IRON (III) ABSORPTION OF

BLENDED SILK FIBROIN MICROPARTICLES

A THESIS SUBMITTED TO THE GRADUATE SCHOOL OF APPLIED SCIENCES

OF

NEAR EAST UNIVERSITY

By LEWIS ODJE GORU

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

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Lewis Odje Goru : Iron (III) Absorption of Blended Silk Fibroin Particles

Approval of the Graduate School of Applied Sciences

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To God Almighty...

ABSTRACT

Silk fibroin blended microparticles were prepared by ionic gelation technique with the aid of a cross-linker Sodium tripolyphosphate (TPP) agent. The microparticles (MPs) were blended with eggshell powder with ratios (0.20g, 0.50g and 0.75g), while the ratio blended with eggshell powder and glycerine with ratios (0.20g, 0.50g and 0.75g). The several ratios of the microparticles were characterized using XRD, SEM, ICP and Iron III ion absorption analysis.

The chemical composition of the eggshell powder investigated using ICP, reviews the presence of calcium (Ca²⁺) and Phosphate (PO₄³⁻) proven it has suitable candidate for biomedical application. The crystallinity of silk fibroin blended microparticles determined by XRD showed various peaks, but at 29.42° it had its highest peaks indicating the presence of Calcite (CaCO₃), Sodium phosphorus oxide hydrate (NaP₃O₁₀(H₂O)₆, Portlandite (Calcium hydroxide (Ca (OH)₂) which are good candidate for bone tissue engineering. Scanning electron microscopy analysis showed smooth homogeneous surface due the silk fibroin, microstructure sparely distributed, rupture due to overlying of microparticles size, shinning surface due to the presence of the phosphate group present in the eggshell. The Iron III ion absorption analysis, reviews that increasing the amount of the eggshell powder in the silk fibroin blended MPs decreases the absorbance behavior, the presence of glycerine causes randomness in absorption behavior of the silk fibroin blended MPs, the presences of glycerine with more amount of eggshell powder causes more randomness in the absorption behavior of the silk fibroin blended MPs.

The result from this study indicates that silk fibroin blended microparticles could be used for different biomedical application.

Keywords: Silk fibroin; Tissue engineering; Absorption; Microparticles; Sodium tripolyphosphate

ÖZET

İpek fibroin mikroküreleri, sodyum triplifosfat varlığında, İyon jelleşme tekniği ile sentezlendi. Toz halinde yumurta kabuğu (0.20 g, 0.50 g, ve 0.75 g) ve / veya gliserin (0.20 g, 0.50 g, ve 0.75 g) ile ipek fibroin, değişik oranlarda karıştırılarak özellikleri farkılıklar gösteren mikroküreler elde edildi. Mikrokürelerin karakterizasyonu, X-Işın difraktometre (XID), Taramalı Elektron Mikroskopu (TEM) ve İndüktif Eşleşmiş Plazma Atomik Emisyon Spektroskopisi (İEPAES) ve Fe (III) absorpsiyon özellikleri kullanılarak yapıldı.

İEPAES sonuçları, yumurta kabuğu kimyasal karışımında kalsiyum (Ca²⁺) ve fosfat (PO₄³⁻) varlığını ispatlamış ve biyomedikal uygulamalarda uygun aday olduğunu ispatlamıştır. XID sonuçları ile yapılan çalışma sonuçları, ipek fibroin mikrokürelerinin kalsiyum karbonat (CaCO₃), sodyum fosfor oksit hidrat (NaP₃O₁₀(H₂O), ve kalsiyum hidroksit (Ca(OH)₂) içerdikleri gözlemlenmiştir. Taramalı elektron mikroskop analizleri, ipek fibroin varlığından dolayı düzgün yüzey içerisine seyrekçe dağıtılmış yumurta kabuğunda mevcut fosfat gruplarını vurguladı. Demir (III) iyon absorpsiyon analizleri sonucunda, toz halindeki yumurta kabuğu varlığının ipek fibroin mikroküreleri içerisindeki varlığının UV absorpsiyonunu azalttığını göstermiştir. Mikroküreler içerisinde ki gliserin varlığı, UV absorbans değerlerinde rastgele farklılıklar göstermiştir.

Bu çalışmanın sonucunda, ipek fibroin –yumurta kabuğu tozu, ipek fibroin-yumurta kabuğu tozu-gliserin karışımlarından, iyonik jelleşme tekniği ile oluşturulan mikrokürelerin demir (III) iyonunu absorbe edebildiğini ve farklı biyomedikal uygulamlarda kullanabilirliğinin olabileceği gözlemlenmiştir.

Anahtar Kelimeler: Ipek Fibroin; Doku mühendisliği; Soğurma; Mikropartiküller; Sodyum tripolifosfat

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LIST OF ABBREVIATIONS

| IG: | Ionic gelation | | |
|---------------------|---|--|--|
| SEM: | Scanning Electron Microscopy | | |
| ICP: | Inductively coupled plasma spectrometry | | |
| XRD: | X - ray Diffraction Analysis | | |
| SF: | Silk fibroin | | |
| MPs: | Microparticles | | |
| ES: | Eggshell | | |
| ESP: | Eggshell powder | | |
| GLY: | Glycerine | | |
| ABS: | Absorbance | | |
| FeCl ₃ : | Iron III chloride solution | | |
| Fe ⁺³ : | Iron III | | |
| Rpm: | Revolution per minute | | |

CHAPTER 1 INTRODUCTION

1.1 General Introduction on Silk Fibroin

Silk fibroin (SF) is a natural polymer processed from different insects and spiders. The best categorized silk is that of the dragline silk from the Nephila clavipes and the cocoon silk from domesticated silk worm Bombyx mori, which had been used in textile-producing companies and production of clinical sutures. It can also be used to prepare scaffold for tissue regeneration (Zhang et al., 2009; Holmes, 2002; Wenk et al., 2011). Bombyx mori consists of a filament core protein silk fibroin (Fibrin) and the glue - like coating composed of non-filamentous hydrophilic protein called sericin, which helps to protect the silk cocoon structure. Sericin makes up twenty to thirty percentages (20 - 30 %) of the silk cocoon mass, it is soluble in both water and alkaline solution. It's separated from the silk cocoons through degumming process. Silk fibroin is characterized by repetitive hydrophobic and hydrophilic peptide sequences (Hofmann et al., 2006); it is composed of heavy and light chain polypeptides of ~ 390 kDa and ~26 kDa respectively, linked by a disulfide bond at the C - terminus of the both subunits. The primary structures of silk fibroin protein are categorized by amino acid roughly 3:2:1 ratio: Glycine (45%), Alanine (30%), and Sericin (12%), its sequence is written as [Gly - Ala - Gly - Ala - Gly - Ser] $_{n}$ as shown in Figure 1.1. The repetitive sequence in hydrophobic residues is dominating the β - sheet structure, forming crystalline regions in silk fibroin fiber and films. The formation of these β - sheet results in insolubility in water, hydrophobic interaction and eventually form into hydrogels.

