Intracellular Microenvironment Responsive Nanogels for Gene Delivery

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Abstract

Gene therapy is a modern technology applied by using therapeutic nucleic acids to treat or prevent diseases. The therapeutic nucleic acids can either be expressed to recombinant proteins for disease treatments or correct the genetic mutations in gene therapy for the treatments of various diseases. However, the development of gene therapy is greatly impeded by the limitation of gene delivery systems. Delivery of gene to eukaryotic cells is a multi-step process. Several barriers including cell membrane, nuclear pore complex (NPC) and intracellular enzymes have inhibited the transfection efficiency of gene. Therefore, the exploring of safe and high efficient gene carriers is essential for the development of gene therapy.

In this thesis, one of the widely studied cationic polymers, polyehtylenimine (PEI), was employed for modifications in the gene delivery application. High molecular weight PEI (HMW PEI) is able to perform high efficient gene transfection to eukaryotic cells due to its good buffering capacity. However, it also has high cytotoxicity to cells owing to its non-biodegradability and its molecular weight related high positive charge density. On the other hand, low molecular weight PEI (LMW PEI) such as PEI800 shows negligible cytotoxicity to cells, but has low transfection efficiency.

In order to reduce the cytotoxicity of PEI-based gene carrier while keep or improve the transfection efficiency, two biodegradable PEI-based gene carriers have been designed and successfully synthesized. The physical and chemical properties of both synthetic carriers were measured. The gene delivery performances of both carriers were also evaluated against Hela and HEK 293 cells. The synthetic biodegradable gene carriers display good gene binding ability, low cytotoxicity and good buffering capacity. In addition, both carriers are able to perform gene transfection with high efficiency.

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Chapter 1 Introduction

1.1 Background

Gene therapy has drawn great attention of researchers in the last decade since it can treat diseases by transferring therapeutic nucleic acids to patients' cells. The first gene therapy experiment was approved by US FDA. Up to 2014, more than 2000 clinical trials of gene therapy have been performed[1]. Gene therapy has been proven to be a promising method for the treatment of various diseases including cancers and other diseases. However, the clinical trial outcomes of gene therapy are greatly related to the performance of the gene delivery system.

Gene delivery to eukaryotic cells is a multiple-step process including cellular uptake, endosomal escape, nuclear trafficking and gene release. Several barriers such as cell membrane, nuclease and nuclear membrane restrain the delivery of nucleic acids. Since the efficiency of transporting naked nucleic acids through tools like gene guns is quite low, gene delivery carriers are required to protect the nucleic acids as well as to improve the transfection efficiency[2].

Different gene vectors have been developed to deliver nucleic acids to perform the gene therapy to cure diseases. Viral and non-viral gene carriers are the two major types of gene carriers that have been developed. Viral gene carriers are used in most of the clinical trials of gene therapy nowadays because of their high efficiency. However, the risks of using viral gene vectors are also high, because it may cause the immunological issues as well as the inflammatory problems[3]. In order to minimize these risks, researchers have developed various non-viral gene carriers. Among non-viral gene vectors, cationic polymers could potentially be an ideal candidate due to its excellent performance in gene delivery.

Polyethylenimine 25K Da (PEI25K) has been considered as one of the most efficient cationic gene carriers for its high transfection efficiency due to its 'proton sponge' effect[4]. In addition, PEI is able to form complexes with nucleic acids through the electrostatic interaction. However, the high cytotoxicity of PEI25K to cells is also caused by its high positive charge density to cells, which becomes the main concern for the development of PEI as an efficient gene carrier. Therefore PEI needs to be modified to reduce the cytotoxicity by reducing its positive charge density. Since the positive charged density is related to the molecular weight of PEI, low molecular weight PEI (LMW PEI) shows a quite low toxicity to cells but suffering low transfection efficiency due to its low DNA binding ability and low buffering capacity[5].

In this research project, I will develop biodegradable PEI-based carriers through cross-linking LMW PEI for efficient gene delivery with low cytotoxicity. The biodegradable PEI-based gene carrier will be synthesized by crosslinking LMW PEI800 with novel biodegradable linkers, 4'4-dithiodibutyric acid (DA). This crosslinked product will break down to low molecular weight molecules after delivering genes into cells and these small molecules will bear low cytotoxicity. In addition, I will improve the transfection efficiency of the synthetic carriers by conjugating a cell penetrating peptide, HIV-1 TAT peptide. This peptide will facilitate cellular uptake and nuclear trafficking of the gene carriers with our synthetic polymers. Both biodegradable PEI-based carriers will be expected to perform high efficient transfection with low cytotoxicity when delivering nucleic acids to cells compared with commercial PEI (PEI25K).

1.2 Aims and Objectives

The aim of this project is to develop a safe and efficient gene delivery system for gene therapy using PEI-based gene carriers. To achieve this aim, this project will focus on two main objectives as follows:

1. To reduce the cytotoxicity of gene carriers to cells, low toxic PEI800 will be cross-linked by a biodegradable linker to form biodegradable polymer with higher DNA binding capability and low cytotoxicity as DNA carrier which will be reduced by cellular enzyme.

2. To improve DNA uptake inside cells, a cell penetrating peptide, HIV-1 Trans-Activator of Transcription (TAT) peptide will be further conjugated with the synthetic biodegradable PEI based gene carrier.

The process of this project is described in Figure 1.1. I will design and synthesize the biodegradable PEI-based gene carriers and then the characterizations and features of synthesized gene carriers will be investigated systematically. Finally, the performance of gene delivery application of synthetic polymers will be evaluated.
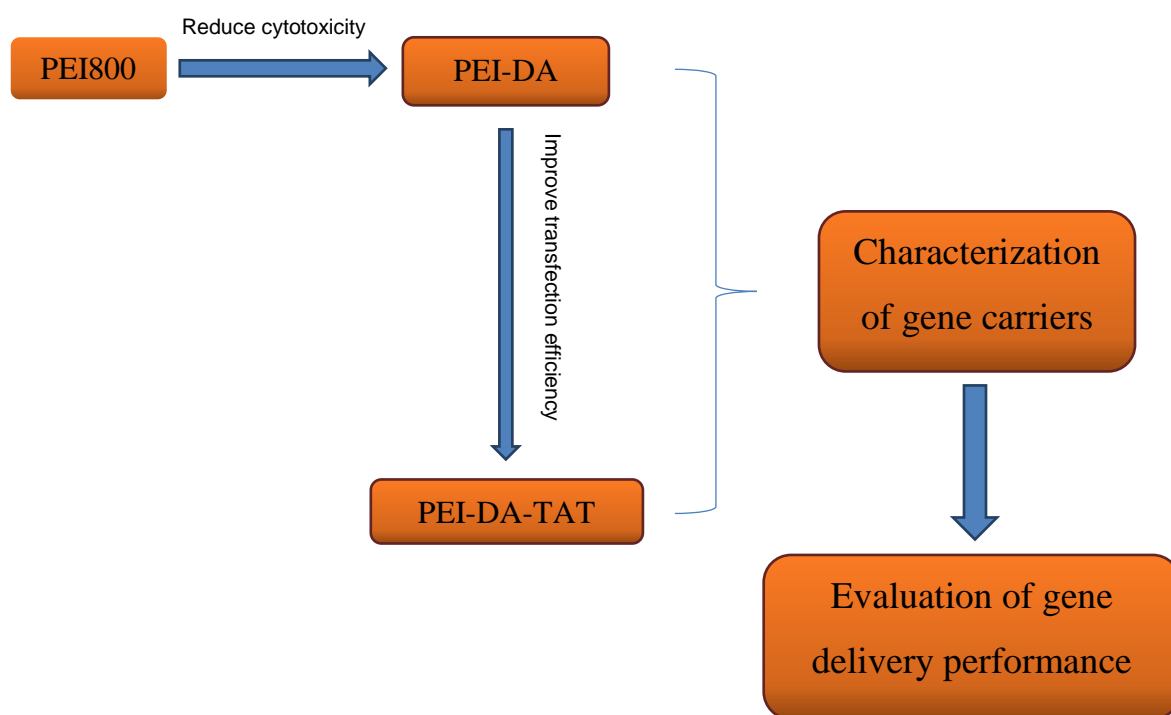
Figure 1.1 The design and experimental structure of the Master project

1.3 Thesis outline

The major research contribution is written in two journal publications.

In Chapter 1, the background, the aim and objectives, structure and thesis outline are introduced.

In Chapter 2, research reviews in recent gene delivery systems and development of PEI based carriers and the applications of cell penetrating peptides are reviewed.

In Chapter 3, a novel biodegradable gene vector has been developed by crosslinking PEI800 with 4'4-dithiodibutyric acid (DA) by carbodiimide chemistry. The synthetic PEI-DA shows neglect cytotoxicity to mammal cells. The transfection efficiency of resulted polymer is comparable that of commercial cationic carrier PEI25K.

In Chapter 4, how to further improve the transfection efficiency of synthetic biodegradable gene delivery by conjugating cell penetrating peptide is described. Here, cell penetrating peptide, HIV-1 Trans-Activator of Transcription (TAT) peptide is applied to from a TAT-labelled intracellular biodegradable PEI-based gene carrier. The results show that the transfection efficiency of the resulted polymer increases after the introduction of TAT by improving the cellular uptake ability as well as nuclear localization ability for the carrier. The cytotoxicity of the resulted polymer is significantly lower than that of PEI25K.

In Chapter 5, the conclusions and the further recommendations of the development of PEI-based gene delivery carriers are described.

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Chapter 2 Literature review

2.1 Introduction

Gene therapy is a modern technology by delivering therapeutic nucleic acid to patients' tissues or cells to fix the genetic problems. The therapeutic nucleic acid can either express recombinant proteins for disease treatments or correct the genetic mutations in cells. Considerable investigations of gene therapy have been conducted since it was conceptualized in 1972[1]. For example, gene therapy has been used in the treatments of various diseases including adenosine deaminase deficiency-severe combined immunodeficiency (ADA-SCID)[2], cancers[3], chronic lymphocytic leukemia (CLL)[4], acute lymphocytic leukemia (ALL)[5] and Parkinson's disease[6].

Over 2000 clinical trials of gene therapy have been conducted from 1989-2014. The number of conducted clinical trials has been increased from 1 in 1989 to 104 in 2014 by 100 times. The increasing clinical trials include treatments of cancer diseases (64.2%), cardiovascular diseases (7.8%), infectious diseases (8.0%), monogenic diseases (9.2%) and others[7]. The treatments of cancer diseases are the major applications of gene therapy. This indicates that the treatments of cancer diseases are the most urgent demand of the gene therapy. However, technical barriers still restrain the successful transformation of gene therapy[8]. Most clinical trials of gene therapy are still carried out in phase I (58.8%) and II (16.8%). Besides, viral vectors with the potential risk of the immunological issues in the treatments[3] are the main vectors used in current gene therapy (more than 54.4%).

In order to facilitate the development of gene therapy application, researchers have put effort onto high efficient non-viral gene vectors. For example, synthetic polymers, as a promising non-viral gene carrier, have been studied for over two decades[9]. However, the lower

transfection efficiency compared with viral gene vectors still cannot meet the requirements for the applications of gene therapy. In addition, the different performances of synthetic gene carriers between *in vitro* and *in vivo* also influence the development of non-viral gene carriers[10]. As shown in Table 2.1, several barriers impact the efficiency of gene delivery to mammal cells, including intracellular and extracellular barriers. For example, cell membrane is the barrier for the uptake of nucleic acids. This limits the amount of gene that could be uptaken by cells. In addition, the nuclear trafficking limits the amount of the nucleic acids to be translated and expressed into recombinant proteins or to fix the mutated gene sequence. To

Table 2.1 The intracellular and extracellular barriers in polymer mediated gene delivery

Intracellular Barriers	*Extracellular Barriers*
1. Gene condensing	1. Stability in solution and biocompatibility with and without ionic molecules
2. Cell membrane	
3. Nucleic acid degradation	2. Anti-nuclease ability
4. Endosomal escape	3. Specific recognition by cells of interest
5. Nuclear trafficking	4. Accumulation of polymers in tissues and cells

overcome these barriers, modified non-viral gene vectors have been synthesized such as cationic polymers[11], silica-based gene carriers[12] and gold-nanoparticle-based gene carriers[13].

Among different gene delivery carriers, the PEI-based gene delivery carrier has drawn great attention due to its high transfection efficiency compared with other non-viral gene delivery carriers. The drawback of PEI-based gene delivery carriers is the high cytotoxicity, which is related to its molecular weight and non-biodegradability in cells. Modifications of the PEI-

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based gene delivery carrier are necessary to improve its performance in gene transfection to eukaryotic cells.

In this review, three different parts were described. In the first part, the process of gene delivery system was described in order to understand the barriers limiting the development of the gene delivery system. In the second part, the development and modifications of one of the most popular cationic gene carriers, polyethylenimine (PEI) were summarised. Finally, cell penetrating peptides with the ability to facilitate the cellular uptake of carriers and nuclear localization ability leading to the improvement of transfection efficiency were highlighted.

2.2 Progress of gene delivery polymers

As shown in Figure 2.1, genes are delivered to mammal cells through a multi-step process, including step 1: the condensation of DNA by interactions between carriers and genes; step 2: cellular uptake through cell membrane by different endocytosis pathways; step 3: escape from the endosome by different mechanisms such as membrane fusion and 'proton sponge' effect;
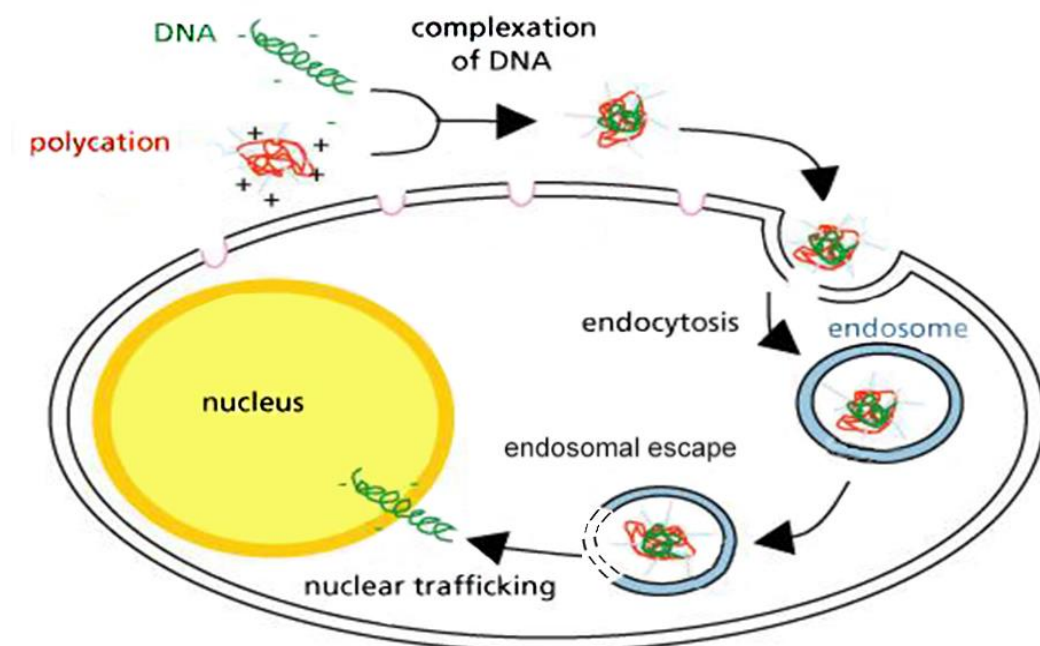
Figure 2.1 Polymer based gene delivery system[14] (modified form Chenoweth et.al)

step 4: nuclear transport through nuclear pore complex (NPC) and step 5: translation and expression of nucleic acids.

Except step 5, all other steps would influence the final transfection efficiency of the whole gene delivery system due to different barriers such as cell membrane, intracellular enzymes and others. Therefore, the understanding of the whole process of gene delivery is the prerequisite for the design and pursuit of efficient cationic gene delivery carriers.

2.2.1 Condensing nucleic acid

In order to protect the nucleic acid during the delivery, the negatively charged gene needs to be interacted and condensed with gene carriers into a suitable particle size ranging around 100 - 200 nm for the cellular uptake[15]. As shown in Figure 2.2, cationic polymers condense the gene through the electrostatic interaction between the positive charged binding groups on the cationic polymers and the negative charged phosphate groups of the gene, and the condensation is an essential factor to influence the particle size of the complex of the carrier/gene. Researchers have found that large molecular weight cationic polymers such as PEI25K with a higher positive charge density could better condense the gene leading to a high efficient gene delivery[16]. However, the high positive charge density is the major factor for high cytotoxicity to cells. In addition, the excess of the positive charged polymers would interact with anionic molecules in tissues or blood stream, and the interaction would cause the inhibition of some cellular processes *in vivo*[17]. Therefore, the control of the positive charge density of gene carriers with high condensing ability but low cytotoxicity is one of the challenges to develop highly efficient gene carriers.
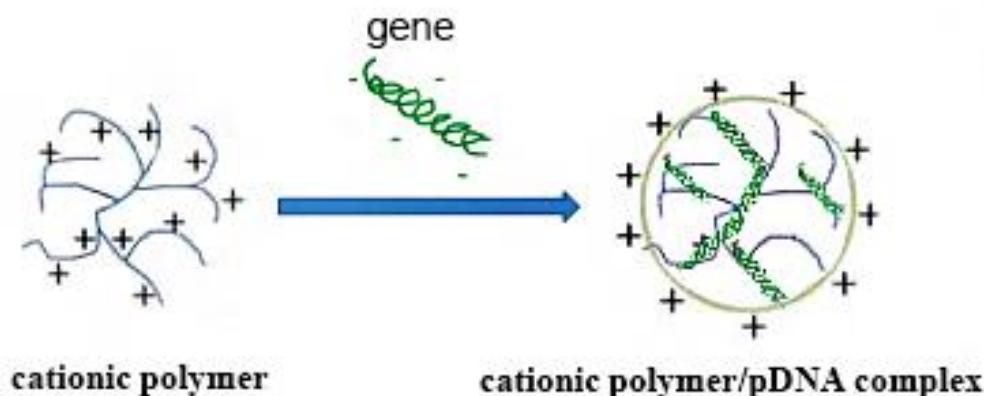
Figure 2.2 The self-assembled cationic polymer/gene structure

2.2.2 Cellular uptake

The first barrier of gene transportation is the cell membrane. Endocytic and non-endocytic pathways are two major cellular uptake pathways. Endocytic pathway of cell uptake is a form of active transport in which large molecules that cannot pass through cell membrane by passive means are transported by engulfing them in an energy–using process[18]. Most cationic gene carriers have been proven to penetrate the cell membrane through the endocytosis pathway and the polymer/gene complexes are later trapped in the intracellular endosome/lysosome[19]. To date, cationic polymers, especially PEI25K, show good cellular uptake ability and this is due to its remaining positive charges after condensation with the gene that interact with negatively charged surface of the cell membrane. Several factors such as the particle size[20], surface charge[21] and ligands on the gene carriers[22] influence the cellular uptake ability of gene carriers. In the study on the influence of the particle size and surface charge, He et al. have reported that particle size and surface charge of gene carriers were more important parameters than the composition of gene carriers[23]. They claimed that a slightly difference of physicochemical properties such as 10 mV-alternation of zeta potential was able to affect the cellular uptake of the polymers. Furthermore, one of the targeting ligands, MC11

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peptide reported by Li et al., facilitated the fibroblast growth factor receptor (FGFR)-mediated cellular uptake in cancer cells[24]. Different synthetic polymers could penetrate cell membrane through different endocytosis pathways such as clathrin-mediated endocytosis, caveolae, macropinocytosis, and phagocytosis[25]. This could cause different performance of the final transfection efficiency[26]. To conclude, the mechanism and efficiency of cellular uptake are dominated by the physical and chemical properties of gene carriers.

2.2.3 Endosomal escape

After the carrier/gene complexes penetrate through cell membrane, it is trapped in the endosome with a pH of 7.0 in the initial stage and 5.0 in the later maturation stage[27]. In the later stage, the endosome grows into lysosome and the externals of lysosome are digested by the hydrolytic enzymes. If the carrier/gene complexes cannot escape from the lysosome, complexes are digested and cleaned out from the cells. Therefore, for a satisfied gene delivery system, gene carriers should facilitate the escape of nucleic acids from the lysosome and also protect the gene from digestion by the intracellular enzymes[28]. Cationic gene vectors could realize the endosomal escape through membrane fusion[29]. Commercial PEI25K showed an excellent escape ability from late endosome through the 'proton sponge' effect leading to its high transfection efficiency[30]. Since the amino groups on PEI are protonated at an acid condition, osmotic swelling occurs because of the influx of chloride ions, and the swelling eventually results in the membranolysis of the endosome/lysosome. However, controversial discussion has been raised about the 'proton sponge' effect[31]. Kulkarni found that the final gene transfection efficiency was not correlated with the positive charge density of non-viral gene vectors by comparing the results of cellular uptake with the level of gene expression[32]. Therefore, the mechanism of endosomal escape of gene carriers needs to be further investigated, and it would help to improve the transfection efficiency of cationic gene carriers.

2.2.4 Nuclear trafficking

After the carrier/gene complexes escape from the endosome/lysosome, the next barrier of gene delivery is how to get through the nuclear membrane and nuclear pore complexes (NPC). The particle size and molecular weight of cargos are two important parameters to affect the entrance through NPC[33]. Carriers with large molecular weight need to display a specific nuclear localization signal (NLS) to overcome the limitation of NPC in active transportation[34-36]. Studies have proven that only around 0.1% of naked gene delivered through microinjection could access to nucleus[33]. Therefore gene carriers have been introduced to facilitate the nuclear trafficking and further improve the transfection efficiency of therapeutic nucleic acids[37]. Strategies of modifications of cationic polymers by conjugating with NLS peptides[38, 39] have been proven to increase the gene transfection efficiency to mammal cells due to the improvement of the nuclear trafficking ability[40, 41].

2.3 Considerations for gene carrier design

To design and synthesize a gene carrier, several issues as shown in Table 2.2 need to be considered. To begin with, the biocompatibility of the gene delivery system is the key factor for the gene delivery system. The solubility is the prerequisite for the gene carrier since the clinical applications of gene therapy are in a liquid environment. In the early stage of the exploring of gene carriers, viral vectors were considered to be good candidates for gene delivery because of their high transfection efficiency. However, the applications of viral vectors were soon been prohibited by their immunogenicity problems. This could cause the rejection response of patients and lead to other diseases[42]. Furthermore, the cytotoxicity to cells needs to be measured *in vitro* and *in vivo*. The consideration of the biodegradability of gene carriers is also important to avoid the accumulation of gene carriers in bloodstream and tissues[43].

