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Non-viral gene transfection in vitro using endosomal pH-sensitive reversibly hydrophobilized polyethylenimine

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ABSTRACT

Reversibly hydrophobilized 10 kDa polyethylenimine (PEI) based on rapidly acid-degradable acetal-containing hydrophobe was designed for nontoxic and highly efficient non-viral gene transfer. Water soluble PEI derivatives with average 5, 9 and 14 units of pH-sensitive 2,4,6-trimethoxybenzylidene-tris(hydroxymethyl)ethane (TMB-THME) hydrophobe per molecule, denoted as PEI-*g*-(TMB-THME)$_n$, were readily obtained by treating 10 kDa PEI with varying amounts of TMB-THME-nitrophenyl chloroformate. Gel retardation assays showed that all PEI-*g*-(TMB-THME)$_n$ derivatives could effectively condense DNA at an N/P ratio of 5/1. Notably, polyplexes of PEI-*g*-(TMB-THME)$_n$ derivatives had smaller sizes (about 100∼170 nm) and higher surface charges (+25 ∼ +43 mV) than the parent 10 kDa PEI at the same N/P ratios ranging from 10/1 to 40/1. MTT assays revealed that these PEI-*g*-(TMB-THME)$_n$ derivatives were practically non-toxic at polymer concentrations used in transfection experiments. The acetal degradation of PEI-*g*-(TMB-THME)$_9$ was shown to be highly pH dependent in which half lives of 1.3, 2.8 and 11 h were determined for pH 4.0, 5.0 and 6.0, respectively, while negligible hydrolysis (<12%) was observed after 24 h at pH 7.4. Gel electrophoresis, dynamic light scattering (DLS) and zeta potential analyses indicated that polyplexes formed at an N/P ratio of 10/1 were dissociated following 5 h incubation at pH 5.0, highlighting the importance of hydrophobic TMB-THME moieties in DNA condensation and supporting that acetal hydrolysis in endosomes would facilitate DNA release. Notably, *in vitro* transfection experiments performed at N/P ratios of 10/1 and 20/1 in HeLa, 293T, HepG2 and KB cells using plasmid pGL3 expressing luciferase as the reporter gene showed that reversibly hydrophobilized PEIs had superior transfection activity to 25 kDa PEI control. For example, polyplexes of PEI-*g*-(TMB-THME)$_{14}$ showed about 235-fold and 175-fold higher transfection efficiency as compared to 10 kDa PEI in HeLa cells in serum-free and 10% serum media, respectively, which were approximately 7-fold and 16-fold higher than 25 kDa PEI formulation at its optimal N/P ratio under otherwise the same conditions. Confocal laser scanning microscope (CLSM) studies confirmed that PEI-*g*-(TMB-THME)$_{14}$ efficiently delivered Cy5-labeled DNA to the nuclei of HeLa cells. These endosomal pH-sensitive reversibly hydrophobilized PEIs have great potentials for safe and efficient non-viral gene transfection.

1. Introduction

In the past decade, non-viral vectors in particular cationic polymers have gained rapidly growing interests for gene transfection in that they present several advantages over viral systems including facile synthesis and tuning of vector structures and properties, large DNA loading capacity, possible repeated administration, and reproducible large-scale pharmaceutical grade production [1–4]. Moreover, polymeric vectors can be modified to achieve prolonged circulation and deliver therapeutic genes to the targeted cells *in vivo* [5–10]. It should be noted, however, that despite their obvious merits, few polymeric systems have advanced to the clinical trials, primarily due to a low transfection activity as compared to the viral counterparts [11,12].

Polyethylenimine (PEI) is one of the most efficient non-viral gene carriers that are able to deliver DNA to a variety of cells due to its unique combination of high charge density and proton sponge

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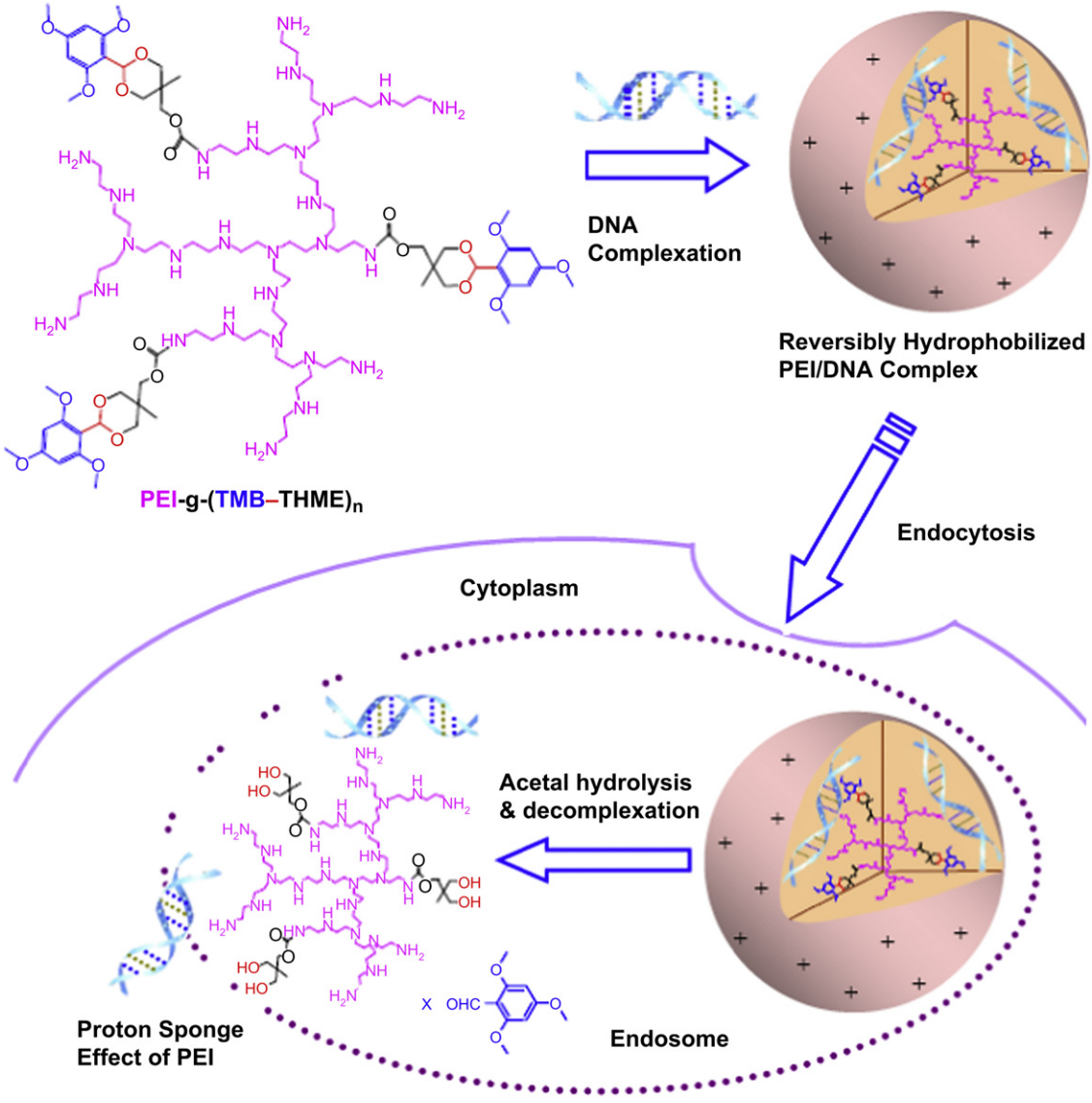
effect [13–15]. The transfection performance of PEI depends on its macromolecular structures and molecular weights, in which 25 kDa branched PEI (denoted as 25 kDa PEI) and 22 kDa linear PEI have turned out to be the best and are currently applied as golden standards for non-viral transfection [16,17]. These PEI reagents are, nevertheless, associated with varying levels of cytotoxicity and furthermore their transfection activity remains moderate as compared to viral vectors. In recent years, based on the fact that low molecular weight PEIs have low cytotoxicity, various types of hydrolytically or reductively degradable PEI polymers and networks have been designed for *in vitro* transfection [18–26]. These degradable PEIs have shown significantly enhanced transfection activity as compared to the parent low molecular weight PEIs, with transfection efficiency approaching or in a few cases exceeding that of 25 kDa PEI control. It should be noted, nevertheless, that the synthesis of degradable PEI polymers and networks are mostly not controlled due to the involvement of coupling reactions between reagents with multiple reactive centers, yielding usually ill-defined vectors in terms of structure as well as molecular weight. Hydrophobic modification represents another effective approach to improve the transfection activity of low molecular weight PEIs [27,28]. For example, Kim reported that cholesterol-modified 1.8 kDa PEI had higher transfection efficiency in CT-26, 293T and A7R5 cells than the parent 1.8 kPEI [29,30]. Klibanov and coworkers reported that dodecylation of 2 kDa PEI resulted in 5-fold higher transfection efficiency relative to that of 25 kDa PEI [31]. Uludag reported that substitution of 2 kDa PEI with aliphatic lipids including caprylic, myristic, palmitic, stearic, oleic and linoleic acids led to a transfection efficiency comparable to 25 kDa PEI [32]. Ramezani and coworkers reported that 10 kDa PEI after conjugation with alkyl-oligoamine had an increased transfection activity in N2A murine neuroblastoma cells to a level similar to that of 25 kDa PEI [33]. The enhanced transfection activity by hydrophobic modification is likely due to its balanced protection and release of DNA as well as enhanced interactions with cellular membranes [34].

