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Enhanced gene delivery using disulfide-crosslinked low molecular weight polyethylenimine with listeriolysin O-polyethylenimine disulfide conjugate

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Abstract

One of the most important requirements for non-viral gene delivery systems is the ability to mediate high levels of gene expression with low toxicity. After the DNA/vector complexes are taken up by cells through endocytosis, DNA is typically contained within the endocytic compartments and rapidly degraded due to the low pH and hydrolytic enzymes within endosomes and lysosomes, limiting its accessibility to the cytosol and ultimately to the nucleus. In this study, the endosomolytic protein listeriolysin O (LLO) from the intracellular pathogen *Listeria monocytogenes* was conjugated with polyethylenimine (PEI) of average molecular weight 25 kDa (PEI25) via a reversible disulfide bond (LLO-s-s-PEI), and incorporated into plasmid DNA condensed with disulfide-crosslinked low molecular weight PEI 1.8 kDa (PEI1.8).

We have investigated and demonstrated that high gene transfection efficiency, which is comparable to that by the most commonly used PEI25, can be achieved by reversibly crosslinking low molecular weight PEI (PEI1.8) using disulfide bonds, with greatly reduced cytotoxicity of the PEI. The reversible incorporation of LLO into the DNA condensates of PEI, through the use of the synthesized LLO-s-s-PEI conjugate, further enhances the transfection efficiency beyond that of DNA condensates with disulfide-crosslinked PEI1.8 alone.

Keywords

Polyethylenimine; Listeriolysin O; Disulfide-crosslink; Non-viral gene delivery

1. Introduction

The success of gene therapy critically depends upon the development of delivery vehicles that can effectively deliver therapeutic genes to target cells with minimal cell toxicity and desirably with some degree of cell selectivity. Viral vector systems in general are considered effective primarily because of their high efficiencies for delivery and expression. However, the use of viruses for gene delivery poses serious problems: they are expensive to produce, difficult to quality-control, potentially damaging to the cells they infect, and may cause potent and adverse immune responses that limit their use [1]. Non-viral gene delivery systems, on the other hand, offer unique advantages as pharmaceuticals over viral vectors, including their safety profiles, lower immunogenicity, and relative ease of preparation and manipulation [2]. Therefore, there continues to be the need to further explore and optimize non-viral vectors for specific gene delivery and therapy applications.

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The gene delivery efficiencies of non-viral vectors, however, are generally low and often do not reach adequate therapeutic levels. Therefore, much of the effort in the non-viral field is devoted to optimizing and enhancing the transfection efficiency of non-viral vectors. There are multiple key steps to be considered for improving the efficiency of non-viral gene delivery systems [3]. To increase the uptake of functionally intact plasmid DNA (pDNA) across the plasma membrane, as well as to promote protection of pDNA in biological environment, negatively charged DNA molecules are most often condensed via electrostatic interaction with cationic transfection reagents. The resulting complexes of pDNA/condensing agent are taken up by cells typically through endocytosis. Following internalization, the normal fate of pDNA is rapid degradation due to the low pH and enzymes within endosomes and lysosomes. One of the most common methods for increasing the *in vitro* transfection efficiency of polymer/DNA complexes is to treat the cells with agents that can alter the default fate of the internalized molecules and/or to breach the membranes of endocytic compartments. Chloroquine, for example, is thought to have a buffering capacity that prevents endosomal acidification, leading to swelling and bursting of the endosomes and thereby has been shown to enhance the transfection activity of polymer/DNA complexes [4]. However, this approach is limited to *in vitro* applications. Adenovirus particles have been used to enhance transfection of polymer/DNA complexes, but are unlikely to be widely used *in vivo* because they may provoke inflammatory responses [5]. Instead of using whole virus particles to enhance cytoplasmic delivery, fusogenic peptides derived from viruses have been used [6], but with limited effectiveness and often requiring large quantities of peptides.

