SWELLING PROPERTIES OF BLENDED SILK FIBROIN BIOFILMS

A THESIS SUBMITTED TO THE GRADUATE SCHOOL OF APPLIED SCIENCES OF NEAR EAST UNIVERSITY

By
AKPOBOME ABIJAH UYOH

In Partial Fulfilment of the Requirments for The Degree of Master of Science in Biomedical Engineering

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Approval of the Graduate School of Applied Sciences

Prof. Dr. İlkay Salihoğlu
Director

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I hereby declare that all information embodied in this document on swelling properties of silk fibroin blend biofilm has been obtained and presented in accordance with academic rules and ethical conduct. I also declare that, as required by these rule and conduct, I have fully cited and referenced all material and results that are not original to this work.

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ABSTRACT

Silk fibroin is a multipurpose biomaterial; it can be easily transformed in different shape, structure and form ranging from scaffolds, hydrogels, biofilms, Micro / Nano particles, non-woven mat for biomedical application. The aim of this study is to use evaporating thermal method to produce silk fibroin biofilm. Silk fibroin was modified with eggshell powder (ESP) and glycerine. The silk fibroin biofilm blend was prepared by evaporation / thermal method at 60^{o}C in an evaporating dish. Scanning electron microscopy analysis (SEM), X – ray diffraction analysis (XRD), inductively coupled plasma spectroscopy (ICP) and swelling test was carried to characterize the silk fibroin biofilm blend.

Swelling test was carried out to investigate the swelling rate of the silk fibroin biofilm in various solvents: Ethanol at pH 7.33; Deionized water at pH 7.0 and Phosphate buffer solution (PBS) at pH 7.4; Hydrochloric acid solution (HCl) at pH 1.0; Sodium hydroxide solution with a pH 12 that is incompatible with the human body. It was observed that increasing the concentration of eggshell powder (ESP) increases the swelling ratio in neutral pH, decreases the swelling ratio in acidic pH and increases the swelling ratio in alkaline pH.

The scanning electron microscopy analysis (SEM) showed that the effect of the glycerine on the eggshell powder (ESP) in the silk fibroin blend has rough aggregation and interconnected fiber particles. XRD analysis showed that crystallinity of the silk fibroin biofilm blend has its highest peak at 29.42^{o} indicating the presence of Calcite ($CaCO_3$). ICP analysis indicated that the presence of Calcium ($Ca^{2+)}$ and Phosphate (PO_4^{-3}) in the eggshell powder (ESP).

The results of this thesis indicate that fabricated silk fibroin blend biofilms with eggshell powder and glycerine, can be good candidates for different biomedical applications.

***Keywords*:** Silk fibroin; Swelling test; Eggshell powder; Glycerine; Evaporation/thermal techniques

ÖZET

İpek fibroin çok amaçlı uygulaması olan bir biyomateryaldir. Farklı şekil ve yapılara kolayca dönüşebilme özelliği var. Bu önemli özelliği ile ipek fibroin, pek çok farklı biyomedikal uygulamalar için iskeleler, hidrojeller, biyofilimler, mikro ve nano parçacıklara dönüştürülebilir. Bu çalışmanın amacı, farklı bir yaklaşım olan, 60^oC'de ısı / buharlaşma metodu ve yumurta kabuğu tozu, gliserinle modifiye edilmiş, ipek fibroin biyofilmleri oluşturmaktır.

Oluşturulan biyofilmlerin karakterizasyonu, X-Işın difraktometre (XID), Taramalı Elektron Mikroskopu (TEM) ve İndüktif Eşleşmiş Plazma Atomik Emisyon Spektroskopisi (İEPAES) ve Şişme özellikleri testleri yapıldı. İEPAES sonuçları, biyofilimlerin kimyasal yapısında kalsiyum (Ca^{2+}) ve fosfat (PO_4^{3-}) varlığını ispatlamıştır. XID sonuçları ile yapılan çalışma sonuçları, ipek fibroin-yumurta kabuğu tozu karışımı biyofilmlerin yapısında kristal yapının arttığı, biyofilimlerin kalsiyum karbonat ($CaCO_3$), sodyum fosfor oksit hidrat ($NaP_3O_{10}(H_2O)$, ve kalsiyum hidroksit ($Ca(OH)_2$) içerdikleri gözlemlenmiştir. Farklı çözeltilerde kullanılarak şişme testi uygulanmıştır. Çözeltiler; pH = 7.33 etanol çözeltisi, deiyonize su pH = 7.00, fosfat tampon çözeltisi pH = 7.40, 0.1 M HCl çözeltisi pH = 1.0, 0.1 M NaOH çözeltisi pH = 12. Yumurta kabuğu tozu miktarı arttıkça pH = 7.00 de şişme oranının arttığı, asidik pH değerinin azaldığı ve bazik pH değerinin arttığı gözlemlenmiştir.

Taramalı elektron mikroskop analizleri, ipek fibroin, yumurta kabuğu tozu ve gliserin varlığında düzensiz dağılımlar ve birbiriyle bağlı fiber parçacıkların varlığı gözlemlenmiştir. XID analizleri sonucunda, en yüksek tepe noktasının 29.42^o gözlenmesi $CaCO_3$ varlığını güçlendirmiştir.

Bu çalışmanın sonucunda, ipek fibroin –yumurta kabuğu tozu, ipek fibroin-yumurta kabuğu tozu-gliserin karışımlarından, buharlaşma / ısı metodu ile oluşturulan biyofilmler, farklı pH değerlerinde, değişik şişme oranına sahip oluyorlar ve içerisinde mevcut kalsit ve fosfat grupları ile biyomedikal uygulamalarda kullanabilecek aday biyomateryaller olarak önerilebilirler.

***Anahtar Kelimeler*:** İpek Fibroin; şişmesi testi; Yumurta kabuğu tozu; Gliserin; Buharlaşma / termal teknikleri

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SEM:	Scanning electron microscopy
XRD:	X – ray diffraction analysis
ICP:	Inductively coupled plasma spectrometry
SF:	Silk fibroin
ES:	Eggshell
ESP:	Eggshell powder
GLY:	Glycerine
NaOH:	Sodium Hydroxide solution
PBS:	Phosphate buffer solution
HCl:	Hydrochloric acid solution
Mpa:	Mega Pascal
RGD:	Rat genome database
Rpm:	Revolution per minute

CHAPTER 1
INTRODUCTION

1.1 Silk Fibroin

Silk is a natural polymer which includes a group of a fibrous protein, produced from cocoons of some species of notable spiders (order Araneae) and silk moth (order Lepidoptera) domestic's arthropods, moths, *Bombyx mori*. The basics proteinaceous molecule in the silk cocoon which is obtained from insects are fibroin and sericin, the fibers of silk are 10 - 25µm in diameters, with each fibers made up of a protein covering coating of hydrophilic gum like protein called sericins (20 - 310KDa) (Kaplan et al., 1998; Zhou et al., 2000; Inoue et al., 2000) which tend to the silk cocoon's structure (Perez-Rigueiro et al., 2000). Twenty five to thirty percent of the silk cocoons mass are made up of sericins, sericins are soluble amino acid, water and alkaline solution, and it is detached from the cocoon via a process known as degumming. Silk Fibroin can be fabricated into films, hydrogels coating, scaffold, sponges, membranes, capsules, micro and nanoparticles for various biomedical applications (Seib and Kaplan, 2012). According to Minoura's research group, the fibroin gotten from *Bombyx mori* silkworm has been investigated as an important biomaterial considered for tissue engineering application. (Altman et al., 2003; Wang et al., 2006; Vepari and Kaplan, 2007; Kundu et al., 2013; Wenk et al., 2011).

Silk Fibroin has been used in the textile industry over the years and as a suture material. The unique mechanical properties of silk together with its biocompatibility, biodegradability, lack of immunogenicity and compatibility with sterilization techniques has made it a good biomaterial for a wide range of biomedical application. Over the decades it has shown its utilization as bio-polymeric scaffold for tissue engineering with excellent biocompatibility with host tissue, its mechanical strength of a biomaterial plays an important role in scaffold designing (Li et al., 2001).

1.2 Properties of Silk Fibroin

Silk fibroin is a protein known for its excellent biocompatibility with host tissue, like its mechanical strength, and biodegradation amongst other polymers.

1.2.1 Structural properties of Silk Fibroin

The fibroin protein consists of layers of antiparallel β sheets. Its primary structure consists of repeating sequence of amino acid (Gly - Ser - Gly - Ala - Gly - Ala)n seen on Figure 1.1 the higher glycine (lesser alanine) content for tight packing of the sheets which contributes to the rigid silk structure and tensile strength. The silk fiber is 10-25µm in diameter, each fiber contains a core protein covered in hydrophilic proteins known as sericins (20-310 kDa) that glue fibers core together. Sericins contain 25 to 35 percent of the silk cocoon mass; it is removed via a process known as degumming (Zhou et al., 2000; Inoue et al., 2000). The core protein contains three chains, heavy chain (~390 kDa), light chain (~26 kDa) and a glycoprotein, P25 (25 kDa) (Inoue et al., 2000).

The fibroin protein gene (H- fib gene) is located on the 25th chromosome of *Bombyx mori* silkworm; it contains two exons and one intron (Zhou et al., 2000; Zhou et al., 2001). The silk polymorphs have been reported, which includes the glandular state that is prior to crystallization (Silk I), the spun silk state which consists of the β-sheet secondary structure (Silk II), and an air/water assembled interfacial silk (Silk III, with a helical structure) (Kaplan et al., 1997; Jin et al., 2003; Motta et al., 2002). The silk I structure is the water soluble state when exposed to heat or any external contact it can easily convert to silk II structure. The primary structure of heavy chain consists of 12 repetitive regions known as the crystalline regions and II non repetitive interspaced regions are known as amorphous regions (Zhou et al., 2001).

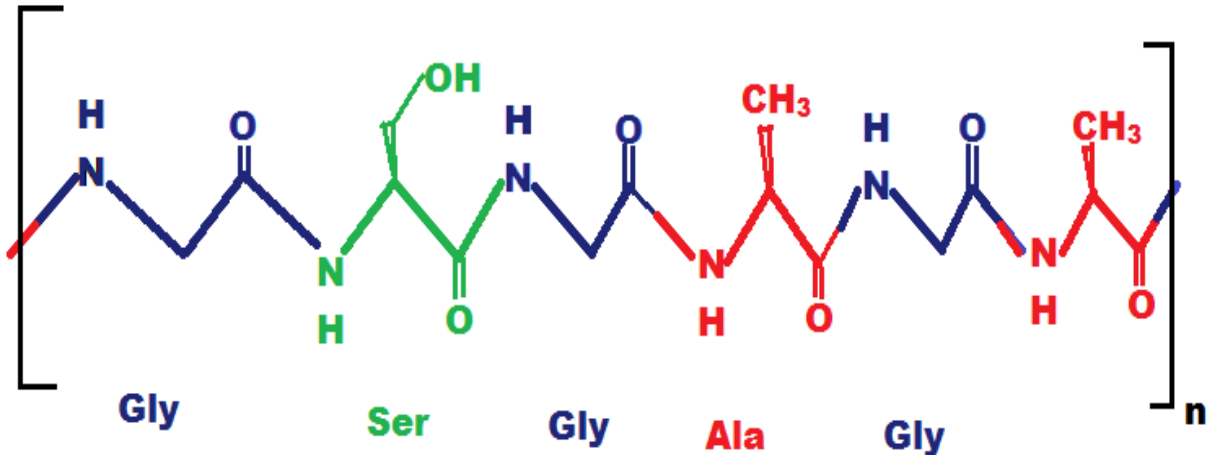

Figure 1.1: Showing the primary structure of silk fibroin amino acid sequence (Gly - Ser - Gly - Ala - Gly - Ala) n (Valluzzi et al., 1999)

1.2.2 Physical properties of Silk fibroin

A silk fiber from Bombyx mori silkworm is yellow in color, a smooth soft surface that is not slippery. It has a triangular cross section with rounded corners, 5-10µm wide (Lewin et al., 2006). The flat surface of the fibrils reflects at different angles, giving the silk its natural shine. Silkworm fibers are naturally excreted from two silkworm glands as a pair of primary filaments (Brin), which are clung together, with sericin proteins that act as glue, to form a bave.

Silk is one of the strongest natural fibers but loses about 20% of its strength when wet. It has a good moisture regain of about 11%, its elasticity is moderate to poor, when elongated, and in small quantity it still remains stretched. Silk fiber exhibit high wear resistance and physiochemical properties making wearing comfortable: the good capability of silk fiber for moisture absorption, air permeability and low electrifilableness (Sashina et al., 2006) and thus susceptible to static cling.

Silk is insoluble in most solvent including water, dilute acid and alkaline solution (Altman et al., 2003). Natural and synthetic silk is known to possess piezoelectric properties in proteins, probably due to its molecular structure (Fukada et al., 1983). An important feature of silk as a biomaterial compared with other fibrous proteins such as collagen is its skilled ability for sterilization (Sugihara et al., 2000). Sterilization of silk fibroin scaffolds by autoclaving does not

change morphology (Meinel et al., 2004) or β- sheet structure when heated to 120°C. Silk fibroin scaffolds can also be sterilized using ethylene oxide (Altman et al., 2003), γ-radiation, or 70% ethanol (Karageorgiou et al., 2004; Li et al., 2006).

1.2.3 Chemical properties of Silk Fibroin

The high proportion (50 %) of glycine, which is a small amino acid, allows tight packing and the supramolecular structure of silk fibers with a width of up to 6.5×10^5nm which contains helically packed nanofibrils 90-170 nm in diameter (Sashina et al., 2006) well-built and resistant to wear. The tensile strength is due to the present of many seeded hydrogen bonds that works when fibers are elongated resisting breakage due to an applied force.

Silk is resistant to most mineral acids, except sulfuric acid. The silk I structure when observed in vitro in aqueous solution, it converts to a β - sheet structure when exposed to methanol, ethanol or potassium chloride (Huemmerich et al., 2006). The silk II structure eliminates water and its insoluble in several solvents including mild acid, alkaline and few chaotropic (Altman et al., 2003).

1.2.4 Mechanical properties of Silk Fibroin

Silk fibroin properties are due to its profound hydrogen bonding, significant crystallinity and hydrophobic nature of the protein. It has a good tensile strength of 300 - 740 Mpa (Shao and Vollrath, 2002) abundant breaking strain and high toughness surpassing other synthetic fiber such as wool and nylon. The geometry and mechanical properties are important design criteria for tissue engineering scaffold, a study carried out by Altman et al. (2003) showed the several tensile strength of various polymer, the tensile strength of silk fibroin is greater than that of Polylactic acid (PLA) with a tensile strength of 28-50 Mpa. In tissue engineering, the scaffold provide initial loading conditions, tendons and bones have a tensile strength of 150 and 160 Mpa respectively, Polylactic acid (PLA) with a low tensile strength cannot provides support for tendons and bones cells to proliferate. Cross linked collagen with tensile strength of 47-72 Mpa still has a low tensile strength for tissues. Therefore silk which has a greater tensile strength than Polylactic acid (PLA) and cross linked collagen, will provide a better cell to tissue support.

