

Block Cationic Polyplexes with Regulated Densities of Charge and Disulfide Cross-Linking Directed To Enhance Gene Expression

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Abstract: A block cationic polyplex, showing a high stability in the extracellular medium and an efficient release of plasmid DNA (pDNA) in the intracellular compartment, was developed by controlling both the cationic charge and disulfide cross-linking densities of the backbone polycations. Poly(ethylene glycol)-poly(L-lysine) block copolymer (PEG-PLL) was thiolated using either of two thiolation reagents, *N*-succinimidyl 3-(2-pyridyldithio)propionate (SPDP) or 2-iminothiolane (Traut's reagent), to investigate the effects of both the charge and disulfide cross-linking densities on the properties of the polyplexes. The introduction of thiol groups by SPDP proceeded through the formation of amide linkages to concomitantly decrease the cationic charge density of PLL segment, whereas Traut's reagent promoted the thiolation with the introduction of cationic imino groups to keep the charge density constant. These thiolated PEG-PLLs were complexed with pDNA to form the disulfide cross-linked block cationic polyplexes, which had the size of approximately 100 nm. Both thiolation methods were similarly effective in introducing disulfide cross-links to prevent the polyplex from the dissociation through a counter polyanion exchange in the extracellular oxidative condition. On the other hand, the efficient release of pDNA responding to the reductive condition mimicking the intracellular environment was only achieved for the polyplex thiolated with SPDP, a system compensating for the decrease in the charge density with the disulfide cross-linking. This distinctive sensitivity toward oxidative and reductive environments was nicely correlated with the remarkable difference in the transfection efficiency between these two types of thiolated polyplexes (SPDP and Traut's reagent types): the former revealed approximately 50 times higher transfection efficiency toward 293T cells than the latter. Obviously, the balance between the densities of the cationic charge and disulfide cross-linking in the thiolated polyplex played a crucial role in the delivery and controlled release of entrapped pDNA into the microenvironment of intracellular compartment to achieve the high transfection efficiency.

Introduction

Despite the current enormous interest in cationic polymer-based gene delivery systems (polyplexes), the rationale to design efficient systems has not yet been obtained. Especially, in vivo gene delivery systems through a systemic route demand a high stability during blood circulation and an efficient gene expression at the target site. Cationic block copolymers with hydrophilic segments, such as poly(ethylene glycol) (PEG), effectively induce a condensation of plasmid DNA (pDNA) upon complexation to form core-shell-type polyplexes with the hydrophilic outer layer surrounding the polyion complex (PIC) core.¹⁻⁵ This provides the increased colloidal stability to the polyplexes in a proteinaceous medium, allowing the substantial increase in their blood circulation time.³ Nevertheless, there is still a possibility

of the block cationic polyplexes to unfavorably dissociate before entering the cytoplasm of target cells through the interaction with negatively charged biomacromolecules in the entity.^{6,7} Thus, a strategy is needed to keep the polyplex structure stable in the extracytoplasmic environment, while inducing the efficient release of entrapped pDNA from the polyplexes after their movement into the cytoplasmic compartment.

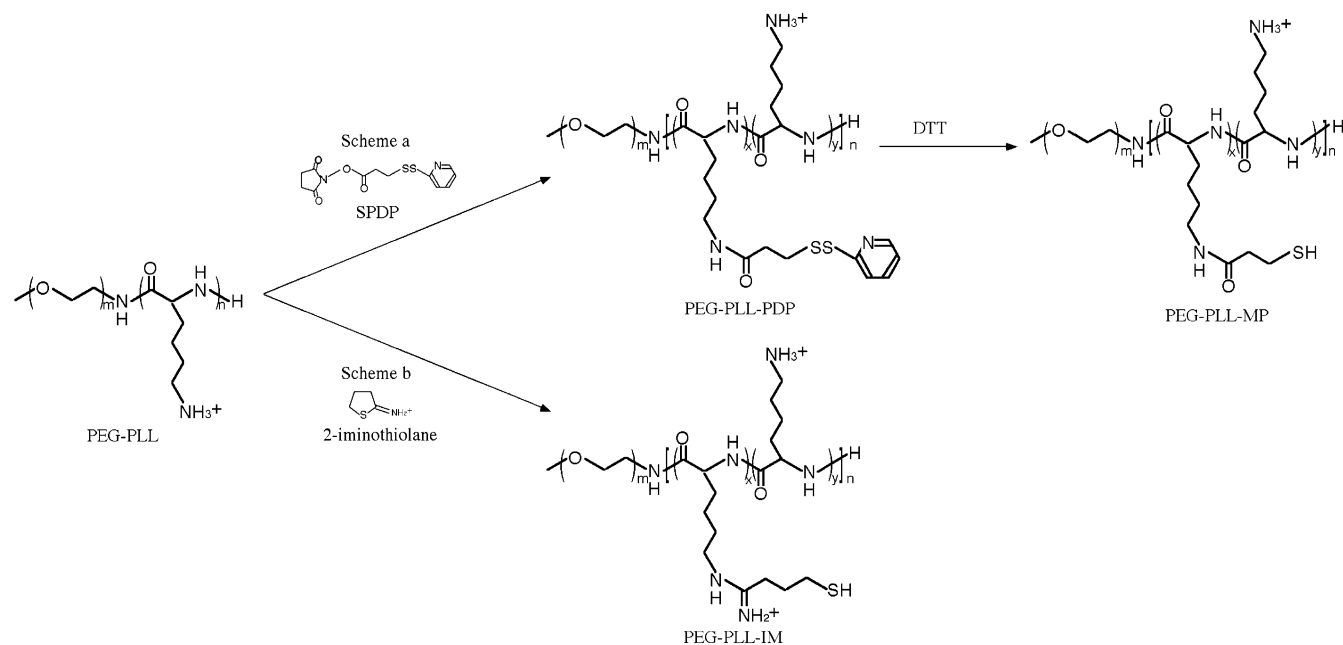
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Scheme 1. Thiolation Schemes of PEG-PLL



