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Bioreducible polymers as a determining factor for polyplex decomplexation rate and transfection

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Abstract

Polyplex formation (complexation) and gene release from the polyplexes (decomplexation) are major events in polymeric gene delivery, however the effect of the decomplexation rate on transfection has been rarely investigated. This study employed mixed polymers of poly(L-lysine) (PLL: MW ~7.4 kDa) and reducible PLL (RPLL) (MW ~6.7 kDa) to design decomplexation rate-controllable $PLL_{100-x}RPLL_x$/pDNA complexes (PRL_x polyplexes). The transfection efficiency of a model gene (luciferase) in MCF7 and HEK293 cell lines increased with increasing x (RPLL content) in the PRL_x polyplexes until peaking at x=2.5 and x=10, respectively, after which point transfection efficiency declined rapidly. In MCF7 cells, $PRL_{2.5}$ polyplex produced 3- or 223-fold higher gene expression than PLL or RPLL polyplexes, respectively. Similarly, the transfection efficiency of PRL_{10} polyplex-transfected HEK293 cells was 3.8- or 67-fold higher than that of PLL or RPLL polyplexes, respectively. The transfection results were not apparently related to the particle size, surface charge, complexation/compactness, cellular uptake, or cytotoxicity of the tested polyplexes. However, the decomplexation rate varied by RPLL content in the polyplexes, which in turn influenced the gene transfection. The nuclear localization of pDNA delivered by PRL_x polyplexes showed a similar trend to their transfection efficiencies. This study suggests that an optimum decomplexation rate may result in high nuclear localization of pDNA and transfection. Understanding in decomplexation and intracellular localization of pDNA may help develop more effective polyplexes.

Keywords

Decomplexation; Nuclear localization; Poly(L-lysine); Polymeric gene carrier; Reducible polymer

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Supporting Information Available. Supporting information includes: details about the synthetic scheme and ^{1}H-NMR analysis of RPLL; Condensation of pDNA with either PLL or RPLL in agarose gel; Normalized transfection efficiencies of $PLL_{100-x}RPLL_x$/pDNA complexes (PRL_x polyplexes) (N/P 5) in MCF7 cells and HEK293 cells; Dose-dependent cytotoxicity of PLL and RPLL in MCF7 and HEK293 cells. This information is available free of charge via the Internet at http://pubs.acs.org.

INTRODUCTION

Interest in polymeric gene carriers has been steadily increased due to their tailored structural characteristics (e.g., charges and architectures) and versatile design components endowing required functionalities (e.g., colloidal stabilization, cellular targetability, endosomal disruption, cytosolic transport, nuclear import, triggering drug release, and biocompatibility).[1–4] Such functionalities have been thought to overcome various extracellular and intracellular barriers encountered in the gene delivery process, anticipating enhanced therapeutic effects of an exogenous gene delivered by polymeric carriers. In addition, it is critical for optimal transfection that the decomplexation to release genes from the carriers should occur at a right intracellular place and a right time.[5] Our group has discovered that high transfection efficiency can be obtained by tuning the pH at which the polyplex is released from the endolysosome and that pH-tunable sulfonamide-based oligomers/polymers loaded in polyplexes affected gene expression but not gene silencing in gene delivery.[6, 7] Despite the recognized significance of intracellular location and timing of gene, the related investigation is seldom conducted.

The release or decomplexation of plasmid DNA (pDNA) from electrostatically-driven polyplexes is mostly induced by competitive interactions with charged biological components which disrupt the ionic bond that binds the gene to the carrier and protects from degradation. For most cationic polyplexes, such as anionic polymers or molecules such as glycosaminoglycans in plasma,[8–10] RNA in the cytoplasm,[11] DNA in the nucleus,[12] heparin,[6, 13, 14] poly(acrylic acid) and sodium dodecyl sulfate in test tubes, it is difficult to control the unpacking process of polyplexes under a given extracellular and intracellular environment because their concentrations cannot be manipulated by extrinsic factors.

Longer polycations generally cause stronger attraction with negatively-charged genes than shorter polycations, resulting in tighter polyplexes.[6, 14, 15] Thus, to facilitate polyanion-induced decomplexation of polyplexes especially in intracellular environments, degradable polymers responding to acidic pHs in the endolysosomes[16] or glutathione in the cytoplasm and the nucleus[14, 17, 18] have been developed. These polyplexes resulted in improved transfection efficiency. But the genes are susceptible to lytic enzymes in the endolysosomes. For pDNA, transfection efficiency could be dependent on the distance between its releasing site and the nucleus due to its poor mobility[19] in intracellular environment. In addition, pDNA is deactivated or degraded by cytosolic nucleases, giving pDNA a 60–90 min half-life in HeLa and COS cells.[20] Therefore, it is hypothesized that the intracellular release rate of genes affects transfection.

It has been generally recognized that the low transfection efficiency of poly(L-lysine) (PLL) polyplexes is caused by lack of endosomolytic activity of PLL. However, PLL polyplexes showed much slower decomplexation rate than poly(cyclooctene-*g*-oligolysine) (i.e., comb-shaped PLL) with nuclear localization signal (NLS), in spite of the fact that the former had a more nuclear uptake than the latter.[21] Non-degradable and degradable PLL polymer-based polyplexes also showed molecular weight (MW)-dependent decomplexation and transfection efficiency.[12, 15] For example, when forming linear polylysine/pDNA complexes, longer polylysine chains, (180 lysine units, MW 28 kDa) showed slower dissociation than shorter polylysine chains, (19 or 36 lysine units, 2.3 kDa or 4.4 kDa, respectively)3 due to the greater positive charge of the longer chains tightly binds the pDNA that interferes the release of the genes from the carrier, thus leading to increased inhibition of RNA synthesis and lower gene expression.[12] However, reducible PLL (RPLL)-based polyplexes showed contradictory *in vitro* transfection results depending on their MWs compared to polyplexes prepared by PLL with comparable MWs. High MW RPLL (187 kDa) showed more effective transfection than PLL (205 kDa),[22] whereas moderate MW

RPLL (65 kDa) showed lower gene transfection efficiency than PLL (54 kDa).[23] Based on these results, it appears that either “too fast” or “too slow” release of genes from polyplexes would bring low transfection efficiency.

This study investigated the effect of the decomplexation rate of pDNA on transfection efficiency. Commercial PLL with 7.4 kDa and synthesized reducible PLL (RPLL) with a comparable MW were selected. Using the polyplexes formed from a model gene and mixed PLL and RPLL, the influence of RPLL content on physicochemical characteristics (e.g., size, surface charge, complexation, and decomplexation) and biological characteristics (e.g., cellular uptake, cytotoxicity, transfection efficiency, and intracellular localization) was studied.

