MARYAME CHANA

IN VITRO VIABILITY ANALYSIS OF BREAST CANCER MCF-7 CELLS BY USING PULLULAN-ACETYL SALICYLIC ACID AND SILK FIBROIN- ACETYL SALICYLIC ACID

NEU 2020

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A THESIS SUBMITTED TO THE GRADUATE SCHOOL OF APPLIED SCIENCES OF

NEAR EAST UNIVERSITY

By

MARYAME CHANA

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

NICOSIA, 2020

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Approval of the Director of Graduate School of Applied Sciences

Prof. Dr. Nadire ÇAVUŞ

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I hereby declare that all the information in this document has been obtained and presented in accordance with the 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.

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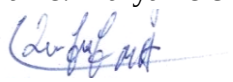
Signature: 
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To my parents…

ABSTRACT

Affecting just over 2 million women per year, furthermore guaranteeing the most substantial cancer-related deaths among women, Breast cancer is the most recurrent of its kind globally. The disease claimed 627,000 women's lives in 2018 alone, this speaking to 15 % of deaths brought about by cancer entirely. The core objective of this research work was to load acetyl salicylic acid (ASA) (Nonsteroidalz Anti-inflammatory drug) with pullulan (PL-ASA) and silk fibroin protein (SF-ASA) nano/micro particles and use it to analyze MCF-7 (differentiated mammary epithelium with estrogen receptors) breast cancer cells viability in vitro. The dropwise suspension polymerization method was used to prepare PL-ASA nano / micro particles, and ionic gelation method was used to prepare SF-ASA nano/micro particles. Characterization of PL-ASA and SF-ASA were analyzed by FTIR spectrophotometer, SEM. The cell viability was measured by using CCK-8 (TEBU-BIO cell counting) following manufacturer's protocol. The MCF-7 cells were incubated with different concentrations of ASA loaded PL and SF nano / micro particles for 48 and 72 hours. The statistical significance was observed for 80 % of PL-ASA and SF-ASA particles between 48 and 72 hours and 40 % in two different incubation periods, respectively. The results of this study demonstrated that, cell viability is only affected at longer incubation times with higher concentrations in non metastatic MCF-7 cell.

Keywords: Breast cancer, MCF-7 , Pullulan, Acetyl Salicylic Acid, Silk Fibroin, Drug delivery system.

ÖZET

Yılda 2 milyondan fazla kadını etkileyen ve ayrıca kadınlar arasında kansere bağlı en önemli ölümleri garanti eden meme kanseri, dünya çapında türünün en fazla tekrarlayan kanseri. Hastalık sadece 2018'de 627.000 kadının hayatına mal oldu ve bu, tamamen kanserden kaynaklanan ölümlerin % 15'ini ifade ediyor. Bu araştırma çalışmasının temel amacı, asetil salisilik asit (ASA) (Nonsteroidal Anti-inflamatuar ilaç) ile pullulan (PL-ASA) ve ipek fibroin proteini (SF-ASA) nano / mikro partiküllerini yüklemek ve bunu MCF- analizinde kullanmaktı. 7 (östrojen reseptörlü farklılaşmış meme epitelyumu) meme kanseri hücreleri in vitro canlılığı. PL-ASA nano/mikro parçacıkları hazırlamak için iyonik jelasyon yöntemi kullanıldı. PL-ASA'nın karakterizasyonu FTIR spektrofotometresi, ve SEM ile analiz edildi. Hücre canlılığı, üreticinin protokolünü takiben CCK-8 (TEBU-BIO hücre sayımı) kullanılarak ölçüldü. MCF-7 hücreleri, farklı konsantrasyonlarda ASA yüklü pullulan ve ipek fibroin nano/mikro parçacıkları ile 48 ve 72 saat süreyle inkübe edildi. İki farklı kuluçka döneminde sırasıyla 48 ve 72 saat arasında PL-ASA ve SF-ASA parçacıklarının %80'i ve % 40'ı için istatistiksel anlamlılık gözlendi. Dolayısıyla, bu çalışmanın sonuçları, hücre canlılığının sadece metastatik olmayan MCF-7 hücrelerinde daha yüksek konsantrasyonlarla daha uzun
inkübasyon sürelerinde etkilendiğini göstermiştir.

Anahtar Kelimeler: Meme kanseri, MCF7, Pullulan, Asetil Salisilik Asit, Ipek Fibroin, İlaç salinim sistemleri

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LIST OF ABBREVIATIONS

ASA:	AcetylSalicylic Acid
ATCC,Canada :	American Type Culture Collection
B. moxi :	*Bombyx Mori*
C_2H_5OH :	Ethanol
$CaCl_2$:	Calcium chloride
CCK-8 :	Cell Counting Kit-8
CHI:	Chitosan
CO_2 :	Carbon dioxide
DMEM/F12:	Dulbecco's Modified Essential Medium and Ham's F-12 medium
DTX. :	Docetaxel
ER. :	Estrogen receptor
FBS. :	Fetal Bovine Serum
FTIR. :	Fourier Transform Infrared Spectroscopy
HER2 :	Human epidermal growth factor receptor 2
$K_4[Fe(CN)_6]$:	Potassium ferric cyanide
MCF-7:	Differentiated mammary epithelium with estrogen receptors
ml:	Milliliter
MRI :	Magnetic resonance imaging
Na_2CO_3 :	Sodium carbonate
NaCl :	Sodium chloride
NPs :	Nanoparticles
NSIFD :	Nonsteroidal Anti-inflammatory Drug
PL :	Pullulan
PR :	Progesterone receptor
PTX:	Paclitaxel
QD:	Quantum-dot
SEM :	Scanning Electron Microscopy
SF :	Silk fibroin
StDev :	Standard deviations
TPP:	Sodium Triphosphate Pentabasic
V :	Volume
VSM :	Vibrating Sample Magnetometer
W :	Weight

CHAPTER 1:

Introduction

As the second leading cause of death internationally, the malignancy of cancer has alarmed the healthcare and healthcare research fields as a major public health concern. To date, researchers continue to praise chemotherapy as one of the most unpreceded cancer treatments but unfortunately cancer cells have created convoluted flagging systems to withstand apoptosis actuated by chemotherapeutics which compromises treatments. The therapeutic index of cancer treatment has been improved by simulating gene therapy with chemotherapy by interrupting various signaling routes of tumor cells dually, however this comes with a major challenge. Simultaneous delivery of genes and drugs usually used in cancer treatment requires specific host carriers due to the physiochemical properties of the genetic material that carries a negative charge. Over the years, innovative researchers have developed various target drug and gene delivery systems for ithe therapy and treatment of different cancers. Quantum-dot (QD) nanoparticles, fibers and structures, cancer targeting dendrimers, multilayered nano structures, mesoporous silica nano particles, polymeric structures iand liposomes are a few examples of the innovative delivery systems that have been designed in the recent years. Self-assembling core-nanomicelles and copolymers have seemingly sparked interest in the medical research field as a result of their biodegradability and biocompatibility properties. these structures have steered cancer killing drugs and gene therapy in a single shell carrier. To illustrate, polypeptide micelle nanoparticles (NPs) ito dually and systematically deliver siRNA-Bcl-2 and docetaxel (DTX) as an effective gene/drug dual-delivery vector were prepared by Cai et al from poly (ethyleneglycol)-b-poly(L-lysine)-b-poly (L-leucine). The data of this study is evidence that a co-delivery system of siRNA i–Bcl-2 and docetaxel have an improved potential invitro in vivo as an antitumor agent when compared to the drug and gene being used separately. Another research approach by innovated by Yin et al displays a co-delivery hyaluronic acid-based amphiphilic conjugate (HA-ss-(OA-gbPEI),HSOP) to deliver AURKA specific siRNA (si-AURKA) as the gene and pactitaxel (PTX) as a triplicated therapy for breast cancer.

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An amphiphilic bifunctional derivative of the biopolymeric material pullulan synthesized by Chen and colleagues by grafting deoxycholic acid and polyethylenine onto the pullulan and doxorubicin was encapsulated by self-assembly process. They discovered that the designed effective chemotherapeutic agent is a promising agent for improving antitumor inefficacy and systemic toxicity for cancer therapy. Sherly et.al has been synthesize and characterize actionized pullulan and diethyl aminoethyl methacrylate grafted dextran. These complexes have been found to be preferable conjugates that act as transfecting agents, have impressive biocompatibility, cytocompatibility, sustained reral clearance with cancerous cells.

Erlotinib loaded silk fibroin nanoparticles with a self-assembling efficiency exhibited low and prolonged cytotoxicity that drug free silk fibroin nanoparticles, this will be an innovative approach that increases the potential in breast cancer treatment. Galam et.al recently encapsulated carboplatin with silk fibroin microparticles as a cancer therapy approach, a staggering spike in dose dependency in absorbance of MCF-7 cell apoptosis was observed through in vitro apoptotic screening. (Galam, Tulay, & Adali, 2020).

