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Design and Characterization of Novel Recombinant Listeriolysin O–Protamine Fusion Proteins for Enhanced Gene Delivery

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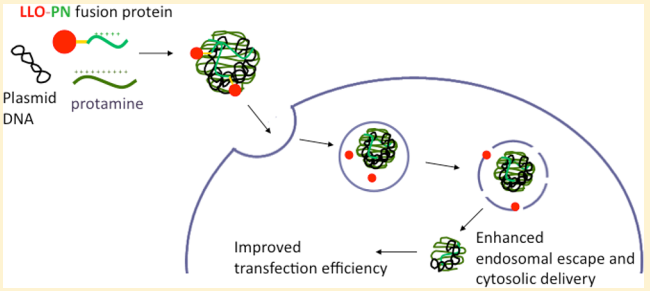
Supporting Information

ABSTRACT: To improve the efficiency of gene delivery for effective gene therapy, it is essential that the vector carries functional components that can promote overcoming barriers in various steps leading to the transport of DNA from extracellular to ultimately nuclear compartment. In this study, we designed genetically engineered fusion proteins as a platform to incorporate multiple functionalities in one chimeric protein. Prototypes of such a chimera tested here contain two domains: one that binds to DNA; the other that can facilitate endosomal escape of DNA. The fusion proteins are composed of listeriolysin O (LLO), the endosomolytic pore-forming protein from *Listeria monocytogenes*, and a 22 amino acid sequence of the DNA-condensing polypeptide protamine (PN), singly or as a pair: LLO-PN and LLO-PNPN. We demonstrate dramatic enhancement of the gene delivery efficiency of protamine-condensed DNA upon incorporation of a small amount of LLO-PN fusion protein and further improvement with LLO-PNPN *in vitro* using cultured cells. Additionally, the association of anionic liposomes with cationic LLO-PNPN/protamine/DNA complexes, yielding a net negative surface charge, resulted in better *in vitro* transfection efficiency in the presence of serum. An initial, small set of data in mice indicated that the observed enhancement in gene expression could also be applicable to *in vivo* gene delivery. This study suggests that incorporation of a recombinant fusion protein with multiple functional components, such as LLO–protamine fusion protein, in a nonviral vector is a promising strategy for various nonviral gene delivery systems.



KEYWORDS: *listeriolysin O, protamine, fusion protein, nonviral DNA delivery*

INTRODUCTION

Nonviral gene delivery vectors, including those based on lipids, polymers, proteins, and peptides, have been studied as attractive alternatives to viral vectors, with advantages such as potentially less immunogenicity and ease of manufacturing as pharmaceuticals.[1−3] The relatively low transfection efficiency of the nonviral vectors has been a major drawback. This limitation is mainly due to their lack of active molecular mechanisms that would otherwise facilitate overcoming multiple biological barriers, including the extracellular environment, plasma membrane, endolysosomes, cytoplasm, and nuclear membrane, all of which exogenous genes must pass through for successful expression of the genes and resulting modification of cellular phenotype.[4−6] Therefore, the success of nonviral vector-mediated gene delivery depends on the development of delivery vectors that can carry DNA protected from the extracellular environment, promote binding and uptake by cells, and actively and efficiently surmount the physical and biological barriers inside cells.

In order to improve transfection efficiency, various functional components have been incorporated into vectors that allow DNA binding and condensation, cellular targeting, endosomal escape, or nuclear import.[7,8] In most cases, each component is assembled by chemical conjugation, for example, conjugation of a receptor-targeting antibody and/or a membrane fusogenic peptide to liposomes or polymers.[9−15] Instead of chemical conjugation, one of the more recent methods for incorporating multiple components into a single vector utilizes genetically engineered fusion proteins containing more than one motif, which has advantages over chemical conjugation in terms of both the relatively straightforward production of large amounts of homogeneous fusion proteins and the design of various fusion proteins with different functional groups with ease. Some studies have reported improved DNA delivery efficiency using genetically engineered vectors with functional components *in vitro*, with low or limited applicability to enhanced gene expression *in vivo*.[16−20]

In this study, we report dramatic enhancement of gene delivery efficiency using two prototypes of genetically engineered fusion proteins incorporated in currently available

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nonviral vectors, characterized and demonstrated *in vitro* and in cultured cells, and tested for their potential utility and applicability *in vivo*. We designed bifunctional recombinant fusion proteins that incorporate listeriolysin O (LLO), the endosomolytic pore-forming protein from *Listeria monocytogenes*, at the N-terminus, and a DNA-condensing cationic peptide sequence derived from protamine (PN), singly or as a pair, at the C-terminus (LLO-PN and LLO-PNPN). Protamine is a positively charged sperm chromatin component that electrostatically binds to and condenses DNA.[21,22] The condensation of large, anionic DNA molecules with cationic protamine improves the cellular binding and uptake as well as the protection of DNA from enzymatic degradation in biological environments. Upon cell binding, most of the nonviral vectors, including condensed DNA polyplexes and DNA lipoplexes, are typically internalized in endocytic compartments and degraded along the endolysosomal pathway unless delivered efficiently from the endolysosome to the cytosol.[7,23] Therefore, promoting endosomal escape of most nonviral vectors confers a significant improvement in transfection efficiency. We chose LLO as a component of our fusion protein as LLO has several properties ideally suited for cytosolic delivery of endocytosed macromolecules: (i) LLO can breach the endosomal membrane and promote the cytosolic delivery of whole *Listeria* bacteria, which are much larger than the size of most, if not all, currently studied nanoscale gene delivery vectors; (ii) LLO is most active at the pH of the endosome (5.5−5.9) but has attenuated activity at the pH-neutral compartment of the cytosol; (iii) LLO has been shown to be degraded relatively rapidly upon reaching the cytosol, further limiting potential damage to cells.[24−26] Thus, cytosolic delivery of macromolecules with LLO can in principle be achieved with relatively limited cytotoxicity, especially that which might result from permeabilization of membranes in a pH-neutral environment such as the plasma membrane and other intracellular organelle membranes.