Several studies have shown that Silk fibroin supports cell attachment and cell proliferation. It is a versatile biomaterial that be conformed to various shapes, structure and dimension. Silk fibroin exhibits notable mechanical properties as well as biocompatibility, proven it as a good biomaterial for tissue engineering. The Fibroin protein is biological material used for artificial skin and several medical applications due to its biodegradability properties (Altman et al., 2003). Silk fibroin was analyzed for several biomedical applications. In an example (Tsubouchi, 1999), silk fibroin based films with a thickness of 10 - 100 µm was developed for quick wound healing

and it could be peeled off causing no harm to the newly formed skin, this application of wound protective membranes made from silk fibroin was examined (Wu et al., 1996). Silk fibroin is believed to be a worthy biomaterial for skeletal tissue engineering because of its excellent oxygen, water vapor permeability and its negligible inflammatory reaction in-vivo (Altman et al., 2003; Silva et al., 2008). Fibroin hydrogel scaffolds were prepared from aqueous solution with 30 % blend of glycerol to enhance in situ bone regeneration (Motta et al., 2004). Microspheres were fabricated from an aqueous solution by laminar jet break - up flow and were examined as platform for controlled drug delivery (Wenk et al., 2008). The assembly process was reported for Silk fibroin particles loaded with small molecule model drugs such as blue, rhodamine B and crystal violet analyzed by aqueous salting out process (Lammel et al., 2010), and it was determined that the elution kinetics of crystal violet is dependent on the secondary structure of the silk fibroin particles. Another approach to design an oral drug delivery system based on accomplishing a silk fibroin to withstand conformational change from random coil to a β - sheet form to impel crystallinity and produce an interpenetrating network (IPN). Various approach was developed, a Silk fibroin based drug delivery system was used: (1) Film and matrix casting with varying composition of silk fibroin, gelatin, glycerin and the model drug and (2) Spray drying of silk fibroin model drug solution.

1.2 Properties of Silk Fibroin

The cocoon of silk fibroin is enclosed in a continuous silk thread whose length can surpass 1 km (Heslot, 1998), it is yellow in colour with flat surface which makes the fibril to reflect at several angles causing its natural shinny appearance, the round end of the fiber are 5 - 10μ m. The structure of silk fibers has a width of 6.5×10^5 nm which contains helically packed Nano fibrils 90 - 170 nm in diameter (Sashina et al., 2006) designed to be resistant to wears and cracks. It's smooth and soft, it losses 20 % of its strength when wet. Silk fiber is a poor conductor of electricity, it is also known to exhibit piezoelectric properties in protein, due to its molecular structure (Fukada et al., 1983).

1.2.1 Biocompatibility

Biocompatibility is the ability of a biomaterial to interact with an appropriate host response in a specific situation in such a way without eliciting any harm or tissue responses to the host tissue (William, 1999). Once a biomaterial is implanted into the host tissue, B cells (Liu et al., 2005), macrophage, dendritic cells (Romai et al., 1966) and mast cell (Zhaoming et al., 1996) from the host tissue immune system are activated and release antibodies and several cytokines targeting antigen epitopes on the biomaterial, it strikes and gets rid of the foreign body by humoral and cellular immune responses. The biocompatibility of silk fibroin materials is very important to consider. Several primary cells and cell lines have been grown successfully on different silk fibroin material to demonstrate a range of biological outcome. However in vitro biocompatibility and degradability of silk fibroin material has been study as an important characteristic of silk fibroin with increasing instability and solubility in-vitro and in-vivo, due to enzymolysis. Long term stability and mechanical property are important for cells that require sufficient time and stiffness to produce their tissue specific matrix, it is important for the silk fibroin material to have low inflammatory responses, low thrombosis formation and low cell toxicity (Bailey, 2013). Therefore it is necessary to adjust the mechanical properties and degradation properties of the silk fibroin material to suit its tissue regeneration.

1.2.2 Biodegradation

Biodegradation behavior of silk fibroin material plays a crucial role in regenerative biomedicine, several *in-vitro* and *in-vivo* studies have shown that the degradation of silk fibroin biomaterial was related to the mode of processing and corresponding content of β - sheet crystalline form (Vepari and Kaplan, 2007).

A biodegradable material can be defined as a material that breaks down *in-vivo*, but with no proof of it dismissed from the body (Vert et al., 1992). Biodegradation of a polymeric material can be attacked by the host biological environment in such as a way the statues of the biomaterial are affected and releases degradation fragments. A material shows preferential surface erosion or bulk erosion depending on its intrinsic properties (water diffusion and degradation rate) and its size (Von-Burkersroda et al., 2002). Such fragments can be moved away from their site of implantation but not necessarily from the body. Biomaterials elicited from the body by natural

pathways or simple filtration resorption *in-vivo* is called bioresorbable biomaterial. Bioresorption is simply reflecting total elimination of the initial biomaterial implanted and any by - product (low molecular weight compounds) Thus, a biodegradable polymer is not certainly biorsesorbable polymer. Bioabsorbable can be dissolved in the body fluids without any molecular degradation; the material can be excreted from the host system.

