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Stability of poly(ethylene glycol)-graft-polyethylenimine copolymer/DNA complexes: influences of PEG molecular weight and PEGylation degree

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Abstract Polyethylenimine (PEI) is one of the most widely investigated cationic polymers for gene delivery. However, PEI/DNA complexes are unstable and tend to aggregate. PEGylation was used to improve the stability. The stability of polymer/DNA complexes was investigated including complexation stability, aggregation stability, sedimentation stability, and nuclease stability. PEI25K/DNA complexes were liable to aggregate to large particles (500–700 nm). The aggregation was proved to be induced by phosphate anion. In the medium without phosphate anion, aggregation was prevented by electrostatic repulsion. Owing to more efficient steric repulsion, PEG2 and PEG5K excelled PEG750 in facilitating copolymers to form stable small polyplexes (below 100 nm) without aggregation regardless of phosphate anion. The steric repulsion predominated over electrostatic repulsion in stabilization.

1 Introduction

The cationic polymer polyethylenimine (PEI) is a widely studied and efficient non-viral gene delivery carrier [1–4]. In physiological pH range, the positively charged amino groups in PEI interact with the negatively charged phosphate groups in DNA by electrostatic attraction and form PEI/DNA complexes as condensed nanoparticles which may protect DNA against nuclease degradation [5–7]. Moreover, the positive surface charge of the nanoparticles at higher N/P ratios may stabilize the complexes as a result of electrostatic repulsion [8, 9]. However, some research groups reported that PEI/DNA complexes tended to aggregate in the presence of salt [4, 10–12], but little information is available concerning the reasons for the aggregation.

To improve stability and biocompatibility, PEI has been modified with nonionic hydrophilic segments, such as poly(ethylene glycol) (PEG) [8, 10, 12–15], pluronic [16], and dextran [11, 17]. Out of these, PEG has been widely used in conjugating to cationic polymers because of its high solubility in water, non-immunogenicity, and enhanced biocompatibility. Various types of PEG–PEI copolymers were extensively investigated [8, 10, 12–15]. However, the surface charge of PEG–PEI/DNA complexes is shielded by the nonionic PEG chains in the outer layer, so the electrostatic repulsion between nanoparticles contributed to the colloidal stability is dramatically reduced. Alternatively, the PEG chains could provide steric repulsion for stabilization. Then, here come the questions: Is the steric repulsion, compared with the electrostatic repulsion, sufficient to maintain the colloidal stability? Does the PEGylation impair the physicochemical properties of the cationic polymer?

In this study, we synthesized three series of PEG-g-PEI copolymers and investigated the influences of PEG molecular weight and PEGylation degree on the stability of polymer/DNA complexes including complexation stability, colloidal stability, and biological stability, so as to develop an optimized PEG–PEI copolymer to be used as a non-viral gene delivery carrier.

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2 Materials and methods

2.1 Materials

Branched PEI (25 KDa) was purchased from Sigma–Aldrich (St. Louis, MO). Linear monomethoxypoly(ethylene glycol)-750 (mPEG750), mPEG2K, and mPEG5K were from Fluka (Buchs, Switzerland). Isophorone diisocyanate (IPDI) was from Huicai (Guangzhou, China). Dibutyltin dilaurate (DBTL) was from Libao (Guangzhou, China). Chloroform (99%), light petrol (99%, 40–60°C), and diethyl ether (99%) were all from Chemical Agent Factory (Guangzhou, China). Chloroform was treated with IPDI for 4 h at 60°C and distilled to remove any traces of water and ethanol.

Plasmid pEGFP-C1, coding for the enhanced green fluorescent protein (EGFP) gene under the control of the CMV immediate-early enhancer/promoter, was a gift from West China University of Medical Sciences (Chengdu, China). The plasmid was amplified in a competent *Escherichia Coli* strain DH5α and purified with a QIAGEN (Chatsworth, CA) kit following the manufacturer's protocol.

2.2 Synthesis of copolymers

PEG-g-PEI copolymers were synthesized using a two-step procedure as described previously [18]. Briefly, as shown in Fig. 1, in the first step of PEG activation, PEG dissolved in pretreated chloroform reacted with IPDI (10–30 molar excess) at 70°C for 10 h. DBTL (0.6–0.7 wt%) was added as a catalyst. Then the polymer was repeatedly precipitated in light petrol and redissolved in chloroform several times to remove excessive IPDI and DBTL. In the second step, the activated PEG and PEI (at given ratios) were refluxed in chloroform with DBTL as a catalyst at 65°C for 24 h. Finally, the solution was concentrated and the polymer was precipitated in a large volume of diethyl ether. The products were dried at reduced pressure. The structure of the copolymers was confirmed by FT-IR and ^{1}H NMR spectroscopy. Molecular weight of the copolymers was calculated according to the integration areas of ethylenimine and ethylene glycol units in the ^{1}H NMR spectrograms.

2.3 Preparation of polymer/DNA complexes

Polymers were diluted with 1× PBS, unless otherwise mentioned. Plasmid DNA was diluted to equal volume with deionized water (20 μg/ml). The polymer solution was added to the DNA solution and pipetted repeatedly, then incubated for 30 min at room temperature. The polymer/DNA ratio was expressed as the N/P ratio, where N represented moles of amino group in PEI and P represented moles of phosphate group in DNA.

2.4 Gel retardation assay

Polymer/DNA complexes were prepared as described above at various N/P ratios, ranging from 0.5 to 6, then incubated for 30 min at room temperature prior to loading onto an EtBr containing 0.8% agarose gel. Electrophoresis was run in

