Nur Pınar SANCAK

DEVELOPMENT OF NOVEL NANOMATERIALS FOR NON-VIRAL GENE DELIVERY

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by

Nur Pınar SANCAK

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APPROVAL PAGE

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Nur Pınar SANCAK

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ABSTRACT

Gene therapy is a medical procedure used to treat diseases caused by nonfunctional genes and it involves replacing those nonfunctional genes with healthy ones that supplied from outside. In gene therapy, both viral and non-viral gene delivery agents are used. The gene delivery agents are also used to produce recombinant proteins for various disease treatments and related researches. In particular, the gene delivery agents are used in the treatments of cancer, diabetes, some genetic diseases along with cellular and regenerative medicine fields.

Although there is a good number of works about non-viral gene delivery agents in the literature, there are quite few researches on boronic acid containing gene delivery agents. In current studies, boronic acid containing gene delivery agents are obtained only by direct modification of synthetic polymers such as polyethylenimine(PEI), Poly(amidoamine) (PAMAM) and amphiphilic polyether (Pluronic-PG) with boronic acid. Still, there are many alternative materials, such as natural polymers, different synthetic polymers and nanoparticles, suitable for developing boronic acid-assisted gene delivery agents.

In this study, it is aimed to develop novel non-viral delivery systems based on boronate ester formation in Human Embryonic Kidney (HEK) 293 Cell line. Within the scope of this study, carbon nanotubes (CNTs) were pre-modified and then, new non-viral gene delivery systems were developed by functionalizing with boronic acid groups in order to increase their gene delivery efficiency. Also, this study was focused on the pEGFPN1 delivery into human embryonic kidney 293 cells (HEK) by using polyamidoamine(PAMAM) dendrimer modified CNTs and boronic acid (BA) modified CNTs at increasing generations (from G1 to G5). The effects of CNTs on cell attachment and viability of human embryonic kidney 293 cell line *in vitro* were measure

by real time cell analysis (RTCA) and WST-1 cell proliferation assay. Results showed that after incubation with two types of CNTs over 24 h, the cellular viability and the metabolic activity of HEK 293 cells decrease at increasing concentrations of CNTs. To detect the pEGFPN1 and CNTs complex formation, agarose gel electrophoresis was used. Agarose gel electrophoresis results demonstrated that each type of CNTs is able to condense DNA to varying degrees at different mass ratios. Fluorescence emitting GFP-transfected cells were observed and counted under a fluorescent microscope. *In vitro* transfection results showed that BA-MWCNTs were more effective than PAMAM-MWCNTs for *in vitro* pEGFPN1 delivery on HEK 293 cells.

In conclusion, this study shows that the gene delivery might be achieved using boronic acid modified carbon nanotubes and also transfection efficiency might be increased by making use of boronic acid-diol interaction. Additionally, Boronic acid modified multiwalled CNTs might be used as gene delivery agents; therefore further *in vivo* studies are required.

Keywords: Gene delivery, carbon nanotubes, polymer, nano material, boronic acid

VİRAL OLMAYAN GEN TAŞIMA UYGULAMALARI İÇİN YENİ NANOMALZEMELERİN GELİŞTİRİLMESİ

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ÖZ

Gen tedavisi, kalıtsal yollarla veya sonradan dış etkenlerle normal işlevini yitiren genler nedeniyle ortaya çıkan hastalıkların tedavisine yönelik dışarıdan normal çalışan genin verilmesi ile yapılan tedavi şeklidir. Gen tedavilerinde viral veya non-viral gen taşıma ajanları kullanılmaktadır. Gen taşıma ajanları ayrıca hastalık tedavisine yönelik veya araştırma alanında rekombinant proteinlerin üretilmesinde kullanılmaktadır. Gen taşıma ajanları özellikle kanser tedavisi, diyabet, çeşitli kalıtsal hastalıklar, hücresel ve rejeneratif tıp alanlarında kullanılmaktadır.

Non-viral gen taşıma ajanları üzerine literatürde pek çok çalışma olmasına karşın, boronik asit fonksiyonel ajanlar üzerine çok az çalışma vardır. Hali hazırda yapılmış olan çalışmalarda boronik asit destekli gen taşıma ajanı sadece sentetik polimerler olan polietilenimin (PEİ), poliamidoamin (PAMAM) ve amfifilik polieter (Pluronic-PG) moleküllerinin boronik asit ile direkt modifikasyonundan elde edilmiştir. Boronik asit destekli gen taşıma ajanı geliştirilebilecek pek çok alternatif malzeme (doğal polimerler, farklı sentetik polimerler ve nanoparçacıklar) non-viral gen taşıma ajanı olarak geliştirilmeyi beklemektedir.

Bu çalışmada, İnsan Embriyonik Böbrek Hücre (HEK 293) hattına boronat ester oluşumuna dayalı yeni non-viral gen taşıma sistemlerinin geliştirilmesi amaçlanmıştır. Bu çalışma kapsamında, nanoparçacıklara ön-modifikasyonlar yapıldı ve daha sonra yeni modifikasyonlar yapılan non-viral gen taşıma ajanları gen taşıma etkinliklerinin daha da arttırılması amacıyla boronik asit gruplarıyla fonksiyonel hale getirilerek yeni non-viral gen taşıma sistemleri geliştirildi. Geliştirilen sistemler, G1'den G5'e artan jenerasyonlardaki PAMAM ve boronik asitle modifiye edilen karbon nanotüpler, HEK 293 hücre hattına GFP plasmidinin aktarımında kullanıldı. Karbon nanotüplerin, HEK hücrelerinin tutunma ve yaşama kabiliyetleri üzerindeki etkileri gerçek zamanlı hücre

analizi ve WST-1 hücre çoğalma analizi deneyleriyle ölçüldü. HEK hücrelerinin artan jenerasyonlardaki karbon nanotüplerle 24 saat inkübe edilmesinin ardından elde edilen sonuçlar, artan jenerasyonlardaki karbon nanotüplerin konsantrasonları arttıkça hücre yaşamının azaldığını ve hücre çoğalmasının yavaşladığını gösterdi. GFP plasmidi ile karbon nanotüplerin kompleks oluşumunu belirlemek için agaroz jel elektroforezi yöntemi kullanıldı. Agaroz jel sonuçları, hem PAMAM hem de boronik asitle modifiye edilen karbon nanotüplerin GFP plasmidi ile farklı kütle oranlarında farklı derecelerde bağlanarak kompleks oluşturduklarını gösterdi. GFP transfekte olan hücreler, yaydıkları floresan ışıma ile floresan mikroskobunda belirlendi ve sayıldı. *In vitro* gen taşıma deneyleri, pEGFPN1'in HEK 293 hücrelerine aktarımında boronik asitle modifiye edilen karbon nanotüplerin daha başarılı olduğunu gösterdi.

Bu çalışma göstermiştir ki, boronik asit modifiyeli karbon nanotüpler kullanılarak gen aktarımı yapılabilir ve boronik asit-diol etkileşimi kullanılarak transfeksiyon verimi artırılabilir. Ayrıca boronik asitle modifiye edilen karbon nanotüpler gen taşıma aracı olarak kullanılabilir, bununla birlikte *in vivo* gen aktarım çalışmalarının yapılması gereklidir.

Anahtar Kelimeler: Gen taşıma, karbon nanotüp, polimer, nano malzeme, boronik asit

To my beloved family

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SYMBOL/ABBREVIATION

BA	Boronic acid
$CaCl_2$	Calcium chloride
Ch	Chitosan
CNT	Carbon nanotube
CO_2	Carbon dioxide
dH_2O	Distilled water
ddH_2O	Double-distilled water
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
E.coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EtOH	Ethanol
FBS	Fetal bovine serum
G1	Generation 1
G2	Generation 2
G3	Generation 3
G4	Generation 4
G5	Generation 5

HCl	Hydrochloric acid
HEK	Human embryonic kidney cells
Kan	Kanamycin
LB Agar	Luria-Bertani agar medium
LB Broth	Luria-Bertani broth medium
MWCNT	Multiwalled carbon nanotube
NaCl	Sodium chloride
NH_2	Amidogen
PAMAM	Polyamidoamine
PBS	Phosphate buffered saline
pDNA	Plasmid deoxyribonucleic acid
PEG	*Polyethylene glycol*
PEI	Polyethylenimine
PLL	Poly-L-lysine
SiRNA	Small interfering ribonucleic acid
SWCNT	Singlewalled carbon nanotube
TBE	Tris-borate-EDT
TE	Tris-EDTA
v/v	volume/volume
w/o	without

CHAPTER 1

INTRODUCTION

1.1 GENE DELIVERY

Gene therapy is a medical procedure used to treat diseases caused by nonfunctional genes and it involves replacing those nonfunctional genes with healthy ones that supplied from outside. Recently, gene therapy has been used in treatment of many diseases such as genetic disorders, infectious diseases and cancer (Jeong, Kim and Park 2007).

Gene therapy can be divided into two categories which are applied either *ex vivo* or *in vivo*, shown in Figure 1.1.

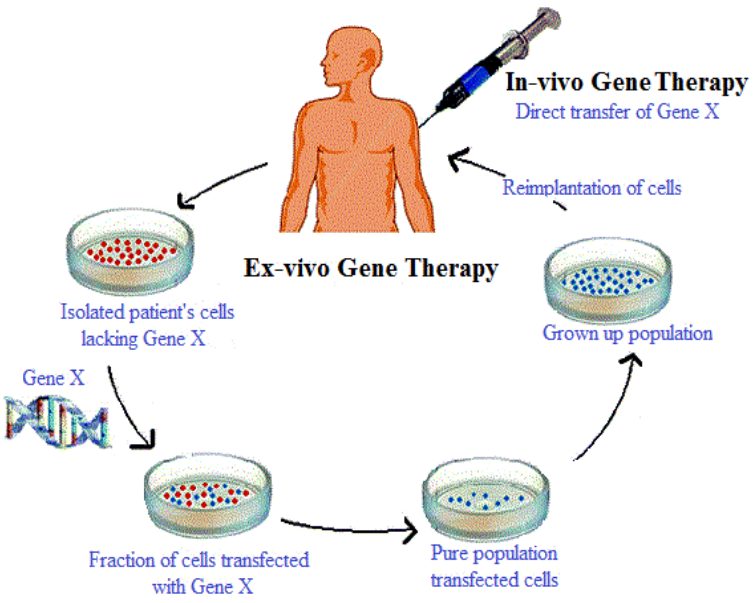

Figure 1.1 *Ex vivo* and i*n vivo* gene therapy

Ex vivo gene therapy is mainly based upon three steps involving previously the isolation of the cells lacking desired gene from either patients or donors, secondly the transferring of desired genes into cultured cells and after expansion of cells *in vitro*, lastly returning the transduced cells to the patient (Naldini 2011). Therefore, *in vivo* gene therapy is performed by using any carrier vehicle in the directly transfer of genetic material into target organs or tissues (Wang et al. 2013).

The main aim about performing gene therapy is not to reorganize the structure of abnormal gene because the knowledge about DNA repair and recombination mechanisms has not been completely clear yet. It is based on the providing of the production of the protein, which is not produced due to abnormal gene, by making changes in genetic material of the cell and modulating gene expression.

Gene delivery is the key of gene therapy. A carrier is required to transfer desired gene into a target cell. Such vehicles of gene delivery are known as vectors. There are different gene transfer systems or gene vectors that have been defined and studied in order to make gene transfer more efficient, specific and suitable for human body (Wang et al. 2013). Additionally, some of significant points in order to achieve efficient gene delivery which are vector safety, specificity of vector to reach the target cell and vector capacity to protect DNA which can be easily degraded by intracellular nucleases that should be considered about all of them because they play an important role during gene transfer.

Herein, choosing a suitable vector is one of the most critical issues in gene delivery. Because there are important points such as cellular uptake, endosomal escape, intracellular cytoplasmic trafficking, DNA release and nuclear uptake that are required to overcome all of them in order to get successful and effective transfection (van Gaal et al. 2011). On the contrary, several cellular processes might be blocked due to using incompatible vehicle to carry gene as a result of toxic effects related to vehicle properties.

Viral and non-viral delivery systems are two major approaches in gene transfer systems which are commonly used. Viral systems including adeno-associated viruses, retroviruses, lentiviruses, herpes viruses and adenoviruses are the successful gene delivery systems (Huang et al. 2011).

Table 1.1 The major types of commonly used viral vectors in gene therapy (Edwards 2014)

Viral Vector	Type and Gene Material	DNA-Carrying Capacity	Key Properties
Retroviruses	Integrates with host chromatin, RNA	8 kb	Efficient transfection *ex vivo* Inefficient transfection *in vivo* Safety concerns
Adenoviruses	Extra chromosomal DNA, dsDNA	30 kb	Highly efficient transfection *in vivo* and *ex vivo* Repeat treatments ineffective due to strong immune response
Adeno-Associated viruses	Either, ssDNA	5 kb	Safety problems owing to potential insertional mutagenesis
Lentiviruses	Integrates with host chromatin, RNA	8 kb	Transfects proliferating and non-proliferating cells Need active transport into cell
Herpex Simplex viruses	Extra chromosomal DNA, dsDNA	40 kb	Effective on many cell types Strong tropism for neurons Difficult to produce in large quantities

The principle of virus-based gene delivery system seems simple and lead to high transfection efficiency and long term gene expression *in vitro*, but numerous problems and risks exist which prevent gene delivery in their usage (Anson 2004). For instance, viruses can easily infect more than one type of cell. Moreover, viruses such as adenoviruses, adeno-associated viruses and lentiviruses lack of selectivity because of having the ability to infect non-dividing cells used for *in vitro* gene delivery in comparison with retroviruses (Bushman 2007). Thus, when viral vectors are used to transfer desired gene into a specific target cancer cell, they might infect healthy cells as well as cancer cells. In contrast to their high transfection efficiency compared with non-

viral vectors (Quong and Neufeld 1998), many clinical trials of viral vectors are limited due to their potential ability to cause mutational infection *via* possible insertion of their genetic material into the host genome and toxic reactions (Jiang et al. 2007; Kim et al. 2009). However, nonviral systems have numerous advantages in compared with viral vectors. Lower immunogenicity, safety, the ability to carry larger DNA molecules are the major advantages of nonviral gene delivery systems.

1.2 NON VIRAL SYSTEMS FOR GENE DELIVERY

Nonviral vector systems are based on the usage of either naked plasmid DNA only or different kinds of molecules that can form complex with DNA such as cationic liposomes or cationic polymers.

One of the simplest and most common nonviral gene delivery system is naked DNA that is able to transfer a gene which has the length of 2–19 kb (Herweijer and Wolff 2003). Although the transfection efficiency of naked DNA only is much lower, having lower immunogenicity of it is the reason why naked DNA only is used in gene delivery. Therefore, *in vitro* transfection studies demonstrated that when naked DNA only is used, transfection efficiency significantly decreased because DNA needs to be protected from the rapid degradation by DNAses in the cytoplasmic trafficking.

The big advantage of using nonviral gene delivery agents is that vectors show low toxicity when they compared to viral gene delivery agents. In addition, most of the nonviral vectors enable to carry larger DNA molecule that have great importance for gene delivery studies, thus many complicated diseases can be treated because of no limitation in size of DNA in the usage of nonviral vectors. Furthermore, some properties of nonviral gene delivery systems which are availability and cost-effectiveness make them more usable.

In general, nonviral gene delivery systems can be classified into two groups according to their application as physical gene delivery systems and chemical gene delivery systems.

1.2.1 Physical Gene Delivery Systems

Physical gene delivery systems are not a novel concept. However, these systems have been developed for successful delivery of nucleic acids. As mentioned, for

successful gene delivery it is necessary that vectors have to overcome biological barriers to enter cells. In order to facilitate the entry of DNA to target cell, physical gene delivery systems are used including microinjection, needle injection, jet injection, gene gun, electroporation, sonoporation and hydrodynamic gene transfer (Wang et al. 2013) Electroporation, microinjection, gene gun and sonoporation are the more common ones in the usage of these systems.

1.2.1.1 Electroporation

Electroporation is a feasible nonviral physical method that uses the high voltage electrical currents onto the target cell for increasing the permeability of the cell membrane and results in the formation of transient pores on the cell membrane and by this way allowing DNA to pass through the specific target cell (Mehier-Humbert and Guy 2005).

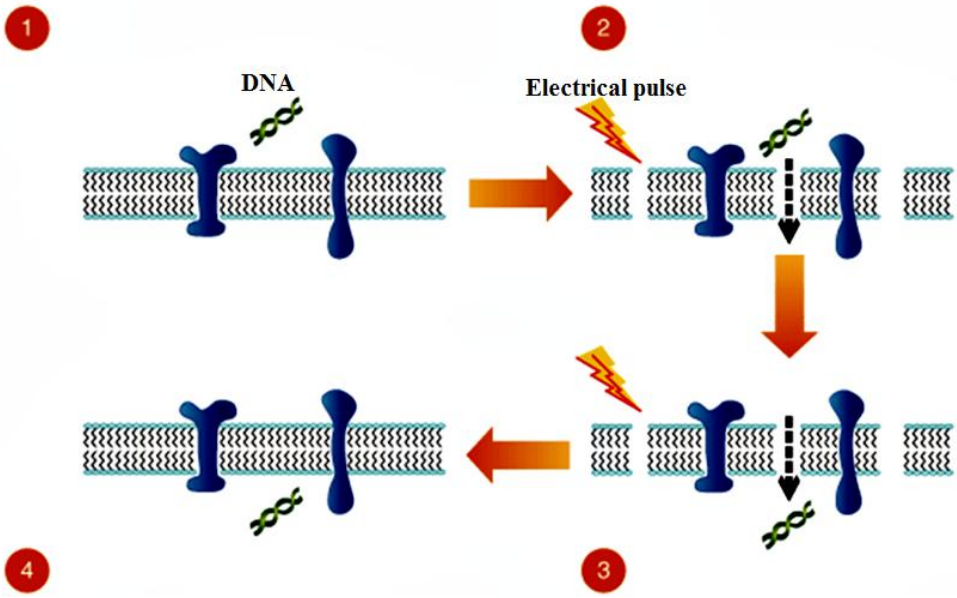

Figure 1.2 The mechanism of DNA vaccination by using electroporation. 1) injection of DNA vaccine into the muscle, 2) pore formation induced by electroporation in cell membrane, 3) entry of DNA vaccine into the cell through the transient pores, 4) after electroporation, expression of encoded antigen by trapped DNA (Wallace et al. 2009).

