol and other substances, which narrow the coronary arteries. The process of this narrowing is termed atherosclerosis, or hardening of the arteries. There are several risk factors associated with this disease. They are diabetes, high blood cholesterol, high blood pressure, obesity, physical inactivity and current cigarette smoking. Nevertheless, people react differently as a result of coronary heart disease. They may have chest pains (angina) for a period of time or a sudden heart attack (complete blockage of the coronary arteries). Angina can be divided into two types. Stable angina is a common form of chest pain due to the coronary arteries being too narrow to supply blood and oxygen. Patients feel pain, pressure or tightness in the middle chest, especially behind the sternum during exercise; however, this type of angina is predictable. In contrast, unstable angina is unpredictable. It causes a short-term chest pain and eventually develops an artery blockage that results in a heart attack. Depending on the level of coronary heart disease, patients may receive treatment in the form of medication, coronary angioplasty and/or coronary artery bypass surgery, but coronary angioplasty is the most common approach used today.

Coronary angioplasty is a common procedure used to reopen narrowed or blocked coronary arteries that allows normal blood flow. "Angio" and "plasty" mean "vessel" and "shaping" in Greek, respectively. The combination of these words indicates "vessel shaping" and that is the purpose of coronary angioplasty. This procedure can be divided into three categories, which are balloon angioplasty, the use of stents and laser angioplasty.[1] When a patient is diagnosed with coronary heart disease and requires coronary angioplasty, the procedure would be performed in a catheterization laboratory. The physician inserts an intravenous line (IV) in the patient's forearm to apply a mild sedative, and the patient receives an anesthetic in the arm, wrist or leg where a catheter will be inserted. The inserted catheter follows the artery to the aorta and reaches the coronary arteries. At this time, an iodine-containing dye is injected into to the heart so the physician can determine the size and locate the blockage by monitoring the image of the dye by coronary angiography. Coronary angiography is an x-ray technique spectifically developed to allow visual inspection of blood flow in and around the heart. It comprises an x-ray tube and a camera, which are rotated around the patient and also moving forward and backward from the patient's head. Once the dye is injected, it selectively blocks x-rays and effectively creates a visual roadmap of the artery along which the catheter is gradually pushed. A guidewire is first inserted to the blockage and followed by a balloon-tipped catheter that is positioned so the balloon lies inside the obstructed region of the artery. During inflation of the balloon, a special syringe pump is connected to the catheter and pumps a mixture of saline and an x-ray contrast agent to inflate the balloon. The contrast material helps the physician visualize the inflation process. Inflation usually starts at 1 or 2 times atmospheric pressure and slowly

increases to 8-12 atm or even 20 atm according to the manufacturer's specifications. The balloon is inflated for one half to two minutes, then deflated and inflated again until the coronary narrowing is relieved. The angioplasty generally is completed in around 1.5 to 2.5 hours. If a stent is employed, it is placed at the tip of the catheter and over the balloon. The inflation is same as in balloon angioplasty but the stent is made of a deformable metal that may be expended within the artery, where it retains its expanded shape and defines the inner diameter of the arterial wall. Laser angioplasty is also similar to the above two methods. The only difference is the use of a laser as a means to burn out plaque in the blood vessel rather than using mechanical methods to reopen the vessel. The advantages of coronary angioplasty over bypass surgery are that patients have shorter recovery time, only local anesthesia is required, invasive surgery is avoided and the cost is low. The current limitation of coronary angioplasty is restenosis. The purpose of this research is to develop stent coatings that inhibit restenosis and thus mitigate related limitations.

1.2 Restenosis

The primary complication of coronary angioplasty is restenosis. This is the process whereby the artery renarrows after angioplasty. Acute occlusions can occur in hours and up to several days after the procedure in 3% to 8% of total cases.[2] Restenosis has a 30% to 50% occurrence rate after three to six months.[3,4] Physicians usually define the reduction of the inner diameter of the coronary artery by more then 50% as restenosis, and angioplasty may be required again if necessary. Indeed, restenosis is a complicated biological response to the arterial injury associated with the insertion and inflation of the

balloon and the stent. It involves the interaction between coronary geometry and cellular response to the local environment, however, we can divide the cause of restenosis into two mechanisms, which are mechanical and cellular responses. These are discussed below.

Mechanical response relates to change of artery geometry. As the balloon is expanded within the coronary artery, it expands the inner diameter of the artery. The components of the blood vessel including smooth muscle cells and endothelial cells are thus strained radially and compressed axially. Once the balloon is removed, these components partially recoil so the vessel returns toward to its original shape as a result of negative remodeling. On the other hand, cellular response is more complicated than mechanical response. The characteristic of the response is the development of a neointimal hyperplasia that reduces the diameter of the lumen. Once the endothelium is injured, platelets in the blood are activated and start to deposit on the endothelium. They express their receptors and secrete enzymes to recruit other cells such as monocytes and lymphocytes to the injury area as well as initiate the deposition of extracellular matrix. Moreover, those signals trigger the migration and proliferation of smooth muscle cells that lead to myointimal thickening. Mechanical response can be eliminated by the insertion of a stent as a support in current practice. Nevertheless, cellular response is difficult to eliminate by stents because cells consider the stent a foreign material and react with it. Stainless steel, nitinol (a nickel-titanium alloy), polylactide compounds and PTFE are common materials for current stents. Current research is focused on drug-eluting stents to minimize cellular response and has demonstrated success via the

use of P-selectin glycoprotein ligand-1,[5] heparin,[6] and paclitaxel.[7]

1.3 Platelets

There are three cell types that exist in blood. They are erythrocytes **(**red blood cells), leukocytes **(**white blood cells) and platelets, where leukocytes have five subgroups. Erythrocytes are responsible for oxygen and carbon dioxide transport, and leukocytes focus on body defense. Platelets are responsible for bleeding control and trigger coagulation that leads to the formation of fibrin when a blood vessel is damaged. Platelets play an important role in the early phase of restenosis. An understanding of platelets and their interaction with an injured blood vessel, specifically the coronary artery after angioplasty for this discussion, may suggest a way to prevent or reduce restenosis after coronary angioplasty.

Platelets are disk-shaped cells with a diameter of 3-4 μm. They contain most of the features of an animal cell such as a cell membrane, receptors and mitochondria but do not contain nuclei. They are produced in the bone marrow and usually have average concentration around 250,000 platelets per microliter in circulation. Platelets only occupy about 0.3% of the total blood volume in comparison with red blood cells that occupy approximate 50% of the total blood volume. Although platelets are small in size, the activation of such small cells is quite complicated. Once they are activated, platelets start to express their surface receptors and shed enzymes to their surroundings that result in platelet adhesion, aggregation and recruitment of other cells. However, they do not play any role when they are not activated.

In the healthy stage, the endothelium in the lumen provides an antiadhesive and anticoagulant surface. It protects the vessel against the deposition of platelets, leukocytes, coagulation factors and other substances. Endothelial cells in the endothelium secrete nitric oxide (NO) to inhibit platelet activation at the same time. However, the endothelium becomes proinflammatory, proadhesive and procoagulant when it is damaged. It secretes von Willebrand factor (vWF) to trigger platelet adhesion and also sheds endothelial microparticles (EMPs) to activate monocytes for further reactions. When platelets are activated, the most important features are shape-change and the formation of pseudopodia, rod like structures that extend from the cell membranes, and that are easy to observe under the microscope. At the same time, they release platelet microparticles (PMPs), which are usually stored in granules until activation, to the local area. Those PMPs contain P-selectin that promotes leukocyte recruitment and provide anionic phospholipid sites for the vitamin K-dependent coagulation factors to assembly that results in coagulation. In addition, platelets also secrete ADP, serotonin and thromboxane A_2 to regulate coagulation and vascular tone, which is the degree of constriction experienced by a blood vessel relative to the maximum expansion. Glycoprotein Ib/IX (GP Ib/IX) and glycoprotein IIb/IIIa (GP IIb/IIIa or integrin $\alpha_{IIb}\beta_3$) are the two most important surface receptors on the platelet surface. GP IIb/IIIa is a fibrinogen receptor that uses fibrinogen to connect platelets together. Meanwhile GP Ib/IX binds to vWF that allows the platelets to adhere on the endothelium. After blood vessel damage, vWF from the endothelium becomes the key for platelet adhesion and aggregation. Circulating platelets start to activate and immobilize on the lumen surface through the binding of GP Ib/IX with vWF to form a

stable layer. Calpain, a thiol protease, receives signals from vWF and regulates the cytoskeletal signal that leads to actin reorganization and shape change.[8] The rest of the platelets in the blood attach to the layer as well. However, this attachment depends on the blinding strength of GP IIb/IIIa and fibrinogen between circulating and immobilized platelets. If the strengths are strong enough, circulating platelets will first attach on immobilized platelets and move along the layer until they form stable aggregation under shear flow. Otherwise, the circulating platelets would detach and return back to the blood. Kulkarni suggests that platelet aggregation is a multistep process that involves vWF exposure, a reversible phase and an irreversible phase of platelet aggregation.[9] In spite of the above platelet aggregation model, Soslau has discovered a unique pathway of platelet aggregation regulated by GP Ib under the effect of thrombin.[10] The pathway associates with internal calcium and a platelet release reaction that does not involve vWF. The existence of the pathway may explain why anti-platelet treatment fails to totally inhibit restenosis.

1.4 Electrostatic Self-Assembly (ESA)

The creation of layer-by-layer thin films has been at the frontier of science and engineering in the recent years, however, nobody recognized the importance of such techniques when Iler first developed this process by alternating absorption of two different charged materials.[11] Since the 1990s', Decher has expanded the work of Iler and demonstrated the capability of the layer-by-layer deposition to fabricate multilayer thin films.[12] The deposition is based on the alternative absorption of polycations and polyanions on charged substrate surfaces. It is possible to monitor the build-up process

using UV/Vis spectroscopy and small angle X-ray scattering (SAXS). The development of this process enables us to modify the substrate surface by adding extra properties provided by the polycations and polyanions.

Electrostatic self-assembly (ESA) (Figure 1.1) is a technique to build-up multilayer films by alternating the adsorption of two oppositely charged materials on a charged surface. It is based on the electrostatic interaction between positive and negative charge to build the layers. This process does not limit the use of materials for either the substrates or multilayers. These materials can be polymers and nanoparticles, cage-structured molecules and rods, as long as they have appropriate charges. In principle, the charged materials can cover the surface, and there is no limitation to the number of layers. The most important part of ESA is the substrate surface modification, for without it, no layer can be formed by electrostatic interaction. The substrate surface is straight forward to clean and carries negative charge by immersing it into concentrated sulphuric acid. This negatively charged substrate is then immersed into the polycation solution. Cationic molecules in the solution are able to attract to the anionic surface under electrostatic interaction. The concentration of the positively charged molecules determines how quickly the negatively-charged surface is covered with cations. Thus, the first cationic monolayer is formed. The resulting surface is washed with water to remove loosely attached charged molecules, and then dipped into the polyanion solution. Anionic molecules are adsorbed, and the surface charge is reversed to form the second monolayer. Repeating the steps of polycation adsorption, wash and polyanion adsorption in a cyclic manner, we are able to fabricate a multilayer thin film coating consisting of two

oppositely-charged molecules with different properties. The formation of such multilayers by ESA offers several advantages over conventional techniques for thin film formation including the following.

- **Tailor-made surface**: ESA processing allows the incorporation of different materials together under electrostatic interaction. Thus, we can custom modify the surface to become biocompatible[13] or antireflective[14] by incorporating the materials having such properties into the multilayer.
- **Manufacturing process at room temperature and atmospheric pressure**: ESA depends only in the interaction between charges and is independent of temperature and pressure. It allows us to process multilayer materials at room temperature and atmospheric pressure.
- **Self-assembly without additional processing:** The ESA process only requires two charged solutions and a substrate. There is no need for vacuum, heat exchange and catalysis in fabrication.
- **Controlled thickness of deposited coatings**: ESA is a technique to build up multilayers and allows deposition of charged layers one-by-one, where the thickness of the layers may be controlled.
- **No substrate size limitation**: The substrate is dipped into charged solutions to trigger electrostatic attraction instead of being exposed to molecules in the gaseous phase. As long as the substrate can be completely immersed into the solutions, the ESA process builds up layers by surface charge reversal. Multilayer films of any shape and size may

be coated. Of particular importance here, our study proves ESA is capable of creating multilayer thin films on the inner surface of dialysis tubing.

