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International Journal of Pharmaceutics 265 (2003) 75–84
international journal of pharmaceutics
www.elsevier.com/locate/ijpharm

Effect of side-chain structures on gene transfer efficiency of biodegradable cationic polyphosphoesters

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Received 18 April 2003; received in revised form 23 July 2003; accepted 23 July 2003

Abstract

Cationic polyphosphoesters (PPEs) with different side-chain charge groups were designed and synthesized as biodegradable gene carriers. Poly(*N*-methyl-2-aminoethyl propylene phosphate) (PPE-MEA), with a secondary amino group ($–CH_2CH_2NHCH_3$) side chain released DNA in several hours at N/P (amino group of polymer to phosphate group of DNA) ratios from 0.5 to 5; whereas PPE-HA, bearing $–CH_2(CH_2)_4CH_2NH_2$ groups in the side chain, did not release DNA at the same ratio range for 30 days. Hydrolytic degradation and DNA binding results suggested that side chain cleavage, besides the polymer degradation, was the predominant factor affected the DNA release and transfection efficiencies. The side chain of PPE-MEA was cleaved faster than that of PPE-HA, resulting poor cellular uptake and no transgene expression for PPE-MEA/DNA complexes in COS-7 cells at charge ratios from 4 to 12. In contrast, PPE-HA/DNA complexes were stable enough to be internalized by cells and effected gene transfection (3400 folds higher than background at a charge ratio of 12). Interestingly, gene expression levels mediated by PPE-MEA and PPE-HA in mouse muscle following intramuscular injection of complexes showed a reversed order: PPE-MEA/DNA complexes mediated a 1.5–2-fold higher luciferase expression in mouse muscle as compared with naked DNA injection, while PPE-HA/DNA complexes induced delayed and lowered luciferase expression than naked DNA. These results suggested that the side chain structure is a crucial factor determining the mechanism and kinetics of hydrolytic degradation of PPE carriers, which in turn influenced the kinetics of DNA release from PPE/DNA complexes and their transfection abilities in vitro and in vivo.

Keywords: Non-viral gene delivery; Sustained release; Polymeric carrier; Polyphosphoester

1. Introduction

Polymer-mediated gene transfer is of great potential in gene medicine, genetic immunization and tissue engineering applications. There are two different types of polymeric gene carriers. One is based on formulations with non-charged polymers, allowing

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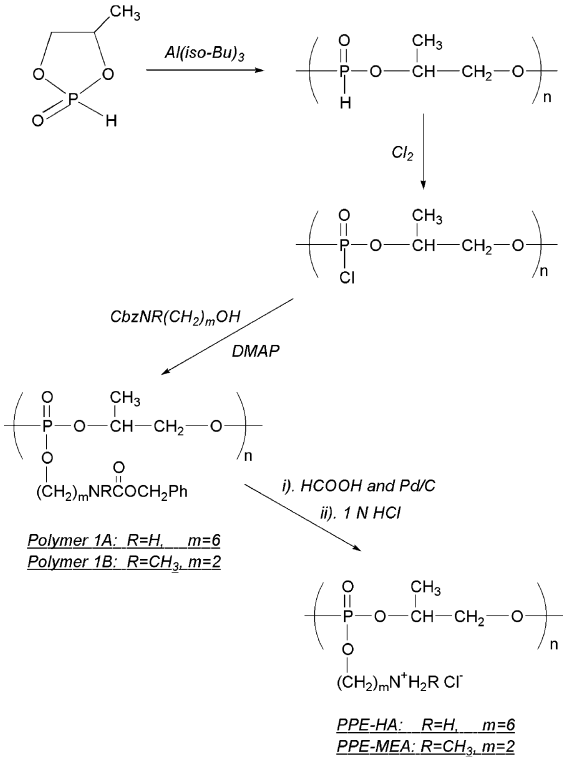
doi:10.1016/j.ijpharm.2003.07.006

localized and extracellular sustained release of plasmid DNA, and is potentially useful for DNA vaccination and tissue engineering applications (Chen et al., 1998; Madsen and Mooney, 2000; Wierzbicki et al., 2002; Klencke et al., 2002). The other predominant type of polymeric gene carrier is based on electrostatic interaction using cationic polymer (Goldman et al., 1997; Garnett, 1999; Roy et al., 1999; Han et al., 2000; Georgantas et al., 2000; Zuber et al., 2001; Mao et al., 2001; Wang et al., 2001, 2002a,b; Davis, 2002; Gebhart et al., 2002). Electrostatic interaction between the cationic polymer and negative DNA molecules results in the formation of complexes or nanoparticles, providing protection to DNA from enzyme degradation and facilitating the cellular uptake of the DNA. The most extensively studied polymeric gene carriers are non-biodegradable polycations, such as polyethylenimine (PEI) or cationic dendrimers. However, when DNA is complexed with these non-degradable polycationic carriers, DNA release might be a rate-limiting step. They provide little or no controlled release function both inside and outside of the cells. Biodegradable and polycationic gene carriers circumvent this potential drawback by providing extracellular and conceivably intracellular sustained release of the DNA. The DNA release kinetics could be adjusted by molecular design of the carrier, which could also potentially influence the intracellular trafficking of the delivered DNA. Such a system might enhance gene transfer efficiency by improving the bioavailability of DNA inside or outside of cells.

