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PAPER

Extraordinarily enhanced gene transfection and cellular uptake by aromatic hydrophobicization to PEI25K

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Different from commonly used alkylation strategies, PEI25K was modified with rigid, highly hydrophobic aromatic moieties to develop superior gene vectors with multiple functions, including structural compatibility with the cell membrane as well as cooperative contribution of electrostatic and hydrophobic interactions to the transfer process. A facile preparation approach was proposed by directly reacting PEI25K with 5-benzyloxyl trimethylene carbonate (BTMC) while omitting reagent activation and catalyst aid. The hydrophobic interactions between PEI-BTMC molecules serves as hydrophobic "locks" to stabilize polyplexes. PEI-BTMC polyplexes were kept good stability in the presence of heparin and DNase. The transfections mediated by PEI-BTMC vectors were better than PEI25K control in different cell lines. Particularly in HeLa cells, such enhancement owing to BTMC attachment can reach even up to 150 times. Experimental data indicated that the highly enhanced transfection mediated by PEI-BTMC was possibly more dependent on the special functions caused by BTMC modification rather than the improved cell-biocompatibility. Confocal laser scanning microscopy (CLSM) studies revealed the considerably higher potency of PEI-BTMC in transporting DNA into HeLa cells in comparison with PEI25K. It is expected that useful information provided in the current study would prompt the advance of PEI-based gene vectors towards practical applications.

Introduction

Design of safe and efficient gene vectors has been an urgent task in the development of gene therapy. In the last decade, polycation vectors have attracted great interest in light of their prominent advantages including large payload, high tolerance towards gene sizes and low immunogenic risk.[1,2] Branched polyethylenimine with a molar mass of 25 kDa (PEI25K), the most representative polycation vector, has demonstrated exciting efficacy in gene delivery though relatively lower than that of viral vectors.[1–4] The high efficacy greatly depends upon its excellent capability in condensing DNA into positively charged nanoparticles, so as to protect DNA from premature degradation and easily penetrate the negatively charged cell membrane.[5,6] However, the high toxicity of PEI25K caused by the high charge density raises great safety concerns, which is the main hurdle against its application in human therapy trials.[7–11]

With increasing understanding about the mechanism of polycation-mediated transfection, a wide variety of safer polycation vectors with versatile structures have been developed in search of superior alternatives to PEI25K. Nevertheless, those delicate designs did not lead to transfection promotion to a significant level yet. In addition, the preparation methods were generally associated with complex chemistry, resulting in considerable difficulties to prepare compositionally and architecturally consistent products.[5,11–13] This issue would adversely affect the therapy performance and sometimes lead to high biotoxicity. Therefore, translating these vehicles into practical applications still remains a great challenge. Provided that the apparent improvements in transfection efficacy and safety profile become realized, to some degree direct modification to PEI25K may be a preferable strategy to develop clinically applicable vectors due to the much easier control over the preparation. Theoretically, this goal may be accessible given the consideration of the inherently high transfection of PEI25K. To date, the relevant studies were intensively directed at biocompatibility improvement *via* partly screening the surface charge of PEI25K by attaching small molecules or polymers.[6,8,12,14] Overall, the transfection enhancement was limited despite the highly improved safety profile.

Gene delivery is a multistep process involving DNA condensation, cellular uptake, endosomal escape, DNA release into the cytoplasm, and transfer of DNA into the nucleus. Like electrostatic force, hydrophobic interaction is supposed to strongly

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affect some of those steps, especially the cell uptake and DNA condensation.[15–19] This opinion is supported by the findings that some uncharged and strongly hydrophobic particles were more readily captured by cell membranes than hydrophilic ones.[20–22] Available reports also suggested that for DNA/vector complexation, the amount of the cationic amphiphile needed to reach the charge inversion was strongly associated with the hydrophobicity of the amphiphile.[23] From the perspective of structural compatibility between cell membrane and vectors, the amphiphilic nature of hydrophobically modified PEI derivatives appeared to be advantageous to endocytosis through the hydrophobic lipid-based plasma membrane. The direct evidence is that lipid-containing transfer agents, INTERFERin and Lipofectamine 2000 were more efficient in delivering siRNA than jetPEI and Metafectene.[17] Taking those into consideration, there is a possibility that amphiphilic PEI derivatives modified with appropriate hydrophobic moieties may afford better transfection together with improved cytotoxicity in comparison with unmodified PEI featured as a highly charged and absolutely water-soluble polycation.

To the end, the transfection by PEI25K derivatives modified with various aliphatic alkyl groups has been intensively explored.[14,16,24–27] Nevertheless, the influence of alkylation on the transfection is still debatable. It is presumably associated with the relatively insufficient lipophilicity of shorter alkyl chains and/or the strong shrinkage tendency of the longer ones in aqueous media, which somewhat compromise the contribution of hydrophobic interactions. Though several rigid and more hydrophobic aromatic molecules such as cholesterol,[28] folate[29,30] and glucocorticoid ligand[31,32] have been conjugated to PEI, those designs were aimed at the introduction of specific biofunctions such as receptor-mediated targeting. Indeed, the aromatic modification towards PEI25K to provide stable and highly hydrophobic surface is rarely reported up to date.

Herein, we report a novel gene vector by coupling hydrophobic groups onto PEI25K surface *via* simple a one-step reaction without any reagent activation and catalyst aid. Specifically, PEI25K was reacted with 5-benzyloxyl trimethylene carbonate (BTMC), affording PEI-BTMC vectors with different compositions in a controlled manner (Scheme 1). An astonishing improvement of transfection activity up to 150 times that of PEI25K was achieved. The relationship between the surface density of BTMC moieties and the biological properties, such as transfection activity, intracellular uptake and cytotoxicity, was systematically explored. It is expected that useful information could be provided in the current study concerning the influence over the transfection of aromatic hydrophobicization towards PEI, and thus prompt the advance of PEI-based gene vectors for practical applications.

