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Creating a unique environment for selecting reactive enzymes with DNA: 'Sticky' binding of oligocation-grafted polymers to DNA

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ABSTRACT

To provide colloidally stable polyplexes formed between pDNA and cationic polymers, cationic polymers have been modified with hydrophilic polymers to form a hydrophilic shell. Block copolymers of cationic and hydrophilic polymers and cationic polymers grafted with hydrophilic polymers are representative designs of such polymers. Here, we report a new design of cationic polymers and oligocationic peptide-grafted polymers. We synthesized 15 kinds of graft copolymers by varying the number of cationic charges of the peptides and their grafting density. We found that graft copolymers with less cationic peptides and less grafting density formed colloidally stable polyplexes. Interestingly, the less cationic graft copolymers bind to excess amounts of pDNA. We also found that the graft copolymers showed selectivity toward reactive enzymes affording the reaction of pDNA with nucleases, while suppressing both the replication of DNA by DNA polymerase and gene expression. The suppression of the replication and expression is considered to result from the high capacity of the graft copolymers for binding with pDNA. The polynucleotides produced by DNA polymerase or RNA polymerase would be captured by the graft copolymers to impede these enzymatic reactions.

1. Introduction

In the field of gene therapy, synthetic cationic polymers have been extensively studied in an effort to replace conventional viral carriers to address safety issues. A variety of polycations including both biopolymers[1–6] and synthetic polymers[7–17] have been examined as nonviral carriers. Polyion complexes (PICs) formed by reacting the above identified polycations with plasmid DNA (pDNA) not only protects the pDNA from degradation by nucleases but also efficiently delivers the anionic pDNA into cells owing to their cationic surface charge. However, PICs usually form large aggregates under physiological ionic conditions, resulting in poor blood circulation.

To improve the colloidal stability of PICs under physiological conditions, diblock copolymers composed of a polycation and a hydrophilic polymer segment, typically polyethylene glycol (PEG), have been widely studied (Fig. 1a).[18–22] When these copolymers are mixed with pDNA, they form polymeric micelles with a PIC core surrounded by a hydrophilic polymer shell. The hydrophilic shell renders the polyplex colloidally stable and reduces its interaction with components within blood to prolong blood circulation. Another representative design of the polycations to improve stability is polycations with graft chains of hydrophilic polymers (Fig. 1b).[23–26] These graft copolymers also form a core-shell-like structure with improved stability. However, when the grafting density of the hydrophilic polymer is high, the polycationic backbone becomes stiff, resulting in a PIC with a unique structure, in which pDNA is extended along the polycationic backbone.[23]

As a new design of polycation for improving the stability of PICs, we have proposed a graft copolymer composed of a neutral main chain and oligocationic graft chains (Fig. 1c).[27–30] We have employed cationic peptides as the graft chains, which are the substrates of intracellular enzymes such as protein kinases[27–29] or proteases[30] specifically activated in disease cells. The enzymatic reaction of the grafted peptides reduces their cationic charge, thus weakening the polyplex to afford a gene expression. This system was successfully applied to cancer cell-specific gene expression.[28] Recently, several groups have reported similar oligocation-grafted hydrophilic polymers that showed good performance as gene carriers with high transfection ability and low cytotoxicity.[31–33]

Abbreviations: PLL, poly-l-lysine; DS, dextran sulfate; PICs, polyion complexes; C/A_{DNA}, charge ratios between cationic charges of the copolymers and anion charges of pDNA; A_{DS}, anion charges of dextran sulfate; A_{total}, total anion charges of pDNA and dextran sulfate.

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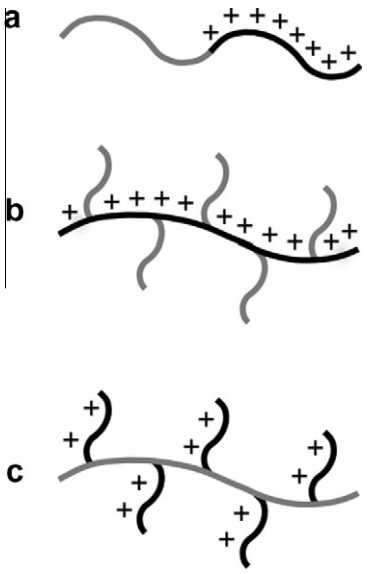


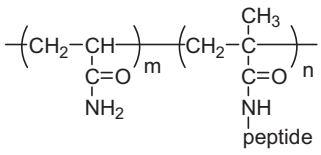
Figure 1. Structures of polycations with hydrophilic polymer segments: a diblock copolymer (a), a polycation grafted with hydrophilic polymers (b), and a hydrophilic polymer grafted with oligocations (c).

Despite the promising features of these new types of graft copolymer, the effects of molecular design on the physicochemical characteristics of the polyplexes and on their susceptibility to enzymes remain poorly understood. To fully characterize the graft copolymers, we synthesized 15 kinds of graft copolymer with various cationic net charges and sequences by varying the grafting density of the cationic peptides (Fig. 2). We investigated in detail the physicochemical characteristics of the polyplexes and the susceptibility of the polyplexes to enzymes including nucleases and polymerases. Consequently, we found that the graft copolymers exhibit quite unique features, that is, a selection of reactive enzymes due to a 'sticky' nature in DNA-binding.

