

Surface-engineering of Poly(ethylene terephthalate) for improved haemocompatibility

By

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A Thesis submitted to the Faculty of Graduate Studies of
The University of Manitoba
in partial fulfilment of the requirements of the degree of

MASTER OF SCIENCE

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Winnipeg

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Abstract

Cardiovascular diseases (CVD) remain the number one cause of mortality globally. Compared with autologous vessels, synthetic vascular grafts such as poly (ethylene terephthalate) (PET) vascular grafts are still widely used to replace or bypass diseased arteries. However, PET is susceptible to thrombosis when in contact with blood.

In this study, “bioactive”/“bioinert” agents – heparin and poly(ethylene glycol) (PEG) were immobilized covalently on chemically inert PET substrate using a special surface modification technique – surface interpenetrating network (IPN) successfully. The improved haemocompatibility of heparin modified PET surface was proved by a platelet adhesion assay. The PEG modified PET substrate also demonstrated decreased platelet adhesion.

Further research has been conducted to immobilize aliphatic chains ended with sulfate and carboxylate groups (existing in heparin) on a model substrate. *In vitro* thrombus formation test indicated an interesting anticoagulating action between those anionic groups with an optimal ratio in the range of (3:1) and (4.5:1).

Acknowledgements

The author wants to express her deepest gratitude to her advisor, Dr. Song Liu, for his advice, guidance and patience during her two-year challenging Master program at the Faculty of Human Ecology, University of Manitoba. He provided his encouragement and support through the study.

The author also heartedly thanks to the members of her thesis committee, Dr. Martin King from the College of Textiles, North Carolina State University and Dr. Harding from the Faculty of Medicine, University of Manitoba, for their professional suggestion and kind help review and comment the thesis for the author.

The author appreciates the helps from Dr. Francis Lin, Faculty of Science, with providing the blood test protocol and the expert advice from Dr. Lena Horne and Dr. Tammi Feltham, Faculty of Human Ecology.

The author acknowledges the financial support from the Natural Sciences and Engineering Research Council of Canada (NSERC) Discovery grant (Grant no.: RGPIN/372048-2009), and the Manitoba Health Research Council (MHRC) Establishment grant.

Finally, the author is indebted to her colleagues in the lab and her parents for their consistent help and generous support.

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Chapter I: Literature Review

1.1 Introduction

1.1.1. Cardiovascular disease

Cardiovascular diseases (CVD) remain the number one cause of mortality globally. In US, for example, CVD accounted for 33.6% deaths in 2007.¹ The total number of Americans who suffer from CVD reached over 60 million in 2003.² CVD can be broadly described as the diseases which affect the heart and blood vessels through atherosclerosis. Both the heart and blood vessels are very important to human body. They function together to control and transport blood which delivers oxygen and nutrients to human organs supporting human life. Once blood vessels are diseased through arteriosclerosis, the results can be fatal. Generally, coronary artery diseases (CAD), referring to arteriosclerosis in coronary arteries,³ occur in small-diameter blood vessels (<6 mm). At first CAD narrow the arteries. These arteries can often be treated with stents or bypass surgery in clinical practice. When balloon angioplasty and stents cannot be used, bypass surgery is often repaired. There are two options for bypass: use of either autologous vessels (eg. Saphenous vein and internal mammary artery) or artificial biomaterials.⁴

The first use of autologous vessels dated back to the beginning of 20th century by Goyanes using the patient's own popliteal vein.⁵ After that, native venous vessels including saphenous veins were considered for the treatment of CVD. Internal,

mammary arteries and radial arterial grafts can also be used for bypass conduit.⁶ Autologous vessels are excellent conduits with patency of 50 % - 70% over 10 years.⁷ Here, the patency indicates the percentage of artificial grafts observed to be fully open for the defined period. The excellent performance of autologous tissues is owing to the tissue compliance on the outer surface and the natural antithrombogenic endothelial lining on the inner surface.⁸ However, the supply of native vessels may not be sufficient.^{9,10} At least 1,400,000 vascular grafts surgeries are needed in United States each year¹¹ while only two thirds of the patients have the suitable autologous vessels for surgery.¹² The autologous bypass vessel may be absent, not suitable on already harvested. In addition, higher surgical costs and morbidity associated with the vessel harvesting must be considered.¹³

As a result, artificial biomaterials, defined as “any substance (other than a drug) or combination of substances synthetic (such as polymers) or natural (such as substances produced by a biological system) in origin, which can be used for any period of time, as a whole or part of a system which treats, augments, or replaces tissue, organ, or function of the body”¹⁴ are still being paid much attention. Research on artificial blood vessels still attracts many researchers and approximately 900 academic articles have been published in the last decade.³

1.1.2 Artificial vascular grafts

Most of the research publications are focused on how to improve the performance of artificial vascular grafts. What are the exact requirements for ideal artificial vascular

grafts?

First of all, artificial vascular grafts should be haemocompatible with human blood. The artificial vascular grafts can thrombose on the inner wall and inhibit the regular blood flow. The human body must not reject the artificial vascular grafts.¹⁵ Based on this, artificial vascular grafts must be non-immunogenic, non-toxic and non-thrombogenic to human body to human blood.¹⁶

Second, artificial vascular grafts should have certain degree of compliance matching that of native vessels. The compliance is also explained as the ratio of incremental volume change to incremental transmural pressure change, expressed as percent of diameter change per millimeter of mercury.³⁵ The nature human blood vessels have a special viscoelastic characteristic due to the anatomy structure.¹⁷ There are three layers in the wall of human blood vessels: tunica interna (mostly endothelial cells), tunica media (mostly smooth muscle cells) and tunica externa (collagen fibers and extracellular matrix). Between the three layers, there are elastic membranes (mostly elastin). Smooth muscle cells control the contractility of human blood vessels while collagen and elastin control the resistance to distension and recoil. The compliance of artificial blood vessels is not ideal, less laminar flow can cause stenosis of the artificial vascular grafts through intimal hyplasia with subsequent thrombosis inside.³ A study performed by Abbott et al¹⁸ showed that the patency of a relatively “stiff” artificial blood vessel was only 37% compared to 85% of the other relatively “compliant” one after 12 weeks in a dog body.

Third, artificial vascular grafts should have certain mechanical stability to sustain the long-term effects of blood pressure. They must not rupture or dilate in long term ideally.

Artificial vascular grafts must be easily manufactured in production, and have easy suturing properties.

In addition to autologous grafts and synthetic grafts, there has also been biological graft. However, they may have possible problems with bacterial contamination and antigenicity. They tend to cause immune reactions. The natural polymers from natural origins may also suffer from source to source variability of properties.³ The long-term durability is also a problem. Compared with this, synthetic polymers are usually inert and non-immunogenic. For these reasons, synthetic polymer vascular grafts have historically been the preferred material for the replacement of diseased blood vessels.

Generally, synthetic vascular grafts refer to commercially available Dacron® and Gore-Tex®.^{19,20} Dacron® is made of polyethylene terephthalate (PET). Gore-Tex® is expanded polytetrafluoroethylene (PTFE). They are both inert and non-degradable. PET was first introduced in 1939 and patented as Dacron® by Dupont in 1950.²¹ After that, PET has been widely used as a biomaterial since it has many advantages. Its high strength and stable performance under continuous mechanical stress endow PET with wide medical applications, especially in cardiovascular surgery.²² The application of PET vascular grafts has a long history. In 1957, PET was first introduced as Dacron grafts for aortic replacement.²³ As an artificial vascular graft material, PET has a unique advantage in that it can be fabricated in special structures. When a design for an artificial vascular graft is made, a certain level of porosity should be considered because the porosity of artificial vascular grafts is needed for cellular ingrowth from surrounding tissue into artificial materials. The sooner the surface of artificial blood vessels is

covered with endothelial cells, the better the healing process is. On the other hand, the pore size of the artificial substrate can not be too large which may cause blood leakage. The fabric structure of PET (Dacron®) is aimed to meet the demand.²⁴ Early vascular grafts belong to the category of woven. Woven grafts have a high bursting strength and can be woven tightly to allow minimal permeability to blood. However, woven grafts were proven to be difficult to handle due to its tight weave. Woven grafts are also less compliant. Here, the compliance is defined as the anastomoses between the stiff synthetic grafts and native vessels.²⁵ Another kind of fabric structure - knit, was applied for PET vascular grafts. It includes weft and warp knits. For weft-knitted vascular grafts, they have better compliance. However, the weft-knitted ones may run and unravel which leads to dilatation and rupturing after implantation. Warp-knitted vascular grafts are designed to resemble woven or weft knitted fabrics in mechanical performance. Meanwhile, the warp-knitted ones do not run, unravel, curl up or fray at edge. The warp-knitted PET vascular grafts are well accepted in clinical practice. On the basis of the main fabrication structure, another type of graft called velour-surface vascular grafts was introduced in 1967 compared to smooth ones.²⁶ Velour structure is emphasizing “filamentous” surface for a material which exposes many filaments on the surface with the aim of increasing tissue incorporation. They are easier to suture and handle. They do not run easily.²⁷ With all of the different fabric structures, PET vascular grafts present different physical properties. The patency rate for aortic bifurcation grafts can be 93% after 5 years.²⁸ Dacron is still the most commonly used material for aortic replacement. It does not show any significant biodegradation, loss of mechanical strength and adverse

reactions.

For the polymer material Gore-Tex® vascular grafts, PTFE was patented by Dupont in 1937 as Teflon and it has been first applied in medical device as artificial heart valves in the early 1960s.²¹ ePTFE has been produced by a paste extrusion process and patented as Gore-Tex® since 1969 which is a microporous material.³ It has been successfully used as vascular grafts because it has the advantages of particular inertness and a special microporous structure which supports tissue adhesion.²⁹ Its electronegative surface can also minimize the reaction with blood components. ePTFE is usually used as femoropopliteal bypass if a synthetic graft is required.

Besides the two kinds of vascular grafts mentioned above, polyurethane (PU)³⁰ is another promising material for artificial vascular grafts because of its good elasticity for potential compliance to tissues. PU was first commercially available in the 1930s as surface coatings and foams.³¹ And PU was first introduced to biomaterial market as a coating for implantable roller pumps.³² Because of its superior compliance, PU-based artificial vascular grafts have attracted more and more interests from researchers. The first PU-based vascular grafts were made of polyester PU by B. Braun Melsungen AG (Melsungen, Germany). However, it was reported that PU-based vascular grafts were susceptible to degradation *in vivo*.³³

Compared to non-degradable synthetic polymer vascular grafts, biodegradable artificial vascular grafts were developed more recently. Polyglycolic acid (PGA) and polylactic acid (PLA) are the two most common bioresorbable substrates under investigation. Those relatively hydrophilic biodegradable polymers are assumed to have

a unique advantage that they could biodegrade without leaving toxic artificial synthetic materials in human body after the completion of in-situ tissue regeneration. However, these biodegradable polymers are prone to undergo aneurysmal dilation and rupture before the generation of a tissue layer with enough strength. It was proved that PGA began to degrade and lose its mechanical strength after several weeks due to its hydrolysis reaction³⁴ before the successful tissue regeneration, which is fatal to human body.

Generally, PET and ePTFE are still the standard synthetic biomaterials to replace the diseased vessels in clinical surgery even if there are many kinds of promising new biomaterials. Compared with ePTFE, PET has good mechanical stability and its special fabrication structure can endow it with different properties. In addition, PET ($1.9 \times 10^{-2} \% \text{ mmHg}^{-1}$) shows better compliance than PTFE ($1.6 \times 10^{-2} \% \text{ mmHg}^{-1}$) while they have the same patency rate in 2 years (both 42%).³⁵ Here, the compliance of artificial grafts has been measured by the ratio of incremental volume change to incremental transmural pressure change, expressed as percent of diameter change per millimeter of mercury. PET is easier to handle and suture.³⁶ The development of PET vascular grafts is still very important and attracts a lot of attention from academia.

With decades of development, PET vascular grafts with large diameter (>6mm) have been improved to remain patent for more than 10 years after implantation. However, when the diameter is smaller than 6 mm, the performance of PET artificial vascular grafts are not ideal. They form blood clots^{37,38} rapidly after implantation due to surface-induced thrombosis. Before discussing surface-induced thrombosis, the

mechanism of blood coagulation and interaction of blood with the foreign material should be explained.

Blood coagulation is a complicated cascade reaction and can be three stages:³⁹ 1. Thromboplastin generation; 2. Thrombin formation activated by thromboplastin; 3. Fibrin formation activated by thrombin. First, the procoagulant reactions in blood environment generate thromboplastin complex. The thromboplastin complex can hydrolyze prothrombin to thrombin. The thrombin can act on fibrinogen and transfer fibrinogen to fibrin thread. At last, fibrin thread mixes with platelets, red blood cells and plasma to form blood clots which is also called thrombosis. There are two ways to generate thromboplastin (**Figure 1.1**): one is intrinsic pathway which is activated by coagulation factors present in the blood; the other is extrinsic pathway which is caused outside of blood vessels.⁴⁰ When the blood contacts with the artificial biomaterials, the blood coagulation belongs to intrinsic pathway. When the blood vessel is injured and the tissue is exposed, the coagulation happens which belongs to the extrinsic pathway. The intrinsic pathway begins with the adhesion of contact factor to activate XII to XIIa. XIIa can open the S-S bond in another factor XI and transfers XI to XIa. Meanwhile, XIIa can activate prekallikrein in blood to kallikrein which can even activate XII. With the help of Ca^{2+} , XIa can activate IX to IXa. At the same time, IXa with Ca^{2+} , phospholipids (existing in platelet membrane) and VIIIa can form VIII complex to activate X to Xa. Xa can finally form thromboplastin complex consisting of Xa, Ca^{2+} and V. In the following stage, the thromboplastin complex hydrolyzes prothrombin (II) to thrombin (IIa). The thrombin transfers fibrinogen to fibrin which is considered as the sign of coagulation

completion. The whole blood coagulation in intrinsic pathway lasts 5-10 mins.

The other pathway, extrinsic pathway, is also called tissue pathway because the blood coagulation is initiated by the tissue thromboplastin (or tissue factor, TF) in tissue fluids. TF and Ca^{2+} work on VII and form VIIa/TF complex followed by the activation of X. Then the process to form the thrombosis is similar to that caused by intrinsic pathway which can be called common pathway. The whole blood coagulation in extrinsic pathway lasts only 15 s. Once the thrombosis of trauma forms, the extrinsic pathway will be replaced by the stable intrinsic pathway.

Table 1.1. Coagulation factors in human blood

| Coagulation factors | | Functions |
|----------------------------|----------------------|---------------------|
| I | Fibronogen | receptor |
| II | Prothrombin | Protease |
| III | Tissue | cofactor |
| IV | Calcium | cofactor |
| V | Proaccelerin | cofactor |
| VII | Proconvertin | Protease |
| VIII | AHF | Protease + cofactor |
| IX | Christmas factor | Protease |
| X | Sturat-Prower factor | Protease |
| XI | PTA | Protease |
| XII | Hageman factor | Protease |
| XIII | FSF | Cofactor |
| Prkallikren | Fletcher factor | Protease + cofactor |

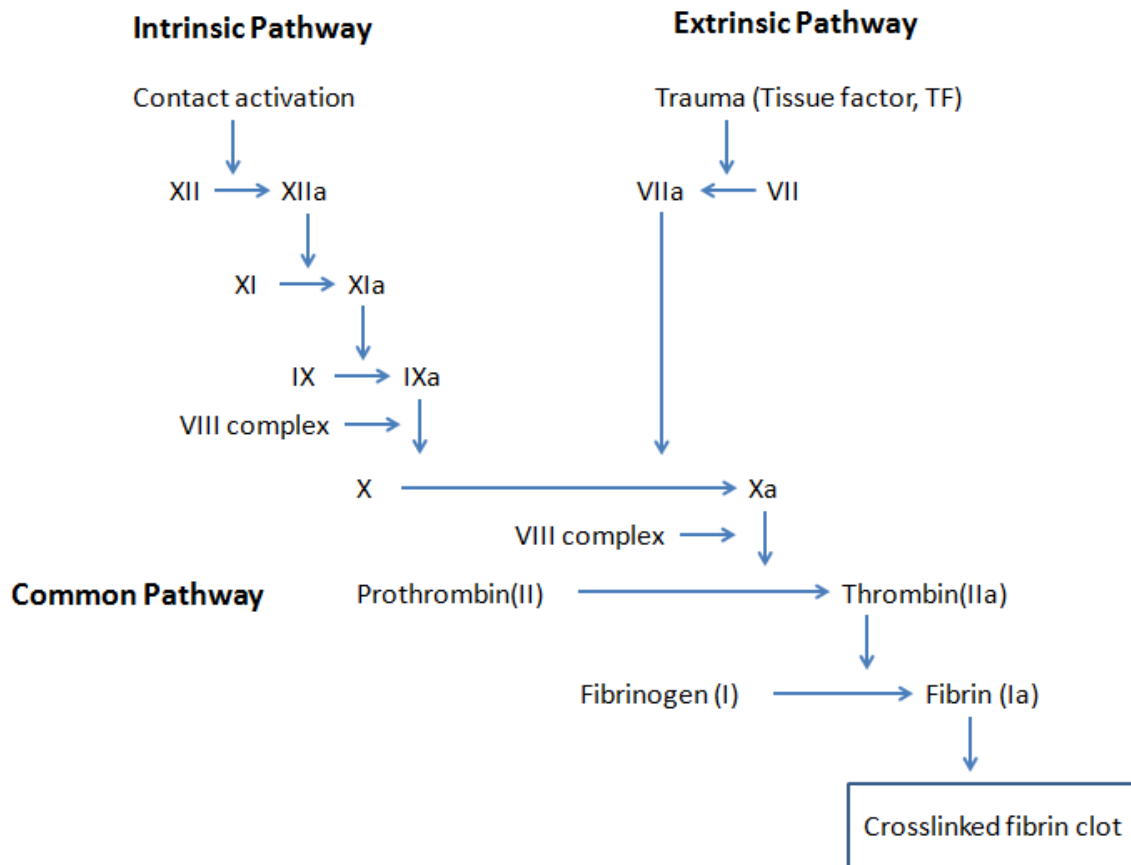


Figure 1.1. Simple scheme of blood coagulation pathways

During the blood coagulation, platelets in human blood also play an important role (**Figure 1.2**). Once the prothombin is activated to thrombin, the thrombin can work on platelets in the blood environment. The platelets can adhere to the trauma on blood vessels. Then the morphology of platelets can further change to the pseudopodia to aggregate other platelets. When the morphology of platelet changes, the platelet is activated to release ADP and β -thromboglobulin (β -TG) which can work on the further platelet aggregation. In addition, the activated platelets can also activate PF3 which is a phospholipid and very important in blood coagulation pathways.

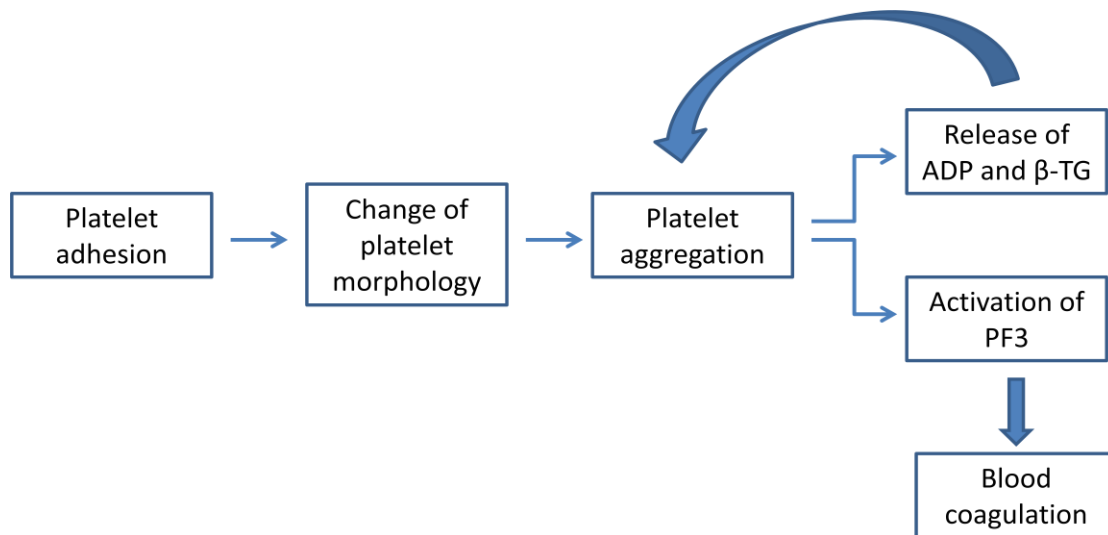


Figure 1.2. Simple scheme of platelet functions

When PET vascular grafts are introduced into human body, the intrinsic pathway of blood coagulation can be activated by the surfaces of artificial PET vascular grafts to yield surface-induced thrombosis. Particularly, when the diameter of PET vascular grafts is smaller than 6mm, the artificial blood vessels are thrombogenic because of the low-flow, high resistance state. This results in graft failure and poor clinical outcomes. Hence, the patency of small-diameter PET vascular grafts needs to be improved. Surface modification of PET vascular grafts is an efficient approach to make the surface of PET vascular grafts more haemocompatible. For surface modification of blood-contact biomaterials, coupling bio-agents to the surface or increasing the hydrophilicity of the surface is the chief method.⁴¹

1.2 Agents used to improve the haemocompatibility of biomaterials

It is the hydrophobic and non-haemocompatible surface of PET that is in contact with

human blood and incites the problem of thrombosis. In view of this, scientists have researched the possibility of introducing haemocompatible and hydrophilic molecules onto the surfaces of artificial biomaterials. The haemocompatible molecules are always referred to “bioactive” molecules since they can improve the haemocompatibility of biomaterials and human endothelial cells growth through their bioactivity.^{42,43} The hydrophilic molecules can inhibit proteins and platelet adhesion on the surface of biomaterials so that these molecules are considered as “bioinert” agents.⁴⁴

1.2.1 Bioactive agents

Heparin is a highly-sulfated glycosaminoglycan. It was isolated from the liver and heart by Mclean in 1916⁴⁵ and proved to have an anticoagulating ability. It is considered as the gold standard anticoagulant because it inhibits the formation of thrombosis effectively. Heparin binds enzyme antithrombin III⁴⁶ to inactivate thrombin (activated factor IIa) or other proteases involved in blood clotting process such as activated factor Xa (**Figure 1.1**). The inactivating rate of these coagulation factors can be increased by 50- to 100- fold due to the binding of heparin. So heparin is an indirect thrombin inhibitor. Heparin has been used to improve the haemocompatibility of stents, vascular grafts and other biomaterials which come into contact with blood and the results were proved to be better than those without heparin surface modification. The plasma recalcification time of PET stents increased dramatically from 58s to 900s after heparin immobilization.⁴⁷ Heparin is also available and proved to be effective in clinical use such as cardiovascular surgery.⁴⁰ Heparin bonded Dacron grafts showed 55% patency

rate compared to 42% (non-bonded ones) after 3 years implantation. Lev et al⁴⁸ followed 238 patients who underwent percutaneous coronary intervention. They applied heparin-coated stents (n=124) and non-coated stents (n=114) to those patients. During 30 days, only one heparin-coated stents (0.8% among n=124) formed thrombosis while seven non-coated stents occurred (6.1% among n=114). The heparin-coated stents showed significantly improved 30-day outcome compared to the non-coated stents.

