ANTIMICROBIAL ACTIVITY AND HEMOCOMPATIBILITY OF CHITOSAN COATED SILK FIBROIN MICRO-FIBERS

A THESIS SUBMITTED TO THE GRADUATE

SCHOOL OF APPLIED SCIENCES

OF

NEAR EAST UNIVERSITY

By

PWADUBASHIYI COSTON PWAVODI

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

NICOSIA, 2017

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I hereby declare that the research work contained in this thesis on the topic: Effect of Antimicrobial and Hemocompatibility Activity on Chitosan Braided Silk fibers is original to the best of our knowledge and has not yet been submitted whether in part or in full for a degree and I also hereby declare that all the information in this document has been obtained and presented in accordance with academic rules and ethical conduct. I also declare that, as required by these rules and conduct, I have fully cited and referenced all materials and results that are not original to this work.

Name, Last name:

Signature:

Date:

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ABSTRACT

Silk fibers are biomaterials that are now used widely in different fields and areas, in biomedical devices and biological products and also employed in treatments, repairs, replacements of damaged tissues and organs also other biomaterials. The main aim of this work is to do a surface modification of the braided silk fibers by coating them with chitosan solution of a particular concentration and then carrying out different tests such as the hemocompatibility test, antimicrobial tests, SEM and XRD on them. The chitosan powder was measured in different concentration and later mixed in 0.1M of acetic acid. The braided silk fibers were coated with chitosan by the layer-by-layer method, later removed and allowed to dry. The chitosan-coated braided silk fibers were observed through an inverted light microscope to see the difference in the diameter between the uncoated and the coated braided silk fibers. Their characterizations have been studied by SEM, and XRD analysis. The silk fibroin microfibers demonstrate effective antimicrobial capability against microbes namely Pseudomonas aeruginosa (ATCC 27853), Enterococcus faecalis (ATCC 29212), Staphylococcus aureus (ATCC 28923), Escherichia coli (ATCC 25922), Bacillus cereus (ATCC 10876) and Candida albicans (ATCC 90028) as examined by the antimicrobial susceptibility tests. Results showed that effective antimicrobial activities are exhibiting higher inhibition ratios. Hemocompatibility results also showed positive values in APTT, PT and INR and other variations because of the samples used. The SEM and XRD result were also positive. All showing the ability of the silk microfibers to be used in wound healing processes.

Keywords: Braided Silk fibers; Chitosan; Hemocompatibility; Antimicrobial activity; Wound Healing

ÖZET

Farklı biyomalzemelerin yanı sıra, ipek fibroin, biyomedikal cihaz ve ürünlerin tasarımında, hasar gören doku ve organların değiştirilmesi, onarımı ve tedavisinde yaygın olarak kullanılmaktadır. Bu çalışmanın temel amacı, örgülü ipek fibroin liflerinin belirli bir konsantrasyonda kitozan çözeltisi ile kaplanarak yüzey modifikasyonu uygulamak, antimikrobiyal özelliklerinin modifiye ederek, kan uyumluluğunun artırmaktır. Kitozan farklı konsantrasyonlarda hazırlanıp, ipek fibroin lifleri tabaka-tabaka yöntemi ile kaplanmışlardır. Bu işlem, eşit zaman aralığında örgülü ipek lifleri homojen bir şekilde kaplanıncaya kadar devam etmiştir. Ters ışık mikroskobu kullanılarak, kitozan kaplı ve kaplanmamış öğrgülü ipek fibroin liflerinin çapları ölçülüp karşılaştırılmıştır. Kitozan kaplı ipek fibroin örgülü liflerinin karakterizasyonu, Taramalı elekron Mikroskobu (TEM), X-Işın Analizi (XIA) yapılarak incelenmiştir. Antimikrobiyal test analizleri ise, Pseudomonas aeruginosa (ATCC 27853), Enterococcus faecalis (ATCC 29212), Staphylococcus aureus (ATCC 28923), Escherichia coli (ATCC 25922), Bacillus cereus (ATCC 10876) ve Candida albicansa (ATCC 90028) karşı gözle görülür antimikrobiyal etkinlik gösterdi. Yapılan kan uyumluluğu testleride kitozan kaplı örgülü ipek fibroin liflerinin kan uyumluğuluğunun yüksek olduğunu gösterdi.Bu çalışma sonucunda, kitozan kaplı örgülü ipek fibroin liflerinin biyomedikal uygulamalarda yara iyileştirici malzeme olarak, yara bezi olarak önerilebilecek önemli bir malzeme olduğu sonucuna varıldı.

***Anahtar Kelimeler*:** Örgülü İpek fibroin lifleri; Kitozan; kan uyumluluğu; antimikrobiyal etkinlik; yara iyileşmesi

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µm:	Micrometer
APTT:	Activated Partial Thromboplastin Time
B. *cereus*:	Bacillus *cereus*
B. *mori*:	Bombyx *mori*
C. *albicans*:	Candida albicans
E. coli:	Escherichia *coli*
E. *faecalis*:	Enterococcus *faecalis*
INR:	International Normalized Ratio
Mins:	Minutes
Mm:	Millimeter
P. *aeroginosa*:	Pseudomonas *aeruginosa*
PT:	Prothrombin Time
S. *aureus*:	Staphylococcus *aureus*
SEM:	Scanning Electron Microscopy
XRD:	X-ray Diffraction

CHAPTER ONE
INTRODUCTION

1.1 Tissue Engineering

Tissue engineering is one of the fields of engineering that is highly and rapidly growing, becoming a vast area of research which is beneficial to the area of biomedical engineering and the medical industry (Badylak and Nerem, 2010; Langer and Vacanti, 1993; Rabkin and Schoen 2002). This field of engineering incorporates and uses the principles of biology and material engineering which helps in the construction of three-dimensional matrices of scaffolds which are used in mimicking and replacing the natural tissue structures and their functions (Lee and Yuk, 2007; Lee and Mooney, 2012; Pawar and Edgar, 2012). These vessels and tissues such as the blood vessels, nerves, muscles and other organs that are damaged are the ones that need to be replaced (Tian et al., 2008; Hosseinkhani et al., 2008). Tissue engineering has become a positive and effectual growing field of engineering that deals with the regeneration of the human tissues (Hosseinkhani and Hosseinkhani, 2008; Hosseinkhani et al., 2007).

This field of engineering involves the use of biomaterials because of their several different properties which allow them to be biocompatible with the biological system of the tissues. These biomaterials come in various forms like the polymers which are in the form of hydrogels, films, fibers and others which are used in fabricaton of other materials that are biocompatible (Subia et al., 2010). Biomaterials such as polymers have two types which could be natural or synthetic, and because of their nature, they undergo different processing technique and technology before they are used (Lee and Shin, 2007; Suh and Matthew 2000; Leclerc et al., 2004; Sionkowska., 2011). For example the silk fibroin is a natural protein polymer, which has been used in recent years by researchers in biomedical applications and other fields, due to its high biocompatibility, low immunogenicity, low bacterial adhesion, slow rate of biodegradability (Omenetto and Kaplan, 2010; Sugihara et al., 2000; Mottaghitalab et al., 2013). Silk fibroin has excellent mechanical properties and structural integrity which makes it suitable to use in bone tissue engineering or used as sutures and other parts of the body (Sofia et al., 2001; Mandal et al., 2012; Yang et al., 2014; Yang et al., 2013).

1.2 Biomaterials

Biomaterials are widely used in different fields and areas. They are employed in biomedical devices, other biological products, in treatments, repairs, replacements of damaged tissues and organs (Stupp and Braun, 1997; Ratner and Bryant, 2004). When biomaterials interact with the biological tissues, there is an activation of the host body defense mechanism against the implanted or foreign material which could lead to contamination in the area of interaction and this leads to thrombosis, necrosis and other reactions (Liu et al., 2004; Gorbet and Sefton, 2004; Furie and Furie, 2005; Horbett, 1993). This response leads to implant failures and rejections by the host body systems (Zimmerli et al., 2004; Darouiche, 2004; Rohde et al., 2007). Due to these effects, biomaterials undergo surface modification or redesign to be able to be used successfully without biological tissues rejecting them after the implantations (Wu et al., 2014).

The surface chemistry of the polymer influences the initial cell behavior as in adhesion, migration, proliferation, differentiation on the polymers (Dalby et al., 2007; Wu et al., 2008; Jeon et al., 2010). The process of the surface functionalization or modification of the biomaterials gives it's specific and desirable properties to be used effectively (Zimmerli et al., 2004; Draouiche, 2004; Rohde et al., 2007; Itoh et al., 2002).

1.3 Silks

Silks are biomaterials which exist in nature, derived naturally and have a unique high class of proteins. These silks are produced by the silk worms, spiders and other insects in their family (Arai et al., 2004). High mechanical strength is one property that the silks possess, which makes them very suitable for use as biomaterials in different areas such as in biomedical applications and other treatments (Fuchs et al., 2006). They also possess the property of biocompatibility, minimal or no immunogenicity when they react with the body system (Horan et al., 2005; Hu et al., 2006), possess low bacterial adhesion (Karageorgiou et al., 2004; Kim et al., 2005), and also possess a stable degradability (Li et al., 2006). These silks most especially the ones got from the *Bombyx mori* (B. *mori)* are processed and used as biomaterials, for example, they have been used as suture materials for some centuries ago (Moy et al., 1991).

The silks represent a unique family of structural proteins which are biocompatible, degradable, mechanically superior. They are susceptible to aqueous or organic solvent processing, and they

can be modified chemically to suit a broad range of biomedical applications. The silk fibroin has been designed at the molecular level at different stages, and this has often resulted in its various classification as a model biomaterial (Jastrzebska et al., 2015). Through the surface modification of the silk fibroin, there have been several studies that have been done and all aimed to improve blood compatibility (Jiang et al., 2010; Elahi et al., 2014; Vepari et al., 2010; Wang et al., 2007; Wang et al., 2011; Gu et al., 2002; Yagi et al., 2011).

Jiang et al., (2010) carried out a research work on the silk fibroin film by synthesizing the zwitterionic phosphobetaine bearing hydroxyl and 8-hydroxy-2-octyl phosphorylcholine to the surface of the silk fibroin film. The work was successful, and the results were positive where the zwitterionic phosphobetaine showed good nonthrombogenicity in the platelet adhesion test (Jiang et al., 2010). Vepari et al., (2010) also worked on the silk fibroin, and he was able to establish that the surface of Polyethyleneglylated silks could be vital in anti-adhesion and anti-thrombosis tests when used as materials for biomedical applications (Vepari et al., 2010).

Silk fibroin coated with ferulic acid was studied by Wang et al., (2007) and he observed that the activated partial thromboplastin, prothrombin time and thrombin time and the whole blood coagulation time all were prolonged largely compared to that of the pure silk fibroin. Moreover, because of that, the improvement of the blood compatibility on the surface of a silk fiber aims at reducing protein adsorption with the eventual goal of decreasing platelet adhesion (Wang et al., 2007). The effect of the surface modification of the silk fibers provides an opportunity for the silk fibers to be used in different areas. The understanding and improvement of the silk fibers have helped greatly in hemocompatibility of the implanted materials designed by natural proteins which are used for biomedical applications (Letchford et al., 2009; Mayer et al., 2009).

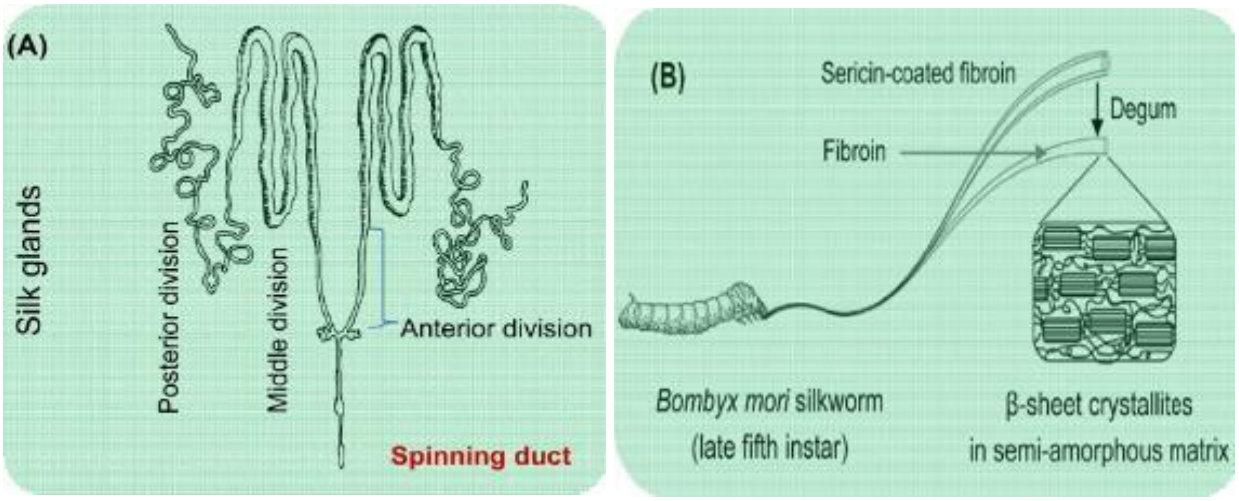
1.3.1 Forms of silk

Silk is a fine, strong fibroin filament that is continuous in nature and also produced by the larva of silk worms which construct their cocoons. The raw silk fiber has impurities such as wax, carbohydrates, inorganic matter, and pigment. The silk fiber has about 70-80% of fibroin and 20-30% of sericin which is the silk gum and removed by processing the silk fiber (Gulrajani, 1992). Since the silk in nature is found to consist two biomacromolecules components which are the fibroin and the sericin which makes the silk easier to be used in different fields spanning from

the medical fields, engineering to the textile industry. Aside the use of silk in the textile industries and the biomedical field, nanotechnologists have also started studies on the fibroin, and it is growing large (Cappello et al., 1994; Chen et al., 2006; Meechaisue et al.,2007; Zoccola et al., 2008; Yin et al., 2009; Zhou et al., 2011; Chutipakdeevong and Ruktanonchai, 2013).

1.3.2 Process of the silk formation

The species of the silkworm that produce the main product of the silk is the *Bombyx mori* (*B. mori*), bulk of the silk is formed from the developmental stage in the cocoon. The silk has two parts the fibroin part and the sericin part. The fibroin part of the silk that is formed are held by the sericin part (Altman et al., 2003; Zhou et al., 2004; Jin and Kaplan, 2003; Vollrath and Porter, 2009; Zhou et al., 2001; Foo et al., 2006; Wilcox et al., 1996).



(a) In vivo processing in silk glands (b) Natural spinning by silkworms

Figure 1.1: Production of fibroin fibers with unique features (Koh et al., 2015)

The process of degumming is used in the removal of the sericin layer and the fibroin which is a shiny fiber and soft is now obtained after the removal of the sericin layer (Tao et al., 2010).

The domesticated silkworm *B. mori* produces silk fibroin fibers which are about 10–25 μm in diameter and consist of two classes of proteins: a light chain which is ~26 kDa and heavy chain which is ~390 kDa and these are present in a 1:1 ratio and linked by a single disulfide bond (Zhou et al., 2000). These proteins are coated with sericin 20–310 kDa, a family of hydrophilic

proteins (Kaplan et al., 1998; Zhou et al., 2000; Yamaguchi et al., 1989; Inoue et al., 2000). The silk fibroin is purified from sericins through the process of boiling the silk cocoons in an alkaline solution (Figure 1A). About twenty-five to thirty percent mass of the silk cocoon is sericin, which is removed during the degumming process.

The silk fibroin from *B. mori* also consists of amino acids of which is primarily 43% glycine (Gly), 30% alanine (Ala) and 12% serine (Ser) (Kaplan et al., 1998). The heavy chain consists of 12 domains that form the crystalline regions in silk fibers, which are interspersed with a primary sequence that is non-repetitive and thus forms organized parts in the fibers. The crystalline portions in the fibers consist of Gly-X repeats, with X being Ala, Ser, Threonine (Thr) and Valine (Val) (Zhou et al., 2001).

