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RESEARCH ARTICLE

Investigation of polyethylenimine-grafted-triamcinolone acetonide as nucleus-targeting gene delivery systems

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Abstract

Background Nuclear membrane is one of the main barriers in polymer mediated intracellular gene delivery. To improve the transgenic activity and safety of nonviral vector, triamcinolone acetonide (TA) as a nuclear localization signal was conjugated with different molecular weight polyethylenimine (PEI).

Methods Different molecular weight PEI [600, 1800, 25 000 (25k)] was conjugated with TA to synthesize PEI-TA by two-step reaction. Their physicochemical characteristics, *in vitro* cytotoxicity and transfection efficiency were evaluated. To investigate the difference of transfection efficiency of various molecular weight PEI-TA, their transfection mechanism was further investigated by confocal microscopy and competition assay. Transgenic expression *in vivo* was evaluated by injection into hepatic portal vein of mice.

Results All PEI-TA could form nanosize polyplexes with DNA and their physicochemical properties resemble each other. Their cytotoxicities were negligible compared to PEI 25k. The order of transfection efficiency was PEI 1800-TA > PEI 600-TA > PEI 25k-TA. A transfection mechanism study displayed that TA could inhibit considerably the transgenic activity of PEI 1800-TA and PEI 600-TA, but that of PEI 25k-TA was not inhibited. It was suggested that PEI 1800-TA and PEI 600-TA might translocate into the nucleus. Confocal microscopy investigation verified this suggestion. The data strongly suggested that the transfection efficiency of PEI 1800-TA *in vivo* was much higher than that of PEI 25k, which was consistent with the results obtained *in vitro*.

Conclusions Low molecular weight PEI-TA could translocate into the nucleus efficiently. PEI 1800-TA presented higher transgenic activity and it has a great potential for gene therapy as a nonviral carrier.

Keywords gene therapy; nonviral vector; nuclear translocation; polyethylenimine

Introduction

Gene therapy may be one of the most important therapies for the next generation. However, the deficiency of safe and efficient methods for gene delivery still remains a critical obstacle to the routine clinic implementation of human gene therapy [1]. Although recombinant viruses are the

most efficient gene delivery vectors currently available, nonviral vectors have several advantages that make them a promising alternative. Over the past few decades, various synthetic vectors, such as poly-L-lysine [2], polyethylenimine (PEI) [3], polyamidoamine (PAMAM) dendrimers [4], have been designed and researched to achieve the level of gene expression and specificity shown by viral ones. Among the numerous of polycations reported, PEI is one of the upmost successful polymers and is used widely for gene delivery because of the 'proton sponge effect' [5].

Gene carriers must traverse multiple extracellular and intracellular obstacles to reach the target cell nucleus, including passage across the plasma membrane, escape from acidic and potentially degradative endocytic vesicles, transport across the nuclear membrane, and release of genetic material in this pathway. Nuclear membrane represents the major barrier to successful nonviral gene delivery. In the past few decades, several strategies have been developed to overcome this barrier, such as cell-penetrating peptides [6], glycosyl residues [7], glucocorticoid (GC) ligand [8] and so on. Using GCs as nuclear localization signals (NLS) to improve transfection efficiency was investigated in several studies [9–13]. Various GCs were conjugated with low molecular weight PEI, and the relationship between GC potency and transfection efficiency was explored [14]. The result obtained indicated that the transgenic expression GC correlated linearly with their potency. The higher the potency of GC substituted on the polymer, the more effective was its transfection. Therefore, a stronger potent GC (triamcinolone acetonide, TA) was grafted on the surface of PAMAM dendrimer, and its transgenic activity was enhanced significantly [15].

In the present study, to gain higher transfection activity, TA was conjugated with various molecular weights PEI [600, 1800, 25 000 (25k)] to create a series of polymeric gene carriers. Their physicochemical properties, cytotoxicities, transfection effects and intercellular localization were examined to obtain the optimal carrier. Transfection *in vivo* was investigated simultaneously to confirm the transgene activity.

Materials and methods

Materials

Polyethylenimine (PEI, branched, 600, 1800 and 25 kDa), 2-iminothiolane (Traut's reagent), fluorescein isothiocyanate (FITC), MTT and ethidium bromide were purchased from Sigma-Aldrich Chemical Co., Ltd (Milwaukee, WI, USA). RPMI 1640, penicillin-streptomycin (PS, 10 000 U/ml), trypsin-ethylenediaminetetraacetic acid (EDTA) (TE, 0.5% trypsin, 5.3 mM EDTA tetra-sodium) were obtained from Gibco BRL (Gaithersberg, MD, USA). Fetal bovine serum (FBS) was purchased from sijiqing Biologic Co., Ltd (Hangzhou, China). Methanesulfonyl chloride was purchased from Jiachen Chemical Co., Ltd. (Shanghai, China). TA was kindly donated by Zhejiang Xianju Pharmaceutical Co., Ltd (Hangzhou, China), and its purity was over 99%. Promega Luciferase Assay Kit containing luciferase cell culture lyses reagent and luciferase substrates was obtained from Promega (Madison, WI, USA). The BCA Protein Assay Kit and Hoechst 33 342 were purchased from Beyotime Institute of Biotechnology (Jiangsu, China). Plasmid DNA (pGL-3, pEGFP-N1) was propagated in *Escherichia coli* DH5α, isolated, and purified using Qiagen Plasmid Mega Kit (Qiagen GmbH, Hilden, Germany). The purity and concentration of DNA were determined by measuring ultraviolet (UV) absorbance at 260 and 280 nm. All other chemicals were of analytical grade.

Synthesis of PEI-TA

PEI-TA was synthesized via a two-step reaction by the slightly modified method from a previous study [16]. First, the 21-hydroxyl group of TA was substituted with mesylates [17]. Briefly, methanesulfonyl chloride (78 μl, 1 mmol) was added dropwise to a solution of TA (217.25 mg, 0.5 mmol) in anhydrous pyridine (5 ml) at 0 °C under N_2 with stirring. After reacting for 5 h, ice water (approximately 100 ml) was added to terminate the reaction. The precipitate was filtered, washed with more ice water, and crude TA-mesylate as a white solid powder was obtained and dried. Using recrystallization from ethanol-acetic ether to purify the crude mesylate. TLC (ethyl acetate/ligroin/methanol = 10/10/1, v/v/v) was performed at the end of the reaction. The product was solubilized in dimethyl sulfoxide (DMSO)-d_6 for ^{1}H-NMR analysis (500 MHz; Bruker, Germany).

Then, TA-mesylate (133.9 mg) and Traut's reagent (34.4 mg) in 2.0 ml anhydrous DMSO were added slowly into 112.5 mg of PEI 600, 1800 or 25k in 2.0 ml anhydrous DMSO, respectively. The reaction was allowed to proceed under N_2 at room temperature under continuous stirring for 4 h. To the reaction mixture, same volume of pure water was added and filtered by 0.45 mm micropore film to remove insoluble impurities. Then dialysed against pure water using dialysis membrane (MWCO 500 for PEI 600, MWCO 1000 for PEI 1800, MWCO 3500 for PEI 25k) for 48 h, the dialysis medium was refreshed every 12 h. A white product (PEI-TA) was obtained after further freeze-drying. The product was solubilized in D_2O for ^{1}H-NMR analysis (500 MHz, Bruker, Ettlingen, Germany). Figure 1 shows the synthetic scheme.