1.2.5. Solubility Properties of Silk Fibroin

Crystalline silk fibroin is insoluble in most solvents and it used to dissolve polymers especially for drug delivery applications, as well as in water. Extremely concentrated salt solutions like Lithium Bromide, Lithium Thiocyanate, Calcium Thiocyanate or Calcium Chloride can dissolve silk fibroin (Kaplan et al., 1997). Electrolyte solutions are capable of slowing down the hydrogen bonds that balance the β - sheets (Phillips et al., 2004), after dissolution, dialysis against water (Deionized water) or buffer is performed to separate the electrolytes. Hexafluoroisopropanol is an expensive and toxic solvent; it is used to process drug delivery material from freeze–dried silk fibroin solution, followed by dissolution of dry silk fibroin in hexafluoroisopropanol (HFIP) (Kirker–Head et al., 2007; Meinel et al., 2006; Karageorgious et al., 2006). The alternative use of aqueous silk fibroin solutions offers tender fabricated drug delivery systems for such biological. Intriguingly, the processing of aqueous instead of hexafluoroisopropanol solution of silk fibroin offers several advantages: the option to load silk fibroin based constructs with drugs, porogens or microparticles that are insoluble in aqueous solutions, recently an introduction for processing the aqueous into fabricated microporous silk fibroin scaffolds by loading paraffin spheres as elutable porogens, as well as Poly (lactic-co- glycolic acid) microparticles to induce a growth factor (Wenk et al., 2009). Further advantages of processing aqueous instead of hexafluoroisopropanol solution of silk fibroin are: the ease of sterilization by filtration and the absence of residual solvents in the fabricated matrix. The common problem with the processing of aqueous silk fibroin solutions still exists, namely the premature reprecipitation into its water insoluble sheet enriched silk II states. Usually highly concentrated silk fibroin solution tends to sum up in a matter of hours to days due to inter and intra molecular interactions of the protein. Several approaches that prevent the formation of a β-sheet structure were studied in order to maintain higher silk fibroin concentrations in a soluble state. For instance, phosphorylation of genetically engineered silk has been shown to increase the total aqueous solubility of the protein through a combination of steric hindrance and charge (Winkler et al., 2000). Recently, the modification of the tyrosine residues in silk fibroin by a diazonium coupling reaction with 4-sulfanilic acid led to a sulfonated silk fibroin derivative that reveals the inhibition of spontaneous protein aggregate or gelatin for more than one year, since unmodified silk fibroin was found to gel within one month (Murphy et al., 2008). Nevertheless, the sulfonated silk fibroin derivative could still transform into a β-sheet enriched structure when treated with methanol. The possibility to control its

solubility properties allows a longer duration of storage of the silk fibroin solution but also for an increase in the silk fibroin concentration without aggregation.

1.2.6. Swelling Properties of Silk Fibroin

The delivery of drugs from matrices such as hydrogels depends partially on the degree of swelling, which in turn relies on the ionization of the network, its degree of crosslinking and its hydrophilic / hydrophobic balance (Peppas and Khare, 1993). Change in polymer compositions can be influenced by the degree of swelling. For instance, an increase in the length of elastin repeating units in the backbone of silk elastin like polymer hydrogels, keeping the length of silk repeating unit constant can result in an increase the degree of swelling due to a decrease in crosslinking density (Haider et al., 2005). This can likely increase the total amount and rate of drug release. The swelling ratio of silk fibroin scaffold has also been shown to decrease with an increase in silk fibroin concentration. Blending silk fibroin with other polymers such as chitosan, (Rujiravanit et al., 2003) hyaluronic acid (Garcia-Fuentes et al., 2008) can lead to an increase in swelling when compared to silk fibroin alone.

1.3 Biological properties of Silk Fibroin

Regardless of the structure, chemical and mechanical properties of silk fibroin, the biological properties are highly considered when fabricating any forms of silk fibroin ranging from scaffold, hydrogel and films. Silk has a high interest in tissue engineering because of its structural strength and biocompatibility with host tissue.

1.3.1 Biocompatibility

Biomaterials show heterogeneity or immunogenicity when implanted into the host tissue. When a foreign material enters the body, B cells (Liu et al., 2005) macrophage, dendritic cells (Romai et al., 1996) and mast cells (Zhaoming et al., 1996) in the immune system are activated and release antibodies and several cytokines targeting antigen epitopes on the biomaterials to attack and get rid of the foreign body by humoral and cellular immune responses. The biocompatibility of silk fibroin porous materials is necessary to consider, several primary cells and cell lines have been successfully grown on various silk fibroin morphology (materials) to show a range of biological outcomes.

Silk fibroin material is biocompatible when studied *in vivo* and *in vitro*, suture made from virgin silk compared with the suture from degummed silk showed a difference in their hypersensitivity (Altman et al., 2003). The inflammatory response of degummed silk fibroin *in vitro* compared with suture of polystyrene and poly (2 - hydroxyethyl methacrylate) showed little adhesion of immuno-competent cells (Santin et al., 1999) virgin silk (fibroin containing sericin gum) is not biocompatible whereas that of degummed silk fibroin (without sericin) was biocompatible (Panilaitis et al., 2003). Studies as showed that once sericin is detached from silk fibroin, it support cell attachment and proliferation for a large range of cell types (Roh et al., 2006; Minoura et al., 1995; Jin et al., 2004). Silk films implanted *in vivo* induced a lower inflammatory response than collagen films and Polylactic acid (PLA) films (Meinel et al., 2005). Silk fibroin non-woven mats implanted subcutaneously in rat induced a weak foreign body response and showed no sign of fibrosis. There was little regulation of inflammatory pathways at the site of implantation by lymphocytes after six months in vivo (Dal et al., 2005). Silk fibroin non-woven mat are biocompatible when studied *in vitro* and *in vivo*, they were biocompatibility in their host tissue.

1.3.2 Biodegradation

Biodegradation is the breakdown of polymeric materials into smaller compounds. The processes vary greatly; the mechanisms are complex, it comprises of the physical, chemistry and biological factors. Depending on the mode of degradation, silk fibroin has enzymatic degradation ability (Arai et al., 2004; Naira and Laurencin, 2007). Enzymes play a significant role in the degradation of silk fibroin, due to their enzymatic degradability, unique Physico-chemical, mechanical and biological properties of silk fibroins have been studied. The enzymatic degradation of biomaterials is a two-step process: firstly adsorption of the enzyme on the surface of the substrate through surface binding domain and the second step is hydrolysis of the ester bond (Naira et al., 2007).

Degradation of biomaterials is necessary for the restoration of the tissue structure and function *in vivo*, control of the rate of degradation is mandatory for functional tissue design, in such way that the rate of scaffold degradation matches the rate of tissue growth (Lanza et al., 2000). Silk fibroin materials retain more than 50 % of its mechanical properties after two (2) months of implantation *in vivo* thus; they are defined as a non - degradable biomaterial by the United States

Pharmacopeia (Horan et al., 2005). A natural polymer such as collagen and silk degrade through the action of proteases. The rate of silk fibroin degradation depends on the structure, morphology, mechanical and biological conditions at the site of implantation. Degradation studies involving the systematic exposure of silkworm silk to enzymes has shown that silk will degrade as a result of proteolysis, with protease begin reported to have a drastic effect (Arai et al., 2004; Horan et al., 2005). A connection between *in vitro* and *in vivo* rate of degradation of silk fibroin fibers has also been studied (Horan et al., 2005). Arai et al. (2004) compared the degradation of silk fibers with silk films when exposed to different amount and type of enzymes. Silk fibroin porous sponge regenerated from *Bombyx mori* fibers degrades differently with different processing conditions (Kim et al., 2005). Silk fibroin degradation can be regulated by change in crystallinity, pores size, porosity and molecular weight distribution (MWD) of silk fibroin (Minoura et al., 1990a). A change in the molecular weight distribution can be gotten by treating silk fibroin under alkaline conditions and heat, while a decrease in the molecular weight distribution can disrupt ordered structures and reduce cross - linkers, potentially resulting in a faster degradation. It will be useful to understand the mechanism and correlation of silk fibroin degradation with mechanical properties.

The degradation of silk fibroin material also depends on the fabrication of the silk fibroin morphology (Biofilms, scaffolds etc.), the degumming procedure may cause unwanted degradation of the biomaterial produced (Jiang et al., 2006), while methanol treatment may significantly reduce the rate of degradation (Minoura et al., 1990b). Fibroin films are reported to experience more significant degradation than fibers (Arai et al., 2004) and aqueous derived silk fibroin scaffolds degrade more rapidly than hexafluoroisopropanol (HFIP) derived scaffolds (Kim et al., 2005), possibly due to increased surface area the rate of degradation is important for tissue engineering applications, and control over the physical form and post - treatment of a silk biomaterial may allow tailoring of the degradation. In the case of bone, the ability of a scaffold to maintain structural integrity over an extended period of time is crucial as it allows mass transport of nutrients and waste products while bone ingrowth, matrix deposition, remodeling and a vascular network is achieved. In circumstances, such as wound healing, more rapid degradation may be desired. Silkworm silks have similar structural characteristics to amyloid (Li et al., 2001) also with dissolved fibroin has been reported to accelerate amyloid accumulation in

mice. The presence of amyloids in the body has been linked with neuro-degenerative diseases including Alzheimer's and Parkinson's diseases.

1.4 Morphology of Silk Fibroin

Silk fibroin is a multipurpose biomaterial, it can be easily be transformed into different shape, structure and form ranging from scaffolds, hydrogels, biofilms, Micro/Nano particles, non-woven mat etc.

1.4.1 Silk fibroin Films

Silk fibroin films can be cast from an aqueous or organic solvent; it can be blended with other polymers like chitosan. Silk films prepared from aqueous silk fibroin solution had oxygen and water permeability depending on the constituent of silk I and silk II structures (Minoura et al., 1990a; Minoura et al., 1990b). Alteration of silk structure was induced by treatment with 50% methanol varying time, a change in the silk structure resulted in improved mechanical and degradability properties of the films (Minoura et al., 1990a). Nano scale silk fibroin films can also be formed from aqueous solution using a layer by layer technique (Wang et al., 2005). Microstructures in films, which are important for increasing surface roughness for cell attachment, were formed by blending the silk films with poly (ethylene oxide) (PEO) (Jin et al., 2004). The rough surfaces were exposed by extracting the poly (ethylene oxide) (PEO) with water, after locking in the beta sheet crystallinity with methanol (Jin, et al., 2004). The roughness was directly related to the content of poly (ethylene oxide) (PEO) used in the process. Fibroblast attachment to silk films has been shown to be higher than collagen films (Minoura et al., 1995a; Minoura et al., 1995b). Silk biofilms employed for skin wound healing in rats, healed in seven days faster with lower inflammatory responses than traditional porcine based wound dressing (Sugihara et al., 2000). It has also been used to improved cell attachment and bone formation, especially when chemically modified with RGD cell binding domains. Silk fibroin films blended with BMP-2 showed increased bone formation compared with the same silk fibroin films without BMP-2 silk fibroin and cellulose films showed increased mechanical strength compared with silk films alone (Freddie et al., 1995).

1.4.2 Silk Fibroin Hydrogel

Hydrogels are three dimensional polymer networks which are physically durable to swelling in aqueous solutions but do not dissolve in these solutions. Hydrogel are biomaterials helps in the delivery of cells and cytokines. Silk fibroin hydrogels have been prepared from aqueous silk solution and are formed from β- sheet structure (Kim et al., 2004; Ayub et al., 1993). An increase in silk fibroin concentration, an increase in temperature, a decrease in pH, and an increase in calcium ions (Ca^{2+}) concentration decreases the time silk fibroin aqueous solution gelatin; hydrogel pore size can be controlled based on silk fibroin concentration and temperature (Kim et al., 2004). Gelation of 3% solution was obtained in two days at pH of 3 to 4 compared with eight days as required from a solution with pH 5-12 (Ayub et al., 1993). Another important factor in gelation includes silk polymer concentration and calcium ion (Ca^{2+}). Hydrogel blend of silk fibroin and gelatin showed a temperature dependent helix coil transition of the gelatin which increases the mechanical properties of the gel composition and temperature dependent properties (Gil et al., 2005a; Gil et al., 2005b) of gelatin-silk fibroin hydrogel were examined for drug delivery purposes. Hydrogel blended with silk and elastin produced a biomaterial called silk-elastin-like protein polymers (SELPs). The water content in silk-elastin-like-protein hydrogels could be managed by the time of gelatin and concentration of the polymer, while the properties were not affected by the ionic strength, temperature or pH (Dinerman et al., 2002a; Dinerman et al., 2002b).

1.5 Eggshell

The eggshell mostly made up of calcium carbonate (95%) and a small amount of organic substance (3.5%) (Nye and Gautron, 2007). The structure of eggshell can be divided into six layers (inner and outside layer) as shown in Figure 1.2.

1.5.1 Structure of Eggshell

The inner layer of the shell membranes makes up the inner most layers (20 μm thick); it has a direct contact with the albumen. The outer membrane which lies directly above the inner membrane is approximately 50μm thick. The inner and outer membrane of the eggshell contains interwoven protein fibers, correspondent to the egg surface providing structural support to the

eggshell (Lammie et al., 2005; Nys and Gautron, 2007). The membrane of eggshell gives strength to the shell and prevents microorganism invasion.

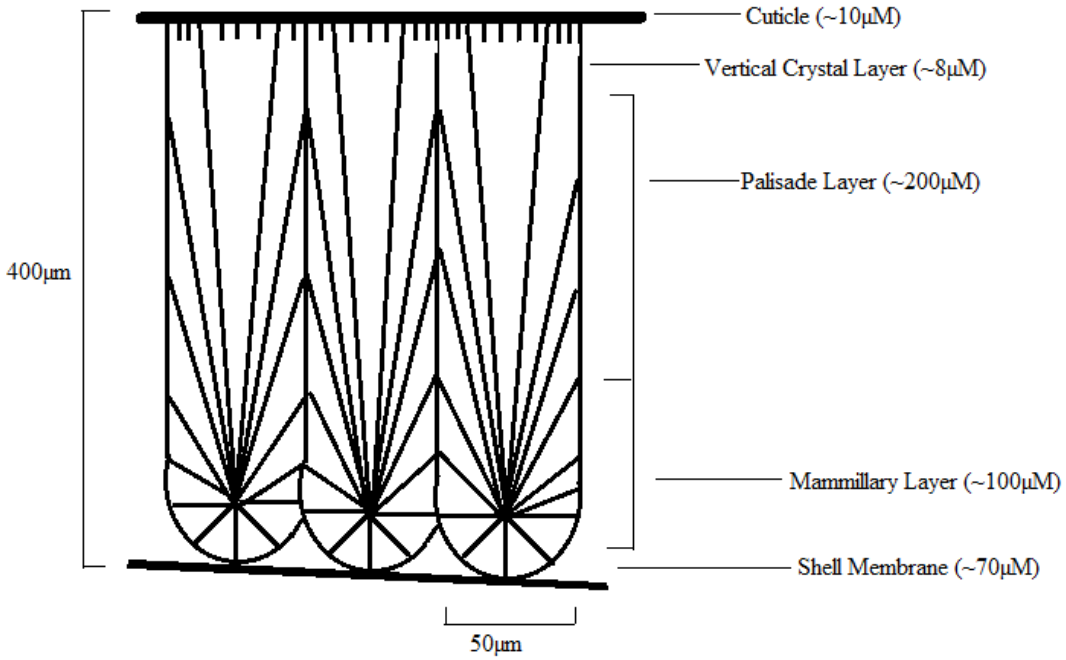

Figure 1.2: Schematic diagram of the different layer within the Eggshell structure (Lammie et al., 2005)

1.5.2. Tissue Engineering Relevance of Biomaterial from Eggshell.

Several biomaterials can be obtained from eggshell such as: Calcium carbonate, Protein (Amino acid) and Hydroxyapatite.

