BLOOD COMPATIBILITY STUDIES OF SILK FIBROIN/ DI ETHYLENE GLYCOL DIMETHACRYLATE BIOFILMS

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Vivian Chisom OKAFOR: BLOOD COMPATIBILITY STUDIES OF SILK FIBROIN/ DI ETHYLENE GLYCOL DIMETHACRYLATE BIOFILMS

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ABSTRACT

The aim of this thesis was to blend pure silk fibroin solution with clopidogrel and a cross-linker {[H2C=C (CH3) CO2CH2CH2]2O} to investigate their properties for future tissue engineering applications. Biofilms were prepared by casting methods at room temperature. Scanning electron analysis, Fourier transform infrared spectroscopy analysis and X-ray diffraction analysis was carried out on the sample containing the highest ratio of the clopidogrel drug to characterize the biofilms.

SEM analysis indicates that cross-linker had an effect on the smoothness of the biofilms.

The samples containing higher amount of clopidogrel showed to be more non-thrombogenic when in contact with fresh human platelet samples showing high lesser prothrombin percentage in higher prothrombin time at 15, 30 and 60 minutes time interval when compared with samples with smaller ration of clopidogrel. The platelet adhesion test was observed using a light microscope and after proper staining using May Grunewald's eosine-methyl blue and Giemsa azur eosin-methylene it was shown to have no platelet adhesion property.

SEM analysis was done on the sample with 0.05g of clopidogrel and surface properties was observed where roughness was shown and little amount of the clopidogrel was seen on the surface of the biofilms. FTIR analysis showed peaks in the pattern showing the functional groups that are present. Also XRD patterns showed that the material is crystalline with the presence of peaks at various points because the silk fibroin was treated with clopidogrel a DEGDMA.

This Silk fibroin/clopidogrel/DEGDMA biofilms could be a potential material for biomedical applications.

Keywords: Silk fibroin; Di (ethylene glycol) di methacrylate; clopidogrel; Biomedical application; Anti-thrombogenicity

OZET

Bu çalışmanın amacı, dietilen glikol dimetakrilat ve klopidegral ile modifiye edilmiş ipek fibroin proteininden, kan uyumluluğu yüksek biyofilm sentez ve karakterizasyonu yapmaktır. Döküm yöntemi ile sentezlenen biyofilmler oda sıcaklığında vakum ortamda kurutuldu. Taramalı Elektron Mikroskobu, Fourier Kızılötesi spektroskopi analizi ve X-ışın kırımı analizleri ile karakterize edilen biyofilmlere, kan uyumluluğu testleride uygulandı.

Örneklerle STA kompakt cihazı ile kanda pıhtılaşma süresi tayini çalışılmıştır. Mevcut klopidegral miktarı arttıkça kan uyumluluğunun arttığı tespit edilmiştir. Yüksek miktarda klopidegral içeren örnekler, PT (saniye), APTT ve INR zamanla azalan değerlere sahip olduğu ve klopidegral miktarı azaldıkça 15, 30 ve 60 dakikada bu değerlerin daha yüksek olduğu gözlemlenmiştir. May Grunewald's eorine-metil mavisi ve Giemsa azur eosin-metilen ile boyanan örnekler Işık mikroskobu ile takip edilip biyofilimler üzerine hiçbir platelet yapışması olmadığı tespit edilmiştir.

Taramalı Elektron mikroskobu ile analizler 0.05 g klopidegral içeren biyofilmlere uygulanmış ve yüzeyin pürüzlü ve çok az miktarda klopidegral içerdiği gözlemlenmiştir. İpek fibroin proteinine ait fonksiyonel grupların varlığı FTIR spektrumunda gözlemlenmiştir. Dietilen glikol dimetakrilatın çapraz bağlayıcı etkisiyle biyofilmin kristal yapısında artış olduğu XRD patterni ile tespit edilmiştir.

Sonuç olarak, ipek fibroin / klopidegral / DEGDMA biyofilmleri, biyomedikal uygulamalar için potansiyel biyomateryaller olarak önerilmektedirler.

Anahtar Kelimeler: İpek Fibroin; Di (etilen glikol) dimetakrilat; klopidegral; Biyomedikal uygulamalar; Anti-thrombojeniklik

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SF Silk fibroin

DEGDMA Di (ethylene glycol) Di methacrylate

SEM Scanning Electroscope Microscopy

FTIR Fourier Transform Infrared Spectroscopy

XRD X-ray Diffraction

APTT Activated partial thromboplastin time

PT prothrombin time

TT Thrombin time

INR International normalized ratio

ADP Adenosine diphosphate

PT % Prothrombin percentage

RPM Rotation per minute

CHAPTER 1

INTRODUCTION

1.1 Silk Fibroin: A Material for Biomedical Application

The silk worm *Bombyx mori* belongs to a family of *Bombicidae* that produces silk fibers; the fibers are delicate twin thread of silk fibrin that tends to be coated with a sericin protective cover (Vasconcelos et al., 2012). This lepidopteran insect (*Bombyx mori*) is of great economic importance that has been of great source of solving biological problems (Mondal et al., 2007). The sericin protein is of great use because of the special properties it has such as an antioxidant, antibacterial, UV resistant, absorbs and releases moisture easily and it inhibits the activity of tyrosine kinase (Gulrajani, 2005). The silk fibrin is a natural polymer that has excellent properties of biocompatibility and great physical properties thus making it a good biological material for biomedical and tissue engineering applications ranging from skin to vascular grafts to substrates for mammalian culture (Minoura et al., 1995a). The fibroin protein has been used for wound dressing applications by regulating the exudates of wound surfaces providing a moist environment that can facilitate re-epitheliazation, remodeling of connective tissue and collagenization (Teramoto et al., 2008). Due to its bio-attuned capacity it also can be a bio-engineering base in the application of tissue wall and membrane repairs, blood vessel, muscle ligament and nerve gadget restoration, bone, tooth and cartilage restoration (Reddy and Prasad, 2011). However, before its use as a biomaterial the gum-like sericin content needs to be washed out during the purification process known as degumming, so as to avoid an opposing immune response when the material is implanted (Haifeng et al., 2006).

1.2 Properties of Silk Fibroin

1.2.1 Chemical Properties of Silk Fibroin

Fibrin created by spiders, larvae of Bombyx mori and many other insects are insoluble proteins. Silk in its raw state comprises of two main proteins, sericin and fibroin. Fibroin is the structural center of silk and a sticky material known as sericin covers it (Dyakonov et al., 2012). The fiber is made up of two cores of fibroin concealed with a layer of sericin (Perez-Rigueiro et al., 2001).

Generally, at a neutral pH silk fibroin is negatively charged and processes an isoelectric property of about 3.8 (Shailesh et al., 2010). The protein's random coil formation changes to a β-sheet formation due to hydrophobic interactions; the β-sheet structure is the main factor responsible for the protein's outstanding mechanical strength (Zhang et al., 2009). The high glycine and alanine components permits tight packing of the sheets, which leads to the silk's rigid structure that cannot be stretched in terms of tensile strength. A blend of this toughness and stiffness makes this protein a suitable material in various areas including biomedicine and textile manufacturing.

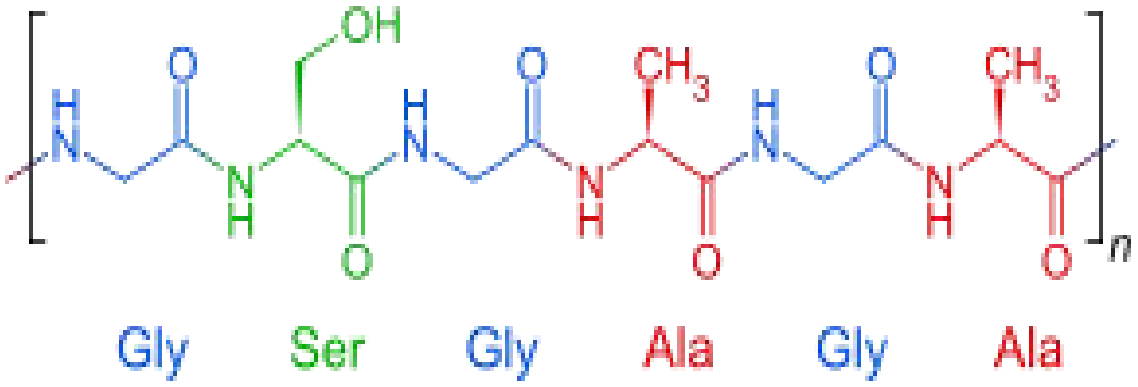

Figure 1.1: Primary structure of SF {Gly-Ser-Gly-Ala-Gly-Ala}$_n$ (Valluzzi et al., 1999)

Fibroin is known to be able to arrange itself in the basic structures namely, silk I, silk II, silk III. Silk I happen to be the natural form of silk fibroin also produced from Bombyx mori silk glands. Silk II is the organization of silk fibroin molecules in spun silk, which has higher strength and is usually used in various commercial applications. Silk III is a structure of fibroin which has been recently discovered, it basically forms in solutions of silk fibroin at an interface like water-oil interface and air-water interface etc. (Valluzzi et al., 1999).

1.2.2 Mechanical Properties

Silk is a versatile biomaterial with outstanding mechanical properties. Fibroin which is a component of the silk biomaterial is a fibrous protein that is composed of a parallel beta-sheet structure, composed of high amino acid with about 43% of glycine, 30% of alanine, and 12% of serine (Nikhom et al., 2012). Due to this unique structure and composition this gave rise to the thermodynamically stable and high tensile strength silk protein (Nikhom et al., 2012). β-sheets acts as a cross-link between the protein molecules, here the regular

structure of these sheets gives rise to a high tensile strength to the spider silk (Gama et al., 2012). Methanol treatment is widely used during the process so as to induce the β-sheet formation though this does not transform all molecular regions. Ethanol, 1-Ethyl-3-[-dimethylaminopropyl] carbodiimide (Alessandrino et al., 2008) hydrochloride (EDC), glutaraldehyde or gepinin also causes the transition from random coils to β-sheet configuration (Perez-Rigueiro et al., 2001; Oguz-Bayraktar et al., 2005;Lin et al., 2008; Silva et al., 2008). Research has shown that a regenerated silk fiber can retain its initial tensile strength for about 21 days within in-vitro culture conditions (Jin and Kaplan, 2003).

1.2.3 Biodegradation of Silk Fibroin

Silk is classified as non-degradable biomaterial according to the US's Pharmacodia definition. However, from research studies done so far, silk materials can be considered as a degradable biomaterial due to the degradation behavior of silk material is usually activated or caused by a foreign body response (Altman et al., 2003; Rossitch et al., 1987; Soong and Kenyon, 1984; Salthouse et al., 1977). Degradation behavior of silk fibroin does not lead to an immunogenic response from the host when compared to synthetic materials. Biodegradation is thus termed as the breaking down of polymer materials or any material into smaller compounds. Silk fibroin is vulnerable to bacterial and enzymatic degradation; the process of degradation tends to vary greatly, and the mechanisms are very complex. Often it comprises of physical, biological and chemical factors. Depending on the mode of degradation, silk fibroins can be classified as an enzymatically degradable polymer (Arai et al., 2004; Naira and Laurencina, 2007) due to the significant role that enzymes play in the degradation of silk fibroins. Due to their enzymatic degradability, unique properties including physico-chemical, mechanical and biological properties of silk fibroins have been extensively studied. Silk fibrin does degrade easily but its degradation lasts over a long period of time. Silk tends to lose its tensile strength within a year in vivo, and would not be recognized at the site of implantation within 2 years. However, the rate of degradation depends on the host recipient and the tissue implantation site.

1.2.4 Biocompatibility

B. mori silk fibers have been in use commercially as suture materials since the end of the 19th century and have proved to be effective biomaterials. There are evidences of adverse biological reactions; this of course raised concerns about the biocompatibility of silk fibers. Sericin, which is a glue-like protein present in the silk cocoon, holds the fibroin fibers together; this has been identified as the source of immunogenic reactions especially of un-degummed silk worm fiber (Soong and Kenyon, 1984; Panilaitis et al., 2003). However, when sericin is associated with the fibers it does bring about significant activation of macrophages (Panilaitis et al., 2003).

In vitro research studies examining macrophage response to fibroin concluded that silk in film (Santin et al., 1999) or fiber form (Panilaitis et al., 2003) did not elicit any significant macrophage activation, however there was macrophage activation in particulate form (Panilaitis et al., 2003), although the authors speculated that the size of the particulate was the cause of the effect. Various in vitro studies have shown that once sericin is extracted, fibroin supports cell attachment and proliferation for a variety of cell types (Gupta et al., 2007; Roh et al., 2006; Servoli et al., 2005, Unger et al., 2004; Minoura et al., 1995a; Jin et al., 2004; Inouye et al., 1998).

According to Minoura, N et all in 1995 stated that silk from the wild silkworm *Antherae apernyi,* contains the RGD sequence, and has been reported to support cell attachment and growth to a greater extent than B. mori silk (Minoura et al., 1995b). The use of water vapor rather than methanol to induce β-sheet transition may support better cell attachment and proliferation (Min et al., 2006).

Another study by Gotoh, K., et al suggested that sulphonated fibroin inhibits the replication of human immunodeficiency virus (HIV) in vitro (Gotoh et al., 2000). Also sericin films have been reported to support the attachment and growth of L929 murine fibroblasts (Minoura et al., 1995b) and human skin fibroblasts (Tsubouchi et al., 2005).

1.2.5 Thrombogenic Properties of Silk Fibrin

With the current development of blood-contacting materials or implantable devices used for health care purposes, it is extremely important to improve the hemocompatibility of the material by surface modification or re-design. At the moment that blood contacts with a foreign biomaterial surface, certain processes occurs which begins from where the material becomes competitive for plasma proteins adsorption and could progress to more complex issues if the blood-contacting material is not blood compatible (Chen et al., 2013).

