Available online at www.sciencedirect.com
ScienceDirect
Bioorganic & Medicinal Chemistry Letters 17 (2007) 2975–2978
Bioorganic & Medicinal Chemistry Letters

A tetra(L-lysine)-grafted poly(organophosphazene) for gene delivery

Yong Joo Jun,[a,c] Jung Hee Kim,[a] Su Jin Choi,[b] Hwa Jeong Lee,[b] Moo Jin Jun[c] and Youn Soo Sohn[a,*]

[a]*Division of Nano Science BK 21, Ewha Womans University, 11-1 Daehyun-Dong, Seodaemun-Gu, Seoul 120-750, Republic of Korea*
[b]*College of Pharmacy, Ewha Womans University, 11-1 Daehyun-Dong, Seodaemun-Gu, Seoul 120-750, Republic of Korea*
[c]*Department of Chemistry, Yonsei University, Shinchon 134, Seoul 120-749, Republic of Korea*

Received 16 January 2007; revised 17 March 2007; accepted 22 March 2007
Available online 25 March 2007

Abstract—In order to develop a new gene delivery vector, a novel cationic poly(organophosphazene) was synthesized by stepwise nucleophilic substitutions of poly(dichlorophosphazene) with a hydrophilic methoxy-poly(ethylene glycol) (MPEG) as a shielding group and a branched tetra(L-lysine), LysLys(LysEt)$_2$, as a cationic moiety. The cationic polymer has shown to form a polyplex by DNA condensation and very low in vitro cytotoxicity probably due to the shielding effect of MPEG, which provides a basis for improving the low gene transfection yield of cationic polyphosphazenes.

Recently, gene delivery systems based on non-viral vectors have attracted much attention in the field of medicine, pharmaceutical sciences, and biotechnology because of their potential utility for medical treatment of genetic disorders, infections, and cancers.[1,2] Viral vectors show more efficient transfer both in vitro and in vivo than non-viral vectors.[2,3] However, some severe disadvantages are associated with the use of viral vectors, such as recombination with wild-type viruses, an immune response against the viral vector, and size limitation of DNA.[4,5] For such reasons, various non-viral vectors over 400 were studied during past two decades,[6] but no suitable cationic polymer useful for efficient and safe gene delivery has been found yet.

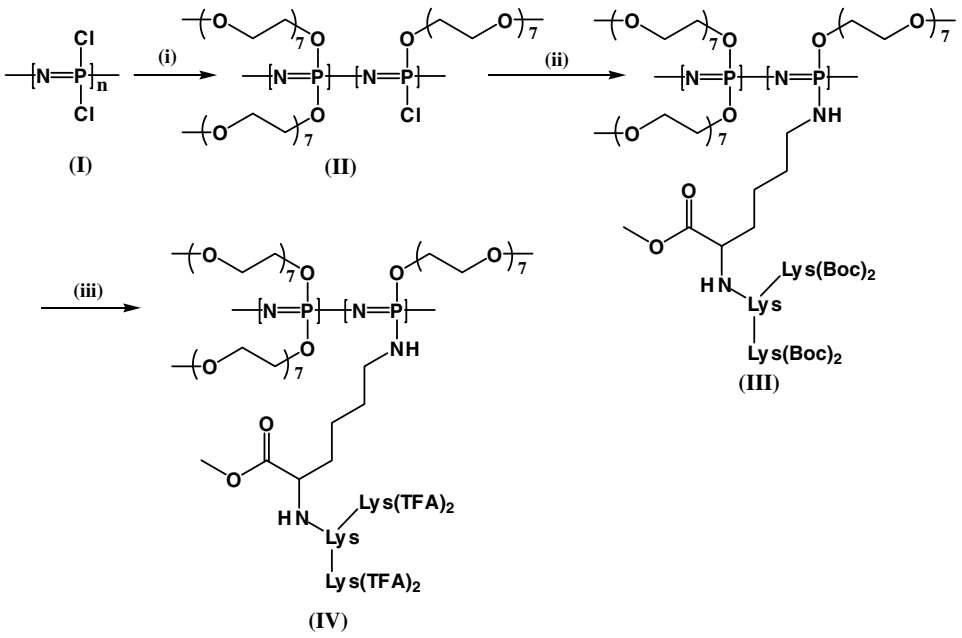
The non-viral vectors bearing L-lysine have been extensively studied since the formation of polyplexes between PLL and DNA was identified.[7] The cationic sites of the protonated primary ε-amine groups of L-lysine grafted to polymers interact electrostatically with negatively charged DNA to form polyplexes, polyelectrolyte complexes. In the case of PLL, it is known that the number of primary amine groups on the PLL backbone is important for the polyplex formation.[8] The high molecular weight of PLL has some advantages as a gene carrier, but its utility is limited due to its high cytotoxicity and tendency to aggregate and precipitate.[9] It is also known that the cationic surface of polyplexes is the main cause for cell cytotoxicity,[4,9] and rapid clearance of the polyplexes from the blood stream.[10] Therefore, introduction of hydrophilic poly(ethylene glycol) to a cationic polymer is a well-known approach to prevent the protein adsorption of polyplexes by hydrophilic surface shielding effect of cationic charges.[9,11] Cationic polyphosphazenes grafted with tertiary amines[12] and their applications as non-viral gene delivery vectors[2,13] were reported in recent years, but all these polyphosphazenes contain only tertiary amines as a side group.

