size than the nuclear pore (~ 9 nm) [35], it is reasonable to assume that the nanoparticles may dissolve in the cytoplasm with significantly lowered calcium and higher phosphate ion concentrations compared to the extracellular environment, releasing free ODN to diffuse into the nucleus through the nuclear pore [36]. Although some punctate fluorescence was still observed, presumably corresponding to the nanoparticles remaining in the endosomal compartment, these results strongly suggest that ODN was successfully released from the nanoparticles in the cytoplasmic compartment due to their dissociation, being consistent with the dialysis experiments as previously mentioned.

3.4. Biological activity

To demonstrate the biological significance of these nanoparticulate carrier systems, the inhibitory activity of ODN and siRNA against pGL3-luciferase gene was evaluated. The inhibitory activity was evaluated from the relative silencing of the pGL3-luciferase expression against the pRL-TK expression used as an internal standard [25] (Fig. 8). Obviously, appreciable silencing of the GL3 luciferase gene expression (up to about 60%) was observed for the siRNA-incorporated nanoparticles prepared over the polymer concentration range from 420 to 700 μg/ml. This is in sharp contrast with the negligible silencing effect of naked siRNA, empty nanoparticles and nanoparticles entrapping non-silencing siRNA used as a control. Furthermore, these results indicate that siRNA in the nanoparticles is indeed released in the cytoplasm keeping its active form. On the other hand, no remarkable inhibition of gene expression was recognized for ODN-incorporated nanoparticles. This result might be attributed to the difference of the target sequences for ODN and siRNA and/or the higher potency of RNAi than ODN [37].

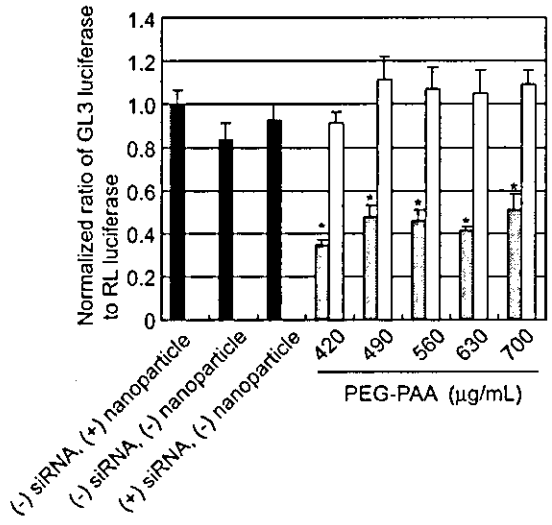

Fig. 8. Biological activities of siRNA incorporated in the hybrid nanoparticles formed at various concentrations of PEG-PAA. Ratios of *Photinus pyralis* GL3 luciferase to *Renilla reniformis* RL luciferase were normalized to cells treated with nanoparticles formed without siRNA. Grey bars indicate the ratios of GL3 to RL in the presence of the nanoparticles entrapping siRNA targeting GL3 luciferase. White bars indicate GL3 to RL in the presence of the nanoparticles entrapping non-silencing siRNA used as a control. The mean ± S.E.M. of the six experiments is presented. Stars indicate significant difference, with $p < 0.01$ (*) between cells treated with GL3 siRNA- and non-silencing siRNA-incorporated nanoparticles.

4. Conclusions

The present study reports on organic–inorganic hybrid nanoparticles composed of block copolymers and CaP crystals, devised for utilization as an ODN or siRNA carrier. The hybrid nanoparticles exhibited diameters in the hectonanometer range, good colloidal stability and high ODN- or siRNA-loading capacity. Furthermore, the incorporation of ODN into the nanoparticles resulted in the enhanced cellular uptake in the in vitro experiment. Finally, the environment-sensitivity against the gap in the calcium and phosphate ion concentrations was revealed, suggesting the intracellular selective dissociation of the hybrid nanoparticles.