In this paper, we report on reversibly hydrophobilized 10 kDa PEIs based on rapidly acid-degradable acetal-containing hydrophobe for nontoxic and marked enhanced non-viral gene transfection (Scheme 1). The commercial 10 kDa PEI has an approximate



Scheme 1. Illustration on reversibly hydrophobilized 10 kDa PEI for efficient intracellular delivery and release of DNA. Hydrophobic modification of 10 kDa PEI enhances its DNA condensation ability and cellular interactions while reversal of hydrophobic modification in endosomes facilitates intracellular release of DNA.

ratio of primary to secondary to tertiary amino groups of 1:1:1 [35]. This acid-degradable hydrophobe uniquely combines the functions of (i) hydrophobic modification, which was assumed to enhance the DNA condensation ability and cellular interactions of 10 kDa PEI [27,28], and (ii) pH-sensitive degradation, which was hypothesized to reverse hydrophobic modification in endosomes and hence facilitate intracellular release of DNA. Inefficient intracellular DNA release is a major barrier for non-viral gene transfection [36,37]. Fréchet and coworkers reported that rapid hydrolysis of cyclic benzylidene acetals at mildly acidic pH of 5.0–6.5 could result in efficient intracellular release of hydrophobic as well as hydrophilic payloads from the nano and micro-particles [38–41]. Inspired by Fréchet's work, we recently prepared rapidly pH-sensitive biodegradable micelles and polymersomes based on benzylidene acetals for triggered release of anti-cancer drugs [42,43]. The present study revealed that benzylidene acetal-modified 10 kDa PEI while being non-toxic mediated up to 175-fold higher transfection efficiency as compared to the parent 10 kDa PEI in HeLa cells, exceeding 16-fold that of 25 kDa PEI. The DNA complexation and release behaviors, cytotoxicity, and gene transfection of reversibly hydrophobilized 10 kDa PEIs were investigated.

2. Materials and methods

2.1. Materials

PEI (10 kDa and 25 kDa), 2, 4, 6-trimethoxybenzaldehyde (TMB, 99%), p-toluenesulfonic acid monohydrate (99%) and p-nitrophenyl chloroformate (p-NC, 97%) were purchased from Alfa Aesar and used as received. 1, 1, 1-tris(hydroxymethyl) ethane (THME, 99%) and dextran sulfate sodium (DSS) were obtained from Aldrich. Tetrahydrofuran (THF) was distilled over sodium wire and CH_2Cl_2 was distilled over CaH_2 prior to use. 4 Å molecular sieves were dried at 110 °C prior to use. 2, 4, 6-trimethoxybenzylidene tris(hydroxymethyl)ethane (TMB-THME) was prepared similar to our previous report [43].

2.2. Preparation of 2, 4, 6-trimethoxybenzaldehyde-tris(hydroxymethyl)ethane-nitrophenyl chloroformate (TMB-THME-NC)

To a solution of TMB-THME (2.31 g, 7.8 mmol) in CH_2Cl_2 (70 mL) were added NEt_3 (2.36 g, 23.4 mmol), pyridine (0.60 g, 7.8 mmol) and 4-nitrophenyl chloroformate (1.565 g, 9.13 mmol). The reaction was allowed to proceed at room temperature (r.t.) for overnight before adding 300 mL of Et_2O (to precipitate $HCl{\bullet}NEt_3$ salt). The solution after filtration was precipitated in cold hexane, filtered and dried *in vacuo* for 2 d. Yield: 60%. ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.30 (d, 2H), δ 7.42 (d, 2H), δ 6.10 (s, 2H), δ 6.00 (s, 1H), δ 4.77 (s, 2H), δ 4.05 (d, 2H), δ 3.83 (s, 6H), δ 3.79 (s, 3H), δ 3.68 (d, 2H), δ 0.90 (s, 3H).

2.3. Synthesis of PEI-g-(TMB-THME)$_n$

10 kDa branched PEI (0.917 g, 0.092 mmol) was dissolved in 4 mL of CH_2Cl_2. A solution of TMB-THME-NC (0.497 g, 1.1 mmol) in 2 mL of CH_2Cl_2 was added dropwise at r.t. The reaction was allowed to proceed for 24 h. The product was isolated by precipitation in cold diethyl ether for three times, dialysis against water to remove *p*-nitrophenol, and freeze-drying. Yield: 73%, ^{1}H NMR (400 MHz, $CDCl_3$) for PEI-*g*-(TMB-THME)$_9$: δ 6.07 (s, 2H), δ 5.93 (s, 1H), δ 4.50 (s, 2H), δ 3.97 (s, 2H), δ 3.81 (s, 6H), δ 3.75 (s, 3H), δ 3.57 (s, 2H), δ 0.74 (s, 3H) δ 3.00–2.50 (PEI). PEI-*g*-(TMB-THME)$_n$ derivatives with average 5, 9, and 14 TMB-THME substituents per PEI molecule were obtained by varying mole feed ratios of TMB-THME to PEI from 9, 12 to 17.

2.4. Characterization

^{1}H NMR spectra were recorded on a Varian Inova 400 MHz spectrometer using $CDCl_3$ as a solvent. The degree of substitution was determined by the ^{1}H NMR. The buffer capacity of PEI-*g*-(TMB-THME)$_n$ was determined by acid-base titration assay over a pH range of 10.0–2.0. In brief, PEI-*g*-(TMB-THME)$_n$ (0.1 mmol N) was dissolved in 5 mL of 150 mM NaCl aqueous solution. The pH of the polymer solution was brought to 10.0 and the solution was titrated with 0.1 M HCl using a pH meter (DELTA 320). For comparison, 10 kDa PEI and 25 kDa PEI was also titrated in the same way. The buffering capacity defined as the percentage of amine groups becoming protonated from pH 7.4 to 5.1, was calculated from equation:

$$\text{Buffer capacity}(\%) = 100(\Delta V_{HCl} \times 0.1\text{M})/N \text{ mol}$$

where ΔV_{HCl}, is the volume of HCl solution (0.1 M) required to bring the pH value of the polymer solution from 7.4 to 5.1, and N mol (0.1 mmol) is the total moles of protonable amine groups in the polymer.

2.5. Particle size and zeta-potential measurements

The polyplexes were prepared at varying N/P ratios from 10/1, 20/1 to 40/1 by adding a HEPES buffer solution (20 mM, pH 7.4) of polymer (600 μL, varying concentrations) to a HEPES buffer solution (20 mM, pH 7.4) of plasmid DNA (150 μL, 37.5 μg/mL), followed by vortexing for 5 s and incubating at r.t. for 30 min. The surface charge and the size of polyplexes were measured at 25 °C with a Zetasizer Nano ZS instrument (Malvern) equipped with a standard capillary electrophoresis cell and dynamic light scattering (DLS, 10 mW He–Ne laser, 633 nm wavelength), respectively. The measurements were performed in triplicate.

2.6. Gel electrophoresis assay

The polyplexes were prepared as above at varying N/P ratios from 1/1 to 8/1. Electrophoresis was carried out on 0.8% agarose gel with a current of 110 V for 40 min in TAE buffer solution (40 mM Tris–HCl, 1 v. % acetic acid, and 1 mM EDTA). The retardation of the complexes was visualized by staining with ethidium bromide.

2.7. Determination of the pH-dependent hydrolysis rate of acetals in the polyplexes

The acetal hydrolysis was determined by UV/Vis spectroscopy by measuring the absorbance at 290 nm according to the previous reports by Fréchet and coworkers [40,41]. The polyplex suspension prepared as above-mentioned was divided into four aliquots and adjusted to pH 4.0, 5.0, 6.0 and 7.4, respectively, by adding 22.5 μL of 4.0 M pH 4.0, 5.0, 6.0 acetate buffer or 7.4 phosphate buffer, while keeping the salt concentration the same. At desired time points, 80 μL aliquot was removed and diluted with 3.5 mL of phosphate buffer (0.1 M, pH 7.4) and the absorbance at 290 nm was measured. In the end, all the samples were completely hydrolyzed by adding two drops of concentrated HCl and were measured again to determine the absorbance at 100% hydrolysis, which was used to calculate extent of acetal hydrolysis.

2.8. Acid-triggered unpacking of PEI-g-(TMB-THME)$_n$ polyplexes

The polyplex suspension prepared at an N/P ratio of 10/1 as above-mentioned was divided into two aliquots. One sample was adjusted to pH 5.0 by adding 4.0 M pH 5.0 acetate buffer while the other sample was added with the same volume of 4.0 M 7.4 phosphate buffer, to keep the same ionic strength. At different time intervals, the samples incubated at pH 5.0 were adjusted back to pH 7.4 with 4 M pH 7.4 phosphate buffer, and then polyplex sizes and zeta potentials were measured. The samples incubated at pH 7.4 were also added with equal volume of 4 M pH 7.4 phosphate buffer before measurement.

Acid-triggered DNA release from polyplexes of PEI-*g*-(TMB-THME)$_n$ was also studied by agarose gel electrophoresis. The polyplexes of PEI-*g*-(TMB-THME)$_{14}$ at an N/P ratio of 10/1 were prepared as above-mentioned. The solution pH was adjusted to pH 5.0 using 4.0 M pH 5.0 acetate buffer. For the control sample at pH 7.4, same volume of 4 M pH 7.4 PB buffer was added. After 4.5 h, 2 μL of DSS (DSS/DNA charge ratio = 40/1) was added and incubated for additional 0.5 h before gel electrophoresis assay.

2.9. MTT assays

The cytotoxicity of PEI-*g*-(TMB-THME)$_n$ conjugates was evaluated in HeLa, 293T, and HepG2 cells by MTT assays. In brief, HeLa, 293T, and HepG2 cells were seeded in a 96-well tissue culture plate at 6000 cells/well in 100 μL DMEM medium containing 10% FBS for 1 d. PEI-*g*-(TMB-THME)$_n$ solutions were added to give varying polymer concentrations and the cells were cultured for additional 2 d. The medium was replaced with 100 μL of fresh medium containing 100 μg MTT and cells were further incubated for 4 h at 37 °C. The medium was aspirated, the MTT-formazan generated by live cells was dissolved in 150 μL of DMSO, and the absorbance at a wavelength of 570 nm of each well was measured using a microplate reader. The relative cell viability (%) was determined by comparing the absorbance at 570 nm with control wells containing only cell culture medium. Data are presented as average ± SD (n = 3).