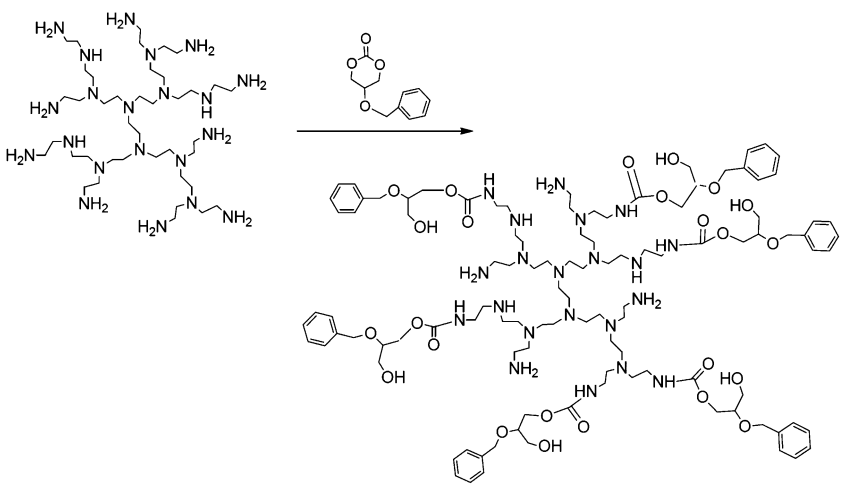
Experimental section

Materials

Branched PEI25K was purchased from Sigma-Aldrich. Dimethylsulfoxide (DMSO) was obtained from Shanghai Chemical Reagent Co. (China). 5-Benzyloxyl trimethylene carbonate (BTMC) was prepared according to ref. 33 and 34. QIAfilter™ plasmid purification Giga Kit (5) was purchased from Qiagen (Hilden, Germany) and GelRed™ was purchased from Biotium (CA, USA). Fetal bovine serum (FBS), Dulbecco's Modified Eagle's Medium (DMEM), Hoechst 33258 and Dulbecco's phosphate-buffered saline (PBS), penicillin–streptomycin, trypsin, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Invitrogen Corp. Micro BCA protein assay kit was obtained from Pierce. Other reagents were analytical grade and used as received.

Synthesis of BTMC-modified polyethylenimine (PEI-BTMC)

PEI-BTMCs were synthesized by direct aminolysis of BTMC in the presence of PEI25K. A typical preparation procedure of PEI-BTMC3 in Table 1 was described as follows: PEI25K (0.26 g, 0.0104 mmol) was dissolved in 2 mL of distilled DMSO, and then BTMC (0.13 g, 0.62 mmol) solution in DMSO (2 mL) was added. The reaction was carried out at 70 °C for 48 h with moderate shaking. The resulting solution was dialyzed (molecular weight cutoff 8000 Da) against water for 48 h. After filtration, the solution was frozen and lyophilized to provide the products.



Scheme 1 Illustration of the preparation and structure of PEI-BTMC.

Characterization of PEI-BTMC

Fourier-transformation infrared (FT-IR) spectra were recorded on a Perkin Elmer-2 spectrophotometer. Prior to the measurements, the samples were pressed into potassium bromide (KBr) pellets. Nuclear magnetic resonance (NMR) spectra of the samples were recorded on a Mercury VX-300 spectrometer at 300 Hz in CD_3OD at the concentrations of ~4% (w/v). The molecular weight distribution (M_w/M_n) of the PEI-BTMCs polymer was measured by size exclusion chromatography (SEC) equipped with a Waters 26 900 separations module and a Waters 2410 refractive index detector. Acetic acid–sodium acetate (pH = 2.9) buffer solution was used as the eluent at a flow rate of 1.0 mL min^{-1}.

The actual molecular weight of PEI25K was measured by the established SEC-MALLS method ($dn/dc = 0.268$) according to ref. 35. The M_w of the used PEI25K was determined to be 24 500 ($M_w/M_n = 1.577$), which was very close to the value of 25 000 g mol^{-1} provided by Sigma-Aldrich Corp. The concentration of BTMC attached onto PEI25K was determined according to the UV absorbance of polymer solution in mixed solvent ($THF : H_2O = 1 : 10$ (v/v)) (500 μg mL^{-1}). The standard calibration curve was experimentally obtained by reading the absorbance at 237 nm of the BTMC solution in mixed solvent ($THF : H_2O = 1 : 10$ (v/v)) with determined concentrations. Thus the actual molar ratio of BTMC to PEI within measured PEI-BTMC was calculated using following equation:

$$\text{molar ratio} = \frac{M_w \times C \times V}{F_w(m - C \times V)}$$

where C is the BTMC concentration in the measured solution; M_w and F_w is the molecular weight of PEI25K and formula weight of BTMC, respectively; V is the total volume of the solution; and m is the weight of the polymer added into the solution.

Cell culture

African Green Monkey SV40-transf'd kidney fibroblast cell line (COS7) and human cervix carcinoma (HeLa) cells were incubated with DMEM containing 1% antibiotics (penicillin–streptomycin, 10 000 U mL^{-1}) and 10% FBS at 37 °C in a humidified atmosphere containing 5% CO_2.

Agarose gel retardation assay

PEI-BTMCs/pDNA complexes at different w/w ratios were prepared by adding appropriate volumes of PEI-BTMC solution (in 150 mM NaCl solution) to 0.1 μg of pGL-3. The obtained complex solutions were diluted by 150 mM NaCl solution to the total volume of 8 μL and vortexed for 30 s before being incubated at 37 °C for 0.5 h. Then 1 μL GelRed™ was added to complexes respectively prior to electrophoresis on 0.7% (w/v) agarose gel which was put in Tris–acetate (TAE) running buffer at 80 V for 1 h. Finally the complexes were observed by an UV lamp with a Vilber Lourmat imaging system (France).

Amplification and purification of plasmid DNA

pGL-3 was used in the experiment. It was transformed as the luciferase reporter genes in *E. coli* JM109 and amplified in the broth media by acid–base titration at 37 °C overnight. The amplified plasmid was first purified by an EndoFree QiAfilter™ Plasmid Giga Kit (5) then dissolved in TE buffer solution and stored at −20 °C. Finally the purity and concentration of plasmids was determined by ultraviolet (UV) absorbance at 260–280 nm.

