POLYETHYLENIMINE – STARCH NANOPARTICLES FOR CANCER GENE THERAPY

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BERKE BİLGENUR KANDEMİR

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ABSTRACT

PEI – STARCH NANOPARTICLES FOR CANCER GENE THERAPY

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Cancer is the most serious disease of in this century and death rates due cancer are increasing rapidly. Since the conventional cancer treatment techniques like chemotherapy, radiation therapy and surgical operations are not sufficiently efficient and they even are harmful for healthy tissues, there is an urgent need for effective treatment techniques with minimal or no side effects. Controlled drug delivery systems are offered as an alternative approach to cancer therapy that helps prevent excessive drug use and may even target the drug to the disease site. It was shown that one of the most efficient therapeutic agents is the use of gene therapy involving siRNA. Cationic nanoparticles are known to be efficient tools in siRNA delivery specificially into the targeted tumor cells. This study aims to develop an autophagic siRNA delivery system to cancer cells using positively charged nanospheres of polyethylenimine(PEI) – starch which possess the required positive charge by employing PEI molecules while lowering the cytotoxic effect of PEI through complexation with starch. PEI-starch nanospheres were prepared by water-in-oil microemulsion method. The size and zeta potential of the PEI-starch nanospheres were characterized by measuring the zeta potential and the particle size, and studying the topography with SEM. Mean diameter of unloaded particles was 60.3 nm and upon loading with siRNA this increased to 84.6 nm. SEM micrographs revealed that the nanospheres were very uniform (NIH Image J was used to measure the diameter using the micrographs) and had smooth surfaces. However, batch-to-batch size variation of the particles was observed.

Zeta potential of PEI-starch nanoparticles increased when the PEI /starch ratios were increased, with the highest surface charge of 8.7 mV (PEI:Starch, 9:1 (w/w)). The encapsulation efficiency of the nanospheres was 1.5 % and this could be increased by optimizing pH conditions of water phase, and also the crosslinking and washing conditions. The nanospheres released 50 % of their siRNA content in the first 3 days and all of the content in a week. Confocal laser scanning microscopy (CLSM) micrographs show the nanoparticles affiliated with MCF 7 cells and additional staining of the nucleus is able to show their location more adequately. Western blot of breast cancer cells treated with EF2K siRNA loaded nanoparticles showed that the EF2K production which leads to proliferation of cancer cells was silenced after the treatment with the siRNA loaded nanoparticles.

Keywords: Polyethylenimine, starch, nanoparticle, siRNA, gene delivery, gene silencing, drug delivery, gene therapy, cancer.

ÖZ

KANSER GEN TERAPİSİ AMAÇLI POLİETİLENİMİN – NİŞASTA NANOPARÇACIKLAR

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Kanser her yıl artış gösteren ölüm oranlarıyla bulunduğumuz yüzyılın en ciddi hastalığı konumundadır. Uygulanmakta olan kemoterapi, radyasyon tedavisi ve cerrahi uygulamalar hem yeterli verime sahip değil hem de sağlıklı hücrelere de zarar vermektedir. Bu yüzden verimli, toksik olmayan ve hızlı sonuç veren tedavine yöntemlerine ihtiyaç duyulmaktadır. İlacın az miktarda daha etkin salımı açısından kontrollü ilaç salım sistemleri kanser tedavisi için önerilen yöntemlerden biridir. Gen terapisi yöntemlerinden ise en etkin olanının siRNA tedavisi olduğu gösterilmektedir. Hedeflenen tümör hücrelerine siRNA salımı gerçekleştirmede katyonik nanokürelerin verimli birer taşıyıcı olarak kullanıldıklarını görmekteyiz. Bu çalışmada, kanser hücrelerinde otofajiye neden olan EF2K siRNA geninin pozitif yüzey yüklü polietilenimin (PEI) – nişasta küreler ile kanser hücrelerine salınımı amaçlanmıştır. PEI pozitif yüklü yapısı ile nanoküreler artı yük kazandırırken, nişasta moleküllerinin ise PEI2den kaynaklanabilecek sitotoksisiteyi azaltması amaçlanmıştır. PEI – nişasta nanoküreler yağ içinde su emülsiyon tekniği ile hazırlanmıştır. Büyüklük ve zeta potansiyel değerleri Taramalı Elektron Mikroskobu (TEM) ile araştırılmıştır. NIH Image J programı nanoküre çapının hesaplanmasında kullanılmıştır. siRNA yüksüz kürelerin ortalama değeri 60.3 nm iken siRNA yüklü nanoküreler ortalama 84.6 nm çapına sahiptirler. TEM sonuçları ayrıca nanokürelerin standart büyüklükte ve pürüzsüz yüzey morfolojisine sahip olduğunu göstermektedir. Zeta potansitel değerleri kürelerdeki PEI /nişasta oranı arttıkça kürelerin yükünün arttığını ve en fazla (PEI:Nişasta, 9:1 (w/w)) 8.7 mV olduğunu göstermektedir. PEI – nişasta nanoküreler siRNA yükleme verimleri ise % 1.5 olup su pazının

pH'ı, çapraz bağlama şartları ve yıkama aşamaları optimize edilerek arttırılabilme olanağına sahiptir. Nanoküreler içeriklerinin % 50'sini ilk 3 günde, diğer % 50'sini ise bir hafta içinde salmışlardır. Konfokal Lazer Taramalı Mikroskobu (KLTM) ile hücre içine girebilmeleri gözlenmiştir ve nanokürelerin hücre etrafında toplandıkları görülmüştür ve ek çekirdek boyamalarıyla nanokürelerin hücre içi veya etrafındaki konumları daha net gözlenebilecektir. Western blot ile kanser hücrelerinin EF2K siRNA yüklü kürelerle tedavi edildikten sonra EF2K seviyelerinin azaldığı gözlemlenmiştir.

Anahtar Kelimeler: siRNA, gen tedavisi, nanoparçacık, polietilenimin, nişasta, kanser, hedefli salım

Dedicated to my lovely family…

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3D	Three Dimensional
ATP	Adenosine triphosphate
Au-Pd	Gold-palladium
BCA	Bicinchoninic assay
BSA	Bovine serum slbumin
CO_2	Carbon dioxide
CLSM	Confocal laser scanning microscope
CPP	Cell penetrating peptide
Da	Dalton
DAPI	4', 6-diamidino-2-phenylindole
DNA	Deoxyribonucleic acid
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulfoxide
DOPC	Di-oleoyl-phosphatidylcholine
E-cadherin	Epithelial cadherin
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
eEF2	Eukaryotic elongation factor 2
EF2	Elongation factor 2
EF2K	Elongation factor 2 kinase
EGF	Endothelial growth factor
EMT	Endothelial to mesenchymal transition
EPR	Enhanced permeation and retention
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
g	Gram
G_1 phase	Gap 1
h	Hour
HPMA	N-(2-Hydroxypropyl)methacrylamide
kDa	Kilo dalton
mAb	Monoclonal antibody
MDR	Multiple drug resistance
min	Minute
Mg	Magnesium

mg	Milligram
mm	Millimeter
mL	Milliliter
nm	Nanometer
M	Molarity
mM	Millimolar
mRNA	Messenger RNA
mTOR	Mammalian target of rapamycin
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
nM	Nanomolar
NP	Nanoparticle
P53	Protein 53
PBS	Phosphate buffer saline
PEG	Polyethylene glycol
PEI	Polyethylenimine
Pen/Strep	Penicillin/streptomycin
PI3	Protease inhibitor 3
PI3K	Protease inhibitor 3 kinase
PLA	Poly(lactic acid)
PLGA	Poly(lactic Acid-co-glycolic acid)
PLL	Poly(l-lysine)
PLLA	Poly(l-lactic Acid)
PMMA	Poly(methyl methacrylate)
RB	Retinoblastoma
RCF	Relative centrifugal force
RISC	RNA induced silencing complex
RNA	Ribonucleic acid
RNAi	RNA interference
RPM	Repeats per minute
S-phase	Synthesis phase
SDS-PAGE	Sodium dodecyl sulphate- polyacrylamide gel electrophoresis
SEM	Scanning electron microscopy
shRNA	Short hairpin RNA
siRNA	Short interference RNA
SMANCS	Poly-(styrene-co-maleyl-half-n-butylate)-neocarzinostatin
TCPS	Tissue culture polystyrene
TGF	Transforming growth factor
TP53	Tumor protein 53
tRNA	Transfer RNA
UV	Ultra violet
v/v	Volume/volume

w/v	Weight/volume
w/o	Water-in-oil
μm	Micrometer

CHAPTER 1

INTRODUCTION

1.1. Importance of Cancer as a Disease

Cancer is probably the most serious disease of century, being major cause of deaths and with more than ten million diagnosis annually (Misra *et.al.,* 2010). Since the disease alters many of the cell signaling mechanisms and thus cellular physiology in various ways, it is a highly complicated and difficult to be understood (Zou *et.al.,* 2005; Reichart *et.al.,* 2008). Although cancer evolves as a localized disease, it is able to spread over to other locations in the body making the local treatments inefficient. Until now, treatments involved clinical and pathological techniques diagnosis was based on morphological analyses such as radiological and histopathological imaging. There are also other treatment techniques which involve chemotherapy, radiation therapy and surgery (Singhal *et.al.,* 2010). Nowadays, as a result of the technological progress, the techniques are being continually improved but are still not optimal. The problems in these therapies involve nonspecific (or systemic) distribution of the therapeutics throughout the body, causing insufficient therapeutic concentrations at the tumor site, and causing multiple drug resistance (MDR), and intolerable cytotoxicity (Das *et.al.,* 2009; Parveen *et.al.,* 2009). Since there are many types of cancer specific for different tissues in the body, current techniques are not capable of handling them equally well in both diagnosis and therapy (Wang *et.al.,* 2008). There is, therefore a serious need novel and efficient treatment techniques which are supported by technological advances that help identify tumor boundaries, identify remaining cancer cells after treatment and also micrometastases. Also there is a great need to construct efficient drug delivery vehicles.

1.1.1. Evolution of cancer

Cancer can be described as a state where division of cells takes place with an abnormal speed. The general characteristic of cancer is the invasion of other tissues using blood, the lymphatic system or the cavities (peritoneum). First they evolve locally in a tissue, then grow and finally spread to other tissues and this spreading process is called metastasis. The cancer type and characteristic

depends on the tissue type in which it first evolves and is named according to this local position they evolve in the body (Figure 1.1).

The cancer cells evolve after several mutations if they cannot be fixed by the cell's own mechanism. The mutated cells keep on dividing. Proliferated mutated cells gain other mutations throughout their life cycle. While they proliferate they gain more mutations. Finally, these mutated cell colonies gain some features which are characteristics of cancer cells; mainly they have rapid and uncontrolled division and proliferation. At a later stage, they metastasize and invade other tissues forming uncontrolled and mutated colonies in those locations (Figure 1.2).

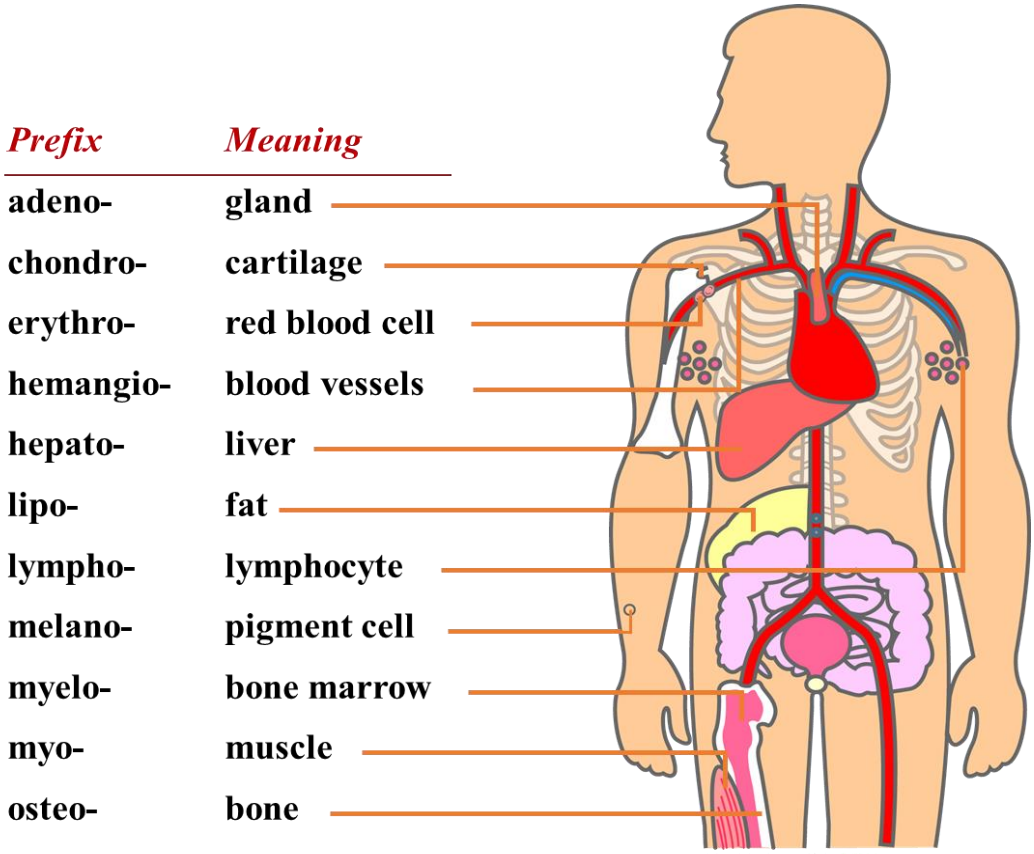


Figure 1.1: Cancer nomenclature according to location in the body (Adapted from National Cancer Institute, 2005)

Defects in detection of DNA damage and DNA repair mechanisms causes genomic instability of cells and this is the fundamental cause of cancer (Hanahan *et.al.*, 2000). The causes of genomic instability can be summarized as telomere dysfunction, loss of cell cycle checkpoint and persistence in DNA damage. The instability of genome causes additional mutations and finally the

cell become malignant (Brown *et.al.*, 2005). This is called "mutator hypothesis of cancer" (Sarasin *et.al.*, 2003).

There are some mechanisms hold by the cell, to inhibit genomic instability. The most important element of these protection mechanisms is tumor suppressor protein p53. The studies show that one of the mostly mutated genes detected in human cancer is TP53 that has been an evidence for its anticancer activity (Levine *et.al.*, 1997). Once activated in response to cellular stresses like DNA damage, hypoxia or proliferative signals, P53 causes apoptosis or cell cycle arrest in cancer cells. The cell cycle arrest is crucial for these kind of cells in order to obstruct uncontrolled proliferation that also means additional mutations, thus increase in oncogenic progresses. In the cells that have these kinds of mutations, p53 is inhibited also, thus inappropriate proliferation continues.

1.1.2. Characteristics of cancer cells

There are mainly six physiologic alterations that occur in malignant cells:

- Self-sufficiency in growth signals
- Resistance to growth inhibitory signals
- Escape from programmed cell death (apoptosis)
- Potential of unlimited replication
- Enhanced angiogenesis
- Tissue invasion and metastasis.

These characteristics is generally abandoned in most of the cancer types (Hanahan *et.al.*, 2000).

