Polyionic complexes from cationic block copolymers and plasmid DNA for gene delivery

By

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under the supervision of Professor Glen S. Kwon

at the University of Wisconsin-Madison

Abstract

Despite the high transfection efficiency of viral vectors, safety concerns hamper their progress in gene therapy clinical trials. As a safer alternative, polymeric non-viral vehicles, on the contrary, suffer from low gene transfer ability which prevents them from translating into practical use. One of the major reasons for the inferior transfection ability is the low colloidal stability of the polyplex particles formed with cationic polymers and pDNA, limiting the capability of the polyplexes to withstand the harsh physiological environments and thus the ability of the particles to reach the therapeutic targets. PEGylation of polyplexes is a common method for improving particle stability; nonetheless, lower transfection activity is always observed with PEGylated polyplexes compared to non-PEGylated counterparts. Different strategies for modifying the surface of polyplex particles based on poly{N-[N-(2-aminoethyl)-2-aminoethyl]aspartamide} (P[Asp(DET)]) cationic polymers through syntheses of different P[Asp(DET)]-based polymers were employed in this investigation aimed at improving the transfection efficiency while maintaining the colloidal stability of polyplexes. A pH-sensitive P[Asp(DET)]-based polyplex system with temporary PEGylation through hydrazone linkage was first tested; the reversibly-PEGylated polyplexes transfected cells more effectively than the irreversibly-PEGylated polyplexes and also demonstrated improved stability relative to the non-
PEGylated particles. Pluronic P85 was then used to synthesize P85-\textit{b}-[Asp(DET)] to introduce an amphiphilic surface to polyplex particles to investigate whether substituting the hydrophilic PEG palisade of polyplex particles with an amphiphilic Pluronic P85 layer could promote the transfection efficiency. Polyplexes with an amphiphilic P85 surface demonstrated higher gene transfer ability than did the PEG-based polyplexes, presumably due to higher cellular uptake of the P85-based particles; however, lower stability was observed with the P85-based polyplexes. To balance the transfection efficiency and colloidal stability of the polyplexes, ternary polyplex particles were prepared with surfaces comprising different ratios of PEG and amphiphilic block copolymers. Salt-induced aggregation of the polyplexes could be substantially reduced when the particle surface was covered with PEG at a 25\% fraction, and the transfection efficiency gradually decreased with the fraction of PEG. These results suggest that the polyplex surface can be tuned to optimize the transfection efficiency and stability for further \textit{in vivo} investigations.
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Chapter 1. Introduction ...................................................................................................... 1
  1.1. Gene therapy ........................................................................................................ 2
  1.2. Viral gene delivery vehicles ................................................................................ 3
  1.3. Non-viral gene delivery vehicles ......................................................................... 4
  1.4. Polyionic complexes .......................................................................................... 5
  1.5. Polymeric gene delivery vehicles ....................................................................... 6
    1.5.1. PEG dilemma ............................................................................................... 8
    1.5.2. Environmentally-sensitive gene delivery vehicles .................................... 9
      1.5.2.1. pH-sensitive gene delivery vehicles ..................................................... 11
      1.5.2.2. Redox potential-sensitive gene delivery vehicles ............................ 13
    1.5.3. Poly(ethylene glycol)-block-poly{N-[N-(2-aminoethyl)-2-aminoethyl]aspartamide} block copolymer for gene delivery ...................... 16
      1.5.3.1. In vitro and in vivo gene delivery ......................................................... 18
      1.5.3.2. Cytotoxicity and biodegradability ...................................................... 19
    1.5.4. Pluronic block copolymers for gene delivery ............................................ 20
  1.6. Research proposal ............................................................................................... 22
    1.6.1. Rationale .................................................................................................... 22
1.6.2. Objectives ........................................................................................................... 23
1.6.3. Specific aims ........................................................................................................ 23


2.1. Introduction ............................................................................................................... 25
2.2. Materials and methods ............................................................................................ 26
  2.2.1. Materials ............................................................................................................ 26
  2.2.2. Methods ............................................................................................................. 27
    2.2.2.1. ¹H, ¹³C NMR, and gel permeation chromatography (GPC) analyses ...... 27
    2.2.2.2. Synthesis of β-benzyl-L-aspartate N-carboxy anhydride (BLA-NCA) ................................................................. 28
    2.2.2.3. Synthesis of P[Asp(Hyd-PEG)]-b-P[Asp(DET)] ......................................................... 30
    2.2.2.4. Synthesis of P[Asp(PEG)]-b-P[Asp(DET)] ........................................................ 32
    2.2.2.5. Synthesis of poly(ethylene glycol)-*block*-poly{N-[N-(2-aminoethyl)-2-aminoethyl]aspartamide} (PEG-b-P[Asp(DET)]) ......... 33
    2.2.2.6. Preparation of polyplexes ................................................................................. 34
    2.2.2.7. Gel retardation and EtBr exclusion assay ..................................................... 34
    2.2.2.8. Particle size and ζ-potential measurements ............................................... 35
    2.2.2.9. Polyplex stability in physiological salt and serum environments ............ 36
    2.2.2.10. PEG release study ....................................................................................... 36
    2.2.2.11. Serum nuclease resistance assay ................................................................. 37
    2.2.2.12. *In vitro* transfection .................................................................................. 37
    2.2.2.13. *In vitro* cytotoxicity .................................................................................. 38
    2.2.2.14. LDH release study ...................................................................................... 38
    2.2.2.15. Statistical analysis ...................................................................................... 39
2.3. Results ................................................................................................................. 39
  2.3.1. Polymer Syntheses ......................................................................................... 39
  2.3.2. Formation of polyplexes .............................................................................. 43
  2.3.3. Polyplex stability in physiological salt and serum environments .......... 46
  2.3.4. PEG Release Study ..................................................................................... 47
  2.3.5. pH-sensitivity of reversibly-PEGylated polyplexes ................................... 49
  2.3.6. Serum nuclease resistance .......................................................................... 50
  2.3.7. In vitro transfection ..................................................................................... 51
  2.3.8. In vitro cytotoxicity ...................................................................................... 53
  2.4. Discussion ........................................................................................................ 55
  2.5. Conclusions ..................................................................................................... 58

Chapter 3. The effects of substituting poly(ethylene glycol) on poly(ethylene glycol)-
block-poly{N-[N-(2-aminoethyl)-2-aminoethyl]aspartamide} with Pluronic P85 on the cellular uptake and transfection efficiency of polyionic complexes prepared with the cationic block copolymers ......................... 60

3.1. Introduction ....................................................................................................... 61
3.2. Materials and methods ..................................................................................... 63
  3.2.1. Materials ...................................................................................................... 63
  3.2.2. Methods ....................................................................................................... 64
    3.2.2.1. $^1$H NMR and gel permeation chromatography (GPC) analyses ........ 64
    3.2.2.2. Synthesis of amino P85 (P85-NH$_2$) ................................................... 64
    3.2.2.3. Syntheses of poly(ethylene glycol)-block-/P85-block-poly {$N$-[N-(2-
          aminoethyl)-2-aminoethyl]aspartamide} (PEG-$b$-/P85-$b$-
          P[Asp(DET)]) ....................................................................................... 65
    3.2.2.4. Preparation of polyplexes .................................................................... 67
    3.2.2.5. Gel retardation analysis ...................................................................... 67
3.2.2.6. Particle size and ζ-potential measurements ........................................... 68
3.2.2.7. In vitro transfection................................................................................. 68
3.2.2.8. In vitro cytotoxicity................................................................................ 69
3.2.2.9. Flow cytometry measurements ................................................................. 69
3.2.2.10. Confocal laser scanning microscopy ..................................................... 70
3.2.2.11. Statistical analysis.................................................................................. 71
3.3. Results............................................................................................................. 71
  3.3.1. Syntheses of PEG-\textit{b}-/P85-\textit{b}-P\{Asp(DET)}........................................... 71
  3.3.2. Formation and stability of polyplexes......................................................... 74
  3.3.3. In vitro transfection efficiency .................................................................... 77
  3.3.4. In vitro cytotoxicity .................................................................................... 79
  3.3.5. Cellular uptake study.................................................................................. 80
  3.3.6. Intracellular trafficking................................................................................ 81
3.4. Discussion......................................................................................................... 83
3.5. Conclusions...................................................................................................... 87

Chapter 4. Particle stability and transfection efficiency evaluations on ternary polyionic complexes with surfaces shielded with different ratios of hydrophilic poly(ethylene glycol) and amphiphilic poly(ethylene oxide)-block-poly(propylene oxide)-block-poly(ethylene oxide) polymers ................. 88

4.1. Introduction....................................................................................................... 89
4.2. Materials and methods.................................................................................... 91
  4.2.1. Materials...................................................................................................... 91
  4.2.2. Methods...................................................................................................... 92
    4.2.2.1 1H NMR and gel permeation chromatography (GPC) analyses .......... 92
4.2.2.2. Synthesis of $\alpha$-methoxy-$\omega$-(2-aminoethyldithio) poly(ethylene oxide)-block-poly(propylene oxide)-block-poly(ethylene oxide) (P(EPE)-SS-NH$_2$) .......................................................... 92

4.2.2.3. Syntheses of poly(ethylene glycol)-block-/P(EPE)-block-/P(EPE)-SS-poly{$N$-$[N$-(2-aminoethyl)-2-aminoethyl]aspartamide} (PEG-$b$-/P(EPE)-$b$-/P(EPE)-SS-P[Asp(DET)]) .......................................................... 94

4.2.2.4. Preparation of polyplexes .......................................................... 96

4.2.2.5. Particle size and $\zeta$-potential measurements ................................ 96

4.2.2.6. In vitro transfection ................................................................. 96

4.2.2.7. In vitro cytotoxicity .................................................................. 97

4.2.2.8. Flow cytometry measurements ............................................... 97

4.2.2.9. Statistical analysis .................................................................. 97

4.3. Results ......................................................................................... 98

4.3.1. Syntheses of PEG-$b$-/P(EPE)-$b$-/P(EPE)-SS-P[Asp(DET)] ............... 98

4.3.2. Formation and stability of binary polyplexes ................................ 101

4.3.3. Reducing environment-sensitivity of P(EPE)-SS-P[Asp(DET)] polyplexes ..... 104

4.3.4. Formation and physical characterizations of ternary polyplexes .......... 105

4.3.5. Stability of ternary polyplexes .................................................. 108

4.3.6. In vitro transfection efficiency and cytotoxicity ............................ 109

4.3.7. Cellular uptake study .................................................................. 113

4.4. Discussion ................................................................................... 114

4.5. Conclusions ................................................................................ 118

---

Chapter 5. General discussion and conclusions ........................................ 120

5.1. Primary findings and conclusions .................................................. 121

5.1.1. Polyplexes with pH-dependent sheddable PEG palisade .................... 121

5.1.2. Polyplexes shielded with Pluronic polymers .................................. 122
5.1.3. Ternary polyplexes shielded with mixture of polyether polymers .......... 123
5.2. Suggestions for future research ......................................................... 125
  5.2.1. Higher stability of reversible linkages ........................................ 125
  5.2.2. Therapeutic genes ..................................................................... 126
  5.2.3. Routes of administration ............................................................ 128
  5.2.4. Specific gene expression .............................................................. 128
5.3. Final remarks .................................................................................. 129

Bibliography ......................................................................................... 131
List of Tables

Chapter 1.

Table 1.1. pH-sensitive polymeric systems for gene delivery. .................................. 12
Table 1.2. Redox potential-sensitive polymeric systems for gene delivery. ................. 15

Chapter 2.

Table 2.1. Characteristics of the polymers synthesized in this study .......................... 43
Table 2.2. Particle sizes of polyplexes formed at N/P = 20 after 12 h incubation in 10 mM Tris-HCl buffer (pH 7.4) containing 150 mM NaCl with or without 10% FBS at 25 °C .......................................................... 47

Chapter 3.

Table 3.1. Characteristics of the polymers synthesized in this study ......................... 73

Chapter 4.

Table 4.1. Characteristics of the polymers synthesized in this study ....................... 101
Table 4.2. Physical characteristics of ternary polyplexes ........................................ 106
List of Figures

Chapter 1.

Fig. 1.1. Structure of PEG-b-P[Asp(DET)] .......................................................... 17

Fig. 1.2. Two-step protonation of the ethylenediamine unit with a distinct conformational change ........................................................................................................... 17

Fig. 1.3. Proposed pathway for the degradation of P[Asp(DET)] through polymer backbone cleavage ............................................................... 19

Fig. 1.4. General structure of Pluronic block copolymer ................................................ 20

Chapter 2.

Fig. 2.1. Schematic illustration of polyplex formation and PEG release from reversibly-PEGylated polyplex in acidic environment ............................................. 26

Fig. 2.2. Synthetic routes of non-PEGylated (nPEG), reversibly-PEGylated (rPEG), irreversibly-PEGylated (irPEG), and PEG-b-P[Asp(DET)] cationic polymers used in this study ........................................................................................................ 29

Fig. 2.3. $^1$H (A) and $^{13}$C NMR (B) spectra of BLA-NCA taken in DMSO-$d_6$ at 25 °C. Differential thermal analysis of BLA-NCA crystals (C). .................. 40

Fig. 2.4. $^1$H NMR spectrum of PBLA taken in DMSO-$d_6$ at 25 °C. ....................... 41

Fig. 2.5. $^1$H NMR spectrum of P[Asp(Hyd-Boc)] taken in DMSO-$d_6$ at 25 °C. ........ 41

Fig. 2.6. $^1$H NMR spectrum of P[Asp(Hyd-Boc)]-b-PBLA taken in DMSO-$d_6$ at 25 °C. .... 41

Fig. 2.7. $^1$H NMR spectrum of P[Asp(Hyd-PEG)]-b-PBLA taken in DMSO-$d_6$ at 25 °C. .... 41

Fig. 2.8. $^1$H NMR spectrum of P[Asp(Hyd-PEG)]-b-P[Asp(DET)] taken in D$_2$O at 80 °C............................................................ 42

Fig. 2.9. $^1$H NMR spectrum of PEG-b-PBLA taken in DMSO-$d_6$ at 25 °C. ............... 42

Fig. 2.10. $^1$H NMR spectrum of PEG-b-P[Asp(DET)] taken in D$_2$O at 80 °C. ............. 42

Fig. 2.11. Gel retardation analysis of polyplexes .......................................................... 43

Fig. 2.12. Ethidium bromide exclusion assay on the polyplexes prepared from nPEG, rPEG, and irPEG polymers. Relative fluorescence intensity vs N/P ratio .......... 44
Fig. 2.13. Cumulant diameters (A) and $\zeta$-potential values (B) of polyplexes formed at various N/P ratios in 10 mM Tris-HCl buffer (pH 7.4) at 25 °C ........................................ 45

Fig. 2.14. Time-dependent particle size change of polyplexes formed at N/P = 20 in 10 mM Tris-HCl buffer (pH 7.4) containing 150 mM NaCl at 25 °C .......................... 46

Fig. 2.15. Time- and pH-dependent PEG release from the rPEG polymer at pH 5 and pH 7.4 at 37 °C .................................................................................................................. 48

Fig. 2.16. Time- and pH-dependent change of $\zeta$-potential at pH 7.4 (A) and pH 5 (B) of polyplexes formed at N/P = 20 at 25 °C .................................................. 49

Fig. 2.17. Gel retardation analysis of naked DNA and rPEG polyplexes after incubation with 10% FBS ........................................................................................................... 51

Fig. 2.18. Transfection efficiency of polyplexes formed at various N/P ratios in MDA-MB-231 cells ........................................................................................................ 52

Fig. 2.19. Cytotoxicity of polyplexes formed at various N/P ratios (A) and LDH release caused by polymers at different amine concentrations (B) in MDA-MB-231 cells ......................................................................................................................... 54

Chapter 3.

Fig. 3.1. Schematic illustration of enhanced cellular uptake by Pluronic-based polyplexes .................................................................................................................. 62

Fig. 3.2. Synthetic routes of Pluronic P85-NH$_2$ (A) and PEG-$_b$-P85-$_b$-P[Asp(DET)] block copolymers (B). ........................................................................................................ 64

Fig. 3.3. $^1$H NMR spectrum of P85-$p$-NPC taken in DMSO-$d_6$ at 25 °C .................. 72

Fig. 3.4. $^1$H NMR spectrum of P85-NH$_2$ taken in DMSO-$d_6$ at 25 °C .................... 72

Fig. 3.5. $^1$H NMR spectrum of PEG-$b$-PBLA taken in DMSO-$d_6$ at 25 °C ................ 72

Fig. 3.6. $^1$H NMR spectrum of P85-$b$-PBLA taken in DMSO-$d_6$ at 25 °C ................ 72

Fig. 3.7. $^1$H NMR spectrum of PEG-$b$-P[Asp(DET)] taken in D$_2$O at 10 °C .............. 73

Fig. 3.8. $^1$H NMR spectrum of P85-$b$-P[Asp(DET)] taken in D$_2$O at 10 °C .............. 73

Fig. 3.9. Gel retardation analysis of polyplexes ................................................................ 74

Fig. 3.10. Cumulant diameters (A), PDI (B), and $\zeta$-potential values (C) of polyplexes formed at various N/P ratios in 10 mM Tris-HCl buffer (pH 7.4) at 25 °C ........ 75
Fig. 3.11. Time-dependent change of particle size (A) and PDI (B) of polyplexes formed at N/P = 10 in 10 mM Tris-HCl buffer (pH 7.4) containing 150 mM NaCl at 25 °C ........................................................................................................ 77

Fig. 3.12. Transfection efficiency of polyplexes formed at various N/P ratios in MDA-MB-231 (A) and A549 (B) cells................................................................. 78

Fig. 3.13. Transfection efficiency of P85-b-P[Asp(DET)] polyplexes formed at N/P = 10 with different concentrations of free P85 in MDA-MB-231 cells .......... 79

Fig. 3.14. In vitro cytotoxicity of polyplexes formed at various N/P ratios (A) and free polymers at different amine concentrations (B) in MDA-MB-231 cells ........ 80

Fig. 3.15. Cellular uptake of Cy5-labeled pDNA polyplexes formed at N/P = 10 in MDA-MB-231 cells. ...................................................................................... 81

Fig. 3.16. Intracellular trafficking analysis by confocal laser scanning microscopy....... 82

Chapter 4.

Fig. 4.1. Schematic illustration of bioreducible ternary polyplex particle......................... 91

Fig. 4.2. Synthetic routes of P(EPE)-SS-NH₂ (A) and PEG-b-/P(EPE)-b-/P(EPE)-SS-P[Asp(DET)] block copolymers (B). ................................................................. 93

Fig. 4.3. ¹H NMR spectrum of P(EPE)-p-NPC taken in DMSO-ᵈ⊖ at 25 °C. ....................... 98

Fig. 4.4. ¹H NMR spectrum of P(EPE)-SS-NH₂ taken in DMSO-ᵈ⊖ at 80 °C. .................... 98

Fig. 4.5. ¹H NMR spectrum of PEG-b-PBLA taken in DMSO-ᵈ⊖ at 25 °C. ....................... 99

Fig. 4.6. ¹H NMR spectrum of P(EPE)-b-PBLA taken in DMSO-ᵈ⊖ at 25 °C. .................... 99

Fig. 4.7. ¹H NMR spectrum of P(EPE)-SS-PBLA taken in DMSO-ᵈ⊖ at 25 °C. ................ 100

Fig. 4.8. ¹H NMR spectrum of PEG-b-P[Asp(DET)] taken in D₂O at 80 °C..................... 100

Fig. 4.9. ¹H NMR spectrum of P(EPE)-b-P[Asp(DET)] taken in D₂O at 10 °C................... 100

Fig. 4.10. ¹H NMR spectrum of P(EPE)-SS-P[Asp(DET)] taken in D₂O at 10 °C.............. 100

Fig. 4.11. Time-dependent particle size change of PEG-b-P[Asp(DET)] polyplexes formed at N/P = 10 with different molecular weights of PEG in 10 mM Tris-HCl buffer (pH 7.4) with 150 mM NaCl at 25 °C........................................... 101
Fig. 4.12. Cumulant diameters (A) and ζ-potential values (B) of polyplexes formed at various N/P ratios in 10 mM Tris-HCl buffer (pH 7.4) at 25 °C ........................... 102

Fig. 4.13. Time-dependent particle size change of polyplexes formed at N/P = 4 in 10 mM Tris-HCl buffer (pH 7.4) containing 150 mM NaCl at 37 °C ..................... 103

Fig. 4.14. Time-dependent change of cumulant diameters of polyplexes formed at N/P = 2 (A) and ζ-potential values of polyplexes formed at N/P = 4 (B) in 10 mM Tris-HCl buffer (pH 7.4) with the presence or absence of 10 mM DTT at 25 °C ........................................................................................................... 104

Fig. 4.15. Particle size (A) and ζ-potential (B) distributions of PEG-b-P(EPE)-b-P[Asp(DET)] ternary polyplexes formed at N/P = 4 with 50% PEG-b-P[Asp(DET)]. ................................................................................................................... 107

Fig. 4.16. Effect of reducing agent on ζ-potential change for ternary polyplexes. ζ-potential values of ternary polyplexes formed at N/P = 4 with different ratios of P(EPE)-SS-P[Asp(DET)] and P(EPE)-b-P[Asp(DET)] in 10 mM Tris-HCl buffer (pH 7.5) after 30 min incubation with the presence or absence of 10 mM DTT at 25 °C ........................................................................................................... 108

Fig. 4.17. Time-dependent particle size change of ternary polyplexes formed at N/P 4 with various compositions of PEG-b-P[Asp(DET)] and P(EPE)-b-P[Asp(DET)] (A) or P(EPE)-SS-P[Asp(DET)] (B) in 10 mM Tris-HCl buffer (pH 7.4) containing 150 mM NaCl at 37 °C ........................................................................................................... 109

Fig. 4.18. Transfection efficiency of binary polyplexes formed at various N/P ratios in MDA-MB-231 (A) and A549 (B) cells ........................................................................................................ 110

Fig. 4.19. Cytotoxicity of binary polyplexes formed at various N/P ratios in MDA-MB-231 (A) and A549 (B) cells ........................................................................................................ 111

Fig. 4.20. Transfection efficiency of ternary polyplexes formed at N/P = 4 with different mixing ratio of PEG- and P(EPE)-based polymers in MDA-MB-231 (A) and A549 (B) cells ........................................................................................................ 112

Fig. 4.21. Cytotoxicity of ternary polyplexes formed at N/P = 4 with different mixing ratio of PEG- and P(EPE)-based polymers in MDA-MB-231 cells ........................................................................................................ 112

Fig. 4.22. Cellular uptake of Cy5-labeled pDNA ternary polyplexes prepared at N/P = 4 with different mixing ratio of PEG- and P(EPE)-based polymers in MDA-MB-231 cells ........................................................................................................ 113
Chapter 5.

Fig. 5.1. Normalized transfection efficiency of polyplexes in this investigation. Transfection efficiency of bPEI polyplexes is set to 100% ................................. 124

Fig. 5.2. 2-Pyridylhydrazone (A) and hydrazone formed with molecule containing aromatic ketone moiety (B)................................................................. 126

Fig. 5.3. Sterically hindered disulfide moieties with more stable thiolytic property........ 126
### List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>$^1$H NMR</td>
<td>proton nuclear magnetic resonance</td>
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<tr>
<td>$^{13}$C NMR</td>
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<td>2-hydroxypyridine</td>
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<td>$P$</td>
<td>p-value</td>
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<tr>
<td>$p$-NPC</td>
<td>4-nitrophenyl chloroformate</td>
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<tr>
<td>P(EPE)</td>
<td>poly(ethylene oxide)-block-poly(propylene oxide)-block-poly(ethylene oxide)</td>
</tr>
<tr>
<td>P(EPE)-b-P[Asp(DET)]</td>
<td>poly(ethylene oxide)-block-poly(propylene oxide)-block-poly{(N-{N-(2-aminoethyl)}-2-aminoethyl)aspartamide}</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>P(EPE)-b-PBLA</td>
<td>poly(ethylene oxide)-block-poly(propylene oxide)-block-poly(ethylene oxide)-block-poly(β-benzyl L-aspartate)</td>
</tr>
<tr>
<td>P(EPE)-p-NPC</td>
<td>poly(ethylene oxide)-block-poly(propylene oxide)-block-poly(ethylene oxide)-p-nitrophenyl carbonate</td>
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<tr>
<td>P(EPE)-SS-P[Asp(DET)]</td>
<td>poly(ethylene oxide)-block-poly(propylene oxide)-block-poly(ethylene oxide)-disulfide-poly{N-[N-(2-aminoethyl)-2-aminoethyl]aspartamide}</td>
</tr>
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</tr>
<tr>
<td>P[Asp(DET)]</td>
<td>poly{N-[N-(2-aminoethyl)-2-aminoethyl]aspartamide}</td>
</tr>
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<td>P85-b-P[Asp(DET)]</td>
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<td>P85-b-PBLA</td>
<td>P85-block-poly(β-benzyl L-aspartate)</td>
</tr>
<tr>
<td>P85-p-NPC</td>
<td>P85-p-nitrophenyl carbonate</td>
</tr>
<tr>
<td>PBLA</td>
<td>poly(β-benzyl L-aspartate)</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
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<tr>
<td>PDI</td>
<td>polydispersity index</td>
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<td>pDNA</td>
<td>plasmid DNA</td>
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<tr>
<td>PEG</td>
<td>poly(ethylene glycol)</td>
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<td>PEO</td>
<td>poly(ethylene oxide)</td>
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<tr>
<td>PEG-b-PLL</td>
<td>poly(ethylene glycol)-block-poly(L-lysine)</td>
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<td>polyethyleneimine</td>
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<td>Abbreviation</td>
<td>Definition</td>
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</tr>
<tr>
<td>PLL</td>
<td>poly(L-lysine)</td>
</tr>
<tr>
<td>PPAA</td>
<td>poly(propylacrylic acid)</td>
</tr>
<tr>
<td>PPO</td>
<td>poly(propylene oxide)</td>
</tr>
<tr>
<td>RES</td>
<td>reticuloendothelial system</td>
</tr>
<tr>
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<td>refractive index</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>rPEG</td>
<td>reversibly-PEGylated</td>
</tr>
<tr>
<td>sc</td>
<td>supercoiled</td>
</tr>
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<td>SCID</td>
<td>severe combined immunodeficiency</td>
</tr>
<tr>
<td>SD</td>
<td>standard deviation</td>
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<tr>
<td>SV40</td>
<td>Simian virus 40</td>
</tr>
<tr>
<td>TFA</td>
<td>trifluoroacetic acid</td>
</tr>
<tr>
<td>THF</td>
<td>tetrahydrofuran</td>
</tr>
<tr>
<td>TRAIL</td>
<td>tumor necrosis factor-related apoptosis-inducing ligand</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet</td>
</tr>
<tr>
<td>UK</td>
<td>United Kingdom</td>
</tr>
<tr>
<td>VEGF</td>
<td>vascular endothelial growth factor</td>
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1 Introduction
1.1. Gene therapy

Gene therapy is a promising approach for treating various intractable diseases, including cancer and hereditary diseases caused by genetic defects. The fundamental concept of gene therapy is the transfer of genetic materials into target cells of a patient for therapeutic purpose.\(^1\) The transferred genetic materials, including both DNA and RNA-based molecules, then correct the genetic disorders by either promoting the expression of naturally occurring proteins for restoring normal protein expression levels, regulating the expression of existing genes, or generating cytotoxic molecules for cell-killing effects.\(^2\)

Compared to conventional therapeutics, gene therapy is considered to be a treatment directly acting at the mutant gene level rather than at the effecting sites of the mutant gene products.\(^3\) Also, it enables therapy based on therapeutic targets which cannot be attacked by small molecules\(^4\) and precludes repeated administrations of proteins or drugs if prolonged gene expression can be achieved.\(^5\) However, due to the susceptibility to rapid enzymatic degradation of nucleic acid-based therapeutics and their anionic and high-molecular-weight nature, it is a great challenge for gene therapeutics to arrive at the target sites while remaining intact and internalize into cells through the plasma membrane, which consists of negatively-charged phospholipids, to elicit therapeutic effects. Therefore, the success of gene therapy highly depends on the development of gene delivery methods or tools which can efficiently, selectively, and safely transport therapeutic genes to the target sites. Unfortunately, a very limited number of vectors have been identified that can fulfill all the mentioned requirements.