These results could be of great significance for ODN or siRNA delivery in vivo. To further enhance the efficacy of the delivery, the ligand conjugation to induce receptor-mediated cellular uptake and the inclusion of endosomotropic reagents into the system are likely to be promising. Furthermore, it should be noted that the hybrid nanoparticles reported here might also find application as a carrier for versatile compounds such as anticancer drugs and proteins, because of the high binding affinity of CaP to these compounds.

Acknowledgements

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References

[1] D. Luo, W.M. Saltzman, Synthetic DNA delivery systems, Nat. Biotechnol. 18 (2000) 33–37.
[2] A.V. Kabanov, P.L. Felgner, L.W. Seymour (Eds.), Self-Assembling Complexes for Gene Delivery, Wiley, Chichester, UK, 1998.
[3] R. Duncan, The dawning era of polymer therapeutics, Nat. Rev., Drug Discov. 2 (2003) 347–360.
[4] Y. Kakizawa, K. Kataoka, Block copolymer micelles for delivery of gene and related compounds, Adv. Drug Deliv. Rev. 54 (2002) 203–222.
[5] A.V. Kabanov, S.V. Vinogradov, Yu.G. Suzdaltseva, V.Y. Alakhov, Water-soluble block polycations for oligonucleotide delivery, Bioconjug. Chem. 6 (1995) 639–643.
[6] S. Katayose, K. Kataoka, Water-soluble polyion complex associates of DNA and poly(ethylene glycol)-poly-(L-lysine) block copolymer, Bioconjug. Chem. 8 (1997) 702–707.
[7] A. Akinc, D.M. Lynn, D.G. Anderson, R. Langer, Parallel synthesis and biophysical characterization of a degradable polymer library for gene delivery, J. Am. Chem. Soc. 125 (2003) 5316–5323.
[8] D.R. Sørensen, M. Leirdal, M. Sioud, Gene silencing by systemic delivery of synthetic siRNAs in adult mice, J. Mol. Biol. 327 (2003) 761–766.
[9] F. Simeoni, M.C. Morris, F. Heitz, G. Divita, Insight into the mechanism of the peptide-based gene delivery system MPG: implications for delivery of siRNA into mammalian cells, Nucleic Acids Res. 31 (2003) 2717–2724.
[10] Y. Kakizawa, A. Harada, K. Kataoka, Environment-sensitive stabilization of core-shell structured polyion complex micelle by reversible cross-linking of the core through disulfide bond, J. Am. Chem. Soc. 121 (1999) 11247–11248.
[11] Y. Kakizawa, A. Harada, K. Kataoka, Glutathione-sensitive stabilization of block copolymer micelles composed of antisense DNA and thiolated poly(ethylene glycol)-block-poly(L-lysine): a potential carrier for systemic delivery of antisense DNA, Biomacromolecules 2 (2001) 491–497.
[12] D. Oupicky, R.C. Carlisle, L.W. Seymour, Triggered intracellular activation of disulfide crosslinked polyelectrolyte gene delivery complexes with extended systemic circulation in vivo, Gene Ther. 8 (2001) 713–724.
[13] D.L. McKenzie, K.Y. Kwok, K.G. Rice, A potent new class of reductively activated peptide gene delivery agents, J. Biol. Chem. 275 (2000) 9970–9977.
[14] M. Yokoyama, T. Okano, Y. Sakurai, H. Ekimoto, C. Shibazaki, K. Kataoka, Toxicity and antitumor activity against solid tumors of micelle-forming polymeric anticancer drug and its extremely long circulation in blood, Cancer Res. 51 (1991) 3229–3336.
[15] N. Nishiyama, Y. Kato, Y. Sugiyama, K. Kataoka, Cisplatin-loaded polymer-metal complex micelle with time-modulated decaying property as a novel drug delivery system, Pharm. Res. 18 (2001) 1035–1041.
[16] N. Nishiyama, S. Okazaki, H. Cabral, M. Miyamoto, Y. Kato, Y. Sugiyama, K. Nishio, Y. Matsumura, K. Kataoka, Novel cisplatin-incorporated polymeric micelles can eradicate solid tumors in mice, Cancer Res. 63 (2003) 8977–8983.
[17] K. Kataoka, M. Yokoyama, G.S. Kwon, T. Okano, Y. Sakurai, Block copolymer micelles as vehicles for drug delivery, J. Control. Release 24 (1993) 119–132.
[18] K. Kataoka, A. Harada, Y. Nagasaki, Block copolymer micelles for drug delivery: design, characterization and biological significance, Adv. Drug Deliv. Rev. 47 (2001) 113–131.
[19] Y. Bae, S. Fukushima, A. Harada, K. Kataoka, Design of environment-sensitive supramolecular assemblies for intracellular drug delivery: polymeric micelles that are responsive to intracellular pH change, Angew. Chem., Int. Ed. 42 (2003) 4640–4643.
[20] Y. Kakizawa, K. Kataoka, Block copolymer self-assembly into monodispersive nanoparticles with hybrid core of antisense DNA and calcium phosphate, Langmuir 18 (2002) 4539–4543.
[21] M. Otsuka, Y. Matsuda, Y. Suwa, J.L. Fox, W.I. Higuchi, A novel skeletal drug-delivery system using a self-setting calcium phosphate cement, J. Pharm. Sci. 83 (1994) 1565–1568.
[22] P.K. Bsjapai, H.A. Benghuzzi, Ceramic systems for long-term delivery of chemicals and biologicals, J. Biomed. Mater. Res. 22 (1988) 1245–1266.
[23] N. Nishiyama, M. Yokoyama, T. Aoyagi, T. Okano, Y. Sakurai, K. Kataoka, Preparation and characterization of self-assembled polymer-metal complex micelle from cis-dichlorodiammineplatinum(II) and poly(ethylene glycol)-poly(alpha,beta-aspartic acid) block copolymer in an aqueous medium, Langmuir 15 (1999) 377–383.
[24] M. Mishra, J.R. Bennett, G. Chaudhuri, Biochem. Pharmacol. 61 (2001) 467–476.
[25] S.M. Elbashir, J. Harboth, W. Lendeckel, A. Yalcin, K. Weber, T. Tuschl, Duplexes of 21-nucelotide RNAs mediate RNA interference in cultured mammalian cells, Nature 411 (2001) 494–498.
[26] D.E. Koppel, Analysis of macromolecular polydispersity in intensity correlation spectroscopy-method of cumulants, J. Chem. Phys. 57 (1972) 4814–4820.
[27] E. Gulari, E. Gulari, Y. Tsunashima, B. Chu, Photon correlation spectroscopy of particle distributions, J. Chem. Phys. 70 (1979) 3965–3972.
[28] G. Kwon, S. Suwa, M. Yokoyama, T. Okano, Y. Sakurai, K. Kataoka, Enhanced tumor accumulation and prolonged circulation times of micelle-forming poly(ethylene oxide-aspartate) block copolymer-adriamycin conjugates, J. Control. Release 29 (1994) 17–23.

