Low Molecular Weight Linear Polyethylenimine-*b*-poly(ethylene glycol)-*b*-polyethylenimine Triblock Copolymers: Synthesis, Characterization, and in Vitro Gene Transfer Properties

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Novel ABA triblock copolymers consisting of *low molecular weight linear* polyethylenimine (PEI) as the A block and poly(ethylene glycol) (PEG) as the B block were prepared and evaluated as polymeric transfectant. The cationic polymerization of 2-methyl-2-oxazoline (MeOZO) using PEG−bis(tosylate) as a macroinitiator followed by acid hydrolysis afforded linear PEI−PEG−PEI triblock copolymers with controlled compositions. Two copolymers, PEI−PEG−PEI 2100−3400−2100 and 4000−3400−4000, were synthesized. Both copolymers were shown to interact with and condense plasmid DNA effectively to give polymer/DNA complexes (polyplexes) of small sizes (<100 nm) and moderate ζ-potentials (∼ +10 mV) at polymer/plasmid weight ratios ≥1.5/1. These polyplexes were able to efficiently transfect COS-7 cells and primary bovine endothelial cells (BAECs) in vitro. For example, PEI−PEG−PEI 4000−3400−4000 based polyplexes showed a transfection efficiency comparable to polyplexes of branched PEI 25000. The transfection activity of polyplexes of PEI−PEG−PEI 4000−3400−4000 in BAECs using luciferase as a reporter gene was 3-fold higher than that for linear PEI 25000/DNA formulations. Importantly, the presence of serum in the transfection medium had no inhibitive effect on the transfection activity of the PEI−PEG−PEI polyplexes. These PEI−PEG−PEI triblock copolymers displayed also an improved safety profile in comparison with high molecular weight PEIs, since the cytotoxicity of the polyplex formulations was very low under conditions where high transgene expression was found. Therefore, linear PEI−PEG−PEI triblock copolymers are an attractive novel class of nonviral gene delivery systems.

1. Introduction

In the past decade, synthetic gene delivery systems based on, for example, cationic liposomes, peptides, and polymers have received rapidly growing interest due to the many advantages they offer over viral systems, such as the lack of specific immune response, no restrictions in the size of DNA, and the ease of large scale production.[1−3] Polyethylenimine (PEI) is one of the most efficient and widely studied synthetic carriers for the delivery of plasmid DNA into cells in vitro and in vivo.[4−7] The high transfection efficiency of PEI/DNA complexes has been ascribed to the capacity of PEI to buffer endosomes, which protects DNA from nuclease degradation and facilitates endosomal escape of PEI/DNA complexes ("proton sponge hypothesis").[4] Further studies have shown that the transfection efficiency and cytotoxicity of PEI/DNA formulations are highly dependent on the macromolecular structure (branched versus linear) and molecular weight of PEI.[8−11] For example, it has been demonstrated that the polyplexes of high molecular weight (HMW) PEI are much more potent and also much more toxic than those of low molecular weight (LMW) PEI (e.g., the transfection efficiency of PEI/DNA complexes follows an order of 70000 > 10000 ≫ 1800[9]). Further, linear PEI/DNA formulations are generally more efficient and less toxic than branched PEI/DNA formulations.[11] It should be noted, however, that compared to the viral delivery systems, the transfection efficiency of PEI polyplexes remains rather low. Moreover, polyplexes of HMW PEI have low colloidal stability and show considerable in vivo toxicity.[12]

In the past several years, modification of PEI to decrease its cytotoxicity and/or improve its transfection efficiency has

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been actively pursued.[13,14] Two major vector modification strategies have been reported. The first involves the conjugation of HMW PEIs with a hydrophilic and biocompatible polymer such as poly(ethylene glycol) (PEG).[15−19] Polyplexes of PEG modified HMW PEI show decreased cytotoxicity and improved colloidal and serum stability. However, the "stealth" effect of PEG often results in a diminished transfection due to decreased cellular association and internalization.[20,21] Cellular uptake of PEGylated polyplexes can be enhanced by the grafting of a targeting ligand at the polyplex surface.[22−25] The second vector modification strategy is directed to the chemical modification of nontoxic LMW PEI to increase its gene transfer efficiency. For example, Klibanov and co-workers reported that hydrophobic modification, for example, dodecylation of part of the primary amino groups of LMW PEI, produces efficient and nontoxic vectors.[26] Water soluble lipopolymer using LMW PEI designed by Lee et al. showed significantly higher gene expression in rabbit myocardium compared to LMW PEI and naked DNA.[27] Studies from other groups showed that covalently linking LMW branched PEIs to HMW polymers or networks largely improves their DNA condensation and/or gene transfer efficiency.[19,28−31] The multiple reactive sites (i.e., primary amine groups) of these branched PEIs, nevertheless, often give rise to complex coupling reactions which make it difficult to synthesize polymers with controlled characteristics.

In this paper, we report novel structurally well-defined *LMW linear* PEI−PEG−PEI triblock copolymers for in vitro gene transfer. These triblock copolymers were designed on the basis of the following reasons: (i) linear PEI has a better-defined structure and gives better transfection than branched PEI,[11] (ii) LMW PEI has a lower toxicity than HMW PEI,[32] (iii) the PEG stealth effect further reduces toxicity and meanwhile improves the colloidal stability of the polymer/DNA complexes,[16] and (iv) linking of two LMW linear PEIs with PEG to a higher molecular weight polymer may improve their DNA condensation and gene transfer efficiency.[29] The copolymers were synthesized by cationic polymerization of 2-methyl-2-oxazoline using PEG−bis(tosylate) followed by acid hydrolysis. Their DNA binding properties were studied using electrophoresis and dynamic light scattering. The in vitro transfection activity as well as the cytotoxicity of PEI−PEG−PEI based polyplexes was evaluated using COS-7 cells and primary bovine endothelial cells (BAECs).