The methods of delivering nucleic acid material into cells can be classified into two major categories: physical transfer of gene into cells without the help of a carrier, such as naked DNA
delivery, and hydrodynamic gene delivery, and vector-based delivery utilizing, for example, viruses, lipids, calcium phosphate, or polymers as the vehicles. In the following sections, we will briefly discuss the current state of viral gene delivery and their safety issues which highlight the needs for the development of non-viral vehicles for plasmid DNA (pDNA) delivery.

1.2. Viral gene delivery vehicles

Among the various kinds of gene carriers, viruses are usually considered to be the most efficient vehicles. Viruses were extensively employed for the early studies of human gene therapy and are still the major subjects being investigated in clinical trials. Until 2012, viral vectors, including adenoviruses and retroviruses, account for more than 60% of the total number of vectors used in gene therapy clinical trials. Despite the favorable gene delivery ability of virus, safety issues are always the major concerns of using virus for gene therapy as serious adverse effects, including patient mortality, have been observed since the first clinical trial more than 20 years ago.

Severe immune response to an adenoviral carrier was reported in a gene therapy clinical trial for ornithine transcarbamylase deficiency in 1999. The immune response eventually caused multiple organ failures and patient death 4 days after the initiation of treatment. Cancer-causing insertional mutagenesis was also reported in a gene therapy clinical trial in France between 1999 and 2005 using retroviral vectors for treating X-linked severe combined immunodeficiency (X-linked SCID) in children. The patients were successfully treated by the gene therapy; however, a leukemia-like condition was observed in 4 of 10 children, and this led
the Food and Drug Administration (FDA) to place a temporary halt in 2003 on all gene therapy trials using retroviral vectors.\textsuperscript{26} Furthermore, in 2008, 1 of 10 children in a similar clinical trial for SCID in the UK also developed leukemia.\textsuperscript{27,28} In the last two decades of gene therapy clinical trials, the FDA has not yet approved any gene therapy product for human use. This lack of approved gene-based therapeutics on the market can be attributed to the intrinsic safety issues of virus-based gene therapy, such as immune responses\textsuperscript{29} and the risk of insertional mutagenesis,\textsuperscript{30,31} which have shifted the attention of researchers to develop safer non-viral alternatives for gene delivery in recent years.

1.3. Non-viral gene delivery vehicles

Non-viral gene delivery vectors, including liposomes,\textsuperscript{13,14} polyionic complexes (polyplexes),\textsuperscript{32,33} polymersomes,\textsuperscript{34,35} are relatively safer substitutes for viral vehicles in terms of immunogenicity and the risk of host genome integration. Moreover, non-viral vectors have higher capacity of nucleic acid cargo, and higher flexibility of chemical production and modification, making them desirable alternatives to viruses.\textsuperscript{36,37} In general, cationic synthetic materials, such as polyethylenimine (PEI),\textsuperscript{16} 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP),\textsuperscript{38} are employed for neutralizing the anionic charges on pDNA molecules and condensing them into sub-micron sized particles through electrostatic interaction. These nano-sized particles allow better cellular uptake of the genetic materials into cytoplasm and subsequent exertion of therapeutic effects. Comparing lipid-based and polymer-based non-viral gene delivery vehicles, polyplexes formed from cationic polymers and anionic nucleic acids possess an advantage in preparation method. The formation of polyplexes usually requires
simple mixing of polymers and nucleic acids. Also, there is no down-sizing process needed as in the preparation of liposomes or polymersomes. In the following sections, we will focus on gene delivery systems using synthetic cationic polymers.

1.4. Polyionic complexes

The formation of polyionic complexes (polyplexes) is achieved by a condensation process of pDNA induced by electrostatic interaction between cationic polymer and anionic pDNA molecules. Due to the difficulty in monitoring the condensation process of pDNA molecules, the major mechanism or driving force of the process is still controversial, but the possible dominant mechanisms can be categorized into two groups: (1) electrostatic interaction between oppositely-charged molecules and (2) increase in entropy through the release of structured water molecules and low-molecular-weight counterions. The condensation process converts the micron-sized pDNA molecules to polyplex particles with sizes generally less than 200 nm based on the coil-globule structural transition of pDNA molecules as demonstrated by dye exclusion and fluorescence microscopy studies. The size reduction and the charge neutralization of pDNA molecules after complexation make the transfer of nucleic acid across the plasma membrane more efficient through endocytosis of the polyplex particles. Nuclease resistance of complexed pDNA is another advantage of the polyplex-based gene delivery systems, especially for systemic delivery applications. While naked pDNA could be degraded by nuclease within minutes, pDNA complexed with cationic polymers was demonstrated to maintain integrity for hours.
In a static light scattering study using polyplexes prepared from poly(ethylene glycol)-block-poly(L-lysine) (PEG-b-PLL) and pDNA, it was estimated that one polyplex particle contained about 1–2 pDNA molecules and the number of pDNA molecules per polyplex particle was dependent on the nitrogen to phosphate (N/P) ratio during mixing and the chain length of the cationic polymer. A general trend of the dependency of physical characteristics of polyplexes on polymer chain length cannot be obtained from contrasting reports in the literature. Despite the uncertainty, polyplex particles can be present as rod-like, toroid-like, or spherical structures and the physical properties of the particles are highly dependent on the chain length of the cationic polymer, polymer charge density, mixing N/P ratio, and ionic strength of the dispersant.

1.5. Polymeric gene delivery vehicles

Synthetic cationic polymers have been extensively used for non-viral gene delivery through the formation of polyplex particles by electrostatic interaction between the polymer and nucleic acid molecules. Cationic or protonatable functional groups, including amino, amidinium, and guanidium groups, are usually found on cationic polymers either locating on the polymer backbone or existing as side chain moieties. Poly(L-lysine) and polyethylenimine (PEI) are two of the commercially available cationic polymers commonly used in the early studies of non-viral gene delivery. As a biodegradable polypeptide, poly(L-lysine) possesses ε-amino groups (pKₐ ~9.5) which are protonated at physiological pH and thus can form polyplex particles upon mixing with pDNA. However, poly(L-lysine)/pDNA polyplexes have been shown to suffer from low transfection efficiency and it is generally accepted that the unsatisfactory efficiency is due to
the lack of endosomal escape ability of the endocytosed polyplex particles. Therefore, poly(L-lysine)/pDNA polyplexes always require the addition of endosomolytic agent, for example, chloroquine, which helps disrupt the endosomal or lysosomal membrane and subsequently allows the polyplexes to escape from the endo/lysosomes,\textsuperscript{58} in order to produce a modest level of transfection activity.\textsuperscript{48,59}

In contrast to poly(L-lysine), PEI has been demonstrated to transfect cells much more efficiently even without the aid of any endosomolytic agents.\textsuperscript{60} The higher transfection ability of PEI is usually ascribed to the presence of the large number of secondary and tertiary amines in addition to the primary amines at high density on the polymer. This structural characteristic allows the protonation level of the polymer to substantially increase from approximately 20 to 45\% when pH drops from 7 to 5. The drop in pH roughly corresponds to the pH change experienced by PEI/pDNA polyplexes after endocytosis. The protonation process basifies the endo/lysosomes, causing the endosomal ATPase proton pumps to transport more protons to the endosomal vesicles to maintain the pH. Influx of protons and the counter ions leads to osmotic swelling and disruption of endosome. Eventually, the polyplex particles can be released into cytoplasm. This is known as the proton sponge hypothesis,\textsuperscript{61} but whether this hypothesis is the mechanism improving transfection activity is debatable.\textsuperscript{52,62} Even though PEI showed gene transfer ability in both \textit{in vitro} and \textit{in vivo} settings,\textsuperscript{16} the transfection efficiency is still lower than that of virus.\textsuperscript{63} Additionally, the non-biodegradable and cytotoxic natures limit the practical use of PEI.\textsuperscript{2,64} As a result, different kinds of cationic polymers have been developed in recent years aiming at enhancing the transfection efficiency and lowering the cytotoxicity. In particular, polymers installed with environmentally-sensitive elements, which are capable of responding to
the change of physiological environment, are one of the focuses in the non-viral gene delivery field.

1.5.1. PEG dilemma

In order to increase the colloidal stability of the polyplex particles under physiological conditions, poly(ethylene glycol)ylation (PEGylation) of particles is a common and effective strategy employed by many researchers.\(^53,65,66\) PEGylation has been extensively used on polymer-,\(^67\) liposome-,\(^68\) or even virus-based delivery vehicles.\(^69\) By forming a dense hydrophilic and sterically hindered PEG palisade around the particle core, the tethered PEG chains can minimize the interaction among particles and/or between particles and biomolecules during systemic circulation.\(^70\) The PEG palisade also allows particles to endure the high salt concentration in physiological environment.\(^66\)

PEGylation can increase the stability of polyplex particles and is important for in vivo applications, but the transfection efficiency of the PEGylated complexes is always shown to be lower than that of the non-PEGylated counterparts.\(^71,72\) It has been suggested that the lower transfection activity caused by PEGylation could be related to lower cellular uptake of particles, diminished endosomal escape of particles, or impaired release of pDNA from the particles.\(^66,71,73\) The compromised transfection efficiency caused by PEGylation is known as the PEG dilemma. Different groups have developed gene-delivering nanoparticles with temporary PEGylation allowing triggered PEG release in response to pH,\(^72\) glutathione level,\(^71\) or enzyme overexpression;\(^74\) and particles with targeting ligands installed at the distal ends of PEG chains\(^75\) aimed at improving transfection efficiency while maintaining particle stability.
1.5.2. Environmentally-sensitive gene delivery vehicles

To improve the transfection ability of non-viral gene delivery systems, researchers have designed and developed dynamic polymeric gene delivery vehicles which are able to respond to external signals such as temperature, ultrasound, and magnetic field. For a delivery system sensitive to an artificially-applied trigger, an external stimulus is usually applied at the therapeutic target site to generate a distinct microenvironment, for example, higher temperature or magnetic flux, in a local region compared to the rest of the body. After administration and during systemic circulation, the specially engineered gene delivery vehicle can then respond to the stimulus to enhance the transfection activity at the corresponding areas either through concentrating the particles, or specifically releasing DNA cargo at the target sites.

Environmentally-sensitive gene delivery vehicles were also developed to respond to the intrinsic physiological stimuli including pH, redox potential, ionic strength, and enzymatic activities. The aim was to promote transfection efficiency at the desired regions by exploiting the specific physiochemical variations between disease sites and healthy tissues or between extracellular and intracellular environments at the target. At the tissue level, for example, tumor tissues have been shown to be more acidic than healthy tissues. The lower pH value of the tumor tissues arises from the rapid growth rate of cancer cells, leading to a high level of glucose consumption and lactic acid accumulation, and insufficient blood supply at the tumor sites. Other than the acidity of tumor tissues, the overexpression of specific enzymes at tumor sites is also considered a unique trigger for stimulus-sensitive gene delivery systems designed to respond at the tissue level. Matrix metalloproteinases (MMPs) are related to tumor progression and substantially overexpressed in cancer tissues. MMP family has once been
suggested to be a promising target for cancer therapy. Although the results in human clinical trials of MMP inhibitors were not impressive, nanoparticles employing MMP-cleavable elements have been widely investigated for drug delivery, gene delivery, and imaging purposes due to their tumor-selectivity.

Apart from responding at the tissue level, polymeric vehicles such as PEI and poly(α-alkyl acrylic acid) were designed to exploit the pH gradient along the endocytic pathway at the cellular level to improve gene delivering ability. Depending on the particle sizes, polyplexes prepared from polyelectrolytes and pDNA commonly internalized into the cells through clathrin-mediated endocytosis or macropinocytosis. As both endocytic pathways can lead the endocytosed particles to endosomal or lysosomal compartments, the polyplexes experience a drop in pH from approximately 7.4 to 5. Polymeric gene delivery vehicles can thus be engineered to undergo protonation, deprotonation, or a charge conversion process in order to disrupt the endo/lysosomal membrane through the proton sponge effect or direct membrane interaction.

It has been reported that the intracellular environment of certain tumor tissues is more reducing than that of normal tissues due to the elevated level of glutathione and which was shown to be responsible for the enhanced survival and resistance to chemotherapy-induced apoptosis of cancer cells. Although variation of glutathione exists between cancer and healthy cells, a redox potential gradient is generally utilized as a physiochemical stimulus at the cellular level, as the extracellular environment is usually considered to be mildly oxidizing while the intracellular milieu is highly reducing. The dramatic redox potential difference across the plasma membrane is attributed to the presence of redox proteins such as thioredoxin and millimolar...
concentration of glutathione inside the cells; in contrast, glutathione only presents in micromolar concentration extracellularly. Although relatively more stable in blood circulation, disulfide linkages can be promptly cleaved, mediated by glutathione and/or redox enzymes in the intracellular reducing environment and therefore they are heavily employed in designing bioresponsive delivery system. Owing to the facile chemistry to introduce reversible linkages to delivery vehicles and no external stimuli are required for triggering the desired response, pH- and redox potential-responsive vehicles have been frequently developed for gene delivery purposes. Several representative gene delivery systems which are capable of reacting to pH or reducing stimulus will be introduced in the following sections.

1.5.2.1. pH-sensitive gene delivery vehicles

Synthetic polymeric systems utilizing pH-sensitive functionalities for enhanced endosomal escape of polyplex particles are summarized in Table 1.1. Cationic polymers capable of buffering at the low pH in the endo/lysosomal compartments can facilitate cytoplasmic delivery of polyplexes. Examples are PEI,16 imidazole-containing cationic polymers,105-107 and poly{N-[N-(2-aminoethyl)-2-aminoethyl]aspartamide} (P[Asp(DET)]),108,109 which will be described in detail in section 1.5.3. Apart from polycations, anionic polymers, poly(propylacrylic acid) (PPAA)110-113 and oligomeric sulfonamides114 also take advantage of the acidity in endosomes to improve transfection efficiency. As the pKa value of PPAA is about 6.0, PPAA deprotonates in the endosomes. The deprotonation increases the hydrophobicity of the polymer which allows the polymer to interact with the endosomal membrane and subsequently cause endosomal
<table>
<thead>
<tr>
<th>Mechanism</th>
<th>Polymer / functional group</th>
<th>Polymer / functional group structure</th>
<th>Reference</th>
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<td>Endosomal escape</td>
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<td></td>
<td></td>
<td>Imidazole group pKa = 6.0</td>
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<td></td>
<td>P[Asp(DET)]</td>
<td><img src="image" alt="Imidazole-containing cationic polymers" /></td>
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<td></td>
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<td>pKa = 6.3 and 9.1</td>
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<td>Ortho ester</td>
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<tr>
<td></td>
<td>dimethoxine)</td>
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</table>

Table 1.1. pH-sensitive polymeric systems for gene delivery.
disruption. Through incorporation in cationic lipoplex formulation, PPAA was shown to enhance transfection efficiency in both in vitro and in vivo studies.61,62

Temporary PEGylation of polyplexes through pH-sensitive chemical linkages, for example, acetal,63,64 hydrazone,65,66 and ortho ester linkages,67 has been tested by different research groups and shown to enhance transfection efficiency through triggered PEG release in acidic environment (Table 1.1). Reversible PEGylation of particles can also be achieved by pH-dependent electrostatic interaction between polyelectrolytes. Bae and coworkers demonstrated that pH-sensitive release of PEG could be obtained based on PEGylation of polyplexes through complexation between cationic PEI/pDNA polyplexes and anionic PEG-β-poly(methacyloyl sulfadimethoxine) polymers.68 All these systems in general are designed to utilize the pH difference between tumor and normal tissues to promote the cellular uptake of polyplexes to tumor cells or to exploit the pH change along the endocytic pathway to facilitate the endosomal escape of particles after the dePEGylation process.

1.5.2.2. Redox potential-sensitive gene delivery vehicles

PEG-detachable gene delivery vehicles can also be obtained using disulfide linkages to connect the PEG chain to the polymer backbone (Table 1.2) to overcome the PEG dilemma by maintaining both colloidal stability and transfection efficiency of polyplex particles.69,70-72 Based on the redox potential gradient across plasma membrane as described in the previous section, polyplexes can be stably protected by the PEG shell during blood circulation as the disulfide linkages are intact in non-reducing condition. Site-specific PEG release through reduction of the disulfide bond can be achieved preferentially inside the cells, minimizing the
transfection hindrance produced by the PEG palisade. However, studies have also demonstrated that the disulfide linkages are not absolutely stable in the extracellular milieu and PEG release could begin before particle endocytosis.\textsuperscript{119} Premature PEG release can occur especially at the extracellular region close to the cellular membrane; a location with an abundance of protein disulfide-isomerase,\textsuperscript{122,123} which catalyzes the sulfhydryl-disulfide exchange, and has a higher concentration of free thiol secreted from the cells.\textsuperscript{121}

High-molecular-weight polycations are commonly used for forming polyplexes with pDNA due to their ability to produce stable particles through effective pDNA condensation. However, low-molecular-weight analogs have been shown to cause lower cytotoxicity and decondense nucleic acid cargo more effectively and thus improve the transfection efficiency.\textsuperscript{124} To take advantages of both high- and low-molecular-weight cationic polymers, disulfide linkage has been employed to connect small amine-containing molecules together to generate high-molecular-weight polycations for gene delivery (Table 1.2).\textsuperscript{125-130} Due to the high molecular weight, the disulfide-linked polycations can form stable polyplexes with pDNA and maintain the particle integrity in the extracellular environment. Once the polyplexes enter the cells, the disulfide linkages on the polymer backbones will be promptly cleaved under the intracellular reducing environment, resulting in a more rapid release process of pDNA and less toxic low-molecular-weight fragments.\textsuperscript{131} Eventually, the bioreducible carriers can achieve comparable transfection efficiency to the high-molecular-weight polycations while improving the biodegradability and solving the cytotoxicity issue at the same time.
<table>
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<th>Mechanism</th>
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<th>Polymer structure</th>
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<td>Temporary PEGylation</td>
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<td>PEG-SS-CWK_{18}</td>
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<td>Reducible polymer backbone</td>
<td>P(-S-CK_{10}C-S-)</td>
<td><img src="image" alt="Structure" /></td>
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<tr>
<td>Polyplex cross-linking</td>
<td>Poly(disulfide amine)</td>
<td><img src="image" alt="Structure" /></td>
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<td>BPEI-SS-S</td>
<td><img src="image" alt="Structure" /></td>
<td>128</td>
</tr>
<tr>
<td></td>
<td>PEG-b-PLL-PDP</td>
<td><img src="image" alt="Structure" /></td>
<td>55,132,133</td>
</tr>
<tr>
<td></td>
<td>PEI polyplex + dithiobis(succinimidyl propionate) (DSP) cross-linker</td>
<td><img src="image" alt="Structure" /></td>
<td>134,135</td>
</tr>
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</table>

Table 1.2. Redox potential-sensitive polymeric systems for gene delivery.
Polyplexes with reversibly cross-linked core can be prepared through complexation between cationic polymers and pDNA, and subsequent oxidation of the pre-installed sulfhydryl moieties or coupling of disulfide-containing cross-linkers (Table 1.2). Core cross-linked polyplex particles have demonstrated high colloidal stability as they have excellent endurance against the polyionic exchange reaction and high salt condition, which are two major triggers for polyplex dissociation; however, the particles could rapidly dissociate in the presence of reducing agents. Furthermore, Kissel and coworkers reported that the blood concentrations of polyplexes after systemic administration could be enhanced through particle cross-linking and the biodistribution profiles could be further modulated by adjusting the cross-linking level of the particles. Collectively, these results suggest that disulfide cross-linking can improve the extracellular particle stability by diminishing the dissociation of polyplexes and promote the release of nucleic acid materials from polyplexes. Also, reversible cross-linking allows the polyplex dissociation process to restore rapidly and preferentially in the intracellular environment after thiolytic cleavages of the disulfide cross-links in the presence of reducing stimulus such as glutathione or redox proteins.

1.5.3. Poly(ethylene glycol)-block-poly{N-[N-(2-aminoethyl)-2-aminoethyl]aspartamide} block copolymer for gene delivery

In 2006, Kataoka and coworkers generated a series of PEG-block-cationic polymers by substituting the flanking benzyl ester groups of PEG-block-poly(β-benzyl L-aspartate) with amine-containing molecules and investigated the in vitro transfection efficiency in cancer and primary cell lines. One of the candidates in that study, PEG-block-poly{N-[N-(2-aminoethyl)-
2-aminoethyl]aspartamide} (PEG-\textit{b}-P[Asp(DET)], Fig. 1.1), demonstrated exceptional gene delivery efficiency, producing comparable transfection efficiency to ExGen500, a commercial product of linear PEI, while generating substantially lower cytotoxicity than both linear and branch PEI.

In another study, the authors ascribed the high transfection ability of PEG-\textit{b}-P[Asp(DET)] to its two pK\textsubscript{a} values (~6.3 and ~9.1) of the two amino groups on the ethylenediamine side chains, allowing two-step protonation with a distinct gauche-anti conformational transition (Fig. 1.2) when the pH drops from neutral in the extracellular environment to acidic in the endo/lysosomal compartments. Furthermore, results obtained from intracellular trafficking studies of P[Asp(DET)]- and poly(L-lysine)-based polyplexes showed the superior transfection efficiency of P[Asp(DET)]-based polyplexes was correlated with their facilitated endosomal escape ability which could be due to the strong buffering and/or direct endosomal membrane destabilizing.
effect based on the pH-dependent protonation event of the ethylenediamine side chains on the P[Asp(DET)] polymers.¹⁰⁹

1.5.3.1. In vitro and in vivo gene delivery

The *in vitro* gene transfer efficiency of PEG-b-P[Asp(DET)] polyplexes has been tested in various cancer cell lines,⁷¹,¹³⁸ primary cells,³³ and multicellular tumor spheroids.¹⁰⁸ In general, P[Asp(DET)] polyplexes prepared at high N/P ratios such as 40–60 could achieve very comparable or even higher transfection activity than PEI, while maintaining minimal toxicity. PEG-b-P[Asp(DET)] polyplexes have also been investigated for *in vivo* applications of pDNA delivery, including bone regeneration,¹³⁹ treatments for vascular lesions,¹⁴⁰ pulmonary arterial hypertension,¹⁴¹ and cornea neovascularization.¹⁴² Consistent with the *in vitro* results, polyplexes prepared at high N/P ratios with excess amounts of cationic polymers were required to achieve effective therapeutic effect *in vivo*; however, the toxicity generated by the polyplexes was still much lower than that of polyplexes prepared with PEI according to histological studies and quantifications of inflammatory cytokine mRNA after treatments.¹⁴¹

The high N/P ratio requirement for PEG-b-P[Asp(DET)] gene delivery system to exert therapeutic effects restricts the *in vivo* applications to local treatments. This limitation imposes a barrier to systemic administration because free polymers may not be able to concentrate at the target sites along with the pDNA polyplex particles. Recently it was revealed that this strict requirement of high N/P ratio for effective transfection was abolished by introducing a cholesteryl moiety to the ω-end of the PEG-b-P[Asp(DET)] block copolymer.¹⁴³ The polyplexes forming at low N/P ratios from cholesterol-modified PEG-b-P[Asp(DET)] and pDNA
demonstrated orders of magnitude higher transfection efficiency than the polyplexes prepared with conventional PEG-b-P[Asp(DET)] in vitro and successfully delayed tumor growth in tumor-bearing mice after systemic administration.

1.5.3.2. Cytotoxicity and biodegradability

Apart from high transfection efficiency, low cytotoxic nature also makes the PEG-b-P[Asp(DET)] block copolymer a strong candidate as a gene delivery vehicle. The low toxicity property of the polymers or polyplexes prepared from PEG-b-P[Asp(DET)] and pDNA has been shown in various in vitro and in vivo studies even at high N/P ratios.\textsuperscript{108,141} Moreover, the risk of time-dependent cytotoxicity caused by P[Asp(DET)]-based polymers was suggested to be minimal based on a pharmacogenomic analysis of global gene expression in transfected cells.\textsuperscript{144} Itaka et al. hypothesized that the lack of toxicity of P[Asp(DET)] was due to the biodegradability of the polymer, and they demonstrated that P[Asp(DET)] could be rapidly degraded at physiological temperature and the degradation products were non-toxic to cells.\textsuperscript{145} Although the exact mechanism for P[Asp(DET)] degradation is unclear, the authors proposed that the biodegradation is likely caused by cleavage of the polymer backbone under physiological conditions as shown in Fig. 1.3, where the five-membered succinimide formation is a critical step for the degradation, based on mass spectrometry analysis.

