BIOMEDICAL APPLICATIONS OF SILK FIBROIN FILMS AND 3D-SCAFFOLDS

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ABSTRACT

Silk from Bombyx mori is a protein-based fiber. Bombyx mori silk fibroin (SF) is one of the most important candidates for biomedical materials based on biocompatibility, biodegradation, processability and excellent mechanical properties. The present study aims at the preparation of silk fibroin for possible applications in tissue engineering. Pure silk fibroin protein was extracted from Bombyx mori silk cocoon.

This graduation project is to explain how silk fibroin is extracted from silk cocoon, purified and film casting process by UV irradiation, and preparation of silk fibroin scaffolds by freeze-drying method at -80^{o}C and -30^{o}C respectively.

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CHAPTER 1

INTRODUCTION:

Silk fibroin is a natural macromolecule extracted from silkworm (Bombyx mori), and has attracted the interest of scientists of various disciplines for a long time. It has a long history of use in medicine, for example, as sutures and artificial ligaments. Silk is mechanically robust biomaterial that offers a wide range of mechanical and functional properties for biomedical applications in the viewpoint of biocompatibility [5] and biodegradability [5].

B. mori silkworm silk fibers have been the primary silk-like material used in biomedical applications particularly as sutures. During decades of use,silk fibers have proven to be effective in many clinical applications [21]. At the same time,some biological responses to the protein have raised questions about biocompatibility.

One of the major difficulties in assessing the biological responses reported to these silk fibers is the absence of detailed characterization of the fibers used including,e xtent of extraction of the sericin,the chemical nature of wax-like coatings sometimes used,and many related processing factors [21]. This variability in source material has resulted in confusion in the literature and in clinical settings concerning the benefits or potential concerns with this class of fibrous protein [21]. For example,of greatest importance is that based on many studies it is clear that the sericin glue-like proteins are the major cause of adverse problems with biocompatibility and hypersensitivity to silk [21]. If sericin is removed,the biological responses to the core fibroin fibers appear to be comparable to most other commonly used biomaterials [21].

Given the benefits of polymer drug delivery implants over traditional periodic systemic administration, the development of biomaterial systems with the necessary properties (biocompatibility, degradation, stabilization, controllability) is paramount [20]. Silk fibroin represents a promising, naturally derived polymer for local, controlled, sustained drug release from fully degrading implants and the polymer can be processed into a broad array of material formats.

Silk biomaterials are covered in the areas of drug delivery systems, especially those that can function as long-term depots. Fundamentals of structure and assembly, processing options, control points and specific examples of implantable silk drug delivery systems (sponges, films) and injectable systems (microspheres, hydrogels) from the 1990s are studied [20]. Owing to its unique material properties, stabilization effects and tight controllability, silk fibroin is a promising biomaterial for implantable and injectable drug delivery applications.

Many promising control points have been identified, and characterization of the relationships between silk processing and material properties and the resulting drug loading and release kinetics will ultimately enhance the overall utility of this unique biomaterial [20]. The ever-expanding biomaterial 'tool kit' that silk provides will eventually allow the simultaneous optimization of implant structure, material properties and drug release behavior that is needed to maximize the cost-efficiency, convenience, efficacy and safety of many new and existing therapeutics, especially those that cannot be delivered by means of traditional administration approaches [20].

Silk fibroin is a kind of protein which is composed of fibers. The silk fibroin fibers from silkworms at room temperature and from an aqueous solution are strong and stiff fibers. Silk must be regenerated into a desirable form to meet specific biomedical applications; film casting and formation of scaffolds. In general, aqueous silk fibroin solution is obtained by dissolving SF

in an electrolyte that contains concentrated natural salts, such as calcium chloride, lithium bromide or potassium bromide.

This project explains how silk is generated, what are its properties, silk fibroin's biochemistry and biomedical applications in film casting and 3D-scaffold generation. The aim of this project is to simplify the production of film casting and by using freeze-drying technique to improve the swelling properties of the 3D-scaffold.

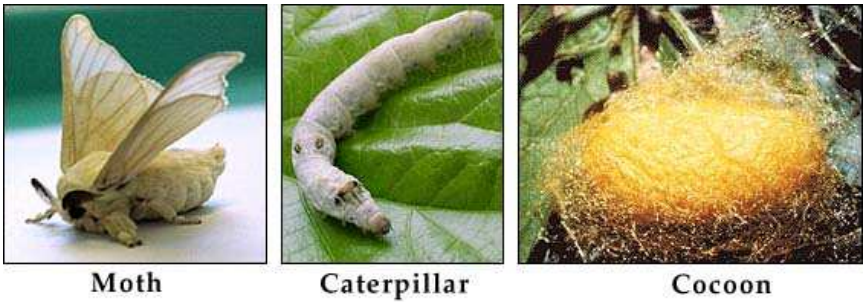
CHAPTER 2

2.1 Bombyx mori:

Silk is produced as the cocoon covering of the silkworm, the pupal form of the Asian or mulberry silk moth, *bombyx mori*. The cocoon is spun by the silk moth caterpillar of a single silk fiber that can be up to several thousand feet in length. To harvest the silk, completed cocoons are boiled or heated to kill the silkworms, then laboriously unwound into single fibers which are plied together and spun into thread or silk yarn [4].