2. Materials and Methods

2.1. Materials. 2-Methyl-2-oxazoline (MeOZO, Aldrich), acetonitrile, and dichloromethane were dried over calcium hydride and distilled under an inert atmosphere. Poly(ethylene glycol) (PEG, M_n (^{1}H NMR) = 3400 g/mol), triethylamine, trimethylamine hydrochloride salt, *p*-toluenesulfonyl chloride (TsCl), and hyperbranched polyethylenimine (M_w = 25000) were purchased from Aldrich. Linear polyethylenimine (M_w = 25000) was purchased from Polysciences, Inc. (Warrington, PA). The plasmid pCMVLacZ, containing a bacterial LacZ gene preceded by a nuclear localization signal under the control of a CMV promoter, was purchased form Sanvertech (Heerhugowaad, The Netherlands). INF7 peptide was synthesized according to the method described by Wagner et al.[33] pDMAEMA (M_w = 1.94 × 10^5 g/mol) was synthesized as described previously.[34]

2.2. Polymer Synthesis. PEI−PEG−PEI triblock copolymers were synthesized by cationic polymerization of 2-methyl-2-oxazoline (MeOZO) using PEG−bis(tosylate) as a macroinitiator followed by hydrolysis.

2.2.1. Synthesis of TsO−PEG−OTs. In a glovebox under a nitrogen atmosphere, TsCl (1.057 g, 5.4 mmol) in CH_2Cl_2 (3.6 mL) was added to a stirred solution of PEG (5.4 g, 1.8 mmol), Et_3N (0.728, 7.2 mmol), and Me_3N·HCl (0.035 g, 0.36 mmol) in CH_2Cl_2 (7.2 mL) at room temperature. The reaction was allowed to proceed for 1 h. The reaction mixture was filtrated to remove insoluble salts. Next, the mother liquor was evaporated and the residue was dissolved in methanol, filtrated, and precipitated in excess diethyl ether. The dissolution/precipitation cycle was repeated once. The macroinitiator was isolated by filtration and dried in vacuo. Yield: 5.2 g. ^{1}H NMR ($CDCl_3$, 300 MHz): δ 2.44 (PEG−$OSO_2C_6H_4C\mathbf{H}_3$, 6H), 3.63 (PEG, 308H), 4.16 ($CH_2C\mathbf{H}_2$-$OSO_2C_6H_4CH_3$, 4H), 7.34 and 7.80 (PEG−$OSO_2C_6\mathbf{H}_4CH_3$, 8H).

2.2.2. Synthesis of PMeOZO−PEG−PMeOZO Triblock Copolymer. In a glovebox under a nitrogen atmosphere, TsO−PEG−OTs (0.752 g, 0.225 mmol), MeOZO (2.0 g, 23.5 mmol), and CH_3CN (10 mL) were charged into a reaction vessel equipped with a stirrer bar ($[MeOZO]_0/[TsO-PEG-OTs]_0$ = 104/1 mol/mol). The vessel was closed, placed in an oil bath thermostated at 70 °C, and stirred for 3 days. After polymerization, the solvent was removed under reduced pressure and the residue was dissolved in $CHCl_3$ and precipitated in excess diethyl ether. Finally, the polymer was isolated by filtration and dried in vacuo. Yield: 2.67 g (97.0%). M_n (^{1}H NMR, $CDCl_3$): 4100−3400−4100. Similarly, employing a higher monomer-to-initiator ratio ($[MeOZO]_0/[TsO-PEG-OTs]_0$ = 208/1 mol/mol), PMeOZO−PEG−PMeOZO triblock copolymer with a higher molecular weight of PMeOZO blocks was obtained. Yield: 2.21 g (93.0%). M_n (^{1}H NMR): 7900−3400−7900.

2.2.3. Synthesis of PEI−PEG−PEI by Acid Hydrolysis of PMeOZO−PEG−PMeOZO. PMeOZO−PEG−PMeOZO 4100−3400−4100 (2.1 g) was dissolved in 12 mL of aqueous HCl (10 wt %) and refluxed overnight under a nitrogen atmosphere at 100 °C. The pH of the reaction mixture was adjusted to 12.5 with 1 M NaOH, which led to precipitation of the polymer. The polymer was isolated by centrifugation, and the precipitate was washed twice with deionized (DI) water and subsequently freeze-dried. Yield: 1.02 g. M_n (^{1}H NMR, DMSO-d_6): 2100−3400−2100. Similarly, the acid hydrolysis of PMeOZO−PEG−PMeOZO 7900−3400−7900 yielded PEI−PEG−PEI triblock copolymer with an M_n value of 4000−3400−4000. A low molecular weight linear PEI polymer was synthesized in an analogous way using methyl tosylate instead of PEG−bis(tosylate) as initiator.

2.3. Acid−Base Titration. The buffering capacity of the synthesized PEI−PEG−PEI triblock copolymers from pH 10 to 3 was determined by acid−base titration. Briefly, 6

mg of polymer was dissolved in 30 mL of 0.1 M NaCl to give a final concentration of 0.2 mg/mL, the pH of the polymer solution was brought to 10 with NaOH, and the solution was subsequently titrated with 0.1 M HCl.

2.4. Biophysical Characterization of Polymer/Plasmid DNA Complexes. *2.4.1. Gel Retardation Assay.* Polymer/plasmid DNA complexes were prepared at varying weight ratios ranging from 0.1875 to 24 in a 5% glucose solution and incubated for 30 min at room temperature. The samples were then electrophoresed through a 1% agarose gel containing ethidium bromide (0.5 μg/mL) at 96 V for 20 min in Tris-acetate buffer (40 mM, 1 mM EDTA, pH 8.0).

2.4.2. Light Scattering and ζ-Potential Measurements. The ζ-potentials and mean diameters of the PEI−PEG−PEI/plasmid DNA complexes in HEPES buffer (5.0 mM, pH 7.4) at 25 °C were determined by employing a Zetasizer 2000 instrument equipped with a DTS5001 cell and dynamic light scattering (DLS) on a Malvern 4700 system, respectively. The polyplexes were prepared by adding a solution of PEI−PEG−PEI with the desired concentration (800 μL of HEPES; concentration ranging from 14.1 to 450.0 μg/mL) to a HEPES buffer solution (200 μL) of plasmid DNA (75 μg/mL) which resulted in polyplexes with polymer/plasmid weight ratios ranging from 0.75 to 24. The dispersions were vortexed for 5 s and incubated at room temperature for 30 min before ζ-potential and size measurements were carried out.