The cytotoxicity of PEI-*g*-(TMB-THME)$_n$ polyplexes prepared at N/P ratios of 10/1 and 20/1 was also evaluated in HeLa, 293T, and HepG2 cells by MTT assays. To facilitate comparison, the experiment was performed using the same protocol as transfection studies. In brief, HeLa, 293T, and HepG2 cells were seeded in a 96-well tissue culture plate at 6000 cells/well in 100 μL DMEM medium containing 10% FBS for 1 d. PEI-*g*-(TMB-THME)$_n$ polyplex dispersions were added and the cells were cultured for 4 h at 37 °C. Next, the polyplexes were removed, 200 μL of fresh culture medium containing 10% serum was added, and the cells were cultured for 2 d. The medium was replaced with 100 μL of fresh medium containing 100 μg MTT and cells were further incubated for 4 h at 37 °C. The relative cell viability (%) was determined as described above.

2.10. In vitro gene transfection

Transfection experiments were performed in HeLa, 293T, HepG2 and KB cells using the plasmid pGL3 as a reporter gene. Transfections were conducted using polyplexes formed at N/P ratios of 10/1 and 20/1. The cells were plated in 24-well plates (cell density 6×10^4 cells/well) and maintained in DMEM supplemented with 10% FBS at 37 °C in a humidified atmosphere containing 5% CO_2 for 1 d. In a standard transfection experiment, the cells were rinsed with PBS and incubated with 100 μL of polyplex dispersion (1 μg of plasmid DNA per well) and 400 μL of culture medium with or without 10% serum for 4 h at 37 °C. Next, the polyplexes were removed, 500 μL of fresh culture medium containing 10% serum was added, and the cells were cultured for 2 d. Luciferase quantification was done using a commercial luciferase assay kit (R-Protagen Co., Ltd) and TD-20/20 Luminometer (Promega, USA). Transfection efficiency was expressed as relative light unit (RLU) per mg of protein. 25 kDa PEI/DNA formulation prepared at an optimal N/P ratio of 10/1 was used as a reference. All the experiments were carried out in triplicate.

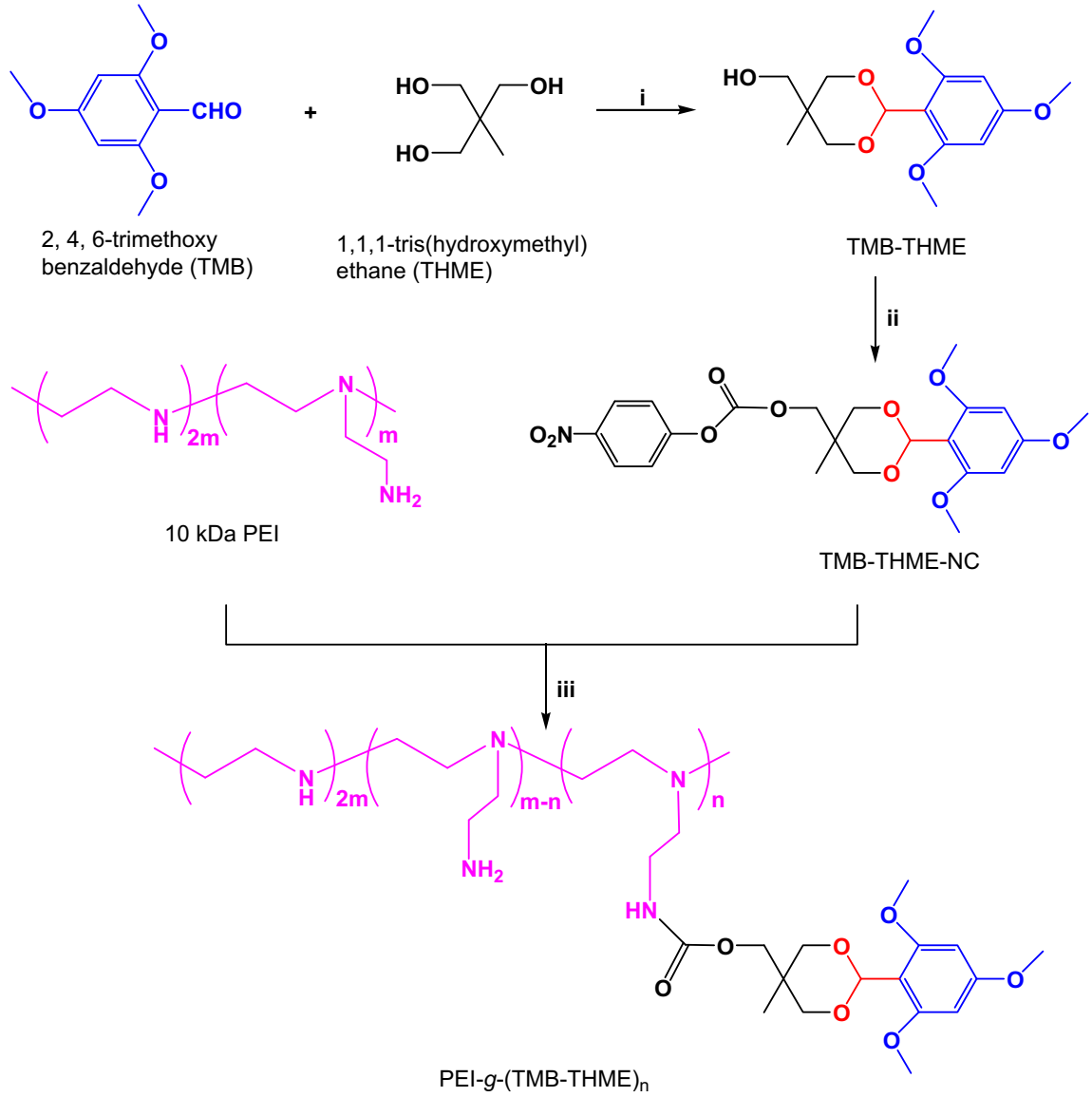
2.11. Intracellular DNA release studies with confocal laser scanning microscope (CLSM)

The cellular uptake and intracellular DNA release behaviors of PEI-*g*-(TMB-THME)$_{14}$ polyplexes at an N/P ratio of 10/1 were studied in HeLa cells with CLSM using Cy5-labeled DNA. The polyplexes of 10 kDa PEI and 25 kDa PEI at an N/P ratio of 10/1 were used as controls. In brief, HeLa cells were plated on coverslips in 24-well plates (cell density 6×10^4 cells/well) and maintained in DMEM supplemented with 10% FBS at 37 °C in a humidified atmosphere containing 5% CO_2 for 1 d. 100 μL of polyplex dispersions (1 μg of Cy5 labeled plasmid DNA per well) were added. The cells were cultured for 4 or 8 h at 37 °C. The polyplexes were removed and the cells were washed with PBS three times and fixed with 4% paraformaldehyde for 15 min. The nuclei were stained with 200 μL of Hoechst 33342 (20 μg/mL) for 15 min at r.t. The cells were observed with a confocal laser scanning microscope (TCS SP5 Leica) following three times rinsing with PBS.

3. Results and discussion

3.1. Synthesis of trimethoxybenzylidene Acetal-modified PEI

Trimethoxybenzylidene acetal-modified PEIs were readily prepared in three steps (Scheme 2). The reaction of 1, 1, 1-tris(hydroxymethyl)ethane (THME) with 2, 4, 6-trimethoxybenzaldehyde (TMB) in THF at r.t. in the presence of catalytic amount of TsOH afforded 2, 4, 6-trimethoxybenzylidene -tris(hydroxymethyl)ethane (TMB-THME) with high yield. ^{1}H NMR showed clearly a singlet at



Scheme 2. Synthesis of PEI-*g*-(TMB-THME)$_n$ conjugates. Conditions: (i) TsOH, molecular sieves, THF, 50 °C, overnight; (ii) p-NC, pyridine, NEt_3, CH_2Cl_2, r.t., overnight; (iii) CH_2Cl_2, r.t., 24 h.

δ 5.98 characteristic of acetal proton and an intensity ratio of signals at δ 6.09 (benzyl protons of TMB), 5.98 and 0.69 (methyl protons of THME) close to 2:1:3 (Fig. S1), corroborating equivalent coupling of TMB and THME. TMB-THME was then activated with 4-nitrophenyl chloroformate (*p*-NC) to give TMB-THME 4-nitrophenylcarbonate (TMB-THME-NC). ^{1}H NMR revealed that the methylene protons at δ 3.54 neighboring to the hydroxyl group vanished while new peaks appeared at δ 4.77 (attributable to the methylene protons next to carbonate) and δ 8.30–8.28 and 7.41–7.39 (assignable to nitrophenyl protons) (Fig. 1A). FT-IR spectrum of TMB-THME-NC showed absorptions characteristic of carbonate at 1760 cm^{-1} and nitro group at 1590 and 1330 cm^{-1} (Fig. S2). Finally, treatment of 10 kDa PEI with TMB-THME-NC brought about trimethoxybenzylidene acetal-modified PEI, denoted as PEI-*g*-(TMB-THME)$_n$ wherein *n* represents average number of TMB-THME hydrophobe per PEI molecule. ^{1}H NMR displayed that besides a broad peak of PEI at δ 2.50–3.00 signals attributable to TMB-THME moieties (δ 4.50) were also detected, while peaks characteristic of nitrophenyl protons disappeared completely (Fig. 1B). The intensity ratio of benzyl protons of TMB, acetal proton, and methyl protons of THME remained to be 2:1:3, indicating that acetals were intact. The average number of TMB-THME hydrophobe per PEI molecule (*n*) could be determined by comparing the integrals of signals at δ 2.50–3.00 and δ 4.50. PEI-*g*-(TMB-THME)$_n$ conjugates with $n = 5$, 9 and 14 were obtained at TMB-THME-NC/PEI mole feed ratios of 9/1, 12/1 and 17/1, respectively (Table 1). FT-IR analysis displayed a peak at 1700 cm^{-1} due to urethane group while absorptions characteristic of carbonate and nitro groups disappeared (Fig. S2), supporting successful synthesis of PEI-*g*-(TMB-THME)$_n$ conjugates. All three PEI-*g*-(TMB-THME)$_n$ conjugates were well soluble in HEPES buffer (pH 7.4, 20 mM) at a tested concentration of 1 mg/mL. Acid-base titration showed that PEI-*g*-(TMB-THME)$_n$ had similar buffer capacity as compared to the parent 10 kDa PEI (Fig. S3 and Table 1). This is likely because TMB-THME is conjugated to PEI mainly through the primary amino groups as a result of their higher reactivity relative to secondary amino groups. The buffer capacity of PEI is, however, mostly determined by its secondary and tertiary amino groups.

