

RESEARCH ARTICLE

Enhanced cytosolic delivery of plasmid DNA by a sulfhydryl-activatable listeriolysin O/protamine conjugate utilizing cellular reducing potential

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Listeriolysin O (LLO), a sulfhydryl-activated pore-forming protein from *Listeria monocytogenes*, was tested and utilized for promoting plasmid DNA (pDNA) delivery into the cytosol of cells in culture. To render pDNA-complexing capability to LLO, the unique cysteine 484 of LLO was conjugated to polycationic peptide protamine (PN) at a 1:1 molar ratio through a reversible, endosome-labile disulfide bond. The sulfhydryl-oxidized LLO construct, LLO-s-s-PN, completely lacked its pore-forming activity, yet regained its original activity upon reduction. The enhanced cytosolic delivery using this construct therefore relies on the requisite reduction of the disulfide bond in LLO-s-s-PN by endogenous cellular reducing capacity. Condensed PN/pDNA complexes incorporating LLO-s-s-PN were tested for their enhanced gene

delivery capability monitoring reporter gene expression in HEK293, RAW264.7, P388D1 cell lines and bone-marrow-derived macrophages in the presence of serum. Dramatic enhancement was observed for all tested complexes with varying weight ratios. The effect was most prominent at 0.64–0.80 (w/w) of PN/pDNA upon replacing 1–4% of PN with LLO-s-s-PN, resulting in approximately three orders of magnitude higher luciferase expression compared to PN/pDNA without apparent toxicity. These results demonstrate that incorporation of endosomolytic LLO into pDNA delivery systems in a controlled fashion is a promising approach of enhancing delivery into the cytosol of target cells in gene delivery strategies.

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Introduction

An expansion of gene therapy applications is a logical consequence of the recent rapid increase in genomic information. The development of efficient non-viral gene delivery systems that can be widely used as an integral part of therapeutic tools, however, is yet to be satisfied despite extensive efforts in the field. One of the most critical drawbacks of non-viral delivery approaches, in comparison with viral vector systems, is their limited transfection efficiency.

Multiple key steps are considered crucial in improving the efficiency of non-viral gene delivery systems. In order to increase the cellular uptake efficiency, cationic polymers and lipids, often with targeting moieties, have been typically used to neutralize and compact polyanionic, large molecular weight plasmid DNA (pDNA).^{1–6} Although these approaches improve the extent of total cellular uptake of pDNA, the low transfection efficiencies and limited effectiveness of the existing non-viral gene delivery systems are largely due to the subsequent cellular processes following the uptake: the transport of genes across cell membranes into the cytosol and eventually into the nucleus. Since most existing non-viral gene delivery schemes deliver pDNA to the

endosomal/lysosomal compartments, likely resulting in rapid degradation of pDNA without overcoming the membrane barrier, significant studies have been devoted to investigating new agents that can breach the membrane barrier, particularly the membranes of the endocytic compartment.^{3,7} One noteworthy strategy is the employment of biological components from viral mechanisms of cell invasion. Either a whole endosome-disrupting adenovirus or viral fusogenic peptides have been conjugated to a non-viral component, reporting a significant increase in the gene expression.^{7–9} Similar membrane-disrupting or fusion-promoting natural or synthetic peptides have been designed and tested.^{10,11} However, these systems often require a large amount of peptides per pDNA complex in order to be effective, partially explaining their limited reports of successes *in vivo*.¹²

Viruses are not the only species that evolved and refined the ability to carry their genomic contents across the membrane barrier of host cells. Several intracellular bacteria have also evolved to possess the mechanism to invade into the cytosol or intracellular compartments of cells,¹³ and thus recently have been utilized to transfer foreign genes. These include genetically engineered *Listeria*,¹⁴ *Shigella*,¹⁵ and *Salmonella*.¹⁶ These bacterial vectors in their present forms share several shortcomings with viral vectors, including potentially high immunogenicity, pathogenicity, and difficulty in targeting and pharmaceutical manufacturing.¹⁷

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For our new gene delivery system reported here, we have exploited the endosomolytic mechanism of LLO from *Listeria monocytogenes* in order to design and develop a non-viral, non-bacterial vector mimicking the genetically engineered *Listerial* gene delivery vector.¹⁴ LLO, a member of the sulfhydryl-activated cytolysin family,¹⁸ is secreted by *Listeria* to breach the phagosomal membranes and to invade into the cytosol, a niche for this intracellular pathogen.¹⁹ Hence, the mechanism of LLO-mediated transfer of macromolecules or particulates is fundamentally different from that of virus-based endosomolytic agents in that LLO is capable of delivering the whole micrometer-sized bacterial particle, which is much larger than the size of a naked or condensed pDNA, from the phagosome to the cytosolic space.¹⁹ A previous study of an enhanced gene delivery system using a similar pore-forming hemolysin, perfringolysin O, conjugated to polylysine/pDNA by a biotin-streptavidin bridge, supports the feasibility of this approach.²⁰ One intriguing feature of the members of sulfhydryl-activated hemolysins, as implied by the name, is that the hemolytic activity of these proteins is abolished upon oxidation of the sulfhydryl group of a unique cysteine amino acid residue in a conserved domain near the C-terminus;^{21–23} an LLO molecule contains only one cysteine at the amino acid position 484 (C484). Therefore, the C484 serves not only as an ideal site for chemical modification and conjugation using sulfhydryl-specific reaction, but also as a convenient regulator of the hemolytic activity. To this LLO C484, we have reversibly conjugated a modified sulfhydryl-reactive protamine (PN) through a disulfide bond at a 1:1 molar ratio. PN, the polycationic moiety of the resulting construct, can complex with pDNA and thus incorporate LLO to the pDNA condensed with PN. The disulfide bond, which is susceptible to cleavage in the environment of endocytic compartments, allows a control of LLO activity in such a way that LLO is active only after the complex is internalized into the endocytic compartments of cells.

Previously, protein co-encapsulated with LLO molecules in pH-sensitive liposomes (*listeriosomes*) was employed in protein delivery into the cytosol of macrophages *in vitro*.²⁴ The LLO-mediated cytosolic delivery of macromolecules has been recently demonstrated to be feasible also in animal models. The *listeriosomes* tested in mice showed strong enhancement in inducing specific cytotoxic T lymphocyte activity against the model antigenic protein.^{24a} In this paper, an LLO-based delivery strategy was tested for enhanced delivery of pDNA into the cytosol. We show that reversibly conjugating LLO to a PN/pDNA complex dramatically enhances the reporter gene transfection efficiency in cell culture models, providing the proof-of-concept of overcoming the endosomal membrane barrier by the use of *Listerial* cell invasion mechanism.

Results

Synthesis and characterization of LLO-s-s-protamine (LLO-s-s-PN)

LLO-s-s-PN was prepared using the synthesis scheme depicted in Figure 1a, which was designed to link LLO and PN via a disulfide bond at a 1:1 molar ratio. As the

PN, an arginine-rich, no lysine-containing cationic polypeptide, has no primary amines that can be utilized for attaching a sulfhydryl-reactive group,²⁵ the only carboxyl group in the C-terminus was used for site-specific conjugation. First, 2-(2-pyridyldithio)ethylamine (PDA) was synthesized by reacting 2-2'-dithiodipyridine and β -mercaptoethylamine hydrochloride, followed by purification with recrystallization. PDA was then coupled to the C-terminal carboxyl group of PN using EDC to yield PN-s-s-pyridine. PN-s-s-pyridine was subsequently reacted with the C484 of LLO. When the reaction mixture was separated using a heparin column with a linear NaCl gradient, LLO, LLO-s-s-PN and PN-s-s-pyridine eluted at 0.7, 1.3 and 1.8 M, respectively (Figure 1b). Two successive affinity columns, therefore, allowed an efficient separation of LLO-s-s-PN from the mixture; excess PN-s-s-pyridine was first removed by a Ni-NTA column, utilizing the histidine-tag moiety present both in LLO and in LLO-s-s-PN molecules, and unreacted free LLO was then separated from LLO-s-s-PN by a heparin column that binds strongly to the cationic PN moiety of the conjugate.