Buffering capacity of the PEI-TA polymers

PEI 600-TA, PEI 1800-TA, PEI 25k-TA water solution (0.2 mg/ml, 30 ml) were prepared, respectively, and pure water was used as a control. After adjusting the initial pH to 10.0 with 0.1 M NaOH if necessary, 25 μl increments of 0.1 M HCl were titrated into the solution and the pH

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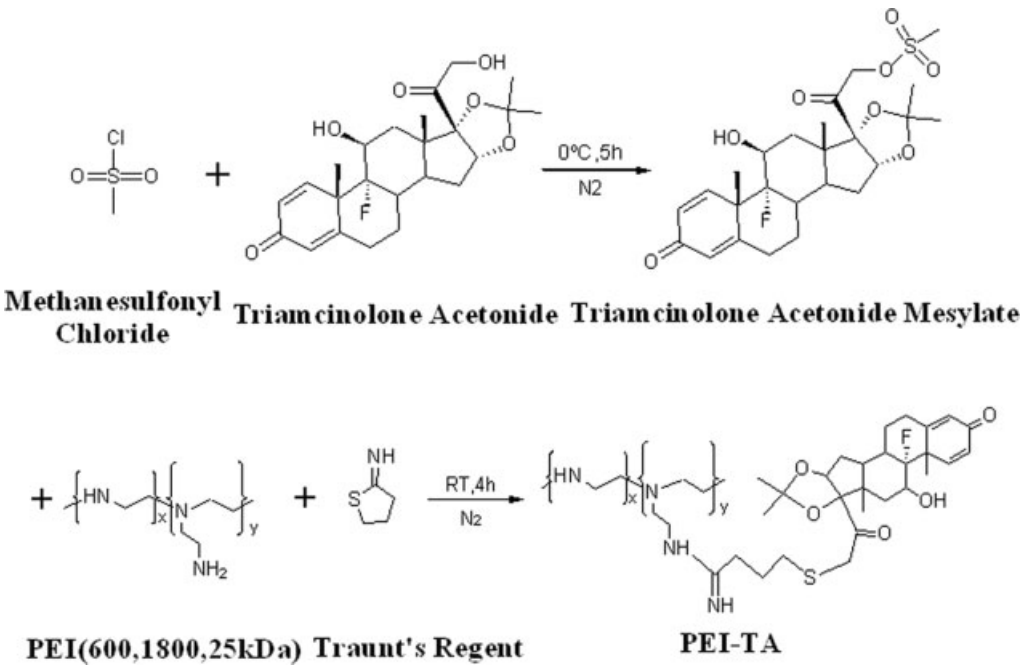

Figure 1. The synthesic scheme of PEI-TA polymers

response was measured by a micro-pH electrode at the same time. The pH values were recorded in the range 10.0–3.0.

Agarose gel electrophoresis

pDNA condensing ability of PEI 600-TA, PEI 1800-TA, PEI 25k-TA were examined by agarose gel electrophoresis. PEI-TA/pDNA polyplexes were prepared at various weight ratios between the polymers and pGL-3 plasmid in HEPES buffered saline (HBS, 25 mM HEPES, 150 mM NaCl, pH 7.4), and the mixtures were incubated for 30 min at room temperature. The samples were electrophoresed on a 1% (w/v) agarose gel stained with 0.25 μg/ml ethidium bromide in TAE buffer at 90 V for 40 min, and analyzed by a UV illuminator to visualize the location of pDNA bands.

Size, ζ-potential measurements and transmission electron microscopy (TEM)

The size and ζ-potential measurements of polyplexes were made using Malvern Zetasizer 3000HAs system (Malvern Instruments Ltd, Malvern, UK). The polyplexes were prepared at a final concentration of 10 μg/ml pDNA at various weight ratios in pure water for size measurements and in HBS (25 mM HEPES, 150 mM NaCl, pH 7.4) for ζ-potential experiments, respectively. The polyplexes were then incubated at room temperature for 30 min. The size and ζ-potential values were presented as the average values of three assays. PEI 1800-TA/pDNA polyplexes were prepared at an optimal weight ratio. The morphologies of resulting polyplexes were observed using TEM (JEM 1230; JEOL, Tokkyo, Japan). One drop of polyplexes was placed on a copper grid and stained with 2% phosphotungstic acid solution for 30 s The grid was allowed to dry further for 20 min and then examined with the electron microscope.

Cytotoxicity assay

The cytotoxicity of the polymers was investigated by MTT assay. Briefly, HEK293 cells and HepG2 cells were seeded at a density of 1×10^4 cells/well in 100 μl of growth medium in 96-well plates (Corning Inc., Lowell, MA, USA), and were incubated for 24 h before adding the polymers. The cells were transfected with PEI-TA/DNA polyplexes at various weight ratios. The PEI 25k and PEI 1800 polyplexes were used as a control at a weight ratio of 1.33 : 1 and 5.3 : 1, respectively [18]. The amount of pDNA was fixed at 0.2 μg/well. The cells were incubated for 24 h at 37 °C. Then the medium was replaced with 20 μl MTT (5 mg/ml) solution and 100 μl of fresh medium without serum and further incubated for 4 h. Subsequently, the medium was removed and 100 μl DMSO was added. After shaking the plate for 20 min, absorbance was immediately measured at 570 nm by using an enzyme-linked immunosorbent assay plate reader (Multiskan Spectrum, Thermo Fisher Scientific Inc., Waltham, MA, USA). Cells incubated without polymers were used as a blank control.

In vitro transfection experiment

HEK293 cells and HepG2 cells were seeded at a density of 1×10^5 cells/well in 24-well plate in RMPI 1640 medium containing 10% FBS, and grown to reach 80% confluence

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prior to transfection. The medium was exchanged with fresh medium with and without 10% FBS. The cells were treated with polyplex solution containing 2 μg of pGL-3 at various weight ratios for 4 h at 37 °C. The final volume was adjusted to 500 μl by medium. After exchanging with a fresh medium with 10% FBS, cell were further incubated for 48 h. Then the growth medium was removed, and the cells were shaken for 30 min at room temperature in 200 μl of reporter lysis buffer. The lysates were transferred into tubes and centrifuged at 13 000 *g* for 5 min. Luciferase activity was measured with a luminometer (Turner Designs Luminometer Model TD-20/20; Promega). The total protein was determined using BCA Protein Assay Kit. The final luciferase activity was expressed as RLU/mg protein.

