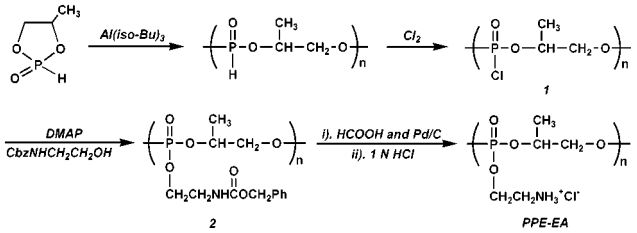
A Novel Biodegradable Gene Carrier Based on Polyphosphoester

Jun Wang, Hai-Quan Mao,* and Kam W. Leong*

Johns Hopkins Singapore, Singapore 117597
Department of Biomedical Engineering
Johns Hopkins University School of Medicine
Baltimore, Maryland 21205

Received April 21, 2001

Gene therapy has been progressively developed with the hope that it will be an integral part of medical modalities in the future.[1] Viral vectors, while efficient in gene transfer in vivo, pose a safety concern unlikely to abate soon, rendering synthetic carriers attractive alternatives. Among existing synthetic vectors, cationic liposomes and polycations such as DC-Chol/DOPE and poly(ethenyleneimine) (PEI) have been extensively studied for gene delivery. However, their toxicity and biocompatibility in vivo still leave much to be desired.[2] Biodegradable polycations are now emerging as a new generation of synthetic carriers. Biodegradable polymers, such as poly[α-(4-aminobutyl)-L-glycolic acid] (PAGA) have been reported recently to mediate gene transfection in vitro and in vivo.[3] Here we report a new biodegradable polymeric carrier, namely, poly(2-aminoethyl propylene phosphate) (PPE-EA) with a phosphate backbone and a β-aminoethoxy side chain. Synthesized in high molecular weights, this polymer binds DNA effectively, degrades over days under physiological condition, and shows remarkably low cytotoxicity.



PPE-EA was synthesized from a precursor polymer **1**, poly(4-methyl-2-oxo-2-chloro-1,3,2-dioxaphospholane), which was obtained by ring-opening polymerization of 4-methyl-2-oxo-2-hydro-1,3,2-dioxaphospholane in the presence of triisobutylaluminum as initiator, followed by chlorination of P−H according to the method described by Penczek et al.[4] The P−Cl bond in polymer **1** is highly reactive to nucleophiles. Reacting **1** with 10% excess of benzyl *N*-(2-hydroxyethyl) carbamate in chloroform using 4-(dimethylamino)-pyridine (DMAP) as a catalyst yielded intermediate polymer **2**. Polymer **2** was purified by reprecipitation in ethyl ether with a recovery yield of 60%. The NMR spectra (^{1}H, ^{13}C, and ^{31}P) indicated that all of the pendant chains were conjugated with the protected ethanolamine. The weight average molecular weight (M_w) of polymer **2** was 8.62×10^4 with a polydispersity index of 1.95 as determined by GPC/LS/RI method (an absolute molecular weight measurement method, d*n*/d*c* calculated was 0.051 mL/g),[5] which corresponded to a number average degree of polymerization (DP_n) of 140.5. Removal of the *N*-benzyloxycarbonyl group was accomplished using the formic acid-Pd/C method.[6] The reaction product was treated with chloric acid and precipitated in an excess amount of acetone. PPE-EA was obtained as white powder (80%). The absence of peaks assigned to benzoxycarbonyl group in ^{1}H spectrum (δ7.2−7.4 and δ5.0−5.1 ppm) and FT-IR spectrum (1716 cm^{-1}) of PPE-EA indicated that the deprotection reaction was complete. ^{31}P NMR spectrum showed a triplet peak (δ1.95−2.1 ppm) corresponding to three different isomeric structures of phosphoesters, similar to that of polymer **2**. The M_w of PPE-EA was 3.03×10^4 with a polydispersity index of 1.66 (GPC/LS/RI method, d*n*/d*c* = 0.075 mL/g, elution buffer: 0.1 M phosphate buffer with 0.15 M NaCl), corresponding to a DP_n of 100.2. The decrease of molecular weight was caused by degradation during the acidic hydrogenation and chloric acid treatment steps. Nevertheless, the molecular weight of PPE-EA prepared by this method was relatively higher than other biodegradable gene carriers. Higher molecular weight could ensure more stable polymer−DNA complexes in aqueous medium. This enhanced stability may be advantageous in many in vivo gene delivery applications.