Previously we reported increased *in vitro* gene expression using LLO that was chemically conjugated to either protamine or polyethylenimine.[27,28] Here, we hypothesized that genetically engineered fusion proteins consisting of LLO and a segment of human protamine can be incorporated into a nonviral gene delivery system with similar or better results in augmenting DNA delivery than those seen with chemically constructed LLO and protamine. We tested the transfection efficiencies of delivery systems incorporating various ratios of such fusion proteins in protamine/DNA polyplexes, and also complexed the system with anionic liposomes to reduce potential nonspecific interactions and further protect the complexes. We report here that such fusion proteins can be prepared, characterized, and incorporated to dramatically enhance the delivery efficiency, ultimately demonstrating the feasibility of new approaches for constructing and improving nonviral vector systems with multiple functionalities.

MATERIALS AND METHODS

Cloning, Expression, and Purification of Fusion Proteins. All chemicals and reagents were purchased from Thermo Fisher Scientific (Waltham, MA, USA) unless otherwise noted. The DNA encoding the polypeptide linker (GGGGSGGGGSRGFFPGGGGSGGGGS) and Arg-8 to Ser-29 of human protamine (RSQSRSRYYRQRQRSRRRRRRS),[29] made by annealing two complementary oligonucleotides (IDT, Coralville, IA, USA), was inserted into the 3′-end of the LLO cDNA in the bacterial expression vector pET29b (EMD Biosciences, Gibbstown, NJ, USA) at restriction sites *Bgl*II and *Not*I to ultimately produce a C-terminal protamine followed by a 6×His tag. To construct the cDNA encoding LLO-PNPN, another cDNA encoding an identical human protamine segment with restriction sites *Not*I and *Xho*I was inserted into the 3′-end of the first protamine cDNA. The fidelity of the resultant construct to the original design was verified by DNA sequencing at the University of Michigan DNA Sequencing Core.

The expression construct containing LLO-PN or LLO-PNPN was transformed into *Escherichia coli* strain BL21(DE3) RIPL (Agilent Technologies, Inc., Santa Clara, CA, USA). Starting cultures from single colonies were grown in 50 mL of LB medium at 37 °C overnight with 30 μg/mL kanamycin and 25 μg/mL chloramphenicol. The starting culture was diluted 1:50 into 2 L of LB medium with 30 μg/mL kanamycin, and incubated at 37 °C until the absorbance at 570 nm, read in an Emax microplate reader (Molecular Devices, Sunnyvale, CA, USA), reached ∼0.7. The culture was induced at 30 °C for 6 h with 1 mM isopropyl β-D-thiogalactopyranoside (IPTG, Invitrogen, Carlsbad, CA, USA), and then centrifuged at 6000*g* for 10 min at 4 °C, and the bacterial cell pellet was frozen at −80 °C until purification. The bacterial pellet was resuspended in lysis buffer (50 mM sodium phosphate, 300 mM NaCl, 20 mM imidazole, 200 μM phenylmethylsulfonyl fluoride (PMSF)) and lysed using a French press (Thermo Spectronic, Madison, WI, USA). The lysate was centrifuged at 10000*g* for 40 min, and the supernatant was incubated with 2 mL of Ni^{2+}-NTA agarose (Qiagen, Valencia, CA, USA) for 2 h. The Ni^{2+}-NTA agarose was washed with a total of 400 mL of wash buffer (50 mM sodium phosphate, 300 mM NaCl, 20 mM imidazole) and eluted with wash buffer containing 250 mM imidazole. The fusion proteins were run in PD-10 desalting columns (GE Healthcare Life Sciences, Piscataway, NJ, USA) for buffer exchange (50 mM sodium phosphate, 300 mM NaCl), and stored in 40% glycerol at −80 °C. The expression of the fusion protein was confirmed by SDS−PAGE with Simply Blue (Invitrogen) staining, and protein concentration was determined by a bicinchoninic acid (BCA) protein assay using bovine serum albumin as the standard (Thermo Fisher Scientific).

Hemolysis Assay. The membrane pore-forming activity of LLO-PN or LLO-PNPN was assessed using an *in vitro* red blood cell (RBC) hemolysis assay as previously described.[30] Briefly, RBCs were washed three times with phosphate-buffered saline (PBS, pH 7.4) and resuspended at a concentration of 2×10^8 cells/mL in MBSE (10 mM MES pH 5.5 containing 140 mM NaCl and 1 mM EDTA) with 2 mM DTT. To 100 μL of RBCs was added 0−100 ng of fusion protein in 100 μL of HEPES-buffered glucose (HBG: 280 mM glucose, 10 mM HEPES, pH 8.4), and the mixture was incubated for 15 min at 37 °C; final pH ≈ 7. The released hemoglobin from lysed RBCs was measured by absorbance at 450 nm in an Emax microplate reader.

Preparation of Plasmid DNA for Transfection Studies. The bicistronic expression plasmid pNGVL3 encoding firefly luciferase and green fluorescent protein (GFP), both under the control of the cytomegalovirus promoter, was a gift from Dr. Gary Nabel (Vaccine Research Center, National Institutes of Health, MD, USA). The plasmid DNA was isolated and purified from *E. coli* using Qiagen Giga Endofree Plasmid Purification kits (Qiagen). Concentrations of plasmid DNA

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Mol. Pharmaceutics 2015, 12, 342−350

were spectrophotometrically determined in a BioTek Synergy HT microplate reader (Winooski, VT, USA) using absorbance at 260 nm, and the ratio of absorbance at 260 to 280 nm was consistently above 1.8.