In recent years many studies have focused and worked extensively to improve the hemocompatibility of biomaterials by surface modification (Chen et al., 2000; Chenoweth et al., 1983; Jindal et al., 1989; Kang et al., 1996; Vallar and Rival, 1996) which aims to reducing protein adsorption with the eventual goal of decreasing platelet adhesion.

Silk fibroin (SF) is a fascinating protein that is widely used for biomaterial applications. SF both in raw and regenerated versions have been used extensively in so many research in the past and ongoing research for various biomedical applications such as cell culture media, sutures (Zhu et al., 2008), anti-thrombogenic materials (Furuzono et al., 1999a; Furuzono et al., 2000b),wound healing applications (Chutipakdeevong et al., 2013), drug delivery applications (Hoffman et al., 2006a; Wang et al., 2007; Wang et al., 2007a; Wang et al., 2007b; Wang et al., 2008c), as biological scaffolds in tissue engineering (Ki et al., 2007; Nogueira et al., 2009), bone application (Hoffman et al., 2007b; Kim et al., 2007; Meinel et al., 2004a; Meinel et al., 2006b), cartilage tissue engineering (Hoffman et al., 2006c; Wang et al., 2005a; Wang et al., 2006b). SF happens to be an attractive promising biomaterial used for even small diameter arterial prostheses (Lovett et al., 2007; Nakazawa et al., 2011).

Unfortunately, pure SF has shown to have poor anti-coagulant activity as a blood contacting material when used alone (Tamada, 2003a). Hence, the issue of how to improve the hemocompatibility of silk fibroin based biomaterials used especially as blood contacting materials is desired by great scholars working in this field, when this important issue is tackled it will lead to a more effective and versatile application of SF. Various assays are used to test for their blood compatible properties today which could range from Hemolytic assay, anti-coagulant assay, platelet adhesion and activation assay blood coagulation time assay (PRT, APTT and PT).

However, the limitation of pure silk fibroin has gradually been overcome by consistent research like that of Vepari et al. who worked on this modification and reported increased anti-adhesion and antithrombotic properties of Poly (ethylene glycol) PEG- modified silk fibroin films. In this study, the silk fibroin film surfaces were PEGylated by reaction with cyanuric chloride-activated poly ethylene glycol (PEG), these surfaces were effective in enhancing anti-adhesion and anti-thrombogenicity on the materials intended for biomedical applications also providing a unique mechanical and tailorable degradation profiles of silk fibroin (Vepari et al., 2010).

Some researchers have studied the effect of heparin-grafting on a plasma treated electrospun silk fibroin fiber so as to investigate the biocompatibility of the SF fibers. They happened to find out using the In vitro coagulation time tests namely; APTT, PT, and TT assays, observations were noted that heparin-modified scaffolds were much higher in terms of blood compatibility than those of the pure silk fibroin scaffolds (Wang et al., 2011).

In addition, Tamada also investigated the sulfation of SF (Bombyx mori) using chlorosulfonic acid in pyridine. It was observed that blood coagulation was prevented by 0.5mg of sulfated fibroin in 1mL of blood when compared to the original pure fibroin which did not show any effect of anti-blood coagulation. It was also claimed that Anticoagulant activity of sulfated fibroin strongly depends on the amount of sulfate groups introduced into the SF material (Tamada, 2004b).

Also Yagi et al. prepared small-diameter vascular grafts of about 1.5mm in diameter and 10mm in length by coating a double-raschel knitted silk fiber graft with SF aqueous solution containing poly(ethylene glycol diglycidyl ether) which acted as a cross linking agent. After eight weeks after implantation of the grafts in rat abdominal aorta, they observed that there was no early formation of thrombosis in the study sample (Yagi et al., 2011).

1.3 Forms of Silk Fibroin

1.3.1 Silk Fibroin as a Bio-Film

Silk based biofilms have been produced in numerous researches, and they do have wide applications *in vivo* and *in vitro*.

Amol R et al work on silk based biofilms with applications as a novel wound healing agent. The bio-modified silk protein was incorporated with *centella asiatica* extract, polyvinyl alcohol and another sample was re-crystallized using 95% formic acid. These materials were arranged in various groups and were used to treat female wistar rats that had undergone acclimatization and surgery (Amol et al., 2012).

Pure silk fibroin films are brittle and stiff in a dry state, thus they need to be modified so as to change their physical and mechanical properties in the applications of a flexible system. Silk fibrin films are highly compatible with the human body. Similar to the human skin in the wet state, silk fibrin biofilms have good oxygen permeability which gives rise for its wide use in biomedical applications. However, pure silk fibrin films are soluble in water because of its random coil structures (Lu et al., 2009).

Silk fibrin based biofilms have been modified in various ways in order to enhance one property or the other. Glycerine (glycerol) is a simple polyol (sugar alcohol) compound which has been used to enhance the mechanical and physical properties of silk fibrin biofilms. Glycerine has the ability to reduce phase separation between polyvinyl alcohol (PVA) and silk in blending or to accelerate silk gelation. Recent studies has shown that blending of silk fibrin and glycerine had efficient result in the enhancing of the films properties, such as the silk fibroin crystallization behavior and elasticity of the films (Lu et al., 2009).

Composite biofilms has been seen recently in many studies; Witoo Luangbudnark et al worked with composite biomaterials. In their study, chitosan was blended with silk fibroin (CS/SF), the morphology, physicochemical properties, and biocompatibility of the fibroblast cells on the blend films was investigated (Luangbudnark et al., 2012).

1.3.2 Silk Fibroin as a scaffold

Vast development has been done in the fabrication of silk fibroin-based scaffolds for tissue engineering application. They have been found to be suitable materials that support adult stem cells differentiation towards ligament lineages along with its excellent biocompatibility and mechanical properties. Silk fibroin based scaffold are often times used in combination with other materials such for its various applications in tissue engineering.

Sponge scaffolds are important materials in tissue engineering applications because they provide a framework of interconnected pores with a high amount of surface area within a defined three-dimensional volume; thus giving room for cell attachment and tissue in growth.

Andreia vasconcelos et al worked with silk fibroin-based scaffolds for wound dressing applications, the scaffolds were blended with elastin and cross linked with genipin. The work was aimed at combining the excellent properties of silk fibroin combined with the elastin protein so they can have the ability to mimic the extracellular matrix (ECM). The samples were assessed for wound healing through the use of human full-thickness skin equivalents, wound burns were induced and samples showed great cytocompatibility with the human fibroblast (Vasconcelos et al., 2012).

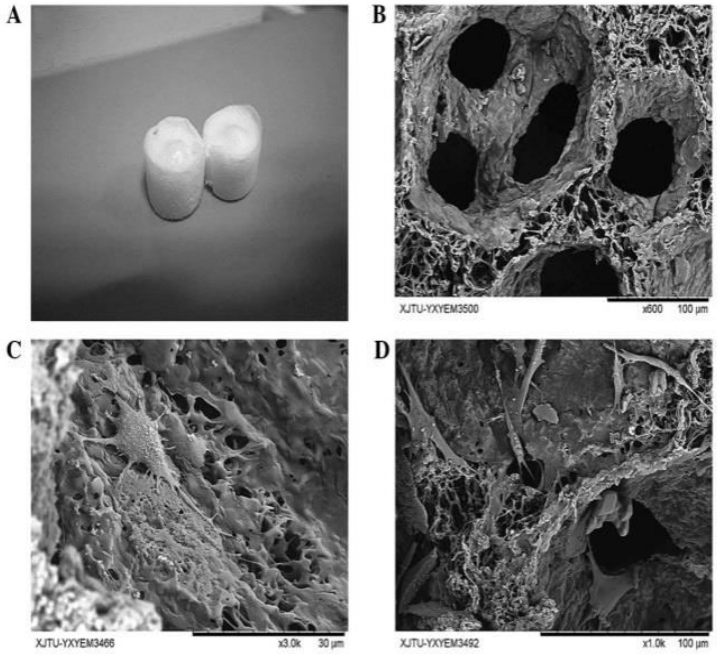

Figure 1.2: Images of a scaffold viewed under an SEM (Ji et al., 2013).

1.3.3 Silk Fibroin as a Nano Particle

A biologically derived and biodegradable Nanoscale delivery agent with no toxic byproducts is to function by allowing the delivery of the therapeutic without compromising efficacy. If the biologically derived carrier has chemical and/or structural characteristics that enhance uptake by the cells, then the carrier would improve efficacy tremendously (Anshu and Gupta, 2010).

A silk fibroin (SF)-based delivery vehicle (Gupta et al., 2009; Kundu et al., 2010; Zhang et al., 2007) is an example of such a biologically derived delivery system that overcomes barriers set by synthetic non-degradable nanoparticles made of silicone (Decuzzi et al., 2010; Tasciotti et al., 2008) or polyethylene glycol (Gabizon and Martin, 1997) and synthetic degradable particles made of polylactic acid and its analogs (Boxes 1 & 2) (Tong et al., 2010).

The biocompatibility properties and controlled degradation of silk fibroin makes it an ideal candidate in nanoscale drug-delivery development. Nano particles are tiny particles that are small enough to penetrate through small capillaries, thus giving room for enhanced cellular uptake of an encapsulated drug or therapeutic molecules (Anshu and Gupta, 2010).

Silk fibroin nanoparticles combined with insulin caused an enhancement in the half-life of insulin present in the human serum by 2.5-times, demonstrating the potential of SF conjugates for peptide/enzyme delivery (Yan et al., 2009). Due to their minute sizes they have great applications in drug delivery systems/applications and they serve in cell targeting for therapy purposes.

1.4 Clopidogrel as an Anti-thrombogenic drug

Clopidogrel anti-platelet agent used in conditions of cerebrovascular diseases, coronary artery diseases and peripheral vascular disease to inhibit blood clots and also to prevent myocardial infarction. Clopidogrel mechanism of action is by inhibiting the ADP receptor on platelet cell membranes. It is a pro-drug; it requires CYP2C19 in the liver for its activation and poor metabolizing of this enzyme may not result to an adequate antiplatelet activity (United States national library, 2015).

Drugs such as rhodamine B and Azoalbumin was embedded in nano-coatings of silk fibroin by Wang (Wang et al., 2007), also drugs like heparin and clopidogrel, where they were applied to vascular systems where they were applied to investigate the thrombogenic material of the modified silk fibroin. This application through this research showed that clopidogrel and paclitaxel were good materials that allowed cell attachment and viability of the human aortic endothelial cells and human coronary artery smooth muscle cells by inhibiting the proliferation of the smooth muscle cells and causing the retardation of the endothelial cells proliferation (Wang et al., 2008c).

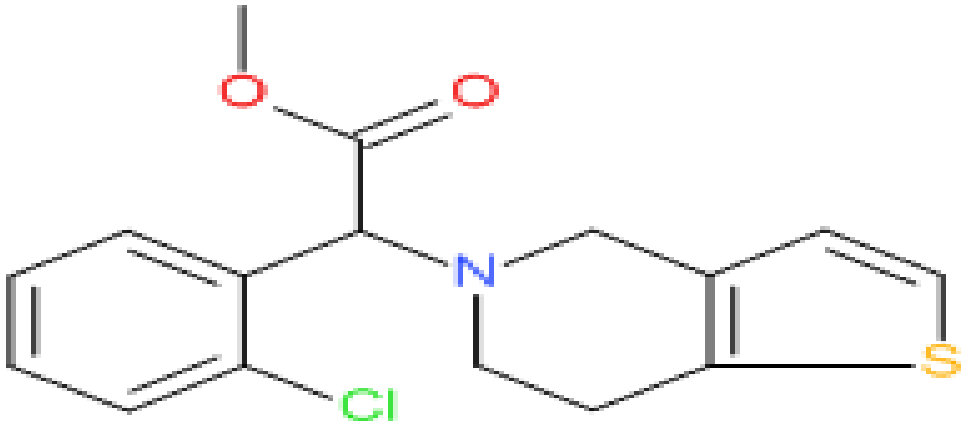

Figure 1.3: Chemical structure of clopidogrel (United States national library, 2015)

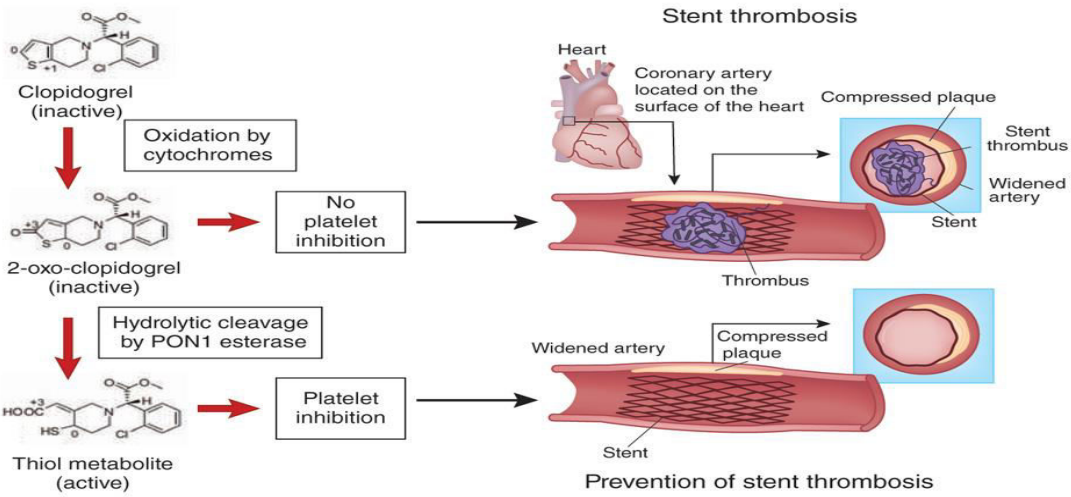

Figure 1.4: Metabolism of clopidogrel in vascular system (Topol and Schork, 2011).