![Proposed pathway for the degradation of P[Asp(DET)] through polymer backbone cleavage.](image)

**Fig. 1.3.** Proposed pathway for the degradation of P[Asp(DET)] through polymer backbone cleavage.\textsuperscript{145}
1.5.4. Pluronic block copolymers for gene delivery

As amphiphilic triblock copolymers with a hydrophobic poly(propylene oxide) (PPO) block situated in between two hydrophilic poly(ethylene oxide) (PEO) blocks (Fig. 1.4), Pluronic block copolymers have been commonly used as pharmaceutical excipients. Over 30 Pluronic block copolymers with various molecular weights of the PPO and PEO blocks, hydrophilic-lipophilic balance (HLB), and critical micelle concentrations (CMCs) are commercially available from BASF Corp. Pluronic polymers have been studied as controlled release and delivery systems for a wide range of cargoes including viruses,\textsuperscript{146} small molecule drugs,\textsuperscript{147,148} and biomacromolecules such as peptides\textsuperscript{149} and nucleic acids.\textsuperscript{150} In terms of gene delivery, Pluronic block copolymers co-administrated as free polymers were shown to facilitate transfection based on different gene delivery methods. It was reported that the expression of naked pDNA could be improved when it was administrated intramuscularly along with free Pluronic P85 polymers.\textsuperscript{151,152} For non-viral carrier-based delivery, free Pluronic polymers promote the transfection ability of various cationic polymers such as poly(N-ethyl-4-vinylpyridinium bromide),\textsuperscript{153} poly(L-lysine),\textsuperscript{154} PEI,\textsuperscript{155} and lipid-based vehicles.\textsuperscript{156}

Pluronic-conjugated polycations were also synthesized and tested for transfection activity. Transfection efficiency of polyplexes based on cationic homopolymers such as PEI demonstrated promising \textit{in vitro} gene delivery efficiency in early investigations. However, the low gene expression level after systemic delivery of polyplexes impaired the practical \textit{in vivo} applications;

![General structure of Pluronic block copolymer.](image)
a consequence probably caused by colloidal instability of the particles.\textsuperscript{65} Despite common use of PEGylation of nanoparticles for improving the colloidal stability of particles, transfection efficiency diminished simultaneously due to the lack of cellular uptake of the PEGylated polyplex particles.\textsuperscript{66,71} Given that the Pluronic polymers are more hydrophobic and better able to interact with the plasma membrane compared to PEG, Pluronic-conjugated polycations were synthesized and employed for transfection studies aimed at overcoming the PEG dilemma.\textsuperscript{157,158} Pluronic-modified polycations possessing different architectures such as graft copolymers and block copolymers were generated using various Pluronic block copolymers and polycations. Transfection efficiency and colloidal stability of the polyplexes prepared with Pluronic-conjugated polycations and pDNA were compared to those of the PEGylated counterparts.

In general, the transfection activity of the Pluronic-based polyplexes was demonstrated to be higher than that of the PEG-based particles, but the Pluronic-based particles tended to aggregate due to the amphiphilicity of the Pluronic palisade surrounding the particles. Different studies demonstrated that the inclusion of free Pluronic polymers during Pluronic-conjugated polycation/pDNA polyplex preparation could enhance the particle stability without compromising the transfection activity, and higher transfection efficiency was observed in some cases.\textsuperscript{150,157-159} The authors suggested that the PPO blocks of the free Pluronic polymers interacted with the PPO blocks of the conjugates based on hydrophobic interaction and the PEO blocks of the free Pluronic would be present on the polyplex surface. The interaction resulted in masking the hydrophobic PPO blocks from aqueous environment and forming more stable polyplex particles.
In summary, Kabanov and coworkers speculated the general mechanisms by which Pluronic polymers assisting the gene expression of the polyplex-delivered pDNA were: First, the interaction between Pluronic polymers and plasma membrane enhance the cellular internalization of polyplex particles.\textsuperscript{153,159} Second, Pluronic polymers promote the translocation of pDNA from the cytoplasm to the nucleus.\textsuperscript{150} Third, the improvement of dispersion stability after the addition of free Pluronic polymers to polyplex particles may further promote transport of polyplex particles into cells.\textsuperscript{159}

1.6. Research proposal

1.6.1. Rationale

In spite of the high transfection efficiency of viral-vectors, safety concerns are still the major barriers for viral-based gene therapy to proceed forward in clinical trials.\textsuperscript{21} As a safer alternative to virus, polymeric non-viral vehicles, on the contrary, suffer from low gene delivering ability which hampers their translation into practical use. Low transfection ability can be caused by, for example, low extracellular stability, poor cellular uptake, insufficient endosomal escape and nuclear transfer of therapeutic genes.\textsuperscript{160} Among these, colloidal stability of the polyplex particles formed with cationic polymers and pDNA can be one of the most crucial prerequisites for successful \textit{in vivo} gene delivery. Particle stability governs the capability of the polyplexes to withstand the harsh physiological environments encountered and thus the ability for the particles to reach the therapeutic targets. PEGylation of polyplexes is a common method to improve their colloidal stability; nonetheless, lower transfection activity is always observed with PEGylated polyplexes compared to non-PEGylated counterparts.\textsuperscript{71,72}
1.6.2. Objectives

The objectives of this dissertation were to develop and physicochemically and biologically characterize polyplex systems based on poly\{N-[N-(2-aminoethyl)-2-aminoethyl]aspartamide\} (P[Asp(DET)]) cationic polymers aiming at overcoming the PEG dilemma and balancing the colloidal stability and transfection efficiency of the polyplexes.

1.6.3. Specific aims

1. To synthesize a pH-sensitive multi-PEGylated cationic polymer which can maintain the PEGylation of polyplex particles at neutral pH while detaching PEG chains from the particles at acidic pH. The colloidal stability of particles can thus be enhanced through PEGylation and release of PEG can occur in response to the drop of pH in the acidic tumor microenvironment or intracellular endo/lysosomal compartments for improving transfection efficiency.

2. To investigate the effects of substituting the PEG block on PEG-\textit{b-}[Asp(DET)] with Pluronic P85 on particle stability, cellular uptake, and transfection activity of polyplexes prepared with the cationic block copolymers.

3. To evaluate the colloidal stability and transfection efficiency of ternary polyplexes with surface covered with different ratios of hydrophilic PEG and amphiphilic poly(ethylene oxide)-\textit{block-poly(propylene oxide)-block-poly(ethylene oxide)} (P(EPE)) polymers.
Poly(ethylene glycol)-detachable polyionic complexes based on poly\{N-[N-(2-aminoethyl)-2-aminoethyl]aspartamide\} cationic polymers for \textit{in vitro} plasmid DNA delivery

2.1. Introduction

PEGylation is considered to be an effective way to improve the colloidal stability of polyplex systems; however, compromised transfection efficiency is always obtained for the PEGylated polyplexes compared to the non-PEGylated counterparts.\textsuperscript{71,72} To overcome the PEG dilemma, we have designed and synthesized a reversibly-PEGylated p[Asp(Hyd-PEG)]-b-p[Asp(DET)] block copolymer with a unique architecture based on a poly(aspartamide) backbone in this chapter. The cationic polymer possessing one block, poly(aspartamide) derivative bearing acylhydrazides (p[Asp(Hyd)]), for multi-PEG conjugation; and another block, poly(aspartamide) derivative bearing cationic ethylenediamine side chains (p[Asp(DET)]), for DNA condensation and endosomal escape, was used as a bioresponsive vehicle aimed at promoting the transfection efficiency and colloidal stability.

The hydrazone linkage employed in PEGylation in this study is pH-sensitive and is stable at neutral pH.\textsuperscript{104} PEGylation using hydrazone bond can provide high stability for the polyplexes during systemic circulation and thus prolonged circulation time can be achieved. On the other hand, the hydrazone linkage is hydrolyzed rapidly at acidic pH and nanoparticles with size smaller than 200 nm are usually shown to employ the clathrin-mediated endocytosis for cell internalization;\textsuperscript{95,161} therefore, the PEG chains are anticipated to dissociate from the polyplexes promptly after endocytosis of the polyplexes as the pH gradually decreases from 7.4 (physiological pH) to 6 (endosomal pH) and then to 5 (lysosomal pH) along the endocytic pathway (Fig. 2.1).\textsuperscript{96,97} Through the release of PEG from the first block of the polymer at acidic pH, the charges on the cationic segment (second block) of the polymer could be more exposed to the negatively charged endosomal membrane. This charge exposure could trigger the disruption
of the endosomal membrane through electrostatic interaction and enhance the endosomal escape of the polyplexes. Furthermore, the reduction in steric hindrance after PEG release could promote efficient release of DNA from the polyplexes following endosomal escape.\textsuperscript{71} By exploiting the decrease in pH along the endocytic pathway and the temporary attachment of PEG through hydrazone linkages, it is expected that the reversibly-PEGylated p[Asp(DET)]-based polycation synthesized here could achieve appreciable polyplex stability without compromising the transfection efficiency.

2.2. Materials and methods

2.2.1. Materials

\(\alpha\)-Methoxy-\(\omega\)-(3-oxopropoxy) poly(ethylene glycol) (PEG propionaldehyde, \(M_n = 5000\) g/mol, \(M_w/M_n = 1.02\)) and \(\alpha\)-methoxy-\(\omega\)-amino poly(ethylene glycol) (PEG-NH\(_2\), \(M_n = 12300\) g/mol, \(M_w/M_n = 1.03\)) were obtained from Nippon Oil and Fats Co., Ltd. (Tokyo, Japan). L-Aspartic acid \(\beta\)-benzyl ester, benzene, branched polyethylenimine (bPEI, \(M_w = 25000\) g/mol), \(n\)-butylamine, dichloromethane (DCM), diethylenetriamine (DET), \(N,N\)-dimethylformamide
(DMF), dimethyl sulfoxide (DMSO), hexane, 2-hydroxypyridine (2-HP), N-methyl-2-pyrrolidone (NMP), sodium cyanoborohydride (NaBH$_3$CN), trifluoroacetic acid (TFA), tetrahydrofuran (THF), $\text{tert}$-butyl carbazate, and triphosgene were purchased from Sigma-Aldrich (St. Louis, MO). $\text{n}$-Butylamine and DET were distilled by conventional methods before use. DCM, DMF, DMSO, hexane, NMP, and THF were purchased as anhydrous grade and used without further purification. Dialysis tubings (MWCOs 1000 and 6–8000) were purchased from Spectra/Por (Rancho Dominguez, CA). Amicon ultra-15 centrifugal filter units (MWCO 30000) were purchased from Millipore (Billerica, MA). The pDNA coding for luciferase in pGL3-C vector with a SV40 promoter (Promega, Madison, WI) was amplified in competent DH5$\alpha$ Escherichia coli cells and purified using PureLink™ HiPure Plasmid Maxiprep Kit (Invitrogen, Carlsbad, CA). Luciferase Assay System Kit, CellTiter-Blue® Cell Viability Assay, CytoTox-ONE™ Homogeneous Membrane Integrity Assay, and ethidium bromide (EtBr) were purchased from Promega (Madison, WI). RC DC Protein Assay Kit was purchased from Bio-Rad (Hercules, CA). RESOURCE S 1 mL cationic exchange column and AKTA FPLC system were purchased from GE Healthcare. MDA-MB-231 human breast cancer cell line was obtained from ATCC.

2.2.2. Methods

2.2.2.1. $^1$H, $^{13}$C NMR, and gel permeation chromatography (GPC) analyses

The $^1$H or $^{13}$C NMR spectra of polymers or molecules were obtained with Varian Unity-Inova 400 MHz or 500 MHz NMR spectrometer (Palo Alto, CA) with temperature regulated at the designated temperature. The concentration of polymers or molecules in NMR solvents was
kept at 10 mg/mL. Chemical shifts were reported in ppm relative to the residual protonated solvent resonance. The polymer molecular weight distributions were monitored using Agilent 1100 series HPLC equipped with TOSOH TSK-gel G3000PWXL and G4000PWXL GPC columns in series with temperature regulated at 40 °C and an internal refractive index (RI) detector. DMF with 10 mM LiCl was used as the eluent at a flow rate of 1 mL/min. The concentration of polymer samples in DMF was maintained at 2 mg/mL for injection. PEG standards were used for calibration.

2.2.2. Synthesis of β-benzyl-L-aspartate N-carboxy anhydride (BLA-NCA)

The synthetic schemes of the polymers used in this study are summarized in Fig. 2.2. β-Benzyl-L-aspartate N-carboxy anhydride (BLA-NCA) was synthesized by the Fuchs-Farthing method using L-aspartic acid β-benzyl ester and triphosgene. Vacuum-dried L-aspartic acid β-benzyl ester (15.0 g, 0.0672 mol) was suspended in dry THF and 0.433 equiv of triphosgene (8.66 g, 0.0292 mol) dissolved in dry THF was added in a stream of argon. The reaction was run at 40 °C for 2 h and the reaction mixture became clear. BLA-NCA was then recrystallized by adding minimal amount of dry hexane to THF. The purified BLA-NCA was stored at -20 °C under argon until use. The molecular structure of BLA-NCA was determined by 1H and 13C NMR spectroscopy, and the purity was monitored by melting point determination with MEL-TEMP II and differential thermal analysis with SDT Q600 (TA Instruments, Lindon, UT) (Fig. 2.3).
Fig. 2.2A. Synthetic routes of non-PEGylated (nPEG), reversibly-PEGylated (rPEG), and irreversibly-PEGylated (irPEG) cationic polymers used in this study.
2.2.2.3. Synthesis of P[Asp(Hyd-PEG)]-b-P[Asp(DET)]

Poly(β-benzyl-L-aspartate) (PBLA) was synthesized as previously described by ring-opening polymerization using β-benzyl-L-aspartate N-carboxy anhydride (BLA-NCA) and n-butylamine as the monomer and initiator, respectively. Briefly, BLA-NCA (1.00 g, 4.02 mmol) was dissolved in 10 mL dry DMSO and n-butylamine (9.80 mg, 0.134 mmol) was quickly added using a cannula. The polymerization was allowed to proceed for 24 h at 40 °C under an argon atmosphere. The product mixture was slowly added into cold diethylether. The precipitated polymer was then subjected to centrifugation. The precipitation was repeated 3 times to purify the polymer. The white crude solid obtained was dissolved in benzene and then freeze-dried. The degree of polymerization of PBLA was determined to be 27 from the peak intensity ratio of the aryl protons of the benzyl groups (C₆H₅−, δ = 7.2–7.3 ppm) to the methyl group protons of n-butylamine (CH₃−, δ = 0.8 ppm) in ¹H NMR spectrum taken in DMSO-d₆ at 25 °C (Fig. 2.4).

To obtain P[Asp(Hyd-Boc)], the side chains of PBLA were substituted with Boc-protected hydrazide through aminolysis reaction. PBLA (650 mg, 0.116 mmol), tert-butyl carbazate (8.26 g, 62.5 mmol, 20 equiv to benzyl groups of PBLA), and 2-hydroxypyridine (3.00 g, 31.5 mmol, 10 equiv) were dissolved in 20 mL dry DMF under argon and stirred for 48 h at 45 °C. The polymer was precipitated in cold diethylether and then dissolved in methanol and dialyzed against methanol using dialysis tubing (MWCO 1000) overnight to remove the 2-
hydroxypyridine. The purified polymer was then freeze-dried in a mixture of methanol and benzene. The degree of substitution was determined to be 92.6% (25/27) from the peak intensity ratio of the \textit{tert}-butyl protons of the Boc-protecting groups ((CH$_3$)$_3$C–, $\delta = 1.2$ ppm) to the methyl group protons of \textit{n}-butylamine (CH$_3$–, $\delta = 0.8$ ppm) in $^1$H NMR spectrum taken in DMSO-$d_6$ at 25 °C (Fig. 2.5).

P[Asp(Hyd-Boc)]-b-PBLA was prepared using P[Asp(Hyd-Boc)] as the macro-initiator to initiate the second polymerization of BLA-NCA. P[Asp(Hyd-Boc)] (240 mg, 0.0386 mmol) was dissolved in 2 mL dry DMF, and BLA-NCA (1.06 g, 425 mmol) dissolved in a mixture of 2 mL dry DMF and 36 mL dry DCM was added in a stream of argon. The reaction mixture was stirred for 96 h at 35 °C under argon. The degree of polymerization was determined to be 106 from the peak intensity ratio of the aryl protons of the benzyl groups (C$_6$H$_5$–, $\delta = 7.2$–7.3 ppm) the methyl group protons of \textit{n}-butylamine (CH$_3$–, $\delta = 0.8$ ppm) in $^1$H NMR spectrum taken in DMSO-$d_6$ at 25 °C (Fig. 2.6). To protect the \textit{N}-terminal amino group of the polymer, P[Asp(Hyd-Boc)]-b-PBLA (820 mg, 0.0293 mmol) was dissolved in NMP and acetic anhydride (324 mg, 3.17 mmol, 100 equiv to polymer) was added under argon. The reaction mixture was stirred at 40 °C for 2 h.

P[Asp(Hyd)]-b-PBLA was obtained through the deprotection of the Boc groups from P[Asp(Hyd-Boc)]-b-PBLA using ice-cold TFA. The reaction mixture was stirred for 30 min at 0 °C. TFA was then removed under vacuum and the polymer was dissolved in DCM, precipitated in diethylether, and freeze-dried in benzene. The resulting polymer, P[Asp(Hyd)]-b-PBLA, had a polydispersity of 1.17 as determined by GPC. P[Asp(Hyd-PEG)]-b-PBLA was then generated by conjugating PEG to the polymer backbone through the acid-sensitive hydrazone linkage. P[Asp(Hyd)]-b-PBLA (200 mg, 7.84 $\mu$mol) and PEG propionaldehyde (455 mg, 0.0910 mmol)
were mixed in DMSO and stirred for 24 h at room temperature. The unconjugated PEG was then removed by washing the polymer mixture in methanol. The number of PEG conjugated to each polymer was quantified to be 6 from the peak intensity ratio of the aryl protons of the benzyl groups of PBLA (C₆H₅, δ = 7.2–7.3 ppm) to the methylene protons of PEG (–OCH₂CH₂–, δ = 3.5 ppm) in ¹H NMR spectrum taken in DMSO-d₆ at 25 °C (Fig. 2.7).

Finally, P[Asp(Hyd-PEG)]-b-P[Asp(DET)] was prepared by substituting the side chains of the second PBLA block of P[Asp(Hyd-PEG)]-b-PBLA with DET through aminolysis. Distilled DET (2.9 mL, 26.7 mmol, 50 equiv to benzyl groups of PBLA block) was added to freeze-dried P[Asp(Hyd-PEG)]-b-PBLA (270 mg, 4.95 µmol) dissolved in 5 mL dry NMP. The reaction mixture was stirred for 30 min at 15 °C under an argon atmosphere. The polymer was then precipitated in diethylether and subsequently dried and dissolved in 1x phosphate buffered saline (PBS, pH 7.4) and dialyzed against PBS at room temperature. The buffer salts and free PEG were then removed by Amicon ultra-15 centrifugal filter units (MWCO 30000) and the polymer as a hydrochloride salt form was then freeze-dried from distilled water. Quantitative introduction of DET was confirmed by the peak intensity ratio of the α-protons of polymer backbone (δ = 4.5 ppm) to the methylene protons of the DET moieties (–CH₂CH₂NHCH₂CH₂–, δ = 2.6–3.6 ppm) in the ¹H NMR spectrum taken in D₂O at 80 °C (Fig. 2.8).

**2.2.2.4. Synthesis of P[Asp(PEG)]-b-P[Asp(DET)]**

The irreversibly PEGylated polycation was synthesized in the similar way described in the previous section. The reversible hydrazone linkage between PEG and the polymer backbone was
reduced by 50 mM NaBH$_3$CN in 1x PBS (pH 7.4) for 24 h at room temperature, purified using Amicon ultra-15 centrifugal filter units (MWCO 30000) and then freeze-dried.

2.2.2.5. Synthesis of poly(ethylene glycol)-block-poly($N$-[N-(2-aminoethyl)-2-aminoethyl]aspartamide) (PEG-$_b$-P[Asp(DET)])

Block copolymer of poly(ethylene glycol)-block-poly($\beta$-benzyl-$L$-aspartate) (PEG-$_b$-PBLA) was synthesized as previously described by ring-opening polymerization using $\beta$-benzyl-$L$-aspartate $N$-carboxy anhydride (BLA-NCA) and $\alpha$-methoxy-$\omega$-amino poly(ethylene glycol) as the monomer and initiator, respectively.$^{163}$ Briefly, vacuum-dried BLA-NCA (1.04 g, 4.18 mmol) was dissolved in 3 mL DMF and 20 mL DCM was quickly added in a stream of argon to lyophilized PEG-NH$_2$ (500 mg, 0.0407 mmol) dissolved in 10 mL DCM. The polymerization was allowed to proceed for 96 h at 35 °C under an argon atmosphere. The product mixture was slowly added into cold diethylether. The precipitated polymer was then subjected to centrifugation. The precipitation procedure was repeated 3 times to purify the polymer. The white crude solid obtained was dissolved in benzene and then freeze-dried. The degree of polymerization of the PBLA block of PEG-$_b$-PBLA was determined to be 80 from the peak intensity ratio of the aryl protons of the benzyl groups of the PBLA block ($C_6H_5–$, $\delta = 7.2$–7.3 ppm) to the methylene protons of PEG ($–OCH_2CH_2–$, $\delta = 3.5$ ppm) in $^1$H NMR spectrum taken in DMSO-$d_6$ at 25 °C (Fig. 2.9). PEG-$_b$-P[Asp(DET)] was subsequently obtained by substituting the benzyl ester side chains of the PBLA block of PEG-$_b$-PBLA with DET through aminolysis reaction as described in section 2.2.2.3. Quantitative introduction of DET was confirmed by the peak intensity ratio of the $\alpha$-protons of polymer backbone ($\delta = 4.5$ ppm) to the methylene
protons of the DET moieties (\(-\text{CH}_2\text{CH}_2\text{NHCH}_2\text{CH}_2\)-, \(\delta = 2.6\text{–}3.6\) ppm) in the \(^1\text{H}\) NMR spectrum taken in \(\text{D}_2\text{O}\) at \(80\) °C (Fig. 2.10).

2.2.2.6. Preparation of polyplexes

The stock solutions were prepared by dissolving the pDNA and synthesized polymers in 10 mM Tris-HCl buffer (pH 7.4) at concentrations of 50 \(\mu\text{g/mL}\) and 10 mg/mL, respectively. Polyplexes at various N/P ratios were formed by adding the polymer solution with different concentrations (1/3 total volume) to the pDNA solution (2/3 total volume), vortexing, and then incubating at room temperature for at least 30 min before experiments. The final concentration of pDNA in all samples was kept at 33.3 \(\mu\text{g/mL}\). The polyplexes were formed in the same way in all experiments unless stated otherwise.

2.2.2.7. Gel retardation and EtBr exclusion assay

Polyplexes with different N/P ratios were loaded on a 0.8% (w/v) agarose gel containing 0.004% ethidium bromide. Electrophoresis was done at 100 V for 45 min with a running buffer of 40 mM Tris-acetic buffer containing 2 mM EDTA. The migrated pDNA was visualized by UV. In the EtBr dye exclusion assay, polyplex samples formed at 33.3 \(\mu\text{g}\) pDNA/mL with different N/P ratios were adjusted to 2.3 \(\mu\text{g}\) pDNA/mL with 10 mM Tris-HCl buffer (pH 7.4) containing 2.5 \(\mu\text{g}\) EtBr/mL and incubated at ambient temperature overnight in dark. The fluorescence intensity of the samples (\(\lambda_{\text{ex}} = 510\) nm and \(\lambda_{\text{em}} = 590\) nm) was measured using a spectrofluorometer (599B, Perkin-Elmer, MA). The reported relative fluorescence intensity (\(F_r\)) was calculated by
\[ F_r = \frac{F_{\text{sample}} - F_0}{F_{100} - F_0}, \]

where \( F_{\text{sample}} \), \( F_{100} \), and \( F_0 \) are the fluorescence intensities of the polyplex samples, the free pDNA without any polymer, and the background without pDNA and polymer, respectively.

2.2.2.8. Particle size and \( \zeta \)-potential measurements

Particle size and \( \zeta \)-potential were determined by dynamic light scattering (DLS) and laser doppler velocimetry, respectively, at 25 °C using Zetasizer Nano-ZS (Malvern Instruments, Worcestershire, UK) equipped with a He-Ne ion laser (\( \lambda = 633 \) nm) for the incident beam at a detection angle of 173°. Polyplex samples formed at 33.3 \( \mu \)g pDNA/mL with different N/P ratios were adjusted to 10 \( \mu \)g pDNA/mL using 10 mM Tris-HCl buffer (pH 7.4). The polyplex samples (750 \( \mu \)L) were transferred to low volume cuvettes for size measurements. Cumulant diameter and polydispersity index (PDI) were determined based on Cumulants analysis by fitting a single exponential to the correlation function and subsequently converting the translational diffusion coefficient to cumulant diameter using the Stokes-Einstein equation:

\[ d(H) = \frac{kT}{3\pi \eta D} \]

where \( d(H) \) is the hydrodynamic diameter, \( D \) is the translational diffusion coefficient, \( k \) is the Boltzmann’s constant, \( T \) is the absolute temperature, and \( \eta \) is the viscosity of the dispersant.

To measure the \( \zeta \)-potential, polyplex samples were transferred to Dip cell (Malvern Instruments, UK) and the electrophoretic mobility was measured, the \( \zeta \)-potential was then calculated by the Henry equation using the Smoluchowski approximation:

\[ \nu = 2\varepsilon \zeta f(Ka)/3\eta \]
where $\eta$ is the viscosity of the dispersant, $\nu$ is the measured electrophoretic mobility, $\varepsilon$ is the dielectric constant of the dispersant, and $f(Ka)$ is the Henry’s function.

### 2.2.2.9. Polyplex stability in physiological salt and serum environments

Polyplex samples were formed at 33.3 $\mu$g pDNA/mL with the same method as described in section 2.2.2.6 except deionized water was used as the medium. After 30 min complexation at room temperature, the samples were diluted to 10 $\mu$g pDNA/mL using deionized water. The salt concentrations were adjusted to 10 mM Tris-HCl and 150 mM NaCl with 10x Tris-HCl buffered saline (pH 7.4) to mimic the physiological salt concentration. The polyplex sizes were monitored using DLS for 12 h at 25 °C, and the results were reported as cumulant diameters. The effect of serum on polyplex size was investigated in the same manner except polyplex samples were formed at 100 $\mu$g pDNA/mL and fetal bovine serum (FBS) with final concentration of 10% (v/v) was added in addition to 150 mM NaCl after 30 min complexation time. The samples were diluted 10 times after 12 h incubation at 25 °C and the polyplex sizes were measured by DLS.

### 2.2.2.10. PEG release study

The reversibly-PEGylated polymer, P[Asp(Hyd-PEG)]-b-P[Asp(DET)], was incubated in 10 mM Hepes buffer (pH 7.4) or sodium acetate buffer (pH 5) at 37 °C for 24 h. Aliquots were withdrawn at specific time points and diluted with ice-cold Hepes buffer (pH 7.4). Samples were then immediately subjected to purification by RESOURCE S 1 mL cationic exchange column and the unbound fractions containing free PEG were saved for subsequent analysis. Briefly, 41.6 $\mu$L of 1 N barium chloride was added to 187.5 $\mu$L of the unbound fraction. 20.8 $\mu$L of 0.1 N
iodide solution was subsequently added and mixed. The samples were allowed to sit at room temperature for 15 min before measuring the absorbance at 535 nm using plate reader (SpectraMax M2e, Molecular Devices, CA). One hundred percent PEG release was calculated based on sample incubated overnight with 0.1 N HCl at 37 °C.

