CATIONIC, α-HELICAL POLYPEPTIDES FOR CELL PENETRATION AND NON-VIRAL GENE DELIVERY

BY

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DISSERTATION

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ABSTRACT

Gene therapy is showing great potentials in treating various kinds of genetic diseases. The development of effective and safe delivery vectors are quite crucial towards gene therapy. Compared with viral vectors, non-viral gene delivery vectors allow the safe delivery of genetic materials with less inherent adverse reactions such as immunogenicity, unexpected viral replication and recombination, and oncogenicity. However, the non-viral vectors usually suffer from the low transfection efficiency due to various challenges and obstacles involved in the non-viral gene delivery process. The goal of my Ph. D. research is to develop the polypeptide-based non-viral gene delivery vectors to achieve excellent cell membrane penetration and gene delivery. Firstly, I modified the complexes from the development of the supramolecular structures via incorporating a sugar containing polypeptide to form the ternary complexes. The ternary complexes contain the membrane target polypeptide, which could facilitate the active targeting to the cells via the mannose-receptor mediated targeting, and the membrane active polypeptide, which could penetrate the cell membrane and allow the efficient gene delivery. Then, I use the chemical modification method to modify the polypeptides with the hydrophobic domain. A library of copolypeptides with different side chains modified by aromatic groups and aliphatic groups are synthesized and screened for cell penetration and gene delivery. This chemical modification strategy significantly enhances the penetration ability of the copolypeptides and allows the efficient penetration via multi-mechanisms: endocytosis, pore formation and the direct membrane translocation without generating pores. The screened copolypeptides could successfully deliver genes into cells.
demonstrated by both in vitro and in vivo experiments. Even though numbers of polypeptides have been successfully demonstrated as the excellent DNA delivery candidates, there still exist various barriers and obstacles in delivering siRNA. One of the limitations is the individual siRNA condensation by the helical polypeptides. To overcome the dearth of polypeptide mediated siRNA delivery, I develop the reversely crosslinked thiolated polypeptide to facilitate the siRNA condensation and significantly improve the stability of siRNA. This strategy allows the tight condensing and protection of the siRNA in the extracellular environment and the release of the siRNA in the intracellular environment. My Ph.D. research aims on the design of the cationic polypeptides as the novel non-viral gene delivery vectors and helps establish the design criteria of the cationic polymers by overcoming various gene delivery barriers.
For my parents, who have always been loving me, and supporting me.
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CHAPTER 1

INTRODUCTION

1.1. Background

Gene therapy is showing great potentials in treating various genetic diseases, such as lysosomal storage disorders and cancers. It allows the therapeutic delivery of the genetic materials, like DNA, mRNA and siRNA, into the target cells to be expressed as the normal proteins, or knockdown the expression of the specific proteins.

Delivery vectors are needed to carry the genes into the cells since either DNA or siRNA cannot individually enter the cells due to the electrostatic repulsion between the negatively charged nuclear acid and the cell membranes. Most of the vectors could be divided into two categories: the viral vectors and the non-viral vectors [1, 2]. For the viral vectors, a certain gene is packaged into a replication-deficient viral particle, which could be delivered into cells later, like retrovirus and adenovirus. They are typically efficient, however, viruses can only deliver very small pieces of DNA into the cells. Besides that, the viral delivery method is labor-intensive and poses the risks of random insertion sites, integration of the viral into the host genome, recombination with natural viruses and the immunogenicity effect [3-6]. Most of the non-viral vectors are the synthetic cationic polymers or lipids. Compared with the viral vectors, the non-viral vectors are attracting more and more attentions these days and have been considered as a kind of safe and ideal alternative to the viral vectors. Currently, various kinds of synthetic materials have been successfully developed as the efficient non-viral gene delivery vectors, such as
polyethylenimine (PEI) [7-12], poly (amidoamine) (PAMAM) [13-18], poly-beta amino ester (PBAE) [19-25], cyclodextrin [26-34], which are all the cationic polymers based non-viral gene delivery vectors, and N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTMA) [35-39], 1,2-dioleoyloxy-3-trimethylammonium propane (DOTAP) [40-42] and 1-(2,3-dioleoyloxypropyl)-2,4,6-trimethylpyridinium lipid (2Oc) [43], which are all the cationic lipids based non-viral gene delivery vectors.

Cell penetrating peptides (CPPs), are sequence-specific short oligopeptides with the length up to 25 amino acids, which can penetrate most of the membranes [44-50]. They typically contain the positively charged amino acids such as lysine or arginine, like HIV-TAT [51-53], Arg9 [54], penetratin [55-58], and melittin [59-61]. Most of the CPPs have the α-helical secondary structures, or can form the α-helix after interacting with phosphate lipid bilayer membranes [62-64]. It has been demonstrated that the helical structure is quite important in terms of the cell penetration ability [65]. Due to their excellent membrane activities, CPPs are able to facilitate the cellular delivery of various kinds of exogenous cargos, including metals, macromolecules (e.g., proteins and nucleic acids), and nanoparticles [66-73]. However, when used as gene transfer agents, CPPs are often too short (fewer than 25 amino acid residues) and lack sufficient cationic charge density, which raise great challenges for CPPs to condense and deliver genes by themselves. As such, they often act as membrane-active ligands incorporated or conjugated to existing delivery vehicles to enhance their delivery efficiencies [74-76].
To overcome the dearth of CPP-mediated non-viral gene delivery, we use the ring-open polymerization method to synthesize the polypeptide. Polypeptides with sufficient backbone length and cationic charge density, such as poly-l-lysine (PLL) and poly-l-arginine (PLR), can individually condense genetic materials, while the gene delivery efficiency is still quite low. It is because the strong cationic charges repulsion on the side chains drives the polypeptides to form the random coil structures, which significantly limit their membrane activity [77, 78]. Then we modify the design of the polypeptide by increasing the side chain length, which successfully reduces the charge repulsion and stabilizes the helical structure [79]. The cationic, water-soluble α-helical polypeptides exhibit the good properties like sufficient positive charge density, which can individually condense and deliver genes. And they also adopt stable α-helical structures which facilitate excellent membrane activity and penetration ability [65, 80-87].

Non-viral gene delivery is the process of gene condensation, membrane targeting, membrane penetration, endosome escape, intracellular unpacking, and functional site localization. There are various barriers and obstacles encountered along the delivery process. Gene condensation is the first and crucial step before entering the cells. Suitable formulation strategy will allow the formation of the stable vector/gene nanoparticles, in which genes could be tightly condensed and away from degradation in the extracellular environment [88-92]. Active membrane targeting is one of the significant obstacles involved in the gene delivery process, which determines the specific recognize and the selectivity of the cells. In that case, various kinds of the targeting ligands, which could be recognized by the membrane receptors have been introduced to the design of the vectors
to facilitate the active membrane targeting, such as the incorporation of the hyaluronic acid (HA) to target the CD44, folate acid to target the some tumor cells and the design of mannosylated vectors to target the macrophages [87, 93-97]. The cell membrane penetration is a predominated barrier for the gene delivery process, which determines the efficiency of the genes taken into the cells. To improve the cell penetration ability, chemical modification method is widely used to optimize the functional groups, charge density and hydrophobicity to promote the interaction with the membrane structure and the ability of insertion into the lipid bilayers [98-107]. Intracellular unpacking, which the intracellular release of the genes, is also an essential step to achieve the successful gene delivery. In the past years, several of the responsive systems have been developed to solve the intracellular unpacking issues via designing the vectors with the responsive to the specific triggers, which thus allows the efficient and on-demand intracellular release of genes [108-114].

1.2. Scope and Organization

The aim of my Ph.D. research is to develop a safe and effective polypeptide-based non-viral gene delivery system. In the following three chapters, I will describe the development and improvement of the functional polypeptides to overcome various gene delivery barriers listed above. The organization of my thesis is briefly described below. Chapter 2 describes the modification of the complexes from the assembly of the supramolecular aspect. The formulation strategy allows the formation of the ternary complexes by incorporating another sugar containing polypeptide with targeting effect to balance the membrane activity and the membrane targeting. Chapter 3 describes the
chemical modification of the helical polypeptides to improve the cell penetration ability. The synthesized copolypeptides with the aromatic domain modification could efficiently penetrate the cells via multi-mechanisms and facilitate the successful in vitro and in vivo gene delivery. Chapter 4 describes the design of the reversely crosslinked thiolated polypeptide, which could individually condense and deliver siRNA, protect siRNA from degradation and achieve the on-demand intracellular release.

1.3. References


CHAPTER 2

ICATION, α-HELICAL POLYPEPTIDE COMPLEXES INDUCE EFFECTIVE GENE TRANSFECTION VIA MANNOSE RECEPTOR-MEDIATED TARGETING MECHANISM

2.1. Introduction

Gene therapy has shown great potentials in treating various genetic diseases, such as cystic fibrosis, diabetes, arthritis, immunological deficiency, and cancer [1-6]. Compared to viral vectors, non-viral gene delivery vectors allow safe delivery of genetic materials with less inherent immunogenicity and oncogenicity. Polycations, capable of condensing the anionic nucleic acids to facilitate their intracellular uptake, are one of the most widely explored non-viral vectors. Although the cationic charge of polycations features strong membrane binding of delivery vehicles and facilitates cellular uptake and transfection of the gene cargos, it meanwhile causes severe associated cytotoxicity [7-9]. Excessive positive charges can ultimately undermine the transfection efficiency [10]. Therefore, it is of particular importance to balance the charge related membrane activity and cytotoxicity in the design of non-viral vectors such that the gene delivery efficiency could be maximized. One promising approach towards this goal is the combinatorial/parallel synthesis that creates a large library of materials and allows identification of the best-performing candidate via screening [11, 12]. This technology, although promising, requires tedious task and often suffers from high cost. Alternatively, covalent modification of existing polycations with various charge-reducing moieties—including saccharides [13], hydrocarbons [14], and poly (ethylene glycol) (PEG) [15-17],
stands as an effective tool to reduce their toxicities. While the modified polycations benefit from improved safety profiles, they typically suffer from diminished gene delivery capabilities [10]. All these challenges thus necessitate a facile and effective strategy for the development of non-viral vectors which can properly balance the membrane activity and toxicity towards maximized gene delivery efficiency.

Cell penetrating peptides (CPPs), exemplified by HIV-TAT, Arg9, penetratin, and melittin, are sequence-specific short oligopeptides that mediate effective membrane penetration and translocation via either energy-dependent endocytosis or energy-independent transduction [18]. Due to their excellent membrane activities, CPPs are able to facilitate the cellular delivery of a variety of exogenous materials, including metals, macromolecules (e.g., proteins and nucleic acids), and nanoparticles [19, 20]. However, when used as gene transfer agents, CPPs are often too short (fewer than 25 amino acid residues) and lack sufficient cationic charge density, which raises great challenges for CPPs to condense and deliver genes by themselves. As such, they often act as membrane-active ligands incorporated or conjugated to existing delivery vehicles to enhance their delivery efficiencies [19, 21, 22]. To address the dearth of CPP-mediated non-viral gene delivery, we recently developed a cationic polypeptide, poly ($\gamma$-4-(((2-(piperidin-1-yl) ethyl) amino) methyl) benzyl-L-glutamate) (PVBLG-8), via a controlled ring-opening polymerization method [23-25] and used PVBLG-8 or its analogues in gene and siRNA delivery [26-28]. PVBLG-8 with stabilized helical structure exhibited desired membrane activity and thus triggered effective cellular uptake as well as gene transfection, which rendered it a better gene delivery vector than traditional oligo-CPPs [23]. However, the
appreciable cytotoxicity of PVBLG-8 at higher concentrations makes it unlikely to strength the gene delivery capabilities by increasing the amount used [26], which thus necessitates alternative approaches to maximize its gene transfer efficiencies without causing additional cytotoxicity.

With an attempt to balance the transfection efficiency and cytotoxicity, we first developed a PVBLG-8-based random copolypeptide (PVBLG-8-r-7) which contains glucosamine side chains that allow mannose receptor targeting/binding as well as reduce the material cytotoxicity via saccharide-mediated charge shielding. Although the cytotoxicity was slightly decreased, this approach shares the similar disadvantage of PEGylation and the resulting copolypeptide demonstrated decreased membrane activity and transfection efficiency compared to PVBLG-8, possibly due to the diminished cationic charge density. Based on such findings, we thus seek alternative strategies which could endow PVBLG-8 with cellular targeting functionality while maintain the membrane activity as well as the gene delivery capability [29]. To this end, poly (γ-glucosamine methyl) benzyl-L-glutamate) (PVBLG-7) [25], a helical polypeptide bearing glucosamine residues, was incorporated to form the PVBLG-8/PVBLG-7/DNA ternary complexes via self-assembly instead of constructing the PVBLG-8-r-7 copolypeptide. We hypothesized that incorporation of PVBLG-7, a cationic helical polypeptide with minimized membrane activity, can strengthen the gene delivery capabilities of PVBLG-8 via mannose-receptor-mediated cellular targeting while will not generate additional cytotoxicity. In various mammalian cell types (HeLa, COS-7, and Raw 264.7) that express mannose receptors [30-32], the cellular uptake level, intracellular kinetics,
transfection efficiency, and cytotoxicity of ternary complexes were explored and compared to PVBLG-8/DNA binary complexes. Upon an optimized combination between membrane activity and cellular targeting, the top-performing formulation with optimal transfection/toxicity balance is identified. This study thus provides insights into the design strategy of safe and effective non-viral vectors for gene delivery.

2.2. Materials and Methods

2.2.1. Materials and cell lines

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO) and used as received unless otherwise specified. Anhydrous tetrahydrofuran (THF), hexane, and dimethylformamide (DMF) were dried by a column packed with 4Å molecular sieves and stored in a glovebox. Dry nitrobenzene (NB) was prepared by treating regular NB with CaH₂ followed by distillation under reduced pressure. Hexamethyldisilazane (HMDS) and 1,5,7-triazabicyclo[4.4.0]dec-5-ene (TBD) were used for controlled ring-opening polymerization of amino acid N-carboxyanhydrides developed by us [25]. γ-(4-Vinylbenzyl)-L-glutamate N-carboxyanhydride (VB-L-Glu-NCA) was prepared as previously reported [23, 33]. Pierce BCA assay kit was purchased from ThermoFisher Scientific (Rockford, IL, USA). Plasmid DNA (pDNA) encoding enhanced green fluorescence protein (EGFP) (pEGFP) was purchased from Elim Biopharm (Hayward, CA, USA). Lipofectamine™ 2000 (LPF), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), and YOYO-1 were purchased from Invitrogen (Carlsbad, CA, USA).
HeLa (human cervix adenocarcinoma cells), COS-7 (African green monkey kidney cells), and Raw 264.7 (mouse leukemic monocyte macrophage cells) were purchased from the American Type Culture Collection (Rockville, MD, USA) and were cultured in Dulbecco’s Modified Eagle Medium (DMEM) (Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin.

2.2.2. Synthesis of PVBLG-8

VB-L-Glu-NCA (58 mg, 0.2 mmol) was dissolved in a mixture of DMF (0.9 mL) and nitrobenzene (30 μL) in a glove box, followed by addition of HMDS (13.3 μL, 0.1 M, M/I=150) and TBD solution (13.3 μL, 0.01 M) in DMF. FTIR was used to monitor the polymerization until the conversion reached 99% (within 48 hours) to obtain poly (γ-(4-vinylbenzyl)-L-glutamate) (PVBLG). $^1$H NMR (500 MHz, CDCl$_3$/TFA-$d$(85:15, v/v), δ, ppm): 7.34 (d, 2H, ArH), 7.20 (d, 2H, ArH), 6.65 (m, 1H, –CH=CH$_2$), 5.72 (d, 2H, –CH=CH$_2$), 5.24 (d, 2H, –CH=CH$_2$), 5.04 (m, 2H, ArCH$_2$O–), 4.59 (m, 1H, α-H), 2.45 (t, 2H, –COCH$_2$CH$_2$–), 2.11 (m, 1H, –COCH$_2$CH$_2$–), 1.93 (m, 1H, –COCH$_2$CH$_2$–).

Tetrabutylammonium fluoride solution (100 μL, 1 M), benzyl chloroformate (50 μL), and $N$,$N$-diisopropylethylamine (DIEA, 50 μL) were added and stirred for 3 h to cleave the N-Si bond and protect the amino end groups. DMF was removed under vacuum, and the resulting polymer was precipitated from cold ethyl ether (45 mL), washed with cold ethyl ether (45 mL × 3), and collected by centrifugation at 4000 rpm. PVBLG was then dissolved in chloroform (30 mL) and oxidized by O$_3$ at -78 °C. Dimethyl sulfide (1 mL) was added and the solution was stirred at RT overnight before the solvent was removed under vacuum. The product poly (γ-(4-aldehydebenzyl-L-glutamate) (PABLG) was
washed with methanol (45 mL × 3) to remove unreacted dimethyl sulfide and other impurities, and collected by centrifugation. \(^1\)H NMR (500 MHz, CDCl\(_3\)/TFA-\(d\) (85:15, v/v)): \(\delta\) 9.86 (s, 1H, ArCHO), 7.95-7.45 (br m, 4H, ArH), 5.16 (br s, 2H, ArCH\(_2\)O–), 4.61 (m, 1H, \(\alpha\)-H), 2.55 (br m, 2H, –COCH\(_2\)CH\(_2\)–), 2.16 (m, 1H, –COCH\(_2\)CH\(_2\)–), 1.98 (m, 1H, –COCH\(_2\)CH\(_2\)–). The obtained PABLG (30 mg) was dissolved in DMF (2 mL), into which 1-(2-aminoethyl)piperidine (150 \(\mu\)L, 10 molar equivalents relative to the Glu repeating unit) was added. After reaction at 50 °C for 24 h, borane pyridine as the reducing agent (133 \(\mu\)L, 10 molar equivalents relative to the Glu repeating unit) was added, and the solution was further stirred at 50 °C for 24 h. HCl (5 M, 1 mL) was added to protonate the amine groups, and the final product PVBLG-8 was dialyzed against water (MWCO = 1 kDa) and lyophilized. \(^1\)H NMR (500 MHz, TFA-\(d\)): \(\delta\) 7.53 (m, 4H, ArH), 5.32 (br s, 2H, ArCH\(_2\)O–), 4.86 (br s, 2H, ArCH\(_2\)NH), 4.53 (s, 1H, \(\alpha\)-H), 4.01 (s, 4H, –OCH\(_2\)CH\(_2\)–), 3.94–3.80 (br m, 6H, –HNCH\(_2\)CH\(_2\)N– and –NCH\(_2\)CH\(_2\)CH\(_2\)CH\(_2\)–), 3.13 (m, 2H, –HNCH\(_2\)CH\(_2\)N–), 2.78 (s, 2H, –COCH\(_2\)CH\(_2\)–), 2.40 (br m, 2H, –COCH\(_2\)CH\(_2\)–), 2.14–1.52 (br m, 6H, –NCH\(_2\)CH\(_2\)CH\(_2\)CH\(_2\)–).