Insects mainly belong to two families, Saturnidae and Bombycidae, which spins silk fibre. Bombyx mori belongs to Bombycidae produces a delicate twin thread of silk fibroin, which is coated by a protective cover of sericin [2].

The domesticated silk worm is a lepidopteran molecular model and an important economic insect that are emerging as an ideal molecular resource for solving a broad range of biological problems. The silkworm produces massive amount of silk protein during the final stage of larval development [2]. Silk protein is a kind of protein like collagen, elastin, keratin, and fibroin is an essential constituent of cocoon filament.



Picture-1: Showing silk moth, silkworm and the cocoon [16].

Picture-2:Silkworm sits on top of many yellow

Picture-3: White cocoons on mulberry leaf [18]. cocoons [17].

Silk proteins are stored in the middle silk gland and they are discharged through the anterior duct and spinneret, at the end of fifth instar . The silk fiber protein is synthesized by silk gland cells and stored in the lumen of the silk glands. Subsequently, it is converted into silk fibres. When the silkworms secrete the liquid silk during the spinning, it passes through the anterior gland and expelled out through the spinneret opening.

Two kinds of silk proteins have been distinguished as major components if silk cocoons, the first is fibroin, a fibrous protein composed of heavy (H) chain, Light (L) chain and glycoprotein linked disulfide bonds [2]. The second protein is the sericin, a natural macromolecular protein, serving as an adhesive to unite fibroin for making silk cocoons of silkworm. The following two figures show the properties of sericin and fibroin [2].

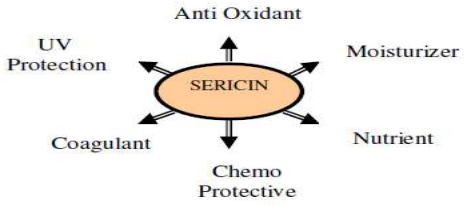


Fig 1. Diagrammatic representation of Attributes of sericin.

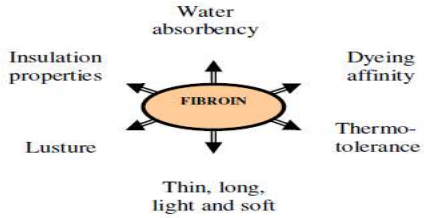


Fig 2. Diagrammatic representation of Attributes of Fibroin.

Figure 1: Diagrammatic representation of attributes of sericine. Figure 2: Diagrammatic representation of attributes of fobrion [2].

Recently, silkworm is being used as biofactory for production of useful protein using silk gland, which has promoted the technological development in sericulture. With all of its background, silkworm can be classified as a value added biomaterial for medical application, application of silk fibroin and sericin as a biomaterial.

2.2 Silk Gland:

Silk gland of B. mori is a typical exocrine gland secreting large amount of silk proteins. It is a paired organ consisting of modified labial/salivary glands located at the two lateral sides under the alimentary canal. Each gland is basically a tube made of glandular epithelium with two rows of cells surrounding the lumen [2].

The cells constituting the gland are huge polyploid cells each with extremely ramified nucleus containing numerous nucleoli. Nuclear ramification develops gradually as the larva grows and reaches conspicuous size in the 4th and 5th instars. Ramification enlarges the nuclear surface and apparently facilitates the transfer of materials related to the silk synthesis between the nucleus and the cytoplasm [2].

According to its morphology and function, the silk gland can be divided into three distinct regions which are shown in Fig 3 on next page (Figure 3) [2].

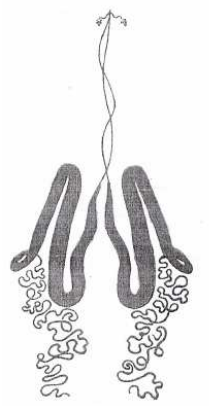


Fig 3. Schematic representation of silk gland of silkworm (*Bombyx mori* L).

Figure 3: Shemactic representation of silk gland of silkworm (Bombyx mori L.) [2]

The posterior part, about 15 cm long and is composed of about 500 secretary cells, which synthesize silk fibroin. The middle silk gland in the lumen of which silk proteins are stored until spinning, is about 7 cm long and contains about 300 secretory cells producing silk sericin, the protein which cements the fibroin thread of the cocoon. The anterior part about 2 cm long is a thin duct composed of about 250 cells with no known secretory function. Bombyx mori silk gland secretes one fibroin and three layers of sericin from the each posterior and middle silk gland in a normal larva.

2.3 Composition of Cocoon Filament

The silk fiber is almost a pure protein fiber composed of fibroin and sericin. The sericine content is more in outer layer of the silk fiber, where in fibroion content is less.

Sericin is chemically a non-filamentous protein [2]. Quantity and nature of sericin are fundamental characteristics in conferring distinctive traits to the cocoon. Sericin is insoluble in cold water, however, it is easily hydrolyzed, where by the long protein molecules brakes down to smaller fractions, which are easily dispersed, or solubilised in hot water [2].

Table-1: Composition of silk in bombyx mori [2].

