İSTANBUL TECHNICAL UNIVERSITY ★ INSTITUTE OF SCIENCE AND TECHNOLOGY

IMMOBILIZATION OF HIRUDIN ON SURFACE OF PTFE TO INCREASE ITS BLOOD COMPATIBILITY

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İSTANBUL TEKNİK ÜNİVERSİTESİ ★ FEN BİLİMLERİ ENSTİTÜSÜ

PTFE YÜZEYLERE KAN UYUMLULUĞUNU ARTTIRMAK İÇİN HİRUDİN TUTUKLANMASI

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FOREWORD

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ABBREVIATIONS

PTFE	**:** Polytetrafluoroethylene
PU	**:** Polyurethane
PE	**:** Polyethylene
ADP	**:** Adenosine Diphosphate
ATU	**:** Antithrombic Unit
CCP	**:** Capacitatively Coupled Plasma
ICP	**:** Inductively Coupled Plasma
RF	**:** Radio Frequency
DC	**:** Direct Current
ECR	**:** Electron Cyclotron Resonance
PSM	**:** Plasma Surface Modification
ATR-FTIR	**:** Attenuated Total Reflection Fourier Transform Infrared
MES	**:** 2-(*N*-morpholino)ethanesulfonic acid
ACD	**:** Acid Citrate Dextrose
H_2	**:** Hydrogen Gas
Ar	**:** Argon Gas
Pa	**:** Pascal
W	**:** Watt
EDC	**:** N-(3-Dimethylaminogrouply)-N-ethly-carbodiimide
NHS	**:** N-Hydroxysuccinimide

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IMMOBILIZATION OF HIRUDIN ON SURFACE OF PTFE TO INCREASE ITS BLOOD COMPATIBILITY

SUMMARY

Blood compatibility is an essential feature for blood-contacting biomaterials, namely blood vessel grafts, artificial heart and catheters. Surface induced thrombosis is one of the major causes for the failure of these biomaterials and can be minimized by altering the surface characteristics.

The aim of this study is to increase the blood compatibility of polytetrafluoroethylene (PTFE), one of the preferred materials for this kind of applications, by a two-step procedure: Firstly, the surface was activated by hydrogen plasma followed by acrylamide attachment and secondly, hirudin, a potent antithrombogenic protein from leeches, was immobilized to the surface. The first part of the study involving the plasma treatment was optimized and different surfaces were characterized by water contact angle measurements and ATR-FTIR. It was seen that the contact angle of the PTFE was decreased from 130° to 59° by optimizing the hydrogen plasma treatment conditions. Then acrylamide solution (25 % w/v; in ethanol/acetone (50 % v/v)) was applied to the surface and subjected to argon plasma treatment (1 min-50 W-13 Pa) for monomer grafting. Water contact angle was further down to 33° and amide groups can be detected by ATR-FTIR analysis. In the second part, hirudin was attached to amide groups on PTFE surface by EDC/NHS activation. Then thrombogenicity (Kinetic Model) test was applied to detect hirudin activity. Test results showed that there is a serious decrease in the clot formation compared with the pristine PTFE samples. Even no clot formation observed in some cases.

As a result, we increased blood compatibility of PTFE surfaces by plasma-induced monomer grafting and hirudin immobilization and obtained an alternative material to be used in medical applications such as vascular graft, catheters etc.

PTFE YÜZEYLERE KAN UYUMLULUĞUNUN ARTTIRILMASI İÇİN HİRUDİN TUTUKLANMASI

ÖZET

Kan uyumluluğu yapay kan damarı, yapay kalpler ve kateterler gibi kan ile temas eden biyolojik malzemeler için gerekli bir özelliktir. Biyolojik malzemelerin yüzeylerinde oluşan pıhtılaşma bu malzemelerin kullanımlarını engelleyen en önemli faktörlerden birisidir. Malzemelerin yüzeylerinde yapılacak olan değişiklikler ile bu problem büyük oranda çözülebilmektedir.

Bu çalışmanın amacı biyomalzeme olarak kullanılabilen PTFE yüzeylerin, önce hidrojen plazma ile aktif hale getirilmesi, daha sonrada akrilamit monomerlerinin ve sülüklerden izole edilen ve pıhtılaşmayı engelleyen bir enzim olan hirudinin yüzeye tutuklanmasıdır. Çalışmanın ilk kısmında, farklı PTFE yüzeylerinde temas açısı ölçümleri ve ATR-FTIR analizleri yapılarak plazma uygulaması için en uygun koşullar belirlendi. Hidrojen plazma koşullarında yapılan optimizasyon ile PTFE yüzeylerin temas açısı 130° den 59° kadar düşürülmüştür. Daha sonra, yüzeylere önce akrilamit çözeltisi (%25 w/v; etanol/aseton (%50 v/v)) , sonrada monomer aşılaması için (1 dk-50 W-13 Pa) Argon plazma uygulanmıştır.Yapılan temas açısı ölçümlerinde açısının 33° kadar düştüğü, ATR-FTIR analizlerinde ise 1665 cm^{-1} civarında amid gruplarının varlığı görülmüştür. Çalışmanın ikinci kısmında EDC/NHS aktivasyonu ile hirudin yüzeydeki amide gruplarına bağlanmıştır. Daha sonra trombus oluşumu (Kinetik Model) testi uygulanarak hirudinin aktivitesi ölçülmeye çalışıldı. Sonuçlar normal PTFE yüzeyleri ile karşılaştırıldığında pıhtılaşmada ciddi bir azalmanın olduğu, hatta bazı ölçümlerde neredeyse hiç pıhtılaşma olmadığını gözlenmiştir.

Sonuç olarak, plazma yöntemine dayalı monomer aşılama ve hirudin tutuklama yöntemiyle PTFE malzemenin yüzeyinin kan uyumluluğunu arttırmayı başardık ve yapay kan damarı, kateter vb. gibi medikal uygulamalarda kullanılabilecek biyo malzemelere bir alternatif elde ettik.

1.INTRODUCTION

Biomaterials research is dedicated to increase the quality and duration of human life by assisting to restore the damaged and non-functional body parts. An important feature of this field is that it requires a multidisciplinary effort to obtain effective results. Since different kinds of materials like metals, ceramics, polymers or composites can be used depending on necessity, good knowledge of materials' properties, how to manipulate and process them, how to characterize them is extremely important. Scientist from life sciences, on the other hand, should be able to define what kind of properties is needed for a specific biomaterial and then involve in the protein/enzyme immobilization, optimization, activity measurements, and so on. Only with this kind of collective effort, a biomaterial of medical value can be designed.

When biomaterial and the body come into contact, the material should be stable and should not release any toxic, carcinogenic or teratogenic substances into the body which result in adverse effects. Furthermore, blood compatibility is very important for blood-contacting biomaterials. Surface induced thrombosis is one of the major causes for the failure of these biomaterials and altering their surface characteristics while preserving the bulk properties is an effective way of increasing their performance.

Our aim is to increase blood compatibility of PTFE surfaces to use them in medical applications. To do that, we generated functional groups (amides) on PTFE surfaces for hirudin immobilization by plasma induced grafting and optimized plasma conditions for H_2 and Ar plasma. Surface characterization of plasma treated PTFE samples was done by water contact angle measurements and ATR-FTIR analysis to check plasma effect. Finally, hirudin was immobilized onto the created functional groups (amides) by using EDC/NHS technique, and finally, activity of immobilized hirudin was tested by using thrombogenicity (Kinetic model) assay.

2.1 Biomaterials

2.1.1 Definition of biomaterials

Biomaterials are synthetic or natural materials that are used in medical devices or in contact with biological systems to treat damaged tissues or organs to restore their functions, or used to replace them. Biomaterials have wide range of applications both medical and nonmedical fields (Table 2.1).

Table 2.1: Uses for biomaterials [1]

Medical uses	Non-medical uses
Artery graft	Arrays for DNA and diagnostics
Breast implant	Bioremediation materials
Cochlear implant	Biosensors
Ear drainage tube	Bioseparations, chromatography
Dental implant	Biofouling-resistant materials
Feeding tube	Biomimetics for new materials
Glaucoma drainage tube	Cell culture
Hydrocephalous shunt	Controlled release for agriculture
Intraocular lens	Electrophoresis materials
Joints (hip, knee, shoulder)	Fuel cells (biomass)
Keratoprosthesis	MEMS
Left ventricular assist device (LVAD)	Muscles (artificial) and actuators
Mechanical heart valve	Nanofabrication
Nerve guidance tube	NEMS
Ophthalmic drug delivery device	Neural computing/biocomputer
Pacemaker	Smart clothing for biowarfare
Renal dialyzer	Yeast array chip
Stent	
Tissue adhesive	
Urinary catheter	
Valve, heart	
Wound dressing	
X-ray guide	
Zirconium knee joint	

2.1.2 History of biomaterials

Biomaterials have being used for along time in our life. Their usage dates far back into ancient times. Artificial eyes, ears, teeth and noses were found in Egypt mummies [2]. However, the early medical implants were destined to fail because people did not have enough information about the concepts related to infection, materials, and the biological reaction to materials. For some scientists, the modern era of medical implants started at 1940 when British ophthalmologist Harold Ridley had an observation on the Spitfire fighter pilots and developed first implantation lens in 1949. Following this discovery, Harold Ridley worked on intraocular lenses (IOLs), Charnley developed the hip implant, Vorhees invented the vascular graft, Kolff was revolutionizing kidney dialysis, and Hufnagel invented the ball and cage heart valve and they all became pioneer of medical biomaterials [1].

2. 1.3 Features of biomaterials

There are several requirements for a material to be used for medical applications. First of all, they should have appropriate mechanical properties to fit its application. A hard tissue implant such as knee joint for example, should not deteriorate or corroded in the body, and withstand the fatigue. A skin implant, on the other hand, should be elastic. Then, they should be sterilized (by autoclave, ethylene oxide, or γ-ray) without degradation. When materials come into contact with a living organism, there are interactions between the biomaterial and a biologically or chemically active, fluid-based medium, so biomaterials and their degradation products can damage cells, cause cancers, or lead to blood clotting. To handle these drawbacks, the materials should be biocompatible that is it should not produce a toxic, injurious, or immunological response in living tissue. Furthermore, size, shape, and porosity properties should be controlled. For example, for the cardiovascular implants, devices should have certain size to avoid clotting, and drug permeability and good release properties for drug delivery are needed [3].

2. 1.4 Types of biomaterials

Biomaterials that are used in medical field can be grouped into four classes depending on their structural and mechanical properties such as metals (Gold, tantalum, stainless steel, Co-Cr, NITI, Ti alloys), ceramics (Alumina, titania, zirconia bioglass, carbon, hydroxyapatite), polymers (Polyethylene(PE),polyurethane(PU), polytetraflouroethylene (PTFE), poly acetal (PA)) , and composites (HA/PE, silica/SR, carbonfiber/ultra high molecular weight). Although metals have high strength, ductility and resistance to wear, they have some limitations such as low biocompatibility, corrosion, high stiffness compared to the tissues and they can release ions that may cause allergic tissue reactions [4]. Ceramics have good biocompatibility, and they are resistant to corrosion and high compression, but brittleness, low mechanical reliability, difficulties in manufacturing, and inelasticity are some of the drawbacks of ceramics. Metals and ceramics are generally used in hard tissue applications (dental applications, bone plates, joint replacement, knee replacement, etc.) because of their structural and mechanical properties. To handle the drawbacks of metals and ceramics, composites materials are developed by using different combinations of metals, ceramics and polymers. Polymers are generally preferred in soft tissue applications (wound dressing, catheters, vascular graft etc.) [2]. Since polytetrafluoroethylene (PTFE) is a polymer and the material used in this study, more emphasis given on polymers as biomaterials in section 2.2.

2.2. Polymers As Biomaterials

2.2.1 Polymers and their medical applications

Polymers commonly used in biomedical devices may be classified in two categories as natural polymers and synthetic polymers depending on their origins. Enzymes, nucleic acids, proteins, cellulose and natural rubber are examples of natural polymers. There are also a large number of synthetic (man-made) polymers consisting of fibers, elastomers, plastics, adhesives, etc. These natural and synthetic polymers have a wide range of application fields in medical area such as extracorporal blood-circulating devices, catheters, blood bags and tubing used for blood transfusion, membranes, hollow fibers, and tubing used for dialysis devices etc. In Table 2.2, Polymers and their medical applications are given.

Table 2.2: Polymers in Medical Applications [5]

Resin	Primary Features	Typical Applications
Polyethylene	Processing ease, chemical resistance	Caps, needle hubs, medical packaging, waste bags
Polypropylene	Autoclavibility, contact clarity	Syringes, specimen collection cups
Gen-purpose polystyrene	Transparency	Petri dishes, labware, test tubes
High-impact polystyrene	Toughness, opacity	Home test kits, diagnostic equipment, housings
Styrene–acrylonitrile	Chemical resistance, transparency, toughness	Diagnostic components, fluid handling devices, flat plate dialyzers
Acrylic	High light-transmission rates, chemical resistance	I.V. components, specimen-collection containers
Acrylonitrile–butadiene–styrene	Toughness, low and high gloss, good processability	Home test kits, housings, surgical staplers, I.V. connectors
Polycarbonate	Chemical and heat resistance, toughness, transparency	Blood centrifuge bowls, cardiotomy reservoirs, profusion devices, hemodialyzers
Polyester	Chemical resistance	I.V. components, catheters, surgical instruments, housings
Poly(vinyl chloride)	Transparency, good scuff resistance	Blood bags, catheters, cannulae, corrugated tubing, renal care products, transfusion supplies, face masks
Polyurethane	Good chemical resistance, toughness, good processability	Catheters, tubing, I.V. connectors, drug delivery systems
Polyetherimide	Autoclavibility, chemical resistance	Sterilization trays
Polysulfone	High heat resistance	Medical trays

2. 2.2 Polymers used in blood-contacting biomaterials

Polymers that are used in cardiovascular applications have a wide range of variety. Devices that are made of with polymers and contacting with blood in cardiovascular applications are grouped into three categories such as devices that are used for permanent replacement in the circulatory system (artificial hearth, hearth valves, vascular grafts); devices that are inserted into a blood vessel for varying time periods of time (catheters, sensors, imaging agents etc.); and extracorporeal devices that are used for removal or return of blood from body (blood oxygenators, hemodialysis units (artificial kidney), etc.). In Table 2.3, blood contacting polymers and applications in devices are given.

Table 2.3: Blood Contacting Polymers [6]

Polymer	Device
Cellulose and derivatives	Membranes for dialysis
Cross-linked collagen	Heart valves
Dextran	Plasma expanders
Human albumin	Plasma expanders
Polyacetal	Heart valves
Polyacrylonitrile	Membranes for dialysis
Polyamides	Catheters, heart valves, membranes for dialysis
Polycarbonates	Syringes, catheters, heart valves
Poly(ethylene terephthalate)	Vascular prostheses
Polypropylene	Syringes, catheters
Polysulfones	Membranes for dialysis
Poly(tetrafluoroethylene)	Vascular prostheses, artificial hearts, catheters
Polyurethanes	Vascular prostheses, artificial hearts, catheters
Poly(vinyl chloride)	Blood bags, catheters
Silicones	Catheters, artificial hearts

Some artificial hearth devices uses a special balloon dilation catheter made of polyvinylchloride (PVC) or polyethylene (PE) [7]. In addition, blood pumps of sac or diaphragm type are made from PVC, polyolefin rubber (Hexsyn), silicone rubber (Silastic), and polyurethane (PU) such as Avcothane, Biomer, and Pellethane are reported [8-10].

