T.C.
REPUBLIC OF TURKEY
HACETTEPE UNIVERSITY
GRADUATE SCHOOL OF HEALTH SCIENCES

Development of Anticancer Drug Loaded Nanoparticle and In-Vitro Evaluation

Pharm. Keishid ABTAHI

Program of Pharmaceutical Technology

MASTER'S THESIS

ANKARA

2023

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Assoc. Prof. Dr. Kıvılcım ÖZTÜRK

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2023

ACCEPTANCE PAGE

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Drug Loaded Nanoparticle and In-Vitro Evaluation

Pharm. Keishid ABTAHI

Supervisor: Assoc. Prof. Dr. Kıvılcım ÖZTÜRK

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ETHICAL DECLARATION

In this thesis study, I declare that all the information and documents have been obtained in the base of the academic rules and all audio-visual and written information and results have been presented according to the rules of scientific ethics. I did not do any distortion in data set. In case of using other works, related studies have been fully cited in accordance with the scientific standards. I also declare that my thesis study is original except cited references. It was produced by me in consultation with supervisor (Assoc. Prof. Dr. Kıvılcım ÖZTÜRK) and written according to the rules of thesis writing of Hacettepe University Institute of Health Sciences.

Pharm. Keishid ABTAHI

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Lastly I want to dedicate this thesis to **Spec. Dr. Mehran NAMI** in loving memory of the life he lived and the impact he made on so many. His passing during the COVID-19 pandemic was a devastating loss, and his loved ones still feel the emptiness his absence has left in their hearts. Though Mehran is no longer with us, his legacy lives on through the memories shared and the lessons taught. He will always be remembered.

Sincerely,

ABSTRACT

ABTAHI K. Development of Anticancer Drug Loaded Nanoparticle and In-Vitro Evaluation, Hacettepe University Graduate School of Health Sciences Pharmaceutical Technology Master of Science Thesis, Ankara 2023. Colorectal cancer is the third most commonly occurring cancer which remains asymptomatic in its early stages, leading to 1.8 million diagnoses per year. If detected at an early stage, it can be treated through chemotherapy, with 5-Fluorouracil being a promising treatment option. However, this medication is accompanied by a significant number of side effects, making the treatment process unpleasant for patients. In this study, 5-FU was loaded into chitosan nanoparticles using an ionic gelation method, with the aim of reducing the drug's side effects and evaluating the cytotoxicity of the prepared nanoparticles. The prepared nanoparticles were characterized in terms of particle size, particle size distribution, zeta potential, morphology, encapsulation efficacy, *in-vitro* release, and *in-vitro* cytotoxicity. The nanoparticles were prepared in a pH of 4.5 using a 5:1 chitosan to tripolyphosphate ratio, resulting in a particle size of 217.03 ± 10.41, a PdI of 0.37 ± 0.11, and a zeta potential of 0.02 ± 0.02. The encapsulation efficacy was calculated as 15.69%, with a sustained release profile of approximately 40% over 24 hours. The cytotoxicity analysis showed that 5-FU-loaded chitosan nanoparticles effectively stopped the proliferation of CT-26 colorectal cancer cells with a lower concentration of 5-FU compared to free 5-FU. In conclusion, the prepared nanoparticles exhibit stable, uniform distribution, appropriate particle size, and efficient encapsulation capability. Furthermore, they demonstrate an initial burst release followed by sustained release after 24 hours, making them a promising candidate for the treatment of colorectal cancer with further in-vivo investigations and clinical trials.

Keywords: Colorectal Cancer, Nanotechnology, 5-Fluorouracil, Chitosan Nanoparticles.

ÖZET

ABTAHI K. Antikanser Etkin Madde Yüklü Nanopartikül Geliştirilmesi ve Formülasyonunun İn-vitro karakterizasyonu, Hacettepe Üniversitesi Sağlık Bilimleri Enstitüsü Farmasötik Teknoloji Programı Yüksek Lisans Tezi, Ankara, 2023. Kolon kanseri, ilk aşamalarında belirtisiz olan ve her yıl 1.8 milyon vakaya yol açan üçüncü en yaygın kanserdir. Erken aşamalarda tespit edilirse, kemoterapi ile tedavi edilebilir ve 5-Fluorourasil bu kanser için umut verici bir tedavi seçeneğidir. Ne yazık ki, bu ilaç çok sayıda yan etkiye neden olur ve hastaların rahatsız edici bir tedavi dönemi yaşamasına neden olur. Bu projede, 5-FU ionik jelasyon yöntemi kullanılarak kitosan nanopartiküllere yüklendi ve hazırlanan nanopartiküllerin sitotoksisitesi değerlendirildi. Hazırlanan nanopartiküller, boyut, boyut dağılımı, zeta potansiyeli, morfolojisi, kapsülasyon etkililiği, in vitro salımı ve in vitro sitotoksisite açısından karakterize edildi. Nanopartiküller pH=4.5'de 5:1 kitosan-tripolifosfat oranı kullanılarak hazırlandı ve nanopartikül boyutu, PdI ve zeta potansiyeli sırasıyla 217.03 ± 10.41, 0.37 ± 0.11 ve 0.02 ± 0.02 olarak hesaplandı. %EE 15.69% olarak hesaplandı ve 24 saatte yaklaşık %40 in vitro sürdürülen salım profili vardı. Sitotoksisite analizine göre, 5-FU yüklü kitosan nanopartiküller, serbest 5-FU ile karşılaştırıldığında daha düşük bir 5-FU konsantrasyonu kullanarak CT-26 kolon kanser hücrelerinin çoğalmasını durdurdu. Sonuç olarak, hazırlanan nanopartiküller istikrarlı ve düzenli dağılıma, uygun boyuta ve etkililikli kapsülasyon kabiliyetine sahiptir. Ayrıca, 24 saat sonra düzenli salımı takiben başlangıçta patlama salımı gösterir, bu da kolon kanserinin *in-vivo* araştırmalar ve klinik denemelerle tedavisi için umut verici bir tedavi olarak görülmesine olanak tanır.

Anahtar kelimeler: Kolon Kanseri, Nanoteknoloji, 5-Fluorouracil, Kitosan Nanopartikül

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SYMBOLS AND ABBREVIATIONS

ºC	Degree Centigrade
5-FU	5-Fluorouracil
ACN	Acetonitrile
CRC	Colorectal Cancer
CSNPs	Chitosan Nanoparticles
CV	Cardiovascular
DAD	Diode Array
DDS	Drug Delivery System
DLS	Dynamic Light Scattering
DMF	Dimetilfurmamide
DMSO	Dimethylsulfoxide
DSC	Differential Scanning Calorimetry
EPR	Endothelial Permeability and Retention
FAP	Familial Adenomatous Polyposis
FAR	Folate Receptor
FDA	Food and Drug Administration
FdUMP	Fluorodeoxyuridine Monophosphate
FdUTP	Fluorodeoxyuridine Triophosphate
HNPCC	Non-Polyposis Hereditary Colorectal Cancer
HPLC	High Performance Liquid Chromatography
HPMA	N-(2-hydroxypropyl)-methacrylamide Copolymer
ICH	International Conference on Harmonization
IV	Intravenous
LbL	Layer by Layer
LOD	Level of Detection
LOQ	Level of Quantification
MMP	Matrix Metalloproteins
MNPs	Magnetic Nanoparticles
MRI	Magnetic Resonance Imaging
MSNs	Mesopores Silica Nanoparticles
NCI	National Cancer Institute
NP	Nanoparticle
nm	Nanometer
PBS	Phosphate Buffer Saline
PCL	Polycaprolactone
PDI	Polydispersity Index
PEG	Polyglutamic Acid
PGA	Polyethylene Glycol
RES	Reticuloendothelial System
SD	Standard Deviation
SEM	Scanning Electron Microscopy
TME	Tumor Microenvironment
TPP	Tripolyphosphate
UV	Ultra Violet
µg	Microgram
µL	Microliter
µM	Micromolar
µm	Micrometer

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1. INTRODUCTION

1.1 Cancer

In recent years, cancer has remained a significant health concern and has caused a lot of deaths in the world in countries all over the world (4). Cancer defined as group of diseases which spread within the body to different organs because of their excessive multiplication. A malignant tumor is an atypical condition in which a group of cells disregards the normal functional constraints regulating cell distribution and grows uncontrollably. Due to a levels of self-sufficiency, malignant cells do not respond to the signals that activate the normal cell cycle, which results in the uncontrolled proliferation and generation of abnormal cells (5). The World Health Organization forecasts that one out of each three people will be diagnosed with cancer in their lifetime (6). Worldwide, an estimated 19.3 million new cancer cases and almost 10.0 million cancer deaths occurred in 2020, according to the Cancer Journal for Clinicians. Female breast cancer has surpassed lung cancer as the most commonly diagnosed cancer, with an estimated 2.3 million new cases (11.7%), followed by lung (11.4%), colorectal (10.0 %), prostate (7.3%), and stomach (5.6%) cancers. Lung cancer remained the leading cause of cancer death, with an estimated 1.8 million deaths (18%), followed by colorectal (9.4%), liver (8.3%), stomach (7.7%), and female breast (6.9%) cancers (7). Early detection and therapy are essential to improving cancer patients' chances of survival. Screening procedures, such as mammograms and colonoscopies, can help detect cancer in its early, most treatable stages. Over the past few decades, cancer mortality has decreased due to better research of cancer pathogenesis and better treatments (4).

1.1.1 Colorectal Cancer

Colorectal cancer, often referred to as colon cancer or rectal cancer, is a malignancy of the colon and or rectum. A staggering 1.8 million new cases are diagnosed annually, making it the third most frequent cancer worldwide. In its early stages, colorectal cancer is frequently asymptomatic; therefore, it is crucial that individuals undergo routine screenings to discover the disease early. Early detection can considerably enhance the efficacy of treatment and raise the likelihood of survival (8).

Adenocarcinomas constitute around 95% of colorectal cancers. Mucinous carcinomas and adenosquamous carcinomas are two examples of the many types of cancer. Carcinogen agents can have direct contact with cells lining the colon and rectum. Five to ten percent of colorectal tumors are linked to recognized genetic disorders. Familial adenomatous polyposis (FAP) and non-polyposis hereditary colorectal cancer (HNPCC) are the two most prevalent forms. An additional 20% of cases occur in individuals with a family history of colorectal cancer. A mutation in tumor suppressor gene APC which is prevalent in people with FAP can cause problems in regulation of cell proliferation and cause number of adenomas at a young age which if left untreated will most probably change into cancer by the age of 40. The usual age of onset for HNPCC is mid-40s and having this type of the cancer raises the risk of a number of other gastrointestinal cancers. HNPCC is characterized by DNA repair gene mutations. These two diseases can cause sporadic colorectal cancer (8).

Several lifestyle variables have been identified as risk factors for colorectal cancer. These include:

1. Consumption of red meat
2. Consumption of processed meat
3. Consumption of two or more alcoholic beverages per day
4. Obesity
5. Low consumption of fruits
6. Consumption of foods containing haem iron

There are several other lifestyle changes which could decrease the risk of colorectal cancer in individuals:

1. Physical activity
2. Consumption of wholegrains
3. Consumption of fiber rich vegetables and fruits
4. Consumption of dairy products
5. Using calcium supplements

Various cancer therapies are available, including surgery, radiation therapy, chemotherapy, immunotherapy, hormone therapy, and targeted therapy. Among the different methods used to treat cancer, surgery is generally the first choice of treatment (6). In practice, chemotherapy, radiation, and their combinations are additional

therapeutic options. These therapeutic methods are strategized according to the type and stage of cancer based on their respective advantages and drawbacks. Chemotherapeutic agents may also be used as first-line treatments to prevent further proliferation of cancer cells and inhibit tumor growth; however, because of their systemic toxicity and unwanted side effects, they have limitations that prevent their safe and effective use. To overcome these problems, nanocarrier-based drug delivery systems (DDS) have been developed to enable fewer side effects and more efficient treatments (4).

1.2 Nanomedicine

In year 2000, the National Institutes of Health (NIH) established a government initiative to finance, coordinate, and advance nanoscale research and development programs. This program was inspired by the significant advances made in nanoscale material science over the last thirty years of the 20th century. The impact of this new program, on research and development in different scientific fields pertaining to human health became immediately apparent. Extensive government funding prompted the initiation of new multidisciplinary studies and the expansion of those already in existence. The rapid convergence of nanoscience and medicine led to the conception of nanomedicine as a new scientific subdiscipline. Subsequently, as described in this part, nanoscience terminology was rapidly and generally enthusiastically adopted by pharmaceutical scientists, leading to the emergence of so-called nano-pharmaceuticals. In general, "nano" became associated with "advanced" quite rapidly, and the pharmaceutical scientific community accepted this new word pretty quickly and started to use it in their scientific publications (9).

Nanomedicine is a branch of research that studies the physical and chemical characteristics of nanoparticles to achieve adjustable physicochemical features of drug carriers and shows promise in improving the biocompatibility, pharmacokinetics, and biodistribution of encapsulated pharmaceuticals, the solubility and permeability of intravenous (IV) drugs, the therapeutic dose, and the toxicities of the encapsulated therapeutic agent (10). If the administered pharmacologically active molecule cannot penetrate the biological barriers between the site of administration and the site of action, any active ingredient cannot be therapeutic. Treating human diseases, especially infectious, cancerous, and inherited ones, presents a formidable task due to

the difficulty of delivering therapeutic molecules to a specific organ, tissue, or kind of cells. The majority of active pharmaceutical ingredients frequently exhibit inadequate bioavailability, poor water solubility, biological degradation, and unintended intrinsic adverse effects. To overcome such drawbacks, the development of novel drug carrier systems is required due to their efficient applicability across various delivery routes, including oral, parenteral, topical, and pulmonary. The introduction of nanotechnology into the medical profession in the form of 'nanomedicines' has made it possible to introduce the notion of 'vectorization,' also known as targeted therapy (11).