A promising approach to realize this strategy is to reversibly cross-link the PIC core of the block cationer polyplex with disulfide bonds cleavable in intracellular reductive environments, where the glutathione concentration is ~ 50 – 1000 times higher than that in extracellular milieu.⁸ Indeed, the utility of this strategy was demonstrated with the PIC of model polyanions,⁹ oligo-DNA,¹⁰ and pDNA,^{7,11,12} showing that the disulfide cross-linking substantially stabilized polyplexes to achieve longevity in the blood circulation. However, the introduction of disulfide cross-links eventually led to the decreased transfection efficiency because of the overstabilization of the complex structure to hinder pDNA release in the intracellular compartment.¹¹ In this regard, the balance between cationic charge and disulfide cross-linking densities of the polyplex should be crucial for the disulfide cross-linked polyplexes achieving the environment-sensitive release of entrapped pDNA in the cytoplasm while maintaining its high stability in an extracellular environment. Nevertheless, previous works regarding the disulfide cross-linking of the polyplexes have not clarified this important issue, directing us to the present study devoting to design the polyplex system with regulated densities of charge and disulfide cross-linking to achieve the enhanced gene expression.

Here the charge and disulfide cross-linking densities of block cationer polyplexes from PEG-poly(L-lysine) block copolymer (PEG-PLL) were regulated by the use of two types of thiolation reagents: *N*-succinimidyl 3-(2-pyridyldithio)propionate (SPDP)¹³ and 2-iminothiolane (Traut's reagent).¹⁴ Thiolation using SPDP proceeds by substituting amide groups for the

ϵ -amino groups of the lysine residue, resulting in the decreased charge density compensated by the introduction of 3-(2-pyridyldithio)propionyl groups, while the charge density of the PLL segments remained unchanged even after thiolation by Traut's reagent, because the reaction was accompanied by the introduction of cationic imino groups. Consequently, the former system, balancing the densities of charge and disulfide cross-linking, achieved for the first time a remarkable increase in the transfection efficiency compared to non-cross-linked control while maintaining its stability in the extracellular environment.

Experimental Section.

Materials. α -Methoxy- ω -amino-poly(ethylene glycol) ($M_w = 12\,000$) was a kind gift from Nippon Oil and Fats Co., Ltd. (Tokyo, Japan). The PEG-PLL block copolymers with an average polymerization degree of PLL segment of 71 were synthesized as previously described.⁴ *N*-Succinimidyl 3-(2-pyridyldithio)propionate (SPDP), dithiothreitol (DTT), and sodium dextran sulfate ($M_w = 25\,000$) were purchased from Wako Pure Chemical Co., Ltd. (Osaka, Japan). 2-Iminothiolane (Traut's reagent) and Micro BCA protein assay reagent kit were purchased from PIERCE Co., Inc. (Rockford, IL). *N*-Methyl-2-pyrrolidone (NMP) was purchased from Aldrich Co., Inc. (Milwaukee, WI). A pGL3 control vector, which was purchased from Promega Co., Inc. (Madison, WI), was used as pDNA in all the experiments. This pDNA was amplified in competent DH5 α *Escherichia coli* and purified using HiSpeed Plasmid MaxiKit purchased from QIAGEN Sciences Co., Inc. (Germantown, MD).

Synthesis of Thiolated PEG-PLL. Introduction of thiol groups to the side chain of PEG-PLL was performed using either a hetero-bifunctional reagent SPDP¹³ (Scheme 1a) or a cyclic thioimide Traut's reagent¹⁴ (Scheme 1b). The typical synthetic procedure is described as follows for the PEG-PLL-PDP (37 mol % substitution): PEG-PLL (51 mg, 1.90 μ mol) and SPDP (60 mg, 192 μ mol) were separately dissolved in NMP containing 5 wt % LiCl (2 mL for PEG-PLL, 6 mL for SPDP). The solution containing SPDP (2.5 mL) was added to PEG-PLL solution (2 mL) and stirred at room temperature for 24 h after the addition of 10% volume of *N,N*-diisopropylethylamine. Then, the mixture was precipitated into an approximately 20-times-excess volume of diethyl ether. The crude precipitate was washed twice with diethyl ether to