1.5.2.1 Calcium Carbonate (Calcite)

The calcified portion constituent of calcium carbonate crystals of the eggshell which begins at the outer membrane can be divided into three layers; the mammillary layer, Palisade layer and the vertical crystal layer (Lammie et al., 2005). The mammillary layer (70μm thick) which forms the inner most layer of the calcified segment of the eggshell goes through the outer membrane via numerous carbonate cones. The initial formation of calcium carbonate crystals takes places at

the mammillary knobs, which are organic core deposited during the egg formation (Lammie et al., 2005).

Calcium is the major mineral component found in eggshell, it is mainly in crystalline form, existing as calcium carbonate ($CaCO_3$), calcium triphosphate and magnesium carbonate. Calcium is found in the human bone (99%) (Jorg et al., 2015), it is responsible for providing structural strength and firmness of the bone in the human body (Vander et al., 1980; Tunick, 1987).

Calcium carbonate of eggshell are substitute for bone, it is biocompatible and has the ability to bond to bone recipient (Dupoirieux et al., 2001), it is nontoxic to the human body, suitable candidate for bone regeneration and dentistry due to its characteristic of cell migration, osteointegration and cell migration which are important step when considering bone regeneration.

1.5.2.2 Hydroxyapatite

Hydroxyapatite $Ca_{10}(PO_4)_6(OH)_2$ has the chemical composition of bone minerals, it is biocompatible to the human body and bioactive (support bone ingrowth and osteointegration) when used in orthopedics, dental and maxillofacial application (Saiz et al., 2007; Rivera et al., 1999). Hydroxyapatite can be in forms of powders, porous blocks and hybrid composite for filling bone defects and voids. It is used when bones are removed or when bone augmentations are needed such as a dental implant. Hydroxyapatite can be used to coat metallic implant to improve their surface properties. It can be produced from the seashell, eggshell and also body fluids.

Hydroxyapatite is formed in eggshell during calcification: a process of rapid biomineralization and bulk mineral found within the eggshell, calcite with a needle like hydroxyapatite in small amount is found in the inner cuticle (Li – Chan and Kim, 2008). The palisade layer (200μm) lies above the mammillary layer and forms the main portion of the calcified layer of the eggshell, in this layer the calcite crystal grows perpendicular to the eggshell membrane. Calcite is the most stable form of calcium carbonate.

1.6 Silk Fibroin Blend Biofilm with Glycerine and Eggshell Powder (ESP)

Films can be formed from Silk fibroin and they are biocompatible with the human body (Altman et al., 2003; Vepari and Kaplan, 2007). Silk fibroin biofilms have good dissolved oxygen permeability in a wet state, which is similar with that of the human skin, proven it suitable for wound dressing and artificial skin application (Minoura et al., 1990).

Silk fibroin films are soluble in water due to its random coil structure. The structural characteristics of the protein should be modified from random coil to β-sheet by heat treatment, mechanical stretching, and immersion in polar organic solvents and curing in water vapor to enhance its biocompatibility. This modification results in aqueous insolubility.

However, Silk fibroin films have shown stiff and brittle in the dry state, exhibiting impressive tensile strength, low elongation and water solubility which limit their application. Hence, the properties of silk fibroin biofilms need to be improved by crosslinking with plasticizers or other materials such as polymers, bioceramic (Ruijuan and Meng, 2013).

Glycerol also known as glycerine or glycerin is a simple polyol (Sugar alcohol) compound, which is odorless, colorless, and viscous liquid with a naturally sweet taste which is nontoxic. It has three hydroxyl groups that are responsible for its solubility in water and its hygroscopic nature. Silk fibroin films show softness and high elasticity when combined with glycerine (Xie and Zhang, 2013).

In this study glycerine and Eggshell powder were blended with silk fibroin to produce a softer and more elastic SF – ESP – GLY blended biofilms and SF – Gly blended biofilm.

1.7 Aim / Objectives of Thesis

1. Applying a thermal casting / evaporation technique to fabricate a stronger and elastic SF-GLY and SF – GLY – ESP blended biofilm for biomedical application.
2. Adding glycerine to the silk fibroin biofilm blend to increase the elasticity of the biofilms and its mechanical properties.
3. Using various solvent with different pH values to investigate the swelling rate of the SF – - GLY and SF – ESP - GLY biofilm blend.

4. Investigating the SF – ESP – GLY biofilm blend characteristic and morphology in respect to it constitute, structure, shape, porosity properties with several analytic procedures; such as scanning electron microscopy (SEM), x-ray diffraction (XRD), induced coupled plasma spectroscopy (ICP) and swelling test were carried on the silk fibroin biofilm blend in this study.

1.7.1 Problem Statement

Research has been carried out in regards to the synthesis of biomaterial from natural sources. Apart from eggshell, other natural sources of hydroxyapatite have been identified that mimics the natural bone composition, such as bovine bone, fish bone, cuttle fish, shell fish, oyster shells and coral. These can be converted into biomaterials (Wu et al., 2013; Sanosh et al., 2009). The constant use of the natural sources of biomaterial excluding the eggshell may lead to their extinction, like corals which have slow growth rate.

Eggshell serves as an excellent biomaterial source because of its sinterability when compared with other calcium phosphate natural sources in terms of hardness, density and cell culture. Cytotoxicity test carried out using osteoblast cell culture proved biocompatibility of eggshell based hydroxyapatite, it showed that eggshell based hydroxyapatite favors adhesion of the osteoblast cells and is non cytotoxic, this is due to the presences of calcium carbonate in the eggshell ($CaCO_3$) (Siva Rama Krishman et al., 2007).

Silk fibroin is natural polymers which as an excellent biocompatibility, biodegradable, tensile properties and can easily conform to the different shape, size and dimension. However, silk fibroin films have shown stiff and brittle in dry state, exhibiting impressive tensile strength, low elongation and water solubility, which limits its application, hence, the properties of silk fibroin biofilms needs to be enhanced by blending with plasticizers such as glycerine or any other material such as polymer, bioceramic, (Ruijuan and Meng, 2013).

CHAPTER 2
MATERIALS AND METHOD

2.1 Materials

The raw domesticated silk worm (*Bombyx mori)* cocoons were purchased from Büyük Han (Great Inn) city Centre Lefkosa, Turkish Republic of Northern Cyprus. The chemicals used for this research are of high quality purchased from reputable companies, Sodium carbonate (Na_2CO_3) used for degumming / purification process were purchased from Sigma – Aldrich, Calcium chloride ($CaCl_2$) used for the dissolution process were purchased also from Sigma – Aldrich , Ethanol was also used for the preparation of the electrolyte solution, Deionized water gotten from Near East University, hospital hemodialysis center was used all through the research, dialysis membrane were purchased from Sigma – Aldrich was used during the dialysis process to enable exchange of ions, glycerine was purchased from a Pharmacy outlet to improve the physical quality of the films and to change its crystalline structure to the β – sheet structure, eggshell was obtained from the market and was grinded to powdered form in the laboratory.

Figure 2.1: *Bombyx mori* silk cocoons

2.2 PREPARATION OF SILK FIBROIN

The process of synthesis raw silk fibroin cocoon to obtain a pure aqueous solution that can be transformed into several shapes and form requires three processes namely:

- Degumming process
- Dissolution process
- Dialysis process

2.2.1 Degumming process

Raw *Bombyx mori* silk cocoons were trimmed into small pieces with a sterilized pair of scissor as shown in Figure 2.1 and treated with 0.1 M of Sodium Carbonate aqueous (Na_2CO_3) solution (Sah and Pramanik, 2010). The purpose of degumming is to remove serine protein; a sticky substance produced by the silkworm that holds the strand of the silk together from the silk fiber structure, this process is also known as scouring.

The aqueous solution is prepared by measuring 5.3g of Sodium Carbonate (Na_2CO_3) with a weight balance; it is dissolved in 500ml of deionized water to form 0.1 M of Sodium carbonate (Na_2CO_3) solution. A gram of silk fibroin fiber were weighed and immersed into 100ml of 0.1 M of Sodium carbonate (Na_2CO_3) solution in a conical flask, a magnetic bar was dropped in the conical flask to enhance a quick removal of the serine protein from the fibers and the conical flask was placed on a magnetic stirrer set at 70^0C to a speed of 1.5 rpm has shown in Figure 2.2, this process has three sessions, and each session is processed for three hours, after each session it is rinsed thoroughly with deionized water to ensure the serine protein is totally removed from the fiber. In completion of the three sessions, the degummed silk fiber is left to dry overnight at room temperature in the laboratory.

Figure 2.2: Degumming / Scouring process

2.2.2 Dissolution process

This process involves the use of chemicals to break down silk fibroin long polypeptide chains into smaller or shorter chain to obtain a pure aqueous solution. This process requires dissolving dried silk fibers in an electrolyte solution containing 29.15ml of ethanol C_2H_5OH, 36 ml of deionized water H_20 and 27.75g of calcium chloride ($CaCl_2$), this substance are stirred in the beaker, a magnetic bar is dropped in for proper mixing into liquid form and the beaker is placed on the magnetic stirrer at 30^oC at 1rpm to dissolve the solution as shown in Figure 2.3 A. When properly dissolved, 6 % of electrolyte solution to the silk fiber weight is measured, the beaker was covered with paraffin to prevent reaction with air while dissolving the silk fibers, and the fibers were dropped at an interval to dissolve in the electrolyte solution. This process continuous until the fiber is completely dissolved and ready for dialysis as shown in Figure 2.3 B.

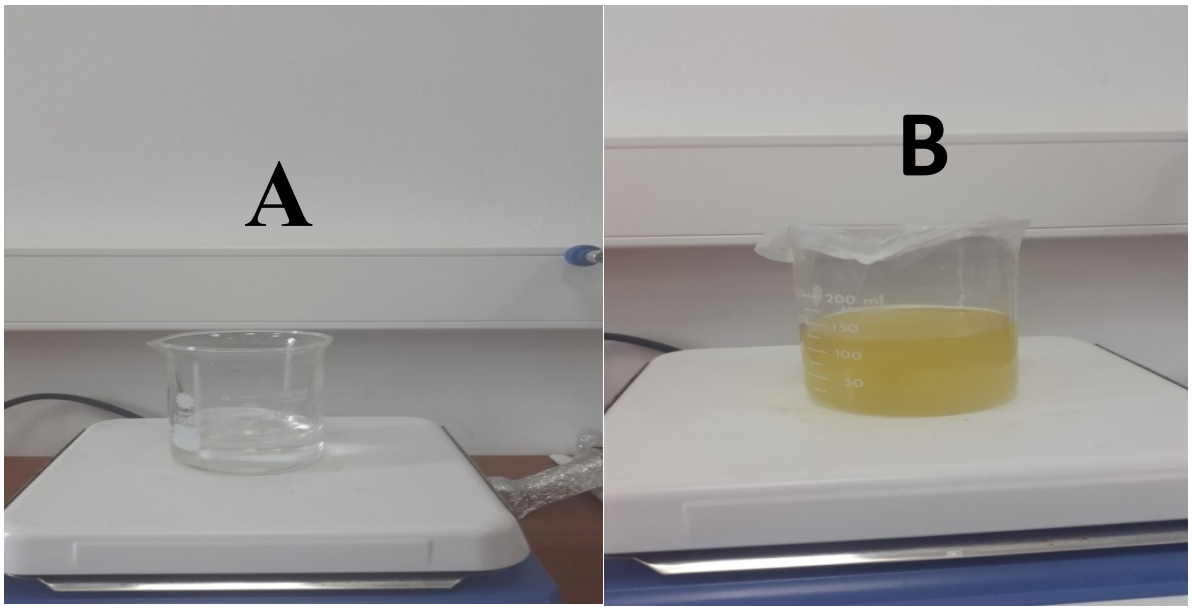

Figure 2.3: Dissolution process

2.2.3 Dialysis process

After the completion of the dissolution process to obtain silk fibroin aqueous solution, the dialysis process is carried out using a dialyzed cellulose membrane - based dialysis semi permeable membrane cassette / tube to enable the exchange of ions from the aqueous solution of the silk fibroin and the deionized water to obtain a pure silk fibroin aqueous solution.

The impure silk fibroin aqueous solution from the dissolution process is poured into the cellulose semi permeable membrane tube with the aid of a funnel, it is tied properly to avoid leakage and placed in a 5000 ml, a magnetic bar is dropped in the beaker and the beaker is placed on the magnetic stirrer at $0^{o}C$ to a speed of 1 rpm as shown in Figure 2.4. This process has three sessions; each session is processed for three hours, at the end of each session the deionized water in the beaker is changed to obtain a pure silk fibroin aqueous solution.

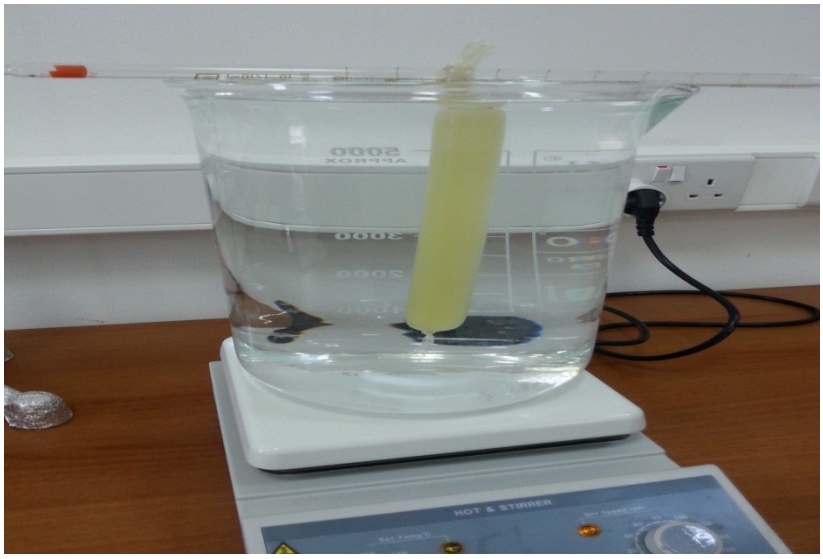

Figure 2.4: Dialysis process

2.3 Preparation of Eggshell Powder

Eggshell were obtained from the market, washed thoroughly with deionized water, left to dry over night at room temperature in the laboratory, it was grind to powdered form after drying and poured into vial as shown on Figure 2.5 below.