We have previously reported a biodegradable gene carrier, poly(2-aminoethyl propylene phosphate) (PPE-EA) (Wang et al., 2001). PPE-EA/DNA complexes release DNA in a sustained manner and enhance gene expression following intramuscular injection (Wang et al., 2002b). The aim of this study is to compare two other polyphosphoester (PPE) carriers in the same series with different side chains, poly(6-aminohexyl propylene phosphate) (PPE-HA) and poly(*N*-methyl-2-aminoethyl propylene phosphate) (PPE-MEA) (Scheme 1), and investigate the effect of the side chain on their degradation kinetics, DNA binding ability, DNA release properties from the complexes, cytotoxicity and transfection ability. Luciferase expression levels in mouse muscle mediated by these two new carriers were also compared.



Scheme 1. Synthetic scheme for PPE-MEA and PPE-HA.

2. Materials and methods

2.1. Materials

4-Methyl-2-oxo-2-hydro-1,3,2-dioxaphospholane was prepared according to the method reported by Lucas et al. and polymerized as reported (Lucas and Scully, 1950; Wang et al., 2001). Carbobenzoxy chloride (−30% in toluene) (TCI, Tokyo, Japan) and Pd/C (10%, Aldrich) were used as received. 4-Dimethylaminopyridine (TCI) was dried under vacuum before use. Benzyl-*N*-(6-hydroxyhexyl) carbamate and benzyl-*N*-methyl-*N*-(2-hydroxyethyl) carbamate were synthesized according to the literature (Greene and Wuts, 1999). Poly-L-lysine (PLL, MW 27 kDa) and polyethylenimine (PEI, MW 25 kDa) were products of Sigma Chemical Co. (St. Louis, MO). PEI was purified by dialysis against water (MWCO (molecular weight cutoff) 3500, Pierce, Rockford, IL). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from

BDH Laboratory Supplies (Poole, Dorset, UK). Dulbecco's modified Eagle's medium (DMEM), L-glutamine, Terrific Broth and ampicillin were purchased from Gibco-BRL (Grand Island, NY). Fetal bovine serum (FBS) was the product of HyClone (Logan, UT). QIAGEN Giga plasmid purification kit was purchased from Qiagen (Hilden, Germany). Luciferase assay system was purchased from Promega (Madison, WI). Bicinchoninic acid (BCA) protein assay kit was purchased from Pierce Chemical Co. (Rockford, IL).

2.2. Polymer characterization

Gel permeation chromatography (GPC) was carried out using a Waters 2690 HPLC module equipped with a Phenomenex® PolySep-GFC-P 4000 HPLC column (Torrance, CA), which was connected to a multiangle light scattering detector (MiniDawn, Wyatt Technology, Santa Barbara, CA) and a differential refractive index detector (Optilab DSP interferometric refractometer, Wyatt Technology). Phosphate buffer (0.1 M, pH 7.4) with 0.15 M NaCl was used as the mobile phase. The ^{1}H, ^{13}C and ^{31}P NMR spectra were measured with a Bruker 400 MHz apparatus (Bruker NMR, Billerica, MA) at 25 °C. Phosphoric acid (85% in water) was used as the external standard for ^{31}P NMR spectra.

2.3. Plasmid DNA

pRELuc plasmid is a 11.9 kb pcDNA encoding firefly luciferase driven by a Rous sarcoma virus promoter inserted into an Invitrogen (San Diego, CA) pREP4 vector (a gift from Dr. R.G. Ulrich, NCI, Frederick, MD). Plasmid pVR1255C is a 6413 bp pcDNA encoding luciferase driven by HCMV promoter (a gift from Dr. Carl J. Wheeler, Vical Inc., San Diego, CA). Both plasmids were amplified in *Escherichia coli* DH5α and purified by QIAGEN Giga plasmid purification kit according to the manufacturer's protocol. The purified plasmids were dissolved in saline and kept in aliquots at a concentration of 1–2 mg/ml.

2.4. Cell culture

Monkey SV40 transformed kidney fibroblast COS-7 cells was obtained from American Type Culture Collection (ATCC), and maintained in complete DMEM (supplemented with 10% FBS, 2 mM L-glutamine, 100 μg/ml of streptomycin, and 100 U/ml of penicillin) at 37 °C in a humidified 5% CO_2-containing atmosphere.

2.5. Animals

Six to 8-week-old female Balb/c mice were obtained and housed in National University of Singapore Animal Holding Unit. Mice were maintained on ad libitum rodent feed and water at room temperature, 40% humidity. All animal procedures were approved by the Animal Care and Use Committees at Johns Hopkins University School of Medicine and National University of Singapore, Faculty of Medicine.

2.6. Synthesis

PPE-HA and PPE-MEA (structures shown in Scheme 1) were synthesized in a similar method as we reported earlier (Wang et al., 2001). As an example, poly(4-methyl-2-oxo-2-hydro-1,3,2-dioxaphospholane) was chloriated in CH_2Cl_2 by introduing Cl_2 until first appearance of a persistent yellow color. The resulted poly(4-methyl-2-oxo-2-chloro-1,3,2-dioxaphospholane) was cooled with ice-water bath, and to this solution was added dimethylaminopyridine (5% excess), followed by adding dropwise a solution of benzyl-*N*-(6-hydroxyhexyl) carbamate (5% excess) through a funnel. The mixture was refluxed for 48 h, washed with 1N HCl twice and water twice. The organic layer was dried over anhydrous magnesium sulfate and concentrated. The precusor product poly(*N*-benzoylcarboxyl-6-aminohexyl propylene phosphate) was obtained as a white glassy solid by pouring the concentrated solution into ether.