MATERIALS AND METHODS

Materials

Poly(L-lysine) hydrogen bromide (PLL·HBr; Mw 12 kDa), dimethyl sulfoxide (DMSO), 4-(2-hydroxy-ethyl)-1-piperazineethanesulfonic acid (HEPES), 3-(4,5-dimethylthiazol- 2-yl)-2,5-diphenyltetrazolium bromide (MTT), D-glucose, cystamine, sodium bicarbonate, recombinant human insulin, ethidium bromide (EtBr), paraformaldehyde (PFA), deuterium oxide (D_2O), dithiothreitol (DTT), heparin sodium salt (139 USP units/mg), Hoechst 33342, RPMI- 1640 medium, Dulbecco’s phosphate buffered saline (DPBS), Dulbecco’s modified eagle’s medium (DMEM), fetal bovine serum (FBS), trypsin-EDTA solution, and penicillin-streptomycin antibiotics were purchased from Sigma-Aldrich (St. Louis, MO). LysoTracker® Green dye and YOYO-1 were received from Invitrogen, Inc. (Carslbard, CA). A pDNA which encodes firefly luciferase (gWiz-Luc or pLuc) was bought from Aldevron, Inc. (Fargo, ND). A decapeptide (Cys-Lys_8-Cys) was synthesized by American Peptide Company (Sunnyvale, CA). Luciferase assay kit and GSH-Glo™ glutathione assay kit were purchased from Promega Corporation (Madison, WI) and BCA protein assay kit was bought from Pierce Biotechnology, Inc. (Rockford, IL). Cy5-labeled pDNA was prepared by using Label IT® nucleic acid labeling kit of Cy5 dye (Mirus Bio LLC; Madison, WI).

Cells and Cell Culture

HEK293 cells (human embryonic kidney cell line) and MCF7 cells (human breast adenocarcinoma cell line) were used in this study. HEK293 cells were cultured in DMEM supplemented with 10% FBS and D-glucose (4.5 g/L). MCF7 cells were cultured in RPMI-1640 supplemented with 10% FBS, D-glucose (2 g/L), and insulin (4 mg/L). The cells were maintained and grown under a humidified air with 5% CO_2 at 37 °C.

Synthesis and Characterization of Reducible Poly(Cys-Lys_8-Cys) (RPLL)

As previously reported,[22, 24] a decapeptide (Cys-Lys_8-Cys) was oxidized to form reducible poly(Cys-Lys_8-Cys) (RPLL) (Figure S1). Cys-Lys_8-Cys (50 mg; 40 μmol) was dissolved in deionized water (DIW; 1 mL) and then cystamine (0.31 mg; 4 μmol) as a chain closer was added into the peptide solution. On adding DMSO (0.33 mL) into the solution, oxidative polymerization of the decapeptide was performed to form RPLL at room temperature (RT). After 1 day polymerization, the synthesized RPLL was dialyzed against DIW by using a dialysis membrane with a molecular weight cut-off (MWCO) 1 kDa for 2 days and then was lyophilized. As shown in Figure S2, the ^{1}H-NMR spectra of RPLL in D_2O were characterized and confirmed by characteristic peaks at δ 1.32 (–CH-C**H**$_2$-CH_2–), δ 1.57 (–CH-C**H**$_2$-CH_2–), δ 2.59 (–CH-C**H**$_2$-NH_2), δ 3.1 (–C**H**$_2$-NH_2CH-CO–), δ 3.25 (–S-CH_2C**H**$_2$-NH_2), δ 3.8 (–S-C**H**$_2$$CH_2$-$NH_2$), δ 4.17 (–NH-C**H**$CH_2$-CO–), δ 4.3 (–$CH_2$-

$NH_2C\underline{\mathbf{H}}$-CO–). Based on ^{1}H-NMR analysis of RPLL, its estimated molecular weight (MW) was 6.7 kDa.

To determine polymer amount used for cell experiments, *in vitro* cytotoxicity of RPLL was evaluated by MTT-based cell viability as previously reported.[7, 14] HEK293 cells and MCF7 cells were seeded at 2×10^3 cells and 5×10^3 cells per well of a 96-well plate, respectively and then were incubated for 24 hr in serum-containing culture medium. The cells were exposed to RPLL having different concentrations (0–100 μg/mL) for 24 hr. Then, MTT solution (10 μL; 5 mg/mL) was treated to the cells in the culture medium (0.1 mL) for additional 4 hr. After discarding the culture medium, DMSO was added to dissolve the formazan crystals which are produced by living cells. Their absorbance was measured at 570 nm to estimate the cell viability and was compared with that of PLL as a non-reducible counterpart.

Preparation and Physicochemical Characterization of Polyplexes

As previously reported,[6, 14, 25] a cationic solution having polymers (*i.e.*, PLL and RPLL) and an anionic solution containing pDNA were separately prepared by using HEPES buffer (20 mM, pH 7.4). Two solutions with an equivalent volume were mixed and then incubated for 30 min at RT. The formed polyplexes (20 μL per 1 μg of pDNA) were further evaluated. Complexation ratio of polyplexes was expressed based on the N/P ratio using amines (N) of polymers and phosphates (P) of pDNA.

Particle sizes and surface charges of polyplexes were evaluated as previously reported.[6, 14, 25] After preparing polyplexes (100 μL; 5 μg pDNA), they were diluted with HEPES buffer (20 mM, pH 7.4) and pDNA in the polyplex solution was 2.5 μg/mL. The polyplexes were then monitored by using a Zetasizer 3000 (Malvern Instrument, UK) with a wavelength of 677 nm and a constant angle of 90° at RT.

The complexation ability of pDNA with PLL and RPLL was evaluated by a gel electrophoresis assay. After loading polyplexes (10 μL; 0.5 μg of pDNA) in 0.8% agarose gel having EtBr, the gel was run in 0.5x TBE buffer at a constant voltage (80 V) for 90 min. A UV illuminator was used to detect whether pDNA was exposed.

Compactness of pDNA was tested by using quenching fluorescent YOYO-1 dye intercalated with pDNA in polyplexes. After pDNA was intercalated with YOYO-1 dye (1 molecule per 10 bases of pDNA), YOYO-1-intercalated pDNA was used to form polyplexes. Fluorescent intensity of YOYO-1 in the polyplexes was measured at 495 nm (excitation) and 515 nm (emission). Compactness (%) of pDNA in polyplexes was calculated as a following equation.

$$\text{Compactness of pDNA}\,(\%)=\left(1-\frac{\text{RFU}_{\text{Polyplex}}-\text{RFU}_{\text{Buffer}}}{\text{RFU}_{\text{pDNA}}-\text{RFU}_{\text{Buffer}}}\right)\times 100\,(\%)$$

where $RFU_{Polyplex}$, RFU_{pDNA}, and RFU_{Buffer} are relative fluorescent units (RFU) of polyplexes, YOYO-1-intercalated pDNA, and HEPES buffer, respectively.

Decomplexation of pDNA from polyplexes was monitored by a gel electrophoresis assay in the presence of DTT and/or heparin. Polyplexes were exposed to 150 mM NaCl solution supplemented with heparin sodium salt (0–100 μg/mL) and/or DTT (20 mM) for 1 hr at 37 °C and a final concentration of pDNA was 25 μg/mL. Then the polyplexes were run and evaluated in 0.8% agarose gel as previously described in the complexation ability of polyplexes.