Electroporation was first used for both *in vitro* and *in vivo* gene delivery and then their applications have been developed based on drug delivery into tumors in

animals and humans (Wang et al. 2013). Additionally, numerous studies with this technique have been applied successfully on various tissues (Heller, Ugen and Heller 2005), such as lung, muscle (Aihara and Miyazaki 1998), liver (Suzuki et al. 1998), skin (Dujardin and Préat 2004), and tumour (Jaroszeski et al. 2004). Recently, the first human trial of gene transfer using electroporation has been reported by Daud et al. that in phase I trial, plasmid interleukin (IL)-12 was delivered by electroporation into the tumors of patients suffering metastatic melanoma (Daud et al. 2008). The results demonstrated that electroporation is a useful physical DNA delivery system having most of advantages such as safety, efficiency and reproducibility.

Despite the advantages of electroporation over its potential, there are two different factors, categorized as physical and biological that are important in the sufficient gene transfer with this technique (Gehl 2003). The type, size, shape, density and location of the target cell and additionally concentration and type of delivered nucleic acid as biological factors; pulse duration and time of discharging, field intensity and electrode geometry as physical factors are some of the important parameters in the use of electroporation which can limit the efficiency of this technique. Furthermore, there are two important problems including difficulties in surgical procedure in the placement of electrodes into the internal tissues and voltage dose applied to tissue. Those might damage the organ and affect genomic DNA stability (Wang et al. 2013) that are needed to be found out to achieve high transfection efficiency depending on the optimization of those parameters.

1.2.1.2 Microinjection

The principle of this technique relies on the injection of nucleic acids directly into cellular cytoplasm and nuclei of a single living cell at a microscopic level by using micropipette (Wang et al. 2013). Microinjection is typically sufficient system that makes possible a single cell injection.

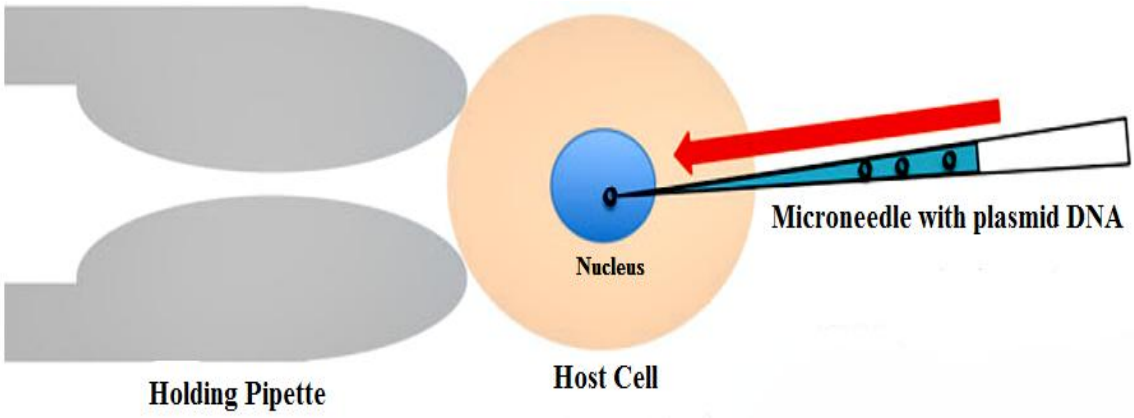

Figure 1.3 An injection of pDNA to the individual cells *via* Microinjection, which uses microneedles to transfer pDNA directly to cell nuclei (Mellott, Forrest and Detamore 2013).

In earliest form of microinjection, living cells were injected using microneedles which made of glass, however the technique were developed by using such combinations of different techniques. For instance, Gill et al. stated that using microneedles coated with microparticles is a good alternative to single microneedles just in the case of microparticles not more than 10 μm in diameter (Gill and Prausnitz 2007). Additionally, microinjection was combined with electroporation in order to delivery of DNA vaccine through the skin (Choi et al. 2010; Daugimont et al. 2010).

There are two main factors including the diameter of the pipette tip and injection timing which are directly related with successful transfection of cells based on microinjection. The shape, size and location of target cells are other important factors that should be considered (Mellott, Forrest and Detamore 2013). In spite of benefits of microinjection, there are still limitations to the use of microinjection caused by challenges in the isolation and immobilization of cells, so performing the system requires specialized training for efficient nucleic acid delivery.

1.2.1.3 Sonoporation

Sonophoresis is a process that makes nanomeric pores in cell membrane to facilitate intracellular delivery of DNA into cells. Pore formation through cell membranes relies on high-intensity ultrasound (Wu 2007). Ultrasound waves stimulate micro-vibrations within the skin epidermis so that the permeability of cells to plasmid or

drug is increased by increasing the kinetic energy (O'Neill and Li 2008; Tomizawa et al. 2013).

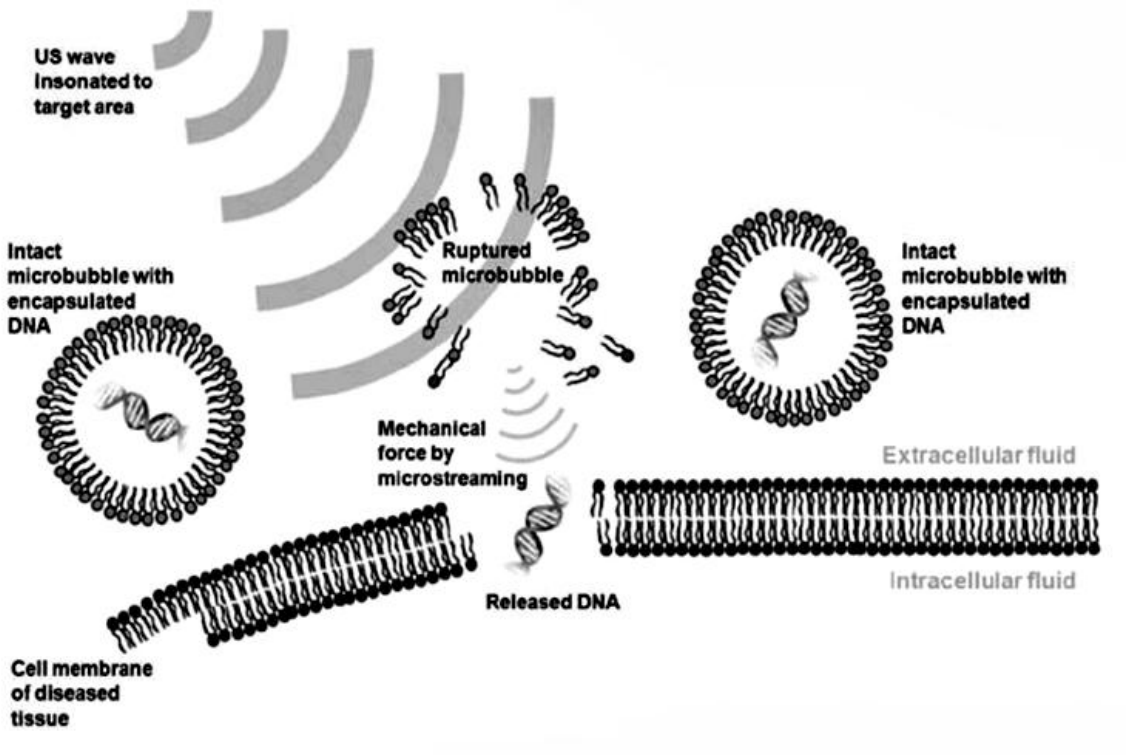

Figure 1.4 The schematic representation of ultrasound induced gene delivery (Kim et al. 2008).

The size and concentration of plasmid DNA can play a major role in facilitating intracellular delivery. While sonoporation is used for increasing drug or gene delivery to diseased tissues *in vivo*, nowadays, ultrasound is widely used in the clinic applications such as enhancement of transdermal absorbsion of drugs or for diagnostic imaging, and kidney stone treatment as well (ter Haar 2007). Moreover, being noninvasive properties of sonoporation enables the use in the large scale application area, particularly in the transdermal absorption of drugs (Mellott, Forrest and Detamore 2013). Despite the advantages of this system over its potential, this system has been limited due to low efficiency of it, especially *in vivo*.

1.2.1.4 Gene Gun

Gene gun technology is a part of physical gene delivery methods that was originally designed for plant transformation and first operated in 1987 by Sanford and his colleagues (Gehl 2003). This technique is often simply referred to as Ballistic DNA injection (bioballistics or biolistics), or DNA-coated particle bombardment. The principle of gene gun relies on the use of particles as carrier that are usually gold, tungsten or silver and the penetration of the plasmid DNA coated particles to target cells or tissue is provided by using the momentum of the particles so that target DNA is transferred into desired cells or tissues, shown in Figure 1.5.

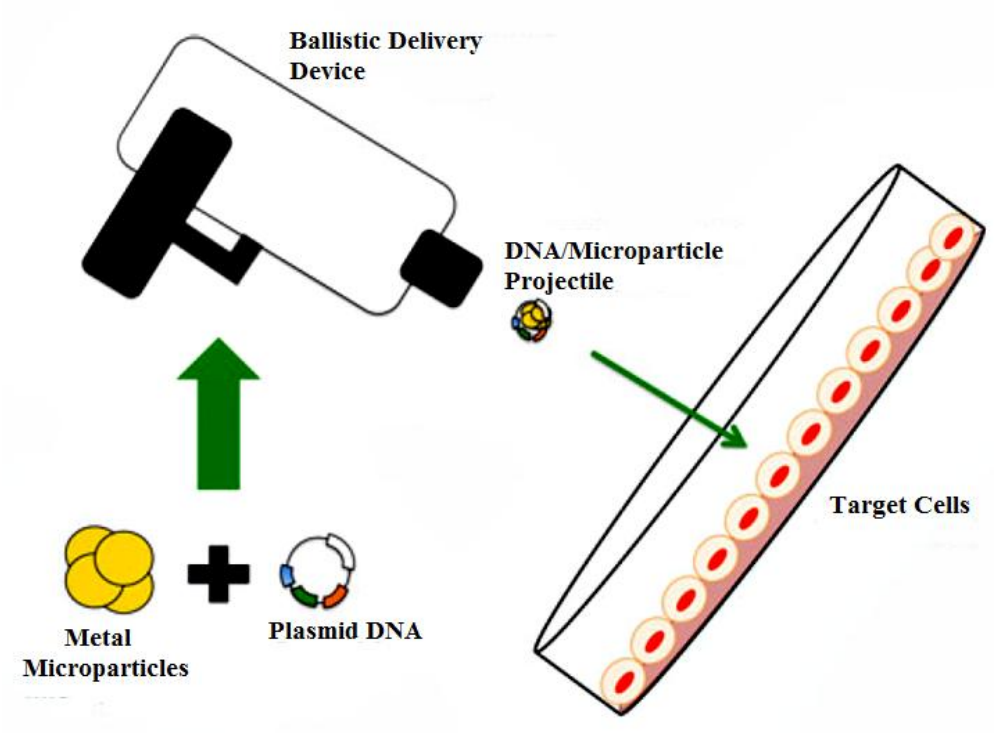

Figure 1.5 Gene delivery using Ballistic DNA injection (Mellott, Forrest and Detamore 2013).

Gene gun is primarily used to enhance gene delivery to diseased tissues *in vivo*. Therefore human clinical trials shown that even if lower DNA dose was applied, immune response highly increased that cells cannot tolerate. Additionally, gene delivery using gene gun is limited by the quantity of delivered nucleic acids even though gene gun has more flexibility for targeting cells. Furthermore, there are also additional

parameters to consider such as the size of particles used as DNA carriers and the gas pressure used for particle movement. On the other hand, there are also benefits using this system in particular formation of DNA vaccines. Moreover, DNA can be easily and effectively transferred into desired organs without any applying surgery (Ibraheem, Elaissari and Fessi 2014).

In general, the main disadvantage of physical nonviral systems is their low transfection efficacy because of the inability of protection DNA from nucleases in systemic circulation. However, most of them are mainly based upon invasion procedures and so physical delivery systems are needed to be developed for further use.

1.2.2 Chemical Gene Delivery Systems

In order to improve non viral gene delivery methods, chemical gene delivery methods have been designed and developed as alternative strategies. In recent years, many investigators have started to use chemical methods for gene delivery because of their advantages compared to physical methods. Chemical gene delivery systems are mainly consist of cationic lipids and cationic polymers such as synthetic or natural polymers, dendrimers, polypeptides and nanoparticles which are more common than physical gene delivery systems (Santos et al. 2010).

Cationic lipids and polymers are two main parts of nonviral gene delivery systems that are positively charged and can form complexes by interacting with negatively charged DNA (Cheung et al. 2010). In addition, having lower immunogenicity, ease of preparation and stable characteristics of cationic lipids and polymers make them more common and favorite gene carriers (Malakooty Poor et al. 2014).

1.2.2.1 Cationic Lipids or Liposome Mediated Gene Delivery

Cationic lipids are designed in essential three parts including hydrophilic positively charged head group, hydrophobic anchor and linker are utilized to target cationic lipids to various cell membranes. Lipid vectors are generated by a combination of plasmid DNA and a lipid solution that result in the formation of a liposome which are also known as lipoplex. The use of liposomes for transfection of DNA into a host cell is called as lipofection. For gene delivery, the complexation process of DNA and cationic lipids is based on the electrostatic interaction that happens spontaneously. Cationic

lipids fuse with the cell membranes of a variety of cell types and introduce the plasmid DNA into the cytoplasm by endocytosis. Studies about liposome mediated gene delivery have shown that lipoplexes are generally stable enough and provide the protection of nucleic acids from degradation in the cellular trafficking.

There are numerous advantages in the use of liposome mediated gene or drug delivery. Primary advantage of liposomes is their low immunogenicity which make them more usable compared to other nonviral systems. Moreover, low toxicity characteristics of liposomes is currently associated with their chemical structure since they are made of only biological lipids (Nayerossadat, Maedeh and Ali 2012). Another advantage of liposomes is the possibility of causing insertional oncogenesis is much lower so that liposome mediated gene delivery makes available to long term gene expression. For instance, when combined with small interfering RNA (siRNA), cationic liposomes may lead to the inhibition of tumor proliferation, inducement of apoptosis, and enhancement of radiosensitivity to tumor cells (Yang W, 2007). Besides the advantages of cationic liposomes, there are also disadvantages including low efficiency. Studies about designing liposomes with having high efficiency have being still continued. Numerous factors can influence the transfection efficiency by liposome mediated gene delivery. However, one concerned issue of gene delivery by liposomes is the charge ratio of cationic lipid to DNA which might be an important factor effecting the transfection efficiency.

N-[1-(2,3-dioleyloxy)propyl]-*N*,*N*,*N*-trimethlylammonium chloride (DOTMA) and N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammoniumethyl sulfate (DOTAP) are most common used cationic lipids. Cationic lipids can be used alone or generating co-lipids. Dioleoylphosphatidylethanolamine (DOPE) is one of the suitable co-lipid which is commonly used. It was shown that DOPE has the potential to enhance transfection efficiency by facilitating liposomes forming and fusion with endosomal membrane (Zuidam and Barenholz 1998). Among them, Felgner J.H. et al had synthesized a hydroxy-containing lipids and they found that transfection efficiency is enhanced using these lipids *via* chemical modification of the hydrophilic positively charged head of cationic liposomes (Felgner et al. 1994). Furthermore, different modification studies can be improved for liposomes to gain a novel characteristics even for drug delivery. For instance, many carcinoma cells express high levels of folate receptors. In the case of liposomes, carrying a drug for cancer, are modified as suitable

to folate, linkage of DNA or DNA-lipid complexes to folate can provide specifically attachment to target cancer cells. Pre-clinical studies have demonstrated that the DNA can easily pass through cell membrane by linking targeting moieties to the liposomes that are in tendency to enter the cell *via* receptor-mediated endocytotic pathway because of linking moiety.

1.2.2.2 Cationic Polymers Mediated Gene Delivery

Nonviral vectors are usually being designed containing high density of cationic sites with the aim of enhancement of their functions. For this purpose, chemical modifications are still being continued.

Cationic polymers are another most used non viral chemical vectors to transfer the desired gene into a target cell. The distinct characteristic of cationic polymers is to contain excessively amine groups which promote their condensing ability to DNA in the case of mixing negatively charged DNA. In addition to extra protonatable functions at neutral pH of cationic polymers, most of them are also biodegradable because of their natural structure.

There are some important points in gene delivery using cationic polymers which must be noticed in order for efficient gene delivery including wrapping to DNA by electrostatic interaction between cationic polymers and DNA, avoiding DNA degradation by nucleases until arrive at target cell or tissue, binding to target cell, entering the cell by endocytosis and endosomal escape (Kundu and Sharma 2008).

Natural polymers, dendrimers, polypeptides and other polymers can be classed under cationic polymers. Poly (L-lysine) (PLL), polyethylenimine (PEI), chitosan and polyamidoamine (PAMAM) are the most known and used cationic polymers in gene delivery (Figure 1.6).

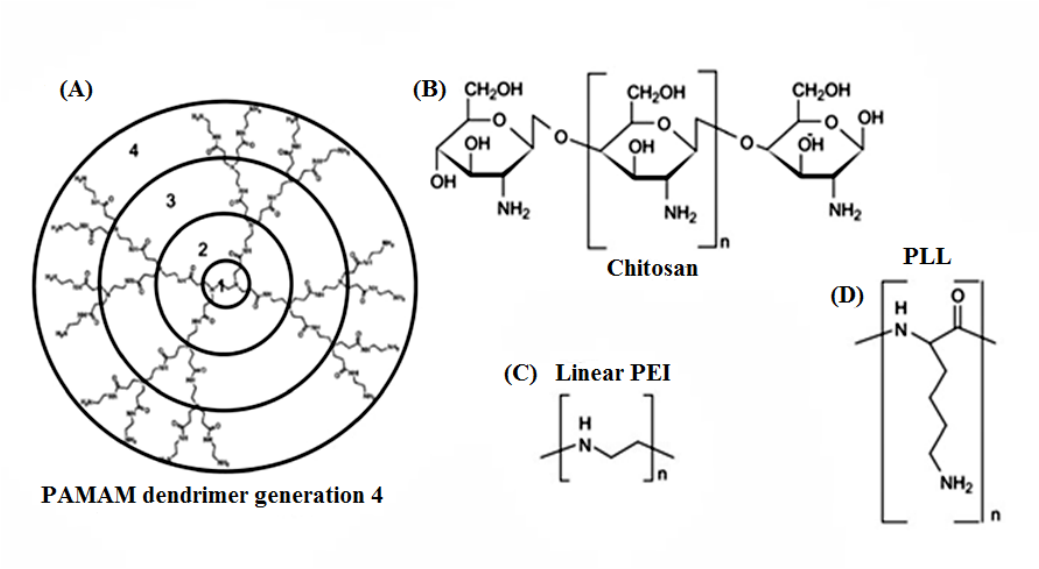

Figure 1.6 Cationic polymers used in gene delivery (A) Poliamidoamine dendrimers(PAMAM)(generation 4), (B) Chitosan, (C) Polyethylenimine (PEI) and (D) Poly(L-lysine)(PLL) (Morille *et al.* 2008).