Among all breast cancer cell lines, Differentiated mammary epithelium with estrogen receptors. Breast cancer cells with positive-Oestrogen receptors (ER) and a multitude of its sub-clones have been studied globally using (MCF-7) cells as they are promising candidates of research.

Using the (MCF-7) cell line for research, differentiated mammary epithelium with estrogen receptors has been propagated for many years by multiple groups and it’s the most regularly used line in laboratory research worldwide as it is usually known to have low metastatic potential, and is a poorly-aggressive and non-invasive cell line.

In experimental works for drug delivery system they used too many biopolymers, nanoparticles, and proteins loaded drugs. pullulan, a naturally occurring homo-polysaccharide with a linear structure is formed by recurring maltotriose units that are amalgamated by α-1, 6 glucosidic bonds. pullulan has favorable characteristics such as non-carcinogenicity, non-immunogenicity, non-mutagenicity and susceptibility to simple modification by chemicals which has drawn a lot of research attention in its use for drug delivery fabrication systems. (Chen et al., 2017).

Verification of synthesis for the drug carrier is shown using the characterization techniques of Scanning Electron Microscopy (SEM), Fourier Transform Infrared Spectroscopy (FTIR).

1.1 Aim of the Study:

This project's primary objective is synthesizing and characterizing PL and SF natural biopolymers micro / nano particles loaded with acetyl salicylic acid (ASA) and a nonsteroidal Anti-inflammatory Drug (NSIFD). The drug delivery system will be used to analyze the apoptotic effects of MCF-7 breast cancer line cell.

1.2 The Importance of the Thesis:

Globally amongst women, breast cancer is the most predominant disease which makes it a major public health concern. With an aim to help prevent death of human beings from breast cancer, the new (SF-ASA and PL-ASA) particles were prepared for the first time ever for treatment of cancer cell line (MCF-7) required by the biopolymers. In our knowledge PL and SF were used for the first time to prepare drug delivery system for breast cancer therapy by using acetic salicylic acid (ASA) as a therapeutic agent on cell line MCF-7. The importance of the thesis is to design a novel drug delivery system from natural available biopolymers such as: PL, SF, and encapsulated acetyl salicylic acid for *in vitro* viability test.

1.3 General objective:

The intended research aim is to synthetize and characterize acetyl salicylic acid loaded pullulan and silk fibroin micro / nano particles and analyze the viability effects of MCF-7 breast cancer cells.

1.4 Specific objective:

- Description of breast cancer disease, therapy and cell line of MCF-7.
- Design a novel drug delivery system by using natural biomaterials and simple methods to be able to minimize side effects during the therapy.
- *In vitro* cell viability of MCF-7 cell line.

1.5 Thesis outline:

Chapter 1 provided a general information, aim, importance, general and specific objective of the thesis. In chapter 2, literature review related to breast cancer, different types of cell lines and biopolymers are explored. The third chapter elaborates on methods and materials used in this work. The fourth chapter renders a general description of the results as well as the discussion section. Chapter 5 draws a conclusion of the research.

CHAPTER 2:

Literature review

2.1 Breast cancer:

The breast is comprised of various tissues extending from adipose tissue to dense breast tissue. Inside these tissues there is a system of projections, each mammary flap is comprised of small, lobules containing milk secreting glands, minuscule channels associate the organs, lobules, and projections, conveying milk from the flaps to the areola. The nipple is situated at the center of the areola, which is the more obscure region that encompasses the areola. lymph and blood vessels additionally pervade the breast where blood feeds the cells. The lymph framework depletes in essence metabolicansties. Breast tissue lymph vessels associate with lymph nodes, the little, bean-molded organs that help battle breast infection.

Breast cancer develops when non-diseased breast tissue cells mutate and over produce beyond control, forming a lump or sheet of tissue called a tumor, these tumors may be malignant or benign. A tumor can be malignant or benevolent. Metastasis is the spread of disease and over produced cells to other parts of the body through the veins or potentially lymph vessels. The three stages of breast cancer, stage I, II, and III depict where the disease is found, how much the disease has developed, and the areas the disease have spread to. In spite of the fact that breast cancer most ordinarily metastasizes close by lymph nodes, It can likewise ispread further through the body to regions, for example, the liver, bones, lungs, and brain. This is called metastatic or stage IV breast cancer growth.

Recurrent breast cancer after the first dose of treatment is indicative of local cancerous malignancy of the lymph nodes.

2.2 Breast cancer cell structure:

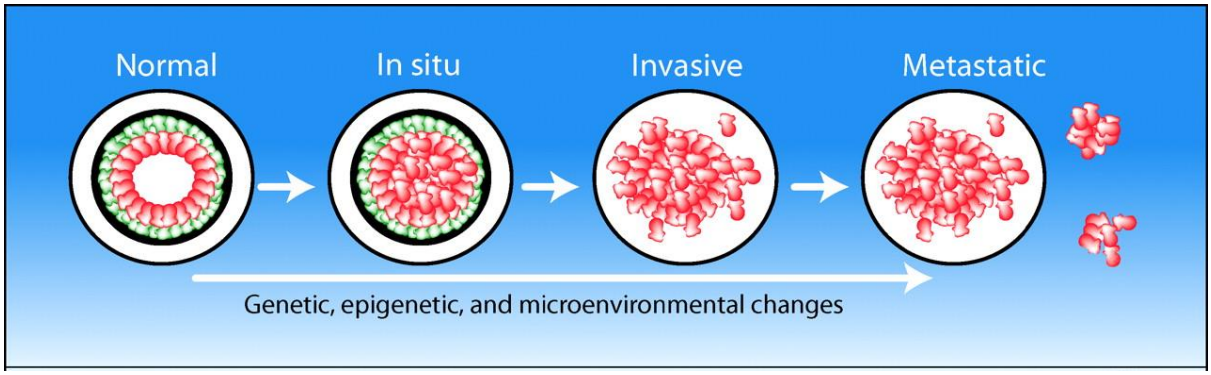

Figure 2.1: Progression of breast cancer cells (Polyak, 2008)

An innovation utilizing (2-disopropylamino ethyl methacrylate) polymer that's highly sensitive to pH as polymer as the internal center and lipid-polyethylene glycol as the external shell exhibited elaborate blood dissemination, upgraded tumor focusing on and entrance, quick intracellular medication and effective hindrance of tumor development was watched (Yao et al., 2019).

To maintain a strategic distance from harm to typical cells, the malignancy treatments ought to be explicit to its target. Chemotherapy, medical surgery and radiotherapy are fundamentally the three treatment modalities for malignant growth. Targeted drug delivery systems with the utilization of biomaterials, especially with the attributes of good biocompatibility, biodegradability, shrewd medication conveyance frameworks, are required for these treatments (Tulay, Galam, & Adali, 2018).

2.3 Types of breast cancer cell lines:

Michigan Cancer Foundation-7 refers to the MCF-7 cancer cell line that was samples from a 69-year-old Caucasian woman in 1970. The full name of the cell line alludes to the organization in Detroit, the origination of where the cell line was set up in 1973 by Herbert Soule and colleagues. MCF-7 held a few attributes of separated mammary epithelium, inclusive of the capacity to process estradiol by means of cytoplasmic estrogen receptors and the ability of shaping vaults.

2.4 Biomaterials used in drug delivery systems:

2.4.1 Silk fibroin protein:

Silk is natural protein produced from silk cocoons of *Bombyx mori* (Adalı & Uncu, 2016). The hydrophobic B-sheet arrangements grant them robust physical, mechanical toughness and strength. Silk is the best biomaterial due to its molecular arrangements allowing to engineer and modify the electrode surface for the biosensor application with biological and chemical functionalities and they stabilize the bio receptors or immobilization incorporated with its active site for the improvements of selectivity and sensitivity the electrodes (Shan et al., 2018). Silk fibroins (SF) are composed of natural proteins (Cao, Wang, & Zhang, 2013). Silk fibroin have low immunogenicity and biocompatibility due to their chemical and physical properties achieved by alternating the polyelectrolyte deposition on the electrode surface for the immobilization of enzyme (Shen, Hu, Guan, & Wang, 2015).

The silk is considered as an outstanding biomaterial because of its great biocompatibility, flexibility, low thrombogenic, biodegradation, high tensile strength, elasticity, and a good degree of toughness that supports bio receptors attachment, support and proliferation (Thu-Hien Luong1 et al., 2015).