The colloidal stability of PEI−PEG−PEI polyplexes was studied using DLS at 37 °C and pH 7.4. The polyplexes were prepared as described above with polymer/DNA ratios of 6/1 and 12/1 (w/w). The ionic strength of the suspension was adjusted to 150 mM NaCl. The particle size was measured after 24 h.

2.5. In Vitro Transfection and Cell Viability Assays. *2.5.1. COS-7 Cells.* Transfection experiments were performed with COS-7 cells (SV-40 transformed African green monkey kidney cells) by using the plasmid pCMVLacZ as a reporter gene as reported previously.[34,35] Two parallel transfection series, one for the determination of the reporter gene expression (β-galactosidase) and the other for the evaluation of cell viability by the XTT assay, were carried out in separate 96 well plates ($\sim 1.0 \times 10^4$ cells per well). Different polymer/plasmid DNA ratios ranging from 0.75/1 to 96/1 (w/w) were used to prepare polyplexes. In brief, the polyplexes were prepared by adding a HBS buffer solution (20 mM HEPES, pH 7.4, 130 mM NaCl, 200 μL) of PEI−PEG−PEI with varying concentrations (from 9.4 to 1200 μg/mL) to a HBS buffer solution (20 mM HEPES, pH 7.4, 130 mM NaCl, 50 μL) of plasmid DNA (50 μg/mL), followed by vortexing for 5 s and incubating at room temperature for 30 min. The incubations of the polyplexes with the cells were performed either in the presence or in the absence of 5.0% serum. In a standard transfection experiment, the cells were rinsed with HBS and incubated with 100 μL of polyplex dispersion (i.e., 1 μg of plasmid DNA per well) and 100 μL of culture (with or without 10% serum) medium for 1 h at 37 °C in a humidified 5% CO_2-containing atmosphere. Next, the polyplexes were removed, 100 μL of fresh culture medium was added, and the cells were cultured for 2 days. The transfection efficiency was determined by measuring the enzyme activity of β-galactosidase using the ONPG assay.[35] In the transfection experiments with the INF7 peptide, prior to the incubation of cells with the polyplexes, to 1 mL of the polyplex dispersion, 20 μL of INF (150 μg/mL) in HBS buffer (20 mM Hepes, 130 mM NaCl) was added. A pDMAEMA/DNA formulation prepared at an optimal polymer/DNA ratio of 3/1 (w/w) was used as a reference.

The number of viable cells was measured using an XTT assay.[36] The XTT value for untreated cells (i.e., cells not exposed to transfection systems) was taken as 100% cell viability.

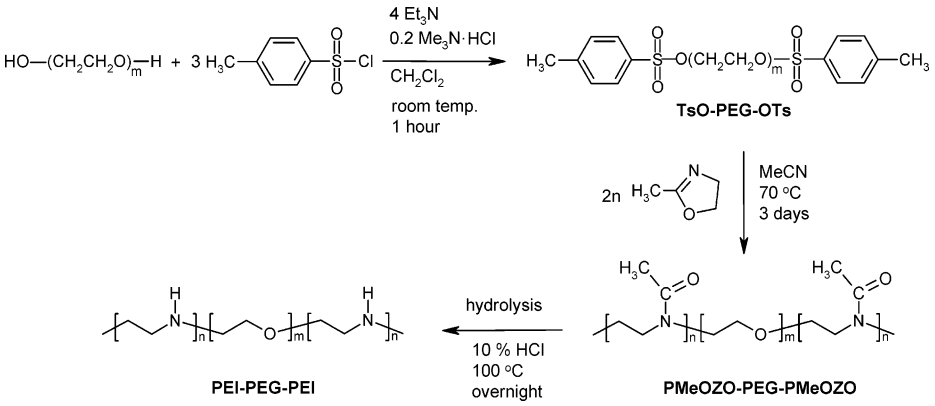
2.5.2. Primary Bovine Endothelial Cells. Transfection experiments were carried out on a primary bovine endothelial cell line (BAEC, Cambrix) between passage numbers 3−9 using a pCMVLuc reporter plasmid. Cells were plated in six well plates and maintained in DMEM (Gibco) supplemented with 10% FBS (Hyclone) at 37 °C in a humidified atmosphere containing 5% CO_2. Transfections were conducted using polymer/pDNA polyplexes at 1.5/1 to 24/1 (w/w) ratios in 5% glucose and a total pDNA concentration of 50 μg/mL. In brief, cells were incubated with 2 mL of media until 70% confluency was reached. At this point, the cells were washed with PBS and 1 mL of cell culture medium was added followed by the addition of polyplexes with a total of 2.0 μg of pDNA per well. After 4 h of incubation, the medium was replaced with 2 mL of fresh media and the cells were cultured for 48 h. Luciferase quantification was done using a luciferase assay system (Promega). The cell viability assay was done after culturing the BAECs in 24 well plates under the same conditions as described above. Polymer/pDNA complexes were added to the wells at 0.5 μg per well. After 44 h of incubation, the cell viability was determined using an MTT assay (Sigma). The viability of these cells was compared to cells that received 5% glucose solution instead of polyplex dispersions.

3. Results and Discussion

3.1. Low Molecular Weight Linear PEI−PEG−PEI Triblock Copolymer Synthesis and Characterization. Linear polyethylenimines are usually prepared by cationic polymerization of 2-oxazoline monomers followed by hydrolysis. To obtain ABA triblock copolymers with low molecular weight (LMW) *linear* polyethylenimine (PEI) as the A block and poly(ethylene glycol) (PEG) as the B block, the macroinitiator PEG−bis(tosylate) (TsO−PEG−OTs) was synthesized and used for initiating the cationic polymerization of 2-methyl-2-oxazoline (MeOZO) (Scheme 1).

