Şerife ÇAKIR DEVELOPMENT OF A KİDNEY-TARGETTED NANOCARRIER FORMULATION AGAINST CISPLATIN INDUCED NEPHROTOXICITY AGU 2019

DEVELOPMENT OF A KIDNEY TARGETTED NANOCARRIER FORMULATION AGAINST CISPLATIN INDUCED NEPHROTOXICITY

A THESIS

SUBMITTED TO THE DEPARTMENT OF BIOENGINEERING
AND THE GRADUATE SCHOOL OF ENGINEERING AND SCIENCE
OF ABDULLAH GUL UNIVERSITY

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF
MASTER OF SCIENCE

By

Şerife ÇAKIR

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SCIENTIFIC ETHICS COMPLIANCE

I hereby declare that all information in this document has been obtained in accordance with academic rules and ethical conduct. I also declare that, as required by these rules and conduct, I have fully cited and referenced all materials and results that are not original to this work.

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M.Sc. thesis titled Development of A Kidney-targetted Nanocarrier Formulation Against Cisplatin Induced Nephrotoxicity has been prepared in accordance with the Thesis Writing Guidelines of the Abdullah Gül University, Graduate School of Engineering & Science.

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ACCEPTANCE AND APPROVAL

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ABSTRACT

DEVELOPMENT OF A KIDNEY-TARGETTED NANOCARRIER FORMULATION AGAINST CISPLATIN INDUCED NEPHROTOXICITY

Şerife Çakır

MSc. in Bioengineering

Department Supervisor:

Assist. Prof. Erkin AYDIN

February 2019

Chitosan is a natural polymer which is less toxic in the body than other synthetic polymers. Chitosan sodium tripolyphosphate (TPP) nanoparticles produced by ionic gelation method are known to be good drug delivery agents for human body tissues, such as brain and kidney. In this study, to eliminate nephrotoxicity in kidney caused by cisplatin, an anti-cancer drug, chitosan-TPP nanoparticles loaded with gene silencing siRNAs were used. In vitro studies have been tested in human kidney cell line Hek293 cells and the toxic effects of nanoparticles were found to be low compared to 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide and 2,3-Bis-(2-Methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide, disodium salt cell cytotoxicity results. The penetration of nanoparticles into the cell was confirmed by fluorescence microscopy and flow cytometry. In vivo studies have shown that nanoparticle injected with siRNA loaded into rats of type 6-8 week in the Balb-c type. Control and siRNA-loaded chitosan nanoparticles cisplatin-treated mice group were used for the animal experiment. After cisplatin injections, creatinine and BUN assays were performed to detect the level changing by nanoparticles after cisplatin-induced siRNA delivery. GAPDH is a control gene and PKCδ, P53, OCT1, OCT2 and γGT genes have important roles in kidney proximal tubule cells. In this study, mRNA levels of these genes were also examined by quantitative PCR. While siRNAs were decreased in the first days of injection, this effect was lost in the following days. Thus, the silencing potential of each siRNA is variable. However, this variability shows a significant change in the study.

Keywords: Chitosan, Nanoparticle, Drug delivery, siRNA, Nephrotoxicity

ÖZET

CİSPLATİN TEMELLİ NEFROTOKSİSİTE KARŞITI BÖBREK HEDEFLİ BİR NANOTAŞIYICI FORMÜLASYONU GELİŞTİRİLMESİ

Şerife Çakır
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Yüksek Lisans Tez Yöneticisi:
Dr. Öğr. Üyesi Erkin AYDIN
Şubat 2019

Kitosan doğal bir polimer olup diğer sentetik polimerlere oranla vücutta daha az toksik etki göstermektedir. İyonik jelasyon metodu ile üretilen kitosan sodyum tripolifosfat (TPP) nanopartiküllerin böbrek ve beyin dokusu gibi insan vücut dokuları için iyi bir ilaç salınım araçları olduğu bilinmektedir. Bu çalışmada bir anti-kanser ilacı olan cisplatinin böbreklere oluşturduğu nefrotoksisiteyi gidermek için, gen susturucu siRNA'larla yüklü kitozan-TPP nanoparçacıkları kullanılmıştır. In vitro çalışmalar human kidney cell line olan Hek293 hücrelerinde denenmiş olup nanoparçacıkların hücreye girişleri ise floresan mikroskobu ve flow sitometri ile doğrulanmıştır. MTT ve XTT sonuçlarına göre nanoparçacıkların toksik etkisi düşük bulunmuştur. In vivo çalışmalara bakıldığında ise, balb-c tip 6-8 haftalık farelere siRNA yüklü nanoparçacık enjeksiyonu yapılmıştır. Sisplatin ile muamele edilmiş fareler kontrol ve siRNA-yüklü kitosan nanopartiküller grubu olarak hayvan grupları kullanılmıştır. Sisplatin enjeksiyonlarından sonra, siRNA-nanopartükül verilmesinden sonra farelerdeki kreatinin ve BUN seviyeleri değişimi incelendi. GAPDH bir kontrol geni olup PKCδ, P53, OCT1, OCT2 ve γGT genleri böbrek proximal tübül hücrelerinde önemli rollere sahiptir. Bu çalışmada bu genlerin mRNA seviyelerine de kantitatif PCR ile bakılmıştır. Enjeksiyonun ilk günlerinde siRNA'lar azalmış iken devam eden günlerde bu etki kaybolmuştur. Böylelikle her siRNA'nın susturma potansiyeli değişkenlik göstermektedir. Fakat bu değişkenlik çalışmada anlamlı bir değişim göstermektedir.

Anahtar kelimeler: Kitosan, Nanopartikül, İlaç Taşıma, siRNA, Nefrotoksisite

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To my

husband…

Chapter 1

Introduction

1.1. Nanomaterials

There have been many publications about nanotechnology and nanoscience in recent years and different definitions have been put forward on the term [1]. Nanometer is defined as one millionth of a millimeter and nanomaterials are usually used to define materials having at least one of their three dimensions in the length scale 1 nm to 100 nm [2]. The fast pace of improvements in the scope of nanotechnology and nanoscience, has canalized novelties in various disciplines of science and engineering. One of these disciplines is pharmaceutical science and technology. Employing nanotechnologies can potentially provide many unprecedented resolutions in diagnosis and treatment of many diseases. When examined, nanomaterials can potentially provide unique and unpredicted [3] pharmaceutical benefits by virtue of their optical, electronic, thermal, chemical, biological and mechanical properties, so that they are called -smart nanomaterials-. Today, several classes of nanomaterials including nanoclays, nanofibers, nanowires, quantum dots, quantum wires, carbon nanotubes, metal-based materials, composites, dendrimers, and nanoparticles are commonly produced for many different applications. They can be prepared under in vitro conditions, hence named synthetic nanomaterials or they can occur naturally. While natural bio/nanomaterials include chitin, silk, gelatin, elastin, keratin or proteins, synthetically formed

nanomaterials include polymeric nanoparticles, such as chitosan, polylactic acid (PLA), polyethylene glycol (PEG) and polylactoglycolic acid (PLGA). The materials considered as nanomaterials have a wide range of physical, chemical and biological properties, which provide them unique advantages in various applications. These properties are indeed the main motivation for using them in these respective applications and they vary with changing synthesis parameters including concentration, pH, or temperature. Aside from pharmaceutical science, nanomaterials are also highly evaluated for disciplines such as chemistry, physics, biology, and electronics. Nanomaterials can also be categorised on the basis of their dimensions: (i) zero-dimension includes nanomaterials which are within the range of 1-100 nm. (ii) One-dimension includes nanotubes, nanorods and nanowires which are needles or rod like shaped within the range of 100 nm-10 μm. (iii) Two-dimension includes nanocoatings, nanofilms and nanolayers, which expose plate like shapes and (iv) three-dimension nanomaterials encompass multilayer nano crystalline structures with three random dimensions. They can comprise of bulk powders, nanowire bundles, multi-nanolayers and nanotubes [4-6].

1.2. Biomaterials

Biomaterial phenomenon expresses medical devices which play a pivotal role in damaged tissues or cells, that are compatible with biological systems [7]. Biomaterials are non-viable products which have unique physical, mechanical, biological properties. They are engineered materials via different methods serve various purposes in biological systems. Biomaterials have been used in many arms of bioengineering including tissue engineering, which include fabricating of synthetic skin, tissue scaffold, body implants such as bone, composites for teeth, and sutures and other surgical devices. Desirable biomaterials provide some substantial benefits for organ or tissues in which they are implanted or delivered. Wound healing, repairing, restoring, modifying and regenerating organ and tissues are some of those benefits mentioned. Cancer therapy, immunmodulation, electrical stimulation, biochemical sensors, drug delivery systems, bioscaffolds, biophotonic, molecular bionics, metallic implant materials, tissue regeneration are some of the examples of biomaterial applications [8]. Biomaterials can be produced synthetically or obtained from natural resources with regards to desired

properties and intended purposes. Chitin, elastin, gelatin, collagen etc are some of natural biomaterials while other polymers including PLA, polycaprolactone (PCL) are examples for synthetic biomaterials. Biomaterials science incorporates the knowledge and practices of several different fields, including physics, chemistry, pharmacy, material science, and various engineering, so it is expanding step-by-step and large scale area, has also increasing concern [9-13]. Biocompability is a critical concept in finding out behavior of biomaterials inside the host organism and host's reaction to implants. It is also substantial to enhancing productivity of biomaterials like medical implants. Biocompatible materials in living tissues or systems are not toxic and do not induce undesirable immune reactions. So they are compatible with biological structures [14]. Scaffolds are unique architectures, which have a pivotal role in tissue regeneration and they should display a good biocompatibility to support cells, providing a suitable surface to allow proliferation in line with tissue regeneration [15]. Biofunctionality is another concept in the sense of biomaterials. Once a biomaterial is inserted in the body, it should not be degraded by biological enzymes, defense systems or tissues contacted with. Although it can lose functionality over time, it should also keep its stability chemically and mechanically during the process. This concept is mostly related to metal biomaterials cause of their degradation that could lead to be detrimental effects for tissue. Biodegradability, bioactivity, bioinert, sterilizable are other concepts which are important. Bioactivity is ability of a biomaterial to featly to be interactive in tissue or organ which is targetted [16]. In virtue of their assertive properties like biocompability, biodegradability and bioactivity, biomaterials structurally and functionally fits to both extracellular matrix (supporting matrix for living cells), cell membrane and general tissue. Thus biomaterials are remarkable materials with these properties within expanding tissue engineering area and its top-line applications [17].

Chapter 2

Cancer

2.1. General Mechanism of Cancer

Cancer is the state of an abnormal fast and uncontrolled cell proliferation. In other definitions, it is a diseases that arise from both genetic factors (genes, mutations) and environmental factors, such as lifestyle and pollution. It has a variety of effective distinguishing properties including (i) activating invasion and metastasis by which could cancer expand other part of the tissues and other organs of the body, (ii) escaping host immunity, (iii) resisting cell death, (iv) sustaining proliferative signaling, (v) inducing angiogenesis, enabling vasculature to spread (vi) genome instability and mutation, (vii) deregulation of cellular energetic, (viii) evading growth suppressors, (ix) tumor promoting inflammation, and (x) enabling replicative immortality (Figure 2.1.) [18, 19]. These decisive features are named hallmarks of the disease and help us to clearly understand evolutionary fabric of the cancer, from gene mutations, like loss of function and pathways to invasion of the body, in the transformation of normal cells into malignant cells [20].

Tumor cells are usually divided into two types as benign and malignant. Benign type does not spread. Malignant type causes potential cancer which could spread near tissues and organs in the body. They redeploy homeostasis by using their own control

mechanisms. Many of malfunctioned proteins play a critical role in these molecular control mechanisms via DNA mutations [21, 22]. Cancers are also diverged into some classes depending on their origin of tissue in the body, including carcinoma, lymphoma, leukemia, brain tumor and sarcoma. They can be genetically inherited or somatic and mostly related to mutation of some important genes, such as p53, PTEN, BRCA genes, RB etc, which get a considerable status in cell cycle [23]. As a nuclear transcription factor and tumor suppressor, p53, could both activate and repress the expression of multiple genes. It is also in contact with cellular proteins and enzymes. P53 has a dominant oncogenic competency for the cell cycle [24]. In cellular stresses, such as hypoxia or DNA damage, p53 tends to pile up in nucleus to play a pro-apoptotic role. Thus, activated p53 can protect the cell from damage via arresting cell or stimulating programmed cell death and it is best known as the warder of the genome [25]. It can be considered that p53 can determine the fate of a cell between supporting survival or promoting death through apoptosis.

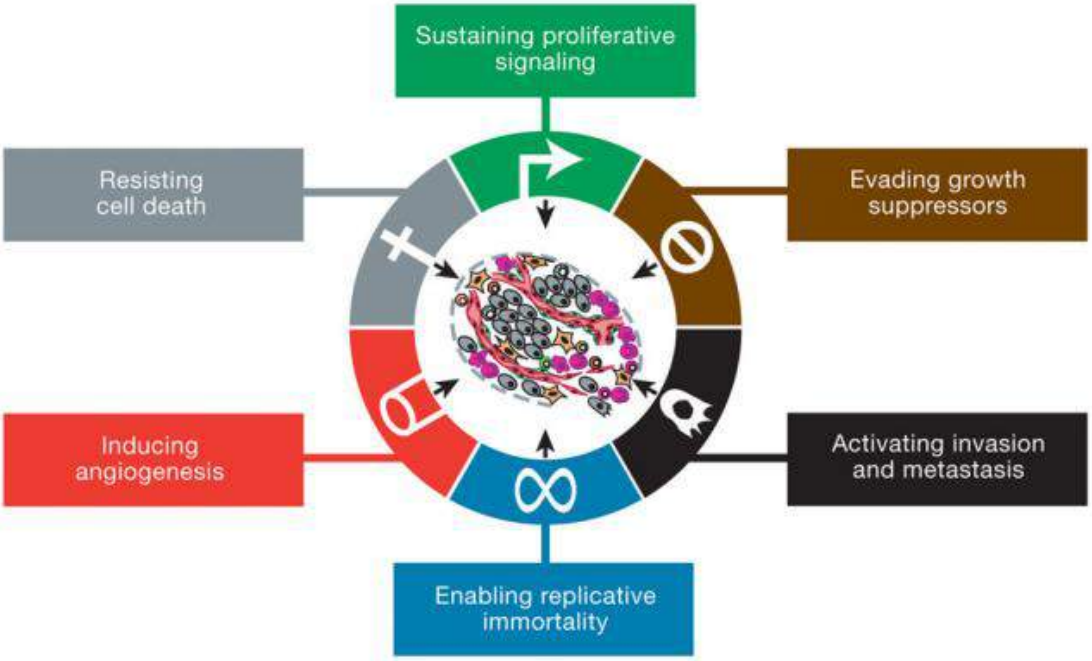

Figure 2.1. Hallmarks of Cancer [19]

2.2. Treatment of Cancer

Cancer treatment strategies vary according to the type of cancer. Chemotherapy, radiation therapy, immunotherapy, surgery, targetted therapy, hormone therapy, stem cell transplantation and precision medicine are considered as the current gold standards of treatment. However, cancer cells could be resistant to therapies. They always make shift and avoid cell death in different ways, besides sometimes dying healthy cells after treatment. They can also alter cell surface proteins, which help them to hide from immune system by using checkpoints of cell cycle, also have genomic instability. Their intracellular pH is usually higher compared to the normal cells, aiding cancer cells in cell migration, cell proliferation and survival, metabolic reprogramming and the other pH dependent processess [26]. Recently, in the context of immune therapy, checkpoint inhibitors are produced to assist T cells. Cyclin-dependent kinases inhibitors are one of them and effective for breast cancer. In addition to that, monoclonal antibodies, adoptive cell transfer, immune system modulators and cancer vaccines are also promising advancements [27]. On the other hand, gene-based therapy could work to inhibit overexpression of responsible genes, causing mutation against cancer. Some drugs like paclitaxel, doxorubicin or cisplatin work on people with cancer via this mechanism [28].

Despite of multifactorious developments in the way of chemotherapy, the effective therapy of cancer disease still resumes unknown. Chemotherapy's major disadvantage is being non-specific. Most chemotherapeutic agents unspecifically target both cancer cells as well as normal healthy cells in the body. The latter often times kill healthy cells of various non-target body sites and causes devastating consequences for the functioning of these tissues and organs.