2.2.3. Synthesis of PVBLG-7

PABLG (30 mg) was dissolved in DMF (2 mL). Glucosamine hydrochloride (200 mg dissolved in DMSO, 10 molar equivalents relative to the Glu repeating unit) was added. After the solution was stirred at 50 °C for 72 h, the polypeptide was reduced by borane pyridine, protonated with HCl, and dialyzed against water using the same method as described above. \(^1\)H NMR (500 MHz, TFA-\(d\)): \(\delta\) 8.35-7.75 (br m, 4H, ArH), 5.54 (m, 2H, OHCHCHCHOH), 5.04 (br m, 2H, ArCH\(_2\)O–), 4.77 (s, 1H, \(\alpha\)-H), 4.49 (br s, 2H,
2.2.4. Synthesis of PVBLG-8-r-7

PABLG (30 mg) was dissolved in DMF (2 mL). Glucosamine hydrochloride (60 mg dissolved in DMSO, 3 molar equivalents relative to the Glu repeating unit) was added. After stirring at 50 °C for 24 h, 1-(2-aminoethyl)piperidine (150 μL, 10 molar equivalents relative to the Glu repeating unit) was added, and the solution was stirred at 50 °C for another 24 h. The polypeptide was then reduced by borane pyridine, protonated with HCl, and dialyzed against water in the same method as described above. $^1$H NMR (500 MHz, TFA-d): $\delta$ 8.10–7.50 (m, 4H, ArH), 5.31 (s, 2H, ArCH$_2$O–), 4.88 (m, 2H, OHCCHCHCHOH) 4.52–4.26 (br m, 3H, ArCH$_2$NH– and α–H ), 4.09-3.81 (br m, 5H,OHCH$_2$CHCHCHCHO–), 3.92–3.60 (br m, 6H, –HNCH$_2$CH$_2$N– and –NCH$_2$CH$_2$CH$_2$CH$_2$–), 3.08 (m, 3H, –HNCH$_2$CH$_2$N– and –NHCH), 2.75 (m, 4H, –COCH$_2$CH$_2$–), 2.40 (br m, 2H, –COCH$_2$CH$_2$–), 2.04–1.52 (br m, 6H, –NCH$_2$CH$_2$CH$_2$CH$_2$–).

2.2.5. Characterization of polypeptides

$^1$H NMR spectra were recorded on a Varian Ul500NB MHz spectrometer. Chemical shifts were reported in ppm and referenced to the solvent proton impurities. Gel permeation chromatography (GPC) experiments were performed on a system equipped with an isocratic pump (Model 1100, Agilent Technology, Santa Clara, CA, USA), a DAWN HELEOS multi-angle laser light scattering (MALLS) detector (Wyatt
Technology, Santa Barbara, CA, USA), and an Optilab rEX refractive index detector (Wyatt Technology, Santa Barbara, CA, USA). Separations were performed using serially connected size exclusion columns (100 Å, 500 Å, 10³ Å, and 10⁴ Å Phenogel columns, 5 µm, 300 × 7.8 mm, Phenomenex, Torrance, CA, USA) at 60 °C with DMF containing 0.1 M LiBr as the mobile phase. The detection wavelength was set at 658 nm, and the MALLS detector was calibrated using pure toluene, which allowed determination of the absolute molecular weights (MWs) instead of calibration using polymer standards. The MWs of polypeptides were calculated according to the dn/dc value of each polymer using the internal calibration system processed by the ASTRA V software (version 5.1.7.3, Wyatt Technology, Santa Barbara, CA, USA). Circular dichroism (CD) measurements were carried out on a JASCO J-700 CD spectrometer (Oklahoma City, OK, USA). Polypeptide was dissolved in DI water at a concentration of 0.1 mg/mL, and was placed in a quartz cell with a pathlength of 0.1 cm. The mean residue molar ellipticity and helicity of each polymer were calculated based on the measured apparent ellipticity following the reported formula [25]:

\[
\text{Ellipticity (} \theta \text{, deg cm}^2 \text{ mol}^{-1} \text{)} = \frac{\text{millidegree} \times \text{mean residue weight}}{\text{pathlength (mm)} \times \text{concentration (mg mL}^{-1} \text{)}}
\]

\[
\text{Helicity (}) \% \text{)} = \frac{100}{39000} \times [\theta_{222}] + 3000 \times 100
\]

2.2.6. Formulation of binary and ternary complexes

Polypeptides and DNA were dissolved in DI water at 0.2 mg/mL. To form binary complexes, polypeptide was added into the DNA solution at various weight ratios
followed by vortex for 30 sec and incubation at RT for 20 min. While for the ternary complexes, PVBLG-8 and PVBLG-7 were mixed at determined weight ratios before they were added to the DNA solution at a fixed PVBLG-8/DNA weight ratio of 15. The mixture was vortexed for 30 sec and incubated at RT for 20 min to obtain the ternary complexes.

2.2.7. Characterization of binary and ternary complexes

A gel retardation assay was first adopted to evaluate the DNA condensation by cationic polymers. Freshly prepared complexes were loaded on a 1% agarose gel at 100 ng DNA/well followed by electrophoresis at 100 V for 30 min. Naked DNA was used as a control, and DNA migration in the agarose gel was visualized by a Gel Doc imaging system (Biorad, Herclues, CA, USA) following staining with ethidium bromide (EB). To quantitatively measure the DNA condensation level, the EB exclusion assay was performed as follows [17]. DNA was first stained with EB at the DNA/EB weight ratio of 10 and RT for 1 h. PVBLG-8, PVBLG-7, or a mixture of them were added into the DNA/EB solution followed by further incubation at RT for 30 min before quantification of the fluorescence intensity ($\lambda_{ex} = 510$ nm, $\lambda_{em} = 590$ nm). The DNA condensation efficiency (%) was calculated according to the following equation:

$$\text{DNA condensation efficiency (\%) } = \left(1 - \frac{F - F_{EB}}{F_0 - F_{EB}}\right) \times 100$$

Where $F_{EB}$, $F$, and $F_0$ denote the fluorescence intensity of pure EB solution, DNA/EB solution with polypeptide, and DNA/EB solution without any polypeptide, respectively.
Particle size and zeta potential of freshly prepared complexes at various weight ratios were also evaluated by dynamic laser scanning (DLS) on a Malvern Zetasizer (Herrenberg, Germany). To evaluate the complex stability, ternary complexes were diluted with PBS (pH 7.0) by 10, 30, 50, and 100 folds, respectively, incubated at 37 °C for 1 h, and subject to DLS measurement.

2.2.8. Cell uptake

To allow visualization and quantification of the cellular internalization, DNA (1 mg/mL) was labeled with YOYO-1 (20 μM) at one dye molecule per 50 bp DNA [34] and used to form complexes as described above. Cells were seeded on 24-well plates at 5×10⁴ cells/well and cultured until they reached confluence. The cell culture medium was replaced with Opti-MEM (500 μL/well) into which complexes were added at 0.5 μg YOYO-1-DNA/well. After incubation at 37 °C for 4 h, cells were washed with PBS containing heparin (20 U/mL) for 3 times to remove the surface-bound cationic complexes [35] and lysed with RIPA lysis buffer (500 μL/well) at RT for 20 minutes. The YOYO-1-DNA content in the lysate was monitored by spectrofluorimetry (λ<sub>ex</sub> = 485 nm, λ<sub>em</sub> = 530 nm) and the protein level was measured using the BCA kit. Uptake level was expressed as ng DNA associated with 1 mg cellular protein. To explore the PVBLG-7-mediated targeting via mannose-receptor recognition, cells were pre-incubated with Opti-MEM (500 μL/well) supplemented with mannose at different concentrations (100 μM, 200 μM, 400 μM, and 800 μM) for 30 minutes prior to the addition of complexes and throughout the 4-h uptake experiment at 37 °C.
The internalization and intracellular distribution of complexes were also observed by confocal laser scanning microscopy (CLSM). HeLa cells cultured on coverslips in 6-well plate were incubated with complexes in Opti-MEM (2 mL) at 1 μg DNA/well. Following incubation for different time (0.5, 1, 2, and 4 h), cells were washed three times with PBS containing heparin (20 U/mL), fixed with 4% paraformaldehyde, and stained with DAPI (10 μg/mL) before observation by confocal laser scanning microscopy (CLSM, LSM700, Zeiss, Germany).

2.2.9. Intracellular kinetics

To explore the cellular uptake pathways of complexes, we performed the cell uptake study at low temperature (4 °C) or in the presence of endocytic inhibitors. To block the energy-dependent endocytosis, the cell uptake study was performed at 4 °C during the 2-h period. Otherwise, cells were pre-incubated with endocytic inhibitors including genistein (100 μg/mL), methyl-β-cyclodextrin (mβCD, 5 mM), wortmannin (10 μg/mL), and chlorpromazine (10 μg/mL) for 30 minutes prior to polypeptide application and throughout the 2-h uptake experiment at 37 °C. The cellular uptake level was determined as described above, and results were expressed as percentage uptake of the control cells which were incubated with complexes at 37 °C for 2 h in the absence of endocytic inhibitors.
To explore the endosomal/lysosomal entrapment, HeLa cells were treated with YOYO-1-DNA-containing complexes (1 μg/mL) for 4 h at 37 °C, stained with Hoechst 33258 (5 μg/mL) and Lysotracker® Red (200 nM), and observed by CLSM.

2.2.10. Membrane activity

The capability of polypeptides to induce membrane disruption was evaluated in terms of the cell uptake level of a hydrophilic, membrane-impermeable dye, fluorescein isothiocyanate (FITC) in its non-reactive form (fluorescein-tris(hydroxymethyl)methanethiourea, FITC-Tris) [18]. Briefly, HeLa cells were seeded on 96-well plates at 1×10^4 cells/well and cultured for 24 h. The medium was replaced with Opti-MEM (100 μL/well), into which polypeptides and FITC-Tris were added at 2 μg/well and 0.2 μg/well, respectively. Free FITC-Tris without polypeptides was added as a control. After incubation at 37 °C for 2 h, cells were washed with PBS containing heparin (20 U/mL) for 3 times and then lysed with the RIPA lysis buffer (100 μL/well). The FITC-Tris content in the lysate was quantified using spectrofluorimetry (λ_ex = 485 nm, λ_em = 530 nm) and the protein level was determined using the BCA kit. Uptake level was expressed as ng FITC-Tris associated with 1 mg cellular protein.

2.2.11. In vitro transfection

Cells were seeded on 24-well plates at 5×10^4 cells/well and cultured in serum-containing media for 24 h before reaching confluence. The culture medium was changed to Opti-MEM (500 μL/well) into which binary or ternary complexes were added at 0.5 μg DNA/well. After incubation at 37 °C for 4 h, the medium was replaced by DMEM
containing 10% FBS (500 μL/well) and cells were further incubated for 48 h before assessment of EGFP expression by flow cytometry. The transfection efficiency was expressed as percentage of EGFP-positive cells (%), and the EGFP expression was also observed by fluorescence microscopy. To future explore the PVBLG-7-mediated targeting effect, cells were incubated with complexes in mannose-supplemented Opti-MEM for 4 h as described above and then cultured in serum-containing media for another 48 h before flow cytometry assessment.

2.2.12. Cytotoxicity

Cells were seeded on 96-well plates at 1×10⁴ cells/well and cultured in serum-containing media for 24 h. The medium was replaced with Opti-MEM (100 μL/well), into which polypeptides or polypeptide/DNA complexes were added at the PVBLG-8 final concentrations of 100, 50, 20, and 10 μg/mL, respectively. After incubation at 37 °C for 4 h, the medium was changed to serum-containing DMEM and cells were further cultured for 48 h before viability assessment by the MTT assay. Results were represented as percentage viability of control cells that did not receive polypeptide or complex treatment.

2.2.13. Statistical analysis

Statistical analysis was performed using Student’s t-test and differences between test and control groups were judged to be significant at *p < 0.05 and very significant at **p < 0.01.
2.3. Results

2.3.1. Synthesis and characterization of PVBLG-8 and PVBLG-8/DNA binary complex

PVBLG-8 was synthesized via HMDS-initiated ring-opening polymerization (ROP) of VB-L-Glu-NCA and subsequent side-chain amination [23, 36] (Fig. 2.1A). HMDS allowed a well-controlled ROP of VB-L-Glu-NCA, as evidenced by the monomodal peaks in the GPC curves (Fig. 2.1B), well-defined MW (degree of polymerization 160 as calculated by GPC), and narrow PDI (<1.2). The conjugation efficiency of 1-(2-aminoethyl) piperidine in PVBLG-8 was determined to be 90% based on $^1$H NMR analysis. PVBLG-8 exhibited excellent solubility in water at pH lower than 9, and it adopted typical α-helical structure with 99% helicity as evidenced by the characteristic double minima at 208 nm and 222 nm in the CD spectrum (Fig. 2.2A). The helical structure of PVBLG-8 notably contributed to its desired membrane activity via the pore formation mechanism, leading to appreciable cellular internalization of the membrane impermeable FITC-Tris, (Fig. 2.2B). The helicity of the PVBLG-8 was remarkably stable against pH change between 1 and 10, indicating that the polypeptide was able to maintain its helicity-dependent membrane activities at both the neutral extracellular pH and the acidic endosomal/lysosomal pH, which thus allowed it to trigger effective intracellular internalization as well as endosomal escape (Fig. 2.2C) [23]. The helicity of PVBLG-8 was also stable against salt concentration increment up to 0.8 M, suggesting that it could remain stable helical structures in the physiological fluids with ionic strength of approximately 0.15 M (Fig. 2.2D). Because of its high molecular weight and cationic charge density, PVBLG-8 was able to condense DNA through electrostatic interactions to form 150-nm polyplexes at the weight ratio higher than 5 (Fig. 2.3A and
B). After condensing DNA to form the binary complex, the helical structure of PVBLG-8 was well maintained (Fig. 2.3C), which thus allowed the complexes to mediate helicity-dependent membrane penetration to trigger cellular internalization and transfection of the gene cargo as shown by the expression of EGFP in transfected cell. The optimal weight ratio was 15 in this experiment (Fig. 2.3D and F). However, sharing the same drawback as other polycations, PVBLG-8 exhibited dose-dependent cytotoxicity due to its membrane activity (Fig. 2.3E). As a result, the cellular uptake level and gene transfection efficiency were reduced when excessive polypeptide was utilized (weight ratio higher than 15).

Fig. 2.1 (A) Reaction scheme of PVBLG-8. (B) GPC curve of PVBLG obtained from

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(Fig. 2.1 cont.) HMDS-initiated polymerization of VB-L-Glu-NCA at the M/I ratio of 150/1. The MW and PDI of the resulting PVBLG were summarized in the figure.

![Graph A](image1.png)

**A** Helicity=99%

![Graph B](image2.png)

**B** Cell uptake level (ng FITC/mg protein)

0 100 200 300 400

PVBLG-8

FITC

![Graph C](image3.png)

**C** Residue molar ellipticity at 222 nm of PVBLG-8 and PVBLG-7 at different pH.

![Graph D](image4.png)

**D** Residue molar ellipticity at 222 nm of PVBLG-8 and PVBLG-7 at different NaCl concentrations.

Fig. 2.2 (A) CD spectrum of PVBLG-8 in DI water (0.1 mg/mL) at pH 7. (B) FITC-Tris uptake level in HeLa cells following co-incubation with PVBLG-8 for 2 h at 37 °C. (C) Residue molar ellipticity at 222 nm of PVBLG-8 and PVBLG-7 at different pH. (D) Residue molar ellipticity at 222 nm of PVBLG-8 and PVBLG-7 at different NaCl concentrations.
Fig. 2.3 (A) DNA condensation by PVBLG-8 at different PVBLG-8/DNA weight ratios as evaluated by the gel retardation assay. N represents naked DNA. (B) Particle size and zeta potential of PVBLG-8/DNA complexes. (C) CD spectra of PVBLG-8/PVBLG-7 mixtures
and their ternary complexes with DNA in DI water at pH 7. (D) Uptake level of PVBLG-8/YOYO-1-DNA complexes in HeLa cells following incubation at 37 °C for 4 h (n=3). (E) In vitro cytotoxicity of PVBLG-8/pEGFP complexes in HeLa cells at different weight ratios as determined by the MTT assay (n=3). (F) In vitro cytotoxicity of PVBLG-8/pEGFP complexes in HeLa cells at different weight ratios as determined by the MTT assay (n=3).

2.3.2. Synthesis and characterization of PVBLG-8-r-7 and PVBLG-8-r-7/DNA complexes

In attempts to potentiate the gene delivery capability of PVBLG-8 while reducing the cytotoxicity, as our first strategy, we developed a PVBLG-8-based random copolypeptide, PVBLG-8-r-7 that contained glucosamine on its side-chain terminal (Fig. 2.4A). We hypothesized that the glucosamine residue would allow active targeting to mannose receptors on specific cell types to promote the gene transfer efficiency. However, in contrary to our hypothesis, the transfection efficiency of PVBLG-8-r-7 was decreased compared to the original PVBLG-8 (Fig. 2.4B), which was likely due to saccharide-mediated shielding of the positive charges of the PVBLG-8 segment (Fig. 2.4D). Such strategy shared the similar shortcoming of PEGylation [37], although the cell tolerability was slightly improved (Fig. 2.4C) due to the charge shielding effect (Fig. 2.4D).
Fig. 2.4 PVBLG-8-r-7 displays diminished transfection efficiency while improved cell tolerability compared to PVBLG-8. (A) Reaction scheme of PVBLG-8-r-7. (B) In vitro transfection efficiencies of PVBLG-8-r-7/pEGFP complexes and PVBLG-8/pEGFP complexes in HeLa cells (n=3). (C) Cytotoxicity of PVBLG-8-r-7 and PVBLG-8 towards HeLa cells as determined by the MTT assay (n=3). (D) Zeta potential of PVBLG-8-r-7/DNA complexes and PVBLG-8/DNA complexes.

