APOPTOTIC AND ANTICANCER POTENTIAL STUDIES OF SILK FIBROIN LOADED CARBOPLATIN PARTICLES

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

By
NANYAK ZINGFA GALAM

In Partial Fulfillment of the Requirements for The Degree of a Doctor of Philosophy in Biomedical Engineering

NICOSIA, 2020

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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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Date: 14/09/2020

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To Xhanfa and Xhandul

ABSTRACT

Cancer of the breast is the 5th leading cause of mortality worldwide. Its management entails the use of anticancer regimen either as adjuvant or neoadjuvant drugs. It is associated with adverse effects resulting in poor compliance as well as collateral damage to bystander cells, thus the need to develop bio-compatible drug carrier system for treatment of cancer. This study set out to create a colloidal drug-delivery system by integrating carboplatin into silk fibroin using the ionic gelation technique then investigate the micro-particle ability to induce programmed cell death on MCF7 adenocarcinoma cells *invitro*. Silk fibroin- carboplatin particles were made and its physical properties examined using FTIR, SEM, mastersizer & bio-degradation studies. The quantity of CP loaded was determined using a UV-VIS spectro-photometric technique. MCF7 adenocarcinoma cells were cultured with 10µg/ml-200µg/ml of SF-CP micro-particles for 24hr, 48hr, and 72 hours. Apoptosis screening was done using the Apostrand Elisa Apoptosis kit. Results obtained were analyzed using the graphpad prism tool. Fourier transform infra-red spectra and studies to determine the release of carboplatin showed successful encapsulation of the drug in silk fibroin. Thus SF-carboplatin microparticle successfully induced apoptosis in MCF7 adenocarcinoma cells *invitro.*

Keywords: *Carboplatin, Breastcancer, Silk fibroin, Ionic Gelation*

ÖZET

Meme kanserinin dünya çapında beşinci önde gelen ölüm nedeni olduğu söylenir. Yönetimi, antikanser rejiminin adjuvan veya neoadjuvan ilaçlar olarak kullanılmasını gerektirir. Yandaş hücrelere zayıf uyumun yanı sıra kollateral hasara yol açan yan etkilerle ilişkilidir, bu nedenle kanser tedavisi için biyo-uyumlu ilaç taşıyıcı sistemi geliştirme ihtiyacı. Bu çalışma, iyonik jelasyon tekniğini kullanarak karboplatini ipek fibroine entegre ederek kolloidal bir ilaç verme sistemi geliştirmeyi amaçladı ve daha sonra MCF7 adenokarsinom hücreleri invitro üzerinde programlanmış hücre ölümünü indükleme için mikro partikül yeteneğini araştırdı. İpek fibroin-karboplatin, FTIR, SEM, mastersizer ve biyo-bozunma analizi kullanılarak sentezlendi ve karakterize edildi. Yüklenen ilacın miktarı bir uv-vis spektrofotometrik teknik kullanılarak belirlendi. MCF7 adenokarsinom hücreleri, sırasıyla 24, 48 ve 72 saat boyunca 10 ug / ml-200 ug / ml SFCP mikropartikülleri ile kültürlendi. Apoptoz taraması Apostrand Elisa Apoptosis kiti kullanılarak yapıldı. Elde edilen sonuçlar graphpad prizma aracı kullanılarak analiz edildi. Fourier dönüşümü kızıl ötesi spektrumları ve karboplatin salınımını belirlemeye yönelik çalışmalar ilacın ipek fibroininde başarılı bir şekilde kapsüllendiğini göstermiştir. Bu nedenle SF-karboplatin mikropartikül, MCF7 adenokarsinom hücrelerinde invitroda apoptozu başarıyla indükledi.

Anahtar Kelimeler*: Karboplatin, Meme Kanseri, İpek fibroin, İyonik Jelleşme*

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LIST OF ABBREVIATIONS

AIDS:	Acquired immune deficiency syndrome
BCL:	B cell lymphoma
BRCA:	Breast cancer gene
DNA:	Deoxyribonucliec acid
EGFR:	Epidermal growth factor receptor
ELISA:	Enzyme linked immunosorbent assay
EPR:	Enhanced permeability and retention effect
FDA:	Food and drug administration
5FU:	Five fluorouracil
GDM:	Geldamycin
HELA :	Human epithelial cell line
HER:	Human epithelial growth factor receptor
HSF:	Silk fibroin hydrosylate
HSP 90:	Heat shock protein 90
MCF-7:	Michigan cancer foundation 7
MDA-MB231 MD:	Anderson metastatic breast cancer 231

MTT: Tetrazolium dye

MYC : Family of regulatory protiens

PAX: Paired box gene

pH: Power of hydrogen

RNA: Ribonucliec acid

SF: Silk fibroin

SFCP: Silk fibroin carboplatin

WHO: World Health Organization

CHAPTER 1
INTRODUCTION

1.1 Thesis Problem

The second highest investigated malignant tumor affecting women in the world is breast cancer. A worldwide gauge of over 2.09 million cases exists out of which 627,000 ladies died in 2018 (WHO, 2019). Breast cancer is presently handled using surgical procedure and chemotherapy (Chou *et al*., 2019). In any case, there are issues identified with chemotherapy because of built up resistance as well as unfavorable impacts of the medication (Al-Zarhani *et al*., 2019). In this way, designing chemotherapeutic medication conveyance frameworks or drug delivery systems are critical for effective cancer treatment. Within the last decade, significant time has been spent in targetable drug delivery frameworks for carcinoma treatment (Khan *et al*., 2017). In spite of the fact that the results after carcinoma treatment have improved, drawbacks like adverse drug effects are as yet a major issue (Singh *et al*., 2017). The pro-drug strategy causes lost dependability and medication action following modification. Colloidal drug conveyance systems are regularly utilized to encase dynamic medications to increase solvency, balance, and adsorption attributes of proteins, similar to silk fibroin (Mc Clements, 2018). As of late, colloidal medication transporter frameworks turned into a developing field for antineoplastic drug delivery systems (Singh *et al*., 2017). Drug dissemination and intracellular up-take are driven by both nanoparticle and microparticle processes in neoplastic cell (Wei *et al*., 2019). They are believed to exploit the non-homogenous makeup of tumor vasculature that is commonly convoluted by broadened endothelial gaps as well as intersections in many areas of the tumor, hence taking advantage of the improved transport and detention reaction also known as the enhanced permeability and retention effect (EPR) thereby guaranteeing confinement of medications toward tumor target site (Brigger, *et al* 2012). The molecules, for example, drugs including nanoparticles, microparticles, and large scale drugs, concentrate inside the tumor tissue in greater amounts contrasted with the traditional tissues. This might be because of the aperture in the cyst vasculature as opposed to traditional tissue. EPR effect plays a significant role in enabling the molecules have access to the cyst site as well as staying present owing to weak lymphatic channel leading to the cyst site (Prabhakar *et al.*, 2013). Varieties of antineoplastic medications are getting utilized considering the reaction of doxorubicin-stacked liposomes and paclitaxel bound albumin. Then again, a number indicated that the medication delivered was not significant (Gao, 2017). This infers particles must be biologically compatible and effectively degradable in biological systems, to evade an aggregation of constituents in the location of the tumor. Since the origin of silk fibroin proteins are from domestic silkworm Bombyx mori (B. Mori) cocoons, they are considered as natural polymers. This essential protein is a key naturally occurring large molecule, utilized to construct anticancer medication conveyance channels (Pham *et al*., 2019). On account of its biocompatibility (Pham *et al.*, 2019. Tulay, *etal* 2018) biodegradability, Haemocompatibility (Adali and Uncu, 2016). Drugs have been encased using silk fibroin (Adali, *et al* 2019). Polymeric nano-and microparticles are regularly made utilizing the essential protein silk fibroin (Tomeh *et al*, 2019) Silk fibroin particles have not just been utilized convey drugs, they were also employed in transporting genes in various types of cancers (Tomeh *et al*, 2019). Worthy of note is the fact that, there is no record of the use of silk fibroin particles in conveying cisplatin which is a subtype of carboplatin inside the cancer cells.

Carboplatin is a platinum containing drug used as an anticancer medication for the treatment of various kinds of malignant growth. Because carboplatin contains bidentate dicarboxylate chelate, they have a lower reaction rate when compared to cisplatin which is used as platinum anticancer medication (Ho *et al*, 2016). Carboplatin exerts its antineoplastic impacts by its interactions with genomic DNA and proteins.

Until this point in time, a variety of techniques have been employed in the production of nano- and microparticles. Techniques like electrospray are considered such strategies (Cao *et al*., 2017). Be that as it may, one of its disadvantages is that more amount of surfactants as well as solvents are needed, the primary reason why the technique is expensive. Moreover, it brings about producing huge amounts of debris, can remain lethal to the vicinity having minimum drug production specificity (Tapia-Hernández *et al*., 2015).

1.2 Aim of the Study

The aims of the study are;

i. To synthesize and characterize silk fibroin microparticles and load them with carboplatin using the ionotropic gelation method.
ii. To evaluate the apoptotic activity of SFCP on MCF-7 breast cancer cells invitro using Apostrand Elisa kit.

In this study we made a null hypothesis that there would not be any significant cell death impact on MCF-7 breast cancer cells in vitro following culture with SFCP.

1.3 Significance of the Study

Evasion of apoptosis has been described as one of the hallmarks of carcinogenesis. The development of a conjugate drug carrier like silk fibroin – Carboplatin particles (SFCP) that can successfully induce apoptosis provides a platform for future functionalization with more drugs or agents such as antibodies to improve drug targeting.

1.4 Limitations of the Study

The study is restricted by the usage of a solitary cell line, MCF7 breast cancer cells. Use of other metastatic cell lines like MDA-MB-231 and normal adenocarcinoma cells like MCF10A would add value to future studies.

1.5 Overview of the Thesis

In this study SFCP particles were synthesized utilizing a procedure known as ionic gelation, characterized as well as its cell death potential investigated using Apostrand Elisa apoptotic kit on MCF-7 breast cancer cells. The particles showed good and sustained apoptotic potential over 72hours.

1.6 Conclusions

Chemotherapy of malignancy is plagued with the issue of poor targeting modalities, deficient streamlining and restriction of the anticancer agent inside the disease cell just as the issues of cytotoxicity to quickly increasing normal cells. Other problems incorporate satisfactory take-up and delivery of the chemotherapeutic agent inside the tumor cells. These difficulties have driven specialists to concentrate on bio-material use in drug conveyance systems. Silk fibroin-based bio-materials have magnificent biocompatibility, biodegradability, and low immunogenicity. These biomaterials additionally have high binding potential for different medications and they can control drug discharge through changing the size and different properties of the protein. It also has established utilization in medication conveyance of a few medications including anticancer medications.

Future possibilities in these studies could incorporate the upgrade of targeting potential with likely functionalization of nano-particles with antibodies to tumor cell markers, just as the blend of a total anticancer routine stacked on a bio-material. Further thought could likewise be given to the multi-functionalization of the silk fibroin bio-materials making it a stage for therapeutic conveyance, checking as well as diagnostics in a concept known as theranostics.

Therefore, the aim of this study was to develop SFN loaded with carboplatin using the ionic gelation method to evaluate its apoptotic effect on MCF-7 breast cancer cell invitro.

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

Malignant tumours are gathering of illnesses described by dysfunctional cell division and gene expression, bringing about impaired regulation of the harmony between cell multiplication and apoptosis. Normal cells are distinct from cancer cells by the expression of cytokine receptors or transcription factors as well as size, functions and structure. This results in abnormal cell growth control, interaction as well as proliferation and integration of the abnormal cell (Siegel *et al.*, 2016). The uncontrolled growth and cell interactions eventually results in the cancer invading surrounding and adjacent tissues ultimately destroying them with its resultant sequelae. Malignant cells may also metastasize via the cardiovascular and lymphatic system. The ability of cancer cells to metastasize as well as its functional and morphological differences distinguishes them from benign masses. Benign masses usually retain the similarity to normal cells morphologically as well as functionally. The loss of function of cancer cells and the invasion of normal tissues may lead to a variety of sequalae, which include albeit not exhaustively; pain, cachexia, immune-suppression, anaemia, and even venous thrombosis which is said to be the leading cause of mortality in cancer (Noble &Pasi, 2010). Several studies have considered the epidemiology, pathophysiology, and progression of cancer. The role of PAX proteins as transcription factors, how they affect embryonic development, their role in governing cell proliferation, self-renewal, failure of apoptosis, movement of embryonic precursor cells and the co-ordination of distinctive differentiation programs has been established. PAX proteins are known to play a vital role in sustaining tissue specific stem-cells by inhibiting end-point differentiation and apoptosis. These properties are exploited by cancer mechanism especially when there is an abnormality in the function of the PAX protein (Lang *et al.*, 2007). Cancer cachexia is a syndrome of nutritional depletion and sequelae of cancer characterized by progressive weight loss. It occurs due to complex communications between the host and the malignancy resulting in the production of catabolic mediators which degrade host tissues, though other factors like age and physical activity may play a significant role (Skipworth*et al.*, 2007). Cancer induced bone pain may be as a result of a combination of inflammatory and neuropathic signals (Urch, 2004). Cancer related anaemia may also occur at the advanced stage of the disease and worsens during chemotherapy and radiotherapy. The anaemia is associated with the activation of immune and inflammatory systems by the malignancy leading to increase production of interleukin 1, tumour necrosis factor, and interferon's which potentially mediate anaemia. (Nowrusian, 2002).

Malignancy is evaluated to be one of the main sources of death. Truth be told, malignant growth causes a larger number of loss lives compared to AIDS, tuberculosis and malaria fever. Besides, the occurrence of death because of malignant growth is much higher in developed nations with it being the second most basic reason for death following cardiovascular sickness in the USA (Jemal *et al*., 2008)

Breast malignancy was accounted for being the 2nd most frequently occurring malignant tumour on earth in 2013 (Desantis *et al*., 2014), furthermore, one in every eight women are expected to develop malignant tumour of the breast making it the 2nd most frequent cause of cancer related mortality amongst women (Baeks & Nelson, 2017). The main risk factor for breast cancer is genetic predisposition, mutations in *BRCA1* and *BRCA2*, which presents as an autosomal dominant inheritance. In addition, epidemiological investigations have related breast malignancy with various predisposing factors including intrinsic oestrogen concentration, early menarche and late menopause (Carmichael, 2006). Along these lines, it has stayed a reason of worry among specialist in health care provision and researchers over the world thus continuous researches are being undertaken to comprehend the pathology of the malignant growth so as to develop better treatment methodologies. Using histological markers breast cancer can be subdivided based on the availability of oestrogen, progesterone, and human epidermal growth factor receptors (EGFR). Those that lack receptors for the hormones and growth factor mentioned above are described as triple negative breast cancer cells (Baeks& Nelson, 2017).

Malignancy treatments must be target specific to keep away from harming normal cells (Philchenkov *et al*., 2004). Ordinarily, surgery, radio and chemo therapies are the three well known methods of elimination of malignancy. Contingent upon the phase of the malignancy, abscissions are employed for evacuation of the cyst or tumors. Be that as it may, before surgery there is a need to apply chemotherapy in other to constrict the cyst prior to removal, and adjuvant chemotherapy to manage the malignant growth cells not removed by surgery. The aforementioned modalities pose both emotional and financial burden to all involved (Folprecht, 2005). There are several adverse effects such as systemic toxicity, collateral injury etc to the use of radiotherapy in the removal of tumors (Wu *et al.*, 2016). In chemotherapy, the treatment methodology relies upon the distinction in malignant growth cell science and normal cell science to accomplish passive or focused tumor targeting so as to improve tumour cell decimation without harming non-cancerous cells. In spite of the fact that chemotherapy is broadly utilized in the treatment of numerous tumours including breast cancer, there is a danger of collateral injury to ordinary cells and systemic toxicity (Johnstone *et al*, 2002). Cisplastin and doxorubicin have shown increase effectiveness when used on oestrogen responsive MCF-7 breast cancer cell lines which were pre exposed to 100pg/ml of oestrogen for three months (Chang & Singh, 2017).

2.2 Breast Cancer Biology

Breast cancers are categorized based on its anatomical location in the breast. The breast is basically made up of milk secreting glands (lobules), ducts (channels through which milk exits to the nipple) and fats. Though there has been varying opinions as regards the presence of cancerous cells in the breast, it is generally accepted that once cells with atypical hyperplasia are seen it denotes the presence of a precancerous entity. These cells can be limited to the lobules and thus called lobular carcinoma *in-situ* or more commonly in the ducts and are referred to as ductal carcinoma *in-situ*. The evolution of these precancerous cells to invasive malignant tumours are poorly understood, however it has been associated with a complex interaction between endogenous (genetic) and exogenous (environmental) factors.

Microscopic metastasis may be seen even with small tumours resulting in the need for adjuvant chemotherapy (Polyak, 2007).

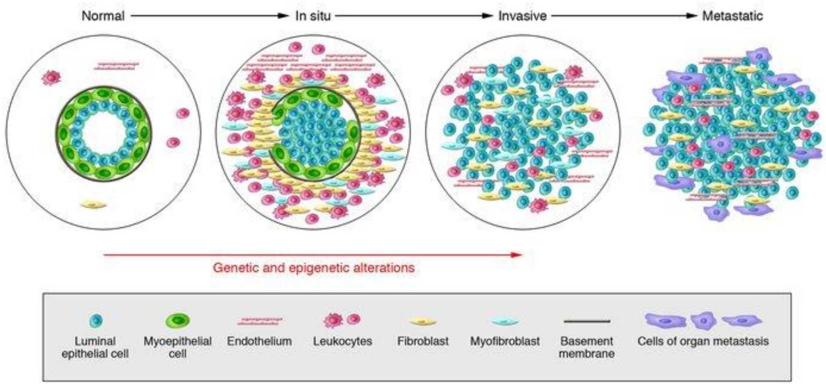


Figure 2.1: Hypothetical image showing four stages of transformation of ductal breast cancer (Polyak, 2007).