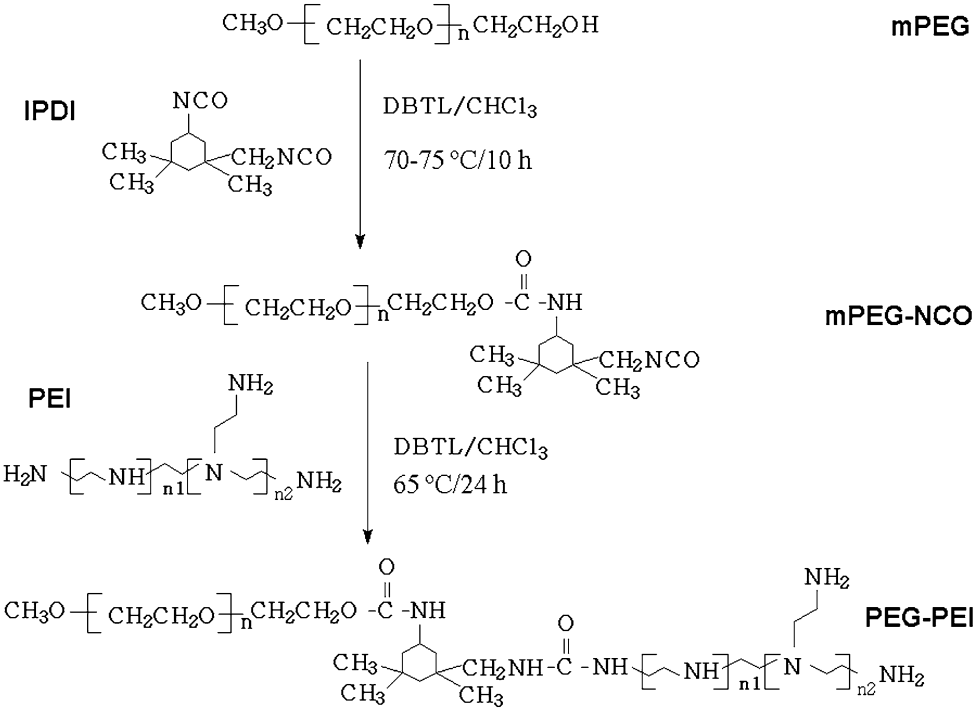

Fig. 1 Synthesis of PEG-PEI copolymers

TBE buffer at a voltage of 90 V for 50 min. DNA retardation was observed using a UV transilluminator.

2.5 Polyanion competition assay

Polymer/DNA complexes were prepared as described above at an N/P ratio of 10 and incubated for 30 min at room temperature, then mixed with 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, and 0.8 IU of heparin/μg PEI as a competing polyanion. The mixture was incubated for 20 min, and then electrophoresis was carried out as above.

2.6 Measurement of particle size and zeta-potential

Polymer/DNA complexes were prepared as described above at various N/P ratios of 1, 2, 4, 6, 10, 20, and 30, and then incubated for 30 min at room temperature. Afterward, the particle size and zeta-potential were measured with a Zetasizer Nano Series 90 (Malvern Instruments, UK) at 25°C as described in Ref. [12].

2.7 Sedimentation stability

Polymer/DNA complexes were prepared as described above at an N/P ratio of 10 and incubated for 30 min at room temperature, then centrifuged at various gravities for 2 min in a UNIVERSAL 32R centrifuge (Hettich Co., Germany). The UV absorbance of the supernatant was measured at 260 nm by a 6131 Biophotometer (Eppendorf Co., Germany). The relative value of A/A_0 was used to evaluate the sedimentation stability of complexes, where A_0 and A denoted the UV absorbance of the supernatant before and after centrifuge, respectively.

2.8 Stability against nuclease and ultrasonication

PEI25K formed complexes with DNA at various N/P ratios of 0.5, 1, 2, 3, and 4. PEG–PEI copolymers formed complexes at an N/P ratio of 4. All the complexes were incubated for 30 min at room temperature. Then 10 μl of naked DNA or complexes (containing 0.4 μg DNA) were mixed with 4 μl of DNase I (0.4 μg) and incubated at 37°C for 30 min. Afterward, 4 μl of 250 mM EDTA was added to the samples and incubated for 10 min to inactivate the nuclease. Subsequently, the samples were treated with 2 μl of heparin (1.25 IU) for 20 min to release DNA. Finally, electrophoresis was carried out as above.

Naked DNA solution was ultrasonicated with a 250 W water bath sonicator (Kudos Co., China) at 40 KHz for 1, 2, 3, 5, 10, and 15 min, respectively. A 10 μl volume of each sample was analyzed by electrophoresis as above. Polymer/DNA complexes were prepared at an N/P ratio of 10 and incubated for 30 min at room temperature, then sonicated under the same conditions. A 5 μl volume of each sample was treated with 5 μl of heparin for 20 min to release DNA. Electrophoresis was carried out as above.

3 Results

3.1 Synthesis of copolymers

Three series of PEG-g-PEI copolymers were synthesized with PEI25K and three different molecular weight mPEGs. In each series of given molecular weight mPEG, PEI25K was grafted with various amounts of mPEG. Composition and molecular weight of PEG–PEI copolymers are presented in Table 1. PEG–PEI copolymers are designated here as *x*–*y*, where *x* denotes the molecular weight of PEG in the copolymers and *y* denotes the weight proportion (%) of PEG in the total initial reactants (PEG and PEI). For instance, 2K-50 indicates that the copolymer was synthesized with mPEG2K and PEI25K fed at 1:1 (w/w). With the three series of PEG–PEI copolymers, we could study the influences of PEGylation degree at given molecular

Table 1 Composition and molecular weight of PEG-PEI copolymers

Sample name	Feed ratio (W_{PEG}/W_{PEI})	PEG content (%)	Number of grafted PEG (n)	MW (Mn)[a]
750-10	mPEG750:PEI = 1:9	13.86	4.14	29.0 K
750-25	mPEG750:PEI = 1:3	19.91	6.39	31.2 K
750-50	mPEG750:PEI = 1:1	44.98	21.02	45.4 K
2K-10	mPEG2K:PEI = 1:9	10.47	1.32	27.9 K
2K-25	mPEG2K:PEI = 1:3	26.96	4.15	34.2 K
2K-50	mPEG2K:PEI = 1:1	53.12	12.75	53.3 K
5K-10	mPEG5K:PEI = 1:9	11.26	0.61	28.2 K
5K-25	mPEG5K:PEI = 1:3	25.08	1.60	33.4 K
5K-50	mPEG5K:PEI = 1:1	52.00	5.19	52.0 K

[a] Calculated based on ^{1}H NMR data

weight PEG (*x*-10, *x*-25, and *x*-50) and PEG molecular weight at given PEGylation degree (750-*y*, 2K-*y*, and 5K-*y*) on the physicochemical properties of the copolymers.

3.2 DNA complexation

To investigate DNA complexation ability of the polymers, agarose gel retardation assay was performed. As shown in Fig. 2a, DNA migration was retarded as the N/P ratio increased. As for PEI25K, complete retardation of DNA was observed at an N/P ratio of 3. In the case of PEG–PEI *x*-10 and *x*-25, similar results were obtained, showing that PEGylation degree of 10 and 25% will not impede the complexation. With regard to PEG–PEI *x*-50, DNA was completely complexed at N/P ratio 4, suggesting that higher PEGylation degree may impede the complexation slightly. Similar results were reported by Petersen and coworkers [12], who found grafting PEI with PEG5K slightly hindered the interaction with DNA. From Fig. 2a, we also learned that in the case of equivalent PEGylation degree, there was no difference in DNA complexation for the PEG–PEI copolymers with different molecular weight PEGs.