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obtain white powder. The polymer was dissolved in 0.01 N HCl solution, dialyzed against distilled water overnight, and lyophilized to obtain the final product. The reaction of PEG-PLL with Traut's reagent was carried out in a similar way, e.g., Traut's reagent (20 mg, 145 μmol) in NMP (2 mL) was added to PEG-PLL (29 mg, 1.10 μmol) in NMP (2 mL) to obtain the PEG-PLL-IM (22 mol % substitution). Thiolated PEG-PLLs with different thiolation degrees were obtained by changing molar ratio of SPDP or Traut's reagent to the ϵ -amino groups of PEG-PLL according to the aforementioned method. The degree of the thiol substitution for each thiolated PEG-PLL was determined from the peak intensity ratio of the methylene protons of PEG (OCH_2CH_2 , $\delta = 3.5$ ppm) to the pyridyl protons of the 3-(2-pyridyldithio)propionyl groups ($\text{C}_5\text{H}_4\text{N}$, $\delta = 7.2$ – 8.3 ppm) or the newly introduced methylene protons of 1-imino-4-mercaptobutyl groups ($\text{HS}-(\text{CH}_2)_3-\text{C}(\text{NH}_2)^+$, $\delta = 2.1$ – 3.4 ppm) in ^1H NMR spectra taken in D_2O at 25 $^\circ\text{C}$.

Preparation of the Cross-Linked Block Cationer Polyplex. Each thiolated PEG-PLL was dissolved in 10 mM Tris-HCl buffer (pH 7.4) at 1–2 mg/mL, followed by the addition of 10% volume of 100 mM DTT solution. After a 30 min incubation at room temperature, the polymer solutions were diluted up to the residual molar concentration of the cationic moieties (primary amino and imino groups) to equal 0.615 $\mu\text{mol}/\text{mL}$ by the same buffer. Then, the polymer solutions were added to a 2-times-excess volume of 50 $\mu\text{g}/\text{mL}$ pDNA solution (0.154 $\mu\text{mol}/\text{mL}$ phosphate groups) to form the polyplex at a charge ratio of 2. The final concentration of pDNA in all the samples was adjusted to 33 $\mu\text{g}/\text{mL}$. Charge ratio was defined as the residual molar ratio of positive charge of PEG-PLL or thiolated PEG-PLL to phosphate group of pDNA. After an overnight incubation at room temperature, the polyplex solutions were dialyzed against 10 mM Tris-HCl (pH 7.4) containing 0.5% dimethyl sulfoxide at 37 $^\circ\text{C}$ for 24 h to remove the impurities, followed by 2 days of additional dialysis for removal of dimethyl sulfoxide. During the dialysis, the thiol groups of thiolated PEG-PLL were oxidized to form the disulfide cross-links. To follow the process of oxidation, the remaining thiol groups in disulfide cross-linked polyplexes were determined by Ellman's method.¹⁵

Size of Cross-Linked Block Cationer Polyplex. To estimate the hydrodynamic diameters of cross-linked block cationer polyplexes, dynamic light scattering (DLS) measurements were carried out using a DLS-7000 instrument (Otuka Electronics Co, Ltd., Hirakata, Japan). An Ar ion laser ($\lambda = 488$ nm) was used as an incident beam. Block cationer polyplex solutions with a charge ratio of 2 were adjusted to have a concentration of 33 μg pDNA/mL. The data obtained at a detection angle of 90 $^\circ$ at 25 $^\circ\text{C}$ were analyzed by the cumulant method to obtain the hydrodynamic diameters and polydispersity indices (μ/Γ^2) of the polyplexes.¹⁶

For AFM imaging of DNA complexes, 4 μL of each sample solution was deposited on a freshly cleaved mica substrate for 30 s. The solution was dried under a gentle flow of nitrogen gas. AFM imaging was performed in tapping mode with standard silicon probes (160 μm in length, Olympus, Tokyo, Japan) on a NVB100 microscope (Olympus) controlled by an operating software of Nanoscope IIIa (Digital Instruments, Santa Barbara, CA). Cantilever oscillation frequency was tuned to the resonance frequency of the cantilever, 260–340 kHz. The 256 \times 256 images were recorded at a 0.5–2 $\mu\text{m}/\text{s}$ linear scanning speed at a sampling density of 4–60 nm^2 per pixel. Raw AFM images have been processed only for background removal (flattening) using the microscope manufacturer's image-processing software.

Dye Exclusion Assay. Block cationer polyplex solutions (33 μg pDNA/mL) prepared at a charge ratio of 2 were adjusted to have 10 μg pDNA/mL with 2.5 μg EtBr/mL and 150 mM NaCl by adding 10 mM Tris-HCl (pH 7.4) buffer containing EtBr and NaCl. The solutions

were incubated at ambient temperature overnight. Fluorescence measurements of sample solutions were carried out at 25 $^\circ\text{C}$ using a spectrofluorometer (FP-6500, Jasco, Tokyo, Japan). Fluorescence intensity of the samples at 590 nm was measured with excitation at 510 nm. Relative fluorescence intensity was calculated as follows:

$$F_r = (F_{\text{sample}} - F_0)/(F_{100} - F_0)$$

where F_{sample} was fluorescence intensity for the samples, F_{100} was free pDNA, and F_0 was background.