PEG−bis(tosylate) was synthesized by *p*-toluenesulfonylation (tosylation) of PEG (M_n = 3400 g/mol) with *p*-toluenesulfonyl chloride in the presence of Et_3N and a catalytic amount of $Me_3N{\cdot}HCl$ in dichloromethane at room temperature, similar to a previously reported method for the tosylation of low molecular weight alcohols.[37] The ^{1}H NMR of purified product showed signals at δ 2.44 (PEG−$OSO_2C_6H_4C\mathbf{H}_3$, 6H), 3.63 (PEG, 308H), 4.16 ($CH_2C\mathbf{H}_2$-$OSO_2C_6H_4CH_3$, 4H), and 7.34 and 7.80 (PEG−$OSO_2C_6\mathbf{H}_4$-CH_3, 8H), respectively. It can be concluded on the basis of

Scheme 1. Synthesis of Linear Polyethylenimine-*b*-poly(ethylene glycol)-*b*-polyethylenimine (PEI−PEG−PEI) Triblock Copolymers



the integrals of the signals at δ 2.44, 4.16, and 3.63 that tosylation of PEG is quantitative yielding PEG−bis(tosylate) (TsO−PEG−OTs). The ring opening polymerization of MeOZO using TsO−PEG−OTs was carried out at 70 °C in acetonitrile for 3 days, which gave by varying the MeOZO/TsO−PEG−OTs ratio PMeOZO−PEG−PMeOZO triblock copolymers in high yield (>97%) with different molecular weights of the PMeOZO blocks. A typical ^{1}H NMR spectrum ($CDCl_3$) of PMeOZO−PEG−PMeOZO is shown in Figure 1a. Resonances at δ 1.98−2.20 and 3.43 are attributed to the methyl and methylene protons of the PMeOZO block, respectively. The singlet at δ 3.63 is assigned to the methylene protons of the PEG block. On the basis of the ratio of the integrals of the signals at δ 3.43 and 3.63, the number average molecular weights for the three blocks can be calculated to be 4100−3400−4100 g/mol, which is close to the target of 4400−3400−4400 g/mol. The hydrolysis of PMeOZO−PEG−PMeOZO was performed in 10 wt % aqueous HCl at 100 °C for 16 h. Upon adjustment of the pH of the hydrolyzed mixture to 12.5, the PEI−PEG−PEI triblock copolymer precipitated and was isolated by centrifugation. This workup procedure eliminates free PEG when present. The ^{1}H NMR in DMSO-d_6 (Figure 1b) displayed two singlets at δ 2.54 and 3.50, which can be attributed to the methylene protons of PEI and PEG blocks, respectively. The ratio of the integrals of these two signals indicated that this triblock copolymer has an average molecular weight of 2100−3400−2100 g/mol (denoted as PEI−PEG−PEI 2100−3400−2100). It should be noted that a small peak at δ 1.98, which could be attributed to residual acetyl groups, is also present. It is estimated on the basis of the relative integrals at δ 1.98 and 2.54 that more than 99% of the acetyl groups have been removed (i.e., residue acetyl groups <1%). Similarly, starting with a MeOZO/TsO−PEG−OTs ratio of 208/1, a PEI−PEG−PEI triblock copolymer with an average molecular weight of 4000−3400−4000 g/mol (denoted as PEI−PEG−PEI 4000−3400−4000) was obtained. In an analogous way, starting from a monotosylated PEG, Kataoka and co-workers synthesized a linear diblock copolymer of PEG and PEI.[38]

The buffering capacity of PEI at low pH may play an important role in the transfection efficiency of PEI/DNA complexes. As proposed by Behr and co-workers, this buffering effect protects DNA from nuclease degradation in the endosomes and facilitates endosomal escape of PEI/DNA complexes (proton sponge hypothesis).[4] Therefore, the buffering capacity of the PEI−PEG−PEI triblock copolymers was investigated by acid−base titration in 0.1 M NaCl aqueous solution. The buffering capacity is defined as the percentage of amine groups becoming protonated from pH 7.4 to 5.1, which can be calculated from the following equation:

$$\text{buffering capacity} = \frac{\Delta H^+_{\text{polymer}} - \Delta H^+_{\text{NaCl}}}{\text{mole N}} \times 100\%$$

wherein $\Delta H^+_{\text{polymer}}$ and ΔH^+_{NaCl} are the moles of H^+ required to bring the polymer solution and 0.1 M NaCl, respectively, from pH 7.4 to 5.1, and mole N is the total moles of amine groups in the polymer. The results (Figure 2) revealed that these low molecular weight PEI−PEG−PEI triblock copolymers have good buffering capacity in the pH 7.4 to 5.1 range (14.2 and 11.1% for PEI−PEG−PEI 2100−3400−2100 and 4000−3400−4000, respectively, and 10.2% for linear PEI 25000).

3.2. Biophysical Characterization of Polymer/Plasmid DNA Complexes. The binding of PEI−PEG−PEI triblock copolymers to DNA was first studied by a gel retardation assay. As shown in Figure 3, both triblock copolymers completely retarded DNA migration at a polymer/DNA ratio at 0.75 (w/w), which corresponds to a nitrogen/phosphate (N/P) ratio of 2.9 for PEI−PEG−PEI 2100−3400−2100 and 3.7 for PEI−PEG−PEI 4000−3400−4000, respectively. A further study of PEI−PEG−PEI/DNA complexes by dynamic light scattering (DLS) (Figure 4) showed that both PEI−PEG−PEI 2100−3400−2100 and 4000−3400−4000 indeed effectively condense DNA into small-sized particles (70−120 nm) at a polymer/DNA ratio ≥0.75/1 (w/w). The ζ-potential profile (Figure 4) showed that polyplexes based on both triblock copolymers have moderate ζ-potentials of approximately +10 mV, which remain constant over a broad range of polymer/DNA ratios (0.75−24/1 (w/w)) and are significantly lower than those observed for PEI 25000 (∼30 mV). This reduced ζ-potential can be ascribed to shielding of the positive surface charge of the polyplexes by the PEG chains. It is speculated that a number of PEI−PEG−PEI triblock copolymers are *only partly involved* (i.e., via only one PEI block) in the condensation of plasmid DNA, which leads to the formation of PEG shielded polyplexes with several short PEI blocks available at the outer surface (Figure