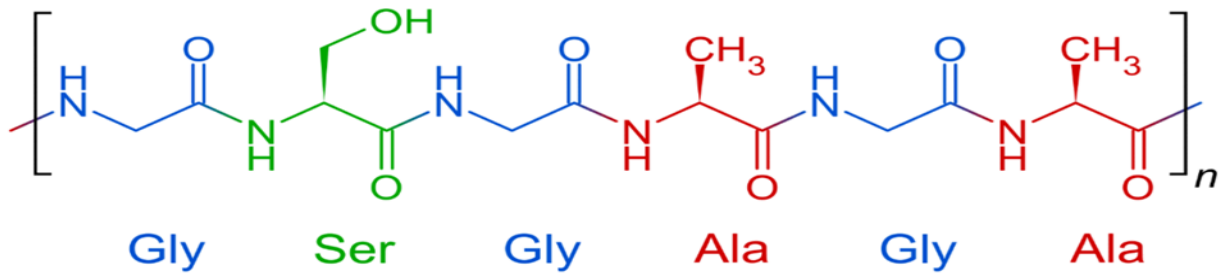

Figure 2.2: Chemical structure of Silk Fibroin (Houacine, Yousaf, Khan, Khurana, & Singh, 2019)

The silk is a biomaterial which is a biocompatible, biodegradable and due to its chemical, physical and mechanical properties is used for the immobilization of bio receptors. In its raw form the fibers of the silk cocoons are held together by a gum like protein substance sericin. The binding protein is removed by the degumming process and the remaining fibroin is used for the immobilization of bio receptors. The fibroin is composed of beta-sheets with a parallel arrangement or amino acid structure with serine (12 %), alanine (30 %) and acid glycine (43 %) and contains fat, wax and mineral salt. The composition of silk fibroin makes for the

immobilization of bio receptors thermodynamically stable, remarkable mechanical tensile strength proteins (Naksupan, Saelim, Pornarin, & Niwat, 2012).U.S FDA approved this biomaterial for medical uses and for designing of biosensor diagnostic equipment. It is also used in scaffolds, applications of tissue engineering and targeted drug delivery systems (Zhang et al., 2017).

2.4.2 Chitosan properties:

Chitosan is a cationic polysaccharide made out of haphazardly conveyed β-(1→4)- connected D-glucosamine (deacetylated unit) and N-acetyl-D-glucosamine (acetylated unit), obtained from marine chitin which is the second most bountiful polysaccharide on the planet, after cellulose. Chitosan is biodegradable, biocompatible and non-harmful, with the goal that it very well may be utilized in clinical applications, for example, antimicrobial and wound mending biomaterials. It likewise utilized as chelating operator because of its capacity to tie with cholesterol, fats, proteins and metal particles. It has positive charges in acidic medium, because of protonation of amino gatherings, and it can tie with negative deposits in the mucin, that lead to improve mucoadhesive properties. Due to chitosan's capacity to work in numerous structures it has numerous regions of enthusiasm inside the clinical business including orthopedic and periodontal applications, tissue building, wound Healing and Drug Delivery (Ibrahim & El-Zairy, 2015)

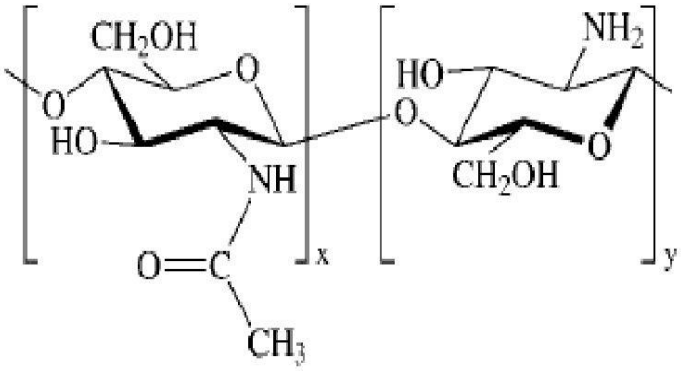

Figure 2.3: Chemical structure of Chitosan (Ibrahim & El-Zairy, 2015)

2.4.3 Pullulan properties:

Among the various drug delivery materials accessible up until now, polysaccharides speak to extremely alluring atoms as they can experience a wide scope of compound alterations, are biocompatible, biodegradable, and have low immunogenic properties. Subsequently, polysaccharides can add to altogether conquer the confinement in the utilization of numerous kinds of medications, including hostile to malignant growth drugs (Posocco et al., 2015).

Pullulan is a naturally occurring polymer delivered monetarily by yeast like growth Aureobasidium pullulans. It is non-harmful, non-immunogenic, non-cancer-causing and non-mutagenic in nature. The structure of pullulan comprises one of a kind linkage design with two α-(1 → 4) and one α-(1 → 6) glycosidic bonds in maltotriose rehashing units (G3). Pullulan invests unmistakable physical qualities because of the nearness of nine hydroxyl bunches on glucopyranose rings of G3 units. It very well may be derivatized in different structures by subbing these hydroxyl gatherings to improve its utilization in biomedical applications. Pullulan and its subordinates are totally investigated for their applications in food and pharmaceutical businesses. Inferable from these uncommon properties, local pullulan and its subordinates have expected application in various diagnostics (Singh, Kaur, Rana, & Kennedy, 2017).

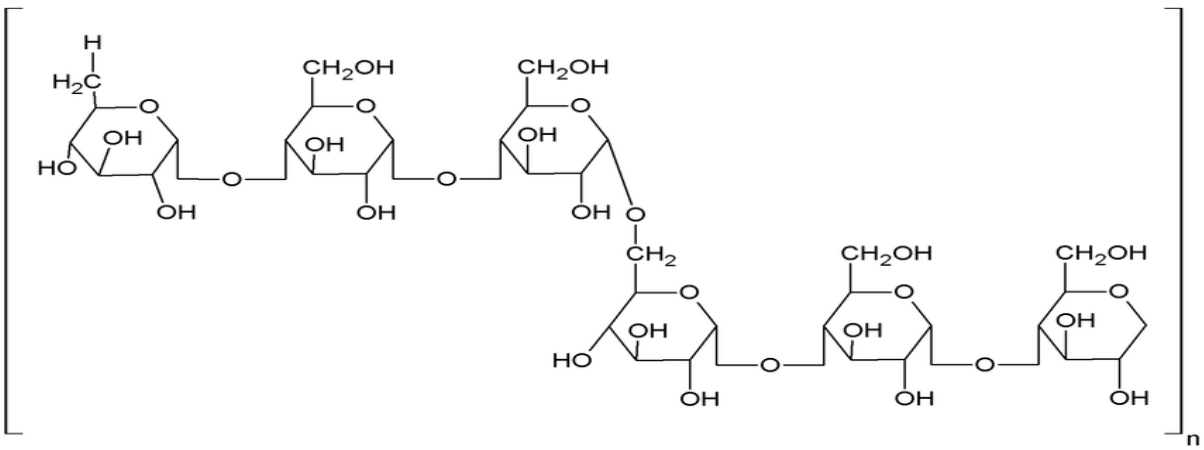

Figure 2.4: Chemical structure of Pullulan (Ferreira, Velasquez, Schaffazick, & Cruz, 2015)

CHAPTER 3:

Materials and methods

3.1 Materials:

*Bombyx Mori (B. moxi)*cocoons obtained from villages in Northern Cyprus, sodium carbonate (Na_2CO_3), potassium ferric cyanide ($K_4[Fe(CN)_6]$), calcium chloride ($CaCl_2$), and ethanol (C_2H_5OH, 98 %), sodium chloride (NaCl), Pullulan (PL) acetic salicylic acid (ASA) MCF-7 cell line was cultured in Dulbecco's Modified Essential Medium and Ham's F-12 medium (DMEM / F12) supplemented with 10 % Fetal Bovine Serum (FBS) and 1 % penicillin / streptomycin mixture 37 in a 5 % carbon dioxide (CO_2) incubator.

3.2 Methods:

3.2.1 Purification of silk fibroin protein:

3.2.1.1 Cleaning process:

The silk is natural abundant protein, nontoxic purify and is obtained from silkworms and it produces the final product of silk fibroin protein through the scientific silk fibroin preparation steps. The cocoons are cheap and they can be purchased from local markets. The surface of the silk cocoons may have unwanted insects and materials like pupa, dust, impurities, and other foreign particles that may affect the result of the experiment so, these parts must be cleaned before cutting into pieces for the next degumming process.

a) Silk cocoons **b) Silk cocoons cut into pieces**

Figure 3.1: Preparation of *Bomboxy Mori* cocoons for degumming

3.2.1.2 Degumming process:

Silk degumming is the process of eliminating the glue protein and sticky substance called sericin which is a glue/gum bond together the silk fiber which have an effect on material characteristics of silk fibroin.