Gel Retardation Analysis. Sodium dextran sulfate (0.83 mM, $M_w = 25\,000$) in 10 mM Tris-HCl (pH 7.4) buffer was added to the same volume of polyplex solutions (33 μg pDNA/mL) prepared at a charge ratio of 2 in 10 mM Tris-HCl (pH 7.4) buffer to have an approximately 20-times-excess charge ratio of sodium dextran sulfate to pDNA in the mixture. The mixed solution was then diluted up to 8.3 μg pDNA/mL by the same volume of 10 mM Tris-HCl (pH 7.4) buffer with or without 50 mM DTT. After an overnight incubation at 37 $^\circ\text{C}$ in a sealed container, 20 μL of each sample (166 ng pDNA) was electrophoresed at 100 V for 1 h on a 0.9 wt % agarose gel in 20 mM Tris-acetic acid buffer containing 10 mM sodium acetic acid (pH 7.8). pDNA in the gel was visualized by EtBr (0.5 $\mu\text{g}/\text{mL}$) staining.

Transfection. 293T cells were plated into six well gelatin-coated culture plates. After a 24 h incubation in 1.5 mL of Dulbecco's modified eagle medium (DMEM) containing 10% serum, the medium was replaced with 1 mL of a transfection medium containing 10% serum and 100 μM hydroxychloroquine. Ninety μL of each polyplex solution (33 $\mu\text{g}/\text{mL}$ pDNA) was then applied to each well for the transfection. The amount of pDNA was adjusted to 3 μg per well. After 24 h of incubation, the medium was replaced with 1 mL of the medium containing 10% serum, followed by an additional 24 h incubation. The luciferase gene expression was then evaluated from the intensity of photoluminescence using Fluoroskan Ascent FL system (Labsystems, Helsinki, Finland). The amount of protein in each well was concomitantly determined using a Micro BCA Protein Assay Reagent Kit.

Effect of Freeze–Thawing Process of Polyplexes on Transfection. Polyplex solutions with a charge ratio of 2 were prepared as described in the preceding section to adjust the pDNA concentration of 33 $\mu\text{g}/\text{mL}$. Each sample was frozen at -20 $^\circ\text{C}$ for 2 h, followed by thawing at room temperature for 30 min. Both nonfrozen and freeze–thawed polyplexes were subjected to the luciferase transfection assay using 293T cells.

Results and Discussion

Thiolation of PEG-PLL. Thiolated PEG-PLLs were synthesized using two types of thiolation reagents, i.e., SPDP and Traut's reagent. The activated ester of SPDP reacts with the ϵ -amino group of lysine residue, resulting in the introduction of the 3-(2-pyridyldithio)propionyl (PDP) groups via amide linkage into the side chain of the PLL segment of PEG-PLL (Scheme 1a). Consequently, the cationic charge density of the PLL segment was decreased through the thiolation. Samples with varying thiolation degree were prepared by this method and abbreviated as PDP-X, where X stands for the percent thiolation (%) of the ϵ -amino groups calculated from the peak intensity ratio of the methylene protons of PEG (OCH_2CH_2 , $\delta = 3.5$ ppm) to the pyridyl protons of the 3-(2-pyridyldithio)propionyl groups ($\text{C}_5\text{H}_4\text{N}$, $\delta = 7.2$ – 8.3 ppm) in ^1H NMR spectrum as typically seen in Figure 1 (PDP-37). Treatment of PDP-X with an excess amount of DTT gave the reduced form having flanking 3-mercaptopropionyl groups, which we abbreviated as MP-X.

As an approach of thiolation with keeping the charge density unchanged, the amidination of ϵ -amino groups of PLL segment

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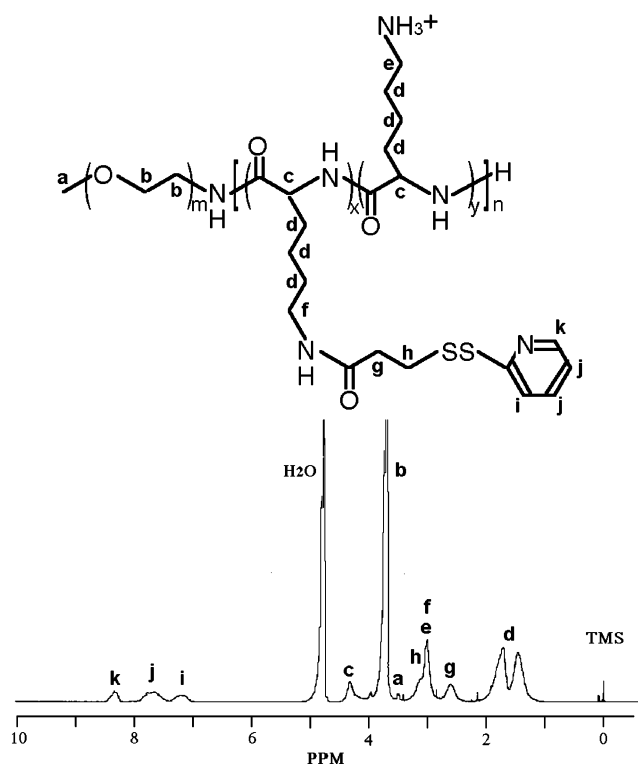


Figure 1. ^1H NMR spectrum of PDP-37 (solvent, D_2O ; temperature, 25°C ; concentration, 10 mg/mL).