1.5 Problem Statement

There is a continuous rise in the need for more research on silk-based biomaterials for biomedical and tissue engineering applications. More work is done in understanding the biocompatibility of these materials which is a priority in tissue engineering materials however we still face few challenges on materials that are intended to stay for a long time without causing any thrombogenic effect or otherwise when it's not needed. This work is centered on the development of silk-based biofilms blended with clopidogrel and cross-linked with Di (ethylene glycol) dimethacrylate that could be a potential material in tissue engineering applications especially in vascular systems applications where blood clotting is not required.

1.6 Aim of the Thesis

The aim of this thesis is to

- Synthesize silk fibroin solution blended with clopidogrel and Di (ethylene glycol) dimethacrylate so as to get a biofilms that will act as non-thrombogenic materials for biomedical applications.
- Perform various characterizations on the samples using FTIR, SEM, and XRD analysis so as to investigate their properties.
- Analyze their thrombogenic properties using blood coagulation test such as PT, APTT, INR and platelet adhesion test.

CHAPTER 2

MATERIALS AND METHODS

2.1 MATERIALS

Bombyx mori silk cocoon were purchased at the local store at buyuk han, they were used to obtain a pure silk fibrin after the purification process, sodium carbonate (Na_2Co_3) was used in the degumming process, calcium chloride ($CaCl_2$) purchased from Sigma Aldrich was used in the dissolution process along with 98% ethanol and de-ionized water in the electrolyte solution. Sigma–Aldrich dialysis-tubing cellulose membrane was used in dialysis of the dissolved silk fibroin. Aldrich 95% Di (ethylene glycol) dimethacrylate was used as a cross linker for the silk fibroin based bio films.

Figure 2.1: Cut raw silk cocoons

2.2 METHODS

2.2.1 Silk Fibroin Purification

2.2.1.1 Degumming process

Degumming process involves the removal of the sericin content from the silk cocoon. First the silk cocoons were cut into small pieces using a pair scissors and then weighed. 0.1M of sodium carbonate was prepared, and was dissolved in 500ml of de-ionized water. The weighed cocoon was placed in a beaker containing the 0.1 M Na_2Co_3 at 1g/100ml (w/v) at -70^{o}c on a magnetic hot plate stirrer at the speed of 1.5 rpm through three sessions for 3

hours each. The degummed silk fibers was washed thoroughly with de-ionized water and dried at room temperature in a clean room environment.

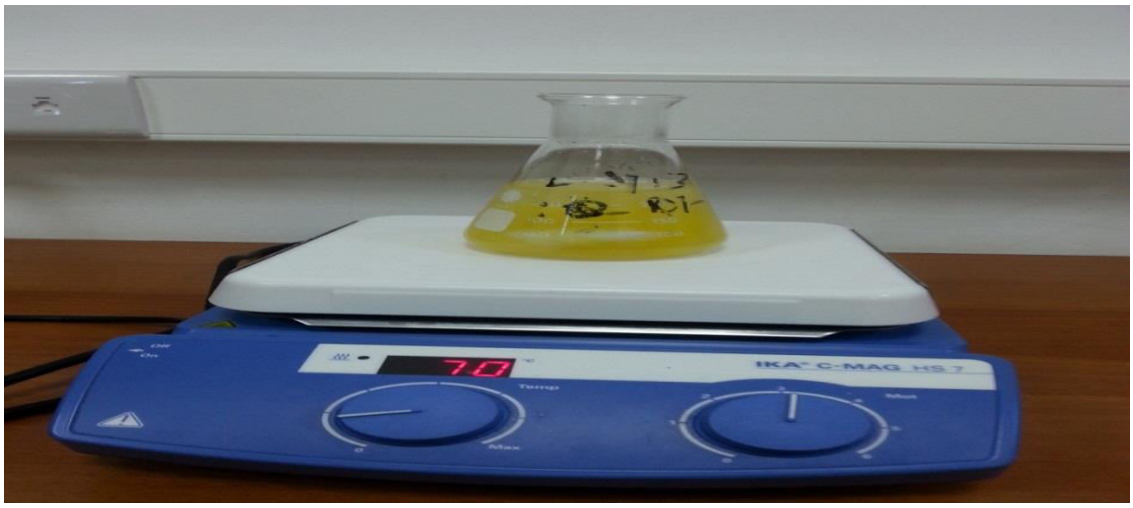

Figure 2.2: Degumming process

2.2.1.2 Dissolution process

This process involves dissolving of the degummed silk fibers so as to have an aqueous solution from the silk fibroin, this works on the principle if breaking up the long polypeptide chain into shorter chain length to get an aqueous silk fibroin solution. This is performed by the dissolving of silk fibrin with nC_2H_5OH:nH_2O:$nCaCl_2$ (2:8:1) molar ratio at 75^oc with continuous stirring in an air tight beaker until a total dissolution of the fiber so as to get an aqueous silk fibroin electrolyte solution.

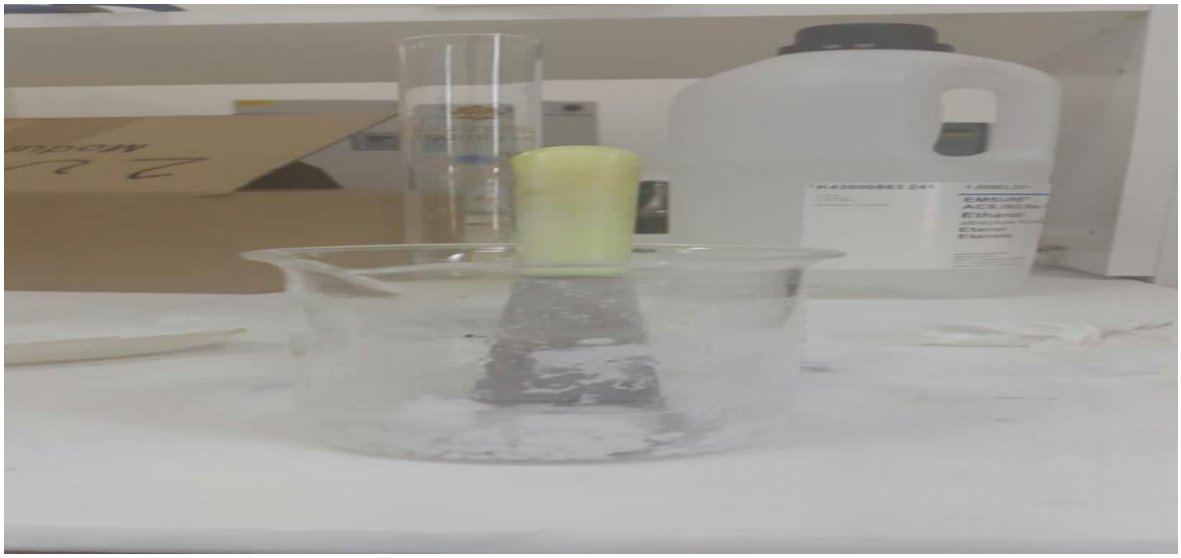

Figure 2.3: Preparation of electrolyte solution ($CaCl_2$, deionized water and ethanol)

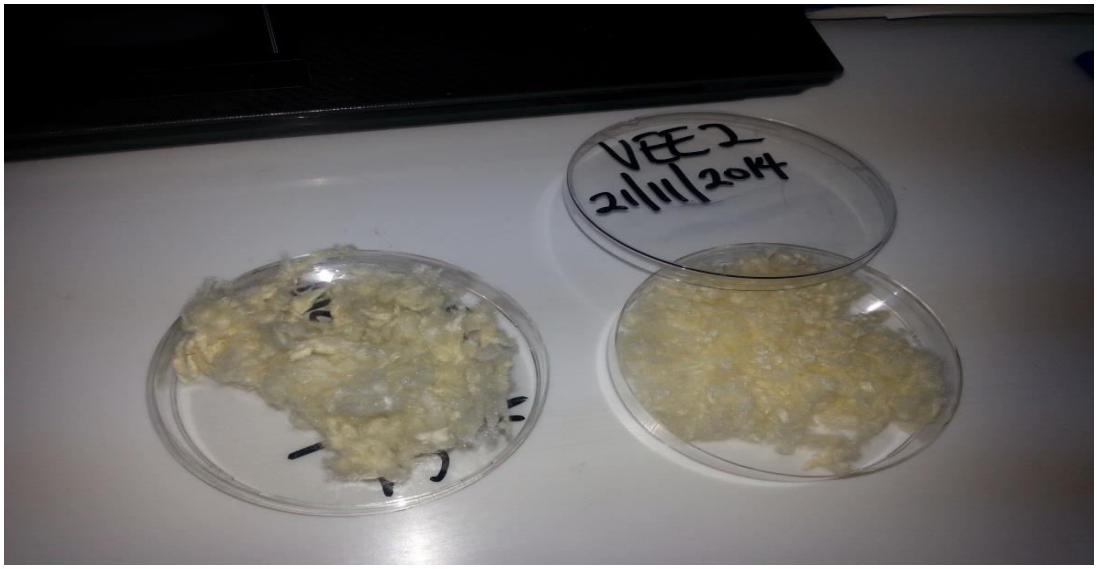

Figure 2.4: Dried degummed silk fibers

Figure 2.5: Prepared electrolyte solution of nC_2H_5OH:nH_2O:$nCaCl_2$ at 75^oc

Figure 2.6: Dissolved 6% silk fibers in the electrolyte solution

2.2.1.3 Dialysis of the aqueous silk solution

This procedure involves the removal of ions obtained during the dissolution process so as to obtain a pure silk fibroin solution. The 6% dissolved silk fibrin solution is poured into a Sigma–Aldrich dialysis-tubing cellulose membrane and tying it properly so as to avoid spillage and this is placed in a 5000 ml beaker containing de-ionized water. This was placed on a magnetic stirrer machine at 0^oC and 1.5 rpm. This will allow the ion to diffuse out of the silk/electrolyte solution through the process of diffusion using the cellulose as a semi-permeable membrane. This procedure was repeated for three times and 3 hours each

with continuous stirring, at every 3 hours the water was changed. At the end of this process a pure silk fibroin solution was ready for the biofilms preparation.

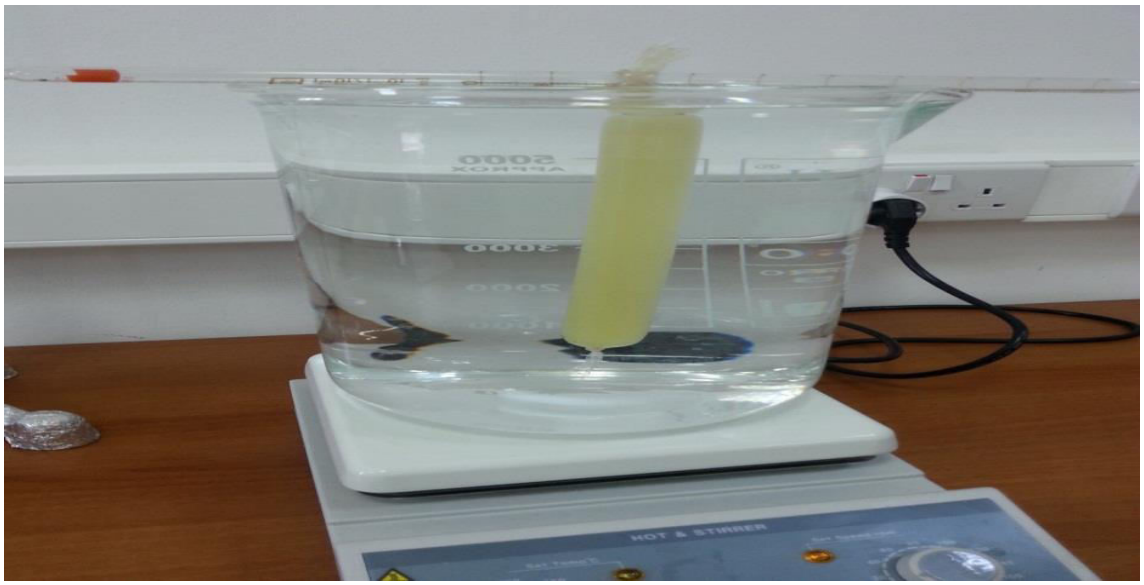

Figure 2.7: Aqueous silk fibroin electrolyte solution in tubing cellulose membrane tube

2.2.2 Biofilms preparation process

To find out optimum conditions of the SF biofilm, clopidogrel was prepared in the presence of Di ethylene glycol di methacrylate which acts as a cross-linking agent and stirred. The heterogeneous solution involved the preparation of 4 ratios of SF/Clopidogrel blend in small glass bottles; they were placed on the magnetic stirrer machine and were stirred using a magnetic Fischer for 5 minutes at 0^{o}C and1.0 rpm.

Table 2.1: Table showing ratios of SF/Clopidogrel blend

Study sample	**SF Solution(ml)**	**Clopidogrel (g)**	**[H2C=C(CH3)CO2CH2CH2]2O (ml)**
S20	2	0.01	0.20
S21	2	0.02	0.20
S22	2	0.03	0.20
S23	2	0.04	0.20

All the blended mixtures was dropped in their respective labeled glass slides, the glass slides were sterilized using methanol to avoid contamination. The samples were placed in a clean dust-free environment to dry for 2 days to allow samples to dry.