In addition, decomplexation kinetics of polyplexes was monitored by a dye-dequenching technique. The polyplexes with YOYO-1-intercalated pDNA (5 μg/mL) were treated with 150 mM NaCl solution having heparin (6.5 μg/mL) and DTT (10 mM) as a decomplexation solution (0.2 mL) at RT. Time-dependent decomplexation was evaluated by changing RFU of YOYO-1-intercalated pDNA exposed from the polyplexes using a plate reader. Free YOYO-1-intercalated pDNA and the decomplexation solution were set as 100% RFU and 0% RFU, respectively.

$$\text{Decomplexation of pDNA}\,(\%)=\frac{\text{RFU}_{\text{Polyplex}}-\text{RFU}_{\text{Buffer}}}{\text{RFU}_{\text{pDNA}}-\text{RFU}_{\text{Buffer}}}\times 100\,(\%)$$

Biological Characterizations of Polyplexes

HEK293 cells and MCF7 cells were seeded at 1×10^5 cells/well and 5×10^5 cells/well (of a 6-well plate), respectively, for cellular uptake, *in vitro* transfection efficiency, and intracellular trafficking study, whereas their cell densities were 5×10^4 HEK293 cells/well and 2.5×10^5 MCF7 cells/well (of a 12-well plate) for *in vitro* cytotoxicity. The seeded cells were incubated for 24 hr in serum-containing culture medium and then were used for further studies.

For *in vitro* transfection study, cells were cultured in a 6-well-plate and the transfection experiments were performed as previously described.[14, 25, 26] One hour before addition of polyplexes, serum-containing culture medium was replaced with serum-free and insulin-free transfection medium. After transfecting with polyplexes, the transfected cells were incubated for 4 hr. The transfection medium was then replaced with serum-containing culture medium and the cells were incubated for additional 44 hr. After the transfection experiments were completed, the cells transfected with pLuc were rinsed with DPBS and then lysed in a reporter lysis buffer. Relative luminescent unit (RLU) and protein content of transfected cells were evaluated by luciferase assay kit and BCA protein assay kit, respectively.

To evaluate whether transfection procedure of polyplex is toxic, *in vitro* cytotoxicity of polyplexes was evaluated by MTT-based cell viability and its experimental procedure mostly followed the aforementioned *in vitro* transfection study except for its cell density and pDNA dose (0.5 μg pDNA per well). After completing transfection procedures for 48 hr, MTT solution (0.1 mL; 5 mg/mL) was added to cells incubated in serum-containing culture medium (1 mL) for additional 4 hr. The *in vitro* cytotoxicity of the polymer, was evaluated using formazan crystals, which are metabolized by live cells. The formazan crystals were dissolved in DMSO and added to the cultured cells; their absorbance was subsequently monitored at 570 nm for converting cytotoxicity of polyplexes.

Cellular uptake of polyplexes was evaluated as previously reported.[6, 14] Polyplexes prepared with YOYO-l-intercalated pDNA (1 μg pDNA per well) were treated to cells. After 4 hr incubation, the cells were detached and fixed with 4% PFA solution. The fluorescent polyplex-containing cells were monitored using flow cytometry (FACScan Analyzer, Becton–Dickinson; Franklin Lakes, NJ) with a primary argon laser (488 nm) and a fluorescence detector (530 ± 15 nm) to detect YOYO-1. Uptake levels of polyplexes in the cells were analyzed with a gated population of living 10,000 cells.

For intracellular trafficking of polyplexes, MCF7 cells were seeded on a coverglass in a six-well plate at 5×10^5 cells/well. Polyplexes prepared with Cy5-labeled pDNA (1 μg pDNA per well) were treated to the cells in serum-free transfection medium. After 3.5 hr

transfection, Hoechst 33342 and LysoTracker®Green dye were added to stain the nuclei and the acidic vesicles (mostly relevant to late endosomes and lysosomes), respectively. At 4 hr incubation with polyplexes, the cells were rinsed with DPBS and fixed with 4% PFA solution for 5 min. The cells were evaluated using a laser scanning confocal microscope. Especially, nuclear localization of Cy5-labeled pDNA was quantified from fluorescence (FL) of two regions of interest (ROIs). The first region of fluorescence was obtained from the whole cell indicated as FL_{cell} and the second region is from the nuclei, FL_{nuclei}. Fluorescence intensity of Cy5-labeled pDNA was measured by using Image J software and the nuclear localization of Cy5-labeled pDNA was calculated from the following equation.[27]

$$\text{Nuclear Localization of pDNA}\,(\%)=\frac{FL_{nuclei}}{FL_{cell}}\times 100\,(\%)$$

Evaluation of intracellular glutathione concentration

HEK293 cells and MCF7 cells were seeded at 5×10^3 cells/well and 2.5×10^4 cells/well (of a 96-well plate), respectively and were cultured for 1 day. After discarding culture medium, the cells were rinsed and then were treated by following GSH Glo™ glutathione assay protocol.

Statistical analysis

The statistical significance of the data was evaluated by conducting an unpaired Student t-test at a confidence level of $p<0.05$.

RESULTS AND DISCUSSION

In order to control the decomplexation rates of pDNA in intracellular environments such as the cytoplasm and nucleus, this study designed pDNA complexes using lysine-based polycation mixtures comprising non-reducible PLL and RPLL as a decomplexation controller of polyplexes. PLL with MW 7.4 kDa (MW 12 kDa of PLL· HBr) and synthesized RPLL with a similar MW of 6.7 kDa were used to minimize MW effects of polycations on size and complexation of polyplexes. In ($PLL_{100-x}RPLL_x$)/pDNA complexes, (100-x)% and x% of primary amines for complexation with phosphate groups in pDNA were from PLL and RPLL, respectively and PRL_x polyplexes as code names were used. Also, PRL_x polyplexes were prepared at a fixed N/P ratio of 5 due to an optimum condition for transfection efficiency of PLL/pDNA complexes[26] and polyplex formation based on electrophoresis (Figure S3).

Effects of RPLL content (*X*) in transfection efficiency of PRL_X polyplexes

Prior to detailed analyses of various physicochemical and biological characteristics of PRL_x polyplexes, we first examined whether the presence of RPLL in PRL_x polyplexes affects their transfection efficiency in MCF7 and HEK293 cells. When we applied PRL_x polyplexes to the cells, their transfection efficiencies increased, had a peak value at x=2.5 for MCF7 cells and x=10 for HEK293 cells and then decreased with increasing concentration of RPLL compared to that of PRL_0 polyplexes (*i.e.*, PLL/pDNA complexes) (Figure 1). Using MCF7 cells, $PRL_{2.5}$ and PRL_5 polyplexes showed 3-fold and 2-fold higher transfection efficiencies than PRL_0 polyplexes, respectively and their differences were statistically significant ($p=0.017$ for $PRL_{2.5}$ polyplexes and $p=0.036$ for PRL_5 polyplexes compared with PRL_0 polyplexes) by unpaired Student's t-test. (Figure 1(a)). PRL_x polyplex-transfected HEK293 cells produced 3.8-fold ($p=0.042$) higher transgene expression using PRL_{10} polyplexes and 2.9-fold ($p=0.048$) and 2.6-fold ($p=0.066$) higher transgene expression using PRL_5 and

$PRL_{7.5}$ polyplexes, respectively, compared to PRL_0 polyplexes (Figure 1(b)). When the transfection efficiencies of PRL_x polyplexes in MCF7 cells were normalized with that of PRL_{100} polyplexes (i.e., RPLL/pDNA complexes), PRL_0, $PRL_{2.5}$, and PRL_5 polyplexes, 74-fold, 223-fold, and 151-fold higher luciferase expression were observed, respectively (Figure S4). For HEK293 cells, PRL_0, PRL_5, $PRL_{7.5}$, and PRL_{10} polyplexes resulted in 23-fold, 67-fold, 60-fold, and 88-fold better gene expression than RPL_{100} polyplexes (Figure S4). The transfection results suggest that RPLL content in PRL_x polyplexes influences the transfection efficiencies. Neither the fast decomplexation of RPLL/pDNA complexes nor the slow decomplexation of PLL/pDNA complexes appear to be optimal for high ransfection efficiency. It seemed that non-degradable PLL helps to slow decomplexation of RPLL-rich PRL_x polyplexes compared to RPLL polyplexes and that degradable RPLL induces faster decomplexation of PLL-rich PRL_x polyplexes than PLL polyplexes. This led to efforts to determine how the change in RPLL content affects the physicochemical and biological characteristics of PRL_x polyplexes.