2.2.1 Breast cancer genetics

Breast cancer has been attributed to several genetic changes affecting any of the following types of genes: (1) Oncogenes- tumour promoting (2) Tumour suppressor genes (3) Modifier genes- involved in DNA repair. All these types of genes are involved in the control of cell proliferation, apoptosis and differentiation. Some genes commonly associated with breast cancer include but are not limited to BRCA1, BRCA2, increase in expression of MYC genes, BCL1 overexpression or mutation of erB2/neu/HER2, others include the over expression of the antiapoptotic BCL2 or the mutated version of TSG101 tumour suppressor gene (Nathanson *et al*.,2001).

2.2.2 Breast cancer vascularity and EPR effect

The breast is supplied blood normally by the internal thoracic artery a branch of the subclavian artery and the axillary artery, however there is neovascularization in cancers resulting in increased regional vascularity of the tumour maintaining a prominent vessel with impaired structural architecture (grubstein*et al*, 2010). This altered architecture results in widened endothelial gaps and decreased lymphatic drainage of solid tumours which forms the underlying basis of passive targeting using small molecules known as enhanced permeability and retention effect (EPR). The small molecules reach the solid tumour via the widened endothelial gaps but find it difficult to exit the tumour site as a result of poor lymphatic drainage (Yin, Liao & Fang, 2014).

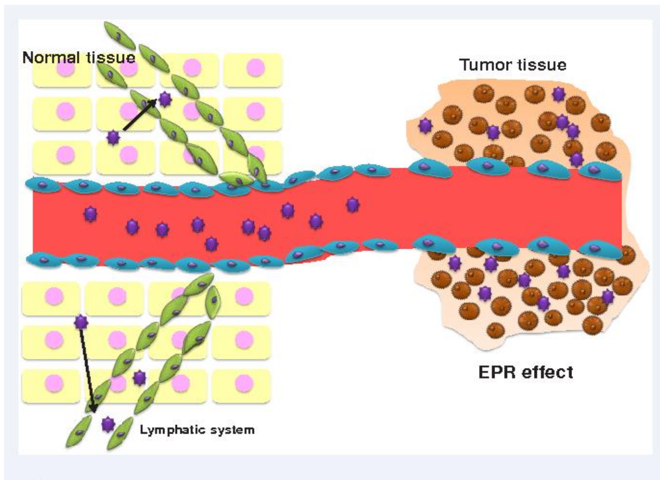


Figure 2.2: image showing the EPR effect.

2.3 Apoptosis and the Hallmarks of Breast Cancer

Simply defined as programmed cell death, is the physiological deletion of aged cells and error prone cells. The process of apoptosis involves both extrinsic and intrinsic pathways characterized by the upregulation of caspase activity, and subsequently cell suicide. Certain morphological and biochemical features are known to accompany apoptosis these includes chromatin condensation, nuclear fragmentation and pyknosis whilst biochemical changes include the activation of caspases, degradation of DNA as well as modification of surface proteins and membranes to enable easy identification of apoptotic cells by phagocytes (Koff *et al.*, 2015). A major hallmark of cancer cells is their ability to avoid the process of apoptosis

(Nguyen *et al.*, 2009) others include rapid proliferation, sustained angiogenesis and tissue invasion and metastasis (Hannahan & Weinberg 2011, Raposo, 2017).

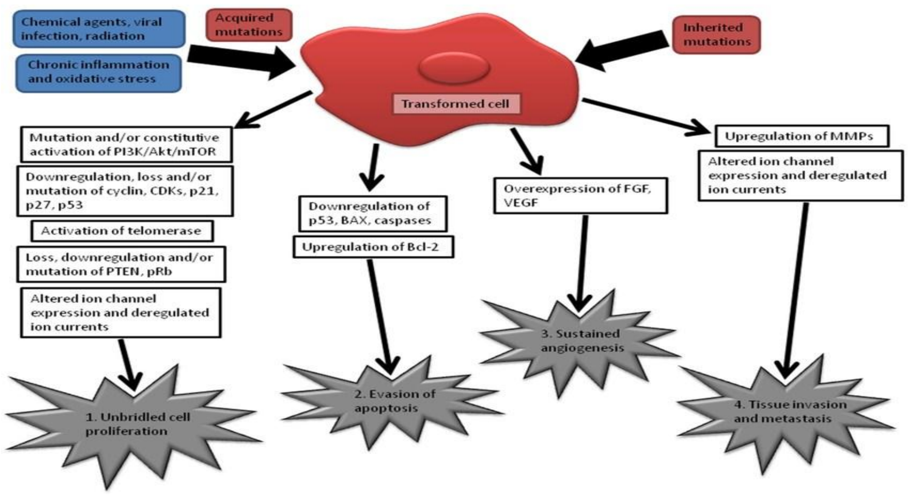


Figure 2.3: diagramatic representation of the hallmark of cancer development. (Raposo, 2017)

2.4 Current Treatment Techniques for Breast Malignancy and Approaches to more Current Technologies

Management of malignant neoplasms of the breast relies upon the sort, stage and spread of the disease to distant sites. Careful surgical excision is the highest quality level for early disease. Chemotherapy all alone or simultaneously with radiotherapy, is normally administered when the malignant growth is progressed or widely metastasized to decrease the spread or militate against the effect of the tumors, so as to improve the quality of life and its expectancy thereof (Simmons *et al*., 2009). Some chemotherapeutic agents usually utilized in treatment of breast malignancy include; docetaxel, paclitaxel and platinum containing agents (cisplatin and carboplatin). Be that as it may, these medications utilized in chemotherapy have a few difficulties that incorporate absence of localization or distinctive bio-distribution and decreased therapeutic index. Also, the chemical agents are confronted with physiological and anatomical barriers and thus very often, higher doses are mandatory. This expands the danger of harming the division of normal cells and systemic toxicity. Amongst the developing innovations in chemical management of malignancy is to effectively deliver drugs utilizing biomolecules. focused medication conveyance frameworks with the utilization of biomolecules, especially those having the attributes of good bio-compatibility and bio-degradability, smart-drug conveyance systems are developed (Torchilin, 2011). The utilization of nanoparticles as medication conveyance framework try to solve these issues by positively modifying the capacity for specific targeting of malignant growth cells, expanding surface area to volume proportion just as lessening systemic toxicity and the crossfire or spectator effect (Hiremath and Hota, 1999).

Common targets for the nanoparticles include receptors expressed by the cancer cells, others include cell-cycle proteins such as cyclin-dependent kinases which govern the cell cycle, they do this together with check point kinases, aurora kinases. This has all been targeted in the management of breast cancer (Otto & Piot, 2017). It is in view of this that different novel drug-delivery systems to include the use of biomolecules as well as nano-particles are employed. Docetaxel loaded poly glycolic lactic acid particles have shown improved efficacy of treatment in taxane resistant triple negative breast cancer cells using mouse models (Bowerman, 2016). There is however a problem of inflammation caused by the debris of the necrotic tumour cells and studies have suggested the use of proinflammatory-resolving mediators like maresin-1 a key mediator of resolution which is synthesized by human macrophages from endogenous decosahexanioc acid. This maresin 1 thus enhances resolution by resolving inflammation and endogenous clearance of tumour cell debris by macrophage phagocytosis (Vatrick *et al.*, 2016), it thus implies that it would be better to use biodegradable, biocompatible and stable nanocarriers as evolved colloidal drug-delivery system in the chemical management of cancer (Boyd, 2008).

2.5 Cytotoxic Drugs used in Breast Cancer Chemotherapy

Cytotoxic drugs used in cancer chemotherapy directly or indirectly cause cell death. They may achieve this feat by either decreasing micro-tubule function, protein synthesis and function or DNA synthesis. Cytotoxic drugs may be cell cycle dependent with the exception of alkylating agents which may act independent of the cycle (Hickman, 1992). A confounding problem to all classes of cytotoxic drugs is the problem of multi-drug resistance, which is a key determinant in the failure of chemical management of cancer. Enhanced efflux of the cytotoxic by ABC transporters on the membranes of the cells have been adduced as a major component in the development of drug resistance and thus the strive by researchers to adopt several approaches including the determination of targets on the surface of this transporters. The utilization of prostaglandin-glycoprotein inhibitors, RNA interference and nano-medicine to counteract this problem (Li *et al.*, 2016). Drugs in this various classes have been used singly or in combination as a regimen to treat breast cancer. These drugs are usually used in combination with other modes of management to cure or reduce the cancer bulk and burden. Several researches are being done daily to enhance the activity of various classes of the cytotoxic drugs on breast cancer cells whilst limiting their toxicity to normal tissue. Geldamycin (GDM) a potent inhibitor of heat shock protein 90 (Hsp90), a protein necessary for folding during protein synthesis was tried as an antitumor drug in breast cancer but later discontinued during clinical trials due to its hepato-toxicity. Further studies have reported enhanced activity of a super paramagnetic iron oxide based polymeric nanocomposite of GDM which showed augmented antineoplastic activity, with increased cellular damage and necrosis of MCF-7 breast adenocarcinoma cells and little or negligible damage to MCF10A normal breast epithelial cells. It does this at lower doses when compared to the pristine drug. It has also been shown via in-vivo studies to have a 2.7-fold delay in tumour progression as well negligible hepatotoxic effect (Prabhu *et al.*, 2017).

Gemcitabine an analogue of deoxycitidine mediates its anti-cancer potential by opposing DNA synthesis ultimately resulting in tumour cell death. It has been used extensively in the management of breast cancer. The conjugation of gemcitabine with a nanoparticle in cancer chemotherapy improved its efficacy and overcame its limitation of a short biological half-life. It also showed less resistance susceptibility (Djawanapelly*et al.*, 2017).

Studies have shown combined drugs with known mechanisms of action with plant extracts or molecules which are said to have some therapeutic effect on the cells. A combination of doxorubicin and a phenolic extract of flaxseed oil which is rich in lignans a form of phytoestrogen on breast cancer cell lines MCF-7 and MDA-MB-231 showed improved induction of apoptosis and cytotoxicity to the cancer cell lines at lower doses when contrasted to the pure form of the drug (Guerreiro *et al*., 2017).

Another study combined cisplastin, a platinum based antineoplastic agent to curcumin (diferuloyl methane) showed a potentiation of the anticancer activity of cisplastin *in vivo* using sprawgdrawley rats' model of breast cancer. It was also shown to ameliorate the nephrotoxic effect of cisplastin, by comparing the renal function as the gross and microscopic pathology of the kidneys of the test group, to the normal control and the cisplatin control group (Kumar *et al*., 2017).

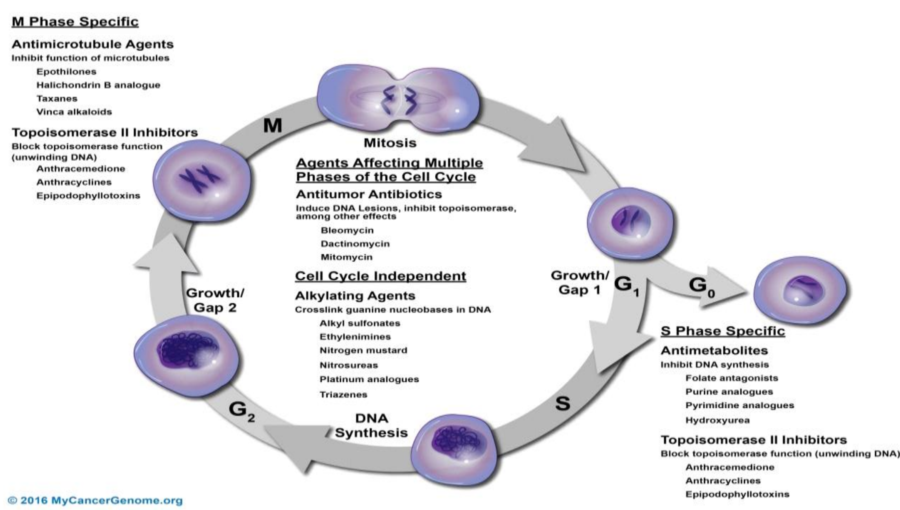


Figure 2.4: Schematic diagram of classification of cytotoxic drugs source: wikipedia.

2.5.1 Carboplatin

Carboplatin which is the drug of interest in the current study is a platinum based antineoplastic drug, which acts by interfering with DNA duplication. It is marketed under the trade name paraplatin. The drug is used for a variety of cancers which include but isn't limited to ovarian, lung, head and neck, breast and brain cancer. Common adverse reactions to Carboplatin include nausea, vomiting, electrolyte dysregulation and anaemia as a result of myelosuppression (Oliver *et al*., 2005).

Myelosuppression is the major drawback of carboplatin and it occurs 21-28 days after the first treatment is given. Carboplatin however has a comparative advantage of having less nephrotoxicity when compared with cisplatin and also because all of its side effects are more easily controlled in comparison to cisplastin. The drug has a bioavailability of 100% and a retention half-life of 30 hours as opposed to the retention half-life of cisplastin which is between 1-3 hours, implying that the drug is more long lasting in circulation as well as within its target site. The drug is administered via the intravenous route and excreted by the kidneys. The clinical ratio of carboplatin is 4:1 of cisplastin as the latter is said to be more potent. Carboplatin has a more stable structure when compared to cisplastin and may be the reason why it has very good bioavailability as well as a high retention time. Its chemistry differs from that of cisplastin due to its bidentate ligand cyclobutane dicarboxylic acid. Its basic mechanism of action is believed to be via aquation hypothesis where it is thought to prevent the duplication of DNA by integrating one or more water molecules whilst displacing an anion necessary for the duplication process. It is also capable of exerting its action via the activation hypothesis implying that it is not limited to a single mechanism of action thus distinguishing it from cisplatin. (Natarajan *et al., 1999).*

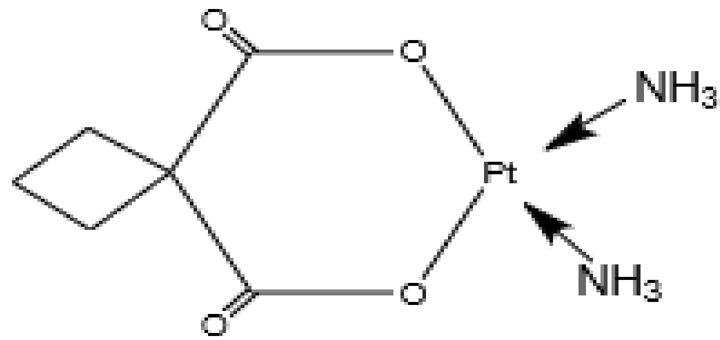


Figure 2.5: Molecular structure of carboplatin ($C_6H_{12}N_2O_4Pt$).

2.6 Biomaterials as Drug Delivery Systems

Chemotherapy being the mainstay in cancer treatment as earlier mentioned has issues with targeted and specific drug delivery thus tempering with the natural pathway and physiology of other surrounding and rapidly dividing cells. This results in apoptosis or cell death, not only in the neoplastic cells but also in normal cells. This phenomenon is known as crossfire or bystander effect. Biomaterials help to surmount this as well as show pH dependent response in targeted drug delivery as a means of overcoming physiological and anatomical barriers (kanamala*et al.*, 2016).

More up to date drug conveyance systems are expected to enhance the adequacy of the medications or chemical drug agents. This is kept up by target specific medication conveyance systems. Intra-venous delivery of medications at solid tumor destinations can be affected by various factors. These factors are identified with the physiology of the tumour, micro and macro- environment of the tumour and the drug-delivery vehicle. More explicitly, the morphological features of the arteries, shifting penetrability of the arterial bed, pH of the micro-environment, the interstitial weight of the necrotic center, and the separation between the tissue mass and the vessels, hypoxia and absence of lymphatic drainage assume an essential job in the effective drug delivery systems (Mathur and Gupta, 2010).

The vast majorities of the bio-materials utilized for drug-delivery systems are polymeric molecules which over the years have appeared to improve drug entrapment and eventually improve efficacy with lower adverse effects. This anyway is not without its setbacks which incorporate issues with the bio-distribution and bringing down the efficacy of the medications. Engineered bio-materials like poly-lactic acid, poly-glycolytic acid, polylactic-coglycolytic acid and collagen have been utilized as fibres, hydrogels, dendrimers, and liposomes to convey drugs (Makadia and Siegel, 2011). Difficulties poised by these group of bio-materials are constrained functionalization, the metabolism, its collection inside cell, bio-availability and eventually the disposal from the body (Langer, 2000). To resolve these issues, research studies has shifted to the utilization of nature's own bio-materials like chitosan and silk fibroin, alginate, peptides, protein's and nucleic acids in drug conveyance systems.

Studies have shown the importance of nano-carriers, the coupling of drugs with specific stimuli triggered drug release system; especially pH triggered drug delivery systems due to the fact that the tumour micro-environment is basically acidic. Thus, the drug and biomaterial wasdesigned to be pH sensitive. The role of protonation, charge reversal or cleavage of bonds in facilitating drug uptake or release is also shown (Kanamala*et al*., 2016).

Polysaccharides of marine origin (alginate, carrageenan, fucoidan and the chitosan family) have been exploited as bio-materials for drug delivery due to their bio-compatibility, bio-degradability and anti- inflammatory activity they also have adhesive and anti-microbial activity. They can also be easily tuned in terms of size and shapes. They also exhibit response dependence to external stimuli such as PH and temperature (Cardoso *et al*., 2016).