After drying, the samples on the glass slides were placed in Petri-dishes containing 70% methanol, the methanol treatment was used to induce the β-sheet on the silk biofilms, the biofilms were removed using sterilized forceps and were placed in sterilized and labeled Petri-dishes and left to dry at room temperature.

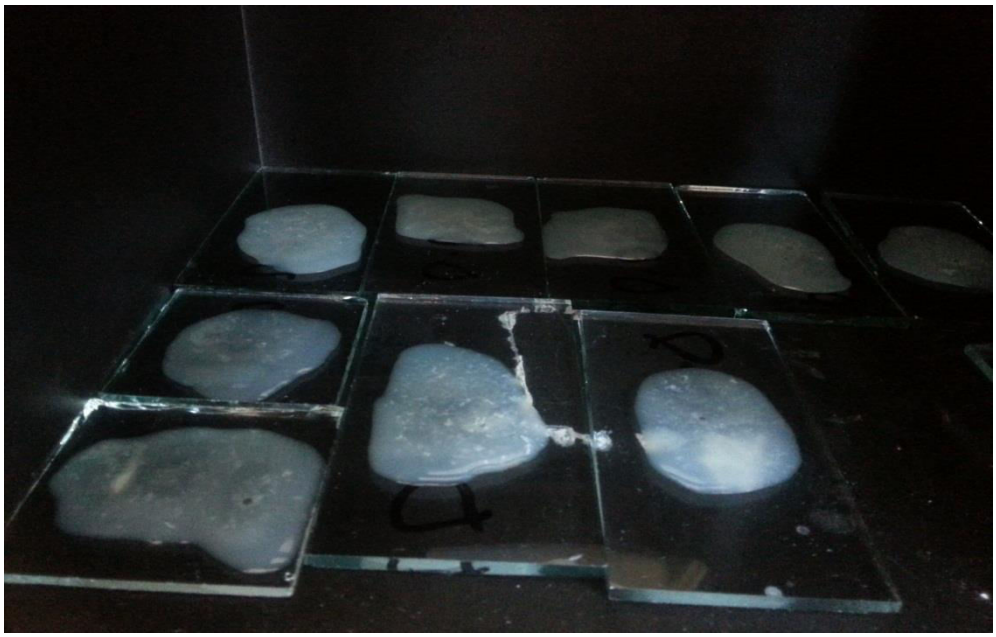

Figure 2.8: Blended biofilms left to dry

2.2.3 Sterilization

The samples were sterilized with hydrogen peroxide prior to further analysis at the sterilization unit of Near East hospital, Lefkosa.

The hydrogen peroxide solution was introduced into a vacuum chamber thereby creating a plasma cloud. Hydrogen peroxide inactivates the microorganism that may be present on the materials by oxidizing their cellular components.

An energy source is required for the process to occur and when it was turned off, water vapor and oxygen were formed, hence absence of toxic residues or harmful emissions. The samples were wrapped before sterilization; the sterilization was maintained at a temperature of 40◦C -50◦C and takes between 45-55 minutes to complete a cycle

2.2.4 *In vitro* Blood coagulation test

The activated Partial Thromboplastin Time percent (APTT) and Pro-Thrombin Time (PT) are indicators that evaluate the efficiency of both the common coagulation pathway and the "Extrinsic" 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. INR is the standardized test, this standardize the result of the PT and APTT no matter the testing result thus making it easy for doctors to understand the clotting times irrespective of the lab and test method. Studies has shown that the shortening of the PTT time might increase the risk of thrombo-embolism.

In this study, SF based biofilms were prepared for plasma coagulation time and was detected by measuring the activated partial thromboplastin time (APTT), prothrombin time (PTT), and INR by STA Compact Hemostasis System equipment, Stagio, US.

Process: 2 study blood samples of healthy persons were used so as to compare the effect of the biofilms in the plasma. The biofilms (S20 and S23) were cut in equal shapes about 1cm^2, samples were incubated with 450 µL healthy human blood plasma in a transparent cuvettes (labeled P1, P2, P3, P4, P5, P6 and P7) with films based on their drug content at static temperatures for 15, 30 and 60 minutes and the clotting times were obtained by the clot detection instrument STA Compact.

Figure 2.9: STA Compact, Stagio, USA

2.2.5 In-vitro Platelet Adhesion Studies

Samples were immersed into the 450µl fresh human platelet-rich plasma (PRP) from healthy a donor (provided by Near East University Hospital, Lefkosa) with a platelet concentration under static condition for 30 minutes. The platelet was centrifuged at 2200 rpm for 5 minutes. The contacting PRP was removed and samples were stained with May Grunewald's eosine-methyl blue for 5 minutes and washed with distilled water. It was then stained with Giemsa Azur eosin-methylene blue solution with 1:9 ratio of Giemsa to PBS solution for 10 minutes and then rinsed with distilled water. The samples were dried on the glass slides for about 15 minutes and they were viewed under the light microscope. The Method was used to determine the platelet adhesion on the surface of biofilms. The adhesion was evaluated by using light microscope for number.

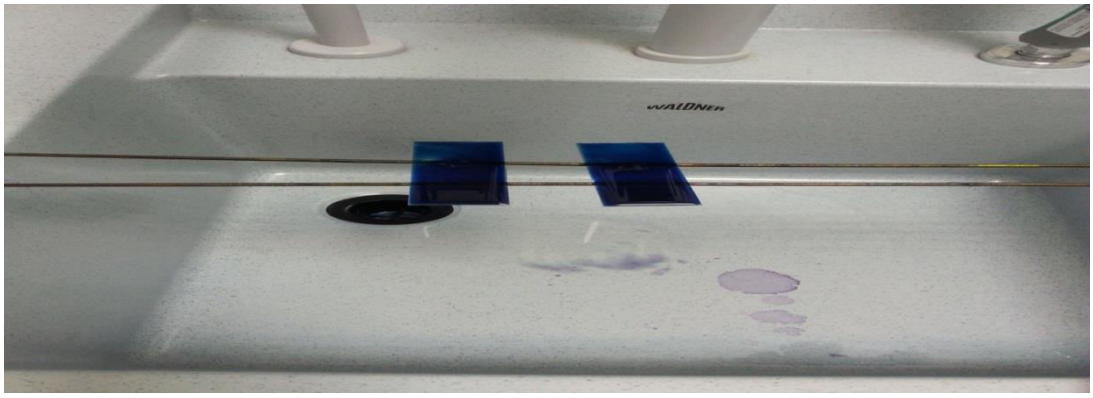

Figure 2.10: May- Grunewald's eosine-methyl blue staining for 5 minutes

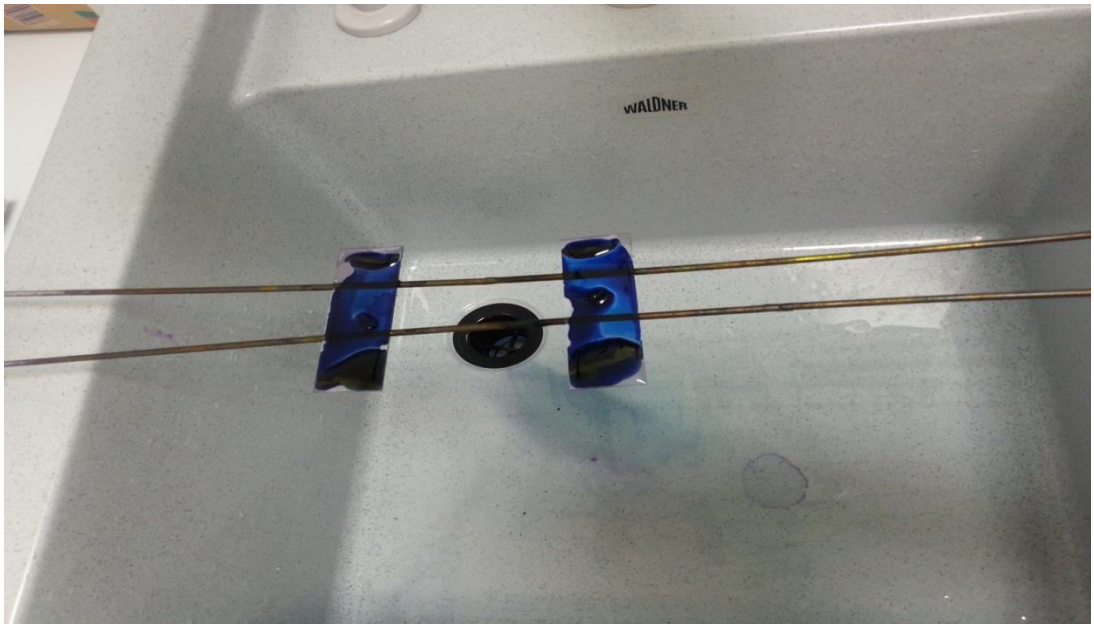

Figure 2.11: Giemsa azur eosin-methylene blue staining for 10 minutes

2.2.6 Scanning Electron Microscopy Analysis

Scanning electron microscopy device work on the principle of using electron beams to obtain images from materials. The electrons interact with atoms in the sample and they produce different type of signals that can be detected by the microscope which contains information about the surface topography and composition of the sample (McMullan, 2006).This analysis was carried out at TUBITAK-Marmara research center at Gebze, Istanbul, Turkey using a SEM M- Jsm- 6510 model at an acceleration voltage 10kV. The device produces images of the samples by focusing a beam of electrons on it and samples were sputter-coated with gold to prevent charging.

2.2.7 Fourier Transform Infrared Spectroscopy (FTIR) Analysis

Fourier transform infrared spectroscopy (FTIR) analysis is used to obtain an infrared spectrum of emission, photoconductivity, absorption or Raman scattering of solid, liquid or gas. This analysis was done using the FTIR K-124 device at TUBITAK-Marmara research center at Gebze, Istanbul, Turkey.

2.2.8 X-Ray Diffraction (XRD) Analysis

X-ray diffraction analysis was carried out at TUBITAK-Marmara research center at Gebze, Istanbul, Turkey using a Shimadzu XRD-M6 model diffractometer with a Cu- X-ray tube (L=1.5405Ao). The diffractometer scans at the rate of 2◦/min within the region of 2θ and resulting diffraction intensity curves obtained was produced.

CHAPTER 3

RESULTS AND DISCUSSION

3.1 *In vitro* Blood coagulation test

This test was done for the purpose of understanding the time taken for the clotting to occur (blood coagulation) so we can evaluate the effectiveness of our materials. The tables below shows the result of the *in vitro* clotting time of prothrombin time (PT), activated partial thromboplastin time (APTT), and INR test.

Table 3.1: Standard clotting time for a healthy person without thinning drugs

APTT	PT (SEC)	PT (%)	INR
23.6 -35.2	11.5-13.5	50-150	0.80-1.10

Table 3.2: Clotting time of study persons at Zero minute

Sample	APTT	PT (SEC)	PT (%)	INR
P1	36.3	13.2	100	1.00
P2	29.1	12.9	106	0.97
P3	30.6	13.1	97	1.02
P4	30.8	13.2	95	1.03
P5	31.9	13.9	86	1.10
P6	32.6	12.8	102	0.99
P7	30.2	13.4	93	1.05

Table 3.3: Results of the blood coagulation test of study samples of 0.01g of clopidogrel

Samples	APTT (SEC)	PT (SEC)	PT (%)	INR
PI				
15 MINUTES	**36.2**	**13.0**	**104**	**0.98**
30 MINUTES	**36.5**	**13.1**	**102**	**0.99**
60 MINUTES	**35.8**	**12.5**	**113**	**0.93**
P2				
15 MINUTES	**29.5**	**13.1**	**102**	**0.99**
30 MINUTES	**29.6**	**12.9**	**106**	**0.97**
60 MINUTES	**29.2**	**12.8**	**107**	**0.96**
P3				
15 MINUTES	**29.8**	**13.5**	**91**	**1.06**
30 MINUTES	**31.2**	**14.6**	**79**	**1.17**
60 MINUTES	**31.1**	**14.4**	**81**	**1.15**
P4				
15 MINUTES	**30.2**	**14.0**	**85**	**1.11**
30 MINUTES	**30.7**	**14.4**	**81**	**1.15**
60 MINUTES	**30.2**	**14.1**	**84**	**1.12**
P5				
15 MINUTES	**31.5**	**13.3**	**94**	**1.04**
30 MINUTES	**31.8**	**14.4**	**81**	**1.15**
60 MINUTES	**32.3**	**15.6**	**70**	**1.27**
P6				
15 MINUTES	**32.7**	**13.3**	**94**	**1.04**
30 MINUTES	**33.3**	**13.1**	**97**	**1.02**
60 MINUTES	**32.7**	**13.4**	**93**	**1.05**
P7				
15 MINUTES	**30.7**	**14.0**	**85**	**1.11**
30 MINUTES	**30.2**	**13.3**	**94**	**1.04**
60 MINUTES	**29.9**	**13.9**	**86**	**1.10**

Table 3.4: Results of the blood coagulation test of study samples of 0.05g of clopidogrel