Figure 2.5: Eggshell and Eggshell powder

2.4 Preparation of Silk Fibroin Blend Biofilm

In this study open dish evaporation / thermal method was used to fabricate the silk fibroin blend biofilms. Open dish evaporation is a process whereby the solvent is placed in an open container such as; Erlenmeyer, evaporating dish, beaker and vial. The container is set on a heat source such as; Steam bath, hot plate, heating mantle and sand bath; and then the solvent dries off.

The different ratios of silk fibroin films were prepared by using a pipette to measure 20ml of silk fibroin aqueous solution; 2ml of glycerine and the various ratios of the eggshell powder as shown on Table 2.1 below, the mixture are poured in a small beaker, a magnet bar is placed in the beaker and its set on a magnetic stirrer at 0^{o}c to 1 rpm for the mixture to blend together.

Table 2.1: Silk Fibroin solution blends, with ESP and GLY.

Sample	Silk Fibroin	Glycerine	Eggshell powder
LS1	20ml	----	----
LG1	20ml	2ml	----
LG2	20ml	1ml	----
L1	20ml	2ml	0.50g
L2	5ml	2ml	0.75g
L3	5ml	2ml	0.10g
L4	5ml	2ml	0.05g

Each of this mixture were poured into an evaporating dish and placed on a magnetic stirrer to dry off at 40^{o}c at 0 rpm until the silk fibroin films and silk fibroin blend biofilm were formed. (3 hours) as shown in Figure 2.6. The samples are placed in a petri dish and taken for sterilization.

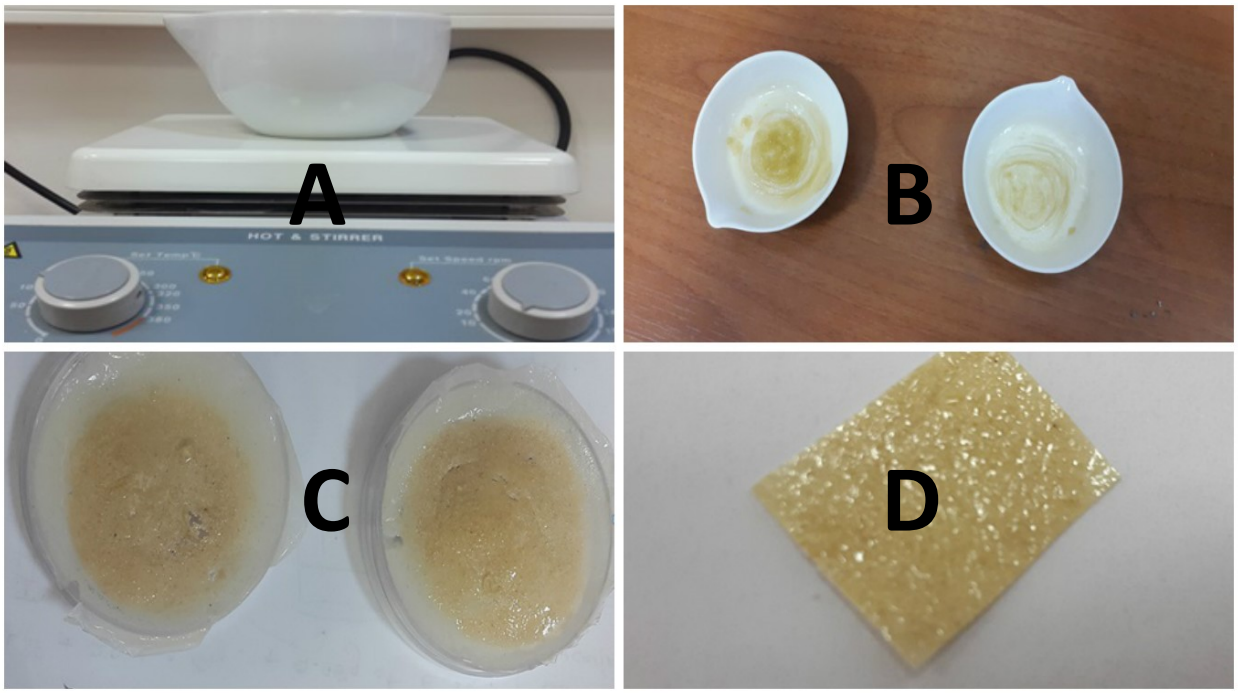

Figure 2.6: SF – ESP - GLY blend biofilm preparation

(A). Evaporation / thermal method.

(B). SF – ESP – GLY blend biofilms in evaporating dish.

(C). SF – ESP – GLY blend biofilms already taken out from the evaporating dish.

(D). SF – ESP – GLY blend biofilms cut into fine piece for characterization.

2.5 Sterilization Process

Hydrogen peroxide sterilization method was used; the sterilization of the samples was carried out in Near East University Hospital sterilization department.

The sterilization chamber is cleaned up; Hydrogen peroxide is injected into the cassette, its vapor diffuses through the chamber (50minutes), exposing the surface of the loaded sterilant and initiates the inactivation of microorganisms, electrical and radio frequency are applied to the chamber.

The excess gas is removed from the chamber in final stage of the sterilization process. The sterilized materials are free from microbes; they can be used immediately or stored for use later.

This process operates in the range of 37 – 44^{o}C and each cycle takes about 75minutes to be completed (William et al., 2008).

2.6 Swelling Test

Silk fibroin films prepared with different constituent and ratio were tested for its swelling behavior in Ethanol (70%) solution, deionized water, phosphate buffer solution (PBS), sodium hydroxide solution (NaOH) and hydrochloric acid (HCl_2).

The dry weight of the silk fibroin films used was 0.20g, during the swelling test they were weighed at interval to know the rate of swelling of the films.

The swelling ratios were calculated using the formula below:

$$\text{Swelling } \% = \frac{\text{Weight(s)} - \text{Weight(dry)}}{Weight\ (dry)} \times 100$$

2.7 Material Characterizations

Silk fibroin biofilm blended were further characterized to study its porosity, composition, surface topography etc.

2.7.1 Scanning Electron Microscope (SEM)

Scanning Electron microscopy was carried out at Tubitak Marmara Research Institute Gebze Istanbul Turkey, using a SEM- Jsm- 6510 model at an acceleration voltage 10kV.

Scanning electron microscopy is an electron microscope which produces images of a sample by focusing beam of electrons. The sample is fixed into the specimen chamber and is electrically conductive at the surface or sputter coated by gold to prevent electrostatic charge. The electrons interact with atom present in the samples, producing several signals containing information of the samples surface topography (size, particles smoothness or roughness) and its composition. (McMullan, 2006).

2.7.2 X – ray Diffraction Analysis (XRD)

X – Ray diffraction is a good technique used to determine the crystallinity of a compound. It distinguished between amorphous and crystalline material E.g. Silk fibroin (which is amorphous and crystalline in nature).

Powder x-ray diffraction analysis was carried out at Tubitak Marmara Research Institution Gebze Istanbul Turkey, using a shimadzu XRD -600 model diffractometer with a CU-X ray tube ($L=1.5405A^0$). The diffractometer scans at a rate of 2^o / minutes within the region of 2 θ resulting in diffraction intensity curves produced.

2.7.3 Inductively Coupled Plasma Spectrometry (ICP-MS)

Inductively coupled plasma mass spectrometry (ICP –MS) is an analytic techniques used to determine the elemental constitute of a material (Ruth, 2005) E.g. Alloys, Plastics, Polymer, Metals and liquid except atmospheric species and noble gases (Oxygen, Nitrogen etc.). (Evan, 2016). It combines a high temperature ranging from 6000-1000 kelvin with a mass spectrometer, converting atoms of the element in the sample's ions; the ions are separated and recognized by the mass spectrometer (Ruth, 2005).

Inductively coupled plasma spectrometry (ICP –MS) was carried out at Tubitak Marmara Research Institution Gebze Istanbul Turkey, using NexON 350Q model of spectrometry.

CHAPTER 3
RESULTS AND DISCUSSION

3.1 Swelling Test

Several ratios of Silk fibroin biofilms were fabricated and placed in various solvent in order to observe their swelling rate and weight in several solvents; ethanol at pH 7.33 which mimic the human urine and the venous blood; deionized water at pH 7.0 and phosphate buffer solution (PBS) at pH 7.4 which is similar to the extracellular fluid of the human bone, heart capillary and arterial blood, hydrochloric acid (HCl) at pH 1.0 which is acidic as in the gastric secretion; sodium hydroxide solution with pH 12 that is incompatible to the human body. The swelling test was carried out to study the behavior of the silk fibroin biofilms blends in several pH conditions

Table 3.1: Ethanol (70%) for Group A samples swelling ratio

Time	LS1	LG1	LG2	L1
5mins	50%	50%	100%	50%
10mins	50%	50%	100%	100%
15mins	50%	50%	50%	100%
30mins	50%	50%	50%	50%
45mins	100%	100%	100%	50%
75mins	50%	100%	200%	100%
105mins	50%	100%	150%	50%

LS1: Silk fibroin biofilm.

LG1: Silk fibroin, glycerin blend biofilm (2ml).

LG2: Silk fibroin, glycerine blend biofilm (1ml).

L1: Silk fibroin, ESP (0.5g) and glycerine blend biofilm.

Table 3.1 shows the swelling behavior of LS1, LG1, LG2 and L1 in 70% ethanol solution (pH 7.33) at different time interval from 5mins to 105mins. The swelling ratio percentages at different time interval on Table 3.1 are represented graphically in Figure 3.1 below.

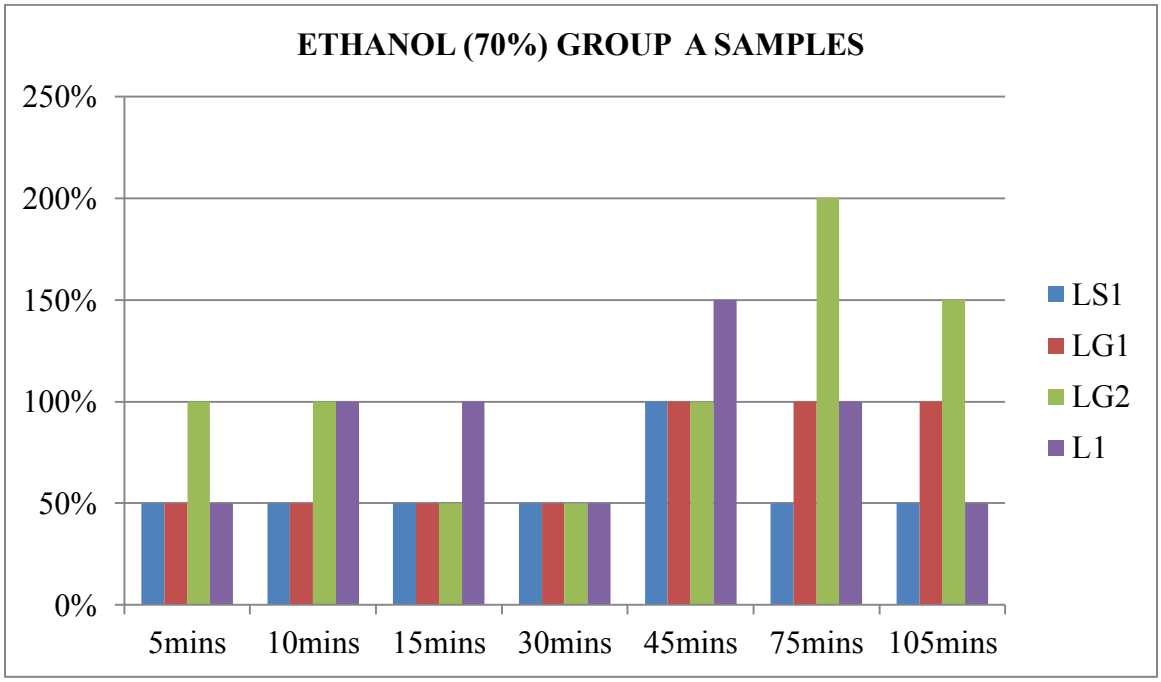

Figure 3.1: Ethanol (70%) swelling behavior of group A samples

Figure 3.1 above graphically explains the swelling ratio percentage of silk fibroin biofilm (LS1) in 70% Ethanol solution (pH 7.33) was stable with an increase at 45 minutes and back to its original swelling ratio, Silk fibroin biofilm blend with glycerine (LG1) swelling behavior was stable with an increased at 45minutes until 105 minutes, Silk fibroin biofilm blend with glycerine (LG2) swelled at randomly with higher value than LG1 with more quantity of glycerine. Silk fibroin biofilms blend with glycerine and eggshell powder (L1, 0.50g) swelled in 70% ethanol solution with an increase at 10 minutes and biodegraded from 75 minutes.

In a nutshell the e silk blend biofilms swelled in 70% ethanol, L1 swelled best in 70% ethanol; the presence of the eggshell enhanced the swelling of L1 in ethanol.

Table 3.12: Ethanol (70%) for Group B samples swelling ratio

Time	L2	L3	L4
5mins	50%	100%	50%
10mins	150%	50%	50%
15mins	50%	50%	50%
30mins	50%	50%	50%
45mins	100%	150%	100%
75mins	200%	50%	100%
105mins	150%	150%	50%

L2: Silk fibroin, ESP (0.75g) and glycerine blend biofilm.

L3: Silk fibroin, ESP (0.1g) and glycerine blend biofilm.

L4: Silk fibroin, ESP (0.05g) and glycerine blend biofilm.

Table 3.12 above shows the swelling behavior of L2, L3 and L4 in 70% ethanol solution (pH 7.33) at different time interval from 5mins to 105mins. The swelling ratio percentages at different time interval on Table 3.12 above are represented graphically in Figure 3.12.

