Biodegradable Poly(ethylene glycol)-*co*-poly(L-lysine)-*g*-histidine Multiblock Copolymers for Nonviral Gene Delivery

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ABSTRACT: The development of biodegradable cationic polymers for use in somatic gene therapy is desirable because degradable polymers have the potential to overcome cellular toxicities that are related to the high charge densities of the polycationic delivery system. Therefore, to produce a biocompatible gene delivery vehicle, we have designed a novel biodegradable, high molecular weight multiblock copolymer (MBC) of the type $(AB)_n$ which consists of repeating units of low molecular weight poly(ethylene glycol) (PEG) conjugated to low molecular weight cationic poly(L-lysine) (PLL). PEG was used not only to impart steric stabilization properties onto the polymer/pDNA complexes but also to introduce biodegradable ester bond linkages into the backbone of the MBCs. Also, to improve the endosome-disrupting capabilities of the polymer, *N*,*N*-dimethylhistidine (His) was coupled at various mole ratios (5 mol % His, 9 mol % His, 16 mol % His, 22 mol % His) to the ϵ-amines of PLL to produce PEG−PLL-*grafted*-His (PEG−PLL-*g*-His) MBCs. Polymer screening revealed that MBCs with 16% His grafted (PEG−PLL-*g*-16% His) (31 kDa) produced the highest transfection efficiency with minimal cytotoxicity in murine smooth muscle cells (A7r5). The MBCs condensed plasmid DNA (pDNA) into nanostructures with an average particle size between 150 and 200 nm with no aggregation and surface charge of ∼4−45 mV. These MBCs also protected pDNA from endonuclease digestion for at least 2 h. The polymers showed exponential decay with a half-life ($t_{1/2}$) of ∼5 h in PBS, pH 7.4 at 37 °C. However, complexes incubated in PBS buffer showed complete stability up to 6 days despite the short polymer $t_{1/2}$. The p*K* of the conjugated imidazoles was found to be 4.75 which would facilitate buffering at low pH environments of the late endosome/lysosome. Finally, the ability of the imidazoles to protonate and destabilize membrane vesicles was investigated by the use of bafilomycin A_1 which showed that the MBCs produced about five times higher transfection efficiency in vitro in A7r5 cells compared to the treated cells. This supports the function of histidine as an endosomal disrupting moiety. Therefore, these results suggest that biodegradable multiblock copolymers are promising candidates for long-term gene delivery.

Introduction

There is an increasing demand for biodegradable polymers due to the large number of biomedical applications such as bioabsorbable surgical sutures, controlled release of drug implants and proteins, medical devices, and wound dressing and for fabricating scaffold in tissue engineering.[1−3] Recently, the need for degradable polymers has spilled over into the field of gene delivery. The use of nonviral gene carriers has been expanding due to many drawbacks of viral vectors including the induction of immunological responses, random insertion of viral sequences into the host chromosomes, recombination events which can lead to virulent viral particles, or limitations associated with DNA encapsulation size.[4,5]

Degradable nonviral gene carriers are required for long-term delivery of a therapeutic gene that would necessitate the removal of the delivery system to circumvent cellular toxicities or organ damage that would occur over an extended time period. In addition, even though polycations such as poly(ethylenimine) (PEI) and protamine have been used extensively in gene delivery systems, these polymers have been shown to induce significant cellular damage.[6,7] Furthermore, there has been evidence that suggest that high molecular weight polymers produce high transfection efficiencies but with the penalty of adversely affecting cell viability due to the high cationic charge densities.[8,9] To date there has been encouraging work done that suggests that the development of biodegradable cationic polymers is appropriate for the advancement of nonviral gene carriers. Lim et al.[10] developed the biodegradable poly[α-(4-aminobutyl)-L-glycolic acid] (PAGA) polymer that produced ∼3-fold higher transfection efficiency in comparison to poly-L-lysine without any adverse effects on cell viability attributed to the degradation and nontoxicity of the polymer. Similarly, Ahn et al. and Petersen et al.[11,12] synthesized water-soluble degradable PEI conjugates that had 3 and 10-fold increases in transfection efficiency with low cytotoxicities, respectively. Thus, the design of high molecular weight, biodegradable polymers holds the promise of increasing the transfection efficiency without affecting cytotoxicity due to the lower charge densities of the low molecular weight byproducts that can be easily removed from the body.

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In the past, poly-L-lysine (PLL) has been widely used in the field of gene delivery because its structure facilitates various modifications of the polymer including conjugation with ligands such as transferrin, epidermal growth factor (EGF), and fusogenic peptides.[13−15] However, the polycationic nature of PLL can affect complex solubility at low N/P ratios (polymer nitrogens: plasmid DNA phosphates), cell viability at high molecular weights, and can lead to serum protein binding to the polymer−DNA complexes. Previously, poly(ethylene glycol) (PEG) has been used in combination with cationic polymers such as PLL for use in gene delivery to increase the pharmacokinetic properties of the polymer−DNA complexes and reduce polymer toxicity.[16−21] PEG is a hydrophilic, nonionic or neutral polyether whose steric stabilization properties impart high solubility, nontoxicity, and nonimmunogenicity to molecules to which PEGs are attached.[22]

Therefore, in an effort to develop a biocompatible, cationic polymer with high transfection efficiency for use in gene delivery, we have designed a novel water-soluble, high molecular weight, biodegradable multiblock copolymer (MBC) of the type $(AB)_n$ consisting of repeating blocks of low molecular weight PEG attached to low molecular weight PLL that was synthesized by ring-opening polymerization of lysine *N*-carboxyanhydride (NCA) to produce MBCs $(PEG-PLL)_x$. In addition to the steric stabilization properties of PEG, the use of homobifunctional PEG in these MBCs has also facilitated the introduction of the biodegradable ester bond functionalities into the backbone of the MBCs thereby conferring degradability to the polymers.

In addition to reducing cellular cytotoxicities, the biodegradable polymer also has to be able to maintain high transfection efficiencies. However, even though PLL has been widely used as a DNA-condensing polycation, it produces low transfection efficiencies since it cannot facilitate endosomal escape of the polymer/pDNA complexes. This is because the p*K* of the primary amines of PLL is above physiological pH whereas the endosomal environment is acidic. Histidine and poly(L-histidine) have both been shown to induce membrane fusion and/or result in increased transfection efficiency of the gene delivery system when conjugated to cationic polymers.[23−25] Furthermore, it has been reported that α-amino groups on histidine residues result in elevation of imidazole p*K* that can favor early endocytic release of complexes.[26] Consequently, the histidine derivative *N*,*N*-dimethylhistidine was conjugated in various mole ratios to the primary ϵ-amines of PLL to produce the final PEG−PLL-*grafted*-His (PEG−PLL-*g*-His) MBCs in an effort to improve the endosomal escape of the complexes after endocytosis.

Experimental Procedures

Materials. All reactions were performed under an atmosphere of dry nitrogen unless otherwise stated. Poly(ethylene glycol) (PEG, M_w 1450) was purchased from Union Carbide Corp. (Danbury, CT). Anhydrous methylene chloride (CH_2Cl_2), succinic anhydride, 4-dimethylamino puridine (DMAP), 1,3-dicyclohexylcarbodiimide (DCC), triphosgene, tetrahydrofuran (THF), hexanes, sodium lump, dimethylformamide (DMF), palladium, 10 wt % on activated carbon (Pd/C), formic acid, dimethyl sulfoxide (DMSO), sodium deuterioxide solution (DNaO), deuterium chloride solution (CID), trifluoroacetic acid (TFA), benzene, Celite, acetone, and ethyl ether (ether) were purchased from Aldrich Chemical Co., Inc. (Milwaukee, WI). Triethylamine (TEA) and dioxane were purchased from J. T. Baker (Phillipsburg, NJ). *N*-Hydroxisuccinimide (NHS) was purchased from Fluka (Milwaukee, WI). H-Lys(Z-OH) (cbz-L-lysine) and *N*,*N*-dimethyl-His-OH (His) were purchased from Bachem Bioscience Inc. (King of Prussia, PA). Poly-L-lysine (PLL M_w 25 600) and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma Chemical Co. (St. Louos, MO). Ethylenediamine (EDA) was purchased from Fisher Scientific (Fair Lawn, NJ). Polystyrene standards purchased from Polysciences, Inc. (Warrington, PA). Dulbecco's Modified Eagle Medium (DMEM), Dulbecco's phosphate-buffered saline (PBS) and trypsin−EDTA were purchased from Gibco BRL (Gaithersburg, MD). Heat-inactivated fetal bovine serum (FBS) was purchased from Hyclone (Logan, UT). Endofree Maxi Plasmid Purification kit was purchased from QIAGEN (Valencia, CA). Luciferase assay system was purchased from Promega Corp. (Madison, WI). Fluorescent activated cell sorting (FACs) was performed using a FACScan flow cytometer, Becton Dickinson (Palo Alto, CA). Propidium iodide (PI) [2,7-diamino-9-phenyl-10-(diethyl aminopropyl)-phenanthridinium iodide methiodide] was purchased from Molecular Probes (Eugene, OR). Bicinchoninic acid (BCA) reagent was purchased from Pierce (Rockford, IL). Protein quantitation was determined with a Bio-Rad model 3550 Microplate reader from Bio-Rad Laboratories (Hercules, CA). ^{1}H NMR spectra were obtained using a Varian Mercury 400, Inc. (Palo Alto, CA) and chemical shifts (δ) were reported in parts per million (ppm). RQ1 RNase-free DNase I enzymes were purchased from Promega Corp. (Madison, WI). PELCO mica disks, 9.9 mm diameter were purchased from Ted Pella, Inc. (Redding, CA). Matrix assisted laser desorption ionization-time-of-flight (MALDI−TOF) data were obtained using a Voyager-DE STR Biospectrometry Workstation from Applied Biosystems (Foster City, CA). Fourier Transform-Infrared (FT-IR) spectra were obtained using a Mattson Galaxy Series 3000 FT-IR from Mattson Instruments Inc. (Madison, WI). The molecular weights of the conjugates were determined by high performance liquid chromatography − size exclusion chromatography (HPLC−SEC) on Shodex OHpak (SB-806M HQ and SB-803 HQ) columns in series using a Shidmadzu system and a Shimadzu RID-10A refractive index detector purchased from Shimadzu (Columbia, MD). pH measurements were determined with a Corning pH meter 340 purchased from Corning Incorporated (Corning, NY). Atomic Force Microscopy (AFM) pictures obtained with a Digital Instruments Nanoscope II SFM model AFM (Santa Barbara, CA).

