pH-Sensitive Cationic Polymer Gene Delivery Vehicle: *N*-Ac-poly(L-histidine)-graft-poly(L-lysine) Comb Shaped Polymer

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Advancing biotechnology spurs the development of new pharmaceutically engineered gene delivery vehicles. Poly(L-histidine) {PLH} has been shown to induce membrane fusion at endosomal pH values, whereas PLL has a well documented efficacy in polyplex formation. Therefore, *N*-Ac-poly(L-histidine)-graft-poly(L-lysine) {PLH-*g*-PLL} was synthesized by grafting poly(L-histidine) to poly(L-lysine) {PLL}. PLH-*g*-PLL formed polyplex particles by electrostatic interactions with plasmid DNA {pDNA}. The mean particle size of the polyplexes was in the range of 117 ± 6 nm to 306 ± 77 nm. PLH-*g*-PLL gene carrier demonstrated higher transfection efficacy in 293T cells than PLL at all equivalent weight ratios with pDNA. The inclusion of chloroquine as an endosomolytic agent enhanced transfection for both PLL and PLH-*g*-PLL gene carriers. PLH-*g*-PLL enhanced β-galactosidase expression compared to PLL, but still increased in efficacy when chloroquine was included.

INTRODUCTION

In the past decade, drug therapies have moved into a realm which includes the use of genes to combat illness. The theory behind the use of genetic material encoding vital proteins is simple; let the patient make their own therapeutic proteins. The efficacy of nonviral gene therapy relies upon the ability to deliver plasmid DNA into a cell nucleus to function as a gene. However, there are many obstacles in utilizing gene delivery for therapeutic treatments, consequently the introduction of new delivery vehicles for transfection continues. Many of the new delivery vehicles have been built on previous designs in an attempt to improve efficacy. One of the problems is the necessity for the ability to introduce plasmid DNA into the specific cell types (*1*−*5*). The other major issue in efficient gene expression is the release of the DNA from the endosomal compartment into the cytoplasm after transfection of the cell has taken place (*1*, *6*). Gene transfer depends on the ability to preferentially target specific cell types and allow release of the DNA into the cytoplasm with limited toxic side effects (*7*). Pharmaceutically engineered gene delivery vehicles cannot provide effective expression if the entire entity is transferred into the lysosomal compartment, which breaks down the plasmid. Although chloroquine has proven to aid in the release of the plasmid DNA into the cytoplasm, it has been found to be toxic and thus cannot be used in vivo. Therefore, there is a growing need for endosomal disrupting agents for enhanced release of the plasmid into the cytoplasm.

Viruses are known for their ability to be extremely efficient in delivering genes to the particular cells that are needed for the survival and progression of the viral species (*8*). Progressing knowledge of the virus and its ability to replicate, and the further understanding of the molecular mechanisms in which its genetic code is integrated into the cell has paved the path for viral based gene delivery (*7*, *9*−*11*). A perfect synthetic virus with no serious health-related side effect concerns led researchers in the direction pointing to the development of cationic polymer-based gene delivery vectors, which mimic the functions of a virus. Cationic polymers have been shown to form complexes (polyplex) with plasmids via ionic interaction (*12*−*16*). Although cationic polymers such as poly(L-lysine) {PLL} have been shown to efficiently condense DNA and protect DNA from nuclease attack, there are some disadvantages (*17*). PLL does not induce or facilitate the endosomal release of DNA. PLL possesses ϵ-amine groups, which can be linked to targeting ligands or pH-sensitive entities giving multifunctional capabilities.

Aggregation and lack of a targeting moiety has been somewhat circumvented by the attachment of other polymers and compounds to PLL forming co-block or comb-shaped polymers. Receptor-mediated endocytosis was found to increase transfection with PLL when the polymer was tethered to galactose, where the endocytosis occurred via the asialoglycoprotein receptor (*13*, *14*, *16*). Also, the transferrin receptor has been shown to be effective in increasing transfection ability (*18*−*20*). Recently, the folate binding protein has been exploited for receptor mediated endocytosis when folic acid has been tethered to cationic polymers (*21*−*24*).

Membrane fusion has been studied for the purpose of creating the opportunity for escape of material from a cellular compartment membrane (*20*, *25*). Viral envelopes have been noted to fuse with the endosomal and/or lysosomal compartment membranes. This fusion of the membrane occurs after the virus protein envelope had been internalized by receptor-mediated endocytosis, and by subsequent exposure to the acidic washes inside the respective compartments. This leads to the release of the

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viral genome into the cytoplasm was as a result of the membrane fusion event (*26*−*29*). While multivalent inorganic cations have been employed for membrane fusion phenomena, cationic polymers have been found to perform the same function with higher efficiencies (*30*, *31*). The cationic polymers of poly(L-lysine) (PLL) and poly(L-histidine) (PLH) were both determined to have membrane fusion capability (*32*). PLL induced fusion occurs only in pH regions outside normal physiological conditions, whereas membrane fusion takes place at physiological pH values when PLH is used. The extent of PLH fusion increases as pH of the environment decreased, coinciding with the biological activity of the endosome (*33*). Histidine residues have been substituted into PLL chains giving pH-sensitive characteristics to the polymer and subsequently increasing transfection (*34*, *35*). In the pursuit of an ideal pharmaceutically engineered polymeric gene delivery system, a *N*-Ac-poly(L-histidine)-graft-poly(L-lysine) comb shaped polymer (PLH-*g*-PLL) was synthesized with the hypothesis of improved endosomal escape via membrane fusion, and subsequently increasing gene expression in cultured cells.