In this context, we have synthesized a novel cationic polyphosphazene bearing a hydrophilic methoxy-poly(ethylene glycol) with molecular weight of 350 (MPEG350) as a shielding group and a branched tetra(L-lysine), LysLys(LysEt)$_2$, as a cationic side group. Various useful properties are expected for this polymer: good shielding effect, good water-solubility, biodegradability,[14] biocompatibility, and effective DNA condensation. In this paper, we describe its synthesis, characterization, and properties as a new gene delivery vector.

The polyphosphazene bearing MPEG350 and the tetral-lysine was synthesized according to the procedure depicted in Scheme 1. Poly(dichlorophosphazene) was prepared according to our procedure.[15] The sodium salt

Keywords: Polyphosphazene; Polyplex; Non-viral vector; Lysine.
* Corresponding author. Tel.: +82 2 3277 2345; fax: +82 2 3277 3419; e-mail: yssohn@ewha.ac.kr

0960-894X/$ - see front matter © 2007 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmcl.2007.03.069



Scheme 1. Synthetic route to the cationic polyphosphazene bearing MPEG350 and a branched tetra(L-lysine) as side groups. Reagents and conditions: (i) −65 °C, 8 h, MPEG–Na; (ii) tetra(L-lysine)[Lys · Lys · $(LysBoc_2)_2$], NEt_3, 50 °C, 2 days; (iii) TFA, 8 h.

of MPEG350 prepared by reaction of MPEG350 (5.25 g, 15 mmol) with a slight excess of sodium hydride in THF at reflux was dropped slowly to the solution of **I** (1.0 g, 8.62 mmol) dissolved in THF. The reaction mixture was stirred for 4 h at −65 °C to afford PEGylated polyphosphazene **II**. Meanwhile, the Boc-blocked tetralysine ethyl ester (6.61 g, 7 mmol) prepared by the literature procedure[16] was dissolved in dry chloroform containing excess amount of dry triethylamine. This tetralysine solution was slowly added to the polymer solution of **II**, and then the mixed solution was stirred at 50 °C for 2 days. The reaction mixture was filtered to remove byproduct salts, and the filtrate was concentrated and reprecipitated twice by a solvent pair of ethanol and diethyl ether. For further purification, the product dissolved in methanol was dialyzed in methanol for 1 day and in water for 1 day using regenerated cellulose membranes (MWCO: 3.5×10^3). The dialyzed solution was freeze-dried to obtain the product (**III**) with the lysine amine groups blocked by Boc. The Boc protecting group in the polymer was removed by reaction with excess of trifluoroacetic acid (TFA) in methylene chloride. The resulting polymer was dialyzed in water for 1 day and then freeze-dried to obtain the final cationic polyphosphazene, $\{NP[MPEG350]_{1.55}[LysLys(Lys(TFA)_2)_2]_{0.45}\}_n$ (**IV**) (Yield: 74%, 6.5 g).