After all the processes of the degumming, the silk fibroin that is obtained has attractive characteristics of tensile strength, toughness, biocompatibility, degradability, thermal instability and they represent the best natural protein fiber with properties better than the synthetic and other natural fibers that are found (Vollrath and Porter, 2009; Vepari and Kaplan, 2007). These fibroin fibers can then be used to fabricate different knitted, braided and non-woven matrices in various processes and techniques. These fibroin fibers are regenerated from the different forms they are subjected into. The dissolved fibroin fibers that are formed from the fibroin solution includes sponges, hydrogels, films, mats, microparticles and microneedles (Vepari and Kaplan, 2007; Rockwood et al., 2011; Tansil et al., 2011; Altman et al., 2002; Liu et al., 2008; Unger et al., 2004; Wang et al., 2006; Tsioris et al., 2012) and they are used as sutures or carriers for controlled release and drug delivery.They can be used as scaffolds in tissue engineering to construct several tissues and organs (Lawrence et al., 2009; Gil et al., 2010; Gil et al., 2010; Yang et al., 2007; Liu et al., 2007).

When different forms of silk fibroin materials are processed, they interact favorably with the biological systems of the body without causing adverse effects in the direction of immunological responses; this shows how high their biocompatibility with the biological environment can be (Hakimi et al., 2007). What makes them biocompatible in their biological environment is their property of biocompatibility, controlled slow degradation rate, hemostatic properties, low antigenicity, non-inflammatory, high oxygen permeability, high drug permeability, resistance against enzymatic cleavage (Talukdar et al., 2011; Wang et al., 2006; Wang et al., 2008; Zhang et al., 2009; Cao and Wang, 2009; Minoura et al., 1995).

The silk fibroin forms a major part of non-bioabsorbable biomedical sutures and has been extensively used since then in the medical field (Kajosu and Bora, 2012; Talukdar et al., 2011). Due to their easy handling and tying capacities, silk sutures are used in eye and lip surgeries, intraoral surgeries and skin wounds (Omenetto and Kaplan, 2010). Their mechanical property is what makes easily to be used for these purposes (Sukigara et al., 2003). They have a thermal stability of the fibers over a wide range of temperatures of up to about 250°C without the loss of their functional integrity (Zhang et al., 2009).

1.3.3 Surface modification

Surface modification is a process that includes the physical adsorption or chemical immobilization of a protein or ligand. The silk surfaces are hydrophobic, and they tend to attract and repel proteins depending upon the pI and hydrophobicity of the protein and also pH of the solution. The differential adsorption of horseradish peroxidase (HRP) to silk fibroin in the form of porous sponges depends on the pH of the solution. The HRP precipitate on the surface when the pI and solution pH are similar (Vepari and Kaplan, 2006).

Surface modification of the silk fibroin can be used to alter cell attachment and impact cell proliferation around the area of interaction. The surface modification that deals with the integrin recognition sequence RGDS increases cell attachment (Pierschbacher and Ruoslahti, 1984).

Silk fibroin fibers were modified with poly (ethylene glycol), and it was observed that there was a decreased attachment of the fibroblasts. (Gotoh et al., 1997).

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1.4 Properties of the Silk Fibers

1.4.1 Biodegradability

The degradation of biomaterials is important in the aspect of full tissue structure restoration and its function in *in vivo* experiments. That is why knowledge of the rate of degradation of the biomaterial is an important feature in the design of functional tissue, such that the rate of the scaffold degradation being formed matches the rate of tissue growth and its functions (Lanza et al., 2000). More than 50% of the mechanical properties of silk fibroin fibers are maintained after two months of implantation; thus, they are defined as a non-degradable biomaterial by the United States Pharmacopeia (Horan et al., 2005). Polymers as poly (lactic acid) (PLA), poly (glycolic acid) (PGA), and PLGA have degradation rates which are related to hydrolysis because of the polyester composition, the purity, and also the conditions they are processed through. The degradation rates of these polymers are controlled by different ratios of polymers with various degradation rates or by their molecular weight altered. (Oh et al., 1999). These polymers by enzymes, such as metalloproteinases (MMPs) show limited degradation. Furthermore, the degradation products of synthetic polymers like the PLA decrease local pH and result in inflammation (Solheim et al., 2000).

1.4.2 Immunological responses

The biological systems have immunological reactions against biomaterials, and these are important points to consider most especially when implanting them on biological systems directly. There were certain differences in the sutures that were made from pure silk compared with those sutures from degummed silk, and these showed differences in hypersensitivity of the silk used as sutures (Altman et al., 2003). The inflammatory response of degummed silk fibroin *in vitro* showed less adhesion of immuno-competent cells when compared with polystyrene and poly (2-hydroxyethyl methacrylate) (Santin et al., 1999). Silk films implanted *in vivo* induced a lower inflammatory response than collagen films and PLA films (Meinel et al., 2005). Silk fibroin non-woven mats in nature were implanted subcutaneously in rats. This resulted in weak foreign body response and no occurrence of fibrosis. There were little reaction and activations of

inflammatory pathways at the implantation site and no invasion by lymphocytes after six months *in vivo* (Dal et al., 2007).

1.4.3 Sterilizability

This is an important feature that must be considered when using silk as a biomaterial, compared with other fibrous proteins such as collagen, because of its versatility of options for sterilization (Sugihara et al., 2000). Sterilization of silk fibroin scaffolds has been done by autoclaving, and this does not change the morphology of the silk fibroin (Meine et al., 2004) or β-sheet structure when heated to 120°C (Furuzono et al., 2004). Comparatively, collagen denatures at these temperatures (Mcclain and Wiley, 1972). Silk fibroin scaffolds can also be sterilized using ethylene oxide, γ-radiation, or 70% ethanol (Karageorgiou et al., 2004; Li et al., 2006).

1.4.4 Biocompatibility

The silk-based biomaterials exhibit processing-dependent biocompatibility (the process of extraction or purification) (Altman et al., 2003). The issues of biocompatibility are visible in virgin silks used as suture material, with reactions ranging from delayed hypersensitivity to acute and chronic inflammatory processes (Soong and Kenyon, 1984). However degummed silk fibers demonstrate minimal inflammatory tissue response, which enables successful implantation and cell culture (Kim et al., 2007; Altman et al., 2007). Degummed silk fibers may be incorporated directly into biomaterial architectures. Further processing steps enable the production of "regenerated silk fibroin solution" for an easier fabrication of biomaterials.

1.4.5 Mechanical properties

Silks fibers mechanically exhibit toughness, elasticity, high strength, and are light weighted (Wang et al., 2006). Their toughness is greater than Kevlar 49, the benchmark in high-performance synthetic fiber technology (Omenetto and Kaplan, 2010). Silk fibroin possesses an anti-thrombotic surface with excellent resistance to high shear stress and blood flow pressure (Kundu et al., 2013). Measures of strength and durability are derived from mechanical properties such as tensile strength and Young's modulus. These properties may be obtained by elongating silk fibers or via techniques such as nanoindentation (Rodrigues et al., 2003; Zimmermann et al., 2000). Silk fibers exhibit high tensile strength, flexibility, and resistance to compressive forces, which makes them suitable for applications requiring considerable tensile strength such as sutures or flexibility such as load-bearing composites (Kucharska et al., 2012).

Also, they possess unique properties including resistance to cumulative deformation.

For example, *B. mori* silk fibers demonstrate a remarkable tensile strength of 0.5 Gpa at an elongation of 15% (Zimmermann et al., 2000). Removal of the water-soluble sericin protein coat from the cocoon yields degummed silk fibers, which exhibit up to a 50% increase in tensile strength. The excellent mechanical properties of silk fibroin make it well conditioned for load-bearing in biomedical applications.(Matsubayashi et al., 2003).

1.4.6 Chemical Functionalization

Chemical functionalization can be employed to yield an improvement of existing properties (physical or biochemical), or to expand the library of potential functionalities.

These can range from chemical modification of amino acid residues to functionalization via insertion of chemical species into silk. The diversity of chemical groups on silk proteins enables site-specific reactions, which allow the addition of different chemical moieties. For example, the covalent decoration of silk fibroin fibers with the integrin recognition sequence Arg-Gly-Asp (RGD) and parathyroid hormone (PTH) improve cytocompatibility of silk (Sofia et al., 2001).

1.5 Chitosan

Chitosan, a naturally occurring polysaccharide, obtained by the alkaline deacetylation of chitin and it is a cationic polysaccharide composed of a 2-amino-2-deoxy-β-D-glucan. This chitin that is deacetylated is present in shells of insects and marine crustacea such as shrimps and crabs. The unique properties of chitosan include availability, biodegradability, biocompatibility, bioactivity, but also non-toxicity, adhesion, and sorption, are the major reasons why it has multiple applications (Ravi., 2000; Pillai., 2001). Another main point of this increasing interest of chitosan is its vast physical forms which can be processed and got by using the appropriate technological process to get it. Chitosan has already been employed in a variety of wide fields such as wastewater treatment, medicine, agriculture, food, paper industry and cosmetics (Khor, 2003; Yuan, 2004; Crini, 2006; Li,1992; Griesbach, 1999).

Chitosan, as a natural polysaccharide is a very important coating substance due to its different important properties such as the antimicrobial activity in wound healing ability, biocompatibility, low toxicity, scar preventing property and its ability to smoothen the surface of various structures. Chitosan films in the medical field have been tested as used for curative wound dressing and also as scaffolds for tissue and bone engineering. The chitosan has reactive functional groups which can be subjected to chemical derivatization allowing the manipulation of mechanical and solubility properties is thereby enlarging its biocompatibility. There have been several studies done which have shown that the biological activity of chitosan depends on its molecular weight and its degree of acetylation. Both parameters of which affect the antimicrobial activity of the chitosan, though the influence of the molecular weight of the chitosan on the antimicrobial activity is far greater than the acetylation degree (Yuan and Robin, 2008).

The addition of chitosan through surface modification of the silk fibers which are processed into sutures is one of the methods used to improve the characteristics of various of such suture materials. In order to impart antimicrobial activity, chitosan was successfully applied on to various suture materials namely polylactic acid sutures (Hu et al., 2009), the polypropylene sutures (Saxena et al., 2011), the polyester sutures (Gupta et al., 2010), the cotton sutures (Shanmugasundaram et al., 2006).

1.5.1 Physical Forms of Chitosan and its Blends (Preparation and Characterization)

Chitosan and its plenty of blends exist different physical forms such as resins, microspheres, hydrogels, membranes and fibers. The selection of one particular physical form of the chitosan depends mainly on the system configuration to be used and the particular applications. The process of shaping chitosan blends into desired physical form starts from mixing the blend components in the liquid form and applying the appropriate shaping method. Chitosan and its blends have been widely researched (Huguet, 1994; Polk, 1994; Liu, 1997; Dumitriu, 1998; Murata, 1999; Tomoaki, 2000; Gonzalez-Rodriguez, 2002; Murata, 2002). The chitosan/alginate microparticles (Kim, 1989; Kim, 1989b) chitosan/xanthan microspheres (Chellat, 2000a) and chitosan/gelatin microspheres (Yao, 1996; Yuji, 1996) have also been reported. A novel natural polymer chitosan/cellulose blend beads were prepared via homogeneous dissolution of chitosan and cellulose in methylmorpholine-N-oxide. The blend microspheres prepared by a spray-drying process have a spherical geometry and a smooth surface morphology (Twu, 2003).

1.2 Objective of the Study

In this study, the pure processed silk fibroin microfibers are coated with the chitosan solution at different concentration through a layer-by-layer process with the chitosan solution and later been dried. After which different tests were carried on the silk fibres, tests such as the physical determination of the change in diameter of the silk fibroin microfibers through the measurement of the silk fibers, and comparing them with the uncoated silk fibres, the hemocompatibility analysis was done to check for change in the blood coagulation activity or clotting time and the antimicrobial tests were also done against bacteria using different microorganisms to check its antimicrobial activity.

1.3 Aim of the Study

This research is aimed at the following:

1. Determining the diameter of the chitosan coated silk fibroin microfibers and comparing them with the diameter of the uncoated ones.
2. Determining the hemocompatibility analysis of the coated silk fibroin microfibers and uncoated ones to check the changes in the coagulation activity in the samples of blood and clotting time.
3. Determining the antimicrobial effect of the coated fibers against microorganisms
4. A general comparison of the coated and uncoated silk fibers through characterization study of the samples of the silk fibroin microfibers.

CHAPTER TWO

MATERIALS AND METHODS

2.1 Materials

The silk micro-fibers were bought from Turkey (Figure 2.1). They have shiny, pure and soft characteristics. Chitosan microparticles were used where 0.2 grams and 0.4 grams of it were measured and used respectively at a 60^0C in different beakers. The acetic acid solution that was used was prepared freshly in the laboratory (0.1M of acetic solution). The digital laboratory hot plate magnetic stirrer, magnetic stirring bars, sensitive precision laboratory analytical electronic weighing scale, and all other glassware used were all from the departmental laboratory and were washed and dried before being used for every experiment.

Figure 2.1: Showing the sample of the silk micro-fibers

2.1.1 The Braiding of the Silk Micro-Fibres

The method that was used to braid the silk fibers was all under the hand knotting technique. The strands of fibers were picked and hand knotted to form one strand. They were later hand crocheted to form a small circle of the silk fiber before they were used for the antimicrobial susceptibility test.

After the silk fibers had been knotted to form one long single strand, they were then coated with the chitosan solution and dried within time intervals that ranged from 30 minutes to 1 hour respectively for the coating time and drying time. After the whole process of coating and drying of the silk fibers, the next process that followed was the measurement of the silk fibers with inverted light microscope to determine the diameter or thickness of the coated and uncoated silk fibers to be able to see the differences between the coated and uncoated silk fibers and how successful the coating was.

2.2 Preparation of the Chitosan Solution

The chitosan was bought from Turkey. It was first prepared by measuring the grams of the chitosan microparticles using the electronic weighing balance. 0.2grams and 0.4grams of the chitosan were measured using the scale. 25ml of freshly prepared diluted 0.1M acetic acid was used to dissolve the grams of the chitosan respectively. The beakers carrying the acetic acid and the chitosan were then placed on the hot plate magnetic stirrer, and magnetic stirring bars were dropped inside the beakers, and the solutions were stirred at a temperature of 60℃ with a rotation of 2. The mixtures were allowed to be stirred for about 20 minutes to 30 minutes. After the chitosan particles were dissolved properly and a viscous chitosan acetate solution formed, it was then allowed to cool down before the dipping process of the fibers were done.

2.3 Coating Process of the Silk Micro-Fibers by the Layer-by-Layer Dipping Method

The next process after the preparation of the solution was the coating process of the silk micro-fibers with the chitosan solution using the layer-by-layer dipping method.

The layer-by-layer dipping method is a process that involves coating of the silk micro-fibers by dipping them in the chitosan acetate solution within different periods of time intervals and allowing them to dry within time intervals too. The silk fibers were tied round different pipettes to one end so that they could be hanged on the beakers both during the dipping process and also during the drying process.

The braided silk micro-fibers were dipped into the solution and allowed for 1 hour for proper coating and deposition on the braided silk micro-fibers and later dried for another period of 1 hour. This procedure was repeated until ensured that there was a homogenous coating of the chitosan on the braided silk micro-fibers. This process was done for both concentrations of the chitosan solution after which further observations and tests were carried out.

The inverted light microscope was used to observe the differences in their thickness, the rate of absorption during the hemocompatibility was also tested, and resistance in the antimicrobial susceptibility test was all also observed.

2.4 The Measurement of the Diameter of the Coated and Uncoated Silk Micro-Fibers

The coated and uncoated braided silk micro-fibers were taken to the Department of the Pharmacy in Near East University where their diameter was measured, and differences were observed to check whether there was any difference or not after the coating process was done. The Inverted light microscope was used to manually observe and measure the differences in the diameter of all the braided silk micro-fibers, along the length of the fibers both for the coated and uncoated braided silk micro-fibers. After the measurement of the fiber, the photographs of the fibers been measured were taken.

2.5 Sterilization Process of both Coated and Uncoated Silk Fibers

Sterilization process was one of the basic processes that were done on the silk micro-fibers before every test and analyses were carried out. This process was done on the silk fibers throughout the time of the research.

All the samples of the fibers were taken to the sterilization unit of Near East Hospital, Lefkosa and were sterilized with hydrogen peroxide before any further analysis.