MATERIALS AND METHODS

Materials. Fmoc-im-benzyl-L-histidine, p-alkoxybenzyl alcohol resin, poly(L-lysine) PLL, ~8000 Da, ethidium bromide, *N*-(2-hydroxyethyl)piperazine-*N*′-{2-ethanesulfonic acid} (HEPES), 3-{4,5-dimethylthiazol-2-yl}-2,5- diphenyltetrazoliumbromide (MTT), chloroquine, O-nitrophenyl-*β*-D-galactopyranoside (ONPG), and *N*-hydroxysuccinimide were all purchased from Sigma Chemical Co. (St. Louis, MO). Piperidine, dimethyl sulfoxide (DMSO), trifluoroacetic acid (TFA), and dicyclohexacarbodiimide (DCC) were purchased from Aldrich (Milwaukee, WI). Plasmid pSV-*β*-gal (EMBL accession no. X65335), DNA size marker, and bacterial JM 109 strain were purchased from Promega (Madison, WI). Fetal bovine serum (FBS), Dulbecco's Modified Eagle's Medium (DMEM), trypsin-EDTA 0.25%, and Dulbecco's phosphate-buffered saline (DPBS) were purchased from Hyclone (Logan, UT). QIAfilter Plasmid Kit was purchased from Qiagen (Boulder, CO). *N*-Hydroxybenzotriazole (HOBT), *N*-*N*-diisopropylethylamine (DIPEA), and 2-(1H−benzotriazole-1-yl)-1,1,3,3-tetramethyluroniumhexafluorophosphate (HBTU) were from AnaSpec, Inc. (San Jose, CA). Dialysis tubing (MWCO 6000−8000) was from Spectrum Medical Industries, Inc. (Los Angeles, CA).

Synthesis of *N*-Acetyl-poly(im-benzyl-L-histidine). Poly(im-benzyl-L-histidine) was prepared by solid-phase peptide synthesis using Fmoc-chemistry. A *p*-alkoxybenzyl alcohol resin was used as a solid support. Briefly, the coupling reaction was conducted with HOBT/HBTU/DIPEA using Fmoc-im-benzyl-L-histidine as a monomer. Piperidine (30%) was used for Fmoc-deprotection. Each coupling and deprotection reaction was repeated 18 times and monitored by ninhydrin tests. The terminal amino group was acylated with acetic anhydride and the polypeptide was detached from the resin with 90% TFA. Cold ether was added to this solution, and the precipitate was washed with ether and dried. The product of this synthesis was a poly-histidine with approximately 18 linked histidines.

Synthesis of *N*-Acetyl-poly(im-benzyl-L-histidine)-NHS ester. *N*-hydroxysuccinimide (0.069 g) and DCC (0.049 g) were added to solution of *N*-acetyl-poly(im-benzyl-L-histidine) (25 mg) dissolved in DMF. The mixture was constantly stirred for 80 min at 30 °C. Dicyclohexylurea (DCU) formed as a solid was removed by filtering-off. Cold ether was added to the solution and the precipitate was obtained. The precipitate was washed further with cold ether.

Synthesis of *N*-Ac-poly(L-histidine)-graft-poly(L-lysine). First *N*-acetyl-poly(im-benzyl-L-histidine)-NHS ester (2.4 mmol) was dissolved in 0.6 mL of DMF, then 5 mg of poly(L-lysine) was added to this solution. After shaking the mixture for 4 h at room temperature, the product was precipitated in cold ether. The im-benzyl group of the imidazole was deprotected by Na/NH_3 method. The polymer was dialyzed overnight using the MWCO 6000−8000 dialysis tubing, followed by freeze-drying. *N*-Ac-poly(L-histidine)-graft-poly(L-lysine) was obtained and ^1H NMR was used to determine conjugation. PLL was determined to have 25% of its ϵ-amine groups tethered to the histidine polymer.

Amplification and Purification of Plasmid DNA (pSV-*β*-gal). Plasmid DNA encoding *β*-galactosidase (pSV-*β*-gal) was used as the reporter gene in this study. The plasmid pSV-*β*-gal was electrophorated into *E. coli* JM 109 bacterial strain using an electrophorator (ECM 600, BTX). The transformed cells were grown in larger quantities (0.5−1 L) of LB broth supplemented with 100 μg/mL ampicillin. The plasmid DNA was purified using the QLAfilter plasmid purification kit from QIAgen and the purified plasmid was diluted in sterilized water. The purity was confirmed by 1% agarose gel electrophoresis followed by ethidium bromide staining and DNA concentration was measured by UV absorption at 260 nm.

Band Retardation. PLH-*g*-PLL/plasmid polyplexes were prepared using 1−100 μg for each molecular weight *N*-Ac-poly(L-histidine)-graft-poly(L-lysine) comb shaped polymer and 1 μg of pSV-*β*-gal. The samples were incubated at room temperature for 30 min to ensure formation of the polyplex. The Easy-Cast Electrophoresis System (Owl Scientific Inc., Woburn, Ma) was setup with a sample buffer TBE (45 mM Tris-borate, 1 mM EDTA, pH 8.0) on a 1% (w/v) agarose gel. The run was taken at 100 V for 90 min, using an Electrophoresis Constant Power Supply (Pharmacia Fine Chemical Model ECPS 3000/150), until the marker and polyplex solutions had run enough length to identify band weights. Following electrophoresis, the gel was stained with ethidium bromide (0.5 μg/mL) for 30 min. Ethidium bromide allows the use of an ultra-violet illuminator to show the location of the DNA and various levels of polyplex formation (*36*).

Ethidium Bromide Displacement. The ability of *N*-Ac-poly(L-histidine)-graft-poly(L-lysine) to effectively condense DNA was monitored by observing the fluorescence quenching of the DNA-ethidium bromide interaction. Solutions were made with 1 mg/mL of DNA and 1 mg/mL of ethidium bromide. The solutions were incubated on the counter for 15 min to ensure interactions between DNA and the ethidium bromide. The range of polymer concentrations tested in ability to quench fluorescence was 0.1−20 mg. The solutions were made to the volume of 1 mL by dilution with a buffer (20 mM Hepes, 0.015 M NaCI, pH 7.4). The complete solutions incubated for 30 min prior to fluorescence testing to allow the Coulombic interactions of the polymer to displace ethidium bromide from the plasmid DNA. The relative fluorescence intensity was measured on an ISS Photon Counting Spectrofluorometer (ISS Inc., Champaign, IL). The spectrofluorometer was operated with an excitation wavelength (λ_{ex}) of 488 nm and emission wavelength (λ_{em}) of 590 nmn (*2*).