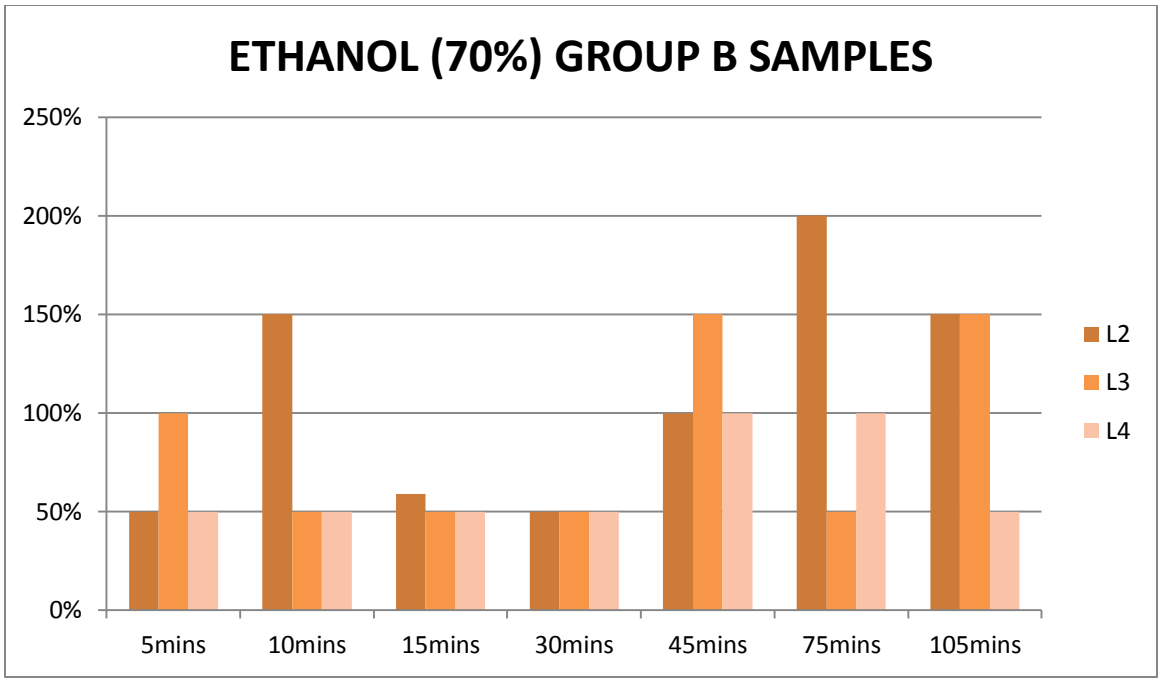

Figure 3.12: Ethanol (70%) swelling behavior graph of group B sample.

Figure 3.12 above graphically explains the swelling ratio percentage of the silk fibroin biofilms blend with glycerine and eggshell powder (L2, 0.75g) swelled in 70% ethanol solution (pH 7.33) at a minimal ratio initially at 5 minutes, from 10 minutes it began to swell randomly. Silk fibroin biofilms blend with glycerine and eggshell powder (L3, 0.10g) swelled in 70% ethanol solution (pH 7.33) at moderate ratio initially at 5 minutes, it became stable until 45 minutes with random swelling behavior until 105 minutes. Silk fibroin biofilms blend with glycerine and eggshell powder (L4, 0.05g) swelled in 70% ethanol solution (pH 7.33) at a minimal stable ratio, increased at 45 minutes and 75 minutes and showed biodegradation at 105 minutes.

In a nutshell, silk fibroin blend biofilms swelling behavior as displayed in Figure 3.12 above indicates the lesser the quantity of eggshell powder in the blend biofilm the more efficient the swelling behavior in ethanol solution (pH 7.33).

Table 3.2: Deionized swelling ratio for Group A samples

Time	LS1	LG1	LG2	L1
5mins	100%	50%	200%	50%
10mins	350%	50%	200%	50%
15mins	150%	50%	150%	100%
30mins	400%	100%	250%	50%
45mins	500%	150%	150%	50%
75mins	500%	50%	50%	50%
105mins	550%	100%	100%	50%

LS1: Silk fibroin biofilm.

LG1: Silk fibroin and glycerine (2ml) blend biofilm.

LG2: Silk fibroin and glycerine (1ml) blend biofilm.

L1: Silk fibroin, ESP (0.5g) and glycerine blend biofilm.

Table 3.2 above shows the swelling behavior of LS1, LG1, LG2, L1 in deionized water (pH 7.0) at different time interval from 5mins to 105mins.The swelling ratio percentages at different time interval on Table 3.2 above are represented graphically in Figure 3.2.

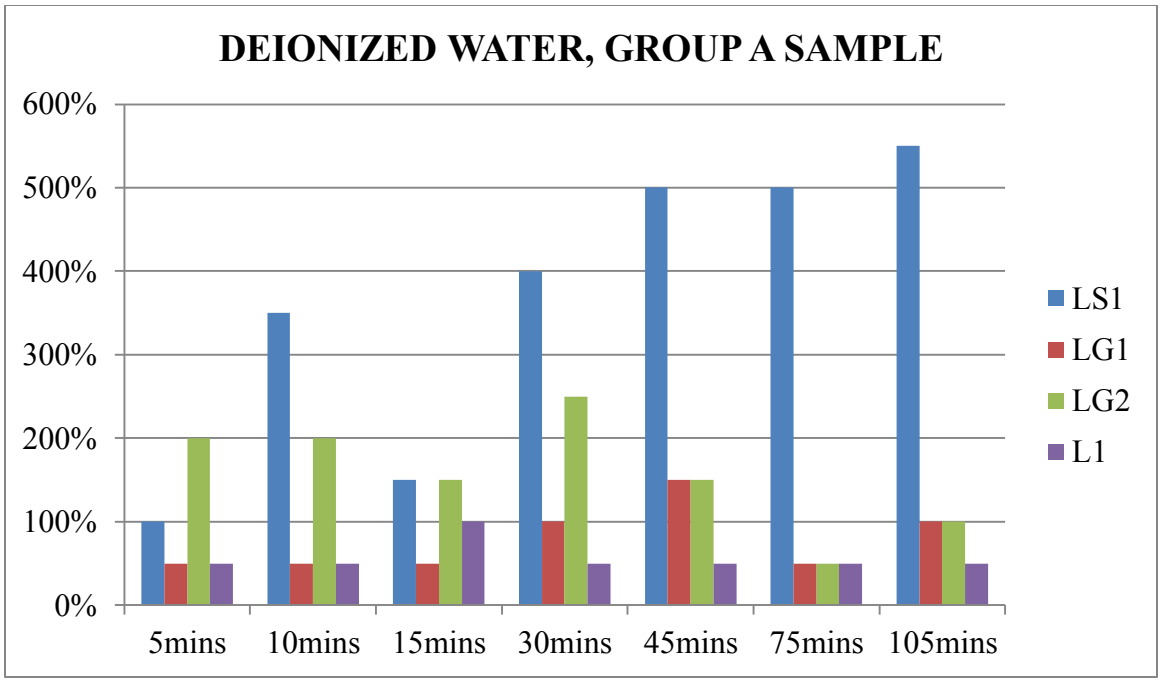

Figure 3.2: Deionized water swelling behavior of group A samples

Figure 3.2 above graphically explains the swelling ratio percentage of the silk fibroin biofilm (LS1) swelled in deionized water (pH 7.0) greatly. Silk fibroin biofilm blend with glycerine (LG2) swelled greatly in deionized water (pH 7.0) compared to the silk fibroin blend with glycerine biofilm (LG1) which indicates that the more the glycerine in pure silk fibroin solution the lesser the swelling ratio in deionized water (pH 7.0). Silk fibroin biofilm blend with glycerin and eggshell powder (L1) swelled within 30minutes and starts to biodegrade

In a nutshell, the silk blend biofilms swelling behavior as displayed in Figure 3.2 indicates that pure silk fibroin biofilm swells greatly in natural solvent (deionized water).

Table 3.21: Deionized water swelling ratio for Group B samples

Time	L2	L3	L4
5mins	100%	50%	50%
10mins	50%	200%	50%
15mins	200%	250%	50%
30mins	300%	150%	50%
45mins	100%	250%	100%
75mins	200%	150%	50%
105mins	150%	150%	100%

L2: Silk fibroin, ESP (0.75g) and glycerine blend biofilm.

L3: Silk fibroin, ESP (0.10g) and glycerine blend biofilm.

L4: Silk fibroin, ESP (0.05g) and glycerine blend biofilm.

Table 3.21 above shows the swelling behavior of L2, L3 and L4 in deionized water (pH 7.0) at different time interval from 5mins to 105mins. The swelling ratio percentages at different time interval on Table 3.21 above are represented graphically in Figure 3.21.

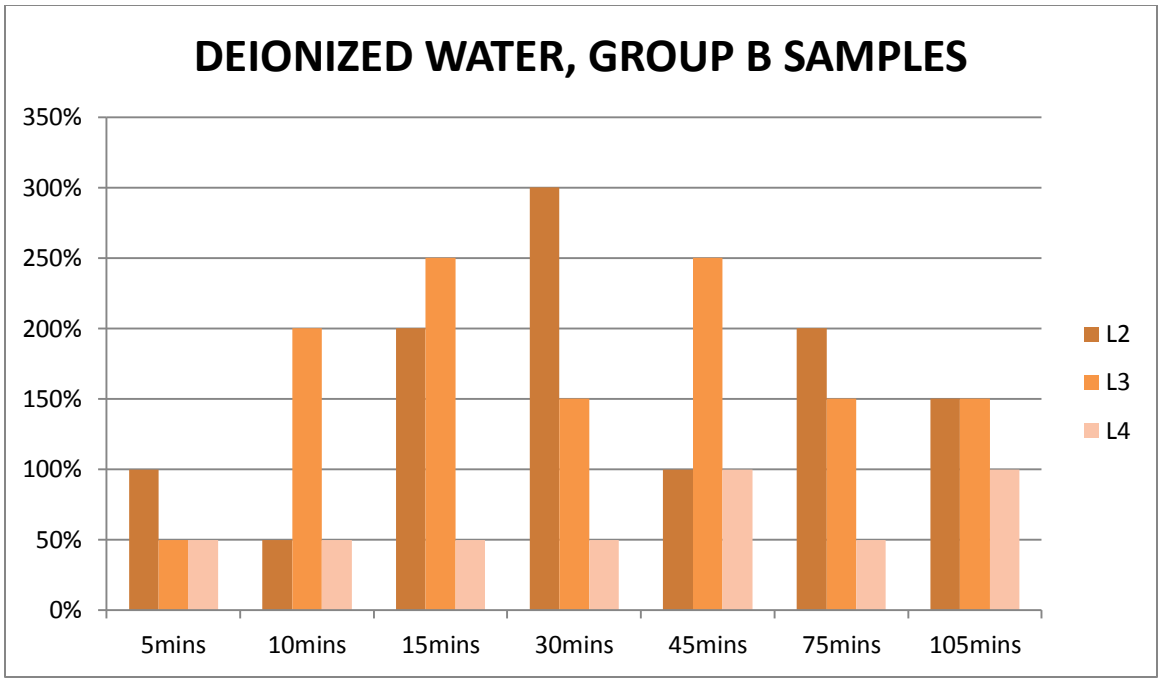

Figure 3.21: Deionized water swelling behavior of group B samples

Figure 3.21 above graphically explains the swelling ratio percentage of the silk fibroin biofilm blend with glycerine and eggshell powder (0.75g) (L2) swelled in deionized water (pH 7.0) at a moderate value, it swell more at 30 minutes and starts to biodegrade from 45minutes. Silk fibroin blend with glycerine and eggshell powder (0.10g) (L3) swelled in deionized water (pH 7.0) at a minimal ratio initially, increased and starts to biodegrade at 75 minutes. Silk fibroin blended with silk fibroin and glycerine (0.05g) (L4) swelled in deionized water (pH 7.0) at a minimal ratio initially and remains stable until 30 minutes, it increases at 45 minutes and starts to biodegrade.

Table 3.3: 0.1 M PBS swelling ratio for Group A samples

Time	LS1	LG1	LG2	L1
5mins	50%	200%	150%	50%
10mins	100%	150%	200%	50%
15mins	150%	400%	150%	50%
30mins	50%	150%	50%	100%
45mins	200%	200%	150%	50%
75min	150%	250%	100%	100%
105mins	150%	300%	50%	50%

LS1: Silk fibroin biofilm.

LG1: Silk fibroin and glycerine (2ml) blend biofilm.

LG2: Silk fibroin and glycerine (1ml) blend biofilm.

L1: Silk fibroin, ESP (0.5g) and glycerine blend biofilm.

Table 3.3 above shows the swelling behavior of LS1, LG1, LG2 and L1 in 0.1M phosphate buffer solution (pH 7.4) at different time interval from 5mins to 105mins. The swelling ratio percentages at different time interval on Table 3.3 above are represented graphically in Figure 3.3.

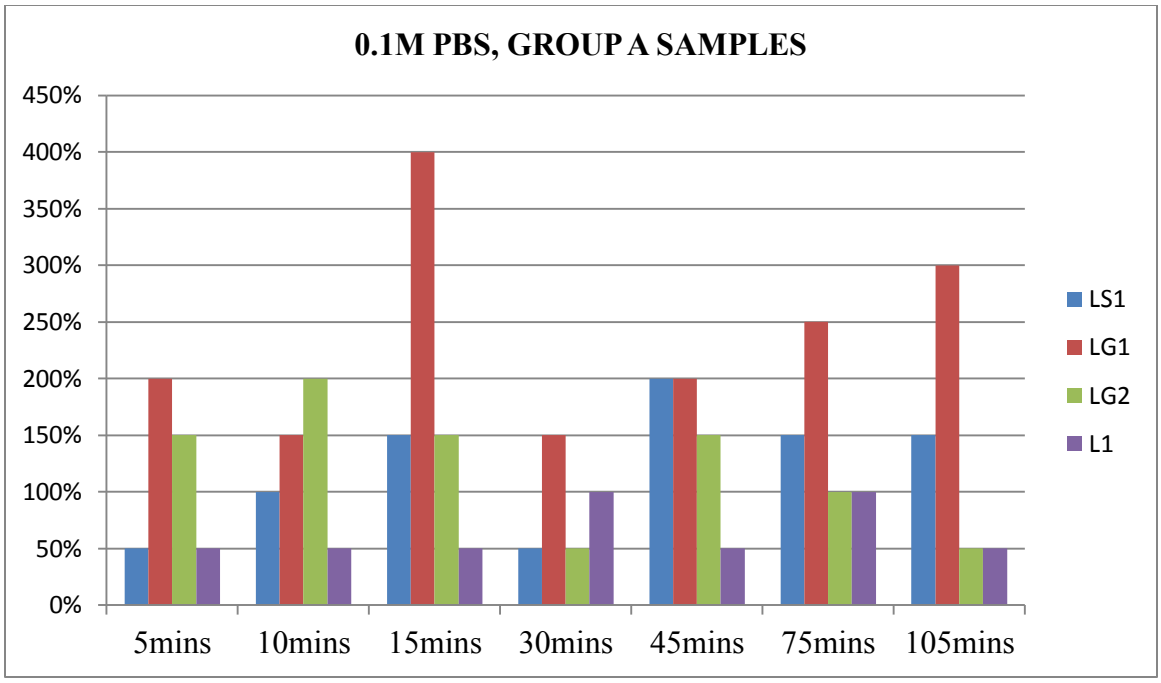

Figure 3.3: 0.1 M PBS swelling behavior of group A samples

Figure 3.3 above graphically explains the swelling ratio percentage of the silk fibroin biofilm (LS1) swelled in PBS (pH 7.4) at a minimal ratio initially, followed by a random swelling and starts to biodegrade at 75 minutes. Silk fibroin biofilm blend with glycerine (LG1) swelled in PBS (pH 7.4) at a high ratio initially, with an incredible swell at 15 minutes, dropped and followed a step like increase pattern. Silk fibroin biofilm blend with glycerine (LG2) swelled in PBS (pH 7.4) at a moderate ratio initially, swelled randomly afterward. Silk fibroin blend with glycerine and eggshell powder (L1) swelled in PBS at a minimal ratio initially with little increase at 30 minutes and 75 minutes.