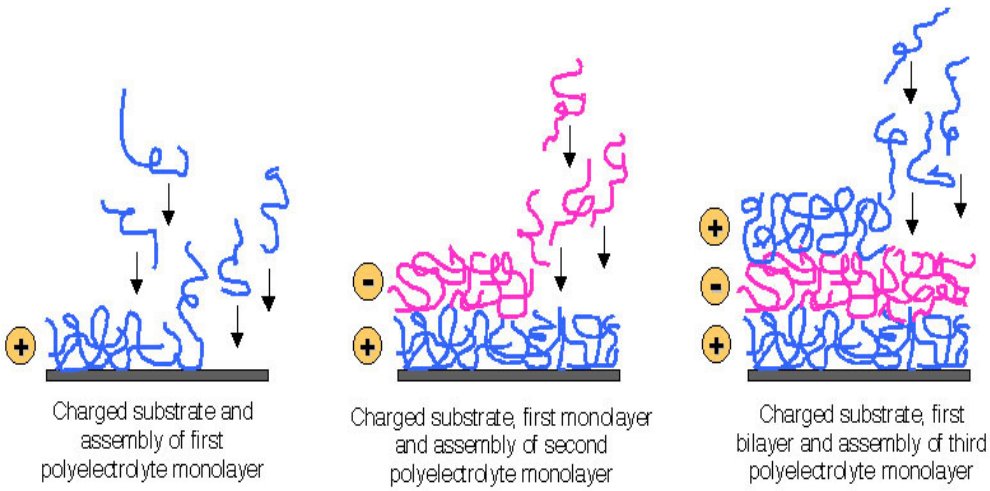

Figure 1.1. The principle of electrostatic self-assembly.

The formation of the multilayer coating is based on the addition of cationic and anionic molecules to the surface. This multilayer can be divided into three zones (Figure 1.2) as Ladam proposed.[15] These are the precursor zone (I), core zone (II) and outer zone (III). The precursor zone is the first few layers near the substrate. The core zone is the main body of the multilayer and the outer zone is the outermost layers. Zones I and III are usually have net charges because zone I gets attached to the substrate surface to provide firm adhesion, and zone III has net charges that are required to recruit opposite charges. In contrast, zone II is a mixture of polyanion and polycation complexes under a 1:1 stoichiometric ratio. These polyelectrolyte complexes are composed of the anionic and cationic groups belonging to different polymer chains, and these chains adhere together

by electrostatic attraction. This three-zone model exists when there are enough layers to compose the coatings and the layers vary from material to material. Otherwise, only Zones I and III exist. By adding layers on the substrate, Zone II would be formed between Zones I and III, and this zone will increase its thickness due to the diffusion of charged molecules from Zone III. The precursor and outer zones consist of only a few layers at this time. It is interesting to notice that the boundaries between Zone I and Zone II and between Zone II and Zone III are fuzzy instead of well defined. This may relate to the nature of the three zones where Zone I and Zone III are in layer from but Zone II is more likely in a mixture of polyelectrolyte complexes due to diffusion of charged materials.

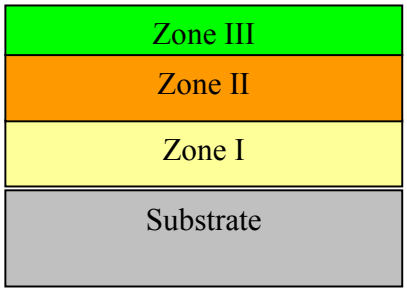

Figure 1.2. Three-zone model of multilayer.

During the fabrication of ESA thin films, the charge density of the substrate surface, the charge of the molecules, the concentration of the molecules and the pH are key parameters that influence the formation of the multilayers. Other than these, salt is another factor that influences the multilayers, though the "so called" salt effect.[16] Salt itself can penetrate into the multilayer and form complexes with both the cationic and

anionic molecules. Such penetration results in an increase in the thickness of the coating. In addition, salt can be used to geometrically smooth the multilayer surface by enhancing the mobility of the charged polymers.

1.5 Motivation of This Research

Coronary angioplasty is a procedure to treat coronary heart disease, but restenosis complicates that procedure. It can start nearly immediately or within three to six months after the procedure. Depending on the level of restenosis, patients may require retreatment that raises medical cost. Researchers are working on different stent designs, stent materials and adding drugs into the stents to overcome this complication, and platelets play an important role in the early stage of restenosis. Other than potentially being useful for the adding of drugs on the stent surface, ESA may be another alternative to fabricate a polymer or other material coating which can reduce restenosis. The use of polyanimo acids and other polymers in self-assembled monolayers enhances the cell growth and adhesion of endothelial cells,[17] fibroblast cells,[18] osteoblast-like cells[19] and even green algae.[20] On the other hand, Serizawa and coworkers have demonstrated that layer-by-layer assembly is capable of fabricating an anticoagulation coating.[21]

The objective of this work is to investigate the possibility of using ESA process to fabricate hemocompatible thin films that can become stent coatings to prevent restenosis. The tasks of this investigation are as follows.

- Design and fabricate polymer thin films formed by ESA process

- Characterize the compositions and morphology of the films using XPS and AFM on glass beads and gold-coated glass, respectively.
- Determine the effect of cytotoxicity of the polymers and ESA coatings on platelets.
- Investigate platelet adhesion on the ESA coatings.
- Measure the effects of clot mass formation that are influenced by ESA coatings.

1.6 Thesis Outline

This thesis is organized to fours chapters. Chapter 1 introduces the background information of current challenges on coronary heart disease and our purpose of using ESA thin films to overcome such challenges,

In Chapter 2, the fabrication and characterization of polymer thin films formed using the ESA process is discussed. We also present the use of soluble polymers and ESA processing parameters. Finally, we show the results of ESA thin films characterized using UV/Vis spectroscopy, x-ray photoelectron spectroscopy (XPS) and atomic force microscopy (AFM).

Chapter 3 focuses on *in-vitro* hemocompatibly tests of the ESA coatings. As platelets are important in restenosis, we demonstrate the ability of the coatings to inhibit platelet adhesion and exhibit low toxicity to the platelets. In addition, we examine the ability of the coatings to inhibit coagulation when whole blood is applied.

The last chapter is Chapter 4, in which we draw conclusions from the present work and suggest an outline of possible future work.

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Chapter 2: Fabrication and Characterization of ESA Films

Electrostatic Self-Assembly (ESA) is a spontaneous thin film fabrication process that is based on the electrostatic attraction between two oppositely-charged aqueous materials which deposit on a charged substrate surface. This process allows the thin film fabrication to be performed under room temperature and ambient conditions, and we are able to control the properties of the films such as thickness, chemical composition, structure and functionalities. Although we are using polyamino acids to fabricate the ESA coatings, inorganic nanoparticles, DNA and proteins are also candidates for the ESA process.

In this study, we investigated the potential of ESA thin films as a hemocompatible material to prevent activation of platelets that leads to coagulation. Two polycations (PVP and PEI) and four polyanions (PMA, Hyaluronic acid, Heparin and Poly-l-glutamic acid) have been used to fabricate eight ESA thin films on polyurethane catheters, 5mm diameter glass beads and gold coated glass. Here we employed X-ray photoelectron spectroscopy (XPS) to characterize the surface compositions of the ESA films on the glass beads and compared the differences of the PVP and PEI coatings. The morphology of the films were examined by atomic force microscopy (AFM). The PVP films showed great consistence in morphology and roughness, but the PEI coatings had a great variation from one to another.

2.1 Fabrication of ESA Thin Films

ESA is a process to produce multilayer thin films by the electrostatic attraction between two different charged materials. The materials can be polymers, ceramics or metals as long as they are soluble in water or in colloid form. The most important part is that all materials must be able to carry either positive or negative charges. Before ESA process, all substrates need to be cleaned in order to remove impurities on the surfaces, and this procedure would modify the surface charge on the substrates

2.1.1 Materials

To fabricate a number of ESA thin films, we used two polycations and four polyanions from six polymers. These combinations enabled us to form eight ESA thin films. The following is a list of polymers that we used:

- Poly(vinylpyrrolidone) (PVP)

  PVP is a water-soluble polymer and in powder-form when it is dry. It has been used as a urinary tract biomaterial to prevent bacterial biofilm formation that may cause blockage of urethral catheters and ureteral stents.[1] In our study, it is used as a cationic carrier to develop a first layer that attaches to the substrate surface and continues to build-up multilayers. We purchased this polymer from Sigma (Cat# 85656-8, St. Louis, MO) with molecular weight (M_w) 55000. Figure 2.1 shows the structure of PVP.

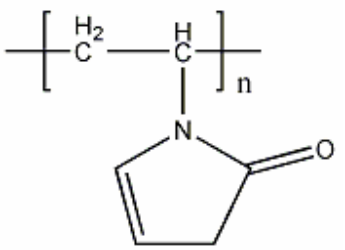

Figure 2.1. Structure of Poly(vinylpyrrolidone) (PVP).

- Poly(ethylenimine) (PEI)

PEI is a viscous polymer purchased from Sigma (Cat# 18197-8, St. Louis, MO) with molecular weight (M_w) 750000. It is widely applied in drug delivery systems to transfer genes into living cells in gene therapy.[2-4] We use it as a positive charge carrier to built-up ESA thin films. The structure of PEI is the following:

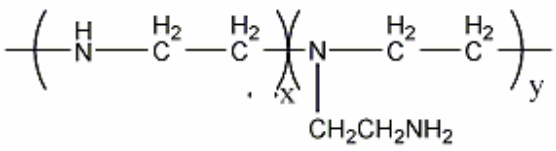

Figure 2.2. Structure of Poly(ethylenimine) (PEI).

- Poly(methacrylic acid) (PMA)

PMA is another viscous polymer purchased from Sigma (Cat# 434493, St. Louis, MO) with molecular weight (M_w) 6500. Researchers are using this polymer with polycaprolactone to improve the cytocompatibility with endothelial cells.[5] This is an anionic carrier for forming ESA films with both PVP and PEI. Figure 2.3 shows the structure of PMA.

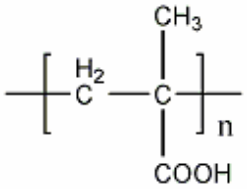

Figure 2.3. Structure of Poly(methacrylic acid) (PMA).

- Poly-l-glutamic acid

Poly-l-glutamic acid is a biocompatible polymer that is commonly used in drug delivery systems[6] and is potential of use in the treatment of thrombosis by controlling the release of clot-dissolving tissue-type plasminogen activator.[7] We purchaed it from Sigma (Cat# P-4761, St. Louis, MO) with molecular weight (M_w) 17000 and used it as a polyanion. The structure is as follows:

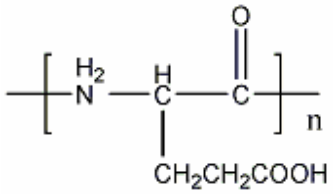

Figure 2.4. Structure of Poly-l-glutamic acid.

- Heparin

Heparin has been used as a clinical antithrombotic agent since the 1940s, and researchers are working on its anticoagulation mechanism.[8] It binds to antithrombin (AT) to form a heparin-AT complex to inactivate coagulate enzymes such as thrombin factor (IIa) and factors Xa, IXa, XIa, and XIIa. Heparin is one of the polyanions that has been employed in the ESA process. We purchased this in powder form polymer from Celsus Laboratories (Cat# PH-43901) with molecular weight (M_v) 13000. Figure 2.5 is its chemical structure.

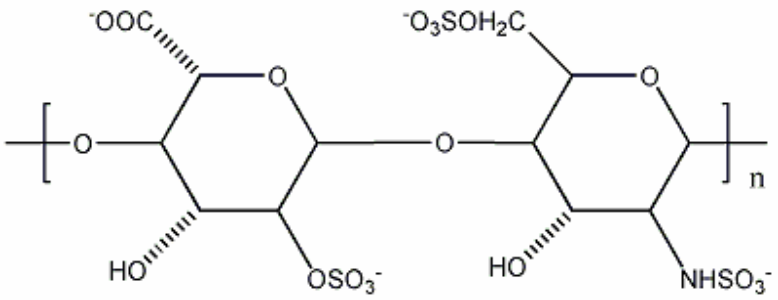

Figure 2.5. Structure of Heparin.

- Hyaluronic acid

Hyaluronic acid is one of the components in the extracellular matrix. It is rich in soft tissues and functions as a lubricant. This material is used to help treat osteoarthritis patients by providing pain relief and improving joint-motion.[9] Hyaluronic acid is our last polyanion that associates with both PVP and PEI to form ESA films and is purchased from Sigma (Cat# H1876, St. Louis, MO). The structure of hyaluronic acid is shown in Figure 2.6.