Table 2.2 Considerations of gene carrier design

1. **Biocompatibility of gene carrier**
   a. Solubility
   b. Safety
   c. Cytotoxicity and immunogenicity
   d. Biodegradability

2. **Gene condensing, protection ability and cell uptake**
   a. Surface charge
   b. Particle size of complexes
   c. Interaction with gene
   d. Gene release

3. **Endosomal escape and nuclear trafficking**
   a. Pathway of endosomal escape
   b. Buffering capacity
   c. Nuclear pore complex entry pathway

The second consideration for gene carrier design is the gene condensing ability and gene protection ability of carriers, which are two important factors to affect the transfection efficiency of the gene delivery carriers. Appropriate particle size and suitable surface charge of the carrier/gene complexes are pursued in order to penetrate through the cell membrane. Carrier/gene complexes are basically formed through two main physical forces, hydrophobic-hydrophilic and cationic-anionic interactions[44]. Both ways can condense the gene into a suitable size for cellar uptake. The condensation and protection ability increase with the increase of these interactions. However, the balance of the condensing ability and the release of gene out of the complex inside the cell is also needed for consideration in a highly efficient gene delivery system[45].

Finally, another challenge for a highly efficient gene delivery system is the escape of carrier/gene complexes from the late endosome. Membrane fusion and 'proton sponge' effect

are two mechanisms for non-viral gene carriers to perform the endosomal escape[29]. An efficient gene carrier should have a strong membrane fusion ability as well as a high positive charge density to guarantee a good buffering capacity. In addition, the nuclear trafficking ability of gene carriers should be considered since efficient nuclear transportation of carrier/gene complexes could lead to the improvement of the final transfection efficiency of gene carriers.

Overall, an ideal gene carrier should have the essential properties including strong gene condensation and protection ability, high cellular uptake ability, low cytotoxicity to cells, excellent performance in facilitate gene endosomal escape and efficient nuclear trafficking. Besides, gene carriers should be able to conjugate different functional components such as targeting receptors[46-48], peptides[49] and sugars[50] to enhance the performance of gene carriers in a gene delivery system.

2.4 PEI-based gene carriers

Polyethylenimine (PEI) has been studied as an efficient cationic non-viral gene carrier candidate owing to its good buffering capacity for endosomal escape. This is considered as one of the key factors for its high transfection efficiency in gene delivery. From the structure of PEI, researchers have found that its buffering capacity is resulted from the high density of the protonated amino groups. As shown in Figure 2.3, the protonated amino groups on PEI lead to the influx of chloride ions and then cause the rupture of endosome structure to achieve the escape of PEI/gene complexes from the endosome/lysosome[16]. This is termed as the 'proton sponge' effect. Gene carried by PEI-based gene carriers is able to avoid the degradation by the intracellular enzymes due to endosomal escape by the 'proton sponge' effect. This phenomena has been recognized as one key contributor for the high transfection efficiency of PEI-based gene carriers[51].
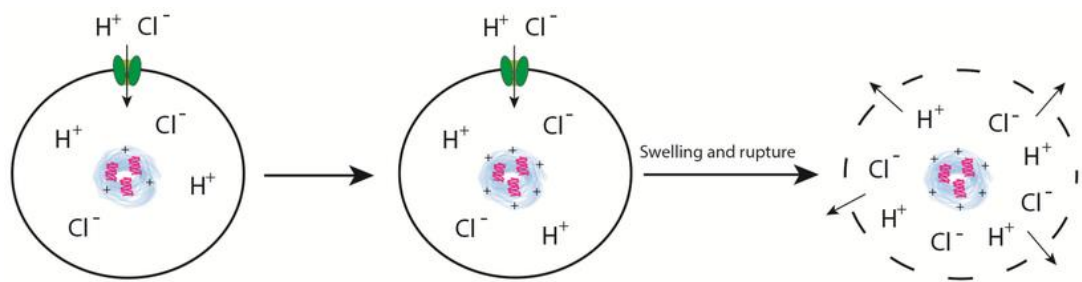
Figure 2.3 Proton sponge effect of PEI[52]

PEI mainly has two forms, branched and linear as shown in Figure 2.4. Branched PEI consists of one-fourth of primary, secondary and tertiary amines and it has been reported to have great potential in gene delivery compared with linear PEI *in vitro* and *in vivo* due to the branched structure[53]. The reason is that different types of the amino groups including primary amino groups, secondary and tertiary amino groups on branched PEI can perform different functions in gene delivery. For example, primary amino groups with positive charge mainly influence the gene binding ability by electrostatic interaction with the negatively charged phosphate groups on nucleic acid.
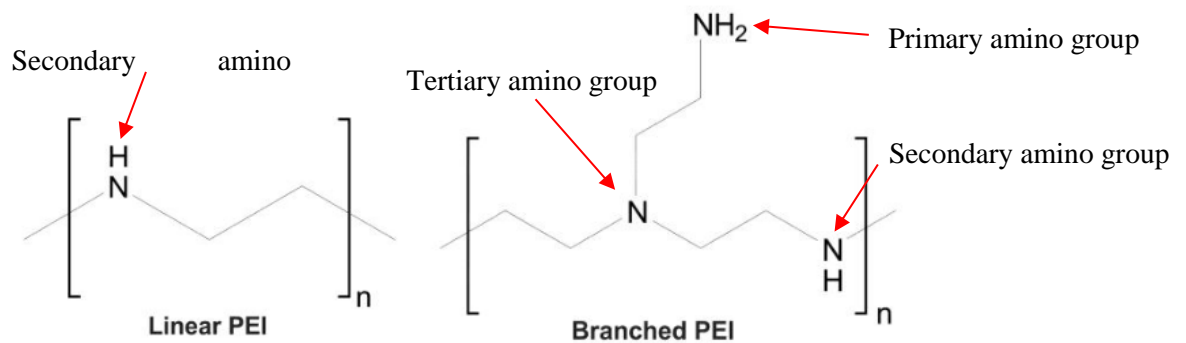
Figure 2.4 Chemical structures of the linear and branched polyethylenimine (PEI)[54]

Secondary and tertiary amino groups have an impact on the transfection efficiency because they can be protonated and help the endosomal escape by causing the endosome/lysosome burst owing to the osmolality changes of the fusion of endosome/lysosome[55, 56]. Due to its higher positive charge density than linear PEI, branched PEI shows better gene condensing

and protection ability as well as the ability to facilitate gene escape from the endosome/lysosome resulting in its high transfection efficiency. On the other hand, its high positive charge density also leads to high cytotoxicity, and this hinders its applications in the gene therapy. The application of linear PEI is also impeded by its relatively low transfection efficiency[53]. In order to overcome these problems, different modifications of PEI have been made to reduce the cytotoxicity of PEI-based gene carriers while keep the high transfection efficiency.

2.4.1 General modifications of PEI-based gene carriers

In order to apply PEI-based gene carriers in the clinical trials for highly efficient gene therapy, different modifications have been investigated in the last decade. The modifications of the amino groups have been normally considered as the general way to expand the applications of PEI-based gene carriers. Different strategies have been investigated including alkylation[57-59] and acylation[60, 61]. A variety of PEI-based gene carriers with high efficiency, high specificity and low toxicity have been synthesized. For example, Forrest et al. reported that buffering capacity of PEI was improved after its primary and secondary amino groups were acetylated, leading to the increase of the final transfection efficiency[62].

Thomas and Klibanov developed a systematic modification of PEI to form PEI derives[16]. As shown in Figure 2.5, different methods of modifications were conducted through A) the quaternization of amines on PEI25K with methyl or ethyl iodides; B) the alkylation of primary and secondary amines on PEI25K with 2-bromocholine; C) the acylation of primary and secondary amines on PEI25K with amino acids including leucine, alanine and histidine; D) the alkylation of primary amines on PEI25K and PEI2K with dodecyl or hexadecyl iodides; and finally E) the quaternization of tertiary amines on PEI25K with hexadecyl iodide.
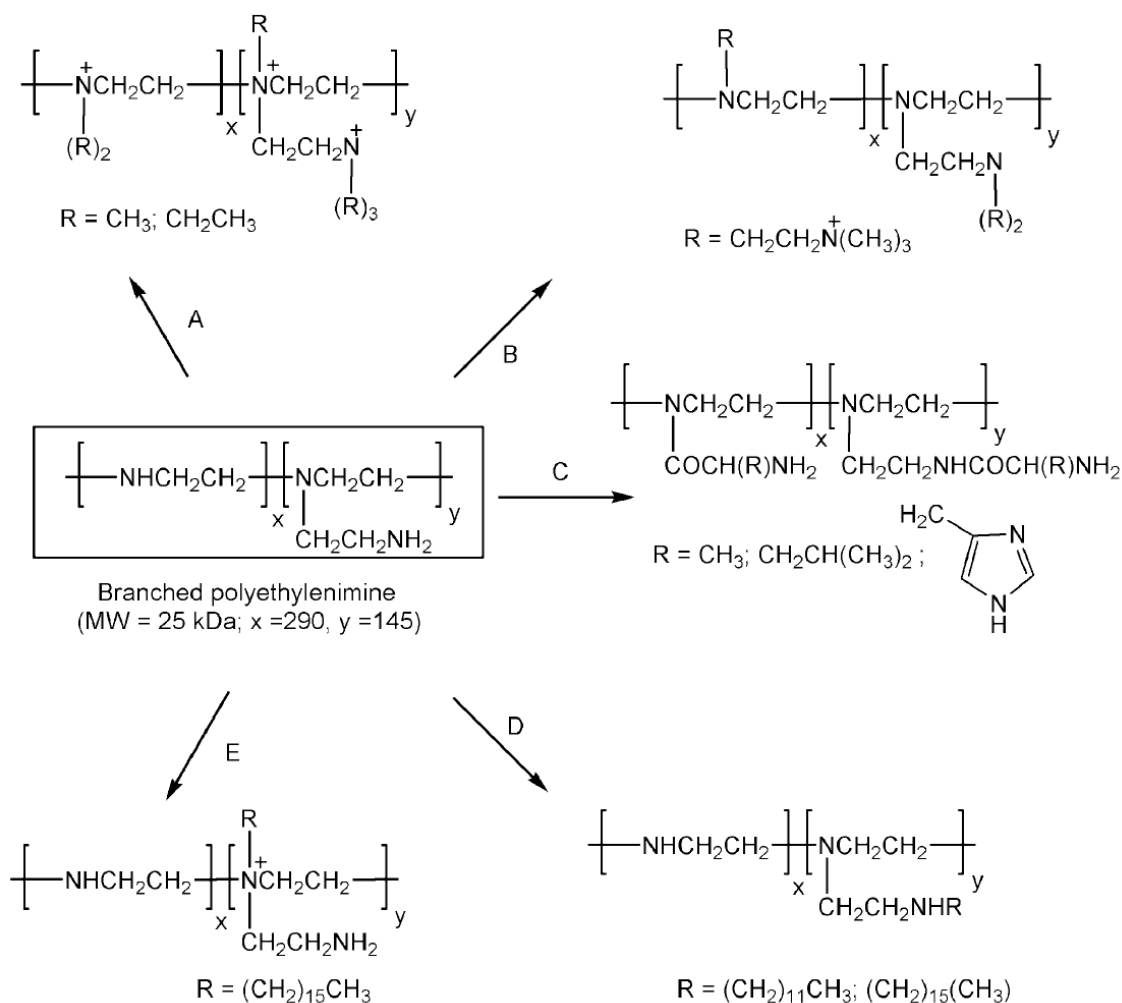
Figure 2.5 Schematic representation of different modifications of PEI[16]

They have found that PEI modified with alanine acrylate and dodecyl had the greatest improvement due to its good hydrophilicity. The resulting carrier also reduced the cytotoxicity. The gene transfection efficiency of resulting polymers was significantly improved. This result also implied that the formation of carrier/gene complex could not only ascribed to electrostatic interaction but also related to hydrophobic interaction.

Forrest et al. reported that poly(ethylene glycol) (PEG) was modified as a hydrophilic and biocompatible part to PEI. After the modification, PEI/PEG copolymer prevented the non-specific interaction because of the shielding effect caused by PEG[63]. Due to PEG's hydrophilicity, chain flexibility, electrical neutrality and absence of functional groups, serum

proteins could not be bound to the PEG modified surfaces. They have synthesized PEI with bifunctional PEG copolymers and the resulting copolymers maintained a high transfection efficiency with low cytotoxicity due to their high water solubility. Similarly, Endres et.al also found that PEI-based gene carriers modified to have good hydrophilicity could condense gene into smaller sizes, whereas more hydrophobic polymers precipitated to larger (>100 nm) particulate gene/carrier complexes[15]. An increase of hydrophilicity also resulted in the increase of the stability of gene carriers, as well as a decrease of cytotoxicity of carriers due to the effective charge shielding by PEG.

Besides, some researchers claimed that the transfection efficiency of PEI-based gene carriers was related to the effective molecular weight of PEI. Higher molecular weight of PEI-based
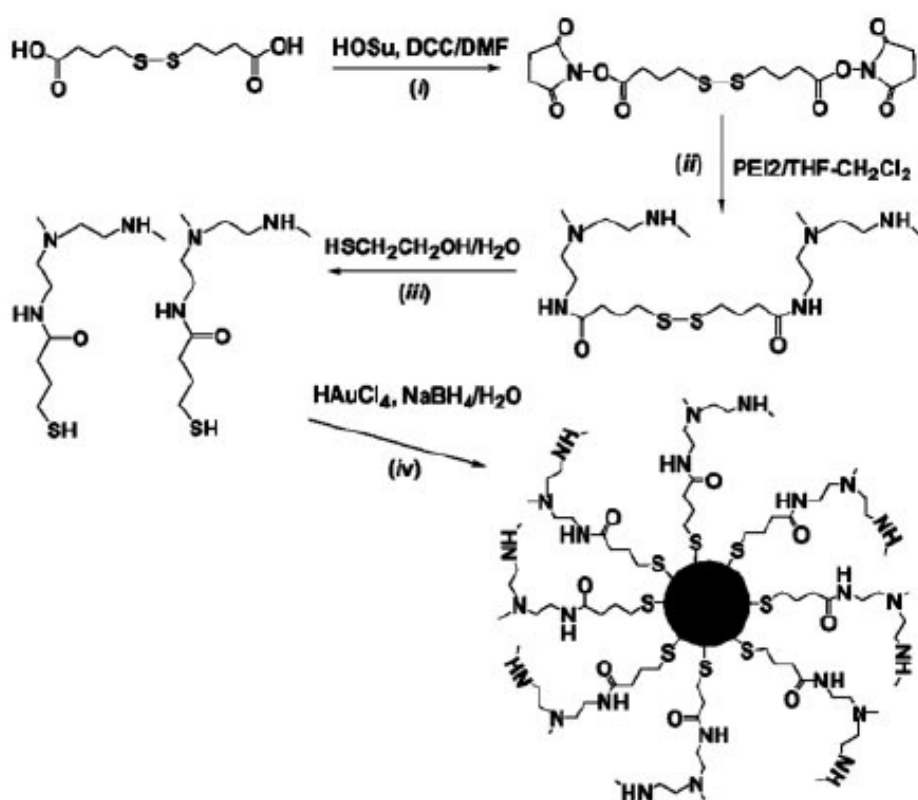
Figure 2.6 Schematic description of the synthesis of PEI2-GNPs[64]

gene carrier may be responsible for the higher gene binding and condensing ability. In order to increase the effective molecular weight, thiol-modified PEI2K was conjugated to gold

nanoparticles (GNPs) to form PEI2-GNPs (shown in Figure 2.6)[64]. The gene transfection efficiency of PEI2-GNPs increased more than 6 folds in COS-7 cells compared with commercial PEI25K. In addition, the transfection efficiency could be further improved by conjugating the PEI2K-GNPs with N-dodecyl-PEI2K. However, the ternary polymer still exhibited moderate toxicity to COS-7 cells.

2.4.2 Biodegradable PEI-based gene carriers

The main barrier of PEI as gene carriers is its high cytotoxicity. Since the main contributor of the toxicity of PEI is its high cationic density, researchers have been exploring methods to prevent the accumulation of cations. Crosslinking low molecular weight PEI by biodegradable linkages has become a promising strategy for PEI modifications[65], since low molecular weight PEI has been proven to have negligible cytotoxicity[66]. Several different linkers have been investigated in the last decade including ester linkage[67, 68], imine linkage[69], carbamate linkage[70] and disulfide linkage[71, 72].

2.4.2.1 Ester linkage

Petersen et al. synthesized an ester bond containing degradable gene carrier using Oligo (L-lactic acid-cosuccinic acid) to crosslink LMW PEI[73]. However, the results showed that the presence of the amide cross-linker could result in slow degradation of the polymers. In addition, the half live was 1.5 months at a physiological condition. This suggested that the resulting carrier was not suitable for gene therapy since accumulation of the polymer in blood stream or tissues might cause adverse effects to patients.
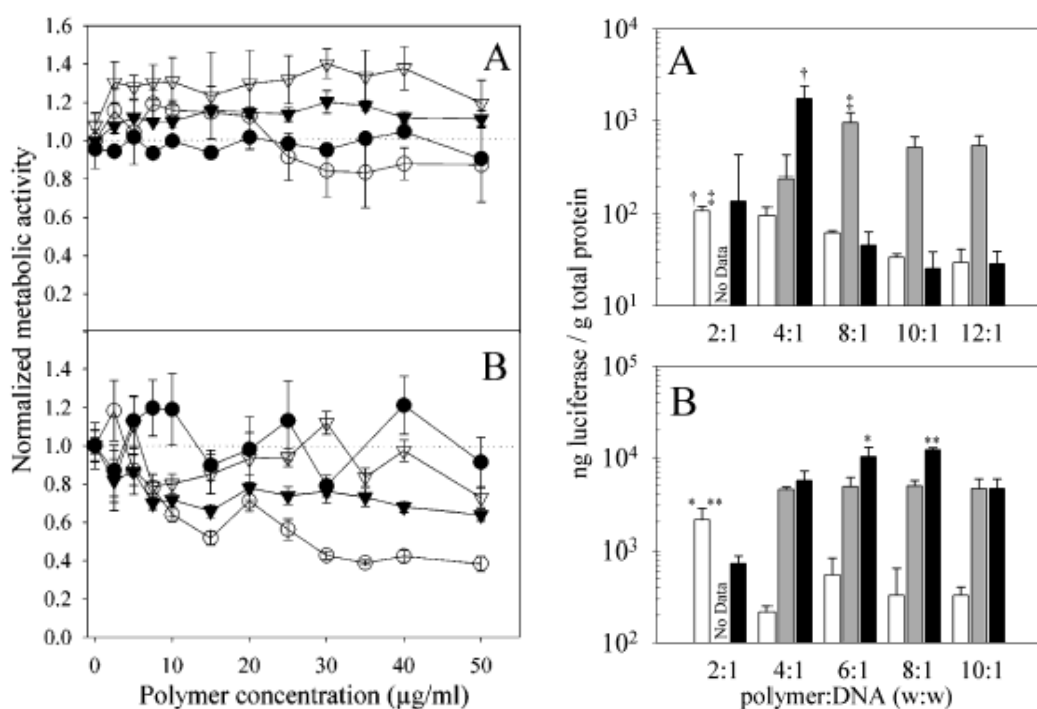
Figure 2.7 Cytotoxicity (Left) and gene delivery activity (Right) of degradable PEI (A) MDA-MB-231 (B) C2C12 cells. Left: 25-KDa PEI (○), 800-Da PEI (●), polymer 1 (▽), and polymer 2 (▼). Right: white bars, 25-KDa PEI; gray bars, polymer 1; black bars, polymer 2[67]

On the other hand, Forrest et al. developed two biodegradable gene carriers synthesized by cross-linking LMW PEI with 1,3-butanediacrylate and 1,6-hexanediacrylate resectively[67]. The two synthetic polymers reduced the half-lives to 4 h and 30 h at a physiological condition respectively. As shown in Figure 2.7, the cell viability of both synthetic polymers increased by over 30% compared with commercial PEI25K (cell viability 50%). Both polymers had a higher transfection efficiency compared with PEI25K in C2C12 cells.

Kim et al. cross-linked LMW PEI with bifunctional PEG containing ester linkages. After synthesizing the polymers, the cell viability and transfection efficiency were evaluated. Their results showed that cell viability remained over 80% after exposure to their polymers, whereas around 40% in presence of PEI25K. In addition, the synthetic polymers showed 3-fold higher transfection efficiency than LMW PEI, however, the efficiency was still lower than that of PEI 25K[56].

2.4.2.2 Imine linkage

Imine-linkage based PEI derivatives can be obtained by employing glutardialdehyde as a linker to crosslink LMW PEI. Kim et al[74] produced the product with a high molecular weight (13-23 KDa). Due to the introduction of glutardialdehyde, an acid labile compound, it had been able to obtain the half-life of 2.5 h and 118 h at pH 5.4 and pH 7.4 respectively. These polymers improved the degradability of the PEI leading to a lower cytotoxicity compared with commercial PEI25K. However, the transfection level of the product was still low.

Recently, Liu et al. developed a biodegradable PEI-based gene carrier, P123-PEI-R13, by crosslinking LMW PEI2K with Pluronic® through carbamate linkages and further conjugated RGD as well as HIV-1 TAT peptide to increase the targeting and cellular uptake ability of the carrier[75]. With the assistance of the targeting ligands, P123-PEI-R13 successfully showed the targeting specificity to HeLa and B16 cells. Higher transfection efficiency with lower cytotoxicity was achieved in both cells comparing PEI25K.

2.4.2.3 Carbamate linkage

Three different LMW PEIs (PEI600, PEI1200 and PEI1800) were reacted with PEG succinimidyl succinate via a carbamate linkage to form the biodegradable PEI-based gene carriers by Ahn et al[56]. The resulting polymers had 3-fold higher transfection efficiency than the initial PEI. In addition, the cell viability of synthetic polymers was over 80%. However, the transfection efficiencies of the synthetic polymers were lower than PEI25K because the cellular uptake of the carrier/gene complexes reduced owing to the hydrophilic nature of PEG.