Preparation of Fusion Protein/Protamine/DNA Complexes and Liposomes. To prepare LLO-PN/protamine/DNA or LLO-PNPN/protamine/DNA complexes, various amounts (0−0.6 mol % of protamine) of LLO-PN or LLO-PNPN were mixed with DNA in HBG and incubated for 20 min at room temperature, and then an equal volume of protamine (Salmine, Sigma-Aldrich, St. Louis, MO, USA) in HBG was added at a weight ratio of 1.2 (which corresponds to a positive/negative charge (+/−) ratio of 1.6), and the complexes were further incubated for 20 min. The final concentration of DNA in the complexes was 150 μg/mL.

In order to prepare complexes associated with negatively charged liposomes, a thin lipid film composed of phosphatidylethanolamine (PE, Avanti Polar Lipids, Alabaster, AL, USA) and cholesteryl hemisuccinate (CHEMS, Sigma-Aldrich) was prepared. PE dissolved in chloroform and CHEMS dissolved in chloroform/methanol (1/1) were mixed at a 2:1 molar ratio and dried to a thin film using a Büchi Rotavapor R-200 rotary evaporator (Büchi Labortechnik AG, Flawil, Switzerland) at 25 °C under <10 mmHg vacuum. The lipid film was hydrated with LLO-PNPN/protamine/DNA complexes by vortexing and sonicating for 30 s twice in a bath-type liposome sonicator (Laboratory Supplies Co., Inc., Hicksville, NY, USA). For each 1 μg of DNA, 7.5 nmol of lipid was used, and the final concentration of lipid was 1.125 mM. For the heat-inactivated negative controls, half of the samples were heated at 75 °C for 10 min after complex formation or liposome association.

Particle Size and Zeta Potential Measurement. The LLO-PNPN/protamine/DNA complexes or liposome(LLO-PNPN/protamine/DNA) were prepared at a DNA concentration of 40 μg/mL in HBG with a 1.6 (+/−) ratio of protamine/DNA. For LLO-PNPN/protamine/DNA, 0.15% of LLO-PNPN was used (keeping the 1.6 (+/−) ratio of protamine/DNA), and 7.5 nmol of lipid film composed of PE and CHEMS was hydrated with the complexes by alternately vortexing and sonicating. The samples were diluted to 5 μg/mL with HBG, and the particle size and zeta potential were determined by quasi-elastic light scattering using a Nicomp 380 ZLS (Particle Sizing Systems, Santa Barbara, CA, USA) zeta potential/particle sizer equipped with an avalanche photodiode detector.

***In Vitro* Transfection.** The murine macrophage-like cell line P388D1 (ATCC, Manassas, VA, USA) was plated in 24-well plates at 1.5×10^5 cells per well and incubated in RPMI-1640 containing 10% FBS, antibiotics (100 units/mL penicillin and 100 μg/mL streptomycin), and 1 mM sodium pyruvate for 24 h before transfection. Cells were grown at 37 °C in a 5% CO_2 humidified atmosphere and were typically ∼70% confluent at the time of transfection. For transfection studies, 300 μL of the transfection sample containing 2 μg of DNA in serum-free or 10% serum-containing RPMI 1640 was added dropwise into each well. All experiments were performed using triplicate samples. After a 4 h incubation with cells at 37 °C, transfection samples were replaced with fresh complete medium and cells were further incubated for 24 h. Thereafter, the medium was removed and the cells were washed once with PBS. The luciferase gene expression in cells was measured using a luciferase assay kit according to the manufacturer's protocol (Promega, Madison, WI, USA). Briefly, cells in each well were lysed with 100 μL of Cell Culture Lysis Buffer (Promega), and lysed cells were transferred to a microcentrifuge tube, vortexed for 10 s, and centrifuged at 12000*g* for 1 min. Then, 20 μL of supernatant was assayed for its luciferase activity with 100 μL of luciferase substrate (Promega) using a BioTek Synergy HT plate reader at 25 °C in luminescence mode. The luciferase activity was expressed as relative luminescence units (RLU) normalized by total cellular protein as determined by BCA assay. Cell viability was monitored by measuring the amount of total cellular protein in each well, in comparison with control wells of untransfected cells without DNA, and is reported as the percentage of cellular protein recovery, determined by BCA assay, after transfection.

***In Vivo* Transfection Study.** Female C57BL/6 mice, 6−7 weeks old, were obtained from Harlan Laboratories (Indianapolis, IN, USA). The mice were kept in filter-topped cages with freely available standard food and water and a 12 h light/dark cycle. The experiment protocols were reviewed and approved by the University Committee on Use and Care of Animals (UCUCA) at the University of Michigan.

Mice in groups of six were intravenously injected via tail vein with 50 μg of DNA per mouse formulated as LLO-PNPN/protamine/DNA, liposome(LLO-PNPN/protamine/DNA), heat-inactivated LLO-PNPN/protamine/DNA, heat-inactivated liposome(LLO-PNPN/protamine/DNA), or HBG buffer only. Mice were sacrificed after 24 h, and spleens, lungs, and livers were harvested, washed with PBS twice at 4 °C, and homogenized with Cell Culture Lysis Buffer. The homogenates were centrifuged at 12000*g* for 10 min at 4 °C, and 20 μL of supernatant was assayed for luciferase activity as described above. The results of the luciferase expression in mice are reported as RLU (from which the RLU of buffer-injected control group was subtracted) per mg of total tissue protein as determined by BCA assay.