PPE-EA underwent degradation in PBS at 37 °C because of the hydrolytic cleavage of the phosphoester bonds in the backbone. The degradation kinetics of PPE-EA was followed by GPC analysis shown in Figure 1. The M_w of PPE-EA dropped 12% in 24 h, and declined gradually to 10,450 (65% decrease) after 7 days. After 10 days of incubation, PPE-EA degraded to oligomers and failed to bind plasmid DNA. It is worth noting that the biodegradation of polyphosphoester with a methoxy or ethoxy side chain would be considerably slower.[7] The results in this study suggest a self-catalytic degradative mechanism involving nucleophilic attack of the phosphate bonds in the backbone by the pendant amino groups. Further investigation of the degradation mechanism is currently ongoing.

PPE-EA was designed to have nontoxic building blocks. The ultimate degradation products are expected to be α-propylene glycol, phosphate, and ethanolamine, all with minimal toxicity profiles. The cytotoxicity of PPE-EA was assessed in a 24-hour cell culture test (COS-7 cells) in comparison with PPE and PEI using the MTT assay.[8] As expected, no significant change in cell morphology and proliferation rate was observed compared with controls (no treatment) after 24 h of incubation with PPE at a dose up to 0.1 mg/mL (Figure 2a). In contrast, PEI and PLL exhibited much higher toxicity, with a LD_{50} below 10 μg/mL for both polymers. Similar results were observed in HEK 293 cells (data not shown).

PPE-EA readily formed complexes with plasmid DNA in PBS or saline, as analyzed by gel electrophoresis. Complete binding of plasmid DNA by PPE-EA was achieved at charge ratios (N/P ratios) of 1.0 and above, suggesting that the majority of the pendant amino groups in PPE-EA were protonated at neutral pH. Plasmid DNA in these complexes was partially protected from enzyme degradation. This was demonstrated by using DNase I as a model enzyme. When incubated with 4 units/mL of DNase I at 37 °C, naked DNA showed significant degradation in 30 min (lane 2 of Figure 2b), whereas DNA recovered from the complexes (N/P of 2) subjected to the same enzymatic treatment remained intact, although some nicking occurred (lane 4 of Figure 2b). This suggested that under physiological condition, where the nuclease concentration is markedly lower than the test condition, such a formulation should render significant protection to the plasmid. Figure 2c showed the complexes also partially protected

* To whom correspondence should be addressed.

(1) Anderson, W. F. *Nature* **1998**, *392*, 25−30. (b) Han, S.; Mahato, R. I.; Sung, Y. K.; Kim, S. W. *Mol. Ther.* **2000**, *2*, 302−317.
(2) (a) Zauner, W.; Ogris, M.; Wagner, E. *Adv. Drug Delivery Rev.* **1998**, *30*, 97−113. (b) Brazeau, G. A.; Attia, S.; Poxon, S.; Hughes, J. A. *Pharm. Res.* **1998**, *15*, 680−684.
(3) (a) Koh, J. J.; Ko, K. S.; Lee, M.; Han, S.; Park, J. S.; Kim S. W. *Gene Ther.* **2000**, *7*, 2099−2104. (b) Maheshwari, A.; Mahato, R. I.; McGregor, J.; Han, S.; Samlowski, W. E.; Park, J. S.; Kim, S. W. *Mol. Ther*. **2000**, *2*, 121−130. (c) Lim, Y. B.; Kim, C. H.; Kim, K.; Kim, S. W.; Park, J. S. *J. Am. Chem. Soc.* **2000**, *122*, 6524−6525.
(4) (a) Penczek, S.; Pretula, J. *Macromolecules* **1993**, *26*, 2228−2233. (b) Biela, T.; Penczek, S.; Slomkowski, S. *Makromol. Chem., Rapid Commun.* **1982**, *3*, 667−671.
(5) Wyatt, P. J. *Anal. Chim. Acta* **1993**, *272*, 1−40.
(6) Zhou, Q. X.; Kohn, J. *Macromolecules* **1990**, *23*, 3399∼3406.
(7) Baran, J.; Penczek, S. *Macromolecules* **1995**, *28*, 5167−5176.
(8) Hansen, M. B.; Nielsen, S. E.; Berg, K. *J. Immunol. Methods* **1989**, *119*, 203-210.

