

PAMAM–Triamcinolone acetonide conjugate as a nucleus-targeting gene carrier for enhanced transfer activity

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ABSTRACT

The excellent transfection efficiency and viability are essential for successful gene therapy. It is suggested that when bound to its glucocorticoid receptor, glucocorticoid steroid can dilate the nuclear pore complexes and facilitate the transport of pDNA into the nucleus. In this research, the two different degrees of substitution of PAMAM–triamcinolone acetonide (PAMAM–TA) conjugates were synthesised for efficient translocation of pDNA into the nucleus. The physicochemical properties of the polyplexes were investigated by agarose gel electrophoresis, Zeta-sizer and TEM. They both could form nano-size polyplexes with pDNA. The polyplexes were very stable and showed excellent buffering capacities, facilitating endosomal escape, and no obvious difference was found between them. The TA-conjugated PAMAM-mediated transfection of luciferase and EGFP genes showed better transfer activity than native PAMAM and was comparable to the PEI 25K (polyethylenimine), and lower cytotoxicity in HEK 293 and HepG 2 cells. Even with 10% serum, their transfer activity was still high relatively. In addition, confocal microscopy examination confirmed that the enhancing mechanism for enhanced gene transfer activity of PAMAM–TA conjugate may involve the nuclear translocation of the polyplex. The low substituted degree of TA to 0.22 did not interrupt its nuclear localization potency. These findings demonstrated that the TA-grafted PAMAM dendrimer is a potential candidate as a safe and efficient gene delivery carrier for gene therapy.

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1. Introduction

Both the advances in molecular biology and biotechnology, along with the completion of the Human Genome Project have led gene therapy to a new level, being an alternative treatment of genetic diseases such as haemophilia, muscular dystrophy or cystic fibrosis [1,2]. As a result, the need to develop efficient, reliable and safe gene (RNA or DNA) delivery systems continues to increase with the development of applications for gene therapy. Viral vectors have been shown to be dominant gene delivery carriers due to their high gene transfer efficiencies. They have been used in the majority of gene delivery studies reported in the literature and about 70% of ongoing clinical trials [3]. However, there still exists the possibility that the viral gene carriers will insert the recombinant virus in the initial coding region of a gene. Furthermore, viruses are inherently immunogenic, leading to difficulty with repeated administrations and the high possibility of immune reactions [4]. On the other hand, Non-viral vectors, provide advantages such as improved safety,

greater flexibility and more controllable manufacturing. But its low transfection efficiency compared to viral vectors hampers the clinical application.

Nuclear membrane is one of the main barriers in polymer-mediated intracellular gene delivery [5]. And it was previously reported that glucocorticoid receptor dilated the nuclear pore to 60 nm and translocated into nucleus when it bound to its ligand, glucocorticoid, as a nuclear localization signal (NLS) [6]. This suggests that the transport of DNA into nucleus may very possibly be facilitated by glucocorticoid.

Poly(amidoamine) (PAMAM) dendrimers were first introduced by Tomalia and co-workers in the mid-1980s [7]. At present, PAMAM dendrimer and polyethylenimine (PEI) have been tested for their potential utility and have exhibited relatively high transfection efficiencies in vitro while PEI showing some promising results in vivo [8]. Dendrimers are core-shell nanostructures with precise structure and low polydispersity, which are synthesized in a layer-by-layer fashion, expressed in generations, around an ethylenediamine or ammonia core unit. The three main properties of dendrimers are nanoscale container properties for drugs or genes, nano-scaffolding properties for producing prodrugs, and biocompatibility for adaptability [9–11]. Interestingly, it was reported that

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the extent of transfection was dependent on the dendritic generation of the PAMAM employed, lower generation PAMAM dendrimers were ineffective for transfection [12]. The high level of control over the dendritic architecture (size, branching density, surface functionality) makes dendrimers been researched as ideal gene carriers in the past decades [13–16].

In our previous research, we synthesized five kinds of glucocorticoid-PEI (GC-PEI) conjugates and discussed the relationship between structure and transfection activity [17]. Choi et al. conjugated dexamethasone, a potent glucocorticoid, to PAMAM G4 dendrimer to facilitate nuclear translocation and enhanced the transfection efficiency [18]. Here, we combined a glucocorticoid of high potency (triamcinolone acetonide, TA) with PAMAM dendrimers to form a novel non-viral gene vectors (PAMAM-TA). And we investigated the polyplex formation, biosafety and gene delivery efficiency, and discovered the impact of substituted degree onto transfection effects. The intracellular localization of PAMAM-TA/pDNA polyplexes was also examined by confocal microscopy.

2. Materials and methods

2.1. Materials

PAMAM dendrimers (ethylenediamine core, G4), polyethylenimine (PEI, branched, 1800 and 25 kDa), 2-iminothiolane (Traut's reagent), fluorescein isothiocyanate (FITC), 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), ethidium bromide were purchased from Sigma-Aldrich Chemical Co., Ltd.

(Milwaukee, WI, USA). RPMI 1640, penicillin-streptomycin (PS, 10,000 U/mL), trypsin-EDTA (TE, 0.5% trypsin, 5.3 mM EDTA tetra-sodium) were obtained from Gibco BRL (Gaithersburg, 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). Triamcinolone acetonide (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 33342 were purchased from Beyotime Institute of Biotechnology (Jiangsu, China). Plasmid DNA (pEGFP-N1 and pGL-3) was kindly provided by Institute of Infectious Diseases, Zhejiang University (Hangzhou, China). The plasmids were propagated in *Escherichia coli* DH5 α , isolated, and purified using Axyprep Plasmid DNA Maxiprep Kit (Axygene Biotechnology Limited, Hangzhou, China). The purity and concentration of DNA were determined by measuring a UV absorbance 260 and 280 nm. All other chemicals were of analytical grade.

2.2. Synthesis of PAMAM-TA

PAMAM-TA was synthesized via a two-step reaction as shown in Fig. 1. Firstly, we substituted its 21-hydroxyl groups with mesylates to activate triamcinolone acetonide. Briefly, methanesulfonyl chloride (41.5 μ L, 0.536 mmol) was added drop wise to a solution of TA (116.5 mg, 0.268 mmol) in anhydrous pyridine (2.5 mL) at 0 °C under N₂ with stirring. After reacting for 5 h at 0 °C, ice water (50 mL) was added. The precipitate was filtered, washed with more ice water, crude triamcinolone acetonide mesylate (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 activation. The product was solubilized in DMSO-*d*₆ for ¹H NMR analysis (500 MHz, Bruker, Germany).