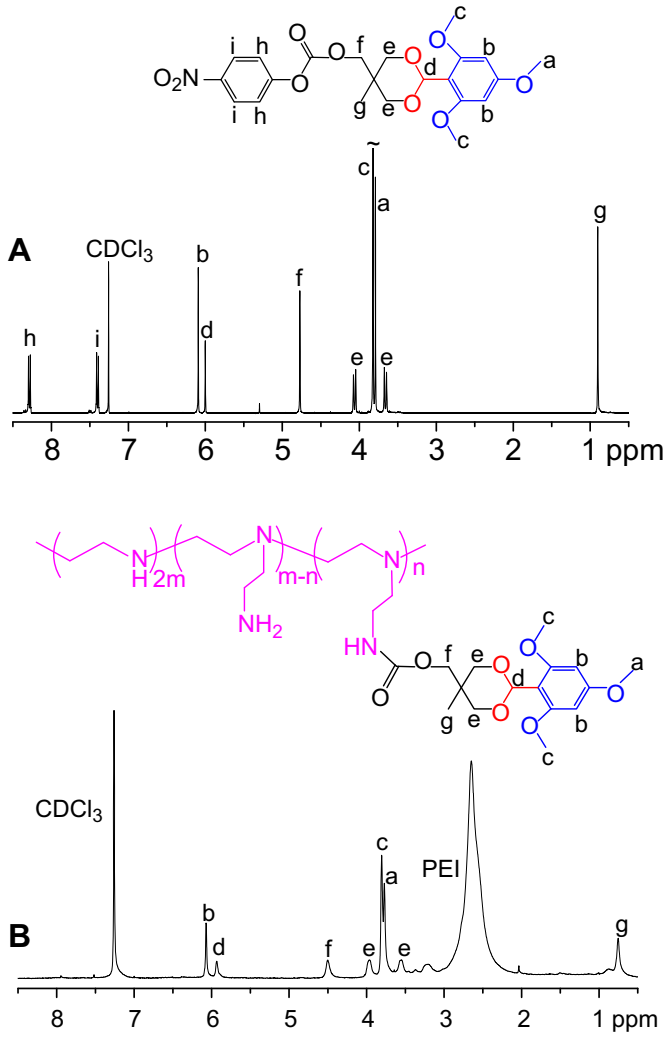


Fig. 1. ^{1}H NMR spectra (400 MHz, $CDCl_3$) of TMB-THME-NC (A) and PEI-*g*-(TMB-THME)$_9$ (Table 1, Entry 2) (B).

3.2. Formation of DNA polyplexes

The plasmid DNA condensation abilities of PEI- *g*-(TMB-THME)$_n$ conjugates were studied using gel electrophoresis, dynamic light scattering (DLS) and zeta potential measurements. Gel retardation assays revealed that all PEI-*g*-(TMB-THME)$_n$ conjugates, along with 10 kDa PEI, were able to completely inhibit DNA migration at an N/P ratio of 5/1 (Fig. 2). DLS results showed that these PEI-*g*-(TMB-THME)$_n$ derivatives condensed DNA into nano-sized particles (89–160 nm) when N/$P \geq$ 10/1, with polydispersities (PDI) ranging from 0.20 to 0.27 (Fig. 3A). The sizes of PEI-*g*-(TMB-THME)$_n$ complexes decreased with increasing N/P ratios from 5/1 to 40/1. Notably, PEI-*g*-(TMB-THME)$_n$ conjugates afforded in general smaller particles than 10 kDa PEI at the same N/P ratios (e.g. 101–124 nm *versus* 162 nm at an N/P ratio of 20/1). The sizes of polyplexes, however, increased with increasing grafting extents of TMB-THME (Fig. 3A). This might be due to that TMB-THME modification of PEI on one hand enhances PEI–PEI and/or PEI-DNA assembly via hydrophobic interactions and on the other hand decreases PEI-DNA ionic interactions as a result of depleted primary amines in PEI and/or increases steric hindrance owing to bulky and rigid nature of TMB-THME. Most likely, at low degree of TMB-THME modification ($n = 5$), increase of hydrophobic interactions dominates steric hindrance and decrease of charge interactions, thereby giving rise to significantly smaller polyplexes as compared to the parent 10 kDa PEI. However, at higher levels of TMB-THME modification, steric hindrance and decrease of charge interactions becomes more pronounced. Interestingly, PEI-*g*-(TMB-THME)$_n$ polyplexes showed also better colloidal stability under 10% serum conditions than the unmodified 10 kDa PEI control (Fig. S4).

Notably, zeta potential measurements revealed that polyplexes of PEI-*g*-(TMB-THME)$_n$ had higher positive charges as compared to those of the parent 10 kDa PEI (Fig. 3B). For example, surface charges of +21.5 mV and +27.9–32.8 mV were observed for polyplexes of 10 kDa PEI and PEI-*g*-(TMB-THME)$_n$ formed at an N/P ratio of 20/1, respectively. The surface charges of PEI-*g*-(TMB-THME)$_n$ polyplexes increased with increasing N/P ratios from 5/1 to 40/1

Table 1
Characteristics of acid-degradable PEI-*g*-(TMB-THME)$_n$ conjugates.

Entry	PEI conjugates	$N_{TMB\text{-}THME}$[a] (design)	$N_{TMB\text{-}THME}$[b] (^{1}H NMR)	buffer capacity[c] (%)
1	PEI-*g*-(TMB-THME)$_5$	9	5	13.9
2	PEI-*g*-(TMB-THME)$_9$	12	9	13.7
3	PEI-*g*-(TMB-THME)$_{14}$	17	14	13.0
4	10 kDa PEI	–	–	13.5
5	25 kDa PEI	–	–	13.9

[a] Designed number of TMB-THME moieties per PEI molecule.
[b] Average number of TMB-THME moieties per PEI molecule determined by ^{1}H NMR.
[c] Determined by acid-base titration.

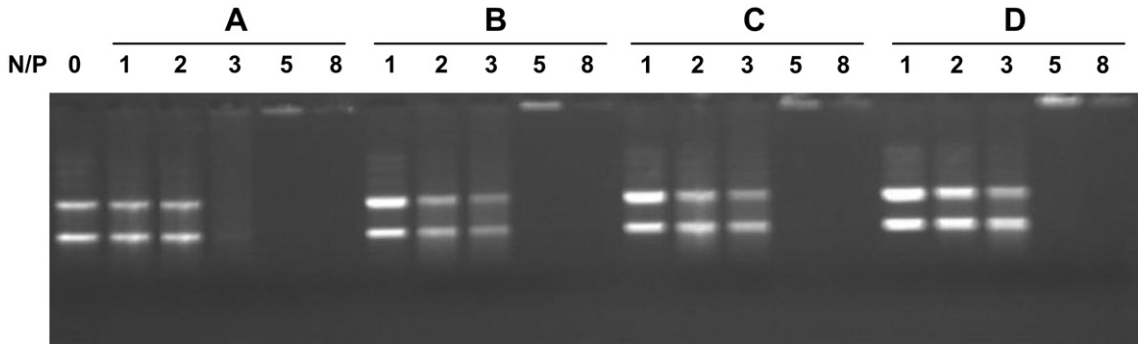


Fig. 2. Agarose gel electrophoresis of polymer/DNA complexes prepared at different N/P ratios ranging from 1/1 to 8/1. (A) 10 kDa PEI; (B) PEI-*g*-(TMB-THME)$_5$; (C) PEI-*g*-(TMB-THME)$_9$; and (D) PEI-*g*-(TMB-THME)$_{14}$.

and grafting extents of TMB-THME (Fig. 3B). The fact that PEI-*g*-(TMB-THME)$_n$ could condense DNA into particles with considerably smaller sizes and higher surface charges as compared to the parent 10 kDa PEI is significant, as effective DNA condensation is a primary requirement for efficient transfection. The better DNA condensation ability of PEI-*g*-(TMB-THME)$_n$ over unmodified PEI is likely due to its largely increased surface charge density resulting from micellization. We reported recently that cationic micelles based on low molecular weight PDMAEMA-PCL-PDMAEMA triblock copolymers are able to complex even with siRNA [44].