10.1021/ja016062m CCC: $20.00
Published on Web 09/01/2001

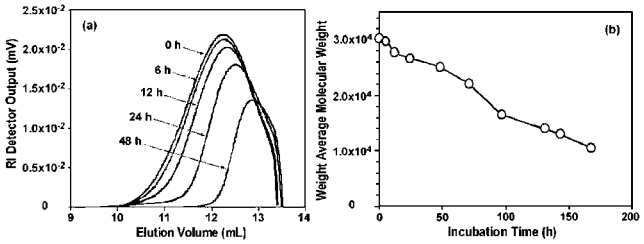


Figure 1. PPE-EA degradation in PBS at 37 °C. (a) Samples were analyzed by GPC with a RI detector. (b) Change of M_w with incubation time (given as absolute molecular weights).

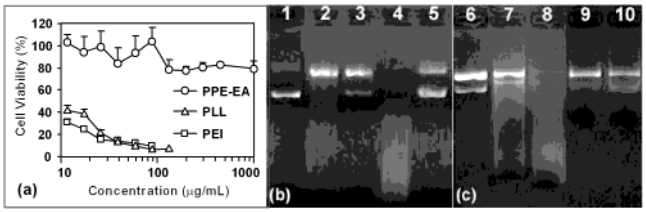


Figure 2. (a). Cytotoxicity of PPE-EA in COS-7 cells in comparison with PLL and PEI. PLL with MW 27 KDa and PEI with MW of 25 KDa were used. Mean ± standard deviation ($n = 6$). (b). Complexes protecting pcDNA against DNase I digestion. Lane 1: pRE-Luciferase; lane 2: pcDNA subjected to 4 units/mL of DNase I digestion for 30 min; lane 3: pcDNA treated with the recovering condition (SDS method: 65 °C overnight with 0.8% SDS); lane 4: DNA recovered from complexes (N/P = 2) after being subjected to same DNase I treatment; lane 5: DNA recovered from complexes (N/P = 2) by SDS method. (c). Complexes protecting pcDNA in complete medium (containing 10% fetal bovine serum). Lane 6: pcDNA; pcDNA was incubated with complete medium at 37 °C for 1 h (lane 7) and 2 h (lane 8); DNA recovered from complexes after incubation in the same medium (lane 9: 1 h; lane 10: 2 h).

DNA when incubated in complete culture medium containing 10% fetal bovine serum (FBS).

As PPE-EA undergoes hydrolytic degradation at pH7.4, plasmid DNA can be released from these complexes under physiological conditions. Such complexes provided a controlled-release system for plasmid DNA. The release rate of DNA was a function of charge ratio. Complexes with an N/P ratio of 1 started to release DNA after 6 h of incubation in PBS at 37 °C and reached complete release after incubation for 4−5 days, as indicated by the electrophoretic analysis (Figure 3). Higher charge ratios lead to slower release of DNA from the complexes. For example, complexes prepared with an N/P ratio of 3 showed an onset of DNA release at day 5, and complete release by day 9. Gel electrophoresis showed that DNA released from the complexes remained intact, although a certain degree of nicking occurred at various time points. It is noted that the electrophoretic mobility of DNA released from the complexes was slightly lower (Figure 3). This could be attributed to complexation of DNA with the degraded PPE-EA oligomers.

