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Effect of multifold charge groups and imidazole-4-carboxaldehyde on physicochemical characteristics and transfection of cationic polyphosphazenes/DNA complexes

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ABSTRACT

To understand the dual influence of multifold charge groups and conjugation of imidazole moiety on the physicochemical characteristics and the transfection activity of polymer complexes, a series of cationic polyphosphazenes based on poly(2-(2-aminoethyoxy)ethoxy) phosphazene (PAEP) with different components of multifold charge groups was synthesized by means of introducing imidazole-4-carboxaldehyde (IC) into PAEP through the formation of Schiff base. Though the polymers with primary amino groups (1°) alone or with abundant primary amino groups could bind DNA more efficiently than the ones with mainly or totally secondary (2°) and tertiary (3°) amino groups, all of the polymers could condense DNA into small particles within 100 nm at the N/P ratio of 24. The cell viability of complexes and the pH buffering capacity of polymers increased with substitution degree of IC increasing. Among all the PAEP-based polymers, the highest transfection activity was found for poly(2-(2-aminoethyoxy)ethoxy/IC)phosphazene (PAEIC) 18 complexes containing 1°, 2° and 3° amines at a ratio of 3.5:1:1 with 18% substitution degree of IC, which indicated that either the coexistence of 1°, 2° and 3° amines or the conjugation of imidazole moiety played an important role in transfection activity. These results suggested that the most efficient gene carrier could be these polymers with 1°, 2° and 3° amines at an appropriate ratio, together with the presence of imidazole moiety in a small fraction.

1. Introduction

Non-viral vector based on cationic polymers has been widely investigated for the delivery of nucleic acids in gene therapy since the cationic polymers could form stable polymer/DNA complexes via electrostatic interaction and protect nucleic acids from enzymatic digestion with low immune response (Wightman et al., 2001; Koping-Hoggard et al., 2003; Ahn et al., 2004). Unfortunately, common challenges using the cationic polymer to deliver gene often involve the high toxicity and limited nucleic acids delivery efficiency (Read et al., 2005). As a result, the development of effective non-viral vector is a key issue for successful gene therapy.

Polyphosphazenes, containing a long-chain backbone of alternating phosphorus and nitrogen atoms with two organic side groups attached to each phosphorus atom, are one of the versatile synthetic polymers. Luten et al. (2003) found it was feasible that the cationic polyphosphazenes bearing tertiary amino groups condensed DNA for effective gene delivery in COS 7 cells. However, the optimal polyphosphazene derivatives with excellent gene transfer efficiency and low cytotoxicity have not been found until today. Regardless of the exact relationship of structure-transfection activity of cationic polymer, the type of charge group is considered to be one of the most important structural parameters for efficient gene delivery (Thomas and Klibanov, 2002; Reschel et al., 2002). It has been reported that the cationic polymers with primary amino groups mediated the highest transfection level among all carriers with other types of charge groups (Wang et al., 2002; Wolfert et al., 1999). Besides, copolymers with primary (1°), tertiary (3°) and/or secondary (2°) amino groups exhibited higher transfection efficiency than a gene carrier bearing primary amino groups alone (Reschel et al., 2002; Sonawane et al., 2003).

The imidazole heterocycle displays a pK_a around 6 thus possessing a buffering capacity in the endolysosomal pH range, and possibly mediating vesicular escape by a "proton sponge" mechanism. Because of this, histidine or other imidazole has been utilized to modify amino groups of polymers so as to improve gene expression in several cases (Benns et al., 2000; Swami et al., 2007; Kim et al., 2003; Roufaï and Midoux, 2001).

In this work, we focused our efforts on investigating the synthesis and the transfection activity of the cationic polyphosphazenes containing different components of charge groups

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ranging from primary to tertiary amines. The imidazole-4-carboxaldehyde (IC) was introduced into the backbone of poly(2-(2-aminoethyoxy)ethoxy)phosphazene (PAEP) by Schiff base reaction between the primary amino groups of PAEP and the aldehyde groups of IC, which would not decrease the amounts of charge groups since the parts of 1° amines for conjugation would be altered to 2° amines during the process of reduction of Schiff base. Thus, different poly(2-(2-aminoethyoxy)ethoxy/IC)phosphazenes (PAEIC) with different components of 1°, 2° and 3° amines (imidazole groups) would be generated after coupling with IC. In addition, PAEIC with different charge groups and different IC substitution degrees were characterized, the biological properties such as cytotoxicity, cellular uptake and transfection activity of complexes were investigated as well.

2. Materials and methods

2.1. Materials

Imidazole-4-carboxaldehyde (IC) was purchased from Lancaster. Branched polyethylenimine (PEI 25 kDa), 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazo-liumbromide (MTT) and 2-(2-aminoethyoxy)ethanol were purchased from Sigma–Aldrich. YOYO-1 was obtained from Invitrogen and Trypan Blue from TianGen. HEK 293T cells (ATCC, Manassas, VA, USA) were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco, USA) with 10% fetal bovine serum (FBS) (Gibco, USA), streptomycine at 40 μg/mL and ampenicillin at 40 U/mL. Cells were maintained at 37 °C in a humidified and 5% CO_2 incubator.