LLO-s-s-PN migrated as a single band (~62 kDa) in non-reducing SDS-PAGE, slightly slower than free LLO (58 kDa) (Figure 2a). Addition of a reducing agent, β ME, in the gel sample buffer shifted the band to 58 kDa, indicating cleavage of the disulfide bond in LLO-s-s-PN. The presence of PN in LLO-s-s-PN was confirmed by urea-based gel electrophoresis with reversed polarity (Figure 2b). A PN band was not detected in a non-reducing gel as LLO-s-s-PN remained intact and stayed in the well, while it migrated as a small molecular weight band, separate from LLO, in a reducing gel. The activity of the chemically modified LLO in LLO-s-s-PN was assayed by monitoring its hemolytic activity toward red blood cells (RBCs) (Figure 2c). The high sensitivity of this assay allows detection of active LLO as low as 2 ng/ml. Addition of 2 μ g LLO to RBCs caused immediate RBC lysis (A, Figure 2c). In comparison, 2 μ g of LLO-s-s-PN, as the C484 of LLO was oxidized and disulfide-bonded, exhibited no detectable LLO hemolytic activity (B, Figure 2c). However, LLO activity in LLO-s-s-PN was fully regained when a reducing agent was present in the assay buffer, after a lag time of several minutes (C, Figure 2c). Similar results were obtained using LLO-s-s-PN complexed with pDNA. When 2 μ g pDNA complex (PN/pDNA w/w ratio = 0.8 with ~1% conjugate) was added to 2 ml of 2×10^8 RBC, which is comparable in dose to the condition used for transfection studies reported below, the hemolysis was similar to that of (B) in Figure 2 with a slightly longer lag time (~500 s) after DTT addition, and no RBC hemolysis without DTT.

We additionally examined the effect of serum on reduction of the disulfide bond in LLO-s-s-PN *in vitro*. RBCs were resuspended in 10% FBS-containing DMEM and incubated with LLO-s-s-PN. Similarly, the conjugate showed activity only in the presence of DTT while no activity was observed for a 4 h period in the absence of DTT (data not shown). The presence of 10% FBS overall retarded and somewhat suppressed the hemolytic activity. The long-term stability of the conjugate, LLO-s-s-PN, was also monitored by the hemolysis assay performed on the samples stored in HBG at 4°C over a 3-month period. No decay of hemolytic activity was observed (data not shown).

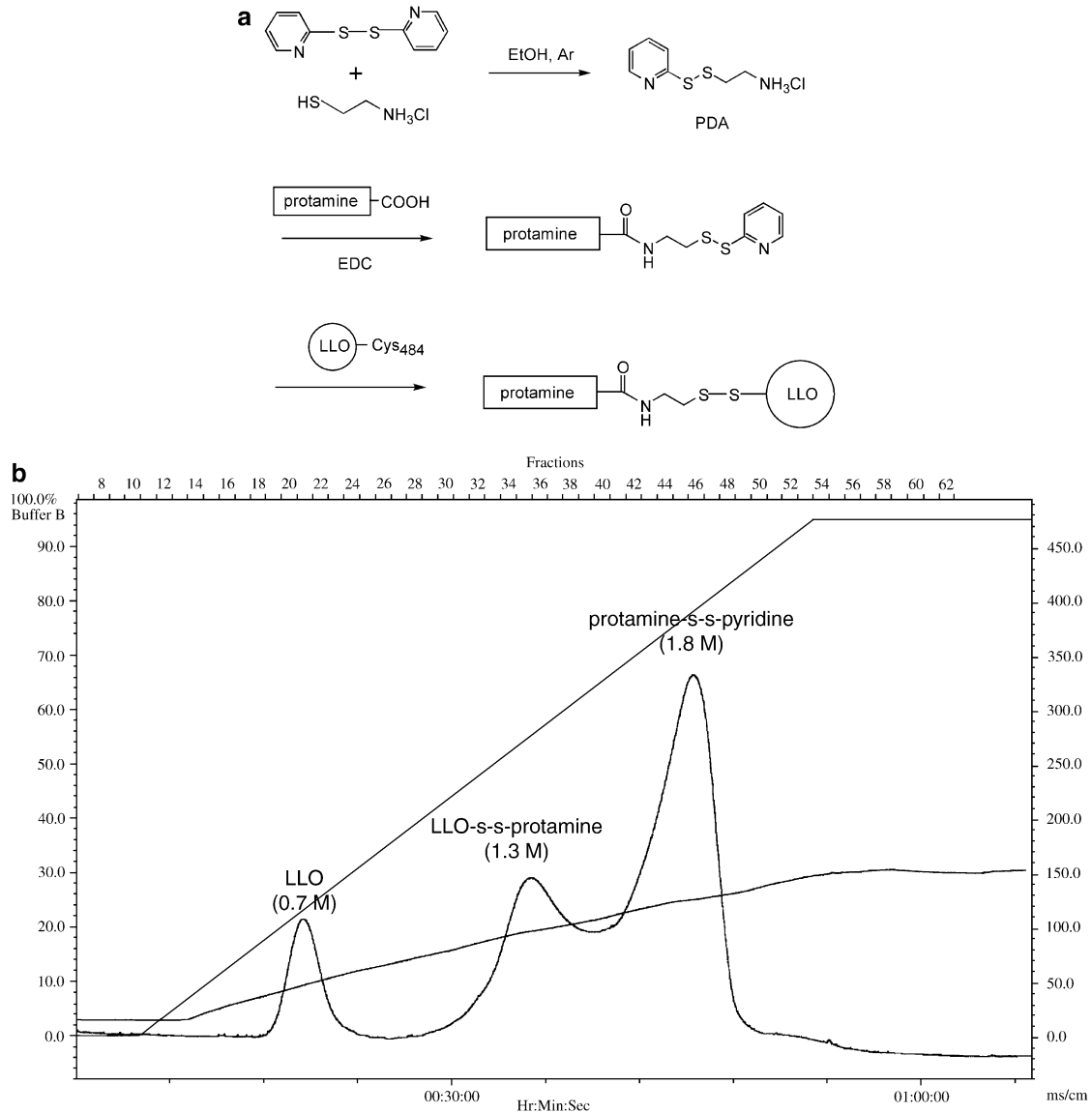


Figure 1 (a) Synthesis scheme of LLO-s-s-PN conjugate. PN-s-s-pyridine was synthesized by attaching 2-(2-pyridylthio)ethylamine (PDA) to the C-terminus of PN using EDC as a coupling agent. The single cysteine 484 of LLO was reversibly conjugated to the modified PN through a disulfide bond to give LLO-s-s-PN. (b) Purification of LLO-s-s-PN. LLO-s-s-PN was purified using two successive affinity columns on FPLC. The chromatogram shows a linear NaCl gradient elution profile from a heparin column of unreacted LLO, LLO-s-s-PN, and excess PN-s-s-pyridine. The latter two peaks were further separated by a Ni-NTA column. Values in parentheses indicate the concentration of NaCl at elution.