The influence of complexation medium was also investigated. As shown in Fig. 2b, in pH 7.4 PBS, both PEI25K and PEG–PEI 2K-25 completely complexed DNA at N/P ratio 3. This result is similar to that in 1× PBS, indicating no distinct impact on the DNA complexation in pH 7.4 PBS. Similar results were obtained in 150 mM NaCl solution and in deionized water (data not shown). In pH 4.0 PBS, complete retardation of DNA was observed at N/P ratio 5 for PEI25K and N/P ratio around 4–5 for PEG–PEI 2K-25. This implies that the complexation is impeded slightly, and the impact on 2K-25 is less than on PEI25K. It was considered that in the low pH environment, protonation of the amino groups in PEI led to an expansion of the polymeric network due to intramolecular charge repulsion [19], inducing a portion of DNA releasing from the complexes. In RPMI 1640 culture medium containing 10% serum, DNA was complexed completely at N/P ratio 4 for PEI25K and N/P ratio around 3–4 for 2K-25. Here, the impeded complexation was observed again, and the DNA complexation for 2K-25 was hindered by serum to less extent compared with that for PEI25K. This finding may provide referenced information for in vitro cell transfection. Surprisingly, some difference was found in 5% glucose solution: DNA migration was incompletely retarded even at a high N/P ratio of 6 for both PEI25K and 2K-25. Further experiments showed that complete retardation of DNA was achieved at N/P ratio around 4 (data not shown), indicating that the complexation was impeded remarkably in 5% glucose solution.

3.3 Complexation stability: competition with polyanion

Heparin was used as a polyanion to compete with DNA for cationic polymers. The amount of heparin at which DNA was released from the complexes was used to evaluate the complexation stability. As shown in Fig. 3, DNA was released from the complexes as the amount of heparin increased. As for PEI25K, complexes were dissociated and DNA was released at a heparin amount of 0.4 IU/μg PEI. For the copolymers with PEG750 and PEG2K blocks at lower PEGylation degree (10 and 25%), DNA was released at the

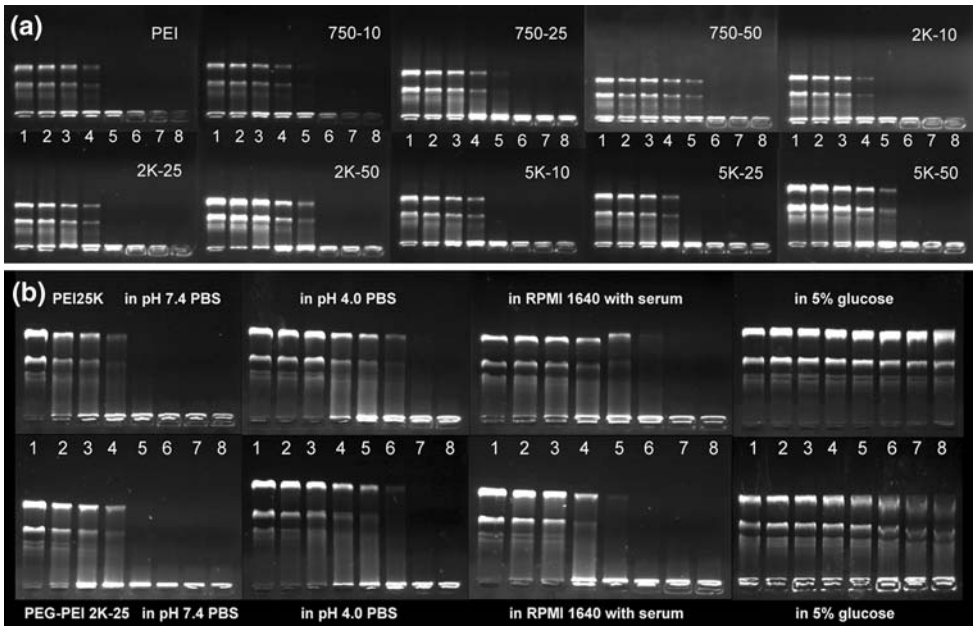

Fig. 2 Agarose gel electrophoresis of polymer/DNA complexes. *Lane 1* plasmid only, *lanes 2–8* N/P = 0.5, 1, 2, 3, 4, 5, and 6. **a** Complexes formed in 1× PBS, **b** Complexes formed in various media

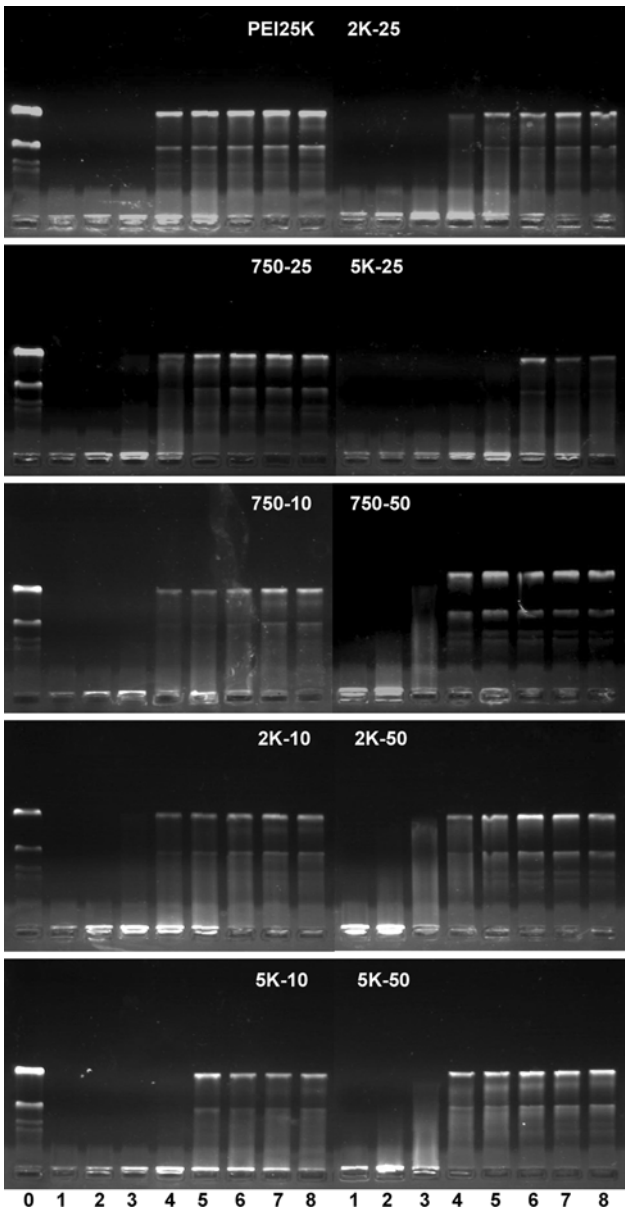

Fig. 3 Stability of complexes against competing polyanion (heparin). *Lane 0* plasmid only, *lanes 1–8* complexes (N/P = 10) were added with 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, and 0.8 IU heparin/μg PEI, respectively

same amount of heparin (0.4 IU/μg PEI). Therefore, the complexation stability of PEG–PEI 750-10, 750-25, 2K-10, and 2K-25 was similar to that of PEI25K, without impact from the PEGylation. With respect to the copolymers with higher PEGylation degree (50%), DNA releasing occurred at a decreased heparin amount of 0.3 IU/μg PEI, suggesting that the complexation with PEG–PEI *x*-50 was less stable than that with PEI25K, probably due to the reduced DNA condensation ability. This is consistent with the result of gel retardation assay. Some researchers also reported that for the polyplexes based on PEGylated PEI25K, increasing the level of PEG grafting reduced the complexation stability [9]. In contrast, for PEG–PEI 5K-10 and 5K-25, DNA was released at a higher heparin amount of 0.5 and 0.6 IU/μg PEI, respectively, showing improved complexation stability. This may be attributed to the unaffected DNA condensation ability, and longer and more flexible side chains of PEG5K with stronger steric repulsion, preventing the access of heparin.