Fluorescence labeling of polymers

The PEI-TA polymers were labeled with FITC as described in a previous study with some modification [19]. PEI-TA polymers were dissolved in PBS (pH 7.4). FITC solution dissolved in DMSO was added dropwise to unlabeled polymer solution. The solution was incubated at room temperature with continuous stirring in the dark overnight. The molar ratio of FITC and polymer taken was 1 : 20. The labeled polymer solution was dialysed against PBS (pH 7.4) for 24 h and then distilled water for 24 h (MWCO 500 for PEI 600, MWCO 1000 for PEI 1800, MWCO 3500 for PEI 25k) until free FITC could not be detected by TLC (chloroform/methanol = 1/1, v/v). The solution was filtered through a 0.22-μm filter and then lyophilized.

Intracellular localization of polyplex

HepG2 cells were seeded at a density of 2×10^5 cells/well on the surface of a cover slide in six-well plates in 2 ml of medium containing 10% FBS and grown to reach 50–60% confluence prior to transfection. The cells were treated with polyplexes solution and further incubated for 4 h. The polyplex solution was prepared by mixing FITC-labeled PEI 600-TA, PEI 1800-TA, PEI 25K-TA and 3 μg of pGL-3 at weight ratio of 10 : 1, 2 : 1 and 2 : 1, respectively. Then, the cell culture medium was exchanged with a fresh medium with 10% FBS. After 24 h, the cells were fixed with 4% paraformaldehyde for 30 min. To stain the cell nucleus, the cells were incubated with Hoechst 33 342 for 15 min at room temperature after washed three times with 2 ml of PBS, and then the cover slips were mounted on glass slides with a drop of 0.1 M glycerine in PBS placed in between to keep the cells from drying out. The cells were analyzed by a confocal fluorescence microscopic system (FV1000-IX81; Olympus, Tokyo, Japan). A UV laser (405 nm excitation) was used to induce the blue fluorescence of Hoechst 33 342 and an argon laser (473 nm) to excite the green fluorescence of FITC.

Competition assay

To investigate whether the transfection efficiency of PEI 600-TA, PEI 1800-TA or PEI 25K-TA were inhibited by TA, TA stock solution (5 μM) prepared in ethanol was added to each well (final ethanol concentration was 1%, v/v) with HepG2 cells and incubated for 30 min before polyplexes were added. The polyplexes at their optimal weight ratio and TA mixture was incubated with cells for 4 h in the absence of 10% FBS. Then, the transfection solution was removed, and the cells were rinsed with PBS. The fresh medium with 10% FBS and TA stock solution were added, and the cells were then incubated for 24 h before assay [13]. Polyplexes and ethanol without TA were added as a control.

In vivo gene transfer

ICR mice (weighing 18–22 g) were purchased from Zhejiang University Laboratory Animal Center and maintained on standard food and water under conventional housing conditions. All animal experiments were carried out in accordance with the Guidelines for Animal Experiments of Zhejiang University. Mice were anesthetized with a peritoneal injection of urethane (1 g/kg). An incision was made in the abdomen, and the portal vein was exposed. PEI 1800-TA/pDNA was prepared in 5% dextrose at weight ratios of 2, and incubated for 30 min, and PEI 25k/pDNA complex as positive control. Each complex in 200 μl of 5% dextrose was injected into the portal vein at a dose of 50 μg pDNA/mouse, and the abdomen was sutured. Mice were sacrificed 24 h later by cervical dislocation. The liver and other organs were harvested [20]. Untreated mice were used as a negative control.

Cryofixation was performed by the immersion of tissues in Tissue-Tek OCT embedding medium (Sakura, Torrance, CA, USA) followed by freezing at −80 °C. Frozen sections of 5 μm thickness were prepared with a cryotome cryostat (CM 1900; Leica, Wetzlar, Germany). Sections were mounted on slides and analyzed by fluorescence microscopy. Single-cell suspensions were prepared by mechanical disruption. The cells were washed thrice and fixed in an 8% buffered paraformaldehyde for 30 min at room temperature, and then resuspended in PBS. The percentage of cells positive for enhanced green fluorescent protein (EGFP) was determined by flow cytometry (Becton-Dickinson Biosciences, Franklin Lakes, NJ, USA) by setting a gate according to the control after excitation with a 488 nm argon laser and detection with a 515–545 nm bandpass filter and 10 000 cells were evaluated in each experiment [21].

Statistical analysis

For statistical analysis, triplicate data were analyzed using one-way analysis of variance. $p < 0.05$ was considered statistically significant. Data are presented as the mean ± SD.

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Results

Synthesis and characterization of PEI-TA polymer

The reaction was composed of two steps. First, the TA 21-OH group was substituted with mesylate. ^{1}H-NMR (TA-mesylate, 500 MHz, DMSO-d_6): 0.81 (s, C-19, CH_3), 1.12 (s, C-25, CH_3), 1.33 (s, C-26, CH_3), 1.47 (s, C-18, CH_3), 3.29 (s, -OSO_2CH_3), 6.00 (s, C-4, CH), 6.22 (m, C-2, CH), 7.28 (d, C-1, CH) (see Supporting information, Figure S1). Then, TA-mesylate was connected with various molecular weights of PEI (Mw 600, 1800, 25k) by Traut's reagent to gain the ultimate products. ^{1}H-NMR (PEI-TA, 500 MHz, D_2O): 0.78 (s, C-19, CH_3), 1.08 (s, C-25, CH_3), 1.36 (s, C-26, CH_3), 1.45 (s, C-18, CH_3), 2.31–3.97 (m, -$NHCH_2CH_2$ NH-), 6.13 (s, C-4, CH), 6.32 (d, C-2, CH),7.42 (d, C-1, CH) (see Supporting information, Figure S2). The degree of TA grafting on each unit of ($NHCH_2CH_2$) in polymer chain was obtained by calculating the peak intensity ratio between the ethyl protons of PEI backbone and 1,2,4-H of TA. It was observed that every 41 ($NHCH_2CH_2$) units conjugated a TA molecule on the backbone of all three PEI. Namely, 0.34 TA residues were found in PEI 600-TA, 1.02 TA residues in PEI 1800-TA and 14.18 TA residues in PEI 25k-TA.

Buffering capacity of the PEI-TA polymer

The buffering capacity of the PEI-TA polymers was examined by acid–base titration by adding 0.1 M HCl to polymer solution. Pure water and PEI 25k were employed as the control (Figure 2). As expected, the buffering capacity of PEI-TA polymers at pH 7.4–6.0 was similar to that of PEI 25k, but relatively high compared to pure water.