Remove of the Cbz groups was accomplished using formic acid and Pd/C according to a reported method (Zhou and Kohn, 1990). Under a nitrogen stream, 300 mg of poly(*N*-benzoylcarboxyl-6-aminohexyl propylene phosphate) was dissolved in 4 ml of anhydrous DMF in a 25 ml round-bottom flask. To this solution was added 1 g of Pd/C catalyst. With vigorous stirring, formic acid (14 ml) was added dropwise over 15 min. The mixture was stirred at room temperature for 14 h and then filtered to remove Pd/C. The catalyst was washed with 20 ml

of 1N HCl, and the filtrate was combined and concentrated under vacuum using a water bath below 50 °C. Ten milliliters of 1N HCl was added to the concentrated solution (~5 ml). The solution was concentrated again as described above to a volume of 1 ml. The resulted solution was added dropwise to 150 ml of acetone cooled to −20 °C. The final polymer PPE-HA was isolated and dried thoroughly under vacuum.

These polymers were characterized by ^{1}H NMR, ^{13}C NMR, ^{31}P NMR. Poly(*N*-benzoylcarboxyl-6-aminohexyl propylene phosphate) (Polymer 1A): ^{1}H NMR (400 MHz, $CDCl_3$, ppm) δ: 1.20–1.40 (7H, –C$\underline{H}_3$, –O$(CH_2)_2$C$\underline{H}_2$C$\underline{H}_2(CH_2)_2$NH–), 1.40–1.52 (2H, –OC$\underline{H}_2CH_2(CH_2)_4$NH–), 1.52–1.68 (2H, –O$(CH_2)_4C\underline{H}_2CH_2$NH–), 3.04–3.20 (2H, –O$(CH_2)_5C\underline{H}_2$NH–), 3.88–4.12 (4H, –OC$\underline{H}_2(CH_2)_5$NH–, –P–OCH–C$\underline{H}_2$O–), 4.52–4.72 (1H, –P–OC$\underline{H}$-CH_2O–), 5.00–5.12 (2H, –C$\underline{H}_2$Ph), 5.24–5.60 (1H, –OCH_2CH_2N$\underline{H}$–), 7.22–7.36 (5H, PhH); ^{13}C NMR (400 MHz, $CDCl_3$, ppm) δ: 18.11 (–$\underline{C}H_3$), 25.42 (–O$(CH_2)_2\underline{C}H_2(CH_2)_3$NH–), 26.57 (–O$(CH_2)_3\underline{C}H_2(CH_2)_2$NH–), 30.13 (–O$CH_2\underline{C}H_2(CH_2)_4$NH–), 30.41 (–O$(CH_2)_4\underline{C}H_2CH_2$NH–), 41.25 (–$\underline{C}H_2$NH–), 66.77 ($\underline{C}H_2$Ph), 68.42 (–O$\underline{C}H_2(CH_2)_5$NH–), 70.13 (–O$\underline{C}HCH_2$–), 73.93 (–OCH$\underline{C}H_2$–), 128.36, 128.81, 137.22 (aromatic carbons), 156.98 ($\underline{C}$=O); ^{31}P NMR (400 MHz, $CDCl_3$, ppm) δ: −3.82 to 0.56.

Poly(6-aminohexyl propylene phosphate) (PPE-HA): ^{1}H NMR (400 MHz, D_2O, ppm) δ: 1.24–1.44 (7H, –CHC$\underline{H}_3$, –O$(CH_2)_2$C$\underline{H}_2$C$\underline{H}_2(CH_2)_2$NH–), 1.57–1.75 (4H, –OCH_2C$\underline{H}_2CH_2CH_2CH_2C\underline{H}_2$N), 2.89–2.97 (2H, –O$(CH_2)_5C\underline{H}_2$N), 3.76–4.44 (5H, –OC$\underline{H}_2(CH_2)_5$N–, –P–OC$\underline{H}$–$CH_2$O–); ^{13}C NMR (400 MHz, D_2O, ppm) δ: 17.32 (–CH$\underline{C}H_3$), 25.71 (–O$(CH_2)_2\underline{C}H_2(CH_2)_3$N), 27.15 (–O$(CH_2)_3\underline{C}H_2(CH_2)_2$N–), 29.80 (–O$CH_2\underline{C}H_2(CH_2)_4$N–), 32.05 (–O$(CH_2)_4\underline{C}H_2CH_2$N–), 39.95 (–O$(CH_2)_5\underline{C}H_2$N–), 69.56 (–O$\underline{C}H_2(CH_2)_5$N–), 70.71 (–O$\underline{C}HCH_2$–), 75.42 (–OCH$\underline{C}H_2$–); ^{31}P NMR (400 MHz, D_2O, ppm) δ: −6.48 to −3.62. $\bar{M}_w = 3.76 \times 10^4$, $\bar{M}_n = 2.79 \times 10^4$.