Hyaluronic acid (HA) is a linear polysaccharide existing in the organism. It is used in tissue engineering due to its cellular functions like attachment and proliferation. Recently, it has been found that sulfated hyaluronic acid can endow biomaterials with an antithrombogenic property due to the heparin-like structure. However, both theories and experiments prove that hyaluronic acid has the heparin-like property only when it is sulfated. Hyaluronic acid alone can not inhibit the formation of thrombosis effectively.⁴⁹ Chen et al has immobilized sulfated hyaluronic acid onto the surface of PET films under UV irradiation. Thrombosis has proven to be reduced by almost 50% after 60min-immersion in human blood with sulfated hyaluronic acid.⁵⁰

Albumin is a very important protein occurring in blood in high concentration. Albumin, fibrinogen and r-globulin are the three primary plasma proteins responsible for initiating a response to blood contacting foreign material. The fibrinogen and r-globulin are prone to activate the platelet adhesion and thrombogenesis.⁵¹ However, albumin presents the opposite property. It has been shown to reduce the platelet adhesion and aggregation and suppress the subsequent thrombus formation.⁵² In *In vivo* experiments, albumin is shown to bind to surfaces of foreign materials and influence the behavior of

the blood contacting medical devices.⁵³ Due to its antithromboresistant ability, adsorbed albumin is applied to adsorb onto the surface of blood-contacting medical devices such as vascular grafts, change the interfacial properties, and further improve the haemocompatibility of those vascular grafts.³⁹ Human albumin impregnated polyester knitted grafts have been proved to have short-term antithrombogenic properties.⁵⁴ However, Nojiri et al tried a long-term experiment using the protein adsorbed PU vascular grafts in a dog model.⁵⁵ The result was totally different from the short-term trial. The protein adsorbed onto the artificial vascular surface reached 1000 Å thick. The long-term outcome of artificial vascular grafts modified with albumin is not ideal.

Gelatin is another kind of protein different from albumin. Gelatin can be produced from partial hydrolysis of collagen. Having a similar structure to collagen, gelatin has many hydroxyl, carboxyl and imine groups.⁵⁶ Since gelatin is a derivative of collagen, it shares almost the same properties as collagen. Both of them are natural polymers and suitable for *in vivo* applications. They can promote cell growth and endothelialization. Due to these properties, they are usually used in tissue engineering.⁵⁷ They can also be used to improve the haemocompatibility of biomaterials.⁵⁸⁻⁶⁰ The reason that gelatin or collagen can be used to improve the haemocompatibility of biomaterials is different from that of heparin or HA. Gelatin and collagen may allow the endothelialization of the surface of biomaterials in a shorter time so that the biomaterial surfaces can mimic the human blood vessels to inhibit the formation of thrombosis.⁶¹ However, there is a concern about both gelatin and collagen. They may accelerate the platelet adhesion before the formation of a layer of endothelial cells on the biomaterial surface. The

immobilization of gelatin on PET increased platelet adhesion from 25% to 40%.⁵⁸ Therefore, anticoagulant modification before the application of gelatin or collagen is necessary.⁶²

These agents mentioned above were the options to improve the haemocompatibility of biomaterials. However, sulphated HA improves the haemocompatibility of artificial vascular grafts due to its similar structure to heparin, but lower thrombin inactivation rate compared to heparin. Gelatin may induce the platelet adhesion which is not ideal for artificial vascular grafts. Albumin only demonstrates the short-term antithrombogenic property. Based on the mechanism and the anticoagulating ability, heparin is a standard and commercial available bioactive agent to improve the haemocompatibility of artificial vascular grafts. It was isolated from the liver and heart by Mclean in 1916⁴⁵ and has a long history of application. There are two important factors for the application of heparin in biomaterials: the method of heparin immobilization and the density of surface immobilized heparin.

1.2.2 Immobilization of heparin

In the recent years, many efforts have been made to immobilize heparin onto different substrates. Heparin can be immobilized onto substrates by forming chemical covalent bonds. Yang et al⁶³ modified PAN membrane with heparin using chitosan as a spacer through forming amide covalent bonds between carboxylic groups in heparin and amine groups in chitosan. The better haemocompatibility of modified PAN membrane was proved through the increased activated partial thromboplastin time (APTT) and

decreased adsorption of plasma proteins when the membrane contacts with blood environment. Lin et al⁶⁴ also applied chitosan to form covalent bonds with heparin to improve the haemocompatibility of PU membrane. The heparin-grafted PU membrane curtailed the protein adsorption, reduced platelet adhesion, elevated thrombin inactivation, and prolonged the blood coagulation time. Therefore, heparin immobilization improved the haemocompatibility of PU membrane. Huang et al⁶⁵ developed a method to covalently bond heparin onto Polysulfone (PSu) sheet and demonstrated a better hydrophilicity. On the other hand, heparin has also been immobilized to substrates through physical coatings or ionic bonds. The coating of heparin and collagen multilayer on intravascular stent was performed successfully by Lin et al and the haemocompatibility of the modified stent was proved by testing with platelets and blood clotting time tests.⁶⁶ Heparin coating on Poly(L-lactide) (PLLA) scaffolds was also achieved successfully by Kurpinski et al and the better haemocompatible property of the modified scaffolds was shown significantly through *in vivo* test.⁶⁷ However, the stability of heparin immobilized through coating method concerns researchers. Sagedal et al investigated the performance of a heparin-coated dialysis filter (AN69 ST).⁶⁸ The conclusion was that the heparin-coated AN69 ST filter did not induce less coagulation as compared to the conventional filter (F x 8). Marconi et al⁶⁹ immobilized heparin onto a commercial ethylene-vinyl alcohol copolymer (EVAL) by covalent bonding as well as ionic bonds for the sake of a better and longer-term haemocompatibility. From *in vitro* experiments, Marconi et al. compared the heparinized EVALs produced by forming covalent bonds with those heparinized EVALs produced by

forming ionic bonds. The APTT of covalently heparin-bonded sample has been increased by 50% compared to that of ionically heparin-bonded samples, possessing a higher degree of haemocompatibility. It was also proved that heparin immobilized through ionic bonds releases from the surface, indicating a poorer stability.

As mentioned above, heparin is a standard anticoagulant to be used on the surface of blood medical devices. Stable covalent bonds is a preferred immobilization way and has the potential for the long-term use of prosthetic vascular grafts.

1.2.3 Surface density of heparin

Besides heparin immobilization, surface concentration of heparin is another important factor closely related to the performance of medical devices. Andersson et al⁷⁰ demonstrated the optimal surface concentration of heparin for stenosis prevention is 1.5-3 $\mu\text{g heparin}/\text{cm}^2$ corresponding to 6-12 antithrombin/ cm^2 . Yang et al⁷¹ tried to improve the haemocompatibility of PSu by chitosan and heparin. It showed that the modified sample adhered less platelets when there was a higher surface density of heparin in the range of 1.5-3 $\mu\text{g heparin}/\text{cm}^2$. Lin et al⁷² introduced heparin to PAN dialysis membrane by different functional groups so as to obtain different surface densities of heparin. Among those samples, the PAN membrane with the highest heparin density---2.74 $\mu\text{g}/\text{cm}^2$ gave the longest blood coagulation time and the least amount of adhered platelets on the surface.

To improve the haemocompatibility of medical devices to a satisfactory level, the concentration of heparin should be taken into account compared to the immobilization

method of heparin.

1.2.4 Bioinert agents

Another kind of strategy applied to improve the haemocompatibility of biomaterials is to create “bioinert” surfaces, namely non-fouling surfaces which can resist the protein adsorption and cell adhesion.^{73,74} To achieve the non-fouling surfaces, the primary force driving the adsorption of proteins to a solid surface---hydrophobic dehydration should be considered.⁷⁵ It results from the hydrophobic interaction between protein and solid surfaces and electrostatic interactions between charged groups on protein and surfaces. Generally, the more hydrophobic material surface is, the more proteins absorbs. Another trend is that the charged surface absorbs the protein with same electric charge. Thus, increasing the hydrophilicity or improving the electric charges of material surface obtains non-fouling surface. It can be achieved by immobilization of special agents on material surface. Poly(ethylene glycol) (PEG), a extremely hydrophilic and uncharged agent, is an example. The long chain of repeat ethylene oxide results in a high dipole which causes extensive hydration to repel proteins on the surface. As a result, PEG has relatively poor binding of proteins and cells^{76,77} such as plasma protein. PEG is also considered as an anti-platelet agent. Additionally, PEG is low toxic and proved by FDA as a biomedical surfactant. The immobilization of PEG is commonly used to build a “bioinert” luminal surface for artificial vascular for a better haemocompatibility.⁷⁸ PEG is used as a “bioinert” agent in application of artificial blood materials to repel plasma protein and platelets.

The antifouling property of PEG depends on many factors, such as the experiment temperature,⁷⁹ the molecular weight of PEG⁴⁴ and the concentration of PEG⁸⁰ on the surface of biomaterials. It is proved that PEG with longer chain length has a better repulsion to protein adhesion.⁸¹

1.2.5 Immobilization of PEG

PEG has been immobilized to several substrates chemically via different functional groups and chemical reactions directly or by the means of pre-activation of the surface such as the use of linkers and coupling agents.⁸² Generally, new functional groups should be introduced to substrate first if the substrate can not react with PEG chain directly. Kim et al⁸³ immobilized PEG with high molecular weight onto PU substrate as described. Isocyanates were used as special functional groups to bond with PEG derivatives. First, diisocyanates reacted with the urea groups (-CO-NH-) on PU substrate. Then free isocyanate group on PU can form bonds with PEG derivatives to achieve the immobilization of PEG. Luo et al⁸⁴ utilized 1,6-hexamethylene diisocyanate as a coupling agent to bind hydroxyl groups on PEG chain onto PU substrate. Since there is no functional group reacting with PEG, a specific group such as isocyanate group should be introduced. Another strategy is to use specific PEG with special functional groups on the ends of long chain. Rana et al⁸⁵ applied PEG functionalized with alkyne groups onto PU substrate through “click” chemistry. The PU substrate was functionalized with azide groups firstly. After that, the alkyne groups on special PEG can react with these azide groups so that PEG has been immobilized to the destination substrate.

As mentioned, proper functional group on the substrate is the prerequisite to apply PEG to improve the haemocompatibility of the biomaterials. Meanwhile, the molecular weight and the reaction conditions should be also considered into the reaction.

1.3 Thermoplastic polymeric material – Poly(ethylene terephthalate)

Thermoplastic polymeric material PET was first commercialized as Dacron® by Dupont in 1939. It is produced by polycondensation reaction of ethylene glycol and either terephthalic acid or dimethyl terephthalate.⁸⁶ The polycondensation process gave high crystallinity to PET material which is very important to its physical property. It endows PET with an excellent tensile strength of 170-180 MPa and a tensile modulus of 14 GPa in oriented form.⁸⁷ In the chemical aspect, PET is an extremely inert material which is not easily degradable. Compared with biological materials which are not autologous, PET is less susceptible to immune reactions to human body. Due to its special physical and inert properties, PET is widely used in medical devices. It has a wide biomedical application in artificial vessels, ligaments,⁸⁸ joint replacement,⁸⁹ implantable sutures⁹⁰ and surgical meshes.⁹¹

1.3.1 Medical application of PET as standard artificial vascular grafts

In the field of artificial vascular grafts, PET, known as Dacron®, has a long history as a replacement to diseased vessels since 1957.²³ After that, PET occurred in 95% of arterial bypass surgeries. There were woven and knit structures for PET vascular grafts. In 1967, a new fabrication structure-velour was introduced to PET vascular grafts²⁶ and

double velour PET vascular grafts were applied in 1978²⁷ to enhance cell ingrowth. Compared to other artificial vascular grafts, PET artificial blood vessels have the unique advantage that it can be fabricated into different structure to improve the performance.

However, as mentioned above, when PET vascular grafts come in contact with blood, especially when the diameter of those grafts is smaller than 6 mm, graft thrombosis tends to occur. To decrease the thrombogenicity, surface modification is an option to introduce bioactive/bioinert agents onto PET vascular grafts to inhibit surface induced thrombosis. It's a challenge to modify the surface of PET since PET is extremely chemical inert, hydrophobic and highly crystalline.

It has been confirmed that the introduction of bioactive/bioinert agents (heparin/PEG) onto the surface of artificial biomaterials can improve their haemocompatibility. Heparin and PEG have been considered for PET substrate. The prerequisite of heparin/PEG-based PET is that there are available functional groups on the surface of PET substrate which can be used to immobilize heparin/PEG. In addition, heparin/PEG should be immobilized stably on the surface of PET substrate. However, PET is an extremely chemically inert material. There are no reactive functional groups on the surface of PET. Due to PET's high hydrophobicity and crystallinity, substrate having functional groups can not diffuse into the long chains of PET to react with PET sufficiently. All of those factors make the durable immobilization of heparin/PEG onto PET substrate difficult.

1.3.2 Surface modification methods of PET to introduce functional groups

Recently, the following techniques have been applied to introduce functional groups on the surface of PET substrate.

One approach is to **coat** PET substrate using compounds with functional groups. Bilsen et al⁹² coated a layer of Poly(ethylene imine) (PEI) to provide $-NH_2$. After that, heparin was immobilized onto the modified PET substrate by another coating layer on the base of PEI and PAA. The modified PET showed improved haemocompatibility. Gericke, et al⁹³ also applied the coating method. First, PET was coated with the amino celluloses to act as a primary anchor layer. Second, the sample was coated with another cellulose polysaccharide layer. After that, the sample continued to be coated with chitosan and heparin. Doliška et al⁹⁴ applied quartz crystal microbalance with dissipation unit (QCM-D) and spinning coating techniques to make PET foil adsorb heparin layer. Fasl et al⁹⁵ used different sulphated polysaccharides similar to heparin molecules as coating materials to improve the haemocompatibility of PET foil. The similar coating method has been applied to modify PET substrate with PEG. Dimitrievska et al⁹⁶ immersed PET fabric into different PEG solutions to obtain different concentration - PEG layers on PET surface. However, it is not convenient to obtain several coating layers. Even if the coating process can be limited in one step and improves the haemocompatibility of PET substrate to a certain extent, the long-term stability of the coating method concerns researchers since the structure is similar to layer by layer.

Another approach for surface modification is to introduce functional groups to PET through **hydrolysis**⁹⁷ or **reduction**.⁹⁸ These two techniques are to make PET substrate

degrade to generate carboxylic and amino groups. Then, the substrate becomes active when it possesses those functional groups on the surface and can be used to immobilize heparin/PEG. However, these techniques may compromise bulk properties of PET. They may cause loss of strength due to the degradation of polymer substrate.

Plasma treatment is very popular in recent years. Plasma modification must be operated with a radio frequency (RF) generator. First, the feed gas, which will provide active particles, is ionized when RF power is applied. This step is to produce plasma. Second, samples are fixed under the glow region. The ionized gas full of active radicals can act on the surfaces of samples and modify the surfaces.⁹⁹

The common plasma treatments used now are oxygen plasma and nitrogen plasma. Oxygen plasma produces carboxyl or even more functional groups on PET surfaces. Nitrogen plasma improves the formation of amide groups. These groups turn the inert surfaces of PET into active ones. The hydrophilicity of PET surfaces is also altered by the increased concentrations of oxygen and nitrogen. Both the chemical composition and the morphology of PET surfaces can be changed by plasma treatments which are beneficial to decrease protein and cell adhesion.^{100,101} Plasma treatment merely modifies a few nanometers of material surfaces so that it won't change the bulk properties of the materials.¹⁰² Kim et al used plasma glow discharge to produce peroxides and introduced carboxylic groups.¹⁰³ Then insulin and heparin were co-immobilized to PET via these carboxylic groups. The final product adhered less platelets showing a better haemocompatibility. However, the aging effect of plasma treatment tends to make the improvement disappear. The surfaces return to their untreated state after a few days. This

problem is very detrimental for the long-term use of PET vascular grafts because the improved haecompatibility should maintain functions for years.

In conclusion, there is no ideal method mentioned above to introduce functional groups to PET and improve its haecompatibility to be suitable for long-term use as artificial vascular graft. The coating of heparin/PEG onto PET surface can be a candidate to improve the haecompatibility of PET substrate. However, the durability of the modified PET in this way can not be ensured. As a result, a durable and non-invasive surface modification for PET substrate to introduce functional groups and immobilize bioactive/bioinert (heparin/PEG) is needed.

A special method called **Thermoplastic semi-interpenetrating network (IPN)** is another technique to improve the surface properties of thermoplastic inert polymers by introducing functional groups. There are two steps: first, the thermoplastic polymer substrate is immersed in the solution containing functional groups for a determined duration. The polymer substrate swells and monomers with functional groups diffuse into the amorphous region. Second, these monomers immediately polymerize once exposed to UV irradiation¹⁰⁴ and form a new crosslinked polymer network within the original thermoplastic polymer substrate. Thus, there is a thermoplastic semi-IPN: the chemically crosslinked introduced functional polymer interlocks with the original physically crosslinked thermoplastic polymer substrate (crystalline areas serve as crosslinking point). Since PET is also a thermoplastic polymer material, IPN method can be applied to modify PET's surface properties. In the previous results in our lab (Dr. Liu's Medical Textile Surface Engineering Lab), Polyacrylamide (PAM) was used to

modify the surface properties of PET substrate through IPN method. Liu et al¹⁰⁵ applied acrylamide (AM) as monomer, N,N'-methylenebisacrylamide (MBA) as crosslinker and benzophenone (BP) as photo initiator to introduce amide functional groups onto PET substrate. First, those substances diffused into swollen surface of PET. Then, those substances polymerized on the surface of PET under UV irradiation. Finally, a new crosslinked polymer network-thermoplastic semi-IPN was formed. The introduced functional polymer is chemically crosslinked to physically interlock with the thermoplastic substrate. Both of the stability and surface concentration were proved to be satisfactory. The mass of PAM immobilized PET remained constant even after 3 days Soxhlet extraction with distilled water. At the same time, the surface concentration of amide as high as 233 nmol/cm² was achieved on PAM immobilized PET. Thermoplastic semi-IPN has the following advantages: IPN method can limit the modification into a shallow region on the surface without compromising the bulk property of the substrate; IPN method can introduce versatile functional groups as well as achieving a high surface density of those functional groups; IPN method can immobilize functional groups very durably onto the substrate. All of these advantages make the the surface modification of PET with heparin/PEG via the IPN method a very good option. With the IPN technique, a large amount of functional groups can be obtained to immobilize an ideal density of heparin or PEG in this way.

Chapter II: Hypotheses and Objectives

Cardiovascular diseases especially CAD, are the leading causes of death in North America. According to the investigation of Tu et al,¹⁰⁶ the annual mortality rates were more than 30 percent among patients with cardiovascular diseases in North America. Since these diseases normally affect small-diameter blood vessels, revascularization of damaged blood vessels is often necessary. Compared to autologous tissues, artificial biomaterials are attractive replacements since autologous tissues are limited by their deficient supply and high surgical morbidity.^{9,37} PET (Dacron®) has become a standard vascular replacement in vascular surgeries since 1957 due to its high strength and stable performance. After 50 years of development, large-diameter PET vascular grafts (>6mm) can remain functioning for more than 10 years after implantation while small-diameter PET vascular grafts (<6 mm) are plagued by early graft thrombosis. Small-diameter grafts thrombose rapidly after implantation because of the low-flow, high resistance conditions.

To improve the haemocompatibility of biomaterials, the common way is to immobilize either bioactive agent---heparin or bioinert agent – PEG. They have different functions. Heparin can inhibit the formation of thrombosis (blood clots) effectively on the surface of grafts while PEG can inhibit the adhesion of proteins and platelets in blood to avoid further aggregation of platelets. Heparin and PEG have been applied to many different substrates presenting functional groups which can react with heparin and PEG. However, PET is extremely chemically inert without any reactive functional group and PET has a high crystallinity which makes surface modification to introduce

functional groups difficult. Therefore, a new technique - IPN method may be useful. IPN method forms a new crosslinked polymer network with functional groups interlocked to PET substrate. This method introduces functional groups to polymer surface without compromising the bulk property of the polymer substrate; it can introduce versatile functional groups as well as achieve a high surface density of those functional groups; at last, it can immobilize functional groups very stably onto the substrate. Based on those advantages, IPN method was chosen as a candidate technique to improve the haemocompatibility of PET.