In vitro transfection of luciferase assay

Transfection of pGL-3 plasmids mediated by PEI-BTMCs in COS7, 293T, HepG2 and HeLa cells was studied and PEI25K was used as positive control. Cells were seeded at a density of 6×10^4 cells per well in the 24-well plate and incubated at 37 °C for 24 h respectively. The complexes at various w/w ratios ranging from 1 to 10 were prepared by adding 1 μg of pGL-3 DNA to appropriate volumes of PEI-BTMC solution (150 mM NaCl solution). The polyplex solutions were vortexed for 30 s and incubated at 37 °C for 0.5 h. The polyplex solutions were diluted to 1 mL by serum-free DMEM. The polyplex solutions were then added to the cell wells. After incubation for 4 h at 37 °C, the cells was washed to remove the remaining polyplexes and fed again by fresh DMEM containing 10% FBS and further incubated at 37 °C for 44 h. The luciferase assay conformed to manufacture's protocols. Relative light units (RLU) were measured by a chemiluminometer (Lumat LB9507, EG&G Berthold, Germany). The total protein was determined according to the BCA protein assay kit (Pierce). Luciferase activity was expressed as RLU mg^{-1} protein. Data were shown as mean ± standard deviation (SD) based on three independent measurements.

Confocal laser scanning microscopy

To study cellular uptake of complexes, fluorescent confocal microscopic images of complexes in HeLa cells were further obtained. HeLa cells were seeded at a density of 1×10^5 cells per well in the 24-well plate with 1 mL of DMEM containing 10% FBS and incubated at 37 °C for 24 h. 1 μg of pGL-3 were fluorescently labeled with 3 μL (1×10^{-5} M) YOYO-1 iodide at 37 °C for 15 min before the complexes at the determined w/w ratio were added and further incubated for 15 min. Then 900 μL of DMEM containing 10% FBS was added and incubated at 37 °C for 4 h. After that, cells in each well were washed five times with PBS and 40 μL hoechst (0.2 mM in DMEM containing 10% FBS) was then added, followed by incubation at 37 °C for 0.5 h. Then cells were photographed by a confocal laser scanning microscope ((Nikon C1-si TE2000, Japan) and finally recorded by EZ-C1 software.

Transmission electron microscopy (TEM)

Transmission electron microscopy (TEM) observation was carried out on a JEM-100CXII at an acceleration voltage of 100 keV. The polymer/pDNA polyplexes were prepared according to the conditions described above. Before visualization, a droplet of the polyplex suspension was placed on a copper grid with Formvar film and stained by a 0.2% (w/v) solution of phosphotungstic acid before measurement.

Cytotoxicity assay

The cell toxicity of PEI-BTMCs and the polymer/pGL-3 polyplexes were examined by MTT assay. The 293T cells and HeLa cells were seeded in the 96-well plate with a density of 6000 cells per well and cultured 24 h in 100 μL DMEM containing 10% FBS respectively prior to adding the polymers. For the assay of polymer/pGL-3 polyplexes, the cells were seeded in the 96-well plates at a density of 1.2×10^4 cells per well and cultured for 24 h. Polymers were complexed with 0.2 μg pGL-3 at different w/w ratios for 0.5 h, which was the same as the transfection conditions. After that, the polyplexes were added to each well for 4 h, the medium was replaced with 200 μL of fresh medium and further cultured for 44 h. Then 20 μL of MTT (5 mg mL^{-1}) solution was added in and further incubated at 37 °C for 4 h. Then the medium was removed and 150 μL DMSO was added. A microplate reader (BIO-RAD 550) was used to measure the absorbance of the medium at 570 nm. The relative cell viability was calculated as follows:

$$\text{Relative cell viability (\%)} = (\text{OD570}_{\text{sample}} - \text{OD570}_{\text{background}})/(\text{OD570}_{\text{control}} - \text{OD570}_{\text{background}}) \times 100\%$$

where $\text{OD570}_{\text{sample}}$ was obtained in the presence of polymers, $\text{OD570}_{\text{control}}$ was obtained in the absence of polymers and $\text{OD570}_{\text{background}}$ was obtained in the absence of polymers and cells. Data are shown as mean ± standard deviation (SD) based on triplicate independent experiments.

Particle size and zeta potential measurement

Particle size and zeta potential were evaluated by dynamic light scattering (DLS) at 25 °C by Nano-ZS ZEN3600 (Malvern Instruments). The complexes at various w/w ratios ranging from 1 to 10 were prepared by adding 1 μg of pGL-3 DNA to appropriate volume of PEI-BTMC solution (in 150 mM NaCl solution). Then the complex solution was vortexed for 30 s before being incubated at 37 °C for 0.5 h and then diluted to 1 mL by 150 mM NaCl solution prior to be measured. Data were shown as mean ± standard deviation (SD) based on three independent measurements.

Polyplex stability in the presence of DNase and heparin

To test the stability of DNA/polymer polyplexes in the presence of serum, the polyplexes of polymer/pDNA (0.1 μg) at w/w = 5 for transfection in 8 μL of 150 mM NaCl solution were treated with 1 μL of EDTA buffer solution (0.5 M) containing 4 mg mL^{-1} heparin for 30 min and then subjected to agarose gel electrophoreses. In a parallel test, after the heparin co-incubation, DNase (1660 U per 1 μg DNA) was added into the mixture solution mentioned above and co-incubated for 15 min prior to agarose gel electrophoresis.

Results and discussion

Syntheses and characterization of PEI-BTMCs

PEI modification methods usually involv reagent activation, rigorous reaction conditions and catalyst aid.[25,36] Those shortcomings would inevitably cause difficulties in yielding compositionally and architecturally consistent products, as well as with the latent hazards originating from toxic residues including the by-products and catalysts. A "green" pathway for PEI modification is highly desired in terms of the stringent safety requirements for future clinical applications.

In the current study, we developed a facile method for aromatic hydrophobicization of PEI25K by one-step aminolysis reaction of 5-benzyloxyl trimethylene carbonate (BTMC) with PEI25K (Scheme 1). Attractively, the reaction can be accomplished in the absence of catalysts mainly due to high reactivity of the amine towards the carbonate groups as well as the large ring-strain of six-membered BTMC. The obtained PEI-BTMCs were purified by dialysis. By adjusting the feed ratios of PEI25K *versus* BTMC, three PEI25K derivatives were eventually obtained and termed PEI-BTMC1, PEI-BTMC2, and PEI-BTMC3, respectively. The SEC curves of three polymers were all monomodal with a moderate distribution (M_w/M_n) of 1.1–1.3. As revealed from SEC profiles (not shown), no small-molecular-weight BTMC residues remained in the products. The actual modification degree can be quantitatively determined by UV-vis spectroscopic analyses, and the resultant data are collected in Table 1.