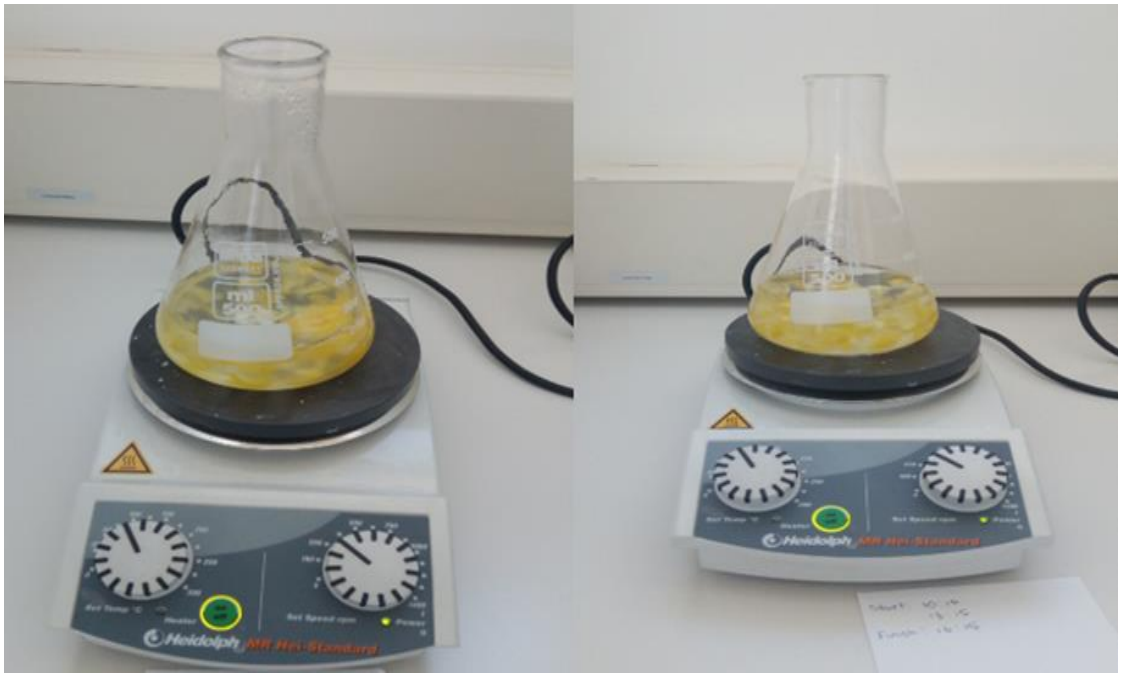

Figure 3.2: Degumming of silk fibroin cocoons with 0.1 M Na_2CO_3

The most common method in degumming is to remove and eliminate the sericin by sodium carbonate. The sericin protein which is the glue-like substance cover or coat the fibroin which can be removed by the thermochemical treatment of the cocoons is also known as degumming. For the preparation of the sodium carbonate solution; we weighed 12 grams of sodium carbonate and mixed it with 200 ml deionized water and then mixed it further by the use of the magnetic stirrer to dissolve homogeneously. We also weighed 2-grams of cut silk cocoons, inserted into the flask and then added 200 ml of sodium carbonate solution.

An electro-spurned magnetic stirrer was used at 75 ℃ for three hours and three rounds. The sodium carbonate solution was changed at each round for three times. The silk fibers were washed with deionized water more than 3 times in each round until the yellow color disappeared and the residue of deionized water was sufficiently clear. After each three-hour degumming process, fibers were unbounded into small pieces and replaced into petri dish overnight to dry at room temperature. This process was followed by the dissolution process.

3.2.1.3 Dissolution process:

The dried silk fiber mixed together with the strong electrolyte solution. The dissolution process was carried out by measuring the ration of $CaCl_2$: C_2H_5OH: nH_2O (calcium chloride (27.79 g)), ethanol (29.13 ml), deionized water (36 ml) at 75 ^oC by a continuous stirring process until the total dissolution. By altering the W/V (weight of the fiber to the volume of electrolyte solution) different aqueous silk fibroin concentrations can be obtained and the dried fibers mix together. After this stage the silk fibers dissolved into the solution.

Figure 3.3: Dissolution and drying process of silk fibroin fibers

3.2.1.4 Dialysis process:

Dialysis is the process of removing salt from the liquid silk fibroin protein solution and includes the final steps for the purification of pure silk fibroin solution. The biopolymer solution was poured into the dialysis tube and then larger beakers were used which totally immersed the dialysis tube and were filled with deionized water.

The dialysis process was carried out for the 48 hours and changed the deionized water within three hours. After 48 hours dialysis processes the pure biopolymer silk fibroin solution was extracted from the dialysate by using the large surge and then poured into the bottle.

Figure 3.4: Dialysis process for silk fibroin protein

3.3 Ionic gelation:

Calvo et al.in 2020 portrayed the IG technique for the nano-encapsulation of proteins. The writers introduced a research with detail for the study of silk fibroin nanoparticles within the sight of sodium tripolyphosphate (TPP). This specific response exploits the cationic conduct of SF within the sight of weakened acids and the polyanionic character of TPP. Emphatically charged amino gatherings crosslink within the sight of contrarily charged phosphate gatherings to frame hydrogels. By continually blending the blend in a particular scope of focuses for every reagent, the nanoparticles show up unexpectedly.

The bases of the technique utilized by Calvo et al. (2020) stay unaltered and have been applied to various sets of counter particles. Figure 3.5 is a schematic portrayal of the IG technique performed by two unique highways, An and B. Highway A shows a method by which the polymeric arrangement is recently blended in with the dynamic head and later added to the counter particle arrangement utilizing a needle siphon. By course B, the polymeric arrangement is first blended in with the dynamic head and later gets the counter particles which are included dropwise. The two courses can be utilized for cationic or anionic polymers (Pedroso-Santana and Fleitas-Salazar, 2020). In our investigation highway A was followed as demonstrated as follows:

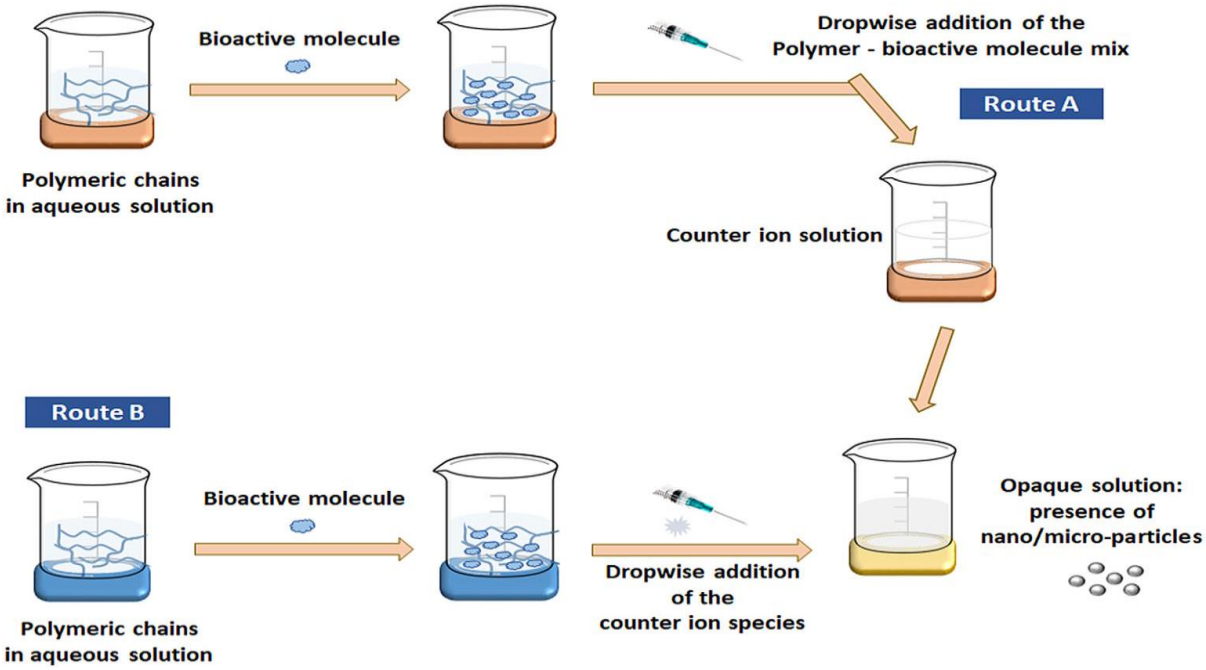

Figure 3.5: General schema of ionic gelation method (Pedroso-Santana & Fleitas-Salazar, 2020)

In this study the ionotropic gelation was used for 2 % PL and 3 % SF solution.