Studies have shown how immunogenic cell death is enhanced when a cell death inducer like oxaliplatin is coupled with nanoparticles developed from known biomaterials (Zhao *et al*, 2016). Another study functionalized graphene oxide with carboxymethyl chitosan and flourescien isothiocyanate and lactobionic acid. The composite loaded with doxorubicin and tested on SMMC-7721 liver cancer cell line showed enhance activity of the drug when compared to the non-functionalized composites. The lactobionic acid functionalized composites selectively induced cell death to the cancer cell lines whereas the non-functionalized composites were toxic to even normal liver cell line L929 (Pan, 2016). Nanographene has been used to develop stimulus responsive drug-delivery system for cancer chemotherapy (Yang *et al*., 2016).

Since most cancer chemotherapy regimens are a cocktail of cytotoxic drugs, there has been efforts to get biomaterials with the capacity to deliver the whole regimen. The combinations of a good biomaterial and nanotechnology have enabled researchers to harmonize the pharmacokinetics of different drugs, thus exploiting their effects of potentiation, synergy and additive properties. This also improves compliance and reduces the burden of multiple administrations on the patients (Hu *et al*., 2016).

Consequent upon the difficulty as well as the need to overcome the resistance poised by certain physiological and anatomical barriers, multifunctional aptamer-based nanoparticles have been exploited. Functional DNA nanostructures loaded with doxorubicin and tested on resistant cancer cells have been shown. The structure comprised of a DNA aptamer and a double stranded DNA, thus forming an aptamer DNA complex with the ability to intercalate into the host cancer cell DNA whilst delivering the drug. This study showed that the aptamer DNA complex loaded with doxorubicin efficiently changed the resistance of human breast cancer cells to doxorubicin (Liu *et al.*, 2016).

2.7 Naturally Occurring Bio-Molecules as Medication Bearers

The requirement for biodegradable organically determined vehicles for drug delivery especially at the nano-scale, with practically no dangerous impact on the surrounding normal tissues as well as no poisonous by-products has been the significant drive into exploring bio-macromolecules. It is accepted that the naturally derived particles or vehicles would have attributes that upgrades take-up, focusing on and maintenance of the captured remedial agents in this manner improving its efficacy. These molecules are derivatives of plants or animal cells (Gupta *et al*., 2009). The biomolecules, for example, silk fibroin and chitosan can beat barriers that synthetic vehicles have found hard to supersede (Decuzzi*et al*, 2010). Ideally the developments of bio-macromolecules as stimulus responsive vehicles are planned for creating smart medication delivery systems (Lorenzo and Colcheiro, 2008). A few investigations have concentrated on utilizing bio-macromolecules as such, including silk fibroin and chitosan framing a three-dimensional scaffold stacked with VEGF for bone tissue engineering (Tong *et al*., 2016) and chitosan-based nanoparticles sustained the delivery of bone morphogenetic protein 2 for bone tissue engineering (Bastami*et al*., 2017). Despite the fact that there are a lot of normally ocurring biomolecules, for example, silk fibroin, chitosan, characteristic collagen, and poly nucleotides, chitosan and silk fibroin will be widely talked about in this study.

2.8 Use of Chitosan as Drug Carriers

A few examinations have been done utilizing chitosan to carry a few anti-cancer agents for treatment in breast malignant growth cells. Chitosan nano-particles were utilized to entrap carboplatin anticancer medication in MCF-7 adenocarcinoma cell line showing optimal entrapment, great targeting on bringing about specific cytotoxicity to the disease cells (Khan *et al*., 2017). Biodegradable chitosan nano-particles encapsulating paclitaxel likewise brought about great apoptotic and anti-cancer activity in triple MDA-MB-231 breast malignancy cell lines (Gupta *et al*., 2017). Targeted medication release studies indicated that the nanoparticles discharged 50% of the stacked medication. It likewise indicated enhanced cytotoxicity on the MDA-MB-231 breast malignant growth cell lines as well as scaled up the antitumor action when contrasted with free taxanes (Gupta *et al*., 2017). These recent studies showed that the nanoparticle-drug complexes have less haemolytic toxicity, enhanced apoptotic activity and more effective anticancer activity when compared with the conventional drug delivery systems.

Chitosan was used as a drug-delivery system to carry a known cytotoxic in drug in combination with a small interfering RNA (SiRNA) molecule to treat cancer cells invitro with increasing efficacy (Xiao & Merlin, 2017). Hyaluronic acid coated chitosan nanoparticles have been demonstrated to enhance the delivery of 5 fluorouracil (5-FU) in tumor cells which exhibit high expression of CD44. It was established that biocompatible and biodegradable hyaluronic acid coated chitosan nanoparticle encapsulating 5FU enhanced the accumulation of 5FU in the tumor cells, particularly those that overexpressed the CD44 antigen. It caused increased apoptosis by inducing mitochondrial damage via increasing the production of reactive oxygen species (Wang *et al*., 2017). In like vain trimethyl chitosan has been used to deliver SiRNA drug conjugates on metastatic breast cancer cell line (MDA-MB-231). They study delivered a conjugation of high mobility protein group 2 (HMGA-2) SiRNA and doxycycline and it showed significant inhibition of the breast cancer cell growth (Eivazy*et al*., 2017).

Solid lipid nanoparticles have been crushed upon one another using chitosan along with hyaluronic acid carapace. They were loaded with paclitaxel and tested on breast cancer cell lines (MCF-7). Results indicated that chitosan- hyaluronic acid coated solid nanoparticle facilitated time-dose dependence, directness of drug transport and cellular uptake by discharge of paclitaxel which then aid its chemotherapy activity (Campos *et al.*, 2017).

Ionic gelation method used in preparing biotinylated N Palmitoyl chitosan and magnetite, was characterized and an in vitro cytotoxic test was conducted. This showed the particles loaded with doxorubicin had anti spread impact on MCF-7 adenocarcinoma cells up until the lowest concentration used (Balan *et al*., 2017).

Smart glucose based focused drug-delivery systems coupled with enzyme sensitive release strategy were developed using magnetic nanoparticles which were grafted to carboxy methyl chitosan and beta cyclodextrin then loaded with prodigiosin. It was tested on MCF-7 adenocarcinoma cell lines and compared with the pristine form. The test drug showed an enhanced efficacy over the breast cancer cell lines than did the pristine form (Rasjegari*et al*., 2017).

Chitosan modified with poly (lactic co glycolic acid) nanoparticles encapsulating epirubicin was tagged with a 5TR1 DNA aptamer against MUC1 receptor. The fabricated nanoparticles with or without the aptamer showed significantly better therapeutic efficiency on MCF-7 breast cancer cell when compared with the free epirubicin, whilst in murine colon cancer cells the targeted nano-particles showed better therapeutic efficiency when compared with the non-targeted nanoparticles carrying epirubicin (Taghavi*et al*., 2017).

Tamoxifen which is known to have poor aqueous solubility and bioavailability, when combined with chitosan and palmitic acid circumvent the earlier listed issues. This was demonstrated in study where a copolymer derived from palmitic acid and chitosan loaded with tamoxifen showed enhanced cytotoxicity and bioavailability when tested invitro with MCF-7 adenocarcinoma cell line (Thotakura, *et al*., 2017).

Curcumin loaded folate modified chitosan nano-particles have been synthesized via a self-assembling process. It was then characterized and tested for cytotoxicity using the MTT assay against MCF-7 breast cancer cell lines. Drug release studies were undertaken and it showed the nanoparticle released curcumin with decreasing PH. The MTT assay showed enhanced cytotoxicity and hence efficacy with the folate modified chitosan nanoparticles (Esfandiarpour *et al*., 2017).

2.9 Silk Fibroin

SF is gotten from the cocoons of insects or the Bombyx mori worms, and in its immaculate crude structure, it comprises of fibroin and sericin. The sericin parcel which is sticky like is normally haemotoxic and must be expelled to upscale the biocompatibility of silk fibroin. The fibroin protein comprises of layers of antiparallel beta sheets of rotating amino acids basically glycine, alanine and serine (Hakimi *et al*, 2007). It comprises of assorted amino acids with various functional groups, which makes it easy to functionalize or include a connection. Silk fibroin can be adjusted by means of hereditary designing to suit wanted attributes for drug conveyance (Megeed*et al*., 2002)

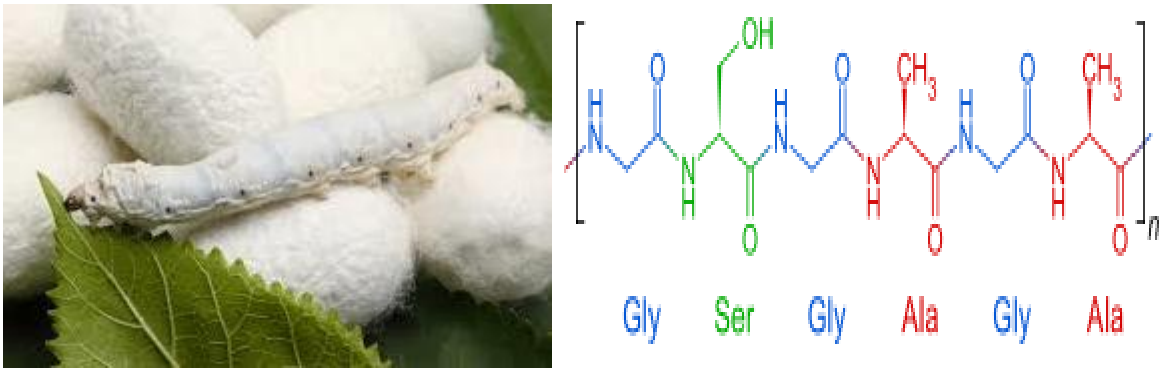


Figure 2.6: Bombyx morii worm, cocoon and the genenal formula for silk fibroin.

2.9.1 Molecular properties of silk fibroin

Silk obtained from bombyx mori worm is made of structural proteins, the fibroin heavy chain (325 kDa) and light chain (25 kDa). A glue-like protein named sericin binds the chains together. Sericins have been associated with immune response it is particularly said to be hemotoxic. It is has higher hydrophylicity when contrasted to fibroin, thus it can be removed by boiling the cut silk fibroin coccoons in alkaline solutions (Altman *et al*., 2003). The heavy chain contains alternating hydrophobic and hydrophilic blocks. The hydrophobic blocks consist of highly ordered sequence repeats of GAGAGS and less ordered repeats of GAGAGX (where X is either Valine or Tyrosine) that make up the crystalline regions of silk fibroin by folding into β-sheets. The hydrophilic part of the core is non-repetitive and short in comparison to the hydrophobic repeat (Altman *et al*, 2003). Its amino acid sequence bestows on silk fibroin the ease of chemical modification and manipulation. Opportunities for chemical modification. Amines, alcohols, phenols, carboxyl groups, and thiols have been explored as potentially reactive side groups for the chemical modification of silk fibroin (Murphy *et al*., 2008). It is believed that by introducing and modifying functional groups in silk fibroin a variety of drugs in different amounts can be added.

2.9.2 Physical properties

1. Molecular Weight

The molecular weight of silk fibroin influences its mechanical properties and bio-degradability, and, hence its quality as a drug-delivery vehicle. If a wide variety of weight ranges are available it would definitely affect the ratio of the silk fibroin to drug, how quickly it would be degraded as well as the drug release kinetics. Silk fibroin prior to processing exhibits large molecular weights. After processing there is a decrease in molecular weight which is proportional to the duration and temperature of boiling the cocoons in alkaline solution. This implies that the longer the duration you boil the lower the resultant molecular weight (Cai *et al.,* 2002). Because this method may lead to production of materials of variable molecular weight and biodegradability, researchers have called for the use of genetically engineered cocoons (Megeed*et al.,* 2002).

2. Crystallinity and Solubility

As earlier mentioned, silk fibroin predominantly consists of hydrophobic blocks. This block of silk fibroin builds up the crystalline regions of the polymer. The do so by their capability to form intermolecular β-sheets. Several treatments ensure the transition of the random coil silk I state of silk fibroin, to silk II conformation, which is highly stable and characterized by a finger print of increased β-sheet. The commonest method of stabilizing silk fibroin by increasing its β sheet content is by treating the protein with methanol. Other methods which achieve this purpose include freeze drying at temperatures lower than -20 degrees Celsius, treatment with water vapour or incubation at high humidity. Increased stability results in decrease water solubility (Monti *et al.*, 1998). It is worthy of note that increase in β-sheet content results in increased mechanical strength, crystallinity and an attendant increase in brittleness of the material. Treatment with methanol has shown the highest percentage of β-sheet transformation. (Min *et al.,* 2006)

Crystalline SF is insoluble in most solvents that are widely used to dissolve polymers for drug-delivery applications, as well as in water. what is usually used to dissolve silk fibroin are high electrolyte solutions, lithium bromide, lithium thiocyanate, calcium thiocyanate or calcium chloride. These electrolyte solutions are able to disrupt the hydrogen bonds that stabilize βsheets (Philips *et al*., 2004).

3. Stability

The stability of materials used as drug delivery vehicles are of utmost importance. Recall that factors necessary for aggregation and of silk fibroin solutions through increasing the β-sheet content had been discussed earlier. Note that untreated silk fibroin solution which is poor in β-sheet content is hygroscopic and are very sensitive to humidity (Min *et al*., 2006). Thus, the β-sheet content is very vital for the purpose of storage and stability. Silk fibroin shows impeccable thermal stability with its structure remaining unaltered at temperatures as high as 140 °C. The glass transition temperature of proteins plays a vital part in protein self-assembly (Baimark*et al.*,2010).

4. Mechanical Properties

Mechanical properties are more important in making drug delivery scaffolds than it is in making nanoparticles. This is due to the fact that they serve the purpose of drug delivery as well as load bearing. Crystalline silk fibroin has outstanding mechanical properties and does not necessarily require crosslinking. It shows remarkable mechanical strength and resilience (Kim *et al.*, 2005).

5. Biodegradation

The drug delivery rate in any drug delivery system is of outmost importance, as this to a large extent determines the bioavailability of the drug. This is usually dependent on the proteolytic degradation of the polymer involved. In using scaffolds for drug delivery particularly as regards tissue regeneration, care must be taken to use materials that would not compromise the strength and the load carrying capacity of the material. While using particles the degradation rate determines the duration of drug release as well as the ability to maintain sustained release of the drug. The crystalline nature of treated silk fibroin makes it a good candidate for drug delivery vehicles as it can be tailored to suite the required release kinetics of the drug. It is also worthy of note that the rate of proteolytic biodegradation of silk fibroin relates directly to β-sheet content (Jin*et al.*, 2005).

2.9.3 Biocompatibility

Silk fibroin has been shown to be very biocompatible. It basically provokes little or no immune response and thus is very safe compared to other materials used for drug delivery (Meinel*et al*., 2005).

2.9.4 Silk fibroin as drug bearers

Silk fibroin derived particles for biomedical applications, laid emphasis on silk fibroin particles demonstrating high accessibility at the site of diseases or target destinations, long term sustained release as well as insignificant harmfulness when utilized as medication conveyance system (Mathur and Gupta, 2010). In this way, SF has kept on creating enthusiasm for biomedical research being a protein and a decent vehicle for drug conveyance, hence its uses as sutures and scaffolds for tissue engineering. Its bio-compatibility, rigidity and thermal stability guarantee that it is a decent candidate for smart-drug discharge products.

The properties of SF make it a perfect candidate for drug-conveyance systems. Silk fibroin nano-particles have been set up by specialists utilizing different techniques which incorporate desolvation, salting out, mechanical communition, electrospraying and supercritical fluid technology and used either as micro-spheres, nano-coatings for drug stacked liposomes, smaller scale coatings for polylactic glycolic acid particles. These are utilized to move micro-molecules, chemicals, genes and growth factors to their ideal site of activity (Zhao, *et al*., 2015).

Silk fibroin coating of lipid nanocarriers or its hybridization with the nano-carriers has been said to greatly assist the drug carriers in overriding several problems and limitations associated with lipid carriers (Gaber *et al.,* 2017). Researchers have also considered the development of a novel carrier in carrier system bringing together the pros in regenerative medicine and nanotechnology thus the development of a system in which mesenchymal stem cells were loaded with silk fibroin nanoparticles encapsulating curcumin for cancer therapy (Perteghella, *et al*., 2017).

2.10 Targeting Tumors Utilizing Silk Fibroin Molecules

Biomaterials employed in targeting tumors could be active or passive. Targeting tumors are considered passive, when they take advantage of the improved transit via the tortuous vessels in the tumours and poor exit due to impaired lymphatic drainage. For this situation, the bio-materials effectively penetrate over the vasculature of the tumor cells because of more extensive holes within the endothelial cells as well as are held at the site of the tumor due to the weak lymphatic channel at the tumor site. This procedure anyway has the issue of varying degrees of penetration among various tumors or diverse tumor destinations and consequently the idea of active tumor targeting has risen. Focused tumor targeting then again may include the conjugation of the nano-particles, ligands and most likely antibodies, aptamers, or radio isotopes which target specific epitopes communicated on the cell surface of the tumors (Iyer*et al*., 2006).

SF nano-particles has demonstrated itself to be a decent possibility for the two types of focusing because of its biocompatibility, flexibility, capacity to shape beta-pleated sheets and self-assemble just as the wide cluster of functional groups it communicates along its length in this manner making it simple for it to be bound to ligands and be further functionalized (Yu *et al*., 2014). In this manner, drug conveyance systems have been made utilizing silk fibroin bio-materials. SF nano-spheres stacked with paclitaxel in lymphatic malignant growth chemotherapy indicated great discharge properties of nine days to about fourteen days following in vitro discharge studies (Chen *et al*., 2012). Silk fibroin nano-spheres stacked with floxuridine with normal size of 210-510nm was tried in vitro utilizing HeLa cells (Yu *et al*., 2014).