However, there are some problems with using heparin. Heparin can lead to side effects such as thrombocytopenia and uncontrolled bleeding if used in high dose. Even though the side effects are rare, reportedly happened in one out of 10 numbers of patients at most,⁴⁰ it is wise to find alternatives to heparin which can avoid the side effects. Based on that, the structure of heparin also has attract more and more attention from researchers.

The rationale of this thesis was based on the following points,

- 1) We developed a surface modification technique – IPN method to improve the surface property of PET substrate on the basis of previous research achieved in our lab. Polyacrylamide and Poly(N-(2-methylbut-3-yn-2-yl)acrylamide) (PMBAA) have been successfully immobilized onto the surface of PET fabrics. We hypothesized that vinyl monomers bearing different functional groups (specifically

Tert-butyl 2-(acrylamido)ethylcarbamate (AEAM-Boc) and *N*-(prop-2-ynyl)acrylamide (NAA)) can also be immobilized onto PET material using the IPN method.

- 2) Once P(AEAM-Boc) and PNAA are successfully immobilized onto PET using the IPN method, we hypothesized heparin and PEG could be durably bonded onto the surfaces with sufficiently high densities through secondary chemical reactions. We also hypothesized that the haemocompatibility of PET can be improved by the introduction of heparin and PEG.
- 3) We constructed a bioactive platform to mimic different functional groups in heparin structure. We hypothesized that –COOH and –SO₃ functional groups in heparin molecule contribute to its anticoagulating performance and there may be an optimum ratio of the two functional groups with which the antithrombogenic activity is the best.

Therefore, a series of synthetic, chemical and *in vitro* biological experiments were designed and carried out to evaluate the modified surfaces and haemocompatibility of modified PET with heparin and PEG as well as the model for alternatives to heparin.

The objectives of this thesis were:

1. To modify PET substrate with bioactive or bioinert substances (heparin & PEG) using thermoplastic semi-IPN method.
2. To conduct both chemical and biological characterizations of the modified PET substrate.
3. To achieve optimal surface concentration of heparin on PET substance and obtain a

better antithrombogenic performance by conducting a systematic study.

4. To further explore antcoagulating function of each functional group (-COOH and -SO₃) in heparin molecule and find out the relationship between antithrombogenicity and the ratio of those functional groups using a bioactive model.

Findings from this thesis would be beneficial in durably and effectively improving the haemocompatibility of PET vascular grafts. They would be also useful in designing and developing a new bioactive agent to replace heparin with a better anticoagulating activity for future vascular grafts development.

Chapter III: Experimental Section

According to the hypotheses and objectives of this study, the whole experimental section can be divided into two parts: Part 1-surface modification of PET using IPN method; Part 2-alternatives to heparin structure. In part 1: **3.2 Surface modification of PET with bioactive agent-heparin; 3.3 Surface modification of PET with bioinert agent-PEG.** In part 2: **3.4 Anticoagulating action between carboxylic and sulfate groups in heparin structure.**

3.1 Materials and Reagents

Surface modification of PET (Part 1):

Poly(ethylene terephthalate) (PET) plain woven fabric (#777H) was purchased from Testfabrics, Inc. The fabrics were Soxhlet-extracted with distilled (DI) water for 24 h to remove detergents and other impurities. The extracted PET fabrics were then air-dried overnight, stored in a desiccator and ready for the following experiments. Ethylenediamine (EAM), acryloyl chloride (AC), di-tert-butyl dicarbonate (Boc₂O), Triethylamine (Et₃N), dichloromethane (CH₂Cl), benzophenone (BP), N,N'-methylenebisacrylamide (MBA), N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC), N-Hydroxy-succinimide (NHS), heparin sodium salt, Methoxypolyethylene glycol azide 2000 (N₃-PEG), acid orange 7 (AO), toluidine Blue dye (TB), mepacrine dye and 3-(4,5-dimethyl-2-thiazolyl)-2,5 diphenyltetrazolium bromide (MTT) were obtained from Sigma-Aldrich (Oakville, ON) and used as received. 2-propynylamine (amine 2, 460 g) was purchased from Atlantic SciTech Group. Human

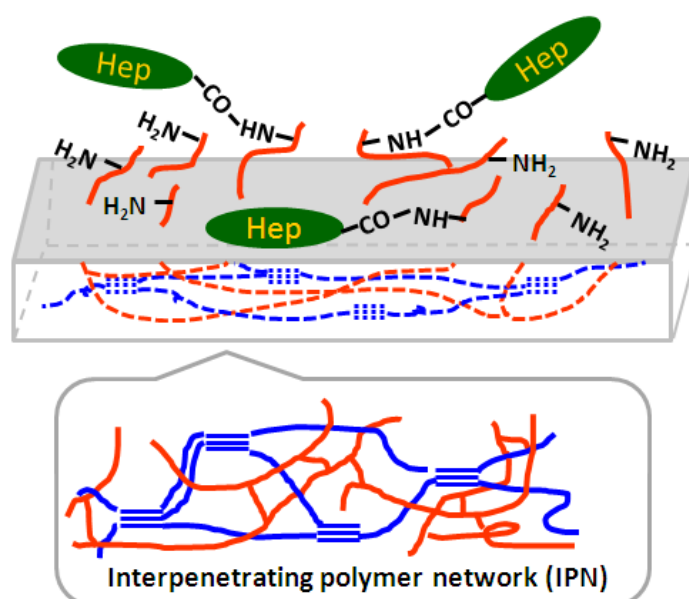
dermal fibroblast cells and fibroblast basal medium were obtained from Cedarlane (Burlington, ON).

Anticoagulating action between carboxylate and sulfate groups in heparin structure (Part 2):

Sodium azide (NaN_3), methyl 6-bromohexanoate (Br-1), lithium hydroxide (LiOH), 6-chlorohexanol (Cl-1), triethylamine (Et_3N), Methanesulfonyl chloride (MsCl), lithium bromide (LiBr), Sodium sulfite (Na_2SO_3), dimethylformamide (DMF), tetrahydrofuran (THF); Thionin acetate (THA) dye was purchased from Sigma-Aldrich (Oakville, ON) and used as received.

3.2. Surface modification of PET substrate with bioactive agent - heparin

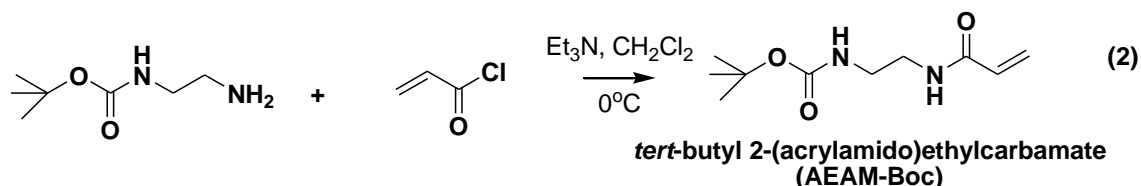
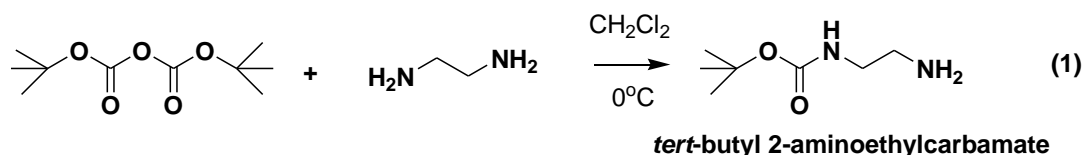
The whole process of modifying PET substrate with the bioactive molecule - heparin was carried out according to **Scheme 3.2.1**.



Scheme 3.2.1. Modification of PET samples with heparin

3.2.1. Synthesis of *tert*-butyl 2-(acrylamido)ethylcarbamate (AEAM-Boc)

Tert-butyl 2-(acrylamido)ethylcarbamate (AEAM-Boc) was synthesized according to a published method.¹⁰⁷ One amine group in EAM was protected by *t*-butoxycarbonyl (Boc) before it reacted with AC (Scheme 3.2.2). Boc₂O (32.8 g, 0.15 mol) dissolving in CH₂Cl (200 mL) was added to a solution of EAM (40 g, 45 mL, 0.66 mol) dissolving in CH₂Cl (200 mL) to obtain *tert*-butyl 2-aminoethylcarbamate (Scheme 3.2.2 (1)). After that, *tert*-butyl 2-aminoethylcarbamate was extracted and dried generating a yield of 21 g (73.61%). AC (11.88 g, 10 mL, 0.1325 mol) dissolving in CH₂Cl (100 mL) was added to a solution of *tert*-butyl 2-aminoethylcarbamate (21 g, 0.1325 mol) and Et₃N (63.6 g, 55 mL, 0.3975 mol) dissolving in CH₂Cl (200 mL) at 0 °C drop by drop under stirring reacting for 24 h to obtain AEAM-Boc (Scheme 3.2.2 (2)). Then AEAM-Boc was extracted and solvent was rotary evaporated to remove generating a yield of 17 g (60.53%). ¹H-NMR (300MHz, CDCl₃, δ): 6.23 (d, *J*=1.35 Hz, 1H); 6.07-6.16 (m, 1H); 5.64 (dd, *J*₁=1.27 Hz, *J*₂=10.08 Hz, 1H); 3.42-3.47 (m, 2H, CH₂); 3.29- 3.35 (m, 2H, CH₂); 1.44 (s, 9H, C(CH₃)₃).



Scheme 3.2.2. Synthesis process of AEAM-Boc

3.2.2 Immobilization of poly(AEAM-Boc) onto PET using the IPN method

PET fabrics were modified with AEAM-Boc by forming an IPN as previously described.^{105,108,109} First, a round PET fabric was swollen in methanol solution containing BP (initiator, 0.55 mol/L), AEAM-Boc (monomer, 2 mol/L) and MBA (crosslinker, 1.5% of monomer) at 40 °C for 60 min. Centrifugation (3 min, 3,500 rpm) of the swollen specimen was used to remove the excess of solution to achieve a pickup of around 33%. The fabric was then exposed to UV irradiation ($\lambda=365$ nm, 60 min) to execute the copolymerization of AEAM-Boc and MBA for the formation of surface IPN. After the polymerization, the sample (named as P(AEAM-Boc)-PET) was extracted with methanol in a Soxhlet-extractor for 24 h, air-dried overnight, and stored in a desiccator for 48 h to obtain a constant weight. The immobilization percentage was calculated according to the following equation:

$$\text{Immobilization Percentage/IP(\%)} = \frac{W_2 - W_1}{W_1} \times 100\% \quad (1)$$

--- Where W1 and W2 are the weights of PET samples before and after the formation of IPN, respectively.

3.2.3 Removal of Boc protecting group on P(AEAM-Boc)-PET

P(AEAM-Boc)-PET samples were cut into small pieces, and hydrolyzed in 4 M HCl/1,4-dioxane (ratio=1:1, v/v) at room temperature to convert NH-Boc groups to amine groups following a previous reported protocol.¹¹⁰ Afterwards, the samples were sequentially rinsed with DI water, saturated NaHCO₃ and DI water to remove excess HCl. The PET sample with amine groups was referred to as PET-NH₂.

3.2.4 Immobilization of heparin onto PET-NH₂

Condensation reaction between carboxylic acid groups of heparin and free amines on PET-NH₂ could result in the heparinization of PET-NH₂.^{111,112} Carboxylic groups of heparin were activated with EDC and NHS as follows: 1 mg heparin, 1 mg EDC and 0.6 mg NHS were added into 500 μ L citric buffer solution (pH=5.6) at 37 °C for certain time (activation time). The PET-NH₂ samples were then immersed in the above solution of succinimidyl ester of heparin at 37 °C for certain time (reaction time). After the reaction, the heparinized PET samples were shaken in 0.1 M Na₂HPO₄ (PH 9.2) for 2 h, followed by sequential washing with 4 M NaCl and DI water for 24 h respectively (rinsing solutions were refreshed for at least 4 times in each of the 24 h washing duration). Finally, the modified samples were freeze-dried overnight. The heparin bonded sample was referred to as PET-Hep.

3.2.5 Instrumental analysis and characterization

Proton Nuclear magnetic resonance ($^1\text{H-NMR}$) spectrum of AEAM-Boc was recorded at room temperature in a 5 mm NMR tube on a Bruker Avance 300 MHz NMR spectrometer. Fourier transform infrared (**FT-IR**) spectra of untreated and modified PET samples were taken on a Nicolet iS10 spectrometer (Thermo Fisher Scientific Inc.) using KBr pellets. X-ray photoelectron spectroscopy (**XPS**) data of untreated and modified PET samples were acquired with a Karto Axis Ultra spectrometer by Kratos Analytical, Inc.

3.2.6 Determination of surface densities of amine and heparin on modified PET

AO dye was used to determine the surface density of amine groups since amine groups can form 1:1 complex with AO at acidic condition. PET-NH₂ samples (2 × 2 cm²) were immersed into 10 mL of AO dye solution (10 mg/mL, pH=3 adjusted by HCl) with constant shaking for 12 h at room temperature. The samples were then rinsed with HCl (pH=3) to remove the residue dye, followed by shaking in NaOH solution (pH=12) for 30 min to remove the non-specifically adsorbed dye on the samples. The absorbance of dye/NaOH solution was measured at 485 nm.^{113,114} Afterwards, the surface density of amine groups corresponding to dye concentration was then calculated from the calibration curve. The measurement was performed in duplicate.

The amount of heparin immobilized on modified PET samples was determined using a colorimetric method.^{115,116} 3 mL of TB solution (25 mg TB dissolving in 500 mL of 0.01 M HCl containing 0.2% NaCl) was diluted with 2 mL Milli-Q ultrapure water.

PET-Hep samples ($1 \times 1 \text{ cm}^2$) were then immersed into the solution for 2 h. 5 mL of n-Hexane was added and agitated by a Vortex mixer for 2 min to ensure uniformity of treatment (the same treatment as in constructing a calibration curve even this step is not indispensable in the case of PET-Hep). After removing the PET-Hep-dye complex from the solution, the absorbance of residue aqueous solution was measured at 631 nm and the amount of heparin immobilized on modified PET samples can be calculated from the previously obtained calibration curve. The measurement was also performed in duplicate.

3.2.7 Stability test of immobilized heparin

To examine the stability of heparin immobilized on the surface of PET substrate, stability test was conducted according to Yang et al.^{63,116} PET-Hep samples ($1 \times 1 \text{ cm}^2$) were immersed in a big beaker containing PBS (pH=7.4, 50 mL) for 1 day. Two pieces of PET-Hep samples were taken out at predetermined times ($t = 4.8, 9.6, 14.4, 19.2$ and 24 hours) and the amount of heparin remained on those samples were determined using the colorimetric method. The heparin remaining can be calculated as the following equation:

$$\text{Heparin remaining(\%)} = \frac{D2}{D1} \times 100\% \quad (2)$$

--- Where D1 and D2 are the surface densities of heparin on PET-Hep samples before and after extraction with PBS solution, respectively.

3.2.8 Platelet adhesion test

Platelet adhesion test was carried out in a static blood environment according to the literature.⁶⁴ Human whole blood was collected from healthy volunteers and mixed with 3.8% sodium citrate solution (ratio of human whole blood and aqueous solution=9:1, v/v) as an anticoagulant. The blood was centrifuged at 1,200 rpm for 15 min at room temperature to obtain platelet-rich plasma (PRP). The number of platelets was determined using a hemocytometer. The platelet concentration of PRP solution was adjusted to 1×10^5 cells/ μ L by adding phosphate buffered saline (PBS, pH=7.4) to PRP. The modified (PET-Hep) and untreated PET samples (1×1 cm²) were equilibrated with PBS (pre-warmed at 37 °C) for 1 h and then immersed into 1 mL of the platelet suspension mentioned above for 2 h at 37 °C with 5% CO₂. After the incubation, the samples were taken out and gently washed with PBS for three times to remove the non-adhered platelets. The platelets remaining in both PRP solution and the the rinsing solution were quantified using the hemocytometer. The extent of platelet adhesion in respect to the PRP control was calculated according to the following equation:

$$\text{Platelet adhesion (\%)} = \frac{P1 - P2}{P1} \times 100\% \quad (3)$$

--- Where P1 is the platelet count in PRP solution before contact and P2 is the total count of platelet in both PRP solution and the rinsing solution after contact.

Analysis of variance (t-test; n=5) of the results was performed with regard to control (untreated PET) at $p < 0.05$, $p < 0.01$ and $p < 0.001$ of significant level.

The distribution of platelets adhered on those PET samples and the morphology of adhered platelets were observed using an OLYMPUS BX60 fluorescent microscope and

a JEOL 840 analytical scanning electron microscope (SEM), respectively. After being removed from the PRP solution, the PET samples were washed with PBS and fixed with 2.5% glutaraldehyde solution (Sigma) for 20 min at room temperature. The samples were washed with PBS again and dehydrated in a series of ethanol/DI water solutions (50, 60, 70, 80, 90 and 100%). Finally a portion of the PET samples was stained in 10 mM mepacrine solution for 90 min at room temperature and the fluorescent images of platelets on those samples were taken under the fluorescent microscope. Another portion of the PET samples was coated with gold in vacuum for SEM observation.

3.2.9 Cytotoxicity test

The cytotoxicity of PET-Hep was evaluated using the MTT assay in Human dermal fibroblast cells (PCS 201-010, ATCC). The cells were cultured on T-25 flask with fibroblast basal medium supplemented with fibroblast serum free media. Culture was kept in an incubator at 37 °C with 5% CO₂ and the medium was changed every 3 days. When the cells grew to 80% of confluence, those cells were detached by adding 1 mL of EDTA and 1 mL of Trypsin. The number of cells was determined by a hemocytometer and the cell concentration of cell suspension can be adjusted by diluting with the culture medium.

MTT assay is a standard test to determine the viability of cells. The mechanism is that cellular enzymes can reduce yellow MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide) to purple crystal formazan. To determine the toxicity of extracts from modified and untreated PET samples, MTT assay was performed as

described.¹¹⁷ The samples were equilibrated with PBS for 1 h and immersed in 2 mL of fresh culture medium for 1, 2 and 4 days, respectively. The culture medium was changed every day and preserved. The fibroblast cells were seeded in a sterile 96-well tissue culture plate with a density of 1.5×10^5 cells/mL using fresh culture medium. After 24 h of incubation, the fresh culture medium was replaced by the medium got at the 1st, 2nd and the 4th day of extraction for another 24 h incubation at 37 °C with 5% CO₂. Subsequently, 10 µL of MTT solution (dissolved in PBS; 5 mg/ml) was added to each well and the well was incubated for 4 h at 37 °C with 5% CO₂. After the formation of purple crystals, excess medium and MTT were removed and 100 µL of dimethyl sulphoxide was added to each well. The well plate was kept in the same incubator for 10 min to dissolve the crystals. The absorbance of the mixed solution was measured at 560 nm using an Opsys MRTM microplate reader (DYNEX Technologies). The cell viability was calculated according to the following equation:

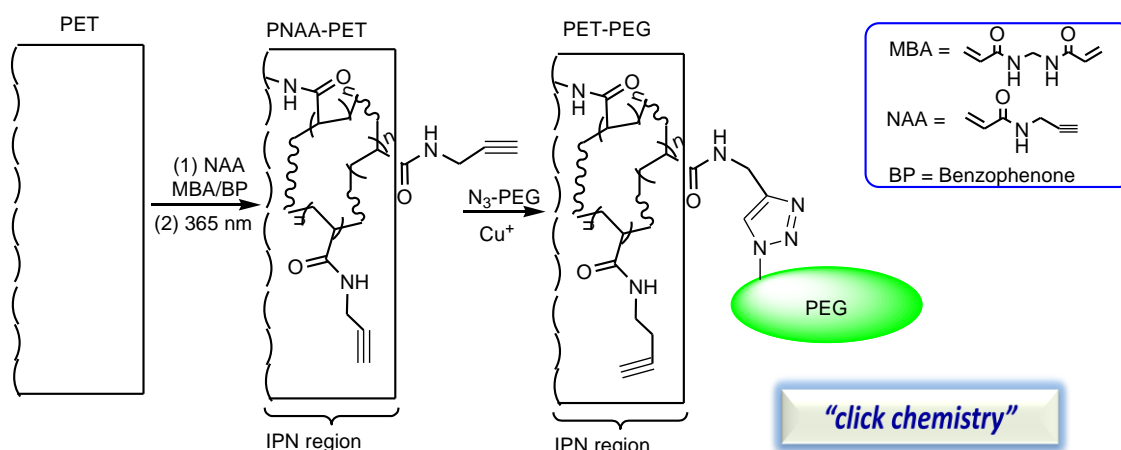
$$\text{Cell viability (\%)} = \frac{D_s - D_b}{D_c - D_b} \times 100\% \quad (4)$$

--- Where D_s, D_b and D_c are the optical densities of formazan crystals produced by sample, blank (medium without cells) and control (medium with cells), respectively.

Analysis of variance (t-test; n=3) of the results was performed with regard to control (untreated PET) at $p < 0.05$ and $p < 0.01$ of significant level.

3.3 Surface modification of PET substrate with bioinert agent - PEG

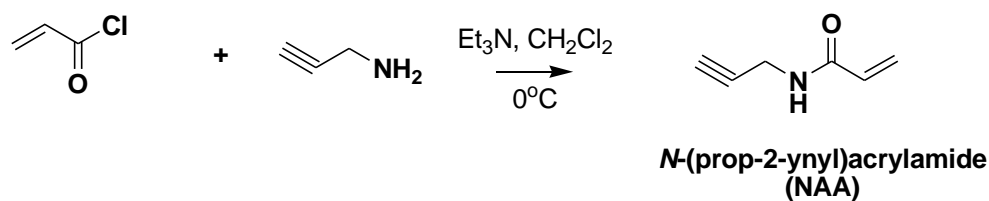
The whole process of PET modifying was carried out as illustrated in **Scheme 3.3.1**.



