# Intracellular Enzyme-responsive Fragmentation of Nonviral Gene Carriers Leads to Polyplex Destabilization and Enhanced Transgene Expression

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A novel cationic oligopeptide has been developed for bioprocessing-triggered nonviral gene delivery systems. Intracellular fragmentation of the carrier in response to endogenous enzyme, furin, led to destabilized polyplexes and enhanced transgene expression.

Various nonviral polymeric gene carriers have been recently proposed for effective gene transfer but are also known to inhibit the transcription efficiency in nuclei due to the strong polyplex compaction. The polyplex compaction is affected by various physical properties of carriers, such as hydrophobicity, hydrophilicity, and molecular weight (MW). Many researchers have been investigating the effect of carrier MW on transfection efficiency.[1–4] Godbey et al. reported that MW 70000 poly(ethyleneimine) (PEI) produced much higher expression levels than low MW PEI in cell culture because of better entry of polyplexes into the cells or stronger protection of pDNA.[1] On the other hand, Schaffer et al. reported that pDNA is dissociated from lower MW poly(L-lysine) more rapidly and that the weak interaction permits larger transcription rate in a cell-free system.[3] According to these findings, intracellular fragmentation of carriers is suspected to result in effective transfection because of the better entry of transgene and the higher transcription rate.

In this work, we designed oligopeptide-type nonviral carriers (Fur-oligopeptides) containing a cleavable sequence for intracellular proprotein convertase, furin. Furin is related to the processing of a wide variety of protein precursors within the secretory pathway and localized at trans-Golgi network, lysosome, and endosome of a broad range of mammalian cells.[5,6] It has been reported that furin recognizes the cleavage-site sequence Arg–X–X–Arg (R–X–X–R).[7] Especially, highly cationic Arg–X–(Lys/Arg)–Arg (R–X–(K/R)–R) is cleaved with 10-fold higher efficiency than R–X–X–R. Fur-oligopeptide, which has a repeating R–X–(K/R)–R sequence, is cationic enough to form polyplexes with nucleic acids electrostatically and to deliver nucleic acids into cells. Fur-oligopeptide is expected to be fragmented in an intracellular environment and lead to high transgene expression by releasing pDNA.

Fur-oligopeptides were synthesized by stepwise elongation of Fmoc amino acids on solid-phase resins. $(RKRKKR)_4C$ has seven cleavage sites.[8] $(RKRKRK)_4C$ is a control sequences without cleavage site. RKKR is a model peptide for the digested fragment.

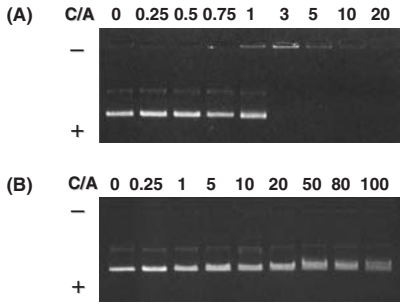


**Figure 1.** EtBr stained agarose gel (0.8%) electrophoresis of $(RKRKKR)_4C$/pT7-Luc (A) and RKKR/pT7-Luc (B) polyplexes at various C/A ratios.

$(RKRKKR)_4C$ formed polyplexes with pDNA completely at C/A ratio of 3 and above (Figure 1A). $(RKRKRK)_4C$ showed the same result (data not shown). On the other hand, RKKR formed polyplex at C/A ratio of higher than 100 (Figure 1B). Mascotti et al. calculated the equilibrium binding constant between oligolysine with various lengths and pDNA by measuring the thermodynamic extent of counter ion release resulted from the polyplex formation.[9] The equilibrium binding constant rapidly increased with increasing oligolysine length. The thermodynamic results support the large difference in polyplex-forming ability of $(RKRKKR)_4C$ (25mer) and RKKR (4mer). These results indicated that Fur-oligopeptide would lose the polyplex-forming ability and release the pDNA when fragmented in cells, which is expected to lead to high gene expression.

Polyplexes must be positively charged for better entry into cells. We measured $\zeta$ potential of $(RKRKKR)_4C$ or RKKR polyplexes. $\zeta$ Potentials of $(RKRKKR)_4C$ polyplexes were around +20 mV at a C/A ratio of 3, while RKKR polyplexes showed negative $\zeta$ potential up to a C/A ratio of 100 (data not shown). The $\zeta$ potential of $(RKRKKR)_4C$ polyplexes is similar to that of PEI/pDNA polyplexes and seems to be enough for gene delivery.[4]

Transient expressions of luciferase gene transfected with Fur-oligopeptides were evaluated. Polyplex solutions were incubated with COS-1 cells for 5 h in the presence of 200 μM chloroquine. Supernatants were removed and replaced with DMEM containing 10% FBS and then cells were cultured for 43 h. Luciferase count per second (CPS) of cells were measured using a luminometer. Obtained luciferase activities were divided by total