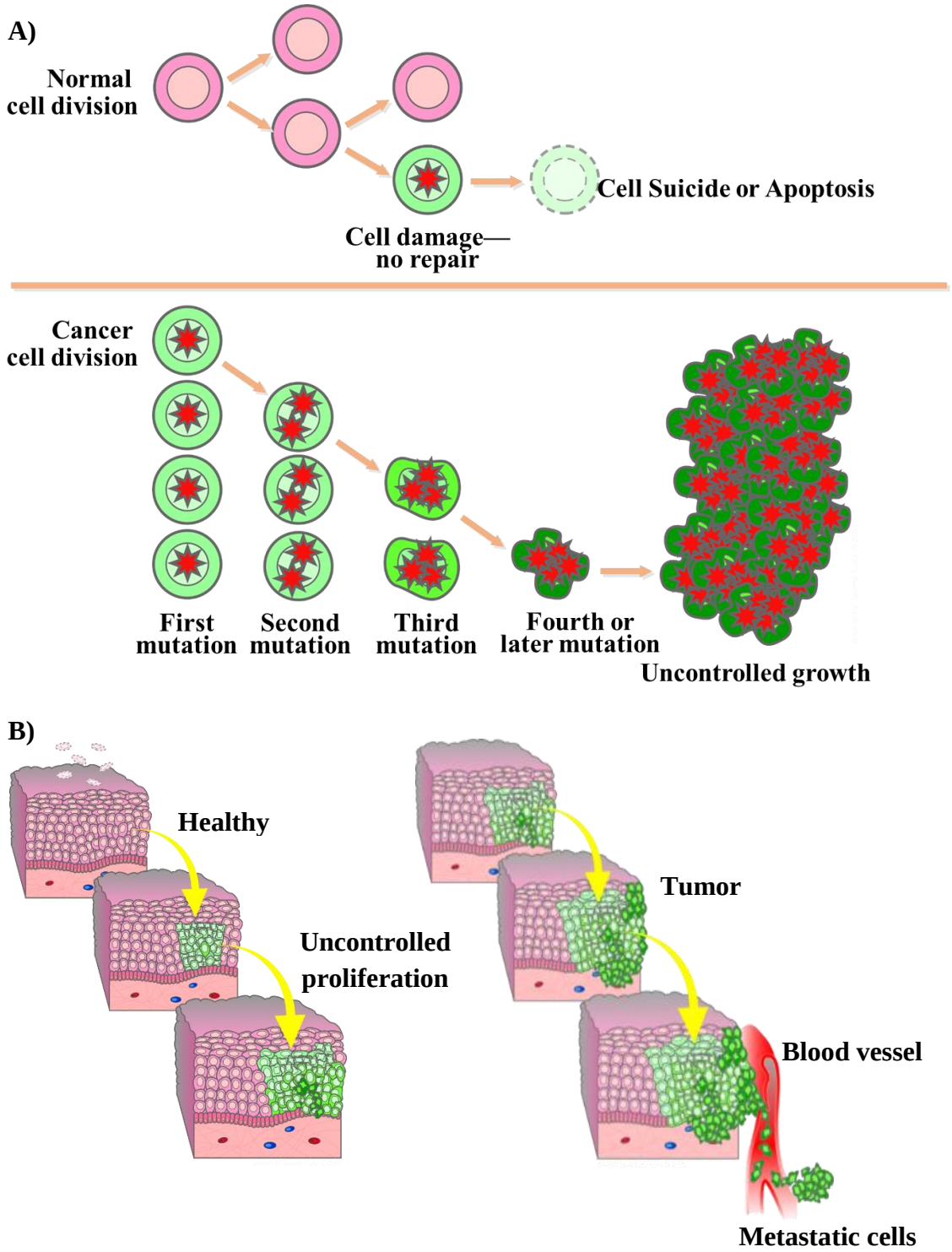


Figure 1.2: Evolution of cancer (A) and metastasis (B) (Adapted from National Cancer Institute, 2005)

Rather than being just a clump of proliferative cells, tumors have complex tissues including various cell types interacting with each other. Normal cells are also present in tumor microenvironment as active participants of tumor formation that form tumor associated stroma. Thus, normal cells play a role in the development of the characteristic behavior of cancer cells. Recently this issue, that tumors cannot be assessed by only cancer cell characteristics, tumor microenvironment must also be taken into consideration and has become a hot topic for cancer researchers (Hanahan *et.al.*, 2011).

Normal tissues have control over growth promoting signals released during specific periods of cell cycle and growth, achieve homeostasis and keep the tissue architecture and function. These signals are generally growth factors that interact with cell surface receptors with intracellular tyrosine kinase functional domains. Once a signal is received, the cells regulate their developmental progress, including survival and energy metabolism. These mitogenic signals are received only at specific steps of cell cycle and growth, at specific locations and from one cell to its neighbors. Moreover, in the environment of a cell, elements of extracellular matrix like functional enzymes play an important role in the secretion and activation of growth factors. In cancer cells, conversely, growth factor ligands are produced independently, and related receptors are also expressed independently. This results in autocrine proliferative stimulation. Tumor cells also trigger tumor associated stroma to supply various growth factors. Additionally, by increasing the amount of receptors on surface of cells, tumor cells make themselves highly responsive to growth factor ligands. Alterations also occur in the structure of these receptors that make them function in a ligand independent manner. Also there may be other alterations in downstream process of this receptor signaling pathway which escapes dependence on ligand interaction, like mutations in B-Raf protein function (Davies *et.al.*, 2010) and PI3 kinase signaling pathway (Yuan *et.al.*, 2008; Jiang *et.al.*, 2009). The cancer cells may also inhibit negative feedbacks of these uncontrolled growth and proliferation events, by gaining mutations in Ras oncoprotein pathway and mTOR kinase as inhibitor of PI3K signaling (O'Reilly *et.al.*, 2006; Sudarsanam *et.al.*, 2010). Since excessive production of the proliferation signals also trigger cell senescence and apoptosis in cells (Lowe *et.al.*, 2004; Evan *et.al.*, 2009; Collado *et.al.*, 2010), most human cancer cells have an optimal level of these genes in order to escape from senescence.

In addition to their support on growth stimulatory events, the cancer cells also act against tumor suppressor genes such as the two main ones that encode RB (retinoblastoma associated) and TP53 proteins.

Other main characteristics of cancer cells are; evasion of contact inhibition, corruption of antiproliferative TGF-β pathway (Bierie *et.al.*, 2006; Massague *et.al.*, 2008; Ikushima *et.al.*, 2010), resistence to apoptosis (by interrupting various mechanisms that control or result in cell death), inhibition of autophagy that mediates cell death, enabling replicative immortality (by hyperactivity of telomerases), and angiogenic effects that involve high expression of VEGF and EGF. Invasion and metastasis are additional futures of cancer cells, which are co-localization mechanisms of cancer cells in different parts of the body. This happens as a consequence of inhibition of cell-to-cell adhesion molecule E-cadherin (Cavallaro *et.al.*, 2004; Berx *et.al.*, 2009)

which make cancer cells anchorage independent and activation of EMT program (endothelial to mesenchymal transition) (Barrallo-Gimeno *et.al.*, 2005; Klymkowsky *et.al.*, 2009; Polyak *et.al.*, 2009; Thiery *et.al.*, 2009; Yilmaz *et.al.*, 2009). Recent studies also show the existence of additional capabilities of cancer cells, like avoidance of immune destruction and deregulation of cellular energetics.

Briefly, in the recent years, the pathogenesis of cancer has been clarified by researchers, that it evolves through multistep mutagenic process. The cancer cells were recognized to have some main common characteristics like uncontrolled and limitless potential of proliferation, self-sufficiency in growth signals, independency from antiproliferative and apoptotic signals. They also trigger stromal cells to support growth factor production for tumor growth, trigger angiogenesis around tumor and blood vessels to supply nutrients and oxygen. They avoid immune detection (Kroemer *et.al.*, 2008) and finally metastasize.

Many of these properties are achieved by cancer cells as a result of genetic alterations, which includes gain-of-function, mutation, amplification and overexpression of oncogenes; in combination with loss-of-function mutation, deletion, epigenetic silencing of tumor suppressors.

Additional to these hallmarks of cancer, Luo *et.al.* proposed some additional properties of cancer cells (2009). These do not initiate tumorigenesis but common characteristics of tumors. These are stress phenotypes that involve DNA damage/replication stress, proteotoxic stress, mitotic stress, metabolic stress, and oxidative stress.

Cellular metabolism of malignant cells are different than normal cells. While normal cells utilize nutrients with aerobic respiration, malignant cells carry out glycolysis which is an anaerobic respiration even in the presence of oxygen. This increases the requirement of cancer cells for glycolysis to produce ATP. This is called "Warburg effect" (Wu *et.al.*, 2009).

1.2. Treatment of Cancer

1.2.1. Anticancer agents

The studies show that, although there are a number of key oncogenes and/or tumor suppressors that were generally mutated in cancer cells, there were also a high number of alterations in various genes that cause oncogenesis (Luo *et.al.*, 2009). For example 20% of all kinases were effective in tumorigenesis (Greenman *et.al.*, 2007), and these various alterations differ due to type of cancer. This negatively affects developments in cancer therapy since almost each type of cancer is related with a different set of mutations.

For this reason, a successful therapy must target common specificities of all cancer types and cause apoptosis, necrosis, senescence, or differentiation. Also the therapies must have a tendency to affect cancer cells more than healthy cells. Current techniques like radiation and chemotherapy

get use of augmented sensitivity of cancer cells to DNA damage but have failed to provide a selective cure.

The targeted therapies on the other hand are more promising with respect to selectivity which makes them more efficient than chemotherapy and radiation.

Considering these requirements for successful therapy, cancer treatment studies were commonly based on therapeutic targeting hallmarks of cancer cells (Figure 1.3). One of them is suppressing oncogene activity and enhancement of tumor suppressor activity. Targeting of protein kinase oncogenes; BCR-ABL (imatinib/Gleevec), EGFR (gefitinib/Iressa, erlotinib/Tarceva), HER2 (trastuzumab/Herceptin) has been proven successful.

In almost all types of tumors the spontaneous DNA damage rate and replication stress are abundant. Mutations in DNA repair pathways cause additional sensitivity to DNA damage (Harper *et.al.*, 2007). The studies to enhance DNA damage stress is proven to be successful like ATM and Chk1 genes. Use of taxol as stress overloading agent was shown to achieve success in treatment of breast and ovarian cancers by stabilizing microtubules.

Another class of targets recently studied are components of protein maturation pathway, like chaperons that enhance protein folding and proteases that turn over unfolded proteins. For example, geldanamycin is used to inhibit ATPase activity which is required for activation of chaperon HSP90. Moreover, bortezomib was used to treat multiple myeloma by inhibiting proteosomal degradation (Richardson *et.al.*, 2006).

Also, temperature elevation is used with chemotherapy to trigger protein unfolding by the help of hyperthermia in treatment of colon, breast, testicular, prostate, liver cancers (Fiorentini *et.al.*, 2006).

A different route for cancer treatment is to inhibit nutrient uptake and/or utilization by causing metabolic and cause oxidative stress and to inhibit angiogenesis. Several kinds of drugs and RNA interference (RNAi) therapies are being designed for enhance these inhibitions in cancer cells. For example dichloroacetate is used to inhibit pyruvate dehydrogenase kinase can selectively cause apoptosis in cancer cells.

On the other hand, RNAi knockdown of lactate dehydrogenase (element of last step of glycolysis) is used to attenuate tumor cell growth. To inhibit angiogenesis, vascular endothelial growth factor (VEGF) is the major target. Anti-VEGF antibody bevacizumab was developed (Ferrara *et.al.*, 2004). Additionally sorafenib and sunitinib that target VEGF receptors are shown to be efficient in treatment of metatstatic renal carcinoma. Imatinib is another anticancer agent successfully used by Yang *et.al.* (2008) to inhibit stromal support of tumor growth.

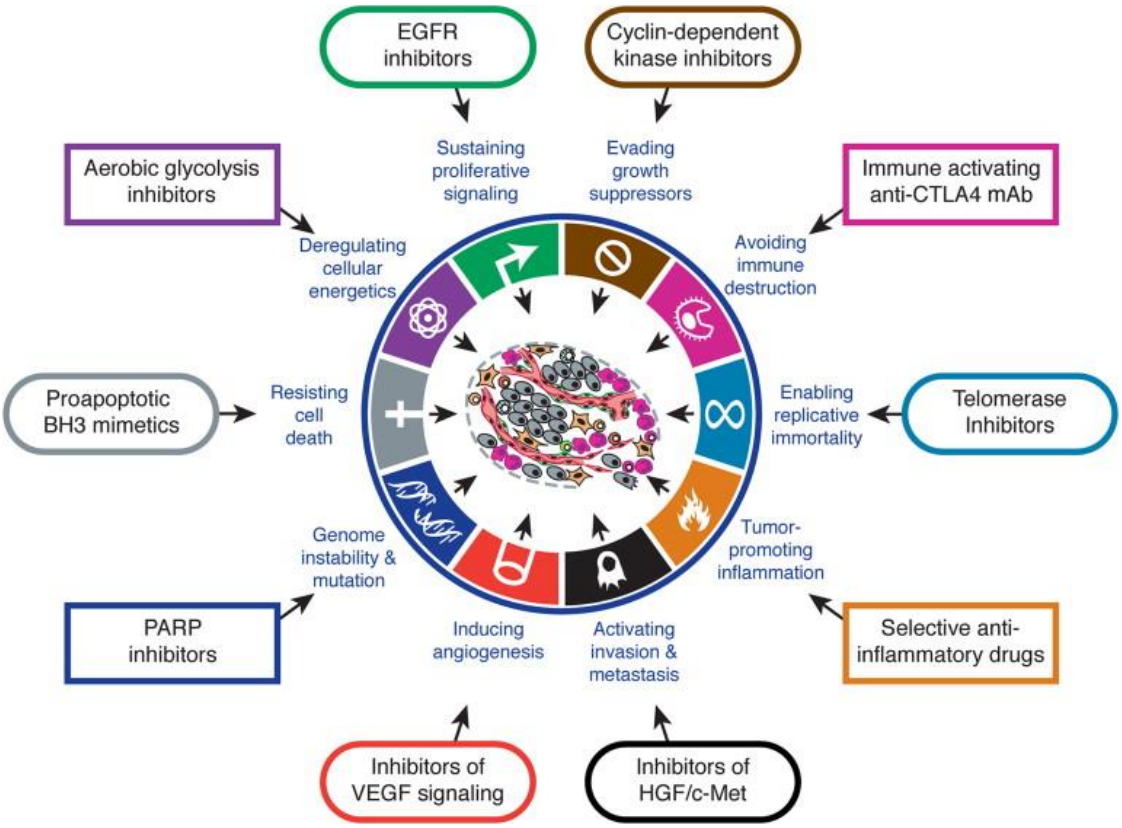


Figure 1.3: Therapeutic targeting of the hallmarks of cancer (Adapted from Hanahan *et.al.*, 2011).

There are various signaling pathways that can be targeted to inhibit growth of cancer cells and to cause apoptosis. The elements of these pathways can be unfunctionalized by RNAi therapy. Also, RNAi is functional for target discovery. As an advantage of RNAi therapy, partial or complete knockdown of target protein can be achieved with RNAi therapy. Moreover, only knowledge of genetic sequence of target gene is essential for treatment, it does not require additional knowledge like gene's cellular function or mutational status.

This therapy can be easily applied to animal models, does not require inhibitor development step. The genome wide screening with RNAi helps discover identical genes that function in cancer development and the ones that are not involved in.

1.2.2. Cancer treatment techniques

The most widely applied cancer therapies are; radiation therapy, chemotherapy and surgery. A technique recently getting popular and promising is targeted therapy, sometimes in combination with gene therapy. The targeted therapy system uses vectors in order to deliver drugs (or genes in gene therapy cases) to the target cancer cells, avoiding drug delivery to non-cancer or healthy cells. This method provides efficiency of the therapy and also lowers the amount of drug that is used per application. Since the administered drug is aimed to the target cells, there is expected to be less non-specific delivery and the concentration of the drug will be maintained after the injection. A decrease in drug concentration can cause drug resistance in cancer cells, so it is important to keep the drug concentration in the range that does not cause drug resistance.

1.2.2.1. Untargeted drug delivery

In normal untargeted drug delivery, the drug is delivered into the body without a carrier system, by various routes like topical, enteral, or parenteral routes according to the required local or systemic effect. According to type of drug, targeted body location or system and structural properties of drug, an efficient delivery route is selected. In topical administration, drug is applied directly to where its action is desired. Eye drops that involve antibiotics for conjunctivitis are examples of topical administration. Enteral routes, on the other hand, involves gastrointestinal pathway for administration and are generally used for supplying drugs for a systemic effect. Parenteral administration also creates systemic effect but involves routes other than the digestive tract. For all of these pathways, there are numerous off target healthy tissues and organs which are affected by drugs which makes this kind of untargeted drug delivery disadvantageous. Since the drug is distributed to a broader range and affecting not only the target system or tissue but all the tissues it can reach, only a small percent of the administered drug can achieve its goal. This kind of drug delivery damages healthy tissues and delays the cure. In order to optimize the physicochemical properties of a drug in the biological tissue, and thus enhance its efficacy and safety, a targeted delivery vehicle is needed. According to the structural features of vector, time and release rate of the drug can be controlled and drug can reach the target site more efficiently.