1.2.3 Structural Properties of Silk Fibroin

The silkworm silk gotten from *Bombyx mori* is made up of fibroin proteins; light chain (~ 26 kDa) and heavy chain (~390 kDa). These core chains are coated with glue like protein called sericin that acts as an envelope to the fibroin fibers together and forms the complex fiber of the cocoon case. The silk fiber is 10 - 25um in diameter, each fiber contains a core protein coated with hydrophilic proteins known as sericin (20 -310 kDa) glue the fiber's core together. Sericin contains 25 to 35 percent of the silk cocoon mass, it causes immunogenic reaction to the body, and therefore they separated by degumming process, boiling the silk cocoon in alkaline solution. There is a kind of proteins that non covalently linked these protein named P25, a 25 kDa glycoprotein (Zhou et al., 2000; Inou et al., 2000; Altman et al., 2003; Tanaka et al., 1999). The modular structure of silk protein comprising of large internal repetitive sequences bound by shorter terminal domains (N- and C- terminal) (Kundu et al., 2013). As shown on Figure 1.1 below the repetitive motif is consisting of sequence of six amino acid residues (Gly - Ala -Gly -Ala - Gly - Ser)n, its sequences forms antiparallel β - sheet leading to the stable mechanical properties of the fiber (Heslot, 1998; He et al., 1999; Asakura et al., 2000; Kim et al., 2005). However the main secondary structures of fibroin are the random coil and amorphous type and antiparallel β - sheet type which is formed through hydrogen bond between adjacent peptide chains (Tsuobi et al., 2001). It has amphiphilic characteristics containing hydrophilic amorphous region and hydrophobic crystalline domains that conforms a beta - sheet secondary structure. The alanine rich regions are accountable for its self-assembly and mechanical stability properties of the biopolymer.



Figure 1.1: Primary structure of *Bombyx mori* silk (Valluzzi et al., 1999)

1.2.4 Mechanical Properties of Silk Fibroin

Silk fibroin has magnificent mechanical properties such as excellent stiffness, strength and toughness as shown in Table1.1. The molecular assembly of the protein in the silk fibers, its hydrophobic domains plays a key role. These domains acquire a great portion of silk fibroin and are accountable for its insolubility, the high fiber strength and thermal stability of the silk fibers which leads to the development of β - sheet secondary structure (Bini et al., 2004). The molecular structures of the silk fibroin fibers are responsible for its biodegradability, bioresorbability, biocompatibility and mechanical properties (Huang et al., 2007).

| Source of Biomaterial | Tenacity | Modulus (GPa) | (%) Strain at |
|-----------------------|------------------------|----------------|---------------|
| | UTS (MPa) | | breakage |
| | | | |
| Bombyx mori silk | 500 (Perez – | 5 – 12 | 19 |
| (with sericin) | Rigueiro et al., 2000) | | |
| | | | |
| Bombyx mori silk | 610 - 690 | 15 – 17 | 4 – 16 |
| (without sericin) | (Perez-Rigueiro | | |
| | et al., 2000) | | |
| | | | |
| Bombyx mori | 740 (Cunniff | 10 | 20 |
| Silk | et al., 1994) | | |
| | | | |
| Collagen | 47 -72 (Pins | 0.0018 - 0.046 | 24 - 68 |
| | et al.,1997) | | |
| | | | |
| Crosslinked Collagen | 47 – 72 (Pins | 0.4 - 0.8 | 12 – 16 |
| | et al., 1997) | | |
| | | | |
| Polylactic acid | 28 - 50 | 1.2 - 3.0 | 2 - 6 |
| | (Engelberg | | |
| | et al., 1991) | | |

Table 1.1: Comparing Bombyx mori Silk and other Polymers Mechanical Properties

1.3 Silk Fibroin Micro particle / Nano particle

Micro and Nano particles have a remarkable high interest in the field of drug delivery due to their ability to deliver any kind of drugs to their target site in the host body for a specific period of time (Vasil ev et al., 2001). Also, nanoparticles and nanoformulations have been used as drug delivery system with great success and nanoparticulate drug delivery system have high potential

for other applications, such as anti - tumor therapy, gene therapy and acquired immune deficiency syndrome (AIDS) therapy, radiotherapy, in the delivery of proteins, antibiotics, virostatics and vaccines as vesicles to pass the blood brain barrier. Nanoparticles provides a huge advantage regarding drug targeting, delivery and release and with their additional potential to combine diagnosis and therapy, emerge as one from the major tools of nanomedicine. Recently, major effort has been studied to develop drug delivery nanospheres for treating various diseases such as cancer, due to the potential for more targeted localization in tumors with active cellular uptake. Micro and nano particles can be fabricated from several synthetic and natural polymers; synthetic polymer for fabricating micro and nano particles are poly (lactic acid) (PLA) and poly (lactic - co -glycolic acid) (PLGA) etc. while natural polymer are chitosan, gelatin, collagen, albumin and the protein silk fibroin.

Silk fibroin protein based micro and nanoparticles provides new innovation for drug delivery due to their distinctive characteristics combined with its biocompatibility, biodegradability, self-assembly, controllable structure and morphology, their tunable drug loading and release properties, which could be regarded as significant advantages compared with the properties of other natural and synthetic polymers (Srihanarn et al., 2011; Wenk et al., 2011). Silk fibroin micro and nano particles could be obtained by several methods: Emulsion - solvent evaporation / extraction methods, phase separation / coacervation, self-assembly, solvent displacement, rapid expansion of supercritical solution, ionic gelation and spray drying (Wang et al., 2010). Selecting each method has an advantage and disadvantage for fabricating micro and nano particles for drug delivery applications.

1.4 Glycerine

Glycerine (Glycerol) also called 1, 2, 3-Propanetriol, glycerin, trihydroxpropane is a sugar alcohol with chemical formulae $C_3H_8O_3$ as shown in Figure 1.2. The three hydroxyl groups result of glycerine hygroscopic nature and solubility in water. It has several characteristics which include: a clear, colorless, hygroscopic, syrupy liquid, high boiling point odorless, sweet taste which is neither harsh nor disagreeable. Glycerine occurs in combined form (triglycerides) in animal fats and vegetable oils (plant form) the fats are obtained via a process called transesterification.



Figure 1.2: Structure of glycerine (Study.com copyright 2013-2015)

1.5 Eggshell

Eggshells are waste from used hatcheries (Hen Bird), home and other sources (Phil and Zhihong, 2009; Amu et al., 2005) and they are available in large quantity to be collected, disposal of egg shell waste can be challenging due to environmental pollution, odor, site of disposal, files and cost of disposal (Phil and Zhihong, 2009). However, it can be refined into other products like fertilizer, artwork, detergent, human and animal nutrition building to produce collagen. Eggshells contain calcium and trace membranes (Phil and Zhihong, 2009; Amu et al., 2005).

The membrane of eggshell consists of collagen as a component; Collagen is extricated and used in various fields such as medicine, biochemical, food, cosmetics and pharmaceutical industries. The egg shell and its membrane make up 10.2% of that total eggshell. The eggshell constitutes of calcified shell and shell membranes including inner and outer membranes as shown in Figure 1.3 below. MacNeil (1997) invented a patent for separating eggshell membranes from eggshell. The organic matter of eggshell and its membrane contains protein as a major constituent of small amounts of carbohydrates and lipids (Burley and Vadehra, 1989). The composition of eggshell is approximately 98.2% calcium carbonate, 0.9% magnesium and 0.9% phosphorous (Phosphate) (Romanoff et al., 1949). Shell membrane contains 69.2 % Protein, 2.7% fat 1.5% moisture and

27.2% ash (MacNeil, 1997). The shell membrane proteins contain approximately 10% of collagen (Froning, 1998).