2. Results and discussion

2.1. Synthesis of polymers

We designed five different peptides composed of glycine and L-arginine (Fig. 2) to examine the effect of net positive charge and the distribution of positive charge in the peptides on the physicochemical characters of the polyplexes. In our previous studies, a peptide with a net charge of +2 was found to form a polyplex.[29] Thus, here we designed peptides with positive charges of +2 or higher. The graft copolymers were obtained by a free radical copolymerization reaction between peptide monomers and acrylamide according to a procedure outlined in our previous report.[27] Three kinds of polymers with different feed molar ratios of the peptide monomers were prepared: **L** (0.5 mol %), **M** (1 mol %), and **H** (2 mol %). Molar ratios of the grafted peptides in the polymers are shown in Table 1.



Copolymer	Peptide	Net charge
1	-WGGGGG**RR**-NH_2	+2
2	-W**RRR**GGGG-NH_2	+3
3	-W**RR**GGGG**R**-NH_2	+3
4	-WG**R**G**R**G**R**G-NH_2	+3
5	-W**RRRRRRR**-NH_2	+7

Figure 2. Chemical structure of graft copolymers and their peptide sequences.

2.2. Colloidal stability of polyplex in PBS

We measured the ζ-potential of the polyplexes formed from the graft copolymers at various charge ratios between cationic charges of the copolymers and anion charges of pDNA (C/A_{DNA}) in phosphate buffered saline (PBS). Figure 3 shows results of the polymer **4** and **5** series which possess +3 and +7 charges, respectively. Poly-l-lysine (PLL; MW = $3 \times 10^4 - 7 \times 10^4$), which was used as a comparison, showed large negative and positive values depending on the C/A_{DNA} ratio. Polymer **5**-**H**, which has the highest grafting density of the most cationic peptide (+7), showed a relatively large positive ζ-potential, ca. +5 mV at C/A_{DNA} ratios of 2 and 4. However, the other polyplexes showed almost neutral values regardless of the C/A_{DNA} ratio. The polyplexes formed from polymer **1**–**3** series also showed small values (Fig. S1).

Then, we evaluated the colloidal stability of the polyplexes formed at a C/A_{DNA} ratio of 4 by monitoring the time course of the change in hydrodynamic diameter of the polyplexes (Fig. 4). In the case of **4**-**L** and **4**-**H**, the sizes of the polyplexes were about 100 nm and did not change during the long-time observation. Polymer **1**-**H**, **2**-**H**, and **3**-**H** also showed the colloidally stable characteristics (Fig. S2). The similarly stable characteristics was also observed in the case of **5**-**L**, while the polyplex of **5**-**H** showed a gradual increase in size, which is similar to the behavior of the polyplex of PLL. The stable characteristics of the polyplexes formed from the representative graft copolymers except for **5**-**H** may indicate that the polyplexes have a core-shell-like segregated structure in which the PIC core formed between pDNA and the grafted peptide is surrounded by the neutral polyacrylamide main chain. However, as observed in **5**-**H**, this weakly segregated structure seems to be lost for polyplexes with a high grafting density of the highly cationic peptide.

2.3. pDNA condensation ability

We evaluated the pDNA condensation ability of the polymer by using an ethidium exclusion assay (Fig. 5). For all kinds of

Table 1
Molecular parameters of graft copolymers

Copolymer	Peptide content		$M_w \times 10^{-4}$ [b]
	mol %[a]	wt %	
1-**L**	0.26	3.1	4.6
1-**M**	0.73	8.3	3.5
1-**H**	1.8	18	2.5
2-**L**	0.27	3.6	5.3
2-**M**	0.60	7.6	3.4
2-**H**	2.3	25	2.1
3-**L**	0.40	5.2	4.9
3-**M**	0.90	11	4.6
3-**H**	2.2	24	2.5
4-**L**	0.37	4.8	6.5
4-**M**	0.84	10	6.8
4-**H**	2.2	24	6.7
5-**L**	0.36	6.5	5.6
5-**M**	0.82	14	4.3
5-**H**	2.1	30	0.4

[a] Determined from the 280-nm absorption of the tryptophan residue.
[b] Determined by GPC in 0.5 M acetate buffer (pH 4.1, containing 0.1 M $NaNO_3$).

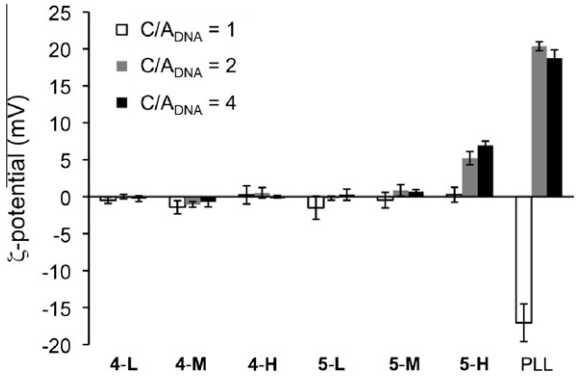


Figure 3. ζ-Potential of polyplexes in PBS. Values represent mean ± SD (n = 3).