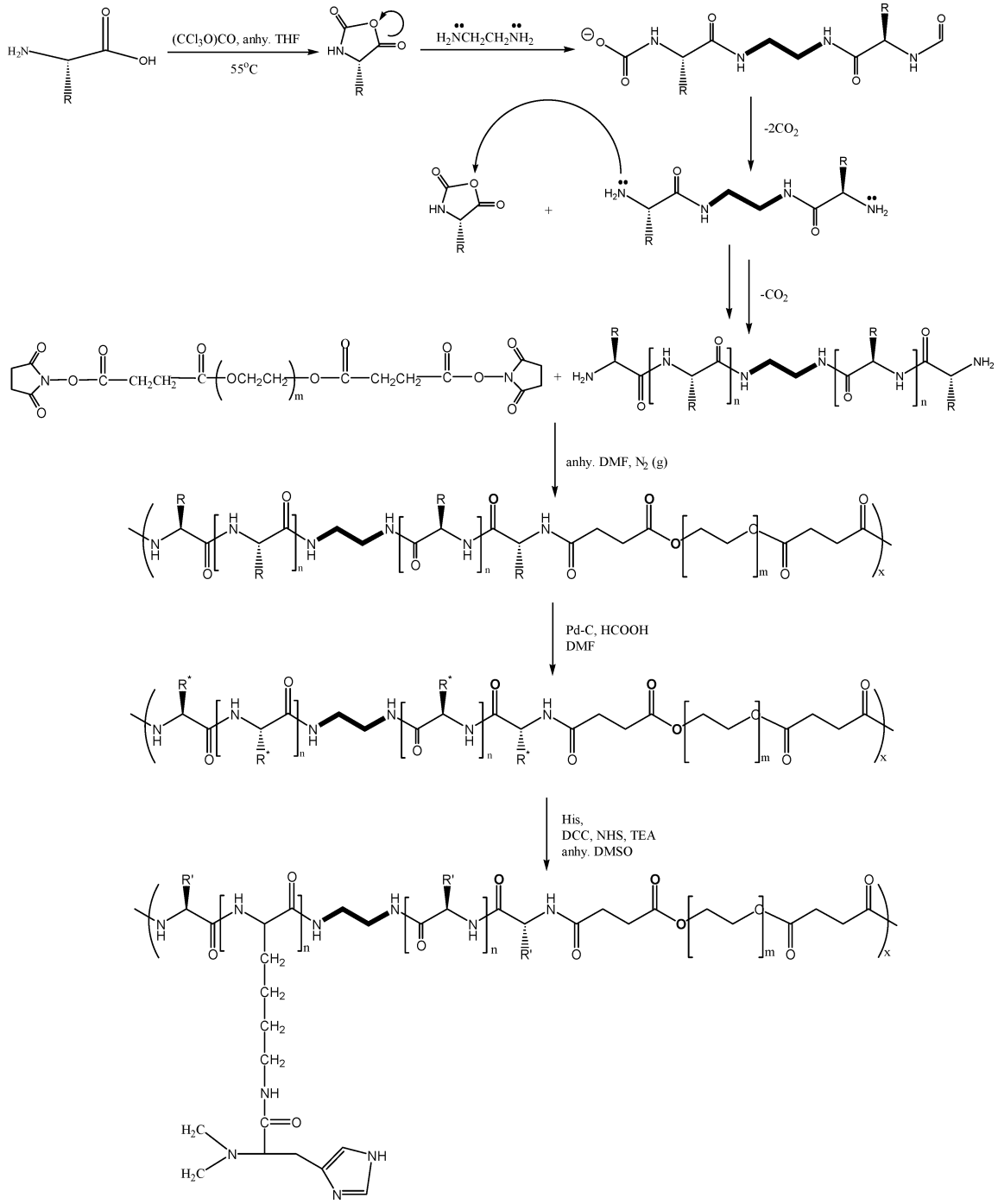
Cell Lines. The murine smooth muscle cell (A7r5) line was purchased from American Type Culture Collection (ATCC) (Manassas, VA) and was cultured in DMEM medium supplemented with 10% FBS and maintained at 37 °C in a humidified atmosphere of 5% CO_2.

Amplification and Purification of pSV-EGFP and pSV−Luc. The pSV-EGFP vector was constructed by subcloning the cassette for the simian virus 40 (SV40) promoter and enhanced green fluorescent protein (EGFP) cDNA obtained from the pCMS-EGFP vector (Clontech) into the p*T*/BH vector that was a kind donation from Dr. Perry B. Hackett (University of Minnesota). The pGL3-Promoter (pSV-Luc) vector was purchased from Promega (Madison, WI). The plasmids were amplified via transformation into JM109 competent cells and purified using a QIAGEN Endofree Maxi Plasmid Purification kit.

Polymer Synthesis. The PEG diol was first converted to a succinic diacid that was subsequently converted to the more reactive homobifunctional NHS ester for nucleophilic substitution with the terminal α-amino group of PLL synthesized in situ to produce the $(PEG-PLL)_x$ MBCs (Scheme 1).

Synthesis of PEG Diacid. PEG diol was dried by dissolving 25 g (17.24 mmol) of polymer in 20 mL of CH_2Cl_2 with stirring. The polymer was precipitated by pouring the concentrated solution into 500 mL anhydrous ether at 4 °C with vigorous stirring and the precipitate was then dried in vacuo.[27] FT-IR (in CH_2Cl_2): broad O−H stretch: polymeric structures, 3333 cm^{-1}; C−H stretch: methylene, 2840 cm^{-1}, C−O−C stretch: ether, 1100 cm^{-1}. The number-average molecular weight (M_n) determined by MALDI−TOF was 1435. ^{1}H NMR

Scheme 1. Synthesis Route for Biodegradable PEG−PLL-*g*-His MBCs[a]



[a] R = cbz-protected ϵ-amine; R* = deprotected ϵ-amine; R′ = free or His-conjugated ϵ-amine.

(400 MHz, D_2O): δ 3.58−3.74 (CH_2), δ 2.00−2.40 (OH) (D_2O exchange study-data not shown). Then, 20 g of anhydrous PEG (27.58 mmol OH groups), 3.45 g of succinic anhydride (34.48 mmol), 3.37 g of DMAP (27.58 mmol), and 3.84 mL of dried TEA (27.58 mmol) were dissolved in 200 mL of dioxane, and the reaction was stirred overnight at room temperature.[28] The reaction solution was concentrated and then added to 200 mL of distilled water that was acidified with 5 N HCl to pH 3.0 followed by extraction with CH_2Cl_2. The combined organic phases were dried with $MgSO_4$, concentrated, precipitated in 400 mL of ether, and dried in vacuo. Reaction completion was monitored by thin-layer chromatography (TLC) that was carried out on silica gel in 2-propanol/30% aqueous ammonia/water (10:2:1).[27] FT-IR (in CH_2Cl_2): broad O−H stretch: carboxylic acid dimers, 3300−2600 cm^{-1} (centered 3000 cm^{-1}); C═O stretch: carboxylic acid, 1750 cm^{-1}. MALDI−TOF: M_n 1746. ^1H NMR (400 MHz, D_2O): δ 3.58−3.74 (PEG CH_2), δ 4.24−4.30 (terminal PEG CH_2), δ 2.60−2.70 (terminal succinate CH_2).

Synthesis of Homobifunctional PEG NHS Ester. First, 10 g of PEG diacid (12.12 mmol of COOH groups), 7.5 g of DCC (36.36 mmol), 4.88 g of NHS (42.42 mmol) were dissolved in 100 mL of anhydrous CH_2Cl_2, and the reaction was stirred overnight at room temperature. The reaction was quenched with 36.36 mmol equi of dH_2O and vacuum filtered. The reaction solution was then concentrated and unreacted NHS was removed by solvent extraction with benzene followed by filtration. The PEG NHS ester was precipitated in ether and dried in vacuo. The *N*-hydroxysuccinimide anion ($^-$OSu) content of the polymer as determined spectrophotometrically in 0.1 M borate buffer, pH 9.0 was 1.75 mmol/g (ϵ_{260} = 8500 M^{-1} cm^{-1} is the extinction coefficient of −Osu).[29] Reaction

completion was monitored by thin-layer chromatography (TLC). FT-IR (in CH_2Cl_2): C=O stretch: succinic anhydride, 1800 and 1740 cm^{-1}. MALDI−TOF: M_n 1946;^{1}H NMR (400 MHz, D_2O): δ 3.60−3.72 (PEG CH_2), δ 4.24−4.30 (terminal PEG CH_2), δ 2.74−2.80 and 2.92−2.98 (terminal succinate CH_2), δ 2.80−2.88 (CH_2 NHS).

Synthesis of cbz-L-Lysine NCA. The facile synthesis of cbz-L-lysine α-amino acid *N*-carboxyanhydrides (NCA's) was performed using a solution of phosgene in THF by the Fuchs−Farthing method.[30,31] Then 10 g of cbz-L-Lysine (35.68 mmol) was dissolved in 100 mL of anhydrous THF in a round-bottom flask equipped with a drying tube and dropping funnel in an oil bath. Following that, 4.23 g of triphosgene (14.27 mmol) was dissolved in 5 mL of THF and added to the flask via dropping funnel and the reaction was heated and stirred at 55 °C for 1 h. The reaction mixture was concentrated and added to ∼300 mL of anhydrous hexane that was stored at 4 °C for 8 h to promote crystallization of the NCAs. The filtered precipitate was redissolved and ∼1 g of charcoal (dried over P_2O_5 in vacuo) was added to the solution to remove the electrophilic acid chloride byproducts. The reaction solution was then filtered through a Celite column and precipitated in anhydrous hexane. The filtered product was then dried in vacuo. FT-IR (in anhydrous CH_2Cl_2): N−H stretch, cbz-L-lysine, 3450 cm^{-1}; C−H stretch, lysine side chain, 3000 cm^{-1}; C=O stretch, anhydride, 1790 and 1855 cm^{-1}; C−O−C stretch, anhydride, 1250 cm^{-1}.

