CHAPTER 1 INTRODUCTION

1.1 Silk Fibroin

Silk fibroin is a natural protein that is derived from silkworms, spiders and other insects. It has been increasingly studied for new biomedical applications due to the high biocompatibility, biodegradability, swelling and remarkable mechanical properties. There are various formats for the silk fibroin such as biofilms, scaffolds, fibers, meshes, membranes, and sponges. These different forms have been proven to support the cell proliferation, adhesion, migration and promote tissue repair *in vivo.* The silk fibroin biofilms are one of the most important forms of the silk fibroin. The usage of silk fibroin biofilms has expanded the use of silk-based biomaterials as promising biofilms for tissue engineering applications ranging from an artificial skin, ligament, connective tissues like skin cell culturing, and also in drug delivery system. The silk fibroin biofilms can exhibit perfect environmental adaptation for cell growth because of the porous structure of the silk fibroin biofilms. This enables integration of cells within the biomaterial. So, the silk fibroin biofilms are useful in a wide range of medical applications.

The silk fibroin is a natural polymer that has been used for several purposes in biomedical and tissue engineering as showed in Fig.1.1, due to its high tensile strength, biocompatibility and other physicochemical properties. It has high capability to forms structures such as biofilm, scaffold, grafts, nano particle, micro particle, and nano fiber. The silk fibers are coated with sericin which is a gum-like protein. Sericin protein works as glue that is intended to maintain the cocoon's structure (Pérez and Rigueiro, 2001), (Liu et al, 2007).

Figure 1.1: Silkworm silk fibroin applications (Kasoju et al, 2012).

1.2 Properties of Silk Fibroin

1.2.1 Chemical Properties

The silk fibroin protein consists of layers of antiparallel beta sheets. Its primary structure mainly consists of the amino acid sequence which is arranged recurrently (Gly-Ser-Gly-Ala-Gly-Ala)$_n$. The high glycine content gives rise to tight packing of the beta sheets, which contributes to silk fibroin's rigid structure that can't be stretched as showed in Fig.1.2. Fibroin is known to arrange itself in three structural forms, silk I, II, and III. Silk I is the natural form of fibroin, as emitted from the *Bombyx mori* silk glands. Silk II refers to the arrangement of fibroin molecules in spun silk, has greater strength and it is often used in various commercial applications. Silk III, which is a newly discovered structure of the silk fibroin (Valluzzi et al., 1999).

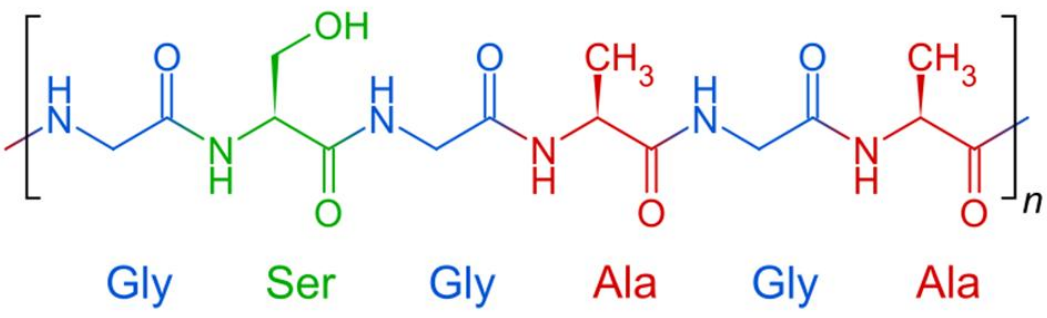

Figure 1.2: Silk fibroin primary structure (Valluzzi et al., 1999)

The fine structure of silk fibroin was investigated by electron microscope and X-ray diffraction method. The testing of silk fibers fragmented by ultrasonic radiation and negatively stained revealed the presence of ribbon-like filaments of well-defined lateral dimensions. Analysis of the expansion of the equatorial reflections in the X-ray diffraction pattern of fibrin yielded similar dimensions for the crystallites lateral extent (Dobb et al, 1967). The membranes were prepared by Bray et al, 2011. The silk fibroin protein supports the implantation of human limbal epithelial (HLE) cells and thus shows significant potential as useful biomaterials for formation ocular surface. This result shows that the silk fibroin membranes are suitable substrate for HLE cultivation and encourages progression in studies in preclinical models (Bray et al, 2011).

1.2.2 Mechanical properties of Silk Fibroin

Silk fibroin is a biomaterial with high significant elasticity, toughness, crystallinity, strength, and resistance to failure in compression. The semi-crystalline regions are the basis for the protein's elasticity. The basis for silk's unique mechanical properties is the combination of the β-sheet crystals and the inter phase between the crystals and the semi crystalline region. The β-sheet structure affects the tensile properties, biodegradation rate and elasticity of the scaffold, so the tailoring of these properties is done in parts with the cross-linking process. The transition depends on the solvent concentration as well as on the period of the time exposed to the solvent. Methanol treatment is used to process and induce β-sheet formation although it does not transform all molecular regions. Ethanol, 1-Ethyl-3-[3-dimethylaminopropyl] carbodiimide is also used to process β-sheet formation (Teo et al., 2006). A silk-fiber matrix was studied as a useful material for tissue engineering anterior cruciate ligaments (ACL). The results show the prepared silkworm fiber matrices, providing unique benefits in terms of mechanical properties as well as biocompatibility and slow biodegradability. These provide suitable biomaterial matrices for the support of adult stem cell differentiation toward ligament lineages (Altman et al, 2002). Studies show that, the regenerated silk fibers can hold their initial tensile integrity under immune deficiency *in vitro* culture conditions (Sukigara et al., 2003). The solvent used for electrospinning can affect the β-sheet formation of the secondary structure of scaffold, which in turn can alter the mechanical properties. Formic acid, HFIP and water were used to electrospin silk scaffolds,

while formic acid and water seem to enhance the mechanical properties of the scaffolds (Pérez-Rigueiro, 2001).

The silk fibroin/chitosan blended biofilms were also prepared by the solvent casting method. The miscibility between silk fibroin and chitosan was examined. The blended biofilm containing 30 wt % chitosan exhibited a maximum increase in density and crystallinity. The tensile strength and initial tensile modulus of blended biofilms were found greatly enhanced with increasing the chitosan content and showed a maximum value at the composition of 30 wt % chitosan (Nagarkar, 2010, p. 3489). By immersing a silk fibroin membrane, water dissoluble silk membrane was prepared. The enzymatic biodegradation behavior, mechanical property, and transparency of silk fibroin membrane in the wet state were investigated. The methanol treatment conditions changed the physicochemical properties (Zarkoob et al, 1998).

1.2.3 Physico-chemical Properties of Silk Fibroin

The physical properties of the polyethylene glycols (PEG1)-Silk fibroin biofilms were studied by Gotoh, (1997). The circular dichroism (C.D.) spectrum of the PEG1-Silk fibroin biofilm showed both positive and negative extreme due to the β-sheet structure. The polarizing microscopic observations and the Differential Scanning Calorimeter (DSC) measurements at rising temperature of the PEG1-Silk fibroin biofilm clarified the thermal behavior of the PEG1-Silk fibroin biofilm. In the DSC thermogram of PEG1-SF, the positions of the endothermic peaks due to the decomposition of silk fibroin and the melting of the PEG chains scarcely shifted compared to the peak position of the silk fibroin having a β-sheet structure and the peak position of PEG1. The result shows that the mutual miscibility between the PEG and the silk fibroin was considered to be very poor. The tensile strength tests showed that the PEG-modification of the silk fibroin improved (Gotoh et al, 1997). By using the IR spectroscopy the silk fibroin/chitosan blended biofilms were examined and the conformational changes of silk fibroin were determined by Kweon, (2001). The effects of the silk fibroin/chitosan blended ratios on the mechanical properties and on the physical properties were investigated. The optimum percentage was 10 – 40 % chitosan containing biofilms for mechanical properties. The Young's modulus and tensile strength were affected by the chitosan contents of the blended biofilms. Silk fibroin/chitosan blended biofilms has good water vapor and oxygen permeability, which makes them useful biomaterials. The

blended biofilm containing 40-50% chitosan showed very high oxygen permeability (Kweon et al., 2001).

The average molecular weight of silk fibroin slightly decreased. After coagulation and washing, transparent biofilms were obtained by blending cellulose and fibroin in all proportions. The crystalline structures of regenerated fibroin and cellulose were β-form and cellulose II, as shown by the characteristic x-ray diffraction profiles. The density values was increased with cellulose content, though less than expected value from a pure additive behavior. Moisture regain increased because of the addition of a small amount of cellulose to the silk fibroin. The results showed that both of elongation and strength at break of silk fibroin biofilms were improved by blending with cellulose. Infrared radiation spectrum exhibited the changes in the skeletal frequencies of the silk fibroin, improving the occurrence of intermolecular interactions between cellulose and fibroin through the hydrogen bond formation (Freddi et al, 1995).

1.2.4 Biodegradation Properties of Silk Fibroin

The stabilization and the release of horseradish peroxidase (HRP) in the silk fibroin biofilms were studied by Lu et al, in 2010. The stability of the protein drugs in the silk fibroin biofilms is attributed to intermolecular interactions between the enzymes and the silk fibroin biofilms, based on the differential scanning calorimetry DSC and the Fourier Transform Infrared Spectroscopy (FTIR). The structural properties of the silk fibroin biofilms molecules, periodic hydrophobic-hydrophilic domains, enabled strong interactions with silk fibroin proteins. The proteolytic biodegradation and dissolution of the silk fibroin biofilms resulted in the bound enzymes release. There was a linear relationship between the silk fibroin biofilms dissolution / degradation and the release of the entrapped protein enzyme as shown in Fig.1.3. The above results show that, silk fibroin materials are carrier for proteins for controllable release kinetics (Lu et al, 2010).