polymers, the condensation ability became more pronounced with an increase in grafting ratio. Each polymer showed higher pDNA condensation ability than its corresponding peptide monomer. Though the peptide of the polymer **1** series has a small positive charge (+2), polymer **1**-**H** was able to induce ethidium exclusion owing to the multivalency of the interaction between a number of the grafted peptides on the polymer and pDNA. Among the examined polymers, the polymer **5** series showed the highest condensation ability, which is almost equivalent to PLL. When the condensation abilities of the polymers were compared at a fixed grafting ratio, the order of the condensation ability was found as follows: **1** <**3** <**4** ∼ **2** <<**5** ⩽ PLL. This order corresponded to the cationic net charge of each peptide. Notably, the charge distribution of the peptides affected the condensation ability; the polymer **3** series showed weaker condensation ability than the polymer **2** and **4** series, despite possessing the same +3 net charge.

The difference in the condensation ability between the polymer **4** and **5** series was significant. This charge dependency in the condensation ability will be important in an enzyme-responsive gene expression system that we have developed.[27–30] When the cationic charges of the grafted peptides are decreased by enzymatic reactions, the condensation ability of the graft copolymers was significantly decreased to activate the gene expression.

2.4. Capacity of graft copolymers in polyplex formation

We evaluated the capacity of the graft copolymers in the polyplex formation with pDNA by using gel retardation assay (Fig. 6). As observed in lanes 1, 6, and 11 (C/A$_{DNA}$ = 1, 2, and 4, respectively), polyplex formation of PLL did not completed even at a C/A$_{DNA}$ ratio = 2 (lane 6) because free pDNA was still detected. However, all three graft copolymers formed polyplex completely at a C/A$_{DNA}$ ratio = 1 (lane 1). These results clearly showed a higher capacity of the graft copolymers in DNA-binding than PLL.

Then we examined the stability of the polyplexes against the addition of dextran sulfate (DS). As mentioned above, an excess amount of PLL was needed to complete the polyplex formation (C/A$_{DNA}$ ratio = 4; lane 11). When DS was added to this polyplex, free pDNA started to appear from an A$_{DS}$/A$_{DNA}$ ratio = 2/1 (lane 13). Thus, the capacity of PLL for both kinds of polyanions is C/A$_{total}$ = 4/3, where A$_{total}$ = A$_{DNA}$ + A$_{DS}$. On the other hand, in the case of **4**-**L** at a C/A$_{DNA}$ ratio = 1, a 4-equivalent amount of DS was needed to generate free pDNA (A$_{DS}$/A$_{DNA}$ ratio = 4/1, i.e., C/A$_{total}$ ratio = 1/5; lane 4). This result again showed that the graft copolymer **4**-**L** can bind to an excess amount of polyanions (at least a 3-equivalent amount in this case).

This excess binding capacity was also observed in **4**-**H**, but its binding capacity was less than **4**-**L**; the critical C/A$_{total}$ ratio at which free pDNA started to appear was 1/3 (A$_{DS}$/A$_{DNA}$ ratio = 2/1;

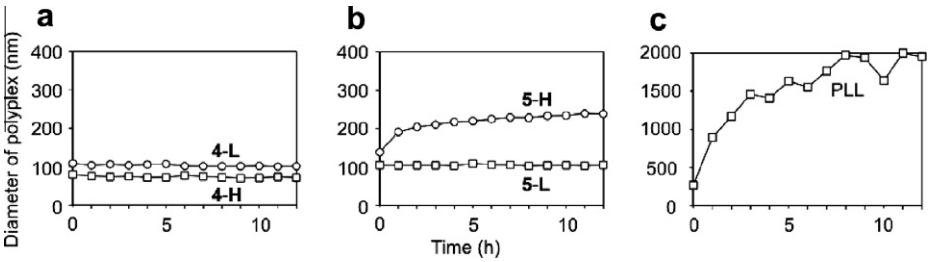


Figure 4. Time courses of changes in hydrodynamic diameter of polyplex in PBS. Each polyplex was formed at a C/A ratio of 4.

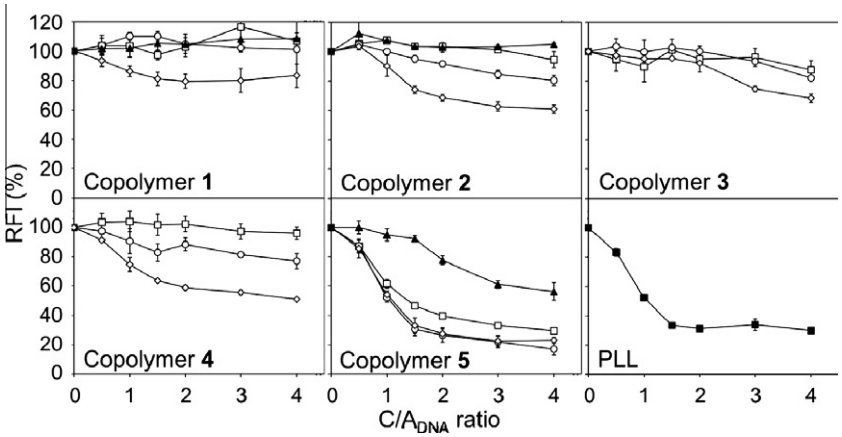


Figure 5. pDNA condensation ability of graft copolymers in PBS. □: **L**, ○: **M**, ◇: **H**, ▲: peptide monomer. Values represent mean ± SD (n = 3).