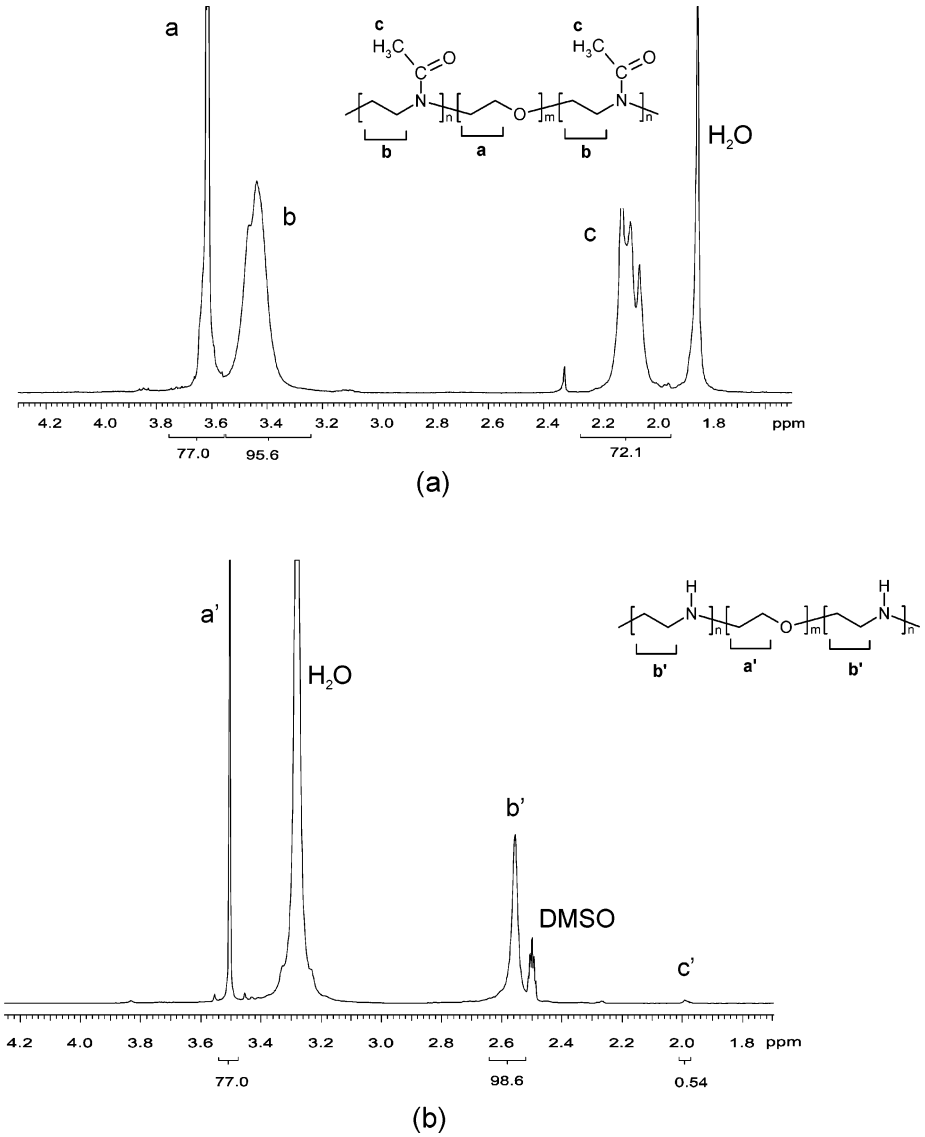


Figure 1. ^{1}H NMR spectra (300 MHz) of PMeOZO−PEG−PMeOZO ($CDCl_3$) (a) and PEI−PEG−PEI (DMSO-d_6) (b). PMeOZO−PEG−PMeOZO was prepared by the ring opening polymerization of 2-methyl-2-oxazoline initiated by TsO−PEG−OTs at a [MeOZO]/[TsO−PEG−OTs] molar ratio of 104/1 at 70 °C for 3 days. Linear PEI−PEG−PEI triblock copolymer was obtained by complete hydrolysis of PMeOZO−PEG−PMeOZO.

5). Thus, the biophysical profiles of polyplexes based on PEI−PEG−PEI triblock copolymers are on one hand distinct from those based on PEI homopolymers, which display high surface charge densities that are associated with high cytotoxicity,[32] and on the other hand different from those based on PEGylated PEIs which have a close to zero ζ-potential and have low transfection efficiencies.[21] Recently, other types of PEG−PEI copolymers such as micelle-forming PEG−PEI−poly(γ-benzyl-L-glutamate) (PBLG) copolymers were designed.[39] Though no transfection results were reported, these amphiphilic cationic copolymers were able to self-assemble with plasmid DNA.

The colloidal stability of PEI−PEG−PEI polyplexes prepared at polymer/DNA ratios of 6/1 and 12/1 (w/w) was studied using dynamic light scattering at 37 °C at pH 7.4. To mimic the physiological conditions, the ionic strength of the suspension was adjusted to 150 mM with NaCl. Interestingly, the size of the polyplexes remained small in the presence of 150 mM NaCl and the polyplexes did not aggregate/swell over a time period of 24 h. This is in contrast with linear PEI/DNA complexes, which grow into large aggregates in less than 1 h when salt is added.[11] Obviously, the presence of PEG leads to a good colloidal stability of PEI−PEG−PEI polyplexes. The improved colloidal stability of PEI−PEG−PEI polyplexes with respect to the PEI/DNA complexes was also supported by the observation that suspensions of PEI−PEG−PEI polyplexes are clear even at high concentrations (e.g., at a DNA concentration of 200 μg/mL), whereas those of PEI polyplexes are turbid and tend to precipitate.

