SUPRAMOLECULAR ARCHITECTURES FORMED BY COPOLYMERS AND CYCLODEXTRINS AND THEIR APPLICATIONS FOR GENE DELIVERY

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Cyclodextrins (CDs) are cyclic oligosaccharides, mainly consisting of 6, 7 or 8 α-D-glucopyranose units (α-CD, β-CD and γ-CD) linked by α-(1,4) bonds. These water-soluble, biocompatible oligosaccharides do not elicit immune responses and have low toxicity in animals and humans. The objective of this thesis was to develop novel CD-based cationic polymers and supramolecular architectures for efficient gene delivery. In this study, a series of novel cationic star polymers consisting of α-CD core and oligoethyleneimine (OEI) arms and cationic supramolecular polyrotaxanes composed of multiple OEI-grafted CDs threaded on various copolymer chains have been synthesized. Results indicated that these cationic polymers not only could efficiently compact plasmid DNA into small nanoparticles, but also showed excellent gene transfection efficiency and low in vitro cytotoxicity in various cell lines. Hence, they are promising non-viral gene delivery vectors and can be widely used in future gene therapy applications.

Keywords: Cyclodextrin, polyrotaxane, oligoethyleneimine, inclusion complexation, polyethyleneimine, non-viral gene delivery vectors
ACKNOWLEDGEMENTS

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SUMMARY

Cyclodextrins (CDs) are cyclic oligosaccharides, consisting mainly of 6, 7 or 8 α-D-glucopyranose units (α-CD, β-CD and γ-CD) linked by α-(1,4) bonds. These water-soluble, biocompatible oligosaccharides do not elicit immune responses and have low toxicity in animals and humans. Over the last few decades, they have been industrially used in many applications such as pharmaceuticals, fragrance, foods and so on. The appearance of CDs is like a doughnut and their inner cavity is hydrophobic. Thus, various guest molecules can be fitted into this hydrophobic cavity of CDs to form non-covalent inclusion complexes (ICs). The ability of CDs to form ICs has been exploited in CD drug formulations. The objective of this thesis was to develop novel cationic star polymers consisting of α-CD core and oligoethylenimine (OEI) arms and soluble, cationic supramolecular polyrotaxanes composed of multiple OEI-grafted CDs threaded on various copolymer chains for efficient gene delivery.

Cationic star polymers can be synthesized by grafting multiple OEI arms of different lengths and structure (ethylenediamine, pentaethylenehexamine, linear PEI with an average molecular weight of 423, and branched PEI with an average molecular weight of 600) to the activated hydroxyl groups of an α-CD ring. In addition, to prepare novel soluble, cationic supramolecular polyrotaxanes, a series of novel polyrotaxanes were synthesized in high yield with copolymers (PPG-PEG-PPG triblock copolymers, PEG-PPG-PEG triblock copolymers or PPG-ran-PEG copolymers) and CDs (α-CDs or β-CDs) based on the block-selected inclusion complexation between the copolymers and CDs, followed by conjugation of 2,4,6-trinitrobenzene sulfonate (TNBS) to both ends of copolymer chains as blocking stoppers. And then, various OEI chains with different chain length and structure
(ethylenediamine, pentaethylenehexamine, linear PEI with an average molecular weight of 423, and branched PEI with an average molecular weight of 600) were grafted onto the above polyrotaxanes to form cationic supramolecular polyrotaxanes with multiple OEI-grafted CDs, leading to the increase of the solubility of the polyrotaxanes. The resulting cationic, soluble star polymers and supramolecular polyrotaxanes were isolated and purified by size exclusion chromatography and their purity was determined by Gel Permeation Chromatography. Their compositions were further characterized by NMR.

Results indicated that all the cationic polymers could efficiently compact plasmid DNA into small nanoparticles, which was confirmed by agarose gel electrophoresis, particle size analysis, and zeta potential measurements, as well as atomic force microscopy imaging. They also showed excellent gene transfection efficiency that was comparable to or even higher than that of branched polyethylenimine (PEI) with molecular weight of 25 K, one of the most effective polymeric gene-delivery carriers studied to date. Moreover, they displayed much lower in vitro cytotoxicity than that of branched PEI (25 K) in various cell lines.

Hence, these novel soluble, cationic star polymers and supramolecular polyrotaxanes can be promising non-viral gene delivery vectors with high gene transfection efficiency and low cytotoxicity, and can be widely used in gene therapy applications.
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# ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ACE</td>
<td>Aminoethylcarbamoyl</td>
</tr>
<tr>
<td>AFM</td>
<td>Atomic force microscopy</td>
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<tr>
<td>CD</td>
<td>Cyclodextrin</td>
</tr>
<tr>
<td>CDI</td>
<td>1,1’-Carbonyldiimidazole</td>
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<tr>
<td>CS-12</td>
<td>N-Dodecylated chitosan</td>
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<td>D₂O</td>
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<td>DMAEA</td>
<td>2-Dimethylaminoethylamine</td>
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<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle’s Medium</td>
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<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<td>EGF</td>
<td>Epidermal growth factor</td>
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<tr>
<td>EtBr</td>
<td>Ethidium bromide</td>
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<td>GCP</td>
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<td>GPC</td>
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<td>KNOB</td>
<td>C-terminal globular domain of the fiber protein</td>
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<td>MuLV</td>
<td>Murine leukaemia viruses</td>
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<td>PPE-EA</td>
<td>Poly(2-aminoethyl propylene phosphate)</td>
</tr>
<tr>
<td>PPO</td>
<td>Poly(propylene oxide)</td>
</tr>
<tr>
<td>RLU</td>
<td>Relative light unit</td>
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<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>SEC</td>
<td>Size exclusion chromatography</td>
</tr>
<tr>
<td>Tf</td>
<td>Transferrin</td>
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<tr>
<td>THF</td>
<td>Tetrahydrofuran</td>
</tr>
<tr>
<td>TMO</td>
<td>Trimethylated chitosan oligomer</td>
</tr>
<tr>
<td>TNBS</td>
<td>2,4,6-Trinitrobenzene sulfonate</td>
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</tbody>
</table>


5. Chuan Yang, Xin Wang, Hongzhe Li, Suat Hong Goh, Jun Li. Synthesis and characterization of polyrotaxanes consisting of cationic α-cyclodextrin threaded on poly[(ethylene oxide)-*ran*-(propylene oxide)] as gene carriers. *Biomacromolecules*, Online published.
CHAPTER ONE

INTRODUCTION

1.1 Research background

Diseases are always one of the most serious threats against the existence of human beings. For centuries, different therapies have been developed to cure various diseases. Recently, gene therapy has been of growing interest in the therapy of some diseases, especially for most hereditary diseases.\(^1\)\(^-\)\(^12\) Gene therapy is a novel approach to treat, cure, or ultimately prevent disease by changing the expression of a person's genes. In other words, it can supply a gene that will lead to biosynthesis of the appropriate functional protein to remedy the defect(s) of the specific gene.\(^2\) However, the lack of efficient, safe and selective carriers for DNA to the nucleus of the target cells is a major limiting factor for the clinical application of gene therapy.

Over the last few decades, cationic polymers are receiving growing attention as gene-delivery carriers. Compared with other gene delivery systems (viral vectors and cationic lipids), cationic polymers for gene delivery are generally economical and
stable, and they can be produced in a large scale and show low host immunogenicity.

By now a great number of polycations have been reported to be able to deliver gene, including homopolymers or derivatives of polyethylenimine (PEI),\textsuperscript{13} poly(L-lysine),\textsuperscript{14} polyamidoamine dendrimers,\textsuperscript{15} Poly(β-amino ester),\textsuperscript{16} polyphosphoester,\textsuperscript{17,18} and chitosan,\textsuperscript{19,20} etc.

Cyclodextrins (CDs), which are cyclic, water-soluble, and biocompatible oligosaccharides, have low toxicity in animals and humans and do not elicit immune responses. They are quite notable due to their unique structures and properties. CDs mainly consist of 6, 7 or 8 $\alpha$-D-glucopyranose units ($\alpha$-CD, $\beta$-CD and $\gamma$-CD) linked by $\alpha$-(1,4) bonds (Figure 1.1).\textsuperscript{21,22} From X-ray structures it appears that the secondary hydroxyl groups (C$_2$ and C$_3$) in CDs are located on the wider edge of the ring and the primary hydroxyl (C$_6$) on the other edge and that the apolar C$_3$ and C$_5$ hydrogens and ether-like oxygens are at the inside of the torus-like molecules. This special structure brings CDs many distinct properties: CDs can dissolve in water because the outside of CDs is hydrophilic, and they can also provide a hydrophobic matrix because the inside cavity of CDs is apolar.\textsuperscript{23} As a result of this cavity, CDs are able to form inclusion complexes with a wide range of guest molecules. Hence, chemical modifications or conjugations and inclusion abilities are easily acquired due to the above chemical structures of CDs. Over the last few decades CDs and their derivatives have been industrially used in many applications such as pharmaceuticals, fragrance, foods and so on.\textsuperscript{21,24}

In 1999, Davis’s group published the first report of using conjugates of CDs in the
delivery of nucleic acid.\textsuperscript{25} Since then, a variety of CD-containing polymers have been developed for gene delivery. In these polymers, CDs either are modified to contain amines and serves as part of the backbone of linear polymers,\textsuperscript{25-27} or are pendent on a polycation (such as PEI) skeleton.\textsuperscript{28} In addition to offering satisfactory efficiency in mediating gene transfection, these CD-modified polycations usually display lower toxicities when compared with their pristine counterparts.

However, the application of CDs in gene delivery system has not been developed sufficiently till now; very little is known about the modification of CDs with oligoethylenimine (OEI) chains and CD-containing supramolecular polyrotaxanes based on inclusion complexation, which are developed and used as gene-delivery carriers.

\textbf{Figure 1.1} Schematic representations of $\beta$-CD. The open and closed arrows point to primary and secondary hydroxyl groups, respectively. The CD architecture is a cup that is $0.79\pm0.01$ nm from top to bottom (primary OH face to secondary OH face), and is slightly larger on the face containing secondary hydroxyl groups. The cavity ($0.47-0.53$, $0.60-0.65$ and $0.75-0.83$ nm for $\alpha$-, $\beta$- and $\gamma$-CD, respectively) and exterior diameters of the CDs ($1.46\pm0.04$, $1.54\pm0.04$ and $1.75\pm0.04$ nm for $\alpha$-, $\beta$- and $\gamma$-CD, respectively, for the faces containing secondary hydroxyl groups) expand as the number of glucopyranoside units increase.\textsuperscript{21,22}

\section*{1.2 Objectives and value of study}

The research reported here was carried out on the cationic star polymer consisting
of α-CD core and OEI arms, the supramolecular structures formed by various copolymers and multiple OEI-grafted CDs, and their applications in gene delivery systems. The specific objectives of this study were:

- To synthesize a series of cationic star polymers by conjugating multiple OEI arms of different lengths and structure ((ethylenediamine, pentaethylenehexamine, linear PEI with an average molecular weight of 423, and branched PEI with an average molecular weight of 600) to an α-CD core, which were isolated and purified using size exclusion chromatography (SEC), and characterize the star polymers using Gel Permeation Chromatography (GPC), $^{13}$C and $^1$H NMR, and elemental analysis;

- To prepare novel polyrotaxanes in high yield with various copolymers (PPG-PEG-PPG triblock copolymers, PEG-PPG-PEG triblock copolymers or PPG-ran-PEG copolymers) and different CDs (α-CD or β-CD) based on the block-selected inclusion complexation between the copolymers and CDs, followed by conjugation of 2,4,6-trinitrobenzene sulfonate (TNBS) to both ends of copolymer chains as blocking stoppers, and characterize them with $^1$H and $^{13}$C NMR;

- To conjugate various OEI chains with different chain length and structure (ethylenediamine, pentaethylenehexamine, linear PEI with an average molecular weight of 423, and branched PEI with an average molecular weight of 600) to the CD rings of the above polyrotaxanes, which were isolated and purified by SEC, and characterize the resulting cationic supramolecular polyrotaxanes using GPC,
13C and 1H NMR, and elemental analysis;

- To condense plasmid DNA (pDNA) into small nanoparticles with the above cationic polymers and confirm the condensation ability of the cationic polymers by agarose gel electrophoresis, particle size analysis, and zeta potential measurements, as well as atomic force microscopy (AFM) imaging.

- To determine the gene transfection efficiency and in vitro cytotoxicity of the complexes formed by the above cationic polymers and pDNA in a variety of cell lines.

This research should provide a series of new carriers for gene delivery systems and extend the applications of cationic supramolecular polyrotaxanes based on CDs to the field of gene therapy. Furthermore, this study could be helpful in clarifying how cationic polymers transfer DNA to the target nuclei.

1.3 Scope of study

This thesis covers the study on the novel cationic star polymers consisting of α-CD core and OEI arms and cationic polyrotaxanes formed by various copolymers and different CDs and their application as carriers in gene therapy. Other supramolecular structures such as rotaxanes, catenanes and polycatenanes and their applications are not considered.

For the various parts of the study, the key is the preparation of novel functional materials for gene transferring. In the following chapters, the preparation processes of these materials are described and their gene-delivery capabilities are determined.
1.4 References


CHAPTER TWO

POLYMERIC GENE DELIVERY VECTORS

2.1 Introduction to gene therapy and gene delivery vectors
2.2 Polyethylenimine
2.3 Poly(L-lysine)
2.4 Polyaminoamine dendrimer
2.5 Poly[(2-dimethylamino)ethyl methacrylate]
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2.10 Cyclodextrin-containing cationic polymers
2.11 References
2.1 Introduction to gene therapy and gene delivery vectors

Genes, which are carried on chromosomes inside cells, are the basic physical and functional units of heredity in every living organism. Humans have between 30,000 and 40,000 genes. They determine obvious traits such as hair and eye color, as well as more subtle characteristics, such as the ability of blood to carry oxygen. Even some complex characteristics, such as physical strength, are considered the result of interactions of a number of different genes along with environmental influences. Strictly speaking, a gene is defined as the segment of deoxyribonucleic acid (DNA) sequence corresponding to a single protein. (or to catalytic or structural RNA molecule for those genes that produce ribonucleic acid (RNA) but not protein). In other words, most genes can carry the instructions that allow cells to produce specific proteins, which are biological macromolecules comprising linear chains of amino acids that affect most of the chemical reactions carried out by the cells. Meanwhile, other genes can produce RNA molecules, which play key roles in protein biosynthesis and gene regulation.

When genes are altered or damaged (known as defective genes) and the encoded proteins are unable to carry out their normal functions, genetic disorders can result. In fact, each of us carry about half a dozen defective genes. Furthermore, most of us remain blissfully unaware that about one in ten persons in the world has, or will develop at some later stage, an inherited genetic disorder, and approximately 2,800 specific conditions are known to be caused by defects (mutations) in just one of the patient's genes.
Recently, growing interests have focused on gene therapy with the increasing knowledge of genes.\textsuperscript{3-15} Gene therapy is a novel approach to treat, cure, or ultimately prevent disease by inserting genes into an individual cell and tissues and changing the expression of the individual’s genes. In detail, the inserted genes will lead to biosynthesis of the appropriate functional protein to remedy the defect(s) of the specific gene. In addition, these genes can encode the protein that is not missing or dysfunctional in the patients to provide different therapeutic benefits. For example, the toxic proteins encoded by specific gene can be introduced into the tumor cell. These proteins can render the malignant cell more sensitive to the cytotoxic therapy than normal ones or protect the normal cells against drug toxicity.\textsuperscript{4}

In the past decades researchers have developed several methods to replace or repair the targeted defective genes: (a) A normal gene may be inserted into a nonspecific location within the genome to replace a nonfunctional gene. This approach is most common. (b) An abnormal gene could be swapped for a normal gene through homologous recombination. (c) The abnormal gene could be repaired through selective reverse mutation, which returns the gene to its normal function. (d) The regulation (the degree to which a gene is turned on or off) of a particular gene could be altered.\textsuperscript{6}

In general, a gene cannot be directly inserted into a person’s cell, and one of the most important factors for successful gene therapy is a carrier molecule, called a vector, which delivers the therapeutic genes into the patient’s target cells. Currently two types of gene delivery vectors have been developed: viral and non-viral.
Viruses are naturally evolved to infect cells and transfer their genetic materials into host cells. In fact, they have a tropism toward certain cell types because they bear molecules on their surface that can interact with membrane receptors and then enter the cells by endocytosis. By their mechanism of infection these viral vectors can easily be released in the cytoplasm by destabilizing or fusing its external coat with the membrane of the endosome. Once entered into the nucleus, the transgene held by the virus can be expressed temporally (episomal state of DNA) or in a stable way (integration into the cell genome). At present the viral vectors developed include retroviruses such as murine leukaemia viruses (MuLV) and lentiviruses, and DNA viruses such as adenoviruses, adeno-associated viruses, Herpes simplex viruses and lentiviruses, which are more efficient than non-viral vectors. However, there are several problems in use: toxicity and immunogenicity, restricted targeting of specific cell types, transient duration of gene expression, low capacity for foreign genes, difficulties in generation of sufficiently high viral titres, and high cost. For these reasons, many researchers have increasingly focused on non-viral vectors as an alternative to viral vectors.

Non-viral vectors mainly include cationic lipids (liposomes) and cationic polymers. Figure 2.1 shows the general procedure for most non-viral gene delivery systems: DNA condensation and complexation, endocytosis, and nuclear targeting/entry. Concretely, firstly negatively charged DNA molecules are condensed and/or complexed with cationic transfection reagents before delivery. Then, these complexes are taken up by cells, usually through endocytosis. Endocytosis is a multistep process
involving binding, internationalization, formation of endosomes, fusion with lysosomes, and lysis. The extremely low pH and enzymes within endosomes and lysosomes usually bring about degradation of entrapped DNA and associated complexes. Finally, DNA that has survived both endocytotic processing and cytoplasmic nucleases must then dissociate from the condensed complexes either before or after entering the nucleus. Once inside the nucleus, the transfection efficiency of delivered DNA is mostly dependent on the composition of the gene expression system.\textsuperscript{25-27} In this process there exist three major barriers to gene delivery: low uptake by target cells, inadequate release of DNA molecules with stability after endocytosis and poor nuclear targeting.\textsuperscript{25,28}

![Figure 2.1](image)

**Figure 2.1** Schematic drawing of DNA delivery pathways with three major barriers: low uptake across the plasma membrane, inadequate release of DNA molecules with limited stability, and lack of nuclear targeting. (A) DNA–complex formation. (B) Uptake. (C) Endocytosis (endosome). (D) Escape from endosome. (E) Degradation (edosome). (F) Intracellular release. (G) Degradation (cytosol). (H) Nuclear targeting. (I) Nuclear entry and expression.\textsuperscript{25}

Cationic lipids are amphiphilic molecules composed of three basic constituents: one or two fatty acid side chains (aryl or alkyl) as hydrophobic moiety, a linker and a hydrophilic amino group as polar headgroup. In aqueous media, the cationic
headgroup promotes interaction with DNA, whereas the hydrophobic moiety assembles into a bilayer vesicular-like structure (liposomes) in the presence of a helper lipid.\textsuperscript{29,30} Freeze-fracture electron micrographs and X-ray diffraction studies show that DNA is sandwiched between many liposomal particles.\textsuperscript{31,32} Recently, many species of cationic lipids have been developed: dioctadecylamidoglycylspermin (DOGS), $N$-[1-(2,3-dioleyloxy)propyl]-$N,N,N$-trimethylammonium chloride (DOTMA), 3-dioleyloxy-$N$-[2-(sperminecarboxamido)ethyl]-$N,N$-dimethyl-1-propanaminium trifluoroacetate (DOSPA), 1,2-dioleoyl-3-trimethylammonium propane (DOTAP), dimethyl dioctadecylammonium bromide (DDAB), dioleoyl phosphatidylethanolamine (DOPE), etc.\textsuperscript{33-47} But there are several problems have not been solved yet: after intravenous injection, some of the cationic lipids can activate the complement so that they are rapidly cleared by liver and spleen;\textsuperscript{48-50} certain proteins such as protein kinase C may also be affected detrimentally by cationic amphiphilies;\textsuperscript{51} toxic effect \textit{in vitro} and \textit{in vivo}, including changes of cells, such as cell shrinking, reduced number of mitoses and vacuolization of the cytoplasm.\textsuperscript{52,53} In addition, non-viral vectors are generally less efficient in delivering DNA and initiating gene expression as compared to their viral counterparts, particularly when used \textit{in vivo} to date.\textsuperscript{15,38}

Cationic polymers are another type of non-viral gene vectors and they have also received increasing attention in the past decade.\textsuperscript{54-67} Cationic polymers can also be classified into two groups: neutral polymers, such as proteins and peptides, e.g. poly(L-lysine) and poly(L-glutamic acid); and synthetic polymers, such as derivatives or homopolymers of polyethylenimine, polyphosphoester, polyamidoamine and
chitosan, etc. These cationic polymers will be reviewed in detail in the following sections.

## 2.2 Polyethylenimine

Polyethylenimine (PEI) is a polymer that has been known for a long time and which has been widely used in processes such as paper production (Polymin®) and wastewater (Epomin®), as well as shampoo manufacturing.\(^6\) This polymer is available in two main forms: linear and branched (Figure 2.2). The branched form is produced by acid-catalyzed polymerization of aziridine monomers, resulting in random branched polymers. Linear forms of PEI are attainable by a similar process, but perform at lower temperature.\(^6\)\(^9\)\(^7\)\(^0\)

In 1995, PEI was first introduced by Behr as gene-delivery carrier,\(^7\)\(^1\) and then it has become one of the gold standards of non-viral gene delivery. Although PEI is available in a broad range of molecular weights (from <1 K to 1600 K), often highly branched PEIs with molecular weight in the range from 25 K to 800 K and linear PEI 22 K are most frequently used in gene delivery.\(^7\)\(^2\)-\(^7\)\(^4\) These PEI/DNA complexes yield
high levels of transgene expression in many cell lines and primary cells under *in vitro*\textsuperscript{75,76} and *in vivo*\textsuperscript{71,77-81} conditions.

The relatively high gene transfection efficiency of PEI is believed to be due in large part to efficient escape from the endocytic pathway through the proton-sponge mechanism. As mentioned above, the endocytic pathway is one of three major barriers to gene delivery, since it leads to the intracellular degradation of DNA. PEI has a high density of amino groups due to every third atom on the PEI backbone being a nitrogen atom. In linear PEI, all of these nitrogen atoms can be protonated, whereas in branched PEI, only two-thirds of them (primary and secondary amino groups) can be charged.\textsuperscript{59} At physiological pH only 15-20\% of the above chargeable nitrogen atoms are protonated.\textsuperscript{82} This unique property makes PEI an extraordinary strong proton sponge. In the presence of PEI in endosome, it has been proposed that endolysosomes are acidified by the action of an ATPase enzyme that actively transports protons from the cytosol into the vesicle. Then, PEI undergoes large changes in protonation during endocytic trafficking. However, the proton-sponge PEI prevents acidification of endocytic vesicles, causing the ATPase to transport more protons to reach the desired pH. The accumulation of protons in the vesicle must be balanced by an influx of counter ions (chloride ions). The increased ion concentration ultimately causes osmotic swelling and rupture of the endosome membrane, which releases the PEI/DNA complexes into the cytosol (Figure 2.3). In brief, PEI enhances intracellular trafficking by buffering the endosomal compartments, thus protecting the DNA from lysosomal degradation by endosomal DNA release via lysosomal disruption.\textsuperscript{7,54,83-85}
High cationic charge density of PEI also contributes to the formation of stable and highly condensed particles (spherical, globular or rod-like structures) by interacting with DNA. This process is supposed to rely predominantly on electrostatic interactions. It is found that the particle size is related to the efficiency of endocytosis and the velocity of cytoplasmatic movement. The complexation of DNA with PEI protects DNA from enzymatic degradation by charge neutralization in subcellular compartments, and promotes DNA movement in cytosol and finally, the nuclear localization for transgene expression. The binding of PEI to DNA is thought to be mainly driven by entropic forces arising from the release of counter ions. The high charge density of PEI enables more counter ions to be released upon binding with DNA, thus forming more stable complexes. In addition, the structure of PEI has a great influence on the formation and the particle size of PEI/DNA complexes. For either branched or linear PEI, it has been found that the molecular weight of PEI affects both condensation behavior and complex size: generally, the complex size decreases with the increase of the molecular weight of PEI in the range of 2-25 K.
Also, complex formation is dependent on the degree of polymer branching. Primary amino groups are known to condense DNA better than other amino groups due to their higher protonation at a given pH. The binding capability of PEI could be correlated to the number of primary amino groups and the complex stability increases with primary amino groups content, thus leading to a higher transfection efficiency.

Beside particle size, the surface charge of PEI/DNA complexes is also an important parameter determining transfection efficiency, as well as other interactions of the complexes. An excess of polycation, as usually used for efficient condensation and tight compaction of DNA, leads to a net positive surface charge of the PEI/DNA complex. The positive surface charge can be measured as a high positive zeta potential, and it has already been found that this positive surface charge usually serves to bind to cells via electrostatic interactions with the negatively charged cell membrane during the cellular uptake process, followed by pinocytosis or endocytosis. This process is also referred to as non-specific adsorptive endocytosis. For the complexes of PEI/DNA, at the N/P ratios (the ratio of amino group in polymer to phosphate group in plasmid DNA is defined as N/P ratio) usually used for complete complexation, they have a zeta potential in the range of 30-35 mV. Moreover, there seem to be no major differences in zeta potential between DNA complexes using different PEIs.