Synthesis of (PEG—PLL)$_x$ MBCs. First, 5 g of cbz-L-lysine NCA (16.32 mmol) was dissolved in 50 mL of anhydrous DMF, followed by addition of 54.6 μL EDA (0.816 mmol) as the initiator and the reaction was stirred for 72 h. Then, 1.57 g of homobifunctional PEG NHS ester (0.816 mmol) was added to the flask and stirred for an additional 48 h.[32] The polymer solution was concentrated, precipitated into ultrapure water, and freeze-dried. The weight-average molecular weight (M_w) of the synthesized cbz-protected (PEG−PLL)$_x$ MBC was determined via HPLC−SEC. The eluent used was HPLC grade DMF at a flow rate of 1.0 mL/min at a cell and column temperature of 40 °C. Polymer and polystyrene standard concentrations of 2.5 mg/mL were prepared and 100 μL injection volumes were used for each run. The MWs were calculated by Class-VP Software for Windows, version 7.1.1. The M_w of the cbz-protected MBCs as determined using the polystyrene standards was found to be 41 000; $M_w/M_n = 3.19$. The protected polymer was then deprotected with formic acid and Pd/C.[33] Then 2 g of the cbz-protected (PEG−PLL)$_x$ MBCs were dissolved in 25 mL of DMF followed by the addition of 6 g Pd/C. Following that, 75 mL of formic acid was then added dropwise through a dropping funnel and the reaction solution was stirred overnight at room temperature and filtered through a Celite column that was rinsed with 0.1 N HCl. The deprotected polymer was then concentrated, precipitated in 500 mL of acetone:ether (1:3), and solvents were partially removed in vacuo for 2−3 h. The polymers were then redissolved in ultrapure water and filtered through a 0.45 μm PVDF filter to remove residual charcoal and freeze-dried. The M_w of the deprotected MBCs as determined using PEG standards was found to be 27 000; $M_w/M_n = 3.12$. The deprotected polymers contained 4.50 mmol/g of ϵ-amine group side chains as determined by the Ninhydrin assay. ^{1}H NMR (400 MHz, D_2O): δ 3.45−3.70 (PEG backbone CH_2), δ 1.20−1.50 (PLL γ-CH_2), δ 1.50−1.90 (PLL β,δ-CH_2), δ 2.80−3.00 (PLL ϵ-CH_2), and 4.10−4.25 (PLL α-CH_2).

Synthesis of PEG—PLL-*g*-His MBCs. His residues were conjugated at various mole ratios (5, 10, 20, 30, and 60 mol %) to the primary ϵ-amines of PLL to produce the final PEG−PLL-*g*-His MBCs. The carboxyl groups on the His residues were first converted to the more reactive NHS ester prior to reaction with the polymer. Then 8.24 mg (0.045 mmol), 16.49 mg (0.090 mmol), 33.00 mg (0.180 mmol), 49.47 mg (0.270 mmol), and 98.90 mg (0.54 mmol) His in separate reaction vessels together with a 3.0 and 3.5 mol excess of DCC and NHS, respectively, were dissolved in 5 mL of dried DMSO, and the reactions were stirred for 1 h at room temperature. Then 100 μL of dried TEA was then added to separate reaction vessels followed by the addition of 200 mg of deprotected (PEG−PLL)$_x$MBCs (0.90 mmol ϵ-NH_2 equivalent) dissolved in 5 mL dried DMSO and the reaction was stirred overnight at room temperature. The reaction solutions were then transferred to polypropylene tubes and centrifuged for 10 min at 13 000 rpm to remove most of the dicyclohexylurea (DCU) byproduct of DCC. The conjugated polymers were then precipitated in 200 mL acetone:ether (1:3) followed by centrifugation at 4200 rpm for 10 min. The solvent was poured off and the polymers were washed several times with ether and dried in vacuo for 6 h to partially remove the ether. The polymers were then redissolved in ultrapure water and filtered through 0.2 μm PVDF filters to remove residual DCU. The His-conjugated polymers were then separated from unconjugated His on PD-10 desalting columns, and the purified polymers were freeze-dried. The degree of His conjugation to the polymer was determined using ^{1}H NMR (400 MHz, D_2O): δ 6.8 and 7.6 (imidazole C2 and C4 respectively).

Polymer Characterization. Polymer Screening and Optimization of PEG—PLL-*g*-His MBCs. The various PEG−PLL-*g*-His MBCs were screened via fluorescent activated cell sorting (FACs), luciferase assay, and MTT assay to evaluate the efficacies of the polymer/pDNA complexes. These assays were also performed in order to determine which conjugate produced the highest transfection efficiency with the least cytotoxicity on A7r5 cells in vitro so that subsequent polymer characterization could be performed on the optimized conjugate. All conjugates were optimized with respect to N/P ratios.

Preparation of Polymer/pDNA Complexes. All polymer/pDNA complexes were prepared freshly prior to use. Polymer stock solutions and the purified pDNA stock solutions had concentrations of 10 and 1.08 mg/mL, respectively, in ultrapure water. Both polymer and plasmid were then diluted to the appropriate concentration depending on the required N/P ratio each to a final volume of 100 μL containing 5% glucose. The pDNA solutions were then added to the polymer solutions in equal volumes and complexation was allowed for 30 min prior to use.

Flow Cytometry. A7r5 cells were seeded at a density of 9 × 10^4 cells/well in a six-well plate and were incubated for at least 24 h prior to transfection. The cells were transfected with PEG−PLL-*g*-His/pSV-EGFP complexes prepared at various N/P ratios. Then 2 μg of pDNA complexes in 2 mL of serum-free DMEM media were added to each well. The cells were then incubated for 4 h in a 5% CO_2 incubator at 37 °C after which the media was replaced with fresh DMEM containing 10% FBS and incubated for an additional 44 h at 37 °C. The cells were washed with 1× PBS buffer, trypsinized and transferred to sterile tubes followed by centrifugation at 1500 rpm for 10 min. The supernatant was poured off, and the cells were resuspended in 1 mL of 1× PBS containing 10% FBS. Then, 10 μL of PI (1.0 mg/mL) fluorescent DNA nuclear marker was added to the cells to determine cell viability. Fluorescence for both EGFP and PI were detected using a FACScan flow cytometer with excitation wavelengths of 488 and 535 nm respectively. The instrument was calibrated with a negative control (nontransfected cells) to identify viable cells and then the transfected cells were analyzed from a gated viable population of 10 000 cells. In all experiments, the viable cells were gated based on cell exclusion of propidium iodide.

Luciferase Assay. A7r5 cells were seeded and transfected with 2 μg of pDNA complexes using the pSV-Luc plasmid as described above. Luciferase assay was performed using a Luciferase kit from Promega and measurements of relative light units (RLU) were performed with a Luminometer from Dynex Technologies Inc. (Chantilly, VA). Protein content was quantitated with BCA reagent carried out with a Bio-Rad Microplate reader at 570 nm.

Cell Viability. Evaluation of cytotoxicity was performed using the MTT assay according to the method of Mosmann.[34] A7r5 cells were seeded at a density of 1.5 × 10^4 cells/well in a 24-well plate and were incubated for at least 24 h prior to transfection. Then 0.5 μg of pDNA complexes was used to transfect the cells as described above. Absorbance was mea-

sured at 570 nm and cell viability was calculated according to the following equation:

$$\text{Cell viability (\%)} = (\text{OD}_{570(\text{sample})}/\text{OD}_{570(\text{control})}) \times 100 \quad (1)$$

Here $\text{OD}_{570(\text{sample})}$ is the absorbance of the transfected cells and $\text{OD}_{570(\text{control})}$ represents the absorbance of the nontransfected cells.

Physicochemical Characterization of Biodegradable PEG—PLL-*g*-His MBCs. Acid—Base Titration. The ability of the gene carriers (PEG−PLL-*g*-His) to protonate and obtain a positive charge over the pH range 12 to 2 was determined by acid−base titration.[24,35] Briefly, 10 mg of each conjugate or PLL (25 600) was dissolved in 10 mL of 150 mM NaCl. One hundred microliters of 1 N NaOH was then added, and the pH of the polymer solution was recorded. The solution was titrated with increasing volumes of 0.1 N HCl and the pH was measured with a Corning pH meter.

Particle Size and ζ Potential. PEG−PLL-*g*-His/pSV-Luc complex size and ζ potential measurements were determined with a Brookhaven Instruments Corp. ZetaPALS (Holtsville, NY) at 25 °C using an ion-argon laser (λ = 677 nm) as the instrument beam at a scattering angle of 90°. For data analysis, the viscosity (0.8905 mPa s) and refractive index (1.333) of pure water at 25 °C were used and measurements for particle size are reported as the effective mean diameters. Smoluchowski's equation was used to calculate the ζ potential values from the electrophoretic mobility.

Atomic Force Microscopy (AFM) of PEG−PLL-*g*-16% His/pDNA Complexes. The surface morphology of PEG−PLL-*g*-16% His/pSV-Luc complexes was determined using a Digital Instrument Nanoscope II SFM instrument using the tapping mode.[36] Then 9.9 mm mica disks were soaked in 33 mM magnesium acetate buffer for 24 h followed by sonication for 30 min in ultrapure water and subjected to glow discharge for 15 s in a vacuum between 100 and 200 mTorr. Then 20 μL of PEG−PLL-*g*-16% His/pSV-Luc complexes (0.1 mg/mL) at 15/1 N/P ratio was then added to the cleaned mica surface for 2 min after which excess solution was gently rinsed off with ultrapure water. The mica surface was then blown dry with nitrogen and mounted onto the microscope stage and the image was acquired using the tapping mode with a low aspect cantilever tip at room temperature.

DNase I Protection Assay with PEG−PLL-*g*-16% His MBCs. To confirm the ability of the PEG−PLL-*g*-16% His MBCs to protect plasmids from endonucleases, DNase I protection assay was performed in vitro.[37] PEG−PLL-*g*-16% His/pSV-Luc complexes were prepared at a 15/1 N/P ratio with a final pDNA concentration of 0.01 mg/mL. 5 units (5U) RQ1 RNase-free DNase I was then added to either naked pSV-Luc (10 μg) or complexes (10 μg pDNA), and the samples were incubated at 37 °C. Then, 100 μL aliquots were removed at 0, 20, 40, 60, and 120 min postincubation and added to separate labeled tubes containing 100 μL of stop solution (200 mM NaCl, 20 mM EDTA, 1% SDS). The tubes were then incubated at 60 °C overnight to dissociate the pDNA from the complexes followed by isolation of pDNA using phenol−chloroform extraction. The pDNA was then precipitated with 70% ethanol and the pellets were redissolved in TE buffer. The samples were electrophoresed and analyzed on a 1% agarose gel that was stained with ethidium bromide (0.5 μg/mL).

