PREPARATION AND CHARACTERIZATION OF SILK FIBROIN/CIPROFLOXACIN/OFLOXACIN SCAFFOLDS

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I hereby declare that all information in this document has been obtained and presented in accordance with academic rules and ethical conduct. I also declare that, as required by these rules and conduct, I have fully cited and referenced all material and results that are not original to this work.

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To my parents….

ABSTRACT

The research work is aimed to synthesize and characterize Silk Fibroin-Triethylene glycol dimethacrylate (SF/TriEGDMA) scaffolds embeded with ciprofloxacin and ofloxacin. The freeze drying technique was used at -20^{o}C. The methanol was used to stabilized β-sheets of the scaffold. Characterization of S.F-Tri(ethylene glycol) dimethacrylate scaffolds were analyzed by Scanning Electron Microscope (SEM) and XRD analysis. A range of licensed microorganisms were used to test the susceptibility rate of crosslinked SF / ciprofloxacin, S.F / oflaxacin scaffolds and pure Silk Fibroin scaffold via Kirby-Bauer Disk Diffusion technique.

The SEM analysis of S.F /TriEGDMA / ciprofloxacin scaffold demonstrated that the structure have heterogenous and porous structure. XRD analysis show that the scaffold has amorphous structure.

The antibacterial susceptibility test of the freeze-dried S.F / TriEGDMA / ciprofloxacin, S.F / TriEGDMA / oflaxacin scaffolds exhibited excellent antibacterial activities against *Bacillus cereus, Escherichia coli, Staphylococcus aureus* and *Candida albicans.*The potent antibacterial activity has been observed by the pure Silk Fibroin scaffold.

The results showed that, S.F / TriEGDMA /Ciprofloxacin and S.F / TriEGDMA / ofloxacin scaffolds possess excellent antibacterial activity against gram-negative bacteria *Escherichia coli* including *Staphylococcus aureus*. The antifungal capability of S.F / TriEGDMA / ciprofloxacin and S.F / TriEGDMA / ofloxacin scaffolds and pure Silk Fibroin scaffold were demonstrated against *Candida albicans* with clear variant zones.

These results indicated that, the composite scaffolds could be suggested suitable for tissue engineering applications.

Keywords: Antibacterial activity, Silk fibroin, Tri ethylene glycol, Ciprofloxacin, Ofloxacin

ÖZET

Bu çalışmanın amacı, Ciprofloxacin ve Ofloxacin antibiyotikleri kullanarak antibakteriyel özellikte İpek Fibrin – Tri-etilenglikol dimetakrilat (İF / TriEGDMA) iskeleleri oluşturmaktır. İskeletler lipofil tekniği ile -20^{o}C de sentezlenmiştir. Morfoloji, kristal yapı taramalı elektron Mikroskopu (TEM) ve X-Işın difraksiyonu yöntemi kullanılarak analiz edilmiştir.

Sentezlenen iskele yapıların heterojen, amorf ve gözenekli yapıları TEM ve X-ışın difraksiyon analizleri ile gözlemlenmiştir. Uygulanan antibakteriyal duyarlılık testi lipofil tekniği ile sentezlenen İF / TriEGDMA / Ciprofloxin ve İF / TriEGDMA / Ofloxacin iskelelerinin *Bacillus cereus, Escherichia coli, Staphylococcus aureus* ve *Candida albicans* karşı mükemmel antibakteriyel duyarlılık göstermiştir. Saf ipek fibroin iskelesininde antibakteriyel özellik gösterdiği analiz edilmiştir.

Sonuçlar, İF / TriEGDMA / Ciprofloxin ve İF / TriEGDMA / Ofloxacin iskelerinin, gram-negatif bakteri *Staphylococcus aureus* içeren *E. coli* karşı mükemmel antabakteriyel özellik gösterdiğini, iskelelerin Atifungal özelliklerinin de *Candida albicans* karşı analiz edilip, etkin olduklarını göstermektedir.

Tüm sonuçlar, yapı iskelelerinin doku mühendisliği uygulamalarında ideal adaylar arasında söz edilebileceğini göstermiştir.

Anahtar Kelimeler: Antibacterial activity, İpek Fibroin, Trietilen glikol, Ciprofloxacin, Ofloxacin

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LIST OF ABBREVIATIONS

S.F:	Silk Fibroin
Gly:	Glycine
Ala:	Alanine
Ser:	Serine
TEGDMA:	Triethylene Glycol Dimethycrylate
SEM :	Scanning electron microscope
FTIR:	Fourier Transform Infrared Spectroscopy
XRD:	X-ray diffraction
***B .mori* :**	*Bombyx mori*
PLA :	Poly Lactic Acid
GLP:	Good Laboratory Practices
PLLA:	Poly (L- Lactic acid)
PEO:	Polyethylene oxide
SELPs:	Silk-elastin-like protein
EDC :	1-ethyl-3-(3- dimethylaminopropyl) carbodiimide hydrochloride
PLGA:	Poly (Lactic-co-glycolic acid)
C. Albicans:	*Candida albicans*
E.coli :	*Escherichia coli*
B. cereus :	*Bacillus cereus*
S. aureus :	*Staphylococcus aureus*
Mm :	Millimeters

CHAPTER 1

INTRODUCTION

1.1 Silk Fibroin

Silk fibroin is a naturally existing polymer present in the glands of silk producing arthropods such as silkworms *(Bombyx mori)*, spiders, mites, and bees. The silk cocoon from silkworms is naturally coated with a gum-like protein known to be sericin sustaining the silk cocoon's structure (Perez-Rigueiro et al., 2001). The gum-like protein removed from the silk cocoon is purified via a process called the degumming process. There are various morphological forms of the silk fibroin such as biofilms, fibers, scaffolds, membranes, and sponges. Silk fibroin has been steadily studied over time with its relevance to biomedical, and tissue engineering as showed in Fig.1.1 possessing some distinct characteristics that include Biodegradability, excellent biocompatibility, swelling and remarkable mechanical properties. These forms of silk fibroin have evidently supported the proliferation of cell, adhesion, migration and also promoted tissue repair in vivo. The biocompatibility of silk fibroin with other biopolymers such chitosan significantly proved its importance in the textile sector.It includes the usage of silk fibroin in medicine triggering interest from various disciplines due to its structure and properties (Sah and Pramanik, 2010).

Silk based materials obtained from silkworms a member of the *Bombycidae* family such as the *Bombyx mori* silk that can also be called mulberry silk. Silk possesses a large molecular weight range of 200-350 kDa including a bulky repeating hydrophobic molecular groups together with a small hydrophilic group (Ayoub et al., 2007). The successful use of silk as a biomaterial from *B .mori* for suturing over the years was reported by Moy et al., 1991. Silk fibroin as a polymer has several advantages over other protein-based biomaterials such that other protein-based biomaterials have a high risk of infection, processes are quite expensive to implement as well as the isolation and purification methods. Silk fibroin biofilms are one of the most emphasized forms of the silk fibroin.

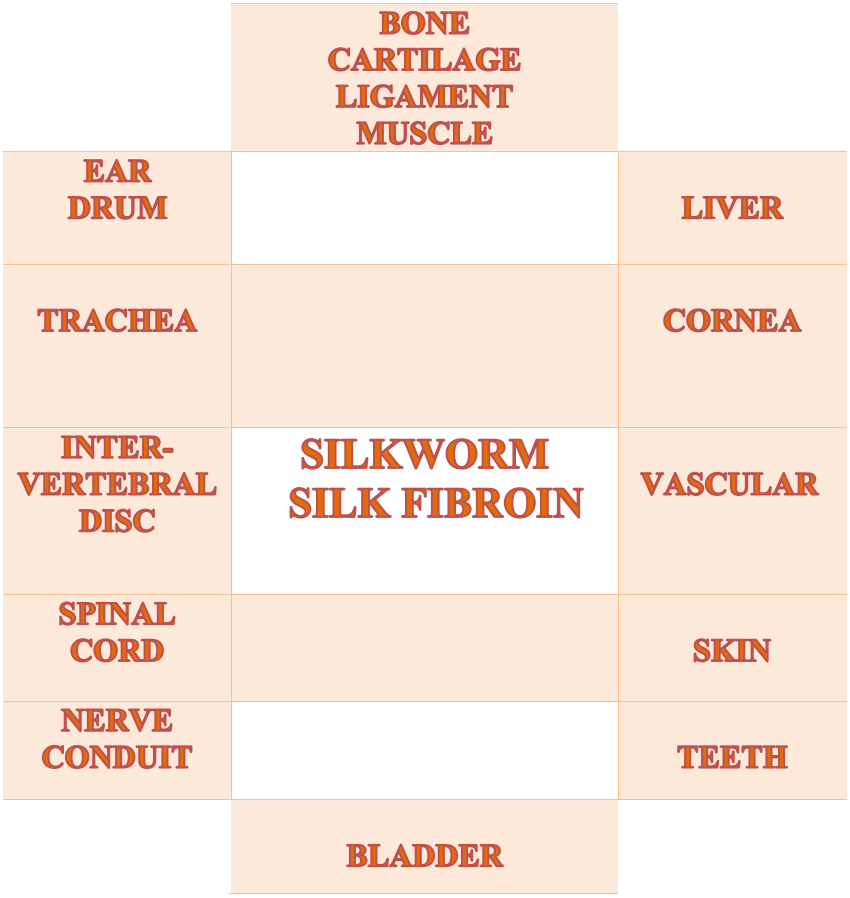

Figure 1.1: Applications of silkworm silk fibroin in tissue Engineering (Kasoju and Bora, 2012)

Silk fibroin biofilms extensively demonstrated its importance in tissue engineering ranging from the fabrication of artificial skin, ligament, connective tissues like culturing of skin cell, and also in drug delivery system. Silk fibroin biofilms studied can be regenerated by dissolving the silkworm cocoon fibers supporting the growth and attachment of human cell line (Gotoh et al., 1997). Another emphasized form of silk fibroin is the silk fibroin based scaffolds that have been used widely for cell culturing and tissue engineering. There are have been numerous studies exploring the use of silk-based scaffolds and porous sponges for biomedical applications (Wang et al., 2006). Silk fibers can be purified routinely using a simple alkali or enzyme based degumming procedure producing sericin free-silk fiber or sponge. There are processing techniques reported yielding the 3-dimensional porous silk fibroin scaffolds with their morphological and functional features that were controlled. The fabrication of silk fibroin scaffolds usually are of traditionally-based manufacturing techniques that include salt leaching, thermally induced phase separation (Tamada, 2005).

1.2 Characteristic Properties of Silk Fibroin

Silk fibroin as a natural polymer is well known to possess some major distinctive properties that are of advantage over other natural polymers in terms of chemical structure, biocompatibility, biodegradability as well as mechanical tensile ability.

1.2.1 Chemical structure and properties of silk fibroin

Silkworm *Bombyx mori,* fibers are about 10 - 25µm in diameter cumbersome and light chain present as 1:1 ratio is linked by a single disulfide bond (Zhou et al., 2000). Studies have shown that silk fibroin belongs to the fibrillar proteins group as well as creatine and collagen (Finkel Shtein et al., 2002). Silk fibroin contains elements in its supramolecular structure with a width up to 6.5×10^5nm consisting of nanofibrils helically packed are 90-170nm in diameter (Altman et al., 2003). The nanofibrils are known to be responsible imparting enhanced strength to the silks. Silk fibers mainly consist of 2 proteins namely sericin and fibroin. Silk contains other amino acids residues in minor amounts as well as other impurities such as fats, waxes, dyes and mineral salts. Silk is coated using a group of hydrophilic proteins known as sericin occupying up to 30% of the silk cocoon's mass removed via the degumming process.

Bombyx mori fibers are made up of residues containing nothing less than 16 amino acids with variation in the ratio of different areas in the supramolecular structure of fibroin. The amino acid structure have repeated areas composed of glycine (45%), alanine (30%) and sericin (12%). The structure demonstrated a rough estimate of 3: 2: 1 ratio possessing the (Gly-Ala-Gly-Ser) sequence (Khan et al., 2009) Shown in fig 1.3. Researchers have shown the three structural arrangement of silk that include silk I, II &III. Silk I is the natural raw form of fibroin found in the *Bombyx mori* silk glands. While Silk II has the arrangement of fibroin molecules in silk with greater strength oftenly applied commercially. Finally, silk III is a newly recognized structure of the silk fibroin comprising of the [Gly-Ser-Gly-Ala-Gly-Ala] sequence (Valluzzi et al., 1999).

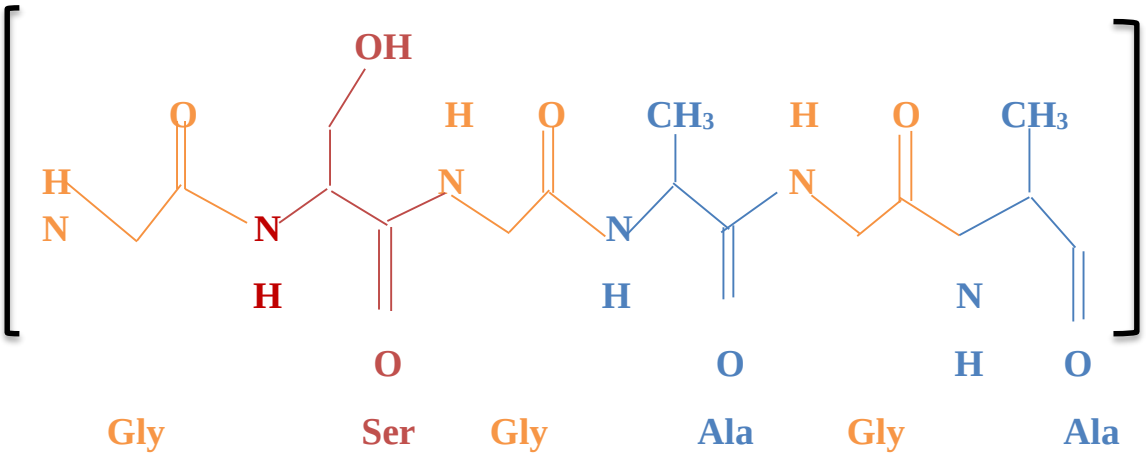

Figure 1.2: Primary structure of fibroin, showing the [Gly-Ser-Gly-Ala-Gly-Ala] sequence (Valluzzi et al., 1999)

1.2.2 Mechanical properties

Silk as a fiber possesses certain mechanical qualities in terms of a balanced modulus as well as elongation ability contributing to the level of toughness. The silk deforms under tensile stress, establishing a reputable performance in fiber technology (Vollrath and Knight, 2001; Vollrath, 2005). Silk also possesses a higher strength-to-density ratio than that steel, the silk spider mainly exhibits a marked strain hardening behavior (Du et al., 2011). The strain hardening behavior or ability has an emphasized level of importance in terms of energy absorption. The wild silkworm also has the strain hardening property including a shape similar to that of spider silks (Rajkhowa et al., 2000). Thus, there are certain implants that have failed due to their poor mechanical properties or higher stress than the optimum concentration at the tissue implant interface. The variation in mechanical properties of silk materials provides a platform of choice made depending on its specificity. In generally the most emphasized choice of selecting a silk-based material are in terms of elasticity, adequate strength and strain hardening.

