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Histidylated cationic polyorganophosphazene/DNA self-assembled nanoparticles for gene delivery

Yongxin Yang [a,b], Zhenghong Xu [b], Shangwei Chen [c], Yu Gao [b], Wangwen Gu [b], Lingli Chen [b], Yuanying Pei [a], Yaping Li [b,*]

[a] *Department of Pharmaceutics, School of Pharmacy, Fudan University, Shanghai 200032, China*
[b] *Center for Drug Delivery System, Shanghai Institute of Materia Medica, Chinese Academy of Sciences, 555 Zuchongzhi Road, Shanghai 201203, China*
[c] *Testing & Analysis Center South Yangtze University, Wuxi 214122, China*

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Abstract

Cationic polyorganophosphazene has shown the ability to deliver gene. To obtain more efficient transfection, His(Boc)-OMe bearing histidine moiety was introduced to synthesize a new derivative of cationic polyphosphazenes with another side group of 2-dimethylaminoethylamine (DMAEA). The poly(DMAEA/His(Boc)-OMe)phosphazene (PDHP) and DNA could self-assemble into nanoparticles with a size around 110 nm and zeta potential of +15 mV at the PDHP/DNA ratio of 10:1 (w/w). The maximum transfection efficiency of PDHP/DNA self-assembled nanoparticles (PHSNs) against 293 T cells was much higher than that of poly(di-2-dimethylaminoethylamine) phosphazenes (PDAP)/DNA self-assembled nanoparticles (PASNs) and PEI 25/DNA self-assembled nanoparticles (PESNs) at the polymer/DNA ratio of 10:1, but the cytotoxicity of PDHP assayed by MTT was much lower than that of PDAP and PEI 25. These results suggested that PDHP could be a good candidate with high transfection efficiency and low cytotoxicity for gene delivery.
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Keywords: Polyphosphazene; Histidine; Cytotoxicity; Gene delivery

1. Introduction

The cationic polymers are one of the most widely investigated non-viral vectors for gene delivery owing to their obvious advantages such as stability in storage, production in large-scale easily, minimal immunogenicity, molecular diversity of chemical or cell specific targeting moieties modification, etc. (Spack and Sorgi, 2001; Merdan et al., 2002). Unfortunately, a major problem associated with cationic polymers is their lower transfection efficiency compared with viral vectors (El-Aneed, 2004). Thus, many efforts have been made to increase their transfection efficiency with low toxicity. Because endosomal escape is thought to be one of the major bottlenecks in non-viral nucleic acid delivery (Singh et al., 2004), a pervasive approach to improve transfection efficiency of cationic carriers is the inclusion or co-application of endosomolytic agents to protect the plasmid from hydrolytic digestion within endosome and/or enable it escape from endosome. The classic endosomolytic agents such as chloroquine (Erbacher et al., 1996; Ciftci and Levy, 2001) or fusogenic peptide (Wagner et al., 1992; Lee et al., 2001) are impractical for in vivo gene therapy because of their cellular toxicity, immunogenicity and side effects. Recently, the polymers that were conjugated with histidine or other moieties containing imidazole group showed a significant enhancement of gene expression without increasing toxicity compared with non-modified polymers (Roufaï and Midoux, 2001; Kima et al., 2003; Mishra et al., 2006; Park et al., 2006; Swami et al., 2007). In this case, histidine or other moieties containing imidazole group could favor polymers/DNA complexes escaping from endosome by a "proton sponge" mechanism, which the imidazole heterocycles display a pK_a around 6, thus possessing a buffering capacity in the endolysosomal pH range, inducing membrane destabilization after their protonation and facilitating complexes releasing to cytosol.

Polyphosphazenes have been investigated for many different biomedical and pharmaceutical applications (Aldini

* Corresponding author. Tel.: +86 21 5080 6820; fax: +86 21 5080 6820.
E-mail address: ypli@mail.shcnc.ac.cn (Y. Li).

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et al., 2001; Zhang et al., 2005; Andrianov et al., 2005; Greish et al., 2005) due to their biodegradability, versatile physicochemical properties and nontoxic degradation products. Polyphosphazenes have not been explored for gene delivery until Hennink's group synthesized and characterized a biodegradable cationic polyphosphazene substituted with side group of 2-dimethylaminoethylamine (DMAEA) (Luten et al., 2003). This poly(di-DMAEA)phosphazene (PDAP) could condense DNA to form positively charged complex nanoparticles, which showed transfection activity in vitro and gene expression at a distant tumor site after intravenous administration (de Wolf et al., 2005). However, few studies have been done for gene delivery using other derivates of polyphosphazenes.