2.2. Plasmid DNA

pEGFP-N2 (4.7 kb) encoding green fluorescent protein driven by immediate early promoter of CMV was purchased from Clontech Laboratories (Palo Alto, CA, USA). pGL-2Luc was purchased from Promega Corp. (Madison, WI, USA). The plasmid DNA (pDNA) was amplified in DH5α strain of *E. coli* and purified by EndFree Plasmid Mega Kit (Qiagen GmbH, Hilden, Germany). pGL-2Luc was labeled with YOYO-1 for cellular uptake. Briefly, 200 μL of pGL-2Luc (0.2 μg/mL) was mixed with 10 μL of 50 μM YOYO-1 and incubated at room temperature for 1 h in the dark.

2.3. Synthesis of polymers

PAEP was synthesized by the method described as the Allock's procedure (Allcock and Chang, 1991) except that poly(dichloro)phosphazene was obtained directly from PCl_5 and NH_4Cl (Carriedo et al., 2003). The imidazole-4-carboxaldehyde (IC) was introduced into poly(2-(2-aminoethyoxy) ethoxy)phosphazene (PAEP) according to the well-known reaction that forms Schiff bases (Chaparro and Vachet, 2003). Briefly, IC was added to a stirred solution of PAEP (0.5 g, 20 mmol) in 10 mL of dry methanol. The stirred mixture was hydrogenated for 12 h under atmospheric pressure at room temperature. The resulting Schiff bases were then reduced using $NaBH_4$ to generate PAEIC with multifold charge groups. The degree of substitution of IC was expected to be 20–100%.

2.4. Buffering capacity

The buffering capacities of PAEP and PAEIC were measured by titration according to the method described by Tseng with PEI as control (Tseng et al., 2005). The polymers were diluted to a final concentration of 2 mg/mL with 0.1N NaCl. The solution was adjusted to pH 8.0 before titration proceeded. An aliquot of 0.1N HCl was successively added into polymer solution (10 mL), and the change in pH was monitored by a pH meter (Sartorius, Germany).

2.5. Preparation and characteristics of polymer/DNA complexes

DNA/polymer complexes employing EGFP-N2 plasmid were prepared at various DNA/polymer ratios (N/P) in the following manner. Polymers were dissolved in distilled water to yield a concentration of 2 mg/mL. While a 50 μL sample of each polymer solution in polypropylene microcentrifuge tubes was gently vortexed, 50 μL of an aqueous solution of DNA (200 μg/mL in distilled water) was added dropwise to each microcentrifuge tubes containing polymer solution (100 μL total volume). Controls employing PEI were prepared in a similar fashion except that they were dissolved in distilled water at concentrations of 1 mg/mL, respectively (N/P ratio, 20: 1). All complexes were incubated at room temperature for 30 min. Then, the formation of complexes was confirmed by electrophoresis on a 1% agarose gel with Tris-acetate-EDTA buffer system (pH 8.0) at 110 V/cm for 45 min. DNA was visualized using ethidium bromide staining. The polymer/DNA ratio (N/P) was defined as the ratio between the moles of the amine groups of PAEP or PAEIC to those of the phosphate ones of DNA.

To investigate the relationship between IC substitution degree and physical characteristics, sizes and ζ potentials of complexes were measured by laser light scattering following their dilution with water by a Nicomp 380/ZLS zeta potential analyzer (Santa, CA, USA).

2.6. BSA-induced aggregation

The possibility of destabilization of complexes by serum proteins was investigated in the presence of bovine serum albumin (BSA, Sigma Co., Ltd.). The pDNA complexes were incubated in the presence of BSA for 3 h with a final DNA concentration of 20 μg/mL (Hashimoto et al., 2006). The concentration of BSA was 0.1, 0.2, 0.3, 0.4 and 0.5 mg/mL. The changes in turbidity at 350 nm upon the addition of BSA were monitored with a UV–vis spectrophotometer (UV-2450, Japan).

2.7. Salt-induced polymer/DNA complexes dissociation

The compaction of DNA into small particles by cationic polymers is necessary during gene transfer process including endocytosis, stability in cytoplasm, cytoplasmic mobility, etc. On the other hand, the binding capacity of polymer to DNA should be appropriate to such an extent that enables DNA release from the carrier and facilitates transportation into the nucleus. To investigate the relative binding interactions of different polymers and DNA, the experiment by salt titration was performed in the presence of PicoGreen (Molecular Probes, Eugene, OR, USA), which is a dsDNA-intercalating dye whose fluorescence is dramatically enhanced upon binding to DNA. Polymer/DNA complexes were prepared as described above and diluted in water to yield a concentration of 300 ng DNA/100 μL solution. 100 μL of the aqueous solution containing the complexes was arrayed into each well of an opaque black 96-well plate. PicoGreen solutions were prepared by diluting the supplied PicoGreen stock solution 200-fold in 10 mM Hepes buffer (pH 7.2) containing various concentrations of NaCl. The PicoGreen solutions were prepared such that the desired salt concentration was achieved upon addition of 100 μL PicoGreen solution to each well containing 100 μL of sample. After 10–15 min, the plate was analyzed using a microplate reader (TACAN infinite F200, Austria). Data was collected using the FITC filter set (excitation 485 nm, emission 535 nm). Fractional dye

