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Screening Nylon-3 Polymers, a New Class of Cationic Amphiphiles, for siRNA Delivery

Venkatareddy Nadithe,[†] Runhui Liu,[‡,§] Bryan A. Killinger,[†] Sara Movassaghian,[†] Na Hyung Kim,[†] Anna B. Moszczynska,[†] Kristyn S. Masters,[§] Samuel H. Gellman,[‡] and Olivia M. Merkel*[,†,∥]

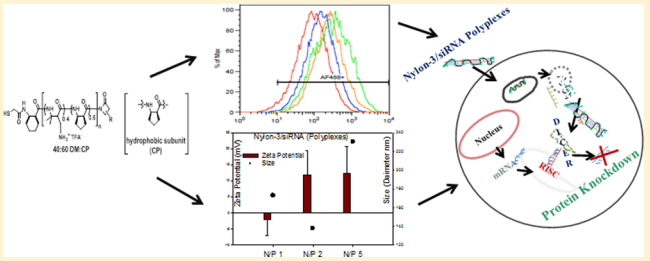
[†]Department of Pharmaceutical Sciences, Eugene Applebaum College of Pharmacy and Health Sciences, Wayne State University, Detroit, Michigan 48201, United States

[‡]Department of Chemistry, [§]Department of Biomedical Engineering, University of Wisconsin, Madison, Wisconsin 53706, United States

[∥]Molecular Therapeutics Program, Barbara Ann Karmanos Cancer Institute, Detroit, Michigan 48201, United States

Supporting Information

ABSTRACT: Amphiphilic nucleic acid carriers have attracted strong interest. Three groups of nylon-3 copolymers (poly-β-peptides) possessing different cationic/hydrophobic content were evaluated as siRNA delivery agents in this study. Their ability to condense siRNA was determined in SYBR Gold assays. Their cytotoxicity was tested by MTT assays, their efficiency of delivering Alexa Fluor-488-labeled siRNA intracellularly in the presence and absence of uptake inhibitors was assessed by flow cytometry, and their transfection efficacies were studied by luciferase knockdown in a cell line stably expressing luciferase (H1299/Luc). Endosomal release was determined by confocal laser scanning microscopy and colocalization with lysotracker. All polymers efficiently condensed siRNA at nitrogen-to-phosphate (N/P) ratios of 5 or lower, as reflected in hydrodynamic diameters smaller than that at N/P 1. Although several formulations had negative zeta potentials at N/P 1, G2C and G2D polyplexes yielded >80% uptake in H1299/Luc cells, as determined by flow cytometry. Luciferase knockdown (20–65%) was observed after transfection with polyplexes made of the high molecular weight polymers that were the most hydrophobic. The ability of nylon-3 polymers to deliver siRNA intracellularly even at negative zeta potential implies that they mediate transport across cell membranes based on their amphiphilicity. The cellular uptake route was determined to strongly depend on the presence of cholesterol in the cell membrane. These polymers are, therefore, very promising for siRNA delivery at reduced surface charge and toxicity. Our study identified nylon-3 formulations at low N/P ratios for effective gene knockdown, indicating that nylon-3 polymers are a new, promising type of gene delivery agent.



KEYWORDS: *nylon-3, siRNA, polyplexes, transfection, RNAi*

INTRODUCTION

Downregulation of gene expression levels by small interfering RNA (siRNA) offers a promising therapeutic approach for various disease conditions by impacting pathogenic mRNAs and promoting the molecular suppression of protein function.[1−4] The critical steps needed for clinical RNAi translation require appropriate packing and shielding of siRNA from various degradation mechanisms, protection from serum enzymes in the bloodstream and extracellular environment during transport across the epithelial cell barrier, delivery into specific cells, defense from endosomal degradation, and release of the siRNA for incorporation into the RNAi machinery to exhibit its mechanism of action.[5] A number of nonviral siRNA formulation approaches, such as liposomal, polymeric, and conjugation strategies (with or without particle-based approaches), have been extensively investigated.[6] The polycation polyethylenimine (PEI) is the most widely tested nonviral delivery system for nucleic acids; however, its high cellular toxicity and the limited available functionalization strategies that allow retention of the buffering capacity of the polymer limit its use as a nucleic acid carrier.[7] In spite of tremendous efforts made during the past decade with nonviral approaches to improve siRNA delivery into cells, tissues, and organs, the main hurdle of balancing toxicity and transfection efficiency still remains.[8] Here, we report a logically designed and synthesized cationic co-polymer-based polymeric delivery system with favorable hydrophilic−lipophilic-balanced properties and low toxicity for efficient siRNA delivery.[9]

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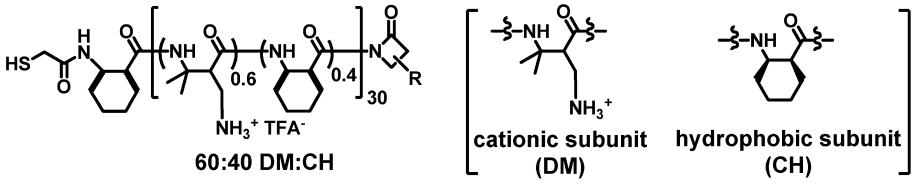
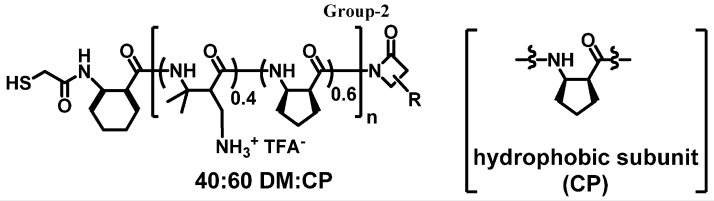
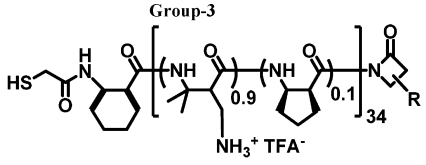
Table 1. Chemical and Physical Properties of Nylon-3 Polymers[a]

Polymer	Number average molecular weight (kDa)	Average degree of polymerization	Fraction of cationic Subunit (DM)	Fraction of hydrophobic subunit (CH or CP)	Average protonable units within one polymer chain
Group-1 					
G1-(6)	6	30	0.6	0.4	18
Group-2 					
G2A- (7.5)	7.5	44	0.4	0.6	18
G2B- (11)	11	66	0.4	0.6	26
G2C- (30	30	180	0.4	0.6	72
G2D- (44)	44	271	0.4	0.6	108
Group-3 					
G3-(7.5)	7.5	34	0.9	0.1	31

[a]All polymers are sequence-random and heterochiral. The R group at the C-terminus of all polymers represents the side chain of either subunit within the co-polymers.

Nylon-3 polymers (poly-β-peptides) have been designed and employed for use in various biological applications, such as biomaterials to promote cell adhesion,[10−13] mimics of host defense peptides possessing antimicrobial activity,[14−17] mimics of lung surfactant proteins,[18] and mimics of natural polysaccharides.[19] These copolymers are sequence-random and heterochiral in nature and synthesized via anionic ring-opening polymerization of β-lactams to incorporate both cationic and lipophilic subunits.[20,21] The physical properties of nylon-3 co-polymers can be tailored by altering the structure of the polymer subunits as well as the subunit ratio. For instance, polymer hydrophobicity can be altered by varying the ratio of cationic (hydrophilic) and hydrophobic subunits, whereas polymer length can be varied by tuning the ratio of co-initiator and total β-lactams or by functionalizing the nylon-3 chain's terminus.[21,22] Lipophilic character was introduced by hydrophobic cyclohexyl (CH) or cyclopentyl (CP) subunits to help mediate siRNA/polymer particle complex formation and cellular uptake. The resulting amphiphilic and biocompatible, protein-like nature of the nylon-3 polymer backbone have directed strong interest toward testing this new class of polycationic materials for nucleic acid delivery.

Here, these polymers are screened for siRNA delivery for the first time. Previously, various amine-containing polymers, such as poly-β-amino esters,[23] poly(L-lysine) (PLL),[24] polypropyleneimines,[25] polyamidoamine (PAMAM) dendrimers,[26] triazine-based dendrimers,[27] and polyethylenimine,[28] were tested but had limited success due to high toxicity and/or immunogenicity. Modifications to decrease the toxicity of these polycations have been accompanied by a loss or reduction in transfection ability. However, the nylon-3 polymer-based materials described here open a new synthetic approach for cationic polymers to incorporate favorable siRNA carrier/delivery characteristics such as solubility in water, positive charges for complexation with negatively charged siRNA at physiologic pH, amphiphilicity, appropriate polymer length, and desired terminal functional groups.