Dynamic Laser Light Scattering. The sizes of the polyplexes formed by increasing weight ratios of PLL and PLH-*g*-PLL were determined using dynamic laser light scattering. Each polyplex was formed by adding a poly-

mer solution and DNA solution (each filtered through 0.2 μm filters) into sterile ddH_20 then vortexing the solution and letting the complex formation take place for 20 min. The polyplexes were formed with PLL in dilute (0.02 mg/mL) and concentrated (0.05 mg/mL) solutions to determine if there was concentration dependence in complexation. The PLH-*g*-PLL polyplex formation was done under a corresponding median concentration. Polyplex diameters were measured using a Lexel Corp model 95 ion-argon laser (Palo Alto, CA) filtered for 514.5 nm, a Brookhaven Instrument Inc. BI-100 M Goniometer, and a BI-2030 Autocorrelator (Holtsville, NY). A Brookhaven correlator program written in QuickBASIC performed all calculations.

Acid−Base Titration. The ability of the gene carriers (PLH-*g*-PLL and PLL) to protonate and obtain a positive charge over a 10 to 2 pH range was determined by acid−base titration. Each polymer was made into a 30 mL solution with a 0.2 mg/mL concentration. The solution was then titrated to a pH of 10 with 1 M NaOH where the pH was measured using a Coming pH meter 340. The solution was then titrated with 1 N HCI given in various volume increments. The pH profile was obtained for each polymer and graphs of the data were generated.

In Vitro Transfection. The 293T transformed immortalized cell line was used for transfection and cell viability tests. The cell line was grown in polystyrene tissue culture flasks containing DMEM supplemented with 10% FBS, penicillin (100 units/mL), streptomycin (100 units/mL), gentamycin (50 μg/mL) in a 37 °C and 5% CO_2 environment. When transfection and/or cell viability experiments were conducted, the cells were plated in 3.5 cm diameter dishes. This was done after the cells had become about 80% confluent in the culture flasks. The cells were decanted of media and washed twice with DPBS and then trypsinized with 0.25% trypsin-EDTA for 8 min or until the cells were observed to detach from the culture flask under a microscope. The cells were then collected, diluted, and counted using a hemocytometer. The appropriate cell concentration (8×10^5 cells/dish) was then placed in the wells in the plating process. For transfection studies, PLL and PLH-*g*-PLL solutions were prepared and filtered through 0.2 μm polycarbonate membrane filters. The polymer solutions were then mixed with pSV-β-gal in ddH_20 and incubated for 20 min at room temperature. The polyplexes were added to media supplemented with 10% FBS. Chloroquine at a 100 pM final concentration was then added to appropriate solutions. The media in each dish was replaced with the transfection solution. The plated cells were then incubated with the polymer/DNA solution for 4 h. After the incubation period was completed, the polyplex solutions were removed and replaced with media. The plated cell culture was then incubated for an additional 36 h.

ONPG Assay for Expressed β-Galactosidase. β-Galactosidase expression from the cells transfected with PLL and PLH-*g*-PLL polyplexes was determined spectrophotometrically at 420 nm using a modified ONPG method (*2*, *36*). Briefly, 300 μL of a 1.33 mg/mL ONPG in a 2× buffer was added to cells, which had been broken down by 300 μL of lysis buffer, and incubated for 4 h. β-Galactosidase hydrolyzes the colorless substrate into yellowish o-nitrophenyl. The absorbance was measured in a 150 μL cuvette at 420 on a Perkin-Elmer UV/VIS/NIR lambda 19 spectrophotometer.

X-gal Assay for Expressed β-Galactosidase. β-Galactosidase expression was also determined by X-gal staining and counting of stained cells. After the 36 h incubation period, the cells were washed twice with ddH_2O and fixed with 1.25% glutaraldahyde for 20 min. The fixed cells were then washed four times with DPBS. The X-gal stain solution was prepared and 1.75 mL was added to the fixed cells. The cells were stained for 4 h until the transfected cells had turned blue. Following staining, the stain solution was removed and the cells were washed twice with DPBS and preserved with a glycerol water solution. The blue cells were then counted under the microscope.

Cell Viability. The cytotoxicity of the polyplexes was determined on 293T cell line using a MTT assay (*2*, *3*). The cells were plated in 3.5 cm diameter dishes at 8×10^6 cells/well. A MTT solution in DPBS was made at 2 mg/mL and filtered. The MTT solution (180 μL) was added to the cell culture 36 h after removal of transfection solution. The MTT solution was removed and 3.5 mL of DMSO was added so that any crystals formed by the living cells due to the presence of MTT would dissolve. The solvating of the crystals by DMSO allows for absorbance to be taken at 570 nm. The absorbance was measured in a 150 μL cuvette on a Perkin-Elmer UV/VIS/NIR lambda 19 spectrometer.

RESULTS

Synthesis of *N*-Ac-poly(L-histidine)-graft-poly(L-lysine). Poly (im-benzyl-L-histidine), an 18-mer linear polymer, was synthesized. There was not any free amine groups open for each step of polymerization, as determined by ninhydrin tests. After the final grafting of linear PLH to PLL was completed, the protecting groups were removed. A schematic of PLH-*g*-PLL synthesis is shown in Figure 1. ^{1}H NMR was used to determine the percent of PLL ϵ-amine groups conjugated to PLH molecules. ^{1}H NMR (D_2O, 300.1 MHz, 300 K): δ 7.45, 6.67 (m, imidazole-H of histidine), 4.36 (m, α-CH of histidine), 4.14 (m, α-CH of lysine), 2.78 (m, β-CH_2 of histidine), 2.52 (m, ϵ-CH_2 of lysine), 1.77 (s, CH_3 of acetyl), 1.64, 1.35, 1.19 (m, $(CH_2)_3$ of lysine. Analysis of proportionality of hydrogen atoms on respective sites indicated that 25% of the PLL ϵ-amine groups were conjugated to linear PLH molecules forming a comb shaped polymer.