1.3 Nanocarriers

According to the International Union of Pure and Applied Chemistry, "nanoparticles" (NPs) typically are particles with any kind of shape with dimensions between 1 and 100 nm. In actuality, this measurement is extended to several hundred nanometers (10, 12).

Nanoscale drug delivery vehicles can contain a wide variety of therapeutic agents, including low molecular weight compounds (hydrophilic and/or hydrophobic), peptides, protein-based compounds, and nucleic acids. By encapsulating these compounds within a nanocarrier, the solubility and stability of the medications can be enhanced, allowing for the reevaluation of potential drugs that were previously overlooked due to poor pharmacokinetics. To keep the medication concentration within the therapeutic window, nanocarriers can release the encapsulated molecules gradually or in response to a localized trigger. Attachment of targeting ligands to the nanocarrier's surface can result in increased uptake by target tissues. The nanocarrier's surface can be modified to extend the blood circulation half-life and change biodistribution. Nanocarriers can cross biological barriers and enter cells due to their small size. The total effect of these qualities is to reduce the systemic toxicity of the therapeutic agent while increasing the agent's concentration in the target location, resulting in a greater therapeutic index for the therapeutic agent (13). The ability of nanocarriers to shield encapsulated pharmaceuticals from instant release, degradation, and undesirable interaction with the biological environment made these dosage forms popular to use them in anticancer drug delivery treatments (12).

Nanocarriers mainly consist of three different types which are organic nanocarriers, inorganic nanocarriers and hybrid nanocarriers.

1.3.1 Organic Nanocarriers

As drug delivery systems, many nanoparticles, including liposomes, micelles, dendrimers, nanotubes, and polymer conjugates, have been created in recent years. As shown below, their composition and structure vary. The selection of system is determined by how they are combined with the drug and the type of drug used for therapy (14).

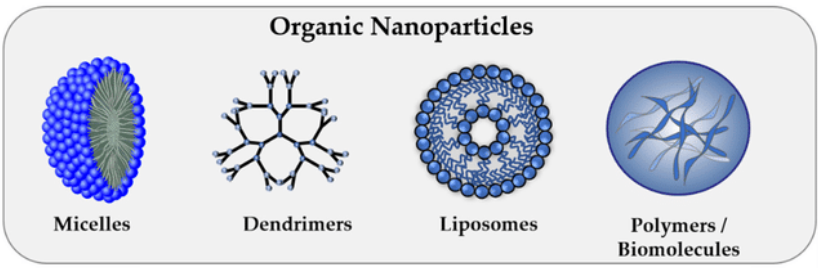

Figure 1. Organic nanoparticles (1)

1.3.1.1 Solid Nanoparticles

The lipophilic drug delivery system using solid lipid nanocarriers is utilized. To make solid lipid nanocarriers, melted solid lipids are dispersed in water and stabilized with emulsifiers, either by micro-emulsification or high-pressure homogenization. Depending on the production conditions and composition, the drug molecules may be integrated into the solid lipid's matrix, shell, or core. Due to its versatility, this solid lipid nanocarrier can overcome the disadvantages of conventional chemotherapy. Conventional solid lipid nanocarriers are rapidly removed by the Reticuloendothelial System (RES) and pose a challenge for extended drug release. Certain next generation nanocarriers, such as nano-structured lipid carrier (a mixture of liquid and solid lipid) and lipid drug conjugates (a water-insoluble carrier molecule), have been discovered to address the disadvantages of conventional solid lipid nanocarriers (15)

Liposomes

To create yet another nano drug delivery method, cholesterol and natural, non-toxic phospholipids can be used to create liposomes, which are essentially tiny phospholipid bubbles with a phospholipid bilayers membrane structure. In spite of drawbacks such as limited entrapment effectiveness, fast drug leakage, high cost, and short half-life, liposomes have significant clinical potential (16). They have a wide

range of applications because they can be administered intravenously, orally, or topically. In addition, the lipid nanoparticles protect pharmaceuticals from degradation caused by water and can be employed to provide a sustained release of active ingredients that are marginally soluble in water (14).

Micelles

In 1913, McBain used the term 'micelle' to refer to the colloidal aggregates generated by combining detergent with water (liquid colloid). They are amphiphilic molecules with a hydrophobic (nonpolar) tail facing the center and a hydrophilic (polar) head in contact with the external solvent (17). Micelles consist of lipids or other amphiphilic compounds, including polymers or polyamino acids, which self-assemble into small nanoparticles with a hydrophobic core (15).

Dendrimers

Dendrimers are spherical macromolecules with well-defined branching structures and surface functional groups that could be modified furthermore. These interactions may involve physical trapping of the drug within the interior of the dendrimer or non-bonding interactions with moieties within the dendrimer cavity. Surface contacts between the drug and dendrimer, which occur through electrostatic interactions or covalent attachment, constitute a second mode of drug-dendrimer interaction. According to the intended use of the drug delivery system, one of these ways is chosen (16). Dendrimers can be utilized in Magnetic Resonance Imaging (MRI), gene delivery, drug delivery, antiviral and vaccine delivery. Due to the toxicity of dendrimers, even though there are several studies on dendrimer-based drug targeting, they cannot be translated from the laboratory to the marketplace. It is anticipated that, in the near future, customization of dendrimers or other technologies will bring solutions to toxicity issues (15).

1.3.2 Polymeric Nanoparticles

Monomers or preformed polymers, as well as natural or synthetic materials, can all be used to produce polymeric NPs, enabling a wide range of potential shapes and properties. Because they are biocompatible and have straightforward formulation parameters, they may be created to provide fine control of a variety of NP characteristics and are typically effective delivery systems (18). Because polymers can

be biodegradable or non-biodegradable and can be synthesized or acquired from natural sources, polymeric nanoparticles offer substantial design flexibility (13). Polymers used for production of nanoparticles are either biopolymers such as albumin, chitosan, and heparin or they are synthetic polymers such as N-(2-hydroxypropyl)-methacrylamide copolymer (HPMA), polystyrenemaleic anhydride copolymer, polyethylene glycol (PEG), and poly L-glutamic acid (PGA) (14).

1.3.2.1 Inorganic Nanocarriers

Inorganic nanocarriers include gold, magnetic nanocarriers, quantum dots, and mesoporous silica etc. The inorganic nanocarriers use their tractable features to their advantage. These inorganic NPs are accurately synthesized and can be designed to have an extensive range of sizes, topologies, and geometries. Biosensing, cell labeling, targeting, imaging, as well as diagnostics, are all viable applications for inorganic nanocarriers. Additionally, inorganic NPs have distinct physical, electrical, magnetic, and optical capabilities, depending to the characteristics of the base material itself (15, 18).

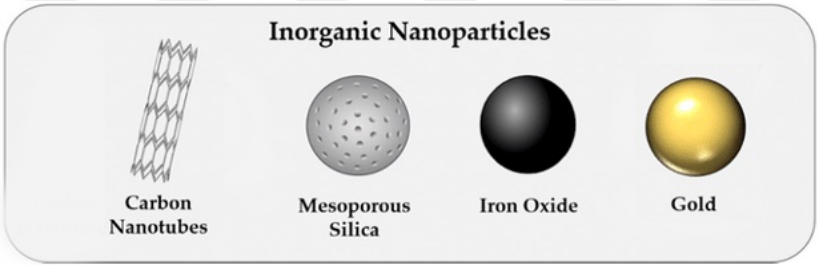

Figure 2. Inorganic nanocarriers (1)

Carbon Nanotubes

Carbon nanotubes are an ideal and prospective source for medication delivery due to their unique biological and physicochemical characteristics. These are hollow structures resembling tubes that include sheets of graphene rolled together at precise angles. Depending on the amount of graphene sheets that are rolled together, the carbon nanotubes may be single-walled or multi-walled. The diameter of the cross section of these tubes can range from 0.4 to 100 nm, while the length is a thousand times the diameter. This carbon nanotubes has numerous uses in the drug administration process due to its unique properties, such as its high aspect ratio,

ultralight weight with large surface area, nanosized needle structure, and distinctive chemical, thermal, mechanical, and electrical capabilities (15).

Gold Nanocarriers

Gold nanoparticles (AuNPs), which have been the focus of the most studies, are utilized in a variety of shapes, including nanospheres, nanorods, nanostars, nanoshells, and nanocages. AuNPs have surface electrons that cycle continuously at a frequency based on their size and shape, causing different photothermal characteristics. AuNPs are also easily functionalized, providing them additional properties and delivery abilities. Gold nanocarriers' optical characteristics are the main reason they're used in biomedicine. It enables many biomolecules, including enzymes, carbohydrates, fluorophores, peptides, proteins, and genes, to bind to the gold nanoparticles. This promotes efficient transport of molecules within the cell by overcoming related obstacles. The primary application of gold nanocarriers facilitates efficient imaging of tumor tissue (15).

Mesoporous Silica

Xerogels and mesoporous silica nanoparticles (MSNs) are the two types of silica utilized in controlled drug delivery systems as carrier systems, they demonstrate various advantages, including biocompatibility, a highly porous structure, and an ease of functionalization. Mesoporous silica has a honeycomb-like structure that allows for the incorporation of additional drug molecules. Silica materials are the nanoparticle carriers most frequently selected for biological applications. Silica xerogels have an amorphous structure with a large porosity and surface area. The porous structure (shape and pore size) is determined by the specifications in production. Drug-loaded silica xerogels are typically created using the sol-gel process. Changes to the synthesis circumstances, such as the ratio of reagents, temperature, concentration of the catalyst, and drying pressure, enables the adjustment of the controlled drug release xerogels' properties. The mechanism by which drugs are loaded into mesoporous silica is chemical or physical adsorption. Diverse types of pharmaceuticals, including antitumor agents, antibiotics, and heart disease drugs, have been incorporated into MNSs through these methods. Typically, drug release is controlled by diffusion. The

possible application of silicalites and mesoporous silica nanoparticles in photodynamic treatment has also been investigated (15, 19).

Iron Oxide

Iron is an essential element in the human body, and iron ions released by iron oxide nanostructures have been demonstrated to be reincorporated into the natural cell metabolism, granting iron oxide NPs a special status among inorganic carriers with respect to toxicity issues. The superparamagnetic property and catalytic activity of iron oxide nanoparticles have made them a highly attractive material class for a wide range of biomedical applications, including so-called 'nanozymes', which are enzyme mimics (1). Due to their advantageous characteristics, iron oxide nanoparticles are the only magnetic nanoparticles (MNPs) approved for clinical use by The United States Food and Drug Administration (FDA). Iron oxide cores can be coated with several shells, including golden, polymeric, silane, or dendrimeric ones, allowing for chemical modification and a straightforward one-step production by alkaline co-precipitation of Fe^{2+} and Fe^{3+}. Furthermore, magnetite and maghemite are present in the human heart, spleen, and liver, demonstrating their biocompatibility and non-toxicity at typical amounts. Predicting an acceptable maximum concentration of MNPs is essential for therapeutic uses. (19).

There are different types of nanomedicines which are approved for clinical use by FDA which are listed below in .

Table 1. List of FDA approved nanomedicines

Drug	Company	Application	Date of first approval
Lipid base nanomedicines			
Doxil®	Janssen	Kaposi's sarcoma, ovarian cancer, multiple myeloma	1995
DaunoXome®	Galen	Kaposi's sarcoma	1996
Am Bisome®	Gilead Sciences	Fungal/protozoal infections	1997
Visudyne®	Bausch and Lomb	Wet age-related macular degeneration, myopia, ocular histoplasmosis	2000
Marqibo®	Acrotech Biopharma	Acute lymphoblastic leukemia	2012
Onivyde®	Ipsen	Metastatic pancreatic cancer	2015
Polymer based nanomedicines			
Oncaspar®	Servier Pharmaceuticals	Acute lymphoblastic leukemia	1994
Copaxone®	Teva	Multiple sclerosis	1996
Peglntron®	Merck	Hepatitis C infection	2001
Eligard®	Tolmar	Prostate cancer	2002
Neulasta®	Amgen	Neutropenia, chemotherapy induced	2002
Abraxane®	Celgene	Lung cancer, metastatic breast cancer, metastatic pancreatic cancer	2005
Cimiza®	UCB	Crohn's disease, rheumatoid arthritis, psoriatic arthritis, ankylosing spondylitis	2008
Plegridy®	Biogen	Multiple sclerosis	2014
Adynovate®	Takeda	Hemophilia	2015
Inorganic nanomedicines			
INFeD®	Allergan	Iron-deficient anemia	1992
DexFerrum®	American Regent	Iron-deficient anemia	1996
Ferrlecit®	Sanofi	Iron deficiency in chronic kidney disease	1999
Venofer®	American Regent	Iron deficiency in chronic kidney disease	2000
Feraheme®	AMAG	Iron deficiency in chronic kidney disease	2009
Injectafer®	American Regent	Iron-deficient anemia	2013

1.4 PEGylation In Nanocarriers

PEGylation, in which polyethylene glycol (PEG) is covalently attached to a molecule, is a well-established approach in the field of targeted drug delivery systems. There are two possible methods of PEGylation: Well-defined conjugated products may be obtained using either 1) random PEGylation in the first generation or 2) site-specific PEGylation in the second generation. PEGylation's purpose is to increase hydrophilicity and decrease the glomerular filtration rate without affecting activity, hence extending the half-life in circulation. The surface modification of nanoparticles with PEGs of various chain length, density, and molecular weight in technologically advanced systems, as well as the incorporation of various targeting moieties (ligands, antibodies, etc.), is one such tactic (16).