However, the huge amount of surface positive charge also results in a rather high toxicity of PEI, which is one of the major limiting factors especially for its in vivo use. Indeed, positively charged complexes can induce erythrocyte aggregation and
trigger the activation of the complement system and removal by the reticulo-endothelial system.\textsuperscript{48} They also have intracellular toxicity — PEI has recently been identified as an apoptotic agent.\textsuperscript{102,103} Although the mechanism has not been fully delineated, it has been found that the cytotoxicity is affected by polymer architecture with increased toxicity observed with an increase in molecular weight and increased branching of the macromolecule.\textsuperscript{68,72,104} Also, the cationic surface charge of the complexes results in numerous unspecific interactions with negatively-charged cellular blood component, vessel endothelia and plasma proteins (such as albumin, fibronectin, immunoglobulines, complement factors or fibrinogen).\textsuperscript{98} These interactions lead to very short plasma half-lives.\textsuperscript{105}

Some researchers ascribe the toxicity of the complexes to excess free PEI in the complexes, but purification of the complexes not only is shown to decrease the cytotoxicity as a result of the removal of excess PEI, but also leads to a decrease in transfection efficiency. In other words, the free PEI substantially contributes to efficient transfection and mediates toxic effects in a dose-dependent manner at the same time. This effect is attributed to the ability of the free polymer to propagate endosomal release, an assumption supported by the fact that the transfection efficiency is re-established after the addition of free PEI.\textsuperscript{106} In addition, an excess of polycation is essential to generate a hydrophilic cationic corona around the complex for sufficient solubilization. Although PEI and DNA alone show excellent aqueous solubilities, their complexes become insoluble at a neutral charge.\textsuperscript{107}

Hence, the structure of PEI has been extensively modified and many novel PEI
derivatives and conjugates have been synthesized as efficient gene-delivery carriers. The aim of these modifications is to reduce cytotoxicity, improve the transfection efficiency, or increase the solubility, biodegradability or chemical homogeneity of PEI.

One of the first and most extensively investigated attempts to modify PEI is the covalent coupling of poly(ethylene glycol) (PEG) chains to the polymer, resulting in block or graft copolymers. Figure 2.4 shows the preparation strategies for the PEGylation of ligand-containing PEI/DNA complexes. To provide such complexes with virus-like transport domain, cell-binding ligands such as transferrin (Tf) or epidermal growth factor (EGF) are covalently linked to the particles, and receptor-specific interaction and endocytosis into target cells is demonstrated (Figure 2.4a). As shown in Figure 2.4b, the PEGylation has been achieved either by condensing DNA with PEG-PEI copolymers (pre-PEGylation) or coupling a PEG layer onto the surface of preformed complexes (post-PEGylation). In contrast to pre-PEGylation methods, post-PEGylation suffers from a rather time consuming sequence of conjugation steps that must be performed on PEI/DNA complexes. In general, the modification of the complexes with PEG results in shielding of the positive surface charges and create a steric barrier against self-aggregation and unfavorable interactions with plasma proteins, and with cellular components in the bloodstream. That is, the shielding of PEG may reduce the undesired and unspecific interactions between the complexes and blood components or non-target cells due to cationic surface charges. Also, modification with PEG improves colloidal
properties of the complexes of PEG-modified polycations and DNA, such as a neutral zeta potential, low cytotoxicity, and little or no tendency for aggregation \textit{in vitro}.\textsuperscript{115} However, attachment of PEG and other steric stabilizers to polycations may interfere with cellular processing, resulting in reduced transfection efficiency \textit{in vivo}, especially at lower doses.\textsuperscript{116-118}

Besides PEG, many polymers have also been used for the surface shielding of PEI/DNA complexes. For example, Pluronic copolymers, which possess the PEO-PPO-PEO structure, are surface-active and capable of self-assembly into micelles in aqueous solutions. It has been found that the Pluronic-PEI copolymer/DNA complexes are sufficiently stable in solution despite complete neutralization, and exhibit elevated levels of transgene expression.\textsuperscript{119}

In addition, various other derivatives and conjugates of PEI with polyacrylic acid (PAA),\textsuperscript{120} dextran\textsuperscript{121} or dextran sulfate,\textsuperscript{122} poly(N-2-hydroxypropyl)methacrylamide) derived copolymers (PHPMA),\textsuperscript{123,124} as well as plasma proteins like transferrin\textsuperscript{125,126} or human serum albumin\textsuperscript{127} etc, have been synthesized in recent years. The aim is either to improve one particular aspect of PEI-mediated transfection (increase solubility of the complexes, enhance endosomolytic activity, improve nuclear transport, and so on) or gain a better insight into the mechanism of transfection (and thus to identify which properties are required to obtain high transfection efficiencies).\textsuperscript{128}
Figure 2.4 (a) Schematic presentation of ligand-containing and shielded PEI/DNA complexes for gene transfer. (b) Preparation strategies for the PEGylation of ligand-containing PEI/DNA complexes. Strategy A: a ligand is covalently conjugated to PEI. Condensation of DNA with this conjugate leads to ligand-containing DNA complexes (step 1) which are subsequently modified with a PEG derivative reacting with free amino groups of the polycation (step 2). Strategy B: DNA is condensed with PEI (step 1) and the resulting complexes are modified by a heterobifunctional PEG derivative which first reacts with amino groups of the polycation (step 2). Subsequently, ligands are incorporated into the complexes by covalent conjugation with the distal end of the polycation (step 3). Strategy C: in a one-step process, DNA is complexed with a mixture of unmodified PEI and pre-synthesized PEG-PEI and ligand-PEG-PEI conjugates.108

2.3 Poly(L-lysine)

Poly(L-lysine) (PLL) is one of the first cationic polymers employed for gene transfer. In 1987, Wu et al. conjugated PLL with asialoorosomucoid, a ligand for the asialoglycoprotein receptor, for hepatocellular gene targeting.129 PLL is a linear polypeptide with the amino acid lysine as the repeat unit (Figure 2.5); thus, it
possesses a biodegradable nature. This property is very useful for in vivo applications. However, the polymer exhibits modest to high toxicity. The PLL/DNA complexes are not only prone to aggregation under physiological conditions, but are also rapidly bound to plasma proteins and cleared from the circulation. To overcome these drawbacks, PEG or dextran has been conjugated to the polymer.

![Chemical structure of Poly(L-lysine)](image)

**Figure 2.5** Chemical structure of Poly(L-lysine)

Targeting ligands, such as transferrin receptor, epidermal growth factor receptor, CD3-T cell receptor, folate receptor and so on, are also linked to the polymer chain (even in early studies) resulting in enhanced transfections. Nevertheless, the naturally occurring receptor ligands are either proteins or complex carbohydrates, which are extremely difficult to obtain consistently in high purity (>98%). These receptor ligands are usually covalently crosslinked to poly(L-lysine), thereby creating novel antigenic epitopes.

Although some PLL/DNA complexes have small particle size and are taken up into cells as efficiently as PEI/DNA complexes, their transfection efficiencies remain several orders of magnitude lower. A potential reason for this is the lack of amino groups with a pKₐ between 5 and 7, thus allowing no endosomolysis and low levels of transgene expression. In other words, different from PEI, at physiological pH the
N-atoms of PLL are nearly fully protonated, resulting in poor escape from the endocytic pathways. The inclusion of targeting moieties or co-application of endosomolytic agent like chloroquine\textsuperscript{141} or fusogenic peptides\textsuperscript{142} may improve reporter gene expression. Another approach can create the desirable proton sponge effect similar to that of PEI/DNA complexes by introducing histidine or other imidazole-containing structures to PLL backbone, thus possessing a buffering capacity in the endolysosomal pH range.\textsuperscript{143} Histidylated PLL shows better transfection efficiency than PLL/chloroquine mixture.\textsuperscript{144}

Overall, it seems unlikely that poly(L-lysine)-based complexes with DNA will find clinical applications because of their relative low transfection efficacy. Hence, poly(L-lysine) has been relegated to a role in mechanistic studies or as a point of comparison to more promising polymers in recent years.

2.4 Polyaminoamine dendrimer

Dendrimers are spheroidal and cascade polymers consisting of a central core molecule which acts as the root from which a number of highly branched, tree-like arms originate in an ordered and symmetric fashion. The degree of branching is expressed in the generation of the dendrimer. Currently the synthesis methods of dendrimers include divergent strategy (starting from a central core) and convergent strategy (starting with what will become the periphery of the molecule building inwards).

The polyaminoamine (PAMAM) dendrimers are polymers containing both tertiary
amines at the branch points as well as primary amines at the termini (Figure 2.6). They are normally based on an ethylenediamine or ammonia core with four and three branch points, respectively. They are synthesized by the divergent method, involving an exhaustive Michael addition of ethylenediamine with methyl acrylate, followed by addition of the resulting ester core to an excess of ethylenediamine. This synthesis gives a high yield of first-generation PAMAM dendrimers, with higher generations arising from repetition of the previous two steps (e.g. G3, G4…). Generation up to 10 can be formed before further assembly is limited by the “de Gennes dense packing” phenomenon. The dendrimers so prepared have a consistent size, structure, and charge characteristic of their generation, i.e. these polymers are well-defined and chemically very homogeneous. In fact, their monodispersity is remarkable, with polydispersity values ($M_W/M_n$) in the range from 1.000002 to 1.005.

Due to the high cationic charge density of primary amine groups on the surface of the polymer, PAMAM dendrimers have been proposed as DNA delivery agents. Haensler and Szoka were first to report the use of PAMAM dendrimers for gene delivery. They found that the sixth-generation dendrimer was better than higher and lower generations by about 10-fold. In fact, PAMAM dendrimers of generation G3 to G10 are all found to form stable complexes with DNA. However, their ability to transfec different cell lines varies, and polymer structure does not directly correlate with the transfection efficiency.

Because of the dendrimer structure, PAMAM dendrimers show high densities of amino groups in the periphery of the molecules. Hence, the complexation process
between dendrimers and DNA does not seem to differ fundamentally from other cationic polymers with high charge density: the terminal primary amino groups of dendrimers interact with the phosphate groups of DNA to form complexes, which protect DNA from degradation.\cite{62,149} The interaction between dendrimer and DNA is based on electrostatic interaction\cite{150} and lacks any sequence specificity.\cite{62} Also, similar to PEI, not all the amino groups of PAMAM dendrimers are protonated at physiological pH, and the lower pKa of the dendrimers cause them to act as proton sponges, enabling more efficient endosomal escape.\cite{83} Furthermore, the addition of cationic excipients such as diethylaminoethyl-dextran (DEAE-dextran) can enhance transfection efficacy.\cite{151}

![Chemical structure of a Polyaminoamine dendrimer (generation 1 and core is ethylenediamine)](image)

Figure 2.6 Chemical structure of a Polyaminoamine dendrimer (generation 1 and core is ethylenediamine)

The high surface positive charges are also related to the dendrimer toxicity. Studies on a solution of rat blood cells shows that a generation dependence of PAMAM dendrimers is observed in haemolysis and changes of erythrocyte morphology.\cite{152}
Moreover, the degree of substitution and the type of amine functionality is important, with primary amines being more toxic than secondary or tertiary amines. The cytotoxicity of the polymers is also found to be generation-dependent, with higher generation dendrimers being the most toxic. Shielding of surface groups has also been used successfully to reduce toxicity, e.g. through covalent attachment of PEG or fatty acids. The modification of terminal groups has been suggested to be more efficient for higher generations dendrimers, as the relatively higher density of non-toxic surface groups may also be more effective in preventing access to potentially toxic core.

In addition, partially degraded PAMAM dendrimers (i.e. a high molecular weight dendrimer missing some branches designated “fractured dendrimer”) seem to be more effective than that of intact dendrimers. Heat treatment of the polymer in some solvents (for example, water or ethanol) causes solvolysis of some amide bonds, resulting in a heterodisperse population of fractured dendrimers that increase transfection efficacy up to 50-fold. The mechanism of the enhancement seems to be twofold. First, the fractured dendrimers have greater flexibility, allowing a more beneficial interaction with the plasmid DNA. Second, fractured PAMAM dendrimer/DNA complexes seem to have enhanced solution stability in comparison to intact PAMAM dendrimer/DNA complexes, which tend to aggregate.

2.5 Poly[(2-dimethylamino)ethyl methacrylate]

Poly[(2-dimethylamino)ethyl methacrylate] (PDMAEMA) is a synthetic polymer
containing side chains with tertiary amino groups (Figure 2.7). It is water-soluble and can form compact complexes with DNA by electrostatic interaction.\textsuperscript{158} The particle size and zeta potential of the PDMAEMA/DNA complexes are dependent on the ratio of the polymer to DNA. Also, as a result it has been found that the complexes with a slightly positive zeta potential (25-30 mV) and a size around 200 nm possess the highest transfection potential.\textsuperscript{159,160} Compared with DEAE-dextran and poly(L-lysine), the transfection efficiency of these complexes is two-fold higher than DEAE-dextran/DNA complexes and eight times than PLL/DNA complexes in the medium in the presence of chloroquine. The cytotoxicity of PDMAEMA is lower than that of PLL, but higher than that of DEAE-dextran.\textsuperscript{160} Interestingly, the transfection efficiency of the complexes is not affected by the presence of serum proteins, even though the presence of serum is known to adversely affect transfection efficiency in other cases.\textsuperscript{160}

![Chemical structure of Poly[(2-dimethylamino)ethyl methacrylate]](image.png)

\textbf{Figure 2.7} Chemical structure of Poly[(2-dimethylamino)ethyl methacrylate]

Earlier workers proposed that PDMAEMA, which like PEI was only partially protonated at physiological pH, facilitated cell transfection by being endocytosed, complexed with DNA, and subsequently acting as a “proton sponge” to burst endosomes/lysosomes and release DNA to the cytosol. It also seemed feasible that the cytotoxicity of PDMAEMA might result from lysosomal bursting, which can induce
cell death. However, the experiment results from Dubrul’s and Jones’s groups are different from the above hypothesis. Dubrue162 et al. synthesized a series of polymethacrylates and found that the buffering properties of the synthetic polymers are not necessarily the decisive element in the transfection process: the polymers containing imidazole groups or acid functions show buffering properties in the pH range of the endosomes (pH 5.5-7.4), in analogy with the “PEI proton sponge”, but they have a lower transfection capacity, probably due to a restricted cell uptake.162,163 Jones and co-workers also found that, although PDMAEMA affected the morphology of late endosomes/lysosomes, it did not physically disrupt them to release their contents to the cytosol. Hence, a more detailed study on the membrane interaction properties and the intracellular trafficking of the PDMAEMA/DNA complexes is needed.164

2.6 Polyphosphazene

Polyphosphazenes are biodegradable cationic polymers, which have been used as synthetic gene-delivery carriers in the past few decades. These biodegradable polymers show lower cytotoxicity and comparable in vitro transfection activities as compared to the non-degradable counterparts. Furthermore, the degradation properties of these polymeric vectors can be employed as a valuable tool to regulate the unpacking and release of the DNA inside the cells.

Polyphosphazenes (Figure 2.8) bearing cationic moieties are synthesized from poly(dichloro)phosphazene, which in turn is obtained by thermal polymerization of
hexachlorocyclotriphosphazene in 1,2,4-trichlorobenzene. Next, either 2-dimethylaminoethanol (DMAE) or 2-dimethylaminoethylamine (DMAEA) side groups are introduced by a substitution reaction.

![Chemical structures of (a) poly(DMAE)-phosphazene, and (b) poly(DMAEA)-phosphazene.](image)

**Figure 2.8** Chemical structures of (a) poly(DMAE)-phosphazene, and (b) poly(DMAEA)-phosphazene.

These polymers are positively charged at physiological pH due to the tertiary amines on the side groups, thus they are able to condense DNA to form positively charged particles. In COS7 cells, the transfection efficiencies of both polyphosphazenes are comparable to PDMAEMA, but their cytotoxicity is lower than that of PDMAEMA. More interestingly, the transfection efficiency of poly(DMAE)-phosphazene is three-fold higher in the absence of serum than in the presence of serum, probably due to the unfavorable interactions between polymer/DNA complexes and serum proteins. In contrast, poly(DMAEA)-phosphazene shows a three-fold lower transfection efficiency in the absence of serum. In addition, it is observed that the polymers can degrade in time under physiological conditions due to hydrolysis of the biodegradable polyphosphazene backbone.\(^{165}\) The results of *in vivo* tumor transfection test of poly(DMAEA)-phosphazene show that the gene expression is primarily confined to tumor tissue, which is remarkable since few cationic
polymers have this property without shielding.\textsuperscript{166}

### 2.7 Poly(β-amino ester)

Linear poly(β-amino ester)s (PAEs), another class of safe, hydrolytically biodegradable polymers for gene delivery, have both tertiary amines and esters in their backbones. The traditional development process of new biomedical polymers includes that polymers are designed one at a time and then individually tested for their properties. Different from this iterative process, linear PAEs can be synthesized by a combinational synthetic strategy, via the conjugation addition of primary amines or bis(secondary amine)s to diacrylates (Figure 2.9). The wide variety of commercially available diamines and diacrylates facilitates parallel synthesis of structurally diverse libraries of polymeric biomaterials. Furthermore, these new synthetic vector families can be screened in cell-based assays without leaving the solution phase.\textsuperscript{167}

![Figure 2.9 Synthesis of linear poly(β-amino ester)s (PAEs) by the conjugate addition of primary or bis(secondary amines) to diacrylates.\textsuperscript{168}](image)

Langer and co-workers have already synthesized several libraries, thousands of PAEs with the semi-automated, solution-phase parallel synthesis.\textsuperscript{167-172} The high-throughput synthesis and screening of these materials not only result in several hundred of polymers that transfect as well as or better than the current gold standard.
— PEI, but also obtain large amounts of structure-function information and identify several structures common in effective polymers. They have also found that polymer molecular weight, polymer chain end-group and polymer/DNA ratio have a significant impact on gene transfer. Through the control and optimization of these parameters, the polymers can successfully mediate gene transfer at levels that surpasses both PEI and Lipofectamine 2000 \textit{in vitro}.\textsuperscript{171-173}

However, in general, linear cationic polyester polymers are reported to degrade very fast in aqueous solution and to release DNA from polymer/DNA complexes within a few hours. This means that the application of linear polyester polymers could be limited for long duration because of its fast degradation rate. Hence, several hyperbranched\textsuperscript{174-176} or crosslinked\textsuperscript{177-179} poly(\(\beta\)-amino ester)s with slow degradation have been developed as gene-delivery carriers recently.

\section*{2.8 Polyphosphates and Polyphosphorimidates}

Both polyphosphates (PPEs) and polyphosphorimidates (PPAs) are water-soluble and cationic polyphosphoesters. These polymers with repeating phosphoester bonds in the backbone are structurally versatile, and biodegradable through hydrolysis and possibly enzymatic digestion at the phosphoester linkages under physiological conditions.\textsuperscript{180}

Polyphosphates (PPEs) bear a phosphoester backbone and the positive charges are introduced by linking residues containing amino groups to the phosphoryl side chain via phosphorus ester bonds (Figure 2.10). The PPEs are appealing for gene delivery
applications because of their potential biocompatibility and similarity to biomacromolecules such as nucleic acids. In 2001, poly(2-aminoethyl propylene phosphate) (PPE-EA) with a β-aminoethoxy side chain was designed and synthesized in high molecular weight and investigated as gene-delivery carrier.\(^{181}\) PPE-EA is designed to have nontoxic building blocks and it can be hydrolyzed to generate α-propylene glycol, phosphate and ethanolamine in physiological condition. Hence, the polymer has significantly low cytotoxicity at a concentration up to 0.1 mg/mL compared to PEI and PLL. PPE-EA mediates a higher level of gene expression, for example, in HEK293 and at the optimal charge ratio PPE-EA transfects cells much more efficiently than PLL. Nevertheless, the transfection efficiency greatly depends on cell type.\(^{181}\)

![Chemical structures of some polyphosphates (PPEs)](image)

**Figure 2.10** Chemical structures of some polyphosphates (PPEs)

A unique feature of PPEs is the capability of controlled release of plasmid from PPE/DNA complexes, achieved as a result of PPE’s degradation. It has been found the side chain structure is a crucial factor determining the mechanism and kinetics of hydrolytic degradation of PPEs.\(^{182}\) Previous work by Penczek demonstrated that the degradation of polyphosphoester with a methoxy or ethoxy side chain was considerably slower.\(^{183}\) However, when the ethoxy side groups are replaced with ethoxyl amino groups, PPE-EA can degrade to oligomers and fail to condense pDNA.
due to the decrease of the net positive charges. Different from PPE-EA, nevertheless, PPE-HA with longer alkylamino groups, degrades much more slowly in PBS. The relatively rapid degradation of PPEs with cationic side groups suggests a self-catalytic degradation mechanism involving nucleophilic attack of the phosphate bonds by the pendent amino groups. This mechanism also leads to a cleavage of the side chain, yielding negatively charged phosphate ions instead.\textsuperscript{180} This result has already been observed during the degradation of PPE-MEA.\textsuperscript{182} Degradation of these PPEs involving the cleavage of the backbone along with the side groups, generating less positively charged products, contributes to the controlled release of pDNA. Moreover, the higher hydrophobicity imparted by the longer alkyl side chain could hinder the nucleophilic attack by hydroxyl ion, resulting in slow degradation kinetics.\textsuperscript{182} Hence, the degradation mechanism, particularly the side chain cleavage, seems to be the dominant factor affecting the DNA release rate of PPE/DNA complexes. This controlled release property can be adjusted by varying N/P ratio as well as the molecular design of the carrier, which in turn can also influence the transfection \textit{in vivo} and \textit{in vitro}.\textsuperscript{182,184,185}

Polyphosphoramidates (PPAs) are series of polymers with a phosphoester backbone containing different charge groups in the side chain connected to the backbone through a phosphoramide bond (Figure 2.11). These PPA carriers with lower cytotoxicity compared with PEI and PLL show charge-dependent transfection abilities and DNA binding capacities.\textsuperscript{186-188} PPA-SP, PPA bearing spermidine side chain, was firstly prepared as a non-viral vector for gene delivery in 2002. It has been
found that gene expression mediated by PPA-SP is greatly enhanced when endolysosomolytic reagent such as chloroquine is used in conjunction. Under the optimized condition, PPA-SP/DNA complexes yield a luciferase expression level closed to PEI (25 K)/DNA complexes in HEK293 cells.\textsuperscript{186}

![Figure 2.11 Chemical structures of some polyphosphoramidates (PPAs)](image)

In fact, the pendent charge groups of PPAs have a great influence on the transfection efficiency: PPAs with primary amino group (PPA-SP and PPA-EA) are the most efficient in transfecting several cell lines,\textsuperscript{186,187} while other PPAs with secondary, tertiary and quaternary amino groups only achieve moderate or low levels of gene expression.\textsuperscript{187} Furthermore, it is worth noting that introducing multiple polymeric carriers with different charged groups (e.g. PPA-SP/PPA-DMA mixture, with primary amino groups and tertiary amino groups) into the same complexes can enhance the transfection efficiency compared with complexes comprising single PPA carrier alone. The mechanism of the higher transfection efficiency mediated by this mixture is unclear till now and it appears to be unrelated to the particle size, zeta potential and DNA uptake.\textsuperscript{188}

2.9 Chitosan
Chitosan, obtained by the partial alkaline deacetylation of chitin, the second most abundant natural polymer, is a non-toxic, biocompatible and biodegradable amino polysaccharide. It is composed of two subunits, D-glucosamine and N-acetyl-D-glucosamine, which are linked by a (1→4) glycosidic linkage (Figure 2.12). Chitosan is widely used as food additive and pharmaceutical excipient. In 1995, preparation of self-assembling polymeric and oligomeric chitosan/DNA complexes was first described by mixing a solution of the respective chitosan with plasmid DNA.

![Chemical structure of chitosans](image)

**Figure 2.12** Chemical structure of chitosans: (A) glucosamine subunits, and (B) N-acetyl glucosamine subunits.

The chitosan backbone of glucosamine subunits shows a high density of amino groups, and requires pH values below 6 to be soluble. In detail, chitosans with high molecular weight (more than 100 K) are dissolved only in dilute acid solution, and the polymers with low molecular weight (such as 22 K) are highly soluble in physiological buffer solution. Chitosan can effectively bind DNA and protect it from nuclease enzymatic degradation via electrostatic interactions of positively charged amino groups of chitosan with the negatively charged phosphate groups of DNA. The stability of chitosan/pDNA complexes have been shown to depend on the molecular weight of chitosan, the ratio of pDNA to chitosan and the preparation medium.
Transfection efficiency mediated by chitosans with molecular weight more than 100 K is less than that of chitosans with molecular weight of 15 K and 52 K. Optimum molecular weight of chitosans is dependent on the cell lines. Chitosans with molecular weight in the range between 10-50 K are excellent as gene transfer reagents. For example, Chitosan with molecular weight 22 K demonstrated higher transfection efficiency than PLL, while its cytotoxicity was lower than PLL.