Previously, the endosomolytic mechanism of listeriolysin O (LLO) from *Listeria monocytogenes* was tested in order to demonstrate the proof-of-concept of a non-viral, non-bacterial vector mimicking the *Listerial* gene delivery vector [7,8]. LLO is secreted by *Listeria* to breach the endo/phagosomal membranes and to invade into the cytosol [9]. Hence, LLO is capable of delivering the bacterial particle, which is much larger than the size of a plasmid DNA, from the endocytic to the cytosolic compartment [9]. LLO's only cysteine, at the amino acid position 484 (C484), is ideal not only as a site for reversible conjugation by sulfhydryl-specific reaction, but also as a site for the regulation of LLO's activity. It has been shown that the attachment of relatively large molecules via disulfide bond inactivates LLO, while the reversibility of the disulfide bond lends itself to being cleaved in the endocytic compartments, thus restoring LLO's activity. Our previous experiments, in which sulfhydryl-reactive thiolated protamine was conjugated at a 1:1 molar ratio to the C484 of LLO, demonstrated that incorporation of protamine-LLO disulfide conjugate (protamine-s-s-LLO) enhanced the gene delivery capability [8]. Protamine was chosen because of its biological relevance and well-characterized sequence, as well as its established ability to electrostatically bind to and condense DNA. Our unpublished preliminary data, however, show that the protamine-s-s-LLO incorporated in the protamine-condensed pDNA, while useful for demonstrating the utility of LLO incorporation for enhanced gene delivery and for *in vitro* usages, may have limited applicability in DNA delivery *in vivo*, justifying and calling for modification of the construct.

In this report, therefore, we have extended our previous work, which was using protamine-condensed pDNA in combination with protamine-s-s-LLO, to testing a cationic polymer with a higher condensing capacity to investigate a potentially better approach to condensing pDNA and incorporating LLO in the polymer/pDNA condensate in a controlled fashion. Polyethylenimine (PEI) is one of the most successful and extensively used synthetic carriers for the delivery of pDNA into cells *in vitro* and *in vivo* [10], and exhibits a relatively high degree of electrostatic interaction with pDNA in comparison with the previously-used protamine. In addition to better condensation of pDNA using PEI, the high transfection efficiency of PEI/DNA complexes has been mostly explained by the endosomal buffering capacity of PEI [10]. High molecular weight PEIs (i.e., PEI of average molecular weight 25

kDa (PEI25)) have been widely used for transfection reagents; however, several groups have reported potential cytotoxicity of high molecular weight PEI against various cell lines [11]. Also, the high affinity of PEI25 for DNA is another important barrier after cytosolic delivery that limits overall transfection efficiency due to the relatively inefficient dissociation of pDNA from PEI once inside cells; several studies have found that gene expression can be enhanced by reducing the polymer/DNA binding strength by decreasing the number of positive charges or molecular weight of cationic polymers [12,13]. Therefore, modification of low molecular weight cationic polymers has been also studied extensively to improve gene transfer efficiency while keeping cytotoxicity manageable [14–22]. In consideration of all these opposing factors, and in order to enhance the efficiency of DNA condensation and gene transfer, we have opted here for the use of low molecular weight PEIs reversibly crosslinked via disulfide bonds to yield high molecular weight polymers.

Several groups have studied the effect of endosomolytic agents, such as chloroquine, on the transfection efficiency of PEI/pDNA condensates. One group showed that chloroquine enhanced the transfection efficiency of PEI-condensed pDNA, while another showed no improvement beyond that mediated by PEI alone [23,24]. In view of these, the effect of PEI/pDNA condensates on LLO activity has yet to be investigated. Since it is known that LLO has increased activity at acidic pH (pH 5.5–6 optimal) [9], it has been of interest to us to test LLO's activity when it is incorporated in the PEI-condensed pDNA which has endosomal proton binding capacity.

In this report, we have aimed to design and investigate an optimal method to incorporate the endosome-disruptive activity of LLO into the PEI-condensed pDNA for enhanced cytosolic delivery of pDNA. For the first goal of incorporating LLO, we synthesized high molecular weight PEI conjugated via a reversible disulfide bond to the unique cysteine of LLO. For the second goal of generating PEI-condensed pDNA, we synthesized disulfide-crosslinkable low molecular weight PEI to generate reversible, high molecular weight PEI as a pDNA-condensing polycation.

