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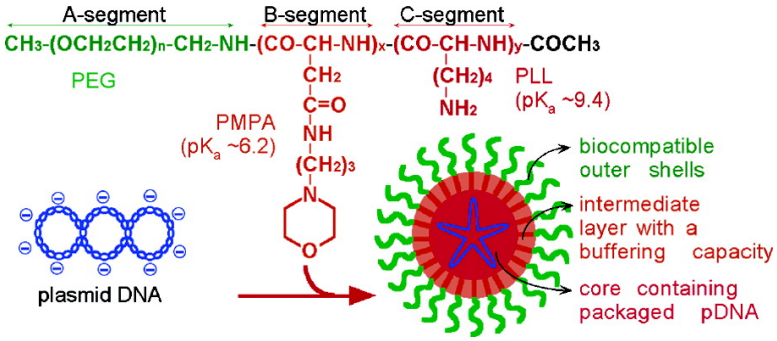

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PEGylated Polyplex Micelles from Triblock Cationers with Spatially Ordered Layering of Condensed pDNA and Buffering Units for Enhanced Intracellular Gene Delivery

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Successful in vivo gene therapy relies on the development of efficient gene vectors. Especially, the synthetic vectors based on cationic polymers have been attracting much attention because of their safety for clinical application and the variety of their chemical design. Nevertheless, the rational design of synthetic vectors remains to be established. Many previous studies have described that polyplexes formed from polycations with a comparatively low pK_a values, such as polyethylenimine (PEI), show a high transfection activity,[1] which has been explained by the proton sponge effect.[2] However, such polycations have a weak affinity to DNA, resulting in the formation of polyplexes that are easily dissociated under physiological conditions. Also, the buffer capacity of polycations may be hampered by their facilitated protonation due to the zipper effect or the neighboring group effect during the complexation process with DNA.[3] These problems could be modulated by the addition of excess polycations (i.e., increasing the N/P ratios[4]) to form polyplexes with a cationically deviated composition. However, it was recently demonstrated that free polycations in such polyplexes substantially contribute to efficient transfection but also mediate toxic effects. Hence, polyplex systems useful for in vivo gene delivery are required to achieve efficient transfection under the condition without free polycations.[5] Also, the gene delivery systems need to be equipped with high stability and biocompatibility. Here, A−B−C type triblock copolymers consisting of three distinctive functional segments were newly designed for constructing gene delivery systems which might not require free polycations to achieve enhanced gene expression but might provide a high stability and biocompatibility. In the present design of triblock copolymers, poly(ethylene glycol) (PEG) was used as the biocompatible A-segment, poly[(3-morpholinopropyl) aspartamide] (PMPA) was used as the low-pK_a B-segment with a buffering capacity, and poly(L-lysine) (PLL) was used as the high-pK_a C-segment to condense the DNA (Figure 1).

The triblock copolymer, PEG−PMPA−PLL, was synthesized by the successive ring-opening polymerization of the *N*-carboxyanhydrides (NCAs) of β-benzyl-L-aspartate (BLA) and ϵ-(benzyloxycarbonyl)-L-lysine (Lys(Z)), initiated by the $-NH_2$ group of α-methoxy-ω-amino PEG (MW 12 000), followed by the aminolysis of the benzyl ester of PBLA using 4-(3-aminopropyl)morpholine and the deprotection of the Z groups of PLL(Z).[6] The triblock copolymer was confirmed to have a narrow molecular weight distribution (M_w/M_n = 1.18), and the number of repeating units of

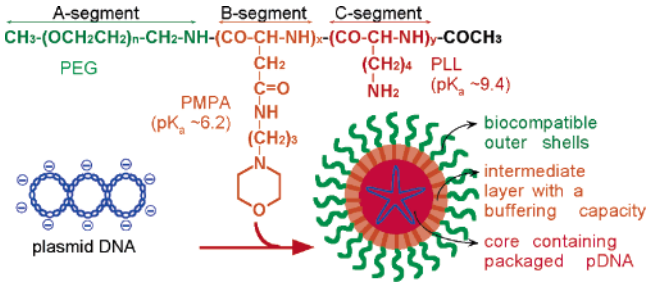

Figure 1. Chemical structure of PEG−PMPA−PLL triblock copolymers and schematic illustration of the hypothesized three-layered polyplex micelles with spatially regulated structure.