2.4.2.4 Disulfide linkage

Disulfide bonds is very promising in clinical applications since it can be cleaved under a high concentration of glutathione in the cytosol or the nucleus, and it can be triggered after escape

from the endolysosomal compartment[76, 77]. Dithiobis(succinimidylprop ionate) (DSP) and dimethyl 3,3'-dithiobispropionimidate (DTBP) were firstly used as the disulfide bond containing biodegradable linkage for synthesis of biodegradable PEI-based gene carriers[78]. PEI800 was cross-linked with DSP and DTBP and the product showed good biodegradability because of the presence of the glutathione in the intracellular environment. It has been demonstrated that the nature of the cross-linkers, the extent of conjugation and the N/P ratio of carrier to gene were three important factors to influence the transfection efficiency of the biodegradable PEI-based gene carriers with disulfide linkages.

Bauhuber et al. prepared a series of PEG-PEI copolymers using disulfide bond-containing agents in order to investigate the influence of the structure on the gene delivery efficiency[79]. The results suggested that the amount of PEG influenced the physicochemical properties of the polymer/gene complexes. With an increase of the amount of PEG, the cellular uptake ability of the product decreased, which led a decrease of the final transfection efficiency.

Recently, Liu et al. synthesized a biodegradable PEI-based gene carrier by 'click chemistry' through the reaction of azide-terminated LMW PEI1800 with a disulfide containing dialkyne[80]. The transfection efficiency of the product was evaluated with and without serum. The results indicated that both with and without serum the degradable PEI-based gene carrier showed higher transfection efficiency and low cytotoxicity compared with commercial PEI (PEI25K) in 293T cells. Furthermore, they also investigated the brush-type degradable PEI-based gene carriers. Their results also showed that the advantages of their biodegradability rendered them ideal candidates as highly efficient gene carriers because they also displayed high transfection efficiency with low cytotoxicity.

2.5 Cell penetrating peptides (CPPs)

Modifications of PEI-based gene carriers with functional ligands such as targeting moieties are another promising strategy to improve the transfection efficiency. The cell penetrating peptide (CPP) is a peptide that can translocate macromolecules through the cell membrane, and it can facilitate the cellular uptake of endocytosis[81]. Table 2.3 lists some of the well-studied CPPs, including HIV-1 Trans-Activator of Transcription (HIV-1 TAT)[82], TransportanTP10[83], Octaarginine (R8)[84, 85] and others. It has demonstrated that the optimal number of residues is around seven to nine for an effective CPP[86]. If the number is more or less than that optimal number, the cellular uptake efficiency significantly reduces[87]. One unknown area of CPPs is the mechanism of the cell interaction with and entry into the cell membrane[92]. Some researchers claimed that the cellular uptake ability of CPPs was related to the negatively charged serum protein such as albumin which helped its interaction with cell membrane[93]. In addition to binding serum proteins, the negatively charged surface of carbohydrates like heparin sulfate proteoglycans (HSPGs) may also be involved in the transport of CPPs through the cell membrane.

Table 2.3 CPPs and their sequence

	Peptide	Sequence	Ref.
1	TP10	AGYLLGKINLKALAALAKKIL	[83]
2	Transportan	GWTLNSAGYLLGKINLKALAALAKKIL	[88]
3	pVec	LLIILRRRIRKQAHAHSK	[89]
4	Penetratin	RQIKIWFQNRRMKWKK	[90]
5	R8	RRRRRRRR	[84, 85]
6	HIV-Tat	YGRKKRRQRRR	[82]
7	KALA	WEAKLAKALAKALAKHLAKALAKALKACEA	[91]

Kogure et al. modified a multifunctional envelope-type nano device (MEND) by using octaarginine (R8) and dioleoylphosphatidylethanolamine (DOPE) in order to enhance the release from the endosome/lysosome[94]. In this research, R8 peptide was used as the positive charged CPP to help the cell uptake of gene through the cell membrane. DOPE was used as a fusogenic lipid to assist the endosomal/lysosomal escape. The DOPE/R8 modified PEI carrier later became popular in the delivery of not only the plasmid DNA[95, 96], but also the siRNA[97] and proteins[98] *in vivo* and *in vitro*.

In addition, Myrberg et al. synthesized a carrier for an anticancer drug, chlorambucil, by conjugating the targeting peptide (PEGA peptide) with a CPP (pVEG) for the application of breast tumor treatment[99]. The results showed that PEGA-pVEG efficiently delivered chlorambucil to the breast tumor cells. In the *in vivo* fluorescence study (as shown in Figure 2.8), fluorescence labelled PEGA-pVEG was found in the vessels of breast tumors. It was reported that little accumulation was also found in non-tumor tissues. These results confirmed
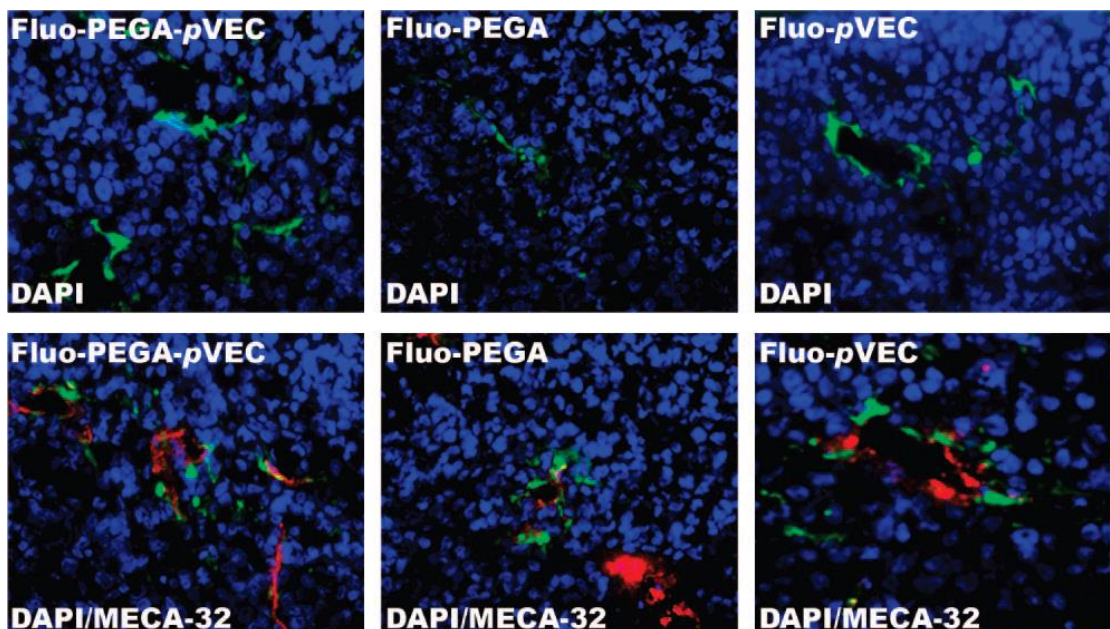
Figure 2.8 Localization of fluoresceinyl peptides in MDA-MB-435 tumors Fluoresceinyl peptides (green) and blood vessels stained with anti-MECA-32 antibody (red) in tumor tissue. Nuclei were detected by DAPI staining (blue).[99]

the excellent performance for the targeted delivery by CPPs. However, because of the high cell penetrating ability of CPP, the conjugation between targeting ligands and CPP may lead to the loss of the specificity of targeting ligands[100].

2.5.1 HIV-1 Trans-Activator of Transcription (TAT) peptide and its applications

Among these CPPs, HIV-1 Trans-Activator of Transcription (TAT) peptide (Tyr-Gly-Arg-Lys-Lys-Arg-Arg-GLn-Arg-Arg-Arg) becomes an attractive because of its ability to facilitate the cellular uptake of different molecules and its nuclear targeting ability[101, 102]. The possible reason for this is the electrostatic traps for the cationic variants and its order of amino acids in HIV-TAT peptide. It has already been demonstrated by applying the TAT peptide in the Chinese hamster ovary (CHO) cells with no galactosyltransferase activity[82]. The TAT peptide has been reported to facilitate the transporting of different materials including nucleic acids, proteins, polymers and other large molecules through the cells as listed in Table 2.4 due to its capacity of cell penetration and nuclear transportation. For example, anticancer drugs, toxins

Table 2.4 List of molecules conjugated with TAT for efficient intracellular uptake

No.	Molecules	Ref.
1	Heterologous proteins	103
2	magnetic mesoporous silica nanoparticles	104
3	Paramagnetic labels	105
4	Technetium and rhenium	106
5	Gold nanoparticles	107
6	Pharmaceutical nanocarriers	108, 109
7	Liposomes	110

and siRNA, have been delivered with the aid of TAT to tumor cells leading to the apoptosis and the breaking of the tumor cells in the last decades. Lee et al. applied the TAT peptide onto chitosan to improve the delivery of the hydrophobic anti-cancer drug, doxorubicin (DOX)[109]. The anticancer effect was improved and more efficient cell internalization was observed for DOX-TAT than free DOX. Significant inhibition of tumor growth in the mice containing CT26 xenograft was also demonstrated using the same chitosan-DOX-TAT complex. Li et al. reported an efficient drug delivery system by conjugating TAT with magnetic mesoporous silica nanoparticles (FMSN)[104]. DNA-toxin anticancer drugs were delivered with the aid of FMSN-TAT *in vitro* and *in vivo*. The delivery efficiency of FMSN-TAT was greatly enhanced.

In addition to the modifications of drug carriers, the TAT peptide was also used to facilitate the cellular uptake of polymer-based gene carriers. Torchilin et al. reported a gene delivery carrier synthesized by the conjugation of liposome with the TAT peptide. The resulting polymer significantly improved the intracellular delivery to a variety of cell lines including H9C2 and BT20 cells[110]. Compared with commercial Lipotectin®, the TAT-liposome/pDNA complex showed a significantly higher gene transfection efficiency but lower cytotoxicity in both NIH 3T3 and H9C2 cells[111].

2.6 Summary

Gene therapy is a promising technology to cure diseases including cancers and genetic diseases in the modern society. However, lack of high-performance gene carriers slows down its development. Although viral gene vectors show high transfection efficiency, its high risk of immunological issues is the main challenge to be applied in clinical settings. Synthetic cationic polymer-based non-viral gene carriers have great potential, however, several factors such as efficiency and biocompatibility still need to be improved.

In this chapter, the mechanism for the gene delivery process was illustrated to help understand how the external gene is uptaken by cells and transports inside the cytoplasm. The barriers of the gene delivery process were critically analysed and the considerations for design of ideal gene carriers were also listed. Cellular uptake and endosomal escape of gene carriers are identified to be two major factors to influence the performance of gene transfection in cells.

Advancements in PEI-based gene carriers are summarised. To develop biodegradable PEI-based gene carriers is a promising strategy to decrease the cytotoxicity of carriers and maintain the high transfection efficiency. HIV-1 TAT peptide is able to improve the delivery efficiency of biomolecules due to its cell membrane penetrating and nuclear transporting ability. The HIV-1 TAT peptide can be conjugated with the synthetic biodegradable gene carriers to overcome the challenging issues of low cellular uptake leading to low transfection efficiency.

Inspired by these research findings, biodegradable PEI-based gene delivery system will be designed in Chapter 3. Modifications of biodegradable PEI with HIV-1 TAT peptide to improve its transfection efficiency will be conducted in Chapter 4.

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Chapter 3 Disulfide Cross-linked Cationic Nanogels as Robust Gene carriers

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Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

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Disulfide Cross-linked Cationic Nanogels as Robust Gene carriers

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Abstract

Gene therapy development is greatly impeded by the availability of highly efficient and safe gene delivery system. In this study, we developed a biodegradable gene delivery carrier, PEI-DA, by crosslinking PEI with a molecular weight of 0.8 KDa (PEI800) with 4'4-dithiodibutyric acid (DA). The synthesized PEI-DA is able to condense plasmid DNA (pDNA) into complexes under 200 nm in a neutral condition. The PEI-DA has demonstrated to display negligible cytotoxicity to both Hela and HEK 293 cells. In addition, PEI-DA assisted pDNA transfected to both Hela and HEK 293 cells more efficiently than PEI with a molecular weight of 25 KDa, a commercial reagent used for gene transfection. Therefore, the PEI-DA could be a promising gene delivery carrier in cancer gene therapy based on its low cytotoxicity and high transfection efficiency.

Key words: gene delivery, PEI, biodegradable, disulfide

3.1 Introduction

Gene therapy has drawn great attention due to its promising applications in the treatments of several different diseases, including cancers[1, 2], vascular diseases[3] and others. However, the progress of gene therapy is impeded by the development of high efficient and biocompatible gene carriers. Due to immunological and genetic issues resulted from viral gene carriers[4], researchers have made extensive efforts into development of the non-viral gene carriers. Cationic polymers, as one type of non-viral gene carriers, have been considered as potential candidates of gene carriers owing to its non-immune response, high genetic material loading capability and its high transfection efficiency.

Polyethylenimine (PEI), known for its 'proton sponge' effect, has been widely investigated for its high gene transfection ability in delivering genetic materials both *in vitro* and *in vivo*[5]. Nonetheless, its application in gene therapy is limited by its high cytotoxicity, which may be due to its non-biodegradability and its high cationic density. Low molecular weight PEI (LMW PEI) showed low cytotoxicity to cells, its ability to transfer genetic materials into cells was also low[6]. Various modifications have been conducted to low molecular weight PEI (LMW PEI) to develop a satisfied gene carrier.

Considering the dependence of transfection efficiency and cell viability on the molecular weight of the PEI, to cross-link LMW PEI with biodegradable linker to form high molecular weight gene carrier could be a good strategy to overcome the high cytotoxicity limiting the development of PEI based gene carrier. Different linkers have been reported to react with LMW PEI to form the gene carriers including ester linkages[7, 8], disulfide linkages[9, 10] and others[11, 12]. Most of the gene carriers prepared with this strategy focused on two major issues. The first one is the formation and the dissociation of gene carrier/gene complexes in extracellular and intracellular environment. And another concern is how to perform high efficient gene transfection with low cytotoxicity[13]. For example, Forrest et al. synthesized the

ester-cross-linked PEI based polymers by crosslinking PEI800 with 1,3-butanediol diacrylate and 1,6-hexanediol diacrylate linked by ester [7]. The gene transfection efficiency of resulted polymers is 2 to 16 fold higher than that of PEI25K. Moreover, the resulted polymers achieve high cell viability in MDA-MB-231 cells and C2C12cells.

Among these biodegradable linkages, disulfide bonds has attracted great attention because it can be cleaved under high concentration of glutathione in the cytosol or the nucleus, and it can be triggered after the escape from the endosomal/lysosomal compartment[14, 15]. Gosselin et al. firstly reported the disulfide bond containing biodegradable gene carrier by the reaction of PEI800 with dithiobis(succinimidy lpropionate) (DSP) and dimethyl·3,3'-dithiobispropionimidate (DTBP). It has been proven that the cytotoxicity of synthetic polymer was reduced significantly compared with commercial PEI25K[16]. However, the transfection efficiency of synthetic polymer is not superior to PEI25K. Recently, Liu et al. synthesized a biodegradable PEI based gene carrier by click chemistry through the reaction of azide-terminated LMW PEI (PEI 1800) with a disulfide containing dialkyne[17]. The transfection efficiency of product was tested with and without serum. The results indicate that both with and without serum the degradable PEI based gene carrier showed low cytotoxicity and comparable transfection efficiency with commercial PEI25K in 293T cells.

Although it has been proven that PEI-based gene carriers through crosslinking LMW PEI with disulfide bonds are able to reduce the cytotoxicity of the synthetic polymers, no significant improvement of the transfection efficiency has been reported compared with non-degradable PEI25K. In this study, 4'4-dithiodibutyric acid (DA), a novel disulfide bond containing biodegradable linker, was firstly applied here to crosslink PEI800. The resulted polymer was characterized regarding the buffering capacity, the physio-chemical properties, and gene binding ability. The gene transfection performance and the cytotoxicity of the

synthetic polymer and commercial PEI25K were evaluated and compared in Hela cells and HEK 293 cells.

3.2. Materials and methods

3.2.1 Materials

PEI800 and PEI25K (MW = 0.8 KDa and 25 KDa), EDC (1-ethyl-3-(3-dimethy-laminopropyl) carbodiimide) and NHS (N-hydroxysulfosuccinimide), GelRed™ and other chemicals/solvents were purchased from Sigma-Aldrich (St.Louis, MO). The QIAGEN Maxi kit was obtained from Qiagen (Boncaster, Australia). The plasma membrane and nuclear labeling kit, nucleic acid stains dimer sampler, fetal bovine serum (FBS), trypsin–EDTA, penicillin–streptomycin (PS) mixture, 3-(4,5-dimethy lthiazol-2-yl)-2,5-diphenyltetrazolium bro-mide (MTT), kanamycin, phosphate buffered saline (PBS) and TAE (Tris–acetate) were purchased from Life Technologies (Mulgrave, Australia).

3.2.2 Methods

3.2.2.1 Preparation of plasmid DNA

The pEGFP-N1 plasmid expressing the enhanced green fluorescent protein (EGFP) was prepared by *Escherichia coli* DH5α strain and extracted using a QIAGEN Midi kit. The integrity and purity of plasmid DNA (pDNA) was analyzed using 1.0 % agarose gel electrophoresis and the DNA concentration was determined using a Jasco UV−vis spectrophotometer (Tokyo, Japan) at the fixed wavelength of 260 nm. The pDNA was further labelled by fluorescent dye (YOYO-1) at a ratio of 1 molecular dye to 100 molar nucleic acid base pairs for the cellular uptake study[18].

3.2.2.2 Synthesis of PEI-DA

The synthesis details of the PEI-DA are described in Scheme 1. PEI800 was reacted with DA using carbodiimide chemistry. To synthesize PEI-DA, NHS/EDC (86.32/143.78 mg, 0.75 mmol) and 4'4-dithiodibutyric acid (89.37 mg, 0.375 mmol) were added to 6 ml DMSO at room temperature and the mixture was stirred for 1 h. 1 ml PEI800 (100 mg/ml) was then added into the mixture. The reaction mixture was stirred at room temperature for a further 16 h. The pH of the resulted reaction mixture was adjusted to 7 by NaOH solution (0.1 M) or HCl solution (0.1 M). The synthetic product was purified by dialysis membrane tubing with a molecular cut off 12-14K Da against deionized water (pH 7) for 3 days. The obtained PEI-DA was lyophilized and further characterized.

3.2.2.3 Characterization of PEI-DA

The Fourier transform infrared spectrometer (FTIR) spectra of PEI800, 4'4-dithiodibutyric acid and PEI-DA were examined using a Thermo NICOLET 6700 Fourier transform infrared spectrometer at room temperature. ^{1}H-NMR experiments were recorded using a 600 MHz Bruker NMR in D_2O.

3.2.2.4 Buffering capacity

The buffering capacity was measured by acid–base titration. Briefly, 50 ml polymer solution (0.05 mg/ml) containing PEI800, PEI 25K and PEI-DA respectively was adjusted to pH 3 with 0.1 M HCl and then 0.1 M NaOH was gradually added until the solution pH reached 11. After the each addition of 0.1M NaOH, the pH was measured by a microprocessor pH meter. 150 mM NaCl was titrated as a control[19].

3.2.2.5 Gene condensing ability of PEI-DA

Different polymer/pDNA complexes were prepared by mixing PEI800, PEI25K and PEI-DA with 1 μg of pDNA (0.2 μg/ml) at various N/P ratios ranging from 0.5 to 50, and then the complexes were diluted to 6 μl and incubated at room temperature for 30 min. Naked pDNA was tested as control. Subsequently, the samples were loaded onto the 0.8 wt % agarose gel containing GelRed™ with Tris-acetate (TAE) running buffer at 80 V for 60 min. The resulted pDNA migration patterns were read under UV irradiation (G-BOX, SYNGENE).

3.2.2.6 Particle size and zeta potential

To measure the apparent particle sizes and zeta potentials, polymer/pDNA complexes were prepared at different N/P ratios from 0.5 to 50 in water. The volume of the samples was 3 ml containing a final pDNA concentration of 5 μg/ml. Then the apparent particle sizes and zeta potentials were examined by Malvern Nano-ZS 90 laser particle size analyzer equipped with ZET5104 cell at room temperature[20]. For particle size analysis, the cumulate method was used to convert intensity–intensity autocorrelation functions to apparent particle sizes according to the Stokes–Einstein relationship[21]. The Smuloschowski model was used to convert electrophoresis mobility to zeta potential. 15 parallel runs were carried out for each measurement and the final data were obtained based on statistical analysis.

3.2.2.7 Cell cytotoxicity

HeLa and HEK 293 cells were cultured in DMEM medium supplemented with 10 % fetal bovine serum (FBS) in 96-well plates (200 μl/well) at a cell density of 1.0×10^5 cells/ml. The cells were allowed to adhere overnight at 37 ℃ in a humidified 5 % CO_2-containing atmosphere. The growth medium was replaced with 200μl fresh medium containing testing carriers including PEI800, PEI25K and PEI-DA at final concentrations of 0.5, 1, 5, 10 and 50 μg/ml. Cells were then incubated for 24 h before 10 μl of MTT (5.0 mg/ml in PBS) was

added to each well to measure the cell viability [22]. After incubating for another 4 h at 37 ℃, the growth medium was replaced by 150 µl of dimethyl sulfoxide (DMSO) to ensure complete solubilization of the formed formazan crystals. Finally, the absorbance of the solution at each well was read using the Biotek Microplate Reader (Biotek, USA) at a wavelength of 595 nm[23].

3.2.2.8 Cellular uptake by confocal laser scanning microscopy (CLSM)

HeLa cells at a concentration of 2×10^5 cells/well were cultured in 6-well plates loaded with cover-glass slides for 24 h. 4 µg YOYO-1 labelled pDNA, which was prepared according to the instruction from Life Technologies, was loaded onto different gene carriers (naked DNA, PEI800 PEI25K and PEI-DA) at an N/P ratio of 10 to form polymer/pDNA complexes. Cells were incubated with polymer/pDNA complexes for another 4 h and then the complexes were removed by washing the cells with PBS three times before fixing with 4 % formaldehyde. The cell membrane and nucleus were separately stained by 100 µl of Alexa Fluor 594 (5 µg/ml) and Hoechst 33258 (2 µM) for 15 min at 37 ℃. The cells were further washed with PBS three times and incubated with 1 ml PBS, and kept at room temperature for further analysis. The fluorescence images were observed from a confocal laser scanning microscope (Leica Confocal 1P/FCS) equipped with a 405 nm diodelaser for Hoechst33258, a 488 nm argon ion laser for YOYO-1 and a 561 nm diode laser for Alexa Fluor 594. The high magnification images were obtained with a 63x objective. Optical sections were averaged 8 times, and all images were processed using the Leica Confocal software[23].