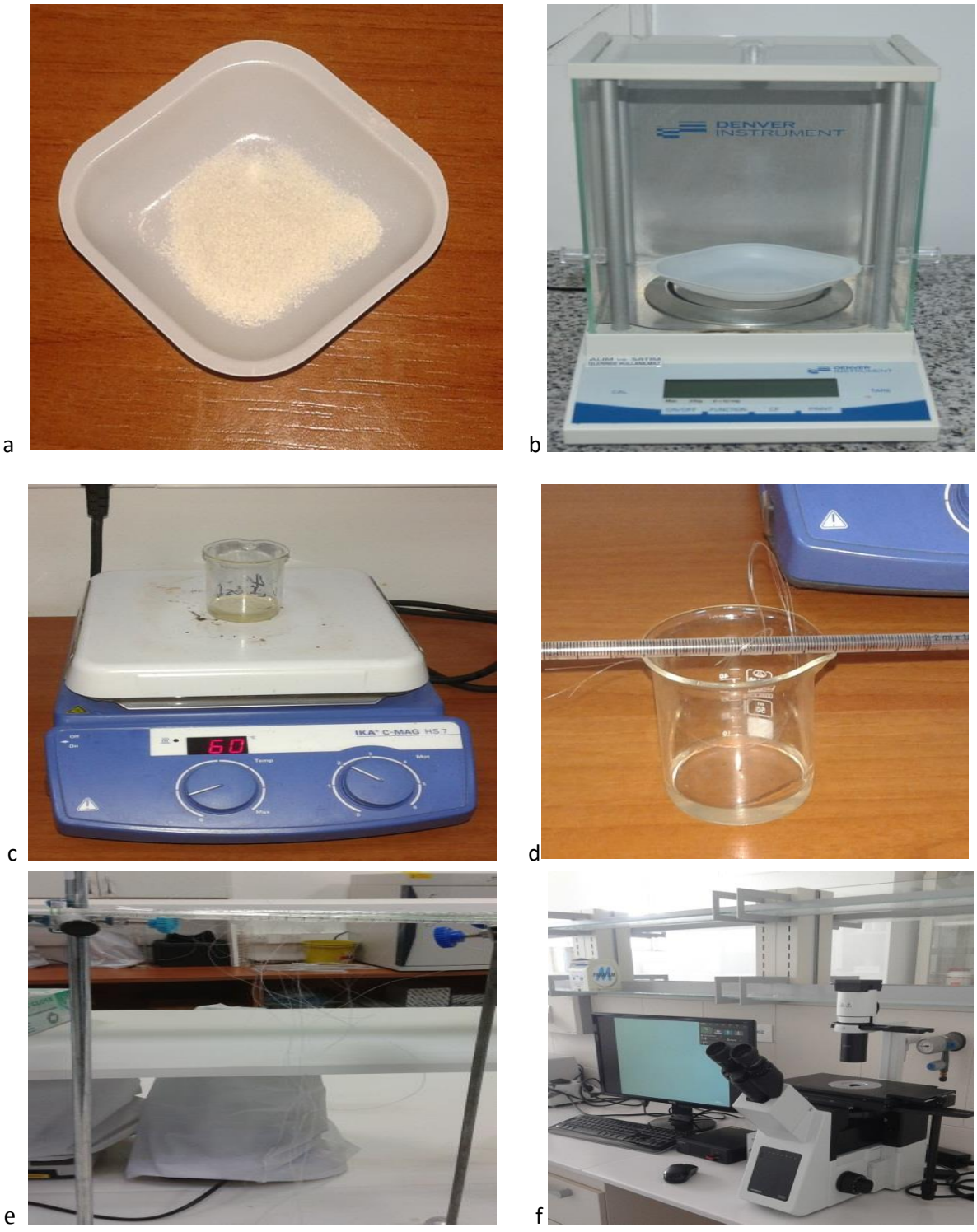


Figure 2.2: Showing all the processes that were followed from the measurement of chitosan to measurement of the diameter of the silk micro-fiber

2.6 Hemocompatibility Analysis on the Silk Micro-Fibers (Coated and Uncoated)

The Hemocompatibility analysis is a very important test carried out on the silk fibers to check for coagulation activity in the blood where prothrombin time in seconds (PTsec), international normalized ratio (INR), prothrombin time (PT%) and the activated partial prothrombin time (APTT) effect are determined and clotting time on the silk fibers to ensure that there is biocompatibility of the blood with the coated and uncoated braided silk fibers. In the microbiology laboratory in the hospital, the coagulation machine was used to analyze the blood samples carrying the cut silk fibers in the blood collection tubes.

The lengths of the silk fibers were measured between 10cm and 15cm and were cut and dropped inside the blood collection tubes carrying the blood samples and then analyzed by the coagulation machine(STA Compact Hemostasis System equipment, Stage, US) and results obtained. However, before this was done the blood samples were centrifuged(Heraeus,Labofuge 400,thermoscientific) for 5mins for a good separation of the plasma from the platelets and red blood cells, in the blood and the centrifuge process was repeated for every time interval.
The PTsec, PT (%), APTT and INR values are all values that are obtained during the coagulation study. The APTT measures and detects abnormalities that are present in a sample of blood that is analyzed. The PT values can indicate the speed of the clotting during the coagulation study, and also shows through the extrinsic pathway how blood sample that is analyzed clots quickly. Moreover, the INR values are obtained based on the values obtained from the PT values. The INR values standardize the PT values that are obtained during the coagulation study.

In this study, silk fibers both coated and uncoated were prepared for analysis, the values from the coagulation study were detected and taken by measuring the activated partial thromboplastin time (APTT), prothrombin time (PTT), and INR by STA Compact Hemostasis System equipment, Stage, US.

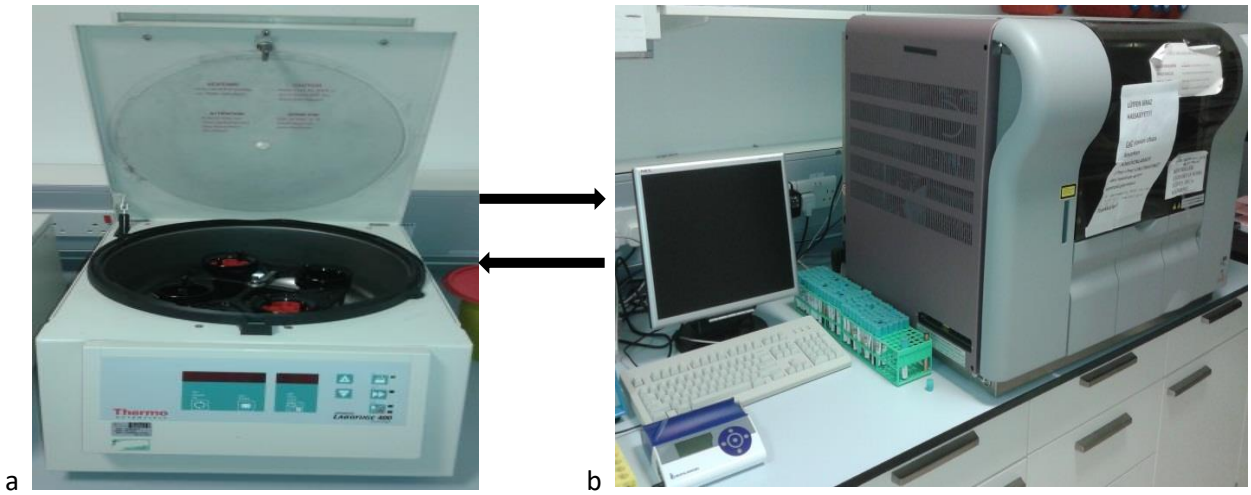


Figure 2.3: Showing the process that was carried out for the hemocompatibility of the silk fibers with the samples of blood

2.7 Antimicrobial Susceptibility Testing On the Silk Fibers

Antimicrobial susceptibility test is carried out to determine the *in vitro* activity of an antimicrobial material that is used against certain microbial species that are used for the tests. The antimicrobial susceptibility test was carried out with six microorganisms, of which 3 of the microorganisms are gram positive, and the other 3 of the microorganisms are gram negative and one of which was yeast or fungus. Namely, the six strains of microorganisms that were used in this study included gram-positive bacteria *Enterococcus faecalis(ATCC 29212), Bacillus cereus ATCC(ATCC 10876), Staphylococcus aureus (ATCC 28923)* and Gram-negative bacteria *Escherichia coli(ATCC 25922), Pseudomonas aeruginosa (ATCC 27853)*and fungi *Candida albicans(ATCC 90028).* The Mueller Hinton agar was the type of agar that was used for the test based on clinical and laboratory standards institutes for antimicrobial susceptibility testing.

The first stage of the susceptibility test is preparing the solid agar medium of Mueller-Hinton agar according to the manufacturer's guideline of 34.0g/L and then sterilizing it by autoclaving at 121 degrees for 15mins. It is then poured into petri dishes to a certain depth of about 4mm, and when it cools down enough, it is then placed in the refrigerator and left overnight for it to solidify. A broth of all the microorganisms is also prepared to a standard value of 0.5 McFarland approximately 10.6 cell/ml which is measured using BD Phoenix spec nephelometer. The agar

plate is labeled according to the name of the microorganisms to be used and using a sterile cotton swab on its surface to inoculate it with 10uL of ID broth containing specific microorganisms.

0.1 grams of each sample was diluted with 1ml of ultra-pure water for us to easily incubate the samples on the sterilized paper discs. Using a sterile tip of cotton buds on the micropipette for each sample to avoid error in the results, 20uL of diluted samples were measured using the micropipette and poured onto the blank disc that has been placed on the inoculated agar.

When all of the samples are carefully placed in the culture media inoculated with the different microorganisms. They are placed at room temperature for 10 minutes and then incubated upside down at 37º for 24hr. After 24hrs results were readily visible.

2.8 Characterization of the Silk Fibers

2.8.1 Scanning Electron Microscopy Analysis

Scanning electron microscope works on the principle of using electron beams to obtain images from materials. The electrons interact with atoms in the samples, and they produce a different type of signals that can be detected by the microscope which contains information about the surface topography and composition of the sample (McMullan, 2006). This analysis was carried out at TUBITAK-Marmara Research Center at Gebze, Istanbul, Turkey using a SEM M- Jsm-6510 model at an acceleration voltage 10kV. The device produces images of the samples by focusing a beam of electrons on it and samples were coated with gold to prevent charging.
Scanning Electron Microscopy is employed to observe surface topography and morphology of the material. The electrons in this analysis scan from the top in a beam fashion, focusing on the sample from one point to another and resulting in refracted electrons.

2.8.2 X-ray Diffraction Analysis

A variety of techniques is available to study the secondary structure of silk in forms ranging from model peptides to natural spun fibers. The crystalline properties of the beta sheets are mainly studied by X-ray diffraction. Early X-ray diffraction studies of native fibers revealed the presence of an orthorhombic unit cell (a=9.40 A in the hydrogen bond direction, b=9.20 in the sheet stacking direction (intersheet distance and c= 6.97 A in the chain and fiber axis direction) which was suggested to arise from antiparallel beta-sheet stacking within the crystalline domains to be able to give proper interpretation of the substance or material used.

CHAPTER THREE

RESULTS AND DISCUSSIONS

3.1 Braiding of the Silk Fibers

The method that was used on the silk fibers was the hand knotting method of the fibers. The strands of fibers were picked and knotted to form one strand which was used throughout the research work. They were later hand crocheted to form a circle also before they were used for the antimicrobial susceptibility test.

After the silk fibers had been knotted to form one long single strand, they were then coated with the chitosan solution and allowed to dry within the time interval that ranges from 30 minutes to 1 hour respectively for the coating time and drying time. After the whole process of coating and drying of the silk fibers, the next process that followed was the measurement of the silk fibers to determine the diameter or thickness of the coated and uncoated silk fibers, to be able to see the differences between the coated and uncoated silk fibers.

3.1.1 Measurement of the Diameter of the Silk Fibers

The measurements were carried out in the Department of the Pharmacy using the Inverted light microscope to observe and measure the differences in the diameter of all the knotted silk fibers both for the coated and uncoated silk fibers. The measurement is done along the length of the fiber. The values were taken from a different region of the length of the coated and uncoated fibers. This was done manually.

The results below showed a clear difference between the coated and uncoated fibers and also compared with the diameter of the single strand of the silk fiber.

The results observed showed a clear difference in the diameters of the silk fibers. This indicates that the chitosan solution that was used to coat the silk fibers increased the diameter of the silk fibers as shown on the table and the graph thereby making the coating process a success.

Below are the results on the table, graph and the images that were taken to show the differences of coated and uncoated microfibers

Table 3.1: Showing the differences in the diameter of both coated and uncoated silk fibers

Single Fiber(µm)	Uncoated Silk Fiber(µm)	0.2g of Chitosan-Coated Silk Fiber(µm)	0.4g of Chitosan-Coated Silk Fiber(µm)
258.3	378.4	354.1	411.6
256.6	382.8	371.4	494.6
259	342.4	355.5	476.3
238	352.8	377	469.3
254.3	343.4	402.7	380.6
253.24	359.96	372.14	446.48

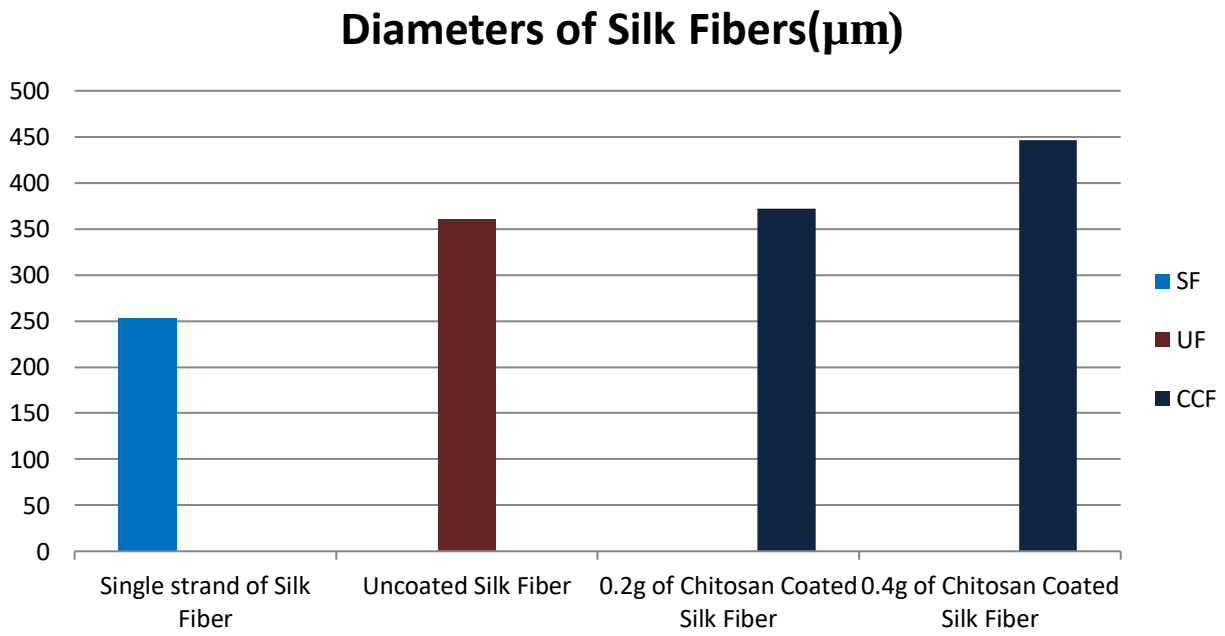


Figure 3.1: Graphical representation of the differences in the diameter of both the single, uncoated and coated braided silk fibers