RESULTS

LLO-Protamine Fusion Proteins: Cloning, Expression, Purification, and Characterization. The DNA encoding the human protamine segment (Arg-8 to Ser-29) was inserted singly or in tandem into the 3′-end of the LLO cDNA and subcloned into the bacterial expression vector pET29b, and the sequences were verified by DNA sequencing. The design of the fusion proteins is shown schematically in Figure 1a. His-tagged LLO-PN or LLO-PNPN fusion proteins were expressed in *E. coli* strain BL21(DE3) RIPL and purified using Ni^{2+}-NTA agarose with a typical yield of 5 mg/L of culture for LLO-PN and 2.5 mg/L for LLO-PNPN. The successful expression and high purity (>95%) of LLO-PN (molecular weight, 63 kDa) and LLO-PNPN (molecular weight, 66 kDa) fusion proteins were confirmed by SDS−PAGE (Figure 1b).

The membrane pore-forming activities of the purified fusion proteins, LLO-PN and LLO-PNPN, were examined by an *in vitro* red blood cell hemolysis assay. The hemolytic activities of fusion proteins were somewhat reduced compared to that of wild type LLO at low concentrations, but similar at relatively high concentrations (≥0.5 μg/mL) and equal at the highest concentration tested (Figure 1c). The relatively attenuated activities of the fusion proteins at the lower concentrations can be explained by the position of the protamine segment (i.e., at the C-terminus of LLO) in the fusion proteins because the C-terminus of LLO has been implicated in binding to cholesterol-containing membranes for subsequent pore formation.[25] The PN-LLO fusion protein, with PN in the N-terminus LLO,

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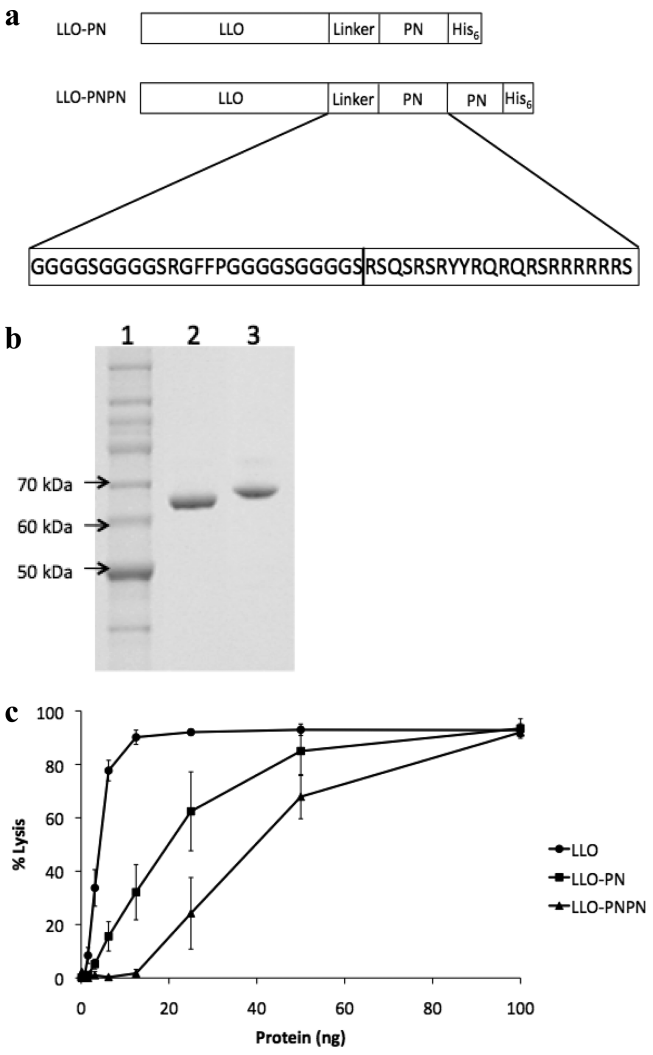

Figure 1. Recombinant LLO-PN and LLO-PNPN fusion proteins and their hemolytic activities. (a) Schematic representation of the recombinant fusion proteins, LLO-PN and LLO-PNPN. The fusion proteins consist of LLO at the N-terminus, linker, one or two copies of a segment of human protamine (PN, residues 8−29), and C-terminal hexahistidine (His_6). (b) SDS−PAGE of expressed and purified LLO-PN and LLO-PNPN fusion proteins. Lane 1: protein molecular weight standards. Lane 2: LLO-PN (63 kDa). Lane 3: LLO-PNPN (66 kDa). (c) Hemolytic activities of LLO, LLO-PN, and LLO-PNPN. Various amounts of LLO or fusion proteins were incubated with RBCs at 37 °C for 15 min, and the release of hemoglobin from lysed RBCs was monitored by the absorbance at 450 nm. The activities of LLO, LLO-PN, and LLO-PNPN were assayed and compared for their ability to perforate membranes by monitoring lysis of RBCs. Protamine alone, without LLO, at comparable amounts did not show any detectable hemolytic activity (not shown in the figure).

exhibited hemolytic activity equal to that of LLO at all concentrations tested (Figure S1 in the Supporting Information).

Transfection Efficiency of Fusion Protein/Protamine/DNA Complexes in P388D1 Cells. In order to investigate the effect of incorporating the LLO-PN and LLO-PNPN fusion proteins into protamine/DNA complexes, the fusion proteins were added to the complexes at 0−0.6 mol % of protamine, keeping the ratio of protamine to DNA constant at 1.2 (w/w), which corresponds to a positive/negative charge ratio of 1.6 (+/−). The murine macrophage-like cell line P388D1 was used to test the *in vitro* transfection efficiencies of the complexes. This cell line was chosen because it is the most challenging cell type for transfection among the cell types used in our previous studies that employed an LLO-protamine chemical conjugate, and also because of the relevance of antigen-presenting cells (APCs) to the clinical application of this gene delivery vector in DNA vaccine delivery applications.[9,27] The luciferase gene expression with either LLO-PN or LLO-PNPN was 2 to 4 orders of magnitude higher than that of protamine/DNA complexes without fusion proteins under serum-free conditions (Figure 2a,b). In the presence of serum, overall the transfection efficiency of all treatments was lower: those of fusion protein/protamine/DNA were only slightly decreased by the presence