Complex formation of PN, LLO-s-s-PN and pDNA

PN/pDNA complexes possessing zeta potentials ranging from negative to positive were prepared by increasing the amounts of PN with constant amounts of pDNA in order to study the influence of polycation/pDNA charge ratio on LLO-s-s-PN-mediated gene transfer efficiency. Zeta potential and particle size of the complexes having PN/pDNA weight ratios of 0.3–2.1 were analyzed (Figure 3a). Throughout all the weight ratios tested, the complexes exhibited an average size of approximately 100 nm, although complexes with zeta potential around charge neutrality exhibited a tendency to aggregate in accordance with previous reports.⁴ Agarose gel retardation assay clearly supported that PN bound to pDNA, neutralized the negative charges, and retarded its electrophoretic mobility in the agarose gel (Figure 3b);

the effect was proportional to the amount of PN added, and complete retardation was observed at a weight ratio of 0.80. These observations are consistent with reported results of ethidium bromide exclusion assay, which examined the exclusion of the DNA-intercalated dye upon addition of PN and condensation of pDNA.²⁶

In order to increase the probability of LLO and condensed pDNA being co-internalized into the endocytic compartment, it is important that the LLO-s-s-PN conjugate stays with the condensate when incorporated in the PN/pDNA complex. To examine whether LLO-s-s-PN complexes with pDNA in the presence of excess PN, three complexes of negative, neutral or positive zeta potential were prepared at PN/pDNA weight ratios of 0.64, 0.80 or 2.14, respectively, with a fixed 1 µg of LLO-s-s-PN which corresponds to 1.2% of overall PN used to

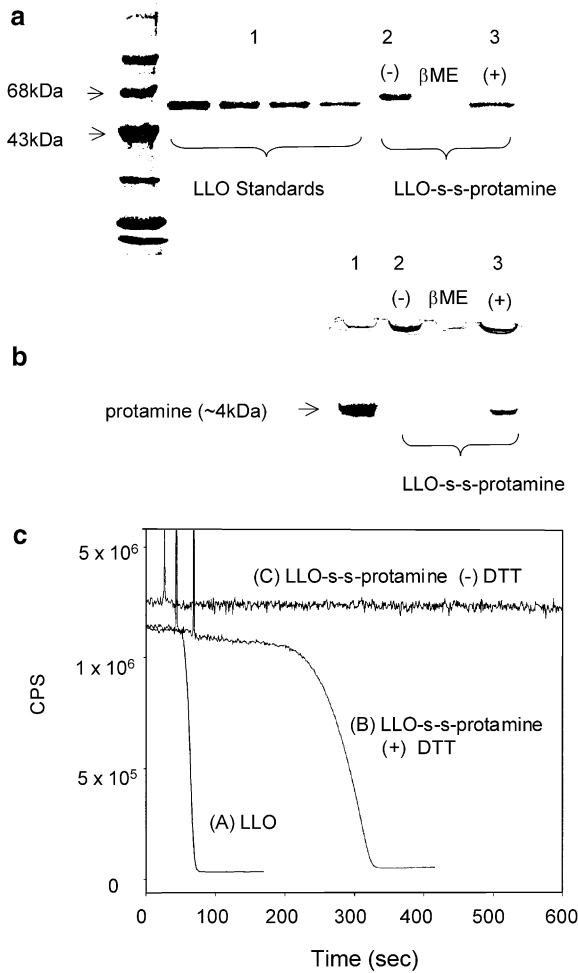


Figure 2 (a) SDS-PAGE of LLO and LLO-s-s-PN with and without βME. Purified LLO-s-s-PN migrated as one band on non-reducing SDS-PAGE (~62 kDa, lane 2), slightly slower than free LLO (58 kDa, lanes 1). The concentration of LLO-s-s-PN was determined by quantitative image analysis utilizing known amounts of LLO standard (lanes 1). The disulfide bond in LLO-s-s-PN was cleaved in the presence of βME in the sample buffer, resulting in free LLO and PN (lane 3). (b) Reverse polarity urea-based gel electrophoresis of PN and LLO-s-s-PN with and without βME. Presence of PN in LLO-s-s-PN was detected by urea-based gel electrophoresis with βME in the sample buffer (lane 3, PN standard: lane 1). LLO-s-s-PN was retained in the well without βME (lane 2). (c) Hemolytic activity assay of LLO and LLO-s-s-PN. To 2 ml of sheep red blood cells (in (A), (B): HBS, pH 7.4, (C): HBS or 10% serum-containing DMEM), (A) 2 μg of LLO, (B) 2 μg of LLO-s-s-PN with 5 mM DTT, (C) 2 μg of LLO-s-s-PN without DTT were added. Dynamic changes in the right-angle light scatter of red blood cells undergoing lysis were measured in a fluorometer.

achieve a weight ratio of 0.80. When LLO-s-s-PN alone was layered on a single-step sucrose gradient (30% sucrose) and ultracentrifuged, LLO activity remained in the upper layer (data not shown). In contrast, when complexes were formed at a weight ratio of 0.64 or 0.80 in HBG or in HBS for 4 h and centrifuged, the activity was found only in the sucrose layer. This strongly indicates that LLO-s-s-PN participated in the complex formation and sedimented into the 30% sucrose layer together with PN/pDNA complex. On the other hand, positive complexes formed at the ratio of 2.1 showed significantly reduced activity sedimenting into the

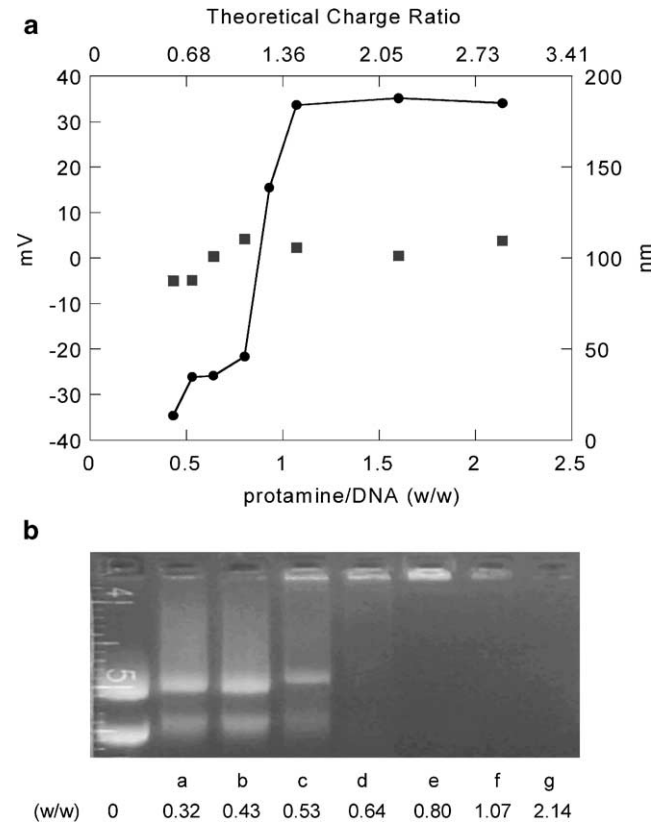


Figure 3 (a) Particle size and zeta potential analysis of PN/pDNA complexes at varying PN/pDNA weight ratios. PN/pDNA complexes were formulated in HBG. Theoretical charge ratios were calculated assuming 3.1 nmol of phosphate negative charge per microgram of pDNA and 4.2 nmol of positive charge per microgram of PN (21 arginine residues per PN sulfate, MW 5 kDa). All the data points are averages of two representative independent measurements. The particle size and zeta potential are indicated as square and circle symbols, respectively. (b) Gel retardation assay of PN/pDNA complexes. PN/pDNA complexes were formulated at the indicated weight ratios in HBG. Complexes were electrophoresed in an ethidium bromide-containing 1.0% agarose gel.

sucrose layer, implicating diminished association of LLO-s-s-PN to PN/pDNA complexes beyond the charge neutral point where the negative phosphate charges of pDNA are occupied with free excess PN (data not shown).