Component:	%:
Fibroin	70-80
Sericin	20-30
Wax matter	0.4-0.8
Carbohydrates	1.2-1.6
Inorganic matter	0.7
Pigment	0.2
Total	100

Sericin of cocoon shell is seperated into two proportions: (1) a-sericin and (2) b-sericin. a-Sericin is present in the outer layer of cocoon shell and b-sericin in the inner layer [2]. The a-sericin contains lesser C and H and somewhat more N and O than the b-sericin. The solubility of a-sericin in the boiling water is more than b-sericin [2].

Silk fibroin secreted in the lumen of posterior silk gland (PSG) of B. mori consists of three protein component: High (H)-chain 350 kDa, Low (L) - chain 26 kDa, and Glycoprotein P25 30 kDa [2]. These three types of fibroin (H-chain, L-chain and P 25) are common among different silk producing insects in Lepidoptera [2].

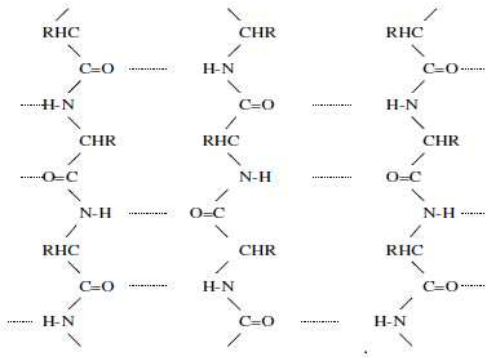


Fig 4. Crystalline structure of the peptide chains in silk fibroin.

Figure 4: Crystalline structure of the peptide chains in silk fibroin [2].

It is shown in Figure-4 that silk fibroin fibers are parallel and they consist of non-crystalline and crystalline regions. The crystalline region tends to be oriented along the fiber axis because the fiber is drawn as it is extruded from the spinnerets of silkworm [2].

CHAPTER 3

3.1 Silk Properties

The fibroin heavy chain of SF contains alternating hydrophobic and hydrophilic blocks similar to those seen in amphiphilic block copolymers [5]. The hydrophobic blocks consist of highly conserved sequence repeats of GAGAGS and less conserved repeats of GAGAGX (where X is either V or Y) that make up the crystalline regions of SF by folding into intermolecular β-sheets [5]. The hydrophilic part of the core is non-repetitive and very short compared to the size of the hydrophobic repeats.

Due to its amino acid sequence, SF provides opportunities for chemical modification. Carboxylic acid side groups from aspartic and glutamic acids, representing 2–3% of the total amino acid content of SF, have been derivatized with primary amines of peptides such as the RGD sequence, with the aim to improve cell adhesion [5]. To expand the range of functionalization, tyrosine residues, representing 10% of the total amino acid content of SF, were modified [5]. (modified silk fibroin)

Such modifications led to changes in SF hydrophilicity and charge and are, therefore, expected to alter the interaction between drug molecules and SF [5]. It is envisioned that by introducing distinct functional groups into SF and by varying the degree of functionalization per SF molecule, a variety of drugs in different amounts can be loaded and released with distinct kinetics, providing a wide range of adjustable drug release systems [5]. In addition to drug delivery contributions, functionalization may also promote cell adhesion and spreading or address cellular signaling pathways through specific cell–matrix interactions [5].

Crystallinity is the basis for the stability of SF [5]. An excessive increase in crystallinity reduces its flexibility and leads to more brittle materials. Crystalline SF is insoluble in most solvents that are widely used to dissolve polymers typical for drug delivery applications, as well as in water [5].

The stability of a polymer is an important feature for the storage of a drug delivery device. Untreated SF matrices are low in β-sheet content, remain hygroscopic and thus highly sensitive to humidity [5] [13]. Incubation at high humidity has been shown to change the conformational

state of SF, leading to increased β-sheet contents. Nevertheless, no systematic investigations exist on the storage stability of SF matrices. SF displays an exceptional thermal stability, being virtually unaffected by temperatures up to 140 °C [5].

The glass transition temperature Tg of proteins is considered to be a major determinant of protein self-assembly. Dry SF films prepared from silk taken from the posterior part of the middle division of the silk gland of the silkworm B. mori demonstrated a Tg of approximately 175 °C, above which there is free molecular movement to transform into the stable β-sheet conformation, showing stability up to around 250 °C [5] [11].

The release of drugs from matrices such as hydrogels depends partially on the degree of ***swelling***, which in turn depends on the ionization of the network, its degree of crosslinking and its hydrophilic/ hydrophobic balance. Changes in polymer compositions can influence the degree of swelling. For instance, an increase in the length of elastin repeating units in the backbone of hydrogels, while keeping the length of silk repeating units constant, can result in an increased degree of swelling due to a decrease in cross-linking density [5].

The compressive strength and modulus of silk fibroin increased with increasing SF concentration [5] [3].

Being a protein, biodegradation of SF predominantly occurs through proteolytic enzymes, with non-toxic degradation products and unproblematic metabolization in vivo [5] [7]. The implantation site, the mechanical environment and the size and morphology of the drug delivery device are likely to affect the degradation rate in vivo [5]. The biodegradation of SF has been described to directly relate to its β-sheet content [5].

Table-2: Comparison of mechanical properties of bombyx mori silk [3].