In vitro transfection activity of PPE-EA/DNA complexes was evaluated in HEK293 cells in comparison with PLL and PEI (Figure 4). Transfection with PPE-EA/DNA complexes (charge ratios between 6 and 8) yielded 45−105-fold higher gene expression than PLL-mediated transfection. In the presence of chloroquine diphosphate (CQ, 100 μM), a reagent known to disrupt endosomal membrane and enhance transfection of DNA complexes trafficked through the endo-lysosomal pathway, transfection efficiency was significantly increased. Transfection efficiency in the presence of 100 μM CQ was 17−260-fold higher than that for transfection without CQ. This enhancement was more pronounced at lower charge ratios. The optimal charge ratio of PPE-EA/DNA complexes showing the highest gene expression level was 4−8, at which ratios PPE-EA transfected cells more efficiently than PLL (68−70-fold higher). This optimal charge-ratio range was severalfolds lower than that required for other biodegradable carriers, suggesting the advantage of a higher molecular weight and more stable gene carrier. Transfection efficiency decreased 40−80% when transfection was performed in complete medium containing 10% FBS, except at a charge ratio of 4 where the transfection was enhanced about 84% in the complete medium.

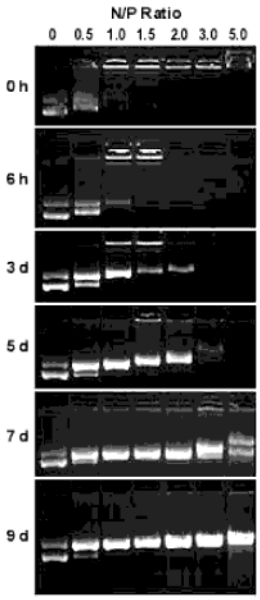


Figure 3. Plasmid DNA released from PPE-EA/DNA complexes prepared with various charge ratios. Complexes were incubated in PBS at 37 °C, and analyzed by electrophoresis (0.8% agrose) at different time points.

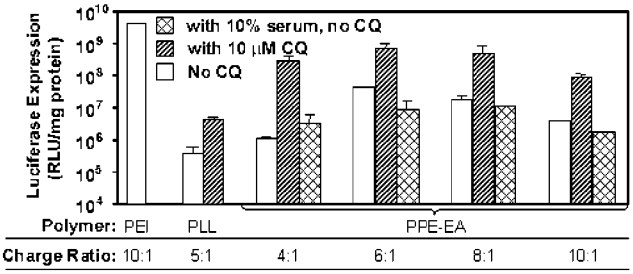


Figure 4. Transfection efficiency of PPE-EA/DNA complexes in HEK293 cells in the absence of CQ (gray bars) and presence of 100 μM CQ (hatched bars) in comparison with PEI- and PLL-mediated transfection. Mean ± standard deviation ($n = 3$).

Transfection mediated by PPE-EA was cell-type dependent. In general, transfection efficiency in other cell lines was lower than that in HEK293 cells. For example, in COS7 cells, PPE-EA mediated about 20-fold higher protein expression than PLL in the presence of CQ and at a charge ratio of 6. In HeLa cells, PPE-EA/DNA complexes only yielded 2-fold higher transfection efficiency than PLL at a charge ratio of 10.

In conclusion, a new biodegradable gene carrier, PPE-EA was designed and synthesized with relatively high molecular weight. A cytotoxicity study suggested the biocompatible nature of the polymer. Complexation between PPE-EA and plasmid protected DNA from nuclease degradation. PPE-EA mediated a higher level of gene expression, but the transfection efficiency greatly depended on cell type. A unique feature of this system was the capability of controlled release of plasmid from PPE-EA/DNA complexes, achieved as a result of PPE-EA degradation. The release-rate of plasmid could be adjusted by varying the charge ratio of PPE-EA to DNA.

Acknowledgment. This work is supported by National Science and Technology Board of Singapore and Johns Hopkins Singapore. We thank Quek Chai-Hoon (IMRE, Singapore) for technical assistance.

Supporting Information Available: Synthesis procedures, NMR spectra, GPC chromatographs, and experimental protocols (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

JA016062M