Effect of LLO-s-s-PN on gene expression in cultured cells

To investigate the effect of LLO-s-s-PN on gene transfer of PN/pDNA complexes with different charge ratios, PN/LLO-s-s-PN/pDNA complexes incorporating fixed amounts of LLO-s-s-PN and pDNA with varying amounts of PN were prepared. The weight ratios of the total PN, free PN plus PN in LLO-s-s-PN, to pDNA used were the same as those in the previous section. The efficiency of gene transfer and gene expression was investigated using luciferase and green fluorescent protein (GFP) reporter genes. A dramatic enhancement of luciferase expression was observed upon LLO-s-s-PN incorporation for all the tested weight ratios of PN to pDNA (Figure 4a). An enhanced LLO effect was observed with increasing amounts of PN up to the

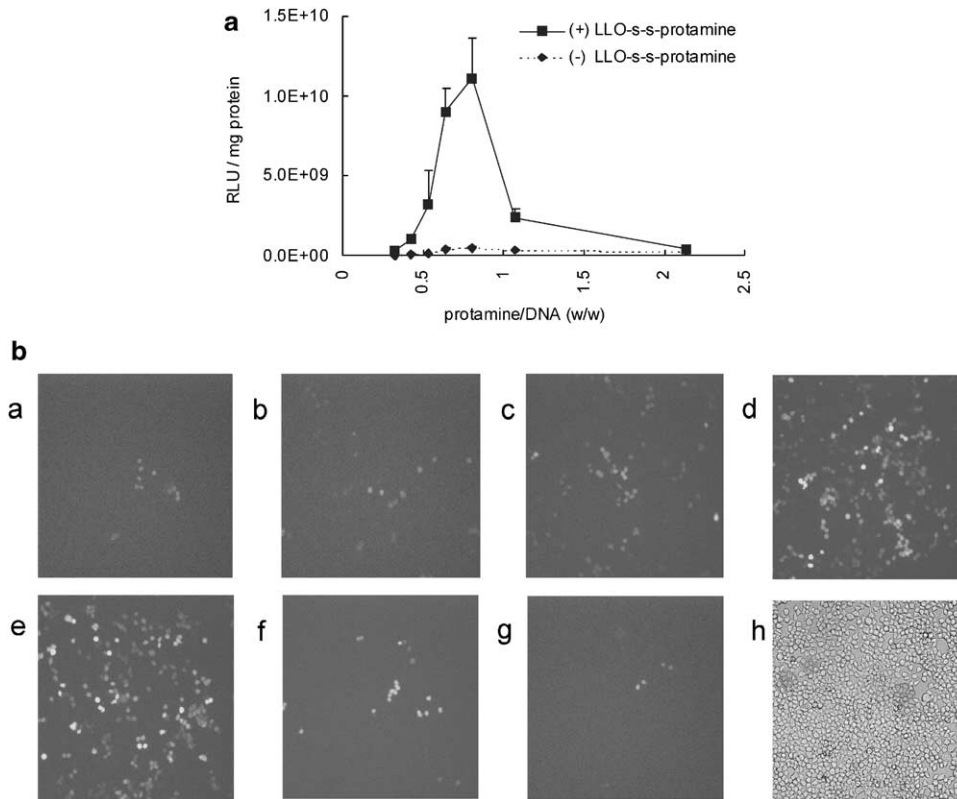


Figure 4 (a) Effect of LLO-s-s-PN on the luciferase gene expression in HEK293 cells at varying PN/pDNA weight ratios. Solid line: luciferase expression 2 days post-transfection using LLO-s-s-PN-containing formulations; LLO-s-s-PN/PN/pDNA complexes incorporating fixed amounts of LLO-s-s-PN (0.06 μg) and pDNA (0.5 μg) and increasing amounts of PN. Dotted line: luciferase expression using control formulations; PN/pDNA complexes without LLO-s-s-PN. All transfection was performed in serum-containing media. (b) LLO-s-s-PN-mediated GFP gene expression in HEK293 cells at varying PN/pDNA weight ratios. LLO-s-s-PN-mediated GFP expression 2 days post-transfection using the LLO-s-s-PN-containing formulations as in (a). PN/pDNA weight ratios for each formulation (a–g) are indicated in Figure 3b. h shows a typical transmission image of cells 2 days post-transfection.

weight ratio of 0.80, which is close to charge neutrality as shown by the zeta potential result (Figure 3a). However, addition of PN beyond this weight ratio resulted in the decline of gene expression. GFP gene expression using complexes at corresponding weight ratios is shown in Figure 4b. Complexes with weight ratios between 0.64 and 0.80 yielded strong GFP expression levels in 30–40% of cells, the highest level achieved under these conditions. In contrast, few green cells were observed when cells were incubated with PN/pDNA complexes containing no LLO-s-s-PN.

In order to investigate the dependence of gene transfer on the dose of LLO-s-s-PN relative to PN/pDNA and to estimate the optimal number of LLO-s-s-PN per complex, 0–12% of PN was replaced with LLO-s-s-PN, keeping the constant ratio of total PN to pDNA at 0.80 (w/w) (Figure 5). Luciferase expression was enhanced by incorporating increasing amounts of LLO-s-s-PN in a dose-dependent fashion in all three cell lines tested. Replacing 1.2% of PN with LLO-s-s-PN produced the highest luciferase expression in HEK293 and RAW264.7 cells. For P388D1 cells, the optimal transfection was achieved at 3.6% substitution. A similar study was carried out for PN/pDNA complexes at a weight ratio of 0.64 in HEK293 cells. The trend was also similar, with 1.2% substitution giving the highest expression (data not shown). No cytotoxicity was observed at these ranges, but increasing the LLO-s-s-PN amount beyond 6% of

total PN resulted in cytotoxicity for all the cell lines, as documented by reduced recovery of total cellular protein. In contrast, when free LLO, comparable in amount to the LLO in 1.2% LLO-s-s-PN substitution, was added to cells together with PN/pDNA complexes, cells started to lyse as early as in 5 min reaching 40% LDH release at 30 min incubation, and with no detectable increase in the luciferase/GFP expression level (data not shown).

Luciferase expression for bone-marrow-derived macrophages (BMM) was also significantly enhanced, typically producing $\geq 0.5 \times 10^7$ RLU/mg at 0.5–2% LLO-s-s-PN incorporation, whereas the control PN/pDNA complexes produced $\leq 1 \times 10^4$ RLU/mg. Recovery of total cellular protein, however, was poor (50–60% of control), and GFP-positive BMMs were typically $\leq 1\%$. While the other cell lines showed no apparent cytotoxicity within these LLO-s-s-PN dose ranges, some BMM started to form blebs about 1 h after transfection, producing delayed LDH leakage which started to appear after 1 h (data not shown).

Using a condition within the optimized range (PN/pDNA = 0.80 w/w with 1.2% of PN replaced with LLO-s-s-PN), LLO-s-s-PN-mediated GFP expression could be observed as early as 9 h post-transfection in HEK293 cells at low intensity (10% of maximum intensity), reached close to the maximum at 24 h, and stayed at that level up to 72 h at which point cells reached confluency (data not