Samples	APTT (SEC)	PT (SEC)	PT (%)	INR
P1				
15 MINUTES	**36.7**	**13.4**	**97**	**1.02**
30 MINUTES	**37.0**	**13.3**	**99**	**1.01**
60 MINUTES	**36.9**	**13.2**	**100**	**1.00**
P2				
15 MINUTES	**29.6**	**13.3**	**99**	**1.01**
30 MINUTES	**29.6**	**13.3**	**99**	**1.01**
60 MINUTES	**2.4**	**13.2**	**100**	**1.00**
P3				
15 MINUTES	**30.7**	**13.6**	**90**	**1.07**
30 MINUTES	**31.0**	**14.0**	**85**	**1.11**
60 MINUTES	**31.0**	**14.7**	**78**	**1.18**
P4				
15 MINUTES	**30.8**	**13.0**	**98**	**1.01**
30 MINUTES	**30.6**	**14.8**	**77**	**1.19**
60 MINUTES	**30.2**	**14.7**	**78**	**1.18**
P5				
15 MINUTES	**31.6**	**14.3**	**82**	**1.14**
30 MINUTES	**31.9**	**14.4**	**81**	**1.15**
60 MINUTES	**31.4**	**16.0**	**67**	**1.31**
P6				
15 MINUTES	**32.8**	**12.2**	**112**	**0.93**
30 MINUTES	**32.7**	**13.3**	**94**	**1.04**
60 MINUTES	**33.0**	**13.5**	**91**	**1.06**
P7				
15 MINUTES	**30.5**	**14.2**	**83**	**1.13**
30 MINUTES	**30.4**	**14.9**	**76**	**1.20**
60 MINUTES	**30.5**	**14.6**	**79**	**1.17**

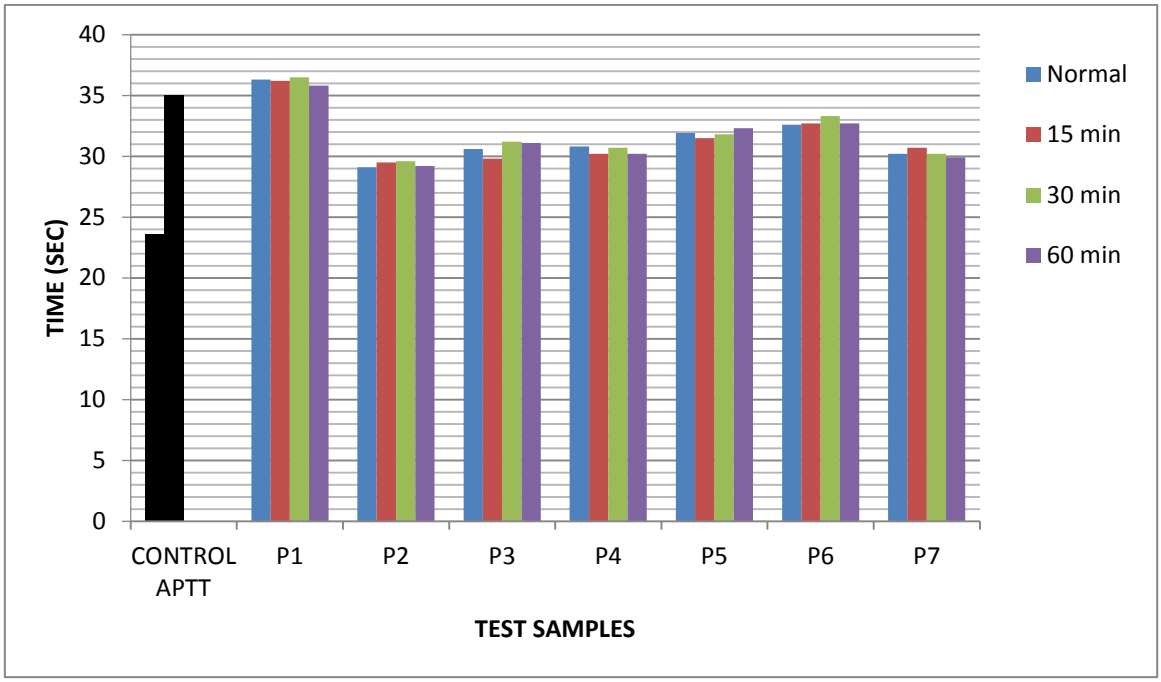

Figure 3.1: Graphical representation of APTT test for 0.01g of clopidogrel samples

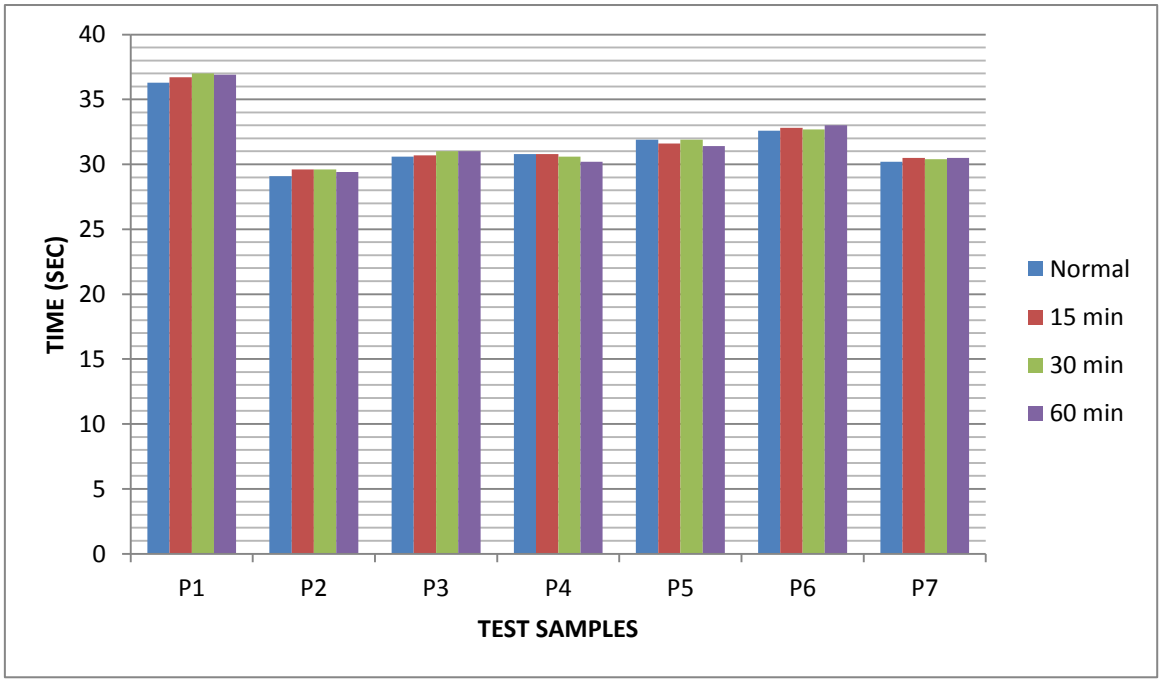

Figure 3.2: Graphical representation of APTT test for 0.05g of clopidogrel samples

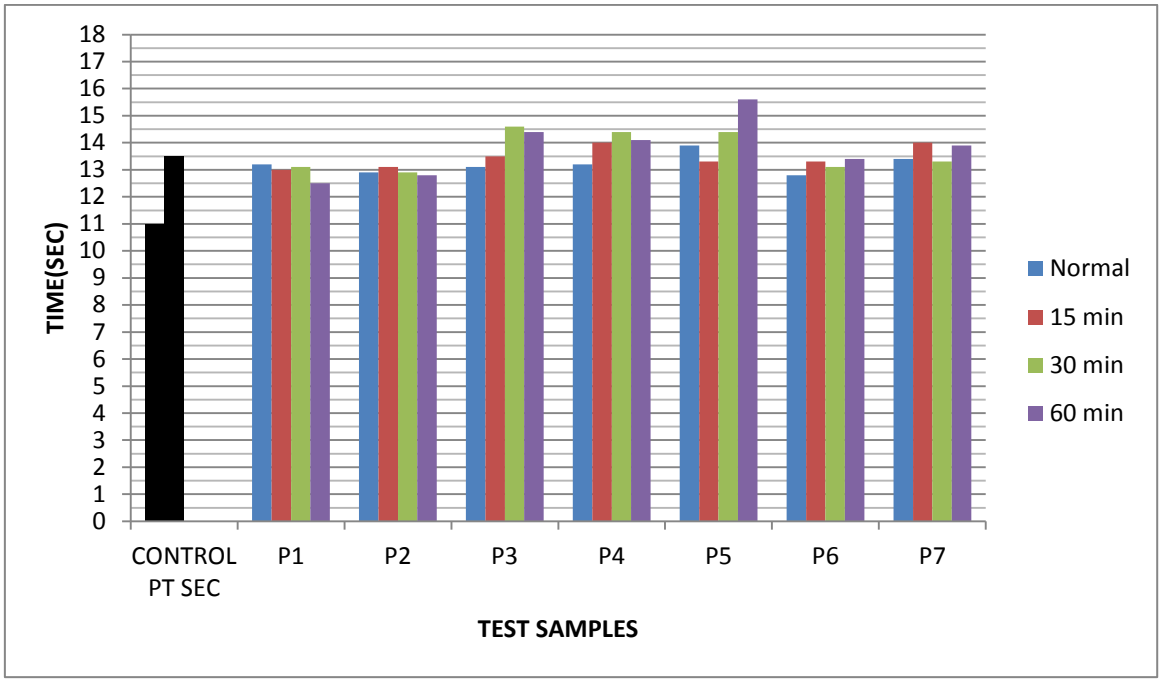

Figure 3.3: Graphical representation of PT (SEC) tests for 0.01g of clopidogrel samples

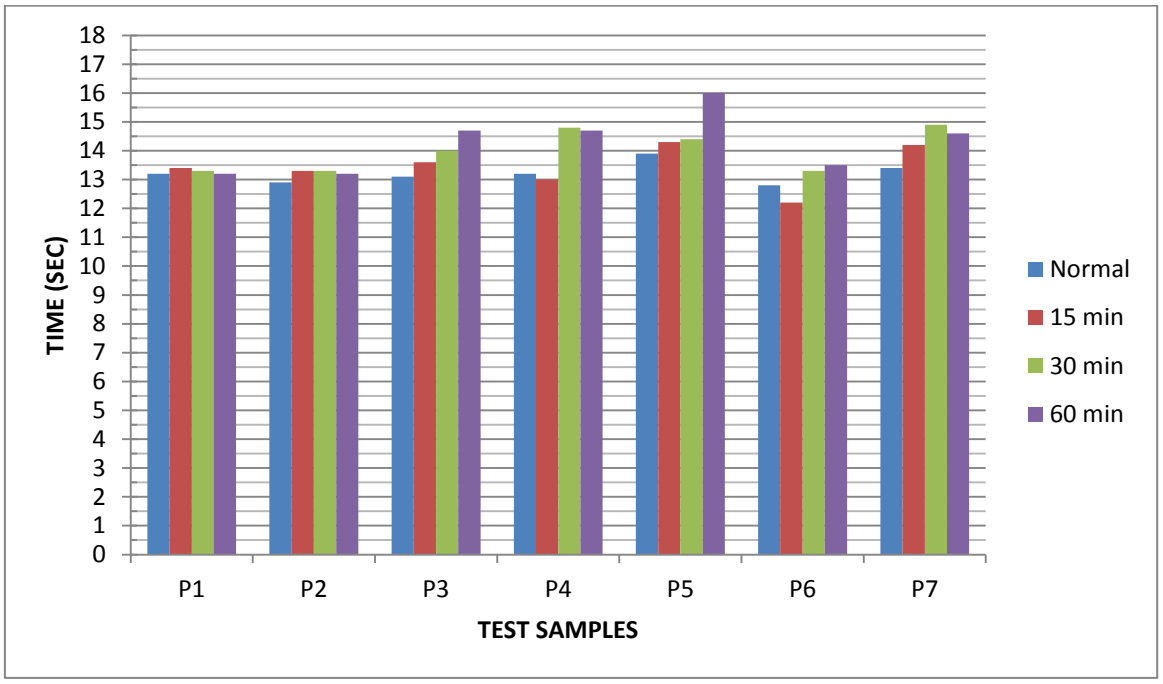

Figure 3.4: Graphical representation of PT (SEC) test for 0.05g of clopidogrel samples

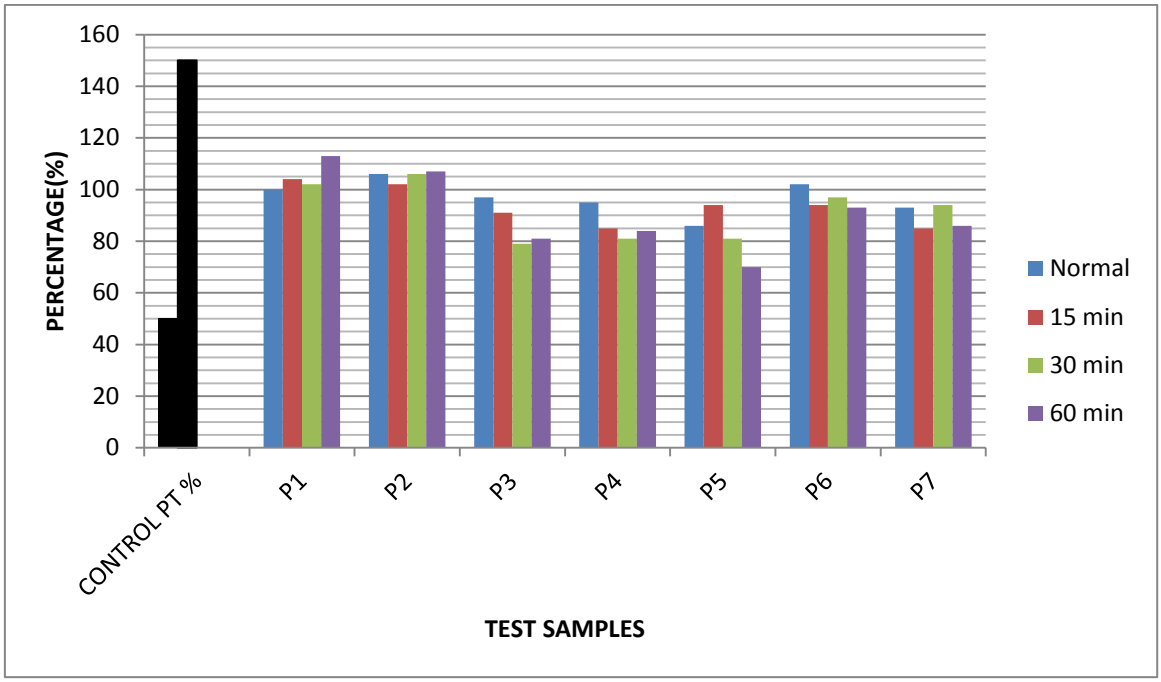

Figure 3.5: Graphical representation of PT (%) test for 0.01g of clopidogrel samples