Polymer Degradation Study of PEG—PLL-*g*-16% His MBCs. The degradation profiles of PEG−PLL-*g*-16% His MBCs were determined in 0.1 M phosphate buffer solution (PBS), pH 7.4. First, 60 mg of polymer was dissolved in 20 mL of PBS and was incubated at 37 °C in a shaker incubator. Then 1 mL aliquot samples were then removed at designated time points and stored at −70 °C for analysis by HPLC−SEC. The M_w of the polymer samples were determined using PEG standards and the eluent used was 0.1% TFA with 0.15 M NaCl in ultrapure water. The samples were filtered through a 0.2 μm filter prior to analysis. The half-life ($t_{1/2}$) of the polymers was determined using the following equation:

$$y = y_0 + a\mathrm{e}^{-bx} \quad (2)$$

Polymer Degradation Study of PEG—PLL-*g*-16% His/pDNA Complexes. The degradation profile of PEG−PLL-*g*-16% His/pSV-Luc complexes was determined in 0.1 M PBS, pH 7.4. PEG−PLL-*g*-16% His MBCs dissolved in 0.1 M PBS, pH 7.4, and was added to 10 μg of pSV-Luc to produce complexes at a 15/1 N/P ratio. The complexes were incubated in a 37 °C shaker incubator and 20 μL aliquot samples were removed at various time points and stored at −70 °C for analysis on a 1% agarose gel that was stained with ethidium bromide (0.5 μg/mL).

Imidazole Protonation of PEG−PLL-*g*-16% His MBCs. The protonation of the imidazole rings of the histidine free base (10 mg/mL) and of the PEG−PLL-*g*-16% His MBCs, 15/1 N/P ratio (10 mg/mL) were determined by measuring the chemical shifts of the C2−H of the histidyl residues vs pH of the solution with ^{1}H NMR spectroscopy in D_2O at 400 MHz.[26,38] Briefly, 10 mg of histidine free base or polymer conjugate was dissolved in 3 mL of D_2O, and the pH was adjusted to below pH 2.0 with 1.0 N CID. The solutions were then titrated with increasing volumes of 0.1 N DNaO and the chemical shifts were measured with a Varian Mercury 400 MHz ^{1}H NMR instrument.

Influence of Endosomal pH on Transfection Efficiency Using PEG—PLL-*g*-16% His MBCs. The ability of the PEG−PLL-*g*-16% His MBCs, 15/1 N/P ratio to disrupt endocytotic vesicles containing PEG−PLL-*g*-16% His/pDNA complexes was investigated by transfecting A7r5 cells in the absence or the presence of bafilomycin A_1, a potent inhibitor of vacuolar ATPases.[39] A7r5 cells were seeded at a density of 9×10^4 cells/well in a 6-well plate and were incubated for at least 24 h prior to transfection. The cells were then transfected with 2 μg of pDNA (PEG−PLL-*g*-16% His/pSV-Luc) complexes at 15/1 N/P ratio as described above in the absence or presence of 200 nM bafilomycin A_1. After 4 h, the transfection media was removed and 2 mL fresh DMEM containing 10% FBS was added and the cells were incubated for an additional 44 h at 37 °C. The luciferase activity was then performed to assay gene expression.

Statistical Tests. Statistical analysis of data performed with GraphPad Prism version 3.02 software using one-way ANOVA with Tukeys post-hoc for p values ≤ 0.05. Data represented with an error indicates sample group mean ± the standard deviation of the mean (σ) used to illustrate the scatter of the data if the distribution of the means approximate a Gaussian distribution.

Results

Low molecular weight PEG diol was converted to the homobifunctional PEG succinic acid that was activated by converting the diacid into the homobifunctional succinimidyl succinate (PEG NHS ester) using DCC as the coupling agent. The use of the succinic acid facilitated the incorporation of the ester bond linkages into the backbone of the copolymer thereby generating the biodegradable polymers. The multiblock copolymers were synthesized by addition of a stoichiometric amount of PEG NHS ester equivalent to the number of moles of initiator used to generate PLL that was synthesized via ring-opening polymerization of lysine NCAs. Subsequent conjugation of the histidine derivatives to the primary ϵ-amines of PLL was performed by first converting the α-carboxyl group of histidine to the reactive acylating intermediate using DCC as the coupling agent which then underwent nucleophilic acyl substitution with the amine (Scheme 1).

After synthesis and purification of the homobifunctional PEG succinic diacid, the expected molecular weight (M_n = 1650) was determined using MALDI−TOF that gave a M_n of 1746. The structure of the diacid was

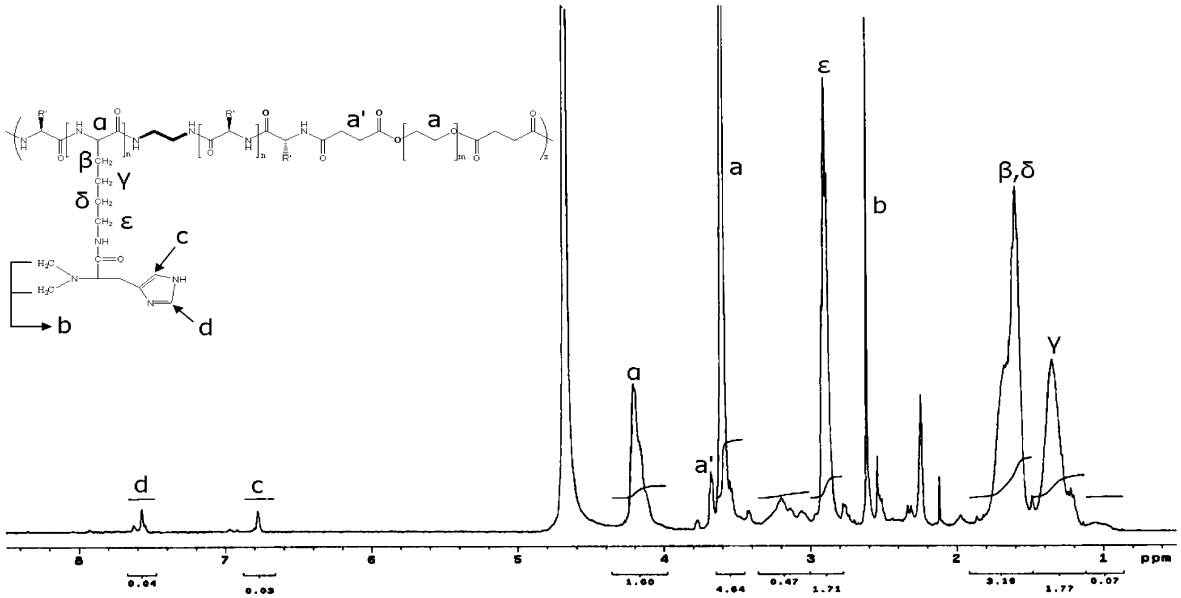


Figure 1. ^{1}H NMR spectrum for PEG−PLL-*g*-His MBCs (D_2O).

verified using both FT-IR and ^{1}H NMR spectroscopy. Liquid FT-IR using anhydrous CH_2Cl_2 as the solvent revealed almost complete disappearance of the broad alcohol stretch at 3333 cm^{-1} and the appearance of the carboxylic acid dimer stretch 3300−2600 cm^{-1} centered at 3000 cm^{-1} and the carboxylic acid stretch at 1750 cm^{-1}. The ester bond of the succinic acid was present at 1100 cm^{-1} even though this bond is not clearly distinguished from the ether bond of both the diol and the diacid. ^{1}H NMR spectroscopy revealed the appearance of the distinguishable terminal PEG CH_2 groups at 4.24−4.30 ppm as well as the terminal succinate CH_2 groups at 2.60−2.70 ppm. The expected molecular weight of the homobifunctional ester (M_n = 1942) was verified using MALDI−TOF that gave a M_n of 1946 for the ester. The structure of the ester was verified using both FT-IR and ^{1}H NMR spectroscopy. Liquid FT-IR using anhydrous CH_2Cl_2 as the solvent revealed the appearance of the carbonyl stretches of the succinic anhydride at 1800 and 1740 cm^{-1} and ^{1}H NMR showed the presence of the additional methylene groups of the NHS at 2.80−2.88 ppm. The synthesized cbz-L-lysine NCA was characterized using liquid FT-IR with anhydrous CH_2Cl_2 as the solvent which showed the appearance of the carbonyl anhydride stretch at 1790 and 1855 cm^{-1} and the C−O−C stretch of the anhydride at 1250 cm^{-1}.

The M_w of the synthesized cbz-protected MBCs was found to be 41 000 with a $(PEG−PLL)_x$ repeating unit of 6 as determined by SEC. After deprotection of the polymers, the M_w was found to be 27 000 with a $(PEG−PLL)_x$ repeating unit of 5−6. The target feed ratio for the degree of polymerization (DP) of PLL per repeating unit was 20 and the actual DP as determined by ^{1}H NMR was 21. The total amount of ϵ-amine group side chains was 4.50 mmol/g of polymer as determined by the Ninhydrin assay. After conjugation and purification of His to the PEG−PLL MBCs, the actual degree of conjugation per repeating unit as determined by ^{1}H NMR was 5% His, 9% His, 16% His, and 22% His that resulted in the following conjugates: PEG−PLL-*g*-5% His, PEG−PLL-*g*-9% His, PEG−PLL-*g*-16% His, and PEG−PLL-*g*-22% His, respectively (Figure 1).

The conjugated His produced distinct chemical shifts 6.8 and 7.6 ppm for C2 and C4 respectively as opposed to unconjugated His that had peaks at 7.3 and 8.5 ppm.

To determine whether the biodegradable MBCs were capable of delivering plasmid DNA to cells for gene expression as well as to select the optimum His-conjugated MBCs for physicochemical characterization, polymers were screened for transfection efficiency and cytotoxicity in vitro. The transfection efficiency was evaluated in murine smooth muscle cells (A7r5) using both flow cytometry and luciferase assay in order to correlate the actual cell populations transfected and the levels of protein produced from these cells. In addition, each conjugate was evaluated as a function of N/P ratios in five unit increments (data not shown), and only optimized ratios for each conjugate are presented. Flow cytometry data showed that the PEG−PLL-*g*-16% His MBC/pDNA complexes resulted in the highest transfection efficiency in which saturation was observed at a 15/1 N/P ratio. These polymers resulted in ∼3- to 4-fold higher percentage of cells in the viable gated population that were positive for EGFP expression in comparison to the optimized PLL control at a 2/1 N/P ratio ($p < 0.05$) (Figure 2A).