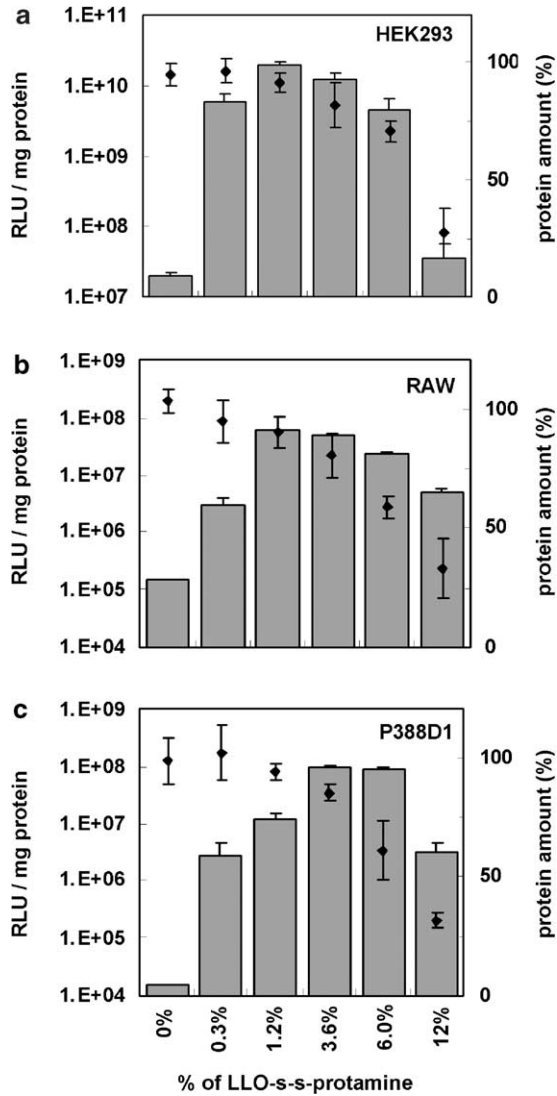


Figure 5 Effect of increasing amounts of LLO-s-s-PN on luciferase expression in three different cell lines. Using the optimal weight ratio of PN/pDNA determined in Figure 4a, varying amounts of PN were replaced with LLO-s-s-PN from 0% to 12% keeping the constant ratio of total PN to pDNA (PN/pDNA=0.8 w/w). HEK293 cells were incubated with 0.5 μ g DNA/well, and RAW264.7 or P388D1 cells with 1 μ g DNA/well. Luciferase activity in the cell lysates was analyzed 2 days post-transfection. Dots indicate the total cellular protein content recovered per well 2 days post-transfection. All transfection was performed in serum-containing media.

shown). To determine the dose dependency of this gene transfer system, the complexes were serially diluted from 1 μ g DNA/well to 30 ng DNA/well in HEK293 cells. Strong luciferase expression was detected even at such low doses (Figure 6b). The expression was dose-dependent in close to a linear fashion up to 0.25 μ g/well, beyond which saturation of the system was observed.

To evaluate the efficacy of these optimized formulations, LLO-mediated gene transfer was compared with commonly used non-viral transfection methods (Figure 6a). Chloroquine (CQ) is known to buffer the acidic environment of endosome and thereby enhances the transfection mediated by pDNA or polyplexes.⁷ Our

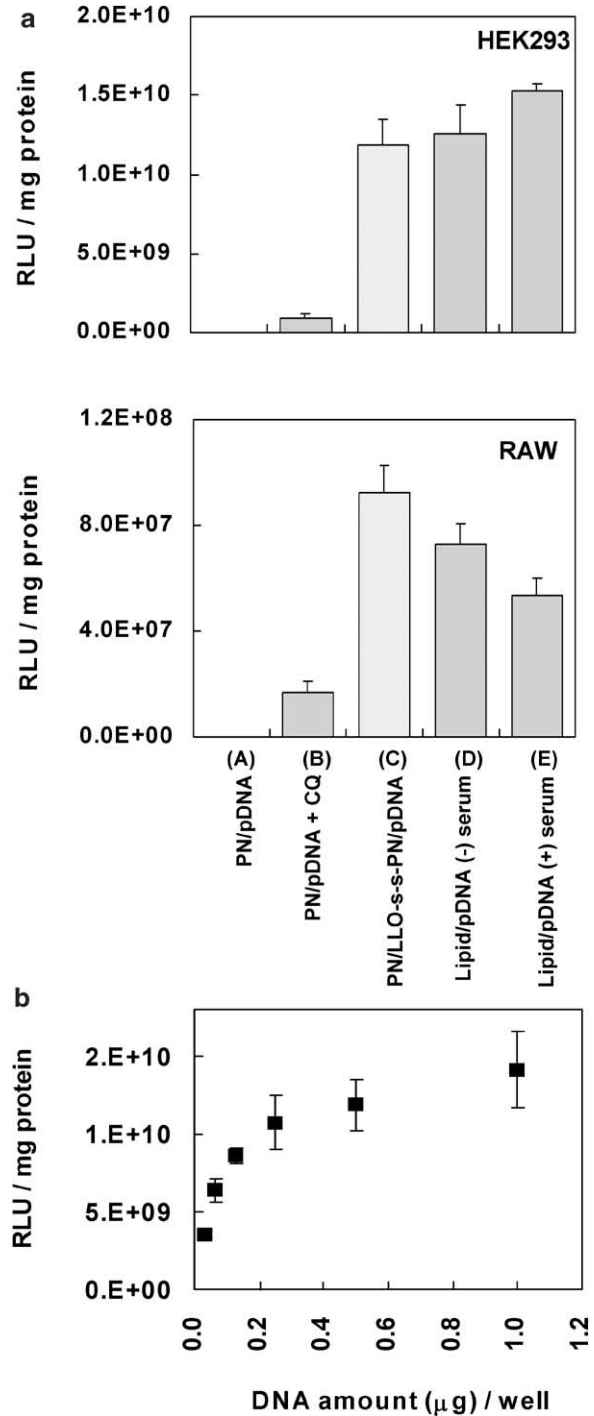


Figure 6 (a) Comparison of LLO-s-s-PN-mediated gene transfer with chloroquine and lipofectamine-mediated transfection in HEK293 and RAW264.7 cells. (A) PN/pDNA complex at 0.80 w/w ratio. (B) PN/pDNA complex (0.80 w/w) co-incubated with 80 μ M chloroquine (CQ) during a 4 h transfection period. (C) LLO-s-s-PN/PN/pDNA complex (0.80 w/w, 1.2% of PN replaced with LLO-s-s-PN). (D) Lipofectamine (lipid)/pDNA complex formulated in OPTI-MEM and transfected in OPTI-MEM. (E) Lipofectamine (lipid)/pDNA complex transfected in 10% serum-containing DMEM. For HEK293 and RAW264.7 cells, 0.5 and 1 μ g DNA/well were used, respectively. Transfection was performed in serum-containing media except for (D). (b) Luciferase reporter gene expression as a function of DNA dose in HEK293 cells. PN/pDNA complexes (0.80 w/w) with LLO-s-s-PN replacing 1.2% of PN were diluted serially down from 1 to 0.03 μ g DNA/well. Luciferase expression was analyzed 2 days post-transfection. All transfection was performed in serum-containing media.

Table 1 Effect of additional agents on LLO-s-s-PN-mediated gene transfer^a

	(+) NH ₄ Cl	(+) BFA	(+) DTNB
HEK293	1.2 (92 ± 1)	0.7 (97 ± 7)	0.6** (84 ± 1)
RAW	1.4 (90 ± 7)	1.2 (81 ± 5)	0.2** (98 ± 1)
P388D1	2.1** (88 ± 10)	1.9* (83 ± 16)	0.6* (79 ± 1)
BMM	8.0** (80 ± 4)	9.8* (72 ± 6)	1.8* (58 ± 1)
Fibroblast	3.7* (70 ± 10)	—	0.6* (100 ± 12)

^aLLO-s-s-PN-mediated transfection was performed in the presence of NH₄Cl (10 mM), BFA (50 nM), or DTNB (5 mM). NH₄Cl and BFA were added 1 h prior to transfection. Data show the ratios of LLO-s-s-PN-mediated luciferase expression in the presence of agents to that in the absence of agents. Numbers in parentheses indicate the percent total cellular protein recovery relative to control cells (no treatment). The decrease or increase over the untreated was statistically significant for the ratios with asterisks ($n=3$, * $P < 0.05$, ** $P < 0.01$).

system was far superior to CQ-mediated transfection of PN/pDNA, and as potent as lipofectamine-mediated transfection, one of the most efficient non-viral transfection agents in cell culture models. Cationic lipid-mediated transfection is often impaired by serum-containing medium.²⁷ In contrast, LLO-s-s-PN-mediated transfection exhibited less toxicity in the presence of serum at the high LLO-s-s-PN dose ranges, maintaining high transfection efficiency (Figure 6a).