2.3.3. Synthesis and characterization of PVBLG-7 and PVBLG-7/DNA binary complex

Since the copolypeptide strategy did not work, we thus sought alternative approaches which can endow PVBLG-8 with cellular targeting functionality while do not
compromise its membrane activity. The self-assembly strategy was adopted which allows convenient preparation of nanostructured complexes from molecular building blocks [29, 38, 39]. Targeting moiety can be incorporated via non-covalent molecular recognition rather than covalent conjugation, such that the integral PVBLG-8 structure can be maintained and the lead complexes can be easily identified via combinatorial selection of substrates [38, 39]. To realize this approach, the targeting building block needs to meet two requirements. First, it should have non-covalent interactions with PVBLG-8 or DNA, allowing it to be integrated into the PVBLG-8/DNA complexes. Second, it should display weak or minimal membrane activity so that it will not induce additional cytotoxicity. With regards to these requirements, PVBLG-7 was developed as a targeting component towards mannose receptors on specific cell types, and the PVBLG-8/PVBLG-7/DNA ternary complexes were constructed via self-assembly to attain non-viral gene delivery (Fig. 2.7A). PVBLG-7 was also synthesized through polymerization of VB-L-Glu-NCA and side-chain amination [25] (Fig. 2.5A), and thus it shared the same molecular weight and PDI as PVBLG-8 (Fig. 2.1B). The conjugation efficiency of glucosamine in PVBLG-7 was determined to be 75% by $^1$H NMR, and the helicity of PVBLG-7 was lower than PVBLG-8 (58%, Fig. 2.5B), presumably due to the lower cationic charge density on the side chain terminal that reduced the intramolecular repulsion, the driving force for the stabilization of helical structure. Because of its lower cationic charge density, PVBLG-7 showed much weaker DNA condensation capacity, as evidenced by the observation that PVBLG-7 condensed only 60% of DNA at a much higher weight ratio of 30 (80% DNA condensed at PVBLG-8/DNA weight ratio higher than 5, Fig. 2.5C and D) and the PVBLG-7/DNA complexes showed lower zeta potential (~20 mV, Fig. 2.6A) than
PVBLG-8/DNA complexes (~40 mV). In accordance with its lower cationic charge
density and helical content, PVBLG-7 exhibited weaker membrane activity and
cytotoxicity (Fig. 2.6B) than PVBLG-8, which indicated that addition of PVBLG-7 may
not induce additional toxicities. We then evaluated the transfection efficiency of PVBLG-
7/DNA complexes at different weight ratios. The results revealed that the optimal
transfection efficiency of PVBLG-7 (~15% EGFP positive cells) was notably lower than
that of PVBLG-8 (~50% EGFP positive cells, Fig. 2.6C).

Fig. 2.5 (A) Reaction scheme of PVBLG-7. (B) CD spectrum of PVBLG-7 in DI water
(0.1 mg/mL) at pH 7. (C) EB exclusion assay showing the condensation of DNA in the
(Fig. 2.5 cont.) ternary complexes. PVBLG-8/DNA weight ratio fixed at 15/1 (n=3). (D) DNA condensation by PVBLG-7 at different weight ratios as evaluated by the gel retardation assay. N represents naked DNA.

Fig. 2.6 PVBLG-7 bears low cationic charge density than PVBLG-8 and thus exhibits weaker DNA condensation capability, lower membrane permeability, and lower cytotoxicity. (A) Particle size and zeta potential of PVBLG-7/DNA complexes. (B) In vitro cytotoxicity of PVBLG-7 in HeLa cells as determined by the MTT assay (n=3). (C) In vitro transfection efficiency of PVBLG-7/pEGFP complexes in HeLa cells at different weight ratios (n=3).

2.3.4. Characterization of ternary complexes

Because PVBLG-8/DNA binary complexes showed maximal cell uptake level and transfection efficiency at the weight ratio of 15 we thus prepared a series of PVBLG-8/PVBLG-7/DNA ternary complexes by fixing the PVBLG-8/DNA weight ratio at 15 while changing the PVBLG-8/PVBLG-7 weight ratio from 1 to 13. Gel retardation assay showed retarded DNA migration in the agarose gel for all the test ternary complexes,
suggesting that DNA could be effectively co-condensed by cationic PVBLG-8 and PVBLG-7 (Fig. 2.7B). An increase in the PVBLG-7 content in the ternary complexes did not significantly alter the DNA condensation level (Fig. 2.7C), indicating that PVBLG-8 with much higher cationic charge density contributed to majority of the DNA condensation in the ternary complexes. Consistently, an increase in the PVBLG-7 content resulted in unappreciable alteration in the particle size and zeta potential (Fig. 2.8A), and the ternary complexes at the PVBLG-8/PVBLG-7/DNA weight ratio of 15/7/1 revealed spherical morphology and diameter of 150-200 nm as shown in the SEM image (Fig. 2.8B). Upon dilution with PBS up to 100 folds, the particle size of ternary complexes (PVBLG-8/PVBLG-7/DNA weight ratio of 15/7/1) maintained unaltered, which indicated their desired stability against salt and dilution (Fig. 2.8C).

Fig. 2.7 (A) Schematic representation of PVBLG-8/PVBLG-7/DNA ternary complexes.
(Fig. 2.7 cont.) (B) DNA condensation by polypeptides as evaluated by the gel retardation assay. (C) EB exclusion assay showing the condensation of DNA in the complexes (n=3).

Fig. 2.8 (A) Particle size and zeta potential of PVBLG-8/PVBLG-7/DNA ternary complexes (PVBLG-8/DNA weight ratio = 15). (B) SEM image of ternary complexes at the PVBLG-8/PVBLG-7/DNA weight ratio of 15/7/1 (bar = 500 nm). (C) Stability of ternary complexes following dilution with PBS at different folds.

2.3.5. Cell uptake and intracellular mechanism

The capability of ternary complexes to deliver DNA intracellularly was evaluated and compared to binary complexes in three different mammalian cell types that express mannose receptors, HeLa, COS-7, and Raw 264.7 [30-32]. PVBLG-8/DNA binary complexes remarkably promoted the internalization level of YOYO-1-DNA, peaking at the PVBLG-8/DNA weight ratio of 15:1 and outperforming LPF/DNA complexes by 3-4 fold (Fig. 2.9). The excellent membrane activity of PVBLG-8 promoted the interaction between complexes and cell membranes, thus triggering efficient cellular internalization that increased with the PVBLG-8/DNA weight ratio up to 15. However, further increase
in the PVBLG-8/DNA weight ratio to 20 and 30 decreased the cell uptake level, mainly due to the excessive membrane activity of PVBLG-8 that caused irreversible cell damage. Therefore, at the optimized PVBLG-8/DNA weight ratio of 15, PVBLG-7 was incorporated to form the ternary complexes. As shown in Fig. 2.9, addition of PVBLG-7 did lead to further improvement in the cell uptake level, mainly attributed to its targeting effect via recognition of mannose receptors. The cell uptake level peaked at the PVBLG-8/PVBLG-7 weight ratio of 15:7, 15:3, and 15:3 for HeLa, COS-7, and Raw 264.7 cells, respectively, which was 1.5-2 fold higher than the PVBLG-8/DNA binary complexes (Fig. 2.9). Further increase of the PVBLG-7 amount decreased rather than increased the cell uptake level, which could result from the competitive binding between mannose receptors and excessive PVBLG-7 that was not associated with the complexes. To further verify the targeting effect, we performed the uptake study in the presence of free mannose at various concentrations. As shown in Fig. 2.10A and B, an increase in the mannose concentration (6-50 molar folds of glucosamine in the ternary complexes, PVBLG-8/PVBLG-7/DNA=15/7/1) led to significantly decreased cell uptake level of the ternary complexes but not the PVBLG-8/DNA binary complexes. Such findings thereby substantiated that PVBLG-7 promoted the cellular internalization of ternary complexes by targeting to cell membranes via recognition of mannose receptors that could be competitively occupied by free mannose.
Fig. 2.9 Uptake level of ternary complexes containing YOYO-1-DNA in HeLa, COS-7, and Raw 264.7 cell following 4-h incubation at 37 °C (n=3). PVBLG-8/DNA weight ratio was maintained constant at 15. N represents naked DNA.

Fig. 2.10 (A) Uptake level of ternary complexes in HeLa cells in the presence of free mannose at various concentrations (n=3). (B) Uptake level of ternary complexes in COS-7 and Raw 264.7 cells in the presence of free mannose at various concentrations (n=3).

The gene transfection efficiency of non-viral vectors is closely related to their
intracellular kinetics, such as the internalization pathway and endosomal escape mechanism [40]. We thus mechanistically probed the intracellular kinetics of the ternary complexes in HeLa, COS-7, and Raw 264.7 cells. By performing the cell uptake study at lower temperature (4 °C) or in the presence of various endocytic inhibitors, we first elucidated the internalization pathway of ternary complexes. Energy-dependent endocytosis was completely blocked at 4 °C; chlorpromazine inhibited clathrin-mediated endocytosis (CME) by triggering the dissociation of the clathrin lattice; genistein and mβCD inhibited caveolae by inhibiting tyrosine kinase and depleting cholesterol, respectively; wortmannin inhibited macropinocytosis by suppressing phosphatidylinositol-3-phosphate [40]. Lowering the temperature resulted in 70% reduction in the cell uptake level, implying that majority of the complexes were internalized via energy-dependent endocytosis. The cell uptake level was also significantly inhibited by genistein, mβCD, and chlorpromazine in all the three cell lines (Fig. 2.11A), which indicated that both the caveolae- and clathrin-mediated pathway were involved during endocytosis of ternary complexes. Wortmannin exerted a cell line dependent inhibitory effect in HeLa and COS-7 cells only but not in Raw 264.7 cells (Fig. 2.11B). Apart from the endocytosis pathway, we also probed the capability of ternary complexes to induce pore formation on cell membranes, an important non-endocytosis mechanism mediated by cationic helical polypeptides [23]. The uptake level of FITC-Tris, a hydrophilic and membrane-impermeable dye, was monitored after co-incubation with ternary complexes to represent the pore formation levels. As shown in Fig. 2.11C, ternary complexes notably enhanced the FITC-Tris uptake level, which was comparable to that of the PVBLG-8/DNA binary complexes at all test PVBLG-8/PVBLG-7 ratios. It therefore indicated that
PVBLG-8, after condensing DNA to form either binary complexes or ternary complexes, maintained its membrane activity that was not counteracted by the incorporation of PVBLG-7. With the optimal formulation of PVBLG-8/PVBLG-7/DNA weight ratio of 15/7/1, we further evaluated the endosomal escape and DNA nuclear transport of ternary complexes in HeLa cells by CLSM. As shown in Fig. 2.11D, YOYO-1-DNA was extensively taken up by HeLa cells post 2-h treatment with ternary complexes, and it was largely separated from Lysotracker® Red-stained endosomes/lysosomes, indicating that they were able to mediate effective endosomal escape. The internalized YOYO-1-DNA was also noted to be distributed to the Hoechst 33258-stained nuclei, suggesting that the ternary complexes could trigger nuclear transport of DNA to initiate gene transcription (Fig. 2.11D).

Fig. 2.11 (A) Uptake level of ternary complexes in HeLa cells at 4 °C or in the presence...
(Fig. 2.11 cont.) of various endocytic inhibitors. (B) FITC-Tris uptake level of HeLa cells following co-incubation with PVBLG-8 and PVBLG-7 at different weight ratios for 2 h at 37 °C (n=3). (C) Uptake level of ternary complexes (PVBLG-8/PVBLG-7/DNA weight ratio of 15/3/1) in the presence of various endocytosis inhibitors in COS-7 and Raw 264.7 cells (n = 3). (D) CLSM images showing the cellular internalization and distribution of PVBLG-8/DNA binary complexes and PVBLG-8/PVBLG-7/DNA ternary complexes in HeLa cells following incubation at 37 °C for 4 h (bar = 20 μm).

2.3.6. In vitro transfection

The gene transfection efficiencies of ternary complexes in HeLa, COS-7, and Raw 264.7 cells were monitored by flow cytometry and compared to those of binary complexes. For the PVBLG-8/DNA binary complexes, maximal transfection efficiency was noted at the PVBLG-8/DNA weight ratio of 15, which was consistent with their cell uptake level. Such result further substantiated our statement that transfection efficiency cannot be improved by keeping increasing the amount of PVBLG-8, largely due to its excessive membrane activity that caused irreversible cell damage. By fixing the PVBLG-8/DNA ratio at such optimized value, we then evaluated the transfection efficiency of ternary complexes containing various PVBLG-7 contents. In the three cell lines that expressed mannose receptors, all the test ternary complexes exhibited significantly higher gene expression level than binary complexes, peaking at the PVBLG-8/PVBLG-7 weight ratios of 15/7, 15/3, 15/3 in HeLa, COS-7, and Raw 264.7 cells, respectively (Fig. 2.12A, B, C and D). Fluorescent images of HeLa cells also revealed higher percentage of GFP-positive cells and higher green fluorescence intensities for ternary complexes compared
to binary complexes (Fig. 2.12E). Such transfection results accorded well with the cell uptake level, again validating our design strategy to improve the transfection efficiency of PVBLG-8 via PVBLG-7-mediated cellular targeting. To further demonstrate the PVBLG-7-mediated mannose receptor targeting effect, we monitored the transfection efficiency of ternary complexes in the presence of free mannose. As shown in Fig. 2.13A B and C, transfection efficiencies of the ternary complexes were largely compromised in the presence of mannose, and when the mannose concentration was increased up to 800 μM (50 molar ratios to glucosamine groups in ternary complex, PVBLG-8/PVBLG-7/DNA = 15/7/1), the ternary complexes exhibited comparable transfection efficiency to binary complexes, suggesting that the PVBLG-7-mediated targeting effect was completely blocked. As a control, binary complexes deprived of the targeting moiety (PVBLG-7) were not influenced by free mannose in terms of transfection efficiency, which served as another evidence for the targeting effect of PVBLG-7.
Fig. 2.12 Ternary complexes mediate higher transfection efficiencies than binary complexes. (A) Transfection efficiency of ternary complexes in HeLa cells at different PVBLG-8/PVBLG-7 weight ratios (n=3). PVBLG-8/DNA weight ratio was kept constant at 15, and for the PVBLG-7/DNA binary complexes, the optimal PVBLG-7/DNA weight ratio of 20 was used. Ternary complexes transfect various mammalian cell lines expressing mannose receptors. Transfection efficiency of ternary complexes in COS-7 cells (B) and Raw 264.7 cells (C) at different PVBLG-8/PVBLG-7 weight ratios (n=3). PVBLG-8/DNA weight ratio was kept constant at 15. For the PVBLG-7/DNA binary complexes, the optimal PVBLG-7/DNA weight ratios of 15 and 20 were used for COS-7 and Raw 264.7 cells, respectively. (D) Representative flow cytometry spectra showing the transfection efficiencies of binary and ternary complexes in HeLa cells. (E) Fluorescent images of HeLa cells transfected with binary complexes (PVBLG-8/DNA weight ratio = 15) and ternary complexes (PVBLG-8/PVBLG-7/DNA weight ratio =
(Fig. 2.12 cont.) 15:7:1) (bar= 100 μm).

Fig. 2.13 (A) Transfection efficiency of binary and ternary complexes in HeLa cells in the presence of free mannose at different concentrations (n=3). In vitro transfection efficiency of ternary complexes (PVBLG-8/PVBLG-7/DNA weight ratio of 15/3/1, B) and binary complexes (PVBLG-8/DNA weight ratio of 15/1, C) in COS-7 and Raw 264.7 cells in the presence of free mannose at various concentrations (n=3).

2.3.7. Cytotoxicity

The cytotoxicity of complexes towards HeLa, COS-7, and Raw 264.7 cells were evaluated by the MTT assay following 24-h treatment. As shown in Fig. 2.14, ternary complexes exhibited a dose-dependent cytotoxicity comparable to that of the binary complexes. Such case verified that PVBLG-7 did not induce additional toxicities to the complexes and it can serve as a non-toxic targeting building block to potentiate the gene transfection. Compared to the free polymers at equivalent concentrations (Fig. 2.15), ternary complexes showed lower cytotoxicity, which was attributed to the partial neutralization of their positive charges by the anionic DNA molecule. More noteworthy
was that at the transfection dose (equal to 1.5 μg PVBLG-8/well), the ternary complexes showed low cytotoxicity (higher than 80% cell viability), which can ensure their safe application towards non-viral gene delivery.

Fig. 2.14 In vitro cytotoxicity of binary and ternary complexes in HeLa, COS-7, and Raw 264.7 cells following 24-h treatment as evaluated by the MTT assay (n=3). PVBLG-8/DNA weight ratio was kept constant at 15.

Fig. 2.15 Cytotoxicity of PVBLG-8/PVBLG-7 mixtures at different weight ratios in
(Fig. 2.15 cont.) HeLa, COS-7, and Raw 264.7 cells following 24-h treatment (n=3).

2.4. Discussion

Synthetic non-viral vectors (lipids and polycations) are widely recognized as promising vectors for gene delivery. However, the poor correlation between the delivery efficiency and safety profiles, especially with regard to chemotoxicity, renders the non-viral vectors with limited applications [10]. Vectors with high transfection efficiency often show high toxicity, while those with low toxicity frequently suffer from low transfection efficiency. One of the main reasons for such poor correlation is probably that different, even conflicting functionalities are required at different stages of the gene delivery processes. For instance, high content of amine moieties in the vectors plays important roles in overcoming the endosomal barrier via a combination of membrane destabilization and “proton-sponge” effect [41]. On the other hand, the cationic charges may induce nonspecific interactions with negatively charged serum components to form thrombi in the capillary, and they may perturb the integral structure of the plasma membranes to induce cytotoxicity or immune responses [42]. It is therefore of great demand to achieve a proper balance between efficiency and toxicity towards the design strategy of non-viral vectors.