2. Materials and methods

2.1. Materials

Low molecular weight PEI (branched, average molecular weight 1.8 kDa: PEI1.8) and high molecular weight PEI (branched, average molecular weight 25 kDa: PEI25) were purchased from Polysciences Inc (Warrington, PA). Dithiothreitol (DTT) was purchased from Fisher Scientific (Hanover Park, IL). Sheep whole red blood cells (RBCs) were purchased from ICN Biomedicals (Costa Mesa, CA). The plasmid pNGVL3 (7.0 kb) containing the cDNAs for green fluorescent protein (GFP) and firefly luciferase under the control of the cytomegalovirus (CMV) promoter was a gift from Dr. Gary Nabel (Vaccine Research Center, National Institutes of Health, MD). The plasmid was amplified in JM-109 competent cells, and isolated and purified with Qiagen Mega Kits following the manufacturer's protocol (Valencia, CA). The concentration of plasmid was determined by measuring the absorbance at 260 nm and its optical density ratio at 260/280 nm was routinely in the range of 1.8 to 1.9.

2.2. Synthesis of thiolated PEI

Ten milligrams of PEI1.8 were dissolved in 0.5 ml of methylene chloride. Aliquots of 0.5 ml containing different amounts of (*N*-Succinimidyl 3-(2-pyridyldithio)-propionate) (SPDP) in methylene chloride were added dropwise to the PEI solution and the mixtures were allowed to react at room temperature for 6 h. Methylene chloride was then removed under vacuum at room temperature. The dried mixtures were extracted by addition of 1 mL of phosphate buffer, followed by filtering through a 0.45 μ m syringe filter. The reaction product, PEI1.8-

pyridyldithiopropionate (PEI1.8-PDP) was purified by size exclusion chromatography (Sephadex G10, 1×35 cm) with deionized water. The collected fractions were assayed for PEI content using Cu^{2+} against PEI1.8 standard at 630 nm. The number of PDP groups per average chain of PEI was determined by monitoring the generation of pyridine-2-thione at 343 nm (molar absorptivity=8080 $\text{cm}^{-1} \text{M}^{-1}$) upon treatment of PEI1.8-PDP at 50 mM DTT. After treating with DTT, thiolated PEI was purified again using Sephadex G10 as described above.

2.3. Oxidation of thiolated PEI1.8 and determination of remaining thiol content

The thiolated PEI1.8 was allowed to oxidize at room temperature at 0.4 mg/mL total concentration of thiolated PEI1.8, and the resulting disulfide bond formation between reduced thiols of PEI1.8 and their crosslinking was calculated based on the content of remaining free thiols, as determined via Ellman's assay. During the oxidation and crosslinking of thiolated PEI1.8, 100 μL of sample solution was added to 150 μL of 0.1 M phosphate buffer in 96-well plates. The color was developed by adding 50 μL of a dithiobis(2-nitrobenzoic acid) (DTNB, Ellman's reagent) solution and incubated for 15 min at room temperature. Absorbance values were obtained with an Emax microplate reader at 405 nm (Molecular Devices, Sunnyvale, CA), and thiol content was calculated using cysteine standards of known concentrations.

2.4. Molecular weight determination of crosslinked PEI1.8

To determine the molecular weight of disulfide-crosslinked PEI1.8, crosslinked polymer was dissolved in 0.5 M sodium nitrate (NaNO_3 , Sigma) to concentrations ranging from 0.1 to 0.5 g/dL, and the viscosities measured using an Ubbelohde capillary viscometer (Cannon, Viscometer No. 75 N956) at 25.0 °C. Reduced (η_{red}) and specific (η_{sp}) viscosities were calculated using the following Eqs. (1) and (2) (η : viscosity of PEI solution; η_0 : viscosity of solvent) [25]: Eq. (1) $\eta_{\text{sp}} = (\eta - \eta_0)/\eta_0$, and Eq. (2) $\eta_{\text{red}} = \eta_{\text{sp}}/c$. The intrinsic viscosity of the polymer solution η_{int} is calculated by extrapolation of the reduced viscosity to infinite dilution (Eq. (3)): Eq. (3) $\eta_{\text{int}} = \eta_{\text{red}}$ for $c \rightarrow 0$. Following the Staudinger–Mark–Houwink relationship (Eq. (4)), a linear relation exists between $\log(\eta_{\text{int}})$ and $\log(M_V)$: Eq. (4) $\eta_{\text{int}} = K_V M^\alpha$. The molecular weight of crosslinked polymer was determined using PEI standards of known molecular weight [26].