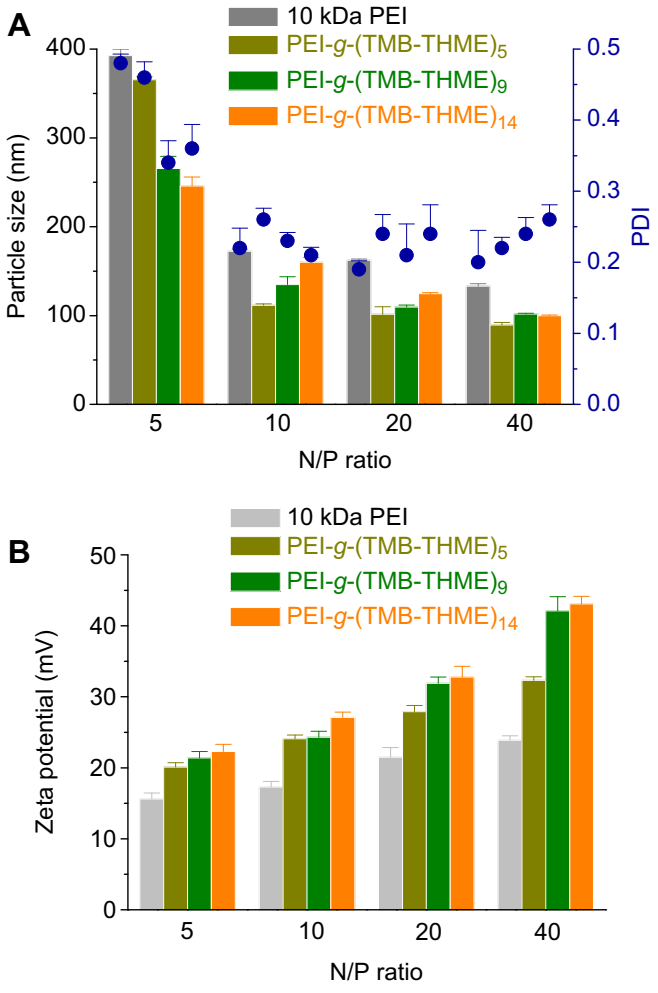


Fig. 3. Average particle size and polydispersity (PDI) (A) and zeta potential (B) of PEI-*g*-(TMB-THME)$_n$/DNA polyplexes prepared at different N/P ratios ranging from 5/1 to 40/1 in HEPES buffer (20 mM, pH 7.4).

3.3. Acid-triggered acetal degradation and Unpacking of polyplexes

The hydrolysis of the acetals in PEI-*g*-(TMB-THME)$_9$/DNA complexes prepared at an N/P ratio of 10/1 was investigated at different pH values (i.e. pH 7.4, 6.0, 5.0 and 4.0). The extent of acetal hydrolysis was conveniently determined using UV/vis spectroscopy by monitoring the absorbance at 290 nm, which is the characteristic absorbance of the hydrolysis product, 2,4,6-trimethoxybenzaldehyde [40,41]. The results showed that the hydrolysis rate of the acetals of PEI-*g*-(TMB-THME)$_9$ polyplexes was highly pH dependent (Fig. 4). While negligible hydrolysis was observed after 24 h at pH 7.4 (<12%), rapid hydrolysis took place at pH 4.0, 5.0 and 6.0, with corresponding half lives of 1.3, 2.8 and 11 h. Within 5 h, approximately 96%, 75%, 33%, and 10% of acetals were hydrolyzed at pH 4.0, 5.0, 6.0 and 7.4, respectively. Interestingly, similar acetals in micelles and polymersomes were shown to degrade much slower at mildly acidic pH of 5.0 and 6.0 [42,43]. The greater pH-sensitivity of the acetals in the PEI-*g*-(TMB-THME)$_n$ polyplexes is most likely due to their easy access of acids.

In the following, we investigated the influence of acetal degradation on DNA release from PEI-*g*-(TMB-THME)$_n$ polyplexes. Gel electrophoresis assays showed that polyplexes of PEI-*g*-(TMB-THME)$_n$ prepared at an N/P ratio of 10/1 following 5 h incubation at

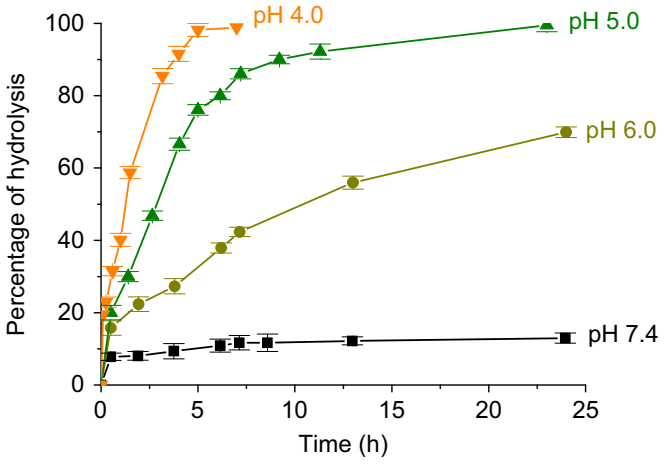


Fig. 4. pH-Dependent hydrolysis of acetals in PEI-*g*-(TMB-THME)$_9$/DNA complexes (N/P ratio = 10/1).

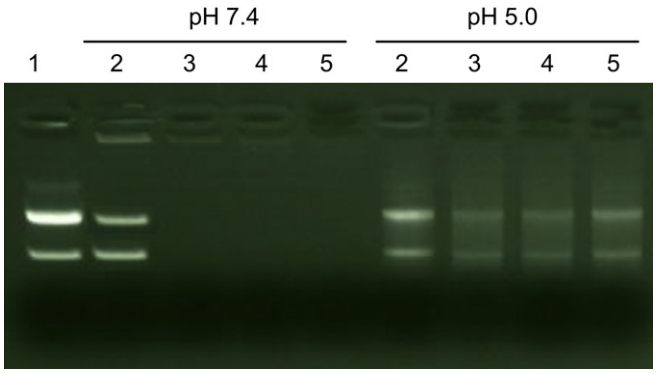


Fig. 5. Agarose gel electrophoresis of PEI-*g*-(TMB-THME)$_n$/DNA complexes (N/P ratio = 10/1) following 5 h incubation at pH 7.4 and 5.0. Lane 1: free DNA; Lane 2: 10 kDa PEI; Lane 3: PEI-*g*-(TMB-THME)$_5$; Lane 4: PEI-*g*-(TMB-THME)$_9$; Lane 5: PEI-*g*-(TMB-THME)$_{14}$. Lanes 2–5 were added with negatively charged DSS at a sodium sulfate/DNA phosphate ratio of 40/1.

pH 7.4 were stable against exchange with 40-fold excess of negatively charged dextran sodium sulfate (DSS) relative to DNA phosphate groups, demonstrating high stability of PEI-*g*-(TMB-THME)$_n$ polyplexes at neutral pH (Fig. 5). In contrast, release of DNA was detected for 10 kDa PEI control under otherwise the same conditions, which further confirms that hydrophobic modification of 10 kDa PEI results in more stable DNA complexes. Interestingly, release of DNA was observed for all three PEI-*g*-(TMB-THME)$_n$ polyplexes following 5 h incubation at pH 5.0 under otherwise the same conditions (Fig. 5), supporting our hypothesis that reversal of hydrophobic modification at mildly acidic pH would facilitate DNA release.

The change in particle sizes and surface charges of PEI-*g*-(TMB-THME)$_9$ polyplexes prepared at an N/P ratio of 10/1 following incubation at pH 5.0 and 7.4 was monitored in time by DLS and zeta potential measurements. Notably, the sizes of polyplexes increased from ca. 130 nm to over 800 nm after 3 h incubation at pH 5.0, while only slight increase of particle sizes was observed for polyplexes incubated at pH 7.4 (Fig. 6A). Accordingly, PEI-*g*-(TMB-THME)$_9$ polyplexes showed significant increase of PDI (>0.7) following 8 h incubation at pH 5.0 (Fig. 6B). In line with particle size changes, zeta

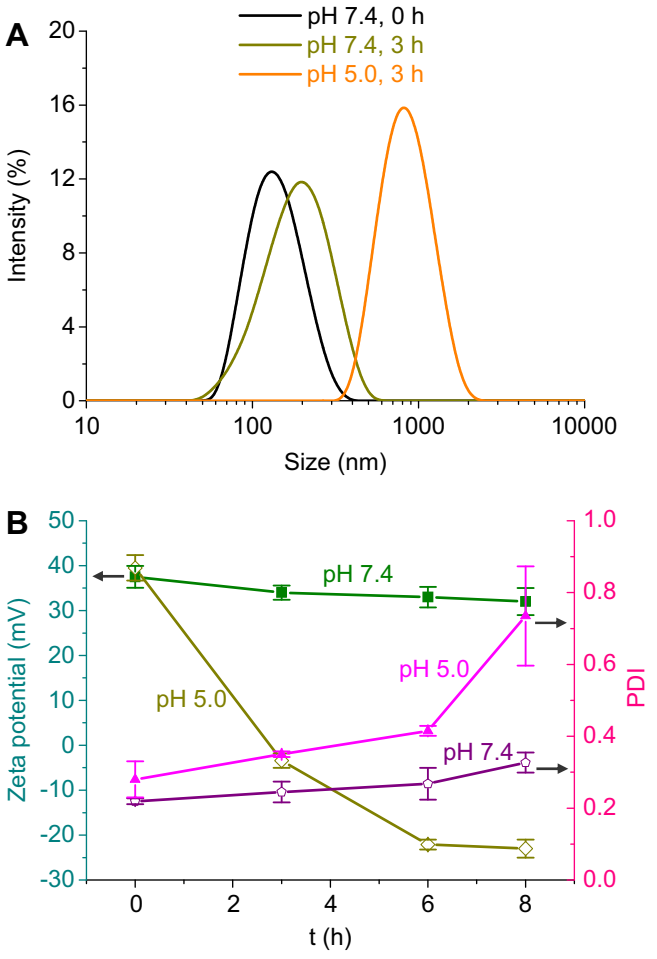


Fig. 6. Change of average particle size (A) and zeta potential and PDI (B) of PEI-*g*-(TMB-THME)$_9$/DNA polyplexes (N/P ratio = 10/1) over incubation time at pH 5.0 and 7.4.