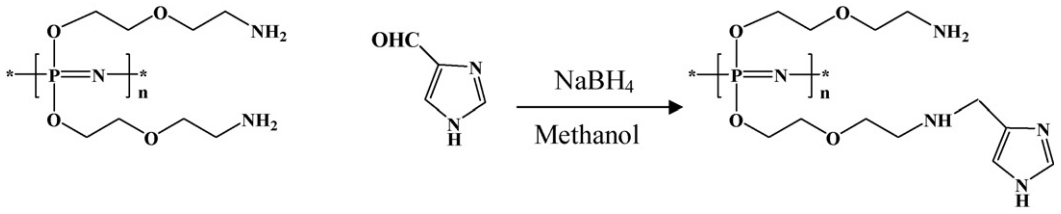

Fig. 1. Synthetic scheme of poly(2-(2-aminoethyoxy)ethoxy/imidazole-4-carboxaldehyde) phosphazene (PAEIC).

exclusion was determined by the following relationship: Dye Exclusion = $1 - (F_{sample} - F_{blank})/(FDNA_{only} - F_{blank})$ (Akinc et al., 2005).

2.8. General transfection protocol

Transfection assays were performed in the following general manner. HEK 293T cells were seeded with complete medium at a seeding density of 1×10^5 cells in 24-well plates prior to transfection. After 24 h, the medium was moved out from each well and replaced with mixture of complexes containing 3 μg DNA and 450 μL growth medium. The cells were incubated for 3 h at 37 °C and 5% CO_2. Following this incubation, the transfection mixture was removed and the cells overlaid with supplemented growth medium and returned to the incubator for a further 48 h before analysis with a flow cytofluorometer (Becton Dickinson, USA).

2.9. Cellular uptake experiments

HEK 293T cells were seeded on 24-well plates at a concentration of 1×10^5 cells/well and grown for 24 h, after which the growth medium was removed and replaced with transfection mixture of fresh growth medium and the complexes employing 3 μg of YOYO-1- labeled plasmid DNA. After incubation for 2 h at 37 °C, the cells were washed with cold PBS twice. Then, the cells were harvested by trypsinization and centrifuged at 3000 rpm. To quench the extracellular fluorescence, the cell suspension was mixed with 25 μL of a 0.4% trypan blue (TB) solution in PBS (Hashimoto et al., 2006). The mean fluorescence intensity (MFI) of the cells was measured by a flow cytofluorometer (Becton Dickinson, USA). A total of 10,000 events were collected per sample. Since the plasmid was labeled with the fluorescent dye, the fluorescence intensity measurements could be directly correlated to the amount of DNA/copolymer complexes. MFI of PEI complexes was normalized to 100.

2.10. MTT assay

To assess the cytotoxicity of PAEP and PAEIC complexes, the assays were performed in triplicate in the following general manner. HEK 293T cells were grown in 96-well plates at an initial seeding density of 2×10^4 cells/well in growth medium. Cells were grown for 24 h in an incubator, after which the growth medium was removed and replaced with fresh complete medium containing DNA/polymer complexes. Complexes were prepared as described above. After 3 h incubation at 37 °C, the transfection mixture was aspirated off, and the cells were treated with 150 μL of DMEM containing 20 μL MTT (5 mg/mL). After incubation at 37 °C in a humidified air atmosphere (5% CO_2) for 4 h, the medium was removed and 150 μL of DMSO was added to each well to dissolve the formazan crystals produced from the reduction of MTT by viable cells. Then, the absorbance of the dimethyl sulfoxide solution from each well was measured at 570 nm using a microplate reader (TACAN, infinite F200, Austria). The results were expressed as percentages relative to control cells (mean ± SD).

2.11. Statistical analysis

Statistical analysis was performed using a Student's *t*-test. The differences were considered significant for $p < 0.05$ and $p < 0.01$ indicative of a very significant difference.