The nano-spheres demonstrated great adherence to the cells, great discharge properties as well as curative and cell murdering effect on the disease cells. SF nano-coating's have been utilized to cover liposomes to alter their energy for sustained active drug delivery. Silk fibroin covered emodin stacked liposomes exhibited that the silk fibroin changed the kinetics of emodin by guaranteeing it was absolutely a diffusional procedure as against swelling. This eased back the discharge and upgraded the efficacy of emodin on the keloid cells (Gobin*et al*., 2006). Research studies explored and improved the potency, conveyance and efficacy of emodin by a tyrosine kinase inhibitor for the treatment of breast malignant growth. The silk fibroin covered liposomal emodin indicated upgraded take-up of emodin by the disease cells bringing about expanded cytotoxicity, drug retention, and diminished pace of discharge and metabolism, accordingly guaranteeing continued discharge and by and large improved efficacy (Cheema *et al*., 2007). Further examinations indicated silk fibroin nano-particles embodying curcumin to latently target HER2 over expressing breast cancer cells as well as HER 2 negative cells with improved apoptotic action, as well as cytotoxicity in both cell lines (Gupta *et al*., 2009).

Active tumor targeting utilizing doxorubicin stacked magnetic silk fibroin nano-particles appeared to enhance the movement and targeting of the refined orthotropic breast cancer cells by the chemotherapeutic agents (Tian *et al*., 2014). Moreover, the silk fibroin stacked doxorubicin indicated expanded release when in contact with the acidic condition of lysosomes of breast malignancy cell lines MCF-7, because of the low pH changing the electrostatic interaction of the silk fibroin protein (Sieb*et al*., 2013). More studies demonstrated utilization of silk protein rods designed and stacked with anastrazole, an FDA approved anti-cancer agent. Its pharmacokinetic information was examined while in-vitro testing it on breast malignant growth cells in Sprague drawley rodent models, utilizing fluid chromatography; this together with mass spectroscopy indicated a zero-order sustained anastrazole discharge for 91 days (Yucel *et al*., 2014).

SF nano-particles have been used to develop magnetic silk core nano shell nanoparticle to deliver curcumin into human breast cancer cells. The magnetic silk core nanoparticles showed sustained release of curcumin with its release rate regulated by varying the concentration of silk fibroin. These particles also showed enhanced cytotoxic and entrapment on cultured human breast adenocarcinoma cell line MDA-MB-231, evaluated by using the MTT and cellular uptake assays (Song *et al*., 2017).

In another study biodegradable eri silk nanoparticles were used to deliver Apo-bovine lactoferin (2% Fe saturated) and Fe- bovine lactoferin (100% iron saturated) to MDA-MB-231 breast cancer cell lines. With the nanoparticles showing sustained and controlled targeted release of the drugs (Patel *et al*., 2016)

Studies have also shown the use of silk fibroin loaded with paclitaxel and conjugated with a recombinant protein (anti- EGFR- iRGD) in the therapy of breast cancer. The nanoparticles were prepared using the carbonamide-mediated coupling procedure. After evaluation of their characteristics they were then tested invivo in a HELA xenograft rat model. The results obtained indicated that the conjugated silk fibroin paclitaxel nanoparticle with the recombinant protein had more targeting and antineoplastic activity in cells with the EGFR antigen expression when compared with the pristine silk fibroin nanoparticles loaded with paclitaxel (Bian*et al*., 2016).

In other studies, self-assembling silk fibroin nanoparticles have been used to demonstrate the release of binary drugs in the treatment of breast carcinoma, in a study done by Li and colleagues, they loaded the silk fibroin with 5FU and curcumin with a loading efficiency of 45% and 15% respectively. The nanoparticles were then tested on cultured murine breast cancer cells for the invitro test and cell apoptosis was measured by flow cytometry an assessment of reactive oxygen species was also undertaken. The invivo test was conducted on mice grafted with the cancer cells and apoptosis was assessed via histology using the haematoxylin and eosin stain. The results indicated an increase in apoptosis by the silk fibroin loaded nanoparticles carrying the binary drugs when compared to the pristine forms of the individual and the combined drugs of 5FU and curcumin. The increase in reactive oxygen species correlated directly with apoptosis and suggested that the nanoparticle binary drug complex induced programed cell death in the cancer cells via stimulating the increase in reactive oxygen species. (Li *et al.*, 2016)

Injectable silk fibroin nano-gel were developed and loaded with doxorubicin characterized and tested on human breast cancer cell line MDA-MB-231 both invitro and invivo. The invitro studies showed that doxorubicin loaded silk nanogel fibers had better outcome in terms of cell viability on the short term when compared with free doxorubicin but had an equivalent outcome with free doxorubicin on the long term. The invivo studies on the other hand showed that the doxorubicin loaded silk nano-gels showed better inhibition of the tumour growth with less normal cellular toxicity and was corroborated by findings on histological studies utilizing the haematoxylin and eosin stain (Wu *et al*., 2016).

Silk fibroin nano-diamond composites have been used to demonstrate the delivery of anticancer drug doxorubicin as well as coexisting imaging potential by fluorescence emission by the diamond component thus suggesting the potential of not only ensuring drug delivery but tracking, monitoring, treatment as well as diagnosing new sites of spread via imaging. This thus provides us with the possibility of having a multifunctional platform for therapy in future (Khalid *et al*., 2016).

2.11 Silk Fibroin and Apoptosis

Apoptosis also known as programmed cell death basically involves the removal of aged cells as well as elimination of cells which contain dysfunctional DNA. Evading this process has been described as the hallmark of all cancers (Nguyen *et al.*, 2009). This implies that any process that could induce programmed cell death in cancers may as well reverse the proliferation of cancer cells and ensure their elimination. Silk fibroin particles have been used in several studies as carriers for anticancer drugs. This brief review aims to examine the apoptotic effect of SF particles.

Mandal and Kundu (2009) demonstrated the apoptotic effect of self-assembled silk sericin/ polaxamer nano-carriers of hydrophilic and hydrophobic drugs for the treatment of cancers including breast cancers. They were able to demonstrate via western blot the up regulation of the proapoptotic Bax protein, decrease in anti- apoptotic Bcl-2 protein and cleavage of PARP as well as demonstrate via confocal microscopic studies apoptosis following staining of MCF-

7 cells with Annexin V. Similarly, Chon and colleagues (2013) were able to demonstrate the apoptotic effect of silk fibroin hydrosylate (HSF) using annexin V / propidium iodide flow cytometry method, as well as a double staining technique in MCF-7 breast cancer cells. They also demonstrated upregulation of Baxprotien, decrease Bcl-2, increase in caspase activity as well as cleavage of PARP, thus determining that HSF achieves its apoptotic effect through mitochondrial caspase dependent pathway.

Mathur and Gupta (2010) in their article alluded to the apoptotic effect of silk fibroin curcumin nanoparticles on MCF-7 breast cancer cells. This was further buttressed by Panda *et al* (2017), in their paper where they highlighted the use of silk fibroin nanoparticles to deliver curcumin to breast cancer cells, with a resultant enhanced uptake of curcumin by the MCF-7 cells, and a corresponding increase in cytotoxic and apoptotic potential. They thus suggested that SF particles be used as potential carriers for anti-tumor drugs in targeted cancer therapy.

Roy and colleagues (2015) used Eri silk fibroin nanoparticles to deliver apo bovine lactoferina known anticancer milk protein to MCF-7 and MDA-MB-231. This was easily internalized by the MCF 7 cells due to the expression of transferrin 1 and transferrin 2 by the cells. Apoptotic screening was performed using the apoptotic marker annexin V to treat and stain the cells. The results of annexin V assay showed higher degree of apoptosis with SF- loaded beta lactoferin treated cells when compared to cells treated with pristine lactoferrin. Lozano-Perez and colleague (2015) also demonstrated the apoptotic effect silk fibroin particles loaded with cisplastin using the known apoptotic marker annexin v.

(Pham *et al.*, 2019) using a DNA gel electrophoresis method demonstrated DNA fragmentation and ladder formation in MCF-7 breast cancer cells treated with cross-linked SF particles loaded with Alpha magostin.

SF particles does not only play a vital role in delivering drugs with apoptotic potential to target cells invitro but it may also act in synergy with this drugs to induce apoptosis in MCF-7 breast cancer cells in-vitro, thus making it a choice vehicle for the delivery of cancer drugs to target cells in future.

2.12 Procedures for Making Silk Fibroin Nanoparticles

Silk fibroin nanoparticles used in drug targeting system has numerous approaches to its production. Here this research would focus on a few of them. Methods used in the development of SF nanoparticles comprises the following; desolvation method, electrospray method, salting out, mechanical communition, supercritical fluid technologies, microdot capillary method, PVA blend method etc. Most of these methods have their pros and cons some of which would be discussed in this study (Zhao *et al*., 2015).

2.12.1 Desolvation method

Desolvation or coaacervation method is said to be the most widely used method for making protein-based nanoparticles, this is as a result of its relatively mild conditions. This method basically lessens protein solubility which results to phase separation and also it involves inclusion of a desolvating substitute usually natural salts or alcohol, which leads to modification shift in the protein configuration hence leading to protein precipitation. An adequate and balanced particle size is achieved via initial process term; thus further inclusion of non-solvent would enhance particle production. In using desolvation method, careful consideration is given to the charge as a net zero charge is said to yield nanoparticles at a rapid as well as productive manner. The PH of the protein solution which is adjustable is tuned based on the desired condition of the particle size and yield required (Sundar *et al*., 2010). Subia and Kundu in their report in 2012 described the preparation of silk fibroin albumin conjugates by desolvation method after which the loaded it with methotrexate and used it on cancer cell lines with optimum entrapment and efficiency (Subia& Kundu, 2012). Loncharoenkal et al in their review article described how organic solvents such as acetone and ethanol were utilized to induce conformational shift in the protein configuration which leads to protein precipitation and coacervation (Lonchaoenkal *et al*., 2014).

2.12.2 Salting out method

Salting out approach is another procedure used in developing nano-particale made from proteins and thus forming coacervates with the solution of protein. This exploits the chemistry of proteins to decrease its solubility in solution. Proteins are known to be made of amino acids which could be either hydrophilic or hydrophobic. When proteins are in solution the hydrophilic portion interacts with water molecules forming hydrogen bonds and stabilizing the proteins whilst the hydrophobic part forms the core of the protein. Deionized salts are known to have higher affinity for water molecules thus increasing the salt concentration in a protein solution would result in the salt interacting with the water molecules competitively against the hydrophilic aminoacids components of the protein hence resulting in a decrease in water barrier between the protein molecules as well as enhanced protein to protein interaction thus aggregating as a result of hydrophobic interactions (Zhao *et al*., 2015).

Lammel and his colleagues in their publication in 2010, described the use of 1.25M potassium phosphate at varying PH to develop silk fibroin nanoparticles which were subsequently loaded with drugs, thus establishing the use of salting out technique to develop a drug delivery system from protein solutions (Lammel *et al*., 2010).

Tian and colleagues also demonstrated the use of salting out to form doxorubicin loaded magnetic silk fibroin nanoparticles, it was subsequently utilized to investigate its activity for targeted drug delivery with the aid of an external magnetic field on multidrug resistant cancer cells (Tain *et al.*, 2014).

2.12.3 Supercritical fluid technologies

This involves the use of fluids above their critical temperature or pressure to micronize solutions. This method circumvents the disadvantages of the conventional method. Supercritical Carbon dioxide is the most widely used supercritical substance and has shown great potential in the field of micronization of materials due to its favorable critical conditions with a critical temperature of 31.1 degrees Celsius and at a critical pressure of 7.38 Mpa, it is also the cheapest amongst materials used in supercritical fluid technologies, it is nontoxic and non-flammable and thus its preference in pharmaceutical and food industries. Though this may be one of the best methods of fabrication of nanoparticles it is relatively expensive and requires more technical expertise when compared to the conventional methods (Zhao *et al*., 2015).

Zhao and colleagues demonstrated the fabrication of silk fibroin nanoparticles with the use of supercritical CO_2 which the published in 2012. The resulting nanoparticles exhibited good spherical shape and mean diameter of 50nm. Characterization of the particles demonstrated conformational transition from the random coil structure to beta sheets thus giving them physical and thermal stability as well as water insolubility before and after alcohol treatment. The particles were then used to carry non-steroidal anti-inflammatory drugs (Zhao *et al*., 2012).

2.12.4 Electro-spraying

This method utilizes electrical forces to atomize liquids. It is an emerging method for rapid and high throughput production of nano-particles (Zhao *et al*., 2015).

Qu and colleagues demonstrated the use of the electrospray method to manufacture silk fibroin nanoparticles using an organic solvent. The particles were shown to be spherical and having an average size of 59nm. The particles were loaded with Cis-dichlorodiamminoplatinum (CDDP) and tested on cancer cell lines in-vitro. The particle drug complex demonstrated sustained drug release and efficient killing of the tumour cells with minimal effect on the normal cell lines (Qu *et al*., 2014).

2.12.5 Mechanical communition

This simply implies the decreasing of solid materials from a certain particle size to a smaller size by crushing, grinding or milling. It however has the defect of long milling times and the presence of impurities (Zhao *et al*., 2015).

2.12.6 Micro emulsion method

This is the thermodynamically stable dispersion of immiscible liquids, aided by the use of surfactants. Micro emulsion could be basically classified into water in oil or oil in water. The method has the advantage of the researcher having a better control of the particle size (Zhoa*et al.*, 2015).

(Myung *et al*., 2008) generated fluorescent silk fibroin nanoparticles using a reverse micro emulsion method. The silk fibroin is dissolved in concentrated lithium bromide solution followed by dialysis. They then mix the coloured dye with the aqueous solution the surfactant used for the micro emulsion is then removed via treatment with ethanol and methanol to yield a colour dye doped silk fibroin Nano particle measuring about 167nm in size. A Fourier transform infra-red spectroscopy showed beta sheet conformation of the nano particles (Zhao *et al*., 2008).

This method however is said to have the problem having to add an organic solvent before removing it with the attendant risk of having residual organic solvent with its possible toxic effects (Sundar *et al*., 2010).

2.12.7 Capillary microdot method

In this method silk fibroin solution is dispensed on a glass slide using micro capillary, the slides are then frozen over the night and freeze dried. The resulting dots are scrapped of the slides and crystallized by methanol treatment. After the crystallization the nano-particles are recruited via centrifugation and rinsed with phosphate buffer saline, it is then characterized. This method was used by Gupta and colleagues to form silk fibroin encapsulated curcumin nano-particles less than 100 nm in size and used against cancer cell line (Gupta *et al*., 2009).

2.12.8 Polyvinyl alcohol blends film method

This process is based on phase separation between SF and polyvinyl alcohol. The silk fibroin and polyvinyl blend solution is dried into a film, then water insoluble particles are fabricated by dissolution of the film in water after which centrifugation is done to rid it of polyvinyl alcohol. Drugs can be loaded into the silk fibroin by mixing the model drug into the original silk fibroin solution. This method was demonstrated by Wang and colleagues to make silk fibroin nano spheres and micro spheres with controllable size and shape (Wang *et al*., 2010).

2.12.9 Ionic gelation method

Ionic gelation method or ion induced method simply involves the interaction of an ionic polymer with oppositely charged ions to induce crosslinking. It thus eliminates the use of harsh chemicals to generate micro or nanoparticles (Ahirrao*et al*., 2013. Kunjachan*et al*., 2014). It has been used to encapsulate probiotics with 100% encapsulation yield. After lyophilization the particles were also shown to protect the lactobacillus from acid media (Sanchez *et al*., 2017). Benavides and colleagues in a study successfully encapsulated thyme and essential oils with an encapsulation efficiency of approximately 85% using ion induction to generate alginate microspheres (Benavides *et al*.,2016). Chitosan indomethacin nanocarriers for intra ocular administration have been produced via ionic gelation using tripolyphosphate (Bandawi, *et al*., 2008). Regenerated silk fibroin has been used to develop injectable hydrogels with laponite nanoparticles using polyelectrolyte complexation method with the resultant effect of increased cell proliferation and osteogenicity in bones (Su*et al*., 2016). Silk fibroin nanoparticles carriers has been shown to successfully encapsulate anticancer drug doxorubicin with 65% encapsulation efficiency after 11 days using the ion induction method (Wang *et al*, 2015). Bearing in mind the problems of ionic gelation which include improper surface morphology, fragile particulate system , low stability (Kujachan*et al*., 2014) and in an attempt to avoid the use of harsh chemicals and enhance the encapsulation efficiency this study focuses on the encapsulation of carboplatin in silk fibroin using ionic gelation method in other to improve the stability of the SFCP particle by formation of beta pleated sheets following lyophilization (Li *et al*, 2001) and enhance encapsulation efficiency.