Material:	**Modulus (Gpa):**	**% Strain at break:**
B.mori silk w/ sericin	5-12	19
B.mori silk w/o sericin	15-17	4-16
B.mori silk average calculations	10	20

3.2 Applications of Silk Fibroin

Film Casting:

Silk fibroin is easily formed into films of thermodynamically stable β-sheets of controllable range of thicknesses (between tens of nanometers and hundreds of micrometers) [9]. These films have excellent optical transparency across visible range and can be easily characterized and functionalized [9].

Techniques of film casting applied to the silk fibroin:

1. Soft Lithograph Based Casting Technique
2. Silk fibroin/Polyurethane Blend Films
3. Ultrathin Silk Fibroin Films
4. Spin-Cast Thin Films
5. Layer-by-Layer Ultrathin Films
6. Nanoimprinting SF Films

- Soft Lithograph Based Casting Technique [9]: This technique enables the fabrication of sub-30nm transverse features in silk fibroin films, when cast at ambient conditions from an aqueous silk solution. The elegance of this method is in its simplicity; the fabrication of such features is completed in the absence of additional harsh chemicals, salts, or high pressures that traditionally accompany most micro and nanofabrication techniques. By employing this simple casting technique, high-quality films that contain a wide spectrum of nano and micropatterns can be fabricated.

- The patterning of silk fibroin films occurs through a modified soft-lithography casting process [9]. Two sets of masters were employed for this purpose.

- During the casting process, 200 mL–1mL of silk fibroin solution is deposited onto a clean, dry master [9]. This solution is then allowed to crystallize in free air at ambient

temperature and pressure. Under these settings, dry films are produced after approximately 16 h [9].

- The patterning of silk fibroin enables the formation of nanometer-scale patterns under ambient conditions of temperature and pressure. The employment of an all-aqueous silk solution, coupled with a simple and repeatable casting technique, results in optically clear, biocompatible, and mechanically tough films [9].

- Silk Fibroin/Polyurethane Blend Films [12]: To blend the water-insoluble and biocompatible polyurethane (PU) with silk fibroin to prepare water-insoluble films as a biomaterial, the ionic liquid, 1-butyl-3 methylimidazolium chloride (BMIMCl), was used to prepare a SF-BMIMCl solution, which was directly blended with PU solution in N-dimethylformamide (DMF).

- 36 g of BMIMCl was completely melted at 100 °C and subsequently 3.42 g of SF was added. The mixture was magnetically stirred at 100 °C for 1 hour and a clear, amber colored SF/BMIMCl solution was obtained. 4 g of PU was dissolved in 30 ml of DMF by mechanical agitation at 80 °C under reflux. Then SF/BMIMCl and PU/ DMF solutions were mixed at various ratios and each mixture was stirred for another half an hour at 80°C. [12]

- The films have anticoagulant characteristics. The blend films exhibit no bands of α-helix [12]. These results indicate that the main conformation of SF in the films was β-sheet, which was induced by treating the films with ethanol instead of methanol. The β-sheet structure and the water-insoluble PU made the SF/PU films insoluble in water [12]

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- Ultrathin Silk Fibroin Films [8]: (Spin Coating and Spin-Assisted Layer-by-Layer Assembly); To fabricate uniform thin and ultrathin polymeric films spin casting and layer-by-layer (LbL) assembly are widely utilized. Spin casting represents the easier

fabrication method (100–1000 nm thick films) for ultrathin silk films while LbL assembly allows for the fabrication of ultrathin (1–100 nm) multilayered films in a step-wise manner similarly to that developed for electrostatically driven LbL. Depending on the nature of components and fabrication conditions, inter-layer interactions may be electrostatic, hydrogen bonding, van der Waals interactions, and short-range hydrophobic interactions [8]

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- *Spin-Cast Thin Films* [8]: Silk films prepared here are stable in organic solvents, such as toluene and acetone, and, after treatment with methanol, their resistance to water treatment increases dramatically resulting in no obvious signs of dissolution observed after water treatment [8].

- The film surface which indicated some aggregation of material in solution prior to spin casting, however, the overall surface was relatively smooth with a surface micro-roughness [8].

- *SA-LbL Ultrathin Films* [8]*:* Unlike for traditional polyelectrolyte LbL multilayers, where there are strong charge inter-layer interactions, the driving force for the assembly of silk fibroin LbL multilayers is mainly short-range hydrophobic interactions [8]. These ultrathin films are not soluble in either water or organic solvents [8].

- Mechanical Properties of Silk Fibroin Films under Compression [8] [9] [12]: Buckling tests, based on the analysis of the buckling instability of ultrathin films on a compliant substrate has been widely exploited because of the simplicity and reliable results with this method. This method has also been recently introduced for the measurement of mechanical properties of ultrathin LbL films and showed reproducible results. To test the mechanical properties of both spin-cast and multilayered films under compressive stresses, compressive stress was applied.

- Mechanical Properties of Silk Fibroin Films under Tensile Stress [8] [9] [12]: Alternative testing of mechanical properties in tensile strain mode was tried on silk fibroin films. The pressure applied to one side of the film causes deflection. The bulging tests have been widely used for measuring the mechanical properties of ultrathin films. It is widely accepted that the bulging test is a robust and consistent method for studying mechanical properties of ultrathin films when it can be applied.