Poly(*N*-benzoylcarboxyl-*N*-methyl-2-aminoethyl propylene phosphate) (polymer 1B): ^{1}H NMR (400 MHz, $CDCl_3$, ppm) δ: 1.12–1.46 (3H, –CHC$\underline{H}_3$), 3.05–3.14 (2H, –OCH_2C$\underline{H}_2$NCH_3–), 3.24–3.48 (3H, –OCH_2CH_2NC$\underline{H}_3$–), 3.84–4.20 (4H, –OC$\underline{H}_2CH_2$NCH_3–, –P–OCH–C$\underline{H}_2$O–), 4.48–4.56 (1H, –P–OC$\underline{H}$–CH_2O–), 5.00–5.12 (2H, –C$\underline{H}_2$Ph), 7.24–7.38 (5H, Ph$\underline{H}$); ^{13}C NMR (400 MHz, $CDCl_3$, ppm) δ: 17.44 (–CH$\underline{C}H_3$), 29.88 (–OCH_2CH_2N$\underline{C}H_3$–), 36.90 (–O$CH_2\underline{C}H_2$NCH_3–), 65.41 (–$\underline{C}H_2$Ph), 66.01 (–O$\underline{C}H_2CH_2$NCH_3–), 66.29 (–O$\underline{C}HCH_2$–), 69.63 (–OCH$\underline{C}H_2$–), 127.56, 128.02, 136.33 (aromatic carbons), 156.19 ($\underline{C}$=O); ^{31}P NMR (400 MHz, $CDCl_3$, ppm) δ: −2.12 to 2.48.

Poly(*N*-methyl-2-aminoethyl propylene phosphate) (PPE-MEA): ^{1}H NMR (400 MHz, D_2O, ppm) δ: 1.23–1.47 (3H, –CHC$\underline{H}_3$), 2.68–2.73 (3H, –OCH_2CH_2NC$\underline{H}_3$), 3.31–3.39 (2H, –OCH_2C$\underline{H}_2$NCH_3), 3.80–4.50 (5H, –OC$\underline{H}_2CH_2$N–, –P–OC$\underline{H}$–C$\underline{H}_2$O); ^{13}C NMR (400 MHz, D_2O, ppm) δ: 17.13, 17.42 (–CH$\underline{C}H_3$), 33.51 (–OCH_2CH_2N$\underline{C}H_3$), 48.89 (–O$CH_2\underline{C}H_2$NCH_3), 64.00 (–O$\underline{C}H_2CH_2$NCH_3), 68.89, 69.44 (–O$\underline{C}$HCH_2–), 76.18, 77.35 (–OCH$\underline{C}H_2$–); ^{31}P NMR (400 MHz, D_2O, ppm) δ: −6.58 to 2.85. $\bar{M}_w = 1.29 \times 10^4$, $\bar{M}_n = 1.02 \times 10^4$.

2.7. Degradation

Polymer (PPE-MEA or PPE-HA) was incubated in 0.1 M phosphate buffer containing 0.15 M NaCl at 37 °C. The molecular weights of degradation products at various time points were determined by gel permeation chromatography using phosphate buffer (0.1 M) with 0.15 M NaCl as the mobile phase (0.5 ml/min) as described above.

2.8. Cytotoxicity assay

The cytotoxicity of PPE-HA and PPE-MEA were evaluated in comparison with two other widely used gene carriers, PLL and PEI. COS-7 cells were seeded in a 96-well plate (Becton–Dickinson, Lincoln Park, NJ) with 50 μl of complete DMEM at a density of 6000 cells per well. Cells were incubated for 24 h at 37 °C followed by adding the solution of polymer (PPE-MEA or PPE-HA) or PLL or PEI (50 μl in complete DMEM) to achieve various carrier concentrations ranging from 0 to 100 μg/ml. Following 24-h incubation, 25 μl of MTT solution (5 mg/ml in PBS) was added, and the plates were incubated at 37 °C for an additional two hours. To each well was added 100 μl of the extraction buffer (20% SDS in 50% DMF) and the plate was incubated overnight at 37 °C. The optical density at 570 nm in each well was measured on a microplate reader (Model 550,

Bio-Rad Laboratories, Hercules, CA) using wells without MTT addition as the blank.

2.9. Preparation of complexes and release of plasmid DNA from the complexes

PPE-MEA/DNA and PPE-HA/DNA complexes were prepared in PBS by mixing pRELuc plasmid DNA (60 μg/ml) with either PPE-MEA or PPE-HA solution at different concentrations to achieve various N/P ratios of 0.5 to 5. The complexes were incubated at room temperature for 30 min, and then incubated at 37 °C. At various time points, complex samples were analyzed for plasmid integrity by gel electrophoresis (0.8% agarose gel).

2.10. Cellular uptake of pDNA

pRELuc plasmid DNA was labeled with [α-^{32}P]-CTP using a nick translation kit (Life Technologies, Rockville, MD) (Rigby et al., 1977), and purified by ethanol precipitation according to manufacture's protocols. COS-7 cells were seeded in 24-well plates 24 h before at a density of 40,000 per well. PPE-HA/[^{32}P]-pDNA or PPE-MEA/[^{32}P]-pDNA complexes at various N/P ratios from 4 to 12 were prepared in saline with the same method as described above and incubated with COS-7 cells either at 37 °C or 4 °C for 4 h. The cells were then washed with PBS, solubilized with 0.5 ml of 1 N NaOH, followed by incubation at room temperature for 2 h. The solutions were added to PCS solution (Amersham-Pharmacia, Arlington Heights, IL), and ^{32}P radioactivity was determined using a scintillation counter (Beckman, Palo Alto, CA).