Silk-based materials manufactured processed from silk fibroin solution is weak as well brittle. The strength of regenerated silk product examined can be improved to the level of the native nature silk fibers (Ha et al., 2005). There are several studies reported which demonstrated that regenerated silk fibers can keep their initial tensile integrity for 21 days within the in-vitro culture conditions (Hyoung-Joon and Kaplan, 2003). Solvents used for electrospinning have an effect on the β-sheet formation of the scaffold's structure that can induce altering mechanical properties. Solvents such as formic acid Hexafluoroisopropanol also water have used to electrospun silk scaffolds. Studies of Water and formic acid activity seem to improve the mechanical properties of scaffolds (Jeong and Park, 2007; Wang et al., 2004).

1.2.3 Biocompatibility

Silk sutures over a period have had a long successive history known in terms of biocompatibility. Recorded incidents of deferred hypersensitivity of silk sutures suggest the presence of a silk-gum like protein called the sericin (Giese et al., 2011). Another investigation was conducted stating that the sericin based materials have shown no established prove to indicate sericin as a source of adverse effects (Zhang et al., 2010). The essence of the detailed investigation was to identify the origin of any cytotoxic non-element specifically in the silk developing an adequate diagnostic method. The treatment of musculoskeletal disease demonstrated the use of silk fibroin bioconjugates for the testing the immunogenicity of silk scaffold. A study of pig model in ligament tissue engineering investigated displayed no evidence or sign of a malfunction in terms of biocompatibility after 24 weeks in an in-vivo culture (Fan et al., 2009).

However, these established reports provided a broad acceptance that adequately degummed sterile silk fibroin products with excellent biocompatibility capacity. Silk products compared manifests good biocompatibility to other commonly used biomaterials Such as collagen and polylactic acid (PLA) (Meinel and Kaplan, 2012). Silk based material have received regulatory approval for the use of biomaterial devices for plastic and reconstructive surgery. In a research under good laboratory practices (GLP), Silk product displayed evident biocompatibility signs with silk-based seri-fascia surgical (Horan et al., 2009). The drawbacks of biocompatibility in various research conducted with silk sutures in the human body has a limited time associated with the period of wound healing. The adaptive immune system based on the location of the implant including the models of the construct is another issue investigated. Another concern that may be noted is the immune reaction in response to degraded products of silk-based materials associated with the size of the silk material as well as their morphologies. The observations made suggests that the degraded products of silk fibroin may induce Amyloidogenesis a condition at which soluble and innocuous protein becomes insoluble protein aggregates known as Amyloid fibrils (Lundmark et al., 2005).

1.2.4 Biodegradation

The degradation of silk studied is based on loss in its mass includes certain changes in terms of morphology and various analysis of the degraded silk products invitro. The United States Pharmacopeia defined any absorbable biomaterial as a material that 'loses most of its tensile strength over of period of 60 days' after implantation. This definition described silk as non-degradable, but silk degrades over a longer period. The tensile strength of silk investigated demonstrated loss in its capacity within a year and was unrecognized at its site of implantation within a period of 2 years. The tensile strength of silk as a biomaterial was tested in an animal model in terms of degradation after implantation for a period. The regenerated silk construct implanted degrades faster than fibers causing the deterioration. Depending on the secondary structure of the silk as a result of preparing regenerated silk materials (Hu et al., 2012).

Biodegradability usually discussed in terms of disintegration following various studies defined biodegradability as the degradability of an implantable polymer by biological element producing fragments. The pieces of the item can readily move away from its usual site via transfer of fluid but usually from the body (Vert et al., 1992). The in vivo degradability evidence of silk products studies revealed the implantation of the silk-based construct was carried out using Lewis rats. The water based 3-D scaffolds showed the disintegration of the structures in few weeks following total disappearance after a period of a year. The significant impact on the degradation of 3-D silk sponge in a host immune system was reported to be mediated by macrophages. Suggesting that silk not being only biodegradable but as well bioresorbable (Wang et al., 2008).

The degradation of the silk-based system is said to be initiated by cells in-vitro, these in-vitro studies have shown a various comparison of biodegradability rate of silk-based materials and structures. The type and concentration of different enzymes at the site of silk material based implant may initiate limitations. Thereby predicting the degradability of silk compared to other synthetic materials that may degrade hydrolytically with variations in hosts (Nair and Laurence, 2007). Silk as a biomaterial has excellent advantages compared to other biomaterials in the aspect of biodegradation. For instance, synthetic biomaterial such as polyglycolides possesses degraded products that are needed to be reabsorbed through metabolic pathways releasing majorly acidic products causing challenges.

The Synthetic biomaterials over time tend to lose certain mechanical properties during degradation. While silk materials can retain its strength over an extended period that is an advantage especially in tissue engineering requiring a slow rate of degradability and load bearing properties. The in-vitro studies of silk have shown proteases such as chymotrypsin attracted the less crystal parts of the proteins to peptides that have the capability to be phagocytosed for further metabolism by the cell. Protease cocktails and chymotrypsin are enzymes produced by macrophages that are capable to degrade silk (Minoura et al., 1990). *B. mori* tend to produce a protease inhibitor in the silk gland embedded within the silk cocoons protecting it against proteolytic degradation prematurely (Kurioka et al., 1999). Silk is said to be a degradable material due to its susceptibility to proteolytic enzymes. Silk compared to dry-spun hot-drawn poly(L-lactic acid) PLLA fibers, and several sutures are absorbable and non-absorbable in the muscle layer surrounding the abdomen of rats (Lam K.H et al., 1995). PLLA and black braided silk reveal signs of degradation after two weeks in Vivo determined by scanning electron microscopy.

1.2.5 Antimicrobial properties of silk fibroin scaffolds

Studies over time have established that fabricated silk fibroin scaffold possesses a mild form of antimicrobial activity, not having a perfect conclusion in terms of preventing all forms of bacterial infection. Certain Reports from research by Lan et al., 2014 investigated the antibacterial capability of silk fibroin using gelatin microspheres loaded with an antimicrobial agent called vancomycin. In this study, the Gelatin microspheres are used as drug carriers carrying the vancomycin (Choy et al., 2008). The vancomycin injected gelatin microsphere/silk fibroin scaffold displayed excellent antimicrobial activity against *Staphylococcus aureus* in vitro. The vancomycin as an antimicrobial agent is active only against Gram-positive bacteria such as *Staphylococcus aureus* reported by Li et al. 2010. Vancomycin as the antimicrobial agent was investigated to have drawbacks majorly in clinical development including long-term and higher parental administration of the vancomycin that can cause harm to the patient & has a high cost of purchase. The disc diffusion method used to test the vancomycin injected gelatin microsphere/silk fibroin scaffold With the vancomycin/silk fibroin scaffold against *S. aureus* & *Escherichia coli*.

The Susceptibility test of the pure Silk fibroin scaffold, conducted against *S. aureus* and *E. coli* incubated at 37°C for 24 hours fulfilled the need for control making comparisons of the inhibition zones. The absence of inhibition zone indicates the lack of antimicrobial activity from the pure silk fibroin scaffold in the culture agar plate (Zhou et al., 2012). The silk fibroin scaffold with vancomycin and the vancomycin injected gelatin microsphere/silk fibroin scaffold was reported to both display inhibition growth zones with semidiameters of 7.75mm & 6.65mm respectively. The antimicrobial activity against *S. aureus* been a gram-positive organism and was determined measuring the difference in the semi-diameter between the inhibition zone and the scaffold structure (Wei et al., 2011). It is proposed that the vancomycin/silk fibroin scaffold possess larger inhibition zones in diameter compared to the vancomycin injected gelatin microsphere/silk fibroin scaffold.

Furthermore using vancomycin as an antimicrobial agent, studies have shown how its therapeutic efficacy can be improved using a fabricated biodegradable gelatin sponge containing varying in contents of β-tricalcium phosphate ceramic controlling the release of vancomycin. However in this research, ciprofloxacin & Ofloxacin are utilized as antimicrobial agents belonging to the class of fluoroquinolones (Ball, 2000; Oliphant and Green, 2002). The blend of Ciprofloxacin and ofloxacin combined with aqueous silk fibroin using Triethylene Glycol Dimethycrylate as a crosslinking agent fabricated the freeze-dried scaffolds. The scaffold's antimicrobial susceptibility against *Escherichia coli, Staphylococcus aureus, Bacillus cereus & Candida albicans* was determined using the disk diffusion method containing various proportions of the antibiotics.

Ciprofloxacin and Ofloxacin are second-generation fluoroquinolone antibiotics with similar characteristics reportedly used to treat a variety of infections including urinary tract infection as well as a respiratory tract infection. Both ciprofloxacin and ofloxacin are quinolones capable of inhibition bacterial cell replication mostly in prokaryotic cells. Ofloxacin is a racemic mixture available in ear drops and eye drops consisting of levofloxacin as the biologically active ingredient. Ciprofloxacin is not considered a first-line agent for viral infections such as common cold. The fluoroquinolone antibiotics has been investigated to possess a broad spectrum of antimicrobial activity against most strains of bacterial pathogens known for gastrointestinal & abdominal infections which includes *Escherichia coli, Haemophilus influenza* e.t.c. Ciprofloxacin as an antimicrobial agent is known for excellent penetration and its availability in both oral & intravenous forms (Brunton et al., 2005).

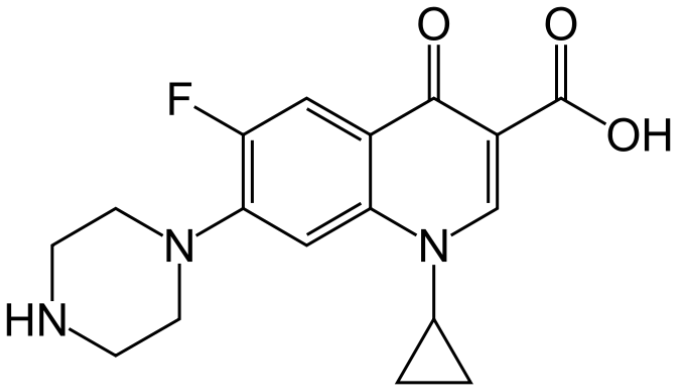

Figure 1.3: Chemical structure of ciprofloxacin

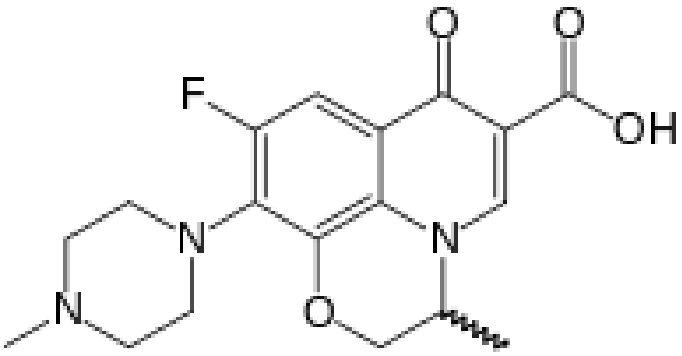

Figure 1.4: Chemical structure of ofloxacin

1.2.6 Swelling properties of silk fibroin

Silk fibroin exhibits swelling as an activity depending on the degree of crosslinking reactions as well as the level of ionization including hydrophilic and hydrophobic balance (Peppas and Khare, 1993). There are certain changes in the concentration of the polymer that was reported to influence the degree of swelling activity. The swelling ability can potentially increase the total amount and rate of drugs released (Haider et al., 2005). The rise in the concentration silk fibroin concentration led to the decrease in the swelling ratio of the silk fibroin scaffolds. The blending of Silk fibroin as a natural polymer with other materials Such as chitosan initiated greater swelling ability (Rujiravanit et al., 2003; Gobin et al., 2005).

1.2.7 Solubility properties of silk fibroin

Natural silk fibers dissolve only in a limited number of solvents when compared to immunoglobulins also known as globular proteins Such as IgA, IgD, IgE, IgG, and IgM. Due to a large amount of intramolecular & intermolecular hydrogen bond with a high level of crystallinity and other physiochemical properties in fibroin (Cai et al., 2002). Studies on Fibroin protein shows how impossible it dissolve in water and majorly in organic solvents but instead swells up to 30-40%. Fibroin dissolves majorly in a concentrated aqueous solution of acids such as formic, sulphuric and in concentrated aqueous, biological and aqueous organic solution of salts such as Libr, $cacl_3$, $Zncl_2$. One of the drawbacks associated with the salt-containing aqueous and Aqueous organic solvents includes long preparation time of dialyzed aqueous solution of fibroin. Other includes enormous consumption rate of power regenerating the solution involved, and the concentration of the salts.

However, fibroin has specific dissolution features in various systems associated with its molecular structure. The dissolution of fibroin in salt solutions is caused due to the interaction of the solvent ions altogether with the functional group of the fibroin macromolecules. The intramolecular & intermolecular hydrogen bonds present in the fibroin structure is reported disrupted as a result of the nucleophilic savage by the anion (Dawsey and Mccormick, 1990). The interaction of the solvent ions with the polar and charged groups of pendent chains of fibroin cause the breakage of the hydrogen bonds between the macromolecules. It was proposed that during the dissolution process, the amorphous parts from fibroin is characterized by a high composition of amino acids residues. The amino acid composition of *Bombyx mori* silk fibroin was examined to experience changes following its recovery from the aqueous Libr (Tsukade et al., 1990).