The 0.1 M sodium triphosphate pentabasic (TPP) solution was prepared by mixing 1.84 g of TPP in 50 mL of pure distilled water.

The optimum composition of samples was shown on Table 3.1.

Sample names	2 % PL (mL)	3 % SF (mL)	0.1 % ASA	0.1 M TPP (mL)
TA01	3	----	1.5	10
SF01	----	3	1.5	10

Table 3.1: Composition of PL-ASA and SF-ASA micro particles

0.1 M of acetic salicylic acid solution was prepared by mixing powder of ASA with 50 ml of pure distilled water.

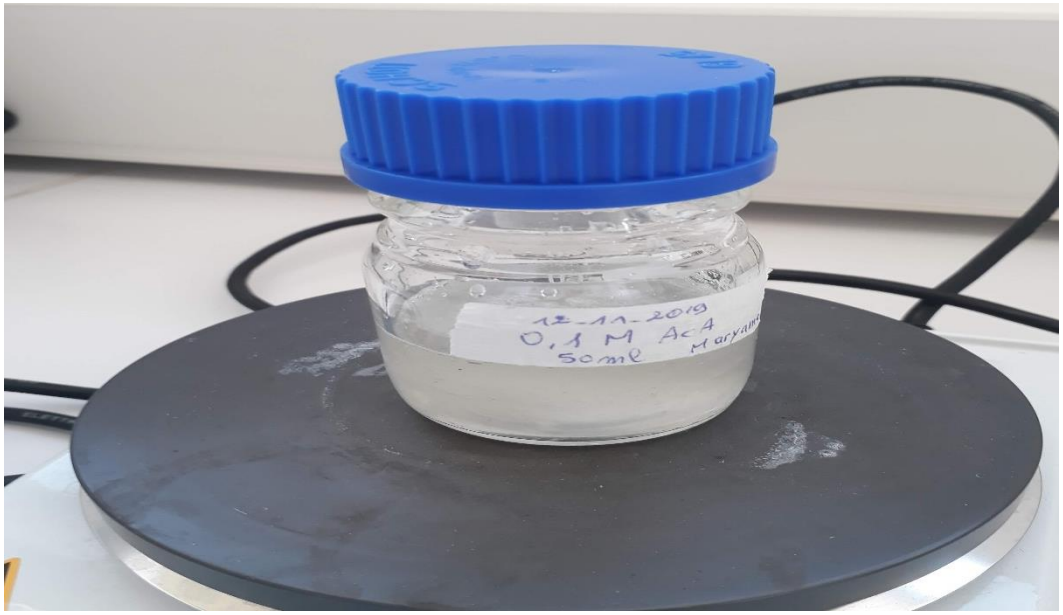

Figure 3.6: Acetic Salicylic Acid solution

3.4 *In vitro* MCF-7 cell viability Analysis:

Cell culture was performed according to the manufacturer's protocol American Type Culture Collection (ATCC, Canada) with slight modification using human breast cancer cell line MCF-7 (differentiated mammary epithelium with estrogen receptors) that were cultured in Dulbecco's Modified Essential Medium and Ham's F-12 medium (DMEM / F12) supplemented with 10 % Fetal Bovine Serum (FBS) and 1 % penicillin/streptomycin mixture 37 in a 5 % Carbon dioxide (CO_2) incubator. Approximately 10,000 cells were seeded in 96 well plates and 24 hours after seeding, MCF-7 cell was cultured with three different concentrations of PL and SF encapsulated ASA, nanoparticles ranging from 90 % to 20 % for 48 and 72 hours. Cell Counting Kit-8 (CCK-8) (TEBU-BIO cell counting) was used to measure the cell viability following manufacturer's protocol. Excel program was used for all statistical analyses.

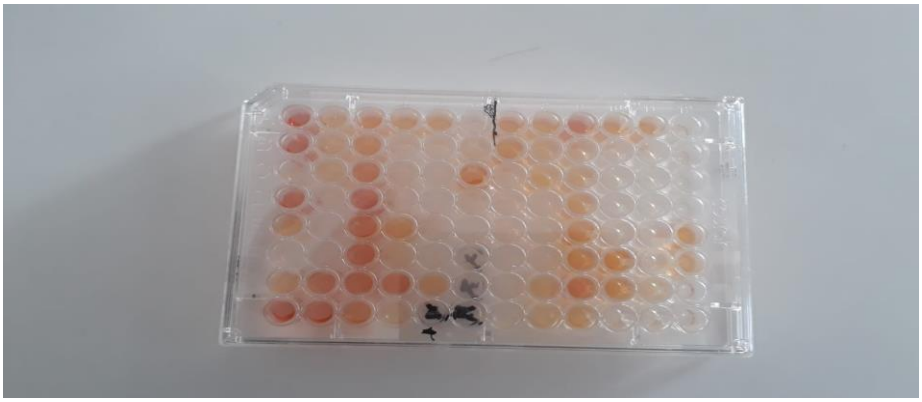

Figure 3.7: *In vitro* viability experiment results both for PL-ASA and SF-ASA after 48h

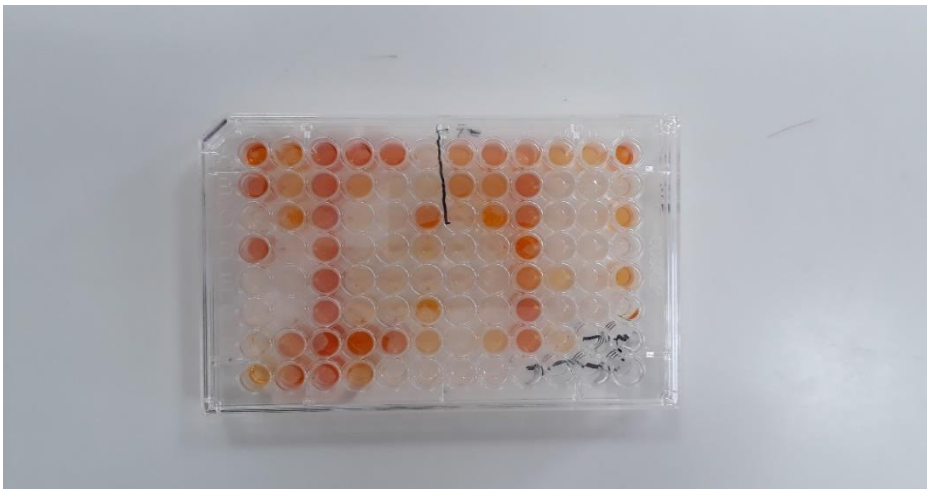

Figure 3.8: *In vitro* viability experiment results both for PL-ASA and SF-ASA after 72h

To read the cell apoptotic effects the plates were placed in the microelectronic reader:

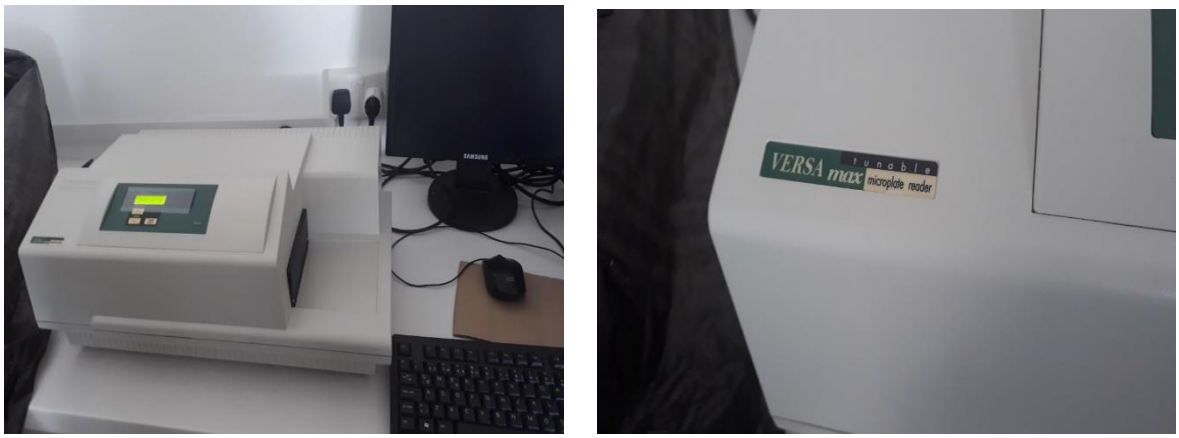

Figure 3.9: VERSA max micro plate reader Tunable

After reading the absorbance values results were analyzed by using excel software.

CHAPTER 4:

Results and discussions

4.1 Results:

The following tables illustrate the mean and standard deviation of the *in vitro* viability of Pullulan-acetyl salicylic acid and silk fibroin-acetyl salicylic acid after 48 hours and 72 hours.