1.2.2.2. Drug Delivery Systems

Drug delivery systems are constructed in order to avoid disadvantages of drug administration routes, like systemic toxicity, inactivation and/or degradation of drugs in circulation systems and unspecificity. Specific and more protective therapy can be provided with targeted drug delivery systems. In these systems, vectors provide controlled (systemic) or sustained release according to their design and composition. These systems control the desired time and duration the drug is

desired to be released (Figure 1.4). The drug levels in blood plasma go beyond optimal therapeutic range which is between maximum desired level and minimum effective level. In order to keep the drug levels in this optimal range, frequent administration with increased dose levels were tried but this caused drug toxicity since drug levels in blood plasma went beyond maximum desired level of drug (Pierigé *et al*, 2008). Drug delivery systems are useful for keeping the drug levels in blood plasma in therapeutic range.

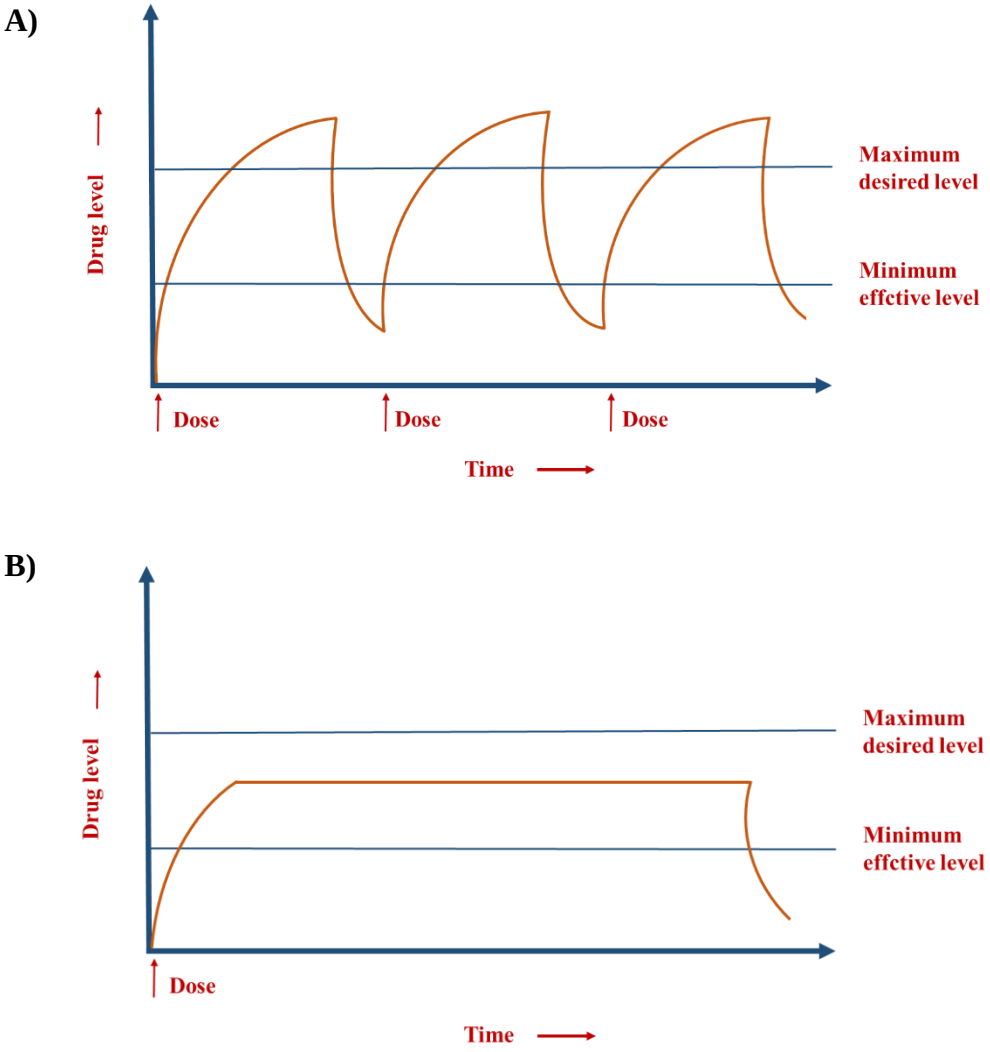


Figure 1.4: Difference between controlled release and conventional systemic release. Red plot shows drug level in plasma. The region between maximum desired level and minimum effective level is optimal therapeutic range.

The type drug release kinetics of drug delivery systems are zero order, first order or Highuchi kinetics. While conventional delivery mechanisms generally obey to first order kinetics, but in order to keep the drug concentration constant in plasma, zero order kinetics must be achieved and this is possible to be provided by a drug delivery system. The mathematical models of these release kinetics are represented in Table 1.1.

Table 1.1: Mathematical formulations of release kinetics and applications

Release kinetic model	Mathematical formula	Application
Zero order	$D_t = k_0 t$	Transdermal systems, matrix tablets with low soluble drugs in coated forms, osmotic systems
First order	$\ln D_t = \ln D0 - k_1 t$	Water soluble drugs in porous matrices
Highuchi	$D_t = k_H t^{1/2}$	Transdermal systems, matrix tablets with water soluble drugs, insoluble matrices
Hixon-Crowel	$D_t^{1/3} = D_0^{1/3} - k_{HC} t$	Tablets that initial geometry is kept constant (ex: multiple planes parallel to each other)

Adapted from Dash, *et.al*., 2010.

The abbreviations in Table 1.1 are as follows; D_t = released drug amount at time t, D_0 = drug amount in the system at time 0, k_0 = zero Order release constant, k_1 = first Order release constant, k_H = Higuchi release constant, k_{HC} = Hixson-Crowell release constant, Zero order release kinetics belongs to systems where the release rate of drug does not depend on its concentration (Hadjiioannou *et al.*, 1993).

When the release rate depends on concentration of drug, the first order release takes place (Bourne, 2002). If the matrix that the drug was embedded is water insoluble, then, Higuchi release kinetics is observed. Finally, for the Hixson-Crowell cube root law takes place when the volume of matrices changes over time (Hixson and Crowell, 1931). These systems are much more useful when the system is a targeted delivery system. This could enhance specificity of drug and prevent its loss to undesired tissues. Drug delivery vectors are aimed to design according to desired release kinetics and to target desired tissue.

Vectors are designed as vehicles that have active or passive targeting mechanism. Passive targeting uses the advantage of structural difference of blood veins in tumor location and the size of the carrier. The enlargement in pore size the blood vessels near the tumor site allows the vehicles

to go out of veins into the tissue, and not to the other sites since the vessels are not permeable enough for the carrier to go out. This is called the Enhanced Permeability and Retention (EPR) effect.

Active targeting aims at specific binding to cells via receptor- ligand relationship. The specific membrane surface receptors of separate cancer cells are identified in many cancer types. These receptors exist only in these cells, or in mucher high amount on the membranes of these cancer cells than on healthy cells. The surface of the vehicle is, therefore, covered with ligands of these specific receptors to enhance binding.

The vectors used in drug delivery can be categorized in variable ways. One such classification is viral and nonviral vectors. Although viral vectors are more efficient in targeting and penetration, they might also be more immunogenic. The nonviral vectors are less or no immunogenic, however the efficiencies are not high as the viral vectors.

The advantage of nonviral vectors is the availability of a wide range of materials and techniques to use in constructing vectors in the desired form and efficiency (Figure 1.5).

Polymers and lipids are mostly used materials used in vector structure. Liposomes are spherical lipid bilayer structures with hollow cores that carry the drug in the core if hydrophilic or in the bilipid layer if hydrophobic. The most promising feature of liposomes is their nonimmunogenic and nontoxic nature. There are a large number of bio or synthetic polymers which are also not immunogenic or toxic. The advantage of polymeric structures when compared to liposomal structure can be listed as higher stability, mechanical strength, high variety and high capacity for modification by many different compounds.

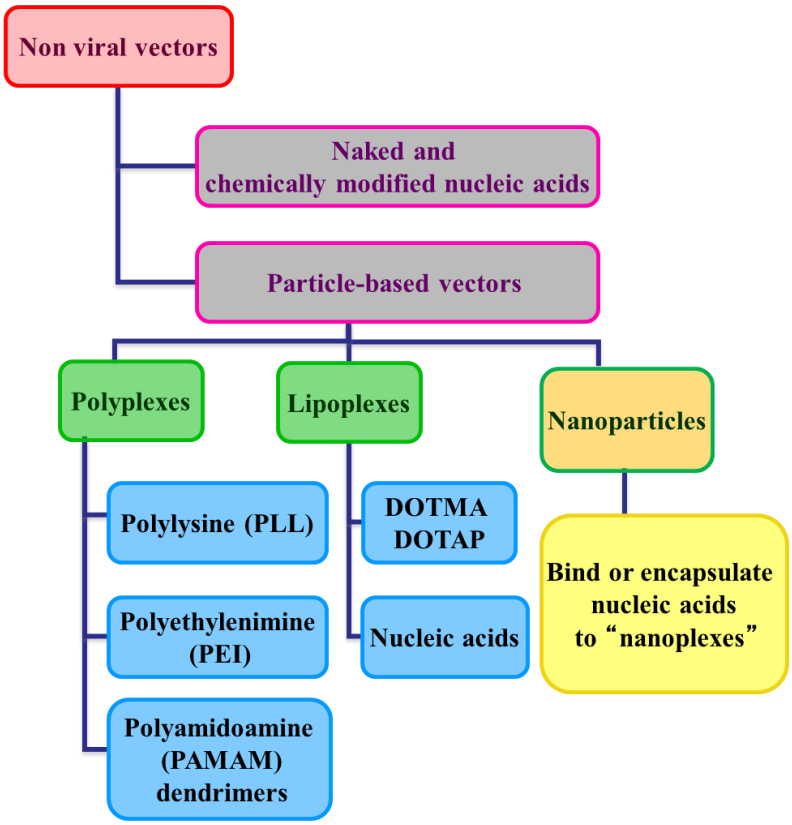


Figure 1.5: Non-viral drug delivery vectors for nucleic acid delivery.

1.2.3. Particulate systems in cancer treatment

Since current cancer treatments damage healthy cells and cause toxicity to patients, the cancer drugs must be delivered to cancer cells selectively. For this reason active and passive targeting of cancer cells are required for cancer treatment. Since tumor vasculature is leaky, EPR effect enables passive targeting of drugs to tumor site (Maeda *et.al*., 2000). Nanocarriers first evolved in market as cancer therapeutics in mid-1990s and they were passively targeted towards cancer tissue. The examples of nanocarrier based cancer drugs on the market are liposomes and PEG – liposome combinations (Torchilin, 2005) that are being used to carry doxorubicin (Batist *et.al*., 2002) and they were used in treatment of Kaposi's sarcoma, breast cancer and ovarian cancer. There are various polymer – protein conjugates that carry styrene maleic anhydride-neocarzinostatin (SMANCS) (for hepatocellular carcinoma), PEG-L-asparaginase (for acute lymphoblastic leukemia), PEG-granulocyte colony-stimulating factor (G-CSF) (prevention of chemotherapy associated neutropenia), and radio immunoconjugates (for non-Hodgkin's lymphoma) and also albumin bound paclitaxel nanoparticles (Peer *et.al*., 2007).

Although the passive targeting carriers are on the market, they have some limitations like failure of nanoparticles to diffuse inside tumors (Park *et.al.*, 2002). Since insufficient amount of drug is delivered to tumor cells, it may induce MDR. To maximize efficiency of these nanocarriers, their surface can be covered with ligands that selectively bind to cancer cell receptors which can trigger uptake of nanoparticles. For example, in a breast cancer model ErbB2 receptors were targeted liposomal doxorubicin was shown to improve therapeutical efficiency.

As targeting agents proteins (generally antibodies and their fragments), aptamers and other receptor ligands (peptides, vitamins, carbohyrates) are being used.

For rapid and effective therapy with nanocarriers, the structures of nanocarriers are also important. Nanocarriers for cancer therapy must first be biocompatible. It should provide high uptake efficiency, and should have a long circulating half-life and it should present low aggregation. Nanocarriers are 1 – 100 nm sized particles (BSI, 2011) that can carry multiple drugs and due to their high surface to volume ratio, sufficient amounts of ligands can be bound on surface of them. They can provide controlled release so keep local drug concentration constant during release. The cancer nanotherapeutics are composed of natural and synthetic polymers and lipids. Their formulations may vary, like polymer conjugates, polymeric nanoparticles or lipid based nanoparticles including liposomes, micelles and dendrimers. Carbon nanotubes, gold nanoparticles are other formations that are being used for cancer therapy (Alonso, 2004; Ferrari, 2005; Duncan, 2006; Couvreur and Vauthier, 2006). There are 12 polymer drug conjugates that are currently in Phase I and II clinical trials (Duncan *et.al*., 2006; Satchi-Fainaro *et.al*., 2006). The commonly used polymers in use for cancer theraphy are N-(2-hydroxypropyl)methacrylamide (HPMA) copolymer, poly(-l-glutamic acid), poly(ethylene glycol) (PEG) and dextran. Usually doxorubicin, paclitaxel, camptotechin and palatinate are studied for polymer-drug conjugation studies.

1.3. A novel therapeutic approach to cancer treatment: Inhibition with siRNA

Since was first discovered in plants and as a defense mechanism against virus infections. Later it was discovered in *Caenorhabditis elegans* and mammalian cells. Small interfering RNA (siRNA) is around 20 – 25 nucleotide long, double stranded oligomer and serves as an endogenous or an exogenous natural defense mechanism against viruses and transposons. It regulates posttranscriptinal mRNA degradation with highly complementary sequences (Tokatlian *et.al*., 2010). Since its discovery it has been widely used for silencing gene expression purposes (Fire *et.al*., 1998; Hammond *et.al*., 2000; Elbashir *et.al*., 2001). It took great interest in cancer therapy research since it had the possibility to silence oncogenes involved in proliferation, survival, invasion, angiogenesis, metastasis, inhibition of apoptosis, and chemotherapy and radiotherapy resistant genes (4-5). Since siRNA and its target are homologues to each other, siRNA silencing

depends on their complementation with their targets specifically and effectively. In the cell double stranded RNA (21-23 nucleotide long) is first produced. Once endonuclease, Dicer, recognize this double stranded RNA, it cleaves it into two fragments which are called siRNA. Later, a protein complex, RNA induced silencing complex (RISC), becomes associated with antisense strands and the new RISC complexes target mRNA (Figure 1.6). Once they find target mRNA and complements it, Argonaute (Ago2) and a RNA endonuclease in RISC complex cleaves the target mRNA. As a result, protein expression is shut down.

siRNA is a part of protein expression control and feedback mechanism in eukaryotic cells. In the cytoplasm, they are in single stranded form. When they recognize their complementary mRNA strands which are translated and secreted in to the cytoplasm, they interact with these compatible strands. RISC factor recognizes and chops up this double stranded oligomer and causes it to degrade the expression of the target protein expression is, therefore, inhibited (Figure 1.6).

This therapeutic approach requires a vector system to protect and carry it because when applied to the patient, there are several problems: degradation or inhibition of siRNA function in the body degradation in serum, non-specific distribution and tissue barriers preventing its transference. In order to protect the siRNA from these extracellular risks, chemical modification with PEG, complex formation with cations and encapsulation in nanoparticles encapsulation are used (Tokatlian *et.al*., 2010).

An ideal siRNA delivery system must provide gene silencing strongly, be biocompatible, biodegradable, nonimmunogenic and bypass rapid hepatic or renal clearance (Ozpolat *et.al*., 2009) as well as specificity to cancer cells and appropriate size for extravasation in tumor site. Nanocarriers have great advantage considering these requirements.

1.3.1. siRNA delivery with nanoparticles

Quantum dots (Tan *et.al*., 2007), magnetic nanoparticles (Lee *et.al*., 2009), biodegredable materials such as natural and synthetic lipids and polymers like poly lactic co-glycolic acid (PLGA), poly lactic acid (PLA) (Shinde, 2007), polyethylenimine (PEI) (Segura *et.al*., 2006), chitosan (Howard *et.al*., 2006), atellocollagen (Minakuchi *et.al*., 2004) have been investigated for use in siRNA nanocarrier construction. Table 1.2 represents nanoparticle formulations that were used for siRNA delivery. The efficiency depends on the particle's surface charge, size, and hydrophobicity. Negatively charged particles are cleared from target location faster than positively charged particles and positive surface charge also enhances circulation time.