In colloidal drug delivery systems, which are intended for human use, biodegradability is an essential criterion. Therefore, PEG-modified biodegradable drug delivery systems having PEG chains covalently attached to the drug delivery system's surface have been produced. The efficiency of the PEG "brush" can influence the biodegradation of biodegradable nanoparticles consisting of poly(lactic-co-

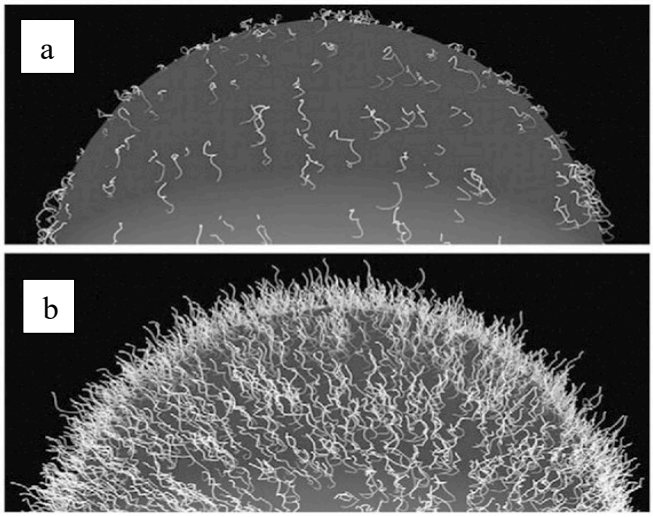

Figure 3. a) The majority of PEG chains are positioned near to the particle's surface. A small amount of PEG chains generated a "mushroom" structure on the surface.

b) The mobility of PEG chains is restrained, and a "brush" configuration with a high concentration of PEG chains on the surface is created. The chains are localized at a distance from the nanoparticle's surface*(3)*.

glycolic acid) (PLGA) or poly(lactic acid) (PLA) in the core as its shown in **Figure 3**(3).

1.5 Targeting In Nanocarrier Systems

The development of effective drug delivery systems based on nanotechnology presents opportunities for a wide range of therapeutic classes, but there is a particular need, for both financial and therapeutic reasons, to develop effective treatments for respiratory, central nervous, and cardiovascular diseases. Due to the restricted capability of nanoparticles to reach target tissues, numerous therapeutic medicines have failed. In addition, it is anticipated that drug delivery systems would provide rapid development prospects for anti-cancer medications, hormones, and vaccines due to their capacity to provide safer and more effective results than conventional approaches. In cancer chemotherapy, for instance, cytostatic medicines affect both malignant and normal cells, hence it is crucial to have a drug delivery mechanism that targets only the malignant tumor. Due to its instability in the biological environment, the drug is also rapidly removed from the body and relatively quickly metabolized, which presents issues. In a similar fashion, certain medications, such as protease inhibitors, bind to proteins in large quantities, preventing their diffusion into the brain and other organs. In drug delivery applications, nanotechnology may not be appropriate for all pharmaceuticals. Specifically, low-efficacy, high-dose active ingredients increase the size of the drug delivery system, making application more challenging (20).

Targeted drug delivery systems are a promising cancer treatment strategy that aims to selectively deliver large dosages of chemotherapeutic medications to tumor cells while reducing their exposure to normal tissues. This can enhance the therapeutic efficacy of the medications and decrease their toxicity. Several strategies exist for targeting drugs to tumor cells, including the use of ligands that bind to specific receptors on the surface of cancer cells, pH-sensitive or temperature-sensitive nanocarriers that are triggered to release their payload in the unique microenvironment of tumors, and nanomedicine formulations that target cancer cell surface receptors. Other tactics include passive drug targeting, which uses the increased permeability and retention (EPR) effect to concentrate pharmaceuticals in tumors, and endothelial cell targeting, which tries to improve drug delivery to the tumor endothelium in order to

impair its blood supply. Triggered drug release involves the stimulation of drug release by external stimuli, such as ultrasound or heat (16).

1.5.1 Passive Drug Targeting

It is currently accepted that the endothelium of blood arteries becomes more permeable compared to a healthy state under specific situations (inflammation/hypoxia, which is typical of malignancies). In order to survive in an oxygen-depleted environment, rapidly growing tumors may recruit new vessels or devour existing blood vessels. Newly generated leaky arteries improve selective penetration of macromolecules ≥ 40 kDa and nano systems into the tumor stroma. Furthermore, NP retention period is lengthened due to the absence of normal lymphatic outflow in tumors. However, due to their relatively brief circulation duration and fast washout from the tumor, small molecule drugs do not benefit from this quality. The pharmacokinetics (prolonged systemic circulation), tumor selectivity, and side effects of small-molecule drugs are all improved by encapsulation in nanoscale drug carriers. **Figure 4** depicts a 'passive' method of tumor targeting that depends on carrier characteristics (size, circulation time, etc.) and tumor biology (vascularity, leakiness, etc.) but requires ligands for specific tissue or organ binding (11).

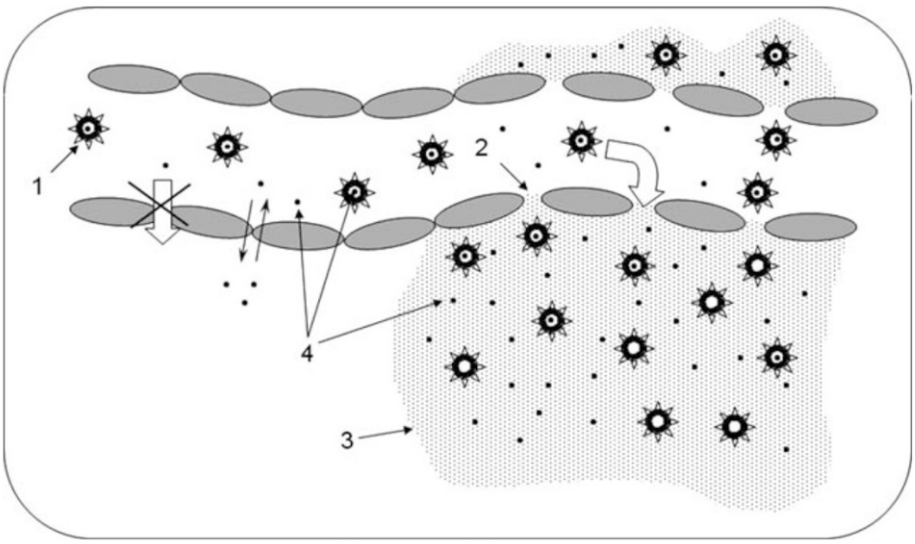

Figure 4. Passive targeting shcematics

1.5.2 Active Drug Targeting

The architecture of the tumor microenvironment (TME) is more complex than that of healthy tissue. Due to the unrestrained proliferation of cancer cells and the release of angiogenic factors, the vasculature and extravascular structure of the tumor environment become excessively disorganized and clogged. In contrast, these structural flaws both contribute to the EPR effect and are the primary cause of uneven tumor accumulation and nanocarrier retention. Active targeting, also known as ligand-mediated targeting, involves the retention and uptake of ligand-bound nanocarriers by disease-specific cells. Ligands that are specific to surface chemicals or receptors that are overexpressed in affected organs, tissues, cells, or subcellular domains need to be selected with care. Actively targeted carriers must be in close proximity to their target to benefit from this enhanced affinity (16). Numerous receptors have been identified, and their antibodies have been produced and studied *in vitro* and *in vivo* with success. Inducing extremely strong ligand-receptor binding, so acting as prospective models for the advancement of active targeting technology. RGD peptide has been shown to interact to alpha-V, beta-3 integrin. These receptors are extensively expressed on both glioma cells and the vasculature of the tumor microenvironment. As a classic example of a ligand, we can mention folic acid (FA), which binds specifically to the folate receptor (FAR) which is also found in TME. In conclusion, tumors can be actively targeted by directly targeting tumor cells, the moderately acidic TME, the vascularization of the TME, and the tumor nucleus (21). Some systems now incorporate numerous targeting modalities in a single nanoparticle (NP) as a result of the development of these technologies. While some of these targeting strategies, such as folic acid, are generalizable, the majority need tumor profiling to establish receptor or ligand overexpression. Additionally, not every targeting of receptors increases specificity. Some receptors that are overexpressed in tumor cell lines are also expressed in healthy tissues, hence reducing therapeutic efficacy.

In addition, there is frequently a trade-off between circulation residence time and cellular absorption. In an effort to improve these properties, NPs have recently been designed with detachable stealth corona systems and charge-reversal systems (negative or neutral charge for circulation, positive charge for uptake). One such approach uses a matrix metalloproteinase (MMP)-degradable linker to connect PEG

to the surface of the nanoparticle: in the cancer microenvironment, the PEG coating is dissolved, exposing a cell-penetrating peptide. Thus, it is possible to create systems that modify a given attribute to optimize for the present delivery barrier (18).

1.6 Nanocarrier Preparation Methods

Finding the optimal preparation procedure for a certain nanocarrier system can be a complex, iterative, multi-step process. The following is a summary of the procedure in general:

1. Identify the objectives: The first stage is to define the objectives of the nanocarrier system, including the type of medicine or therapeutic agent to be delivered, the cells or tissues to be targeted, and the desired pharmacokinetic and pharmacodynamic qualities.
2. Polymer screening: Depending on the objectives, a number of polymers can be screened for their ability to synthesize stable and functional nanocarriers. Biocompatibility, water solubility, and functionalizability should be considered while selecting polymers.
3. Method of preparation selection: Several nanocarrier preparation methods can be selected based on the screening results. The approach must have the capability to generate monodisperse, stable, and well-defined nanocarriers.
4. Optimization of preparation conditions: Once a preparation process has been determined, the parameters, including polymer concentration, stirring speed, pH, and temperature, must be tuned to get the desired attributes of the nanocarriers.
5. Characterization of the nanocarriers: Nanocarriers must be described in terms of their size, shape, surface charge, drug load, and release profile.
6. Evaluation *in vitro* and *in vivo*: The manufactured nanocarriers should be assessed for their ability to deliver the drug or therapeutic agent to the targeted cells or tissues, as well as their safety and toxicity profile.
7. Scale-up and formulation: Once the optimal preparation method has been determined, the process may be scaled up and nanocarriers can be created for use in preclinical and clinical trials. The real approach may vary depending on the exact objectives, materials, and methods employed. Additionally, many

testing and optimization cycles may be necessary to generate a final product that fulfills the intended criteria.

The following are examples of frequently utilized manufacturing methodologies (22):

1. Ionic gelation
2. Solvent casting
3. Freeze-drying
4. Layer-by-layer deposition (LbL)
5. Electrospinning
6. Three-dimensional (3D) printing
7. Reverse micellar method
8. Ionic gelation with radical polymerization
9. Emulsification
10. Desolvation
11. Nanoprecipitation
12. Spray drying

1.6.1 Ionic Gelation

In the preparation of chitosan nanoparticles, ionic gelation, which involves the intermolecular interaction of macromolecules with opposing charges, is a common technique. This method is preferred due in part to its simplicity and lack of equipment requirements. This approach employs the cross-linker tripolyphosphate (TPP), which can interact with cationic chitosan via electrostatic forces. In the procedure of ionic gelation, solutions of the polymer and cross-linker are produced at the proper concentrations. The TPP-chitosan complex is created by adding TPP to the chitosan solution, and nanoparticles are obtained by adding the polyanionic TPP solution to the acidic chitosan solution in a stepwise manner. Based on the chelation of positive and negative ions, spherical nanoparticles are produced. Protein-based active compounds were able to be loaded onto chitosan nanoparticles due to the process's minimal difficulty. In a study done by Fernandez-Urrusuno et al (23) for the preparation of insulin-loaded chitosan nanoparticles, insulin was combined with TPP solution and then added to the chitosan solution while stirring continuously. In this

work, chitosan hydrochloride salts (Secure 210 Cl and Protasan 110 Cl) were utilized as polymer, and the effects of molecular weight and degree of deacetylation on particle size, zeta potential, and active ingredient loading efficiency were examined. The end result was nanoparticles with a particle size of 300-400 nm and a zeta potential of 25-54 mV. The insulin loading was increased to 55% using this strategy. Due to the impact of chitosan's amine groups on the loading efficiency of gelation, it was found that the method's efficacy is dependent on deacetylation of chitosan.

1.7 Polymers Used in Nanocarrier Preparation

There are different types of polymers used in nanocarrier preparations, polymers are a versatile and promising material for application in nanocarriers for drug administration due to their biocompatibility and ability to be functionalized with a wide range of compounds. The formation of nanocarriers such as nanoparticles, liposomes, dendrimers, and hydrogels can be accomplished by the use of various polymers. Each of these nanocarriers possesses distinctive features that make them appropriate for various sorts of medications and applications.

