Facilitated Disassembly of Polyplexes Composed of Self-assembling Amphiphilic Polycations Enhances the Gene Transfer Efficacy

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Amphiphilic polycations, p(DMAPAA-*co*-MPC-*co*-SA)s, with various cation contents were designed as novel micelle-like nonviral gene carriers. The carriers led to a higher transgene expression in COS-1 cells three times as high as the case of the linear-type polycations. The enhanced transgene expressions were found to be related to the disassembly of plasmid DNA/carrier polyplexes. It can be concluded that a topological factor of the carriers greatly influences on the gene expression efficiency.

A variety of polycations with different chemical structures have been found to be effective gene carriers but still have lower efficiency than the viral vectors.[1–3] To improve it, each step in the gene transfer such as cellular uptake of polyplexes,[4] their release from the endosome,[5] and the localization into the nucleus[6] have been studied. In contrast, the transcription of the polyplexes, which is the last step of the gene transfer, was not well understood. We previously reported the enhanced gene expression by introducing nonionic hydrophilic groups to conventional poly(L-lysine) carrier.[7] This chemical modification was found to effectively suppress the polyplex compaction, and hence its transcription efficiency in the nuclei was greatly improved. In the present study, we have tried to improve the intranucleus transcription efficiency by changing the molecular topology of the polycation carriers from linear-type to micelle-like for the first time. Our working hypothesis was that the micelle-like polycations interact with DNA molecule with less entanglement than the linear-type ones. In order to prepare both liner-type and micelle-like carriers which have similar properties other than the topology, slight amount of hydrophobic moieties was introduced to the conventional linear polycations. The hydrophobized polycations are known to form a kind of micelle based on the self-aggregation mechanism.[8] A series of amphiphilic polycations containing 2-methacryloyloxyethylphosphoryl choline (MPC) were synthesized, and the micelle forming property and the gene transfection ability were investigated. Although MPC has been reported to act as a component of block polymer-type carriers, we selected this monomer as a nonionic hydrophilic monomer as previously reported.[9,10]

Random copolymerization of *N*-[3-(dimethylamino)propyl] acrylamide (DMAPAA), MPC, and stearyl acrylate (SA) were carried out in methanol using AIBN as an initiator ($M/I = 200$) at 60 °C for 4 h, dialyzed against methanol, and lyophilized (Figure 1). The compositions were determined by ^{1}H NMR. The copolymers were expressed as p(DMAPAA*x*-*co*-MPC*y*-*co*-SA*z*), where *x*, *y*, and *z* represent the molar composition of each monomers. DMAPAA content of the copolymers was similar to the monomer ratio in feed. The molecular weight determined by gel permeation chromatography (GPC) of

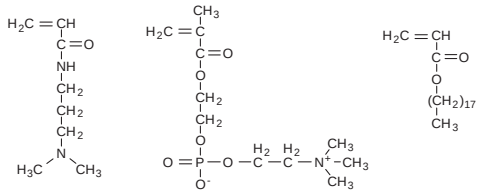

Figure 1. Chemical structures of used monomers.

p(DMAPAA46-*co*-MPC53-*co*-SA1), p(DMAPAA62-*co*-MPC37-*co*-SA1), p(DMAPAA40-*co*-MPC60), and p(DMAPAA62-*co*-MPC38) were, 52,000, 42,000, 102,000, and 65,000, respectively.

The critical micelle concentration (cmc) of the obtained polymers were measured using pyrene as hydrophobic probe molecules at various polymer concentrations from 0.01 to 1.0 g L^{-1}.[11] The fluorescent sensitization with an increased concentration was observed only for p(DMAPAA-*co*-MPC-*co*-SA)s, and red shift from 334 to 343 nm was observed, indicating that the amphiphilic polycations formed micelle-like structures in water. The cmc of p(DMAPAA46-*co*-MPC53-*co*-SA1) and p(DMAPAA62-*co*-MPC37-*co*-SA1) was 3.1 and 6.6 mg L^{-1}, respectively. Their particle sizes determined by DLS of 100 μg L^{-1} in filtered-Dulbecco's Modified Eagle's Medium were 280 ± 28 and 290 ± 34 nm, respectively.

pCMV-Luc endcoding luciferase gene and the copolymers were incubated in TE buffer for 30 min at room temperature resulting in the polyplex formation. The charge ratio of mixed plasmid DNA and polymers (C/A ratio) was ranged from 0.5 to 10.0. The agarose gel electrophoresis showed that the DNA formed polyplex at the C/A ratio higher than 1.0 completely. COS-1 cells were transfected with the polyplexes by the chloroquine method.[12]