Control Groups:

A4: Acetyl Salicylic Acid (ASA) in 48 hours; **A7:** Acetyl Salicylic Acid (ASA) in 72 hours (1; 10; 50)

P4: Pullulan+TPP in 48 hours; **P7:** Pullulan+TPP in 72 hours (25:75); (50:50); (75:25)

S4: Silk fibroin+TPP in 48 hours; **S7:** Silk fibroin+TPP in 72 hours (25:75); (50:50)

Test Groups:

PA4: Pullulan+ASA+TPP in 48 hours; **PA7:** Pullulan+ASA+TPP in 72 hours

P41: P71 (25:75); P42; P72 (50:50); P43; P73 (100:1)

SA4: Silk fibroin+ASA+TPP in 48 hours; **SA7:** Silk fibroin+ASA+TPP in 72 hours

S41: S71 (25:75); S42; S72 (50:50); S43; S73 (100:1)

	Control groups				Test groups	
	A4	A7	P4	P7	PA4	PA7
10 :90	3,030	2,351	***	***	***	***
	3,033	2,353	***	***	***	***
	3,041	2,355	***	***	***	***
	3,064	2,356	***	***	***	***
	3,075	2,358	***	***	***	***
	3,077	2,360	***	***	***	***
	3,084	2,362	***	***	***	***
	3,085	2,364	***	***	***	***
Mean	3,061	2,357				
StDev	0,023	0,004				
25 :75	***	***	1,033	1,849	1,863	3,552
	***	***	1,041	1,851	1,865	3,555
	***	***	1,064	1,855	1,867	3,557
	***	***	1,075	1,859	1,870	3,559
	***	***	1,076	1,861	1,873	3,561
	***	***	1,084	1,863	1,877	3,565
	***	***	1,084	1,867	1,879	3,566
	***	***	1,085	1,870	1,880	3,568

Mean			1,068	1,859	1,872	3,560
StDev			0,020	0,007	0,007	0,006
50 :50	0,740	0,539	0,891	1,213	1,753	3,355
	0,745	0,541	0,899	1,215	1,755	3,357
	0,755	0,543	0,900	1,218	1,756	3,358
	0,765	0,545	0,909	1,220	1,759	3,361
	0,765	0,547	0,910	1,223	1,762	3,363
	0,778	0,550	0,914	1,224	1,764	3,365
	0,780	0,551	0,920	1,227	1,766	3,366
	0,781	0,553	0,922	1,230	1,768	3,368
Mean	0,764	0,546	0,908	1,221	1,760	3,362
StDev	0,016	0,005	0,011	0,006	0,005	0,005
75 :25	***	***	0,919	1,502	***	***
	***	***	0,923	1,506	***	***
	***	***	0,927	1,508	***	***
	***	***	0,929	1,511	***	***
	***	***	0,931	1,513	***	***
	***	***	0,937	1,516	***	***
	***	***	0,937	1,521	***	***
	***	***	0,939	1,523	***	***
Mean			0,930	1,513		
StDev			0,007	0,007		
100 :1	3,328	2,070	***	***	2,893	3,023
	3,330	2,072	***	***	2,896	3,026
	3,336	2,074	***	***	2,899	3,028
	3,338	2,076	***	***	2,900	3,030
	3,340	2,079	***	***	2,901	3,032
	3,340	2,081	***	***	2,909	3,033
	3,342	2,084	***	***	2,910	3,035
	3,344	2,085	***	***	2,912	3,037
Mean	3,337	2,078			2,903	3,031
StDev	0,006	0,006			0,007	0,005

Table 4.1: Mean and standard deviation of PL-ASA particles *in vitro* viability studies after 48h and 72h.

	Control groups				Test groups	
	A4	A7	S4	S7	SA4	SA7
10 :90	3,030	2,351	***	***	***	***
	3,033	2,353	***	***	***	***
	3,041	2,355	***	***	***	***
	3,064	2,356	***	***	***	***
	3,075	2,358	***	***	***	***
	3,077	2,360	***	***	***	***
	3,084	2,362	***	***	***	***
	3,085	2,364	***	***	***	***
Mean	3,061	2,357				
StDev	0,023	0,004				
	***	***	2,523	2,187	1,133	2,860

25 :75	***	***	2,525	2,189	1,134	2,863
	***	***	2,525	2,192	1,136	2,864
	***	***	2,528	2,194	1,139	2,866
	***	***	2,530	2,196	1,141	2,867
	***	***	2,533	2,197	1,430	2,868
	***	***	2,534	2,198	1,144	2,869
	***	***	2,535	2,199	1,147	2,870
Mean			2,529	2,194	1,176	2,866
StDev			0,005	0,004	0,103	0,003
50 :50	0,740	0,539	3,027	2,421	0,541	1,142
	0,745	0,541	3,028	2,424	0,542	1,145
	0,755	0,543	3,030	2,425	0,544	1,146
	0,765	0,545	3,033	2,429	0,546	1,148
	0,765	0,547	3,036	2,432	0,547	1,150
	0,778	0,550	3,038	2,434	0,549	1,153
	0,780	0,551	3,039	2,437	0,550	1,155
	0,781	0,553	3,044	2,439	0,553	1,156
Mean	0,764	0,546	3,034	2,430	0,547	1,149
StDev	0,016	0,005	0,006	0,006	0,004	0,005
75 :25	***	***	***	***	***	***
	***	***	***	***	***	***
	***	***	***	***	***	***
	***	***	***	***	***	***
	***	***	***	***	***	***
	***	***	***	***	***	***
	***	***	***	***	***	***
	***	***	***	***	***	***
Mean						
StDev						
100 :1	3,328	2,070	***	***	0,721	0,612
	3,330	2,072	***	***	0,723	0,613
	3,336	2,074	***	***	0,724	0,615
	3,338	2,076	***	***	0,726	0,616
	3,340	2,079	***	***	0,728	0,620
	3,340	2,081	***	***	0,729	0,622
	3,342	2,084	***	***	0,730	0,623
	3,344	2,085	***	***	0,734	0,624
Mean	3,337	2,078			0,727	0,618
StDev	0,006	0,006			0,004	0,005

Table 4.2: Mean and standard deviation of SF-ASA particles *in vitro* viability studies after 48h and 72h.

This chart demonstrates the *in vitro* cell viability absorbance result of PL-ASA particles with breast cancer cell line MCF-7. A4, A7 and P4, P7 are the control groups while PA4, PA7 are the test groups. As it is shown in the chart the test group absorbance is lower than the control groups which can signify that the MCF-7 cells are dying in proportion with a high absorbance of control groups.

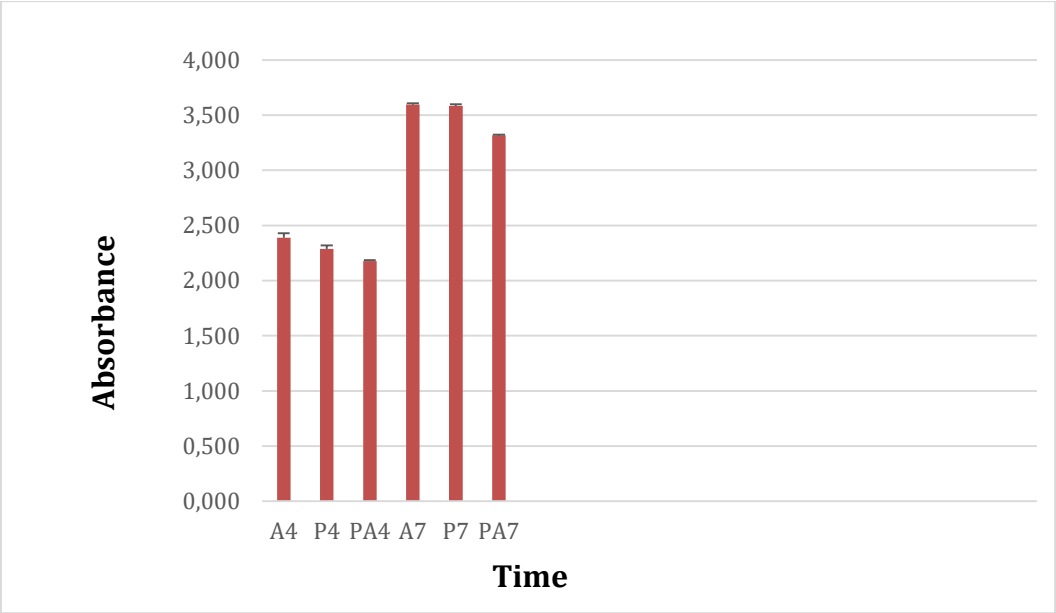

Figure 4.1: *In vitro* cell viability absorbance of PL-ASA particles with breast cancer cell line MCF-7

The following chart present the *in vitro* cell viability absorbance results of PL-ASA particles with breast cancer cell line MCF-7. A4, A7 and SF4, SF7 are the control groups while SFA4, SFA7 are the test groups. As it's shown in the chart the test group absorbance is lower than the control groups which means that the MCF-7 cells are dying in proportion with a high absorbance of control groups.