1.2.2.2.1 Poly (L-lysine)

Poly (L-lysine) is one of the nonviral cationic polymers that has the ability to easily wrap DNA because of having extremely amine groups in its natural structure (Laemmli 1975). Poly (L-lysine) provides both the protection to DNA from extracellular degradation via generating polyelectrolyte complexes and increasing the interaction of negatively charged molecules on cell surface. While generally low molecular weight PLLs are not usable for gene delivery due to lower transfection ability, high molecular weight PLLs cause high cellular toxicity when it was used for gene delivery even if it provides higher transfection efficiency (Choi et al. 1998).

1.2.2.2.2 Polyethylenimine (PEI)

Polyethylenimine (PEI) is another nonviral cationic polymer that also used as gene delivery agent. PEI can be synthesized at different lengths as linear or branched and modified by using different functional groups (Gosselin, Guo and Lee 2001). However, it is required that when a gene is transferred using PEI, PEI have to possess an optimum weight in order for both protection of DNA and delivery of DNA. Additionally, it is known that high molecular weight PEI has toxicity on cell viability (Fischer et al. 1999) because, PEI causes accumulation of polymers on cell surface due

to its extremely positive charge (Kircheis et al. 2001). Moreover, the existence of positive charge on polymer/DNA complex can cause non-specific interactions between PEI and plasma or cell membrane proteins. For finding out this problem, receptor mediated endosytosis is used for DNA complexes (Kichler 2004). For this purpose, RGD-peptides are commonly used as targeted ligand in cellular attachment via integrins. In the recent research, PEI/DNA complex has been coated with hydrophilic poly ethylene glycol involved 17.7 pairs of carboxylic acid and succinate so that coating of the DNA complexes by PEG-Suc(poly ethylene glycol- succinate) has recharged their surface to negative, and effectively protected them from the albumin-induced aggregation. As a result of the injection of those biocompatible DNA complexes with ligand into mouse tail vein, very high gene expression was seen in tumor, lung, and liver (Sakae et al. 2008). On the other hand, different modifications on PEI were generated in order to increase biocompatibility and biodegradability of PEI.

1.2.2.2.3 Chitosan (Ch)

Chitosan is a linear cationic aminopolysaccharide produced by deacetylation of chitin that is also an alternative to PEI via bio-compatible and biodegradable properties (Romøren, Thu and Evensen 2002). Complexation mechanism in chitosan based gene delivery relies on the encapsulation which differs from other cationic polymers mediated gene delivery systems. It means that therapeutic gene is encapsulated by chitosan layer and moves though cell membrane. Additionally, it was shown that the release of DNA from chitosan changes depending on the *in vitro* conditions. Different studies are being continued for the increase the low transfection efficiency of chitosan. For instance, to increase the transfection efficiency of chitosan, recently chitosan-graft-polyethylenimine (CHI-g-PEI) copolymer has been used and it was shown that chitosan-graft-polyethylenimine (CHI-g-PEI) copolymer shows lower toxicity than PEI(25kDa), higher interaction with DNA and higher efficiency at gene delivery in HeLa, 293T and HepG2 cells (Jiang et al. 2007).

1.2.2.2.4 Polyamidoamine (PAMAM) Dendrimers

Dendrimers are other members of cationic polymers that are the most sufficient nonviral gene delivery agents because of condensing DNA via electrostatic interactions to form complexes. Dendrimers are described as highly effective nonviral vectors for *in vitro* transfection because synthesis of increasing branches of dendrimers provides more

binding surface for DNA to dendrimer thus DNA binding capacities of dendrimers are increased which means increasing gene delivery efficiency *in vitro* (Bielinska et al. 2000). Moreover, biocompability and biodegradability are the two reasons why PAMAM dendrimers are the most favored nonviral gene delivery agents. Also, PAMAM dendrimers are safer for cell viability which means non-toxic in comparison with other cationic polymers. However, there are some studies that prove the cytotoxic effects of PAMAM dendrimers associated with generation (Kukowska-Latallo et al. 1996; Roberts J.C. 1996) and therefore, PAMAM dendrimers that are low generation, have low toxicity and high transfection efficiency are still being researched (Shieh et al. 2008). In the recent modification studies, PAMAM dendrimers with lipids (two octadecyl chains) were combined so that more efficient gene transfer was made by depending on increased PAMAM generation (Kono et al. 2012).

1.2.3 CARBON NANOTUBES

One of the major branches of nanotechnology is CTNs that are discovered at 1991. CTNs are a novel class of nanomaterials that used as a new carrier for the delivery of biomolecules, such as DNA or proteins into mammalian cells. Furthermore, CTN based delivery system enables to carry drug for cancer therapy.

1.2.3.1 Properties of Carbon Nanotubes

CTNs are categorized into two major groups as single-walled CTNs and multi-walled CTNs depending on the number of graphene layers. While single-walled CTNs are composed of a rolled monolayered graphene, multi-walled CTNs are composed of a rolled multiple layers of graphene sheets. The diameter is ranging from 0.4 to 2 nm for single walled CTNs and from 1.4 to 100 nm for multi-walled CTNs (Cheung et al. 2010). Both types of CTNs have such excellent electronic properties such as highly heat conduction and electrical conduction which make CTNs more useable. Multiwalled CTNs have many advantages including multiple layers of graphene which provide larger length and higher DNA binding capacity that have extended their usage. Also, recent studies based on the use of CTNs to carry a gene showed that while single-walled CTNs were found to be less toxic than multi-walled CTNs at the cellular level, multi-walled CTNs were found to be more efficient in gene delivery (Kam, Liu and Dai 2006). Apart from their attractive optical and electrical properties, the use of CTNs for

nano-biotechnology applications is increased by exploring the potential biological properties of CTNs.

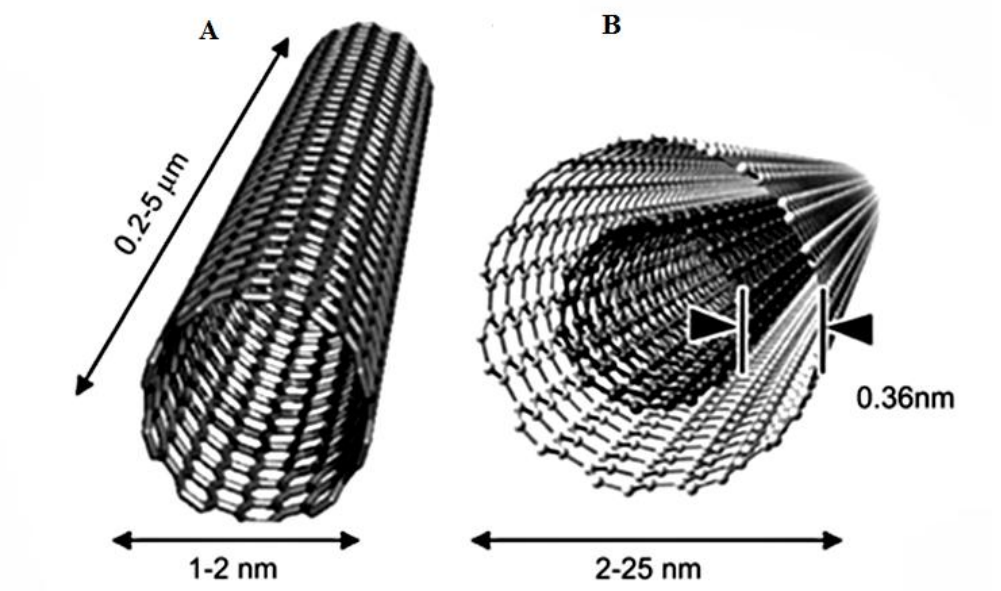

Figure 1.7 The schematic diagram of CTNs A) single-walled CTN B) multi-walled CTN indicating typical dimensions of length, width, and separation of graphene layers in multi-walled CTNs (Reilly 2007).

As described above, vectors have to possess some important properties which are primary requirements for efficient gene delivery.These properties are optimum buffering capacity for endosomal escaping, the ability to condensing with DNA and reducing cytotoxicity (Thankappan et al. 2011). For that purpose, various modification studies have still being carried out to make carriers having these properties. Additionally, vector biocompatibility and the interaction between vector and different types of biological molecules such as DNA, RNA, proteins, and peptides are being developed by this way. The results based on modification studies demonstrated that there are several areas which CTNs could be used indeed. CTNs have a great importance in nanotechnology because of their chemical, physical and also electrical structures.

1.2.3.2 Modifications of Carbon Nanotubes

Covalent and non-covalent functionalizations are two major approaches, which are used to functionalize CTNs. In the case of covalent functionalization of CTNs, because of molecular composition of CTN that is extremely hydrophobic, large hydrophobic surface of it is chemically changed with the aim of generating an anchor site (Behnam et al. 2013), whereas CTNs are altered *via* coating using surfactants, synthetic polymers and biopolymers in non-colavent functionalization (Karimi et al. 2015). Therefore covalent modifications are applied in some studies, non-covalent surface modifications are more common.

Recent research has focused on the development of CTNs with the different chemical modifications for gene delivery (Pantarotto et al. 2004). Chemical modification studies are very important because, designing a new gene carrier that has the potential of higher transfection and lower cytotoxicity is the only way to achieve efficient gene delivery. As it has been reported, several chemical groups that can be used in the modification of CTNs do not reduce their attractive chemical and physical properties (Liu et al. 2013). In this point, one of the potential negative effects of chemical modifications might be related with the solubility of CTNs that is a concern must be controlled for efficient gene delivery. Furthermore, it was shown that the solubility of CTNs affect gene delivery which changes depending on their surface modification (Gao et al. 2006). Another effect of chemical modification is its induced toxicity. To prevent the negative effect of carrier over cell viability, such chemical modification studies have been tried to make with less toxic carrier.

1.2.3.3 Applications of Carbon Nanotubes for Gene Delivery

Cationic polymer functionalized CTNs are explored and are shown to be sufficient DNA carriers (Chen et al. 2012). Polyaminoamine (PAMAM) and polyethilenimin(PEI) dendrimers modified CTNs have been widely used as gene delivery agents for *in vitro* nonviral gene delivery. For instance, Yang et al. demonstrated that cellular toxicity has been reduced and cellular uptake of the nanoparticles has been enhanced just in case the surfaces of multiwalled CTNs were modified with polyamidoamine dendrimer. In addition to this, Qin et al. found that CTNs which are covalently functionalized with PAMAM dendrimers have the capability to effectively transfer pEGFPN1 into HeLa cells in comparison with pure

PAMAM dendrimers (Qin et al. 2011). Besides, another study shown that generation of PAMAM that linked over multiwalled CTNs, or size of carbon nanomaterial and molecular weight of carbon nanomaterial highly influences transfection efficiency and stability of the complex (Malakooty Poor et al. 2014).

1.2.3.3.1 Mechanisms of Carbon Nanotubes/DNA Complexation and Cellular Trafficking

The process of DNA and CTN complexation mechanism is suggested that electrostatic interaction happens between CTNs with negatively charged plasmid DNA. Recent studies have shown that the main aspect about being complex is that DNA wraps CTN (Cheung et al. 2010). Following this binding, as suggested, CTNs may pass through cell membrane via fusion with the cell membrane. Additionally, many investigators have suggested that as a major cellular internalization pathway for biological molecules, endocytosis is also the main pathway in the cellular uptake mechanism for CNTs. Despite the fact that phagocytosis and diffusion are also thought to be alternative mechanisms (Pérez-Martínez et al. 2011).

The uptaken mechanisms of CTNs/DNA complexes are, first, the attachment of CTNs to the cell surface that is provided by nonspecific interaction between CTNs and cell surface, second, endocytosis into endosomes, which there are mainly two important acceptable possible mechanisms such as fluid-phase endocytosis and receptor mediated endocytosis including clathrin-mediated endocytosis and/or caveolae-mediated endocytosis, third, releasing DNA from endosomes which is the most important step for transferring DNA because after DNA release, lysosomal degradation of DNA can be triggered, and fourth, location of the transgene to the nucleus, if the gene can escape lysosomal degradation (Zhang et al. 2004). After that time, CTNs and DNA are separated in cytoplasm. Seperated CTN particules are degraded *via* enzymatic oxidation by peroxidase enzymes including myeloperoxidase, horseradish peroxidase (HRP) and hydrogen peroxidase (H_2O_2) that play major role in the biodegradation of CTNs *in vitro* and *in vivo* (Zhao, Allen and Star 2011).

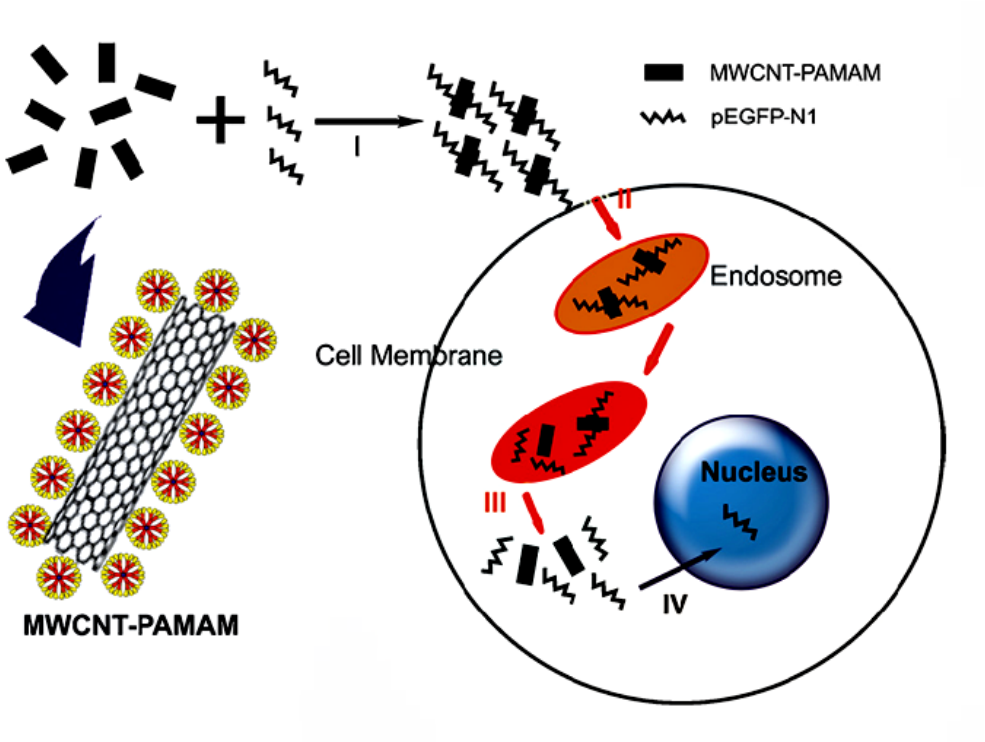

Figure 1.8 pEGFPN1 delivery via MWCNT-PAMAM. I) complexation of pEGFPN1 and MWCNT-PAMAM, II) cellular uptake of MWCNT-PAMAM/pEGFPN1 hybrids *via* endocytosis, III) endosomal escape and DNA release, IV) translocation to nucleus. (Qin et al. 2011)

Among the achievement of PAMAM modified CTNs for gene delivery, the recent study has shown that boronic acid modified dendrimers are a good alternative to unmodified dendrimers as gene delivery agents. It is considered that these polymers enable to increase *in vitro* gene delivery (Peng et al. 2010). Also, past studies suggested that the main contribution of modificaiton of CTNs is to enhance the binding of DNA to the cell surface, to prevent DNA degradation in cytoplasm and to facilitate the DNA escape from endosomes (Wang et al. 2013), which are the main requirements for effective gene delivery.

1.2.3.3.2 The Role of Boronic Acid for Gene Delivery

A boronic acid is an alkyl or aryl substituted boric acid containing a carbon–boron bond belonging to the larger class of organoboranes. Boronic acid generates borate esters by binding reversible to saccharides 1,2- and 1,3-diol that are located on mammalian cell surface. This interaction between boronic acid and small sugars on cell surface which is attached to many different proteins on cell surface, also known as

receptors, can be utilized in the treatment of diabet and cancer (Matsumoto et al. 2009). Recently, Ellis et al. have been studied the binding ability of boronic acid to related saccharides on cell surface such as sialic acid which is the aim of this study have been attributed to involvement of high levels of sialic acid on glycocalyx of cancer cells (Dube & Bertozzi 2005). Furthermore, it was shown that phenylboronic acid binds to saccharides on cancer cells stronger than other saccharides (Springsteen 2002). All of stated above are the evidences of boronic acid that might be used as agent in the selective targeting of cancer cells in therapy.

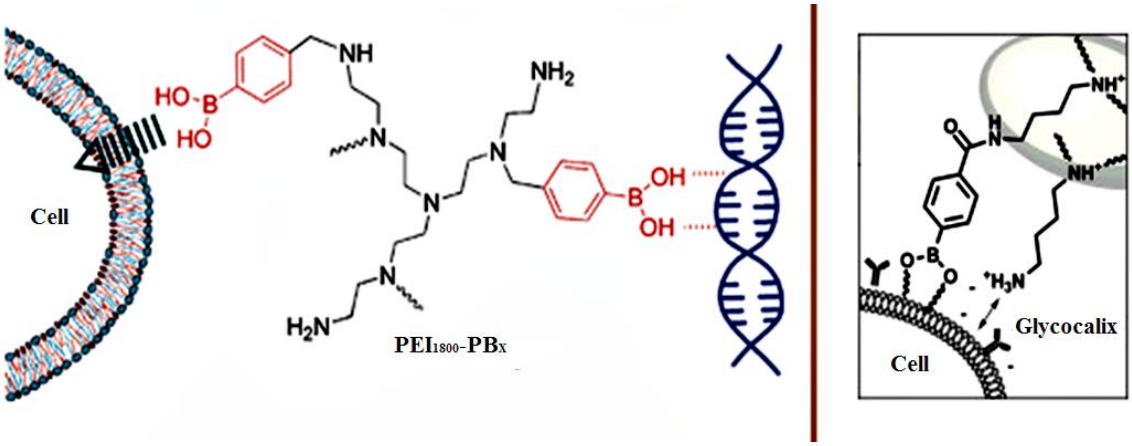

Figure 1.9 The figure of interaction between boronic acid/DNA and boronic acid/cell surface (Piest & Engbersen 2011).