Biodegradable polymers are the most preferred type of polymers for drug delivery systems. Biodegradable polymers are one form of biomaterials that decompose into harmless byproducts and regular metabolites. The two categories of biodegradable polymers are natural polymers, such as cellulose, chitosan, polypeptides, and proteins, and synthetic polymers, such as poly (glycolic-co-lactic) acid (PLGA), poly (lactic acid) (PLA), poly (glycolic acid) (PGA), poly (caprolactone) (PCL), aliphatic polycarbonates, and polyphosphazenes.

1.7.1 Chitosan

Polysaccharides are natural biopolymers that have a wide range of applications in the field of biomedicine due to their unique properties, including biodegradability, biocompatibility, and high availability. One such polysaccharide is chitosan, which is derived from chitin, a natural polymer found in crustaceans, fungi, and arthropods. Chitin can be obtained through acid treatment and alkaline extraction and can then be deacetylated to produce chitosan. Chitosan is a cationic polysaccharide that is composed of glycosidic linkages between glucosamine units. It is highly versatile and can be produced in various forms, including fibers, sponges, and powders. Researchers

have found that chitosan has many advantages, including non-toxicity, controlled release, biodegradability, biomimetics, and stability. Additionally, chitosan can be chemically and structurally modified to improve its properties. Due to its many benefits, chitosan is widely used in a variety of industries, including biomedical science, material science, bioengineering, and pharmaceutical science. One of the most promising applications of chitosan is in the development of nanoparticles for drug delivery systems. Chitosan nanoparticles (CSNPs) have been found to have a wide range of advantageous properties, including pore size, surface-to-volume ratio, and surface reactive groups. Researchers have developed a number of different methods for preparing CSNPs, considering a wide range of variables, including size, stability, retention duration, and drug loading capacity. Some of the most commonly used methods include ionic gelation, nanoencapsulation and chemical modification. These methods allow for the creation of CSNPs that are tailored to specific applications, such as targeted drug delivery and sustained release. Chitosan is a versatile and biocompatible polysaccharide that has many potential applications in a wide range of industries. Its potential for use in drug delivery systems, in particular, has attracted significant attention from researchers worldwide (24). Chitosan may have poor mechanical and thermal qualities and may not be as stable in fluids with fluctuating pH and ionic strength. Consequently, nanoscale-sized materials (such as nanoparticles (NPs), nanosheets, nanorods, nano-capsules, nanofibers, etc.) can be added to chitosan as fillers to improve its biological and physicochemical properties. Nanoparticles show a variety of advantageous properties, including pore size, surface-to-volume ratio, and surface reactive groups. In the biomedical field, the potential of utilizing nanoparticles as nanocarriers that can encapsulate medications, convey them to the target region, and provide a controlled release has been exploited extensively (2). Ohya et al.(25) were the ones who initially characterized chitosan nanoparticles in 1994 for the purpose of the circulatory delivery of 5-fluorouracil. Since then, they have been widely synthesized using a range of techniques and modified in a variety of ways in consideration of a wide range of variables, including size, stability, retention time, and drug loading capacity. Nanoencapsulation and chemical modification are the two primary methods for creating chitosan nanoparticles loaded with drugs.

Chitin is a semi-crystalline -(1-4)-linked N-acetyl-d-glucosamine homopolymer. This copolymer is composed of the building blocks -(1-4)-2-amino-d-glucose and -(1-4)-2-acetamido-d-glucose. Chitosan is a partly deacetylated chitin-based derivative. It is distinguished from other polysaccharides by its nitrogen content. **Figure 5** shows the structural of chitin and chitosan. The presence of amino and hydroxyl groups in the structure of chitosan enables the production of polymeric derivatives of this material.

Researchers have spent years studying chitosan nanocomposites and have developed a number of different approaches to synthesis, considering a wide range of variables including size, stability, retention duration, and drug loading capacity. Among these different methods which are used to prepare CSNPs, ionic gelation is one of the most used methods because of its advantages such as controllability of process, convenience, non-toxicity and not having any organic solvent in the procedure (26). These systems use tight junctions on cells to enter into the cell and deliver macromolecules into desired sites (27). Also CS nanoparticles have mucoadhesive properties which helps to release the drug payloads in sustained release manner (28). Chitosan and its derivatives can be used to enhance peptide and protein-based drug delivery systems. Additionally, chitosan has been widely studied in drug delivery to the brain. It can increase the permeability of drugs through the blood-brain barrier because it affects the tight junction. Due to the positive surface charge, chitosan nanoparticles can be absorbed by negatively charged cell membranes, hence extending their residence time on nasal mucosa. As a result, the transport of medications from

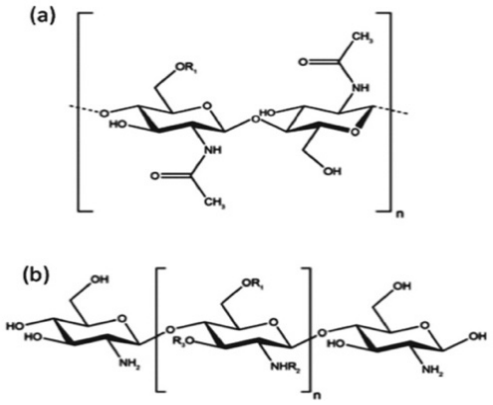

Figure 5. (a) structure of chitin (b) structure of chitosan (2)

the nasal passages to the brain is optimized. By modifying the surface of chitosan nanoparticles with tumor-targeting peptides such as chlorotoxin and transferrin, the capacity to target a brain tumor is increased even more (29).

1.8 5-Fluorouracil

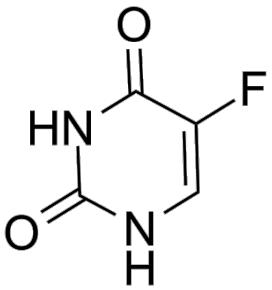

Figure 6. Chemical structure of 5-Fluorouracil

There are different synthesized chemical compounds which are used in cancer treatment which are approved by FDA in 2022. One of the first chemicals which got approved in 1962 to be used as an antimetabolite in colorectal cancer (CRC) treatment and still is in use is 5-Fluorouracil (5-FU) (**Figure 6**). Since approval by FDA, 5-FU is one of the most commonly administered first-line agents which is used alone or in combination of other anticancer agents in clinical practice to treat many solid tumors of digestive origin (colorectal, anal, pancreatic, esophageal, gastric and ampullary tumors) and other cancer types (i.e. breast, cervix and head and neck cancers) (30, 31).

5-Fluorouracil is one of the few antineoplastic medications that have been rationally formulated based on understanding of tumor biochemistry and is clinically effective.

This small molecule is more acidic and even more soluble in aqueous medium than its endogenous pyrimidine counterpart (32). 5-FU is synthesized by adding a fluorine atom to uracil in C-5 position and upon reaching the cell it uses facilitated transport mechanism which is used to take uracil into the cell then it is converted to three different active metabolites: fluorodeoxyuridine monophosphate (FdUMP), fluorodeoxyuridine triphosphate (FdUTP) and fluorouridine triphosphate (FUTP). 5-Fuorouracil is intracellularly activated to a 5'-monophosphate nucleotide (FUMP) either directly by 5-phosphoribosyl-1-pyrophosphate (PRPP) or in two steps via conversion to fluorouridine by uridine phosphorylase and then to FUMP by uridine kinase. FUMP is converted to FUDP, which is then reduced to F-dUDP by

ribonucleotide reductase, resulting in the creation of FdUMP. It may also be directly metabolized to FUdR and FdUMP by thymidine phosphorylase and thymidine kinase, respectively. FdUMP forms a ternary complex with thymidylate synthase and N5, N10-methylenetetrahydrofolate, the folate cofactors. Thus, 5-FU blocks dUMP to thymidylate conversion (TMP) (33).

These active metabolites disrupt DNA synthesis by interacting with thymidylate synthase enzyme and also it integrates into ribosomal RNA (rRNA) and causes reprograming of translation in cell which prevents the cancer cell from reproducing and making vital proteins, which leads to cell death (31).

Administration of 5-FU with conventional drug delivery systems has major limitations which leads to different problems in clinical use. For example, non-specific delivery which can cause systemic toxicity, poor dissolution thus sub-optimal absorption and short plasma half-life and low bioavailability which leads to higher doses of 5-FU resulting in more side effects. In addition, tumors developing resistance limits the conventional delivery system use (27, 34).

Fluorouracil toxicity manifests in numerous ways. Initial signs include nausea and anorexia. The next symptoms include stomatitis and diarrhea. Mucosal ulcerations of the digestive tract can cause diarrhea, shock, and death. Myelosuppression is the principal toxicity of IV bolus regimens. Infusional 5-FU toxicity is proportional to dose and period of exposure. DPD catalyzes the first stage of 5-FU degradation. In a tiny proportion of the population, DPD activity is lower than the population average. The administration of 5-FU to these patients can result in severe, life-threatening toxicity. Unfortunately, in the majority of instances, this hereditary predisposition is not identified until patient starts to use 5-FU therapy.(35)

The current work aims to study characterization and *in-vitro* properties of 5-FU loaded CS nanoparticles prepared using ionic gelation method with controlled PH and sonification of nanoparticles.

2. MATERIAL & METHOD

2.1 Materials

2.1.1 Used Chemicals for the Experiment

Acetonitrile (ACN)	Sigma-Aldrich, USA
Methanol (Met OH)	Sigma-Aldrich, USA
PBS pH 7,4 Tablets	Sigma-Aldrich, USA
Protosan®	Sigma-Aldrich, USA
Tripolyphosphate	Sigma-Aldrich, USA
5-Fluorouracil (5-FU)	Sigma-Aldrich, USA

2.1.2 Used Biological Substances

CT26 Cell line

2.1.3 Used Devices

Flow Cytometry Device (FACSAria II)	BD Biosciences, USA
Multi Magnetic Stirrer (RT10)	IKA, China
Differential Scanning Calorimeter (Q-100)	TA Instruments, USA
Precision Balance (XS 105)	Mettler Toledo, Switzerland
HPLC Device (Agilent 1260 Infinity Diode Array Detector)	Agilent Technologies, USA
HPLC Column (Restek Roc C18 5m, 250x4.6mm)	Restek, France
Lyophilizer (FreeZone 4.5)	Labconco, USA
Micropipette	Eppendorf, Germany
Particle Size and Zeta Potential Meter (Zetasizer NanoZS)	Malvern, UK
Plate Reader (Spectra Max Plus)	Molecular Devices, USA
Centrifuge (FC5718R)	Ohaus, Canda
Centrifuge Device (Universal 320)	

Scanning Electron Microscope (Quanta 400F Field Emission)	FEI Company, USA
Ultra-Pure Water System (Milli Q)	Millipore, USA
Ultrasonic Bath (AL 04-12)	Advantage-Lab, Switzerland
Vortex (Vortex 4 Basic)	IKA, China
Horizontal Shaker Water Bath	Hettich, Germany

2.2 Method

2.2.1 Determination of Ultraviolet (UV) Spectrum For 5-FU Using High Pressure Liquid Chromatography (HPLC)

For the characterization of 5-FU in HPLC UV and visible light spectrum wavelengths (190-800 nm) were analyzed and the wavelength with the highest absorbance was chosen as the suitable wavelength for HPLC analysis. For this process 5-FU was dissolved in water in 50 µg/mL concentration and used in for spectrum determination.

2.2.2 Development and Validation of Methods for Determining the Amount of Active Ingredients Using HPLC

For 5-FU determination HPLC method was developed by modifying the method used by Moshera Samy et al. (36) which reverse phase HPLC method using polar mobile phase was used and details regarding the method are given in **Table 2**.

Table 2. HPLC conditions for determination of 5-FU

HPLC Device	Agilent 1260 Infinity Diode Array Detector HPLC
Mobile Phase	Distilled water: Acetonitrile (90:10)
Injection Volume	20 µL
Flow Rate	1 mL/min
Colon	C_{18} (250x4.6 pore size: 5 µm)
Colon Temperature	25 ± 1 °C
Wavelength	275 nm
Detector	Diode Array (DAD)- UV
Analysis Type	Isocratic (consistent mobile phase)
Concentration Range	0,5-100 µg/mL

Analytical Method Validation

HPLC method for 5-FU determination is validated according to International Conference on Harmonization (ICH) Q2(R1) guidelines in accordance with parameters given below:

- Linearity
- Accuracy
- Precision
- Sensitivity
- Specificity
- Stability

Linearity

According to guidelines, linearity is defined as the "degree to which the assay exhibits a linear relationship between the response and the analyte concentration within a given range." In order to calculate it, one main stock solution of 5-FU was prepared by dissolving 5-FU powder in water:methanol (9:1) solution in 1 mg/mL concentration and this stock solution was used to prepare 7 different concentrations of 5-FU. For preparation of these solutions stock solution was diluted using water:methanol (9:1) mixture to prepare 100, 50, 25, 10, 1, 0.5 and 0.05 µg/mL concentrations of 5-FU (n=3). The calibration line was plotted using the obtained peak areas against the concentrations of the solutions.

Accuracy

Accuracy in an experiment refers to the degree to which the results of an experiment or measurement agree with the true or accepted value. International Conference on Harmonization (ICH) Q2(R1) guidelines recommends that the accuracy should be determined by analyzing the samples at the low, middle, and high levels of the calibration range. In order to determine the in-experimental accuracy, standard solutions were prepared at three different concentrations (low, medium, high; 10, 50, 100 µg/mL) in the calibration curve, three of each concentration (n=6), and measurements were carried out consecutively on the same day.