Figure 1.3: Structure of Egg shell (www.chemistry view.org)

1.6 Importance of Biomaterials from Eggshell in Tissue Engineering.

Calcium carbonate, hydroxyapatites are biomaterials that can be derived from eggshell.

1.6.1 Calcium Carbonate (Calcite)

Calcium carbonate is the abundant form of inorganic calcium in human body, approximately 1.5 - 2.2 % of the body weight, about 99% present in bone. Its primary function is to provide structural integrity and strength for bones, also maintaining normal function of muscle, nerve and secretory organ (Vander et al., 1980; Tunick, 1987). It is found in the calcified portion of eggshell which proceeds into the outer membrane, it can be divided into three layers; the mammillary layer, palisade layer and the vertical crystal layer (Lammie et al., 2005). The mammillary layer (70 μ m thick) which forms the inner most layer of the calcified portion of the

eggshell penetrates the outer membrane by means of numerous carbonate cones. The beginning of the formation of calcium carbonate crystals take place at the mammillary knobs, which are organic cores deposited during the egg formation (Lammie et al., 2005) the calcite is the most stable form of calcium carbonate; it forms elongated structures called columns, palisades, or crystallite

The calcium carbonate in hen eggshell has similar mineral composition of that of coral. The calcium carbonate from eggshell and coral has been used to treat bone defect in dentistry and orthopedics due to its excellent characteristics such as cell proliferation, osteointergration, cell migration and other important step in bone regeneration in a converted form as hydroxyapatite and in its natural aragonite form (Durmus et al., 2008; Dupoirieux et al., 1994; Schopper et al., 2003).

1.6.2 Hydroxyapatite

Hydroxyapatite composed of 70% of the bone is a bioceramics commonly used for bone implantation, due to its excellent bioactivities. The used of calcium phosphate salts to successfully replace and grow bone tissue are known for many years since it was discovered, calcium phosphate plays a major role in the inorganic phase of hard tissues, such as bones and teeth, which is important to medical researcher to consider this salt as a method of improving healing in bone regeneration (De Groot, 1993). It is formed into 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). Calcite is the most stable form of calcium carbonate. Apart from eggshell, there are other natural sources of calcium carbonate and hydroxyapatite such as bovine bones and fish bones, cuttle fish shells, oyster and corals (Sanosh, 2009). A large presence of calcite contributes to a better mechanical property and strength of eggshell based hydroxyapatite.

1.7 Aim / Objective of Thesis

- 1. To fabricate microparticles using ionic gelation method.
- 2. To study the morphology and particle sizes of the microparticles.
- 3. Fabricating better quality of microparticles by blending silk fibroin with eggshell powder and glycerine inventing new materials for tissue engineering applications.
- Using iron chloride (FeCl₃) as a solvent and an UV visible spectrophotometer for absorption of Fe⁺³ ions analyzes.
- The blended silk fibroin MPs were investigated in respect of its sensitivity, constitute, shape, size, structure, porosity, dimension and further analytical procedure such as; Scanning electron microscopy (SEM), X- ray diffraction analysis (XRD), Induced coupled plasma spectroscopy (ICP) and Iron III ion absorption.

CHAPTER TWO MATERIALS AND METHODS

2.1 Materials used

Bombyx mori cocoons were purchased from store at Büyük Han (Great Inn) Girne kapisi Lefkosa, Turkish Republic of Northern Cyprus with further purification procedure to produce aqueous silk fibroin solution. The chemicals used for this research are of high standard purchased from a reliable company. Sodium carbonate (Na₂CO₃) used for degumming / purification process were purchased from Sigma – Aldrich, Calcium chloride (CaCl₂) used for the dissolution process were purchased also from Sigma – Aldrich, Ethanol used for the preparation of the electrolyte solution, Deionized water got from Near East University, Hospital Lefkosa, Dialysis membrane from Sigma – Aldrich used during the dialysis process to enable exchange of ions. Sodium triphosphate pentabasic (Na₅P₃O₁₀) were gotten from Sigma – Aldrich, used during the formation of the micro particles. Iron chloride used for the absorption analysis was gotten from Sigma - Aldrich, eggshell was obtained from the market, glycerine was obtained from a reliable Pharmacy to improve the micro particles, Glass wares such as conical flask, measuring cylinder, beaker were used all through the research, they were properly washed and arranged in the oven to dry.

2.2 Methods

Several procedures are carried out to obtain pure silk fibroin aqueous solution.

2.2.1 Silk Fibroin Purification Process

Purification of silk fibroin requires several procedures namely:

2.2.1.1 Cutting of Bombyx mori Cocoons

Dried *Bombyx mori* cocoons as shown on Figure 2.3 A is shaped into small squares with a pair of sterilized scissor on preparation for degumming procedure.

2.2.1.2 Degumming

Degumming is a procedure of boiling the small pieces of silk cocoon in an aqueous solution to separate sericin protein from the silk fiber structure (Bradford, 1976).

The aqueous solution is obtained by measuring 5.3 g of Sodium carbonate (Na_2CO_3) with a weighing balance; it is dissolved in 500 ml of deionized water to form 0.1 M of Sodium carbonate (Na_2CO_3) solution. The silk cocoons were poured into a conical flask that contains 0.1M of Sodium carbonate (Na_2CO_3) solution 1g/ 100ml, a magnetic bar was placed in the conical flask and the conical flask was placed on a magnetic stirrer at 70^oC to a speed of 1.5 rpm as shown on Figure 2.1 below. The procedure was repeated three times; each session comprises of three hours, the fibers were washed with deionized water thoroughly to ensure the total removal of serine protein from the fiber and replacing 0.1M of Sodium carbonate (Na_2CO_3) solution at the end of each session. Then it is left to dry overnight to obtain silk fiber, the linted silk fiber obtained are shown on Figure 2.3 C.