An increase in BTMC modification degree was observed with increasing molar ratio of BTMC *versus* PEI25K in the feed, and they correlated well with each other except for PEI-BTMC3. In this case, the actual modification degree of 51.2 was lower than the molar feed ratio of 60. Owing to the increased hydrophobicity upon BTMC modification, a fraction of polymers with high modification degree precipitated during the dialysis process. Therefore, the molar ratio of 51.2 can be approximately viewed as the threshold with regard to the water-solubility of the obtained products.

The structures of the obtained PEI-BTMCs were verified by NMR and IR analyses. From IR spectra (spectra not shown), a strong absorbance from carbonyl groups ($\nu_{C=O}$) appears at 1652 cm^{-1} and the peak at 1386 cm^{-1} is attributed to the stretching frequency of amide C–N (ν_{C-N}). A bending vibration of the carbamate N–H (δ_{N-H}) at 1549 cm^{-1} could be observed. All the characteristic resonances from BTMC and PEI moieties were clearly detectable in the typical ^1H NMR spectrum of PEI-BTMC2 (Fig. 1). In addition to the characteristic signals in the region 2.49–2.61 ppm belonging to PEI, the appearance of a resonance at 7.30 ppm attributed to benzene protons together with the signal centered at 4.62 ppm belonging to the methylene protons of a benzyl group, strongly confirms the successful BTMC modification to PEI25K.

Table 1 Reaction condition and results of PEI-BTMC preparation

Sample	Feed molar ratio (PEI/BTMC)	Composition (PEI/BTMC)[a]
$PEI\text{-}BTMC_1$	1 : 20	1 : 19.4
$PEI\text{-}BTMC_2$	1 : 40	1 : 39.6
$PEI\text{-}BTMC_3$	1 : 60	1 : 51.2

[a] Data were determined according to the UV-vis spectroscopy analyses.

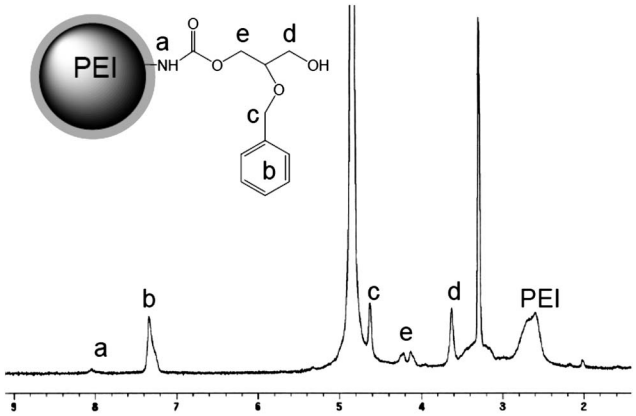


Fig. 1 ^{1}H NMR spectrum of PEI-BTMC2 in CD_3OD.

In vitro cytotoxicity

Biocompatibility is one of the critical properties for gene vectors with regard to the *in vivo* applications. Studies have revealed that free polycations rather than DNA-combined polycations are indeed the major cause of acute toxicity of polycation/DNA transfer systems.[8] To preliminarily evaluate the safety profile of PEI-BTMCs, *in vitro* cytotoxicity tests were conducted in 293T and HeLa cells by MTT assay using PEI25K as the control.

PEI25K showed the highest toxicity in the two cell lines and the effect depended upon the concentration (Fig. 2). In comparison, PEI-BTMCs exhibited substantially enhanced biocompatibility in both cell lines. The charge density has been documented as a key factor to affect the cytotoxicity of polycations. The reduced cytotoxicity upon BTMC modification may be associated with the consequently decreased surface amine content. In addition to the concentration dependency, the cytotoxic effect was found to be composition dependent. The larger the modification degree, the lower the cytotoxicity for PEI-BTMCs. The decreased amount of primary amines with increasing modification degree could account for such composition-dependent features. In contrast, nearly no difference in cell biocompatibility was detected among the acetylated PEI25K derivatives with different modification degrees.[37] The different findings in the two cases were likely caused by the stronger hydrophobicity of aromatic BTMC moieties, which made the influence more pronounced. Fig. 2 also includes the cytotoxicity data of the polyplexes with pGL-3 plasmid. The polyplexes displayed higher safety profiles. The positive charge of the PEI-BTMCs was partly counterbalanced by the negative charge of DNA, thus reducing the toxicity. Relatively, three PEI-BTMC polyplexes showed lower cytotoxicity than PEI25K polyplexes formed at the optimal transfection w/w ratio of 1.3.

DNA-binding assay

DNA-binding ability is of great importance for cationic vectors in terms of effective payload and protection of DNA from environmental damage.[14] The polyplexes formed at various polymer : DNA weight (w/w) ratios were subjected to electrophoretic analyses to detect the retardation ratio required to inhibit the migration of pGL-3 plasmids. For the unmodified PEI25K, the mobility of pGL-3 plasmids could be entirely retarded upon reaching a w/w ratio of 0.2 (N/P ratio = 1.5). Gel retardation assays revealed that all PEI-BTMC conjugates still had fairly satisfactory DNA-binding ability with a low