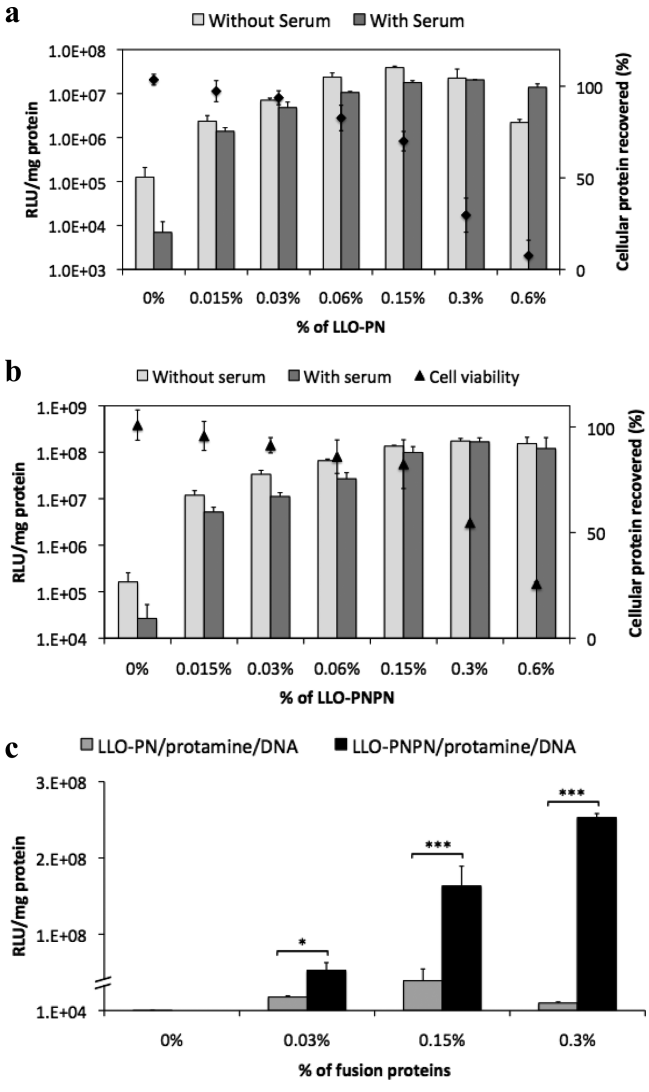

Figure 2. Effect of various amounts of LLO-PN or LLO-PNPN incorporated in protamine/DNA condensates on transfection efficiency and cell viability in P388D1 cells. Increasing amounts of LLO-PN (a) or LLO-PNPN (b) were mixed with protamine/DNA complexes while maintaining a 1.6 (+/−) charge ratio. P388D1 cells were incubated with the complexes (2 μg of DNA/well) in the absence or presence of serum. Luciferase activity of cell lysates was determined 24 h after transfection. Dots indicate cell viability, i.e., the mean ± SD total cellular protein recovered after transfection. (c) Comparison of the transfection efficiencies of LLO-PN/protamine/DNA and LLO-PNPN/protamine/DNA in P388D1 cells in serum-free medium. (* $p < 0.05$, *** $p < 0.001$) ($n = 3$, mean ± SD).

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Mol. Pharmaceutics 2015, 12, 342−350

of serum, while that of protamine/DNA without fusion protein was drastically reduced (Figure 2a,b). With increasing amounts of LLO-PN or LLO-PNPN beyond 0.3 mol % of protamine, we observed a concomitant increase in cytotoxicity, as indicated by the reduced recovery of total cellular protein after transfection. The cytotoxicity of both LLO-PNPN and LLO-PN was minimal when incorporated at up to 0.3 mol %, and the cytotoxicity of LLO-PNPN remained lower than that of LLO-PN when incorporated beyond 0.3 mol % of protamine.

The effect of changing the ratio of full-length protamine to DNA on gene delivery was also examined with various amounts of protamine (0.1−2.4 protamine/DNA (w/w)) and a fixed amount of LLO-PN (0.15%); luciferase gene expression was enhanced with increasing amounts of protamine up to a 1.2 weight ratio, with no further enhancement in expression beyond the 1.2 ratio (Figure S2 in the Supporting Information). While both LLO-PN and LLO-PNPN markedly enhanced the luciferase gene expression of protamine/DNA complexes, the gene expression with LLO-PNPN was 3- to 4-fold more enhanced compared to that with LLO-PN (Figure 2c) at the same mol % incorporated. Therefore, LLO-PNPN was exclusively used for further transfection experiments, presented below.

We then investigated whether the transfection efficiency and cytotoxicity would be modified and controlled by further complexing the LLO-PNPN-containing cationic polyplexes with anionic liposomes, similar to the previously reported LPD systems.[31−33] The association of analogous gene delivery vehicles with anionic liposomes has been shown to reduce potential nonspecific interactions with plasma proteins relative to those of positively charged complexes.[34] To assess the effect of liposome association, a base condensate formulation consisting of 1.2 (w/w) protamine/DNA with incorporated LLO-PNPN (at 0.15 mol %) was used, since this composition struck an optimal balance of significantly enhanced gene expression level with low cytotoxicity as shown in Figure 2a. The average diameter of the complexes without liposomes was 166 (±47) nm, and zeta potential was 20.1 (±0.9) mV. The theoretical net negative charge ratio (0.88 (+/−)), predicted to result from the addition of 7.5 nmol of anionic PE:CHEMS liposomes/μg of DNA to the cationic polyplexes (theoretical ratio 1.6 (+/−)), was confirmed by measuring the zeta potential of the final liposome-containing complexes: −27.9 (±2.0) mV. The average diameter of the formulation increased to 242 (±94) nm when complexed with liposomes.