Erbacher et al. investigated chitosan and lactosylated chitosan vectors for their transfection efficiencies in vitro. In their studies, the complexes of chitosan with DNA are found to transfect effectively HeLa cells, independent of the presence of 10% serum. Also, the transfection of the complexes is not enhanced by using endosomolytic agents such as chloroquine to facilitate escape of DNA from the lysosomal compartment. More interestingly, the gene expression is observed to gradually increase over time (from 24-96 h). At 96 h, chitosan is found to be 10-times more efficient than PEI mediated transfection. Roy and co-workers described an immunoprophylactic strategy using oral allergen-gene immunization to moderate peanut antigen-induced murine anaphylactic responses. Oral administration of DNA nanoparticles synthesized by complexing pDNA with chitosan results in transduced gene expression in the intestinal epithelium, thus indicating the probable use of chitosan/DNA nanoparticles in murine anaphylactic responses.

To increase transfection efficiency, trimethylated chitosan oligomers (TMO) was prepared through quaternization of oligomeric chitosan as demonstrated by Thanou et al. These cationic polymers have been characterized and tested for their efficiency
on transfecting COS1 cells and Caco2 cells. When chitosan and quaternized chitosan oligomers are mixed with DNA, they spontaneously form complexes. All synthesized derivatives show excellent solubility in water at different pH values. Furthermore, the complexes have been tested for specific targeting to Hep-G2 cells and for expression of the β-gal reporter gene. The complexes efficiently transfect the Hep-G2 cells, possibly by internalization via the galactose receptor presenting on the cellular surface of Hep-G2 cells, as proposed by Murata.\textsuperscript{198} Actually, the transferrin receptor is one of the first to be exploited for receptor-mediated gene delivery. The number of transferrin molecules attached to each nanoparticle surface varies according to the molecular weight of the polymer.\textsuperscript{199} Compared to non-modified chitosan, the method results in a fourfold enhanced transfection efficiency, depending on the cell line. Conjugation of C-terminal globular domain of the fiber protein (KNOB) to chitosan-DNA nanoparticles also results in a 130-fold increase in the transfection efficiency in HeLa cells and several fold in HEK293 cells.\textsuperscript{199}

In addition, Lee and Kim et al synthesized hydrophobically modified chitosan with deoxycholic acid and demonstrated efficient transfection of chitosan/DNA complexes into COS7 cell lines. The deoxycholic acid-modified chitosan can self-associate to form micelles of a mean diameter of 160 nm. The transfection efficiency of the complexes is enhanced compared to that achieved by pristine DNA, but lower than that achieved by Lipofectamine.\textsuperscript{200,201} Park and co-worker used galactosylated chitosan-graft-PEG (GCP) as a DNA vector, and the transfection efficiency using GCP/DNA complexes was very low, mainly because of interaction with plasma
leading to dissociation of GCP/DNA complexes.\textsuperscript{202} \textit{N}-dodecylated chitosan (CS-12) was also synthesized and incorporated with DNA to form a self-assembly complex by Yau et al.\textsuperscript{194} The incorporation of CS-12 can enhance the thermal stability of DNA, but no analysis of transfection efficiency \textit{in vitro} and \textit{in vivo} has been published by the author at the moment.

\textbf{2.10 Cyclodextrin-containing cationic polymers}

Cyclodextrins (CDs) are torus shaped cyclic D-gluco-oligosaccharides produced from starch by enzymatic degradation. Although CDs containing between 6 to 12 D(+)-glucopyranose units attached by \(\alpha\)-(1,4) glucosidic bonds have been isolated, only those containing 6 (\(\alpha\)-CD), 7 (\(\beta\)-CD) or 8 (\(\gamma\)-CD) residues in a oligomer structure are currently used (Figure 2.13). CDs have an amphiphilic structure, which consists of a hydrophobic inner cavity and hydrophilic exterior and is capable of interacting with a large variety of guest molecules to form non-covalent inclusion complexes. Moreover, these water-soluble, biocompatible oligosaccharides do not elicit immune responses and have low toxicity in animals and humans. Hence, they are industrially used in many applications such as pharmaceuticals, fragrance, foods and so on.\textsuperscript{203,204}

A new class of linear, CD-based polymers was introduced by Davis and co-workers in 1999 for gene delivery applications.\textsuperscript{205} Since then, numerous CD-containing polymers have been used as delivery vehicles for nucleic acid. The linear cationic \(\beta\)-CD-based polymers are synthesized by condensation of difunctionalized CDs monomers and difunctionalized co-monomers, thus their polymer structure can be
methodically controlled. The interaction between the polymers and DNA can form compact complexes with diameter about 100 nm. The transfection efficiency of the β-CD-based polymers is comparable to that of PEI and lipofectmine, at the same time they show low in vivo and in vitro toxicity.205-207 The effect of CD size (β- or γ-CD), charge center (amidine or quaternary ammoniums), and charge density on gene-delivery efficiency and polymer toxicity have also been investigated. As a result, it is found that large size of CD, long distance of cationic center to CD and high content of CDs in the polymer are favorable to increase transfection efficiency and reduce cytotoxicity. The quaternary ammonium analogues exhibit lower gene expression values and similar toxicities to their amidine analogues.208-210

![Chemical structure of cyclodextrins (CDs): α-CD, β-CD, and γ-CD.](image)

**Figure 2.13** Chemical structure of cyclodextrins (CDs): α-CD, β-CD, and γ-CD.

Uekama and colleagues synthesized a series of PAMAM dendrimer-CD and PAMAM-modified dendrimer-CD conjugates by grafting CD (α-, β- and γ-CD) to generations of PAMAM dendrimers (G2, G3, and G4) or PAMAM-modified dendrimers (G2) to guide rational design of CD-containing non-viral gene carriers.211-214 Linear and branched PEIs grafted with β-CD were also synthesized and efficiently delivered nucleic acids to cultured cells with low toxicity than the unmodified pristine PEIs.215,216
A key feature of the complexes of CD-containing polymer with DNA is that the particles can be readily surface-modified by the formation of inclusion complex (Figure 2.14). In a series of publications Davis and co-workers have demonstrated PEGylation of the complexes by modification with adamantane-PEG conjugates (the adamantane and CD form inclusion complex with high association constant). This self-assembly approach to the complexes modification has also been used to functionalize the complexes with targeting ligands such as galatose, transferring and insulin for cell-specific uptake.

**Figure 2.14** (A). Schematic of inclusion compound formation. (B). Schematic for post-DNA-complexation PEGylation by inclusion compound formation by PEG-AD and ligand-PEG-AD. PEG is conjugated to adamantane. Adamantane forms an inclusion complex with β-cyclodextrin on the complex surface and brings with it PEG or PEG-L to decorate the complex and provide steric stabilization and targeting.

In addition, Tang et al. used CDs and CD derivatives to crosslink low molecular
branched PEI to form high molecular weight cationic polymers, which displayed lower cytotoxicity and high gene transfection in cultured cells.\textsuperscript{222,223} Recently, soluble supramolecular inclusion complexes formed by threading \(\alpha\)- or \(\gamma\)-CD over linear cationic homopolymer or copolymer have been synthesized and examined as gene carries. The inclusion complexes show high transfection efficiency and significantly lower cytotoxicity.\textsuperscript{224,225} Ooya et al. also introduced aminoethyl- carbamoyl (ACE) groups to the polyrotaxanes consisting of \(\alpha\)-CD and poly(ethylene glycol), and they found that the introduction of ACE groups enhanced the opportunity of complexion with DNA.\textsuperscript{226} Later, they reported the use of dimethylaminoethyl- modified \(\alpha\)-CDs threaded onto a PEO chain and capped by cleavable end groups. The cleavage of the end groups cause the dethreading of \(\alpha\)-CDs and rapid release of DNA in cells, but the tertiary amines conjugated to the \(\alpha\)-CDs may not be efficient in DNA complexation and gene delivery.\textsuperscript{227}

2.11 References


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CHAPTER THREE

CATIONIC STAR POLYMERS CONSISTING OF α-CYCLODEXTRIN
CORE AND OLIGOETHYLENIMINE ARMS AS NON-VIRAL GENE
DELIVERY VECTORS

3.1 Introduction

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3.1 Introduction

Cationic polymers are the major type of non-viral gene delivery vectors investigated in the past decade.\textsuperscript{1-6} A large number of polycations have been reported to be able to effect gene transfection, including homopolymers or copolymers of polyethyleneimine (PEI),\textsuperscript{7} poly(L-lysine),\textsuperscript{4} polyamidoamine,\textsuperscript{8} poly(L-glutamic acid),\textsuperscript{9} polyphosphoester,\textsuperscript{10,11} and chitosan.\textsuperscript{12,13} Among these polymers, PEI homopolymers with molecular weights (MW) higher than 25 K are currently the most popular polymers used as gene carriers. They are considered the gold standard for polymeric non-viral gene delivery due to their high transfection efficiency, but the rather high toxicity of these PEI homopolymers strictly limits their application in gene therapy. Meanwhile, it is generally believed that PEI homopolymers with a MW less than 1.8 K shows low gene delivery ability but is less toxic.\textsuperscript{14}

Cyclodextrins (CDs) are a series of cyclic oligosaccharides composed of 6, 7, or 8 D(+)--glucose units linked by α-1,4-linkages and named α-CD, β-CD, or γ-CD, respectively. They are biocompatible, and do not elicit immune responses and have low toxicities in animal and human bodies.\textsuperscript{15} Since 1999, a class of linear and CD-based polymers were introduced by Davis and co-workers for the delivery of nucleic acids.\textsuperscript{16-20} Most of these polymers contained amines and CDs in the polymer backbone. Uekama’s group also conjugated CDs (α-, β- or γ-CD) to polyamidoamine dendrimers to enhance gene transfection.\textsuperscript{21,22} Further, Davis’ and Pack’s groups modified PEI with β-CD, grafting multiple β-CD molecules to linear or branched PEI (MW 25 K).\textsuperscript{20,23} These CD-grafted PEIs delivered nucleic acids efficiently to cultured cells with lower
toxicity than the pristine PEI (MW 25 K). Recently, Tang et al. also used CDs to crosslink low molecular weight branched PEI (MW 600) to form high molecular weight cationic polymers (average MW 61 K), which displayed lower cytotoxicity and high gene transfection in cultured cells.24 Most recently, we reported the synthesis of novel cationic supramolecules composed of multiple oligoethyleneimine-grafted β-CD that are threaded and blocked on a polymer chain as a new class of polymeric gene delivery vectors.25 In contrast to the conventional cationic polymers containing a long sequence of covalently bonded repeating units, the novel supramolecular gene carriers were designed based on a new mechanism, where many cationic cyclic units were threaded upon a polymer chain to form an integrated supramolecular entity to function as a macromolecular gene vector, which showed excellent DNA binding ability, low cytotoxicity, and high gene transfection efficiency.

In this study, we synthesized a series of new cationic star polymers where many oligoethyleneimine (OEI) arms of different lengths are attached to an α-CD core. We found that not only do these new α-CD-OEI star polymers show much lower cytotoxicity, but the gene transfection efficiency is similar to that of branched PEI (MW 25 K). We also investigated the effect of the length of the OEI arms of the star polymers, and found that the transfection efficiency of the α-CD-OEI star polymers improved with the increase of the chain length of the OEI arms.

3.2 Experimental section

3.2.1 Materials
Pentaethylenehexamine was obtained from Fluka. α-cyclodextrin was purchased from Tokyo Kasei incorporation. Ethylenediamine, linear PEI with molecular weight of 423 (OEI-9), branched PEI of molecular weight of 600 (OEI-14) and branched PEI (MW 25 K) were purchased by Aldrich. D$_2$O used as solvent in the NMR measurements was also obtained from Aldrich. Qiagen kit and Luciferase kit were purchased from Qiagen and Promega, respectively. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), penicillin, and streptomycin were obtained from Sigma.

### 3.2.2 Synthesis of cationic α-CD-OEI star polymers

The details of the procedure for the preparation of star polymer 2 are given below as a typical example. α-CD (0.414 g, 0.4 mmol, 94% purity) was dried at 120 °C in vacuum overnight. When the flask cooled, 40 mL dry DMSO was injected under nitrogen. After all α-CD was dissolved, the DMSO solution of α-CD was added dropwise during a period of 6 h under nitrogen to 40 mL of anhydrous DMSO solution in which 1,1’-carbonyldiimidazole (CDI) (5.84 g, 36 mmol) was dissolved, and the mixture was stirred overnight under nitrogen at room temperature. Then, a mixture of 400 mL THF and 1700 mL Et$_2$O was poured in the resulting solution to precipitate the product. The precipitate was centrifuged and washed with THF 3 times. The resulting sticky solid was dissolved in 40 mL DMSO and this solution was slowly added dropwise during a 3 h period into 12.55 mL (43.2 mmol) of pentaethylenehexamine which was separately dissolved in 40 mL of DMSO with
stirring at room temperature, and stirred overnight. 900 mL THF was poured into the reaction mixture to precipitate the product. The precipitate was centrifuged and washed 3 times with THF, and the resulting crude product was purified by size exclusion chromatography (SEC) on a Sephadex G-50 column using DI water as eluent. Finally, 0.1891 g white solid 2 was obtained.

In the preparation of star polymer 4, dichloromethane (DCM) was used to precipitate the product instead of THF. Then, the reaction mixture was filtered and the solid was washed 3 times with DCM. Finally, the resulting crude product was purified by size exclusion chromatography (SEC) on a Sephadex G-50 column using DI water as eluent.

The yields and analytical data for all four products are given below.

**α-CD-OEI star polymer 1.** Yield, 21%. $^1$H NMR (400 MHz, D$_2$O, 22 °C): $\delta$5.14 (s, broad, 6H, H(1) of CD), 3.47-4.62 (m, broad, 36H, H(3), H(6), H(5), H(2) and H(4) of CD), 2.98-3.47 (t, broad, 12H, CONCH$_2$ of ethylenediamine), 2.86 (s, broad, 12H, NCH$_2$ of ethylenediamine). Anal. Calcd for C$_{53}$H$_{95}$N$_{12}$O$_{36}$$\cdot$6H$_2$O: C, 40.59; H, 6.81; N, 10.27. Found: C, 40.35; H, 6.30; N, 10.07.

**α-CD-OEI star polymer 2.** Yield, 17%. $^1$H NMR (400 MHz, D$_2$O, 22 °C): $\delta$4.98 (d, broad, 6H, H(1)H of CD), 3.40-4.64 (m, broad, 36H, H(3), H(6), H(5), H(2) and H(4) of CD), 2.92-3.40 (t, broad, 14H, CONCH$_2$ of pentaethylenehexamine), 2.65 (m, 123H, NCH$_2$ of pentaethylenehexamine). Anal. Calcd for C$_{111}$H$_{237}$N$_{41}$O$_{37}$$\cdot$4H$_2$O: C, 47.50; H, 8.81; N, 20.41. Found: C, 47.66; H, 8.96; N, 20.71.

**α-CD-OEI star polymer 3.** Yield, 29%. $^1$H NMR (400 MHz, D$_2$O, 22 °C): $\delta$5.12 (d,
α-CD-OEI star polymer 4. Yield, 19%. \(^{1}\text{H NMR}\) (400 MHz, D\(_2\)O, 22 °C): δ5.11 (s, broad, 6H, H(1)H of CD), 3.46-4.60 (m, broad, 36H, H(3), H(6), H(5), H(2) and H(4) of CD), 3.00-3.46 (m, broad, 10H, CONCH\(_2\) of OEI-14), 2.67 (m, 264H, NCH\(_2\) of OEI-14). Anal. Calcd for C\(_{179}\)H\(_{409}\)N\(_{74}\)O\(_{35}\)•24H\(_2\)O: C, 43.97; H, 9.53; N, 19.44. Found: C, 42.63; H, 8.65; N, 20.79.

3.2.3 Measurements

Gel permeation chromatography (GPC) Gel permeation chromatography (GPC) analysis was carried out with a Shimadzu SCL-10A and LC-10AT system equipped with a Sephadex G-75 column (size: 2.5 × 32 cm), a Shimadzu RID-10A refractive index detector. 1× PBS buffer solution was used as the eluent. Fractions were collected per 1 mL and were further detected with a HORIBA SEPA-300 high speed accurate polarimeter at wavelength 589 nm with cell length 10 cm and response 2 s.

\(^{1}\text{H NMR spectra}\) The \(^{1}\text{H NMR}\) spectra were recorded on a Bruker AV-400 NMR spectrometer at 400 MHz at room temperature. The \(^{1}\text{H NMR}\) measurements were carried out with an acquisition time of 3.2 s, a pulse repetition time of 2.0 s, a 30° pulse width, 5208-Hz spectral width, and 32 K data points. Chemical shifts were referred to the solvent peaks (δ = 4.70 ppm for D\(_2\)O).
**13C NMR spectra** The $^{13}$C NMR spectra were recorded on a Bruker AV-400 NMR spectrometer at 100 MHz at room temperature. The $^{13}$C NMR measurements were carried out using composite pulse decoupling with an acquisition time of 0.82 s, a pulse repetition time of 5.0 s, a 30° pulse width, 20,080-Hz spectral width, and 32 K data points.

**Plasmid** The plasmid used was pRL-CMV (Promega, USA), encoding *Renilla* luciferase, which was originally cloned from the marine organism *Renilla reniformis*. All plasmid DNAs were amplified in *E. coli* and purified according to the supplier’s protocol (Qiagen, Hilden, Germany). The quantity and quality of the purified plasmid DNA was assessed by optical density at 260 and 280 nm and by electrophoresis in 1% agarose gel. The purified plasmid DNA was resuspended in TE buffer (10 mM Tris-Cl, pH 7.5, 1 mM EDTA) and kept in aliquots at a concentration of 0.5 mg/mL.

**Cells and Media** All cell lines were purchased from ATCC (Rockville, MD). COS7 and HEK293 cells were maintained in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum, 100 units/mg penicillin, 100 µg/mL streptomycin at 37 °C and 5% CO2. Opti-MEM reduced serum medium, DMEM medium was purchased from Gibco BRL (Gaithersburg, MD).

**Gel Retardation Experiments** Each star polymer was examined for its ability to bind pRL-CMV through gel electrophoresis experiments. All the star polymer stock solutions were prepared at a nitrogen concentration of 1 mM in distilled water and the pH was adjusted to 7.4. Solutions were sterile filtered (0.2 µm) and stored at 4 °C. pRL-CMV (0.2 µg; 2 µl of a 0.1 µg/µl in TE buffer) was mixed with an equal volume
of polymer at nitrogen/phosphate (N/P) ratios between 0 and 10. Each mixture was vortexed and incubated for approximately 30 min at room temperature and then analyzed on 1% agarose gel containing 0.5 µg/mL ethidium bromide (EtBr). Gel electrophoresis was carried out in TAE running buffer (40 mM Tris-acetate, 1 mM EDTA) with a current of 80V for 40 min in a Sub-Cell system (Bio-Rad Laboratories, CA). DNA bands were visualized and photographed by a UV transilluminator and BioDoc-It imaging system. Both of them were purchased from UVP Inc, USA.

**Cell viability assay** Two cell lines (HEK293 and COS7) were cultured in DMEM medium supplemented with 10% FBS at 37 °C, 5% CO₂, and 95% relative humidity. For cell viability assay, the cells (15,000 cells/well for HEK293, and 10,000 cells/well for COS7) were seeded into 96-well microtiter plates (Nunc, Wiesbaden, Germany). After 24 h, culture media were replaced with serum-supplemented culture media containing serial dilutions of polymer and the cells were incubated for 24 h. 10 µl sterile filtered MTT (5 mg/mL) stock solution in PBS was added to each well, reaching a final concentration of 0.5 mg MTT/mL. After 5 h, unreacted dye was removed by aspiration. The formazan crystals were dissolved in 100 µl/well DMSO and measured spectrophotometrically in a microplate reader (Spectra Plus, TECAN) at a wavelength of 570 nm. Six wells were treated together as a group. The relative cell growth (%) related to control cells cultured in media without polymer was calculated by \([A]_{\text{test}}/[A]_{\text{control}}\times100\%\).

**In Vitro transfection and luciferase assay** Transfection studies were performed with HEK293 and COS7 cells using the plasmid pRL-CMV as reporter gene. In brief,
24-well plates were seeded with cells at a density of $5 \times 10^4$/well 24 h before transfection. The star polymer/DNA complexes at various N/P ratios were prepared by adding the star polymer into DNA solutions dropwise, followed by vortexing and incubation for 30 min at room temperature before transfection. At the time of transfection, the medium in each well was replaced with reduced-serum medium or normal medium. The complexes were added into the transfection medium and incubated with cells for 4 h under standard incubator conditions. After 4 h, the medium was replaced with 500 µl of fresh medium supplemented with 10% FBS, and the cells were further incubated for an additional 20 h under the same conditions, resulting in a total transfection time of 24 h. Cells were washed with PBS twice, lysed in 100 µl of cell culture lysis reagent (Promega, Cergy Pontoise, France). Luciferase gene expression was quantified using a commercial kit (Promega, Cergy Pontoise, France) and a luminometer (Berthold Lumat LB 9507, Germany). Protein concentration in the sample was analyzed using a bicinchoninic acid assay (Biorad, CA, USA). Absorption was measured on a microplate reader (Spectra Plus, TECAN) at 570 nm and compared to a standard curve calibrated with BSA samples of known concentration. Results are expressed as relative light units (RLUs) per milligram of cell protein lysate (RLU/mg protein).

**Dynamic light scattering and zeta-potential measurements** Measurements of particle size and zeta potential of the complexes were performed using a Zetasizer Nano ZS (Malvern Instruments, Southborough, MA, USA). Complex solutions (100 µl) containing 3 µg of pDNA (pRL-CMV) were prepared at various N/P ratios.
ranging from 2-30. The mixture was vortexed for 20 s, incubated for 30 min at room temperature and diluted in 1 mL of the distilled water before being analyzed on a Zetasizer. The size measurement was performed at 25 °C at a 90° scattering angle. The mean hydrodynamic diameter was determined by cumulative analysis. The zeta potential measurements were performed using a capillary zeta potential cell in automatic mode.

Confocal microscopy For confocal microscopy, the plasmid pEGFP-N1 (Clontech Laboratories Inc., USA), encoding a red-shifted variant of wild-type green fluorescence protein (GFP), was used to examine the GFP expression in HEK293 cells. HEK293 cells were seeded onto lab-Tek 4-chambered coverglass (Nalge-Nalge international, USA) at density of 5×10^4 cells/well in 500 µl of complete DMEM. After 24 hours, transfection was undertaken with 2 µg EGFP plasmid. Each chamber was transfected in 0.3 mL reduced serum Opti-MEM media. 20 µl of the star polymer/DNA suspension was added per well. After 4 h, the transfection media was removed and the cells washed. After 20 h of further incubation in serum-containing media, the wells was washed with phosphate-buffered saline (PBS) and imaged under a laser scanning confocal microscope (LSM 410, Carl Zeiss, USA). GFP fluorescence was excited at 488 nm and emission was collected using a 515 nm filter.

Atomic force microscopy (AFM) A Digital Instruments D3000 Atomic Force Microscopy in a tapping mode was employed to image the nanoparticle samples. Briefly, silicon disks were soaked in 50% acetone for a minimum of 2 h and rinsed with distilled water. When the silicon disks were completely dry, 20 µl of star
polymer/DNA complexes containing 1 µg of pRL-CMV at N/P ratios 0, 2 and 10 were placed on the silicon surface for 2 min after which the complexes was carefully removed with a piece of tissue paper. All the AFM images were obtained with a scan rate of 0.5 or 1 Hz over a selected area of 10×10 µm or 5×5 µm. Image analysis was performed using Nanoscope software after removing the background slope by flatting images.

3.3 Results and discussion

3.3.1 Synthesis and characterization of cationic α-CD-OEI star polymers

Each α-CD ring contains six glucose units, therefore having many hydroxyl groups, which can be activated and grafted with multiple oligoethylenimine (OEI) arms of different lengths to form a cationic star polymer. As shown in Scheme 3.1, the hydroxyl groups of α-CD were activated with 1,1’-carbonyl diimidazole (CDI), followed by reaction with large excess of OEI to give the α-CD-OEI star polymers. OEIs with different chain lengths, ethylenediamine (n = 1), pentaethylenehexamine (n = 5), OEI-9 (n = 9), and OEI-14 (n = 14), were grafted to α-CD to give α-CD-OEI star polymers 1, 2, 3 and 4, respectively. To ensure that there was no intra- or intermolecular crosslinking, the molar ratio of CDI or OEI to α-CD was more than 100 in the above grafting reactions.

Figure 3.1 shows the size exclusion chromatograms of α-CD-OEI star polymers 1 – 4 in comparison with free α-CD. α-CD has relative small molecular size, which was eluted out at the low molecular weight region of the column. The four α-CD-OEI star
polymers were eluted out at higher molecular weight region of the column, and showed an increase in molecular weight with increasing the OEI arm length, i.e., the molecular weight followed the order of $\alpha$-CD < 1 < 2 < 3 < 4. Each star polymer showed a unimodal peak and this peak was eluted out at the same position in both RI and OR diagrams. The results indicate that the $\alpha$-CD-OEI star polymers were pure and there were no intra- or intermolecular crosslinking byproducts.

Scheme 3.1 Synthesis procedures and the structures of $\alpha$-CD-OEI star polymers.
Figure 3.1 Size exclusion chromatograms of α-CD (a), and α-CD-OEI star polymers 1 (b), 2 (c), 3 (d), and 4 (e), detected with refractive index (RI) and optical rotation (OR), respectively.