The dissolution process also increased the glycine and alanine residues of the hydrophobic regions initiating the amorphous areas that decrease after the precipitation of the polymer. Studies also show the dissolution temperature is raised to 70°C enabling the degradation of macromolecules present (Furuhata et al., 1994).

1.3 Morphological Forms of Silk Fibroin

1.3.1 Silk fibroin films

Silk fibroin films are casted products from aqueous organic solvents and can be formed as well from the blending of other polymers. Silk films were investigated to be prepared from an aqueous silk fibroin possessing oxygen and water vapor permeability depending on the content of the silk structure (Minoura et al., 1990). The use of 50% methanol treatment on silk films structure was proposed exhibiting different properties of the film such as mechanical & degradability properties. The layer-by-layer technique is a method in which nanoscale silk films formed from an aqueous solution (Wang et al., 2005). Ultrathin films are reported to be stable due to hydrophobic interactions and predictability in the films thickness that be could obtain as a result of solution control conditions. These films have been studied over time to support the human mesenchymal cells adhesion and proliferation.

However, studies have established that microstructures in biofilms are of advantage for increasing surface roughness. Cell attachment is formed by blending silk with polyethylene oxide PEO (Jin et al., 2004). The exposure of these rough surfaces initiated the extraction of PEO with water, locking the β-sheet crystallinity with methanol. Several studies have investigated the use of mammalian and insect cells in terms of attachment to silk fibroin films compared to collagen films. It has been proposed that use of silk films in the healing of skin wounds in rat healed up within a period of 7days. Displaying a minimal inflammatory response compared to the traditional porcine-based wound dressing. Silk was also reported to improve the attachment of cells and bone formation when coupled with Bone morphogenetic protein BMP-2 displaying increase bone formation varied with silk fibroin films (Karageorgiou et al., 2004).

1.3.2 Silk fibroin hydrogels

Silk fibroin hydrogel is a three-dimensional polymer formed as a result of the sol-gel transition of silk fibroin solution in an aqueous solution initiated in the presence of acids. They are referred to also as biomaterials capable of durability in terms of swelling in aqueous solutions. The sol-gel transition is increased and enhanced by increasing the concentration of the protein present, temperature and the addition of ca^{2+}. Several studies have evidently demonstrated that silk fibroin hydrogels were prepared from the aqueous silk fibroin solution obtained from the β sheet structure (Kim et al., 2004). The rate of gelation in the solution depends on the PH of the silk solution influencing the rate at which the gel in the solution. It Gelation is a process developed using 3% solution within a period of 3-4 days compared with another solution formed within eight days possessing a PH range of 5-12 (Ayub et al., 1993). Some factors are also considered essential for gelation such as the concentration of the silk polymer and the concentration of Ca ions. Hydrogel pore is studied to be one of the factors examined in gelation as a process; others include fibroin concentration and its temperature. Suggesting that an increase in the concentration of silk fibroin solution gave a rise in temperature by decreasing the PH level that also affect the time for gelation.

However gelation can be induced by supplementing silk solutions using a non-ionic surfactant investigated with the addition of poloxamer that can reverse the sol-gel transition (Kang et al., 2000). Several studies show that hydrogels mixed with gelatin have established a temperature-dependent helix-coil transition of the gelation and its impact on the rheological and mechanical properties of the gel. Silk fibroin hydrogels blended with gelatin possesses a composition and temperature depended on properties that were studied for a drug delivery purpose (Gil et al., 2005). The use of benfotiamine for oral delivery depends on the concentration of the silk present in the fibroin-glycerol. Studies have shown the usefulness of silk hydrogels with the good mechanical quality involving the manufacture of load-bearing scaffolds for tissue engineering that includes cartilage tissue regeneration (Chao et al., 2010).

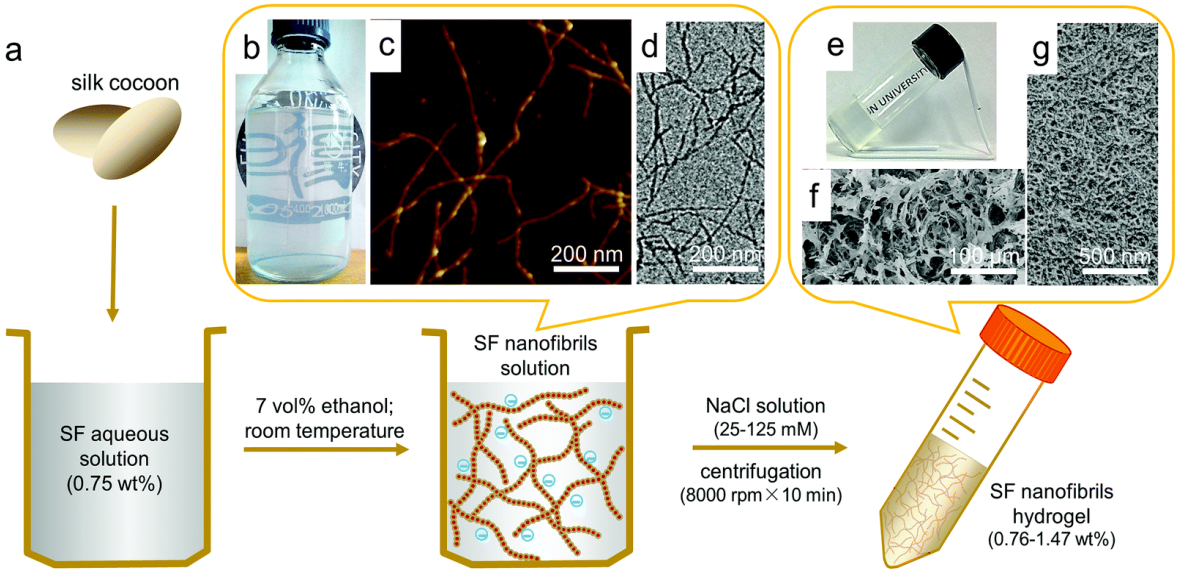

Figure 1.5: Schematic representation of silk nanofibrils based hydrogels formation

Silk fibroin hydrogels are fabricated into various forms of monosaccharides such as ribose, fructose, glucose, and mannose. The hydrolysis of trichlormethiazide reported was observed to be dependent on the number of hydroxyl groups present on various monosaccharide molecules (Hanawa et al., 2000). The attachment of Osteoblast-like cells onto 2% (w/v) silk fibroin hydrogels was investigated evidently showing characteristic features of adhesion and biocompatibility. The addition of 30% glycerol to the silk fibroin hydrogel has been studied to result in the increase of cell proliferation. A study conducted for critical femur defects in rabbits has revealed silk fibroin hydrogels causes a greater trabecular bone volume. As well as a thickness that is higher in minerals in bone formation compared to poly (D, L lactide-glycolide) (Fini et al., 2005). The combination of silk fibroin hydrogels with elastin initiates the formation of biomaterials called silk-elastin-like protein polymers (SELPs). The content level of water in the SELPs hydrogels is managed using the time of gelation and concentration of the polymer without any impact altering certain properties including the strength of the ions and the temperature. These SELPs hydrogels are quite important in the release of some essential molecules such as vitamin B_{12} and theophylline (Dinerman et al., 2002).

1.3.3 Silk fibroin scaffolds

The 3-D porous scaffolds are known to be suitable structures used for tissue culturing because of its ability to mimic the in-vivo physiological microenvironment. Silk-based scaffolds are structures manufactured using some fabricating methods such as freeze-drying, porogen leaching technique (Li et al. , 2001). The porous sponge silk scaffolds are of great importance in tissue engineering in terms of proliferation, migration of cells, attachment of cells including nutrient-waste transport. Studies have shown that solvent-based sponges were manufactured via gas foaming, porogens and lyophilisation (Nazarov et al., 2004). The pore sizes of the silk scaffolds, either small or large pores depend on the choice of the fabricating technique involved. One of the commonly used methods of preparing the 3-dimensional fibroin scaffolds is the freeze-drying method (Nazarov et al., 2004). The level of porosity in the freeze-dried fibroin scaffold was below 70% (Li et al., 2001).

However, several studies show that freeze-dried scaffolds seem not suitable for cell migration, proliferation, and expansion. A more improved freeze-drying method is established known to be the freeze-drying/foaming technique used to fabricate silk fibroin scaffold with high porosity and interconnected pores > 100µm obtained from 6% concentration. Factors such as freezing temperature, freezing rate and level determines the pore size and porosity of the fabricated scaffold. These factors Initiates a certain degree of relationship and interaction between temperature & pore formation thereby obtaining a porous structure >100µm micron in diameter (Li et al., 2001).

The glass transition zone of the aqueous silk fibroin observed between -20°C to -34°C shows that the higher the freezing temperature, with time the larger crystal ice formed. Thereby creating larger pores due to a longer freezing time (Nazarov et al., 2004). The devised novel method suggested by Rina Nazarov et al. involves combining the freeze-drying and gas foaming method using varying concentration of fibroin forming stable frozen structure at -20°C. The frozen, composite structure was placed first in the air at 20°C for several minutes before proceeding to the stage of lyophilization making the structure partly thaw. The thawing time and temperature also are factors determining the fabrication of the porous structure indicating the freeze-drying/foaming technique is a simple and adequate method used for preparing silk fibroin scaffolds for tissue engineering.

Moreover, there are other fabricating techniques that include electrospinning, and rapid prototyping technologies that are newly developed, electrospinning as a fabricating technology fabricates nano-scale non-woven materials. This method involves the use of high voltage power source with a translating and spinning mandrel including the polymer chosen illustrated in figure 1.6 showing a typical electrospinning setup. In this fabricating technique, the fiber is formed due to the electrostatic repulsion possess charges at the surface of the solution droplet. The force produced by the electric field is between the needle tip and the target (Alessandrino et al., 2008). Electrospinning involves some parameters used to manage the process modifying the scaffold's properties such as the solution concentration, the voltage induced, flow rate, and the air gap distance. The usage of silk as a polymer forming electrospun silk fibroin scaffolds have parameters. They include electric field, the type solvent, solution concentration, air gap distance controlling the diameters of fibers produced (Sukigara et al., 2003). Several studies have shown that silk fibroin electrospun nanofibers developed a random coil structure with β-sheet formation achieved by the treatment of the scaffold using methanol or a cross-linking agent (Vepari, 2007).

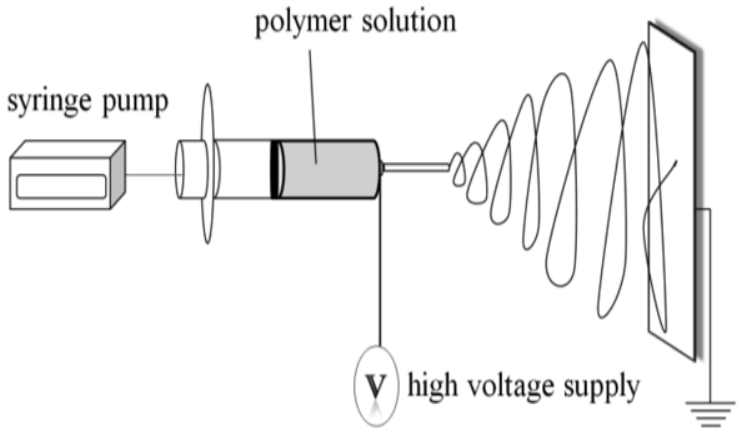

Figure 1.6: A typical electrospinning setup for creating aligned fibrous scaffolds (Teo and Ramakrishna, 2006)

However, repeated freezing procedure and thawing processes tend to increase the pore size of silk scaffolds from the ranging from 60µm to 250µm (Li et al., 2001). Studies have also shown that fabrication techniques such as solvent casting and gas foaming gain better control over the scaffold structure porosity. Porogen leached 3-D silk scaffold is examined to be of relevance in tissue-engineering applications especially in the aspect of bone tissue & cartilage tissue engineering (Meinel et al., 2005). Silk fibroin scaffolds blends with other biomaterials forming a composite silk fibroin scaffold possesses excellent mechanical and biological qualities obtained by the incorporation of inorganic compounds or organic fillers (Hokugo et al., 2006). The composite-based scaffold has the advantage of the excellent tensile stress and a significant challenge in terms of the composite compatibility.The design of some components examined cause poor compatibility initiating uniform mixtures as well as adverse tissue reactions (Wang, 2003).

Silk composite scaffolds were also reviewed to be fabricated by incorporating milled silk particles enhancing its biocompatibility and establishing an outstanding improved modulus below 50KPa to about 2.2 Mpa (Rajkhowa et al., 2010). These composite scaffolds can further undergo further modification including strengthening the silk fibers improving the modulus to about 13MPa (Mandal et al, 2012). The improvement of these mechanical properties may possess the ability sufficient to regenerate trabecular bone but still need to have certain practical requirements in terms of load bearing. Silk composite with knitted silk mesh is studied acting as ligament scaffolds by which a uniformed distribution of cells were observed after 24 weeks post implantation (Fan et al., 2008). Various findings were made recommending the eligibility of silk composites with mechanical properties for highly emphasized tissue engineering applications.

1.3.4 Silk fibroin scaffolds blended with cross-linking agents

Several studies over time established that a biomaterial such as silk fibroin has the capability for various advanced biomedical applications. Such as wound cover materials and tissue engineering scaffolds formation (Dal et al., 2005). The three-dimensional framework as a morphological form of silk fibroin can be applied successfully to tissue engineering matrix as well as tissue inducing materials and cultural cell substrates. The freeze-drying technique is established as one of the conventional methods proposed in the preparation of porous *Bombyx mori* silk fibroin materials. The use of cross-linking agent 1-ethyl-3-(3- dimethyl aminopropyl) carbondimide hydrochloride (EDC) in a research was investigated obtaining a cross-linked silk fibroin material. The uncross-linked porous scaffold was observed to possess a random coil structure. The cross-linked structure containing EDC cross-linking agent produced a α-helix structure as well as an increased in porosity in the silk fibroin material indicating that the EDC stimulated silk fibroin to fabricate a α-structure.