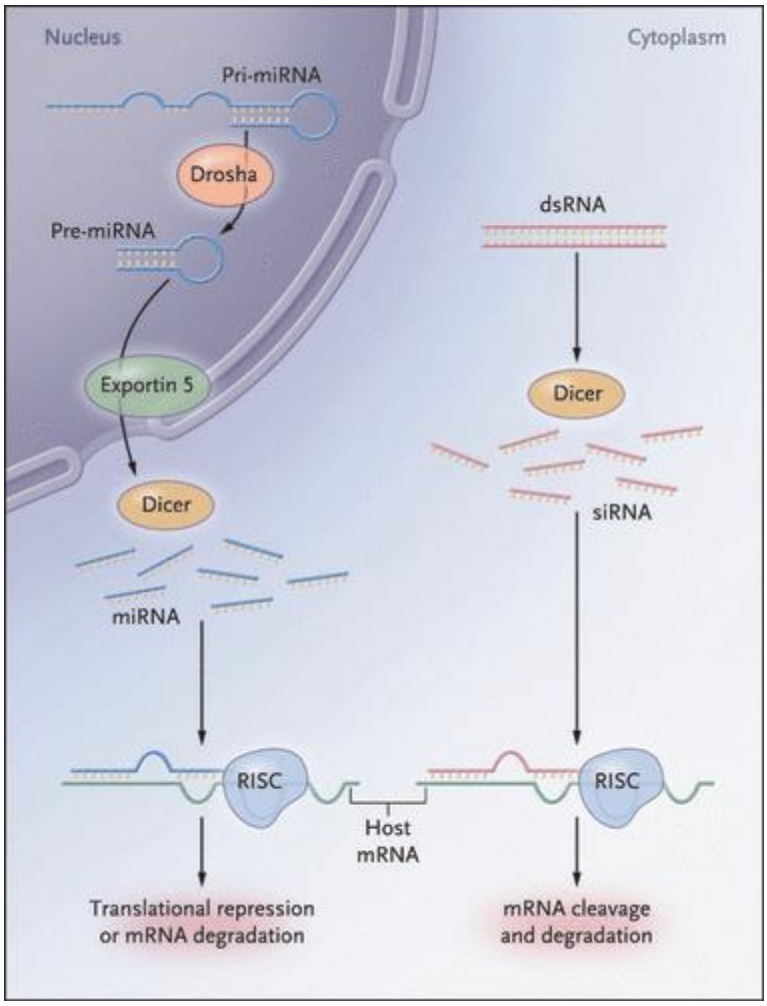


Figure 1.6: The RNA interference process in mammalian cells. miRNA refers to microRNA and long precursor microRNA is named as pre-miRNA. First, RNase III endonuclease Drosha cleaves pre-miRNA in the nucleus (~70 nucleotide long segments). Exportin-5 carries them to cytoplasm. There, Dicer cleaves them and mature miRNA are formed. Doubled stranded RNA (dsRNA) is cleaved by Dicer into siRNA fragments which are incorporated into RISC. After miRNA binds to RISC, translational repression and mRNA degradation occurs (Adapted from Ozpolat *et.al*., 2009).

Particles up to 100 nm can escape from reticuloendothelial system clearanceand may have longer circulation period compared to particles bigger than 100 nm. The requirements of a siRNA nanocarrier can be listed as;

- Prevention of siRNA degradation
- Delivery of sufficient concentrations of siRNA to target location
- Specificity to target cells (with high-affinity ligands)
- To have a controlled release kinetic
- Nontoxicity

Targeted therapies for siRNA delivery is based on recognition and, if possible, internalization of the nanoparticles. For this purpose, specific ligands and antibodies are linked on the surface of nanoparticles (Pirollo *et.al*., 2008).

Techniques to enhance internalization involve peptide-polymer-lipid complexes (Schaffert *et.al*., 2008; Kim *et.al*., 2009), conjugations or complexations of cell penetrating peptides (CPP) (Muratovska *et.al*., 2004; Moschos *et.al*., 2007) with modification of ligands for receptor mediated endocytosis (Rozema *et.al*., 2007; Pirollo *et.al*., 2008).

Once the siRNA (and the carrier) are in the cells, they are internalized in the endosomal system with a low pH that destroys the siRNA; here the siRNA has to be protected from endosomal degradation. In order to avoid endosomal degradation, fusogenic peptides (Oliveira *et.al*., 2007; Hatakeyama *et.al*., 2009), acid responsive polymers or lipids were used to equilibrate acidic pH of endosomes and protect siRNA from acid degredation (Rozema *et.al*., 2007; Convertine *et.al*., 2009).

One of the pH responsive polymers is PEI and it is the most widely used polymer for this purpose and functions by complexing with the siRNA thus stabilizing it against pH or enzymatic degradation (Schaffert *et.al*., 2008; Kim *et.al*., 2009). Also, some chemical modifications could be applied to siRNA to target it to the mRNA (Akhtar *et.al*., 2007; Rao *et.al*., 2009).

Table 1.2: Selected nanoparticles that have been used for siRNA delivery.

Category of particle	Type, form and composition of carrier	Comments
Lipid complex	Cationic liposomes	Lung toxicity
	Neutral liposomes (DOPC)	Biodegradable-nontoxic
	Lipoplexes	
	Stable nucleic acid-lipid particles	
Conjugated polymers	Polymer-functional peptides	
	Polymer-lipophilic molecules (e.g., cholesterol)	
	Polymer-PEG	
Cationic polymers	Chitosan	Biodegradable-nontoxic
	Atellocollagen	Biodegradable-nontoxic
	PEG	Cytotoxicity
	Cyclodextrin	
	Poly-L-lysine	

Adapted from Ozpolat *et.al*., 2009

1.3.2. Apoptotic effect of EF2K siRNA on breast cancer cells

Eukaryotic elongation factor-2 kinase (eEF2K) is a calmodulin dependent enzyme that inhibits eukaryotic elongation factor-2 (eEF2) by phosphorylating it. Normally the function of eEF2 is to trigger translocation of pepdidyl t-RNA from ribosomal A to P site during peptide chain elongation (Wu *et.al*., 2009). Phosphorylation of eEF2 by eEF2K decreases the enzyme's affinity to bind peptidyl t-RNA and elongation is terminated. Thus, protein synthesis is inhibited. Cancer cells prefer to prevent protein synthesis since it requires large amount of energy produced by cells. The cancer cells prefer to use this energy for survival instead.

eEF2K activity was shown to be in increased amounts in breast cancer specimens when compared with noral adjacent tissue (Chafouleas *et.al*., 1981). In more recent studies, the cells which have enhanced proliferation, eEF2K was active, like breast cancer cells (Parmer *et.al*., 1998), malignant glioma cells (Bagaglio *et.al*., 1994), and HL60 leukemia cells (Nilsson *et.al*., 1995). Conversely, non-proliferating cells do not exhibit active EF2K, while its activation was shown in cells in G1/S transition stage of cell cycle (Parmer *et.al*., 1997).

Moreover, Tekedereli *et.al*. (2012) has shown that eEF2K regulated cell growth, clonogenicity and down regulating eEF2K expression using liposomal siRNA inhibited orthopedic tumor growth in breast cancer model, and it even enhanced efficacy of chemotherapy in athymic female nu/nu mice. In the study it was mentioned that the down modulation of eEF2K leaded to a decrease in expression levels of c-Myc, cyclin D1 and increase in that of p27. It was also showed that the basal activities of c-Src, FAK and Akt was decreased by down regulation of eEF2K.

1.4. Polyethylenimine – Starch Nanoparticles for EF2K siRNA delivery to Cancer Cells

One of the highly efficient polymers in siRNA delivery was shown to be polyethylenimine (PEI) (Urban-Klein *et.al*., 2005). PEI has a positive charge due to the amine groups in its structure, it was studied as DNA-PEI complexes and shown to be highly efficient compared to other positively charged polymer types like chitosan and poly-l-lysine (Figure 1.7). Additional to it's highly attachment capability to cell surface, the transfection efficiency of PEI can also be explained with "proton sponge effect" of PEI (Akinc *et.al*., 2005).

Two forms of PEI, linear and branched, are available. Acid-catalyzed polymerization of aziridine monomers results in randomly branched PEI. Linear PEI is formed by a similar process but at a lower temperature (Tomalia *et.al*., 1985; Godbey *et.al*.,1999). Ethyl-amine repeating units in PEI are the groups that provide high water solubility to PEI. As a most important characteristic of PEI, it is very cationic, results from protonation potential of amino nitrogens in every third atom. Every fifth or sixth amino nitrogen is in protonated form at pH 7.4 (Suh *et.al*., 1994). Nucleic acid interactions shift the protonation profile of PEI but still every second to third nitrogen is protonated

at pH 7.4 and for this reason, compared to other positively charged polymers like poly-l-lysine and chitosan, it has a higher buffering capacity over a broad range (Boussif *et.al*., 1995; Tang *et.al*., 1997). Various molecular weights of PEI (e.g., 0.7, 2, 25, 50, 70, 800 kDa), and several PEI derivatives like ethoxylated PEI or epichlorohydrin-modified PEI are also available (Boussif *et.al*., 1996; Kichler *et.al*., 1999).

The binding of PEI-nucleic acid complexes usually happen via electrostatic interactions with negatively charged cell membrane and this is followed by endocytosis or pinocytosis (Duncan *et.al*., 1979; Leonetti *et.al*., 1990). Recently interactions with positively charged PEI-nucleic acid complexes and proteoglycans on the cell surface were demonstrated (Labat-Moleur *et.al*., 1996; Mislick *et.al*., 1996) and the interaction was indicated as an essential step for internalization of the particles (Szoka *et.al*., 2000).

After the cell engulfs an ingredient, independent of the type of mechanism (non-specific adsorptive endocytosis, phagocytosis, or receptor mediated endocytosis) it is taken up into endosomes or other internal vesicles of the cells and the particle still remains separated from cytoplasm by lipid membrane. Following this, normal cellular trafficking directs the particles to lysosomes which have an acidic pH and in which nucleic acids are unstable and is degraded. PEI provides proton-sponge effect and neutralizes the acidic pH, protects siRNA from acidic degradation, and triggers release of undamaged siRNA to cytoplasm. But the disadvantage of PEI is its cytotoxicity that results also from its positive charge due to electrostatic interactions with negatively charged cell membranes (Fischer *et.al*., 2002).

A number of studies are being investigated to carry siRNA to target cells by use of PEI (Lungwitz *et. al.*, 2005; Zinthchenko *et. al*., 2008). One of the studies showed that the delivery of siRNA to target receptor was successfully achieved in rats by PEI-siRNA complexes and target receptor was selectively silenced (Tan *et. al.*, 2004). (Whitehead- knocking down barriers: advances in siRNA delivery). In another study, PEI-siRNA complexes were administered intraperitoneally in a subcutaneous mouse tumor model and the target protein was silenced successfully while tumor growth was reduced (Urban-Klein *et.al.*, 2004). Additional studies were focused on reducint toxicity of PEI by various modifications in PEI structure (Thomas *et.al.*, 2005; Richards Grayson *et.al.*, 2006; Werth *et.al*., 2006).

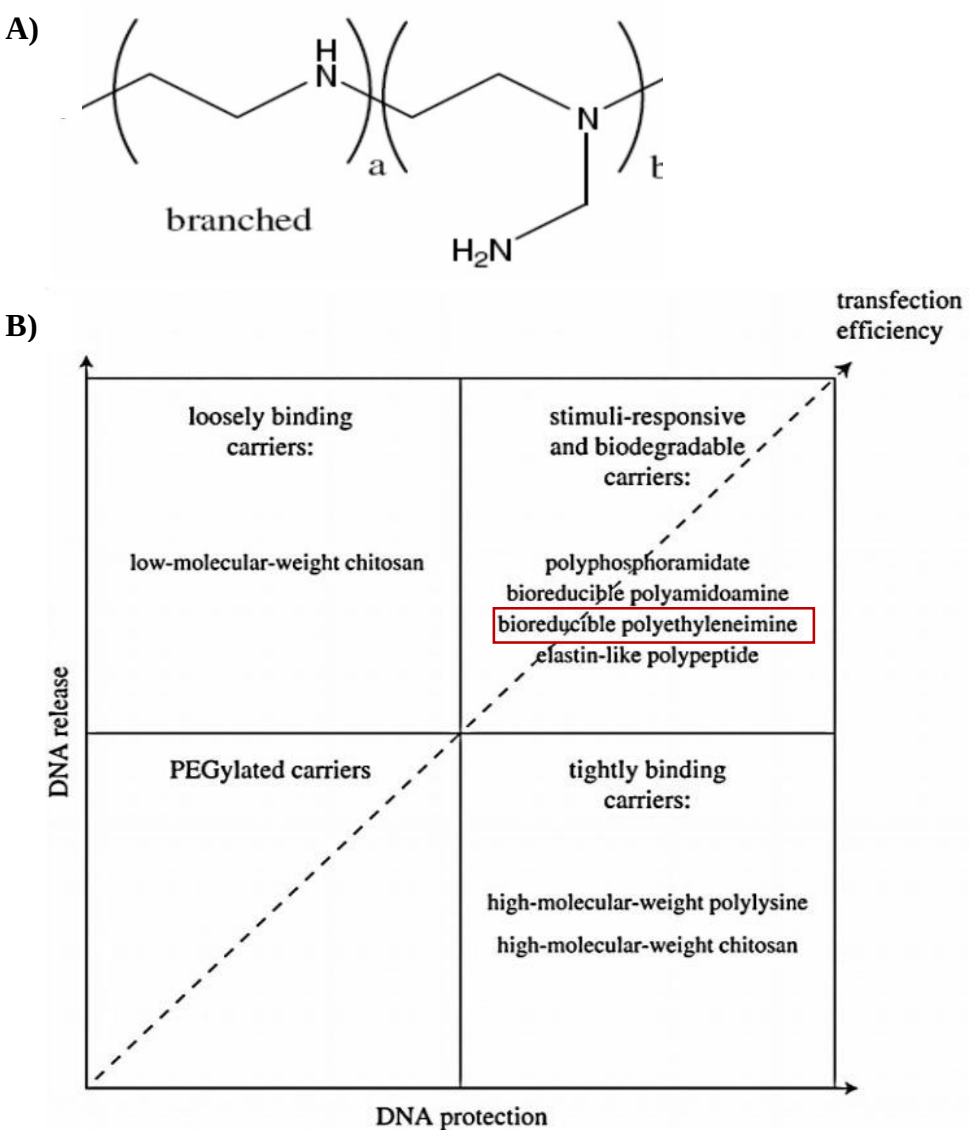


Figure 1.7: Structure of PEI (A) and comparison of DNA protection and release of PEI with other positively charged polymers (adapted from Grigsby *et.al*., 2009) (B).

The aim of this study was to construct a vector that efficiently delivers apoptotic siRNA into the target cancer cell. In this study, PEI/starch nanospheres were prepared to deliver EF2K siRNA to MCF 7 breast cancer cells.

The target protein was chosen to be the elongation factor 2 kinase (EF2K) which phosphorylates EF2 and activates it, which plays a role mainly in protein expression. Inhibition of EF2 can decrease speed of protein translation, thus required growth of cells, therefore leads to autophagy, followed in later steps by apoptosis (Tekedereli *et.al*., 2012).

An important characteristic of an efficient delivery vector is its transfection efficiency. It should recognize, attach, and transfect the target cell in order to release its payload efficiently. Positive surface charge enhances transfection efficiency since it helps attach the negatively charged cell surfaces (Richards *et.al*., 2006), and this can be provided by using positively charged polymers on the surface or in the whole structure of the vector. In this study, PEI serves both to complex with starch to form a particle and also help the particle attach cell surfaces.

The vector composed of PEI and siRNA can be designed to decrease its toxicity. This can be achieved by combining PEI with a nontoxic (uncharged) polymer. An optimal amount of nontoxic polymer could decrease the toxicity that amine groups of PEI cause. In a study, starch was used to decrease toxicity of highly positive polymer poly (l-lysine) (PLL) for construction of a gene carrier (Suyao *et.al*., 2004).