3.4 Particle size of the complexes

Particle size of the complexes is influenced markedly by N/P ratios. It is generally considered that DNA is condensed more tightly as the N/P ratio increases, leading to a smaller particle size [6, 7]. In our experiments, no particle was detected by the instrument at lower N/P ratios (N/P = 1 and 2), maybe due to inefficiency of the polymers to condense DNA into particles, or forming loose and unconsolidated complexes that could not be distinguished from the bulk solution. At N/P ratio 4, particles were detected and the particle size decreased with increasing N/P ratios, as shown in Table 2. The particle size of the complexes formed with PEI25K was in the range of 500–700 nm or so, while the complex size for most PEGylated PEI dramatically decreased (<200 nm). Since no significant difference was found in DNA condensation ability between PEI25K and PEG–PEI copolymers via gel retardation assay, the large particles for PEI25K should not be ascribed to incomplete DNA condensation. We speculate that the large particles are aggregates from small particles. Most PEG–PEI copolymers formed small particles without aggregation, maybe attributed to the steric repulsion from the electroneutral and flexible PEG chains.

For PEG2K and PEG5K, at an N/P ratio of 10, copolymers with higher PEGylation degree (25 and 50%) formed complexes with the size below 100 nm. In contrast, the complex size for the copolymers with lower PEGylation degree (10%) increased (190 ± 46 nm for 2K-10 and 147 ± 38 nm for 5K-10). This indicates that the steric repulsion against aggregation becomes remarkable with increasing PEGylation degree.

For PEG750, at lower N/P ratios (N/P = 4 and 6), copolymers formed aggregated complexes (500–900 nm), probably due to incomplete compaction and the poor steric repulsion of PEG750 with short chains. Thus, the steric repulsion is relevant to the molecular weight of PEG besides PEGylation degree. Short PEG chains (PEG750), even at high content (50%), can not provide sufficient steric repulsion against aggregation. However, at N/P ratios above 10, complex size for PEG–PEI 750-*y* decreased below 100 nm, suggesting that incompletely condensed complexes were liable to aggregate.

Preliminary experiments showed that PEI25K formed large polyplexes in 1× PBS, so the influence of

Table 2 Particle size (nm) of polymer/DNA complexes prepared in 1× PBS determined by dynamic light scattering (*DLS*) ($n = 3$)

N/P ratio	4	6	10	20	30
PEI25K	715 ± 115	645 ± 109	591 ± 104	583 ± 88.2	533 ± 107
750-10	961 ± 202	879 ± 203	128 ± 41	83.0 ± 15.4	88.7 ± 13.6
750-25	828 ± 187	807 ± 159	122 ± 34.1	77.2 ± 18.8	72.1 ± 17.1
750-50	721 ± 133	548 ± 129	98.3 ± 23.7	69.9 ± 18.7	60.3 ± 19.5
2K-10	386 ± 76.2	186 ± 43.7	190 ± 46.4	80.3 ± 22.4	87.1 ± 19.5
2K-25	161 ± 49.7	107 ± 31.2	85.3 ± 19.9	75.3 ± 33.2	66.9 ± 22.6
2K-50	108 ± 31.6	91.4 ± 25.7	72.4 ± 17.2	59.7 ± 13.7	60.8 ± 13.9
5K-10	191 ± 41.5	175 ± 46.3	147 ± 38.0	85.7 ± 17.8	68.7 ± 29.6
5K-25	103 ± 16.8	86.4 ± 33.5	70.7 ± 8.55	79.7 ± 11.2	59.4 ± 6.39
5K-50	94.7 ± 11.6	72.5 ± 9.18	73.2 ± 9.32	55.2 ± 7.86	40.1 ± 5.17

Table 3 Particle size (nm) of polymer/DNA complexes prepared in various media[a] determined by DLS ($n = 3$)

Polymer	Medium	N/P ratio		
		10	20	30
PEI25K	pH 7.4 PBS	555 ± 125	548 ± 130	546 ± 125
	pH 4.0 PBS	447 ± 87.8	523 ± 132	535 ± 115
	Deionized water	45.1 ± 13.1	43.3 ± 12.1	42.4 ± 10.4
	150 mM NaCl	97.7 ± 30.0	61.7 ± 13.6	52.7 ± 13.3
	5% glucose	54.3 ± 14.8	51.5 ± 13.7	42.7 ± 10.9
PEG-PEI 2K-25	pH 4.0 PBS	75.6 ± 18.1	71.0 ± 18.2	70.9 ± 16.5
	150 mM NaCl	69.1 ± 18.6	50.1 ± 16.2	57.7 ± 13.7
	5% glucose	71.5 ± 19.1	55.5 ± 16.2	55.8 ± 15.7

pH 7.4 PBS composition: 50 mM KH_2PO_4, 40 mM NaOH

pH 4.0 PBS composition: 57 mM Na_2HPO_4, 42 mM citric acid

[a] 1× PBS composition: 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4

Table 4 Particle size (nm) of different polymer/DNA complexes prepared in 1× PBS determined by DLS ($n = 3$)

N/P ratio	10	20	30
Homopolymer PEI25K	591 ± 104	583 ± 88.2	533 ± 107
Mixture PEI25K/mPEG2K	513 ± 104	565 ± 106	555 ± 106
Copolymer PEG-PEI 2K-25	85.3 ± 19.9	75.3 ± 33.2	66.9 ± 22.6

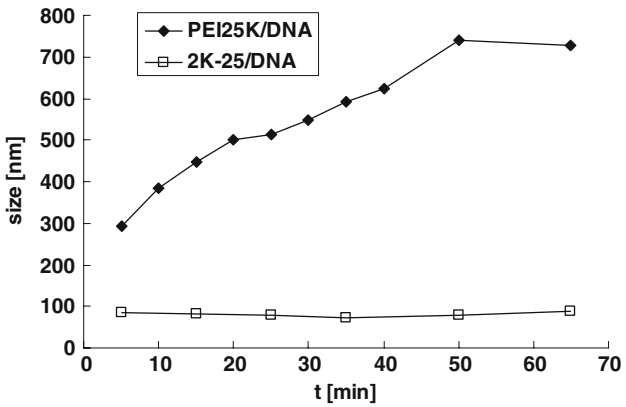

Fig. 4 Dynamic process of the particle size of the polyplexes after complexation with PEI25K and PEG-PEI 2K-25 in 1× PBS (N/P = 10), determined by DLS

complexation medium with different concentration of phosphate anion was also investigated. As shown in Table 3, in the media of pH 7.4 and 4.0 PBS containing abundant phosphate anion, PEI25K complexed with DNA to form large particles that were 400–600 nm in diameter. While in the media without phosphate anion such as deionized water, 5% glucose and 150 mM NaCl, the diameters of the PEI/DNA complexes were smaller than 100 nm. We concluded previously that there was no significant difference in DNA condensation ability between various complexation media investigated via gel retardation assay, thus the PEI/DNA complexes exhibited large size in PBS were not due to incomplete DNA condensation. We believe once again that the large particles are aggregates from small particles. Moreover, the aggregation is induced by phosphate anion. However, the particle sizes of the complexes formed with copolymer 2K-25 in various media were all below 100 nm, indicating better stability of the complexes, owing to the steric repulsion from PEG blocks against aggregation.