The dependence of the transient expression on the C/A ratio was shown in Figure 2. Each polymer showed no cytotoxicity. As we previously proposed, the transgene expression was greatly affected by the polymer compositions due to the balance of the cationicity and nonionic hydrophilicity.[13] In the present study, the carriers having about 40% of cation contents showed higher gene expression, irrespective of the SA contents. When copolymers with similar DMAPAA contents were compared, p(DMAPAA-*co*-MPC-*co*-SA)s showed about three times higher expression than the p(DMAPAA-*co*-MPC)s. On the other hand, cellular uptake of polyplexes was not affected by the SA unit at all, and the amount of ^{32}P-labeled pDNA delivered using p(DMAPAA46-*co*-MPC53-*co*-SA1) and p(DMAPAA40-*co*-MPC60) was 13.2 ± 1.9 and 16.3 ± 0.6 ng/1.0×10^4 COS-1 cells, respectively. In addition, there was no big difference in the intracellular distribution when compared under a fluorescent micro-

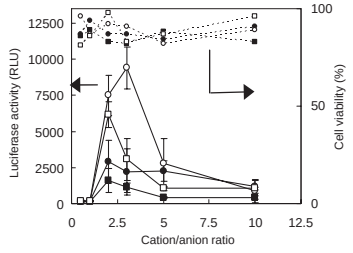

Figure 2. Expression of luciferase gene introduced to COS-1 cells using p(DMAPAA46-*co*-MPC53-*co*-SA1) (○), p(DMAPAA62-*co*-MPC37-*co*-SA1) (□), p(DMAPAA40-*co*-MPC60) (●), and p(DMAPAA62-*co*-MPC38) (■) and the cell viability.

scope (date not shown).

These results indicate that the higher efficiency of p(DMAPAA46-*co*-MPC53-*co*-SA1) was resulted from the efficient transcription of the transgene complexing with the micelle-like carriers after their internalization. We then examined the dissociation tendency of the polyplexes. The exchange reaction of DNA with anionic macromolecules, such as mRNA, sulfated sugars, and nuclear chromatin existing as essential cellular components was reported to occur during the intracellular trafficking.[12] A given amount of potassium polyvinyl sulfate (PVSK) was added to the polyplex (C/A ratio = 3). The unit mole ratio of PVSK and cationic copolymer was set to 0.8:1. The reaction mixture was incubated for 30 min at room temperature and evaluated on 0.8% agarose gel (Figure 3). PVSK did not affect on the mobility of free DNA. The clear difference in the easiness of polyplex dissociation was observed. Complete release of DNA from the polyplexes with p(DMAPAA46-*co*-MPC53-*co*-SA1) was observed by adding PVSK solution. In comparison, dissociation of the p(DMAPAA40-*co*-MPC60)/pDNA polyplexes has not occurred at all. The micelle-like structure is considered to facilitate the release of plasmid DNA from the polyplexes even in the intracellular environment.

In vitro transcription/translation experiment using TNT® T7 coupled Reticulocyte Lysate System (Promega Corporation, USA) was carried out for the p(DMAPAA46-*co*-MPC53-*co*-SA1)/pDNA polyplex suspentions in the presence or absence of PVSK. Briefly, reaction lysate (25 μL TNT® Rabbit Reticulocyte Lysate, 0.5 μL Amino Acid Mixture (1 mM), 2 μL TNT® Reaction Buffer, 1 μL TNT® T7 RNA Polymerase, 2 μL Rnase inhibitor (27.2 u/μL)) was added to pT7-Luc/carrier polyplex suspension (C/A ratio = 3) and incubated for 90 min at 37 °C, and the luciferase activity was measured. The luciferase activities for p(DMAPAA46-*co*-MPC53-*co*-SA1) /pDNA polyplex and free DNA were 1.0×10^5 and 5.1×10^5 RLU, respectively. The p(DMAPAA46-*co*-MPC53-*co*-SA1) suppressed the direct transcription of the plasmid DNA. On the other hand, when

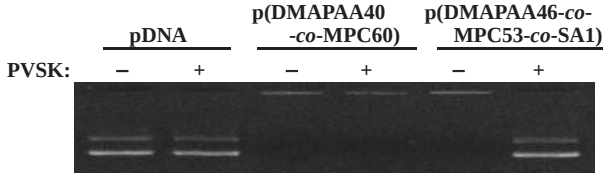

Figure 3. Disassembly of p(DMAPAA40-*co*-MPC60)/pDNA and p(DMAPAA46-*co*-MPC53-*co*-SA1)/pDNA polyplexes in the presence or absence of 0.8 mol % PVSK relative to the DMAPAA unit moles.