This result was also confirmed with the luciferase assay which showed that transfection with PEG−PLL-*g*-16% His MBC/pDNA complexes produced ∼4- to 5-fold higher protein expression in comparison to the PLL control ($p < 0.01$) (Figure 2B).

The higher transfection efficiency of the PEG−PLL-*g*-16% His MBCs is most probably due to the presence of the histidine residues that are able to facilitate endosomal buffering with subsequent release of pDNA into the cytosol followed by nuclear translocation. The higher His content of the PEG−PLL-*g*-16% His MBCs resulted in greater transfection efficiency in comparison to the 0%, 5%, and 9% His-conjugated polymers. Even though the 9% His-conjugated polymers resulted in relatively high transfection efficiency at 30/1 N/P ratio, this level of protein produced was not statistically significant in comparison to PLL ($p > 0.05$). In addition, the decrease in transfection efficiency of the PEG−PLL-*g*-22% His MBCs indicates that complex conformation is also important for transfection efficiency. The un-

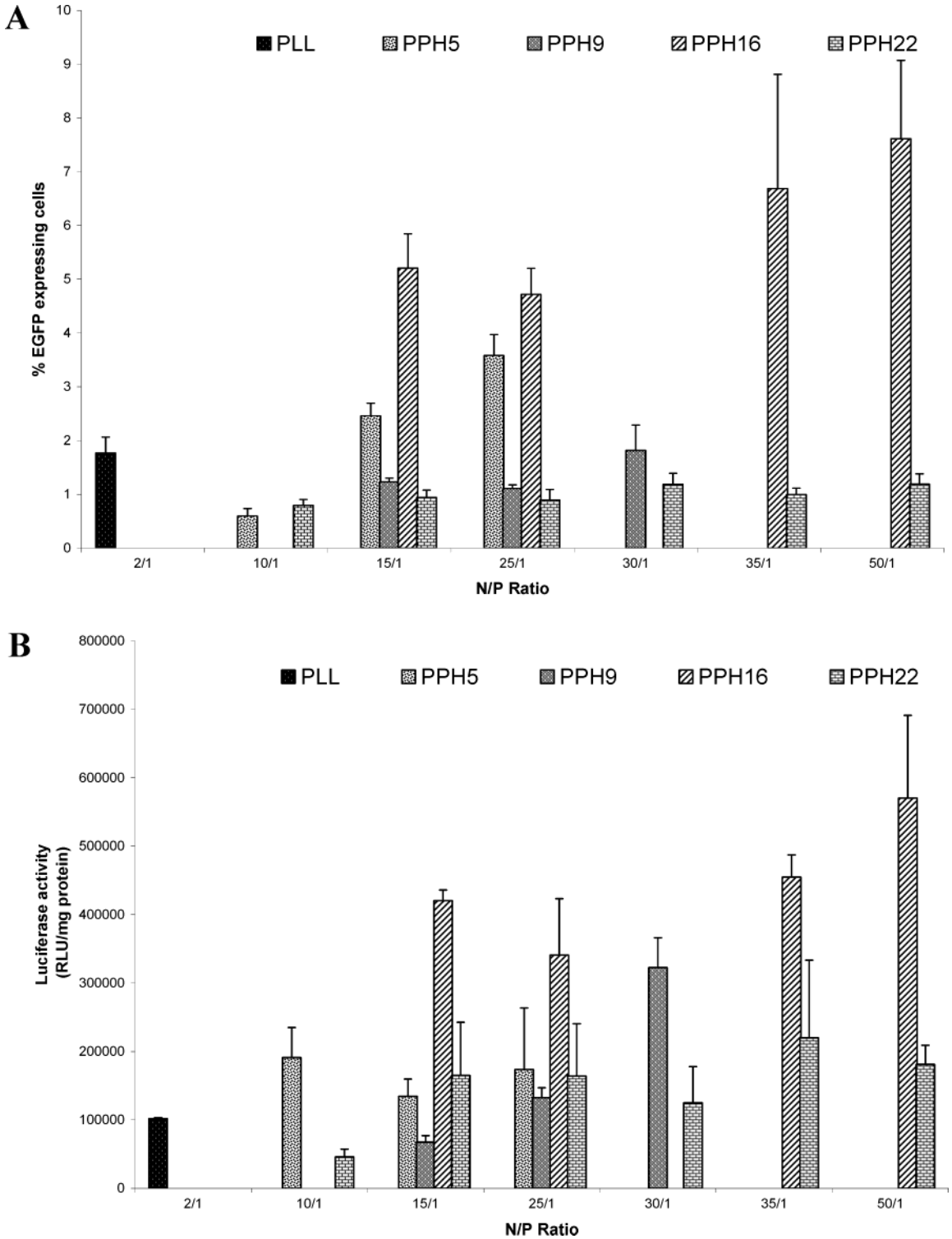


Figure 2. Evaluation of transfection efficiency of PEG−PLL-*g*-His MBCs with A7r5 cells at different N/P ratios. (A) Flow cytometry using pSV-EGFP/polymer complexes. (B) Luciferase assay using pSV-Luc/polymer complexes. Luciferase data for PEG−PLL-*g*-0% His MBCs no greater than PLL control at all N/P ratios. Data reported as mean ± SD, $n = 3$.

charged imidazole rings at physiological pH are probably stacked on the interior of the polymer/pDNA complexes where the amount of His residues present would dictate the degree of stacking and hence the size and conformation of the complexes that inadvertently impact the endocytic process. Therefore, a balance between cationic charge and imidazole rings is probably required for optimum transfection efficiency. The higher His content of the PEG−PLL-*g*-22% His MBCs therefore suggests an altered conformation of the complexes that ultimately affected the transfection efficiency of the polymers despite increasing the buffering capacity.

To determine the cytotoxicity of these optimized polymers in vitro, the cell viability was evaluated in A7r5 cells using the MTT assay. The data indicate that most of the synthesized conjugates do not significantly affect the cell viability of A7r5 cells in comparison to PLL at 2/1 N/P ratio, which results in a decrease in cell viability to ∼70% (Figure 3).

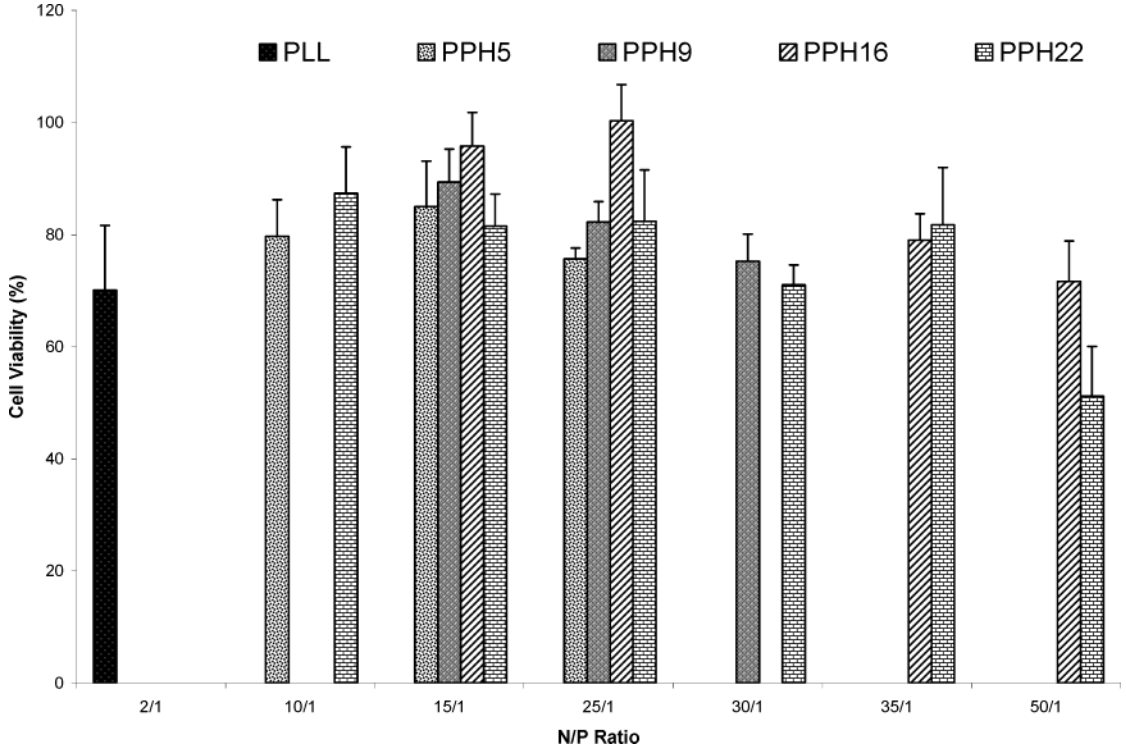


Figure 3. Cell viability data for biodegradable PEG−PLL-*g*-His MBCs with A7r5 cells at different N/P ratios with MTT assay. Data reported as mean ± SD, $n = 3$.

In addition, the PEG−PLL-*g*-16% His MBCs produced almost no cytotoxicity at 15/1 and 25/1 N/P ratios, even though these polymers in conjunction with the other conjugates affect the cell viability of the cells at N/P ratios above 25/1. The decrease in cytotoxicity of these MBCs can probably be attributed to hydrolytic and enzymatic degradation of the high molecular weight polymers that leads to low molecular weight PEG and PLL blocks. Low molecular weight PLL blocks affect aggregation and fusion of phospholipid vesicles to a less extent in comparison to high molecular weight polymers.[40]

The conjugation of histidine moieties to the multiblock copolymers should impart pH-sensitive characteristics to the polymers resulting in a proton buffering effect within the endosomal/lysosomal compartments of the cell. Therefore, to demonstrate the ability of the histidine moieties conjugated to the polymers to undergo protonation of both the tertiary amines on the histidine derivatives and the imidazole rings, acid−base titration of the polymer conjugates was performed. The acid−base titration profiles of PLL, PEG−PLL-*g*-5% His, PEG−PLL-*g*-9% His, PEG−PLL-*g*-16% His, and PEG−PLL-*g*-22% His MBCs were obtained with 0.1 N HCl solution (Figure 4).