2.2.2.11. Serum nuclease resistance assay

Polyplex samples were formed as described in section 2.2.2.6 using deionized water. pH was adjusted to 7.4 with 10x Tris-HCl buffer (100 mM, pH 7.4). FBS with final concentration of 10% (v/v) was added to samples before incubation at 37 °C. Naked DNA treated with the same conditions was used as a control. At specific time points, naked DNA or polyplex sample was withdrawn and 1 µL of 5 M EDTA was added to stop the nuclease activity. Samples were kept frozen at -20 °C until electrophoresis. Twenty USP units of heparin were used to release pDNA from the polyplexes and incubated for 30 min at room temperature prior to electrophoresis.

2.2.2.12. In vitro transfection

MDA-MB-231 human breast cancer cell line was cultured in DMEM supplemented with 10% FBS, 100 IU penicillin, and 100 µg/mL streptomycin. Cells were seeded at 30000 cells/well on 24-well plates and incubated for 24 h. After the cells reached to about 60% confluence, polyplex solutions (30 µL) were prepared with different polymers at varying N/P ratios, diluted with 270 µL of fresh culture medium containing 10% FBS, and then added to the cells. The pDNA amount for each well was kept at 1 µg. The polyplexes were allowed to incubate with the cells for 6 h before refreshing with 1 mL of DMEM with 10% FBS. The cells were then
maintained at 37 °C for another 42 h before luciferase assay. For luciferase assay, the cells were washed with 1x PBS (1 mL) after medium removal. Cell Culture Lysis Reagent (140 μL) was then added to each well and the cells were incubated at room temperature for 15 min. One hundred μL of luciferase substrate was added to 20 μL of the lysate. The luminescence intensity was measured by an Orion microplate luminometer (Berthold Detection Systems, Oak Ridge, TN). The obtained luciferase expression was then normalized with the amount of total proteins present in the lysates determined by the RC DC Protein Assay Kit.

2.2.2.13. In vitro cytotoxicity

MDA-MB-231 cells were seeded at 3000 cells/well on 96-well plates in DMEM containing 10% FBS and incubated for 24 h. Polyplex solutions (10 μL) prepared at different N/P ratios were diluted with fresh medium (90 μL) and then added to the cells. The cells were incubated with polyplexes or free polymers for 6 h before refreshing medium. The cells were further incubated for 18 h. CellTiter-Blue® Cell Viability Assay was then carried out according to the manufacturer’s protocol. The fluorescence signals (λ_ex = 560 nm and λ_em = 590 nm) were measured 3 h after adding the dye. The results were represented as percentages of cell viability determined using untreated cells.

2.2.2.14. LDH release study

MDA-MB-231 cells were seeded at 3000 cells/well on 96-well plates in DMEM containing 10% FBS and incubated for 24 h. Medium was removed and cells were washed with 100 μL 1x PBS. Serum-free DMEM (90 μL) with polymer solutions (10 μL) prepared at different amine
concentrations were subsequently added to the cells. The cells were incubated with the polymer solutions for 6 h. CytoTox-ONE™ Homogeneous Membrane Integrity Assay was then carried out according to the manufacturer’s protocol. Maximum LDH release was determined from cells treated with the lysis solution supplied from the assay kit. Results were reported as percentages of maximum LDH release.

2.2.2.15. Statistical analysis

The experimental data were analyzed by unpaired Student's t-test. $P < 0.05$ was considered as significant.

2.3. Results

2.3.1. Polymer Syntheses

The synthesis of $\text{P[Asp(Hyd-PEG)]-b-P[Asp(DET)]}$ was carried out as shown in Fig. 2.2. The monomer, BLA-NCA, used for polymerization was first synthesized and purified through recrystallizations. The structure was confirmed by NMR, and the purity was evaluated by melting point determination ($129.5–130.7 \degree C$) and differential thermal analysis (Fig. 2.3). PBLA was synthesized as the first block of the polymer. The degree of polymerization was estimated to be 27 by $^1\text{H}$ NMR (Fig. 2.4). To introduce hydrazide moieties to the polymer for subsequent PEGylation, Boc-protected hydrazide was used to prevent undesired initiation at the free hydrazide moieties rather than the terminal amine during the second polymerization. Since the conversion result using Boc-hydrazide alone was not satisfactory, 2-hydroxypyridine (2-HP) was utilized to raise the conversion degree. $^1\text{H}$ NMR confirmed that the conversion was 92.6% when
2-HP was used (Fig. 2.5). The resulting polymer, P[Asp(Hyd-Boc)], was then employed as the macro-initiator to start the second polymerization of PBLA. The degree of polymerization of the second PBLA block was estimated to be 106 (Fig. 2.6). GPC revealed that the molecular weight distribution of the polymer after each polymerization step to be unimodal. Boc protecting groups were removed from P[Asp(Hyd-Boc)]-b-PBLA, and PEG was subsequently conjugated to P[Asp(Hyd)]-b-PBLA through hydrazone bond formation between the deprotected hydrazide and the aldehyde group of PEG propionaldehyde. After purification, GPC showed that there were no free PEGs present, and $^1$H NMR estimated that there were about 6 PEGs conjugated per

![Fig. 2.3](image)

$^1$H (A) and $^{13}$C NMR (B) spectra of BLA-NCA taken in DMSO-$d_6$ at 25 °C. Peaks are indicated with letters and numbers corresponding to protons and carbons in the structure, respectively. Differential thermal analysis of BLA-NCA crystals (C).
polymer molecule (Fig. 2.7). The aminolysis of the second block of PBLA with DET was carried out at low temperature (15 °C) for very short time (30 min) to prevent the release of PEG from the polymer with the presence of excess amine. $^1$H NMR confirmed the aminolysis reaction was successful and there were 5–6 PEG chains attached to each of the reversibly-PEGylated
polymer (Fig. 2.8). The irreversibly-PEGylated polymer, P[Asp(PEG)]-b-P[Asp(DET)], was synthesized in the same manner with the hydrazone linkages between PEG chains and the polymer backbone reduced by NaBH₃CN at the final step. PEG-b-P[Asp(DET)] was synthesized as a control polymer. ^1H NMR showed that the degree of polymerization of the P(Asp) block was 80 and the aminolysis reaction with DET was successful (Fig. 2.9 and 2.10). The physical characterizations of each polymer are summarized in Table 2.1.

**Fig. 2.8.** ^1H NMR spectrum of P[Asp(Hyd-PEG)]-b-P[Asp(DET)] taken in D₂O at 80 °C.

**Fig. 2.9.** ^1H NMR spectrum of PEG-b-PBLA taken in DMSO-δ₆ at 25 °C.

**Fig. 2.10.** ^1H NMR spectrum of PEG-b-P[Asp(DET)] taken in D₂O at 80 °C.
<table>
<thead>
<tr>
<th>Polymer</th>
<th>Code</th>
<th>DP of 1st P(Asp) block</th>
<th>DP of 2nd P(Asp) block</th>
<th># PEG conjugated</th>
<th>$M_w/M_n$</th>
<th>Total MW (g/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P[Asp(Hyd)]-b-P[Asp(DET)]</td>
<td>nPEG</td>
<td>22</td>
<td>105</td>
<td>0</td>
<td>1.10</td>
<td>28000</td>
</tr>
<tr>
<td>P[Asp(Hyd-PEG)]-b-P[Asp(DET)]</td>
<td>rPEG</td>
<td>27</td>
<td>106</td>
<td>6</td>
<td>1.31</td>
<td>58000</td>
</tr>
<tr>
<td>P[Asp(PEG)]-b-P[Asp(DET)]</td>
<td>irPEG</td>
<td>27</td>
<td>106</td>
<td>6</td>
<td>1.31</td>
<td>58000</td>
</tr>
<tr>
<td>PEG-b-P[Asp(DET)]</td>
<td>-</td>
<td>80</td>
<td>-</td>
<td>1</td>
<td>1.07</td>
<td>31200</td>
</tr>
</tbody>
</table>

Table 2.1. Characteristics of the polymers synthesized in this study. $^a$ Determined by $^1$H NMR. $^b$ Determined by GPC. $^c$ Calculated based on the HCl salt form of the polymers.

2.3.2. Formation of polyplexes

Polyplexes were formed by mixing each polymer solution with pGL3C pDNA solution directly at different N/P ratios. Agarose gel electrophoresis showed that the bands of free pDNA disappeared at N/P = 2, while the pDNA was completely retarded at N/P = 3 for all polyplexes (Fig. 2.11). This suggests that all pDNA molecules were successfully trapped inside the polyplex particles. As the N/P ratio here was calculated solely based on the number of amino groups on the polymer excluding free hydrazide groups and the results are similar to that obtained from homopolymer containing only amino groups from DET side chains, it reveals that the free hydrazide does not participate in DNA condensation at pH 7.4. These results are consistent with the published results. EtBr molecules are known to emit fluorescence upon interaction with DNA. Also, during pDNA condensation there is a coil-to-globule structural

![Fig. 2.11. Gel retardation analysis of polyplexes.](image)
transition and the globular structure inhibits the intercalation of DNA by EtBr resulting in decreased fluorescence emission; therefore, EtBr exclusion assay was employed to determine the condensation degree of pDNA at different N/P ratios quantitatively. The fluorescence data obtained using different polyplexes are shown in Fig. 2.12. All polyplexes exhibit similar patterns in terms of pDNA condensation ability. The relative fluorescence intensity dropped from N/P = 0 to N/P = 1.5 and reached a plateau at N/P = 2, which indicates that pDNA achieves maximum condensation at N/P = 2 for which the ratio of positive charge to negative charge is 1:1 as DET side chain is mono-protonated at pH 7.4. These results are consistent with the results obtained using gel electrophoresis.

Physical characteristics of polyplexes are closely related to their therapeutic performance; thus, it is essential to determine the size and ζ-potential of the polyplexes. The DLS measurement data are shown in Fig. 2.13A. The size of polyplexes gradually decreased to 70–90 nm when N/P ≥ 2 for the reversibly-PEGylated (rPEG) and irreversibly-PEGylated (irPEG) polyplexes. With the non-PEGylated (nPEG) polyplexes, at least N/P = 4 was required to bring
the size down to the same range. At low N/P ratio range (0.5–3), the sizes of the rPEG and irPEG polyplexes were below 150 nm without aggregation; however, the size of the nPEG polyplexes was significant different from that of the PEGylated polyplexes. The size of the nPEG polyplex went up to about 2700 nm at N/P = 2, which represents significant aggregation at this N/P ratio. As the ethylenediamine side chain is only mono-protonated at pH 7.4, the overall charge of the polyplexes formed at N/P = 2 should be theoretically neutral. This was also confirmed by ζ-potential estimation that the ζ-potential of particles at N/P = 2 was very close to zero (Fig. 2.13B). At this critical N/P ratio and without the steric hindrance provided by PEG, the polyplexes experienced the minimal repulsive force among one another and the counterion screening effect caused the neutral polyplex particles to aggregate.

**Fig. 2.13.** Cumulant diameters (A) and ζ-potential values (B) of polyplexes formed at various N/P ratios in 10 mM Tris-HCl buffer (pH 7.4) at 25 °C (mean ± SD, n = 3).

The ζ-potential measurement data shown in Fig. 2.13B reveals that the ζ-potential of the polyplexes increased with N/P ratio. All polyplexes have close-to-neutral ζ-potential at N/P = 2 and the ζ-potential values reached plateau at about N/P = 5. The values maintained the same up
to N/P = 20. The ζ-potential values were significant different between the PEGylated and non-PEGylated polyplexes. The ζ-potentials of the PEGylated (rPEG and irPEG) polyplexes were kept below +10 mV and were much lower than +30 mV obtained with the non-PEGylated (nPEG) polyplexes. This result suggests that the charge on the cationic block of the polymer could be effectively shielded by the PEG moieties.

2.3.3. Polyplex stability in physiological salt and serum environments

To investigate the stability of the polyplexes under physiological salt condition, polyplexes were incubated in 150 mM NaCl solution and their sizes were monitored using DLS for 12 h. As shown in Fig. 2.14, the addition of salt promptly induced the nPEG polyplexes to aggregate.

The size increased from around 70 nm to micron range within 1 h after the addition of salt. In the case of rPEG or irPEG polyplexes, a rapid size increase could not be observed, which implies that PEGylation was necessary to keep the polyplexes intact, at least to prevent aggregation.
under physiological salt condition. For the PEGylated polyplexes, the size of the irPEG polyplexes stayed the same while the size of the rPEG polyplexes grew from 70 nm to about 150 nm after 12 h incubation. Although the size of the rPEG polyplexes increased, it was still much smaller than that of the nPEG polyplexes after salt addition. The impact of serum on particle stability was also investigated using DLS. During the 12 h incubation with 10% FBS, the nPEG polyplexes aggregated significantly and particle size distribution became very polydispersed. In contrast, the PEGylated polyplexes showed higher stability in serum-containing medium. The particle sizes of the irPEG polyplexes incubated in serum-containing and serum-free medium were very comparable and stayed below 100 nm after 12 h incubation, while the size of the rPEG polyplexes increased to about 500 nm (Table 2.2). The increase in size of the rPEG polyplexes observed in both experiments might be due to the dynamic nature of the polymer as hydrazone linkages between PEG and polymer backbone are still hydrolysable at neutral pH.

<table>
<thead>
<tr>
<th>Polyplexes</th>
<th>Cumulant diameter (nm) ± SD w/o 10% FBS</th>
<th>Cumulant diameter (nm) ± SD w/ 10% FBS</th>
</tr>
</thead>
<tbody>
<tr>
<td>nPEG</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>rPEG</td>
<td>150.7 ± 26.8</td>
<td>495.9 ± 37.1</td>
</tr>
<tr>
<td>irPEG</td>
<td>86.4 ± 8.2</td>
<td>79.1 ± 5.1</td>
</tr>
</tbody>
</table>

Table 2.2. Particle sizes of polyplexes formed at N/P = 20 after 12 h incubation in 10 mM Tris-HCl buffer (pH 7.4) containing 150 mM NaCl with or without 10% FBS at 25 °C (mean ± SD, n = 3). nd: not determined due to significant aggregation and high polydispersity.

2.3.4. PEG Release Study

As PEGylation of polyplex is required for prolonged systemic circulation and rapid release of PEG inside the cells is preferable for high transfection efficiency, it is necessary to monitor
the PEG release from the polymer at different pH. To quantitatively measure the release of PEG at different pH, the reversibly-PEGylated polymer was incubated at 37 °C for 24 h at pH 7.4 and pH 5 to mimic the physiological and endo/lysosomal pH, respectively. The release profiles at pH 7.4 and pH 5 are shown in Fig. 2.15. A more rapid release of PEG at the initial stage of the experiment was observed at pH 5 compared to pH 7.4. Fifty percent of the total amount of PEG was released within 30 min at pH 5, while the amount of PEG release remained below 50% after 8 h at pH 7.4. Although it is still unclear that how many PEG chains are required to stabilize the polyplex during circulation, it is true that the rPEG polymer shows the ability to maintain 50% PEG at pH7.4 for 8 h. Moreover, the polymer could release the PEG shield very rapidly at pH 5, which corresponds to the acidic pH at the endo/lysosomal compartments. This indicates that the polyplexes may be able to benefit from the intracellular prompt release of PEG to facilitate the endosomal escape of the polyplexes and to promote the release of pDNA from the polyplex particles.

![Fig. 2.15. Time- and pH-dependent PEG release from the rPEG polymer at pH 5 and pH 7.4 at 37 °C (mean ± SD, n = 3).](image-url)
2.3.5. pH-sensitivity of reversibly-PEGylated polyplexes

The value of polyplex surface potential is related to the amount of PEG present on the polyplex. A PEG layer with higher PEG density on the polyplex should be able to shield the charge more effectively and result in lower $\zeta$-potential. On the other hand, when PEG chains are detached, the charge of the polyplex will be more exposed to the surroundings and higher $\zeta$-potential should be observed. As hydrazone linkage is hydrolyzed at a faster rate at acidic pH than at neutral pH, monitoring the polyplex surface potential would be useful for confirming PEG conjugated through hydrazone bond is released from the polyplexes more rapidly at low pH. The time-dependent change of $\zeta$-potential value of different polyplexes was shown in Fig. 2.16. As buffer salts could affect $\zeta$-potential measurement, it is more reliable to compare the change of $\zeta$-potential of polyplexes at the same pH. Fig. 2.16A shows that the amount of PEG released from the rPEG polyplexes at pH 7.4 caused significant difference in $\zeta$-potential compared to the irPEG polyplexes only at $t = 4$ h ($^*P < 0.05$) but not within the first 3 h. However, Fig. 2.16B reveals that the $\zeta$-potential of the rPEG polyplexes rapidly changed at pH

![Fig. 2.16.](image)

**Fig. 2.16.** Time- and pH-dependent change of $\zeta$-potential at pH 7.4 (A) and pH 5 (B) of polyplexes formed at N/P = 20 at 25 °C (mean ± SD, $n = 3$, $^*$ indicates statistically significant difference with irPEG group with $P < 0.05$).
These results suggest that the rate of PEG release from the polyplexes at pH 5 was higher than that at pH 7.4 and the PEG release at pH 5 within the first 4 h could contribute to significant increase in $\zeta$-potential.

2.3.6. Serum nuclease resistance

Another challenge for polyplex to successfully deliver therapeutic genes to targets is to protect the DNA from degradation by the abundant nuclease present throughout the human body. Therefore, the ability of the polyplexes to resist serum nuclease degradation was investigated. The reversibly-PEGylated polyplexes were incubated at 37 °C in 10 mM Tris-HCl buffer (pH 7.4) containing 10% FBS. Polyplex samples were withdrawn at the indicated time point. The DNA was released from the polyplexes through polyion exchange reaction using heparin as the polyanion. As shown in Fig. 2.17, the supercoiled (sc) form of naked pDNA disappeared completely after 1 h incubation and all the DNA was changed to the open circular (oc) and linear forms. The band intensity of the oc DNA and linear DNA remained about the same for 4 h and then started to decrease. DNA fragments due to DNA degradation appeared as smear on the agarose gel started to appear at 4 h and the DNA was completely degraded after 24 h incubation as only DNA fragments could be observed at this time point. Compared to the naked DNA, the polyplexes showed a very different result. As shown in the gel image, the polyplexes protected the DNA from degradation by serum nuclease for at least 24 h under the experimental conditions. There were no DNA fragments and linear DNA observed at any time points. Also, there was no significant increase in the intensity of the bands of the oc DNA, implying that most of the pDNA remained as the intact sc form with the complexation with polymer.
Fig. 2.17. Gel retardation analysis of naked DNA and rPEG polyplexes after incubation with 10% FBS. oc DNA and sc DNA represent open circular DNA and supercoiled DNA, respectively.

2.3.7. In vitro transfection

The transfection efficiency of the polyplexes formed using the synthesized polymers, nPEG, rPEG, and irPEG, was compared to that of the polyplexes formed using PEG-b-P[Asp(DET)] and branched polyethylenimine (bPEI) in MDA-MB-231 breast cancer cell line. bPEI is a control commonly used for comparing transfection efficiency, while PEG-b-P[Asp(DET)] is a diblock copolymer that showed promising in vitro and in vivo gene delivery ability and negligible toxicity. The polyplexes were formed at N/P = 10, 20, and 40 for the transfection experiment. bPEI polyplexes were formed at N/P = 10 as determined to be the optimal N/P ratio for transfection without causing massive cell death. Fig. 2.18 shows that the most prominent transfection agent in this study was the nPEG polyplex. This agreed with our hypothesis that polyplexes without PEGylation should show better transfection ability compared to their PEGylated counterparts. The polyplexes formed with the irPEG polycations showed the lowest transfection efficiency which is 5 orders of magnitude lower than that of the non-PEGylated polyplexes in this experiment. With the same number of PEG chains, but conjugated through pH-
sensitive hydrazone linkage, the rPEG polyplexes at various N/P ratios demonstrated about 4 to 5 orders of magnitude higher transfection efficiency than that of the irPEG polyplexes (*P < 0.01) and 1 order of magnitude lower compared to that of the nPEG particles. This indicates that polyplexes can achieve a more comparable level of transfection to the non-PEGylated polyplexes through the reversible attachment of PEG chains to the polyplexes even there is a high density of PEG molecules protecting the polyplexes. Moreover, the nPEG and rPEG polyplexes showed higher and similar transfection efficiency compared to bPEI, respectively, while causing minimal toxicity which will be discussed below. Compared to the extensively-investigated PEG-b-p[Asp(DET)] polyplexes, the rPEG polyplexes have an advantage in transfection efficiency and the difference was significant at lower N/P ratios, e.g. 10 or 20 (*P < 0.01). When N/P ratio increased, however, the transfection ability of the diblock copolymer polyplexes became more comparable to that of the rPEG polyplexes. Within the N/P ratio range tested, increasing N/P
ratio did not affect the transfection efficiency of the nPEG and rPEG polyplex systems substantially. High transfection level could be achieved as low as N/P = 10 for the nPEG and rPEG polyplexes. On the contrary, N/P = 40 was required for PEG-b-p[Asp(DET)] polyplexes to achieve a more comparable result to the rPEG polyplexes.

2.3.8. In vitro cytotoxicity

To evaluate the cytotoxicity of the polyplexes formed with the polymers synthesized, cytotoxicity assays based on cellular metabolism and LDH release were carried out using MDA-MB-231 cells. bPEI was used as a control for both assays. The relative metabolism of cells treated with different kinds of polyplexes at the same N/P ratio range used in transfection experiment is shown in Fig. 2.19A. Among the polyplexes formed with the P[Asp(DET)]-based polycations, nPEG, rPEG, and irPEG, no significant difference in toxicity was observed. Almost 100% cell viability based on metabolism was observed even at N/P = 40, which was the maximum N/P ratio tested, in MDA-MD-231 cell line. The data reveal that PEGylation of P[Asp(DET)]-based polycations did not affect the toxicity caused of the polymers under the tested conditions, and this might be due to the intrinsic low toxicity profile of the P[Asp(DET)]-based polymers. In contrast, the cytotoxicity caused by bPEI polyplexes was significantly higher than P[Asp(DET)]-based polyplexes at every N/P ratio tested. At the N/P ratio used in transfection experiment, N/P = 10, cell viability dropped to 50% after bPEI polyplex treatment. The toxicity increased with the amount of bPEI used. The cell viability further diminished to 30% at N/P = 20 and less than 10% at N/P = 40.
Fig. 2.19. Cytotoxicity of polyplexes formed at various N/P ratios (A) and LDH release caused by polymers at different amine concentrations (B) in MDA-MB-231 cells (mean ± SD, n = 3, *P < 0.001).

It is well known that cationic polymers can interact with and disrupt the cellular membrane. LDH release through the leaky cell membrane damaged by cationic polymers could be used to evaluate the membrane toxicity caused. The membrane toxicity produced by different free polymers at a range of amine concentration is shown in Fig. 2.19B. At amine concentration of 100 µM, bPEI caused about 80% LDH release. The percentage increased to almost 100% when 500 or 1000 µM of bPEI was used. For the P[Asp(DET)]-based polymers, they all showed significant lower membrane toxicity (< 30%) compared to bPEI (*P < 0.001), although there was a trend that the LDH release percentage increased with the amine concentration. As a general difference among the different P[Asp(DET)]-based polymers within the tested concentration range was not present, it cannot be concluded whether PEGylation reduces the membrane cytotoxicity of P[Asp(DET)]-based polymers. The LDH release assay result was consistent with the metabolic toxicity result that the P[Asp(DET)]-based polymers causing minimal and significantly lower toxicity compared to bPEI.
2.4. Discussion

To develop a gene delivery vehicle for \textit{in vivo} applications, PEGylation of the vehicle is necessary for increasing the systemic circulation time and physiological stability. Although PEGylation can enhance the polyplex stability and facilitate the escape from the recognition and clearance by the reticuloendothelial system (RES), it hampers the transfection efficiency of the vehicle. This is probably due to the decreased cellular uptake of the polyplexes and the difficulty in releasing DNA intracellularly as a result of the steric hindrance of PEG molecules. In this regard, a pH-sensitive multi-PEGylated cationic polymer with a unique architecture was synthesized and tested for \textit{in vitro} gene delivery efficiency in this study. The diblock copolymer comprises two functional segments: a hydrazide functionalized block, P[Asp(Hyd)], for multi-PEGylation through pH-sensitive hydrazone linkages; and a cationic block, P[Asp(DET)], for DNA condensation and enhanced endosomal escape. PEGylation of the cationic polymer through hydrazone linkages was restricted to one of the two blocks of the polymer, while the second cationic block was kept intact for DNA condensation. This unique synthesis strategy is different from the common way many investigators employed for multi-PEGylation by conjugating PEG chains randomly on cationic polymers using reactive amines as the reaction sites.\textsuperscript{53,72,165} The advantages of this restricted PEGylation approach include maintaining the cationic charge density of the polymers after PEG conjugation and minimizing the steric hindrance produced by PEG during DNA condensation. These may affect the overall polyplex stability and eventually the transfection efficiency. PEG propionaldehyde with a molecular weight of 5000 g/mol was employed in this study as studies showed that 5000 g/mol PEG was sufficient to reduce particle sizes, effective for charge shielding and polyplex stabilization in physiological ionic strength
environments compared to PEG with lower molecular weight.\textsuperscript{53} Moreover, PEG with molecular weight of 5000 g/mol was demonstrated to be effective for micelle-type nanoparticles to minimize plasma protein interaction.\textsuperscript{166} The reversibly-PEGylated polymer prepared here was expected to provide polyplex stability through PEGylation in extracellular environment while releasing the PEG molecules as soon as the polyplexes encounter the acidic pH in intracellular compartments, such as endosomes and lysosomes, in order to maintain high transfection efficiency.

The success of incorporation of pDNA into polyplexes using the polymers synthesized was shown in gel retardation (Fig. 2.11) and EtBr exclusion analyses (Fig. 2.12). The polyplexes prepared were about 70–90 nm in size at N/P $\geq$ 5 (Fig. 2.13A), thereby making them suitable for further in vivo applications. Moreover, the $\zeta$-potential of the polyplex was effectively shielded by the high density PEG layer with a reduction of $\zeta$-potential from +30 mV to +10 mV as shown in Fig. 2.13B. Note that the $\zeta$-potential of polyplexes formed from DNA and diblock copolymer, PEG-b-p[Asp(DET)], was in the same range (+5 to +10 mV).\textsuperscript{138} The relatively small positive values imply that the PEG shielding is still not 100\% complete. The unsuccessful further reduction of $\zeta$-potential by the higher density PEG palisade produced by the multi-PEGylated polymer can be due to the lower-molecular-weight PEG (5000 g/mol) employed in this study compared to the 12000 g/mol PEG used for the synthesis of PEG-b-p[Asp(DET)]. Even with higher PEG density, the thinner PEG palisade may just provide a comparable charge shielding effect to the thicker PEG layer with lower density. The small positive $\zeta$-potential value also suggests that there will still be nonspecific interaction between the polyplex particles and negatively charged biomolecules inside body; therefore, PEG with higher molecular weight
could be employed for multi-PEGylation in order to further reduce the surface charge. However, it may also lower the overall transfection efficiency of the vehicle.