GENE DELIVERY

[29] E. Taillandier, J. Liquier, Infrared-spectroscopy of DNA, Methods Enzymol. 211 (1992) 307–335.
[30] E. Fernandez, F.J. Gil, M.P. Ginebra, F.C.M. Driessens, J.A. Planell, Calcium phosphate bone cements for clinical applications: Part I. Solution chemistry, J. Mater. Sci. 10 (1999) 169–176.
[31] D.E. Clapham, Calcium signaling, Cell 80 (1995) 259–268.
[32] A.C. Guyton, Human Physiology and Mechanisms of Disease, 5th ed., Saunders, Philadelphia, PA, 1992.
[33] O. Zelphati, F.C. Szoka Jr., Intracellular distribution and mechanism of delivery of oligonucleotides mediated by cationic lipids, Pharm. Res. 13 (1996) 1367–1372.
[34] W. Zauner, A. Kichler, W. Schmidt, K. Mechtler, E. Wagner, Glycerol and polylysine synergize in their ability to rupture vesicular membranes: a mechanism for increased transferrin-polylysine-mediated gene transfer, Exp. Cell Res. 232 (1997) 137–145.
[35] J.P. Leonetti, N. Mechiti, G. Degols, C. Gagnor, B. Lebeleu, Intracellular-distribution of microinjected antisense oligonucleotides, Proc. Natl. Acad. Sci. U. S. A. 88 (1991) 2702–2706.
[36] P.L. Paine, L.C. Moore, S.B. Horowitz, Nuclear envelope permeability, Nature 254 (1975) 109–114.
[37] A. Grunweller, E. Wyszko, B. Bieber, R. Jahnel, V.A. Erdmann, J. Kurreck, Comparison of different antisense strategies in mammalian cells using locked nucleic acids, 2′-*O*-methyl RNA, phosphorothioates and small interfering RNA, Nucleic Acids Res. 31 (2003) 3185–3193.