3.2.2.9 Gene transfection

HeLa and HEK 293 cells were seeded in 24-well plates and cultured in complete DMEM supplemented with 10 % fetal bovine serum (FBS) at 37 ℃ in a humidified 5 % CO_2 incubator. After 24 h incubation, when the confluent percentage of the cell culture reached 80

%, the medium was replaced with 200 μl fresh culture medium in the absence of FBS. Meanwhile, the polymer/pDNA complexes prepared by incubating PEI800, PEI25K and PEI-DA with pDNA at an N/P ratio of 10 at room temperature for 30 min, were added to each well. After another 6 h incubation, the culture medium was replaced by 1 ml fresh complete cell culture medium with 10 % FBS and the cells were further incubated for another 42 h[23].

3.2.2.10 Green fluorescent protein (GFP) expression measurement by flowcytometry

The extent of GFP expression was evaluated by flowcytometry. The green fluorescence intensity was detected directly by a FACSCalibur flowcytometer (Becton Dickinson), and the transfection efficiency was calculated by the percentage of positive cells, using non-transfection cells (mock cells) as the negative control. Briefly, after transfecting pEGFP-N1 in cells by different carriers (naked pDNA, PEI800, PEI25K and PEI-DA), Hela cells and HEK 293 cells were harvested after trypsin digestion after 48 h post-transfection. Cells were washed with PBS buffer, and centrifuged at 1000 rpm for 5 min. The cells were stained by propidium iodide (400 μl, 0.5 μg/ml) in 1x PBS. Approximately 1 to 2×10^4 cells were analyzed at the rate of 200-500 cells per second. CellQuest3.3 software was used for data analysis[24].

3.2.2.11 Statistical analysis

Data obtained from our experiments were represented as mean ± SE (standard error). Statistical analysis of the experimental data was performed using a two-sample, two-tailed t-test. $p < 0.05$ is considered to be significant[25].

3.3 Results and discussion

3.3.1 Synthesis and characterization of PEI-DA

Comparing with PEI25K, low molecular weight PEI (LMW PEI) such as PEI800 is less toxic to cells. However, the transfection efficiency of LMW PEI is also low. In order to improve the transfection efficiency of LMW PEI, disulfide bonds were introduced to LMW PEI by the reaction of DA with PEI800 using an EDC/NHS coupling system. Carboxyl groups on DA were reacted with amino groups on PEI800. The feed ratio of the carboxyl groups on DA to the amino groups on PEI800 was controlled at 1:10 to optimise the formation of cross-linked PEI800 network. As shown in Figure 3.1, the FTIR spectrum of PEI-DA is compared with PEI800 and DA to confirm the successful synthesis of PEI-DA. PEI800 shows the characteristic peaks at 1664 cm^{-1} and 1041 cm^{-1}, indicating the NH_2 vibration and the C-N stretching respectively. For the spectrum of DA, the specific signal of 890 cm^{-1} is contributed by the disulfide bonds. The appearance of the signal at 1639 cm^{-1} and 1544 cm^{-1} in the spectrum of PEI-DA are ascribed to C=O stretching (amide I band) and N-H deformation (amide II band). This confirms the formation of amide bond by the reaction between the carboxyl groups on DA and the amino groups on PEI800. Meanwhile, the signal at 879 cm^{-1} in the PEI-DA is the evidence of the successful introduction of the disulfide bond after the reaction[15]. In addition, ^{1}H-NMR was used for a further confirmation of the chemical structure of PEI-DA. As shown in Figure 3.2, the chemical shifts located within the range of 2.4 to 2.6 ppm (close to the linker) and 3.3 to 3.6 ppm (far from linker) represent the H-atom of PEI800, while the presence of the chemical shift signal at δ 2.0, 2.8 and 3.6 ppm confirms the introduction of DA.

3.3.2 Buffer capacity

Buffering capacity of gene carriers is normally considered to play an important role in the cellular uptake and the endosomal escape. It is one key parameter to affect the transfection efficiency in gene delivery[26, 27]. The buffering capacity of cationic polymers is mainly contributed by the density of the positive charges on the polymers[28]. Hence, the investigation of positive charge density is crucial to develop good gene carriers. The buffering capacity of PEI-DA was measured by back titration from pH 3 to 11. As shown in Figure 3.3, PEI-DA shows better buffering capacity than PEI800. In addition, the buffering capacity of PEI-DA in the range of pH from 5-7 corresponding to the pH change in early endosome is similar to that of PEI25K. Our study demonstrates that the crosslinking of PEI800 by DA increases the positive charge density of the polymer leading to the improvement of the buffering capacity. Therefore, the good buffering capacity of PEI-DA may promote endosomal escape of PEI-DA/gene complexes and eventually lead to highly efficient gene transfection[28].

3.3.3 Gene binding ability of PEI-DA

Gene binding ability is the prerequisite for efficient gene delivery. It is recognized that positive charge density is one key factor to influence the formation of the polymer/pDNA complex[29]. The gene binding ability of PEI800, PEI25K and PEI-DA were performed at different N/P ratios by gel electrophoresis using naked pDNA as a negative control. As shown in Figure 3.4, the amount of free pDNA molecule migrating in the gel decreases with an increase of the N/P ratio of PEI-DA to pDNA, and pDNA can be completely retarded at the N/P ratio between 1 and 5. PEI25K shows a significantly strong gene binding ability that pDNA can be completely condensed at a low N/P ratio of 0.5. PEI800 shows a weaker ability to condense pDNA. The better gene binding ability of PEI-DA compared with PEI800 is contributed by the higher positive charge density after crosslinking by DA. The gene binding

ability of PEI-DA is comparable with other reported biodegradable PEI-based gene carriers. For example, the disulfide cross-linked PEI (PEI-SS$_x$) retarded pDNA migration at an N/P ratio of 2[14].

3.3.4 Particle size and zeta potential

The entry of incompatible molecules into cells depends on the size of polymers and the elasticity of cell membrane. Particles in different sizes penetrate cell membrane via various pathways, including endosome-mediated endocytosis; clathrin-mediated endocytosis; caveolae-mediated endocytosis and micropinocytosis[30]. A well-controlled particle size of polymer/gene complex is essential for gene delivery. It is reported that the optimal size for gene delivery to cells ranges from 100 to 200 nm[31]. PEI-DA/pDNA complexes samples were prepared by mixing PEI-DA with pDNA at various N/P ratios from 0.5 to 50. The particle sizes of PEI-DA/pDNA complexes were measured by dynamic light scattering (DLS). As shown in Figure 3.5a, the mean particle size of PEI-DA/pDNA complexes decrease from 520 nm to 105 nm with an increase of the N/P ratios from 0.5 to 50 and similar results were achieved with the complexes prepared by commercial PEI25K and pDNA. However, the condensation of pDNA by PEI800 does not lead to small particles. The mean particle size of PEI800/pDNA complexes at the N/P ratio of 50 is still at around 400 nm, which means that PEI800 is not suitable for efficient gene delivery. After crosslinking PEI800 with DA, the gene condensation ability of PEI-DA is improved. Although the particle sizes of PEI-DA/pDNA complexes are slightly larger than those of PEI25K/pDNA complexes, it is still in the range for efficient entry into cells. The particle size results of PEI-DA/pDNA is even better than some other reported biodegradable PEI-based gene carriers, such as the disulfide cross-linked PEI (PEI-SS$_x$) with the particle size around 400 nm[14].

Zeta potential is another crucial factor to affect the cellular uptake and transfection efficiency of gene carriers. It is believed that particles with a positively charged surface could implement a better translocation through cell membrane due to the interaction with the negatively charged proteoglycans on the surface of cells[32]. The zeta potentials of PEI-DA/pDNA complexes prepared at pH 7 at different N/P ratios were evaluated. The formation of the complexes by pDNA and cationic polymers is dominated by the electrostatic interaction. Therefore, the zeta potential results of the polymer/pDNA complexes can further confirm the gene binding ability of the polymers. As shown in Figure 3.5b, the mean zeta potential of PEI-DA/pDNA complexes is negative (-15.02 mV) at the initial N/P ratio of 0.5, and then increases with an increase of the N/P ratio from -1.64 mV (at the N/P ratio of 1) to 20.21 mV (at the N/P ratio of 50). This result confirms the gel electrophoresis result of PEI-DA that PEI-DA is able to fully retarded pDNA at the N/P ratio between 1 and 5. As comparison, the zeta potentials of PEI25K/pDNA complexes always remain positive from the N/P ratio of 0.5 due to its high positive charge density.

The results of particle sizes and zeta potentials of PEI-DA/pDNA complexes demonstrates that after crosslinking PEI800 with DA, the gene binding ability is improved due to the increase of the positive charge density on the polymers as more PEI800 has been packed in the synthesized polymer.

3.3.5 Cell viability of PEI-DA

The main concern for applications of PEI-based gene carriers is the high cytotoxicity. To evaluate the cell viability in the presence of PEI-DA, the 3-(4,5-dimethy lthiazol-2-yl)-2,5-diphenyltetrazoliumbro-mide (MTT) assay was conducted. Hela and HEK 293 cells were cultured with a range of PEI-DA concentrations from 0.5 to 50 ug/ml. The cytotoxicity of PEI25K and PEI800 were also measured as references. Figure 3.6 illustrates the cell viability

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results after exposure to PEI25K, PEI800 and PEI-DA for Hela cells (a) and HEK 293 cells (b) after 24 h incubation. PEI-DA is found to have negligible cytotoxicity to either Hela cells or HEK 293 cells at the range of testing concentrations with the cell viability above 85 %. As comparison, PEI (PEI25K) shows a significant cytotoxicity against Hela cells and HEK 293 cells and the cell viabilities are lower than 30 % for both Hela and HEK 293 cells at the concentration of 50 µg/ml. Exposure to PEI800 results in high cell viability against both Hela and HEK 293 cells. Since disulfide bonds can be cleaved by glutathione within the reductive intracellular environment[15], PEI-DA can be degraded into PEI800 after transferring into cells, and the degradation products have low cytotoxicity as well. As comparison, the cell viability of the reported disulfide cross-linked PEI (PEI-SS$_x$) reduced to be lower than 20 % with the polymer concentration above 50 µg/ml[14]. In contrast, the cell viability in the presence of 50 µg/ml of PEI-DA still remains more than 80 %. The results demonstrate that crosslinking PEI800 with DA is a better strategy to reduce the cytotoxicity of PEI-based carriers.

3.3.6 Cellular uptake of PEI-DA/pDNA complex

In order to evaluate the cellular uptake performance of PEI-DA/pDNA complex, pDNA was labelled by YOYO-1 with green fluorescence. PEI-DA/pDNA complex was prepared at an N/P ratio of 10 and delivered into Hela cells, and naked DNA, PEI800 and PEI25K were also tested as references. The cell membrane and nuclei of cells were dyed with Alexa Fluor 594 in red fluorescence and Hoechst 33258 in blue fluorescence respectively. After 6 h transfection of pDNA in cells, images were taken by a confocal fluorescence microscopy. As shown in Figure 3.7, pDNA with the green fluorescence label complexed by PEI25K and PEI-DA can be seen in the cytoplasm and nucleus of Hela cells. However, it is observed that pDNA with green fluorescence signal is less when it is transferred by PEI800, and naked pDNA without carriers also performs poor cellular entry to Hela cells. It is clearly shown that the cellular uptake performance of PEI-DA is similar to that of commercial PEI25K and is much better

than that of PEI800. This demonstrates that the cellular uptake ability of PEI-DA improves after crosslinking PEI800 with DA due to the enhancement of the gene binding and condensing ability.

3.3.7 Cell transfection

The gene transfection efficiency of PEI-DA was quantified using flow cytometry based on the amount of expressed green fluorescent protein (GFP). The measurement was conducted through transfecting PEI-DA complexed pEGFP-N1 plasmid at an N/P ratio of 10 in Hela and HEK 293 cells. pEGFP-N1 plasmid delivered via PEI800 and PEI25K were used as references and naked pDNA without carriers was also employed as a control. As shown in Figure 3.8 and 3.9, the transfection efficiency of the PEI-DA/pDNA complex is comparable with that of the PEI25K/pDNA complex in Hela cells (26.77 % vs 23.34 %) as well as in HEK 293 cells (23.67 % vs 25.22 %). PEI800 shows quite low transfection efficiency (2.59 % in Hela cells and 1.53 % in HEK 293 cells).

It is demonstrated that after cross-linking low molecular weight PEI with DA, the synthetic PEI-DA is able to perform an efficient gene transfection. The high efficient can be resulting from: 1) the increased positive charge density leading to the improvement in gene binding and condensation; 2) the increased buffering capacity resulting in similar performance of the endosomal escape as PEI25K. As a comparison, the gene transfection efficiency of the reported disulfide cross-linked PEI (PEI-SS$_x$) is 2-3 times higher than that of PEI25K[14]. Although PEI-DA exhibits similar transfection efficiency as PEI25K, and its efficiency is lower than the PEI-SS$_x$, the cytotoxicity of PEI-DA is much lower.

3.4 Conclusions

In this study, a novel disulfide bond containing PEI based gene carrier, PEI-DA, was successfully synthesized and applied in gene delivery. PEI800 was reacted with DA to form

PEI-DA with disulfide bond. PEI-DA was characterized to confirm the success of synthesis. PEI-DA was then complexed with pDNA for further investigation. The particle size of PEI-DA/pDNA complexes is lower than 200 nm beyond the N/P ratio of 10, suggesting that PEI-DA is able to condense pDNA at a low N/P ratio of carrier/pDNA. DNA migration experiment demonstrates that PEI-DA has better gene binding and condensation ability than PEI800. It is also shown that the buffering capacity of PEI-DA is similar to that of PEI25K, and much higher than that of PEI800. PEI-DA performs a comparable gene transfection similar to PEI25K with the efficiency of 26.77 % in Hela cells and 23.67 % in HEK 293 cells. Both Hela and HEK293 cells have very high cell viability (above 85 %) in the presence of PEI-DA in the cell culture medium, which proves that PEI-DA is a non-toxic gene carrier. The high transfection efficiency and low cytotoxicity of PEI-DA indicate that PEI would be a promising candidature for efficient gene delivery in the applications of gene therapy. Future studies will focus on further improvement of the transfection efficiency of the biodegradable gene carrier by monitoring its degradation kinetics at different gene delivery stages including cellular uptake and endosomal escape.