The pH-responsive nature of the PEG release from the polymer was confirmed by PEG release study and ζ-potential measurements under different pH. The polyplexes or free polymers were incubated at pH 7.4 and 5 to mimic the physiological pH and acidic endo/lysosomal pH, respectively, in these experiments. At neutral pH, the amount of PEG attached to the polymer maintained at more than 50% for about 8 h at 37 °C, while the PEG chains were released from the polymer at a much faster rate at acidic pH (50% release within 30 min) (Fig. 2.15). The increase in ζ-potential of the reversibly-PEGylated polyplexes accompanying the PEG release at acidic pH was also confirmed in this study (Fig. 2.16). The more rapid release of PEG chains from the polymers at acidic pH compared to neutral pH indicates that the polyplexes would be more stable at extracellular neutral pH and prompt detachment of PEG chains could occur at the acidic compartments (e.g. endosomes, or lysosomes) inside the cells. The dissociation of PEG chains from the polyplex could increase the exposure of the cationic segments of the polymers to the endosomal membrane causing membrane disruption and thus further facilitating endosomal escape of the polyplexes.

Although the rPEG polyplexes showed 1 order of magnitude lower transfection efficiency compared to the nPEG polyplexes, they demonstrated more than 1000 times higher efficiency at every single N/P ratio tested than the irPEG polyplexes which possess the same amount of stably linked PEG chains (Fig. 2.18). The in vitro transfection experiment also revealed that the transfection efficiency of the rPEG polyplexes was similar to that of PEG-b-p[Asp(DET)] polyplexes at N/P = 40 and significantly higher at lower N/P ratios. As studies showed that PEG-
b-P[Asp(DET)] is a promising \textit{in vitro} and \textit{in vivo} gene delivery agent,\textsuperscript{108,141} the pH-sensitive multi-PEGylated polymer could also be a valid candidate for future \textit{in vivo} experiments. The success of a non-viral gene delivery vector is also governed by the toxicity caused. It is very common that cationic gene delivery vectors possess certain level of cytotoxicity due to their positive charges. The cytotoxicity caused by the polymers synthesized in this study and bPEI was evaluated based on the cell metabolism (Fig. 2.19A) and membrane integrity (Fig. 2.19B) after polyplex or polymer treatments. In these two studies, bPEI showed significant higher toxicity compared to the P[Asp(DET)]-based polymers. Furthermore, there was very low toxicity caused by the P[Asp(DET)]-based polymers even at high N/P ratios or high amine concentrations. One of the advantages of PEGylation of cationic polymer is to reduce the toxicity,\textsuperscript{53} but no reduction in toxicity could be observed by PEGylation of P[Asp(DET)]-based polymers in this study. The main reason could be the intrinsic negligible toxicity of P[Asp(DET)]-based polymers.

2.5. Conclusions

In summary, a bioresponsive gene delivery vehicle with unique architecture aimed at achieving satisfactory polyplex stability and polyplex transfection efficiency has been synthesized and tested in the present chapter. The results demonstrated that the PEG release through hydrazone linkage cleavage from the polymer is pH-sensitive. Majority of the PEG chains can be maintained on the polymer backbone for a long period of time at neutral pH, while the release of PEG is faster at acidic pH. This pH-sensitive nature allows temporary PEGylation of the reversibly-PEGylated polyplexes for maintaining stability and achieving pH-dependent
PEG detachment for higher transfection efficiency compared to the irreversibly-PEGylated counterparts.
The effects of substituting poly(ethylene glycol) on poly(ethylene glycol)-block-poly{N-[N-(2-aminoethyl)-2-aminoethyl]aspartamide} with Pluronic P85 on the cellular uptake and transfection efficiency of polyionic complexes prepared with the cationic block copolymers.

This chapter has been published in full: Lai, T. C.; Kataoka, K.; Kwon, G. S. Pluronic-based cationic block copolymer for forming pDNA polyplexes with enhanced cellular uptake and improved transfection efficiency. *Biomaterials* 2011, 32, 4594–4603.
3.1. Introduction

A PEG-based diblock catiomer, poly(ethylene glycol)-block-poly\{N-[N-(2-aminoethyl)-2-aminoethyl] aspartamide\} (PEG-\textit{b}-P[Asp(DET)]), has been shown to be an excellent delivery vehicle for pDNA as promising results have been obtained in transfection, cytotoxicity,\textsuperscript{33} and biodegradability studies.\textsuperscript{145} Although polyplexes prepared from PEG-\textit{b}-P[Asp(DET)] showed clear advantages in transfection performance and toxicity over polyplexes prepared from other cationic polymers, for example, poly(L-lysine),\textsuperscript{138} branched PEI, and linear PEI,\textsuperscript{108} PEG-\textit{b}-P[Asp(DET)] polyplexes are always required to be prepared at high N/P ratios for transfection in order to surpass its competitors, and the polyplexes usually internalized into the cells at a much lower level compared to the polyplexes formed with the non-PEGylated P[Asp(DET)] homopolymers.\textsuperscript{33,71} This implies that further modifications on PEG-\textit{b}-P[Asp(DET)] are required to boost its performance especially at low N/P ratios. As an amphiphilic triblock copolymer with a hydrophobic poly(propylene oxide) block situated in between two hydrophilic poly(ethylene oxide) blocks, Pluronic block copolymer has been shown to interact with plasma membrane, lower the microviscosity of the membrane, and promote cellular uptake of small molecules\textsuperscript{167} and biomacromolecules including peptides\textsuperscript{149} and nucleic acids.\textsuperscript{150} Different Pluronic polymers, including P85,\textsuperscript{150} L92,\textsuperscript{158} and P123,\textsuperscript{168} have been used to conjugate to cationic polymers aiming at improving the delivery of nucleic acid-based materials. Enhanced delivery was observed with those Pluronic-based carriers; however, free Pluronic polymers are usually necessary to be included in the formulations to promote transfection efficiency and to allow the formation of colloidally stable polyplexes.
In the present chapter, we employed the amphiphilic nature of Pluronic P85 to investigate whether the cellular internalization and transfection efficiency of polyplexes could be increased by replacing the hydrophilic PEG block with amphiphilic Pluronic P85 on the PEG-\textit{b}-P[Asp(DET)] diblock copolymer (Fig. 3.1). P85 was chosen as its HLB (hydrophilic-lipophilic balance) value of 16 is a mid-range value within the Pluronic family. Also, certain degree of hydrophilicity is required for maintaining colloidal stability of particles as it is speculated that the Pluronic chains are located at the surface of the particles after polyplex formation. P85-\textit{b}-P[Asp(DET)] was synthesized using P85 amine as the initiator and the synthesized polymer was confirmed to be able to condense pDNA to produce nano-sized polyplex particles. The transfection efficiency and toxicity of the polyplexes formed with P85-\textit{b}-P[Asp(DET)] were evaluated and compared to those of the PEG-\textit{b}-P[Asp(DET)] polyplexes.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{Fig3.png}
\caption{Schematic illustration of enhanced cellular uptake by Pluronic-based polyplexes.}
\end{figure}
3.2. Materials and methods

3.2.1. Materials

\(\alpha\)-Methoxy-\(\omega\)-amino poly(ethylene glycol) (PEG-NH\(_2\), \(M_n = 5200\) g/mol, \(M_w/M_n = 1.04\)) was obtained from Nippon Oil and Fats Co., Ltd. (Tokyo, Japan). Pluronic P85 was kindly provided by BASF Corp. (Florham Park, NY). L-Aspartic acid \(\beta\)-benzyl ester, benzene, branched polyethylenimine (bPEI, \(M_w = 25000\) g/mol), 1,3-diaminopropane, dichloromethane (DCM), diethylenetriamine (DET), \(N,N\)-dimethylformamide (DMF), dimethyl sulfoxide (DMSO), hexane, \(N\)-methyl-2-pyrrolidone (NMP), 4-nitrophenyl chloroformate (\(p\)-NPC), tetrahydrofuran (THF), and triphosgene were purchased from Sigma-Aldrich (St. Louis, MO). DET was distilled by conventional methods before use. DCM, DMF, DMSO, hexane, NMP, and THF were purchased as anhydrous grade and used without further purification. \(\beta\)-Benzyl-L-aspartate \(N\)-carboxy anhydride (BLA-NCA) was synthesized by the Fuchs-Farthing method using L-aspartic acid \(\beta\)-benzyl ester and triphosgene as described in section 2.2.2.2. Dialysis tubings (MWCOs 1000 and 6–8000) were purchased from Spectra/Por (Rancho Dominguez, CA). The pDNA coding for luciferase in pGL4 vector with a CMV promoter (Promega, Madison, WI) was amplified in DH5\(\alpha\) Escherichia coli cells and purified using PureLink\textsuperscript{TM} HiPure Plasmid Maxiprep Kit (Invitrogen, Carlsbad, CA). Luciferase Assay System Kit, CellTiter-Blue\textsuperscript{®} Cell Viability Assay, and ethidium bromide (EtBr) were purchased from Promega (Madison, WI). RC DC Protein Assay Kit was purchased from Bio-Rad (Hercules, CA). MDA-MB-231 human breast cancer cell line and A549 human lung cancer cell line were obtained from ATCC.
3.2.2. Methods

3.2.2.1. $^1$H NMR and gel permeation chromatography (GPC) analyses

Analyses were performed as described in section 2.2.2.1.

3.2.2.2. Synthesis of amino P85 (P85-NH$_2$)

Syntheses of the polymers used in this study are outlined in Fig. 3.2. Amino P85 (P85-NH$_2$) was prepared as previously described with modifications.$^{169}$ $p$-NPC (98 mg, 0.467 mmol) was dissolved in benzene and then added dropwise to P85 (2.15 g, 0.467 mmol) dissolved in benzene. The reaction mixture was stirred for 24 h at room temperature under an argon atmosphere. After reaction, benzene was evaporated under vacuum. The crude product was dissolved in methanol, precipitated in dry ice-chilled diethylether, and then subjected to centrifugation. The precipitation procedure was repeated at least 3 times to ensure the complete removal of unreacted $p$-NPC. The white solid obtained was dissolved in benzene and lyophilized. The degree of

![Fig. 3.2. Synthetic routes of Pluronic P85-NH$_2$ (A) and PEG-b-/P85-b-P[Asp(DET)] block copolymers (B).](image-url)
activation was determined to be 78% from the peak intensity ratio of the aryl protons of the nitrophenyl group (NO$_2$–C$_6$H$_4$–, $\delta$ = 7.5 and 8.25 ppm) to the methyl protons of P85 (–CH$_3$, $\delta$ = 1.0 ppm) in $^1$H NMR spectrum taken in DMSO-$d_6$ at 25 °C (Fig. 3.3). The activated P85 was subsequently reacted with 1,3-diaminopropane to obtain P85-NH$_2$. P85-$p$-nitrophenyl carbonate (P85-$p$-NPC) (1.08 g, 0.226 mmol) and 200 equiv of 1,3-diaminopropane (3.8 mL, 45.5 mmol) were dissolved in methanol. P85-$p$-NPC was added to 1,3-diaminopropane slowly and the reaction was allowed to proceed for 24 h at room temperature. The reaction mixture was transferred to a dialysis tubing (MWCO: 1000) and dialyzed extensively with methanol for 1 week. The product was then recovered by lyophilization from benzene. The degree of amine functionality was confirmed to be about 50% from the peak intensity ratio of the methylene protons of the propylamino moieties (–CH$_2$CH$_2$CH$_2$–, $\delta$ = 1.4–1.5 and 2.9–3.0 ppm) to the methyl protons of P85 (–CH$_3$, $\delta$ = 1.0 ppm) in $^1$H NMR spectrum taken in DMSO-$d_6$ at 25 °C (Fig. 3.4). P85 molecules without amine functionality were not separated from P85-NH$_2$ at this stage. The purification would be done after PBLA polymerization described in the following section.

3.2.2.3. Syntheses of poly(ethylene glycol)-block-/P85-block-poly{N-[N-(2-aminoethyl)-2-aminoethyl]aspartamide} (PEG-$b$-/P85-$b$-P[Asp(DET)])

Poly(ethylene glycol)-block-poly(β-benzyl-L-aspartate) (PEG-$b$-PBLA) was synthesized as previously described by ring-opening polymerization using β-benzyl-L-aspartate N-carboxy anhydride (BLA-NCA) and α-methoxy-ω-amino poly(ethylene glycol) as the monomer and initiator, respectively.$^{163}$ Briefly, BLA-NCA (835 mg, 3.35 mmol) dissolved in 1.5 mL DMF and
10 mL DCM was added to PEG-NH$_2$ ($M_n = 5200$ g/mol, 146 mg, 0.0281 mmol) dissolved in 5 mL DCM. The polymerization was allowed to proceed for 96 h at 35 °C under an argon atmosphere. P85-\textit{block}-poly(β-benzyl-L-aspartate) (P85-\textit{b}-PBLA) was synthesized with the same procedure while P85-NH$_2$ was used as the initiator of the polymerization. The product mixture was slowly added into diethylether. The precipitated polymer was then subjected to centrifugation. The precipitation procedure was repeated 3 times to purify the polymer. Free P85 without amine functionality was also removed during this purification step. The white crude solid obtained was dissolved in benzene and then freeze-dried. The degree of polymerization of the PBLA block of PEG-\textit{b}-PBLA was determined to be 106 from the peak intensity ratio of the aryl protons of the benzyl groups (C$_6$H$_5$—, δ = 7.2–7.3 ppm) to the methylene protons of PEG (—OCH$_2$CH$_2$—, δ = 3.5 ppm) in $^1$H NMR spectrum taken in DMSO-$d_6$ at 25 °C (Fig 3.5). The degree of polymerization of the PBLA block of P85-\textit{b}-PBLA was estimated to be 105 using the methyl protons of P85 (—CH$_3$, δ = 1.0 ppm) for peak integration (Fig 3.6).

PEG-\textit{b}-P[Asp(DET)] was obtained by substituting the side chains of PBLA block with DET through aminolysis reaction as previously described.$^{71}$ Freeze-dried PEG-\textit{b}-PBLA (202 mg, 7.49 μmol) was dissolved in 4 mL NMP and added dropwise to 4.3 mL (39.6 mmol) distilled DET (50 equiv to benzyl groups of PBLA segment) dissolved in 4 mL NMP. The reaction mixture was stirred for 0.5 h at 15 °C under an argon atmosphere. The polymer was then precipitated in diethylether and subsequently dried and dissolved in 1x PBS (pH 7.4) and dialyzed against PBS 3 times in a dialysis tubing (MWCO: 6–8000) at 4 °C. The polymer solution was then dialyzed against distilled water overnight at 4 °C, lyophilized, and the polymer was collected as a hydrochloride salt form. P85-\textit{b}-P[Asp(DET)] was prepared from P85-\textit{b}-PBLA with the same
procedure. The substitution was confirmed by the disappearance of the aryl protons of the benzyl groups (\(C_6H_5–\), \(\delta = 7.2–7.3\) ppm) and the emergence of methylene protons of the DET moieties (\(-CH_2CH_2NHCH_2CH_2–\), \(\delta = 2.6–3.6\) ppm). Quantitative introduction of DET was also confirmed by the peak intensity ratio of the \(\alpha\)-protons of polymer backbone (\(\delta = 4.5\) ppm) to the methylene protons of the DET moieties (\(-CH_2CH_2NHCH_2CH_2–\), \(\delta = 2.6–3.6\) ppm) in the \(^1\)H NMR spectrum taken in D$_2$O at 10 °C (Fig. 3.7 and 3.8).

3.2.2.4. Preparation of polyplexes

The stock solutions were prepared by dissolving pDNA and the synthesized polymers in 10 mM Tris-HCl buffer (pH 7.4) at concentrations of 50 \(\mu\)g/mL and 10 mg/mL, respectively. Polyplexes at various N/P ratios were formed by adding polymer solution with different concentrations (1/3 total volume) to pDNA solution (2/3 total volume), vortexing, and then incubating at room temperature for at least 30 min before experiments. The final concentration of pDNA in all samples was kept at 33.3 \(\mu\)g/mL. The polyplexes were formed in the same way in all experiments.

3.2.2.5. Gel retardation analysis

Polyplexes with different N/P ratios were loaded on a 0.8% (w/v) agarose gel containing 0.004% ethidium bromide and electrophoresed at 100 V for 45 min with a running buffer of 40 mM Tris–acetic buffer with 2 mM EDTA. The migrated pDNA was visualized by UV.
3.2.2.6. Particle size and ζ-potential measurements

Particle size and ζ-potential were determined by dynamic light scattering (DLS) and laser doppler velocimetry, respectively, at 25 °C using Zetasizer Nano-ZS (Malvern Instruments, Worcestershire, UK) equipped with a He-Ne ion laser (λ = 633 nm) for the incident beam at a detection angle of 173°. Polyplex samples formed at 33.3 μg pDNA/mL with different N/P ratios were adjusted to 10 μg pDNA/mL using 10 mM Tris-HCl buffer (pH 7.4). Polyplex samples (50–100 μL) were transferred to micro cuvettes for size measurements, while 750 μL of each sample was used for measuring ζ-potential using Dip cell (Malvern Instruments, UK). Cumulant diameters and ζ-potential values of polyplex samples were then estimated and analyzed as described in detail in section 2.2.2.8.

3.2.2.7. In vitro transfection

MDA-MB-231 human breast cancer cell line and A549 human lung cancer cell line were cultured in DMEM and RPMI 1640, respectively, supplemented with 10% FBS, 100 IU penicillin, and 100 μg/mL streptomycin. MDA-MB-231 was seeded at 50000 cells/well and A549 was seeded at 30000 cells/well on 24-well plates and incubated for 24 h. After the cells reached to about 60% confluence, polyplex solutions (30 μL) were prepared with different polymers synthesized at varying N/P ratios, diluted with 270 μL of fresh culture medium containing 10% FBS, and then added to the cells. The pDNA amount for each well was kept at 1 μg. The polyplexes were allowed to incubate with the cells for 24 h. The polyplex-containing medium was then removed and 1 mL of fresh DMEM with 10% FBS was added to each well. The cells were then maintained at 37 °C for another 24 h before luciferase assay. For luciferase
assay, the cells were washed with 1x PBS (1 mL) after medium removal. Cell Culture Lysis Reagent (140 μL) was then added to each well and the cells were incubated at room temperature for 15 min. One hundred μL of luciferase substrate was added to 20 μL of the lysate. The luminescence intensity was measured by an Orion microplate luminometer (Berthold Detection Systems, Oak Ridge, TN). The obtained luciferase expression was then normalized with the amount of total proteins present in the lysates determined by the RC DC Protein Assay Kit.

3.2.2.8. *In vitro* cytotoxicity

MDA-MB-231 cells were seeded at 5000 cells/well on 96-well plates in DMEM containing 10% FBS and incubated for 24 h. Polyplex solutions (10 μL) prepared at different N/P ratios or free polymer solutions with different amine concentrations were diluted with fresh medium (90 μL) and then added to the cells. The cells were incubated with polyplexes or free polymers for 24 h before refreshing medium. The cells were incubated for another 24 h and the CellTiter-Blue® Cell Viability Assay was carried out according to the manufacturer’s protocol. The fluorescence signals ($\lambda_{ex} = 560$ nm and $\lambda_{em} = 590$ nm) were measured 4 h after adding the dye. The results were represented as percentages of cell viability determined using untreated cells.

3.2.2.9. Flow cytometry measurements

pDNA was labeled with Label IT® Tracker™ Intracellular Nucleic Acid Localization Kit, CyTM5 (Mirus, Madison, WI) according to the manufacturer’s protocol. MDA-MB-231 cells were seeded at 100000 cells/well on 6-well plates in DMEM containing 10% FBS and incubated for 24 h. Polyplex solution prepared with Cy5-labeled pDNA for each polymer at N/P = 10 was
added to the cells after dilution with fresh DMEM medium containing 10% FBS and incubated for 4 h. The concentration of pDNA was kept at 2 μg/well. After removing the polyplex-containing medium, the cells were rinsed with 1x PBS 3 times. The cells were trypsinated with 500 μL of trypsin-EDTA and collected by centrifugation at 300 g for 2 min. The cell pellets were resuspended in 500 μL PBS. The Cy5 fluorescence intensity of each sample was then measured using BD LSR II cytometer (BD Biosciences, Franklin Lakes, NJ) equipped with a 633 nm laser for excitation.

3.2.2.10. Confocal laser scanning microscopy

MDA-MB-231 cells were seeded at 100000 cells/well on sterile coverslips on 6-well plates in DMEM containing 10% FBS and incubated for 24 h. Polyplex solution was prepared with 2 μg Cy5-labeled pDNA for each sample at N/P = 10. The polyplexes were added to the cells after dilution with fresh DMEM medium containing 10% FBS and incubated for designated time periods. Polyplex containing medium were then removed and the cells were rinsed with PBS. After staining the acidic late endosomes and lysosomes with 75 nM LysoTracker Green (Invitrogen, Carlsbad, CA) in DMEM for 30 min at 37 °C, the cells were rinsed again with 1x PBS and then fixed with 4% formaldehyde for 10 min at room temperature. The cells were washed with PBS and the cell nuclei were stained with 3 mM DAPI (Invitrogen, Carlsbad, CA) in PBS for 5 min at room temperature. The cells were rinsed with PBS and the coverslips were mounted on glass slides with SlowFade mounting medium (Invitrogen, Carlsbad, CA) and then the microscopy was done using FluoView 1000 confocal microscope (Olympus, Tokyo, Japan).
with a 100x objective. The excitation wavelengths used were 405 nm for DAPI (blue), 488 nm for LysoTracker Green (green), and 635 nm for Cy5 (red).

3.2.2.11. Statistical analysis

The experimental data were analyzed by unpaired Student's t-test. $P < 0.05$ was considered as significant.

3.3. Results

3.3.1. Syntheses of PEG-b-/P85-b-P[Asp(DET)]

The block copolymers of PEG-\textit{block}-poly(aspartamide) and P85-\textit{block}-poly(aspartamide) were synthesized according to Fig. 3.2. Initially, P85 was modified to introduce a primary amine for polymerization. P85 was activated with 1 molar equivalent amount of 4-nitrophenyl chloroformate in order to minimize the generation of P85 with both hydroxyl ends activated. The activated P85 was then reacted with excess amount of 1,3-diaminopropane to prevent P85-dimer formation. After purifications, $^1$H NMR showed that the activation percentage of P85 was about 78% for the first step (Fig. 3.3), while the amine functionality was approximately 50% after the second reaction (Fig. 3.4). GPC analysis confirmed that the obtained P85 with terminal amino group contained no dimers and the molecular weight polydispersity index was determined to be 1.11. The ring-opening polymerization of BLA-NCA was then carried out using $\alpha$-methoxy-$\omega$-amino poly(ethylene glycol) and the modified P85 amine as the initiators to prepare PEG-\textit{b-}PBLA and P85-\textit{b-}PBLA, respectively. The degrees of polymerization of the PBLA blocks were estimated to be 106 for PEG-\textit{b-}PBLA and 105 for P85-\textit{b-}PBLA (Fig. 3.5 and 3.6). GPC
Fig. 3.3. $^1$H NMR spectrum of P85-\(\gamma\)-NPC taken in DMSO-\(d_6\) at 25 °C.

Fig. 3.4. $^1$H NMR spectrum of P85-NH$_2$ taken in DMSO-\(d_6\) at 25 °C.

Fig. 3.5. $^1$H NMR spectrum of PEG-\(b\)-PBLA taken in DMSO-\(d_6\) at 25 °C.

Fig. 3.6. $^1$H NMR spectrum of P85-\(b\)-PBLA taken in DMSO-\(d_6\) at 25 °C.

measurements revealed that the molecular weight polydispersity indices were 1.09 and 1.18 for PEG-\(b\)-PBLA and P85-\(b\)-PBLA, respectively. The introduction of diethylenetriamine (DET) to
the PBLA blocks was done by aminolysis reaction with excess amount of DET relative to the benzyl ester groups of PBLA at low temperature. After protonation of the DET side chains and purification using dialysis at 4 °C to prevent degradation of the polymers, $^1$H NMR obtained with the obtained polymers showed that the removal of benzyl ester groups was complete and the substitution rates for both PEG-b-P[Asp(DET)] and P85-b-P[Asp(DET)] were close to 100% (Fig. 3.7 and 3.8). Table 3.1 summarizes the compositions and physical characteristics of the synthesized polymers.

![Fig. 3.7. $^1$H NMR spectrum of PEG-b-P[Asp(DET)] taken in D$_2$O at 10 °C.](image1)

![Fig. 3.8. $^1$H NMR spectrum of P85-b-P[Asp(DET)] taken in D$_2$O at 10 °C.](image2)

<table>
<thead>
<tr>
<th>Polymer</th>
<th>MW (g/mol)$^a$</th>
<th>Initiator $M_w/M_n$</th>
<th>-NH2 %</th>
<th>DP of P(Asp)$^b$</th>
<th>$M_w/M_n^c$</th>
<th>Total MW (g/mol)$^d$</th>
</tr>
</thead>
<tbody>
<tr>
<td>P85-b-P[Asp(DET)]</td>
<td>4600</td>
<td>1.11$^e$</td>
<td>50$^b$</td>
<td>105</td>
<td>1.18</td>
<td>29600</td>
</tr>
<tr>
<td>PEG-b-P[Asp(DET)]</td>
<td>5200</td>
<td>1.04$^a$</td>
<td>98.4$^a$</td>
<td>106</td>
<td>1.09</td>
<td>30300</td>
</tr>
</tbody>
</table>

Table 3.1. Characteristics of the polymers synthesized in this study. $^a$ Information provided by manufacturers. $^b$ Determined by $^1$H NMR. $^c$ Determined by GPC. $^d$ Calculated based on the HCl salt form of the polymers.
3.3.2. Formation and stability of polyplexes

Polyplexes were formed by adding stock polymer solution to pGL4 pDNA solution directly at different N/P ratios. To confirm the polyplex formation between the pDNA and polymers, PEG-\(b\)-P[Asp(DET)] or P85-\(b\)-P[Asp(DET)], agarose gel electrophoresis was employed. As shown in Fig. 3.9, the amount of free pDNA decreased with N/P ratio. Complete pDNA retardation was observed at N/P = 2 and 2.5 for P85- and PEG-based polyplexes, respectively, which indicates that no free pDNA was present and all pDNA was trapped inside the polyplex particles at N/P ratio > 2. As the N/P ratio was calculated based on the total number of primary and secondary amino groups on the polymers and it has been reported previously that there is around 50% protonation of the DET side chains at pH 7.4,\(^{108}\) N/P = 2 would be the theoretical stoichiometric mixing ratio between the pDNA and polymers and the minimal ratio of complete pDNA retardation. The results obtained are similar to published results.\(^{71}\) After confirming the polyplex forming ability of the polymers, the hydrodynamic diameters and polydispersity indices (PDI) of the polyplexes formed at various N/P ratios were measured by DLS and the results are shown in Fig. 3.10A and 3.10B, respectively. Within the N/P ratio range tested, all the polyplexes maintained sizes below 90 nm. The sizes of polyplexes with N/P > 5 stayed the same.