Various nanocarrier based drug delivery systems have been used to encapsulate therapeutic drugs, plasmids or siRNA as potential drug carriers [29]. Since tumor area has rather irregular vascularity and both high blood fluidity and interstitial pressure in tumor tissue. Lately, various approaches have been proved helpful regarding variance of anti-cancer drugs and their access in tumor area via novel support systems which boost effective therapy. One of these approaches uses Enhanced Permeability and Retention (EPR) Effect which makes possible to accumulate drugs or protein-polymer conjugates, micelles, dendrimers etc. into tumor area via weakness of blood vessels

(vascularisation), enhanced extravasations (excessive permeability at the area), lack of drainage of lymphatic system (retention) in the tumor microenvironment. Over the past decade, EPR effect has been highly evaluated in nanomedicine in order to improve promising anticancer agents and treatment of diseases including cancer. EPR effect has been observed in several cancer types such as breast, lung, skin, and pancreas and ovarian, in animal models through accumulation of nanoparticles at the cancer sites [30-32]. The notion of arranging site-specific anticancer drug delivery is based on targeting. A selective targeting is important to achieve for carrying complex or conjugates with drug of interested, which delivers to target biosites (tumor area) in a specific way. The drugs selected effectively, target a specific site in a controlled way, selectively in certain therapeutic frame, which prevent untowanted effects on normal cells while increasing the therapeutic concentration at intended area. So it is called targetted drug delivery system [33]. Targetted drug delivery aims enhancing bioavailability and drug cumulation at intended target site, preventing drug elimination or degradation by the host immune elements, at avoiding accumulation of the drug at normal healthy cells to avoid toxicity. The drug can be degradable via immune system, toxicity arising from some delivery vehicles and non-controllable residence time in bloodstream, could be counted as disadvantages of drug delivery systems.

A drug-loaded nanoparticle intended for delivering its ingredients to the desired body parts, can be of different sizes. Thus, during targetted and controlled drug delivery with nanoparticle, size is an important parameter to consider. However, it is also important which type of chitosan nanoparticle used is substantial. For instance, in delivery of chitosan nanoparticles in liver for human hepatocellular carcinoma cells, between 68 and 103 nm with mean diameter are used by Kou et al. in 2017 [34]. Microparticles are particles about 1000 nm or higher in diameter and used for targeting lungs due to their accumulation at this tissue [35]. Generally nanoparticles with less than 200 nm accumulate in spleen and liver without being destroyed by macrophages in blood stream [36]. However the so called mesoscale nanoparticles with approximately 400 nm mean diameters tend to localize in renal proximal tubules when they are intravenously injected [37]. This study is also based on size-dependent targetted and controlled siRNA delivery by chitosan nanoparticles on kidney.

2.3. Cisplatin

Cisplatin is a well-established platin-derivative drug that is used in chemoterapy of many cancer types such as breast, bladder, lung, testicular and ovarian cancers. It's molecular weight is 301,1 gm/mol and density is 3.74 g/cm^3 and water solubility is 2.53 g/L [38].

The mechanism of biochemical action of cisplatin has been reported to be mostly taken into the cell by passive diffusion. In recent studies, it has been observed that membrane proteins responsible for copper transport affect the concentration of cisplatin in the cell. Cisplatin is an anti-neoplastic drug that prevents cellular division and development. Cisplatin prevents the proliferation of cancer cells, slowing their development and spreading in the body. Cisplatin is a chemotherapeutic agent that is commonly used in the treatment of many solid tumors. Despite the good response to cisplatin in testicular cancers, therapeutic efficacy is limited due to toxicity or drug resistance in other solid tumors. Super oxide anions and the reactive oxygen derivatives such as hydroxyl radicals by creating the most damage to the kidney [39].

Because cisplatin is excreted from the kidney, it accumulates more in the kidneys than in other organs. The most important of the side effects of cisplatin is nephrotoxicity. With several pathogenic mechanisms, the drugs cause the kidney injury, including well-known toxicity effect [40]. Due to actively accumulation on renal epithelial cells and proximal tubule cells, kidney cells experience a massive cytotoxicity effects after cisplatin treatment. Recently, studies on cisplatin are reported that there are some important membrane transporter proteins acting in cisplatin uptake into the kidney cells [41]. Copper transporter (CTR1), Organic Cation Transporter (OCT2) and multidrug extrusion transporter 1 (MATE1) are transporter proteins which are expressed from membrane cells [42]. These transporters are crucial mediators of cellular uptake of drugs such as cisplatin, and they have important roles in toxicity mechanism of cisplatin.

In this study, five important genes which act in proximal tubule cells, OCT1, OCT2, p53, PKCδ and γGT are targetted via siRNA-loaded chitosan-TPP nanocarriers. Translations of these genes are simultaneously silenced via anti-siRNA molecules. Thus, it is thought that the gene silencing mechanism can reduce cisplatin-based nephrotoxicity in vivo conditions with the study. Knockdown of OCT genes greatly

reduces cellular uptake of cisplatin. In addition, preventing of translation of p53, PKCδ and γGT, which are responsible for the activation of apoptotic mechanisms, will also give time for DNA repair in cells. OCT (also known as SLC22) is an organic cation mediator that helps transport of cations across the cell and is also a member of carrier protein family. The protein provides uptake of cationic drugs in the human kidney cells [43]. Another important protein is known as the guardian of the genome, p53, is a tumor suppressor protein which is responsible in cell cycle check points. When inactivating or mutations in p53 pathway, it causes cancer [44]. PKCδ is a protein which regulates many important cell activities such as cell survival, apoptosis and proliferation. It is shown PKCδ inhibiton ensures to ruin cancer cell proliferation [45]. Γ-glutamyl transpeptidase (γGT) is a cellular transferase that catalysis transfers of functional groups of glutathione. The enzyme is widely located in many of tissues and organs of the body, such as kidney, pancreas and liver. There are a few reports that the enzyme is related renal cancers [46]. The enzyme is key enzyme which is expressed lower level in kidney of people with renal cancer.

Chapter 3

3. Nanoparticles

The nanoparticle as a delivery system vehicle is an ever-growing research area. They have enormous potentials in diagnosis as well as therapy. Nanoparticles can be designed

to carry various ingredients including small molecules, therapeutic proteins, signal molecules, genes, and RNA interference agents such as siRNA. A nanoscale drug carrier, offers a lot of opportunities such as altering their blood circulation periods, regulating biodistribution by targeting, protecting the enclosed ingredients they carry, and stimulating the efficacy of therapy [47]. Nanomaterial devices could imitate or change biological procedures like tissue scaffold structure. Besides the enormous diversity of structures and unique properties, they can classified as nanofibers, nanocapsules, nanomembranes, nanochips, nanobots, nanomachines, nanotubes etc, as some of the examples of nanoscale devices for drug delivery [48].

Polymers have attracted the attention of scientists over the past few decades because of their applications due to their biodegradable structure, effectively controlled and site-specific delivery and release drugs, power of tissue engineering. Nanoparticles are macromolecular nanomaterials which can have a colloidal or firm structure with a, generally spherical geometry and they diversify in range from 1 to 1000 nm. However structures with sizes less than 200 nm have been preferred as a drug delivery device in most nanomedicine applications. A drug could be entrapped, encapsulated, dissolved by, or attached to a nanoparticle depending on the preparation method. So they have different features to achieve effective encapsulation for the best drug route. Nanoparticles are considered as a safer means of carrying of anticancer drugs than their conventional systemic free form application (Figure 3.1.) [49]. Polymeric nanoparticles are a class of nanocarriers and have a polymeric backbone which generally forms a biodegradable and biocompatible monomer, becoming special for targeting. Polymers are very favorable substances to produce nanoparticles [50]. Due to their different chemical groups which they have, they are non-cytotoxic, they have biodegradable structures and they show low immunogenic responses [51]. Polymeric nanoparticles have ester and amide bonds which enable to hydrolyze in vivo, and hydrophilic and hydrophobic regions which also enable to drug attachment to nanoparticle surface. The polymers including poly alkylcyanoacrylate, polymethylidene malonate, poly-L-lactide (PLA) and polyethylene glycol (PEG) are greatly investigated synthetic and biodegradable polymers. Also some of polymers, such as chitosan, chitin, and dextran are obtained from natural and biological sources. As well as copolymers which have different properties and that form via combination various polymers, like PLA-block-PEG copolymers, they demonstrate less toxicity and more biocompatible than uniform

polymeric structure [52]. Copolymers are also the best options for less easily desorption from the cell surface, that the situation is substantive. Polymers are covalently bound to another in this state. Polyethylenimine and PEG are widely used polymers for grafting and blocking with chitosan in different applications [53]. Due to its polycationic nature, chitosan could be modified easily. The conjugation of several polymers or functional groups, provide suitable features such as optical, electrical, magnetic or physiochemical. However, there are some shortcomings from used methods, so techniques are not employed totally effective. Moreover, copolymerization relies on monomers of polymer structure; it affects singular reactivity of each monomer [54].

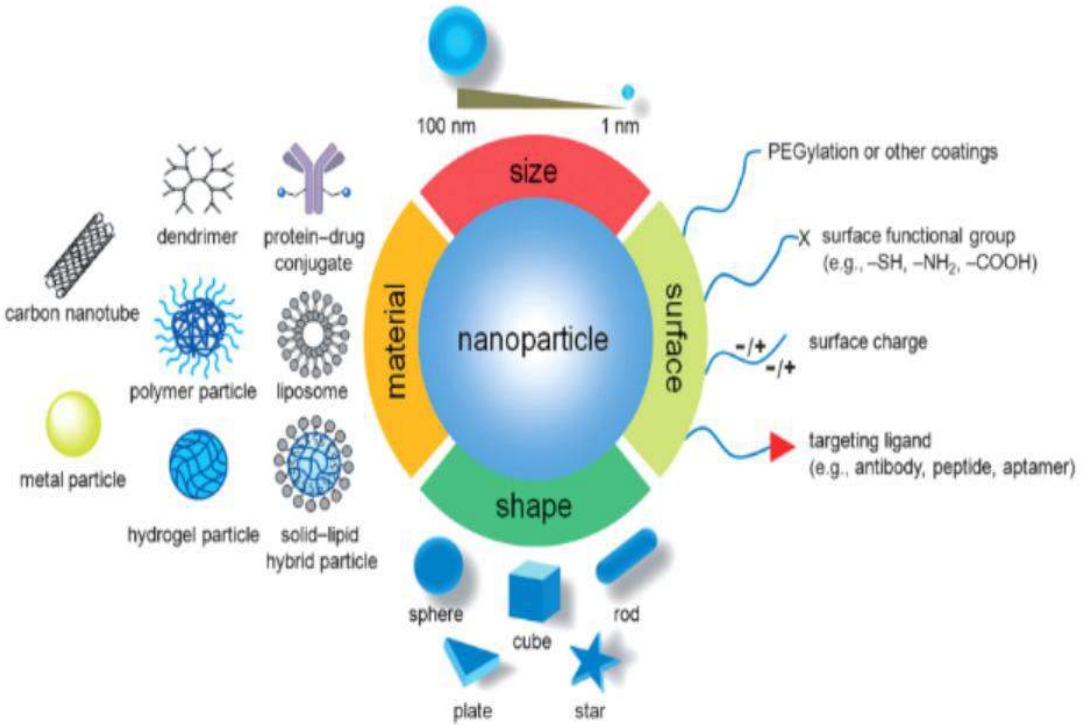

Figure 3.1. Nanoparticle has been explored as carriers for drug delivery in cancer therapy [55].

Chapter 4

Chitin and Chitosan Polymers

4.1. Chitin as a natural polymer

Chitin $(C_8H_{13}O_5N)_n$ or poly (β-(1→4)-N-acetyl-D-glucosamine) is a long chain natural homopolymer (Figure 4.1.), also an ample polysaccharide which is produced in enormous quantities in nature by crustaceans and fungi. Therefore, it is widely available for many uses. Chitin is a derivative of glucose. Besides component of exoskeleton of insects and crustaceans, it is also a factor of cell wall in living organisms, such as fungi [56]. It displays several desirable capabilities such as fortification, durability, dietary effect on regulation of plasma cholesterol levels as a food supplement in chitosan form, antibacterial and antioxidant activity, anti-inflammatory and anticancer effect, and food preservation [57]. Chitosan is a chitin derivative, which is obtained by deacetylation of chitin under alkaline conditions. Depending on the application chitosan may have varying degrees of deacetylation, up to 100% or less [58]. Chemical structure determines biological properties of chitosan biopolymer. Although chitin has low solubility and chemically no reactivity as a biopolymer in water, chitosan and its derivatives are soluble, that is the important limiting driver for application in biological systems owing to biocompatibility and nontoxic nature.

Chitin and chitosan have also potential as biomaterials for use in tissue engineering. They have been successfully implemented in tissue engineering of nerves and blood

vessels via their generation into tubular forms. For instance, chitin-based scaffolds, resulting preparation methods, are all around outputs which could be optimized for various novel objectives. Chitin-based nanomaterials have suitable pore size distribution, biodegradability, structural collectivity, non toxic property, biocompability, adhesion property and capable of contact with cells and biological processess such as migration and proliferation [59]. On the other hand, wound healing, cancer diagnosis, wound which made of chitosan-based materials, antiaging cosmetics and vaccine adjuvant are the other applications of these polysaccharides in biomedicine research through their unique biochemical properties. Recently, chitosan has been in focus of investigators for its many nanotechnology applications including nanoparticles, nanovesicles, nanocomposites and nanofilms [60].

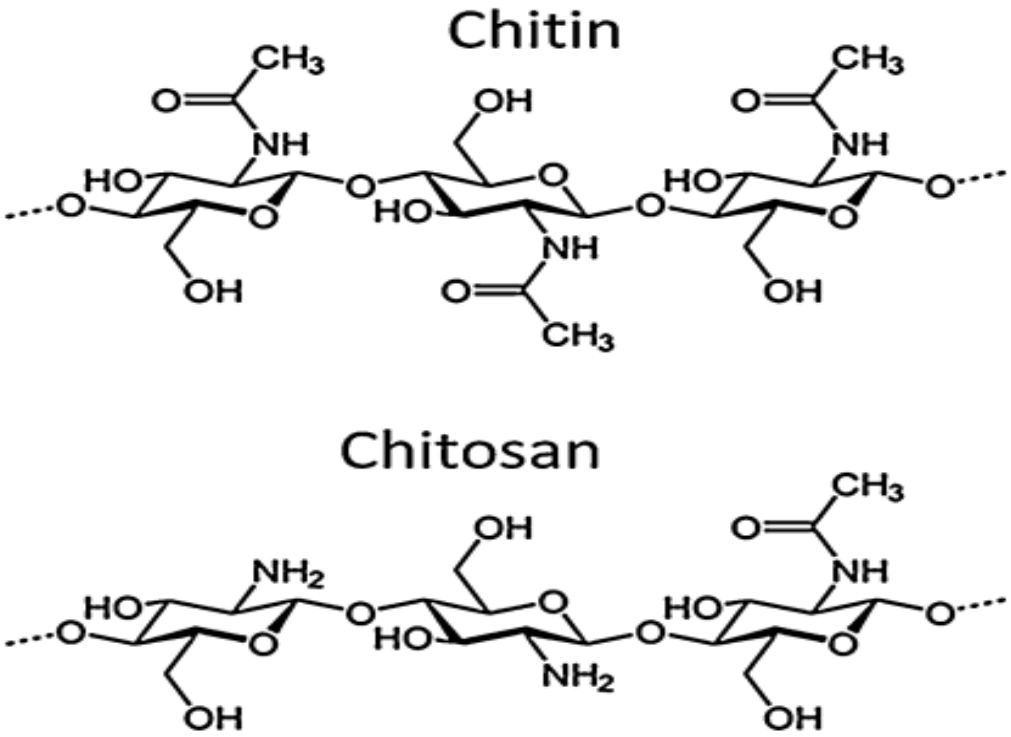

Figure 4.1. Structure of chitin and chitosan [61].

4.2. Chitosan nanoparticles

Chitosan is a viable option for drug delivery applications due to its non-toxic, low allergenic and immunostimulating properties and biodegradability. Its versatile

applications mostly depend on its several drug formulations like matrix, tablet, micro- or nanoparticles [62]. Recently, chitosan has been used in many applications as a viable biomedical product. They can be listed as synthetic skin and nerves to treat burn damages, artificial heart, tendon and bone etc. Chitosan has polycationic amino groups in its linear polysaccharide structure. Chitosan also remedies some disadvantages of chitin polymer, due to its complex structure, challenge in extraction and limited solubility. Thus, it is relatively the best drug carrier, scaffold in tissue engineering and wound healing material along with its unique properties [63]. Chitosan, by its polycationic chemistry provides cellular delivery of drugs, genes, proteins, peptides or other negatively charged nucleic acids such as siRNA and DNA into various cells, which drive stable blocks and complexes [64]. Furthermore, chitosan nanoparticle expedites cargos and saves their integrity during delivery and also allows controlled targetted delivery of their cargoes. Positively charged chitosan molecule is used to form a nanoparticle structure by various methods. Several techniques can be employed for preparation of chitosan nanoparticles, such as ionic gelation and self-assembly, microemulsion or ultrafine milling. Ionic gelation is the most frequently used among them. In this method, sodium tripolyphosphate (TPP), an agent, that has negatively charged polyanions, complexes with chitosan. In the process, TPP is added dropwise on the chitosan solution at various proportions under continuous stirring at room temperature [65]. Thus, chitosan-TPP nanoparticles are generated by ionic cross linking process to yield complexes with different diameters depending on process conditions [66]. The chitosan-TPP nanoparticles have spherical shape that could make possible physically and chemically entrap and encapsulate proteins, antibodies, inhibitor or activators to act in different biological pathways. The formation of chitosan-TPP nanoparticles is influenced by various parameters. At the foremost deacetylation degree of chitosan, TPP and chitosan ratios, solvent concentrations, pH, reaction time, temperature, stirring speed, chitosan molecular weight and ionic strength are the topline factors which affect the resulting particle properties like size and zeta potential [67].