Recently, there have been many reports in developing non-viral gene delivery vectors using a combinatorial/parallel synthesis approach to construct large libraries of materials with unique molecular structures. For instance, Anderson et al. [12] created a library of over two thousand unique molecules via Michael addition reactions between
varieties of diacrylates and amines. In a similar approach, Barua et al. [43] generated a library of eighty compounds via ring-opening polymerization of diglycidyl ethers by amines. Often, the only rational design in the library approach is the inclusion of cationic amines in the molecular building block that allows DNA condensation, and the success of it mainly relies on the screening process to identify leading materials with appropriate balance between gene transfection efficiencies and cytotoxicity. Despite the success, the library approach is labor intensive and leads to high cost. In comparison, rational modification of existing star materials to impart additional functionalities against the transfection barriers and reduce the cytotoxicity would display lower risk and higher likelihood of success. Multi-functionalization via multi-step conjugation is one of the most commonly used strategies, among which PEGylation of polycations serves as a practical solution to reduce the material toxicity by partly shielding the surface positive charges [17, 37]. However, significant reduction of the transfection efficiency is inevitable, mainly because of the reduced cellular uptake level and the impaired capacity to aid endosomal escape. Charge-conversion materials, which can undergo the charge conversion from positive to neutral/negative post-transfection, demonstrates a promising strategy in realizing the full potential of cationic materials while minimizing the cytotoxicity post-transfection [10, 44-46]. Despite the success of the afore-mentioned approaches, they all involve complex chemistries, which may raise difficulties in quality control and significant batch-to-batch variation. With diverse functionalities incorporated in a single material, it was also difficult to modulate the relative amount of each functional moiety towards the optimal combination.
In comparison to these reported approaches, we in the current contribution developed a membrane-permeable and mannose-receptor targeting ternary complex via a facial and modular self-assembly strategy. The complexes were based on effective cationic helical polypeptides we recently identified through a library approach [23]. PVBLG-8 was capable of inducing effective membrane destabilization/disruption, which can thus facilitate cellular internalization and endosomal escape to mediate effective gene transfection. Because excessive membrane activity of PVBLG-8 at high doses would cause irreversible cell damage, it is unlikely to strength its transfection capabilities by keeping increasing the PVBLG-8 amount. As such, PVBLG-7, an analogue to PVBLG-8 that bears glucosamine residues and displays minimal membrane activities, was introduced to the ternary complexes to allow mannose receptor-mediated cellular targeting and correspondingly potentiate the transfection efficiency of PVBLG-8. Such hypothesis was substantiated by the enhanced cellular uptake level and transfection efficiencies in various mannose receptor-expressing cell types; such effect was completely inhibited in the presence of free mannose that competitively occupied the mannose receptors, which further verified the targeting capability of PVBLG-7. The incorporation of PVBLG-7 did not compromise the membrane activity of PVBLG-8, and it did not bring additional cytotoxicity to PVBLG-8 due to its low membrane activity. These results collectively indicated that within the self-assembled ternary complexes, each individual building block worked synergistically and performed its intended roles without impairing the functionalities of remaining components. In a direct comparison, a random copolypeptide PVBLG-8-7 demonstrated decreased rather than increased transfection efficiency compared to PVBLG-8, which was attributed to the compromised
membrane permeability of the PVBLG-8 moiety by the PVBLG-7 segment. As such, the self-assembly approach adopted herein is advantageous over the strategy that diverse functionalities are assembled in a single polymer where they influence each other. Because of the simplicity and adjustability of the self-assembly approach, a proper balance between the membrane activity and targeting capability can also be easily determined by modulating the relative amount of each functionality, thus identifying a cell-line specific formulation for the ternary complexes towards maximized gene transfection efficiency yet minimized cytotoxicity.

During the design of the self-assembled complexes, the second component used together with PVBLG-8 should meet the following three requirements. First, it should have non-covalent interactions with PVBLG-8 or DNA, allowing it to be integrated into the PVBLG-8/DNA complexes. Second, it should display weak or minimal membrane activity so that it will not induce additional cytotoxicity. Third, it should carry a specific functionality that PVBLG-8 does not have, such as cell targeting or the nuclear transport capability. PVBLG-7 perfectly meets such requirements. It bears proper cationic charge densities so that it can co-condense DNA with PVBLG-8 to form stable ternary complexes; the low membrane activity of PVBLG-7 will not cause addition damage to the cell membranes. The glucosamine side chains on PVBLG-7 allow active targeting to mannose receptor expressing cells to potentiate the cellular internalization level. It is desired that PVBLG-7 should adopt α-helical conformation, because polypeptides with helical structure have stronger DNA binding affinity than the random-coiled analogues [46]. Our results showed that the optimal combination of membrane activity and targeting
capacity was different in various mannose receptor-expression cell types (HeLa, COS-7, and Raw 264.7). To this regard, the self-assembly approach was more effective and less labor-consuming than multi-step chemical reactions in terms of varying the relative amount of each functionality. As a result, the top-performing ternary complexes with maximal synergistic effect were identified in the three test cell types, which represented a dramatic improvement over the commercial reagent LPF. Using the self-assembly strategy, polypeptides with various side chains can also be incorporated to a single complex to exhibit multifunctionalities and thus overcome the multiple cellular barriers against transfection. For instance, polypeptides containing imidazole side groups can further promote endosomal escape; polypeptides containing SV40 and TAT side chains can potentially facilitate the nuclear targeting and transport; other targeting ligands (e.g., folate, transferrin, biotin) can also be used to target different cell types.

2.5. Conclusion

We demonstrated a convenient, flexible, and modular self-assembly approach in developing effective and safe non-viral gene delivery vectors, which was achieved by combining a highly membrane-penetrating while toxic cationic helical polypeptide (PVBLG-8) with a less cationic, relatively inert, and cell-targeting polypeptide (PVBLG-7). Within the PVBLG-8/PVBLG-7/DNA ternary complexes, the membrane activity of PVBLG-8 and the cellular targeting capability of PVBLG-7 worked synergistically without compromising each other. As such, a proper balance between the membrane activity and the targeting efficiency was easily identified in each certain cell type by modulating the relative amount of each individual component, and thus the gene
transfection efficiency of PVBLG-8 was maximized without inducing additional cytotoxicity. Such approach described here would serve as an effective tool in overcoming the efficiency-toxicity inconsistency of cationic non-viral gene delivery vectors; it is easy to handle, does not involve complex synthetic chemistry, and can be easily optimized by changing the building blocks (e.g. incorporation of another polypeptide bearing nuclear localization signals), which we believe will provide an important addition to the existing efforts in identifying cell-specific gene delivery vectors.

2.6. References


CHAPTER 3

POLYPEPTIDES MODIFIED WITH AROMATIC DOMAIN FOR IMPROVED CELL PENETRATION AND GENE DELIVERY

3.1. Introduction

Gene therapy is attracting more and more attentions for the treatment of various genetic diseases these days via the delivery of the genetic materials into the target cells to facilitate the expression or the knockdown of the specific gene [1-3]. Non-viral gene delivery vectors have emerged as promising alternatives to viral vectors because of their minimal immunogenicity, improved biocompatibility and chemical flexibility. However, their clinical applications have been hampered by their low transfection efficiency resulting from severe extracellular and intracellular barriers including the difficulty in binding to the cell surface, limited cell penetration level, endosome entrapment and associated toxicity caused by excessive charges [4-6].

Cationic polymers and lipids are the two major categories of non-viral gene delivery vectors [7, 8]. Polyplexes formed by cationic polymers and DNA are usually more stable than those formed by lipids [9]. Among various polycations, cationic polypeptides have attracted more and more attention because of their innate ability to adopt ordered conformations such as α-helices, which endows them with excellent membrane activities [10-15]. Compared with traditional cell penetration peptides (CPPs), cationic polypeptides with sufficient backbone length can independently condense and delivery genes due to their high molecular weights (MWs) and cationic charge density.
We recently developed a cationic polypeptide, poly (γ-(4-(((2-(piperidin-1-yl)ethyl) amino) methyl) benzyl-L-glutamate) (PVBLG-8), via a controlled ring-opening polymerization method and used PVBLG-8 or its analogues to achieve efficient gene and siRNA delivery [14]. PVBLG-8 adopted the stabilized helical structure, which contributed to its excellent membrane penetrating property via the pore formation mechanism. Besides that, the high molecular weight (MW) and sufficient cationic charge density allowed the condensation of genes, which rendered it a better gene delivery vector than traditional oligo-CPPs for gene delivery to mammalian cells [16, 17]. Even though PVBLG-8 has been demonstrated as desired gene delivery candidate due to its excellent membrane activity, its excessive positive charges and membrane permeability may lead to irreversible damage to cell membranes [16]. Thus the rational design and modification on the polypeptide structure and mechanistic study on the relationship between the structure and the property would optimize the gene delivery efficiency.

It is well known that CPPs adopt either inherent helical structures or form can helices after interacting the cell membranes. It typically contains a large number of Arginine (Arg) residues and the guanidine groups of the Arg residues are contributing to the penetration efficiencies, which is due to the interaction with the sulfate groups of glycosaminoglycans on cell membranes [18-20]. It is because the penetration efficiency of such CPPs can be self-activated by hydrophobic content, which is a rigid amphiphilic structure to interact with the lipid bilayers to promote membrane permeation and facilitate the membrane translocation. This provides the design strategy for the synthetic
polypeptides by increasing the hydrophobicity of the side chains or the backbone of polymers to promote their interaction with phospholipids [10, 21-25]. Among various kinds of the hydrophobic contents, aromatic groups have been reported to perform superior cell penetration and self-activation property than aliphatic groups when interacting with the cell membrane [26]. It has been demonstrated that the aromatic groups have more favorable free energy of insertion into the phospholipids [27, 28]. In addition, it has also been reported that the translocation of traditional CPPs is closely related with aromatic domain, such as that the incorporation of tryptophan residue will promote the cell internalization via an energy-independent non-endocytosis pathway [18, 26, 29, 30]. Motivated by these understandings, we developed a new series of cationic, a-helical copolypeptides by incorporating various kinds of aromatic domain to the side chains of PVBLG-8, attempting to elucidate the effect of the aromatic domain modification of polypeptide structure and maximize the cell penetration and gene delivery efficiency. The gene delivery efficiency of the copolypeptides modified by aromatic domain would be firstly explored in HeLa and B16F10 cell lines for the screening of the top performing material in the library and optimization of the transfection conditions. Then the in vivo gene transfection efficiency would be demonstrated by delivering the genes into the murine melanoma tumor models from via both topical and systemic delivery method.

### 3.2. Materials and Methods

#### 3.2.1. Materials and cell lines
All chemicals were purchased from Sigma-Aldrich (St. Louis, MO) and used as received unless otherwise specified. Anhydrous tetrahydrofuran (THF), hexane, and dimethylformamide (DMF) were dried by a column packed with 4Å molecular sieves and stored in a glovebox. Dry nitrobenzene (NB) was prepared by treating regular NB with CaH₂ followed by distillation under reduced pressure. Hexamethyldisilazane (HMDS) and 1, 5, 7-triazabicyclo [4.4.0] dec-5-ene (TBD) were used for controlled ring-opening polymerization of amino acid N-carboxyanhydrides developed by us. γ-(4-Vinylbenzyl)-l-glutamate N-carboxyanhydride (VB-l-Glu-NCA) was prepared as previously reported [4, 14]. Pierce BCA assay kit was purchased from ThermoFisher Scientific (Rockford, IL, USA). Plasmid DNA (pDNA) encoding luciferase (pCMV-Luc) was purchased from Elim Biopharm (Hayward, CA, USA). 3-(4, 5-dimethylthiazol-2-yl)-2, 5- diphenyl-2H-tetrazolium bromide (MTT), and YOYO-1 were purchased from Invitrogen (Carlsbad, CA, USA). Pierce BCA assay kit was purchased from ThermoFisher Scientific (Rockford, IL, USA).

HeLa (human cervix adenocarcinoma cells) and B16F10 (murine melanoma cells) were purchased from the American Type Culture Collection (Rockville, MD, USA) and were cultured in Dulbecco’s Modified Eagle Medium (DMEM) (Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin.

Female C57BL/6 mice (8-10 week old) were obtained from Charles River Laboratory (Wilmington, MA, USA) and were housed in a germ-free environment with four mice per cage. Mice were given access to food and water and exposed to a 12:12 h
light–dark cycle (7:00 am–7:00 pm) at 25 ± 1 °C. The animal experimental protocol was approved by the Institutional Animal Care and Use Committees (IACUC) of University of Illinois at Urbana–Champaign.

3.2.2. Instrumentation

1H NMR spectra were recorded on a Varian U500 (500 MHz) spectrometer. Electrospray Ionization mass spectrometry (ESI-MS) was performed on a Waters Quattro II Mass Spectrometer.

Tandem gel permeation chromatography (GPC) experiments were performed on a system equipped with an isocratic pump (Model 1100, Agilent Technology, Santa Clara, CA, USA), a DAWN HELEOS 18-angle laser light scattering detector (also known as multi-angle laser light scattering (MALLS) detector, Wyatt Technology, Santa Barbara, CA, USA) and an Optilab rEX refractive index detector (Wyatt Technology, Santa Barbara, CA, USA). The detection wavelength of HELEOS was set at 658 nm. Separations were performed using serially connected size exclusion columns (100 Å, 500 Å, 103 Å and 104 Å Phenogel columns, 5 μm, 300 × 7.8 mm, Phenomenex, Torrance, CA, USA) at 60 °C using DMF containing 0.1 M LiBr as the mobile phase. The MALLS detector was calibrated using pure toluene with no need for external polymer standards and can be used for the determination of the absolute molecular weights. The molecular weights (MWs) of all polymers were determined based on the dn/dc value of each sample calculated offline by using the internal calibration system processed by the ASTRA V software (version 5.1.7.3, Wyatt Technology, Santa Barbara, CA, USA).
Circular dichroism (CD) measurements were carried out on a JASCO J-700 CD spectrometer (Oklahoma City, OK, USA). The polymer samples were typically prepared at concentrations of 0.1 mg/mL for CD analysis unless otherwise specified. The solution was placed in a quartz cell with a path length of 0.1 cm. The mean residue molar ellipticity of each polypeptide was calculated based on the measured apparent ellipticity according to the reported formulas: Ellipticity ($[\theta]$ in deg cm$^2$ dmol$^{-1}$) = (millidegrees $\times$ mean residue weight)/(path length in millimeters $\times$ concentration of polypeptide in mg ml$^{-1}$). The helicity of the polypeptides was calculated using the following equation:

$$\text{helicity} = (\frac{-[\theta_{222}] + 3,000}{39,000}) [15].$$

Lyophilization was performed on a FreeZone lyophilizer (Labconco, Kansas City, MO, USA).

3.2.3. Synthesis of Naphthalene-L-Glu-NCA

N, N, N’, N’-tetramethylguanidine (8.5 mL, 67.75 mmol) was added slowly to a stirred mixture of L-glutamic acid (5.00 g, 33.98 mmol), L-glutamic acid copper (II) complex copper (II) salt (8.63 g, 16.82 mmol), DMF (32 mL) and water (4.75 mL). 1-(Chloromethyl)naphthalene (10 mL, 70.96 mmol) was added in one portion, the mixture was heated at 40 Celsius for 24 h. Acetone (300 mL) was then added and stirred for 20 h to get a fine precipitate. The solids were collected by suction and washed by acetone. The solids were then added into a newly made EDTA disodium salt solution and stirred for 20 h. The product was then collected by suction and washed by water. Isopropanol-water mixture (50 mL, 1:1, v/v) was then added to remove copper complex for 3 times. The isopropanol was removed under vacuum and the water was removed via lyophilization to
obtain white product naph-L- glu (4g). In a dried 250 mL two-neck round bottom flask, naph-L-glu (1 g) was added and dried under vacuum for 2 h. Anhydrous THF (30 mL) was added to the flask, along with phosgene (10 % Toluene Solution, 3 mL), the mixture was kept stirring at 50 Celsius for 2 h under the protection of drying tube and aluminum foil. Most THF was then removed under vacuum to obtain a yellow solid. The solid was dissolved with THF and centrifuged to remove the insoluble, then recrystallized with THF-hexane mixture (hexane: THF = 5:1, v/v) three times in glove-box.

3.2.4. Synthesis of Anthracene-L-Glu-NCA

N, N, N’, N’-tetramethylguanidine (8.5 mL, 67.75 mmol) was added slowly to a stirred mixture of L-glutamic acid (5.00 g, 33.98 mmol), L-glutamic acid copper (II) complex copper (II) salt (8.63 g, 16.82 mmol), DMF (32 mL) and water (4.75 mL). 9-(Chloromethyl)anthracene (10 mL, 70.96 mmol) was added in one portion, the mixture was heated at 40 Celsius for 24 h. Acetone (300 mL) was then added and stirred for 20 h to get a fine precipitate. The solids were collected by suction and washed by acetone. The solids were then added into a newly made EDTA disodium salt solution and stirred for 20 h. The product was then collected by suction and washed by water. Isopropanol-water mixture (50 mL, 1:1, v/v) was then added to remove copper complex for 3 times. The isopropanol was removed under vacuum and the water was removed via lyophilization to obtain yellow product anth-L-glu (3.2g). In a dried 250 mL two-neck round bottom flask, anth-L-glu (1 g) was added and dried under vacuum for 2 h. Anhydrous THF (30 mL) was added to the flask, along with phosgene (10 % Toluene Solution, 3 mL), the mixture was kept stirring at 50 Celsius for 2 h under the protection of drying tube and aluminum foil.
foil. Most THF was then removed under vacuum to obtain a yellow solid. The solid was dissolved with THF and centrifuged to remove the insoluble, then recrystallized with THF-hexane mixture (hexane: THF = 5:1, v/v) three times in glove-box.

3.2.5. Synthesis of P0

VB-L-Glu-NCA (58 mg, 0.2 mmol) was dissolved in a mixture of DMF (0.9 mL) and nitrobenzene (30 μL) in a glove box, followed by addition of HMDS (13.3 μL, 0.1 M, M/I=150) and TBD solution (13.3 μL, 0.01 M) in DMF. FTIR was used to monitor the polymerization until the conversion reached 99% (within 48 hours) to obtain poly (γ-(4-vinylbenzyl)-L-glutamate) (PVBLG). Tetrabutylammonium fluoride solution (100 μL, 1 M), benzyl chloroformate (50 μL), and N,N-diisopropylethylamine (DIEA, 50 μL) were added and stirred for 3 h to cleave the N-Si bond and protect the amino end groups. DMF was removed under vacuum, and the resulting polymer was precipitated from cold ethyl ether (45 mL), washed with cold ethyl ether (45 mL × 3), and collected by centrifugation at 4000 rpm. PVBLG was then dissolved in chloroform (30 mL) and oxidized by O3 at -78 °C. Dimethyl sulfide (1 mL) was added and the solution was stirred at RT overnight before the solvent was removed under vacuum. The product poly (γ-(4-aldehydebenzyl-L-glutamate) (PABLG) was washed with methanol (45 mL). The obtained PABLG (30 mg) was dissolved in DMF (2 mL), into which 1-(2-aminoethyl) piperidine (150 μL, 10 molar equivalents relative to the Glu repeating unit) was added. After reaction at 50 °C for 24 h, borane pyridine as the reducing agent (133 μL, 10 molar equivalents relative to the Glu repeating unit) was added, and the solution was further stirred at 50 °C for 24 h. HCl (5
M, 1 mL) was added to protonate the amine groups, and the final product P0 was dialyzed against water (MWCO = 1 kDa) and lyophilized.