3. Results

3.1. Synthesis and characteristics of polymer

The imidazole group is often coupled with polymers in order to improve the endosomal escape of gene carriers. Frequently, the covalent attachment of imidazole group to the amines of polymers is performed through carbodiimide reaction via NHS-active-ester intermediates (Kim et al., 2003). However, such modification of

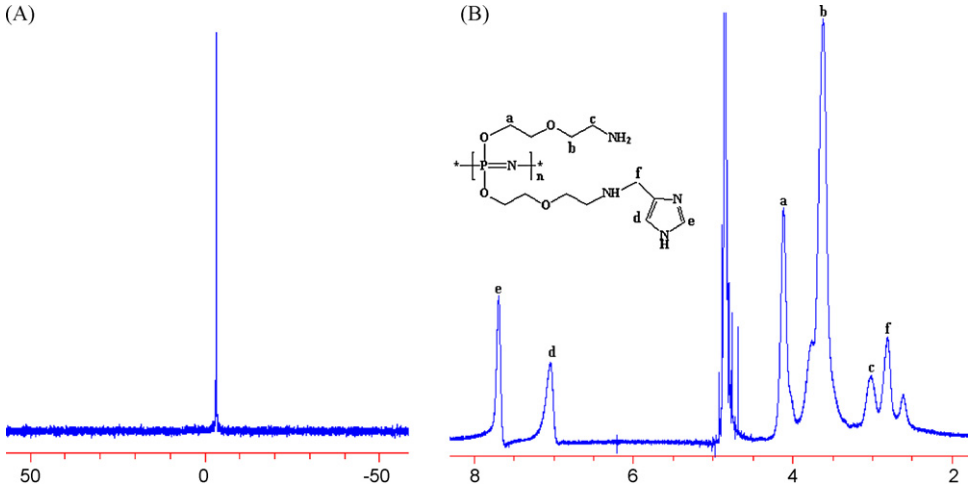

Fig. 2. ^{31}P NMR (A) and ^{1}H NMR (B) spectra of PAEIC.

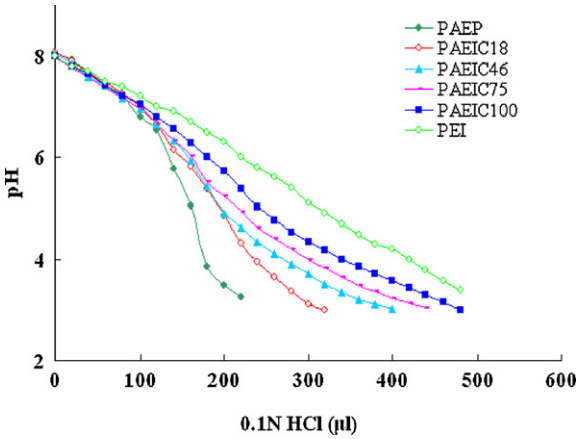

Fig. 3. Titration curves of polymer solutions (2 mg/mL). The polymer solutions were adjusted to pH 8.0 before titration with an aliquot of 0.1N HCl.

amino groups in polymers would decrease the amounts of charge groups and the DNA binding strength of the vectors. Because of this, imidazole group was introduced to the backbone of PAEP in a different way in this work. IC containing imidazole groups was reacted with the primary amino groups of PAEP through several routes including the formation of Schiff base linkages, which can be reduced with cyanoborohydride to create stable secondary amine bonds. By means of the incorporation of imidazole groups, the primary amines were altered to secondary amines without decreasing the amounts of charge groups, and the additional tertiary amines were generated from the moiety of IC. Therefore, the derivates of PAEP comprised of different ratios of 1°, 2° and 3° amino groups with different substitution degrees of IC were synthesized by the reaction as shown in Fig. 1. All the polymers grafted with imidazole group were obtained as yellow viscous elastic solids, which were soluble in water. The polymers were characterized by means of multinuclear (^{1}H, ^{31}P) NMR spectroscopy. The ^{1}H and ^{31}P NMR spectra of PAEIC were shown in Fig. 2. The substitution degree of IC was calculated from the integration ratio of IC and 2-(2-aminoethyoxy)ethanol appearing at 7.6 and 4.2 ppm, respectively. In this work, four polymers including PAEIC100, PAEIC75, PAEIC46 and PAEIC18 were synthesized, whose substitution degree of IC was 100%, 75%, 46% and 18%, respectively.

3.2. Buffering capacity

Fig. 3 showed that the buffering capacities of all PAEIC polymers were higher than PAEP in the pH range of 5–7 after coupling with IC. Even PAEIC18, with lowest substitution degree of IC as 18%, presented advantage of buffering capacity over unmodified PAEP. The buffering capacities of PAEIC polymers increased with degree of substitution of IC increasing. PAEIC100, which was grafted with IC by 100%, exhibited superior buffering capacity than unmodified PAEP and other modified polymers. Therefore, the introduction of imidazole group could be responsible for the increment of buffering capacity of PAEIC polymers. Besides, the presence of primary amines and the newly added secondary and tertiary amines, which were protonable at lower pH, made the proton sponge propensity of the ensuing polymers retained within a wide range of pH.

3.3. Preparation and characteristics of polymer/DNA complexes

The preparation of pDNA complexes was carried out by simple-mixing of the polymers with pDNA solutions in water. The behavior of pDNA complexes with different IC substitution degree varied slightly in gel retardation assay. The N/P ratio, at which the polymers could bind the DNA efficiently, was 3, 3, 4.5, 4.5 and 4.5 for PAEP, PAEIC18, PAEIC46, PAEIC75 and PAEIC100, respectively (data not shown). The results implied that the DNA binding capacities of polymers were depressed when the substitution degree of IC of the polymers was equal to or over 46%.