- Molecular Structure of Films [8]: The presence of the β-sheets in the films was confirmed. Although broad maxima are observed for the spin-cast films that are indicative of a disordered structure which is a clear indication of the presence of two preferential intermolecular interactions in the lateral packing of the backbones with crystalline spacings.

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- Nanoimprinting SF Films [10]: *Optofluidics*, is already undergoing evolution, finding applications to an ever-increasing range of problems, including varieties of biological sensing and detection [10]. Optofluidics was developed as a fusion of microfluidics and photonics to enable compact, novel optical modulation technologies. The union of optical and fluidic confining structures led optofluidic devices to be applied to sensing problem, especially looking toward highly parallel, sensitive and low analyte volume applications [10].

- A further development of the optofluidics, introduced here through the use of silk [10], is to activate the constituent material of the device to make it chemically sensitive to species flowed past it. Typically, optofluidic devices are fabricated from materials usually found in photonics or microfluidics such as silica, silicon, polydimethylsiloxane or polymethacrylmethacrylate and other polymers. These materials, while possessing suitable and well-characterized optical and material properties are not inherently chemically sensitive or specific.

- Nanoimprinting is a high-throughput lithography technique in which a mold is pressed onto a thermoplastic material heated above its glass-transition temperature [10].

- Nanoimprinting of silk fibroin films is performed via two processes which depend on adjusting the silk fibroin film glasstransition temperature [10].

- To nanoimprint films at ambient humidity, a hot embossing process is employed whereby a silk film is pressed on to a heated master pattern. To demonstrate the utility of silk nanoimprinting for use in biophotonic sensing applications, a self-sensing optofluidic device was constructed. The silk solution was doped with lysed red blood cells and cast a film on a glass slide. Then using the room temperature nanoimprinting method, 600 grooves/mm grating was imprinted in the hemoglobin doped silk film and annealed with methanol to preserve the imprinted grating and eliminate water solubility [10].

In this project, SF films were formed by UV irradiation using 100% and 50% transmittance. Films were kept at room temperature.

Silk Fibroion Scaffolds:

Silk fibroin is an important polymer for scaffold designs, forming biocompatible and mechanically robust biomaterials [1]. Various technologies have been employed to produce 3-D porous SF scaffolds including foaming, particle leaching and electrospinning [1]. SF scaffolds have been investigated for application in various tissues such as tendon, bone, cartilage and neural tissues [1]. Techniques used to produce SF 3D-scaffolds [19] [1] [14];

- Gas foaming--- Gas-foamed scaffolds use gas bubbles to create porous polymer scaffolds
- Particle leaching---gelatin particle leaching with polylactic acid
- Electrospining---sintering

- SF porous sponges---interconnected pores

Silk Fibroin Based Porous Materials:

Porous three-dimensional materials, and the network structure materials, are composed of interconnected or closed pores [6]. Porous three-dimensional biomaterials provide a microenvironment for attachment, increase surface area, support a large cell mass, form an extracellular matrix and play an important role in manipulating cell functions in regenerative medicine.

Silk Fibroin Porous Sponges:

Porous sponges are important tissue engineering materials. Regenerated SF solutions have been utilized in the preparation of porous sponges [6]. SF porous sponges can be obtained using porogens, gas forming, and freeze-drying, freeze-drying/foaming, electrospun fibers [6].

Porous silk fibroin sponges have applications in the following described areas; [6]

1-Bone and Cartilage:

Bone tissue is a specialized form of connective tissue, which is composed of calcified extracellular matrix and bone cells including osteoprogenitor, osteoblasts, osteocytes and osteoclasts. The bone matrix consists of both an organic and inorganic matrix. The biodegradability, distinguishing mechanical properties, and low inflammatory response of SF ensure its role as one of the promising porous materials for osteogenic applications.

Fibers electrospun from aqueous solution of silk fibroin, polyethylene oxide and bone morphogenetic protein-2 were prepared as a scaffold for human mesenchymal stem cells; in vitro culture in osteogenic media led to the formation of bone-like tissue [6]. Addition of

hydroxylapatite nanoparticles to the SF solution prior to electrospinning produced fibers with the nanoparticles embedded inside and was found to improve bone formation [6]. In vivo implantation of electrospun silk fibroin fibers in calvarial defects in mice facilitated the complete healing of the defect with new bone within 12 weeks [6].

2-Skin Tissue:

Studies showed that SF porous material can accelerate wound healing, improve adhesion and spreading of normal human keratinocytes and fibroblasts, upgrade the growth and development of skin tissue [6].

3-Vascular Grafts:

SF porous matrices have potential as vascular graft matrices due to their ability to support the attachment, proliferation and differentiation of vascular cells and resist shear stress and pressure from simulated blood flow. Fibers electrospun from aqueous solutions of bombxy mori silk fibroin and polyethylene oxide were used as scaffolds for human aortic endothelial cellsand human coronary artery smooth muscle cells; in both cases in vitro culture in endothelial growth medium led to the formation of vascular tissues within a week [6].

4-Nerve Grafts:

Peripheral nerve repair represents a common clinical challenge, and the current gold standard for treating large nerve defects involves the implantation of nerve auto-grafts that is limited by graft availability, secondary deformities, and potential differences in tissue structure and size.