CHAPTER 2

MATERIALS & METHODS

2.1 CARBON NANOTUBES

Multi-wall CTNs (MWCNT) were bought with NH_2 modification on the surface from Cheap Tubes Company in USA.

2.2 SYNTHESIS and MODIFICATION OF PAMAM DENDRIMERS

There are two main ways to have more than one generation PAMAM grown on MWCNT surfaces. One of them is to modify MWCNT surface step by step until desired number of generations of PAMAM is grown on the surface; other one is to synthesize PAMAM dendrons first, then these grown dendrimer generations are added directly to that of desired molecules, in this case, the MWCNTs. In order to have PAMAM dendrimer on MWCNT surface by second way, there are three required steps: first step is esterification; then, amination process should be carried, as a last step, one should carry addition of PAMAM generation reaction to have one generation grown PAMAM on MWCNT surface. In addition, there are some abbreviations for indication of PAMAM dendrimer growing steps. For instance, MWCNT itself without any substituion on it is abbreviated as MWCNT G0, once this nanotubes are aminated, then they are called as MWCNT G0.5; if addition of PAMAM generation is complited for that MWCNT G0.5 nanotubes, it is called as MWCNT G1 which refer to “Generation 1”.

2.2.1 Synthesis of G2 Polyamidoamine (PAMAM) Dendron

Approximnately 50 ml G1 PAMAM dendron and 75 ml methanol were mixed, other than that solution, 250 ml methanol and 200 ml methylacrylate were mixed in 1000 ml volumetric flask. These two solutions are let to cool for half an hour at -20°C. Then, flask was sit into salt-ice bath. While magnetic stirrer is mixing the cold solution,

G1 PAMAM dendron was added slowly. It is stirred for a week or for ten days. Work up process consists of evaporation of solvent by means of rotation and vaporization instrument. In order to purify further, 30-40 ml methanol were added to the flask and then evaporation was done.

2.2.2 Esterification Process

100 ml methyl metacrylate and 100 ml methanol was mixed in 500 ml volumetric flask. In order to protect MWCNTs from crosslinking, mixture was rest at -20°C for half an hour. MWCNTs were also cooled, then they were mixed. Work up step was carried by means of methanol.

2.2.3 Amination Reaction

100 ml ethanol and 100 ml ethylenediamine were mixed in 500 ml in volumetric flask. Approximately 100 mg MWCNT was solved in 100 ml ethanol, these two mixtures were put in -20°C separately for half an hour. Cooling process increases selectivity during chemical reaction. Cooled two solutions were mixed in a flask which was placed in ice-salt bath and rest in -20°C for two days. In order to carry work up for this solution, it undergoes centrifugation process to get aminated MWCNTs separately. Purification of seperated MWCNTs centrifugation process was done repeatedly with pure methanol.

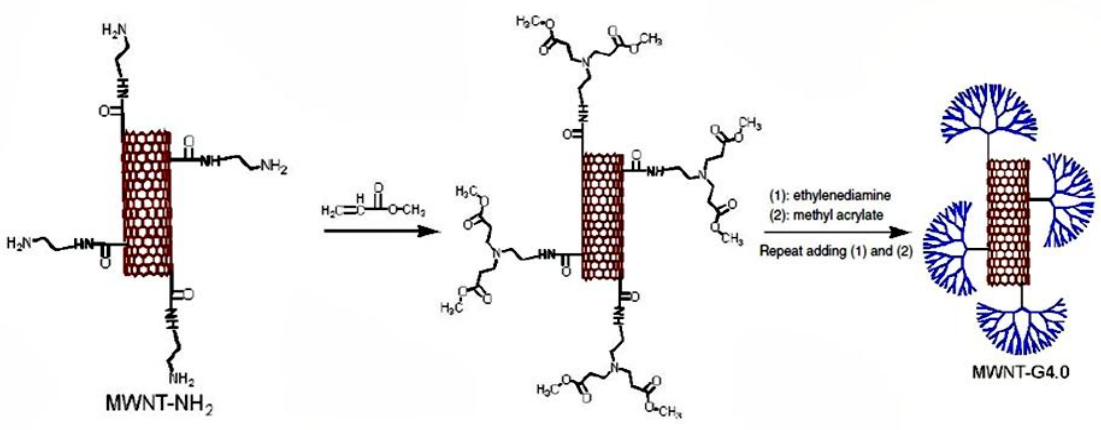

Figure 2.1 Experimental route of synthesis and modification of PAMAM-MWCNT.

Also, G3, G4 and G5 PAMAM dendrimers were synthesized and modified using the same procedure as described above.

2.2.4 Reactions of The Addition of Boronic Acid upon PAMAM-MWCNTs

0.165 g 4-carboxyphenylboronic acid, 0.4 g n-hydroxysuccinimide (NHS), 0.5 g EDC and 50 ml methanol were mixed in 100ml volumetric flask. Solid components were solved by means of magnetic stirring at room temperature (RT). Then approximately 100 mg modified MWCNT was added. It was let overnight in oil bath fixed at 40°C.

2.3. SOLUBILITY STUDIES of MODIFIED MWCNTs

The dispersibility of MWCNTs were studied in different solvents including DMEM (Gibco), DMEM+%10 FBS mixture (Gibco, Sigma), Ethanol (%96, Sigma), dH_20, ddH_20 and 1X PBS (Dulbecco's Phosphate Buffered Saline, Lonza). 1 mg of different MWCNTs that are with the both different modifications as PAMAM and Boronic acid and different generations from G1 to G5 was added to 1 mL of all solutions chosen for dispersibility studies. All solutions were dispersed in an ultrasonic bath (Bandalin Sonorex, Germany) for 30 min at RT and then ultrasonic probe (Bandalin Sonoplus, Germany) was applied for 5 min at RT. The aggregation in solution of the modified MWCNTs was observed under the invert microscope (Carl Zeiss AG, Oberkochen, Germany). Afterwards the best solution that provided better dispersion was determined comparing with others and was stored at 4 ^{0}C until needed. Prior to use, nanotube solutions were briefly mixed using vortex once again.

2.4 CULTURE of HEK CELL LINE

HEK 293 cell line was gifted by Yeditepe University, Department of Genetics and Bioengineering, and stored in liquid nitrogen tank (-196^{0}C) for future use. Previously 10 ml of warmed up DMEM (Dulbecco's Modified Eagle Medium, Gibco) at 37 ^{0}C was transferred into the 15 ml falcon tube (Greiner). Next, cryovial tube was taken from the liquid nitrogen tank and it was transferred into the 37 ^{0}C water bath and shaken slowly until melting. Then, the contents in the cryovial were transferred to the 15 ml falcon tube including DMEM as quickly as possible and centrifuged at 2000 rpm for 5 min. After centrifugation, the supernatant was discarded to get rid of DMSO (Dimethyl

Sulfoxide, Applichem) and other unwanted cellular products from the medium. Pellet is the remaining part that was resuspended by finger mix. After that cells were seeded into a 25 cm^2 tissue culture flask (Greiner) in 10 ml DMEM containing 10% (v/v) FBS (Fetal Bovine Serum; Gibco), 1% penicilin streptomycin (Biochrom,Germany). Finally, cells were incubated in a humidified atmosphere of 95% air and 5% CO_2 at 37 ^{0}C and the flask cap was kept flexible in order to allow circulation of CO_2 into flask. After one day, medium was refreshed in order to eliminate the dead cells.

After reaching % 70-80 cell confluency, which means spreading of cells by attaching the surface of the flask, subculture process should be done. First, DMEM (Dulbecco's Modified Eagle Medium, Gibco), PBS (Dulbecco's Phosphate Buffered Saline, Lonza), FBS (Fetal Bovine Serum; Gibco) and Trypsin-EDTA solution (Gibco) were warmed up to 37 ^{0}C at water bath. Medium was removed by a sterile pipette and cells were washed with 5 ml of PBS in the flask. Then, PBS was removed, cells were trypsinized with pre-warmed 2 ml of 0.25 % Trypsin/EDTA solution and cells were incubated in incubator containing 5% CO_2 at 37 ^{0}C for 3-4 min. Next, cells were observed under the inverted microscope. When the cells were detached on the flask surface, 1 ml of FBS was added in the flask to neutralize the activity of the trypsin. The detached cells were transfered into 15 ml centrifuge tube and then were washed twice with DMEM by centrifugation at 2000 rpm for 5 min. After centrifugation, supernatant was discarded until 2 ml of the cell suspension remained at the bottom of the tube. Pellet was dissolved in 2 ml of the cell suspension by finger mix. 20 µl of the cell suspension and 20 µl of 0.4 % Trypan blue solution (Sigma) were mixed and the cells were counted with hemocytometer. After counting cells, cells were seeded at a density of 20.000 cells/cm^2 into a cell culture flask including DMEM (Dulbecco's Modified Eagle Medium, Gibco) containing 10% (v/v) FBS, 1% penicilin streptomycin, in a humidified atmosphere of 95% air and 5% CO_2 at 37 ^{0}C. Cells were subcultured twice a week for continuous culture.

2.5 REAL TIME CELL ANALYSIS (RTCA)

To determine the optimum concentrations of different generations of modified CTNs on HEK 293 cell viability, the Real-Time Cell Analyzer System (RTCA; xCELLigence, Roche) was used that is based on measurement of the attachment, spreading and proliferation of the cells at the experimental condition by using the

system. HEK293 cells were seeded at a density of $1x10^4$ cells/well in duplicate 96 E-plate which is integrated with microelectrodes at the bottom and pre-incubated for 24 h in 200 µl of DMEM including 10% FBS, 1% penicillin streptomycin, in a humidified atmosphere (95% air and 5% CO_2 at 37 ^{0}C). After pre-incubation, the culture medium was removed from all wells. 100 µl of new culture medium containing 10% FBS was added to each wells. 1 mg/mL stock solutions of two different types of CTNs which are PAMAM-MWCNTs and BA-MWCNTs with increasing generations from G1 to G5 were prepared freshly in DMEM. Cells were treated with two different types of CTNs with different generations at increasing concentrations, from 5µg/ml to 75 µg/mL by serial dilution. Also, two wells in each E-plate that consist of the same amount of HEK293 cells in culture medium were used as control. Then, cells were observed at 24, 48 and 72 h periods. The effect of CTNs on cell viability was detected in real time by using impedance measurements that is based on the interaction between living cell and microelectrodes which cover the bottom of the plate.

2.6 CELL PROLIFERATION ASSAY (WST-1)

In duplicate 96-well plates, $10x10^4$ HEK 293 cells/ well were seeded and grown in 100 µl of DMEM medium that consists of 1% penicillin streptomycin and 10 % FBS, for 24 h in a humidified atmosphere of 95% air and 5% CO_2 at 37 ^{0}C. PAMAM-MWCNTs and BA-MWCNTs were prepared at the concentration of 1 mg/mL in 1X PBS (Dulbecco's Phosphate Buffered Saline, Lonza) solution. When the cells had reached 70% confluence, 100 µl of both PAMAM-MWCNTs and BA-MWCNTs were added to the following final concentrations: 1, 2.5, 5, 15, 25 µg/mL. After 24 h incubation with CTNs, 10 µl of WST-1 reagent (Roche, Germany) was added to the wells and incubated for 2 h at 37 ^{0}C. After that, absorbance was measured at 450 nm by using an ELISA reader (BioRad, USA).

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2.7 TRANSFORMATION of pEGFPN1 INTO COMPETENT *E. coli (DH5-Alpha)*

2.7.1 pEGFPN1

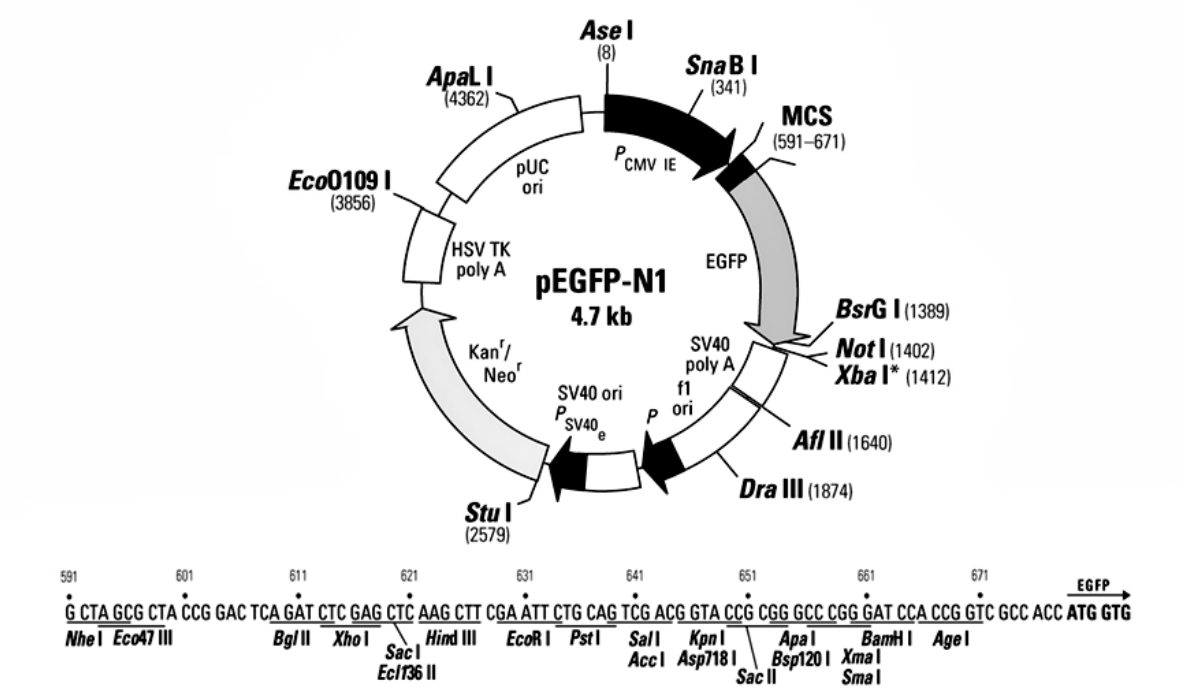

Figure 2.2 Restriction map and multiple cloning sites (MCS) of pEGFPN1 vector.

2.7.2 Preparation of $CaCl_2$ Solution

To prepare 50 mM $CaCl_2$ solution in 100 mL, 0,55 g of $CaCl_2$ was weighed and then dissolved in 100 mL dH_2O. After obtaining completely homogenized solution, it was filtered through 0,2 μm filter and stored at 4°C for future use.

2.7.3 Preparation of LB Broth

To prepare 500 mL of LB broth, 10 gr LB broth powder (BD Biosciences) including NaCl, tryptone, yeast extract were dissolved in 500 mL distilled water in cleaned and sterilized round bottle with cap by shaking. The top of the cap of round bottle was covered with aluminum foil and labeled with autoclave tape. It was sterilized by autoclaving at 121°C for 15 min. After removing the solution from the autoclave, the

broth solution was allowed to cool at RT. When it was cooled, the round bottle was labeled and stored at 4°C by sealing with parafilm.

2.7.4. Preparation of LB Agar

In order to make 500 mL of LB agar, 17.5 gr LB agar powder (BD Biosciences) including NaCl, tryptone, yeast extract and agar were dissolved in 500 mL distilled water in sterile erlenmayer flask by boiling to completely dissolve the powder. The top of the erlenmayer flask was covered with aluminum foil and labeled with autoclave tape. It was sterilized by autoclaving at 121°C for 15 min. After removing the solution from the autoclave, the agar solution was allowed to cool to 55°C. The pouring plates process was carried out in sterile laminar flow cabinet by working near a bunsen burner. 2,5 mL of kanamysin (Biochrom,Germany) was added into 500 mL of LB agar solution and it was swirled to mix. ~20 mL of LB agar was poured per 10 cm polystyrene petri dish (BD Biosciences). The lids were partially removed on the plates and it was allowed to cool until solidified. After solidification, the plates were inverted and the bottoms of plates were labelled with antibiotic and date. Finally, plates were stored at 4°C by sealing with parafilm.

2.7.5 Preparation of Calcium Shocked Competent Bacterial Cells

The *E. coli* (DH5α) stock culture was plated out on LB agar applying streak-plate technique. After incubation overnight at 37°C, a single bacterial colony was picked and subcultured into 5 ml LB broth (w/o antibiotics) using a sterile loop and incubated at 37°C, 200 rpm overnight. 1 mL of this overnight liquid culture was then added into 200 mL LB broth medium (w/o antibiotics) in a 500 ml glass bottle with lid and incubated in a shaker at 37°C until an absorbance of 0.3 - 0.6 OD at 600 nm was reached. After detection of OD at 600 nm, the culture was cooled down on ice for 10 min. The culture was divided equally into two different 50 mL sterile tubes and centrifuged at 3600 rpm for 15 min at 4°C. After discarding the supernatant, the pellet was resuspended in a 10 mL of ice cold 1.0 M $CaCl_2$ slowly by pipetting. The pellets were combined at this step and then centrifuged at 1200 rpm for 10 min. After carefully discarding the supernatant, the pellet was resuspended in a 6,5 mL of ice cold 1.0 M $CaCl_2$ and incubated at 0°C for 1,5 h. After the incubation, 1,2 mL of 80 % autoclaved

glycerol was added and incubated on ice for 10 min. Finally, the cells were aliquoted at 200 µl volume into 1,5 mL microtubes and stored -80°C.

2.7.6 Transformation of pEGFPN1 into Competent DH5α E. coli using Heat Shock

Before starting the transformation experiment, all reagents were cooled down. Competent cell vial was thawed on ice. 2 µl of 0,5 M β-merkapto ethanol was added to tube and mixed by tapping gently. 50 ng of pEGFPN1 (Amaxa, Lonza) was gently added into 200 µl of competent cell and incubated on ice for 30 min. After that, the tube was incubated for 30 sec in 42 ^{0}C of heat block and then it was immediately placed on ice for 2 min and left at RT. 450 µl of pre-warmed LB medium was added to vial and incubated at 37 ^{0}C for 1 h at 200 rpm and then, tube was left on ice. 100 µl of cell suspension was plated out on LB agar plates containing 50 µg/mL kanamycin. The plates were inverted and incubated at 37°C overnight. After overnight incubation, plates were stored at 4°C for future use.