2.11. In vitro transfection protocol and luciferase assay

In vitro transfection of COS-7 cells with PPE-MEA/DNA or PPE-HA/DNA complexes was evaluated using luciferase as a marker gene. Complexes were prepared with pRELuc plasmid at various N/P ratios in saline as described above. Cells were seeded 24 h prior to transfection into a 24-well plate (Becton–Dickinson) at the density of 4×10^4 per well with 1 ml of complete medium. At the time of transfection, the medium in each well was replaced with 1 ml of serum free DMEM. Complexes at a DNA dose of 3 μg/well were incubated with the cells for 4 h at 37 °C. The medium was replaced with 1 ml of fresh complete medium and cells were further incubated for 48 h. All the transfection tests were performed in triplicate. After the incubation, cells were permeabilized with 200 μl of cell lysis buffer (Promega Co., Madison, WI). The luciferase activity in cell extracts was measured using a luciferase assay kit (Promega Co.) on a luminometer (Lumat LB 9507, EG&G Berthold, Bad Wildbad, Germany). The light units (LU) were normalized against protein concentration in the cell extracts, which was measured using BCA protein assay kit (Pierce).

2.12. Luciferase expression in mouse muscle

Balb/c mice were injected intramuscularly into bilateral tibialis anterior muscles with 40 μl of complexes containing 1 μg of pVR1255C with either PPE-MEA or PPE-HA at a N/P ratio of 0.5 (six species per group). Groups of mice receiving same volume of naked DNA and saline injection served as the controls. At various time-points, the muscles were isolated and homogenized with 0.5 ml of Reporter Lysis Buffer (Promega Co.), followed by a freeze–thaw cycle. The extraction mixture was then centrifuged at 13,000 × *g* for 5 min and 10 μl of the supernatant were used for light emission measurement for a period of 10 s. Reconstituted luciferase (Promega Co.) was used as a standard.

3. Results and discussion

3.1. PPE synthesis and degradation

The synthetic scheme for cationic PPE-MEA and PPE-HA is shown in Scheme 1. The synthesis started with preparation of the precursor polymer, namely poly(1,2-propylene *H*-phosphonate), which was obtained by ring opening polymerization of a cyclic monomer, 4-methyl-2-oxo-2-hydro-1,3,2-dioxaphospholane. Following the method of P–H chlorination described by Penczek et al. (Penczek and Pretula, 1993) we obtained a precursor polymer, poly(4-methyl-2-oxo-2-chloro-1,3,2-dioxaphospholane), containing P–Cl bonds that are highly reactive

to nucleophiles. Reacting this precursor with excess amount of benzyl-*N*-(6-hydroxyhexyl) carbamate or benzyl-*N*-methyl-*N*-(2-hydroxyethyl) carbamate with 4-dimethylamino-pyridine (DMAP) as a catalyst yielded intermediate polymer with Cbz-protected amino groups in the side chain with a recovery yield of around 60% for both polymers. The NMR spectra (^{1}H, ^{13}C and ^{31}P) indicated that all the pendant chains were conjugated with the protected amino groups. Removal of Cbz group was accomplished using the formic acid-Pd/C hydrogenation method. The reaction product was treated with chloric acid and precipitated in excess amount of acetone. PPE-MEA and PPE-HA were obtained as white powder with yields over 80%. The absence of peaks assigned to benzoxycarbonyl group in ^{1}H spectrum (δ: 7.2–7.4 and 5.0–5.1 ppm) and FT-IR spectrum (1716 cm^{-1}) indicated that the deprotection reaction was complete.

PPE-MEA and PPE-HA underwent hydrolytic degradation when incubated in PBS at 37 °C. This is likely due to the hydrolytic cleavage of the phosphoester bonds in the backbone as well as the side chain. The initial $\bar{M}_w$ of PPE-MEA and PPE-HA determined by GPC were 1.29×10^4 and 3.76×10^4, respectively. The slightly lower molecular weight of PPE-MEA comparing with that of PPE-HA is most likely due to the faster degradation during deprotection of PPE-MEA. Some pendent groups of PPE-MEA are also cleaved during deprotection, which generated phosphate groups instead, as evidenced by the splitting peaks of C in ^{13}C NMR spectrum of PPE-MEA (Fig. 1) for methyl (δ: 17.13, 17.42, –CH<u>C</u>H$_3$), methylene (δ: 68.89, 69.44, –O<u>C</u>HCH$_2$–), and methylidyne (δ: 76.18, 77.35, –OCH<u>C</u>H$_2$–) groups of the isopropyldiol residues due to different chemical environments between –P–OH and –P–OCH$_2$CH$_2$N–.

The degradation kinetics of both PPEs was followed by GPC analysis shown in Fig. 2. $\bar{M}_w$ of PPE-HA dropped 50% in 20 days from 3.76×10^4 to 1.87×10^4, followed by another 20% decrease (to 1.48×10^4) in the next 30 days, showing a decreased degradation rate with time. This is similar to another reported polyphosphoester carrier PPE-EA (Wang et al., 2001). In contrast, PPE-MEA degraded at a much slower rate. $\bar{M}_w$ of PPE-MEA decreased only 28% after 50 days of incubation under the same conditions.