They developed a novel biomimetic design of the silk fibroin-based nerve graft (silk fibroin graft) which was composed of a silk fibroin-nerve guidance conduit inserted with oriented SF filaments [6]. The SF graft was used for bridge implantation across a 10-mm long sciatic nerve

defect in rats, and the outcome of peripheral nerve repair at six months post-implantation was evaluated by a combination of electrophysiological assessment [6].

5-Ligaments and Tendons:

Bone marrow-derived mesenchymal stem cells and anterior cruciate ligament fibroblasts on combined SF porous scaffolds for ligament tissue engineering application were studied to compare the cellular responses [6]. Bone marrowderived mesenchymal stem cells were found to be a better cell source than anterior cruciate ligament fibroblasts [6].

6-Drug Delivery:

Drug delivery technologies were used in modifying drug release profile, absorption, distribution and elimination for the benefit of improving product efficacy and safety, as well as patient convenience and compliance.

SF has been suggested as a platform for drug delivery either in the form of films or as genetically engineered silk-elastine hydrogels and other silk fibroin or silk fibroin blended hydrogels. Some kinds of SF Porous 3D scaffolds were also prepared for drug delivery [6].

Current efforts in the area of drug delivery include the development of targeted delivery in which the drug is only active in the target area of the body (for example, in cancerous tissues) and sustained release formulations in which the drug is released over a period of time in a controlled manner from a formulation. Types of sustained release formulations include liposomes, drug loaded biodegradable microspheres and drug polymer conjugates [6].

CHAPTER 4

4.1 Materials:

Silk cocoons were purchased from traditional market. Na_2CO_3 was purchased from E.Merck D-6100 Darmstadt and ethanol was purchased from Selim ve Oğlu. Ltd. $CaCl_2$, KH_2PO_4 (used in acidic buffer solution) and K_2HPO_4 (used in phosphate buffer solution) were purchased from Sigma-Aldrich. In addition, distilled and ultra-pure water were obtained from the Medicine Faculty of Near East University.

4.2 Experimental:

4.2.2 Preperation of Pure Silk Fibroin Biomaterial:

Bombyx mori cocoons are cut into small pieces before starting the purification of silk fibroin.

- Silk fibroin cocoon cut into small pieces.

4.2.2.1 <u>Degumming:</u>

0.1M 100mL Na_2CO_3 solution is added to 1.000g of cocoon pieces. Degumming is the process of purification where sericine is separated from fibroin by stirring on a magnetic stirrer. Degumming is done multiple times (approximately 5-7 times) for 3hours. Each time the solution is filtrated and 0.1M Na_2CO_3 is added again.

- Degumming on a magnetic stirrer

- After every degumming, filtration is done to remove sericine and change Na_2CO_3 solution. Later the protein is washed to remove all the remaining of sericine.

Preparation of Na_2CO_3: 500mL of ultra pure water is added to 5.2995g Na_2CO_3 and agitated.

When sericin is completely removed (fibers can be seen), fibroin is washed with distilled and ultra pure water. Fibroin is placed on a petridish and ovened in 37^{o}C for 1 day.

- Silk fibroin after degumming. Small pieces are removed from sericine and and proteins fiber structure can be seen. (on the left is yellow cocoons SF, on the right white cocoons SF)

After the protein is completely dry, electrolyte solution is added. Fibroin is liquidized with an electrolyte solution ($CaCl_{2)}$ on the magnetic stirrer for couple of hours.

Preparation of electrolyte solution: $n_{CACl_2} : n_{C_2H_5OH} : n_{H_2O}$

1 : 2 : 8

All substances are mixed, ultra pure water is added and the solution is agitated.

4.2.2.2 Dialysis:

The ions are extracted from the silk fibroin by distilled-water based dialysis. Protein is placed in a cellulose membrane in a large beaker. This process is done for three days and the water is changed 3times a day during the dialysis.

- Water-based dialysis of liquidized silk fibroin (in an electrolyte solution; the solution is placed in a cellulose membrane)

Purification process is ended by filtrating the liquidized fibroin after dialysis.

4.2.2.3 Film casting:

A very thin layer of liquefied pure silk fibroin is placed on a glass plate. UV irradiation is used to form the films. (λ = 320nm)

Films are produced in a natural environment at room temperature. It is observed that 100% transmittance had better results with 40 minutes.

4.2.2.4 3D-scaffolds:

Purified silk fibroin is used. By using 2mL syringes, 1mL of silk fibroin is withdrawn into the syringe. Syringes are placed in both -80^{0}C and -30^{0}C deep freezers. Also, at the same time using %99.08 chilled ethanol %70 ethanol was prepared and placed in both the freezers. We need ethanol to be at the same temperature as scaffolds because if it is at a higher temperature, scaffolds dissolve in it immediately.

Swelling properties of the scaffolds are observed in phosphate buffer solution and in acid buffer solution.

Preparation of phosphate buffer solution (pH=7.4) and acid buffer solution pH=1.2):

For phosphate buffer solution K_2HPO_4 (known 174.2gmol^{-1}) m is calculated by m=n/v and for acid buffer solution KH_2PO_4 (known 136.09gmol^{-1}) m is calculated by m=n/v. Adjusting the pH was done by dipping basic solution into acid solution using pH-meter.