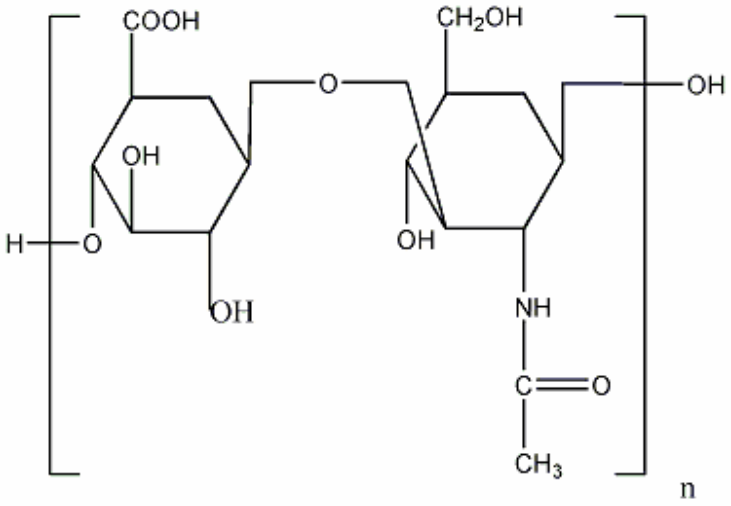

Figure 2.6. Structure of Hyaluronic acid.

2.1.2 Substrate Modification

For ESA process, it is important to modify the substrate surface. The modification is to clean and remove impurities from the substrate surface prior the ESA process, and it would modify the surfaces to have charges. Without modification polyelectrolytes cannot adhere on the substrate by electrostatic attraction. We used 5mm-diameter glass beads (silicon dioxide), gold-coated glass and polyurethane catheters as substrates for the ESA process. To use them, we performed the following modifications

5mm-diameter glass beads (Table I) were immersed in a mixture of concentrated sulphuric acid and hydrogen peroxide in 7:3 volume ratio for 30 minutes at room temperature to remove impurities and created negative charges. After the immersion, the beads were washed extensively with deionized water. The beads were directly put into the ESA dipping machine (Nanostater V, nanoStrata Inc, Tallahassee, FL) for ESA processing without further modification.

Gold-coated glass (Table I) was first immersed in acetone for three minutes and then washed extensively with deionized water. Later, the glass put into ethanol for another three minutes and rinsed with deionized water again. After the above procedures, the gold-coated glass was dried under nitrogen and directly ran through ESA process after modification.

Finally, polyurethane catheters (Table I) were sectioned into small pieces with 2mm long and placed into 50 vol % sulphuric acid for 1 minute to remove impurities and created

negative charges.. All catheters were rinsed with deionized water intensively and then used in the ESA process without further modification.

Table I: Surface modifications on substrates

Substrates	Modifications
5mm glass beads	Immersed in a mixture of concentrated sulphuric acid and hydrogen peroxide in 7:3 volume ratio for 30 minutes at room temperature
Gold-coated glass	Immersed in acetone for three minutes and then in ethanol for three minutes
Polyurethane catheters	Immersed 50 vol % sulphuric acid for 1 minute

2.1.3 ESA Process

Eight ESA polymer thin films were prepared from two polycations (PVP and PEI) and four polyanions (PMA, Poly-l-glutamic acid, Heparin and Hyaluronic acid) on glass beads, gold-coated glass and polyurethane catheters using Nanostater V dipping machine (source). For the ESA process, all thin films were fabricated under the same procedures except pH value. Substrates were immersed in both polyanions and polycations for three minutes to allow both charges to have enough time to interact together and build-up multilayers. Before each polyelectrolyte immersion, the substrates were rinsed with pH adjusted deionized water, which was close to the pH value of both polyelectrolytes, for 45 seconds and three times. This procedure washed off polymers that were loosely attached on the substrate surface and prevents contamination of the polymer solutions. In addition, the water pH should be closely matching the pH value of both solutions, otherwise, the conformation of the polyelectrolytes would be changed which reduced the interaction strength of both charges. The alternating layer-by-layer absorption in the dipping machine built 30 bilayers, in which 1 bilayer is a combination of a positive and a

negative layer, of ESA thin films, and the outermost layer was polyanion. The conditions of ESA processing are shown in Table II.

Table II: ESA Thin Films Fabrication Conditions

Polymer Combinations	**Concentration of Polymer Solutions (M)**	**pH of Polymer Solutions and Deionize Water**	**Dipping Time (min)**
PVP/PMA	10^{-3}	5±0.1	3
PVP/Poly-l-glutamic acid	10^{-3}	5±0.1	3
PVP/Heparin	10^{-3}	7.2±0.1	3
PVP/Hyaluronic acid	10^{-3}	5±0.1	3
PEI/PMA	10^{-3}	5±0.1	3
PEI/Poly-l-glutamic acid	10^{-3}	8±0.1	3
PEI/Heparin	10^{-3}	7.2±0.1	3
PEI/Hyaluronic acid	10^{-3}	9±0.1	3

2.2 Characterization of ESA Thin Films

Once the ESA thin films were fabricated, they were characterized. All coatings were very thin and essentially colorless. Spectroscopy was the technique that we employed in the investigation. Here we used x-ray photoelectron spectroscopy (XPS) to examine the surface compositions, and atomic force microscopy (AFM) to characterize surface morphology after the ESA process.

2.2.1 X-ray Photoelectron Spectroscopy (XPS)

X-ray photoelectron spectroscopy (XPS) (Figure 2.7) or so called electron spectroscopy for chemical analysis (ESCA) is an electron spectroscopic method to determine the quality of elements on a sample surface. It was first developed by K. Siegbahn and his research group in the mid 1960s. The principle of this spectroscopy is using x-ray as an excitation source to knock out electrons from the orbitals of elements that are located on a sample surface. Those electrons or photoelectrons are detected by a sensor and output data is used to generate a plot of binding energies that are associated to the oxidation state of the elements on the sample surface. In common operation, a sample is placed in a high vacuum chamber, and x-rays will knock out electrons of the elements at the sample surface. The energy of the electrons (binding energy) is analyzed by an electrostatic analyzer and the electrons are detected by a multichannel detector. According to the binding energy and electrons, XPS generates a spectrum of binding energies with a series of peaks associated with the oxidation state of the elements. Although the shape of the peaks may vary due to chemical environment, the peaks of each binding energy are characteristic of each element, allowing determination of the composition on the sample surface. In addition, XPS can scan the sample up to 20 atomic layers in depth. Such advantages allow us to use XPS to determine the compositions of multilayer[10] and biomaterials.[11]

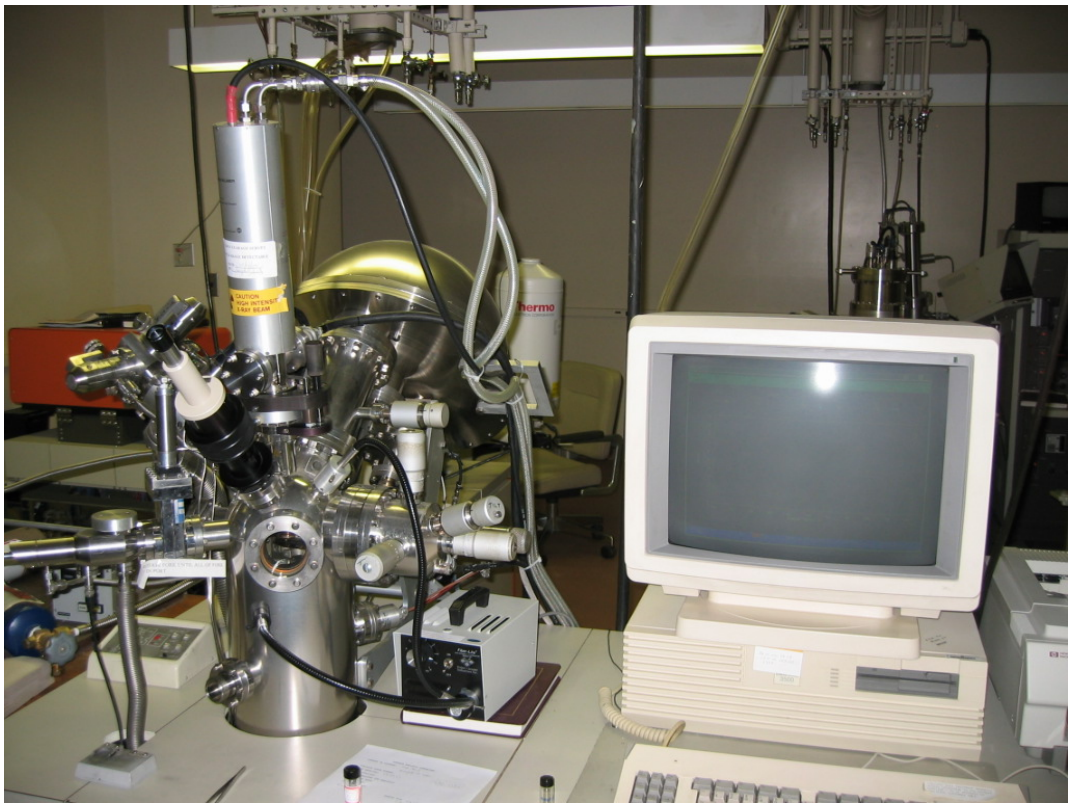

Figure 2.7. Image of XPS (left) and computer analyzer (right).

5mm diameter glass beads that were coated with eight ESA coatings were placed in a desiccator for several days to remove moisture. Analytical experiments were performed using a Perkin-Elmer-5400 XPS equipped with Mg X-ray source at 1200.53eV. The spectra of each coating were taken at 44.75eV and 17.9eV for wide angle scan and small angle energy scan, respectively, and the energetic resolution with calibration were at 0.9eV Ag 3D. Throughout the experiments, each scan was taken at 45^{o} take-off angle and 54^{o} incident angle.

The results of XPS wide-angle scan for the ESA coatings were shown in Figure 2.8. According to the graph, silicon peaks existed at 100 eV and about 160 eV. These peaks

representing Si were from the substrate which was silicon dioxide, and the ESA film was thin enough to allow the XPS to detect the substrate composition. The carbon peak at about 280 eV was from the carbon in the polymer chains of the polyanion and the polycation in each ESA thin film. On the other hand, the oxygen peak at around 530 eV was the mixture of oxygen from the glass bead and the coating. Although we knew carbon and oxygen exist on the surface based upon the XPS results, it was difficult to determine if the ESA coating was successfully fabricated on the substrate or not because these two elements could be contaminations from the atmosphere. To address this issue, we looked for specific elements that were used to compose the polymers such as nitrogen and sulphur. The nitrogens were in PVP, PEI, Poly-l-glutamic acid, Heparin and Hyaluronic acid and the sulphur (Figure 2.9) from Heparin were good indicators to identify the ESA coatings and the specific polymers.

XPS is a powerful tool to determine surface composition. During our experiments, we found the differences between the ESA coatings composed of PEI and the coatings that contain of PVP (Figure 2.10). The areas of Si peaks for PEI coatings were smaller than those in PVP coatings. As the peak areas in XPS represent the relative amount of the elements on the substrate surface, the small areas of Si peaks for PEI coatings indicated that the PEI coatings had more polymers and were thicker than the PVP coatings with larger Si peak areas. In addition, oxygen peak areas of the PEI coatings were smaller than those in the PVP films. Such area reduction simply told us that XPS was detecting more oxygen from the films whether than from the substrate itself. Moreover, the carbon peak areas of the PEI coatings were larger than the PVP coatings which meant

that the PEI films were thicker than the PVP films. ESA films were successfully fabricated on glass beads and XPS was able to detect specific polymers in the films based upon the composition elements such as nitrogen and sulphur. XPS data suggested the PEI coatings were thicker than the PVP coatings, however, it might due to the PEI coatings were denser then the PVP coatings. To confirm the thickness issue of the thin films, we have to use ellipsometery to determine the thickness of the coatings

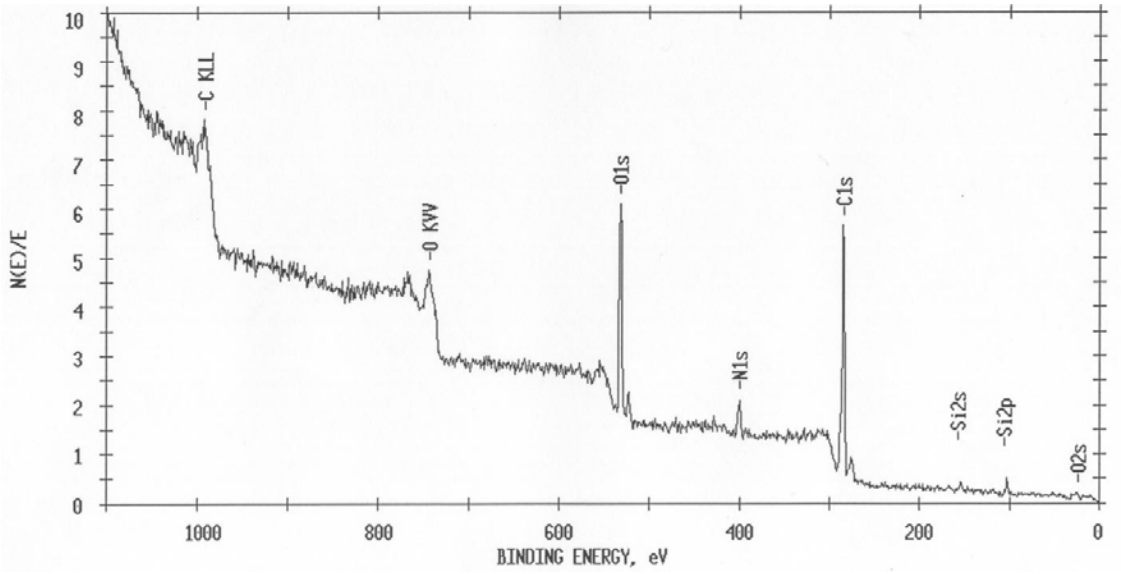

Figure 2.8. XPS wide-angle scan of PEI/PMA coating on glass bead.