Scheme 3.3.1. Modification of PET samples with PEG

3.3.1 Synthesis of *N*-(prop-2-ynyl)acrylamide (NAA)

N-(prop-2-ynyl)acrylamide (NAA) was synthesized according to a published method.^{118,119} AC (22 mL, 0.273 mol, 98%) dissolving in CH₂Cl (200 mL) was added to a solution of 2-propynylamine (16 g, 0.285 mol) and Et₃N (41 mL, 0.299 mol) dissolving in CH₂Cl (200 mL) at 0 °C drop by drop under stirring reacting for 24 h. The product was extracted and the solvent was rotary evaporated to remove. After that, the product was purified by flash column chromatography on silica gel (received from Selecto Scientific Georgia, USA) generating a yield of 21.6 g (74.48%). The synthesis process was shown as **Scheme 3.3.2**.



Scheme 3.3.2. Synthesis of NAA

3.3.2 Immobilization of poly(NAA) onto PET using the IPN method

PET fabrics were modified with NAA by forming an similar IPN to P(AEAM-Boc)-PET as previously described above. First, a round PET fabric was swollen in methanol solution containing BP (initiator, 0.55 mol/L), NAA (monomer, 2 mol/L) and MBA (crosslinker, 1.5% of monomer) at 40 °C for 2 h. Centrifugation (3 min, 3,500 rpm) of the swollen specimen was used to remove the excess of solution. The fabric was then exposed under UV irradiation ($\lambda=365$ nm, 2 h) to execute a photopolymerization of NAA and MBA for the formation of surface IPN. After the polymerization, the sample (named as PNAA-PET) was extracted with methanol in a Soxhlet-extractor for 24 h, air-dried overnight, and stored in a desiccator for 48 h to obtain a constant weight. The immobilization percentage was calculated according to the **equation 1** above.

3.3.3 Immobilization of N₃-PEG onto PNAA-PET

PNAA-PET was immersed in the N₃-PEG solution (0.01 M, t-BuOH/H₂O = 1:1) and Na ascorbate (40% mol) and Cu²⁺ (10% mol) were added subsequently to initiate the “click” reaction. After 2 h, the sample was taken out and rinsed with water and ethanol. Then the sample was immersed in ethanol again and continuously shaken overnight to remove the physically adsorbed N₃-PEG. The PET samples with PEG were referred to as PET-PEG.

3.3.4 Instrumental analysis and characterization

The similar instruments were used to detect the surface modification. Proton Nuclear magnetic resonance ($^1\text{H-NMR}$) spectrum of AEAM-Boc was recorded at room temperature in a 5 mm NMR tube on a Bruker Avance 300 MHz NMR spectrometer. Water Contact angle measurements of untreated PET and modified PET were carried out by a standard contact angle goniometer (Model 200, with DROP image standard) from RaméHart Inc.

3.3.5 Determination of PNAA on the surface of PET substrate - “Click” linkage between PNAA-PET and dansyl azide dye

PNAA-PET fabric was immersed in a solution of dansyl azide dye (shown in **Figure 4.2.2.**) (0.01 M, t-BuOH/H₂O = 1:1) and Na ascorbate (40% mol) and Cu²⁺ (10% mol) were added subsequently to initiate the “click” reaction. After 2 h, the sample was taken out rinsed with water and ethanol. Then the sample was immersed in ethanol again and continuously shaken overnight to remove the physically adsorbed fluorescent dye. To produce the control sample, the same procedure was repeated except that Cu²⁺ was added. Then the samples were observed under UV light.

3.3.6 Biological test (Platelet adhesion test)

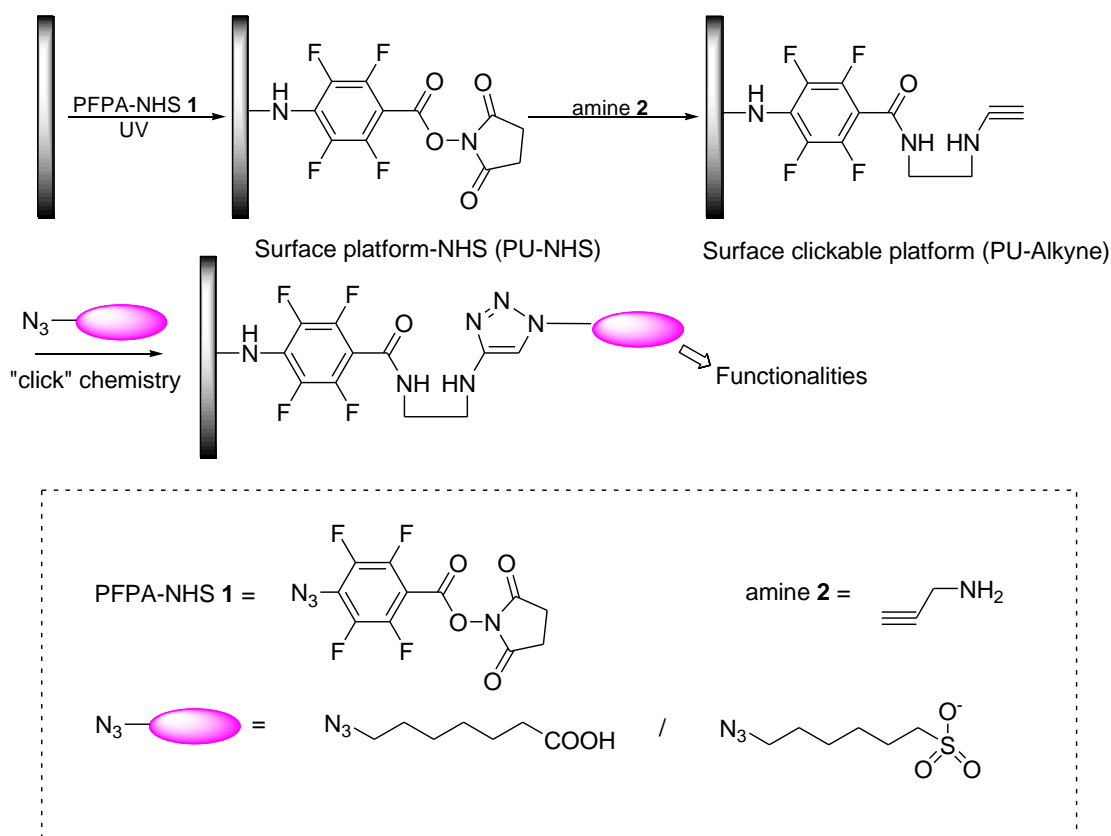
Similar platelet adhesion test was carried out in a static blood environment according to the literature⁶⁴ as mentioned above. Human whole blood was collected from healthy volunteers and mixed with 3.8% sodium citrate solution (ratio of human whole blood

and aqueous solution=9:1, v/v) as an anticoagulant. The blood was centrifuged at 1,200 rpm for 15 min at room temperature to obtain platelet-rich plasma (PRP). The number of platelets was determined using a hemocytometer. The platelet concentration of PRP solution was adjusted to 1×10^5 cells/ μL by adding phosphate buffered saline (PBS, pH=7.4) to PRP. The modified (PET-PEG) and untreated PET samples ($1 \times 1 \text{ cm}^2$) were equilibrated with PBS (pre-warmed at 37 °C) for 1 h and then immersed into 1 mL of the platelet suspension mentioned above for 2 h at 37 °C with 5% CO₂. After the incubation, the samples were taken out and gently washed with PBS for three times to remove the non-adhered platelets. The platelets remaining in both PRP solution and the the rinsing solution were quantified using the hemocytometer. The extent of platelet adhesion in respect to the PRP control was calculated according to the **equation 4**.

Analysis of variance (t-test; n=3) of the results was performed with regard to control (untreated PET) at $p < 0.05$, $p < 0.01$ and $p < 0.001$ of significant level.

3.4 Anticoagulating action between carboxylate and sulfate groups in heparin structure

The whole process to explore the special anticoagulating action between carboxylate and sulfate groups in heparin structure was shown in **Scheme 3.4.1**. Substances with sulfate and carboxylic functional groups were immobilized onto the same substrate through chemical reactions.



Scheme 3.4.1. Schematic illustration of surface “clickable” platform

3.4.1 Synthesis work

Synthesis of PFPA-NHS

Perfluorophenyl azide (PFPA-NHS) was prepared according to known procedures.¹²⁰ Briefly, methyl 2,3,4,5,6-pentafluorobenzoate was added into sodium azide (NaN_3) dissolving in the mixture of acetone and H_2O . The product of this step was methyl 4-azido-2,3,5,6-tetrafluorobenzoate (N_3 -PFPA). After that, F-PFPA was hydrolyzed by NaOH in the mixture of methanol and H_2O solution to obtain 4-azido-2,3,5,6-tetrafluorobenzoic acid (PFPA-COOH). Then PFPA-COOH was added

to NHS/ dicyclohexylcarbodiimide (DCC) solution to react with NHS and formed the expected product PFPA-NHS (yield: 99%).

Synthesis of azido compounds with different functional groups (N₃-COOH & N₃-SO₃)

Synthesis of 6-azidohexanoic acid (N₃-COOH) was carried out according to the known procedures described in the literature.¹²¹ Sodium azide (NaN₃) in DMF reacted with methyl 6-bromohexanoate (Br-1) to obtain N₃-1. After that, N₃-1 was hydrolyzed by lithium hydroxide (LiOH) in the mixture of water and methanol to obtain N₃-COOH (Shown in **Scheme 3.4.2 (a)**).

Sodium 6-azidohexane-1-sulfate (N₃-SO₃) was first synthesized in our lab (**Scheme 3.4.2 (b)**) using the similar method to synthesis of sodium 6-chlorohexane-1-sulfinate (Cl(CH₂)₆SO₃Na).¹²²

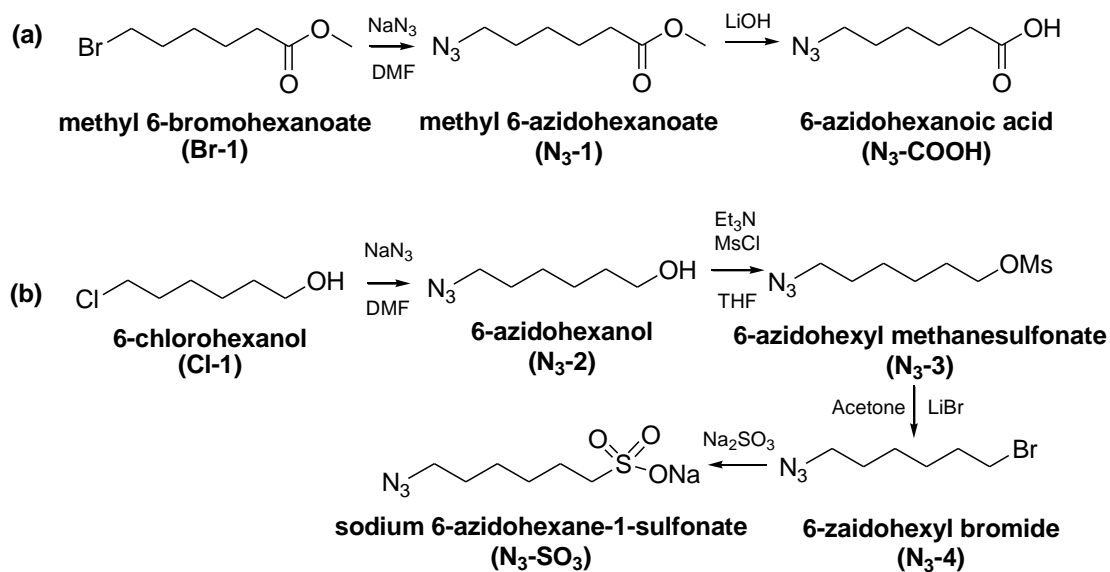
6-azidohexanol (N₃-2)¹²³ Sodium azide (NaN₃) (2.86 g, 44.0 mmol) was added to a solution of DMF (75 mL) in a 250 mL round-bottomed flask with a magnetic stirring bar. 6-chlorohexanol (Cl-1) (5.46 g, 40.0 mmol) was then added to the flask and heated to 80 °C for 24 h under nitrogen. After that, the reaction flask was cooled to room temperature and water (75 mL) was added. The mixture solution was extracted with diethyl ether and the organic phases were kept followed by washing with water. The organic solution was then dried by Na₂SO₄ and concentrated using a rotary evaporation to obtain N₃-2.

6-azidohexyl methanesulfate (N₃-3)¹²³ N₃-2 (2.58 g, 18.0 mmol) and triethylamine (Et₃N) (2.53 g, 25.0 mmol) was dissolved in THF. Methanesulfonyl chloride (MsCl) (2.86 g, 25.0 mmol) dissolving in THF was added to the mixture solution mentioned

above drop by drop under stirring. The reaction was last for 2 h and water was added to eliminate excess MsCl. After that, the mixture solution was extracted with diethyl ether. The organic layer was kept and washed with HCl, water, NaHCO₃ and water. The organic solution was then dried by Na₂SO₄ and concentrated using a rotary evaporator to obtain N₃-3.

6-zaidohexyl bromide (N₃-4)¹²³ N₃-3 (3.67 g, 16.6 mmol) dissolving in acetone was added to a solution of lithium bromide (LiBr) (2.94 g, 33.8 mmol) dissolving in acetone reacting for 24 h. The reaction solution was cooled by adding iced water. After that, the mixture solution was extracted with diethyl ether. The organic layer was kept and washed with water and brine. The organic solution was then dried by Na₂SO₄ and concentrated using a rotary evaporator to obtain N₃-4.

Sodium 6-azidohexane-1-sulfate (N₃-SO₃) Sodium sulfite (Na₂SO₃) (1.24 g, 9.8 mmol) and N₃-4 (2.19 g, 11.0 mmol) were added to an ethanol-water mixture (30 mL of ethanol, 20 mL of water) and the resulting biphasic solution was heated to reflux for 4 h under vigorous stirring. Next, the mixture was cooled to room temperature and the upper aqueous phase was separated and extracted with chloroform (3 × 10 mL) in order to eliminate residual N₃-4. The obtained aqueous solution was evaporated to dryness and the resulting mixture was suspended in 80 mL boiling ethanol and filtered in hot condition. The filtrate was evaporated in vacuo until precipitation commenced and cooled in a water-ice bath. The white precipitate was filtered, concentrated and dried in vacuo to give 1.5 g of N₃-SO₃ (Yield, 69 %).



Scheme 3.4.2. Strategy for the preparation of azido-functionalized compounds (a) synthesis process of N₃-COOH; (b) synthesis process of N₃-SO₃

3.4.2 Introduction of compounds with different functional groups to PU film substrate

To introduce functional groups (-SO₃ and -COOH) to PU substrate film, Perfluorophenyl azide (PAPF) platform (**Scheme 3.4.1**) developed in our lab was applied. According to the “clickable” surface platform, N₃-SO₃ and N₃-COOH were immobilized onto PU film via “click” chemistry. The whole process can be presented as **Scheme 3.4.1**.

Preparation of PU-NHS

The immobilization of PFPA-NHS onto PU film was carried out according to the

protocol.¹²⁴ First, the surface of untreated PU film was covered evenly by PFPA-NHS solution (16mg dissolving in 4 mL of methanol). The samples with NHS solution were then dried in an oven at 60 °C for 20 min. After that, the samples were irradiated under UV light (365 nm) for 10 min and washed by ethanol for three times to remove excess PFPA-NHS. The PU samples modified by PFPA-NHS were referred to as PU-NHS.

Preparation of PU-Alkyne

PU-NHS samples were immersed in a solution of amine-2 dissolving in acetonitrile (ACN, CH₃CN) (50 mM) and reacted overnight at room temperature. Afterward, the film sample was rinsed thoroughly with ethanol, air dried in the dark and stored at 4 °C until use. The resulting films were referred to as PU-Alkyne.

Preparation of PU modified with different functional groups

PU-alkyne was immersed in the N₃ compound solution (0.01 M, t-BuOH/H₂O = 1:1) and Na ascorbate (40% mol) and Cu²⁺ (10% mol) were added subsequently to initiate the “click” reaction. After 2 h, the sample was taken out and rinsed with water and ethanol. Then the sample was immersed in ethanol again and continuously shaken overnight to remove the physically adsorbed N₃ compound. N₃ compound solution can be N₃-SO₃, N₃-COOH and mixture of N₃-SO₃ and N₃-COOH as a determined molar ratio. The PU films with different functional groups were referred to as PU-SO₃, PU-COOH and PU (A:B = molar number of N₃-SO₃: molar number of N₃-COOH).

3.4.3 Instrumental analysis and characterization

Nuclear magnetic resonance (**NMR**) spectra of synthesized compound was recorded at room temperature in 5 mm NMR tubes on a Bruker Avance (^1H : 300 MHz; ^{13}C : 75 MHz) NMR spectrometer. Attenuated total reflectance (**ATR**) spectra of untreated and modified PU samples were taken on a Nicolet iS10 spectrometer (Thermo Fisher Scientific Inc.) using KBr pellets. X-ray photoelectron spectroscopy (**XPS**) data of untreated and modified PU samples were acquired with a Kratos Axis Ultra spectrometer by Kratos Analytical, Inc. Contact angle measurements of untreated PU and modified PU were carried out by a standard contact angle goniometer (Model 200, with DROP image standard) from RaméHart Inc.

3.4.4 Determination of functional groups on the surface of modified PU films

During the experiments, different functional groups have been introduced onto the surface of PU films. Those functional groups were investigated by different methods as following.

Determination of alkynyl groups in PU-Alkyne using an indirect dye method

PU-Alkyne film was immersed in a solution of dansyl azide dye (shown in **Figure 4.3.4.**) (0.01 M, t-BuOH/H₂O = 1:1) and Na ascorbate (40% mol) and Cu²⁺ (10% mol) were added subsequently to initiate the “click” reaction. After 2 h, the sample was taken out rinsed with water and ethanol. Then the sample was immersed in ethanol again and continuously shaken overnight to remove the physically adsorbed fluorescent dye. To produce the control sample, the same procedure was repeated except that Cu²⁺ was

added. Then the samples were observed under UV light.

Surface density of carboxylic groups on PU-COOH

THA Dye was used to determine the surface density of carboxylic groups as reported previously.¹²⁵ The PU-COOH was immersed in 5 mL of THA dye solution (0.1 mg/mL, dissolving in ethanol) shaking for 12 h at room temperature. Then the modified PU film was removed and the absorbance of residue dye solution after diluting by 5 times was measured at 605nm. The amount of THA dye in residue solution can be calculated from the previously obtained calibration curve. Since carboxylic groups can form 1:1 complex with THA dye, the surface density of carboxylic groups on the surface of PU-COOH film was obtained from the difference between the residue dye solution and initial dye solution. The measurement was performed in duplicate.

3.4.5 In vitro thrombus formation

The in vitro thrombus formation test was performed according to literatures.^{50,126} The whole blood was collected from healthy volunteers. The fresh blood was mixed with ACD solution as ratio (9:1, v/v). ACD solution was obtained by dissolving 2.45 g of anhydrous D-glucose, 2.20 g of sodium citrate dihydrate and 0.08 g of citric acid monohydrate in 100 mL of DI water. The blood with ACD solution was then added to the surface of modified PU film followed by adding 0.02 mL of 0.1 M CaCl₂ solution. After incubation at 37°C for certain periods, the samples were washed by DI water gently. The samples with thrombus formed on the surface were then fixed by 2.5%

glutaraldehyde dissolving in PBS solution. After air-drying, the samples were weighed by electronic balance and the weight of thrombus on the surface could be calculated.

Chapter IV: Results and Discussion

In accordance with Chapter III Experimental Section, this study can be divided into two parts: surface modification of PET via the IPN method for improved haemocompatibility; the other one is the exploration on alternatives to heparin structure. In the following, “**4.1 Surface modification of PET substrate with bioactive agent-heparin**” and “**4.2 Surface modification of PET substrate with bioinert agent-PEG**” are related to surface modification of PET. “**4.3 Anticoagulating action between carboxylate and sulfate groups in heparin structure**” is related to the research on anticoagulating functions of different functional groups in heparin structure.

4.1. Surface modification of PET substrate with bioactive molecule - heparin (Some of this part has been submitted to Macromolecular Chemistry and Physics)

In this part and the following part (**4.2 Surface modification of PET substrate with bioinert agent-PEG**), woven PET fabric was used through the whole surface modification process. Although warp-knitted PET structure for artificial vascular grafts has advantages in some domains, woven structure is still accepted in PET vascular grafts. Compared with knit structure, the surface of woven one is more regular and easier to handle during the experiments. Therefore woven PET was applied during the surface modification part.

4.1.1 Surface analysis and characterization

Synthesis of AEAM-Boc

The functional monomer AEAM-Boc was synthesized in 2 steps as shown in **Scheme 3.2.2** and its NMR spectrum (**Figure 4.1.1**) was identical to the report one.¹⁰⁷ The single peak at 1.44 ppm is caused by the three methyl groups in Boc structure. The two peaks at 3.29-3.47 ppm prove the methene groups. And the peaks at 5.64, 6.07-6.16 and 6.23 ppm are related to the hydrogen in vinyl structure.

¹H-NMR (300MHz, CDCl₃, δ): 6.23 (d, *J*=1.35 Hz, 1H); 6.07-6.16 (m, 1H); 5.64 (dd, *J*₁=1.27 Hz, *J*₂=10.08 Hz, 1H); 3.42-3.47 (m, 2H, CH₂); 3.29- 3.35 (m, 2H, CH₂); 1.44 (s, 9H, C(CH₃)₃).