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

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Abstract: A block catiomer 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 catiomer 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 catiomer 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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(1) (a) Katayose, S.; Kataoka, K. *Advanced Biomaterials in Biomedical Engineering and Drug Delivery Systems*; Springer: Tokyo, 1996; pp 319−320. (b) Katayose, S.; Kataoka, K. *Bioconjugate Chem.* **1997**, *8*, 702−707.
(2) Wolfert, M. A.; Schacht, E. H.; Toncheva, V.; Ulbrich, K.; Nazarova, O.; Seymour, L. W. *Hum. Gene Ther.* **1996**, *7*, 2123−2133.
(3) Harada-Shiba, M.; Yamauchi, K.; Harada, A.; Takamisawa, I.; Shimokado, K.; Kataoka, K. *Gene Ther.* **2002**, *9*, 407−414.
(4) Kakizawa, Y.; Kataoka, K. *Adv. Drug Delivery Rev.* **2002**, *54*, 203−222.
(5) Kwok, K. Y.; McKenzie, D. L.; Evers, D. L.; Rice, K. G. *J. Pharm. Sci.* **1999**, *88*, 996−1003.
(6) Oupicky, D.; Howard, K. A.; Konak, C.; Dash, P. R.; Ulbrich, K.; Seymour, L. W. *Bioconjugate Chem.* **2000**, *11*, 492−501.
(7) Oupicky, D.; Carlisle, R. C.; Seymour, L. W. *Gene Ther.* **2001**, *8*, 713−724.

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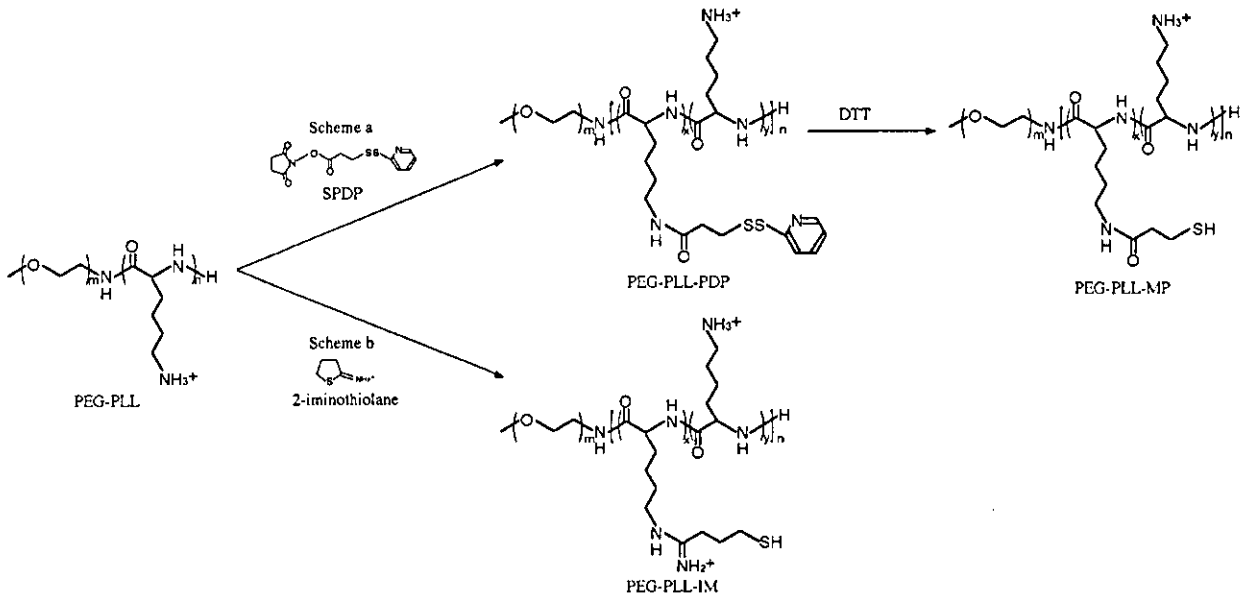
Scheme 1. Thiolation Schemes of PEG-PLL