In this task of screening for potential siRNA delivery materials, we logically designed and synthesized heterochiral nylon-3 co-polymers for siRNA complexation and particle formation. Promising nylon-3 co-polymer-based polycations were screened for efficient siRNA delivery and selected for further investigation based on their physicochemical characteristics, such as particle size, surface charge, and buffering capacity. In vitro biological evaluation was performed with H1299/Luc cells to test cytotoxicity, polyplex uptake was quantified and the uptake route was assessed by flow cytometry, endosomal release was detected by confocal microscopy, and gene knockdown efficiency was assessed at the protein level using luciferase expression assays.

MATERIALS AND METHODS

Materials. Nylon-3 polymers were synthesized via anionic ring-opening polymerization (ROP) from racemic β-lactams and characterized as described previously.[11] Briefly, a mixture of β-lactam monomers and co-initiator (4-*tert*-butylbenzoyl chloride) in THF was treated with lithium bis(trimethylsilyl)-amide (LiHMDS), and the reaction was stirred at room temperature for 6 h. The reaction mixture was removed from the glovebox, and the reaction was quenched by addition of

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methanol. A white precipitate was collected after adding pentane to the reaction mixture and centrifugation. The collected white solid was subjected to another two cycles of dissolution/precipitation in THF/pentane to give the Boc-protected polymer that was characterized by gel permeation chromatography (GPC) using THF as the mobile phase. The Boc protecting groups were removed with trifluoroacetic acid (TFA) at room temperature to afford the deprotected polymer as an amine salt. Diethyl ether (Et_2O) was added to the reaction mixture to precipitate out a white solid that was collected after centrifugation. The collected white solid was subjected to another two cycles of dissolution/precipitation in MeOH/Et_2O to give the final deprotected polymer that was used in this study. Three different partially 2′-O-methylated siRNA duplexes used for polyplex preparation were bought from Integrated DNA Technologies Inc. (Coralville, IA, USA). Sequences of siLuc (specific, anti-luciferase sequence), control (nonspecific, negative control sequence), and fluorescently labeled siRNA (Alexa Fluor-488 siRNA and TYE-546-labeled siRNA) were synthesized as described before.[29] Luciferase assay reagent and cell culture lysis reagent (CCLR) were purchased from Promega (Madison, WI, USA). HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), D-glucose, sodium bicarbonate, and RPMI-1640 were purchased from Sigma-Aldrich (St. Louis, MO, USA). FBS (fetal bovine serum), trypsin-EDTA (ethylenediaminetetraacetic acid), and penicillin−streptomycin were purchased from Hyclone, Thermo Scientific (Waltham, MA, USA). MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), SYBR Gold dye, and Lipofectamine 2000 (LF) were bought from Life Technologies (Carlsbad, CA, USA), and polyethylenimine 25 kDa (PEI) was obtained from BASF (Ludwigshafen, Germany).

Polymer Characteristics. Synthesized polymers were broadly categorized into three different groups (G1−G3; Table 1) based on the fraction of cationic subunit and the ring size of the hydrophobic subunit (five- or six-member ring) within the polymer chains. In terms of cationic subunit content, the G3 group has the highest content (90%), followed by G1 (60%) and G2 (40%). The hydrophobic subunit varied between 10 and 60%, with the least hydrophobic content (10%) being in G3, followed by G1 (40%) and G2 (60%). While keeping the cationic and hydrophobic content constant, polymers with molecular weights varying between 7.5 and 44 kDa were synthesized within group G2.

N/P Ratio. The ratio of the number of nitrogens in the polymer to phosphates in siRNA (N/P) was calculated by using the information from Table 1 and the equation below. Both the specific anti-luciferase and the scrambled siRNA duplex used in these experiments comprise 52 nucleotides.

$$\text{N/P} = \text{amount of polymer (grams)} / (\text{amount of siRNA in moles} \times \text{number of nucleotides in siRNA duplex} \times \text{protonated unit mass in grams/mole})$$

Preparation of Polymer−siRNA Complexes (Polyplexes). The polymer and siRNA solutions were prepared with filtered 10 mM HEPES buffer (pH 7.2) (0.22 μm filter: Nalgene syringe filter, Sigma-Aldrich, St. Louis, MO, USA). Polyplexes (polymer/siRNA mixtures) at different N/P ratios were formed by pipetting and quickly mixing equal volumes of siRNA (50 μL) and polymer (50 μL) into a microcentrifuge tube. The polyplexes were then briefly vortexed for 30 s and incubated for 30 min at room temperature to equilibrate and permit stable complex formation. Polyplexes were freshly prepared for every experiment.

SYBR Gold Dye Condensation Assay. To test the siRNA condensation ability for various polymers at different N/P ratios (2, 5, 10, 15, and 20), a previously reported SYBR Gold assay was applied.[30] SYBR Gold dye was diluted to 4× in duplex buffer (100 mM potassium acetate and 30 mM HEPES in diethylpyrocarbonate (DEPC) treated water, pH 7.4). The polyplexes were prepared at different N/P ratios and pipetted into an opaque FluoroNunc 96-well plate (Nunc, Thermo Fisher Scientific, Waltham, MA, USA). Twenty five microliters of SYBR Gold solution was added to each well, and the plate was incubated for 10 min in the dark. Fluorescence caused by dye intercalation to siRNA duplexes and its quenching in polyplexes was measured as a relative fluorescence value using a fluorescence plate reader (BioTek Synergy 2, PerkinElmer, Waltham, MA, USA) at 495/537 nm excitation/emission. The percentage of uncomplexed siRNA was calculated by dividing the polyplex fluorescence by the fluorescence of free intercalated siRNA.

Cells and Cell Culture. Luciferase-expressing nonsmall cell lung cancer cells (H1299/Luc) were cultured in RPMI-1640 media supplemented with FBS (10%), HEPES (10 mM), sodium bicarbonate (1.5 g/L), D-glucose (4.5 g/L), sodium pyruvate (1 mM), and penicillin−streptomycin (1%). The cells were subcultured, maintained, and grown under humidified air with 5% CO_2 at 37 °C.

MTT Cell Viability Assay. To test the free polymer's cytotoxicity, experiments were conducted by adding different concentrations of the polymer solutions from every group to H1299/Luc cells plated 24 h prior to the treatment. Free polymer concentrations were made by directly dissolving the nylon-3 polymers or PEI 25 kDa, which served as a control, in complete RPMI-1640 media. Concentrations ranging from 1 to 20 μg/mL were evaluated. In comparison, the highest polymer concentrations used for the transfections ranged from 1 to 1.8 μg/mL for N/P ratio 2 and 40 pmol siRNA. H1299/Luc cells were seeded in 96-well plates at a density of 10 000 cells per well and incubated overnight with 5% CO_2 at 37 °C in a humidified atmosphere. After 24 h of incubation, the media was removed, and each well was rinsed with 200 μL of phosphate buffered saline (pH 7.4). Cells were incubated for another 3 h after adding 100 μL of MTT-containing media (0.5 mg/mL in RPMI-1640 media) to each well. After the media was completely removed, 200 μL of dimethyl sulfoxide (DMSO) was then added into each well to dissolve the purple formazan crystals formed by active mitochondria in the treated cells. The absorption at 570 nm was quantified using a microplate reader (BioTek Synergy 2). Results are shown as the average of triplicates ± standard deviation (mean value ± standard deviation) as the percent of viable cells relative to 100% viability, represented by cells cultured in the absence of polymers.

Size and Zeta Potential Measurements of Polyplexes. Size and zeta potential characterizations were performed using a Nanosizer Nano ZS (Malvern Instruments). Polyplex size was measured by transferring 100 μL of polyplexes containing 20 pmol of siRNA to a low volume UV cuvette (Brand GmbH, Germany). Following size measurements, zeta potential measurements were performed by diluting the same 100 μL of polyplexes to 750 μL with filtered 10 mM HEPES buffer

(pH 7.4) and transferring the dilution into a Zeta Cell (Zetasizer Nano series, Malvern, UK). Scattered light was detected at an angle of 173° in backscatter mode to minimize the noise in the measurements caused by any dust particles in the dispersing diluents. For size distributions, viscosity and refractive index values of distilled water at 25 °C were used as input parameters and calculated by a general purpose size algorithm. The zeta potential was calculated by the Smoluchowski approximation. Multimodal size analysis was performed on each sample measurement, consisting of 10 runs of 10 s each. Each measurement of zeta potential consists of at least 30 runs. Values given are the average of three separate experiments performed in triplicate ± standard deviation.