Effects of RPLL on particle size, surface charge, and complexation/compactness of PRL_x polyplexes

The compactness and surface charge of pDNA polyplexes are affected by complexation conditions (e.g., dose of polymers, N/P ratios) and polymer characteristics (e.g., architectures, charge density, flexibility, hydrophobicity).[5] Although the MW of RPLL used in this study is similar to that of PLL, the chain structure of RPLL is different from that of PLL (Figure S1). Thus, it is important to determine whether RPLL causes significant changes in particle size, surface charge, and complexation of PRL_x polyplexes compared to those of PLL polyplexes.

The particle size of RPL_0 polyplex was about 100 nm in diameter. Particle sizes were slightly increased with increasing RPLL content in PRL_x polyplexes, growing to about 120 nm for PRL_5 polyplex ($p=0.02$) and then to around 150 nm ($p<0.01$) for PRL_x polyplexes with x=10–50 (Figure 2). It might be due to the weaker pDNA complexation of RPLL compared with PLL resulting from the lower charge density of RPLL (157 Da per one primary amine) than PLL (129 Da per one primary amine). This conclusion is supported by the large particle size (250 nm diameter) of RPL_{100} polyplex.

PRL_0 polyplex had a zeta-potential about 40 mV. Introducing RPLL to PRL_x polyplexes, decreased the surface charge to about 35 mV at x=5 ($p=0.53$) and then reached a saturated value (~20 mV) at x=10–100 ($p<0.01$) (Figure 2). This observation is due to the presence of one carboxylic group associated with the cysteine on each decapeptide unit which negates the positive charge of the primary amine on the lysine. The presence of free carboxylic groups in RPLL provides a possible explanation for the increased particle size of PRL_x polyplexes with higher RPLL content.

Condensation of pDNA in PLL and RPLL polyplexes (N/P 5) prepared in a HEPES buffer was confirmed by a gel electrophoresis method in 0.8% agarose gel (Figure S3). At N/P 5, both cationic polymers completely shielded pDNA on the polyplex surface. In addition, the compactness of pDNA in PRL_x polyplexes was monitored by tracking quenching of YOYO-1- intercalated pDNA. As shown in Figure 3, the fluorescent intensity of unquenched YOYO-1- intercalated pDNA was set to 0% and complete quenching was given a value of 100%. All tested PRL_x polyplexes (x=0–100) showed above 90% compactness of pDNA. The results indicate that different amounts of RPLL did not cause serious difference in the compactness of pDNA in PRL_x polyplexes.

Effects of RPLL in cellular viability and cell uptake of PRL_x polyplexes

The surface charge and particle size of polyplexes influence cellular uptake which can further affect transfection efficiency. Also, excessively high positive surface charges of polyplexes could damage the cell membrane resulting in cellular toxicity and leading to reduced transfection efficiency. Thus, cellular uptake studies of PRL_x polyplexes were performed by flow cytometry in MCF7 and HEK293 cells (Figure 4) and cytotoxicity of PRL_x polyplexes was evaluated by MTT-based cell viability assay (Figure 5).

After forming PRL_x polyplexes (x=0–12.5) with YOYO-1-intercalated pDNA, the cells were incubated with the polyplexes in serum-free and protein-free transfection medium for 4 hr. It was expected that cellular uptake of PRL_0 polyplexes would be much higher than those of other PRL_x polyplexes (x=2.5–12.5) because PRL_0 polyplexes had much higher positive surface charges than other PRL_x polyplexes (x=2.5–12.5). However, with both the cells, PRL_x polyplexes showed similar uptake regardless of the amount of RPLL present (Figure 4). The results indicate that different transfection efficiencies of PRL_x polyplexes (Figure 1) might be not caused by the cellular uptakes of PRL_x polyplexes (Figure 4).

The cytotoxicity of polycations is influenced by their length, charge, degradability, hydrophobicity, and architecture[28] and strongly affects the cytotoxicity, and thus transfection efficiency, of their polyplexes. PLL· HBr with MW 12 kDa showed more toxicity than RPLL 6.7 kDa in MCF7 and HEK293 cells (Figure S5). The IC_{50} of PLL· HBr was about 80 μg/mL for MCF7 cells and about 65 μg/mL for HEK293 cells. RPLL showed negligible cytotoxicity with above 80% cell viability in the tested range of polymer (0~100 μg/mL). Different cytotoxicities of PLL and RPLL might result from different degradation characteristics in intracellular environments because RPLL is degraded much more quickly than RPLL in the cytoplasm and the nucleus.

The cytotoxicity of PRL_x polyplexes during a 48 hr transfection process was evaluated by an MTT-based cell viability assay using MCF7 and HEK293 cells. PRL_0 and PRL_{100} polyplexes had about 88% and almost 100% cell viability against MCF7 cells, respectively. The difference might be caused by lower toxicity of RPLL than PLL. However, cell viability in the presence of PRL_x polyplexes (x=2.5–12.5) differed only negligibly from PRL_0 polyplexes. When applying the polyplexes into HEK293 cells, all tested PRL_x polyplexes (x=0–100) were non-toxic compared to untreated control cells. Thus, the findings indicate that PRL_x polyplex-dependent transfection efficiencies (Figure 1) were not influenced by the toxicity results of PRL_x polyplexes.

Effects of RPLL in decomplexation of pDNA from PRL_x polyplexes

pDNA must be decomplexed from the polyplex within the cell if the encoded protein is to be expressed. If pDNA release is too slow or absent gene expression will hardly occur due a lack of accessibility of transcriptional machinery to pDNA.[5] Also, if polyplexes release pDNA too early, it may not reach the nucleus due to its limited mobility and vulnerability to enzymatic degradation.[5, 19] Although PLL and RPLL had similar MW, their polyplexes caused very different transfection efficiencies. PLL/pDNA complexes (N/P 5) showed 74-fold and 23-fold higher luciferase expression than RPLL/pDNA complexes (N/P 5) in MCF7 and HEK293 cells, respectively (Figure 1 and Figure S4). With the hypothesis of the presence of relationship between the release rate of pDNA from a given system and transfection, decomplexation of pDNA from PRL_x polyplexes were monitored in heparin- and DTT-containing NaCl solution.

First, PRL_x polyplexes (x=0, 5, 10, 20, 50, and 100) were incubated in 150 mM NaCl solution with DTT (20 mM) and different concentrations of heparin (0–100 μg/mL) at 37°C for 1 hr and then were electrophoresed in 0.8% agarose gel with EtBr. The stronger anionic

character of heparin induced the release of weaker anionic pDNA when interacting with polyplexes.[14] When reducible bonds in a polycation exist, DTT degrades disulfide bonds of reducible polymeric backbone. The static interaction strength with pDNA of the fragmented polycations become weaker, resulting in facilitated pDNA release.[14] As shown in Figure 6, 50 μg/mL of heparin induced pDNA exposure of PRL_0 polyplexes (i.e., PLL polyplexes) in the loading well and 75 μg/mL of heparin caused complete decomplexation of pDNA from PRL_0 polyplexes. However, pDNA complexed in PRL_{100} polyplexes (i.e., RPLL polyplexes) was exposed at 0 μg/mL and was released at 10 μg/mL of heparin. With increasing the amount of RPLL in PRL_x polyplexes, the required heparin concentration for complete decomplexation of pDNA was decreased. However, pDNA release from PRL_x polyplexes with x=0–10 is difficult to discern under these experimental conditions due to the static analysis of the experiment.