The purpose of this work is to develop a novel derivate of poly(organo)phosphazene with more efficient transfection activity and low cytotoxicity for gene delivery. In this work, a new cationic derivative of poly(organo)phosphazene by introducing His(Boc)-OMe and DMAEA as side groups was synthesized. The synthesis, characteristics, cytotoxicity and transfection activity of poly(DMAEA/His(Boc)-OMe)phosphazene (PDHP) were investigated. The influence of histidine moiety on transfection activity of PDHP was discussed as well.

2. Materials and methods

2.1. Materials

All the reagents (analytical grade) were purchased from Shanghai Chemical Reagents Corp., unless otherwise noted. NH_4Cl was dried in a dissicator over P_2O_5 (Panreac). Tetrahydrofuran (THF) was treated with KOH and distilled twice from Na in the presence of benzophenone. Petroleum ether refers to that fraction with a boiling point in the range 60–90 °C. PCl_5 was purified by sublimation. Sulfamic acid (HSO_3NH_2), 2-dimethylaminoethylamine (Aldrich), PEI 25 K (Aldrich), $CaSO_4{\cdot}2H_2O$, His(Boc)-OMe (GL Biochem Ltd.) and 1,2,4-trichlorobenzene were used as purchased. pEGFP-N1 (4.7 kb) encoding green fluorescent protein driven by immediate early promoter of CMV was purchased from Clontech Laboratories (Palo Alto, CA, USA). The plasmid DNA (pDNA) was amplified in DH5α strain of *E. coli* and purified by EndFree Plasmid Mega Kit (Qiagen GmbH, Hilden, Germany).

2.2. Cell cultures

The 293 T (human embryonic kidney) cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and were grown in DMEM containing 10% fetal bovine serum (FBS), 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.

2.3. Synthesis of poly(DMAEA/His(Boc)-OMe)phosphazene (PDHP)

PDHP was synthesized from poly(dichlorophosphazene) (PDCP), which was carried out and purified according to Carriedo's method (Carriedo et al., 2003). The polymer PDHP was obtained as follows, briefly, a solution of His(Boc)-OMe (1.95 g) and 4 ml triethylamine in dry tetrahydrofuran (50 ml) was added dropwise to a stirring solution of polydichlorophosphazene (2.1 g) in dry tetrahydrofuran (150 ml). The mixture was stirred for 24 h at room temperature. Then, fourfold excess DMAEA was added and the mixture was stirred for another 6 h at 0 °C and 42 h at room temperature. The insoluble hydrochloride was filtered, the filtrate was concentrated under reduced pressure. The polymer was isolated by precipitating the concentrate in petroleum ether. For further purification, the polymer was dissolved in THF and precipitated again in petroleum ether. In order to remove unreacted molecules and inorganic salt, the solution was dialyzed for 2 days against ultrapure water using cellulose dialysis membranes (molecular weight cutoff: 3500, Spectrum Co.) and against THF for 1 day. THF was removed by reduced pressure, and the polymer was dissolved in water and collected by lyophilization. PDAP was synthesized according to Hennink's method as well (Luten et al., 2003).

2.4. Characterization of PDHP

^1H nuclear magnetic resonance (NMR) and ^{31}P NMR spectra were recorded on a Varian Mercury Plus-400 NMR spectrometer (Varian, USA). The chemical shifts were given relative to tetramethsilane or 85% H_3PO_4 as an external standard. The spectra were recorded in $CDCl_3$. The molecular weight and distribution were determined by Waters 600 gel permeation chromatograph (GPC) with acetonitrile/water/trifluoro acetic acid (10/90/0.05, v/v) as the eluent at a flow rate of 0.5 ml/min. The calibrations were peptide standards.

2.5. Preparation and characterization of PDHP/DNA self-assembled nanoparticles (PHSNs)

In order to prepare PHSNs, an aqueous solution of pEGFP-N1 (200 μg/ml) of 100 μl was added dropwise to 300 μl distilled water with different concentration of PDHP and vortexed for 10 s. The mixture was kept 30 min at room temperature to allow the formation of PHSNs. As control, PDAP/DNA self-assembled nanoparticles (PASNs) and PEI 25/DNA self-assembled nanoparticles (PESNs) were also prepared by the same process as described for PHSNs above. The particle size and ξ potential of PHSNs were measured using a Nicomp 380/ZLS zeta potential analyzer (Particle Sizing System, USA). Complex formation was confirmed by electrophoresis for 45 min at 110 V/cm in a Tris–acetate–EDTA buffer system (pH 8.0), and DNA was visualized using ethidium bromide staining.