However, the cross-linked silk fibroin scaffold/material displayed a distinct decreasing rate in water solubility as a result of the active cross-linking reaction in the silk fibroin structure. During this research, the cross-linking agent Triethylene Glycol Dimethacrylate (TEGDMA) $C_{14}H_{22}O_6$ was employed to stir 6% aqueous silk fibroin/ciprofloxacin blends. Following the freeze-drying procedure of the composite yielding a Freeze-dried scaffold structure. Triethylene Glycol Dimethacrylate is a hydrophilic bifunctional methacrylic crosslinking agent providing a high crosslink density. It has the capability to impart other properties such as crosslinking monomer to polymers including flexibility, adhesion, heat resistance e.t.c.

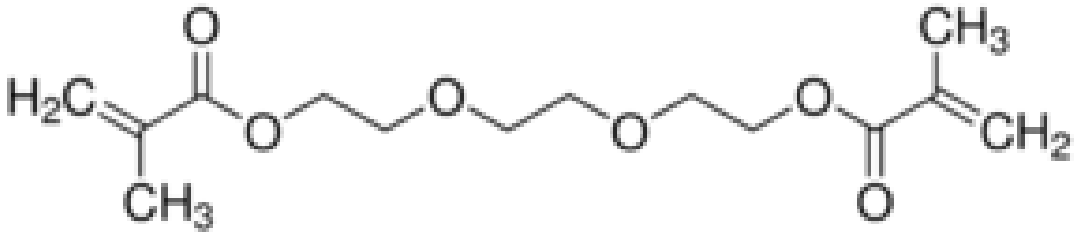

Figure 1.7: Structure of Triethylene glycol dimethacrylate

1.3.5 Silk fibroin particles

Microparticles has gained a wide range of research interest in the field of drug delivery due to their capacity to deliver different drugs targeted for various parts of the body for a particular period. A range of synthetic and natural polymers such as poly(lactic acid) (PLA), silk fibroin were studied to be part of microparticles structure (Freiberg and Zhu, 2004; Rudra et al., 2011). Silk fibroin has been explored being a versatile biomaterial for the formation of microparticles, due to its superior biocompatibility capacity, adequate mechanical properties as well as its slow rate of biodegradation (Vepari and Kaplan, 2010). Silk fibroin based microparticles initiates options for drug delivery due to their unique structure and morphology as well as excellent biocompatibility. The release ability of silk fibroin particles has become an advantage compared to other natural & synthetic biomaterials (Shi & James, 2011; Wenk et al., 2011). It was reported that silk fibroin micro and nanoparticles could be generated by several methods such as evaporation /extraction method, phase separation as well as self-assembly (Wang et al., 2010). Each of these methods possesses several advantages and disadvantages thereby selecting the method becomes a factor in fabricating microparticles/nanoparticles for drug delivery application.

However, silk fibroin microspheres can be prepared using lipid vesicles inform of templates showing the fat removal is due to the effect of methanol or sodium chloride, thereby inducing β-sheet structure of about 2μm in diameter. The usage of the laminar jet for fabricating silk fibroin spheres has been investigated using aqueous silk fibroin solution from a vibrating nozzle at controlled frequency and amplitude. The methanol treatment or exposure to the water vapor had both confirmed inducing the β-sheet content. The use of chemical reagents such as acetone, methanol is a drawback proved to hurt the aspect of cell proliferation and growth (Cheng et al., 2004). Some investigations carried out, has revealed Silk microparticles possess a certain level of relevance to scaffolds.These milled particles have the ability to strengthen structures reinforcing and improving the mechanical properties as well as biological outcomes in the drug delivery application (Rajkhowa et al. , 2010).

1.4 Aim of Thesis

Silk fibroin as a natural existing biomaterial has been highlighted in the various development of tissue engineering applications including musculoskeletal implants and cellular proliferation and drug delivery. These applications have major limitation concerns in the aspect of microbial contamination mostly at surgical sites of implantation. Therefore, this research will focus on the fabrication of antibiotics blended silk fibroin scaffolds with antimicrobial capability crosslinked using Triethylene Glycol Dimethacrylate. But first, the challenge of fabricating scaffolds with antimicrobial activity is discussed by blending aqueous silk fibroin with fluoroquinolone antibiotics ciprofloxacin & ofloxacin using Triethylene Glycol Dimethacrylate as the crosslinking agent following the freeze-drying procedure. The antimicrobial susceptibility of the blended silk fibroin scaffolds including pure silk fibroin scaffold as control is tested against various microbial pathogens. The morphology of the fabricated structures can be examined using SEM Scanning electron microscopy, FTIR Fourier Transform Infrared Spectroscopy, and X-ray Diffraction Analysis.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials and Methods

The raw domesticated *Bombyx Mori*. Cocoons obtained from Büyük Han Northern Cyprus were cut into pieces and purified to obtain pure silk fibroin protein furtherly discussed in section 2.2.3. Sodium carbonate Na_2CO_3 used in the degumming purification process was acquired from Sigma-Aldrich. Calcium chloride ($CaCl_2$) also purchased from Sigma-Aldrich was utilized in the dissolution of the silk fibroin fibers along with ethanol and deionized water in the preparation of electrolyte solution. The Dialysis membrane obtained from sigma-Aldrich will prevent most proteins of molecular weight 12,000 or greater to pass through. The tube is of 16 mm in diameter and has an average flat width 25 mm (1.0 inc.) with a capacity of ~60 ml/ft. Triethylene Glycol Dimethacrylate $C_{14}H_{22}O_6$ purchased from sigma-Aldrich was implored for crosslinking in the preparation of silk fibroin scaffold incorporated with 2-fluoroquinolone antibiotics ciprofloxacin & ofloxacin obtained from Pharma Mondial Ltd.

Figure 2.1: Raw silk cocoons

2.2.1 Purification of Silk Fibroin

2.2.1.1 Degumming

The degumming procedure is a technique used in the removal and separation of the sericin protein glue from the silk fiber structure. In this procedure, 0.1M of sodium carbonate (Na_2CO_3) solution 1g/100ml (w/v) was prepared in a conical flask containing the silk cocoons. The hot plate is set to 75°C spinning at the speed of 1.5 rpm for three sessions, three hours each as shown in fig 2.2. The degummed silk fibers were washed using deionized water and rinsed thoroughly. The remains of the sericin were removed from the silk and left to dry overnight in the laboratory at room temperature to obtain silk fibers shown in figure 2.3.

Figure 2.2: Degumming process

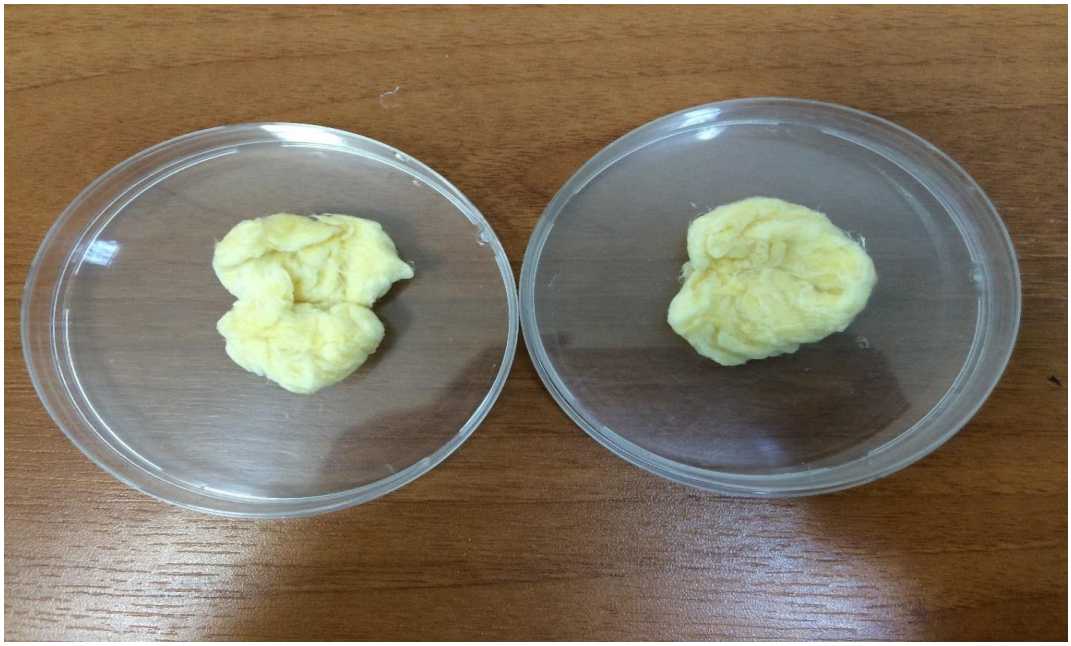

Figure 2.3: Degummed silk fibroin after the degumming process left to dry at room temperature

Figure 2.4: Silk fibers in petri dishes

2.2.2 Dissolution process of silk fibers

It is the total dissolution of silk fibroin fibers involving the disintegration or breakdown of long length polypeptide chains into shorter chains obtaining an aqueous form of silk fibroin.This procedure was carried out by blending the silk fibroin fibers into the prepared $C_2H_5OH : H_2O : CaCl_2$ electrolyte solution (2:8:1) molar ratio. The hot plate stirrer was set to 75°C with continuous stirring Until the total dissolution of fibers shown in figure 2.6. The process resulted in an electrolyte solution blended with silk fibroin, altering the w/v (weight of the fibers to the electrolyte solution's volume) including different silk fibroin concentrations (2% , 3% , 4% , 5% , 6%).

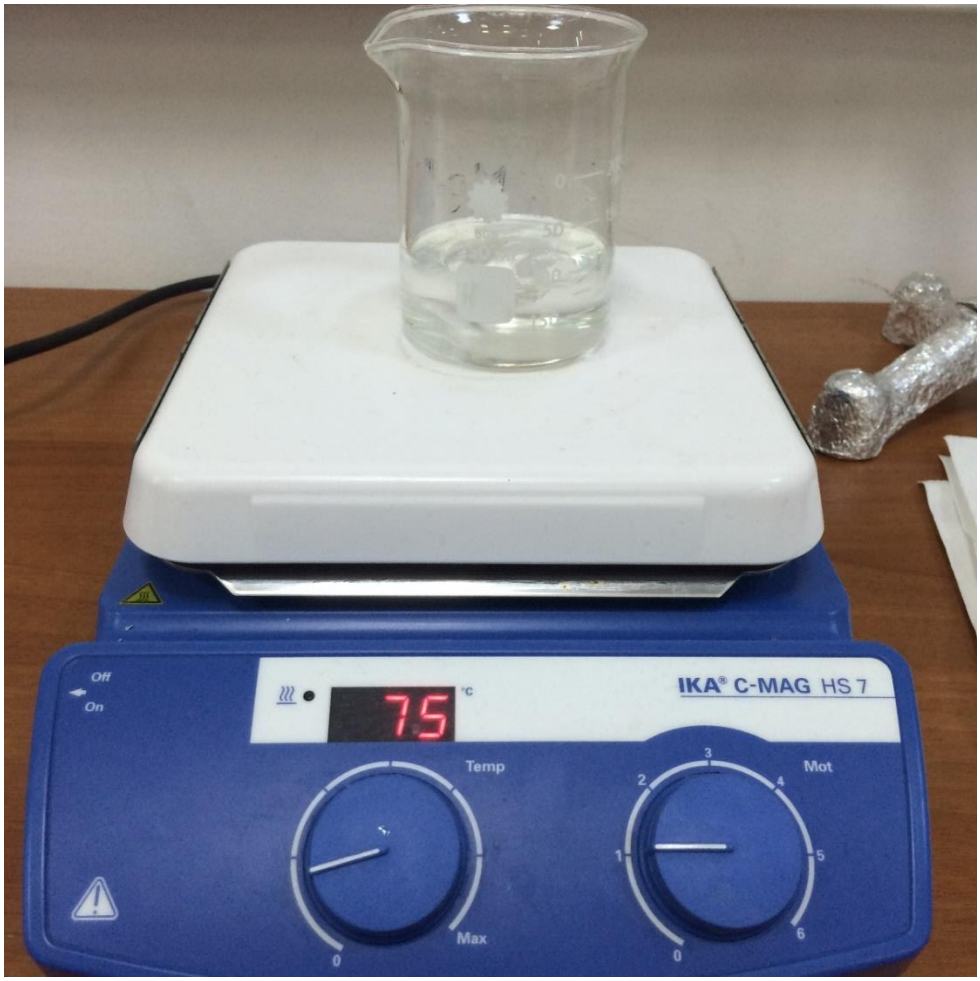

Figure 2.5: The prepared $C_2H_5OH : H_2O : CaCl_2$ electrolyte solution **(2:8:1)** solution