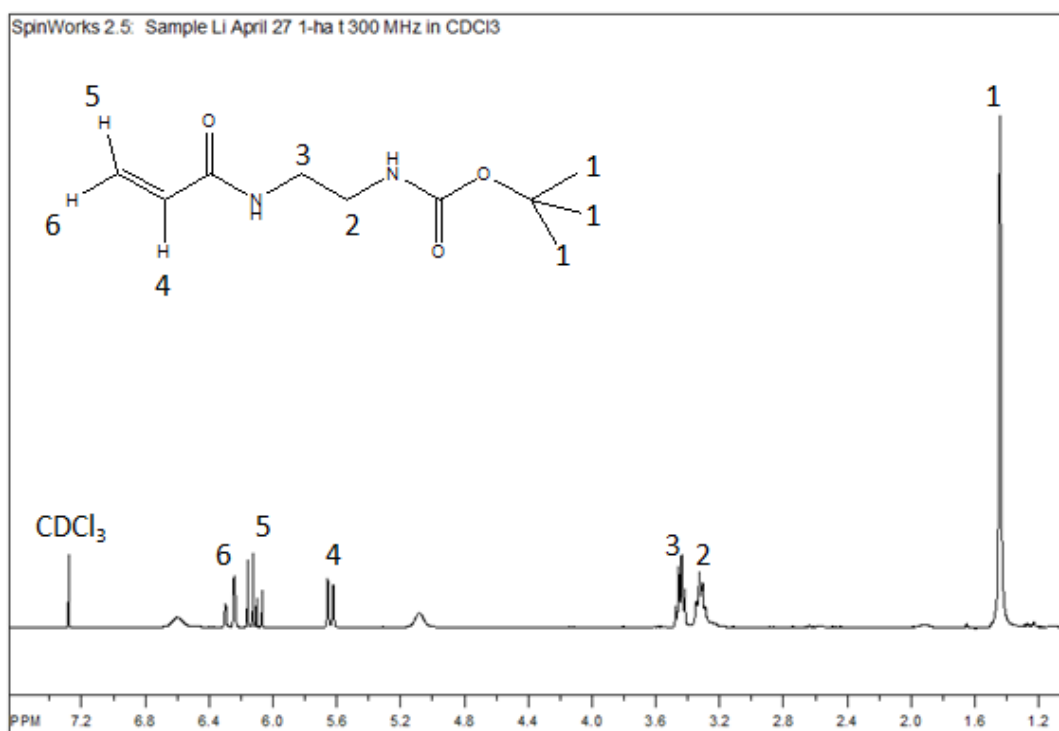


Figure 4.1.1. ¹H-NMR spectrum of AEAM-Boc

Immobilization of AEAM-Boc on PET via the IPN method

AEAM-Boc was immobilized onto PET using the non-destructive IPN method. During the process of IPN formation, centrifuge was used before UV irradiation to help swelling solution diffuse into PET uniformly, giving an IP (%) of 7% according to **equation 1**. Otherwise, non-uniform diffusion of swelling solution causes non-uniform distribution of functional groups on PET surface, even a bulk of polymer containing functional groups after polymerization on the surface.

Confirmation of IPN on P(AEAM-Boc)-PET

FT-IR spectra revealed the successful immobilization given the new peaks at 1697 cm^{-1} and 1654 cm^{-1} characteristic of AEAM-Boc amide, which are obvious in the subtracted spectrum (**Figure 4.1.2 (C)**).

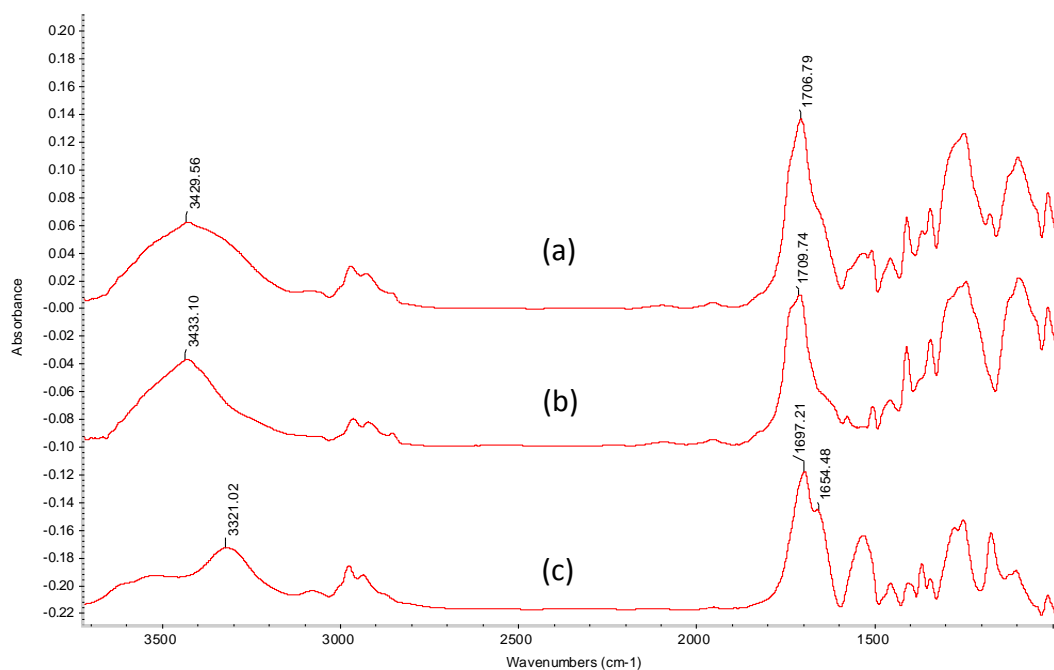


Figure 4.1.2. FT-IR spectra of (a) PET modified by AEAM-Boc through IPN method

(P(AEAM-Boc)-PET) (b) Untreated PET (c) subtraction between P(AEAM-Boc)-PET and untreated PET.

Recycle of IPN swelling solution for P(AEAM-Boc)-PET substrate

AEAM-Boc was synthesized in the Medical Textile Surface Engineering Lab. In the lab setting, it took 4 days to synthesis only 17 g of AEAM-Boc which was consumed once in the preparation of swelling solution. For the purpose of recycling the swelling solution of AEAM-Boc, a series of trials have been carried out.

As shown in **Figure 4.1.3**, IP (%) values were almost the same after several times of reuse of AEAM-Boc swelling solution. The average IPs (%) obtained from 1st swelling solution to 4th swelling solution were 7.32%, 7.83%, 7.79% and 7.37%. Only on the 5th use of swelling solution, IP (%) jumped to 8.19%, which may be caused by the percentage change of constitutes in swelling solution after several-time use. The jump might be also a result of higher pick-up. As a result, the swelling solution can be reused at least 4 times to get similar IP values on PET substrate. The recycle of swelling solution makes IPN method more convenient. It is also considered to be friendly to environment.

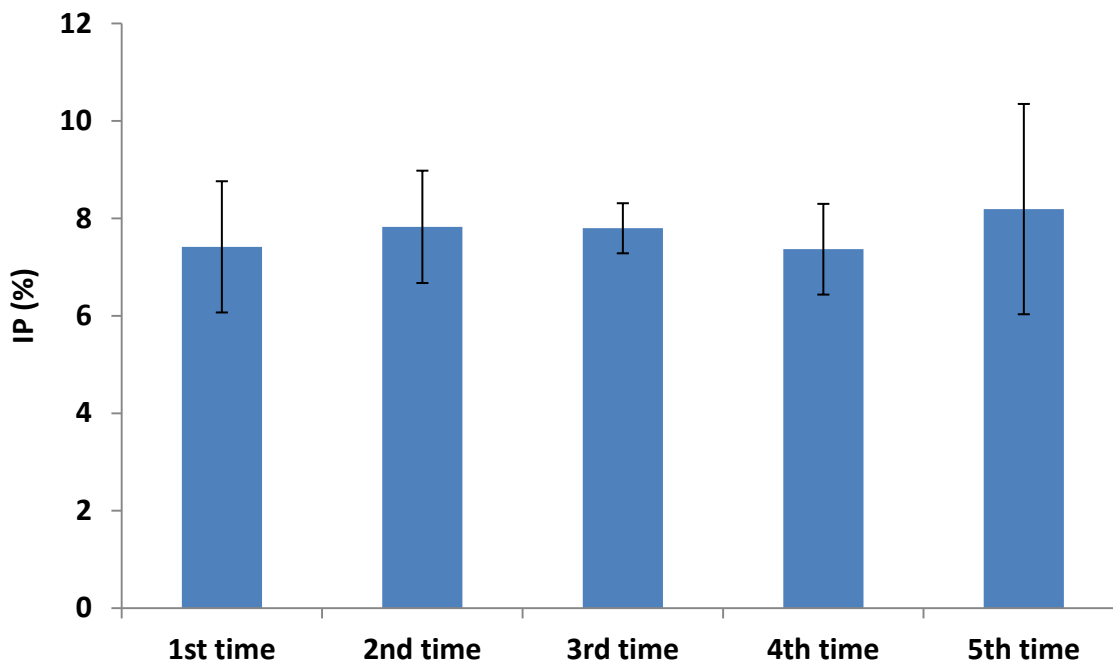


Figure 4.1.3. The relationship between recycle time of AEAM-Boc swelling solution and IP (%)

Determination of surface densities of amine and heparin on modified PET

P(AEAM-Boc)-PET was then hydrolyzed in 4 M HCl solution to generate free amine groups for further functionalization with heparin. The amounts of amine groups on PET samples were determined using a colorimetric assay. As shown in **Figure 4.1.4 (a)**, surface amine group increased almost linearly with the duration of hydrolysis within the studied hydrolysis period (1-5 hours). The depth of color on the samples was also consistent with the titration results: the higher the surface density of amine groups, the more AO deposited onto the substrate by forming 1:1 complex with free amine, the deeper the color (**Figure 4.1.4 (b)**). After the first hour of hydrolysis, the heterogeneous hydrolysis proceeded as a pseudo first order reaction in the presence of excess amount of

HCl. However, the fabric structure of PET substrate could be compromised when hydrolysis time was more than 2 h and even macroscopic damage was visualized after 5 h hydrolysis in 4 M HCl.

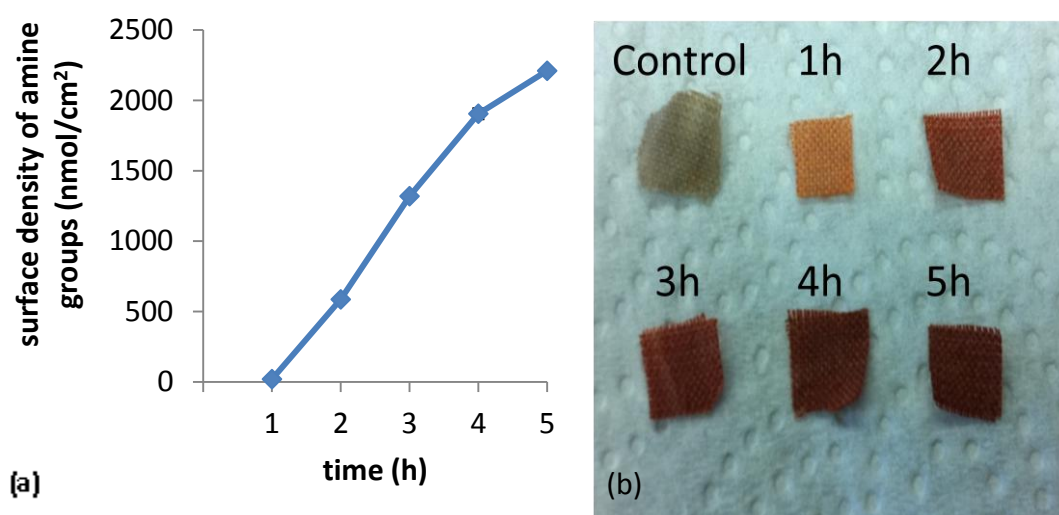


Figure 4.1.4. (a) The relationship between the surface density of amine groups generated by hydrolyzing P(AEAM-Boc)-PET and different hydrolysis periods; (b) images of samples dyed with AO.

To effect the covalent bonding of heparin for full coverage on surfaces, the surface density of anchoring amine group is generally in the range of 10 to 400 nmol/cm².^{63,72,127} The surface amine density reached 586.7 nmol/cm² after 2 h hydrolysis, which is more than double of the high end of the above range (219.5 nmol/cm²) and may be sufficient for the bonding of heparin considering that the bulky heparin may cover up certain amount of amine underneath negating the need of very high density of amine groups. So, PET-NH₂ obtained from 2 h hydrolysis of P(AEAM-Boc)-PET was used for the

following heparin immobilization and characterization.

Table 4.1.1. Surface densities of heparin on PET-Hep

| PET-Hep Samples (activation time + reaction time) | 10 min+4 h | 10 min+24 h | 2 h+24 h |
|---|-----------------|-----------------|-----------------|
| Surface density of heparin ($\mu\text{g}/\text{cm}^2$) | 0.93 ± 0.20 | 1.54 ± 0.20 | 2.40 ± 0.10 |

XPS measurements

XPS spectra of PET-NH₂, PET-Hep and untreated PET are listed in **Figure 4.1.5**. Compared with the spectrum of untreated PET, a new N 1s peak at 399.55 eV appeared in that of PET-NH₂, lending support to the successful immobilization of P(AEAM-Boc) on PET. From the high-resolution N 1s spectrum (inset in **Figure 4.1.5 PET-NH₂**), two different N peaks could be seen indicating the existence of two different types of amines in PET-NH₂. Carbamate (Boc protected NH₂) and amide N corresponded to the peak at 399.53 eV and the other peak at 401.35 eV was from free NH₂ generated from the removal of the protective functional group Boc. According to atomic concentration (%) in XPS data, the conversion ratio from Boc protected NH₂ to free NH₂ (top most layers detectable in XPS) can be calculated as 48.1 %.

The formation of amide bonding between heparin and PET-NH₂ was facilitated by activating the carboxylic acid groups of heparin with NHS and EDC. Both activation and reaction periods affected the amount of heparin bonded onto PET-NH₂. As shown in

Table 4.1.1, extended activation (2 h) and reaction (24 h) durations led to higher surface density of heparin ($2.40 \mu\text{g}/\text{cm}^2$). The reaction time was not further extended since it was reported that heterogeneous heparinization of aminized surfaces reached a plateau after 20-24 h of reaction.¹²⁷ In addition, $2.40 \mu\text{g heparin}/\text{cm}^2$ falls in the reported optimal surface density of heparin for stenosis prevention ($1.5\text{-}3 \mu\text{g heparin}/\text{cm}^2$).⁷⁰ $2.40 \mu\text{g heparin}/\text{cm}^2$ can be converted to $4.05 \text{ nmol repeating units}/\text{cm}^2$ (the repeating unit of heparin $\text{C}_{12}\text{H}_{19}\text{NO}_{20}\text{S}_3$ has a MW of $593 \text{ g}/\text{mol}$). Hence, the conversion yield from free NH_2 ($586.7 \text{ nmol}/\text{cm}^2$) to heparin repeating unit can be calculated as $4.05/586.7 = 0.69\%$. The reason for the low conversion ratio is manifold. First, heterogeneous reactions always present extremely low yield due to less chance of molecule collision hence reaction. Second, the bulky heparin molecule, once bonded onto the surface through one amide bond from one of its more than 20 repeating units, hinders the approaching of other heparin molecules to the un-reacted free amine groups in close vicinity.

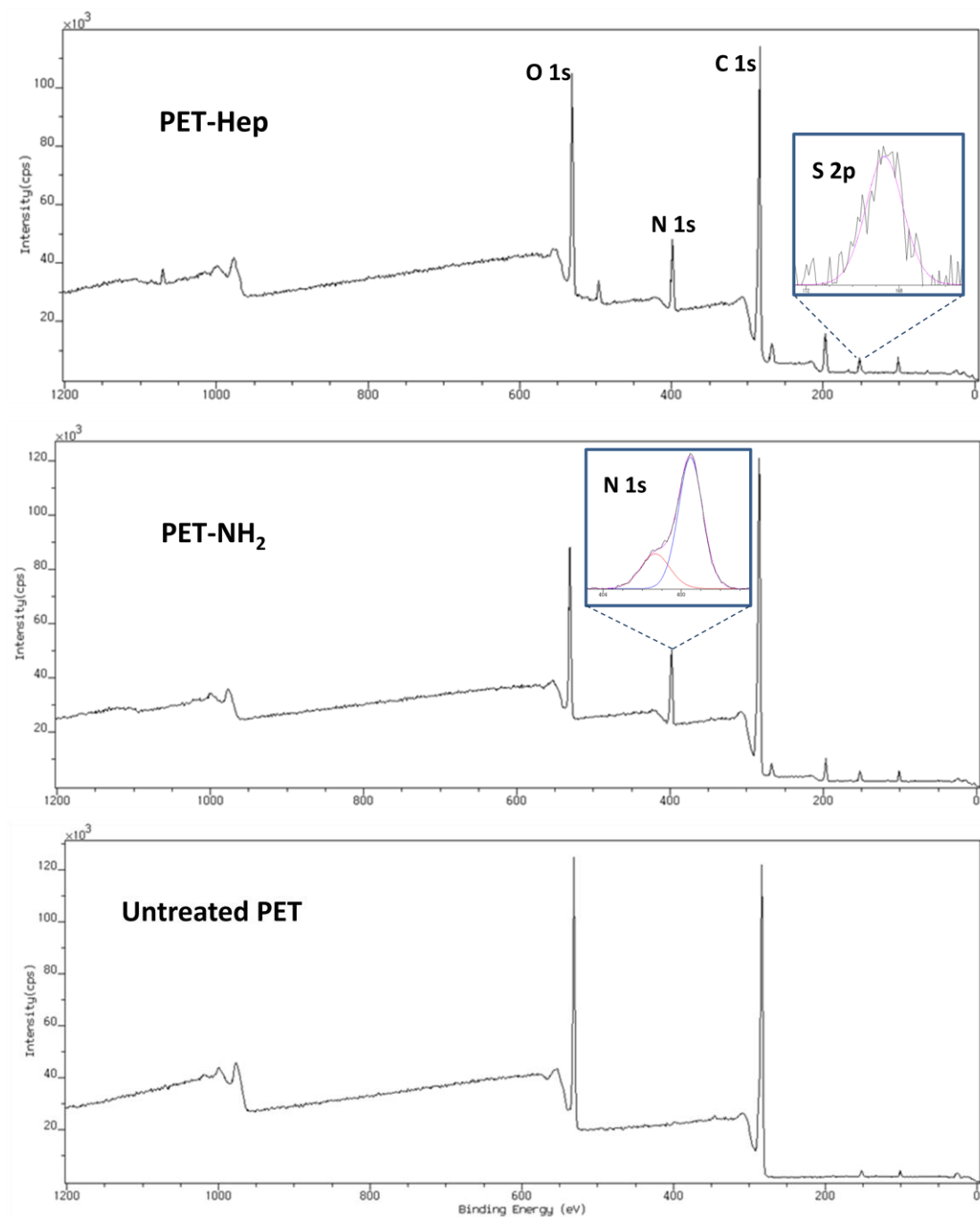


Figure 4.1.5. XPS spectra of PET samples (from top to bottom: PET-Hep; PET- NH₂ got from 2 h hydrolysis of P(AEAM-Boc)-PET and untreated PET)

After the immobilization of heparin, the N/C (%) derived from XPS measurements decreased from 13.27% for PET-NH₂ to 11.59% (**Table 4.1.2**). This drop can be

explained by the immobilization of heparin. Since the surface of PET-NH₂ was covered by heparin the mole ratio of nitrogen to carbon of which (8.3%) is lower as compared to 13.27% on the surface of PET-NH₂. The mole percent of sulfur element on the surface of PET-Hep was almost four times that of the background signal (0.06% on the surface of untreated PET), indicating a successful bonding of heparin on the PET substrate. The high resolution of S 2p spectrum is shown in **Figure 4.1.5** as an inset.

Based on S/N (**Table 4.1.2**), we can also get the conversion percentage as follows:

Suppose x percent of free amine catches one heparin repeating unit (realistically it is not the case since once a repeating unit is caught, the whole chain is immobilized. However, for the purpose of comparing with the colorimetric quantification data, we can use the molecular weight of the repeating unit since the final results are based on the same denominator in both cases), then $S/N = 3x/(2+x) = 0.23/8.76$, x can be solved from the equation as 1.76%. This number is around 2.55 times of the conversion yield (0.69%) derived from the colorimetric quantification data. It is because XPS detects all the heparin molecules sitting on the topmost layer and not all the nitrogen atoms shielded by heparin, the underestimated denominator results a higher estimation of conversion yield.

Table 4.1.2. Surface atomic concentrations (%) of PET samples measured by XPS

| Samples | Atomic Concentration (%) | | | | N/C (%) | S/N (%) |
|---------------------|--------------------------|------|------|-------|---------|---------|
| | C 1s | N 1s | S 2p | O 1s | | |
| Untreated PET | 77.83 | 0.26 | 0.06 | 21.85 | 0.34 | -- |
| PET-NH ₂ | 75.13 | 9.97 | 0.05 | 14.85 | 13.27 | -- |
| PET-Hep | 75.58 | 8.76 | 0.23 | 15.43 | 11.59 | 2.63 |

4.1.2 Biological evaluation of PET-Hep

Platelet adhesion test

Platelet adhesion is an early sign of blood clotting cascade. Thus, platelet adhesion test has been widely adopted to evaluate the haemocompatibility of blood contacting medical devices. Plasma suspension containing platelets was first brought in close contact with test samples for certain time and platelet adhesion on the samples was then quantified. The anticoagulating performance partly depends on the platelet adhesion ratio: during a predetermined period, the more platelets adhere on the samples, the more likely thrombosis will occur *in vivo*. Surface modification can be an effective way to improve the haemocompatibility of substrates by avoiding activation and aggregation of platelets. In our case, untreated PET and PET modified with heparin samples were immersed in PRP suspension for 2 h. As shown in **Figure 4.1.6 (B)**, platelet adhesion on untreated PET samples reached almost 50%. It is similar to commercial Dacron vascular grafts.¹¹⁷ As a highly-sulfated glycosaminoglycan, heparin can effectively work on heparin-binding proteins such as antithrombin III.⁴⁶ The formed heparin-protein complex can further bind with thrombin and prevent thrombin from forming fibrin network. Platelet adhesion on PET-Hep samples was only 11.33% (**Figure 4.1.6 (B)**), nearly a quarter of platelets on untreated PET samples. It is in accordance with the anticoagulation feature of heparin. On the other hand, the result proves that heparin has been effectively immobilized onto PET substrate with well retained bioactivity using the IPN surface modification technique.