PRL_x polyplexes (x=0–10) were exposed to a weakly reducing decomplexation solution (i.e., 150 mM NaCl solution with DTT (10 mM) and of heparin (6.5 μg/mL)) at RT and their pDNA release kinetics were monitored by de-quenching of YOYO-1-intercalated pDNA. Compared to the decomplexation kinetics of pDNA from PRL_0 polyplexes, pDNA releases from PRL_x polyplexes (x=2.5–10) were slightly accelerated with increasing RPLL content (Figure 7). These static and dynamic decomplexation results may support the transfection efficiency data of PRL_x polyplexes and also indicate significance of decomplexation optimization regarding release of pDNA from polyplexes.

Transfection results of PRL_x polyplexes (Figure 1) showed the highest transfection efficiency at a different amount of RPLL in PRL_x polyplexes depending on cell type. That is, $PRL_{2.5}$ polyplexes and PRL_{10} polyplexes showed maximum transfection efficiency in MCF7 and HEK293 cells, respectively. This result might be explained by different decomplexation rates of PRL_x polyplexes which are further influenced by cell-dependent intracellular glutathione levels, which may accelerate RPLL degradation and thus pDNA release. In fact, tumor cells may have about 4-fold more intracellular glutathione levels than normal cells.[29] And although tumor cell lines are originated from a identical organ, intracellular glutathione levels are different.[30] Thus, to understand clearly why MCF7 cells required a smaller amount of RPLL in PRL_x polyplexes to reach maximum transfection efficiency than HEK293 cells, intracellular glutathione levels of these two cells were monitored by a GSH-Glo glutathione assay protocol. As shown in Figure 8, MCF7 and HEK293 cells had 51.7 ± 0.7 nmol/25000 cells and 4.0 ± 0.1 nmol/5000 cells, respectively. As expected, MCF7 cells tumor cell line represented higher glutathione contents in the cells than the non-cancerous HEK293 cells. When applying same PRL_x polyplexes, lower intracellular glutathione concentrations may cause slower decomplexation rate of PRL_x polyplexes. Thus, to achieve similar decomplexation rate of PRL_x polyplexes in MCF7 cells, PRL_x polyplexes in HEK293 cells require higher RPLL contents because higher RPLL contents induce faster decomplexation rate of PRL_x polyplexes. These factors help to explain why that $PRL_{2.5}$ polyplexes and PRL_{10} polyplexes reached a maximum transfection efficiency in MCF7 and HEK293 cells, respectively.

In addition, when PLL with higher MWs than 7.4 kDa (used in this study) is applied for PRL polyplexes, RPLL content in the polyplexes should be increased to represent similar decomplexation rate of PLL 7.4 kDa-based PRL polyplexes because higher MW PLL makes stronger binding affinity with pDNA.

Effects of RPLL in intracellular localization of PRL_x polyplexes

After pDNA is internalized into cells, nuclear localization of pDNA is a significant step required for transfection to take place. The polyplexes of polylysine having 28 kDa (about 180 repeating units) delivered pDNA in the nucleus and perinuclear area, whereas

polylysines with 19 and 36 repeating units (about 2.3 kDa and 4.4 kDa, respectively) delivered pDNA in the nucleus.[5, 12] Lower MW polylysine-mediated polyplexes expressed higher transfection efficiency than higher MW polylysine-mediated polyplexes.[12] However, when using PLL with an intermediate MW (e.g., PLL with MW 7.4 kDa used in this study) to form polyplexes, it is unclear whether pDNA is localized only in the nucleus or not. It is also unclear how degradable polymers influence intracellular localization of pDNA.

Thus, to understand these significant localization issues of pDNA delivered with degradable polycations, pDNA was labeled with Cy5 and the nucleus and acidic vesicles (i.e., late endosomes and lysosomes) were stained with Hoechst 33342 and LysoTracker™ Green, respectively. As shown in Figure 9, all tested PRL_x polyplexes-transfected MCF7 cells showed similar distributions of acidic vesicles in the cytoplasm based on similar fluorescent intensity of LysoTracker™ Green. The presence of strong acidic vesicles was caused by weak endosomolytic function of PLL and RPLL. For intracellular distribution of pDNA, it was dependent on RPLL contents in PRL_x polyplexes. In PRL_0, $PRL_{7.5}$, and PRL_{10} polyplexes, pDNA was mostly localized in the cytoplasm and little pDNA was found in the nucleus. However, it seemed that pDNA in $PRL_{2.5}$ and PRL_5 polyplexes distributed almost evenly throughout the cytoplasm and the nucleus.

To clearly understand the nuclear localization of pDNA in PRL_x polyplex-transfected MCF7 cells, the fluorescent intensity of pDNA was separately measured in the whole cell and in the nucleus (Figure 10). When transfecting MCF7 cells with PRL_0 polyplexes, 22% of pDNA was found in the nucleus and the remaining 78% in the cytoplasm and acidic vesicles. Similarly, $PRL_{7.5}$ and PRL_{10} polyplexes-transfected MCF7 cells had around 25% of pDNA in the nucleus. These PRL_x polyplexes (x=0, 7.5, and 10) delivered 3–4-fold more pDNA in the cytoplasm and acidic vesicles than in the nucleus, but, as estimated in confocal microscope, $PRL_{2.5}$ and PRL_5 polyplexes delivered 40~42% of pDNA in the nucleus which is 1.8–1.9-fold more than that of PRL_0 polyplexes. The different nuclear localization of pDNA may cause their different transfection efficiencies.

Most researches for developing effective polymeric gene carriers have focused on new polymers with functionalities (e.g., colloidal stability, receptor-targetability, endosomolytic activity, nuclear translocation, quick pDNA release, and cytocompatibility) that can overcome various extracellular and intracellular hurdles during a journey of polyplexes from an administration site to the nucleus as a subcellular organelle of interest.[1, 2] Although the significance of timely release of pDNA in the cytosol or the nucleus in polymeric transfection efficiency is recognized,[5] the relationship between decomplexation rate, intracellular localization, and transfection efficiency of pDNA delivered with polymeric carriers has been widely ignored or not investigated. This study has shown that the pDNA contents in the nucleus is influenced by decomplexation rates of pDNA from polyplexes, and in turn influences the transfection efficiency of the polyplexes. These findings will give greater understanding in polymer-based gene delivery systems and will help to design effective functionalized polyplexes.