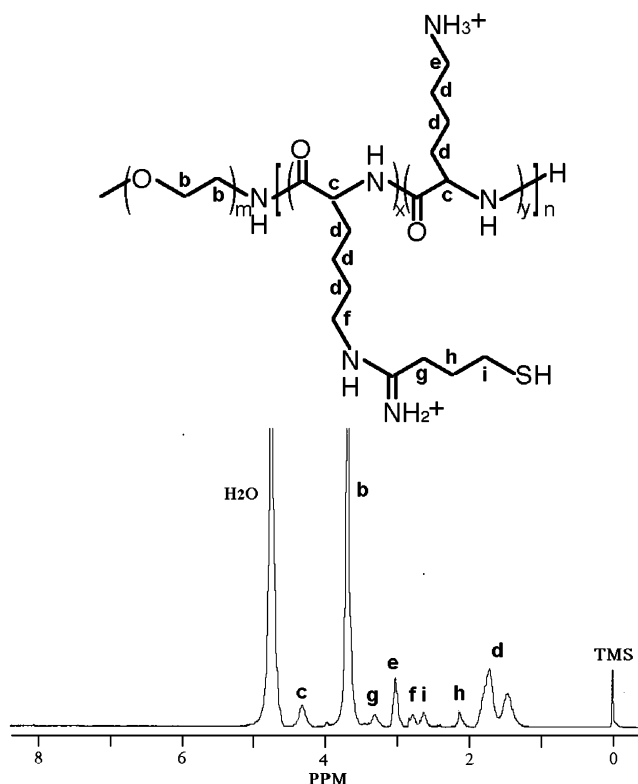


Figure 2. ^1H NMR spectrum of IM-22 (solvent, D_2O ; temperature, 25°C ; concentration, 10 mg/mL).

was done by Traut's reagent to introduce thiol groups with cationic imino moieties as seen in Scheme 1b. ^1H NMR spectrum shown in Figure 2 (IM-22) clearly indicates the introduction of both thiol and imino moieties in the sample from the peaks of newly introduced methylene protons of 1-imino-4-

Table 1. Cumulant Diameters and Polydispersity Indices ($\mu\Gamma^2$) of Block Cationer Polyplexes

	cumulant diameter [nm]	polydispersity index ($\mu\Gamma^2$)
PEG-PLL polyplex (non-cross-linked)	102	0.16
IM-X polyplexes (cross-linked)		
IM-9	102	0.15
IM-22	100	0.16
MP-X polyplexes (cross-linked)		
MP-5	103	0.14
MP-13	106	0.14
MP-28	103	0.13
MP-37	115	0.19

mercaptobutyl groups from Traut's reagent ($\text{HS}-(\text{CH}_2)_3-\text{C}(\text{NH}_2^+)^-$, $\delta = 2.1-3.4\text{ ppm}$). Thiolated polymers containing 1-imino-4-mercaptobutyl groups were abbreviated as IM-X, where X stands for the percent thiolation.

Formation of Cross-Linked Block Cationer Polyplex. The block cationer polyplexes from pDNA and thiolated PEG-PLL were prepared at the charge ratio of 2, where no free pDNA was observed in an agarose gel retardation analysis (data not shown). To determine the process of oxidative cross-linking, the polyplex samples were subjected to Ellman's test for the estimation of residual thiol groups. After 3 days of dialysis, the amount of remaining thiol groups was calculated to be less than 10% of the initial value for all the samples, indicating that the core of block cationer polyplexes was cross-linked through covalent disulfide bonds between the side chain of PLL segment of PEG-PLL.

The size and shape of the cross-linked polyplexes were then evaluated by DLS and AFM measurements. Table 1 summarizes the cumulant diameters of the polyplexes determined by DLS, indicating that all of the block cationer polyplexes, regardless of the cross-linking and charge densities, have diameters around 100 nm with a moderate polydispersity between 0.13 and 0.19. This is in line with the results of the atomic force microscopy (AFM) of the dried polyplexes on a mica disk as seen in Figure 3. A toroidal structure in the size range of 60–100 nm and a rodlike structure with a long axis of 150–300 nm were observed by AFM. Our previous work on polyplex examination by static light scattering revealed that the block cationer polyplexes of PEG-PLL prepared at a charge ratio of 2 is likely to contain a single pDNA molecule in each of the polyplexes without any secondary coalescence.¹⁶ Present AFM imaging of the polyplexes is reasonably consistent with this previous estimation based on the static light scattering analysis.