A promising approach to realize this strategy is to reversibly cross-link the PIC core of the block catiomer 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 catiomer 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 DH5α *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 heterobifunctional reagent SPDP[13] (Scheme 1a) or a cyclic thioimidate Traut's reagent[14] (Scheme 1b). The typical synthetic procedure is described as follows for the PEG-PLL-PDP (37 mol % substitution): PEG-PLL (51 mg, 1.90 μmol) and SPDP (60 mg, 192 μmol) 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

(8) Meister, A.; Anderson, M. E. *Annu. Rev. Biochem.* **1983**, *52*, 711−760.
(9) Kakizawa, Y.; Harada, A.; Kataoka, K. *J. Am. Chem. Soc.* **1999**, *121*, 11247−11248.
(10) Kakizawa, Y.; Harada, A.; Kataoka, K. *Biomacromolecules* **2001**, *2*, 491−497.
(11) Trubetskoy, S. V.; Loomis, A.; Slattum, M. P.; Hagstrom, E. J.; Budker, G. V.; Wolff, A. J. *Bioconjugate Chem.* **1999**, *10*, 624−628.
(12) McKenzie, D. L.; Kwok, K. Y.; Rice, K. G. *J. Biol. Chem.* **2000**, *275*, 9970−9977.
(13) Carlsson, J.; Drevin, H.; Axen, R. *Biochem. J.* **1978**, *173*, 723−737.
(14) King, T. P.; Li, Y.; Kochoumian, L. *Prepr. Protein Conjugates* **1978**, *17*, 1499−1506.

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, δ = 3.5 ppm) to the pyridyl protons of the 3-(2-pyridyldithio)propionyl groups (C_5H_4N, δ = 7.2−8.3 ppm) or the newly introduced methylene protons of 1-imino-4-mercaptobutyl groups ($HS-(CH_2)_3-C(NH_2^+)^-$, δ = 2.1−3.4 ppm) in ^{1}H NMR spectra taken in D_2O at 25 °C.

Preparation of the Cross-Linked Block Catiomer 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 μmol/mL by the same buffer. Then, the polymer solutions were added to a 2-times-excess volume of 50 μg/mL pDNA solution (0.154 μmol/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 μg/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 °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 Catiomer Polyplex. To estimate the hydrodynamic diameters of cross-linked block catiomer polyplexes, dynamic light scattering (DLS) measurements were carried out using a DLS-7000 instrument (Otuka Electronics Co. Ltd., Hirakata, Japan). An Ar ion laser (λ = 488 nm) was used as an incident beam. Block catiomer 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° at 25 °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 × 256 images were recorded at a 0.5−2 μm/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 catiomer 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 °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:

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

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 °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 μg/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 μg/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 μg/mL. Each sample was frozen at −20 °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, δ = 3.5 ppm) to the pyridyl protons of the 3-(2-pyridyldithio)-propionyl groups (C_5H_4N, δ = 7.2−8.3 ppm) in ^{1}H 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

(15) Riddles, P. W.; Blakeley, R. L.; Zerner, B. *Anal. Biochem.* **1979**, *94*, 75−81.
(16) Itaka, K.; Yamauchi, K.; Harada, A.; Nakamura, K.; Kawaguchi, H.; Kataoka, K. *Biomaterials* **2003**, *24*, 4495−4506.