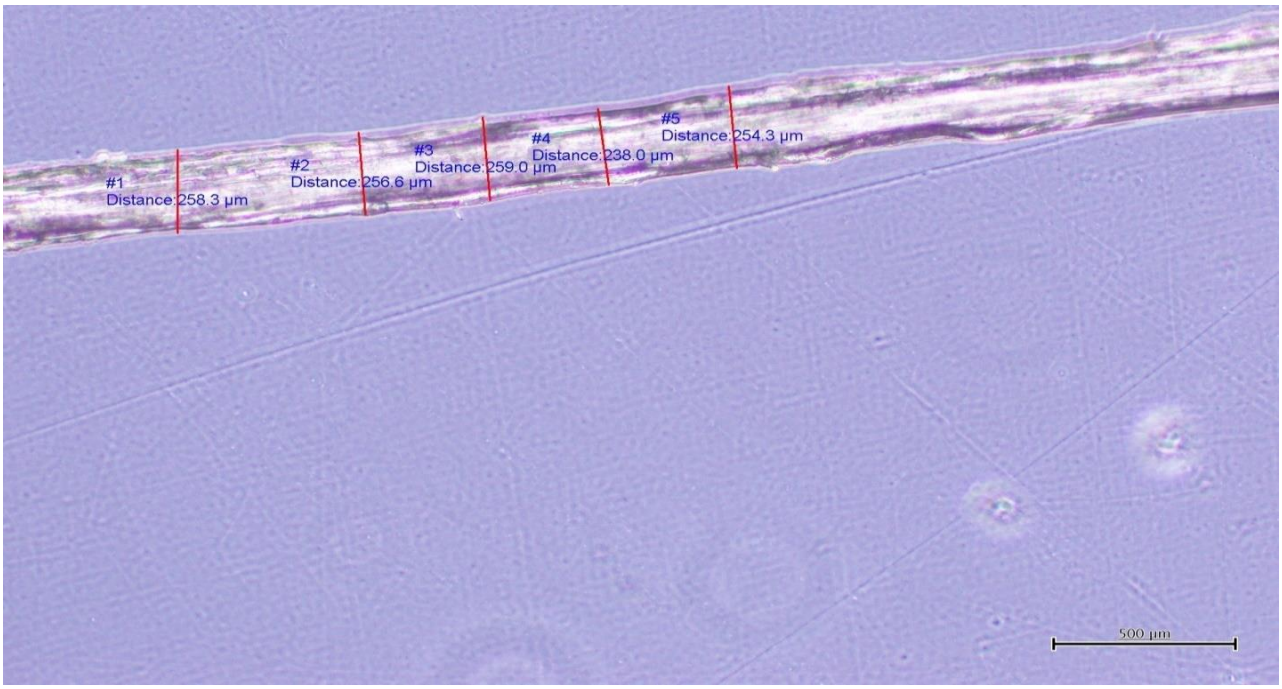


Figure 3.1.1: Shows the photograph of an uncoated single strand of silk fiber with different diameters on its length

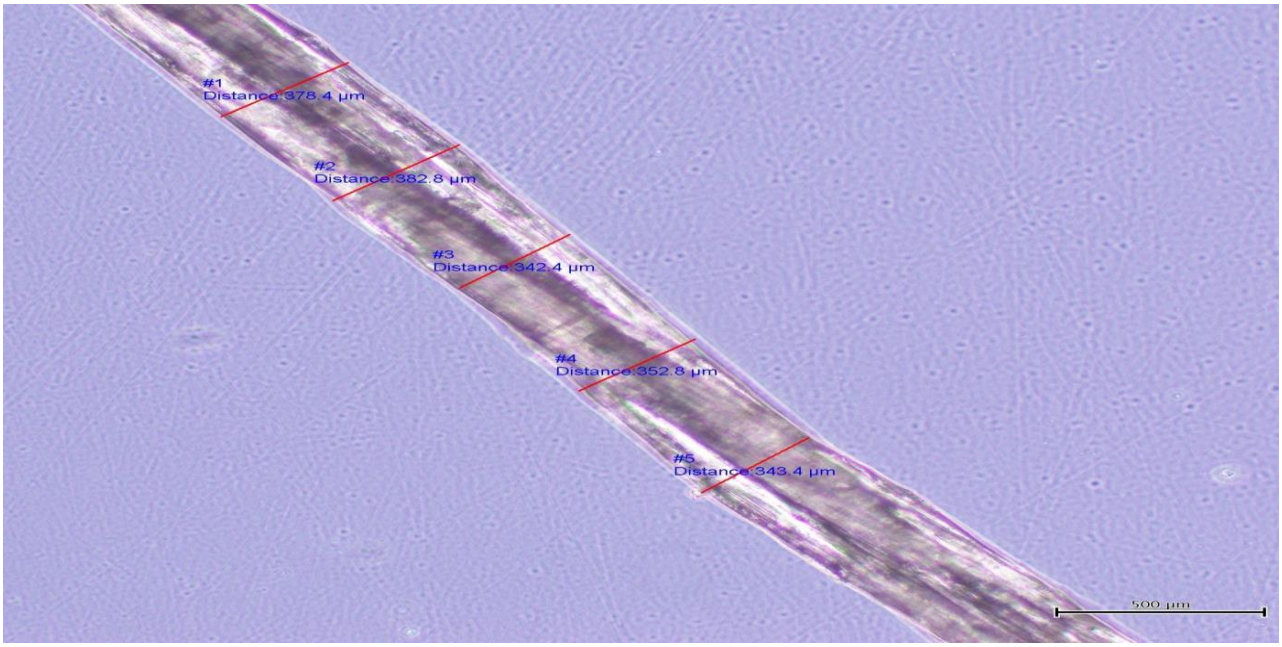


Figure 3.1.2: Shows the photograph of uncoated braided silk fiber with the different diameters on its length

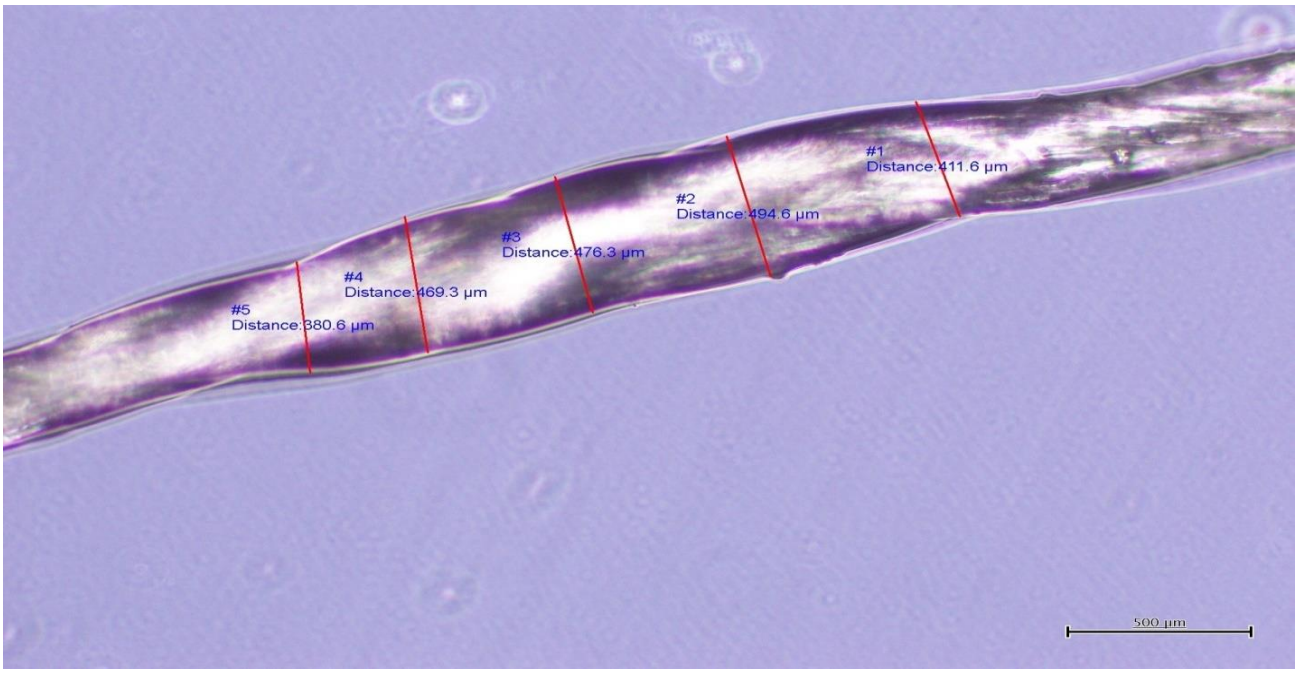


Figure 3.1.3: Shows the photograph of 0.2grams of chitosan coated braided silk fiber with different diameters along its length

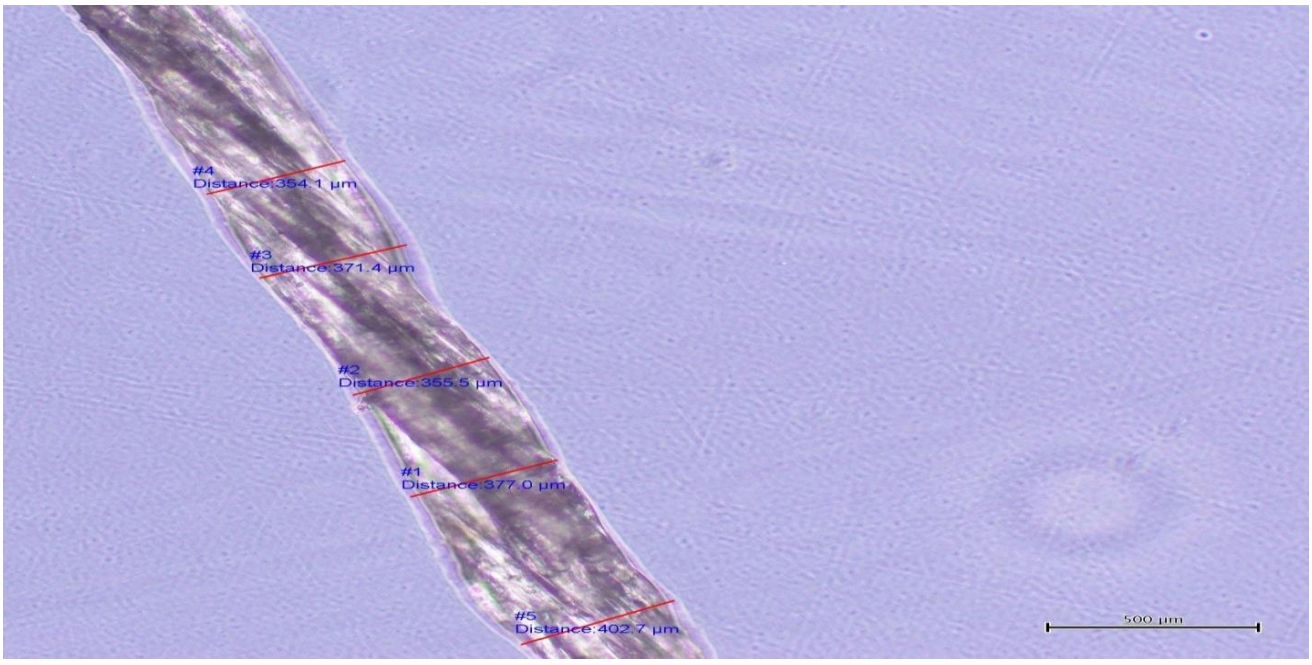


Figure 3.1.4: Shows the photograph of 0.4grams of chitosan coated braided silk fiber with different diameters along its length

3.2 Characterization of the Silk Fibers

Hemocompatibility test, antimicrobial susceptibility test, Scanning Electron Microscopy(SEM) analysis, X-ray Diffraction Analysis were all carried out in the characterization of the samples of the silk fibers both for the coated and uncoated.

3.2.1 Antimicrobial Susceptibility Test Results

The twelve results of all the samples showed a zone of inhibition after 24 hours, and the results were all positive after their incubation. The uncoated silk fibers served as the negative control groups, and all showed zones of inhibition against all the microorganisms that were used for the susceptibility tests.

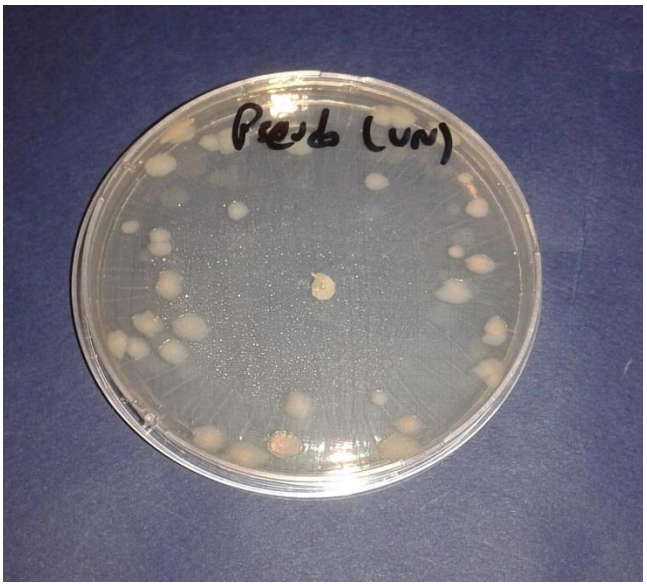
The coated silk fibers with chitosan solution which is the positive control group showed larger zones of inhibition against all the microorganisms which were used more than the uncoated silk fibers.

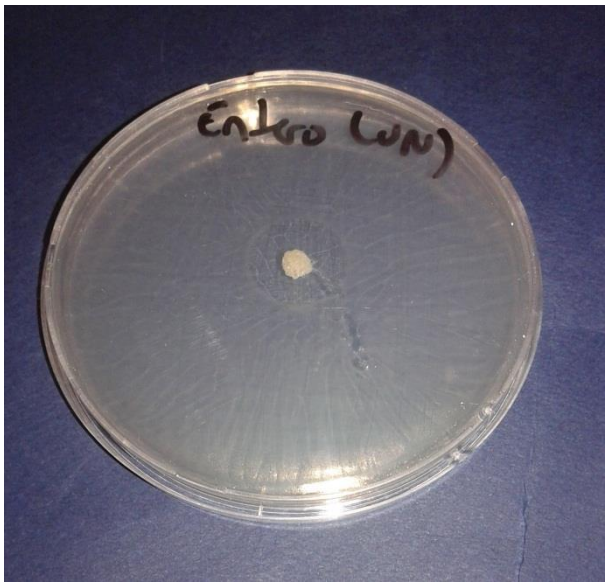
The Table and figures below show the different results of the diameter of the zone of inhibition on all the microorganisms.