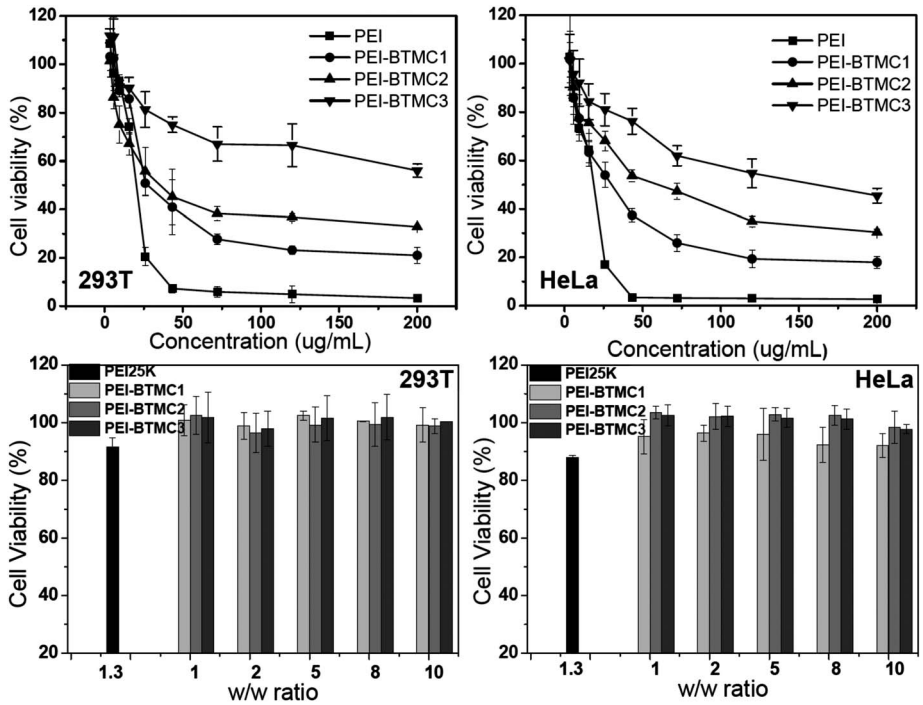


Fig. 2 Cytotoxicity of PEI-BTMCs in 293T and HeLa cells in comparison with that of PEI25K (upper); and cytotoxicity of PEI-BTMC/pGL-3 plasmid polyplexes in 293T and HeLa cells at various w/w ratios (bottom). The plasmid concentration was fixed at 1 μg mL^{-1}. (Data shown as mean ± SD ($n = 3$).)

retardation w/w ratio of 1 (Fig. 3), though lower than that of PEI25K. Previous work led to a conclusion that the primary amine groups of PEI were significantly important for DNA compacting.[15] The lowered DNA-binding ability upon BTMC modification should be due to the reduced availability of amine groups.[36,38]

Somewhat interestingly, with regard to the retarding w/w ratio detected, there was no significant difference in the DNA-binding ability between three vectors irrespective of the modification degree. From another perspective, the corresponding N/P retarding ratios were 6.45, 5.46, and 5.00, showing even a slightly increasing trend in DNA-binding ability with increasing modification degree. This finding appears to be contrary to the established opinion that the reduced availability of amine groups would attenuate the ability of polycations to integrate with DNA, and consequently larger amounts of polymers are required to inhibit DNA migration.[36,38]

It is suggested that the electrostatic attraction may not be the only driving force to inhibit pGL-3 migration upon complexation. There exist other forces contributing to the stable association of PEI-BTMCs with DNA. According to the structure of PEI-BTMC as illustrated in Scheme 1, the additional forces should be associated with the introduced hydroxyl and/or benzyloxyl groups attached to PEI25K. In our recent research, the hydroxyl coupling onto PEI25K were shown to apparently weaken the DNA affinity.[39] Some studies also showed that hydrophobic moieties attached to PEI can provide a more powerful binding to DNA than hydroxyl groups.[39,40] Thus, it is naturally deduced that the presence of benzyl groups plays an important role in preserving the stability of pGL-3/PEI-BTMC polyplexes *via* hydrophobic forces. Note that the bases of a double-stranded DNA, which might be the only component of DNA that can hydrophobically interact with BTMC, normally form a strong interaction with adjacent bases inside the double helix, and thus are not likely to be available for interaction with BTMC groups. It is thus assumed that the hydrophobic attraction between benzyl groups may play an important role in the polyplex stabilization, working as locks against polyplex disintegration.[40] Such hydrophobic contribution to polyplex stability would somewhat counteract the negative effect of reducing electrostatic attraction to DNA. As a result, when those two forces were at a comparable level, the DNA-binding ability would present little dependency upon the modification degree of BTMC. In fact, in the case of hydrophobic modification towards several low-molecular-weight PEIs having weaker electrostatic attraction than PEI25K, the DNA-binding abilities of the modified derivatives were reported to approach or even exceed those of the parent PEIs.[17,41]

On the other hand, it should be noted that the slightly lowered DNA-binding ability of PEI-BTMCs compared to PEI25K may not be disadvantageous to the transfection. That is because a suitable gene-delivery system has to meet two contradictory requirements, including both adequate DNA protection under the extracellular conditions and the efficient intracellular DNA release. In many recent studies, lack of an active DNA-release mechanism inside cells due to the strong binding ability of PEI25K has been proposed to be a limitation against the intracellular transfection.[42–45] In this regard, properly lessening the DNA/PEI25K interaction by BTMC modification may possibly favor the transfection since it would lead to relatively easier intracellular detachment of DNA from vectors.

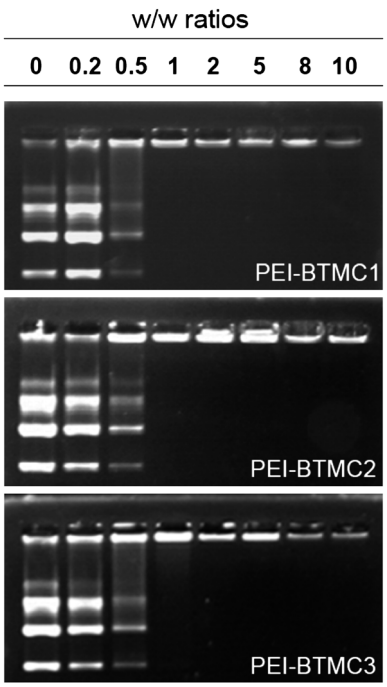


Fig. 3 Agarose gel electrophoresis retardation assay of PEI-BTMCs. Plasmid pGL-3 (100 ng) was mixed with polymers at different w/w ratios.

Zeta-potential of PEI-BTMC/pGL-3 polyplexes

The positive surface charge of DNA/polymer polyplexes would facilitate their approach to the negatively charged cell membranes and thus promote the endocytosis.[5,6] Herein, pGL-3 plasmid was used to evaluate the influence of BTMC modification on the polyplex zeta potential.