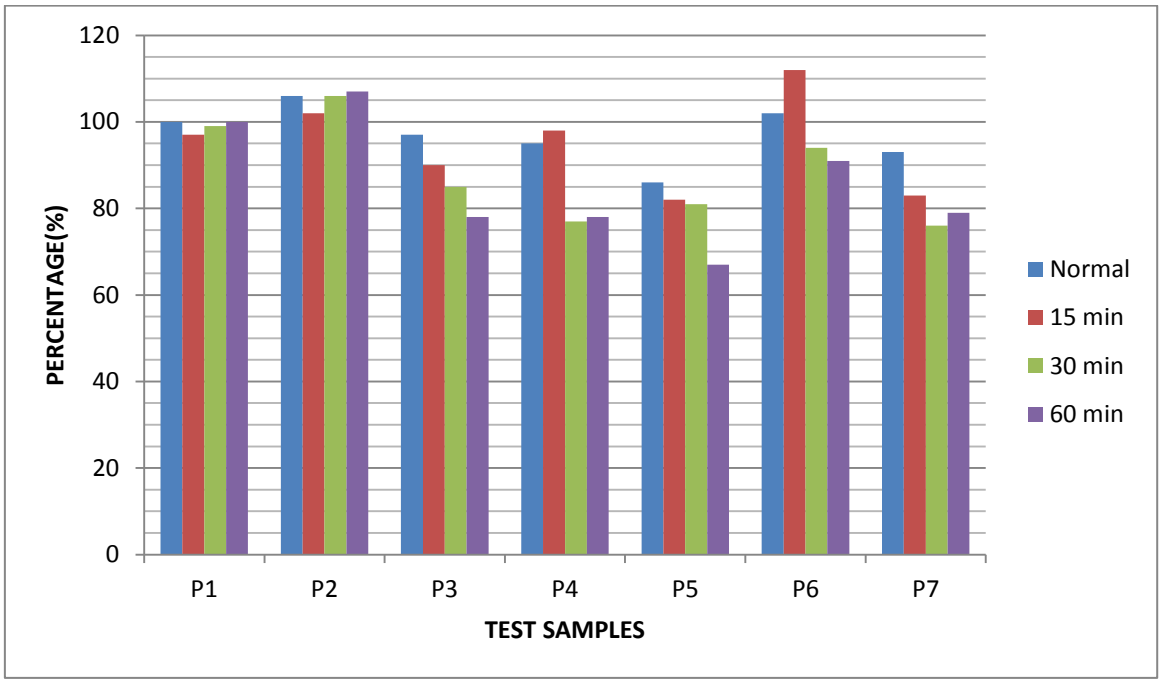

Figure 3.6: Graphical representation of PT (%) test for 0.05g of clopidogrel samples

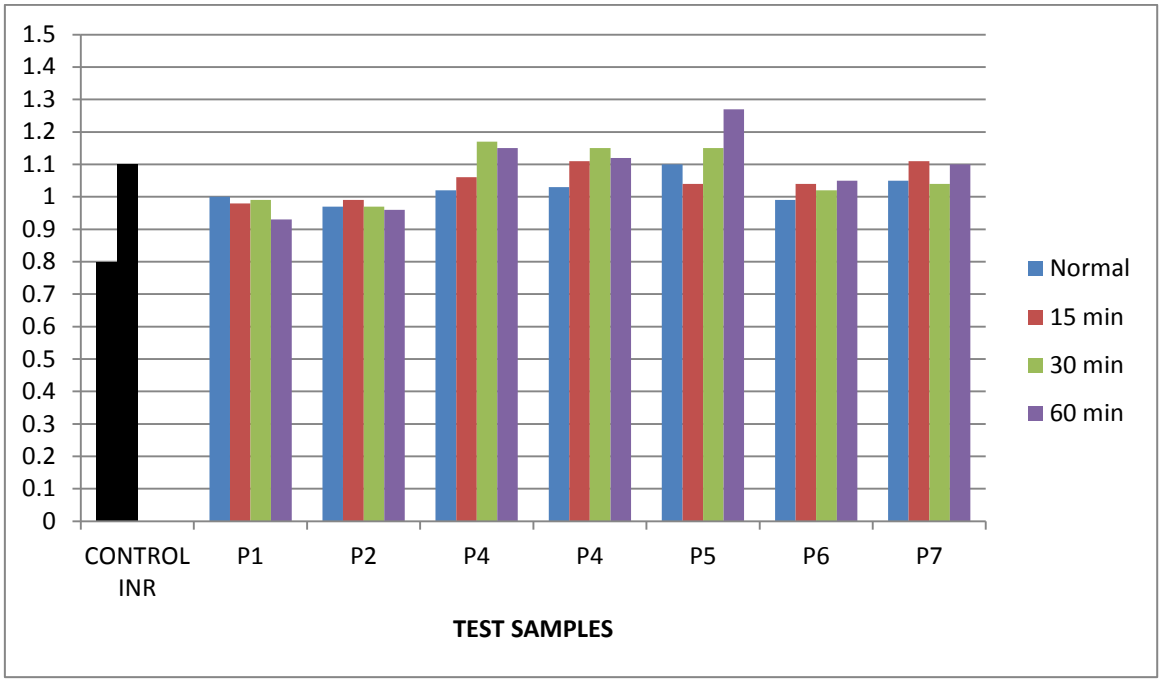

Figure 3.7: Graphical representation of INR test for 0.01g of clopidogrel samples

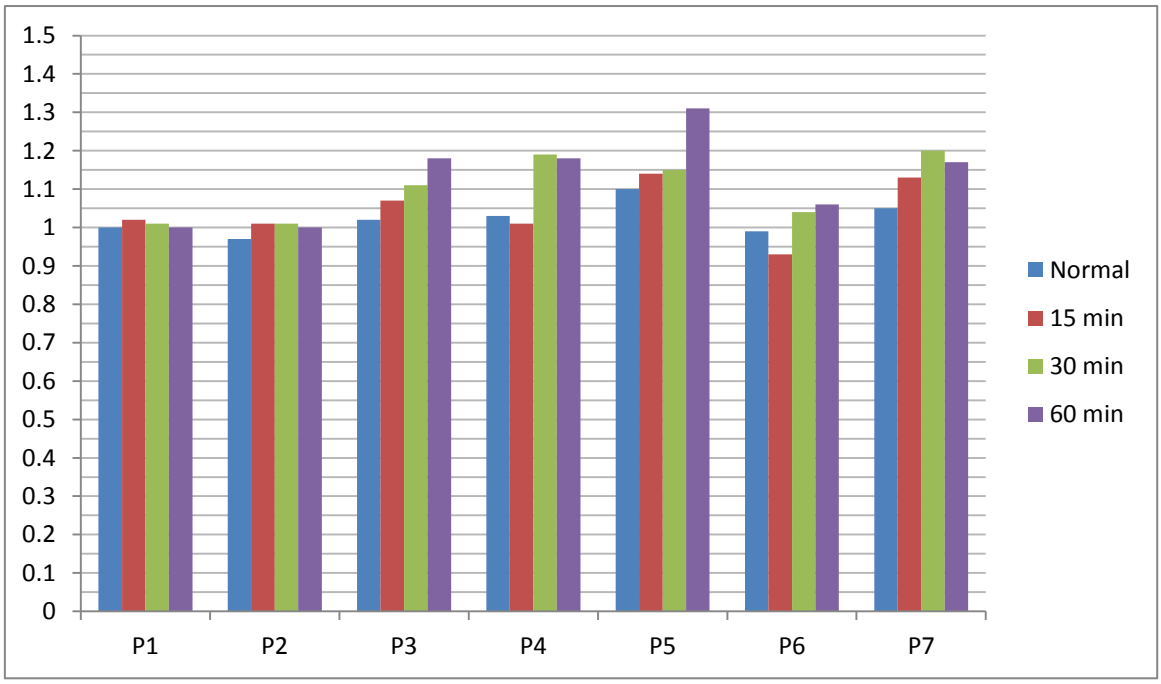

Figure 3.8: Graphical representation of INR test for 0.05g of clopidogrel samples

The samples were evaluated for their blood compatibility, using Hemocompatibility assays such as APTT, PT, INR and Platelet adhesion test. Blood samples of healthy persons were centrifuged and the derived plasma was labeled P1 to P7, plasma samples without the biofilms were used as the control samples and those containing the cut biofilms were used as the study groups. The result showed that plasma samples with clopidogrel had clotting time that was slightly increased from the person's normal clotting time at zero minute and a greater increase occurred with the plasma samples with 0.05g of clopidogrel blended films. The black bar present on the graphs represents the standard ranges of the normal PT tests. The standard APTT time ranges from 23.6-35.2 seconds, which varies from lab to lab and country to country. Thus any result that reads higher than 35.2 seconds is considered to be having great blood coagulation as a result of anti-coagulant activity or any blood thinning drug. Plasma samples containing the 0.01g and 0.05g of clopidogrel blended films were observed to have slight increase of the APTT when compared to their standard APTT ranges at zero minute, however, the number readings shown on the table 3.3 and 3.4 and the graphical representation on figure 3.1 and 3.2 especially in the APTT test showed very little significance in the increase and that could be as a result of the low amount of clopidogrel blended with the silk film. This same effect occurred in the study of Tamada, where he researched on the sulfation of Silk fibroin (Bombyx mori) using chlorosulfonic acid in pyridine. He observed that blood coagulation was prevented by 0.5mg of sulfated fibroin in 1mL of blood, while original fibroin did not show any effect. He also claimed that Anticoagulant activity of sulfated fibroin is strongly dependent on the amount of sulfate groups introduced the silk fibrin sample (Tamada, 2004b).

Based on standard medical range, PT% ranges from 50 -150%. However, various figures depending on the hospital or country could be given but they are all slightly different from themselves. As the PT percentage approaches 150 and above, it shows a great blood coagulation activity and vice versa. For all blood samples it can be observed that when 0.01g of clopidogrel was placed in the plasma at different times, the PT percentage dropped from the healthy persons standard ranges to a lower percentage with P5 in table 3.3 and 3.4 recording the highest reduction even after adding of 0.05g of clopidogrel contained-biofilms in the fresh plasma. All other samples showed a reduction as well which could be seen, with some higher than the other.

PT SEC recorded showed an increase in time when the samples were placed in 0.01g and 0.05g of clopidogrel. The Normal range of PT SEC ranges from 11-13.5 seconds and an increase in the time indicates an intake of an anti-coagulant drug or food that enhances anticoagulation activity. Due to the presence of clopidogrel which is an anticoagulant drug the effect was seen despite its minute amount in the silk fibroin biofilms, with P5 in table 3.3 and 3.5 also recording the highest time and P2 recording the lowest, all other showed an increase in time when 0.01g of clopidogrel blended biofilms were added and further increased when 0.05g of clopidogrel blended biofilms were placed in the plasma. Though the values may differ in their effect with clopidogrel, this could be attributed to the difference in genetic and bodily physiology.

An increase in INR result above 1.1 based on the standard values from few laboratories, indicates anticoagulation activity, also its value is dependent on the PT sec, PT percentage and APTT values, so therefore patients with an increased values of PT and APTT, following a decreased PT percentage will have a high INR result which indicates poor blood clotting and those with low PT and APTT following an increase in PT percentage will show a reduced INR result which indicates blood coagulation activity. Based on the result gotten from the blood coagulation test this effect could be observed in table 3.3 and 3.4 respectively.

Even with all clear limitations samples containing 0.05g of clopidogrel showed more anticoagulation activity compared to samples with 0.01g of clopidogrel, owing to the fact that clopidogrel which act as an anticoagulant to blood samples was higher in quantity in those samples biofilms that were more effective.

3.2 Platelet Adhesion Test

After proper staining of the films and drying followed by viewing under a light microscope, there was no visible platelet adhesion on all of the biofilms stained; this is probably due to the effect of the Di (ethylene glycol) di methacrylate and the clopidogrel present in the film as shown in Figure 3.9 and figure 3.10. This result could also be linked to the Studies by Vepari et al. who also reported increased anti-adhesion and antithrombotic properties of Poly (ethylene glycol) modified silk fibroin films (Vepari et al., 2010).