Figure 2.1: Degumming procedure

2.2.1.3 Dissolution

This procedure involves the transformation of the silk fiber using a chemical procedure to break down its long polypeptide chains into smaller or shorter chain to obtain its aqueous solution. This process involves the use of an electrolyte solution, it is prepared by measuring 29.15 ml of ethanol (C_2H_5OH), 36 ml of deionized water (H_2O) and 27.75 g of calcium chloride ($CaCl_2$) mixed in a beaker, a magnetic bar is placed in the beaker and the beaker is placed on the magnetic stirrer at 70°C at 1 rpm to obtain a pure electrolyte solution as shown in Figure 2.3A below. The silk fibers were dropped at intervals into the pure electrolyte solution. This process continued until the fiber dissolved completely as shown in Figure 2.3 B below.



Figure 2.2: Dissolution procedure

2.2.1.4 Dialysis of Aqueous Silk Fibroin

The dialysis procedure is the last stage in the purification of silk fibroin which involves removal of ions within the solution obtained during the dissolution procedure. A carboxymethyl cellulose semi permeable membrane tube is used which enables exchange of ions, the silk fibroin aqueous solution is poured into the carboxymethyl cellulose semi permeable membrane tube carefully and putting the tube in a 5000 ml beaker filled with deionized water, a magnetic bar is placed in the beaker and the beaker is placed on the magnetic stirrer at 0° C to a speed of 1.5 rpm as shown in

Figure 2.3 E. This procedure was repeated three times; each for three hours, with a change of the deionized water at the end to obtain a pure silk fibroin aqueous solution.



Figure 2.3: Schematic diagram of Silk Fibroin synthesis

2.2.2 Eggshell Powder Preparation

Eggshell was obtained from the market, it was washed properly with deionized water and left to dry over night at room temperature in the laboratory. After drying it was processed into powdered form as shown on Figure 2.4 B.



Figure 2.4: Eggshell and Eggshell powder

2.2.3 Microparticles Preparation process

Silk fibroin microparticles can be fabricated using several methods; emulsion-solvent evaporation/extraction method, phase separation, self-assembly, solvent displacement, rapid expansion of superficial solution, spray drying, sieving method and ionic gelation method (Wang et al., 2010), each method has its advantage and disadvantage, therefore when selecting any method for the fabrication of microparticles their advantages and disadvantages should be considered.

In this study, microparticles were prepared by ionotropic gelation or ionic gelation method which is based on the ability of polyelectrolytes to cross-link in the presence of counter ions as shown in Figure 2.5, Sodium triphosphate pentabasic ($Na_5P_3O_{10}$) is used as the counter ion to fabricate the microparticles (Poonam et al., 2012). 0.1M of Sodium triphosphate pentabasic ($Na_5P_3O_{10}$) was prepared by measuring 7.36g in a weighing balance machine and dissolve it in 200ml of deionized water. In this study, two sets of microparticles were fabricated; the first set blend with the bioceramic (eggshell powder) with silk fibroin and the second set blend with the bioceramic (eggshell powder), silk fibroin and glycerin to increase the quality and quantity of the microparticles, the ratios are shown in Table 2.1.

Polyelectyte Solution

(Silk fibroin (+) + Eggshell)

 \downarrow

Added drop carefully under magnetic stirrer using pipette

 \downarrow

Counter ion solution

(Sodium propolyphosphate (-))

 \downarrow

Microparticles Formation.

Figure 2.5: Illustrating the basic step in the process of IG. (Poonam et al., 2012)

| Study | Silk fibroin (SF) | Glycerine | Eggshell | 0.1M TPP | |
|---------|-------------------|-----------|----------|----------|--|
| Samples | (ml) | (Drops) | Powder | Solution | |
| | | | (grams) | (ml) | |
| | | | | | |
| S1 | 2ml | | 0.20g | 50ml | |
| S2 | 2ml | | 0.50g | 50ml | |
| S3 | 2ml | | 0.75g | 50ml | |
| | | | | | |
| | | | | | |
| | | | | | |
| SG1 | 2ml | 20 drops | 0.20g | 50ml | |
| SG2 | 2ml | 20 drops | 0.50g | 50ml | |
| SG3 | 2ml | 20 drops | 0.75g | 50ml | |
| | | | | | |

Table 2.1: Samples with Silk Fibroin blended microparticles

The three different ratios of eggshell powder (0.20g, 0.50g and 0.75g) were blend with 2ml of silk fibroin and 20 drops of glycerine, the other three different ratios of eggshell powder (0.20g, 0.50g and 0.75g) were blend with 2ml of silk fibroin. As displayed in Figure 2.6 below they were placed separately in different beakers on a magnetic stirrer for proper blending with a magnetic bar in it. 50 ml each of 0.1M Sodium triphosphate pentabasic (Na₅P₃O₁₀) was poured into a separate beaker, the mixture of one of the ratios was taken with a pipette and dropped carefully, it was carried out for every ratio, every drop gave a visible cloud which settled at the bottom of the beaker. The micro particles was filtered after 24 hours and was dried at room temperature in the laboratory over night. After drying, it was grinded into powder and poured carefully in a plastic vial and labeled carefully. The labeled samples are free for microbes before further characterization.



Figure 2.6: Illustrating the basic procedure in fabricating SF blended MPs

2.2.4 Iron III Absorption Assay

This analysis was carried out using T60 UV – Visible spectrophotometer machine at 356 λ .

UV- visible spectroscopy is a device used in analytical chemistry for quantitative determination of different analytes like transit metal ions, highly linked organic compound and some biological macromolecules. Solid, liquid and gasses can be analyzed.

Different ratio (0.025M, 0.05M, 0.075M and 0.1M) of Iron III chloride (FeCl₃) was measured respectively, poured into a beaker containing pure water, a magnetic bar was dropped inside the beaker and placed on a magnetic stirrer at 0°C at a speed of 2.0 rpm for 4 hours as shon in Figure 2.7 A. After dissolving the liquid, it was poured into a bottle, the bottle was wrapped with foil paper to prevent solidification of the liquid, reaction with light and to keep it at room temperature as shown in Figure 2.7 B.

For the UV - visible spectrophotometer measurement, pure water was poured into a cuvette to rinse it thoroughly, the water was poured away, pure water of about 2ml was poured into the cuvette, the transparent side of the plastic cuvette was wiped to avoid stain or finger print to

allow passage of the UV light through the cuvette. The lid of the UV – visible spectrophotometer was opened, the cuvette containing the pure water was placed in the sample chamber, the wavelength was set at 356 λ and the auto zero bottom was click followed by the start bottom. 0.1g of the MPs was measured, 10ml of any of the ratios of iron III chloride (FeCl₃) was measured and poured into a beaker as shown in Figure 2.7 C, the MPs and iron III chloride solution (FeCl₃) were mixed together at interval of (5mins, 15mins, 30mins, 45mins, 60mins and 90mins), measure 1ml out of the mixture poured it into the cuvette as shown in Figure 2.7 D, open the lid, placed it in the sample chamber, click on the start bottom, when the machine beeps the absorbance result will be displayed on the screen, the result were written down and the process continued until the end with the different samples of the MPs and FeCl₃ ratios.