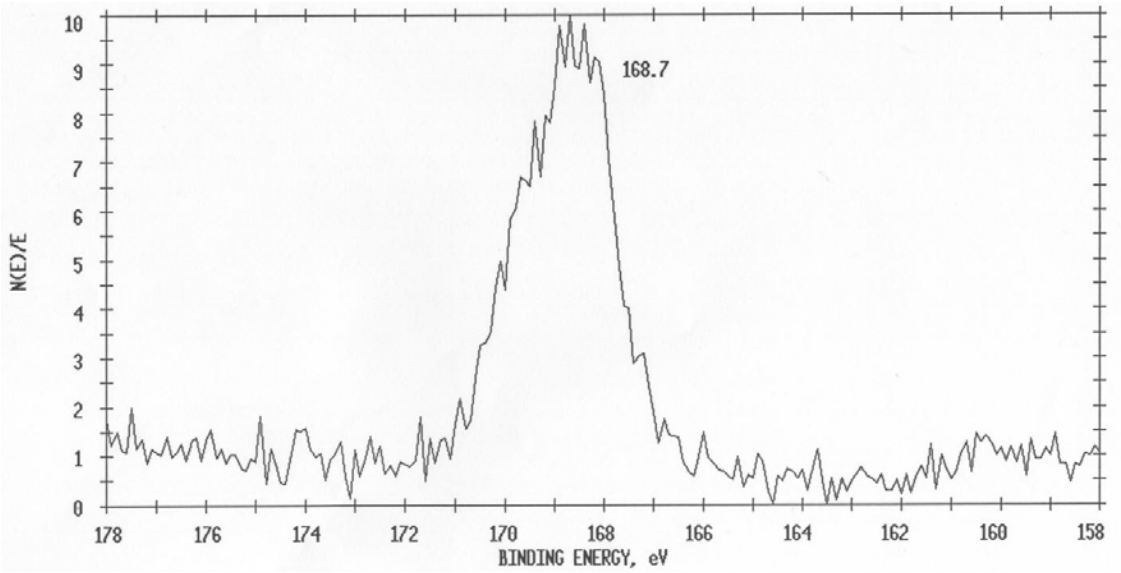

Figure 2.9. XPS small-angle scan of PEI/Heparin coating on glass bead showing sulphur peak at 168.7eV.

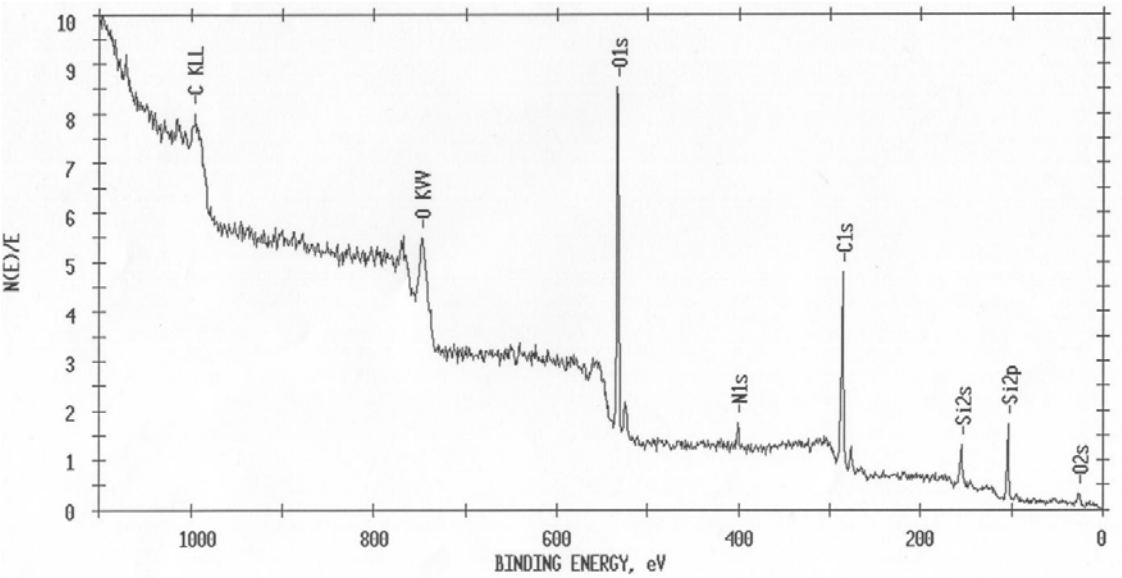

Figure 2.10. XPS wide-angle scan of PVP/PMA coating on glass bead.

2.2.2 Atomic Force Microscopy (AFM)

Other than XPS, atomic force microscopy (AFM) (Figure 2.11) is another technique for us to characterize the ESA films that we have fabricated previously. AFM is a member of the scanning probe microscopy (SPM) family. AFM utilizes a shape tip made from either Si or Si_3N_4 to scan the sample surface back and forth in a close contact form to measure the interaction between the tip and the sample. During the scanning phase, AFM has a feedback mechanism that enables a piezo-electric scanner to maintain the tip at constant force to obtain height information or keeps the tip at constant height to obtain force information above the sample surface. By recording the location of the tip and force/height information, AFM itself is able to produce the surface topography of the sample down to nanometer resolution. Such resolution helps us determine the morphology of the materials on the surface according to different applications. Currently, contact, non-contact and tapping mode are the popular ways to characterize surface morphology. In the contact mode, the tip scans the sample surface in close contact and maintains a mean force at 1nN between the tip and surface to generate surface topography. On the other hand, the non-contact mode produces surface topography by maintaining the tip at 50-150 angstroms above the surface during scanning. The tapping mode is somewhat between the contact and non-contact mode. This mode oscillates a cantilever around its resonant frequency by using a piezoelectric crystal with an oscillating amplitude larger than 20nm. This oscillation allows the tip to alternate contact with the surface and maps the surface morphology. AFM is employed in characterizing polymer morphology,[12] self-assembled monolayers[13] and biomaterials.[14]

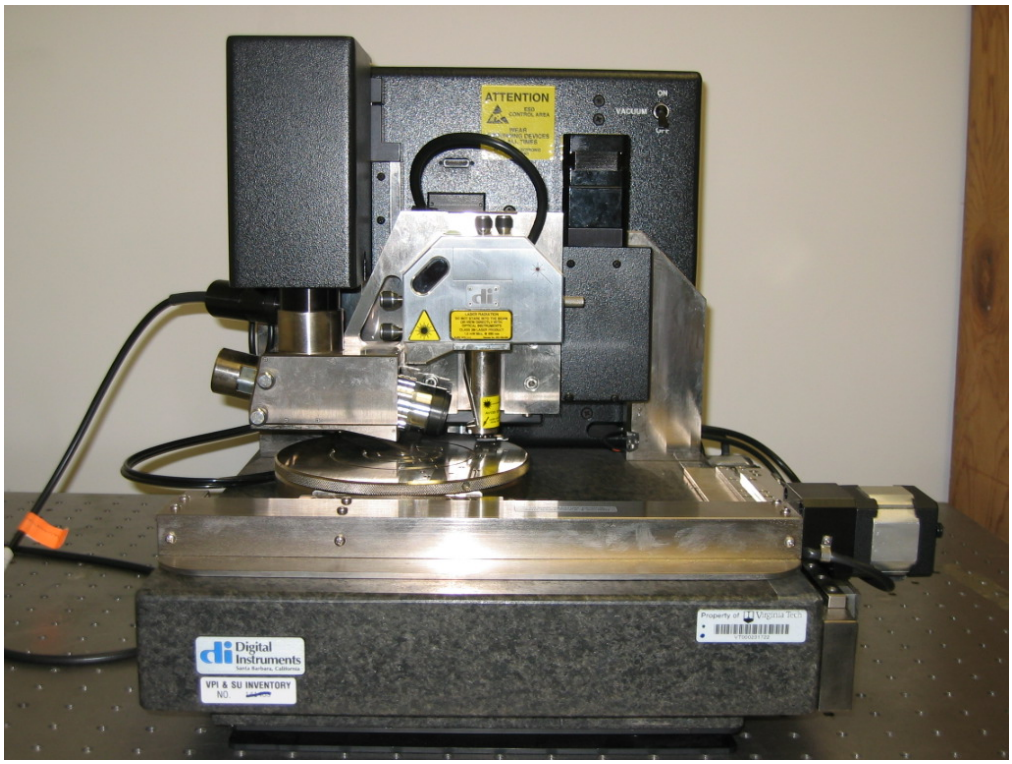

Figure 2.11. Image of atomic force microscopy.

In characterizing ESA thin films on gold coated glass, we employed a Nanoscope III (Digital Instruments, Inc.) atomic force microscope and used it at room temperature in ambient conditions at tapping mode. The advantages of tapping mode are enable us to image sample surfaces that are easily damaged, and loosely hold to their substrate. It overcomes problems associated with frictions, adhesion, and electrostatic forces. During operation, the tip ran under the cantilever frequency (usually 250-325 kHz). We scanned the films at the center of the glass sample with a scan size in of 2, 2.5 and 5 μm and the scanning rate was 1 Hz.

Figure 2.12 was the phase image of PVP/Heparin that we have fabricated for the study. For phase image, a probe is oscillated vertically above the sample surface. As the probe

lightly taps the surface, the phase signal changes in compare with the phase signal of the driving oscillator. AFM uses these signals to generate phase images with bright and dark regions, where the regions represent different compositions. The PVP/Heparin image indicated the film was uniform and the grains on the film were homogeneously without sign of damages or defects. Moreover, the film was in same colour region. It suggested the film has same compositions without contaminations. The roughness analysis indicates all PVP coatings have similar roughness which range around 1nm (data no shown). Figure 2.13 is the phase image of PEI/PMA. The image had similar features as PVP samples. In contrast, the PEI/Poly-L-glutamic acid coating (Figure 2.14) showed a completely different morphology. The gains on the surface had different sizes, shapes and align in one direction. The film was not as uniform as the PVP/Heparin coating. In addition, the PEI coatings had a great range of roughness ranging from 0.5nm to 14nm (data no shown). Although all thin films were fabricated under the same process, it is interesting to observe that polymers interacted differently at various pH values. Most PVP coatings were fabricated at pH 5, except PVP/Heparin was at pH 7.2. On the other hand, PEI coatings were processed under a wide range of pH from 5 to 9. PEI and PVP were both fabricated with Heparin at pH 7.2. PVP/Heparin showed the typical morphology of PVP coatings that we fibricated, but PEI/Heparin showed a completely different morphology in comparison with other PEI coatings (data not shown). Shiratori suggested pH was the factor that affected ESA coating thickness and morphology.[15] pH affected the degree of ionization of an adsorbed polymer chains that resulted in the variation of thickness. Also pH might modify the conformational state of a multilayer surface. Once the surface was dominated by loop and tail segments, the

surface became rough. On the other hand, the domination of train segments would produce smooth surface. Polymer diffusion among the layers also changes the thickness of the thin film.[16] PEI is a copolymer instead of a homopolymer like PVP. The blocks of PEI change their conformation and charge density at various pH, but PVP may not change too much. At the same time, pH changes the conformation and charge density of polyanions as well. The combination of polymer composition and pH value lead the ESA process to produce different morphology with a variety of polycation/polyanion formulations.

According to AFM results, PVP coatings were uniform. PEI coatings, however, had morphologies that were different to each other. All these different surface morphologies were due to polymer composition and pH values during the ESA process.