2.6. Evaluation of cytotoxicity

The cytotoxicity of PDHP was compared with that of PDAP and PEI 25 by the MTT assay. Briefly, 293 T cells were seeded at a density of 2×10^4 cells per well in 150 μl growth medium in 96-well microtitre plates and incubated for 24 h. Growth medium was replaced by fresh serum-free DMEM medium containing polymers (5, 25, 50, 100 and 200 μg/ml). Cells were incubated

for additional 4 h and the medium was replaced with 150 μl of growth medium prior to the addition of 20 μl per well of MTT solution (5 mg/ml). The plate was incubated for an additional 4 h at 37 °C in a 5% CO_2 incubator. Then, MTT containing medium was removed, and 150 μl of DMSO was added to dissolve the crystals formed by living cells. Absorbance was measured at 570 nm using a microplate reader (ELX800, BIO-TEX Instrument, Inc.). The cell viability (%) was calculated and compared with the cells treated with PBS only.

2.7. In vitro transfection

293 T cells were seeded at a density of 1×10^5 cells per well in 24-well plate and incubated for 24 h at 37 °C under a 5% CO_2 atmosphere prior to transfection. Then, the culture medium was replaced with 500 μl serum-free DMEM medium containing either naked DNA or polymer/DNA self-assembled nanoparticles. After incubation 4 h, the cells received growth medium and incubated for an additional 48 h. After the growth medium was removed, the cells were rinsed and resuspended in PBS to determine fluorescence intensity using a FACS Calibur (Becton Dickinson, USA) equipped with an argon ion laser and a red diode laser. The analysis of cells (1×10^4) was performed by CellQuest software. All transfection experiments were performed in triplicate. Cell viability of polymer/DNA complex nanoparticles was also evaluated by MTT assay at 4 h after transfection.

2.8. Statistical analysis

Statistical analyses were performed using a Student's *t*-test. The differences were considered significant for p value < 0.05.

3. Results and discussion

3.1. Synthesis and characteristics of PDHP

Polydichlorophosphazene (PDCP), the main intermediate in the synthesis of water-soluble PDHP, is usually synthesized by the ring-opening polymerization of hexachlorotriphosphazene [$N_3P_3Cl_6$] (Allcock and Kugel, 1965) or the living cationic polymerization of phosphoramines at ambient temperature (Allcock et al., 1996). But these methods showed no molecular weight control, broad polydispersities, high cost and inconvenient operation, etc. In this paper, a simple and convenient onepot synthesis of PDCP in THF solution was carried out to obtain PDCP with narrow polydispersities according to Carriedo's method (Carriedo et al., 2003). His(Boc)-OMe rather than histidine was chosen as one of side groups because of high reactive polar phosphorus–chlorine bonds (P–Cl) in PDCP, where a free carboxylic group and two nucleophilic site (N-α and N-im of histidine) of histidine would participate in chlorine substitution easily to yield cross-linking polymer. The introduction of DMAEA, another substituent group, was based on the assumption that high density of amine groups of DMAEA enabled PDHP to form tighter and smaller nanoparticles with DNA through charge interactions. Due to the high reactivity of DMAEA, the synthesis of mixed substituent polymer was accomplished by introduction of His(Boc)-OMe residue first, followed by treatment with an excess DMAEA to produce an approximate ratio of the two groups (His(Boc)-OMe:DMAEA = 0.4:1.6, molar ratio).

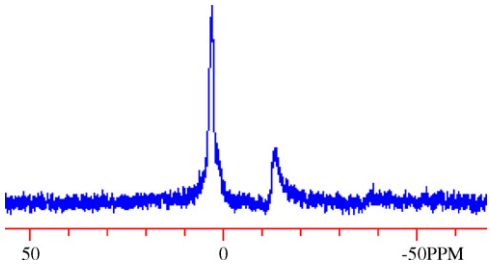

Fig. 1. ^{31}P NMR spectra of poly(Boc-His-OMe/DMAEA)phosphazene.