0.8 mol % of PVSK was added to this system, the activity was greatly recovered and reached 4.4×10^5 RLU, which is about 85% of the result for the free DNA. In contrast, p(DMAPAA40-*co*-MPC60)/pDNA shows 1.5×10^5 and 0.7×10^5 RLU in the presence or absence of PVSK, respectively. These results indicate that suppressed transcription can be recovered only for p(DMAPAA46-*co*-MPC53-*co*-SA1), which is in a good agreement with the above agarose gel electrophoresis (Figure 3). The easiness of dissociation of the polyplexes strongly correlates with the total transgene expression in vitro as shown in Figure 2. However, these results do not necessarily indicate the polyplex disassembly in the cells before the transcription. It is also possible that the polyplexes are directly recognized by the transcription factors without the disassembly. When the luciferase activities in in vitro system for p(DMAPAA46-*co*-MPC53-*co*-SA1) (1.0×10^5 RLU) and p(DMAPAA40-*co*-MPC60) (0.7×10^5 RLU) in the absence of PVSK (see above) were compared, the direct transcription was also improved slightly by adding just 1% of stearyl group in the linear polycation. The micelle-type amphiphilic carriers were able to improve the intracellular transcription via both of these mechanisms.

It was found that DNA complexing with the copolymers having stearyl residues is much more easily replaced with added PVSK than the case of the linear-type copolymers (Figure 3). The amphiphilic copolymers were found to form micelle-like structure. These carriers promote the transcription caused by easiness of DNA release from the polyplexes and by less entanglement. These results suggest that the factor of carrier topology is important to detemine the expression efficiency.

References and Notes

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1 S. C. De Smedt, J. Demeester, and W. E. Hennink, *Pharm. Res.*, **17**, 113 (2000).

2 C. Kneuer, M. Sameti, U. Bakowsky, T. Schiestel, H. Schirra, H. Schmidt, and C. M. Lehr, *Bioconjugate Chem.*, **11**, 926 (2000).

3 J. H. Jeong, S. H. Song, D. W. Lim, H. Lee, and T. G. Park, *J. Controlled Release*, **73**, 391 (2001).

4 G. Y. Wu and C. H. Wu, *Biochemistry*, **27**, 887 (1988).

5 O. Boussif, F. Lezoualc'h, M. A. Zanta, M. D. Mergny, D. Scherman, B. Demeneix, and J. P. Behr, *Proc. Natl. Acad. Sci.*, **92**, 7297 (1995).

6 C. K. Chan and D. A. Jans, *Hum. Gene Ther.*, **10**, 1695 (1999).

7 T. Yamaoka, T. Kimura, R. Iwase, and A. Murakami, *Macromol. Biosci.*, **2**, 437 (2002).

8 I. Lee and K. Akiyoshi, *Biomaterials*, **25**, 2911 (2004).

9 J. K. Lam, Y. Ma, S. P. Armes, A. L. Lewis, T. Baldwin, and S. Stolnik, *J. Controlled Release*, **100**, 293 (2004).

10 M. Licciardi, Y. Tang, N. C. Billingham, S. P. Armes, and A. L. Lewis, *Biomacromol.*, **6**, 1085 (2005).

11 S. Katayose and K. Kataoka, *Bioconjugate Chem.*, **8**, 702 (1997).

12 P. Erbacher, A. C. Roche, M. Monsigny, and P. Midoux, *Bioconjugate Chem.*, **6**, 401 (1995). A mixture of 50 μL of polyplex suspension and 50 μL of chloroquine solution (200 μM) was added to monolayer culture of 1.0×10^4 seeded cells in a single well of a 96-well muitiplate, incubated for 8 h, and then cultured in a fresh medium for another 40 h. Luciferase activity of the cell lysate in Trion-X100 solution was measured on a Lumicounter ATP-300 (ADVANTEC MFS, INC., USA).

13 T. Yamaoka, H. Iwata, N. Hamada, H. Ide, and Y. Kimura, *Nucleic Acids Symp. Ser.*, **31**, 229 (1994).

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