3.2.6. Synthesis of P1 to P8

VB-glu-NCA and Naph-glu-NCA at determined molar ratios were dissolved in DMF (1.0 mL) and Nitrobenzene (30 μL), followed by adding HMDS and TBD. After the completion of the polymerization, the solution was then taken out of glove-box and precipitated by methanol. Diisopropylethylamine and CbzCl was added to cleavage the N-Si bond and protect the amino end groups. The polymer was precipitated using hexane/ether (1/1) and collected by centrifuge.

PNLG-r-PABL copolymer was dissolved in chloroform (30 mL) at -78 Celsius. O2 was bubbled into the solution for 1 min followed by bubbling of O3 until the solution became blue indicating the reaction was completed. O3 was then replaced by O2, which was bubbled into the solution for another 2 min until the solution became colorless. The solution was then degassed and backfilled with nitrogen. Dimethyl sulfide was then added to the mixture. The solution was stirred at room temperature overnight and solvent was removed under vacuum. The product was purified by stirring in methanol. The resulting polymer was reacted with 1-(2-aminoethyl)piperidine in DMF (2 mL) at 50 Celsius for 24 h. Borane-pyridine was then added to the solution and stirred for another 24 h.

3M HCl (1 mL) was added to the solution and kept stirring for 10 min. The
polymer was then purified by dialysis against DI water in a dialysis bag with a molecular weight cut-off (MWCO) of 1000 (3 days, DI water changes every 12 h). The final solution was lyophilized for 2 days to obtain the polymer P1. P2 to P8 were synthesized by the similar method.

3.2.7. Synthesis of Rhodamine labeled P0 to P8

Rhodamine isothiocyanate and ethylenediamine were dissolved in DMF at the molar ratio of 1:20. TEA was added (5 molar equivalents to the amine group), and the reaction was allowed to proceed at RT for 24 h from light. Residual ethylenediamine and DMF were removed by vacuum-drying, and the polymer was washed 3 times with ether to completely get rid of residual ethylenediamine, thus achieving the RhB-NH2. PABLG, PNLG-PABLG, PALG-PABLG and PLeu-PABLG (50 mg) were dissolved in 2 mL DMF, into which a mixture of RhB-NH2 and 1-(2-Aminoethyl) piperidine (molar ratio 1:20) was added. Reaction was allowed at 50 °C for 24 h, borane pyridine as the reducing agent (5-10 molar equivalents) was added, and the resulting solutions was further stirred at 50 °C for 24 h. 3 M HCl (3 mL) was then added to protonate the amine groups, and the soluble RhB-P0 to RhB-P8 were dialyzed against water before lyophilization.

3.2.8. Formulation and characterization of polypeptides and plasmid DNA complexes

Polypeptides and plasmid DNA were dissolved in deionized water at 1 mg/mL. Complexes were formed by adding polypeptides into DNA solutions at various N/P ratios followed by vortex for 30 sec and incubation at RT for 20 min. Particle size and zeta
potential of complexes at various N/P ratios were evaluated by dynamic laser scanning (DLS) on a Malvern Zetasizer (Herrenberg, Germany).

The DNA condensation by cationic polypeptides were evaluated by gel retardation assay. Freshly prepared complexes were loaded on a 1% agarose gel at 100 ng DNA/well followed by electrophoresis at 100 V for 30 min. Naked DNA was used as a control, and DNA migration in the agarose gel was visualized by a Gel Doc imaging system (Biorad, Herclues, CA, USA) following staining with ethidium bromide (EB). To quantitatively evaluate the DNA condensation level, EB exclusion assay was performed as follows [12]. DNA was first stained with EB at the DNA/EB weight ratio of 10 at RT for 1 h. Polypeptides solutions were added into the DNA/EB solutions followed by further incubation at RT for 30 min before quantification of the fluorescence intensity ($\lambda_{ex}$ = 510 nm, $\lambda_{em}$ = 590 nm). The DNA condensation efficiency (%) was calculated according to the following equation:

$$\text{DNA condensation efficiency} \% = (1 - \frac{F}{F_0}) \times 100$$

Where $F_{EB}$, $F$, and $F_0$ denote the fluorescence intensity of pure EB solution, DNA/EB solution with polypeptide, and DNA/EB solution without any polypeptide, respectively.

3.2.9. Cell penetration of cationic copolyepptides

Cells were seeded on 96-well plates at $1 \times 10^4$ cells/well and cultured for 24 h or 48 h before they reached confluence. The medium was refreshed with serum-free
DMEM, and RhB-P0 to RhB-P8 were added 2 μg/well. After incubation at 37 °C for 4 h, the cells were washed with cold PBS containing 20 U/mL heparin for 3 times, which could completely remove surface-bound cationic proteins from cells. Cells were then lysed with RIPA lysis buffer at RT for 20 min before assessment of fluorescent content by spectrofluorimetry ($\lambda_{\text{ex}} = 540$ nm, $\lambda_{\text{em}} = 625$ nm) and protein level using the BCA kit. Uptake level was expressed as μg Rhodamine labeled polypeptides associated with 1 mg of cellular protein.

3.2.10. Cell uptake of polypeptides/DNA complexes

The intercalating dye YOYO-1 (20 μM) was first mixed with plasmid DNA at one dye molecule per 50 bp DNA labeled [31]. The labeled DNA was incubated at room temperature for 20 minutes, followed by the addition of different polypeptides to form the complexes. Cells were seeded on 24-well plates at $5 \times 10^4$ cells/well 24 h prior to uptake until they reached confluence. The cell culture medium was refreshed with serum-free DMEM (500 μL/well) and the complexes were added to each well (0.5 μg DNA/well). After incubation at 37 °C for 4 h the cells were washed with PBS containing heparin (20 U/mL) for 3 times to remove the surface-bound cationic complexes from cells. Cells were then lysed with RIPA lysis buffer (500 μL/well) at room temperature for 20 minutes shaking before assessment of YOYO-1-DNA content using spectrofluorimetry ($\lambda_{\text{ex}} = 485$ nm, $\lambda_{\text{em}} = 530$ nm) and protein level using BCA kit. Uptake level was expressed as ng DNA associated with 1 mg protein.

3.2.11. Cell penetration and DNA internalization mechanisms
Cells were seeded on 96-well plates at $1\times10^4$ cells/well and cultured for 24 h or 48 h before they reached confluence. The medium was refreshed with serum-free DMEM, followed by the addition of polypeptides or complexes as described above. After incubation at 37 °C and 4 °C for 2 h the cells were washed with PBS containing heparin (20 U/mL) for 3 times to remove the surface-bound cationic complexes from cells. Cells were then lysed with RIPA lysis buffer (500 μL/well) at room temperature for 20 minutes shaking before assessment of RhB labeled polypeptides ($\lambda_{ex} = 540$ nm, $\lambda_{em} = 610$ nm) and YOYO-1-DNA content using spectrofluorimetry ($\lambda_{ex} = 485$ nm, $\lambda_{em} = 530$ nm) and protein level using BCA kit. Results were expressed as percentage of control cells where incubated at 37 °C.

Cells were seeded on 96-well plates at $1\times10^4$ cells/well and cultured for 24 h or 48 h before they reached confluence. The medium was refreshed with serum-free DMEM containing different endocytosis inhibitors as methyl-β-cyclodextrin (mβCD, 5 mM), wortmannin (10 μg/mL) and chlorpromazine (10 μg/mL) for 30 minutes prior to polypeptide application and throughout the 2 h uptake experiment at 37 °C, followed by the addition of polypeptides or complexes as described above.

3.2.12. Membrane activity

The capability of polypeptides to induce membrane disruption was evaluated in terms of the cell uptake level of a hydrophilic, membrane-impermeable dye, fluorescein isothiocyanate (FITC) in its non-reactive form (fluorescein-tris (hydroxymethyl) methanethioureia, FITC-Tris) [32]. Briefly, cells were seeded on 96-well plates at $1\times10^4$
cells/well and cultured for 24 h. The medium was replaced with Opti-MEM (100 μL/well), into which polypeptides and FITC-Tris were added at 2 μg/well and 0.2 μg /well, respectively. Free FITC-Tris without polypeptides was added as a control. After incubation at 37 °C for 2 h, cells were washed with PBS containing heparin (20 U/mL) for 3 times and then lysed with the RIPA lysis buffer (100 μL/well). The FITC-Tris content in the lysate was quantified using spectrofluorimetry (λ_ex = 485 nm, λ_em = 530 nm) and the protein level was determined using the BCA kit. Uptake level was expressed as ng FITC-Tris associated with 1 mg cellular protein.

3.2.13. CLSM images

To visualize the intracellular delivery of polypeptide/DNA complexes, HeLa cells were incubated in serum-free DMEM in a 6-well plate with P0/DNA and P3/DNA complexes at 1 μg DNA/well. Following incubation for 4 hours, cells were then washed with cold PBS, fixed with 4% paraformaldehyde, stained with DAPI (2 μg/mL), and subjected to observation using CLSM (LSM700, Zeiss).


Cells were seeded on 96-well plates at 1×10^4 cells/well and incubated for 24 h prior to transfection studies. The medium was replaced by Opti-MEM or serum containing DMEM, into which polypeptide/DNA complexes at various weight ratios were added at 0.1 μg DNA/well. After incubation for 4 h, the complexes were removed and fresh media were added. Cells were further cultured for 20 h, following by determination of luciferase expression using a Bright-Glo Luciferase assay kit and
cellular protein level using a BCA kit. Results were expressed as relative luminescence unit (RLU) associated with 1 mg of protein.

3.2.15. Cytotoxicity measurements

Cells were seeded on 96-well plates at $1 \times 10^4$ cells/well and cultured until they reached confluence. The medium was replaced with serum-free DMEM (100μL/well), into which the polypeptide itself and polypeptide/DNA complexes were added at different polymer concentrations (100, 50, 20, 10 μg/mL). After incubation at 37 °C for 4 hours, the medium was refreshed with serum-containing media and future cultured for another 20 hours before testing cell viability using the MTT assay. The cytotoxicity results were expressed as percentage viable cells of control cells.

3.2.16. In vivo transfection

B16F10 cells were prepared as single cell suspension, 50 μl of $2 \times 10^6$ cells in PBS was injected subcutaneously into mice. Tumor size was measured using a vernier caliper. When the tumor sizes reached 100 mm$^3$, mice were randomly divided into six groups to minimize the differences of body weights and tumor sizes among groups (N = 4). The complexes were prepared in HEPES buffered glucose (20mM HEPES, pH = 7.2, 5% glucose) by adding polypeptides into plasmid DNA at the weight ratio of 10. In each group, complexes containing a dosage of 10 μg pCMV-Luc plasmid DNA was injected intratumorally three times every four hour. At 48 h time point post the last injection, mice were sacrificed. Tumor tissues were taken out and homogenized in Promega cell lysis buffer. The supernatant was collected via centrifugation (12,000 g/min) at 4 °C for the
quantification of luciferase activity and protein content. The transfection efficiency was expressed as RLU per mg protein.

Mice bearing B16F10 melanoma tumors were randomly divided into five groups to minimize the differences of body weights and tumor sizes among groups (N = 4). The complexes were prepared in HEPES buffered glucose (20mM HEPES, pH = 7.2, 5% glucose) by adding polypeptides into plasmid DNA at the weight ratio of 20. PEG-PLG was then added at the PEG-PLG/DNA weight ratio of 10 to form the complexes with coating. In each group, complexes containing a dosage of 20 µg pCMV-Luc plasmid DNA was injected intravenously. At 24 h time point post the injection, mice were sacrificed and tumor tissues were taken out and homogenized in Promega cell lysis buffer. The supernatant was collected via centrifugation (12,000 g/min) at 4 °C for the quantification of luciferase activity and protein content. The transfection efficiency was expressed as RLU per mg protein.

3.2.17. Statistical analysis

Statistical analysis was performed using Student’s t-test and differences were judged to be significant at *p<0.05 and highly significant at **p<0.01.

3.3. Results and Discussion

3.3.1 Synthesis and characterization of the polypeptides

P0 was synthesized via a controlled ring-opening polymerization of VB-L-Glu NCA following side chains amination. All of the random copolypeptides were synthesized via a controlled ring-opening co-polymerization of VB-L-Glu NCA and
hydrophobic domain modified NCA (Scheme 3.3). The NCA modified with hydrophobic domain was synthesized via monoesterification of glutamate (Scheme 3.1) and subsequent ring close reaction with phosgene (Scheme 3.2). By introducing various categories and amount of hydrophobic groups including benzene, naphthalene, anthracene and isopropyl groups into PVBLG-8 with the chain length ratio of 1:4 and 1:1 (hydrophobic chain length: positive charge chain length), we could develop a library of copolypeptides with various charge density and hydrophobic domains, named as P0 to P8 (Table 1). All the polypeptides were characterized by GPC and NMR to determine the final structure and summarized in Table 2. All the polypeptides were soluble in water and adopted typical α-helical secondary structures as indicated by the characteristic two-minimum ellipticity at 208 nm and 222 nm (Fig.3.1). The helicity of polypeptides were calculated by the following equation: helicity = (−[θ]_{222} + 3,000)/39,000, where [θ]_{222} is the mean residue ellipticity at 222 nm. All the copolypeptides exhibited relatively high helicity (65%-95%) in DI water. The introduction of aromatic groups leads to the increase of hydrophobicity as well as steric effect, which slightly affect the helicity of polypeptides.
Scheme 3.1 Synthetic route of (A) Naph-L-Glu and (B) Anth-L-Glu.
Scheme 3.2 Synthetic route of (A) Naph-L-Glu NCA monomer, (B) Anth-L-Glu NCA monomer and (C) Leu-L-NCA monomer.
Scheme 3.3 Synthetic route of copolypeptides modified with aromatic domain.

Table 1. Structures of polypeptides modified with aromatic domain

<table>
<thead>
<tr>
<th>Name</th>
<th>Structure</th>
<th>R</th>
<th>x</th>
<th>y</th>
</tr>
</thead>
<tbody>
<tr>
<td>P0</td>
<td>PVBLG-8</td>
<td>-</td>
<td>0</td>
<td>50</td>
</tr>
<tr>
<td>P1</td>
<td>PBLG_{10}-PVBLG-8_{40}</td>
<td>Benzene</td>
<td>10</td>
<td>40</td>
</tr>
<tr>
<td>P2</td>
<td>PBLG_{25}-PVBLG-8_{25}</td>
<td>Benzene</td>
<td>25</td>
<td>25</td>
</tr>
<tr>
<td>P3</td>
<td>PNLG_{10}-PVBLG-8_{40}</td>
<td>Naphthalene</td>
<td>10</td>
<td>40</td>
</tr>
<tr>
<td>P4</td>
<td>PNLG_{25}-PVBLG-8_{25}</td>
<td>Naphthalene</td>
<td>25</td>
<td>25</td>
</tr>
<tr>
<td>P5</td>
<td>PALG_{10}-PVBLG-8_{40}</td>
<td>Anthracene</td>
<td>10</td>
<td>40</td>
</tr>
<tr>
<td>P6</td>
<td>PALG_{25}-PVBLG-8_{25}</td>
<td>Anthracene</td>
<td>25</td>
<td>25</td>
</tr>
<tr>
<td>P7</td>
<td>PLLeu_{10}-PVBLG-8_{40}</td>
<td>Isopropyl</td>
<td>10</td>
<td>40</td>
</tr>
<tr>
<td>P8</td>
<td>PLLeu_{25}-PVBLG-8_{25}</td>
<td>Isopropyl</td>
<td>25</td>
<td>25</td>
</tr>
</tbody>
</table>
Table 2. Properties of polypeptides modified with aromatic domain

<table>
<thead>
<tr>
<th></th>
<th>M/I[a]</th>
<th>Mn (Mn*)×10^3[b,c]</th>
<th>PDI[c]</th>
<th>Composition[d]</th>
</tr>
</thead>
<tbody>
<tr>
<td>P0</td>
<td>(50+0)/1</td>
<td>25.0 (23.9)</td>
<td>1.13</td>
<td>PVBLG-852</td>
</tr>
<tr>
<td>P1</td>
<td>(10+40)/1</td>
<td>22.6 (21.4)</td>
<td>1.08</td>
<td>PBLG25-PVBLG-843</td>
</tr>
<tr>
<td>P2</td>
<td>(25+25)/1</td>
<td>17.3 (17.8)</td>
<td>1.10</td>
<td>PBLG25-PVBLG-824</td>
</tr>
<tr>
<td>P3</td>
<td>(10+40)/1</td>
<td>25.4 (21.9)</td>
<td>1.06</td>
<td>PNGL12-PVBLG-846</td>
</tr>
<tr>
<td>P4</td>
<td>(25+25)/1</td>
<td>19.0 (19.0)</td>
<td>1.17</td>
<td>PNGL25-PVBLG-825</td>
</tr>
<tr>
<td>P5</td>
<td>(10+40)/1</td>
<td>21.2 (22.4)</td>
<td>1.20</td>
<td>PALG2-PVBLG-838</td>
</tr>
<tr>
<td>P6</td>
<td>(25+25)/1</td>
<td>20.9 (20.3)</td>
<td>1.09</td>
<td>PALG25-PVBLG-827</td>
</tr>
<tr>
<td>P7</td>
<td>(10+40)/1</td>
<td>23.5 (20.7)</td>
<td>1.12</td>
<td>PLLLeu13-PVBLG-815</td>
</tr>
<tr>
<td>P8</td>
<td>(25+25)/1</td>
<td>14.1 (15.8)</td>
<td>1.07</td>
<td>PLLLeu26-PVBLG-811</td>
</tr>
</tbody>
</table>

[a] Feed ratio of (NCA containing hydrophobic domains + VB-L-Glu-NCA)/HMDS.

[b] Obtained Mw for P0 to P8 (expected Mw *).

[c] Obtained Mw and PDI were determined by GPC.

[d] The composition was determined by ^1H NMR spectroscopy.