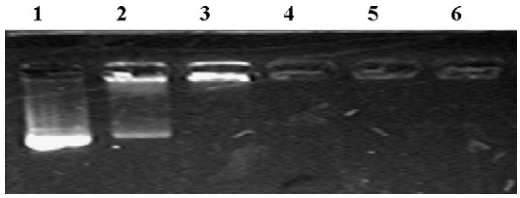

Fig. 2. Gel retardation assay of PHSNs at various PDHP/DNA ratios (w/w) using 1% agarose in Tris–acetate running buffer (pH 8.0). lane 1, DNA control; lanes 2–6, PDHP/DNA = 0.5:1, 1:1, 3:1, 5:1 and 10:1, respectively.

The ^{31}P NMR spectrum of PDHP showed a strong signal at 3.2 ppm and a relatively weak signal at −13.4 ppm (Fig. 1), which were attributed to –NP(DMAEA)$_2$ and –NP(DMAEA/His(Boc)-OMe)-systems, respectively. The Mw and Mn (relative to peptide standards) of PDHP were 17.1 and 15.2 kDa, respectively.

3.2. Characteristics of PHSNs

The DNA retardation assay by electrophoresis indicated that PDHP could form compact nanoparticles when the weight ratio of PDHP/DNA was over 1:1 (Fig. 2). The particle size and zeta potential of polymer/DNA self-assembled nanoparticles were considered to be two of important parameters for efficient gene delivery. The proper size could facilitate the internalization of polyplexes into cells (Mahato et al., 1997). The effect of PDHP/DNA ratio on the particle size was examined in Table 1, which showed that PDHP efficiently condensed DNA into nanoparticles with average sizes in a range of 100–130 nm at ratio of 1:1 and over 1:1. The positive surface charges of polymer/DNA self-assembled nanoparticles allowed them to

Table 1
Particle size of PHSNs at various PDHP/DNA ratios ($n = 3$)

PDHP/DNA ratio (w/w)	Average particle size (nm)
0.5	232.9 ± 5.8
1	107.4 ± 7.4
5	115.3 ± 11.2
10	122.7 ± 6.1
20	129.6 ± 2.3

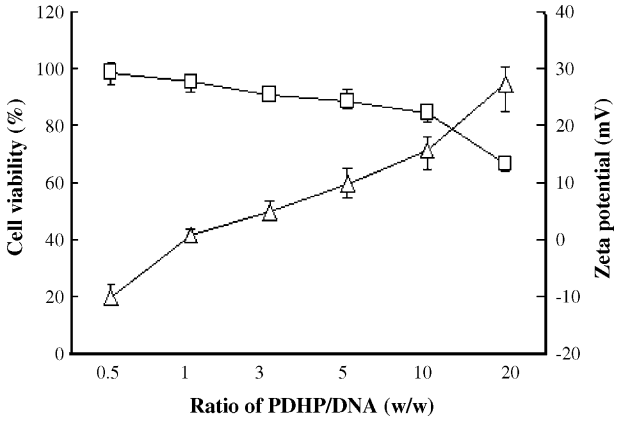

Fig. 3. Effect of PDHP/DNA ratio (w/w) on the cell viability (□) and ξ potential (△) of PHSNs.

bind to the negatively charged cell membranes (Mislick and Baldeschwieler, 1996) and entered into cytosol to obtain efficient transfection. As shown in Fig. 3, the zeta potential of PHSNs increased sharply from 0.81 to 27 mV when the ratio was up from 1:1 to 20:1. So, PHSNs prepared at higher PDHP/DNA ratios were thought to be helpful for gene transfer into cells.

3.3. Cell viability

The low cytotoxicity is one of essential characteristics of optimal gene delivery systems for in vivo application. The cytotoxicity of PDHP was examined against 293 T cell line by MTT assay (Fig. 4). PDAP and PEI 25 were used as control. The cytotoxicity was found to be a function of the polymer/DNA ratio and increased with ratio increasing. As shown in Fig. 4, the cell viabilities of PDHP was about 77% at the concentration of 50 μg/ml, much higher than 41% of PDAP and 30% of PEI 25. The histidine moiety, a building block of proteins, might be one of the factors that reduced toxicity of PDHP. The cytotoxicity of PHSNs was assessed by MTT assay as well (Fig. 3). The cell viability of PHSNs was over 85% when PDHP/DNA ratio was under 10:1. While the cell viability dropped to 66% at the ratio of 20:1 with a high zeta potential. The positive surface charges could facilitate the attachment of complexes onto the negatively charged proteoglycans of cell membranes and cellular entry. On the other hand, exorbitant density of cation would contact directly to cell membrane leading to higher cytotoxicity. As shown in Fig. 3, two curves of cell viability and zeta potential displayed reverse tendency with ratio of PDHP/DNA increasing, and finally intersected at a point. The corresponding ratio of the intersection was 12:1, which was so close to the optimal transfection ratio of 10:1. It meant that we could extrapolate the optimal ratio of polymer/DNA once the information of cell viability and zeta potential of nanoparticles were known.