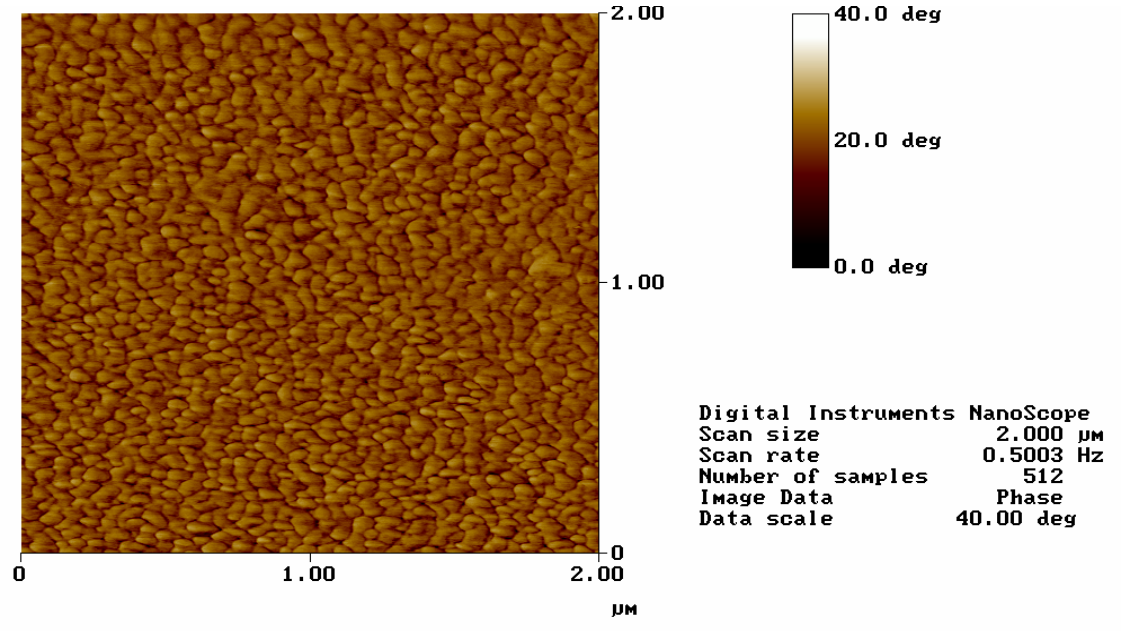

Figure 2.12 AFM phase image of PVP/Heparin thin film at 30 bilayers.

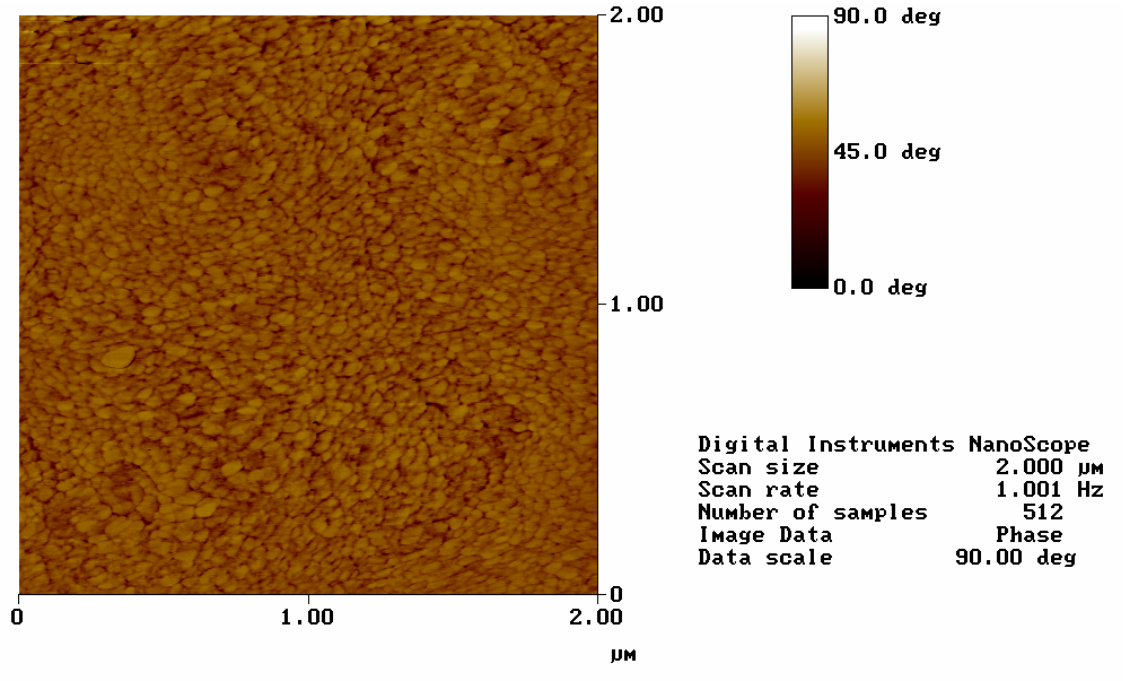

Figure 2.13 AFM phase image of PEI/PMA thin film at 30 bilayers.

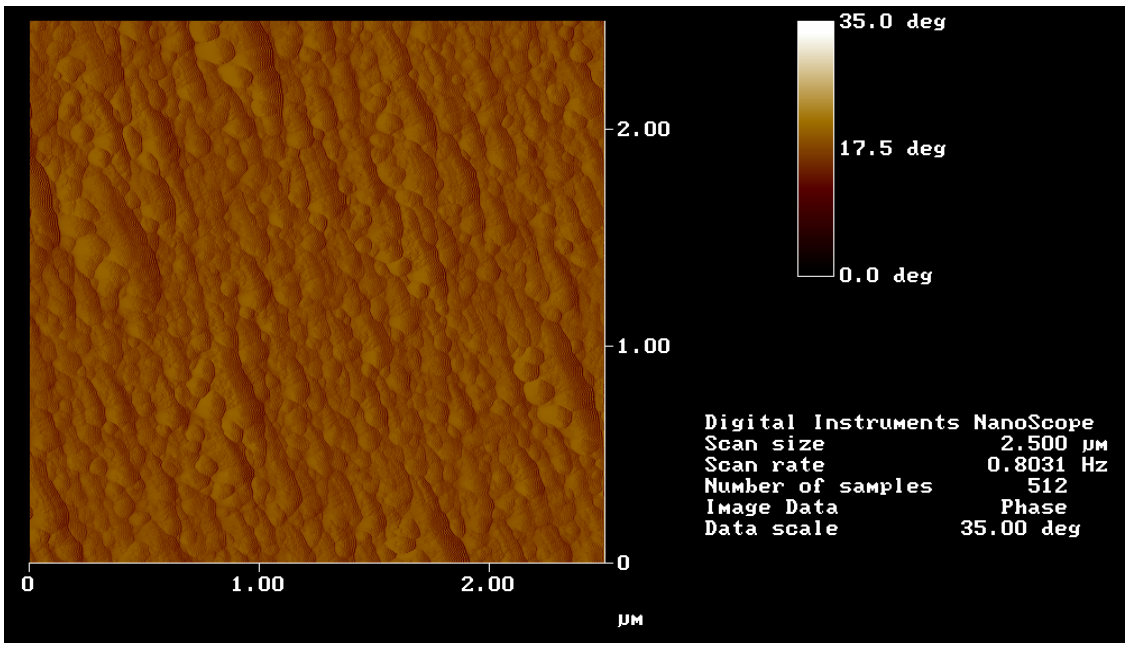

Figure 2.14 AFM phase image of PEI/Poly-l-glutamic acid at 30 bilayers.

2.3. References

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Chapter 3: *In-Vitro* Hemocompatible Tests on ESA thin films

Restenosis is the process of re-narrowing of the coronary artery after coronary angioplasty is performed. Researchers use different stent designs, materials and even drug-eluting stents to reduce the occurrence of restenosis. When a device is implanted into a human body, the cells at the particular implant area would be activated and responsed to the device, however, the activation and response are vary from human to human, and organ to organ. For the blood vessel itself, the responses of platelets, smooth muscle cells, and blood proteins after angioplasty is performed lead to restenosis. It is important for us to develop a material that reduces the chance of restenosis, and here we would like to introduce a term hemocompatible, which means blood compatible, for the materials. A hemocompatible material should achieve several tasks as follows.

1. **The material should not have any unfavorable interaction with platelets that results in activation**. Since platelets contact with the material surface, platelets are activated and secrete enzymes to activate other cells in the local area that result in restenosis. A material that can minimize interaction with platelets would reduce the chance of restenosis.
2. **The material should not have interactions with the blood proteins**. Blood proteins play significant roles in coagulation and aggregation between cells. In a healthy stage, the proteins are inert, but would be activated once an implant is inserted or the blood vessel is damaged. So a material with a

property involving reduction of protein activations could prolong the life and performance of a stent.

3. **The material should not be toxic to humans**. Stents are considered be long-term implanted devices. Any material composing the bulk or coating of the stents must not have any effect that harms the human body. Toxic material damages local cells and tissue, and finally requires extra surgery for replacement of the stent. As a result, the toxicity of the material should be tested with the cells at the implant area and carefully evaluated.

In pervious chapters, we have already discussed the theory, fabrication and characterization of ESA thin films. These films show potential of being hemocompatible coatings for use on stents. Eight different ESA films have been fabricated on polyurethane catheters and glass beads in order to test their hemocompatibility. Our hemocompatibility tests have three sections with aims to evaluate the coatings based on the above criteria. First, the ESA films on the catheters are incubated with equal amounts of platelet suspension to study the adhesion property of the coatings. Platelet morphology and the amount on each film then would be exanimated under SEM. Clot mass formation is a test to understand the interactions between blood proteins and the ESA coatings on glass beads during their incubation with whole blood. We measure the dry clot mass on each bead as the performance measure of the coatings. Less mass on the surface means the better the coating is. Finally, lactate dehydrogenase (LDH) is an enzyme in cells that is released to the surrounding environment when the cell membrane is damaged. Six polymers were dissolved in a

buffer solution and diluted in a series of concentrations for tests. Each polymer solution is incubated with equal amounts of platelet suspension, and the LDH release from platelets was measured by using a microtiter plate reader (ELISA reader). The amount of LDH release correlates with the toxicity of each polymer. Base on the above three tests, we are able to evaluate the hemocompatible properties of the ESA films *in vitro*.

3.1 Platelet Adhesion

In our *in vitro* studies, we fabricated eight ESA thin films on polyurethane catheters and incubated them with platelet suspension. These tests are performed to provide information about the adhesion and activation properties of platelets to ESA films and the change in platelet morphology which is a sign of platelet activation. We hydrolysis our ESA coatings can minimize platelet adhesion.

3.1.1 Method

50ml of healthy horse blood was obtained from Virginia-Maryland Regional College of Veterinary Medicine and collected by using a 60ml syringe. 600ul of 15% EDTA was added to the blood as an anticoagulant. The blood was centrifuged for 10 minutes at 150g to obtain a mixture of serum and platelets relatively free of red blood cells. The mixture centrifuged for 15 minutes at 400g to obtain a platelet pellet at the bottom of the polypropylene tube during the spin. The platelet pellet was transfered to a new polypropylene tube and resuspended with 20ml of PBS, and centrifuged again for 15 min at 400g. The supernatant was carefully removed and the new platelet pellet suspended

in 5ml of PBS. The concentration of the platelet suspension was determined by passing the suspension via a cell counter (CELL-DYN 3700, Abbott Laboratories) and finally diluted to $4x10^7$ platelets/ml. Each ESA film coated catheter was incubated with 200µl of the new platelet suspension in a 96-well culture plate at 37^oC for 30 minutes with constant rotation. After incubation, the catheters were washed in PBS to remove loosely attached platelets and kept in fixative for a week. Platelet adhesion on both coating and control catheters were imaged by a SEM.

3.1.2 Results

According to the SEM images, polyurethane catheters with the ESA coatings showed great differences in comparison with control catheters. Figure 3.1 was an SEM image of a control catheter with scale at 10µm and magnification at $2.02x10^3$. The image showed there were large amount of platelets (small and irregular shaped cells on SEM) adhered on the surface. All platelets had changed their shape with the formation of pseudopodia surrounding their cell membranes. On the other hand, catheters with ESA films have only a small amount of platelets on the surface. Figure 3.2 was the typical results of our ESA thin films. Although platelets were all activated as in the control catheter, the great difference in the amount of platelets indicated the ESA coatings improved the surface property of the catheters and resulted in an inhibition of the adhesion and activation of platelets. For our fabrication process, polyanions were always the outmost layer. That meant our ESA thin films were mostly covered by negative charges. Furthermore, most cells had negative charges at their cell membranes. We suggested the electrostatic repulsion between the negatively charged ESA film and the cell membrane inhibited

platelet adhesion on the catheter rather than promote cell adhesion (Figure 3.3). Likewise, we could alter the outermost layer be polycations and promote cell adhesion for other applications.[1]

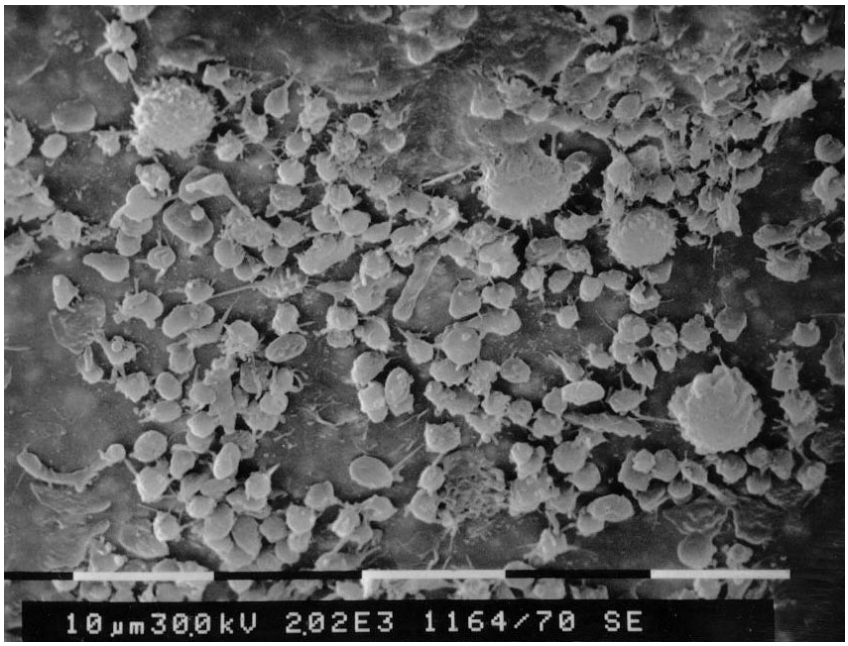

Figure 3.1. SEM image of platelet adhesion on control polyurethane catheter with scale at 10µm.