![Fig. 3.1 CD spectrum of P0 to P8 in DI water (0.1 mg/mL) at pH 7.](image)

3.3.2 Cell penetration ability mediated by cationic α-helical polypeptides

To evaluate the cell penetration property of copolypeptides, rhodamine labeled polypeptides were prepared by conjugating rhodamine-NH₂ to the aldehyde groups on the
side chains and incubating with cultured HeLa cells in serum-free media at both 37 °C for 2 h. As a screen for cell penetration level, the content of internalized polypeptides were quantified and normalized using fluorescence spectrometer. As shown in Fig.3.2A, compared with P0 (homopolypeptide), all the synthesized copolypeptides with hydrophobic domain modification revealed notably increase in cell penetration by up to three folds. It has also been demonstrated that aromatic domain outperformed aliphatic ones in cell penetration via the comparison between P1 to P6 (aromatic functionalized copolypeptides) and P7 to P8 (aliphatic functionalized copolypeptides), which is mainly due that the aromatic residues have more favorable free energies of internalization into the cell bilayer membrane. Among all the copolypeptides, P3, with the incorporation of naphthalene with 20% (percentage of chains containing naphthalene groups), exhibit top performance with largest internalization amount, revealing that the substitution of positive charges for aromatic domain will allow significant promotion in mediating enhanced cell penetrating property and the substitution groups and ratios need optimizations to maximize the cell penetration property.

To explore the mechanism involved in the cell penetration process, HeLa cells were incubated with P0 to P8 at 4 °C, which the endocytosis energy was blocked. Compared with the cell penetration level at normal temperature, homopolypeptides (P0) and copolypeptides (P1 to P6) revealed definitely different story. As shown in Fig. 3.2, significant inhibition (61%) was observed for P0 when incubating with cells at low temperature, indicating that the endocytosis was the major mechanism in mediating the cellular internalization. However, most of the cell penetration level of P1 to P8
maintained when blocking endocytosis energy, demonstrating that the major mechanism for copolypeptides to enter cells was energy-independent non-endocytosis.

Fig. 3.2 Cell penetration level of RhB-P0 to RhB-P8 in HeLa cells following incubation for 2 h at 4 °C and 37 °C (n=3).

To further demonstrate the detailed endocytosis mechanisms involved in the cell penetration process, we mechanismically probed the intracellular kinetics. HeLa cells were incubated with P0 to P8 in the presence of various endocytic inhibitors. mβCD inhibited caveolae by depleting cholesterol; chlorpromazine inhibited clathrin-mediated endocytosis (CME) by triggering the dissociation of the clathrin lattice; wortmannin inhibited macropinocytosis by suppressing phosphatidyl inositol-3-phosphat. As shown in Fig. 3.3A, B&C, the cell penetration level of P0 was significantly inhibited by mβCD, chlorpromazine and wortmannin, however, only wortmannin could slightly inhibit the cell penetration level of the polypeptides modified with hydrophobic domain. The results indicated that the caveolae-, clathrin- and macropinocytosis mediated pathway were
involved during endocytosis of the homopolypeptide, P0. However, for the copolypeptides modified with hydrophobic domain, only wortmannin exerted inhibitory effect.

Fig. 3.3 Cell penetration level of polypeptides in the presence of various endocytic inhibitors such as (A) mβCD (B) chlorpromazine and (C) wortmannin (n=3).

Apart from the endocytosis pathway, we also probed the ability of the polypeptides to induce pore formation on the cell membranes, which is an important non-endocytosis mechanism mediated by cationic helical polypeptides. We investigated the membrane activity of all the synthesized polypeptides using fluorescein isothiocyanate/tris (hydroxymethyl) methane methanethioureia (FITC-Tris), which is a
membrane-impermeable dye (nonreactive after reacting with Tris), as a marker to evaluate pore formation level. All the copolypeptides with the positive charges substituted by aromatic domain exhibited reduced FITC-Tris uptake level compared with positive charges non substituted P0, revealing that the reduced charge density induce decreased pore formation level (Fig. 3.4). It could be preliminarily hypothesized that the aromatic domain functionalized polypeptides outperformed homopolypeptides with significantly increased cell penetration level and reduced endocytosis and pore formation, which is presumably because the direct membrane translocation without generating pores mechanism contributed by the aromatic domain mediate most of the cellular internalization level.

![Graph showing FITC-Tris uptake level in HeLa cells following co-incubation with polypeptides for 2 h at 37 °C (n=3).](image)

Fig. 3.4 FITC-Tris uptake level in HeLa cells following co-incubation with polypeptides for 2 h at 37 °C (n=3).

To further demonstrate the hypothesis, P0 (DL) and P3 (DL), the random coil analogues of P0 and P3 were prepared by the racemic D,L-NCA. It notably showed that
the random coiled polypeptides exhibited little pore formation ability (Fig. 3.5C) due to
the limited helicity (Fig. 3.5A). By comparing the cell penetration ability of P0 (DL) and
P3 (DL) at the reduced temperature, we thus noticed that the random coiled P3 showed
notably higher cell penetration level (Fig. 3.5B), which indicated that besides endocytosis
and pore formation, there existed another non-endocytic and non-pore formation
mechanism mediating the copolypeptides entering the cells.

Fig. 3.5 (A) CD spectrum of P0 and P3 in both helical and random coiled type in DI
water (0.1 mg/mL) at pH 7. (B) Cell penetration level of RhB-P0 and RhB-P3 in both
helical and random coiled type at both 37 °C and 4 °C (n=3). (H) FITC-Tris uptake level
in HeLa cells following co-incubation with P0 to P3 in both helical and random coiled
type for 2 h at 37 °C (n=3).

3.3.3 Characterization of the complexes formed by the DNA and polypeptides

To develop this library of copolypeptides as potentially efficient gene delivery
vectors, plasmid DNA encoding luciferase (pCMV-Luc) was used to form a series of
polypeptide/DNA complexes (Scheme 3.4). Gel retardation assay indicated retarded DNA
migration in the 4% agarose gel for all the complexes formed by polypeptides and DNA, meaning that DNA could be effectively condensed by cationic polypeptides due to their polycationic properties (Fig. 3.6). As the Fig.3.7 shown, higher than 88% of the DNA would be condensed into the complexes when the weight ratios of the polypeptide to DNA higher than 16. All the polypeptides thus were able to condense plasmid DNA to form the 160-nm polyplexes with the positive surface charges at the weight ratio higher than 8 (Fig.3.8).

Scheme 3.4. Illustration of cellular internalization mechanisms of polypeptide/DNA complexes.
Fig. 3.6 DNA condensation by P0 to P8 at different polypeptide/DNA weight ratios as evaluated by the gel retardation assay. N represents naked DNA.

Fig. 3.7 EB exclusion assay showing the condensation of DNA in the complexes at various weight ratios (n=3).
3.3.4 Cell uptake and intracellular mechanism

The ability of the copolypeptides to deliver DNA into the cells were evaluated and compared with the homopolypeptide. Similar to the results of cell penetration level, the incorporation of hydrophobic domain promoted the DNA uptake level by 2-3 folds. P3/DNA complexes remarkably exhibited the highest internalization level of YOYO-1-DNA (Fig. 3.9). Similar to the results of cell penetration mechanism, the DNA uptake inhibition level at the low temperature exhibited the similar trend. The cell uptake results at low temperature revealed that complexes formed with copolypeptides functionalized
with aromatic domain could partially bypass endocytosis pathway compared with homopolypeptides (Fig.3.9), indicating promising potential to overcome endosomal entrapment, which is the most severe gene delivery obstacles. These results successfully validated the proposed hypothesis that the polypeptides modified with aromatic groups will mediate effective cell internalization and DNA uptake.

Fig. 3.9 Uptake level of complexes containing YOYO-1 DNA in HeLa cells following incubation for 4 h at 4 °C and 37 °C (n=3).

<table>
<thead>
<tr>
<th>Name</th>
<th>vs P0</th>
<th>Inhibition percentage at low temperature(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P0</td>
<td>1</td>
<td>71.4</td>
</tr>
<tr>
<td>P1</td>
<td>1.4</td>
<td>45.4</td>
</tr>
<tr>
<td>P2</td>
<td>1.2</td>
<td>51.1</td>
</tr>
<tr>
<td>P3</td>
<td>2.7</td>
<td>42.2</td>
</tr>
<tr>
<td>P4</td>
<td>2</td>
<td>44.7</td>
</tr>
<tr>
<td>P5</td>
<td>1.5</td>
<td>39.9</td>
</tr>
<tr>
<td>P6</td>
<td>1.3</td>
<td>45.4</td>
</tr>
<tr>
<td>P7</td>
<td>1.6</td>
<td>60.3</td>
</tr>
<tr>
<td>P8</td>
<td>1.1</td>
<td>47.9</td>
</tr>
</tbody>
</table>

By labeling DNA with YOYO-1 and nuclear with DAPI, HeLa cells were treated with complexes formed with Rhodamine labeled polypeptides and YOYO-1 DNA, with P0 and P3 as examples. As shown in Fig. 3.10, both complexes revealed the accumulation of green fluorescent signal and red fluorescent signal in the intracellular plasma environment and the accumulation of the YOYO-1-DNA in the cell nuclear, demonstrating the intracellular internalization, unpacking and the nuclear transportation of DNA mediated by the polypeptides. For the P3/DNA complexes, green fluorescence
spread to the entire intracellular environment in a permeated manner, indicating that most of the DNA were delivered into the cytoplasm via the passive diffusion of the complexes

Fig. 3.10 CLSM images showing the cellular internalization and distribution of RhB-polypeptides/YOYO-1 DNA complexes in HeLa cells following incubation at 37 °C for 4 h (bar = 20 μm).

3.3.5 In vitro transfection

The transfection efficiencies of polypeptide/DNA complexes at various weight ratios were evaluated in HeLa cells by monitoring the luminescence intensity. As shown in Fig. 3.11A, copolypeptides modified with hydrophobic domain exhibited enhanced luciferase level compared with homopolypeptide (P0) and P3 reached the highest transfection efficiency at the weight ratio of 10. Such result also demonstrated the
superiority of P3 in terms of gene transfection. To further investigate the gene transfection performance in serum-containing cell growth media, HeLa cells were treated with P0/pCMV-Luc and P3/pCMV-Luc complexes at different polypeptide/DNA weight ratios in serum-containing environment for 4 h. Luciferase expression level was tested 24 h post transfection. It is noted that transfection efficiency of P0 was significantly inhibited in the presence of serum even after the re-optimization of the formulation, while for P3, there was no significant reduced level of the transfection efficiency between serum-free and containing conditions after increasing the amount of the polypeptides in the complexes, indicating the importance of aromatic groups on the complexes stabilization against serum (Fig. 3.11B).

Fig. 3.11 (A) \textit{In vitro} transfection efficiency of polypeptide/pCMV-Luc complexes in HeLa cells at different weight ratios (n=3). (B) \textit{In vitro} transfection efficiency of P0/pCMV-Luc and P3/pCMV-Luc complexes in HeLa cells at different weight ratios following incubation at both serum-free and serum-containing DMEM (n=3).
3.3.6 Cytotoxicity

The cytotoxicity of complexes towards HeLa cells were evaluated by the MTT assay following 24-h treatment. As shown in Fig. 3.12A, homopolypeptide exhibited largest cytotoxicity and the copolypeptides with reduced charge density showed increased percentage of viable cells. It is because that the positive charges of polypeptides were partially substituted with aromatic domain and the membrane activity was eliminated, which presumably lead to the decreased cytotoxicity compared with P0 with large charge density. Compared to the free polypeptides at equivalent concentrations (Fig. 3.12B), polypeptide/DNA complexes showed lower cytotoxicity, which was mainly due to the partial neutralization of their positive charges by the anionic plasmid DNA. At the transfection dose (equal to 10 μg/mL polypeptides), the complexes exhibited higher than 90% cell viability, which can ensure the safe application towards transfection.

Fig. 3.12 (A) *In vitro* cytotoxicity of polypeptides in HeLa cells following 24-h treatment as evaluated by the MTT assay (n=3). (B) *In vitro* cytotoxicity of polypeptides/DNA
(Fig. 3.12 cont.) complexes in HeLa cells following 24-h treatment as evaluated by the MTT assay (n=3). The weight ratio of polypeptides to DNA was fixed at 10.

3.3.7 In vitro gene transfection in melanoma cells

Upon demonstrating the ability of P3 to facilitate the gene delivery into melanoma tumors, we then evaluated the in vitro gene transfection efficiency in B16F10 cells. As shown in Fig. 3.13A, P3/DNA notably improved the gene transfection efficiency in melanoma cells compared with P0/DNA complexes, which was in agreement with the transfection results in HeLa cells. The potent gene delivery efficiency of P3/DNA complexes in the serum containing environment was again elucidated, which maintained 90% of the transfection efficiency in the serum-free conditions after re-optimization and substantially outperformed P0/DNA complexes (Fig. 3.13B).

Fig. 3.13 (A) In vitro transfection efficiency of P0/pCMV-Luc and P3/pCMV-Luc complexes in B16F10 cells at different weight ratios (n=3). (B) In vitro transfection efficiency of P0/pCMV-Luc and P3/pCMV-Luc complexes in B16F10 cells at different weight ratios following incubation at serum-containing DMEM (n=3).
3.3.8 *In vivo gene transfection in melanoma tumors*

The ability of P3 to mediate the delivery of plasmid DNA in vivo was then investigated in a mouse bearing melanoma tumor. In this model, B16F10 cells were xenograft to the flanks of C57BL6 mice. Firstly, we evaluated the intracellular delivery of the plasmid DNA into the tumor cells after the topical injection. Cy5 labeled pCMV-Luc/polypeptide complexes were intratumorally injected into tumor tissues 4 h before imaging. As Fig. 3.14, complexes labeled with Cy5 penetrate into tumor cell nuclear 4 h after treatment while naked DNA could not enter the intracellular plasma and nuclear, indicating the potential for future luciferase expression.

![Fig. 3.14 CLSM images of melanoma tissues treated topically with Naked DNA and P3/Cy5-DNA. Red, Cy5 labeled DNA. Blue, DAPI stained nuclear. Bar = 50 μm.](image)
To evaluate the transfection efficiency of P3/DNA in tumor tissues, 10µg pCMV-Luc in complexes were intratumoral injected three times every one hour. Complexes formed by PLL/DNA and PEI (25k)/DNA were evaluated as control groups. The other two groups were administered intratumorally with naked DNA and HEPES buffered glucose as negative controls. After 48 h of the last injection, complexes based on aromatic domain modified polypeptides (P3) displayed approximately 40-fold higher luciferase activity compared with complexes of PLL (Fig.3.15). Even though PEI (25k) mediated higher expression efficiency compared with P3, it was well known that high Mw PEI was a kind of quite toxic cationic material.

Fig. 3.15 *In vivo* luciferase activity in tumor tissues after intratumoral treatment of P3/DNA complexes (n=4).

We then investigated the *in vivo* transfection efficiency of P3/DNA complexes following intravenous injection. The complexes were formed by mixing P3 and DNA at the weight ratios of 20 and then PEG-PLG were coated to the surface at the PEG-PLG/DNA weight ratios of 10. Complexes containing 20µg pCMV-Luc were
intravenously injected. Complexes formed by P3/DNA (no coating) were evaluated as control groups. The other two groups were administered intravenously with naked DNA and HEPES buffered glucose as negative controls. After 24 h of the last injection, P3/DNA (no coating group) exhibited limited expression of luciferase in the tumors. This low gene delivery efficiency was caused by the positive surface charges of the complexes, which had the potential to attract and bind to the negatively charged proteins in the blood when circulation. To improve in vivo gene delivery, we modified the surface of the P3/DNA complexes with PEG-PLG, which was a coating material containing two parts. The first part was a poly-L-glutamic acid (PLG), which was negatively charged and capable of binding to the positively charged surface of the polyplexes. The other part was polyethylene glycol (PEG), which was neutral and was able to overcome the RES clearance. Compared with no coating group, the coated complexes exhibited almost 20 times higher transfection efficiency in tumors.

Fig. 3.16 Comparison between coating and no coating polyplexes for in vivo luciferase transfection after intravenous treatment of P3/DNA complexes (n=4).
3.4. Conclusion

In conclusion, we have developed a library of copolypeptides with hydrophobic domain modification and studied the mechanism involved in the cell penetration and gene delivery. By substitution of positive charges with aromatic domains, copolypeptides successfully promote the cell penetration property and gene delivery efficiency via firstly bypassing endosytosis pathway, which will limit DNA releasing by endosomal entrapment, and then reducing pore formation level and membrane activity from safety aspect. This is the first strategy from the mechanism-motivated structure design to overcome obstacles existing in the non-viral gene delivery. These helical copolypeptides demonstrated high capacity and efficiency in delivering DNA in serum free conditions and also the tolerance in the serum-containing environment. They could also mediate successful expression of DNA in melanoma tumor tissues after both the intratumoral and the intravenous treatment, showing great potential when used as various gene therapeutic systems.

3.5. References


CHAPTER 4

REDOX-RESPONSIVE, REVERSIBLY-CROSSLINKED THIOLATED CATIONIC HELICAL POLYPEPTIDES FOR EFFICIENT siRNA ENCAPSULATION AND DELIVERY

4.1. Introduction

RNA interference (RNAi) has recently emerged as a promising therapeutic strategy for the treatment of various genetic disorder-related diseases by silencing the expression of therapeutically relevant target genes in a precise and specific manner [1]. Small interfering RNA (siRNA) duplexes, usually 20-25 base pairs in length, are oligonucleotides that can regulate silencing of the target genes by triggering sequence-specific mRNA degradation [2-5]. Despite their efficiency, their high molecular weight, hydrophilicity, and negative charge prevent siRNA from penetrating cell membranes. siRNA also suffer from degradation by ubiquitous RNase [6-8]. Therefore, effective carriers which can protect siRNA from degradation and facilitate their delivery to the cytosol are crucial to effective RNAi.

In the past decade, a variety of materials, such as cationic polymers, lipids, proteins, and peptides [9-15], were explored as siRNA delivery vectors. Although the cationic polymers facilitate cellular internalization, they enter the cells mainly via the endocytosis mechanism, which thereafter experience endosomal entrapment and lysosomal degradation. Cell penetrating peptides (CPPs), such as HIV-TAT, Arg9, penetratin, and melittin, are sequence-specific, membrane-active short oligopeptides that
can efficiently enter the cytosol via direct membrane penetration. However, CPPs are usually too short (with 25 amino acid residues or less) and lack sufficient cationic charge density to efficiently condense and deliver siRNA. As such, they often function as membrane-active ligands conjugated to existing delivery vehicles to improve the delivery efficiency.