Figure 2.7: Schematic diagram showing the process of Iron III absorption analysis

2.2.6 Samples Characterization

2.2.6.1 X – ray Diffraction Analysis (XRD)

X-ray powder diffraction is an analytical technique mainly used for phase identification of crystalline material, providing information on cell unit dimensions. The analyzed material is finely ground and placed in the sample holder (Bish and Post 1989). X – Ray diffraction analysis was carried out in TUBIKAT Marmara Research Institution Istanbul Turkey, making use of shimadzu XRD 600 which scans at 2° / minutes within a region of 20 which results in diffraction curves been released.

2.2.6.2 Scanning Electron Microscopy (SEM)

Scanning electron microscopy analysis was carried out in TUBIKAT Marmara Research Institute Istanbul Turkey using SEM- Jsm- 6510 model microscope at an acceleration voltage of 10kV.

Scanning electron microscope is a technique for investigating the structural surface of a biological sample (Duckette and Ligrone, 1995; Minoura et al., 1995; Motta et al., 1994) with a beam of electron focused on the sample; the samples are sputtering coated in gold to prevent electrostatic charges.

2.2.6.3 Inductively Coupled Plasma Spectrometry (ICPS)

Inductively coupled plasma spectrometry was carried out in TUBIKAT Marmara Research Institute Istanbul Turkey using NexON350Q model spectrometry; with a flame temperature range from 6000 - 10000.

The inductively coupled plasma spectrometry is a device used to analyzed or determine the composition of a material such as polymer, metals, plastics, liquid etc. (Evans, 2016). It is a type of emission spectroscopy that uses inductively coupled plasma to release excited atoms and ions which produce electromagnetic radiation at wavelength identify a particular element (Stefansson et al., 2007; Mermet, 2005).

CHAPTER THREE RESULTS AND DISCUSSION

3.1 Iron III Absorption Assay.

Various molarity of iron III chloride (FeCl₃) solution were made (0.025M, 0.05M, 0.075M, 0.1M), 0.1g of the MPs were poured into10ml of each of the moles of FeCl₃ solution, 1ml were taking at intervals and using a UV- visible spectrophotometer to check it absorbance at 356 λ .

The purpose of the absorption analysis is to determine absorbance behavior of the silk fibroin blended MPs using FeCl₃ solution.

| Time | 0.025M of | 0.05M of | 0.075M of | 0.1M of |
|--------|-------------------|-------------------|-------------------|-------------------|
| | FeCl ₃ | FeCl ₃ | FeCl ₃ | FeCl ₃ |
| 5mins | 0.136 | 0.075 | 0.778 | 0.485 |
| 15mins | 0.010 | 0.117 | 0.863 | 0.451 |
| 30mins | 0.017 | 0.249 | 0.846 | 0.340 |
| 45mins | 0.067 | 0.243 | 0.447 | 0.155 |
| 60mins | 0.337 | 0.232 | 0.475 | 0.104 |
| 90mins | 0.146 | 0.213 | 0.515 | 0.286 |

| Table 3.1: $Fe^{+3}A$ | Absorption behavior | of SF &0.20g | ESP MPs (S | 51) |
|------------------------------|---------------------|--------------|------------|-----|
|------------------------------|---------------------|--------------|------------|-----|

The table above shows the absorbance of SF & 0.20g ESP MPs (S1) in several molarities of FeCl₃ solution with different time intervals from 5 minutes to 90 minutes. The data's on Table 3.1 above are explained graphically on Figure 3.1.



Figure 3.1: Graph of SF & 0.20g ESP MPs (S1) Absorption

As observed from Figure 3.1, the absorbance behavior of silk fibroin MPs blended with eggshell powder in various molarities of FeCl₃ solution; in 0.025M of FeCl₃ solution, it absorbed randomly in a very low value, in 0.05M of FeCl₃ solution, it starts to absorb little initially, increased from 15 minutes and starts decreasing from 45 minutes down in a step-like pattern, in 0.075M of FeCl₃ solution, it starts to absorb at very high value, with random increase and decrease, from 45 minutes it absorbed upward in a step-like pattern, in 0.1M of FeCl₃ solution, it absorbed randomly and starts to decrease from 30 minutes and increased at 90minutes.

Nevertheless, silk fibroin MPs blended with ESP (0.20g) absorbed in the various molarities of Iron III chloride solution, it absorbed efficiently in 0.075M and 0.1M of FeCl₃ as shown on Table 3.1 and Figure 3.1 above. This indicates the higher the concentration of FeCl₃ the more efficient the silk fibroin blended MPs absorption behavior in lower amount of eggshell powder.

| Time | 0.025M of | 0.05M of | 0.075M of | 0.1M of |
|--------|-------------------|-------------------|-------------------|-------------------|
| | FeCl ₃ | FeCl ₃ | FeCl ₃ | FeCl ₃ |
| 5mims | 0.081 | 0.174 | 0.330 | 0.398 |
| 15mins | 0.066 | 0.117 | 0.103 | 0.100 |
| 30mins | 0.145 | 0.164 | 0.232 | 0.062 |
| 45mins | 0.179 | 0.154 | 0.122 | 0.316 |
| 60mins | 0.205 | 0.236 | 0.080 | 0.216 |
| 90mins | 0.427 | 0.426 | 0.482 | 0.389 |

Table 3.2: Fe⁺³ Absorption behavior SF, 0.20g ESP & GLY MPs (SG1)

The table above shows the absorbance of SF, ESP 0.20g & GLY (SG1) in several molarities of FeCl₃ solution with different time intervals from 5 minutes to 90 minutes. The data's on Table 3.2 above are explained graphically on Figure 3.2 below.