2.7.7 Preparation of Miniculture

A single bacterial colony was picked from previously transformed *E. coli* (DH5α) plate using a sterile loop and added into 5 mL sterile LB broth including 3 µl kanamysin and incubated in a shaker at 37°C, 200 rpm for 8 h.

2.7.8 Preparation of Midiculture

Following mini culture steps, after incubation for 8 h, 200 µl of bacterial broth culture was transferred into 100 mL of sterile LB broth containing 60 µl of kanamysin and incubated in a shaker at 37°C, 200 rpm overnight.

2.7.9 Purification of pEGFPN1

The transformed pEGFPN1 was purified using the QIAGEN Plasmid Midi and Maxi Kits (Qiagen). Before starting the purification experiment, RNase A solution was added to Buffer P1, Buffer P2 was checked for SDS precipitation due to low storage temperatures and Buffer P3 was pre-chilled at 4°C. The bacterial culture was divided equally into two different 50 mL sterile tubes and centrifuged at 6000 rcf for 15 min at 4°C to harvest the bacterial cells. After centrifugation, the pellet was obtained and the

supernatant was discarded. The bacterial pellet was resuspended in 4 mL Buffer P1. Next, 4 mL of Buffer P2 was added into tube and mixed by vigorously inverting the sealed tube 4–6 times and incubated at RT for 5 min. 4 mL of chilled Buffer P3 was then added immediately and mixed by vigorously inverting 4–6 times, and then incubated on ice for 15 min. The tubes were centrifuged at 20,000 rcf for 30 min at 4°C. While centrifugation step was being performed, QIAGEN-tip 100 was equilibrated by applying 4 mL Buffer QBT and it was allowed the column to empty by gravity flow. After that, the supernatant was applied to the QIAGEN-tip and it was entered the resin by gravity flow. The QIAGEN-tip was washed with 20 mL (2 x 10) ml Buffer QC and then 5 ml of Buffer QF was added to elute DNA. 3.5 mL of isopropanol at room-temperature was added to the eluted DNA and centrifuged immediately at 15,000 rcf for 30 min at 4°C for precipitation of DNA. After centrifugation, the supernatant was carefully decanted. DNA pellet was washed with 350 µl of room-temperature 70% ethanol and centrifuged at 15,000 rcf for 10 min. After centrifugation, the supernatant was carefully decanted without disturbing the pellet. The pellet was air dried for 2 h and then the DNA was redissolved in 40 µl of 1X TE buffer (pH 8.0).

2.8 FORMATION of pDNA-MWCNT COMPLEXES

The plasmid (pEGFPN1, Amaxa) was expressed, isolated and purified as described before (2.7.6, 2.7.7, 2.7.8, 2.7.9). Two different types of CTNs which are PAMAM-MWCNTs and BA-MWCNTs with increasing generations from G1 to G5 were used for complexation with pEGFPN1. 1µg of pEGFPN1 was mixed with different mass ratios of CTNs at increasing mass ratios from 1:2 to 1:200 and complexes were incubated 37 ^{0}C for 1 h using Dry Block Heating Thermostat (Biosan, Latvia). The complexation ratio was analyzed by agarose gel electrophoresis.

2.9 AGAROSE GEL ELECTROPHORESIS

2.9.1 Preparation of 0,5X TBE

0,5X TBE was prepared from 10X TBE stock solution (Tris-borate-EDTA, Thermo) by diluting 50 ml 10X TBE with 950 ml distilled water.

2.9.2 Preparation of Agarose Gel

1.2 g agarose powder (Sigma) dissolved in 120 ml of 0,5X TBE buffer by boiling in a microwave oven (Beko MD1510) in order to make 1% agarose gel. Before adding 6 µl of Pronasafe Nucleic acid staining solution (Conda, Spain) into agarose solution, it was cooled down and then poured directly into the horizontal casting tray.

2.9.3 Running Agarose Gel

10 µl of each samples of pEGFPN1/PAMAM-MWCNT complexes were mixed with 3 µl of 6X Loading Dye (Thermo Scientific, USA) and 10 µl of each samples of pEGFPN1/BA-MWCNT complexes were mixed with 3 µl of 6X Loading Dye (Thermo Scientific, USA). 1 µl only pEGFPN1 were used as marker for control that were mixed with 4 µl of 6X Loading Dye (Thermo Scientific, USA). The agarose gel was covered by filling with 0,5X TBE Buffer. The gel was loaded with different complexes and run at 65V for 1 h. After resulting time, the resuting gel was imaged under UV.

2.10 *IN VITRO* TRANSFECTION

2.10.1 Preparation of Poly-L-lysine Coated 96 well plates

Poly-L-Lysine is one of the extracellular matrix components that also used to coat slides to promote attachment of cells. Poly-L-Lysine enhances electrostatic interaction between negatively-charged ions of the cell membrane and positively-charged surface ions of attachment factors on the culture surface. When adsorbed to the culture surface, it increases the number of positively-charged sites available for cell binding. 2 mg/mL stock solution of poly-l-lysine was diluted to 50 µg/mL using 1X PBS. 100 µl of diluted poly-l-lysine was added into each well. The plates were left for 2 h at RTin clean bench. After incubation, the solution was aspirated and each well was washed with 1 mL 1X PBS for 5 min following 3 cycles and then plates were air dried for overnight in clean bench. After overnight incubation, the plates were covered with aluminum foil and stored at 4 ^{0}C until use.

2.10.2 In vitro Transfection Experiment

Before starting transfection experiment, HEK 293 cells were cultured in poly-L-lysine coated duplicate 96-well plates ($10x10^4$ cells/well) with 200 µl of DMEM medium that consists of 1% penicillin streptomycin and 10 % FBS. PAMAM-MWCNTs and BA-MWCNTs were freshly prepared at the concentration of 1 mg/mL in 1X PBS solution and diluted by serial dilution. When the cells had reached 70% confluence, the medium was replaced with 100 µl fresh DMEM containing 10% FBS. For transfection experiment, firstly, 1 µg of pEGFPN1 was diluted in 12,5 µl 1X Opti-Mem (Gibco, USA) applying spin down for 15-25 sec and pre-incubated at 15 min at RT. Each types of CTNs that are different mass ratios were diluted in 12,5 µl 1X Opti-Mem by vortexing for 3-5 times and then pre-incubated at 15 min at RT. After 15 min incubation at RT, pEGFPN1 was added into CTNs. A series of PAMAM-MWCNTs/ pEGFPN1 and BA-MWCNTs/pEGFPN1 complexes with different mass ratios (pEGFPN1: CNTs, 1:2, 1:3, 1:4, 1:5, 1:10 (µg)) were prepared and diluted in 1X Opti-Mem using the same procedure described as above. Then, each sample was incubated for 1 h at 37 ^{0}C to form complex. After 1 h incubation at 37 ^{0}C, complexes were added to wells and cells treated with complexes were incubated for 12 h in a humidified atmosphere of 95% air and 5% CO_2 at 37 ^{0}C. After 12 h, the cells were washed with 150 µl PBS (1X) once, the culture medium was renewed and allowed to grow for another 24 h with fresh DMEM containing 1% penicillin streptomycin and 10 % FBS at 37 ^{0}C in a humidified atmosphere containing 95% air and 5% CO_2. GFP-transfected cells that emitted fluorescence were observed and counted under a fluorescent microscope (Carl Zeiss AG, Oberkochen, Germany). The transfection efficiency was calculated as a percentage of fluorescent cells out of the total number of cells. For the positive control, Fugene (3µl) (Roche, USA) and pEGFPN1 (1µl, 1µg/µl) were diluted in 1X Opti-Mem and incubated with the cells in DMEM containing 1% penicillin streptomycin and 10 % FBS for 24 h. Additionally, for negative control, naked pEGFPN1 (1µg in 200 µl fresh DMEM) was also diluted in 1X Opti-Mem and incubated with HEK 293 cells.

CHAPTER 3

RESULTS

3.1 DISPERSION OF PAMAM-MWCNTs and BA-MWCNTs

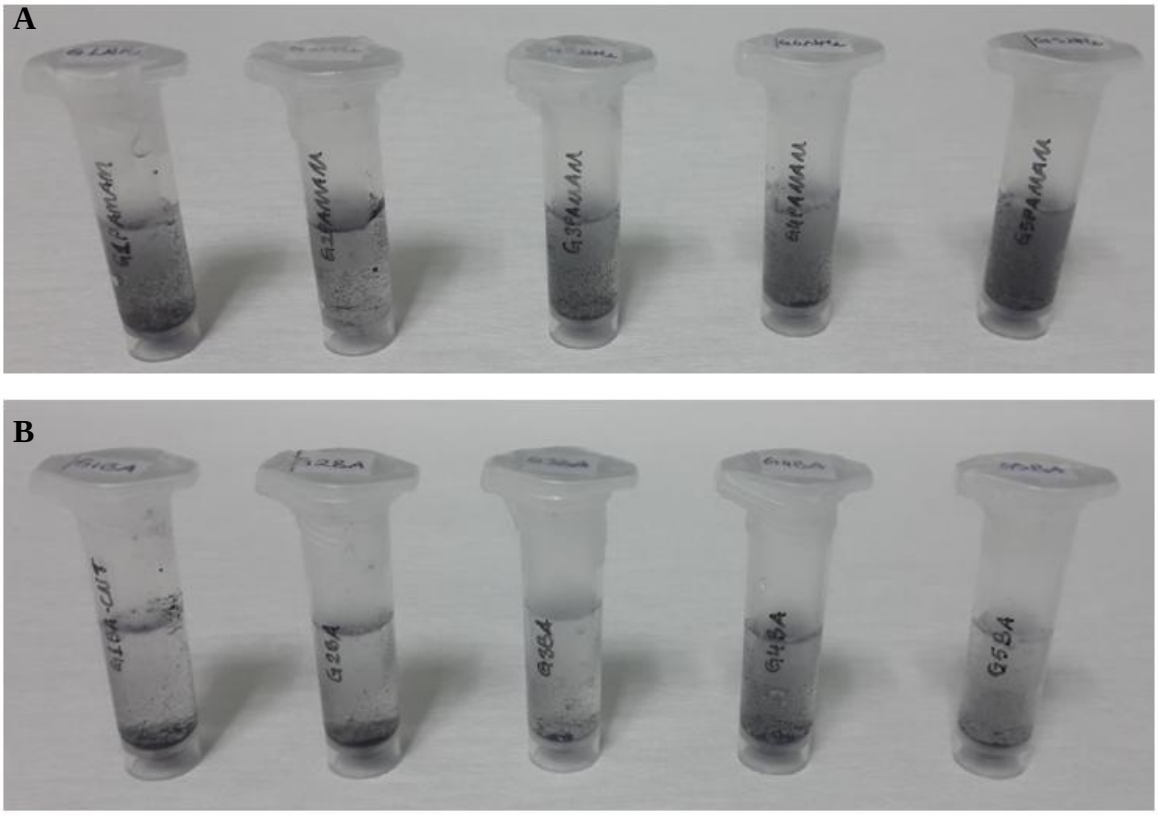

Figure 3.1 Dispersion of 1 mg/mL stock solutions of PAMAM-MWCNTs and BA-MWCNTs in 1X PBS before the sonication treatment. A) PAMAM-MWCNTs B) BA-MWCNTs.

The preperation of 1 mg of both PAMAM-MWCNTs and BA-MWCNTs including five generation in 1 mL of 1X PBS solution was shown in figure 3.1. As seen in figure 3.1, after adding of CNTs into PBS solution, both PAMAM-MWCNTs and BA-MWCNTs that are five different generations aggregated in PBS.

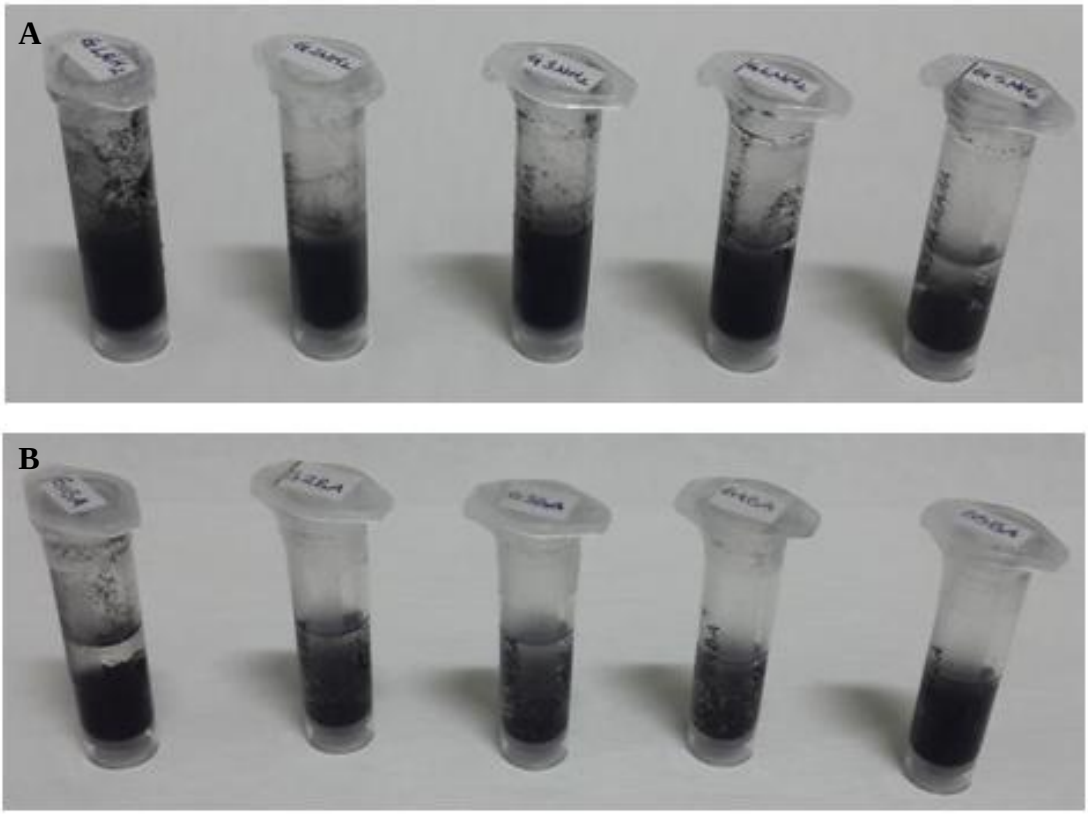

Figure 3.2 Dispersion of 1 mg/mL stock solutions of PAMAM-MWCNTs and BA-MWCNTs in 1X PBS after the sonication treatment. A) PAMAM-MWCNTs B) BA-MWCNTs.

In figure 3.2, it was observed that after sonication treatment, the dispersibilities of CNTs were better than unsonicated condition.

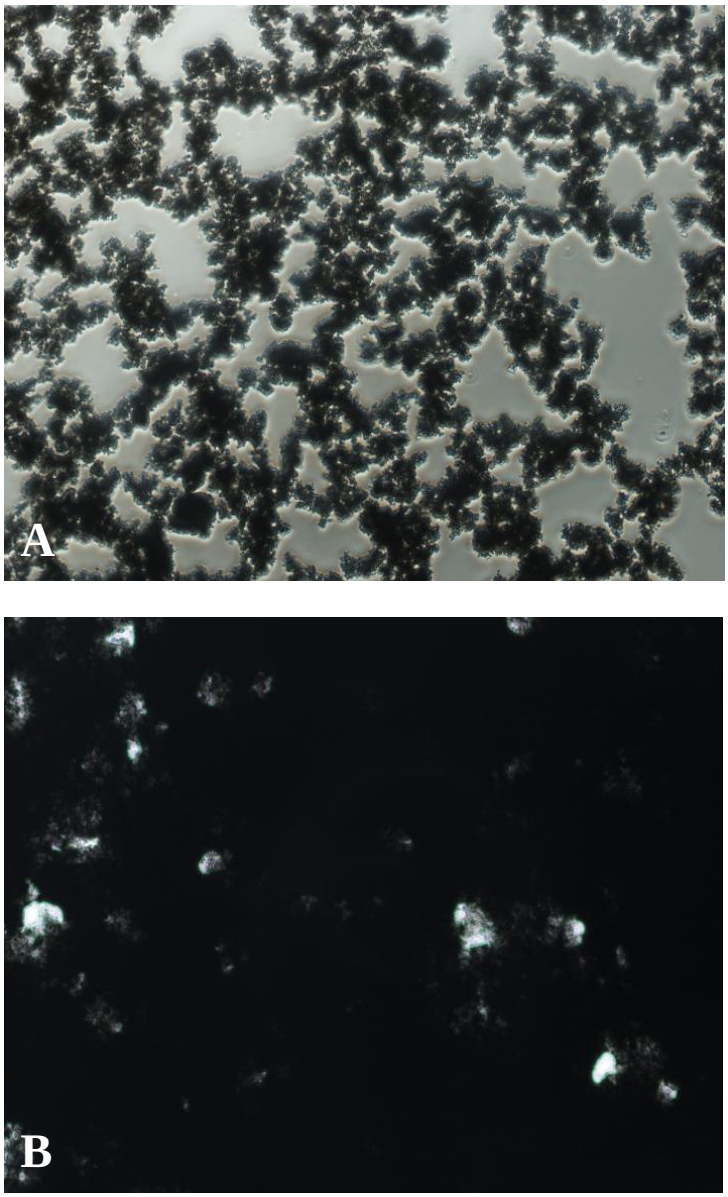

Figure 3.3 Representation of the dispersion of 1 mg/mL of G5BA-MWCNTs in 1X PBS under invert microscope (10X magnification). A) before the sonication treatment, B) after the sonication applying at utrasonic bath for 30 min and sonic probe for 5 min.

The dispersion of 1 mg/mL of G5BA-MWCNTs in 1X PBS under invert microscope at 10X magnification was shown in figure 3.3. In figure 3.3, while clumps of CNTs in 1X PBS were observed before the sonication treatment, clouds of CNTs in 1X PBS were observed which means the obtained homogenous dispersion of CNTs in 1X PBS after sonication treatment.