Mechanical valves and bioprosthetic heart valves are two kinds of commonly used heart valve prostheses. Polymer valves are generally preferred for temporary replacement. The polymers used in mechanical valves are a Dacron or poly(tetrafluorethylene) (PTFE), and a poly(ethylene terephtahalate)-covered stent for bioprosthetic hearth valves[7]. Polymer valves such as seamless trileaflet valves have been made of PUs [11].

Vascular grafts that are made of polymeric materials are among the most successful artificial organs and frequently used in permanent implantations. Vascular grafts whose diameter is >6 mm is generally used in medical applications, because vascular grafts whose diameter is <4 mm have a possibility to form thrombus. To handle this drawback, scientists have been working on modification of the surface biomaterial. The polymers most often used are polyester (Dacron) fiber in woven, knitted, and velour types, or porous expanded PTFE (Telfon, Gore-Tex, Impra) fabrics [12-13].Their porous structure induces the formation of new pseudoneointima, resulting in the prevention of blood leakage.

When myocardial activity or electrical conducting pathways of a hearth is dysfunctional or unreliable, cardiac pacemakers are used to supply electrical stimulation to the heart to store its function again. Silicone and PUs such as Pellethane are used in the wire lead insulation of pacemakers because of their blood compatibility and durability [14].

Blood oxygenators, are used as extracorporeal heart–lung machines, to replace the heart temporarily in open-heart operations. There are two types of blood oxygenators, the bubble type and the membrane type. The former, in which blood is directly contacted with oxygen and carbon dioxide, is composed of soft plasticized PVC film or rigid polycarbonate (PC); in the latter blood is oxygenated via a polymeric membrane such as PP, silicone rubber, PTFE, or polysulfone [7].

There are many different kinds of intravascular catheters. For intravenous (IV)-administration indwelling catheters PE, PP, silicone rubber, and PU are used. Other types of catheters made of PU, PVC or Teflon-coated Dacron are also reported [7]. Balloon-tipped catheters are another type of catheters and used to monitor blood pressure.

2.2.3 Polytetrafluoroethylene (PTFE)

Polytetrafluoroethylene (PTFE) was discovered by R. Plunkett in 1938 [15]. The molecular structure consists of carbon chain in center covered by fluorine sheath to

protect carbon chains from chemical attacks by forming a helix around carbon atoms (Figure 2.1) [16].

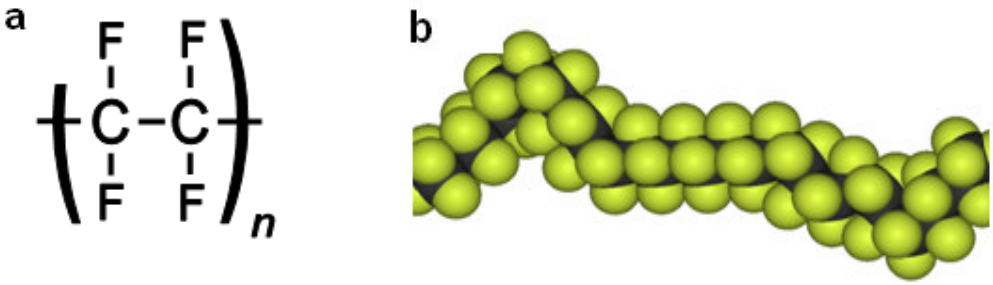


Figure 2.1: Polytetrafluoroethylene (PTFE) structure a) linear form, b) 3-D form

Since C-F bonds are very stable, the polymer has a high stability even when it is heated above its melting point. Its' melting point changes between 300- 380˚ [17], and its density is ca 2.13-2.19 g/cm^3. PTFE has a high chemical resistance and insoluble in all organic solvents except in some certain fluorinated liquids such as perfluorinated kerosenes at temperatures approaching the melting point of the polymer, and incapability of specific interactions because of high crystallinity (>90%). Furthermore, PTFE is a tough, flexible material of moderate tensile strength (2500–3800 psi, i.e., 17–21 MPa) at 238C. The friction coefficient of PTFE is low and is about 0.07. In literature, coefficient of friction is reposted to be in the range of 0.02-0.10 for polymer to polymer, and 0.09-0.12 for polymer to metal [5]. PTFE has also weathering resistance, so it is not wetted by water or has a very low adsorption (0.005%) [5] and high dielectric strength, low dielectric constant. All these and other properties of polytetrafluoroethylene were given in details by Sperati [18].

PTFE seems a good polymer for medical applications because of its properties such as low coefficient of friction, hardness, high thermal stability, and chemical resistance, but its biocompatibility should be improved to be used in cardiovascular applications to avoid thrombus formation on the surface.

2.3. Biocompatibility and Material-Blood Interactions

2.3.1 Biocompatibility

When materials come into contact with a living organism, there are interactions between the material and a biologically or chemically active, fluid-based medium, and if the material is not biocompatible, the material itself, its degradation products or additives can damage cells, cause cancers, or lead to blood clotting. Therefore, biocompatibility may be defined as the ability of a material to perform with appropriate host response in a
specific application without adversely and significantly affecting the body and, without the material itself suffering any adverse effects [19,20]. When the material is integrated into the body, it should not induce thrombus formation, immune response, inflammatory reaction, or infection; and it must be nontoxic, noncarcinogenic, and nonmutagenic [21]. Materials contacting the blood and not inducing thrombus formation, inflammatory reaction or infection, and nontoxic as mentioned before is called as blood compatible that is one of the specific definition of biocompatibility. If the material is used as a tissue replacement then it should be tissue compatible which is another specific definition of the biocompatibility. Blood compatibility is also one of the most important properties of biomedical polymers.

2.3.2 Material-Blood interactions

2.3.2.1 Blood clotting mechanism

Blood is composed of three main elements such as liquid medium, plasma, and cellular elements, which are subdivided into red blood cells (erythrocytes), white blood cells (leukocytes), and platelets. When the material comes into contact with blood, first event occurs between material and blood is redistribution of interfacially bound water and ions, and rapid adsorption of plasma proteins. These processes influence subsequent interactions of blood cells, especially platelets and leukocytes, with proteinated surfaces.

Adsorption of plasma results in activation of blood coagulation cascade. Activation of blood coagulation cascade leads to the polymerization and crosslinking of fibrin at the blood interface. There are different coagulation pathways that are initiated by absorbed proteins depending on the nature of the surface. The overall scheme of blood coagulation is summarized in Figure 2.2.

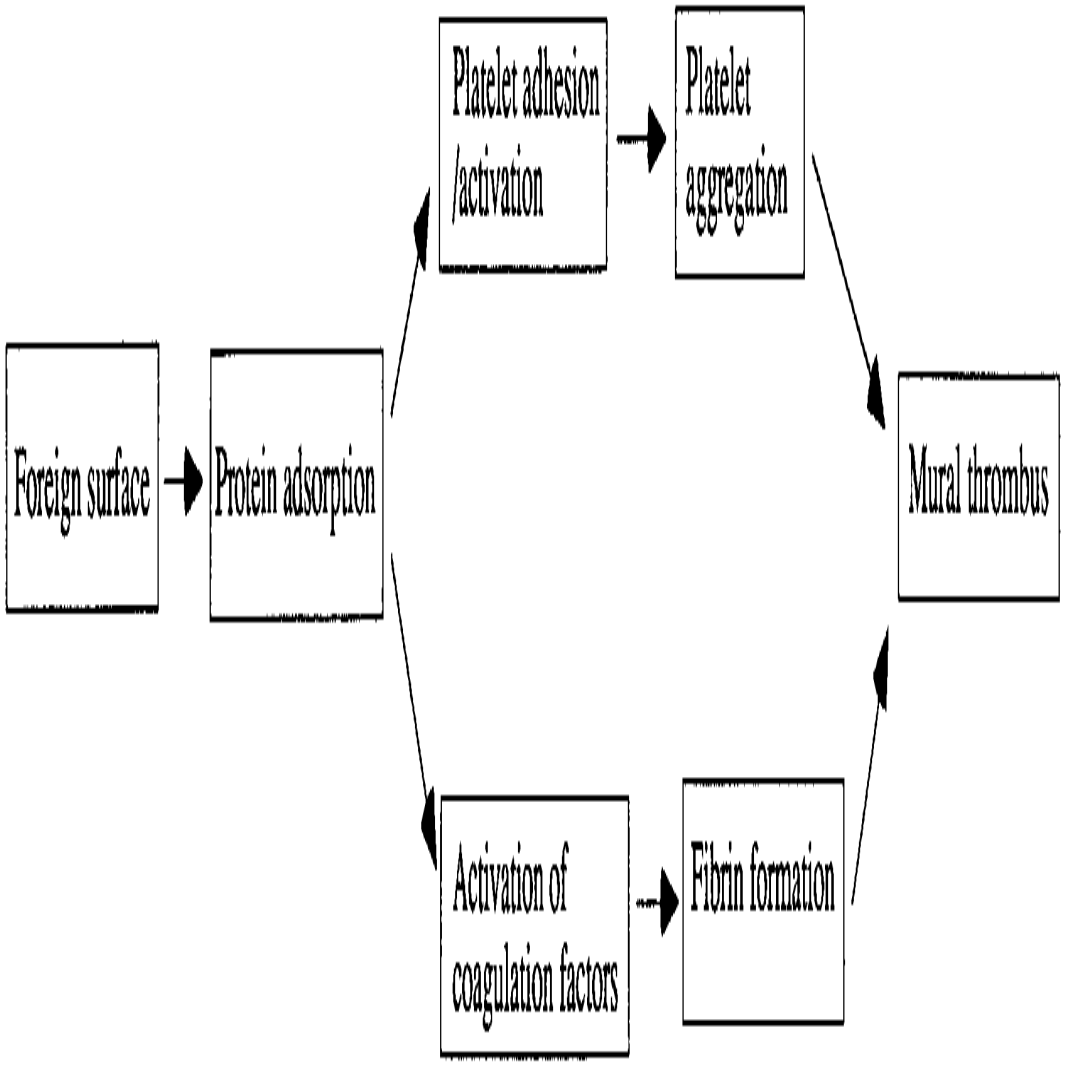


Figure 2.2: Thrombus formation on the material surfaces [7]

There are two types of plasma protein adsorption at the blood–material interaction as seen in Figure 2. 2. The first one is adsorption of albumin, fibrinogen, or γ-globulin from the blood plasma, and the second one is platelet and leukocyte adhesion which result in the polymerization of fibrin onto the surface.

Platelet ahdesion/activation

Platelets are small disc-type elements containing a variety of granules. Platelets adhere to materials through pseudopodia, some internal changes occur in the platelets, which result in releasing granules. Released granules attract more platelets and attracted platelets start to stick each other. This is called as platelet aggregation. This platelet aggregation then result in initiating thrombus. Adenonsine diphosphate (ADP), and thromboxane A_2 are the two most important released components which are very effective and potent substance for platelet aggregation.

Activation of coagulation factors

Activation of blood coagulation factors is another coagulation pathway. Enzymes, lipids and ions are needed to activate blood coagulation factors and to form fibrin. Thrombin that is the main coagulation factor polymerizes the soluble fibrinogen monomer into an insoluble crosslinked fibrin network. Actually, fibrin is formed in response to three independent mechanisms such as platelet adhesion and activation like mentioned before and extrinsic pathways, or intrinsic pathways of coagulation factor activation like in Figure 2.3. The extrinsic pathway is activated by blood exposed to tissue factors (collagen, membrane constituents, lipids, and proteins). In the intrinsic pathway, blood is exposed to artificial surfaces, where various coagulation factors are adsorbed and converted in a chain reaction. The intrinsic and extrinsic pathways differ in initiation mechanism, but stimulate thrombin the same way. The activation of coagulation factors involves platelet phospholipids and Ca^{2+} ions.

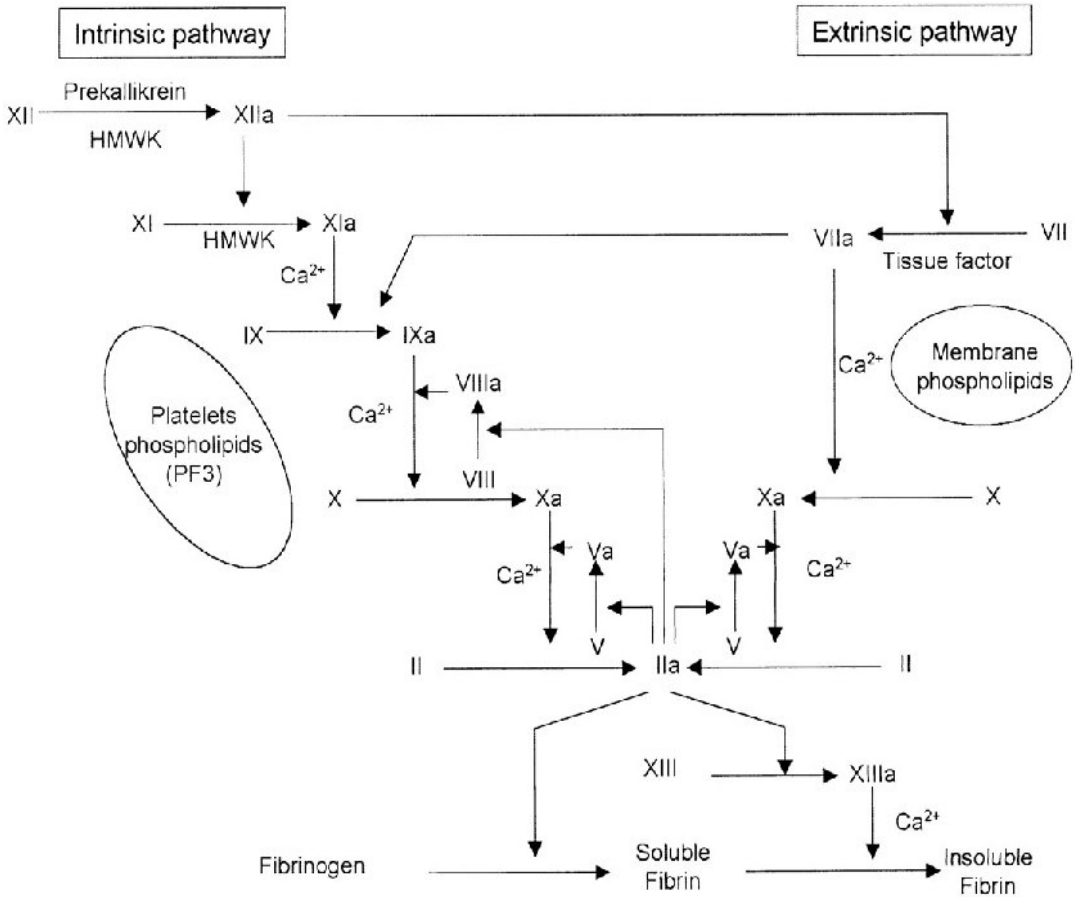


Figure 2.3: Coagulation Pathways [6]

Complements and fibronectins

In addition to plasma proteins, platelets, and coagulations factors, there are two additional factors affect blood compatibility of biomaterials known as complements and fibronectins. Complements are the primary humoral mediator of antigen–antibody

reactions, and therefore are related to immunity. If complements are activated, leukocytes will adhere to artificial surfaces. These leukocytes and activated complements may also attract platelets and help in the formation of mural thrombus. Fibronectins are glycoproteins found on the surface of various cells and present in platelet granules. Fibronectins act as adhesive proteins to bind cells to other cells and artificial substrates. They have affinity for fibrinogen, fibrin, and platelets. Fibronectins also appear to be important in mural thrombus.