Flow Cytometric Quantification of Cellular Uptake of Polyplexes. The cellular uptake of the fluorescently labeled polyplexes (Alexa Fluor-488 siRNA) was measured using flow cytometry. The day before the transfection, 50 000 cells were seeded in 48-well plates and grown for 24 h. The cells were then transfected with polyplexes containing either 30 or 40 pm of siRNA. The final volume per well was 200 μL, and the siRNA concentration was 150 or 200 nM, respectively. After 5 h of transfection, cells were washed with phosphate buffered saline (PBS) and detached using 0.25% trypsin-EDTA (Hyclone Fisher Thermo Scientific, USA). Detached cells were washed twice with 1 mL of PBS by centrifuging (400 g for 5 min) and resuspending the cells. For flow cytometry analysis, the cells were resuspended in 400 μL of sterile PBS and transferred to BD Falcon FACS tubes (Fisher Scientific, USA). For each sample, 10 000 events were counted on a BD LSR II Analyzer (BD Biosciences, USA) with an excitation laser of 488 nm. The cellular uptake fluorescence intensity from each sample was obtained and was quantified as the average fluorescence intensity ± standard deviation of triplicate samples from the gated, viable cells.

Route of Cellular Uptake of Polyplexes. To study the route of polyplex uptake, 50 000 H1299/Luc-cells were incubated with different types of specific uptake inhibitors,[31] such as chlorpromazine (10 μg/mL), nystatin (10 μg/mL), wortmannin (12 ng/mL), and methyl-β-cyclodextrin (3 mg/mL), for 1 h followed by incubation with G2D polyplexes containing Alexa Fluor-488 siRNA for 5 h. As controls, cells were also transfected with Alexa Fluor-488 siRNA and LF or PEI 25 kDa, and untreated cells served as blank control. After the incubation period, the cells were either trypsinized (unquenched samples) or treated with a 0.4% trypan blue solution for 5 min (quenched samples) before they were trypsinized. Subsequently, the cells were washed and subjected to flow cytometric detection of siRNA uptake as described above.

Endosomal Entrapment. To determine if polyplexes of G2D experience endosomal entrapment after intracellular uptake or are released from the endolysosmal compartment, 30 000 H1299/Luc-cells were seeded per chamber on chamber slides with 8 chambers. The cells were transfected 24 h later with polyplexes prepared at N/P ratio 5 using G2D or PEI 25 kDa polymers and 20 pmol of TYE-546-labeled siRNA per chamber. The polyplexes were added to a total volume of 200 μL. As a positive control, the commercially available transfection reagent LF was used, and untreated cells served as a negative control. Cells were incubated for 4 h at 37 °C and 5% CO_2 before the endolysosomes were stained with 75 nM Lysotracker Green (Life Technologies, Grand Island, NY) for 1 h in the presence of the polyplexes. Subsequently, the cells were washed and fixed on the slides with a 4% paraformaldehyde solution. Samples were embedded with FluorSave (Calbiochem, Merck Millipore, Darmstadt, Germany). A Leica SPE laser scanning confocal microscope equipped with a spectral detector (Leica Microsystems, Wetzlar, Germany) was used for confocal microscopy. TYE-546-labeled siRNA was excited with a solid-state laser (Pavilion Integration Corporation, San Jose, CA) with an excitation wavelength of 532, and Lysotracker was excited with a sapphire solid-state laser (Coherent Inc., Santa Clara, CA) with an excitation of 488 nm. Fluorescence emission of TYE-546 was detected in channel 1 at 560 nm, and the emission of the Lysotracker was detected in channel 2 at 510 nm. All images shown were acquired using a 40× objective with a 0.75 numerical aperture (Leica Microsystems, Wetzlar, Germany).

Acid–Base Titration Curves. To determine the buffering ability of different polymers, titration curves were generated to measure the polymer's ability to be protonated over the pH range from 12 to 2 by titration with 0.1 N HCl. Each polymer (2.5 mg) was weighed and dissolved in 5 mL of 18.2 MΩ distilled water. The pH of the polymer-containing solution (0.5 mg/mL) was adjusted above 12 with 1 M NaOH and was allowed to stabilize for 5 min. Subsequently, it was titrated with standard 0.1 N HCl until the pH of the polymer solutions decreased to 2. The pH value was detected with a pH meter using an electrode at 25 °C (Accumet Basic, Fisher Scientific, USA). Polymers that show smaller changes in pH values during the HCl titration possess a greater buffering ability.

Transfection. Prior to transfection, H1299/Luc cells were seeded in 48-well plates at a density of 20 000 cells per well in 400 μL of medium and incubated for 24 h at 37 °C in humidified atmosphere with 5% CO_2. On the day of transfection, 100 μL of RPMI-1640 medium and 100 μL of polyplexes (N/P ratio 2) made of PEI 25 kDa or nylon-3-polymers with different amounts of siRNA (30 or 40 pmol) were added to each well. Lipofectamine lipoplexes served as a transfection control, and untreated cells (100% luciferase expression) were used for normalization of the results. After 5 h of incubation, the media containing polyplexes or lipoplexes was removed and replaced with 500 μL of fresh RPMI-1640 culture medium. The cells were grown for a total of 72 h prior to measuring luciferase expression. The medium was then discarded, and the cells were washed with 200 μL of PBS (pH 7.4). Cells were then lysed in 100 μL of CCLR for 10 min at 37 °C. Luciferase activity was quantified by injection of 50 μL of luciferase assay buffer, containing 10 mM luciferin, into each well of an opaque 96-well plate. The wells had been prefilled with 20 μL of cell lysate each. Relative light units (RLU) were measured with a plate luminometer (BioTek Synergy 2, VT, USA) to quantify the cellular luciferase activity. All experiments were performed in quadruplicate and were representative of three independent experiments. Results are given as mean values of replicates of four ± standard deviation. The transfection efficiency was expressed as percentage of knockdown compared with the expression level in nontransfected control cells.

Statistical Testing. Statistical testing between samples was performed via two-way ANOVA and Bonferroni post-test, assuming unknown variances with an alpha level of 0.05.

■ RESULTS AND DISCUSSION

A small set of nylon-3 polymers was designed, synthesized, and evaluated for siRNA delivery. The first critical step was to

evaluate their siRNA condensation efficiency. Condensation protects siRNA from degradation in the in vitro and extracellular environments. It was recently reported that polyplex stability depends on the molecular reorganization of these complexes based on their thermodynamic properties.[32] Therefore, the N/P ratios of all formulations tested here were optimized with regard to their physicochemical parameters and cellular uptake.

Condensation. A strong complexation of polymer with siRNA prevents siRNA release inside the cytoplasm and its incorporation into RISC complex, whereas a weak complexation prematurely releases siRNA, causing degradation before polyplex endocytosis.[33] The SYBR Gold assay showed that the tested nylon-3 polymers were able to condense siRNA effectively. Condensation was observed even at a low N/P ratio (2) and showed a strong ability of these polymers to interact with siRNA. Although the exact interaction mechanism between nylon-3 polymers and siRNA is unknown, we hypothesize that it is due to a combination of electrostatic and hydrophobic interactions resulting from their structural properties.[32] For pDNA, condensation was considered to be complete when 90% of the phosphodiester backbone charge was neutralized.[34] Since siRNA is a comparably short molecule, this threshold of 90% condensation was easily achieved in our studies with small amounts of cationic nylon-3 polymers. All of the polymers showed greater than 95% condensation at N/P ratios higher than 5 (Figure 1). This confirmed that only small

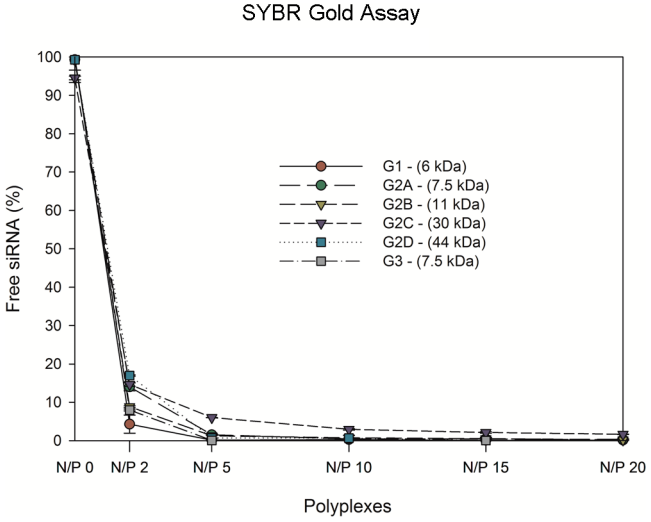

Figure 1. Condensation profiles of siRNA complexed at different N/P ratios using varied nylon-3 polymers with average molecular weights ranging from 6 to 44 kDa, as indicated.

amounts of polymers were needed to pack siRNA and minimize unwanted side/toxic effects resulting from higher amounts of cationic polymers in siRNA delivery.