Particle size and surface charge are two of the factors that have impact on the internalization of polymer/DNA complexes by non-specific endocytosis. Complexes less than 200 nm in size with a positive surface charge enable them internalize efficiently. As shown in Table 1, the particle sizes of PAEP and PAEIC complexes were less than 100 nm, and there was no significant difference between them at the N/P ratio of 24. The average ζ potentials of majority of complexes were about 20 mV except PAEIC75 and PAEIC100 complexes with ζ potentials of 13–14 mV. It was clear that the incorporation of IC more than 75% exerted little effect on the particle sizes of complexes at N/P ratio of 24, but showed apparent influence on the surface charge of complexes.

3.4. Stability and unpacking of pDNA complexes

Upon intravenous injection of cationic pDNA complexes, even in the transfection medium containing serum, interactions between cationic particles and negatively charged blood components could reduce the zeta potential, promote aggregation of complexes and result in poor cellular uptake and consequently inefficient transfection activity. When the concentration of BSA was less than 0.2 mg/mL, PAEIC complexes exhibited higher stability than PAEP complexes. Although PAEP could condense DNA into smaller particles, their higher surface charge (Table 1) increased non-specific interactions with BSA and resulted in obvious increase in turbidity (Fig. 4) at the presence of serum less than 0.2 mg/mL. Possibly, the modification of PAEP with IC changed the structure and positive charge of PAEIC/DNA complexes, which could benefit the stability of the PAEIC/DNA complexes. These findings were in agreement with the results of reference (Hashimoto et al., 2006).

At more than 0.2 mg/mL of BSA, all of the complexes displayed a significant increase in turbidity (Fig. 4), in particular for PAEIC46/DNA complexes. The results suggested that the modification of IC to PAEP could enhance the stability of complexes in the low concentration of BSA. However, higher concentration of BSA could provoke the instability of complexes as well.

Table 1
Physicochemical and biological properties of polymers or polymer/DNA complexes.

Polymer	DS of IC (%)	Ratio of amino groups (%)			Retarding ratio (N/P)	Buffering capacity[a] (μL)	Size (nm)	ζ Potential (mV)	Transfection efficiency (%)
		1°	2°	3°					
PAEP	0	100	0	0	3	70	85.1 ± 5.9	20.8 ± 2.4	57.1 ± 8.8
PAEIC18	18	63.6	18	18	3	90	90.6 ± 7.8	21.3 ± 4.7	84.4 ± 7.8
PAEIC46	46	33.3	33.3	33.3	4.5	100	99.3 ± 8.9	20.7 ± 2.6	53.3 ± 5.4
PAEIC75	75	14.3	42.8	42.8	4.5	120	93.1 ± 6.4	13.2 ± 3.5	46.9 ± 7.6
PAEIC100	100	0	50	50	4.5	140	95.8 ± 5.8	14.3 ± 2.3	59.3 ± 4.3

[a] The buffering capacity was calculated from the cumulative volume of 0.1N HCl added when the value of pH changes from 7 to 5.

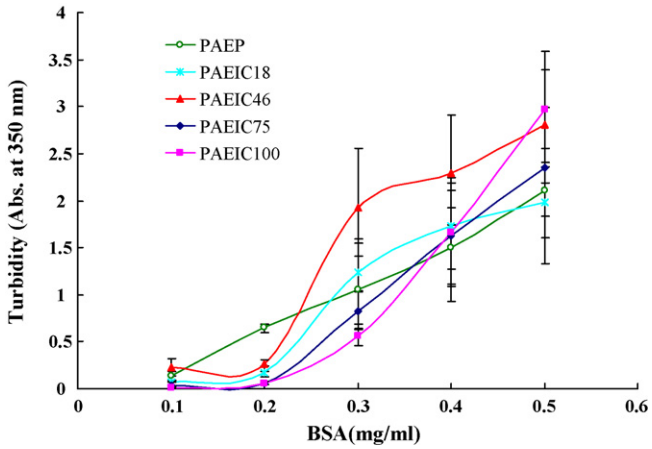

Fig. 4. Turbidity of pDNA complexes at 3 h after incubation with BSA. The pDNA complexes were prepared at N/P ratio of 24.

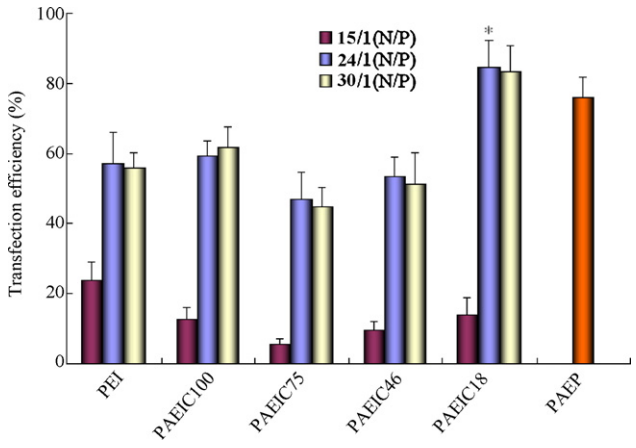

Fig. 6. The transfection efficiency of HEK 293T cells mediated by PAEP or PAEIC complexes at various polymer/DNA ratios (N/P) in growth medium, PEI complexes at the N/P ratio of 20 as control group ($n = 3$, error bars represented standard deviation). $^{*}p < 0.05$ compared with the transfection of PAEP, PAEIC100, PAEIC75 and PAEIC46 complexes at the N/P ratio of 24, respectively.