Particle size of the complexes formed in 1× PBS with homopolymer PEI25K, copolymer 2K-25, and simple mixture of mPEG2K and PEI25K (1:3, w/w) was compared in Table 4. Complex size for mixed polymers was similar to that for homopolymer PEI25K (500–600 nm), but different from that for copolymer 2K-25 (below 100 nm). Therefore, simply mixing PEI with PEG could not provide efficient steric repulsion against aggregation.

Particle size of the complexes (N/P = 10) formed in 1× PBS was measured over time. As shown in Fig. 4, during

measurement, complex size for PEI25K increased from 300 to 700 nm, indicating aggregation of the complexes. In contrast, complex size for PEG–PEI 2K-25 remained constant below 100 nm, showing excellent stability against aggregation.

3.5 Zeta-potential of the complexes

Zeta-potential of the complexes gradually increased with increasing N/P ratios. As shown in Table 5, at lower N/P ratios (N/P = 1 and 2), no condensed complexes formed and the zeta-potential was negative (−20 to −40 mV), mainly due to the phosphate groups of DNA backbone. The zeta-potential increased to nearly electrical neutrality as DNA was completely condensed. As the N/P ratio continued to increase, the complexes associated with excessive polycations and exhibited positive zeta-potential. However, the zeta-potential did not increase further at N/P ratio above 10, suggesting that complex formation reached equilibrium state. Meanwhile, Table 5 revealed that, for condensed complexes at various N/P ratios, the zeta-potential for PEG–PEI copolymers remarkably reduced compared with that for PEI25K. This implies that the flexible nonionic PEG chains in the outer layer of the complexes are able to shield the positive charge of the inner polycation/DNA core. The negative zeta-potential of the complexes for both PEI25K and PEG-PEI copolymers inflected to positive at N/P ratio 6 or so. Therefore, the charge shielding effect of PEG had only marginal influence on the polymers to condense DNA.

For PEG2K and PEG5K, at an N/P ratio of 10 or above, copolymers with higher PEGylation degree (25 and 50%) formed complexes with the zeta-potential reduced to +1 to +2 mV, compared with +10 to +12 mV for PEI25K. In contrast, the copolymers with lower PEGylation degree (10%) formed complexes with the zeta-potential reduced only to +3 to +6 mV. Hence, the charge shielding effect augmented with increasing PEGylation degree.

Table 6 Zeta potential (mV) of PEI25K/DNA complexes formed in various media determined by LDA ($n = 3$)

N/P ratio	10	20	30
Deionized water	+7.93 ± 1.81	+9.64 ± 1.46	+9.81 ± 1.75
5% glucose	+10.1 ± 1.15	+11.3 ± 1.96	+13.4 ± 2.10
150 mM NaCl	+25.8 ± 4.61	+24.3 ± 4.68	+25.6 ± 4.15
pH 7.4 PBS	−12.2 ± 1.96	−11.1 ± 1.86	−9.40 ± 1.64
pH 4.0 PBS	−17.8 ± 2.76	−13.1 ± 1.59	−4.13 ± .916

For PEG750, the zeta-potential of the complexes only reduced to +5 to +6 mV, regardless of the PEGylation degree, suggesting the relatively poor shielding effect of short PEG chains. Thus, the charge shielding effect is relevant to the molecular weight of PEG as well as PEGylation degree. Short PEG chains (PEG750), even at high content (50%) and a high N/P ratio of 30, can not further reduce the zeta-potential of the complexes.

The influence of complexation medium on the zeta-potential of the complexes for PEI25K was shown in Table 6. In the medium without phosphate anion, such as deionized water, 5% glucose, or 150 mM NaCl, the zeta-potential was positive, due to the excess of polycations. In contrast, in pH 7.4 and 4.0 PBS containing abundant phosphate anion, the zeta-potential at various N/P ratios dramatically reduced to negative (yet exhibiting increasing tendency with increasing N/P ratios). Therefore, the negative zeta-potential may be ascribed to the complexes adsorbing the multivalent phosphate anion with high density of negative charge. Hence, the zeta-potential of the complexes is influenced by ionic species and ionic strength of the complexation medium. Since the surface charge of the complexes contributes to the interaction with cell membrane, and then transfection efficiency, this finding may provide referenced information for in vitro cell transfection. As for the copolymer 2K-25, the zeta-potential in various

Table 5 Zeta potential (mV) of polymer/DNA complexes prepared in 1× PBS determined by laser Doppler anemometry (LDA) ($n = 3$)

N/P ratio	1	2	4	6	10	20	30
PEI25K	−38.7 ± 6.69	−23.7 ± 4.47	−18.3 ± 2.47	2.51 ± 0.267	9.70 ± 1.62	11.5 ± 2.03	12.2 ± 2.74
750-10	−23.3 ± 4.91	−12.1 ± 2.24	−7.52 ± 1.21	−0.313 ± 0.0411	4.44 ± 0.572	4.98 ± 0.969	6.30 ± 1.17
750-25	−33.6 ± 6.40	−20.9 ± 4.81	−18.8 ± 2.28	−4.56 ± 0.516	5.24 ± 0.618	5.16 ± 1.13	5.53 ± 0.904
750-50	−26.6 ± 4.87	−13.4 ± 2.86	−6.78 ± 1.17	−2.37 ± 0.276	2.60 ± 0.383	5.84 ± 1.61	5.31 ± 0.921
2K-10	−34.1 ± 6.31	−26.9 ± 5.83	−6.66 ± 1.01	2.96 ± 0.352	3.39 ± 0.407	4.86 ± 0.927	6.51 ± 1.016
2K-25	−32.7 ± 5.68	−6.17 ± 1.32	−4.60 ± 0.609	−0.957 ± 0.151	2.36 ± 0.361	1.74 ± 0.384	1.83 ± 0.331
2K-50	−28.6 ± 5.41	−16.4 ± 3.82	−0.45 ± 0.0657	0.198 ± 0.0271	0.790 ± 0.120	0.843 ± 0.186	1.78 ± 0.321
5K-10	−39.9 ± 7.81	−35.5 ± 7.01	−2.96 ± 0.414	−0.914 ± 0.110	3.53 ± 0.485	2.53 ± 0.560	4.93 ± 0.883
5K-25	−40.5 ± 7.92	−28.8 ± 5.67	−7.18 ± 1.71	−1.03 ± 0.131	1.07 ± 0.193	1.87 ± 0.347	2.31 ± 0.329
5K-50	−38.0 ± 6.48	−13.4 ± 2.86	−2.31 ± 0.364	0.170 ± 0.0187	0.373 ± 0.0448	1.43 ± 0.268	1.19 ± 0.220

media remained constant (data not shown), showing better stability, owing to the charge shielding effect from PEG blocks.