2.5. Acid titration of PEI1.8, crosslinked PEI1.8, and PEI25

A total of 2 mg of each polymer was dissolved in 5 mL of Milli-Q water (Millipore, Bedford, MA) and adjusted to pH 11.5 with 1 M NaOH. Aliquots (5 μL) of 1 M HCl were added, and the solution pH was measured with a pH meter (Accumet AB15, Hudson, MA) after each addition.

2.6. Preparation of pDNA and polymer complexes

Stock PEI and pDNA solutions were prepared before experiments at various weight ratios of PEI to pDNA. The complexes were prepared by adding, under agitation, appropriate amounts of the polycation in 75 μL of HBG (10 mM Hepes buffer containing 5% glucose) to 7.5 μg of pDNA in 75 μL of the same buffer. The resulting solutions containing the complexes were incubated at room temperature for 20 min.

2.7. Agarose gel retardation and particle size measurement

The extent and effectiveness of pDNA condensation by disulfide-crosslinked PEI1.8 was evaluated by a gel retardation assay. Cross-linked PEI1.8 and pDNA solutions were mixed at various w/w ratios (PEI/pDNA) and incubated for 20 min at room temperature to allow complex formation. The PEI/pDNA complexes were resolved in a 1% (w/v) agarose gel pretreated with 0.5 mg/mL ethidium bromide in Tris-boric acid-EDTA (TBE; 45 mM Tris, 45 mM borate, 1 mM EDTA) buffer at 80 V. Particle sizes of PEI/pDNA complexes were

measured by dynamic light scattering using a particle sizer (NICOMP 380ZLS, Santa Barbara, CA) equipped with an avalanche photodiode detector.

2.8. Conjugation of LLO to polymer

PEI25 was thiolated with SPDP and purified by size exclusion chromatography (Sephadex G10, 1×35 cm) with deionized water. This pyridyldithiol-activated PEI25 was reacted with LLO and followed by filtering through 0.45 µm syringe filter. The level of LLO conjugation was determined by pyridine-2-thione absorbance assay at 343 nm. The average number of LLO molecules per chain of PEI25 was calculated based on the amount of pyridine-2-thione generated after pyridyldithiol-activated PEI25 reacted with the thiol group of LLO.

2.9. Hemolysis assay

The membrane pore-forming activity of LLO and LLO-s-s-PEI25 was tested by an *in vitro* red blood cell (RBC) hemolysis assay. Briefly, RBCs were washed three times with HBS (pH 7.4) and were re-suspended 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, 10 ng of LLO or LLO-s-s-PEI was added. Dynamic changes in the right-angle light scatter (at 590 nm) of RBCs undergoing lysis were measured following the method used previously [8].

2.10. Cell culture

HEK (human embryonic kidney) 293 cells were cultured in DMEM containing glutamine (GIBCO) supplemented with 10% heat-inactivated FBS (GIBCO) and antibiotics (100 units/ml penicillin and 100 µg/ml streptomycin, Sigma). Cells were grown at 37 °C in humidified air containing 5% CO₂ and passaged every 3 days. The cells (3×10^5 per well) were plated on Costar 6-well tissue culture plates 24 h before transfection. Immediately before the initiation of transfection experiments, the medium was removed from each well, and the cells were washed twice with DMEM without serum and antibiotics, and treated with the complexes.

2.11. Transfection and reporter gene expression

To each well 0.5 ml of the transfection medium (DMEM without serum, containing 1 µg pDNA complexed with PEI) was added, followed by incubation at 37 °C in a humidified atmosphere (5% CO₂) for 4 h. The transfection medium was then removed, and the cells were further incubated under the same conditions in complete medium for 24 h. Thereafter, the medium was removed and the cells were washed twice with Dulbecco's PBS without CaCl₂ and MgCl₂ (Sigma, St. Louis, MO). The cells were assayed for luciferase activity following the manufacturer's protocol (Perkin Elmer, Waltham, MA) and the results expressed as relative luciferase activity per mg of protein. Total protein was measured using the BCA (bicinchoninic acid, Pierce, Rockford, IL) assay.

2.12. Cytotoxicity assay in vitro

The toxicity of the PEI/pDNA complex to cells during transfection was evaluated by the XTT assay. Briefly, HEK 293 cells were plated on 96-well plates at 9×10^3 cells/well and grown to 40–70% confluence, at which point the culture media was replaced with 0.5 mL of fresh DMEM supplemented with transfection media. After 4 h of incubation, the media was replaced with fresh culture media and grown for an additional 24 h, at which point the media was replaced with 0.5 mL of fresh media containing 50 µL of XTT solution (1 mg/ml) and then incubated for 2 h. The absorbance of the formazan product formed by viable cells was measured spectrophotometrically at 595 nm in an Emax microplate reader. The percent viability of cells monitored by the XTT assay was determined relative to untreated cells.