CONCLUSION

Polyplexes prepared with a mixture of PLL and RPLL were used to understand the relationship between decomplexation rate, intracellular localization, and transfection efficiency. Although the size and surface charge of PRL_x polyplexes were influenced by the RPLL content, these characteristics did not influence cellular uptake of PRL_x polyplexes. However, the highest transfection efficiency of PRL_x polyplexes in this study may be caused by an optimal decomplexation rate (faster than that of PLL polyplexes and slower than that of RPLL polyplexes) and more nuclear localization of pDNA. These findings will help to

design decomplexation-tunable polyplexes made of various combinations of non-degradable and degradable polymers.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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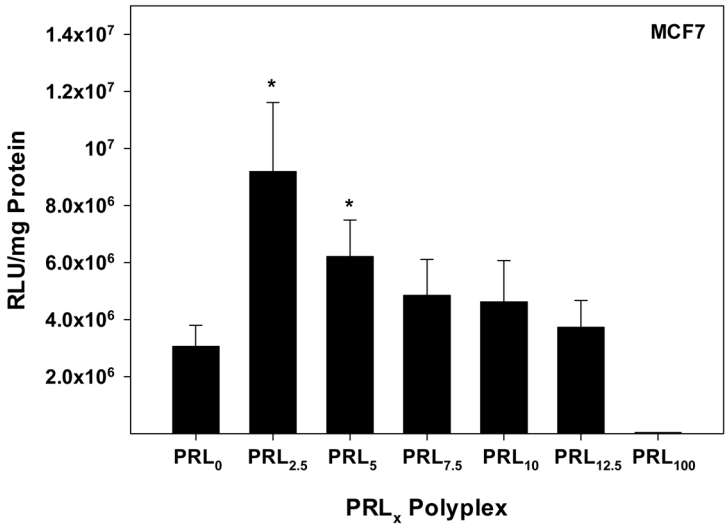

Figure 1(a)

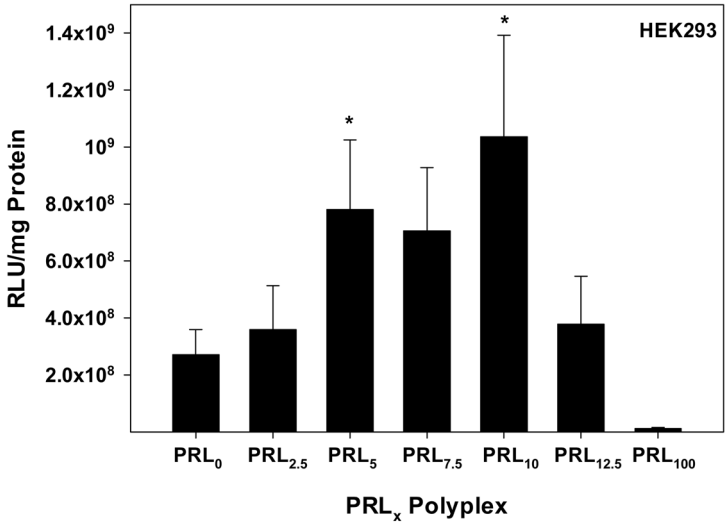

Figure 1(b)

Figure 1.

Transfection efficiencies of $PLL_{100-x}RPLL_x$/pDNA complexes (PRL_x polyplexes) (N/P 5) in (a) MCF7 cells and (b) HEK293 cells. PLL/pDNA and RPLL/pDNA complexes were denominated as PRL_0 and PRL_{100} polyplexes, respectively. * means $p<0.05$ compared with PRL_0 polyplex by unpaired Student t-test. (Mean ± Standard Error; $n\geq4$)

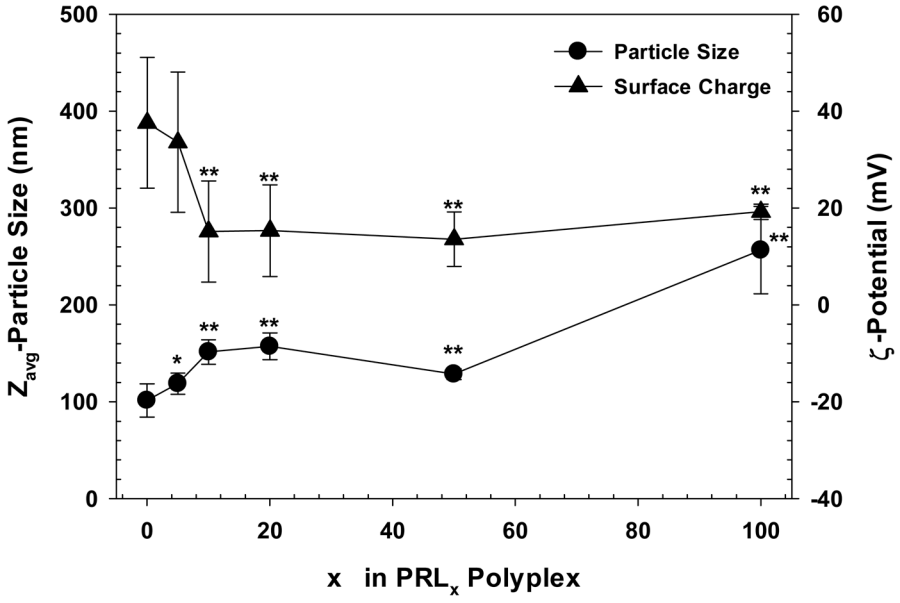

Figure 2.

Particle size and surface charge of PRL_x polyplexes (N/P 5) in HEPES buffer (25 mM, pH 7.4). * and ** mean $p<0.05$ and $p<0.01$, respectively, compared with PRL_0 polyplex by unpaired Student t-test. (Mean ± Standard Deviation; n = 10)

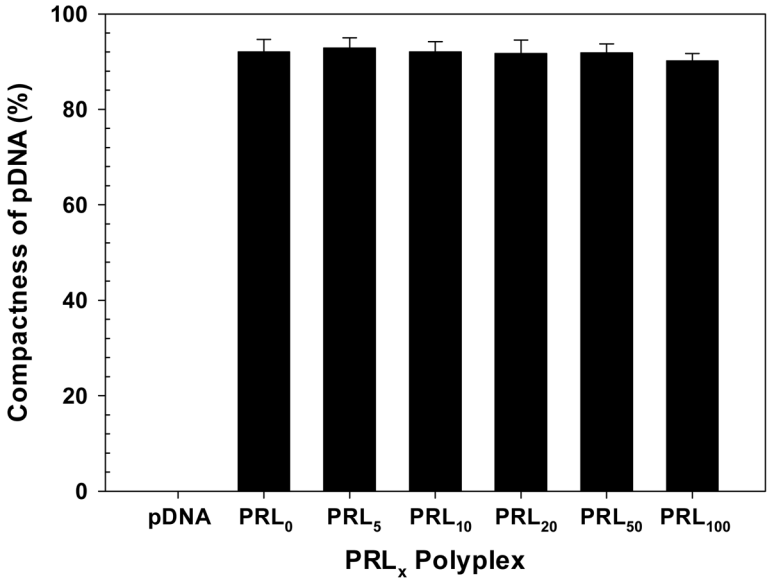

Figure 3.
Compactness (%) of pDNA in PRL_x polyplexes (N/P 5) prepared in a HEPES buffer (25 mM, pH 7.4). (Mean ± Standard Deviation; n = 3)