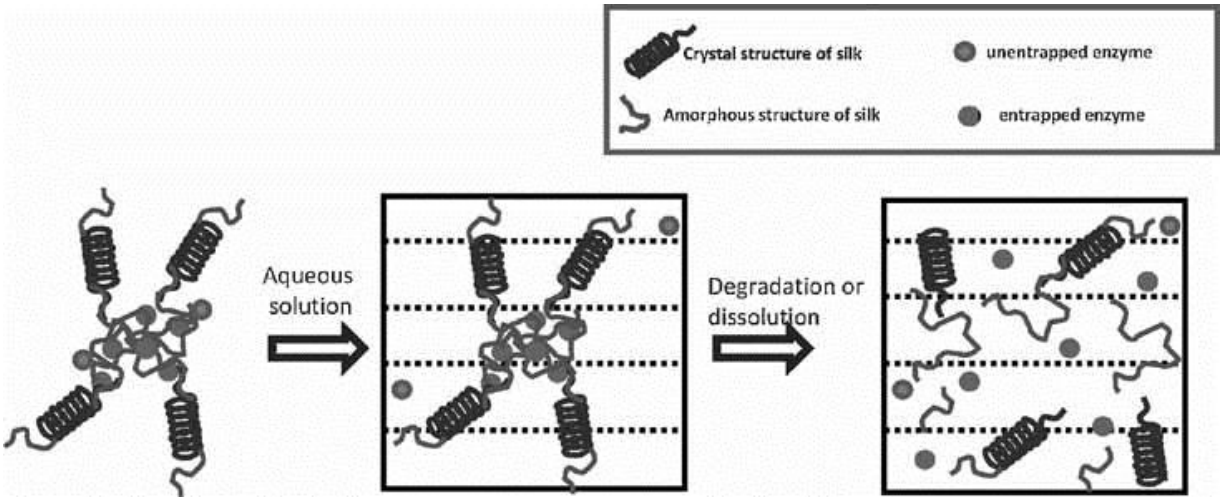

Figure 1.3: Stabilization and release of enzymes for SF biofilms (Lu et al, 2010)

Silk fibroin is considered as biodegradable materials due to its vulnerability to bacterial and enzymatic biodegradation. It will lose most of its tensile strength in a year in vivo, and will be unrecognizable at the implantation site within two years. The rate of biodegradation depends on the tissue implantation site. Studies show that proteases will cleave the protein at the less-crystalline regions (Alessandrino et al, 2008). The solvent used to electrospun the silk fibroin may affect the silk fibroin biofilms biodegradation of *in vitro* as well as *in vivo*. The electrospinning from an aqueous solution instead of an organic solvent like Hexafluoroisopropanol (HFIP) can increase the silk fibroin biodegradation rate while promoting the cell penetration and proliferation. Methanol treatment can significantly decrease the biodegradation rate (Alessandrino et al, 2008), (Zarkoob et al., 2000). The biodegradation rates of these polymers cannot be tailored at high rate as that of the silk fibroin. The collagen which is a vastly used biomaterial degrades between 1 to 4 weeks and sometimes long than 4 weeks depending on the cross-linking process (Sukigara et al, 2004). The synthetic polymer, poly (lactic-co-glycolic acid) (PLGA) (85:15) usually biodegrades within 26 weeks, while PLGA (50:50) biodegrades between 6 and 8 weeks *in vitro* (Jin et al., 2002, Jin et al., 2004). The silk fibroin, have the ability to modified to have similar biodegradation rates by altering the type of the solvent for electrospinning (Alessandrino et al, 2008).The thermal biodegradation of the silk fibroin biofilms were shifted because of the chitosan enhanced β-sheet conformation of silk fibroin. Enzymatic biodegradation, flexibility, and swelling also increased by the content of the silk fibroin protein in the blended biofilms.

The fibroblast cells spread by dendritic extensions on the chitosan / Silk Fibroin biofilms. The cell-cell interactions were checked by cultivation with fibroblast cells. The

biocompatibility of the blended biofilms was evaluated. All the biofilms showed high biocompatibility and no cytotoxicity by MTT assay (3-(4,5-Dimethylthiazol-2-yl)-2, 5-Diphenyltetrazolium Bromide). The result shows that the silk fibroin biofilms had possibility to apply as a supporting biomaterial for artificial tissue modifications (Luangbudnark et al., 2012). The thermal analysis by Moraes et al, in 2010 showed that the silk fibroin is thermally stable and that when its amount in the blend increases, the temperature at which biodegradation is initiated also increases (Moraes et al., 2010).

The biodegradation behavior of the silk fibroin biomaterials in human body is important and vital for growth of the tissues. The regulations of biodegradation behavior by altering the degree of crosslinking have been done by Xu et al., in 2011. The results showed that the degradation rate of cross-linked silk fibroin biofilms was inversely proportional to the degree of cross-linking. This experimental field would provide a new direction in the controlling of the biodegradation period time for specific tissue engineering application (Xu et al, 2011).

1.2.5 Biocompatibility of Silk Fibroin

The biocompatibility property of silk fibroin protein is one of the important reasons for the wide range of application on the biomedical field. The SD rat dermal was cultured cells on the silk fibroin biofilm by using SD rat dermal. The cell grown in shape of curve and proliferation process activity of the cells in the silk fibroin extracts from the Cholecystokinin Octapeptide (CCK-8) test kit. The result showed that the cells cultured on the 50 kGy-irradiated biofilm grown faster than that on the 25kGy-irradiated biofilm and the control. The silk fibroin irradiated with higher gamma ray dose could stimulate the cells growth and proliferation (Jin et al, 2012).

The silk fibroin mixed by the sulfonated silk fibroin (SSF) and highly biocompatible new kind of composite materials silk fibroin biofilms obtained by Ma et al., in 2006. The anticoagulant activity was characterized with prothrombin time (PT), the activated partial thromboplastin time (APTT), and thrombin time (TT), which all increased remarkably the clot times exceeded the measurement limit of clot detection instrument. The cell compatibility of the composite silk fibroin biofilms was evaluated through the cell morphologies on the silk fibroin biofilms and the cell viability by methyl thiazolyl tetrazolium (MTT) assay. The adhesion of the platelet was also investigated as one of the blood compatibility parameters. The result shows that the SF/SSF composite biofilms were a

potential material for tissue engineering matrix and blood compatible materials (Ma et al, 2006).

The silk fibroin – PEG based materials were prepared by Serban et al., in 2011. These blends are very cytocompatible, were crosslink within a few seconds by the chemical reaction between maleimides and thiols presented on the PEGs and have the potential to extra stabilize through β-sheet formation by the silk fibroin. The silk fibroin-PEG based materials shows longer biodegradation times and decreased swelling (Serban et al, 2011).

Recombinant human-like collagen (RHLC) was mixed with fibroin to prepare biocompatible biofilm as the scaffold material for the hepatic tissue engineering applications (Hu et al, 2006). The solution was used to blend the RHLC with the silk fibroin in order to enhance the blended biofilms hydrophilicity and biocompatibility, whereas maintaining elasticity, X-ray diffraction (XRD) and Fourier transform infrared spectroscopy (FTIR) analysis shows that hydrogen bonds were formed between the silk fibroin and the RHLC. The scanning electron microscope (SEM) test data confirmed that homogeneous microstructures were still retained after the introduction of silk fibroin with RHLC. Contact angle measurements showed that the hydrophilicity of the silk fibroin/RHLC biofilms was greater after RHLC was added. The suitability and the proliferation of the cell cultures on fibroin/RHLC biofilms were significantly enhanced compared to the pure silk fibroin biofilms or tissue culture plates.

1.2.6 Swelling Properties of Silk Fibroin Biofilms

The degree of swelling in the water of blended biofilms was independent on the methanol treatment or water temperature. The addition of silk fibroin into S-PVA biofilms promoted the permeation of the neutral salts (Yamaura et al, 1990). The mixed protein-based hydrogels has been prepared by blending gelatin (G) with the amorphous silk fibroin and promoting beta-crystallization of the silk fibroin with subsequent exposure to the methanol or methanol/water solutions. The introduction of beta crystals in the silk fibroin serves to extend the solid and stabilize the hydrogel network like behavior of these thermally responsive materials to elevated temperatures beyond the helix transition of gelatin. The unique temperature and the composition dependent properties of the gelatin silk fibroin hydrogels have been investigated by Gil et al., in 2005. The result shows that it's useful for stimuli-responsive drug delivery vehicles applications (Gil et al, 2005).

The protein/synthetic polymer hybrid interpenetrating polymer networks (IPNs) of the poly (N-isopropylacrylamide) (PNIPAAm) with the *Bombyx mori* silk fibroin have been prepared by using methanol treatments as showed in Fig. 1.4. Those IPNs having the beta sheet crystalline structure of the silk fibroin show improved loss moduli and storage. The effect of silk fibroin beta sheet networks on the IPNs copolymerized with acrylic acid (AAc) P (NIPAAm-co-AAc) / SF IPNs is compared with that on the PNIPAAm/SF IPNs, and the parameters controlling the deswelling kinetics of the IPNs are studied (Gil et al, 2007).