Figure 3.2 shows the $^{13}$C NMR spectra of α-CD-OEI star polymer 2 in comparison with its precursors free α-CD and pentaethylenehexamine. In Figure 3.2c, all peaks attributed to the α-CD core and the grafting pentaethylenehexamine arms were observed. The peak at δ 158.4 ppm corresponds to the carbon of the urethane groups that link the pentaethylenehexamine chains to the α-CD core. Compared with free α-CD, the peak of C-6 of the α-CD core of star polymer 2 shifted from 60.9 to 64.3 ppm, which may be because of the grafting of the pentaethylenehexamine arms. In fact, of the three types of hydroxyl groups of α-CD, those at the 6-position (primary hydroxyl groups) are the most nucleophilic and more subject to the modification under these weak basic conditions.26
Figure 3.2 ¹³C NMR spectra of α-CD (a), pentaethylenehexamine (b), and α-CD-OEI star polymer 2 (c) in D₂O.

Figure 3.3 shows the ¹H NMR spectra of α-CD-OEI star polymers 1 – 4 in comparison with α-CD. In the spectra of Figure 3.3b - 3e, the signals for both α-CD core and grafting OEI arms were observed, while the peaks were much broadened due to the restriction of the molecular motion by the grafting of OEI to α-CD core. From the ¹H NMR spectra, the average number of OEI chains grafted onto each α-CD (x) was estimated. About 5.8 molecules of ethylenediamine were grafted onto the α-CD core in 1, and about 6.8 molecules of pentaethylenehexamine were grafted onto the α-CD core in 2; nearly every glucose unit of the α-CD core was grafted with one ethylenediamine or pentaethylenehexamine. In the meantime, about 3.4 molecules of OEI-9 were grafted on the α-CD core in 3, and about 5.0 molecules of OEI-14 were grafted on the α-CD core in 4. It is likely that the longer OEI chains resulted in more
difficult grafting because of the spatial hindrance. However, the branched structure of OEI-14 increased the number of OEI arms grafted on the α-CD core in star copolymer 4, probably because it contained higher number of primary amine groups.

![Image of NMR spectra](image)

**Figure 3.3** $^1$H NMR spectra of α-CD (a) and α-CD-OEI star polymers 1 (b), 2 (c), 3 (d), and 4 (e) in D$_2$O.

### 3.3.2 Formation of cationic α-CD-OEI star polymer/DNA complexes

The ability of the cationic α-CD-OEI star polymers to condense plasmid DNA (pDNA) into particulate structures was confirmed by agarose gel electrophoresis, particle size and zeta potential measurements, as well as AFM imaging.

The formation of α-CD-OEI star polymer/DNA complexes was examined by their electrophoretic mobility on an agarose gel at various ratios of amino-group (in α-CD-OEI star polymer) to phosphate group (in pDNA), defined as N/P ratio. Figure 3.4 shows the gel retardation results of cationic α-CD-OEI star polymer/DNA complexes.
complexes with increasing N/P ratios in comparison with branched PEI (25 K). \(\alpha\)-CD-OEI star polymers 1, 2, and 3 showed decreasing pDNA condensation capability; they complexed with pDNA efficiently at N/P ratios of 1.5, 2.5, and 4, respectively, due to the decreasing primary amino density with increasing the OEI length. However, \(\alpha\)-CD-OEI star polymer 4, where the arms were branched OEI-14, could inhibit the migration of pDNA at N/P ratios of 2, showing similar DNA condensation capability to branched PEI (25 K). Therefore, \(\alpha\)-CD-OEI star polymers 1 and 4 have stronger DNA condensation capability due to the relatively higher density of primary amino group. These results are also supported by Kissel and co-workers.\(^{27}\)

Figure 3.4 Electrophoretic mobility of plasmid DNA in the complexes between \(\alpha\)-CD-OEI star polymers and plasmid DNA in comparison with PEI/DNA complex at various N/P ratios.

Figure 3.5 shows the particle size and zeta potential of \(\alpha\)-CD-OEI star
polymer/DNA complexes in comparison with PEI (25 K)/DNA complex at various N/P ratios. As shown in Figure 3.5a, all four α-CD-OEI star polymers could efficiently compact pDNA into small nanoparticles. Generally, the particle size decreased with an increase in N/P ratio until the N/P ratio was between 6 and 8. The particle size remained in the 100 – 200 nm range after the N/P ratio reached 8. All α-CD-OEI star polymers and PEI (25 K) formed nanoparticles with pDNA with comparable sizes at high N/P ratios. The surface net charge of the complexes of pDNA with α-CD-OEI star polymers increased from negative to positive as the N/P ratio increased from 0 to 8 and stabilized at N/P ratio of 10 and above (Figure 3.5b).

![Figure 3.5](image)

**Figure 3.5** Particle size (a) and zeta potential (b) of the complexes between α-CD-OEI star polymers and plasmid DNA in comparison with PEI/DNA complex at various N/P ratios.

Figure 3.6 shows representative tapping mode AFM images of naked DNA and the complexes between α-CD-OEI star polymer 4 and pDNA at N/P of 2 and 10. The AFM image of naked plasmid DNA revealed a supercoiled structure of pDNA. At N/P ratio 2, supercoiled plasmid DNA could still be identified under AFM while some of
the pDNA was condensed to nanoparticles by α-CD-OEI star polymer. Compared with the partial condensation at N/P ratio of 2, the pDNA could be tightly packed and totally formed nanoparticles at N/P ratio of 10. The results indicate that the complexation of pDNA by α-CD-OEI star polymer led to the formation of compact nanoparticles.

Figure 3.6 Atomic force microscopy (AFM) images of the supercoiled plasmid DNA (a), and the complexes between α-CD-OEI star polymer 4 and DNA at N/P ratios of 2 (b) and 10 (c).

3.3.3 Cytotoxicity of cationic α-CD-OEI star polymers

Cytotoxicity of polymeric gene vectors may be an important factor that affects the transfection efficiency. Figure 3.7 shows the results of in vitro cytotoxicity of α-CD-OEI star polymers analyzed by the MTT method in two cell lines (HEK293 and COS7). As shown in Figure 3.7, all the products showed a dose-dependent effect on cytotoxicity. The LD_{50} values, which represent concentration of the polymers resulting in 50% inhibition of cell growth, were calculated. The LD_{50} value of PEI in COS7 was 12 µg/mL, while the LD_{50} values of α-CD-OEI star polymers 1, 2, 3 and 4 were 451, 519, 560, and 88 µg/mL, respectively. A similar trend was also observed in HEK293 cells. Hence, all the four α-CD-OEI star polymers exhibited much lower
toxicity in both cell lines than that of PEI (25 K). It may be attributed to the reduction of the density of amino groups and the introduction of $\alpha$-CD in the star polymers. It is thought that the high amino density and high molecular weight are the reasons of the high cytotoxicity of PEI (25 K). It is noted that $\alpha$-CD-OEI star polymer 4 showed higher cytotoxicity than that of the other three star polymers. This is probably due to the higher amino density and the branch nature of OEI-14, which is more similar to the structure of the branched PEI (25 K).

![Graph](image)

**Figure 3.7** Cell viability assay in HEK293 (a) and COS7 (b) cell lines. The cells were treated with various concentrations of $\alpha$-CD-OEI star polymers 1, 2, 3 and 4, and PEI (25 K) for 24 hours in a serum-containing medium. Cell viability was determined by the MTT assay and expressed as a percentage of control, that is, the untreated cell cultures.

3.3.4 *Transfection efficiency of cationic $\alpha$-CD-OEI star polymers*

*In vitro* transfection efficiency of $\alpha$-CD-OEI star polymer/DNA complexes was assessed using luciferase as a marker gene in HEK293 and COS7 cells. Figure 3.8 shows the gene transfection efficiency of $\alpha$-CD-OEI star polymers for DNA delivery.
in the absence and presence of serum compared with those of branched PEI (25 K) and naked pDNA (ND). The transfection results in both cells lines demonstrated that, among the α-CD-OEI star polymers examined, the transfection efficiency generally increased following the order $1 < 2 < 3 < 4$, with more pronounced enhancements from star polymers 3 to 4. This indicates that, for the linear OEI arms, the transfection efficiency was dependent upon the chain length of the OEI grafted onto α-CD core. In the case of α-CD-OEI star polymer 4, the primary amino groups of the branched OEI increased its binding ability and complex stability, thus led to much higher transfection efficiency.28-29

**Figure 3.8** *In vitro* gene transfection efficiency of the complexes of cationic α-CD-OEI star polymer/DNA in comparison with that of PEI (25 K) or naked DNA (ND), in HEK293 (a and b) and COS7 (c and d) cells in the absence and presence of serum. Data represent mean ± standard deviation (n=3).

In both cell lines in the absence of serum, with the increase in N/P ratio from 10 to 30, the transfection efficiency mediated by PEI (25 K) decreased dramatically, most
likely due to its high cytotoxicity, while that mediated by α-CD-OEI star polymers increased slightly or remained no significant changes (Figure 3.8, a and c). The transfection efficiency of complexes formed by star polymer 4 was even higher than that of branched PEI (25 K) at most N/P ratios. Particularly, the transfection efficiency mediated by star polymer 4 was 50 times higher than that of PEI (25 K) at N/P ratio of 30 in HEK293 cells, and star polymers 2 and 3 also showed higher transfection efficiency than PEI (25 K) under the same conditions (Figure 3.8, a).

The gene transfection efficiency showed more cell type dependency under serum conditions. The transfection efficiency mediated by α-CD-OEI star polymer 4 was comparable to that of PEI (25 K) in HEK293 cells, while generally lower than that of PEI (25 K) in COS7 cells (Figure 3.8, b and d).

Confirmation of the gene delivery capability of α-CD-OEI star polymer 4 was also obtained by fluorescence microscopy (Figure 3.9). Plasmid pEGFP-N1 encoding green fluorescence protein (GFP) was used to examine the GFP expression in HEK293 cells. GFP expression could not be detected when the transfection was mediated by naked DNA, which was used as a negative control (data not shown). Strong fluorescence signal could be observed when transfections were mediated by either α-CD-OEI star polymer 4 or PEI at N/P ratio of 10. However, when the transfection was mediated by PEI (25 K), the GFP expression in HEK293 cells was stronger than that mediated by α-CD-OEI star polymer 4.
Figure 3.9 The confocal microscopy images of transfected HEK 293 cells. The transfections were mediated by (a) $\alpha$-CD-OEI star polymer 4 and (b) PEI (25 K) at N/P ratio of 10 in the absence of serum using green fluorescence protein gene as a reporter gene. The same field of cells was observed by Nomarski optics (right panel) or by fluorescence microscope (left panel) to visualize GFP expression.

3.4 Conclusion

A series of novel cationic $\alpha$-CD-OEI star polymers have been synthesized by conjugating multiple oligoethylenimine (OEI) arms onto an $\alpha$-CD core as non-viral gene delivery vectors. The star polymers were fully characterized in terms of their molecular structures by using SEC, $^{13}$C and $^1$H NMR, and elemental analysis. The $^{13}$C NMR spectra provided a strong evidence to show that the conjugation of OEI arms mainly occurred at the primary hydroxyl groups of $\alpha$-CD. $\alpha$-CD-OEI star polymers 1, 2, and 3 contained linear OEI arms with chain lengths ranging from 1 – 9 ethylenimine units, while $\alpha$-CD-OEI star polymers 4 contained arms of branched OEI of 14 ethylenimine units.

All the four $\alpha$-CD-OEI star polymers could inhibit the migration of pDNA on
agarose gel through formation of complexes with pDNA, and the complexes formed nanoparticles with sizes ranging from 100 – 200 nm at N/P ratios of 8 or higher. The star polymers displayed much lower in vitro cytotoxicity than that of branched PEI (25 K).

The α-CD-OEI star polymers showed excellent gene transfection efficiency in HEK293 and COS7 cells. Generally, the transfection efficiency increased with an increase in the OEI arm length. Star polymer 4, with the longest and branched OEI arms, showed the highest transfection efficiency among the four star polymers, which was comparable to or even higher than that of branched PEI (25 K). The novel α-CD-OEI star polymers with different OEI arms can be promising new non-viral gene delivery vectors with low cytotoxicity and high gene transfection efficiency for future gene therapy applications.

3.5 References


CHAPTER FOUR

CATIONIC SUPRAMOLECULES COMPOSED OF MULTIPLE OLIGOETHYLENIMINE-GRAFTED β-CYCLODEXTRINS THREADED ON A POLY[(ETHYLENE OXIDE)]-POLY[(PROPYLENE OXIDE)]-POLY[(ETHYLENE OXIDE)] TRIBLOCK COPOLYMER CHAIN FOR EFFICIENT GENE DELIVERY

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4.1 Introduction

Supramolecular architectures composed of chain-interlocked macrocycles such as rotaxanes and catenanes have attracted tremendous interest because of their unique structures as well as the potential as building blocks for a variety of functional nanomaterials.\textsuperscript{1-8} Since the first syntheses of polyrotaxanes with multiple \(\alpha\)-cyclodextrin (\(\alpha\)-CD) rings threaded over a polymer chain,\textsuperscript{9,10} increasing attention has been focused on the studies of the supramolecular structures\textsuperscript{10-19} and their properties for electronics\textsuperscript{20-22} and biomaterials applications.\textsuperscript{23-27}

Cationic polymers have been the most promising non-viral gene delivery vectors investigated in the past decade.\textsuperscript{28-33} A great number of polycations have been reported to effect gene transfection, including homopolymers or derivatives of polyethylenimine (PEI),\textsuperscript{34} poly(L-lysine),\textsuperscript{31} polyamidoamine,\textsuperscript{35} poly(L-glutamic acid),\textsuperscript{36} polyphosphoester,\textsuperscript{37,38} and chitosan.\textsuperscript{39,40} In contrast to the conventional polycations with long sequences of covalently bonded repeating units, we recently have designed novel cationic supramolecules with multiple oligoethylenimine-grafted CDs that are threaded and blocked on a polymer chain.\textsuperscript{41} Herein, we demonstrate that the cationic supramolecular gene delivery vectors show good DNA binding ability, low cytotoxicity, and high gene transfection efficacy that is similar to that of PEI (25 K), one of the most effective gene delivery polymers studied to date.\textsuperscript{33}

4.2 Experimental section

4.2.1 Materials

Pluronic L64 PEO-PPO-PEO triblock copolymer 1 was supplied by BASF, Germany. The copolymer has a chain composition of \(\text{EO}_{13}\text{PO}_{30}\text{EO}_{13}\), a number-average molecular weight of 2900, and an EO content of 40 wt %. The molecular
characteristics were confirmed using GPC and $^1$H NMR spectroscopy, which were found to be within the specification of the supplier. 2,4,6-Trinitrobenzene sulfonic acid solution and pentaethylenhexamine were purchased from Fluka. 2,4,6-Trinitrobenzene sulfonic acid solution was neutralized with NaOH solution before use. Tris(2-aminoethyl)amine, ethylenediamine, and oligoethylenimine (polyethylenimine with low molecular weight, $M_n = 423$) were obtained from Aldrich. $\beta$-CD was purchased from Tokyo Kasei Inc. DMSO-$d_6$ and D$_2$O used as solvent in the NMR measurements were also obtained from Aldrich.

4.2.2 Synthesis

4.2.2.1 Preparation of PEO-PPO-PEO Tetra(amine)

PEO-PPO-PEO triblock copolymer 1 ($M_n = 2,930, 2.985$ g, 1.02 mmol) was heated overnight in a flask at 80 °C in vacuum. When the flask cooled, 15 mL of anhydrous DMF was injected under nitrogen. After 1 was dissolved, the DMF solution of 1 was added dropwise during a period of 6 h under nitrogen to 15 mL of anhydrous DMF solution in which 1,1’-carbonyldiimidazole (CDI) (1.65 g, 10.2 mmol) was dissolved, and the mixture was stirred overnight under nitrogen at room temperature. Then, the resulting solution was slowly added dropwise during a period of 3 h into 15.54 g (102 mmol) of tris(2-aminoethyl)amine which was dissolved in 15 mL of anhydrous DMF with stirring at room temperature, followed by stirring the mixture overnight. DMF was removed by vacuum evaporation, and the resulting mixture was dissolved in CHCl$_3$ and washed with H$_2$O for 3 times to remove excess tris(2-aminoethyl)amine. After removing CHCl$_3$, the product was further purified on a Sephadex LH-20 column using methanol as eluent, to give 2.315 g of 2 as viscous liquid (yielded, 69%). $^1$H NMR (400 MHz, DMSO-$d_6$, 22 °C): $\delta$ 3.33-3.54 (m, 107H and 91H, -CH$_2$CH$_2$O- of
PEO block and -CH₂CHO- of PPO block), 3.13 (m, 4H, OCONCH₂), 2.87 (m, 4H, OCONCCH₂), 2.65 (m, 8H, CNCH₂), 2.49 (m, 8H, CH₂N), 1.05 (d, 91H, –CH₃ of PPO block). Anal. Calcd for C₁₅₈H₃₂₂N₈O₆₀•3H₂O: C, 56.70; H, 9.88; N, 3.35. Found: C, 56.42; H, 9.94; N, 3.40.

4.2.2.2 Preparation of β-CD-PEO-PPO-PEO Polyrotaxane

PEO-PPO-PEO tetra(amine) 2 (0.4 g) was added to 266 mL of β-CD aqueous solution (0.03 g/mL) together with 0.6 g of NaHCO₃ for adjusting the pH of the solution. The reaction mixture was ultrasonicated for 20 min and stirred overnight at room temperature. The inclusion complex 3 was formed as white precipitate. To block the ends of the inclusion complex, 3.36 g of sodium salt of 2,4,6-trinitrobenzene sulfonic acid was added into the mixture and stirred overnight. The reaction mixture was centrifuged and the precipitate was washed 3 times with water. The resulting wet solid was dissolved in 30 mL of DMSO and poured into 450 mL of MeOH to precipitate the product. The precipitate was collected and washed 3 times with MeOH. The precipitate was dissolved in 30 mL of DMSO again and poured into 500 mL of H₂O to precipitate the product. The resulting precipitate was centrifuged and washed 3 times with H₂O. Finally, the resulting wet solid was freeze dried to give the pure polyrotaxane 4 as yellow solid (yield, 2.033 g, 90%). ¹H NMR (400 MHz, DMSO- d₆, 22 °C): δ 9.24 (s, 4H, meta H of phenyl), 8.93 (s, 4H, meta H of phenyl), 5.75 (s, 90H, O(2)H of CD), 5.70 (m 90H, O(3)H of CD), 4.83 (s, 90H, H(1)H of CD), 4.43 (d, 90H, O(6)H of CD), 3.00-4.00 (m, 540H, H(3), H(6), H(5), H(2) and H(4) of CD, 107H, -CH₂CH₂O- of PEO block, 91H, -CH₂CHO- of PPO block), 1.04 (m, 91H, -CH₃ of PPO block). Anal. Calcd for C₇₂¹H₁₂₂₃N₂₀O₅₃₂•5₃H₂O: C, 44.10; H, 6.82; N, 1.43. Found: C, 43.62; H, 6.86; N, 1.69.
4.2.2.3 Preparation of cationic multiple OEI-grafted β-CD-PEO-PPO-PEO polyrotaxane

The procedures for the preparation of 5b from polyrotaxane 4 are given below as a typical example. Polyrotaxane 4 (0.261 g, 0.015 mmol) was dried at 40 °C in vacuum overnight. When the flask cooled, 40 mL of dry DMSO was injected under nitrogen. After 4 was dissolved, the DMSO solution of 4 was added dropwise during a period of 6 h under nitrogen to 40 mL of anhydrous DMSO solution in which 1,1’-carbonyldiimidazole (CDI) (3.60 g, 22.2 mmol) was dissolved, and the mixture was stirred overnight under nitrogen at room temperature. A mixture of 300 mL of THF and 600 mL of Et2O was poured into the resulting solution to precipitate the product. The precipitate was centrifuged and washed 3 times with THF. The resulting wet solid was dissolved in 40 mL of DMSO and this solution was slowly added dropwise during a period of 3 h into 8.60 mL (29.6 mmol) of pentaethylenehexamine that was dissolved in 40 mL of DMSO with stirring at room temperature, followed by stirring the mixture overnight. Next, 900 mL of THF was poured into the reaction mixture to precipitate the product. The precipitate was centrifuged and washed 3 times with THF, and the resulting crude product was purified on a Sephadex G-50 column using deionized water as eluent. Finally, 0.333 g of brown solid 5b was obtained (yield, 61%).

The yields and analytical data for all four cationic polyrotaxanes are given below.

**Cationic polyrotaxane 5a.** Yield, 39%. 1H NMR (400 MHz, D2O, 22 °C): δ 7.89 (s, 8H, meta H of phenyl), 5.04 (d, broad, 90H, H(1)H of CD), 2.94-4.63 (m, broad, 540H, H(3), H(6), H(5), H(2) and H(4) of CD, 101H, -CH2CH2O- of PEO block, 45H, -CH2CHO- of PPO block, 170H, CONCH2 of ethylenediamine), 2.74 (m, 170H, NCH2 of ethylenediamine), 1.12 (d, 91H, -CH3 of PPO block). Anal. Calcd for
C$_{976}$H$_{1732}$N$_{190}$O$_{617}$•130H$_2$O: C, 41.36; H, 7.09; N, 9.39. Found: C, 40.61; H, 6.58; N, 11.10.

_Cationic polyrotaxane 5b_. Yield, 61%. $^1$H NMR (400 MHz, D$_2$O, 22 °C): δ 7.98 (s, 8H, meta H of phenyl), 5.02 (d, broad, 90H, H(1)H of CD), 3.00-4.62 (m, broad, 540H, H(3), H(6), H(5), H(2) and H(4) of CD, 101H, -CH$_2$CH$_2$O- of PEO block, 45H, -CH$_2$CHO- of PPO block, 135H, CONCH$_2$ of pentaethylenehexamine), 2.69 (m, 1214H, NCH$_2$ of pentaethylenehexamine), 1.09 (d, 91H, -CH$_3$ of PPO block). Anal. Calcd for C$_{1463}$H$_{2976}$N$_{425}$O$_{599}$•130H$_2$O: C, 45.70; H, 8.48; N, 15.47. Found: C, 45.19; H, 7.99; N, 16.01.

_Cationic polyrotaxane 5c_. Yield, 44%. $^1$H NMR (400 MHz, D$_2$O, 22 °C): δ 7.98 (s, 8H, meta H of phenyl), 4.98 (d, broad, 90H, H(1)H of CD), 2.96-4.60 (m, broad, 540H, H(3), H(6), H(5), H(2) and H(4) of CD, 101H, -CH$_2$CH$_2$O- of PEO block, 45H, -CH$_2$CHO- of PPO block, 86H, CONCH$_2$ of OEI-9), 2.55 (m, 1532H, NCH$_2$ of OEI-9), 1.08 (d, 91H, -CH$_3$ of PPO block). Anal. Calcd for C$_{1573}$H$_{3288}$N$_{467}$O$_{575}$•130H$_2$O: C, 46.47; H, 8.90; N, 16.10. Found: C, 45.86; H, 8.43; N, 16.80.

4.2.3 Measurements

_Gel permeation chromatography (GPC)_ Gel permeation chromatography (GPC) analysis for PEO-PPO-PEO triblock copolymer was carried out with a Shimadzu SCL-10A and LC-10ATVP system equipped with two Phenogel 5 μm, 50 and 1000 Å columns (size: 300 × 4.6 mm) in series and a Shimadzu RID-10A refractive index detector. THF was used as eluent at a flow rate of 0.30 mL/min at 40 °C. Monodispersed poly(ethylene glycol) standards were used to obtain a calibration curve.
GPC analysis for cationic polyrotaxanes was carried out with a Shimadzu SCL-10A and LC-10AT system equipped with a Sephadex G-75 column (size: 2.5 × 32 cm), a Shimadzu RID-10A refractive index detector. 1× PBS buffer solution was used as the eluent. Fractions were collected per 1 mL and were detected with a HORIBA SEPA-300 high speed accurate polarimeter at wavelength 589 nm with cell length 10 cm and response 2 s.

**1H NMR spectra** 1H NMR spectra were recorded on a Bruker AV-400 NMR spectrometer at 400 MHz at room temperature. The 1H NMR measurements were carried out with an acquisition time of 3.2 s, a pulse repetition time of 2.0 s, a 30° pulse width, 5208-Hz spectral width, and 32 K data points. Chemical shifts were referred to the solvent peaks (δ = 2.50 ppm for DMSO-d$_6$ and δ = 4.70 ppm for D$_2$O).

**Plasmid** The plasmid used was pRL-CMV (Promega, USA), encoding *Renilla* luciferase, which was originally cloned from the marine organism *Renilla reniformis*. All plasmid DNAs were amplified in *E. coli* and purified according to the supplier’s protocol (Qiagen, Hilden, Germany). The purity and concentration of the purified plasmid DNA were determined by absorption at 260 and 280 nm and by agarose gel electrophoresis. The purified plasmid DNA was resuspended in TE buffer (10 mM Tris-Cl, pH 7.5, 1 mM EDTA) and kept in aliquots at a concentration of 0.5 mg/ml.

**Gel retardation experiments** All polymer stock solutions were prepared at a nitrogen concentration of 1 mM in distilled water and the pH was adjusted to 7.4. pRL-CMV (0.2 µg in 2 µl TE buffer) was mixed with the polymer at nitrogen/phosphate (N/P) ratios from 0 to 10. Each mixture was vortexed and incubated for approximately 30 min at room temperature, and analyzed on 1% agarose gel in TAE running buffer (40 mM Tris-acetate, 1 mM EDTA) for 40 min at 80 V in a Sub-Cell system (Bio-Rad Laboratories, CA). The gel was stained with
ethidium-bromide (0.5 μg/ml) and the DNA bands were visualized and photographed by a UV transilluminator and BioDoc-It imaging system (UVP Inc, USA).

