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Carboxymethyl Poly(L-histidine) as a New pH-Sensitive Polypeptide To Enhance Polyplex Gene Delivery

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Received March 3, 2008
Accepted August 1, 2008

Abstract: Carboxymethyl poly(L-histidine) (CM-PLH) as a new pH-sensitive polypeptide has enhanced polyplex gene delivery. Agarose gel retardation assay and zeta potential measurement proved that the anionic CM-PLH at physiological pH coated the PEI/DNA binary complexes. The resulting CM-PLH/PEI/DNA ternary complexes showed the gene expression value 300 times higher than that of the PEI/DNA binary complexes. These results suggest that the synergistic effect of the pH-sensitive imidazole groups at endosomal pH and the anionic carboxymethyl groups at physiological pH in the CM-PLH enhanced polyplex gene delivery.

Keywords: Carboxymethyl poly(L-histidine); pH-sensitive polypeptide; polyplex; gene delivery

Nonviral gene delivery systems based on a polycation/DNA complex, that is, polyplex, have attracted great attention in recent years. The polyplexes protect DNA from nuclease degradation and have a nanoscale size enough to enter the cell through endocytosis. Amine-containing polycations such as poly(L-lysine),[1] poly[2-(dimethylamino)ethyl methacrylate],[2] and polyamidoamine-dendrimer[3] have been proposed as carriers for genetic material because they readily form complexes with DNA. Especially, pH-sensitive polycations such as poly(ethylenimine) (PEI)[4−6] or imidazole-containing polymer,[7−9] which is able to capture protons entering an endosome, have been used to achieve efficient release of the delivered material from endosomes. These polyplexes are taken up by cells through adsorptive endocytosis due to their positively charged character, leading to effective gene expression.[10,11] However, the surface cationic charge induces problems in in vivo conditions: nonspecific interactions with nontarget tissues and blood components such as serum proteins.[12−14] To alleviate these problems, anionic polymers

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(1) Wu, G. Y.; Wu, C. H. Receptor-mediated in vitro gene transformation by a soluble DNA carrier system. *J. Biol. Chem.* **1987**, *262*, 4429–4432.

(2) van de Wetering, P.; Cherng, J. Y.; Talsma, H.; Crommelin, D. J.; Hennink, W. E. 2-(Dimethylamino)ethyl methacrylate based (co)polymers as gene transfer agents. *J. Controlled Release* **1998**, *53*, 145–153.

(3) Haensler, J.; Szoka, F. C., Jr. Polyamidoamine cascade polymers mediate efficient transfection of cells in culture. *Bioconjugate Chem.* **1993**, *4*, 372–379.

(4) Boussif, O.; Lezoualc'h, F.; Zanta, M. A.; Margny, M. D.; Scherman, D.; Demeneix, B.; Behr, J. P. A versatile vector for gene and oligonucleotide transfer into cells in culture and *in vivo*: Polyethylenimine. *Proc. Natl. Acad. Sci. U.S.A.* **1995**, *92*, 7297–7301.

(5) Thomas, M.; Klibanov, A. M. Enhancing polyethylenimine's delivery of plasmid DNA into mammalian cells. *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99*, 14640–14645.

(6) Sonawane, N. D.; Szoka, F. C., Jr.; Verkman, A. S. Chloride accumulation and swelling in endosomes enhances DNA transfer by polyamine-DNA polyplexes. *J. Biol. Chem.* **2003**, *278*, 44826–44831.

(7) Midoux, P.; Monsigny, M. Efficient gene transfer by histidylated polylysine/pDNA complexes. *Bioconjugate Chem.* **1999**, *10*, 406–411.

(8) Pack, D. W.; Putnam, D.; Langer, R. Design of imidazole-containing endosomolytic biopolymers for gene delivery. *Biotechnol. Bioeng.* **2000**, *67*, 217–223.

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

(10) Cherng, J. Y.; van de Wetering, P.; Talsma, H.; Crommelin, D. J.; Hennink, W. E. Effect of size and serum proteins on transfection efficiency of poly((2-dimethylamino)ethyl methacrylate)-plasmid nanoparticles. *Pharm. Res.* **1996**, *13*, 1038–1042.