3.2 REAL TIME CELL ANALYSIS (RTCA)

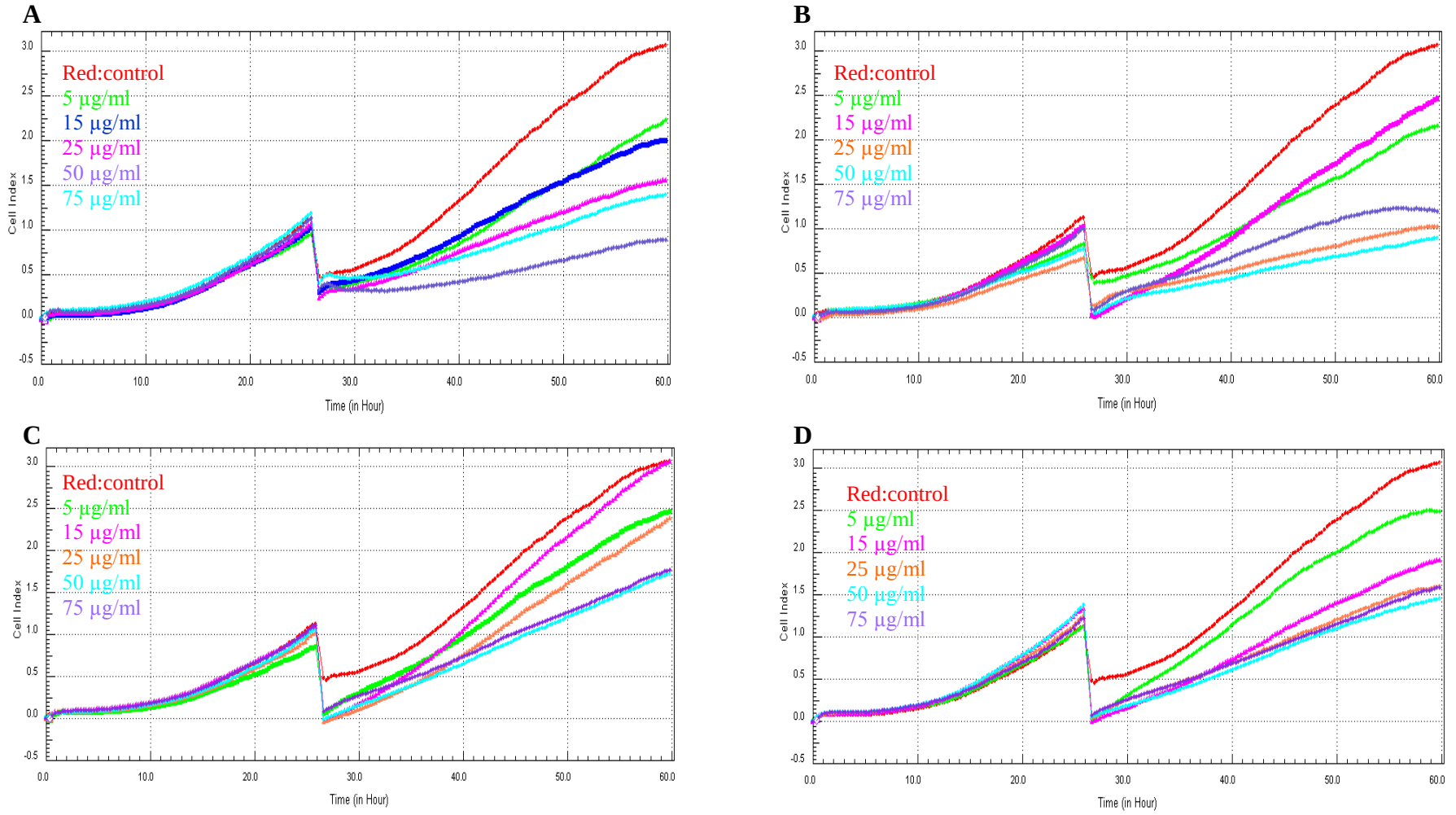

Figure 3.4 Growth curve of HEK 293 cells treated with different types of MWCNT for 60 hours *in vitro* culture. A) G1PAMAM-MWCNT, B) G2PAMAM-MWCNT, C) G3PAMAM-MWCNT, D) G4PAMAM-MWCNT.

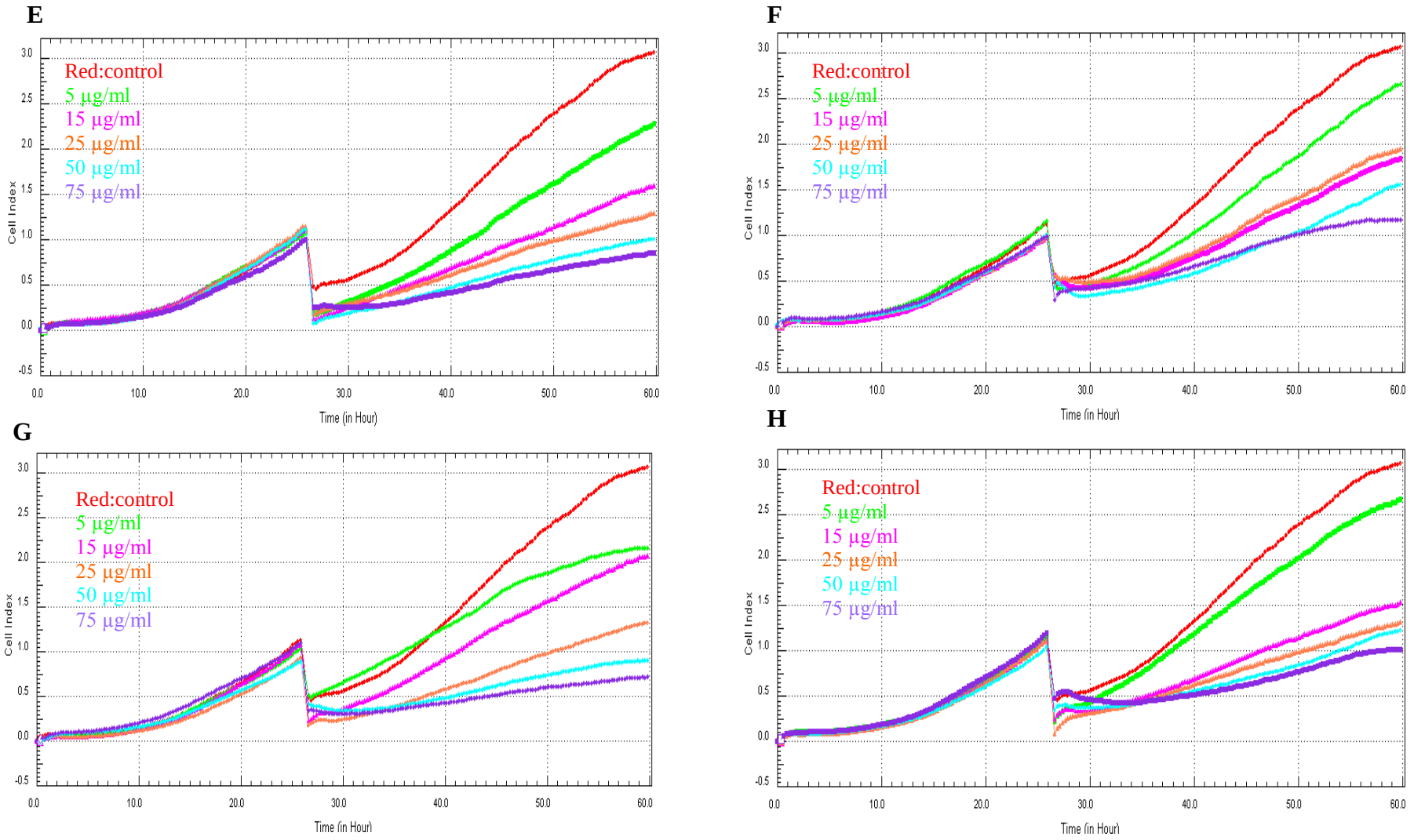

Figure 3.4 Growth curve of HEK 293 cells treated with different types of MWCNT for 60 hours *in vitro* culture. E) G5PAMAM-MWCNT, F) G1BA-MWCNT, G) G2BA-MWCNT, H) G3BA-MWCNT.

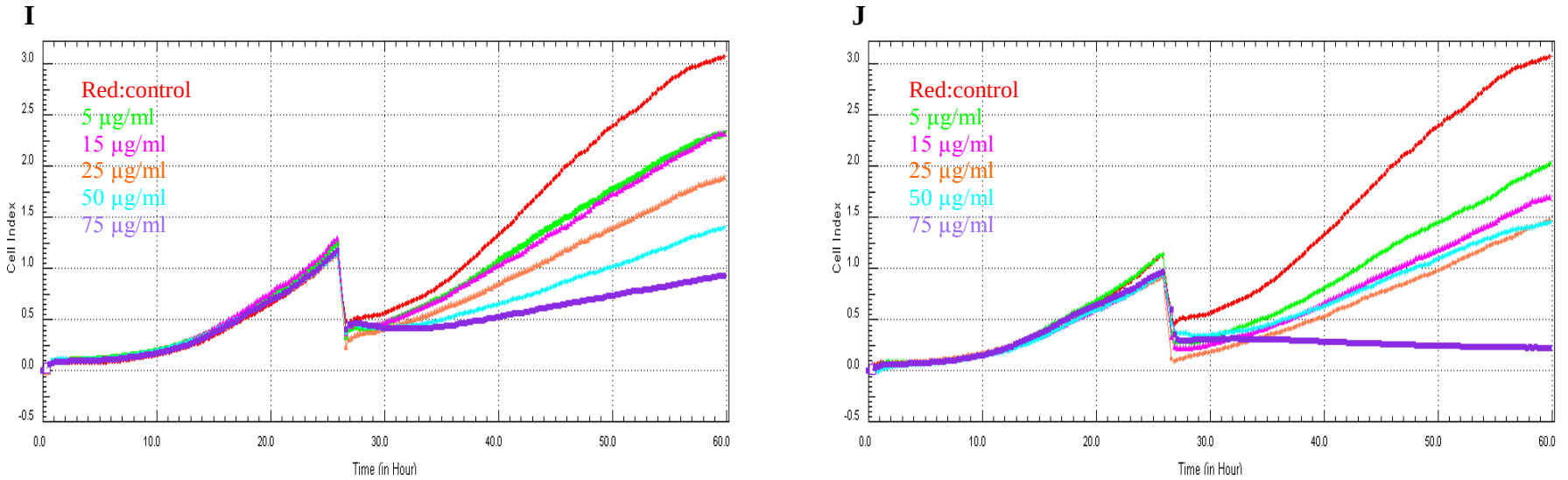

Figure 3.4 Growth curve of HEK 293 cells treated with different types of MWCNT for 60 hours *in vitro* culture. I) G4BA-MWCNT and J) G5BA-MWCNT. Red line shows growht curve of HEK 293 cells untreated with CTNs in all graphs. The optimum CTN concentration to treate in the further experiments was determined as ranging between 5-25 µg/mL at the end of 60 hours incubation period.

As seen in figure 3.4, two different types of CTNs with different five generations had similar effects on HEK 293 cells. According to graphs in figure 3.4, a significant reduction in cell index was observed at high concentrations of CTNs in RTCA system and the optimum CTN concentration that enables to live almost entire cells in cell culture was determined as ranging between 5-25 µg/mL at the end of 60 hours incubation period.

3.3 CELL PROLIFERATION ASSAY (WST-1)

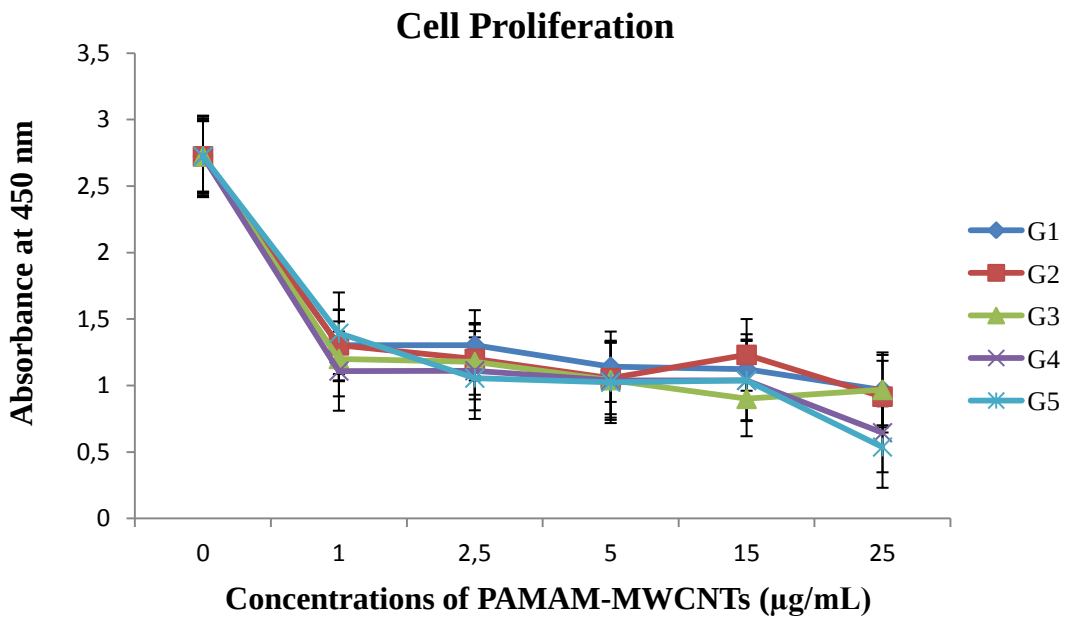

Figure 3.5 Dose-dependent proliferation of HEK 293 cells treated with PAMAM-MWCNTs.

Dose dependent proliferation of HEK 293 cells treated with PAMAM-MWCNTs shown in the figure 3.5. PAMAM-MWCNTs have inhibitory effect over HEK cells proliferation. Based on our findings, after incubation of HEK cells with PAMAM-MWCNTs at increasing concentrations for 24 h, cell attachment and growing were reduced to 48% of the total cells that the best ratio given cells treated with PAMAM-MWCNTs to live and after increased one more generation of G1PAMAM-MWCNTs, cell number decreased to 47% even the concentration is the same that 1 µg/mL of G2PAMAM-MWCNTs. We observed that cell proliferation reduced at the increasing concentrations (1 µg/mL, 2,5 µg/mL, 5 µg/mL, 15 µg/mL, 25 µg/mL) of G1PAMAM-MWCNTs following the ratio of 48% > 47% > 42% > 41% > 35%. Results showed that when concentration of G2PAMAM-MWCNTs was 1 µg/mL, 47% cells were alive that exhibited similar life ratio with G1PAMAM-MWCNTs at the 2,5 µg/mL concentration. Similarly we observed that while 38% cells were alive when concentration of G3PAMAM-MWCNTs was 5 µg/mL that shown similar life ratio with G4PAMAM-MWCNTs at the 15 µg/mL concentration. Whereas 40% of total cells were detected as

viable in cell culture at the 1 µg/mL concentration of G4PAMAM-MWCNTs, almost 24% of the total cells were attached to the surface at the 25 µg/mL concentration of G4PAMAM-MWCNTs after the incubation period. A highest concentration (25 µg/mL) with the highest generation (G5) of PAMAM-MWCNTs lead to about 80% decrease in cell viability and about 20% cells were alive after the incubation with 25 µg/mL concentration of G5PAMAM-MWCNTs. In general, cell proliferation decreased as long as concentrations of PAMAM-MWCNTs were increased.

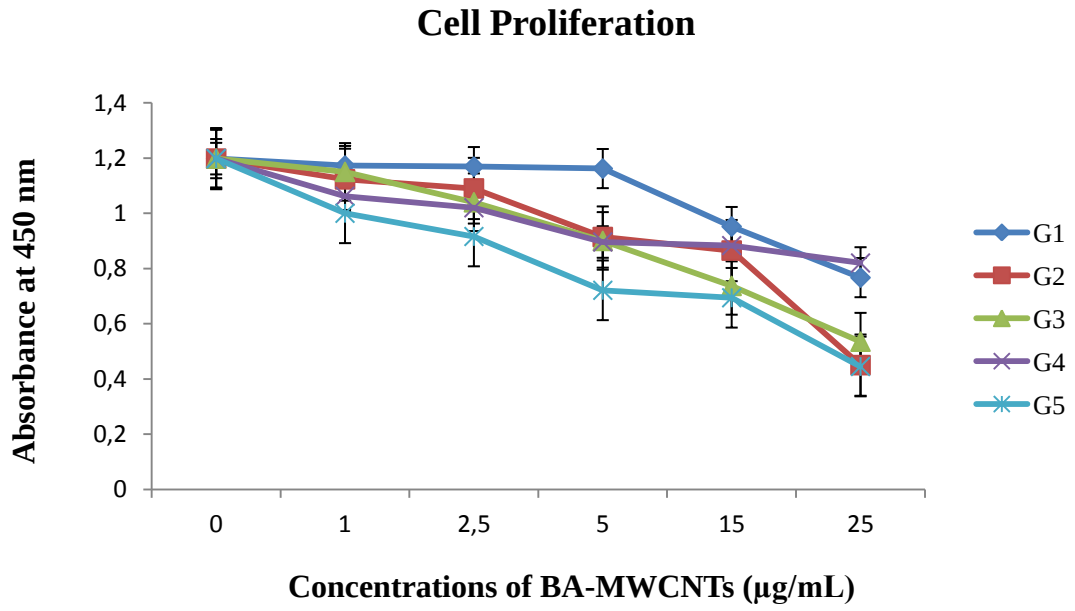

Figure 3.6 Dose-dependent proliferation of HEK 293 cells treated with BA-MWCNTs.

Dose dependent proliferation of HEK 293 cells treated with BA-MWCNTs shown in the figure 3.6. As shown in figure 3.6, we observed that BA-MWCNTs have also inhibitory effect over HEK cells proliferation after treated HEK cells with BA-MWCNTs with increasing generations for 24 h incubation and the proliferative activity of HEK cells decreased with the increase of BA-MWCNTs concentrations. Depending on our results, it can be said that cell attachment and growing were almost similar to the control cells at lower concentrations than 5 µg/mL of G1BA-MWCNTs, however cell attachment and growing decreased at the increasing generations of BA-MWCNTs, when the concentration was lower than 5 µg/mL. A low concentration (1 µg/mL) of

G1BA-MWCNTs lead to about 2% decrease in cell viability and about 63% cells were alive after the incubation with 25 µg/mL concentration of G1BA-MWCNTs. While 94% of total cells were detected as viable in cell culture at the 1 µg/mL concentration of G2BA-MWCNTs, almost 37% of the total cells were attached to the surface at the 25 µg/mL concentration of G2BA-MWCNTs. Likewise, whereas the concentration of G3BA-MWCNTs was 1 µg/mL, 96% cells were viable and when the concentration of G3BA-MWCNTs increased to 25 µg/mL, proliferation of HEK cells decreased to 44%. Similarly, 88% cells were alive at the lowest concentration (1 µg/mL) of G4BA-MWCNTs; therefore 63% cells were able to continue their proliferative activity at the 25 µg/mL concentration of G4BA-MWCNTs. Also, it was observed that while G5BA-MWCNTs at the low concentration (1 µg/mL) cause to 83% reduction of cell number, after incubated with G5BA-MWCNTs at increased concentration (25 µg/mL), 36% of total cells were attached to the surface which it was similar to cell decrase of G2BA-MWCNTs at the same concentration.