Particle size and zeta-potential measurements The particle sizes of the polymer/pRL-CMV complexes were determined using a N4 Plus Submicron Particle Sizer (COULTER, USA). Complex solutions (100 μl) containing 20 μg of DNA were prepared at various weight ratios ranging from 2 to 20. After 30 min incubation, the complex solutions were diluted to a final volume of 1 ml prior to measurements. Scattering light was detected at 90°, running of 200 s at room temperature. The data obtained were analyzed in the Unimodal Analysis mode. Zeta-potential measurements were carried out using a Zeta Plus zeta-potential analyzer (BIC, USA) at 25 °C. Five hundred microliter of complex solutions containing 100 μg of pRL-CMV were prepared at various N/P ratios ranging from 2 to 30. After 30 minutes incubation, the complex solutions were diluted to 1.5 ml of the final volume prior to measurements. The sampling time was set to automatic mode. Zeta-potential values were presented as the average of 10 runs.

Cell viability assay The cytotoxicity of the cationic polyrotaxanes in comparison with PEI (25 K) was evaluated using MTT assay in L929 and HEK293 cell lines. The cells were cultured in the medium supplemented with 10% FBS at 37 °C, 5% CO₂, and 95% relative humidity. For cell viability assay, the cells were seeded in a 96-well plate at a density of 10,000 cells/well. After 24 h, culture media were replaced with serum-supplemented culture media containing serial dilutions of polymers and the cells were incubated for 24 h. Then, 10 μl of sterile filtered MTT stock solution in PBS (5 mg/ml) was added to each well, reaching a final MTT concentration of 0.5 mg/ml. After 5 h, unreacted dye was removed by aspiration. The formazan crystals were dissolved in DMSO (100 μl/well) and the absorbance was measured using a
microplate reader (Spectra Plus, TECAN) at the wavelength of 570 nm. The relative cell viability (%) was related to control cells cultured in media without polymer. All experiments were conducted for six samples and averaged.

**In vitro transfection and luciferase assay** Transfection studies were performed with HEK293 cells using the plasmid pRL-CMV as reporter gene. In brief, 24-well plates were seeded with cells at a density of $5 \times 10^4$/well 24 h before transfection. The polymer/DNA complexes at various N/P ratios were prepared by adding the polymer into DNA solutions dropwise, followed by vortexing and incubation for 30 min at room temperature before the transfection. At the time of transfection, the medium in each well was replaced with reduced-serum medium or normal medium. The complexes were added into the transfection medium and incubated with cells for 4 h under standard incubator conditions. After 4 h, the medium was replaced with 500 µl of fresh medium supplemented with 10% FBS, and the cells were further incubated for an additional 68 h under the same conditions, resulting in a total transfection time of 72 h. Cells were washed with PBS twice, lysed in 100 µl of cell culture lysis reagent (Promega, Cergy Pontoise, France). Luciferase gene expression was quantified using a commercial kit (Promega, Cergy Pontoise, France) and a luminometer (Berthold Lumat LB 9507, Germany). Protein concentration in the samples were analyzed using a bicinchoninic acid assay (Biorad, CA, USA). Absorption was measured on a microplate reader (Spectra Plus, TECAN) at 570 nm and compared to a standard curve calibrated with BSA samples of known concentration. Results are expressed as relative light units (RLUs) per milligram of cell protein lysate (RLU/mg protein).

4.3 Results and discussion
Scheme 4.1 shows the synthesis procedures and the structures of the cationic polyrotaxanes (5a, 5b, and 5c). α-, β-, and γ-CD consist of 6, 7, and 8 glucose units, respectively.\textsuperscript{42} Oligoethylenimine-grafted β-CD was selected as the building block, because β-CD is larger than α-CD and could therefore accommodate grafting with more chains of oligoethylenimine (OEI); the ethylenimine unit has a high cationic density because every third atom is an amino nitrogen that can potentially be protonated.\textsuperscript{43} Pluronic triblock copolymer 1, with a number-average molecular weight ($M_w$) of 2900 and a 40 wt% content of poly(ethylene oxide) (PEO) was selected as the threading polymer. Copolymer 1 has a central poly(propylene oxide) (PPO) block of 30 PO units, and two flanking PEO blocks of 13 EO units. β-CD is known to selectively thread around PPO segments.\textsuperscript{44} Therefore, in complex 3 and polyrotaxane 4, PPO segments were covered with 13 β-CD rings, whereas PEO segments were free of complexation. This provided some free space for β-CD rings to move along the polymer chain in polyrotaxane 4, allowing more efficient grafting of OEI to β-CD — a dense coverage of β-CD on the polymer might be spatially unfavorable to the grafting reaction. For blocking the ends of the copolymer to prevent the dethreading of β-CD, whose cavity is relatively large, we designed a bifunctional end group as shown in 2 and 3, that could react with two molecules of 2,4,6-trinitrobenzene sulfonate (TNBS) to form a bulky stopper that is big enough to trap the threaded β-CD on the polymer chain.
Scheme 4.1 Synthesis procedures and structures of cationic supramolecules with multiple OEI-grafted β-CD rings.

As shown in Scheme 4.1, OEIs with different chain lengths, ethylenediamine (k = 1), pentaethylenehexamine (k = 5), and linear oligoethylenimine with average molecular weight of 423 (OEI-9, k = 9), were grafted to the polyrotaxane 4 to give
OEI-grafted polyrotaxanes 5a, 5b, and 5c, respectively. To ensure that there was no intra- or inter-molecular crosslinking, a large excess of OEI (100 times of β-CD) was used in the grafting reactions. Of the three types of hydroxyl groups in β-CD, those at the 6-position (primary hydroxyl groups) are the most nucleophilic, and are thought to be modified under these weak basic conditions. This is also in accordance with the fact that the maximum number of grafted OEI per β-CD did not exceed 7, the number of the glucose units of β-CD.

Figure 4.1 shows the GPC diagram of the PEO-PPO-PEO triblock copolymer 1. The elution curve showed a single unimodal peak. The number-average molecular weight was found to be 2930, which is in accordance with the value given by the supplier. The copolymer was found to be nearly monodisperse, with a polydispersity of 1.04.

**Figure 4.1** GPC diagram of PEO-PPO-PEO triblock copolymer 1. The following molecular weight data were obtained: $M_n = 2930$, $M_w/M_n = 1.04$.

Figure 4.2 shows the $^1$H NMR spectra of polyrotaxane 4 in comparison with β-CD and PEO-PPO-PEO tetra(amine) 2 in DMSO-$d_6$. In Figure 4.2c, the peaks for β-CD, EO and PO segments of the triblock copolymer, and the 2,4,6-trinitrophenyl end group were all observed, while they were broadened as compared with the respective free counterparts in Figure 4.2a and b. This is due to the restricted molecular
movement of the components in the polyrotaxane. Quantitative comparisons between the integral intensities of the peaks of $\beta$-CD and those of threading copolymer segments gave the compositions of the polyrotaxanes. In other words, the numbers of $\beta$-CD rings threaded in a single polyrotaxane chain could be determined. It was found that 13 molecules of $\beta$-CD, on average, were threaded and blocked on the PEO-PPO-PEO triblock copolymer.

Figure 4.2 $^1$H NMR spectra of $\beta$-CD (a), PEO-PPO-PEO tetra(amine) 2 (b), and polyrotaxane 4 in DMSO-$d_6$.

Figure 4.3 shows the size exclusion chromatography (SEC) diagrams of 5a, 5b, and 5c in comparison with free $\beta$-CD. $\beta$-CD has a relatively small molecular size, which was eluted out at the low molecular weight region of the column, and was detected by RI and OR. There was no detection by UV (419 nm) because $\beta$-CD has no UV absorption. In contrast, all three cationic polyrotaxanes 5a, 5b, and 5c were eluted out
at the high molecular weight region of the column, and were detected by RI, UV (419 nm), and OR at the same time. The results indicate that the cationic polyrotaxanes 5a, 5b, and 5c had large molecular size because there were 13 molecules of OEI-grafted β-CD threaded and blocked on the polymer chain forming an integrated supramolecular entity. The OEI-grafted β-CD rings were capped by the 2,4,6-trinitrobenzene groups, so the cationic polyrotaxanes were detected by UV at 419 nm.

Figure 4.3 Size exclusion chromatograms of β-CD (a), cationic polyrotaxanes 5a (b), 5b (c), and 5c (d) detected with refractive index (RI), UV at 419 nm, and optical rotation (OR), respectively.

Figure 4.4 shows the $^1$H NMR spectra of 5a, 5b, and 5c in comparison with β-CD. In the spectra of 5a, 5b, and 5c, the signals for β-CD, the grafting OEI, the threading PEO-PPO-PEO triblock copolymer, and the end capping groups were all observed, while the peaks were much broadened because of the restriction of the molecular
motion by the chain interlocking. The average number of OEI chains grafted onto each β-CD (y) was estimated from the $^1$H NMR spectra. About 6.7 molecules of ethylenediamine were grafted onto each β-CD in 5a, nearly every glucose unit of β-CD being grafted with one ethylenediamine. Approximately 5.3 molecules of pentaethylenehexamine were grafted onto each β-CD in 5b, and about 3.4 molecules of OEI-9 were grafted on each β-CD in 5c. It is clear that the longer the OEI chain, the lower the number of OEI chains could be grafted onto each β-CD.

![Figure 4.4](image)

**Figure 4.4** $^1$H NMR spectra of β-CD (a), cationic polyrotaxanes 5a (b), 5b (c), and 5c (d) in D$_2$O.

### 4.3.2 Formation of cationic polyrotaxane/DNA complexes

The ability of the cationic polyrotaxanes to condense plasmid DNA (pDNA) into particulate structures was confirmed by agarose gel electrophorsis, particle size analysis, and zeta potential measurements. Figure 4.5a shows the gel retardation
results of cationic polyrotaxane/DNA complexes with increasing N/P ratios, in comparison with PEI (25 K). Cationic polyrotaxane 5a could inhibit the migration of the pDNA at N/P ratios of 1.5 and above, while 5b and 5c could completely complex the pDNA at N/P ratios of 2 and above. Therefore, the cationic polyrotaxanes developed in this work have similar or slightly better DNA condensation ability compared to PEI (25 K).

![Image of cationic polyrotaxanes binding to DNA](image)

**Figure 4.5** Binding ability of cationic polyrotaxanes to DNA and the particle size and zeta-potential of their complexes with DNA in comparison with PEI (25 K). (a) Electrophoretic mobility of plasmid DNA in the complexes; (b) Particle size of the complexes; and (c) Zeta-potential of the complexes.

All three cationic polyrotaxanes could efficiently compact pDNA into small nanoparticles, as shown in Figure 4.5b. Generally, the particle size decreased with an
increase in N/P ratio until the N/P ratio was 4 to 6. The particle size remained in the 150 to 250 nm range after the N/P ratio reached 6. At low N/P ratios, the pDNA complexes with PEI (25 K) had larger particle sizes than those with cationic polyrotaxanes, but decreased between 100 and 150 nm after the N/P ratio reached 10 (Figure 4.5b). The surface net charge of complexes of pDNA with cationic polyrotaxanes increased from negative to positive as the N/P ratio increased from 0 to 4, and reached a plateau at the N/P ratio of 4 and above (Figure 4.5c).

4.3.3 Transfection efficiency of cationic polyrotaxanes

The transfection efficiency of cationic polyrotaxane/DNA complexes was assessed using luciferase as a marker gene in HEK293 cells. Figure 4.6 shows the gene transfection efficiency of cationic polyrotaxanes for DNA delivery in the absence and presence of serum, in comparison with those of PEI (25 K) and naked pDNA (ND). We found that the cationic supramolecules showed high gene transfection efficacy in the absence as well as in the presence of serum, comparable to that of PEI (25 K), one of the most effective gene delivery polymers studied to date. Luciferase expression by transfection with cationic polyrotaxanes was several orders higher than that mediated by ND.

The transfection efficiency mediated by cationic polyrotaxanes was dependant upon the chain length of OEI grafted onto β-CD, and follow the order of 5c > 5b > 5a in most cases. Therefore, it is preferable to graft longer OEI onto polyrotaxanes for more efficient gene transfection. In the absence of serum, the optimal N/P ratio for most efficient transfection for 5b and 5c was 5 : 1, which is a very low N/P ratio. In contrast, in the presence of serum, the transfection efficiency increased with the N/P ratio, reaching an optimal transfection efficiency at N/P ratio of 25 to 35. It is
interesting that the gene transfection efficiency of \textbf{5b} and \textbf{5c} in the presence of serum was significantly higher than that in the absence of serum. This indicates that the cationic polyrotaxane/DNA complexes are stable enough in the complete medium, implying the new cationic supramolecules may be advantageous for \textit{in vivo} applications.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure4.png}
\caption{In vitro gene transfection efficiency of the complexes of cationic polyrotaxane/DNA in HEK293 cells in comparison with that of PEI (25 K) or naked DNA, in the absence (a) and presence (b) of serum. The transfection efficiency of PEI (25 K) was obtained with the optimal N/P ratio of 10. Data represent mean ± standard deviation (n = 3).}
\end{figure}

\subsection{4.3.4 Cytotoxicity of cationic polyrotaxanes}

Cytotoxicity of polymeric gene vectors may be an important factor that affects the transfection efficiency. Our studies showed that the cationic polyrotaxanes were much less toxic than PEI in cell cultures. Figure 4.7 shows the cytotoxicity of cationic polyrotaxanes \textbf{5a}, \textbf{5b}, and \textbf{5c} in L929 cells and HEK293 cells in comparison with PEI (25 K). Generally, all three cationic polyrotaxanes showed significantly lower
cytotoxicity than PEI (25 K) in both cell lines. The high amino density and the high molecular weight may be the reasons of the toxicity of PEI. Although cationic polyrotaxanes 5a, 5b, and 5c were grafted with OEI, the very different chain architectures from that of PEI may result in lower cytotoxicity. Generally, the amino density of cationic polyrotaxanes 5a, 5b, and 5c is lower than PEI. Despite the lower amino density, the cationic polyrotaxanes had similar or slightly better DNA binding capacity.

![Cytotoxicity of cationic polyrotaxanes](image)

**Figure 4.7** Cytotoxicity of cationic polyrotaxanes 5a, 5b, and 5c in L929 cells (a) and HEK293 cells (b) in comparison with PEI (25 K). Data represent the mean ± standard deviation (n = 6).

It was reported that polyimino carbonate-containing β-CD, β-CD-modified PEI, and polyamidoamine dendrimer conjugates with CDs have been synthesized for enhanced gene transfection. These conjugates or derivatives are basically composed of amino-bearing polymers covalently bonded with CDs. Our cationic polyrotaxanes
are different, where many small OEI-grafted β-CD units are threaded and blocked upon a polymer chain to form an integrated macromolecular entity.

Ooya et al. also reported the use of dimethylaminoethyl-modified α-CDs threaded onto a PEO chain and capped by cleavable end groups.\(^{50}\) In their cases, α-CD only contains tertiary amines, which may not be as efficient in DNA complexation and gene delivery. Our design using oligoethylenimine-grafted β-CDs threaded on a PEO-PPO-PEO triblock copolymer may have the following advantages. First, the low spatial hindrance presented by the spare PEO segment permits efficient grafting of OEI chains onto β-CDs. Second, the OEI-grafted β-CD rings can freely move along both PPO and PEO segments in solution, giving flexibility for efficient complexation with DNA. Finally, our system has a lot of “flapping” OEI chains with many primary and secondary amines, which may also be beneficial for interaction with DNA and/or cell membranes. These factors may contribute to the transfection efficiency that is equal to PEI (25K) at the optimized N/P ratio and molecular weight, and is 2 — 4 orders higher than the dimethylaminoethyl-modified α-CDs polyrotaxane system.\(^{50}\) However, the work of Ooya et al. was more focused on release of the DNA in cells by dethreading through cleavage of the end groups, which addressed a different but very important aspect of gene-delivery design.

4.4 Conclusion

In this study, we have successfully demonstrated a smart materials design of cationic supramolecular polyrotaxane entities for efficient gene delivery. When properly designed, the structures and conformations of the cationic polyrotaxanes can be further controlled in terms of the density of amino groups and the flexibility of the
supramolecules to give gene carriers with different properties for a wide range of applications.

4.5 References:


42. Bender, M. L.; Komiyama, M. *Cyclodextrin Chemistry*, Springer-Verlag, Berlin, Germany, **1978**.


CHAPTER FIVE

OLIGOETHYLENIMINE-MODIFIED POLYROTAXANES COMPOSED OF α-CYCLODEXTRINS THREADED ON POLY[(PROPYLENE OXIDE)]-POLY[(ETHYLENE OXIDE)]-POLY[(PROPYLENE OXIDE)] TRIBLOCK COPOLYMER FOR EFFICIENT GENE TRANSFER

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5.3.4 Cytotoxicity of cationic polyrotaxanes
5.1 Introduction

Over the last few decades, supramolecular architectures have intrigued researchers because of their unique structures and remarkable properties such as molecular recognition, self-assembly, self-organisation and kinetic and thermo-dynamic complementarity. Polyrotaxane, formed by multiple macrocycles threading over a polymeric chain, is such an example. Since the first polyrotaxane was synthesized in 1992, growing interests have been shown in the study of the supramolecular structure of the polyrotaxanes and their potentials for electronical and biomedical applications.

Currently, cationic polymers have gained much attention for their potential use as nonviral gene carriers. Compared with other gene delivery systems (viral vectors and cationic lipids), cationic polymers for gene delivery are generally economical and stable, and they can be produced in a large scale and show low host immunogenicity. By now a great number of polycations have been reported to be able to deliver gene, including homopolymers or derivatives of polyethylenimine (PEI), poly(L-lysine), polyamidoamine, poly(L-glutamic acid), polyphosphoester, and chitosan.

For conventional polycations containing long sequences of covalently bonded repeating units, the mobility of their molecular chains will decrease with an increase
of their repeating units and molecular weight in the solution state. In contrast, under
the same condition the macrocycles in polyrotaxanes will rotate and/or slide along the
polymeric chain freely, which can improve the mobility of cationic ligands linked to
the macrocycles and enhance the interaction of the cationic ligands and receptor
DNA.\(^{27,28}\) In 2004, Ooya et al. introduced aminocarbamoyl (ACE) groups to
polyrotaxanes consisting of \(\alpha\)-CD and poly(ethylene glycol), and found that the
introduction of ACE groups enhanced the opportunity of complexion with DNA.\(^{29}\)
They also reported the use of dimethylaminoethyl-modified \(\alpha\)-CDs threaded onto a
PEO chain and capped by cleavable end groups. The cleavage of the end groups cause
the dethreading of \(\alpha\)-CDs and rapid release of DNA in cells, but the tertiary amines
conjugated to the \(\alpha\)-CDs may not be efficient in DNA complexation and gene
delivery.\(^{30}\) Our group also designed and synthesized cationic supramolecules
composed of multiple oligoethylenimine-grafted \(\beta\)-CDs threaded on a polymeric
chain, these supramolecules showed good DNA binding ability, low cytotoxicity and
high gene-transfection efficiency in HEK293 cells.\(^{31}\)

In this study, we synthesized a variety of novel cationic polyrotaxanes containing
PPO-PEO-PPO triblock copolymer, \(\alpha\)-CD and various linear or nonlinear
oligoethylenimine (OEI) chains for gene delivery. In both HEK293 and COS7 cells,
these polyrotaxanes showed low cytotoxicity and high transfection efficiency which is
similar to that of branched PEI (25 K), one of the most effective gene-delivery
polymers studied to date. Especially, they displayed sustained gene delivery capability
in HEK293 cells in both serum and serum free condition, while the transfection
efficiency of PEI (25 K) decreased dramatically with the increasing expression duration.

5.2 Experimental section

5.2.1 Materials

Pluronic-R PPO-PEO-PPO triblock copolymer ($M_n = 1,990$, $M_w/M_n = 1.04$) was obtained by Aldrich. This polymer has chain composition of PO$_8$EO$_{23}$PO$_8$. The molecular characteristics were determined by combination of GPC and $^1$H NMR results, which were found to be within the specification of the supplier. 2,4,6-Trinitrobenzene sulfonic acid solution and pentaethylenehexamine was obtained from Fluka. 1,1′-Carbonyldiimidazole (CDI) and $\alpha$-cyclodextrin were purchased from Tokyo Kasei incorporation. Ethylenediamine, linear PEI with molecular weight of 423 (OEI-9), branched PEI with molecular weight of 600 (OEI-14) and branched PEI (25 K) were also supplied by Aldrich. DMSO-$d_6$ and D$_2$O used as solvent in the NMR measurements were also obtained from Aldrich. Qiagen kit and Luciferase kit were purchase from Qiagen and Promega, respectively. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazodium bromide (MTT), penicillin, and streptomycin were obtained from Sigma.

5.2.2 Synthesis

The procedures for preparation of $5b$ from PPO-PEO-PPO triblock copolymer $\mathbf{1}$ are given below as a typical example.
5.2.2.1 Synthesis Preparation of PPO-PEO-PPO bis(amine)

Pluroic-R PPO-PEO-PPO triblock copolymer 1 \( (M_n = 1990, 2.0 \text{ g, 1 mmol}) \) was heated in a flask at 80 °C in vacuum overnight. When the flask cooled, 15 mL of anhydrous DMF was injected under nitrogen. After all of 1 was dissolved, the DMF solution of 1 was added dropwise over a period of 6 h under nitrogen to 15 mL of anhydrous DMF solution in which CDI (1.622 g, 10 mmol) was dissolved, and the mixture was stirred overnight under nitrogen at room temperature. The resulting solution was slowly added dropwise during a period of 3 h into 24.0 g (400 mmol) of ethylenediamine which was dissolved in 15 mL of anhydrous DMF with stirring at room temperature and stirred overnight. Excess ethylenediamine and DMF was removed by vacuum evaporation. The resulting viscous solution was purified by size exclusion chromatography (SEC) on a Sephadex LH-20 column using methanol as eluent. Finally, 1.80 g viscous liquid 2 was yielded (83%). \(^1\)H NMR (400 MHz, DMSO-\(d_6\), 22 °C): \( \delta \) 3.25-3.71 (m, 92H and 48H, -CH\(_2\)CH\(_2\)O- of PEO block and -CH\(_2\)CHO- of PPO block), 3.22 (s, 4H, CONCH\(_2\) of ethylenediamine), 2.04 (s, 4H, N\(_\text{CH}_2\) of ethylenediamine), 1.04 (s, 48H, –CH\(_3\) of PPO block).

5.2.2.2 Preparation of \( \alpha\)-CD-PPO-PEO-PPO polyrotaxane

0.5 g of the resulting PPO-PEO-PPO bis(amine) 2 was added to 59 mL \( \alpha\)-CD saturated solution (0.145 g \( \alpha\)-CD/mL H\(_2\)O), and 0.75 g NaHCO\(_3\) was added to adjust the pH value of the solution. The reaction mixture was ultrasonicated for 20 min and stirred at room temperature overnight. Then, 3.15 g of sodium salt of picrylsulfonic acid was added to the mixture and stirred overnight. Subsequently, 200 mL H\(_2\)O was
poured into the reaction mixture to precipitate the product. The precipitate was centrifuged and washed 3 times with water. The resulting wet solid was dissolved in 30 mL DMSO and poured into 500 mL CH₃COOCH₂CH₃ to precipitate the product. The precipitate was centrifuged and washed 3 times with CH₃COOCH₂CH₃. The resulting wet solid was dissolved in 30 mL DMSO again and poured into 500 mL H₂O to precipitate the product. The resulting precipitate was centrifuged and washed with H₂O for 3 times. Finally, the resulting wet solid was dried by freeze (liquid nitrogen) in vacuo and 1.76 g pure polyrotaxane 4 was yielded (89 %). ¹H NMR (400 MHz, DMSO-d₆, 22 °C): δ 8.89 (s, 2H, meta H of phenyl), 8.77 (s, 2H, meta H of phenyl), 5.62 (s, 47H, O(2)H of CD), 5.47 (m, 47H, O(3)H of CD), 4.75 (s, 47H, H(1)H of CD), 4.38 (s, 47H, O(6)H of CD), 2.80-4.00 (m, 282H, H(3), H(6), H(5), H(2) and H(4) of CD, 92H, -CH₂CH₂O- of PEO block, 48H, -CH₂CHO- of PPO block), 1.04 (s, 48H, -CH₃ of PPO block).

5.2.2.3 Preparation of cationic multiple OEI-grafted α-CD-PPO-PEO-PPO polyrotaxane

The resulting polyrotaxane 4 (0.2028 g, 0.02 mmol) was dried at 40 °C in vacuum overnight. When the flask cooled, 40 mL dry DMSO was injected under nitrogen. After all 4 was dissolved, the DMSO solution of 4 was added dropwise over a period of 6 h under nitrogen to 40 mL of anhydrous DMSO solution in which CDI (2.27 g, 14 mmol) was dissolved, and the mixture was stirred overnight under nitrogen at room temperature. A mixture of 300 mL THF and 600 mL Et₂O was poured into the resulting solution to precipitate the product. The precipitate was centrifuged and
washed 3 times with THF. The resulting wet solid was dissolved in 40 mL DMSO and this solution was slowly added dropwise over a period of 3 h into 4.90 mL (16.8 mmol) of pentaethylenehexamine which had been dissolved in 40 mL of DMSO with stirring at room temperature and stirred overnight. 900 mL THF was poured in the reaction mixture to precipitate the product. The precipitate was centrifuged and washed 3 times with THF, and the resulting crude product was purified by size exclusion chromatography (SEC) on a Sephadex G-50 column using DI water as eluent. Finally, 0.186 g brown solid 5b was yielded (46%).