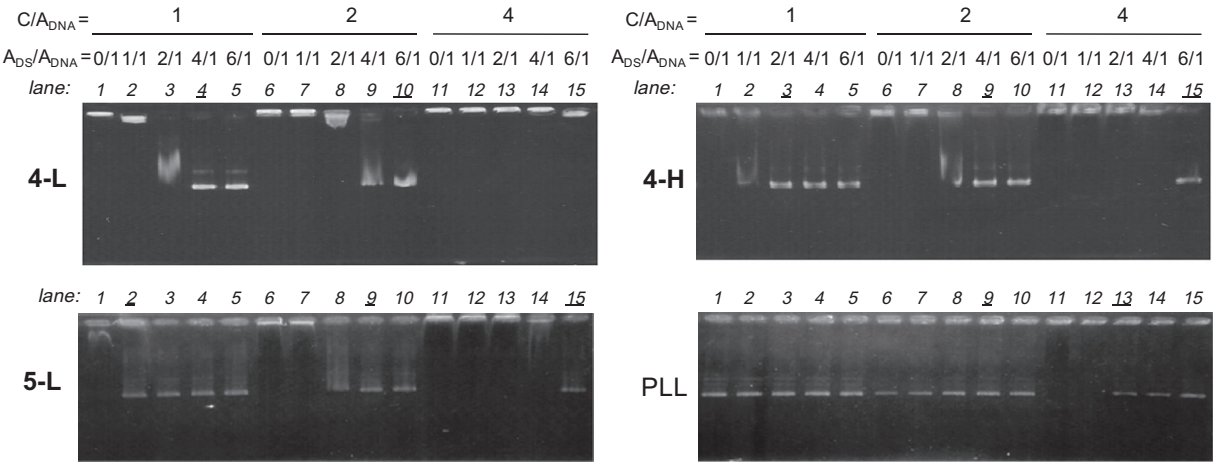


Figure 6. Capacity of graft copolymers for binding with polyanions (pDNA and DS). The underlined lane numbers show the critical C/A_{total} ratios at which free pDNA started to appear. $A_{total} = A_{DNA} + A_{DS}$.

lane 3). The binding capacity of **5**-**L** was even lower; the critical C/A_{total} ratio was 1/2 (A_{DS}/A_{DNA} ratio = 1/1; lane 2). These results indicated that the binding capacity of the graft copolymers increases with a decrease in both the grafting density and the cationic charge of the peptides.

The critical C/A_{total} ratios of all **L**-series polymers are summarized in Table 2 (Fig. S1 for the result of the gel electrophoresis analysis). The binding capacity of the polymers was roughly proportional to the decreasing cationic net charge of the grafted peptides. The excess capacity of the graft copolymers should be ascribed to their unique structure. Figure 7 schematically shows the difference in polyplex structures between normal polycations and the graft copolymers. Normal polycations including PLL forms tight polyplex due to the high cationic charge density along the chain.[34–37] In the graft copolymers, however, each cationic peptide is connected by the long hydrophilic polymer main chain, so that the cationic peptides are spread in a wide volume owing to the exclusion volume of the main chain. Because of the exclusion volume, the graft copolymer will not adopt a compact globular conformation to completely neutralize the anionic charge of pDNA. The remaining free cationic peptides can bind to other pDNA, resulting in the excess binding capacity of the graft copolymers.

Table 2
Critical C/A_{total} of each graft copolymer

Copolymer	C/A_{DNA}	Critical C/A_{total}[a]
	1	1/5
1-**L**	2	2/7
	4	4/>7
	1	1/5
2-**L**	2	2/7
	4	4/>7
	1	1/3
3-**L**	2	2/5
	4	4/7
	1	1/5
4-**L**	2	2/5
	4	4/>7
	1	1/2
5-**L**	2	2/3
	4	4/7
	1	1/<1
PLL	2	2/<1
	4	4/3

[a] A C/A_{total} ratio at which free pDNA started to appear after addition of DS to the polyplex formed at each C/A_{DNA} ratio.

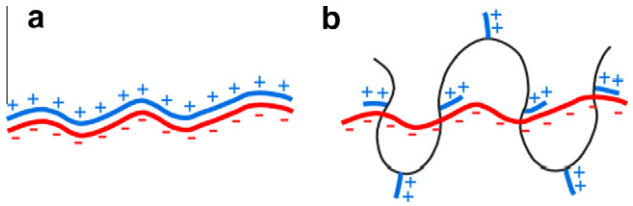


Figure 7. Difference in the polyplex structure of (a) usual polycations and (b) graft copolymers.