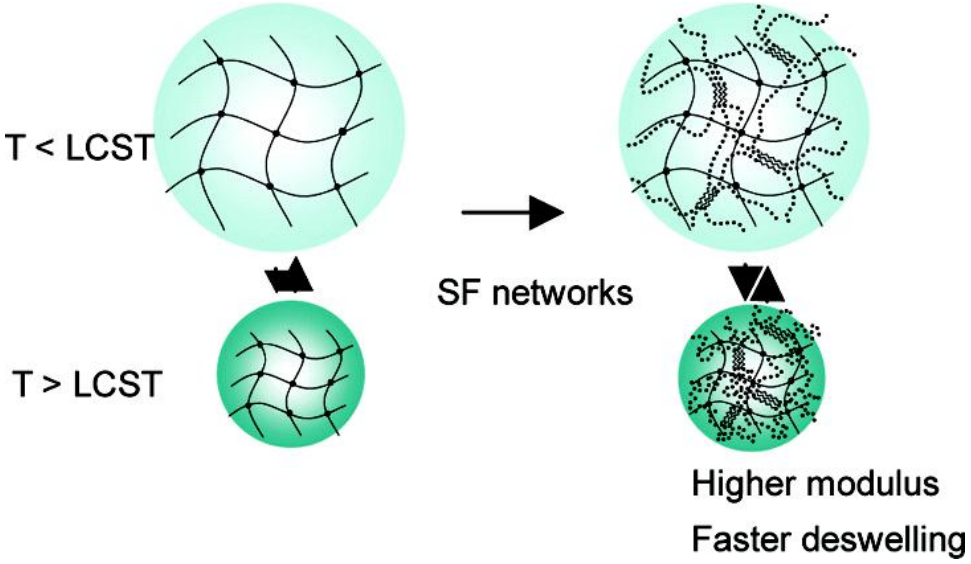

Figure 1.4: Effect of SF interpenetrating networks on swelling/deswelling kinetics and rheological properties of poly (N-isopropylacrylamide) hydrogels (Gil et al, 2007).

1.2.7 Solubility of Silk Fibroin

The imidazolium-based ionic liquid solvents was investigated for the regeneration and dissolution of silkworm (*Bombyx mori*) silk fibroin by Phillips et al., in 2004. The dissolution of the silk fibroin in the ionic liquid was analyzed by using wide-range-X-Ray scattering. The dissolved silk fibroin protein was processed into 100 μm-thicks, two-dimensional biofilms. The solvent, methanol or acetonitrile, has an impact on both the secondary structure of the silk fibroin protein and the topography of the biofilms (Phillips et al, 2004). The solubility of silk fibroin in aqueous-salt, organic media, and aqueous-organic, was analyzed by Sashina et al., in 2006. The functions of the secondary structural organization of the silk fibroin in

solutions and in the solid state after the recovery from different solutions were analyzed (Sashina et al, 2006).

1.2.8 Blood Clotting Measurements

Platelets and the immune cells responded differently to the different silk fibroin biofilms obtained by different processing protocols and stabilization. The data which presented in the work of Motta et al., demonstrate that the bioactivity can be influenced by altering the chemistry, such as the specific process which is used in the preparation of the materials used to assess biological responses, or by the source of silk protein (Motta et al, 2009).

1.3 Forms of Silk Fibroin

1.3.1 Silk Fibroin Scaffolds

The cell transplantation using biodegradable scaffolds offers the possibility to replace organ function and to regenerate new tissues. The biodegradable, biocompatible polymers play very important role in the organ regeneration as temporary substrates to transplant the cells which allow the attachment and growth of the cells, and also retention of differentiated function. The processing techniques have been enhanced to manufacture reproducibly scaffolds with high porosities for the cell seeding and large surface areas for the cell attachment. These scaffolds have been utilized to demonstrate the feasibility and regenerating of several tissue and organs as showed in Fig. 1.5 (Thomson et al, 1995).

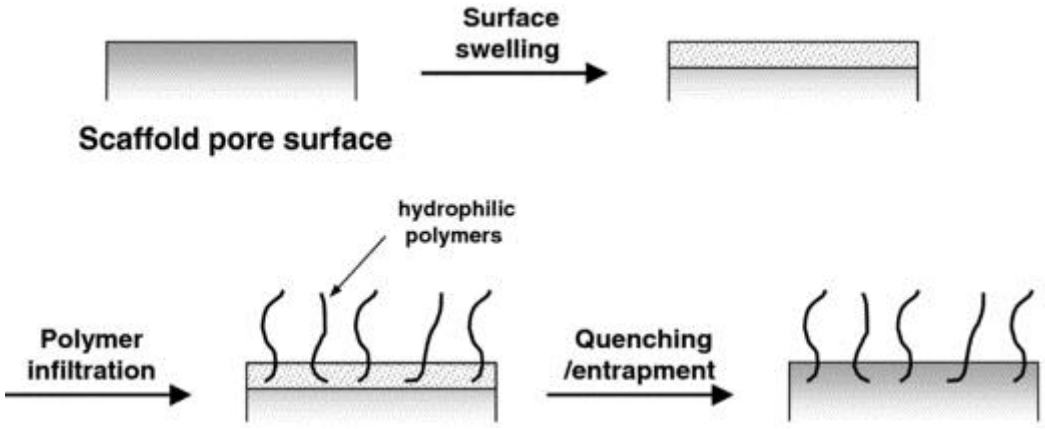

Figure 1.5: Hydrophilic polymer entrapments on a scaffold's surfaces (Oh, 2013.)

The silk fibroin protein can also be used for the production of porous silk fibroin scaffolds for different applications of tissue engineering (Sah et al, 2010). The silk fibroin concentration and processing method affects the three-dimensional scaffold structure on bone tissue formation by osteogenic differentiation of human adipose tissue derived stem cells (hASC). These resulted in a very similar structure with bone tissue that was formed in all silk fibroin scaffold groups (Correia et al, 2003). The silk fibroin has a useful and unique combination of properties, including excellent mechanical performance and good biocompatibility. The relationship between biodegradation behavior and the secondary structure of the silk fibroin scaffolds was indicated in the study of Hu et al., 2012. The scaffolds with different secondary structure were prepared by controlling the freezing temperature degree and by the treatment with ethanol or carbodiimide. By using FTIR, the quantitative proportions of each secondary structure were obtained, and each sample was then biodegraded in the vitro with collagenase IA for 18 days. The results showed that the high content of the β-sheet structure leads to a low biodegradation rate. The random coil region in the silk fibroin biomaterial is also biodegraded, while the crystal region stills stable and the amount of the β-sheet structure increases during the incubation (Hu et al, 2012).

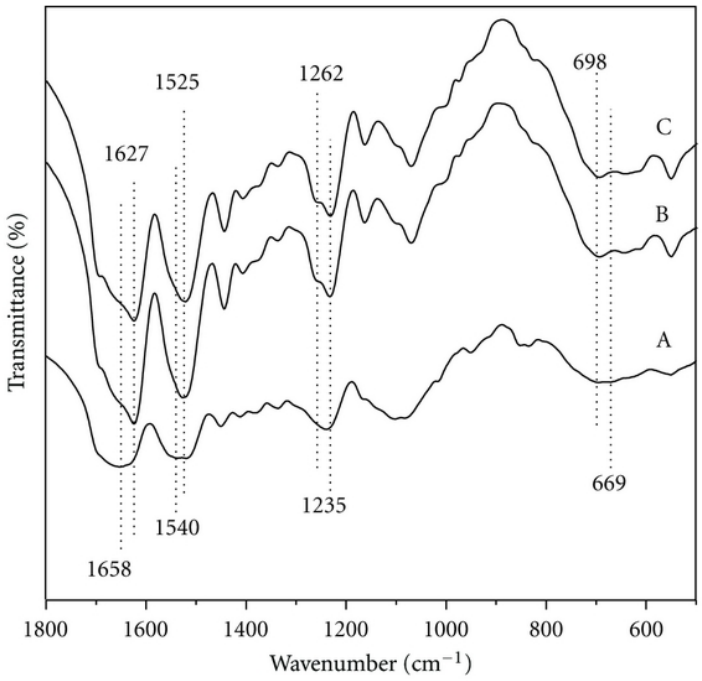

Figure 1.6: FTIR spectra of porous SF scaffold incubated with collagenase (Hu, (2012)).

As a suitable biomaterial for anterior cruciate ligaments (ACL) tissue engineering a silk-fiber matrix was studied by Altman et al., 2002. The matrix intended to match the mechanical requirements and complexity of the human ACL. The results showed that the suitably prepared silk fiber matrices, away from giving unique benefits in terms of low biodegradability and biocompatibility and also mechanical properties, proper biomaterial matrices can be provided for supporting the adult stem cell differentiation toward ligament lineages (Altman et al, 2002). The silk fibroin were combined with elastin protein and resulted in a scaffold which mimics the extracellular matrix (ECM). Genipin was used as a cross-linker by Vasconcelos et al., 2012 and obtained scaffolds with smaller pore size reduces swelling ratio, biodegradation and release rates. The composition has a great effect on the physical properties of the scaffold. The cytocompatibility with human skin fibroblasts along with the healing improvements make these scaffolds suitable for wound dressing applications (Vasconcelos et al, 2012). The silk fibroin scaffolds were prepared by leaching and freeze-drying methodologies. The results indicated that the antiparallel β-pleated sheet (silk-II) conformed in the silk fibroin scaffolds. The scaffolds developed are proposed to be suitable for use in the cartilage tissue-engineered scaffolding and meniscus (Yan et al, 2012). The benefit of using silk fibroin and chitosan blend (SFCS) biological scaffolds was investigated for the purpose of applications in cartilage tissue engineering with tracheal tissue reconstruction. Cartilage generation on chondrocyte–scaffold constructed with or without a perichondrium wrapping was checked and the capability of these scaffolds as cell carrier systems for chondrocytes was examined. The results showed that, in tracheal transplant properties, similar to those of the fully functional native tracheal (Zang et al, 2011). Cell affinity is one of the important issues required for developing materials of the tissue engineering. For this purpose, the silk fibroin protein was coated on the PHBHHx biofilms and its porous scaffolds. The results shows that the SF modified PHBHHx material is a potential material applicable in the cardiovascular tissue engineering (Sun, 2009).