(11) Kabanov, A. V.; Astafieva, I. V.; Maksimova, I. V.; Lukanidin, E. M.; Georgiev, G. P.; Kabanov, V. A. Efficient transformation of mammalian cells using DNA interpolyelectrolyte complexes with carbon chain polycations. *Bioconjugate Chem.* **1993**, *4*, 448–454.

(12) Dash, P. R.; Read, M. L.; Barrett, L. B.; Wolfert, M. A.; Seymour, L. W. Factors affecting blood clearance and in vivo distribution of polyelectrolyte complexes for gene delivery. *Gene Ther.* **1999**, *6*, 643–650.

(13) Plank, C.; Mechtler, K.; Szoka, F. C., Jr.; Wagner, E. Activation of the complement system by synthetic DNA complexes: a potential barrier for intravenous gene delivery. *Hum. Gene Ther.* **1996**, *7*, 1437–1446.

10.1021/mp800094b CCC: $40.75
Published on Web 08/14/2008

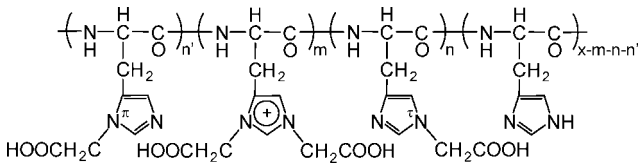

Figure 1. Chemical structure of carboxymethyl poly(L-histidine) (CM-PLH).

such as poly(acrylic acid)[15] or hyaluronic acid[16] have been used to coat the surface of polyplexes.

We have already reported the synthesis of carboxymethyl poly(L-histidine) (CM-PLH; Figure 1) as a new pH-sensitive polypeptide at endosomal/lysosomal pH.[17] The pH-sensitive polypeptide possesses imidazole groups for the large capacity of proton buffering at endosomal/lysosomal pH as well as anionic carboxymethyl groups at physiological pH. Furthermore, CM-PLH exhibits hemolytic activity at endosomal/lysosomal pH as well as no significant effect on a rapid aggregate formation of serum proteins at physiological pH. The resulting properties attributed to both pH-sensitive imidazole groups and anionic carboxymethyl groups have led us to use CM-PLH to enhance the polyplex gene delivery. Here we report the enhancement of the polyplex gene delivery by CM-PLH as a new pH-sensitive polypeptide. The anionic CM-PLH at physiological pH is expected to coat the PEI/DNA binary complexes, resulting in the formation of the ternary complexes. This paper describes the efficient gene delivery mediated by the resulting CM-PLH/PEI/DNA ternary complexes in comparison with the PEI/DNA binary complexes; detailed studies on the mechanism of the transfection activity are outside the scope of the present study.

To deliver DNA into cytoplasmic space, as shown in Figure 2,[18] we examined whether CM-PLH with higher content (53 mol %) of the carboxymethyl groups formed the ternary complex with DNA and PEI (Figure 2A). Lanes 2, 6, and 10 contain the PEI at an N/P ([nitrogen]$_{PEI}$/[phosphate]$_{DNA}$) ratio of 15, 30, and 45 in the absence of CM-PLH. The resulting bands are retained at the origin at pH