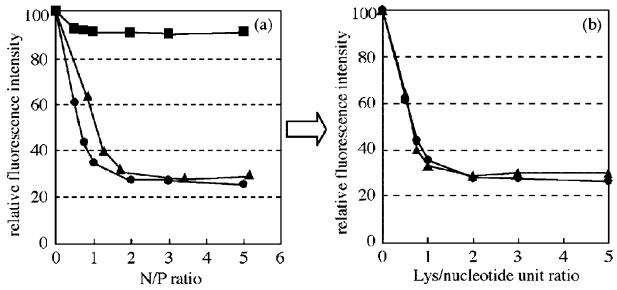

Figure 2. Interaction of PEG−PLL (circle), PEG−PMPA (square), and PEG−PMPA−PLL (triangle) copolymers with pDNA in 10 mM PBS (pH 7.4) + 150 mM NaCl, evaluated by dye exclusion assay. (a) In this figure, the *X*-axis represents the N/P ratio, where N stands for the total of MPA and Lys units. (b) In this figure, the *X* axis represents the Lys/nucleotide unit ratio.

PMPA and PLL was calculated to be 36 and 50, respectively, from the ^{1}H NMR. Diblock copolymers, PEG−PLL with 48 PLL units and PEG−PMPA with 39 PMPA units, were used as comparative samples in this study. The formation of polyplex micelles from these block cationers was confirmed by a gel retardation assay (Figure S4, Supporting Information).[6] Also, the interaction between the di- or triblock copolymers and plasmid DNA (pDNA) was evaluated by an ethidium bromide (EtBr) exclusion assay (Figure 2a). In the case of PEG−PLL (pK_a 9.4), the fluorescence intensity was decreased to 20% of that of the naked pDNA at the N/P ratio of 2. In contrast, the system of PEG−PMPA, having a cationic segment with a lower pK_a value (pK_a 6.2), maintained relatively high fluorescence (>90%) over a wide range of N/P ratios, suggesting that PEG−PMPA lacks the capacity to condense pDNA to a level detectable by this assay. On the other hand, PEG−PMPA−PLL exhibited an 80% decrease in fluorescence at the N/P ratio of 3. Interestingly, the fluorescence profile of PEG−PMPA−

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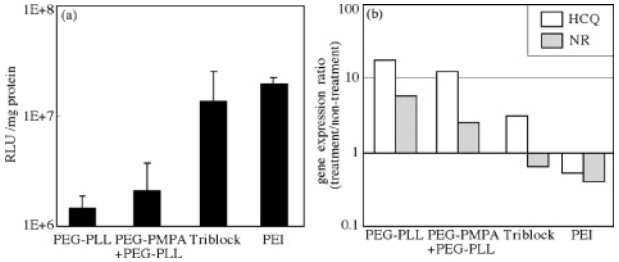

Figure 3. In vitro transfection of luciferase gene to HeLa cells by polyplex micelles from di- or triblock copolymers. HeLa cells were incubated with each micelle in the medium containing 10% serum for 24 h, followed by an additional 24 h incubation without the micelles. (a) The polyplex micelles were prepared at a Lys/nucleotide ratio of 2, and the PEI/pDNA was prepared at the corresponding N/P ratio to the PEG−PMPA−PLL/pDNA. (b) The effects of HCQ and NR on the TE of the polyplexes were evaluated. The PEI polyplex was prepared at the N/P ratio of 10.

PLL/pDNA was almost identical to that of PEG−PLL/pDNA when the N/P ratio was converted to the Lys/nucleotide unit ratio (Figure 2b). Presumably, in the complex of PEG−PMPA−PLL/pDNA, the PLL segment may predominantly contribute to the pDNA condensation. This assumption was confirmed by ^{1}H NMR measurement of PEG−PMPA−PLL/pDNA [Lys/nucleotide ratio = 2 (N/P ratio = 3.4)] in deutereted phosphate-buffered saline (pD 7.4, 150 mM NaCl), in which the chemical shifts assigned to the PLL segment completely disappeared but those assigned to the PMPA segments remained in the spectrum (Figure S6, Supporting Information).[6] This result is consistent with the hypothesis that the PEG−PMPA−PLL/pDNA may form three-layered polyplexes as illustrated in Figure 1. Also, the complete disappearance of PLL peaks from the NMR spectrum suggests that PEG−PMPA−PLL in free form may be minimal in the solution. The size and ζ-potential of the PEG−PMPA−PLL complexes at the Lys/nucleotide ratio of 2 were determined to be 88.7 nm and 7.3 mV, respectively, comparable to those obtained from the PEG−PLL complexes at the N/P ratio of 2 (91.7 nm and 2.1 mV, respectively). The particle size of approximately 100 nm is consistent with the condensed structure of pDNA, and the low absolute value of the ζ-potential suggests the formation of the PEG palisade surrounding the polyplex core.