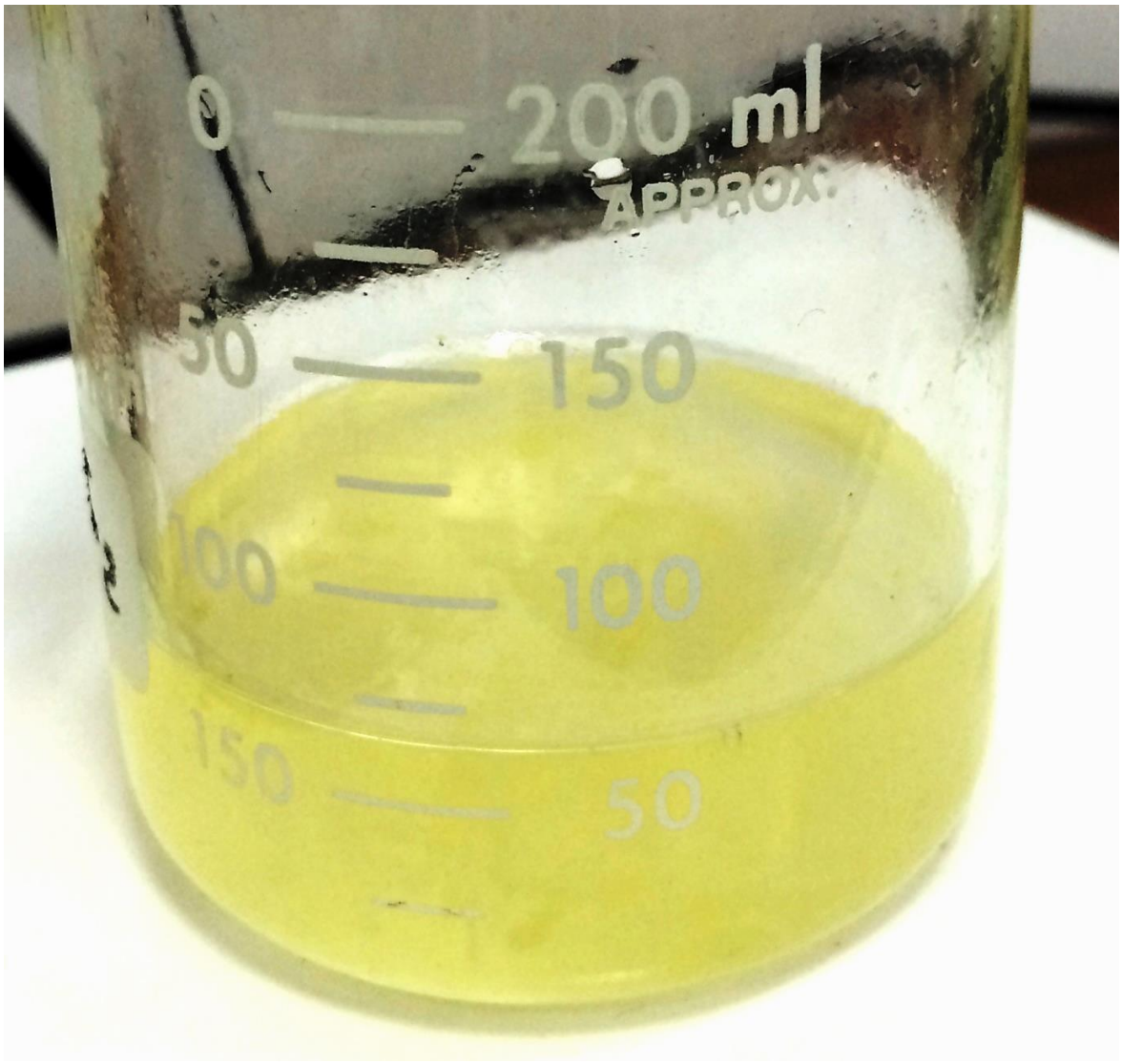

Figure 2.6: Continous dissolution of silk fibroin fibers

2.2.3 Dialysis

After the dissolution procedure, the aqueous silk fibroin blended with the electrolyte solution was dialyzed using carboxymethyl cellulose semipermeable membrane tube. The procedure was done by placing the membrane in a large beaker (5 liters) which was with filled deionized water. The dialysis process allows the diffusion of ions from the silk/electrolyte solution through the membrane to the water. The dialysis procedure was repeated several times at different periods 1, 3, 6, 9, 12 hours) with continuous stirring as shown in figure 2.7 obtaining a pure aqueous silk fibroin.

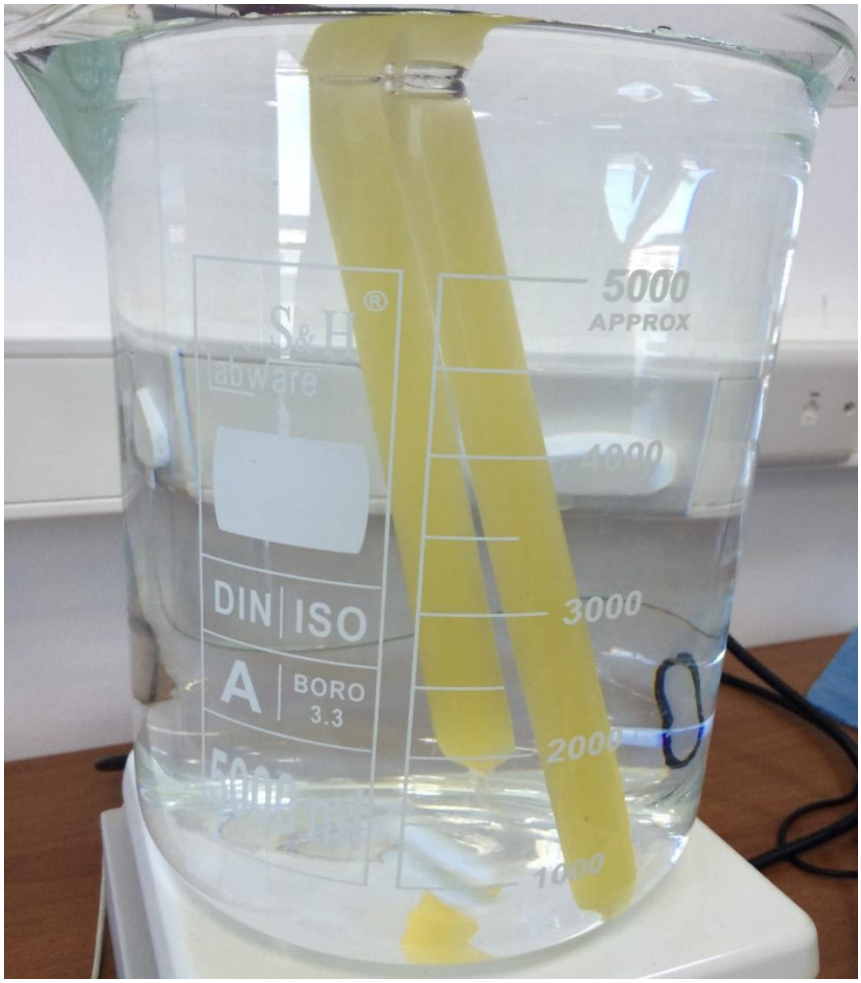

Figure 2.7: Dialysis of the aqueous silk fibroin with deionized water

Figure 2.8: Pure aqueous silk fibroin obtained after the dialysis process

2.2.4 Preparation of silk fibroin/ciprofloxacin/ofloxacin scaffolds using triethylene glycol dimethacrylate as a cross-linking agent

The aqueous silk fibroin obtained after the dialysis process was cross-linked using triethylene glycol dimethacrylate $C_{14}H_{22}O_6$. The aqueous solution was blended with Ciprofloxacin and ofloxacin belonging to the class of fluoroquinolones antibiotics, combined in varying proportions as shown in Table 2 & 3 respectively. A total of 12 samples were prepared including silk fibroin cross-linked with Triethylene Glycol Dimethacrylate only as control samples. The crosslinked silk fibroin/ciprofloxacin solution was poured into syringes and kept in a freezer at a temperature of -20°C for 8 hours. The freeze-dried scaffold formed in the syringe was allowed to thaw at room temperature and was immersed in chilly 70% methanol. The structure obtained was removed from methanol to dry at room temperature as shown in figure 2.10.

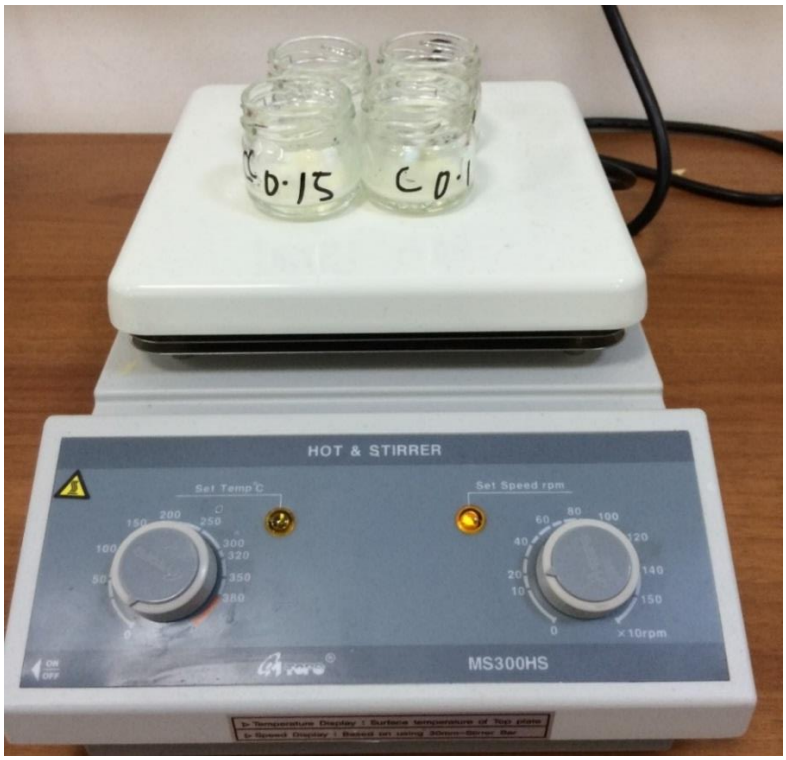

Figure 2.9: The Blending of aqueous silk fibroin and Ciprofloxacin/ofloxacin in varying proportions using TEGDMA $C_{14}H_{22}O_6$

Table 2.1: Ratios of aqueous silk fibroin blended with ofloxacin

Silk fibroin samples	Cross-linker volume $C_{14}H_{22}O_6$ TEGDMA	Floxin (Grams)	Freezing Temperature	Silk fibroin volume	Final concentration of Silk Fibroin
S.F 1	0.15ml	0.05g	-20°C	2 ml	3.2 %
S.F 2	0.15ml	0.1g			
S.F 3	0.15ml	0.15g			
S.F 4	0.15ml	0.20g			

Table 2.1 & Table 2.2 shows various ratios of the blended composite solution including constant ratio of TEGDMA, S.F volume and concentration as well as variant quantities of ofloxacin and ciprofloxacin.

Table 2.2: Ratios of aqueous silk fibroin blended with ciprofloxacin

Silk fibroin samples	Cross-linker volume $C_{14}H_{22}O_6$ TEGDMA	Cipro (Grams)	Freezing Temperature	Silk fibroin volume	Final concentration of Silk Fibroin
S.F 5	0.15ml	0.05	-20°C	2 ml	3.2%
S.F 6	0.15ml	0.1			
S.F 7	0.15ml	0.15			
S.F 8	0.15ml	0.20			

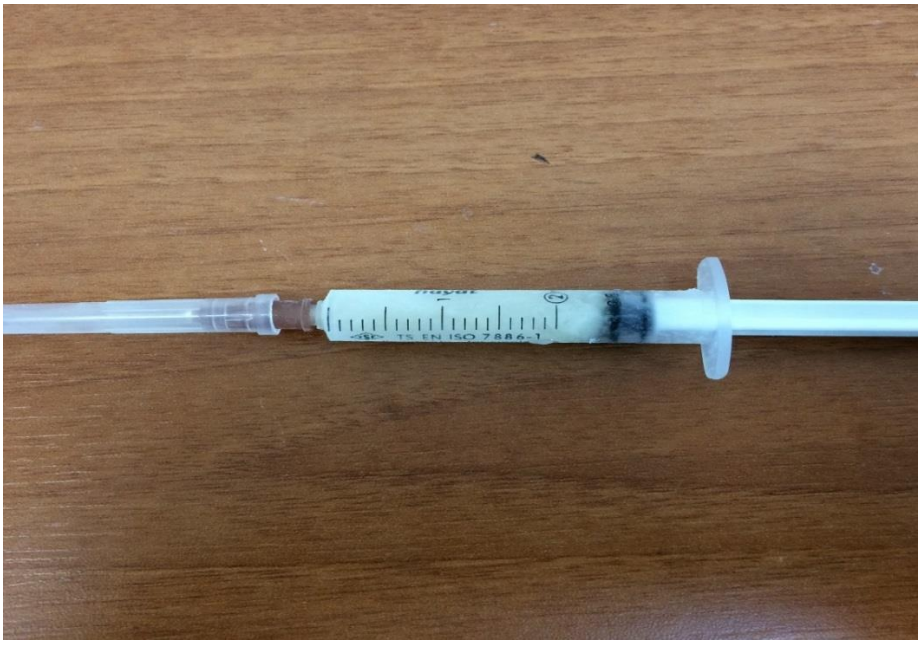

Figure 2.10: Silk fibroin scaffold froze in the syringe

Figure 2.11: Freeze-dried silk fibroin scaffolds

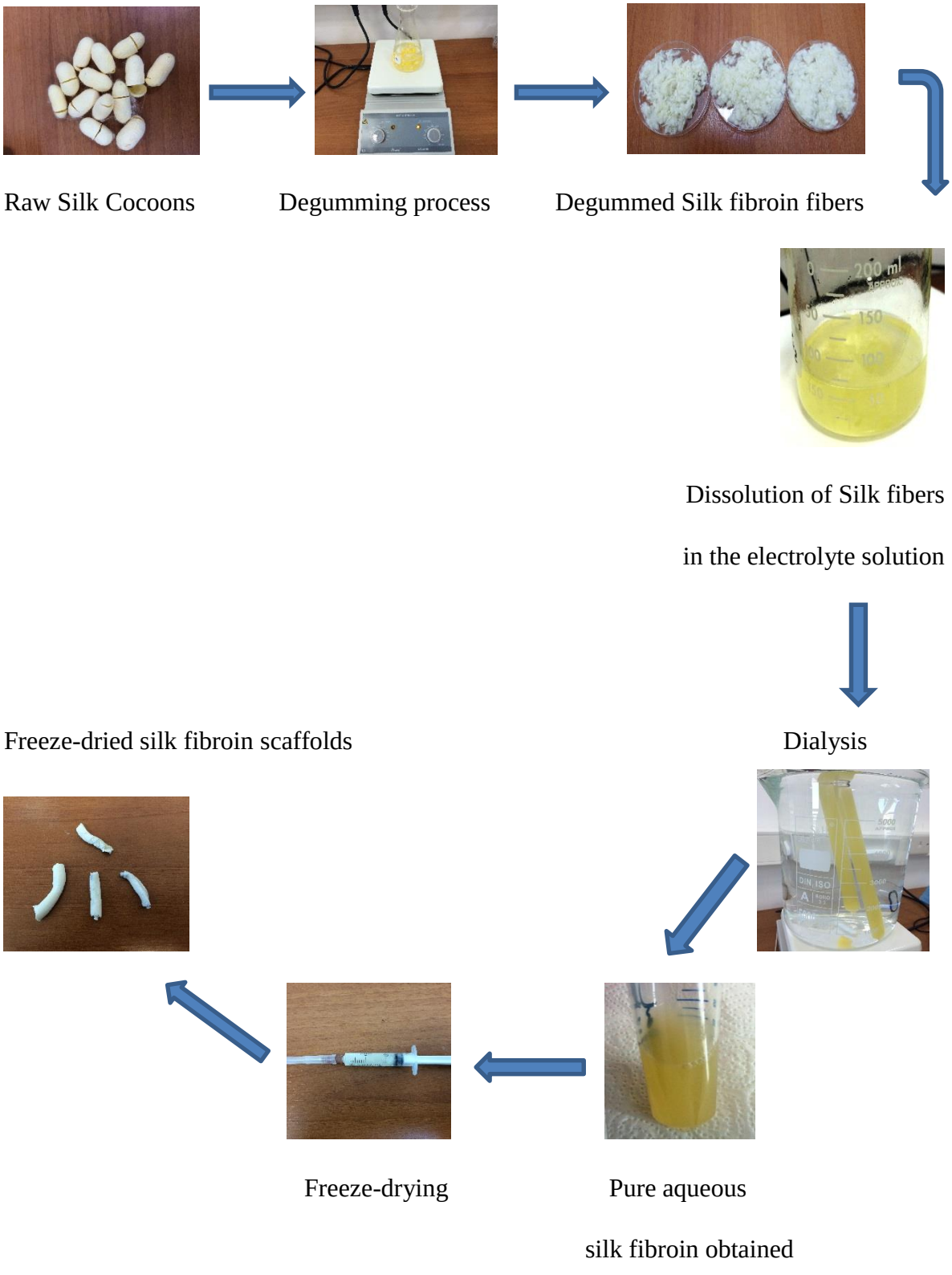

Figure 2.12: Silk fibroin purification process

2.2.5 Silk fibroin with different concentration and crosslinking

The concentration of aqueous Silk fibroin obtained was increased from 2% w/v, 3% w/v, 4% w/v, 5% w/v and 6% w/v in the ($CaCl_2$:H_2O:C_2H_5OH) electrolyte solution. In this experiment, the SF solution concentration after dialysis decreased to half of the original as well as an increase in the volume. Due to water leakage from the dialysis procedure of 2%, 3%,4%,5% and 6% aqueous silk fibroin solution, the SF solution becomes 2%, 2.5% and 3% all w/v after dialysis.