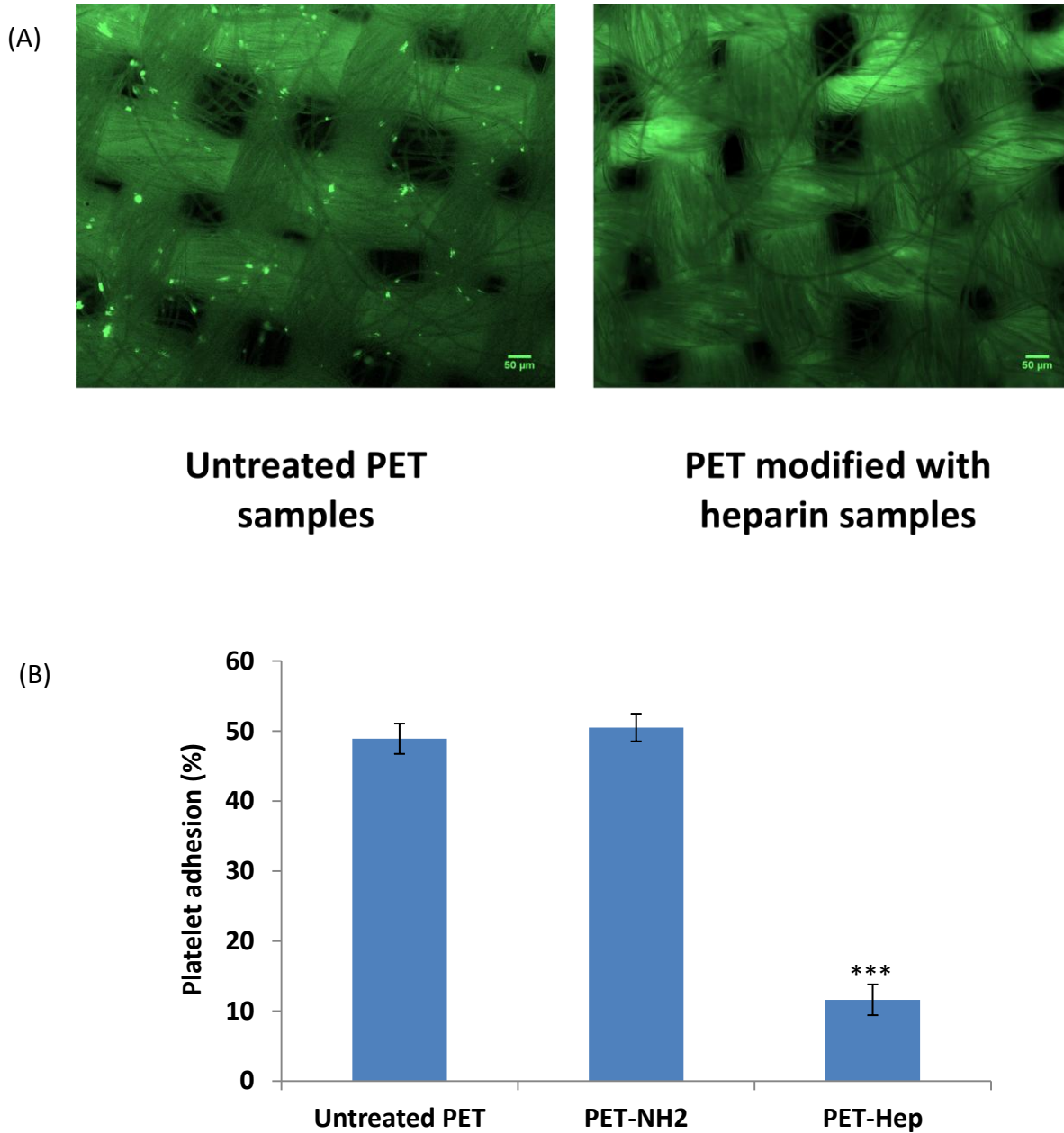


Figure 4.1.6. (A) Fluorescence images of platelets on untreated PET sample (left) and PET-Hep sample (right) at $\times 260$ magnification. Platelets were labelled by Mepacrine dye; (B) Quantification of platelet adhesion on control (untreated) and modified (PET-NH₂ & PET-Hep) samples. The diagram includes t-test results (n = 5) with respect to untreated PET (*p < 0.05, **p < 0.01 and ***p < 0.001)

To observe the distribution of platelets adhering on modified and untreated PET samples, platelets were labelled with mepacrine dye. Fluorescence images were taken and shown in **Figure 4.1.6 (A)**. There were significantly more platelets on the surface of untreated PET than the modified PET even though the distribution of platelets was not so uniform. Those images agree with the quantification results of platelet adhesion.

The morphology of modified and untreated PET samples after incubation in platelet suspension was further investigated by SEM. As shown in **Figure 4.1.7**, untreated PET was covered by platelets, which is consistent with the observation of adhered platelets under fluorescent microscope. Signs of pseudopodia could even be observed on the untreated PET (**Figure 7 b & c**). Again, platelets could hardly be spotted on the surface of PET-Hep.

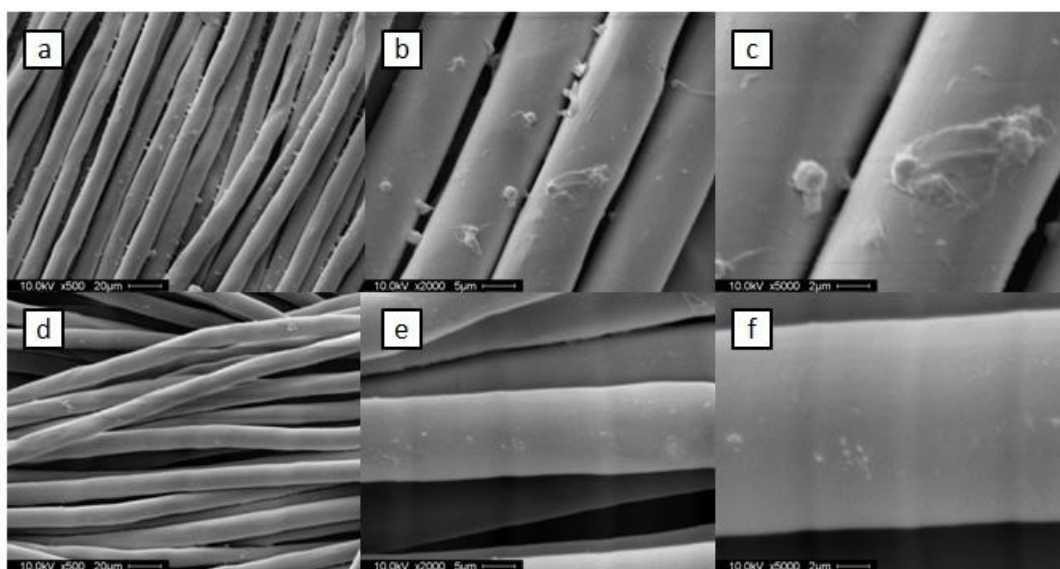


Figure 4.1.7. SEM images of platelet adhesion after 2h of incubation on (a) untreated PET sample at $\times 500$ magnification (b) untreated PET sample at $\times 2000$ magnification (c)

untreated PET sample at $\times 5000$ magnification (d) PET modified with heparin sample at $\times 500$ magnification (e) PET modified with heparin sample at $\times 2000$ magnification (f) PET modified with heparin sample at $\times 5000$ magnification

Cytotoxicity test

After being cultured in the extraction of PET-Hep, the biological response of fibroblast cells was evaluated using MTT assay. As shown in **Figure 4.1.8**, the relative cell viability in PET-Hep extracts was higher than 80%, indicating that the PET-Hep extracts are not toxic to the cells.¹¹⁷ Ke et al studied the cytotoxicity of AEAM-Boc on HEK293 cells and observed 80% cell viability in the presence of AEAM-Boc at a concentration as high as 20 $\mu\text{g/mL}$.¹²⁸ Since AEAM-Boc polymer is durably immobilized and covered with biocompatible heparin molecules in the case of PET-Hep, low cytotoxicity of PET-Hep extract is as expected.

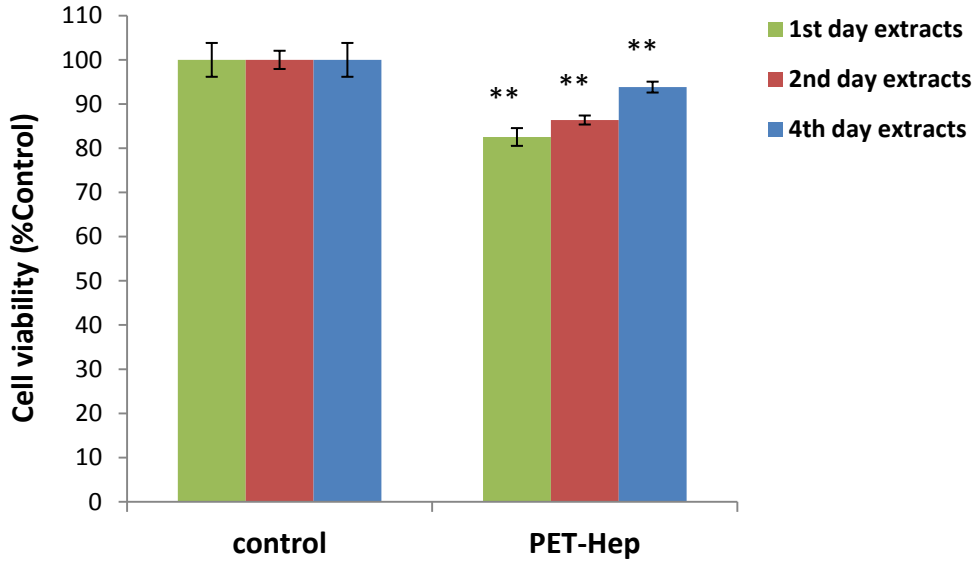


Figure 4.1.8. MTT assay for fibroblast cells cultured in control (cell medium) and in the medium with extracts from PET-Hep sample. The diagram includes t-test results (n = 3) with respect to control (*p < 0.05 and **p < 0.01).

4.1.3 Stability of immobilized heparin on PET-Hep

To examine the stability of immobilized heparin, PET-Hep samples were immersed into PBS (pH=7.4) solution for 1 d (24 h). Two pieces of those samples were removed from PBS solution every few hours (4.8 h) and the remaining heparin was quantified by TB O dye titration. The percent of released heparin increased gradually with the immersion duration and the release curve plateaued at around 5% after 14.4 h (**Figure 4.1.9**). The small portion of heparin loss was probably due to unremoved physically adsorbed heparin on PET-Hep. The fact that more than 94% of heparin immobilized on PET-Hep could sustain 24 h extraction in PBS solution is due to the covalent nature of

the bonding between heparin and PET substrate. The finding accords with the previous reported durability of heparin grafted on polyurethane films.¹²⁹ The bioactive and durable PET-Hep possesses the potential to be used as vascular grafts and further research is needed to validate its used in small-diameter vascular prosthesis.

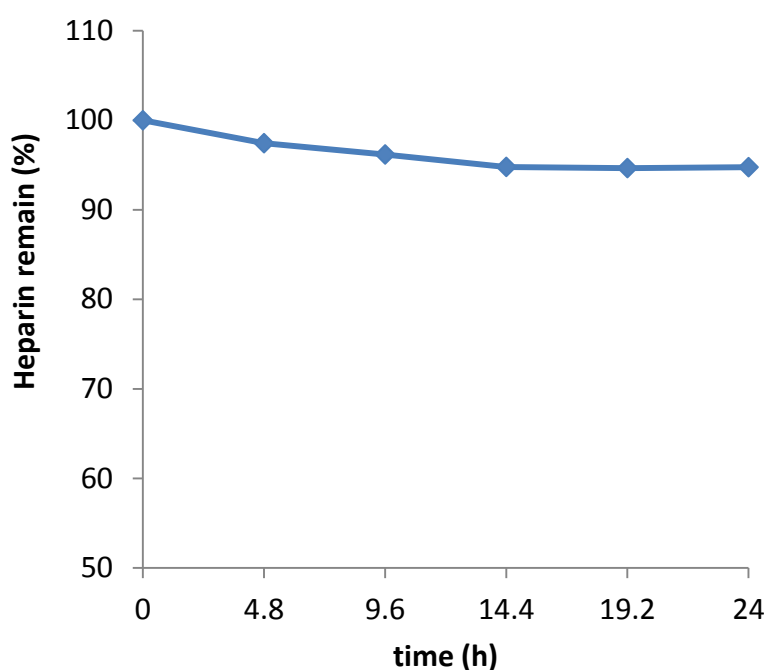


Figure 4.1.9. The stability of heparin immobilized on PET-Hep samples.

4.2 Surface modification of PET substrate with bioinert agent - PEG

4.2.1 Surface analysis and characterization

Synthesis of NAA

The synthesized NAA was characterized on a Bruker Avance NMR spectrometer. The

spectrum was corresponding to the literature.¹¹⁸ As shown in **Figure 4.2.1**, the three peaks from the range of 5.66-6.35 ppm prove the vinyl structure. The peaks at 4.1-4.2 ppm are multiple which is caused by the hydrogen in methyl groups. The peak at 2.24 ppm resonates triply due to the special alkyne structure.

¹H-NMR (300MHz, CDCl₃, δ): 6.28-6.35 (m, 1H, C=CH); 6.10-6.20 (m, 1H, CH=C); 5.66-5.73 (m, 1H, C=CH); 4.1-4.2 (m, 2H, CH₂); 2.24 (t, $J_1=2.55$ Hz, $J_2=5.09$ Hz, C≡CH).

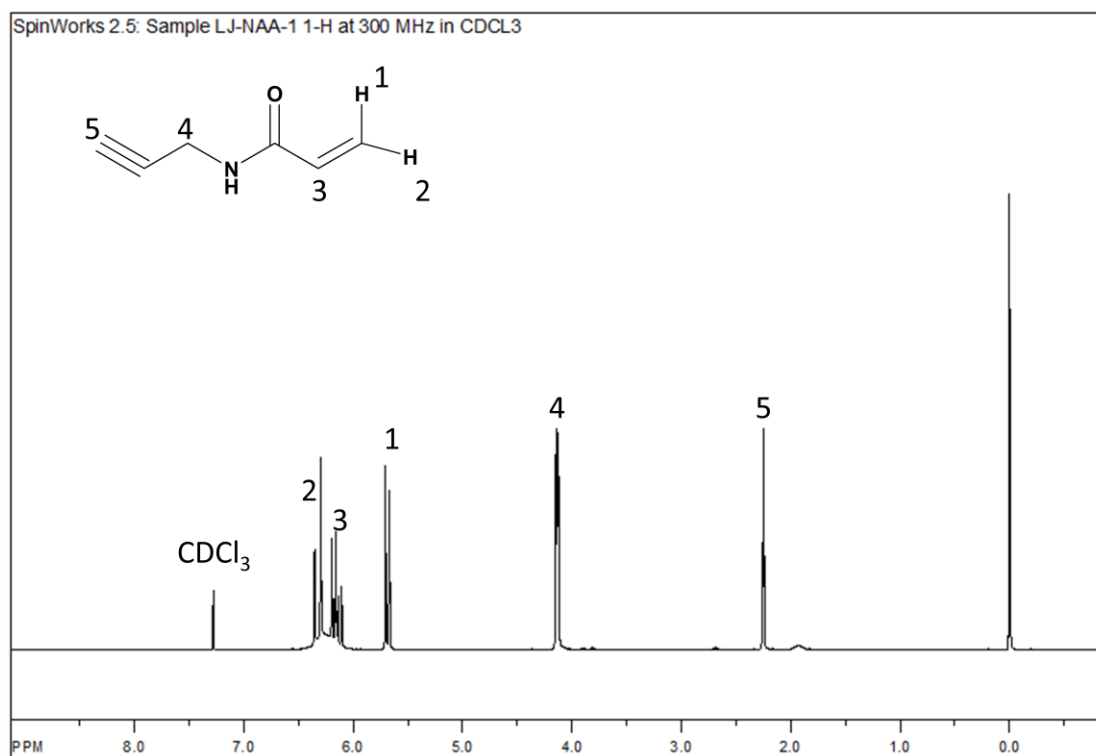


Figure 4.2.1. ¹H-NMR spectrum of NAA

Immobilization of NAA on PET via IPN method

NAA was immobilized onto PET using the non-destructive IPN method. The same centrifugation procedure mentioned in surface modification of PET with heparin was

applied to help decrease non-uniform distribution of functional groups during the process of IPN formation, giving an IP (%) of 5% according to **equation 1**.

Confirmation of IPN on PNAA-PET - “Click” linkage between PNAA-PET and dansyl azide dye

Since no significant difference was observed in ATR spectrum between PNAA-PET and untreated PET (data not shown), a dansyl-azide dye was used via the “click” chemistry method to validate the surface alkynyl groups on PNAA-PET (**Figure 4.2.2**). After thorough rinse, uniform and green fluorescence occurred on “click” PNAA-PET samples (**Figure 4.2.2(b)**), while only blue auto fluorescence was showed on the control sample (untreated PET) (**Figure 4.2.2(a)**). This result indicates the successful “click” linkage reaction which also proves the successful formation of PNAA-PET platform with uniformly distributed alkynyl groups. It also confirms that the IPN method has uniformly immobilized NAA onto the surface of PET substrate.

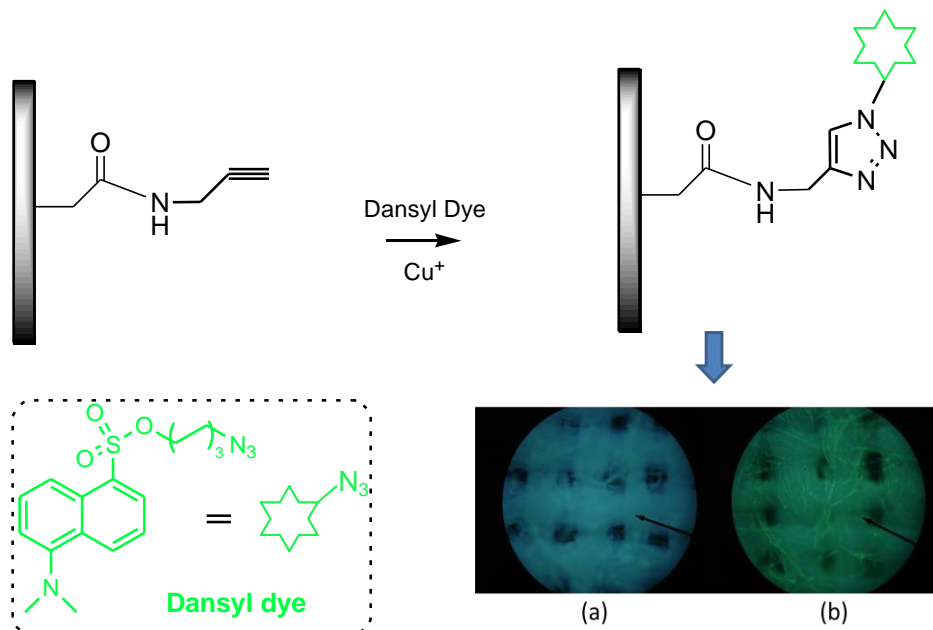


Figure 4.2.2. Characterization of PNAA-PET samples. Visualization of PNAA-PET (b) and untreated PET (a, control) after “click” linkage with dansyl dye.

Contact angle Measurements

The surface functionalization of PET fabric was characterized by water contact angle measurements. As shown in **Table 4.2.1**, the static water contact angle of hydrophobic untreated PET was first measured as 107.0°. The hydrophilicity of PET substrate was improved by introduction of the amide bond in NAA. Afterwards, the hydrophilicity of PET substrate was improved by the introduction of PEG. PEG is proved to be a strongly hydrophilic substance.⁷⁶ The difference between water contact angles suggests the successful immobilization of NAA and N₃-PEG to PET substrate.

Table 4.2.1. Water contact angle of untreated and modified PU samples

| samples | water contact angles (°) |
|---------------|--------------------------|
| untreated PET | 107.0 ± 5.5 |
| PET-NAA | 9 s disappear |
| PET-PEG | 3 s disappear |

4.2.2 Biological evaluation of PET-PEG

Platelet adhesion test

The similar platelet adhesion test has been done to PET-PEG samples as mentioned above in PET-Hep part. The most platelet adhesion was observed on the surface of untreated PET with a percentage of 50%. After the immobilization of NAA onto PET, the platelet adhesion fell to 25% due to the hydrophilic amide bonds in NAA structure. Platelet adhesion on PET-PEG samples continued to fell to only 8.89%, even less than that on PET-Hep samples (11.33%). It is reasonable because PEG is an antiplatelet agent while heparin is considered as a good anticoagulant. On the other hand, the result proves that surface modification of PET substrate with bioinert molecule-PEG using the IPN technique has been successful.