All solutions had an initial pH range from 11.55 to 11.78 with 1.0 N NaOH. The initial high pH values between pH 11.78 and 8.0 represent protonation of the ϵ-amine groups of PLL as well as the tertiary amines of the histidine residues present on the conjugates. The steep slope of PLL suggests no buffering capacity below pH 8.0 as all the amine groups have already been protonated. However, the histidine-conjugated polymers show increasing buffering capacity of the polymers with increasing histidine content as shown by the decreasing slopes of the curves. This can be contributed to the protonation of the relative amounts of imidazole groups present on the different conjugates where the PEG−PLL-*g*-22% His MBCs showed the highest buffering capacity due to the highest histidine content of the polymers. These data are in agreement with previous reports in which partially substituted PLL with histidine polymers possessed both DNA condensing and pH buffering abilities.[24]

The particle size distribution of the polymer/pDNA complexes formed from the optimized PEG−PLL-*g*-His MBCs with pSV-Luc was determined by dynamic light scattering. The data showed that the majority of the complexes remained relatively constant between ∼150−200 nm in effective diameter at various N/P ratios in comparison to PLL 2/1 N/P ratio which had an average particle size of ∼114 nm (Figure 5A).

However, a significant increase in particle size for the PEG−PLL-*g*-22% His/pDNA complexes at 15/1, 25/1, and 30/1 N/P ratios was observed. The larger particle size of the complexes formed from the conjugates in comparison to PLL is probably due to the presence of the histidine residues whose bulky imidazole rings would affect complexation and hence complex size. In addition, the relatively discrete complexes indicate that the PEG blocks are able to sterically stabilize the complexes in solution so that no aggregation is observed. The steric stabilization effects of PEG are also observed for the ζ potential of the complexes. The ζ potential of complexes formed with PLL at 2/1 N/P ratio is ∼20 mV in comparison to the complexes formed with the PEG−PLL-*g*-5% His MBCs that have a ζ potential of ∼7 mV at 10/1 N/P ratio (Figure 5B).

The lower ζ potential values are due to the shielding effect of the PEG blocks. The ζ potential of the complexes range from ∼7 to 45 mV depending on the conjugate.

The degree of pDNA complexation and the resulting complex morphology was studied using atomic force microscopy (AFM) with the PEG−PLL-*g*-16% His MBCs complexed with pSV-Luc at a 15/1 N/P ratio. The AFM

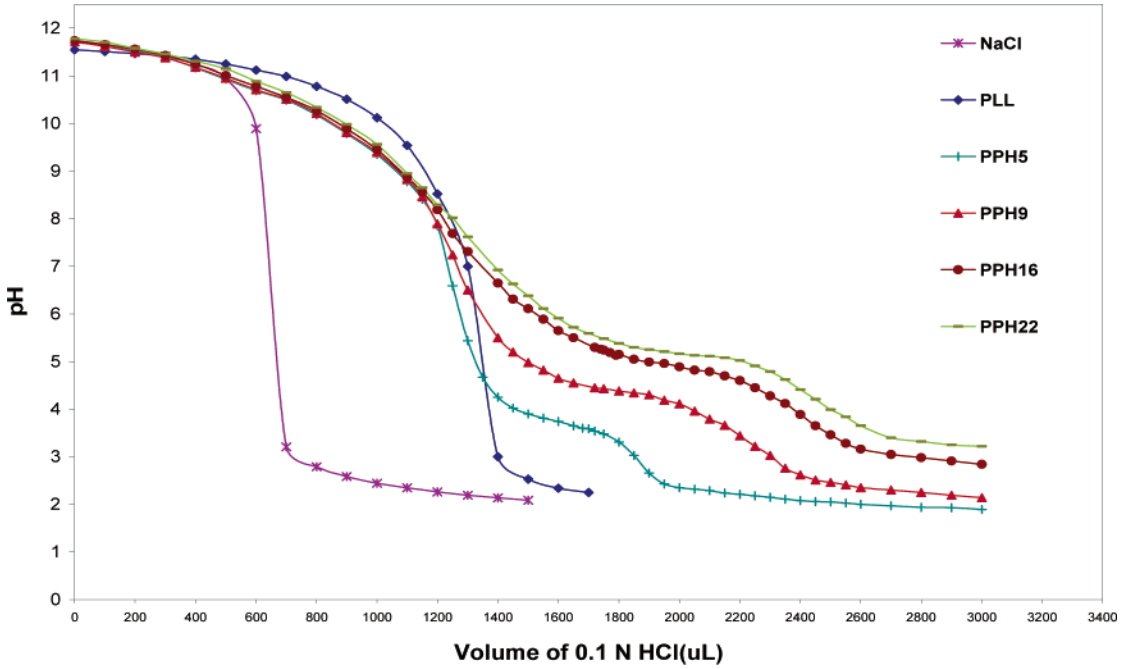


Figure 4. Acid−base titration profile of PEG−PLL-*g*-His MBCs with 0.1 N HCl solution.

data showed uncomplexed, supercoiled pDNA bound to the mica surface (Figure 6A).

The addition of polymer to the DNA resulted in complete complexation of the pDNA after 30 min without any agglomeration of complexes in solution, as can be seen by the homogeneous array of relatively small, discrete complexes on the surface of the mica. There was also no uncondensed pDNA observed on the mica surface after complexation with the cationic polymer (Figure 6B). Further analysis of a single complex showed that the complexes had a roughly spheroidal shape that was ∼100 nm in both length and width (Figure 6C). The discrete condensed complexes on the mica surface indicate that the PLL blocks within the MBCs are sufficient to condense the pDNA in solution. These data also suggest that the presence of the PEG blocks within the MBCs is sufficient to prevent aggregation of complexes in solution due to the steric stabilization effects of the PEG. The size of the complex on the mica surface approximately corresponds to the average particle size of the complexes in solution i.e., 100 vs 150 nm, respectively.

Plasmid DNA must be protected from degradation by endonucleases prior to its final destination within the cell.[41] The ability of the MBCs to protect the nucleic acids from endonuclease digestion was investigated using a DNase I protection assay with PEG−PLL-*g*-16% His MBCs. The result shows that the PEG−PLL-*g*-16% His MBCs are able to protect pDNA from degradation by the endonuclease DNase I for up to 2 h (Figure 7, lane 10).

The naked pDNA was almost fully degraded by DNase I in 20 min, as shown by the smear in lane 2, and in 40 min, all the naked pDNA was completely degraded. This is opposed to the complexed pDNA that was reisolated from the complexes which was protected at all time points from endonuclease digestion as shown by the intact pDNA bands on the stained agarose gel (Figure 7, lanes 6−10). Thus, this assay was able to clearly demonstrate that the MBCs can fully protect condensed pDNA from endonuclease activity.

One of the most important aspect of these novel multiblock copolymers is the presence of the ester linkages that confer biodegradability and hence biocompatibility onto the polymers. This will abrogate the problems of increased transfection efficiency observed with high molecular weight polymers that result in a concomitant increase in cytotoxicity.[8] Thus, the degradation profile of the PEG−PLL-*g*-16% His MBCs was determined in 0.1 M PBS, pH 7.4 at 37 °C. The data showed that the initial M_w of the His-conjugated MBCs at time 0 h was 31 000 as determined by SEC using PEG standards. Shortly after dissolution in buffer, the polymer began to degrade relatively quickly. In 4 h, the polymer had degraded to 17 000, which is a little over half of its M_w and in 24 h the MBCs had completely degraded into its constituent PEG and PLL blocks (data not shown). Overall, the MBCs exhibited exponential decay and after fitting the data to a three-parameter exponential equation, the half-life ($t_{1/2}$) of the MBCs was found to be ∼5 h in buffer solution. This type of degradation profile is consistent with published reports of "self-destroying polymers" in which the main chain cleavages occur via nucleophilic attack of the amine groups of the polymer itself resulting in probably both *intra*molecular and *inter*molecular aminolysis.[10] To determine the stability of polymer/pDNA complexes in buffer solution, PEG−PLL-*g*-16% His/pSV-Luc complexes were incubated in 0.1 M PBS, pH 7.4 at 37 °C. The results showed that incubation of the complexes up to 6 d resulted in no release of pDNA (Figure 8).

Thus, polymer degradation has little impact upon complex stability after pDNA condensation in buffer solution.

The protonation of the histidine free base and PEG−PLL-*g*-His MBCs was determined by H^1 NMR spectroscopy by recording the C2−H peak shifts as a function of pH that is proportional to the degree of imidazole protonation.[42] The p*K* of the imidazole free base was found to be 5.72 whereas the p*K* of the histidine imidazole in PEG−PLL-*g*-16% His MBCs was 4.75 (Figure 9).