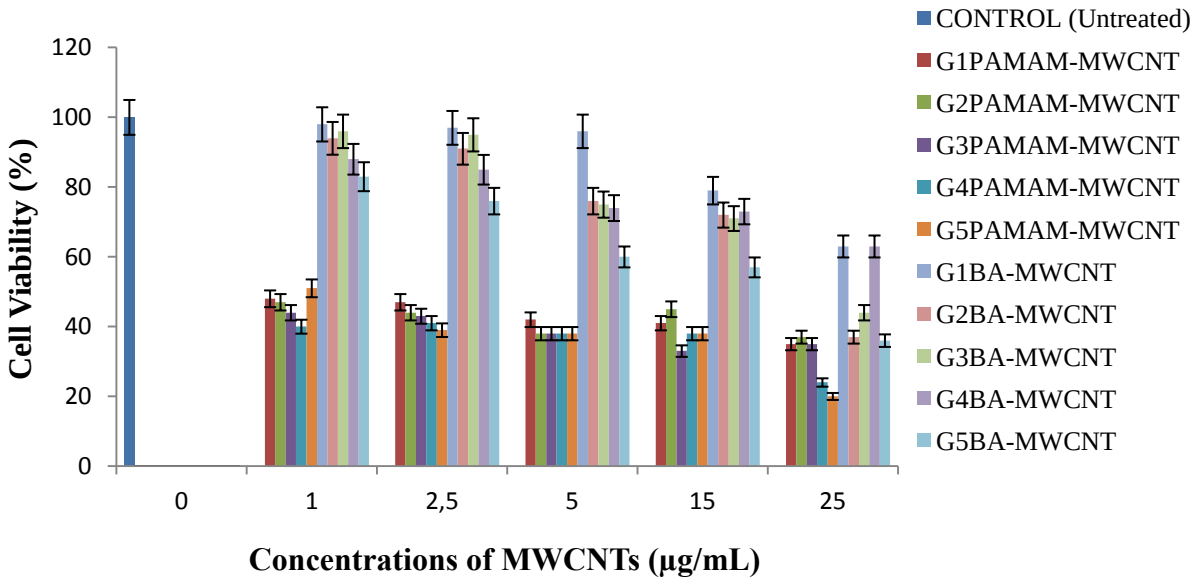

Figure 3.7 Viability of HEK293 cells after incubated with both PAMAM-MWCNTs and BA-MWCNTs at increasing concentrations for 24 h in cell culture medium.

Dose-dependent proliferation results of HEK cells treated with both PAMAM-MWCNTs and BA-MWCNTs were combined in Figure 3,7. The cellular viability is

calculated as a percentage from the viability of the control cells. The viability of the control cells is considered 100%.

3.4 AGAROSE GEL ELECTROPHORESIS

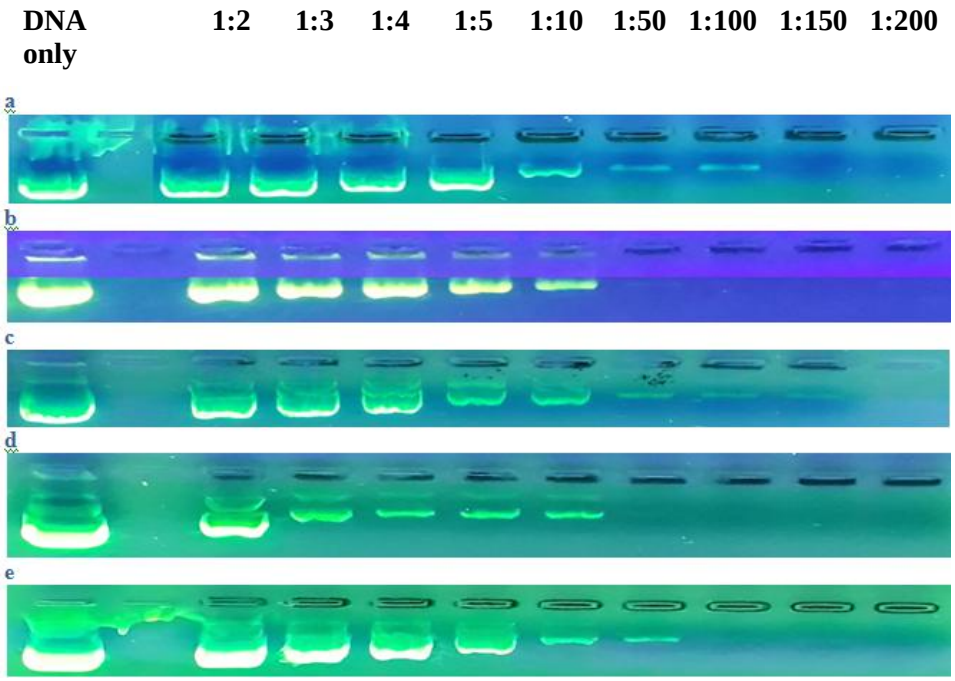

Figure 3.8 Gel electrophoresis of the PAMAM-MWCNTs/ pEGFPN1 complexes at different mass ratios. DNA only: 1 µg of pEGFPN1, a) G1PAMAM-MWCNT, b) G2PAMAM-MWCNT, c) G3PAMAM-MWCNT, d) G4PAMAM-MWCNT, e) G5PAMAM-MWCNT.

To determine pEGFPN1 and multiwalled CTNs complex formation, agarose gel electrophoresis was performed. In figure 3.8, agarose gel electrophoresis results of the PAMAM-MWCNTs/pEGFPN1 complexes at different mass ratios were shown that each generation of PAMAM-MWCNTs is able to condense DNA to varying degrees at different mass ratios. According to our gel electrophoresis results, pEGFPN1 completely binds to PAMAM-MWCNTs at mass ratios between 1:100 and 1:200 when PAMAM-MWCNTs are on generation 2, generation 4 and generation 5. As seen in the figures 3.8, the DNA binding capacities of each five generations of PAMAM-MWCNTs decreased while mass ratio is lower than 1:10, therefore when mass ratio increased from 1:5 to 1:10, the DNA binding capacities of each five generations of PAMAM-

MWCNTs increased and additionally generation 3 of PAMAM-MWCNTs and generation 4 of PAMAM-MWCNTs have similar band at mass ratio of 1:10 in compared with the others and control that included only 1 µg/µl concentration of pEGFPN1.

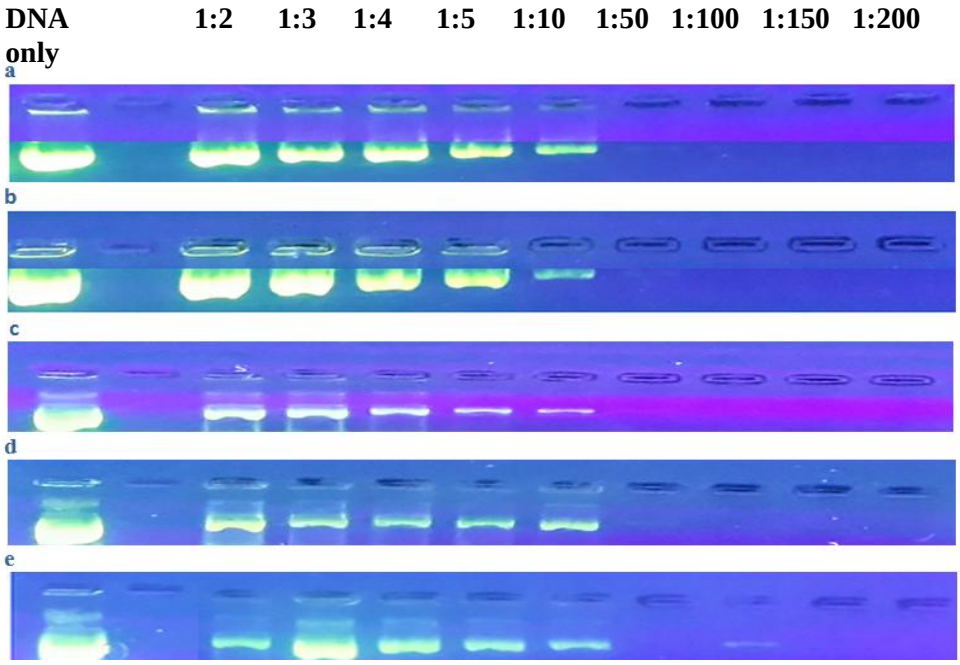

Figure 3.9 Gel electrophoresis of the BA-MWCNTs/pEGFPN1 complexes at different mass ratios. DNA only: 1 µg of pEGFPN1, a) G1BA-MWCNT, b) G2BA-MWCNT, c) G3BA-MWCNT, d) G4BA-MWCNT, e) G5BA-MWCNT.

In figure 3.9, agarose gel electrophoresis results of the BA-MWCNTs/ pEGFPN1 complexes at different mass ratios were shown that each generation of BA-MWCNTs is able to condense DNA to varying degrees at different mass ratios. According to our gel electrophoresis results, pEGFPN1 completely binds to different five generations of BA-MWCNTs at mass ratios between 1:50 and 1:200. Based on the gel electrophoresis result, it was observed that generation 1 of BA-MWCNTs and generation 2 of BA-MWCNTs have the lowest DNA binding capacities compared the others. When the levels of green fluorescent emission of pEGFPN1 that formed complexes with five different generations of BA-MWCNTs compared to control, which included only 1 µg/µl concentration of pEGFPN1, generation 3 of BA-MWCNTs and

generation 4 of BA-MWCNTs were similar and additionally were lower than the others. Likewise, it was observed that the levels of green fluorescent emission of pEGFPN1 decreased rapidly with the increase of BA-MWCNTs generations as seen in bands of generation 1, generation 2 and generation 5 until mass ratio increased to 1:10.

3.5 *IN VITRO* TRANSFECTION of pEGFPN1

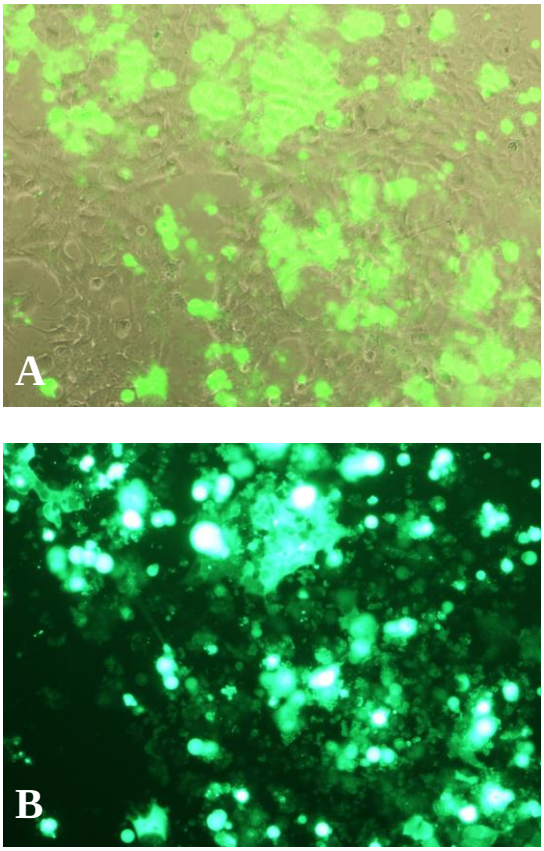

Figure 3.10 *in vitro* transfection of pEGFPN1 for control (10X magnification). A) The merged image and B) fluorescence of HEK293 cells by using Fugene at 1:3 ratio.

In Figure 3.10, the fluorescence and merged images of HEK cells transfected with pEGFPN1 using X-tremeGENE 9 DNA transfection reagent were shown.

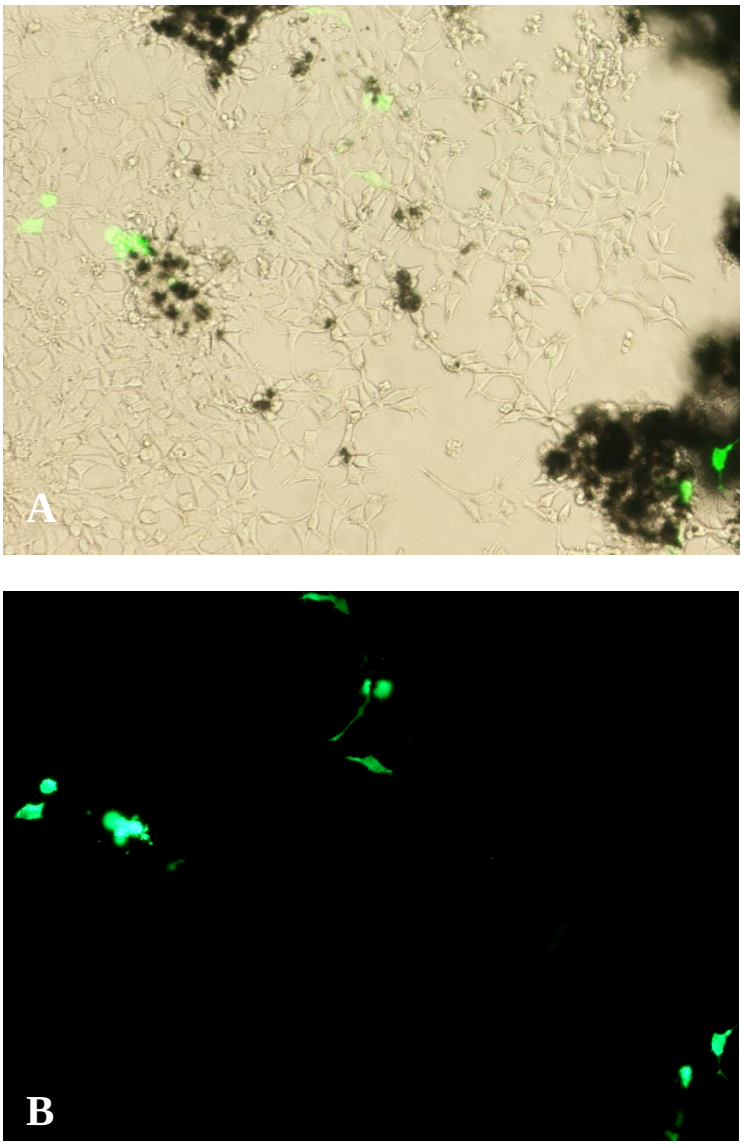

Figure 3.11 *In vitro* transfection of pEGFPN1 using G3BA-MWCNT (10X magnification). A) The merged image and B) fluorescence of HEK 293 cells successfully transfected with pEGFPN1 gene by using G3BA-MWCNT at 1:10 mass ratio.

In Figure 3.11, the fluorescence and merged images of HEK cells transfected with pEGFPN1 using G3BA-MWCNTs were shown. It was found that G3BA-MWCNT can succesfully deliver pEGFPN1 into HEK cells when the pEGFPN1/DNA complexation ratio was 1:10 and the pEGFPN1 has been successfully expressed.

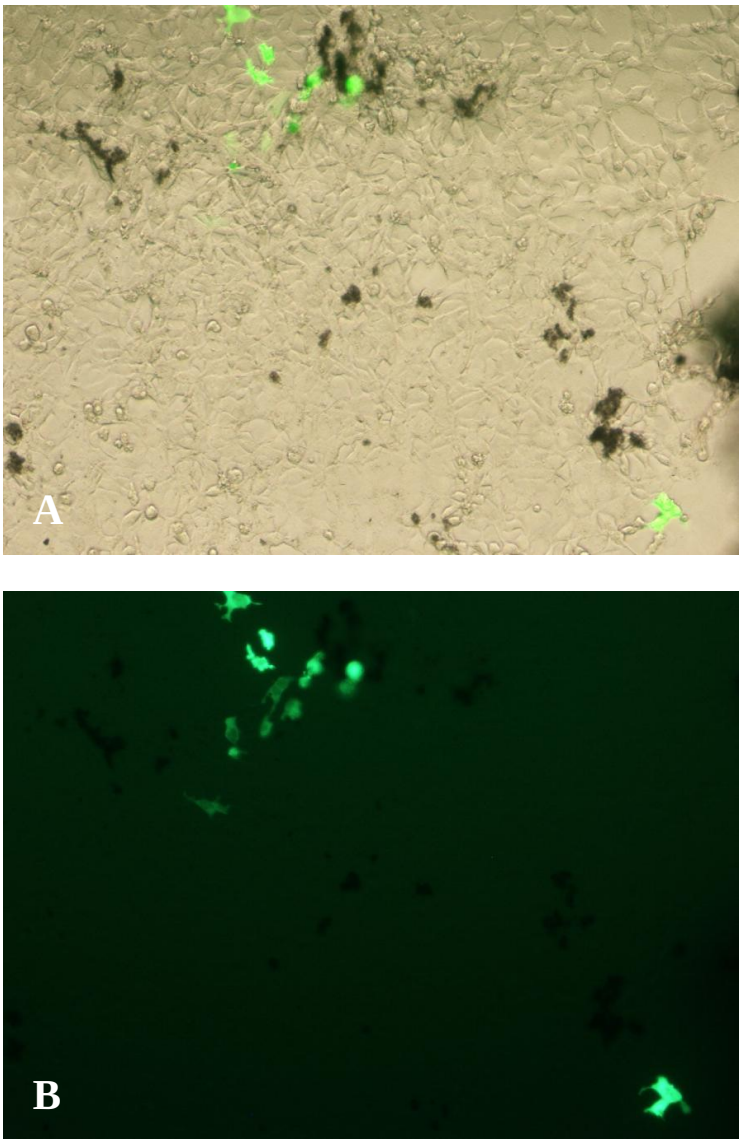

Figure 3.12 *in vitro* transfection of pEGFPN1 using G4BA-MWCNT (10X magnification). A) The merged image and B) fluorescence of HEK 293 cells successfully transfected with pEGFPN1 gene by using G4BA-MWCNT at 1:10 mass ratio.