A. *Pseudomonas aeruginosa*(ATCC 27853)
B. *Enterococcus faecalis*(ATCC 29212)
C. *Staphylococcus aureus*(ATCC 28923)
D. *Escherichia coli*(ATCC 25922)
E. *Bacillus cereus*(ATCC 10876)
F. *Candida albicans* (ATCC 90028)

Figure 3.2.1: Shows the inhibition zones for the sample of Uncoated Crocheted silk fibroin microfibers against both gram positive and gram negative microorganisms a)*Pseudomonas aeruginosa* b) *Enterococcus faecalis c) Staphylococcus aureus d) Escherichia coli e) Bacillus cereus f) Candida albicans*

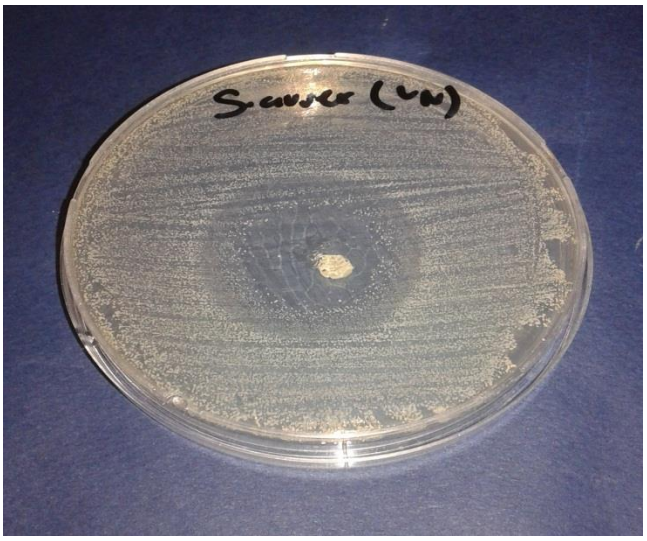
a)

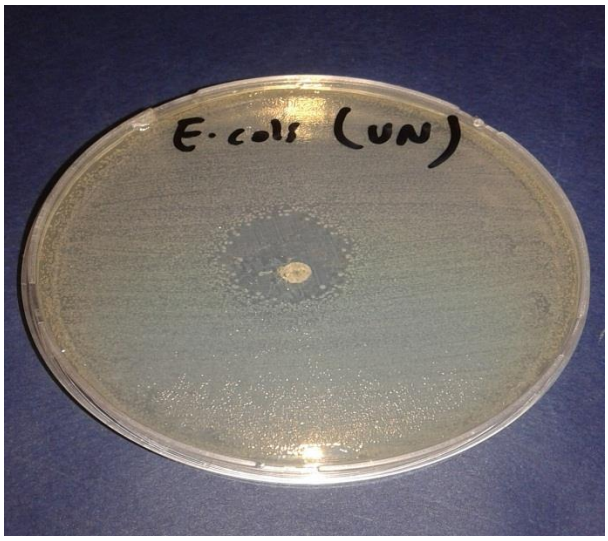




b)

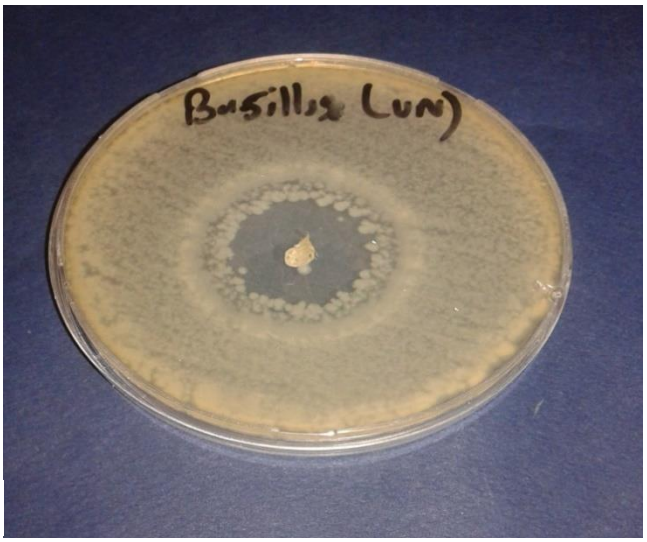
c)

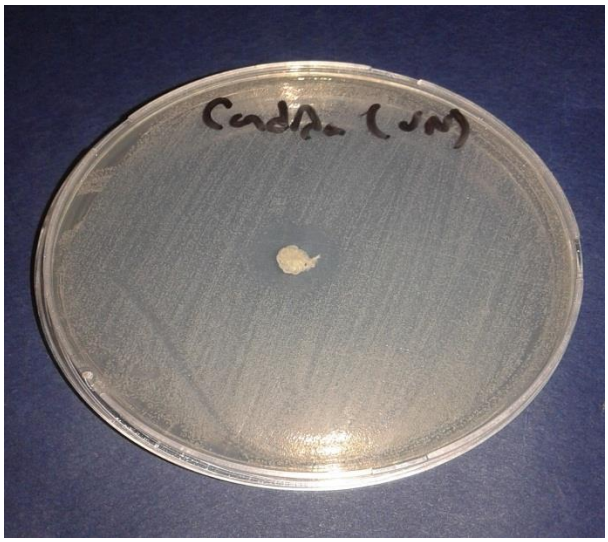




d)

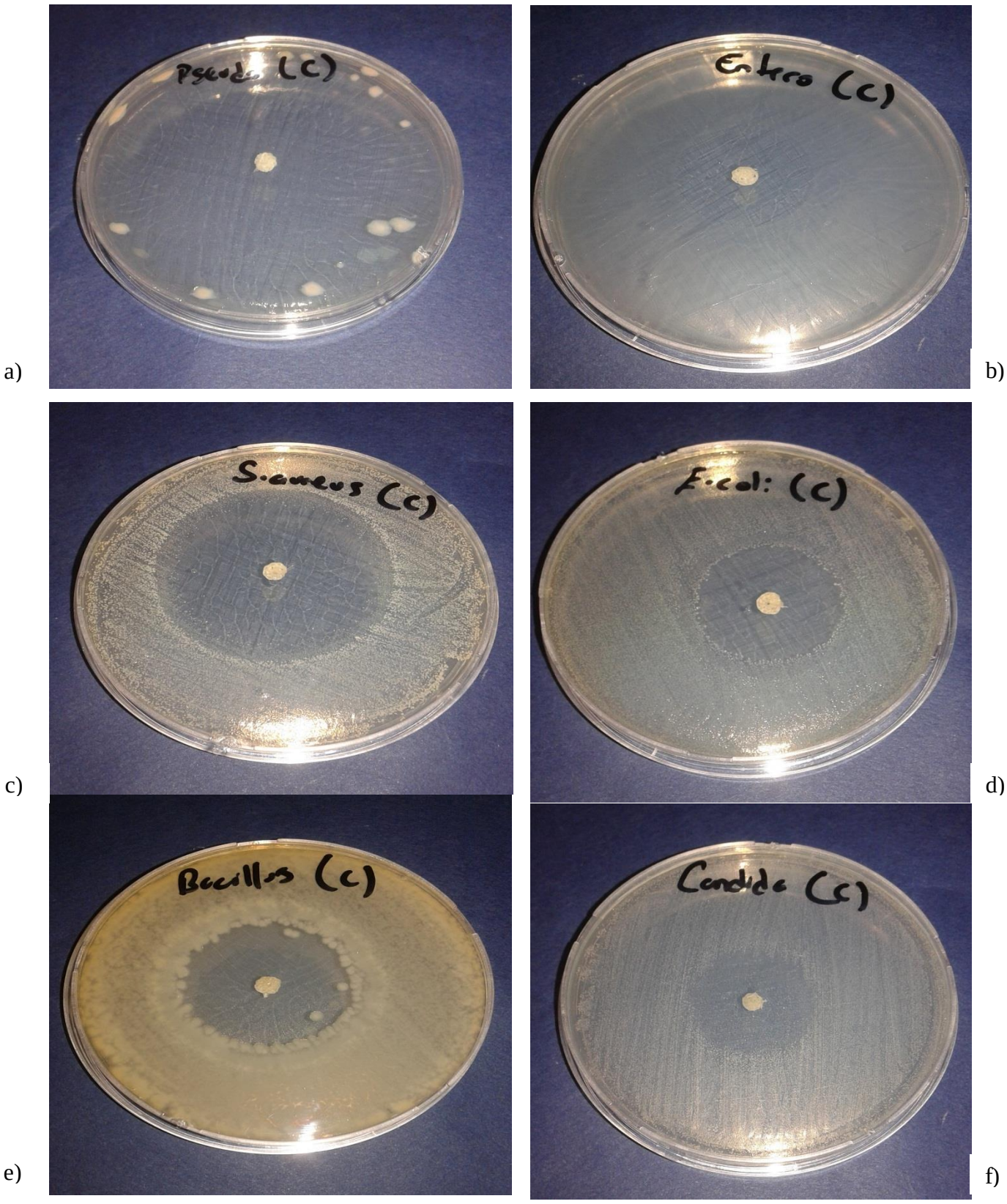
e)





f)

Figure 3.2.2: Shows the inhibition zones for the sample of coated crocheted silk fibroin microfibers against both gram positive and gram negative microorganisms



a) b) c) d) e) f)

a)*Pseudomonas aeruginosa* b) *Enterococcus faecalis c) Staphylococcus aureus*
d) Escherichia coli *e) Bacillus cereus* *f) Candida albicans*

Table 3.2: Showing the ratio differences in mm as a result of the inhibition zones formed by the antimicrobial activity of both the coated and uncoated silk micro-fibers

Micro-organisms	Uncoated Silk Fibers(mm)	Coated Silk Fibers(mm)
C.albicans	20	31
S.aureus	36	50
E.coli	19	35
B.cereus	25	36
E.faecalis	16	38
P.aeroginosa	55	66

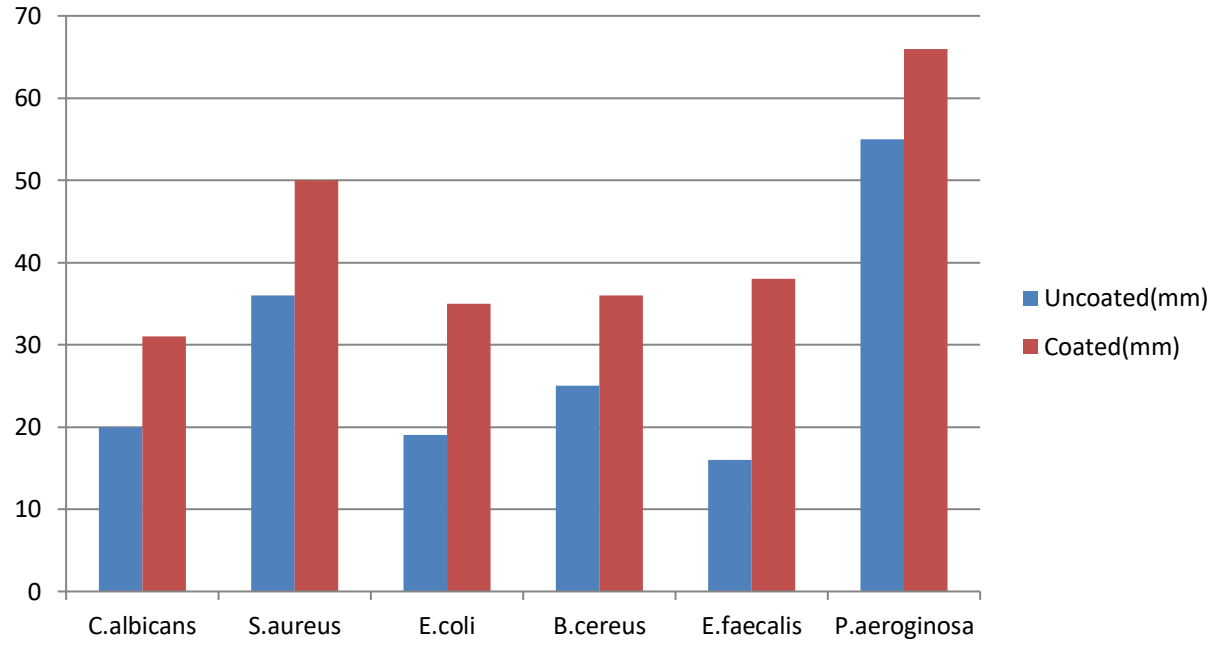


Figure 3.2.3: A graphical representation of the different effects of the antimicrobial activity of the silk fibers both coated and uncoated on the microorganisms

3.2.2 Hemocompatibility Test

Invitro Blood coagulation test

This test and analysis were done to be able to determine the anticoagulant activity of the biomaterial that is the uncoated and chitosan coated silk fibers. Certain markers were taken to be able to check the anti-coagulant activity of these fibers, which are the activated partial thromboplastin time (APTT), prothrombin time (PT), international normalized ratio (INR). Ethical and proper permissions were taken from the Hospital and head of the department of blood coagulation and urine analysis before samples of blood were used.

These were used to determine the hemocompatibility of the silk fiber through the different clotting times that were analyzed. The normal standard clotting time or values vary from one region to another.

Below are the results that were obtained from the analysis.

Table 3.3: Showing the normal standard clotting time for a healthy person without thinning drugs

APTT(sec)	PT (sec)	PT(%)	INR
23.6-35.2	**11.5-15**	**70-120**	**0.80-1.20**

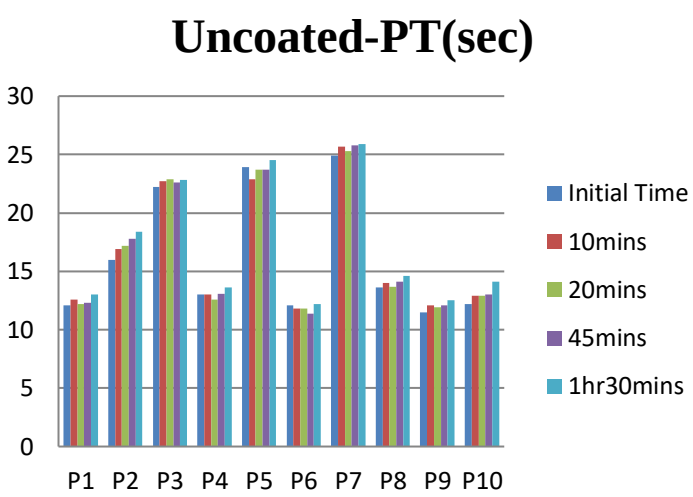


Figure 3.3.1: Graphical representation of PTsec test for uncoated silk fibers where there is an increase in the value of pt but with variations due to factors from the sample of blood used.

PT(SEC)	Initial Time	10mins	20	45	1hr30mins
P1	12.1	12.6	12.2	12.3	13
P2	16	16.9	17.2	17.8	18.4
P3	22.2	22.7	22.9	22.6	22.8
P4	13	13	12.6	13.1	13.6
P5	23.9	22.9	23.7	23.7	24.5
P6	12.1	11.8	11.8	11.4	12.2
P7	24.9	25.7	25.3	25.8	25.9
P8	13.6	14	13.7	14.1	14.6
P9	11.5	12.1	11.9	12.1	12.5
P10	12.2	12.9	12.9	13	14.1

Table 3.3.1: Showing result of PTsec test for uncoated silk fibers

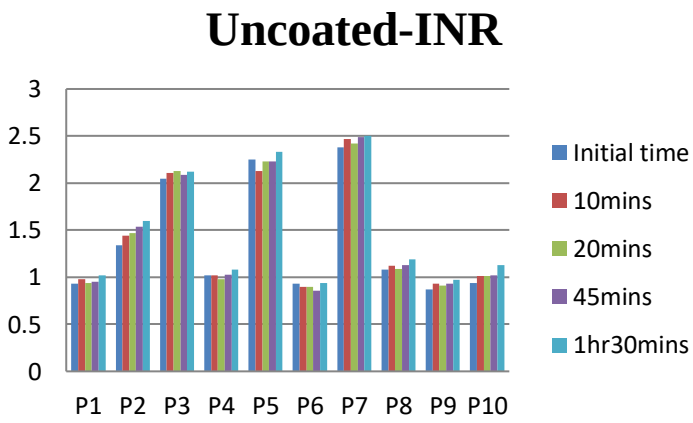


Figure 3.3.2: Graphical representation of INR test for uncoated silk fibers where there is an increase in the value of inr but with variations due to factors from the sample of blood used.

INR	Initial time	10mins	20mins	45mins	1hr 30mins
P1	0.93	0.98	0.94	0.95	1.02
P2	1.34	1.44	1.47	1.54	1.6
P3	2.05	2.11	2.13	2.09	2.12
P4	1.02	1.02	0.98	1.03	1.08
P5	2.25	2.13	2.23	2.23	2.33
P6	0.93	0.9	0.9	0.86	0.94
P7	2.38	2.47	2.42	2.49	2.5
P8	1.08	1.12	1.09	1.13	1.19
P9	0.87	0.93	0.91	0.93	0.97
P10	0.94	1.01	1.01	1.02	1.13

Table 3.3.2: Showing result of INR test for uncoated silk fibers

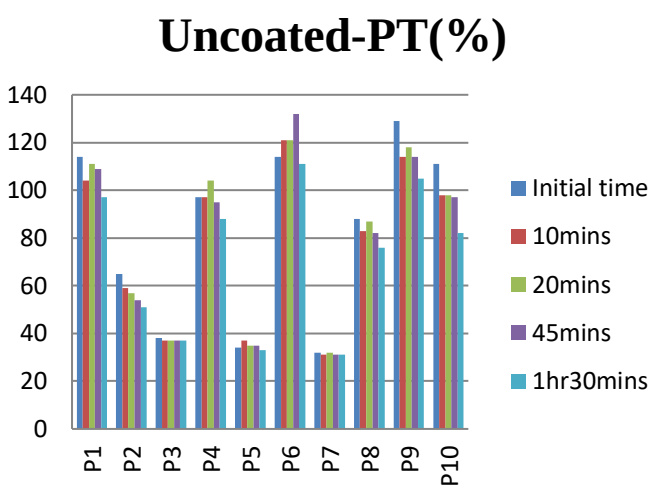


Figure 3.3.3: Graphical representation of PT(%) test for uncoated silk fibers where there is an increase in the value of ptsec but with variations due to factors from the sample of blood used.