Then, the conjugation reaction was followed by the procedures as previously report with some modification [18,19]. Traut's reagent and TA-mesylate (2 equiv. or

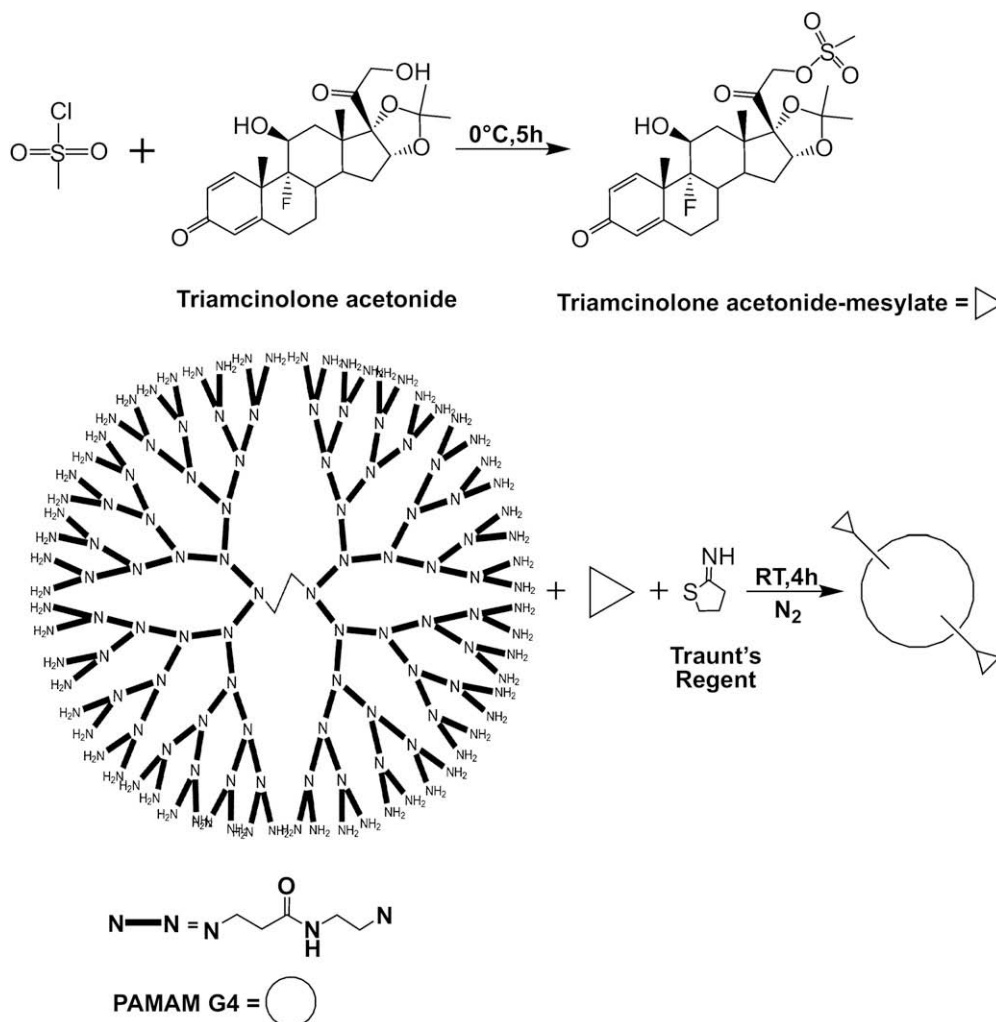


Fig. 1. Synthetic scheme of PAMAM-TA polymers.

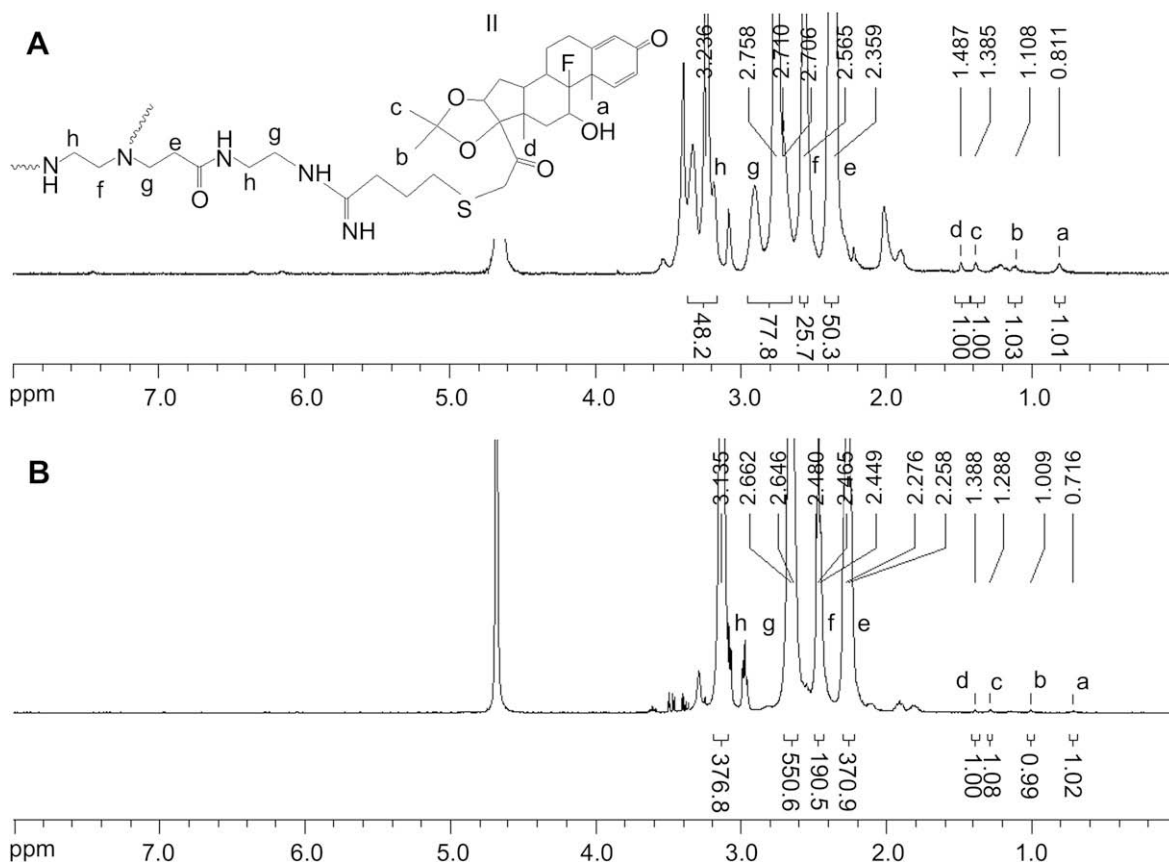


Fig. 2. The ¹H NMR spectrum of PAMAM-TA-H (A) and PAMAM-TA-L (B).

1 equiv. to PAMAM G4) in 3.0 mL anhydrous DMSO were added slowly with 1 equiv. of PAMAM in 2.0 mL anhydrous DMSO. The reaction was allowed to proceed under N₂ 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 dialyzed against pure water using dialysis membrane (MWCO 3500) for 48 h, the dialysis medium was refreshed every 12 h. A white product (PAMAM-TA) was obtained after further freeze-drying. The structure of product was confirmed by using ¹H NMR (500 MHz, Bruker, Germany, D₂O).

2.3. The buffering capacity of the PAMAM polymers

Polymer solution was prepared in a 50 mL flask (0.2 mg/mL, 30 mL) and pure water was used as a control. After adjusting the initial pH to 10.0 with 0.1 M NaOH if necessary, 25 mL increments of 0.1 M HCl were titrated into the solution and the pH response was measured by a micro-pH electrode at the same time. The whole pH variation was recorded from 10.0 to 3.0.