The LLO-PNPN/protamine/DNA gene delivery systems were compared with negative and positive controls in the presence and absence of serum (Figure 3). As a negative control, heat-inactivated formulations were also prepared in order to assess the LLO-mediated enhancement of the transfection efficiency; these heat-inactivation conditions (75 °C for 10 min) abolish LLO's hemolytic activity,[32] which provides an ideal negative control with all the components present in the complexes except the activity of LLO. Lipofectamine was used as a positive control in order to examine the relative efficiency of the gene delivery system compared to a commonly used DNA delivery vector. The luciferase gene expression with heat-inactivated LLO-PNPN/protamine/DNA or liposome(LLO-PNPN/protamine/DNA) in P388D1 was significantly decreased compared to that without heat inactivation ($p < 0.0001$), while heat inactivation did not affect the transfection efficiency of liposome-(protamine/DNA) without LLO-PNPN. These results suggest

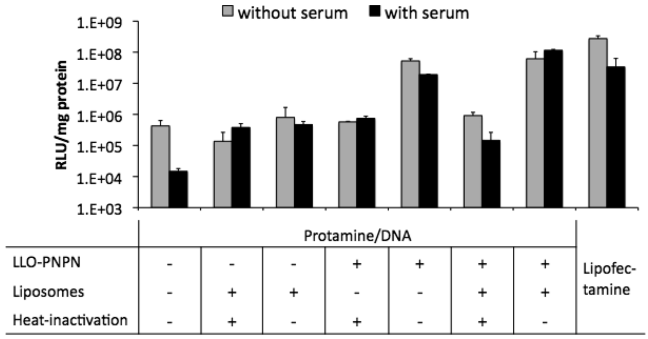

Figure 3. Effect of anionic liposomes on transfection efficiency of LLO-PNPN/protamine/DNA complexes in P388D1 cells. The complexes were prepared with LLO-PNPN (incorporated at 0.15% of protamine) and used to hydrate lipid films composed of PE and CHEMS (7.5 nmol of lipids/μg of DNA) by vortexing and sonication. Protamine/DNA complexes and liposome(protamine/DNA) without LLO-PNPN were also prepared for comparison. As a negative control, samples were heat inactivated at 75 °C for 10 min in order to abolish LLO's hemolytic activity; Lipofectamine was used as a positive control. Plasmid DNA in various formulations was incubated with P388D1 cells at 2 μg of DNA per well without or with 10% serum, and luciferase activity of cell lysates was assayed 24 h after transfection (n = 3, mean ± SD).

that LLO-PNPN plays a key role in the enhancement of transfection efficiency as well as that heat inactivation does not negatively impact the rest of the complex including all other factors such as particle size, charge, and thus the stability and cellular uptake of the complexes. In the presence of 10% serum, the transfection efficiency of the tested gene delivery systems containing LLO-PNPN was comparable to or better than the luciferase gene expression achieved using Lipofectamine; luciferase activity with LLO-PNPN/protamine/DNA was similar to that with Lipofectamine ($p > 0.05$), while luciferase activity with liposome(LLO-PNPN/protamine/DNA) was higher than that with Lipofectamine ($p < 0.01$). Lipofectamine showed significantly higher transfection efficiency in the absence of serum ($p < 0.0001$), and therefore showed generally better performance than the tested gene delivery systems under no-serum conditions ($p < 0.001$).

***In Vivo* Luciferase Gene Expression.** A limited set of preliminary *in vivo* experiments were performed to investigate the feasibility of the LLO-PNPN fusion protein-based gene delivery vector for *in vivo* application; LLO-PNPN/protamine/DNA with or without liposomes was intravenously administered to C57BL/6J mice, and luciferase activity was measured in spleen, liver, and lung. Although this system was initially intended and characterized in macrophage-like cells for DNA vaccine applications as its potential immediate use, and despite the fact that the carriers have not been designed or optimized for *in vivo* systemic gene delivery, we tested using an iv administration route whether the augmentation of gene expression can be achieved in any tissue in comparison with its heat-inactivated counterpart. The luciferase gene expression from the liposome (LLO-PNPN/protamine/DNA) formulation was detected in spleen and lung, with a higher expression level in spleen, while luciferase activity produced by LLO-PNPN/protamine/DNA was only detected in lung (Figure 4). Overall the expression level in these tissues examined was low using the 0.15% LLO-PNPN tested in these preliminary experiments, and there was no detectable expression in liver above that in liver of control animals. Consistent with their *in*

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Mol. Pharmaceutics 2015, 12, 342−350

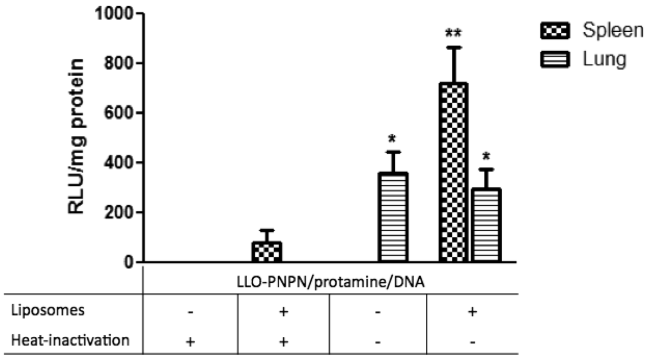

Figure 4. *In vivo* luciferase gene expression. LLO-PNPN/protamine/DNA or liposome(LLO-PNPN/protamine/DNA) was injected intravenously into mice (50 μg of DNA/mouse), and mice were sacrificed 24 h following injection. The spleens and lungs were harvested and homogenized in lysis buffer, and the supernatants were assayed for luciferase activity (n = 6, mean ± SEM). The RLU of the buffer-injected control group was subtracted from the RLU of each sample. * $p < 0.05$, ** $p < 0.01$ (compared to heat-inactivated liposome(LLO-PNPN/protamine/DNA).

vitro transfection efficiencies, heat-inactivated controls *in vivo* showed much lower or nondetectable luciferase gene expression in spleen or lung.