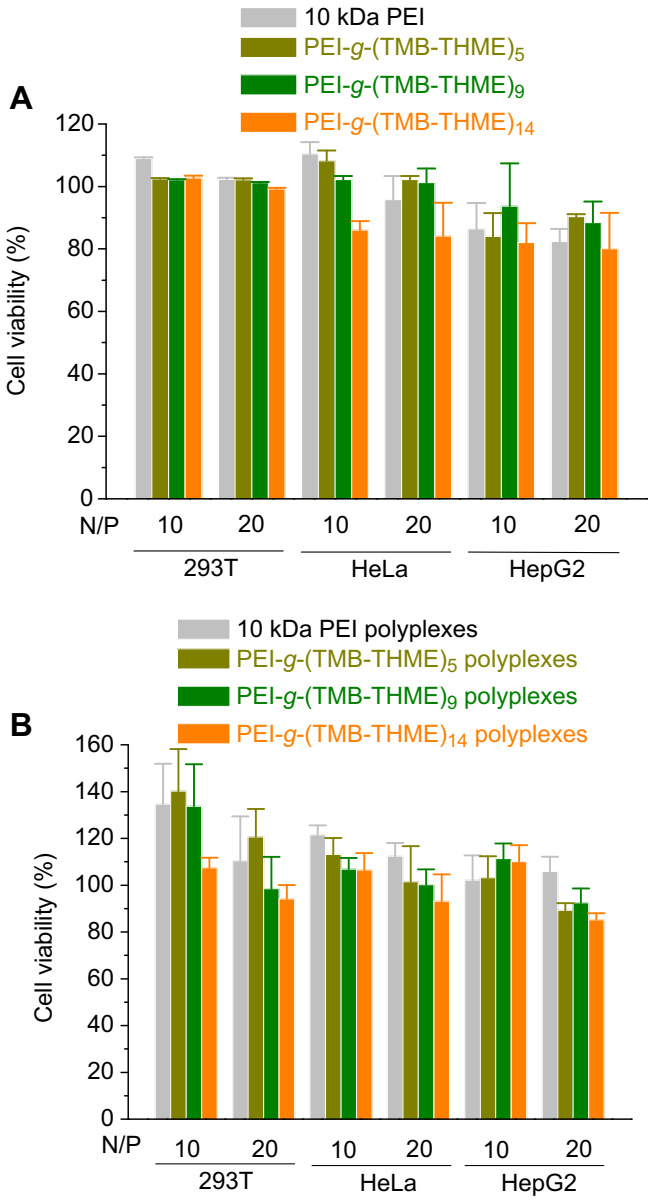


Fig. 7. Cytotoxicity of PEI-*g*-(TMB-THME)$_n$ conjugates and polyplexes in 293T, HeLa and HepG2 cells determined by MTT assays ($n = 5$). (A) PEI-*g*-(TMB-THME)$_n$ at polymer concentrations corresponding to N/P ratios of 10/1 and 20/1 in the transfection experiments; and (B) PEI-*g*-(TMB-THME)$_n$ polyplexes formed at N/P ratios of 10/1 and 20/1.

potential measurements revealed that PEI-*g*-(TMB-THME)$_9$ polyplexes turned into negative charges after 3 h incubation at pH 5.0 and decreased to over −20 mV after 6 h (Fig. 6B). In comparison, little change of zeta potentials was observed at pH 7.4 even after 8 h incubation. It is evident from these results that TMB-THME modified 10 kDa PEI forms smart DNA complexes that are stable at pH 7.4 but are rapidly dissociated to release DNA under a mildly acidic condition mimicking that of endosomes due to hydrolysis of acetals with reversal of hydrophobilization.

3.4. Cytotoxicity and in vitro gene transfection

The cytotoxicity of PEI-*g*-(TMB-THME)$_n$ conjugates as well as PEI-*g*-(TMB-THME)$_n$ polyplexes was investigated in 293T, HeLa and HepG2 cells by MTT assays. Notably, all three PEI-*g*-(TMB-THME)$_n$ derivatives, along with 10 kDa PEI, exhibited low cytotoxicities to 293T, HeLa and HepG2 cells (cell viability > 80%) at polymer concentrations corresponding to N/P ratios of 10/1 and 20/1 applied in the following transfection experiments (Fig. 7A). Moreover, low cytotoxicities (cell viability > 85%) were also observed for PEI-*g*-(TMB-THME)$_n$ polyplexes formed at N/P ratios of 10/1 and 20/1 (Fig. 7B).

The *in vitro* transfection activity of PEI-*g*-(TMB-THME)$_n$ was evaluated in HeLa, 293T, HepG2 and KB cells using pGL3 as a reporter gene at N/P ratios of 10/1 and 20/1. Interestingly, polyplexes of all three reversibly hydrophobilized PEIs showed markedly enhanced transfection efficiencies in HeLa cells as compared to those of the parent 10 kDa PEI in serum-free medium (Fig. 8A). For example, polyplexes of PEI-*g*-(TMB-THME)$_5$, PEI-*g*-(TMB-THME)$_9$, and PEI-*g*-(TMB-THME)$_{14}$ formed at an N/P ratio of 20/1 mediated 135-fold, 104-fold and 235-fold higher levels of gene expression as compared to those of 10 kDa PEI, respectively, which were approximately 3-fold, 2.5-fold and 7-fold higher than 25 kDa PEI formulation at its optimal N/P ratio under otherwise the same conditions (Fig. 8A). Under 10% serum conditions, PEI-*g*-(TMB-THME)$_{14}$/DNA complexes at an N/P ratio of 10/1 displayed 175-fold enhancement of gene transfection efficiency relative to 10 kDa PEI formulation, which exceeded 16 times that of 25 kDa PEI (Fig. 8B). It should be noted that this remarkable enhancement of transfection activity of PEI is unprecedented. Klibanov reported significant improvement of transfection activity of 2 kDa PEI via dodecylation to a level of 5-fold that of 25 kDa PEI [31]. While, most other studies showed that modification of low molecular weight PEIs affords

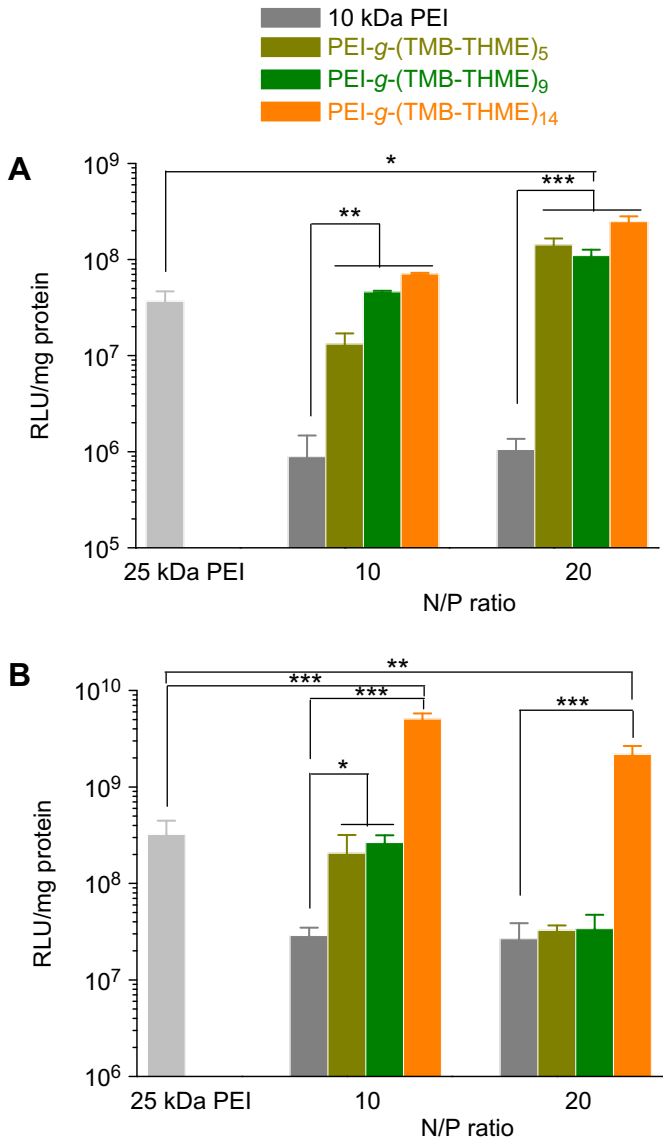


Fig. 8. Transfection efficiencies of PEI-*g*-(TMB-THME)$_n$ polyplexes in HeLa cells at N/P ratios of 10/1 and 20/1 in serum-free medium (A) and 10% serum-containing medium (B). 25 kDa PEI formulation at its optimal N/P ratio of 10/1 and 10 kDa PEI formulations at N/P ratios of 10/1 and 20/1 were used as controls (Student's *t*-test, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$).