In this study, starch was selected to be used as a nontoxic biopolymer. Hydrogel structure of starch was thought to be an appropriate property drug delivery purposes (Santander-Ortega *et.al*., 2009; Malafaya *et.al*., 2006). For this reason, starch was selected to be used in this study.

Since both polymers are (PEI and starch) hydrophilic, a nanoparticle composed of them is expected to be unstable in a hydrophilic environment. In order to enhance the stability of the nanoparticle in blood, an additional stabilization (crosslinking) step was required in the construction progress. If amine groups of PEI in the outer surface of the nanoparticles were to be crosslinked with a crosslinker, it would decrease the charge and the cytotoxic effects of PEI while providing stability to the nanoparticles as a result of the crosslinkage.

Genipin was shown to be an efficient and nontoxic crosslinker among other generally used protein crosslinkers like glutaraldehyde and formaldehyde (Sung *et.al*., 1999) and like gluteraldehyde it crosslinks protein structures by binding two amine groups together (Liang *et.al*., 2002). For this reason genipin was chosen to be used as the crosslinker of primary amine groups of PEI in and on the surface of PEI – starch nanoparticles (Figure 1.8).

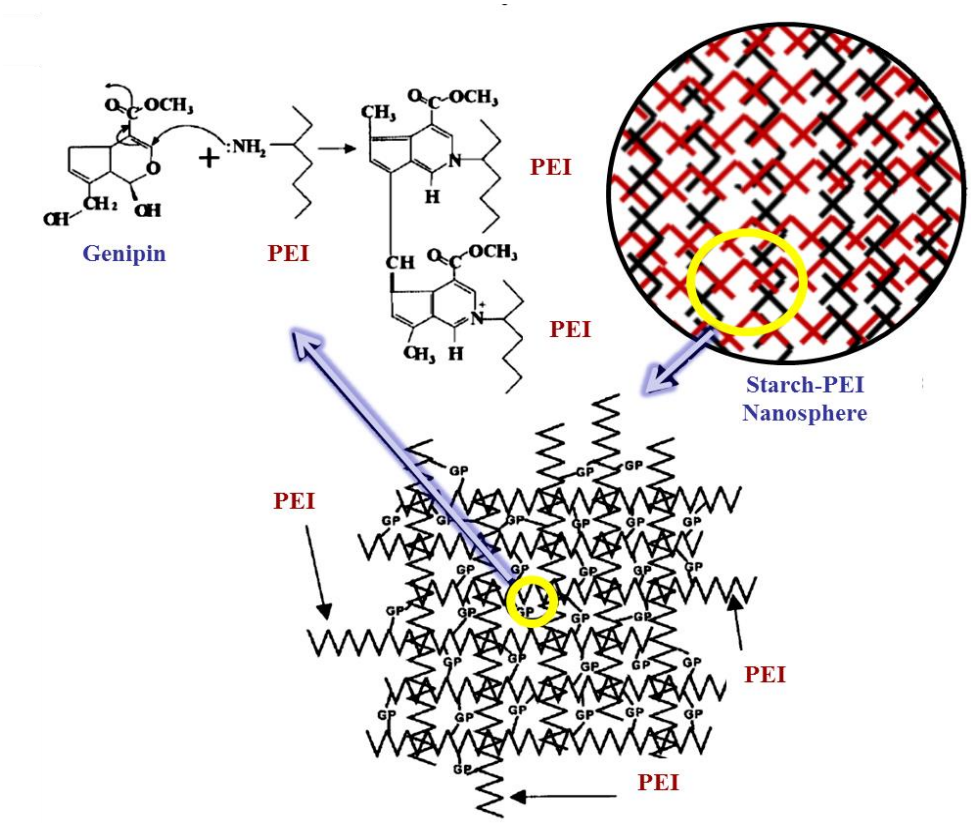


Figure 1.8: Crosslinking of PEI with Genipin.

The spheres were designed to follow the route shown in Figure 1.9 at the end of which siRNA is separated into single strands. The single stranded siRNA would then find the target mRNA in cell and bind to it. RISC factor would arrive and chop up this combined structure and protein expression would be inhibited.

In order to complete this route, physicochemical properties of the PEI – Starch nanoparticles had to be optimized. In this study, characterization and efficiency tests crucial for this siRNA delivery approach were carried and the results were discussed.

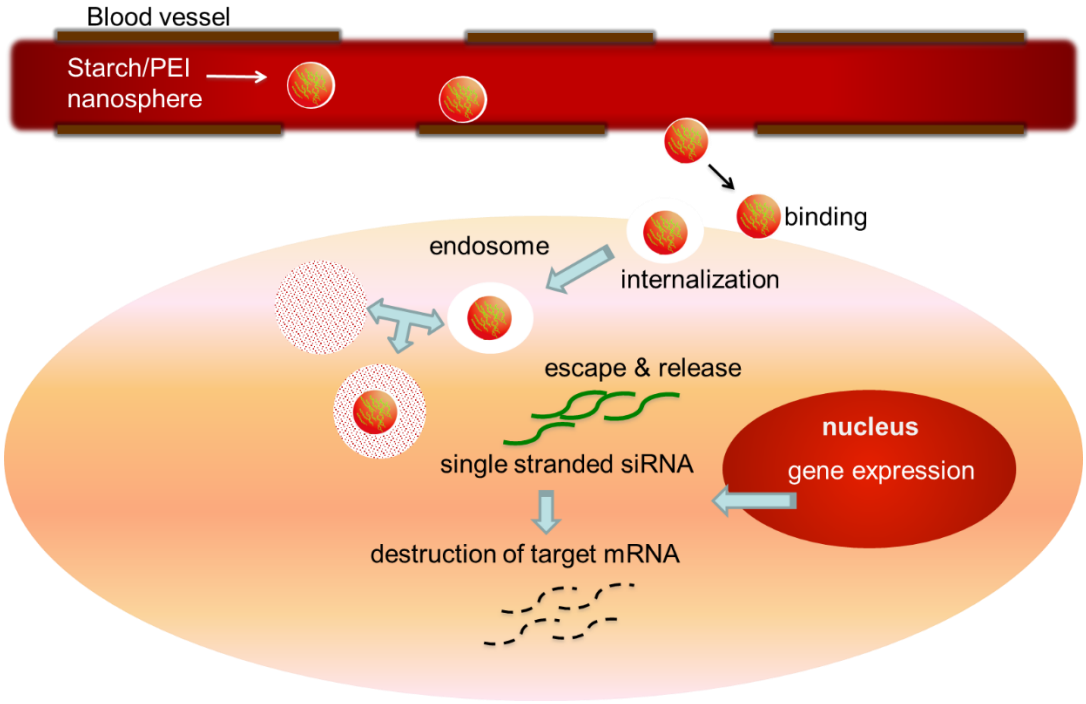


Figure 1.9: siRNA delivery route of PEI-Starch nanospheres

Since there is not a study present in literature about a drug delivery vector composed of a blend of PEI and starch, PEI-starch nanoparticles used in this study is proposed as a novel carrier system for drug delivery. This novel carrier system was first used in this study to deliver EF2K siRNA to MCF 7 breast cancer cells.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

Polyethylenimine (PEI, branched), Elongation factor 2 kinase siRNA (EF2K siRNA), insulin human recombinant, insulin (from bovine pancreas), fluorescein isothiocyanate isomer I (FITC), Tween 80, corn oil, penicillin/streptomycin were purchased from Sigma-Aldrich Corporation (USA). Starch from potato (soluble, extra pure) was purchased from MERCK. Mini Sart single use filter unit (0.20 μm, for filtering water phase in nanoparticle preparation procedure) was purchased from Sartorius Stedim Biotech (Germany). Genipin was purchased from Wako Chemicals (USA).

Human breast cancer cells (MCF7) were obtained from American Type Culture Collection (No: HTB-22™, ATCC). PAGEr Precast Gels, Dulbecco's Modified Eagle Medium (DMEM, low glucose), and fetal bovine serum (FBS) were purchased from Lonza (Switzerland). L-Glutamine was purchased from Biochrome (Germany) and vancomycin was purchased from Hospira (USA). RIPA Buffer (10x) was purchased from Cell Signalling Technology (USA). Protease inhibitor Coctail was purchased from BM (Ankara). BCA Protein Assay Kit was purchased from Novagen-EMD4Biosciences (EMD Millipore, USA). EF2K siRNA, Alexa-555 labelled siRNA, Control siRNA, EF2K primary antibody, anti-rabbit secondary antibody were gifts from Dr. Bülent Özpolat, MD Anderson Cancer Center (USA).

2.2 Methods

2.2.1. Preparation of Polyethylenimine-Starch Nanoparticles

Polyethylenimine-starch (PEI-starch) nanoparticles were prepared by water-in-oil (w/o) microemulsion method. Water phase contained 5% Tween 80 as surfactant, PEI (10 mg/mL) and starch (10 mg/mL), which were all dissolved in distilled water. The water phase was added dropwise into corn oil (oil phase) in a proportion of 1:20 while the corn oil was being stirred on magnetic stirrer at 700 rpm. The emulsion was stirred overnight in order to make the particle sizes uniform. The nanoparticles were separated from oil phase by centrifuging the emulsion at 15 000 rpm (24 150 RCF) for 40 min (rotor: 15156-H Sigma, 3K30). Later the nanoparticles were washed with distilled water and centrifuged. Supernatant was discarded and pellet with the nanoparticles was resuspended and incubated in genipin solution (% 0.5, in PBS) for 48 h for crosslinkage. The nanoparticles were washed twice with distilled water and ethanol, at centrifugation conditions of 15 000 rpm for 40 min (rotor: 15156-H Sigma, 3K30). The last pellet was resuspended in distilled water and frozen at -20°C for lyophilization. The nanoparticles were lyophilized for 10 h and observed under SEM (FEI Quanta 200 FEG SEM, USA).
The size and charge measurements of the nanoparticles were made with Brookheaven ZetaPALS Zeta Potential and Particle Size Analyser (USA) at METU Central Laboratory.

2.2.1.1. Preparation of PEI-Starch nanoparticles with different PEI/starch (w/w) ratios

Since the nanoparticles differ in their charge and cytotoxicity due to their PEI content, nanoparticles with different PEI/starch ratios were prepared in order to choose the optimum ratio for less cytotoxicity and appropriate positive charge. The PEI and starch solutions which were the components of water phase were mixed in different PEI:starch ratios (PEI:starch (w/w) 9:1, 3:1, 1:1, 1:3, 1:9). The charges of PEI-starch nanoparticles with different PEI/starch ratios were determined with Malvern Nano ZS90 Zeta Potential and Mobility Measurement Instrument (UK). Cytotoxicities of each batch were tested with MTT cell viability assay (Mosmann *et.al*., 1983). The moles of amine groups in PEI per moles of phosphate groups in siRNA (N/P ratios) (Zhao *et.al.*, 2009) were calculated for each of the PEI:starch ratios. They were 0.4, 1, 2, 3 and 4 for the PEI:starch (w/w) ratios of 1:9, 1:3, 1:1, 3:1 and 9:1 respectively.

2.2.1.2. Preparation of control siRNA, EF2K siRNA, Alexa-555 labelled siRNA Loaded PEI-Starch Nanoparticles

Control siRNA, EF2K siRNA, Alexa-555 labelled siRNA loaded PEI-starch nanoparticles were prepared separately, by adding siRNA into the water phase (1%, siRNA:PEI (w/w), 1:10 in RNase/DNase free water). siRNA loaded nanoparticles were prepared according to the procedure described in section 2.2.1.1. Since the zeta potential measurement of the unloaded nanoparticles with 9: 1 PEI: starch ratio found was to be positive (8.7 mV), this ratio was used in this preparation method.

2.2.2. Characterization

2.2.2.1. Nanoparticle Topography with SEM

EF2K siRNA loaded PEI-strach nanoparticles were applied onto carbon tapes (Electron Microscopy Sciences, USA), attached to SEM stubs, and Au-Pd sputter coating (10 nm) was performed under vacuum before the SEM study with QUANTA 400F Field Emission SEM (Netherland). The diameters of the nanoparticles were measured from the SEM images using the Image J software (NIH).

2.2.2.2. Particle Size Distribution and Surface Charge of PEI – Starch Nanoparticles

Size distribution and charge of empty and siRNA loaded PEI-starch nanoparticles were measured by Brookhaven ZetaPALS Zeta Potential and Particle Size Analyzer (USA). The nanoparticles were not lyophilized for this step. After the last centrifugation of washing step, the pellet of nanoparticles was resuspended in distilled water and loaded into size and charge measurement cuvettes, respectively.

2.2.2.3. Release kinetics of PEI- Starch Nanoparticles

In the release tests, insulin (5,800 Da) which is soluble in physiological pH (pH 7.365) was used as model drug due to its similarity in molecular weight with a typical siRNA (≈ 6,000 Da). Insulin loaded PEI-Starch nanoparticles were prepared in order to analyze the release behavior of nanoparticles. First, FITC was bonded to insulin (Sigma Aldrich, AJH 8/08) in order to obtain insulin concentration dependent spectroflurometric absorbance from the release medium. The PEI-starch nanoparticles were prepared according to the procedure in Section 2.2.1. Genpin crosslinking step was done in order to crosslink amine groups in insulin, instead of amine groups

in siRNA. The FITC tagged insulin (FITC-insulin) was loaded into nanospheres by adding the determined amount of FITC-insulin in water phase (1:10, FITC- insulin:PEI, (w/w)). The nanoparticles were resuspended in distilled water (1 mL) after the last washing step, then moved into dialysis tubing (SnakeSkin, MW 10,000 cut off, pleated, Thermo Scientific, USA). Ultrapure water was used as release medium (5 mL). This medium was kept shaking in Innova shaker incubator (Eppendorf-New Brunswick, USA) at 37ºC, 25 rpm for a week. The amount of FITC-insulin that was released into distilled water was measured by Shimadzu Spectrofluorophotometer RF-5000 each day and loading efficiency of the nanoparticles was estimated according to total released FITC-insulin amount in a week.

2.2.3. *In vitro* Studies

2.2.3.1. Determination of Cytotoxicity of PEI-starch Nanoparticles

MCF7 breast cancer cell line (passage no 5) were cultured in Dulbecco's Modified Eagle Medium (DMEM, low glucose) supplemented with 1% L-glutamine, 10% fetal bovine serum, 1% penicillin/streptomycin and 1% vancomycin. The cells were maintained in an incubator with 5% CO_2 at 37°C (Sanyo MCO-17AIC, Japan). When the cells became confluent, they were detached from the flask by treatment with trypsin-EDTA solution (3 mL, 0.05% in PBS) for 5 min. Then culture medium (6 mL, supplemented with %1 L-glutamine, 10% fetal bovine serum, 1% penicillin/streptomycin and 1% vancomycin) was added to the flask to inhibit trypsin activity. The cell suspension was centrifuged (3000 rpm, 5 min) and the pellet was resuspended in the medium (2 mL, 1% penicillin/streptomycin, 1% vancomycin, 10% FBS) (SOP-METUBM02, 2006). The cells were counted with Chemometec NucleoCounter (Denmark) and 10,000 cells were seeded onto 24 well tissue culture plates (TCPs).

Cell numbers were determined with the MTT assay (Mosmann *et.al*., 1983). The 24 well plates seeded with 10,000 MCF7 cells were incubated (5% CO_2, 37°C) for 4 h in order to achieve cell attachment on TCPS. The medium was removed and PEI-starch nanoparticles which were suspended in the culture medium in increasing concentrations (25, 50, 75, 100 µg particles/mL) were added to the wells containing the cells which were then incubated (5% CO_2, 37°C) for 24 h. The medium was removed, the wells were washed twice with sterile PBS and then MTT solution (1 mL) was put into each well and incubated for 3 h (5% CO_2, 37°C) for formazan crystal formation. The MTT solution was gently removed in order to prevent cell detachment, acidified isopropanol (1 mL) was put in the wells and formazan crystals were dissolved. Aliquots of formazan solution (200 µL) were put in a 96- well plate in triplicates. Absorbances were measured at 550 nm against the blank (acidified isopropanol) using a UV spectrophotometer (Thermo Scientific Multiscan Spectrum, Type 1500, USA) and the absorbances were converted to number of cells by a calibration curve (Appendix A.1). The results were evaluated by comparing the

experimental group with the control group at the same time points. All the experiments were conducted in triplicate and cell-free medium was used as the blank.