Precision

The reliability of an analytical procedure can be thought of as its precision. It refers to the degree of concordance between independent analytical results acquired

under specified conditions. The precision of an analytical method is determined by calculating the coefficient of variation (CV) for a set of samples of the same concentration, measured in sequence, using a sufficient number of samples for a statistically valid evaluation. Repeatability (the degree to which identical findings are achieved when the same measurements are repeated under the same conditions) and reproducibility (the degree to which the same results are obtained by different analysts using the same method on the same sample) are used to convey precision. For 5-FU HPLC method precision analysis three different solutions with 10, 50, 100 µg/mL concentrations (low, medium, and high) were analyzed and their %CV for repeatability and reproducibility were calculated with the given formula.

$$\%\,Coefficient\ of\ Variation\ = \frac{Standard\ Deviation\ (SD)}{Average\ (X)} \times\ 100$$

Equation 1. % CO formula

Sensitivity

The analytical method's sensitivity is measured in two ways: the limit of detection (LOD) and the limit of quantification (LOQ). Under defined conditions, the limit of quantification is the lowest concentration that can be quantitatively measured by the analytical method with an acceptable degree of accuracy and precision. Under defined conditions, the limit of detection is the lowest concentration that can be qualitatively assessed by the analytical procedure. LOD's acceptable signal-to-noise ratio is 3, while LOQ's is 10.

Specificity

The specificity of an analytical technique refers to its capacity to evaluate the active ingredient in the presence of contaminants and excipients. In this regard, solutions of the auxiliary compounds to be involved in the production of nanoparticles were produced and analyzed at the concentrations to be found in the formulation.

Stability

In order to demonstrate the stability of 5-FU throughout the release studies, the solutions in phosphate buffer (PBS) medium in pH 7.4 at 100 µg/mL concentration were kept at 37 °C for 24 and 48 hours and analyzed. It has been shown statistically whether the difference between the results is significant or not.

2.2.3 Preparation of Chitosan Nanoparticles

The nanoparticles were synthesized via ionic gelation method (28). In this study Protasan® which is a salt form of chitosan was used to prepare chitosan nanoparticles. Different concentrations of chitosan polymer were studied as shown in **Table 3** and lastly the main formulation with the best nanoparticle size and best encapsulation efficacy was chosen to move forward as shown in **Table 4**.

Briefly, 15, 25 and 50 mg of CS was dissolved in double purified water and the pH was adjusted to 4.5 by using 1% acetic acid solution. 15 mg of 5-FU powder was dissolved in 9:1 water-methanol mixture to make a solution with a concentration of 5.25 mg/ml. Using a micro-syringe, 2.85 ml of the prepared 5-FU solution was then gradually added to 7.14 ml of CS solution. Under mechanical stirring, 10 ml of sodium tripolyphosphate solution with a concentration of 0.5 mg/ml was added to CS/5-FU solutions at 700 rpm. The reaction was held at room temperature for 1 hour. After 1 hour of stirring in room temperature, produced solution containing chitosan nanoparticles were transferred into a centrifugal tube with glycerin in the bottom of the tube and then, tube was centrifuged at 10,000 rpm for 30 minutes at 4ºC. Schematic scheme of the process is shown **Figure 7**.

The same procedure was used to synthesize empty Chitosan nanoparticles (CS-NPs) but instead of 2.85 ml of 5-FU solution, 2.85 ml of double-distilled water was added into the mixture.

Table 3. Different concentrations used for CSNP formulation

	CS (mg/ml)	TPP (mg/ml)
Formulation 1	1.5	0.5
Formulation 2	2.5	0.5
Formulation 3	5.0	0.5

Main formulation which was used throughout the thesis project which was chosen because of the acceptable nanoparticle characterizations are shown in **Table 4**.

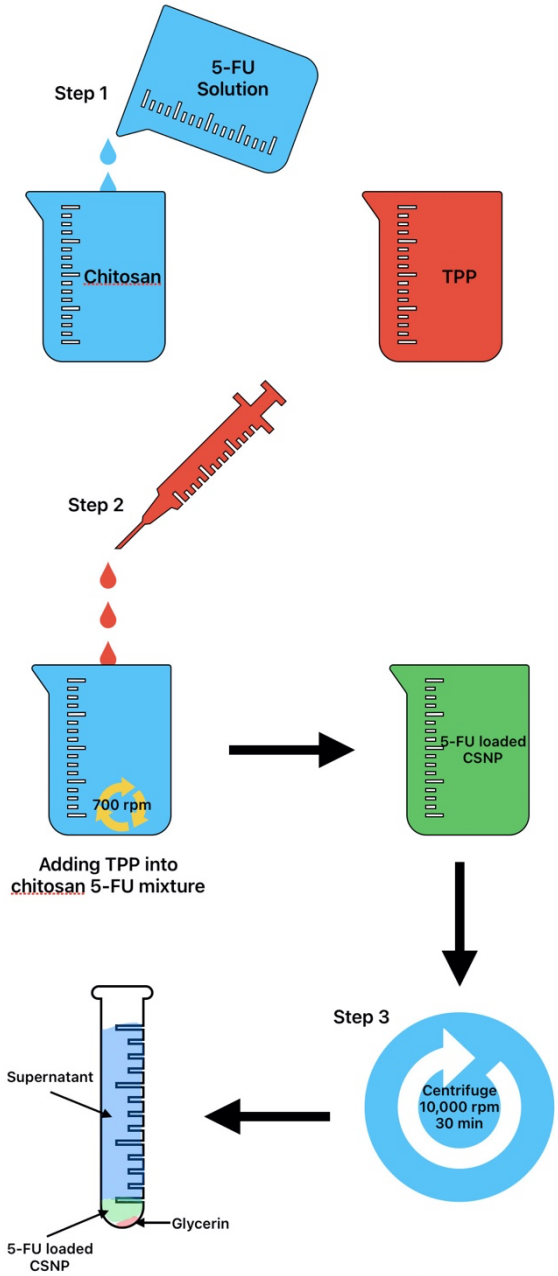

Figure 7. Schematic scheme of 5-FU loaded CSNP preparation

Table 4. Concentrations and Volumes used in chitosan formulation

	Concentration	Volume
CS	3.5 mg/ml	7.15 ml
TPP	0.5 mg/ml	10 ml
5-FU	5.25 mg/ml	2.85 ml

2.2.4 Characterization of Nanoparticles

Determination of Particle Size, Polydispersity Index and Zeta Potential

Nanoparticles were assessed using Zetasizer (Nano-ZS) (Malvern Instruments, England). Particle size, polydispersity index (PDI) and zeta potential of blank CSNP and 5-FU loaded CSNP were determined at 25°C. Particle size and polydispersity index were measured using the principle of dynamic light scattering (DLS) and Zeta potential is measured using the principle of laser Doppler electrophoresis. DLS measures fluctuations in scattered light of the particles moving in Brownian motions while the laser doppler electrophoresis method measures the frequency changes during the scattering of the laser sent to the nanoparticles in a liquid medium under a variable electrical field. All of the measurements for this experiment were triplicated.

Morphological Properties of Nanoparticles

After completing Zetasizer analysis, two batches one with 5-FU loaded nanoparticles and another one with empty nanoparticles were synthesized and sent to Middle East Technical University's central laboratory (METU MERLAB) to be inspected using a Scanning Electron Microscopy (SEM) (NovaTM NanoSEM 430). This analysis was utilized to examine the morphological aspects of nanoparticles. Nanoparticles were adhered to metal sheets using double-sided tape. The samples were then coated with 100 Å gold using a brand coating device (BIORAD). Afterwards, the surface morphologies were examined at different magnifications such as 25000x, 50000x, 100000x by sending electrons on the samples with the Quanta 400F Field Emission SEM device.

Differential Scanning Calorimetry (DSC) Analysis

Differential Scanning Calorimetry (DSC) was used on lyophilized 5-FU loaded nanoparticles, empty chitosan nanoparticles and also physical mixture of Protosan, TPP and 5-FU to determine different thermal properties such as glass-liquid transition temperature, melting point, and phase transition temperature. Also this method is used to verify the encapsulation of 5-FU into chitosan nanoparticles (37). Measurements were made using Q-100 DSC (TA Instruments, USA) in Hacettepe University Faculty of Pharmacy Drug and Cosmetic R&D and Quality Control Laboratories (HÜNİKAL). All of the mentioned lyophilized nanoparticles and physical mixtures were weighed in appropriate amount (5 mg) and placed in aluminum sample containers and DSC analysis temperature range was between 0°C to 300°C with gradual increase of 10°C per minute.

Determination of Encapsulation Efficacy

In order to provide the basis for *in-vitro* studies which will be carried out in this thesis project the encapsulation efficacy analysis was done to determine the amount of active substance loaded into the produced nanoparticles. Indirect method was used to determine the encapsulation efficacy of 5-FU loaded chitosan nanoparticles. After producing the nanoparticles with the above-mentioned method, the supernatant was collected and filtered using 0.22 µm filter then the amount of unloaded 5-FU was determined using HPLC analysis. The encapsulation efficacy was determined using below formulation:

$$EE(\%) = \frac{(weight\ of\ total\ 5\ FU) - (weight\ of\ free\ 5FU\ in\ supernatant)}{weight\ of\ total\ 5FU} \times 100$$

Equation 2. $Encapsulation\ Efficacy\ calculation\ equation$

2.2.5 *In-vitro* Release Analysis

In order to determine the amount of 5-FU released from chitosan nanoparticles *in-vitro* drug release analysis was done. In this regard temperature-controlled water bath shaker was used. Release analysis was done in 37±0.1 °C and 105 spm (stroke

per minute) for 24 hours. 5-FU loaded nanoparticles were prepared and centrifuged then distributed in 14 ml of PBS (PH=7.4) solution which represents physiological PH of the body. Then they were separated into 14 different 1 ml falcon tubes and placed into the water bath. In the intended time intervals (30 minutes, 1,2,4,6,12,24 hours) two of the tubes were collected, centrifuged and their supernatant were filtered using 0.22 µm filter and analyzed using HPLC for the released 5-FU three times each and mean of the results were used for calculation. For this analysis sink condition was calculated and 1 ml of release media was decided for the release study.

2.2.6 Cell Culture Studies

Determination of Effective Doses of Active Substance in Colon Adenocarcinoma Cells

Preparation of Cells

For *in-vitro* studies on 5-FU loaded chitosan nanoparticle cytotoxicity effect CT-26 mouse colon adenocarcinoma cells were used, in order to prepare cells for this analysis, cells which were frozen at -196 °C using liquid nitrogen were kept in a 37 °C water bath until the freezing solutions (DMSO-FBS) were thawed. When the dissolution was completed, it was taken into 10 ml RPMI medium containing 10% FBS, 1% penicillin-streptomycin and glutamic acid. After centrifugation at 1800 rpm for 10 minutes, the supernatant was removed, and the precipitated cells were dissolved in the same RPMI mixture and transferred to T-25 cell flasks. Cells were checked under a light microscope and after 24 hours, cells showing adhesion were observed, and proliferation was observed by changing the medium.

Cytotoxicity Studies on CT26 Cells

CT-26 cells were seeded in 96 well cell plates the night before and adhesion was ensured in an incubator at a constant temperature of 37 °C with 5% CO_2. After adhesion the cell culture containing FBS was cleaned away and each formulation solution consisting of 5-FU solution, blank CSNP and 5-FU loaded CSNP was added in a non-serum containing media, after 6 hours formulation was cleared away and cells were washed using PBS solution twice. The cytotoxic effect was quantitatively measured 24 hours, 48 hours, and 72 hours after the samples which were applied. After incubation, 25 µL of MTT solution was added to the wells and incubated for another 4 hours. After incubation, 80 µL of 23% SDS dissolved in 45% dimethylformamide

(DMF) (pH: 4.7) was added (38). The plates were incubated for approximately 16 hours at 37 °C in an incubator and spectral absorbance measurements were taken at 570 nm.

Statistical Evaluation

The results acquired for the thesis are presented as mean standard deviation. There were at least three repetitions of each experiment run. The data were examined using Prism 7 (Graphpad) software and the proper statistical tools (such as ANOVA, Chi-square, and/or Student's t test). instances when a difference was thought to be substantial at $p < 0.05$.

3. RESULTS

3.1 Determination of Ultraviolet (UV) Spectrum For 5-FU

5-FU solution was prepared at a concentration of 50 µg/mL. This solution was analyzed using high-pressure liquid chromatography (HPLC) methods, as described in the section "Development and validation of methods for determining the amount of active ingredients using high-pressure liquid chromatography (HPLC)" in the "Materials and Methods" section. The three-dimensional UV spectra of the active ingredient was obtained, and it was shown that the wavelengths with the highest absorption was 275 nm with selected mobile phase (**Figure 8**.)