3. Results and discussion

3.1. Synthesis and characterization of disulfide-crosslinked PEI

In order to synthesize a reversibly crosslinked PEI from small molecular weight PEI using disulfide-crosslinking, thiol groups were conjugated to amine groups of PEI1.8 utilizing SPDP. In order to introduce thiol groups to PEI1.8, different molar ratios of thiolating agent SPDP with respect to PEI1.8 were first tested. With the reaction condition of 3 molar-excess of SPDP to PEI1.8, and 6 h incubation (see Methods, Section 2.2), an average of 2 thiol groups per PEI1.8 was achieved. The free thiol-containing PEI1.8 was then allowed under an oxidizing condition to form larger molecular weight, disulfide-crosslinked PEI1.8 while the spontaneous oxidation reaction was monitored by following remaining free thiol content using Ellman's assay at various time points. Fig. 1(a) shows the free thiol content of thiolated PEI1.8 as a function of time upon disulfide bonding formation as a result of spontaneous oxidation; the decreasing amount of free thiol groups was monitored as expressed in this figure in percent of the initial total free thiol amount over a time period of 144 h. The amount of free thiols during a typical reaction procedure dropped with an apparent $t_{1/2}$ of approximately 6 h. The average molecular weight of crosslinked PEI1.8 polymer after 96 h of reaction, determined based on relative viscosity measurements (see Methods, Section 2.4), was approximately 20 kDa.

After the synthesis of disulfide-crosslinked PEI1.8 (s-s PEI1.8) of apparent average molecular weight of 20 kDa was achieved using the thiolated low molecular weight PEI1.8, we then further investigated its physicochemical properties and also tested its gene delivery capacity in comparison to 25 kDa PEI (PEI25) and unmodified 1.8 kDa PEI (PEI1.8), with particular focus on their relative cytotoxicities and gene transfection efficiencies, before we investigated the three polymers with LLO incorporation.

It is known that PEI has the capacity to bind protons, during which the endosomal acidification process can promote endosomal escape of polymer/pDNA complexes, most likely by the previously-hypothesized osmolytic effect, the so-called "proton sponge effect" [10]. Thus the proton binding capacity of PEI/pDNA complexes has been reported to correlate to transfection efficiency accordingly [27]. The proton binding capacity of the disulfide-crosslinked PEI1.8 of apparent molecular weight ~20 kDa, was monitored in the acid-base titration assay (Fig. 1 (b)) and compared with that of PEI25 as a reference polymer. Its slope in the pH range 5.1–7.4 of acid-base titration curves was similar to that of PEI25. This proton binding capacity of s-s PEI1.8 and PEI25 would be beneficial for enhanced gene expression using pDNA/PEI condensates, while its effect is unclear toward the enhancement by LLO-mediated endosomal escape, as investigated in section 3.5.

3.2. Characterization of disulfide-crosslinked PEI1.8 (s-s PEI1.8) and plasmid DNA complex

In order to be effective as carriers for gene delivery, polymers must possess the capacity to condense pDNA for protection from degradation and for efficient cellular uptake. We therefore investigated the ability of our modified PEI polymer to condense plasmid DNA and inhibit its migration in agarose gel electrophoresis; complexes were formed at various PEI to pDNA weight ratios, ranging from 0.06/1 to 0.77/1, and electrophoresed in an agarose gel. Fig. 1(c) shows the gel retardation results of PEI/pDNA complex with pDNA. The s-s PEI1.8/pDNA complexes showed complete retardation at a weight ratio of 0.5, which was similar to that achieved by PEI25.

The effective diameters of the PEI/pDNA complexes, using the three forms of PEI formed at the PEI/pDNA weight ratios corresponding to their optimum transfection efficiency, were measured by dynamic light scattering. Complexes composed of s-s PEI1.8 showed effective diameters in the range of 100–150 nm at a PEI/pDNA ratio of 6.45 (w/w), whereas unmodified

PEI1.8 produced complexes with diameters of roughly 890 nm; the apparent size of pDNA condensates with PEI25 was approximately 120 nm (Fig. 1(d)).