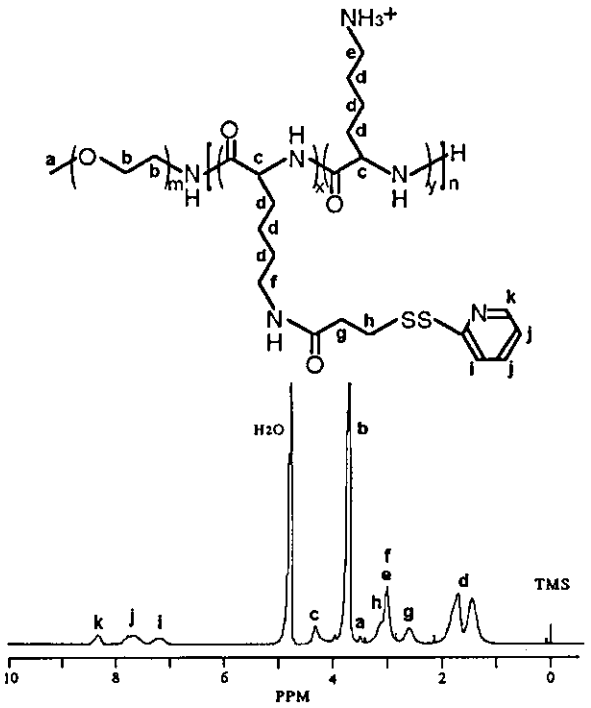


Figure 1. ^{1}H NMR spectrum of PDP-37 (solvent, D_2O; temperature, 25 °C; concentration, 10 mg/mL).

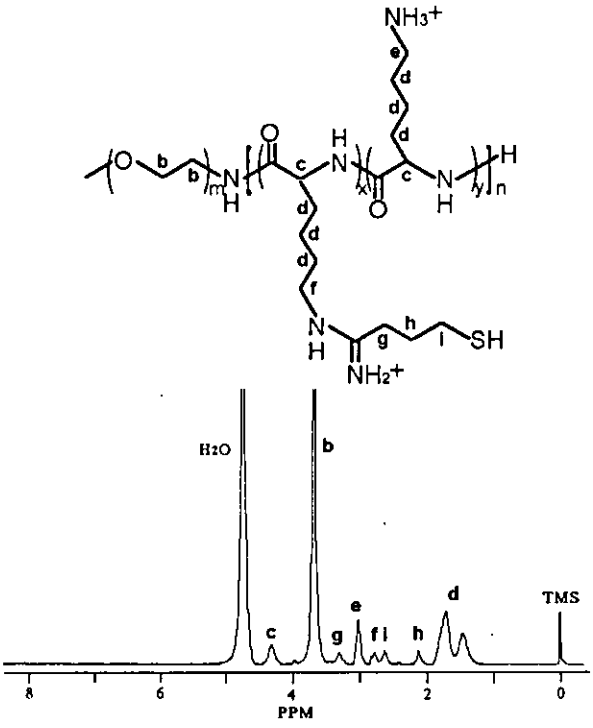


Figure 2. ^{1}H NMR spectrum of IM-22 (solvent, D_2O; temperature, 25 °C; concentration, 10 mg/mL).

was done by Traut's reagent to introduce thiol groups with cationic imino moieties as seen in Scheme 1b. ^{1}H 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-mercaptobutyl groups from Traut's reagent ($HS-(CH_2)_3-C(NH_2^+)^-$, $\delta = 2.1-3.4$ ppm). Thiolated polymers containing 1-imino-4-mercaptobutyl groups were abbreviated as IM-X, where X stands for the percent thiolation.

Table 1. Cumulant Diameters and Polydispersity Indices (μ/Γ^2) of Block Catiomer Polyplexes

	cumulant diameter [nm]	polydispersity index (μ/Γ^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

Formation of Cross-Linked Block Catiomer Polyplex. The block catiomer 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 catiomer 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 catiomer 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 catiomer 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

(17) LePecq, J.-B.; Paoletti, C. *J. Mol. Biol.* **1967**, *27*, 87−106.
(18) Itaka, K.; Harada, A.; Nakamura, K.; Kawaguchi, H.; Kataoka, K. *Biomacromolecules* **2002**, *3*, 841−845.