It is noteworthy that smearing of the pDNA bands was observed for the graft copolymer before the critical C/A_{total} ratios (e.g., lanes 2, 3, 8, and 9 in **4**-**L**, and lanes 2 and 8 in **4**-**H**). To our knowledge, such smearing is unusual for normal polycations, including PLL. The reason for the smearing is considered to be because of a release of pDNA from the polyplex during electrophoresis. This 'sticky' nature of the binding between the copolymers and pDNA should be distinguished from apparently irreversible binding observed in usual polycations.[38] Recently, Harada et al. reported a similar smearing of pDNA that was complexed with a PLL having multi PEG-arms.[39] A common feature between their polymers and our graft copolymers is the presence of bulky hydrophilic polymer segments. These bulky segments would prevent the formation of a tight polyplex.

2.5. Susceptibility of polyplex to nucleases

We evaluated the susceptibility of the polyplexes to endonuclease DNase I, which degrades pDNA in a non-sequence-specific manner. The polyplex of each graft copolymer was formed at an excess C/A_{DNA} ratio of 4. The degradation of pDNA was monitored by the increase in UV absorption at 260 nm. As shown in Figure 8a, the degradation of pDNA was strongly suppressed by the complexation with PLL. In the case of the polymer **5** series, the degradation was suppressed as strongly as PLL, regardless of the peptide grafting ratios. However, the polyplexes of the polymer **4** series resulted in degradation even at the highest grafting ratio, **4**-**H**.

Then, we evaluated the susceptibility of the polyplexes to another type of nuclease, restriction enzymes. The polyplexes were formed at various C/A_{DNA} ratios, and then two restriction enzymes (*Xba*I and *Hind*III) were added. After 2 h, a large excess amount of polyanion (DS) was added to dissociate the polyplex, and then the solution was subjected to a gel electrophoresis analysis

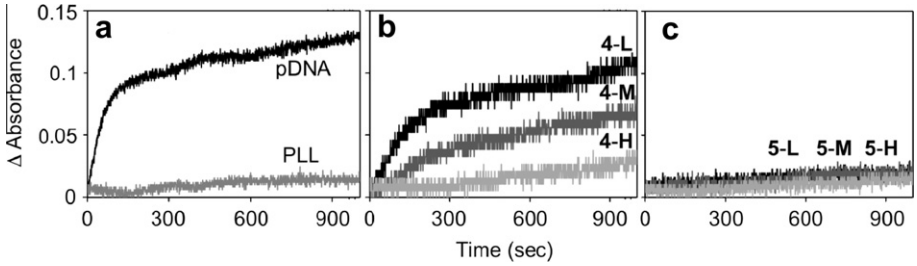


Figure 8. Susceptibility of polyplexes to DNase I monitored by an increase in UV absorption.

(Fig. 9). As observed in lane 4, the two enzymes produced two kinds of fragments, 5.4 (F1) and 1.7 kbp (F2) from the free pDNA. In the case of the polymer 5 series, however, only linear and open circular DNA were detected, indicating that the protection from the enzymatic cleavage was observed through the polyplex formation. However, in the case of the polymer **4** series, the protection from the enzymatic cleavage was only observed in **4**-**H** at high C/A_{DNA} ratios.

Therefore, the susceptibility of the polyplexes to the examined two types of enzymes shows a similar trend, that is, the polyplex becomes more susceptible to these enzymes with decreases both in the cationic net charge of the peptides and grafting density of the peptides. The molecular mechanisms of the digestion by the two types of enzymes were different; DNase I cleaves pDNA in a non-sequence-specific manner, while the restriction enzymes are sequence-specific enzymes that slide along pDNA to find a target sequence.[40] Thus, the polyplexes formed between the graft copolymers and pDNA is not so tight and even affords the sliding motion of the restriction enzymes along pDNA.

2.6. Susceptibility of polyplex to DNA polymerase

We then evaluated the susceptibility of the polyplexes to a DNA amplification reaction catalyzed by DNA polymerase by using a real-time PCR system.[41] Figure 10 shows DNA amplification curves for the polyplexes formed at varying C/A_{DNA} ratios (1, 2, and 4). Free pDNA showed half-maximal cycle numbers of 10. PLL completely suppressed the amplification reaction when the C/A_{DNA} ratio exceeded 2, while it showed almost no suppression at a C/A_{DNA} ratio of 1, indicating the presence of a substantial amount of free pDNA. This result corresponds to the low binding capacity of PLL estimated in Figure 6.

We compared three polymers of the **1**, **3**, and **5** series with different peptide charges. The better suppression was observed in the polymers of the **1** and **5** series than the **3** series, which have a positive charge midway between the charges of the polymers of the **1** and **5** series. The other polymer series with +3 peptide charges (**2** and **4** series) similarly show the weaker suppression than the **1** and **5** series (Fig. S4).

To explain the weakest suppression of the **3** series, we have assumed following two determining factors of the PCR suppression, which has an opposite dependence on the positive charges of the peptides. The reduced suppression ability from the polymers of the **5** and **3** series should be determined by the increasing accessibility of DNA polymerase to pDNA with decreasing peptide charge owing to the looser polyplex formation. On the contrary, the improved suppression from the polymers of the **3** to **1** series seems to be governed by the higher DNA-binding capacity of the polymers of the **1** series, as observed in Table 2. The polymers of the **1** series would interact with more newly synthesized DNA strands, resulting in more efficient suppression of the amplification reaction.