3.3. Transfection efficiency and cytotoxicity of disulfide-crosslinked PEI1.8 (s-s PEI1.8)/pDNA condensate

The above-described, three types of PEI polymers, PEI1.8, s-s PEI1.8 and PEI25, were tested for their relative gene delivery capacity and corresponding cytotoxicity using luciferase reporter gene expression and cell viability investigated at different polymer/pDNA (w/w) ratios. The transfection efficiency in HEK 293 cells using different forms of PEIs at selected w/w ratios is shown in Fig. 2(a). Transfection efficiency was measured as luciferase enzyme activity and normalized to total cell protein content. The data showed that s-s PEI1.8 mediated a significantly higher level of gene expression than the PEI1.8 in HEK 293 cells at all the polymer/pDNA ratios tested. Both forms of PEI1.8 exhibited increasingly higher transfection with increasing polymer/pDNA ratio (from 2.6 to 12.9 PEI/pDNA ratios). With increasing polymer/pDNA ratios, however, high molecular weight PEI, PEI25, resulted in lower transfection efficiency. This opposite adverse trend of transfection efficiency by PEI25 with higher PEI/pDNA ratios, in contrast with that mediated by PEI1.8, could be due to several reasons, including high cytotoxicity of PEI25 (as shown below) and/or slow release of pDNA from the polymer/pDNA complex. The overall polymer/pDNA binding could be enhanced by the use of s-s PEI1.8, which has high molecular weight because of the reversible disulfide-crosslinking of low molecular weight PEI. However, its binding strength can be lowered when disulfide bonds are cleaved to generate low molecular weight PEI in a reducing environment inside cells.

Relative cell viability during transfection under the conditions used in Fig. 2(a) was evaluated on the basis of XTT assay in comparison with that of cells not receiving any transfection reagents. High molecular weight PEI in general has been reported to have higher cytotoxicity than its low molecular weight PEI counterpart [11]. HEK 293 cells were treated with polymer/pDNA complexes and incubated for 4 h, and the resulting cytotoxicity was assayed 20 h later. The PEI25/pDNA condensates exhibited high cytotoxicity, reducing cell viability to 50% or less at the concentrations used in transfection studies (Fig. 2(b)). In comparison, the pDNA condensed with PEI1.8 exerted a relatively low cytotoxic effect, showing more than 60% cell viability even at the highest PEI/pDNA ratio tested. The s-s PEI1.8, which is similar to PEI25 in terms of its molecular weight, exhibited a cytotoxicity profile that was drastically different from that of PEI25 and rather similar to that of PEI1.8. The results indicate that the disulfide-crosslinked PEI1.8 is significantly less toxic than PEI25, maintaining the low cytotoxicity of uncrosslinked PEI1.8 despite its apparent high molecular weight upon crosslinking, while keeping transfection efficiency close to that mediated by PEI25.

3.4. Synthesis and characterization of LLO-s-s-PEI conjugate

In order to ensure that LLO is complexed with the condensed pDNA, stays in stable association until uptake, and then delivered and co-internalized into endocytic compartments, we chose and conjugated high molecular weight PEI (PEI25) to LLO via disulfide linkage, which was prepared similarly to the previously-reported LLO-s-s-protamine [8]. As a result of the high affinity of PEI25 for plasmid DNA in comparison with protamine, it is expected that LLO of LLO-s-s-PEI construct would therefore indirectly be tightly associated with the pDNA. The conjugation yield after disulfide bond formation between LLO and PEI25 was determined by monitoring the generation of pyridine-2-thione at 343 nm as different concentrations of LLO solution was added for reaction. With increased amount of LLO added to pyridyldithiol-activated PEI25 solution, the absorbance at 343 nm due to the generated pyridine-2-thione was increased proportionally. As a result, we achieved to synthesize LLO-s-s-PEI25 in which LLO was conjugated at a 1:1 molar ratio to pyridyldithiol-activated PEI25. Lack of LLO-mediated