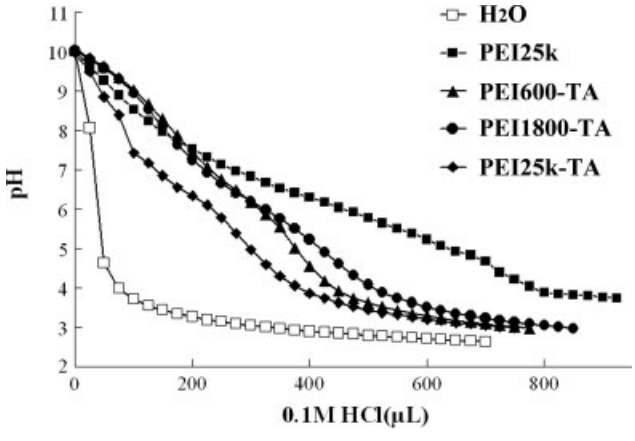

Figure 2. Measurement of buffer capability the three PEI-TA polymers by acid–base titration. PEI 25k and pure water were used as a control. Solutions were titrated with 0.1 M HCl from 10.0–3.0

Agarose gel electrophoresis

As shown in Figure 3, the ability of PEI-TA polymers to condense pDNA was examined by using a DNA retardation assay by agarose gel electrophoresis with PEI-TA/pDNA weight ratios in the range 0–5.0. The resulting polymers comprising PEIs of different molecular weight displayed different DNA binding capabilities. PEI 600-TA could completely retard DNA migration at a polymer/DNA weight ratio of 1.5, and PEI 1800-TA and PEI 25k-TA at 0.8.

Polyplex size, ζ-potential measurements and TEM

Effectively transfection efficiency of gene carrier depends on the ability to condense negatively-charged pDNA into nanoparticles with positive charges so as to enter easily into the cell. To better understand DNA condensation, we used dynamic light scattering to examine the particle size (Table 1). As expected, all the polymers condensed DNA into nanosized particles. After being complexed with DNA, PEI 1800-TA and PEI 25k-TA exhibited small hydrodynamic diameters approximately 200 nm above a weight ratio of 1, although their diameters could not be measured at a weight ratio of 0.5 (data not shown). According to PEI 600-TA, the diameter was relatively larger at low weight ratio. However, with the weight ratio increasing to 10, the size of the PEI 600-TA/pDNA polyplex decreased and stabilized at approximately 200 nm. The abrupt size increments may be caused by polyplex aggregation at a low weight ratio as a result of the reduced DNA condensation properties [3,22].

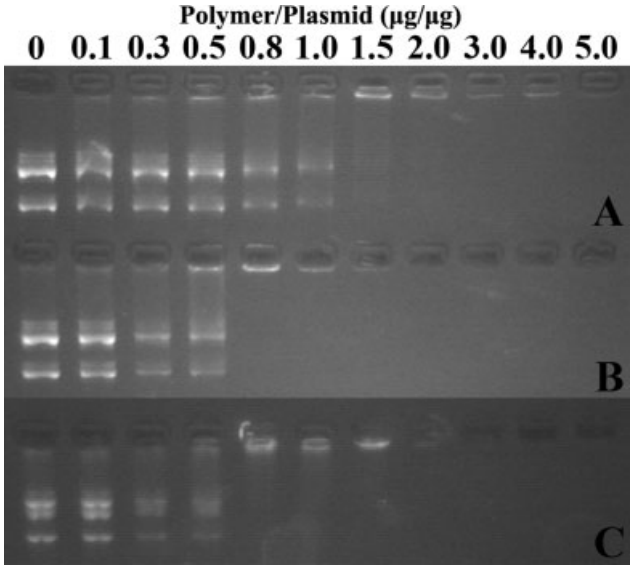

Figure 3. Agarose gel electrophoresis retardation examination of PEI-TA/pDNA polyplexes at various weight ratios: PEI 600-TA (A), PEI 1800-TA (B) and PEI 25k-TA (C). The mixtures were incubated at room temperature for 30 min and electrophoresis on 1% (W/V) agarose gel and stained with ethidium bromide

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Table 1. Particle size of the pDNA complexes with PEI-TA ($n = 3$)

	Weight ratio	Size (nm)
PEI600-TA	2 : 1	421.33 ± 99.85
	6 : 1	371.67 ± 56.3
	10 : 1	176.33 ± 8.08
	14 : 1	164.33 ± 6.43
PEI1800-TA	1 : 1	184.33 ± 11.15
	2 : 1	263.67 ± 32.02
	3 : 1	212.00 ± 41.57
	4 : 1	168.33 ± 5.69
PEI25k-TA	1 : 1	268.67 ± 16.07
	2 : 1	189.00 ± 20.52
	3 : 1	202.67 ± 24.99
	4 : 1	215.00 ± 22.61

Date are the mean ± SD of three independent experiments.

Table 2. ζ-potential of the pDNA complexes with PEI-TA ($n = 3$)

	Weight ratio	ζ-potential (mV)
PEI600-TA	1 : 1	−22.36 ± 1.77
	2 : 1	6.23 ± 1.16
	6 : 1	20.21 ± 1.61
	10 : 1	27.34 ± 1.42
	14 : 1	33.96 ± 1.97
PEI1800-TA	0.5 : 1	−17.77 ± 1.45
	1 : 1	16.95 ± 1.80
	2 : 1	29.34 ± 1.01
	3 : 1	29.33 ± 2.06
	4 : 1	33.01 ± 1.01
PEI25k-TA	0.5 : 1	−18.59 ± 0.75
	1 : 1	17.82 ± 1.85
	2 : 1	30.27 ± 1.39
	3 : 1	28.25 ± 1.36
	4 : 1	33.22 ± 1.77

Date are the mean ± SD of three independent experiments.

The morphology of PEI 1800-TA/pDNA polyplex observed by TEM is shown in Figure 4. The polymers formed well-defined, spherical complexes with pDNA at the representative weight ratio of 2 : 1. Sizes determined by TEM were significantly similar to that measured by dynamic light scattering.

The ζ-potential values of the polyplexes were also estimated at various weight ratios. A positive change of polyplex is considered to be important for adhering to the negatively-charged cellular membrane, leading to efficient intracellular trafficking [23]. The surface charges of the polyplexes were measured and are shown in Table 2. The ζ-potential values of polyplexes were negative at weight ratio of 1 : 1 for PEI 600-TA and 0.5 : 1 for PEI 1800-TA and PEI 25k-TA, and abruptly increased to become positive at 2 : 1 and 1 : 1. The values reached approximately 30 mV at the ranges over weight ratio 10 : 1 for PEI 600-TA and 2 : 1 for PEI 1800–TA and PEI 25k-TA, demonstrating stable complexes formed with pDNA.