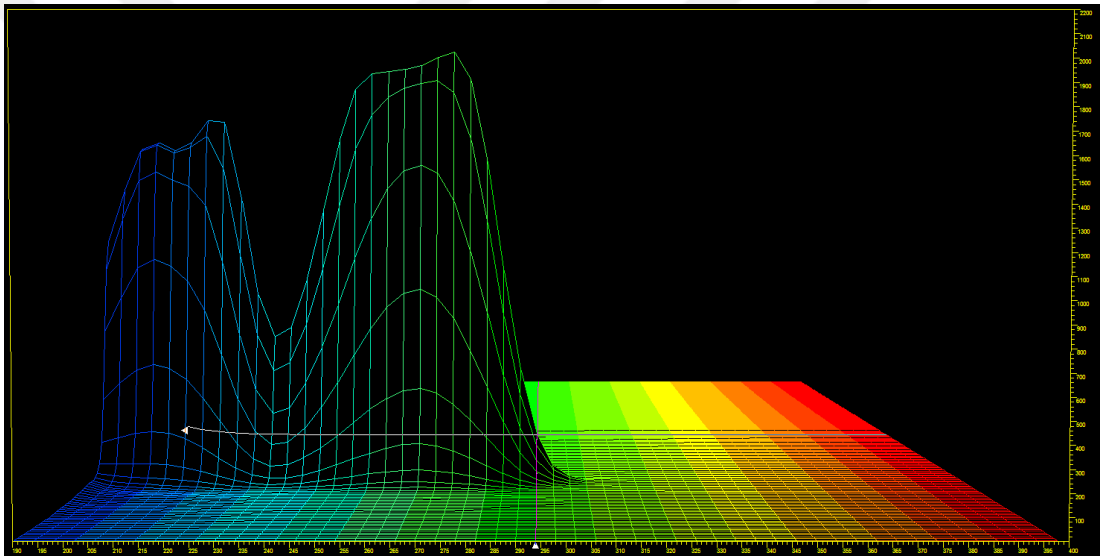

Figure 8. 3D spectrum for 5-FU (190-800 nm)

3.1.1 Development and Validation of Method for Determining the Amount of Active Ingredient Using HPLC

As mentioned in section 2.2.2 “Development and Validation of Methods for Determining the Amount of Active Ingredient using HPLC” of materials & method part. The retention time for 5-FU using 9:1 Water:ACN as mobile phase were measured as 3.46 minutes.

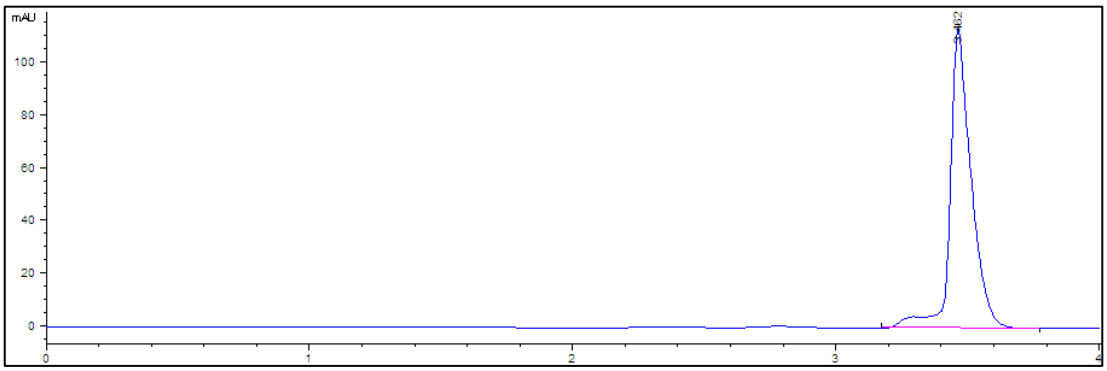

Figure 9. HPLC Chromatograph for 5-FU (50 µg/mL)

The findings regarding the validation of the methods developed for the quantification of active substances according to the International Conference on Harmonization (ICH) Q2(R1) Guidelines are given below, respectively:

Linearity

The findings of section 2.2.2 "Development and Validation of Methods for Determining the Amount of Active Ingredients Using HPLC" regarding linearity have been presented below, and they reveal a remarkable level of accuracy. The results demonstrate a correlation coefficient (R^2) value of 0.9999, indicating a high degree of linearity between the measured concentration and stock solution. These results are highly encouraging and suggest that the model is well-suited for further analysis.

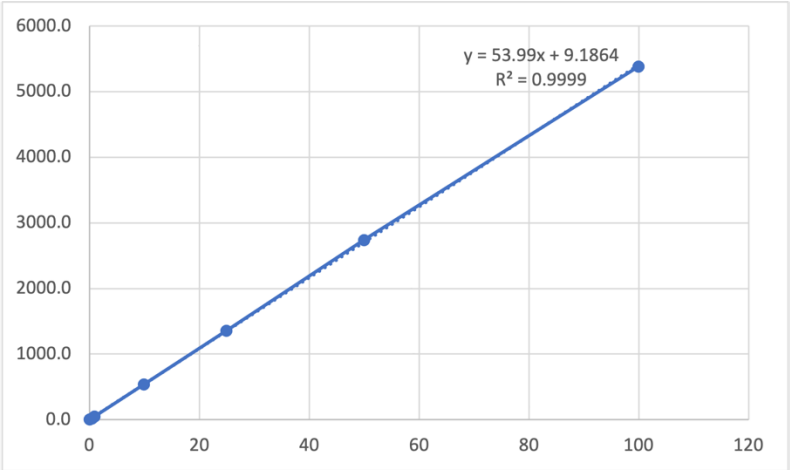

Figure 10. Calibration graph and its equation for 5-FU linearity validation

Accuracy

The findings of section 2.2.2 "Development and Validation of Methods for Determining the Amount of Active Ingredients Using HPLC" regarding accuracy of developed method for 5-FU HPLC analysis which was done with six (n=6) repeats are shown below in **Table 5**. As shown, the accuracy of the developed assay methods has been demonstrated by finding the recovery value of 5-FU using HPLC between 99% and 102%.

Table 5. % Recovery Values for 5-FU using HPLC analysis method

Stock Solution Conc.	Measured Con.	%SD	% Recovery Value
10	9.924	1.041	99.24
50	50.251	0.25	100.50
100	99.79	0.193	99.79

Precision

The repeatability and reproducibility results for 5-FU are shown in the table below (**Table 6** , **Table 7**). It is seen that the % coefficient of variation (%CO) values calculated for this method are not more than 2%, and the reproducibility and reproducibility of HPLC methods have been demonstrated. Results are expressed as mean and standard deviation (SD).

Table 6. repeatability values for HPLC analysis of 5-FU

5-FU conc.	AUC ± SD	Measured conc. ± SD	% CO
10	540.33 ± 1.52	9.83 ± 0.03	0.28
50	2739.00 ± 4.00	50.56 ± 0.07	1.46
100	5400.00 ± 4.58	99.84 ± 0.08	0.08

Table 7. reproducibility values for HPLC analysis of 5-FU

	Used conc. (µg/mL)	Measured Conc. (µg/mL) ± SD	%CO
On the same day	10	9.91 ± 0.12	1.17
	50	50.56 ±0.07	0.15
	100	99.74 ± 0.08	0.09
On different days	10	9.95 ± 0.08	0.84
	50	50.49 ± 0.09	0.18
	100	99.94 ± 0.10	0.10

Stability

Stability analyses results are shown below (**Table 8**).

Table 8. Stability analysis values for 5-FU

5-FU	t = 0	t = 24 hr	t = 48 hr
AUD	5390.00 ± 3.00	5388.67 ± 7.78	5393.33 ± 10.79

3.2 Particle Size, Polydispersity Index and Zeta Potential Analysis

During the formulation experiments, nanoparticles were produced using the method described in the "Preparation of 5-FU loaded chitosan nanoparticle formulations" section of the "Materials and Methods" section. Results of analysis of blank and 5-FU loaded chitosan nanoparticles using NanoZS device are given below in **Table 9**. and graphs of zeta potential and size of blank CSNP and formulation (2) CSNP are given in **Figure 11**, **Figure 12**, **Figure 13**, **Figure 14** respectively. These analyzes were repeated three times and results are given as average ± SD.

Table 9. Particle size, PDI and zeta potential of different formulations

	Size (nm)	PDI	Zeta
Blank CSNP	214.00 ± 3.21	0.28 ± 0.02	1.11 ± 0.11
Formulation 2	217.03 ± 10.41	0.37 ± 0.11	0.02 ± 0.02
Formulation 3	481.00 ± 7.21	0.24 ± 0.01	1.14 ± 0.16

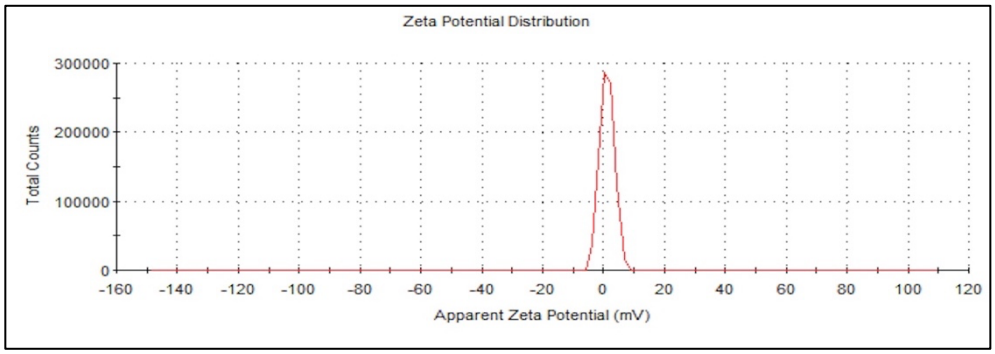

Figure 11. Blank CSNP zeta potential

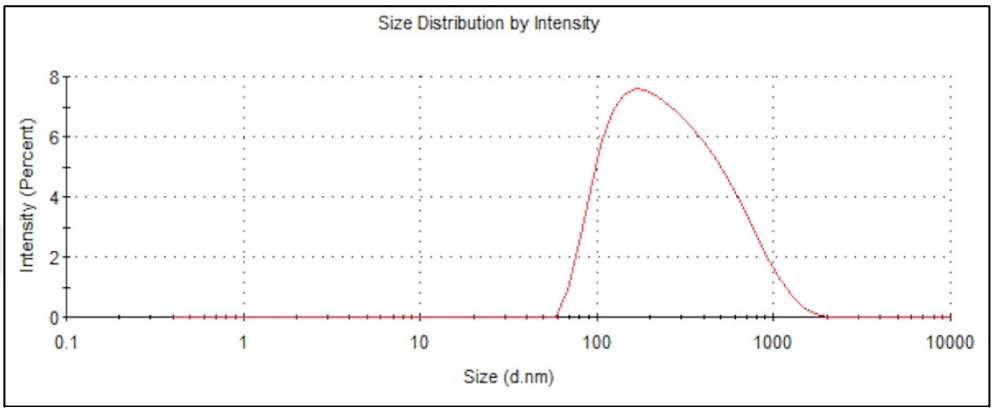

Figure 12. Blank CSNP size distribution

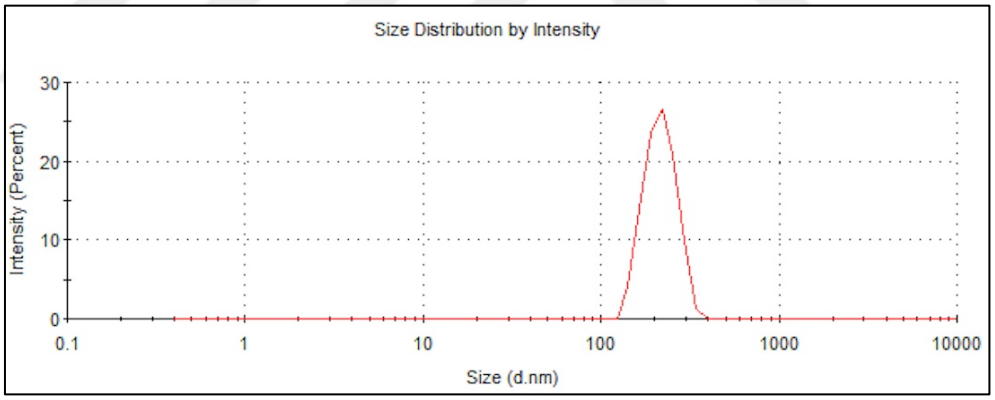

Figure 13. 5-FU loaded CSNP formulation (2) size distribution

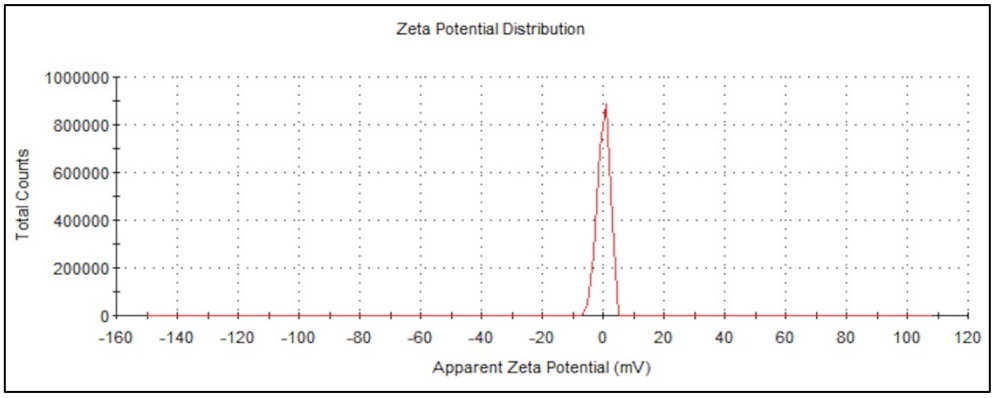

Figure 14. 5-FU loaded CSNP formulation (2) zeta potential

3.3 Morphological Properties of Nanoparticles

After determining the formulation parameters for nanoparticle formulations, blank and 5-FU loaded nanoparticles were prepared for characterization studies. SEM analysis carried out at METU MERLAB at a magnification of 100,000x, the results are presented in Figure X. The images taken show that the size of the particles measured using the scale below is approximately 170 nm and also there was no significant difference between 5-FU loaded and blank CSNPs and shape and size is consistent with the particle size results obtained with Nano ZS (**Figure 15**).