To further investigate the mechanistic aspects of LLO-s-s-PN-mediated gene transfer into the cytosol, transfection was performed in media containing an agent that neutralize the acidic compartments of cells, NH₄Cl and bafilomycin A (BFA), or a membrane-impermeant sulfhydryl blocker, DTNB (Table 1). The presence of NH₄Cl in the transfection media slightly enhanced luciferase expression in all the cell types tested ($P < 0.01$ for P388D1 and BMM). Similar trends were observed using BFA, except for that of HEK293 ($P < 0.05$ for P388D1 and BMM). On the other hand, DTNB suppressed the expression significantly for the three cell lines, while it induced a slight increase in BMM (Table 1). In primary cultures of fibroblast, the augmentation of gene expression upon incorporation of LLO-s-s-PN was approximately 100-fold while the effect of NH₄Cl or DTNB was similar to that observed in the three cell lines (Table 1).

Discussion

The well-characterized PN sequence²⁵ allowed us to design an appropriate conjugation scheme (Figure 1); two proteins conjugated at 1:1 molar stoichiometry through a disulfide bond. Since lysine, a commonly used conjugation moiety, is not present in salmon or herring PN, and the N-terminus is largely a secondary amine of proline, the carboxylic acid on the C-terminus of PN was chosen for conjugation of a sulfhydryl-reactive group. PDA, an amine-containing pyridyl-disulfide linker, was highly effective for this purpose, quantitatively yielding PN-s-s-pyridine using EDC as a coupling agent. PDA is easy to synthesize and highly water soluble, making it an ideal tool for creating a sulfhydryl-reactive moiety in

proteins. Utilization of two successive affinity columns for LLO-s-s-PN purification in the final step also greatly facilitated the synthesis procedure. Our unpublished data show that when polylysines, synthetic cationic peptides with less defined molecular weights, were used in combination with *N*-succinimidyl-3-(2-pyridyldithio)propionate (SPDP), heterogeneous products with more than one LLO molecule conjugated per polylysine were formed, leading to inconsistent products and transfection results. Thus, these controlled conjugation and purification processes are important for achieving reproducible formulations, essential for pharmaceutical manufacture and consistent therapeutic effect. The highly repeated arginine sequences of PN are hypothesized to contain nuclear localizing signals as implied by its major biological function of condensing sperm DNA and delivering the DNA into egg nuclei after fertilization.²⁶ This characteristic, along with its relative homogeneity, should render PN better-suited than other synthetic polycations as a pDNA condensing agent for gene delivery.

LLO is a member of the 'sulfhydryl-activated' hemolysin family as a biochemical modification of the unique cysteine residue with a bulky reagent abrogates the hemolytic activity, which is readily reversed upon removal of the attached modifying moiety.^{21–23} This unique characteristic of the cysteine was exploited in the design of our delivery system; a ~4 kDa PN molecule was attached to the C484 through a reversible disulfide linkage to form hemolytically inactive LLO-s-s-PN. The original LLO activity then could be retrieved when LLO was released from PN upon reduction. The data demonstrate that the pore-forming activity of LLO-s-s-PN was completely lost as the cysteine was oxidized and that, upon addition of a reducing agent, LLO activity was fully regained after a short lag time in the *in vitro* assay (Figure 2c). This lag time is probably due to the time required for DTT to reduce the disulfide bond of LLO-s-s-PN. Thus, it is clearly shown that the hemolytic activity can be controlled by the redox state of the C484 of LLO. As the C484 of biochemically purified LLO from *Listeria monocytogenes* is readily oxidized and requires reduction for LLO activity, its redox state may be a regulator of LLO utilized by *Listeria* during infection in nature, along with the pH dependency of the LLO hemolytic activity.¹⁸ In comparison, the previously reported delivery system by Gottschalk *et al* used a biotin-streptavidin bridge to incorporate perfringolysin O to polylysine/pDNA complex,²⁰ requiring a non-specific biotinylation procedure that in many cases results in the loss of protein function or heterogeneous end products. Moreover, the incorporated perfringolysin O is functionally active in the complex, thus producing high toxicity at their optimized condition due most likely to the active pore formation on the cell surface. In contrast, as the activity of LLO is highly regulated in our LLO-s-s-PN construct, the LLO-s-s-PN-mediated system gave an optimal expression without showing any apparent toxicity, although some cytotoxicity was observed at a dose range about five-fold above the optimal LLO dose (Figure 5).

While the particle size was not appreciably affected by increasing PN/pDNA weight ratios (Figure 3a), the effect of LLO-s-s-PN on augmenting gene expression was most prominent when the PN/pDNA complexes were well

condensed (Figure 4) with retarded mobility in agarose gel (Figure 3b). It is generally speculated that the uptake amounts of complexes are higher when complexes are more condensed with polycations and close to charge neutral or positively charged.⁶ On the other hand, the trend in our system of reduced gene expression for the more positively charged complexes is presumably due to the possibility that the association extent of LLO-s-s-PN with PN/pDNA decreases beyond the charge neutrality point, since free PN complexes more tightly with pDNA. The binding affinity of LLO-s-s-PN to pDNA is most likely lower than that of PN to pDNA due to the bulky LLO moiety; this is supported by the data that the LLO-s-s-PN elutes from the cation exchange heparin resin at 1.3 M salt concentration, whereas PN-s-s-pyridine elutes at 1.8 M (Figure 1b).

The well-defined 1:1 molar ratio conjugation allowed us to estimate the average number of LLO-s-s-PN molecules incorporated per PN/pDNA complex. Substituting only 1.2% of PN with LLO-s-s-PN was sufficient to produce the luciferase expression nearly three orders of magnitude enhanced over the PN/pDNA complex in HEK293 and RAW264.7 cell lines (Figure 5). This corresponds to approximately eight LLO-s-s-PN molecules per pDNA molecule of 7 kbp. Taking the estimation that each polycation/pDNA complex of 50–200 nm contains multiple pDNA molecules,²⁸ 40–160 LLO-s-s-PN molecules are in each PN/pDNA complex and are necessary for the efficient endosome lysis and consequent delivery of pDNA complex into the cytosol. As it requires ~50 molecules of streptolysin O, a homologous pore-forming hemolysin, to oligomerize to form a functional pore in a single RBC,²⁹ it is implied that there exists sufficiently active reduction process in the cellular compartments following the internalization of a PN/pDNA complex carrying 40–160 LLO-s-s-PNs.

Studies on biochemically purified LLO have shown increased LLO activity at lower pH.^{30–32} Both the extent of cytosolic delivery of protein using LLO-containing liposomes in BMM and the escape of *Listeria monocytogenes* from phagosomes of BMM were diminished by raising the pH of endosomal/lysosomal compartments either using NH₄Cl, a weak base, or BFA, a H⁺-ATPase inhibitor, which supports that the LLO endosomolysis activity is optimal at a low pH environment of the endocytic pathway.^{24,30} Contrary to our hypothesis based on these results, the transfection in the three transformed cell lines tested was rather increased by the addition of NH₄Cl or BFA in most cases (Table 1). This is potentially due to several factors operating toward the enhancement of transfection in an unacidified endocytic compartment: (i) the elevation of endosome luminal pH may kinetically disfavor enzymatic degradation of pDNA; (ii) the disulfide reduction essential to this system is chemically more favorable at neutral than at acidic pH, since redox reactions generally require deprotonation of the thiol group for nucleophilic attack.³³ The situation, moreover, is more complicated if one considers endosomal enzymatic cleavage of disulfide bonds that is also pH-dependent, and requires careful mechanistic studies. In addition, the recombinant LLO used in this study showed significant hemolytic activity even at neutral pH although pH-dependent.