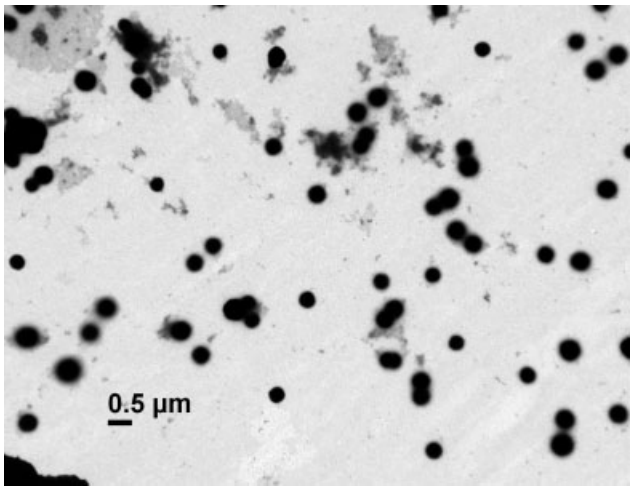

Figure 4. Transmission electron microscope of PEI 1800-TA with pDNA at weight ratios of 2 : 1. Polyplexes were negatively stained by a 2% aqueous solution phosphotungstic acid for 30 s. Scale bar = 500 nm

Cytotoxicity assay

The cytotoxicity of PEI-TA was examined on HEK293 cells and HepG2 cells by the MTT assay. PEI 1800 and PEI 25k were used as a control at weight ratios of 5.2 and 1.33, respectively. As shown in Figure 5, PEI 1800 was relatively nontoxici, although PEI 25k demonstrated serious cytotoxicity and its relative cell viability (RCV) was approximately 60% at the optimal weight ratio. In general, all PEI-TA polymers exhibited a dose-dependent cytotoxicity. At low weight ratios, no significant cytotoxicity was found among these polymers. However, along with the increasing weight ratio, all polymers showed evident toxicity and indicated less than 20% RCV above a weight ratio of 30. There was no significant difference of RCV among the three polymers. The three polymers have relatively low cytotoxicity compared to PEI 25k. Interestingly, the toxicity of PEI25K-TA was substantially lower than that of native PEI 25k.

In vitro transfection experiment

The transfection efficiency of the three PEI-TA polymers in HEK293 cells and HepG2 cells was shown in Figure 6. All the cells were treated *in vitro* by 2 μg/well pDNA complexed with PEI-TA. Gene transfer efficiency was measured as luciferase enzyme expression and normalized to total cell protein. PEI 25k was used as positive control.

The result indicated that PEI 600-TA reached the maximum transgenic expression at weight ratio of 10, whereas PEI 1800-TA and PEI 25k-TA reached their maximum at a relatively lower weight ratio of 2. The transfection efficiency of PEI 1800-TA at its optimal weight ratio was significantly higher than that of PEI 25k, which has been known to be the most potent PEI. Unexpectedly, however, decreased transaction was observed with PEI 25k-TA polymer, which was also lower than that of PEI 1800-TA and PEI 600-TA. The order of transfection efficiency in the two cells was PEI 1800-TA > PEI 600-TA > PEI 25k-TA.

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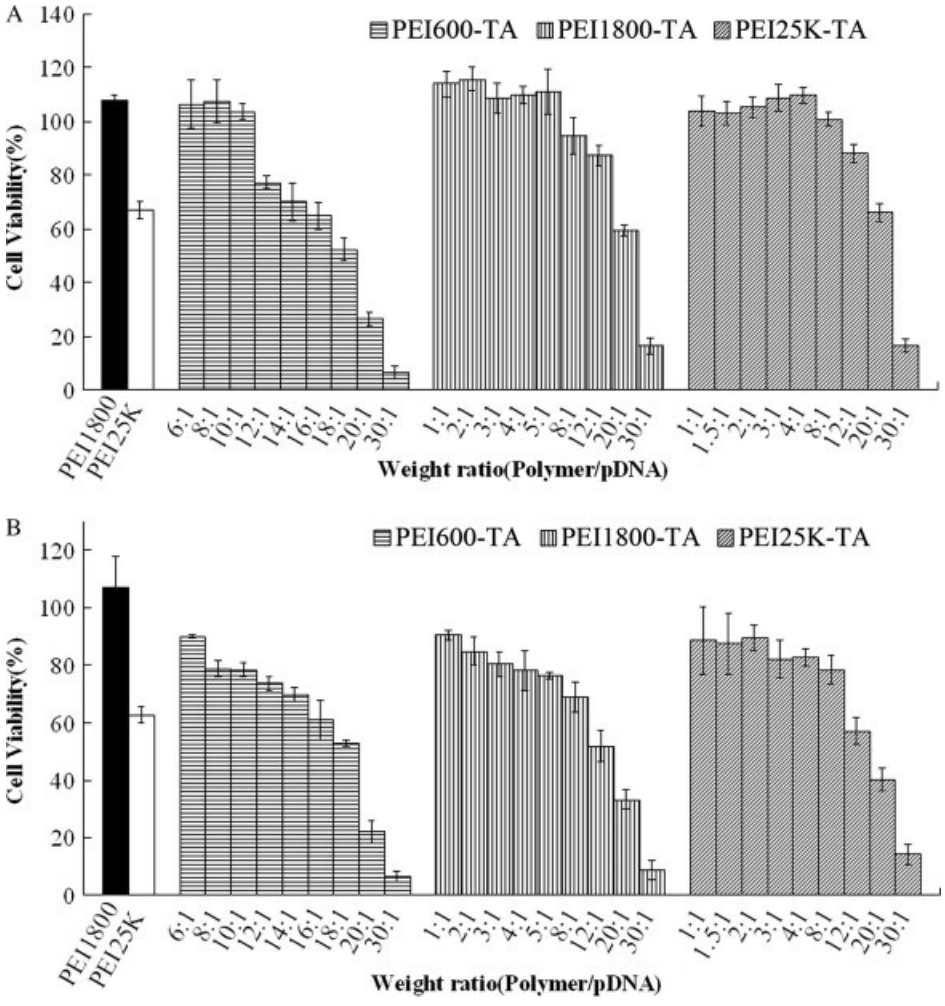

Figure 5. Cytotoxicity of PEI-TA/pDNA polyplexes in HEK293 cells (A) and HepG2 cells (B). PEI 1800 and PEI 25k polyplexes were used as a control at a weight ratio of 5.3 and 1.33, respectively. The total amount of pDNA added to a 96-well plate was 0.2 μg/well. Cell viability was assayed by the MTT assay. Each data points represent the mean ± SD of three experiments

It is well known that cationic particles aggregate significantly in the presence of serum protein. As shown in Figure 7, transfection efficiency of all PEI-TA polymers was dropped significantly in 10% serum in HepG2 cells, indicating that PEI-TA/pDNA polyplexes might aggregate in 10% serum, hindering their transgenic activity as a result. However, luciferase expression of PEI 1800-TA was still relatively higher than other polymer, which supports its potential for application *in vivo*.

Intracellular localization of polyplex

To investigate the subcellular compartment in which the PEI-TA polymers resided, we labeled these polymers with FITC, and the nucleus with Hoechst 33 342, and the representative image was shown in Figure 8. It was revealed that there was a considerable difference between low and high molecular weight PEI. The transfected FITC-labeled PEI 600-TA and PEI 1800-TA were localized in blue-labeled nuclear compartments, whereas transfected PEI 25k-TA was only found around the nucleus. These results indicated that TA residues could efficiently translocate PEI 600-TA and PEI 1800-TA into the nucleus more than PEI 25k-TA.

Competition assay

Furthermore, the competitive study was performed in the presence of TA as a glucocorticoid receptor competitor in HepG2 cells at the optimal weight ratio. The cells were preincubated with TA for 30 min, and both the transfection solution and culture medium contained TA to saturate glucocorticoid receptor. As shown in Figure 9, the gene transfer activities of PEI 600-TA and PEI 1800-TA was decreased drastically, although PEI 25k and PEI 25k-TA were not suppressed much more in the presence of TA. This might mean that TA residues did not facilitate PEI 25k-TA to enter into the nucleus, and had no significant influence on its transgenic activity.