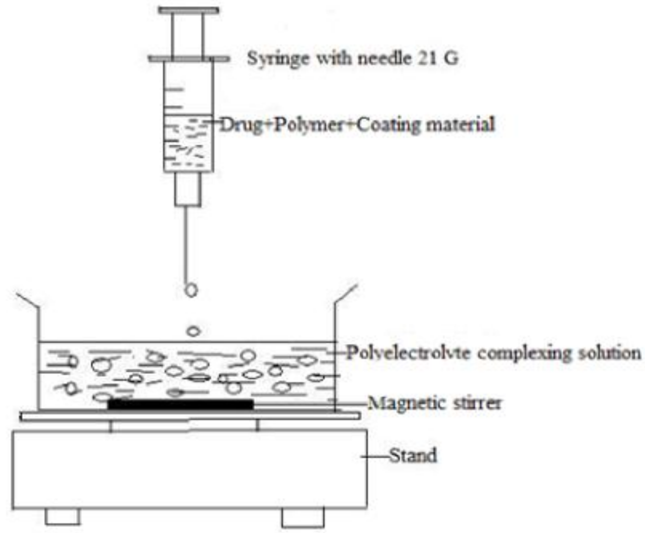


Figure 2.7: Image of the process of ionic gelation

CHAPTER 3
MATERIALS AND METHOD

3.1 Materials

Silk fibroin used was of the domesticated mulberry cocoons gotten from Buyuk Han in the North of Cyprus. Apostrand Elisa apoptosis kit (Enzo, Switzerland), MCF-7 breast cancer cells (ATCC® HTB-22™,Manassa, Virginia, US), 10 % FBS Thermofisher, Grand Island, New York, US, 1% penicillin / streptomycin (Thermofisher, Grand island, New York, US), insulin in DMEM / F12 (thermofisher US), $C_6H_{12}N_2O_4Pt$, Sigma Aldrich, Germany, Na_2CO_3, Sigma Aldrich, Schneldorf, Germany,sodium triphosphate pentabasic(TPP) Sigma Aldrich, $CaCl_2$, Sigma Aldrich, Schneldorf, Germany, C_2H_5OH, Sigma Aldrich, Schneldorf, Germany and dialysis membrane (cut off MW 12,400) with full average diameter 16 mm and flat width 25mm capacity of 60ml/ft were purchased from Sigma Aldrich. All other reagents were of analytical grade and also purchased from Sigma Aldrich.

3.2 Preparation of Pure Silk Fibroin

The process of making pure silk fibroin involves degumming, dissolution and dialysis respectively.

3.2.1 Degumming

The degumming of silk involves the cleavage of peptide bonds either by hydrolytic or enzymatic methods of the sericin in silk fibroin. This can be done either in an acidic, alkaline or neutral condition. In this study we employed the hydrolytic method of boiling off in an alkaline solution of sodium bicarbonate solution. 5g of cut and cleaned silk cocoons was boiled with 500ml of 0.1 molar sodium bicarbonate at 70-degrees Celsius for three hours on the magnetic stirrer at a speed of 1.5rpm, this was repeated three times with the silk washed with ultra-pure water after each episode.


a


b


c

Figure 3.1: (A) Silk cocoons (B) Clean and cut cocoons (C) Process of degumming

3.2.2 Dissolution

The dissolution achieves changing of the physical state of silk fibroin from a solid to liquid form by breaking the polypeptide chains using strong electrolyte solution consisting of calcium chloride, water and ethanol in a ratio of 1:8:2 calculated as 27.75 g of calcium chloride, 29.15mL of absolute ethanol and 36ml of deionized water. The required grams to get 6% at 6g/100mL w/v was calculated and mixed on the magnetic stirrer and heated at 75 degrees Celsius until complete dissolution is achieved.

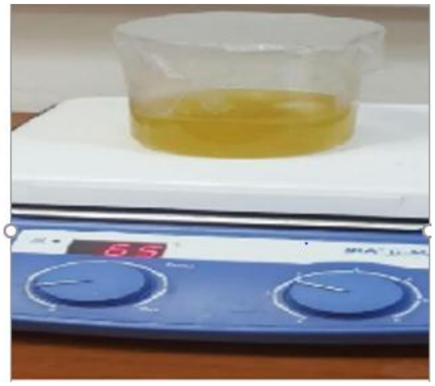


Figure 3.2: Process of dissolution

3.2.3 Dialysis

Dialysis is done to remove the ions involved in the dissolution process, and consequently yield pure silk fibroin. A semipermeable membrane tube or cassette is used to perform the dialysis of liquid silk fibroin against deionized or ultra-pure water in other to allow the diffusion of the ions and salt into the water compartment from the silk fibroin. In this study a snake skin dialysis membrane with molecular cut off (MCO) 7000 was used over nine hours in three divided rounds. The silk fibroin is measured and put into the dialysis membrane, a 2000mL beaker is filled with ultra-pure water and the tube containing the silk fibroin is placed into the beaker which contains a magnetic stirring bar and is placed on the stirrer at 1rpm for three hours after which the water is changed and the process repeated two more times. The pure silk fibroin is then removed with the aid of a syringe.

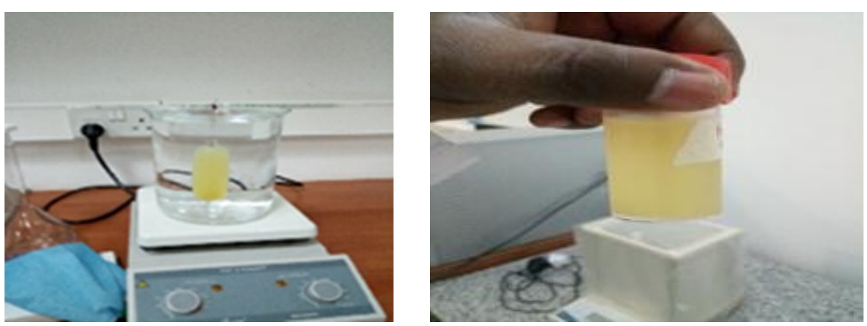


Figure 3.3: Showing the process of dialysis and pure silk fibroin respectively

3.3 Incorporation of Drugs and Synthesis of Particles

In this work the direct incorporation method of the drug is used, the drugs are dissolved into a known quantity of pure silk fibroin and subsequently made into nanoparticles using an ionic gelation method based on the usage of a cross linker known as sodium triphosphate pentabasic (TPP). Since silk fibroin has strongly polar side groups, it does not require activation before conjugation with drugs or other molecules. The drug was added and stirred vigorously with the aid of a magnetic stirrer till it was well mixed.

3.3.1 Synthesis of microparticles

The solution of the SF and drugs are then added drop wise with the aid of a micrometer gauge syringe while stirring into 50mL of 0.1M TPP Solution. The mixture is then ultra-sonicated, and centrifuged at 400rpm for 10minutes. The top most layers are collected separately, making it possible to employ spectroscopy to investigate the quantity of carboplatin present which would then enable the calculation of the amount of loaded carboplatin.

The collected nanoparticles are washed with ultra-pure water and sterilized using an autoclave.

Calculations are done by:

$$\% \text{ Encapsulation} = \frac{weightofdruginmicroparticle}{weightofdrug} \times 100 \qquad (4.1)$$

3.4 Particle Characterization

3.4.1 Particle size analysis

Mastersizer 2000 version 5.60 serial no: MAL100704 (Malvern, UK) was of Middle East Technical University (METU) Central Laboratory located in Ankara, Turkey and used to analyze the size of the SFCP microparticle.

3.4.2 SFCP microparticles morphology

Scanning electron microscope (S-3400N: Hitachi, Japan) was used to detect the surface morphology while gold nanoparticles were utilized in the encrustation of particles. The voltage for photography was 15KV. Experiments were carried out at room temperature. Mastersizer 2000 (Malvern, UK) was used to decide the particle size during distribution.

3.4.3 Biological degradation test

At 37°C, 0.2mg/ml of protease was employed to investigate the biological degradation of SFCP nanoparticles. Thus, biological degradation can be determined by:

$$Biodegradation\ (weight\ loss)\% = \frac{weight\ (t)}{initial\ weight} x100 \quad (4.2)$$

3.4.4 Fourier Transforms Infrared Spectroscopy (FTIR)

Fourier transform infrared spectroscopy is employed on silk fibroin nanoparticles to know its configuration and evolution including the encasing of carboplatin in silk fibroin, using a Perkin Elmer FTIR spectroscopy.

3.5 *In-vitro* Drug Release Profile

The *in-vitro* release profile is assed via dialysis method. The SFCP (2g) micro-particles is sealed in a snake skin dialysis membrane MCO 10,000 and dialyzed against phosphate buffer at pH of 4.8 mimicking the acidic nature of the tumour microenvironment and 7.4 mimicking physiological pH respectively. The amount of carboplatin released is assessed with the aid of UV spectroscopy using an indirect method described by Basotra and colleagues (Basotra *et al*., 2013) this is done while keeping the volume of PBS constant over a known time interval.

3.6 Cell Culture

Human adenocarcinoma cell line MCF-7 was used for intracellular accumulation and cytotoxicity studies. Breast adenocarcinoma cells were maintained inside an incubator at 37 degree Celsius, with air supplemented with CO2 (5%),10% PBS, 1% penicillin/streptomycin, 4 mg insulin in DMEM/ F12 in a humidified atmosphere and incubated. The media was changed every other day until it reached 70% confluence after which it was passaged.

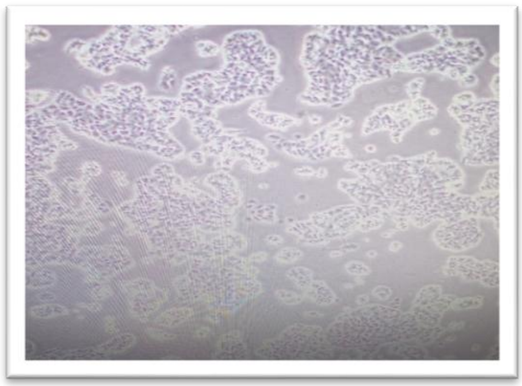


Figure 3.4: MCF7 cells at > 60% confluence

3.6.1 Apoptosis evaluation

Kit used in this process is known as Apostrand Elisa apoptosis kit, the cells are seeded in a 96 well plate and the culture media for the experimental and control groups are used. A micro plate reader is used to assess the denatured DNA as a result of formamide action on the cells. Apostrand Elisa kit is sensitive to the DNA of Cells during apoptosis by formamide denaturation, this was only possible by the utilization of single DNA strand having monoclonal antibody. 96 well plates were prepared and a minimum of 5000 cells was implanted. A day later, the cells are expected to have seeded properly and the optimum density achieved. The cells were then incubated with 50,100,150 and 200µg/mL SFCP nanoparticles for 24-72hours specifically. Positive controls are cultured cells of carboplatin and the negative control was without carboplatin. Percentage of cytotoxicity was expressed as IC_{50}. The results for apoptosis were calculated as well as standard error of mean ± (SEM).

3.7 Statistical Analysis

Data acquired were indicated by standard error mean ±. One-way variance analysis was used to test for group significant differences; having statistical importance of 0.05 level of probability using graphpadinstat tool.

CHAPTER 4
RESULTS AND DISCUSSION

4.1 Encapsulation of Silk Fibroin Carboplatin Particles (Synthesis and Characterization)

Basically the research purpose was to construct silk fibroin-carboplatin microparticle as a vehicle for focused drug delivery utilizing ionic-gelation approach. The reaction scheme showing interaction between SF, TPP, and CP is shown in Figure 4.1. Several methods have been used in the past to encapsulate carboplatin, however, none of the data used showed the use of ionic gelation approach. The distribution of the silk fibroin carboplatin microparticle was achieved using Mastersizer of about 0.02-2000µm having a sensitivity and obscuration of 12.24%. Silk fibroin carboplatin microparticle used has a volume of 0.0728%. Therefore, the mean value for the volume weighted and surface weighted are seen in Table 4.1 as 40.629µm and 79.695µm.

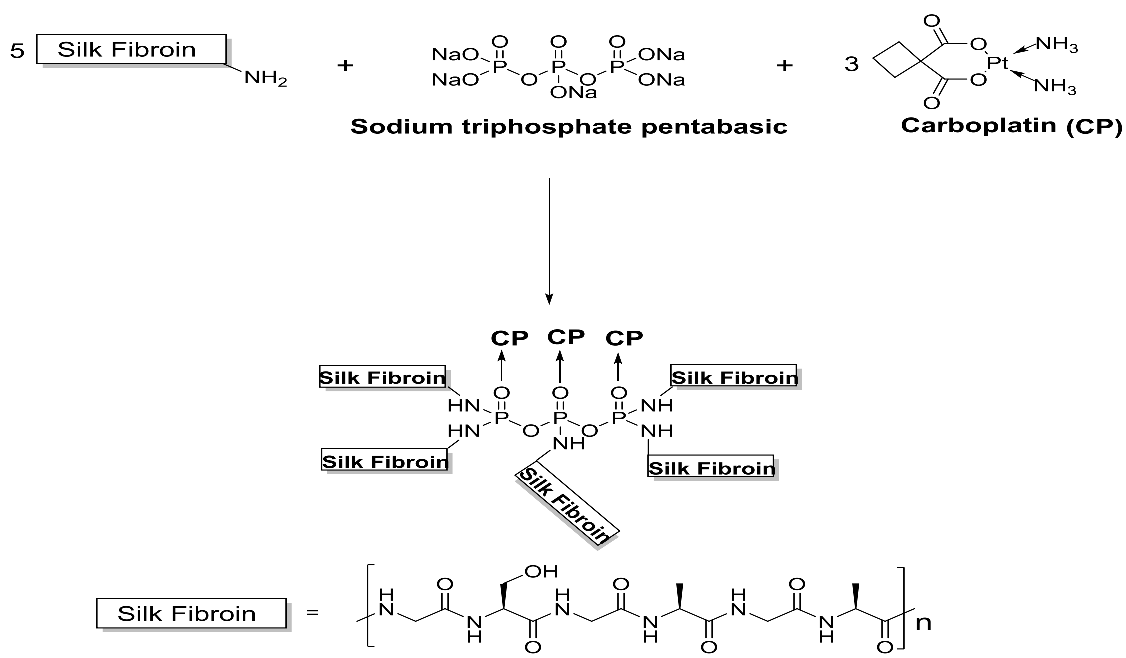


Figure 4.1: Reaction scheme of the crosslinking between SF, CP and TPP

Table 4.1: Master Sizer Analysis of Particle Size Distribution

Size Distribution Analysis of Silk Fibrion Carboplatin Microparticle	
Residue Weighted	0.802%
Sensitivity	Normal
Size range	0.02-2000µm
Concentration	0.0728% Volume
Specific surface area	$0.184 m^2/g$
Volume Weighted Mean D (4, 3)	79.695µm
Surface Weighted Mean D (3, 2)	40.629µm
E44d (0.1)	22.212µm
d (0.5)	61.728µm
d (0.9)	146.080µm

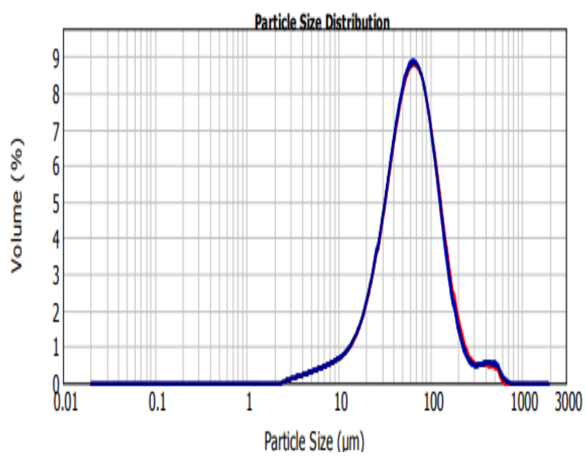
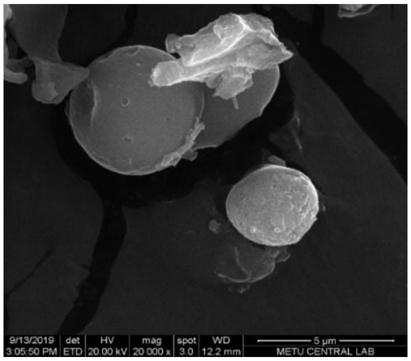
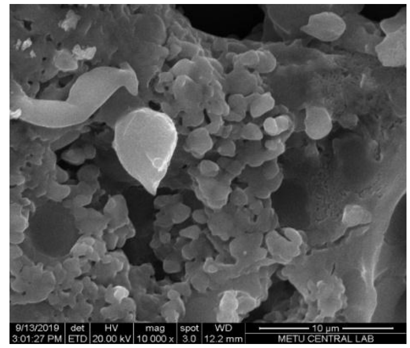


Figure 4.2: Size distribution graph of Silk fibroin carboplatin microparticle

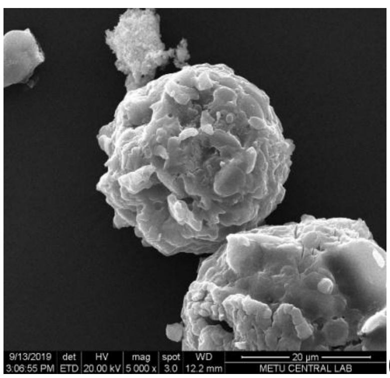
SEM was used for the morphological analysis of Silk fibroin carboplatin microparticle. From the micrograph provided by the SEM from the silk fibroin carboplatin microparticle, it showed that the shape of the analyte is spherical (Figure 4.3a), nonetheless, the particles showed a size range of 5-146.08µm. Globules presented in figure (4.3b) consist of little pseudospherical particle with aggregate forms (4.3c).



(a)



(b)



(c)

Figure 4.3: Micrographs of SFCP from Scanning electron microscope (**a**) 5µm, (**b**) 10µm (**c**), 20µm,

4.2 Biodegradation Analysis

SFCP microparticles were analyzed using solution of protease (0.2mg/100ml H_2O) at 37^{o}C. Table 1 & 2 showed the synthesis condition and biodegradation profile of the silk fibroin carboplatin microparticle. SFCP showed faster degradation with increase in the amount of carboplatin in the particle. The rate of biodegradation is determined by the quantity or amount of carboplatin in the silk fibroin carboplatin microparticle in the reaction as proven by the result.