There was no statistically significant difference among the overall condensation behavior of all polymers tested (p = 0.351); however, there was a significant decrease in fluorescence for all polymers at N/P ratio 2 compared to free siRNA and at N/P 5 compared to N/P 2 ($p < 0.001$). Even though increasing the molecular weight of polymers within group G2 (G2A to G2D) was expected to cause more efficient siRNA condensation at low N/P ratios, there were no significant differences (p = 0.529). These results demonstrate that the amount of polymer used to condense the siRNA (N/P ratio) had an effect, whereas the molecular weight has no significant influence on condensation.

Cell Viability. One major concern with cationic-based polymers is their toxicity. Drug and gene delivery systems need to possess no or minimal toxicity in vitro and in vivo to become candidates for clinical translation.[35] The cell viability profiles of the different nylon-3 polymers showed minimal toxicity (Figure 2). Cell viability was greater than 85% at polymer

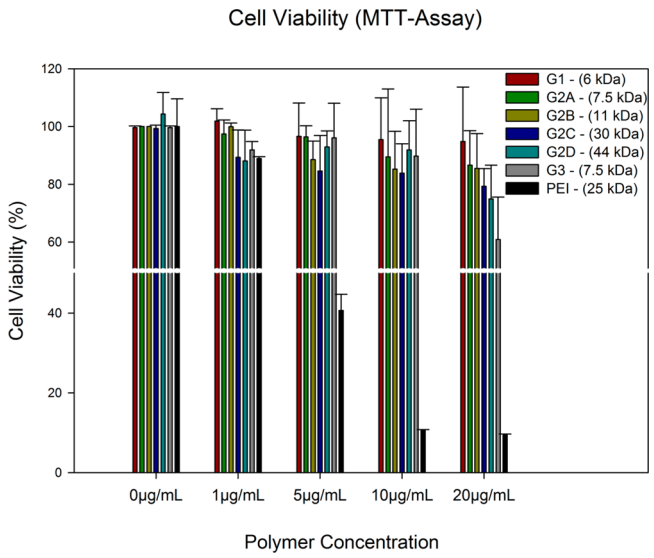

Figure 2. Cytotoxicity profiles of different nylon-3 polymers (average molecular weight ranging from 6 to 44 kDa) in comparison to PEI 25 kDa on H1299/Luc cells with increasing concentrations (0–20 μg/mL) after incubating for 24 h at 37 °C.

concentrations less than 10 μg/mL. This polymer concentration was much higher than the concentrations necessary for effective siRNA transfection, which ranged from 1 to 1.8 μg/mL for N/P ratio 2 and 40 pmol siRNA, depending on the polymer, and did not cause any pronounced cellular toxicity. The G1 polymer showed relatively low toxicity in comparison to that of polymers in the G2 or G3 groups. In general, increasing the molecular weight resulted in a trend of increasing toxicity for G2 polymers (G2D > G2C > G2B > G2A) at the highest concentration (20 μg/mL). This extrapolation implies that toxicity increases with an increase in the molecular weight among polymers with identical subunit composition. In contrast, the G3 polymer, despite having a lower molecular weight than G2B, G2C, and G2D, showed greater toxicity at 20 μg/mL, reflecting that toxicity also increases with increased cationic content (G3 vs G1 and G2A). Polycation-mediated toxicity was reported previously to arise from both loss of outer plasma membrane integrity and subcelluar mitochondrial membrane potential, leading to pore formation.[36] It is therefore possible that the toxicity of this type of polymer may be further reduced by optimizing the content of cationic subunits within the polymers chains, by using other conjugation strategies, or by introducing favorable structural groups for future siRNA delivery system design.[37] However, a critical balance should be considered between cationic charge density for siRNA complexation and a rational design that decreases polymer toxicity. While cationic charge mediates siRNA complexation, we show here that group 2 polymers efficiently deliver siRNA intracellularly at only 40% cationic subunits. We hypothesize, therefore, that polymers with an increased percentage of

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hydrophobic subunits and less than 40% cationic subunits may still be efficient and possess reduced toxicity.

Statistical analysis showed that there was not a significant difference in the toxicity profiles among polymers G1, G2A, G2B, and G2C at all concentrations tested ($p > 0.1$). However, polymers G2D and G3 showed a significant increase in toxicity at 20 μg/mL when compared to control cells cultured in the absence of polymers ($p = 0.003$ and 0.002, respectively). Even though high molecular weight polymer G2D seemed to cause higher toxicity at the highest concentration (20 μg/mL), statistical analysis showed that the G2 polymers (G2A, G2B, G2C, and G2D) were not significantly different in terms of their impact on cell viability ($p = 0.136$). PEI 25 kDa (control polymer) showed significant ($p < 0.001$) cellular toxicity (less than 50%) even at low concentrations (5 and 10 μg/mL) of the polymer. At the highest concentration tested (20 μg/mL), the cell viability decreased to less than 10%. Importantly, broadly used, high molecular weight PEI[38] was significantly more toxic ($p < 0.001$) at all tested concentrations than that of the nylon-3 polymers.

Particle Size. Appropriate particle size and surface charge favors the entry of siRNA into cells. It was reported that a high excess of cationic polymer (high N/P ratio), such as PEI in polyplexes, leads to high surface charges (+ 25 mV) and condenses nucleic acids into small particles.[39] It was also previously shown that endocytosis depends on size, with smaller size particles being taken up more easily by cells.[38,40−42] Particle size analysis of polyplexes showed that the size and polydispersity of the complexes depended on the N/P ratio (Figure 3). At an N/P ratio of 5, polymer G1 showed a smaller particle size (117 ± 16 nm) than that at an N/P ratio of 1 (180 ± 33). Among G2 polymers, polymer G2A with an N/P ratio of 5 showed a smaller size (120 ± 2 nm) with a lower PDI (0.15), indicating that slightly higher N/P ratios were required for smaller complexes, in contrast to 25 kDa branched PEI, which shows a minimum in hydrodynamic diameter at N/P 2.[32] The size distribution plots by scatter intensity can be found in the Supporting Information. There was also a clear decreasing size trend for the G2A polymer when the N/P ratio was increased from 1 to 2 and 5. Only in the case of G2A were the polyplex sizes significantly smaller at N/P 5 compared to those at N/P ratios 1 and 2 ($p < 0.001$). Polymers G2B, G2C, and G2D all formed relatively small complexes below 260 nm, which is a size range overlooked by phagocytosing macrophages.[43] Interestingly, increasing the molecular weight (G2B < G2C < G2D) or increasing the N/P ratio of these polyplexes did not have a significant effect on the hydrodynamic diameter. Additionally, there were no significant differences among polymers of different subunit composition but similarly low molecular weight (G1, G2A, and G3). Polymer G3 showed a particularly small particle size at N/P 2, which is comparable to the behavior of PEI 25 kDa.[32]

Whereas the G1 polymer formed the smallest polyplexes at N/P ratio 5, G2C polyplexes were the smallest at N/P ratio 2. Increasing the molecular weight in this category may help to further lower the size of the particles. Among the G2 polymers, increasing the molecular weight and varying the N/P ratio did not have a consistent or significant influence on particle size, however. Even though the N/P ratio is a critical parameter and is commonly increased to decrease polyplex sizes, it was recently described that there may be an optimal formulation at rather low N/P ratios which can yield small polyplexes. Increasing the N/P ratio beyond this optimal value was shown

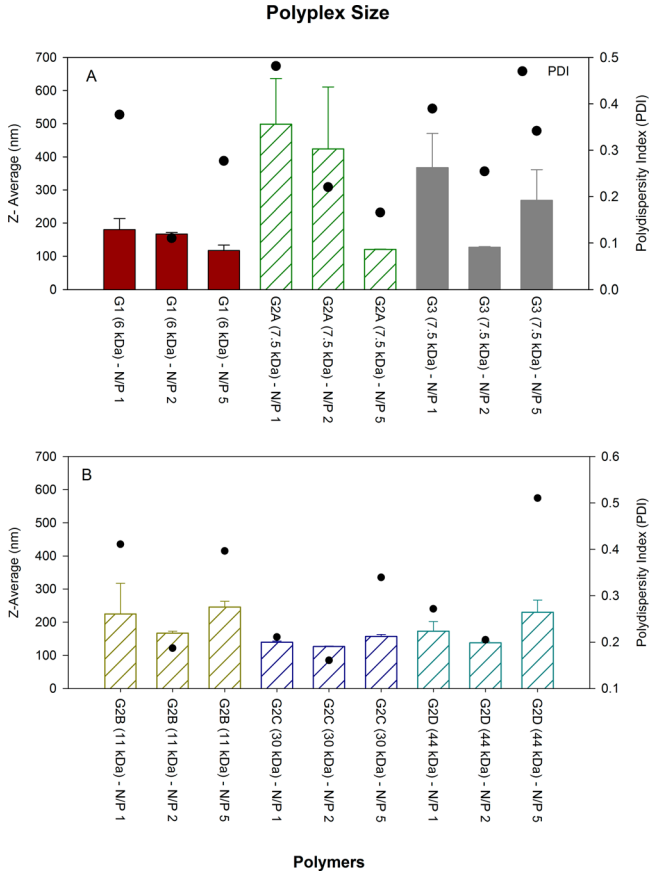

Figure 3. Polyplex (nylon-3/siRNA) particle size and polydispersity index (PDI) at different N/P ratios (1, 2, and 5) after incubation for 30 min at room temperature. (A) Polymers G1, G2A, and G3. (B) Polymers G2B, G2C, and G2D.

to cause larger hydrodynamic diameters and polydispersity indices (PDIs).[32] This finding is in-line with the particularly low size distributions (PDIs) observed for all nylon-3 formulations except G2A at N/P 2.