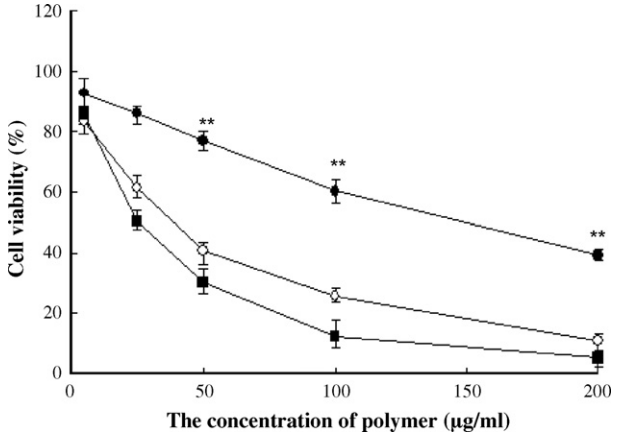

Fig. 4. Cell viability of 293 T cells after incubation with PDHP (●), PDAP (◯) and PEI (■) in serum-free DMEM medium for 4 h at 37 °C in a 5% CO_2 incubator. Cytotoxicity was measured by MTT assay and expressed as the percentage of cell viability by setting the untransfected cells as 100%. Data represented the means of triploid sample ± S.D. ($n = 3$). *$p < 0.05$ and **$p < 0.01$ compared with PADP.

3.4. In vitro transfection experiment

Transfection experiment was performed against 293 T cell line in the absence of serum using a plasmid containing a reporter gene encoding enhanced green fluorescence protein (EGFP). The total EGFP fluorescence intensity of 1×10^4 cells was used as an index of transfection efficiency. The effect of amount of PDHP on the transfection efficiency of PHSNs was shown in Fig. 5. The transfection activity of PHSNs displayed a parabolic tendency with PDHP per well increasing. PHSNs achieved the maximum transfection efficiency at 25 μg PDHP per well with 2.5 μg DNA per well. The enhanced transfection of PHSNs was attributed to DNA dosage per well increasing with PDHP per well increasing. The transfection of PHSNs decreased with PDHP per well increasing overly, which might be due to high concentration of PDHP per well resulting in the cytotoxicity increasing as shown in Fig. 4. As a result, the transfection was performed at 2.5 μg DNA per well, and analyzed at 48 h after transfection. The effect of polymer/DNA ratio on the transfection efficiency of PHSNs against 293 T cells was shown in

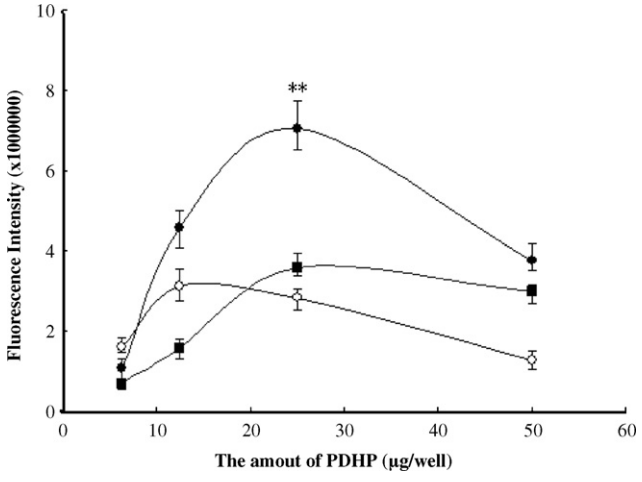

Fig. 5. The transfection efficiency of PHSNs at the polymer/DNA ratio of 5:1 (◯), 10:1 (●) and 20:1 (■) with different amount of PDHP against 293 T cells. After incubation 4 h with PHSNs, the cells received growth medium and incubated for an additional 48 h. The fluorescent intensity of EGFP (1×10^4 cells) was measured by FACS Calibur. **$p < 0.01$ compared with PHSNs at polymer/DNA ratio of 5:1 and 20:1.