2.2.3.2. PEI – Starch Nanoparticle Uptake by MCF7 cells

MCF 7 cells were used to determine penetration of nanoparticles into the cells. Culture medium was DMEM low glucose supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin (100 unit), 1% vancomycin and 1% l- glutamine.

MCF 7 cells were seeded at a density of 100,000 into each well of 24-well plate and they were incubated in culture medium for 24 h (5% CO_2, 37°C). Then, Alexa 555 labeled siRNA loaded PEI – starch nanoparticles (0.2 nmol siRNA/well) suspended in the culture medium were added to the wells containing the cells. After 24 h of incubation, cells were fixed with paraformaldehyde (4%, 1 mL), stained with 4',6-diamidino-2-phenylindole (DAPI) and FITC-phalloidin for nucleus and cytoskeleton, respectively.

Fluorescent stains, FITC phalloidin and DAPI were used after 100 and 500 fold dilution, respectively, of the stock solution in BSA-PBS solution (0.1 %, w/v).

After incubation, medium was removed and Triton X in PBS (1 % v/v, 1 mL) was added onto the cells. Cells were kept at room temperature for 5 min to achieve permeation and then cells were washed with PBS. Solution of BSA in PBS (1% w/v, 1 mL) was added to the cells and incubated for 30 min at 37°C. FITC-Phalloidin (1 mL) was then added in each well and incubated at 37°C for 1 h. Then cells were washed with PBS again. After staining process, nanoparticles and cells were examined under fluorescence microscope (Olympus IX-70, Japan) and confocal microscope (Leica DM2500, Germany). FITC-Phalloidin was detected after excitation at 532 nm and emission at 605-635 nm. In order to study particle penetration into the cells, z-stacks were obtained.

2.2.3.3. Transfection efficiency of EF2K siRNA loaded PEI –Starch Nanoparticles

The transfection efficiency of EF2K siRNA loaded PEI-Starch nanoparticles were analyzed with Western blotting (Brunette *et.al*., 1981). MCF 7 cells were used to achieve transfection of nanoparticles into the cells. The cells were treated with EF2K siRNA loaded PEI-Starch nanoparticles in conditions described in Section 2.2.3.2. After 24 h of incubation with the nanoparticles, the cells in each well were washed with PBS and then trypsinized as described in section 2.2.3.1. After the last centrifugation, the cells were resuspended in PBS. The cell suspension was centrifuged again (3000 rpm, 5 min). After moving out supernatants, pellets that include cells were resuspended with 50 µL of RIPA Buffer containing 10% protease inhibitor cocktail. The pellet tubes and lysis buffer were kept on ice. The pellet tubes were vortexed for 30 sec for 3 times, with 10 min intervals. The tubes were centrifuged (13 000 rpm, 10 min) and supernatants that include proteins were moved into new tubes. Protein concentrations of each

sample were determined by BCA Assay (Smith *et.al*., 1985) and the concentrations were equalized. After loading buffer was added into each sample, the samples were loaded into wells of 4-12 % PAGEr Gold precast gels (Lonza, USA) and gel electrophoresis was run at 50 V until samples leave wells completely. Then, for the resolving gel, the voltage was increased to 100 V and gel electrophoresis was run until the dye which shows the location of samples reached near the bottom of the gel electrophoresis cassette. The gel was taken out and prepared for western blotting. Western blotting was done at 22 V overnight. The membrane was stained first with EF2K primary antibody, then with anti-rabbit secondary antibody. Finally, film was developed to compare thickness of separate bands demonstrating each sample.

CHAPTER 3

RESULTS AND DISCUSSION

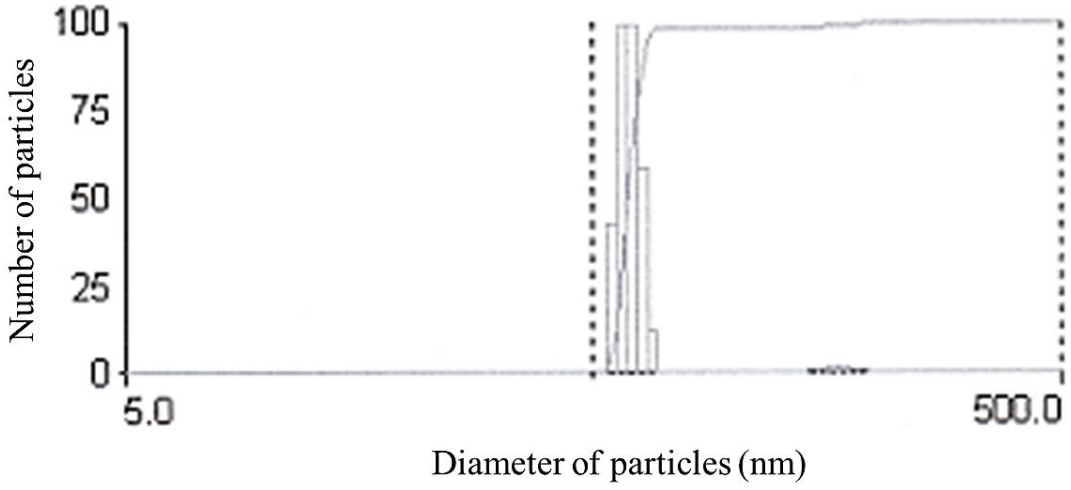
3.1. Preparation of PEI – Starch Nanoparticles

3.1.1. Size and Zeta potential of PEI-Starch Nanoparticles

The mean diameter of PEI:Starch nanoparticles (unloaded) was 60.3 nm (Figure 1A) and for EF2K siRNA loaded nanoparticles it was 84.6 nm (Figure 1B). Thus, there was a significant increase in size after siRNA was loaded into the nanoparticles. This can be a result of the increase in the concentration of water phase during nanoparticle preparation, which involves siRNA. Also, negatively charged phosphate groups of siRNA interact with the positively charged amine groups in PEI, and since genipin crosslinks the amine groups of PEI, this causes less crosslinking. A decrease in crosslinking causes an increase in the particle size.
A previous study about crosslinking efficiencies of gelatin microspheres (Liang *et.al*., 2002) showed that the size of genipin crosslinked gelatin microspheres was smaller than that of glutaraldehyde crosslinked counterparts, as a result of heterocyclic crosslinking mechanism of genipin. This crosslinking structure results more intense crosslinking than gluteraldehyde with network crosslinking structure.

The diameter of EF2K siRNA loaded nanoparticles (84.6 nm) is an appropriate size for passive targeting to solid tumors due enhanced permeability and retention (EPR) effect in which enhanced permeability of veins in the vicinity of the tumor tissue enables extravasation of the nanoparticles at such sites (Brannon-Peppas *et.al*., 2012).

A)



B)

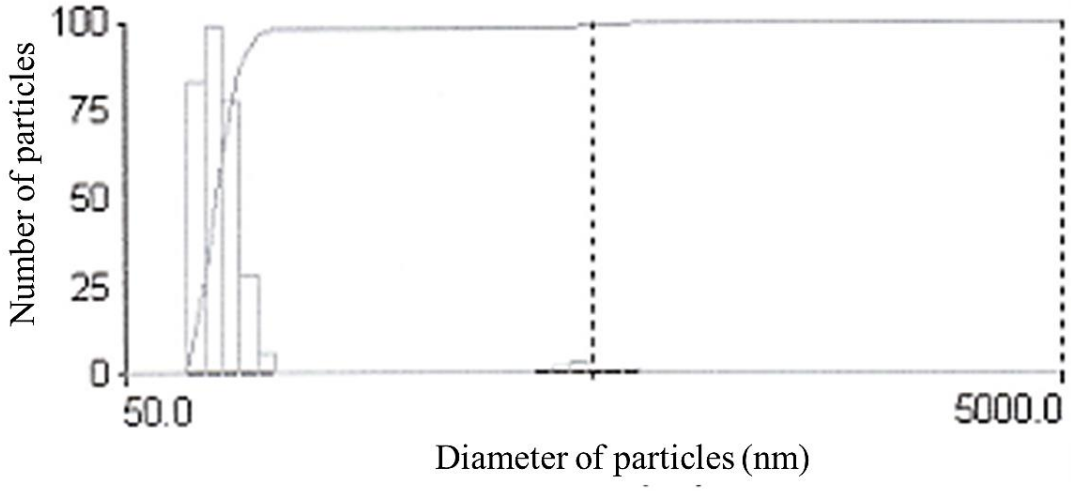


Figure 3.1: Size distribution of PEI-starch nanoparticles A) Unloaded (60.3 nm) and B) EF2K siRNA loaded (84.6 nm).

Surface charge is quite crucial for attachment of nanoparticles to cells and high transfection efficiency is achieved with particles with positive surface charge (Witstrand *et.al*., 2006).

For this reason, PEI-starch nanoparticles with different PEI/starch (w/w) ratios were constructed and their Zeta potentials were measured (Figure 3.2). Increasing PEI amount was shown to increase the surface potential of nanoparticles.

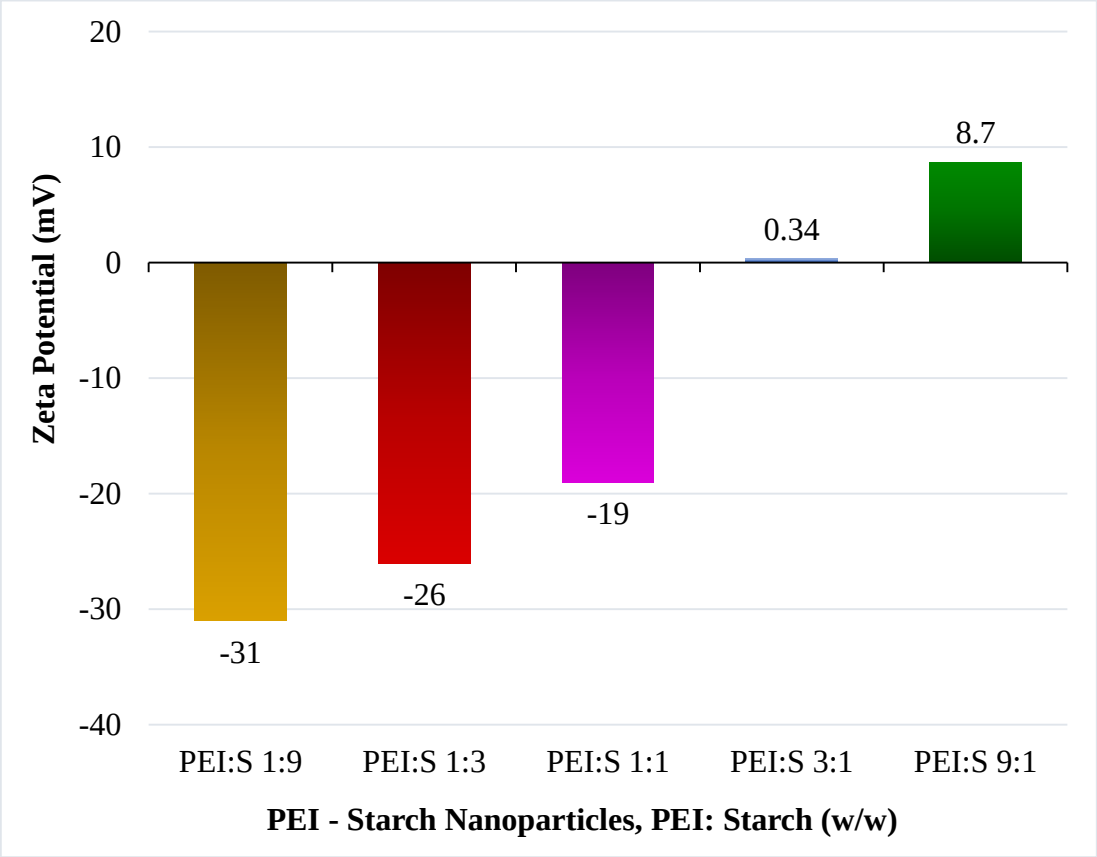


Figure 3.2: Surface potential of PEI-starch nanoparticles with different PEI: starch ratios (PEI: S refers to PEI: Starch ratio (w/w)).

It is observed that, as expected, as the PEI content increases (from 1:9 to 9:1), so does the Zeta Potential of the nanoparticle surfaces (from -3 to 8.4 mV).
Since positive surface charge enhances the interaction of nanoparticles with cells, NP 9:1 is the most advantageous of all others. Since it is shown that positive charge also has a disadvantage; it causes cytotoxicity (Hunter *et.al.*, 2010), the cell viabilities of MCF 7 cells that are treated with PEI-starch nanoparticles in different PEI: starch (w/w) ratios were also tested with MTT cell viability assay for their cytotoxicity.

3.1.2. Morphology and size of PEI- starch nanoparticles

SEM micrographs of siRNA loaded PEI-starch nanoparticles, with 9:1 PEI: starch (w/w) ratio (Figure 3.3) were obtained and from them their diameters were measured with NIH Image J program. Since Zeta sizer measurements showed that loaded particles were larger compared to unloaded particles, they were also measured with SEM. The mean diameter of this loaded particle was found as 58±17 nm.

For siRNA loaded PEI-starch nanoparticles, the mean diameter in zeta size measurements (84.6 nm) is approximately 20 nm higher than Image J mean diameter calculations (58±17 nm). This deviation could arise due to agglomeration since zeta size measurements cannot detect particle agglomeration and if clumps are involved in mean diameter calculations then it would be larger.

The process conditions in emulsion based nanoparticle preparation method like concentration of water and oil phases, the rotation speed of magnetic mixer used in emulsification and emulsification duration effect directly the size of nanoparticles (Liang *et.al*., 2002). In this study, genipin is an additional parameter that affect size. The concentration of genipin and duration of crosslinking are also important parameters for size of PEI-starch nanoparticles. The size of particles can be modified according to the application aimed at.

The clumping of particles can be controlled by surfactants (Menon *et.al*., 2011). So the type and concentration of surfactant can be optimized in order to decrease or prevent clumping of nanoparticles.

3.2. Release kinetics of PEI- starch nanoparticles

The release tests to substitute for siRNA release were done using FITC-labeled insulin loaded PEI-starch nanoparticles. Insulin was preferred due to its comparable molecular weight (~5,800 Da) with siRNA (~13,000 Da). During the release studies, the particles were incubated in a shaking water bath at 37 °C in a dialysis tubing (with a 10,000 Da cut off). Amount of insulin in the release medium was determined daily by measuring the absorbance of FITC in the medium by spectrofluorometry at λ_{ex} 490 nm and λ_{em} 525 nm daily (Calibration curve of FITC-insulin is presented in Appendix A.2). The cumulative insulin released is presented in Figure 3.4. The concentration on 8^{th} day was assumed as the final release concentration since in following three days the FITC fluorescence did not changed at all.

Nanoparticles released 50% of their content within 2 days, and all of their content in 8 days. This suggests that PEI-starch nanoparticles can achieve a prolonged release of a molecule the size of siRNA.

The temperature and pH of release medium have been reported to strongly affect drug release kinetics of nanoparticles (D'Souza *et.al*., 2005; Faisant *et.al*., 2006). Additionally, the ratio of (particle mass exposed to release medium/volume of release medium) (w/v) has been shown to affect release kinetics since rate of pH change resulting from degradation products (generally decrease pH of medium) can differ among different volumes of release medium and this pH

change can cause changes in release profile, like increasing release rate and triggering burst release (Close *et.al*., 2010).