The yields and analytical data for all four cationic polyrotaxanes are given below.

**Cationic polyrotaxane 5a.** Yield, 77%. $^1$H NMR (400 MHz, D$_2$O, 22 °C): δ 8.38 (s, 2H, meta H of phenyl), 8.01 (s, 2H, meta H of phenyl), 5.03 (d, broad, 47H, H(1) of CD), 2.92-4.59 (m, broad, 282H, H(3), H(6), H(5), H(2) and H(4) of CD, 92H, -CH$_2$CH$_2$O- of PEO block, 48H, -CH$_2$CHO- of PPO block, 124H, CONCH$_2$ of ethylenediamine), 2.74 (s, 124H, NCH$_2$ of ethylenediamine), 1.12 (s, 48H, -CH$_3$ of PPO block).

**Cationic polyrotaxane 5b.** Yield, 46%. $^1$H NMR (400 MHz, D$_2$O, 22 °C): δ 8.43 (s, 2H, meta H of phenyl), 8.04 (s, 2H, meta H of phenyl), 5.07 (d, broad, 47H, H(1)H of CD), 3.00-4.57 (m, broad, 282H, H(3), H(6), H(5), H(2) and H(4) of CD, 92H, -CH$_2$CH$_3$O- of PEO block, 48H, -CH$_2$CHO- of PPO block, 78H, CONCH$_2$ of pentaethylenehexamine), 2.74 (m, 937H, NCH$_2$ of pentaethylenehexamine), 1.11 (s, 48H, -CH$_3$ of PPO block).

**Cationic polyrotaxane 5c.** Yield, 54%. $^1$H NMR (400 MHz, D$_2$O, 22 °C): δ 8.39 (s,
2H, meta H of phenyl), 8.02 (s, 2H, meta H of phenyl), 5.00 (d, broad, 47H, H(1)H of CD), 2.96-4.61 (m, broad, 282H, H(3), H(6), H(5), H(2) and H(4) of CD, 92H, -CH₂CH₂O- of PEO block, 48H, -CH₂CHO- of PPO block, 36H, CONCH₂ of OEI-9), 2.72 (m, 823H, NCH₂ of OEI-9), 1.11 (s, 48H, -CH₃ of PPO block).

**Cationic polyrotaxane 5d.** Yield, 66%. ¹H NMR (400 MHz, D₂O, 22 °C): δ 8.34 (s, 2H, meta H of phenyl), 7.96 (s, 2H, meta H of phenyl), 4.96 (d, broad, 47H, H(1)H of CD), 2.94-4.62 (m, broad, 282H, H(3), H(6), H(5), H(2) and H(4) of CD, 92H, -CH₂CH₂O- of PEO block, 48H, -CH₂CHO- of PPO block, 55H, CONCH₂ of OEI-14), 2.57 (m, 1827H, NCH₂ of OEI-14), 1.08 (s, 48H, -CH₃ of PPO block).

### 5.2.3 Measurements

**Gel permeation chromatography (GPC)** Gel permeation chromatography (GPC) analysis for PPO-PEO-PPO triblock copolymer was carried out with a Shimadzu SCL-10A and LC-10ATVP system equipped with two Phenogel 5 μm, 50 and 1000 Å columns (size: 300 × 4.6 mm) in series and a Shimadzu RID-10A refractive index detector. THF was used as eluent at a flow rate of 0.30 mL/min at 40 °C. Monodispersed poly(ethylene glycol) standards were used to obtain a calibration curve.

GPC analysis for cationic polyrotaxanes was carried out with a Shimadzu SCL-10A and LC-10AT system equipped with a Sephadex G-75 column (size: 2.5 × 32 cm), a Shimadzu RID-10A refractive index detector. 1× PBS buffer solution was used as the eluent. Fractions were collected per 1 mL and were detected with a HORIBA
SEPA-300 high speed accurate polarimeter at wavelength 589 nm with cell length 10 cm and response 2 s.

**1H NMR spectra** The 1H NMR spectra were recorded on a Bruker AV-400 NMR spectrometer at 400 MHz at room temperature. The 1H NMR measurements were carried out with an acquisition time of 3.2 s, a pulse repetition time of 2.0 s, a 30° pulse width, 5208-Hz spectral width, and 32 K data points. Chemical shifts were referred to the solvent peaks (δ = 4.70 ppm for D2O and 2.50 ppm for DMSO-d6).

**13C NMR spectra** The 13C NMR spectra were recorded on a Bruker AV-400 NMR spectrometer at 100 MHz at room temperature. The 13C NMR measurements were carried out using composite pulse decoupling with an acquisition time of 0.82 s, a pulse repetition time of 5.0 s, a 30° pulse width, 20,080-Hz spectral width, and 32 K data points.

**Basic hydrolysis of cationic polyrotaxane 5c** The basic hydrolysis was conducted at room temperature by adding 50.6 mg of cationic polyrotaxane 5c to a 5 wt% of content of NaOH solution (2.5 mL, 1.25 mol/L). The resulting hydrolyzed product was sampled and detected by GPC at 24 h, 48 h and 96 h, respectively. Prior to measurements, all the samples were neutralized to pH 7.3.

**Plasmid** The plasmid used was pRL-CMV (Promega, USA), encoding *Renilla* luciferase, which was originally cloned from the marine organism *Renilla reniformis*. All plasmid DNAs were amplified in *Escherichia coli* and purified according to the supplier’s protocol (Qiagen, Hilden, Germany). The purity and concentration of the purified plasmid DNA were determined by absorption at 260 and 280 nm and by
agarose gel electrophoresis. The purified plasmid DNA was resuspended in TE buffer (10 mM Tris-Cl, pH 7.5, 1 mM EDTA) and kept in aliquots at a concentration of 0.5 mg/mL.

**Cells and Media** All cell lines were purchased from ATCC (Rockville, MD). COS7 and HEK293 cells were maintained in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum, 100 units/mg penicillin, 100 µg/mL streptomycin at 37 °C and 5% CO₂. Opti-MEM reduced serum medium, and DMEM medium were purchased from Gibco BRL (Gaithersburg, MD).

**Gel Retardation Experiments** All polymer stock solutions were prepared at a nitrogen concentration of 1 mM in distilled water and the pH was adjusted to 7.4. pRL-CMV (0.2 µg in 2 µL TE buffer) was mixed with polymer at N/P ratios from 0 to 10. Each mixture was vortexed and incubated for approximately 30 min at room temperature, and then analyzed on 1% agarose gel in TAE running buffer (40 mM Tris-acetate, 1 mM EDTA) for 40 min at 80V in a Sub-Cell system (Bio-Rad Laboratories, CA). The gel was stained with ethidium bromide (0.5 µg/mL) and the DNA bands were visualized and photographed by a UV transilluminator and BioDoc-It imaging system (UVP Inc., USA).

**Cell viability assay** Two cell lines (COS7 and HEK293) were cultured in the DMEM medium supplemented with 10% FBS at 37 °C, 5% CO₂, and 95% relative humidity. For cell viability assay, the cells were seeded in a 96-well microtiter plate (Nunc, Wiesbaden, Germany) at a density of 10,000 cells/well for COS7 cells and 15,000 cells/well for HEK293 cells. After 24 h, culture media were replaced with
serum-supplemented culture media containing serial dilutions of the polymers and the cells were incubated for 24 h. Then, 10 μL of sterile filtered MTT stock solution in PBS (5 mg/mL) was added to each well, reaching a final concentration of 0.5 mg/mL. After 5 h, unreacted dye was removed by aspiration. The formazan crystals were dissolved in DMSO (100 μL/well) and the absorbance was measured using a microplate reader (Spectra Plus, TECAN) at the wavelength of 570 nm. The relative cell viability (%) was related to control cells cultured in media without polymer. All experiments were conducted for six samples and averaged.

**In vitro transfection and luciferase assay** Transfection studies were performed with COS7 and HEK293 cells using the plasmid pRL-CMV as reporter gene. In brief, 24 h before transfection, 24-well plates were seeded with cells at a density of 5×10⁴/well. The polymer/DNA complexes at various N/P ratios were prepared by adding the polymer into DNA solutions dropwise, followed by vortexing and incubation for 30 min at room temperature before the transfection. At the time of transfection, the medium in each well was replaced with reduced-serum medium or normal medium. The complexes were added into the transfection medium and incubated with cells for 4 h under standard incubator conditions. After 4 h, the medium was replaced with 500 μL of fresh medium supplemented with 10% fetal bovine serum (FBS), and the cells were further incubated for an additional 20 h (longer incubation time of 44 h and 68 h for sustained gene delivery study) under the same conditions, resulting in a total transfection time of 24 h (longer total transfection time of 48 h and 72 h for sustained gene delivery study). Cells were washed twice
with PBS and lysed in 100 µL of cell culture lysis reagent (Promega, Cergy Pontoise, France). Luciferase gene expression was quantified using a commercial kit (Promega, Cergy Pontoise, France) and a luminometer (Berthold Lumat LB 9507, Germany). Protein concentration in the samples was analyzed using a bicinchoninic acid assay (Biorad, CA, USA). Absorption was measured on a microplate reader (Spectra Plus, TECAN) at 570 nm and compared to a standard curve calibrated with bovine serum albumin (BSA) samples of known concentration. Results are expressed as relative light units per milligram of cell protein lysate (RLU/mg protein).

**Dynamic light scattering and zeta-potential** Measurements of particle size and zeta potential of the complexes were performed using a Zetasizer Nano ZS (Malvern Instruments, Southborough, MA, USA) with a laser light wavelength of 633 nm at a 173° scattering angle. Complex solutions (100 µl) containing 3 µg of pDNA (pRL-CMV) were prepared at various N/P ratios ranging from 2-30. The mixture was vortexed for 20 s, incubated for 30 min at room temperature and diluted in 1 mL of distilled water before being analyzed on the Zetasizer. The size measurement was performed at 25 °C in triplicate. The deconvolution of the measured correlation curve to an intensity size distribution was accomplished using a non-negative least squares algorithm. The Z-average hydrodynamic diameters of the particles were given by the instrument. The Z-average size is the intensity weighted mean diameter derived from a Cumulants or single exponential fit of the intensity autocorrelation function. The zeta potential measurements were performed using a capillary zeta potential cell in automatic mode using the same samples for the particle size measurements.
Confocal microscopy For confocal microscopy imaging of the gene transfection, the plasmid pEGFP-N1 (Clontech Laboratories Inc., USA), encoding a red-shifted variant of wild-type green fluorescence protein (GFP), was used to examine the GFP expression in HEK293 cells. HEK293 cells were seeded onto lab-Tek 4-chambered coverglass (Nalge-Nane international, USA) at density of 5×10⁴ cells/well in 500 µL of complete DMEM medium. After 24 hours, transfection was undertaken with 2 µg EGFP plasmid in 0.3 mL of reduced-serum Opti-MEM medium in each well. At the time of transfection, 20 µL of cationic polyrotaxane 5b-DNA complex solution was added in each well. After 4 h, the transfection media was removed and the cells were washed with fresh complete DMEM medium. After 20 h of further incubation in serum-containing complete DMEM medium, the cells were washed with phosphate-buffered saline (PBS) and imaged under a laser scanning confocal microscope (LSM 410, Carl Zeiss, USA). GFP fluorescence was excited at 488 nm and emission was collected using a 515 nm filter.

AFM A Digital Instruments MultiMode-AFM with Nanoscope IV controller in a tapping mode was employed to image the nanoparticle samples. Briefly, silicon disks were soaked in 50% acetone for a minimum of 2 h and rinsed with distilled water. When the silicon disks were completely dried, 20 µL of cationic polyrotaxane 5b-DNA complexes containing 1.0 µg of pRL-CMV at N/P ratios 0, 2, and 10 were placed on the silicon surface for 2 min, followed by removing the complex solutions carefully with a piece of tissue paper. All the AFM images were obtained with a scan rate of 0.5 or 1 Hz over a selected area of 2×2 µm. Image analysis was performed
5.3 Results and discussion

5.3.1 Synthesis and characterization of cationic polyrotaxanes

Scheme 5.1 synthesis procedures and the structures of multiple OEI-modified cationic α-CD-PPO-PEO-PPO polyrotaxanes 5a, 5b, 5c and 5d.

Scheme 5.1 shows the synthesis procedures and the structures of the cationic polyrotaxanes (5a, 5b, 5c and 5d). Firstly, for conversion of both of the terminal hydroxyl groups of the PPO-PEO-PPO triblock copolymer 1 to amino groups, the
hydroxyl groups were activated with CDI, followed by reaction with a large excess of ethylenediamine to give PPO-PEO-PPO bis(amine) 2. These copolymers were allowed to react with saturated solution of α-CD and a large excess 2,4,6-trinitrobenzene sulfonate (TNBS) in sequence to form polyrotaxanes 4. Finally, various linear or nonlinear OEIs with different molecular weights were grafted to polyrotaxane 4 to give the corresponding cationic polyrotaxanes (5a, 5b, 5c and 5d).

Figure 5.1 shows the 1H NMR spectra of polyrotaxane 4 with reference to α-CD and PPO-PEO-PPO bis(amine) 2 in DMSO-d6. In Figure 5.1c, the peaks for α-CD, EO and PO segments of the triblock copolymer, and the 2,4,6-trinitrophenyl end groups were all observed, while they were broadened as compared with the respective free counterparts in Figure 5.1a and b. This is due to the restricted molecular movement of the components in the polyrotaxane. Quantitative comparisons between the integral intensities of the peaks of α-CD and those of threading copolymer segments gave the compositions of the polyrotaxanes. It was found that 8 α-CD rings, on average, were covered and blocked on the PPO-PEO-PPO triblock copolymer in one polyrotaxane 4.

Figure 5.2 shows the size exclusion chromatograms of the cationic polyrotaxanes in contrast to free α-CD. α-CD has relative small molecular size, which was eluted out at the low molecular weight region of the column, and was detected by RI and OR. There was no detection by UV (419 nm) because α-CD has no UV absorption. In contrast, all four cationic polyrotaxanes were detected by RI, UV (419 nm), and OR at the same time. It is found that they eluted out at higher molecular weight region of the
column due to their large molecular size. Comparing to the other synthetic polyrotaxanes, 5a eluted out late, which is in agreement with its smaller molecular size. Each cationic polyrotaxane showed a unimodal peak and this peak eluted out at the same position in all three spectra. This result indicates that these polyrotaxanes are pure with no intra- or intermolecular crosslinked byproducts.

Figure 5.1 $^1$H NMR spectra of $\alpha$-CD (a), PPO-PEO-PPO bis(amine) (b), $\alpha$-CD-PPO-PEO-PPO polyrotaxane (c) in DMSO-$d_6$. 

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Figure 5.2 GPC trace of α-CD and cationic polyrotaxane 5a, 5b, 5c and 5d with their respective refractive index (RI), UV at 419 nm and Optical rotation (OR) curve.

Figure 5.3 shows the $^{13}$C NMR spectra of the cationic polyrotaxane 5b with reference to free α-CD and pentaethylenehexamine. In Figure 5.3c, all peaks attributed to α-CD and grafting OEI were observed clearly. The peak at δ 158.2 ppm corresponds to the carbon of carbonyl groups conjugated OEI chains to α-CD rings. Additionally, compared to free α-CD, the peak of C-6 on the α-CD rings of 5b shifted from 60.7 ppm to 64.3 ppm. It may be attributed mainly to the grafting of OEI. In fact, of the three types of hydroxyl groups of α-CD, those at the 6-position (primary hydroxyl groups) are the most nucleophilic and are thought to be modified under the weak basic conditions.32
Figure 5.3 $^{13}$C NMR spectra of $\alpha$-CD (a), pentaethylenehexamine (b), and cationic polyrotaxanes 5b (c) in D$_2$O.

Figure 5.4 shows the $^1$H NMR spectra of the cationic polyrotaxanes with reference to $\alpha$-CD. In the spectra of 4b-e, the signals for both $\alpha$-CD and grafting OEI were observed. The peaks were much broadened due to the restriction of molecular motion by the grafting OEI units. From the $^1$H NMR spectra, the average number of OEI chains conjugated to each $\alpha$-CD ($y$) was estimated. About 5.3 molecules of ethylenediamine were grafted onto each $\alpha$-CD in 5a and about 5.0 molecules of pentaethylenehexamine were grafted onto each $\alpha$-CD in 5b, near to one OEI chain per glucose unit of $\alpha$-CD. About 2.3 molecules of OEI-9 were grafted on each $\alpha$-CD in 5c and about 3.5 molecules of OEI-14 were grafted on each $\alpha$-CD in 5d. From 5a, 5b to 5c, it is clear that the longer the OEI chain, the less number of OEI chains could be
conjugated to each α-CD. It can be attributed to the influence of the steric hindrance of OEI chains on the conjugating reaction. But, for 5d, more primary amino groups from branched structure of OEI-14 participated in the conjugation reaction, leading to the increased number of OEI chains conjugated to each α-CD.

![Diagram](image)

**Figure 5.4** $^1$H NMR spectra of α-CD (a), and cationic polyrotaxanes 5a (b), 5b (c), 5c (d) and 5d (e) in D$_2$O.

### 5.3.2. Degradation of cationic polyrotaxane 5c under basic condition

Figure 5.5 shows the GPC trace of cationic polyrotaxane 5c and its basic degradation products at 24 h, 48 h, and 96 h, with reference to α-CD, with their respective RI, UV at 419 nm and OR curve. From Figure 5.5b to d, it is evident that on the OR curves the elution time of the degradation products were much longer than
those of cationic polyrotaxane 5c, increasing with an increase of degradation period, indicating the declining trend of molecular size. The degradation product at 96 h eluted out at the same position of α-CD, suggests that not only the bulky stoppers, TNBS moieties, of the cationic polyrotaxane 5c have been cut off and the OEI-9-grafted α-CD rings had slid out of the polymeric chain, but the carbamic bonds were cleaved and OEI-9 moieties removed under the strong basic condition. On the UV curves of Figure 5.5b-d, the peaks eluting out at about 440 min represents the TNBS residues.

![Figure 5.5](image)

**Figure 5.5** GPC trace of cationic polyrotaxane 5c (a) and its basic hydrolysis products at 24 h (b), 48 h (c), and 96 h (d), in comparison with α-CD (e), with their respective refractive index (RI), UV at 419 nm and optical rotation (OR) curve.

5.3.3 *Formation of cationic polyrotaxane/DNA complexes*

The ability of the cationic polyrotaxanes to condense plasmid DNA (pDNA) into
particulate structures was confirmed by agarose gel electrophoresis, particle size and zeta potential measurements, as well as AFM images. It is known that the DNA condensation capability of cationic polymers is one of the prerequisites to be a gene carrier. To confirm the formation of the synthesized cationic polyrotaxane/DNA complexes, agarose gel electrophoresis was performed and retardation of DNA mobility examined. Figure 5.6a shows the gel retardation results of cationic polyrotaxane/DNA complexes with increasing N/P ratios compared to branched PEI (25 K). Cationic polyrotaxane 5a could compact pDNA entirely at the low N/P ratio of 1, while 5b and 5c could inhibit the migration of pDNA at N/P ratio of 2 and above. This indicates that cationic polyrotaxanes with linear OEI units have similar or slightly better DNA condensation ability compared to PEI (25 K). For 5d, complexes with pDNA complete only at N/P ratio of 3 and above, probably because of the branched OEI units in this cationic polyrotaxane.

Figure 5.6b and c shows the particle size and zeta potential of cationic polyrotaxane/DNA complexes with reference to PEI (25 K)/DNA complex at various N/P ratios. In Figure 5.6b, all four cationic polyrotaxanes efficiently compacted pDNA into small nanoparticles. Generally, their mean particle size decreased sharply with increasing N/P ratio from 2 to 6. After a N/P ratio of 6 was reached, the particle size varied within 85-165 nm. In the case of the complex formed by PEI (25 K), its hydrodynamic size reached a plain around 120-160 nm at N/P ratio of 4 and above. Within the range of N/P ratio 10-30, the particle size of PEI (25 K)/DNA complex was higher than that of the cationic polyrotaxanes at the same N/P ratio.
Zeta potential measurements are indicative of the surface charge of polymer/DNA particles, and a positive surface charge of untargeted polymer is necessary for binding to anionic cell surface, which consequently facilitates cell uptake. As shown in Figure 5.6c, the surface net charge of the complexes of pDNA with PEI and 5d increased abruptly from negative to positive as the N/P ratio increased from 0 to 4 and stabilized at N/P ratio of 10 and above. Also, though the surface net charge of the complex of 5a, 5b and 5c with pDNA was positive and beyond 8 mV at N/P ratio 2, they stabilized within 20~28 mV and were lower than that of PEI (25 K)/DNA complex, which reached an almost constant value around 33 mV, from N/P ratio of 8 to 30.

![Figure 5.6](image)

**Figure 5.6** Binding ability of cationic polyrotaxanes to DNA and the particle size and zeta potential of their complexes with DNA in comparison with PEI (25K). (a). Electrophoretic mobility of plasmid DNA in the complexes; (b). particle size of the complexes; and (c). zeta potential of the complexes.

Figure 5.7 showed representative tapping mode AFM images of naked DNA and
cationic polyrotaxane 5b/DNA complexes at N/P ratio of 2 and 10. Results from the AFM study showed that the complexation of DNA by 5b led to the formation of compact nanoparticle. In Figure 5.7a, loose, supercoiled structure of pDNA could be found when the pDNA was not condensed by polymer. At N/P ratio of 2, supercoiled plasmid DNA could still be identified under AFM while some of the pDNA was condensed to nanoparticles by 5b. Compared to this partial condensation at N/P ratio of 2, the same amount of pDNA could be tightly packed and formed pDNA complexes at N/P ratio of 10 completely. Moreover, it was found that the diameter of the nanoparticles in Figure 5.7c ranged within 110-160 nm, which is in agreement with the dynamic light scattering results.

Figure 5.7 Atomic force microscopy (AFM) images of the supercoiled plasmid DNA (a), and cationic polyrotaxane 5b/DNA complex at N/P = 2 (b) and N/P = 10 (c).

5.3.4 Cytotoxicity of cationic polyrotaxanes

Cytotoxicity of polymeric gene vector may be an important factor that affects the transfection efficiency. Figure 5.8 showed the results of in vitro cytotoxicity of the cationic polyrotaxanes analyzed by MTT method in two cell lines (COS7 and HEK293). As shown in Figure 5.8a, all the synthesized cationic polymers and PEI (25
K) showed a strong dose-dependent effect on cytotoxicity and the cytotoxicity of the polymers was much lower than that of PEI (25K). For example, at concentration of 62.5 μg/ml, COS7 cells only showed approximately 7% cell viability when incubated with PEI (25 K). In the meanwhile, in the case of 5a, 5b and 5c, which were grafted with linear OEI chains, their relative growth rate in COS7 cells showed more than 70% viability under the same condition. 5d, which was grafted with branched OEI-14, exhibited nearly 50% cell viability at this concentration. LD50 value was also calculated to further compare the cytotoxicity of the cationic polymers with PEI (25 K). The LD50 value of PEI (25 K) in COS7 was 25 μg/ml, while those of cationic polyrotaxanes 5a, 5b, 5c and 5d were 155, 135, 140 and 55 μg/ml, respectively, which were much lower than that of the PEI (25 K). These results may be attributed to the reduction of amino density resulted from the supramolecular structure of the polyrotaxanes since high amino density and the high molecular weight may be the reasons of the toxicity of PEI (25 K). A similar trend was also observed in HEK293 cells (Figure 5.8b).
5.3.5 Transfection efficiency of cationic polyrotaxanes

In vitro transfection efficiency of complexes formed between pDNA and cationic polyrotaxanes was assessed utilizing a transient expression of luciferase reporter in both HEK293 and COS7 cells. Figure 5.9 shows the gene transfection efficiency of cationic polyrotaxanes for DNA delivery compared with those of branched PEI (25 K) and naked pDNA in the absence and presence of serum (ND), in both COS7 and HEK293 cells. The structure of polymers play an important role in the transfection efficiency. In COS7 cells the transfection efficiency mediated by cationic polyrotaxanes was dependent upon the chain length of the OEI conjugated to α-CD. Among the polymers examined, increased OEI length produced greater transfection efficiency following the order \(5\text{c}>5\text{b}>5\text{a}\), but the transfection capability of \(5\text{d}\) did not follow this rule probably because of its branched OEI chains.
The relationship of transfection efficiency with the chain length of OEI was not strong in HEK 293 cells. The transfection efficiency mediated by 5b was quite similar to PEI at various N/P ratios in the absence of serum. Compared with PEI (25 K), 5b and 5c showed a similar or higher gene delivery capability in the presence of serum. Especially, when the transfection was conducted at N/P ratio of 30, the transfection efficiency of 5b and 5c in the presence of serum was 10-fold and 7-fold more than that of PEI (25 K), respectively.

Figure 5.9 In vitro gene transfection efficiency of the complexes of cationic polyrotaxanes in comparison with that of PEI (25 K) and naked DNA (ND), in COS7 cell in the absence and presence of serum (a) and (b); in HEK293 cell in the absence and presence of serum (c) and (d). Data represent mean ± standard deviation (n=3).