The present polymer is very soluble in water as well as in most of polar organic solvents and fully characterized by means of multinuclear (^1H, ^{31}P) NMR spectroscopies, GPC, DLS, ζ potential, and elemental analyses. All the synthetic reactions were monitored using ^{31}P NMR spectroscopy. The ^{31}P NMR spectra of the PEGelated intermediate **II** and the final polymer **IV** are depicted in Figure 1. The stepwise substitution reactions were continued until only two major peaks of –N–P–O– and –O–P–O– were observed at 2.87 and −2.87 ppm, respectively. The molar ratio of PEG and tetrapeptide was determined by ^1H and ^{31}P NMR integration, which was well in accord with the elemental analysis data.[17] The number average molecular weight (M_n) and weight average molecular weight (M_w) of the polymer were measured in a water/acetonitrile mixture (80:20) by GPC using polyethylene oxide standard ($M_n = 1.4 \times 10^4$, $M_w = 3.8 \times 10^4$, and PDI = 2.79).

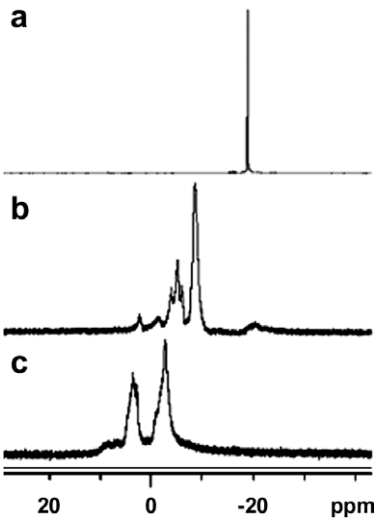


Figure 1. ^{31}P NMR spectra of poly(dichlorophosphazene) (a), the PEGylated intermediate (b), and the final product, $[NP(MPEG350)LysLys(LysEt)_2]_3$(c).

We have performed the particle size analysis and ζ potential measurements for the present polymer to determine its shielding capability and gene adduct formation. Polyplex formation of plasmid DNA with our cationic polymer was studied by DLS and zeta

potential measurements at various N/P ratios (0, 0.5, 1, 2, 3, 4, and 8) in FBS-free media at room temperature. The size condensation of polyplexes was observed and the average diameter was about 120 nm at N/P ratios of 3, 4, and 8, and remained stable for 8 h. The sizes of gene and neutral polyplexes (N/P = 1) could not be detected by DLS because of their low scattering yield at N/P = 0 and the formation of unstable aggregates at N/P = 1. The surface charge of the polyplex was studied by ζ potential measurements at various N/P ratios (0, 0.5, 1, 2, 3, 4, and 8). The ζ potential of the polyplex increased with increasing N/P ratio from −63 mV at N/P = 0 ratio to +27 mV at N/P = 4 and 8. Such a ζ potential value of +27 mV at N/P = 4 and 8 is unexpectedly high, but a similar value of +25 mV at N/P = 4 was recently observed for dendritic PLL PEGylated[18] to the same degree (60%) as our present polymer bearing 59% PEG of the polymer. Consequently, successful DNA condensation and shielding effect of PEG group for the present polymer were characterized and the results are shown in Figure 2. The conceptual diagram for the formation of polyplex by this cationic polymer is schematically shown in Figure 3.