In a nutshell, the silk fibroin blend biofilms swelling behavior as displayed in Figure 3.3 above indicates, the silk fibroin blend with glycerine LS1 with more glycerine swells best in 0.1M PBS (pH 7.4)

Table 3.3 1: 0.1M PBS swelling ratio for B samples

Time	L2	L3	L4
5mins	100%	100%	50%
10mins	150%	100%	50%
15mins	50%	200%	100%
30mins	200%	50%	50%
45mins	50%	150%	100%
75mins	200%	150%	150%
105mins	100%	250%	100%

L2: Silk fibroin, ESP (0.75g) and glycerine blend biofilm.

L3: Silk fibroin, ESP (0.1g) and glycerine blend biofilm.

L4: Silk fibroin, ESP (0.05g) and glycerine blend biofilm.

Table 3.31 above shows the swelling behavior of L2, L3 and L4 in 0.1M phosphate buffer solution (pH 7.4) at different time interval from 5mins to 105mins. The swelling ratio percentages at different time interval on Table 3.3.1 are represented graphically in Figure 3.3.

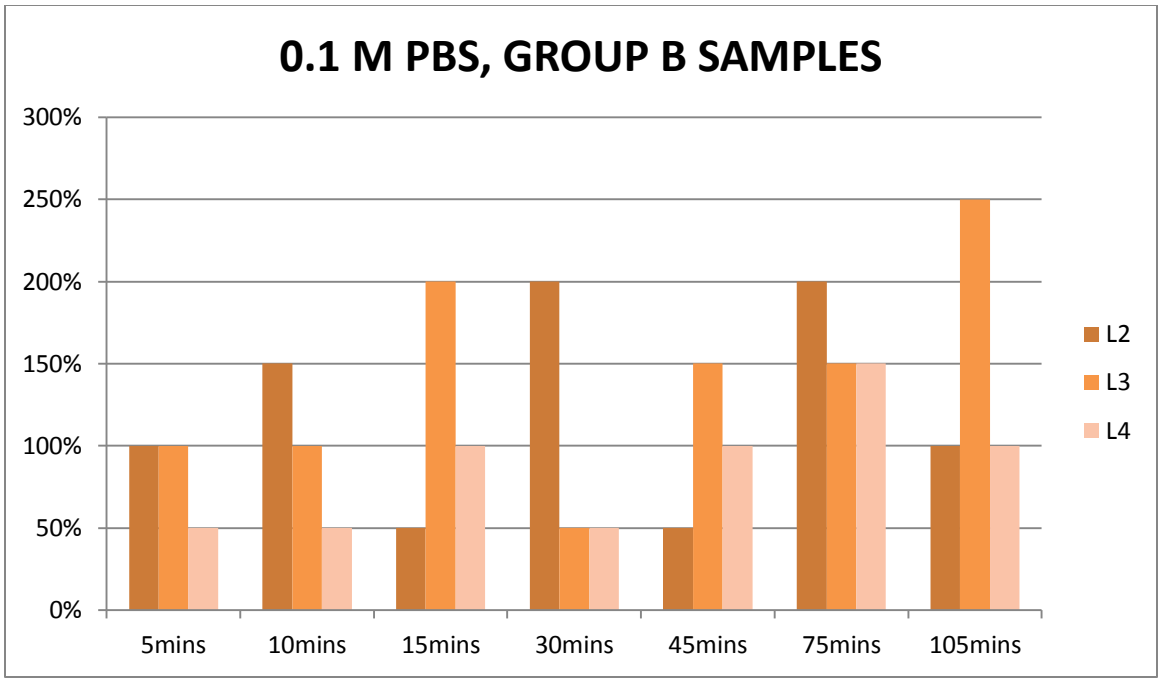

Figure 3.31: 0.1M PBS swelling behavior of group B samples

Figure 3.31 above graphically explains the swelling ratio percentage of the silk fibroin biofilm blend with glycerine and eggshell powder (L2) (0.75g) swelled in PBS (pH 7.4) at a moderate ratio initially, and experience random swelling afterward. Silk fibroin biofilm blend with glycerine and eggshell powder (L3) (0.10g) swelled in PBS at moderate ratio initially, increased and decreased randomly. Silk fibroin biofilm blend with glycerine and eggshell powder (L4) (0.05) swelled minimal initially, increased and decreased randomly.

Table 3.4: 0.1M HCl swelling ratio for A samples

Time	LS1	LG1	LG2	L1
5mins	50%	50%	300%	200%
10mins	150%	50%	250%	150%
15mins	150%	50%	300%	250%
30mins	400%	100%	100%	300%
45mins	250%	100%	250%	100%
75mins	250%	50%	100%	350%
105mins	200%	50%	100%	250%

LS1: Silk fibroin biofilm.

LG1: Silk fibroin and glycerine (2ml) blend biofilm.

LG2: Silk fibroin and glycerine (1ml) blend biofilm.

L1: Silk fibroin, ESP (0.5g) and glycerine blend biofilm.

Table 3.4 above shows the swelling behavior of LS1, LG1, LG2 and L1 in 0.1M hydrochloric acid solution (pH 1.0) at different time interval from 5mins to 105mins. The swelling ratio percentages at different time interval on Table 3.4 above are represented graphically in Figure 3.4.

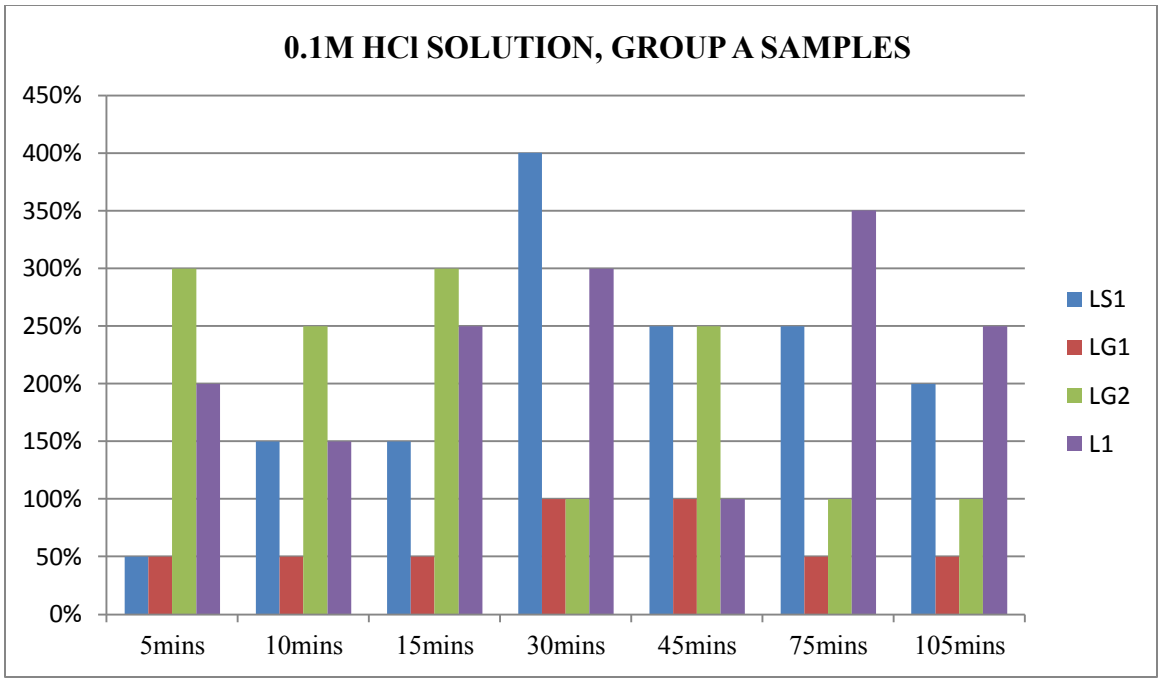

Figure 3.4: 0.1M HCl swelling behavior of group A samples

Figure 3.4 graphically explains the swelling ratio percentage of the silk fibroin biofilm (LS1) swelled in HCl solution (pH 1.0) at a minimal value initially, increased and decreased randomly. Silk fibroin biofilm blend with glycerine (LG1) swelled in HCl solution (pH 1.0) at a stable minimal ratio initially, increased at 45 and 75 minutes, further experience biodegradation. Silk fibroin biofilm blend with glycerine (LG2) swelled in HCl solution (1.0) at a high value initially, increased, decreased randomly and starts to biodegrade at 75minutes. Silk fibroin biofilm blend with glycerine and eggshell powder (L1) (0.50g) swelled in HCl solution (pH 1.0) at a high value initially, increased and decreased randomly.

In a nutshell, the silk fibroin blend biofilms as displayed in Figure 3.4 above swelled greatly in HCl solution (pH 1.0) excluding silk fibroin biofilm blend with glycerine LG1 with a slow swelling behavior.

Table 3.4 1: 0.1 M HCl solution swelling ratio for group B sample

Time	L2	L3	L4
5mins	50%	50%	100%
10mins	50%	150%	50%
15mins	100%	100%	100%
30mins	150%	150%	150%
45mins	100%	50%	100%
75mins	50%	50%	50%
105mins	50%	150%	100%

L2: Silk fibroin, ESP (0.75g) and glycerine blend biofilm.

L3: Silk fibroin, ESP (0.1g) and glycerine blend biofilm.

L4: Silk fibroin, ESP (0.05g) and glycerine blend biofilm.

Table 3.41 above shows the swelling behavior of LS1, LG1, LG2 and L1 in 0.1M phosphate buffer solution at different time interval from 5mins to 105mins. The swelling ratio percentages at different time interval on Table 3.4.1 are represented graphically in Figure 3.4.1.

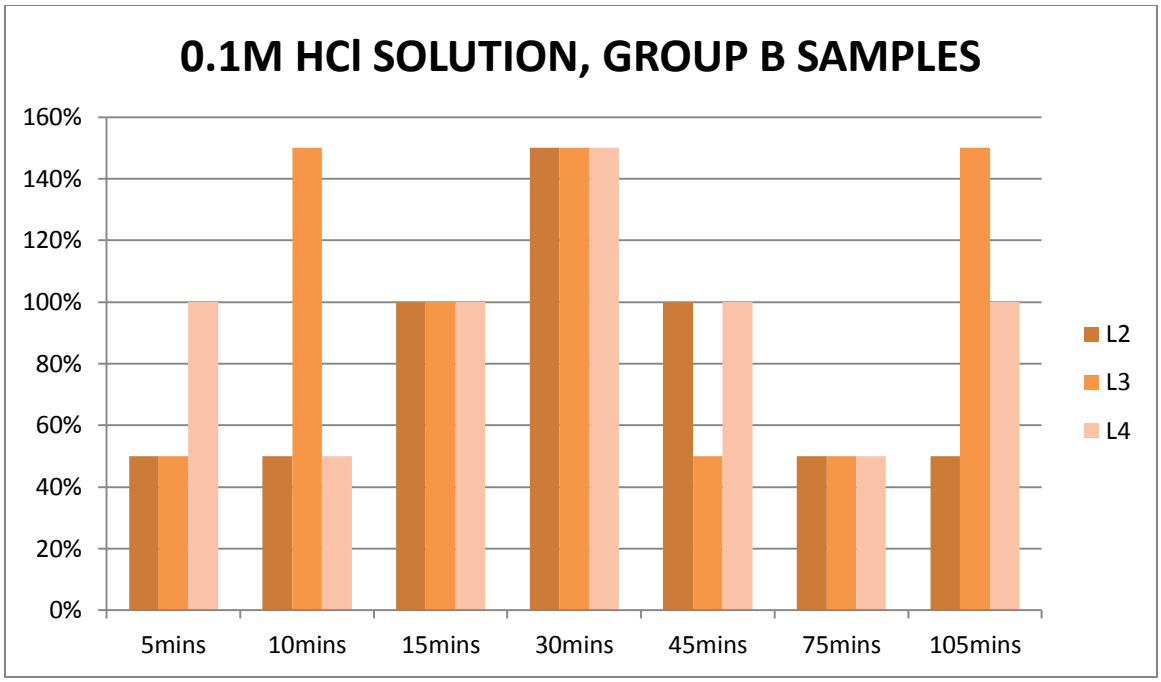

Figure 3.4.1: 0.1M HCl solution swelling behavior of group B samples

Figure 3.1 above graphically explains the swelling ratio percentage of silk fibroin biofilm blend with glycerine and eggshell powder (L2, 0.75g) swelled in HCl solution (pH 1.0) at a minimal ratio initially, increased and starts to biodegrade at 45 minutes. Silk fibroin biofilm blend with glycerine and eggshell powder (L3, 0.10g) swelled in HCl solution (pH 1.0) at a minimal ratio initially, increased and decreased uniformly and starts to biodegrade from 45minutes. Silk fibroin biofilm blend with glycerine and eggshell powder (L4, 0.05g) swelled n HCl solution at a moderate ratio initially, increased and decreased randomly.

In a nutshell, the silk fibroin blend biofilms swelling behavior as displayed in Figure 3.41 swelled greatly in HCl solution (pH 1.0).

Table 3.5: 0.1 M NaOH solution Group A samples

Time	LS1	LG1	LG2	L1
5mins	50%	150%	200%	200%
10mins	150%	300%	300%	150%
15mins	200%	250%	300%	250%
30mins	400%	250%	200%	300%
45mins	450%	200%	200%	100%
75mins	350%	150%	300%	350%
105mins	450%	150%	200%	250%

LS1: Silk fibroin biofilm.

LG1: Silk fibroin and glycerine (2ml) blend biofilm.

LG2: Silk fibroin and glycerine (1ml) blend biofilm.

L1: Silk fibroin, ESP (0.50g) and glycerine blend biofilm.

Table 3.5 above shows the swelling behavior of LS1, LG1, LG2 and L1 in 0.1M sodium hydroxide solution (pH 12) at different time interval from 5mins to 105mins. The swelling ratio percentages at different time interval on Table 3.5 above are represented graphically in Figure 3.5.