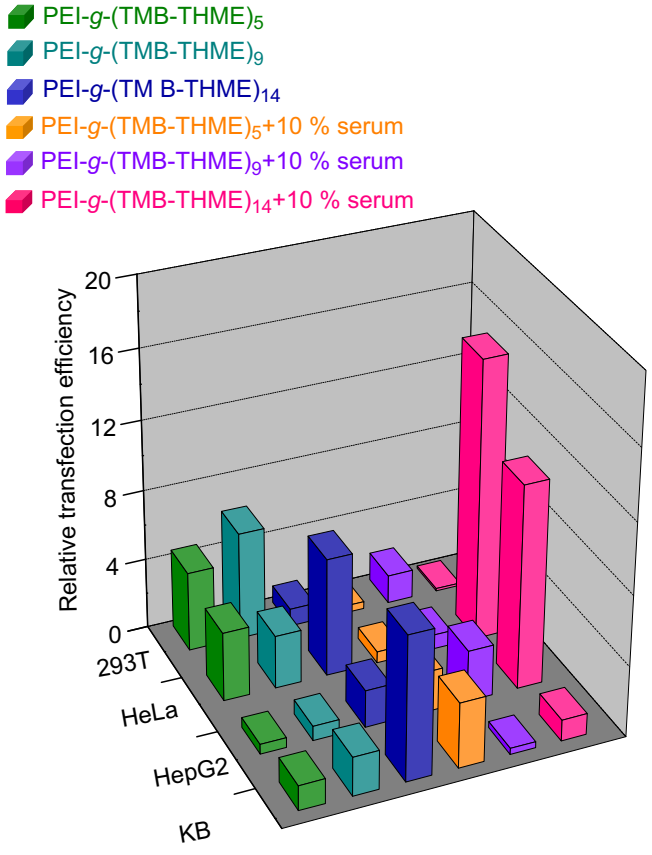


Fig. 9. Comparative transfection of polyplexes based on PEI-*g*-(TMB-THME)$_n$. Transfection experiments were performed using 293T, HeLa, HepG2 and KB cells in the presence or absence of 10% serum. Transfection efficiency was normalized to that of 25 kDa PEI polyplexes at an N/P ratio of 10/1 under corresponding transfection conditions. The data given are average values of three measurements.

transfection efficiency slightly higher than or comparable to that of 25 kDa PEI [28,30,32].

The transfection activity of cationic polymers is often dependent on their molecular structure, transfection medium and cell line. Fig. 9 shows the normalized transfection efficiencies of PEI-*g*-(TMB-THME)$_n$ polyplexes in four different cell lines. Notably, high transfection efficiencies were also observed in 293T, HepG2 and KB cells. For example, PEI-*g*-(TMB-THME)$_5$ and PEI-*g*-(TMB-THME)$_9$ mediated approximately 4.5 and 6 times higher levels of gene expression than 25 kDa PEI, respectively, in 293T cells in the absence of serum. PEI-*g*-(TMB-THME)$_{14}$ afforded 8-fold higher transfection in KB cells in the absence of serum and 12-fold higher transfection in HepG2 cells in the presence of 10% serum than corresponding 25 kDa PEI controls. Despite its slightly lower buffer capacity, PEI-*g*-(TMB-THME)$_{14}$ demonstrated the best transfection activity in HeLa, HepG2 and KB cells, confirming the delicate effects of hydrophobic modification (from DNA condensation, cellular uptake, to DNA release inside cells) on gene transfection.

In the following, the cellular uptake and intracellular DNA release behaviors of PEI-*g*-(TMB-THME)$_{14}$ polyplexes formed at an N/P ratio of 10/1 were studied in HeLa cells with CLSM using Cy5-labeled DNA (Fig. 10). The nuclei were stained with Hoechst 33342 (blue). Interestingly, considerable amount of DNA (red) has been delivered to the perinuclear region of HeLa cells following 4 h transfection with polyplexes of PEI-*g*-(TMB-THME)$_{14}$ (Fig. 10A). Much more DNA was observed inside cells when increasing transfection time to 8 h (Fig. 10B). It is important to note that significant amount of DNA was colocalized within the nuclei of HeLa cells (Fig. 10B), supporting efficient DNA delivery to the cell nuclei. In comparison, negligible DNA was observed in HeLa cells

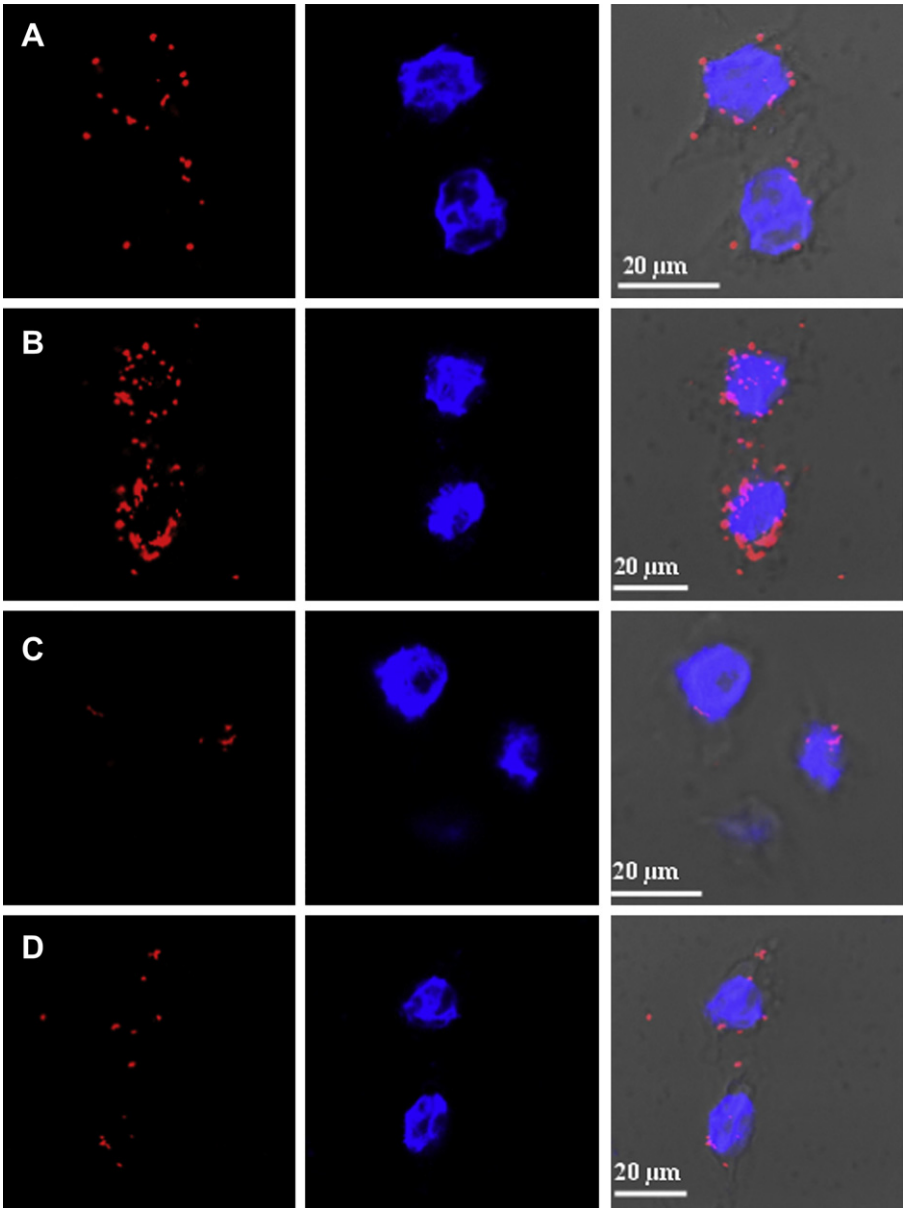


Fig. 10. CLSM images of HeLa cells transfected with Cy5-labeled pDNA polyplexes of PEI-*g*-(TMB-THME)$_{14}$ at an N/P ratio of 10/1 in the presence of 10% serum (1 μg DNA/well). 10 kDa PEI and 25 kDa PEI polyplexes at an N/P ratio of 10/1 were used as controls. Cells were incubated with Cy5-labeled pDNA polyplexes for 4 or 8 h. For each panel, images from left to right show Cy5-labeled pDNA (red), cell nuclei stained by Hoechst 33342 (blue), and overlays of two images. The bar represents 20 μm (A) PEI-*g*-(TMB-THME)$_9$ polyplexes, 4 h; (B) PEI-*g*-(TMB-THME)$_9$ polyplexes, 8 h; (C) 10 kDa PEI polyplexes, 8 h; (D) 25 kDa PEI polyplexes, 8 h. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

following 8 h transfection with the unmodified 10 kDa PEI polyplexes (Fig. 10C). The cells transfected with 25 kDa PEI control showed stronger DNA fluorescence than those with 10 kDa PEI, but far less DNA fluorescence as compared to PEI-*g*-(TMB-THME)$_{14}$ polyplexes (Fig. 10D). These results confirm that PEI-*g*-(TMB-THME)$_{14}$ mediates superior intracellular DNA delivery to 25 kDa PEI.

The markedly enhanced transfection activity of PEI-*g*-(TMB-THME)$_n$ conjugates is most likely because they have uniquely satisfied two contradictory requirements, *i.e.* adequate DNA protection in the extracellular conditions and efficient release of DNA under the intracellular environments. The balance of DNA protection and release has been a long challenge for non-viral gene transfection [6,34]. Lack of an active DNA release mechanism inside cells is one of the major barriers accounted for the low transfection efficiencies of many current systems [2,12,45]. The reversible hydrophobilization has appeared to be an appealing approach to elegantly resolve the stability dilemma of polymeric gene delivery systems. Furthermore, it should be noted that reversal of hydrophobic modification in endosomes also yields hydrophilically modified PEIs with reduced cytotoxicity.