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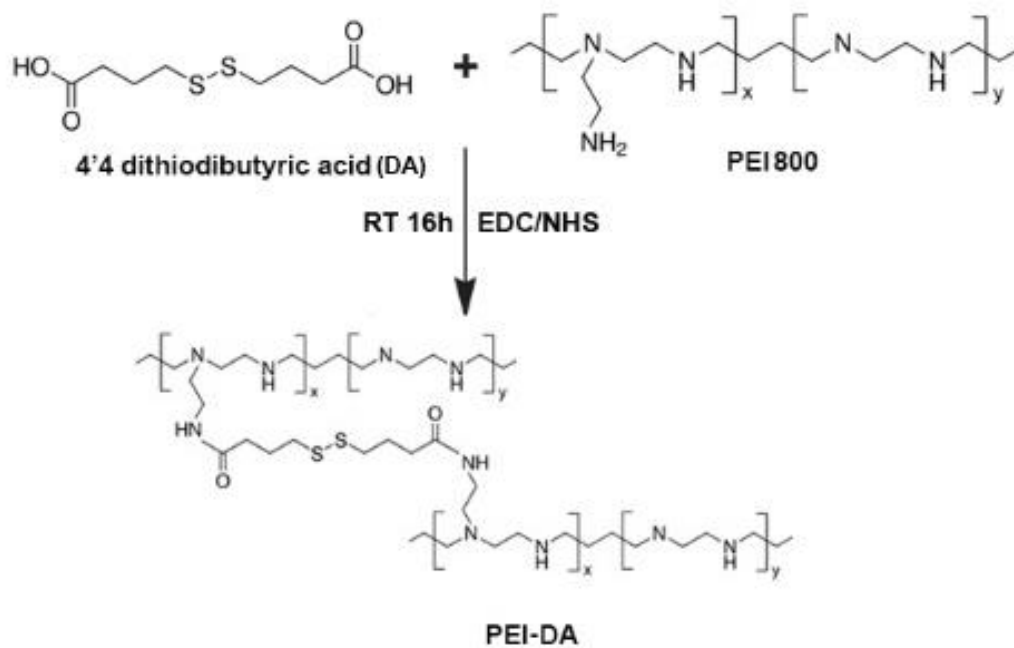
Scheme 3.1 Schematic description on the synthesis of PEI-DA
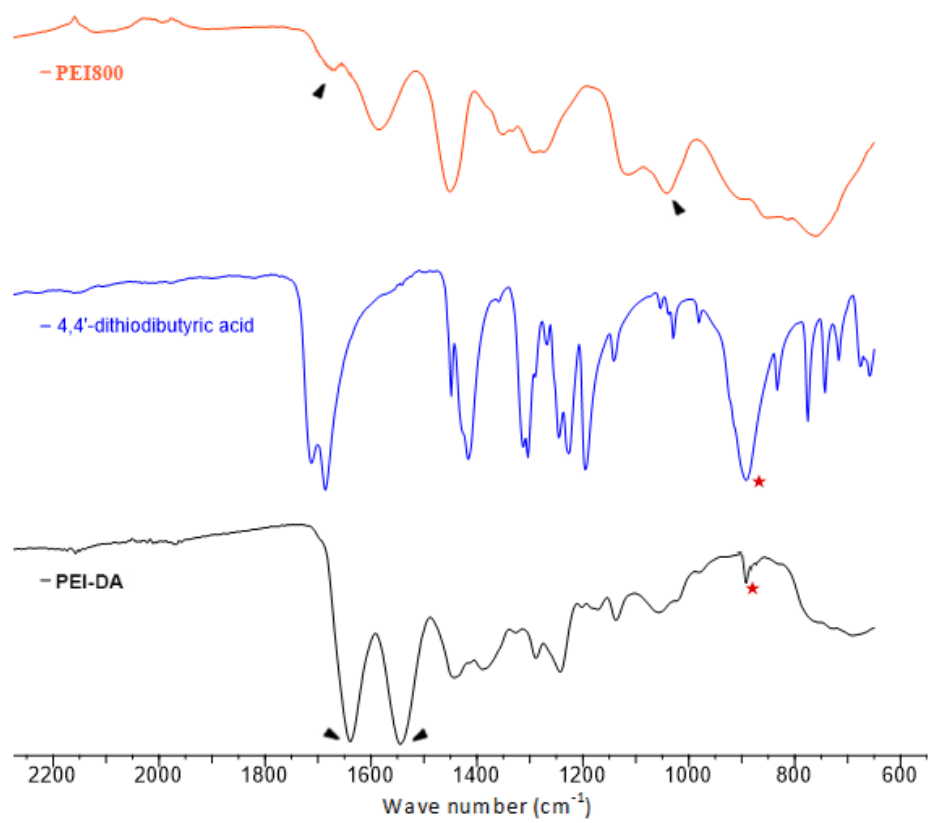
Figure 3.1 FTIR spectrometry of PEI800, 4'4-dithiodibutyric acid and PEI-DA
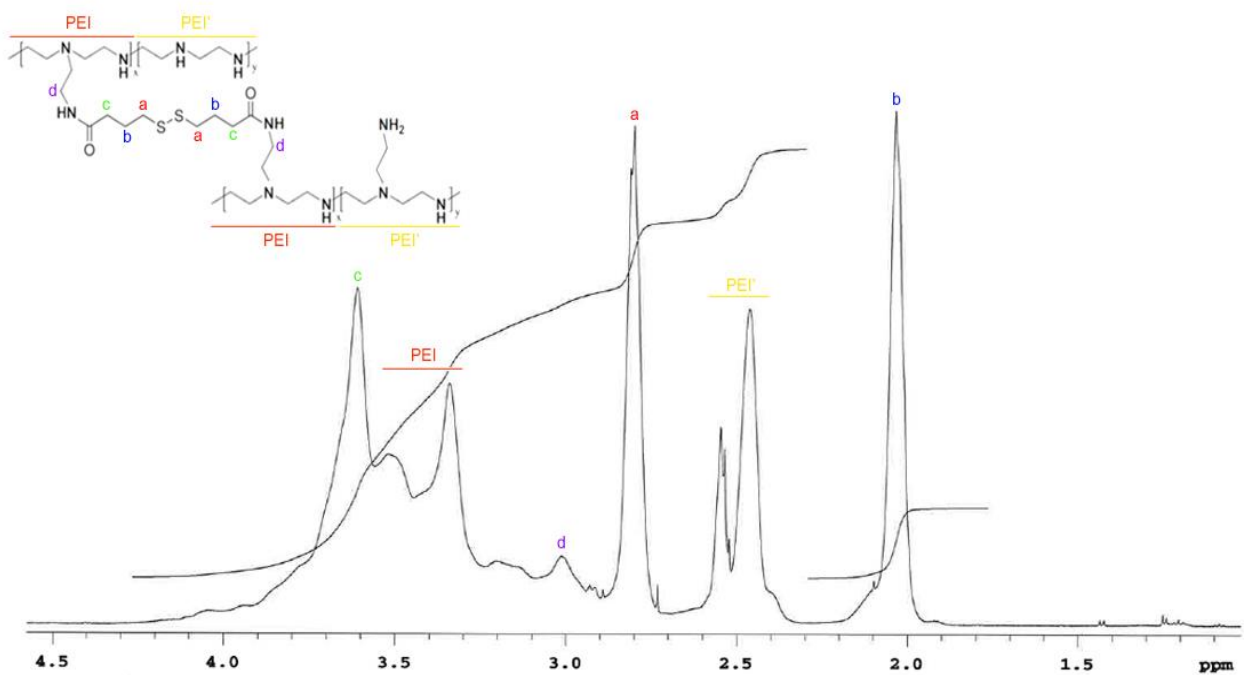
Figure 3.2 NMR spectrometry of PEI-DA
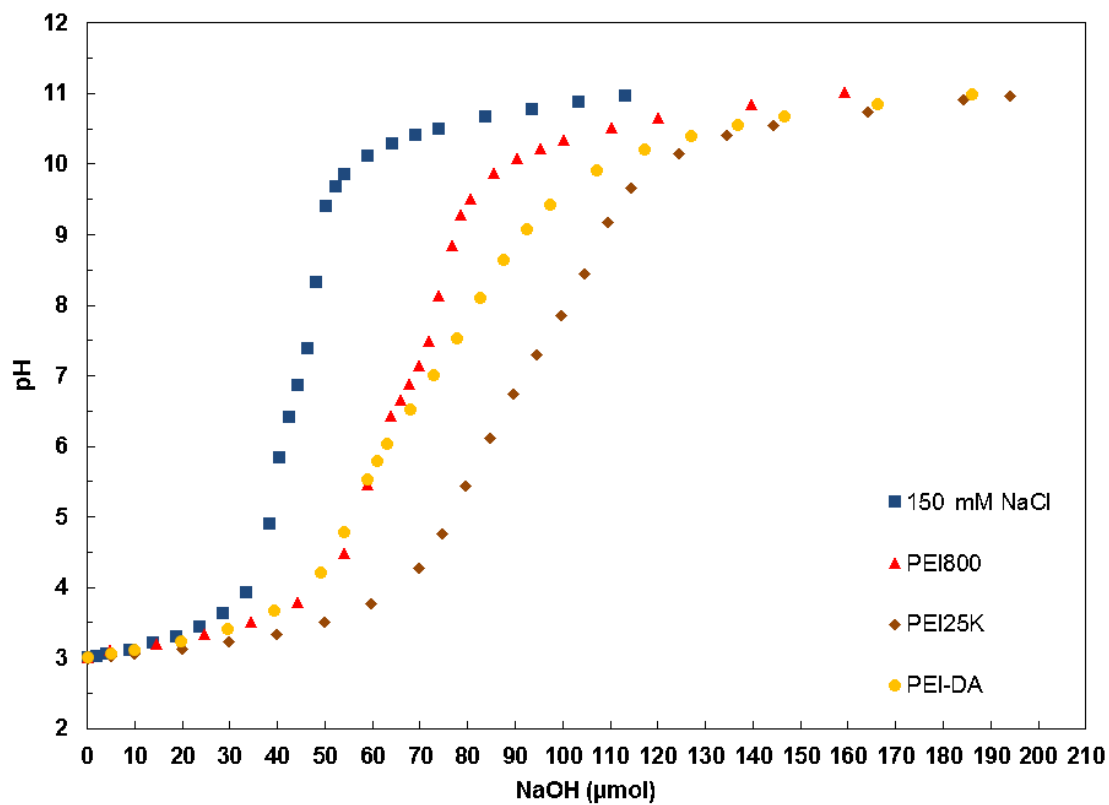
Figure 3.3 Buffer capacity of PEI800, PEI25K and PEI-DA solution at 25 ºC 1 atm. 150 mM NaCl was used as the control.
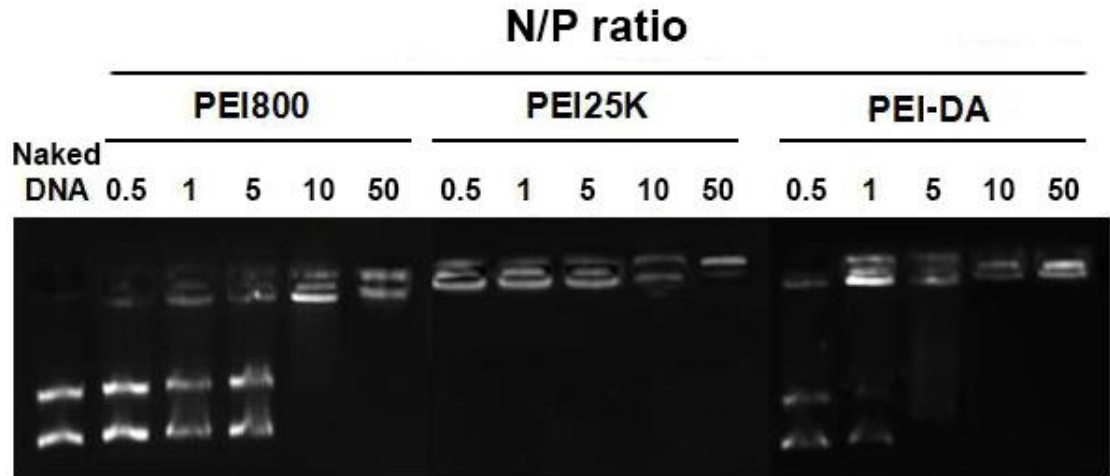
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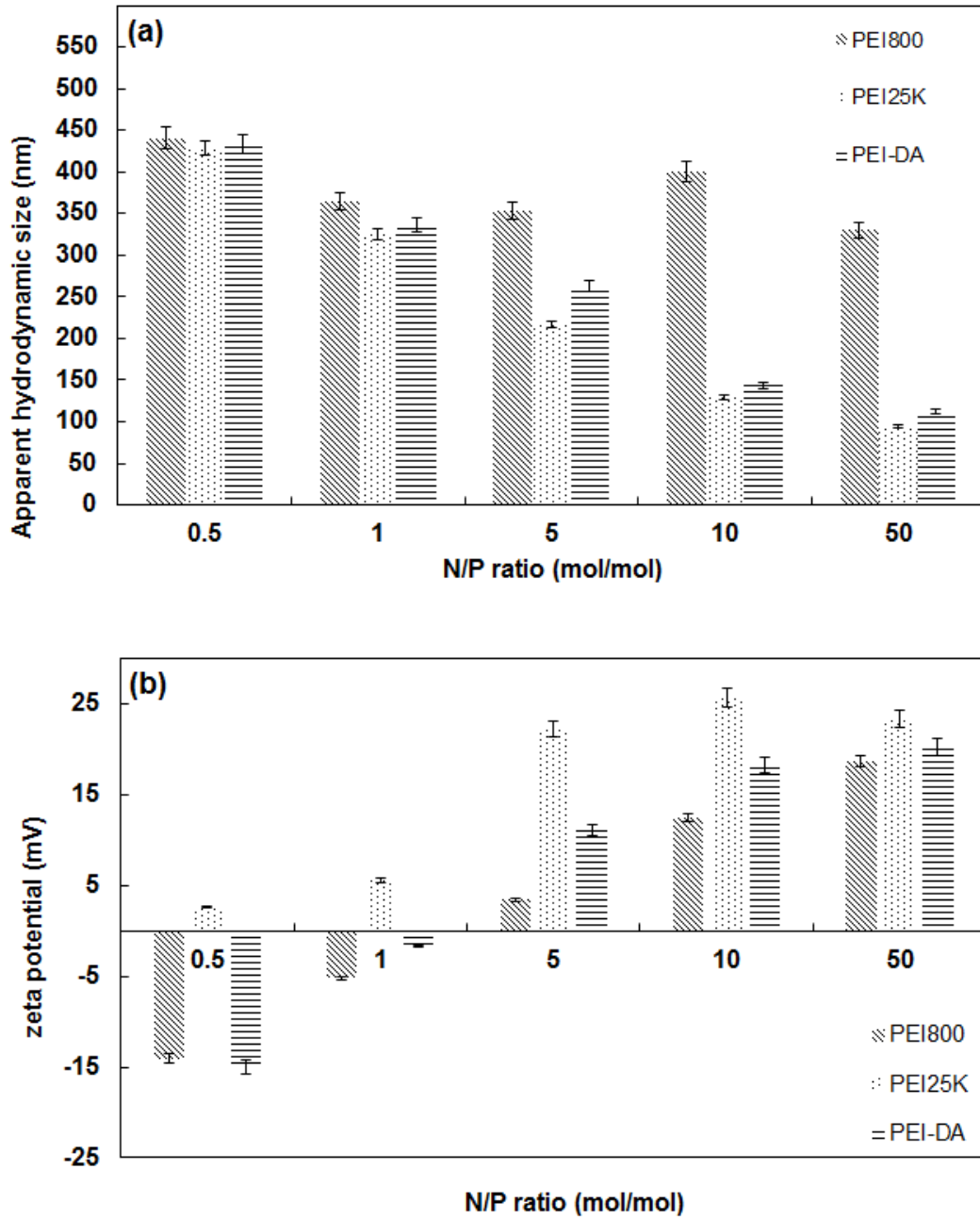
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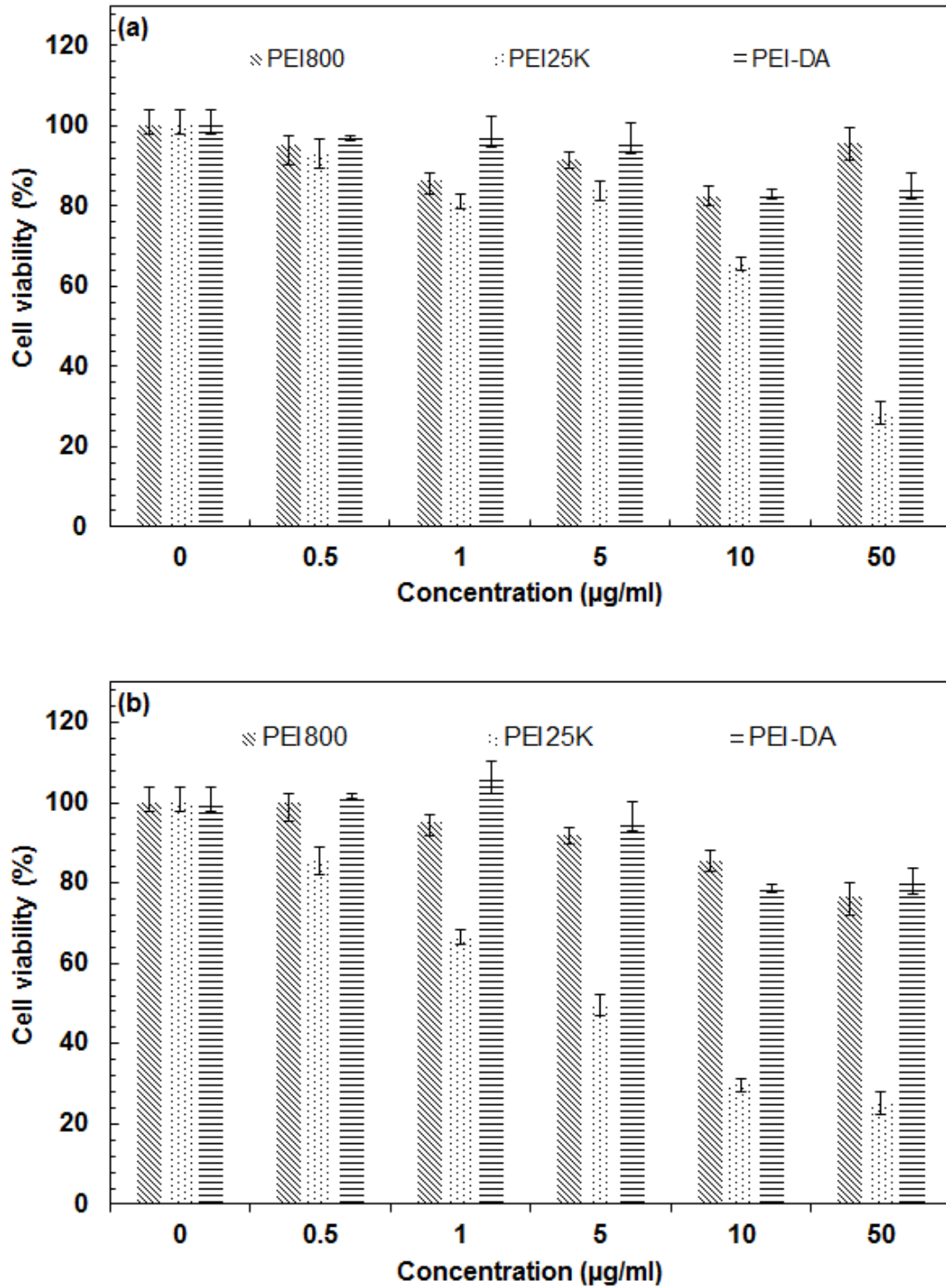
Figure 3.6 Cell viability of Hela cells (a) and HEK 293 cells (b) after exposing to PEI800, PEI25K and PEI-DA.
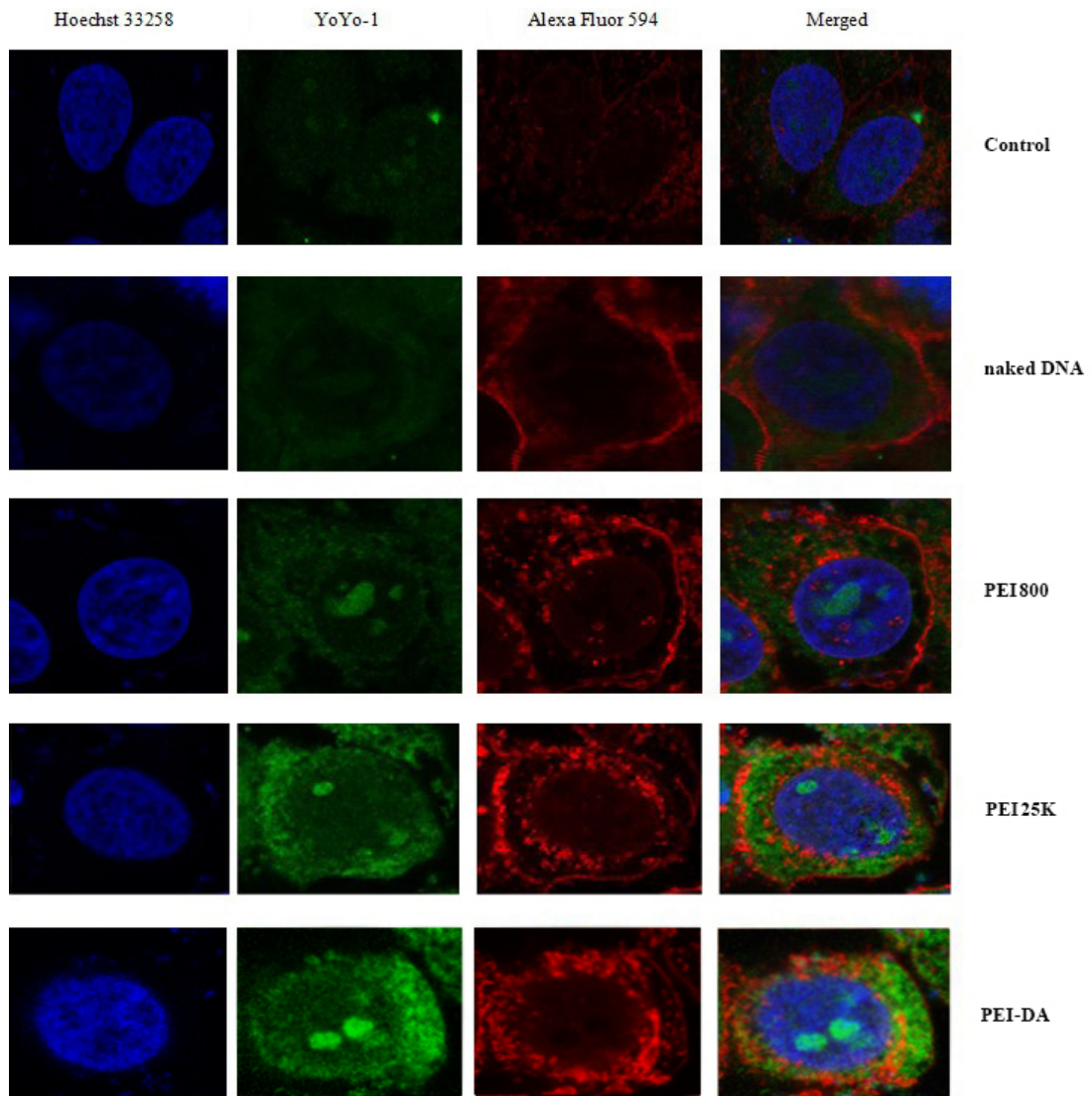
Figure 3.7 Hela cell uptake of YOYO-1-labelled pDNA complexed with different gene delivery carriers: PEI800, PEI25K and PEI-DA at an N/P ratio of 10. Mock cells were used as control, and naked pDNA was used as the reference.
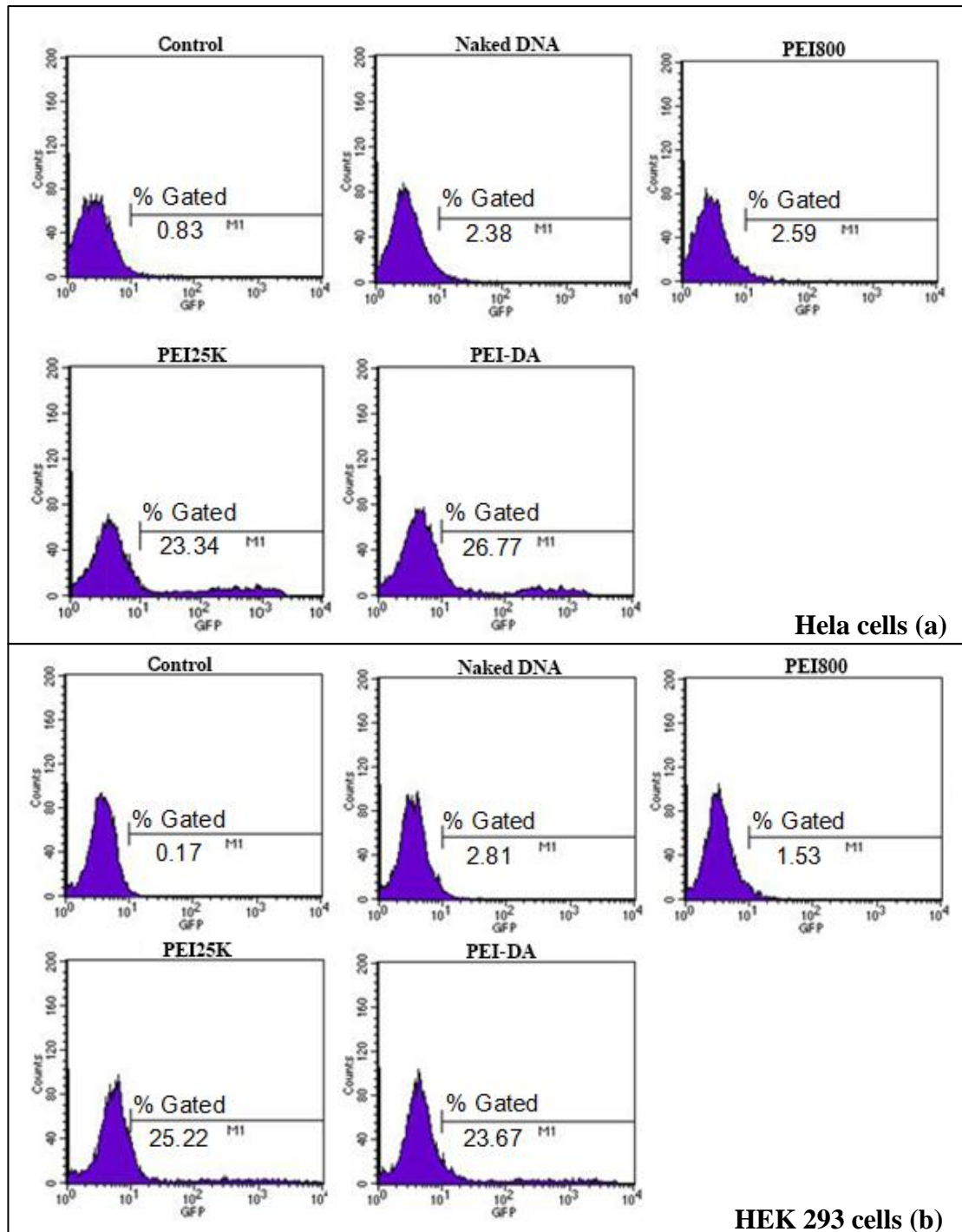
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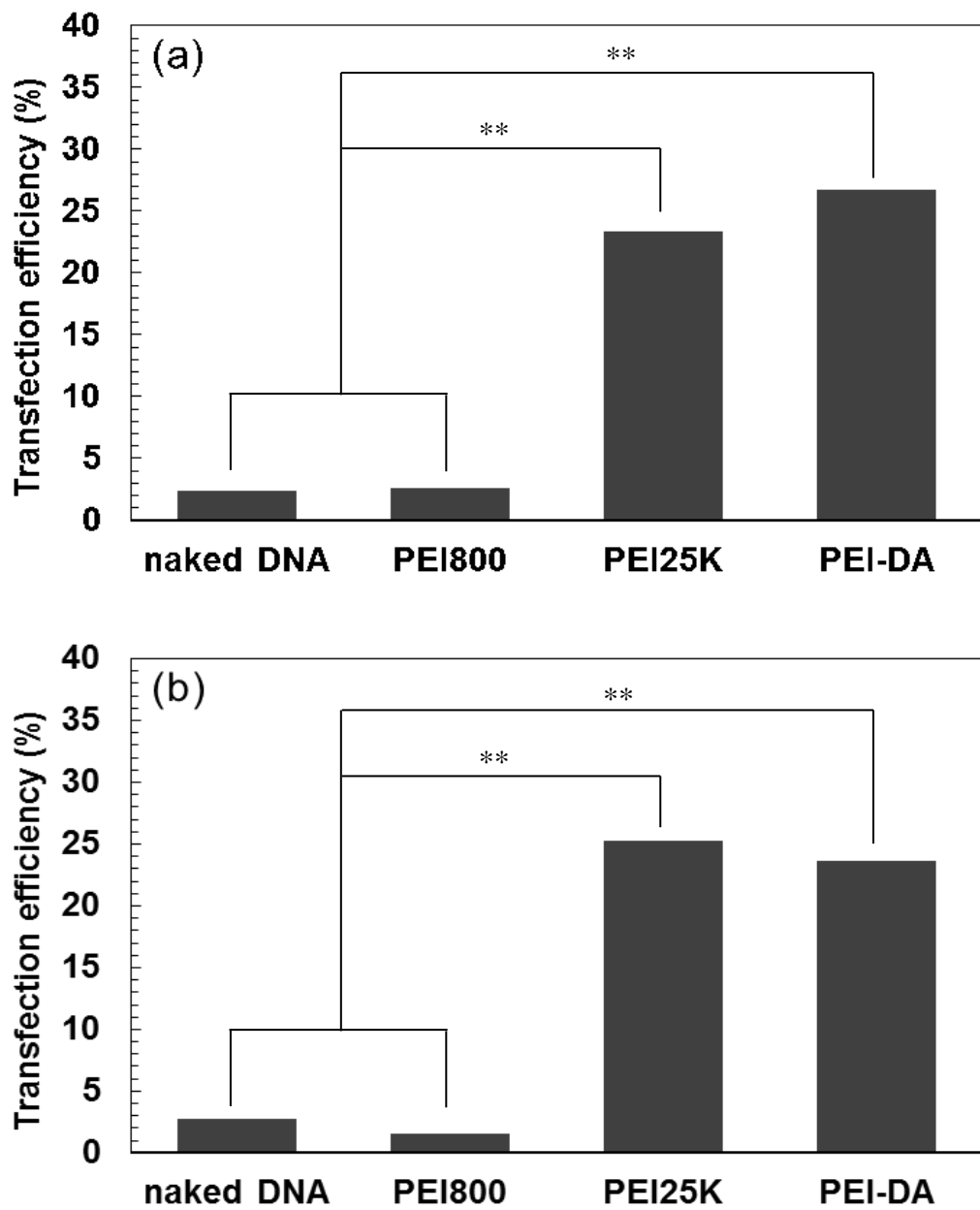
Figure 3.9 Transfection efficiency of naked DNA, PEI800, PEI25K and PEI-DA when delivering pDNA to Hela cells (a) and HEK 293 cells (b) at an N/P ratio of 10.