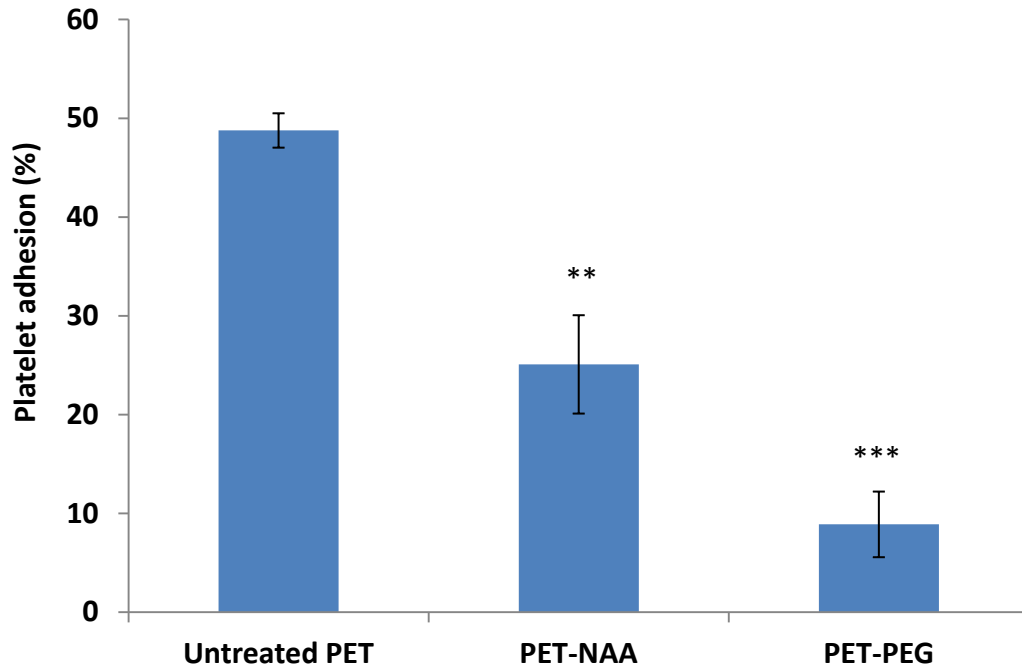


Figure 4.2.3. Quantification of platelet adhesion on control (untreated) and modified (PNAA-PET & PET-PEG) samples. The diagram includes t-test results (n = 3) with respect to untreated PET (*p < 0.05, **p < 0.01 and ***p < 0.001)

4.3 Anticoagulating action between carboxylate and sulfate groups in heparin structure

As mentioned above, heparin is a well-established anticoagulant and has been applied for many substrates for years. The haemocompatibility of biomaterials has been improved after the immobilization of heparin. However, problems related to heparin rise in recent years.

Large doses of heparin are required for clinical anticoagulation which can lead to

several side effects.¹³⁰ Thrombocytopenia is an important problem caused by heparin. In 1958, unusual thrombi affecting the lower limb arteries in some patients treated with heparin was first found by American vascular surgeons Weismann and Tobin.¹³¹ In mid-1970s, the unusual syndrome has been identified as heparin-induced thrombocytopenia syndrome (HIT).¹³² 5%-10% of patients who are under an anticoagulant therapy can suffer severe HIT especially in the first three weeks of the therapy.¹³³ Antithrombotic therapy using heparin can also cause bleeding episode.¹³⁴

Moreover, the bioactivity of heparinized surfaces can be compromised by the formation of covalent bonds between heparin and the substrate.¹³⁵ A part of the functional groups in heparin structure possessing anticoagulating activity are consumed to form the covalent bonds with the substrate. *In vivo*, endogenous heparinases may eventually degrade the attached heparin in long-term use.¹³⁶ Herein, searching for a heparin-like chemical substance to replace heparin has been paid much attention.

Heparin is a highly-sulfated glycosaminoglycan. Due to its special polysaccharide confirmation, heparin can bind with ATIII to form a complex. Afterwards, the complex can inactivate coagulation factor II and X to inhibit the blood coagulation. However, the exact anticoagulating mechanism of heparin, which part of heparin bind with ATIII, is still under discussion. There are hydroxyl, sulfate and carboxyl functional groups in the structure of heparin. Sulfate groups have been proved to be responsible for the antithrombogenic activity of heparin. Many efforts have been directed to investigate the haemocompatibility of sulfate polymers.^{50,83,135,138,139} However, Lv et al¹³⁷ mentioned that both sulfate and carboxyl groups are very important for the blood compatibility of

heparin. Lv et al drew a conclusion that sulfate groups can inhibit the activity of thrombin and carboxyl groups can depress the activated partial thromboplastin.¹³⁷ Only a few reports^{137,139} have worked on both sulfate and carboxyl groups immobilized on the same substrate. No one has even explored the optimal ratio of sulfate and carboxyl groups.

As heparin is not a perfect anticoagulant, alternatives to heparin have been investigated. Most of the research has been done on substances containing sulfate groups. However, it has been proved that carboxyl functional groups in heparin structure affect the anticoagulating activity.¹³⁷ It is hypothesized that carboxylic groups could exert anticoagulating action with sulfate groups to provide alternatives with a similar bioactivity to heparin.

To explore alternatives to heparin with both sulfate and carboxylic functional groups as well as their haemocompatibility, a bioactive “Click” platform established in Medical Textile Surface Engineering Lab can be adopted. The platform was based on “Click Chemistry”.¹⁰⁹

“Click Chemistry”,¹⁴⁰ especially the Cu(I)-catalyzed azide alkyne cycloaddition (“click”), is an extraordinarily powerful strategy for the preparation of functional polymers¹⁴¹ because of its reaction specificity, quantitative yields, and good functional group tolerance. Several current reviews address the development and modification of polymers using “Click Chemistry”.^{142,143}

Through the “Click” platform, many different bioactive functional groups can be immobilized onto the substrate including sulfate and carboxylate functional groups.

Both of them are mixed in the reaction solution and react with the same substrate. After that, the antithrombogenicity of the modified substrate with sulfate and carboxylic groups can be determined by biological test. Meanwhile, the ratio of those two different functional groups on the substrate can be adjusted by the ratio of those functional groups in “click” reaction solution.¹⁴⁴ As a result, the optimal ratio of sulfate and carboxylic groups to mimic the heparin structure can be investigated by utilizing the “click” platform.

In this part, PU film was chosen to be the model substrate. As mentioned above, PU is a promising vascular graft material because of its good thermal stability and elasticity. PU material is less inert to react with functional groups compared with PET. On the other hand, film is flatter than fabric which makes film easier to handle in tests. Film is not as porous as fabric so that film cannot leak liquids. In a model study, PU film is convenient and proper.

4.3.1 Surface analysis and characterization

Structural analysis of synthesized compounds (N₃-COOH & N₃-SO₃Na)

The ¹H-NMR spectrum of N₃-COOH was characterized on a Bruker Avance NMR spectrometer. The spectrum is corresponding to the spectrum described in the literature.¹²¹ The peaks related to the ethyl groups in ester structure were not observed in NMR spectrum, indicating the complete hydrolysis.

¹H-NMR (300MHz, CDCl₃, δ): 3.30 (t, *J*=6.84 Hz, CH₂); 2.39 (t, *J*=7.41 Hz, CH₂); 1.59-1.74 (m, 2CH₂); 1.37-1.50 (m, CH₂) (data not shown). The synthesis of N₃-COOH

was proved to be successful.

Both ^1H and ^{13}C NMR spectra of $\text{N}_3\text{-SO}_3$ synthesized in our lab were collected on a Bruker Avance NMR spectrometer. It corresponds to the NMR spectra of the similar structure $\text{Cl}(\text{CH}_2)_6\text{SO}_3\text{Na}$ discussed in literature.¹²² As shown in **Figure 4.3.1**, the peaks in the range of 1.36-1.85 ppm are the resonances of hydrogens in methene groups. The peak at 3.37 ppm splits triply due to the azido groups in neighborhood. The peak at 2.9 ppm is caused by the hydrogen close to sulfate group. In **Figure 4.3.2**, there are six peaks suggesting six carbon atoms in the compound. From those results, the synthesis of $\text{N}_3\text{-SO}_3$ can be confirmed.

^1H -NMR (300MHz, D_2O , δ): 3.37 (t, $J=6.9$ Hz, CH_2); 2.91-2.99 (m, CH_2); 1.72-1.85 (m, CH_2); 1.36-1.57 (m, CH_2). ^{13}C -NMR (75MHz, D_2O , δ): 56.14, 55.95, 32.76, 32.24, 30.51, 28.89.

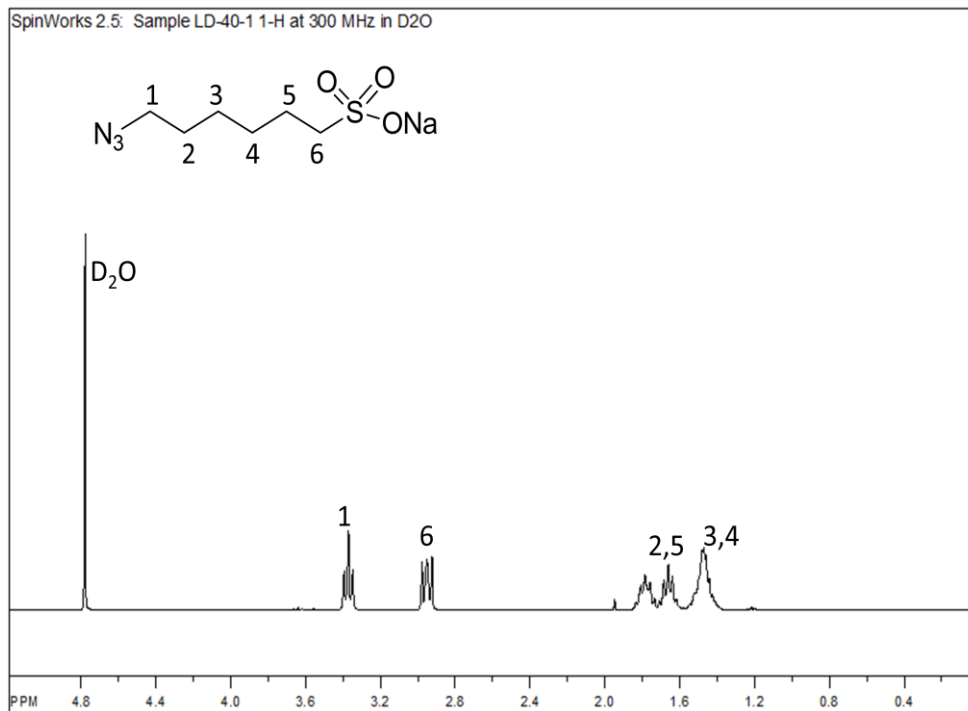


Figure 4.3.1. ¹H-NMR spectrum of N₃-SO₃

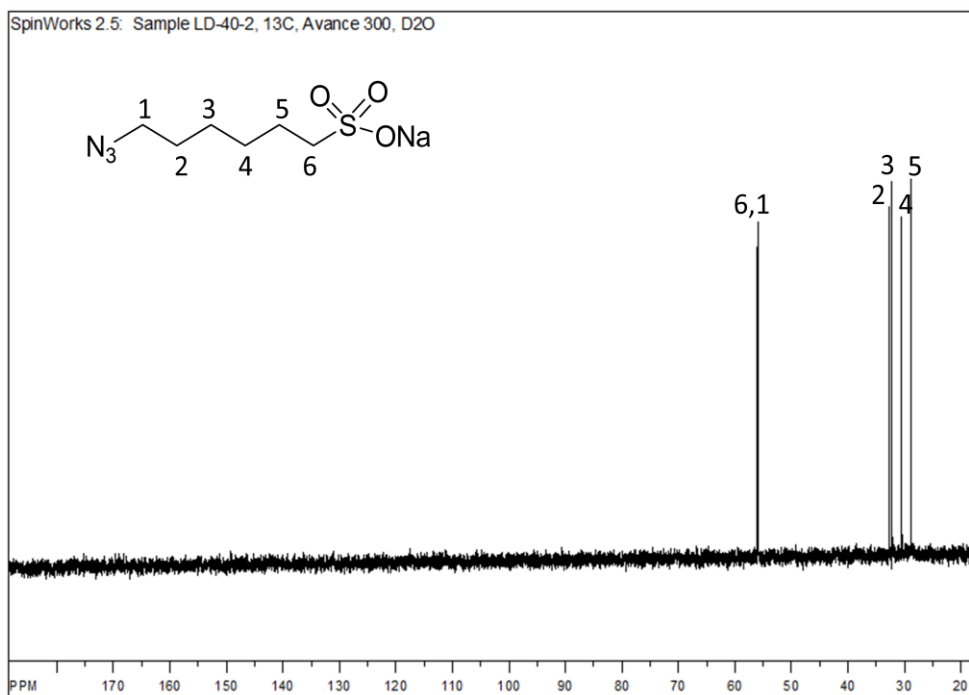


Figure 4.3.2. ¹³C-NMR spectrum of N₃-SO₃

Introduction of compounds with different functional groups to PU film substrate

ATR spectra to confirm PU-NHS The first step in modification of PU film was to obtain PU-NHS. Based on PU-NHS, amine 2 was immobilized and surface clickable platform (PU-Alkyne) was achieved which contained “clickable” alkynyl groups. However, either PU-NHS or PU-Alkyne doesn't show significant peaks in ATR spectra, an indirect method was adopted to confirm the modification. PU-NHS reacted with 2-azido-ethylamine and the product (PU-Azide) should possess an azido group which is obvious in ATR spectrum. After that, untreated PU, PU-NHS, PU-Azide and PU-Azide (back of the film) were observed using a Nicolet iS10 spectrometer (Thermo Fisher Scientific Inc.)(**Figure 4.3.3**). In the spectrum of PU-Azide (**Figure 4.3.3(c)**), a distinct peak appeared at 2102.15 cm^{-1} (ascribing to $-\text{N}_3$ group) in contrast to that of PU-NHS (**Figure 4.3.3(b)**) while there was no azido peak in the spectrum of PU-Azide (back), suggesting the physically adsorbed 2-azido-ethylamine was washed off completely and the successful covalent attachment of 2-azido-ethylamine on PU-NHS. On the other hand, it also indicates the successful modification of PU film to obtain PU-NHS. The ATR spectrum difference between PU-NHS, PU-Azide and PU-Azide (back) evidently demonstrated that the first step in modification of PU film was successful and PU-NHS formed.

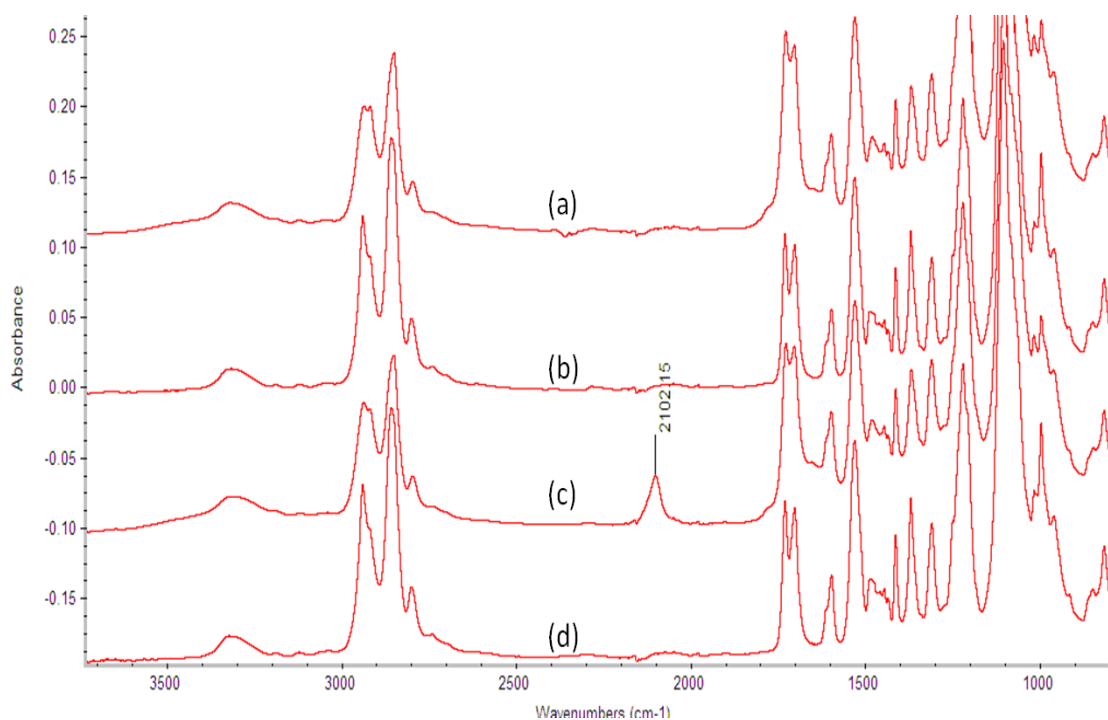


Figure 4.3.3. ATR spectra of (a) untreated PU; (b) PU-NHS; (c) PU-Azide; (d) PU-Azide (back).

Determination of alkynyl groups in PU-Alkyne was carried out using a dye method. Similar to PNAA-PET part, there was no significant difference between the ATR spectra of PU-NHS and PU-Alkyne (data not shown), we adopted an indirect method to determine the alkynyl group. A dansyl-azide dye was attached with alkynyl groups onto PU-Alkyne via the “click” reaction (**Figure 4.3.4**). After rinse process, only blue auto fluorescence occurred on the control sample, determining there was no alkynyl groups to bind dansyl dye (**Figure 4.3.4 (b)**). Uniform and green fluorescence was observed on “click” PU-Alkyne samples (**Figure 4.3.4 (a)**), indicating the successful “click” linkage reaction and the successful formation of PU-Alkyne with uniformly distributed alkynyl groups.

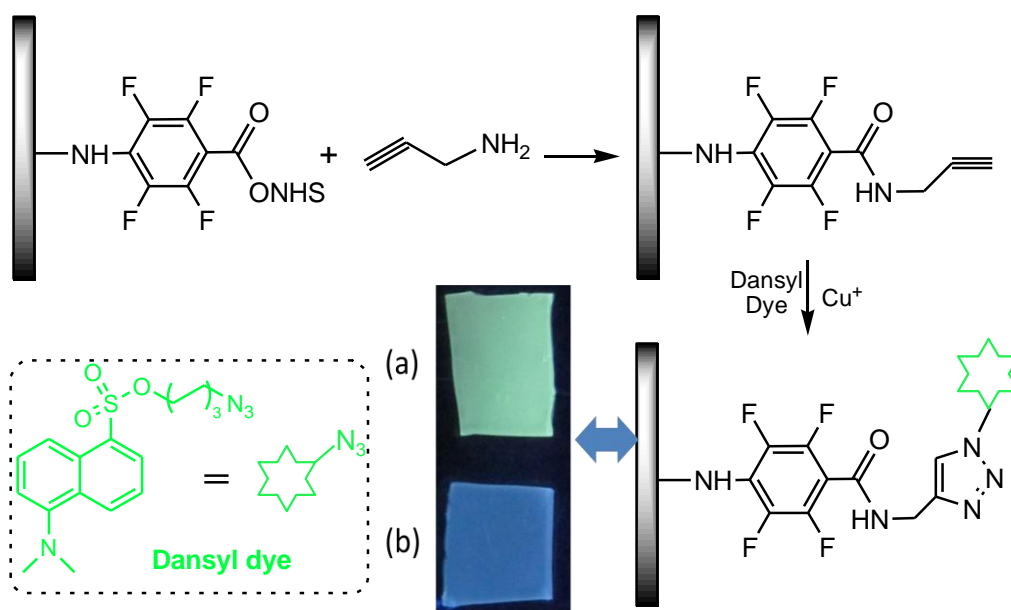


Figure 4.3.4. Formation and characterization of PU-NHS and PU-Alkyne samples. Visualization of PU-Alkyne (a) and PU-NHS (c, control) after “click” linkage with dansyl dye.

Determination of carboxylic groups on PU-COOH using THA dye method

After the successful immobilization of alkynyl groups on PU film, carboxylate and sulfate compounds containing azido groups were “clicked” onto the same substrate via binding to alkynyl groups. They were referred to PU-COOH and PU-SO₃. **The surface density of carboxylic groups** on PU-COOH samples was determined using a colorimetric titration technique as described in the experimental section.