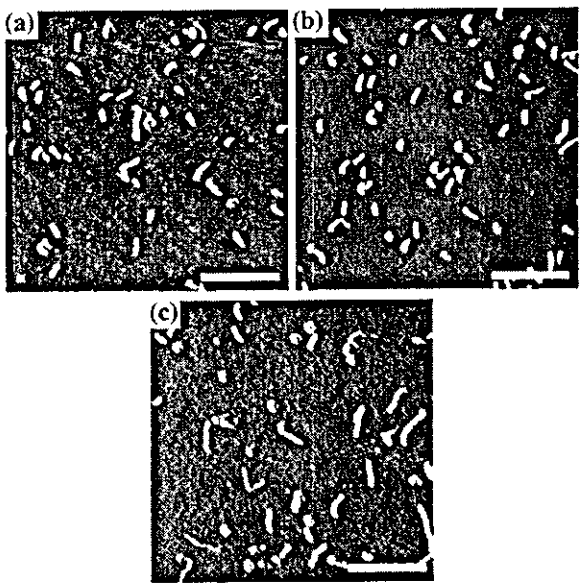


Figure 3. AFM images of block catiomer polyplexes. (a) PEG-PLL polyplex (non-cross-linked), (b) IM-22 polyplex (cross-linked), and (c) MP-28 polyplex (cross-linked). Images are shown in amplitude mode, and all the scale bars are equivalent to 500 nm.

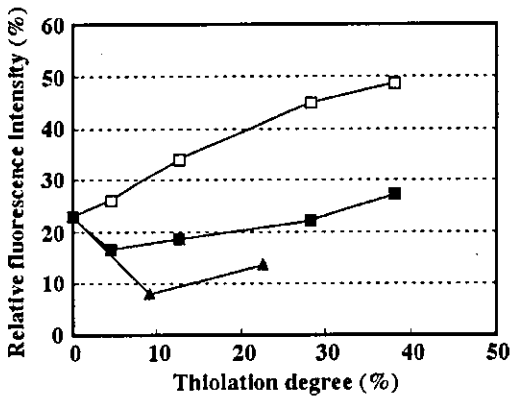


Figure 4. Effect of thiolation degree of non-cross-linked and cross-linked block catiomer polyplexes on the fluorescence intensity of EtBr. Fluorescence measurements were carried out as described in the Experimental Section. (□) PDP-X polyplex, (■) MP-X polyplex, (▲) IM-X polyplex.

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 Catiomer 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

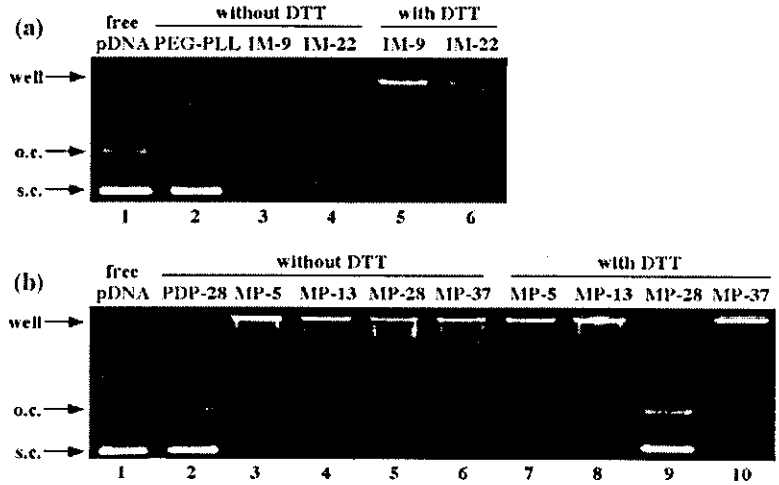


Figure 5. Agarose gel retardation analysis. Each sample was mixed with dextran sulfate (M_w = 25 000, ca. 20 times excess charge ratio to pDNA) and incubated at 37 °C for 24 h, followed by gel electrophoresis (final concentration of pDNA: 8.3 μg/mL). The reductive condition was prepared by adding DTT (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, fee 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).