2.4. Agarose gel electrophoresis

Polyplexes were prepared at various weight ratios between each of 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 on a UV illuminator to show the location of the DNA.

2.5. Size and ζ-potential measurements

The size and ζ-potential values of polyplexes were determined by Malvern Zetasizer 3000HAs system (Malvern Instruments Ltd., U.K.). Polyplexes were formed at a final concentration of 10 μg/mL pDNA at various weight ratios in water for size measurements and in HBS (25 mM HEPES, 150 mM NaCl, pH 7.4) for ζ-potential experiments, respectively. The size and ζ-potential values were presented as the average values of 3 assays.

2.6. Transmission electron microscope (TEM)

The morphology of polyplexes with an optimal weight ratio was observed using TEM (JEM 1230, JEOL, Japan). One drop of polyplex 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 was then examined with the electron microscope.

2.7. In vitro transfection experiment

We next examined the ability of PAMAM-TA to transfect human embryonic kidney 293 cells and human liver carcinoma HepG 2 cells using plasmid DNA that contain the firefly luciferase and EGFP gene. The cells were seeded at a density of 1×10^5 cells/well in 24-well plate in RPMI 1640 medium containing 10% FBS, and

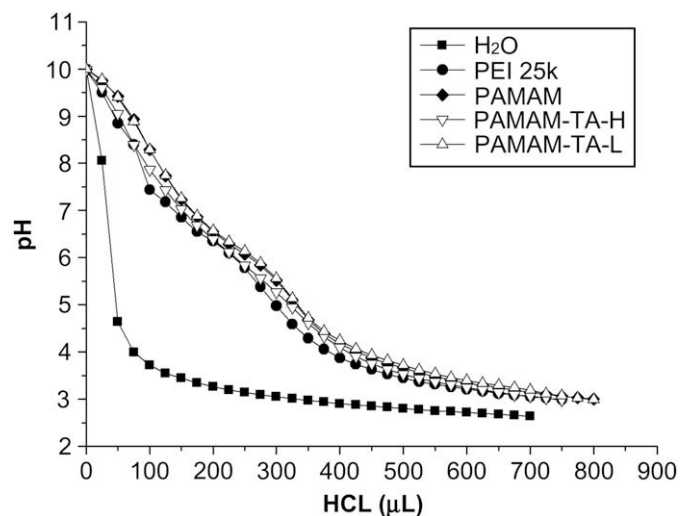


Fig. 3. Determination of the buffer capacity of PEI 25K and PAMAM-TA series by acid-base titration. Solutions containing each polymer (0.2 mg/mL) were adjusted to pH 10.0 and then titrated with 0.1 M HCl from 10.0 to 3.0.

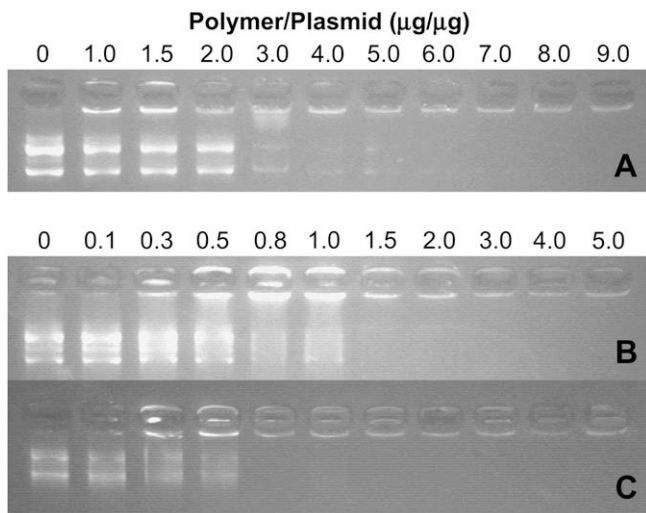


Fig. 4. Agarose gel electrophoresis retardation assay of polyplexes at various weight ratios: PAMAM (A), PAMAM-TA-H (B), PAMAM-TA-L (C). The mixtures were incubated at room temperature for 30 min and electrophoresis on 1% (W/V) agarose gel and stained with ethidium bromide.

grown to reach 80% confluence prior to transfection. Before transfection, the medium was exchanged with fresh medium with or without 10% FBS. The cells were treated with polyplex solution containing 2 μg of pDNA at various weight ratios for 4 h at 37 °C and the final volume was adjusted to 500 μL by medium. After exchanging with a fresh medium with 10% FBS, cells 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 rpm for 5 min. Luciferase activity was measured with a luminometer (Turner Designs Luminometer Model TD-20/20, Promega). The total protein was determined by BCA protein assay kit. The final luciferase activity was expressed as RLU/mg protein. Inverted fluorescent microscope (Leica DMI 4000 B, Germany) was used to observe the EGFP expression of the polyplexes in 293 cells.

2.8. Cytotoxicity assay

The cytotoxicity of the polymers was measured by MTT assay. Briefly, HEK 293 cells and HepG 2 cells were seeded at a density of 1×10^4 cells/well in 100 μL of growth medium in 96-well plates (Corning), and were incubated for 24 h before adding the polymers. One day later, the cells were transfected with polyplexes at various weight ratios. The PEI 25K and PEI 1800 polyplexes were as control at weight ratios of 1.33:1 and 5.3:1 [20]. 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) solutions and 100 μL of fresh medium

Table 1

Particle size of the pDNA complexes with PAMAM, PAMAM-TA-H and PAMAM-TA-L. Each data is the mean \pm SD ($n = 3$).

	Weight ratio	Size
PAMAM	2:1	236.3 \pm 7.6
	3:1	185.4 \pm 11.7
	4:1	110.5 \pm 7.3
	5:1	133.3 \pm 6.4
	6:1	167.0 \pm 2.0
PAMAM-TA-H	1:1	275.3 \pm 19.2
	1.5:1	196.3 \pm 17.6
	2:1	161.3 \pm 5.5
	3:1	157.7 \pm 8.5
	4:1	167.3 \pm 10.2
PAMAM-TA-L	1:1	248.7 \pm 1.5
	1.5:1	182.7 \pm 3.1
	2:1	187.0 \pm 5.3
	3:1	181.0 \pm 7.0
	4:1	180.3 \pm 2.3

Table 2

ζ -potential of the pDNA complexes with PAMAM, PAMAM-TA-H and PAMAM-TA-L. Each data is the mean \pm SD ($n = 3$).

	Weight ratio	ζ -potential (mV)
PAMAM	2:1	-22.4 \pm 1.8
	3:1	-7.0 \pm 1.2
	5:1	17.5 \pm 2.3
	6:1	18.4 \pm 1.9
	7:1	21.4 \pm 2.7
PAMAM-TA-H	0.5:1	-19.8 \pm 1.4
	1:1	-3.9 \pm 2.4
	2:1	14.8 \pm 1.6
	3:1	18.0 \pm 1.9
	4:1	18.5 \pm 2.2
PAMAM-TA-L	0.5:1	-16.8 \pm 1.4
	1:1	1.3 \pm 1.0
	2:1	15.6 \pm 1.0
	3:1	18.9 \pm 1.3
	4:1	19.5 \pm 1.7

without serum and further incubated for 4 h. After that, the medium was removed and 100 μL DMSO was added. After shaking the plate for 20 min, absorbance was immediately measured at 570 nm using an ELISA plate reader (Thermo Multiskan Spectrum, USA). Cells incubated without polymer were used as a blank control.