Zeta Potential. At an N/P ratio of 1 (irrespective of the group), all of the polyplexes showed negative zeta potentials (Figure 4). The polyplexes made from polymer G3 were the most negatively (−21 ± 10 mV) charged particles. Among the

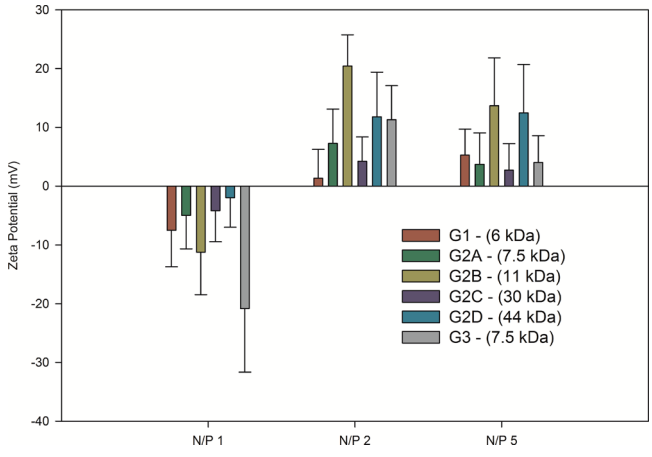

Figure 4. Zeta potential of nylon-3/siRNA polyplexes incubated for 30 min at room temperature at different N/P ratios (1, 2, and 5).

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G2 polymers, the negative charge seemed to show a decreasing trend as the molecular weight of the polymer increased (almost neutral for G2D). The repulsive forces between the negatively charged cell membrane and the negative particle are usually believed to counteract efficient cellular entry. Negative zeta potentials may indicate that the complexes were loosely condensed, causing larger particle sizes,[22] which is in-line with the hydrodynamic diameters found here for N/P 1. All of the polyplexes at N/P ratios of 2 and 5 showed a positive surface charge irrespective of the polymer composition. At N/P 2, the G1 polyplexes showed the least positive zeta potential (1.3 ± 5 mV), which increased (to 5.2 ± 4.4 mV) at N/P 5. The G2 (G2A, G2B, G2C, and G2D) and G3 polymers showed a slightly higher positive charge at N/P 2 compared to that at N/P 5, which is comparable with findings for PEI/siRNA complexes.[32] Statistical analysis showed that there were significant differences among the category of the polymers (G1, G2 or G3) in terms of changes in the zeta potential ($p < 0.298$). The differences were significant for the changes according to the N/P ratio ($p < 0.001$ between N/P 1 and 2 and $p < 0.001$ between N/P 1 and 5); however, the differences were insignificant between N/P ratios 2 and 5 ($p = 0.487$). Comparing polyplexes at N/P 1 and 2 indicated that the sizes for those formulations were similar; however, the surface charge reversed from negative to positive. Within each polymer, the N/P ratio therefore needed to be optimized for siRNA delivery to find the ideal particle characteristics, in terms of size and zeta potential. Particles with high zeta potentials were shown to have lesser particle aggregation and thus higher colloidal stability due to electrostatic repulsion between particles.[39] However, strongly positive surface charges may damage the plasma membrane, preventing recovery after transient transfection conditions (5 h) and thus may increase toxicity. With the current synthetic design of the nylon-3-based polymers, toxicity-decreasing subunits and functional groups can be easily incorporated even if formulations with higher zeta potentials are required to achieve efficient protein knockdown.[15,24]

Cell Uptake. In general, cellular uptake improved with increasing N/P ratios from 1 to 2 and 5 (Figure 5). The G1 polymer, at an N/P ratio of 1, showed an uptake of polyplexes into 22% of the cells treated, which increased to 86% at an N/P ratio of 5. The polyplexes derived from G2A and G2B polymers showed 27 and 14% cellular uptake at N/P ratio 1, which increased to 88 and 85% at N/P ratio 2, respectively. Even at a low N/P ratio of 1, in spite of negative zeta potentials, the high molecular weight polymers (G2C and G2D) showed greater than 80% cellular uptake of the polyplexes, which increased further to greater than 95% uptake at N/P ratio 5. In the case of the G3 polymer, cellular uptake was N/P ratio-dependent also. G3 polyplexes showed less than 2% uptake at N/P ratio 1, which significantly increased to 50% at N/P ratio 5. It has been long thought that increasing the surface charge of polyplexes and their efficacy of nucleic acid delivery were proportional and associated with the problem of increased cytotoxicity.[44] In contrast to this hypothesis, we observed efficient siRNA delivery at negative zeta potentials here. This may be attributed to the hydrophobic moieties of the nylon-3 polymers and is a very promising property of these formulations. These materials were previously shown to exhibit micelle-type structures.[45] This property of amphiphilic polymers has been reported previously to be an important parameter for the formulation of very efficient siRNA delivery systems.[29,46] These results also suggest that the cationic content was important for siRNA

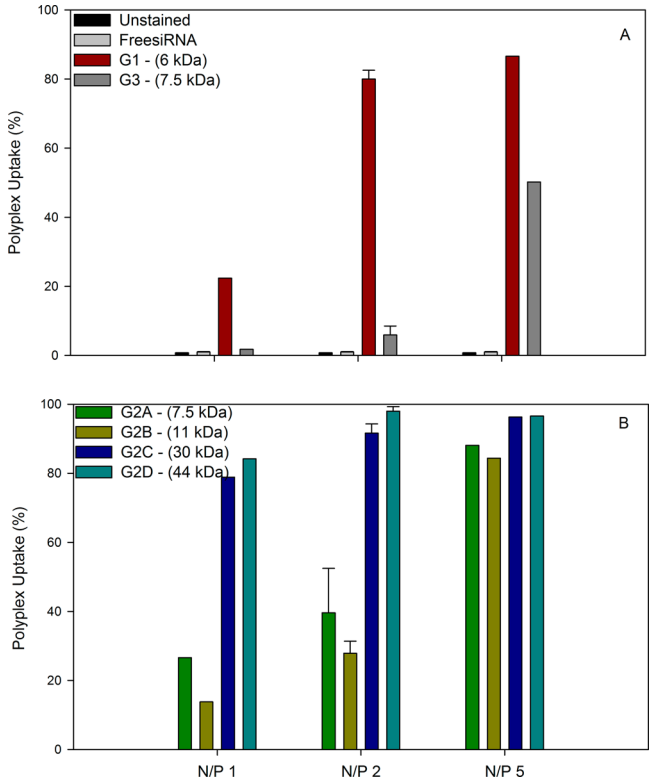

Figure 5. In vitro cellular uptake of Alexa Fluor-488-labeled siRNA polyplexes by H1299/Luc cells at different N/P ratios (1, 2, and 5) and 20 pmol of siRNA when incubated for 6 h at 37 °C. (A) Untreated cells, free siRNA, and polymers G1 and G3. (B) Polymers G2A, G2B, G2C, and G2D.

condensation, whereas the 5- or 6-member ring structure in the hydrophobic subunit may mediate interaction with the lipid bilayer of cell membranes and therefore is also an important feature in the design of nylon-3 polymer-based siRNA delivery systems. It is possible that the particle's surface charge as well as particle−cellular interactions, such as interaction with anionic cell-surface proteoglycans and hydrophobic interactions with lipid bilayers, plays an important role in polyplex uptake.[47−49] Interestingly, the smallest polyplexes obtained at N/P 2, which were formulated with G1, G2C, and G2D, were most efficient in terms of uptake in spite of their negative charge. It seems, therefore, that particles made of amphiphiles that are smaller than 200 nm can interact with cellular membranes and can be successfully taken up. In this context, it is surprising to find in our study that highly positive zeta potentials and the side effect of increased cytotoxicity are not prerequisites for efficient nonviral siRNA delivery with nylon-3 polymers. Also, it was confirmed that an optimization of the N/P ratio in terms of surface charge and cellular uptake was necessary and that this N/P ratio may be rather low.[32]

There was a statistical significance in cellular uptake of the polyplexes between N/P ratio 1 and 5 of G3 polyplexes ($p < 0.001$). Polyplexes made of G2C or G2D showed significantly increased uptake compared to that of G3 polyplexes at all N/P ratios ($p < 0.001$). These results indicate that the N/P ratio indeed plays a significant role in tuning the uptake of the polyplexes.