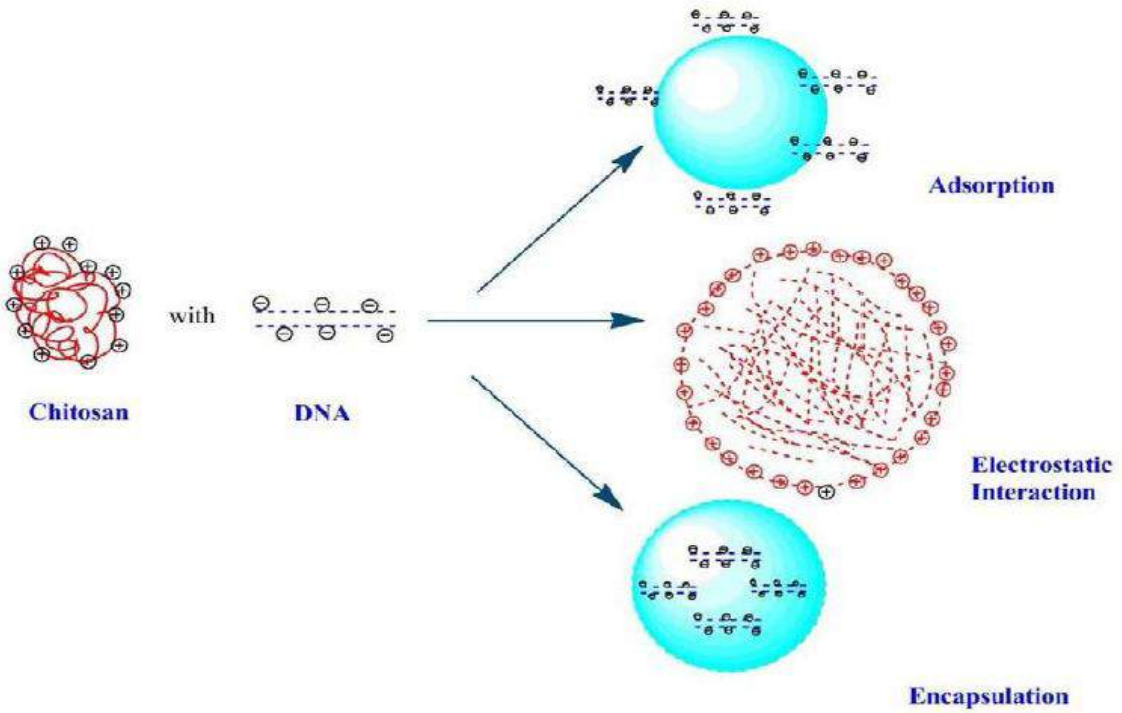

Figure 4.2.. Synthesis of chitosan-based DNA/siRNA nanoparticles using different methods [68].

4.3. Properties of Chitosan Nanoparticles

Chitosan is an eco friendly biopolymer in that it has solubility in several mildly acidic solvents, such as acetic acid and formic acid. Therefore, use of any organic solvents is not required for nanoparticle synthesis. It has free amino groups in polymeric structure, so it displays positively charge. Under mechanical stirring, chitosan solution interacts with polyanions like tripolyphosphate or anionic surfactants like SDS and complexes with them to produce self assembled nanoparticles [69]. Chitosan nanoparticles are used in several pharmaceutical applications and biomedical purposes, such as drug delivery and release, gene therapy, tissue engineering, weight loss. Their properties can be chemically developed by boosting their solubility in water through modifying chitosan-based nanoparticles. Although chitin and chitosan display totally the same chemical molecular structure, degree of acetylation feature has the crucial factor between two polymers residues. The value represents a ratio of glucosamine and *N*-acetyl glucosamine bonds. Thus, it is known that their solubility in solution could change [70].

Considering that physicochemical properties, molecular weight is another trait which like the degree of deacetylation, can affect the solubility of chitosan polymers. It changes *N*-acetylglucosamine in chitosan structure that is crucial for inter-and intermolecular effect. When chitosan solubility ascends according to acetylation of residues, it could be resulted a fewer yield. The physiochemical features of chitosan nanoparticles can be determined by Fourier transform infrared (FTIR) spectroscopy, Scanning electron microscope (SEM), X-Ray Diffraction (XRD), Dynamic light scattering (DLS), Transmission electron microscopy (TEM) etc. Mean particle size and size distribution, surface properties, polydispersity index (PDI) and zeta potential as behavior in certain pH solution, are important parameters in determining chemical, physical and biological nature of chitosan nanoparticle. These parameters are also affected by temperature of reaction under mild conditions, concentration of chitosan and ionic agent ratio, magnetic stirring and incubating time in room temperature, controlled ultrasonication, purification and centrifuge influence structurally chitosan stability [71].

4.3.1. Effect of Ultrasonic Agitation

The stability of chitosan nanoparticles in solutions are substantially based on preparation methods of these particles. Ultrasonic agitation is one method which provides effective ultrafine spherical chitosan nanoparticles to prevent agglomeration and allows favorable sized particles. After ionic gelation technique, particles are dispersed in a media and then mean particle size is adjusted under controlled ultrasonic waves using an ultrasonic bat h ultrasonic homogenizer. In this method, amplitude (%) and application duration are crucial parameters for determining the resulting particle size which is of prime.

4.3.2. Effect of Magnetic Stirring

In mild acid solutions such as that of acetic acid, chitosan has a high solubility. Chitosan and TPP chemically crosslink each other, by binding ammonium groups of chitosan and anionic bonds from TPP [72]. Mechanical stirring is an effective way of preparation of chitosan nanoparticles by crosslinking. Magnetic stirring allows dissolution of chitosan in media smoothly. Especially, drug-chitosan conjugates require mechanical treatment

like stirring. Duration of magnetic stirring should be determined according to chitosan type thereby morphology, solubilisation, molecular weight and degree of deacetylation (DD) % [73].

4.4. Methods for preparation of Chitosan nanoparticles

4.4.1. Ionic Gelation Method

Ionic or ionotropic gelation is a moderately simple complexation technique for preparing chitosan nanoparticles. The method utilizes crosslinking between chemical groups of chitosan and ionic agent form the nanoparticles. It is important to determine several reaction conditions like mixing duration and concentrations of agents to be mixed. These factors affect the size of final particles, surface charge of them and size distribution of nanoparticles. In the method, there is no requirement for use of any organic solvent enabling avoiding from degradation of residues before the delivery of cargo into organism [74]. However, the parameters effecting nanoparticles should be evaluated in an effort to optimize the method, which alters successful formation of nanoparticles. The assembled nanoparticles also may tend to aggregate in solution. In due course of stirring, mechanical energy generates from reaction, this could be highly possible increasing electrostatic repulsion energy. Finally positive surface charged molecules are induced to remove each other, and then nanoparticle acumulation occurs. This kind of aggregation cause solution nondispersive, particle distribution is irregular and not monodisperse. At this point, factors such as stirring speed and duration are really important when process implements. It will affect the performance of controlled preparation of chitosan-TPP nanoparticle and reaction yield. Chitosan nanoparticles may have desirable size in literature range from 50 nm to 1000 nm [75].

4.4.2. Microemulsion Method

The microemulsion method or covalent cross-linking method provides creating nanoparticles by a constricted distribution of particle size in enormous amounts of solvents. Microemulsions are isotropic, transparent, clear, thermodynamically fixed

solutions of oil, water and a surfactant or cosurfactant as a stabilizer under simple mild conditions. The complex is natively mixed of hydrocarbons and olefins. They have generally spherical driblets which are made of separation between two phase, diffused and lasting phases. The driblets could be ranged from 1 to 100 nm or 10 to 50 nm which are composed by a reverse micellar process and have three solubilisation regions: the apolar continuum, the micellar interface and the intramicellar water pool. Since therefore, due to their physicochemical disposition, microemulsion can maintain an environment by itself that harbors both hydrophilic and lyophilic complexes conformably. The microemulsion system has recently shown to attract a lot of interest [76].

4.4.3. Self-Assembling Method

In this method, novel chemical groups are needed to self association of nanoparticles despite simple building. The technique is used easily and to get suitable broad-scale. In an aqueous media, self-associating nanoparticles occur amphiphilic co-polymer which has supramolecular core-shell buildings. The structure has solubility and disperses in liquid media. The structure also provides entrapment of drugs due to its hydrophobic kernel. Self-assembling nanoparticles have high stability, and their surface properties can be adjusted in order to define new properties such as mucoadhesive features and penetration abilities. With their amphiphilic bioadhesive copolymer nature, they could penetrate or permeate that enable influential interaction with parts of tissues, such as corneal sites of eye [77, 78]. Nanopigments, nanoclusters, nanofilms and nanoparticles could be produced by the technique [79].

4.4.4. Ultrafine Milling/Grinding Method

It is an influential technique to form a nanopowder by using a ball-milling. There are a few reports on chitosan nanopowders producing which is used by the method. In this method, chitosan powders are used as the starting material. It may provide production of large nanoparticles [80]. In comparison to other methods for preparation of chitosan nanoparticles, such as ionic gelation and microemulsion techniques, ultrafine milling allows hypolipidemic applications with large concentrations [81].

4.4.5. Emulsification Solvent Diffusion/Evaporation Method

In this technique, organic solvent has a fractional miscibility. The emulsion is attained by adding organic state into mixture of chitosan-stabilizing agent while stirring and then high-pressure homogenization is applied. The method can include hard processing circumstances. Emulsion evaporation is widely used for encapsulation of drug inside water-insoluble polymer. Before encapsulation, an emulsion is prepared, following evaporation of the solvent so as to produce nano- or microspheres. Many drugs confirmed by the FDA, are prepared by these method [82].

4.4.6. Complex Coacervation Method

The method comprises two happened existing phases. The former is intense coacervate phase that has abounding ingredients in colloids. The latter is supernatant phase that is diluted and has fewer ingredients in colloids. Thus, the technique is separated into both easy and complex coacervation. Compared to other microencapsulation processes, when coacervation method is used under moderate conditions, eventually favorable latent occurs regarding microencapsulation without working on tough conditions.

4.4.7. Polyelectrolyte complex (PEC)/ self-assembly polyelectrolyte

A complex is occured by self-assembly about positively charged polymer and polyanionic polymer. Chitosan nanoparticles can be formed by polyelectrolyte complexation. In aqueous solution, reaction spontaneously forms, so there is usually no need for any initiator or toxic agents. Due to positively charged chitosan, chitosan can create polyelectrolyte complexes with negatively charged polyanions. For instance, DNA, hyaluronate and dextran sulphate are polyanions and prepared for chitosan nanoparticle formulation with easy and moderate self-assembly polyelectrolyte method. Another example of the method is chitosan/dextran sulphate encapsulating amphotericin B as a drug [83].

4.4.8. Coprecipitation Method

The method suits chitosan molecules which serve as a drug transporter for controlled and prolonged releasing and drug delivery. In technique, through dehydrating the solvent, grafting *D, L*-lactic acid is used for preparation of chitosan nanoparticles. The chitosan nanoparticles have particles which are uniform and monodisperse. Approximately 10 nm diameter of particles is occured. Recently, albumin encapsulation with a great efficacy has been possible via coprecipitation method [84].

Chapter 5

5. Material/Methods

5.1. Materials

5.1.1. Chitosan Nanoparticle Preparation

High purity (HP) of Chitosan ($C_{18}H_{35}N_3O_{13}$, Poly(D-glucosamine), deacetylated chitin , white mushroom, HP grade, mol wt Mv 60,000-120,000 Da, Degree of acetylation (DD) ≤ 40 mol %, 740063-50g) and tripolyphosphate (TPP, 72061-100g, p.a., ≥ 98.0 % (T))

and Sigma Aldrich Company. Glacial Acetic Acid (100%, CH_3COOH, Mw 60.05 g/mol) is purchased from Merck from Germany. Experimental study is done in the Bioengineering Department of Abdullah Gul University, Kayseri, Turkey. 10 ml injector (Genject, single use syringe, sterile, non-toxic, pyrogens free), 0.20 µm syringe filter (GVS life sciences, sterile), 50 ml centrifuge tubes (PP, DNase, RNase, human DNA and pyrogens, non-cytotoxic, graduated, screw cap, sterilized, IsoLab) are used in experiment. All chemicals are analytical grade. Cryovials are used for preparation of chitosan nanoparticles on magnetic stirrer (Selecta multimatic-9N and Heidolph). Ultrasonic bath (Bandelin, Sonorex) is used for the stabilizing size of nanoparticles. The solutions are mixed by vortex (Velp scientifica) and autoclaved sterile tips are then dried inside Memmert Drying Owen). Acid solutions are prepared inside Delta fume hood.

5.1.2. PEGylation of Chitosan Nanoparticles

Polyethyleneglycol (PEG) 2000 (Succinyl, 17928, Sigma-5G) was used for PEGylation process of chitosan nanoparticles. Glacial Acetic acid, NHS and EDC (purum≥98, 0, Sigma, 03450-1G) were used for the process. Lyophilisator (LabConco) was used to transform PEGylated chitosan into powder form. 20 ml capped glass bottle was used for PEGylation. Snake skin dialysis tubing (3500 MWCO, Thermo scientific, 68035) was used for the process.

5.1.3. FITC-Conjugation of Chitosan

Fluorescein sodium salt (F6377-100G, Sigma Aldrich), High purity chitosan (Sigma, Mv 60,000-120,000 Da, Degree of acetylation ≤ 40 mol %, 740063-50g) and 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC, Sigma, 03450-1G), Snake skin dialysis tubing (3500 MWCO, Thermo scientific, 68035) were used for the process.

5.1.4. Cell Culture

HEK 293 cells adherent kidney cell lines (ACCT, CRL-1573) was kindly provided by Asst. Prof. Ahmet EKEN, Department of Medical Biology, Erciyes University, Kayseri,

Turkey. Phosphate buffered saline (PBS) was prepared with tablets Sigma (P4417-100 tab) using ultrapure water and autoclaved (Nüve, steam sterilizer) and stored at +4◦C until use. All cell culture experiments were performed in laminar flow. The cells were monitored by Leica (ZEISS Primo Vert) inverted microscope within inclose well plates (6 or 96 well) or 75 cm^2 flasks. Cells were cultured in 6 well plates and were incubated at 37◦C in a humidified 5 % CO_2 atmosphere (Panasonic, MCO-170MUV-PA). TrypLE Express (Gibco) was used for subculture. Fetal Bovine Serum (FBS) (Biological Industries) is stored at -20◦C. L-glutamine (Gibco) was an irresolute essential amino acid required in cell culture. Sodium pyruvate (Gibco, 100 mM) is usually added to cell culture media as a carbon source in addition to glucose.

5.1.5. Characterization of Chitosan Nanoparticles

Size, size distribution and PDI (polydispersity index) values were validated by dynamic light scattering (DLS, Malvern, zeta sizer) with disposable cuvette. Graphs were created by OriginProLab programs. PEGylated chitosan was confirmed by Fourier-transform infrared spectroscopy (FTIR, Nicolet6700, and Thermo Scientific).

5.2. Methods

5.2.1. Preparation of Chitosan-TPP-siRNA Nanoparticles

5.2.1.1. Preparation of gPEG-chitosan polymer

Obtaining PEG grafted chitosan polymer is based upon study of Prego et al [85]. 80 mg High Purity chitosan is dissolved in %0.175 acetic acid solution. Then 14,2 mg O-Methyl-O'-succinylpolethylene glycol 2000 (mPEG) (Sigma, 17928-5G-F) and 1.6 mg N-Hydroxysuccinimide (NHS) (130672-25G, Sigma) are added inside the mixture on a magnetic stirrer. When the solution stirring, 21,7 mg N-3-Dimethylaminopropyl-N′-ethylcarbodiimide hydrochloride) (EDC) (03450-1G, Sigma) is slowly drop-by-drop added in the mixture on magnetic stirring. The mixture is stirred 22 hours at room

temperature. After the process, the mixture is dialyzed with distilled and autoclaved water and freeze-dried with lyophilisator. The powder is stored at +4◦C for further analysis and characterisation [86]. At the storage process, PEG-chitosan powder is in a petri dish and covered parafilm and aluminum folio.