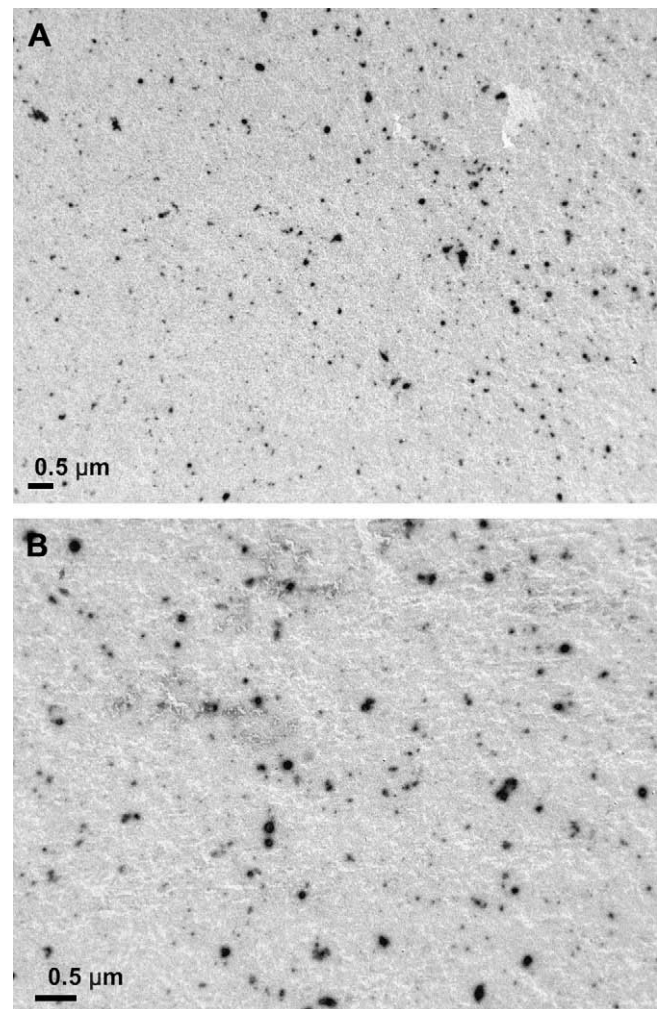


Fig. 5. Morphology of PAMAM polyplex (A) and PAMAM-TA-H polyplex (B) at weight ratio of 5 and 3 as observed by transmission electron microscope. The particles were negatively stained by a 2% aqueous solution phosphotungstic acid for 30 s.

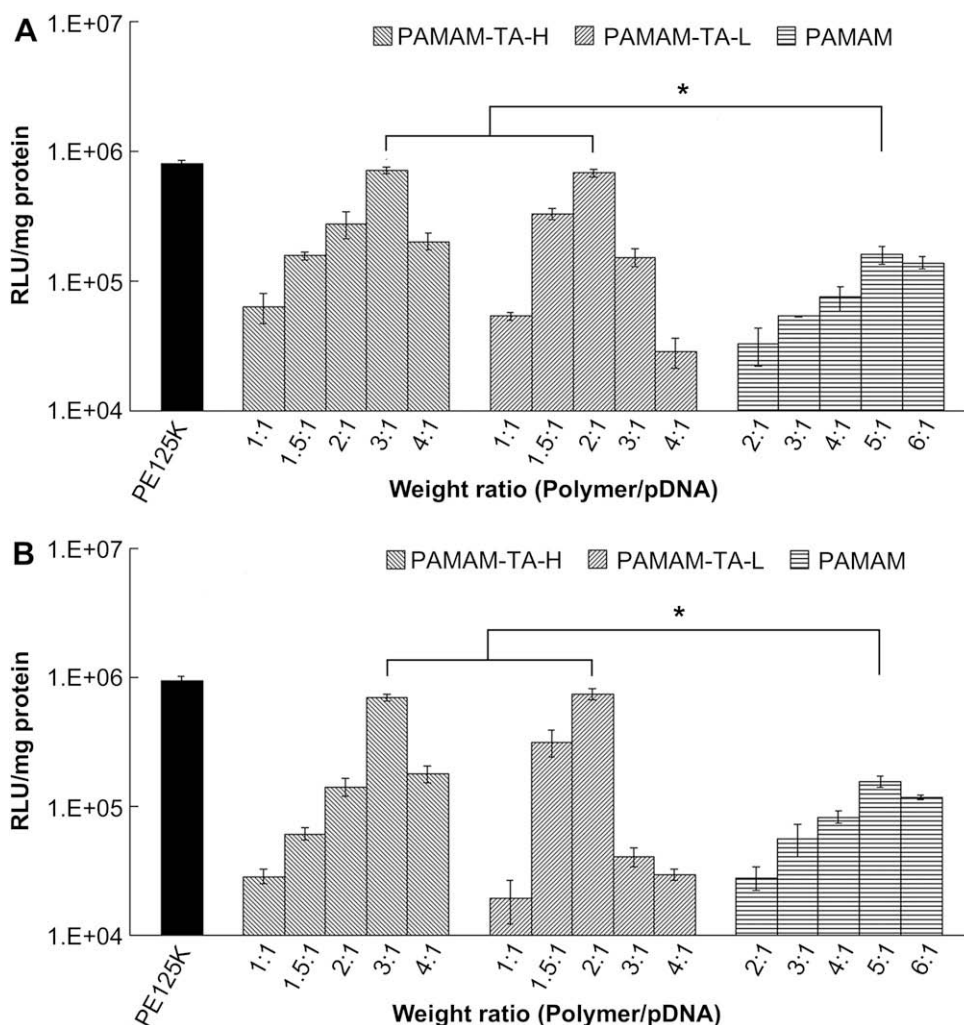


Fig. 6. Transfection efficiency of PAMAM and PAMAM-TA polymers in HEK 293 cells (A) and HepG 2 cells (B). PEI 25K was used as control. The polyplexes at various weight ratios containing pGL-3 were added to 1×10^5 cells/well in 24-well plate for 4 h. The pDNA's concentration was 2 $\mu\text{g}/\text{well}$. Cells were harvested after 48 h transfection, luciferase activity was examined. Relative light units (RLU) were shown as means \pm SD of triplicates. (*) indicated statistically significant difference ($P < 0.05$).

2.9. Fluorescence labeling of dendrimers

The dendrimers were labeled with FITC as former report with some modification [17,21]. PAMAM or PAMAM-TA was dissolved in PBS (pH 7.4). FITC solution dissolved in DMSO was added dropwise to unlabeled dendrimers solution. The molar ratio of dendrimer and FITC taken was 1:20. The solution was incubated overnight at room temperature with continuous stirring in the dark. The labeled dendrimers solution was dialyzed against PBS (pH 7.4) for 48 h and then distilled water for 24 h 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.