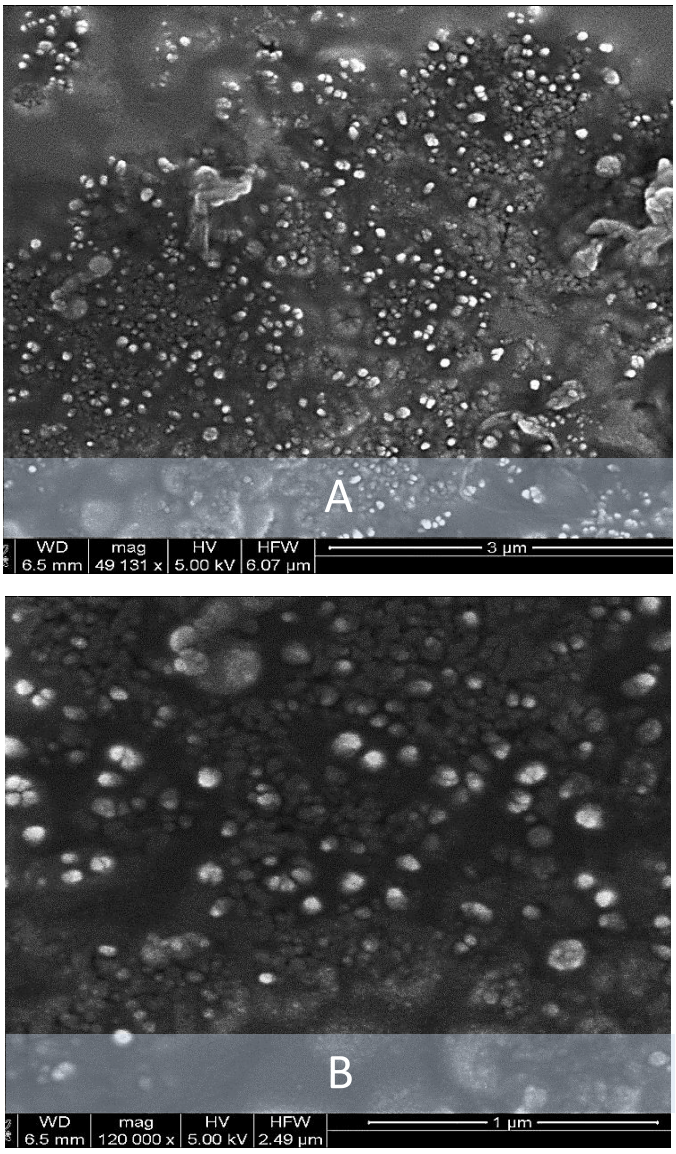


Figure 3.3: Morphology of PEI – starch nanoparticles. Magnifications: A) x 49,131 and B) x 120,000.

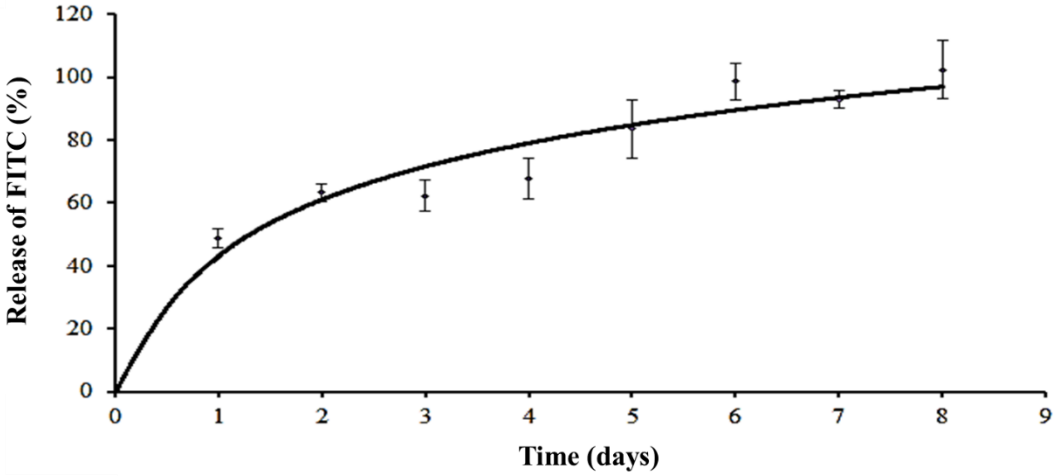


Figure 3.4: Release of FITC-labelled insulin from PEI-starch nanoparticles. Fluorescence intensity was measured at λ_{ex} = 490 nm and λ_{em} = 525 nm.

Generally buffers (PB, PBS, TE, etc.) at biological pH (pH 7.4) are being used in release studies (Patil *et.al*., 2009; Rujitanaroj *et.al*., 2011; Chen *et.al*., 2012).
Alexa-555 labelled siRNA can be used instead of insulin to obtain a more realistic data.
Genipin was preferred in order to crosslink amine groups of PEI. Since genipin has bulky heterocyclic crosslinking structure the relaxation happens more slowly compared to glutaraldehyde crosslinking (alternative amine crosslinker) which has a network crosslinking structure. A Fickian diffusion is more closely achieved with genipin crosslinked microspheres (Liang *et.al*., 2002). That means slower degradation of nanoparticles, which is required in controlled release systems, is possible with genipin crosslinking.

3.3. *In vitro* Studies

3.3.1. Cytotoxicity of PEI-Starch Nanoparticles

Although a positive surface charge increases transfection efficiency, increasing this positive charge also leads to increasing cytotoxicity by these nanoparticles. In order to choose an optimal surface charge with an acceptable level of cell viability, PEI-starch nanoparticles with different PEI: starch (w/w) ratios were tested for their cytotoxicities (Figure 3.5). The viabilities observed with MCF7 cells were 90.7, 86.6, 85.6, 81.9, and 78 % for 1:9, 1:3, 1:1, 3:1, 9:1 ratios of PEI: Starch (w/w), respectively (Calibration curve of MCF 7 cells is presented in Appendix B).
This shows that cell viability decreases gradually with increasing PEI content in the composition of the nanoparticle. Although PEI-starch nanoparticles with 9:1 PEI: Starch ratio (NP 9:1) were

the most cytotoxic (78 % viability) compared to other, less toxic, nanoparticles, it was advantageous to use because it is the only one among them with a positive surface charge (Zeta potential 8.7 mV).

Among a large number of polycations, 25 kDa branched polyethylenimine is the most prominent with its higher condensation ability due to high amount of primary amine groups in its structure, thus, higher efficiency in transfection and higher cytotoxicity (Parhamifar *et.al*., 2010). Recent studies show that polyplex induced cytotoxicities depend on complicated mechanisms that involve necrotic and apoptotic pathways and need to be studied further (Hunter *et.al*., 2006). A way to decrease cytotoxicity was shown to blend the polycations with anionic polymers.

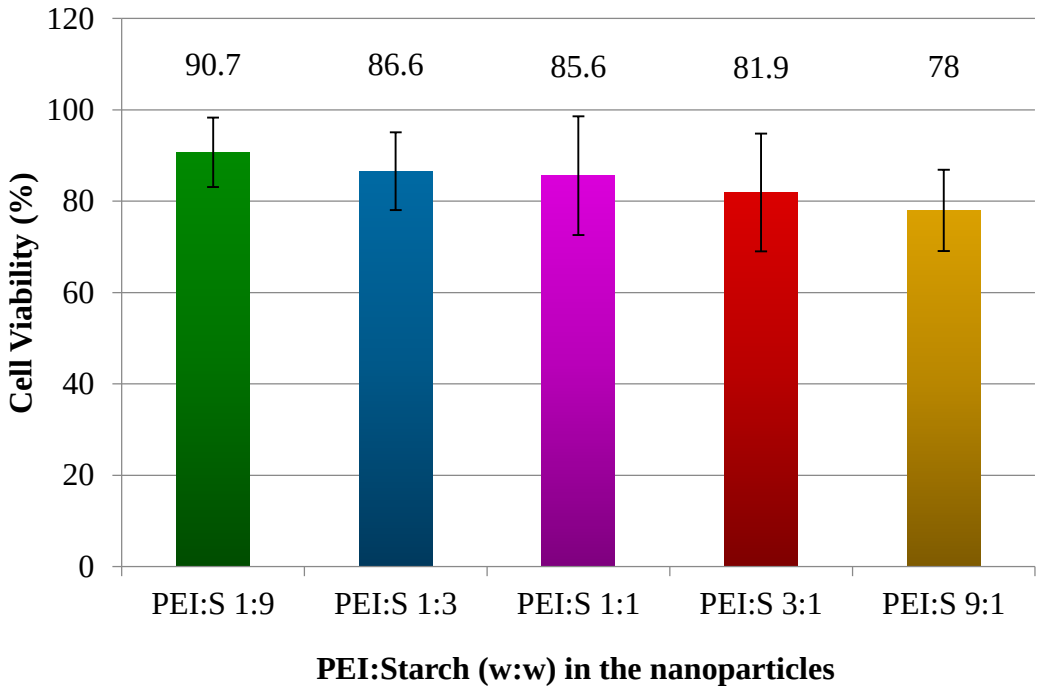


Figure 3.5: Viability of MCF 7 cells treated with unloaded PEI – starch nanoparticles with PEI: starch (w/w) ratios of 1:9, 1:3, 1:1, 3:1 and 9:1.

Starch, as a polar polysaccharide, was previously used in combination with cationic polymers both to bind specific ligands in order to achieve specific gene delivery (Thiele *et.al*., 2010) and to optimize the charge of cationic polymers (Suyao *et.al*., 2004). In this study, starch was used in order to both provide spacing in nanoparticle structure and balance excessive presence of positive charges of PEI with the hydroxyl groups in its structure. As a result, as PEI amount decreased after injection with starch, a higher percent of cell viability is achieved.

3.3.2. Transfection of MCF 7 cells treated with Alexa-555 siRNA loaded PEI– Starch nanoparticles

After translation, the proteins are released into the cytoplasm to join their own cellular pathways, and the silencing of proteins by siRNA is achieved in the cytoplasm (Hunter *et.al*., 2010). For this reason, PEI-starch nanoparticles had to release their siRNA content in the cytoplasm. In order for the nanoparticles to effectively release EF2K siRNA into the cytoplasm, they had to transfect the target MCF 7 cells.

In order to observe the movement and uptake of the nanoparticles by MCF 7 cells, PEI-starch nanoparticles were loaded with Alexa-555 labelled siRNA and used to treat the cells. The cells were observed with CLSM (Figure 3.6).
In this figure phalloidin that is excited at 532 nm shows the actin filaments, and therefore, the cytoskeleton of the MCF 7 cells, (green). Red fluorescence originates from PEI-starch nanoparticles loaded with Alexa-555 labelled siRNA. Since Alexa-555 is excited both at 488 nm, and also at 635 nm it gives both red and blue fluorescence, respectively.
It is observed that the PEI-starch nanoparticles were concentrated around the MCF 7 cells. Yellow or orange colors on the cells could be due to the nanoparticles inside the cells. These show that the nanoparticles were affiliated with the cells, but this is not specifically an indicator of uptake by the cells. Z- stack images could be an additional support of uptake. In this experiment, MCF 7 cells were very concentrated (100,000 cells /well in 24 well plate) and because of their clumping nature they were very thick to obtain z-stacks and x60 images.
As a future study, in order to show uptake, less concentrated MCF 7 cells that are treated with Alexa – 555 labelled siRNA loaded nanoparticles can be used, and stained with DAPI and phalloidin and both fluorescence microscopy and z-stack (CLSM) micrographs can be obtained. That can show the location of the nanoparticles around nucleus and in combination with z-stack images these can be a reliable indicator of nanoparticle uptake.
Previously, Gilleron *et.al*. (2013) concluded that only a minor fraction of internalized siRNA could escape from multivesicular system after cellular uptake of siRNA loaded liposomes. This indicates that escape from endosomal compartments is the critical step in efficiency of gene silencing. In order to observe intracellular pathway of PEI-starch nanoparticles and release of siRNA, fluorescence labelled siRNA loaded PEI-starch nanoparticles could be observed with confocal microscopy and in order to visualize siRNA localization in intracellular organelles, probe labelling methods could be used additionally as Sahay *et.al*. and Guilleron *et.al*. (2013) mentioned in their studies with liposomes.

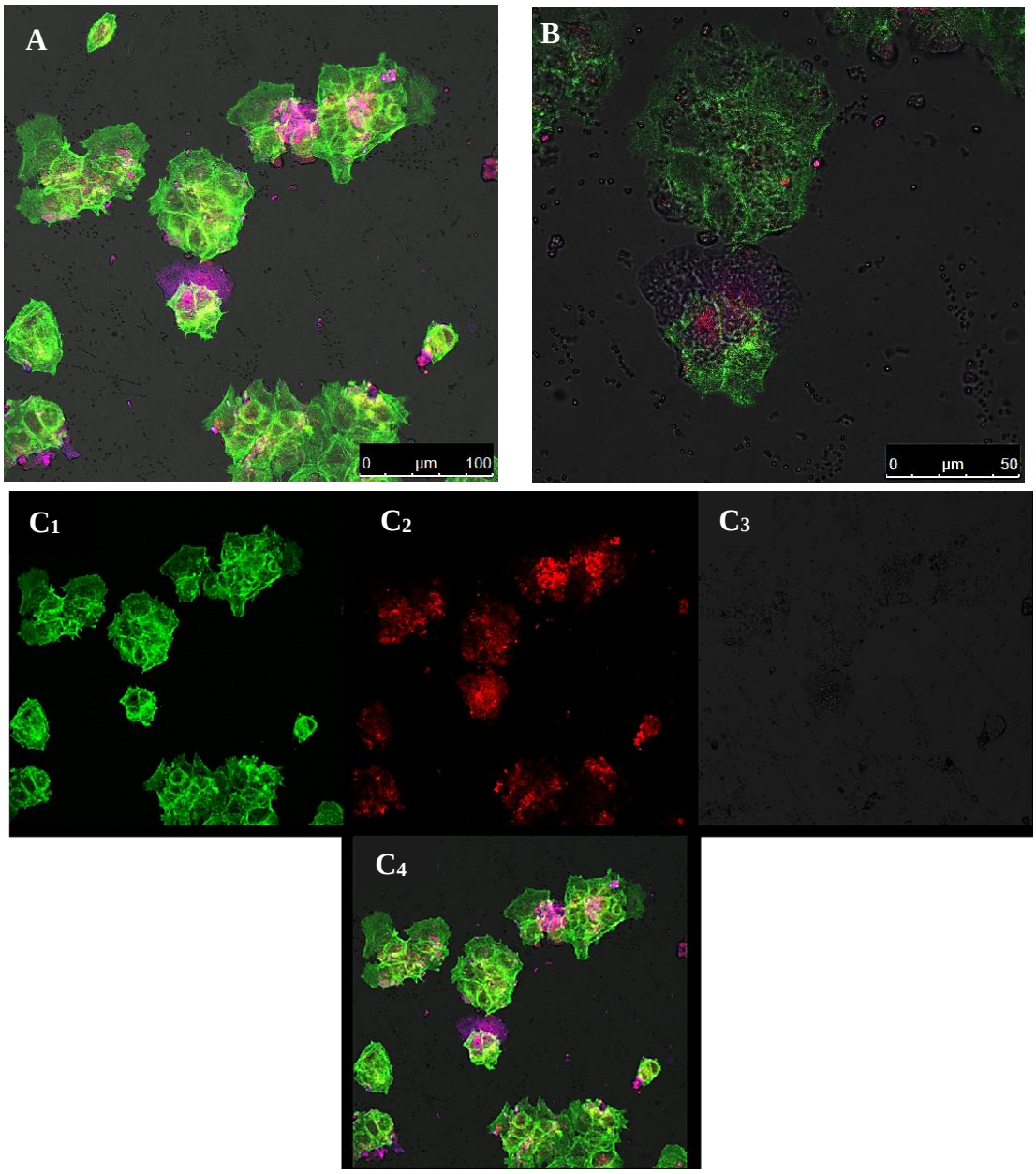


Figure 3.6: Confocal microscopy of MCF 7 cells treated with Alexa-555 labelled siRNA loaded PEI-starch nanoparticles. A) x 20, B) x 40 and x 20 C_1) fluorescence image of MCF 7 cells at 532 nm, C_2) fluorescence image of Alexa-555 labelled siRNA loaded PEI-starch nanoparticles at 488 nm, C_3) transmission image of MCF 7 cells,
C_4) overlay of C_1 and C_2

3.3.3. Efficiency of delivery of EF2K siRNA by PEI-Starch nanoparticles to MCF 7 cancer cells

The efficiency of treatment of PEI-starch nanoparticles with MCF 7 cancer cells was studied with western blot analysis (Figure 3.7). EF2K siRNA and MCF 7 cells were chosen for the western blot test since it is shown that EF2K siRNA is the inhibitor of breast cancer cell growth (Tekedereli *et.al*., 2012). Untreated MCF 7 cells and MCF 7 cells treated with unloaded nanoparticles were used as the negative controls.