In vitro cell transfection activity of the present polyplex tested at various N/P ratios was examined at different transfection time. Polyplexes were formed by plasmid DNA and the polymer at various N/P (0, 1, 4, 8, 12, 24, 36, and 48) ratios in FBS-free media for 30 min at room temperature. The amount of plasmid DNA used for the polyplex formation was fixed at 1 μg at each N/P ratio. Following the treatment of cells with polyplexes for 2, 4, and 6 h, the media were replaced by 500 μL of media containing 10% FBS, and the cells were incubated for 2 days. Figure 4 shows the luciferase activity of the cells. The present polyplex shows a bell-type curve at the transfection activity, which has been similar to recently reported other non-viral gene delivery systems such as PEI22 polyplexes and p(DMAEA)-pzz polyplexes.[2,13] The transfection activity increased with increasing transfection time up to 6 h in the range of

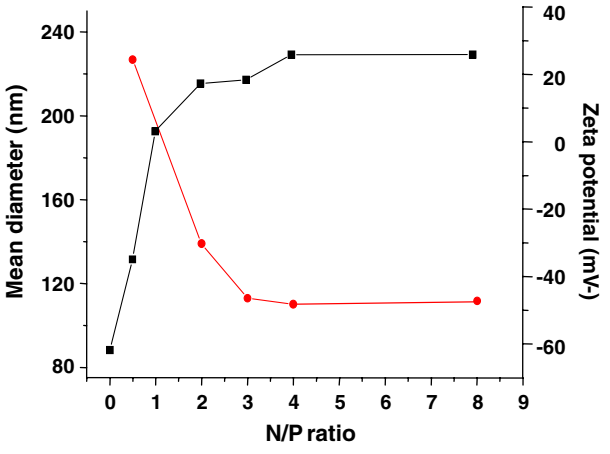


Figure 2. The mean diameter (●) and ζ potential (■) of the polyplex prepared at various N/P ratios. In case of N/P = 0 and N/P = 1, the size of polyplex could not be detected because of their low scattering yield and low aggregation stability of neutral particles.

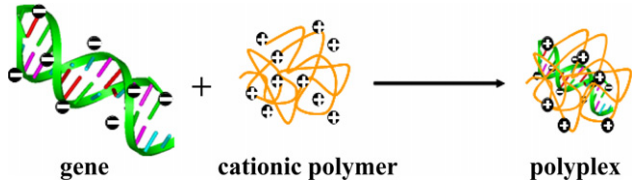


Figure 3. Schematic representation of the polyplex formed by the cationic polymer with negatively charged DNA.

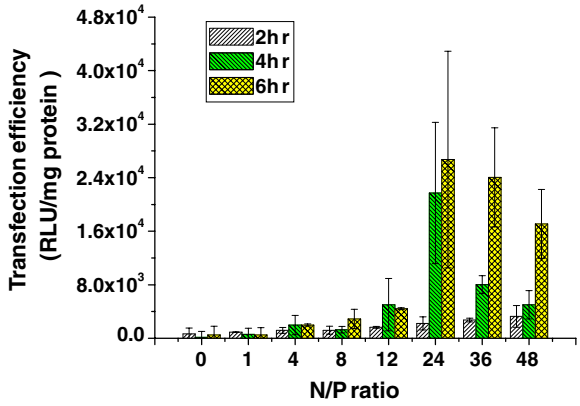


Figure 4. The gene transfection efficiency depending upon the N/P ratio.

N/P ratios from 4 to 48. For 4- and 6-h incubations, the maximum transfection efficiency was observed at N/P ratio of 24. The maximum transfection yield of this polymer is remarkably lower compared with the previous report,[13] probably due to the insufficient number of available cationic amine sites of the tetralysine groups grafted to the phosphazene backbone. Therefore, in order to improve transfection yield of this polymer, it seems necessary to increase the tetralysine content of the polymer.

In vitro cell cytotoxicity test was performed using SRB staining assay according to the previous report.[19] As is seen from Figure 5, the present cationic polymer shows almost no in vitro cytotoxicity against the SK-OV3 cell line up to the concentration of 10 mg/ml. Approximately 20% reduction in cell viability was observed at N/P ratio of 48 (8 mg/ml polymer) after 4- and 6-h incubations. In the case of 2-h incubation, relatively lower reduction in cell viability with increasing amount of polymer was observed than 4- and 6-h incubations.