It has been reported that the degradation of PPE with a methoxy or ethoxy side chain is considerably slower (Baran and Penczek, 1995). The relatively fast degradation of the cationic polyphosphates suggests a self-catalytic degradation mechanism involving nucleophilic attack of the phosphate bonds by the pendant amino groups. This mechanism also likely led to a cleavage of the side chain, rendering negatively charged phosphate ions instead. Such a negatively charged backbone would shield further attack of OH$^-$ on P=O, and result in a decreased degradation in the latter incubation stage. Following this reasoning, the slower degradation rate of PPE-MEA than that of PPE-HA observed by GPC also suggested that the side chain of PPE-MEA might be cleaved at a faster rate.

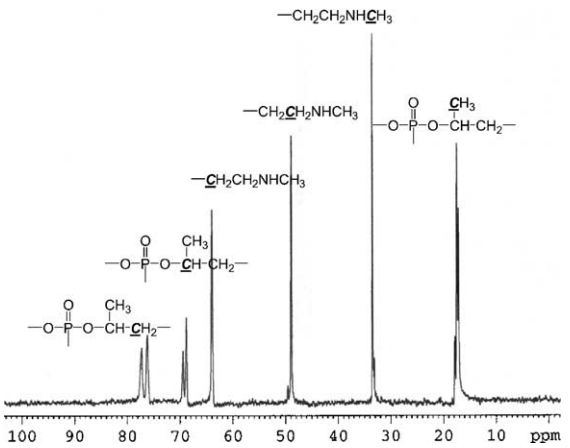


Fig. 1. ^{13}C NMR of PPE-MEA (400 MHz, D_2O).

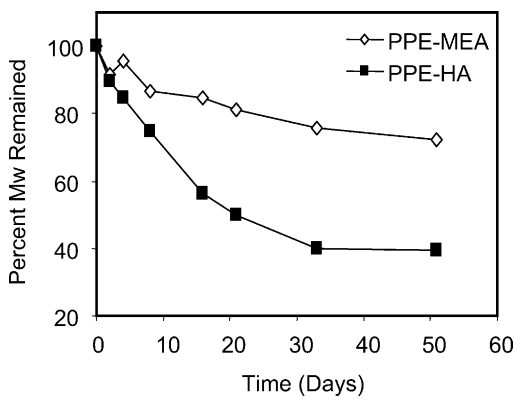


Fig. 2. Degradation profiles of PPE-MEA and PPE-HA in PBS at 37 °C.

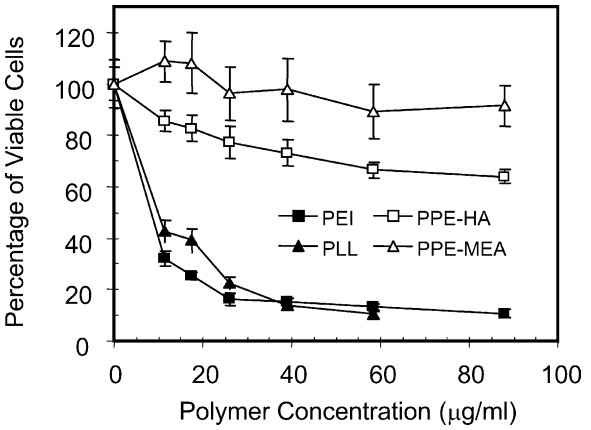


Fig. 3. Cytotoxicity of PPE-MEA and PPE-HA in COS-7 cells in comparison with PLL and PEI. Mean ± S.D. ($n = 6$).

3.2. Cytotoxicity of PPEs

Cytotoxicity of a polymeric gene carrier is important for practical considerations. It may also affect the gene expression in hindering or interfering with the transcription and translation processes in the cells. Previously reported PPE-EA carrier showed minimal toxicity to COS-7, HEK293 cells and good biocompatibility in mouse muscle because of the nontoxic building blocks and degradation products (Wang et al., 2001, 2002b). The in vitro cytotoxicity of PPE-MEA and PPE-HA as analyzed by the MTT assay showed similar favorable results (Fig. 3). In this assay, the incubation time of polymer with cells were extended to 24 h as opposed to the typical 4-h incubation in order to highlight the low cytotoxicity of PPEs (Wang et al., 2001). PPE-MEA exhibited minimal toxicity in COS-7 cells, while PPE-HA showed slightly higher toxicity, but it was still much lower than that of PLL and PEI. The LD_{50} of PEI and PLL in this assay was below 20 μg/ml, while the viability of cells cultured with 88 μg/ml of PPE-HA still remained above 70%. The cytotoxicity of these polymers to HEK293 cells was similar to that in COS-7 cells (data not shown).

3.3. DNA release profiles

Both PPE-MEA and PPE-HA could bind DNA efficiently. Complete binding of plasmid DNA occurred at minimum charge (N/P) ratios of 1 and 3 for PPE-HA and PPE-MEA, respectively (Fig. 4, 0 h). DNA release properties of these two carriers were significantly different (Fig. 4). No DNA release was detected for up to 30 days from PPE-HA/DNA complexes (N/P ratio >1) at 37 °C in PBS. This is probably due to the higher hydrophobicity imparted by the longer alkyl side chain that hinders the nucleophilic attack by hydroxyl ion. Matching this hypothesis, PPE-MEA with a shorter side chain yielded a faster DNA release than PPE-HA.