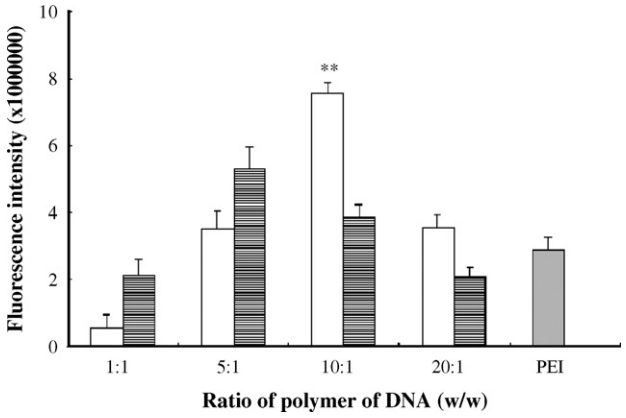

Fig. 6. Effect of polymer/DNA ratio on the transfection efficiency of PHSNs (white bars) and PASNs (stripped bars) against 293 T cells. PESNs (gray bar) at the ratio of 10:1 as another control group. Transfection was performed at a dose of 2.5 μg DNA and analyzed at 48 h after transfection. The fluorescent intensity of EGFP (1×10^4 cells) was measured by FACS Calibur. $^{**}p < 0.01$ compared with PASNs.

Fig. 6. The optimal transfection of PHSNs was obtained at the polymer/DNA ratio of 10:1. It was much higher than the transfection efficiency of control group (PASNs and PESNs). The less EGFP expression of PHSNs was observed at the ratio of 5:1 possibly due to inadequate positive charge of nanoparticles, and the number of imidazole moiety was not enough to induce the proton sponge effect. At the ratio of PDHP/DNA (10:1), the optimization of the balance between positive surface charge of nanoparticles that participate in effective complex with DNA and the amount of imidazole heterocycles responsible for endolysosomal escape of the complexes enables PDHP to mediate a maximum transfection efficiency with low toxicity (Putnam et al., 2001). When the PDHP/DNA ratio increased from 10:1 to 20:1, the transfection efficiency of nanoparticles dropped to a lower level, which could be resulting from higher cytotoxicity. As for PASNs, the transfection efficiency reached maximum at PDAP/DNA ratio of 5:1, then gradually decreased with the ratio increasing. A possible explanation was the sharp increase of cell toxicity when the ratio of PDAP/DNA increased from 5:1 to 10:1–20:1. The cell transfected with naked (non-condensed) plasmid DNA barely expressed the reporter gene, which demonstrated that plasmid DNA without any carrier had very low transfection efficiency as reported in our previous work (Gao et al., 2007). The fluorescent images of transfected cells also confirmed EGFP fluorescence intensity analysis above (Fig. 7).

4. Conclusions

In this study, a novel cationic derivate of polyphosphazene containing histidine moiety, which was used as "proton sponge", was synthesized and characterized. This new polymer and DNA could self-assemble into nanoparticles, which enhanced gene transfection activity with much lower cytotoxicity compared with PDAP/DNA complex nanoparticles (without histidine