3.3. In Vitro Transfection Activity and Cytotoxicity of PEI−PEG−PEI Triblock Copolymer Polyplexes. To evaluate the transfection potential of linear PEI−PEG−PEI triblock copolymers, their complexes with plasmid pCMVLacZ expressing β-galactosidase were incubated with COS-7 cells. Various polymer/plasmid ratios ranging from 0.75/1 to 96/1 (w/w) were used, and the transfection experiments were performed using different media, that is, in the presence or absence of 5.0% serum. Interestingly, as shown in Figure 6a, the polyplexes of PEI−PEG−PEI

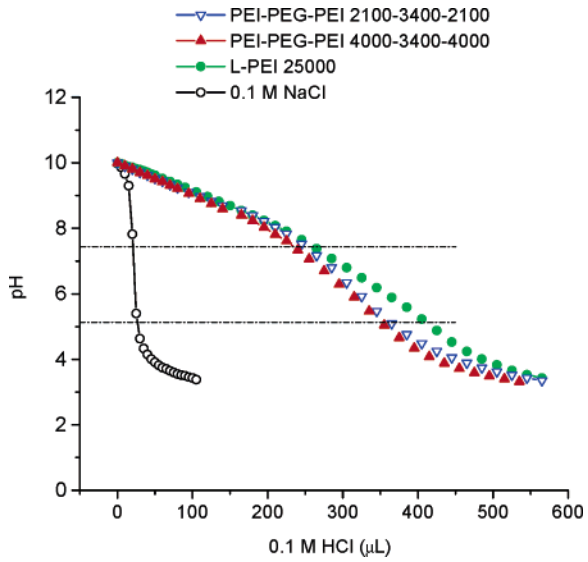


Figure 2. Titration curves obtained by titrating aqueous solutions of PEI−PEG−PEI triblock copolymers (0.2 mg/mL) in 0.1 M aqueous NaCl (pH 10, adjusted with NaOH) with 0.1 M HCl. As a reference, the titration curve of linear PEI 25000 is also presented.

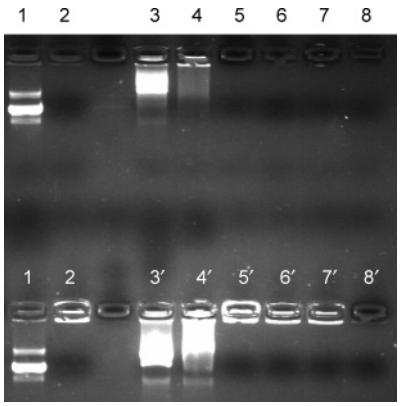


Figure 3. Agarose gel electrophoresis of PEI−PEG−PEI/plasmid complexes prepared at different polymer/plasmid ratios. Lane 1, free DNA; lane 2, linear PEI 25000/DNA complexes at N/P = 10/1; lanes 3−8, PEI−PEG−PEI 2100−3400−2100/DNA complexes with polymer/DNA ratios (w/w) of 0.1875, 0.375, 0.75, 1.5, 3, and 6; and lanes 3′−8′, PEI−PEG−PEI 4000−3400−4000/DNA complexes with polymer/DNA ratios (w/w) of 0.1875, 0.375, 0.75, 1.5, 3, and 6.

4000−3400−4000 gave a remarkable level of transfection. The transfection efficiency first increased and then decreased with increasing polymer/plasmid ratios, in which an optimal transfection efficiency of approximately 110% relative to pDMAEMA was observed at a polymer/plasmid ratio of 12/1 (w/w) (corresponding to an N/P ratio of 60/1) in the absence of serum. This high level of transgene expression only slightly decreased when serum proteins were present in the transfection medium (Figure 6a). The polyplexes based on the lower molecular weight triblock copolymer, PEI−PEG−PEI 2100−3400−2100, were also able to transfect COS-7 cells. The transfection efficiency increased with increasing polymer/plasmid ratios, reaching an efficiency of 25% relative to pDMAEMA/DNA formulation at a polymer/DNA ratio of 96/1 (w/w).[40] INF7 peptide is an effective endosome disruptive compound and tremendously enhances the gene transfection activity of, for example, poly(L-lysine)/DNA complexes.[33] The addition of INF7 did not enhance the transfection efficiency of the PEI−PEG−PEI polyplexes

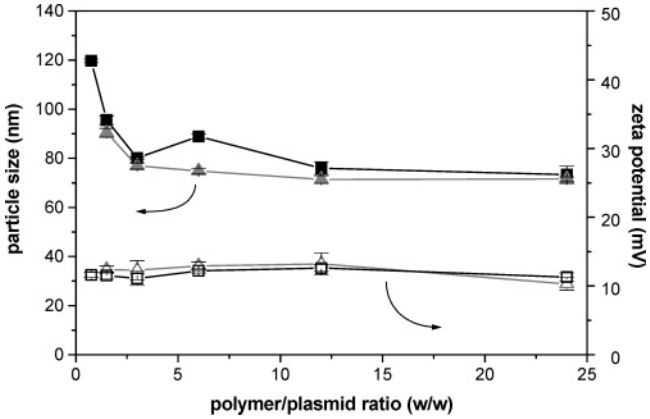


Figure 4. Average diameter (filled triangles, PEI−PEG−PEI 2100−3400−2100; filled squares, PEI−PEG−PEI 4000−3400−4000) and ζ-potential (open triangles, PEI−PEG−PEI 2100−3400−2100; open squares, PEI−PEG−PEI 4000−3400−4000) of polyplexes of PEI−PEG−PEI triblock copolymer as a function of the polymer/DNA ratio.

(data not shown). This indicates that for PEI−PEG−PEI triblock copolymer polyplexes endosomal escape is not a limiting factor in the transfection process. Likely, as shown in Figure 2, the polymers have sufficient buffering capacity to escape the endosomal compartment.