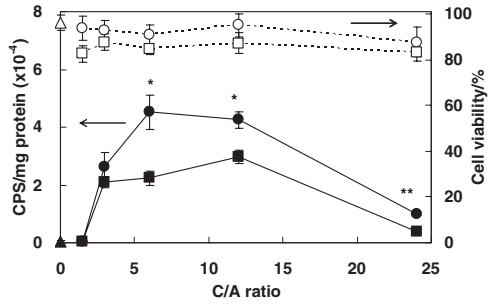


**Figure 2.** Transient luciferase expression (closed symbols) and cell viability (open symbols) in COS-1 cells transfected using $(RKRKKR)_4C$ (circles) and $(RKRKRK)_4C$ (squares) for 5 h in the presence of 200 μM chloroquine. (Triangles) represents the results for naked pCMV-Luc ($^*P < 0.01$, $^{**}P < 0.001$).

protein content of the cell lysates and expressed as CPS/mg protein (Figure 2). Cell viability was also assessed by the total protein in each cells. As is clearly shown, $(RKRKKR)_4C$ showed improved luciferase expression compared with a control $(RKRKRK)_4C$ with the same MW and amino acid composition. No cytotoxicity was observed in both cases. $(RKRKKR)_4C$ sequences in polyplexes is believed to be digested by intracellular furin, and this sequence-specific digestion brought about the polyplex destabilization.

We investigated the destabilization of Fur-oligopeptides/pDNA polyplexes in response to the digestion by furin in a cell-free system. Fur-oligopeptides were mixed with 200 ng pDNA in furin digestion buffer at C/A = 5 and incubated for 30 min. Five units of furin were added to 8 μL polyplex solutions and incubated for 1, 6, 12, and 24 h at 37 °C. Destabilization of polyplexes was evaluated by two experiments shown below. First, we perfomed anion-exchange assay with 0.5 equiv of potassium poly(vinyl sulfate) (PVS-K). When PVS-K was added to polyplex solutions, pDNA is replaced with PVS-K, and free pDNA is released. Reaction mixtures containing the free DNA were analyzed on EtBr-stained agarose gel (0.8%) electrophoresis. EtBr showed high fluorescence intensity by intercalating in free pDNA (Figure 3, Lane 1) but the intensity is completely suppressed when mixed with $(RKRKKR)_4C$/pDNA polyplexes because of their strong compaction (Figure 3, Lane 2). The fluorescence intensity was recovered by treating polyplexes with furin, and the intensity increased with the incubation time due to loosed compaction (Figure 3, Lane 3–6). Furthermore, release of free pDNA from digested polyplexes was observed, when the furin reaction time was longer than 12 h. Second, we evaluated the transription efficiency of the furin-treated polyplexes using an in vitro transcription/translation assay. Luciferase activity for $(RKRKKR)_4$/pT7-Luc polyplexes were increased to 30- and 100-fold after furin treatment for 1 and 6 h, respectively.[10] These results suggested that the enhanced gene expression shown in Figure 2 was resulted from the destabilization of highly compacted polyplex by intracellular furin digestion.

Katayama et al. reported polymeric carriers including substrates for protein kinase.[11] The substrate was phosphorylated by kinase, and then the charge of carriers decreased. As a result, the carriers lose polyplex-forming ability with pDNA, and GFP expression in NIH-3T3 cells was enhanced in response to the forskolin stimulation. However, since the positive charge density

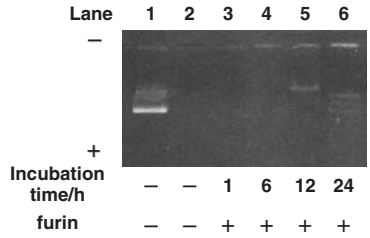


**Figure 3.** PVSK-induced disassembly of polyplexes treated with furin for 0, 1, 6, 12, and 24 h. Lane 1: free pCMV-Luc; lanes 2–6: $(RKRKKR)_4C$/pCMV-Luc polyplexes.

of this carrier is very low, the carrier cannot deliver pDNA into cells by itself and needs HVJ-E envelope. In contrast, $(RKRKKR)_4C$ can deliver pDNA into cells by itself and is fragmented to short peptides in response to intracellular furin. Other groups have reported biodegradable PEI derivatives.[12,13] These carriers might also facilitate intracellular release of pDNA from polyplexes, but remaining low MW PEI fragments are still non-biodegradable as is different from our Fur-oligopeptides.

In general, oligopeptide-type carriers have a high potential for useful gene carriers because various types of functional sequences such as receptor-binding sequences, NLS sequences, and digestive sequences can be combined. Increasing the MW of the carrier might be effective for pDNA delivery to cells[5] but higher MW carriers are reported to be more cytotoxic than the lower MW ones.[4] The optimum MW of Fur-oligopeptides are now studying.

In conclusion, Fur-oligopeptide has been newly developed as a gene carrier which is fragmented by intracellular furin, and the destabilized polyplexes are effectively transcibed in cells. This intracellular fragmentation of cationic carriers is a novel strategy for non-viral gene delivery.

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