2.10. Intracellular localization of polyplex

HepG 2 cells were seeded at a density of 2×10^5 cells/well on the surface of a cover slide in 6-well plate in 2 mL medium containing 10% FBS before transfection. After incubating for 24 h, the cells were treated with polyplexes solution and further incubated for 4 h. The polyplex solutions containing 3 μg of pGL-3 were prepared by mixing FITC-labeled PAMAM, PAMAM-TA-H, PAMAM-TA-L and pDNA at weight ratios of 5:1, 3:1 and 2:1, respectively. Then, the cell grown 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 nuclei, the cells were incubated with Hoechst 33342 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 with a confocal fluorescence microscopic system (Olympus FV1000-IX81, Tokyo). A UV laser (405 nm excitation) was used to induce the blue fluorescence of Hoechst 33342 and an argon laser (473 nm) to excite the green fluorescence of FITC.

2.11. Statistical analysis

Statistical analysis was performed using one-way analysis of variance (ANOVA). Differences between groups were considered statistically significant at $P < 0.05$ (*).

3. Result and discussion

3.1. Synthesis and characterization of PAMAM polymer

It was reported that GC can facilitate the transgenic expression as a nuclear translocation signal [22–24]. Recently, different commonly used GCs were conjugated to low molecular weight PEI and their transfection activity was examined. The present study investigated the possibility of creating a potent transfection agent by grafting TA onto the dendritic surface of the PAMAM dendrimer (generation 4), which is commercially available at a relatively low cost and contains a reasonable number of tertiary amines that are believed to contribute to the endosome-buffering effect.

The 21-hydroxy group of TA is not required for pharmacological activity and was therefore a reasonable choice for conjugation to a polycation. The TA was reacted with methanesulfonyl chloride under mild condition to generate C₂₁-substituted TA [25]. Triamcinolone acetonide mesylate (TA-mesylate): ¹H NMR (500 MHz, DMSO-d₆) δ 0.81 (s, C-19, CH₃), 1.12 (s, C-25, CH₃), 1.33 (s, C-26, CH₃),

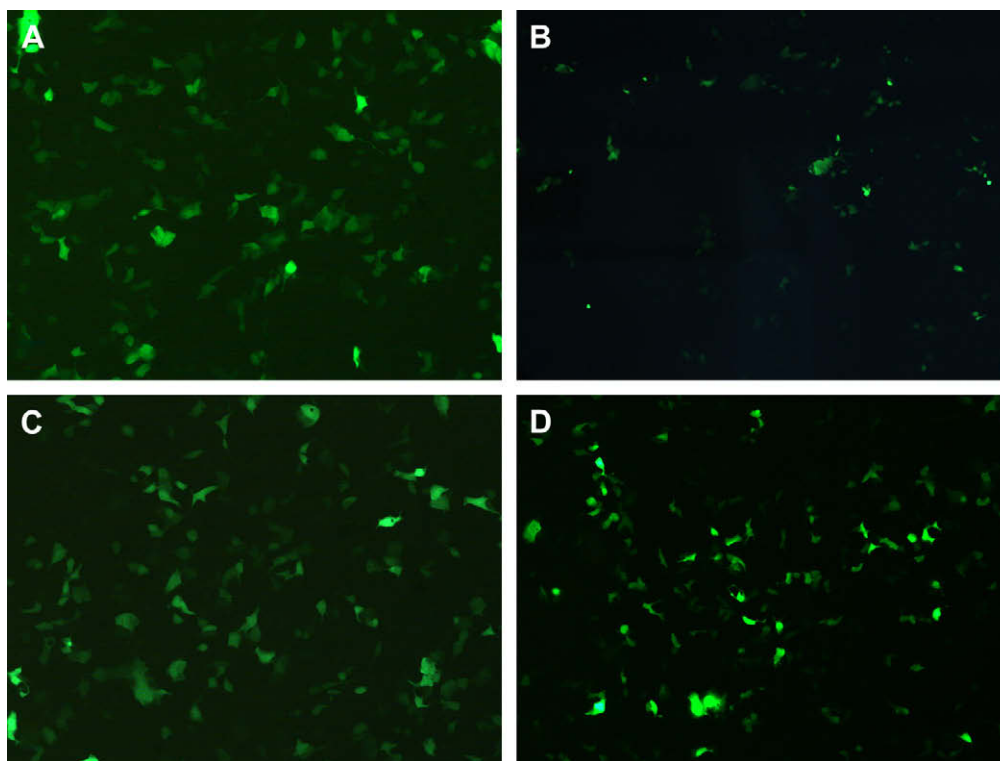


Fig. 7. Comparison of transfection efficiency of HEK 293 cells mediated by PEI 25K (A), PAMAM (B), PAMAM-TA-H (C), PAMAM-TA-L (D) at optimal weight ratio using EGFP report gene. HEK 293 cells (1×10^5 cells/well) were treated with polyplex containing $2 \mu\text{g}$ of pDNA containing EGFP gene.

1.47 (s, C-18, CH_3), 3.29 (s, $-\text{OSO}_2\text{CH}_3$), 6.00 (s, C-4, CH), 6.22 (m, C-2, CH), 7.28 (d, C-1, CH). Then a one-pot reaction between PAMAM, 2-iminothiolane (Traut's reagent) and TA mesylate yielded PAMAM-TA polymer. The proportion of reagents was controlled different with 2 or 1 equiv. Traut's reagent and TA mesylate to 1 equiv. PAMAM so that the substituted degree of TA was different from each other. The synthesis of PAMAM-TA polymer was confirmed by proton NMR spectrum. The ^1H NMR spectra were shown in Fig. 2.

Two different degrees of substitution of PAMAM polymers were synthesized. The number of grafted TA was calculated by comparing the NMR peak intensities between the protons of PAMAM and TA. It was observed that 1.65 TA residues were found in high substituted PAMAM polymer (PAMAM-TA-H) and 0.22 TA residues in low one (PAMAM-TA-L).

3.2. The buffering capacity of the PAMAM polymers

PAMAM has similar protonation capability with its grafted polymers (Fig. 3) both at relatively high and low substitution degrees. All of them also appear to buffer better at neutral medium. Since molecules entering cells on endocytic pathways will experience a drop in pH from neutral to 5 [26], buffer capability of therapeutic gene vectors is vital to the molecules escaping the endosomes of cells. The proton sponge nature of polymers ensures buffering inside the endosomes, resulting in the degradation of lysosomes so that the genes can be protected and functional. The two kinds of polymers had relatively high buffer capability in the pH range of 4–7 compared to pure water so that we considered they are sufficiently suitable for gene transfection.