Band Retardation Assay. The resulting gel electrophoresis when PLH-*g*-PLL complexed with pSV-β-gal was prepared and viewed with the aid of ethidium bromide association fluorescence (not shown). With an exclusion of the two terminal lanes, PLH-*g*-PLL concentration was increased compared to a constant amount of DNA. The increasing concentrations of this polymer decreased the electrophoretic mobility of plasmid DNA. No mobility of the DNA was observed at the polymer/DNA weight ratios above 20 (w/w). This decrease in electrophoretic mobility of the pDNA is due to the increasing DNA condensation and compaction.

Ethidium Bromide Displacement Assay. The ability of *N*-Ac-poly(L-histidine)-graft-poly(L-lysine) to condense DNA was tested with the ethidium bromide displacement assay. The more effective the polymer was in condensing plasmid DNA, the lower the relative fluorescence would be. The quenching of ethidium bromide/DNA interaction fluorescence by PLH-*g*-PLL was monitored by a spectrofluorometer. From Figure 2 it can be seen that the increasing concentrations of PLH-*g*-PLL increased the quenching of ethidium bromide fluorescence. At a low polymer/DNA ratio, there is little exclusion of ethidium bromide demonstrating minimal complexation and condensation. The data shows that increasing polymer concentration increases the ability to condense

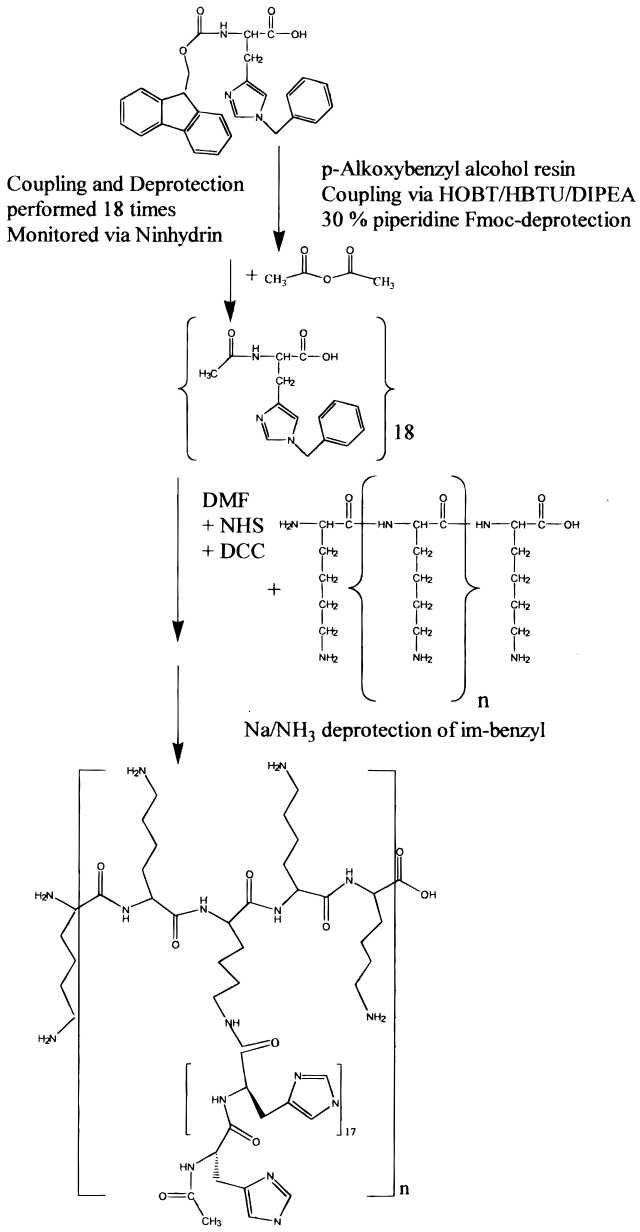

Figure 1. Schematic for synthesis of *N*-Ac-poly(L-histidine)-graft-poly(L-lysine) {PLH-*g*-PLL}.

DNA and subsequently hinders interactions with ethidium bromide. This is in good agreement with band retardation data.

Dynamic Laser Light Scattering. The particle size of polyplexes was determined by dynamic laser light scattering. The PLL polyplex particles were formed by increasing polymer/DNA weight ratios and in concentrated and dilute solutions. The size change with increasing polymer amount (PLL and PLH-*g*-PLL) at constant DNA can be seen in Figure 3. When PLH-*g*-PLL was used to compact pDNA, the polyplex size decreased as polymer concentration increased (Figure 3). PLH-*g*-PLL did not compact pDNA as effectively as PLL, however at higher weight ratios, PLH-*g*-PLL was just as effective as PLL. The polyplex size did not drastically decrease further at higher weight ratios.