Ethidium bromide (EtBr) is known to form an intercalating complex with double helical polynucleotides to show a striking enhancement in its fluorescence intensity.¹⁷ This enhanced fluorescence was quenched upon the condensation of DNA through the complexation with cationic component due to the hindered intercalation of EtBr into double-stranded structure of DNA molecules. Thus, EtBr exclusion assay was frequently utilized to estimate the degree of pDNA condensation in ion complexes.^{17,18} As seen in Figure 4, non-cross-linked polyplexes

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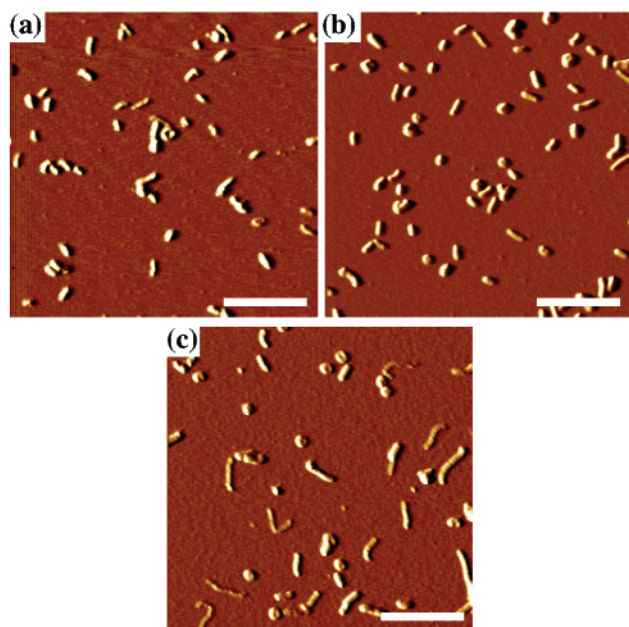


Figure 3. AFM images of block cationer polyplexes. (a) PEG-PLL polyplex (non-cross-linked), (b) IM-22 polyplex (cross-linked), and (c) MP-28 polyplex (cross-linked). Images are shown in amplitude mode, and all the scale bars are equivalent to 500 nm.

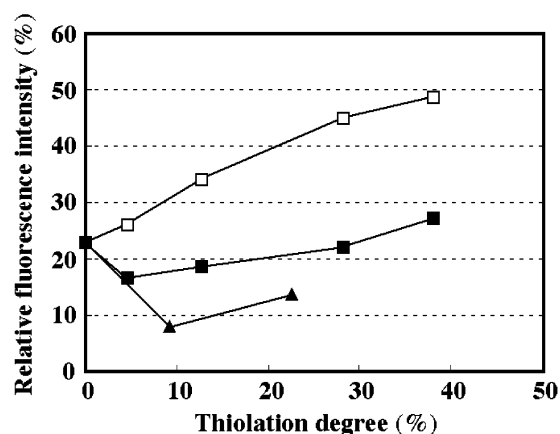


Figure 4. Effect of thiolation degree of non-cross-linked and cross-linked block cationer polyplexes on the fluorescence intensity of EtBr. Fluorescence measurements were carried out as described in the Experimental Section. (□) PDP-X polyplex, (■) MP-X polyplex, (▲) IM-X polyplex.

from PDP-X showed a monotonic increase in the EtBr fluorescence with an increase in the thiolation degree, indicating a lowered tendency of dye exclusion. This result demonstrates that the decreased charge density of PLL segment through the reaction with SPDP led to a lowered capacity to induce pDNA condensation. On the other hand, the disulfide cross-linked polyplexes from MP-X had a substantially lowered fluorescence intensity than the corresponding non-cross-linked polyplexes from PDP-X and revealed the fluorescence intensity the same as the polyplex from PEG-PLL (nonthiolated polymer), indicating their characteristic to compensate for the decreased cationic charge with disulfide cross-linking. Furthermore, the cross-linked polyplexes from IM-X with a fixed charge density exhibited even lower fluorescence intensity than the cross-linked MP polyplexes with a decreased charge density, showing that the additional disulfide cross-linking to the electrostatic interaction further promotes the condensation of pDNA.

Stability of Cross-Linked Systems. The stability of the cross-linked polyplexes against the counter polyanion exchange reaction may play a key role in pDNA release from the polyplexes in the intracellular compartment and was thus evaluated by the agarose gel electrophoresis as seen in Figure 5. The effects of cross-linking and charge densities on counter polyanion-induced dissociation of the polyplexes in nonreductive and reductive conditions were evaluated by this method. Obviously, in the nonreductive condition (Figure 5a, lane 2 and Figure 5b, lane 2), the bands of the free pDNA (super-coil and open-circular forms) were observed for the non-cross-linked systems (PEG-PLL and PDP-28 polyplexes). On the contrary, the free pDNA bands were not detected in the condition without DTT (Figure 5a, lanes 3 and 4 and Figure 5b, lanes 3–6) for all of the cross-linked systems (IM- and MP-polyplexes), indicating the substantial stabilization of the cross-linked polyplexes against the exchange reaction. Noteworthy is the behavior of MP-28 in the reductive medium containing 25 mM DTT (Figure 5b, lane 9): The free pDNA bands were only clearly observed for this system among all of the cross-linked polyplexes. This result indicates that the release of pDNA through the cleavage of the disulfide cross-linking occurs effectively with the polyplex designed to optimally balance the densities of charge and disulfide cross-linking.