1.3.2 Silk Fibroin Grafts

A novel biomimetic design of the silk fibroin-based nerve graft has been developed that was composed of the silk fibroin-nerve guidance conduit (NGC) mixed with the oriented silk fibroin filaments. The silk fibroin graft was used to bridge the implantation through a 10-mm long the sciatic nerve problems in the rats. The results show that, the peripheral nerve was repair at 6 months after the implantation was examined by the combination of the electro-physiological assessment. The examined morphological parameters show that the silk fibroin

grafts could improve the peripheral nerve regeneration for treating the large peripheral nerve defects. The results indicated that there is a high possibility of using themodern developed nerve grafts as a promising in state of nerve auto grafts (Yang et al, 2007).

Chitosan grafting caused a β-sheet to random coil conformational transition of the silk fibroin and important changes in the thermal behavior. The results were reported in the work of Freddi et al., 2006 showed that, the possibility of the enzymatically initiated protein with polysaccharide grafting for the production of a bio-based, polymers (Freddi et al, 2006). The smaller vascular grafts were made from the synthetic biomaterials, particularly those smaller than 5 mm in the diameter, are associated with a high percent of thrombosis. The silk fibroin provides scaffolds the antithrombotic surface for different cell types in the tissue engineering (Enomoto et al, 2010).

1.3.3 Silk Fibroin Nanoparticles

The nano-particles were prepared from the silk fibroin by using different techniques and methods (Kundu et al., 2010). The silk worm *Antheraea mylitta* and domesticated *Bombyx mori* have been used to prepare nanoparticles. These nanoparticles have been investigated, considering the size of the particle, stability, surface charge and the morphology along with its release of growth factors and cellular uptake. The nanoparticles were spherical in shape, stable, negatively charged, 150–170 nm in average diameter and did not impose any toxicity and exhibited mostly silk II (β-sheet) structure. The studies about the cellular uptake show that, accumulation of the fluorescence isothiocyanate conjugated silk fibroin nanoparticles in the cytosol of murine squamous cell carcinoma cells. The VEGF release from the silk fibroin nanoparticles showed a significantly sustained release more than 3 weeks, signifying the potential application in medical side as a growth factor delivery system (Kundu et al, 2010). The silk fibroin nanoparticles were prepared by Kundu et al., 2010 from the liquid silk fibroin by using polar aprotonic organic solvents and water-miscible protonic solvents. The results showed that the biodegraded peptide chains of the regenerated silk fibroin is collected heterogeneously or homogeneously to form the looser globular structure in the silk fibroin aqueous solution (Kundu et al, 2010). The color dye-doped silk fibroin nanoparticles fabricated by using the micro emulsion method by Zhang et al., 2007. These nanoparticles were 167 nm in diameter. The size distributions were calculated and the morphology of the nanoparticles was also determined. The observed stability of the loaded fluorescent molecules in the silk fibroin nanoparticles showed that it can be used as an important and new device for

the molecular imaging and bioassays. The low biodegradation of silk combined with its biocompatibility, the nano-scale size, their capacity to encapsulate fluorescent dye, may have a great effect and applications in the various biological applications (Zhang et al, 2007). In the work of the Myung et al., 2008, the silk fibroin nanoparticles were prepared by a solution-enhanced dispersion by supercritical CO_2 (SEDS). The resulting nanoparticles showed spherical shape, a smooth surface, and constricted particle size dispersal with a mean diameter of the particle approximately 50 nm nanoparticles. The (indomethacin) IDMC–SF nanoparticles after ethanol treatment showed a significantly sustained release more than 2 days. These studies show that, the silk fibroin nanoparticles are biocompatible carrier for drugs delivery system (Myung et al, 2008). The capillary-microdot technique silk fibroin-derived curcumin nanoparticles show effectiveness against breast cancer cells and are suitable to treat *in vivo* the breast tumor by local, sustained and long-standing biodegradable therapeutic delivery system (Zhao et al, 2012). The silk fibroin nanoparticles had the ability to overcome barriers set by synthetic non degradable nanoparticles made of the silicone, biodegradable polylactic acid–polyglycolic, and polyethylene glycol and acid polymers. (Gupta et al, 2009). The silk fibroin was conjugated with the methoxypoly (ethylene glycol) derivatives to prepare the silk fibroin nanoparticles by Kweon et al., 2010. The conjugation of the silk fibroin with PEG was examined with the various instrumental analyses techniques. The amino acid and nuclear magnetic resonance spectrometry analysis showed that the tyrosine and the serine residues in silk fibroin were reacted with PEG and resulted in increasing of molecular weight. The shapes and the sizes of the silk fibroin nanoparticles observed by transmission electron microscope were ranged approximately 150-400 nm in diameter and spherical morphology (Kweon et al, 2010).

1.3.4 Silk Fibroin Micro Particles

The size distribution and particle size of the regenerated silk fibroin microspheres prepared by the silk fibroin molecular chains mild self-assembling are strongly affected by the ethanol additive amount, the freezing temperature and the concentration of the silk fibroin protein (Cao et al, 2007). The silk fibroin microspheres were obtained with predictable and controllable range sizes from 0.2 to 1.5 μm, as showed in Fig. 1.7.

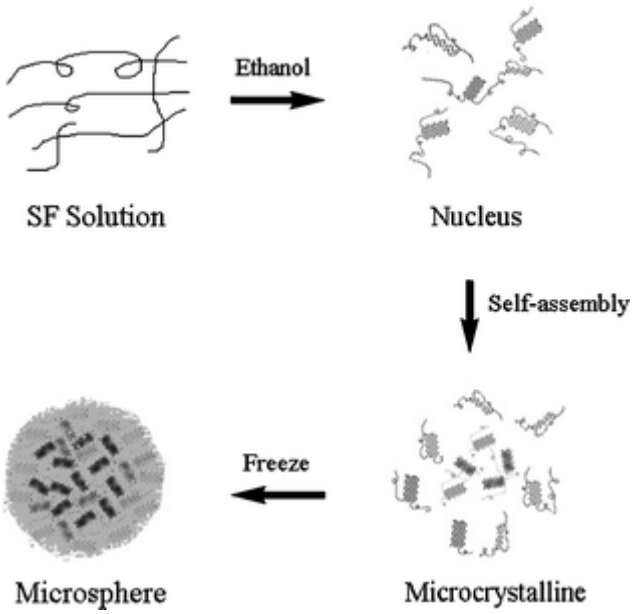

Figure 1.7: The preparation of regenerated SF microspheres (Cao et al, 2007).

The submicronic particles or / and the microspheres were prepared by the spray dryer method. The development of submicronic particles with transition from the random coil to the β-sheet structure during spray dryer treatment has been examined. The swelling ratio is dependent on the pH of the solution. Morphologically, SFMP particles, average 2-10 lm in size, and SFMP was spherical in shape. The final microspheres applied to immobilization of drugs (Yeo et al, 2007). The Cross-linked and the non-cross-linked silk fibroin microspheres use the simple water-in-oil emulsion solvent diffusion method were studied by Imsombut et al., 2010. The silk fibroin micro particles were smooth in surface and spherical in shape. The silk fibroin microsphere sizes were found to depend upon the various process parameters. Genipin crosslinked and non crosslinked of the silk fibroin microspheres contained porous structures. The genipin crosslinking induced the conformational transition of the silk fibroin from random coil to β-sheet form but the size and shape of the silk fibroin microparticles does not changed. These silk fibroin microspheres useful and suitable microcarriers for hydrophilic drug delivery system (Imsombut et al, 2010).

The drug-loaded silk fibroin spheres with or without polyethylene glycol diglycidyl ether prepared with a water-in-oil emulsion solvent diffusion method were prepared. The (PEGDE) crosslinking, and also effects of the PEGDE ratio the homogenizing speed and the drug release behaviors of the silk fibroin spheres were examined. The results showed that the desired drug release profiles of silk fibroin spheres can be design by adjusting the PEGDE ratio and the particle size (Baimark, 2010).

The laminar jet breaks-up of an aqueous silk fibroin solution spheres, have great encapsulation efficiencies and sustained release kinetics that helps to preserve the bioactivity of the embedded growth factor, with a great sustained release profile. The applications of these spheres vary from the delivery of growth factors for tissue repair. By using the Sephadex G-25 gel filtration chromatography and preparing silk fibroin microsphere particles (SFMP) were simply prepared by spray dryer as showed in Fig 1.8. (Wenk, 2008).