hemolytic activity of LLO-s-s-PEI25 and its reducibility with reducing condition was evaluated using red blood cells (RBCs) as a substrate for LLO activity. In this RBC hemolysis-based LLO activity assay shown in n process by focusing 3, a fixed equal amount (10 ng) of LLO-s-s-PEI25 was added to 2 mL of RBCs with or without 5 mM DTT. Lysis of RBCs by LLO released from the LLO-s-s-PEI25 under the disulfide reducing condition (with DTT) and under non-reducing condition (without DTT) was assayed by monitoring the change in right angle light scattering at 590 nm wavelength. LLO-s-s-PEI25 exhibited no detectable hemolytic activity when no DTT was present; no hemolytic activity of LLO in the synthesized LLO-s-s-PEI25 without reduction of the disulfide bond was expected since it is known that oxidation of LLO's only cysteine (C484) with sufficiently bulky molecules results in LLO inactivation. However, the LLO hemolytic activity of LLO-s-s-PEI25 was regained after the addition of DTT (Fig. 3) as shown by the rapid decrease in right angle light scattering of RBC upon addition of DTT to LLO-s-s-PEI25 incubated with RBC after a lag time (~50 s); in a reducing environment, the disulfide bond of LLO-s-s-PEI25 is designed to be cleaved between the C484 of LLO and the PEI, and LLO is released in its active form.

3.5. Effect of LLO-s-s-PEI conjugate on gene expression

The gene transfection efficiency was enhanced by reversible incorporation of LLO into PEI/pDNA complexes through the use of the synthesized LLO-s-s-PEI conjugate (Fig. 4). To investigate the dose-dependency of the amount of LLO-s-s-PEI incorporated in the PEI/pDNA complex (expressed as w/w % of LLO-s-s-PEI25 relative to the total PEI in the PEI/pDNA complex), different amounts of LLO-s-s-PEI were added to polymer-containing solutions and pre-mixed before condensate formation with pDNA. For this experiment, the weight ratio of PEI to pDNA tested was 6.45. Incorporation of LLO-s-s-PEI at only 1% (w/w) of total PEI enhanced luciferase expression (Fig. 4) close to two-fold over no LLO-s-s-PEI (0%) in PEI/pDNA condensates. This enhancement by LLO-s-s-PEI was achieved with no detectable additional cytotoxicity monitored by XTT assay; there was no significant difference in cytotoxicities between cells incubated PEI/pDNA with 1% (w/w) LLO-s-s-PEI and without LLO-s-s-PEI. The positive effect of LLO-s-s-PEI indicates that the disulfide bond of LLO-s-s-PEI incorporated in the PEI/pDNA complex is reduced upon internalization by cells, with resultant enhanced pDNA delivery from the endocytic compartment via LLO activity released from the PEI/pDNA condensates. The effect observed here of LLO in the form of LLO-s-s-PEI incorporated in PEI/pDNA, although significant without any induction of cytotoxicity, was similar to that previously observed with LLO-s-s-protamine, but not as dramatic as that using LLO-s-s-protamine incorporated in protamine/pDNA condensates [8]. With the data presented in Figs. 2 and 4, we demonstrate that reversible incorporation of LLO into PEI/pDNA condensates, through the use of a synthesized LLO-s-s-PEI conjugate, further enhances the transfection efficiency beyond that of PEI alone, regardless of PEIs used for condensation. First we tested the transfection efficiency in HEK 293 cells using the three different forms of PEIs at selected w/w ratios (2.58, 6.45 and 12.9), and s-s PEI1.8 gave a significantly higher level of gene expression than the PEI1.8 at all three polymer/pDNA ratios tested. Both, unmodified and crosslinked, forms of PEI1.8 exhibited higher transfection than PEI25 (n process by focusing 2) at 12.9 polymer to pDNA w/w ratio. Then, to test the effect of synthesized LLO-s-s-PEI conjugate on transfection with PEIs, different amount of LLO-s-s-PEI was incorporated in the PEI/pDNA complex, in which the weight ratio of PEI to pDNA was fixed at 6.45. (n process by focusing 4). The data showing that the enhancement in gene expression by LLO incorporation was more prominent in protamine/pDNA than in PEI/pDNA could presumably be due to already enhanced gene expression with PEI as a condensing agent and strong proton binding capacity of PEI, along with the optimum activity of LLO at acidic pH. These results also suggest that more investigations regarding the pH-dependent activity of LLO with non-viral gene carriers are needed for developing an optimal LLO-mediated DNA delivery system.