Acid−Base Titration. The acid−base titration profile was obtained for PLL, PLH, and PLH−PLL, and shown in Figure 4. The initial high pH protonation of the ϵ-amine groups of PLL can be seen as a horizontal trend above pH 8. However, the titration curve trend turns

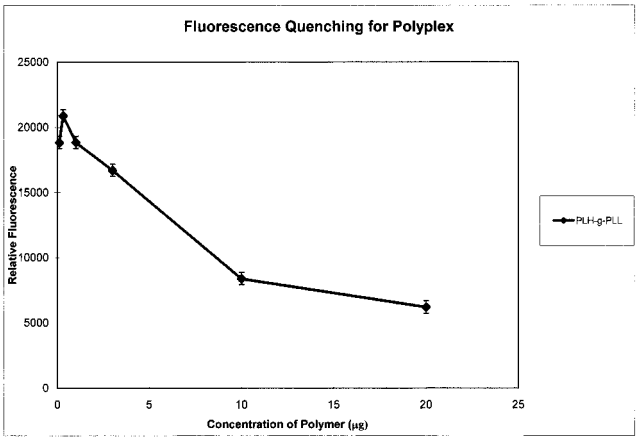

Figure 2. Effect of polymer concentration on DNA condensation. The solutions were incubated for 30 min to ensure polyplex formation. Error bars are the standard deviation of data points for the same polyplex solution.

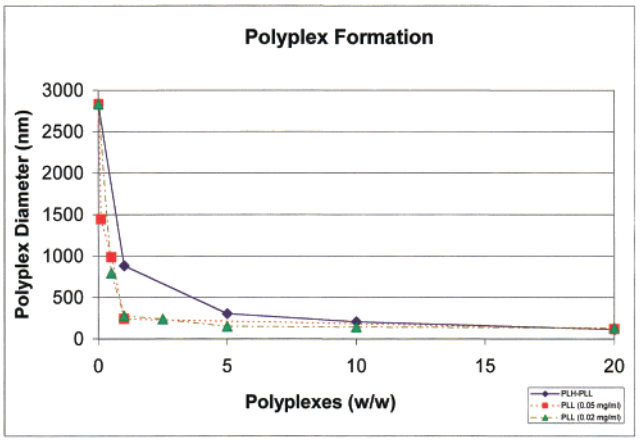

Figure 3. Effect of polymer concentration on particle size of pSV-*β*-gal polyplexes prepared using PLL and *N*-Ac-poly(L-histidine)-graft-poly(L-lysine). The solutions were incubated for 30 min to ensure polyplex formation. Error bars are the standard deviation of data points for the same polyplex solution taken at different times.

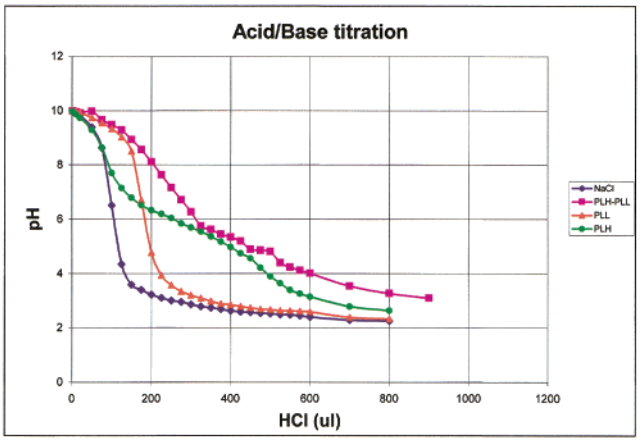

Figure 4. Acid−Base titration profile of 0.1 M NaCI, PLL, PLH, and PLH−PLL.

nearly vertical suggesting little buffering capacity of PLL. Unlike PLL, PLH showed considerable buffer capacity over almost the entire pH. The imidazole of histidine has a pK_a around 6.0 and this pK_a indicates that PLH protonates and becomes a strong polycation in the endosomal pH range. When PLH and PLL are combined, the titration curve gives the basic buffering capacity of

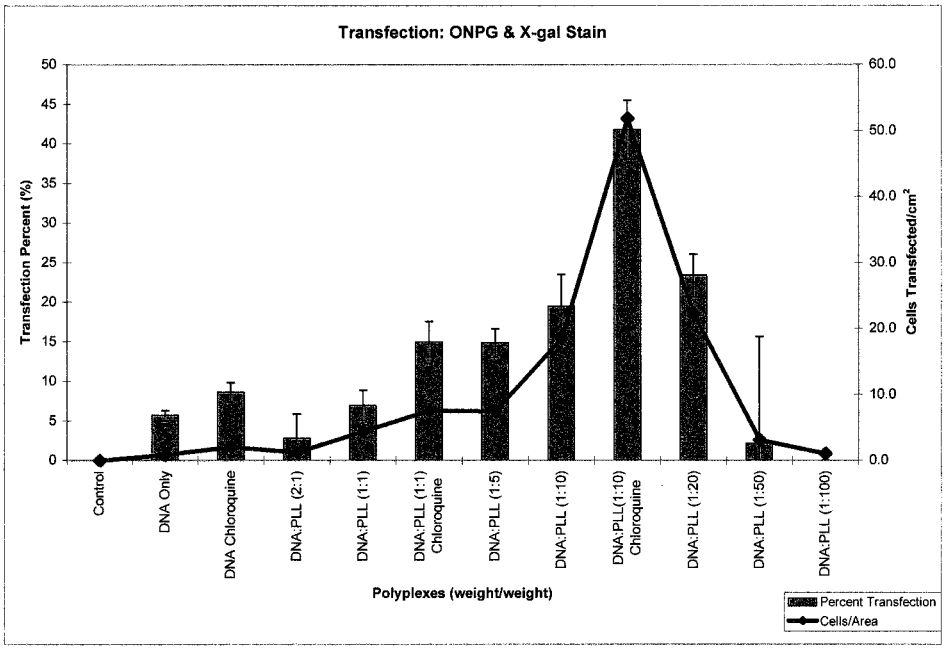

Figure 5. Effect of polymer concentration efficiency of polyplexes prepared with PLL and pSV-β-gal. Transfections were carried out using 293T cultured cells. The *x*-axis defines the polymer/DNA weight/weight ratio, with poly(L-lysine) (PLL) and with or without the aide of chloroquine. The left *y*-axis is obtained from absorbance from ONPG assay given in transfection percent, which is the absorbance percentage of that obtained by a calcium phosphate precipitation positive control. The right *y*-axis is defined as cells transfected/cm^2 counted after X-gal staining. Results are presented as mean ± standard deviation of three assays.