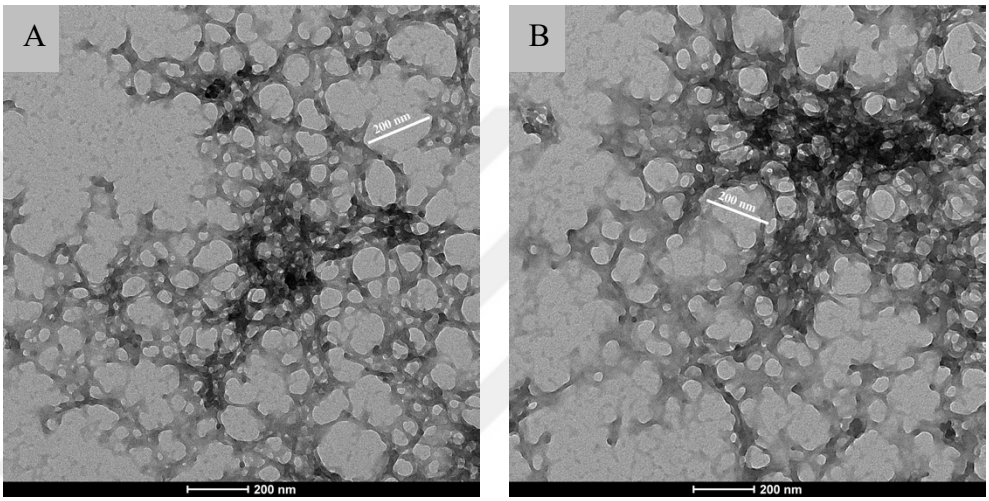

Figure 15. SEM analysis results A) blank chitosan nanoparticles B) 5-FU loaded chitosan nanoparticles

3.4 Differential Scanning Calorimetry (DSC) Analysis

Differential scanning calorimetry (DSC) analysis was performed to investigate the thermal properties of 5-FU loaded chitosan nanoparticles, blank chitosan nanoparticles, physical mixture of materials and 5-FU powder. The main objective of the DSC analysis was to confirm the encapsulation of 5-FU in the chitosan nanoparticles and to determine the transition temperatures such as glass transition temperature, phase transition temperature and melting point. The samples were analyzed using a Q-100 DSC device as described in “Differential Scanning Calorimetry (DSC) analysis” section of “Material and Method”. The results obtained from the DSC analysis were used to confirm the successful encapsulation of 5-FU in the chitosan nanoparticles and to understand the thermal behavior of the samples (**Figure 16**).

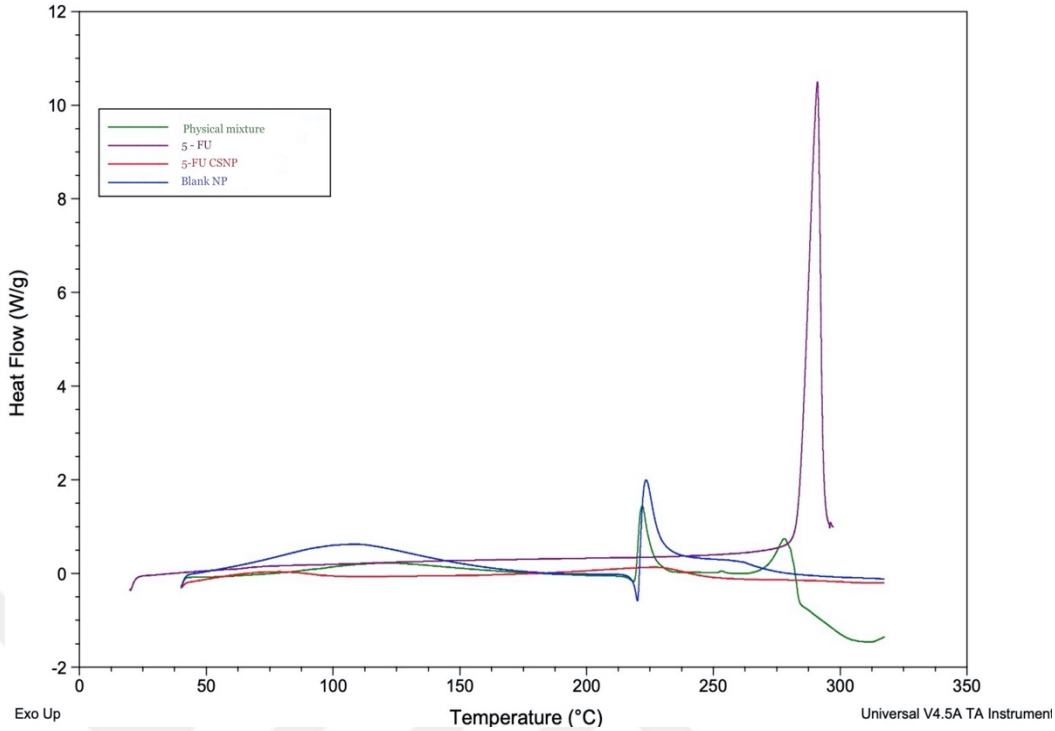

Figure 16. DSC thermogram for 5-FU loaded chitosan, blank chitosan, physical mixture, and 5-FU

3.5 Determination of Encapsulation Efficacy

Analysis results for encapsulation efficacy of 5-FU loaded chitosan nanoparticles were determined with the method mentioned in "Determination of Encapsulation Efficacy" part of method as are shown as %EE in **Table 10** encapsulation efficacy for chitosan nanoparticle were 15.69%.

Table 10. %EE of chitosan nanoparticles

Nanoparticle formulation	Encapsulation efficacy %
5-FU loaded chitosan nanoparticles	15.69

3.6 *In-vitro* Release Analysis

As described in "*In-vitro* release analysis" part of method, nanoparticles were prepared and analyzed in PBS solution (PH=7.4) for period of 24 hours and results for release analysis are shown in **Figure 17**.

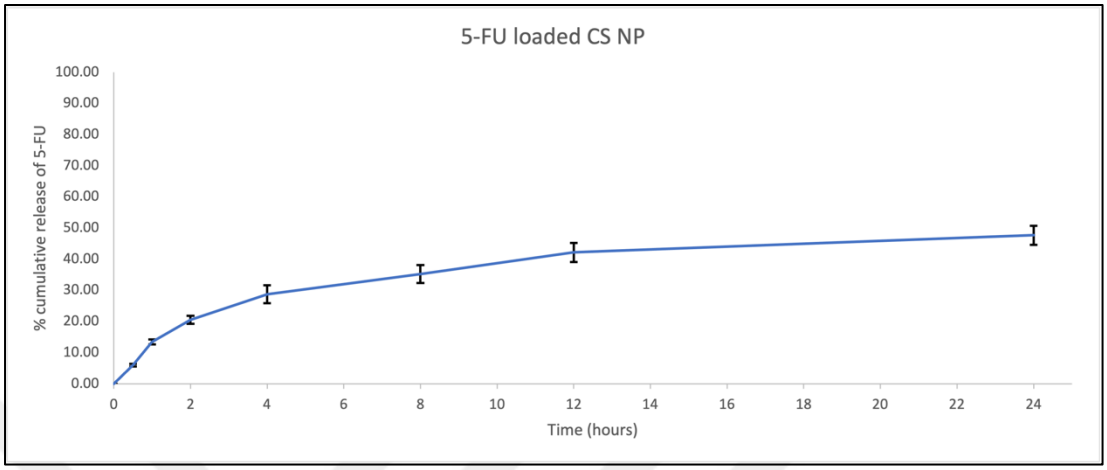

Figure 17. 5-FU loaded CSNP *in-vitro* release graph

3.7 Cytotoxicity Studies on CT26 Cells

As stated in the section 2.2.6 " Determination of Effective Doses of Active Substance in Colon Adenocarcinoma Cells" of the materials and methods section, CT-26 cells were incubated with effective substance solutions at the determined concentrations for 48 hours. After incubation, MTT analysis was performed and IC50 values were calculated using Prism 7 program based on cell viability and for 5-FU, IC50 was 2.91, 4.76 and 0.01µM for 24, 48 and 72 hours respectively. Results of cell viability percentages after incubation with different solutions are given below in **Figure 18**, **Figure 19**, and **Figure 20**.

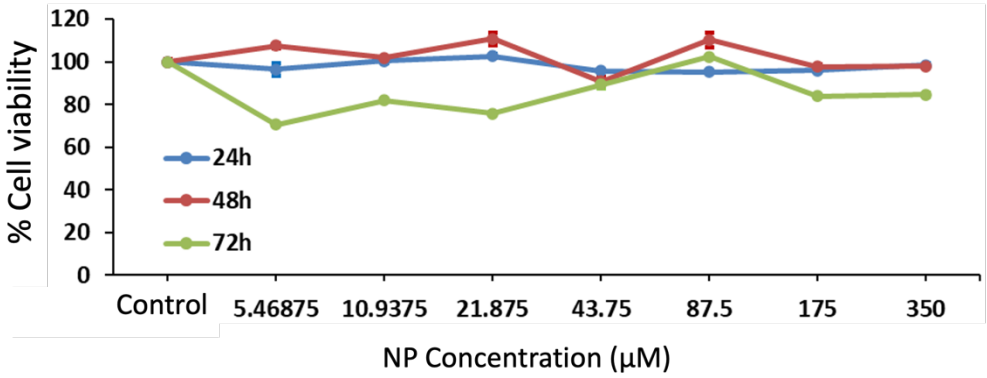

Figure 18. Cell viability % for blank CSNP

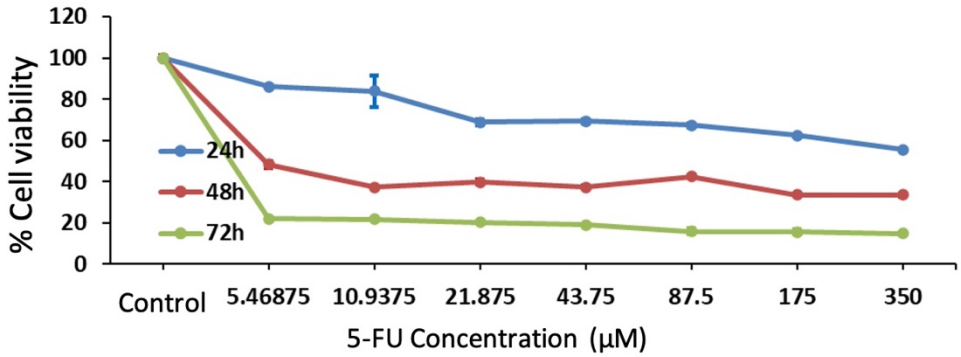

Figure 19. Cell viability % for 5-FU solution

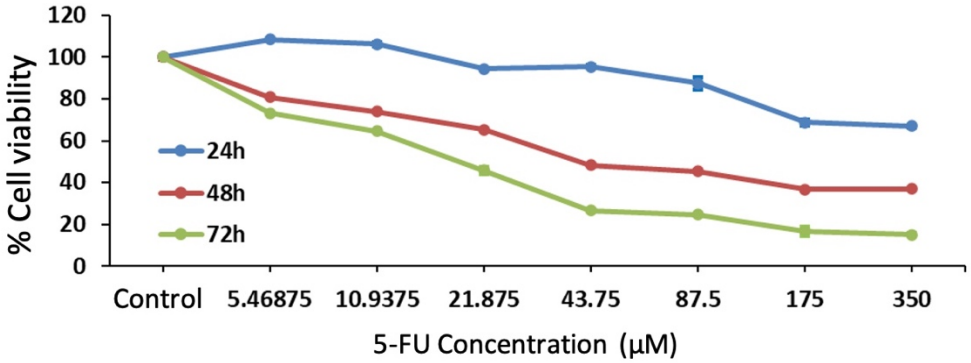

Figure 20. Cell viability % for 5-FU loaded CSNPs

Blank nanoparticles were also investigated in terms of cytotoxic effects of polymer on CT-26 cells. Results showed that chitosan, which is the polymer used to prepare nanoparticle formulations, does not affect cell viability.

It was observed that, 5-FU solution was decreased the cell viability to 20 % levels after 72 hours incubation for all concentration points. 5-FU solution was also decreased cell viability to around 40% levels independently from concentration. 24 hours incubation of 5-FU solution with cells caused gradually decrease in cell viability, it was found as 50% at highest drug concentration (350 µM). 5-FU solution and 5-FU loaded CSNPs showed similar cytotoxicity pattern at 24 and 48 hours, however due to the controlled drug release 5-FU at lower drug concentration levels (5.47, 10.94, 21.875 µM) higher cell viability was obtained with 5-FU loaded CSNPs.

4. DISCUSSION

In the scope of the thesis studies, a nanoparticular drug carrier system using chitosan nanoparticles loaded with 5-FU has been designed in order to be used in treatment of colorectal cancer and has been successfully developed through *in-vitro* and in-vivo characterization studies. The findings obtained in the scope of the thesis studies have been discussed scientifically in this section and compared with literature.