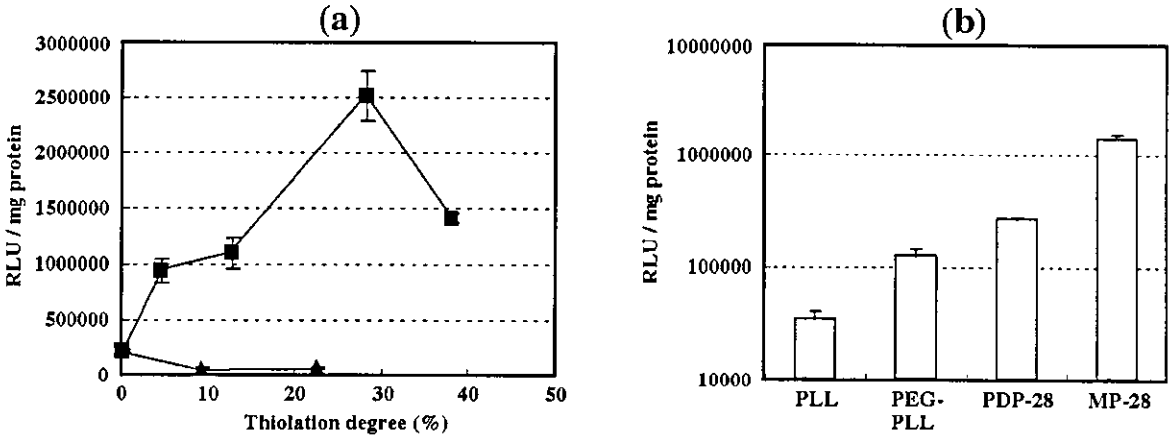


Figure 6. Transfection of luciferase gene to 293T cells by the block catiomer polyplexes. 293T cells were incubated with the polyplexes in the medium containing 100 μM hydroxychloroquine (HC) and 10% serum for 24 h, followed by an additional 24 h culture without the polyplexes and HC in the medium containing 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 catiomer 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

(19) Talsma, H.; Cherng, J.-Y.; Lehrmann, H.; Kursa, M.; Ogris, M.; Hennink, W. E.; Cotten, M.; Wagner, E. *Int. J. Pharm.* **1997**, *157*, 233–238.
(20) Cherng, J.-Y.; Wetering, P.; Talsma, H.; Crommelin, D. J. A.; Hennink, W. E. *Pharm. Res.* **1997**, *14*, 1838–1841.
(21) Anchordoquy, T. J.; Carpenter, J. F.; Kroll, D. J. *Arch. Biochem. Biophys.* **1997**, *348*, 199–206.

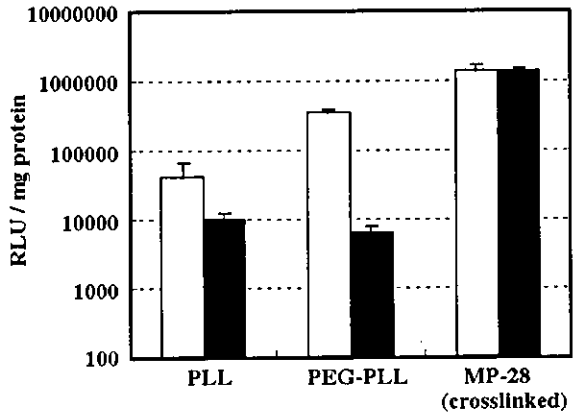


Figure 7. Effect of freeze–thawing on the transfection efficiency of polyplexes. Open bar: nonfrozen sample. Closed bar: freeze–thawed sample. All the polyplex samples were frozen at −20 °C for 2 h, followed by thawing at room temperature for 30 min. PLL represents poly(L-lysine) homopolymer (M_w = 18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

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.

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