CHAPTER 5

RESULTS AND DISCUSSIONS

Laboratory studies showed that silk cocoons can easily be processed. After purification of silk fibroin, it is cast on a thin glass plate and placed under UV for film formation. The resulting film adhered to the plate.

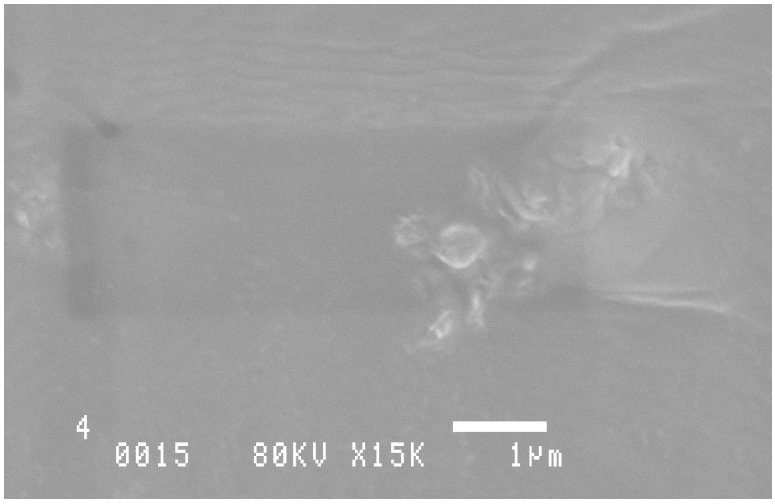
- SF film generated under %100 transmittance UV

The film has excellent surface quality and optical transparency. This technology of film casting of silk fibroin has simplifies the process of casting films

Table-5: (λ=320nm) UV irradiation of films transmittance table

	Transmittance:	**Time:**	**Comments:**
SF-1	100%	30min	transparency was good
SF-2	100%	40min	shown best results
SF-3	50%	30min	film was not formed
SF4	50%	40min	film was not smooth enough

SEM (Scanning Elecetron Micrograph) of SF2 film:



At 100% transmittance in 40 minutes, the SF2 showed a very smooth surface, high stability and transparency.

3D-Scaffolds:

In this project, 1mL of liquefied pure silk fibroin was withdrawn into an injector and freeze drying process was applied. Freeze drying process applied on silk fibroin scaffolds in two different temperatures; -30^{o}C and -80^{o}C. After four hours in the freezer, the scaffolds were formed. They were treated with 70% ethanol which was at the same temperature as the scaffolds. It was observed scaffolds treated with ethanol at room temperature were dissolving immediately. The scaffolds which were freeze-dried at -30^{o}C were treated with ethanol at -30^{o}C and the scaffolds which were freeze-dried at -80^{o}C were treated with ethanol at -80^{o}C. The swelling studies of scaffolds are tabulated below.

- Silk fibroin scaffolds on the right one took from -30^{0}C deep freezer, on the left the swelling process of scaffolds.

Table-3: Swelling studies of silk fibroin scaffolds in acid buffer solution at 25^{o}C, pH=1.2.

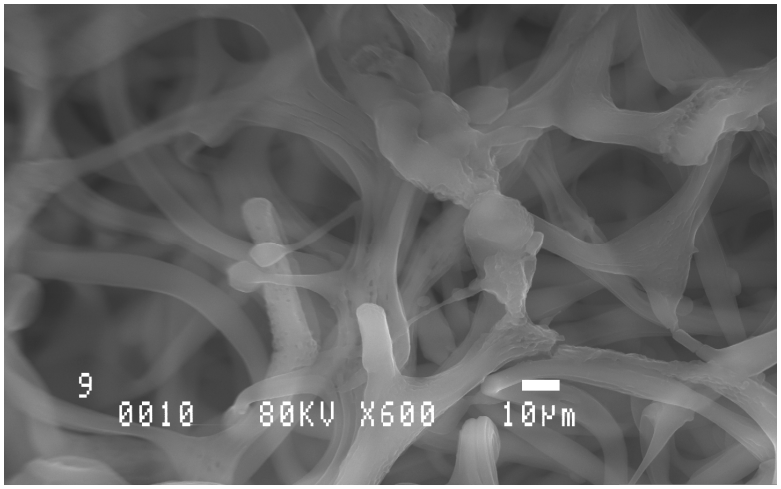
Time	-80°C silk fibroin scaffold	% Swelling	-30°C silk fibroin scaffold	%Swelling
0 min (w_i)	0.007 g	---	0.005g	---
5 min	0.039 g	457.142%	0.021 g	320%
15 min	0.038 g	442.857%	0.013 g	160%
25 min	0.033 g	371.428%	0.020 g	300%
35 min	0.030 g	328.571%	0.018 g	260%
30+35 min	0.044 g	528.571%	0.021 g	320%
60+35 min	0.043 g	514.285%	0.026 g	420%
90+35min	0.047 g	571.428%	0.037 g	640%
3 hours	0.051 g	628.571%	0.023 g	360%
1 day	0.044 g	528.571%	0.032g	540%
1 day	0.049 g	600%	0.023 g	360%
2 days	0.049 g	600%	0.035 g	600%

Table-4: Swelling studies of silk fibroin scaffolds in phosphate buffer solution at 25^{o}C, pH=7.4