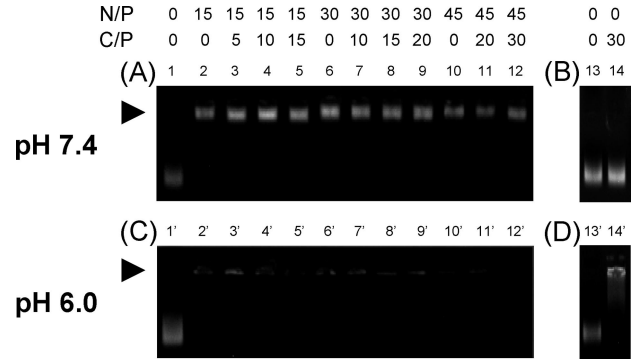

Figure 2. Analysis of the pH-dependent formation of CM-PLH/PEI/DNA ternary complexes by agarose gel electrophoresis (A, C). CM-PLH/PEI/DNA mixtures were loaded at pH 7.4 (lanes 1−12) or pH 6.0 (lanes 1′−12′): lane 1 (and 1′), DNA alone; lanes 2 (and 2′), 6 (and 6′), and 10 (and 10′), PEI/DNA mixtures, where the unit ratios relative to nitrogen atoms of PEI per phosphate group of DNA (N/P) are 15, 30, and 45; other lanes, CM-PLH/PEI/DNA mixtures at different C/P ([carboxymethyl]$_{CM\text{-}PLH}$/[phosphate]$_{DNA}$), which is the unit ratio relative to carboxymethyl groups of CM-PLH per phosphate group of DNA, and N/P ([nitrogen]$_{PEI}$/[phosphate]$_{DNA}$) ratios as indicated. As a control, a CM-PLH/DNA mixture (B, D) was loaded at pH 7.4 (lanes 13 and 14) or pH 6.0 (lanes 13′ and 14′): lane 13 (and 13′), DNA alone; lane 14 (and 14′), the CM-PLH/DNA mixture, where the C/P ratio is 30. Solid arrowhead indicates the well where each sample was loaded.

7.4, indicating the PEI/DNA complex formation. In the presence of CM-PLH, the resulting bands (lanes 3, 4, 5, 7, 8, 9, 11, and 12) were also retained at the origin. In the case of the CM-PLH/DNA mixture (Figure 2B), on the other hand, the band was completely migrated into the plus pole of the gel, which suggested that the CM-PLH and DNA exhibited no complex formation owing to the almost complete deprotonation of the imidazole groups. Taking these results into account, no DNA was released from the PEI/DNA complex by competitive exchange with the deprotonated CM-PLH as polyanion. From these results, however, the formation of CM-PLH/PEI/DNA ternary complexes is unclear, so that we measured the zeta potential of the resulting complexes. The zeta potentials of PEI/DNA complexes were positive ($\pm$15.8 $\pm$ 1.5 mV), whereas those in the presence of CM-PLH were almost neutral (0.0 $\pm$ 2.4 mV). The decrease in the zeta potentials by adding CM-PLH suggests that the CM-PLH coated the PEI/DNA binary complexes resulting in ternary complexes.

At pH 6.0, on the other hand, the resulting CM-PLH/PEI/DNA ternary complexes (lanes 3′, 4′, 5′, 7′, 8′, 9′, 11′, and 12′) as well as the PEI/DNA binary complexes (lanes 2′, 6′, and 10′) were not apparently stained (Figure 2C). In the case of the CM-PLH/DNA mixture, the band was retained at the origin (Figure 2D). These results suggest that the protonation of the CM-PLH as well as the PEI enhanced at endosomal pH. The resulting CM-PLH is considered to behave as a

(14) Ward, C. M.; Read, M. L.; Seymour, L. W. Systemic circulation of poly(L-lysine)/DNA vectors is influenced by polycation molecular weight and type of DNA: differential circulation in mice and rats and the implications for human gene therapy. *Blood* **2001**, *97*, 2221–2229.

(15) Trubetskoy, V. S.; Wong, S. C.; Subbotin, V.; Budker, V. G.; Loomis, A.; Hagstrom, J. E.; Wolff, J. A. Recharging cationic DNA complexes with highly charged polyanions for in vitro and in vivo gene delivery. *Gene Ther.* **2003**, *10*, 261–271.

(16) Ito, T.; Iida-Tanaka, N.; Niidome, T.; Kawano, T.; Kubo, K.; Yoshikawa, K.; Sato, T.; Yang, Z.; Koyama, Y. Hyaluronic acid and its derivative as a multi-functional gene expression enhancer: Protection from non-specific interactions, adhesion to targeted cells, and transcriptional activation. *J. Controlled Release* **2006**, *112*, 382–388.