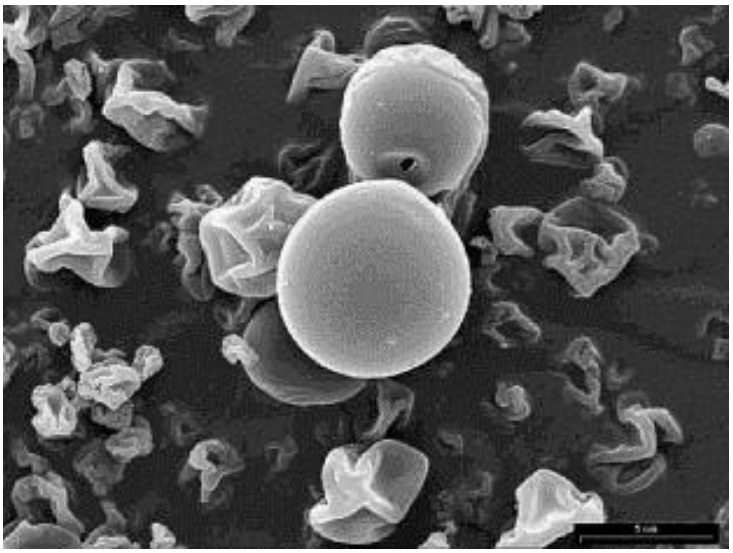

Figure 1.8: The simple preparation of micro spheres from pure silk fibroin (Wenk, (2008)).

1.3.5 Silk Fibroin Biofilms

The silk fibroin biofilms were prepared from aqueous solutions of the silk fibroin protein polymer and crystallinity of the biofilms was induced and controlled by the methanol treatment (Hofmann et al, 2006). The properties of the blend biofilms obtained by mixing the silk fibroin and polyacrylamide (PAAm) were examined. The DSC curves of SF/PAAm blend biofilms showed overlapping of the main thermal transitions characteristic of the individual polymers. The peak of dynamic loss modulus of silk fibroin at 193°C gradually shifted to lower temperature in the blended biofilms (Freddi et al, 1999).

The effect of the different organic solvents on the thermal characteristics and the structure of the silk fibroin biofilms were studied. The silk fibroin biofilms prepared by using evaporating technique by S. Prasong, and K. Nualchai. They were treated the films with different organic solvents. The results showed that the secondary structures of the silk fibroin biofilms were

changed after treatment silk fibroin biofilms with the ethyl acetate, ethanol, and methanol, except acetone. Methanol showed the higher effect to improve the stability of the silk fibroin biofilms compared to other solvents. The ethyl acetate, ethanol, and methanol could be used to enhance the stability of the silk fibroin biofilms (S. Prasong et al, 2001). To understand the effects of the casting solvents on the silk fibroin biofilm properties, the formic acid (FA), water (W), and trifluoroacetic acid (TFA) are used as solvents as showed in Fig 1.9. The significant biodegradation of the silk fibroin was observed for TFA-based biofilm compared to the TA-based biofilms and water. The silk fibroin biodegradation result showed in a significant reduction in modulus of TFA-based biofilms and tensile strength. Compared to the water, TFA-based silk fibroin biofilms demonstrated lower water solubility (Rajkhowa et al., 2011). The study demonstrated that the small variation in the random coil conformations and β-sheet percentage resulted in significant change in the rates of the enzymatic biodegradation without any change to their tensile properties (Rajkhowa et. al., 2011).

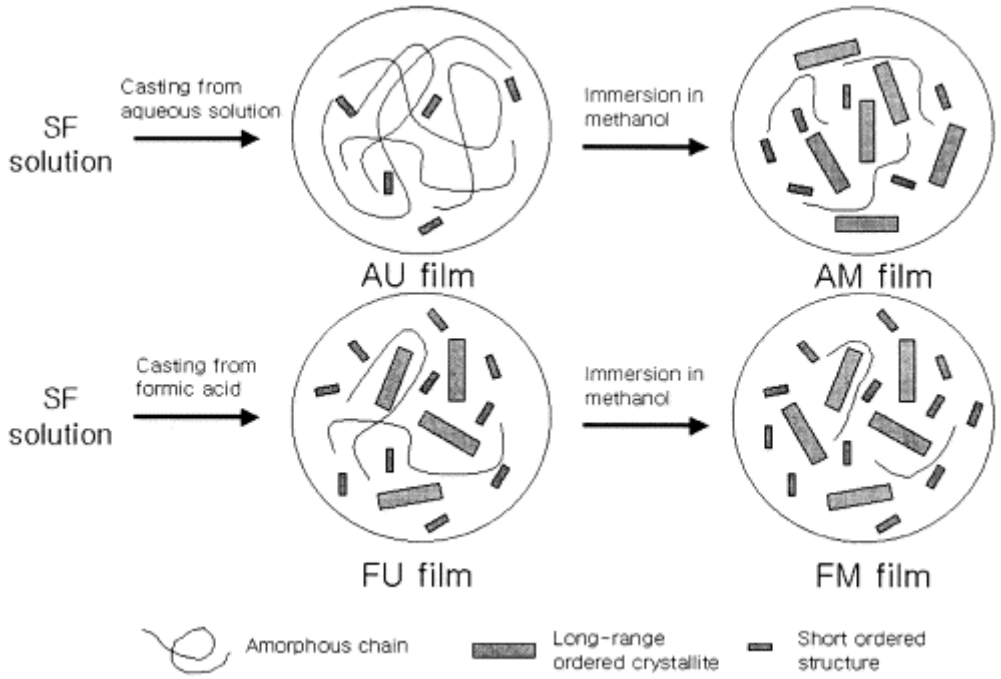

Figure 1.9: Crystallite formations of the SF biofilms induced by formic acid and methanol (Rajkhowa et al, 2011).

The effect of thin silk fibroin biofilms on the glial cells growth of peripheral neurons and electro physiologic properties were examined by Benfenati et al., 2012. The result shows that, the silk fibroin biofilms are a favorable substrate to support *in vitro* on neuron. The studies of the silk fibroin biofilms shows that, the adhesion of peripheral neuron culture and

the neurite out growth bioelectrical properties of astrocytes were improved. By functionalization with the specific trophic molecules, the result was indicate that the NGF-silk fibroin biofilms enable to modulation of firing properties of cultured neurons and to increased neurites outgrowth also showed the bioelectrical properties of astrocytes modulated specifically (Benfenati et al, 2012). The probability of obtaining silver nanoparticles on of silk fibroin biofilms was studied by Sashina et al., 2009. The size of silver particles on the fiber surface was evaluated and a comparative assessment of the effects of reducing the agents on the morphology was also examined (Sashina et al, 2009). The silk fibroin biofilms are very suitable for imaging applications (Vepari et al, 2007, Ma et al, 2006). The cell culture system methodology for the silk fibroin biofilm is a scalable for fast assessments of cell-silk fibroin biofilm surface interactions. To study differences in the responses of cells and cell proliferation for alignment, it is very important to use the surface patterned silk fibroin biofilms (Lawrence et al, 2009, Patel et al, 2010).

The cells were cultured on both flat silk biofilm substrates and micro-patterned, and then assessed through scanning electron microscopy. The silk biofilm *in vitro* culture system shows a customizable experimental setup useful to the study of the cell-surface interactions on the biomaterial substrate (Lawrence et al, 2012). The silk fibroin biofilms supported the metabolic activity and adherence of the PC12 cells by K-Casrin transformer organism and in combination with the nerve growth factor (NGF), supported neurite outgrowth during the PC12 cell differentiation (Uebersax et al, 2007). The controlled release of fluorescein-iso-thio-cyanate (FITC)-labeled dextran's from the methanol-untreated and treated silk fibroin biofilms was modeled to characterize the mechanisms and release kinetics. By using linear regression relationship between percent of the entrapped FITC-dextran particles and molecular weight, it was presented for the diffusion model for the simulating release of FITC-dextran of varied molecular weights from the methanol-untreated and treated silk fibroin biofilms (Hines et al, 2011). The ultra thin multilayer biofilms of silk fibroin were fabricated by spin-assisted layer-by-layer and spin coating assembly. The biofilms mechanical properties were studied both in the compression and the modes tensile. These properties are occurred by the highly crystalline β-sheets, serving as physical crosslinks and reinforcing fills. The silk fibroin biofilms with proper mechanical strength have abroad and potential applications in biodevices, coatings for artificial skin, and implants (Jiang et al, 2007).

1.4 Problems Statement

The modification of new biocompatible biomaterials with high nonthrombogenic properties and very low platelet adhesion for biomedical applications such as drug delivery and tissue engineering started from decades. For our knowledge, the UV-induced photopolymerization technique was not applied by using photoinitiator in the biofilm formation of silk fibroin. The research will focus on the improvement of swelling, biodegradability and blood compatibility properties of silk fibroin biofilms irradiated by UV.

1.5 Aim of the Thesis

The aim of this thesis is to synthesize silk fibroin / N, N' methylene diacrylamide biofilms by using UV-induced photopolymerization technique. The morphology of the synthesized biofilms is characterized by using SEM, XRD patterns. The evaluation of their swelling properties both in acid and phosphate buffer solution is examined. Their biodegradability properties in protease enzyme are evaluated. The blood compatibility and platelet adhesion tests are also applied for the investigation of blood compatibility properties.