Table 4.2 Silk fibroin – Carboplatin micro particles synthesis conditions

SAMPLE	3% SILK FIBRION(ML)	CARBOPLATIN(ML) 10%V/V	TPP (0.1M) ML
SFCP1	10	0.01	5O
SFCP2	10	0.05	50
SFCP3	10	0.10	50

Sodium Triphosphate Pentabasic: TPP

Table 4.3 Silk Fibroin Carboplatin biodegradation analysis using protease solution at 37°C

Time w(hr)	SFCP1(μg)	SFCP2(μg)	SFCP3(μg)
0.00	100	100	100
0.25	90.88	86.30	83.40
0.50	82.64	79.22	75.76
0.75	73.40	69.85	66.41
1.00	60.87	56.20	50.22
1.25	59.41	49.23	42.35
1.50	52.73	43.32	30.72
1.75	45.53	38.90	13.65
2.00	36.90	30.24	05.44
2.30	27.23	18.84	0
2.75	18.35	05.67	0
3.00	04.23	0	0
12	0	0	0
24	0	0	0
25	0	0	0
26	0	0	0

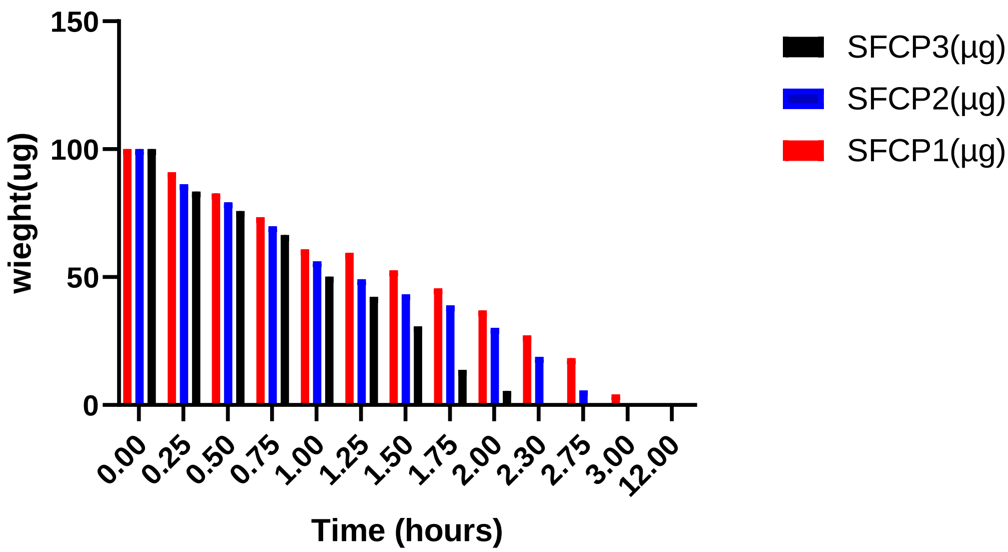
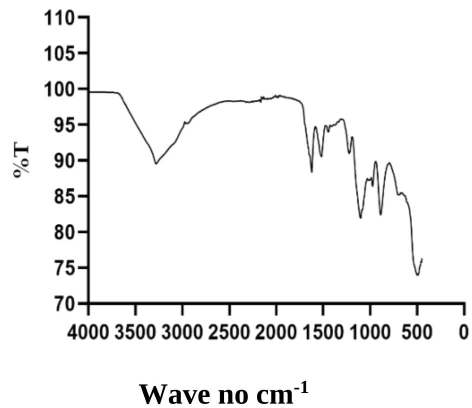


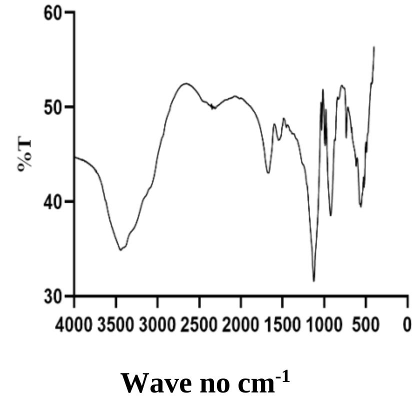
Figure 4.4: Graph showing Silk Fibroin Carboplatin biodegradation analysis using protease solution at 37°C

4.3 FTIR Spectra Analysis

Fourier transforms infrared demonstrated (FTIR) distinctive silk fibroin indications. Silk fibroin and carboplatin results from FTIR analysis are in agreement with studies in the past (Lozano-Perez *et al*., 2005; Jose *et al*., 2016). SFCP particle exhibited a decrease in transmittance; perhaps due to the ring-like form of carboplatin, thereby resulting in an increase in absorbance an invariably a decrease in transmittance by the SFCP. SFCP particles tracing demonstrated high absorptivity and low transmittance in contrast to silk fibroin alone. However, SFCP particles and silk fibroin indicated peaks expressive of OH group at 3452cm^{-1} (Figure 4.4a) and 3273cm^{-1} for silk fibroin alone (Figure 4.b). Another peak showed 1680cm^{-1} indicated a carbonyl functional group for SFCP microparticles while 1622cm^{-1} peak indicated amide1 for silk fibroin particles. Observable secondary bends of NH were seen at 1554cm^{-1} for SFCP and 1517cm^{-1} for silk fibroin particles. SFCP microparticles peak for carbonyl group was seen at 1123cm^{-1}. All spectral shift, characteristics and features are that of carboplatin stacked silk fibroin particles (Lozano-Perez *et al*., 2015).



(a)



(b)

Figure 4.5: FTIR-spectrum showing transmittance against wave number $cm^{-1}$for silk fibroin protein microparticles (a) and (b) spectrum for SFCP micro-partictles.

4.4. Encapsulation Percentage of SFCP Microparticles

Encapsulation percentage was investigated by weighing the drug within the particle divided by the quantity of the feeding drug through the indirect procedure used by Basotra and colleagues (Basotra*et al*,2013). The starndard deviation of particle synthezised and encapsulation percentage through ionic gelation was calculated. Thus, mean encapsulation percentage of carboplatin in SF utilizing TPP to induce ionic gelation was 83.22±0.07. Therefore, the process of ionic gelation procedure of carboplatin encapsulation in silk fibroin protein was demonstrated for the first time in this study.

4.5 Drug Release of SFCP

The cumulative drug release was measured at varying pH for the synthetized particles for over 48hr;(figure 6). Particles indicated an initial fast rate of drug release and afterwards, became steady with time. At acidic pH, a higher rate of drug concentration was released. There was a significant fast delivery with successive regularization of the amount from the particles which agrees with previous studies (Perez et al, 2015). Results therefore, verifies study by Subia and colleagues(Subia, *et* al, 2013) and thus conform with the properties of anticancer candidate treatment approach considering that cancer microenvironment is known to be acidic.

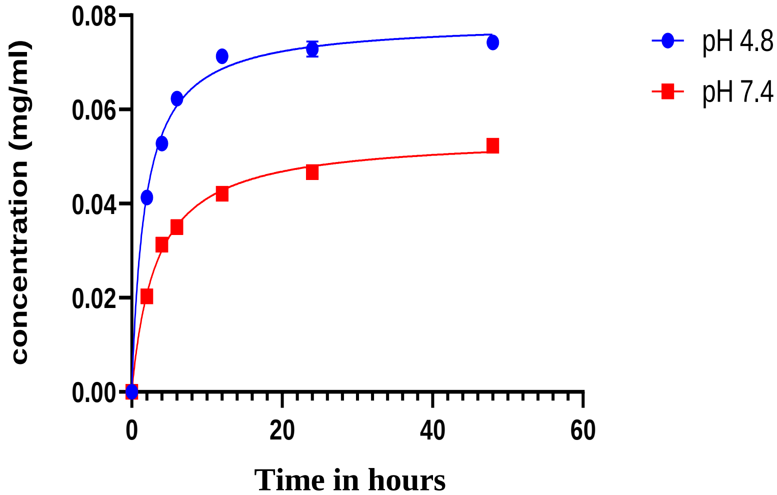


Figure 4.6: Graph showing CP release from SFCP microparticlesover time in hours.

4.6 SFCP Particles and Apoptotic Activity

Student's t test and analysis of variance were used to analyze the absorbance Values recorded. Incubation results of SF-CP on MCF-7 adenocarcinoma cell lines at various time-intervals 24, 28 and 72 hours at 450nm. Significant apoptotic activity at 95% confidence interval was observed on SF-CP particles prepared by inoic gelation procedure over the control experiment (figure 7). At varying incubation times, there was no significant difference recorded. Therefore, this may show a balance in the activity of the drug over 72hr. SFCP particles has an IC_{50} of 9.23µg/mL.

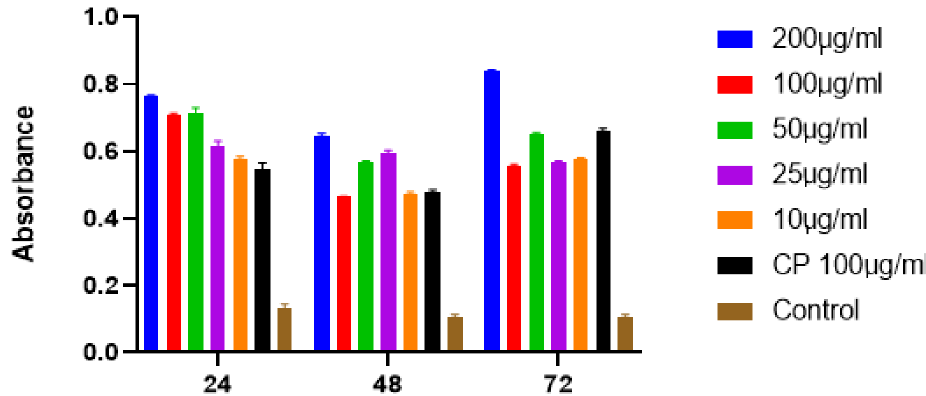


Figure 4.7 SFCP particles ANOVA analysis on MCF-7 cell lines after 24, 48 and 72hr.

Apoptotic activity and absorbance 450nm are directly proportional and thus, indicated an optimal activity at 72hr which was dose dependent. There were statistical significance ($p<0.00001$) on the concentration of SFCP microparticles when contrasted with the control experiment. No significant difference was demostrated between apoptotic activity of positive control and SFCP microparticles, considering that when apoptosis fails to occur, cancer develops (Nguyen *et al*., 2009). Thus, prodecures that can alter apoptosis can also reverse the spread of cancer cells. There are many studies in the past where silk fibroin particles were employed as target transporters for anticancer drug delivery. Silk fibroin when encased with natural materials or products inhibited cancer proliferation and causes apoptosis in breast cancer (Mathur & Gupta, 2010; Panda *et al*., 2017). This research study is the first to investigate and prove encapsulation of SFCP microparticles with carbiplatin anticancer drug by ionic gelation procedure that indicated apoptotic activity at 95% significant confidence interval compared to control experiment. Therefore, this result shows an uninterrupted activity over a period of three days; considering the efficiency of SFCP microparticles as having apoptotic reaction on MCF-7 breast cancer cell lines.

CHAPTER 5
CONCLUSION

5.1 Conclusions

The particles synthesized shown in Table 4.1 as 40.629 µm and 79.695 µm as mean values of volume weighted and surface weighted is above the range of the nanoparticle (Singh *et al.*, 2009) and is consistent with the sizes of silk fibroin microparticles though larger than nanoparticles synthesized by Subia and colleagues(Subia *et al*, 2014),particles of this size would also easily utilize the EPR effect to access the tumour site due to widened endothelial gaps as well as poor lymphatic drainage in solid tumours (Meng *et al*, 2011). Utilization of such particles may have problems as regards the heterogeneity of the tumour vasculature. Particularly when comparing primary tumours and secondaries that result from them. These being a result of the fact that secondary tumour do not have as much vascularization as the primary tumours. Secondary tumours however are associated with more macrophages a component of the mononuclear phagocyte system. This fact confers on our vehicle the silk fibroin microparticles some advantages since studies have shown that modification of the bio-distribution profile of cytotoxic and cytostatic drugs attached to biodegradable carriers, precede in a way that ensures its delivery to the mononuclear phagocyte system (Brigger*et al.*, 2012*)*. This implies that our drug does not only present a platform for passive targeting of primary tumours but may be very important in the management of secondaries as well. The results of the percentage encapsulation suggest good encapsulation using the ionic gelation method; this may be due to the cross linker effect of TPP (Patil *et al*, 2012). Though electro-spray method have been used to synthesize silk fibroin cisplatin particles it has been described as very expensive utilizing a lot of chemicals and producing residual waste which may be harmful (Tapia-Hernandez *et al*, 2015) Ionic gelation on the other hand requires fewer chemicals with very little or no harmful residue generated .The biodegradation profile shows that biodegradation of SFCP progresses faster with increase in the carboplatin concentration relative to the SF and TPP, suggesting that the degradation of the SFCP can be regulated by varying the amount of carboplatin solution. The cumulative drug release over a 48hour period was characterized by a rapid release phase followed by normalization of the concentration of drug exuded from the particles synthesized. Micro-particles synthesized however showed a pH-dependent release with more of the CP released at acidic pH, this corraburates the study done by Subia and colleagues (Subia *et al*, 2014).

Apoptosis which is in other words 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 hallmark 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 the silk fibroin particles are good transporters of anticancer drugs. To date, a number of studies have used silk fibroin encapsulated drugs or natural products to test the proliferation and apoptotic activities in breast adenocarcinoma cells. SFCP showed an outstanding MCF7 cell death activity, showing a 95% confidence interval as to the control experiment, with no significant differences between the doses and the pristine drug. This showed the effectiveness of the drug at the end of 72-hour period. This therefore, shows the promising *in vitro* apoptotic action of SFCP on MCF-7 breast cancer cells.

5.2 Conclusion

This study successfully established the use of ionic gelation method to synthesize SFCP microparticles for the first time. The indirect technique utilized by Basotra and colleagues was used to determine the amount of CP encapsulated (Basotra *et al*, 2013). The distinctive attributes of SF and carboplatin was affirmed using Fourier transform infrared spectra. Drug cumulative release profile was of the primitive feature of antineoplastic agents favouring an acidic micro environment an important feature for passive drug targeting, bearing in mind that the tumour microenvironment is traditionally acidic. There was good apoptotic activity shown *in vitro* against MCF7 breast adenocarcinoma cell line, which is the hallmark for ensuring cancer cell death. SFCP particles created via ionic gelation method promises to present an excellent platform for cancer chemotherapy, with prospects for further functionalization for drug targeting and theranostics.

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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 "APOPTOTIC AND ANTICANCR POTENTIAL STUDIES OF SILK FIBROIN LOADED CARBOPLATIN 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: Assoc. Prof. Dr.
Name Surname: Pınar Tulay

Signature:

Role in the Research Project: Supervisor

APPENDIX 2

SIGNED SIMILARITY REPORT

Ph.D

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☐	AUTHOR	TITLE	SIMILARITY	GRADE	RESPONSE	FILE	PAPER ID	DATE
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☐	Nanyak Galam	Acknowledgments	0%	--	--	▢	1376967053	31-Aug-2020
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Assoc. Prof. Pinar Tulay

APPENDIX 3

RAW DATA ENCAPSULATION

Synthesis – 10mg 0f carboplatin was loaded on SF (10mg/ml)

Loading dose= 10mg

Concentration in nanoaggregates = loading dose – concentration in supernatant

Absorbance was read at 706nm

Appendix 3: Raw data for ionic gelation method

S/N	ABSORBANCE	CONCENTRATION IN SUPERNATANT(MG/ML)	CONCENTRATION IN PARTICLES (MG/ML)
1	0.260	1.668	8.332
2	0.261	1.674	8.326
3	0.259	1.661	8.339

APPENDIX 4

RAW DATA DRUG RELEASE

Appendix 4a: Drug release at pH 4.8

S/N	2 hours	4 hours	6 hours	12 hours	24 hours	48 hours
1	4.10	5.20	6.20	7.10	7.10	7.41
2	4.20	5.40	6.20	7.20	7.35	7.43
3	4.10	5.20	6.30	7.09	7.40	7.43

Appendix 4b: Drug release at pH 7.4

S/N	2 hours	4 hours	6 hours	12 hours	24 hours	48 hours
1	2.00	3.10	3.50	4.18	4.70	5.20
2	2.00	3.20	3.60	4.20	4.72	5.30
3	2.10	3.10	3.51	4.25	4.60	5.19

APPENDIX 5

DESCRIPTIVE STATISTICS FOR DRUG RELEASE

Appendix 5a: Descriptive statistics for drug release at pH 4.8

	2 hours	4 hours	6 hours	12hours	24 hours	48 hours
Number of values	3	3	3	3	3	3
Minimum	4.100	5.200	6.200	7.090	7.100	7.410
Maximum	4.200	5.400	6.300	7.200	7.400	7.430
Range	0.1000	0.2000	0.1000	0.1100	0.3000	0.02000
Mean	4.133	5.267	6.233	7.130	7.283	7.423
Std. Deviation	0.05774	0.1155	0.05774	0.06083	0.1607	0.01127
Std. Error of Mean	0.03333	0.06667	0.03333	0.03512	0.09280	0.006506

Appendix 5b: Descriptive statistics for drug release at pH 7.4

	2 hours	4 hours	6 hours	12 hours	24hours	48 hours
Number of values	3	3	3	3	3	3
Minimum	2.000	3.100	3.500	4.180	4.600	5.190
Maximum	2.100	3.200	3.600	4.250	4.720	5.300
Range	0.1000	0.1000	0.1000	0.07000	0.1200	0.1100
Mean	2.033	3.133	3.537	4.210	4.673	5.230
Std. Deviation	0.05774	0.05774	0.05508	0.03606	0.06429	0.06083
Std. Error of Mean	0.03333	0.03333	0.03180	0.02082	0.03712	0.03512