In Vitro Transfection Efficiency of Cross-Linked Block Cationer Polyplex. To assess the environmental sensitivity in the intracellular compartment, transfection of luciferase gene (pGL3) to cultured 293T cells was then carried out using these cross-linked polyplexes (Figure 6a). The cross-linked polyplexes of IM series, showing no release of entrapped pDNA in the electrophoretic assay under the reductive environment, resulted in an even lower transfection than the non-cross-linked control (PEG-PLL). This indicates that an additional introduction of disulfide cross-links to the polyplex with an appreciably high cationic charge density works synergistically to prevent pDNA release from the polyplex even in the reductive intracellular environment, resulting in a lowered transfection efficiency. Notably, the cross-linked polyplexes of MP series, the system compensating for the decrease in the charge density with the disulfide cross-linking, revealed an increased transfection efficiency compared to PEG-PLL, taking a maximum efficiency at MP-28 to achieve 1 order of magnitude higher luciferase expression than PEG-PLL. Note that this is reasonably consistent with their capability of pDNA release in the reductive environment as demonstrated in electrophoretic assay (Figure 5b). Progressive increase in gene expression with an increased thiolation degree up to 28% for MP series may be ascribed to the facilitated release of pDNA from the polyplex in the intracellular reductive environment due to the decreased charge density of ion-complexed polycation segment. On the other hand, MP-37 apparently revealed a lowered gene expression compared to MP-28. Release of pDNA from MP-37 is likely to be impeded even in the intracellular reductive environment, presumably due to the overstabilization through the excessive disulfide cross-linking. As seen in Figure 6b, PDP-28, the non-cross-linked control of MP-28, revealed only a 2 times higher transfection efficiency than PEG-PLL, indicating that the lowered charge density is not the main reason for the significantly higher efficiency of the MP-28 polyplex. It is reasonable to assume that disulfide cross-links may inhibit the MP-28

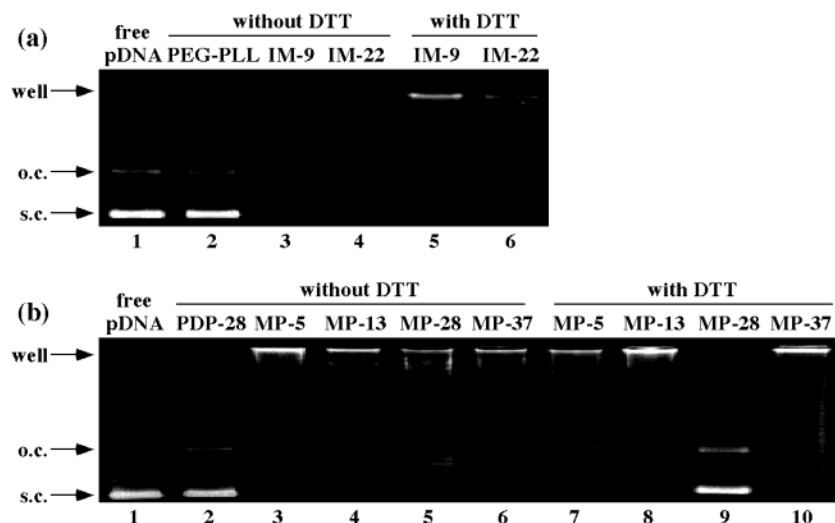


Figure 5. Agarose gel retardation analysis. Each sample was mixed with dextran sulfate ($M_w = 25\,000$, ca. 20 times excess charge ratio to pDNA) and incubated at $37\text{ }^\circ\text{C}$ for 24 h, followed by gel electrophoresis (final concentration of pDNA: $8.3\ \mu\text{g/mL}$). The reductive condition was prepared by adding DTT (25 mM). (a) Retardation assay for IM series. From left: lane 1, free pDNA; lane 2, PEG-PLL polyplex; lanes 3 and 4, IM-X polyplex without DTT; lanes 5 and 6, IM-X polyplexes with 25 mM DTT. (b) Retardation assay for MP series. From left: lane 1, free pDNA; lane 2, PDP-28; lanes 3–6, MP-X without DTT; lanes 7–10, MP-X with 25 mM DTT. (s.c., supercoiled; o.c., open circular form of pDNA).