In Figure 3.12, the fluorescence and merged images of HEK cells transfected with pEGFPN1 using G4BA-MWCNTs were shown. It was found that G4BA-MWCNT can succesfully deliver pEGFPN1 into HEK 293 cells when the pEGFPN1/DNA complexation ratio was 1:10 and the pEGFPN1 has been successfully expressed.

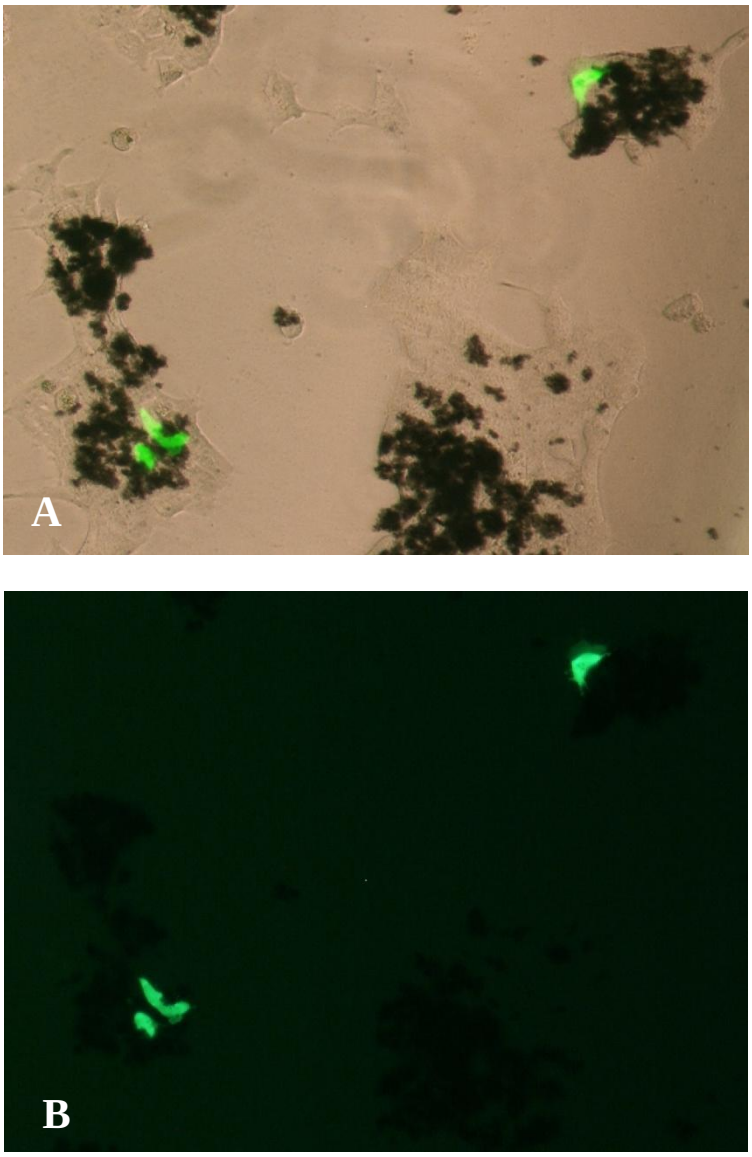

Figure 3.13 *in vitro* transfection of pEGFPN1 using G3PAMAM-MWCNT (10X magnification). A) The merged image and B) fluorescence of HEK 293 cells successfully transfected with pEGFPN1 gene by using G3PAMAM-MWCNT at 1:10 mass ratio.

In Figure 3.13, the fluorescence and merged images of HEK 293 cells transfected with pEGFPN1 using G3PAMAM-MWCNTs were shown. It was found that G3PAMAM-MWCNT can succesfully deliver pEGFPN1 into HEK 293 cells when the pEGFPN1/DNA complexation ratio was 1:10 and the pEGFPN1 has been successfully expressed.

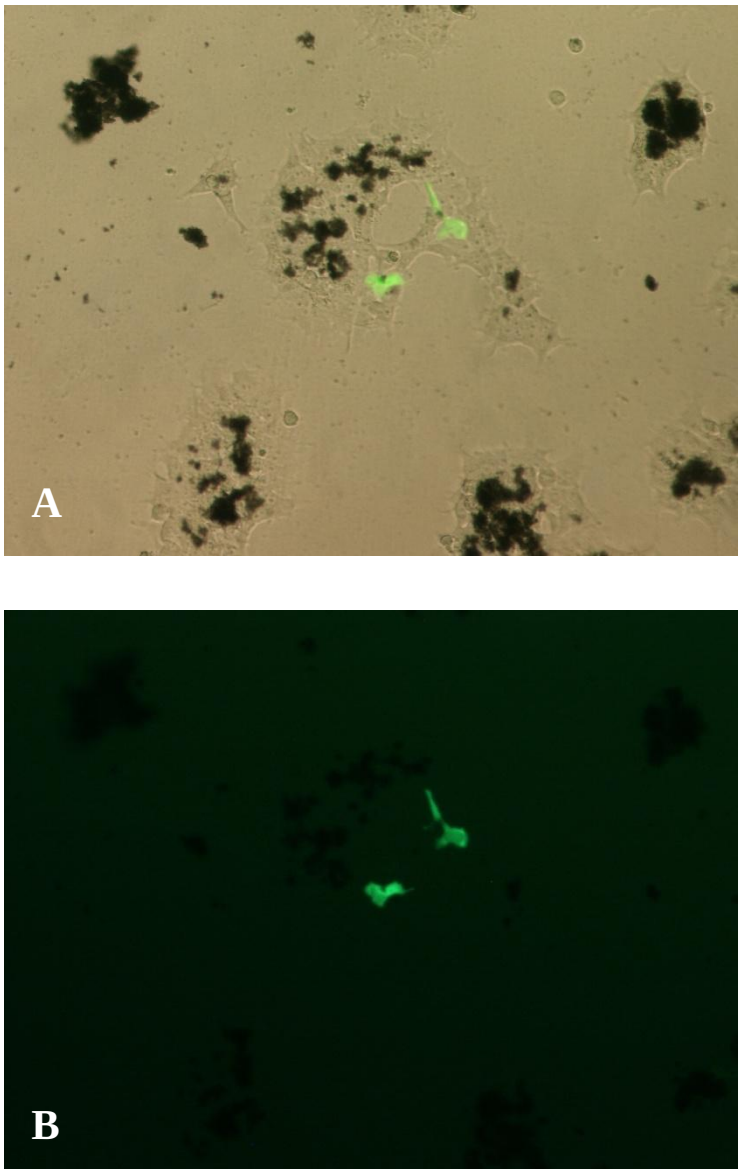

Figure 3.14 *in vitro* transfection of pEGFPN1 using G4PAMAM-MWCNT (10X magnification). A) The merged image and B) fluorescence of HEK 293 cells successfully transfected with pEGFPN1 gene by using G4PAMAM-MWCNT at 1:10 mass ratio.

In Figure 3.14, the fluorescence and merged images of HEK 293 cells transfected with pEGFPN1 using G4PAMAM-MWCNTs were shown. It was found that G4PAMAM-MWCNT can succesfully deliver pEGFPN1 into HEK 293 cells when the pEGFPN1/DNA complexation ratio was 1:10 and the pEGFPN1 has been successfully expressed.

CHAPTER 4

DISCUSSION AND CONCLUSION

Transfection is a transferring process of foreign DNA into nucleus of cultured host cells mediated through vectors. Transfection studies since the beginning of gene therapy trials have demonstrated that the usage of any kind of vector, viral or nonviral, is necessary for the effective gene delivery (Dang and Leong 2006). This necessity is also supported by naked DNA transfection results that transfection efficiency of naked DNA is much lower. There are two types of vectors as viral and nonviral that are chosen considering their effectiveness to deliver a gene. Viral based vector systems are retroviruses, adenoviruses, adeno-associated viruses, herpes viruses, pox viruses and lentiviruses that have been widely used to carry gene during the past years and successful gene delivery has been obtained. These viral based systems provide high efficiency of gene delivery and long term gene expression (Munier et al. 2005). Despite the advantages of these systems over their potential, there are the potential risk factors in the usage of these systems such as immunological and toxicological effects including the stimulation of inflammatory system that results in the degeneration of transduced tissue, causing mortality due to toxin production, the limitation in the capacity of viral vector to carry a target gene size (Anson 2004; Gardlík 2005; Munier et al. 2005; Nagasaki and Shinkai 2007). Because of these reasons, many researchers tended to use nonviral vectors for gene delivery. Since nonviral vectors are generally considered to be safer than viral vectors and however their infection efficacy is significantly less (Bieber et al. 2002).

CTNs are one of the subclasses of nanotechnology that have important properties which make them usable in variety areas. As it is widely known, successful *gene delivery* relies on safety and efficiency of vector, therefore, CTNs are one of the well-studied nanomaterial to be utilized *in vitro* gene delivery as a vehicle. So that the application of CTNs as a delivery vehicle has become possible *via* studies that based on

investigation of CTNs abilities including the mechanism of cell surface interaction an cell penetration. Yang et al. (2011) conducted a study to investigate whether or not there is a relation between the physical/chemical properties of different types of CTNs, transfection efficiency and toxicity. Result of the study confirmed that physical and chemical properties of CTNs are related to the intracellular delivery of plasmid DNA and cytotoxicity (Yang et al. 2011). Furthermore, it was investigated two types of CTNs, either single walled CTNs or multiwalled CTNs, which can be used to carry any molecule.

In this study, we investigated an *in vitro* transfection of pEGFPN1 in Human Embryonic Kidney (HEK 293) cell line using increasing generations (G1-G5) both of polyamidoamine (PAMAM) dendrimer modified CTNs and boronic acid (BA) modified CTNs and proposed to associate the role of boronic acid with gene delivery. To investigate also the effect of multiwalled CTNs in gene delivery into HEK 293 cells, we chose two types of multiwalled CTNs that one of them is modified by using polyamidoamine (PAMAM) dendrimers and the other one is modified by using boronic acid. Good transfection efficiency of PAMAM modified CTNs has been already proven by Qin et al. (Qin et al. 2011), but there have been no any study carried out about transfection efficiency of boronic acid modified multiwalled CTNs. However there is only a study by Peng et al., showing that combining of boronic acid groups with polyethylenimine was resulted in the enhancement of the gene delivery capability of pure polyethylenimine and also partially decreasing in toxicity of pure polyethylenimine. Therefore, we wanted to see whether there was a correlation between boronic acid and CTN mediated gene delivery. For this purpose, PAMAM-MWCNT and Boronic Acid-MWCNT were used as carrier of pEGFPN1 plasmid DNA for the delivery into HEK 293 cells. Control experiments were carried out through the incubation of cells in the absance of CTNs and Fugene transfection reagent was used as a positive control. In paralel experiments, HEK 293 cells were incubated for 48 hours with increasing generations of two types of CTNs and transfection efficiencies were analyzed by fluorescence microscopy.

HEK 293 cell line was chosen for our *in vitro* gene delivery experiments because, these cells are one of the most promising cell types for gene delivery due to their availability, easily preparation to culture and relatively simple requirements for *in vitro* expansion. In spite of the fact that HEK 293 cells had no problem growing in flasks, they had a difficult time attaching to well plates in refreshment process used for

gene delivery experiments. In order to overcome this problem, well plates were coated with poly-l-lysine (PLL) before transfection experiments for better attachment of cells to culture surface. After coating well plates using poly-l-lysin (PLL), we observed that cells could successfully attach in 96-well plates.

To determine the non toxic but effective increasing generation concentrations of both PAMAM-MWCNTs and BA-MWCNTs on HEK 293 cell viability, real time cell analyzer system was used. Depending on all graphs at figure 3.4, the optimum CNT concentration that enables to live almost entire cells in cell culture was determined as ranging between 5-25 μg/mL during 60 hours incubation period.

Cell viability was confirmed by cell proliferation assay using WST-1 in order to detect viable cells in response to CTN treatment. We established similar experimental set up with RTCA to compare the results. As seen in the figure 3.5 and 3.6, both PAMAM-MWCNTs and BA-MWCNTs have similar inhibitory effect over HEK 293 cell proliferation. Based on our findings, cell attachment and proliferation are similar to the untreated control cells at lower concentrations than 5 μg/mL of BA-MWCTNs, therefore almost half of the cells were attached to the surface as a result of treatment with lower concentrations than 5 μg/mL of PAMAM-MWCTNs. Additionally, there are three parameters including concentration, generation and type of multiwalled CTNs to compare the effect of CTNs over cell viability. First, our results showed that cell proliferation decreased at increasing concentrations of each two types of multiwalled CTNs. Second, cell proliferation decreased at increasing generations for two types of multiwalled CTNs depending on increasing concentration. And third, PAMAM-MWCNTs were determined to show more inhibitory effect on the proliferation of cells in comparison with BA-MWCNTs. Consequently, PAMAM-MWCNT on generation 5 (G5) and at the 75 μg/mL concentration was found to have the highest cytotoxic effect on cells. The observed higher inhibitory effect of G5PAMAM-MWCNTs over cell viability might also be attributed to the high quantity of amine groups which are known to possess efficient inhibitory activity. Moreover, 2 μg/mL concentration of G1BA-MWCNT was determined as showing the lowest inhibitory effect on cell viability. As a result of WST-1 assay, it was determined that the inhibitory effect of BA-MWCNTs on cell proliferation increase following the sequence of G1BA-MWCNTs < G3BA-MWCNTs < G2BA-MWCNTs < G4BA-MWCNTs < G5BA-MWCNTs at the increasing concentration range. On the other hand, it can easily said that there was no significant decrease on WST-1 results observed between two generations, which

decreased cell number was lower at G3BA-MWCNTs than G2BA-MWCNTs, that is attributed to dispersive properties of multiwalled carbon nanotubes.

Also, we considered that their toxicity at cellular level might increase due to non-solubility characteristics of CTNs. It means that when CTNs used as gene carrier *in vitro*, CTNs are not completely dissolved in many types of medium due to creating clumps in cell culture medium. So, imbalanced dispersion of CTNs in cell culture might cause the enhancement of toxicity. Herein, solubility of multi-walled CTNs which are directly related to toxicity of CTNs is the fundamental problem that might affect efficient gene delivery. If homogenous dispersion of CNTs in culture medium could be obtained, then a gene might be transferred into cells with a high efficiency and decreased mortality.

In this point, several fundamental issues about toxic effect of CTNs remain to be addressed for enhancing gene delivery in usage. To prevent cellular toxicity, various chemical modification studies have been carried out to make less toxic CTNs. Suitable surface modification of the nanotubes is required for enhancement of their dispersion in various solvents (Mittal 2011). Since, one of the most important challenges of working with CTNs as gene carrier is dispersion. As it is well known, CTNs are classes of nano-scale materials and aggregate in the solvent due to chemical interactions between them. Moreover, there are such solvents (except surfactants) which CTNs generally cerate clumps in it (Sanz et al. 2011).

This study also includes the investigation of DNA binding capacity of multiwalled CTNs. In the literature, the binding mechanism of pDNA onto CNTs were studied by Singh et al. (Singh et al. 2005), despite this there is no any study in the investigation of binding pDNA to boronic acid modified multiwalled CTNs. As mentioned above, only one study has been conducted by Peng et al. including the enhancement of transfection efficiency based on the interaction between phenylboronic acid and polyethilenimine. In this respect, this study aimed to contribute to this area with new knowledge by comparing the both DNA binding capacity and transfection efficiency of phenylboronic acid modified multiwalled CTNs. To determine the formation of the complex of pEGFPN1 and multiwalled CTNs, agarose gel electrophoresis was used. Agarose gel electrophoresis results have shown that each type of multiwalled CTNs is able to condense DNA to varying degrees at different mass ratios as seen in figures 3.7 and 3.8, which determined based on the retardation of pEGFPN1/MWCNTs complexes in agarose gel at whole mass ratio ranges. According

to our gel electrophoresis results, pEGFPN1 completely binds to both PAMAM-MWCNTs and BA-MWCNTs at mass ratios between 1:50 and 1:200. As seen in the figures 3.7 and 3.8, the DNA binding capacities of both G3PAMAM-MWCNTs and G3BA-MWCNTs have similar at the same mass ratios while mass ratio is lower than 1:10. Also, 1:2, 1:3, 1:4 and 1:5 mass ratios of each ten generations did not get success to completely condense pEGFPN1 which means they were unable to electrostatically neutralize pEGFPN1's negative charge. Therefore, we observed that DNA binding capacity of both PAMAM-MWCNTs and BA-MWCNTs increased until generation 3 and there was no significantly decrease in fluorescence levels on G4 of both PAMAM-MWCNTs and BA-MWCNTs. A significantly decrease in fluorescence levels observed on G5 of both PAMAM-MWCNTs and BA-MWCNTs which is attributed to DNA did not efficiently wrap multiwalled CNTs as a result of increasing outer diameter by increased generations. Also, we observed that G3/G4PAMAM-MWCNTs and G3/G4BA-MWCNTs had very similar band densities in the agarose gel which also means had similar binding capacity in the interaction with pEGFPN1, we decided to use two generations as G3 and G4 of both PAMAM-MWCNTs and BA-MWCNTs in the following transfection experiments.

According to our *in vitro* pEGFPN1 transfection results, it can be said that G3BA-MWCNTs at 1:10 mass ratio show the best transfection efficiency and the results also confirmed by agarose gel electrophoresis. We also observed low GFP gene expression levels at the mass ratio of 1:4 and 1:5 of G3PAMAM-MWCNTs and G3BA-MWCNTs. In general, BA-MWCNTs were more effective than PAMAM-MWCNTs for *in vitro* pEGFPN1 delivery on HEK 293 cells which is because of better penetration to cell surface provided by boronic acid enhances the uptake of multiwalled CNTs. On the other hand, *in vitro* pEGFPN1 transfection efficiencies of both PAMAM-MWCNTs and BA-MWCNTs are lower than control group that performed using X-tremeGENE 9 DNA transfection reagent.

In conclusion, in our research, we modified MWCNTs using boronic acid-diol interaction in order to gain carbon nanotubes specificity in the penetration of cell membrane and similarly expected that BA-MWCNTs show better penetration and higher transfection efficiency than PAMAM-MWCNTs. Additionally, we achieved to transfect HEK 293 cells with pEGFPN1 by PAMAM-MWCNT and Boronic acid-MWCNT at varying transfection efficiency, cytotoxicity and cell viability. Also,

Boronic acid modified multiwalled CNTs might be used as gene delivery agents; therefore further *in vivo* studies are required.

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