3.6 Sedimentation stability

DNA has absorption at 260 nm. Pilot experiments revealed that DNA condensed in complexes also showed absorbance at 260 nm (but not the same value as free DNA), while PEI25K and PEG–PEI had no absorption at that wavelength. Therefore, the absorbance of the supernatant signified the presence of DNA. As the complexes for PEI25K centrifuged at 2500×*g* for 2 min, the A/A_0 value decreased to 0.012 (Fig. 5a), indicating complete sedimentation of the complexes. Under the same conditions, the A/A_0 value for PEG–PEI 2K-25 was 0.766. Even centrifuged at 22000×*g* for 2 min, the complexes remained at a value of 0.383. Hence, PEG–PEI copolymers, compared with PEI25K, formed complexes with better sedimentation stability, maybe due to the steric repulsion from PEG blocks.

Sedimentation stability of the complexes for PEI25K formed in various media was shown in Fig 5b. After centrifugation at 10000×*g* for 2 min, complete sedimentation occurred for the complexes with large size formed in pH 7.4 and 4.0 PBS, similar to that in 1× PBS. Interestingly, complexes with small size (similar to that for PEG–PEI 2K-25) formed in 150 mM NaCl solution and deionized water, however, precipitated ($A/A_0 < 0.081$) under the same condition. On this condition, the A/A_0 value for PEG–PEI 2K-25 was 0.540 (Fig. 5a). Therefore, the sedimentation stability is independent of particle size. Surprisingly, the A/A_0 value for the complexes formed with PEI25K in 5% glucose was 0.978 after centrifugation at 10000×*g* for 2 min. Even centrifuged at 22000×*g* for 10 min, the complexes remained at a value of 0.688. Considering the results of gel retardation assay, we speculate that DNA is loosely complexed in 5% glucose and the supernatant may exist free DNA that will not precipitate from the solution by centrifugation.

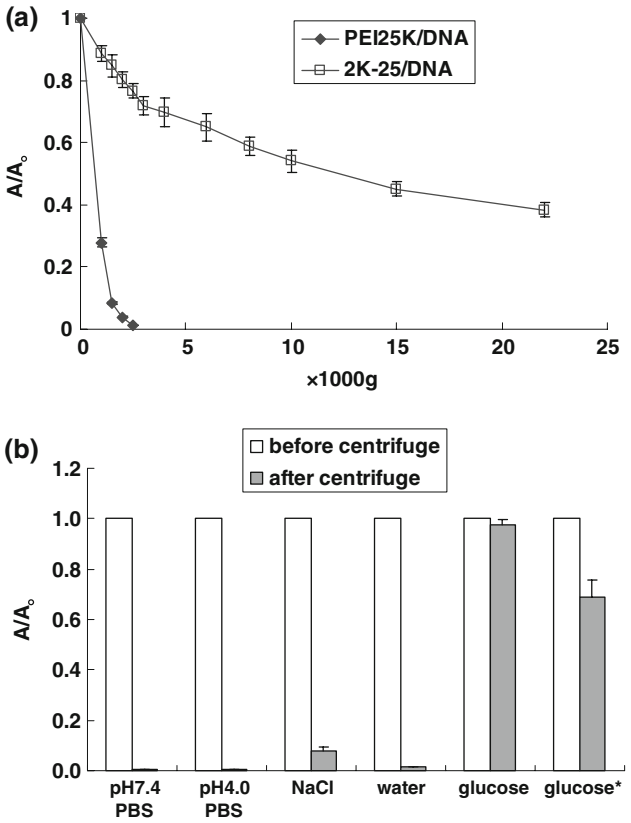

Fig. 5 Sedimentation stability of complexes (N/P = 10) **a** Complexes prepared in 1× PBS centrifuged at various gravities for 2 min; **b** PEI25K/DNA complexes formed in various media centrifuged at 10000×*g* for 2 min (* centrifuged at 22000×*g* for 10 min)

3.7 Stability against nuclease and ultrasonication

Complexes formed with various polymers were treated with DNase I to investigate the nuclease stability. As shown in Fig. 6, DNA was completely complexed with PEI25K at N/P ratio 4 and no migration of DNA was observed (Lane 2). The condensed DNA could be released by heparin and

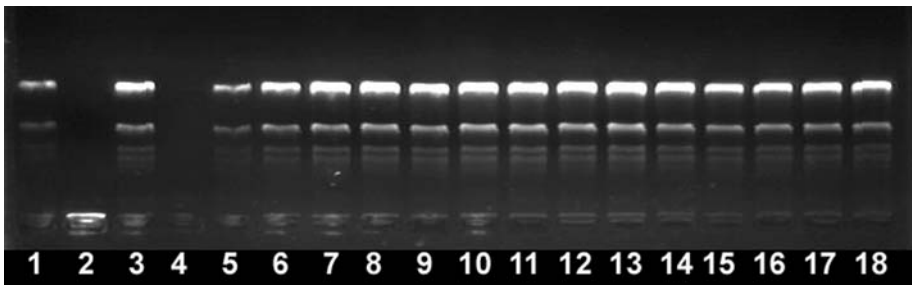

Fig. 6 Stability of polymer/DNA complexes against DNase I. *Lane 1* plasmid only; *lane 2* PEI25K/DNA complexes (N/P = 4); *lane 3* PEI25K/DNA complexes (N/P = 4) + heparin; *lane 4* naked DNA + DNase I + heparin; *lanes 5–9* PEI25K/DNA complexes (N/P = 0.5, 1, 2, 3, 4) + DNase I + heparin; *lanes 10–18* PEG-PEI/DNA complexes (N/P = 4, 750-10, 750-25, 750-50, 2K-10, 2K-25, 2K-50, 5K-10, 5K-25, 5K-50) + DNase I + heparin