5.2.2. Preparation of siRNA-loaded Chitosan-TPP Nanoparticles

5.2.2.1. Ionic Gelation Method

The objective of this step is to synthesize through self-assembly chitosan nanoparticles in presence of chitosan and the polyanionic agent TPP [87] [88]. In this context, polymer and TPP concentration are the major parameters affecting the properties of resulting nanoparticles [89]. A 3 mg/ml chitosan solution was prepared by weighting 30 mg HP chitosan and dissolving in 10 ml 0,175 % acetic acid in a 50 ml centrifuge tube. [90], by occasionally mixing on magnetic stirrer.

TPP is prepared 1 mg/ml by dissolving 1mg into autoclaved and distilled water. While separately dissolving both TPP and Chitosan solutions, sterile autoclaved 2 ml eppendorf (IsoLab) is taken and 2 ml chitosan solution is put inside for centrifugation 10.000, 12.000 and 13.000 g 15◦C and 10, 15 and 20 minutes. 300 µl chitosan solutions is taken from supernatant and put inside a cryovial on magnetic stirrer. The spherical chitosan-TPP nanoparticles are obtained by mixing 120 µl TPP on 300 µl chitosan solutions dropwise while 800 rpm mechanical stirring for 30 minutes for gelation and then overnight. Time is the crucial parameter here to obtain uniform chitosan-TPP nanoparticles [91, 92]. Final chitosan: TPP ratio was 300:120 ratios (2,5:1) in the nanoparticles synthesis solution [93-95].

5.2.2.2. Adding siRNA-TPP in PEG-Chitosan Solution

A siRNA mixture is prepared with siRNAs, OCT, p53, PKCδ and γGT. They are highly expressed in cancer cells. OCT (also known as SLC22) is an organic cation mediator that helps transport of cations across the cell and is also a member of carrier protein family. The protein provides uptake of cationic drugs in the human kidney cells [43]. Another important protein is known as the guardian of the genome, p53, is a tumor

suppressor protein which is responsible in cell cycle check points. When inactivating or mutations in p53 pathway, it causes cancer [44]. PKCδ is a protein which regulates many important cell activities such as cell survival, apoptosis and proliferation. It is shown PKCδ inhibiton ensures to ruin cancer cell proliferation [45]. Γ-glutamyl transpeptidase (γGT) is a cellular transferase that catalysis transfers of functional groups of glutathione. The enzyme is widely located in many of tissues and organs of the body, such as kidney, pancreas and liver. There are a few reports that the enzyme is related renal cancers [46]. The enzyme is key enzyme which is expressed lower level in kidney of people with renal cancer. In this process, firstly siRNA mixture is added in TPP solution with 3 µl volumes in 420 µl chitosan: TPP mixture. P53, PKCδ, γGT and OCT1/2 are mixed with equal volumes as 3 µl in TPP when stirring chitosan continuously.

5.2.3. Ultrasonication of Chitosan Nanoparticles

After ionic gelation method, cross-linked chitosan-TPP nanoparticles are treated by ultrasonic bath (Bandelin Sonorex) 10 minutes without heat. To avoid excessive heating an ice packs in ultrasonication bath. Timing of sonication is substantial factor determining size, size distribution and PDI values of created chitosan-TPP nanoparticles. Even though sonication can relatively decrease size and PDI, exceed the time also affects an agglomeration/aggregation in media [71, 96]. On the other hand, cryovials are placed in order to shake or topple in water, and caps of them are wrapped by parafilm to prevent any water or solution leakage. While working the ultrasonic bath, cover of the machine, could be closed to decrease discomfort noise. While sonication process, unavoidably there is generating of bubbles in solution. When the characterization is being done, the laser light from zeta sizer will not properly read the particles because of bubbles. Thus, definitely incubate would be done after every sonication for removing undesirable bubbles in room temperature or preferably at +4◦C. After size measurement besides sonication process should be incubated at room temperature or +4◦C for stabilizing of chitosan nanoparticles.

5.2.4. Synthesis of FITC-Conjugated Chitosan

A fluorescein dye, fluorescein isothiocyanate (FITC), is widely used for labeling and tracking of nanoparticles in cells. Here, the dye-conjugated chitosan powder preparation is explained in detail. 25 mg high purity chitosan is dissolved in 25 ml of %0,175 acetic acid solution and pH is adjusted 6 with 1M NaOH. To this solution, 10 mg/ml of FITC in ethanol and 21 mg of EDC are added. The mixture is stirred at room temperature for 12 hours. After 12 hours, the mixture is dialyzed for 2 days and get powder polymer from lyophilisator(freeze dried). Finally, a FITC-conjugated chitosan powder is obtained. It is noticed that the powder is highly affected by light. After freeze-drying, petri dish should be covered aluminum folio and stored at +4◦C for further analysis.

5.2.5. Hek293 Cell Culture

5.2.5.1. Cell Thawing

Firstly, before and after working inside a safety cabinet, the cabinet should be cleaned with %70 ethanol. Whole equipment, which is used inside the cabinet, is also properly cleaned with alcohol, then materials can be placed inside. Moreover, if it is needed, before the working, ultraviolet (UV) is activated for 15 minutes. It absolutely should be activated after work for sterilizing. When activating UV, laboratory should be empty. While placing materials into cabinet, they should be closed and alcoholed. Whole tips should be filtered and closed. Any material that has been opened outside is not placed in the cell cabinet.

Dulbecco Modified Eagle Medium (DMEM, 500 ml) is not a completed media and contains sodium pyruvate. Fetal Bovin Serum(FBS), Penicilin/Streptomycin and L-Glutamine are taken out -20◦C dissolved with necessary amounts. Preferentially, these important nutrients can be aliquoted into 15 or 50 ml conicals as less than stock solutions. 50 ml FBS, 6 ml L-Glutamine and 6 ml Penicilin/Streptomycin are added respectively in 500 ml DMEM. The media is called completed media. The media is filtered by 0,22 μm syringe filter and stored at +4◦C for next step cell culture. PBS solution is prepared one day ago and autoclaved for sterilization.

The cells, Hek293, are adherent cell line. They are stored in -80◦C inside a cryovial. A

cryovial is dissolved quickly at room temperature. Then, it is alcoholed and placed inside a cabinet. 5 ml media (FBS and nutrients free) is set in a 15 ml conical and cells that are dissolved are added inside media. The mixture is pipetting well and centrifuged 300 g (1637 rpm) 7 minutes. After discarding supernatant, the pellet is dissolved with 1 ml completed media. 15 ml completed media is measured with the help of a 25 ml serological pipette into 75 cm^2 flask. It should be noted that thawing date, cell name, subculture number and person who cultured cells are written on flask before put incubator. The flask is incubated then 37◦C %5 CO_2 and should be checked everyday for cell growth. Before the culturing, the completed media should be taken out +4◦C and should be warm in 37◦C set a water bath.

5.2.5.2. Subculture of Hek293 Cells

The flask is taken from the out incubator. When the cells reach high intensity as %90-100 in flask, cells create a misty layer bottom of flask. This layer can be apparent with a naked eye. The visual observation is also an evident for cell growth. Although the cells create this misty layer, it should be confirmed also with an inverted microscope.
The flask is up righted inside cabinet. The media is removed with serological pipette without touching the attached cells on flask bottom. 20 ml PBS are added on cells without touching the layer, after 1 minute, slowly removed from flask. FBS in completed DMEM inhibits the activity of trypsin. Washing with PBS, is necessary for preventing this unwanted situation. With a new serological pipette, 3 ml trypsin is added on cells and stored 3 minutes inside incubator for activity of the enzyme. After the step, whether cells separate from flask bottom or not, it is confirmed with a microscope. On cells, it is added 20 ml FBS-free DMEM, and centrifuged 300 g 7 minutes with a conical. The same process in thawing cells, supernatant is discarded and the cells are dissolved with 1 ml media and cultured in a flask. After labeling, the flask is stored CO_2 incubator.

5.2.5.3. Cell Freezing

With the same procedure in cell thawing and subculturing, after obtaining a cell pellet, the pellet is dissolved in 950 µl DMEM (FBS free or completed) and then added 50 µl

dimethyl sulfoxide (DMSO, a freezing agent) in a cryovial. Almost immediately, the cryovial is placed in a frosty box which contains isopropanol. After labeling, the box is stored at -80◦C.

5.2.5.4. Cytotoxicity Assays

Cytotoxicity studies indicate whether a substance has a cytotoxic potential for a cell. Due to adaptation to animal experiments and ease of application, cell based cytotoxicity studies has become frequently preferred alternatively to animal experiments in laboratories. Cytotoxicity, depending on the dose of the substance examined and the duration of exposure, is a phenomenon that damages the cells varying degrees. Cells may die as a result of events, such as apoptosis, autophagy and necrosis when expose to the cytotoxic agent, or they may lose their proliferation properties [97]. After cytotoxicity processes, cell viability degree and amount of cytotoxic substance are determined. Colorimetric, luminescence and enzymatic methods are cytotoxicity assays. In colorimetric methods, it is used tetrazolium salts, such as (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) (MTT), and 2,3-bis-(2-metoksi-4-nitro-5-sülfofenil)-2H-tetrazolyum-5-karboksianilid (XTT). The methods are based on measurements with colour changing or specifically cell staining using dyes like crystal violet or neutral red.

5.2.6. XTT Assay

For this assay, Biological Industries XTT (20-300) Assay is taken out -20◦C and immediately, it is dissolved sterile XTT activating reagent and XTT solution. It should be avoided re-freezing these chemicals. The test procedure contains cultivation of cells in a 96 well plate, adding XTT reagent and incubation for 2-24 hours. During incubation an orange color is formed and intensity of the color change can be measured with a spectrophotometer or ELISA reader. The aim of the assay is measurement and quantify of mitochondria enzyme activity of intense cells in each well, with higher concentration of dye.

The cells are cultivated in a 96 well plate. Each well contains 100 µl DMEM and plate is incubated 37◦C %5 CO_2. In plate it should be present positive background that

contains just cells and media. After defrost XTT chemicals and pipetting, 0.1 ml activation solution and 5 ml XTT reagent are mixed in an eppendorf. Then 50 µl XTT mixture reaction solutions is added for each well and incubated for 24 hours. Before incubation plate is shaked gently for mixing dye and media equally. After 24 hours absorbance of samples is measured at 450-455 nm. In this assay, it is measured 4 hours, 24 hours and 48 hours for confirmation.

5.2.7. MTT Assay

Sigma Thiazolyl Blue Tetrazolium Bromide (MTT, M2128-10 g) is used for this assay. 1 mg/ml MTT is prepared with DMEM media. With basic steps, the cells are cultivated in a 96 well plate, seed 100 µl DMEM for each well and added 10 µl yellow MTT solutions inside, then incubated 37◦C %5 CO_2 for both 4 hours and 24 hours. After incubation, purple formazan salt crystals are formed. The absorbance of samples is measured and quantified Varioskan (Thermo scientific) microplate reader at 550 nm. However, MTT washing solution is prepared before exposure cells. MTT washing solution is prepared with 950 µl isopropanol and 50 µl Triton-X-100, well mixing.

5.2.8. Cellular Uptake of Chitosan-TPP Nanoparticles

5.2.8.1. Flow Cytometry

The Hek293 cells are cultivated in 6 well plate. After 24 hours, 100 µl FITC-CH-NPs in media are employed for 4 hours. The media is removed from each well. The plate is washed for three times with sterile PBS solution. The cells are transformed suspended state in an eppendorf. The cell mixture is dissolved in 1 ml staining buffer (% 2 FBS in PBS). The solution is analyzed by Flow cytometry.

5.2.8.2. Florescence Microscopy

The Hek293 cells are cultivated in 24 well plate. After 24 hours, 100 µl FITC-CH-NPs in media are employed for 4 hours. The media is removed from each well. The plate is washed for three times with sterile PBS solution. The plate is fixed by %4

parafolmaldehyde solution in 10 minutes. After the step, the plate is washed for three times with sterile PBS solution. 1 mg DAPI is dissolved 1 ml PBS, then it is diluted one by one hundred and 200 µl is employed on well in 10 minutes. After DAPI staining, the cells are washed by PBS in 1 minute. The plate is analyzed by Zeiss Axio Florescence Microscope.

5.2.8.3. ELISA Reader Florescence

The Hek293 cells are cultivated in 96 well plate. After 24 hours, 100 µl FITC-CH-NPs (FITC-Chitosan-Nanoparticles) in media are employed for 4 hours. The media is removed from each well. The plate is washed for three times with sterile PBS solution. Then 100 µl lysis solutions (950 µl Isopropanol and 50 µl Triton-X-100) is employed for each well in 5 minutes and quantified by florescence ELISA reader.

5.2.9. Maximum Dosage Day Detection and Animal Groups

In this study, three animal groups are planned for the further experiments. MDD (maximum dosage day) is found fifth (5 days) from earlier our experiments. In first groups of animals, 100 µl (0,1 ml) PBS (or saline) are injected to tail vein of mice, which are anaesthetized by ketamine/xylasin agent. After 4 hours, it is injected intraperitoneally 15 mg/kg cisplatin to these mice. Cisplatin should be mixed by PBS before injection. In second groups of animals, mice which are anaesthetized by ketamine/xylasin agent are injected empty chitosan nanoparticles. After 4 hours cisplatin 15 mg/kg cisplatin are injected to mice. In the last group of animals, the same quantity, 7 animals, siRNA-loaded chitosan nanoparticles are mixed by 0,1 ml PBS, are injected to tail vein of mice. After 4 hours, cisplatin 15 mg/kg are injected to mice intraperitoneally. All mice are sacrificed on 5 days and gathered kidneys and bloods of mice. Blood samples will be used creatinine and BUN detection. Kidney samples will be used mRNA assays.

5.2.10. RNA isolation from Kidney Tissues

The kidney sample from were sacrificed mice homogenized in 1 ml RiboEx for total RNA isolation with probe sonicator on ice. All steps were employed accordingly Hybrid-R RNA purification kit protocol. After homogenization step, centrifuge process (10 000 g 15 minutes and 30 seconds) was started. Chloroform was used for phase separation, RBI buffer was used for RNA binding, and SWI buffer was used for RNA washing twice. Finally Nuclease free water was used for RNA elution in the final step. The concentration of the isolated RNA samples was measured Nano2000 instrument and the samples were stored at -80 for further analysis.

5.2.11. Creatinine and BUN (Blood Urea Nitrogen) Detection

As mentioned in kit protocol (SpinReact), working reagents are mixed accordingly sample quantities. After blanking, while urea read is employed at 340 nm absorbance, creatinine read is employed at 492 nm absorbance. After reading, ΔA values are calculated according to absorbance results of each kit. The results are converted to a bar graph on figure 6.1.6.1. and 6.1.7.1. Figure 6.1.7.1. demonstrates creatinine and BUN level after injection.

5.2.12. Real Time PCR (Quantitative PCR, qPCR)

Before quantitative PCR (Real Time PCR), there should be converted RNA to cDNA. Therefore, RNA samples were converted to cDNA with High Capacity cDNA Reverse Transcription kit (Applied Biosystem, Thermo Fisher Scientific). Master mix and template RNA quantities and cycle properties were shown in table 5.1.12.1.

	Component	**Quantity (µl)**
Master Mix	10xRT Buffer	2 µl
	25x dNTP mix (100mM)	0,8 µl
	10x RT random primer	2 µl
	Reverse Transcriptase	1 µl
	Water	4,2 µl

Template RNA	RNA (μg/μl)	10 μl

Table 5.1.12.1. cDNA synthesis components and quantities.

For Real time PCR (qPCR), Maxima SYBR Green/Fluorescein qPCR master mix (Thermo Scientific) was used. Primers were designed according to table 5.1.12.2. gene sequences.

OCT1	Forward 5′-GGTGAATGCTGAGC-3′ Reverse 5′-ACATCTCTCTCAGGTGCCCG-3′
OCT2	Forward 5′-CAATGGCCTATGAGATAGTCT-3′ Reverse 5′-GCAGCAACGGTCTCTCTTCTT-3′
p53	Forward 5′-GGGCCCGTGTTGGTTCATCC-3′ Reverse 5′-CCGCGAGACTCCTGGCACAA-3′
PKCδ	Forward 5′-TCTGGGAGTGACATCCTAGACAACAACGGG-3′ Reverse 5′-CAGATGATCTCAGCTGCATAAAACGTAGCC-3′
γGT	Forward 5′-GGAGAGAGTTTCTGCCCATCCATAC-3′ Reverse 5′-GCGGCTGGGTGGGTGGT-3′
GAPDH (internal control)	Forward 5′-ACAGTCCATGCCATCACTGCC-3′ Reverse 5′-GCCTGCTTCACCACCTTCTTG-3′

Table 5.1.12.2. Primers 6 genes for real time PCR, GAPDH, housekeeping gene.