Also, the cellular uptake in terms of the scattered fluorescence intensity per cell was quantified by normalizing the peak heights to the percentage maximum on the *Y* axis

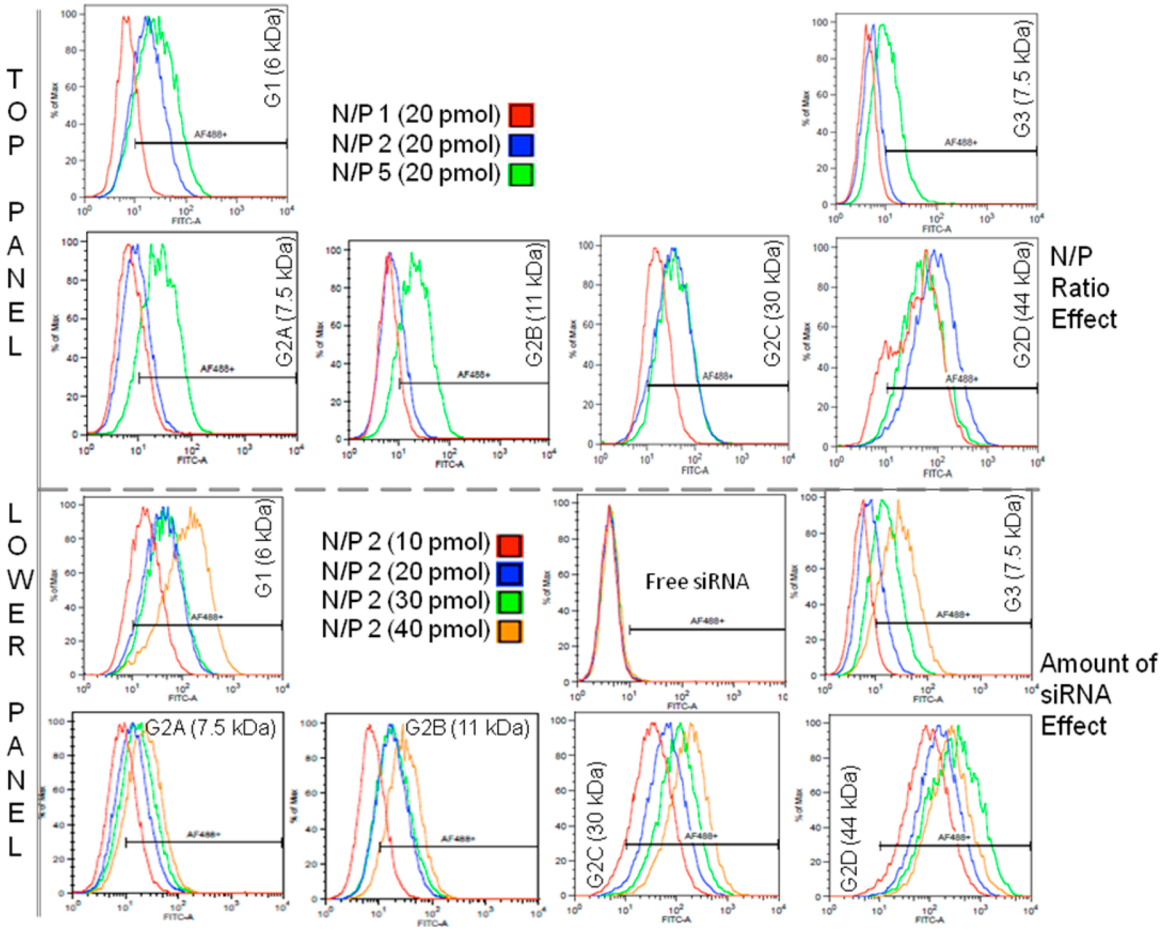

Figure 6. Forward scatter of Alexa Fluor-488-labeled siRNA polyplexes in H1299/Luc cells at different (top) N/P ratios (1, 2, and 5) and (lower) amounts of siRNA (10, 20, 30, and 40 pmol) when incubated for 6 h at 37 °C.

(Figure 6). In the top panel (N/P ratio effect), the overlaid histograms show that the higher intensity corresponds to higher amounts of siRNA uptake among G1, G3, G2A, and G2B when formulated at N/P ratio 5 compared to that at N/P ratios 1 and 2. The fluorescence intensity was already increased at N/P 2 compared to that at N/P 1 for G2C polyplexes, whereas no difference was observed between N/P 2 and 5. At all N/P ratios (1, 2, and 5), strong fluorescence intensity was measured for G2D polyplexes, indicating the profound effect of molecular weight for uptake efficiency. The lower panel shows cellular uptake at a constant N/P ratio of 2 and increasing amounts of siRNA (10−40 pmol per well), as indicated by fluorescence intensity. It was confirmed that free labeled siRNA had insignificant uptake, even with increasing amounts of siRNA (10−40 pmol). The fluorescence intensity or siRNA uptake increased with an increasing amount of siRNA (10−40 pmol) among all of the polyplexes (G1, G2A, G2B, G2C, G2D, and G3). This indicated that higher amounts of siRNA can help at any given N/P ratio to increase the amount of siRNA delivered to H1299/Luc cells. However, in the case of G2D polyplexes, the fluorescence intensity was highest at 30 pmol of siRNA and decreased at 40 pmol. This could be an indication of saturation of polyplex uptake for the very efficient formulation, G2D. In general, siRNA uptake increased with an increase in the N/P ratio and also, for a given N/P ratio, with an increasing amount of siRNA among these tested nylon-3 polymer/siRNA polyplexes. Comparing group 2 polymers, an increase of the molecular weight also caused an increase in uptake efficiency with regard to the percentage of positive cells (Figure 5) and the amount of siRNA delivered per cell (Figure 6).

Route of Cellular Uptake. Transfection efficiency may also depend on the endocytosis pathway of these polyplexes. Therefore, we evaluated whether the nylon-3 polyplexes were taken up in a manner similar to that other polyplexes, mainly caveolae-mediated endocytosis, or behave more like lipoplexes and followed a preference of clathrin-mediated endocytosis.[50−53] Cellular uptake expressed as median fluorescence intensity (MFI) in the presence of different inhibitors is shown in Figure 7. Our results show a higher MFI in cells incubated with G2D polyplexes when compared to that with PEI 25 kDa polymer. Chlorpromazine is known to inhibit the formation of new clathrin-coated pits by preventing clathrin protein recycling. Methyl-β-cyclodextrin inhibits cholesterol-dependent clathrin-mediated glycolipids, is involved in lipid raft depletion,[54] and also selectively inhibits caveolae-mediated endocytosis.[55] Since chlorpromazine and methyl-β-cyclodextrin had the strongest impact on cellular uptake of siRNA/G2D polyplexes and significantly ($p < 0.001$) decreased the MFI when compared to that of G2D polyplexes in the absence of inhibitors, our results show that G2D polyplexes are predominantly internalized via cholesterol-dependent clathrin-mediated endocytosis and only partially by macropinocytosis and caveolae-mediated endocytosis. The observed decrease in MFI in both quenched and unquenched cells ranged between 90 and 95% compared to that for G2D polyplexes. Wortmannin and nystatin, which inhibit cellular uptake by macropinocytosis

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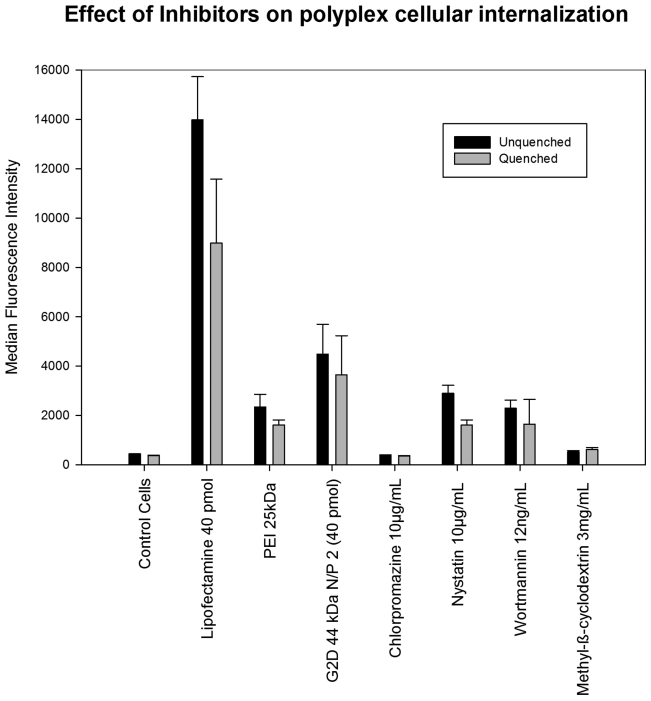