APPENDIX 6

Appendix 6: Raw data absorbance vs time

Time (hours)	200µg/ml	100µg/ml	50µg/ml	25µg/ml	10µg/ml	CP (100µg/ml)	Control
24	0.764	0.704	0.720	0.600	0.575	0.535	0.125
	0.766	0.702	0.700	0.620	0.573	0.541	0.130
	0.768	0.715	0.728	0.628	0.586	0.568	0.147
48	0.650	0.465	0.569	0.580	0.473	0.470	0.102
	0.642	0.466	0.570	0.590	0.470	0.472	0.100
	0.652	0.467	0.571	0.603	0.479	0.486	0.116
72	0.841	0.545	0.653	0.570	0.574	0.662	0.100
	0.839	0.556	0.650	0.565	0.572	0.650	0.115
	0.843	0.559	0.656	0.569	0.582	0.668	0.100

APPENDIX 7

ABSORBANCE VS TIME

Appendix 7: Absorbance Vs Time

	200µg/ml	100µg/ml	50µg/ml	25µg/ml	10µg/ml	CP 100µg/ml	Control
24hours	0.766±0.0020	0.707±0.0070	0.716±0.0144	0.616±0.0144	0.578±0.0070	0.548±0.0176	0.134±0.0115
48 hours	0.648±0.0053	0.466±0.0010	0.570±0.0010	0.591±0.0115	0.474±0.0046	0.476±0.0087	0.106±0.0087
72 hours	0.841±0.0020	0.555±0.0074	0.653±0.0030	0.568±0.00265	0.576±0.0053	0.660±0.0092	0.105±0.0087

APPENDIX 8

ONE WAY ANALYSIS OF VARIANCE ABSORBANCE VS TIME

Appendix 8: Transmittance of Pristine Silk Fibroin

Table Analyzed	**Data 2**				
Data sets analyzed	A-G				
ANOVA summary					
F	19.78				
P value	<0.0001				
P value summary	****				
Significant diff. among means (P < 0.05)?	Yes				
R squared	0.8945				
Brown-Forsythe test					
F (DFn, DFd)	0.7821 (6, 14)				
P value	0.5978				
P value summary	ns				
Are SDs significantly different (P < 0.05)?	No				
Bartlett's test					
Bartlett's statistic (corrected)					
P value					
P value summary					
Are SDs significantly different (P < 0.05)?					
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Treatment (between columns)	0.7235	6	0.1206	F (6, 14) = 19.78	P<0.0001
Residual (within columns)	0.08532	14	0.006095		
Total	0.8088	20			
Data summary					
Number of treatments (columns)	7				
Number of values (total)	21				

APPENDIX 9

TRANSMITTANCE FOR SFCP MICROPARTICLES

Created as New Dataset cm^{-1}	Sample 039 By Administrator Date Friday %T	September 06 2019
4000	44.72	
3999	44.72	
3998	44.72	
3997	44.72	
3996	44.71	
3995	44.71	
3994	44.71	
3993	44.7	
3992	44.7	
3991	44.7	
3990	44.7	
3989	44.7	
3988	44.7	
3987	44.69	
3986	44.69	
3985	44.68	
3984	44.68	
3983	44.68	
3982	44.68	
3981	44.67	
3980	44.67	
3979	44.67	
3978	44.66	

3977	44.66
3976	44.66
3975	44.66
3974	44.66
3973	44.65
3972	44.65
3971	44.65
3970	44.65
3969	44.65
3968	44.64
3967	44.64
3966	44.64
3965	44.63
3964	44.63
3963	44.62
3962	44.62
3961	44.62
3960	44.62
3959	44.62
3958	44.62
3957	44.62
3956	44.61
3955	44.61
3954	44.6
3953	44.6
3952	44.59
3951	44.58
3950	44.57

3949	44.57
3948	44.56
3947	44.56
3946	44.57
3945	44.57
3944	44.57
3943	44.57
3942	44.57
3941	44.57
3940	44.57
3939	44.57
3938	44.56
3937	44.55
3936	44.53
3935	44.52
3934	44.51
3933	44.51
3932	44.52
3931	44.53
3930	44.54
3929	44.53
3928	44.53
3927	44.52
3926	44.52
3925	44.51
3924	44.5
3923	44.49
3922	44.49

3921	44.48
3920	44.47
3919	44.47
3918	44.47
3917	44.48
3916	44.49
3915	44.49
3914	44.49
3913	44.49
3912	44.49
3911	44.47
3910	44.46
3909	44.44
3908	44.43
3907	44.42
3906	44.41
3905	44.4
3904	44.39
3903	44.38
3902	44.39
3901	44.42
3900	44.44
3899	44.45
3898	44.43
3897	44.42
3896	44.41
3895	44.4
3894	44.4

3893	44.41
3892	44.41
3891	44.39
3890	44.36
3889	44.34
3888	44.35
3887	44.36
3886	44.35
3885	44.33
3884	44.31
3883	44.32
3882	44.35
3881	44.36
3880	44.35
3879	44.32
3878	44.31
3877	44.32
3876	44.33
3875	44.32
3874	44.29
3873	44.27
3872	44.28
3871	44.3
3870	44.3
3869	44.27
3868	44.26
3867	44.27
3866	44.29

3865	44.29
3864	44.27
3863	44.25
3862	44.25
3861	44.24
3860	44.23
3859	44.22
3858	44.21
3857	44.2
3856	44.21
3855	44.24
3854	44.26
3853	44.22
3852	44.15
3851	44.13
3850	44.15
3849	44.18
3848	44.17
3847	44.16
3846	44.16
3845	44.15
3844	44.14
3843	44.12
3842	44.12
3841	44.12
3840	44.14
3839	44.15
3838	44.15

3837	44.12
3836	44.1
3835	44.09
3834	44.1
3833	44.11
3832	44.1
3831	44.08
3830	44.07
3829	44.07
3828	44.07
3827	44.06
3826	44.04
3825	44.02
3824	44.02
3823	44.04
3822	44.06
3821	44.04
3820	44
3819	43.99
3818	44.02
3817	44.04
3816	44.02
3815	43.97
3814	43.94
3813	43.95
3812	43.96
3811	43.96
3810	43.94

3809	43.95
3808	43.95
3807	43.93
3806	43.89
3805	43.87
3804	43.89
3803	43.93
3802	43.93
3801	43.89
3800	43.84
3799	43.84
3798	43.86
3797	43.87
3796	43.86
3795	43.84
3794	43.83
3793	43.83
3792	43.83
3791	43.81
3790	43.8
3789	43.79
3788	43.78
3787	43.77
3786	43.76
3785	43.75
3784	43.74
3783	43.73
3782	43.73

3781	43.73
3780	43.72
3779	43.71
3778	43.7
3777	43.69
3776	43.69
3775	43.68
3774	43.66
3773	43.64
3772	43.64
3771	43.64
3770	43.63
3769	43.61
3768	43.6
3767	43.6
3766	43.6
3765	43.59
3764	43.56
3763	43.55
3762	43.55
3761	43.54
3760	43.53
3759	43.51
3758	43.49
3757	43.48
3756	43.46
3755	43.45
3754	43.43

3753	43.42
3752	43.42
3751	43.41
3750	43.39
3749	43.36
3748	43.34
3747	43.35
3746	43.38
3745	43.38
3744	43.33
3743	43.27
3742	43.24
3741	43.25
3740	43.23
3739	43.18
3738	43.14
3737	43.13
3736	43.15
3735	43.19
3734	43.22
3733	43.24
3732	43.21
3731	43.15
3730	43.1
3729	43.08
3728	43.07
3727	43.05
3726	43.04

3725	43.04
3724	43.04
3723	43.04
3722	43.04
3721	43.02
3720	43.01
3719	43
3718	42.99
3717	42.98
3716	42.98
3715	42.96
3714	42.94
3713	42.92
3712	42.89
3711	42.86
3710	42.84
3709	42.83
3708	42.82
3707	42.81
3706	42.79
3705	42.77
3704	42.76
3703	42.75
3702	42.74
3701	42.71
3700	42.68
3699	42.65
3698	42.63

3697	42.61
3696	42.58
3695	42.55
3694	42.52
3693	42.5
3692	42.49
3691	42.48
3690	42.47
3689	42.45
3688	42.39
3687	42.34
3686	42.32
3685	42.31
3684	42.29
3683	42.26
3682	42.23
3681	42.2
3680	42.16
3679	42.12
3678	42.1
3677	42.1
3676	42.1
3675	42.06
3674	42.01
3673	41.99
3672	41.99
3671	41.98
3670	41.95

3669	41.89
3668	41.85
3667	41.84
3666	41.82
3665	41.79
3664	41.75
3663	41.71
3662	41.67
3661	41.62
3660	41.58
3659	41.54
3658	41.51
3657	41.48
3656	41.42
3655	41.36
3654	41.31
3653	41.27
3652	41.22
3651	41.17
3650	41.13
3649	41.08
3648	41.04
3647	41.01
3646	40.97
3645	40.95
3644	40.92
3643	40.89
3642	40.85

3641	40.8
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3639	40.71
3638	40.66
3637	40.61
3636	40.55
3635	40.5
3634	40.44
3633	40.38
3632	40.33
3631	40.29
3630	40.28
3629	40.26
3628	40.22
3627	40.19
3626	40.19
3625	40.19
3624	40.18
3623	40.16
3622	40.15
3621	40.15
3620	40.13
3619	40.08
3618	40.02
3617	39.97
3616	39.94
3615	39.92
3614	39.9

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3612	39.8
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3610	39.7
3609	39.65
3608	39.61
3607	39.57
3606	39.53
3605	39.49
3604	39.44
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3602	39.34
3601	39.29
3600	39.25
3599	39.2
3598	39.16
3597	39.11
3596	39.07
3595	39.03
3594	38.99
3593	38.94
3592	38.9
3591	38.85
3590	38.8
3589	38.77
3588	38.73
3587	38.7
3586	38.67

3585	38.63
3584	38.6
3583	38.57
3582	38.53
3581	38.49
3580	38.46
3579	38.42
3578	38.39
3577	38.35
3576	38.32
3575	38.28
3574	38.24
3573	38.2
3572	38.15
3571	38.1
3570	38.04
3569	38
3568	37.97
3567	37.95
3566	37.94
3565	37.92
3564	37.9
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3562	37.83
3561	37.8
3560	37.77
3559	37.74
3558	37.71

3557	37.68
3556	37.65
3555	37.61
3554	37.58
3553	37.55
3552	37.52
3551	37.48
3550	37.45
3549	37.41
3548	37.37
3547	37.34
3546	37.31
3545	37.29
3544	37.27
3543	37.25
3542	37.23
3541	37.2
3540	37.17
3539	37.14
3538	37.11
3537	37.08
3536	37.06
3535	37.03
3534	37.01
3533	36.98
3532	36.95
3531	36.92
3530	36.89

3529	36.86
3528	36.83
3527	36.8
3526	36.77
3525	36.74
3524	36.72
3523	36.7
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3520	36.63
3519	36.61
3518	36.58
3517	36.56
3516	36.53
3515	36.5
3514	36.47
3513	36.44
3512	36.41
3511	36.39
3510	36.37
3509	36.35
3508	36.32
3507	36.3
3506	36.27
3505	36.24
3504	36.21
3503	36.18
3502	36.16

3501	36.14
3500	36.12
3499	36.1
3498	36.08
3497	36.05
3496	36.03
3495	36.01
3494	35.99
3493	35.96
3492	35.94
3491	35.92
3490	35.89
3489	35.87
3488	35.84
3487	35.82
3486	35.8
3485	35.77
3484	35.75
3483	35.73
3482	35.7
3481	35.67
3480	35.65
3479	35.63
3478	35.6
3477	35.58
3476	35.56
3475	35.54
3474	35.51

3473	35.49
3472	35.46
3471	35.43
3470	35.39
3469	35.36
3468	35.33
3467	35.31
3466	35.29
3465	35.27
3464	35.25
3463	35.23
3462	35.2
3461	35.18
3460	35.15
3459	35.13
3458	35.11
3457	35.09
3456	35.06
3455	35.04
3454	35.02
3453	35
3452	34.98
3451	34.96
3450	34.94
3449	34.92
3448	34.91
3447	34.9
3446	34.89

3445	34.88
3444	34.88
3443	34.88
3442	34.87
3441	34.87
3440	34.87
3439	34.87
3438	34.87
3437	34.88
3436	34.88
3435	34.89
3434	34.9
3433	34.91
3432	34.93
3431	34.94
3430	34.95
3429	34.97
3428	34.98
3427	35
3426	35.01
3425	35.02
3424	35.03
3423	35.04
3422	35.05
3421	35.06
3420	35.07
3419	35.09
3418	35.1

3417	35.11
3416	35.11
3415	35.12
3414	35.12
3413	35.13
3412	35.14
3411	35.14
3410	35.15
3409	35.16
3408	35.16
3407	35.17
3406	35.17
3405	35.18
3404	35.17
3403	35.17
3402	35.17
3401	35.17
3400	35.17
3399	35.17
3398	35.18
3397	35.19
3396	35.19
3395	35.19
3394	35.19
3393	35.19
3392	35.19
3391	35.19
3390	35.2

3389	35.21
3388	35.22
3387	35.23
3386	35.24
3385	35.25
3384	35.26
3383	35.27
3382	35.28
3381	35.29
3380	35.3
3379	35.32
3378	35.34
3377	35.36
3376	35.38
3375	35.41
3374	35.44
3373	35.46
3372	35.49
3371	35.51
3370	35.54
3369	35.57
3368	35.6
3367	35.64
3366	35.69
3365	35.73
3364	35.77
3363	35.8
3362	35.84

3361	35.88
3360	35.91
3359	35.95
3358	35.98
3357	36.02
3356	36.05
3355	36.09
3354	36.12
3353	36.14
3352	36.17
3351	36.19
3350	36.22
3349	36.25
3348	36.28
3347	36.31
3346	36.34
3345	36.36
3344	36.38
3343	36.4
3342	36.43
3341	36.44
3340	36.46
3339	36.48
3338	36.5
3337	36.52
3336	36.55
3335	36.57
3334	36.59

3333	36.61
3332	36.62
3331	36.64
3330	36.66
3329	36.67
3328	36.68
3327	36.69
3326	36.71
3325	36.72
3324	36.74
3323	36.75
3322	36.77
3321	36.78
3320	36.8
3319	36.81
3318	36.82
3317	36.83
3316	36.84
3315	36.85
3314	36.86
3313	36.87
3312	36.88
3311	36.88
3310	36.89
3309	36.89
3308	36.9
3307	36.9
3306	36.91

3305	36.92
3304	36.94
3303	36.95
3302	36.96
3301	36.98
3300	36.99
3299	37
3298	37
3297	37.01
3296	37.02
3295	37.02
3294	37.03
3293	37.04
3292	37.05
3291	37.06
3290	37.08
3289	37.09
3288	37.11
3287	37.12
3286	37.13
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1223	42.9
1222	42.83
1221	42.76
1220	42.68
1219	42.6
1218	42.52
1217	42.44
1216	42.35
1215	42.27
1214	42.18
1213	42.1
1212	42.02
1211	41.96
1210	41.91
1209	41.87
1208	41.84
1207	41.83
1206	41.81

1205	41.78
1204	41.74
1203	41.68
1202	41.62
1201	41.54
1200	41.45
1199	41.37
1198	41.28
1197	41.17
1196	41.05
1195	40.93
1194	40.81
1193	40.68
1192	40.54
1191	40.41
1190	40.28
1189	40.14
1188	40
1187	39.86
1186	39.72
1185	39.58
1184	39.43
1183	39.29
1182	39.16
1181	39.02
1180	38.88
1179	38.74
1178	38.6

1177	38.47
1176	38.33
1175	38.21
1174	38.1
1173	37.98
1172	37.87
1171	37.74
1170	37.62
1169	37.48
1168	37.34
1167	37.22
1166	37.11
1165	37
1164	36.9
1163	36.8
1162	36.68
1161	36.56
1160	36.41
1159	36.28
1158	36.15
1157	36.02
1156	35.9
1155	35.79
1154	35.7
1153	35.61
1152	35.52
1151	35.44
1150	35.37

1149	35.3
1148	35.22
1147	35.13
1146	35.01
1145	34.84
1144	34.63
1143	34.4
1142	34.15
1141	33.89
1140	33.64
1139	33.43
1138	33.27
1137	33.14
1136	33.04
1135	32.95
1134	32.86
1133	32.74
1132	32.58
1131	32.42
1130	32.25
1129	32.07
1128	31.91
1127	31.78
1126	31.69
1125	31.62
1124	31.57
1123	31.56
1122	31.58

1121	31.62
1120	31.68
1119	31.78
1118	31.91
1117	32.06
1116	32.22
1115	32.41
1114	32.61
1113	32.82
1112	33.04
1111	33.27
1110	33.52
1109	33.77
1108	34
1107	34.21
1106	34.41
1105	34.57
1104	34.7
1103	34.82
1102	34.94
1101	35.04
1100	35.15
1099	35.25
1098	35.37
1097	35.47
1096	35.58
1095	35.71
1094	35.85

1093	36
1092	36.15
1091	36.32
1090	36.48
1089	36.64
1088	36.78
1087	36.93
1086	37.07
1085	37.2
1084	37.33
1083	37.48
1082	37.63
1081	37.77
1080	37.91
1079	38.07
1078	38.25
1077	38.43
1076	38.62
1075	38.82
1074	39.03
1073	39.24
1072	39.46
1071	39.7
1070	39.96
1069	40.22
1068	40.5
1067	40.79
1066	41.1

1065	41.4
1064	41.7
1063	41.99
1062	42.28
1061	42.56
1060	42.84
1059	43.13
1058	43.42
1057	43.7
1056	43.99
1055	44.3
1054	44.63
1053	44.99
1052	45.37
1051	45.79
1050	46.23
1049	46.67
1048	47.1
1047	47.53
1046	47.94
1045	48.35
1044	48.75
1043	49.13
1042	49.49
1041	49.81
1040	50.09
1039	50.31
1038	50.46