Figure 3.2: Graph of SF, 0.20g ESP & GLY MPs (SG1) absorption

As observed from Figure 3.2, the absorbance behavior of silk fibroin MPs blended with 0.20g ESP and glycerine in various molarities of FeCl₃ solution; in 0.025M of FeCl₃ solution, it starts to absorb little at 5 minutes, decreases at 15 minutes, at 30 minutes it increases gradually until 90 minutes, in 0.050M of FeCl₃ solution, it starts to absorb at a minimal values at 5 minutes, decreased, increased, from 45 minutes, it starts to increase until 90 minutes, in 0.075M of FeCl₃ solution, it starts to absorb initially at a moderate value, with a random increase and decrease, In 0.1M of FeCl₃ solution, it starts to absorb initially at a moderate value, with a random increase and decrease and decrease.

Nevertheless, silk fibroin MPs blended with 0.20g ESP and GLY absorbance in various molarities of FeCl₃ solution, but 0.025M and 0.05M absorbed efficiently as shown in Table 3.2 and Figure 3.2, also the blended MPs experience a higher absorbance at 90 minutes in all the molarities of FeCl₃ solution.

| Time | 0.025M of | 0.05M of | 0.075M of | 0.1M of |
|--------|-------------------|-------------------|-------------------|-------------------|
| | FeCl ₃ | FeCl ₃ | FeCl ₃ | FeCl ₃ |
| 5mins | 0.123 | 0.066 | 0.585 | 0.045 |
| 15mins | 0.298 | 0.310 | 0.068 | 0.189 |
| 30mins | 0.068 | 0.370 | 0.109 | 0.379 |
| 45mins | 0.044 | 0.406 | 0.206 | 0.494 |
| 60mins | 0.107 | 0.420 | 0.061 | 0.506 |
| 90mins | 0.074 | 0.488 | 0.338 | 0.487 |

Table 3.3: Fe⁺³Absorption behavior of SF &075g ESP MPs (S3)

The table above shows the absorbance of SF & 0.75g ESP MPs (S3) in several molarities of FeCl₃ solution with different time intervals from 5 minutes to 90 minutes. The data's on Table 3.3 above are explained graphically on Figure 3.3.



Figure 3.3: Graph of SF & 0.75g ESP MPs absorbance

As observed from Figure 3.3, the absorbance behavior of silk fibroin MPs blended with 0.75g ESP in various molarity of FeCl₃ solution; In 0.025M of FeCl₃ solution, it starts to absorb at a minimal value, it starts to experience random increase and decrease, in 0.050M of FeCl₃ solution, it starts to absorb at a minimal value, it experience a gradually increase from 10 minutes until 90 minutes, in 0.075M of FeCl₃ solution, it starts to absorb at a high value, it experience a random increase and decrease absorbance from 15 minutes until 90 minutes, in 0.1M of FeCl₃ solution, it starts to absorb at a minimal value, it starts to experience a random increase and decrease in its starts to absorb at a minimal value, it starts to experience a random increase and decrease in its starts to absorb at a minimal value, it starts to experience a random increase and decrease in its absorbance.

Nevertheless, silk fibroin MPs blended with 0.75g ESP absorbed in the various molarities of $FeCl_3$ solution, but absorbed efficiently in 0.050M of $FeCl_3$ solution as shown on Table 3.3 and Figure 3.3. The results indicates the more the eggshell powder the less efficient its absorbance in $FeCl_3$ solution.

| Time | 0.025M of | 0.05M of | 0.075M of | 0.1M of |
|--------|-------------------|-------------------|-------------------|-------------------|
| | FeCl ₃ | FeCl ₃ | FeCl ₃ | FeCl ₃ |
| 5mins | 0.079 | 0.093 | 0.050 | 0.626 |
| 15mins | 0.044 | 0.153 | 0.096 | 1.342 |
| 30mins | 0.068 | 0.262 | 0.136 | 1.588 |
| 45mins | 0.046 | 0.294 | 0.210 | 0.406 |
| 60mins | 0.073 | 0.331 | 0.154 | 0.463 |
| 90mins | 0.156 | 0.245 | 0.076 | 0.347 |

Table 3.4: Fe⁺³Absorption behavior of 0.75g ESP + GLY (SG3)

The table above shows the absorbance of SF, 0.75g ESP & GLY MPs (SG3) in several molarities of FeCl3 solution with different time interval from 5 minutes to 90 minutes. The data's on Table 3.4 above are explained graphically on Figure 3.4.



Figure 3.4: Graph of SF, 0.75g ESP & GLY (SG3) absorption

As observed from Figure 3.4 above the absorbance behavior of silk fibroin MPs blended with 0.75g ESP and glycerine in various molarities of FeCl₃ solution; in 0.025M, 0.050M and 0.075M of FeCl₃ solution, starts to absorb at a minimal value and starts to experience random increase and decrease in its absorbance, in 0.1M of FeCl₃ solution starts to absorb at a high value, increases until 30 minutes, it starts to experience a random absorption from 45 minutes until 90 minutes.

Nevertheless, silk fibroin MPs blended with 0.75g of ESP and glycerine absorbs in the various molarities of FeCl₃ solution, but absorbed efficiently in 0.1M of FeCl₃ solution as shown on Table 3.4 and Figure 3.4.

3.2 X-Ray diffraction analysis

The X- ray diffraction analysis was carried out to characterize the structural crystallinity pattern of the silk fibroin micro particles blends. The crystallinity of G1sample as displayed on figure 3.5 below, shows its highest peaks at $2\theta = 29.42^{\circ}$, on Figure 3.5.1 below the highest peaks at 2θ = 29.42° exhibits characteristics of calcite (CaCO₃) and sodium phosphorous oxide hydrate (Na₅P₃O₁₀ (H₂O)₆. Calcite (CaCO₃) due to the presence of the eggshell powder in the silk fibroin blended MPs, that of the sodium phosphorous oxide hydrate (Na₅P₃O₁₀ (H₂O)₆ is as a result of the sodium triphosphate pentabasic (Na₅P₃O₁₀) used during the fabrication of the silk fibroin blended MPs. Several peaks of calcite (CaCO₃) and sodium phosphorus oxide hydrate (Na₅P₃O₁₀ $(H_2O)_6$ was displayed on Figure 3.5.1 at $2\theta = 23.12^\circ$, $2\theta = 39.54^\circ$ and others. Similar peaks of calcite (CaCO₃) at $2\theta = 29.42^{\circ}$ as shown on figure 3.5.1, was displayed on a previous study of Boniface et al (2015). At $2\theta = 23.12^{\circ}$ and $2\theta = 34.10^{\circ}$ on Figure 3.5.1 displayed sodium phosphorous oxide hydrate (Na₅P₃O₁₀ (H₂O)₆ and portlandite (Calcium hydroxide) (Ca (OH) ₂), previous study of Eric et al. (1999) has similar peaks displaying portlandite (Calcium hydroxide) (Ca (OH) 2) on Figure 3.5.1 Calcite (CaCO3) sodium phosphorous oxide hydrate (Na₅P₃O₁₀ $(H_2O)_6$ and portlandite (Calcium hydroxide) (Ca (OH) ₂) were all displayed at $2\theta = 47.06^{\circ}$ on Figure 3.5.1 below.