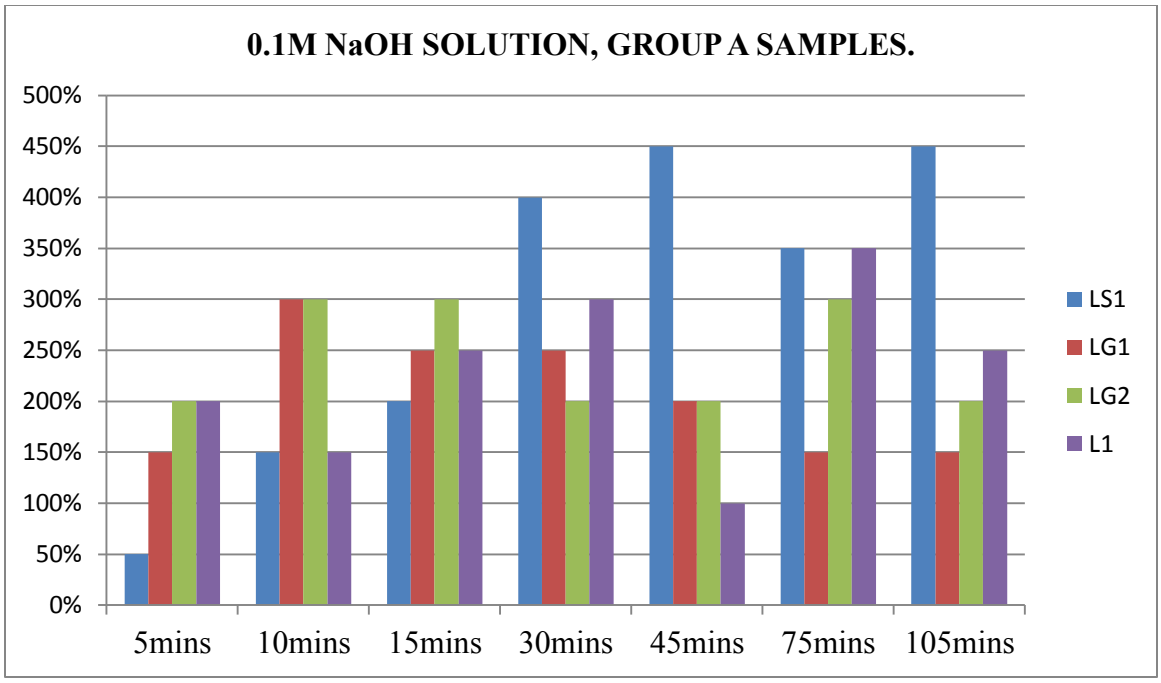

Figure 3.5: 0.1M NaOH swelling behavior of group A samples

Figure 3.1 above graphically explains the swelling ratio percentage silk fibroin biofilm (LS1) swelled in NaOH solution (pH 12) at a minimal ratio initially, increased rapidly, decreased at 75 minutes and increased at 105 minutes. Silk fibroin biofilm blend with glycerine (LG1) swelled in NaOH solution (pH 12) at a moderate ratio initially, increased and decreased and starts to biodegrade at 45minutes. Silk fibroin biofilm blend with glycerine (LG2) swelled in NaOH solution (pH 12) at a moderate ratio initially, increased and decreased randomly. Silk fibroin biofilm blend with glycerine and eggshell powder (L1, 0.50g) swelled in NaOH solution (pH 12) at a high moderate ratio initially, it increased and decreased randomly.

In a nutshell, the biofilm swelling behavior as display in Figure 3.5 above swelled greatly in NaOH solution (pH 12), especially pure silk fibroin biofilm and silk fibroin blend with glycerin and eggshell powder (L1, 0.50g).

Table 3.5 1: 0.1 M NaOH solution swelling ratio Group B samples

Time	L2	L3	L4
5mins	150%	200%	100%
10mins	250%	250%	150%
15mins	100%	450%	50%
30mins	100%	250%	100%
45mins	150%	250%	150%
75mins	100%	400%	50%
105mins	100%	300%	100%

L2: Silk fibroin, ESP (0.75g) and glycerin blend biofilm.

L3: Silk fibroin, ESP (0.1g) and glycerine blend biofilm.

L4: Silk fibroin, ESP (0.05g) and glycerine blend biofilm.

Table 3.51 above shows the swelling behavior of L2, L3 and L4 in 0.1M sodium hydroxide solution (pH 12) at different time interval from 5mins to 105mins. The swelling ratio percentages at different time interval on Table 3.5.1 above are represented graphically in Figure 3.5.1.

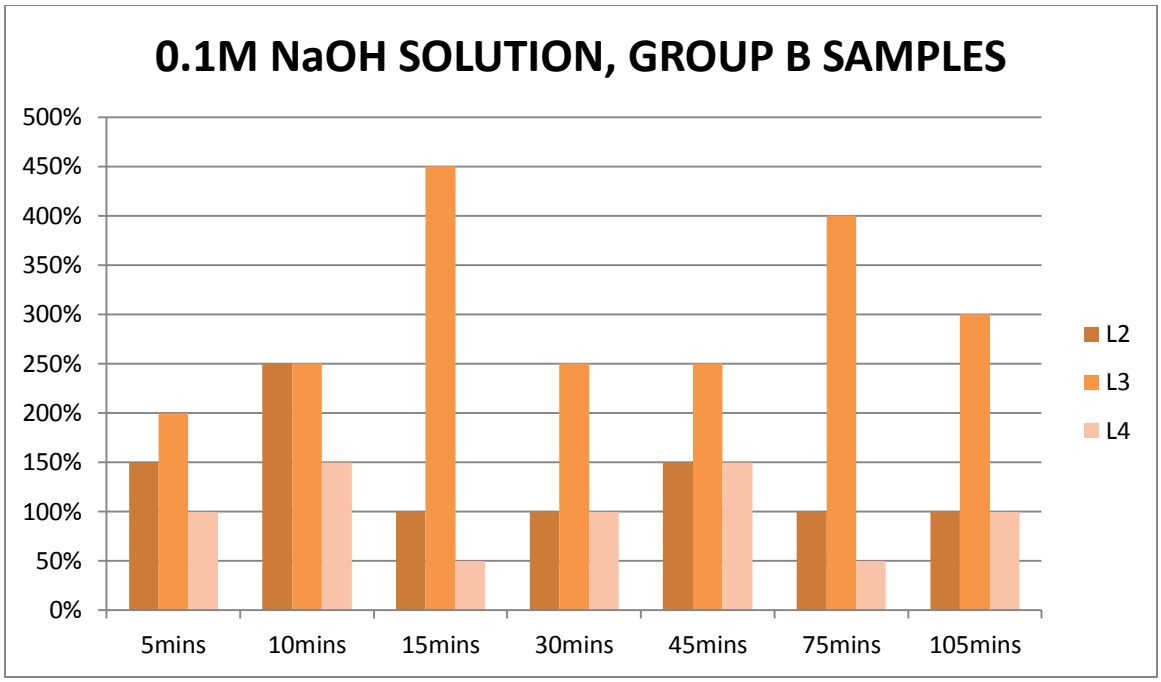

Figure 3.52: 0.1M NaOH swelling behavior of group B samples

Figure 3.52 above graphically explains the swelling ratio percentage of the silk fibroin biofilm blend with glycerine and eggshell powder (L2, 0.75g), swelled in 0.1M NaOH solution (pH 12) at a moderate ratio initially, increased and decreased randomly. Silk fibroin biofilm blend with glycerine and eggshell powder (L3, 0.10g), swelled in 0.1M NaOH solution at a moderate ratio initially, increased and decreased randomly. Silk fibroin biofilm blend with glycerine and eggshell powder (L4, 0.05g), swelled in 0.1M NaOH solution at a minimal ratio initially value, increased and decreased randomly.

In a nutshell, the silk fibroin blend biofilms swelling behavior as displayed in Figure 3.51 above swelled moderately in NaOH solution (pH 12), L3 swelled best in pH 12.

3.2 Scanning Electron Microscopy

This analysis was carried out to understand the porosity, size and surface roughness of the silk fibroin biofilms blend. The morphology of the samples was examined on the scanning electron microscope at 10kv for several magnifications as shown in Figure 3.6 and 3.6.2, it shows rough aggregation, interconnected fiber particles due the presence of the crosslinker glycerine $C_3H_8O_3$ and the incorporation of eggshell powder which is almost distributed in the entire film. In Figure

3.6, the long fine flakes like particle were formed by silk fibroin showing the protein β- sheet because the crosslinker and the bioceramic are insoluble in pure silk fibroin solution and sit at the center of the biofilms leaving the silk fibroin at the edge of the biofilm. In Figure 3.6, 3.6.3 and 3.64 gives a clearer view of the rough aggregation and interconnected fiber because they have higher magnification than Figure 3.6 and 3.6.2.