2.3 Analysis of Antimicrobial Susceptibility Test of Ciprofloxacin/Ofloxacin Silk Fibroin Scaffolds

2.3.1 Inoculum preparation

Mueller Hinton agar is a microbiological growth medium prepared to determine the antimicrobial susceptibility of the scaffolds whether Susceptible (S), Intermediate (I) and Resistant® against strains of microbial pathogens. The strains of microorganisms include *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923, *Bacillus cereus* ATCC 10876 & *Candida albicans* ATCC 90028. The turbidity of the test suspension containing the inoculums is adjusted to 0.5Mcfarland standard for 15minutes following inoculation onto the Mueller-Hinton agar and incubated at 37°C for 18-24hrs.

2.3.2 Disk diffusion method

The antimicrobial susceptibility test of the cross-linked silk fibroin scaffolds incorporated with Ciprofloxacin and Ofloxacin antibiotics, as well as the ordinary silk fibroin structure was conducted using Kirby-Bauer disk diffusion technique. The antimicrobial test of the scaffolds was investigated in the Department of Clinical Microbiology & Infectious diseases Near East Hospital, North Cyprus. The inoculums containing the strains of microorganisms inoculated using cotton swab was carried out via the Stokes method onto the plates containing solidified Mueller Hinton agar medium. The Stokes method is used dividing the plates into halves containing different proportions of the antibiotics present in the silk fibroin scaffolds shown in Table 3.1 & 3.2. The pure S.F scaffold, as well as S.F containing antibiotics scaffolds, was sliced into uniform sizes using sterile surgical blades (Carbon

steel). The sliced scaffolds were placed into plates containing the inoculums with complete contact with the agar medium. Incubation of these plates was carried out at 37°C for 24hrs exhibiting clear variant zones measured to the nearest millimeters (mm) using a ruler.

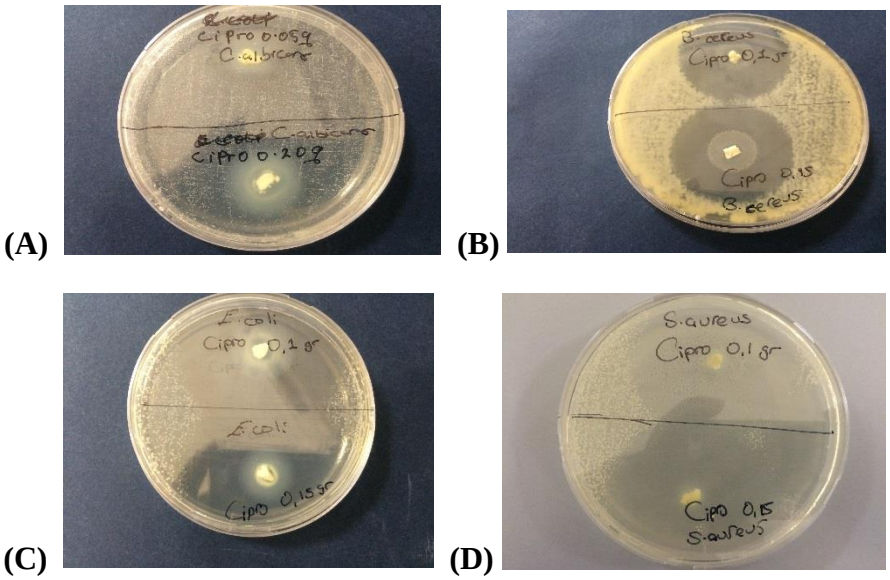

Figure 2.13: Inhibition zones of silk fibroin/Ciprofloxacin scaffolds against (A) *C. albicans* , (B) *B. cereus*, (C) *E. coli* and (D) *S. aureus*

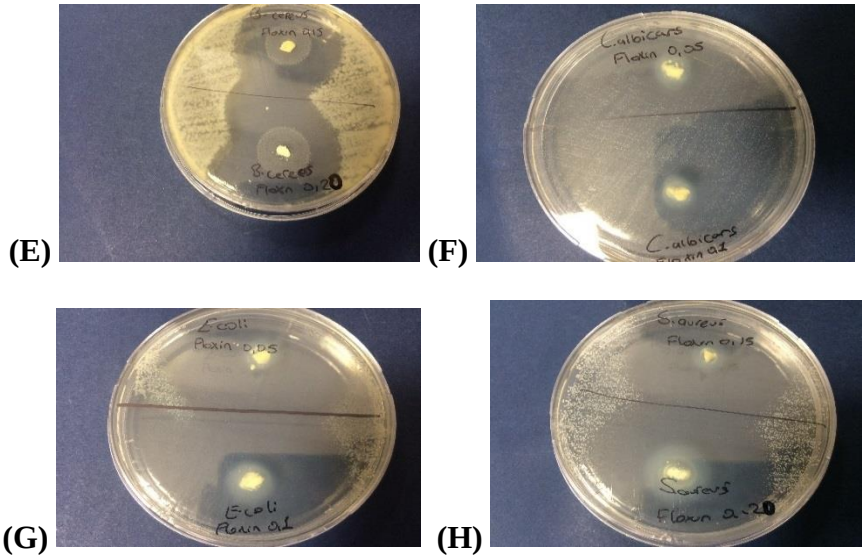

Figure 2.14 : Inhibition zones of Silk Fibroin/Ofloxacin Scaffolds against (E) *B. cereus* (F) *C. albicans* (G) *E. coli* and (H) *S. aureus*

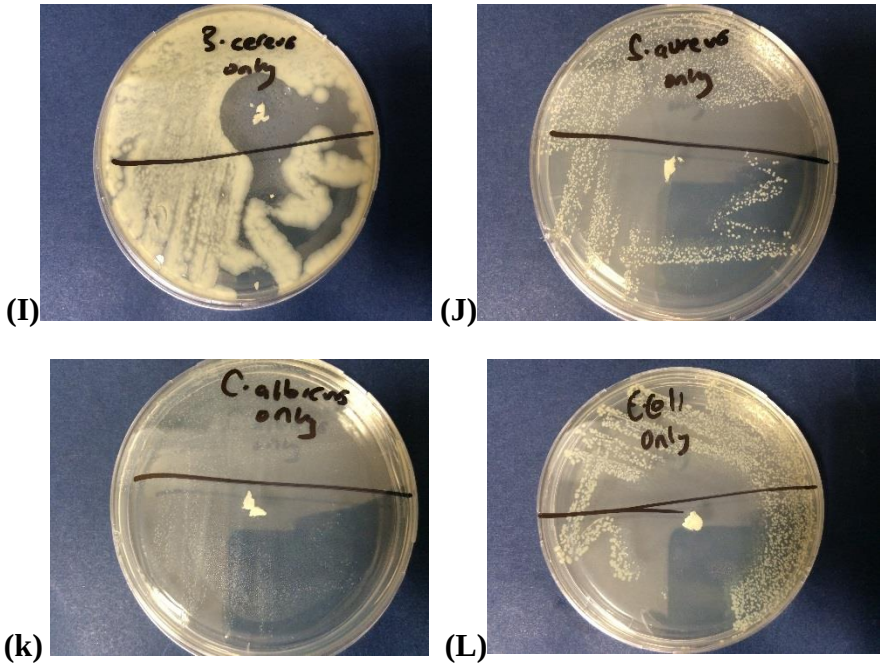

Figure 2.15 : Inhibition zones of free silk scaffolds against (I) *B. cereus* (J) *S. aureus* (K) *C. albicans* and (L) *E. coli*

2.4 Scanning Electron Microscope (SEM) Analysis

The surface and cross-section morphologies of the silk fibroin/ciprofloxacin scaffolds were observed at different magnifications using an SEM JSM-6510 model, photographed at 10kV acceleration voltage in TUBITAK-MAM Marmara research center Arastirma Merkezi Gebze, Istanbul, Turkey. A scanning electron microscopy (SEM) is an electron microscope that is used to produce images of sample involving beam of electrons. The beam of electrons is produced at the top of the lens passing through electromagnetic lenses focusing down towards the sample scanning through it and deflecting the beam in X & Y axes.

2.5 X-ray Diffraction (XRD) Analysis

X-ray diffraction analysis can be used to investigate a sample structure using spatial distribution including intensities of X-radiation interacting with the electrons of the sample & X-rays are diffracted.

The x-ray diffraction analysis was executed in TUBITAK-MAM Marmara research center using Shimadzu XRD-6000 model diffractometer of X-ray voltage source 40kV at a current of 40 mA with Cu X-ray tube (λ=1.5405 A° (10-10 meter). The diffraction intensity curves obtained, was produced scanning at the rate of 2◦/min within the scanning region of 2θ. This procedure was carried out using the method proposed by Jawarska et al.

2.6 Fourier Transform Infrared Spectroscopy (FTIR) Analysis

The FTIR Analysis is an approach employed to initiate infrared spectrum of absorption, emission as well as Roman Scattering of a photon of the sample (solid, liquid or gas). It involves the conversion of raw data into an actual spectrum (Griffiths and Hasseth, 2007). The infrared spectra of the cross-linked S.F scaffolds containing ciprofloxacin were measured using FTIR spectrophotometer carried at TUBITAK-MAM Marmara research center.

CHAPTER 3

RESULTS AND DISCUSSION

3.1 Antimicrobial Susceptibility Test Result of Silk fibroin/Ciprofloxacin and Ofloxacin Scaffolds

The inhibition zones shown in Figure 2.1, 2.2 & 2.3 following 18- 24 hours incubation at 37°C is as a result of antibiotics released from the crosslinked silk fibroin structure containing ciprofloxacin & ofloxacin antibiotics. The pure silk fibroin scaffold was fabricated as well as a control displaying clear zones against the four microbial strains. The measured variant inhibition zones exhibited by the antibiotics containing silk fibroin scaffold is shown in Table 3.1, and 3.2 including clear areas produced by the pure silk fibroin scaffold are all measured to the nearest millimeters.

Table 3.1: Inhibition zones of ciprofloxacin present in the silk fibroin scaffolds

Amount of Cipro (Grams)	Inhibition zones of *S. aureus*	Inhibition zones of *B. cereus*	Inhibition zones of *C. albicans*	Inhibition zones of *E. coli*
0.05	40mm	39mm	10mm	60mm
0.1	55mm	40mm	15mm	65mm
0.15	60mm	47mm	20mm	70mm
0.20	65mm	52mm	30mm	80mm

Table 3.2: Inhibition Zones of Ofloxacin present in the silk fibroin scaffolds

Amount of Floxin (grams)	Inhibition zone of *S. aureus*	Inhibition zone of *B. cereus*	Inhibition zone of *C.albicans*	Inhibition zone of *E. coli*
0.05	50mm	41mm	21mm	60mm
0.1	57mm	43mm	24mm	67mm
0.15	60mm	45mm	27mm	71mm
0.20	62mm	50mm	35mm	75mm

Table 3.3: Inhibition zones of free silk fibroin scaffold against the following microorganisms

Free S.F Scaffolds	Inhibition zone of *S. aureus*	Inhibition zone of *B. cereus*	Inhibition zone of *C. albicans*	Inhibition zone of *E. coli*
	46mm	40mm	30mm	51mm