One of the unique strategies of our pDNA delivery system lies in the prerequisite reduction of the disulfide

bond in LLO-s-s-PN by a cell and thus controlled activation of the endosomolytic activity of LLO inside the endocytic compartments of a cell. The strong enhancement of gene expression observed upon LLO-s-s-PN incorporation clearly indicates that, upon association with cells en route to lysosomes in the internalization pathway, the disulfide bonds of LLO-s-s-PNs in the PN/pDNA complex are indeed reduced allowing activated LLO molecules to be released from the complex, subsequently forming a pore in the membranes of the endocytic compartment and resulting in enhanced delivery of pDNA into the cytosol. The exact locale and the mechanism of reductive activity is not clear and remains to be an important issue for further studies. There is substantial biological evidence for the reductive cleavage of disulfide bonds acting on the endocytosed substrates.^{33–35} Reduction of the disulfide bond between diphtheria toxin subunits has been suggested to occur at an early stage of endocytosis by cell-membrane-associated protein disulfide isomerase (PDI), based on the observation that anti-PDI antibodies and a membrane impermeant sulfhydryl blocker, DTNB, inhibited the toxin-mediated cytotoxicity.^{36,37} When LLO-s-s-PN-mediated transfection was performed in the presence of DTNB, we also observed partial inhibition of gene expression in all the three cell lines tested (Table 1). This indicates that some portion of LLO-s-s-PN molecules in fact get reduced at an early stage of endocytosis in these transformed cell lines, although this result alone does not allow us to definitively pinpoint the locale of the reduction process occurring at the cell surface and in the endocytic compartments.³⁶ Significance of the cell surface enzyme-mediated reduction in the endocytosed material, however, is unclear. Comparison of cytotoxicity and LDH release between free LLO and conjugated LLO-s-s-PN strongly suggests that the reduction process of LLO-s-s-PN is more dominant after the complex is internalized; reduced, active LLO administered along with PN/pDNA complexes caused severe fast-acting toxicity to HEK293 cells, as early as ~5 min, with 40% LDH release within 30 min, whereas the same dose of LLO-s-s-PN incorporated into PN/pDNA complexes produced high gene expression without any cytotoxicity throughout the culture. The cytotoxicity only seen at high LLO-s-s-PN concentration per pDNA perhaps indicates either infrequent but some events of disulfide bond reduction of cell-surface-bound conjugates or too much disruption of endocytic/lysosomal compartment integrity.

More recently, disulfide reduction of endocytosed material in endosomes/lysosomes of antigen presenting cells (APCs) was clearly demonstrated to be a critical step in antigen presentation.³⁸ The presence of presumptive redox enzyme responsible for this process has only recently been identified and characterized (γ -interferon inducible lysosomal thiol reductase, GILT) in the endosome/lysosome of APCs.³⁹ Expression of related proteins in cell types other than APCs remains to be investigated, although expression of GILT has been reported to be inducible in fibroblasts, keratinocytes, and endothelial cells.⁴⁰ In this regard, it is of interest to compare the transfection results and responses to BFA, NH₄Cl, and DTNB between the three cell lines and the primary culture of BMM. BMM exhibited a different mode of cytotoxicity profile as well from that of HEK293,

with delayed LDH leakage that started only after 1 h. This may be due to cell-type-dependent variation where the reduction predominantly occurs in a cell, as has been suggested by others.^{41,42} Unique cellular responses peculiar to the primary culture of macrophages, however, cannot be ruled out,^{43,44} in addition to fundamental dissimilarities between transformed *versus* normal primary cells. In primary cultured fibroblasts, however, the gene expression with and without NH₄Cl or DTNB treatment was similar to that in the transformed cell lines, suggesting unique cell-type dependency further. These are ongoing studies that need to be addressed, as it could lead to an unexpected pitfall when the system is applied to *in vivo*, which would certainly more reflect phenomena observed in primary rather than transformed cells.

In conclusion, we have demonstrated that reversible incorporation of LLO into PN/pDNA complex, through the use of the synthesized LLO-s-s-PN conjugate, dramatically enhances the reporter gene transfection efficiency in cell culture models. The requirement of only a small amount of LLO-s-s-PN per pDNA suggests great potentials of the LLO-mediated delivery of macromolecules across the membrane barrier, which is perhaps much more efficient than that mediated by other existing endosome-disrupting agents. Our non-viral delivery system can easily be scaled up for animal experiments, which are under investigation for DNA vaccine applications. Our system also shows the potential usage of cellular reducing capability in targeting therapeutic macromolecules into the cytosol. The application of a reversible disulfide bond to a prodrug strategy will become more useful and meaningful as the mechanism of cellular reduction process is elucidated on a molecular basis for each cell or tissue type.

Materials and methods

Chemicals

Protamine (PN) sulfate (Grade III, Clupeine), 2,2'-dithiodipyridine, β -mercaptoethylamine hydrochloride, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), β -mercaptoethanol (β ME), chloroquine (CQ) and NH₄Cl were purchased from Sigma. Dithiothreitol (DTT) was obtained from Fisher Scientific. Sheep whole red blood cells (RBCs) were purchased from ICN Biomedicals. 5,5'-Dithiobis(2-nitrobenzoic acid) (DTNB) was purchased from Pierce. Bafilomycin A1 (BFA) was obtained from Calbiochem.

Recombinant LLO purification

Recombinant LLO was overexpressed in *E. coli* BL21(DE3) transformed with pET29b which carried a six histidine-tagged LLO gene *hly* (a kind gift from Dr Daniel Portnoy, University of California, Berkeley, CA, USA).⁴⁵ The protein was purified using Ni-NTA superflow (Qiagen, CA) on an FPLC system (Bio-Rad, Biologic HR Chromatography System) as previously described.⁴⁶ Typically, from a 1 l culture, ~20 mg of purified LLO was obtained. Protein purity was analyzed by SDS-PAGE with Coomassie staining, and protein concentration was determined by a BCA-protein assay (Pierce, IL, USA) using bovine serum albumin as a standard.

2-(2-Pyridylidithio)ethylamine (PDA)

PDA was synthesized by the procedure described by Connor and Schroit.⁴⁷ Briefly, 2,2'-dithiodipyridine (3.96 g, 18 mmol) was dissolved in 15 ml of 10% (v/v) glacial acetic acid in ethanol, and β -mercaptoethylamine hydrochloride (1.02 g, 9 mmol) dissolved in 7 ml of ethanol was gradually added while mixing under argon. The reaction was allowed to proceed for 3 h at r.t. After removing the resulting precipitates, the supernatant was evaporated *in vacuo* to yellow oil. The oil was then precipitated and washed by the addition of 50 ml of diethylether and was redissolved in 10 ml of ethanol (three times) to give a white powder. Yield: 1.16 g, 58%. ¹H-NMR (500 MHz, CD₃OD) δ : 3.16 (t, 2 H), 3.32 (t, 2 H), 7.34 (t, 1 H), 7.67 (d, 1 H), 7.82 (t, 1 H), 8.56 (d, 1 H).

Protamine-s-s-pyridine (PN-s-s-pyridine)

Protamine sulfate (40 mg) (~8 μ mol) was reacted with PDA hydrochloride (22 mg, 100 μ mol) using EDC (31 mg, 160 μ mol) at r.t. for 2 h in 5 ml of MES buffer (0.1 M, pH 4.9). The reaction mixture was purified by extensive dialysis in 0.5% acetic acid and 10% methanol and lyophilized to give the product as white amorphous. Yield: 42 mg, 97%. Purity of PN-s-s-pyridine was confirmed by both reverse phase HPLC and gel electrophoresis. The column employed was a 4.6 \times 250 mm, 5 μ m Alltima (Alltech) C8 column. PN and PN-s-s-pyridine in phosphate-buffered saline solution were applied onto the column equilibrated with 5% acetonitrile aqueous solution containing 0.1% trifluoroacetic acid, and was then eluted using a gradient of acetonitrile (5–37.5% over 30 min). The flow rate was set at 1 ml/min, and the elution was monitored at 215 nm.