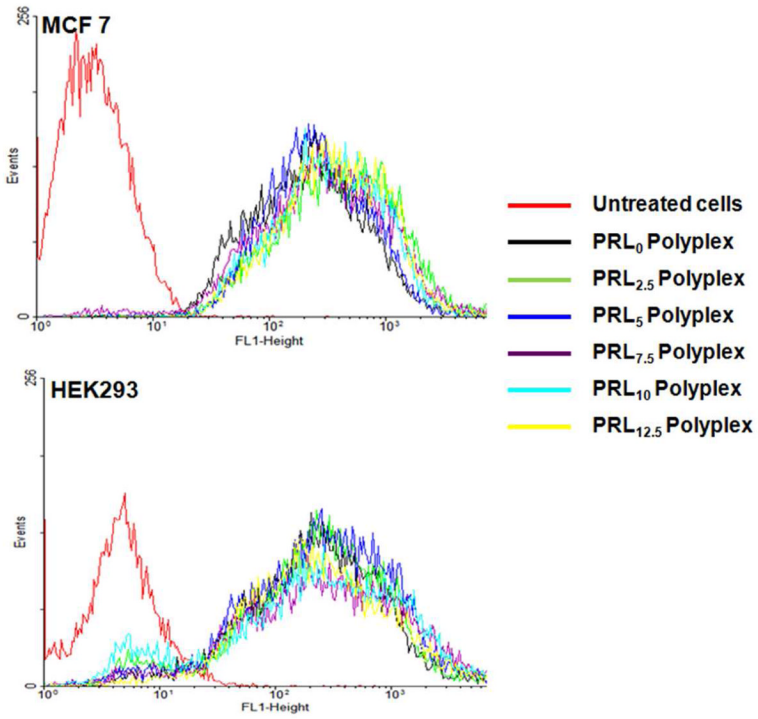

Figure 4.
Cellular uptake of PRL_x polyplexes (N/P 5) after 4 hr transfection in (a) MCF7 and (b) HEK293 cells.

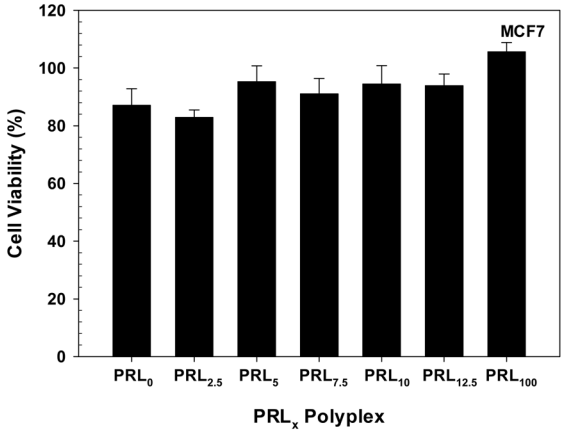

Figure 5(a)

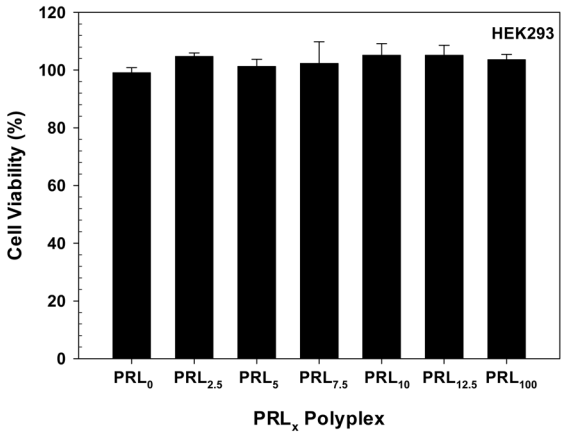

Figure 5(b)

Figure 5.
Cytotoxicity of PRL_x polyplexes (N/P 5) after 48 hr transfection in (a) MCF7 and (b) HEK293 cells. (Mean ± Standard Error; n = 6)

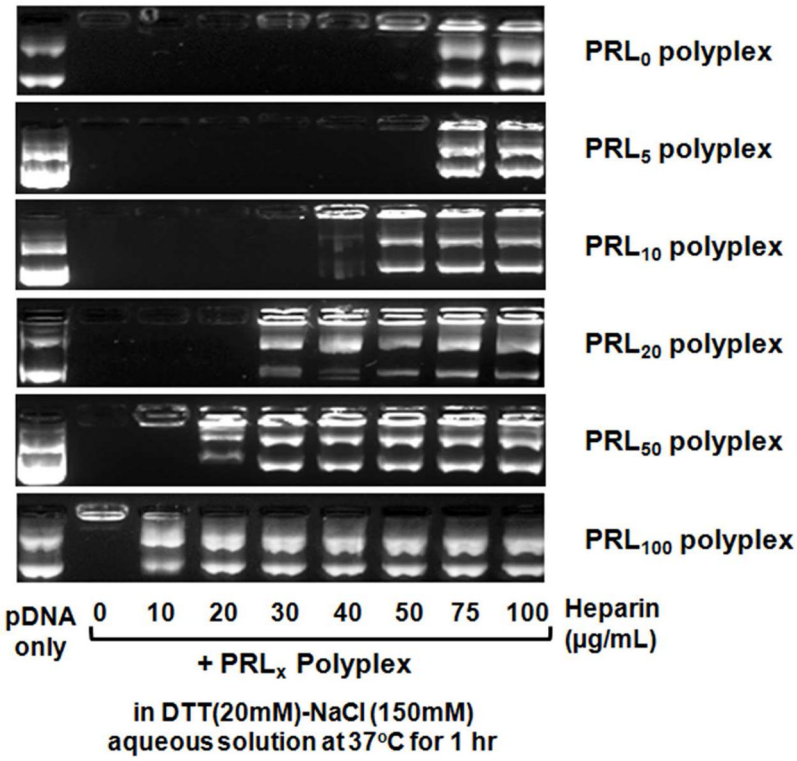

Figure 6.

Decomplexation (or pDNA release) of PRL_x polyplexes (N/P 5). After the polyplexes with pDNA (25 μg/mL) were exposed to DTT (20 mM) and heparin (0–100 μg/mL) in 150 mM NaCl aqueous solution at 37°C for 1 hr, the polyplex solution was loaded into 0.8% agarose gel and then was electrophoresed at 80 V for 90 min.

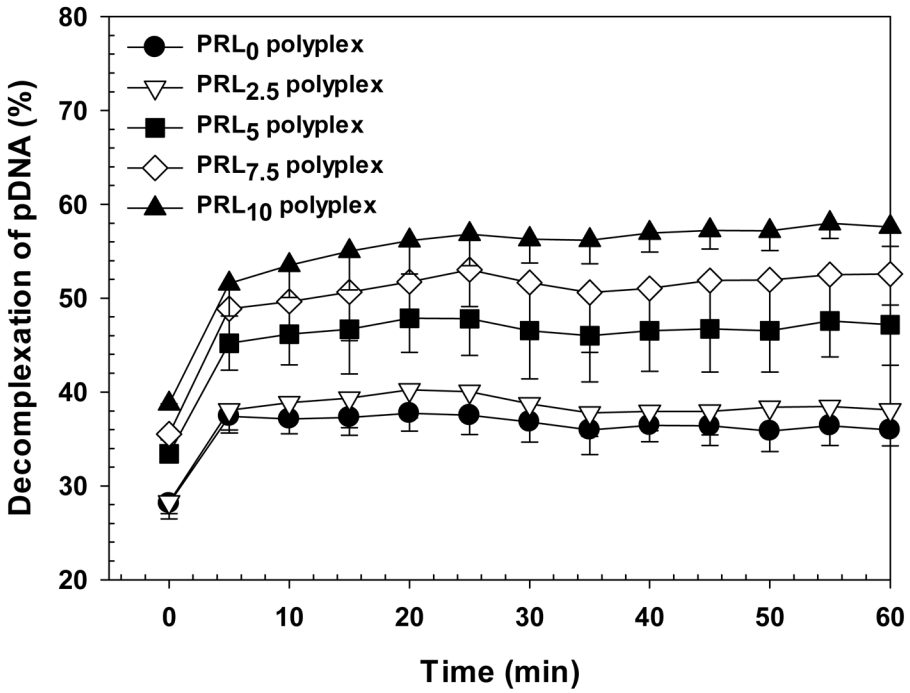

Figure 7.