4. Conclusions

We have demonstrated that reversible hydrophobic modification of 10 kDa PEI results in highly potent yet nontoxic gene transfer vectors. The reversible hydrophobilization has elegantly resolved the stability dilemma of cationic polymer-based gene delivery systems in that hydrophobic modification enhances polyplex stability in extracellular environments while reversal of hydrophobic modification in endosomes via acid-triggered acetal degradation causes rapid de-stabilization of polyplexes to actively release DNA inside cells. Lack of active DNA release mechanism in cells is a general barrier for efficient non-viral gene transfection. Moreover, reversal of hydrophobic modification inside cells leads to also reduced cytotoxicity. It should further be noted that this hydrophobic modification approach is straightforward and yields PEI-based vectors with well-defined structures and controlled extents of TMB-THME substitution. These reversibly hydrophobilized PEI vectors are highly promising for safe and efficient non-viral gene transfection.

Acknowledgments

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Appendix. Supplementary information

Supplementary data related to this article can be found online at doi:10.1016/j.biomaterials.2011.08.017.

References

[1] Luo D, Saltzman WM. Synthetic DNA delivery systems. Nat Biotechnol 2000; 18:33–7.
[2] Park TG, Jeong JH, Kim SW. Current status of polymeric gene delivery systems. Adv Drug Deliv Rev 2006;58:467–86.
[3] Pack DW, Hoffman AS, Pun S, Stayton PS. Design and development of polymers for gene delivery. Nat Rev Drug Discov 2005;4:581–93.
[4] De Laporte L, Rea JC, Shea LD. Design of modular non-viral gene therapy vectors. Biomaterials 2006;27:947–54.
[5] Jeong JH, Kim SW, Park TG. Molecular design of functional polymers for gene therapy. Prog Polym Sci 2007;32:1239–74.
[6] Schaffert D, Wagner E. Gene therapy progress and prospects: synthetic polymer-based systems. Gene Ther 2008;15:1131–8.
[7] Kircheis R, Wightman L, Robitza B, Ogris M, Brunner S, Wagner E. Polymer-based systems for tumor-targeted gene delivery. Cancer Gene Ther 2000;7: 1206.
[8] Demeneix B, Hassani Z, Behr JP. Towards multifunctional synthetic vectors. Curr Gene Ther 2004;4:445–55.
[9] Morille M, Passirani C, Vonarbourg A, Clavreul A, Benoit JP. Progress in developing cationic vectors for non-viral systemic gene therapy against cancer. Biomaterials 2008;29:3477–96.
[10] Kim WJ, Yockman JW, Jeong JH, Christensen LV, Lee M, Kim YH, et al. Anti-angiogenic inhibition of tumor growth by systemic delivery of PEI-*g*-PEG-RGD/pCMV-sFlt-1 complexes in tumor-bearing mice. J Control Release 2006; 114:381–8.
[11] Wong SY, Pelet JM, Putnam D. Polymer systems for gene delivery-past, present, and future. Prog Polym Sci 2007;32:799–837.
[12] Mintzer MA, Simanek EE. Nonviral vectors for gene delivery. Chem Rev 2009; 109:259–302.
[13] Boussif O, Lezoualch 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: 7297–301.
[14] Abdallah B, Hassan A, Benoist C, Goula D, Behr JP, Demeneix BA. A powerful nonviral vector for in vivo gene transfer into the adult mammalian brain: polyethylenimine. Hum Gene Ther 1996;7:1947–54.
[15] Akinc A, Thomas M, Klibanov AM, Langer R. Exploring polyethylenimine-mediated DNA transfection and the proton sponge hypothesis. J Gene Med 2005;7:657–63.
[16] Godbey WT, Wu KK, Mikos AG. Size matters: molecular weight affects the efficiency of poly(ethylenimine) as a gene delivery vehicle. J Biomed Mater Res 1999;45:268–75.
[17] Lungwitz U, Breunig M, Blunk T, Gopferich A. Polyethylenimine-based non-viral gene delivery systems. Eur J Pharm Biopharm 2005;60:247–66.
[18] Russ V, Elfberg H, Thoma C, Kloeckner J, Ogris M, Wagner E. Novel degradable oligoethylenimine acrylate ester-based pseudodendrimers for in vitro and in vivo gene transfer. Gene Ther 2008;15:18–29.
[19] Petersen H, Merdan T, Kunath F, Fischer D, Kissel T. Poly(ethylenimine-co-L-lactamide-co-succinamide): a biodegradable polyethylenimine derivative with an advantageous pH-dependent hydrolytic degradation for gene delivery. Bioconjug Chem 2002;13:812–21.
[20] Jeong JH, Christensen LV, Yockman JW, Zhong ZY, Engbersen JFJ, Kim WJ, et al. Reducible poly(amido ethylenimine) directed to enhance RNA interference. Biomaterials 2007;28:1912–7.
[21] Liu J, Jiang XL, Xu L, Wang XM, Hennink WE, Zhuo RX. Novel reduction-responsive cross-linked polyethylenimine derivatives by click chemistry for nonviral gene delivery. Bioconjug Chem 2010;21:1827–35.
[22] Peng Q, Zhong ZL, Zhuo RX. Disulfide cross-linked polyethylenimines (PEI) prepared via thiolation of low molecular weight PEI as highly efficient gene vectors. Bioconjug Chem 2008;19:499–506.
[23] Breunig M, Lungwitz U, Liebl R, Goepferich A. Breaking up the correlation between efficacy and toxicity for nonviral gene delivery. Proc Natl Acad Sci USA 2007;104:14454–9.
[24] Thomas M, Ge Q, Lu JJ, Chen JZ, Klibanov AM. Cross-linked small polyethylenimines: while still nontoxic, deliver DNA efficiently to mammalian cells in vitro and in vivo. Pharm Res 2005;22:373–80.
[25] Wang Y, Chen P, Shen J. The development and characterization of a glutathione-sensitive cross-linked polyethylenimine gene vector. Biomaterials 2006;27:5292–8.
[26] Luten J, van Nostruin CF, De Smedt SC, Hennink WE. Biodegradable polymers as non-viral carriers for plasmid DNA delivery. J Control Release 2008;126: 97–110.
[27] Liu ZH, Zhang ZY, Zhou CR, Jiao YP. Hydrophobic modifications of cationic polymers for gene delivery. Prog Polym Sci 2010;35:1144–62.
[28] Incani V, Lavasanifar A, Uludag H. Lipid and hydrophobic modification of cationic carriers on route to superior gene vectors. Soft Matter 2010;6: 2124–38.
[29] Lee M, Rentz J, Han SO, Bull DA, Kim SW. Water-soluble lipopolymer as an efficient carrier for gene delivery to myocardium. Gene Ther 2003;10:585–93.
[30] Han SO, Mahato RI, Kim SW. Water-soluble lipopolymer for gene delivery. Bioconjug Chem 2001;12:337–45.
[31] Thomas M, Klibanov AM. Enhancing polyethylenimine's delivery of plasmid DNA into mammalian cells. Proc Natl Acad Sci USA 2002;99:14640–5.
[32] Neamnark A, Suwantong O, Bahadur KCR, Hsu CYM, Supaphol P, Uludag H. Aliphatic lipid substitution on 2 kDa polyethylenimine improves plasmid delivery and transgene expression. Mol Pharm 2009;6: 1798–815.
[33] Dehshahri A, Oskuee RK, Shier WT, Hatefi A, Ramezani M. Gene transfer efficiency of high primary amine content, hydrophobic, alkyl-oligoamine derivatives of polyethylenimine. Biomaterials 2009;30:4187–94.
[34] Grigsby CL, Leong KW. Balancing protection and release of DNA: tools to address a bottleneck of non-viral gene delivery. J Roy Soc Interface 2010;7: S67–82.
[35] von Harpe A, Petersen H, Li YX, Kissel T. Characterization of commercially available and synthesized polyethylenimines for gene delivery. J Control Release 2000;69:309–22.

[36] Lechardeur D, Verkman AS, Lukacs GL. Intracellular routing of plasmid DNA during non-viral gene transfer. Adv Drug Deliv Rev 2005;57:755–67.
[37] Ruponen M, Honkakoski P, Ronkko S, Pelkonen J, Tammi M, Urtti A. Extracellular and intracellular barriers in non-viral gene delivery. J Control Release 2003;93:213–7.
[38] Cohen JA, Beaudette TT, Cohen JL, Brooders KE, Bachelder EM, Frechet JMJ. Acetal-modified dextran microparticles with controlled degradation kinetics and surface functionality for gene delivery in phagocytic and non-phagocytic cells. Adv Mater 2010;22:3593–7.
[39] Broaders KE, Cohen JA, Beaudette TT, Bachelder EM, Frechet JMJ. Acetalated dextran is a chemically and biologically tunable material for particulate immunotherapy. Proc Natl Acad Sci USA 2009;106:5497–502.
[40] Gillies ER, Jonsson TB, Frechet JMJ. Stimuli-responsive supramolecular assemblies of linear-dendritic copolymers. J Am Chem Soc 2004;126:11936–43.
[41] Gillies ER, Frechet JMJ. pH-responsive copolymer assemblies for controlled release of doxorubicin. Bioconjug Chem 2005;16:361–8.
[42] Chen W, Meng FH, Cheng R, Zhong ZY. pH-Sensitive degradable polymersomes for triggered release of anticancer drugs: a comparative study with micelles. J Control Release 2010;142:40–6.
[43] Chen W, Meng F, Li F, Ji S-J, Zhong Z. pH-responsive biodegradable micelles based on acid-labile polycarbonate hydrophobe: synthesis and triggered drug release. Biomacromolecules 2009;10:1727–35.
[44] Zhu CH, Jung S, Luo SB, Meng FH, Zhu XL, Park TG, et al. Co-delivery of siRNA and paclitaxel into cancer cells by biodegradable cationic micelles based on PDMAEMA-PCL-PDMAEMA triblock copolymers. Biomaterials 2010;31:2408–16.
[45] Mastrobattista E, van der Aa M, Hennink WE, Crommelin DJA. Artificial viruses: a nanotechnological approach to gene delivery. Nat Rev Drug Discov 2006;5:115–21.