The zeta-potentials gradually increased from 20 to 35 mV with increasing w/w ratios of PEI-BTMC to pGL-3 (Fig. 4). Nevertheless, there was a subtle difference in the zeta potential amongst three vectors when the same w/w ratio was applied. Generally speaking, PEI modification with non-charged groups would inevitably reduce the surface charge. Consequently in many

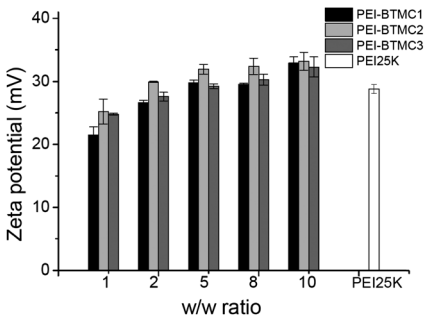


Fig. 4 Zeta potentials of PEI-BTMC/pGL-3 (1 μg mL^{-1}) polyplexes at different w/w ratios with PEI25K control at w/w ratio of 1.3 : 1. Data were as mean ± SD ($n = 3$).

cases, polyplex formulations with highly modified polycation vectors would result in relatively lower zeta potentials.[14] The contrary result observed herein may be associated with the difference in binding ability among three vectors. Due to the contribution to polyplex stability from hydrophobic interaction, fewer amine groups are needed to tightly bind DNA, which offsets the reduced availability of amine groups after BTMC modification. Supporting evidence for this explanation is that PEI25K modified with relatively more hydrophobic propionic anhydride possessed higher polyplex potential than acetic anhydride-modified PEI25K despite the same degree of modification.[14]

Particle size of PEI-BTMC/pGL-3 polyplexes

To effectively mediate the endocytosis, cationic polymers need to condense string-like DNA into compact nanoparticles.[46] In the current study, DLS was utilized to measure the hydrodynamic size of PEI-BTMC/pGL-3 polyplexes at different w/w ratios. As observed in Fig. 5A, all three PEI-BTMCs could condense pGL-3 plasmids into compact nanoparticles with the mean sizes ranging from 250 to 350 nm. When the w/w ratio was below 2, the higher the BTMC modification degree, the smaller was the particle size. Nevertheless, with further increase of the w/w ratio, a relatively smaller particle size was presented for PEI-BTMC2 polyplexes among the three polyplexes. Under conditions with excess polymers, more likely it is the combination of electrostatic attraction and hydrophobic interaction that cooperatively affected the particle size of formed polyplexes. As a consequence, complexation with moderately modified PEI-BTMC2 gave rise to a relatively more compact structure. The morphologies of PEI-BTMC/pGL-3 plasmid (w/w = 5) polyplexes were further revealed by TEM images, included in Fig. 5B. The images indicated that the polyplexes were dispersed as separate entities with a spherical shape. The particle size of PEI-BTMCs/DNA complexes was about 50–70 nm measured by TEM.

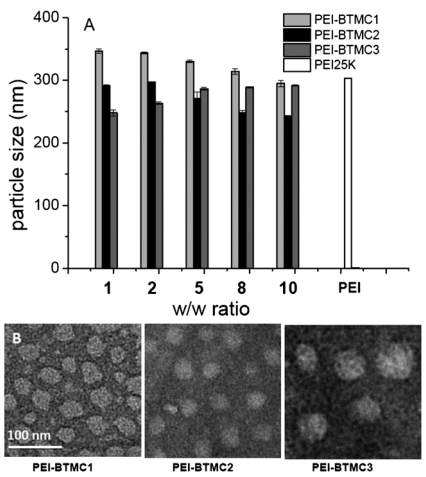


Fig. 5 (A) Mean particle size of PEI-BTMC/pGL-3 (1 μg mL^{-1}) polyplexes with different w/w ratios compared with PEI25K control at w/w ratio of 1.3 : 1. Data shown as mean ± SD ($n = 3$). (B) TEM images of polymer/pGL-3 plasmid polyplexes (w/w = 5).

Polyplex stability in the presence of DNase and heparin

Polycations can self-assemble with negatively charged DNA *via* electrostatic interactions, to condense the string-like DNA into compact nanoparticles. The resulting polyplexes provide protection for genes from serum nuclease digestion and allow for facile cellular uptake *via* a non-specific endocytotic pathway. Serum proteins are the major components in the blood and have been identified as a key element that destabilizes the polyplexes after intravenous injection.[47] To assess the stability of the polyplexes against exchange reactions, agarose gel electrophoreses were challenged in serum-imitated conditions with the presence of heparin and/or DNase. Here, heparin was used on behalf of negatively charged serum components.[39,48] In all the experiments, the DNA amounts were identical. As shown in Fig. 6A, released DNA from PEI25K polyplexes at the optimal transfection w/w ratio of 1.3 was already seen on the gel as a smear when exposed to heparin, suggesting the gradual detachment of DNA from the polyplexes loosened by heparin. After DNase addition, the smear disappeared due to rapid degradation. Compared with the PEI25K/DNA (w/w = 1.3), stronger DNA affinity was observed when increasing the w/w ratio to 5. Nevertheless, PEI25K can mediate the highest transfection performance at w/w = 1.3, which suggested that the DNA protection may be not the only factor in determining the transfection efficacy. For instance, the dose-limiting toxicity was assumed to largely influence the transfection at higher polymer concentrations. Overall, PEI-BTMC polyplexes displayed good stability in terms of the fluorescence intensity in gels after the incubation with heparin. In comparison, it seemed that BTMC modification would result in a somewhat reduced capability to prevent the polyplexes from dissociation by heparin and DNase.

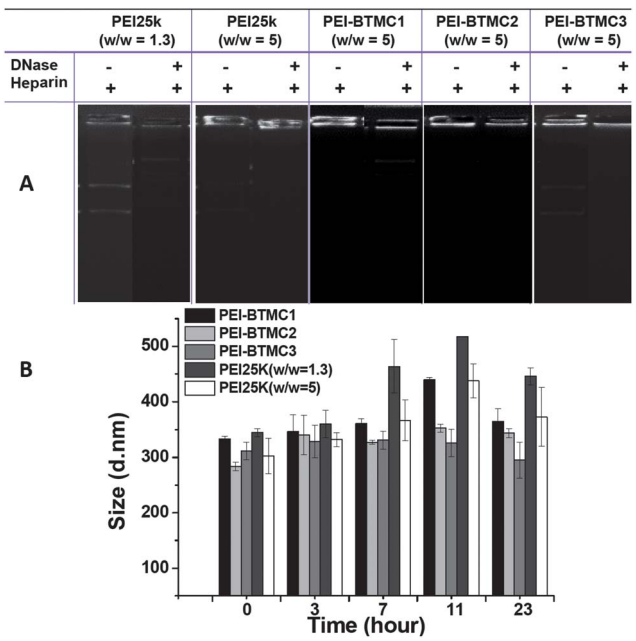


Fig. 6 (A) Agarose gel electrophoreses of DNA/polymer polyplexes incubated with heparin and/or DNase; (B) variation of polymer/DNA polyplex size as a function of standing time in PBS solution.