LLO-s-s-protamine (LLO-s-s-PN)

LLO (3 mg, 0.05 μ mol) was reacted with 1 mg of PN-s-s-pyridine (~0.2 μ mol) in 10 ml of HEPES-buffered saline (HBS: 10 mM HEPES, 140 mM NaCl, pH 7.4) for 4 h at 4°C. The reaction mixture was filtered through a 0.2 μ m membrane to remove particulates, and purified using two successive affinity columns on an FPLC system. First, excess PN-s-s-pyridine was separated by a Ni-NTA superflow column (Qiagen, CA). LLO and LLO-s-s-PN were eluted with 400 mM imidazole and the mixture was directly injected into a heparin column (Amersham Pharmacia Biotech, NJ, USA). LLO-s-s-PN was then separated from unreacted free LLO using a NaCl step gradient. The purified conjugate was then dialyzed overnight in HEPES-buffered glucose (HBG; 280 mM glucose, 10 mM HEPES, pH 7.4) at 4°C, aliquoted and kept at –80°C. The purity of the conjugate was confirmed by SDS-PAGE (4–20% gradient), and urea-based gel electrophoresis with reversed polarity (15% acrylamide) adapted from a published report.⁴⁸ The concentration of the conjugate was determined by comparing the reduced conjugate with known amounts of LLO standard on SDS-PAGE using Metamorph Image Analysis System (Universal Imaging Corporation, PA, USA).

Hemolysis assay

The membrane pore-forming activity of LLO and LLO-s-s-PN was tested by an *in vitro* RBC hemolysis assay as previously described.⁴⁹ Briefly, RBCs were washed three

times with HBS (pH 7.4) and were resuspended at a concentration of 10^8 cells/ml in HBS or in 10% serum-containing Dulbecco's modified Eagle medium (DMEM). To 2 ml of RBCs with or without 5 mM DTT, 2 μ g of LLO or LLO-s-s-PN was added. Dynamic changes in the right-angle light scatter of RBCs undergoing lysis were measured in a fluorometer (Jobin Yvon-Spex Instruments SA., NJ, USA). Both the excitation and emission monochromators were set to 590 nm with slit widths of 1 nm.

Cell culture

Human embryonic kidney (HEK) 293 and murine macrophage P388D1 cell lines were purchased from the American Type Culture Collection. Murine macrophage RAW264.7 cell line was a gift from Dr Joel Swanson (University of Michigan). HEK293 and RAW264.7 cells were cultured in complete DMEM containing 10% heat-inactivated fetal bovine serum (FBS, Gibco), non-essential amino acid, 100 U/ml penicillin and 100 U/ml streptomycin. P388D1 cells were cultured in complete RPMI-1640 supplemented with 1 mM sodium pyruvate, FBS, and antibiotics. Cells were maintained at 37°C in a 5% CO₂ humidified atmosphere. Bone marrow cells were extracted from femurs of 4–12-week-old Balb-C mice (Harlan Laboratories, IN, USA). Cells were cultured in bone marrow media in 5% CO₂ at 37°C. Bone marrow media were prepared using DMEM supplemented with 20% FBS, 30% L-cell culture supernatant, and antibiotics.⁵⁰ Primary cultures of rat fibroblast from choroid plexus were prepared by dissection of choroid plexus of 1–2-day-old rats, pronase/Trypsin-DNase I digestion and differential adhesion to culture dishes (fibroblasts kindly provided by Dr D Smith, University of Michigan). The fibroblasts were grown in 10% FBS-containing RPMI medium and passaged twice before transfection experiments.

Expression vectors

The plasmid pNGVL3 (7.0 kbp) coding for green fluorescent protein (GFP) and firefly luciferase under the promoter of cytomegalovirus was a gift from Dr Gary Nabel (Vaccine Research Center, National Institutes of Health, MD, USA). pDNA was isolated from *E. coli* using a Qiagen Plasmid Maxi kit. Concentration of pDNA was determined spectrophotometrically using absorbance at 260 nm.

Complex preparation

PN/LLO-s-s-PN/pDNA complexes, at weight ratios of total PN to pDNA ranging from 0.32. to 2.14, were prepared by vortexing equal volumes of pDNA and PN premixed with LLO-s-s-PN in HBG. The final pDNA concentrations in the complexes were typically 40 μ g/ml. DNA complexes were incubated for 30 min on ice before transfection.

Sucrose gradient fractionation of complexes

PN/pDNA complexes (w/w = 0.64, 0.80, 1.21) containing 8.4 μ g of DNA and 1 μ g of LLO-s-s-PN were formulated in 175 μ l of HBG. After a 4 h incubation on ice, complexes were layered on 175 μ l of 30% sucrose in an ultracentrifuge tube. The gradient was then centrifuged at 35 000 rpm (100 000g) in a Sorvall RP55-S rotor for 30 min at 4°C. HBG and sucrose fractions were then separated, and monitored for the level of LLO hemolytic

activities by RBC hemolysis assay. The same study was also performed in the presence of salt, in which the complexes were formulated in HBG, and after 30 min, an equal volume of HEPES-buffered saline (280 mM NaCl, 10 mM HEPES, pH 7.4) was added.

Particle size and zeta potential measurements

PN/pDNA complexes were prepared at a pDNA concentration of 40 μ g/ml in HBG with varying amounts of PN, then diluted to 5 μ g/ml with HBG after 30 min. The particle size of complexes was determined by quasi-elastic light scattering on a zeta potential/particle sizer (NICOMP 380ZLS) equipped with an avalanche photodiode detector. Zeta potential was measured for the complexes formulated in a similar way at a final DNA concentration of 10 μ g/ml in HBG.

Transfection and reporter gene expression

Cells were plated in 24-well plates at 3.3×10^4 cells/well for HEK293 cells, and 1.0×10^5 cells/well for RAW264.7, P388D1, and bone-marrow derived macrophages (BMM) 24 h prior to transfection. BMM were collected using chilled phosphate-buffered saline without calcium and magnesium at the end of 5-day cell culture period and transfected on day 6. Typical cell densities at the time of experiments were ~70% confluent. Transfections were performed in 175 μ l/well of complete media containing 10% FBS. Twenty-five microliters of pDNA complexes in HBG, typically at 0.5–1 μ g pDNA/well, was added dropwise. All assays were done in triplicate. The effects of NH₄Cl, BFA, and DTNB on transfection were studied by adding 10 mM, 50 nM, and 5 mM of agents, respectively, to transfection media during the transfection period. NH₄Cl and BFA were added 1 h prior to transfection. Lipofectamine-mediated transfection (Life Technologies, MD, USA) was performed according to the manufacturer's instructions, and the ratio of pDNA to lipid was optimized for each cell line. After 4 h incubation at 37°C, the media were replaced with fresh complete media. After 48 h, cells were washed once with 0.5 ml of phosphate-buffered saline. Cells were lysed with Reporter Lysis Buffer (Promega, WI, USA) and subjected to one freeze–thaw cycle. The lysates were collected, centrifuged, and 20 μ l of the supernatant was assayed for its luciferase activity using 100 μ l of luciferase substrate (Promega) in an AutoLumat LB953 luminometer (EG&G Berthold). The relative luminescence units (RLU) of each sample were monitored for 10 s. Luciferase activity in the cell lysates was normalized by total cellular protein content determined by BCA assay. GFP expression was monitored concurrently during the 48 h culture period using fluorescence microscopy equipped with GFP excitation and dichroic filter cube (Zeiss Axiovert; Chroma Filters). The percentage of fluorescent cells was determined by the average count of positive cells in three different random fields, as analyzed by the integrated fluorescence intensity per cell utilizing the Metamorph Image Analysis System (Universal Imaging Co., IL, USA).

Cytotoxicity study

Lactate dehydrogenase (LDH) leakage from the cells was determined by the LDH toxicology assay kit from Sigma, and the cytotoxicity was calculated based on a formula

provided by the manufacturer and expressed as a percentage of cell death based on the control levels.

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