3.3. Agarose gel electrophoresis

The self-assembly of PAMAM polymer and pDNA was examined by agarose gel retardation assay. The DNA was condensed by the

positively charged polymer at increasing weight ratios. Non-migration of pDNA in the gel indicated the formation of cationic polyplexes by PAMAM polymers. As shown in Fig. 4, PAMAM were able to retard pDNA completely at a weight ratio of 4. PAMAM-TA-H and PAMAM-TA-L can retard pDNA from a weight ratios of 1.5 and 0.8, respectively. The result showed that more PAMAM-TA-H was needed to condense pDNA completely than PAMAM-TA-L. The reason was possibly that more primary amines on the surface of PAMAM-TA-H were substituted by TA so that less positive-charge primary amines can complex with negative-charge pDNA phosphates.

3.4. Polyplex size, ζ -potential measurements and transmission electron microscope (TEM)

The particle size and zeta potential of the polyplexes were examined at the various weight ratios (Tables 1 and 2). With increasing PAMAM polymer/DNA weight ratios, the particle size decreased and the zeta potential increased, showing that polymers with more charge could more efficiently condense DNA. As shown in Table 1, above weight ratio of 4, the size of PAMAM/pDNA was found to reach around 100 nm and no further reduced. In the case of PAMAM-TA polymer, the sizes of the polyplexes were less than 200 nm above weight ratio of 1.5 which was similar with PAMAM polyplex. The results indicated that all PAMAM polymers can concentrate pDNA into nanoparticles of around 100 nm in diameter. The size of the polyplex was confirmed by TEM (Fig. 5). At the weight ratio 5 and 3, the complexes of pDNA with PAMAM and PAMAM-TA-H exist in the form of spherical nanoparticles of about 100 nm which is consistent with the result of dynamic light scattering. Polyplexes with size at 100–200 nm are thought to be suitable for an efficient gene delivery [27].

Positively charged surfaces of polyplexes are thought to be important for their adsorption to negatively charged cellular membranes followed by cellular uptake through internalization mechanisms [28]. As shown in Table 2, the net surface charge of the

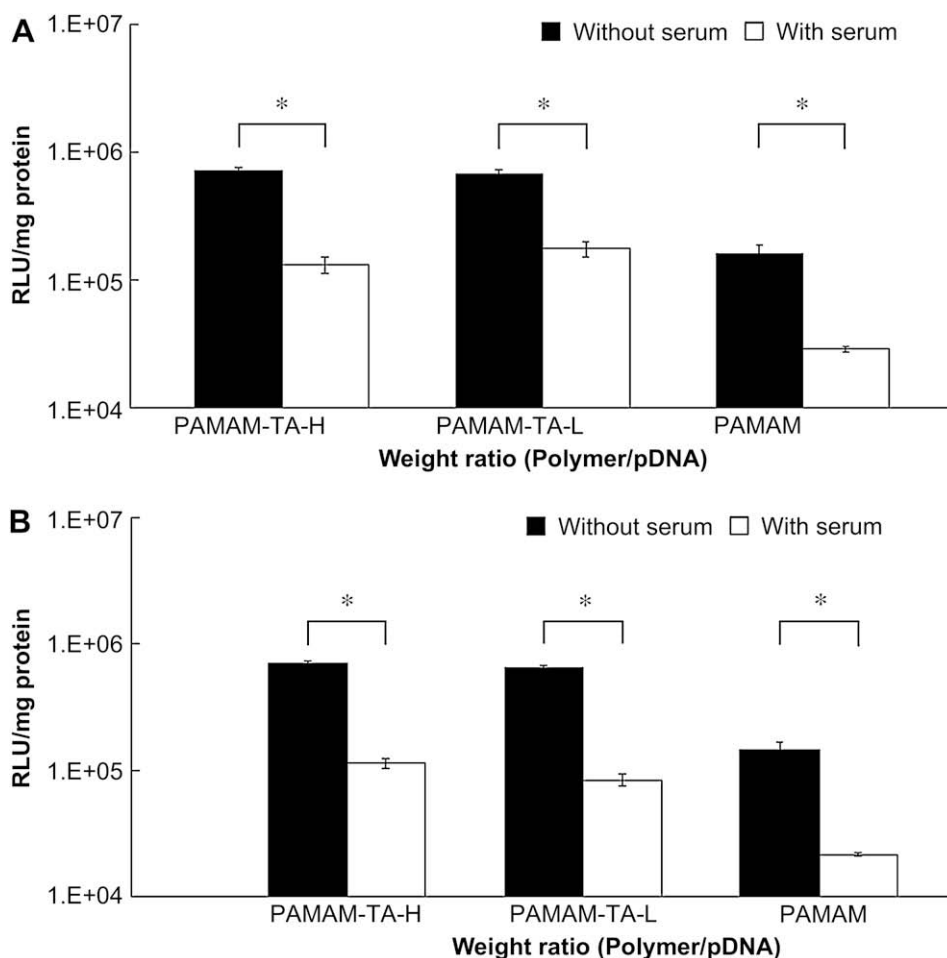


Fig. 8. Comparison of transfection efficiency for HEK 293 cells (A) and HepG 2 cells (B) at 2 µg/well in the presence of 10% serum among PAMAM-TA-H, PAMAM-TA-L and PAMAM at optimal weight ratio of 3, 2 and 5, respectively. Luciferase activity was shown as means \pm SD ($n = 3$). (*) indicated statistically significant difference ($P < 0.05$).

polyplex was negative at low weight ratio, but the zeta potentials of all polyplexes were gradually increased in accordance with the increase of charge ratios. Then, the zeta potential values of PAMAM and PAMAM-TA polyplexes were maintained constantly about 20 mV over weight ratio of 5 and 2. The zeta potential of all PAMAM polymers will give rise to similar affinity for anionic cell surfaces and in turn facilitate uptake into the cell.

3.5. *In vitro* transfection experiment

Polycation modified with glucocorticoid (GC) have been recently reported to be effective gene carriers and those polymers showed a high level of transfective activity with nuclear translocation [18,19,22–24,29–31]. The common characteristic of those systems is thought to be that the GC residues are on the surface of the polymers and their transfection activity was enhanced remarkably. And their transfection efficiency was correlated closely with their GC residue's binding affinity with glucocorticoid receptor (GR) [17]. We deduced that if GC residue could be more potent, the transfective activity might be more pronounced. Thus, triamcinolone acetonide (TA) which has great binding affinity with GR [32] was conjugated with PAMAM dendrimer, known as low toxicity and locating in cytoplasm [33], in order to achieve significant gene delivery potency.

The transfection activity of the newly synthesized PAMAM polymers was investigated in HEK 293 and HepG 2 cells using pGL-3 and pEGFP-N1 reporter gene. PEI 25K polyplexes were prepared at the optimal weight ratio (1:33) demonstrating high transfection

efficiency as positive control. PAMAM polyplexes were prepared at various weight ratios ranging from 2 to 6, and PAMAM-TA polyplexes were from 1 to 4.