Chapter 4 Cell Penetrating Peptide Labelled Intracellular Microenvironment Responsive Nanogels for Advanced Gene Delivery

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     iv.   the candidate's stated contribution to the publication is accurate (as detailed above);

     v.   permission is granted for the candidate in include the publication in the thesis; and

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Cell Penetrating Peptide Labelled Intracellular Microenvironment Responsive Nanogels for Advanced Gene Delivery

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Abstract

Highly efficient gene delivery vehicles are pursued to progress gene therapy into clinical applications. In this study, we developed a cell penetrating peptide-labelled biodegradable gene carrier for efficient external gene transfection. The carrier was prepared by coupling Polyethylenimine 800Da (PEI800), 4'4-dithiodibutyric acid (DA) and HIV-1 Trans-Activator of Transcription (TAT) peptide in the presence of EDC/NHS. The resulted polymer, PEI-DA-TAT was able to condense plasmid DNA (pDNA) into a complex with a hydrodynamic size of around 150 nm under a neutral condition. PEI-DA-TAT showed negligible cytotoxicity to both Hela and HEK 293 cells. The new carrier performed better in regard to transfection efficiency in comparison with non-labelled biodegradable PEI (PEI-DA) as well as commercial PEI (PEI25K). Our findings suggest that PEI-DA-TAT should be a promising carrier to be applied in gene therapy.

Key words: gene delivery, biodegradable, polyethylenimine, HIV-1 TAT

4.1 Introduction

A surging interest in the development of highly efficient gene delivery vehicles has been seen with the ultimate goal for their use in clinical gene therapy. It has been demonstrated that therapeutic nucleic acids delivered into cells can treat various diseases including cancers[1] and cardiovascular disease[2]. Efficient gene delivery vehicles are required to help transport therapeutic genes into cells since a series of barriers in the eukaryotic cells including cellular uptake, endosome escape and nuclear transport have impended transfection of the naked nucleic acid. Viral and non-viral gene carriers have been explored to overcome these barriers[3, 4]. However, immunological and inflammatory issues have restricted the application of viral gene carriers, even though they exhibit higher transfection efficiency than non-viral carriers. On the other hand, synthesized non-viral gene carriers often show moderate to high toxicity in eukaryotic cells[3].

Polyethylenimine (PEI), a cationic non-viral gene carrier, has been investigated for its excellent gene protection from intracellular enzyme degradation and high transfection efficiency associated with the 'proton sponge' effect[5, 6]. However, it shows high cytotoxicity owing to its high cationic charge density, which is related to a high molecular weight and its non-biodegradability. Such cytotoxicity has prevented PEI from its application in gene therapy. It has been reported that low molecular weight PEI (LWM PEI) such as PEI800 has a low cytotoxicity while the transfection efficiency is also low[7]. To harness the low cytotoxicity of LMW PEI, strategies have been adopted, including modifying LMW PEI with polyethylene glycol (PEG)[8], targeting ligands or hydrophobic groups[9] and cross-linking LMW PEI with different linkages[10].

Among these strategies, cross-linking LMW PEI with a biodegradable linkage could be very promising to achieve low cytotoxicity and high transfection efficiency[11]. Disulfide bonds that can be cleaved by glutathione (GSH) in the cytosol or the nucleus have become one of good

biodegradable linkage candidatures. In addition, the degradation of disulfide bonds is triggered after the escape of carriers from the endolysosomal compartment[12], which ensure the endosomal escape process of PEI-based gene carriers. Various reducible PEI-based gene carriers have been prepared by different linkers with disulfide bonds such as N,N'-cystamine bisacrylamide (CBA)[13, 14], dithiobis(succinimidyl propionate) (DSP)[15], and dimethyl-3,3'-dithiobis(propionimidate) (DTBP)[15]. Although these disulphide bond-containing PEI products can be biodegradable, some other issues remain to be resolved. For example, products reacted by LMW PEI with CBA showed poor gene binding and condensing ability resulting from the low molecular weight of the product (lower than 10 KDa) and its unregulated structure due to the Michael addition[16]. Connecting PEI with DSP results in reduction of the amount of the primary groups on PEI due to the formation of amide bonds leading to the drop of the gene binding for the carrier[15].

One of the biocompatible materials, cell-penetrating peptides (CPPs), can facilitate the translocation of macromolecules like proteins and polymers through the biological membrane in mammal cells[17, 18]. However, the mechanism for internalization of the CPPs is still not clear. Researchers have proposed several different hypotheses, such as micropinocytosis[19] and lipid raft mediated cellular uptake[20, 21]. HIV-1 Trans-Activator of Transcription peptide (HIV-1 TAT) is one of the most popular CPPs, since it represents a protein transduction domain as well as a nuclear localization sequence (NLS)[22, 23]. TAT has been grafted to different carriers. Lee et al.[24] reported a hybrid-synthesized drug carrier through chemical conjugation of doxorubicin (DOX) with TAT to the chitosan backbone. The anticancer efficiency of the resulted carrier was enhanced and the efficiency of cell internalization was improved compared with free DOX. Significant inhibition of tumor growth in CT26 xenograft-bearing mice after treating by the TAT-modified carrier/DOX complex was also found. Moreover, Li et al reported an efficient drug delivery system by conjugating TAT with magnetic

mesoporous silica nanoparticles (FMSN)[25]. DNA-toxin anticancer drugs were delivered by the FMSN-TAT in vitro and in vivo. In addition to modification of drug carriers, the TAT peptide was also used to improve the cellular uptake ability of polymer based gene carriers. Torchilin conjugated the liposomes with TAT to deliver external gene into H9C2 cells and BT20 cells in vitro[26]. The transfection efficiency of TAT conjugated liposomes significantly increased compared with commercial Lipotectin®. Even though TAT has been applied in modifications of different carriers, there is no report so far on TAT-labelled intracellular biodegradable PEI-based carriers for gene delivery with high transfection efficiency and low cytotoxicity.

In this study, HIV-1 TAT labelled intracellular biodegradable PEI (PEI-DA-TAT) was synthesized by cross-linking LMW PEI (PEI800) with 4'4-dithiodibutyric (DA) and then conjugated with HIV-1 TAT. We hypothesize that the transfection efficiency of PEI-DA-TAT should be improved since TAT could improve the cellular uptake ability of the gene carrier. In addition, the cytotoxicity of PEI-DA-TAT should be low since the disulfide bond would be cleaved after it is exposed to GSH inside the cells and degraded into LMW PEI which has negligible toxic to cells. PEI-DA-TAT was evaluated for its gene binding ability, cytotoxicity, cellular uptake and gene transfection efficiency in Hela and HEK 293 cells.

4.2 Materials and Methods

4.2.1 Materials

PEI800 and PEI25K (MW = 0.8 KDa and 25 KDa), EDC (1-ethyl-3-(3-dimethy-laminopropyl) carbodiimide), NHS (N-hydroxysulfosuccinimide), Fluorescein isothiocyanate (FITC), Gel red and other chemicals/solvents were purchased from Sigma-Aldrich (St.Louis, MO). HIV-1 TAT peptide (Tyr-Gly-Arg-Lys-Lys-Arg-Arg-GLn-Arg-Arg-Arg) was

purchased from GenicBio (Shanghai, China). The QIAGEN Maxi kit was obtained from Qiagen (Boncaster, Australia). The plasma membrane and nuclear labeling kit, nucleic acid stains dimer sampler, fetal bovine serum (FBS), trypsin–EDTA, penicillin–streptomycin (PS) mixture, kanamycin, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), phosphate buffered saline (PBS) and TAE (Tris–acetate) were purchased from Life Technologies (Mulgrave, Australia).

4.2.2 Methods

4.2.2.1 Preparation of plasmid DNA

The pEGFP-N1 plasmid expressing the enhanced green fluores-cent protein (EGFP) was prepared in *Escherichia coli* DH5α strain and extracted using a QIAGEN Midi kit. The integrity and purity of plasmid DNA (pDNA) were analyzed using 1.0 % agarose gel electrophoresis and the DNA concentration was determined using a Jasco UV–vis spectrophotometer (Tokyo, Japan) at the fixed wavelengths of 260 nm. The pDNA was further labelled by fluorescent dye (YOYO-1) at a ratio of 1 molecular dye to 100 molar nucleic acid base pairs for the cellular uptake study.

4.2.2.2 Synthesis of PEI-DA-TAT

The synthesis details of the PEI-DA-TAT are described in Scheme 1. Firstly, PEI-DA was synthesized. PEI800 was interacted with DA using carbodiimide chemistry. To synthesize PEI-DA, NHS/EDC (86.32/143.78 mg, 0.75 mmol) and DA (89.37 mg, 0.375 mmol) were added to 6 ml DMSO at room temperature and the mixture was stirred for 1h. PEI800 (100 mg, 100 mg/ml) was then added to the mixture. The pH of the reaction mixture was adjusted to 7 by adding NaOH (0.1 M) or HCl (0.1M). The mixture was further reacted for 16 h at room temperature. After the reaction, the product was dialyzed against deionized water (pH 7) for 3 days. The obtained PEI-DA polymer was lyophilized and then characterized.

The HIV-1 TAT (Tyr-Gly-Arg-Lys-Lys-Arg-Arg-GLn-Arg-Arg-Arg) peptide was conjugated with PEI-DA using an NHS/EDC coupling system. Briefly, 0.3 mg (1 %) and 3 mg (10 %) TAT peptides were added to 5 ml MilliQ water at room temperature. EDC/NHS in 1.4 fold of the molar weight of TAT were added separately (23.44/39.04 mg for 1 % and 234.37/390.38 mg for 10 %). The mixture was stirred at room temperature for 1 h and then PEI-DA (30 mg) was added to the mixture. The reaction was stirred for 16 h, and the pH value of the reaction mixture was adjusted to 7.0 by adding NaOH (0.1 M) or HCl (0.1M) and the mixture solution was further dialyzed against deionized water (pH 7.0) for 3 days. The obtained PEI-DA-TAT 1 % and PEI-DA-TAT 10 % polymers were lyophilized and then characterized. To confirm the success of the conjugation of TAT peptide, FITC-labelled TAT conjugation to PEI-DA was conducted. Briefly, 0.01 % FITC label was grafted with TAT peptide (30 mg). Then 0.3 mg (1 %) and 3 mg (10 %) FITC labelled TAT peptides were reacted with 30 mg PEI-DA at the same condition described above in the EDC/NHS system. The PEI800, PEI-DA, FITC labelled TAT peptide and PEI-DA-TAT (concentration of 1 mg/ml) were evaluated with UV-vis at the wavelength of 495 nm at room temperature.

4.2.2.3 Fourier transform infrared spectroscopy (FTIR)

The Fourier transform infrared spectrometer (FTIR) spectra of PEI800, DA, HIV-1 TAT peptide, PEI-DA, PEI-DA-TAT 1 % and PEI-DA-TAT 10 % were monitored using a Thermo NICOLET 6700 Fourier transform infrared spectrometer at room temperature under reflection mode.

4.2.2.4 Acid–Base Titration Assays

The buffering capacity was measured by acid–base titration. Briefly, 50 ml of sample solution in the concentration of 0.05 mg/ml including PEI800, PEI25K, PEI-DA, PEI-DA-TAT 1 % and PEI-DA-TAT 10 % was adjusted to pH 3 with 0.1 M HCl and then 0.1 M NaOH was

gradually added until the solution pH reached 11. After the each addition of 0.1M NaOH, the pH was measured by a microprocessor pH meter. 150 mM NaCl was titrated as a control[27].

4.2.2.5 DNA condensing ability of PEI-DA-TAT

Different polymer/pDNA complexes were prepared by mixing PEI800, PEI25K, PEI-DA, PEI-DA-TAT 1 % and PEI-DA-TAT 10 % with pDNA (0.2 µg/ml) at various N/P ratios ranging from 0.5 to 50. Then the complexes were diluted to 6 µl and incubated at room temperature for 30 min. Subsequently, the samples were loaded onto the 0.8 wt % agarose gel containing GelRed™ with Tris-acetate (TAE) running buffer at 80 V for 60 min. The resulted pDNA migration patterns were read under UV irradiation (G-BOX, SYNGENE).

4.2.2.6 Particle sizes and zeta potentials

Particle sizes and zeta potentials of a gene carrier are related to the efficiency of its cellular uptake ability. To achieve this optimal condition, various polymer/pDNA complexes were prepared at different N/P ratios from 0.5 to 50. Particle sizes and zeta potentials were examined by a Malvern Nano-ZS 90 laser particle size analyzer equipped with ZET5104 cell at room temperature. For particle size analysis, the cumulate method was used to convert intensity–intensity autocorrelation functions to apparent particle sizes according to the Stokes–Einstein relationship[28]. The Smuloschowski model was used to convert electrophoresis mobility to zeta potentials. 15 parallel runs were carried out for each measurement and the final data were obtained based on statistical analysis.

4.2.2.7 Evaluation of cytotoxicity

HeLa cells and HEK 293 were cultured in DMEM medium supplied with 10 % FBS in 96-well plates (200 µl/well) at a cell density of 1.0×10^5 cells/ml. After incubation, the cells were allowed to adhere overnight at 37 ℃ in a humidified 5 % CO_2-containing atmosphere. The growth medium was replaced with 200µl fresh medium. Then different testing carriers

(PEI800, PEI25K, PEI-DA, PEI-DA-TAT 1 % and PEI-DA-TAT 10 %) were added at the concentrations of from 0.5 to 50 µg/ml respectively. Cells were then incubated for 24-72 h before 10 µl of MTT (5 mg/ml in PBS) was added to each well to measure cell viability [29]. After incubating for another 4 h at 37 ℃, the growth medium was replaced by 150 µl of dimethyl sulfoxide (DMSO) to ensure complete solubilization of the formed form-azan crystals. Finally, the absorbance was determined using the Biotek Microplate Reader (Biotek, USA) at a wavelength of 595 nm[30].

4.2.2.8 Cellular uptake by confocal laser scanning microscopy (CLSM)

HeLa cells at a concentration of 2×10^5 cells/well were cultured in 6-well plates loaded with cover-glass slides for 24 h. 4 µg YOYO-1 labelled pDNA (YOYO-1 labelled pDNA was pre-pared according to the instruction from Life Technologies) was loaded onto different gene carriers (naked DNA, PEI800 PEI25K, PEI-DA PEI-DA-TAT 1 % and PEI-DA-TAT 10 %) at an N/P ratio of 10 to form polymer/pDNA complexes. Cells were incubated with polymer/pDNA complexes for another 4 h and then the complexes were removed by washing the cells with PBS three times before fixing with 4 % formaldehyde. The cell membrane and nucleus were separately stained by 100 µl of Alexa Fluor 594(5 µg/ml) and Hoechst 33258 (2 µM) for 15 min at 37 ℃. The cells were further washed with PBS three times and incubated with 1 ml PBS, and kept at room temperature for further analysis. The fluorescence images were observed from a confocal laser scanning microscope (Leica Confocal 1P/FCS) equipped with a 405 nm diodelaser for Hoechst33258, a 488 nm argon ion laser for YOYO-1 and a 561 diode laser for Alexa Fluor 594. The high magnification images were obtained with a 63x objective. Optical sections were averaged 8 times to reduce noise, and images were processed using the Leica Confocal software[30].

4.2.2.9 Gene transfection

HeLa and HEK 293 cells were seeded in 24-well plates and cultured in complete DMEM supplemented with 10 % fetal bovine serum (FBS) at 37 ℃ in a humidified 5 % CO_2 incubator. After 24 h culturing, the confluent percentage of the cell culture reached 80 %. The medium was replaced with fresh 200 μl culture medium in the absence of FBS. Meanwhile, the polymer/pDNA complexes prepared by incubating PEI800, PEI25K, PEI-DA, PEI-DA-TAT 1 % and PEI-DA-TAT 10 % with pDNA at an N/P ratio of 10 at room temperature for 30 min were added to each well. After 6 h incubation, the cultured medium was replaced by 1 ml fresh complete culture medium with 10 % FBS and the cells were further incubated for another 42 h[30].

4.2.2.10 Green fluorescent protein (GFP) expression

GFP expression level was evaluated by flowcytometry. The green fluorescence intensity was also detected directly by a FACSCalibur flowcytometer (Becton Dickinson), and the transfection efficiency was calculated by the percentage of positive cells, using non-transfection cells (mock cells) as the negative control. Briefly, cells with pEGFP-N1 transfected with carriers including (naked pDNA, PEI800, PEI25K, PEI-DA, PEI-DA-TAT 1 % and PEI-DA-TAT 10 %) were harvested from trypsin digestion after 48 h post-transfection. One million cells were washed with PBS buffer, and centrifuged at 1000 rpm for 5 min. The cells were stained by propidium iodide (400 μl, 0.5 μg/ml) in 1x PBS. Approximately 1 to 2×10^4 cells were analyzed at the rate of 200-500 cells per second. CellQuest3.3 software was used for data analysis[31].

4.2.2.11 Statistical analysis

Data obtained from our experiments were represented as mean ± SE (standard error). Statistical analysis of the numerical variables was performed using a two-sample, two-tailed t-test. A value of $p < 0.05$ is considered to be significant[32].

4.3 Results and discussion

4.3.1 Synthesis and characterization of PEI-DA-TAT

In this study, 4'4-dithiodibutyric acid (DA) was first reacted with LMW PEI (PEI800) through the EDC/NHS coupling system, through formation of amid bonds between carboxyl groups and amino groups of PEI, to synthesize the intracellular biodegradable PEI-DA. The feed ratio of carboxyl groups on DA to amino groups on PEI800 was contributed to be around 1 to 10 to facilitate formation of the cross-linked PEI800 network. Next, HIV-1 TAT peptide was further conjugated to PEI-DA by EDC/NHS coupling to prepare PEI-DA-TAT. After purification, successful synthesis of PEI-DA-TAT was confirmed by the FTIR spectra (Figure 4.1). For PEI800, the characteristic peaks at 1664 cm^{-1} and 1041 cm^{-1} indicate the NH$_2$ vibration and the C-N stretching. For PEI-DA, because of conjugation of the carboxyl group on DA and the amino group on PEI800, the signals at 1639 cm^{-1} and 1544 cm^{-1} are assigned to C=O stretching (amide I band) and N-H deformation (amide II band)[12]. Meanwhile, the signal at 879 cm^{-1} remains for the PEI-DA, which is corresponding to disulfide bonds from DA. PEI-DA-TAT shows a similar FTIR result as non-TAT labelled PEI-DA, and the possible reason is that there is no new functional group introduced after the conjugation of TAT peptide. Therefore, there is no distinguished difference in the FTIR results between PEI-DA and PEI-DA-TAT.

In order to verify success in conjugation of the TAT peptide, FITC labelled PEI-DA-TAT (FITC-PEI-DA-TAT 1 % and FITC-PEI-DA-TAT 10 %) were synthesized and characterized by UV-vis at a wavelength of 495 nm. PEI-DA, PEI800 and FITC labelled TAT peptide were also measured as the references. The absorbance of PEI-DA-TAT at 495 nm is 0.098 (PEI-DA-TAT 1 %) and 0.152 (PEI-DA-TAT 10 %), which are significantly higher than PEI-DA (0.003) and PEI800 (0.004), but lower than FITC-TAT with an absorbance of 1.346. This confirms conjugation of the TAT peptide to PEI-DA. Combining the FTIR results with UV absorbance of PEI-DA-TAT, we confirm the successful introduction of the disulfide bonds and conjugation of the TAT peptide for the synthesized samples.

4.3.2 Buffering capacity

Buffering capacity is considered to be related to the endosomal escape ability of gene carriers. This is a key parameter to affect the transfection efficiency in gene delivery[6, 33]. The PEI-DA-TAT was back titrated to measure the buffering capacity from pH 3 to 11. The buffer capacities of PEI25K, PEI800 and PEI-DA were evaluated as references. In Figure 4.2, PEI-DA shows a broader buffering capacity than LMW PEI (PEI800), and is comparable to the buffering capacity with commercial PEI (PEI25K). PEI-DA-TAT 1 % and PEI-DA-TAT 10 % show the similar buffering capacity as PEI-DA. This indicates that conjugation of a small amount of TAT peptides has no significant change in the buffering capacity. The increased buffering capacity of PEI-DA-TAT and PEI-DA compared with LMW PEI may be mainly contributed from cross-linking of PEI800 by DA. The good buffering capacity of PEI-DA-TAT indicates it should be a suitable carrier for gene delivery, since it can facilitate gene escape from the late endosome/lysosome and maintain the integral structure for gene delivery.