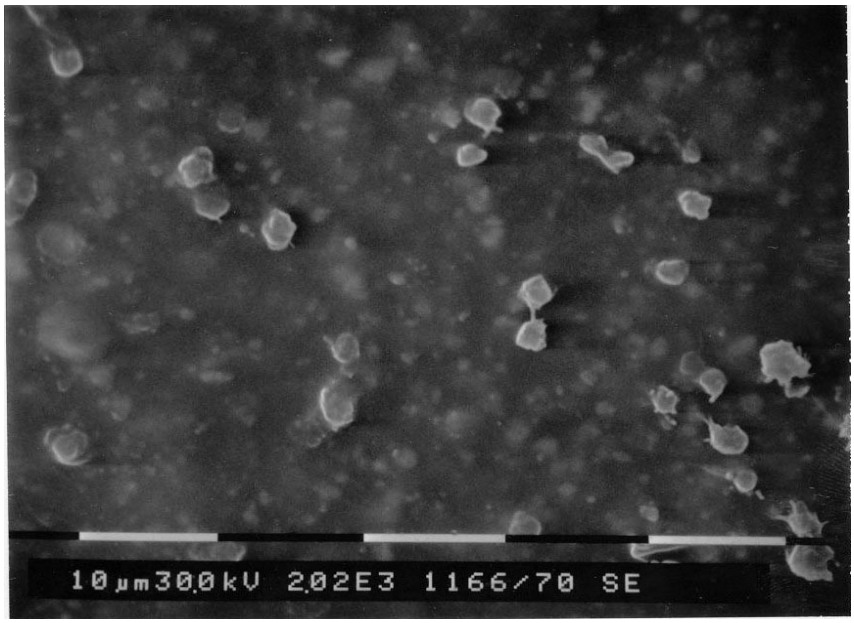

Figure 3.2. SEM image of platelet adhesion on PVP_Heparin thin film coated catheter with scale at 10µm.

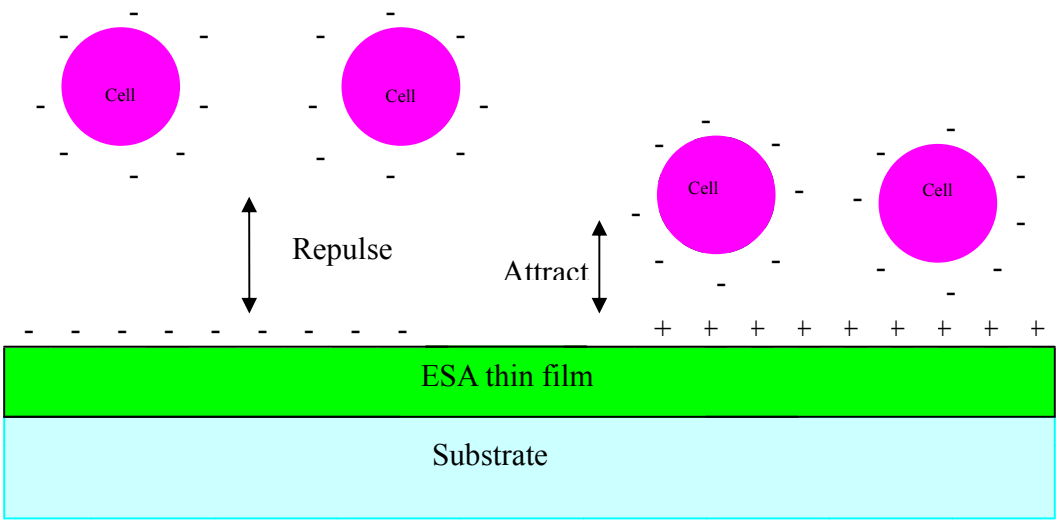

Figure 3.3. Schematic diagram showing the anti-cell adhesion and promote cell adhesion property of ESA thin film having negatively charged and positively charged outmost layer, respectively.

3.2 Clot Mass Formation

A thrombus is a clot found within a blood vessel of a living human. The procoagulant and anticoagulant proteins in the bloodstream are in equilibrium in a healthy person. Nevertheless, this equilibrium can be broken down by factors such as diseases and stimulation from implanted materials, and these unwelcome factors trigger coagulation that leads to thrombus. Coagulation is a cascade process that involves a number of proteins and cells working together. It is a very complicated process, and its mechanism is not fully known. In prevention of the procoagulant property of a stent, the stent surface should be modified to minimize the responses from the cells and the proteins in blood. Layer-by-layer assembly of polycations and polyanions on the stent surface may alter the affinity of the stent to the cells and the proteins. Testing this hypothesis, eight ESA thin films have fabricated on glass beads incubated with whole blood at 37^{o}C for 30 minutes to investigate the interaction between ESA films and whole blood in terms of the formation of a clot on the bead surface.

3.2.1 Method

All ESA coated glass beads were stored in a desiccator and their weights were recorded until the values become constant. Each dry bead is incubated with 250µl of whole blood without anticoagulant at 37^{o}C for 30 minutes plus constant rotation. All beads are taken out to allow dry under room temperature and pressure for another 30 minutes after incubation. Beads are stored in the desiccator again and the new weights are recorded with the clot on bead surface until the values become constant to determinate the mass of

the clot on every glass bead. One-way ANOVA is performed by using Minitab R14 to determine the statistical significant of the ESA thin films. P values smaller than 0.05 are considered as significant.

3.2.2 Results

The results of the ESA coatings showed great improvement in inhibiting the formation of clots. Control beads triggered coagulation after ten minutes of incubation and the blood turned into a solid (data not shown). On the other hand, glass beads coated with the eight ESA films did not stimulate coagulation or only a small portion of clot was formed on the surface of the glass bead (Figure 3.4). The blood incubated with the coated glass beads was maintained in liquid form through out the incubation and turned into solid after the coated beads were removed. The PEI films had the best performance that inhibited the formation of clots on glass bead surface. Although the clot mass on the PEI films was about 1.5mg, it should be noticed that we never washed all glass beads after incubation. The 1.5mg on the PEI coatings was from the blood that originally adhered on the bead surface. No solid clot was observed on any of the PEI films.

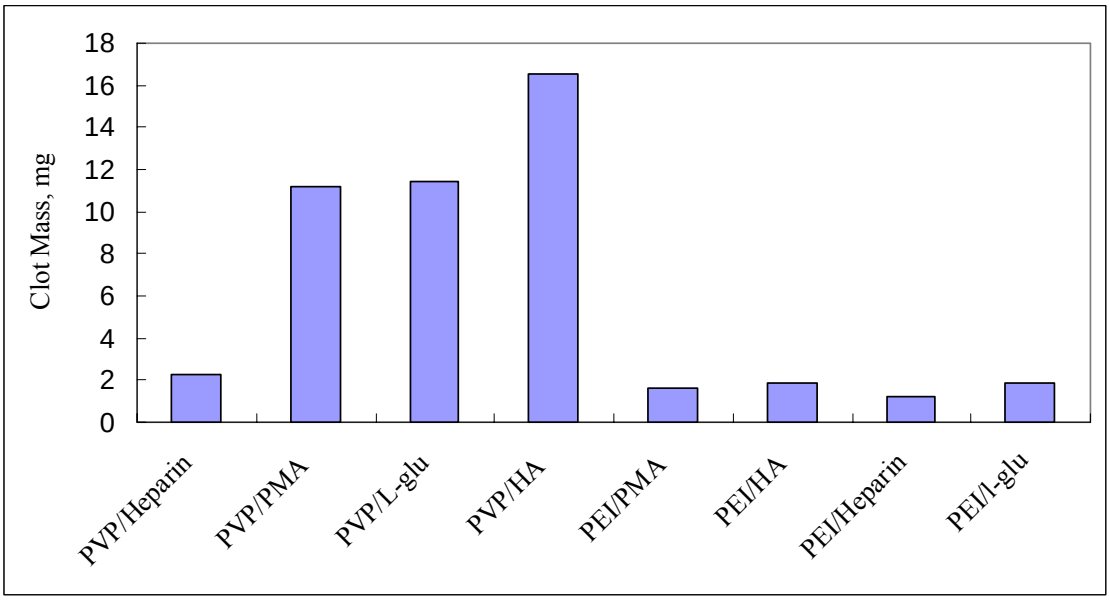

Figure 3.4. Average clot mass on the ESA thin films after incubation with whole blood.

The PVP thin films, however, showed different performance from the PEI coatings. Most of the PVP films had a solid blood clot on the glass bead surface except PVP/Heparin that had the same anti-clot property as the PEI coatings. The mass of the clot on the PVP films ranged from 11mg to 16mg. The one-way ANOVA test (Figure 3.5) revealed PVP/Heparin had no significant difference among the PEI films, but other PVP films had significant difference in comparison with the PVP/Heparin and the PEI coatings. Heparin is a natural anticoagulant. It binds to antithrombin (AT) to form a heparin-AT complex to inactivate coagulate enzymes such as thrombin factor (IIa) and factors Xa, IXa, XIa, and XIIa.[2] Heparin incorporated with both PVP and PEI exhibits an anticoagulation property that indicated heparin was active in inhibiting coagulation enzymes, and our hypothesis was true for the Heparin coatings. PMA, Hyaluronic acid and Poly-L-glutamic acid were not used as an anticoagulant in the literature, but they had partial anticoagulation properties when combined with PVP and complete anticoagulation

with PEI, respectively. Although PVP films were thinner than PEI films based on XPS data in Chapter 2, the thickness of the coatings did not play an important role in the formation of clot mass because Heparin in both types of films yields the same result. Serizawa and coworkers state that suitable polymer species generate anticoagulant activity.[3] In our study, we agree with this statement because PMA, Hyaluronic acid and Poly-L-glutamic acid perform better with PEI than PVP. PEI is a linear polymer and more flexible than PVP. It may incorporate more polyanions than PVP that results in higher negative charge density. We suggested high negative charge density inhibited platelet adhesion as in section 3.1 and also probably inactivated the conformational change of blood proteins, which protein conformational change is the prerequisite for cell-cell adhesion. This study showed that a surface could become anticoagulate by adding polyanions to be the outermost layer even though these polyanions did not have any anticoagulant property. The charge density of the polyanions would be the key to evaluate the performance of the anticoagulation and was determined by the interaction with polycations. Strong interaction resulted in higher negative charge density and was able to stop coagulation *in vitro*.

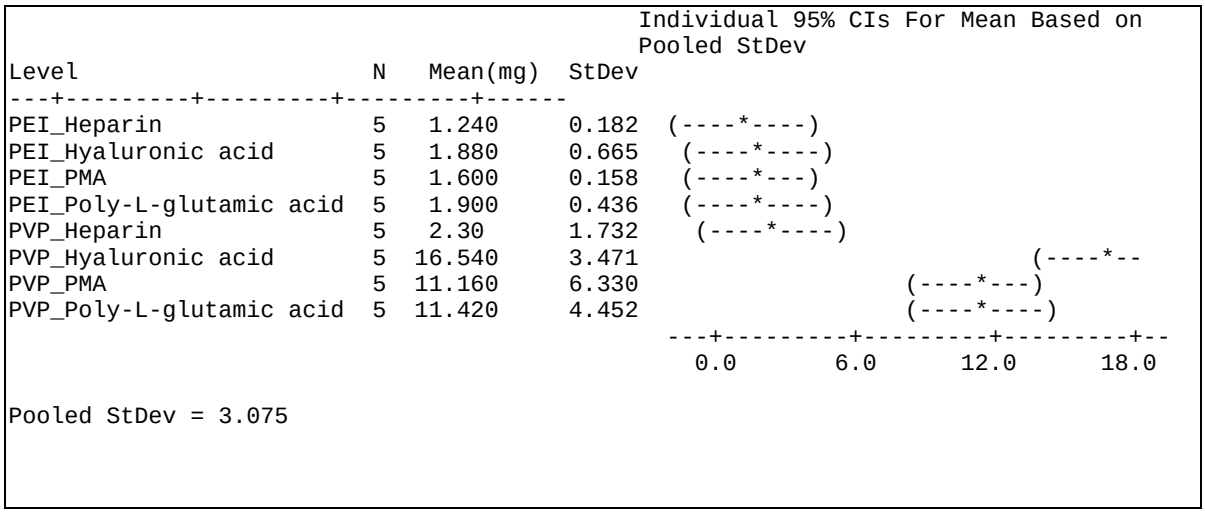

Figure 3.5. One-way ANOVA test showing the statistical significance of each ESA coating at 95% confidence interval.