PLL and the endosomal pH buffering capacity of PLH. This result is in good agreement with previous reports where partially substituted PLL with histidine polymers had both DNA condensing and endosomolytic properties (*37*). Figure 4 supports the previous data that PLH-*g*-PLL retains the complexation and compaction ability associated with PLL, but also induces endosomal pHL protonation.

Transfection. Transfection was performed on 293T cells with the polyplexes prepared at different polymer/DNA ratios using poly(L-lysine) and *N*-Ac-poly(L-histidine)-graft-poly(L-lysine). Transfection efficiency was determined using ONPG and X-gal methods. Furthermore, each series of experiments included a calcium precipitation transfection solution as a positive control where its transfection ability was considered to be 100%. The absorbances taken from the polymer solutions were compared to those obtained from the calcium precipitation method, and reported as a percent of calcium precipitation. Figure 5 shows the effect of PLL/DNA weight ratio on transfection in vitro. For a given amount of DNA (9 μg/well), an increase in PLL concentration increased the transfection to a maximum with 20 weight ratio. Subsequent increase in polymer concentration decreased activity of β-galactosidase due to cell death. This trend was obtained by both assay schemes employed. The change in the absorbance is directly proportional to the β-galactosidase enzyme productivity of the transfected cells.

The absorbance increased for each polymer concentration when chloroquine was included in the transfection solution. β-Galactosidase concentration was a maximum when chloroquine was included and polyplexes were prepared at a polymer/DNA ratio of 10 (w/w) (Figure 5).

The effect of PLH-*g*-PLL/DNA weight ratio on transfection was studied using pSV-β-gal complexed with PLH-*g*-PLL. Transfection solutions had increasing amounts of polymer at a constant amount of DNA up to a maximum PLH-*g*-PLL/DNA ratio of 20. An increase in PLH-*g*-PLL polymer concentration increased the transfection (Figure 6). Transfection solutions containing PLH-*g*-PLL were compared to those that included only PLL. PLH-*g*-PLL showed better transfection efficiency for equivalent weight ratios of PLL. For equivalent weight ratios, PLH-*g*-PLL has less cationic polymer molecules. A less amount of polymer molecules was taken to prove that poly(histidine) grafted to PLL increased the transfection efficiency of the gene delivery vehicle.

The corresponding PLH-*g*-PLL transfection solutions were also given the endosomal disrupting agent chloroquine to determine if there would be any further endosomal escape of the polyplexes. Enhanced transfection efficiency, above that provided by the histidine residues, was observed for each of the PLH-*g*-PLL polyplex systems in the presence of chloroquine. The increase in transfection when chloroquine was included demonstrates that the grafted histidine polymers are not improving the endosomal escape potential to a maximum. However, PLH grafts improved the efficacy of the PLL cationic polymer molecules. The use of the ONPG and X-gal stain methods gave supporting data for comparison. Multiple assays indicating similar transfection profiles supports the hypothesis that grafting PLH to PLL enhances endosomal escape by the gene delivery vehicle and therefore increased the ability of the transfected cell to express the encoded protein.

Cell Viability. The efficacy of a gene carrier is partly dependent on the corresponding toxicity of the polymer and polyplex solutions. The wells containing cells in only media with no treatment of polymer or polyplexes were used as a positive control with a cell viability of 100%. The cell viability was determined for the cells treated with PLL/DNA polyplexes prepared at different weight ratios of PLL ranging from 1 to 100 (Figure 7). The solutions with increased amount of PLL over 20 times the amount of DNA were toxic to 293T cells. Although the increase in PLL concentration enhanced transfection, it was also toxic to the cells. The relative cell viability of

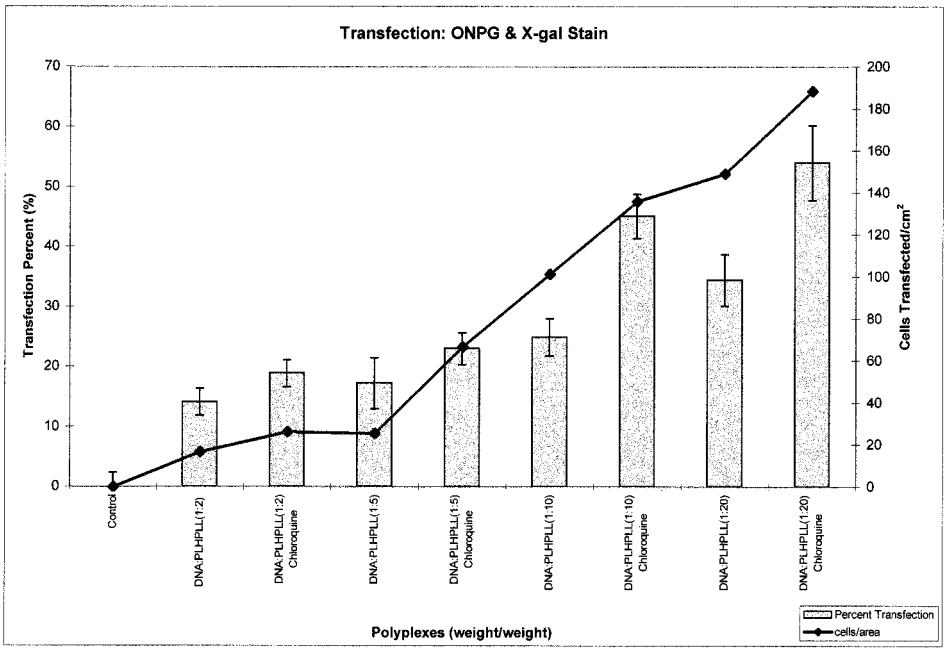

Figure 6. Effect of polymer concentration on transfection efficiency on 293T cells with polyplexes prepared using *N*-Ac-poly(L-histidine)-graft-poly(L-lysine) {PLH-*g*-PLL} and pSV-*β*-gal. The *x*-axis defines the polymer/DNA weight/weight ratio, *N*-Ac-poly(L-histidine)-graft-poly(L-lysine) {PLH-*g*-PLL} and with or without the aide of chloroquine. The left *y*-axis is obtained from absorbance from ONPG assay given in Transfection Percent, which is the absorbance percentage of that obtained by a calcium phosphate precipitation positive control. The right *y*-axis is defined as cells transfected/cm^2 counted after X-gal staining. Data are represented as the mean ± SD of three assays.