4.3.3 Characterization of PEI-DA-TAT/pDNA complexes

Gene binding ability is the prerequisite for an efficient gene delivery system. PEI-DA-TAT, a cationic polymer, can be used to condense and protect the gene materials to improve the cellular uptake. To evaluate the cell condensing ability of PEI-DA-TAT 1 % and PEI-DA-TAT 10 %, polymer/DNA complexes prepared at different N/P ratios from 0.5 to 50 were electrophoresed in agarose gel using naked pDNA as reference and compared with PEI-DA, PEI800 and PEI25K. As shown in Figure 4.3, non-TAT labelled biodegradable PEI based gene carrier, PEI-DA shows a gene binding ability better than LMW PEI (PEI800), but slightly lower than PEI25K. It can fully retard pDNA beyond the N/P ratio of 3. The increase in the gene binding ability can be contributed from the increase in the positive charge density after cross-linking PEI800. Furthermore, 1 % TAT labelled biodegradable carrier, PEI-DA-TAT 1 %, completely binds pDNA between N/P ratios of 1 and 5. With a higher ratio of TAT peptide in the conjugated polymer, PEI-DA-TAT 10 % binds pDNA thoroughly beyond the N/P ratio of 1. TAT peptide is a positively charged peptide and it is able to facilitate of the electrostatic interaction with the negatively charged pDNA[34]. This could explain the increased of the gene binding ability of PEI-DA-TAT 10 % compared with PEI-DA-TAT 1 %. The gel electrophoresis result indicates that the gene binding ability of PEI-DA-TAT 10 % is improved compared with PEI-DA and PEI800. This improved gene condensing ability suggests that it may improve the cellular uptake efficiency and protect pDNA from intracellular enzymatic attacks[35].

The physical properties of PEI-DA-TAT/pDNA complexes were investigated through measurements of particle size and zeta potential. The particle size of gene/carrier complex is an essential parameter. It is reported that the particle size of the gene carrier and gene complexes in the range around 100-200 nm is optimal for gene delivery[36]. The particle sizes of PEI-DA-TAT 1 % and PEI-DA-TAT 10 % with pDNA were determined using dynamic

light scattering (DLS). As shown in Figure 4.4a, the general trend is the size of carrier/pDNA complexes prepared from PEI25K, PEI-DA, PEI-DA-TAT 1 % and PEI-DA-TAT 10 % reduces with an increase in N/P ratios, except for PEI800/pDNA complexes, which has a size more than 350 nm even at the N/P ratio of 50. PEI-DA, after cross-linking PEI800 using DA to form high molecular weight polymer, shows better pDNA condensation ability than PEI800, but still slightly poorer than PEI25K. PEI-DA-TAT 1 % and PEI-DA-TAT 10 % shows similar particle sizes to PEI-DA from the N/P ratio of 0.5 to 50. At the N/P ratio of 10, PEI25K, PEI-DA, PEI-DA-TAT 1 % and PEI-DA-TAT 10 % have a similar size of around 150 nm after complexing with pDNA, which falls in the optimal range for gene delivery. Beyond the N/P ratio of 10, these four vehicles are able to condense pDNA to a smaller size. However, the change in the particle size is less significant than the smaller N/P ratios. The particle size of the carrier/pDNA complexes prepared by PEI-DA, PEI-DA-TAT 1 % and PEI-DA-TAT 10 % indicates that conjugation of the TAT peptide has no influence on the size of carrier/pDNA complexes and the size of the complexes is mainly depended on the molecular weight of the carriers.

The surface of the mammalian cell membrane is negatively charged, and it is normally considered that positively charged gene delivery vehicles can facilitate cellular uptake and hence improve the gene transfection efficiency[37]. Zeta potentials of the PEI-DA-TAT/pDNA complexes at different N/P ratios from 0.5 to 50 were evaluated at pH 7. As shown in Figure 4.4b, with an increase of the N/P ratios, zeta potentials of PEI-DA-TAT 1 % and PEI-DA-TAT 10 % increases from -17 mV and -16 mV respectively to around 20 mV for both PEI-DA-TAT 1 % and PEI-DA-TAT 10 %. The zeta potential change becomes insignificant as the N/P ratio increases beyond 10. In contrast, PEI25K maintains a positive value for zeta potential at the lowest N/P ratio of 0.5. Commercial PEI (PEI25K) has been proven to have an excellent pDNA condensing ability because of its high positive charge density[38], which

explains its positive zeta potential even in a low N/P ratio. The zeta potential of TAT-labelled PEI-DA is similar to that of non-TAT-labelled PEI-DA at all N/P ratios, and this indicates that the conjugation of TAT has no significant on the surface charge of the carrier/pDNA complexes. It can be concluded that PEI-DA-TAT is able to condense pDNA into small particles with a positive charge for cellular uptake at and beyond an N/P ratio of 10.

4.3.4 Cell toxicity of PEI-DA-TAT

PEI25K has shown an excellent performance in regard to condensation of pDNA, the particle size and the surface charge of the gene/carrier complexes (Figure 2-4). However, cytotoxicity is the major hindrance of PEI25K in gene delivery application[36]. The cytotoxicity of PEI-DA-TAT 1 % and PEI-DA-TAT 10 % was evaluated against Hela and HEK 293 cells. The cell viability was measured by the MTT assay at various polymer concentrations (0.5 to 50 µg/ml). Figure 4.5 shows the cell viability results for Hela cells (a) and HEK 293 cells (b) after incubation of cells with polymer solution for 24 h. It can be seen that PEI800 is nontoxic to both cell types even at the highest concentration of 50 µg/ml. This justifies our choice of this LMW PEI as the initial material. After cross-linking of LMW PEI with DA, and conjugation with TAT, the resulting products, PEI-DA, PEI-DA-TAT 1 % and PEI-DA-TAT 10 % show the similar cell viability results at all tested concentration for both cell types. This suggests that cross-linking with DA and conjugation with TAT peptide do not change the low cytotoxicity property of PEI800. This result also confirms that the positively charged TAT is non-toxic to cells responded by Baoum et al[34]. In contrast, commercial PEI (PEI25K) shows a strong toxicity to both Hela and HEK 293 cells and the cell viabilities are lower than 30 % in both Hela and HEK 293 cells at a polymer concentration of 50 µg/ml. The low cytotoxicity of PEI-DA-TAT compared with commercial PEI (PEI25K) suggests that PEI-DA-TAT could be employed a safe carrier for gene in the mammalian cells.

4.3.5 Cellular uptake of PEI-DA-TAT/pDNA complexes

To evaluate the cellular uptake of the PEI-DA-TAT/pDNA complex and pDNA distribution inside the cell after cellular uptake, pDNA dyed with YOYO-1 was transfected into Hela cells at an N/P ratio of 10. The cell membrane was dyed with Alexa Fluor 549 and the nucleus was dyed with Hoechst 33258 to distinguish the distribution of pDNA. The images of cellular uptake were taken under a confocal microscope after 6 h transfection. As shown in Figure 4.6, green fluorescence signals representing pDNA samples are found in both cytoplasm and nucleus of Hela cells when pDNA is delivered by PEI25K, PEI-DA, PEI-DA-TAT 1 % and PEI-DA-TAT 10 %. The total pDNA uptake by cells is the sum of pDNA in the cytoplasm and nucleus, and digested pDNA by the enzymes inside the cells. PEI800 has a much less denser positive charge than PEI-DA and PEI25K. A larger size of the PEI800/pDNA complex excludes its entry through the cell membrane, leading to very weak green fluorescence signals. The naked DNA has a poor cell membrane penetration due to its negative charge and large hydrodynamic size. It may also be digested by the enzymes inside the cells to small fragments and moved out from the cells, which does not exhibit green fluorescence signals. It is noted that when PEI25K is employed, the majority of green fluorescence signal is located in the cytoplasm in the Hela cells and a very small amount of green signals in the nucleus. The PEI/pDNA complex escaping out of late endosomes may not be able to transport to the nucleus due to the lack of the assistant for the nucleus penetration and its large hydrodynamic size[39]. However, PEI-DA, PEI-DA-TAT 1 % and PEI-DA-TAT 10 % appear to have distinguished green signals inside the nucleus. The cellular uptake ability of gene carriers is one of the most important factors to influence the gene transfection efficiency[40]. With more pDNA translocated in the nucleus when delivering by PEI-DA and PEI-DA-TAT, more pDNA could be expressed in to target proteins and the transfection efficiency of PEI-DA and PEI-DA-TAT may be improved.

4.3.6 Cell transfection

The complexes of PEI-DA-TAT and fluorescence reported gene pEGFP-N1 were prepared at an N/P ratio of 10 to evaluate the transfection efficiency. The PEI-DA-TAT/pDNA complexes were applied to Hela and HEK 293 cells and the transfection efficiency was determined by flowcytometry after 48 h transfection. Commercial PEI (PEI25K), LMW PEI (PEI800), and PEI-DA were used comparison. As shown in Figure 4.7, non-TAT labelled PEI-DA shows a similar transfection efficiency compared with PEI25K in both Hela (26.77 % vs 24.23 %) and HEK 293 cells (23.67 % vs 25.22 %). As discussed before, PEI-DA and PEI25K show similar results in the particle size and zeta potential which could be the reason for their similar transfection efficiencies. In addition, similar buffering capacity of PEI-DA to PEI25K also indicates that after cross-linking PEI800 with DA, PEI-DA obtain similar positive charge density to PEI25K leading to similar 'proton sponge' effect. However, with the introduction of DA, PEI-DA shows a much lower cytotoxicity compared with PEI25K. This suggests that PEI-DA should be a better gene carrier in the application of gene delivery. On the other hand, the transfection efficiency of LMW PEI (PEI800) is quite low in Hela cells (~10 fold less than PEI-DA) and HEK 293 cells (~16 fold less than PEI-DA). As shown Figure 4.6, the cellular uptake of PEI800/pDNA complex is poor, which could explain the lower transfection efficiency of PEI800. The transfection efficiency of PEI-DA-TAT 1 % at a low ratio of conjugated TAT shows similar transfection efficiency as PEI-DA. This indicates that the TAT peptide has little contribution for gene transfection at a lower ratio. The cellular uptake is dominated through the electrostatic interaction-mediated endocytosis. However, PEI-DA-TAT 10% has shown better transfection efficiency in both Hela and HEK 293 cells. The transfection efficiency improves by about 8 % for Hela cells and 7 % for HEK 293 cells. The improvement of the transfection efficiency is similar to the that from another report on a TAT-modified gene delivery carrier prepared by Torchilin et al[41]. The transfection efficiency

of the TAT modified liposome as the gene carrier was increased by 5-10 % in NIH/3T3 and H9C2 cells. A higher percentage of TAT in the PEI-DA-TAT polymer may contribute to higher cellular uptake of the PEI-DA-TAT/pDNA complex due to its ability to facilitate translocation of the complex through the cell membrane, and the TAT peptide on the PEI may also help the PEI/pDNA complex penetrate into the nucleus due to its nuclear localization ability[25].

4.4 Conclusions

In this study, we report a novel intracellular biodegradable gene delivery carrier, PEI-DA-TAT, for high efficient gene delivery with low cytotoxicity. PEI-DA-TAT shows negligible impact on the physical properties compared with non-TAT labelled PEI-DA and it is able to retard pDNA into suitable size for gene delivery. Gene transfection efficiency to Hela and HEK 293 cells of PEI-DA-TAT 10 % is higher than commercial gene carrier (PEI25K) contributed by the increase of intracellular uptake ability with the conjugation of HIV-1 TAT and the nuclear targeting ability of TAT peptide. In addition, PEI-DA-TAT has shown low toxicity to Hela cells and HEK 293 cells resulting from its biodegradable disulfide bond, introduced by DA. This makes PEI-DA-TAT a promising gene vector with safety. The satisfied nucleic acid loading ability, low toxicity, good cellular uptake ability and high gene transfection efficiency render PEI-DA-TAT to be an efficient and safe delivery vector for potential gene therapy.