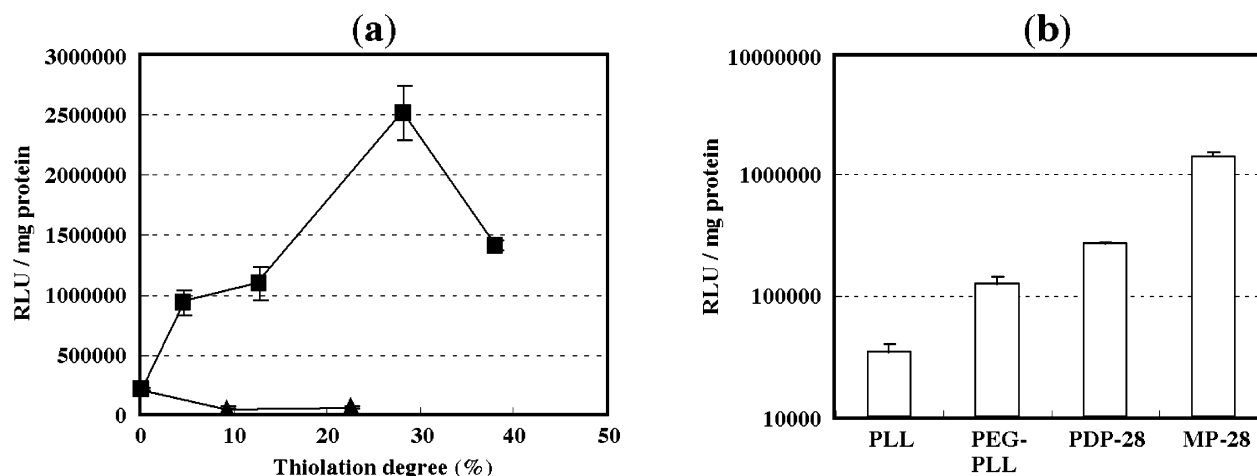


Figure 6. Transfection of luciferase gene to 293T cells by the block cationer polyplexes. 293T cells were incubated with the polyplexes in the medium containing $100\ \mu\text{M}$ hydroxychloroquine (HC) and 10% serum for 24 h, followed by an additional 24 h culture without the polyplexes and HC in the medium containing 10% serum. (a) Effect of the thiolation degree on the transfection efficiency. (■) MP-X polyplex, (▲) IM-X polyplex. (b) Progressive increase in transfection with a modulation in the structure of PLL-based polyplexes. PLL (poly(L-lysine) homopolymer, $M_w = 18\,000$) was used as the control polyplex.

polyplex from disintegrating in the extracytoplasmic environment, allowing the effective intracellular delivery of pDNA. Subsequently, the cleavage of the disulfide bond in the reductive intracellular compartment triggers the efficient release of pDNA from the MP-28 polyplex, because it should induce a substantial decrease in the association force in the polyplex, a system compensating the decreased charge with disulfide cross-linking.

From a practical viewpoint, long-term storage of gene carriers in a regulatory condition, including powdered and frozen formulations, is a crucial issue. Nevertheless, the conventional lipoplexes and polyplexes are not tolerant of freezing, resulting in a significant loss in the transfection efficiency in the absence of particular saccharide compounds as lyoprotectants.^{19–21} As

seen in Figure 7, the disulfide cross-linking (MP-28) completely protects the transfection capacity of the block cationer polyplexes even after the simple freeze–thawing without using any protective reagents. This is in a sharp contrast to the significantly lowered transfection efficiency of non-cross-linked polyplexes after the freeze–thawing process. The disulfide cross-linking obviously prevents the polyplex from the structural change induced by freezing stress. Indeed, there was no substantial change in AFM images of the cross-linked polyplexes before and after the freeze–thawing process (data not shown).

Conclusions

Here, the disulfide cross-linked polyplex is shown to undergo the environment-sensitive release of pDNA through the regulated

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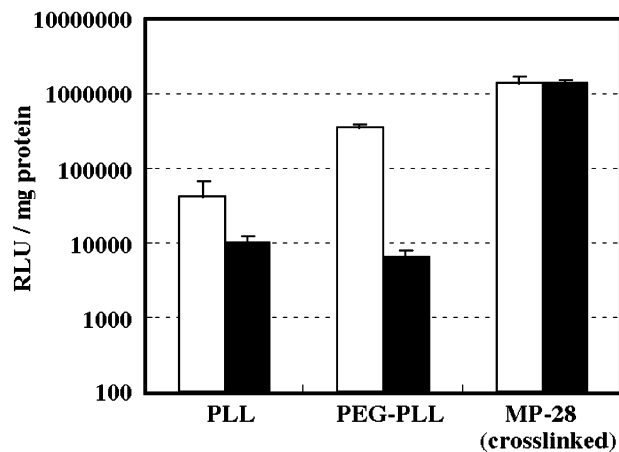


Figure 7. Effect of freeze–thawing on the transfection efficiency of polyplexes. Open bar: nonfrozen sample. Closed bar: freeze–thawed sample. All the polyplex samples were frozen at $-20\text{ }^{\circ}\text{C}$ for 2 h, followed by thawing at room temperature for 30 min. PLL represents poly(L-lysine) homopolymer ($M_w = 18\ 000$).

cleavage of disulfide bond in the reductive condition. The cross-linked polyplexes with a lowered charge density (MP-X polyplexes), in particular MP-28, achieved significantly higher transfection than those with a fixed charge density (IM-X

polyplexes). The presence of the optimal thiolation degree (28%) in MP-X supports our hypothesis that the efficient transfection in the disulfide cross-linked polyplexes can be achieved by controlling the balance between the cationic charge and disulfide cross-linking densities. Our results should provide a comprehensive knowledge of designing the environment-sensitive polyplex systems with high stability in extracellular environments and an effective releasing capacity of pDNA in intracellular compartments for the efficient transfection. Our recent animal study revealed an appreciable gene expression in mouse liver for intravenously injected MP-28, and detailed results will be reported elsewhere in near future.

Acknowledgment. This work was financially supported by the Core Research Program for Evolutional Science and Technology (CREST) from the Japan Science and Technology Corporation (JST) as well as by Special Coordination Funds for Promoting Science and Technology from the Ministry of Education, Culture, Sports, Science and Technology of Japan (MEXT). We express our appreciation to Mr. Hiroshi Nagano, The University of Tokyo, and Mr. Shigeto Fukushima, Nippon Kayaku Co., Ltd., Japan, for their help with the polymer synthesis.

JA0379666