2.4. How to Prevent Clot Formation

2.4.1 Immune system

Since biomaterials contact with the biologically or chemically active, fluid-based medium in the body, coagulation must be prevented. Otherwise, people come across with serious health problems, even they may pass away. The body has a defense system against coagulation that result in disruption in system functions. If any coagulation attempt exists, immune system becomes active. Then, fibrinolytic enzyme plasmin and its proenzyme, plasminogen which are in charge to dissolve fibrin in the body, start to dissolve fibrin.

2.4.2 Anticoagulants

Anticoagulants such as Antithrombin III, Heparin, Prostaglandins,hirudin, urokinase and several drugs like Aspirin and dipyridamole also prevent formation of fibrin by inhibiting steps that are shown in Figure 2.4. Antithrombin III is a lipoprotein in the body, and the most potent inhibitor of coagulation. Heparin is a carbohydrate that contains sulfate and sulfonate groups, and functions as an anticoagulant by binding to and activating antithrombin to inhibit thrombin. Prostaglandins are long-chain hydroxyunsaturated fatty acids, and also prevent platelet aggregation, so clot initiation does not start. Drugs such as aspirin and dipyridamole also act as anticoagulants, and inhibit platelet aggregation.

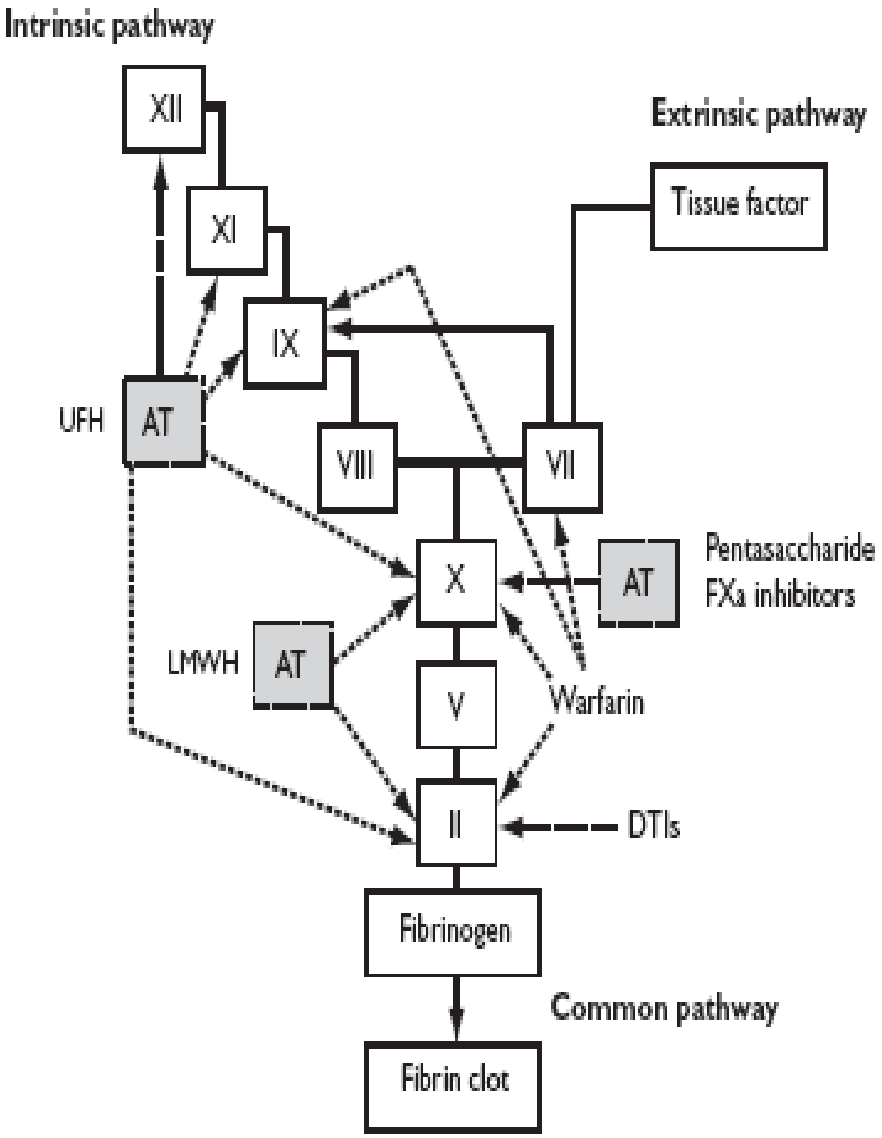


Figure 2.4: Anticoagulation Pathways

Biomaterials should be biocompatible as mentioned until now, so coagulation must be prevented on the material surface by anticoagulants. To make a material blood compatible, variety of surface modification techniques have been studying to create useful surfaces to immobilize anticoagulants such as heparin, urokinase and hirudin..

2.5. Surface Modification Techniques for Polymers

2.5.1 Chemical modification of polymer surfaces

Surface modifications should not affect the bulk properties of polymers very much, but create surfaces with desired properties. The chemical treatment of polymers for surface modification includes reactions as oxidation, halogenation, and other standard chemical reactions.

Surface oxidation techniques include the use of corona discharge, ozone, hydrogen peroxide, nitrous acid, alkaline hypochloride, UV irradiation, oxidizing flame, and chromic acid. The reactions result in initiation of the formation of hydroperoxides, that catalyze the formation of aldehydes and ketones and then, acids and esters. If surface-oxidized polyethylene coated with a thin film that is produced by using, vinylidene chloride, acrylonitrile, and acrylic acid terpolymers becomes impermeable to oxygen and more resistant to grease, oil, abrasion, and high temperatures[5] .
Alkali and acid treatments is another chemical method used for surface modification of polymers. When alkali and acid treatments applied to the polymers, containers suitable for storing light-sensitive compounds, improved adhesion to polyethylene and nylons, antifogging lenses can be produced. Surface halogenation also result in increased adhesion to polar surfaces [5].

2.5.2 Surface modification of polymers by grafting

Modified polymers surfaces can also be created by grafting [22]. There are two methods for producing grafted polymer surfaces: direct coupling of existing polymer molecules to the surface and graft polymerization of monomers to the surface

2.5.2.1 Polymer coupling

In polymer coupling, polymers are directly used for surface modifications in stead of monomers to obtain functional groups. Two theories exist to graft a polymer to a surface

for blood contacting polymers. First theory, grafting a hydrophilic polymer to the target surface in order to separate blood proteins [23-25]. Then second theory is to confer the biological properties of the grafted macromolecule (for example heparin) to the surface [26].

2.5.2.2 Graft polymerization

Graft polymerization involves the radical polymerization of acrylic or vinylic monomers onto the polymeric surface, and then producing peroxides by using these monomers. There are different kinds of methods used for graft polymerization onto different substrate surfaces such as direct chemical modification [27], ozone [28,29], gamma rays [30], electron beams [31], glow discharge (low temperature plasma) [32-34], corona discharge [35-36], and UV irradiation [37].

2.6. Plasma and Plasma Treatment

2.6.1 Plasma

Plasma is a quasi-neutral gas and known as fourth state of matter. It includes both electrons and ions as well as neutrals, atomic and molecular species that occur because of presence of an electromagnetic field. Plasma mainly generated by electric field, but magnetic field, combustion and nuclear reactions can also generate plasma. Different kinds of reactions happen in plasma such as excitation, ionization and dissociation. The excitation means increasing internal energy and translation to a higher state. If the energy is given more than required that is enough for excitation, electrons that are weekly bounded are removed form an atom and result in ionization. Excitation and ionization can also occur because of the reactions by electron collision, ion collision, neutral particle collision and radiation. Dissociation occurs as a result of inelastic collision of a molecule with an electron, ion or photon. When neutral fragments, either hot or in an excited state, hit the substrate surface they affect the process chemistry.

As an example; the various active species generated in a CCP reactor are shown in Figure 2.5 [38].

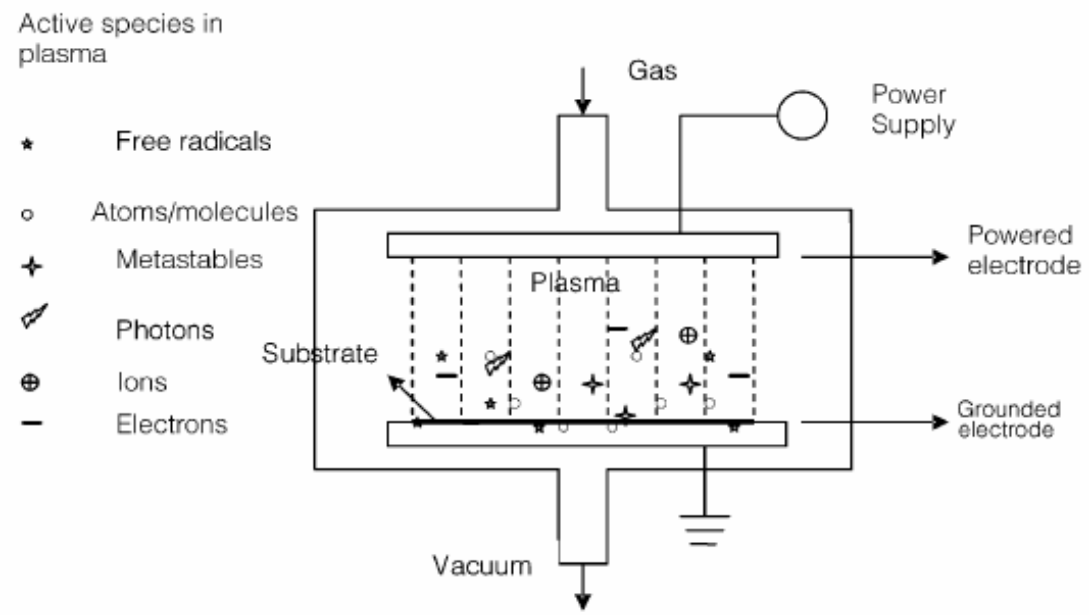


Figure 2.5: Schematic diagram of capacitatively coupled reactor

2.6.2 Plasma sources

There is a variety of plasma sources that can be grouped into 3 main categories such as gaseous, metallic, and laser-based plasma sources (Table 2.4) [39].

Table 2.4: Plasma Sources

1. Gaseous plasma sources
-Radio frequency (RF) glow discharge
-Electron cyclotron resonance (ECR)
-Corona discharge atmospheric arc
2. Vacuum arc plasma source
3. Laser plasma source

In our study, RF glow discharge plasma source is used for the surface modification of PTFE, so the characteristics of this plasma source, such as the electron temperature, ion temperature, electron density, and uniformity will be discussed more than other techniques in the next section.

2.6.2.1 Gaseous plasma sources

RF glow discharge plasma source

RF glow discharge plasma is a gaseous plasma, and gaseous plasma is ignited by applying a potential through the gas, and the breakdown potential that depends on the pressure and discharge gap width. For example, the relationship between the breakdown potential of air and pressure is given in Figure 2.6.

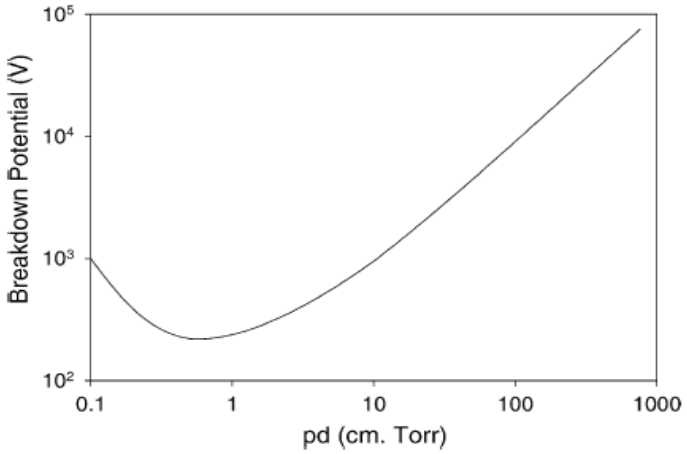


Figure 2.6: Breakdown potential versus pressure and discharge gap [39]

A change in this value leads to an increase of the critical breakdown electric field. There are four regions between the current and voltage in a low-pressure gas discharge such as (1) dark or Townsend discharge, (2) normal glow, (3) abnormal glow, and (4) arc discharge where the plasma becomes highly conductive like in Figure 2.7.

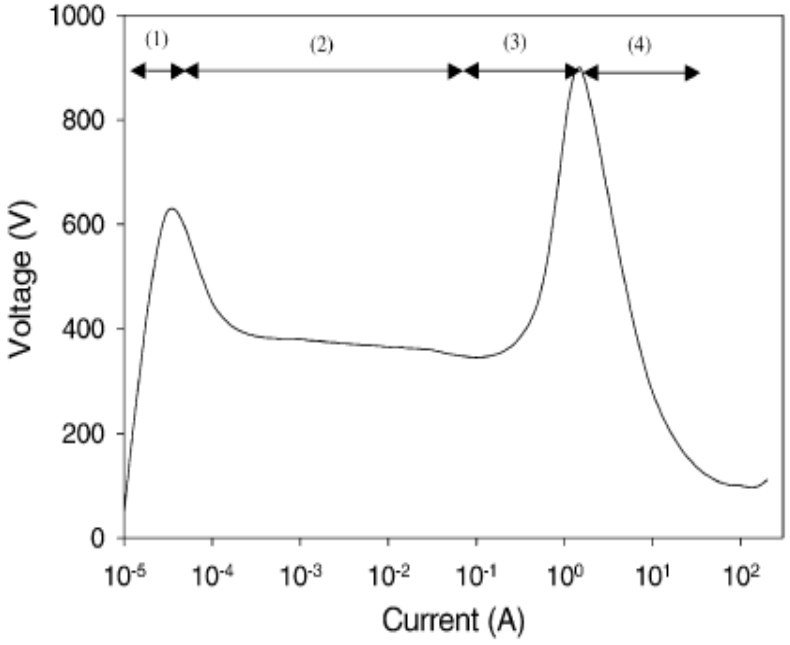


Figure 2.7: Current–voltage characteristics of a low-pressure gas discharge at 1 Torr [39]

Plasma sources such as direct current (DC), RF glow discharge (rfGD), and electron cyclotron resonance (ECR) are operated at low-pressure as the breakdown electric field is smaller and the current is more controllable. These plasma sources can generate large area uniform plasma with a well controlled electron density.