(17) Asayama, S.; Kato, H.; Kawakami, H.; Nagaoka, S. Carboxymethyl poly(L-histidine) as a new pH-sensitive polypeptide at endosomal/lysosomal pH. *Polym. Adv. Technol.* **2007**, *18*, 329–333.

polycation in endosome. The CM-PLH property change of the polyanion at physiological pH into the polycation at endosomal pH is therefore expected to innovate on the polypeptide functions for polyplex gene delivery.

To clear the effect of the CM-PLH, we examined the gene expression mediated by the CM-PLH/PEI/DNA ternary complexes. Figure 3 shows the transfection of luciferase gene to HepG2 cells by the ternary complexes.[19] It is worth noting that the gene expression by the PEI/DNA binary complexes increased in the presence of the CM-PLH. Namely, the CM-PLH/PEI/DNA ternary complexes showed higher gene expression value. It should be noted that the CM-PLH/PEI/DNA ternary complex at a C/P and N/P ratio of 15 and 30, respectively, showed the gene expression value 300 times higher than that of the PEI/DNA binary complexes. This is the maximum increase, which is presumably due to the maximum negative zeta potential (−2.5 mV) in the ternary complexes. The CM-PLH/PEI/DNA ternary complexes with negative zeta potential exhibited higher colloidal stability in serum, as compared to the PEI/DNA binary complexes with positive zeta potential (Figure S-1, Supporting Information). Furthermore, the CM-PLH/PEI/DNA ternary complexes caused negligible hemolysis at physiological pH, resulting in negligible cytotoxicity during the transfection, whereas the hemolytic activity increased significantly at endosomal pH (Figure S-2, Supporting Information). This is probably due to the increase of the zeta potential (±16.9 ± 3.4 mV from 0.0 ± 2.4 mV at pH 7.4) as well as the decrease of the particle size (100 ± 5 nm from 675 ± 96 nm at pH 7.4) of the CM-PLH/PEI/DNA complexes at pH 6.0. These results suggest that the gene delivery by the CM-PLH/PEI/DNA ternary complexes depends on the CM-PLH properties of both anionic carboxymethyl groups for suppression of the nonspecific interaction of serum proteins and pH-sensitive imidazole groups for membrane disruptive ability at endosomal pH.

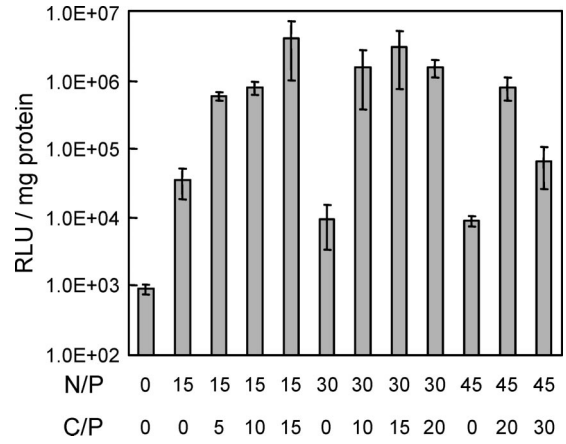

Figure 3. Transfection of luciferase gene to HepG2 cells by CM-PLH/PEI/DNA ternary complexes. The CM-PLH/PEI/DNA ternary complexes were prepared at different C/P and N/P ratios as indicated. The PEI/DNA binary complexes were prepared at the N/P ratio of 15, 30 or 45. The cells (1×10^4 cells/well) were transfected by adding 200 ng of plasmid DNA complexed with the polycations for 1 day in the presence of 10% FBS. The gene expression was determined 2 days later as RLU normalized by protein concentrations. Symbols and error bars represent the mean and standard deviation of the measurements made in paired samples ($n = 3$).