CHAPTER 2 MATERIALS AND METHODS

2.1 Materials

Bombyx Mori. Cocoons used to get pure silk fibroin protein as showed in Fig. 2.1. After purification processes which are described in section 2, sodium carbonate, Na_2CO_3 was used purchased from Sigma Aldrich. Calcium chloride, $CaCl_2$ and ethanol used also purchased from Sigma Aldrich. Dialysis membrane (cut off M.W. 12,400) this tubing will prevent most proteins of molecular weight 12,000 or greater to pass through own membrane, when full average diameter 16 mm and average flat width 25 mm (1.0 inc.) capacity of ~60 ml/ft. purchased from Sigma-Aldrich. N, N'-methylene diacrylamide $C_7N_{10}H_2O_2$, used as cross linker mixing with silk fibroin for biofilms preparation also purchased from Sigma-Aldrich.

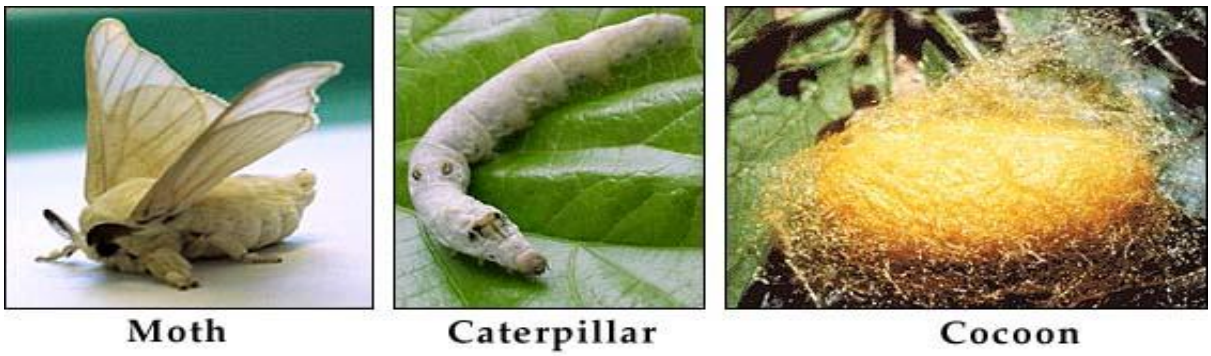

Figure 2.1: Raw silk fibroin cocoons.

2.2 Methods

2.2.1 Acetic Acid Buffer Solution Preparation (APS)

The glacial acetic acid was diluted to get 0.5 M of acetic acid solution then pH fixed on 1.2 by HCL, where the NaOH working to keep the value of pH at 1.2. If the pH value raising over this point it will reversed back by NaOH.

2.2.2 Phosphate Buffer Saline Solution Preparation (PBS)

The formulation used in this study of the PBS contains the following constituents as show in Table 2.1.

Table 2.1: Phosphate Buffer Saline Contents

Salts	**Concentrations**	**Concentrations**
(--)	(mmol/L)	(g/L)
NaCl	137	8.01
KCl	2.7	0.20
$Na_2HpO_4*2H_2O$	10	1.78
KH_2PO_4	2.0	0.27
pH	7.4	7.4

The pH of the PBS solution is adjusted to 7.4 by adding either sodium hydroxide NaOH or Hydrochloric acid (HCl) depending on the pH value where it's became more than 7.4.

2.2.3 Silk Fibroin Purification

2.2.3.1 Degumming Process

Degumming process used to treatment silk fibroin, for extraction of sericin amino acid from cocoons. In this process, silk cocoons were added in 0.1M sodium carbonate Na_2CO_3 solution 1g/100ml w/v and stirred on the hot plate at 75^oC with1.5 rpm as shown in Fig 2.2. Degumming process carried out through three sessions of time, three hours per each session, and then the produce of degummed silk is washed with pure water. Finally, degummed silk fibroin dried in laboratory at room temperature to get the silk fibers as show in Fig 2.3.

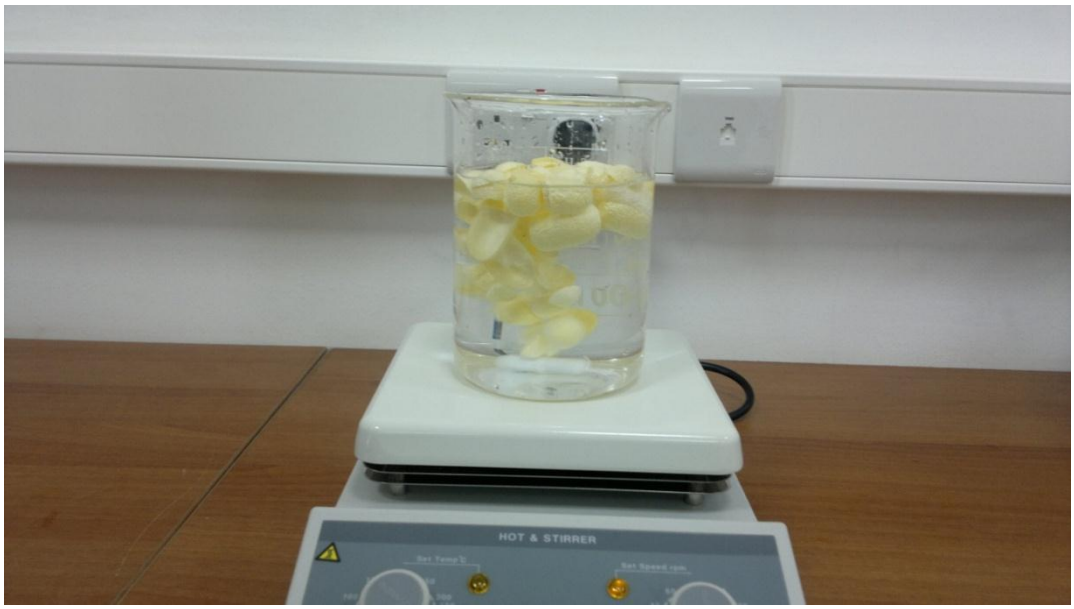

Figure 2.2: Degumming process

Figure 2.3: Degummed silk fibers

Figure 2.4: Degummed silk fibers in dry case

2.2.3.2 Dissolution Process

The dissolution process used for dissolving the silk fibroin to have an aqueous form of silk fibroin, the benefit of this process is to break the long polypeptide chains into shorter chains lengths. This process prepared by blending silk fibroin with n_{C2H5OH}:n_{H2O}:$n_{CaCl2,;}$ (2:8:1) molar ratio at 75^{o}C with continuous stirring until the complete dissolution. After that the electrolyte solution with silk fibroin protein has been obtained as showed in Fig. 2.5.

Figure 2.5: Silk fibers dissolving in the electrolyte solution

2.2.3.3 Dialyses

After dissolution process which an aqueous electrolyte solution produced with silk fibroin, the dialysis process started. The need of dialyses process was to remove the ions to get pure silk fibroin solution. The dialyses process done by pouring the aqueous electrolyte solution into a carboxymethyl cellulose semipermeable membrane tube and putting the membrane in the large beaker 5 liters in volume filled with distilled water. According to the membrane properties the ions will diffuse through the membrane to the water as showed in fig. 2.6. This process repeated six times with different periods of time (1, 3, 6, 9, 12, 12 hours) with continuous stirring, after that the pure aqueous silk fibroin formation obtained, now the silk fibroin ready to using it in the biofilm preparations and other applications.

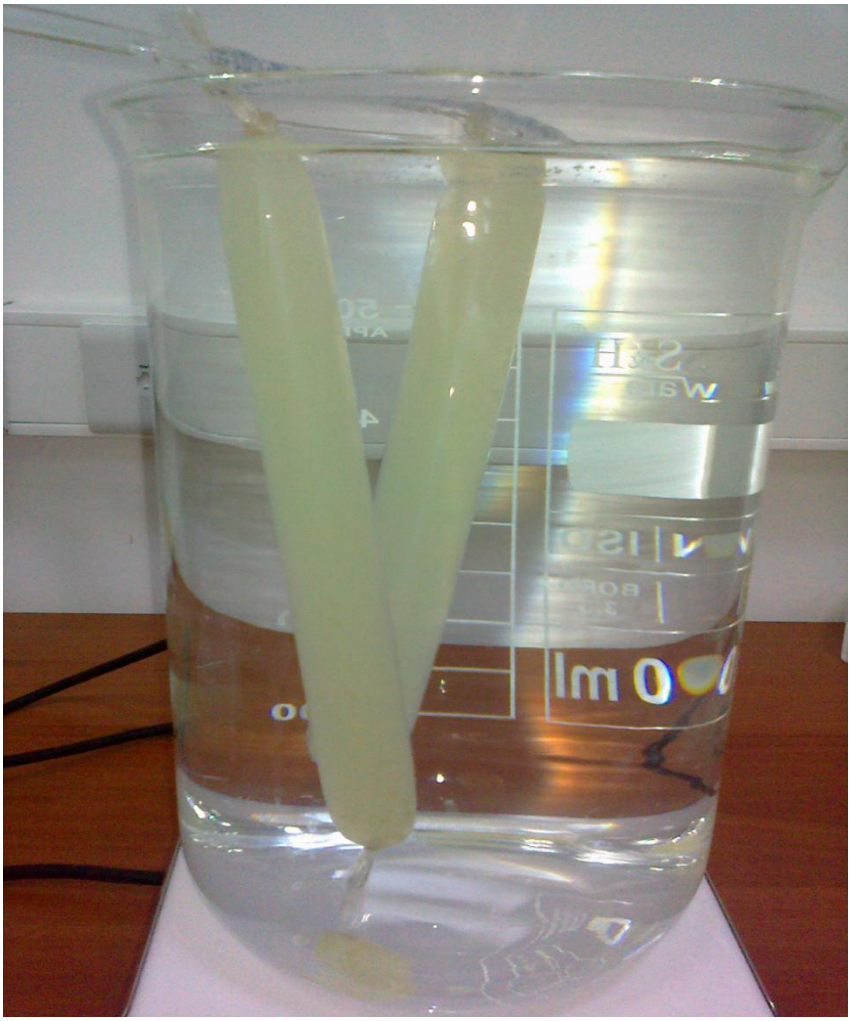

Figure 2.6: Dialysis of the aqueous silk fibroin with distilled water.