RF is able to produce a large volume of stable plasma, so it is often chosen as a source for Plasma Surface Modification (PSM). The RF discharges can be grouped as capacitive coupling and inductive coupling based on RF power. Plasma produced with capacitive coupling called as capacitively coupled plasma (CCP) and inductively coupled plasma (ICP) if it is produced with inductive coupling. In the ICP reactor, a magnetic field is created around the coil where passing electric current generates plasma. Since the electrodes are kept outside the reaction chamber (Figure 2.8), ICP reactors are free from contaminants such as impurities introduced to the plasma process [39].

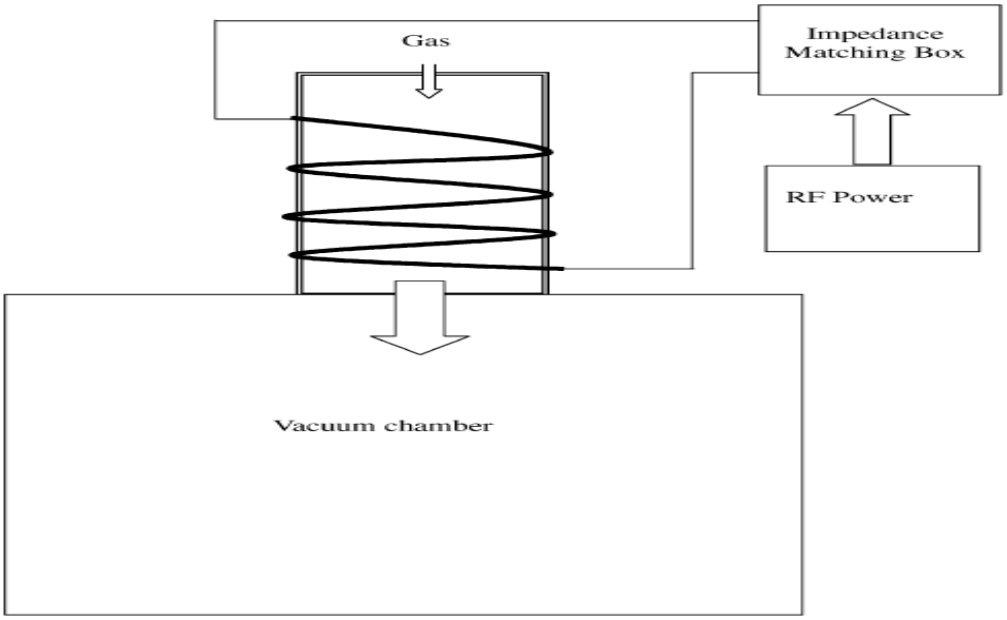


Figure 2.8: Schematic diagram of inductively coupled reactor

In the CCP reactor, one of the two metal electrodes is connected to the power supply and the other one is grounded like in Figure 2.5.

13.56 MHz is generally used in RF glow discharge and the pressure during discharge changes between 10^{-3} and 100 Torr [40,41]. The electron density in RF glow discharge in low-pressure (10^{-3} to 1 Torr) varies from 109 to 1011 cm^{-3}, whereas the electron density in medium pressure (1–100 Torr) can reach 1012 cm^{-3} [42]. The electron temperature is several eV and the ion temperature is very low [39].

Electron cyclotron resonance (ECR)

ECR plasma source is operated at a pressure between 10^{-5} and 10^{-3} Torr which means plasma is generated at low pressure. To produce ECR plasma, Microwave power at 2.45 GHz is used. To achieve the ECR conditions, there are coils occur around chamber to supply magnetic field. When electrons come into contact with the magnetic field, they start to rotate around the magnetic field lines. To obtain a high density plasma, magnetic field should be adjusted to match the cyclotron frequency of the electrons.

Corona discharge

Corona discharge produces corona effect which mean formation of high energy electromagnetic fields close to charged thin wires or points. Corona discharge system contains two electrodes whose sizes are different from each other like in Figure 2.9 [38].

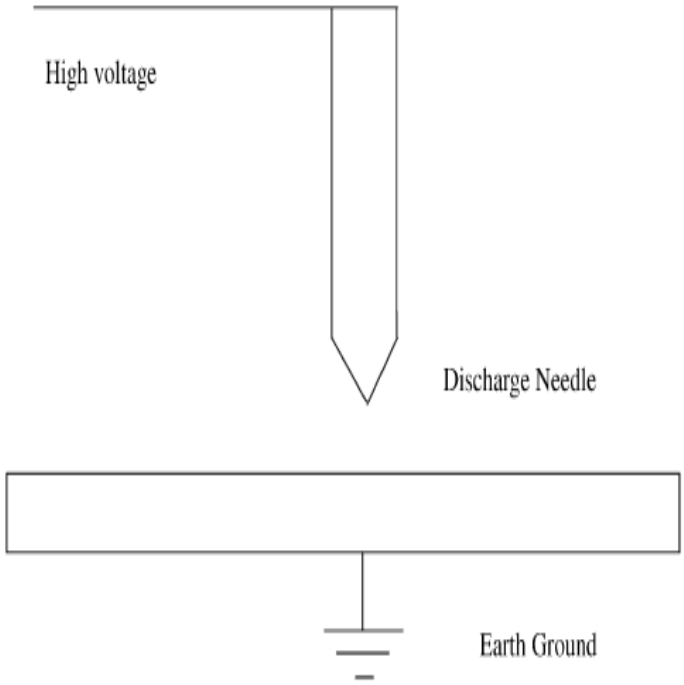


Figure 2.9: Corona Discharge Plasma System

Anode has a small characteristic compared to cathode and strong electric field because of high voltage. The material to be treated is placed on the cathode and the discharge is usually carried out in atmospheric air.

Atmospheric arc

Plasma spray torch is commonly used atmospheric arc plasma. The schematic of a plasma spray torch is given in Figure 2.10. It consists of an outer grounded and water-

cooled shield as an anode, and gas inlet components and sticky-type cathode with a conical tip. To obtain plasma the discharge current and power density should be very high. Pressure of electrically conductive gas to develop a plasma is about 10^5 Pa, and temperature T > 8000 K [38].

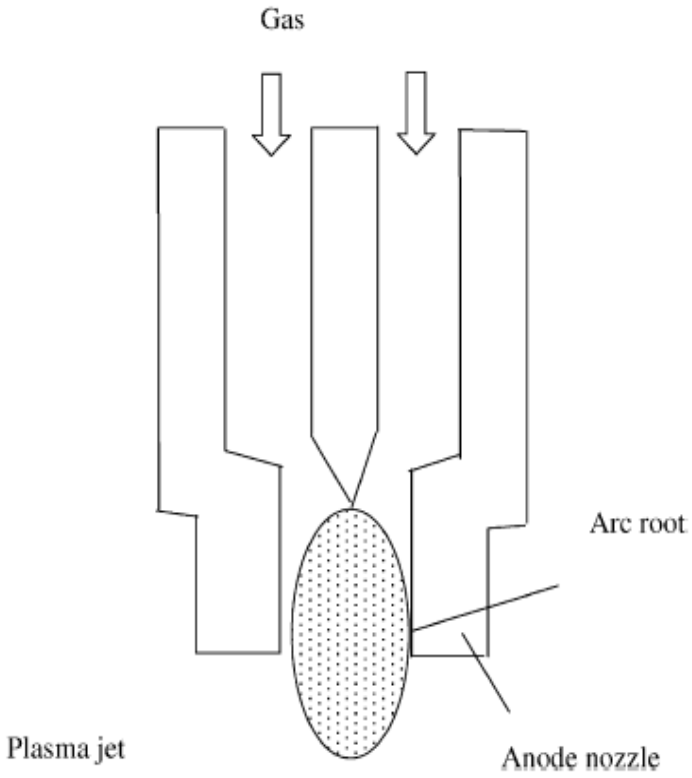


Figure 2.10: Atmospheric Arc Plasma System

2.6.2.2 Vacuum arc plasma source

Vacuum arc plasma source includes two parts such as plasma production unit and macro-particle filter like in Figure 2.11 [38]. If a high voltage is applied, arc discharge between the cathode and anode is started. The arc discharge current is located at the cathode surface which forms non-stationary locations of extremely high current density. High density current occurs because of high power density that result in a phase transformation from solid cathode material to ionized plasma.

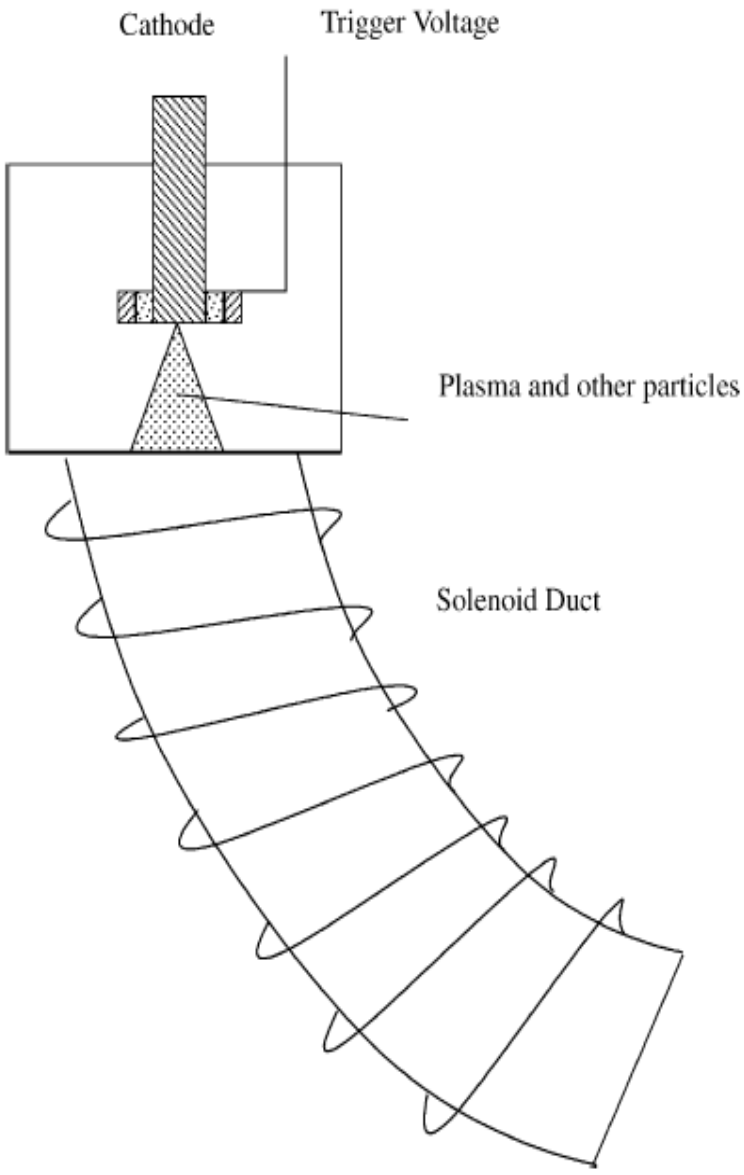


Figure 2.11: Vacuum Arc Plasma System

2.6.2.3 Laser plasma source

In the Laser Plasma Source, the plasma is generated by interactions between high-density laser pulses and the solid as shown Figure 2.12 [38].

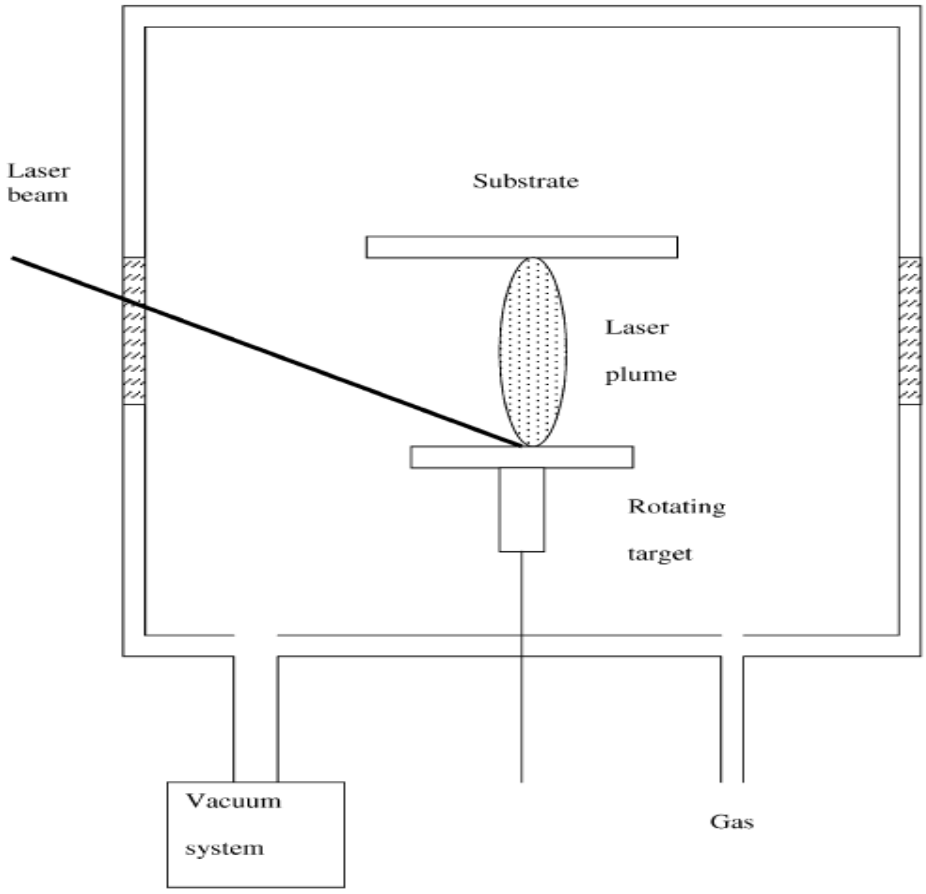


Figure 2.12: Laser Plasma System

2.6.3 RF plasma treatment

2.6.3.1 Surface modification

Surface modification of polymers by plasma treatment is achieved using gases such as H_2, O_2, N_2, argon and helium [38]. When plasma treatment to a surface is applied, enhanced adhesion ability, and surface wettability, and reduced surface friction can be achieved. Furthermore, removal of surface contaminants and weakly bound polymer

layers, etching and substitution of chemical groups on the surface that permit covalent bonding can also achieved by plasma treatment.

Removal of surface contaminants

To clean polymer surfaces from contaminants such as air pollutants, fingerprints, oxide layers, weakly bonded surface layers and other surface additives, low-pressure plasma is applied to the polymer surface. The choice of gas used for contaminant removal depends on the nature of the contaminant and the substrate. For example, It is possible to remove contaminations by oxidation of organic contaminants with oxygen plasma or by reduction of oxides or sulphides by hydrogen plasma [43].

