Enhanced Expression of Foreign Gene Transferred to Mammalian Cells *in vitro* Using Chemically Modified Poly(L-lysine)s as Gene Carriers

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Poly(L-lysine) (PL) is chemically modified on the basis of the commonly essential structures of effective gene carriers, which we previously found, in order to improve the insufficient gene-transferring efficacy of PL. The PL-derivatives were then subjected to gene transfer with the osmotic shock procedure. Poly(N$^{\varepsilon}$-trimethyl-L-lysine-*co*-serine) (PtmLS) showed much higher transient gene expression than PL with low cytotoxicity while poly(N$^{\varepsilon}$-trimethyl-L-lysine) and poly(l-lysine-*co*-serine) did not. These results strongly support the importance of both of the nonionic hydrophilic groups and quaternary ammonium groups of the gene carriers.

Nonviral gene carriers[1] such as water-soluble polycations,[2-5] various liposomes, or other spherical shaped gene carriers have recently been attracting great attention in the field of gene therapy and molecular biology, because they may possibly be safer even *in vivo* usage than the conventionally used viral carriers. Among them, water-soluble polycations are expected as biodistribution-controllable gene carriers in comparison with the spherical shaped carriers that would be rapidly accumulated in the reticuloendothelial system in the body. Until now, various polycations have been reported to induce a high gene expression[2-5] but the structural requirements for the effective carriers have not yet been well understood. This interfers with advancement of molecular design of the carrier polycations. Recently, we found that the essential chemical structure of the effective gene carriers is having "both" of nonionic hydrophilic groups and highly dissociated cationic groups under the physiological conditions.[6]

Poly(L-lysine) (PL) is the most widely studied gene carriers and has been used as the base material for biologically active gene carriers having bio-derived moieties like transferrin[7] or asialoglycoprotein.[8] On the other hand, the gene introduction efficiency of PL itself was reported to be quite low,[9,10] resulting in the difficulties for developing effective PL-derived gene carriers. In the present paper, PL was chemically modified based on our finding described above in order to develop a novel base materials for the biodegradable gene carriers.

Poly(lysine-co-serine) (PLS) which is a random copolymer of L-lysine and serine with the unit composition of 3/1 was also used. The lysine residues of PL and PLS were methylated by use of dimethyl sulfate in order to impart them high basicity, giving Poly(N$^{\varepsilon}$-trimethyl-L-lysine) (PtmL) and Poly(N$^{\varepsilon}$-trimethyl-L-lysine-*co*-serine) (PtmLS), respectively.[11] The complete conversion of primary ammonium groups of the lysine residue to trimethylammonium groups was confirmed by the amino acid analysis. PLS and PtmL possesses either hydroxyl groups or quaternary ammonium groups, respectively, while PtmLS has both of them (Figure 1). The molecular weight of the PL, PLS, PtmL, and PtmLS measured by GPC was 18700, 23400, 24800, and 30000, respectively.

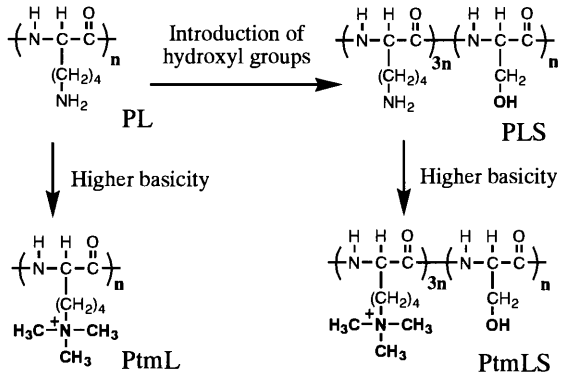

Figure 1. Chemical structures of the polypeptides used.