As shown in Fig. 6, the PAMAM, PAMAM-TA-H and PAMAM-TA-L polyplexes exhibited the maximal transfection efficiency at the weight ratio of 6, 3 and 2, respectively. The transfection efficiency of PAMAM-TA-H and PAMAM-TA-L showed essentially more than 4 times as that of native PAMAM, which was comparable to PEI 25K at the optimal conditions. The transfection efficiency of native PAMAM (G4) was over 5–6 times less than that of PEI 25K in both cells, which was similar with the former report [34]. The difference of optimal weight ratio between PAMAM-TA-H and PAMAM-TA-L might be caused by the charge density difference between them. The primary amines on the surface of PAMAM-TA-H polymer were substituted by TA more than that on PAMAM-TA-L, as a result, higher amounts of polymers with higher degree of substitution was needed to achieve higher transgenic expression. The result corresponded with the result of agarose gel electrophoresis. The weight ratio of the highest transgenic expression was higher than that of polyplex formation in agarose gel electrophoresis. This may suggest that lower amounts of polymers were sufficient to neutralize DNA but not to transfect cells properly. Therefore, excess PAMAM polymer molecules would be needed to completely enclose DNA and form a positively protective shield around DNA which would facilitate transfection [35].

Meanwhile, PAMAM-TA-H and PAMAM-TA-L possessed a similar high level of luciferase expression at respective optimal weight ratio without significant difference. The degree of substitution of TA from

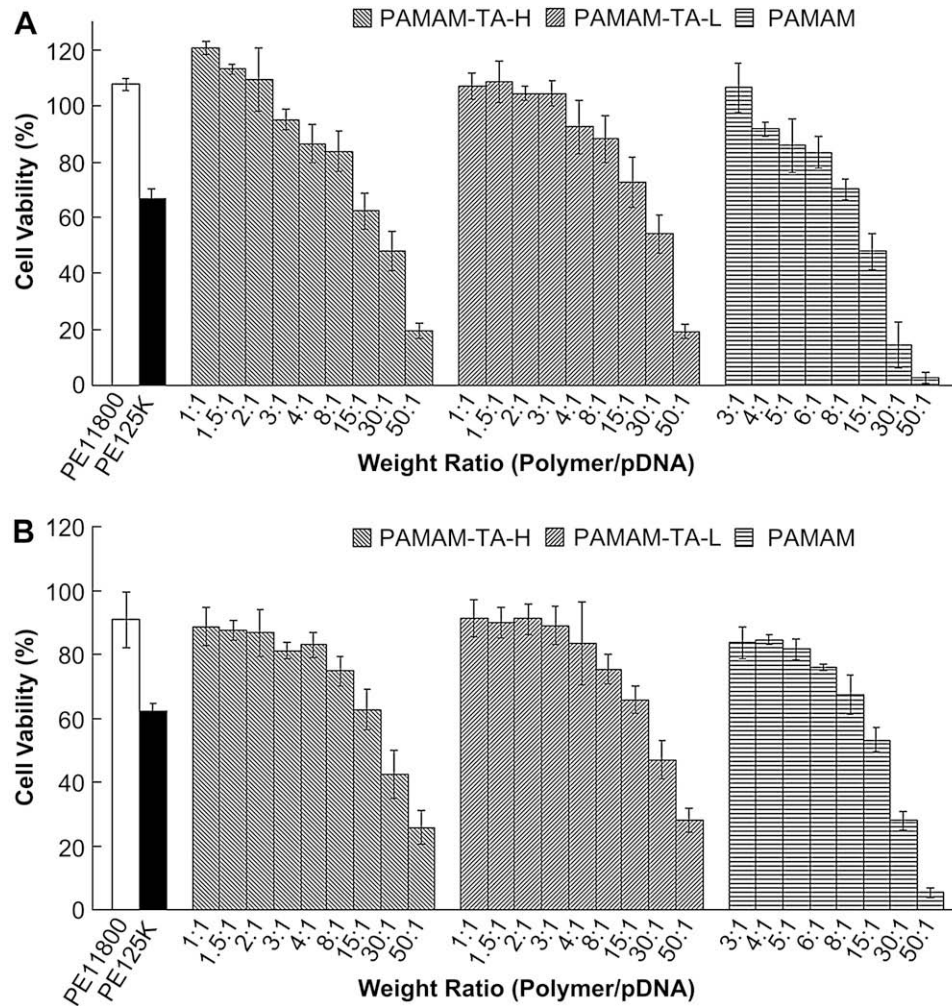


Fig. 9. MTT assay for cytotoxicities of PAMAM-TA-H, PAMAM-TA-L and PAMAM polyplexes at various weight ratios in HEK 293 (A) and HepG 2 (B) cells. PEI 25K and PEI 1800/pDNA complexes were prepared at 1.33:1 and 5.3:1 of weight ratio, respectively. The amount of pDNA was fixed at 0.2 $\mu\text{g}/\text{well}$. The data points represent the mean \pm SD of three experiments.

0.22 to 1.65 on PAMAM backbone was not affected with their gene delivery potency. It meant that they were efficient equally as transfection agents, and even at low TA substitution, those PAMAM derivatives still showed relatively excellent transgenic activity.

The EGFP reporter gene expression in HepG 2 cells also confirmed this result (Fig. 7). The two TA substituted PAMAM dendrimers could lead to similar EGFP expression, which were much higher than that of native PAMAM and comparable to PEI 25K.

Serum is generally believed to impair gene transfer activity through pDNA degradation, slight cellular uptake and/or dissociation of pDNA from polyplexes [36]. In order to further evaluate the potency of PAMAM-TA polymers, their transfection efficiency in the presence of 10% serum was examined at the optimal weight ratio in both cells. The results (Fig. 8) indicated that luciferase expression of all PAMAM polymers was decreased with serum, but the two PAMAM-TA polymers were still higher significantly than PAMAM in both cells. It suggests that they were more suitable for application in vivo to obtain satisfying transfection.

3.6. Cytotoxicity assay

In general, the ultimate success of polycations as gene delivery carriers is characterized by maximal efficiency and minimal toxicity. The cytotoxicity of PAMAM polymer at various weight ratios was

evaluated by MTT assay in HEK 293 and HepG 2 cells. As controls, PEI 25K, PEI 1800 and PAMAM were used. In Fig. 9, PEI 25K displayed serious cytotoxicity and the relative cell viability (RCV) of PEI 25K were around 60% at its best weight ratio. And PEI 1800 was relatively nontoxic. Meanwhile, the RCV of PAMAM was decreased with the increase of weight ratio, showing obvious cytotoxicity at a weight ratio of 8 which was similar to that of PEI 25K. In comparison with PEI 25K and PAMAM, PAMAM-TA dendrimer indicated relatively high cell viability (over 80%) in both cells at all tested weight ratios. With the increase of weight ratio, all PAMAM dendrimer showed increasing cytotoxicity, but the toxicity of PAMAM-TA was relatively lower than that of native PAMAM which meant a promising clinical application. There were no significant differences of cytotoxicity between PAMAM-TA-H and PAMAM-TA-L. The cytotoxicity of cationic polymers is considered to be a consequence of damages from interaction with plasma membrane or other cellular compartments. Therefore, the fact that the cytotoxicity of PAMAM is higher than that of PAMAM-TA suggests that charge density of PAMAM dendrimers is over its derivatives.