Chapter 6

Conclusions and Future Prospects

6.1. Conclusions

We synthesized siRNA-loaded PEG-coated chitosan nanoparticles, which measured around 400 nm. The chitosan nanoparticles are also tagged with a florescence dye (FITC, Fluorescein isothiocyanate) for the tracking our nanoparticles on kidney cells or body by florescence imaging. FTIR analysis confirmed both PEG and FITC conjugate chitosan polymer. It reveals chemical bonding from the synthesized polymer. Here PEG (Polyethylene glycol) is a hydrophilic crosslinking conjugate tool for suitable for drug delivery. It provides to avoid the destruction nanoparticles by phagocytosis in blood stream circulation. The ionic gelation method was used to form 400 nm chitosan nanoparticles. Then they are confirmed by dynamic light scattering (DLS). But before the method, centrifugation process was performed. Before adding TPP and siRNA mixture to chitosan, chitosan powder was added into an organic acid, acetic acid solution. The solution was sonicated by a sonic probe or ultrasonic waves. The synthesized nanoparticles were suitable for storage at + 4 ^{o} C before injection. In following days, the nanoparticle solutions were measured by DLS for sure size was not changed at these circumstances. Thus, the nanoparticles could be stayed freezer around 3 days before injection without aggregation in solution. In vitro studies were performed with human kidney Hek293 cell line. To confirm kidney accumulation of synthesized nanoparticles in kidney, before injection to mice, we used florescence imaging with

cryosection kidney tissues from a negative control mice group, three mice. Additionally, from these mice group, kidney tissues were collected for flow cytometry. There was a significant result from flow cytometry, all FITC-conjugated chitosan nanoparticles uptake by the kidney cells. Our chitosan nanoparticles accumulated kidney. We injected intraperitoneally 15 mg/kg of cisplatin into two groups of Balb-c 6-8 week's male mice. We weighed all mice groups before cisplatin injection into them. First was control, after cisplatin injection 3 days and 5 days, blood samples were collected for creatinine and BUN level detection. Kidney tissues were also collected for RNA isolation, mRNA level detection in further real time PCR. Creatinine and BUN assays from this first control groups showed us cisplatin negatively affected more kidneys, it causes apoptosis in kidney. So, we decided to inject our nanoparticles into mice groups at day 5. Cisplatin-treated free nanoparticles in PBS (saline) mice group, cisplatin-treated mice group PEG-coated siRNA-loaded chitosan-TPP nanoparticles in PBS and only cisplatin-treated mice group were used for the main animal experiment. Injections were performed injecting intravenous by tail vein. Then creatinine and BUN assays were performed to detect the level changing by nanoparticles after cisplatin-induced siRNA delivery. Finally gene expression levels were investigated by real time PCR. PRCδ, OCT1, γGT and p53 genes were used in gene expression. At γGT gene exhibited increasing expression as statistically significant. The other genes were also changed after siRNA-loaded chitosan nanoparticles.

As nanometer size, 400 nm was accumulated specifically kidney proximal tubule cells [37]. In the study were not used chitosan nanoparticles as drug carrier for kidney tissue but mesoscale nanoparticles especially hold by proximal epithelial tubule cells on a different mice group. They also used fluorescence tracking for the biodistribution of particles on mice body. They used PEG polymer for this purpose and we also used it. They get a great visual fluorescence image from tissues. We could not weight the nanoparticle-treated mice but they also performed that important physical property after injection. They checked mice on IVIS imaging to track particles in body. We could not get the chance to use the technology. Not only the kidney tissues but also they could get the chance to track their carriers on the body easily.

RNA interference technology is the important approach for the drug delivery in kidney tissues. SiRNA-loaded nanoparticles are suitable in vivo applications. They are also targetted by the renal proximal cells with different mice group. In the study, siLRP2 and unspecifically target siRNA molecules were used [98]. Some renal genes were searched

by the qPCR in the study. Then the different cells were used than us. Their siRNAs targeted specifically renal tubular epithelial cells and showed really significant reducing gene expression compared to non target siRNA without using a siRNA delivery carrier. They also injected into mice tail vein like us. While using 7,5 mg/kg siRNA amounts, but we used less than this, 1,1 mg/kg. This may not be enough for siRNA delivery into mice. But next studies we will increase amounts to get much better results. SiRNA effect was longed at 96 h but gene expression was augmented compared to untargeted siRNA molecules at 144h.

Chitosan oligomers could be favorable carriers in renal drug delivery. Here the study was used approximately 490 nm and they observed kidney accumulation of their FITC-conjugated chitosan oligomers. But our chitosan nanoparticles were around 430 nm in diameter. They showed especially in kidney accumulation compared to the other organs [99].

The study is the first publish about PEG-coated and siRNA-loaded chitosan-TPP nanoparticles on BALB-c mice and targeted kidney proximal epithelial tubule cells. It was shown that kidney was the place accumulation of siRNA-loaded chitosan nanocarrier molecules at 400 nm in diameter. Additively, the study is also first searching gene expression levels the five crucial genes in kidney. Then creatinine and BUN assays from blood serums were used in other diseases except for cisplatin-induced nephrotoxicity [100]. The previous study used the assays for chronic renal failure.

6.1.1. Characterisation of Chitosan-TPP-siRNA Nanoparticles

Malvern NanoZS is used for size distribution of chitosan-TPP nanoparticles. As mentioned above, it is delivered a nanoparticle which has around 400 nm mean size. This is achieved with ionic gelation method for preparation of chitosan nanoparticles. In total, 420 µl nanoparticle solution is prepared with 120 µl TPP and 300 µl chitosan (2,5:1 chitosan: TPP rate). For this study, it is preferred that nanoparticles have PDI values less than 0,4. This means highly monodisperse nanoparticles with peak 1 size distribution and intensity percent. Figure 6.1.1.2. shows a size distribution graph after nanoparticles formation process. Peak 1 is based on for size dependent nanoparticle treatment. Table 5.1.12.2. also shows which nanoparticle formation is suitable for nanoparticle solution treatment on cell line, with PDI values, Peak 1 and result quality

for the sample nanoparticle solutions. All the samples are supernatants from centrifuged chitosan-TPP nanoparticle mixture after gradual sonication process. Result quality is acceptable measurement for sample and is obtained from incubation inside cuvette at room temperature. Figure shows PEGylated chitosan polymer and normal HP chitosan FTIR analysis. PEG, PEGylated chitosan and HP chitosan alone are analyzed separately to confirm PEG-conjugation by HP chitosan (SD, standard deviation).

Samples	Peak 1 (nm)	Intensity %	PDI	Average size (d.nm)	Result Quality
1	423,2	100,0	0.257	325,8±SD	Good
2	389,8	100,0	0,387	400,4±SD	Good
3	388,3	100,0	0,373	459,5±SD	Good

Table 6.1.1. Three samples of prepared HP chitosan nanoparticles. It is shown PDI, average size and measurement result quality of samples.

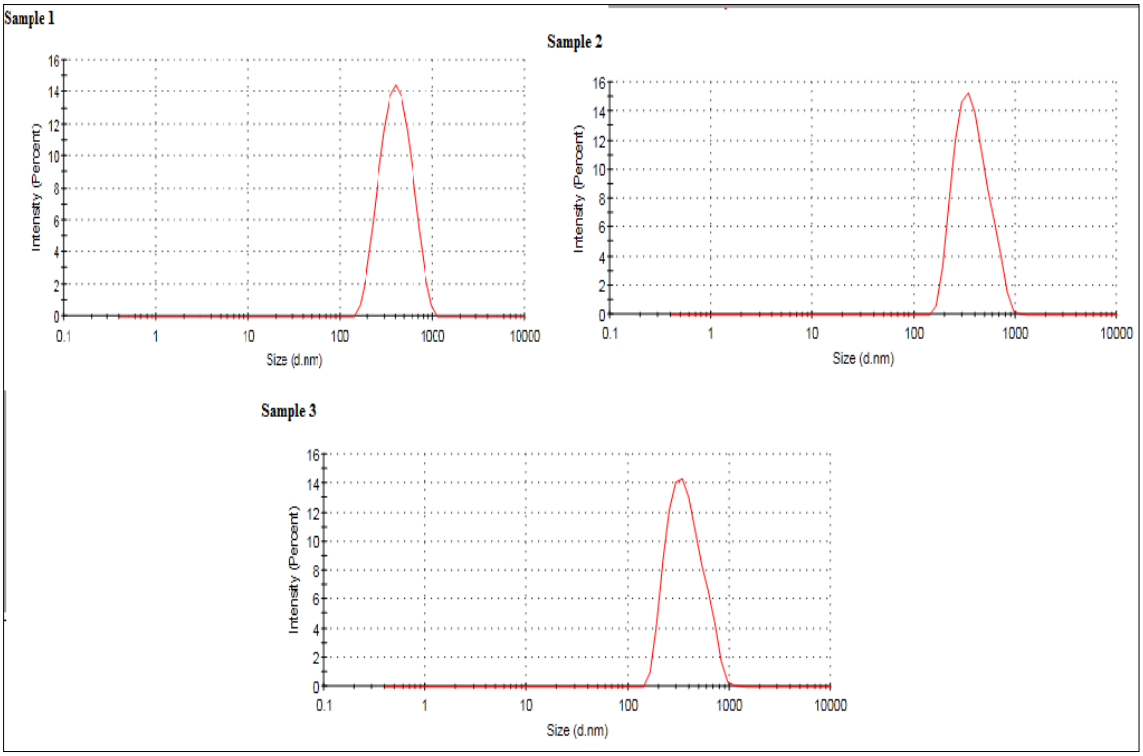

Figure 6.1.1.2. Size distribution by intensity of chitosan nanoparticles. Axis implies intensity percent, ordinate implies size diameter in nm.

6.1.2. Characterisation of FITC-conjugated-PEG- Chitosan for Tracking Nanoparticles

After dialysis which is applied for preparation of FITC-conjugated chitosan powder. To confirm binding florescence dye to chitosan molecule, FTIR analysis is used to characterize FITC dye, HP chitosan and freeze-dried FTIC-chitosan powder. Figure 6.1.2.1. and 6.1.2.2. shows that functional groups and fingerprint groups of samples from FTIR measurement. All samples are powder states. FTIR is an analytical instrument that identifies chemical property of liquid or powder materials by using infrared light. The data is compared to world library and detected closest match-up. Transmittance % is PEGylated chitosan, HP chitosan and only PEG FTIR % transmittance results are also shown in Figure 6.1.2.3.

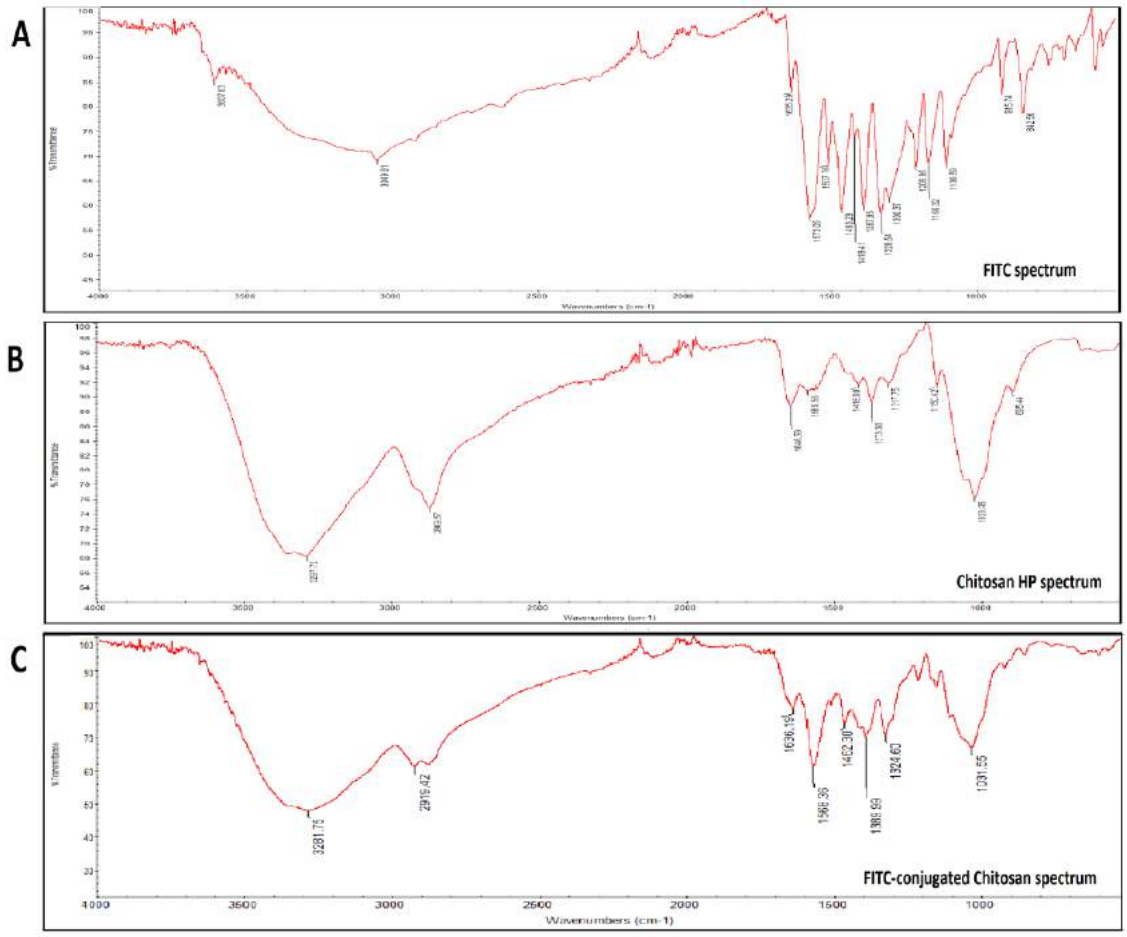

Figure 6.1.2.1. A) FTIR spectrums of FITC dye; B) both FITC and HP chitosan and C) HP chitosan spectrum.

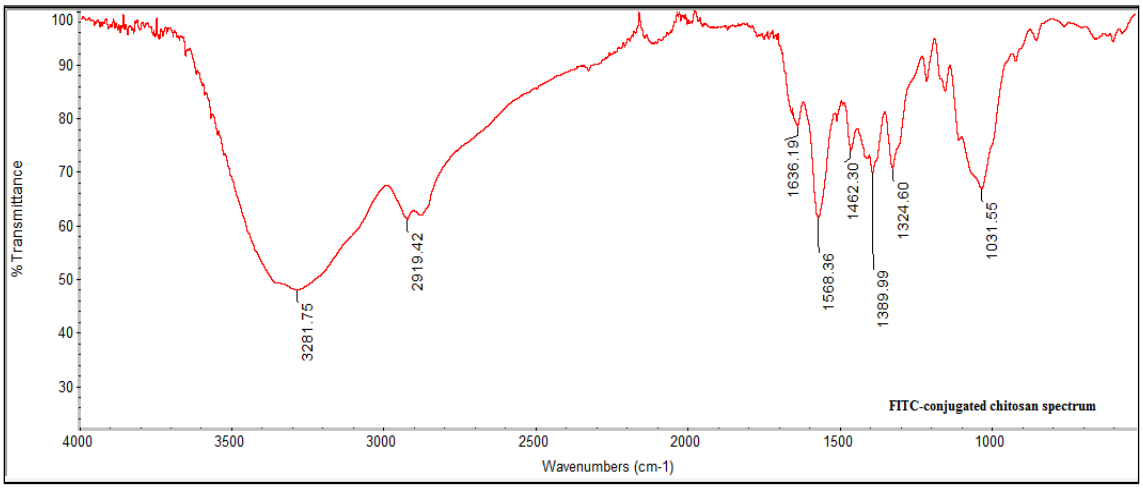

Figure 6..1.2.2. FITC-conjugated HP chitosan FTIR spectrum is shown.