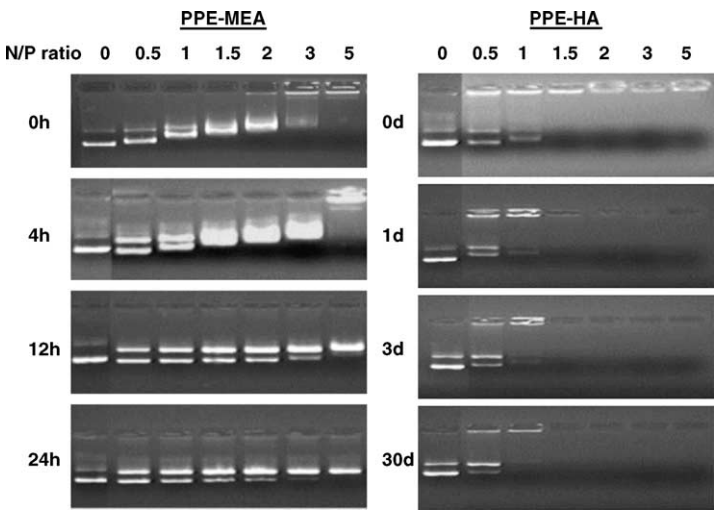


Fig. 4. Electrophoretic mobility of DNA released from complexes prepared at various N/P ratios. Complexes were incubated in PBS at 37 °C, and analyzed on a 0.8% agrose gel at various time points.

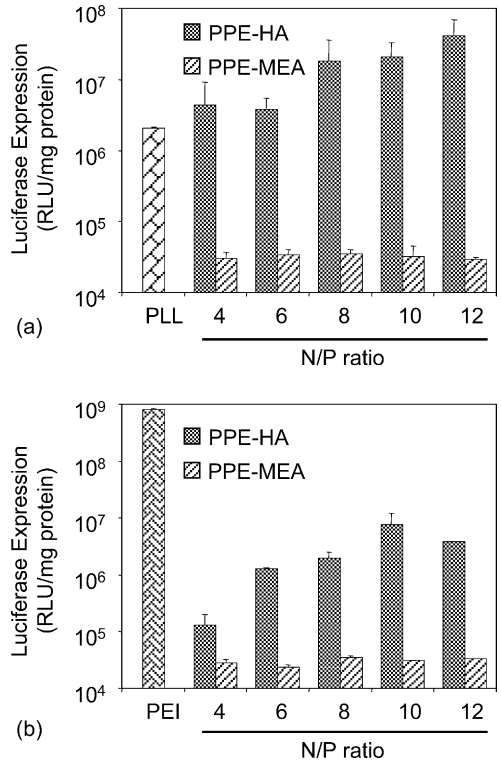


Fig. 5. Transfection efficiency of PPE-MEA/DNA and PPE-HA/DNA complexes in COS-7 cells in the presence of 100 μM CQ (a) and in the absence of CQ (b). Mean ± S.D. ($n = 3$).

DNA was completely released in several hours and the release rate was dependent on the N/P ratio. Higher ratios led to slower release of DNA from the complexes. This faster DNA release from PPE-MEA/DNA complexes is likely a result of side-chain cleavage in PPE-MEA. As the side chain of PPE-MEA is cleaved rapidly, the positive charges are replaced with negative charges (phosphate ion). This resulted in a less positive charged carrier, therefore reduced the binding ability of carrier to DNA. Taken together, the degradation mechanism, particularly the side chain cleavage, seemed to be the dominant factor affecting the DNA release rate from PPE/DNA complexes.

3.4. Transfection efficiency in vitro

Transfection activities of PPE-MEA/DNA and PPE-HA/DNA complexes were evaluated in COS-7 cells. As shown in Fig. 5, in the presence of chloroquine diphosphate (CQ, 100 μM), an efficient reagent to disrupt endosomal membrane, PPE-HA/DNA complexes (N/P ratios between 4 and 12) yielded luciferase expression from 4×10^6 to 4×10^7 RLU/mg protein, which was 2–20 folds higher than PLL-mediated transfection, though significantly lower (100 folds or more) than PEI-mediated transfection. In the absence of CQ (Fig. 5b), as expected, transfection efficiency of PPE-HA/DNA complexes was 3–30 folds lower as compared with that in the presence of CQ. PPE-MEA/DNA complexes, in contrast, showed no transfection ability at N/P ratios of 4–12 with or without the addition of CQ.

To understand the difference of transfection ability between PPE-MEA and PPE-HA, The particle size was measured using a Zetasizer® 3000. Minimal difference was found between two polymers when N/P ratios were from 6 to 12. The average sizes for PPE-HA/DNA and PPE-MEA/DNA complexes were 388±32 nm and 403±27 nm, respectively. Aggregates (1080 nm) were only found with PPE-MEA/DNA complexes at N/P ratio of 4, while at the same ratio, PPE-HA/DNA complexes was smaller, giving particle size of 567 nm in average. We further investigated the cellular uptake of DNA mediated by these two carriers. DNA uptake by COS-7 cells was determined by incubating cells with complexes containing ^{32}P-radio labeled DNA. A parallel experiment was performed at 4 °C using the same protocol. The values obtained at 4 °C accounted for the non-specific cell adsorption of the complexes on the cell membranes, and were taken as the background for the uptake study. DNA uptake after subtraction of background was shown in Fig. 6. Surprisingly, DNA uptake mediated by PPE-MEA was very low and similar to naked DNA. This is probably because that PPE-MEA was quickly dissociated from DNA in the media due to the rapid side-chain cleavage of PPE-MEA. DNA uptake mediated by PPE-HA/DNA complexes ranged from 9 to 14% depending on charge ratio, which correlated to the higher transfection ability to COS-7 cells as compared with that of PPE-MEA/DNA complexes.