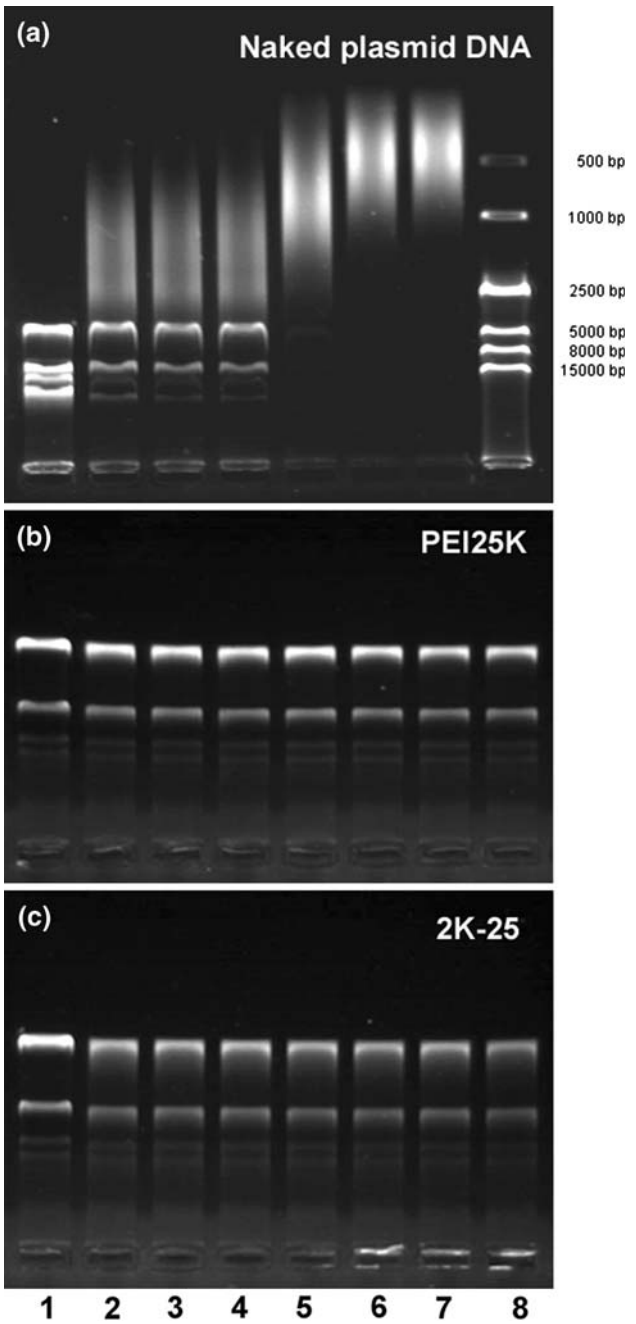

Fig. 7 Stability against ultrasonic **a** effect of ultrasonic on naked plasmid DNA. *Lane 1* plasmid only; *lanes 2–7* naked DNA ultrasonicated for 1, 2, 3, 5, 10, and 15 min, respectively; *lane 8* DNA ladder. **b** and **c** Protection against ultrasonic. *Lane 1* plasmid only; *lanes 2–8* polymer/DNA complexes (N/P = 10) ultrasonicated for 0, 1, 2, 3, 5, 10, and 15 min, respectively, then released by heparin

migrated as free DNA (Lane 3). Naked DNA was completely degraded when treated with DNase I (Lane 4). Complexes formed with PEI25K at given N/P ratios provided protection against nuclease (Lane 5–9). The intensity of the DNA band in lane 5 fainted slightly, suggesting part of the DNA was degraded due to the incomplete complexation at lower N/P ratio. Bright DNA bands were obtained at N/P ratios above 1, indicating more intact DNA survived from nuclease degradation with increasing N/P ratios. All the PEG–PEI copolymers provided sufficient protection against DNase I even at a low N/P ratio of 4 (Lane 10–18). Therefore, PEGylation did not impair the nuclease stability. Similar results were reported by Brus and co-workers [20].

Ultrasonication is one of the common techniques to prepare nanoparticles. As a premise, ultrasonication stability is required for the samples. Feasibility of ultrasonication for polymer/DNA nanoparticles was investigated. As shown in Fig. 7a, naked DNA sonicated within 3 min was partially destructed to small pieces of nucleic acid. When sonicated more than 5 min, the plasmid DNA was completely destructed to nucleic acid debris around 500 bp. DNA complexed with polymers was sonicated under the same conditions. After treated with heparin, the plasmid DNA was released and migrated as intact (Fig. 7b, c), suggesting both PEI25K and PEG–PEI copolymers were able to provide sufficient protection against ultrasonic.

4 Discussion

It has been reported that conjugating PEG to PEI improved the stability and biocompatibility of the complexes with DNA as gene delivery system [8, 10, 12–15]. In this study, we synthesized three series of PEG–PEI copolymers and investigated the influence of PEG molecular weight and PEGylation degree on the stability of polymer/DNA complexes.

In our work, PEG and PEI blocks were directly linked together with a coupling reagent. Grafting PEG onto PEI with diisocyanate as a coupling reagent such as hexamethylene diisocyanate (HMDI) was reported previously [21]. We chose IPDI as a coupling reagent because of its good stability, safety and low toxicity. Moreover, the reactivity of the two isocyanate groups in the IPDI molecule is different. The alicyclic isocyanate group is more reactive than the aliphatic one [22]. In the presence of excessive IPDI, mPEG reacts chiefly with the alicyclic isocyanate group rather than the aliphatic one, avoiding mPEG-IPDI-mPEG byproducts. In contrast, the two isocyanate groups of HMDI have the same reactivity, due to its symmetric molecular structure.

By particle size measurements, we found that PEI25K/DNA complexes formed large particles (500–700 nm) in the medium containing phosphate anion but small particles (below 100 nm) in the medium without phosphate anion. Some research groups reported that PEI/DNA complexes tended to aggregate in the presence of salt [4, 10–12]. Therefore, the large particles may be aggregates from small particles. It was reported that counterions in the medium may

induce electrostatic attraction between like-charged polyelectrolytes by ionic bridging and cause aggregation [23, 24]. Moreover, multivalent counterions were more effective in mediating ionic bridging compared with monovalent counterions [25]. Considering the positively charged PEI25K/DNA complexes and the negative multivalent phosphate anions in the medium, we suppose the aggregation is induced by the bridging effect of the phosphate anion.

Glodde et al. [9] reported that nonaggregating particles were formed only if there was enough repulsion (electrostatic or steric) between the individual particles. Without steric repulsion from PEG, the aggregation stability of PEI25K/DNA complexes mainly relies on electrostatic repulsion. In the medium without phosphate anion, small particle size was observed without aggregation owing to electrostatic repulsion, whereas in the medium containing phosphate anion, the electrostatic repulsion was insufficient to prevent the aggregation induced by phosphate anion, leading to large particle size. Moreover, the nonaggregated complexes formed in the medium without phosphate anion still precipitated after centrifugation. Hence, the electrostatic repulsion provides minor contribution to the stabilization. In contrast, PEG–PEI copolymers formed small-sized complexes (below 100 nm) without aggregation regardless of phosphate anion. Therefore, the steric repulsion from PEG blocks is sufficient to prevent the aggregation induced by phosphate anion. Similar conclusion has been reported [8–10, 12]. Additionally, the sedimentation stability was improved after PEGylation. Hence, the steric repulsion, rather than electrostatic repulsion, mainly contributes to the stabilization.