Transgene expression *in vivo*

Finally, the gene delivery of PEI 1800-TA was tested *in vivo*. EGFP plasmid was mixed with PEI 1800-TA

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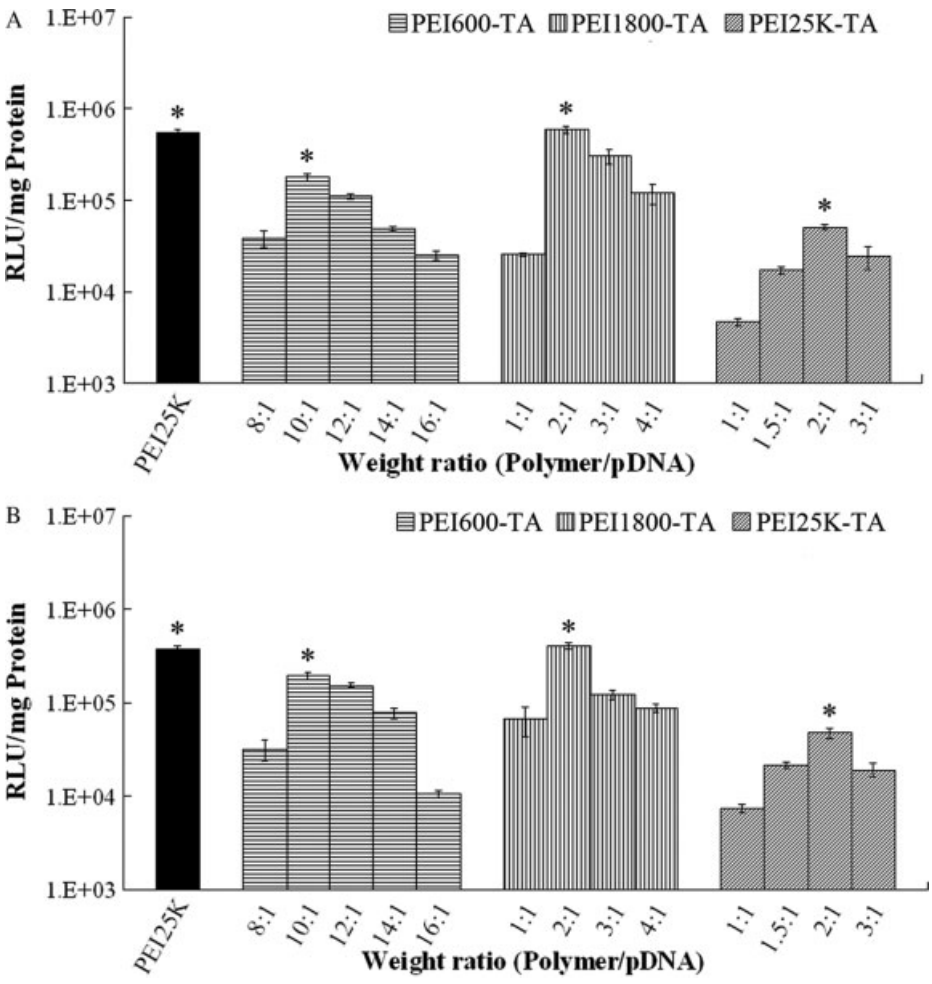

Figure 6. Gene transfer activity of PEI-TA polyplexes in HEK293 cells (A) and HepG2 (B) at various weight ratios. The concentration of pGL-3 was 2 μg/well. PEI 25k was used as a control. Each value represents the mean ± SD of three experiments. *Statistically significant difference ($p < 0.05$)

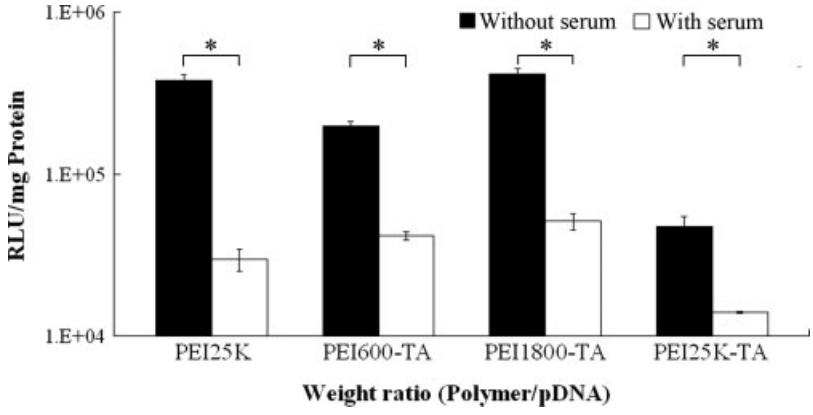

Figure 7. The transfection result in HepG2 cells in the presence of 10% serum. The PEI-TA polyplexes were prepared at the optimal weight ratios, respectively. Each value represents the mean ± SD of three experiments. *Statistically significant difference ($p < 0.05$)

and injected into mice via the portal vein. After 1 day, the liver and other organs were excised and the section was made, which was then observed under fluorescence microscopy. The number of fluorescent colonies in liver of PEI 1800-TA/pDNA was higher than that of PEI 25k/pDNA (Figures 10A and 10B). There was no background fluorescence (Figure 10C). GFP expression was not found in other organs (data not shown). To quantify transfection *in vivo* of PEI 1800-TA, The GFP expression was monitored by flow cytometry (Figure 11). PEI 1800-TA demonstrated a substantially higher transfection outcome in liver than PEI 25k