PT(%)	Initial time	10mins	20mins	45mins	1hr 30mins
P1	114	104	111	109	97
P2	65	59	57	54	51
P3	38	37	37	37	37
P4	97	97	104	95	88
P5	34	37	35	35	33
P6	114	121	121	132	111
P7	32	31	32	31	31
P8	88	83	87	82	76
P9	129	114	118	114	105
P10	111	98	98	97	82

Table 3.3.3: Showing result of PT(%) test for uncoated silk fibers

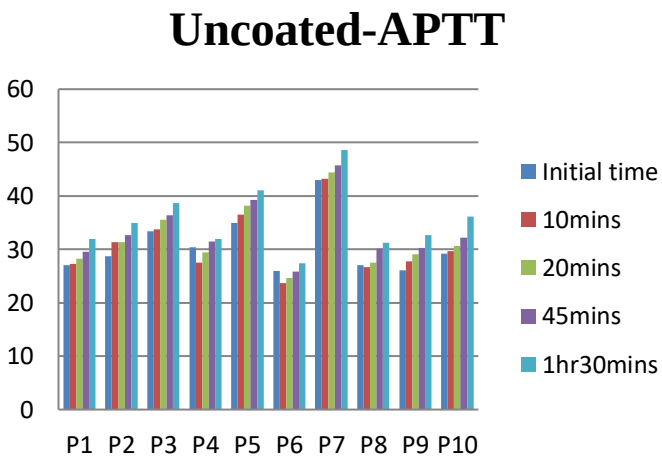


Figure 3.3.4: Graphical representation of APTT test for uncoated silk fibers where there is an increase in the value of aptt but with variations due to factors from the sample of blood used.

APTT	Initial time	10mins	20mins	45mins	1hr 30mins
P1	27	27.3	28.2	29.6	32
P2	28.7	31.4	31.3	32.7	35
P3	33.4	33.7	35.5	36.4	38.7
P4	30.4	27.5	29.4	31.5	32
P5	35	36.5	38.2	39.3	41.1
P6	26	23.7	24.6	25.9	27.4
P7	43	43.2	44.4	45.77	48.6
P8	27	26.7	27.5	30.2	31.2
P9	26.11	27.8	29.1	30.3	32.7
P10	29.2	29.7	30.6	32.2	36.2

Table 3.3.4: Showing result of APTT test for uncoated silk fibers

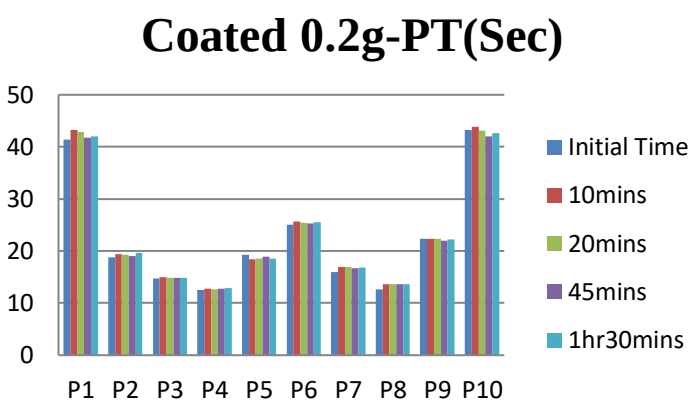


Figure 3.3.5: Graphical representation of 0.2g PTsec test for coated silk fibers where there are changes in values but with variations comparing with the initial values due to factors from the sample of blood used.

PTsec (0.2g)	Initial Time	10mins	20mins	45mins	1hr 30mins
P1	41.4	43.2	42.9	41.8	42
P2	18.8	19.4	19.3	19	19.6
P3	14.7	15	14.9	14.8	14.9
P4	12.5	12.8	12.7	12.8	12.9
P5	19.3	18.4	18.5	18.9	18.5
P6	25.1	25.7	25.4	25.3	25.5
P7	15.9	16.9	16.9	16.7	16.8
P8	12.7	13.6	13.6	13.6	13.6
P9	22.3	22.4	22.4	22	22.2
P10	43.2	43.8	43.1	42	42.6

Table 3.3.5: Showing result of 0.2g PTsec test for coated silk fibers

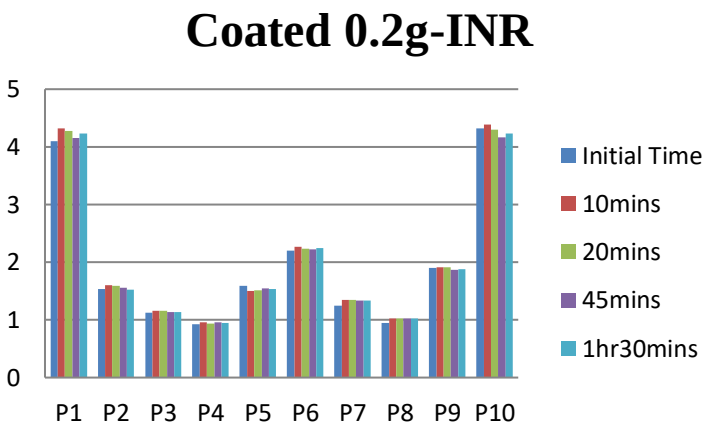


Figure 3.3.6: Graphical representation of 0.2g INR test for coated silk fibers where there is an increase in the value but with small variations due to factors from the sample of blood used.

INR	Initial Time	10mins	20mins	45mins	1hr 30mins
P1	4.1	4.32	4.28	4.15	4.23
P2	1.54	1.6	1.59	1.56	1.52
P3	1.13	1.16	1.16	1.14	1.14
P4	0.93	0.96	0.94	0.96	0.95
P5	1.59	1.5	1.51	1.55	1.53
P6	2.2	2.27	2.23	2.22	2.24
P7	1.25	1.35	1.35	1.33	1.34
P8	0.95	1.03	1.03	1.03	1.03
P9	1.9	1.91	1.91	1.87	1.88
P10	4.32	4.39	4.3	4.17	4.23

Table 3.3.6: Showing result of 0.2g INR test for coated silk fibers

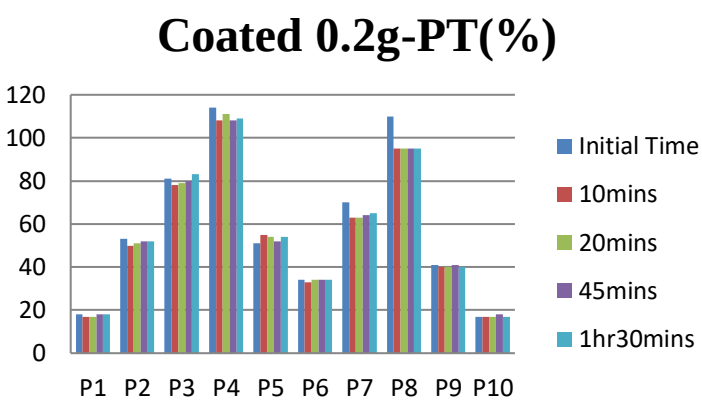


Figure 3.3.7: Graphical representation of 0.2g PT(%) test for coated silk fibers where there is an increase in the value but with little variations due to factors from the sample of blood used.

PT(%)	Initial Time	10mins	20mins	45mins	1hr 30mins
P1	18	17	17	18	18
P2	53	50	51	52	52
P3	81	78	79	80	83
P4	114	108	111	108	109
P5	51	55	54	52	54
P6	34	33	34	34	34
P7	70	63	63	64	65
P8	110	95	95	95	95
P9	41	40	40	41	40
P10	17	17	17	18	17

Table 3.3.7: Showing result of 0.2g PT(%) test for coated silk fibers

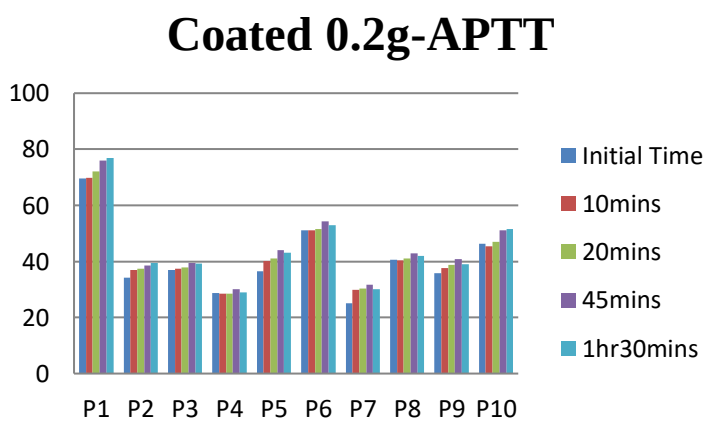


Figure 3.3.8: Graphical representation of 0.2g APTT test for coated silk fibers where there is a remarkble increase in the values but with variations due to factors from the sample of blood used.

APTT	Initial Time	10mins	20mins	45mins	1hr 30mins
P1	69.5	69.9	72.2	76	76.8
P2	34.3	36.9	37.5	38.6	39.4
P3	37	37.5	37.8	39.5	39.3
P4	28.7	28.4	28.6	30.1	29
P5	36.6	40.2	41.1	44	43
P6	51.2	51	51.5	54.2	53
P7	25.1	29.9	30.3	31.8	30
P8	40.6	40.4	41	42.8	42
P9	35.9	37.6	38.7	40.8	39
P10	46.2	45.3	46.9	51.2	51.5

Table 3.3.8: Showing result of 0.2g APTT test for coated silk fibers

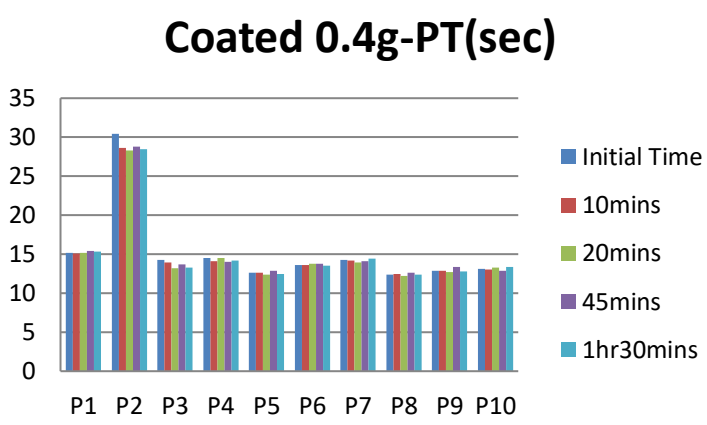


Figure 3.3.9: Graphical representation of 0.4g PTsec test for coated silk fibers where there is an increase in the value but with less variations due to factors from the sample of blood used.

PTsec (0.4g)	Initial Time	10mins	20mins	45mins	1hr 30mins
P1	15.2	15.1	15.2	15.4	15.3
P2	30.4	28.6	28.3	28.8	28.5
P3	14.3	13.9	13.2	13.7	13.3
P4	14.5	14.1	14.5	14	14.2
P5	12.6	12.6	12.4	12.9	12.5
P6	13.6	13.6	13.8	13.8	13.5
P7	14.3	14.2	13.9	14.1	14.4
P8	12.4	12.5	12.2	12.6	12.4
P9	12.9	12.9	12.7	13.4	12.8
P10	13.1	13	13.3	12.9	13.4

Table 3.3.9: Showing result of 0.4g PTsec test for coated silk fibers

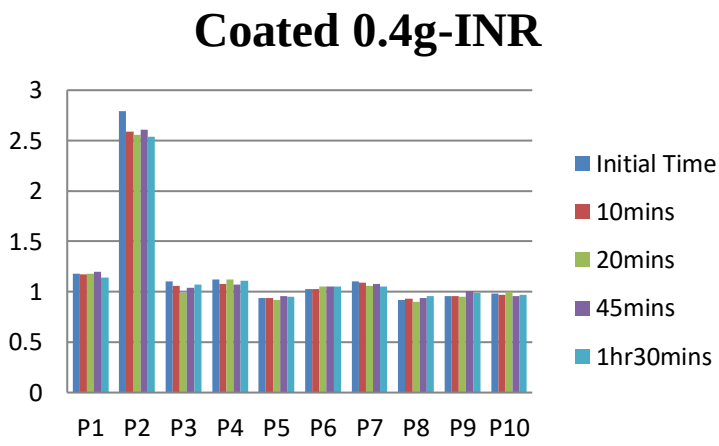


Figure 3.3.10: Graphical representation of 0.4g INR test for coated silk fibers where there is an increase in the value but with little variations due to factors from the sample of blood used.

INR	Initial Time	10mins	20mins	45mins	1hr 30mins
P1	1.18	1.17	1.18	1.2	1.14
P2	2.79	2.59	2.56	2.61	2.54
P3	1.1	1.06	0.99	1.04	1.07
P4	1.12	1.08	1.12	1.07	1.11
P5	0.94	0.94	0.92	0.96	0.95
P6	1.03	1.03	1.05	1.05	1.05
P7	1.1	1.09	1.06	1.08	1.05
P8	0.92	0.93	0.9	0.94	0.96
P9	0.96	0.96	0.95	1.01	0.99
P10	0.98	0.97	1	0.96	0.97

Table 3.3.10: Showing result of 0.4g INR test for coated silk fibers

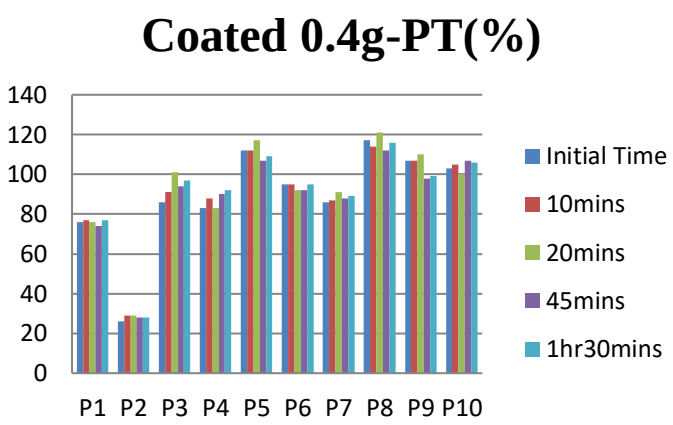


Figure 3.3.11: Graphical representation of 0.4g PT(%) test for coated silk fibers where there is an increase in the value of but with variations due to factors from the sample of blood used.

PT(%)	Initial Time	10mins	20mins	45mins	1hr 30mins
P1	76	77	76	74	77
P2	26	29	29	28	28
P3	86	91	101	94	97
P4	83	88	83	90	92
P5	112	112	117	107	109
P6	95	95	92	92	95
P7	86	87	91	88	89
P8	117	114	121	112	116
P9	107	107	110	98	99
P10	103	105	100	107	106

Table 3.3.11: Showing result of 0.4g PT(%) test for coated silk fibers

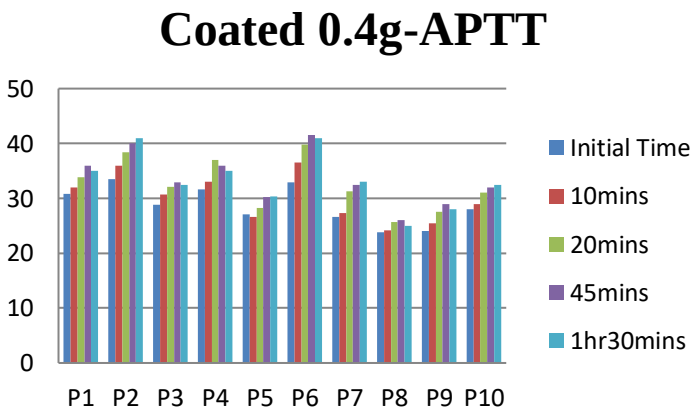


Figure 3.3.12: Graphical representation of 0.4g APTT test for coated silk fibers where there is an increase in the values but with great variations due to factors from the sample of blood used.