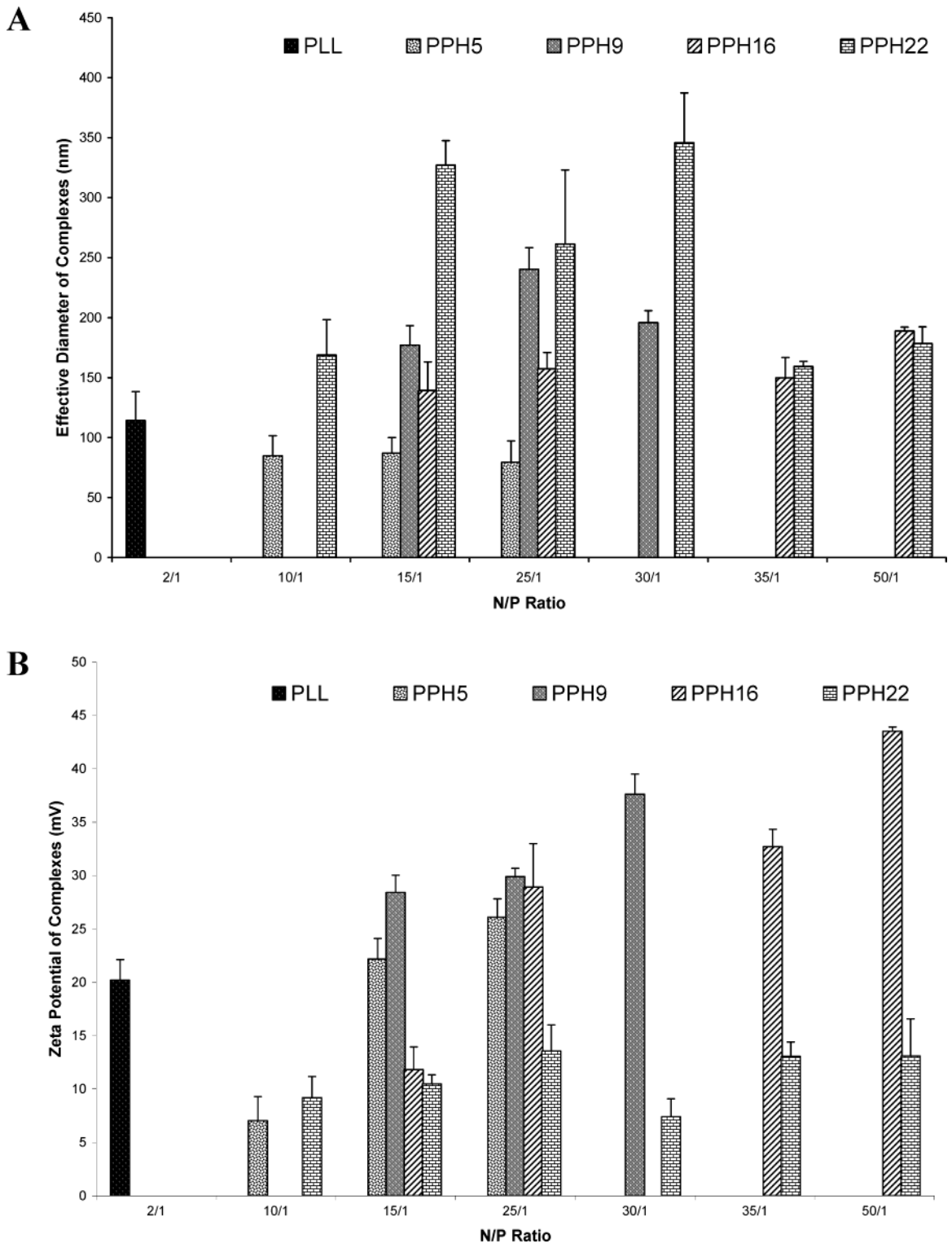


Figure 5. (A) Effective diameter of PEG−PLL-*g*-His MBC/pDNA complexes at different N/P ratios as determined by dynamic light scattering. (B) ζ potential of PEG−PLL-*g*-His/pDNA complexes at different N/P ratios as determined by laser Doppler anemometry. Data reported as mean ± SD, $n = 3$.

The decrease in p*K* of the imidazole group suggest that proton buffering of the endocytic vesicles containing the complexes probably occurs in either the late endosomes or the lysosomes.

The conjugation of histidine moieties to the MBCs is expected to result in membrane destabilization resulting in disruption of endocytotic vesicles.[23] To determine whether the His moieties conjugated to the MBCs contribute to endosomal escape of the complexes, A7r5 cells were transfected with PEG−PLL-*g*-16% His/pDNA complexes in the absence or the presence of bafilomycin A_1, a potent inhibitor of vacuolar ATPases.[43] The result of the assay showed that transfection of the cells in the presence of bafilomycin A_1 resulted in ~8-fold decrease in luciferase expression in comparison to the untreated transfected cells and comparable to the transfection data for cells transfected with polymers containing no histidine residues (Figure 10).

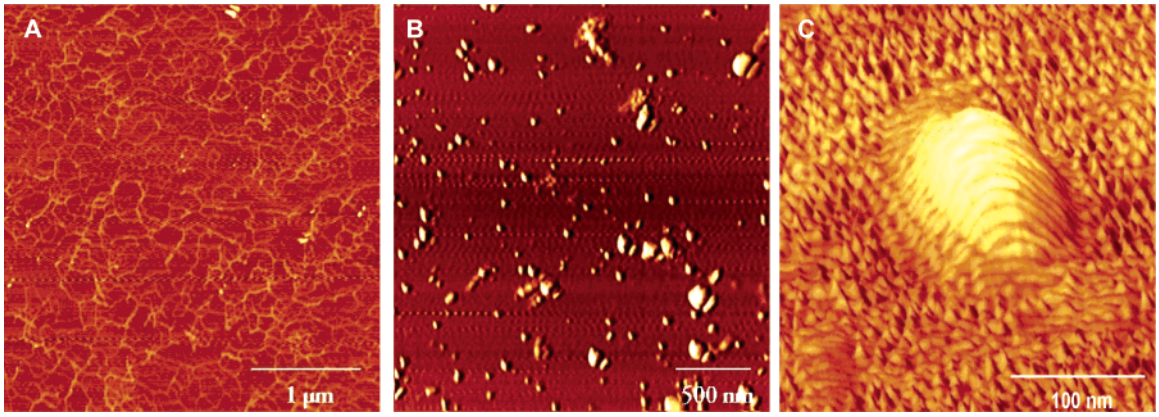


Figure 6. Atomic force microscopy (AFM) images of plasmid DNA condensation with PEG−PLL-*g*-16% His MBCs at 15/1 N/P ratio bound to the mica surface in air. (A) Plasmid DNA prior to complexation with polymer. (B) PEG−PLL-*g*-16% His MBC/pDNA complexes after 30 min incubation at room temperature. (C) Morphology of a single PEG−PLL-*g*-16% His MBC/pDNA complex.

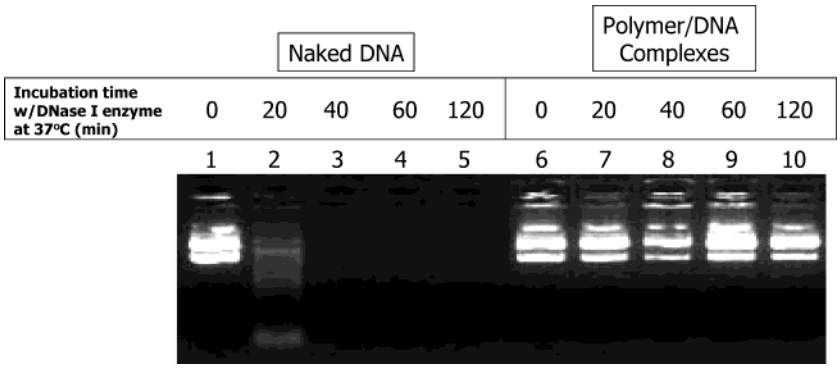


Figure 7. DNase I protection assay for PEG−PLL-*g*-16% His MBCs at 15/1 N/P ratio. Dissociated and reisolated plasmids from polymer/pDNA complexes after exposure to DNase I followed by electrophoresis on a 1% agarose gel at 80 V for 35 min.

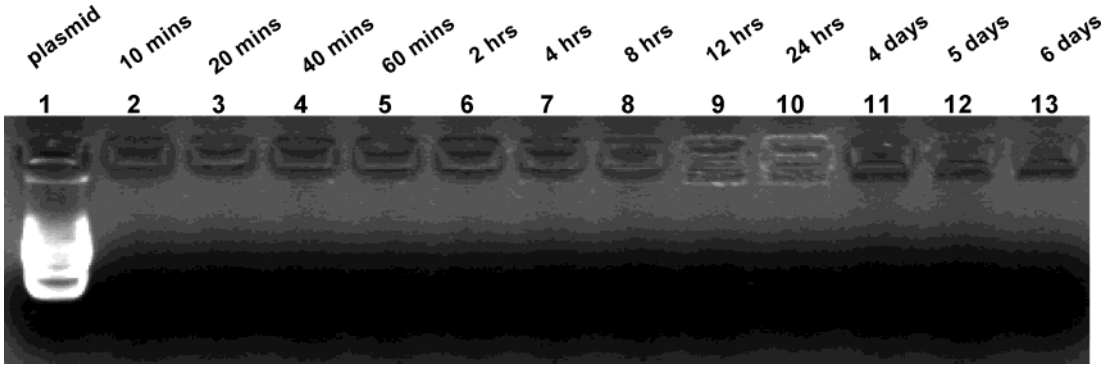


Figure 8. Degradation study of PEG−PLL-*g*-16% His MBC/pDNA complexes at 15/1 N/P ratio. Polymer dissolved in 0.1 M PBS, pH 7.4 buffer followed by addition of pDNA in water to form complexes incubated at room temperature for 30 min. Polymer/pDNA complexes were then incubated at 37 °C in a shaker incubator and 20 μL aliquots of complexes were withdrawn at different time points followed by storage at −70 °C. Samples were electrophoresed on a 1% agarose gel at 80 V for 35 min.

These data suggest that protonation of histidine imidazole rings is required for endosomal escape of complexes after endocytosis.

Discussion

In this study, biodegradable cationic PEG−PLL-*g*-His MBCs have been synthesized to produce biocompatible gene delivery vehicles that increase transfection efficiencies as well as overcome the hurdles of cellular toxicity that are frequently observed with high molecular weight polymers having high charge densities. In the synthesis of these MBCs, the highest degree of His conjugation obtained was 22% per repeating PLL unit, even though the highest target conjugation was 60%. The low conjugation efficiency could be due to steric hindrance of neighboring imidazole rings and methyl groups present on the histidine derivatives that limit the approach of additional histidine residues toward the ϵ-amines of PLL. The presence of the histidine residues as well as PEG chains resulted in complete ethidium bromide exclusion, i.e., complete pDNA condensation at a N/P ratio of 5/1 for lower His-conjugated polymers (5% and 9%) and 10/1 for higher His-conjugated polymers (16% and 22%) determined by gel retardation assay (data not shown). This suggest that even though complete pDNA condensation occurs at a higher N/P ratio as compared to PLL, the polymers are still able to