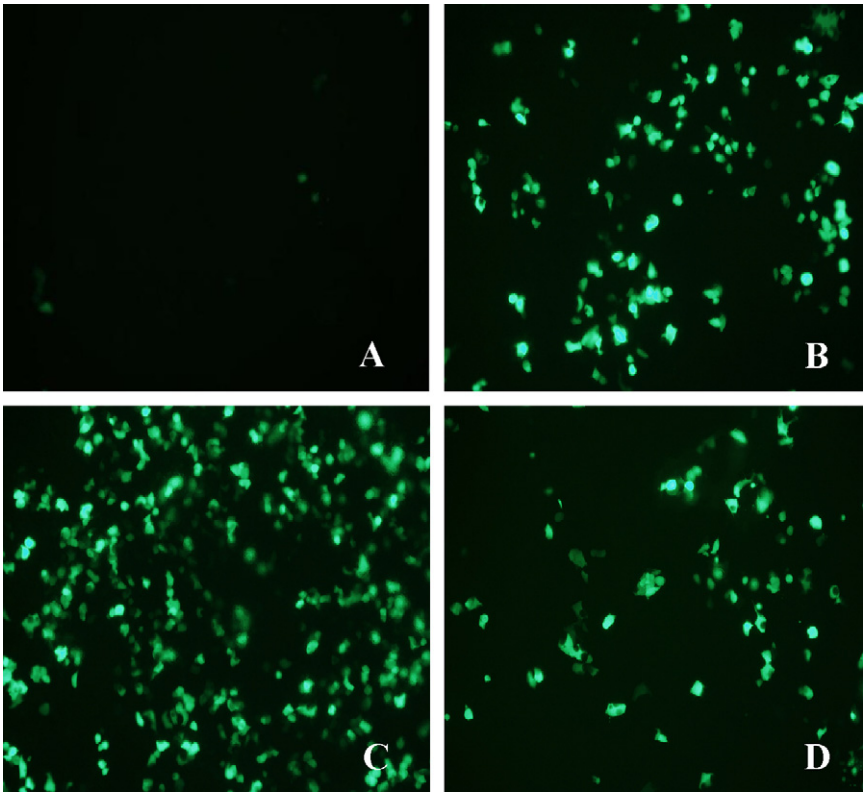

Fig. 7. Images of 293 T cells transfected with polymer/DNA self-assembled nanoparticles as observed under fluorescent microscope (20× magnification). The pictures corresponding to (A) cells transfected by naked plasmid DNA; (B) cells transfected by PASNs at polymer/DNA ratio of 10:1; (C) cells transfected by PHSNs at polymer/DNA ratio of 10:1; (D) cells transfected by PESNs at polymer/DNA ratio of 10:1. Transfection was performed at a dose of 2.5 μg of DNA for all groups and analyzed at 48 h after transfection.

moiety as endosomolytic agent). Therefore, PDHP could be presented as a promising cationic polymer for gene delivery. Further studies would focus on evaluating in vivo transfection efficiency of this cationic derivate of polyphosphazene.

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References

Aldini, N.N., Caliceti, P., Lora, S., Fini, M., Giavaresi, G., Rocca, M., Torricelli, P., Giardino, R., Veronese, F.M., 2001. Calcitonin release system in the treatment of experimental osteoporosis. Histomorphometric evaluation. J. Orthop. Res. 19, 955–961.

Allcock, H.R., Kugel, R.L., 1965. Synthesis of high polymeric alkoxy- and aryloxy-phosphonitriles. J. Am. Chem. Soc. 87, 4216–4217.

Allcock, H.R., Crane, C.A., Morrissey, C.T., Nelson, J.M., Reeves, S.D., Honeyman, C.H., Manners, I., 1996. Living cationic polymerization of phosphoramines as an ambient temperature route to polyphosphazenes with controlled molecular weights. Macromolecules 29, 7740–7747.

Andrianov, A.K., Marin, A., Roberts, B.E., 2005. Polyphosphazene polyelectrolytes: a link between the formation of noncovalent complexes with antigenic proteins and immunostimulating activity. Biomacromolecules 6, 1375–1379.

Carriedo, G.A., Alonso, F.J.G., Gomez-Elipe, P., Fidalgo, J.I., Alvarez, J.L.G., Presa-Soto, A., 2003. A simplified and convenient laboratory-scale preparation of 14^N or 15^N high molecular weight poly(dichlorophosphazene) directly from PCl_5. Chem. Eur. J. 16, 3833–3836.

Ciftci, K., Levy, R.J., 2001. Enhanced plasmid DNA transfection with lysosomotropic agents in cultured fibroblasts. Int. J. Pharm. 218, 81–92.

de Wolf, H.K., Luten, J., Snel, C.J., Oussoren, C., Hennink, W.E., Storm, G., 2005. In vivo tumor transfection mediated by polyplexes based on biodegradable poly(DMAEA)-phosphazene. J. Control. Release 109, 275–287.

El-Aneed, A., 2004. An overview of current delivery systems in cancer gene therapy. J. Control. Release 94, 1–14.

Erbacher, P., Roche, A.C., Monsigny, M., Midoux, P., 1996. Putative role of chloroquine in gene transfer into a human hepatoma cell line by DNA/lactosylated polylysine complexes. Exp. Cell Res. 225, 186–194.

Gao, Y., Gu, W.W., Chen, L.L., Xu, Z.H., Li, Y.P., 2007. A multifunctional nano device as non-viral vector for gene delivery: in vitro characteristics and transfection. J. Control. Release 118, 381–388.