4. Conclusion

We have in this report investigated and demonstrated that enhanced gene transfection efficiency, which is comparable to that by the most commonly used PEI25, can be achieved by incorporating two unique strategies that overcome several key barriers in gene delivery: by reversibly crosslinking low molecular weight PEI (PEI1.8) using disulfide bonds, gene expression was enhanced with greatly reduced cytotoxicity; additionally, reversible incorporation of the endosomolytic function of LLO into the PEI/pDNA condensates, through the use of the synthesized LLO-s-s-PEI conjugate, further enhances the transfection efficiency beyond that of PEI alone. Further refinement of the delivery system in regards to the choice of polymers and their crosslinking will be necessary for optimal condensation of plasmid DNA and for controlled incorporation of LLO activity, which maximizes the effect of LLO and crosslinked polymer and at the same time keeps polymer-induced cytotoxicity minimal.

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Fig. 1.

Characterization of thiolated and disulfide-crosslinked PEI1.8: (a) Oxidation of thiolated PEI1.8 and formation of disulfide-crosslinked PEI1.8 (s-s PEI1.8). The amount of remaining free thiols upon disulfide-crosslinking of thiolated PEI1.8 was monitored as a function of incubation time using Ellman's assay; (b) pH titration of disulfide-crosslinked PEI1.8 (s-s PEI1.8) to monitor its proton binding capacity in comparison with PEI25. Aliquots of 1 M HCl solution were added from pH 11.5 PEI solutions, and the change of pH was monitored for s-s PEI1.8 and PEI25; (c) Gel retardation assay of PEI/pDNA complex ((1) s-s PEI1.8, (2) PEI1.8, and (3) PEI25). Lane 1, DNA ladder; Lane 2, pDNA only; Lane 3–7, PEI/pDNA complex at

weight ratio of PEI to pDNA =0.06, 0.12, 0.25, 0.5, and 0.77, respectively. (d) Particle size of PEIs at weight ratio of PEI to pDNA =1.29, 2.58, and 6.45.

Fig. 2.

Transfection with three different forms of PEIs (different molecular weights and different crosslinking: s-s PEI1.8; PEI1.8; PEI25) (a) The effect of disulfide-crosslinking of PEI1.8 on luciferase gene expression in HEK 293 cells at different PEI/pDNA weight ratios; HEK 293 cells were transfected with 1 μ g of pDNA complexed with PEIs (disulfide-crosslinked PEI1.8 (s-s PEI1.8); PEI1.8; s-s PEI25) ($n=3$, error bars represent standard deviation); (b) The effect of various forms of PEIs on cell viability in HEK 293 cells at different PEI/pDNA weight ratios. Cytotoxicities of pDNA condensates with the three forms of PEIs (s-s PEI1.8, PEI1.8, and PEI25) as in (a) above, incubated at varying w/w ratios of PEI to pDNA during transfection, were monitored by XTT assay. ($n=3$, error bars represent standard deviation).

Fig. 3.

LLO activity of LLO-s-s-PEI monitored by *in vitro* red blood cell hemolysis assay. LLO-s-s-PEI25 was assayed for its ability to lyse red blood cells (RBCs) by monitoring the change in scattering of 590 nm light by 2×10^8 RBCs/ml in a Spex Fluoromax-2 spectrofluorometer. LLO-s-s-PEI25 exhibited no detectable hemolytic activity (top curve A). Hemolytic activity of LLO-s-s-PEI25 was regained after incubation with 5 mM DTT (curve B). Hemolytic activity of wild type LLO without any modification was shown for a positive control (bottom curve C). The complete restoration of LLO's activity by DTT is indicated by the sigmoidal decrease in the scatter of light by the RBCs, beginning with a lag time of ~50 s. after the addition of LLO. Y-axis represents the relative light scattering of RBCs.

Fig. 4.

The effect of increasing amounts of LLO-s-s-PEI25, incorporated in the PEI/pDNA condensates, on luciferase gene expression in HEK 293 cells. The amount of LLO-s-s-PEI25 incorporated in the PEI/pDNA complex (expressed as w/w % of LLO-s-s-PEI25 relative to PEI in the PEI/pDNA complex) was increased using various forms of PEI (PEI1.8, s-s PEI1.8, and PEI25) at PEI/pDNA weight ratio of 6.45 ($n=3$, error bars represent standard deviation).