As for the relative binding interactions of different polymers and DNA, reduced dye exclusions of all of complexes with salt concentration increasing were observed in Fig. 5, which indicated that high concentration of salt could destabilize these complexes. Under physiological salt concentration (0.15 M), all complexes showed good stability with over 90% dye exclusion. However, PAEP and PAEIC complexes showed less stability in the presence of higher salt concentration, particularly for PAEIC 100 and PAEIC 18, the dye exclusion in 1 M NaCl was only 52% and 56%, respectively, which could result from the difference of structure and physical characteristics of polymers. It seemed like that there was no correlation between the substitution degree of IC and extent of instability of complexes induced by salt titration.

3.5. In vitro transfection efficiency

The in vitro transfection ability of complexes was evaluated in HEK 293T cells at the N/P ratio from 15 to 30 using EGFP-N2 plasmid. As shown in Fig. 6, all of the complexes exhibited very low level of gene expression at the N/P ratio of 15, which could result from less positive charges of complexes at this ratio. Much higher transfection activities of all of the complexes were observed at the N/P ratio of 24. While the N/P ratio increased from 24 to 30, their level of gene expression remained stable without significant changes. In view of the higher cytotoxicity at higher N/P ratio, the optimum N/P ratio of complexes for cell transfection was finally determined at 24. At N/P ratio of 24, PAEIC18 complexes achieved the highest transfection efficiency of 81%, not only much higher than PAEP complexes, but also slightly higher than that of PEI 25K complexes (Fig. 7). PAEIC75 complexes mediated the lowest level of gene expression. The results showed that the transfection efficiency of complexes was not directly proportional to the substitution degree of IC.

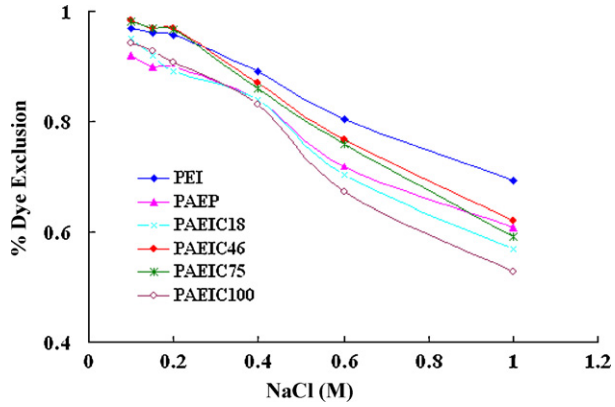

Fig. 5. Assessment of polymer/DNA binding affinity by salt titration. Binding affinity was determined by exclusion of the dsDNA-intercalating dye PicoGreen. Polymer/DNA complexes were prepared at the N/P ratio of 24.

3.6. Cellular uptake

The ability of the cellular uptake of DNA mediated by different complexes was qualified by flow cytometry. In order to know the correlation of cellular uptake and the gene expression, complexes were formed at the same polymer/DNA ratios as used in above transfection experiment. Fig. 8 showed that PAEIC100 complexes mediated the highest level of cellular uptake, which was significantly higher than that of other complexes. With the highest transfection efficiency, PAEIC18 complexes exhibited the second highest level of cellular uptake. Much lower cellular uptake of PAEIC46 and PAEP complexes indicated their uptake-limited. The discrepancy of PAEIC100 complexes between higher level of cellular uptake and relatively lower zeta potential suggested that the uptake into cells is not solely dependent on surface charges of complexes. Further investigation would focus on the mechanism of cellular uptake of complexes.

3.7. In vitro cytotoxicity

The introduction of imidazole group is expected to improve the buffering capacity and decrease the cytotoxicity of polymers since the imidazole ring could offer potent biocompatibility as a side chain in histidine. The results of MTT assay showed the obvious influence of IC substitution degree on the cell viability of the complexes (Fig. 9). The toxicity decreased with the substitution degree of IC on PAEP increasing. With the cell viability of 85%, PAEIC100 complexes presented much lower cytotoxicity than that of unmodified PAEP complexes and PEI 25K complexes with the cell viability of 62% and 56%, respectively. It seemed like that lower cytotoxicity of PAEIC100 complexes than other complexes was a consequence of incorporation of imidazole moiety and less positive charges.