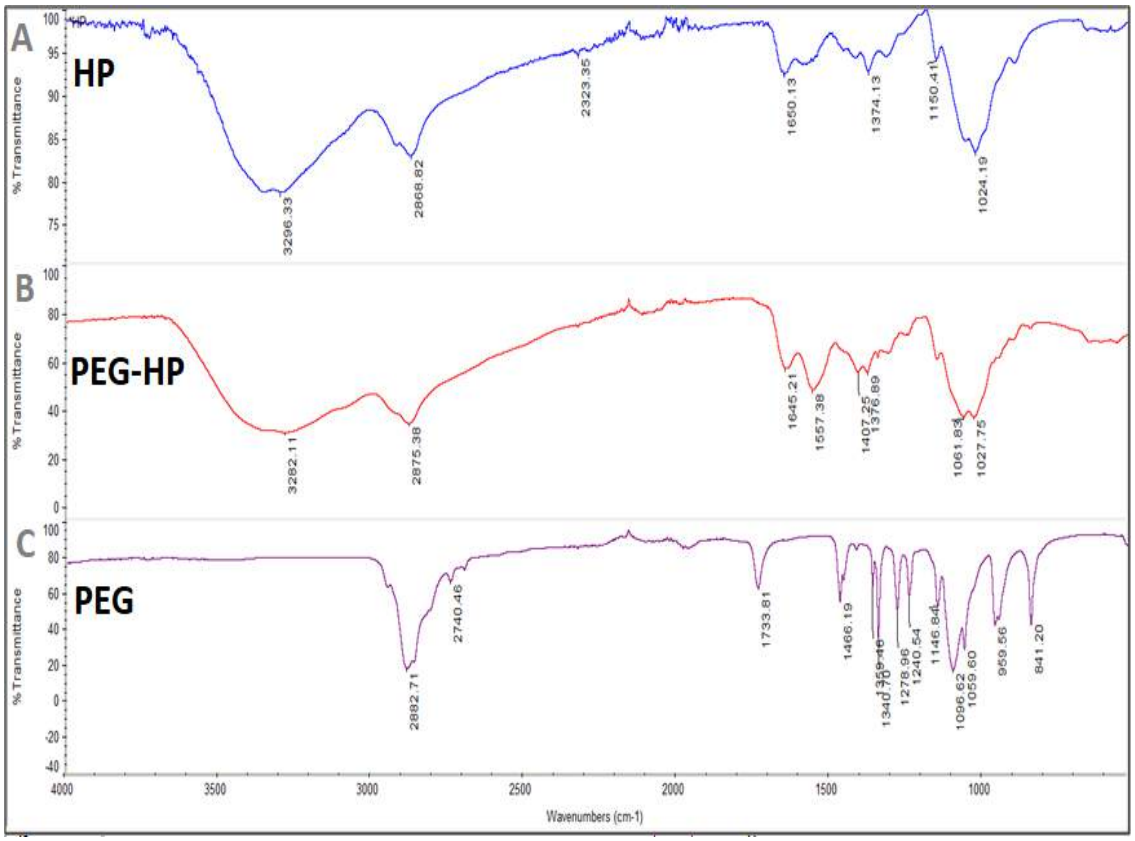

Figure 6.1.2.3. Three FTIR spectrums of PEG, PEGylated HP chitosan and only HP with wavenumbers

6.1.3. Encapsulation Efficacy of Chitosan-TPP-siRNA nanoparticles

After siRNA-loading on chitosan nanoparticles and measurement their size distribution by intensity, how much siRNA is loaded should be determined by a Nanodrop measurement. 420 µl chitosan-siRNA mixtures are measured by Malvern NanoZS(UK). After measurement, unbounded siRNA is detected in the mixture. Firstly, the mixture is centrifuged, 13 000 g 50 minutes 15◦C. then the supernatant harbors unbounded RNA molecules. 1 µl supernatant is enough for measurement. It is observed that around % 90 siRNA molecules are attached on chitosan nanoparticles, depending on their concentration µg/µl. To confirm concentration result, five measurements is taken for average concentration of RNA by using Nanodrop.

To detect encapsulation efficacy of siRNA-loaded chitosan nanoparticles, an equation was used after concentration measurement. Figure 6.1.3.1. shows the equation.

$$\frac{\text{sample siRNA concentration-supernatant siRNA concentration}}{\text{sample siRNA concentration}} \text{X100} = \text{Encapsulation Efficacy (\%)}$$

Figure 6.1.3.1. Equation which is used for the encapsulation efficacy of siRNA-loaded chitosan nanoparticles.

As sample siRNA concentration is 1,329 µg/µl and supernatant measurement is 0,248 µg/µl. Thus encapsulation efficacy of chitosan nanoparticles is found around 83-90 %.

6.1.4. Cytotoxicity Assay

These assays are used for cell proliferation intensity, after exposure of chitosan nanoparticles on Hek293 human kidney cell line. Accordingly the results absorbance of samples, there is not significantly difference in treatment of nanoparticles on the cell line. While MTT assay is applied 24 and 48 hours, XTT assay is implemented 4h, 24 h and 48 hours in a plate. Figure 6.1.4.1. is obtained from absorbance of samples, and shows both MTT and XTT cell proliferation percentage bar graph. Figure 6.1.4.1. shows 4 h MTT assay. The experiments are employed three times for confirmation.

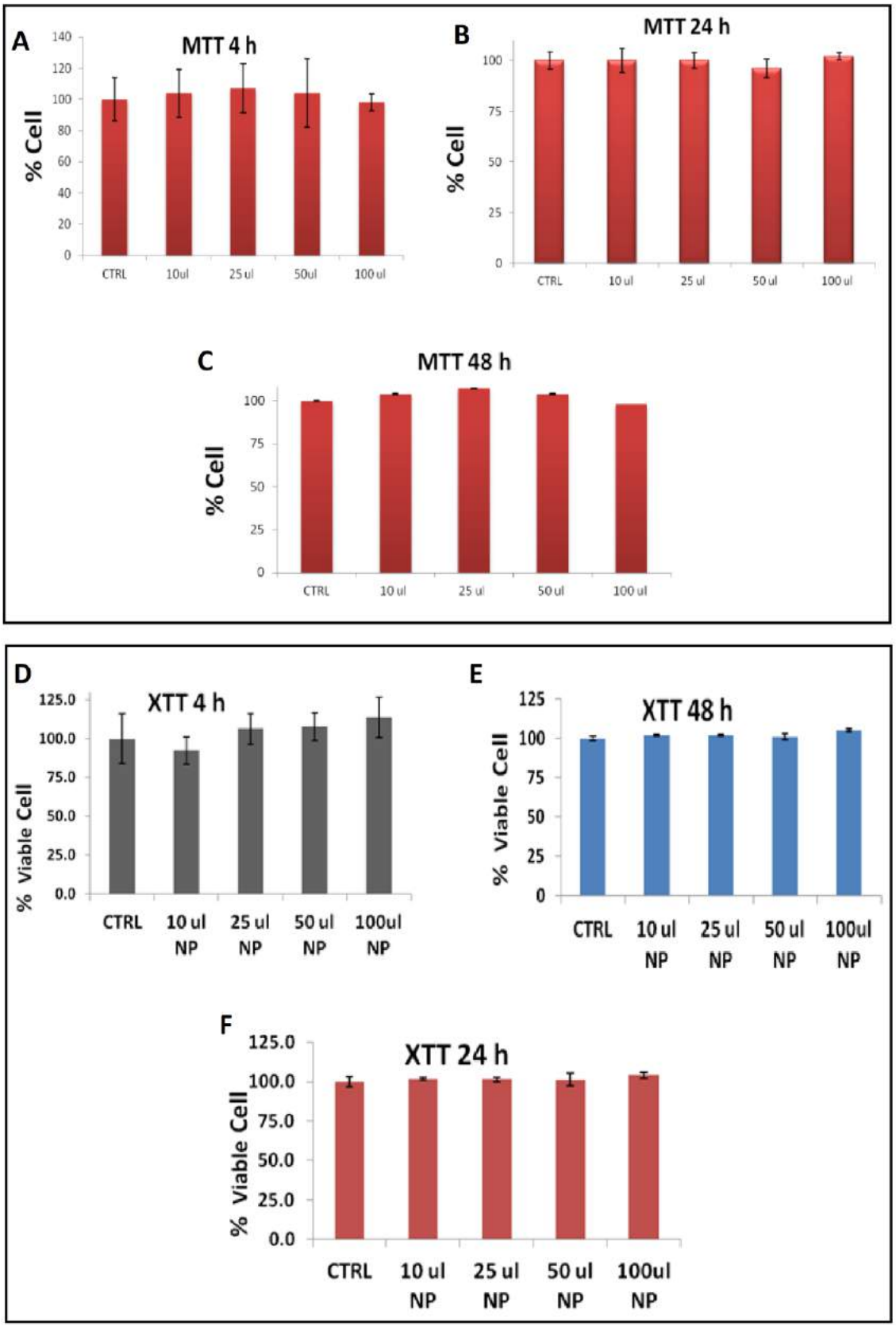

Figure 6.1.4.1. XTT (A,B,C) and MTT (D,E,F) results, graph bar.

6.1.5. Florescence Imaging and Absorbance of Nanoparticles Uptake by The Hek-293 Cells

Addition to cytotoxicity assays, in this study, there is also used florescence absorbance and flow cytometry for confirmation of nanoparticle cellular uptake in Hek293 cells with some gradual steps in a plate. It should be detected that FITC dye conjugated chitosan-TPP nanoparticles enter inside the cell. The cells are stained with DAPI for imaging cell nucleus and cell location in well for further merge analysis. Figure 6.1.5.2. shows florescence microscopy image of FITC-conjugated chitosan nanoparticles treated on Hek293 cell line. Figure 6.1.5.1. shows that mean florescence intensity (MFI) of nanoparticles and FITC-conjugated chitosan nanoparticle graph bar.

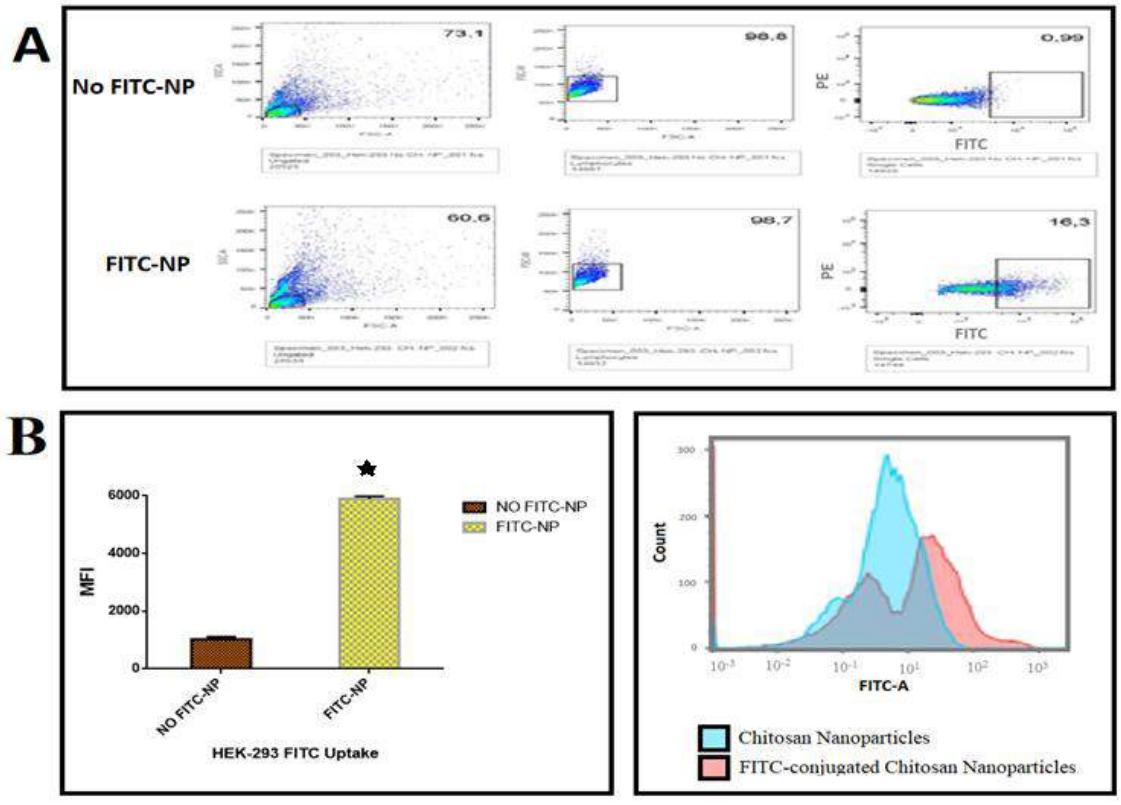

Figure 6.1.5.1. Flow cytometry measurement of FITC-conjugated chitosan nanoparticles treated on Hek293 cell line. A) Flow cytometry dot plot B) Mean fluorescence intensity and Histogram , Fıtc-NP and No Fıtc-NP. Asteriks show statisticly significant p=0.05.

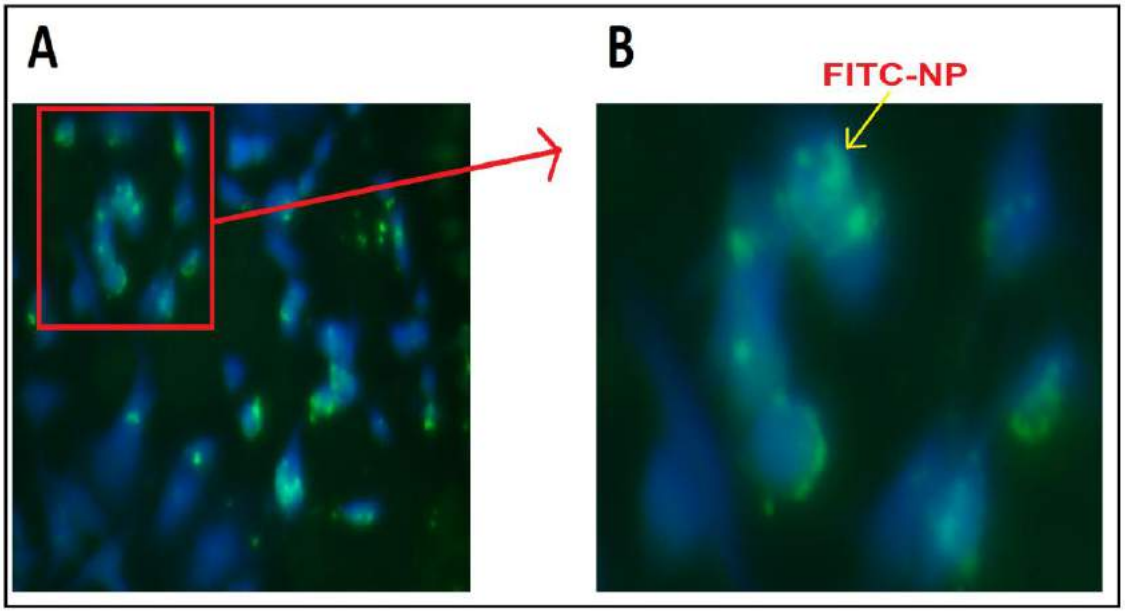

Figure 6.1.5.2. Florescence microscopy image of FITC-conjugated chitosan nanoparticles treated on Hek293 cell line. A) 10 X B) 40X

6.1.6. Cisplatin-induced Nephrotoxicity in mice and Creatinine&BUN level detection

After weighing, 15 mg/kg cisplatin was injected to Balb-c type mice intraperitoneally. Blood samples were collected from tail vein in 3, 5, 7 and 9 days after injection (Figure 6.1.6.1). Then creatinine and BUN (Urea) level were detected from samples. Figure 6.1.6.2. shows Urea and Creatinine results on different days. It is clear that there is an increase on 5 days compared to first injection day (zero, 0 day).

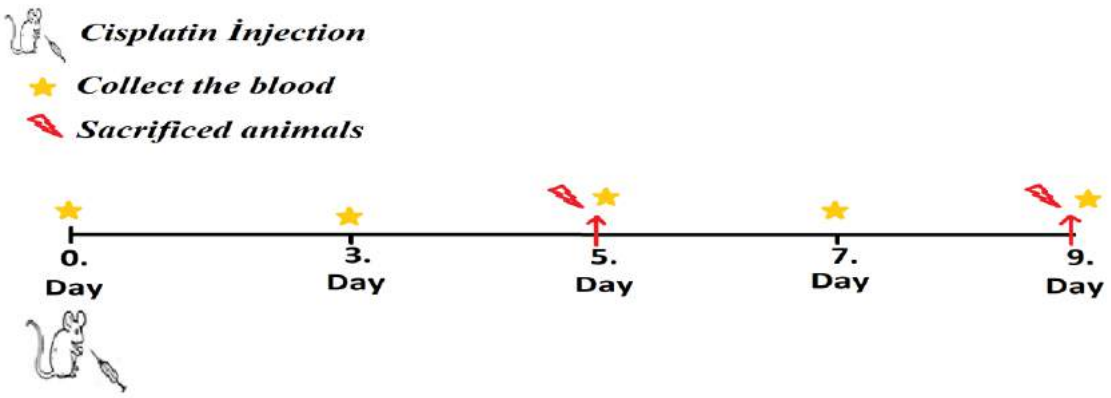

Figure 6.1.6.1. Cisplatin injection and blood collection days were applied in Balb-c mice.