We recently developed a cationic polypeptide poly (γ-4-((2-(piperidin-1-yl) ethyl) amino) methyl) benzyl-L-glutamate) (PVBLG-8) for gene delivery. PVBLG-8 with its stabilized helical structure exhibits excellent membrane activity, which has been shown to facilitate intracellular internalization and endosomal escape of DNA cargos [16-20]. In comparison to plasmid DNA, siRNA molecules have fewer charged groups, lower molecular weight and higher stiffness, which makes it difficult for them to form stable complexes with cationic polymers, especially with helical polypeptides with rigid, rod-like structures [19, 21]. siRNA loosely bound to helical polypeptide can still be attacked by nucleases and is therefore vulnerable to enzymatic degradation before reaching the target site. Therefore, it is of particular importance to improve the siRNA condensation efficiency of cationic, helical polypeptides.

Herein, we designed and synthesized a thiolated cationic helical copolypeptide, poly (γ-4-((2-(piperidin-1-yl) ethyl) aminomethyl) benzyl-L-glutamate)-r-poly(γ-4-((2-mercaptoethyl) amino methyl) benzyl-L-glutamate) (PVBLG-8-SH). The cationic PVBLG-8 segment affords positive charges to loosely condense the oppositely charged siRNA, and the pendent thiol groups on the side chain terminals can be crosslinked to
form compact complexes that allow stable siRNA encapsulation as well as protection against nuclease-assisted siRNA degradation. The potent membrane activity of the helical PVBLG-8-SH promotes intracellular delivery of siRNA cargos that can be promptly released in the cytosol upon redox-triggered disulfide cleavage to allow efficient mRNA knockdown. The physicochemical properties of the polypeptide/siRNA complexes, intracellular kinetics, gene silencing efficiency, and cytotoxicity were evaluated. This study thus provides insights into the design of cationic helical polypeptides for siRNA delivery.

4.2. Materials and Methods

4.2.1. Materials and cell lines

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) and used as received unless otherwise specified. Anhydrous tetrahydrofuran (THF), hexane, and dimethylformamide (DMF) were dried by a column packed with 4Å molecular sieves. Nitrobenzene was treated by CaH2 followed by distillation under reduced pressure. γ - (4-Vinylbenzyl)-L-glutamate N-carboxyanhydride (VB-L-Glu-NCA) was prepared as previously reported [22]. PVBLG-8 was synthesized following previously published procedures as its molecular weights (MWs) were determined by gel permeation chromatography (GPC) [17].

Pierce BCA assay kit and 5, 5’-dithio-bis-(2-nitrobenzoic acid) (DTNB) were purchased from ThermoFisher Scientific (Rockford, IL, USA). LipofectamineTM 2000 (LPF) and 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-2H-tetrazolium bromide (MTT) were purchased from Invitrogen (Carlsbad, CA, USA). siRNA against the GL2 luciferase
gene (siGL2) with target sequence 5’-CGT ACG CGG AAT ACT TCG A-3’ was purchased from Thermo Fisher Scientific Inc. (Waltham, MA, USA). The negative control siRNA containing scrambled sequences were purchased from Qiagen (Valencia, CA, USA). siRNA labeling kit with the FAM dye was purchased from Life Technologies (Grand Island, NY, USA). Bright-Glo luciferase assay kit was purchased from Promega (Madison, WI, USA).

HeLa-Luc cells stably expressing GL2 luciferase were purchased from the Signosis, Inc. (Santa Clara, CA, USA) and were cultured in Dulbecco’s Modified Eagle Medium (DMEM) (Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS) and 1% hygromycin B (Roche, Indianapolis, IN, USA).

4.2.2. Instrumentation

$^1$H NMR spectra were recorded on a Varian U500 MHz spectrometer. Infrared spectra were recorded on a Perkin-Elmer 100 serial FTIR spectrophotometer calibrated with polystyrene film (Perkin-Elmer, Santa Clara, CA, USA). Lyophilization was performed on a FreeZone lyophilizer (Labconco, Kansas City, MO, USA).

4.2.3. Synthesis and characterization of PVBLG-8-SH

VB-L-Glu-NCA (29 mg, 0.1 mmol) was dissolved in a mixture of DMF (1 mL) and nitrobenzene (30 µL) in a glove box, followed by addition of hexamethyldisilazane (HMDS) (20 µL, 0.1 M, M/l=50) and 1,5,7-triazabicyclo[4.4.0]dec-5-ene (TBD) solution (20 µL, 0.01 M) in DMF. FTIR was used to monitor the polymerization until the
conversion reached 99% (24 h) to obtain poly(γ-(4-vinylbenzyl)-L-glutamate) (PVBLG). Tetrabutylammonium fluoride solution (1 M in THF, 50 µL), benzyl chloroformate (25 µL), and N,N-diisopropylethylamine (DIEA, 25 µL) were added and the solution was stirred for 3 h to cleave the N-Si bond and protect the terminal amino groups. The solution was then concentrated under vacuum and the resulting polymer was purified by precipitation from cold ethyl ether. PVBLG was then dissolved in chloroform (15 mL) and oxidized by O₃ at -78 °C. Dimethyl sulfide (1 mL) was added and the solution was stirred at room temperature (RT) overnight before most solvent was removed under vacuum. The product poly (γ-(4-aldehydobenzyl-L-glutamate) (PABLG) was then purified by precipitation from methanol and dried under vacuum. The obtained PABLG (15 mg) was dissolved in DMF (1 mL), 2-aminoethanethiol hydrochloride (6 mg dissolved in DMSO, 0.5 molar equivalents relative to the aldehyde) was added. After stirring at 50 °C for 24 h, 1-(2-aminoethyl) piperidine (50 µL, 6.7 molar equivalents relative to the aldehyde) was added, and the solution was stirred at 50 °C for another 24 h. After reduction at 50 °C for another 24 h using borane-pyridine complex, the final polypeptide was purified through ultrafiltration (molecular weight cut-off = 3 kDa) against water filled with nitrogen at 4 °C and lyophilized. \(^1\)H NMR (500 MHz, TFA-d):
\[
\begin{align*}
\delta & \quad \text{m, 4H, ArH), 5.30 (br s, 2H, ArCH2O−), 4.88 (br s, 2H, ArCH2NH), 4.49 (s, 1H, α–H), 3.99 (s, 4H, -OCH2CH2-), 3.92-3.83 (br m, 6H, -HNCH2CH2N- and -NCH2CH2CH2CH2CH2-), 3.15 (m, 2H, -HNCH2CH2N-), 2.75 (s, 2H, -COCH2CH2-), 2.37 (br m, 2H, -COCH2CH2-), 2.14-1.52 (br m, 6H, -NCH2CH2CH2CH2CH2- and -CH2-SH)}
\end{align*}
\]
4.2.4. Quantitation of the thiol groups by Ellman’s reagent

Ellman’s reagent was prepared by dissolving 5, 5’-dithio-bis-(2-nitrobenzoic acid) (DTNB) in phosphate buffer (0.5 M, pH = 8) at the concentration of 0.3 mg/mL. Ellman’s reagent (0.3mg/mL, 50 μL) was added into the PVBLG-8-SH solution (0.5 mg/mL, 50 μL) which was incubated for 20 min in the dark at RT before measurement of absorbance at 405 nm [23].

4.2.5. Preparation and characterization of crosslinked polypeptide/siRNA complexes

siRNA and polypeptide were dissolved in DEPC-treated water at 0.1 mg/mL and 1 mg/mL, respectively, and then mixed at the polypeptide/siRNA weight ratios of 5, 10, 15, 20, 30 and 40 followed by pipetting for 30 s and incubation in the glass vial filled with O₂ at 37 °C for 2 h. As the control group, un-crosslinked complexes were formed by mixing polypeptide and siRNA and incubation in the glass vial filled with N₂ at 37 °C for 15 min. The obtained crosslinked and un-crosslinked complexes were characterized for size and zeta potential by dynamic laser scanning (DLS) on a Malvern Zetasizer (Herrenberg, Germany). The gel retardation assay was used to evaluate the siRNA condensation by polypeptides. Complexes were loaded on a 4% low-melting agarose gel at 200 ng siRNA/well followed by electrophoresis at 70 V for 60 min. Naked siRNA was used as a control, and siRNA migration in the agarose gel was visualized by a Gel Doc imaging system (Biorad, Herclues, CA, USA) following staining with ethidium bromide (EB). To quantitatively measure the percentage of siRNA condensed, the EB exclusion assay was performed as follows [24]. siRNA solution was prepared in 140 mM NaCl and 20 mM HEPES at pH 7.2 and polypeptide was dissolved in DEPC-treated water at 1
The crosslinked complexes prepared as described above were stained with EB at the siRNA/EB weight ratio of 10 and RT for 1 h before quantification of the fluorescence intensity ($\lambda_{\text{ex}} = 510$ nm, $\lambda_{\text{em}} = 590$ nm). The siRNA condensation efficiency (\%) was calculated according to the following equation:

$$\text{siRNA condensation efficiency (\%) } = (1 - \frac{F - F_{EB}}{F_0 - F_{EB}}) \times 100$$

Where $F_{EB}$, $F$, and $F_0$ represent the fluorescence intensity of pure EB solution, siRNA/EB solution, and siRNA/EB/polypeptide solution, respectively.

In order to evaluate the siRNA release from the crosslinked complexes, complexes were mixed with GSH at various concentrations (4.5 $\mu$M represents the extracellular GSH concentration and 10 mM represents the intracellular GSH concentration) and incubated up to 120 min. The percentage of siRNA released from the complexes and the particle sizes were quantified as described above.

4.2.6. Stability of siRNA

In order to evaluate the stability of siRNA following RNase treatment, free siRNA and polypeptide/siRNA complexes were treated with fetal bovine serum at a final concentration of 50 % for 4 h at 37 °C. Heparin was added to dissociate the siRNA from the complexes, and samples were immediately loaded on a 4% agarose gel followed by electrophoresis at 70 V for 1 h. The siRNA stability was visualized by a Gel Doc imaging system following staining with EB.
4.2.7. In vitro cell uptake

To allow visualization and quantification of cellular internalization, siRNA was labeled with the FAM dye using the silencer® siRNA labeling kit and used to form complexes with polypeptide as described above. HeLa-Luc cells were cultured in serum-containing DMEM, seeded in 96-well plates at $1 \times 10^4$ cells/well, and cultured for 24 h before cell uptake. The medium was replaced by serum-free DMEM (100 μL/well) and crosslinked PVBLG-8-SH/FAM-siRNA complexes were added at 20, 40, 60, 80 and 100 nM siRNA/well. After incubation at 37 °C for 4 h, cells were washed with cold PBS containing heparin (20 U/mL) for 3 times and lysed with the RIPA lysis buffer containing 0.5% SDS which could dissociate FAM-siRNA from the lipo- and polyplexes (100 μL/well) at RT for 20 minutes [25, 26]. The FAM-siRNA content in the lysate was determined by spectrofluorimetry ($\lambda_{ex} = 492$ nm, $\lambda_{em} = 518$ nm) and the total protein level was determined by the BCA kit. Uptake level was expressed as the amount of FAM-siRNA associated with 1 mg cellular protein.

To further evaluate the cell uptake mechanism, the cell uptake study was performed at 4 °C during the 2-h period to block the energy-dependent endocytosis. The cell uptake level was determined as described above, and results were expressed as percentage uptake of the control cells which were incubated with crosslinked complexes at 37 °C for 2 h.

We then probed the pore formation ability of the complexes by monitoring the uptake level of fluorescein-tris(hydroxymethyl)methanethiourea (FITC-Tris), which is
the non-reactive form of a hydrophilic and membrane-impermeable dye. HeLa-Luc cells were cultured in serum-containing DMEM, seeded in 96-well plates at 1×10^4 cells/well, and cultured for 24 h. The medium was replaced by serum-free DMEM (100 μL/well) into which crosslinked PVBLG-8-SH and FITC-Tris were added at 2 μg/well and 0.2 μg/well, respectively. Free FITC-Tris without polypeptides was added as a control. After incubation at 37 °C for 2 h, cells were washed with cold PBS containing heparin (20 U/mL) for 3 times and lysed with the RIPA lysis buffer (100 μL/well). The FITC-Tris content in the lysate was determined by spectrofluorimetry (λ<sub>ex</sub> = 485 nm, λ<sub>em</sub> = 530 nm) and the total protein level was determined by the BCA kit. Uptake level was expressed as ng FITC-Tris associated with 1 mg cellular protein.

Internalization and intracellular distribution of FAM-siRNA in HeLa-Luc cells were further visualized by confocal laser scanning microscopy (CLSM). HeLa-Luc cells cultured on coverslips in 6-well plate were treated by complexes in serum-free DMEM (2 mL) at 60 nM FAM-siRNA/well. Following incubation at 37 °C for 4 h, cells were washed three times with PBS containing heparin (20 U/mL), fixed with 4% paraformaldehyde, and stained with DAPI (10 μg/mL) and Lysotracker® Red (200 nM) before observation by CLSM (LSM700, Zeiss, Germany).

To explore the serum-resistant ability of the crosslinked complexes, cells were incubated with complexes in DMEM containing 10% FBS throughout the 4-h uptake experiment at 37 °C, and the uptake level was quantified as described above.
4.2.8. *In vitro gene knockdown*

HeLa-Luc cells were cultured in serum-containing DMEM, seeded in 96-well plates at $1 \times 10^4$ cells/well, and cultured for 24 h before the transfection study. The medium was replaced by serum-free DMEM (100 μL/well) and polypeptide/siGL2 complexes were added at predetermined concentrations (20, 40, 60, 80 and 100 nM siRNA/well). After incubation at 37 °C for 4 h, the medium was replaced by DMEM containing 10% FBS (100 μL/well) and cells were further incubated for 24 h before quantification of luciferase expression using a Bright-Glo luciferase assay kit and measurement of the cellular protein level by a BCA kit. Luciferase expression level was evaluated as relative luminescence unit (RLU) associated with 1 mg of cellular protein (RLU/mg protein). The silencing efficiency was indicated as the percentage of luciferase expression levels of control cells which did not receive complexes treatment. To verify specific gene knockdown, identical experiments were performed for polypeptide/scr siRNA (scrambled siRNA) complexes.

4.2.9. *Cell viability assay*

HeLa-Luc cells were seeded in 96-well plates at $1 \times 10^4$ cells/well and cultured in serum-containing DMEM for 24 h. The medium was replaced by serum-free DMEM (100 μL/well) into which complexes were added at the siRNA concentrations and the polypeptide/siRNA weight ratios in accordance with the afore-mentioned transfection process. After incubation at 37 °C for 4 h, the medium was replaced by serum-containing DMEM and cells were further incubated for another 20 h. Cell viability was then evaluated by the MTT assay, and results were represented as percentage viability of
control cells that did not receive complex treatment.

4.2.10. Statistical analysis

Statistical analysis was performed using Student’s t-test and differences between test and control groups were judged to be significant at *p < 0.05 and very significant at **p < 0.01.

4.3. Results

4.3.1. Synthesis and characterization of PVBLG-8-SH

PVBLG-8-SH was synthesized via ring-opening polymerization (ROP) of VB-L-Glu-NCA initiated by HMDS and subsequent side-chain reductive amination by 2-aminoethanethiol and 1-(2-aminoethyl)piperidine (Fig. 4.1B and 4.1C) [22]. HMDS allowed a well-controlled ROP of VB-L-Glu-NCA, as demonstrated by the well-defined MW (degree of polymerization was 52 as calculated by GPC compared with the designed M/I of 50). The percentage of thiol groups on the PVBLG-8-SH before crosslinking was determined to be 4.7% (molar ratio) and 0.09% (molar ratio) after crosslinking using the Ellman’s reagent, indicating that before crosslinking, an average of 2.4 thiol groups had been successfully conjugated to each polypeptide molecular chain and after crosslinking, most of the thiol groups were converted to disulfide bonds. The low incorporation efficiency of thiol groups was because they were incorporated by the reductive amination reaction between 2-aminoethanethiol hydrochloride and the benzaldehyde groups on polypeptide side chains. Before the reducing agent borane was added, 6.7 equiv. 1-(2-aminoethyl)piperidine was also added. Since the reaction between aldehyde and amine is
reversible, two amines attached on polypeptide side chains will reach equilibrium. PVBLG-8-SH adopted typical α-helical structure as evidenced by the FTIR analysis showing the typical amide bands of α-helix at 1650 and 1553 cm⁻¹, and the helical structure of the thiolated polypeptide was maintained after the crosslinking and nanocomplex assembling (Fig. 4.1D). Such results thus indicated that the thiolated polypeptide might be able to maintain the helix-dependent membrane activity after crosslinking of the pendent thiol groups.
Fig. 4.1 (A) Schematic representation of PVBLG-8-SH/siRNA crosslinked complexes.

(B) Reaction scheme of PVBLG-8-SH. (C) FTIR spectra of PVBLG-8-SH and PVBLG-8-SH/siRNA complexes before and after crosslinking.

4.3.2. Formulation and characterization of PVBLG-8-SH/siRNA crosslinked complex
The formulation included a two-step process, charge-charge interaction followed by oxidative crosslinking (Fig. 4.1A). First, anionic siRNA was added into the solution of α-helical cationic polypeptide PVBLG-8-SH. The charge-charge interactions between cationic polypeptide and anionic siRNA led to the formation of loose complexes with relatively large particle size (~500 nm, Fig. 4.2A) and positive zeta potential value (~20 mV, Fig 4.2B). To allow crosslinking of the PVBLG-8-SH/siRNA complex, the loose complex was incubated at 37 °C for 2 h in the glass vial filled with O2 when the pendent thiol groups were oxidized to form intra- and intermolecular disulfide. Condensed nanocomplexes were thus yielded which possessed smaller size (~200 nm, Fig. 4.2A). All of the nanocomplexes showed PDI values lower than 0.3, indicating a relatively homogenous size distribution.