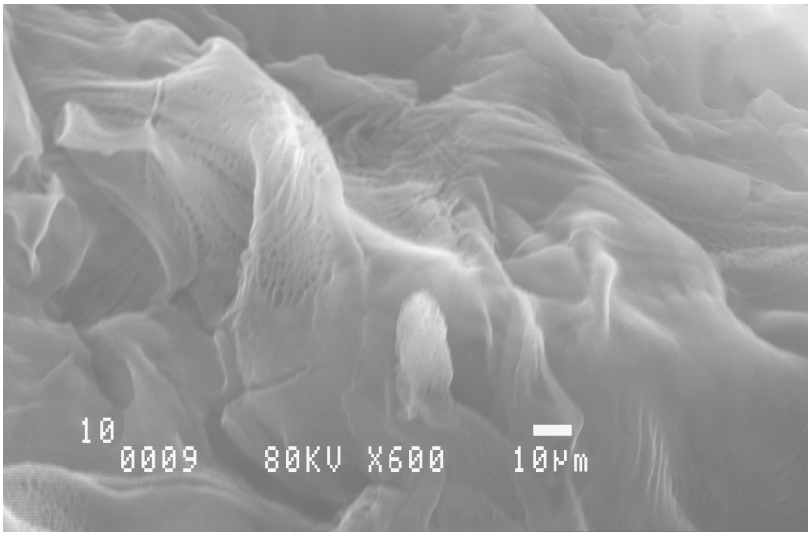
Time	-80°C silk fibroin scaffold	%Swelling	-30°C silk fibroin scaffold	%Swelling
0 min(w_i)	0.009 g	----	0.006 g	----
5 min	0.054 g	500%	0.013g	116.66%
15 min	----	----	0.039 g	550%
25 min	0.066 g	633.333%	0.037 g	516.66%
35 min	0.068 g	655.555%	0.035g	483.33%
30+35 min	0.057 g	533.333%	0.036 g	500%
60+35 min	0.034 g	277.777%	0.037 g	516.66%
1 hour	0.033 g	266.66%	0.039 g	450%
1 hour	0.043 g	377.77%	0.044 g	630.33%
3 days later	0.057 g	533.333%	0.0509 g	748.330%
3 hours	0.057 g	533.33%	0.047 g	683.33%
2 hours + 30 min	0.053 g	488.88%	0.046 g	666.66%
1 day	0.046 g	411.11%	0.062 g	933.33%
1 day	0.059 g	555.55%	0.053 g	783.33%
2 days	0.069 g	666.66%	0.054 g	800%

In Table-3 it is shown that scaffolds freeze-dried at -80^{o}C have shown a higher swelling rate than -30^{o}C scaffolds. The same result was observed in the Table-4. However, it can be concluded that scaffolds have higher swelling rate in a basic environment.

SEM micrograph of the scaffold prepared at -80^{o}C:



SEM micrograph of the scaffold prepared at -30^{o}C:



Pore sizes are greater in 3D-SF scaffold prepared by freeze-drying at -80^{o}C than the scaffold prepared at -30^{o}C. This affects the swelling properties of the scaffold which is very important in controlled release drug delivery systems.

Despite the excellent properties of SF that make it an attractive biomaterial for biomedical applications, a number of challenges remain. As a natural product the properties of silk may vary between both species and individuals of the same species [3]. Moreover, inconsistencies in the degumming process may render the quality control of SF delivery systems and predictions for

their release kinetics difficult [3]. Genetically engineered SF proteins (modified SF) may overcome such deficiencies.

CHAPTER 6

CONCLUSIONS

- Silk fibroin was successfully purified

- Film casting by UV irradiation shows remarkable results with a smooth and transparent film

  -SEM micrographs of the film shows excellent β-sheet formation

- 3D-scaffolds prepared by freeze-drying technique at -80^{o}C shows perfect swelling behavior than the scaffold prepared at -30^{o}C

  -SEM micrographs of the scaffold shows greater pore sizes with the scaffold prepared at -80^{o}C

Swelling properties of silk fibroin scaffolds can be improved. Improvement of the stability of silk fibroin films is an important future prospect with better mechanical properties. SF can be processed into diverse morphologies to meet different needs in biomedical applications. Surface modification of films can be an improvement for biocompatibility. Modified silk fibroin is in need for tissue engineering applications.

B. mori silk fibers are composed primarily of two types of proteins: (1) sericin,the antigenic gum-like protein surrounding the fibers and (2) fibroin,the core filaments of silk comprised of highly organized beta-sheet crystal regions and semi-crystalline regions responsible for silk's elasticity compared to fibers of similar tensile integrity [21]. Silk has been used in biomedical applications for centuries primarily for the ligation of wounds. Virgin silk suture (containing sericin) induces hypersensitivity in patients,causing a Type I allergic reaction [21]. Exposure to silk debris may sensitize patients to silk causing adverse allergic reactions when silk is used as suture material. Silk fibroin elicits a foreign body response following implantation in vivo [21].

Yet the response is comparable to the most popular synthetic materials in use today as biomaterials,and is dependent upon the implantation site and model used for investigation. silk fibroin when utilized as films,foams and fibers,may offer a ‘new’ alternative biomaterial for use as matrices in tissue engineering where mechanically robust,lon g-term degradable materials are needed [21].

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