2.2.4.1 Biofilms preparation

By blending 2ml of silk fibroin solution with the cross linker $C_7N_{10}H_2O_2$, the blended solution were poured over a piece of glass and but the piece under UV wave in dark chamber and UV irradiation started. The process has been continuing until the biofilm formed. Both wave lengths have been applied and compared in all characterizations. The biofilms that has been irradiated with short wavelength prepared earlier than the one irradiated with the long wavelength. The crosslinker content also affect the duration of irradiation. As the crosslinker amount increases in the blend solution the duration decreased as showed in Fig.2.7. Different amount of crossilnkers were used during the study (25, 50,125,150 micro liter).

Figure 2.7: UV-irradiation chamber of photopolymerization.

2.2.4.2 Methanol Treatment

The biofilms prepared by UV-irradiation was washed and methanol was poured on the surfaces to fix the secondary structure and to convert the random coils to β-sheet structure by hydrogen bonding as show in figure 2.8.

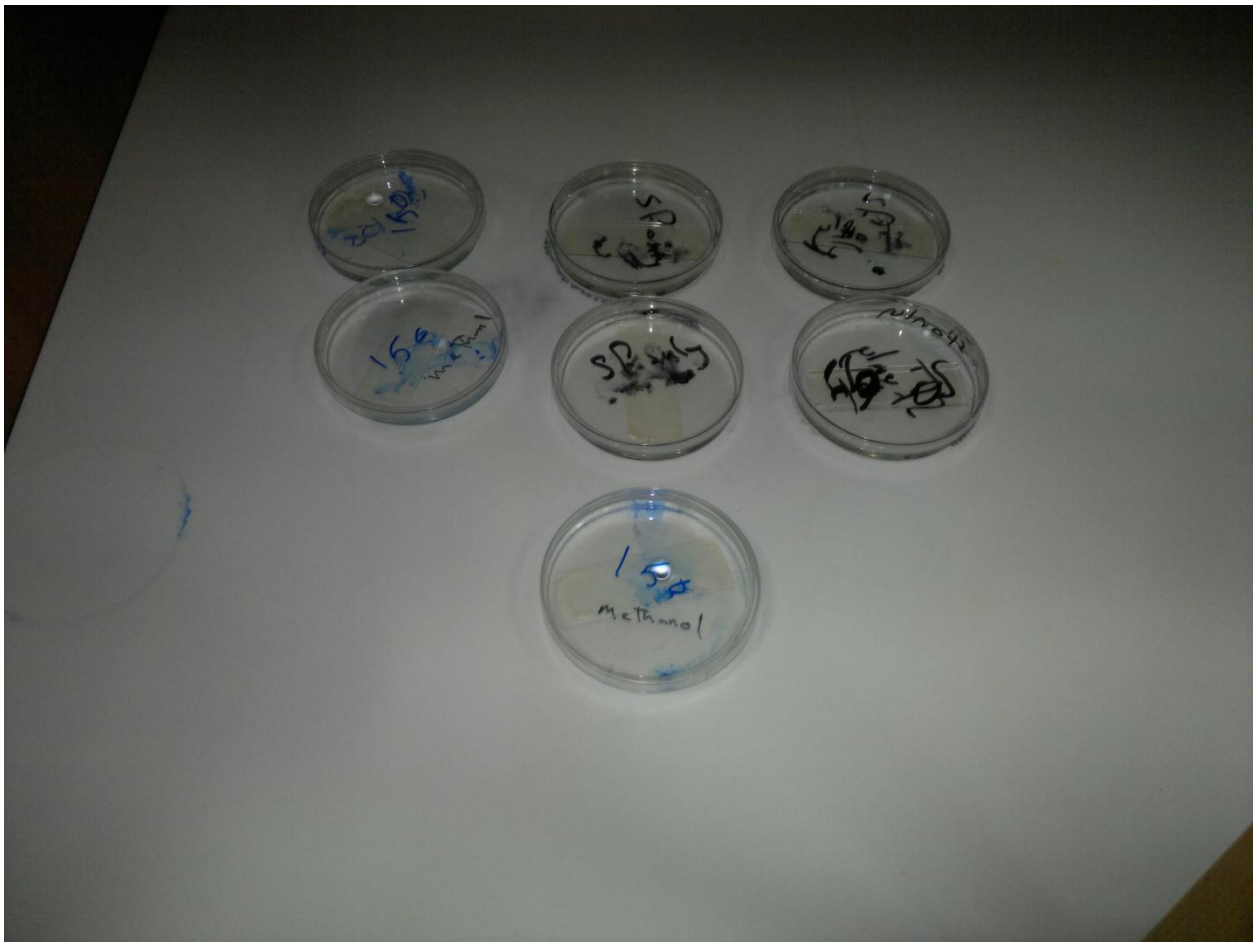

Figure 2.8: Silk fibroin biofilms under methanol treatment.

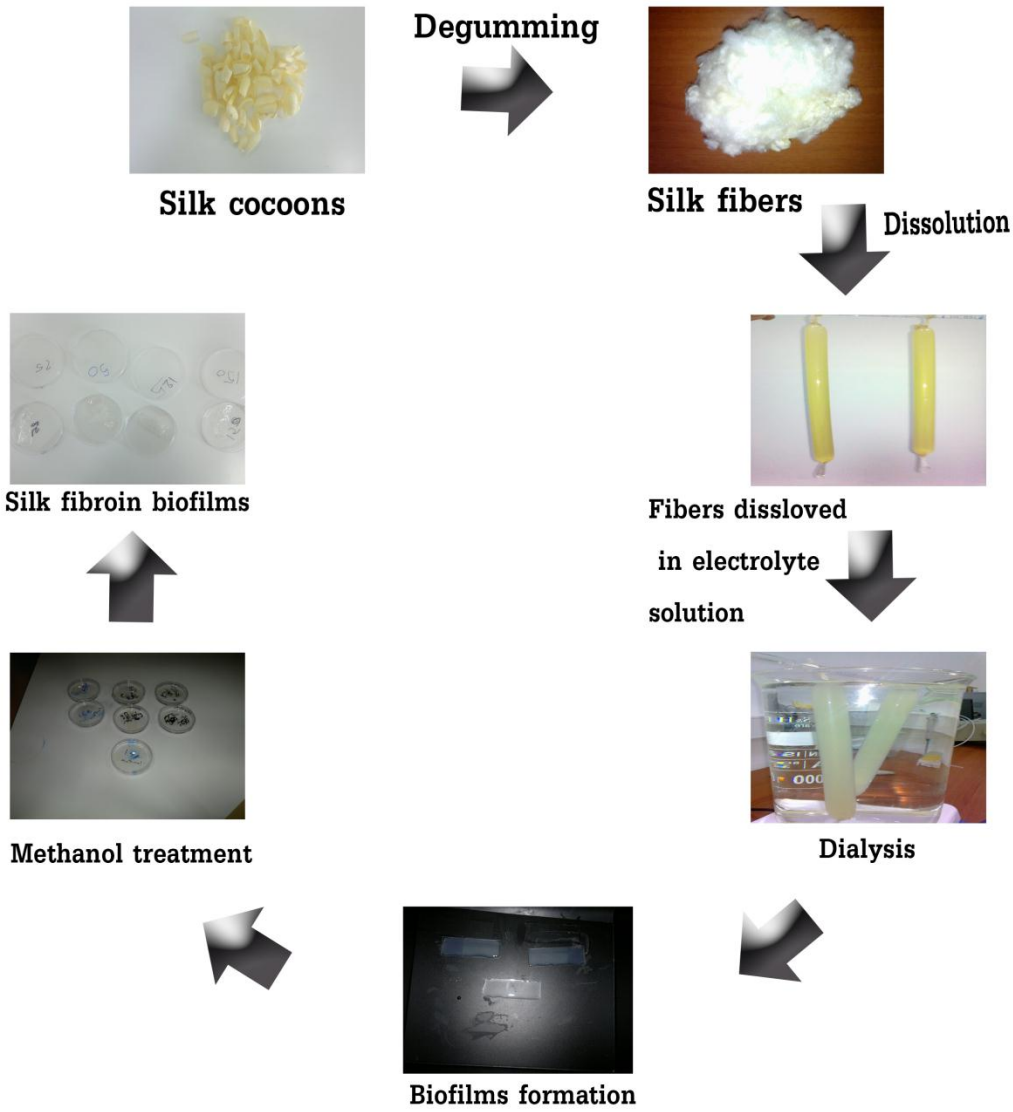

Figure 2.9: Silk purification process flowcharts.

2.2.5 Protein Concentration Calculation

The protein which extracted from the silk cocoons calculated practically by taking 1 ml of aqueous silk fibroin solution which produced from dialysis process and by applying heat (37^{o}C) on it a biofilm of the silk fibroin protein was formed. By weighting the biofilm the exact protein content in 1ml can be calculated.

2.2.6 Swelling

The silk fibroin biofilms prepared under different conditions and methods tested to check for their swelling properties in the ABS and PBS solutions as showed in Fig. 2.10.