Figure 7. Cellular uptake of siRNA/nylon-3 polyplexes at N/P ratio 2 and 40 pmol in the presence of uptake inhibitors and in comparison to that of LF and PEI 25 kDa.

and by caveolae-mediated cellular uptake, only caused 30−50% decreases in cellular uptake in unquenched cells and 50−60% in quenched samples. These results show that chlorpromazine and methyl-β-cyclodextrin most strongly inhibited polyplex cellular uptake compared to wortmannin and nystatin, indicating that nylon-3 polyplexes are efficiently taken up by clathrin-mediated endocytosis. Additionally, lipid raft-mediated uptake may play an important role for their internalization, as suggested for siRNA lipoplexes previously.[56] It was previously shown that the cellular uptake route of nanoparticles depends on their size. Particles around 200 nm can be internalized via clathrin-mediated endocytosis, whereas 500 nm particles favor caveolae-mediated endocytosis.[57] On the basis of the size of G2D polyplexes, we hypothesize that the particles are in a suitable size range for clathrin-mediated endocytosis. It was also reported in the past that DOTAP lipoplexes are mainly taken up by clathrin-mediated endocytoses, whereas PEI polyplexes no longer show transfection efficacy if caveolae-mediated endocytosis is blocked.[50] Therefore, we conclude that the amphiphilic nylon-3 polyplexes share similarity with lioplexes with regard to their uptake route. Additionally, Lu et al. recently reported that their lipoplexes also were mainly taken up by clathrin-mediated endocytosis; however, depletion of cholesterol from the cell membrane prevented functional siRNA delivery. The authors therefore suggested that a fusogenic mechanism between lipoplexes and cell membranes was responsible for efficient siRNA-mediated gene knockdown after nonviral delivery.[56] Since there are also reports in the literature that explain how clathrin-mediated endocytosis can be affected by cholesterol depletion,[58,59] cholesterol depletion cannot generally be interpreted as inhibition of lipid rafts. However, a lipid-mediated or fusognic uptake mechanism as described for polyplexes seems to play an important role for amphiphilic polyplexes as well.

Endosomal Entrapment. The endolysosome can be a substantial barrier to efficient cytosolic siRNA delivery,[60] and cellular uptake of siRNA does not necessarily result in gene knockdown. For example, if siRNA is entrapped in the endolydodome after endocytosis, then it is not available for incorporation in the RNA-induced silencing complex (RISC), a necessary step for induction of the RNAi machinery.[61] To achieve efficient gene knockdown, endosomal escape is considered to be essential for most polyplex-based siRNA delivery systems. Cationic polymers with design features that incorporate better buffering moieties, such as histidine or imidazole, were shown to facilitate escape from the endosome.[41,62,63] It was also reported that unmodified low molecular weight PEI showed slightly lower buffering capacity in comparison to that of high molecular weight PEI.[64] This property, referred to as the proton sponge, has often been cited to explain the endosomal release of nucleic acids after transfection with PEI.[65] Therefore, we assessed uptake into endolysosomes and release from the latter in a colocalization experiment (shown in yellow) with fluorescent siRNA (shown in red) and Lysotracker (shown in green). All samples showed some extent of colocalization, which is interpreted as siRNA being present in endolysosomes. Figure 8A depicts the pattern

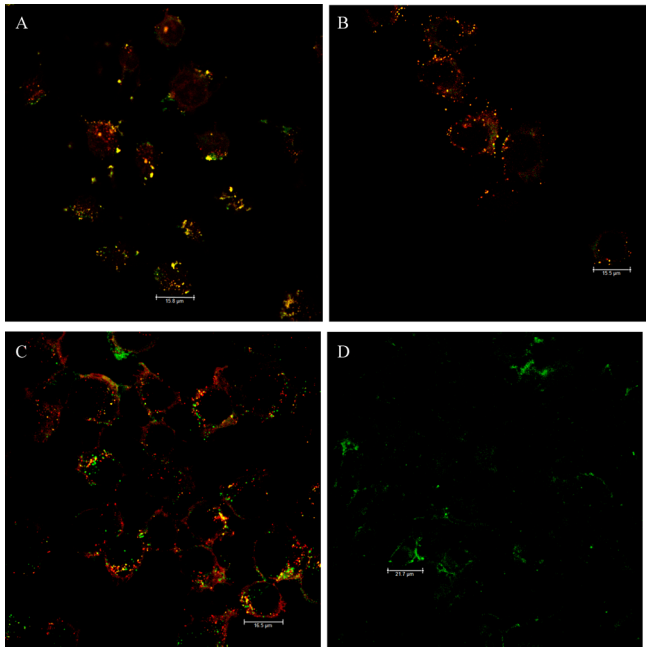

Figure 8. Confocal images of intracellular uptake of siRNA (red) mediated by (A) G2D, (B) LF, or (C) PEI in comparison to (D) cells treated with Lysotracker but without nanoparticles. Endosomal entrapment is shown by colocalization with Lysotracker (yellow). Empty endolysosomes are shown in green.

of intracellular uptake of siRNA delivered with polymer G2D and shows colocalization with endolysosomes but also shows free siRNA with an even distribution throughout the cytoplasm. The pattern of siRNA delivered with LF (Figure 8B) is comparable in the cytosolic distribution but shows smaller yellow dots, which depict siRNA in the endolysosomal compartment. Figure 8C clearly shows colocalization of siRNA delivered with PEI and Lysotracker, but it also shows a substantial amount of siRNA attached to the outer cell membrane that is not endocytosed yet. While the cells

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transfected with LF and G2D showed released siRNA in the cytoplasm that was evenly distributed, PEI, in contrast, is known to adhere strongly to the cell surface, which was also shown in the cell micrographs. It can be suggested from the results shown here that even the siRNA that is taken up by clathrin-mediated endocytosis is efficiently released to the cytoplasm. The proposed fusogenic properties of the nylon-3 polyplexes could have a beneficial effect. Additionally, lipid raft-mediated uptake, as suggested, would not deliver the siRNA to the endolysosome at all. All of these aspects explain the efficient cytoplasmic delivery of siRNA via G2D polyplexes.

Acid−Base Titration Curves of Nylon-3 Polymers. Another property of polycations that promotes endosomal release of nucleic acid cargo is their buffering capacity. PEI has been considered to be ideal cationic polymer in terms of optimal buffering capacity that aids in endosomal escape because of its high concentration of amine groups.[66] The G2C and G2D polymers showed a comparable proton buffering capacity as that of PEI within the experimental pH range (Figure 9). Polymers G2A and G2B showed the least buffering capacity among all polymers tested. The G1 and G3 polymers showed better buffering capacity compared to that of G2A and G2B but less compared to that of PEI at pH values above 7. Below pH 7, however, they behaved comparably to PEI.

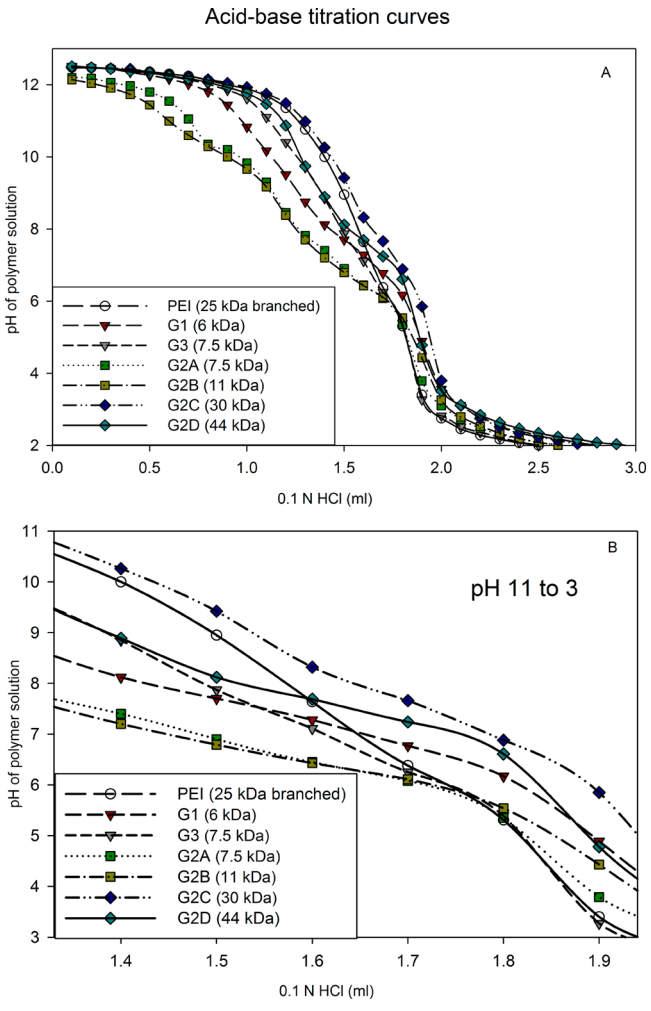

Figure 9. Buffering ability of different nylon-3 polymers when titrated with 0.1 N HCl and pH of the polymer solution measured at room temperature. (A) pH 12−2. (B) pH 11−3.