2.7. Cell-free gene expression from polyplex

To examine the effect of the unique structure of the polyplex on gene expression processes, we used cell-free gene expression system. As shown in Figure 11, PLL suppressed the gene expression to 52% and 15% from the expression of free pDNA at C/A_{DNA} ratios of 2 and 4, respectively. Although **4**-**H** showed the least ability in both the condensation ability (Fig. 5) and DNase I resistance (Fig. 8) than PLL, the suppression was more pronounced in **4**-**H**; 33% and 4% at C/A_{DNA} ratios of 2 and 4, respectively. The similar high suppression of gene expression was also seen in **2**-**H** and

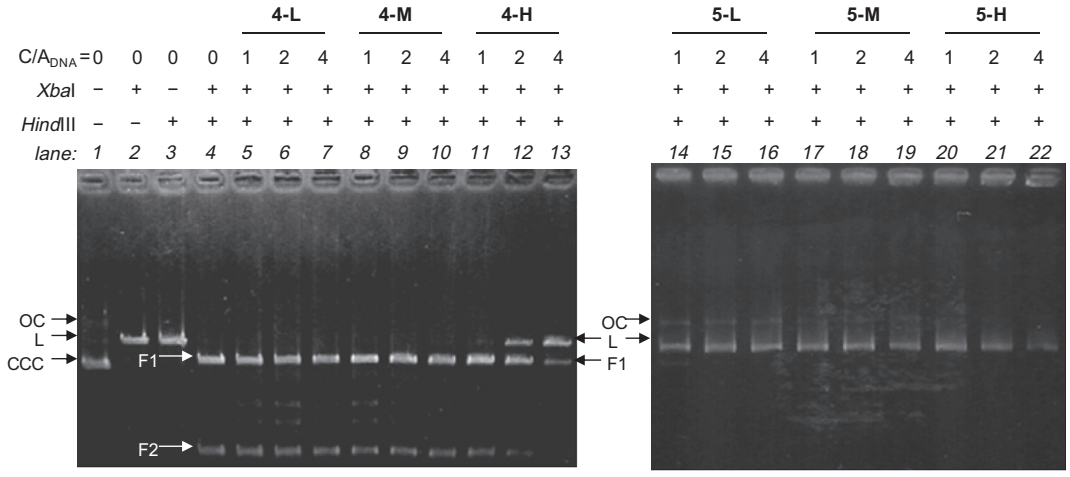


Figure 9. Susceptibility of polyplex to restriction enzymes: (*Xba*I and *Hind*III). OC (open circular), L (linear), CCC (super coiled), F1 and F2 (digested fragments).

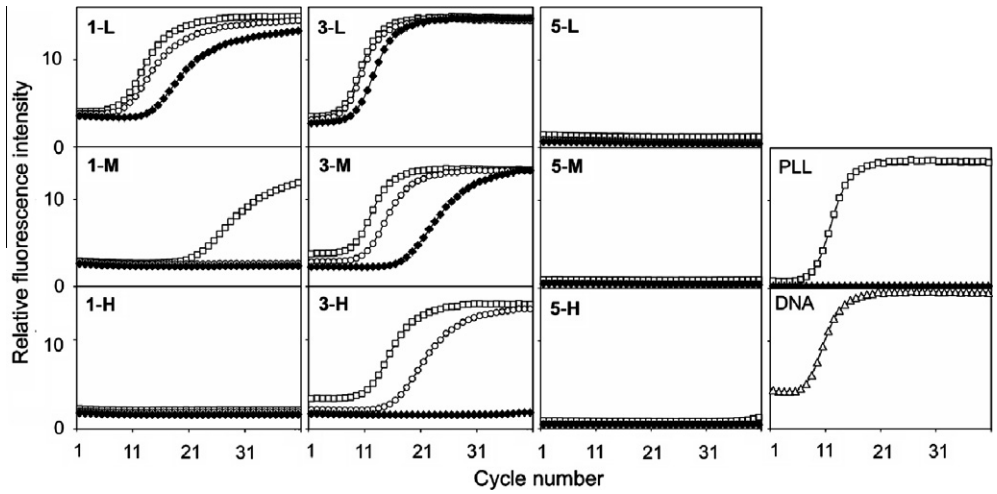


Figure 10. Susceptibility of polyplex to DNA polymerase monitored by real-time PCR system. Polyplexes were formed at a C/A_{DNA} ratio = 1 (open squares), 2 (open circles), and 4 (closed diamonds), respectively.

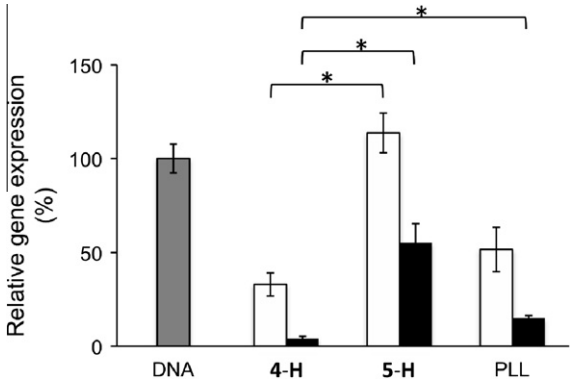


Figure 11. Efficiency of gene expression from polyplexes in a cell-free gene expression system. Polyplexes were formed at a C/A_{DNA} ratio = 2 (open bar) and 4 (closed bar), respectively. $^{*}P$ <0.05.