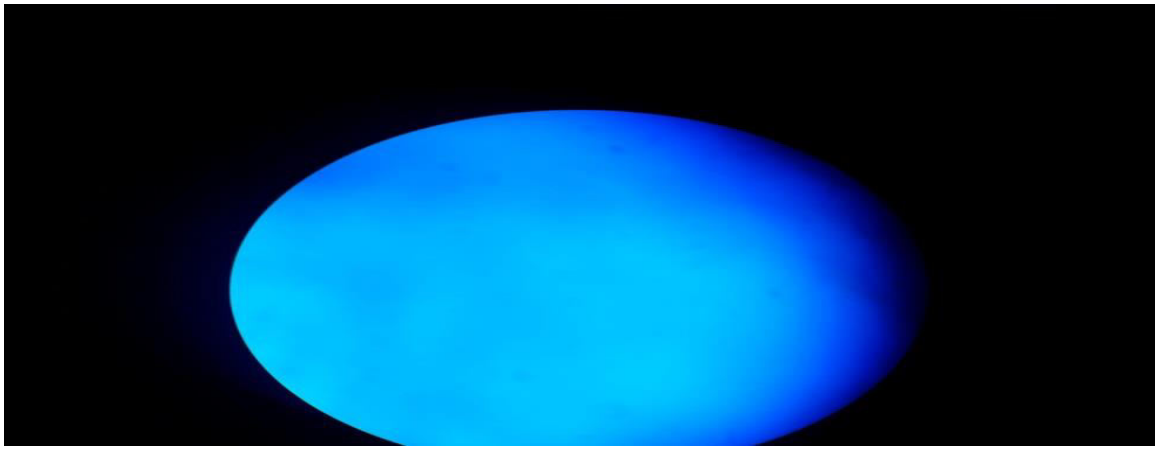

Figure 3.9: Stained biofilms with 0.01g of clopidogrel viewed under the light microscope

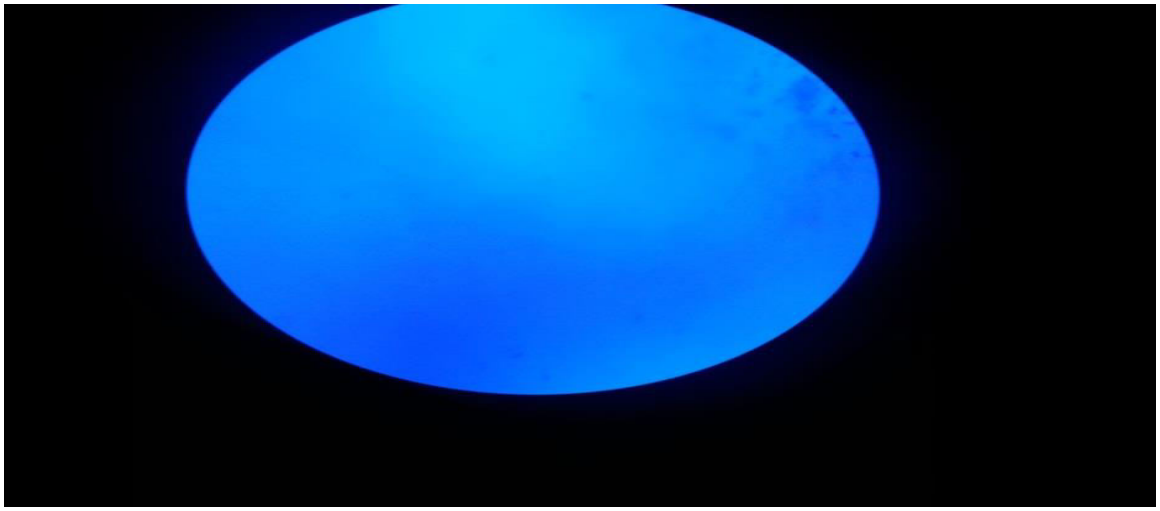

Figure 3.10: Stained biofilms with 0.05g of clopidogrel viewed under the light microscope

3.3 Scanning Electron Microscopy Analysis

After analysis of the samples, the surface characteristics can be seen at different magnifications and resolutions shown in figure 3.11 a, b, c, d, e and f. Surface roughness existed in the films due to the presence of the crosslinker (Di ethylene glycol dimethacrylate) and this surface roughness could also be a good factor to reduce platelet adhesion which is desired for the intended application (Zingg et al., 1982). The cross-linker showed to be insoluble in the biofilms leading to a granule-like formation on the films. Figure 3.11b showed images of particles of clopidogrel on the surface of the biofilms. Figure 3.11 d, e, and f showed the porosity of the films in greater magnifications.