4.1 Development and Validation of Methods for Determining the UV Spectrum of Active Ingredient and Quantification by HPLC

In this study method for 5-FU HPLC analysis was designed and validated according to related articles and International Conference on Harmonization (ICH) Q2(R1) guidelines. The UV spectrum related to the active substance is presented in Figure **8** and wavelength which had the highest absorbance was determined as 275 nm. In a study conducted by Ana Cristina de Mattos et al. (39), the wavelength at which the highest absorbance was reported as 265 nm and also in a study by Shashank Tummala et al. (40) which the absorbance of 5-FU was 269 nm and also for the last article regarding absorbance Li Sun et al. (28) reported highest absorbance as 265 nm for 5-FU and considering these articles and determined data 275 nm was in range for 5-FU and for rest of the study this UV absorbance was used.

In the context of the studies, method for determining the amount of 5-FU active substance was developed using HPLC and was successfully validated. In this regard the method was developed by modifying the method presented in the literature by Ana Cristina de Mattos et al. (39) for HPLC determination of 5-FU. Chromatograph for 5-FU using HPLC was given in **Figure 9** and retention time using mobile phase Water:ACN (9:1) was 3.52 minutes.

The linearity studies were carried out in the concentration range of 0.05-100 µg/mL. It was determined that there was a linear ratio between the standard solution concentration and the peak area obtained from the HPLC analysis in the studied concentration range. The findings related to the analysis are given in **Figure 10** and the r^2 values for the calibration curves of the active substances were found to be 0.999 As reported in the literature (41) and also accepted by relevant health authorities and guidelines, the stronger the relationship between variables, the closer the r^2 value is to

1. Based on these findings, the linearity of the methods for the working concentration range has been demonstrated.

For accuracy validation studies results are given in **Table 5** and % recovery value for 5-FU using HPLC method is in the range of 99% and 102% which shows determined concentrations of the solution using HPLC is valid according to US pharmacopeia and International Conference of Harmonization (42).

In order to evaluate the precision of determination method three solutions with concentrations of 10, 50 and 100 µg/mL were analyzed for repeatability and reproducibility which the data are given in **Table 6** and **Table 7** respectively. According to analyzed data % Coefficient of Variation is the most important value for validation of this method, according to literature written of validation of HPLC methods by Ghulam A Shabir (42) the %CV should be less than 2% for a method to be validated and out data for repeatability and reproducibility are shown %CV for less than 2% for solutions analyzed on the same day or between different days using HPLC method which shows the validation of this method.

Sensitivity analyses were done using 10 µg/mL concentration and their AUC for 5-FU, by using the HPLC instrument LOD and LOQ were determined as 28 ng/mL and 96 ng/mL respectively for this method. According to this evaluation for 5-FU chosen method was validated to ensure the determination of 5-FU in nanogram concentration in any solution.

Chitosan and TPP, excipients utilized in formulation, were examined using HPLC techniques created for each active ingredient as part of selectivity studies. It was discovered that the excipients had no effect on the peaks of the active substance when the peaks obtained with these analyses and the peaks acquired as part of encapsulation efficiency and release tests were compared. It has been established that the developed procedures are specific to the formulations and active ingredients.

Stability analysis were done using PBS solution with pH=7.4 and in 37°C for 24 and 48 hours for 100 µg/mL concentration and results for AUC in 0, 24 and 48 hours are given in **Table 8** which shows no differences ($p<0.05$) between different time frames which shows 5-FU is stable until 48 hours in the given medium.

As a result of these studies, the determined HPLC method for 5-FU evaluation was validated according to ICH guideline and used for upcoming analysis in the thesis.

4.2 Formulation of Chitosan Nanoparticles

4.2.1 Preparation of Chitosan Nanoparticles

In the context of this study chitosan was used as FDA approved polymer which is a biocompatible and biodegradable polymer used to synthesize hydrophilic nanoparticles to be used as drug delivery systems. Additionally, chitosan is an excellent polymer for transporting peptides, proteins, oligonucleotides, and plasmids since it is non-toxic and tissue compatible. It prevents bioactive compounds from being degraded by enzymes and chemicals as they pass through epithelial cells. 5-FU was encapsulated into the chitosan nanoparticles using a method which was modified from an article by P. Calvo et al. (43) chitosan nanoparticles were formed using ionic gelation method. This method was further modified using different mass ratios of CS and TPP with different PH value which was used by Aline Martins dos Santos et al. (44). Lastly the formulation 2 which produced CSNP with desired particle size, polydispersity index and zeta potential which was given in **Table 9** was used as main formulation. In regard to given formulation concentrations in **Table 3** formulation 1 which had 3:1 (CS:TPP) concentration ratio caused aggregation while producing the formulation, it was in line with article by Moshera Sami et al. (36) which produced CSNPs using different CS:TPP ratios and 3:1 ratio formulation caused aggregation and sedimentation as well while producing the nanoparticles.

Formulation 2 and 3 were continued to be assessed in order to choose the best formulation regarding particle size, PdI and zeta potential.

4.2.2 Characterization of Prepared Chitosan Nanoparticles

Particle Size, PDI and Zeta Potential of Nanoparticles

Particle size, PDI index and zeta potential analysis results for both blank and 5-FU loaded prepared chitosan nanoparticles for the context of thesis were given in **Table 9**. in section 3.2 “Particle Size, Polydispersity Index and Zeta Potential Analysis”. Formulation 1 was aggregated while mixing the TPP and CS because the ratio between CS and TPP was 3:1 which causes aggregation of CSNP as in the article done by M. Samy et al. (27) suggested the ratio of CS to TPP should be around 4 or more to see the nanoparticle formation. In an article written by Li Sun et al. (28) particle size, PdI and zeta potential of nanoparticles were in range with the nanoparticles produced in this study which states that there is no significant increase

in particle size after 5-FU loading but there is a decrease in zeta potential of the nanoparticle because 5-FU is a potentially negatively charged molecule but CSNPs are positively charged molecules and when 5-FU is loaded into the CSNPs zeta potential of the prepared nano molecule decreases from 1.11 ± 0.11 to 0.02 ± 0.02 and this measurement is aligned with the article.

Formulation 2 was selected as the chosen formulation for next steps because formulation 3 which had 10:1 (CS:TPP) ratio was not in the desired particle size range (100-250 nm) with 481 nm particle size.

In an article written by A.M. dos Santos et al. (44) particle size of chitosan nanoparticles prepared in pH=4.5 size of nanoparticles were measured around 200nm for a ratio of 4:1 of CS:TPP formulation with a PdI of 0.3 which was in line with the findings of this study, although they did not prepare nanoparticles with 5:1 ratio but their conclusion was the increase in size with increase in the ratio CS:TPP and the findings in this study shows 217 ± 10.41 nm for CSNP loaded with 5-FU which is confirms their conclusion.

For the decided chitosan nanoparticle zeta potential was 1.11 for blank nanoparticle and 0.02 for 5-FU loaded nanoparticle, pH was decreased to 4.5 in order to increase the zeta potential for more stable nanoparticle formulation but the increase was not significant because of Protasan® salt formulation which is used in this study and for further studies this needs to be taken into consideration.

Morphological Properties of Nanoparticles

When the SEM images that are presented in **Figure 15** were analyzed, it was discovered that the dimensions of the chitosan nanoparticles that were prepared with the final formulation parameters, as measured by the scale that is located underneath the figure, were compatible with the particle size results that were obtained using Nano ZS (approximately 200 nm). It is important to note that the diameters of the particles seen by SEM were sometime lower than those seen by Nano ZS, and this was true for both empty nanoparticles and nanoparticles loaded with 5-FU. For the purpose of Nano ZS analysis in particular, particle measurement is typically carried out in an aqueous solution, which encourages the particles to expand. On the other hand, for SEM, particle measurement is carried out under dry conditions, which results in the particles

being smaller. The findings were in line with the article written by A.M. dos Santos et al. (44) for morphological properties of CSNP.

Differential Scanning Calorimetry (DSC) Analysis

DSC was used to determine thermal characteristics and verify encapsulation of 5-FU in prepared CSNP during *in-vitro* characterization investigations of nanoparticles. Examining the DSC thermogram shown in **Figure 16** reveals that the prominent melting peaks found at 225 and 280 for CS and 5-FU, respectively, are located at those corresponding temperatures. When the active components and the polymer were physically mixed together, it resulted in the observation of peaks with similar characteristics. When 5-FU was loaded onto chitosan nanoparticles, DSC analysis did not provide any particular peaks which were in line with an article done by M. Samy et al.(27). This suggests that the 5-FU have been encapsulated into the nanoparticles effectively (45). It was found that the melting point peak that was obtained for 5-FU was consistent with the research that had been done before by S. Tummala et al.(40).

Determination of Encapsulation Efficacy

Following the procedures outlined in "Determination of Encapsulation Efficacy" (Section 2.2.4.), the quantity of 5-FU loaded into chitosan nanoparticles was calculated. Encapsulation efficacy percentage is provided in Section 3.5, titled " Determination of Encapsulation Efficacy " (**Table 10**).

For the prepared formulation %EE was 15.69% which was less than what was anticipated but it was in accordance with the article written by M. Samy et al. (27). Encapsulation percentage of 15.69% was used for all of the *in-vitro* release studies done in this project. EE% for chitosan differs when the concentration and ratios of CS and TPP are different and also when pH of the preparation medium is changed (44).

4.3 *In-Vitro* Release Analysis

The "*In-vitro* Release Analysis" section of Section 2.2.5. describes how the *in-vitro* release tests were carried out. **Figure 17** provides the graph of the % cumulative release as a function of time. When the release profile is investigated, it is discovered that the drug release begins with a "burst effect." It is possible that this is the result of 5-FU molecules being adsorbent to the surface of chitosan nanoparticles (46). In an study conducted by Li Sun et al. (28) the same type of drug release from nanoparticles

were observed. The burst effect continues with sustained release effect for 24 hours and it can maintain the concentration of the 5-FU in release medium and cause longer anticancer effect using loaded nanoparticle rather than free drug. The %cumulative release of around 40% for this study is aligned with a study done by M. Samy et al.(36) and it shows the correlation of release of 5-FU from chitosan nanoparticle in a sustained manner in PBS with pH=7.4 but as its shown in **Figure 17** all of the loaded 5-FU is not released from chitosan and this is shown in a study done by Y. Herdiana et al. (47) shows different release patterns from chitosan nanoparticles in different pH mediums and CS:TPP ratios.

4.4 Cytotoxicity Studies on CT26 Cells

The efficacy of drug-loaded nanoparticles in cell culture was investigated by first establishing the optimal drug concentrations for treatment of colorectal cancer cells in culture and then assessing the cytotoxicity of these nanoparticles *in vitro*. IC_{50} concentrations for 24-, 48- and 72-hours incubation of CT-26 cell line for 5-FU were calculated as 2.91, 4.76 and 0.01 μM respectively. 5-FU is a potent anticancer drug against CT-26 cells and treatment with 5-fluorouracil (5-FU) significantly decreases the viability of CT26 cells *in vitro*, as evidenced by a concomitant decrease in cell proliferation and induction of apoptosis (48).

Due to the aggregation problem of chitosan nanoparticles on cell culture studies, modified MTT method was used in thesis study (49). Blank nanoparticles were also investigated in terms of cytotoxic effects of polymer on CT-26 cells. Results showed that chitosan does not affect cell viability. MTT results showed that, 5-FU loaded CSNPs are effectively released the drug and showed cytotoxic effect as aimed. Although it seems that, 5-FU is more effective than 5-FU loaded CSNPs at lower concentrations (5.47, 10.94, 21.875 μM), this situation might be explain with controlled drug release of nanoparticles, which is fundamental for passive drug targeting and provides high tolerability of treatment by patients.

5. CONCLUSION

One of the most common approaches of treating cancer is chemotherapy. Anticancer medications are largely cytotoxic chemicals, which not only destroy cancer cells but also harm normal cells, hence treatment is not without serious side effects. In an effort to reduce or eliminate these harmful side effects, chemotherapeutic drugs are being tested using different nanoparticular drug delivery systems. In the scope of this thesis study, 5-FU anticancer drug was loaded into chitosan nanoparticles and different aspects of this approach was investigated and the highlights of the results are as below:

- HPLC determination method for 5-FU was developed and validated in order to determine different concentrations of 5-FU according to ICH guidelines.
- Chitosan nanoparticles with desired particle size which enables better cellular uptake, sustained release and also passive targeting were prepared with loaded 5-FU.
- Chitosan nanoparticles were analyzed for zeta potential, and it is shown that zeta potential of particles is positive which causes easier cellular uptake.
- For the produced formulation zeta potential was low (1.01) which means it will have long term stability problems and it needs to be investigated in future studies in order to increase the zeta potential and produce more stable nanoparticles.
- Encapsulation efficacy of the chitosan nanoparticles were low (~17%) but they showed promising effectiveness against colorectal cancer cells (CT-26) which can be promising for future studies regarding better encapsulation and better anticancer efficacy using lower doses of 5-FU.
- *In-vitro* release from chitosan nanoparticles were evaluated and it was shown that encapsulation of 5-FU in nanoparticular systems could increase the release time of the drug and cause less frequent dose administration also causing passive targeting of the drug which results in less side effects and more patient satisfaction.

- *In-vitro* cell cytotoxicity of the nanoparticles were investigated, and it's shown that chitosan nanoparticles are not toxic and do not cause a decrease in cell viability % and because of their biocompatible and biodegradable nature it's safe to use them as drug carrier nano systems.
- For future studies it is recommended to do in-vivo studies to show the effect of 5-FU in a biological environment in order to investigate the passive targeting efficacy and cytotoxicity effect on healthy tissues.

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