In conclusion, a new cationic polyphosphazene bearing MPEG350 and a tetralysine, $LysLys(LysEt)_2$, as side groups has been prepared for a gene delivery vector, which was found to form a polyplex with DNA. The size condensation of the polyplex was observed at 120 nm and the surface charge was characterized to be approximately 27 mV. The cationic polymer exhibited almost no in vitro cytotoxicity against the SK-OV3 cell line probably due to the shielding effect of the PEG groups. However, the present polyplex exhibited relatively low transfection yield probably because of the insufficient number of cationic amine sites of the tetralysine groups

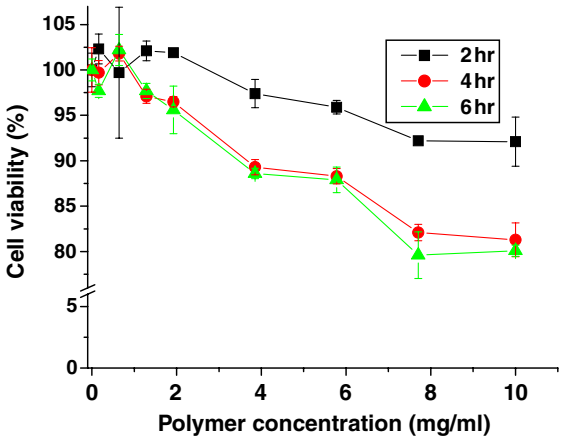


Figure 5. Cytotoxic effect of the present cationic polymer against the SK-OV3 cell line after 2-, 4-, and 6-h incubations at 37 °C. Cell viability was determined by SRB staining assay. Each data point represents the mean ± SD (n = 3).

grafted to the polymer backbone, and further study is underway to improve transfection yield by increasing the cationic sites of the polymer.

Acknowledgments

This work was supported by a Korean Research Foundation Grant (KRF-2004-005-C00090), by the SRC program of the Korea Science and Engineering Foundation through the Center for Intelligent Nano-Bio Materials at Ewha Womans University (Grant R11-2005-008-01002-0), and by Ministry of Commerce, Industry and Energy of Korea.