Sputtering and etching

Sputtering is removal of materials from the surface by chemical reactions and one of the simple methods for surface treatment. During the sputtering process, plasma is generated by using inert gases such as argon and neon. The ions inside the plasma are accelerated towards the substrate by the applied electric field. When the ions come into contact with the surface atoms of substrate, they make elastic and inelastic collisions with them, so they transfer an amount of their energies to the surface atoms. If the surface atoms gain the enough energy, they escape from the surface into vacuum chamber. Therefore, contaminations can be removed with enough sputtering time from surface. There are kinds of interaction between plasma and polymer, namely modification and degradation. If the modification dominates, the properties of the polymer will chance due to ion beam interaction, but if the degradation dominates, the polymer surface will etched off.

Substitution of chemical groups

Surface characteristic of polymers can be modified by using RF plasma treatment by substitution chemical groups that present on the surface of polymers. To modify the

surface different process gases can be used. These process gases may incorporate large varieties of chemical groups such as hydroxyl, carbonyl, carboxylic, amino or peroxyl groups. Gases that are used to generate plasma for substitution of chemical groups are reactive while inert gases are in use for plasma-induced grafting.

2.6.3.2 Plasma- Induced grafting

Functional groups and reactive sites are needed for RF plasma-induced grafting. Firstly, free radicals are formed by using inert gases, and then monomers are introduced on to these reactive sites to form grafted polymer. In plasma-induced grafting, material is added into the backbone of polymer instead of functionally modifying it.

2.6.3.3 Plasma polymerization

In plasma polymerization, gases used for plasma are the monomers for polymerization, so gases in the plasma undergo polymerization through a free-radical initiation process. Methane, ethylene, propylene, fluorocarbon monomers and organosilicon compounds can be polymerized by this method. In Table 2.5, some of the gases used for plasma and polymerization is given with their applications.

Table 2.5: Plasma Gases and Applications [38]

Plasma gas	Application
Oxidizing gases (O_2, air, H_2O, N_2O)	Removal of organics by oxidation and to leave oxygen species in the polymer surface
Reducing gases (H_2, mixtures of H_2)	Replacement of F or O in surfaces, removal of oxidation-sensitive materials, conversion of contaminants to low molecular weight species that do not polymerize or re-deposit on adjacent surfaces
Noble gases (Ar, He)	To generate free radicals in surfaces to cause cross-linking or to generate active sites for further reaction
Active gases (NH_3)	To generate amino groups
Fluorinated gases (CF_4, SF_6 and other perfluorinated gases)	To make the surface inert and hydrophobic
Polymerizing gases (monomer gases for direct polymerization, Ar or He pretreated)	Polymerization of layers onto substrates by direct polymerization or by grafting on Ar or He pretreated polymer surface

3. MATERIALS & METHODS

This study includes two parts. In the first part, functional groups (amino groups) were obtained on PTFE surfaces for hirudin immobilization by plasma induced grafting. To do that, optimum conditions for H_2 and Ar plasma treatment were determined by examining the effect of power, time and pressure of the plasma conditions. After the optimum conditions for plasma treatment was determined, acrylamide monomers were used for grafting. Furthermore, to see the effect of plasma treatment on the PTFE surfaces, surface characterization of treated PTFE samples were done by water contact angle measurements, and ATR-FTIR analysis.

In the second part, hirudin was immobilized onto the created amide groups by using EDC/NHS technique, and finally, activity of immobilized hirudin was tested by using thrombogenicity (Kinetic model) assay.

3.1 Materials and Laboratory Equipment

3.1.1 Equipment

The laboratory equipment used during this study is listed in Appendix A.

3.1.2 Chemicals, enzymes, and buffers

The chemicals, enzymes and PTFE are given in Appendix B together with their suppliers. The compositions and preparation of buffers and solutions are given in Appendix C.

3.2 Methods

3.2.1 Sample preparation

Firstly, PTFE samples (thickness: 0.025mm) were cut into square pieces (2.5 x 2.5 cm), and then they were incubated in acetone for 24 hours at room temperature to remove contaminants from the surface.

3.2.2 Optimization of plasma conditions

Effect of power, time and pressure of the hydrogen plasma treatment, and argon plasma treatment on the surface characteristic of PTFE was investigated by water contact angle measurements and ATR-FTIR analysis. RF power was set to 25, 50, 75,100 and 125 W, time were set to 1,2,5,8 and 12 min, and pressure was set to 5,11,13,21 and 45 Pa for H_2 plasma optimization by changing only one parameter while keeping others constant. Optimization of the argon plasma treatment on PTFE surfaces were also done by applying the same conditions used for H_2 plasma. To determine the effect of plasma on the surface characteristic of PTFE, water contact angle measurements and ATR-FTR analysis were done.

3.2.3 Contact angle measurements

To understand the effect of plasma on hydrophilicity of PTFE surfaces, water contact angle measurements were done by using distilled water. The water was dropped onto three different regions of surface and then angles were averaged.

3.2.4 Functional group (amino group) formation

To obtain amino groups on the surface of prepared PTFE samples, samples were firstly exposed to hydrogen plasma treatment. RF glow discharge plasma source was used for plasma generation. Hydrogen plasma treated samples then exposed to air for peroxide formation (2-2.5 h). Acrylamide solution (25% w/v; in ethanol/acetone (50 % v/v)) was then applied to the surface. Then, PTFE samples were dried at 37˚C for 30 minutes. Dried samples were subjected to argon plasma treatment for monomer grafting to form functional groups. Finally, samples were washed in distilled water at 37˚C on a shaker for 17-18 hours to remove unbound chemicals.

3.2.5 ATR-FTIR analysis

To check if there exists any new functional group on the surface of treated PTFE to be used hirudin immobilization, ATR-FTIR analysis were done. Groups on the surface of pristine PTFE and treated PTFE were compared.

3.2.6 Hirudin immobilization

To immobilize hirudin on amino groups on the PTFE samples, EDC-NHS activation was used. This is a well known method for the attachment of carboxyl groups to amino groups. To do this, 5µl of NHS (0.19 mM) solution, 20.5 µl of EDC (0.38 mM) solution are dissolved in 5 ml of MES buffer. Hirudin (626 ATU) was added into the mixture. Prepared enzyme solution was then put onto the treated PTFE surface and incubated overnight at 4˚C by gently mixing on orbital shaker (50 rpm) for immobilization. Finally, hirudin immobilized PTFE surfaces washed with PBS buffer (0.1 M, pH: 7.4) for 5 hours at 4˚C on orbital shaker to remove excess and unbound enzymes.

3.2.7. Effect of enzyme concentration

To determine the enzyme concentration that is enough to prevent clot formation in a particular area, we tried different enzyme concentration (156, 313, 625 ATUs). Thrombogenicity test procedure was applied to these measurements and effects of different enzyme concentrations on clot formation were analyzed.

3.2.8 Thrombogenicity test

To measure the activity of immobilized hirudin, a thrombogenicity test that is also called as Kinetic Model was used [44]. Acid Citrate Dextrose (Appendix C, ACD, 170 µl) solution added into 1.6 ml of fresh human blood. Three PTFE sample prepared for the thrombogenicity test. The first one was pristine PTFE (Control 1), the second one was plasma treated PTFE (Control 2), and third one was plasma treated and enzyme immobilized PTFE. Firstly, these samples were weighted and put into NaCl solution for 30 minutes at room temperature. Then 200 µl of blood-ACD solution was put onto the PTFE samples. To start clot formation, 20 µl of $CaCl_2$ (0.1 M) solution was added to each sample and mixed.

After 1 hour of incubation, 5 ml of distilled water put to end the clot formation. The clot formation was fixed with formaldehyde solution (5 ml, 37 %) with a 5 min. incubation. Finally, samples were washed with distilled water, dried between tissue paper and weighted.

4. RESULTS AND DISCUSSION

4.1 Plasma Optimization and Contact Angle Measurements

Wettability and adhesion ability of PTFE surfaces can be increased by the plasma treatment. This is important because adsorption of acrylamide solution on the surface of PTFE is essential for functional group formation. Therefore, hydrophilicity of surface, solubility of monomer in a solvent, and solvent adsorption by PTFE surface are all important and should be optimized maximum for enhanced functional group formation to immobilize enzymes, proteins etc.

In our study, we examined the power, time and pressure effect on the plasma, and how hydrophilicity of PTFE surfaces is affected by these conditions by means of water contact angle measurements. Pristine PTFE has a hydrophobic character which is proved with our measurement as given Table 4.1.

Table 4.1: Contact Angle of Pristine PTFE

	Contact Angle(˚)		
	Left	**Right**	**Average**
1. Measurement	136,87	135,15	136,01
2. Measurement	126,03	124,58	125,30
3. Measurement	118,72	120,53	119,62
		Average	126,98

The changes in the hydrophilicity of PTFE surfaces depending on the change in the power, time, and pressure of H_2 plasma treatment are given Table 4.2, Table 4.3, and Table 4.4, respectively.

Table 4.2: Change in Contact Angle Depending on Power (H_2 Plasma)

Pressure=13 Pa, t=1 min	Contact Angle(˚)		
Power	**Left**	**Right**	**Average**
25 watt	80,91	79,69	80,30
50 watt	82,61	82,90	82,76
75 watt	71,61	72,46	72,04
100 watt	59,69	60,91	60,30
125 watt	58,75	58,92	58,83

Table 4.3: Change in Contact Angle Depending on Time (H_2 Plasma)

Pressure=13 Pa, Power= 125 Watt	Contact Angle(˚)		
Time	**Left**	**Right**	**Average**
1 min	82,61	82,90	82,76
2 min	63,19	61,88	62,54
3 min	57,27	55,65	56,46
5 min	63,47	62,89	63,18
12 min	63,02	62,21	62,62

Table 4.4: Change in Contact Angle Depending on Pressure (H_2 Plasma)

Power=125 W, t=2 min	Contact Angle(˚)		
Pressure	**Left**	**Right**	**Average**
5.1 Pa	63,47	64,55	64,01
11 Pa	63,19	61,88	62,54
13 Pa	60,27	58,12	59,20
21 Pa	61,23	62,00	61,62
45 Pa	89,74	87,12	88,43

These results clearly show us that there is a serious change in the water contact angle of H_2 plasma treated PTFE surfaces. Power, time and pressure are important parameters of plasma treatment and play an important role on the hydrophilicity of PTFE. In H_2 plasma treatment, hydrophilicity of surface increases depending on the increase in power (Figure 4.1). At 125 W, contact angle decreased from ca 127 ˚ to ca 59˚ compared to pristine PTFE.

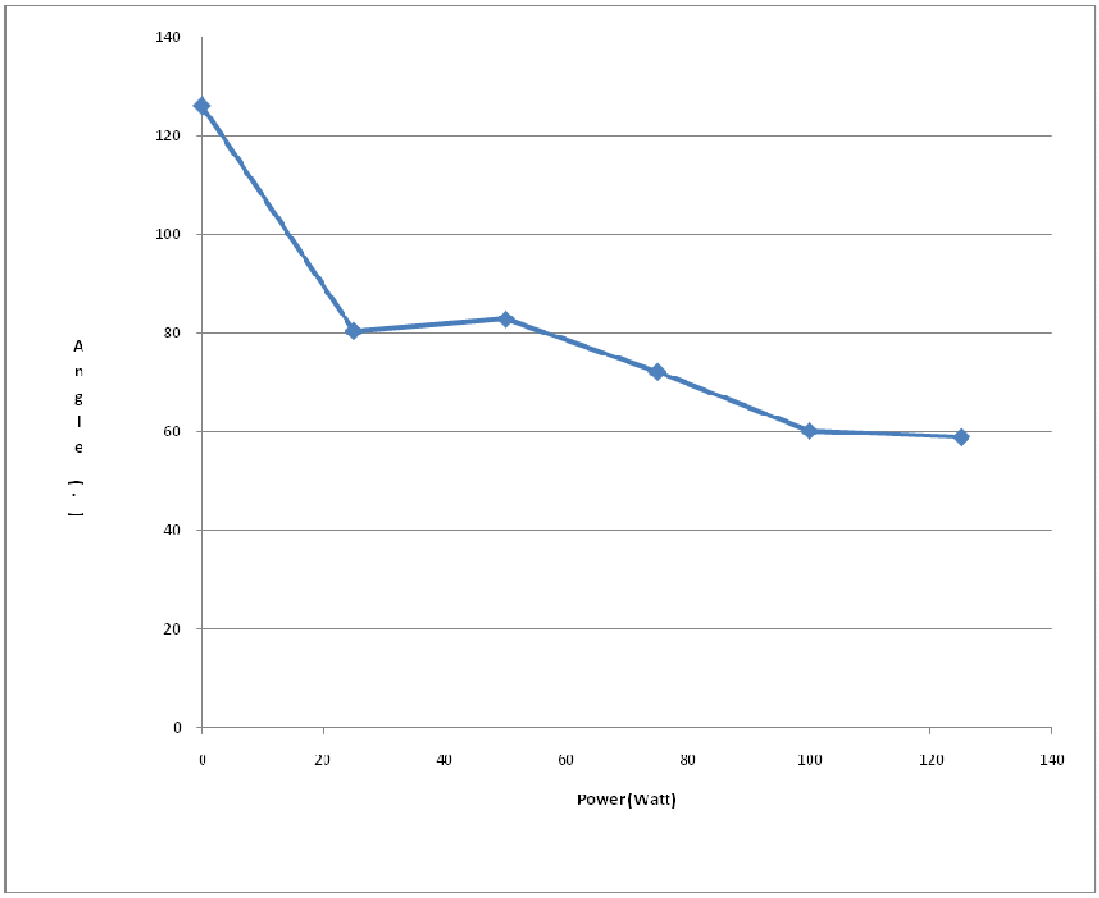


Figure 4.1: Power- Contact Angle Relation in H_2 Plasma Treatment

Time is another parameter that affects the plasma treatment results, so the hydrophilicity of the surface. After, optimum condition for power was set to 125 W in H_2 plasma treatment, the effect of time was investigated at a time interval of 1-12 min. It was seen that, 2-3 min of plasma treatment is enough and, there is no notable changes afterwards (Figure 4.2) .At t=2-3 min. contact angle was found to be between 56-62˚.

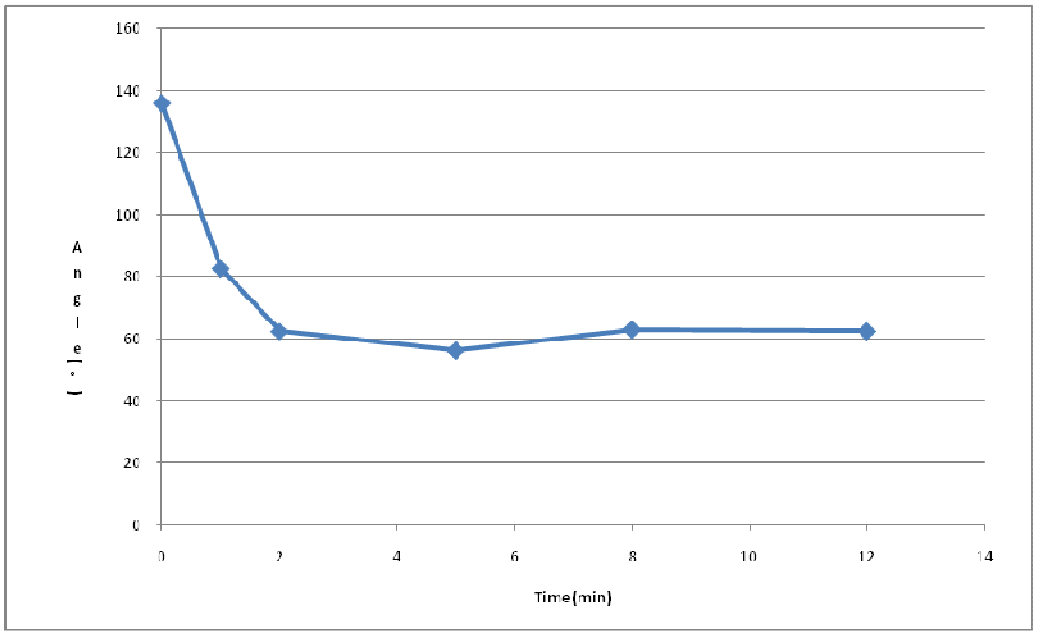


Figure 4.2: Time - Contact Angle Relation in H_2 Plasma Treatment

When the effect of Plasma pressure on the hydrophilicity was examined, it was seen that ca 13 Pa is the most useful range for plasma treatment. Hydrophilicity increases until 13 Pa then it starts to decrease again (Figure 4.3).