However, all of the polymers possessed buffering capacity within the experimental pH range, 5−8, most likely due to the amino groups in their structures. The buffering performance of the polymers decreased in the following order: G2C ∼ G2D > G1 ∼ G3 > G2A ∼ G2B. Often times, loss of buffering ability is observed after grafting chemical structures such as poly(ethylene glycol) (PEG) to cationic polymers in order to reduce their toxicity.[46,67] In our studies, with introduction of hydrophobic subunits, we did not generally observe a decreased buffering ability of polymers containing a low percentage of cationic monomers (group 2). Although polymers G2A and G2B had decreased buffering capacity, the increase of the molecular weight (polymers G2C and G2D) led to increased buffering ability. Buffering capacity may not exclusively mediate gene knockdown, but it triggers endosomal escape if polyplexes are taken up into the endolysosomal compartment. Interestingly, one of the polymers (G2D) that exhibited high buffering capacity also showed efficient transfection (Figure 10) efficiency.

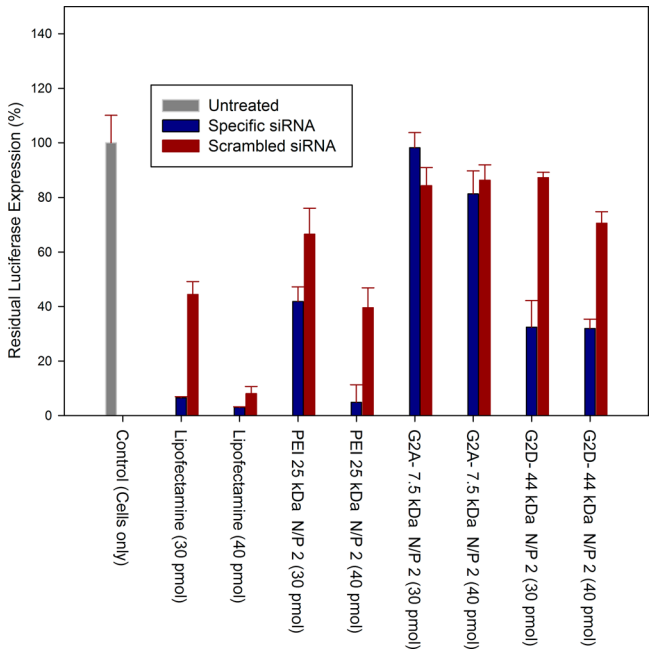

Figure 10. Luciferase protein knockdown in H1299/Luc cells 72 h after transfection with 30 and 40 pmol of siRNA/nylon-3 polyplexes at N/P ratio 2 in comparison to that with LF and PEI 25 kDa.

Luciferase Protein Knockdown. Once the polyplexes enter the cell cytoplasm and/or escape the endosome, the complexes need to dissociate, and the siNRA needs to be incorporated into RISC to degrade specific mRNA.[68] Even though polyplexes made of the low molecular weight polymers G1 and G3 showed cellular uptake (Figure 5), we did not observe any protein knockdown at the tested N/P ratios (data not shown). G1 polyplexes exhibited the most favorable particles sizes, which may explain their more efficient cellular delivery. G3 polyplexes, on the other hand, despite having a higher charge density within the polymer, were not efficiently taken up. The increase of the amount of siRNA only slightly increased the delivery with G3, whereas increasing the N/P ratio to 5 had a positive effect. However, further increasing the N/P ratio and thus the excess of polymer G3 may not be useful due to G3's most unfavorable toxicity profile (Figure 2). The

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low knockdown efficiency of G1, which demonstrated effective siRNA delivery, may be attributed to weaker or slower decomplexation kinetics in the intracellular environment.[69] It is possible that in the case of G1 polyplexes siRNA was not available for incorporation into RISC. Strategies such as introducing bioreducible polymeric groups in the nylon-3 structure may enhance the decomplexation ability and thus the transfection efficiency.[70,71] The ability of selected (G2A and G2D) nylon-3/siRNA-based polyplexes to mediate luciferase protein knockdown in H1299/LUC cells as compared to its level in untreated cells is shown in Figure 10. Significant knockdown was observed only with high molecular weight amphiphilic polymer G2D, composed of 40% cationic subunits and 60% hydrophobic subunits. The lower molecular weight polymer G2A did not achieve any significant gene knockdown compared to nonspecific siRNA, even at high siRNA concentrations (30 and 40 pmol of siRNA in 200 μL). Cells treated with G2A polyplexes showed 98 ± 6 and 84 ± 7% residual luciferase expression after transfection with specific and nonspecific siRNA, respectively (at N/P 2 and 30 pmol), indicating that the slightly decreased protein expression was negligible and may not have been caused by an RNAi effect. Even at higher siRNA amounts (40 pmol), the remaining luciferase expression was 81 ± 8 and 86 ± 6% for specific and nonspecific siRNA, respectively, reflecting that increasing the amount of siRNA may have little effect on protein knockdown with this specific polymer/siRNA formulation. A similar trend was observed with groups 1 and 3 polymers (data not shown). Protein expression levels for LF-treated cells were found to be 6.5 ± 0.4 and 3 ± 0.2% after transfection with specific luciferase siRNA and 45 ± 5 and 8 ± 3% with nonspecific siRNA at 30 and 40 pmol of siRNA, respectively. Cells treated with PEI 25 kDa polyplexes at N/P 2 expressed a residual percentage of 42 ± 5 and 5 ± 6% after transfection with specific luciferase siRNA and 67 ± 10 and 40 ± 7% after transfection with nonspecific siRNA at 30 and 40 pmol of siRNA, respectively. Even though the knockdown efficiency seemed to be greater after transfection with LF and PEI, profound toxicity associated with these formulations was clearly observed in cells treated with nonspecific siRNA formulations. However, as the molecular weight within group 2 polymers increased to 44 kDa, protein knockdown was clearly observed with G2D polyplexes at N/P 2 with 30 and 40 pmol of siRNA per well. Residual protein expression was found to be 33 ± 10 and 32 ± 4% with specific luciferase siRNA (67 and 68% knockdown) and 87 ± 2 and 70 ± 4% with nonspecific siRNA at 30 and 40 pmol of siRNA, respectively. This indicates the potential of the G2D polymer for further development and modification for efficient siRNA delivery. The knockdown efficiency of the G2D polyplexes at N/P ratio 2, with 30 and 40 pmol of siRNA, was statistically significant when compared to control cells ($p < 0.001$).

Altogether, G2 polymers showed promise for efficient luciferase protein knockdown at comparably low toxicity. These results indicate the importance of structural features and appropriate molecular weights for nylon-3 polymers in siRNA delivery.

CONCLUSIONS

Many cationic polymers exhibit great potential for siRNA delivery. However, their clinical application is limited by high toxicity or relatively low transfection efficacy. Here, we showed the gene knockdown ability of cationic nylon-3 polymers with low toxicity and at a low N/P ratio. These polymers are especially promising for pulmonary nucleic acid delivery as they can mimic lung surfactant proteins.[18] The simple synthesis and structure versatility of nylon-3 polymers enable further introduction of various chemical functionalities and tuning of multiple chemical and physical properties to circumvent various barriers for siRNA delivery in the future development of nonviral vectors. Bioactivity-enhancing chemical properties, such as molecular weight, charge density, hydrophilicity/hydrophobicity, buffering capacity, which accounts for endosomal escape, complex stability, and bioreducibility, can be further tailored to optimize this new class of nylon-3-based nonviral vectors for siRNA delivery. Additionally, introduction of fluorescent labels for tracing[45] and ligands for receptor targeting can be accomplished easily by functionalization of the C-terminus of nylon-3 polymers. Future studies on the mechanistic elucidation of gene knockdown will also help to better understand and design nylon-3 polymers for use as promising siRNA delivery vectors.

ASSOCIATED CONTENT

Supporting Information

Size distribution plots of all polyplexes at N/P 2. This material is available free of charge via the Internet at http://pubs.acs.org.

AUTHOR INFORMATION

Corresponding Author

*Phone: 1-313-577-1523. Fax: 1-313-577-2033. E-mail: olivia.merkel@wayne.edu.

Notes

The authors declare no competing financial interest.

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