3.7. Intracellular localization of polyplex

After modified by TA, the transgenic activity of PAMAM dendrimer was improved drastically in both cells. The results

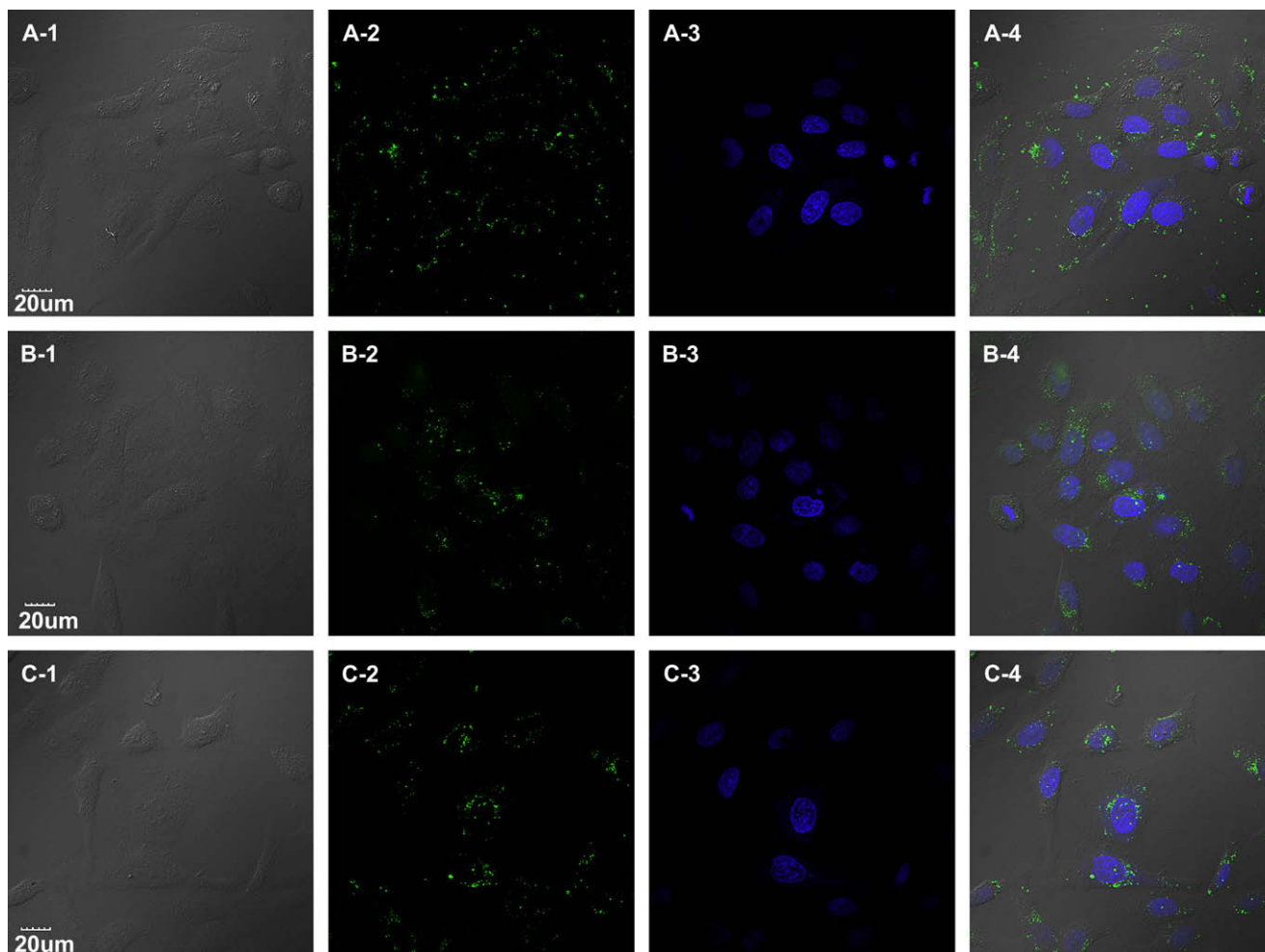


Fig. 10. Intra-cellular localization of FITC-labeled PAMAM (A), PAMAM-TA-H (B), PAMAM-TA-L (C) polyplexes (green) in HEK 293 cells. Hoechst 33342 was used to stain the nucleus (blue). Cells were fixed 24 h after transfection and visualized by confocal microscopy. The image 1 was the respective transmitted light images, 2 was the cellular nucleus, 3 was labeled dendrimer and 4 was the merged images. Scale bar: 20 μm .

indicated that TA residues as nuclear localization signal (NLS) might translocate the PAMAM polymer/pDNA into nucleus so that transgenic expression was enhanced. In order to confirm the suggestion, PAMAM and PAMAM-TA-H labeled by FITC were complexed with pGL-3 at optimal weight ratios and transfected to 293 cells for 4 h. After further 20 h incubated at 37 °C, intracellular localization of polyplex was examined by laser confocal microscopy.

As presented in Fig. 10, there was a considerable difference between PAMAM and PAMAM-TA polymers. PAMAM dendrimer was only accumulated in cytoplasm but in the nucleus which was consistent with the former reports [33,36]. Both PAMAM-TA polymers were found inside the nucleus region which stained in blue. The result clearly indicated that high and low degrees of substitution of PAMAM-TA could translocate into the nucleus equally. The low substituted TA on PAMAM could also guide pDNA into nuclear translocation efficiently. This efficient translocation into the nucleus may lead to the higher transfection efficiency of PAMAM-TA at a lower polymer concentration.

4. Conclusion

PAMAM (G4) dendrimer conjugated TA (PAMAM-TA) was synthesized to improve its transfection efficiency and reduce

cytotoxicity. In order to examine the contribution of the substitution degree of TA, low substituted PAMAM-TA was synthesized, too. They both could form nano-size polyplexes with pDNA, which were investigated by agarose gel electrophoresis, Zeta-sizer and TEM. And their physicochemical properties resembled each other. MTT assay showed that their cytotoxicity was lower than that of native PAMAM and PEI 25K significantly. The two PAMAM-TA polyplexes both represented greatly enhanced transgenic activity in comparison with native PAMAM on various cells, even with 10% serum. The investigation of the polyplex localization showed that PAMAM polyplex was internalized into cytoplasm, but PAMAM-TA-H and PAMAM-TA-L polyplex both were found to be inside nuclei. This meant that TA conjugation to PAMAM dendrimer could accelerate intra-nuclear location, finally leading to satisfying transfection efficiency. The low substituted degree of TA to 0.22 did not interrupt its nuclear localization potency. TA is a versatile pharmacological drug as an effective anti-inflammatory anti-rheumatoid arthritis and anti-anaphylaxis reagent. The alteration of gene expression may also be beneficial to these diseases gene therapy. PAMAM-TA may have synergistic effect such as enhanced transfection and anti-inflammatory, anti-rheumatoid arthritis, anti-anaphylaxis effect. In summary, PAMAM-TA with excellent transfection efficiency and high cell viability is highly promising as a nuclear gene delivery carrier.

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Appendix

Figures with essential colour discrimination. Certain figures in this article, in particular Figs. 7 and 10, are difficult to interpret in black and white. The full colour images can be found in the on-line version, at doi:10.1016/j.biomaterials.2009.07.036.

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