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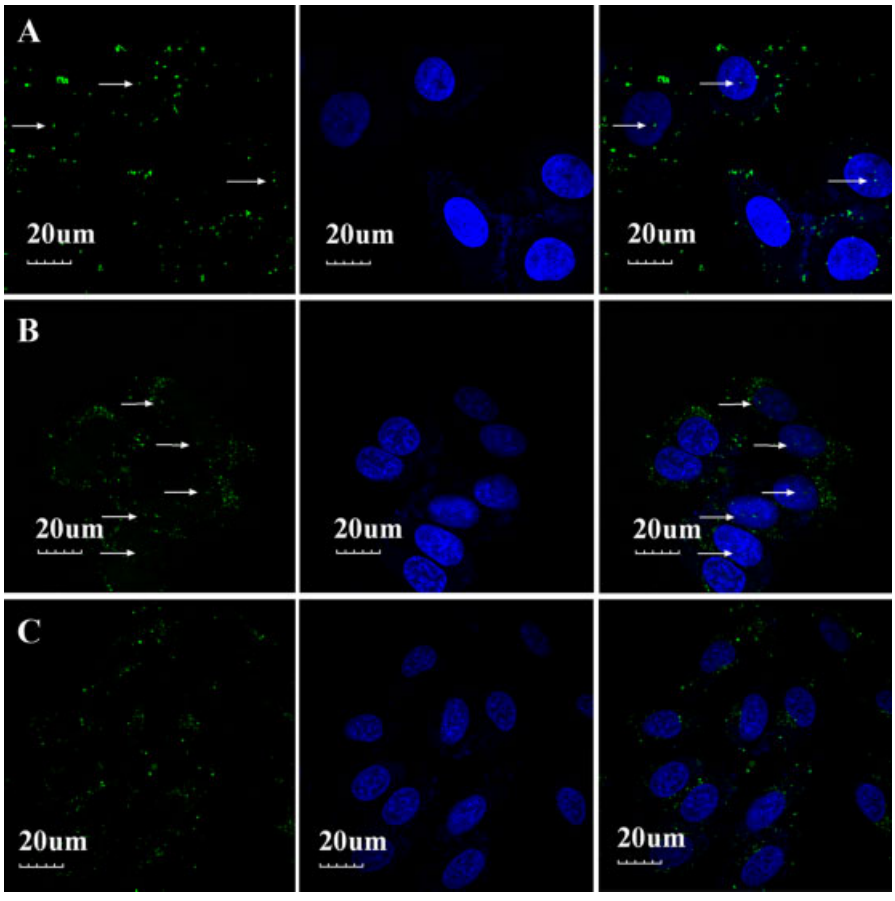

Figure 8. Nuclear localization of PEI-TA polyplexes in HepG2 cells. PEI-TA polymers were labeled with FITC (green) and the nucleus was stained by Hoechst 33342 (blue). The cells were scanned with a confocal fluorescence microscope. (A) PEI 1800-TA; (B) PEI 600-TA; (C) PEI 25k-TA. Left: FITC-labeled PEI-TA polymers; middle: Hoechst 33342 labeled nucleus; right: merged images of the left and middle rows. Scale bar = 20 μm

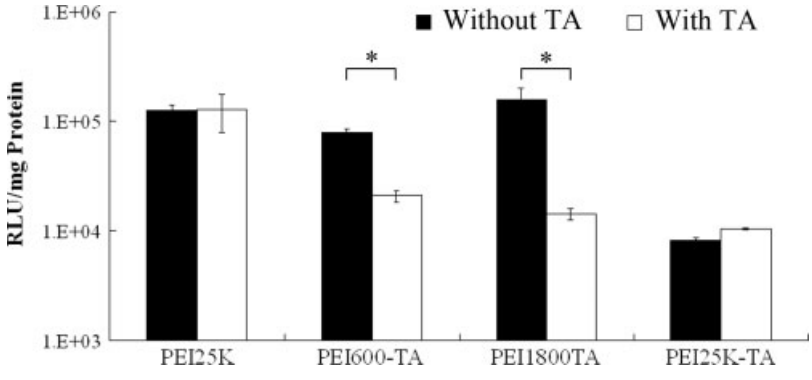

Figure 9. Effects of competitors TA on the transfection efficiency of the PEI-TA polyplexes in HepG2 cells at their optimal weight ratios. The luciferase activity without competitors was used as a control. Each value represents the mean ± SD of three experiments. *Statistically significant difference ($p < 0.05$)

(Figure 11A, curve 3 versus curve 2). The average number of fluorescent cells of PEI 1800-TA was as two-fold higher than that of PEI 25k (Figure 11B). Negative control of liver cells (Figure 11A, curve 1) and other organs showed no fluorescence (data not shown). The above results concerning transfection efficiency as a measure of GFP expression clearly indicated the excellence of PEI 1800-TA over PEI 25k and thereby their potential as a gene carrier *in vivo*.

Discussion

After entering into cell, GC could bind with glucocorticoid receptors, which locate in cytoplasm, and then translocate into the nucleus. In addition, the nuclear pore is dilated up during this process to facilitate the translocation [24]. Thus, GC as NLS could be conjugated with various polycation to enhance its transgenic activity. For combining with polycation, the 21-OH of GC is an

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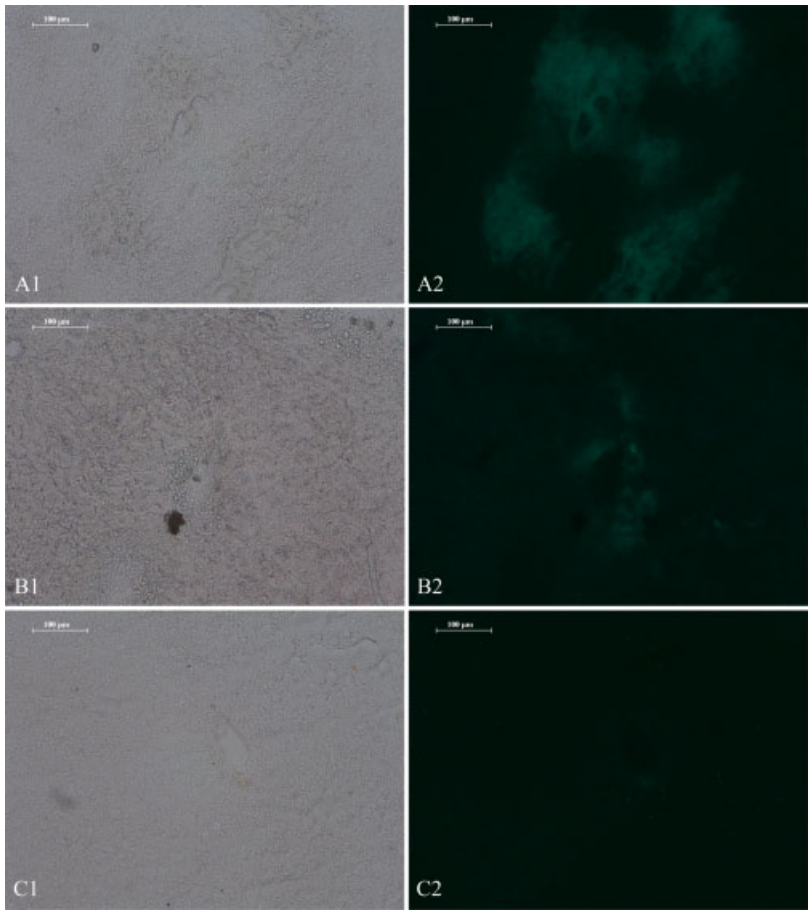

Figure 10. The qualitative evaluation of gene expression *in vivo*. Gene expression in liver of mice treated with PEI 1800-TA/pDNA (A), PEI 25k/pDNA (B) and negative control (C), 24 h after portal vein administration. Frozen sections (5 mm thick) were examined by fluorescent microscopy. Image 1 in (A–C) is the respective bright-field light image; image 2 in (A–C) shows GFP expression in the liver. Scale bar = 100 mm