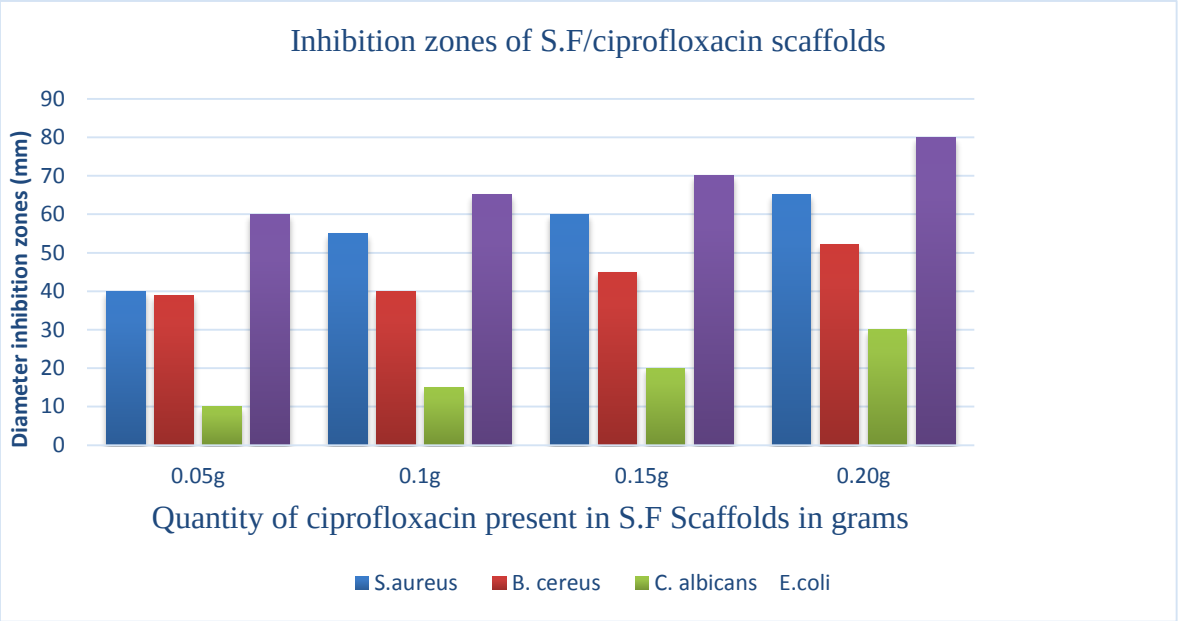

Figure 3.1: Proportions of ciprofloxacin in silk fibroin scaffolds and inhibition zones against *S. aureus*, *B. cereus*, *C. albicans* & *E. coli*

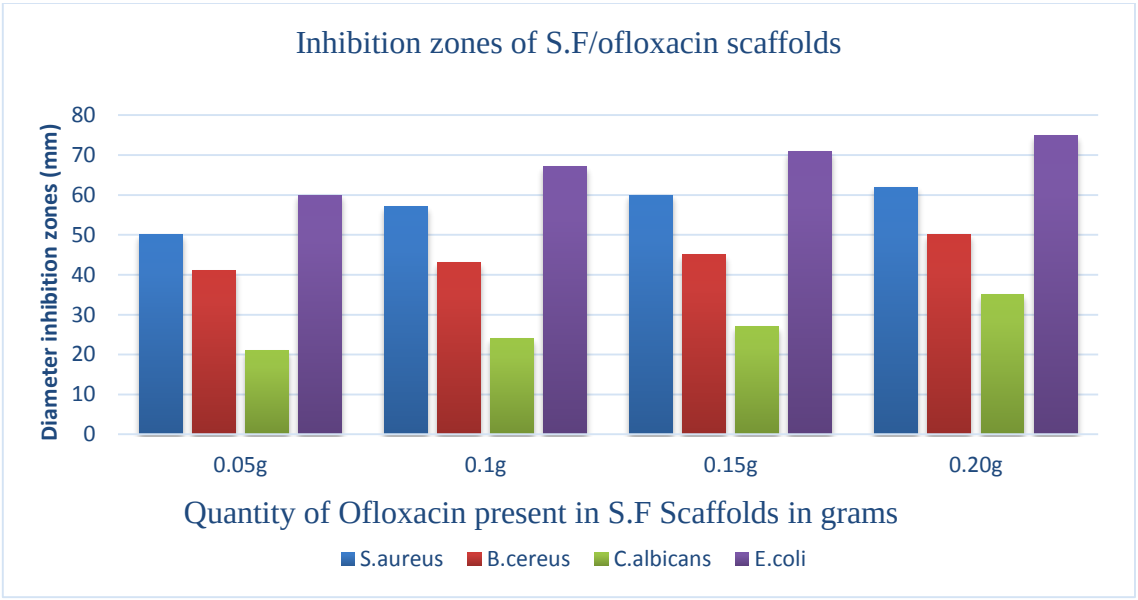

Figure 3.2: Proportions of Ofloxacin in silk fibroin scaffolds and Inhibition zones against *S. aureus*, *B. cereus*, *C. albicans* & *E. coli*

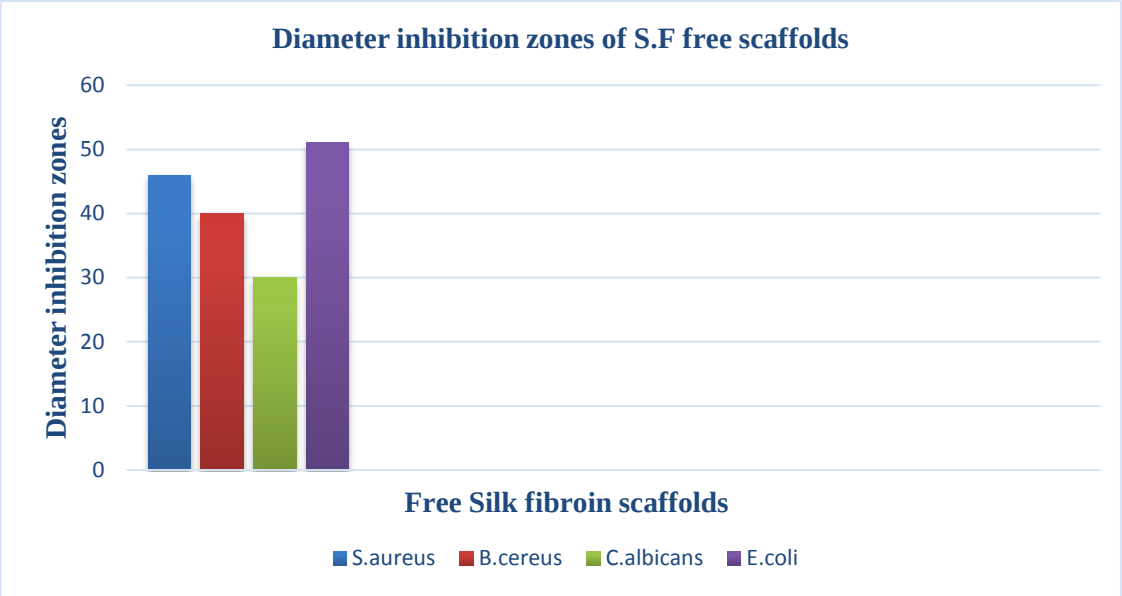

Figure 3.3: Inhibition zones of Free silk fibroin scaffolds against *S. aureus, B. cereus, C. albicans* & *E. coli*

The antimicrobial activity of the silk fibroin/ciprofloxacin and ofloxacin scaffolds crosslinked using Triethylene Glycol Dimethacrylate $C_{14}H_{22}O_6$ as a cross-linking agent was investigated via Kirby-Bauer disk diffusion technique making use of Mueller-Hinton agar as a microbiological growth medium. Table 3.1, 3.2 & 3.3 revealed the antimicrobial activities of silk fibroin blended antibiotic scaffolds (Cipro/Floxin) in various proportions measured in grams was incubated for 24 hours at 37°C. The Inhibition zones shown in figure 2.13, 2.14 &2.15 is as a result of the antibiotics released from the crosslinked silk fibroin/ciprofloxacin and ofloxacin scaffolds. The surface area of the inhibition zones was noted to expand following the rise in quantity (grams) of Ciprofloxacin and ofloxacin antibiotics present in the silk fibroin scaffolds. The strains of microbial pathogens inoculated onto the Mueller-Hinton agar containing the scaffolds includes. *Staphylococcus aureus, Bacillus cereus, Escherichia coli* & *Candida albicans* .

However, the antimicrobial susceptibility test revealed that silk fibroin blended antibiotic scaffolds pronounced the largest inhibition zone against the gram negative bacteria *Escherichia coli* ATCC 25922. Another massive inhibition zone was exhibited against *Staphylococcus aureus* ATCC 25923. It can be observed from Table 3.1 and 3.2 that the crosslinked silk fibroin scaffolds containing different proportions of ciprofloxacin and ofloxacin displayed antifungal activity against *Candida albicans.* The prepared pure silk fibroin scaffold as well demonstrated a reasonable antimicrobial capacity against *Escherichia coli, Staphylococcus aureus, Bacillus cereus* and *Candida albicans* indicating that the pure silk fibroin scaffold possesses a certain level of antimicrobial capability.

3.2 SEM Analysis

Morphological features of silk fibroin/ciprofloxacin scaffolds were studied at different magnifications using the scanning electron microscope (SEM).

3.2.1 SEM analysis of silk fibroin/ciprofloxacin scaffolds

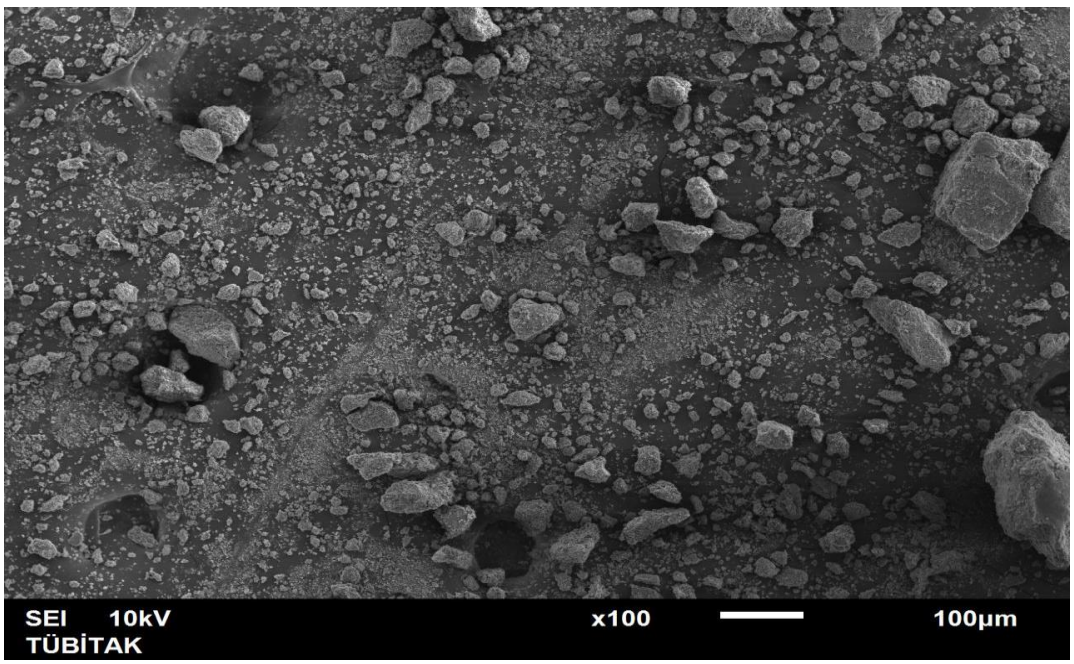

Figure 3.4: SEM micrograph of silk fibroin/ciprofloxacin scaffold

The SEM micrograph in figure 3.4 reveals the surface morphology and structure of the crosslinked silk fibroin/ciprofloxacin scaffold. It can be observed from the SEM micrograph that the surface of the cross-linked silk fibroin/ciprofloxacin scaffold has dominant aggregated roughly scattered particle structures varying in size as well as a moderate porosity level. Therefore, the roughly distributed particle structures on the surface of the scaffold as in figure 3.5 indicates that the surface can be suitable for cell attachment.

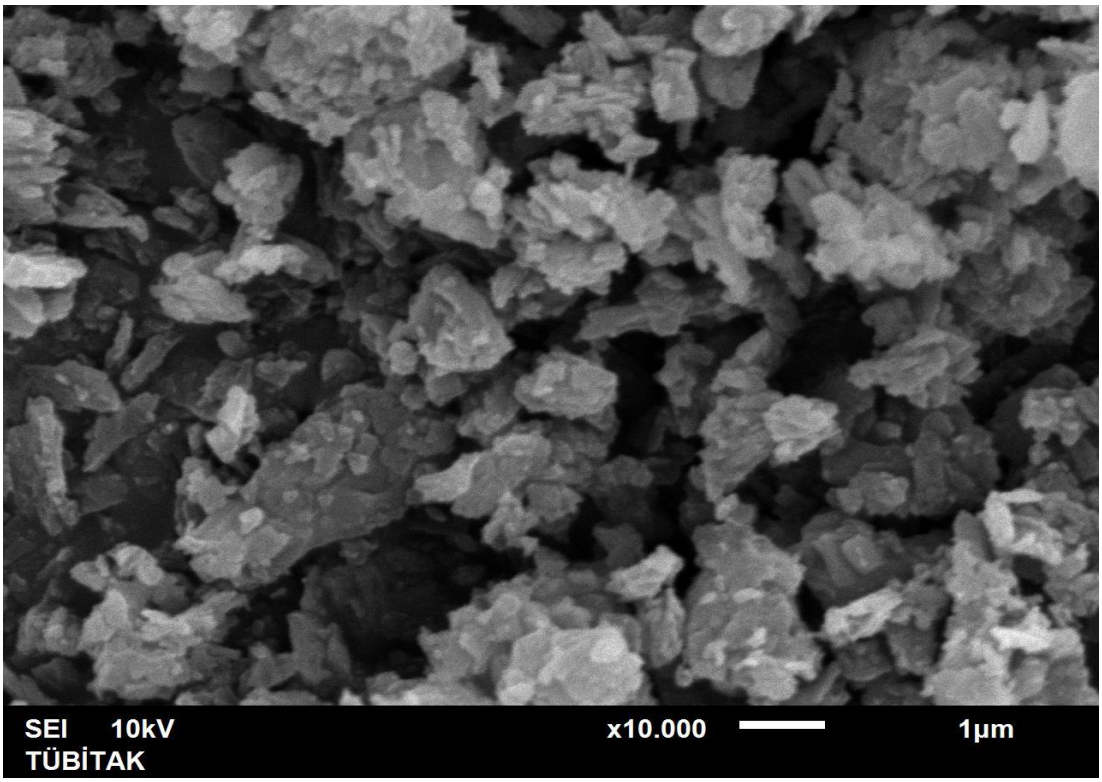

Figure 3.5: SEM micrograph of silk fibroin/ciprofloxacin scaffold showing its rough particle surface structure