References and notes

1. (a) Mulligan, R. C. *Science* **1993**, *260*, 926; (b) Ruponen, M.; Honkakoski, P.; Rönkkö, S.; Pelkonen, J.; Tammi, M.; Urtti, A. *J. Control. Release* **2003**, *93*, 213; (c) Blaese, R. M. *Sci. Am.* **1997**, *6*, 91; (d) Prud'homme, G. J. *J. Gene Med.* **2000**, *2*, 222.
2. 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. *J. Control. Release* **2003**, *89*, 483.
3. (a) Rots, M. G.; Curiel, D. T.; Gerritsen, W. R.; Haisma, H. J. *J. Control. Release* **2003**, *8*(7), 159; (b) Blits, B.; Boer, G. J.; Verhaagen, J. *Cell Transplant.* **2002**, *11*, 593; (c) McTaggart, S.; Al-Rubeai, M. *Biotechol. Adv.* **2002**, *20*, 1; (d) Mah, C.; Byne, B. J. *Clin. Pharmacokinet.* **2002**, *41*, 901.
4. Park, T. G.; Jeong, J. H.; Kim, S. W. *Adv. Drug Deliv. Rev.* **2006**, *58*, 467.
5. (a) Lee, M.; Kim, S. W. *Pharm. Res.* **2005**, *22*, 1; (b) Verma, I. M.; Somia, N. *Nature* **1997**, *389*, 239.
6. Breyer, B.; Jiang, W.; Zhou, L.; Paul, R.; Feng, T.; He, T. C. *Curr. Gene Ther.* **2001**, *1*, 149.
7. Laemmli, U. K. *Proc. Natl. Acad. Sci. U.S.A.* **1975**, *72*, 4288.
8. Kwoh, D. Y.; Coffin, C. C.; Lollo, C. P.; Jovenal, J.; Banaszczyk, M. G.; Mullen, P.; Phillips, A.; Amini, A.; Fabrycki, J.; Bartholomew, R. M. *Biochim. Biophy. Acta* **1999**, *1444*, 171.
9. (a) Choi, Y. H.; Liu, F.; Kim, J. S.; Choi, Y. K.; Park, J. S.; Kim, S. W. *J. Control. Release* **1998**, *54*, 39; (b) Liu, G.; Grossmann, G. A.; Pasumarthy, M.; Perales, J. C.; Cooper, M. J.; Hanson, R. W. *J. Biol. Chem.* **2001**, *276*, 34379.
10. Ogris, M.; Brunner, S.; Schuller, S.; Kircheis, R.; Wagner, E. *Gene Ther.* **1999**, *6*, 595.
11. (a) Katayose, S.; Kataoka, K. *Bioconjugate Chem.* **1997**, *8*, 702; (b) Toncheva, V.; Wolfert, M. A.; Dash, P. R.; Oupicky, D.; Ulbrich, K.; Seymour, L. W.; Schacht, E. H. *Biochim. Biophys. Acta* **1998**, *1380*, 354; (c) Jeong, J. H.; Kim, S. W.; Park, T. G. *Bioconjugate Chem.* **2003**, *14*, 473; (d) Ahn, C. H.; Chae, S. Y.; Bae, Y. H.; Kim, S. W. *J. Control. Release* **2004**, *94*, 567.
12. Allcock, H. R.; McIntosh, M. B.; Klingenberg, E. H.; Napierala, M. E. *Macromolecules* **1998**, *31*, 5255.
13. deWolf, H. K.; Luten, J.; Snel, C. J.; Oussoren, C.; Hennink, W. E.; Storm, G. *J. Control. Release* **2005**, *109*, 275.
14. (a) Lee, B. H.; Lee, Y. M.; Sohn, Y. S.; Song, S.-C. *Macromolecules* **2002**, *35*, 3876; (b) Song, S. C.; Lee, S. B.; Jin, J.-I.; Sohn, Y. S. *Macromolecules* **1999**, *32*, 2188; (c) Allcock, H. R.; Fuller, T. J.; Matsumura, K. *Inorg. Chem.* **1982**, *21*, 515.
15. Sohn, Y. S.; Cho, Y. H.; Baek, H. G.; Jung, O. S. *Macromolecules* **1995**, *28*, 7566.
16. (a) Greenstein, J. D.; Winitz, M. *Chemistry of the Amino Acids*; John Wiley and Sons: New York, 1961, pp 925–942; (b) Jones, J. *Amino Acid and Peptide Synthesis*; Oxford University Press: Oxford, 1992, pp 31–38; (c) Gillies, E. R.; Jonsson, T. B.; Fréchet, J. M. J. *J. Am. Chem. Soc.* **2004**, *126*, 11936; (d) Driffield, M.; Goodall, D. M.; Smith, D. K. *Org. Biomol. Chem.* **2003**, *1*, 2612.
17. Elem. Anal. Calcd for $[NP(C_{15}H_{31}O_8)_{1.55}(C_{33}H_{55}O_{14}N_8F_{12})_{0.45}]$ (%): C, 44.51; H, 7.15; N, 6.26. Found(%): C, 44.53; H, 7.24; N, 5.78. ^{31}P NMR [D_2O, δ]: 2.87 (s), −2.84 (s). ^{1}H NMR (D_2O, δ): 1.25–1.6 (br, 4.08H); 1.6–1.8 (br, 2.65H); 1.8–1.9 (br, 2.6H); 2.95 (br, 1.91H); 3.33 (s, 4.5H); 3.57 (br, 4.05H); 3.65 (s, 30H).
18. Männistö, M.; Vanderkerken, S.; Toncheva, V.; Elomaa, M.; Ruponen, M.; Schacht, E.; Urtti, A. *J. Control. Release* **2002**, *83*, 169.
19. Skehan, P.; Storeng, R.; Scudiero, D.; Monks, A.; McMahon, J.; Vistica, D.; Warren, J. T.; Bokesch, H.; Kenney, S.; Boyd, M. R. *J. Natl. Cancer Inst.* **1990**, *82*, 1107.