3.3 LDH Cytotoxicity

Lactate dehydrogenase (LDH) is a stable cytoplasmic enzyme present in all cells. The release of this enzyme is thought to be proportional to the damage of the cell membrane, and the amount of release is proportion to the cell lysed. According to this relationship, we are able to evaluate the cytotoxicity of implanted materials to specific cells, which is the cytotoxic potential of the ESA polymer thin films to platelets in this study. LDH cytotoxicity is a colorimetric assay and measures the amount of LDH release in terms of colour intensity. In theory, the cell supernatant collected from a suspension of cells contains a certain amount of LDH after incubation with tested materials in a period of time showing individual cell membrane damage via enzyme release. The LDH converts lactate to pyruvate by reduction of NAD^+ and the hydrogen reduced from NAD^+ reacts with a yellow tetrazolium to salt become a red formazan salt (Figure 3.6). The amount of LDH in the culture supernatant is proportional to the amount of cell death, and correlates to the amount of formazan salt as well. By measuring the light intensity of

the formazan salt, we can determine the percentage of LDH release as a cytotoxicity indicator of our ESA thin films and the polymers that fabricate the ESA films. In our study, we study the high and low-control of LDH release from healthy horses, the cytotoxicity of polymer solutions in a series of concentrations and the cytotoxicity of selected ESA coatings on glass beads against horse platelets.

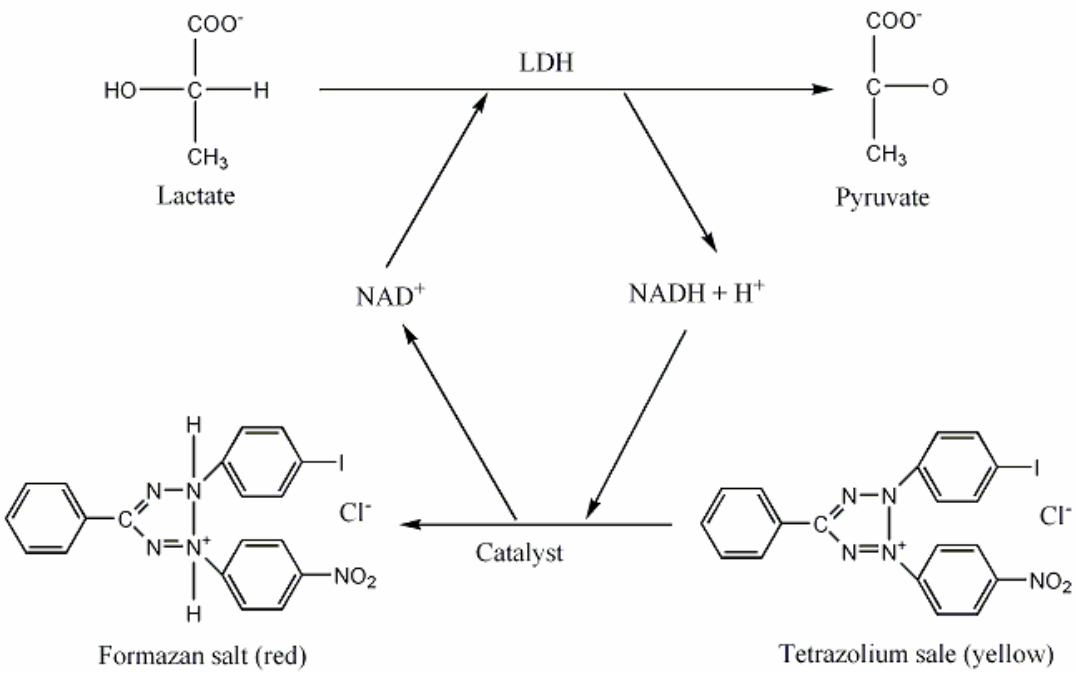

Figure 3.6. Colorimetric assay of LDH measure the color intensity of formazan salt based the release of LDH.

3.3.1 Methods

Platelet suspensions were prepared the same as in section 3.1.1 but with different horses. Six polymers were dissolved in PBS and diluted to five different concentrations based on a log scale from 2000μg/ml to 0.2μg/ml (equal volume of the polymer solutions and platelet suspension were mixed together to form the final concentrations of the polymers

from 1000μg/ml to 0.1μg/ml). 150μl of polymer solutions were mixed with 150μl of platelet suspension at $4x10^7$ platelets/ml in triplicate and incubated for two hours at 37°C with constant rotation in a 96-well culture plate. The incubation also included triplicate of the high-controls (150μl of platelet suspension at $4x10^7$ platelets/ml was mixed with 150μl of 1% Triton X-100) and the low-controls (300μl of platelet suspension at $2x10^7$ platelets/ml). After incubation, the mixtures were centrifuged at 200g for 10 minutes. 100μl of the supernatants were removed from the suspensions and reacted with 100μl of the LDH agents (purchased from Roche) in dark for 30 minutes. Finally, the mixtures were placed in a microtiter plate reader (ELISA reader, Spectro-Max Plus 384, Molecular Devices, CA) and recorded the light intensity corresponding to each polymer solution. To investigate the LDH release associated with various ESA coatings, the procedures were the same as the experiments for polymer solutions, but there were some modifications. ESA coated glass beads were incubated with 250μl of platelet suspension at $4x10^7$ platelets/ml. The high-controls were 125μl of 1% Triton X-100 plus 125μl of platelet suspension at $8x10^7$ platelets/ml. The low-controls were 250μl of platelet suspension at $4x10^7$ platelets/ml. We also investigated the variation of both high and low-controls via three horses. The final volume of each control was 300μL and platelet concentration was $4x10^7$ platelets/ml. The cytotoxicity percentage was calculated by the readings form the ELISA and the following equation.

$$Cytotoxicity(\%) = \frac{ExperimentalValue - LowControl}{HighControl - LowControl} \times 100$$

3.3.2 Results

In our high and low-control study, there was a hypothesis that all horses should have constant high and low-control values for our cytotoxicity calculation. Three healthy horses were selected and draw blood in three consecutive days to conduct this study. The three days study showed the high and low-controls are changing day after day for the same horse (Figure 3.7 and 3.8). The controls among the three horses were never been the same. As a result, we could not have a standard high and low-control to calculate the cytotoxicity of polymer solutions and ESA coatings and must calculate the cytotoxicity in each experiment. The variation of both the high and low-controls were from two sources. First our platelet suspension did not only contain platelets but also had different amount of red blood cells (Figure 3.9) and white blood cells (Figure 3.10). The concentrations of these cells were varying day by day and also these cells releaseed LDH when the cell membranes were damaged. The extra LDH released from both red and white blood cells influence the LDH release from platelets, which affected the high and low-controls. In addition, physiological conditions of the horse during blood extraction were another consideration. Horse 3 at day 1 was under stress during blood extraction. Thus the high and low-controls were high then the controls from horse 1 and 2 at day 1 who did not under stress during extraction. The physiological conditions of the horse during extraction were mostly influenced by the handling procedures. Any mistake during handling would induce change in physiological conditions that resulted in higher LDH release. Although we did not achieve the standard high and low-control for LDH cytotoxicity study, the cytotoxicity of different materials were still able to compare each other because cytotoxicity was a relative percentage of LDH release based on high

and low-control rather then looking for the absolute value of cell death.

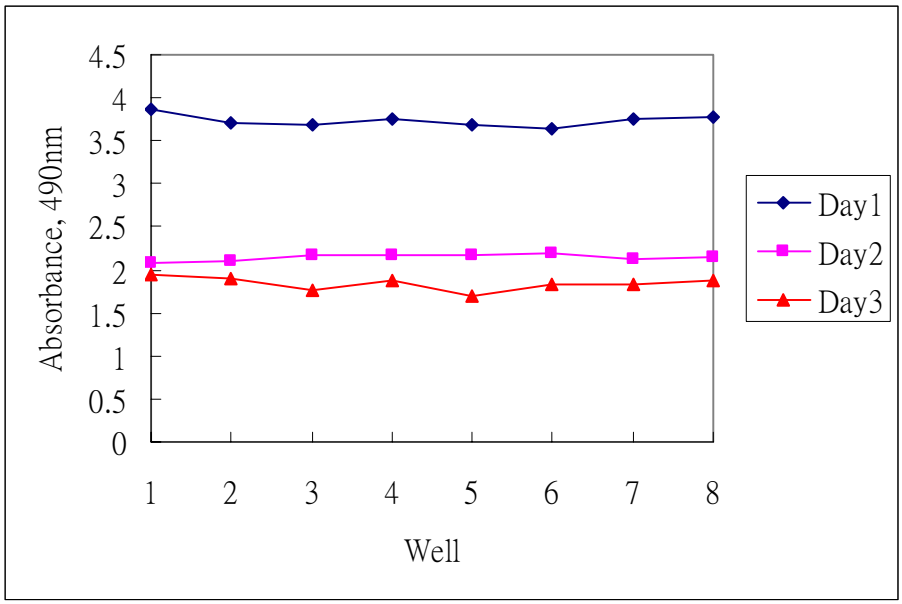

Figure 3.7. The absorption of LDH release of Horse 3 high controls. Wells refer to the well number in the 96-well culture plate.

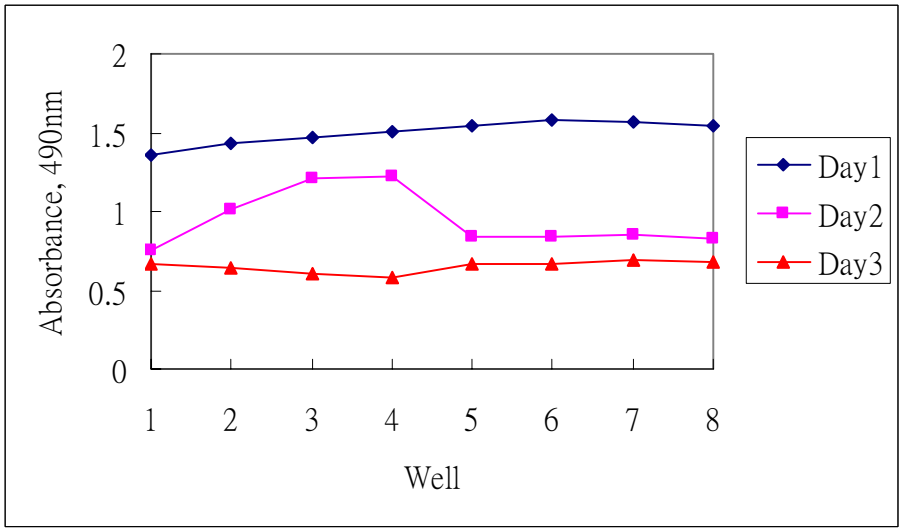

Figure 3.8. The absorption of LDH release of Horse 3 low controls. Wells refer to the well number in the 96-well culture plate.

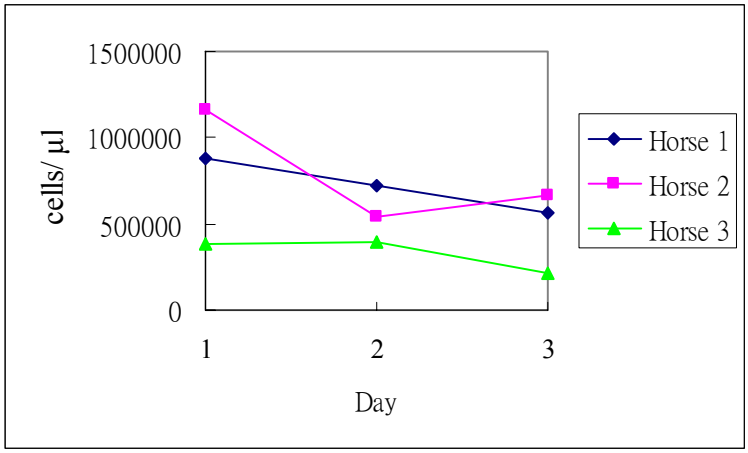

Figure 3.9. Red blood cell concentration from day 1 to 3 for Horse 1, 2 and 3.