APTT	Initial Time	10mins	20mins	45mins	1hr 30mins
P1	30.8	32	33.8	36	35
P2	33.5	35.9	38.4	40	41
P3	28.8	30.7	32.1	32.9	32.4
P4	31.6	33	37	36	35
P5	27.1	26.6	28.3	30.2	30.3
P6	32.9	36.5	39.8	41.5	41
P7	26.6	27.3	31.3	32.4	33
P8	23.8	24.2	25.7	26	25
P9	24	25.5	27.5	29	28
P10	28	29	31.1	32	32.5

Table 3.3.12: Showing result of 0.4g APTT test for coated silk fibers

The coated and uncoated silk fiber samples were evaluated for their blood compatibility, using the hemocompatibility assays such as APTT, PT, and INR. Samples of the blood were centrifuged and used, the blood samples without the fibers were used as controls, and then the fibers were later cut and dropped into the samples and used as the study groups.

The result from the samples having the silk fibers showed a clotting time higher than the initial clotting time obtained from the samples that had no fibers inside. There were variations in the results obtained due to the different state of the blood samples where there could be ones lacking some clotting factors such as vitamin K. The different tables and their bars show a graphical representation of all the values obtained. The standard APTT time ranges from 23.6-35.2 seconds; this standard value varies from country to country or hospital to hospital. Moreover, in case the value obtained is greater than the standard values then we can say that there is great coagulation activity and thus anticoagulant drugs are needed.

PT% ranges from 70 -120%. As the PT percentage approaches 120 and above, it shows a great blood coagulation activity, and vice versa and drugs are needed to help reverse the situation.

The normal range of PT(sec) ranges from 11.5-15 seconds. Any increase in the time indicates an intake of an anti-coagulant drug or food that helps enhances the anticoagulation activity.

An increase in INR result above 0.80-1.20 based on the standard values from few laboratories,

Indicates anticoagulation activity, also its value is dependent on the PT sec, PT percentage and APTT values, so, therefore, patients with an increased values of PT and APTT, following a decreased PT percentage will have a high INR result which indicates poor blood clotting and those with low PT and APTT following an increase in PT percentage will show a reduced INR result which indicates blood coagulation activity. All these results are showing that the biomaterial silk fibers that were used are highly biocompatible with the blood because of their activity in the blood causing enhanced anticoagulant activity.

3.2.3 Scanning Electron Microscopy Analysis (SEM)

Scanning Electron Microscopy (SEM) was adopted to analyze the surface morphology of the silk braided sutures. The Scanning Electron Microscopy analysis was done at TÜBİTAK- Marmara Research Center at Gebze, Istanbul, Turkey by using a SEM Model Jsm- 6510 model at an acceleration voltage 10kV.

The result of the SEM at a magnification of X100 shows the braided silk fibers and how well the coatings were done on the surface of the fibers with points of knots clearly seen from the picture. At a magnification of X250 at 14.7mm, there is a clear separation of the coated material from the coating material.A clear view of the braided fibers and the chitosan substance that was used to coat the fibers. Moreover, at same magnification but at 15.3mm, the fibers were properly shown with their different coatings.

At a magnification of X500 and also at a magnification of X1000, the picture shows how smooth the surface of the braided fibers is as a result of the coating from chitosan.

At a magnification of X2500, X5000 and X10000, the pictures show the coating substance in their degradable form and how they degrade with time. All these results showed clearly the morphology of the braiding and coating that was done.

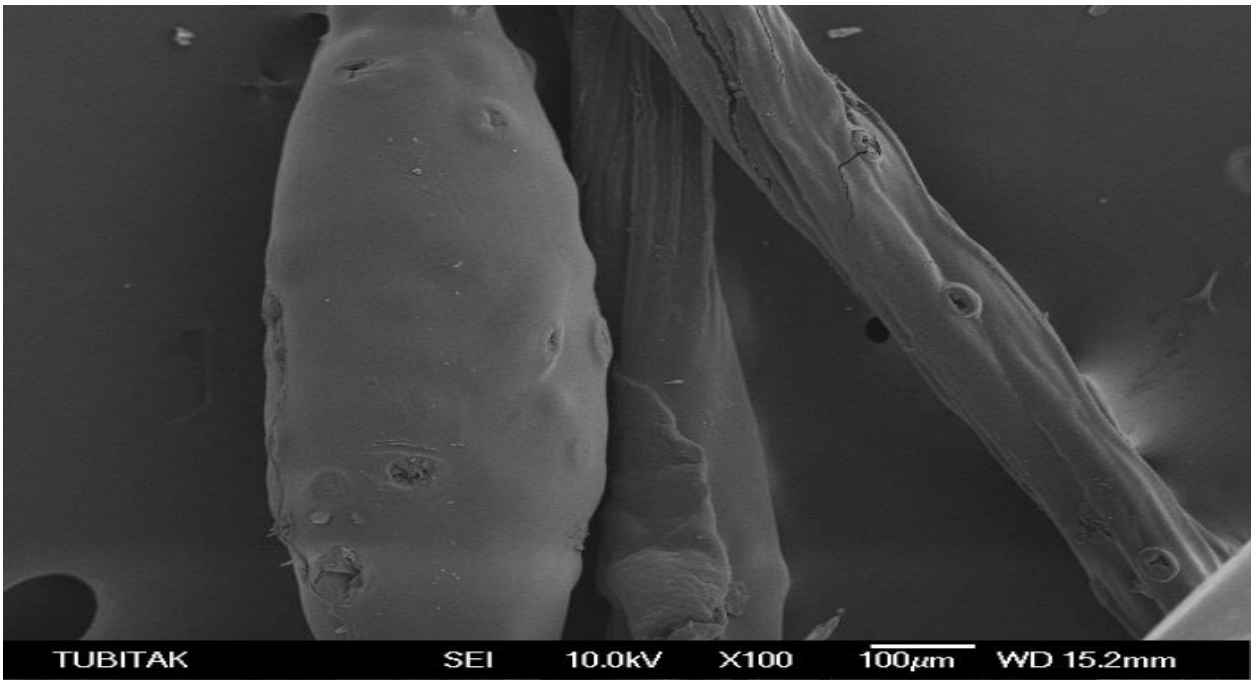


Figure 3.4.1: Showing SEM micrograph of coated silk fiber of 100µm at X100 magnification

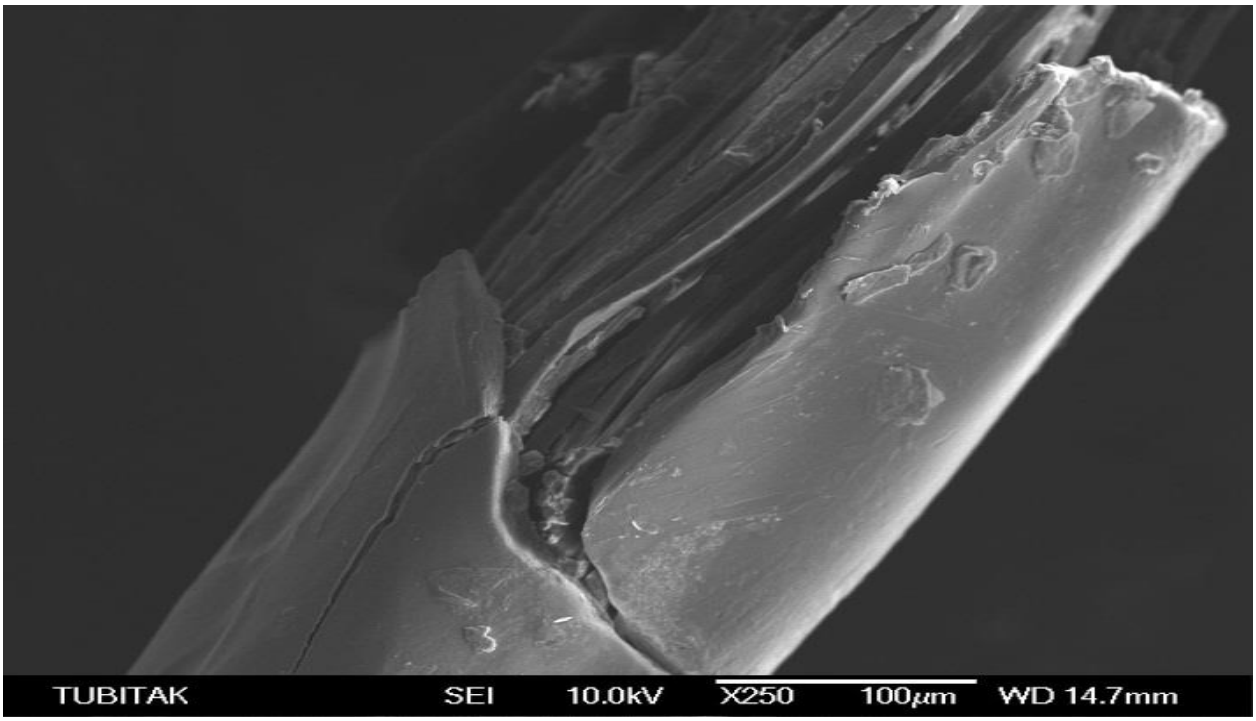


Figure 3.4.2: Showing SEM micrograph of coated silk fiber of 100µm at X250 magnification

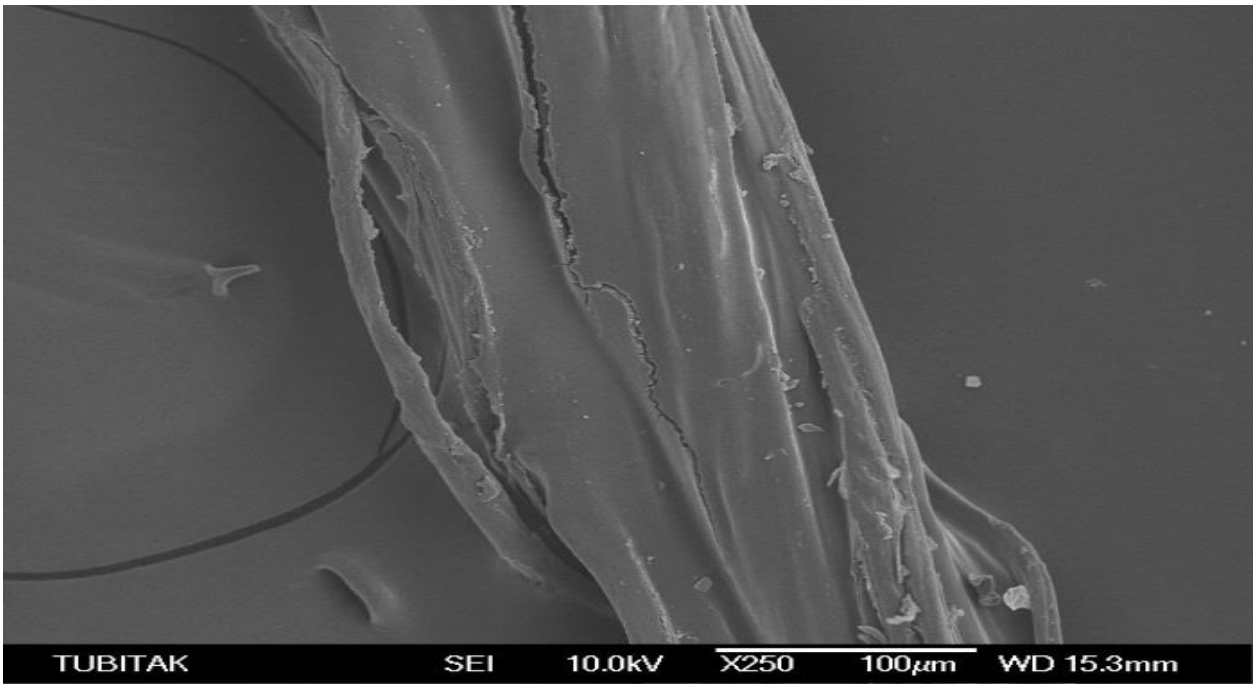


Figure 3.4.3: Showing SEM micrograph of coated silk fiber of 100µm at X250 magnification

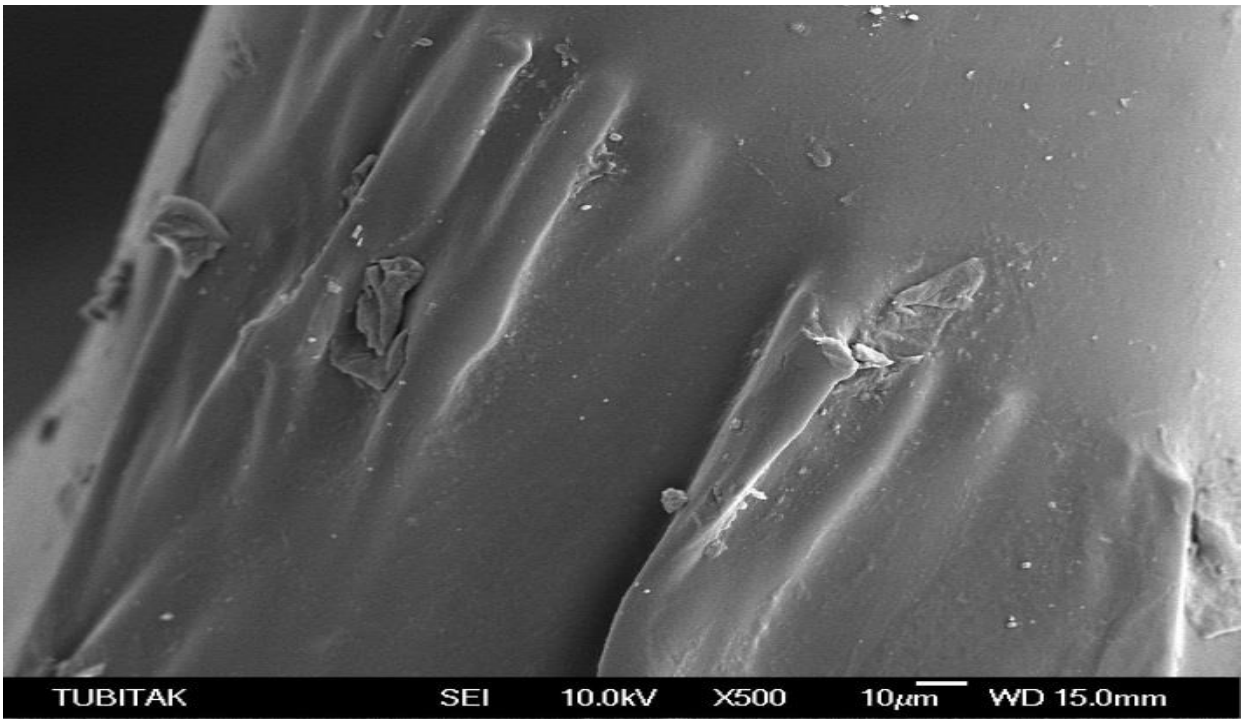


Figure 3.4.4: Showing SEM micrograph of coated silk fiber of 10µm at X500 magnification

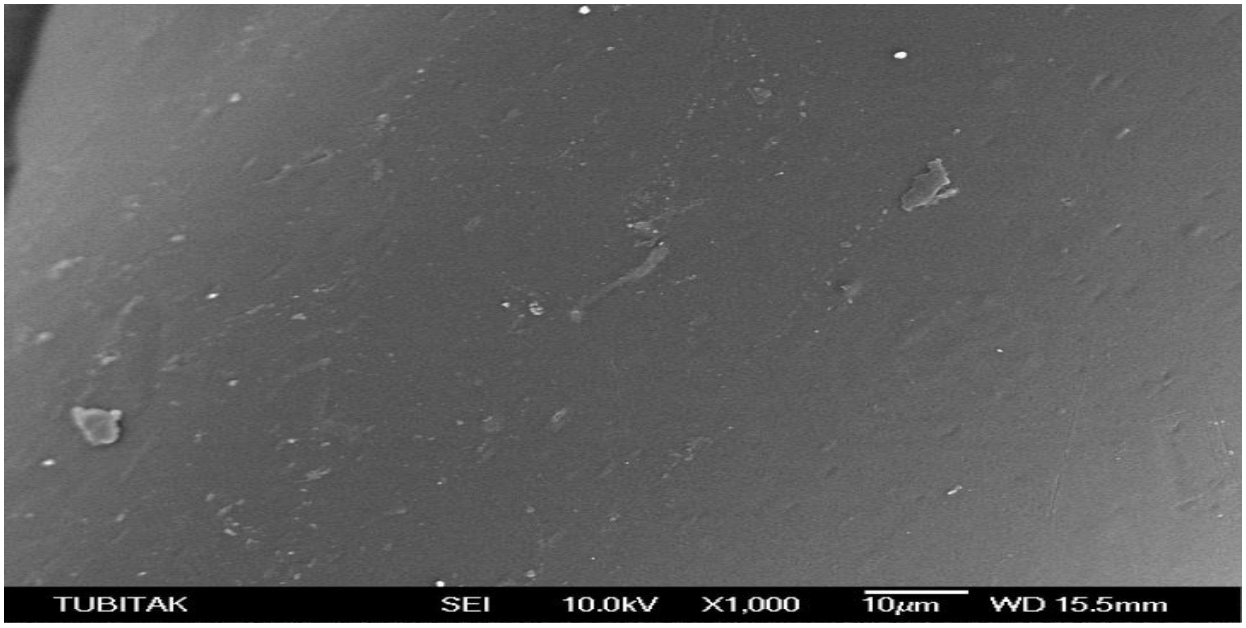


Figure 3.4.5: Showing SEM micrograph of coated silk fiber of 10µm at X1000 magnification