![Fig. 3.9. Gel retardation analysis of polyplexes.](image)
at about 70 nm for both PEG-\(b\)-P[Asp(DET)] and P85-\(b\)-P[Asp(DET)] polyplexes. Moreover, there was no aggregation observed for the polyplexes formed at stoichiometric mixing ratio (N/P = 2), implying that the PEG and P85 chains on the polyplex surface could minimize the interaction among polyplex particles even the particles possessed neutral charges.

![Graph A](image1)

**Fig. 3.10.** Cumulant diameters (A), PDI (B), and \(\zeta\)-potential values (C) of polyplexes formed at various N/P ratios in 10 mM Tris-HCl buffer (pH 7.4) at 25 °C (mean ± SD, \(n = 3\)).

The \(\zeta\)-potential of the polyplexes was determined by laser-Doppler electrophoresis. As seen in Fig. 3.10C, the \(\zeta\)-potential increased with N/P ratio of the polyplexes. The \(\zeta\)-potential was
almost neutral (~0 mV) at N/P = 2. This is consistent with the stoichiometry at this N/P ratio as all negative charges on the pDNA molecules are neutralized by the positive charges on the polymer molecules with no free polymer theoretically present. The ζ-potential values of both PEG-\textit{b}-P[Asp(DET)] and P85-\textit{b}-P[Asp(DET)] polyplexes leveled off at N/P = 5 and the values stayed the same up to N/P = 20. The maximum ζ-potential values obtained in this study are +10 mV and +15 mV for PEG-\textit{b}-P[Asp(DET)] and P85-\textit{b}-P[Asp(DET)] polyplexes, respectively. The difference in maximum ζ-potential values reveals that the charge shielding efficiency of the PEG layer may be higher than that of the P85 layer surrounding the polyplex particles.

The stability of the polyplexes was examined under physiological salt concentration of 150 mM NaCl based on particle size measurements. If the polyplexes are not stable, salt-induced aggregation will occur due to the loss of electrostatic repulsion after the charges on the polyplexes are screened by salt. The sizes of the polyplexes in dispersant containing 150 mM NaCl were monitored for 12 h at 25 °C by DLS. The size measurement was done every 30 min after salt addition. Both polyplexes formed with PEG-\textit{b}-P[Asp(DET)] and P85-\textit{b}-P[Asp(DET)] showed growth in size within the 12 h time period. The size of the PEG-\textit{b}-P[Asp(DET)] polyplexes increased from 100 nm to about 140 nm, while the P85-\textit{b}-P[Asp(DET)] polyplexes showed a more significant size increase from 120 nm to 270 nm (Fig. 3.11A). Although the size increased substantially for the P85-\textit{b}-P[Asp(DET)] polyplexes, the PDI remained below 0.18 after 12 h incubation (Fig. 3.11B). This indicates that the uniform size distribution of the polyplexes prepared from P85-\textit{b}-P[Asp(DET)] could still be maintained with the growth in particle size. The particle size polydispersity did not change significantly for PEG-\textit{b}-P[Asp(DET)] polyplexes.
Fig. 3.11. Time-dependent change of particle size (A) and PDI (B) of polyplexes formed at N/P = 10 in 10 mM Tris-HCl buffer (pH 7.4) containing 150 mM NaCl at 25 °C (mean ± SD, n = 3).

3.3.3. *In vitro* transfection efficiency

The effect of substituting PEG with P85 on the block copolymers on gene delivery ability of the polyplexes was evaluated from the luciferase gene expression in cells after transfection with plasmid pGL4. The transfection experiments were carried out in MDA-MB-231 human breast cancer cell line and A549 human lung cancer cell line using polyplexes prepared from PEG-\(b\)-P[Asp(DET)] and P85-\(b\)-P[Asp(DET)] at various N/P ratios. Polyplexes formed with bPEI at N/P = 10 was used as a control as this N/P ratio was determined to be optimal N/P ratio for transfection without causing massive cell death. P85-\(b\)-P[Asp(DET)] polyplexes shows highest transfection efficiency in the study with MDA-MB-231 cells and are efficient vehicles for pDNA delivery at both low and high N/P ratios (Fig. 3.12A). The transfection efficiency of P85-\(b\)-P[Asp(DET)] polyplexes did not increase dramatically with N/P ratios. Within the N/P ratio range tested, the luciferase expressions are within 1 order of magnitude. Furthermore, P85-\(b\)-P[Asp(DET)] polyplexes achieved higher luciferase expression than did the bPEI polyplexes at
every single N/P ratio. On the other hand, PEG-\textit{b}-P[Asp(DET)] polyplexes showed lower transfection ability than P85-\textit{b}-P[Asp(DET)] polyplexes, and the gene expression level obtained with PEG-\textit{b}-P[Asp(DET)] polyplexes was highly dependent on the N/P ratio at which the polyplexes formed. Quantitatively, the luciferase expression level was about 3 orders of magnitude higher for P85-\textit{b}-P[Asp(DET)] polyplexes at N/P 5, while the difference diminished to 1 order of magnitude at N/P = 40 in MDA-MB-231 cells. A549 cells were also used for comparing the transfection efficiency of different polyplexes (Fig. 3.12B) and a similar tendency was observed. The difference in luciferase gene expression was clearly shown between PEG-\textit{b}-

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig3.12.png}
\caption{Transfection efficiency of polyplexes formed at various N/P ratios in MDA-MB-231 (A) and A549 (B) cells (mean ± SD, \(n = 3, *P < 0.005\)).}
\end{figure}

P[Asp(DET)] and P85-\textit{b}-P[Asp(DET)] polyplexes at low N/P ratios, and the expression level became more comparable at high N/P ratios. Free P85 block copolymers at various concentrations were also added to the P85-\textit{b}-P[Asp(DET)] polyplex (N/P =10) solution for
transfection study; however, significant improvement in transfection efficiency could not be obtained (Fig. 3.13).

3.3.4. *In vitro* cytotoxicity

The toxicity caused by the PEG-\(b\)-P[Asp(DET)], P85-\(b\)-P[Asp(DET)], and bPEI polymers was evaluated using MDA-MB-231 cells based on the change of cellular metabolism. The study was done with different polyplexes at the N/P ratio range (N/P = 5–40) used in transfection study. As shown in Fig. 3.14A, P85-\(b\)-P[Asp(DET)] polyplexes showed slightly higher toxicity than did the PEG-\(b\)-P[Asp(DET)] polyplexes; however, they could still maintained about 90% cell viability at N/P = 40. On the contrary, polyplexes formed with bPEI caused significant toxicity to the cells. The cell viability decreased to 15% at N/P = 20 and almost 0% at N/P = 40.

The intrinsic toxicity of free polymers was also investigated. No substantial toxicity caused by free PEG-\(b\)-P[Asp(DET)] or P85-\(b\)-P[Asp(DET)] polymers was observed even up to amine concentration of 1000 \(\mu\)M, while the bPEI polymers elicited appreciable toxicity towards the
cells (Fig. 3.14B). Fifty percent growth inhibition was observed at amine concentration of approximately 100 µM and complete growth inhibition was obtained at 250 µM.

![Graph showing in vitro cytotoxicity of polyplexes formed at various N/P ratios (A) and free polymers at different amine concentrations (B) in MDA-MB-231 cells.](image)

**Fig. 3.14.** *In vitro* cytotoxicity of polyplexes formed at various N/P ratios (A) and free polymers at different amine concentrations (B) in MDA-MB-231 cells. 100% cell viability was determined from untreated cells. bPEI was used as control (mean ± SD, n = 3).

### 3.3.5. Cellular uptake study

The cellular uptake of polyplexes prepared with different cationic polymers at N/P = 10 by MDA-MB-231 cells was quantified by flow cytometry. After 4 h incubation with the cells, the polyplexes formed with P85-\textit{b}-P[Asp(DET)] showed highest level of cellular internalization based on the mean fluorescence intensity detected from the Cy5-labeled pDNA, which was 3 times higher than that of bPEI polyplexes and about 10 times higher than that of the polyplexes formed with PEG-\textit{b}-P[Asp(DET)] (Fig. 3.15A). The overall trend of cellular uptake of polyplexes is the same as the one obtained with transfection experiments. Comparing to PEG-\textit{b}-P[Asp(DET)], P85-\textit{b}-P[Asp(DET)] promoted a more narrow distribution and a higher level of the amount of polyplex uptake within the cell population (Fig. 3.15A and B).
Fig. 3.15. Cellular uptake of Cy5-labeled pDNA polyplexes formed at N/P = 10 in MDA-MB-231 cells. Flow cytometric data reported as mean of the relative fluorescence intensity ± SD (n = 3, *P < 0.005, **P < 0.001) (A). Flow cytometric histogram profiles of different polyplexes showing the distribution of Cy5 fluorescence intensity (B).

3.3.6. Intracellular trafficking

To gain more insight into the intracellular localization of polyplexes after cellular uptake, confocal laser scanning microscopy was employed to collect images of cells incubated with polyplexes for different time durations. The cell nuclei were stained with DAPI (blue). The pDNA was labeled with Cy5 (red), while the late endosomes and lysosomes were stained with LysoTracker Green as shown in green in the confocal images. Colocalization of pDNA and late endosomes/lysosomes produced yellow pixels due to the overlapping of the red and green fluorescence. As seen in Fig. 3.16, larger amount of red fluorescence could be observed with P85-b-P[Asp(DET)] polyplexes relative to the PEG-based polyplexes at all the time points tested in this study, and this is consistent with the results obtained from the flow cytometry analysis. The polyplexes prepared from P85-b-P[Asp(DET)] interacted with the cellular membrane as fast as 30 min after polyplex addition as majority of the polyplexes was found to be localized at the
cell membrane (Fig. 3.16A). The polyplexes progressively arrived at the late endosomes and lysosomes between 2 and 4 h as more yellow pixels were observed at 4 h after incubation started (Fig. 3.16C). After 24 h, a fraction of the polyplexes successfully escaped from the acidic vesicles as red fluorescence was detected in the cytoplasm and around those vesicles. Yellow pixels could still be seen at this time point, which represents partial escape of the polyplexes from the late endosomes and lysosomes (Fig. 3.16D). There was no substantial difference observed in terms of intracellular trafficking between the polyplexes formed with PEG-b-P[Asp(DET)] and P85-b-P[Asp(DET)] except the total amount of Cy5 fluorescence detected.

**Fig. 3.16.** Intracellular trafficking analysis by confocal laser scanning microscopy. Polyplexes containing Cy5-labeled pDNA (red) were formed with P85-b-P[Asp(DET)] (A–D) and PEG-b-P[Asp(DET)] (E–H) at N/P 10 and incubated for different time periods (A, E: 0.5 h, B, F: 2 h, C, G: 4 h, D, H: 4 h and subsequent 20 h polyplex free incubation) with MDA-MB-231 cells before images were taken. Cell nuclei and late endosomes/lysosomes were stained with DAPI (blue) and LysoTracker Green (green), respectively.
3.4. Discussion

PEGylated polyplexes have been extensively studied for delivering nucleic acid materials ultimately for therapeutic purposes. On the one hand PEGylation enhances the colloidal stability of polyplexes and minimizes the recognition of the polyplexes by the reticuloendothelial system (RES), but on the other hand PEGylation reduces the interaction between the polyplexes and the target cells and which leads to hampered cellular uptake of the polyplexes and subsequently diminished transfection efficacy. In this regard, the PEG block of PEG-\(b\)-P[Asp(DET)] was substituted with amphiphilic Pluronic P85 to investigate whether the cellular uptake and transfection efficiency could be improved through promoting polyplex particle and cell membrane interaction.

P85-\(b\)-P[Asp(DET)] was prepared by the polymerization of BLA-NCA using P85 modified with amine functionality as the initiator and the subsequent aminolysis reaction to substitute the benzyl ester protecting groups with diethylenetriamine (DET) for introducing cationic moieties (Fig. 3.2). The complexation of pDNA by the polymers synthesized, PEG-\(b\)-P[Asp(DET)] and P85-\(b\)-P[Asp(DET)], was confirmed by gel retardation analysis as shown in Fig. 3.9. Complete retardation was observed at N/P > 2, which corresponds to N\(^{+}\)/P > 1 at physiological pH 7.4 due to the mono-protonated state of the DET cationic side chains. Physical properties such as particle size and \(\zeta\)-potential were characterized. The polyplexes formed with both block copolymers have sizes at about 70 nm when N/P \(\geq\) 5 (Fig. 3.10A). Although close-to-neutral \(\zeta\)-potential was observed at the stoichiometric N/P = 2 (Fig. 3.10C), no aggregation could be observed based on the size measurements, and this reveals that both PEG and P85 chains can provide a certain level of protection for the polyplexes when the particles are experiencing minimal repulsive force at
this critical N/P ratio. The values of ζ-potential of the polyplexes leveled off at N/P 5 and stayed at around +10 mV and +15 mV for PEG-b-P[Asp(DET)] polyplexes and P85-b-P[Asp(DET)] polyplexes, respectively. As previously reported that the ζ-potential can go up to +30 mV for particles prepared from the non-PEGylated P[Asp(DET)] homopolymers, the ζ-potential result suggests that the PEG and P85 chains on the surface of the particles can effectively shield the charges on the particles, while the PEG chains provided a better charge shielding effect as lower ζ-potential was observed. The lower charge masking ability of P85 can be owing to its lower molecular weight (PEG: 5200 g/mol and P85: 4600 g/mol) and its amphiphilic nature, which may result in a thinner P85 palisade surrounding the polyplex compared to PEG.

Polyplex stability is important for \textit{in vitro} and especially crucial for \textit{in vivo} gene delivery. The effect of introducing P85 to polyplex surface on stability of polyplexes in solution with physiological salt concentration was monitored for 12 h based on the changes of particle size (Fig. 3.11A) and polydispersity index (Fig. 3.11B) as salt can screen the charges on the polyplexes and minimize the charge repulsion force among polyplex particles. The PEG-based polyplexes demonstrated superior stability compared to the P85-based polyplexes. The increase in particle size and change in polydispersity index were small for the PEG-based polyplexes, while the size increased about 2 times for the polyplexes prepared with P85-b-P[Asp(DET)] within the 12 h period. The lower stability of the P85-based polyplex may be related to the more hydrophobic P85 layer surrounding the polyplex which provides less protection to the polyplex particle against salt-induced aggregation compared to the more hydrophilic PEG layer. When the particles lose charge repulsion in the physiological salt environment, they will have higher chance to interact with one another, and at the same time the hydrophobicity of the P85 chains
may lead the polyplex particles to come together based on hydrophobic interaction. This result allows us to know that further modifications on this polyplex system to improve the stability are necessary for future \textit{in vivo}, especially systemic, applications.

The substitution of the PEG block with Pluronic P85 on the block cationomer contributed to enhanced transfection efficiency of the polymers in particular when the polyplexes were formed at low N/P ratios. A high transfection efficiency could be achieved in both MDA-MB-231 and A549 cell lines with polyplexes prepared with P85-b-P[Asp(DET)] at low N/P ratio, and increased N/P ratio did not contribute to better transfection result (Fig. 3.12). On the contrary, the transfection ability of the PEG-based polyplexes was highly dependent on N/P ratio. PEG-based polyplexes prepared at high N/P ratios, for example N/P = 40, are required to achieve results comparable to the P85-based polyplexes. Study has shown that free cationic polymers exist when N/P ratio is higher than 4 for this specific PEG block copolymer.\textsuperscript{143} This implies that large excess amount of the PEG-b-P[Asp(DET)] polymers is required to coexist with the polyplexes in order to achieve high transfection, which will impose an obstacle for \textit{in vivo} applications because injected materials are diluted rapidly in physiological environments and it may be impossible for large amount of free polymers to concentrate at the target sites. As P85-b-P[Asp(DET)] polyplexes were shown to possess high transfection ability even the N/P ratio was low as 5 and without the presence of large excess amount of polymers, this could be an advantage of using this gene delivery vehicle for \textit{in vivo} applications. Previous studies have shown that including free Pluronic block copolymers in different polyplex formulations (branched PEI, Superfect,\textsuperscript{170} and poly(2-N-(dimethylaminoethyl) methacrylate\textsuperscript{158}) could enhance the transfection; nonetheless, no increase in transfection efficiency was observed in this study.
using the combination of free P85 and P85-\textit{b}-P[Asp(DET)] as the free Pluronic and polyplex-forming agent, respectively (Fig. 3.13). Although no definite conclusion can be drawn whether free Pluronic can promote better transfection of P85-\textit{b}-P[Asp(DET)] polyplexes at this stage, it is possible that the testing conditions may play a crucial role for utilizing free Pluronic polymer as a transfection enhancer.

The mechanism for the improved transfection performance by P85-\textit{b}-P[Asp(DET)] polyplexes was further elucidated by the analyses of cellular uptake and intracellular trafficking of the polyplexes. It was confirmed by both flow cytometry (Fig. 3.15) and confocal microscopy (Fig. 3.16) that the cellular internalization of P85-\textit{b}-P[Asp(DET)] polyplexes was much more efficient than that of the PEG-based polyplexes. P85-\textit{b}-P[Asp(DET)] polyplexes started interacting with the cell membrane extensively within 30 min and the polyplexes accumulated inside the cells at a much higher level during the 24 h time course than did the PEG-\textit{b}-P[Asp(DET)] polyplexes. All these data are consistent with the hypothesis that the amphiphilic characteristic of P85 substantially facilitates the cellular uptake of the polyplex particles by increasing the interaction between the particles and the cellular membrane. On the one hand the amphiphilic nature of P85 helps improve the internalization of particles, but on the other hand it is possible that amphiphilic P85 may cause excess disruption of the cell membrane and lead to undesired toxicity or cell death. Significant toxicity caused by P85-\textit{b}-P[Asp(DET)] polyplexes or the free polymers could not be observed in this study based on the cellular metabolic level (Fig. 14), suggesting that P85-\textit{b}-P[Asp(DET)] is an efficient synthetic delivery vehicle for pDNA with low toxicity.
3.5. Conclusions

In this chapter, we demonstrated that substituting the hydrophilic PEG layer with an amphiphilic Pluronic P85 layer on the surface of polyplex could lead to higher transfection efficiency of the P[Asp(DET)]-based pDNA delivery system. The amphiphilic nature of the P85 shell promoted cellular uptake of the polyplex particles and subsequently increased the transfection ability of the particles. Another advantage of the P85-based polyplex system is high transfection efficiency is achievable at low N/P ratios, and this is especially beneficial for in vivo applications. However, it has been shown in this study that the stability with respect to salt-induced aggregation of the P85-based polyplexes is lower than that of the PEG-based polyplexes; therefore, further improvements on the P85-based polyplex stability may be required before it can be used for in vivo pDNA delivery.
Particle stability and transfection efficiency evaluations on ternary polyionic complexes with surfaces shielded with different ratios of hydrophilic poly(ethylene glycol) and amphiphilic poly(ethylene oxide)-block-poly(propylene oxide)-block-poly(ethylene oxide) polymers

4.1. Introduction

In the previous chapter, we demonstrated that amphiphilic Pluronic P85 could be used to substitute the hydrophilic poly(ethylene glycol) (PEG) block on poly(ethylene glycol)-block-poly{N-[N-(2-aminoethyl)-2-aminoethyl]aspartamide} (PEG-b-P[Asp(DET)]) to improve the transfection ability of polyplexes formed with the cationic polymers and pDNA, especially at low N/P ratios. However, it was also shown that the Pluronic P85-based polyplex nanoparticles suffered from salt-induced aggregation and showed more significant increase in particle size in physiological salt environment compared to the PEG-based particles. Kabanov and coworkers improved the colloidal stability of the Pluronic-based polyplex particles by including free Pluronic polymers during the polyplex formation. The utilization of free Pluronic in those studies also improved the overall transfection efficiency of the polyplex systems.\textsuperscript{150,157,159} Unfortunately, it was shown in previous chapter that free Pluronic could not improve the transfection level of the P[Asp(DET)]-based polyplexes, and preliminary studies on particle stability suggested that addition of free Pluronic polymers to polyplexes could not stabilize the particles. Therefore, in order to balance the stability and transfection efficiency of the polyplexes, ternary polyplexes consisting of PEG-b-P[Asp(DET)], poly(ethylene oxide)-block-poly(propylene oxide)-block-poly(ethylene oxide)-block-poly{N-[N-(2-aminoethyl)-2-aminoethyl]aspartamide} (P(EPE)-b-P[Asp(DET)]), and pDNA were prepared and investigated whether the colloidal stability of the ternary complexes could be improved by incorporating PEG-based polymers for complexation while maintaining a satisfactory level of transfection.

Another aim of this study was to explore if the transfection ability of the present ternary complex system could be further improved by replacing P(EPE)-b-P[Asp(DET)] used in ternary
complexation with a bioreducible P(EPE)-SS-P[Asp(DET)] polymer, which has a redox
potential-responsive disulfide linkage located between the P(EPE) polymer and the cationic
block. It has been demonstrated that the reduction of disulfide bond could occur intracellularly in
the reducing glutathione-rich cytoplasm\textsuperscript{103} or extracellularly due to the presence of free thiols
secreted by cells\textsuperscript{121} or protein disulfide-isomerase on the cellular membrane.\textsuperscript{122,123} Moreover,
several reviews suggested that amphiphilic polymers and polyelectrolytes (cationic/anionic
polymers) interact with the lipid bilayer using different mechanisms,\textsuperscript{171,172} thus, the co-presence
of those two species at close proximity might lead to a more effective membrane perturbation. If
the cleavage of disulfide linkage occurs near the cell membrane surface or along the endocytic
pathway after particle internalization, the detachment of P(EPE) polymers from the polyplex
particles may allow higher cellular uptake or more efficient endo/lysosomal escape of the
particles through the combined membrane-disturbing effects of the amphiphilic P(EPE) and
cationic polymers.

In this chapter, we describe the syntheses of two P(EPE)-based block copolymers, P(EPE)-
b-P[Asp(DET)] and bioreducible P(EPE)-SS-P[Asp(DET)], and show that both of them can form
ternary polyplexes with additions of PEG-\textit{b}-P[Asp(DET)] and pDNA. Characterizations on
particle stability at physiological conditions, sensitivity to reducing environment, and
transfection ability of the ternary complexes were performed. The ternary complexes revealed
higher stability against salt-induced aggregation compared to the P(EPE)-based binary polyplex
particles. Moreover, the ternary polyplexes with P(EPE)-detachable property demonstrated
improved transfection efficiency compared to the non-bioreducible counterparts.
4.2. Materials and methods

4.2.1. Materials

α-Methoxy-ω-amino poly(ethylene glycol) (PEG-NH$_2$, $M_n = 12300$ g/mol, $M_w/M_n = 1.03$) was obtained from Nippon Oil and Fats Co., Ltd. (Tokyo, Japan). Amphiphilic triblock copolymers, α-methoxy-ω-amino poly(ethylene oxide)$_{27}$-block-poly(propylene oxide)$_{40}$-block-poly(ethylene oxide)$_{27}$ (P(EPE)-NH$_2$, $M_n = 4800$ g/mol, $M_w/M_n = 1.10$) and α-methoxy-ω-hydroxy poly(ethylene oxide)$_{27}$-block-poly(propylene oxide)$_{40}$-block-poly(ethylene oxide)$_{27}$ (P(EPE)-OH, $M_n = 4800$ g/mol, $M_w/M_n = 1.07$), were synthesized by Advanced Polymer Materials Inc. (Montreal, Canada). L-Aspartic acid β-benzyl ester, benzene, branched polyethylenimine (bPEI, $M_w = 25000$ g/mol), cysteamine, dichloromethane (DCM), diethylenetriamine (DET), $N, N$-dimethylformamide (DMF), dimethyl sulfoxide (DMSO), dithiothreitol (DTT), hexane, $N$-methyl-2-pyrrolidone (NMP), 4-nitrophenyl chloroformate ($p$-NPC), tetrahydrofuran (THF), and triphosgene were purchased from Sigma-Aldrich (St. Louis, MO). DET was distilled by conventional methods before use. DCM, DMF, DMSO, hexane, NMP, and THF were purchased as anhydrous grade and used without further purification. β-
Benzyl-L-aspartate $N$-carboxy anhydride (BLA-NCA) was synthesized by the Fuchs-Farthing method using L-aspartic acid β-benzyl ester and triphosgene as described in section 2.2.2.2. Dialysis tubings (MWCOs 1000 and 6–8000) were purchased from Spectra/Por (Rancho Dominguez, CA). The pDNA coding for luciferase in pGL4 vector with a CMV promoter (Promega, Madison, WI) was amplified in DH5α *Escherichia coli* cells and purified using PureLink™ HiPure Plasmid Maxiprep Kit (Invitrogen, Carlsbad, CA). Luciferase Assay System Kit, CellTiter-Blue® Cell Viability Assay, and ethidium bromide (EtBr) were purchased from Promega (Madison, WI). *RC DC* Protein Assay Kit was purchased from Bio-Rad (Hercules, CA). MDA-MB-231 human breast cancer cell line and A549 human lung cancer cell line were obtained from ATCC.

4.2.2. Methods

4.2.2.1 $^1$H NMR and gel permeation chromatography (GPC) analyses

Analyses were performed as described in section 2.2.2.1.