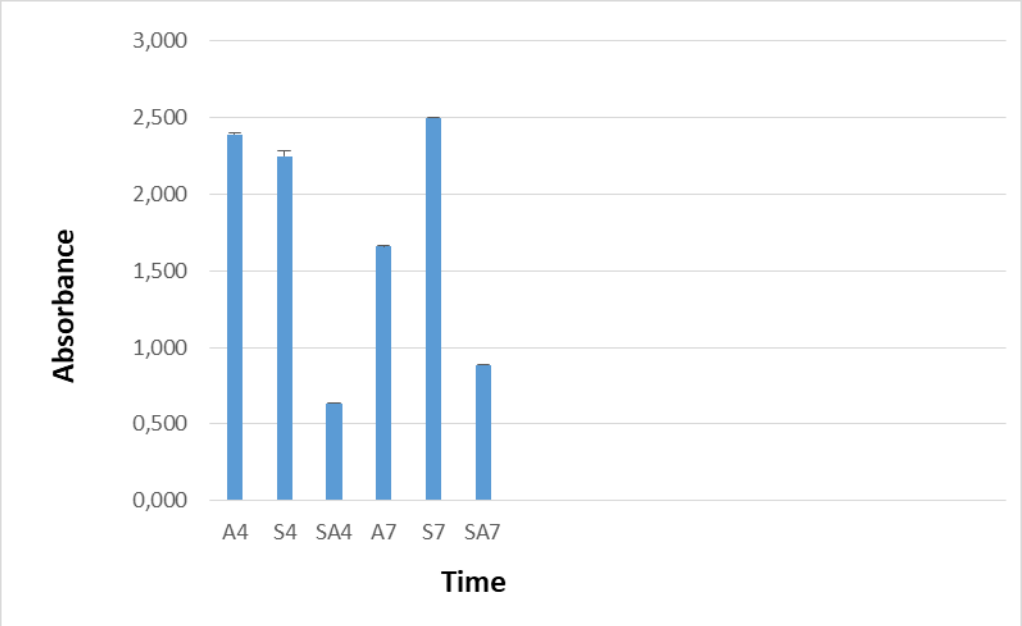

Figure 4.2: *In vitro* cell viability absorbance of SF-ASA particles with breast cancer cell line MCF-7

The scanning electron microscope (SEM) illustrates the shape of the particles. Because of the lyophiliation (freeze-drying which consists of removing water from a liquid, pasty or solid product, using deep-freezing, then vacuum evaporation of the ice without melting it) the shape of SF particles is not very clear and their spherical structure is presented in the scale of 20 µm.

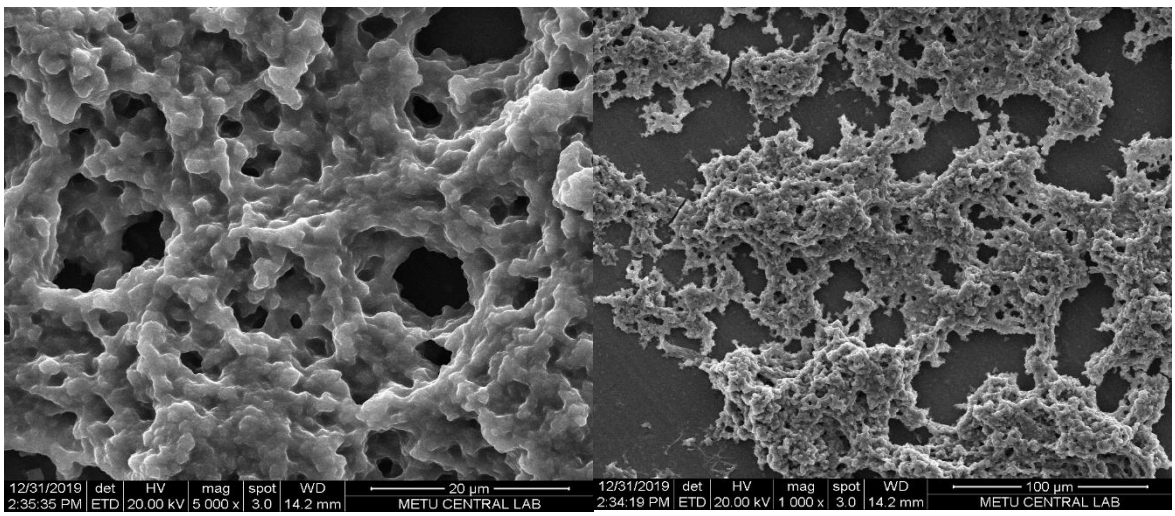

Figure 4.3: Silk fibroin SEM

The scanning electron microscopy (SEM) technique shows the shape of PL particles. It is clear when comparing the size of SF particles with PL particles based on the scale used that PL particles are smaller than SF particles which is a positive property of PL within the scope of this study.

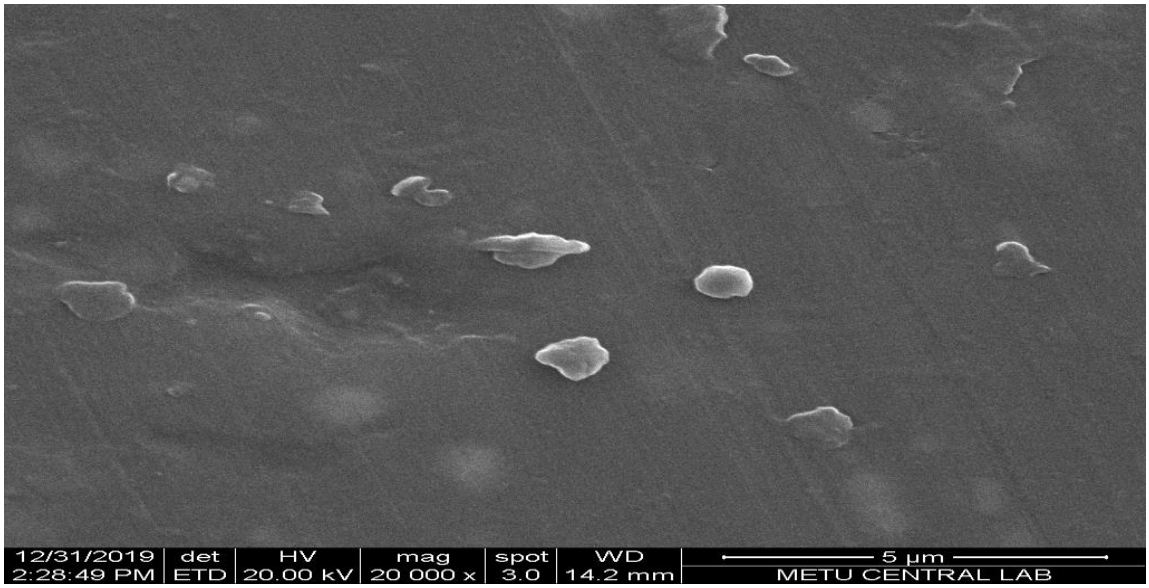

Figure 4.4: Pullulan SEM

Fourier transformer infrared results provided data through these diagrams concerning the characteristic peaks of PL, SF and ASA which showed that the functional groups of this study have shown an improvement

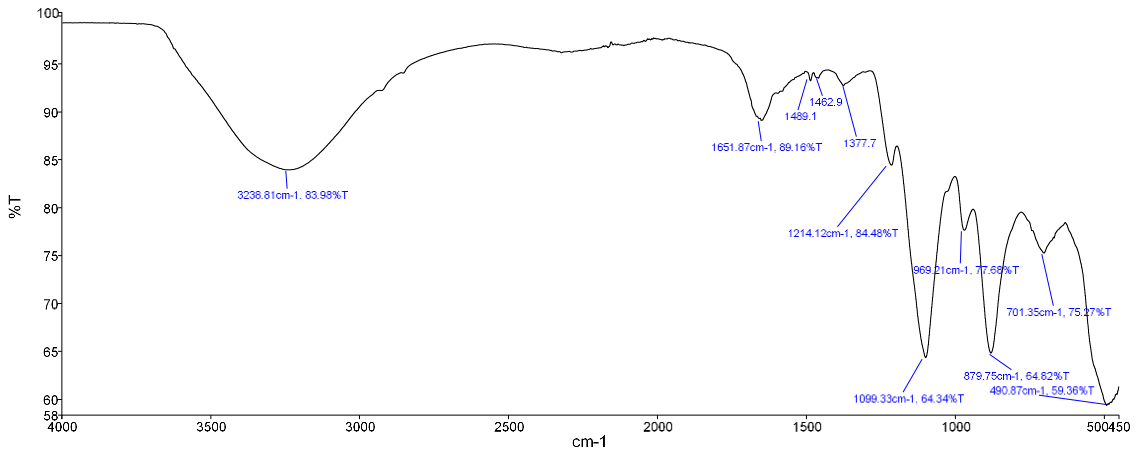

Figure 4.5: Silk fibroin loaded acetyl salicylic acid FTIR results

In this FTIR analysis characteristic peaks of SF are observed on the spectrum of SF-ASA:

- 1657 cm-1 corresponding to amide I
- 1525 cm-1 corresponding to amide II
- 1245 cm-1 corresponding to amide V

Characteristic peaks of Acetylsalicylic acid are as follows:

- 1605.49 cm-1
- 3100-1100 cm-1 acetyl salicylic acid molecule and its oxyanion.