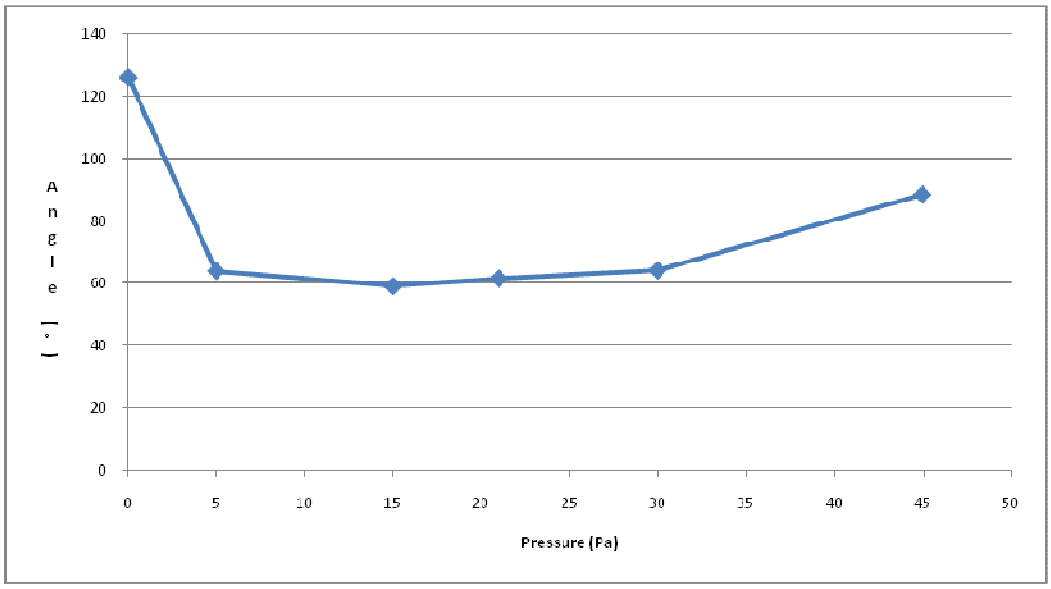


Figure 4.3: Pressure - Contact Angle Relation in H_2 Plasma Treatment

The most hydrophilic surface with H_2 plasma was obtained at 125 W, 2 min, and 13 Pa (=6.1 sccm). All PTFE samples were first treated in these optimum conditions for hirudin immobilization.

Then conditions for Ar plasma treatment, which was used for graft polymerization, were optimized. Power, time and pressure effects were given Table 4.5, Table 4.6, and Table 4.7, respectively.

Table 4.5: Change in Contact Angle Depending on Power (Ar Plasma)

Pressure=26 Pa, t=5 min	Contact Angle(˚)		
Power	Left	Right	Average
25 watt	73,98	73,55	73,77
50 watt	64,45	63,09	63,77
75 watt	83,24	82,11	82,68
100 watt	93,27	93,48	93,38
125 watt	110,77	110,37	110,57

Table 4.6: Change in Contact Angle Depending on Time (Ar Plasma)

Pressure=26 Pa, Power=50 watt	Contact Angle(˚)		
Time	Left	Right	Average
1 min	71,12	72,87	72,00
2 min	68,3	69,02	68,66
5 min	64,45	63,09	63,77
8 min	64,56	62,23	63,40
12 min	65,19	66,31	65,75

Table 4.7: Change in Contact Angle Depending on Pressure (Ar Plasma)

Power=50 W, t=2 min		Contact Angle(˚)	
Pressure	Left	Right	Average
10 pascal	52,96	53,51	53,24
26 pascal	69,38	69,80	69,59
30 pascal	71,48	70,87	71,17

Optimum plasma conditions of Ar plasma was found to be similar to that of H_2 plasma except for power. Effect of time (Figure 4.4), and pressure effect (Figure 4.5) is almost the same. An increase in pressure, causes a decrease in mean free path, velocity, and energy of atoms and electrons, so hydrophilicity decreases in high pressures. However any increase in power above 50 W (Figure 4.6) results in a decrease in hydrophilicity in Argon plasma treatment. This is probably due to the inert nature of Argon. it increases surface roughness with increasing power. On the other hand, Hydrogen is a reactive gas and modify surface with substitution, so does not affect roughness as in the case of Argon plasma.

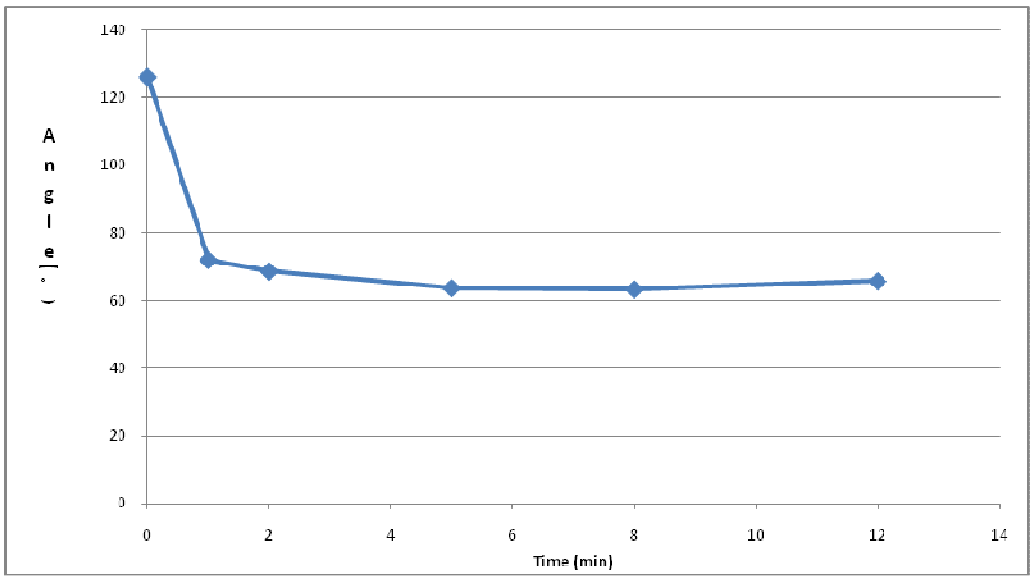


Figure 4.4: Time - Contact Angle Relation in Ar Plasma Treatment

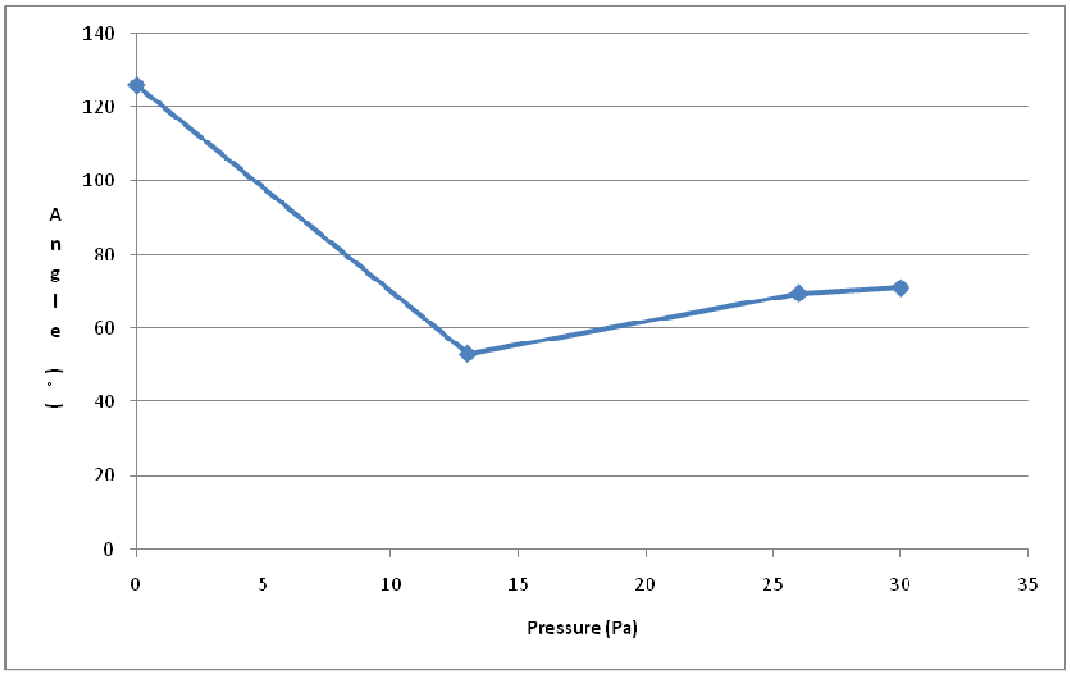


Figure 4.5: Pressure - Contact Angle Relation in Ar Plasma Treatment

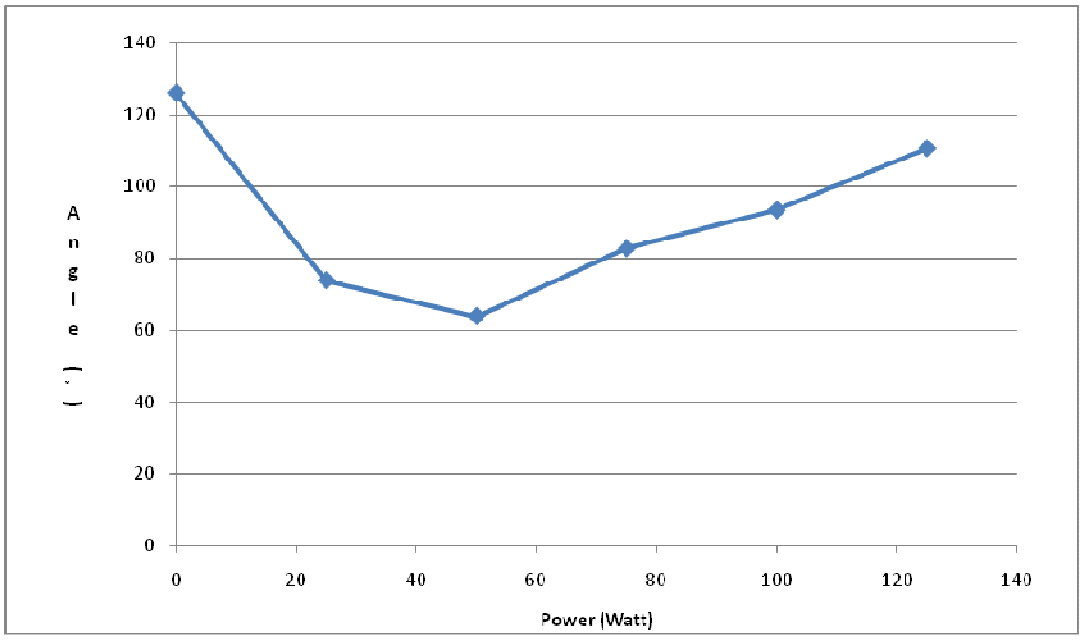


Figure 4.6: Power - Contact Angle Relation in Ar Plasma Treatment

In our study optimum conditions for Argon plasma induced grafting was determined as accepted as 50 W, 1 min, and 13 Pa. These results are consistent with the literature [45].

4.2 Functional Group Formation and ATR-FTIR Analysis

Since H_2 is a reactive gas, it results in substitution of florine atoms by hydrogen. Then, treated surface was exposed to air and peroxides (R-O-O-R'), and hydroperoxides (R-O-O-H) were formed mainly by the reaction of water in the air with the broken bonds (free radicals) on the surface after plasma treatment (Figure 4.7).

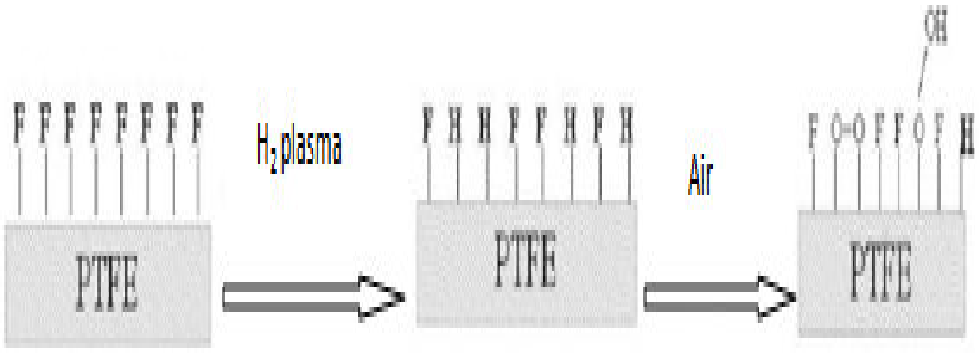


Figure 4.7: Peroxide (R-O-O-R') and Hydroperoxide Formation (R-O-O-H)

To introduce amino groups to the surface, acrylamide solution (25% w/v; in ethanol/acetone (50 % v/v)) was put on the surface and adsorbed by PTFE surfaces. Ar plasma treatment was then applied in the optimum conditions set in Section 4.2 and unbound acrylamide was washed away. Presence of new functional groups at the surface of PTFE was analyzed by ATR-FTIR spectra (Figure 4.8).