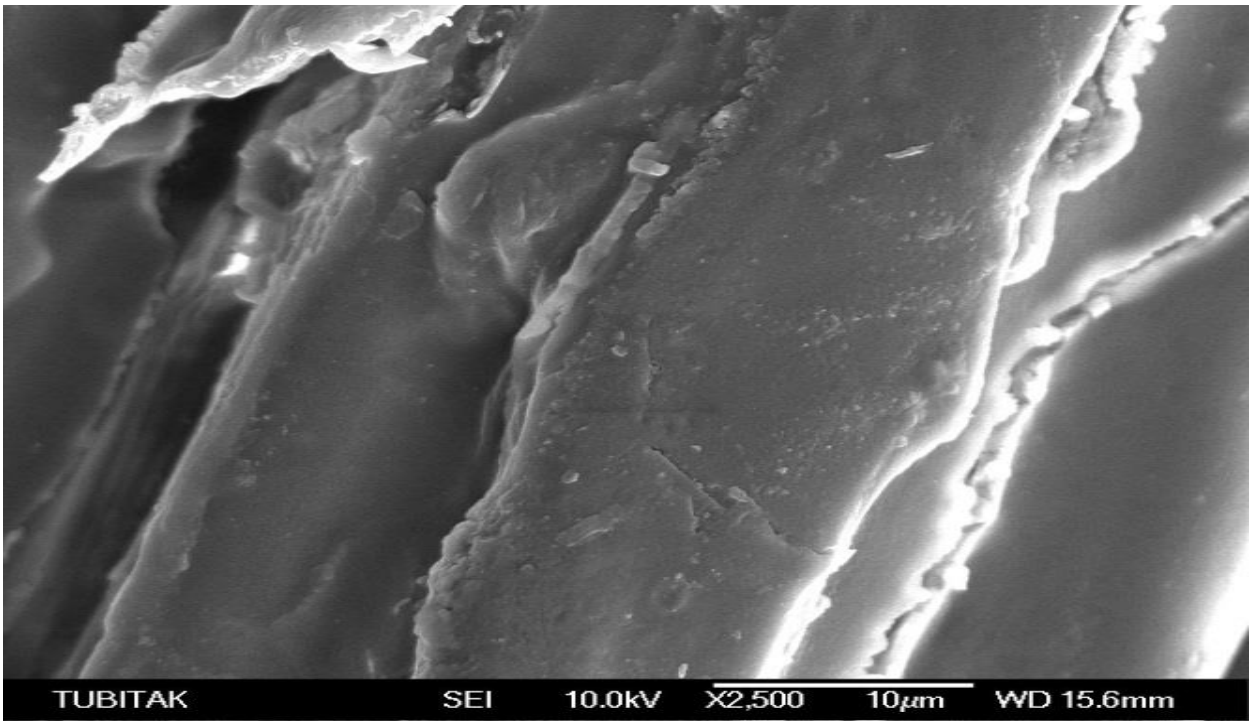


Figure 3.4.6: Showing SEM micrograph of coated silk fiber of 10µm at X2500 magnification

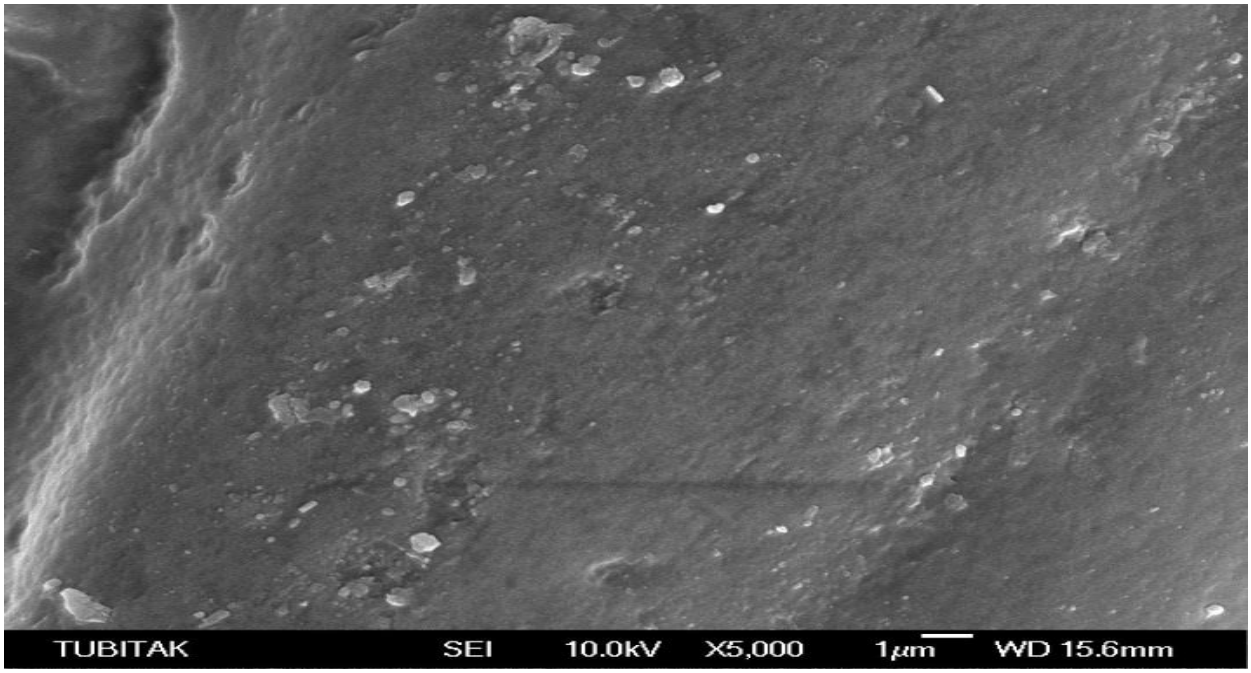


Figure 3.4.7: Showing SEM micrograph of coated silk fiber of 1µm at X5000 magnification

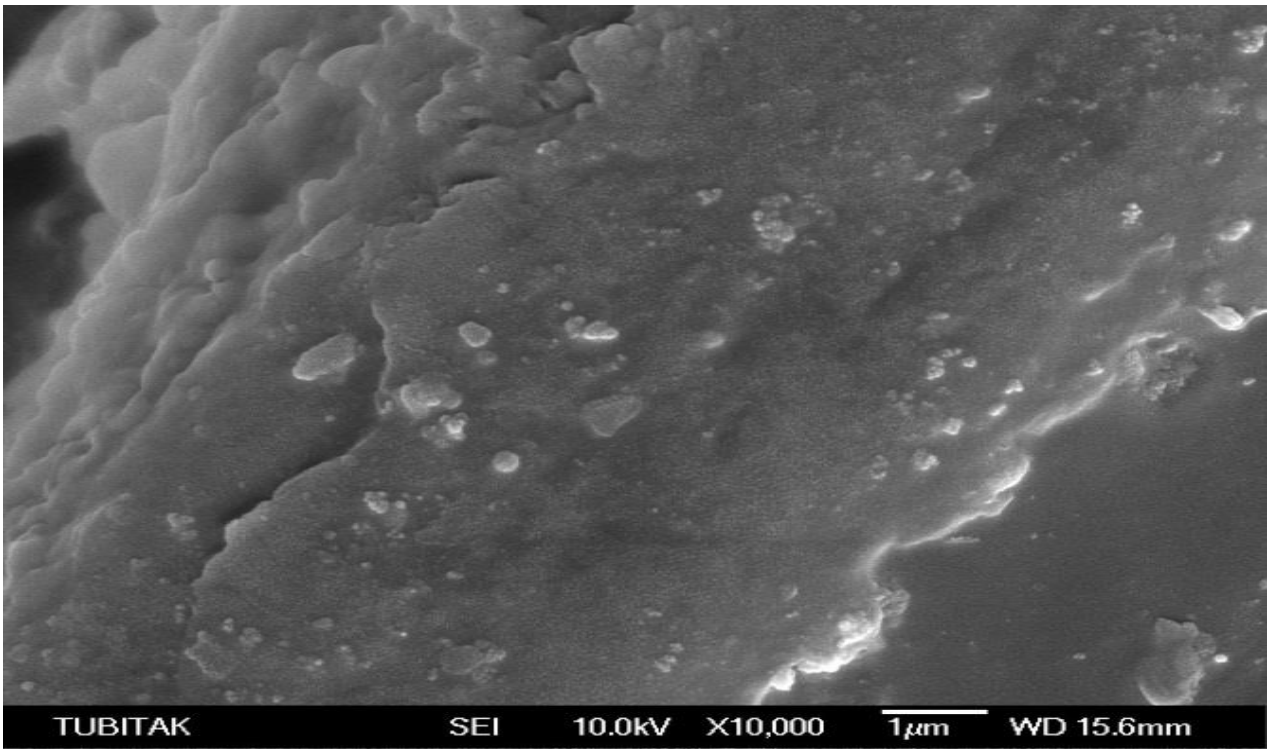


Figure 3.4.8: Showing SEM micrograph of coated silk fiber of 1µm at X10000 magnification

3.2.4 X-ray Diffraction Analysis (XRD)

XRD is a technique that is used to be able to determine the crystalline structure and the crystallinity nature in biomacromolecules or polymers. The crystalline phase in the biomacromolecules or polymers has distinct peaks arising from the diffraction of the X-ray. We can obtain the general structural information on the crystalline phase of the biomacromolecules or polymers, i.e., the unit cell parameters, the crystalline size and the crystallinity. The graph showed that the crystallinity and the crystalline nature of the silk fibers were affected by the coating substance which is the chitosan. Moreover, the chitosan gave the silk fibers an ordered structure.

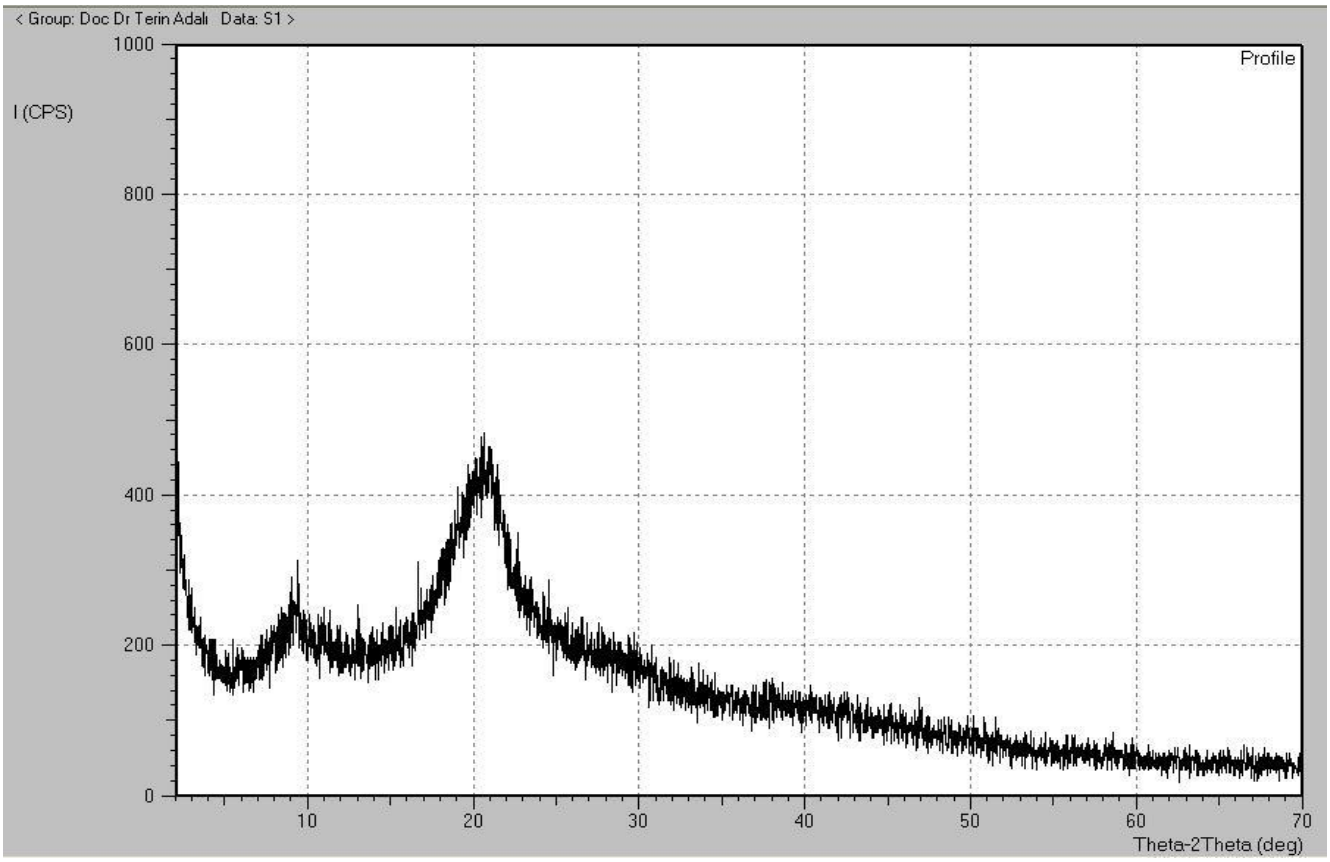


Figure 3.5: Showing the x-ray diffraction study that was carried out on the samples to study the crystalline structure of the silk fiber coated with chitosan.

The XRD pattern is shown in Figure 3.5 above and the strongest peak was observed at 2Θ= 21º, smaller peaks at 9º, 12º, 14º, 17º, 23º and 25º were also noted from the XRD raw data.

The peaks at 9º and 21º showed an interaction between silk fibers and chitosan. Pure silk fibers have been shown to be amorphous with no visible peaks. The XRD patterns results are in line with results from previous studies of Agostini de Moraes et al., where pure SF film showed an XRD pattern of amorphous nature.

3.3 Applications of the Study

Over decades of use, silk fibers have shown to be effective in many clinical applications. Tsubouchi (Wu et al., 1996) developed a wound dressing material from silk fibroin based biomaterial which is used to accelerate healing and can be removed without damaging the newly formed skin. Because of the properties of Silk proteins such as good biocompatibility, biodegradability and bioresorbability, it has promising advantages over other materials. Their physical, chemical properties can also be easily modified to achieve other characteristics such as mechanical and degradation characteristics (Stitzel et al., 2006). The silk sericin part of the silk fibers and the fibroin part are prospective wound healing agents and are anti-oxidant and also considered as bio-adhesive mediators of the human body (Dandin and Kumar, 2007). In the clinical treatment of skin defect, silk fibroin functions as useful material that promotes collagen synthesis and re-epithelialization. Furthermore, silk fibroin was considered to be proper for the generation of biomedical products such as blended materials because of its minimal adverse effects on the immune system (Rajendrana et al., 2012).

CHAPTER FOUR
CONCLUSION

4.1 Conclusion

The results that have been obtained and observed from this work showed that surface modification of the fibers increased its biocompatibility with the blood. Moreover, also increased its antimicrobial activity against microorganisms and bacteria and this is vital when used in tissues, wounds, and scaffolds. This work also showed that the silk fibers have antimicrobial activity in themselves but needs to be modified with other substances to be able to make them suitable for use in different biomedical applications.

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