4. Discussion

Several experiments had been executed in this work to characterize the biological and physicochemical properties of complexes and to yield considerable information on the interaction of PAEP

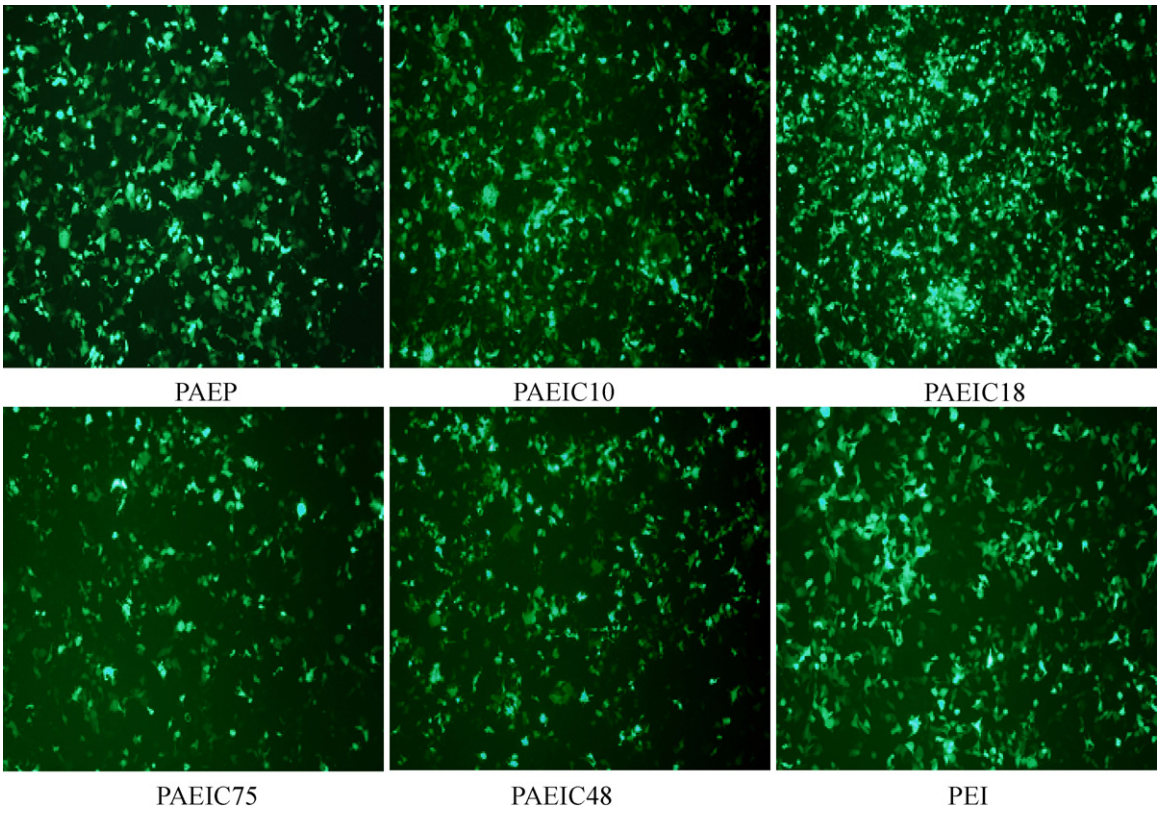

Fig. 7. Images of 293 T cells transfected with polymer/DNA complexes observed under fluorescent microscope (20× magnification) at the N/P ratio of 24.

derivates with DNA (Table 1). Firstly, we investigated the influence of charge strength on the properties of complexes containing different ratios of 1°, 2° and 3° amino groups resulting from different substitution degree of IC. The primary amino-based PAEP and 63.6% primary amino-based PAEIC18 were more effective for DNA condensation, retarding DNA at the N/P ratio of 3. In contrast, PAEIC46, PAEIC75 and PAEIC100 were relatively poor for DNA condensation with the retarding ratio of 4.5. All of the polymers could produce discrete complexes nanoparticles of less than 100 nm without obvious difference at the N/P ratio of 24. Although the conjugation of IC with PAEP did not diminish the amounts of charge groups, the zeta potentials of PAEIC75 or PAEIC100 complexes based on mainly 2°, 3° amino groups and less 1° or without 1° amino group obviously decreased at N/P ratio of 24, possibly due to the weaker alky of 2° amino group and imidazole groups than 1° amino group. It suggested that the ratio of 1° amino groups in polymer showed an effect on the efficiency of DNA binding and positive charge.