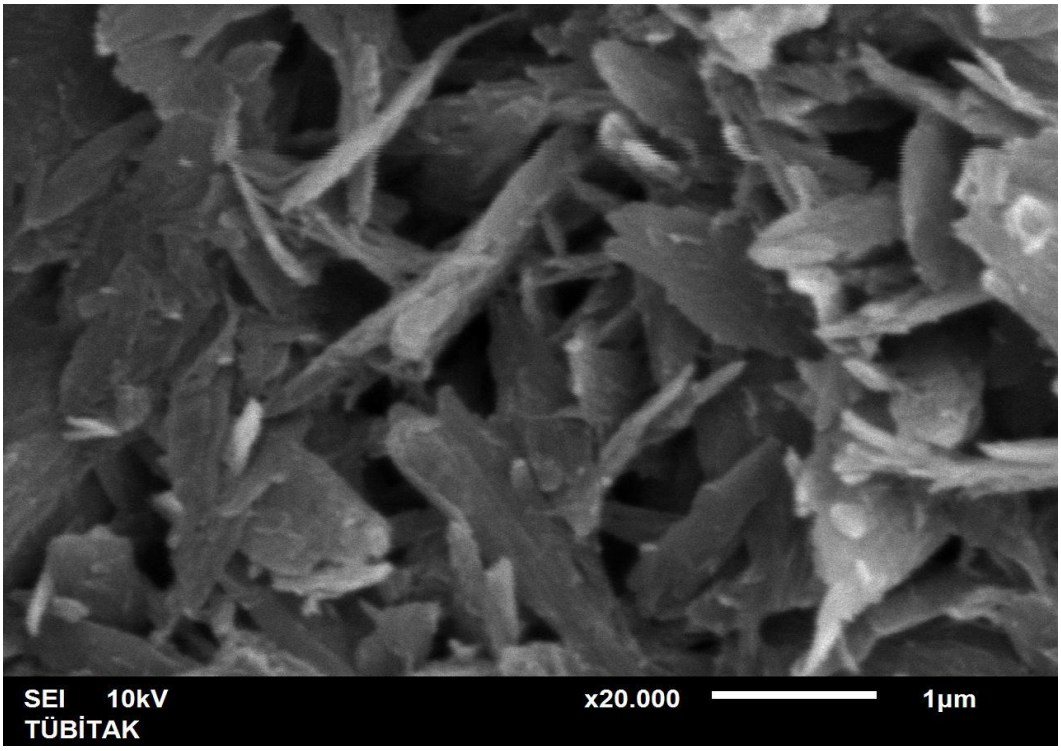

Figure 3.6: SEM micrograph of silk fibroin /ciprofloxacin scaffold cross-linked with Tri-ethylene glycol dimethacrylate $C_{14}H_{22}O_6$

Figure 3.6 shows the SEM micrograph of the silk fibroin/ciprofloxacin scaffold crosslinked using Triethylene Glycol Dimethacrylate $C_{14}H_{22}O_6$. The figure reveals a uniform porous aggregated sheet with anomalous-Shaped structures in clusters possessing thick, smooth surface as a result of the interaction between the crosslinking agent and silk fibroin/ciprofloxacin blend.

3.3 X-ray Diffraction (XRD) Result Analysis

The various structural changes of the cross-linked silk fibroin/ciprofloxacin scaffold were determined by the X-ray diffraction analysis shown in figure 3.7. The figure displays the pronounced peaks exhibited by the silk fibroin/ciprofloxacin scaffold cross-linked with Triethylene Glycol Dimethacrylate $C_{14}H_{22}O_6$. The XRD Pattern presented various diffraction peaks shown in 2θ, namely 12°, 25°, 27°, 30°, 44.5° and 64.5° respectively. The diffraction peaks at (2θ=25°) & (2θ=27°) of the crosslinked silk fibroin/ciprofloxacin scaffold shows correspondence to silk I crystalline structure due to the use of methanol inducing β-sheets formation of the structure. Ethanol present in the electrolyte solution preparation used to dissolve silk fibers also yielded effect on the structure's crystallinity. It is said that ciprofloxacin remained in its crystalline nature due peaks at 12°, 25° & 30° which suggests the scaffold is stable and has a low rate of solvent solubility.

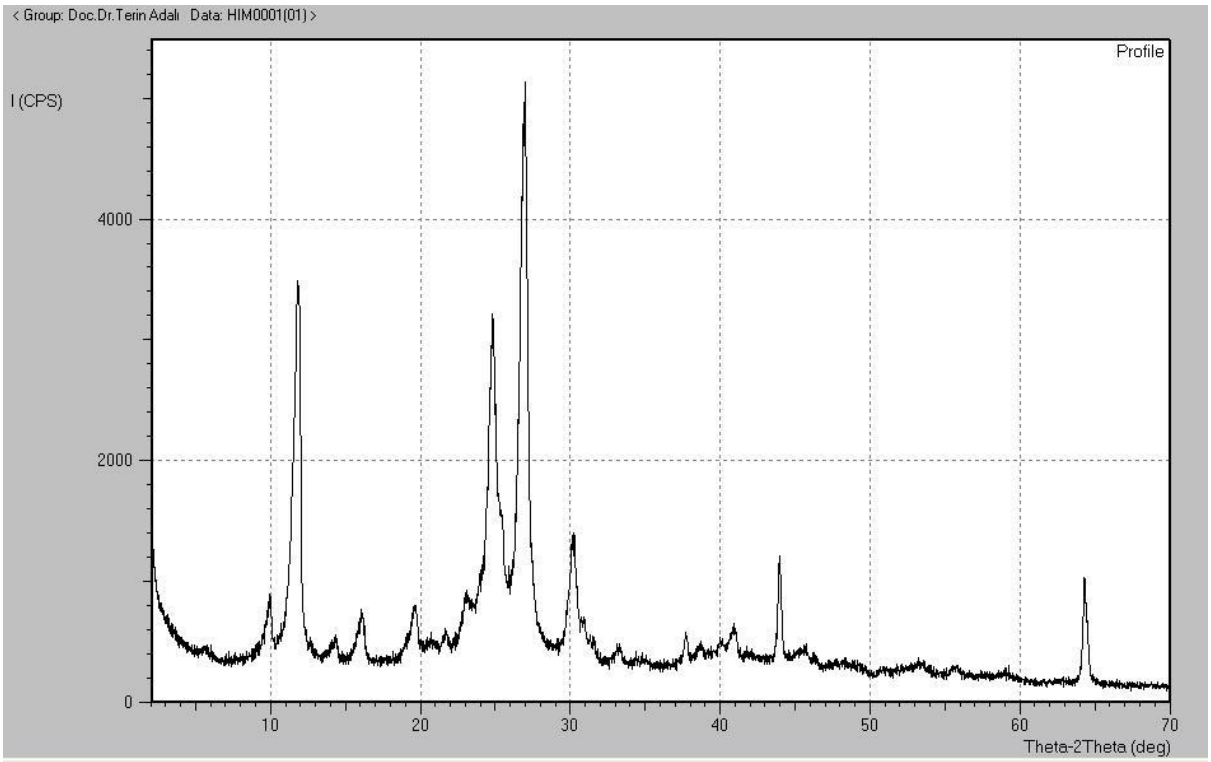

Figure 3.7: X-ray diffraction pattern of silk fibroin/ciprofloxacin scaffold cross-linked with triethylene glycol dimethacrylate $C_{14}H_{22}O_6$

3.4 Fourier Transform Infrared Spectroscopy (FTIR) Result Analysis

The figure 3.8 shown below displays results from the FTIR analysis of cross-linked silk fibroin/ciprofloxacin scaffold. Due to the presence of the amide groups in silk fibroin protein structure, the characteristic absorbance peak seen around 1624.5cm^{-1} is as a result of absorption peaks of the peptide backbone of amide I (C=O) group. The absorbance peak exhibited at 1712.6cm^{-1} is due to the presence of amide II (N─H) group existing in the silk fibroin structure. These characteristic absorbance peaks indicated the existence of hydrogen-bonded NH group in the composite silk fibroin scaffold. The conformation of *B.mori* silk fibroin is characterized by the β-sheet absorption peaks observed at 1624.5cm^{-1} due to the effect of methanol treatment on the cross-linked silk fibroin/ciprofloxacin scaffold. Figure 3.8 also demonstrated absorbance peaks at 1467.4 cm^{-1} and 1494.8 cm^{-1} due to the existence of Carbonyl group (C─O) present in ciprofloxacin blend of the silk scaffold.

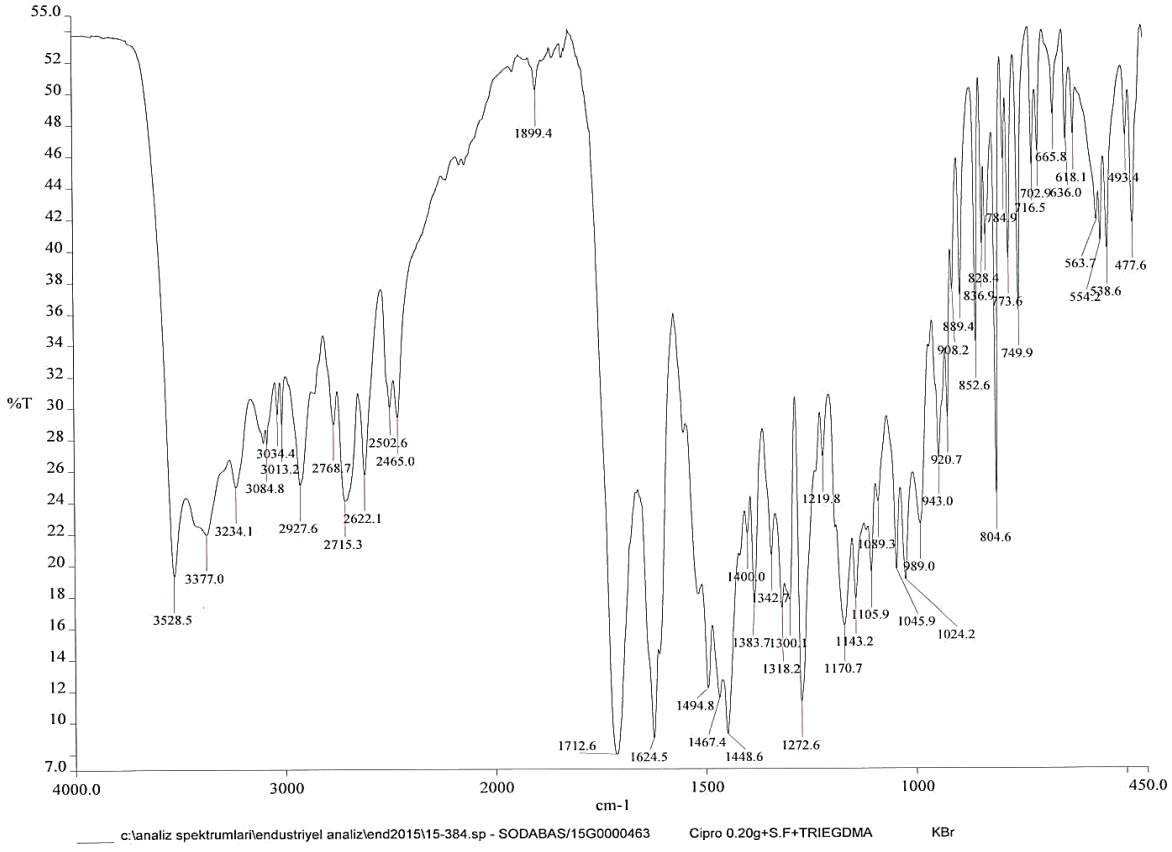

Figure 3.8: FTIR spectra of cross-linked silk fibroin/ciprofloxacin scaffold

CHAPTER 4

CONCLUSION

The various proportions of crosslinked silk based scaffolds containing different ratios of ciprofloxacin were prepared by the freeze-drying technique at ─20°C. Factors such as temperature, concentration of crosslinking agent, polymer composition, and freezing rate are the factors influencing the formation of cross-linked silk fibroin/ciprofloxacin and ofloxacin scaffolds. The antimicrobial susceptibility of the silk fibroin/ciprofloxacin and ofloxacin scaffolds as well as pure silk fibroin scaffold was tested via Kirby-Bauer disk diffusion technique against four microbial strains namely *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923, *Bacillus cereus* ATCC 10876 *& Candida albicans* ATCC 90028. Different inhibition zones were exhibited by the scaffolds containing variant proportions of the antibiotics. It was observed that the higher amount of ciprofloxacin and ofloxacin present in the silk-based scaffold, the larger the inhibition zones exhibited by the antibiotic silk fibroin scaffolds. The structure loaded with 0.20g of both antibiotics produced the most significant inhibition zones of 75mm and 80mm against *Escherichia coli* ATCC 25922 indicating that the bacteria is Susceptible (S) to cross-linked silk fibroin/ciprofloxacin and ofloxacin scaffolds. *Candida albicans* ATCC 90028 exhibited clear zones of 27mm and 30mm as a result of 0.20g ciprofloxacin and ofloxacin present in the silk-based scaffold. These findings suggest that the crosslinked silk fibroin/ciprofloxacin scaffolds are capable of antimicrobial activities against *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923, *Bacillus cereus* ATCC 10876 *& Candida albicans* ATCC 90028.

According to the SEM analysis of the cross-linked silk fibroin/ciprofloxacin scaffold, the role of the crosslinker was shown smoothing the surface of the composite structure also possessing rough particle structures. It also revealed porous aggregated flat anomalous-shaped sheets. The sparsely rough distributed particle surface indicates the suitability of the scaffold for cell attachment.

The XRD pattern analysis, displayed various diffraction peaks of ($2\theta=25°$) & ($2\theta=27°$) showing a correspondence to the native silk I crystalline structure. The crystalline structure is as a result of the β─sheet structure present in the silk/ciprofloxacin scaffold due to the use

of methanol treatment. Peaks at 12°, 25° & 30° shows the evident presence of an unchanged form of ciprofloxacin in the silk-based scaffold.

Lastly, the FTIR analysis showed the composition of silk fibroin/ciprofloxacin scaffold as well as the unchanged structure of silk fibroin as a result of characteristic absorbance peak seen around 1624.5cm^{-1}. The confirmation of ciprofloxacin present in the silk-based scaffold was revealed by characteristic absorbance peaks 1467.4 cm^{-1} and 1494.8 cm^{-1} due to the presence of Carbonyl bond group (C─O).

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