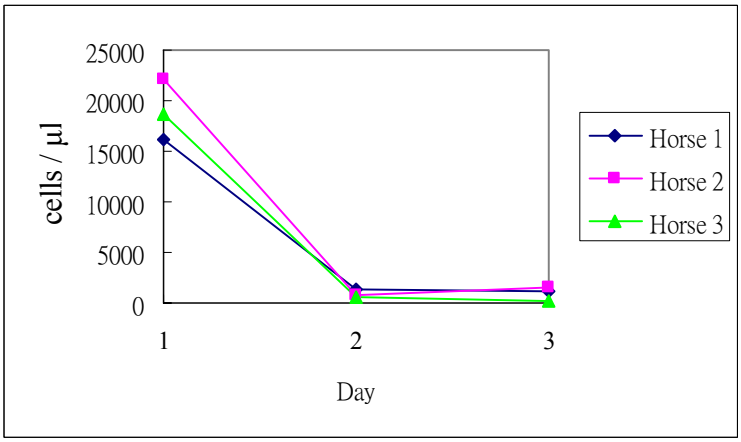

Figure 3.10. White blood cell concentration from day 1 to 3 for Horse 1, 2 and 3.

Our cytotoxicity study on polymer solutions showed no cytotoxicity of polyanions except Hyaluronic acid had 2% cytotoxicity at 1000µg/ml (Figure 3.11). In addition, only PEI was cytotoxic at 1000µg/ml and 100µg/ml with values at 46% and 2%, respectively (Table III). These results revealed that polymer-induced cytotoxicity only occurs at high concentration and for specific polymers. We also studied the cytotoxicity of PVP/Heparin and PVP/Poly-L-glutamic acid. Both thin films had cytotoxicity only at 0.32% which indicated the ESA thin films might not be as likely to damage platelets (Table IV).

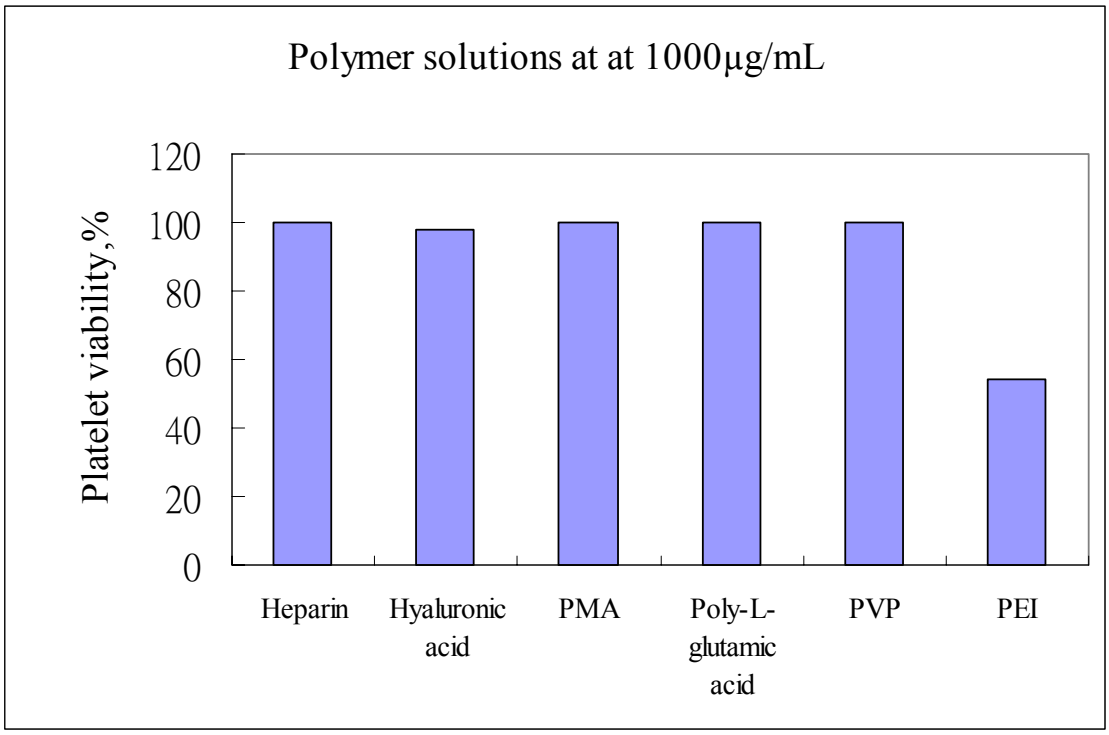

Figure 3.11. Platelet viability at 1000μg/mL of polymer solutions.

Table III. Cytotoxicity (%) of the polymer solutions at various concentrations.

Polymers / Concentrations	Hyaluronic Acid	PEI
1000μg/mL	2% ± 1.12%	46% ± 4.55%
100μg/mL	-	2% ± 1.58%

TableIV. Cytotoxicity (%) of the ESA thin films.

	ESA coatings	
	PVP/Heparin	PVP/Poly-L-glutamic aicd
Cytotoxicity (%)	0.32% ± 0.03%	0.32% ± 0.01%

We suggest when polymers mix with platelet suspension, they have chances to meet with platelets. Polyanions carry negative charges that probably induce electrostatic repulsion between polymers and platelets. On the other hand, polycations have positive charges

that cause electrostatic attraction between polymers and platelets because platelets have negative charges at their cell membrane (Figure 3.12). Once attraction occurs, the interactions of polycations with membrane proteins and phospholipids disturb the membrane structure and function.[4,5] This influence leads to membrane damage and finally cell death. Moreover, interactions between polycations and cell membrane induce erythrocyte lysis.[6] Polymer charge density and flexibility are another two parameters that govern cytotoxicity of the polymers. Polycations with high charge density are likely to attach to cells, and a flexible polymer chain is more likely to attach to cells than a rigid polymer chain because the flexible polymer chain can increase the chance of electrostatic attraction between the positive charges on the polycations and the negative charges on cell membrane. Ferruti and coworkers have demonstrated polymers that contain tertiary amines exhibit a lower cytotoxicity than those polymers having primary or secondary amines.[7]

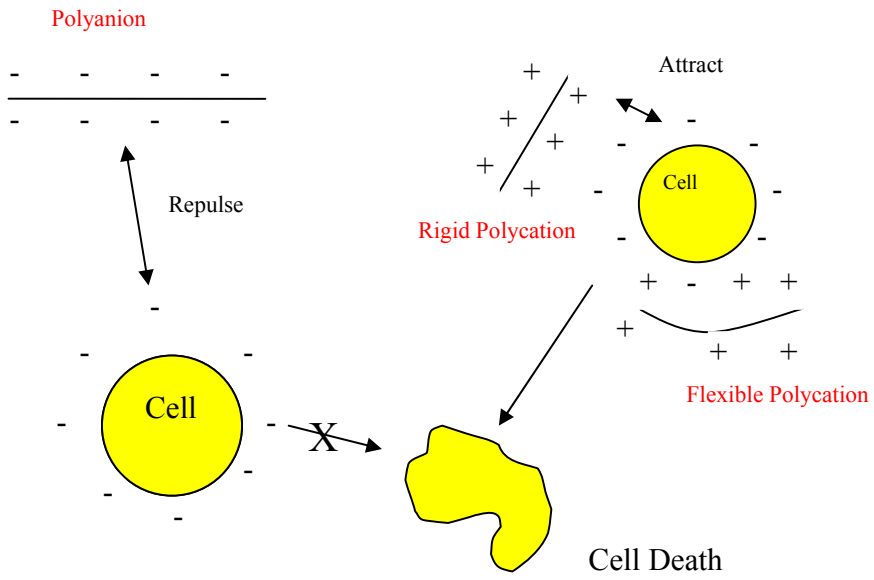

Figure 3.12 Schematic diagram showing electrostatic attraction between polycation and cell, and electrostatic repulsion between polyanion and cell. The attraction finally leads to cell death.

In our study, PEI is a copolymer with linear branches that makes it more flexible than PVP. It has higher positive charge density because PEI has secondary and tertiary amines in the backbone and primary amines at the branches (Figure 2.2). Although PVP has side groups along its backbone, the side groups are more bulky than the linear branches in PEI. These bulky side groups limit the motion of PVP and PVP acts more rigid. The tertiary amines of PVP are located between the backbone and the side groups that reduces the chance of tertiary amines explore to cell membranes. As a result, PEI is more cytotoxic than PVP. Our results are the same as those in the literature that show PEI is highly cytotoxic.[8, 9] Polyanions in our study do not have cytotoxic effect on platelets probably due to the negatively charges on the polymer chains. Choksakulnimitr and coworkers show polyanions do not have cytotoxic effect,[10] although different cell types were used in experiments. This evidence may reveal the low

cytotoxicity of polyanions may apply to different cells.

The ESA thin films had low LDH release, but the values were higher than for polymer solutions. It was because the platelet suspension concentration that incubated with the ESA thin films were two times of the suspensions that incubate with the polymer solutions. Higher cell concentration should have higher LDH release. The assumption seems correct, but our ESA films had polyanions as the outermost layers. These negatively charged layers might repulse cell adhesion, inactivated blood proteins and acted as free polymer form in liquid phase. Furthermore, the polymers that fabricated the ESA thin films were in the concentrations less than 0.2μg/ml. Thus, our ESA coatings did not have significant cytotoxicity effect on platelets.

3.4 Conclusions

ESA thin films fabricated from two polycations and four polyanions were successfully coated on polyurethane catheters and 5mm diameter glass beads. The *in vitro* hemocompatibility tests demonstrated the ESA thin films inhibited platelet adhesion, inactivated blood protein response for coagulation, while the PEI films were the lowest clot masses, and did not have significant cytotoxicity effect to platelets. A replicating study using three horses showed it was difficult to establish standard high and low-control to calculate the cytotoxicity of polymer solutions and ESA coatings due to the LDH release from white and red blood cells in platelet suspension and physiological conditions of the horse during blood extraction. The hemocompatible properties of the ESA films might probably due to the fact that the outermost layers of the ESA films were the polyanions. However, the three hemocompatiblity tests were the primary tests to

evaluate the possibility of using ESA thin films as hemocompatible coatings for stents. Further investigation is required to understand the charge effect to platelet adhesion, coagulation and cell membrane.

3.5 References

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Chapter 4: Conclusions and future work

Electrostatic self-assembly (ESA) is a technique to fabricate nanoscale thin films, which may be potential to fabricate polymer thin films that prevent restenosis. This thesis demonstrated the feasibility of the ESA process to fabricate eight potentially hemocompatible polymer thin films.

XPS and AFM were able to characterize the ESA films on glass beads and Au-coated glass respectively. XPS indicated the ESA films were successfully coated on glass beads by showing specific element peaks like nitrogen and sulphur as indicators of the films. All PEI films were thicker than the PVPs because the silicon peaks in the PEIs were smaller than the PVPs. PVP coatings were homogeneously distributed on gold-coated glass according to AFM images. On the other hand, PEIs had different morphologies at various pH values. The differences of the PVPs and PEIs form XPS and AFM images revealed diffusion among the polymer layers plays an important role in film thickness. Moreover, pH was the factor that affects ESA coating thickness and morphology.

Three hemocompatibility tests had been performed on the fabricated ESA coatings. All ESA coatings were able to inhibit the adhesion of platelets on polyurethane catheters to the same degree, and the coatings inhibited coagulation. Our results showed the PEI thin films had the lowest clot masses (~2mg). Finally, cytotoxicity tests demonstrated polycations were cytotoxic but only at high concentration. Polyanions showed no

similar cytotoxic effects as polycations. The ESA thin films are low cytotoxic (0.32%) too. According to the above results, the PEI thin films were the best hemocompatible films. The hemocompatible properties of the ESA thin films might come from the negatively charged outermost layer by electrostatic repulsion between negative charges on the platelet membrane and the ESA films.

This thesis explored the possibility of using ESA polymer thin films being as alternative to cytotoxic drugs to prevent restenosis. Future work should be considered includes the following.

- Surface charge density of the ESA films.
- Thickness of the ESA films using ellipsometry.
- Charge effect on ESA film surface. Can cationic charge inhibits platelet adhesion, coagulation and cytotoxicity to platelets?
- Investigate the blood proteins response to ESA thin films.
- The level of the expression of the platelet receptors such as glycoprotein Ib/IX, glycoprotein IIb/IIIa and P-selectin should be determined. These are key components for platelet aggregation and their expressions relate to platelet activation.
- Other than cell surface receptors, platelet microparticles are released during platelet activation. This expression can give us more insight about the interaction between ESA films and platelets.

- Blood proteins play important role in coagulation. The detection of these proteins allows us to understand which proteins are inhibited by ESA films.
- Red blood cells, white blood cells and endothelial cells exist in the inner surface of blood vessels. We should determine the cytotoxic effect of ESA films to these cells.
- *In vivo* hemocompatible tests should be performed to investigate the effect of ESA coatings to animal bodies.