Decomplexation (or pDNA release) kinetics of PRL_x polyplexes (N/P 5) using YOYO- 1-labeled pDNA. The polyplexes with pDNA (5 μg/mL) were exposed to DTT (10 mM) and heparin (6.5 μg/mL) in 150 mM NaCl aqueous solution (0.2 mL) at RT. (n = 3; Mean ± Standard Deviation).

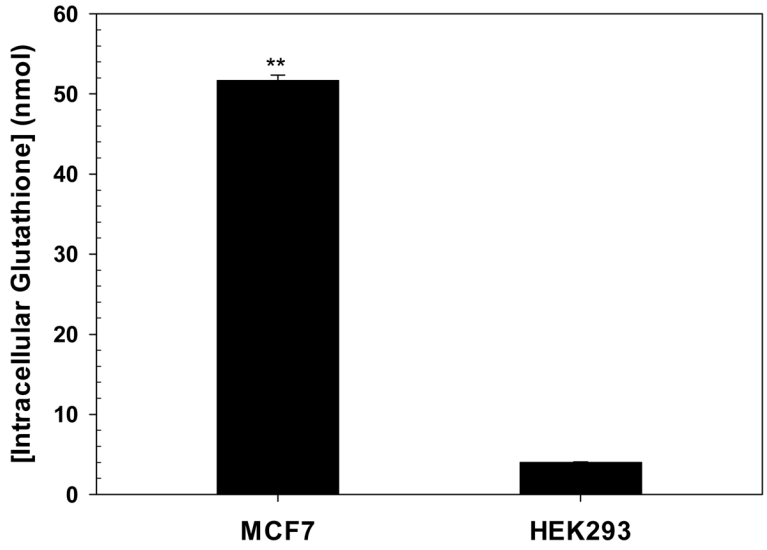

Figure 8.

Intracellular glutathione levels of MCF7 and HEK293 cells. After seeding 25000 or 5000 cells into a well for MCF7 or HEK293 cells, the cells were culture for 24 hr and then were evaluated. ** means $p<0.01$ compared with HEK293 cells by unpaired Student t-test. (Mean ± Standard Error; n = 3)

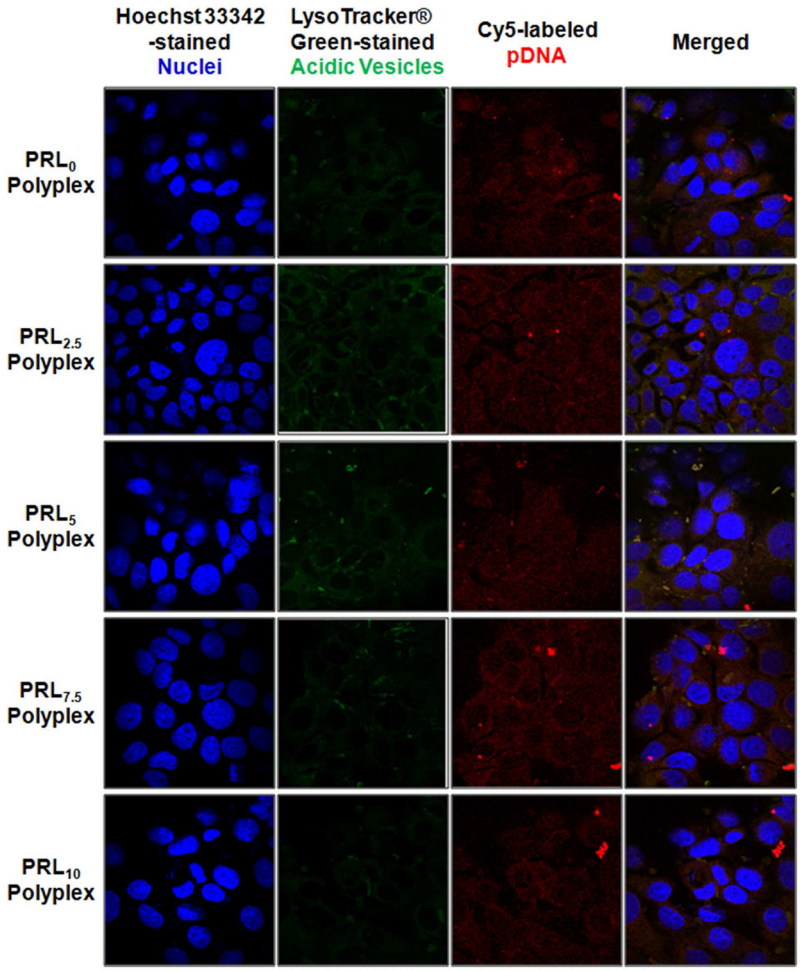

Figure 9.
Intracellular distribution of Cy5-labeled pDNA delivered by PRL_x polyplexes (N/P 5) at 4 hr post-transfection in MCF7 cells.

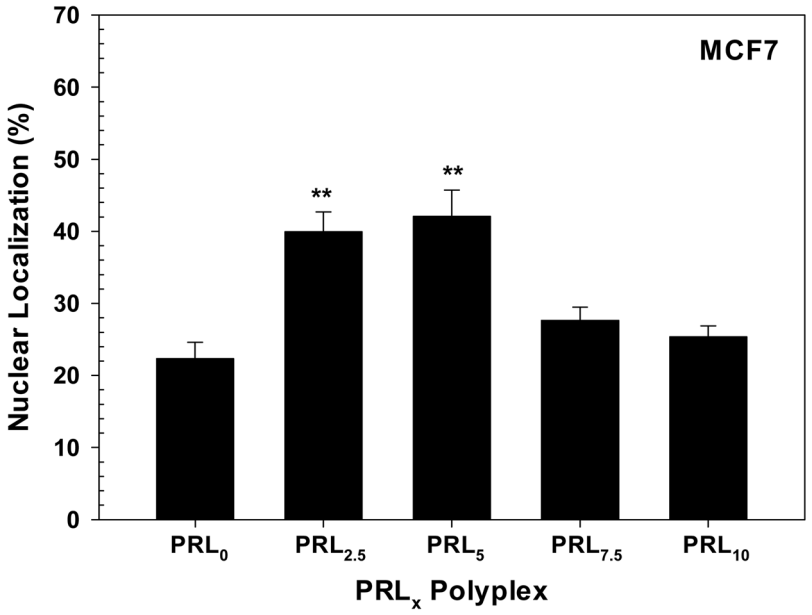

Figure 10.

Nuclear localization of Cy5-labeled pDNA delivered by PRL_x polyplexes (N/P 5) at 4 hr post-transfection in MCF7 cells. Nuclear localization was calculated using relative fluorescence intensity ratio in cell and the nucleus (n = 10; Mean ± Standard Error).