Calcium carbonate is the most stable form of calcium carbonate in nature (Yoshioka and Kitano, 1985). It's constituent in eggshell powder is about 95% of the total mass of eggshell (Nys and



Gautron, 2007). Portlandite is a rare oxide mineral, occurs naturally in form of calcium hydroxide (Ca (OH) $_2$). It is the calcium analogue of brucite (Mg (OH) $_2$) (Palache et al., 1944).

Figure 3.5: XRD diffractogram of SF + GLY + 0.20g ESP MPs



Figure 3.6: XRD diffractogram showing SF + GLY + 0.5g ESP MPs showing crystallinity pattern and chemical composition of the MPs at different peaks

3.3 Scanning Electron Microscopy

Scanning electron microscopy is a technique that describes the shape, size, surface and morphology of silk fibroin micro particles blends. The figures below describe the morphology of G1 sample carried out under electron microscope at 10Kv at different µm and magnification for a better description and understanding. In Figure 3.6 and 36.1, it showed smooth, homogeneous surface due to the presences of silk fibroin, rough network of microstructure of the micro particles due to the presences of glycerin and eggshell powder. The rupture in figure 3.7.1 are due to presence of the phosphate group present in the eggshell powder. In Figure 3.7.2, 3.7.3, and 3.7.4 it shows small aggregation sparely distributed on the surface.



Figure 3.7: SEM micrograph SF +GLY + 0.20g ESP MPs x100



Figure 3.7.1: SEM micrograph SF +GLY + 0.20g ESP x250



Figure 3.7.2: SEM micrograph SF + GLY + 0.20g ESP MPs x500



Figure 3.7.3: SEM micrograph SF + GLY + 0.20g ESP MPs x1.000



Figure 3.7.4: SEM micrograph SF + GLY + 0.20g ESP MPs x2.500

3.4 Inductively Coupled Plasma Spectrometry

Inductively coupled plasma spectrometry is an analysis carried to identify the composition of the eggshell powder, Calcium (Ca²⁺) 34. 1% and Phosphate (PO₄³⁻) 0.3 % were identified in the eggshell powder sample.

Calcium performs a key role in a vast range of biological function, in skeletal mineralization such as teeth and bone formation, muscles and nerve formation etc. Calcium is the fifth most abundant element in the human body, which is only available to the human via dietary products. Osteoporosis is known as weak or porous bone disorder associated with fracture of the hips, wrist and other bone part. Proper intake of calcium supplement and diet reduces the risks of osteoporosis (Riggs and Melton 1995). Regular intake of calcium reduces hypertension and reduces the risk of increased blood pressure (McCarron and Reusser 1999; Allender et al., 1996).

Phosphate (PO_4^{3-}) is an inorganic chemical and a salt of the phosphoric acid, in the human body phosphorus combine with oxygen to form phosphate. Phosphate is the structural component of

the bone and teeth in every living begin biological system. This structure composed of crystalline calcium phosphate in form hydroxyapatite.

Calcium phosphate forms hydroxyapatite, which form the material properties of the bone (Wang et al., 2006). The mineral composition of calcium phosphate $[Ca_{10} (PO_4)_6 (OH_2)]$ can be formed in some bioceramic E.g. Coral, sea urchin as hydroxyapatite (Pegg et al., 1987). Calcium and phosphate combine in various ground process in the skeletal system, they metabolized works with osteoblasts, osteocytes and extracellular matrix proteins (Qin et al., 2004) to mineralize osteoid as it is deposited. Calcium phosphate can be used as biomaterial in dental implants, orthopedics and bone defects (Doremus, 1992; Best et al., 2008; Vallet – Regi, 2001).

Chapter 4 Conclusion

Silk fibroin has showed great interest in biomedical application due to its versatile properties that can be conformed into various shape, structure and dimension. It has a good mechanical properties and it has proven to be biocompatible in host tissue, when blended with other material such as: polymers, bioceramics etc.

In this study SF - ESP - GLY MPs and SF - ESP MPs blends were fabricated with ionic gelation technique with sodium triopolyphosphate (TPP) to produce microparticles with better mechanical properties for biomedical applications. The microparticles were made into two categories, each with the three different ratios; the silk fibroin microparticles blended with eggshell powder with ratios (0.20g, 0.50g and 0.75g) and the silk fibroin microparticles blended with 20 drops of glycerine and eggshell powder with ratios, (0.20g, 0.50g, and 0.75g). The SF – ESP – GLY MPs produce more microparticles than that of the SF – ESP MPs. Several analysis were carried out to investigate the silk fibroin blended microparticles such as; Iron III ion (FeCl₃) absorption assay, scanning electron microscopy (SEM), X- ray diffraction analysis (XRD) and induced coupled plasma spectroscopy. The iron III ion analysis showed good absorption behavior with the silk fibroin blended MPs; by increasing the amount of eggshell powder (S3) the absorbance rate has been decreased of silk fibroin blending MPs, S3 absorbed better in 0.050M of FeCl₃ solution at optimal absorbance of 0.488 at 90 minutes, unlike S1, it absorbed best in 0.075M and 0.1M at optimal absorbance respectively, 0.863 at 15 minutes and 0.485 at 5 minutes. The presences of glycerine causes more randomness in absorbance of the silk fibroin blended MPs as in SG1 and SG3; SG1 absorbed best in 0.025M and 0.050M of FeCl₃ solution with optimal absorbance respectively 0.427 and 0.426 at 90 minutes, SG3 absorbed best in 0.1M of FeCl₃ solution with it optimal absorbance value 1.588 at 30 minutes. In a nutshell the amount of eggshell powder affects the absorbance of the silk fibroin blended MPs, further studies should be carried out with more samples for longer duration for a better understanding of the absorbance behavior of the silk fibroin blended MPs.

In conclusion, the result showed that for further characterization SF - ESP - GLY - MPs and SF - ESP MPs are suitable candidates for biomedical applications such as bone filler (Defect and

voids) and dentistry filler, further studies should be carried for a better understanding of its applications.

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