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Timing-controlled Decompaction of Polyplexes In Vivo Greatly Enhances Transgene Expression

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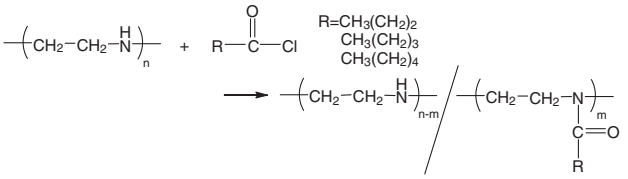
Compaction extent of polyplexes was successfully regulated by cold treatment in buffer solution using thermoresponsive gene carriers composed of linear poly(ethyleneimine) (l-PEI) and alkyl side chains. Plasmid DNA (pCMV-Luc) was transfected to COS-1 cells using these carriers with different cold treatments. The luciferase expression was greatly enhanced when cells were treated at 4 °C in well-defined timing. This was a direct observation of how intracellular destabilization in regulated timing is important for nonviral gene transfection.

There has been significant interest in synthetic polycation as a non-viral gene carrier and in gene therapy.[1] Several polycationic carriers, such as poly(ethyleneimine) (PEI),[2] poly(L-lysine) (PLL),[3] and chitosan,[4] have been developed due to various advantages over viral vectors. Among them, PEI is one of the most widely studied gene carriers because of its high efficiency gene expression.[2] Furthermore, mechanism analysis for efficient gene transfer including cellular uptake, lysosomal escape, and nuclear transport has been widely carried out. Although the decompaction or dissociation of polyplexes is believed to be important for gene expression, studies of this are not well developed because it is not easy to control these phenomena in cells.

Recently, thermoresponsive polymers have received much attention as intelligent materials for various applications. Poly(*N*-isopropylacrylamide) (PNIPAAm) is one of the most typical thermoresponsive polymers.[5] A block copolymer consisting of poly(L-lactic acid) and poly(ethylene glycol)[6] and poly(amino acid)s[7] have been also reported. Kurisawa et al. reported that thermoresponsive copolymer, poly[*N*-isopropylacrylamide-*co*-2-(dimethylamino)ethyl methacrylate-*co*-butyl methacrylate], showed high transfection efficiency.[8,9] A PEI-*graft*-PNIPAAm copolymer was synthesized as a thermoresponsive carrier by Bisht et al.[10] Lavigne et al. reported that high gene expression using PEI–PNIPAAm conjugates as a carrier occurred below the LCST.[11] We report herein synthesis and timing-controlled gene transfection by use of new thermoresponsive PEI derivatives as gene carriers. PEI derivatives were synthesized by the reaction of l-PEI ($M_w = 22000$) with various carboxylic acid chlorides in chloroform at room temperature for 48 h (Scheme 1). In this study, butyryl chloride, propanoyl chloride, and hexanoyl chloride were used for the synthesis of PEI derivatives. The synthesis of PEI derivatives is summarized in Table 1 and the introduction ratio was determined by ^{1}H NMR. PEI-C4 was soluble in water at room temperature. PEI-C5 and PEI-C6 were insoluble in water.

Figure 1 shows the transfection efficiency of PEI derivative/pCMV-Luc complexes. Complexes were formed by mixing PEI derivatives with pCMV-Luc at several cation/anion (C/A) ratios. The transfection efficiency was determined by luciferase activity in COS-1 cells at 37 °C. PEI homopolymer as a control showed high transfection efficiency with increasing C/A ratio. The transfection efficiency of PEI-C4 at high C/A ratios of 24 and 48 was almost the same as that of PEI. It was demonstrated that the transfection efficiency was not affected by the introduction into the side chain of PEI. For PEI-C5 and PEI-C6, low transfection efficiencies were observed because of their low solubility in water.

Figure 2 shows photographs of 1 wt% solution of PEI-C4 at 4 and 37 °C. The transparent solution at 4 °C became opaque at 37 °C. The turbidity change took place sharply in both heating and cooling processes. This result showed that PEI-C4 was



Scheme 1.

Table 1. Synthesis of PEI derivatives

Sample	Chloride	Yield/%	Introduction ratio[a]/%
PEI-C4	Butyryl chloride	55	64
PEI-C5	Propanoyl chloride	64	67
PEI-C6	Hexanoyl chloride	59	58

[a]Determined by ^{1}H NMR.

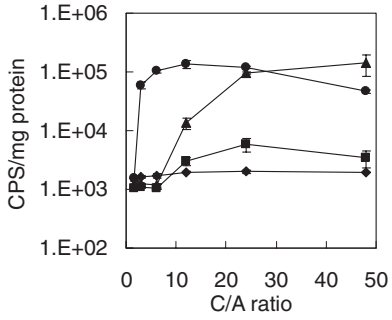


Figure 1. Transfection efficiency determined by luciferase activity in COS-1 at 37 °C. The polyplexes composed of pCMV-Luc (100 ng) and polycations (C/A 48–1.5) in FBS (−) DMEM were added to culture medium for 1×10^4 cells per well in the presence of 100 μM chloroquine. ●: PEI, ▲: PEI-C4, ■: PEI-C5, and ◆: PEI-C6. Values are shown as means ± standard deviations.