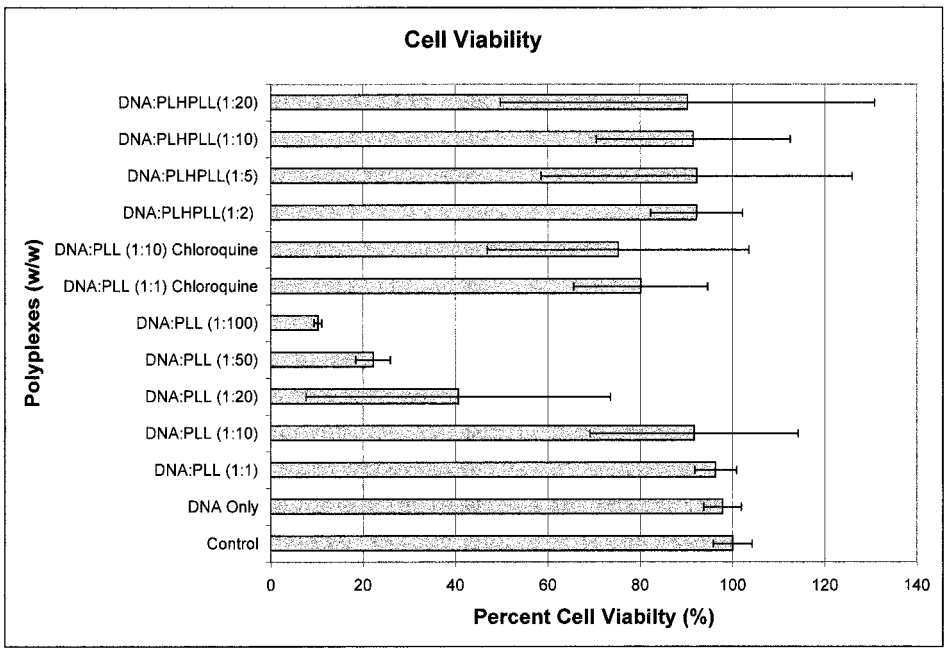

Figure 7. Cell viability percent of 293T cultured cells transfected with polyplexes prepared with pSV-*β*-gal and either PLL or PLH-*g*-PLL. PLL represents poly(L-lysine), PLHPLL represents *N*-Ac-poly(L-histidine)-graft-poly(L-lysine). The *x*-axis also indicates if transfection solution contained chloroquine. The error bars are the standard deviation of three assays.

the polyplexes prepared at a PLL/DNA weight ratio of 10 was ~80%. Generally, there was increase in toxicity with increase in polymer concentration. However, at the optimal PLL/DNA ratio of 10, the cell viability stayed in a range acceptable and greater than 70%.

PLL/DNA polyplexes prepared at w/w ratios of 1 and 10 were studied for toxicity in the presence of chloroquine. The inclusion of chloroquine in the solution increased the toxicity, while only ~40% cell viability for the cells treated with polyplexes with PLL/DNA of 20 w/w.

The toxicity of PLH-*g*-PLL polyplexes was also determined using 293T cells (Figure 7). The toxicity of the solutions was increased as the polymer concentration in the polyplex vector was increased. However, the relative cell viability was still greater than 80%.

DISCUSSION

Nonviral vectors require the entry of plasmid DNA into the cells and then further entry into the nucleus to function as a gene. These nonviral vectors must overcome at least four limiting barriers. The first barrier is

targeting the gene delivery vehicle to the desired area in vivo, while avoiding degradation by the physiological defense system. Also, by limiting which cells will endocytose the vector, the specific cells needed to produce the encoded protein will have a higher probability of successful transfection. The second barrier is the need to introduce DNA into the specific desired cell type efficiently and safely. Efficiency is in the level of transfection and transgene expression, which includes the ability to preferentially target specific cell types and allow pDNA release into the cytoplasm with limited toxic side effects (*1*). Without an in vivo targeting capability, the future of gene delivery would be limited to ex vivo methods of transfection. The introduction of in vivo procedures lacking an effective targeting moiety could lead to widespread random insertion of the genetic material into cells all over the human body. The third barrier is the release of DNA from the endosomal compartment. The plasmid DNA in the pharmaceutically engineered gene delivery vehicle cannot provide effective expression if the entire entity is transferred into the lysosomal compartment and subsequently degraded (*38*). Fusogenic peptides derived from different viruses have been used to enhance the escape of gene delivery vehicles from the endosome (*20*). These peptides are amino acid sequences found on virus particles that have pH dependent conformations allowing for cytoplasm targeting. Along these lines, other pH sensitive components such as chloroquine and poly(histidine) have been utilized with increased transfection efficiency (*34, 35, 39*). The fourth barrier is the translocation of the DNA into the nucleus. The transfer of the genetic material into the nucleus can be accomplished with a nuclear localization signal peptide. However, this nuclear localization step in gene delivery is not fully understood and steps are continuing to progress to advance this area (*40*).