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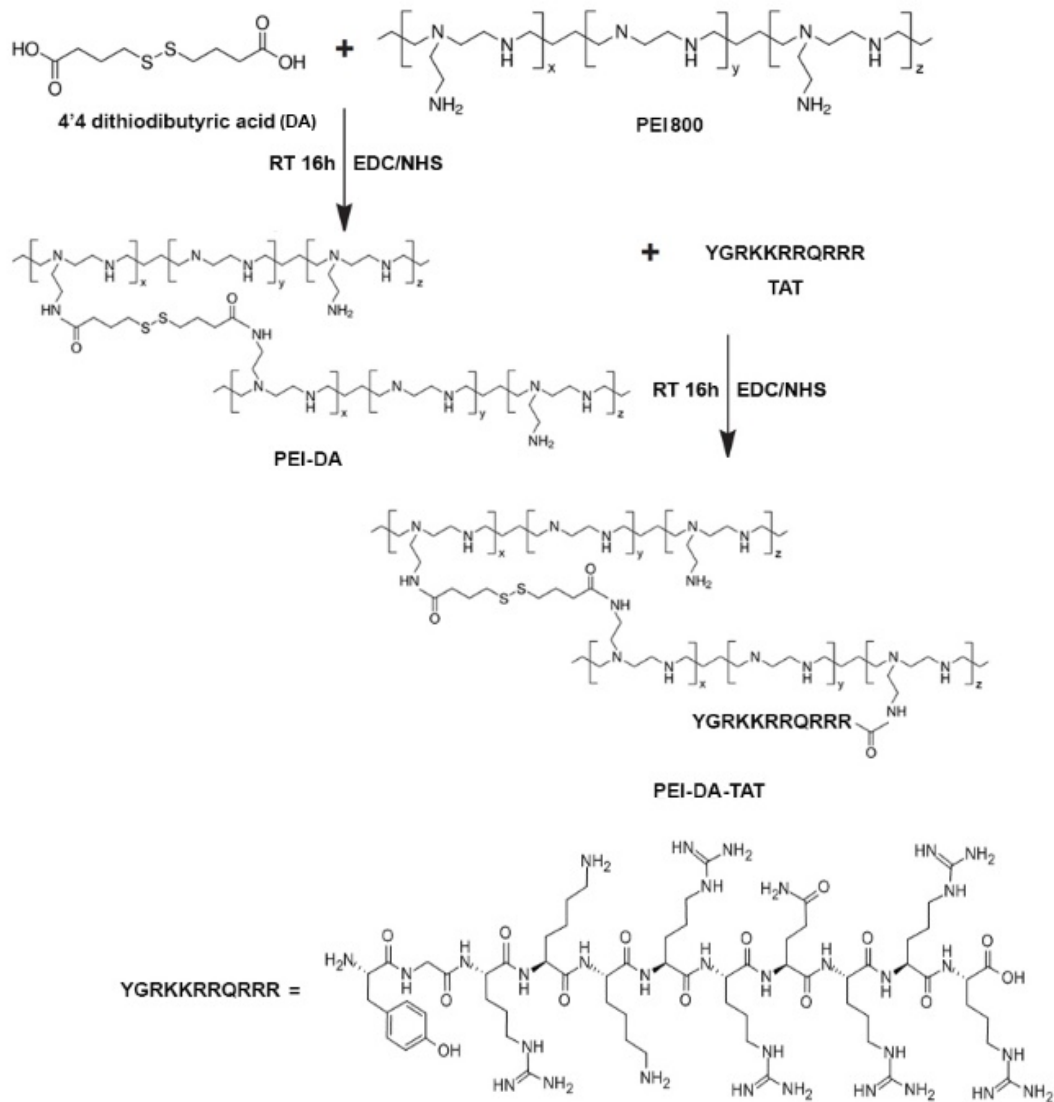
Scheme 4.1 Schematic description on the synthesis of PEI-DA-TAT
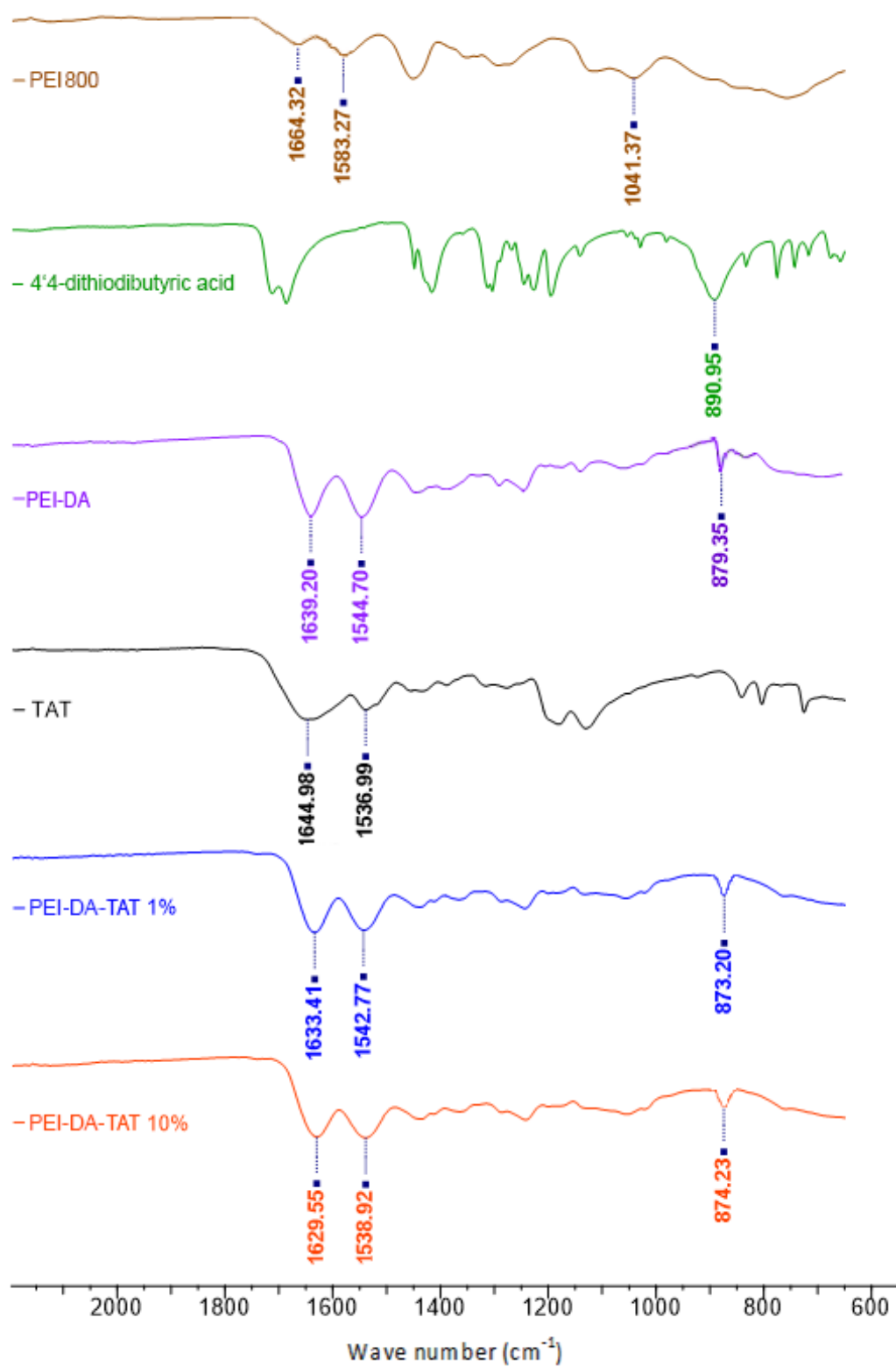
Figure 4.1 FTIR spectra of PEI800, 4'4-dithiodibutyric acid, TAT, PEI-DA, PEI-DA-TAT 1 % and PEI-DA-TAT 10 %
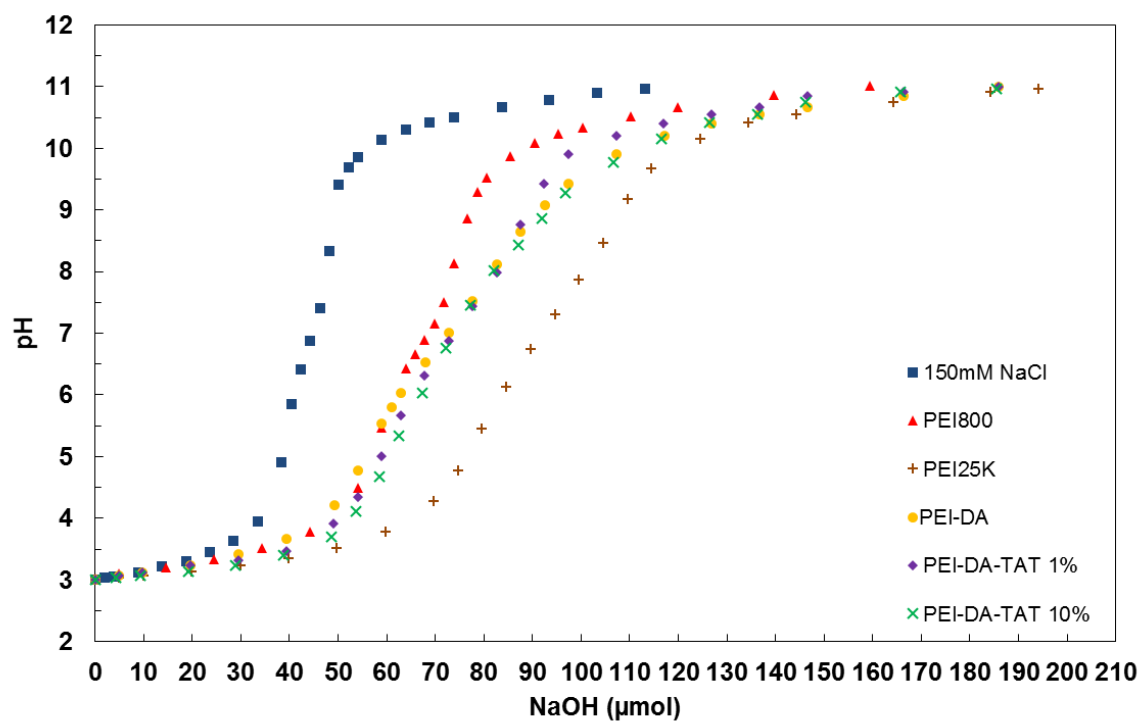
Figure 4.2 Buffering capacities of PEI800, PEI25K, PEI-DA, PEI-DA-TAT 1 % and PEI-DA-TAT 10 % at 25 ºC 1 atm. 150 mM NaCl was used as the control.
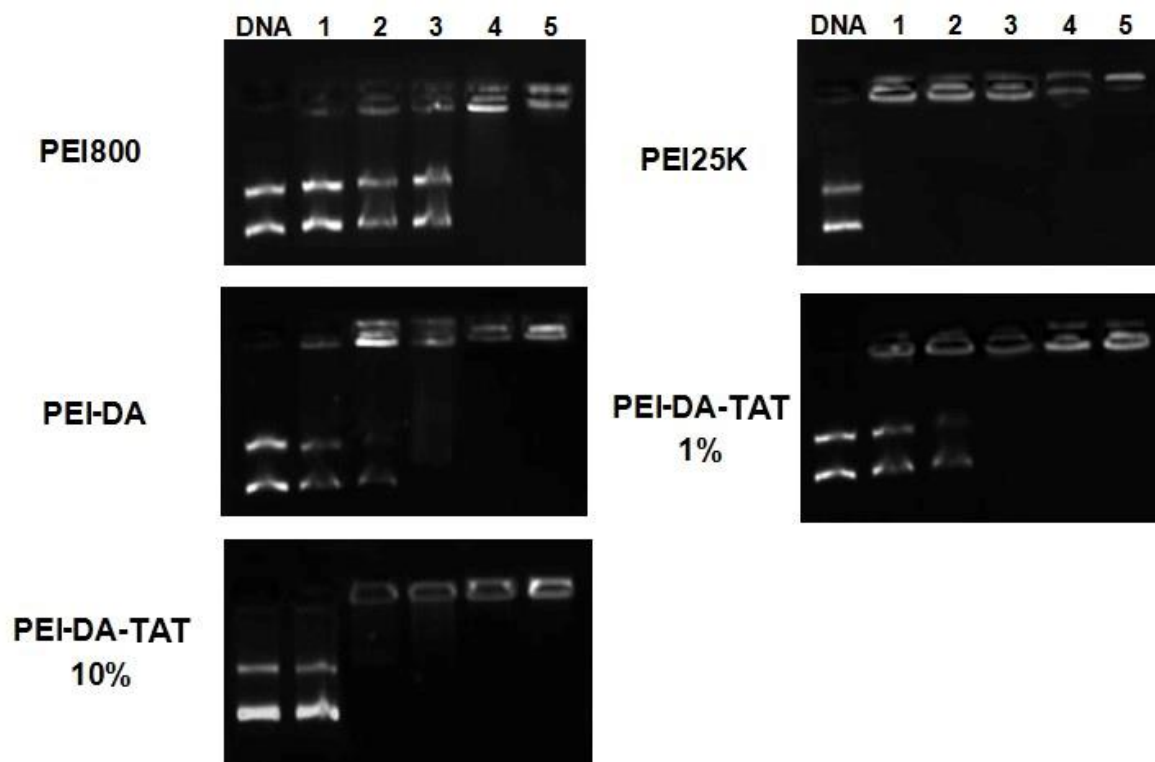
Figure 4.3 Evaluation of nucleic acid binding and protection capability of PEI800, PEI25K, PEI-DA, PEI-DA-TAT 1 % and PEI-DA-TAT 10 % at different N/P ratios (N/P ratios of 0.5, 1, 5, 10 and 50 from lane 1-5). The first lane, naked DNA, was used as reference. Exposure time: 400 ms.
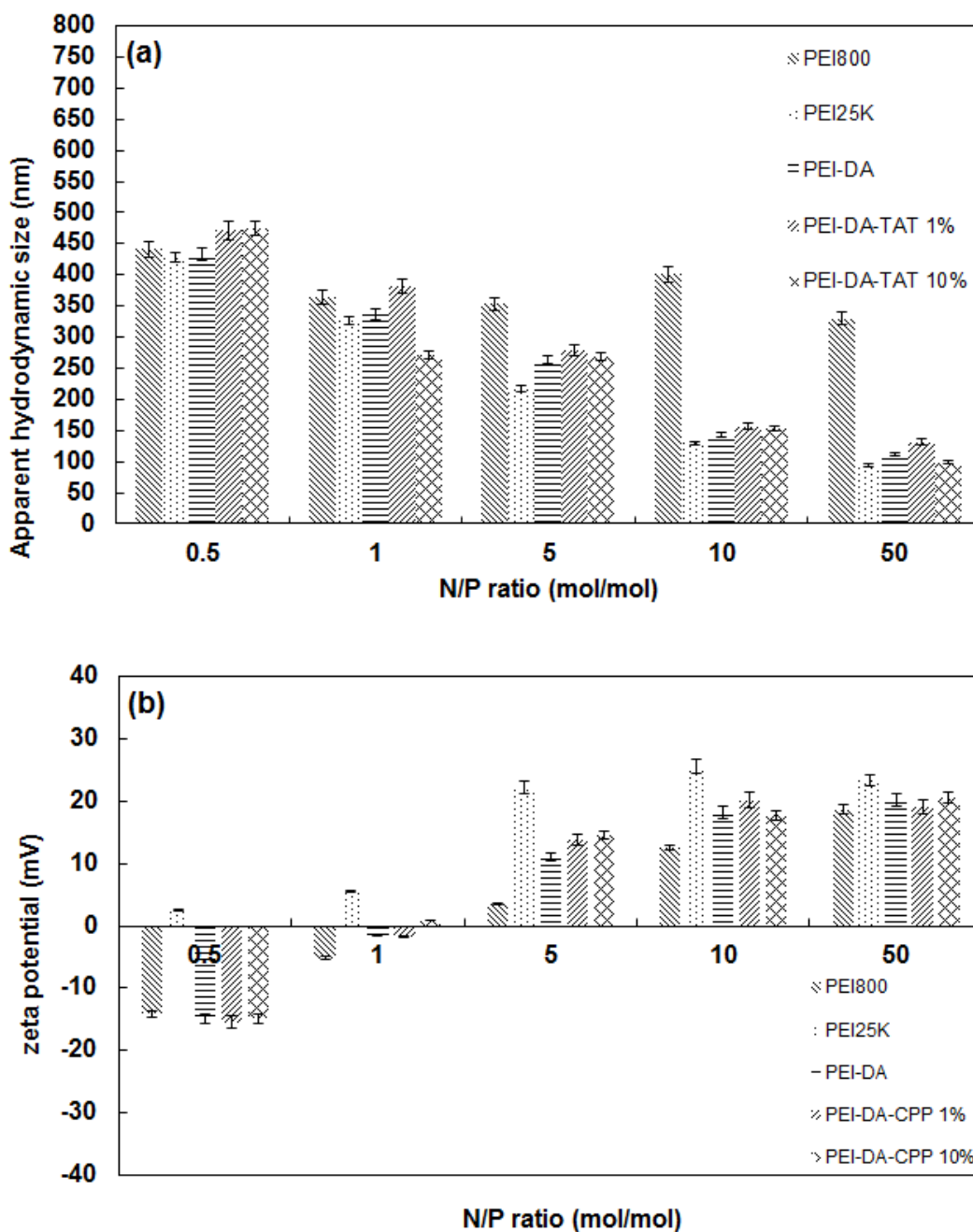
Figure 4.4 Apparent hydrodynamic sizes (a) and zeta potentials (b) of carriers/pDNA complexes prepared by mixing PEI800, PEI25K, PEI-DA, PEI-DA-TAT 1 % and PEI-DA-TAT 10 % with pDNA at different charge ratios (N/P) of carrier to pDNA at pH 7.0 and 25 °C. For all measurements, the pDNA concentration was fixed at 5 µg/ml.
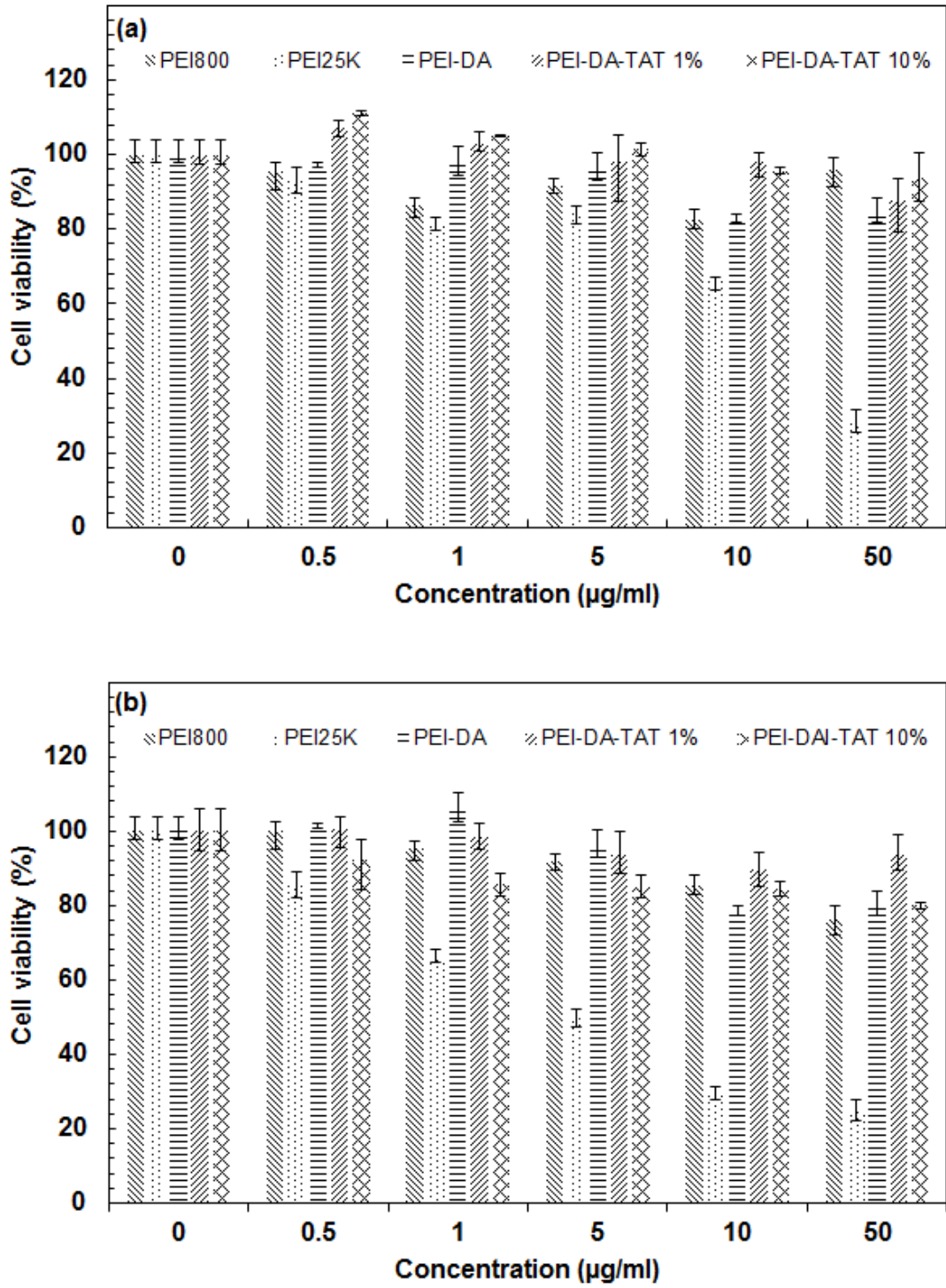
Figure 4.5 Cell viability of Hela cells (a) and HEK 293 cells (b) after exposing to PEI800, PEI25K, PEI-DA, PEI-DA-TAT 1 % and PEI-DA-TAT 10 %.
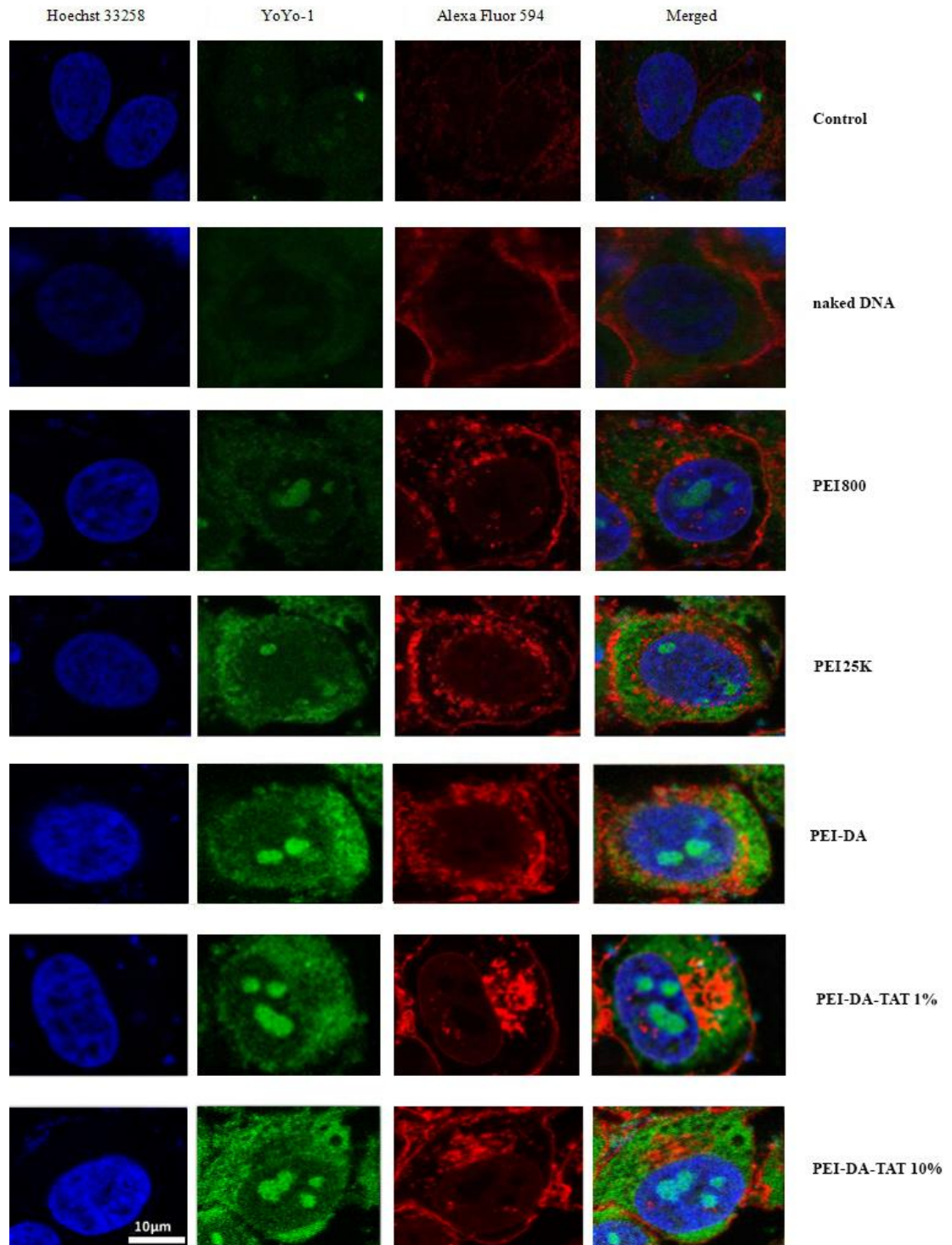
Figure 4.6 Hela cell uptake of YOYO-1-labelled pDNA complexed with different gene delivery vectors: PEI800, PEI25K, PEI-DA, PEI-DA-TAT 1 % and PEI-DA-TAT 10 % at an N/P ratio of 10. Mock cells were used as control, and naked pDNA was used as the reference.
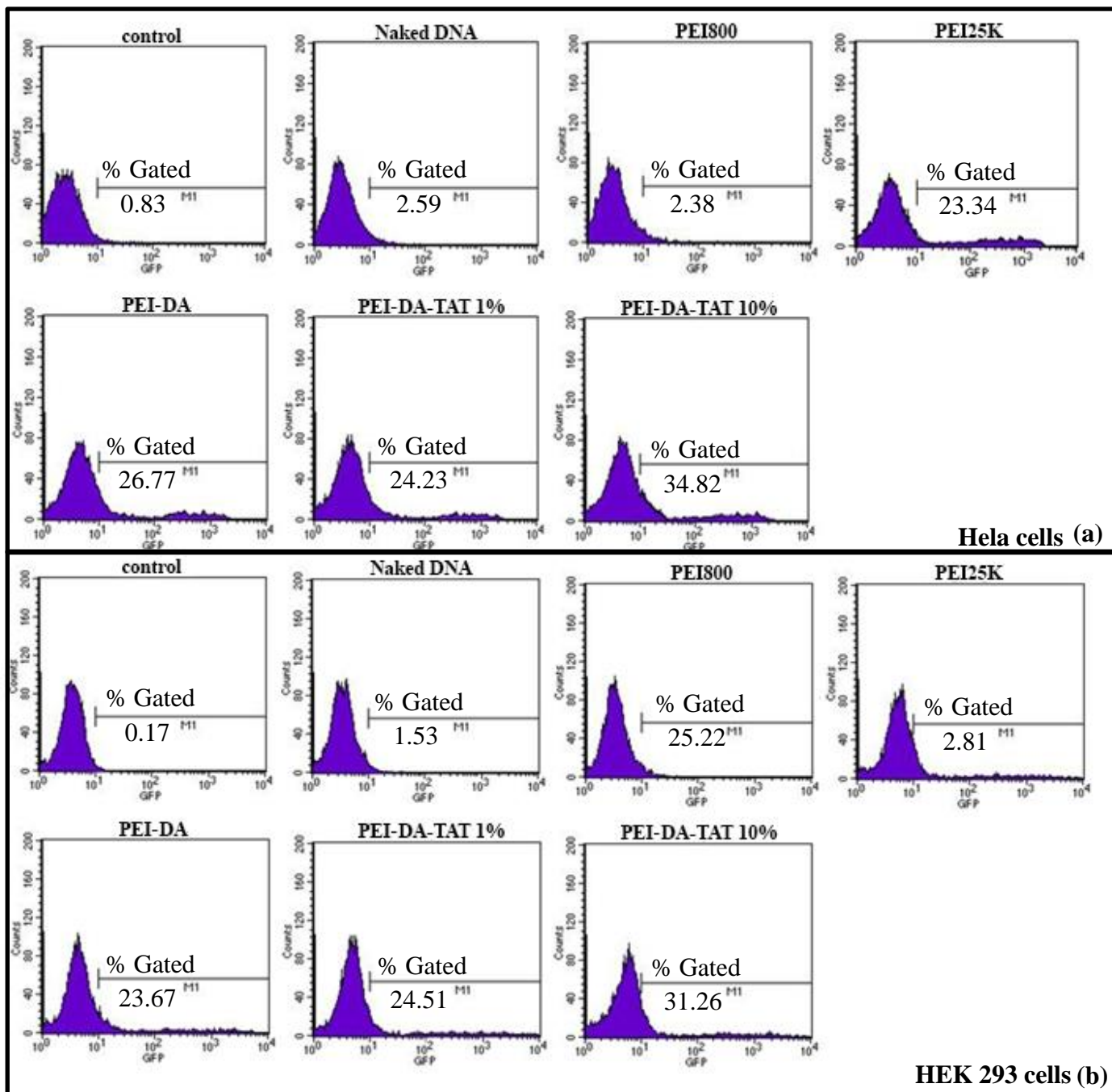
Figure 4.7 Flow cytometry of GFP expression by various polymer/pDNA complexes prepared by PEI800, PEI25K, PEI-DA, PEI-DA-TAT 1 % and PEI-DA-TAT 10 % at an N/P ratio of 10 in Hela cells (a) and HEK 293 cells (b). Mock cells were used as control, and naked pDNA was used as the reference.

Chapter 5 Conclusions and future directions

5.1 Conclusions

The evolution of therapeutic nucleic acids and genetic materials has promoted the development of gene therapy. However, the progress is prohibited by the lack of satisfied gene carriers. Various delivery systems have been developed to meet the requirements for the satisfied gene delivery based on two major concerns: 1. How to reduce the cytotoxicity of gene carriers to eukaryotic cells; 2. How to perform high efficient gene delivery process.

PEI25K has been proven to be a good candidature for the efficient gene delivery. However, the applications of PEI25K are greatly limited by the molecular weight related cytotoxicity to cells. Besides, the efficiency of PEI-based gene carriers is still lower than that of viral-based gene carriers. In order to solve these problems, two biodegradable PEI-based gene carriers were designed and successfully synthesized in this project.

To reduce the cytotoxicity of PEI-based gene carriers, an intracellular biodegradable PEI-based polymer (PEI-DA) was synthesized by crosslinking LMW PEI800 with disulfide bond containing linker, 4'4-dithiodibutyric acid. The synthetic polymer shows good biocompatibility, good gene condensing and protection ability as well as good buffering capacity. All of these make it a promising candidature as an efficient gene carrier. More importantly, the cytotoxicity of the synthetic carrier decreased significantly by over 50% compared with commercial PEI25K, while keep the similar transfection efficiency to PEI25K. These results indicate that the synthesized biodegradable PEI-DA is able to achieve the objective to reduce the cytotoxicity and remain the transfection efficiency.

Furthermore, in order to improve the transfection efficiency of PEI-DA, HIV-1 TAT peptide, one of the well-studied CPPs, was introduced to PEI-DA. After conjugating PEI-DA with TAT peptide, the physical and chemical properties of PEI-DA-TAT are found to be similar to

those of PEI-DA. No significant change was observed in the biocompatibility, gene condensing ability, buffering capacity and cytotoxicity between PEI-DA-TAT and PEI-DA. In addition, the transfection efficiency of PEI-DA-TAT is further improved from 26.77% to 34.82% in Hela cells and 23.67% to 31.26% in HEK 293 cells. It demonstrates that the conjugation of TAT peptide is able to improve the transfection efficiency of PEI-based gene carriers without changing their physical and chemical properties. This could be a promising strategy to improve the efficiency of gene delivery by PEI-based gene carriers.

5.2 Future directions

Based on the conclusions in this thesis, the modification of PEI-based gene delivery system is a promising method to develop the gene carriers for safe and high efficient gene delivery. However, the gene delivery process varies from different cell lines. In this case, more different cell lines are recommended to be measured for the gene delivery of the reported PEI-based carriers to gather a better understanding of the design and further development of PEI-based gene delivery system. Finally, in order to improve the specificity of PEI-based gene delivery system, targeting ligands such as folic acid or other ligands targeting to specific cells are recommended to introduce to the resulted PEI-based gene carriers to facilitate the applications of PEI-based gene carriers in targeting gene delivery. However, just like other well defined gene delivery carriers, the reported PEI-based gene delivery carriers still need to be examined *in vivo*, since the gene delivery *in vivo* is normally sophisticated. Therefore, the resulted PEI-based gene carriers need to be evaluated *in vivo* or even clinical trials in the future.