DISCUSSION

Recognizing the importance of equipping and enabling a nonviral gene delivery system with functional components for the efficient transport of DNA through multiple biological barriers, genetically engineered fusion proteins that consist of diverse functional motifs have recently been studied as potentially effective and relatively safe nonviral vectors. In this report, in order to demonstrate the proof-of-concept of utilizing a well-defined multifunctional recombinant fusion protein as a component of gene delivery vectors, we designed and purified fusion proteins containing two functional components, LLO and a segment of human protamine, which can bind to DNA and facilitate its endosomal escape, resulting in an enhanced transfection efficiency of protamine/DNA complexes in cultured cells.

Previously, we observed an increase in transfection efficiency using LLO chemically conjugated via disulfide bond to protamine (LLO-s-s-protamine) or with 25 kDa polyethylenimine (LLO-s-s-PEI).[27,28] The fusion constructs characterized in the current study, LLO-PN and LLO-PNPN, are hemolytically active as long as the single cysteine of LLO is reduced. The relative hemolytic activity of LLO-PN was comparable to that of LLO-s-s-PEI in the presence of DTT, and that of LLO-PNPN was lower than that of LLO-s-s-PEI. The highest transfection efficiencies were achieved in the previous studies when LLO-s-s-protamine was incorporated at 1.2% of protamine in protamine/DNA complexes, or LLO-s-s-PEI at 1% of PEI in PEI/DNA complexes, respectively. In the current study, only 0.15% of LLO-PNPN was needed for a dramatic increase in luciferase gene expression, which is approximately 10-fold less than the amount that was required with the chemical conjugates of LLO and polycation in the previous studies to achieve roughly equivalent transfection levels.

The 0.15 mol % incorporation of LLO-PNPN corresponds to approximately two LLO-PNPN molecules per 7 kbp plasmid DNA. The currently accepted model for their mechanism of pore formation suggests that the family of cholesterol-dependent pore-forming cytolysins to which LLO belongs generally requires 33−50 monomers per pore.[25] Perales et al. have calculated that each polycation/DNA complex having an average diameter of 50−200 nm contains from 5 to 20 plasmid DNA molecules.[35] If each LLO-PNPN/protamine/DNA complex with an average diameter of 150−200 nm contains 15−20 plasmid DNA molecules, and approximately 30−40 LLO-PNPN molecules are in each protamine/DNA complex, then 0.15% LLO-PNPN is theoretically sufficient for pore formation in endosomal membranes.[25]

It is not clear why the level of enhancement is higher using the fusion proteins compared with the chemical conjugates. Two factors might be responsible: (1) the reduction of cysteine in the fusion proteins could be more efficient once taken up by cells; (2) the release of LLO activity from the complex might be more efficient than that of LLO-s-s-PEI or LLO-s-s-protamine. The noteworthy difference in the relative transfection efficiencies of the LLO-PNPN fusion protein vs LLO-s-s-protamine or LLO-s-s-PEI may be at least partly due to the fact that LLO has a unique cysteine at amino acid position 484 (C484); oxidation of the sulfhydryl group with a sufficiently bulky moiety abolishes the activity of LLO.[24] The attachment of protamine or PEI via a disulfide bond using the sulfhydryl of C484 reversibly inactivates LLO, and upon reduction of this disulfide inside cells LLO's hemolytic activity is restored. While conjugation of a polycation via a disulfide bond may be a reasonable strategy for regulating LLO's activity, potential variations in reduction processes inside cells may result in incomplete reactivation of LLO or differences in the intracellular locale of LLO reactivation.[36,37] In either case, a relatively higher quantity of LLO-s-s-polycation may therefore be needed to see an improvement in transfection efficiency. In comparison, although their hemolytic activities are lower than that of LLO, very small amounts of the fusion proteins (0.015−0.6%) were shown to be sufficient for the improvement of transfection efficiency with minimal toxicity (Figure 2).

In order to test and potentially control the release of LLO from LLO fusion proteins and thus from the DNA complexes upon uptake by cells, we also examined whether the LLO-PN fusion proteins can be further optimized by targeted cleavage of the PN fragment from LLO within the endolysosomal pathway, similarly to that which we have done with LLO-s-s-polycation conjugates, by introducing a cathepsin D cleavable linker into the fusion protein between LLO and PN (Figure 1a). We hypothesized that if LLO's dissociation from protamine/DNA complexes is promoted inside endolysosomes via hydrolysis by cathepsin D, hypothetically exposing the C-terminus of LLO and thus restoring its maximum pore-forming activity, then this would allow more efficient release and endosomal escape of DNA and result in improved exogenous DNA expression. To test this hypothesis, we designed LLO-PN with a cathepsin D recognition peptide sequence from the B-chain of insulin, RGFFP. As a negative control with a noncleavable linker, the two hydrophobic amino acids (FF) were mutated to positively and negatively charged amino acids (RE), thus keeping the same length and net charge of the fusion protein. We also constructed an N-terminal PN and LLO fusion protein (PN-LLO), which was almost as hemolytically active as parent LLO and relatively more active than LLO-PN and LLO-PNPN at the lower concentrations tested (Figure 1c and Figure S1 in the Supporting Information). When we compared the transfection efficiencies of protamine/DNA complexes with each fusion protein (LLO-PN with cathepsin D cleavable linker, LLO-PN

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Mol. Pharmaceutics 2015, 12, 342−350

with control linker, and PN-LLO) the luciferase gene expression results were not significantly different (Figure S3 in the Supporting Information), suggesting that the presence of the cathepsin D cleavable linker or the position of the PN fragment does not affect the transfection efficiency. These results are perhaps reflective of the fact that, unlike the LLO-s-s-polycation conjugates, which are completely and yet reversibly inactivated, the LLO-PN fusion proteins do not require a reactivation step inside cells for restoration of their functional activity.