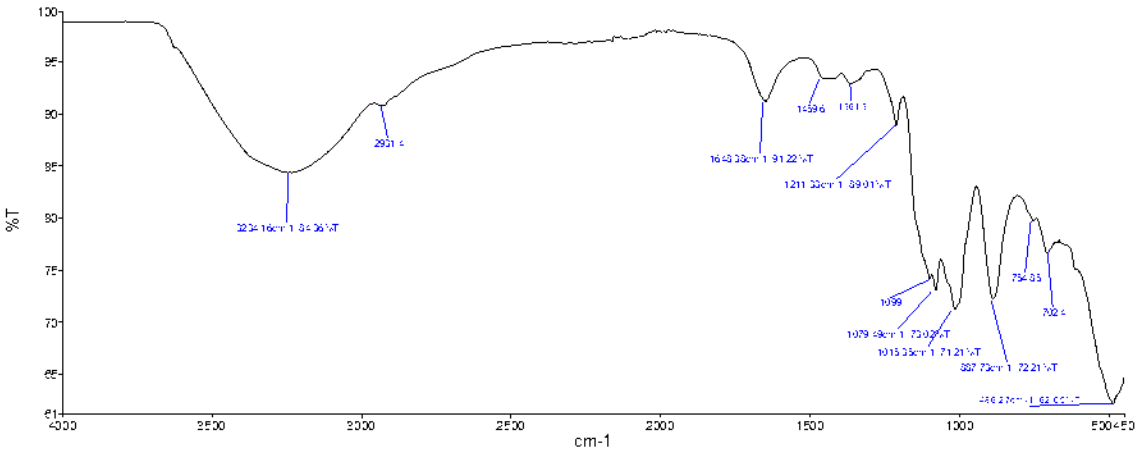

Figure 4.6: Pullulan FTIR results

In this FTIR analysis characteristic peak of PL are:

- 3234 cm-1
- 2931.4 cm-1
- 1648.38 cm-1
- 1361.3 cm-1
- 1015 cm-1
- 887.7 cm-1

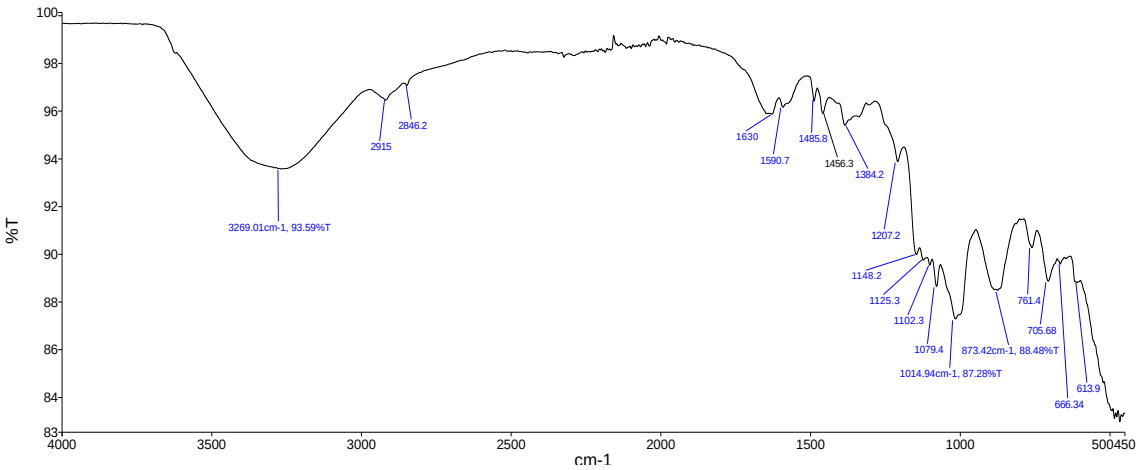

Figure 4.7: Pullulan loaded acetyl salicylic acid FTIR results

In this FTIR analysis characteristic peak of PL are observed on the spectrum of PL-ASA:

- 3234 cm-1
- 2931.4 cm-1
- 1648.38 cm-1
- 1361.3 cm-1
- 1015 cm-1

Characteristic peaks of Acetylsalicylic acid are as follows:

- 1605.49 cm-1
- 3100-1100 cm-1 acetyl salicylic acid molecule and its oxyanion.

4.1 Discussions:

The results of the percentage encapsulation suggest that the ionic gelation method had greater efficiency in drug loading when compared with the dissolution method using acetone. This may be due to leaching of the drug by acetone as well as the cross-linker effect of TPP allowing for incorporation of more functional groups in the silk fibroin protein. Ionic gelation requires fewer chemicals with very little or no harmful residual generated.

Apoptosis, which is otherwise called programmed cell death is primarily the removal of aged cells or the elimination of cells with dysfunctional DNA. Evading this process has been described as the characteristic of all cancers. This implies that whatever causes apoptosis in cancers can also alter the proliferation of cancer cells and ensure their elimination. Studies in the past have demonstrated that the Pullulan and Silk fibroin particles are good transporters of anticancer drugs. In this study, PL-ASA and SF-ASA demonstrated an outstanding cancer cell

death activity, showing a 95 % confidence interval as to the control experiment, with no significant differences between the doses and the percentage amount of the drug. This showed the effectiveness of the drug at the end of 72 hour-period. As a result, it can be concluded that both PL-ASA and SF-ASA are promising particles for *in vitro* apoptotic action on MCF-7 breast cancer cells.

CHAPTER 5:

Conclusion

5.1 Conclusion:

It is possible to state that this research has successfully established the first use of ionic gelation method on PL and SF micro particles.

It was clearly observed that there was good apoptotic activity shown *in vitro* against MCF-7 breast adenocarcinoma cell line, using SEM and FTIR methods which is the hallmark for ensuring cancer cell death.

This research has explored that PL-ASA and SF-ASA particles created via ionic gelation method promise to present an excellent platform for the use of cancer chemotherapy therapy, with prospects for further functionalization for drug targeting (Galam et al., 2020).

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Appendices

Appendix 1: Ethical approval document

NEAR EAST UNIVERSITY

ETHICAL APPROVAL DOCUMENT

Date:12/08/2020

To the Graduate School of Applied Sciences,

For the thesis project entitled as "IN VITRO VIABILITY OF BREAST CANCER MCF-7 CELLS BY USING PULLULAN – ACETYL SALICYLIC ACID AND SILK FIBROIN – ACETYLSALICYLIC ACID PARTICLES", the researchers declare that they did not collect any data from human/animal or any other subjects. Therefore, this project does not need to go through the ethics committee evaluation.

Title: Doç. Dr.
Name Surname: Terin Adalı

Signature: 

Role in the Research Project: Supervisor

Appendix 2: Similarity report

NEAR EAST UNIVERSITY

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AUTHOR	TITLE	SIMILARITY	GRADE	RESPONSE	FILE	PAPER ID	DATE
Maryame Chana	abstract	0%	--	--		1381276192	07-Sep-2020
Maryame Chana	Acknowledgment	0%	--	--		1381276071	07-Sep-2020
Maryame Chana	conclusion	0%	--	--		1381276302	07-Sep-2020
Maryame Chana	results	1%	--	--		1381277043	07-Sep-2020
Maryame Chana	introduction	8%	--	--		1381276407	07-Sep-2020
Maryame Chana	materials and methods	9%	--	--		1381276821	07-Sep-2020
Maryame Chana	Thesis	12%	--	--		1381277407	07-Sep-2020
Maryame Chana	Literature Review	15%	--	--		1381276547	07-Sep-2020

Assoc. Prof. Dr. Terin Adalı