Kinetics of expression is also important for gene delivery. Figure 5.10 shows the time-dependent changes of gene expression of the cationic polyrotaxane 5b and 5c in comparison with that of PEI (25 K) at N/P ratio of 10 in HEK293 cells, and the transfection efficiency was monitored for 3 days. While the transfection efficiency
mediated by PEI (25 K) decreased with the increasing expression duration no matter in the absence or in the presence of serum, 5b and 5c showed a sustained gene delivery capability. In both serum and serum free condition, increases in transfection efficiency could be found in HEK293 cells transfected with 5b and 5c/pRL-CMV when the expression duration increased from 24 h to 48 h, and then to 72 h. These results may be mainly attributed to the supramolecular structure of these polymers: in these cationic polyrotaxanes, the OEI-grafted \( \alpha \)-CD rings can rotate and/or move along the polymeric chain freely, and this flexibility may enhance the interaction of with DNA and/or cellular membrane.\(^{28}\)

![Graph](image)

**Figure 5.10** *In vitro* gene transfection efficiency of the complexes of cationic polyrotaxane 5b and 5c in comparison with that of PEI (25 K) at N/P ratio of 10 in the absence (a) and presence (b) of serum at different expression duration (24 h, 48 h and 72 h) in HEK293 cells. Data represent mean ± standard deviation (n=3).

Confirmation of the gene delivery capability of cationic polyrotaxane 5b was also obtained by fluorescence microscopy in contrast with that of PEI (25 K) (Figure 5.11). Plasmid pEGFP-N1 encoding green fluorescence protein (GFP) was used to examine
the GFP expression in HEK293 cells. Strong fluorescence signal could be observed when transfections were mediated by either 5b or PEI at N/P ratio of 10. Also, when the transfection was mediated by PEI, the GFP expression in HEK293 cells was stronger than that of mediated by 5b. GFP expression could not be detected when the transfection was mediated by naked DNA, which was used as a negative control (data not shown).

Figure 5.11 The confocal microscope images of transfected HEK293 cells. The transfections were mediated by (a) 5b and (b) PEI (25 K) at N/P ratio of 10 in the absence of serum using green fluorescence protein gene as a reporter gene. The same field of cells was observed by Nomarski optics (right panel) or by fluorescence microscope (left panel) to visualize GFP expression.

5.4 Conclusion

In this study, a series of water soluble cationic polyrotaxanes containing PPO-PEO-PPO triblock copolymer, α-CD and various OEI chains were synthesized and investigated for gene delivery. Cytotoxicity studies showed that these cationic polyrotaxanes displayed significantly low cytotoxicity in comparison with branched
PEI (25 K), owing to the low positive charge density resulted from the supramolecular structure of the polyrotaxanes. For the cationic polyrotaxanes with linear OEI chains, an increased OEI length produced greater transfection efficiency in COS7 cells. In HEK293 cells, 5b and 5c showed a similar or higher gene delivery capability in the presence of serum, and even 10-fold and 7-fold more than that of branched PEI (25 K) at N/P ratio of 30, respectively. More interestingly, in both serum and serum free condition, the cationic polyrotaxane 5b and 5c displayed the sustained gene delivery capability in HEK293 cells, while the transfection efficiency of PEI (25 K) decreased dramatically with the increasing expression duration. These transfection results can be attributed to the structure of the above cationic polyrotaxanes. Such a supramolecular structure enables the OEI-grafted α-CD rings to rotate and/or move along the polymeric chain freely, and this flexibility may enhance the interaction of the polyrotaxanes with DNA and/or cellular membrane.

Therefore, these cationic polyrotaxanes have a high potential as novel nonviral gene carriers, especially for continuous, large dose of in vivo administration, due to their low cytotoxicity, high and sustained gene delivery capability, which are crucial factors in clinical uses.

5.5 References


CHAPTER SIX

SYNTHESIS AND CHARACTERIZATION OF POLYROTAXANES
CONSISTING OF CATIONIC \(\alpha\)-CYCLODEXTRIN THREADED ON
POLY\([\text{ETHYLENE OXIDE}-r\text{-PROPYLENE OXIDE}]\) AS GENE
CARRIERS

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6.1 Introduction

Over the last few decades, the development of non-viral gene carriers with low cytotoxicity and high transfection efficiency has been the hardest task for gene delivery systems, and hundreds of cationic polymers were developed as gene carriers in numerous laboratories around the world.\textsuperscript{1,2} Compared with viral vectors and cationic lipids, cationic polymers for gene delivery are generally economical and stable. They can be produced in a large scale and show low host immunogenicity. So far, a great number of cationic polymers have been reported to be able to deliver genes, including homopolymers or derivatives of polyethylenimine (PEI),\textsuperscript{3} poly(L-lysine),\textsuperscript{4} polyamidoamine,\textsuperscript{5} poly(L-glutamic acid),\textsuperscript{6} polyphosphoester,\textsuperscript{7} chitosan,\textsuperscript{8} and cyclodextrins (CDs).\textsuperscript{9-13}

Cyclodextrins (CDs) are a series of cyclic oligosaccharides composed of 6, 7, or 8 D(+) glucose units linked by $\alpha$-1,4-linkages and named $\alpha$-, $\beta$-, or $\gamma$-CD, respectively. Recently, increasing attention has been focused on studies of supramolecular structures of polyrotaxanes formed by CDs threaded on a polymer chain\textsuperscript{14-22} and their applications in biomaterials.\textsuperscript{23-25} In contrast to conventional polymers containing long sequences of covalently bonded repeating units, the macrocycles in polyrotaxanes can rotate and/or slide along the polymeric chain freely.\textsuperscript{26,27} We recently reported the synthesis of a novel cationic supramolecule composed of multiple oligoethylen-
imine-grafted β-CD that are threaded and blocked on a polymer chain as a new class of polymeric gene delivery vectors. The novel supramolecular gene carriers contain many cationic cyclic units that are threaded upon a polymer chain to form an integrated supramolecular entity to function as a macromolecular gene vector, which showed excellent DNA binding ability, low cytotoxicity, and high gene transfection efficiency HEK293 cells. Yui and co-workers also reported the use of dimethylaminoethyl-modified α-CDs threaded onto a poly(ethylene oxide) (PEO) chain and capped by cleavable end groups for gene delivery. The cleavage of the end groups caused the dethreading of α-CDs and rapid release of DNA in cells, but the tertiary amine conjugated to the α-CD rings might not be efficient in DNA complexation and gene delivery.

We previously found that a random copolymer, poly[(ethylene oxide)-ran-(propylene oxide)] (P(EO-r-PO)) can form inclusion complexes with α-CD. Although there are propylene oxide (PO) units randomly placed in the polymer backbone, the copolymer still can penetrate the smallest cavity of α-CD to form inclusion complexes. It was concluded that α-CD can overcome the energy barrier in passing over a PO unit or a short PO segment, and then form a stable inclusion complex with ethylene oxides (EO) units of the copolymer. In this chapter, we report the synthesis and characterization of a series of polyrotaxanes consisting of multiple cationic α-CD rings threaded on P(EO-r-PO) copolymer as a new gene carriers. The design of the gene carriers was based on the inclusion complex formation between α-CD and P(EO-r-PO) copolymer. In the polyrotaxane gene carriers, the cationic
α-CD rings only resided selectively on the EO segments of the P(EO-r-PO) chain, which may increase the mobility of α-CD rings through rotating and/or sliding along the copolymer as well as the flexibility of the polyrotaxane, enhancing the interaction of the cations of α-CD rings with DNA and/or cellular membranes. The polyrotaxane gene carriers showed high transfection efficiencies in a variety of cell lines, while exhibiting much lower cytotoxicity than branched high molecular weight PEI.

6.2 Experimental section

6.2.1 Materials

The P(EO-r-PO) copolymer \( (M_n = 2,370, \frac{M_w}{M_n} = 1.06) \) was supplied by Aldrich. This copolymer had a chain composition of EO_{41}PO_{10}. The molecular characteristics were confirmed using GPC and \(^1\)H NMR spectroscopy, which were found to be within the specification of the supplier. 2,4,6-Trinitrobenzene sulfonic acid (TNBS) solution and pentaethylenehexamine (OEI-5) was obtained from Fluka. 1,1’-Carbonyldiimidazole (CDI) and α-cyclodextrin were purchased from Tokyo Kasei incorporation. Ethylenediamine (OEI-1), linear PEI with molecular weight of 423 (OEI-9), branched PEI with molecular weight of 600 (OEI-14) and branched PEI (molecular weight 25 K) were supplied by Aldrich. DMSO-\( d_6 \) and D\(_2\)O used as solvent in the NMR measurements were obtained from Aldrich. Qiagen kit and Luciferase kit were purchase from Qiagen and Promega, respectively. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazodium bromide (MTT), penicillin, and streptomycin were obtained from Sigma.
6.2.2 Synthesis

The procedure for synthesis of polyrotaxanes consisting of multiple cationic α-CD rings threaded on P(EO-r-PO) copolymer is shown in Scheme 6.1. The following describes the details of the synthesis of 5b from P(EO-r-PO) copolymer 1 as a typical example.

6.2.2.1 Preparation of P(EO-r-PO) bis(amine)

P(EO-r-PO) copolymer 1 ($M_n = 2,370$, 1.90 g, 0.80 mmol) was heated in a flask at 80 °C in vacuum overnight. When the flask cooled, 15 mL of anhydrous DMF was injected under nitrogen. After all of 1 was dissolved, the DMF solution of 1 was added dropwise during a period of 6 hours under nitrogen to 15 mL of anhydrous DMF solution in which CDI (1.30 g, 8.0 mmol) was dissolved, and the mixture was stirred overnight under nitrogen at room temperature. The resulting solution was slowly added dropwise during a period of 3 hours into 4.8 g (80 mmol) of ethylenediamine which was separately dissolved in 15 mL of anhydrous DMF with stirring at room temperature, followed by stirring the mixture overnight. Excess ethylenediamine and DMF was removed by vacuum evaporation. The resulting viscous solution was purified by size exclusion chromatography (SEC) on a Sephadex LH-20 column using methanol as eluent. Finally, 1.42 g of viscous liquid 2 was obtained (yield, 70%). $^1$H NMR (400 MHz, DMSO-$d_6$, 22 °C): δ 3.25-3.71 (m, 168H and 33H, methylene of EO segments, and methylene and methine of PO segments), 3.23 (s, 4H, -CH$_2$- of -CONHCH$_2$-), 2.01 (s, 4H, -CH$_2$- of -CH$_2$NH$_2$), 1.06 (d, 33H,
6.2.2.2 Preparation of $\alpha$-CD-P(EO-r-PO) polyrotaxane

P(EO-r-PO)-bis(amine) 2 (0.20 g) was added to 27.6 mL of $\alpha$-CD saturated aqueous solution (0.145 g/mL) that contained 0.30 g of NaHCO$_3$ for adjustment of the pH value of the solution. The reaction mixture was ultrasonicated for 20 min and stirred at room temperature overnight. Then, 0.55 g of sodium salt of 2,4,6-trinitrobenzene sulfonic acid solution was added and stirred overnight, followed by pouring 100 mL of water into the reaction mixture. The precipitate was centrifuged and washed 3 times with water. The resulting wet solid was dissolved in 20 mL of DMSO and poured into 300 mL of MeOH to precipitate the product. The precipitate was centrifuged and washed 3 times with MeOH. The resulting wet solid was dissolved in 20 mL of DMSO again and poured into 300 mL water to precipitate the product. The resulting precipitate was centrifuged and washed 3 times with water. Finally, the resulting wet solid was freeze dried and 0.59 g of pure polyrotaxane 4 was obtained (yield, 52%). $^1$H NMR (400 MHz, DMSO-$d_6$, 22 °C): $\delta$ 8.93 (broad, s, 4H, meta H of phenyl), 5.63 (s, 66H, O(2)H of CD), 5.47 (m, 66H, O(3)H of CD), 4.75 (s, 66H, H(1) of CD), 4.41 (s, 66H, O(6)H of CD), 3.17-3.840 (m, 396H, H(3), H(6), H(5), H(2), and H(4) of CD; 168H, methylene of EO segments; 33H, methylene and methine of PO segments), 1.07 (broad, s, 33H, -CH$_3$ of PO segments).

6.2.2.3 Preparation of cationic multiple OEI-grafted $\alpha$-CD-P(EO-r-PO) polyrotaxane

The resulting polyrotaxane 4 (0.206 g, 0.015 mmol) was dried in a flask at 40 °C in vacuum overnight. When the flask cooled, 40 mL of anhydrous DMSO was injected
under nitrogen. After all 4 was dissolved, the DMSO solution of 4 was added dropwise during a period of 6 hours under nitrogen to 40 mL of anhydrous DMSO solution in which CDI (2.56 g, 16.35 mmol) was dissolved, and the mixture was stirred overnight under nitrogen at room temperature. A mixture of THF (300 mL) and Et₂O (600 mL) was poured into the resulting solution to precipitate the product. The precipitate was centrifuged and washed 3 times with THF. Then, the resulting wet solid was dissolved in 40 mL of anhydrous DMSO and this solution was slowly added dropwise during a period of 3 hours into 5.70 mL (19.62 mmol) of pentaethylenehexamine which was dissolved in 40 mL of anhydrous DMSO while stirring at room temperature, followed by stirring the mixture overnight. THF (900 mL) was poured into the reaction mixture to precipitate the product. The precipitate was centrifuged and washed 3 times with THF, and the resulting crude product was purified by size exclusion chromatography (SEC) on a Sephadex G-50 column using deionized water as eluent. Finally, 0.169 g of brown solid 5b was obtained (yield, 41%).

The yields and analytical data for all four cationic polyrotaxanes synthesized in this work are given below.

**Cationic Polyrotaxane 5a.** Yield, 35%. ¹H NMR (400 MHz, D₂O, 22 °C): δ 8.38 (s, 2H, meta H of phenyl), 7.99 (s, 2H, meta H of phenyl), 5.03 (s, broad, 66H, H(1) of CD), 2.95-4.61 (m, broad, 396H, H(3), H(6), H(5), H(2), and H(4) of CD; 168H, methylene of EO segments; 33H, methylene and methine of PO segments; 138H, methylene of -CONHCH₂-), 2.78 (s, 138H, methylene of -CH₂NH₂), 1.08 (d, 33H,
-CH₃ of PPO block).

**Cationic Polyrotaxane 5b.** Yield, 41%. $^1$H NMR (400 MHz, D₂O, 22 °C): $\delta$ 8.36 (s, 2H, meta H of phenyl), 7.97 (s, 2H, meta H of phenyl), 4.95 (s, broad, 66H, H(1)H of CD), 2.94-4.58 (m, broad, 396H, H(3), H(6), H(5), H(2), and H(4) of CD; 168H, methylene of EO segments; 33H, methylene and methine of PO segments; 106H, methylene of -CONHCH₂-), 2.67 (m, 1268H, methylene of pentaethylenehexamine), 1.07 (s, 33H, -CH₃ of PO segments).

**Cationic Polyrotaxane 5c.** Yield, 30%. $^1$H NMR (400 MHz, D₂O, 22 °C): $\delta$ 8.37 (s, 2H, meta H of phenyl), 7.98 (s, 2H, meta H of phenyl), 4.98 (d, broad, 66H, H(1)H of CD), 2.97-4.62 (m, broad, 396H, H(3), H(6), H(5), H(2), and H(4) of CD; 168H, methylene of EO segments; 33H, methylene and methine of PO segments; 57H, methylene of -CONHCH₂-), 2.67 (m, 1305H, methylene of OEI-9), 1.08 (d, 33H, -CH₃ of PO segments).

**Cationic Polyrotaxane 5d.** Yield, 48%. $^1$H NMR (400 MHz, D₂O, 22 °C): $\delta$ 8.36 (s, 2H, meta H of phenyl), 7.98 (s, 2H, meta H of phenyl), 4.98 (d, broad, 66H, H(1)H of CD), 2.97-4.61 (m, broad, 396H, H(3), H(6), H(5), H(2), and H(4) of CD; 168H, methylene of EO segments; 33H, methylene and methine of PO segments; 69H, methylene of -CONHCH₂-), 2.56 (m, 1293H, methylene of OEI-14), 1.09 (d, 33H, -CH₃ of PO segments).
6.2.3 Measurements

**Gel permeation chromatography (GPC)** GPC analysis for P(EO-co-PO) copolymer was carried out with a Shimadzu SCL-10AVP and LC-10ATVP system equipped with two Phenogel 5 μm, 50 and 1000 Å columns (size: 300 × 4.6 mm) in series and a Shimadzu RID-10A refractive index detector. THF was used as eluent at a flow rate of 0.30 mL/min at 40 °C. Monodispersed poly(ethylene glycol) standards were used to obtain a calibration curve.

Size exclusion chromatography (SEC) analysis for cationic polyrotaxanes was carried out with a Shimadzu SCL-10AVP and LC-10ATVP system equipped with a Sephadex G-75 column (size: 2.5 × 32 cm), a Shimadzu RID-10A refractive index detector and a Shimadzu SPD-10AVP UV-Vis detector. PBS buffer solution (1×) was used as the eluent. Fractions were collected per 1 mL and their optical rotation (OR) were further measured using a HORIBA SEPA-300 high speed accurate polarimeter at wavelength 589 nm with cell length of 10 cm and response time of 2 s.

**1H NMR spectra** The 1H NMR spectra were recorded on a Bruker AV-400 NMR spectrometer at 400 MHz at room temperature. The 1H NMR measurements were carried out with an acquisition time of 3.2 s, a pulse repetition time of 2.0 s, a 30° pulse width, 5208-Hz spectral width, and 32 K data points. Chemical shifts were referred to the solvent peaks (δ = 4.70 ppm for D2O and 2.50 ppm for DMSO-d6).

The 13C NMR spectra were recorded on a Bruker AV-400 NMR spectrometer at 100 MHz at room temperature. The 13C NMR measurements were carried out using composite pulse decoupling with an acquisition time of 0.82 s, a pulse repetition time
of 5.0 s, a 30° pulse width, 20,080-Hz spectral width, and 32 K data points. The chemical shifts were referred to an external standard.

**Plasmid** The plasmid used was pRL-CMV (Promega, USA), encoding *Renilla* luciferase, which was originally cloned from the marine organism *Renilla reniformis*. The plasmid DNA was amplified in *Escherichia coli* and purified according to the supplier’s protocol (Qiagen, Hilden, Germany). The purity and concentration of the purified plasmid DNA were determined by absorption at 260 and 280 nm and by agarose gel electrophoresis. The purified plasmid DNA was resuspended in TE buffer (10 mM Tris-Cl, pH 7.5, 1 mM EDTA) and kept in aliquots at a concentration of 0.5 mg mL⁻¹.

**Cells and Media** All cell lines were purchased from ATCC (Rockville, MD, USA). COS7, HEK293, and BHK-21 cells were maintained in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 units/mg penicillin, and 100 μg/mL streptomycin at 37 °C and 5% CO₂. MES-SA and SK-OV-3 cells were grown in McCoy’s 5a medium with 1.5 mM L-glutamine supplemented with 10% heat-inactivated fetal bovine serum, 100 units/mg penicillin, and 100 μg/mL streptomycin at 37 °C and 5% CO₂. Reduced-serum Opti-MEM medium, DMEM medium, and McCoy’s 5a medium were purchased from Gibco BRL (Gaithersburg, MD, USA).

**Gel Retardation Experiments** Each polymer was examined for its ability to bind pRL-CMV plasmid DNA through agarose gel electrophoresis experiments. All polymer stock solutions were prepared at a nitrogen concentration of 1 mM in
distilled water and the pH was adjusted to 7.4. pRL-CMV (0.2 μg in 2 μL TE buffer) was mixed with polymer at nitrogen/phosphate (N/P) ratios from 0 to 10. Each mixture was vortexed and incubated for approximately 30 min at room temperature, and analyzed on 1% agarose gel containing 0.5 μg/mL ethidium bromide (EtBr). The gel electrophoresis was carried out in TAE running buffer (40 mM Tris-acetate, 1 mM EDTA) for 40 min at 80V in a Sub-Cell system (Bio-Rad Laboratories, CA, USA). The DNA bands were visualized and photographed by a UV transilluminator and BioDoc-It imaging system (UVP Inc., USA).

**Cell viability assay** The cytotoxicity of the cationic polyrotaxanes in comparison with PEI (25 K) was evaluated using MTT assay in COS7 and HEK293 cell lines. The cells were cultured in complete DMED medium supplemented with 10% FBS at 37 °C, 5% CO₂, and 95% relative humidity. The cells were seeded in a 96-well microtiter plate (Nunc, Wiesbaden, Germany) at a density of 10,000 and 15,000 cells/well for COS7 and HEK293, respectively. After 24 h, culture media were replaced with serum-supplemented culture media containing serial dilutions of polymers, and the cells were incubated for 24 h. Then, 10 μL of sterile filtered MTT stock solution in PBS (5 mg/mL) was added to each well, reaching a final MTT concentration of 0.5 mg/mL. After 5 h, unreacted dye was removed by aspiration. The formazan crystals were dissolved in DMSO (100 μl/well), and the absorbance was measured using a microplate reader (Spectra Plus, TECAN) at the wavelength of 570 nm. The relative cell viability (%) related to control cells cultured in media without polymers was calculated with \( \frac{[A]_{\text{test}}}{[A]_{\text{control}}} \times 100\% \), where \([A]_{\text{test}}\) is the absorbance of the wells
with polymers and $[A]_{\text{control}}$ is the absorbance of the control wells. All experiments were conducted for six samples and averaged.

**In vitro transfection and luciferase assay** Transfection studies were performed in five cell lines (HEK293, COS7, BHK-21, MES-SA and SK-OV-3 cells) using the plasmid pRL-CMV as the reporter gene. In brief, 24 h before transfection, 24-well plates were seeded with cells at a density of $5 \times 10^4$/well. The polymer-DNA complexes (2.0 µg DNA/well for HEK293 and COS7 cells and 1.0 µg DNA/well for the other cell lines) at various N/P ratios were prepared by adding the polymer into DNA solutions dropwise, followed by vortexing and incubation for 30 min at room temperature before the transfection. At the time of transfection, the medium in each well was replaced with reduced-serum Opti-MEM medium or normal complete DMEM medium. The complexes were added into the transfection medium and incubated with cells for 4 h under standard incubator conditions. After 4 h, the medium was replaced with 500 µL of fresh medium supplemented with 10% fetal bovine serum (FBS), and the cells were further incubated for an additional 20 h under the same conditions, resulting in a total transfection time of 24 h. Cells were washed with PBS twice, lysed in 100 µL of cell culture lysis reagent (Promega, Cergy Pontoise, France). Luciferase gene expression was quantified using a commercial kit (Promega, Cergy Pontoise, France) and a luminometer (Berthold Lumat LB 9507, Germany). Protein concentration in the samples was analyzed using a bicinchoninic acid assay (Biorad, CA, USA). Absorption was measured on a microplate reader (Spectra Plus, TECAN) at 570 nm and compared to a standard curve calibrated with
BSA samples of known concentration. Results are expressed as relative light units per milligram of cell protein lysate (RLU mg\(^{-1}\) protein).

**Dynamic light scattering and zeta-potential** Measurements of particle size and zeta potential of the complexes were performed using a Zetasizer Nano ZS (Malvern Instruments, Southborough, MA, USA) with a laser light wavelength of 633 nm at a 173° scattering angle. Complex solutions (100 µl) containing 3 µg of pDNA (pRL-CMV) were prepared at various N/P ratios ranging from 2-30. The mixture was vortexed for 20 s, incubated for 30 min at room temperature and diluted in 1 mL of distilled water before being analyzed on the Zetasizer. The size measurement was performed at 25 °C in triplicate. The deconvolution of the measured correlation curve to an intensity size distribution was accomplished using a non-negative least squares algorithm. The Z-average hydrodynamic diameters of the particles were given by the instrument. The Z-average size is the intensity weighted mean diameter derived from a Cumulants or single exponential fit of the intensity autocorrelation function. The zeta potential measurements were performed using a capillary zeta potential cell in automatic mode using the same samples for the particle size measurements.

**Confocal microscopy** For confocal microscopy imaging of the gene transfection, the plasmid pEGFP-N1 (Clontech Laboratories Inc., USA), encoding a red-shifted variant of wild-type green fluorescence protein (GFP), was used to examine the GFP expression in HEK293 cells. HEK293 cells were seeded onto lab-Tek 4-chambered coverglass (Nalge-Nane international, USA) at density of 5×10\(^4\) cells/well in 500 µL of complete DMEM medium. After 24 hours, transfection was undertaken with 2 µg
EGFP plasmid in 0.3 mL of reduced-serum Opti-MEM medium in each well. At the
time of transfection, 20 μL of cationic polyrotaxane 5c-DNA complex solution was
added in each well. After 4 h, the transfection media was removed and the cells were
washed with fresh complete DMEM medium. After 20 h of further incubation in
serum-containing complete DMEM medium, the cells were washed with phosphate-
buffered saline (PBS) and imaged under a laser scanning confocal microscope (LSM
410, Carl Zeiss, USA). GFP fluorescence was excited at 488 nm and emission was
collected using a 515 nm filter.