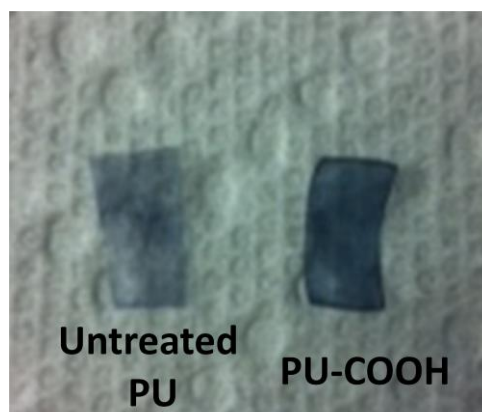


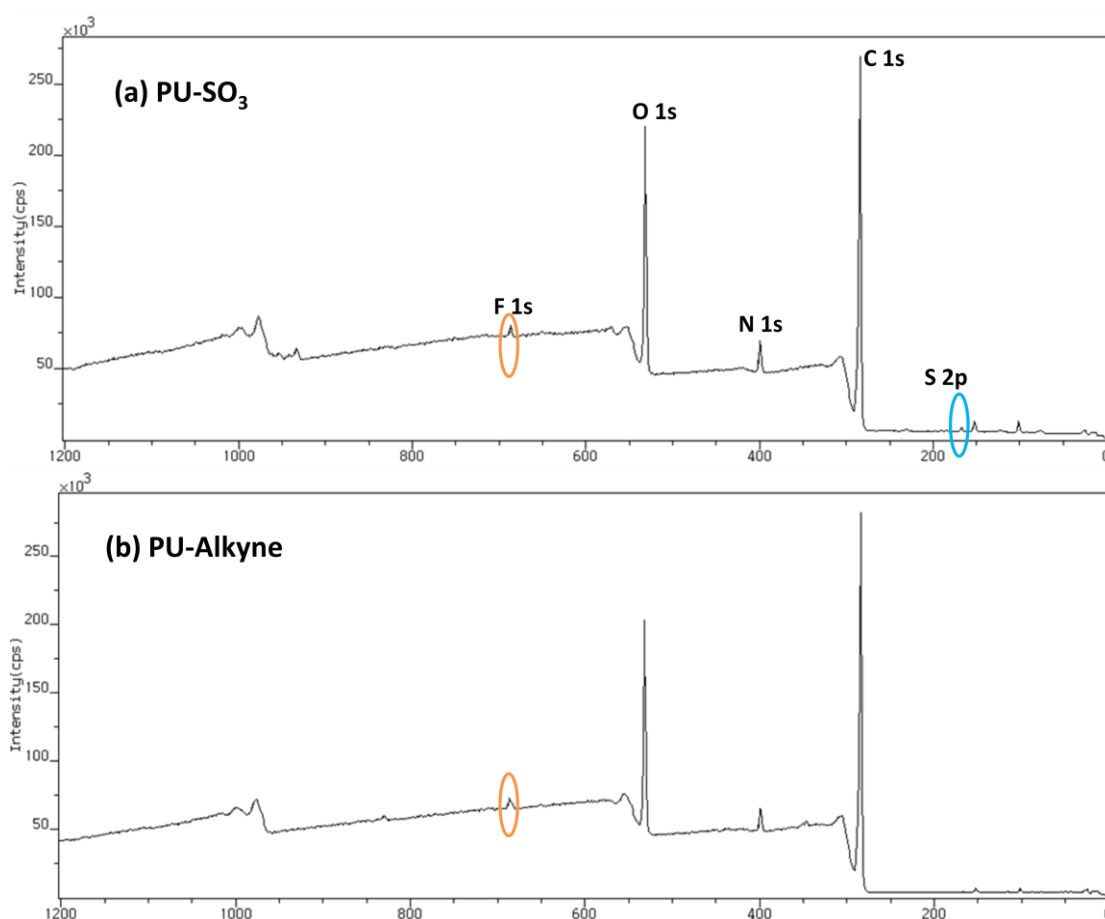
Figure 4.3.5. Images of untreated and modified PU samples dyed with THA dye

Untreated PU and PU-COOH samples were immersed in THA dye for 12 h. Afterwards, PU-COOH presented dark blue color since carboxylic groups on PU-COOH sample formed a complex with the THA dye. However, there was almost no change on untreated PU (**Figure 4.3.5**). The direct difference between untreated PU and PU-COOH confirms the successful immobilization of N_3 -COOH on PU substrate. The exact amount of carboxylic groups was calculated via calibration curve and gave a value of 1.62 nmol/cm^2 on PU-COOH sample.

XPS Measurements of sulfonic groups on PU-SO₃

Since a dye method was merely used to determine sulfonic groups, another measurement - XPS was adopted to confirm the sulfonic groups on PU-SO₃. Modified PU samples (PU-SO₃ and PU-Alkyne) and untreated PU samples were all observed using XPS measurements as described in experimental section. In the spectrum of PU-Alkyne (**Figure 4.3.6 (b)**), there was a distinct new peak occurring at 666 eV corresponding to the element of fluorine (F). It resulted from the first step in the modification of PU film -

the immobilization of PFPA-NHS onto PU film and formation of PU-NHS. In the structure of PFPA-NHS, there is F element. Then F occurs in PU-NHS and the following PU-Alkyne. In the spectrum of PU-SO₃ (**Figure 4.3.6 (C)**), a peak at 168 eV (S element) was detected as expected, suggesting the successful immobilization of N₃-SO₃ onto PU film. Meanwhile, the F atomic concentration decreased from 0.76% to 0.34% (**Table 4.3.2**). It is reasonable that there is no F element in the structure of N₃-SO₃. Once N₃-SO₃ was attached to PU-Alkyne, the F atomic concentration would be dragged down by the addition of new element - Sulfur (S).



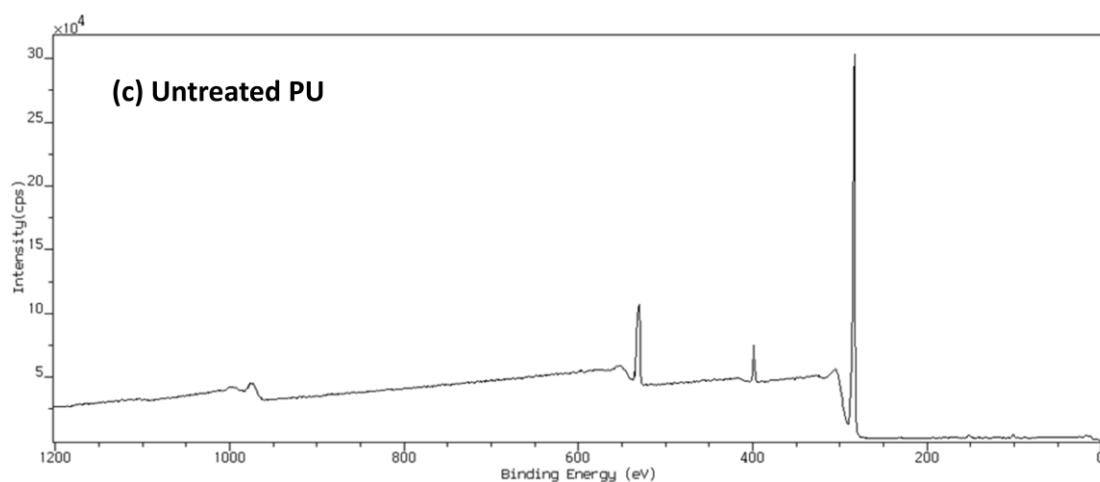


Figure 4.3.6. XPS spectra of untreated and modified PU samples: (a) PU-SO₃; (b) PU-Alkyne and (c) untreated PU.

Table 4.3.1. Surface atomic concentrations (%) of PET samples measured by XPS





| samples | Atomic Concentration (%) | | | | |
|--------------------|--------------------------|------|------|-------|------|
| | C 1s | N 1s | S 2p | O 1s | F 1s |
| Untreated PU | 89.87 | 3.79 | -- | 6.34 | -- |
| PU-Alkyne | 82.11 | 2.69 | 0.06 | 14.38 | 0.76 |
| PU-SO ₃ | 80.54 | 2.97 | 0.29 | 15.86 | 0.34 |

Contact angle Measurements

The surface functionalization of PU films was characterized by water contact angle measurements. As shown in **Table 4.3.3**, the static water contact angle of PU-Alkyne was first measured to find that it decreased from 97.6° (untreated PU) to 87.8°. Afterwards, the hydrophilicity of PU substrate was further improved by introduction of

carboxylic groups and sulfonic groups, suggesting the successful immobilization of N_3 -COOH and N_3 -SO₃ to PU films. Compared with PU-COOH, PU-SO₃ presented a lower water contact angle due to the stronger hydrophilicity of sulfonic groups. The water contact angle test confirms the whole modification of PU films.

Table 4.3.2. Water contact angle of untreated and modified PU samples

| | water contact angles (°) | |
|--------------------|--------------------------|--|
| untreated PU | 97.6 ± 0.3 |  |
| PU-alkyne | 87.8 ± 0.3 |  |
| PU-COOH | 83.3 ± 0.6 |  |
| PU-SO ₃ | 81.7 ± 0.4 |  |

4.3.2 Biological evaluation of modified PU samples

In vitro thrombus formation test

To explore the bioactivity of each functional group in heparin molecule, -COOH and -SO₃ were immobilized onto PU films as described in experimental section. Then in vitro thrombus formation test were used to determine the anticoagulating performance of surfaces of modified PU samples according to the method proposed by Imai and Nose.¹⁴⁵ First, untreated PU, PU-COOH, PU-SO₃ and PU-SO₃-COOH (SO₃:COOH=3:1) were investigated by in vitro thrombus formation test. The sample PU-SO₃-COOH (SO₃:COOH=3:1) was to imitate the ratio of SO₃ : COOH in heparin molecule. After predetermined periods of incubation, the thrombus weights formed on the surfaces of samples were obtained and the results are shown in **Figure 4.3.7**. It is no doubt that untreated PU has the poorest blood biocompatibility among the four samples. Almost 25 mg of thrombus formed on the surface after 1 h incubation. After immobilization of -COOH and -SO₃ groups onto untreated PU, the antithrombogenicity of untreated PU was enhanced due to the anionic surface.¹²⁰ It can be seen clearly that PU-COOH surfaces presented worse antithrombogenicity than PU-SO₃ surfaces. However, an interesting sign was observed that after the immobilization of mixed functional groups (SO₃:COOH=3:1), PU-SO₃-COOH has the least thrombus weight of all. At the first 30 min, there was almost no thrombus formed on the surface. It is similar to PU substrate modified with heparin mentioned by Ito.¹²⁰ From the data, it is concluded that -COOH contributes to the anticoagulating performance of heparin. Not only -SO₃ but also -COOH can prevent the formation of thrombus on the substrate. Meanwhile, we were

wondering whether there is a relationship between the antithrombogenicity and the ratio of $-\text{SO}_3$ and $-\text{COOH}$.

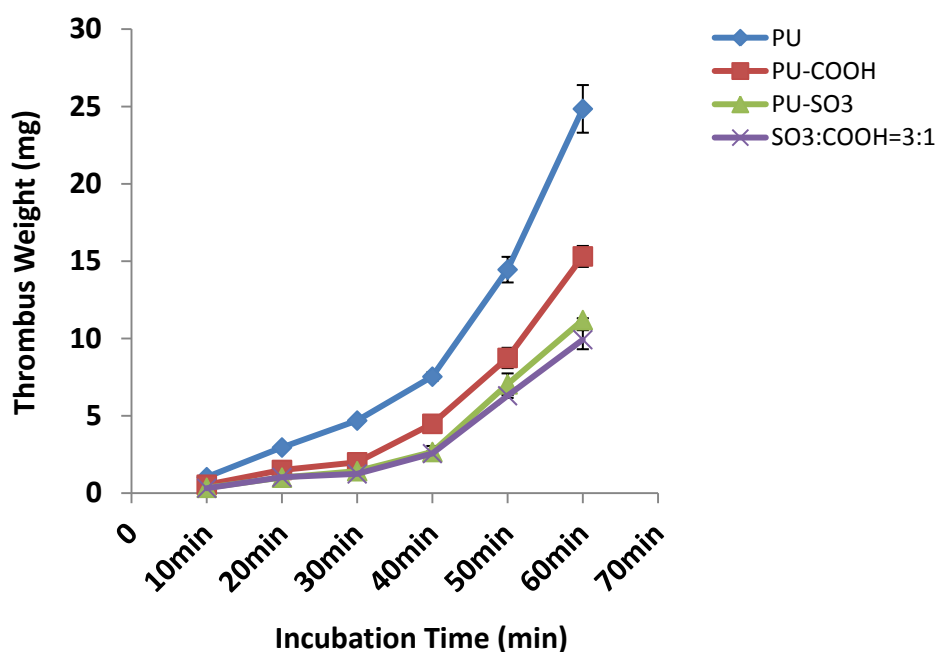


Figure 4.3.7. Thrombus formation on untreated and modified PU samples (PU-SO₃; PU-COOH and PU-SO₃-COOH (3:1)).

To explore the relationship between the antithrombogenicity and the ratio of $-\text{SO}_3$ and $-\text{COOH}$, another *in vitro* thrombus formation test was carried out. PU-COOH, PU-SO₃ and PU-SO₃-COOH (SO₃:COOH=3:1; 4.5:1 & 6:1) were applied this time (**Figure 4.3.8**). Here, the modified PU samples with the ratio of SO₃:COOH (< 3:1) showed poor antithrombogenic activity (not shown in **Figure 4.3.8 (A)**). Among all the samples, PU-COOH formed the most thrombus (almost 16mg) during 60min, indicating worse antithrombogenic ability compared with those containing sulfate groups. Interestingly,

modified PU film containing sulfate groups only did not present the best antithrombogenicity. PU-SO₃-COOH (SO₃:COOH=3:1 & 4.5:1) showed less thrombus on the surface than both PU-COOH and PU-SO₃. As the percentage of -SO₃ on the surface increased (SO₃:COOH=4.5:1), the material display a even better antithrombogenicity than other samples, suggesting an improved anticoagulating relation between carboxylate and sulfate groups. However, when the percentage of -SO₃ on the surface kept increasing (SO₃:COOH=6:1), the thrombus weight increased, suggesting that the special anticoagulating relation should be controlled by the ratio of sulfate and carboxylate groups. Here, results of statistical analysis on samples after 60min were shown in **Figure 4.3.8 (B)**. Modified PU films were compared with PU-COOH and PU-SO₃ samples, respectively. All the modified PU films (SO₃:COOH=6:1,4.5:1 & 3:1) were also compared with each other using the same statistical analysis software (not shown in **Figure 4.3.8 (B)**), showing a significant difference between each other.

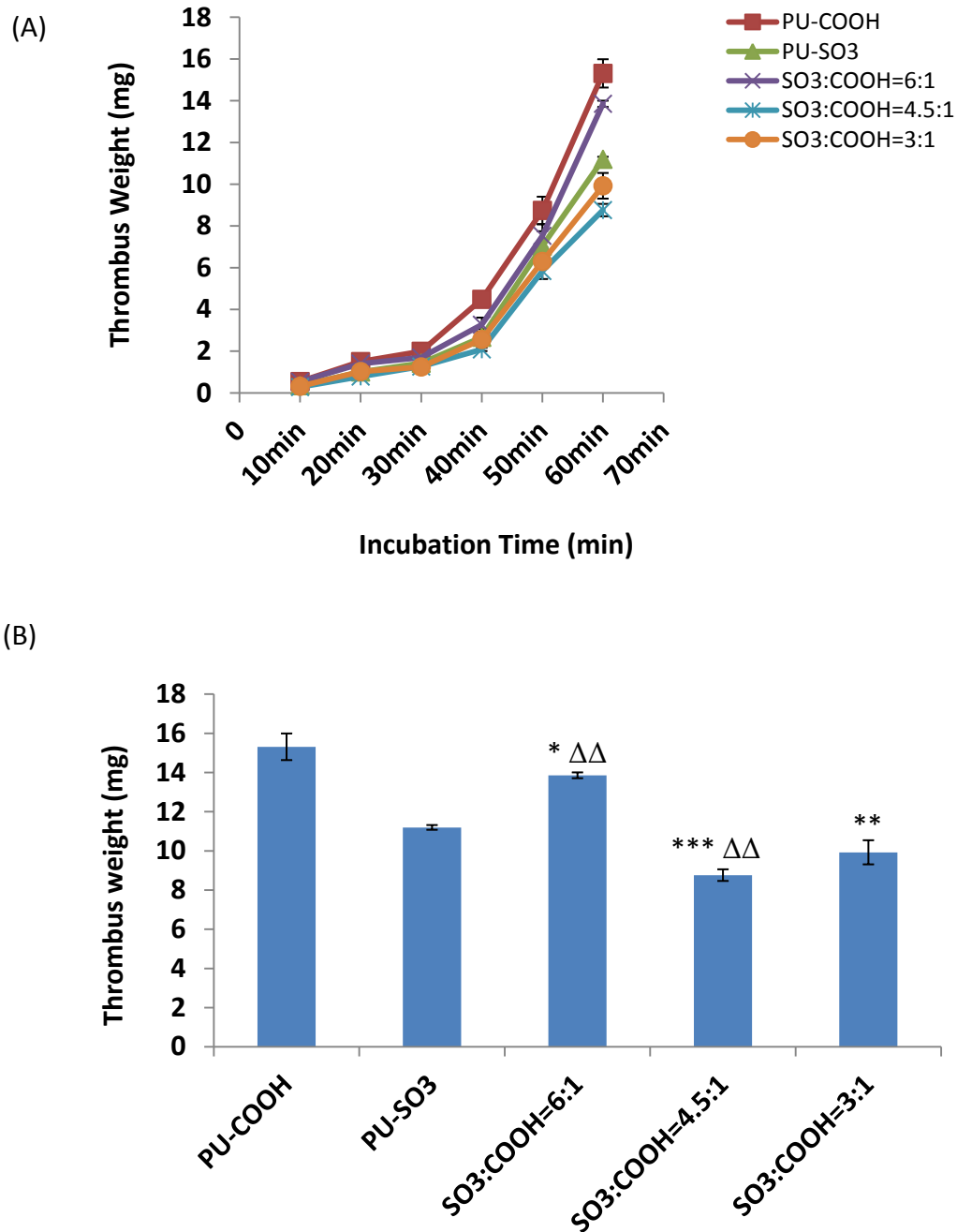


Figure 4.3.8. (A) Thrombus formation on different modified PU samples (PU-COOH; PU-SO₃ and PU-SO₃-COOH(3:1; 4.5:1 & 6:1)); (B) Thrombus formation on different modified PU samples (PU-COOH; PU-SO₃ and PU-SO₃-COOH(3:1; 4.5:1 & 6:1)) after 60 min incubation. The diagram includes t-test results (n = 3) with respect to PU-COOH (*p < 0.1, **p < 0.05 and ***p < 0.01) & PU-SO₃ (Δp < 0.05 and ΔΔp < 0.01).

Based on the results of *in vitro* thrombus formation test, it can be concluded that there is a relationship between the antithrombogenicity and the ratio of $-\text{SO}_3$ and $-\text{COOH}$. However, the anticoagulating action between sulfate and carboxylate groups cannot be linear since the thrombus weight did not keep decreasing as the percentage of sulfate groups increased. Combining with the results from *in vitro* thrombus formation test and mathematical function, the relationship between antithrombogenic activity and the ratio of sulfate and carboxylic groups is assumed to have an optimal point. At the beginning, as the percentage of sulfate groups increases, the antithrombogenicity increases. It keeps increasing until the ratio reaches the optimal value. After that, the antithrombogenicity decreases as the percentage of sulfate groups continues to increase. The curve corresponds to the results of *in vitro* thrombus formation test. Herein, the optimal ratio of $-\text{SO}_3$ and $-\text{COOH}$ for antithrombogenic activity is in the range of (3:1) and (4.5:1).

Chapter V: Conclusion and Future Work

Thousands of bypass graft surgeries are performed each year. When the supply of autologous vessels is insufficient, synthetic grafts become necessary and important. When those artificial vascular grafts made of synthetic polymers such as PET come in contact with blood, they can be thrombogenic which is especially true for small-diameter grafts due to the property of artificial material. Therefore, artificial vascular grafts need improvements to make them more haemocompatible with human blood. In other words, the luminal surface of synthetic vascular grafts directly contacting with blood should inhibit platelet adhesion and thrombosis formation.

It has been known that surface modification of the luminal surface with a bioactive agent like heparin or bioinert agent like PEG is a common way to improve the haemocompatibility of artificial vascular grafts such as PET. However, PET is an extremely inert material which does not have functional groups to bind with those haemocompatible agents directly. And PET has high crystallinity which is not easy to introduce foreign functional groups to PET's long chain. The immobilization of the haemocompatible agents onto PET durably and efficiently is a challenge.

The results from this study indicated that a special surface IPN technique introduced functional groups onto PET substrate by forming a three-dimensional polymer network and the following immobilization of haemocompatible agents was achieved by binding with the functional groups successfully. First, durable heparinization of PET (PET-Hep) was obtained by using the IPN technique. A vinyl functional monomer AEAM-Boc was polymerized and crosslinked in the swollen surface of PET to form a durable IPN so to

anchor amine functional groups onto chemically inert PET allowing the introduction of heparin on the surface through covalent bonding. The formed PET-Hep presented significantly less platelet adhesion in respect to untreated PET (around ¼) and was also nontoxic to the tested fibroblast cells. Because of the covalent nature of heparin binding on PET substrates, more than 94% of immobilized heparin could sustain 24 h extraction in PBS solution. The heparin engineered PET substrate holds the potential to be used in small-diameter vascular grafts. The same IPN method was applied to modify PET material with another kind of bioinert molecules-PEG. The PEG with azido functional groups was chemically bonded to the inert PET substrate modified with alkynyl groups. The surface hydrophilicity of PET-PEG was enhanced significantly. Compared with PET-Hep (11.33%), a better platelet adhesion result of PET-PEG (8.89%) was achieved due to the antiplatelet property of PEG molecules. The surface modification technique by forming thermoplastic semi-IPN could be a potential method to improve the haemocompatibility of artificial vascular grafts material via the introduction both bioactive and bioinert agents. Exploration of heparin molecular structure was also performed in this study. Sulfate and carboxylate functional groups were fixed to the same substrate successfully in a bioactive platform developed in Medical Textile Surface Engineering Lab. Without considering the polysaccharide confirmation of heparin, both of the two functional groups were proved to affect the anticoagulating performance of heparin. There was a special anticoagulating effect between those two functional groups. Furthermore, the optimal ratio of sulfate and carboxylate groups for antithrombogenic activity was investigated and assumed in the range of (3:1) and (4.5:1) through *in vitro*

thrombus formation test.

Developing less thrombogenic vascular grafts would be the ultimate goal of this research. The findings from this thesis show the IPN method is a good candidate to improve PET synthetic material with better durable haemocompatibility. Since PET is the material used in this thesis, future work can be the study of other synthetic thermoplastic material via the same IPN method. The haemocompatibility of those synthetic vascular grafts materials using IPN method to introduce bioactive/bioinert agents can be performed since IPN method could be applied to several thermoplastic polymers such as PU and ePTFE. In addition, the exact optimal ratio of sulfate and carboxylate groups (more different ratio points) based on the similar polysaccharide structure of heparin can be explored to develop an alternative to heparin. It is believed that the development of small-diameter artificial vascular grafts with long-term patency will make a valuable contribution to improving the cardiovascular surgery and subsequently improving patient outcomes.

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