Fig. 4.2 Particle size (A) and zeta potential (B) of PVBLG-8-SH/siRNA complexes before and after crosslinking.
The agarose gel retardation assay revealed that non-thiolated PVBLG-8 was unable to tightly condense siRNA as evidenced by the siRNA migration in electrophoresis even at high PVBLG-8/siRNA weight ratio up to 40 (Fig. 4.3B), according well with our previous findings [19]. In comparison, crosslinked complexes formed from the thiolated polypeptide, PVBLG-8-SH, led to retarded siRNA migration in the 4% agarose gel at the PVBLG-8-SH/siRNA weight ratio higher than 5, suggesting that siRNA could be effectively and individually entrapped by the helical, thiolated polypeptide after crosslinking of pendent thiols to form intra- and inter-molecular disulfide (Fig. 4.3A). Un-crosslinked complexes formed by PVBLG-8-SH as a control were unable to condense siRNA, which further substantiated the importance of crosslinking in yielding compact complexes (Fig. 4.3C). Such observation was further demonstrated by a quantitative EB exclusion assay, wherein crosslinked PVBLG-8-SH afforded markedly higher siRNA condensation efficiency (60%) than PVBLG-8 (2%) and un-crosslinked PVBLG-8-SH (6%) (Fig. 4.3D).
Fig. 4.3 (A) Gel retardation assay showing siRNA condensation by PVBLG-8 (A), un-crosslinked PVBLG-8-SH (B), and crosslinked PVBLG-8-SH (C) at various polymer/siRNA weight ratios. N represents naked siRNA. PVBLG-8-SH was crosslinked at 37°C for 2 h after polymer/siRNA complexes were formed. (D) Condensation efficiency of siRNA at various polymer/siRNA weight ratios as quantified by the EB exclusion assay (n=3).

The ability of crosslinked PVBLG-8-SH complexes to protect siRNA from nuclease digestion was then evaluated by incubating the crosslinked PVBLG-8-SH/siRNA complexes with 50% FBS that contained digestive RNase. After dissociating
the encapsulated siRNA using heparin, we then evaluated the siRNA integrity via electrophoresis on a 4% agarose gel.

As shown in Fig. 4.4A, siRNA encapsulated in the crosslinked complexes remained intact upon the treatment of RNase compared with that encapsulated in the un-crosslinked complexes (Fig. 4.4B), indicating that crosslinked complex formed by PVBLG-8-SH was able to completely prevent the nucleolytic degradation of siRNA while complex formed by PVBLG-8 revealed limited siRNA stability.

\[ \text{A} \quad \text{PVBLG-8-SH} \quad \text{B} \quad \text{PVBLG-8} \\
\text{+serum/heparin} \quad \text{+serum/heparin} \]

Fig. 4.4 Crosslinked PVBLG-8-SH complexes protect siRNA from nucleolytic degradation. Stability of siRNA in crosslinked PVBLG-8-SH complexes (A) or PVBLG-8 complexes (B) following treatment with serum for 4 h. N1 represents naked siRNA without serum treatment and N2 represents naked siRNA treated with serum.

4.3.3. Uptake profile of the PVBLG-8-SH/siRNA crosslinked complex

As shown in Fig. 4.5A and B, crosslinked complexes remarkably promoted the internalization level of FAM-siRNA (over thousands fold v.s. naked FAM-siRNA) which peaked at the PVBLG-8-SH/siRNA weight ratio of 20:1 and the siRNA concentration of
60 nM. Further increase in the siRNA concentration up to 80 nM and 100 nM did not lead to elevation in the intracellular internalization level. Compared with LPF as a commercial transfection reagent, a 2-3-fold higher uptake level was also noted, which could be attributed to the potent membrane activity of the helical PVBLG-8-SH to mediate intracellular cargo delivery. With the optimal formulation of crosslinked complexes (PVBLG-8-SH/siRNA weight ratio of 20), we further illustrated the intracellular uptake mechanism. The cell uptake study was first performed at 4 oC when energy-dependent endocytosis was totally inhibited. As shown in Fig. 4.5C, lowering the temperature led to ~70% inhibition of the cell uptake level, indicating that most of the complexes were internalized via energy-dependent endocytosis. Because endocytosis often leads to endosomal entrapment, it was indicated that the polypeptide nanocomplexes may be able to mediate effective endosomal escape. In addition to the endocytosis mechanism, we also probed the pore formation ability of the complexes by monitoring the uptake level of FITC-Tris, which is a hydrophilic and membrane-impermeable dye. As shown in Fig. 4.5D, crosslinked complexes significantly increased the FITC-Tris uptake level, indicating that the complexes had the capability to induce pore formation on cell membrane and accordingly facilitate direct penetration of siRNA cargos, which is another important mechanism mediated by cationic helical polypeptides. We further evaluated the capability of complexes to mediate endosomal escape via observation by CLSM. As shown in Fig. 4.5E, green fluorescence (FAM-siRNA) largely separated from red fluorescence (Lysotracker Red-stained endosomes/lysosomes), indicating effective endosomal escape of the nanocomplexes. The green fluorescence was uniformly distributed in the cytoplasm, which again substantiated that the complexes were
extensively internalized by HeLa-Luc cells post 4-h incubation and they had escaped out from endosomal entrapment.

When used in vivo, interference raised by serum often serves as an obstacle against the efficiency of non-viral gene delivery vectors [24]. As such, the ability of crosslinked PVBLG-8-SH complexes to mediate intracellular delivery of siRNA was further probed in the presence of serum. Because of the interference raised by serum, highest cell uptake level was noted at the optimal PVBLG-8-SH/siRNA ratio of 40, which represented a 75% uptake level of that under serum-free condition (Fig. 4.5F). In comparison, a dramatic reduction in the cell uptake level was observed for both the PVBLG-8/siRNA complexes (63%) and the un-crosslinked PVBLG-8-SH/siRNA complexes (54%) in the presence of serum, which could be attributed to the aggregation and destabilization of the loose complexes upon non-specific adsorption of negatively charged serum proteins onto the positively charged complexes. Such discrepancy between the crosslinked and un-crosslinked complexes thus indicated that crosslinking of the polypeptide via disulfide bonding endowed the complexes with improved resistance against serum by allowing stable and tight nanostructures with lower surface charges.
Fig. 4.5 (A) Uptake level of FAM-siRNA containing crosslinked PVBLG-8-SH complexes in HeLa-Luc cells following 4-h incubation at 37 °C at various polymer/siRNA weight ratios (siRNA concentration fixed at 60 nM) (n=3). (B) Uptake level of FAM-siRNA-containing crosslinked PVBLG-8-SH complexes in HeLa-Luc cells following 4-h incubation at 37 °C at various siRNA concentrations (polymer/siRNA ratio fixed at 20) (n=3). Naked FAM-siRNA (N) and LPF/siRNA complexes were used as controls at the siRNA concentration of 60 nM. (C) Uptake level of FAM-siRNA-containing crosslinked PVBLG-8-SH complexes in HeLa-Luc cells at 4 °C (n=3). (D) FITC-Tris uptake level of HeLa-Luc cells following co-incubation with PVBLG-8-SH for 2 h at 37 °C (n=3). (E) CLSM images of HeLa-Luc cells following treatment with PVBLG-8-SH/FAM-siRNA crosslinked complexes at 37 °C for 4 h (siRNA concentration of 60 nM; PVBLG-8-SH/siRNA weight ratio of 20) (bar = 20 μm). The nuclei were stained with DAPI, and the endosomes/lysosomes were stained with Lysotracker Red. (F)
(Fig. 4.5 cont.) Uptake level of polymer/FAM-siRNA complexes in HeLa-Luc cells following 4-h incubation with or without serum at 37 °C (n=3). siRNA concentration was fixed at 60 nM while the polymer/siRNA weight ratios was optimized at 40.

4.3.4. Redox-responsive siRNA release profiles

The release profile of crosslinked complexes was evaluated as a function of glutathione (GSH) concentration. GSH is an intracellular reducing reagent that is wildly present in the cytosol at the concentration of around 10 mM and rarely present in the extracellular environment (with a low concentration of around 4.5 µM). As shown in Fig. 4.6A, at the GSH concentration of 10 mM, the encapsulated siRNA was completely released within 60 min, while at the GSH concentration of 4.5 µM and 0 mM, only a small portion of siRNA (3% ~ 5%) was released within 120 min. As shown in Fig. 4.6B, in the presence of 10 mM GSH, the complex size increased dramatically from 200 nm to 500 nm within 20 min, indicating that the cleavage of the disulfide bond led to the transformation from tight complexes to loose complexes. The size further increased to over 1000 nm within 60 min, indicating that thorough disruption of the complex structure, which was in accordance with the complete siRNA release within 60 min. These results demonstrated that the crosslinked PVBLG-8-SH/siRNA complexes were stable in the extracellular environment while could rapidly release the siRNA cargo under reductive conditions in the intracellular environment.
Fig. 4.6 (A) siRNA release from complexes upon treatment with GSH of various concentrations (n=3). (B) Particle size of PVBLG-8-SH/siRNA upon treatment with GSH (10mM).

4.3.5. Crosslinked complexes induce effective gene silencing with low cytotoxicity

In consistence with their cell uptake level, maximal gene silencing efficiency was noted for the crosslinked PVBLG-8-SH complexes at the siRNA concentration of 60 nM and weight ratio of 20 (Fig. 4.7A), which was comparable to that of LPF as one of most widely used commercial reagent. Further increase in the siRNA concentration and polypeptide/siRNA weight ratio did not lead to further improvement in the gene knockdown efficiency. As shown in Fig. 4.7B, crosslinked complexes mediated gene knockdown efficiency of as high as 60% while un-crosslinked complexes and PVBLG-8/siRNA complexes only led to gene knockdown efficiency of 40%. Compared to their un-crosslinked counterpart and the complexes formed with PVBLG-8, the crosslinked complexes exhibited strongest ability in terms of mediating gene knockdown (Fig. 4.7B), which again substantiated the significance of disulfide crosslinking towards stabilized
siRNA encapsulation as well as redox-triggered intracellular siRNA release that ultimately contributed to improved RNAi efficiency. As a control, complexes containing scr siRNA did not induce appreciable luciferase knockdown, which indicated that the observed gene knockdown was due to the sequence-specific RNA interference rather than material toxicity.

Cytotoxicity assessment using the MTT assay further revealed that after 24-h treatment, the crosslinked PVBLG-8-SH complexes showed minimal cytotoxicity (viability higher than 85%) at the siRNA concentration of 60 nM used for the previous transfection studies. This result further ensured the safety of the crosslinked complexes when applied as siRNA delivery vectors (Fig. 4.7C).
Fig. 4.7 (A) Normalized luciferase GL2 level of HeLa-Luc cells following incubation with PVBLG-8-SH/siRNA crosslinked complexes at various siRNA concentrations and PVBLG-8-SH/siRNA weight ratios (n=3). (B) Normalized luciferase GL2 level of HeLa-Luc cells following incubation with different polymer/siRNA complexes. Scr siRNA represents crosslinked PVBLG-8-SH complexes containing scrambled siRNA. siRNA concentration was fixed at 60 nM and the PVBLG-8-SH/siRNA weight ratio was fixed at 20 (n=3). (C) Cytotoxicity of PVBLG-8-SH/siRNA crosslinked complexes in HeLa-Luc cells (n=3).
4.4. Discussion

Synthetic non-viral vectors, especially cationic polymers and lipids, have been widely developed and applied as siRNA delivery vectors. However, stability of the nanocomplexes formed merely through electrostatic interactions remains low in the biological milieu mainly due to the smaller size and linearity of siRNA when compared with plasmid DNA, which thus serves as a major obstacle for in vivo application [5]. The poor stability of the nanocomplexes is a major impediment especially when cationic helical polypeptides are used as siRNA vectors because the polypeptide adopts a linear and rigid rod-like structure which further weakens the interaction with siRNA. Our previous study [19] showed that siRNA cannot be independently condensed by the cationic, helical polypeptide even though they can form loose complexes and the free polypeptide can also facilitate the intracellular delivery of siRNA by generating pores on cell membranes. Moreover, since siRNA could not be tightly condensed by the polypeptide, it is vulnerable to enzymatic hydrolysis by ubiquitous nucleases in the body when the formulation is subjected to use for in vivo RNAi.

Recently there have been multiple reports in developing stabilized polymer/siRNA complexes by either increasing the positive charge density of polymers or the negative charge density of siRNA. Increasing positive charge density of polymers can indeed improve the interactions between polymer and siRNA, which however, will generate undesired charge-associated toxicity at the meantime [27]. Alternatively, increasing the molecular weight and negative charge density of siRNA by polymerizing the siRNA stands affords an effective strategy to improve complexation [28, 29]. Park et
al. reported that polymerized siRNAs (poly-siRNA) could be successfully prepared using a disulfide linkage at the 5’ end of each siRNA strand and could be complexed by the cationic carriers to form stable complexes [30]. Despite the success in stabilizing the complexes with polycations, the polymerized siRNA suffers from potential risk in stimulating IFN-α response and thus safety remains as a serious concern for this strategy [29, 30]. Additional strategies also include chemical conjugation of siRNA to the delivery vector via cleavable disulfide bonding [31] and the co-condensation with ionic crosslinking agents such as sodium triphosphate (TPP) [32]. The incorporation of another polyanion that provides additional interaction forces with polycations to co-encapsulate the siRNA cargo is one of the most commonly used strategies to stabilize the cationic polymer/siRNA complexes. However, the introduction of the third component renders the complexes with excessive complexity which makes the optimization of the nanostructure difficult [33].

Compared with these reported approaches, we developed a two-step formulation strategy to allow helical polypeptide-mediated tight encapsulation of siRNA and stabilization of polypeptide/siRNA complexes. Thiol groups were introduced to the side chains of a smart polypeptide PVBLG-8 that has been demonstrated to possess excellent membrane activity. Loose complexes were first formed via charge interactions between oppositely charged polypeptide and siRNA, and the tight and stable complexes were subsequently formed upon crosslinking of the thiol groups to form intra- as well as inter-molecular disulfide. This formulation strategy could be applied to the delivery of most kinds of siRNA except those modified by thiol groups. The achieved disulfide bonds not
only features augmented siRNA binding and stabilized nanostructure, but also allows redox-responsive siRNA release in the cytosol. As such, integrity of the siRNA cargo against nuclease degradation was markedly improved, and the stability of the nanocomplexes against serum was also greatly enhanced, which collectively contributed to the notably enhanced RNAi efficiency. When there have been large body of works reported utilizing additional polyanionic component or increasing the positive charge density of cationic polymers to achieve stable formulation with siRNA, this two-step formulation strategy reported in this paper offers an alternative, potentially move controllable, pathway to achieve stable siRNA delivery nanocomplexes. There are several advantages of using this disulfide cross-linking strategy to achieve stably formulated siRNA delivery complex over multi-component, charge complex. The rigid rod-like structures of the helical polypeptide are well maintained following the formulation process, thus its excellent membrane activity is largely preserved for successful siRNA delivery. In addition, the release of siRNA from the complex is better controlled through GSH triggered reduction of the disulfide bond. When the disulfide bonds are cleaved, the nanostructure hold tightly via disulfide cross-linking becomes unstable and siRNA becomes more susceptible to release. This potentially provides a means for active control over siRNA release, in contrast to other siRNA delivery vehicles based on charge interactions with uncontrollable siRNA release profiles and unknown siRNA release mechanism.
4.5. Conclusion

In this study, we developed a cationic, helical, and thiolated polypeptide which can form stable nanocomplexes with siRNA through a two-step formulation process, the charge interaction between polypeptide and siRNA and post crosslinking of the pendent thiol groups on polypeptide side chains. The crosslinked nanocomplexes feature tight encapsulation of siRNA to maintain stable nanostructures at the extracellular compartment while they facilitated siRNA release in the cytosol upon redox-triggered disulfide cleavage. More importantly, formation of the crosslinked nanocomplexes did not compromise the helical secondary structure of the polypeptide, thus its excellent membrane activity was well maintained to mediate effective cellular internalization as well as endosomal escape of the siRNA cargo. This crosslinked polypeptide formulation would thus serve as a promising approach to address several critical challenges in non-viral siRNA delivery.

4.6. References


APPENDIX
CHARACTERIZATION OF NCA MONOMERS AND POLYPEPTIDES

$^1$H NMR Spectrums

$^1$H NMR spectrum of PVBLG in CDCl$_3$/TFA-$d$ (85:15, v/v).
$^1$H NMR spectrum of PABLG in CDCl$_3$/TFA-$d$ (85:15, v/v).
$^1$H NMR spectrum of PVBLG-8 in TFA-$d$. 

![NMR Spectrum Image](image-url)
$^1$H NMR spectrum of PVBLG-7 in TFA-$d$. 
$^1$H NMR spectrum of PVBLG-8-\(r\)-7 in TFA-\(d\).
$^1$H NMR spectrum of Naph-L-Glu in CDCl$_3$
$^1$H NMR spectrum of Anth-1-Glu in CDCl$_3$
$^1$H NMR spectrum of Naph-L-Glu NCA in CDCl$_3$
$^1$H NMR spectrum of Anth-L-Glu NCA in CDCl$_3$
$^1$H NMR spectrum of Leu-$\text{L}$ NCA in CDCl$_3$
$^1$H NMR spectrum of PBLG-r-PVBLG(10/40) in CDCl$_3$
$^1$H NMR spectrum of PBLG-r-PVBLG(25/25) in CDCl$_3$
$^1$H NMR spectrum of PNLG-r-PVBLG(10/40) in CDCl$_3$
$^1$H NMR spectrum of PNLG-r-PVBLG(25/25) in CDCl₃
$^1$H NMR spectrum of PALG-r-PVBLG(10/40) in CDCl$_3$
$^1$H NMR spectrum of PALG-r-PVBLG (25/25) in CDCl$_3$
$^1$H NMR spectrum of PLeu-r-PVBLG(10/40) in CDCl$_3$
$^1$H NMR spectrum of PLeu-r-PVBLG(25/25) in CDCl$_3$
$^1$H NMR spectrum of P0 in TFA-$d$. 

![H NMR spectrum of P0 in TFA-$d$.]
$^1$H NMR spectrum of P1 in TFA-\textit{d}
$^1$H NMR spectrum of P2 in TFA-$d$. 

![H NMR spectrum of P2 in TFA-$d$.]
$^1$H NMR spectrum of P3 in TFA-$d$. 
$^1$H NMR spectrum of P4 in TFA-$d$. 

![H NMR spectrum of P4 in TFA-$d$.]
$^1$H NMR spectrum of P5 in TFA-$d$. 

![NMR spectrum of P5 in TFA-$d$.](image)
$^1$H NMR spectrum of P6 in TFA-$d$. 
$^1$H NMR spectrum of PVBLG-8-SH in TFA-$d$. 