The swelling ratios calculated by

$$\text{Swelling Ratio } \% = \frac{\text{Ws} - \text{Wd}}{\text{Wd}} * 100\% \quad (1)$$

Where Ws the weight of the biofilm which it changed after each test measurement

W_d the weight of biofilm in dry state before test.

Figure 2.10: Swelling tests for the silk fibroin biofilms.

2.2.7 Biodegradation

After silk fibroin biofilms prepared with different conditions and methods they were tested to check their biodegradation properties. For biodegradation test, 0.3 g/mL of protease enzyme as show in figure 2.11. The silk fibroin biofilms tested by embedding them into enzyme solution and weigh for different durations. The temperature was fixed to 37^{o}C

The biodegradation percentage calculated by this equation.

$$\text{Biodegadation Ratio} = \frac{\text{Wd}}{\text{Ws}} * 100\% \quad (2)$$

where Wd = Weight after biodegradation, Ws = Biofilms weight before biodegradation.

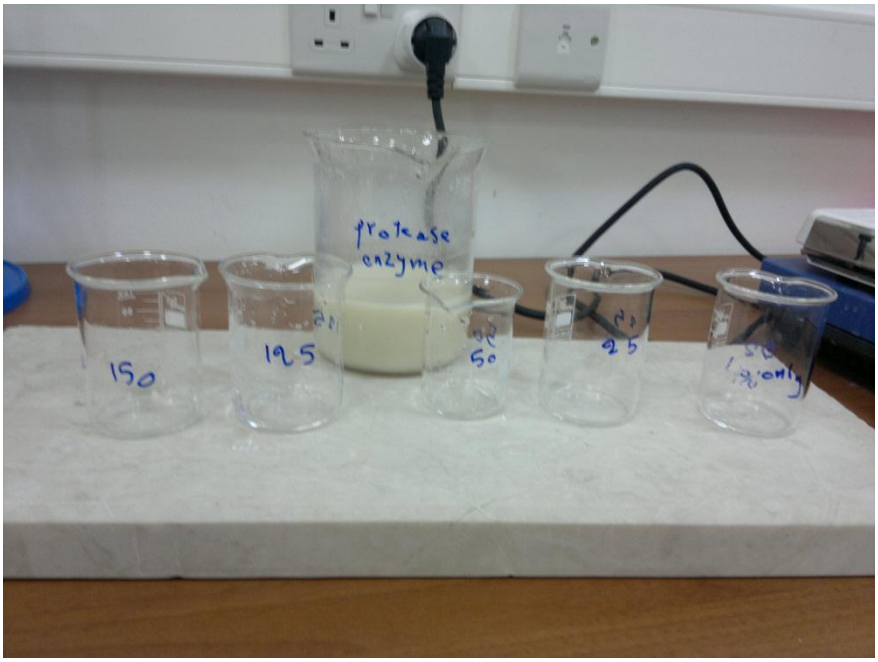

Figure 2.11: Biodegradation tests for SF biofilms with protease enzyme

2.2.8 X-Ray Diffraction analysis (XDR)

Powder X-ray diffraction was carried out at TUBITAK-MAM- Gebze, by using a Shimadzu XRD-600 model diffractometer with Cu X-ray tube (L= 1.5405 A).

By using Jawarska el al method the crystallinity index calculated.

$$\text{CrlPeak} = \frac{I110 - Iam}{I110} \quad (3)$$

2.2.9 In-vitro Coagulation Time Test

The activated Partial Thromboplastin Time percent (act PTT %) and ProThrombin Time (PTT) are indicators of evaluating the efficiency of both the common coagulation pathway and the "Extrinsic" pathway is determined by the use of APTT combined with prothrombin time. In this work we focused on intrinsic and extrinsic pathways of the classical blood coagulation pathway as showed in Fig.2.12.

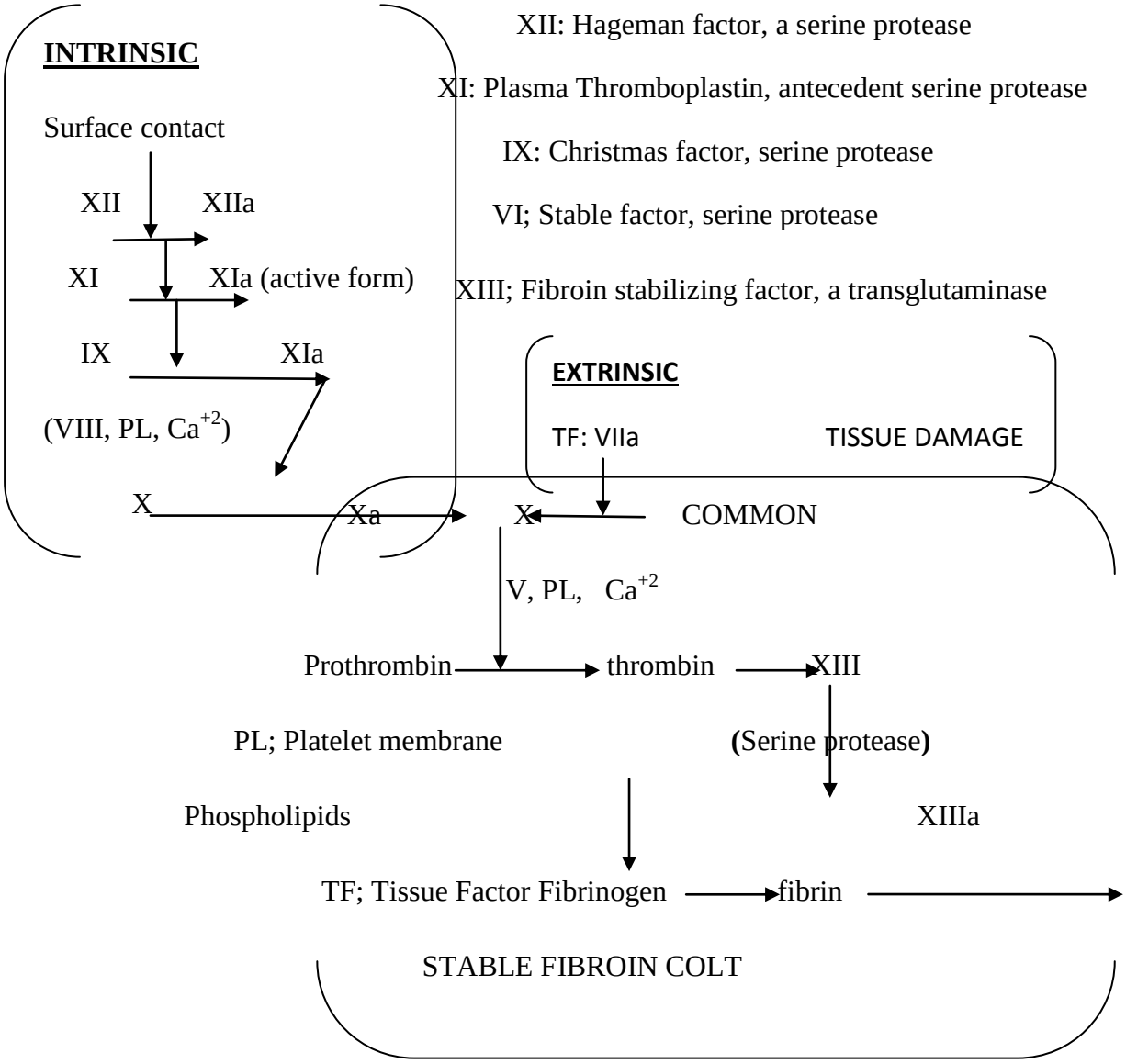

Figure 2.12: The three pathway that make up the classical blood coagulation pathway

The prothrombin time and its derived measure International Normalized Ratio (INR) are measures of the extrinsic pathway of coagulation. They are used to determine the bloods clotting tendency. IRN is the prothrombin ratio raised to the power of the International Sensitivity Index (ISI) (Korte et al, 2000) indicated that the shortening of the PTT might increase the risk of thromboembolism.

In this study, the effect of crosslinker $C_7H_{10}N_2O_2$ on SF biofilms for plasma coagulation were detected by measuring the activated partial thromboplastin time (APTT), prothrombin time (PTT), and INR by STA Compact Hemostasis System equipment, Stago, US.

Measurement; The 1 cm^2 square biofilm samples were incubated with 150 µL healthy human blood plasma in a transparent cuvettes at 37^o C for 3 minutes and the clotting times were obtained by the clot detection instrument STA Compact.

2.2.10 In-vitro Platelet Adhesion Studies

Samples were immersed into the fresh human platelet rich plasma (PRP) from healthy donors (provided by Near East University Hospital, North Cyprus) with a platelet concentration close to physiological (about 2 x 10^5 µl) for 15 minutes under static conditions at 37^oC. The contacting PRP was removed and samples washed in ultra-pure water.

The Peripheric Seaming Method was used to determine the platelet, red blood cells and white blood cells adhesion on the surface of biofilms. The adhesion was evaluated by Electron microscope for number, adhesion morphology and platelet micro-particle formation.

The steps of peripheric seaming method;

Step 1: 1- Direct drying technique was used with May Grünwald.

2- Incubation time was 5 minutes.

3- Washed with distilled water.

Step 2: 1- Direct drying technique was used with May Grünwald.

2- Incubation time was 8 minutes.

3- Washed with distilled water.

Step 3: 1- Incubation time is 1 minute in distilled water.

2- Drying process was applied.

Step 4: Electron Microscope was used for further investigation.