The neutral or negatively charged nonviral gene carriers have poor ability to interact with the cell membrane. In this study, even without any targeting ligands,[20,21] efficient gene delivery was achieved. The total gene expression depended on the incubation time (Figure S-3, Supporting Information). This is probably due to the internalization of the CM-PLH/PEI/DNA ternary complexes by the nonspecific endocytosis.[22] Furthermore, the gene expression by the PEI/DNA polyplexes coated with other anionic polypeptides such as poly(L-aspartic acid), which were not protonated at endosomal pH for lack of imidazole groups, was lower than that by the CM-PLH/PEI/DNA polyplexes (Figure S-4, Supporting Information). Taking these results into account, the

(18) A typical procedure is as follows: Calf thymus DNA, purchased from Sigma Chemical Co. (St. Louis, MO), was dissolved in PBS (−) at 1.13 mg/mL. The resulting DNA stock solution was added to the polymer solutions in 50 mM sodium phosphate buffer (pH 7.5 or pH 6.0) at various polymer/DNA ratios. The final diluted concentration of DNA was adjusted to 50 μg/mL. After 30 min of incubation at room temperature, each sample (corresponding to 0.5 μg of DNA) was mixed with a loading buffer and loaded onto a 1% agarose gel containing 1 μg/mL of EtBr. Gel electrophoresis was run at room temperature in 50 mM sodium phosphate buffer (pH 7.5 or pH 6.0) at 50 V for 10 min. The DNA bands were visualized under UV irradiation.

(19) A typical procedure is as follows: In a typical 96-well plate experiment, 1×10^4 cells/well HepG2 cells were transfected in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated FBS by the addition of 15 μL of PBS (−) containing 200 ng of plasmid DNA encoding the modified firefly luciferase (pGL3-Control Vector; from Promega Co.) and complexed with polycations. The number-average molecular weight of the PEI (from Sigma Chemical Co.) used in these experiments is ~60,000. After 1 day of incubation, the medium was removed and the cells were further incubated for 2 days in the Dulbecco's modified Eagle's medium supplemented with 10% FBS. Then, the cells were subjected to the luciferase assay (Promega kit) according to the manufacturer's instruction. Luciferase activities were normalized by protein concentrations and are presented as relative light unit (RLU). Protein concentrations were determined by BCA protein assay kit (Pierce) according to the manufacturer instruction.

(20) Asayama, S.; Sekine, T.; Kawakami, H.; Nagaoka, S. pH-Dependent dissociation of carbohydrate ligand polycations from DNA ternary complexes. *Chem. Lett.* **2006**, *35*, 100–101.

(21) Asayama, S.; Sekine, T.; Kawakami, H.; Nagaoka, S. Design of aminated poly(1-vinylimidazole) for a new pH-sensitive polycation to enhance cell-specific gene delivery. *Bioconjugate Chem.* **2007**, *18*, 1662–1667.

(22) Matsui, H; Johnson, L. G; Randell, S. H; Boucher, R. C. Loss of binding and entry of liposome-DNA complexes decreases transfection efficiency in differentiated airway epithelial cells. *J. Biol. Chem.* **1997**, *272*, 1117–1126.

synergistic effect of the imidazole and carboxymethyl groups of the CM-PLH is considered to succeed in the efficient gene delivery.

In conclusion, we have demonstrated the enhancement of the polyplex gene delivery by CM-PLH as a new pH-sensitive polypeptide. The anionic CM-PLH at physiological pH coated the PEI/DNA binary complexes, resulting in the formation of the ternary complexes. The resulting CM-PLH/PEI/DNA ternary complexes mediated the efficient gene delivery which was considered to have depended on the pH-sensitive imidazole and anionic carboxymethyl properties of the CM-PLH. In this study, the new pH-sensitive polypeptide CM-PLH has enhanced the gene delivery mediated by PEI/DNA complexes. CM-PLH is a promising polypeptide as an enhancer of polyplex gene delivery.

Acknowledgment. This work was partially supported by a Grant-in-Aid (No. 05-037: Research for Promoting Technological Seeds) from Japan Science and Technology Agency.

Supporting Information Available: Figures S-1−S-4 depicting colloid stability, effects of pH on hemolytic activity, effects of incubation time on transfection efficiency, and transfection of luciferase gene. This material is available free of charge via the Internet at http://pubs.acs.org.

MP800094B