Several groups have reported that the modification of cationic polymers with PEG results in a considerable reduction in transfection activity due to an inefficient cellular uptake.[20,21] The high transfection activity of polyplexes based on PEI−PEG−PEI 4000−3400−4000 can be attributed to their relatively low cytotoxicity (Figure 6b, discussed below) and positive surface charge as revealed by ζ-potential measurements (Figure 4). Kissel and co-workers studied HMW branched PEI conjugated with different PEGs and found that the cytotoxicity and ζ-potential of their polyplexes play a key role in the transfection efficiency; that is, the polyplexes with low cytotoxicity and high positive ζ-potential give the best transfection.[41] Furthermore, in the PEI−PEG−PEI triblock copolymers, the PEG chain acts as a linker for the LMW linear PEIs. It has been reported that the coupling of LMW *branched* PEI to a HMW PEI resulted in increased transfection.[19,28−30]

The in vitro cytotoxicity of polyplexes based on PEI−PEG−PEI triblock copolymers was studied as a function of polymer/plasmid ratio (w/w) by using the XTT assay. LMW linear PEI 2100 and high molecular weight (HMW) PEI 25000 were used as references. In agreement with the literature, LMW linear PEI based DNA formulations have a very low cytotoxicity (Figure 6b). All cells retained their metabolic activity at a polymer/plasmid ratio up to 12/1 (w/w). PEI−PEG−PEI 2100−3400−2100 formulations were nontoxic at polymer/DNA ratios up to 24/1 (w/w) (Figure 6b). At high polymer/plasmid ratios of 48/1 and 96/1 (w/w), some toxicity was observed (the cell viabilities were 85 and 70%, respectively). It should be noted that, in the presence of 5% serum, polyplexes of both PEI 2100 and PEI−PEG−PEI 2100−3400−2100 were essentially nontoxic at polymer/plasmid ratios up to 96/1 (w/w) (data not shown). Polyplexes of PEI−PEG−PEI 4000−3400−4000 exhibited a higher cytotoxicity than those of PEI−PEG−PEI 2100−3400−2100 (Figure 6b). However, at a polymer/plasmid ratio

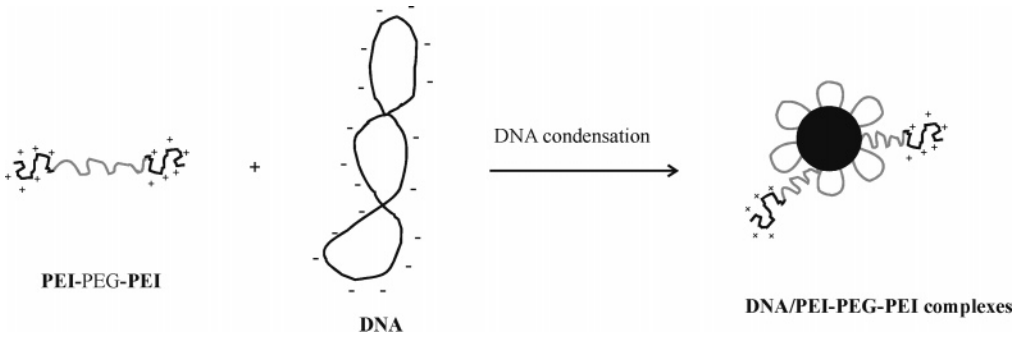


Figure 5. Possible structure of polyplexes formed from plasmid DNA condensation using PEI−PEG−PEI triblock copolymers.

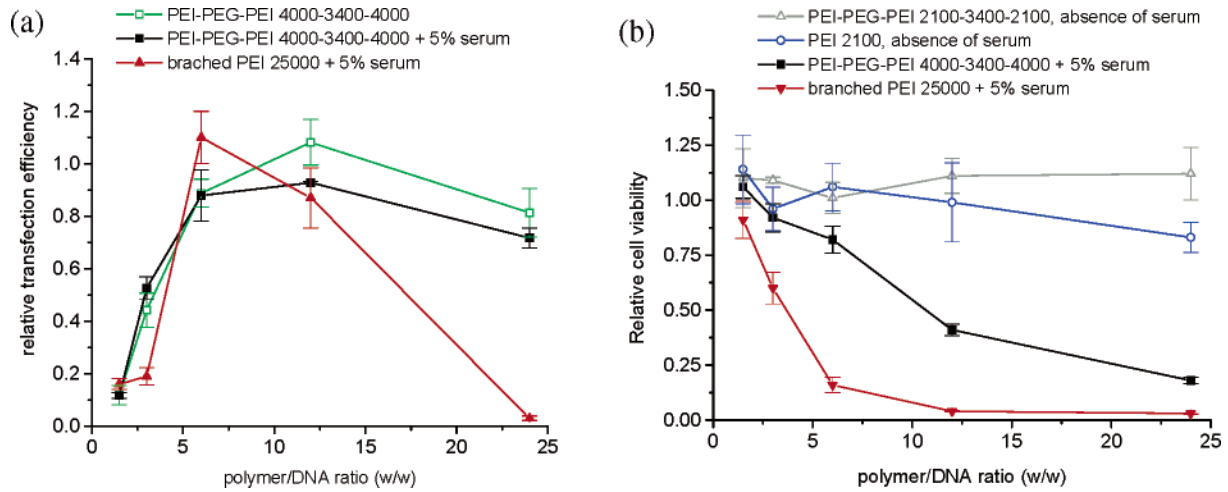


Figure 6. Transfection efficiency (a) and cytotoxicity (b) of PEI−PEG−PEI/plasmid complexes using various polymer/DNA ratios (w/w) in COS-7 cells. Transfection efficiencies were normalized to that of pDMAEMA/DNA complexes at a ratio of 3/1 (w/w) in the absence of serum (i.e., the transfection efficiency of pDMAEMA polyplexes in the absence of serum at their optimal polymer/DNA ratio of 3/1 (w/w) was taken as 1.0). Cell viability was determined by the XTT assay. For comparison, transfection efficiency and cell viability data of polyplexes of branched PEI 25000 in the presence of 5% serum are also given. The data were expressed as mean values (± standard deviations) of three experiments.