Tracking the PEI-BTMC polyplex size as a function of incubation time provided us with further information about the complex's stability from another perspective. An insignificant change of the PEI-BTMC polyplex size was detected over 23 h standing in PBS solution (Fig. 6B). Relatively, the size of PEI25K polyplexes presented an evident fluctuation during the observation period. The results suggested that the undesired effect of PEI-BTMC polyplex aggregation was minimal.

In vitro transfection

As for the alkylation strategy for PEI modification, whether the transfection is increased or decreased by conjugation with hydrophobic molecules is still controversial.[14,16,24–27] Contrary results regarding the influence of modification degree and alkyl chain length were obtained in those studies. It is assumed to be associated with many factors including lipophilicity of alkyl chains and the shrinkage of alkyl chains in aqueous medium. Hence, the present study provides a chance to further understand the influence of hydrophobic modification on the transfection, from a sharply different perspective by incorporating rigid and highly hydrophobic aromatic moieties onto PEI25K. The transfections mediated by PEI-BTMCs were investigated firstly in 293T cells and HeLa cells. In the luciferase assay, PEI25K is well-established as the gold standard and was used as the control for comparison at its optimal w/w ratio of 1.3.[3]

As shown in Fig. 7, all PEI-BTMC polyplexes displayed apparently higher transfection activities than PEI25K polyplexes in both 293T and HeLa cells. In particular, such transfection enhancement due to BTMC modification presented more profoundly in HeLa cells. The maximal luciferase expression level mediated by PEI-BTMC1, PEI-BTMC2, and PEI-BTMC3 was 10, 30, and 150 times that of PEI25K, respectively. Among the three derivatives, PEI-BTMC2 displayed the best transfection in 293T cells while PEI-BTMC3 did in HeLa cells. Specifically, the transfection activity of PEI-BTMC2 was 6-fold higher than that of PEI25K in 293T cells.

Such marked deviation between the PEI-BTMC-mediated transfections in normal 293T and cancerous HeLa cells naturally raises the interesting question of if PEI-BTMCs could mediate highly efficient transfection in other cancerous cells. Thus a comparative study was further performed by investigating the PEI-BTMC-mediated transfection in Cos 7 and cancerous HepG2 cells (Fig. 8). The maximum transfection efficiency with PEI-BTMCs was 12-fold higher than PEI25K control in Cos 7 cell, whereas in HepG2 cells the former was slightly higher than the latter. The BTMC modification did not lead to marked improvement of transfection in cancerous HepG2 cells.

Though the findings were inconsistent with our expectations, the extraordinarily high transfection activity presented in cancerous HeLa cells was after all very encouraging as compared with the data reported so far for polycation-mediated transfection.[24] Such a fascinating result prompted us to explore the possible mechanism for the ultrahigh transfection efficiency of PEI-BTMC in HeLa cells.

In vitro transfection assay in the early stage

Effective gene transfection mediated by polycation vectors depends largely upon the ability of vectors to condense DNA into positively charged compact polyplexes *via* electrostatic attractions. Nevertheless, excessive surface charge would produce detrimental damage to the cells and lead to a compromised transfection in many cases. Hence, there is a possibility that the highly enhanced transfection efficiency after BTMC modification is associated with the concurrently improved cytotoxicity. To judge this assumption, a quantitative comparison was performed in HeLa cells between the transfections mediated by PEI25K and PEI-BTMC2 with the modest modification degree at their optimal transfection w/w ratios after just 1 h co-incubation. After 1 h transfection, the cell viabilities were at a similar level of above 80% for both cases, as revealed by MTT assay. The quantitative assay showed that the luciferase expression level mediated by PEI-BTMC2 after 1 h transfection was still remarkably higher than that of PEI25K. Specifically, that by PEI-BTMC2 (2.8×10^6 RLU mg^{-1}) was around 100 times higher than that of PEI25K (3.0×10^4 RLU mg^{-1}). Thus, the data indicates that the reduced cytotoxicity may not be the main factor responsible for the highly improved transfection mediated by PEI-BTMCs.

Cellular uptake of PEI-BTMC polyplexes in HeLa cell

For the majority of reported PEI25K derivatives, the modification would shield the surface charge and consequently reduce the damage to cells. Nevertheless, to some extent, such biotoxicity improvement may be achieved at the expense of endocytosis efficiency and DNA binding ability, which are strongly associated with the transfection efficiency. For example, highly surface-

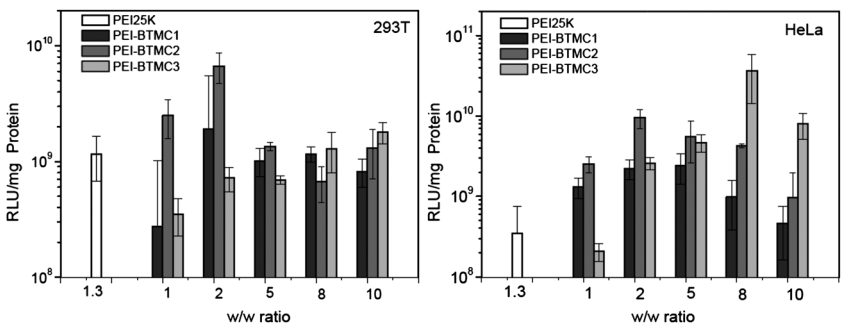


Fig. 7 Transfection efficiency of PEI-BTMC/pGL-3 polyplexes at different w/w ratios in 293T and HeLa cells in comparison with PEI25K control at w/w 1.3. pGL-3 (1 μg mL^{-1}) was used as the reporter plasmid. Data shown as mean ± SD ($n = 3$).