The next parameter studied in depth was the substitution degree of IC on the properties of complexes. As we expected, the pH buffering capacity of polymers increased with substitution degree of IC increasing. PAEIC100, with 100% substitution degree of IC, displayed superior pH buffering capacity than other polymers. In addition, the modification of IC to PAEP enhanced the stability of complexes in the low concentration of BSA, which suggested that the hamper of aggregation of complexes could facilitate efficient cellular uptake and consequently effective transfection activity. On

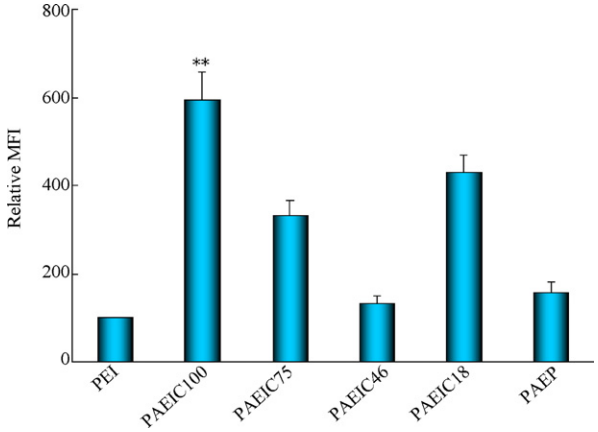

Fig. 8. Cellular uptake of polymer complexes on HEK293 T cells at the N/P ratio of 24. MFI of PEI complexes prepared at the N/P ratio of 20 was normalized to 100 ($n = 3$, error bars represent standard deviation). $^{**}p < 0.01$ compared with the relative MFI of PAEP, PAEIC18, PAEIC46 and PAEIC75 complexes at the N/P ratio of 24, respectively.

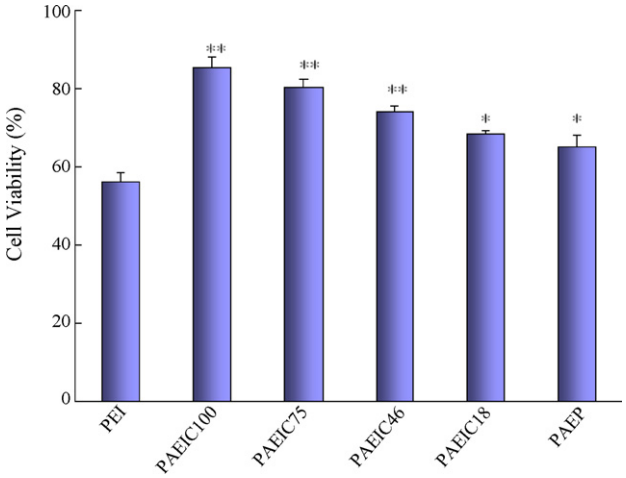

Fig. 9. Cytotoxicity of polymer complexes on HEK293 T cells measured by MTT assay at the N/P ratio of 24 except PEI complexes at the ratio of 20 ($n = 3$, error bars represent standard deviation). $^{*}p < 0.05$ or $^{**}p < 0.01$ compared with the cytotoxicity of PEI complexes.

the other hand, the stability of complexes induced by salt titration seemed not to be correlated with the substitution degree of IC.

Finally, the biological activity of complexes was investigated in detail. The extent at which the cell viability of polymer/DNA complexes improved was a function of the increasing substitution degree of IC, which could be attributed to the biocompatibility of imidazole moiety as well as lower positive charges for PAEIC 75 and PAEIC 100 complexes. PAEIC 100 complexes exhibited the highest level of cellular uptake, while the trendline that the uptake level of complexes increased with substitution degree of IC increasing was not observed. Inferior to PAEIC100 complexes in terms of cellular uptake and pH buffering capacity, PAEIC18 complexes mediated the highest transfection efficiency, which suggested that transfection efficiency is not always in a good agreement with cellular uptake data and pH buffering capacity. The significant improvement of transfection efficiency mediated by PAEIC18 complexes compared to that of unmodified PAEP complexes could be ascribed to appropriate positive charge resulting from the ratio of 1°, 2° and 3° amino groups at 3.5:1:1. Meanwhile, the newly added secondary and tertiary amino groups could make PAEIC18 more protonatable at lower pH and interact more closely with DNA than unmodified PAEP. More efficient endosomal escape due to 18% substitution degree of IC and suitable unpacking of PAEIC18/DNA complexes thus more efficient nuclear translocation could be the reasons accounting for their superior transfection activity as well. With higher level of cellular uptake and excellent pH buffering capacity, the level of gene expression mediated by PAEIC100 complexes was not consistently high in transfection experiment. The discrepancy suggested that the limit-step of efficient transfection for PAEIC100 complexes could be low positive charge and downstream barriers rather than cellular uptake and endosomal escape.

5. Conclusion

In this study, PAEP-based polymers with similar molecular weight containing different ratios of 1°, 2° and 3° amino groups were synthesized and characterized. The results showed that the efficiency of DNA binding was mainly dominated by 1° amines rather than 2° or 3° amines. When the substitution degree of IC increased over 75%, the ζ potentials of complexes diminished while the particle size of complexes was the same as others at the N/P ratio of 24. In addition, the cytotoxicity of complexes and the pH buffering capacity of polymers were a function of the substitution degree of IC. The highest transfection activity was found for PAEIC18 complexes containing 1°, 2° and 3° amine at a ratio of 3.5:1:1 with 18% substitution degree of IC. Consideration of the information presented in this work leads us to believe that the most efficient gene carrier will be based on cationic polymers with 1°, 2° and 3° amine at an appropriate ratio, together with the presence of imidazole moiety in a small fraction.

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