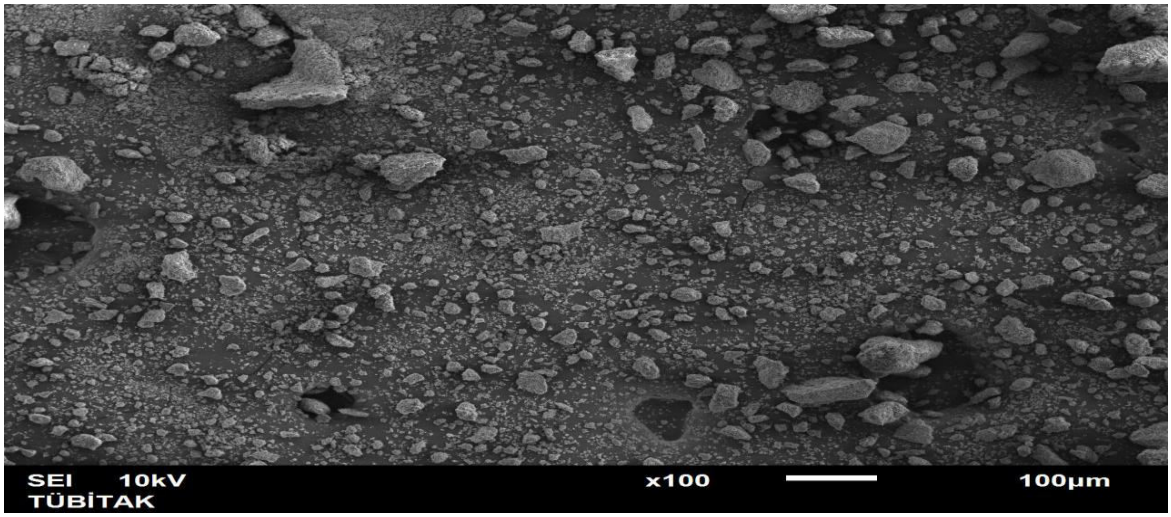

Figure 3.11(a): SEM of SF+0.20ml DEGDMA+0.05g of clopidogrel at x100

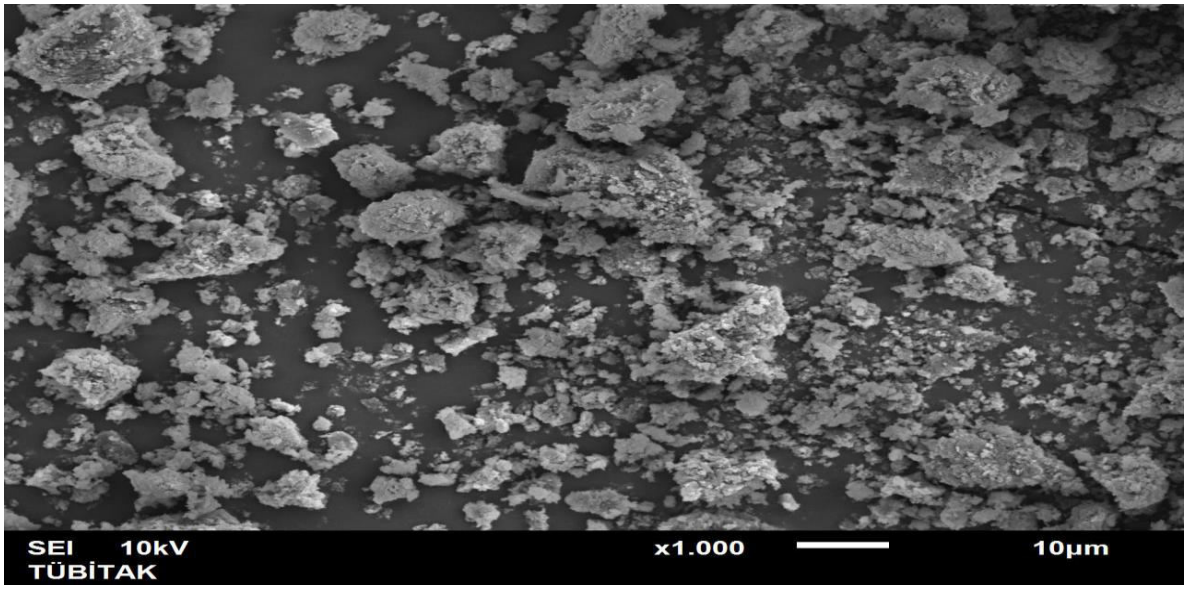

Figure 3.11(b): SEM of SF+0.20ml DEGDMA+0.05g of clopidogrel at x1000

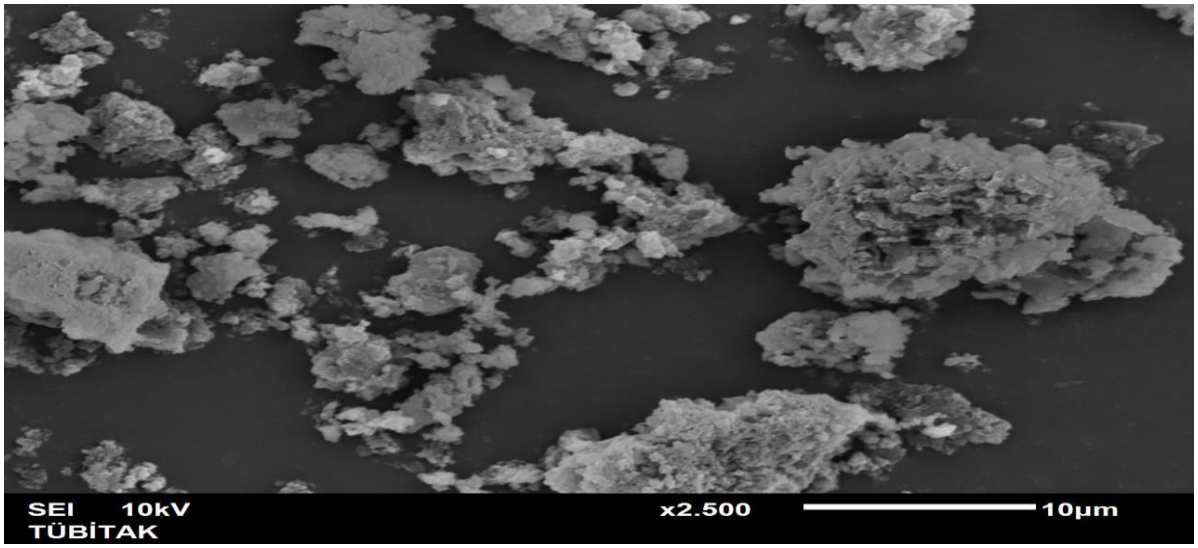

Figure 3.11(c): SEM of SF+0.20ml DEGDMA+0.05g of clopidogrel at x2500

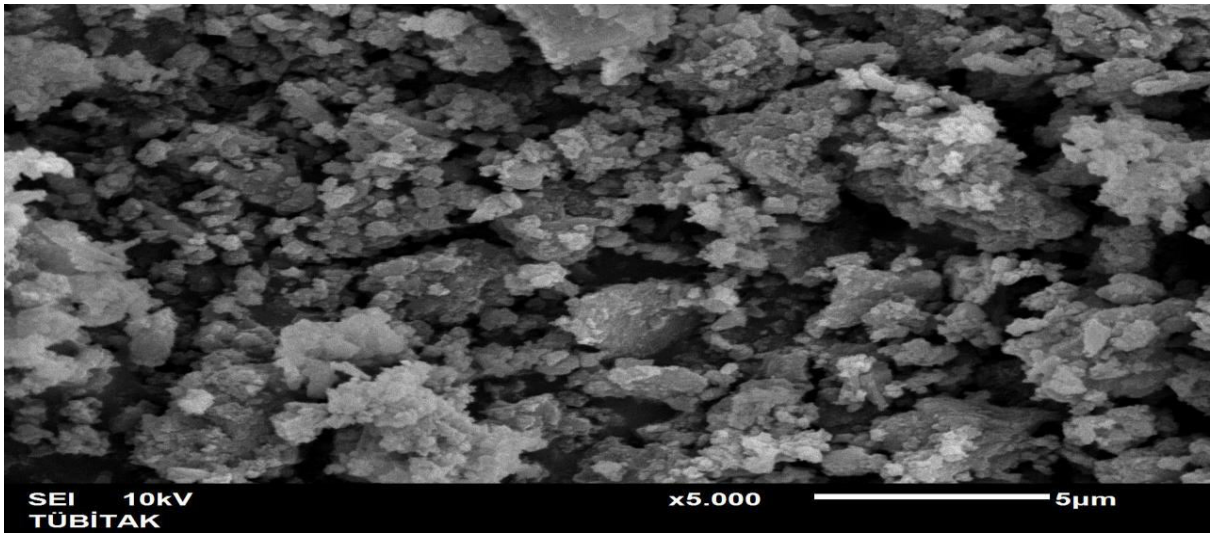

Figure 3.11(d): SEM of SF+0.20ml DEGDMA+0.05g of clopidogrel at x5000

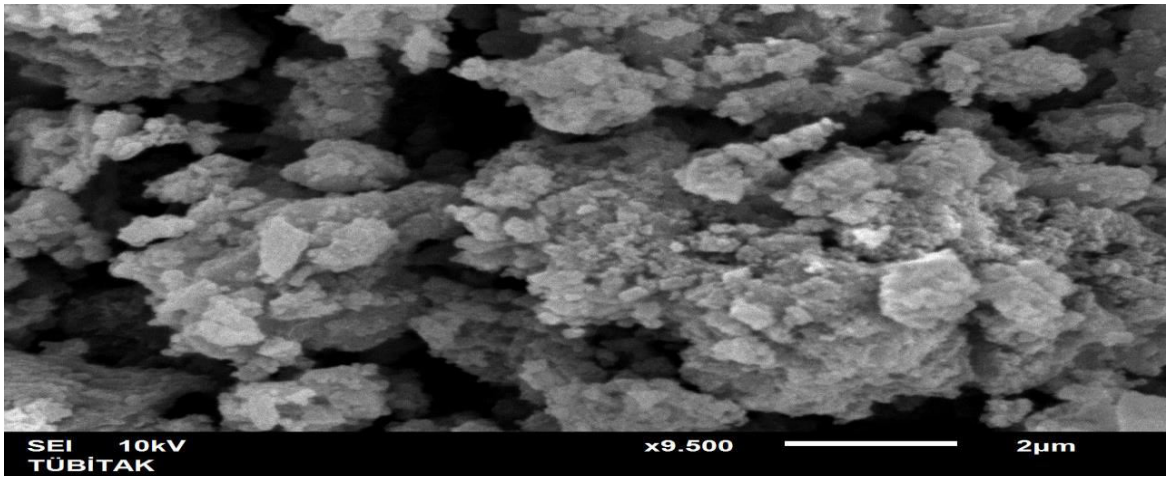

Figure 3.11(e): SEM of SF+0.20ml DEGDMA+0.05g of clopidogrel at x9500

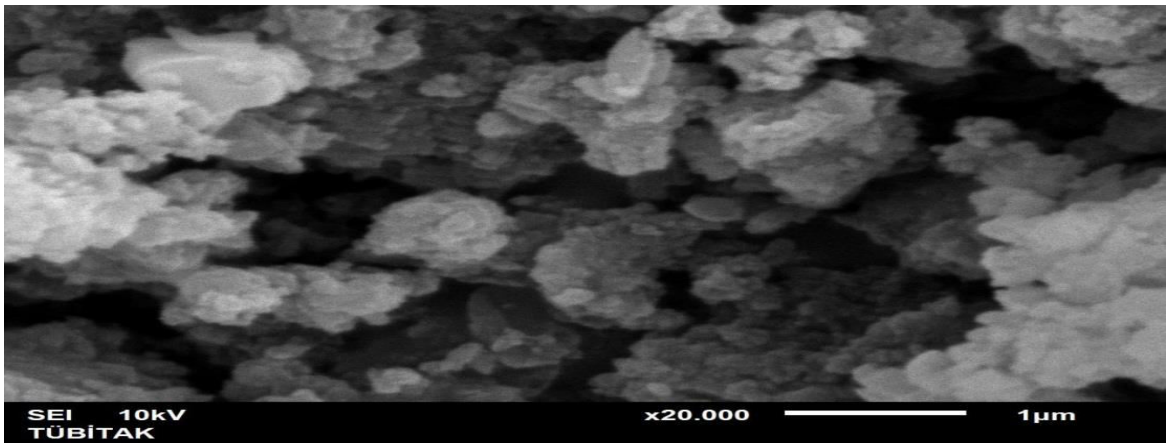

Figure 3.11(f): SEM of SF+0.20ml DEGDMA+0.05g of clopidogrel at x20000

3.4 X-ray Diffraction (xrd) Analysis

X-ray diffraction study was carried out on the samples so as to study the crystalline structure of the silk fibrin blend biofilms and also to study the changes of the crystallinity of the blend. The diffractometer scans at the rate of 2◦/min within the region of 2θ and resulting in diffraction intensity curves. The result in Figure 3.13 showed peaks at 12^o, 14^o, 22^o, 27^o, 29^o, 44^o and 64^o, the high peak at 27^o and 44^o and 64^o suggests the presence of silk fibroin. The peaks at 12^o and 14^o showed an interaction between silk fibrin and clopidogrel. Pure silk fibroin has been shown to be amorphous with no visible peaks as shown in figure 3.12. The XRD patterns results is in line with results from previous studies of Agostini de Moraes et al, where pure SF film showed an XRD pattern of amorphous

substances, with the absence of crystallinity peaks, which is a characteristic of a silk I secondary structure (Moraes et al., 2010). The change in the crystallinity of the blend films is possibly due to interactions between SF, clopidogrel and the cross-linker chains that can interact by hydrogen bonding, thus stabilizing the SF molecular structure (She et al., 2008).

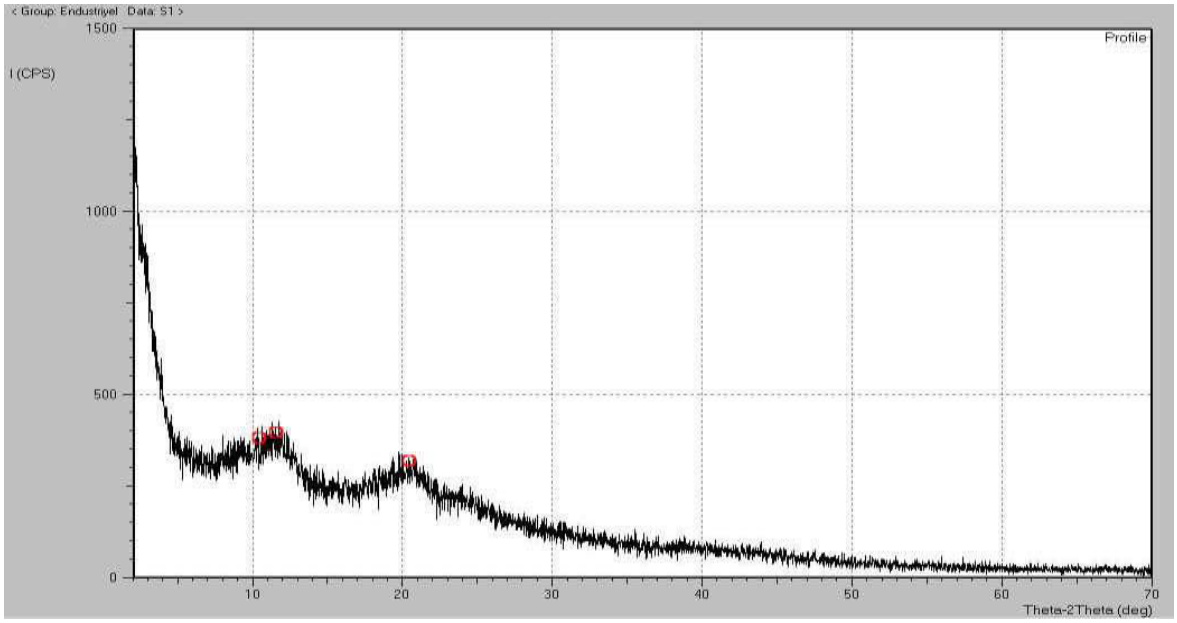

Figure 3.12: XRD analysis of pure silk fibroin

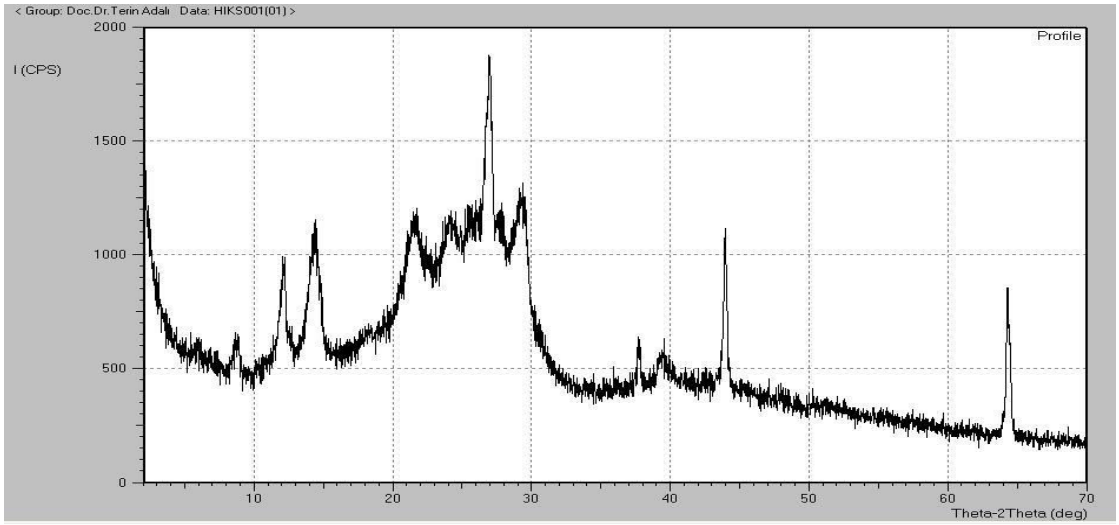

Figure 3.13: XRD analysis of Silk fibroin+0.20ml DEGDMA+0.05g clopidogrel

3.5 Fourier Transform Infrared Spectroscopy (FTIR) Analysis

The FTIR results in Figure 3.14 showed peaks at 3429.2 cm-1 and 2955.9 cm-1 corresponds to the OH frequency vibrations, of clopidogrel, the sharp peak at 1720.0 cm-1 correlates with the CH_3 in the amide group of silk fibroin. The absorption bands at 1120.2 cm-1; this relates with the C-O-C bonds which also corresponds with C-O stretching. The presence of amide 1 and amide 2 showed at peaks in 1637.3 cm-1 and 1453.5 cm-1 that showed the presence of silk protein presence in the film. Less stable structure was observed as a result of the blends due to the presence of well defined absorbtion bands.

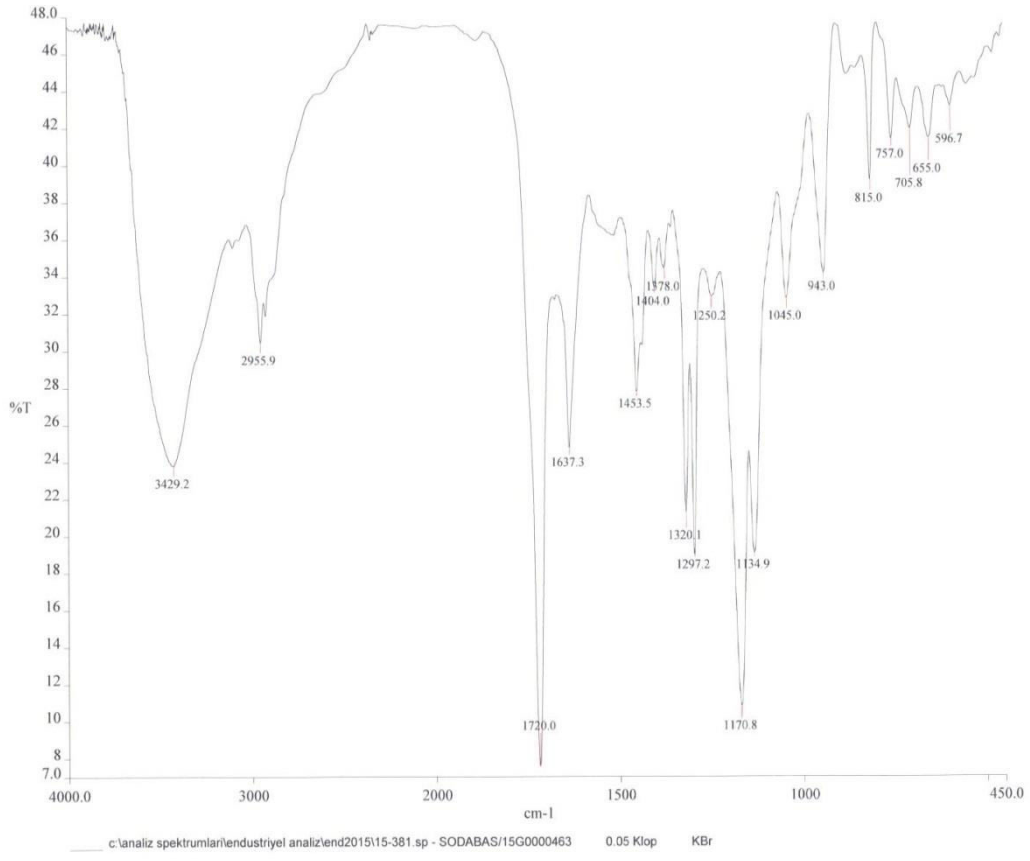

Figure 3.14: FTIR analysis of Silk fibroin+0.20ml DEGDMA+ 0.05g of clopidogrel

CHAPTER 4

CONCLUSION

Silk fibers have long been a material of great attention for many researchers in various field of biomedical application. For the purpose of understanding its thrombogenic properties, silk fibroin was used in a standard ratio and blended with varying dosage of clopidogrel which is an anti-coagulant drug.

7 study samples (P1 –P7) were used for both the control and study group. The control group had no clopidogrel modified biofilms in them and they were tested at zero minute using the STA compact device, and the same 7 study plasma samples were tested with 0.01g and 0.05g of clopidogrel blended biofilms. During my experiment it was observed that 0.20g of clopidogrel blended with silk fibroin produces no films at all which proved very difficult until the ratio of the clopidogrel was reduced to 0.01g to 0.05g. Still with the minute amount of the clopidogrel the effect of the clopidogrel on the APTT, PT, INR and PT % could be seen in a small level. Therefore, with a more advanced film production technique the SF/clopidogrel blended film could be very effective in biomedical application. Platelet adhesion test showed no platelet adhesion which is desired for the material.

The characterization of the films were done only on samples containing 0.05g of clopidogrel due to financial constraints, in the XRD, the film was observed to be crystalline because of the presence of the cross linker. The modified film showed peaks at 12^{o}, 14^{o}, 22^{o}, 27^{o}, 29^{o}, 44^{o} and 64^{o}, the high peaks at 27^{o} and 44^{o} and 64^{o} suggests the presence of silk fibroin. SEM analysis showed great amount of roughness which can enhance cell attachment, also the porosity of the film and particles of clopidogrel was observed. FTIR analysis showed the functional groups present in the biofilms, the presence of Amide I and Amide II groups were observed and the result showed the less stable nature of the films that resulted from the presence of well- defined absorbtion bands

In conclusion, this work is an original work and with the result obtained from this work it showed that it has great prospects as a biomedical material. However, it could be worked on more in the future by implementing an advanced biofilm production technique, so as to modify SF with a larger amount of clopidogrel dosage for a more effective result.

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