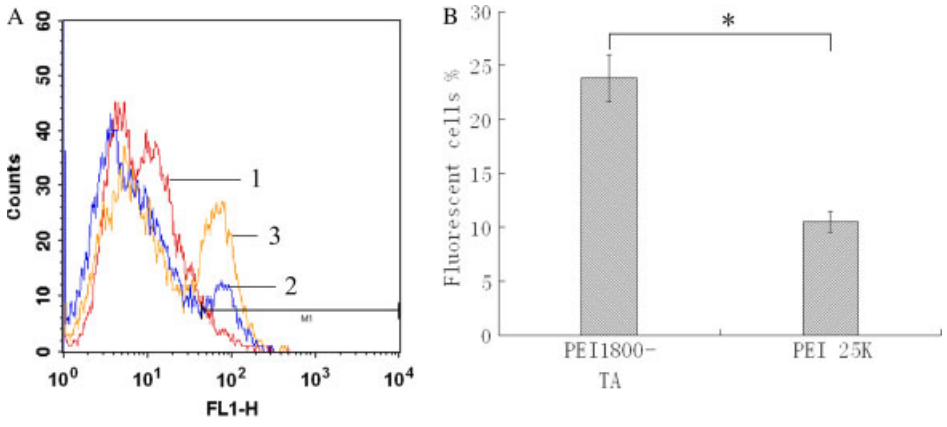

Figure 11. Cell transfection *in vivo* with PEI 1800-TA/pEGFP-N1 complexes and PEI 25k/pEGFP-N1 complexes was quantified by flow cytometry. (A) Flow cytometry data (i.e. the number of fluorescent cells and fluorescence intensity on the EGFP channel) for liver. 1, Negative control; 2, fluorescence of cells treated with PEI 1800-TA/pEGFP-N1 complexes; 3, fluorescence of cells treated with PEI 25k/pEGFP-N1 complexes. (B) Average percentage of EGFP positive cells in liver treated with PEI 1800-TA and PEI 25k respectively. The results are the mean ± SD of at least three mice

ideal choice for substitution because it is not required for pharmacological activity. In the present study, TA was conjugated with various molecular weights PEI to create PEI-TA polymers, and their physicochemical properties and transfection efficiency were investigated.

The study of physico-chemical properties indicated that these polymers had excellent buffering capacity in the endosomal pH range as a result of the interior amines of the PEI backbone. The buffer capabilities of PEI 600-TA, PEI 1800-TA and PEI 25k-TA were almost identical to

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each other. We reasoned that the substituted degree of TA in every ($NHCH_2CH_2$) unit was similar, and that the excessive presence of free prime amines results in similar buffer capability.

Agarose gel electrophoresis showed that the required polymer/pDNA weight ratios were not identical. More PEI 600-TA was needed to condense pDNA completely and PEI 1800-TA and PEI 25k-TA showed relatively higher DNA binding capabilities. The sizes and ζ-potential of PEI 600, PEI 1800 and PEI 25k polyplexes reached extreme values to form stable complexes at different weight ratios, which was consistent with the result of DNA retardation assay by agarose gel electrophoresis.

The transfection efficiency study showed that the optimal weight ratio of the three PEI-TA polymers was different. This result was consistent with the gel retardation result demonstrating that PEI 600-TA has a relatively lower DNA binding capacity among the three polymers. At optimal weight ratios, the three polymers were able to condense pDNA efficiently into nanoparticles, and the ζ-potential of polyplexes reached the approximate maximal value. This means that the size and positive charge of particles might be correlated with gene transfection [25]. It has been reported that a compact and defined-size structure at 100–200 nm was considered to facilitate endocytosis [26], and the positive charge facilitates nonspecific association with the negatively-charged membranes of cells followed by cellular uptake through internalization mechanisms [27].

Low molecular weight PEI decorated with GC demonstrates excellent gene expression [8–14]. After being modified by TA, however, various molecular weights PEI showed different transfection efficiency. The polyplexes prepared with low molecular weight PEI showed enhanced transfection efficiency, although that of PEI 25k-TA was diminished. Therefore, we further investigated the transfection mechanism of PEI-TA aiming to explain the transgenic activity change of different PEI-TA.

Confocal microscopy showed that PEI 600-TA and PEI 1800-TA could accumulate efficiently in the nucleus. This indicated that TA residues could more efficiently translocate low molecular weight PEI into the nucleus than high molecular weight PEI. This finding explains why low molecular weight PEI polyplexes showed obvious transfection efficiency [21]. However, PEI 25k-TA failed to translocate into nucleus, so that its transfection efficiency was diminished rather than enhanced.

Competition transfection experiments were performed by adding TA as a glucocorticoid receptor competitor. In the presence of TA, transgenic activity of low molecular weight PEI-TA was inhibited considerably, but that of PEI 25k-TA was not affected obviously. Therefore, we suggest that the enhancing mechanism of PEI 600-TA and PEI 1800-TA for gene transfer activity might be ascribed to glucocorticoid receptor-mediated nuclear translocation. After conjugation with TA, low molecular weight PEI polyplex could be carried into the nucleus and result in a pronounced transfection efficiency. However, PEI 25k-TA polyplexes did not display an inhibited transfection activity in the presence of TA. This result means that conjugated TA residues did not facilitate PEI 25k to enter into the nucleus, and had no significant influence on its transgenic activity.

A similar decrease was revealed in other studies [28,29]. Branched PEI 25k-ligand conjugate also did not reveal significantly higher gene transfer activity than branched PEI 25k in another study [30]. Furthermore, the toxicity of PEI 25k-TA was attenuated according to the above cytotoxicity experiment, and this could be explained by a reduced interaction of endogenous nucleic acids with the modified PEI.

It has been reported that the structure and other properties of synthetic gene carriers, including size, shape, side groups and charge, affect complexation of nucleic acids, intracellular pathways, and hence the overall delivery efficiency [31–33]. Thus, the intracellular fates of polyplexes for controlled therapeutic effects could be tuned by structural modification of the polymer [34,35]. This means that the molecular structure could determine its intracellular localization and nuclear delivery of PEI-TA might be found to be related to the molecular weight of PEI.

In vivo gene transfer requires not only the ability of a vector to introduce gene into the cells, but also an efficient delivery of gene to the vicinity of the cells. After administration into the blood circulation, the pDNA complex interacts with various cells and molecules, such as serum proteins [36,37] and erythrocytes. The cationic nature of the complex attracts negatively-charged cells and molecules that alter the physico-chemical properties of the complexes. When injected into the portal vein of mice, PEI 1800-TA and PEI 25k/pEGFP-N1 complexes showed a relatively high transgene expression in liver. Thus, the data strongly suggested that the transfection efficiency of PEI 1800-TA was much superior to that of PEI 25k. This is consistent with the result obtained *in vitro*. The transgene expression of complexes was not found in other tissues [21]. This suggests that the interaction between complexes with high ζ-potential and various cells in blood might influence their transfection activity. TA has a versatile pharmacological action as a potent corticosteroid. It could be used as antasthmatic or anti-rheumarthritis reagent. Normally, it has a low risk of serious or long-term side-effects if used under normal medical supervision. Therefore, it can be concluded that PEI 1800-TA is a promising candidate as a gene carrier.

Supporting Information

Supporting information may be found in the online version of this article.

Acknowledgements

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