4.2.2.2. Synthesis of α-methoxy-ω-(2-aminoethylthio) poly(ethylene oxide)-block-poly(propylene oxide)-block-poly(ethylene oxide) (P(EPE)-SS-NH$_2$)

Syntheses of the polymers used in this study are outlined in Fig. 4.2. Disulfide containing P(EPE) monoamine (P(EPE)-SS-NH$_2$) was prepared from P(EPE)-OH as previously described with modifications.$^{169}$ Fifty equivalent of $p$-NPC (660 mg, 3.14 mmol) was dissolved in benzene and then added dropwise to P(EPE)-OH (301 mg, 0.0627 mmol) dissolved in benzene. The reaction proceeded for 24 h at room temperature under an argon atmosphere. Benzene was then
evaporated under reduced pressure and the crude product was dissolved in methanol, precipitated in dry ice-chilled diethylether, and then subjected to centrifugation. The precipitation and centrifugation procedure was repeated 3 times to ensure the complete removal of unreacted p-

NPC. The white solid obtained was dissolved in benzene and lyophilized. The degree of activation was determined to be about 100% from the peak intensity ratio of the aryl protons of the nitrophenyl group (NO$_2$–C$_6$H$_4$–, $\delta$ = 7.5 and 8.25 ppm) to the methyl protons of P(EPE) (–CH$_3$, $\delta$ = 1.0 ppm) in $^1$H NMR spectrum taken in DMSO-$d_6$ at 25 °C (Fig. 4.3). The activated P(EPE) was subsequently reacted with cysteamine to obtain P(EPE)-SS-NH$_2$. P(EPE)-$p$-nitrophenyl carbonate (P(EPE)-$p$-NPC) (0.17 g, 0.0342 mmol) and 200 equiv of cysteamine (0.54 g, 7 mmol) were dissolved in methanol and mixed. The reaction was allowed to proceed for 48 h at room temperature. The GPC result showed that there was no dimer (P(EPE)-SS-
P(EPE)) present after 48 h reaction and the reaction mixture was transferred to a dialysis tubing (MWCO: 1000) and dialyzed extensively against methanol. The product was then lyophilized from benzene. The degree of amine functionality was confirmed to be about 80% from the peak intensity ratio of the methylene protons of the cysteamine moieties (–CH₂CH₂SSCH₂CH₂–NH₂, δ = 2.7–2.9 ppm) to the methyl protons of P(EPE) (–CH₃, δ = 1.0 ppm) in ¹H NMR spectrum obtained in DMSO-d₆ at 80 °C (Fig. 4.4). GPC was used again to monitor the molecular weight distribution after dialysis and it showed that P(EPE)-SS-P(EPE) dimers were formed during dialysis; however, the dimeric molecules were not separated from P(EPE)-SS-NH₂ at this stage as they did not affect subsequent polymerization reaction. The dimers would be removed by selective precipitation after the polymerization of PBLA described in the following section.

### 4.2.2.3. Syntheses of poly(ethylene glycol)-block-P(EPE)-block-P(EPE)-SS-poly{N-[N-(2-aminoethyl)-2-aminoethyl]aspartamide} (PEG-b-P(EPE)-b-P(EPE)-SS-P[Asp(DET)])

Block copolymer of poly(ethylene glycol)-block-poly(β-benzyl-L-aspartate) (PEG-b-PBLA) was synthesized similarly as previously described in section 3.2.2.3. β-Benzyl-L-aspartate N-carboxy anhydride (BLA-NCA) and α-methoxy-ω-amino poly(ethylene glycol) were used as the monomer and initiator, respectively.¹⁶³ Vacuum-dried BLA-NCA (1.22 g, 4.90 mmol) dissolved in 3 mL DMF and 20 mL DCM was quickly added in a stream of argon to lyophilized PEG-NH₂ (Mₙ = 12,300 g/mol, 502 mg, 0.0408 mmol) dissolved in 10 mL DCM. The polymerization was allowed to proceed for 96 h at 35 °C under an argon atmosphere. P(EPE)-block-poly(β-benzyl-L-aspartate) (P(EPE)-b-PBLA) and P(EPE)-SS-poly(β-benzyl-L-aspartate) (P(EPE)-SS-PBLA) were synthesized with the same procedure while P(EPE)-NH₂ and P(EPE)-SS-NH₂ were used as
the initiator of the polymerization, respectively. The product was precipitated by adding the reaction mixture into diethylether and then subjected to centrifugation. The precipitation procedure was repeated 3 times to purify the polymer. P(EPE)-SS-P(EPE) dimer without amine functionality was also removed during this purification step. The degree of polymerization of the PBLA block of PEG-b-PBLA was determined to be 101 from the peak intensity ratio of the aryl protons of the benzyl groups of the PBLA block (C₆H₅−, δ = 7.2–7.3 ppm) to the methylene protons of PEG (–OCH₂CH₂−, δ = 3.5 ppm) in ¹H NMR spectrum taken in DMSO-d₆ at 25 °C (Fig. 4.5). The degrees of polymerization of the PBLA blocks of P(EPE)-b-PBLA and P(EPE)-SS-PBLA were estimated to be 100 and 104, respectively using the methyl protons of P(EPE) (–CH₃, δ = 1.0 ppm) and the aryl protons of the benzyl groups (C₆H₅−, δ = 7.2–7.3 ppm) of the PBLA block for peak integration (Fig. 4.6 and 4.7).

The side chains of the PBLA block of the synthesized PEG-b-PBLA were then substituted with DET through aminolysis reaction to obtain PEG-b-P[Asp(DET)].¹¹ Freezedried PEG-b-PBLA (512 mg, 0.0155 mmol) was dissolved in 12 mL NMP and added dropwise to 8.5 mL (78.3 mmol) distilled DET (50 equiv to benzyl group of PBLA block) dissolved in 8 mL NMP. The reaction mixture was stirred for 0.5 h at 15 °C under an argon atmosphere. The polymer was then precipitated in diethylether and subsequently dried and dissolved in cold 0.01 N HCl and dialyzed against 0.01 N HCl 3 times (2 h each) in a dialysis tubing (MWCO: 6–8000) at 4 °C. The polymer solution was then dialyzed against deionized water overnight at 4 °C, lyophilized, and the polymer was collected as a hydrochloride salt form. P(EPE)-b-P[Asp(DET)] and P(EPE)-SS-P[Asp(DET)] were obtained with the same method. Quantitative introduction of DET was confirmed by the peak intensity ratio of the peak intensity ratio of the α-protons of
polymer backbone ($\delta = 4.5$ ppm) to the methylene protons of the DET moieties ($-\text{CH}_2\text{CH}_2\text{NHCH}_2\text{CH}_2-$, $\delta = 2.6$–$3.6$ ppm) in the $^1$H NMR spectrum taken in D$_2$O at 10 or 80 °C (Fig. 4.8–4.10).

4.2.2.4. Preparation of polyplexes

The stock solutions were prepared by dissolving pDNA and synthesized polymers in 10 mM Tris-HCl buffer (pH 7.4) at concentrations of 50 $\mu$g/mL and 10 mg/mL, respectively. Binary polyplexes were prepared as previously described in section 3.2.2.4. Ternary polyplexes were formed using the same procedure except two different polymer solutions were thoroughly mixed before adding to pDNA solution. The percentage of individual polymer used in forming ternary complexes was calculated based on the amino group percentage of the corresponding polymer at N/P = 4, which was fixed for all ternary polyplexes in this study.

4.2.2.5. Particle size and $\zeta$-potential measurements

Particle size and $\zeta$-potential measurements were done at 25 °C or 37 °C as previously described in section 3.2.2.6.

4.2.2.6. In vitro transfection

Transfection experiments on MDA-MB-231 and A549 cell lines were similarly carried out as described in section 3.2.2.7. The polyplex incubation time was reduced to 4 h and the post-transfection incubation time prior to luciferase assay was increased to 44 h as it has been
determined in section 3.3.5 that the polyplex particles could internalize into cells efficiently in 4 h incubation.

4.2.2.7. In vitro cytotoxicity

MDA-MB-231 cells and A549 cells were seeded at 5000 cells/well and 3000 cells/well, respectively, on 96-well plates in the corresponding medium containing 10% FBS and incubated for 24 h. Polyplex solutions (10 μL) prepared at different N/P ratios were diluted with fresh medium (90 μL) and then added to the cells. The cells were incubated with the polyplexes for 4 h before exchanging medium. The cells were incubated for another 44 h and CellTiter-Blue® Cell Viability Assay was carried out according to the manufacturer’s protocol. The fluorescence signals ($\lambda_{ex} = 560$ nm and $\lambda_{em} = 590$ nm) were measured 4 h after adding the dye. The results were represented as percentages of cell viability determined using untreated cells.

4.2.2.8. Flow cytometry measurements

Cellular uptake of polyplex particles was evaluated using MDA-MB-231 cells as described in section 3.2.2.9. All polyplexes were formed at N/P = 4 as to be consistent with the in vitro transfection and cytotoxicity experiments.

4.2.2.9. Statistical analysis

The experimental data were analyzed by unpaired Student's t-test. $P < 0.05$ was considered as significant.
4.3. Results

4.3.1. Syntheses of PEG-b-/P(EPE)-b-/P(EPE)-SS-P[Asp(DET)]

The syntheses of the cationic block copolymers were described in Fig. 4.2 and the physical characterizations of the polymers were summarized in Table 4.1. Disulfide containing amphiphilic P(EPE) monoamine was synthesized from activation of the terminal hydroxyl groups of P(EPE)-OH with p-NPC (Fig. 4.3) and subsequent reaction between the activated P(EPE) with cysteamine (Fig. 4.4). During this subsequent reaction, the amino group of cysteamine first reacted with the activated P(EPE) to form a carbamate ester; the free thiol group at the other end of cysteamine then reacted with another cysteamine molecule to form a disulfide linkage. Excess amount of cysteamine was employed to prevent the formation of dimeric P(EPE)-SS-P(EPE) while favoring the formation of P(EPE)-SS-NH$_2$, which was then used to initiate the polymerization of BLA-NCA. $^1$H-NMR showed that the degree of polymerization of

Fig. 4.3. $^1$H NMR spectrum of P(EPE)-p-NPC taken in DMSO-$d_6$ at 25 °C.  

Fig. 4.4. $^1$H NMR spectrum of P(EPE)-SS-NH$_2$ taken in DMSO-$d_6$ at 80 °C.
the PBLA block of each polymer was approximately 100 (Fig. 4.5–4.7) and GPC confirmed that
the molecular weight distribution of each polymer was unimodal with $M_w/M_n$ equal to or below
1.13. The obtained copolymers were then subjected to aminolysis reaction with DET, followed

![Fig. 4.5. $^1$H NMR spectrum of PEG-$b$-PBLA taken in DMSO-$d_6$ at 25 °C.](image1)

![Fig. 4.6. $^1$H NMR spectrum of P(EPE)-$b$-PBLA taken in DMSO-$d_6$ at 25 °C.](image2)

![Fig. 4.7. $^1$H NMR spectrum of P(EPE)-SS-PBLA taken in DMSO-$d_6$ at 25 °C.](image3)
by purification, protonation of the DET side chains, and lyophilization. $^1$H-NMR revealed that the benzyl ester protecting groups were completely removed from the obtained cationic copolymers and the substitution rates for DET were close to 100% (Fig. 4.8–4.10).

**Fig. 4.8.** $^1$H NMR spectrum of PEG-$b$-P[Asp(DET)] taken in D$_2$O at 80 °C.

**Fig. 4.9.** $^1$H NMR spectrum of P(EPE)-$b$-P[Asp(DET)] taken in D$_2$O at 10 °C.

**Fig. 4.10.** $^1$H NMR spectrum of P(EPE)-SS-P[Asp(DET)] taken in D$_2$O at 10 °C.
Table 4.1. Characteristics of the polymers synthesized in this study. \textsuperscript{a} Information provided by manufacturers. \textsuperscript{b} Determined by \textsuperscript{1}H NMR. \textsuperscript{c} Determined by GPC. \textsuperscript{d} Calculated based on the HCl salt form of the polymers.

<table>
<thead>
<tr>
<th>Polymer</th>
<th>MW (g/mol)</th>
<th>Initiator</th>
<th>$M_w/M_n$</th>
<th>-NH2 %</th>
<th>DP of P(Asp)</th>
<th>$M_w/M_n$</th>
<th>Total MW (g/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P(EPE)-b-P[Asp(DET)]</td>
<td>4800</td>
<td>1.10</td>
<td>70\textsuperscript{a}</td>
<td>100</td>
<td>1.13</td>
<td>28500</td>
<td></td>
</tr>
<tr>
<td>P(EPE)-SS-P[Asp(DET)]</td>
<td>4800</td>
<td>1.07</td>
<td>80\textsuperscript{b}</td>
<td>104</td>
<td>1.13</td>
<td>29600</td>
<td></td>
</tr>
<tr>
<td>PEG-b-P[Asp(DET)]</td>
<td>12000</td>
<td>1.03</td>
<td>97.8\textsuperscript{c}</td>
<td>101</td>
<td>1.09</td>
<td>36200</td>
<td></td>
</tr>
</tbody>
</table>

4.3.2. Formation and stability of binary polyplexes

To investigate the effect of different molecular weights of PEG on particle stability of PEG-\textit{b}-P[Asp(DET)] polyplexes, the stability in physiological salt condition of PEG-\textit{b}-P[Asp(DET)] polyplexes prepared with PEG with molecular weight of 5000 or 12000 g/mol was evaluated based on particle size change by DLS measurements. Fig. 4.11 displays that higher-molecular-weight PEG enhanced the particle colloidal stability with almost no size change for 12 h under the tested conditions; thus, PEG with molecular weight of 12000 g/mol was used hereafter in

![Figure 4.11](image-url)
this study. Polyplexes at different N/P ratios were then formed with either PEG-\(b\)-P[Asp(DET)], P(EPE)-\(b\)-P[Asp(DET)], or P(EPE)-SS-P[Asp(DET)], and the cumulant diameters of the polyplexes are shown in Fig. 4.12A. Within the N/P ratio range tested, all the polyplexes possess sizes below 110 nm. The sizes of polyplexes with N/P > 4 maintained at around 70 to 80 nm for all polyplexes. A larger particle size but no aggregation was observed for the polyplexes formed at N/P = 2, which has been determined to be the stoichiometric mixing ratio as approximately 50% of the amino groups of the DET side chains were protonated at pH 7.4,\textsuperscript{108} indicating that both the PEG and P(EPE) chains on the polyplex surface could help prohibit the interaction among neutrally charged polyplexes under the conditions tested.

![Fig. 4.12. Cumulant diameters (A) and \(\zeta\)-potential values (B) of polyplexes formed at various N/P ratios in 10 mM Tris-HCl buffer (pH 7.4) at 25 °C (mean ± SD, \(n = 3\)).](image)

The \(\zeta\)-potential of the polyplexes was evaluated, and the results are shown in Fig. 4.12B. All polyplexes displayed the same trend of \(\zeta\)-potential change with N/P ratio. The \(\zeta\)-potential increased rapidly in the range of N/P = 1–4 and was almost neutral at stoichiometric N/P ratio of 2. The increase in \(\zeta\)-potential value subsided above N/P = 4 and the values stayed at about the
same up to N/P = 32. Moreover, Fig. 4.12B demonstrates that in general the \( \zeta \)-potential values of P(EPE)-b-P[Asp(DET)] and P(EPE)-SS-P[Asp(DET)] polyplexes were higher than those of PEG-b-P[Asp(DET)] polyplexes. The overall smaller \( \zeta \)-potential values of PEG-b-P[Asp(DET)] polyplexes revealed that the PEG chains provide a more efficient charge shielding effect. All the P(EPE)-b-P[Asp(DET)] polyplex characterization results obtained in this study were similar to the results obtained using polyplexes formed with P85-b-P[Asp(DET)] in the previous chapter.

Polyplex colloidal stability was investigated based on the salt-induced particle aggregation at 37 °C using DLS. Polyplex particles (N/P = 4) formed with PEG-b-P[Asp(DET)] showed minimal increase in size during the 1 h incubation. As shown in Fig. 4.13, the cumulant diameter was maintained below 100 nm. However, the stability of the polyplexes formed with P(EPE)-b-P[Asp(DET)] or P(EPE)-SS-P[Asp(DET)] was inferior to that of PEG-b-P[Asp(DET)] polyplexes. Rapid particle aggregation was observed and the cumulant diameters increased to about 800 to 900 nm in 1 h.

**Fig. 4.13.** Time-dependent particle size change of polyplexes formed at N/P = 4 in 10 mM Tris-HCl buffer (pH 7.4) containing 150 mM NaCl at 37 °C (mean ± SD, n = 3).
4.3.3. Reducing environment-sensitivity of P(EPE)-SS-P[Asp(DET)] polyplexes

In order to investigate whether the P(EPE) block copolymers could be released under a reducing environment specifically due to the cleavage of the disulfide bond on the P(EPE)-SS-P[Asp(DET)] polymers, the changes in particle size and ζ-potential of polyplexes were monitored in non-reducing (0 mM DTT) and reducing (10 mM DTT) environments at 25 °C. Ten millimolar DTT is commonly used to mimic the intracellular reducing environment in the cytoplasm. To look at the size change after the release of the P(EPE) shell, neutral polyplexes were prepared at stoichiometric N/P ratio of 2. Significant increase in particle size for P(EPE)-SS-P[Asp(DET)] polyplexes in reducing milieu was shown in Fig. 4.14A. In contrast, no such aggregation was observed for the same polyplexes under non-reducing environment, which implies that the P(EPE) chains were predominantly stayed on the particle surface. As control polyplexes, PEG-b-P[Asp(DET)] and P(EPE)-b-P[Asp(DET)] polyplexes (N/P = 2) were incubated with 10 mM DTT and there was no change in particle size observed.

![Fig. 4.14. Time-dependent change of cumulant diameters of polyplexes formed at N/P = 2 (A) and ζ-potential values of polyplexes formed at N/P = 4 (B) in 10 mM Tris-HCl buffer (pH 7.4) with the presence or absence of 10 mM DTT at 25 °C (mean ± SD, n = 3).](image-url)
**ζ-potential** of the bioreducible polyplexes prepared at N/P = 4 in the absence or presence of 10 mM DTT was also measured to further confirm the detachment of P(EPE) block copolymers from the particles under reducing environment. Instantaneous increase in ζ-potential (from +11.5 mV to +23.3 mV) was observed for P(EPE)-SS-P[Asp(DET)] polyplexes within 5 min after 10 mM DTT treatment and the ζ-potential values were about the same up to 30 min (Fig. 4.14B). On the other hand, no such rapid change in ζ-potential was observed for P(EPE)-SS-P[Asp(DET)] polyplexes in the absence of DTT or PEG-b-P[Asp(DET)] and P(EPE)-b-P[Asp(DET)] polyplexes after the addition of DTT. These results correlate with the redox potential-dependent size change of the polyplexes and reveal that the release of P(EPE) chains from the P(EPE)-SS-P[Asp(DET)] polyplexes was specific to reducing environment and occurred rapidly.

### 4.3.4. Formation and physical characterizations of ternary polyplexes

As it has been shown in sections 3.3.2 and 4.3.2 that the P(EPE)-based (or P85-based) polyplexes possess lower colloidal stability compared to the PEG-based counterpart especially at elevated temperature, ternary polyplexes comprised various ratios of PEG-b-P[Asp(DET)] and P(EPE)-b-P[Asp(DET)], and pDNA were prepared aiming at improving the stability of particles and maintaining a satisfactory level of transfection. Furthermore, P(EPE)-SS-P[Asp(DET)] was used instead of P(EPE)-b-P[Asp(DET)] for ternary complex formation to examine whether the site-specific release of P(EPE) chains from the polyplexes could further boost the transfection efficiency based on the possibility of higher cellular uptake and cellular membrane disruption contributed by free P(EPE) amphiphilic copolymers.
Ternary polyplexes were formed at N/P = 4 for all experiments as it has been shown that free polymers exist at N/P > 4 and a low N/P ratio may be more beneficial to *in vivo* experiments in terms of transfection efficiency and cytotoxicity.\textsuperscript{143} The physical characteristics of the ternary complexes are summarized in Table 4.2. The sizes of all particles were around 70 nm with low PDI (< 0.14). The $\zeta$-potential measurements of the complexes reveal that the addition of PEG-$b$-P[Asp(DET)] could reduce the $\zeta$-potential of the polyplexes. The reduction in $\zeta$-potential values increased with the amount of PEG-$b$-P[Asp(DET)] used. At a 75% PEG-$b$-P[Asp(DET)] composition in the ternary complexes, the $\zeta$-potential was +2.6 mV and +3.6 mV for the PEG-$b$-/P(EPE)-$b$-P[Asp(DET)] and PEG-$b$-/P(EPE)-SS-P[Asp(DET)] ternary polyplexes, respectively. These values are very comparable to the +2.0 mV $\zeta$-potential of PEG-$b$-P[Asp(DET)] binary polyplex at N/P = 4. Moreover, both the particle size and $\zeta$-potential distributions were unimodal for all ternary polyplexes. The representative size and $\zeta$-potential distributions of PEG-$b$-

<table>
<thead>
<tr>
<th>Polymer fraction</th>
<th>PEG-$b$-P(EPE)-$b$-P(EPE)-SS-</th>
<th>Size (nm) ± SD</th>
<th>PDI ± SD</th>
<th>$\zeta$-potential (mV) ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>25%</td>
<td>75% (-b-)</td>
<td>66.5 ± 0.1</td>
<td>0.105 ± 0.003</td>
<td>4.2 ± 1.4</td>
</tr>
<tr>
<td></td>
<td>75% (-SS-)</td>
<td>70.9 ± 3.6</td>
<td>0.106 ± 0.036</td>
<td>7.0 ± 1.2</td>
</tr>
<tr>
<td>50%</td>
<td>50% (-b-)</td>
<td>70.0 ± 1.6</td>
<td>0.078 ± 0.017</td>
<td>4.1 ± 1.0</td>
</tr>
<tr>
<td></td>
<td>50% (-SS-)</td>
<td>70.6 ± 1.0</td>
<td>0.093 ± 0.020</td>
<td>4.5 ± 0.6</td>
</tr>
<tr>
<td>75%</td>
<td>25% (-b-)</td>
<td>73.1 ± 3.1</td>
<td>0.139 ± 0.052</td>
<td>2.6 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>25% (-SS-)</td>
<td>71.4 ± 0.7</td>
<td>0.101 ± 0.008</td>
<td>3.6 ± 0.4</td>
</tr>
</tbody>
</table>

*Table 4.2. Physical characteristics of ternary polyplexes (mean ± SD, n = 3).*
/P(EPE)-b-P[Asp(DET)] ternary complexes containing 50% PEG-b-P[Asp(DET)] are shown in Fig. 4.15.

![Graph A](image1.png)

![Graph B](image2.png)

**Fig. 4.15.** Particle size (A) and \( \zeta \)-potential (B) distributions of PEG-b-/P(EPE)-b-P[Asp(DET)] ternary polyplexes formed at N/P = 4 with 50% PEG-b-P[Asp(DET)].

The redox potential-sensitivity of PEG-b-/P(EPE)-SS-P[Asp(DET)] ternary polyplexes at various composition ratios was examined by looking at the \( \zeta \)-potential change 30 min after the addition of 10 mM DTT. The change in \( \zeta \)-potential of the ternary complexes is shown in Fig. 4.16. Increase in \( \zeta \)-potential due to the release of P(EPE) chains from polyplexes in reducing condition was observed for ternary complexes prepared at the 25% PEG-b-P[Asp(DET)] composition only, but not at 50 and 75%. One possible reason could be the PEG chains shielded the increase of charges after P(EPE) detachment for ternary polyplexes at the higher PEG percentage compositions.
4.3.5. Stability of ternary polyplexes

The stability of the ternary complexes was assessed by monitoring the particle size change at 37 °C in the physiological salt condition of 150 mM NaCl for a 12 h period and the results are shown in Fig. 4.17. As a control, PEG-b-P[Asp(DET)] binary polyplex prepared at N/P = 4 showed a minimal increase in size. The cumulant diameter increased slightly from 90 nm to around 100 nm. PEG-b-/P(EPE)-b-P[Asp(DET)] ternary complexes showed increase in size ranging from 15 to 50 nm during the 12 h period depending on the amount of PEG-b-P[Asp(DET)] used for complexation. The change in size of particles decreased with the percentage of PEG-b-P[Asp(DET)] employed for ternary complex preparation. As seen in Fig. 4.17B, the changes in size of the PEG-b-/P(EPE)-SS-P[Asp(DET)] ternary complexes at different mixing ratios showed similar tendency as those of the non-reducible ternary complexes.

**Fig. 4.16.** Effect of reducing agent on ζ-potential change for ternary polyplexes. ζ-potential values of ternary polyplexes formed at N/P = 4 with different ratios of P(EPE)-SS-P[Asp(DET)] and PEG-b-P[Asp(DET)] in 10 mM Tris-HCl buffer (pH 7.5) after 30 min incubation with the presence or absence of 10 mM DTT at 25 °C (mean ± SD, n = 3).
Fig. 4.17. Time-dependent particle size change of ternary polyplexes formed at N/P 4 with various compositions of PEG-\textit{b}-P[Asp(DET)] and P(EPE)-\textit{b}-P[Asp(DET)] (A) or P(EPE)-SS-P[Asp(DET)] (B) in 10 mM Tris-HCl buffer (pH 7.4) containing 150 mM NaCl at 37 °C. Percentage represents the fraction of PEG-\textit{b}-P[Asp(DET)] used based on charge %. Results are expressed as means of triplicate experiments (Note: error bars omitted for clarity of the graphs).

4.3.6. \textit{In vitro} transfection efficiency and cytotoxicity

It has been shown in previous study\textsuperscript{150} and chapter 3 that substituting PEG with Pluronic P85 on the surface of polyplex particles could enhance the transfection efficiency. P(EPE)-based polyplexes were used as an analog of P85-based polyplexes for examining their transfection ability in this study. The transfection efficiency of the binary polyplexes was first evaluated in MDA-MB-231 human breast cancer cell line and A549 human lung cancer cell line. As shown in Fig. 4.18A and 4.18B, both P(EPE)-\textit{b}-P[Asp(DET)] and P(EPE)-SS-P[Asp(DET)] polyplexes achieved much higher luciferase expression compared to the PEG-based polyplexes in both tested cell lines. The transfection efficiency of the P(EPE)-based polyplexes did not show a dramatic N/P ratio-dependent increase and high transfection level was achieved at N/P ratio as low as 4. In contrast, high N/P ratio was necessary for the PEG-based polyplexes to obtain a comparable transfection efficiency to the P(EPE)-based polyplexes. Similar trend was observed
in the study comparing the P85-based and PEG-based polyplexes in the previous chapter. It was hypothesized that the introduction of disulfide linkage between the P(EPE) polymer and the cationic P[Asp(DET)] block could enhance transfection efficiency of the polyplexes. Performance difference between P(EPE)-b-P[Asp(DET)] and P(EPE)-SS-P[Asp(DET)] polyplexes in terms of gene delivery ability is shown to be varied in the 2 tested cell lines. Overall a 3 to 9-fold higher transfection was observed with P(EPE)-SS-P[Asp(DET)] polyplexes in MDA-MB-231 cells, and approximately a 2-fold improvement was achieved in A549 cells.

**Fig. 4.18.** Transfection efficiency of binary polyplexes formed at various N/P ratios in MDA-MB-231 (A) and A549 (B) cells (mean ± SD, *n = 3, *P < 0.05).