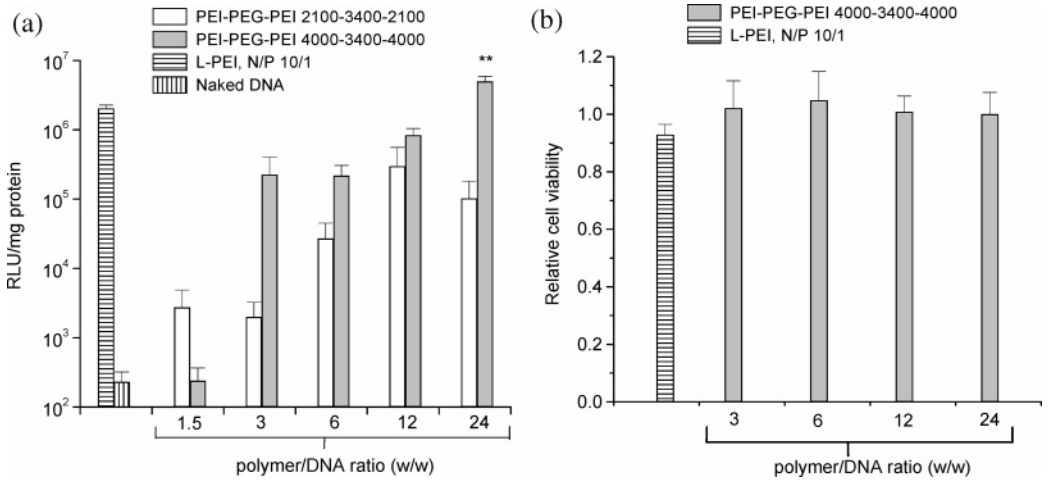


Figure 7. Transfection efficiency (a) and cytotoxicity (b) of polyplexes based on PEI−PEG−PEI triblock copolymers in the presence of 10% FBS in primary bovine endothelial cells (BAECs). The transfection experiments were performed using a pCMVLuc reporter gene (2.0 μg per well) with polymer/DNA ratios varying from 1.5/1 to 24/1 (w/w). Cell viability was determined by the MTT assay. For comparison, transfection efficiency and cell viability data of polyplexes of linear PEI 25000 at an N/P ratio of 10/1 in the presence of 10% FBS are also given (**$p < 0.01$, PEI−PEG−PEI 4000−3400−4000 vs L-PEI).

of 6/1 (w/w) at which high transgene expression was obtained, the cell viability was about 80%. As a comparison, for polyplexes of branched PEI 25000, less than 20% of the cells remained viable at a polymer/plasmid ratio of 6/1 (w/w), under conditions where these polyplexes showed the best transfection (Figure 6a). Therefore, these LMW linear PEI−PEG−PEI triblock copolymers are far less cytotoxic than HMW PEIs which can be ascribed to the following. First, the surface charge of the polyplexes is partly shielded by the PEG chains. As reported previously, PEGylated cationic polymers are in general less toxic than the unmodified version.[16] Second, the PEI chains in the triblock copolymers

are linear and short. It has been reported that linear PEI is less toxic than branched PEI[11] and LMW PEI is less toxic than HMW PEI.[10]

The gene transfer properties of these PEI−PEG−PEI triblock copolymers were also evaluated using primary bovine endothelial cells (BAECs) and plasmid DNA encoding for a luciferase reporter gene in the presence of 10% serum. As shown in Figure 7a, polyplexes based on both PEI−PEG−PEI triblock copolymers were capable of transfecting BAECs. The polyplexes of PEI−PEG−PEI 4000−3400−4000 exhibited a higher level of gene expression than PEI−PEG−PEI 2100−3400−2100 polyplexes, which is in agreement with transfection results obtained using COS-7 cells. The transfection efficiency of PEI−PEG−PEI 4000−3400−4000 based polyplexes increased with increasing polymer/DNA ratio (from 1.5/1 to 24/1 (w/w)). Notably, at a polymer/DNA ratio of 24/1 (w/w), polyplexes of PEI−PEG−PEI 4000−3400−4000 showed a 3-fold higher luciferase reporter gene expression over linear PEI 25000/DNA complexes (N/P = 10/1). Moreover, Figure 7b revealed that PEI−PEG−PEI 4000−3400−4000 polyplexes are not toxic for BAECs at all polymer/DNA ratios under the applied transfection conditions. For instance, cells incubated with PEI−PEG−PEI 4000−3400−4000 polyplexes formed at a polymer/DNA ratio of 24/1 (w/w) showed a viability of 100 ± 7.6%. The linear PEI 25000 polyplexes, on the other hand, exhibited a slight cytotoxicity (cell viability 93 ± 3.6%).

Hence, it appeared that triblock-type copolymers of PEI−PEG−PEI possess great advantages for gene transfer in vitro. First, *LMW linear* PEI has low cytotoxicity and the conjugation with PEG further reduces its cytotoxicity, rendering more biocompatible carriers. Second, the linking of LMW PEIs by PEG has resulted in an increase of molecular weight, which could be part of the reason for enhanced transfection efficiency. Third, the transfection of this triblock system is not influenced by the presence of serum in the transfection medium, contrary to HMW PEI polyplex formulations for which a large decrease in transfection is generally observed. Fourth, the moderate ζ-potential of these PEI−PEG−PEI polyplexes facilitates efficient cellular uptake, which could explain why PEI−PEG−PEI triblock copolymers have superior transfection activity as compared with PEG modified HMW PEIs. Furthermore, polyplexes of PEI−PEG−PEI show also highly improved colloidal stability, which is desired for in vivo applications.

4. Conclusions

We have demonstrated that low molecular weight linear PEI−PEG−PEI triblock copolymers are a novel class of efficient polymeric carriers for DNA delivery in vitro in the presence of serum. Good colloidal stability, lack of serum inhibition on transfection activity, and low cytotoxicity of polyplexes based on PEI−PEG−PEI triblock copolymers make them very promising for in vivo gene transfer. In the future, the macromolecular structure and molecular weights of PEI and PEG blocks of these copolymers will be varied to achieve optimal transfection efficiency and safety profile, which may eventually lead to a new generation of synthetic vectors for in vitro, ex vivo, and in vivo gene delivery.

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