The experiment was performed in two different sets and one of the sets was studied in triplicates in order to test precision of results (Appendix C). When the β-actin levels were compared it was observed that the intensities of the bands that correspond to β-actin differed slightly. This probably was caused by unequal loading of proteins into the polyacrylamide gel, or, by inefficient separation of the proteins from cell lysates.

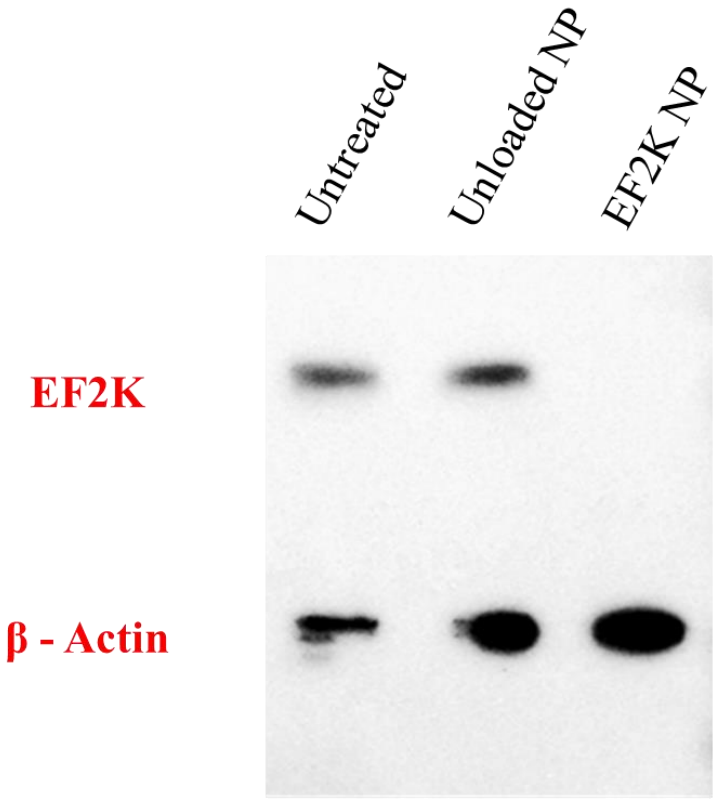


Figure 3.7: Western blot membrane images of MCF 7 cells treated for 72 h with various batches of EF2K siRNA loaded nanoparticles (NP1, NP2, NP3) (100 nM EF2K siRNA and 100,000 cells per well).

The amounts of negatively charged phosphate groups in the chemical structure of siRNA and positively charged amine groups in PEI play an important role in binding efficiency of PEI to siRNA. For this reason, N/P ratio (the ratio of moles of amine groups of cationic polymers to moles of phosphate groups of genetic material) has become an important parameter for transfection efficiency (Grayson *et.al*., 2006; Zhao *et.al*., 2009; Utsuno *et.al*., 2010).

For the studies made by 25 kDa branched polyethylenimine, it was shown that N/P ratios from 4 to 10 had a quite strong binding between PEI and DNA (Grayson *et.al*., 2006). The N/P ratio of PEI-starch nanoparticles with 9:1 PEI: starch ratio (w/w) was 4 in this study and this made the nanoparticles a good carrier for siRNA. According to western blot data, the PEI-starch nanoparticles transfected MCF 7 cells efficiently. This supports the idea that N/P ratio 4 is suitable for siRNA transfection.

CHAPTER 4

CONCLUSION

A novel siRNA carrier to initiate apoptosis in breast cancer cells was aimed to be constructed. In literature 25 kDa branched polyethylenimin was studied in myriad of gene delivery studies and shown to be the most efficient polycation in gene delivery (Neu *et.al*., 2005). The disadvantage of the polymer was its cytotoxic behavior. Many different approaches were studied and continuing to be studied in order to decrease cytotoxicity of PEI mainly depending on causing structural changes in structure of PEI (Kircheis *et.al*.,2001). Among these studies, PEI-starch blend was not studied before and due to the natural and anionic structure of starch, it is thought that it would efficiently make complex with PEI and decrease its cytotoxicity. Considering this, PEI-starch nanoparticles were aimed to be constructed as nanocarrier for delivery of apoptotic siRNA into cancer cells. In order to check if their physical structuras are suitable for siRNA delivery, characterization tests were done. Zeta size measurements show that they are in suitable size range for EPR effect of tumor site and endocytosis. SEM micrographs and NIH Image J size measurements were consistent with data obtained from Zeta sizer. Zeta potential measurements revealed that their charge were increasing as PEI content in them increased. The PEI-starch nanoparticles with 9:1 PEI:starch (w/w) ratio showed optimal characteristic futures. It had a positive surface charge and desirable size was also obtained for this ratio as NIH Image J calculations revealed. The cytotoxicity was also tested and percent of cell viability was also in desirable range. The uptake and transfection efficiencies were checked with CLSM and western blot. The nanoparticles were shown to affiliate with MCF 7 breast cancer cells and they were able to silence EF2K protein almost totally.

These results show that the PEI-starch nanoparticles are optimal in size, charge, release kinetics and efficiency in delivery of their content, which make them a novel siRNA delivery vector.

4.1. Future Work

Characterization and efficiency of nanoparticles were tested. Although the results are very promising, some of them may have to be repeated in order to increase reproducibility. For example, gel electrophoresis should be repeated in order to show EF2K decrease with equal β–actin levels.

Although molecular weights of insulin and a typical siRNA are similar, there is a considerable difference between their 3D structures. For this reason, the release studies must also be repeated by using Alexa-555 labelled siRNA loaded nanoparticles. Also, the release media may be changed

from ultrapure water to 50% FBS, in order to mimic the environment that the nanoparticles are aimed to be delivered in body.
In order to show uptake more accurately, the samples can be stained with DAPI and locations of nanoparticles around DAPI could be observed with fluorescence microscopy. When these images were compared with CLSM images, we can learn much more about location of nanoparticles in or around cells.

PEI – starch nanoparticles have potential to be an efficient vector for siRNA delivery to cancer cells. Additional tests and studies should be performed for development of the nanoparticles. PEI-starch nanoparticles are aimed to be delivered intravenously. For this reason, interaction of nanoparticles with blood ingredients should be checked by an additional hemacompatibility test.

The next step of development for the nanoparticles may be coating of nanoparticles with functional ligands specific to target cancer type, thus, achieve active targeting. Aptamers could be used in order to construct a highly selective surface around nanoparticles.

A final step would be animal studies. Tumor gained nude mice could be used to test the efficiency of the nanoparticles in localization and also in treatment.

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APPENDIX A

INSULIN CALIBRATION CURVE

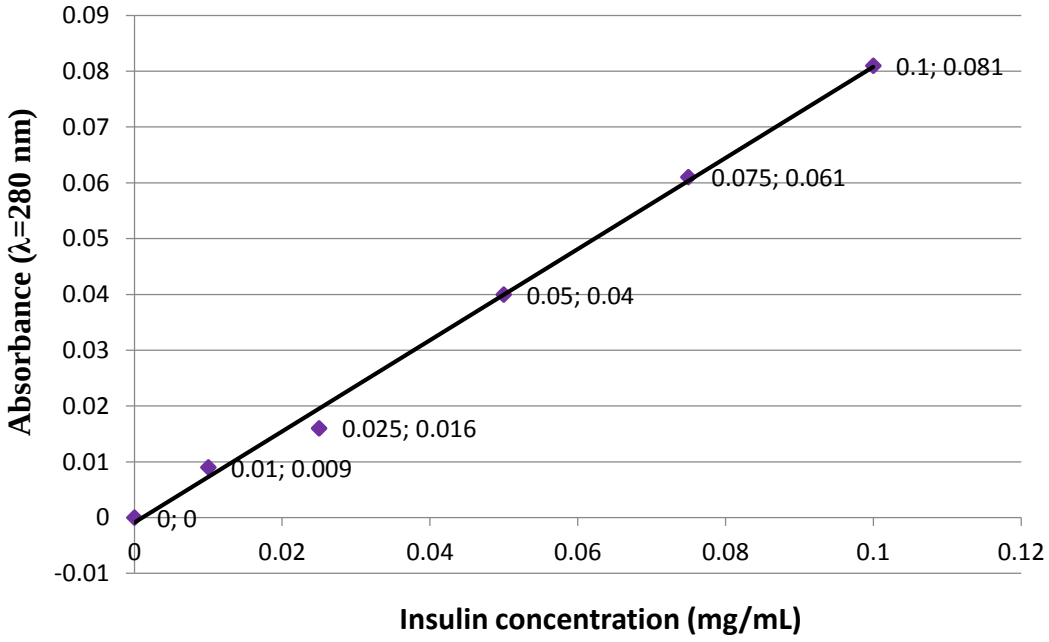

Figure A.1: Insulin calibration curve for release studies (pH 7.4, 10 mM)

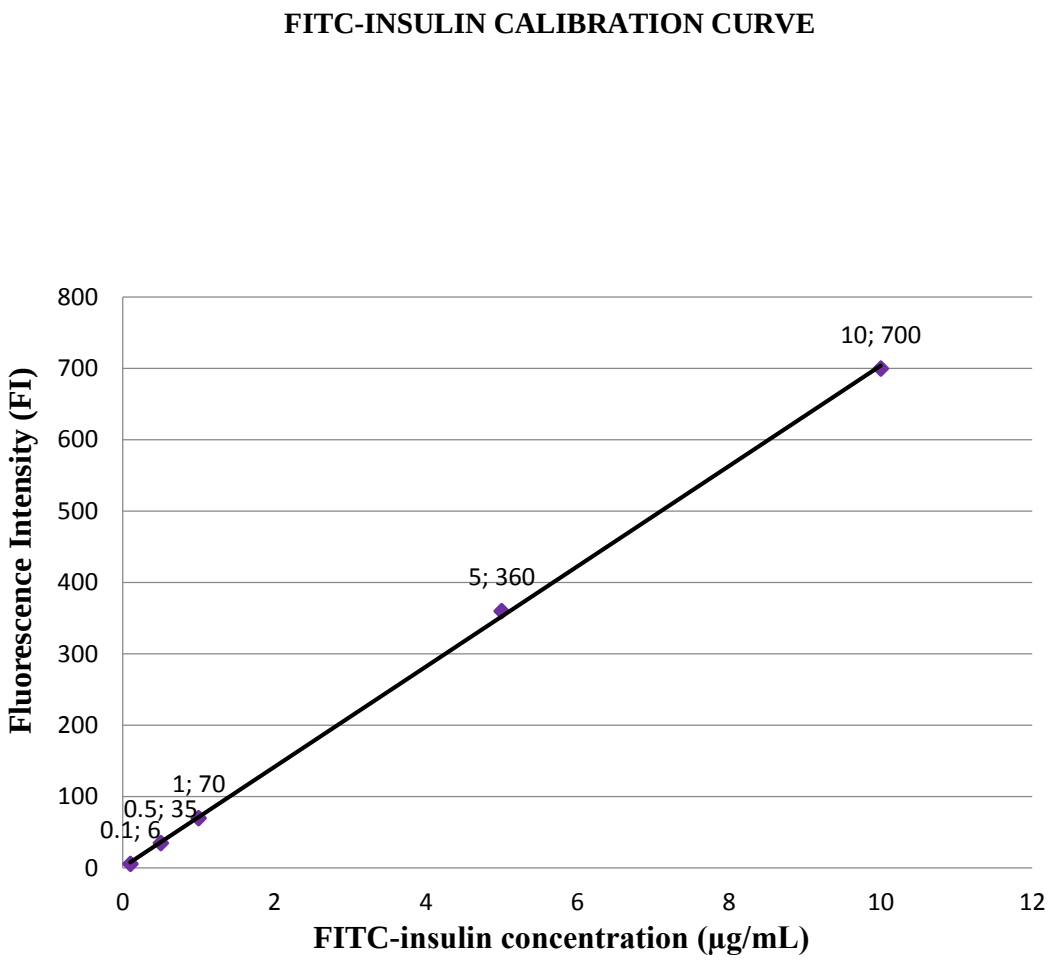

Figure A.2: Calibration curve of FITC-insulin for analysis of release kinetics of PEI-starch nanoparticles

APPENDIX B

CALIBRATION CURVE OF MCF 7 CELLS

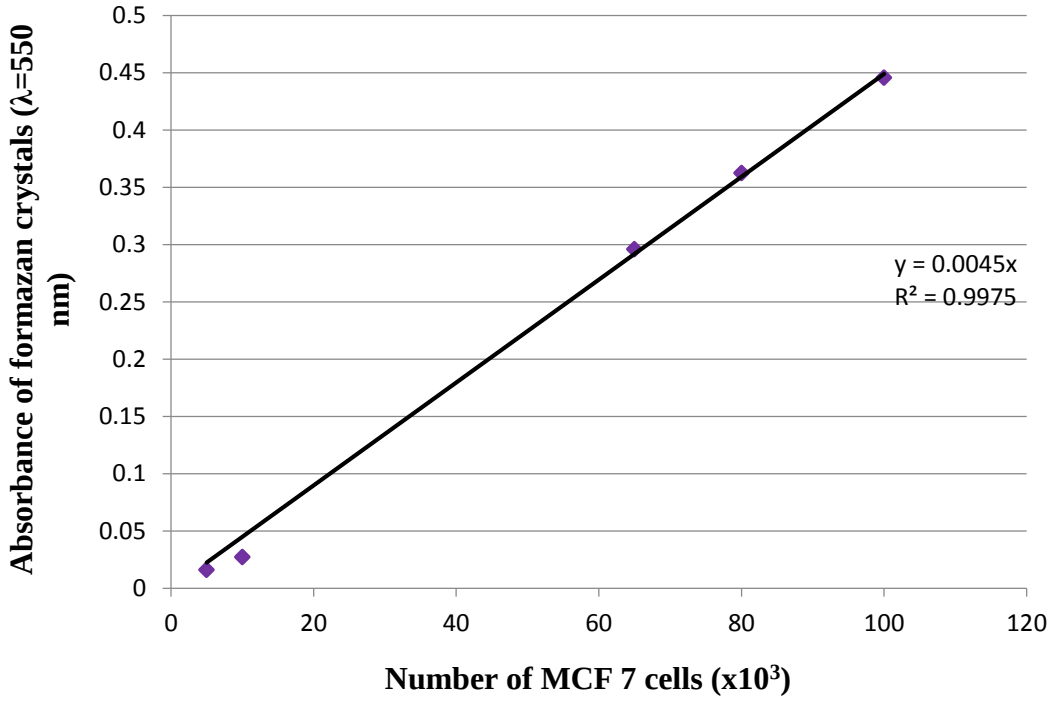

Figure B.1: MCF 7 calibration curve for MTT cell viability assay.

APPENDIX C

WESTERN BLOT IMAGE

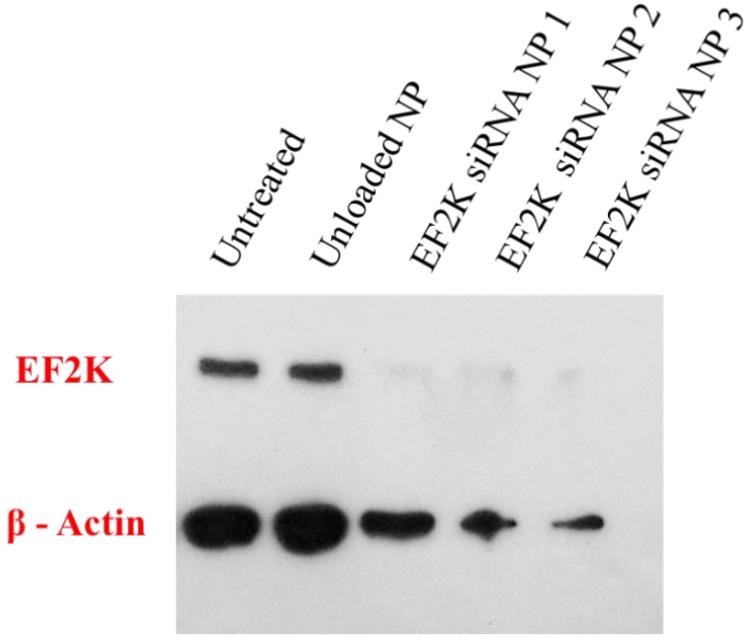

Figure C.1: Western blot image of MCF 7 cells treated with EF2K siRNA loaded nanoparticles (NP) for 72 h (100 nM EF2K siRNA and 100 000 cells per well