The surface charge of complexes is described with zeta-potential. Electrostatic repulsion increases as the absolute value of zeta-potential increases, giving rise to improved colloidal stability. We found that complexes formed with PEI25K, though with higher zeta-potential, were liable to aggregate. Nevertheless, PEG-PEI formed stable complexes with smaller size (below 100 nm), resistant to aggregation even at nearly electrical neutrality. These demonstrate again that steric repulsion but not electrostatic repulsion contributes mainly to the stabilization.

5 Conclusion

In this study, we synthesized three series of PEG-g-PEI copolymers. Compared with PEI25K, the complexation ability and complexation stability for the copolymers with lower PEGylation degree remained nearly unchanged, but slightly declined at 50% PEGylation degree. Complexes for PEI25K tended to form aggregates induced by phosphate anion. PEG2K and PEG5K excelled PEG750 in providing efficient steric repulsion to prevent aggregation. Therefore, the optimal copolymer PEG-PEI 2K-25 may be a promising carrier for in vitro transfection.

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References

1. Boussif O, Lezoualc'h F, Zanta MA, Mergny MD, Scherman D, Demeneix B, et al. A versatile vector for gene and oligonucleotide transfer into cells in culture and in vivo: polyethylenimine. Proc Natl Acad Sci USA. 1995;92(16):7297–301.
2. Godbey WT, Wu KK, Mikos G. Size matters: molecular weight affects the efficiency of poly(ethylenimine) as a gene delivery vehicle. J Biomed Mater Res. 1999;45(3):268–75.
3. Kunath K, Harpe A, Fischer D, Petersen H, Bickel U, Voigt K, et al. Low-molecular-weight polyethylenimine as a non-viral vector for DNA delivery: comparison of physicochemical properties, transfection efficiency and in vivo distribution with high-molecular-weight polyethyleneimine. J Control Rel. 2003;89(1): 113–25.
4. Wightman L, Kircheis R, Rossler V, Carotta S, Ruzicka R, Kursa M, et al. Different behavior of branched and linear polyethylenimine for gene delivery in vitro and in vivo. J Gene Med. 2001;3(4):362–72.
5. Godbey WT, Wu KK, Mikos AG. Poly(ethylenimine) and its role in gene delivery. J Control Rel. 1999;60(1):149–60.
6. Lungwitz U, Breunig M, Blunk T, Gopferich A. Polyethylenimine-based non-viral gene delivery systems. Eur J Pharm Biopharm. 2005;60(2):247–66.
7. Neu M, Fischer D, Kissel T. Recent advances in rational gene transfer vector design based on poly(ethylene imine) and its derivatives. J Gene Med. 2005;7(8):992–1009.
8. Park MR, Han KO, Han IK, Cho MH, Nah JW, Choi YJ, et al. Degradable polyethylenimine-alt-poly(ethylene glycol) copolymers as novel gene carriers. J Control Rel. 2005;105(2):367–80.
9. Glodde M, Sirsi SR, Lutz GJ. Physiochemical properties of low and high molecular weight poly(ethylene glycol)-grafted poly(ethylene imine) copolymers and their complexes with oligonucleotides. Biomacromolecules. 2006;7(1):347–56.
10. Sung SJ, Min SH, Cho KY, Lee S, Min YJ, Yeom YI, et al. Effect of polyethylene glycol on gene delivery of polyethyleneimine. Biol Pharm Bull. 2003;26(4):492–500.
11. Tseng WC, Fang TY, Su LY, Tang CH. Dependence of transgene expression and the relative buffering capacity of dextran-grafted polyethylenimine. Mol Pharm. 2005;2(3):224–32.
12. Petersen H, Fechner PM, Martin AL, Kunath K, Stolnik S, Roberts CJ, et al. Polyethylenimine-graft-poly(ethylene glycol) copolymers: influence of copolymer block structure on DNA complexation and biological activities as gene delivery system. Bioconjug Chem. 2002;13(4):845–54.
13. Petersen H, Kunath K, Martin AL, Kissel T. Star-shaped poly (ethylene glycol)-block-polyethylenimine copolymers enhance DNA condensation of low molecular weight polyethylenimines. Biomacromolecules. 2002;3(5):926–36.
14. Banerjee P, Weissleder R, Bogdanov A. Linear polyethyleneimine grafted to a hyperbranched poly(ethylene glycol)-like core: a copolymer for gene delivery. Bioconjug Chem. 2006;17(1): 125–31.
15. Ahn CH, Chae SY, Bae YH, Kim SW. Biodegradable poly(ethylenimine) for plasmid DNA delivery. J Control Rel. 2002;80(1–3):273–82.

16. Gebhart CL, Sriadibhatla S, Vinogradov S, Lemieux P, Alakhov V, Kabanov V. Design and formulation of polyplexes based on pluronicpolyethyleneimine conjugates for gene transfer. Bioconjug Chem. 2002;13(5):937–44.
17. Tseng WC, Jong CM. Improved stability of polycationic vector by dextran-grafted branched polyethyleneimine. Biomacromolecules. 2003;4(5):1277–84.
18. Zhang X, Pan SR, Hu HM, Wu GF, Feng M, Zhang W, et al. Poly(ethylene glycol)-block-polyethylenimine copolymers as carriers for gene delivery: Effects of PEG molecular weight and PEGylation degree. J Biomed Mater Res A. 2008;84(3):795–804.
19. Harpe A, Petersen H, Li Y, Kissel T. Characterization of commercially available and synthesized polyethylenimines for gene delivery. J Control Rel. 2000;69(2):309–22.
20. Brus C, Petersen H, Aigner A, Czubayko F, Kissel T. Physicochemical and biological characterization of polyethyleniminegraft-poly(ethylene glycol) block copolymers as a delivery system for oligonucleotides and ribozymes. Bioconjug Chem. 2004;15(4):677–84.
21. Petersen H, Fechner PM, Fischer D, Kissel T. Synthesis, Characterization, and Biocompatibility of Polyethylenimine-graft-poly(ethylene glycol) Block Copolymers. Macromolecules. 2002;35:6867–74.
22. Tassel X, Barbry D, Tighzert L. A new blocking agent of isocyanates. Eur Polym J. 2000;36:1745–51.
23. Huang C, Cruz MO. Polyelectrolytes in multivalent salt solutions: monomolecular versus multimolecular aggregation. Macromolecules. 2002;35(3):976–86.
24. Jiang T, Wu J. Ionic effects in collapse of polyelectrolyte brushes. J Phys Chem B. 2008;112(26):7713–20.
25. Dautzenberg H, Kriz J. Response of polyelectrolyte complexes to subsequent addition of salts with different cations. Langmuir. 2003;19(13):5204–11.