3-**H** (Fig. S5). The effective suppression of these polymers, **2-H**, **3**-**H**, and **4-H** is probably attributable to their larger binding capacity with polyanions; they would interact with transcribed mRNA strands to suppress the gene expression. This gene suppression depending on the larger capacity would be further supported by the weaker suppression of **5**-**H** than these three polymers because **5**-**H** has a lower capacity than they do.

Because **5-H** has a lower molecular weight than the other polymers (Table 1), there was a concern that the low suppression of **5-H** may result from its low molecular weight. However, as shown in Figure S5, **5-L,** which has a similar molecular weight with the other polymers, shows weaker suppression than the other polymers. As we discussed in Table 2. **5**-**L** has a lower binding capacity than the other polymers. Thus, the low gene suppression both in **5**-**L** and **5**-**H** would result from their low binding capacities.

These gene expression results corresponded to those of the DNA amplification reaction at which the larger capacity of the polymer **1** series suppressed the amplification reaction more than the polymer **3** series (Fig. 10). The excess binding capacity of the graft copolymers resulting from their 'sticky' nature is a key for the suppression in these two reactions because they are accompanied by the production of the polynucleotides strands. The unique binding mode of the graft copolymers enables the enzyme-responsive gene expression system, which we have previously reported.[27] In this system, the graft copolymers should satisfy two conditions: suppressing gene expression while affording enzymatic reactions to grafted peptides.

3. Conclusions

The density of the cationic charge of a grafted peptide and the grafting density of the peptide determined the structure of the corresponding polyplex. When both cationic net charge and grafting density are high, the graft copolymers condensed pDNA as tightly as PLL, resulting in the formation of unstable polyplexes. However, the graft copolymers with less cationic charge and less grafting density formed colloidally stable polyplexes with loosely condensed pDNA. The graft copolymers bind to an excess amount of pDNA owing to the prevention of the tighter condensation by the bulky neutral main chain. The binding of the graft copolymers to pDNA is not a strong binding but has a 'sticky' nature that can be dissociated by gel electrophoresis. This unique binding mode of the graft copolymers to pDNA creates an environment that selects the reactive enzymes; the graft copolymers afforded the nucleases reactions, while it suppressed DNA amplification and gene expression. The results obtained here will serve as a basis to design an enzyme-responsive gene expression system for a next-generation gene carrier.

4. Experimental

4.1. Synthesis of peptides

Peptides were synthesized according to standard Fmoc-chemistry procedures. The obtained crude peptides were purified by reverse-phase liquid chromatography (dC_{18} OBD™ 5 μm 19 × 100 mm Column) using a linear gradient at a flow rate of 3.0 mL/min with an acetonitrile/water mobile phase containing 0.1% trifluoroacetic acid. The purified peptides were then lyophilized to obtain a white powder. The obtained peptides were identified by MALDI TOF-MS (PerSeptive Biosystems).

4.2. Synthesis of graft copolymers

The copolymers were prepared according to procedures outlined in our previous report.[27] The molar ratios of the grafted peptides in the polymers were determined from the 280 nm absorption of the L-tryptophan residue. The molecular weights of the copolymers were determined by gel permeation chromatography (GPC). GPC

analysis was performed on a Shimadzu GPC system with two columns (TSKgel G5000PW$_{XL}$, G6000PW$_{XL}$) at room temperature. Acetate buffer in the amount of 0.5 M (pH 4.1, containing 0.1 M $NaNO_3$) (elution rate, 0.45 mL/min) and polyethylene oxides were used as an eluent and standards, respectively.

4.3. Plasmid DNA

A plasmid DNA, pCMV-luc, containing a firefly luciferase cDNA driven by a CMV promoter was prepared following a procedure outlined in our previous report.[42] In a cell-free protein expression experiment, a plasmid DNA, pT7-luc (Promega), containing a firefly luciferase cDNA driven by a T7 promoter was used.

4.4. Measurements of ζ-potential and hydrodynamic diameter

To prepare polyplexes, 500 μL of pCMV-luc solution (0.005 μg/μL in PBS) was mixed with 500 μL of the copolymer solution (PBS) to adjust the C/A$_{DNA}$ ratios (the final concentration of pDNA = 2.5 μg/mL). Then the solution was incubated for 2 h at room temperature. After incubation, the ζ-potential and hydrodynamic diameter of the polyplexes were determined by using a Zetasizer (Nano-ZS-K, Malvern Instruments, UK) equipped with a He/Ne laser at a detection angle of 173° and a temperature of 25 °C.