**Atomic Force Microscopy** A Digital Instruments MultiMode-AFM with Nanoscope
IV controller in a tapping mode was employed to image the nanoparticle samples.
Briefly, silicon disks were soaked in 50% acetone for a minimum of 2 h and rinsed
with distilled water. When the silicon disks were completely dried, 20 μL of cationic
polyrotaxane 5c-DNA complexes containing 1.0 μg of pRL-CMV at N/P ratios 0, 2,
and 10 were placed on the silicon surface for 2 min, followed by removing the
complex solutions carefully with a piece of tissue paper. All the AFM images were
obtained with a scan rate of 0.5 or 1 Hz over a selected area of 2×2 μm. Image
analysis was performed using Nanoscope software after removing the background
slope by flating images.

### 6.3 Results and discussion

#### 6.3.1 Synthesis and characterization of cationic polyrotaxanes

Scheme 6.1 shows the synthesis procedures and the structures of the cationic
polyrotaxanes (5a, 5b, 5c and 5d). First, P(EO-r-PO)-bis(amine) 2 was prepared from
P(EO-PO) random copolymer 1, which has a number-average molecular weight (MW) of 2370 and an 80 mol % content of ethylene oxide (EO) segments. The two hydroxy end groups were activated with 1,1'-carbonyldiimidazole (CDI), followed by reaction with excessive ethylenediamine to give 2. It is known that P(EO-r-PO) can form inclusion complexes with α-CD, although there were PO segments randomly placed in the copolymer backbone, and a PPO homopolymer can not form a complex with α-CD because the PPO chain is too large to penetrate the small inner cavity of α-CD.\(^{30}\) We interestingly found that α-CD molecules are able to overcome the energy barrier in passing over a PO unit or a short PO segment and then form a stable inclusion complex with EO segments of the P(EO-r-PO) random copolymer.

Scheme 6.1 Synthesis procedures and the structures of multiple OEI-grafted cationic polyrotaxanes (5a, 5b, 5c and 5d).
Polypseudorotaxane 3 was formed between P(EO-r-PO)-bis(amine) 2 and α-CD, and polyrotaxanes 4 was synthesized by adding 2,4,6-trinitrobenzene sulfonate (TNBS) to 3, forming two bulky stoppers to block the two ends of the inclusion complex, in which about 12 α-CD rings are trapped on the copolymer chain. Finally, linear or nonlinear oligoethyleneamines (OEIs) with different MW, ethylenediamine ($k = 1$), pentaethylenehexamine ($k = 5$), linear OEI with MW of 423 (OEI-9, $k = 9$), and branched OEI with MW of 600 (OEI-14, $k = 14$), were grafted to the α-CD molecules of polyrotaxanes 4 to give cationic polyrotaxanes 5a, 5b, 5c and 5d, respectively.

6.3.2. Molecular Characterization of Cationic Polyrotaxanes.

Figure 6.1 shows the size exclusion chromatography (SEC) profiles of the cationic polyrotaxanes with reference to pristine α-CD. The elution curves for all four cationic polyrotaxanes were recorded against refractive index (RI), UV-vis absorption (Abs) at 419 nm, and optical rotation (OR), while that for pristine α-CD was recorded against RI and OR since it has no UV-vis absorption. As shown in Figure 6.1, α-CD has relative small molecular size, which was eluted out at the low MW region of the column. In contrast, all four cationic polyrotaxanes were eluted out at higher MW region of the column due to their larger molecular sizes, and were detected by RI, Abs, and OR at the same time, indicating the cationic polyrotaxanes comprise the 2,4,6-trinitrophenyl (TNP) ends and cationic α-CD units. Compared to the other synthetic polyrotaxanes, 5a was eluted out a bit later, which corresponds to its smaller molecular size than the other cationic polyrotaxanes, since 5a is grafted with the
shortest ethylenediamine chains. Each cationic polyrotaxane showed a single peak in the SEC profiles, indicating that the cationic polyrotaxanes are pure and there was no intra- or intermolecular crosslinking.

**Figure 6.1** Size exclusion chromatograms of pristine \( \alpha \)-CD and cationic polyrotaxane 5a, 5b, 5c, and 5d detected using refractive index (RI), UV-vis absorption (Abs) at 419 nm, and optical rotation (OR).

Figure 6.2 shows the \(^{13}\)C NMR spectra of cationic polyrotaxane 5b in comparison with pristine \( \alpha \)-CD and pentaethylenehexamine. In Figure 6.2c, all peaks attributed to \( \alpha \)-CD, the grafting OEI, and the threading copolymer were observed clearly; and the peaks were broadened because all components of the cationic polyrotaxane formed an integrated macromolecular system which restricts their molecular motion. The peak at \( \delta \) 158.1 ppm corresponds to the carbon of carbonyl groups, which links the OEI chains to \( \alpha \)-CD rings. Compared to pristine \( \alpha \)-CD, the peak of C-6 of \( \alpha \)-CD in 5b shifted from 60.8 to 64.4 ppm. This is evidence that the grafting of OEI chains
happened mainly at the 6-position hydroxyl groups. In fact, of the three types of hydroxyl groups of α-CD, those at the 6-position (primary hydroxyl groups) are the most nucleophilic and are thought to be modified under the weak basic conditions.  

![Diagram of α-CD](image)

**Figure 6.2** $^{13}$C NMR spectra of pristine α-CD (a), pentaethylenehexamine (b), and cationic polyrotaxanes 5b (c) in D$_2$O.

Figure 6.3 shows the $^1$H NMR spectra of polyrotaxane 4 with reference to pristine α-CD and P(EO-$r$-PO) bis(amine) 2 in DMSO-$d_6$. In Figure 6.3c, the peaks for α-CD, EO and PO segments of the triblock copolymer, and the 2,4,6-trinitro-phenyl end groups were all observed, while they were broadened as compared with the respective free counterparts in Figure 6.3a and b. This is due to the restricted molecular movement of the components in the polyrotaxane. Quantitative comparisons between the integral intensities of the peaks of α-CD and those of threading copolymer
segments gave the compositions of the polyrotaxanes. It was found that 12 α-CD rings, on average, were covered and blocked on the P(EO-\(r\)-PO) copolymer in one polyrotaxane 4.

Figure 6.3 \(^1\)H NMR spectra of α-CD (a), P(EO-\(r\)-PO) bis(amine) (b), and α-CD-P(EO-\(r\)-PO) polyrotaxane (c) in DMSO-\(d_6\).

Figure 6.4 shows the \(^1\)H NMR spectra of the cationic polyrotaxanes with reference to pristine α-CD. In the spectra of (b) – (e) in Figure 6.4, the signals for α-CD, the grafting OEI chains, the threading copolymer, and the TNP ends were observed, while the peaks were much broadened due to the restriction of the molecular motion by molecular interlocking and the grafting of the OEI units. From this \(^1\)H NMR spectra, the average number of OEI chains grafted to each α-CD was estimated. Corresponding to cationic polyrotaxane 5a, 5b, 5c, and 5d, the number of the OEI chains grafted to one α-CD molecule in these cationic polyrotaxanes is about 4.2, 4.8,
2.6, and 3.1, respectively. Generally, the longer the OEI chain, the less number of OEI chains could be grafted to each α-CD ring due to the influence of the steric hindrance of OEI chains on the grafting reaction. However, for 5d, more primary amino groups from branched structure of OEI-14 could participate in the grafting reaction, resulting in the increasing number of OEI chains grafted to each α-CD ring.

![Figure 6.4](#)

**Figure 6.4** $^1$H NMR spectra of prisine α-CD (a), and cationic polyrotaxanes 5a (b), 5b (c), 5c (d) and 5d (e) in D$_2$O.

6.3.3 Formation of cationic polyrotaxane/DNA complexes

The ability of the cationic polyrotaxanes to condense plasmid DNA (pDNA) into particulate structures was confirmed by agarose gel electrophoresis, particle size and zeta potential measurements, as well as AFM images.

DNA condensation capability is a prerequisite for polymeric gene vectors. In this
study, the complexation of cationic polyrotaxanes with DNA was analyzed using agarose gel electrophoresis. Figure 6.5 shows the gel retardation results of cationic polyrotaxane/DNA complexes with increasing N/P ratios in comparison with branched PEI (25 K)/DNA complex. Cationic polyrotaxanes 5a, 5c, and 5d could compact pDNA entirely at the low N/P ratio of 1.5 to 2.0, while PEI (25 K) could inhibit the migration of pDNA at N/P ratio of 2 and above, indicating that these polyrotaxanes have slightly better DNA condensation ability than PEI (25 K). Different from the other cationic polyrotaxanes, 5b showed lower ability to condense DNA since the migration of DNA was only retarded at N/P ratio of 3 and above.

![Figure 6.5](image)

**Figure 6.5** Electrophoretic mobility of plasmid DNA in the complexes between cationic polyrotaxanes and DNA in comparison with PEI/DNA complex at various N/P ratios.
Figure 6.6 shows the particle size and zeta potential of cationic polyrotaxane/DNA complexes in comparison with PEI (25 K)/DNA complex at various N/P ratios. In Figure 6.6a, all four cationic polyrotaxanes could efficiently compact pDNA into small nanoparticles. The diameters of the complexes formed by 5a, 5b, and 5c with DNA decreased sharply from more than 500 nm to around 200 nm with the increase of N/P ratio from 2 to 4, similar to the case of PEI (25 K)/DNA complex. The hydrodynamic size of 5d/DNA complex only varied within the range of 100 to 200 nm with the increase of N/P ratio from 2 to 30. After N/P ratio reached 6, all four cationic polyrotaxanes and PEI (25 K) could condense DNA into nanoparticles with diameters ranged within 100 to 200 nm.

Zeta potential is an indicator of the surface charge of polymer/DNA nanoparticles, and a positive surface charge allows an electrostatic interaction between negatively charged cellular membranes and the positively charged complexes.32 As shown in Figure 6.6b, the surface net charge of the complexes of pDNA with PEI (25 K) and cationic polyrotaxanes 5a, 5b or 5c increased dramatically as the N/P ratio increased from 2 to 4 and stabilized at N/P ratio of 10 and above. The zeta potential of 5d/DNA complex only varied within the range of 13 to 27 mV with the increase of N/P ratio from 2 to 30. After N/P ratio reached 10, the zeta potential of the complexes of pDNA with all four cationic polyrotaxanes and PEI (25 K) was strongly positive and varies within the same range (20 – 30 mV), which results in a similar affinity for cell surface.33
Figure 6.6 Particle size (a) and zeta potential (b) of cationic polyrotaxanes/DNA complexes in comparison with PEI (25 K)/DNA complex at various N/P ratios.

Figure 6.7 shows representative taping mode AFM images of naked pDNA and cationic polyrotaxane 5c/DNA complexe at N/P ratio of 2 and 10. The images obtained clearly demonstrate significant morphological differences when different N/P ratios were applied, as well as the formation of compact nanoparticles. In Figure 6.7a, loose, supercoiled structure of pDNA could be observed when pDNA was not condensed by a cationic polymer. At N/P ratio of 2, supercoiled plasmid pDNA could still be identified under AFM while small and compact nanoparticles were formed at the same time. Compared to this partial condensation at N/P ratio of 2, the same amount of pDNA could be tightly packed and completely formed pDNA complexes in the form of spherical nanoparticles at N/P ratio of 10.
6.3.4 Cytotoxicity of cationic polyrotaxanes

Cytotoxicity is one of the most important factors to be considered in selecting polymeric materials as gene carriers. Figure 6.8 shows the results of *in vitro* cytotoxicity studies of cationic polyrotaxanes in two cell lines (HEK293 and COS7) using MTT assay. As shown in Figure 6.8, all the cationic polyrotaxanes showed a dose-dependent effect on cytotoxicity. It is worth noting that the cationic polyrotaxanes with linear OEI chains exhibited less toxicity in both cultured HEK293 and COS7 cells than the PEI control. The slopes of the dose-response cytotoxicity curves were much steeper for PEI (25 K) than those for 5a, 5b and 5c. One possible reason is that the introduction of cyclodextrin and copolymer results in the lower density of amino groups and the high density of amino groups is always considered as an important factor leading to high cytotoxicity of PEI.\(^{34}\) Likewise, the cytotoxicity of 5d was similar to that of PEI (25 K), which can also be attributed to its higher density of amino groups because 5d was grafted with higher MW branched OEI-14. These results also appeared to be supported by the calculated LD\(_{50}\) values: in COS7 cell lines, the LD\(_{50}\) value of PEI (25 K) was less than 25 µg/mL, while those of cationic...
Polyrotaxanes 5a, 5b, 5c, and 5d were 97, 308, 170, and 36 µg/mL, respectively. The similar trend was also observed in HEK293 cell lines.

![Figure 6.8](image)

**Figure 6.8** Cell viability of cationic polyrotaxanes in (a) HEK293 and (b) COS7 cells in comparison with PEI (25 K).

6.3.5 Transfection efficiency of cationic polyrotaxanes.

In *vitro* gene transfection efficiency of cationic polyrotaxane/DNA complexes was assessed using luciferase as a marker gene in HEK293 and COS7 cells. Figure 6.9 shows the gene transfection efficiency of cationic polyrotaxanes compared with those of branched PEI (25 K) and naked pDNA (ND) at various N/P ratios in the absence and presence of serum in HEK293 and COS7 cells.

In HEK293 cells (Figure 6.9, a and b), the transfection efficiencies mediated by cationic polyrotaxanes were comparable to or even higher than those of branched PEI
When the transfection was conducted in the absence of serum, 5c constantly exhibited the highest transfection efficiencies among the four cationic polyrotaxanes, which were also higher than the PEI control at low and high N/P ratios. From 5a to 5c, the transfection efficiency increased with an increase in the length of the OEI grafted to α-CD. However, 5d, the one with longest OEI grafted, showed the lowest transfection efficiency. This is much different from PEI, where higher MW always results in higher transfection efficiency. When the transfection medium was supplemented with 10% FBS, 5b exhibited the highest transfection efficiencies among the four cationic polyrotaxanes. The transfection efficiencies mediated by 5a, 5b, and 5c increased with increasing N/P ratio, and finally became much higher than those of PEI at high N/P ratio, while the transfection efficiency of PEI dropped at higher N/P ratios. Particularly, 5b displayed approximately 10 times higher transfection efficiency than that of the PEI control at N/P of 20 to 30. Again, 5d showed the lowest transfection efficiency among the four cationic polyrotaxanes except at N/P ratio of 10.

In COS7 cells (Figure 6.9, c and d), however, 5d showed the highest transfection efficiency among the four cationic polyrotaxanes at low N/P ratio (10 to 20), while 5b was the best for gene transfection at higher N/P ration (25 and 30). Generally, the transfection efficiencies mediated by cationic polyrotaxanes were comparable to or slight lower than those of branched PEI (25 K) in both reduced-serum medium and complete medium.
The in vitro transfection efficiencies of cationic polyrotaxane 5b were also evaluated in three other cell lines including BHK-21, SK-OV-3, and MES-SA cells at various N/P ratios, in comparison with those of branched PEI (25 K) at N/P of 10, at which branched PEI (25 K) usually exhibits the highest transfection efficiency. Like other transfection hosts such as HEK293 and COS7, the Syrian golden hamster kidney fibroblast cell line BHK-21 has been also widely used to evaluate in vitro gene transfection efficiency mediated by cationic polymers. The human cell lines SK-OV-3 (ovarian carcinoma) and MES-SA (uterus sarcoma) are derived from human tumor cells, which could serve as excellent candidates for tumor cell transfection investigations. As shown in Figure 6.10, the gene transfection efficiencies of 5b were dependant on serum condition of the culture medium in different cell lines. In
BHK-21 cells, the transfection efficiencies of $5b$ improved with the increase in N/P ratio in serum condition, while its transfection efficiencies in serum-free condition were lower at high N/P ratio. In SK-OV-3 cells, the transfection efficiencies of $5b$ at N/P ratio of 15 to 40 were similar to that of the PEI control in both serum-free and serum conditions. In MES-SA cells, the transfection efficiencies of $5b$ were higher than that of the PEI control in serum-free condition, while those of $5b$ were much lower than that of the PEI control. Hence, when the cationic polyrotaxanes are properly designed with different compositions, density of the amino groups, lengths of OEI grafted, and flexibility of the main chains, the properties of the gene carriers may be altered to suit gene delivery in a wide range of different cells with high gene transfection efficiencies.

Finally, confirmation of the gene delivery capability of the cationic polyrotaxane $5c$ was also obtained by fluorescence microscopy in contrast with that of PEI (25 K). Plasmid pEGFP-N1 encoding green fluorescence protein (GFP) was used to examine the GFP expression in HEK293 cells. As shown in Figure 6.11, strong fluorescence signal could be observed when transfection was mediated by either $5c$ or PEI at N/P ratio of 10. However, when the transfection was mediated by PEI, the GFP expression in HEK293 cells was stronger than that mediated by $5c$. In contrast, GFP expression could not be detected when the transfection was mediated by naked DNA, which was used as a negative control.
Figure 6.10 *In vitro* gene transfection efficiency of the complexes of cationic polyrotaxane 5b in comparison with that of PEI (25 K) in BHK-21 cell (a), SK-OV-3 cell (b), and MES-SA cell (c), in the absence and presence of serum. Data represent mean ± standard deviation (n=3).

Figure 6.11 The confocal microscope images of transfected HEK293 cells. The transfections were mediated by (a) 5c and (b) PEI (25 K) at N/P ratio of 10 in the absence of serum using green fluorescence protein gene as a reporter gene. The same field of cells was observed by Nomarski optics (right panel) or by fluorescence microscope (left panel) to visualize GFP expression.
6.4 Conclusion

In the present study, four supramolecular cationic polyrotaxanes (5a, 5b, 5c, and 5d) with multiple OEI-grafted cationic α-CD rings threaded and blocked on a P(EO-r-PO) copolymer chain have been successfully synthesized and characterized. In the cationic polyrotaxanes, approximately 12 cationic α-CD rings were threaded on the P(EO-r-PO) copolymer with a MW of 2370 and an EO/PO molar ratio of 4:1, while the cationic α-CD rings were grafted with liner OEI of MW up to 423 for 5a, 5b, 5c, and with branched OEI of MW of 600 for 5d. All four cationic polyrotaxanes could efficiently bind pDNA and for nanoparticles with sizes of 100 to 200 nm, which were suitable for cell uptake. In vitro cytotoxicity studies showed that cationic polyrotaxanes 5a, 5b, and 5c exhibited much lower cytotoxicity than high MW branched PEI (25 K), while cationic polyrotaxane 5d with branched OEI chains of higher MW (MW 600) exhibited similar cytotoxicity to high MW branched PEI in both HEK293 and COS7 cells. The cationic polyrotaxanes displayed high gene transfection efficiencies in a number of cell lines such as HEK293, COS7, BHK-21, SK-OV-3, and MES-SA., and showed strong cell type and serum condition dependency. In serum condition, cationic polyrotaxane 5b displayed approximately 10 times higher transfection efficiency than that of the PEI control at N/P of 20 to 30. It is thought that the OEI-grafted α-CD rings were only located selectively on EO segments of the P(EO-r-PO) chain in the polyrotaxanes, while PO segments were free of complexation. Such structure increased the mobility of the cationic α-CD rings and
the flexibility of the polyrotaxanes, which enhanced the interaction of the cationic α-CD rings with DNA and/or cellular membrane.

Our studies have demonstrated that the new cationic polyrotaxanes have high gene delivery capability a variety of cell lines. When properly designed, the structure, conformation and composition of the cationic polyrotaxanes may be controlled to give gene carriers with different properties for a wide range of potential gene therapy applications.

6.5 References


7.1 Conclusions

In this study, novel cationic star polymers consisting of α-CD core and oligoethylenimine (OEI) arms and a series of soluble, cationic supramolecular polyrotaxanes composed of multiple OEI-grafted CDs threaded on various copolymer chains were prepared and characterized, and these new polymer systems were used as DNA carriers in the gene delivery systems.

To prepare the above novel cationic star polymers, multiple OEI arms of different chain length and structure (ethylenediamine, pentaethylenehexamine, linear PEI with an average molecular weight of 423, and branched PEI with an average molecular weight of 600) were conjugated onto an α-CD ring core. These star polymers were isolated and purified using size exclusion chromatography (SEC), and characterized using Gel Permeation Chromatography (GPC), $^{13}\text{C}$ and $^1\text{H}$ NMR, and elemental analysis.
It was found that all the α-CD-OEI star polymers could inhibit the migration of pDNA on agarose gel through formation of complexes with pDNA, and the complexes formed nanoparticles with sizes ranging from 100 – 200 nm at N/P ratios of 8 or higher. The complexation was supposed to rely predominantly on electrostatic interactions between positively charged amino groups of OEI arms of the star polymers with the negatively charged phosphate groups of DNA. The α-CD-OEI star polymers displayed much lower in vitro cytotoxicity than that of branched polyethylenimine (PEI) of molecular weight 25 K due to the introduction of α-CD in the star polymers. They also showed excellent gene transfection efficiency in HEK293 and COS7 cells since the amino groups on the OEI arms of the star polymers increased the binding ability and complex stability, thus leading to much higher transfection efficiency.

To synthesize cationic, soluble CD-containing supramolecular polyrotaxanes, firstly, a series of new polyrotaxanes were synthesized in high yield with copolymers (PPG-PEG-PPG triblock copolymers, PEG-PPG-PEG triblock copolymers or PPG-ran-PEG copolymers) and CDs (α-CDs or β-CDs) based on the block-selected inclusion complexation between the copolymers and CDs, followed by conjugation of 2,4,6-trinitrobenzene sulfonate (TNBS) to both ends of copolymer chains as blocking stoppers. These polyrotaxanes were purified by SEC and their compositions were characterized by $^1$H and $^{13}$C NMR; secondly, multiple OEI chains with different chain length and structure (ethylenediamine, pentaethylene- hexamine, linear PEI with an average molecular weight of 423, and branched PEI with an average molecular weight
of 600) were grafted onto the CD rings of the above polyrotaxanes. These resulting soluble supramolecular polyrotaxanes were purified by SEC and their compositions were characterized by using GPC, $^{13}$C and $^1$H NMR, and elemental analysis.

All the cationic, soluble supramolecular polyrotaxanes could efficiently condense plasmid DNA (pDNA) into small nanoparticles due to the electrostatic interactions between supramolecular polyrotaxanes with pDNA. They also showed excellent gene transfection efficiency that was comparable to or even higher than that of branched PEI (25 K), one of the most effective polymeric gene-delivery carriers studied to date. In addition, they displayed much lower \textit{in vitro} cytotoxicity than that of branched PEI (25 K) in a variety of cell lines.

Overall, in this study, the compositions of the cationic, soluble supramolecular polyrotaxanes could be varied by changing different cyclodextrins (α-CD or β-CD) and various copolymers (PPG-PEG-PPG triblock copolymers, PEG-PPG-PEG triblock copolymers or PPG-ran-PEG copolymers). Thus, the density and distribution of the cationic charges were changed. These changes were proved to have a great influence on the gene tranfection efficacy and cytotoxicity in the study. Generally, the supramolecular polyrotaxanes with longer grafting linear OEI chains showed good gene transfection efficiency and higher cytotoxicity. Nevertheless, the cationic polyrotaxanes with various copolymer chains showed distinctly different performances in various cell lines.

Besides the above cationic supramolecular polyrotaxanes, single cationic OEI-grafted α-CD ring (i.e. star polymer consisting of α-CD core and OEI arms) was
also prepared and investigated as gene carrier in the study. With an increase of grafting arm length, the transfection efficiency and cytotoxicity of the synthesized cationic star polymers also increased. Especially, the cationic star polymer grafting with branched OEG with MW of 600 has the largest density of cationic charge. Although the transfection efficiency of the cationic star polymer was comparable to that of branched PEI (25 K), its cytotoxicity is almost the same with the PEI control.

7.2 Future work

It is well known that each gene-delivery carrier has strengths and limitations, e.g. immune responsiveness may limit the usefulness of one carrier, inability to be concentrated to be adequately titrated to another, etc. Hence, while one vector may be best suited to a particular situation, none of these vectors is clearly superior to the others for general use. In this study, I not only synthesized a variety of cationic polymers for efficient gene delivery, but also demonstrated a “smart design” of supramolecular polyrotaxanes. This “smart design” means that the structure, conformation and composition of the synthetic cationic supramolecular polyrotaxanes can be further modified and controlled in terms of the density of the amino groups and the flexibility of the polyrotaxanes. Thus, novel gene-delivery carriers with different properties will be developed for various applications. Also, for the cationic supramolecular polyrotaxanes I have obtained, determination of transfection efficiency and in vitro cytotoxicity will be carried out in more cell lines, to find out the specific situation in which the supramolecular polyrotaxanes perform well.
PEGylation is the most extensive modification method for many cationic polymers since it will increase the solubility of the complexes formed by cationic polymers with DNA and reduce the cytotoxicity of the polymers.\textsuperscript{2-4} Moreover, during the PEGylation process, targeting ligands such as galatose, transferring and insulin are always used for cell-specific uptake.\textsuperscript{5-7} Davis and co-workers have demonstrated post-PEGylation of the complexes by modification with adamantane-PEG conjugates.\textsuperscript{8} For the star polymers I have synthesized, PEGylation, including pre-PEGylation and post-PEGylation, can also be carried out, and targeting ligands will be introduced to the cationic polymer-DNA complexes at the same time.

In addition, in the future, the biodegradable blocking groups will be used, thus the cationic supramolecular polyrotaxanes can be degraded in the cell and the cytotoxicity is expect to be reduced. The determination of toxicities and gene transfection efficiency \textit{in vivo} of the cationic supramolecular polyrotaxanes as DNA vectors will be carried out.

7.3 References


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