Greish, Y.E., Bender, J.D., Lakshmi, S., Brown, P.W., Allcock, H.R., Laurencin, C.T., 2005. Low temperature formation of hydroxyapatite-poly(alkyloxybenzoate)phosphazene composites for biomedical applications. Biomaterials 26, 1–9.

Kima, T.H., Ihma, J.E., Choia, Y.J., Nahb, J.W., Cho, C.S., 2003. Efficient gene delivery by urocanic acid-modified chitosan. J. Control. Release 93, 389–402.

Lee, H., Jeong, J.H., Park, T.G., 2001. A new gene delivery formulation of polyethylenimine/DNA complexes coated with PEG conjugated fusogenic peptide. J. Control. Release 76, 183–192.

Luten, J., van Steenis, J.H., van Someren, R., Kemmink, J., Schuurmans-Nieuwenbroek, N.M.E., Koning, G.A., Crommelin, D.J.A., van Nostrum, C.F., Hennink, W.E., 2003. Water-soluble biodegradable cationic polyphosphazenes for gene delivery. J. Control. Release 89, 483–497.

Mahato, R.I., Rolland, A., Tomlinson, E., 1997. Cationic lipid-based gene delivery systems: pharmaceutical perspectives. Pharm. Res. 14, 853–859.

Merdan, T., Kopeček, J., Kissela, T., 2002. Prospects for cationic polymers in gene and oligonucleotide therapy against cancer. Adv. Drug Deliv. Rev. 54, 715–758.

Mishra, S., Heidel, J.D., Webster, P., Davis, M.E., 2006. Imidazole groups on a linear, cyclodextrin-containing polycation produce enhanced gene delivery via multiple processes. J. Control. Release 116, 179–191.

Mislick, K.A., Baldeschwieler, J.D., 1996. Evidence for the role of proteoglycans in cation-mediated gene transfer. Proc. Natl. Acad. Sci. U.S.A. 93, 12349–12354.

Park, J.S., Han, T.H., Lee, K.Y., Han, S.S., Hwang, J.J., Moon, D.H., Kim, S.Y., Cho, Y.W., 2006. N-acetyl histidine-conjugated glycol chitosan self-assembled nanoparticles for intracytoplasmic delivery of drugs: endocytosis, exocytosis and drug release. J. Control. Release 115, 37–45.

Putnam, D., Gentry, C.A., Pack, D.W., Langer, R., 2001. Polymer-based gene delivery with low cytotoxicity by a unique balance of side-chain termini. Proc. Natl. Acad. Sci. U.S.A 98, 1200–1205.

Roufaï, M.B., Midoux, P., 2001. Histidylated polylysine as DNA vector: elevation of the imidazole protonation and reduced cellular uptake without change in the polyfection efficiency of serum stabilized negative polyplexes. Bioconjug. Chem. 12, 92–99.

Singh, R.S., Goncalves, C., Sandrin, P., Pichon, C., Midoux, P., Chaudhuri1, A., 2004. On the gene delivery efficacies of pH-sensitive cationic lipids via endosomal protonation: a chemical biology investigation chembiol. Chem. Biol. 11, 713–723.

Spack, E.G., Sorgi, R.S., 2001. Development non-viral DNA delivery systems for cancer and infectious disease. Drug Discov. Today 6, 186–197.

Swami, A., Aggarwal, A., Pathak, A., Patnaik, S., Kumar, P., Singh, Y., Gupta, K.C., 2007. Imidazolyl-PEI modified nanoparticles for enhanced gene delivery. Int. J. Pharm. 335, 180–192.

Wagner, E., Plank, C., Zatloukal, K., Cotten, M., Birnstiel, M.L., 1992. Influenza virus hemagglutinin HA-2 N-terminal fusogenic peptides augment gene transfer by transferring–polylysine–DNA complexes: toward a synthetic virus-like gene-transfer vehicle. Proc. Natl. Acad. Sci. U.S.A. 89, 7934–7938.

Zhang, J.X., Qiu, L.Y., Jin, Y., Zhu, K.J., 2005. Physicochemical characterization of polymeric micelles constructed from novel amphiphilic polyphosphazene with poly(*N*-isopropylacrylamide) and ethyl 4-aminobenzoate as side groups. Colloid Surf. B: Biointerf. 43, 123–130.