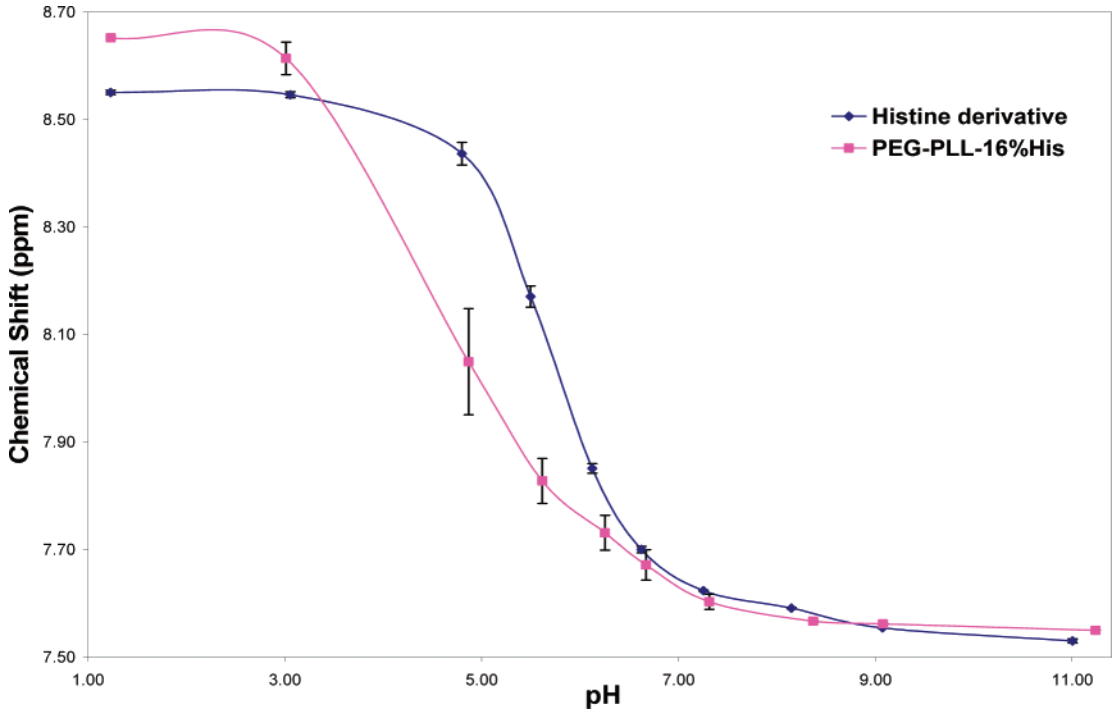


Figure 9. Acid−base titration of free *N*,*N*-dimethylhistidine and PEG−PLL-*g*-16% His MBCs for determination of histidine pK_a using 0.1 N NaOD and 0.1 N DCl. Chemical shifts determined using ^{1}H NMR. Data reported as mean ± SD, $n = 3$.

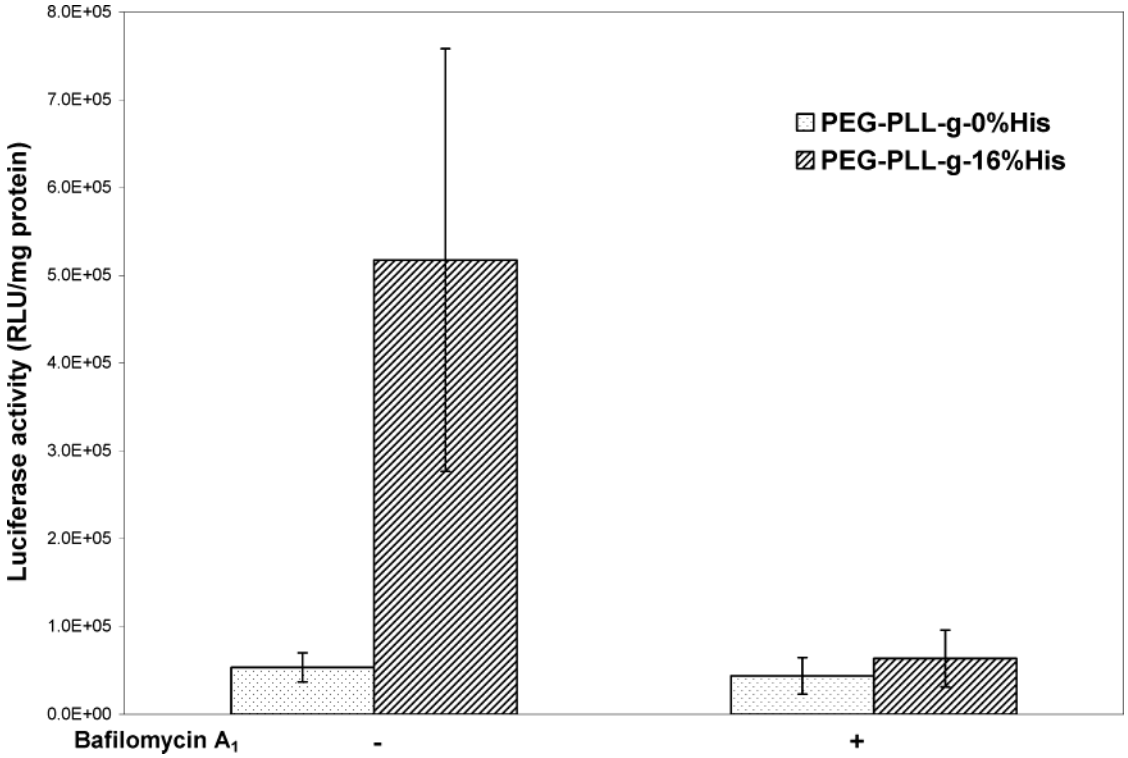


Figure 10. Influence of bafilomycin A_1 on the transfection efficiency of A7r5 cells using PEG−PLL-*g*-0% His and PEG−PLL-*g*-16% His MBCs via the luciferase assay. Data reported as mean ± SD, $n = 3$.

adequately condense pDNA that is consistent with other PLL-conjugated carriers.[44]

The transfection data revealed the unexpected findings that even though conjugation of histidine moieties to the MBCs increase the transfection efficiencies of the polymer, the transfection efficiencies are not linearly correlated with histidine content. The decrease in transfection efficiency and increase in particle size of the 22% His-conjugated MBCs indicates that complex size and conformation are also important for optimum complex efficacy. Thus, a balance between cationic charge, imidazole content, and complex size of the particles must be obtained in order to achieve a favorable result.

The pH profile for the PEG−PLL-*g*-5% His MBCs indicates that proton buffering occurs at pH 4.0−3.0 whereas PEG−PLL-*g*-9% His, PEG−PLL-*g*-16% His, and PEG−PLL-*g*-22% His MBCs show proton buffering at ∼pH 5.5−4.0. These data suggest that the proton

buffering capacity of the conjugates is well below the endosomal pH range from 5.8 to 6.4 reported for internal steady-state pH (pH_i), which is in contrast to steady-state pH_i for lysosomes that range from ~5.2−5.5 for isolated lysosomes and 3.8−4.8 for lysosomes in intact cells.[45] Thus, proton buffering of complexes formed with these MBCs probably occur either in the late endosome or the lysosomes.

The particle size for the conjugates is consistent with both the presence of the histidine residues as well as the PEG blocks as is demonstrated by the size increase for the PEG−PLL-*g*-His MBCs in comparison to PLL complexes, with the exception of the PEG−PLL-*g*-5% His MBCs. The decrease in particle size of the 5% His-conjugated polymers could be due to the lower histidine content and alternate conformation in comparison to the other conjugates. PEG is a heavily hydrated polymer that has great conformational flexibility that would form a hydrophilic corona around the complex and in doing so increase the particle size of the complexes.[22] In addition, the ζ potential of the conjugates is lower in comparison to PLL at similar N/P ratios due to the nonionic hydrophilic PEG layer that is able to shield some of the surface charges of the complexes. Also, not only were the polymer complexes sufficient to fully protect the pDNA from endonuclease digestion, but also, upon release, the pDNA showed the same plasmid form as the control, i.e., supercoiled pDNA. Thus, MBCs produced complexes that resulted in the delivery of intact plasmids.

Biodegradable cationic polymers containing ester linkages degrade easily if the polycations are based on amines that can undergo an aminolysis reaction with the ester linkages. This observation was consistent with the findings of Lim et al.[10] who reported that the polyester, PAGA, had a $t_{1/2}$ of 30 min due to nucleophilic attack of the ester linkages by the amines of the polymer's lysine residues. However, the results from the degradation profile showed that the polymer had a 10-fold increase in $t_{1/2}$ in comparison to PAGA. The increase in $t_{1/2}$ of the conjugated polymer is probably due to the increased crystallinity of the higher molecular weight polymer in comparison to the PAGA polymer (M_w 3300). However, once the polymer has condensed the pDNA, the amine groups are no longer available to react with the ester linkages and so there is an increased complex stability regardless of the polymer half-life in buffer solution. Also, the complex stability in serum will be investigated in the future to determine the release profile in the presence of serum esterases that will readily hydrolyze the ester linkages.

The p*K* of the imidazole in the free base and PEG−PLL-*g*-16% His MBCs was 5.72 and 4.75, respectively. These data were consistent with acid−base titrations which showed that protonation of the histidine conjugates were probably occurring in the pH range of 5.5−4.0. The decrease in imidazole p*K* upon conjugation to the polymer indicates that the quartenary amine on the His derivative significantly reduces protonation of the imidazole ring thereby resulting in an overall decrease in p*K* of the MBCs. These data suggest that release of polymer/pDNA complexes from endocytic vesicles probably occur from either the late endosomes or from the lysosomes. However, there is a general belief that the escape of complexes from the early endosomes results in an increase in gene expression, since this would limit exposure of the pDNA to digestive lysosomal enzymes. In contrast, Luby-Phelps and Lukacs et al.[46,47] have reported that complexes larger than 54 nm showed almost no mobility in the cytoplasm, unless they were actively transported by the microtubular and microfibril networks and that pDNA mobility appeared to be size-dependent. These observations according to Pack and Forrest[10] suggests that escape of polyplexes from the early endosomes that are "far" from the nucleus have a small probability of reaching the nuclear envelope whereas polyplexes escaping from the lysosomes into the cytosol will be much closer to the nuclear envelope and will therefore have a greater probability of reaching the nuclear envelope resulting in gene expression. Our data are consistent with this hypothesis in that even though the p*K* of the conjugated MBCs is relatively low, late proton buffering of the endocytic compartment still results in efficient gene expression.

In conclusion, these results suggest that biodegradable multiblock copolymers for gene delivery are efficient carriers that can increase the transfection efficiency without adversely affecting the cell viability. These polymers which exhibit steric stabilization properties due to the presence of the PEG blocks can be used for local as well as systemic delivery. In addition, the high molecular weight MBCs can result in a significant increase in $t_{1/2}$ of the biodegradable polymers because of the increase in crystallinity that should produce more effective transfection efficiencies. Furthermore, the conjugation of targeting moieties to these polymers can be used to increase the specificity of the polymer and in doing so reduce nonspecific uptake of the complexes.

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