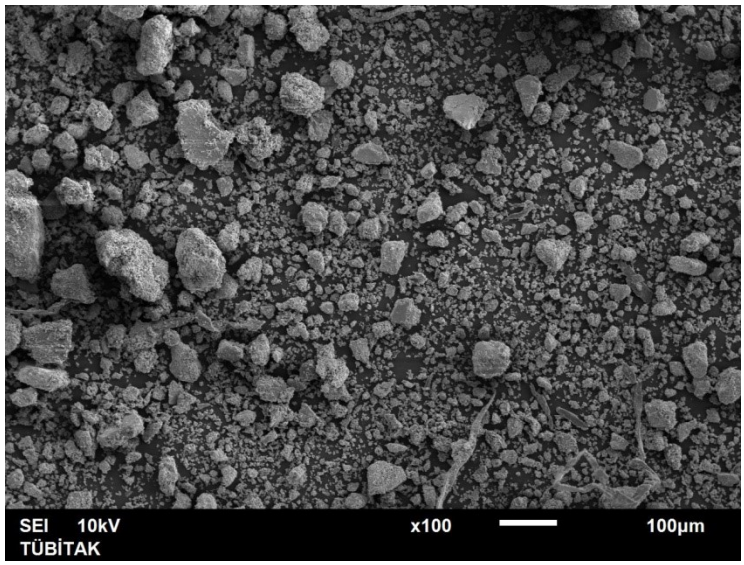

Figure 3.6: SEM micrograph Silk Fibroin + Glycerine + 0.50g ESP BF x 100

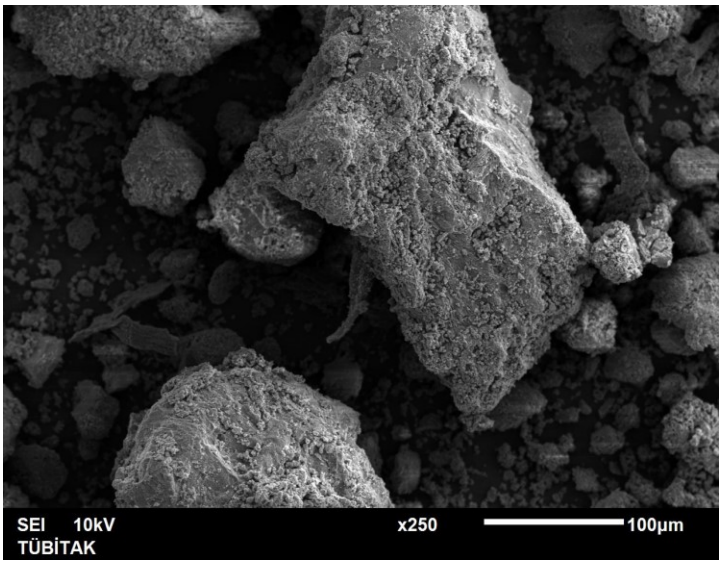

Figure 3.6.1: SEM micrograph of Silk Fibroin + Glycerine + 0.50g ESP BF x 250

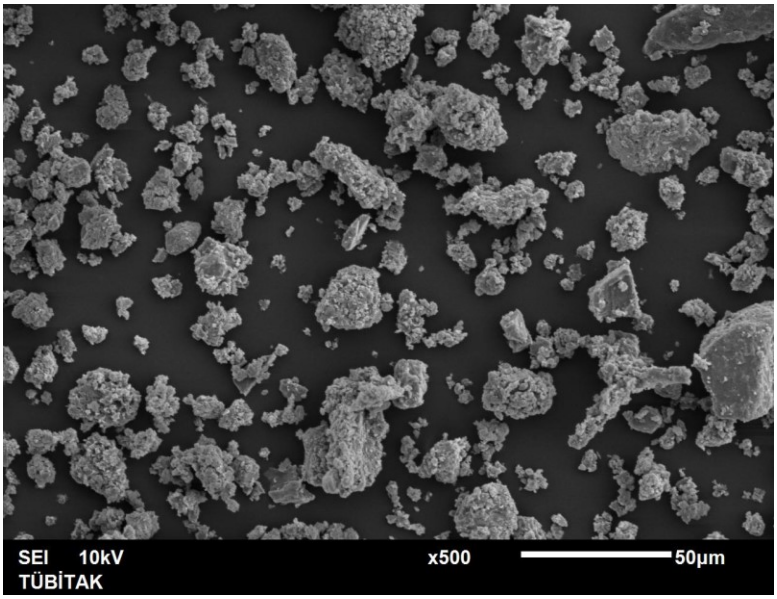

Figure 3.6.2: SEM micrograph Silk Fibroin + Glycerine + 0.50g ESP BF x 500

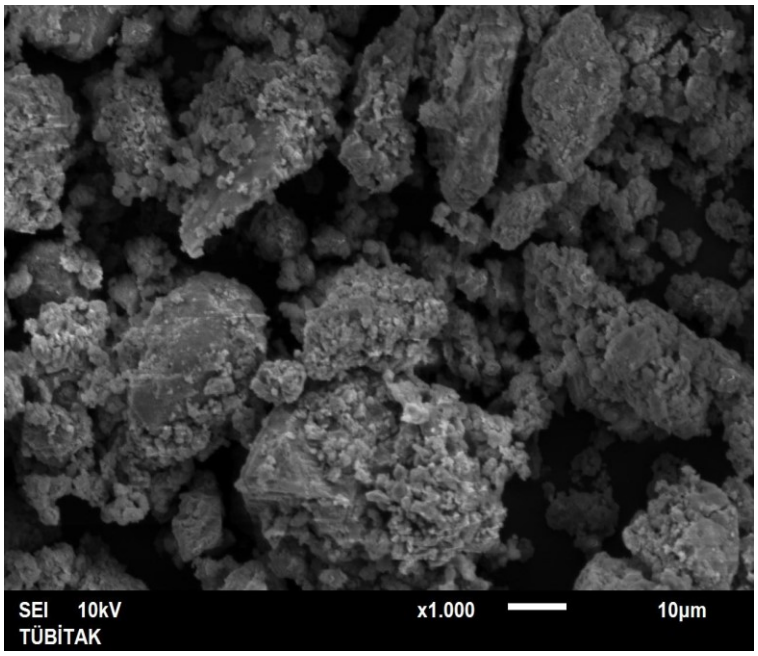

Figure 3.6 3: SEM micrograph Silk Fibroin + Glycerine + 0.5g ESP BF x 1.000

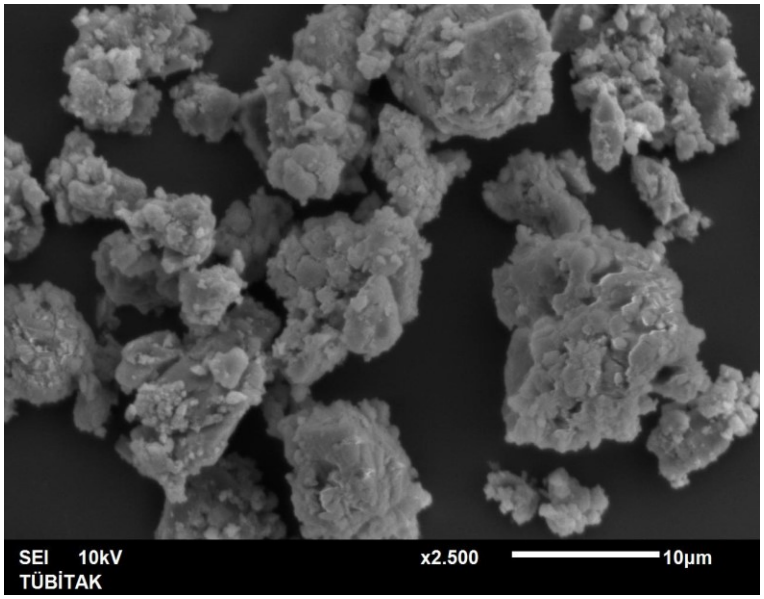

Figure 3.6.4: SEM micrograph Silk Fibroin + Glycerine + 0.5g ESP BF x 2.500

3.3 X – ray Diffraction Analysis (XRD)

The X – ray diffraction analysis was carried out to determine the crystallinity pattern of the silk fibroin films blend, the crystallinity showed its highest peak at $2\theta = 29.42^{o}$ as shown in Figure 3.7 and on Figure 3.7.1 below $2\theta = 29.42^{o}$ displayed characteristic of calcite ($CaCO_3$) and several other peaks at $2\theta = 39.46^{o}$, 47.58^{o}, previous study shown similar peaks of calcite (Eric et al., 1999). Silk fibroin showed its crystallinity between $2\theta = 10^{o}$ to 20^{o} as shown in previous study (Song et al., 2003). The highest peak shows the presence of calcite which increased the crystallinity of the biofilm at $2\theta = 29.42^{o}$, calcite is a carbonate mineral and the most stable form of calcium carbonate (Yoshioka and Kitano, 1985) which is the major composition of an egg shell. Calcium carbonate (Calcite) of hen eggshell has comparable mineral composition to coral, which has been used in orthopedics and dental application for so many years in converted form of hydroxyapatite. The discovery of biomaterials for bone tissue replacement has soared due to the numbers of individual in need for a bone replacement (Li and Tjong, 2011). The biomaterials for bone tissue replacement, should be able to support the weight of the human body before been used as an implant; (Durmus et al., 2008) it should be compatible with several cell features, like: osteointergration, cell migration, cell proliferation and other step required for bone regeneration (Schopper et al., 2003).

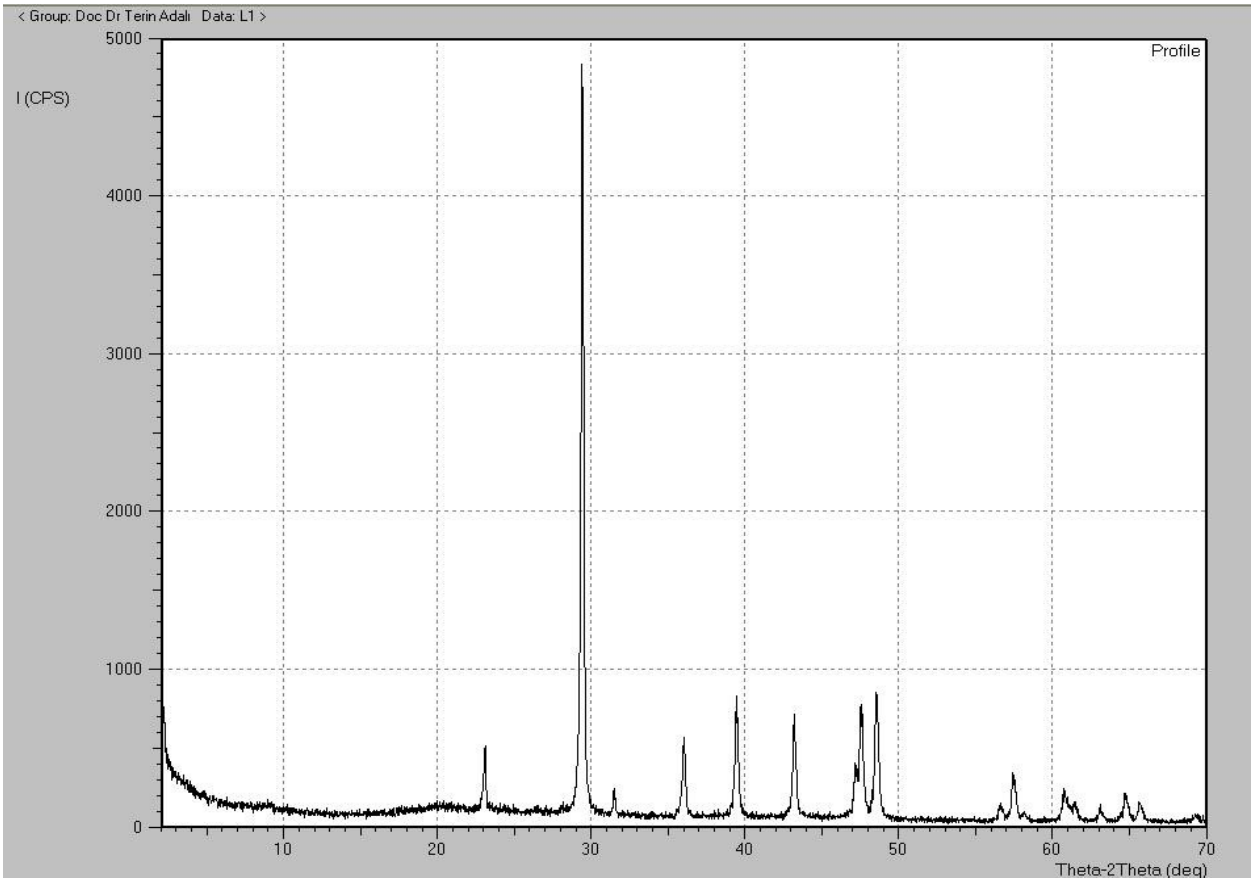

Figure 3.7: XRD diffractogram of Silk fibroin biofilm + glycerine + 0.5g eggshell powder (ESP) BF.

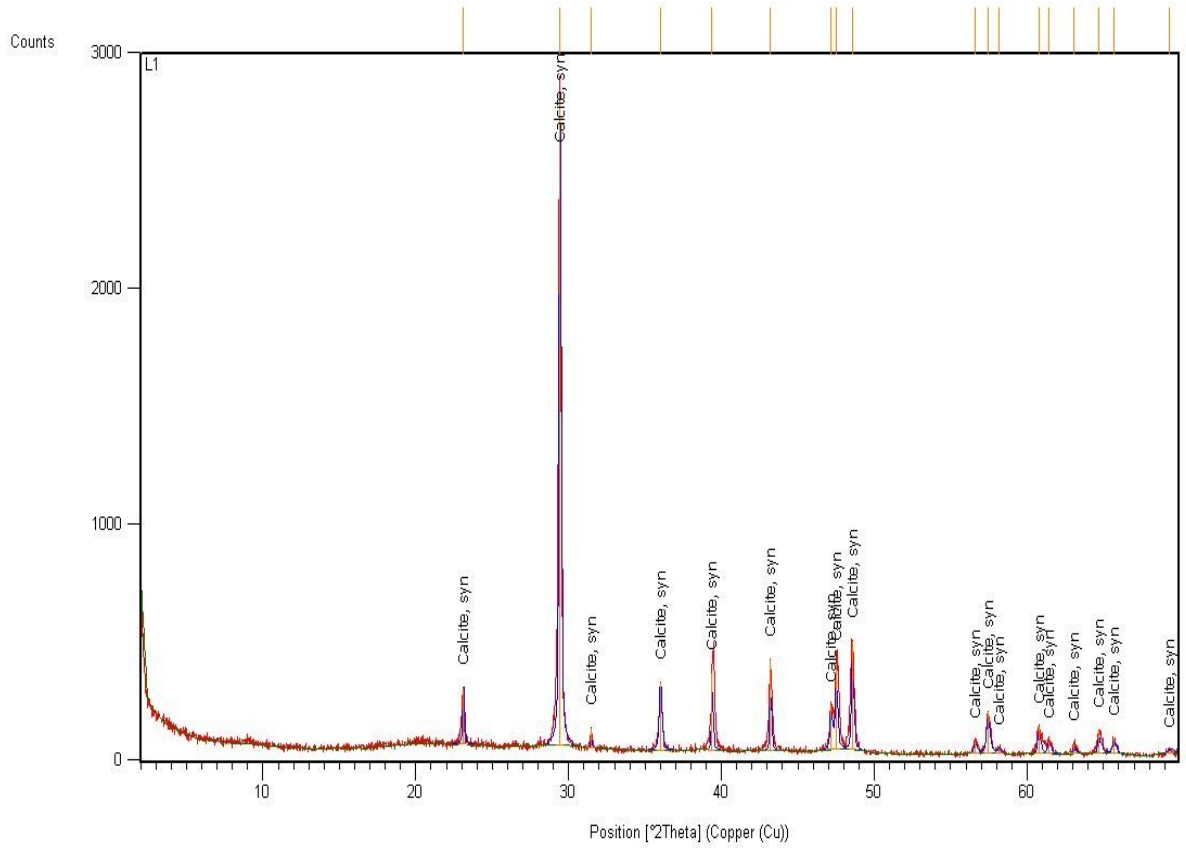

Figure 3.7.1: XRD diffractogram of silk fibroin + glycerine +0.5g Eggshell powder BF, showing crystallinity pattern and chemical composition of the biofilms at different peaks.

3.4 Inductively Coupled Plasma Spectrometry

The inductively coupled plasma spectrometry was carried out to determine the composition of eggshell powder, 34.1% Calcium (Ca^{2+}) and 0.3 % of Phosphate (PO_4^{3-}) was discovered in the eggshell powder.

Calcium (Ca^{2+}) is the most abundant mineral composition in the human body, it is important for the formation of bones and teeth. The muscles, nerves, heart and blood clotting system also need calcium to function. It can be found in dietary supplement, food and medication for preventing calcium deficiency (Ross, et al., 2011). Calcium deficiency can result to osteoporosis (Weak bones), Osteomalacia (Soft bones accompanied with pain), rickets (Softening of bone in young

ones) and several others diseases ranging from reducing the risk of high blood pressure in pregnant women also deceasing the risk of cardiovascular diseases (Bostick et al., 1999).

Phosphate (PO_4^{3-}) is a salt of phosphorus acid, in the human body the phosphorus combine with oxygen to form phosphate. Bone contains 85% of phosphate, while the other percentages are used in the cell to produce energy. The teeth and bones needs phosphate for formation, also that of the nerve tissue and muscles contraction (James, 2016). The quantity of phosphate in the blood affect the level of calcium in the blood, calcium and phosphate in the blood works in opposite proportion, as the quantity of calcium increases the quantity of phosphate deceases. The hormone called parathyroid (PTH) regulate the amount of calcium and phosphate in the blood.

Calcium combines with phosphate to form hydroxyapatite which is the mineral constitute of the bone, the mineral composition of some coral like Sea urchin are natural form of hydroxyapatite (Pegg et al., 1987) Calcium phosphate [$Ca_{10}(PO_4)_6(OH_2)$] is the most functional biocompatible material which is mostly found in the bone. It can be used to coat material for dental and orthopedic applications (Breme et al., 1995); it shows better biological affinity and activity compared to other bioceramics such as zirconia etc. It can be used in several applications which includes; dental implants, percutaneous device, periodontal treatment, bone defect, orthopedics and cranio maxillofacial reconstruction (Doremus, 1992; Best et al., 2008; Vallet-Regi 2001).

CHAPTER 4
CONCLUSION

Silk fibroin swells water, it swells moderately in acidic, neutral pH medium, alkaline medium. In this study by fixing the ratio of silk fibroin and glycerine at constant value, different amounts of eggshell powder were used to synthesize blend biofilm.

Two SF + GLY blend biofilm and four SF + GLY + ESP blend biofilm samples were made, swelling test were carried against five solvents, Ethanol (70%) with pH 7.33, Deionized water with pH 7.0, 0.1M Phosphate buffer solution with pH 7.4, 0.1M Hydrochloric acid solution with pH 1.0 and 0.1 M Sodium hydroxide with pH 12. It was observed that, the presence of glycerine and eggshell powder (ESP) enhanced the swelling properties of the pure silk fibroin in ethanol solution as seen in L1 and LG2. Increasing the ratio of the ESP as in L2, an optimum swelling percentage of 300 were observed at 30 minutes hence, the silk fibroin blends swelled considerably in deionized water. As seen in L3 a reduced, ratio of the ESP in the silk fibroin blend showed a significant swelling percentage in PBS and NaOH solutions while the four SF+GLY+ESP biofilm blend swelled significantly in 0.1M HCl solution which is an acidic media. Further studies should be conducted over longer periods to establish the swelling and biodegradation pattern of this silk fibroin biofilm blend for biomedical application.

The chemical composition of the eggshell powder (ESP) determined by ICP, results showed that of Calcium ($Ca^{2+)}$ and Phosphate (PO_4^{3-}), group's constituent present in eggshell powder (ESP) has proven it to be a good candidate for orthopedic and dental implant application for tissue engineering.

The crystallinity of the silk fibroin biofilm blend determined by XRD showed its highest peak at $2\theta = 29.42^{o}$, Peaks indicated the presence of calcite ($CaCO_3$), several other peaks of calcite are $2\theta = 39.46, 47.58$). Previous study showed similar peaks of calcite ($CaCO_3$) (Eric et al., 1999).

The scanning electron microscopy analysis (SEM) showed rough aggregation, interconnected fiber particles and long flake like particles. The roughness and interconnected characteristics of

the silk fibroin blends indicated that it can support cell proliferation, cell integration and cell attachment, which are characteristic of a biomaterial for bone regeneration application.

In conclusion, the study showed that the silk fibroin biofilm blend swelled in various solvents.

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