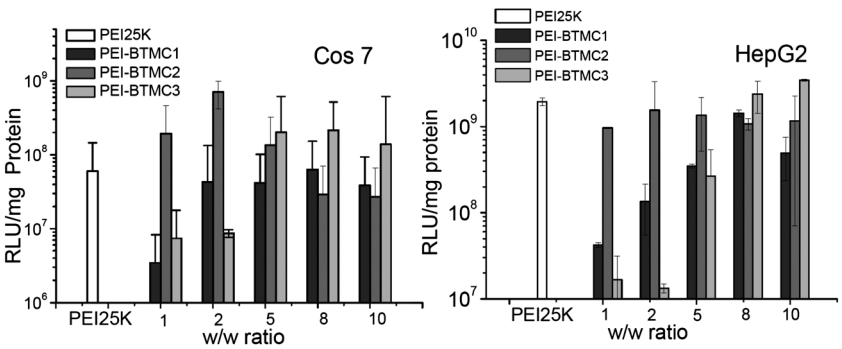


Fig. 8 Transfection efficiency of PEI-BTMC/pGL-3 polyplexes at different w/w ratios in Cos 7 and HepG2 cells compared with PEI25K control at w/w 1.3. pGL-3 (1 μg mL^{-1}) was used as the reporter plasmid. Data shown as mean ± SD ($n = 3$).

alkylated PEI25K did not transfect the cells regardless of the incubation period.[24] They formed larger polyplexes and had low or negative zeta potential, which decreased the ability of the polyplexes to interact with and penetrate the cell membrane.

To visually examine the influence of BTMC modification on the cellular uptake, HeLa cells were incubated with pGL-3 polyplexes at the optimal w/w ratios for 4 h and then observed by confocal laser scanning microscopy (CLSM). The pGL-3 plasmids within the polyplexes were labeled with YOYO-1 iodide, presenting green fluorescence. The nuclei were stained by Hoechst 33258 and presented blue to distinguish from the green fluorescence. PEI-BTMC2 with the modest modification degree was used as the typical model and PEI25K was taken as the control. The initial cell densities were identical in both cases for comparison purposes.

Tracking the DNA/PEI-BTMC2 provided images sharply different from those observed for PEI25K/pGL-3 polyplexes (Fig. 9). Green pGL-3 plasmids exhibited an even distribution not only in the cytoplasm but also in the blue-labeled nuclear areas in the case of PEI-BTMC2 transfection. In comparison, PEI-BTMC2 showed considerably higher potency to transport pGL-3 plasmids into the cells, as revealed by the much stronger fluorescence intensity. The CLSM observation presented a good positive correlation with the transfection results. It is suggested that the highly enhanced transfection activity upon BTMC modification may have a strong association with the outstanding potency of PEI-BTMC to mediate the cellular uptake.

The polymer compositions would affect not only the adsorption of polyplexes onto the cell membrane but also the penetration through it. The electrostatic attraction should be a powerful driving force for the access of polyplexes to the cell membrane. Nevertheless, it was reported that hexyl- or dodecyl-modified PEI interacted strongly with phospholipids, whereas the unmodified PEI did not.[36,49] At this point, the structural compatibility between BTMC-modified PEI25K and the biological membrane may be an important factor that affects the transmembrane process.[17] Although it is still too early to draw a conclusion about the mechanism of PEI-BTMC-mediated transfection, the ultrahigh transmembrane potency of PEI-BTMC polyplexes observed herein is very encouraging.

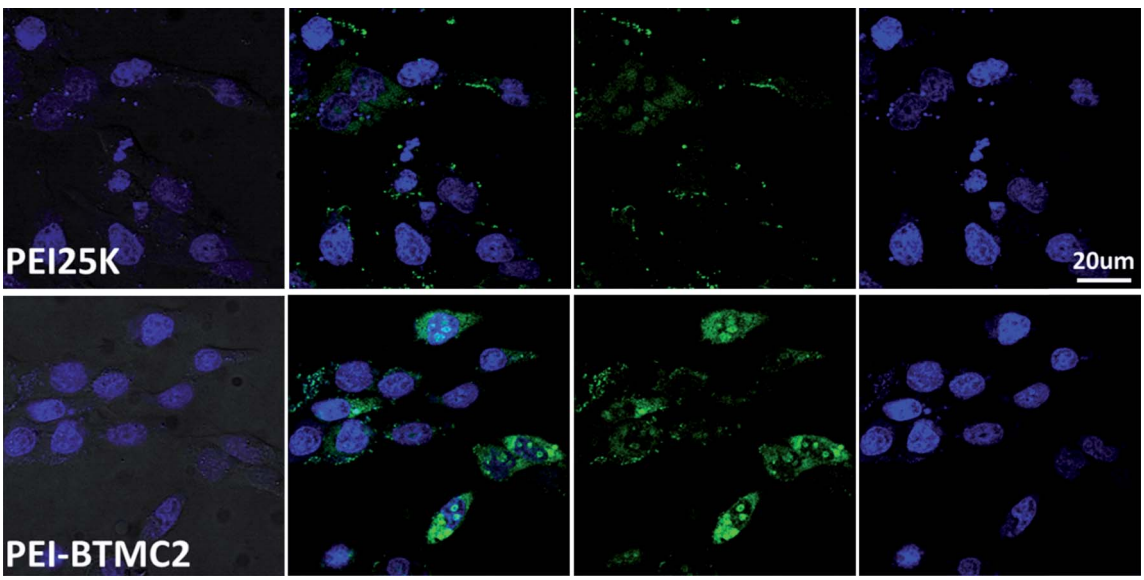


Fig. 9 Fluorescent confocal microscopic images of pGL-3(1 μg mL^{-1}) polyplex distribution in HeLa cells (PEI-BTMC2, w/w ratio = 2; PEI25K, w/w ratio = 1.3). pGL-3 plasmids were stained green by YOYO-1 and the nuclei of cells were stained blue by Hoechst 33258.

Conclusions

In the current study, a series of novel polycation gene vectors of PEI-BTMCs were facilely prepared in a controlled manner. In addition to the substantially improved cell biocompatibility, the designed PEI-BTMCs displayed ultrahigh transfection efficiency in the observed cell lines. Particularly in the cancerous HeLa cells, the transfection efficiency was up to 150-fold higher than that transfected with PEI25K polyplexes. Although the mechanism of PEI-BTMC-mediated transfection is not yet fully understood, the improved cell biocompatibility after BTMC modification appears not to be the main factor responsible for the highly enhanced transfection. Alternatively, such transfection enhancement is proposed to have a strong association with the special functions caused by BTMC modification such as hydrophobic "locks" to stabilize polyplexes, relatively easier release of DNA inside cells and in particular the highly enhanced transmembrane capacity.

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