4.5. Ethidium bromide exclusion assay

To prepare polyplexes, 1.25 μL of ethidium bromide solution (0.1 μg/μL in H_2O) was added to 5 μL of pCMV-luc solution (0.1 μg/μL in H_2O), and the solution was incubated for 15 min at room temperature. The volume was adjusted to 50 μL with PBS. Then, 50 μL of the copolymer solution (PBS) was mixed with the CMV-luc plasmid solutions to adjust the C/A$_{DNA}$ ratios (the final concentration of pDNA = 5.0 μg/mL). The polyplexes were then incubated for 2 h at room temperature. After incubation, the fluorescence intensity of the polyplex solutions at 590 nm (excitation at 531 nm) was measured at 37 °C using a spectrofluorometer (ARVO SX, PerkinElmer, Waltham, MA, USA). The relative fluorescence intensity (RFI) was calculated as RFI = $(F_{sample}-F_0)/(F_{100}-F_0) \times 100$, where F_{sample}, F_{100}, and F_0 represent the fluorescence intensity of the polyplex samples, free pDNA without polycations, and background, respectively.

4.6. Binding capacity of graft copolymer

To prepare polyplexes, 5 μL of pCMV-luc solution (0.01 μg/μL in PBS) was mixed with 5 μL of the copolymer solution (PBS) to adjust the C/A$_{DNA}$ ratios (the final concentration of pDNA = 5.0 μg/mL). The polyplexes were then incubated for 2 h at room temperature. After incubation, 0.5 μL of DS solution was added to the polyplex solutions to adjust the A$_{DS}$/A$_{DNA}$ ratios (the final concentration of pDNA = 5.0 μg/mL). The mixed solutions were then incubated for 15 min at room temperature. Then, each mixed solution was subjected to electrophoresis at 100 V for 30 min on a 1.0 wt % agarose gel with a running buffer of 40 mM Tris-acetate buffer containing 1.0 mM EDTA. The migrated pDNA was visualized by soaking the gel in distilled water containing Sybar Gold (Molecular Probes, Leiden, Netherlands).

4.7. Resistance of polyplex to endonuclease

To prepare polyplexes, 50 μL of pCMV-luc solution (0.05 μg/μL of H_2O) was mixed with 50 μL of the copolymer solution (H_2O) to adjust the C/A$_{DNA}$ ratio to 4 (the final concentration of pDNA = 5.0 μg/mL). Then, 90 μL of the polyplex solution and 10 μL of reaction buffer of 400 mM Tris–HCl (pH 7.5) containing 80 mM $MgCl_2$ and 50 mM DTT, were mixed, and incubated for 2 h at room temperature. Then, 1 μL of DNase I solution (1 U/μL) was added to the polyplex solutions. The absorbance of the polyplex solutions at 260 nm was monitored at 37 °C using a UV/Vis spectrometer (UV-2550, SHIMADZU, Japan).

4.8. Resistance of polyplex to restriction enzymes

To prepare polyplexes, 4 μL of pCMV-luc solution (0.01 μg/μL in H_2O) was mixed with 4 μL of the polymer solution (H_2O) to adjust the C/A$_{DNA}$ ratios (the final concentration of pDNA = 5.0 μg/mL). Then, 1 μL of reaction buffer containing 50 mM NaCl, 10 mM Tris–HCl, 10 mM $MgCl_2$ and 1 mM DTT (pH 7.9) was added to the polyplex solutions. The polyplexes were then incubated for 2 h at room temperature. After incubation, 0.5 μL (10 U) of *Xba*I and *Hin*dIII were added to the polyplex solutions, and the copolymer solutions were incubated for 2 h at 37 °C. Then, an excess amount of DS was added to the polyplex solutions, which were then incubated for 15 min at room temperature. After incubation, each mixed solution was subjected to electrophoresis.

4.9. DNA amplification assay

To prepare polyplexes for DNA amplification experiments by using a real-time PCR system, 2 μL of pCMV-luc solution (0.01 μg/μL in H_2O) was mixed with 2 μL of the copolymer solution (H_2O) to adjust the C/A$_{DNA}$ ratios (the final concentration of pDNA = 5.0 μg/mL). Then, the polyplex solutions were diluted with 4 μL of sterile water. The polyplexes were then incubated for 2 h at room temperature. After incubation, 1 μL of primer solutions (5′-AAGATGGAACCGCTGAGA-3′ and 5′-GCAGCCAACCGTCTTC-3′, 4 mM each) and 10 μL of SYBR Premix Ex Taq™ II (TaKaRa, Japan) were added to the polyplex solutions. Then, PCR was performed on pCMV-luc for 40 cycles of 95 °C for 5 s, 55 °C for 10 s, and 72 °C for 10 s, and DNA amplification curves were plotted.

4.10. Cell-free protein expression experiment

To prepare polyplexes, 5 μL of T7-luc plasmid solution (0.06 μg/μL in H_2O) was mixed with 5 μL of the copolymer solution (H_2O) to adjust the C/A$_{DNA}$ ratios (the final concentration of pDNA = 30 μg/mL). The polyplexes were then incubated for 2 h at room temperature. After that, 0.5 μL of 1 mM methionine and 20 μL of TNT Mix solution containing reticulocyte lysate (Promega) were added to the polyplex solutions. Then, the mixture was incubated for 30 min at 30 °C. Next, 100 μL of luciferin substrate was added to the polyplex solutions, and chemiluminescence was measured using a luminometer (MiniLumat LB 9506, EG & G Berthold, Germany).

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Supplementary data

Supplementary data (capacity of graft copolymers for binding with polyanions (pDNA and DS)) associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2011.12.025.

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