In order to optimally deliver DNA to the cytosol, the fusion proteins and the condensed DNA should be internalized together by the cells. With the hypothesis that the number and/or density of positively charged amino acid residues in the LLO−protamine fusion proteins affects their interaction with the protamine/DNA complexes as well as the efficiency of LLO-mediated endosomal release of DNA, we tested fusion proteins with one protamine segment, LLO-PN (22 amino acids, 12 arginines), and two protamine segments, LLO-PNPN (44 amino acids total, 24 arginines). With LLO-PNPN, the luciferase gene expression in P388D1 cells was 3- to 4-fold more enhanced than that with LLO-PN, while simple co-incubation and addition of wild type LLO to protamine/DNA complexes did not produce significant enhancement of *in vitro* transfection efficiency. Upon intravenous injection into mice, LLO-PNPN/protamine/DNA produced significant luciferase expression in lung, while no detectable expression was seen in other organs. The *in vitro* and *in vivo* transfection results using nonliposomal protamine/DNA condensates support our hypothesis and suggest that with 24 arginines the interaction between LLO-PNPN and DNA is fairly strong and remains bound to protamine/DNA complexes in the circulation.

That the relative luciferase expression was highest in lung we attribute to the net positive charge of the LLO-PNPN/protamine/DNA complexes. The positive charge of protamine is necessary for the neutralization and condensation of DNA as well as protection of DNA from degradation.[21,22] The interaction of cationic surfaces of complexes with the negatively charged plasma membrane can also induce cellular uptake, resulting in relatively high transfection efficiency, but the rapid and nonspecific interactions between positively charged vectors and negatively charged plasma components, including red blood cells, form agglutinates that can result in the highest gene expression in lung after intravenous injection.[31,38] In order to reduce these unwanted interactions, we associated the complexes with anionic liposomes to change the theoretical net charge from positive to negative. Interestingly, the *in vitro* transfection efficiency with anionic liposome(LLO-PNPN/protamine/DNA) was higher than that with cationic LLO-PNPN/protamine/DNA or the cationic lipid formulations with Lipofectamine, with its heat-inactivated negative control showing low levels of transfection comparable to protamine/DNA complexes without LLO-PNPN (Figure 3). Further studies are needed to determine whether this is true for other cell types that are not known for high uptake of negatively charged liposomes. The macrophage-like cells used in the current cell culture studies take up anionic liposomal particles avidly, and thus provide some indication as to whether the anionic liposome(LLO-PNPN/protamine/DNA) could be potentially used for DNA vaccine carriers once they are optimized in the future studies for their distribution and uptake in animals.

The gene expression tested in mice was not in any way ideal in terms of tissue distribution and gene expression. However, it clearly supports that the effect of LLO-PNPN is positive in terms of the gene expression level when compared with the counterpart control formulation with heat-inactivated LLO-PNPN (Figure 4). After intravenous injection of liposome-(LLO-PNPN/protamine/DNA) into mice, the highest gene expression was observed in spleen followed by lung, consistent with a reduction in nonspecific interactions between the vector and serum components that carry a net negative surface charge (Figure 4). Following intravenous injection, foreign particles are generally recognized and phagocytosed by APCs of the reticuloendothelial system.[39] The fenestrated endothelia that line the capillaries of the spleen and liver allow particles to diffuse into these tissues where they would encounter and be taken up by resident macrophages, which are likely the primary cells transfected by the vector. This *in vivo* result (highest luciferase expression in spleen) is consistent with our previously reported transfection results using LLO-LPDII composed of protamine/DNA complexed with anionic liposomes containing encapsulated LLO[32] with some differences in the expression levels in various organs potentially due to differences in the rate of clearance or biodistribution dependent on liposome composition and size.[40,41] The protamine/DNA cationic polyplexes without liposomes, and their minimal or undetectable gene expression when iv injected, indicate that the complex may be unstable *in vivo*, aggregate in circulation, and possibly be trapped in lung capillaries. When the protamine/DNA complex with LLO-PNPN was taken up by lung, however, it showed detectable expression in comparison with its heat-inactivated counterpart control, assuming that their distribution and uptake were the same.

In conclusion, we have demonstrated that the incorporation of a recombinant LLO-protamine fusion protein in protamine/DNA complexes, with and without further complexation with anionic liposomes, dramatically enhances their gene transfection efficiency in cultured cells. We also present preliminary data showing that the enhancement in gene expression would also be observed in an animal model. This initial study suggests that this recombinant fusion protein with multiple functional domains is a potential candidate to be efficiently and effectively incorporated in various nonviral DNA carrier platforms for improving their transfection efficiency. It is conceivable that with further modification and characterization the LLO-protamine chimeras could be tailored to achieve specific aims, such as increasing/decreasing the PN-derived polypeptide's affinity for DNA, mutagenesis of LLO to further limit potential damage to nonendosomal membranes, greater and more controlled efficiency of endosomal escape, and less immunogenicity toward the vector components. Furthermore, other functional domains such as targeting ligands or nuclear-localization signals can also be added or replaced and expressed as a single recombinant protein with relative ease. Thus, this recombinant fusion protein, as an essential component of a nonviral vector, has inherent flexibility and may be improved upon by incorporating multiple functional components into optimally designed, currently existing or future gene delivery systems applicable to *in vivo* gene therapy.

■ ASSOCIATED CONTENT

Supporting Information

Figures depicting hemolytic activity of PN-LLO, effect of LLO-PN on transfection efficiency at various protamine/DNA

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Mol. Pharmaceutics 2015, 12, 342−350

weight ratios, and effect of cathepsin D cleavable linker on transfection efficiency. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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