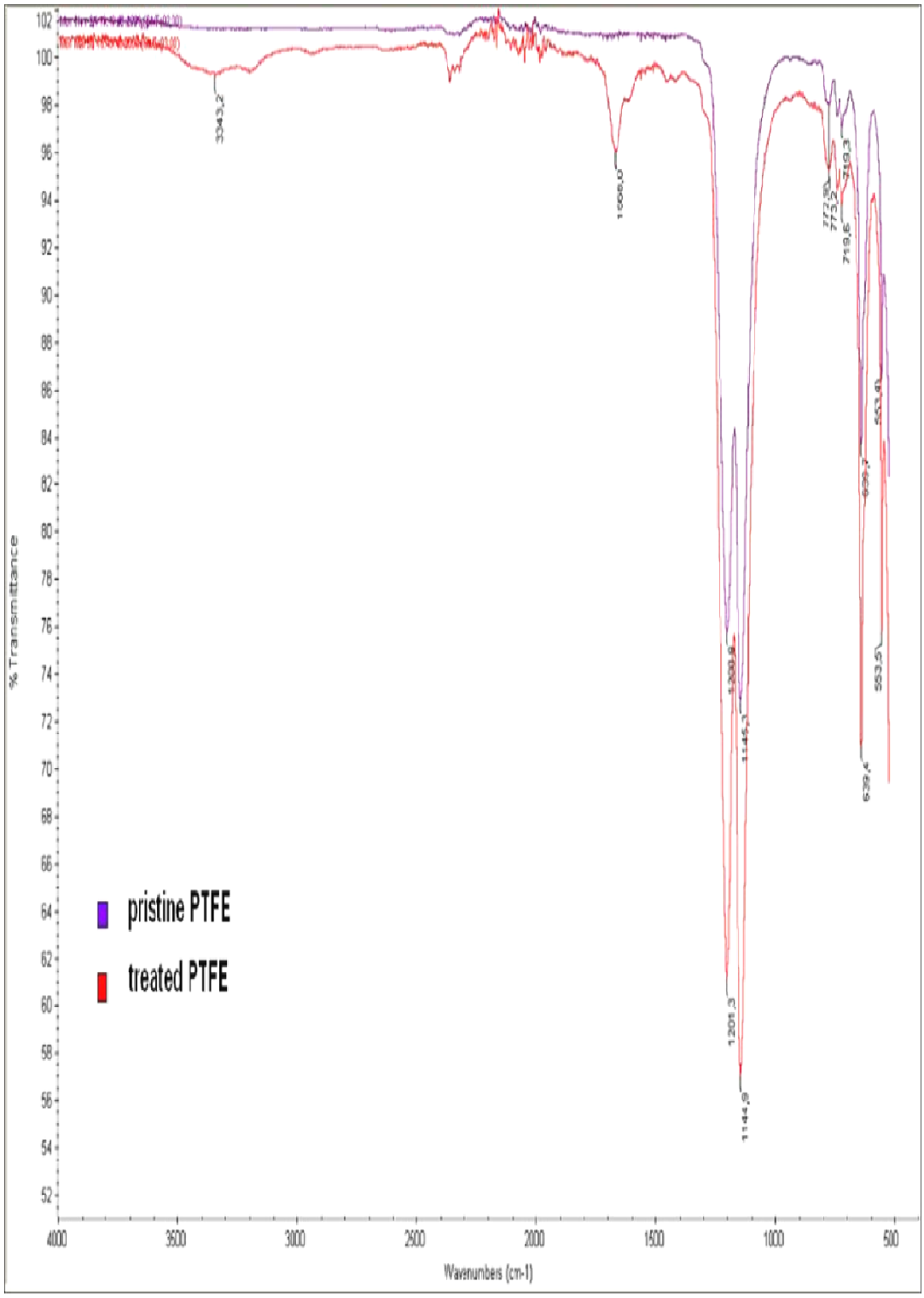


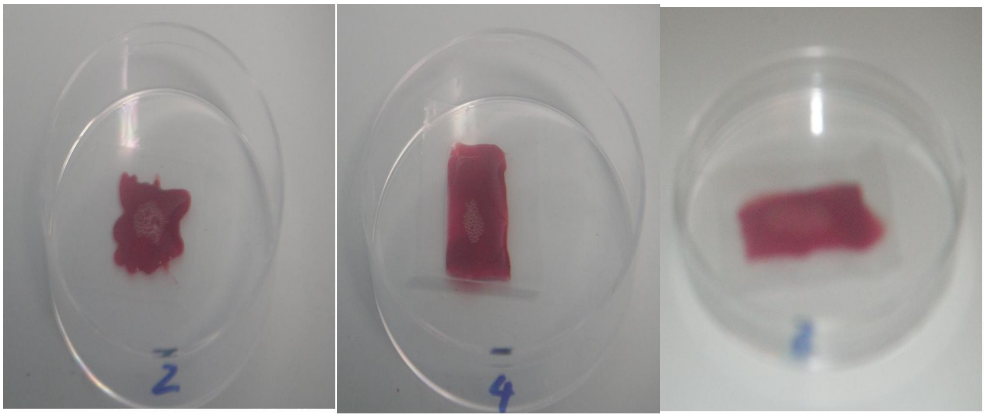
Figure 4.8: ATR-FTIR Analysis

As it can be observed from the FTIR results there is a new peak at ca 1665 cm^{-1} which corresponds to amide groups ($O=C-NH_2$). Therefore, it can be said that functional group formation for enzyme immobilization was successfully achieved.

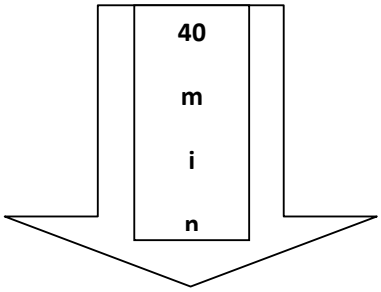
There are important points to obtain functional amino groups on the surface. Selection of solvent is one of them; the solvent must be able to solve monomers(acrylamide in our case) with high efficiency, and at the same time, it should be adsorbed by polymer(PTFE in our case) sufficiently. When water, which is one of the greatest solvent of acrylamide, was used as solvent, acrylamide grafting was not successful, because adsorption of water by PTFE is not good as acetone and ethanol as mentioned in [46]. When aqueous acrylamide solution was put on PTFE surface, they tend to accumulate in an area or form separate droplets but did not spread evenly on the surface. This affected the yield of polymer grafting. When Ethanol/Acetone mixture started to be used, it was observed that the monomer solution could spread evenly on PTFE surface and gave better results. Another thing to be considered is the air exposure step after the H_2 plasma treatment. If acrylamide is directly put on the surface without air exposure, acrylamide grafting can not be done successfully. Hydroperoxide formation due to air exposure is crucial before proceeding to the next step. This finding is consistent with the literature [47].

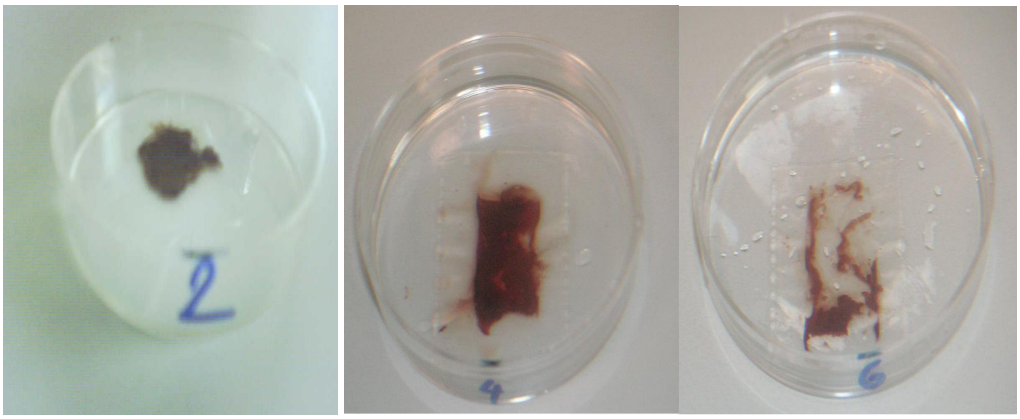
4.3 Thrombogenicity Test

In thrombogenicity test, two controls were selected, pristine PTFE (Control 1) and plasma treated PTFE (without any protein, Control 2) and the results were presented in Figure 4.9 and Figure 4.10.In the case of pristine PTFE, there was a problem originated from the high hydrophobicity of PTFE. Blood did not spread over the surface, so clot formation occurs only where it is put (Figure 4.9a). There is clot formation in large area of plasma treated PTFE (Control 2) compared with the pristine PTFE. As plasma treated PTFE is hydrophilic, blood was adsorbed by PTFE and spread to a larger area. We observed that there is a big decrease in the clot formation on the hirudin immobilized PTFE surfaces.



a **Control 1** **Control 2** **hirudin immobilized PTFE**





b **Control 1** **Control 2** **hirudin immobilized PTFE**

Figure 4.9: Thrombogenicity test 1(a) before coagulation starts (b) after the coagulation

Different enzyme concentrations resulted in different amounts of clot formation. In Table 4.8, weight difference on PTFE samples depending on different enzyme concentrations were given.

Table 4.8: Change in Clot Formation Depending on Enzyme Amount

15 µl Enzyme Solution(156 ATUs)			
(60 min)	**initial weight(mg)**	**last weight (mg)**	**Difference (mg)**
Plasma Treated PTFE	22,50	93,30	70,80
Enzyme Immobilized PTFE	22,80	106,80	84,00
30 µl Enzyme Solution(313 ATUs)			
(60 min)	**initial weight(mg)**	**last weight (mg)**	**Difference (mg)**
Plasma Treated PTFE	23,50	106,90	83,40
Enzyme Immobilized PTFE	26,60	68,30	41,70
60 µl Enzyme Solution(626 ATUs)			
(60 min)	**initial weight(mg)**	**last weight (mg)**	**Difference (mg)**
Plasma Treated PTFE	22,80	107,30	84,50
Enzyme Immobilized PTFE	25,50	51,50	26,00

These results showed that 60 µl enzyme solution (626 ATUs) is more efficient to prevent clot formation than 30 µl (313 ATUs) and 15 µl (156 ATUs) of enzyme solution did not show any antithrombogenicity effect when compared with the control on a 2.5 cm^2 surface area. Depending on the increase in enzyme, clot formation decreases like in Figure 4.10.

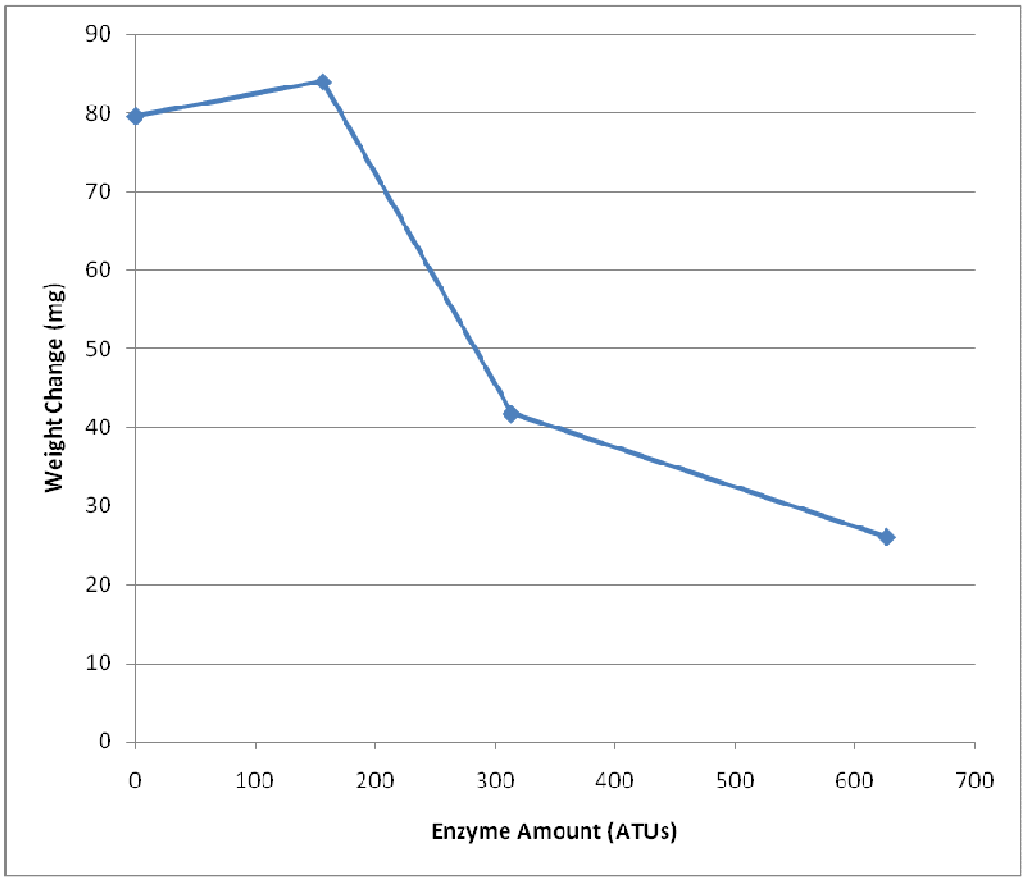
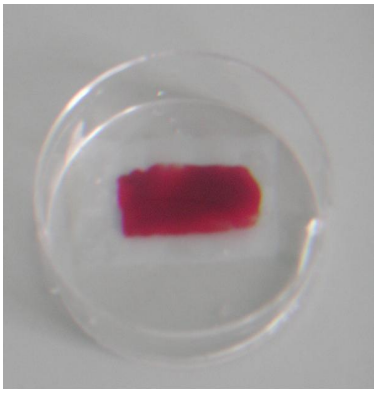
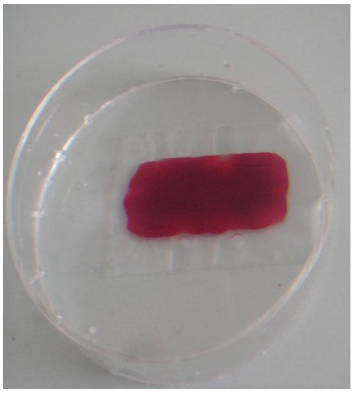


Figure 4.10: Weight Change versus Enzyme Amount

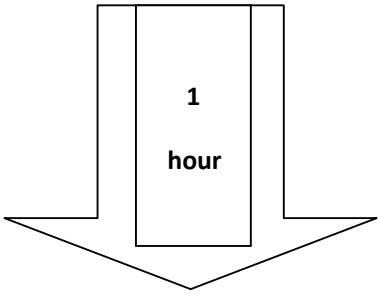
While there is a big decrease in weight difference, which occurs because of clot formation, with the increased enzyme amount from 125 to 626 ATUs, decrease in weight difference slows down after ca 400 ATUs which mean we were almost reached to suitable enzyme amount for a 2.5 cm^2 surface area.

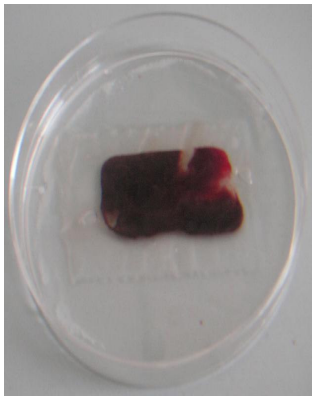
After determination of suitable enzyme amount, we repeated our experiments and checked the reproducibility of experiments. In the most of these experiments, we observed that there is a big decrease in the weight of PTFE samples which are enzyme immobilized compared with the pristine or just plasma treated PTFE samples. Even in some cases, there is no clot formation like in Figure 4.11.





hirudin immobilized PTFE **Control 2**





hirudin immobilized PTFE **Control 2**

Figure 4.11: Thrombogenicity test 2

Antithrombogenic activity of immobilized hirudin can be clearly seen in Figures 4.9 and 4.11. The result was obtained in Figure 4.9 were taken before optimization of the procedure, but even these results show the difference between hirudin immobilized PTFE and controls. There are many reasons why some clot formation occurred in previous tests (Figure 4.9) compared with the later results (Figure 4.11):

- It is sometimes difficult to set the plasma conditions to optimum values and this can affect the properties and monomer grafting. Therefore, the surface should be checked by FTIR and water contact angle tests before protein immobilization study.