In this study, *N*-Ac-poly(L-histidine)-graft-poly(L-lysine) {PLH-*g*-PLL} cationic polymer was synthesized (Figure 1) and characterized in terms of DNA condensing, particle size of polyplex, toxicity, and transfection in vitro (*4, 5, 36*). PLH-*g*-PLL is a PLL backbone with 25% of its ϵ-amine group's graft with PLH by analysis with ^{1}H NMR. The reactions were monitored via ninhydrin assay to indicate if there were primary amines present. The use of mass spectral analysis would have been a better way to do a more thorough characterization of intermediates in the polymer synthesis. However, the reaction scheme does not allow other side reactions, although it may change the polydispersity of the final product. The linking of PLH to PLL was a limiting step in this synthesis; therefore, proton-NMR was used to determine the conjugation percentage by comparing histidine and lysine residues. The polydispersity of PLH may not be exact to yield an 18 mer polymer, but with accurate weighing of reagents and careful synthesis the ninhyrdrin assay should indicate complete reactions.

Higher weight ratios of PLH-*g*-PLL/plasmid DNA effectively retarded the electrophoretic mobility (data not shown). This suggests that PLH-*g*-PLL did not relinquish the ability of PLL to condense DNA. These data was consistent with other conjugated PLL carriers (*2, 34*).

Both polymers efficiently condensed DNA into small polyplexes. PLL and PLH-*g*-PLL exhibited fluorescence quenching with increasing polymer concentration as determined from the ethidium bromide displacement assay (Figure 3). This fluorescence quenching shows increasing DNA compaction with an increase in polymer concentration. This supports the evidence provided by the retarded gel electrophoreses.

The PLH-*g*-PLL showed a similar compaction profile with less magnitude in comparison to PLL. The optimal condensation of PLH-*g*-PLL was higher with a polymer/DNA ratio of 20. PLH-*g*-PLL polymer had less ability to condense DNA, as poly(L-histidine) is not charged at pH 7.4 and does not complex with plasmid at neutral pH (*35*). However, polyplexes of diameter 117 nm were formed when 20 times excess polymer was used (Figure 4). This happens because at lower ratios of PLH-*g*-PLL/pDNA, PLH-*g*-PLL has 25% of the ϵ-amine groups unavailable for protonation. Also histidine residues are attached to PLL affects a low weight ratio compaction of DNA by decreasing the amount of amines for a given weight.

PLL as shown in Figure 4 had the ability to protonate at pH values above physiological conditions and also showed a steep decrease through neutral to acidic pH. The PLH profile was indicative of a neutral polymer at high pH and began to protonate at pH 6.5−5.0 and buffered the system through this range. The pH, where buffering capability occurred, was that of physiological endosomal pH values. The resulting profile obtained from PLH with PLL was observed to be a summation of the two titrations, and indicates the addition of both properties with negligible loss of either compaction or endosomnal protonation. Protonation of histidine polymers within the physiological pH range is significant for endosomal disruption, where the pK_a of polyhistidine and the titration profile are consistent with desired properties given by previous reports (*28, 33−35*). Poly(L-histidine) has been shown to induce fusion of lipid bi-layers in a slightly acidic medium upon protonation of the imidazole groups by increasing 16 interactions between the polymeric cation and the negatively charged membrane phospholipid (*32, 33*). In addition, PLH was found to be more fusogenic in acidic medium than PLL.

The inclusion of chloroquine increased the transfection efficiency, which is consistent with previously reported results (*2, 5, 39*). The transfection ability of the polyplex reached a maximum value for the vector complexed with PLH-*g*-PLL, and when chloroquine was also added to the system (Figure 6). Both PLL and PLH-*g*-PLL demonstrated increasing transfection efficiency as the amount of polymer increased, β-galactosidase concentration in 293T cells increased. All polymer solutions increased transfection efficacy when chloroquine was added to aid in endosomal escape (*39*). PLH-*g*-PLL/DNA complexes prepared at weight ratio of 20 was most effective, especially when chloroquine was included. Our findings on enhanced transfection with PLH-*g*-PLL is in good agreement with Midoux and Monsigny (1999) (*35*) who demonstrated that the partial substitution of poly(L-lysine) ϵ-amine groups with histidine residues significantly enhanced transfection. In their studies, optimal conditions for transfection was found when PLL substitution level was 38 ± 5% (*34*). The additional increase in transfection with inclusion of chloroquine demonstrates that there is still room for improvement in the polymeric gene carrier. The endosomal disruption of PLH-*g*-PLL is a step in the right direction for enhanced gene transfer. Scientists have also reported that the amphipathic peptide HSWYG {Gly-Leu-Phe-His-Ala-Ile-Ala-His-Phe-Ile-His-Gly-Gly-Trp-His-Gly-Leu-Ile-His-Gly-Trp-Tyr-Gly} containing five histidine residues, also permeabilizes cell membranes at endosomal pH values (*38*). The generation of cationic charges via protonation of several imidazole groups inside the endosome is a suitable approach to allow DNA to be released into the cytosol (*34*). Therefore, the transfection efficiency will be increased from the enhanced ability of DNA to escape the endosomal com-

partment and be translocated into the nucleus for transcription.

CONCLUSION

The pharmaceutically engineered pH-sensitive *N*-Ac-poly(L-histidine)-graft-poly(L-lysine) comb shaped polymer {PLH-*g*-PLL} showed improved transfection in 293T cells in comparison to a poly(L-lysine) {PLL} control, possibly due to enhanced endosomal release of DNA. The endosomal escape usually occurs via a membrane fusion, buffering, or a nonspecific endosomotropic function. There was successful transfection and transgene expression for higher weight ratios of *N*-Ac-poly(L-histidine)-graft-poly-(L-lysine) comb shaped polymer. The pursuit of viable gene delivery vehicles should continue in biocompatible polymer designs until a nonviral gene delivery vector is produced with high efficiency in cell targeting and transport into the cellular cytoplasm leading to efficient expression of transfected pDNA.

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