The complex formation of these polypeptides with pEGFP-N1 plasmid DNA encoding Enhanced Green Fluorescent Protein derived from *Aequorea victoria* (Clontech Laboratories Inc., Palo Alto, CL) was monitored on an agarose gel electrophoresis. Each polypeptide was found to form complexes with DNA in a very similar manner as follows. All of the added DNA formed complex with polypeptides at C/A ratio of 1.0 and the total electrostatic charge of the complex became positive around at C/A ratio of 3.0. C and A in the C/A ratio indicate the molar quantity of the cationic groups of polycation and the phosphate groups of DNA.

pEGFP-N1 plasmid was transfected into COS-1 cells by the osmotic shock procedure using these carriers[12,13] and the transient expression of EGFP was evaluated under the fluorescent microscope (ex.: 488 nm and em.: 507 nm). The percent transient expression was expressed as the percentage of the number of cells emitting fluorescence to the total cell number. The dependence of the transient expression of the gene introduced using the polypeptides on the C/A ratio is shown in Figure 2. PL and PtmL showed no transient expression of EGFP irrespective of the C/A ratio, suggesting that the impartment of high basicity to PL is not effective in enhancing transfection efficacy. On the other hand, a little enhancement of EGFP expression was observed in the case of PLS especially around at the C/A ratio of 10 but no statistical difference was observed. In contrast, PtmLS induced much higher transient expression. The percent transient expression was rapidly increased at C/A ratio of 3.0 which is in a good agreement with the fact that the total charge of the PtmLS/DNA complex became positive at the C/A>3.0. The big difference between the efficacy of PLS and PtmLS indicates an important role of the quaternary ammonium groups in the presence of the

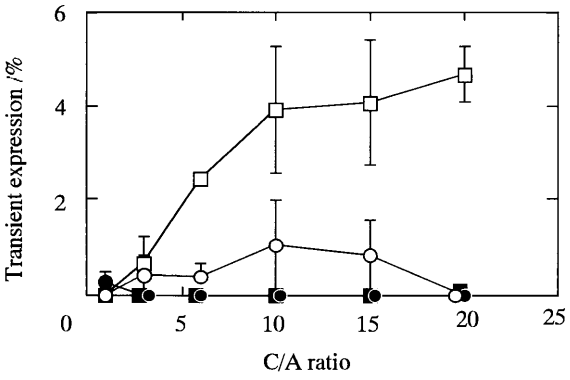

Figure 2. Transient expression of EGFP gene introduced to COS-1 cells by the osmotic shock procedure using (●) PL, (○) PLS, (■) PtmL, and (□) PtmLS.

hydrophilic residues. The reason for the low efficacy of the primary ammonium groups is unclear but the same phenomenon was observed in the case of dextran derivatives with primary or tertiary ammonium groups as is previously reported.[13]

The amount of FITC labeled DNA taken up by COS-1 cells was in the range of 0.6–1.9ng/10^4 cells irrespective of the used polypeptides and PtmLS shows the lowest ingestion of DNA. It suggests that the difference in the transient expressions was caused at some point following the internalization of the complexes into the cells. The detailed mechanism is unclear yet but one possibility is the difference in the recognition of complexes by the transcription factors. We recently found that the compaction of the complex was greatly affected by the nonionic hydrophilic groups of the polycations by measuring the fluorescent intensity of the complexes of FITC-DNA and various polypeptides.[14] Then, the complexes of peptides and plasmid DNA encoding luciferase were subjected to the in vitro transcription/translation system using rabit reticulocyte lysate (Promega Co., WI). The activity of produced luciferase in the case of PLS and PtmLS was 3.48 and 2.31 times larger than PL and PtmL, respectively, indicating the efficacy of the serine residues introduced to the polypeptides. While the trimethylation of PL increased the efficiency slightly but no statistical difference was observed. Since this must not be a single reason for the high transferring ability of the PtmLS, the fate of polypeptide/DNA complexes after internalization by the cells including their dissociation and transportation should be studied further.

The cytotoxicity of the four polycations after the osmotic shock procedure at C/A=6 was comparable (cell viability > 75%) and much lower than that of currently used cationic dextran (cell viability <20%). PtmLS is, therefore, promising materials as a novel water-soluble nonviral gene carrier with higher gene expression than original PL and the lower cytotoxicity. In addition, a preliminary study on the other gene transfer system using chloroquine treatment revealed that the gene transferring ability of PtmLS to Hela is markedly higher than the other polypeptides.

In the present paper, the essential structures for effective gene carriers, which we previously reported, was proved by using PL-derivatives and a novel biodegradable gene carrier, PtmLS, was successfully developed. It may be possible to enhance the efficacy further by optimizing the side group compositions of PtmLS. Moreover, an introduction of the other amino acid residue, like cysteine, which is capable for the biological modifications by various functional group-specific coupling reactions would be also promising. Not only biological modifications but also the optimization of the chemical structures of the polymers should be carried out in designing effective gene carriers.

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