The cytotoxicity caused by the binary polyplexes was also evaluated based on the cellular metabolism in MDA-MB-231 (Fig. 4.19A) and A549 (Fig. 4.19B) cell lines. In general, PEG-b-P[Asp(DET)] and P(EPE)-b-P[Asp(DET)] polyplexes showed more than 85% cell viability at all tested N/P ratios in the tested cells. The toxicity level produced by P(EPE)-SS-P[Asp(DET)] polyplexes laid between PEG-b-P[Asp(DET)] or P(EPE)-b-P[Asp(DET)] polyplexes and bPEI.
polyplexes. In detail, at low N/P ratios, the toxicity of P(EPE)-SS-P[Asp(DET)] polyplexes was negligible and comparable to that of the other two P[Asp(DET)]-based polyplexes. At N/P = 32, higher toxicity was observed and the cell viability of the P(EPE)-SS-P[Asp(DET)] polyplex treated cells decreased to 77% and 44% in MDA-MB-231 and A549 cells, respectively; however, the toxicity caused was still lower than that produced by bPEI, which showed around 22% cell viability in both cell lines.

![Graph A](image1.png)  
![Graph B](image2.png)  

**Fig. 4.19.** Cytotoxicity of binary polyplexes formed at various N/P ratios in MDA-MB-231 (A) and A549 (B) cells (mean ± SD, n = 3).

The transfection efficiency of ternary polyplexes (N/P 4) was then analyzed in MDA-MB-231 (Fig. 4.20A) and A549 (Fig. 4.20B) cells. Both PEG-b-/P(EPE)-b-P[Asp(DET)] and PEG-b-/P(EPE)-SS-P[Asp(DET)] ternary complexes showed similar trend of transfection ability which decreased with the percentage of PEG-b-P[Asp(DET)] composition used. The P(EPE)-detachable ternary polyplexes demonstrated 2-fold and 1 to 2 order of magnitude increase in transfection compared to the non-bioreducible ternary complexes in A549 and MDA-MD-231 cells, respectively. The toxicity of the ternary polyplexes was also examined in the same cell
lines. Due to the low N/P ratio (N/P = 4) used for forming ternary complexes, no significant toxicity was observed for all tested ternary polyplexes. The cells could maintain more than 90% cell viability after ternary complex treatments (Fig. 4.21).

![Graph A](image1.png)  ![Graph B](image2.png)

**Fig. 4.20.** Transfection efficiency of ternary polyplexes formed at N/P = 4 with different mixing ratio of PEG- and P(EPE)-based polymers in MDA-MB-231 (A) and A549 (B) cells (mean ± SD, n = 3, * indicates statistically significant difference between P(EPE)-b- and P(EPE)-SS- groups with P < 0.05).

![Graph A](image3.png)  ![Graph B](image4.png)

**Fig. 4.21.** Cytotoxicity of ternary polyplexes formed at N/P = 4 with different mixing ratio of PEG- and P(EPE)-based polymers in MDA-MB-231 (A) and A549 (B) cells (mean ± SD, n = 3).
4.3.7. Cellular uptake study

Cellular internalization of different polyplexes was evaluated by measuring Cy5 fluorescence intensity from MDA-MB-231 cells using flow cytometry after 4 h treatments of polyplexes prepared with Cy5-labeled pDNA at N/P = 4 with different polymers. The Cy5 fluorescence intensity decreased with the amount of PEG-b-P[Asp(DET)] polymers used for ternary complex preparation while the PEG-b-P[Asp(DET)] binary polyplexes showed lowest cellular uptake. Furthermore, both the binary and ternary complexes formed with P(EPE)-SS-P[Asp(DET)] demonstrated higher cellular uptake ranging from 1.4- to 2.4-fold difference compared to the polyplexes formed with P(EPE)-b-P[Asp(DET)] (Fig. 4.22). The bioreducible nature might allow the P(EPE)-SS-P[Asp(DET)] polyplexes to enter the cells more effectively through releasing P(EPE) polymers from the polyplexes.

Fig. 4.22. Cellular uptake of Cy5-labeled pDNA ternary polyplexes prepared at N/P = 4 with different mixing ratio of PEG- and P(EPE)-based polymers in MDA-MB-231 cells (mean ± SD, n = 3, *P < 0.005, **P < 0.001.).
4.4. Discussion

In this chapter, we prepared ternary polyplexes surrounded by various ratios of hydrophilic PEG and amphiphilic P(EPE) polymers using different block cationomers based on a poly{\(N\)-[\((2\text{-aminoethyl})\text{-2-aminoethyl}]\text{aspartamide}\)} backbone (PEG-\(b\)-P[Asp(DET)]) and P(EPE)-\(b\)-P[Asp(DET)]) aimed at balancing the stability and transfection efficiency of the polyplex particles. Moreover, the bioreducible P(EPE)-SS-P[Asp(DET)]) polymer was employed for polyplex formation to assess whether the transfection ability of the current system could be further enhanced by the redox potential-dependent release of free P(EPE) polymers from the polyplexes.

The P[Asp(DET)]-based cationic block copolymers were synthesized by the ring-opening polymerization of BLA-NCA using different initiators and the subsequent aminolysis reaction with DET to introduce ethylenediamine cationic moieties (Fig. 4.2). Amphiphilic PEO-\(b\)-PPO-\(b\)-PEO polymers (P(EPE)) with structure similar to Pluronic P85 having one hydroxyl terminal protected with methoxy group were used instead of modified Pluronic P85 for polymerization to ensure the structure of the synthesized polymers by restricting the initialization of polymerization from only one end of the amphiphilic P(EPE) polymers. All the synthesized cationic polymers were able to form polyplexes with pDNA with size less than 110 nm as shown in Fig. 4.12A. The sizes were reduced to 70 to 80 nm in the N/P ratio range of 4–32. The maximum \(\zeta\)-potential measured for the P(EPE)-based polyplexes (with or without disulfide linkage) at the tested N/P ratios was higher than that of PEG-based particles (Fig. 4.12B), which implies that the PEG layer provides a better charge shielding effect than does the P(EPE) layer and this is consistent with the previous results comparing PEG- and P85-based polyplexes (section 3.3.2). The better charge
shielding effect may attribute to the higher molecular weight of the PEG used; moreover, the
amphiphilic nature of P(EPE) may contribute to interaction among the polyplexes and free
colymers due to hydrophobic interaction. This can eventually lead to the higher $\zeta$-potential
values observed for the P(EPE)-based polyplexes.

Intracellular release of free P(EPE) polymer molecules from polyplex particles may further
improve the transfection ability of the present system. In order to examine whether the P(EPE)-
SS-P[Asp(DET)] polyplexes possess the specificity in releasing the P(EPE) polymers from the
particles, the changes in size and $\zeta$-potential in non-reducing and reducing conditions were
investigated. Substantial increase in size was observed for P(EPE)-SS-P[Asp(DET)] polyplexes
at N/P = 2 in a reducing milieu (Fig. 4.14A). The observed aggregation demonstrated that the
P(EPE) was successfully released from the particles in a reducing environment as the detachment
of P(EPE) caused the loss of steric hindrance provided by the P(EPE) palisade and at the same
time the electrostatic repulsive force was minimal among particles at N/P = 2. Such an increase
in size was not observed in a non-reducing environment. The detachment of P(EPE) from the
polyplexes was also confirmed with the prompt increase in $\zeta$-potential in a reducing environment
but not under a non-reducing condition (Fig. 4.14B). The changes observed imply that the
disulfide linkage is stable in the absence of reducing agent but the release of P(EPE) is almost
instantaneous once the particles encounter a reducing environment.

The micellization process of Pluronic polymers has been suggested to be temperature-
sensitive. The temperature-dependency is due to the rapid dehydration of the poly(propylene
oxide) block and which becomes more hydrophobic with increasing temperature.$^{173,174}$ This
dehydrating property of poly(propylene oxide) may lead to polyplex instability at temperature
above the room temperature (e.g. 37 °C) when the polyplex surface is covered with the
amphiphilic P(EPE) polymers. To investigate the stability of polyplexes at elevated temperature,
37 °C was chosen as this temperature is more relevant to the physiological and in vitro testing
conditions. It can be observed in Fig. 4.13 that the stability of the P(EPE)-based polyplexes
against salt-induced aggregation was much lower than that of the PEG-based polyplexes. The
increase in size was confirmed to be much more rapid at the physiological temperature of 37 °C
than at 25 °C (Fig. 4.13 and 3.11). The instability at elevated temperature means that
improvements on colloidal stability of the P(EPE)-based polyplexes may be necessary for in vivo
applications. Although a lower stability was observed for the P(EPE)-based particles,
significantly higher transfection efficiency could be achieved compared to the PEG-based
polyplexes especially at low N/P ratios (Fig. 4.18). It can also be seen that there is limited N/P
ratio dependency for P(EPE)-based polyplexes in transfection efficiency, which demonstrates a
clear advantage over the PEG-based counterpart as no excess amount of free polymers are
necessary for high level of transfection. Smaller amount of polymers used can also contribute to
lower toxicity caused to the target cells. The higher transfection level obtained with P(EPE)-SS-
P[Asp(DET)] polyplexes compared to P(EPE)-b-P[Asp(DET)] polyplexes suggests that there is
improvement in transfection through the redox potential-triggered release of free P(EPE)
polymers for the binary polyplex system; however, the degree of enhancement is cell line-
dependent.

In order to benefit from both the PEG- and P(EPE)-based polymers, ternary polyplexes
containing the PEG-based, P(EPE)-based cationic polymers, and pDNA were prepared at N/P =
4 and examined. The ternary polyplexes showed a size of around 70 nm and the \( \zeta \)-potential
decreased with the proportion of the PEG-b-P[Asp(DET)] polymers used (Table 4.2). All size and ζ-potential distributions of the ternary complexes were unimodal (Fig. 4.15) which imply that the population of the polyplex particles more likely consisted of single species instead of mixtures containing individual PEG-based and P(EPE)-based polyplexes. Higher stability was hypothesized as one of the benefits of the ternary polyplexes. The dramatic aggregation observed with P(EPE)-based binary polyplexes at 37 °C in the physiological salt environment was successfully prohibited by adding PEG-b-P[Asp(DET)] during ternary complex formation. Change in size was still detectable but significantly reduced to a 15 to 50 nm increase over a 12 h period depending on the amount of the PEG-b-P[Asp(DET)] polymers used (Fig. 4.17). Similar change in the particle size was observed for both ternary complexes formed with P(EPE)-b-P[Asp(DET)] or P(EPE)-SS-P[Asp(DET)] polymers. This reveals that majority of the P(EPE) chains stayed on the particle surface during the 12 h incubation time under the tested conditions.

The transfection ability of the ternary complexes gradually decreased with the amount of PEG-b-P[Asp(DET)] polymers employed for complex formation (Fig. 4.20). The decrease of transfection is probably due to the lower cellular uptake of the particles (Fig. 4.22) when PEG polymers became more dominant on the surface of the ternary polyplexes. Comparing the reducible and non-reducible ternary polyplexes, the reducible ones demonstrated a 2-fold and 1-2 orders of magnitude higher transfection ability in A549 (Fig. 4.20B) and MDA-MB-231 cells (Fig. 4.20A), respectively. The better transfection efficiency of the bioreducible ternary complexes is likely to be related to the release of free P(EPE) polymers under reducing environment. As it has been shown that the reduction of disulfide linkage could occur extracellularly in cell-containing systems by thiol-containing cell surface proteins\textsuperscript{122,123} or cell-
secreted free thiols and which might affect the cellular uptake of disulfide-possessing nanoparticles\(^1\) \(^{121}\) flow cytometry was used to monitor the cellular internalization of different polyplexes into MDA-MB-231 cells in order to predict the possible sites of disulfide reduction and to further elucidate the cause of the higher transfection shown by the bioreducible polyplexes in this study. As seen in Fig. 4.22, all particles prepared with the reducible P(EPE)-SS-P[Asp(DET)] internalized more into the cells than did the non-reducible ones. These results suggest that at least partial cleavage of the disulfide bonds occurred outside the cells in the 4 h incubation period and the release of P(EPE) polymers subsequently promoted the internalization of the particles. Therefore, the increase in cellular uptake might be one of the reasons for the higher transfection efficiency observed with the bioreducible binary and ternary polyplexes. Although the difference in particle internalization level between the reducible and non-reducible ternary complexes became more marginal when the PEG-b-P[Asp(DET)] fraction in the complexes reached 50 and 75\%, a higher transfection efficiency was still observed with the reducible ternary complexes in MDA-MD-231 cells. This indicates that other mechanisms causing the improved transfection or alteration of intracellular trafficking of the particles due to the presence or release of free P(EPE) polymers could not be ruled out.

4.5. Conclusions

Utilizing two different block catiomers, we have prepared ternary pDNA polyplexes with surfaces covered by hydrophilic PEG polymers and amphiphilic P(EPE) polymers at various ratios for balancing particle stability and transfection efficiency in this chapter. In general, the incorporation of PEG-b-P[Asp(DET)] to ternary complex formation substantially enhanced the
stability of the particles but decreased the transfection ability of the polyplexes at the same time. To further improve the gene delivery efficiency of the present ternary polyplex system, ternary complexes possessing regulated P(EPE) detachment ability was developed by employing the bioreducible P(EPE)-SS-[Asp(DET)] cationic polymer. The redox potential-sensitive ternary complexes not only maintained similar colloidal stability as the ternary complexes without disulfide linkages but also demonstrated a 2-fold to 2 orders of magnitude improvement in transfection efficiency.
5 General discussion and conclusions
5.1. Primary findings and conclusions

Different strategies for modifying the surface of polyplex particles formed with poly{\(N\)-[(2-aminoethyl)-2-aminoethyl]aspartamide} P[Asp(DET)] cationic polymers and pDNA were investigated in the present dissertation aimed at improving the transfection efficiency while maintaining colloidal stability of the particles through syntheses of different P[Asp(DET)]-based polymers. The use of P[Asp(DET)] as the pDNA condensing block is due to its excellent transfection efficiency demonstrated in both \textit{in vitro} and \textit{in vivo} studies, low cytotoxicity,\textsuperscript{33,108,141} and high biodegradability.\textsuperscript{145} A major drawback of the P[Asp(DET)]-based gene delivery system is the strict requirement of high N/P ratio (up to N/P = 80 in \textit{in vivo} studies\textsuperscript{139,141}) for high transfection activity, which significantly impairs the use of this polyplex system for \textit{in vivo} applications. Consequently, improvement of transfection efficiency at low N/P ratios was also addressed. To utilize the physiochemical variations of specific (micro)environments inside the human body for promoting transfection efficiency, hydrazone linkage and disulfide linkage for responding to pH difference and redox potential variation, respectively, were employed during the surface modification of polyplexes. The major findings in chapters 2–4 are summarized as follows.

5.1.1. Polyplexes with pH-dependent sheddable PEG palisade

To address the PEG dilemma issue, a pH-dependent multi-PEGylated P[Asp(DET)]-based cationic polymer was synthesized and investigated for gene transfer efficiency. It was demonstrated that the integrity of polyplex particles could be maintained in a 12 h period at neutral pH with minimal increase in hydrodynamic diameter. The hydrolysis of hydrazone
linkages as revealed by PEG release from the polymers or polyplex particles was shown to be pH-dependent and more rapid in an acidic environment. For transfection activity, the reversibly-PEGylated polyplexes exhibited more comparable efficiency to the non-PEGylated polyplexes than did the irreversibly-PEGylated counterparts. Moreover, all the tested P[Asp(DET)]-based cationic carriers caused much less toxicity to cells compared to bPEI as shown by both cellular metabolism and membrane integrity evaluations. The improved transfection of the reversibly-PEGylated particles is presumably due to the better endosomal escape and/or smoother intracellular pDNA release from polyplexes after PEG detachment from the polyplexes. Without further investigation on the cellular internalization of polyplexes, the possibility cannot be excluded that enhanced transfection caused by higher cellular uptake of particles is due to extracellular PEG release.

5.1.2. Polyplexes shielded with Pluronic polymers

To obtain a balance between facile polymer synthesis and high transfection efficiency compared to the pH-sensitive multi-PEGylated polymer generated in chapter 2, the PEG block of the diblock copolymer, PEG-b-P[Asp(DET)], was substituted with amphiphilic Pluronic P85. The purpose was to promote cellular uptake of the polyplexes while maintaining particle stability. P85-shielded polyplexes demonstrated no aggregation when prepared at the stoichiometric N/P ratio of 2 at which the polyplexes experience minimal repulsive force. When the charges on particles were further screened by the addition of NaCl, significant size change (~150 nm) was observed in a 12 h period at room temperature while PEGylated counterparts
showed only 40 nm increase in particle size. This suggests that the P85 shell can provide a certain level of polyplex stabilizing effect but not as effective as the PEG shell.

Although the colloidal stability of the P85-based polyplexes is lower than that of the PEG-based particles, the transfection activity of the P85-based polyplexes was substantially higher, especially at lower N/P ratios of 5 and 10. Subsequently, it was observed that the higher transfection efficiency of P85-based particles might be related to the more rapid cellular uptake of the P85-based particles than that of the PEG-shielded polyplexes. The P85-based polyplexes were able to interact with cellular membrane extensively in 30 min. As the P85-based gene delivery system showed orders of magnitude higher transfection efficiency than did the PEG-based particles at low N/P ratios while maintaining similar toxicity profile, investigations for \textit{in vivo} applications are justified but colloidal stability may need to be further improved in that case.

5.1.3. Ternary polyplexes shielded with mixture of polyether polymers

Ternary polyplexes consisting of pDNA and different ratios of PEG-\text{-}b\text{-}P[Asp(DET)] and P(EPE)-\text{-}b\text{-}P[Asp(DET)] cationic polymers were prepared intending to improve the stability of the P85-based binary polyplexes as described in chapter 3. The particle stability was dramatically ameliorated when PEG-\text{-}b\text{-}P[Asp(DET)] was included at a 25% fraction of total polymer during preparation of ternary complexes. Although the particle stability could be increased with the fraction of PEG-\text{-}b\text{-}P[Asp(DET)] used, the transfection efficiency of the polyplexes decreased simultaneously. Assuming that the co-presence of amphiphilic block copolymers and polyelectrolytes can promote higher lipid membrane interaction or disruption, a bioreducible analog, P(EPE)-\text{SS}\text{-}P[Asp(DET)], was synthesized. The analog was used for preparing ternary
polyplexes which could utilize the redox-potential gradient across plasma membrane to release P(EPE) amphiphilic block copolymers from the polyplexes intracellularly. The bioreducible ternary complexes demonstrated similar stability compared to the non-reducible ones; and the transfection activity of the bioreducible ternary complexes was shown to be higher by 2-fold to 2 orders of magnitude. The increase in transfection efficiency shown by the bioreducible ternary complexes could not be solely attributed to intracellular release of P(EPE) polymers. The bioreducible ternary complexes internalized into cells more effectively than did the non-reducible ternary complexes, suggesting that reduction of the disulfide linkages and thus release of P(EPE) chains from the polyplexes might partially occur in extracellular milieu.

In summary, different surface modification strategies using polyether-based polymers and/or environmentally-responsive linkages for improving the transfection activity and particle stability of the P[Asp(DET)]-based polyplex gene delivery system have been investigated in this

![Normalized transfection efficiency of polyplexes in this investigation. Transfection efficiency of bPEI polyplexes is set to 100% (mean ± SD, n = 3).](image-url)

**Fig. 5.1.** Normalized transfection efficiency of polyplexes in this investigation. Transfection efficiency of bPEI polyplexes is set to 100% (mean ± SD, n = 3).
dissertation. The normalized transfection efficiency of all polyplexes studied is shown in Fig. 5.1. Most of the tested polyplexes demonstrated comparable gene transfer ability to bPEI polyplexes while keeping the cytotoxicity relatively low. Furthermore, compared to PEG-b-P[Asp(DET)] polyplexes, the polyplexes developed in this dissertation could significantly enhance the transfection activity at low N/P ratios, which could be beneficial for in vivo applications.

5.2. Suggestions for future research

5.2.1. Higher stability of reversible linkages

Various studies reported that acylhydrazone linkages can be substantially hydrolyzed even at neutral pH\textsuperscript{175,176} while disulfide linkages can also be cleaved in extracellular environments.\textsuperscript{177} Consistent with the observations that hydrazone was not absolutely stable at neutral pH from other studies, it was observed at pH 7.4, the PEG chains were partially detached from the pH-sensitive PEGylated polymer investigated in chapter 1 even in the short incubation time of an hour. Moreover, as was shown in chapter 4, the bioreducible ternary polyplexes internalized into the cells at a higher level compared to the non-reducible ones, implying that partial reduction of the disulfide linkages occurred extracellularly, which promoted higher cellular uptake of the particles. These observations imply that the environmentally-sensitive linkages employed in the studies may experience premature cleavage in the physiological environment before reaching the target sites. Therefore, improvements on the stability of the reversible linkages may be required to better spatially control the cleavage and restrict it to the desired sites.
To generate a more efficient hydrazone linkage that is more stable at neutral pH while having higher hydrolytic activity at acidic pH, options for hydrazone formation are molecules containing aromatic ketone\textsuperscript{104} rather than aliphatic aldehyde and/or polymer backbone functionalized with 2-pyridylhydrazide\textsuperscript{72} instead of acylhydrazide (Fig. 5.2). For disulfide linkage, a methyl or phenyl group can be introduced to the $\alpha$-position of the disulfide bond (Fig. 5.3). The modified structure adjacent to the disulfide linkage can provide steric hindrance to the disulfide moiety and prevent the attack of thiolate anions. It was reported that the hindered disulfide bonds possess several fold higher stability than the unhindered ones under \textit{in vivo} conditions\textsuperscript{177-179}.

5.2.2. Therapeutic genes

Currently a common observation is synthetic cationic polymers showing comparable or even several fold higher transfection activities than PEI, but the observed gene transfer level seems to be the upper limit of the non-viral field and is still much inferior compared to the transfection
ability of viruses. In order to bring the non-viral gene delivery vectors to practical use, factors other than polymer chemistry should be considered to maximize the therapeutic effects of these gene carriers. The proper selection of therapeutic gene is one of the critical factors that can compensate for the unsatisfactory gene delivery ability of non-viral vectors. In general, only a portion of the whole population of cells can express the target protein after transfection using non-viral vehicles, which may be one of the conditions limiting the transfection efficiency of these vehicles. In this regard, therapeutic molecules possessing bystander effect or attacking extracellular targets such as cell surface receptors or extracellular molecules may provide significant therapeutic effect even when the percentage of transfected cells is low.

Fang and coworkers reported that cancer cells transiently transfected with plasmid expressing tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) protein could kill themselves and their neighboring untransfected cells.\textsuperscript{180} TRAIL is a type II transmembrane protein which can induce apoptosis via interaction with death receptors.\textsuperscript{181} The bystander killing effect observed suggests that TRAIL-transfected cells can express TRAIL proteins on their cell surface, which subsequently interact with the death receptors on the cell surface of neighboring cells and induce apoptosis. Apart from TRAIL, polyplex-delivered plasmid expressing soluble form of vascular endothelial growth factor (VEGF) receptor has also been shown to delay tumor growth based on the inhibitory effect of the secreted soluble receptors on extracellular VEGF molecules.\textsuperscript{133} These results imply that therapeutic effect can still be achieved even if only a fraction of the whole population of cells is successfully transfected. The choice of therapeutic molecules requiring partial transfection of the whole cell population to elicit therapeutic effect may compensate for the low transfection efficiency of non-viral carriers.
5.2.3. Routes of administration

Systemically administered polyplex particles were shown to be rapidly cleared from blood circulation with half-lives in the order of minutes. Even with PEGylation, polyplexes promptly dissociated in the blood stream and non-specifically localized in lungs or liver depending on their physical characteristics while only a small fraction could arrive at the target sites.\textsuperscript{134,135} In addition to the intrinsic low transfection activity of polyplexes, high concentration of polyplex particles at the disease sites is desirable to maximize the therapeutic effect.

To this end, dosing routes alternative to systemic administration could be considered to augment the therapeutic feasibility of polyplex gene delivery systems. Compared to intravenous injection, intratracheal or intraperitoneal administration may be a more valid dosing option for non-viral gene delivery as local administration minimizes the dilution effect of systemic administration. Local administration also reduces the chance of undesired localization of the delivered materials to organs or regions other than the target sites. Intratracheal delivery of polyplex particles carrying plasmids expressing human adrenomedullin, a vasodilatory peptide, was reported to produce remarkable therapeutic effects in an animal model with pulmonary arterial hypertension.\textsuperscript{141} Furthermore, the tumor load of mice bearing advanced-stage peritoneal ovarian cancer was shown to be significantly lower after intraperitoneal treatments of polyplexes carrying diphtheria toxin suicide gene relative to that of untreated mice.\textsuperscript{182}

5.2.4. Specific gene expression

Targeting ligands, such as antibodies, peptides, and small molecules, have been extensively used to conjugate onto nanoparticles, aimed at promoting therapeutic effect at the target sites.
The strategy is especially appropriate for anti-tumor therapies as tumor cells overexpress certain biomarkers which make them distinguishable from normal tissues.\textsuperscript{183} However, biodistribution studies of polymer- and lipid-based nanoparticles demonstrated that the targeted particles and non-targeted counterparts displayed similar distribution profiles; the authors claimed that the enhanced anti-tumor therapeutic outcomes of the targeted nanoparticles were due to the higher cellular uptake of targeted nanoparticles relative to the non-targeted particles.\textsuperscript{184,185} This indicates that specific localization of nanoparticles may not be necessarily improved by installing targeting ligands, and the activity of the therapeutic agents may not be restricted at the target sites.

As an advantage of the pDNA-based gene delivery system, the plasmid construct can be easily engineered to enhance the site-specificity of gene expression. Tissue-specific promoter sequence can be introduced upstream of the target gene sequence in a plasmid construct to act as an on/off switch for transcription of the corresponding gene. Promoter sequence of mesothelin (a cell surface glycoprotein overexpressed in ovarian cancer cells) was ligated into a luciferase reporter gene plasmid and shown to preferentially promote the gene expression in ovarian cancer cells but not in other cancer cell types.\textsuperscript{182} In another study of polyplex-based VEGF therapy, a plasmid construct containing the enhancer sequence of the erythropoietin gene locating upstream of the VEGF therapeutic gene was demonstrated to magnify the VEGF expression specifically in ischemic myocardium under hypoxic condition.\textsuperscript{186}

5.3. Final remarks

To summarize, polymer chemistry is one of the critical factors governing the development of polymeric non-viral gene delivery carriers. Modifications of the particle surface of polyplexes
based on cationic P[Asp(DET)] polymers through the use of different P[Asp(DET)]-based block copolymers have been investigated and their transfection efficiency was shown to be superior to polyplexes prepared with PEG-\textit{b}-P[Asp(DET)] especially at low N/P ratios. The enhanced transfection activity of the polyplex systems in this investigation warrant further studies on \textit{in vivo} applications with consideration of improving the stability of reversible linkages employed and proper selection of administration routes and therapeutic genes regulated by site-specific promoters.
Bibliography


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144