1037	50.52
1036	50.46
1035	50.26
1034	49.91
1033	49.41
1032	48.81
1031	48.23
1030	47.79
1029	47.62
1028	47.76
1027	48.11
1026	48.56
1025	49.02
1024	49.45
1023	49.87
1022	50.28
1021	50.68
1020	51.05
1019	51.36
1018	51.59
1017	51.75
1016	51.84
1015	51.87
1014	51.84
1013	51.76
1012	51.64
1011	51.48
1010	51.28

1009	51.04
1008	50.77
1007	50.47
1006	50.14
1005	49.8
1004	49.44
1003	49.07
1002	48.69
1001	48.32
1000	47.95
999	47.59
998	47.24
997	46.91
996	46.62
995	46.38
994	46.22
993	46.13
992	46.08
991	46.03
990	45.98
989	45.96
988	46.09
987	46.46
986	47.08
985	47.82
984	48.54
983	49.13
982	49.53

981	49.73
980	49.78
979	49.71
978	49.55
977	49.34
976	49.09
975	48.82
974	48.53
973	48.23
972	47.92
971	47.59
970	47.26
969	46.91
968	46.56
967	46.21
966	45.87
965	45.52
964	45.17
963	44.83
962	44.5
961	44.18
960	43.87
959	43.57
958	43.29
957	43.02
956	42.77
955	42.53
954	42.32

953	42.12
952	41.93
951	41.76
950	41.6
949	41.45
948	41.31
947	41.18
946	41.04
945	40.9
944	40.75
943	40.6
942	40.45
941	40.3
940	40.15
939	40
938	39.86
937	39.72
936	39.57
935	39.44
934	39.31
933	39.19
932	39.07
931	38.97
930	38.87
929	38.79
928	38.71
927	38.64
926	38.6

925	38.55
924	38.52
923	38.51
922	38.51
921	38.53
920	38.57
919	38.61
918	38.67
917	38.74
916	38.83
915	38.94
914	39.06
913	39.2
912	39.34
911	39.49
910	39.66
909	39.82
908	39.98
907	40.15
906	40.32
905	40.49
904	40.67
903	40.85
902	41.03
901	41.21
900	41.38
899	41.57
898	41.77

897	41.97
896	42.17
895	42.39
894	42.62
893	42.87
892	43.12
891	43.39
890	43.66
889	43.93
888	44.21
887	44.49
886	44.76
885	45.02
884	45.27
883	45.5
882	45.71
881	45.9
880	46.07
879	46.22
878	46.34
877	46.44
876	46.5
875	46.53
874	46.52
873	46.49
872	46.45
871	46.44
870	46.46

869	46.49
868	46.52
867	46.56
866	46.62
865	46.7
864	46.82
863	46.99
862	47.22
861	47.49
860	47.79
859	48.09
858	48.39
857	48.68
856	48.95
855	49.21
854	49.46
853	49.68
852	49.89
851	50.08
850	50.25
849	50.41
848	50.55
847	50.69
846	50.8
845	50.89
844	50.96
843	51.01
842	51.04

841	51.05
840	51.04
839	51.01
838	50.98
837	50.93
836	50.88
835	50.84
834	50.8
833	50.78
832	50.78
831	50.79
830	50.81
829	50.83
828	50.84
827	50.85
826	50.85
825	50.86
824	50.88
823	50.9
822	50.92
821	50.94
820	50.96
819	50.99
818	51.01
817	51.04
816	51.08
815	51.13
814	51.19

813	51.26
812	51.33
811	51.4
810	51.47
809	51.54
808	51.6
807	51.66
806	51.72
805	51.78
804	51.84
803	51.9
802	51.95
801	52
800	52.04
799	52.08
798	52.12
797	52.15
796	52.18
795	52.21
794	52.23
793	52.26
792	52.28
791	52.3
790	52.3
789	52.3
788	52.3
787	52.3
786	52.3

785	52.31
784	52.3
783	52.28
782	52.26
781	52.24
780	52.21
779	52.2
778	52.18
777	52.16
776	52.14
775	52.11
774	52.09
773	52.06
772	52.04
771	52.03
770	52.03
769	52.02
768	52.02
767	52.02
766	52.02
765	52.03
764	52.03
763	52.04
762	52.05
761	52.05
760	52.05
759	52.05
758	52.04

757	52.03
756	52
755	51.95
754	51.89
753	51.81
752	51.73
751	51.63
750	51.51
749	51.35
748	51.17
747	50.95
746	50.7
745	50.41
744	50.09
743	49.72
742	49.32
741	48.87
740	48.39
739	47.92
738	47.48
737	47.11
736	46.86
735	46.76
734	46.81
733	47
732	47.28
731	47.6
730	47.93

729	48.26
728	48.56
727	48.84
726	49.07
725	49.27
724	49.43
723	49.56
722	49.65
721	49.73
720	49.79
719	49.87
718	49.93
717	49.98
716	50
715	49.99
714	49.98
713	49.96
712	49.93
711	49.89
710	49.85
709	49.81
708	49.76
707	49.72
706	49.67
705	49.63
704	49.58
703	49.53
702	49.49

701	49.45
700	49.4
699	49.37
698	49.33
697	49.28
696	49.22
695	49.17
694	49.12
693	49.07
692	49.01
691	48.95
690	48.89
689	48.82
688	48.75
687	48.68
686	48.61
685	48.53
684	48.44
683	48.34
682	48.24
681	48.15
680	48.05
679	47.96
678	47.87
677	47.78
676	47.69
675	47.6
674	47.51

673	47.43
672	47.37
671	47.39
670	47.55
669	47.75
668	47.82
667	47.65
666	47.33
665	47.05
664	46.87
663	46.76
662	46.7
661	46.64
660	46.57
659	46.52
658	46.47
657	46.43
656	46.4
655	46.36
654	46.33
653	46.27
652	46.21
651	46.16
650	46.12
649	46.06
648	46.01
647	45.96
646	45.92

645	45.86
644	45.81
643	45.77
642	45.74
641	45.7
640	45.65
639	45.6
638	45.56
637	45.52
636	45.47
635	45.44
634	45.4
633	45.35
632	45.29
631	45.23
630	45.17
629	45.1
628	45.02
627	44.92
626	44.83
625	44.72
624	44.6
623	44.49
622	44.37
621	44.23
620	44.08
619	43.93
618	43.83

617	43.79
616	43.84
615	43.96
614	44.11
613	44.25
612	44.36
611	44.46
610	44.53
609	44.57
608	44.59
607	44.62
606	44.65
605	44.65
604	44.63
603	44.6
602	44.56
601	44.49
600	44.38
599	44.26
598	44.12
597	43.96
596	43.79
595	43.62
594	43.47
593	43.31
592	43.12
591	42.94
590	42.75

589	42.56
588	42.35
587	42.15
586	41.95
585	41.73
584	41.5
583	41.28
582	41.06
581	40.83
580	40.59
579	40.37
578	40.2
577	40.04
576	39.91
575	39.81
574	39.77
573	39.76
572	39.75
571	39.77
570	39.81
569	39.83
568	39.82
567	39.79
566	39.76
565	39.7
564	39.63
563	39.56
562	39.52

561	39.48
560	39.45
559	39.45
558	39.47
557	39.51
556	39.55
555	39.63
554	39.72
553	39.81
552	39.87
551	39.95
550	40.05
549	40.14
548	40.23
547	40.34
546	40.46
545	40.58
544	40.67
543	40.74
542	40.81
541	40.86
540	40.89
539	40.93
538	40.98
537	41.04
536	41.12
535	41.27
534	41.5

533	41.76
532	42.02
531	42.26
530	42.45
529	42.56
528	42.55
527	42.44
526	42.25
525	42.02
524	41.79
523	41.62
522	41.55
521	41.53
520	41.54
519	41.62
518	41.77
517	41.97
516	42.18
515	42.42
514	42.72
513	43.06
512	43.42
511	43.78
510	44.12
509	44.43
508	44.73
507	45.07
506	45.43

505	45.74
504	45.96
503	46.12
502	46.23
501	46.3
500	46.33
499	46.33
498	46.27
497	46.1
496	45.83
495	45.55
494	45.35
493	45.25
492	45.21
491	45.22
490	45.28
489	45.39
488	45.59
487	45.87
486	46.19
485	46.47
484	46.69
483	46.86
482	46.99
481	47.05
480	47.07
479	47.1
478	47.16

477	47.21
476	47.22
475	47.24
474	47.27
473	47.29
472	47.34
471	47.43
470	47.58
469	47.74
468	47.89
467	48.06
466	48.24
465	48.42
464	48.6
463	48.79
462	48.97
461	49.13
460	49.3
459	49.51
458	49.73
457	49.9
456	50.04
455	50.19
454	50.36
453	50.52
452	50.66
451	50.83
450	51

449	51.16
448	51.29
447	51.4
446	51.5
445	51.62
444	51.77
443	51.93
442	52.06
441	52.14
440	52.23
439	52.35
438	52.48
437	52.57
436	52.6
435	52.6
434	52.58
433	52.57
432	52.58
431	52.6
430	52.59
429	52.55
428	52.51
427	52.49
426	52.5
425	52.53
424	52.58
423	52.68
422	52.84

421	53.06
420	53.33
419	53.57
418	53.69
417	53.72
416	53.79
415	53.89
414	54.02
413	54.17
412	54.38
411	54.61
410	54.77
409	54.86
408	55
407	55.21
406	55.49
405	55.79
404	56.1
403	56.34
402	56.41
401	56.38
400	56.38

APPENDIX 10

TRANSMITTANCE FOR PRISTINE SF

cm^{-1}	%T
4000	99.56
3999	99.56
3998	99.55
3997	99.55
3996	99.55
3995	99.56
3994	99.56
3993	99.56
3992	99.56
3991	99.56
3990	99.56
3989	99.56
3988	99.56
3987	99.55
3986	99.55
3985	99.55
3984	99.55
3983	99.55
3982	99.56
3981	99.56
3980	99.55
3979	99.55
3978	99.55

3977	99.54
3976	99.54
3975	99.55
3974	99.55
3973	99.55
3972	99.55
3971	99.55
3970	99.55
3969	99.55
3968	99.55
3967	99.55
3966	99.55
3965	99.55
3964	99.55
3963	99.55
3962	99.55
3961	99.56
3960	99.56
3959	99.56
3958	99.56
3957	99.56
3956	99.56
3955	99.56
3954	99.55
3953	99.55
3952	99.55
3951	99.55
3950	99.55

3949	99.56
3948	99.56
3947	99.56
3946	99.56
3945	99.56
3944	99.56
3943	99.56
3942	99.57
3941	99.57
3940	99.57
3939	99.57
3938	99.56
3937	99.56
3936	99.55
3935	99.55
3934	99.54
3933	99.54
3932	99.55
3931	99.55
3930	99.55
3929	99.55
3928	99.55
3927	99.55
3926	99.55
3925	99.56
3924	99.56
3923	99.56
3922	99.56

3921	99.56
3920	99.56
3919	99.56
3918	99.56
3917	99.56
3916	99.56
3915	99.56
3914	99.56
3913	99.56
3912	99.56
3911	99.56
3910	99.56
3909	99.56
3908	99.55
3907	99.55
3906	99.55
3905	99.55
3904	99.55
3903	99.55
3902	99.56
3901	99.56
3900	99.56
3899	99.56
3898	99.56
3897	99.55
3896	99.55
3895	99.55
3894	99.55

3893	99.55
3892	99.56
3891	99.57
3890	99.57
3889	99.57
3888	99.56
3887	99.55
3886	99.54
3885	99.54
3884	99.54
3883	99.55
3882	99.55
3881	99.56
3880	99.57
3879	99.57
3878	99.57
3877	99.56
3876	99.55
3875	99.54
3874	99.54
3873	99.55
3872	99.55
3871	99.54
3870	99.54
3869	99.55
3868	99.55
3867	99.54
3866	99.54

3865	99.54
3864	99.54
3863	99.55
3862	99.55
3861	99.54
3860	99.54
3859	99.54
3858	99.55
3857	99.55
3856	99.56
3855	99.55
3854	99.55
3853	99.55
3852	99.56
3851	99.57
3850	99.56
3849	99.55
3848	99.55
3847	99.54
3846	99.53
3845	99.53
3844	99.53
3843	99.53
3842	99.54
3841	99.54
3840	99.54
3839	99.54
3838	99.55

3837	99.55
3836	99.55
3835	99.54
3834	99.54
3833	99.54
3832	99.54
3831	99.55
3830	99.55
3829	99.54
3828	99.54
3827	99.54
3826	99.54
3825	99.54
3824	99.54
3823	99.53
3822	99.53
3821	99.53
3820	99.54
3819	99.54
3818	99.54
3817	99.54
3816	99.54
3815	99.54
3814	99.55
3813	99.54
3812	99.54
3811	99.53
3810	99.53

3809	99.53
3808	99.52
3807	99.52
3806	99.52
3805	99.52
3804	99.52
3803	99.51
3802	99.51
3801	99.51
3800	99.51
3799	99.51
3798	99.51
3797	99.51
3796	99.52
3795	99.53
3794	99.53
3793	99.53
3792	99.52
3791	99.52
3790	99.51
3789	99.52
3788	99.52
3787	99.53
3786	99.53
3785	99.53
3784	99.53
3783	99.53
3782	99.53

3781	99.53
3780	99.52
3779	99.52
3778	99.52
3777	99.51
3776	99.51
3775	99.51
3774	99.51
3773	99.51
3772	99.51
3771	99.51
3770	99.51
3769	99.51
3768	99.52
3767	99.52
3766	99.53
3765	99.53
3764	99.52
3763	99.52
3762	99.51
3761	99.51
3760	99.51
3759	99.51
3758	99.51
3757	99.5
3756	99.5
3755	99.49
3754	99.48

3753	99.48
3752	99.47
3751	99.48
3750	99.48
3749	99.48
3748	99.49
3747	99.51
3746	99.52
3745	99.52
3744	99.51
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624	84.95
623	84.89
622	84.83
621	84.79
620	84.77
619	84.74
618	84.69

617	84.6
616	84.48
615	84.37
614	84.29
613	84.24
612	84.2
611	84.18
610	84.16
609	84.13
608	84.11
607	84.11
606	84.12
605	84.13
604	84.12
603	84.11
602	84.11
601	84.08
600	84.04
599	83.99
598	83.95
597	83.91
596	83.87
595	83.83
594	83.81
593	83.79
592	83.77
591	83.75
590	83.71

589	83.65
588	83.57
587	83.49
586	83.41
585	83.35
584	83.3
583	83.26
582	83.23
581	83.16
580	83.04
579	82.89
578	82.77
577	82.65
576	82.54
575	82.43
574	82.34
573	82.25
572	82.17
571	82.09
570	82.02
569	81.9
568	81.75
567	81.58
566	81.39
565	81.19
564	80.97
563	80.76
562	80.57

561	80.37
560	80.12
559	79.85
558	79.57
557	79.3
556	79.06
555	78.85
554	78.65
553	78.4
552	78.1
551	77.82
550	77.61
549	77.42
548	77.22
547	77.02
546	76.86
545	76.73
544	76.57
543	76.38
542	76.17
541	76
540	75.88
539	75.82
538	75.79
537	75.73
536	75.62
535	75.54
534	75.51

533	75.48
532	75.42
531	75.37
530	75.35
529	75.34
528	75.32
527	75.28
526	75.22
525	75.13
524	75.03
523	74.97
522	74.95
521	74.94
520	74.89
519	74.82
518	74.74
517	74.65
516	74.55
515	74.48
514	74.42
513	74.36
512	74.29
511	74.28
510	74.34
509	74.39
508	74.37
507	74.28
506	74.18

505	74.09
504	74.02
503	74
502	74.02
501	74.05
500	74.06
499	74.09
498	74.13
497	74.15
496	74.12
495	74.09
494	74.08
493	74.03
492	73.95
491	73.9
490	73.89
489	73.91
488	73.94
487	74
486	74.11
485	74.2
484	74.22
483	74.22
482	74.26
481	74.32
480	74.41
479	74.54
478	74.7

477	74.83
476	74.9
475	74.96
474	75.04
473	75.09
472	75.09
471	75.08
470	75.13
469	75.18
468	75.23
467	75.28
466	75.36
465	75.41
464	75.42
463	75.46
462	75.58
461	75.72
460	75.84
459	75.89
458	75.96
457	75.98
456	75.92
455	75.85
454	75.82
453	75.81
452	75.88
451	76.06
450	76.31

APPENDIX 11

CURRICULUM VITAE

PERSONAL INFORMATION

Surname, Name : Galam Nanyak
Nationality : Nigerian
Date and Place of Birth : 13 June 1980, Plateau
Marital Status : Married

EDUCATION

Degree	Institution	Year of Graduation
M.Sc.	Human Physiology, University of Jos	2014
MBBS	Medicine and Surgery	2006

WORK EXPERIENCE

Year	Place	Responsibilities
2019-present	Dept of Physiology, University of Jos	Nursing std Coordinator
2016-present	Faculty of Basic Medical Sc. Board Member	University of Jos
2011- 2013	Dept of Physiology PG committee	Asst. Coordinator
2010 - 2014	Faculty Alumni Committee Member	University of Jos
2010- 2014	Faculty of Medical Sciences Staff adviser	University of Jos
2008 - 2016	Faculty of Medical Sciences, Board Member	University of Jos

LANGUAGES SPOKEN

English, Hausa, Tarok

PUBLICATIONS IN PEER REVIEW JOURNALS IN COVERAGE OF SCI, SCIE, AND SCOPUS

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