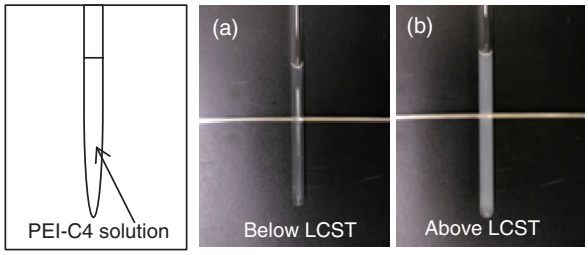


Figure 2. Photographs of the 1 wt % polymer solution of PEI-C4 at (a) 4 and (b) 37 °C.

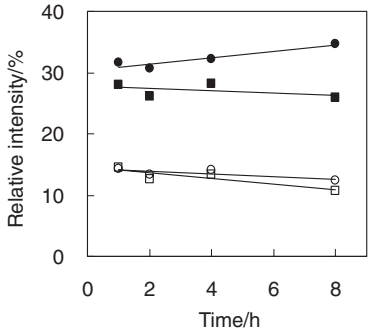


Figure 3. Relative fluorescence intensities of complexes depending on the temperature. Fluorescein-labeled plasmid DNA was complexed with PEI or PEI-C4. Complexes were incubated at 4 or 37 °C for various times. ●: PEI-C4 (4 °C), ■: PEI-C4 (37 °C), ○: PEI (4 °C), and □: PEI (37 °C).

thermoresponsive polymer. The LCST of PEI-C4 was estimated around 30 °C.

Relative fluorescence intensities of complexes depending on the temperature were examined (Figure 3). Fluorescein-labeled pCMV-Luc (F-pCMV-Luc) was complexed with PEI or PEI-C4 at the C/A ratio of 24 and complexes were incubated at 4 or 37 °C for 1, 2, 4, and 8 h. A gradual increase in the relative fluorescence intensity of PEI-C4/F-pCMV-Luc complex by treating at 4 °C was found, whereas such increase was not observed when the complex was incubated at 37 °C. In the case of PEI, the change of relative fluorescence intensities was not observed. This change must be because of the decompaction of the polyplexes resulting from the increased hydrophilicity of the PEI-C4. The temperature lower than the LCST caused a conformational change of PEI-C4 and made the complex unstable.

Effects of the post-transfection cold procedure on the luciferase expression are shown in Figure 4B. Relative gene expression was calculated as follows: (CPS/mg protein with the cooling procedure)/(CPS/mg protein without the cooling procedure). When cells were treated at 4 °C for 6 h at 24 h post transfection, the relative gene expression increased 2.3 times (Figure 4A, ▲). This kind of enhancement was not observed for the PEI (Figure 4A, open marks). The cold treatment for 2 h did not affect the expression at all (Figure 4A, ●). This may be due to insufficient decompaction of the polyplexes. The internalized complexes are considered to be decompacted as is shown in Figure 3 and were transcribed, resulting in high gene expression. When cells were cold treated for 6 h at 6 h post transfection, the

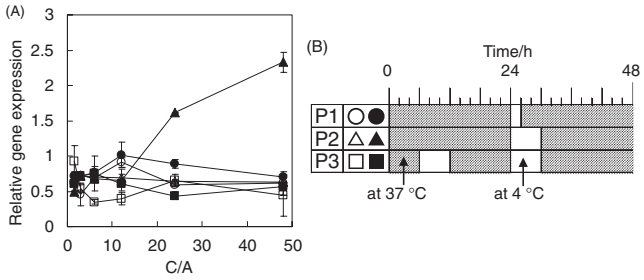


Figure 4. Relative gene expression depending on temperature (A). Cells were incubated with complexes composed of pCMV-Luc (100 ng) and polycations (C/A 48–1.5) in FBS (−) DMEM. PEI-C4 (closed symbol) and PEI (open symbol) were used. The cooling procedure is shown on panel B.

expression enhancement was not observed even for the 6 h cold treatment at 24 h post transfection (Figure 4A, ■), suggesting that the decompaction at a too early stage in the intracellular trafficking of polyplexes suppressed the gene expression completely.

In conclusion, new thermoresponsive polymers based on PEI were used for controlling the intracellular decompaction of the polyplexes. Thermoresponse was found in polymer solution prepared by the reaction of butyryl chloride with PEI. The stability of PEI-C4/F-pCMV-Luc complex was clearly affected by cold treatment in a buffer solution. Furthermore, high gene expression was achieved by well-defined cold treatment procedure. Our system will be useful for mechanistic analysis of the intracellular behavior of polyplexes for efficient polymeric carrier-based gene transfer.

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