- At the beginning of the experiments distilled water with pH 5.5 was used for enzyme immobilization. EDC-NHS immobilization is best done at pH 5.5 and adversely affected by buffers. Then MES buffer which is suitable for this reaction to take place was obtained and started to be used in the experiments. It is possible that enzyme immobilization yield is increased by MES buffer usage.

- As It can be seen from Figure 4.9, clot formation generally occurs at the interface of hydrophobic and hydrophilic regions. Coagulation factors may assemble easier in these region and the clot formation is more likely.

5.CONCLUSION

PTFE is an attractive material for biomedical applications, and especially for vascular grafts, catheters etc. This is due to its high inertness, good mechanical properties and high oxygen permeability which are crucial for this type of applications. But the surface properties of PTFE is not very suitable to be used as it is. In catheters, for example, when pristine PTFE is used, an antithrombogenic agent should be given to the patient to prevent surface induced blood coagulation. In vascular grafts, pristine PTFE may lead to surface induced thrombosis and failure of the graft. The aim of this thesis is to increase the blood compatibility of PTFE to improve its performance as a blood-contacting biomaterial. For this, first the acrylamide grafting via plasma treatment was optimized, then hirudin, a potent antithrombogenci protein, was immobilized on the surface.

Firstly, the effect of power, time and pressure of plasma treatment on the surface modification, thus hydrophilicity of PTFE was determined. Parameters for H_2 plasma and Argon plasma gave similar results except for power. When power value exceed 50 W in Argon plasma, a decrease in hydrophilicity was observed as opposed to H_2 plasma treatment. A serious decrease in water contact angle of PTFE surfaces can be obtained by optimizing the hydrogen plasma treatment conditions. Water contact angle can be further down by monomer grafting. Solvent is found to be an important parameter for monomer grafting because a good solvent may solve a large amount of acrylamide, but if it is not adsorbed by PTFE efficiently, monomer grafting severely reduced. Hirudin, a good antithrombogenic agent, was successfully immobilized on PTFE surface by EDC/NHS activation. To measure the activity of immobilized hirudin, thrombogenicity (Kinetic Model) test was applied and it was seen that immobilized hirudin retain its activity after immobilization.

As a result, we increased blood compatibility of PTFE surfaces by plasma-induced monomer grafting and hirudin immobilization. This material should now be tested in

animal models to have a better idea about its potential in medical applications such as vascular graft, catheters etc.

REFERENCES

[1] Ratner D. Buddy., Bryant Stephanie J., 2004. BIOMATERIALS: Where We Have Been and Where We are Going, *Annu. Rev. Biomed. Eng.* **6**:41–75

[**2**] **S. Ramakrishna., J. Mayer., E. Wintermantel., Kam W. Leong.,** 2001. Biomedical Applications Of Poylmer-composite materials*, Composite Science and Technology*, **61** 1189-1224

[3] Stamatialis Dimitrios F. et al., 2008.Medical applications of membranes.Drug delivery,artificial organs and tissue engineering*, Journal of Membrane Science.* **308** 1–34

[4] Speidel MO, Uggowitzer PJ., 1998.Biocompatible nickel-free stainless stell to avoid nickel allergy, Materials in Medicine*,*Switzerland, *vdfHochschulverlag AG an der ETH zurich.* p. 191-208

[5] Chanda M., Salil R., 2006**.** Plastics Technology Handbook,*Taylor & Francis Group*

[6] T.Avramoglou., J.Jozefonvicz., M.Jozefowicz. Polymeric Biomaterials*,*611 – 646

[7] Dumitriu S., 2001.Polymeric Materials 2nd Edition, *Marcel Dekker.*

[8] Aebischer P. et al., 1992**.** Materials Science and Technology, Vol. 14, *Medical and Dental Materials*, Germany

[9] Halpren B. D., Tong Y. C., 1989**.** Polymers: Biomaterials and Medical Applications, *Wiley-Interscience*: New York

[10] Hasircl N., 1991**.** High-Performance Biomaterials; Szycher, M. Ed.; *Technomic: Lancaster,* PA, 71–90.

[11] Russell F. B. et al., 1980**.** Organs, *Trans. Am. Soc. Artif. Intern. 26*, 66

[**12] Lee H.; Neville K.,** 1971**.** Handbook of Biomedical Plastics, *Pasadena Technology: Pasadena,* CA

[13] Lyman, D. J. et al., 1981.Biomedical and Dental Applications, Vol. 14, *Polymer Science and Technology*; Plenum: New York

[14] Devanathan, T. et al., 1980. Med. Dev. Artif. Organs, *Biomat. 8*, 369

[15] Plunkett, R. J. (to Kinetic Chemicals Inc.) U.S. Patent 2 230 654, 1941

[16] Bunn, C. W., 1955. J. Polym. Sci., *16*, 332.

[17]Khanna, Y. P. et al., 1990.Macromolecules, *23*, 2488

[18] Sperati, C. A., 1989. Polymerization and Depolymerization, Polymer Handbook 3rd ed, V1, *John Wiley & Sons*, New York,

[19] Wilimas D. F., de Putter C.,1988. Advances in biomaterials, *Elsevier*, Amsterdam

[20] Williams, D. F., 1981.Fundamental Aspects of Biocompatibility, *CRC: Boca Roton,* FL.

[21] Von Recum A.F.,1986.Handbook of Biomaterials Evaluation: Scientific, Technical, and Clinical Testing of Implant Materials; Macmillan:New York

[22] Y. Uyama, K. Kato, Y. Ikada., 1998.Surface modification of polymers by grafting. *Adv. Polym. Sci. 137*:1

[23] A. Kishida, K. Mishima, E. Corretge, H. Konishi, Y.Ikada., 1992. Interactions of poly(ethylene glycol)-grafted cellulose membranes with proteins and platelets. *Biomaterials,13*:113

[24] N. P. Desai., J. A. Hubbell., 1991. Biological responses to polyethylene oxide modified polyethylene terephthalate surfaces.*J. Biomed. Mater. Res. 25*:829.

[25] D. K. Han., K. D. Park., G. H. Ryu., U. Y. Kim., B. G. Min., Y. H. Kim., 1996. Plasma protein adsorption to sulfonated poly(ethylene oxide)-grafted polyurethane surface. *J. Biomed.Mater. Res. 30*:23

[26] A.P. Zhu, M. Zhang, J. Wu, J. Shen, 2002.Covalent immobilization of chitosan/heparin complex with a photosensitive hetero bifunctional crosslinking reagent on PLA surface, *Biomaterials* 23 (23) 4657–4665

[27] M. Ceuk, M. Sacak., 1996.Grafting of acrylamide–methacrylic acid mixture onto poly(ethylene terephthalate) fibers by azobisisobutyronitrile. *J. Appl. Polym. Sci. 59*:609

[28] F. C. Loh, K. L. Tan, E. T. Kang, K. G. Neoh, M. Y. Pun., 1995. Near-UV radiation induced surface graft copolymerization of some O3-pretreated conventional polymer films, *Eur.Polym. J. 31*:481

[29] J. Buchenska., 1995.Modification of polyamide fibers (PA6) by grafting polyacrylamide (PAM). *J. Appl. Polym. Sci. 58*:1901.

[30] Y.-E. Fang, X. P. Lu, S. Z. Wang, X. Zhao, F. Fang.,1996. Study of radiation-induced graft copolymerization of vinyl acetate onto ethylene-co-propylene rubber. *J. Appl. Polym. Sci. 62*:2209.

[31] S. Tsuneda, K. Saito, S. Furusaki, T. Sugo., 1995. Highthroughput processing of proteins using a porous and tentacle anion-exchange membrane. *J. Chromatogr A. 689*:211

[32] H. Y. Chuang, J. D. Andrade., 1985.Immunochemical detection by specific antibody to thrombin of prothrombin conformational changes upon adsorption to artificial surfaces. *J. Biomed. Mater. Res. 19*:813

[33] S. D. Lee, G. H. Hsiue, C. Y. Kao, P. C. Chang., 1996. Artificial cornea: surface modification of silicone rubber membrane by graft polymerization of pHEMA via glow discharge, *Biomaterials 17*:587

[34] A. S. Hoffman., 1984. Ionizing radiation and gas plasma (or glow) discharge treatments for preparation of novel polymeric biomaterials, *Adv. Polym. Sci. 57*:141

[35] A. Kishida, H. Iwata, Y. Tamada, Y. Ikada., 1991. Cell behavior on polymer surfaces grafted with non-ionic and ionic monomers, *Biomaterials 12*:786

[36] J. H. Lee, H. W. Jung, I. K. Kang, H. B. Lee., 1994. Cell behavior on polymer surfaces with different functional groups, *Biomaterials 15*:705

[37] E. T. Kang, K. L. Tan, K. Kato, Y. Uyama, Y. Ikada., 1996. Surface modification and functionalization of polytetrafluoroethylene films. *Macromolecules 29*:6872

[38] N. Gomathi, A. Sureshkumar, Sudarsan Neogi., 2008.RF plasma-treated polymers for biomedical applications*, Current Science, Vol. 94, No. 11*

[39] P.K. Chu[a], J.Y. Chen, L.P. Wang, N. Huang.,2002.Plasma-surface modification of biomaterials, *Elsevier* R 36 143–206

[40] Y.P. Raizer, M.N. Shneider, N.A. Yatsenko., 1995. Radio-Frequency Capacitive Discharges, *CRC Press*, Boca Raton, FL.

[41] S.-G. Park, B.-H. Oh, M. Sohn, J. Kim., 2000., Surf. Coatings Technol. **133/134** 509

[42] S. Miyake, Y. Setsuhara, Y. Sakawa, T. Shoji., 2000. Surf. Coatings Technol. **131/ 171**

[43] Lee, C., Kim, H. W. and Kim, S., 2007.Organic contaminants removal by oxygen ECR plasma. *Appl. Surf. Sci.*, **253**, 3658–3663

[44] Imai Y, Nose Y., 1972. A new method for evaluation of antithromogenicity of materials. J Biomed Mater Res, 6:165–72

[45] Chen-Yuan Tu, Chiu-Ping Chen, Yi-Chieh Wang, Chi-Lan Li, Hui-An Tsai, Kueir-Rarn Lee,Juin-Yih Lai.,2004.Acrylamide plasma-induced polymerization onto expanded poly(tetrafluoroethylene) membrane for aqueous alcohol mixture vapor permeation separation. *European Polymer Journal*, *Volume 40, Issue 7,* Pages 1541-1549

[46] Chi-Lan Li., Chen-Yuan Tu., N. Inagaki., Kueir-Rarn Lee.,Juin-Yih Lai., 2006. Plasma-Induced Solid-State Polymerization Modified Poly(tetrafluoroethylene) Membrane for Pervaporation Separation of Aqueous Alcohol Mixtures. *Journal of Applied Polymer Science*

[47] CY Tu., YL Liu., KR Lee., JY Lai., 2005. Surface grafting polymerization and modification on poly(tetrafluoroethylene) films by means of ozone treatment. *Elsevier ,Volume 46, Issue 18*, Pages 6976-698

APPENDICES

Appendix A

Laboratory Equipment

Pipettes	Eppendorf 10 µL 100 µl, 1000 µl, 2500 µl
pH meter	Mettler Toledo MP220
Pure water systems	USF Elga UHQ-PS-MK3, Elga Labwater
Shaker (37˚C)	Certomat S II
Orbital Shaker (4˚ C)	Heidolph Duomax 1030
RF Power Generator	PFG COOE/Hüttinger
Vacuum Equipment	TSY-HF450/PECVD350/MMPS20/C
	Genertec International Corporation
Contact Angel	Cam200 KSV
ATR-FTIR	NICOLET 6700
Balances	Precisa XB220A
	Precisa BJ610C

Appendix B

Chemicals

Ethanol	Sigma-Aldrich
Acetone	Sigma-Aldrich
Acrylamide	Sigma-Aldrich
N-Hydroxysuccinimide	Sigma-Aldrich
N-(3-Dimethylaminogrouply)-N-ethly-carbodiimide	Sigma-Aldrich
NaOH	Riedel-de Haën
$NaH_2PO_4.2H_2O$	Sigma-Aldrich
$Na_2HPO_4.2H_2O$	J.T. Baker
$C_6H_8O_7.H_2O$	Sigma-Aldrich
$Na_3C_6H_5O_7 . 2H_2O$	LaCheMa
$C_6H_{12}O_6$	Sigma-Aldrich
NaCl	Carlo Erba Reagent
$CaCl_2.2H_2O$	MERCK
CH_2O	J.T.Baker
$C_6H_{13}NO_4S.H_2O$	Sigma-Aldrich

ENZYMES AND PTFE

rHirudin	Sigma-Aldrich
Polytetrafluoroethylene (PTFE)	Sigma-Aldrich

Appendix C

Solutions and Buffers

Acrylamide Solution (% 25 w/v, %50 v/v Ethanol/Acetone)(30 ml)

- 15 ml Ethanol
- 15 ml Acetone
- 7.5 gr Acrylamide

N-Hydroxysuccinimide NHS Solution (5 ml)

- 5 ml Distill Water
- 5.4 mgr NHS

2-(*N*-morpholino)ethanesulfonic acid (MES) Buffer (0.05 M) (pH:5.5) (400 ml)

- 3.90 gr MES Buffer
- 400 ml Distill Water
- 0.1 M NaOH Solution

- Add NaOH solution until pH reaches to 5.5

Phosphate Buffer Saline (PBS) Buffer (1 M) (pH: 7.39) (100 ml)

- 0.416 gr $NaH_2PO_4.2H_2O$
- 1.25 gr $Na_2HPO_4.2H_2O$
- 0.85 gr NaCl
- 100 ml Distill Water

- To obtain 10 ml, 0.1 M PBS, add 9 ml of distill water to 1 ml of 1 M PBS buffer.

rHirudin Stock Solution (60 µl)(626 ATU)

- Add 1ml of 0.1 M PBS buffer into the rHirudin bottle (104378 ATU)
- Take 350 µl of rhirudin solution (36571 ATU) from bottle
- Divide 350 µl of rhirudin solution (36571 ATU) into eppendorfs? that contain 60 µl of rhirudin solution (626 ATU)
- Save both the main stock of 650 µl and 60 µl rhirudin solutions at (-20˚C)

Acid Citrate Dextrose (ACD) Solution (75 ml)

- 0.59 gr $C_6H_8O_7 .H_2O$
- 1.65 gr $Na_3C_6H_5O_7 . 2H_2O$
- 1.84 gr $C_6H_{12}O_6$
- 75 ml Distill Water

Sodium Chloride (NaCl) Solution (30 ml) (% 0.5)

- 0.15 gr NaCl
- 30 ml Distill Water

Calcium Chloride ($CaCl_2$) Solution (0.1 M) (30 ml)

- 0.44 gr $CaCl_2.2H_2O$
- 30 ml Distill Water

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