3.5. Gene expression in mouse muscle mediated by PPE/DNA complexes

The in vivo gene transfer efficiency of the PPE-MEA/DNA and PPE-HA/DNA complexes was

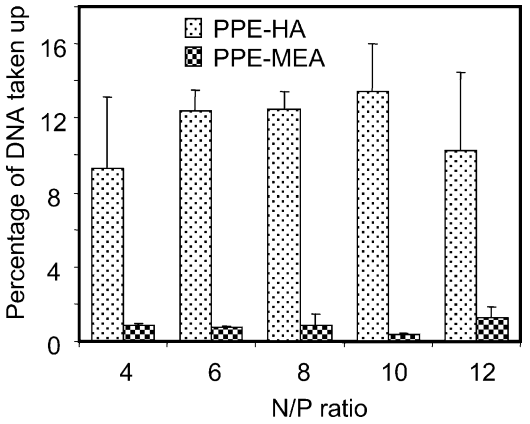


Fig. 6. Cellular uptake of [α-^{32}P] labeled pDNA mediated by PPE carriers at various N/P ratios in COS-7 cells.

evaluated in mouse muscle using the luciferase plasmid VR1255C (Fig. 7). N/P ratio of 0.5 was applied to this study because previous study showed that complexes at high ratios did not transfect well in muscle (Wang et al., 2002b). In this experiment, naked DNA injection showed a peak expression of 3.58 ng of luciferase per muscle at day 7, and the expression leveled off between 2 and 4 weeks. However, gene expression levels mediated by PPE-MEA and PPE-HA showed a reversed order as compared with their in vitro transfection results. PPE-MEA/DNA complexes yielded a higher luciferase expression of 5.12–5.39 ng per muscle at the day 3 and week 4 time points, corresponding to about 1.5–2-fold higher gene expression than naked DNA group at the same time points. However, PPE-HA/DNA complexes showed a delayed and lower

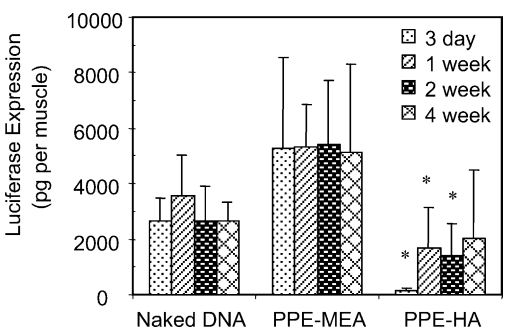


Fig. 7. Luciferase expression in mouse muscle after intramuscular injection of naked DNA and PPE/DNA complexes ($n = 5$–6). (*) $P < 0.05$ when compared with PPE-HA group.

gene expression as compared with naked DNA, with the highest expression of 2.01 ng per muscle at week 4, and the lowest expression of 140 ng per muscle at day 3.

Cationic liposomes have been reportedly as a hindrance rather than assistance in mediating muscle transfection (Yang and Huang, 1996; Cohen et al., 2000). Many cationic polymer/DNA complexes also yielded lower efficiency than naked DNA in transfecting muscles (Wolff et al., 1992; Rolland and Mumper, 1998). Non-ionic polymers such as polyvinyl pyrrolidone and poloxamers are effective carriers in this application, presumably by protecting the DNA from enzyme degradation and providing a sustained and localized release of DNA. The mechanism by which DNA/polymer nanoparticles or complexes failed to mediate efficient transgene expression in muscle remains unclear. One plausible scenario is that the nanoparticles or complexes were taken up by cells in the tissue and transported to other sites. Wang et al. (2001) have shown that PEI/DNA complexes were transported to brain stem in a retrograde transport manner following intramuscular injection of the nanoparticles, while no gene expression was detected at the injection sites. It remains to be tested if PPE-HA/DNA nanoparticles were transferred out of the injection site through a similar manner.

If the polymer carrier is biodegradable, polymer/DNA nanoparticles or complexes could act as a depot for sustained and local release at the injection site, and potentially prolong the gene expression in the muscle. PPE-EA and PPE-MEA are two examples of such a carrier system. PPE-MEA and PPE-EA (Wang et al., 2002b) underwent degradation over a period of a few hours to days, and yielded a sustained release of DNA. This in turn effected higher gene expression than naked DNA.

In conclusion, cationic polyphosphates, PPE-MEA and PPE-HA, with the same backbone but different charge groups, show significant difference in degradation kinetics, DNA release and transfection ability both in vitro and in vivo. The determinant factor appears to be rate of cleavage of the side-chain and the degradation of PPE. This study demonstrated the versatility of this series of biodegradable carriers, and provided a new approach to modulate gene expression through molecular design of the gene carriers.

Acknowledgements

This work was supported by Agency for Science, Technology and Research, Singapore and the Johns Hopkins Singapore.

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