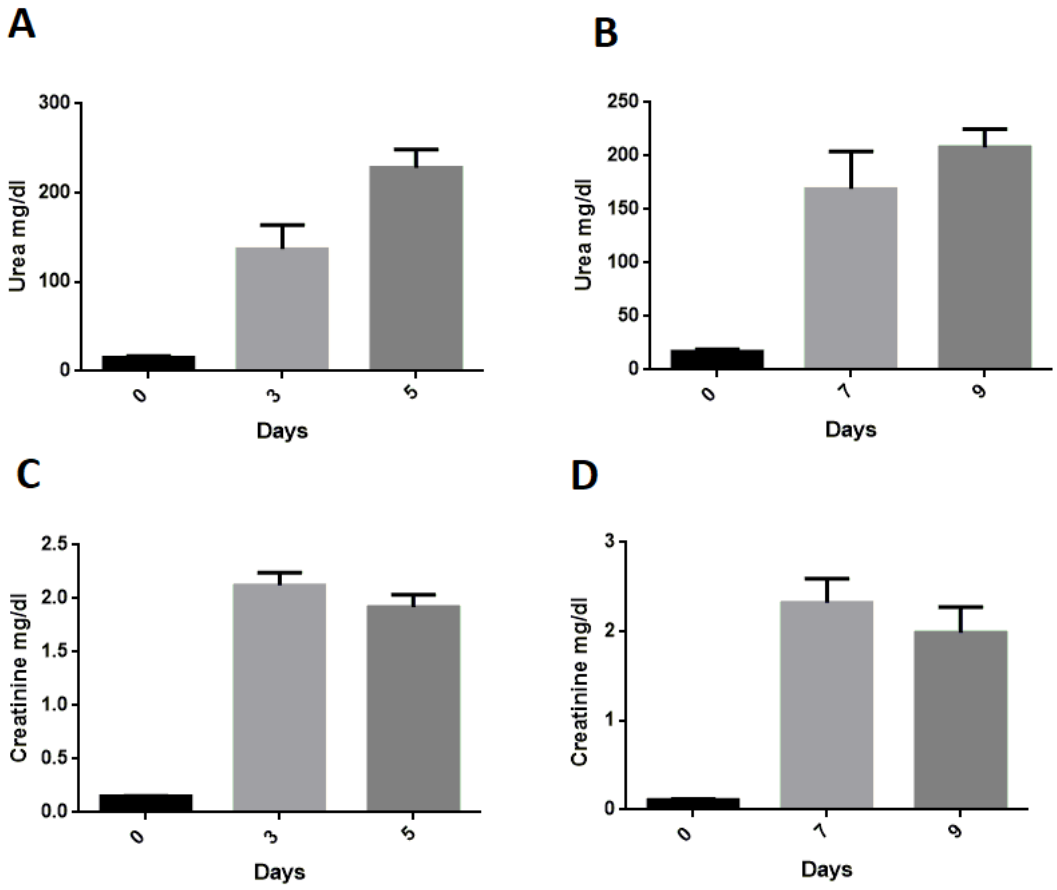

Figure 6.1.6.2. Urea (BUN) and Creatinine level on 5 days with ± Standard deviations. Control means mice groups which were not injected siRNA-loaded chitosan nanoparticles. Free NP means empty nanoparticle injected mice groups. SiRNA+NP means siRNA-loaded chitosan nanoparticle injected mice groups. Error bars are also shown on graph.

6.1.7. Creatinine & Urea levels in mice with Cisplatin-induced Nephrotoxicity

15 mg/kg cisplatin was injected to Balb-c type mice intraperitoneally. Blood samples were collected (Figure 6.1.7.1.). Then creatinine and BUN (Urea) level were detected from samples (Figure 6.1.7.2.) Mice were sacrificed on 5 days which were observed the highest cisplatin effect on mice. Blood samples were collected for creatinine and BUN detection.

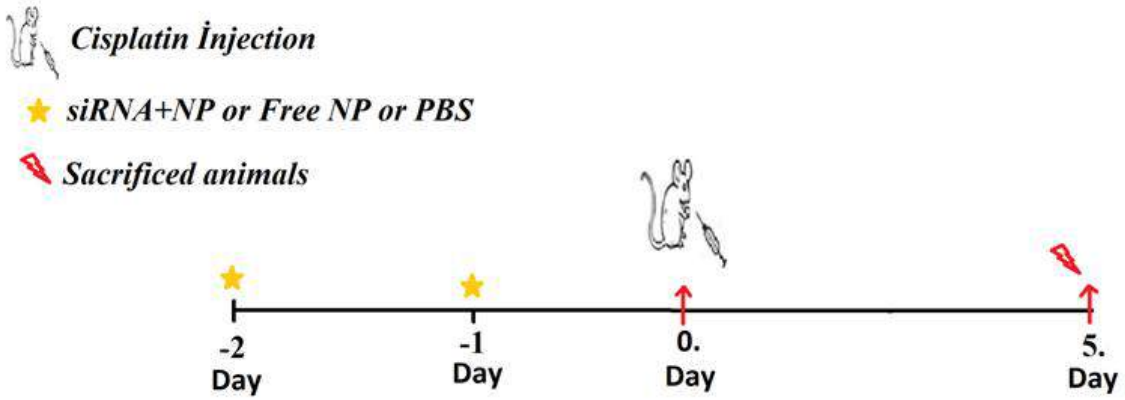

Figure 6.1.7.1. Cisplatin,,siRNA-Nanoparticles,free Nanoparticles and PBS injection and blood collection days were applied in Balb-C mice.

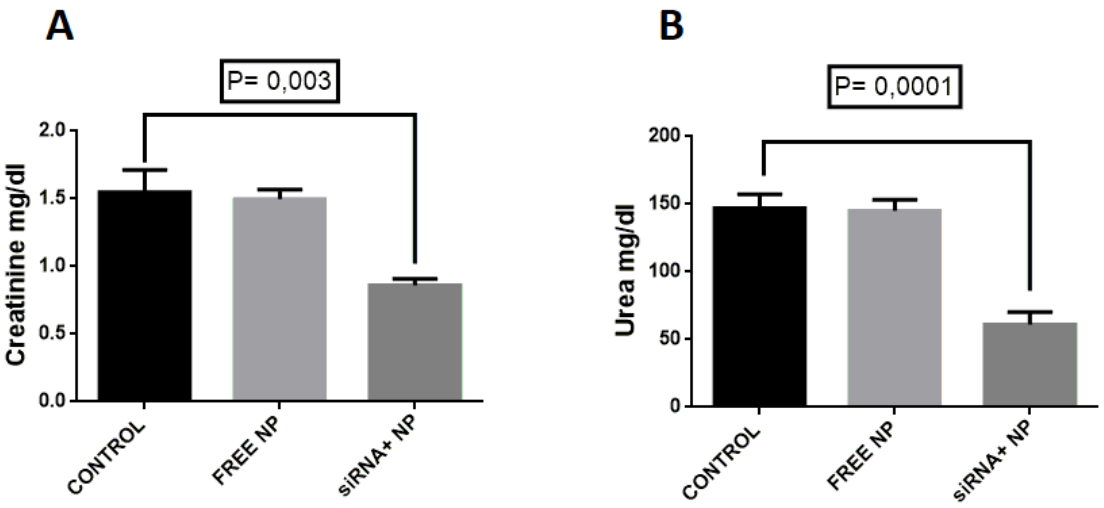

Figure 6.1.7.2. Urea (BUN) and Creatinine levels with ± Standard deviations after injection, p values demonstrates statistically significant result. Control means mice groups which were not injected siRNA-loaded chitosan nanoparticles. Free NP means empty nanoparticle injected mice groups. SiRNA+NP means siRNA-loaded chitosan nanoparticle injected mice groups. Error bars are also shown on graph.

6.1.8. Gene Expression Levels In Mice With Cisplatin-Induced Nephrotoxicity

15 mg/kg cisplatin was injected to Balb-c type mice intraperitoneally. They were sacrificed on 5 days. Collected kidneys were separated dorsally with the help of a sterile lancet. Some part of kidney was collected in RiboEx solution for RNA isolation; some part of kidney was stored at -80◦C for western blotting and remaining kidney part was stored in 4 % formaldehyde solution for immunohistochemistry assays. RNA samples were used for cDNA synthesis. From the synthesized cDNA samples, Real time PCR was employed for each mouse groups as triplet. Figure 6.1.8.1. shows mRNA levels from 4 genes, γGT6, OCT1, P53 and PKCδ. OCT2 did'nt work on Q-PCR.

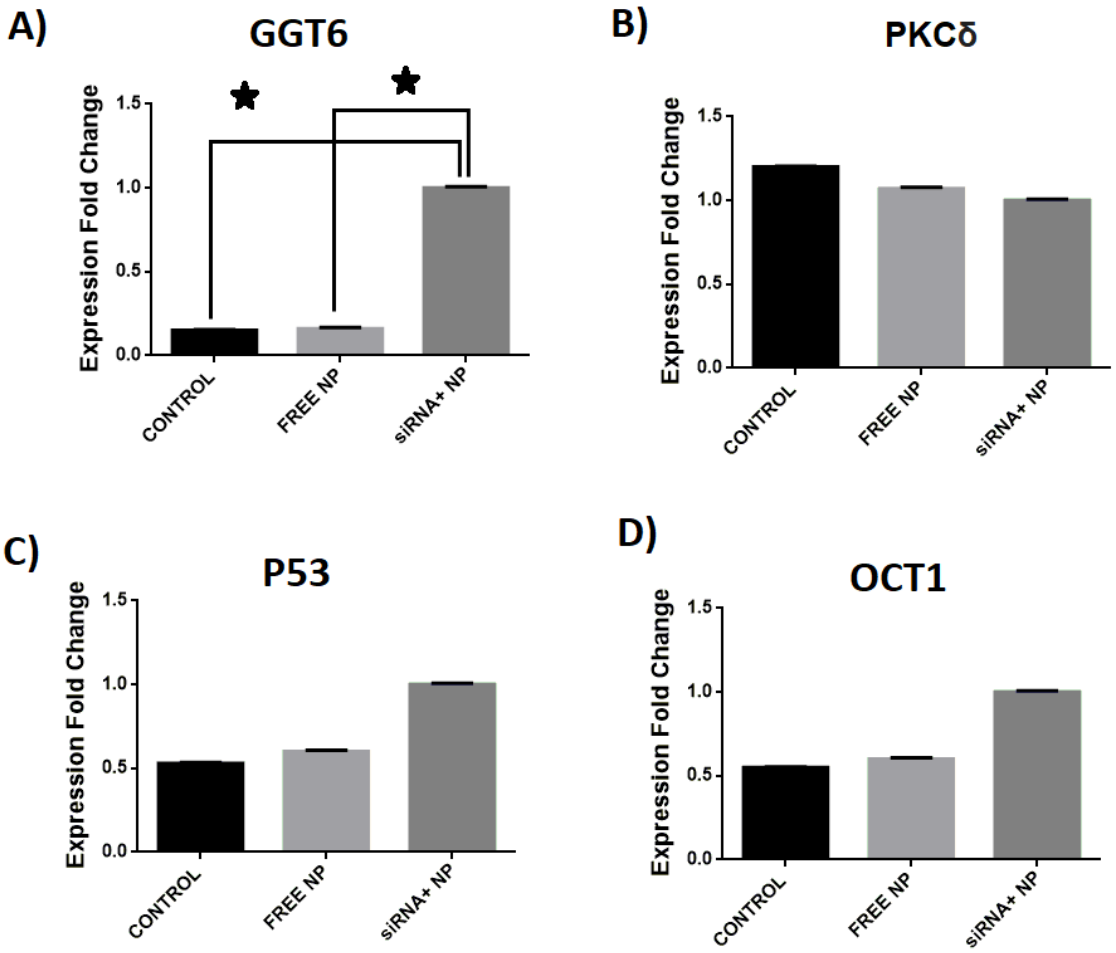

Figure 6.1.8.1. Real time PCR results, GGT6, PKCδ, P53 and OCT1 genes mRNA levels after injection. Control means mice groups which were not injected siRNA-loaded chitosan nanoparticles. Free NP means empty nanoparticle injected mice groups. SiRNA+NP means siRNA-loaded chitosan nanoparticle injected mice groups. Asterisks refer to statistically significance of bar graph. Error bars are also shown on graph.

6.2. Future Prospects

Chitosan and its derivatives have been curiously used for delivery of genes, drugs and molecules in different biomedical applications over the last decades [101]. In the literature, there have been investigated and modified several chitosan nanoparticles on cell lines. Chitosan-TPP nanoparticles are polymer-based nanocarriers which are used controlled and targetted drug delivery. Here, size is important parameter to detect nanoparticle for their aims in several applications.

FITC-chitosan nanoparticles are prepared by ionotropic gelation method and assayed on human adenocarcinoma cell line A549 [102]. Around 200 nm sized FITC-labeled nanoparticles are characterized by confocal microscopy and cellular uptake studies. After incubation process, it is demonstrated that while FITC-chitosan concentration increases from 0,4 to 0,8 mg/ml, rate of cellular uptake of the nanoparticles also increases 10 μg/mg to 30 in 4 hours. Thus, there is a significant cellular uptake when using chitosan nanoparticles through the cells.

Another study showed there are several reports about targeting human kidney cell line by chitosan nanoparticles. In one of them, human embryonic kidney cells are used for targeting curcumin-loaded chitosan nanoparticles by approximately 200 nm mean size. At lower concentration curcumin-loaded chitosan nanoparticles show a low cytotoxicity on Hek293 cell line [103]. The results from the study it is clearly understood chitosan-based nanoparticles do not display clastogenic property on cells.

Nanoparticles at mesoscale approximately 400 nm average size in diameter are largely delivered to renal proximal tubule epithelium. It has revealed the importance of nanoparticles as promising nanocarriers in order to remedy of kidney diseases including cancer [37].

For the past decades, RNAi technologies have been used in drug delivery for treatment

of human kidney-based diseases for medical purposes, although there are some challenges of delivery and in vivo targeting. SiRNA-loaded chitosan-TPP nanoparticles can stay in the specifically kidney tissues. Thus they have been attracted by investigators as a delivery system. Particularly proximal tubule epithelial cells are places accumulating chitosan-siRNA nanoparticles. In mice, chitosan nanoparticles are administered by intravenous injection to reach the kidneys. Moreover, molecular weight of chitosan polymer is also an important parameter that affects the delivery. The synthesized nanoparticles can stay in the human body more than two days. Thus the project is related kidney nephrotoxicity and is also based on the research. In the research, when knockdown of some genes which are expressed by proximal tubule endothelial cells in kidney, can be providing a therapeutic approach for several kidney-based human disorders [104].

Just as we expected, empty nanoparticles did not affect the target mRNA levels of siRNAs. SiRNA-loaded nanoparticles also made a difference in all types of siRNA. It is clear that we wanted to reach nanoparticles to the kidney cells and they create an effect there. After uptake of siRNAs, they suppressed target mRNAs, thereby target mRNA levels decreased. But when siRNA effect was all over, the cells produced them more than normal level. So the cell was trying to compensate for their lack.

In our further studies, we will inject siRNA-loaded chitosan nanoparticles on mice in earlier days than fifth, based on recent publish. In this manner, after injection more siRNA effect on mRNA levels in proximal tubule cells will be investigated.

The protein levels of the kidney tissues obtained by our study will be examined by western blotting, and the immunohistochemical tests of the tissues will be performed by TUNEL and caspase 3 assays. In the light of the information obtained, chitosan-based nanoparticle strategy may shed light on a possible treatment approach that reduces or prevents cisplatin nephrotoxicity.