In vitro transfection efficiency (TE) of the PEG−PMPA−PLL/pDNA at the Lys/nucleotide ratio of 2 was evaluated against HeLa cells. Notably, PEG−PMPA−PLL/pDNA revealed 1 order of magnitude higher TE than PEG-PLL/pDNA (Figure 3a), which was comparable to that of the PEI/pDNA at the corresponding N/P ratio, without showing appreciable cytotoxicity (Figure S9, Supporting Information).[6] On the other hand, the TE of the system composed of (PEG−PMPA + PEG−PLL)/pDNA, where the contents and the repeating units of the PMPA and PLL segments were nearly equal to PEG−PMPA−PLL, was almost the same as that of PEG−PLL. Also, the polyplexes of PEG−PMPA showed no transfection activity over a wide range of N/P ratios (data not shown). These results strongly indicate the importance of aligning in tandem two types of polycations with different pK_a values in a single polymer strand. To study the mechanism of the transfection, the effects of hydroxychloroquine (HCQ) and nigericin (NR) on transfection behavior were investigated. HCQ is known to increase the TE of the polyplexes lacking a buffering capacity, whereas NR could decrease the TE of the polyplexes showing the proton sponge effect.[7] The PEG−PMPA−PLL/pDNA showed less effect of HCQ on enhancing the gene expression compared with the PEG−PLL/pDNA, while it showed an appreciable decrease in the TE in the presence of NR (Figure 3b). Similar trends were also confirmed for 293T cells (Figure S8, Supporting Information). These biological results are consistent with the hypothesis that the enhanced TE of the PEG−PMPA−PLL/pDNA may be attributed to the proton sponge effect. Thus, the buffering capacity of PMPA segment appears to be maintained in the PEG−PMPA−PLL/pDNA under the condition with low Lys/nucleotide ratio. The preferential contribution of the PLL segment to the DNA condensation may ensure the presence of the uncomplexed PMPA segment, even at a comparatively low N/P ratio, to work as a buffering unit.[6]

Nonviral gene vectors used in vivo must have a high stability to be tolerated under harsh conditions in the body. In our previous studies, polyplexes based on PEG−PLL showed a high serum tolerability[8] and prolonged blood circulation.[9] Although the PLL segments form stable polyplexes with pDNA, the transfection activity might be inefficient due to the lack of a proton buffering capacity. In contrast, polycations with a lower pK_a have a buffering capacity for the enhanced transfection but demand a high N/P ratio to achieve a high efficacy. Polyplexes formed at a high N/P ratio may not be useful for in vivo transfection due to stability and toxicity concerns.[5] The result reported here led to the novel design of nonviral gene vectors, overcoming the problems of conventional systems based on the proton sponge concept, using the A−B−C type triblock copolymers, PEG−PMPA−PLL (Figure 1). The results are consistent with the hypothesis that PEG−PMPA−PLL might form three-layered polyplex micelles consisting of a core of pDNA/PLL polyion complexes, an intermediate layer of PMPA segments with a buffer capacity, and an outer shell of biocompatible PEG segments. The PEG−PMPA−PLL polyplexes showed a significantly enhanced transfection activity through the buffering capacity of the PMPA segment, while efficiently compacting pDNA by the PLL segment. Importantly, this increased transfection was achieved under the condition where free or loosely associated polycations are assumed to be minimal, facilitating the future utility of this polyplex micelle for in vivo gene delivery.

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Supporting Information Available: Synthetic method and characterization of triblock copolymers as well as additional data on the physicochemical and biological properties of the PEG−PMPA−PLL/pDNA. This material is available free of charge via the Internet at http://pubs.acs.org.

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