BIBLIOGRAPHY

1. Lead, J.R., et al., Nanomaterials in the environment: Behavior, fate, bioavailability, and effectsAn updated review. Environmental Toxicology and Chemistry, 2018. 37(8): p. 2029-2063.
2. Buzea, C., I.I. Pacheco, and K. Robbie, Nanomaterials and nanoparticles: Sources and toxicity. Biointerphases, 2007. 2(4): p. Mr17-Mr71.
3. Kabir, E., et al., Environmental impacts of nanomaterials. J Environ Manage, 2018. 225: p. 261-271.
4. Jeevanandam, J., et al., Review on nanoparticles and nanostructured materials: history, sources, toxicity and regulations. Beilstein J Nanotechnol, 2018. 9: p. 1050-1074.
5. Zhang, C., et al., Zero-dimensional, one-dimensional, two-dimensional and three-dimensional biomaterials for cell fate regulation. Adv Drug Deliv Rev, 2018. 132: p. 33-56.
6. N.T., J., R. N.T., and K. S., Zero-dimensional, one-dimensional, two-dimensional and three-dimensional nanostructured materials for advanced electrochemical energy devices. Progress in Materials Science, 2012. Volume 57, (Issue 4, May 2012,): p. Pages 724-803.
7. Othman, Z., et al., Understanding interactions between biomaterials and biological systems using proteomics. Biomaterials, 2018. 167: p. 191-204.
8. Dziki, J.L., et al., Extracellular Matrix Bioscaffolds as Immunomodulatory Biomaterials<sup/>. Tissue Eng Part A, 2017. 23(19-20): p. 1152-1159.
9. Asghari, F., et al., Biodegradable and biocompatible polymers for tissue engineering application: a review. Artif Cells Nanomed Biotechnol, 2017. 45(2): p. 185-192.
10. M.A., J., Principles of Regenerative Medicine (Third Edition), in Biocompatibility and Bioresponse to Biomaterials. 2019: Academic Press. p. 675-94.
11. Sun, Q., et al., Functional biomaterials towards flexible electronics and sensors. Biosens Bioelectron, 2018. 119: p. 237-251.
12. Shan, D., et al., Polymeric biomaterials for biophotonic applications. Bioact Mater, 2018. 3(4): p. 434-445.
13. A., Y., et al., Biocompatible and electroconductive polyaniline-based biomaterials for electrical stimulation. European Polymer Journal, 2018. 11.
14. Teodorescu, M., M. Bercea, and S. Morariu, Biomaterials of PVA and PVP in medical and pharmaceutical applications: Perspectives and challenges. Biotechnol Adv, 2018.
15. Cao, B., et al., Bacteriophage-based biomaterials for tissue regeneration. Adv Drug Deliv Rev, 2018.
16. Gritsch, L., et al., Polylactide-based materials science strategies to improve tissue-material interface without the use of growth factors or other biological molecules. Mater Sci Eng C Mater Biol Appl, 2019. 94: p. 1083-1101.
17. Tchobanian, A., H. Van Oosterwyck, and P. Fardim, Polysaccharides for tissue engineering:

Current landscape and future prospects. Carbohydr Polym, 2019. 205: p. 601-625.
18. Hanahan, D. and R.A. Weinberg, The hallmarks of cancer. Cell, 2000. 100(1): p. 57-70.
19. Hanahan, D. and R.A. Weinberg, Hallmarks of cancer: the next generation. Cell, 2011. 144(5): p. 646-74.
20. Horne, S.D., S.A. Pollick, and H.H. Heng, Evolutionary mechanism unifies the hallmarks of cancer. Int J Cancer, 2015. 136(9): p. 2012-21.
21. Weinberg, R.A., The retinoblastoma protein and cell cycle control. Cell, 1995. 81(3): p. 323-30.
22. Paramio, J.M., et al., PTEN tumour suppressor is linked to the cell cycle control through the retinoblastoma protein. Oncogene, 1999. 18(52): p. 7462-8.
23. Barnes, R.P., et al., DNA Polymerase Eta Prevents Tumor Cell Cycle Arrest And Cell Death During Recovery from Replication Stress. Cancer Res, 2018.
24. Ozaki, T. and A. Nakagawara, Role of p53 in Cell Death and Human Cancers. Cancers (Basel), 2011. 3(1): p. 994-1013.
25. Menendez, S., S. Camus, and J.C. Izpisua Belmonte, p53: guardian of reprogramming. Cell Cycle, 2010. 9(19): p. 3887-91.
26. White, K.A., B.K. Grillo-Hill, and D.L. Barber, Cancer cell behaviors mediated by dysregulated pH dynamics at a glance. J Cell Sci, 2017. 130(4): p. 663-669.
27. Tong, C.W.S., et al., Recent Advances in the Treatment of Breast Cancer. Front Oncol, 2018. 8: p. 227.
28. Brannon-Peppas, L. and J.O. Blanchette, Nanoparticle and targeted systems for cancer therapy. Advanced Drug Delivery Reviews, 2012. 64: p. 206-212.
29. Reddy, L.H., Drug delivery to tumours: recent strategies. Journal of Pharmacy and Pharmacology, 2005. 57(10): p. 1231-1242.
30. Natfji, A.A., et al., Parameters Affecting the Enhanced Permeability and Retention Effect: The Need for Patient Selection. Journal of Pharmaceutical Sciences, 2017. 106(11): p. 3179-3187.
31. Enhanced permeability and retention effect for selective targeting of anticancer nanomedicine: are we there yet? Drug Discov Today Technol, 2012. 9(2): p. e71-e174.
32. Nakamura, Y., et al., Nanodrug Delivery: Is the Enhanced Permeability and Retention Effect Sufficient for Curing Cancer? Bioconjugate Chemistry, 2016. 27(10): p. 2225-2238.
33. Jain, V., S. Jain, and S.C. Mahajan, Nanomedicines Based Drug Delivery Systems for Anti-Cancer Targeting and Treatment. Current Drug Delivery, 2015. 12(2): p. 177-191.
34. Kou, C.H., et al., Preparation and characterization of the Adriamycin-loaded amphiphilic chitosan nanoparticles and their application in the treatment of liver cancer. Oncol Lett, 2017. 14(6): p. 7833-7841.
35. Deshmukh, M., et al., Biodistribution and renal clearance of biocompatible lung targeted poly(ethylene glycol) (PEG) nanogel aggregates. J Control Release, 2012. 164(1): p. 65-73.
36. Na, J.H., et al., Precise Targeting of Liver Tumor Using Glycol Chitosan Nanoparticles: Mechanisms, Key Factors, and Their Implications. Mol Pharm, 2016. 13(11): p. 3700-3711.
37. Williams, R.M., et al., Mesoscale nanoparticles selectively target the renal proximal tubule epithelium. Nano Lett, 2015. 15(4): p. 2358-64.

38. Dasari, S. and P.B. Tchounwou, Cisplatin in cancer therapy: molecular mechanisms of action. Eur J Pharmacol, 2014. 740: p. 364-78.
39. Wang, D. and S.J. Lippard, Cellular processing of platinum anticancer drugs. Nat Rev Drug Discov, 2005. 4(4): p. 307-20.
40. Naughton, C.A., Drug-induced nephrotoxicity. American Family Physician, 2008. 78(6): p. 743-750.
41. Miller, R.P., et al., Mechanisms of Cisplatin nephrotoxicity. Toxins (Basel), 2010. 2(11): p. 2490-518.
42. Ciarimboli, G., Membrane transporters as mediators of Cisplatin effects and side effects. Scientifica (Cairo), 2012. 2012: p. 473829.
43. Motohashi, H. and K. Inui, Organic cation transporter OCTs (SLC22) and MATEs (SLC47) in the human kidney. AAPS J, 2013. 15(2): p. 581-8.
44. Vieler, M. and S. Sanyal, p53 Isoforms and Their Implications in Cancer. Cancers (Basel), 2018. 10(9) 10(9):288
45. Berardi, D.E., et al., PKCdelta Inhibition Impairs Mammary Cancer Proliferative Capacity But Selects Cancer Stem Cells, Involving Autophagy. J Cell Biochem, 2016. 117(3): p. 730-40.
46. Priolo, C., et al., Impairment of gamma-glutamyl transferase 1 activity in the metabolic pathogenesis of chromophobe renal cell carcinoma. Proc Natl Acad Sci U S A, 2018. 115(27): p. E6274-E6282.
47. Bertrand, N., et al., Cancer nanotechnology: The impact of passive and active targeting in the era of modern cancer biology. Advanced Drug Delivery Reviews, 2014. 66: p. 2-25.
48. Singh, R. and J.W. Lillard, Nanoparticle-based targeted drug delivery. Experimental and Molecular Pathology, 2009. 86(3): p. 215-23.
49. Brigger, I., C. Dubernet, and P. Couvreur, Nanoparticles in cancer therapy and diagnosis. Advanced Drug Delivery Reviews, 2012. 64: p. 24-36.
50. Barratt, G.M., Therapeutic applications of colloidal drug carriers. Pharm Sci Technolo Today, 2000. 3(5): p. 163-71.
51. Nimesh, S., N. Gupta, and R. Chandra, Cationic polymer based nanocarriers for delivery of therapeutic nucleic acids. J Biomed Nanotechnol, 2011. 7(4): p. 504-20.
52. Malam, Y., M. Loizidou, and A.M. Seifalian, Liposomes and nanoparticles: nanosized vehicles for drug delivery in cancer. Trends in Pharmacological Sciences, 2009. 30(11): p. 592-599.
53. Pezzoli, D., et al., Chitosan-Graft-Branched Polyethylenimine Copolymers: Influence of Degree of Grafting on Transfection Behavior. Plos One, 2012. 7(4).
54. Bhavsar, C., et al., Functionalized and graft copolymers of chitosan and its pharmaceutical applications. Expert Opin Drug Deliv, 2017. 14(10): p. 1189-1204.
55. H., H., et al., Nanoparticle decoration with surfactants: Molecular interactions,assembly, and applications. Surface Science Reports, 2017. 72(1): p. 1-58.
56. Rinaudo, M., Chitin and chitosan: Properties and applications. Progress in Polymer Science, 2006. 31(7): p. 603-632.
57. Koide, S.S., Chitin-chitosan: Properties, benefits and risks. Nutrition Research, 1998. 18(6): p. 1091-1101.

58. Younes, I. and M. Rinaudo, Chitin and chitosan preparation from marine sources. Structure, properties and applications. Mar Drugs, 2015. 13(3): p. 1133-74.
59. Elieh-Ali-Komi, D. and M.R. Hamblin, Chitin and Chitosan: Production and Application of Versatile Biomedical Nanomaterials. Int J Adv Res (Indore), 2016. 4(3): p. 411-427.
60. Wan, S., et al., Chitosan-modified lipid nanovesicles for efficient systemic delivery of l-asparaginase. Colloids Surf B Biointerfaces, 2016. 143: p. 278-84.
61. van den Broek, L.A., et al., Chitosan films and blends for packaging material. Carbohydr Polym, 2015. 116: p. 237-42.
62. Rampino, A., et al., Chitosan nanoparticles: preparation, size evolution and stability. Int J Pharm, 2013. 455(1-2): p. 219-28.
63. Cheung, R.C., et al., Chitosan: An Update on Potential Biomedical and Pharmaceutical Applications. Mar Drugs, 2015. 13(8): p. 5156-86.
64. Katas, H. and H.O. Alpar, Development and characterisation of chitosan nanoparticles for siRNA delivery. J Control Release, 2006. 115(2): p. 216-25.
65. Sreekumar, S., et al., Parameters influencing the size of chitosan-TPP nano- and microparticles. Sci Rep, 2018. 8(1): p. 4695.
66. Stie, M.B., et al., Delivery of proteins encapsulated in chitosan-tripolyphosphate nanoparticles to human skin melanoma cells. Colloids Surf B Biointerfaces, 2018. 174: p. 216-23.
67. Xu, Y. and Y. Du, Effect of molecular structure of chitosan on protein delivery properties of chitosan nanoparticles. Int J Pharm, 2003. 250(1): p. 215-26.
68. J., T., S. K., and D.P.K. . Chitosan in the Light of Nanobiotechnology: A Mini Review. J Biomed Tech Res., 2015.
69. Thongngam, M. and D.J. McClements, Characterization of interactions between chitosan and an anionic surfactant. Journal of Agricultural and Food Chemistry, 2004. 52(4): p. 987-91.
70. Sorlier, P., et al., Relation between the degree of acetylation and the electrostatic properties of chitin and chitosan. Biomacromolecules, 2001. 2(3): p. 765-72.
71. Gokce, Y., et al., Ultrasonication of chitosan nanoparticle suspension: Influence on particle size. Colloids and Surfaces a-Physicochemical and Engineering Aspects, 2014. 462: p. 75-81.
72. Kalliola, S., et al., The stability of green nanoparticles in increased pH and salinity for applications in oil spill-treatment. Colloids and Surfaces a-Physicochemical and Engineering Aspects, 2016. 493: p. 99-107.
73. Khanmohammadi, M., H. Elmizadeh, and K. Ghasemi, Investigation of Size and Morphology of Chitosan Nanoparticles Used in Drug Delivery System Employing Chemometric Technique. Iranian Journal of Pharmaceutical Research, 2015. 14(3): p. 665-75.
74. Fabregas, A., et al., Impact of physical parameters on particle size and reaction yield when using the ionic gelation method to obtain cationic polymeric chitosan-tripolyphosphate nanoparticles. Int J Pharm, 2013. 446(1-2): p. 199-204.
75. Masarudin, M.J., et al., Factors determining the stability, size distribution, and cellular accumulation of small, monodisperse chitosan nanoparticles as candidate vectors for anticancer drug delivery: application to the passive encapsulation of [(14)C]-doxorubicin. Nanotechnol Sci

Appl, 2015. 8: p. 67-80.
76. Shukla, T., et al., Biomedical applications of microemulsion through dermal and transdermal route. Biomed Pharmacother, 2018. 108: p. 1477-494.
77. Vaishya, R.D., et al., Controlled ocular drug delivery with nanomicelles. Wiley Interdiscip Rev Nanomed Nanobiotechnol, 2014. 6(5): p. 422-37.
78. Thipparaboina, R., et al., Micellar carriers for the delivery of multiple therapeutic agents. Colloids Surf B Biointerfaces, 2015. 135: p. 291-308.
79. Zhu, L., et al., An eco-friendly synthesized mesoporous-silica particle combined with WSe2-graphene-TiO2 by self-assembled method for photocatalytic dye decomposition and hydrogen production. Sci Rep, 2018. 8(1): p. 12759.
80. V., S., et al., Effects of Some Parameters on Particle Size Distribution of Chitosan Nanoparticles Prepared by Ionic Gelation Method. Journal of Cluster Science 2013. 24(3): p. 891–903.
81. Zhang, W., et al., The hypolipidemic activity of chitosan nanopowder prepared by ultrafine milling. Carbohydr Polym, 2013. 95(1): p. 487-91.
82. Le, T.M.H., et al., Preparation of drug nanoparticles by emulsion evaporation method. Apctp-Asean Workshop on Advanced Materials Science and Nanotechnology (Amsn08), 2009. 187-88
83. Tiyaboonchai, W. and N. Limpeanchob, Formulation and characterization of amphotericin B-chitosan-dextran sulfate nanoparticles. International Journal of Pharmaceutics, 2007. 329(1-2): p. 142-49.
84. Bhattarai, N., et al., Chitosan and lactic acid-grafted chitosan nanoparticles as carriers for prolonged drug delivery. International Journal of Nanomedicine, 2006. 1(2): p. 181-87.
85. Prego, C., et al., Chitosan-PEG nanocapsules as new carriers for oral peptide delivery. Effect of chitosan pegylation degree. J Control Release, 2006. 111(3): p. 299-308.
86. Parveen, S. and S.K. Sahoo, Long circulating chitosan/PEG blended PLGA nanoparticle for tumor drug delivery. Eur J Pharmacol, 2011. 670(2-3): p. 372-83.
87. E., M., et al., Chitosan: a vehicle to carry active drug molecules into the diseased periodontal site. Guident Journal, 2015. p.232-3.
88. Calvo, P., et al., Chitosan and chitosan/ethylene oxide-propylene oxide block copolymer nanoparticles as novel carriers for proteins and vaccines. Pharm Res, 1997. 14(10): p. 1431-6.
89. Desai, K.G., Chitosan Nanoparticles Prepared by Ionotropic Gelation: An Overview of Recent Advances. Crit Rev Ther Drug Carrier Syst, 2016. 33(2): p. 107-58.
90. Yousefpour, P., et al., Preparation and comparison of chitosan nanoparticles with different degrees of glutathione thiolation. Daru, 2011. 19(5): p. 367-75.
91. Debnath, S.K., et al., Development and evaluation of Chitosan nanoparticles based dry powder inhalation formulations of Prothionamide. PLoS One, 2018. 13(1): p. e0190976.
92. Wenny R., et al., The effect of temperature and chitosan concentration during storage on the growth of chitosan nanoparticle produced by ionic gelation method. International Seminar on Fundamental and Application of Chemical Engineering 2017.
93. Tsai, W.H., et al., EGFR-targeted photodynamic therapy by curcumin-encapsulated chitosan/TPP nanoparticles. Int J Nanomedicine, 2018. p. 903-16.

94. Ozbas-Turan, S. and J. Akbuga, Plasmid DNA-loaded chitosan/TPP nanoparticles for topical gene delivery. Drug Deliv, 2011. 18(3): p. 215-22.
95. Gwak, S.J., et al., Chitosan/TPP-hyaluronic acid nanoparticles: a new vehicle for gene delivery to the spinal cord. J Biomater Sci Polym Ed, 2012. 23(11): p. 1437-50.
96. Tang, E.S., M. Huang, and L.Y. Lim, Ultrasonication of chitosan and chitosan nanoparticles. Int J Pharm, 2003. 265(1-2): p. 103-14.
97. Elmore, S., Apoptosis: a review of programmed cell death. Toxicol Pathol, 2007. 35(4): p. 495-516.
98. Eadon, M.T., et al., In Vivo siRNA Delivery and Rebound of Renal LRP2 in Mice. J Drug Deliv, 2017. 2017: p. 4070793.
99. L.Zhen, et al., Chitosan oligomers as drug carriers for renal delivery of zidovudine. Carbohydrate Polymers, 2011: p. 2284–290.
100. Jing, S.B., et al., Effect of chitosan on renal function in patients with chronic renal failure. J Pharm Pharmacol, 1997. 49(7): p. 721-3.
101. Saranya, N., et al., Chitosan and its derivatives for gene delivery. Int J Biol Macromol, 2011. 48(2): p. 234-8.
102. Huang, M., et al., Uptake of FITC-chitosan nanoparticles by A549 cells. Pharm Res, 2002. 19(10): p. 1488-94.
103. Zheng, Y., et al., Cytotoxicity and Genotoxicity in Human Embryonic Kidney Cells Exposed to Surface Modify Chitosan Nanoparticles Loaded with Curcumin. AAPS PharmSciTech, 2016. 17(6): p. 1347-352.
104. Gao, S., et al., Megalin-mediated specific uptake of chitosan/siRNA nanoparticles in mouse kidney proximal tubule epithelial cells enables AQP1 gene silencing. Theranostics, 2014. 4(10): p. 1039-51.