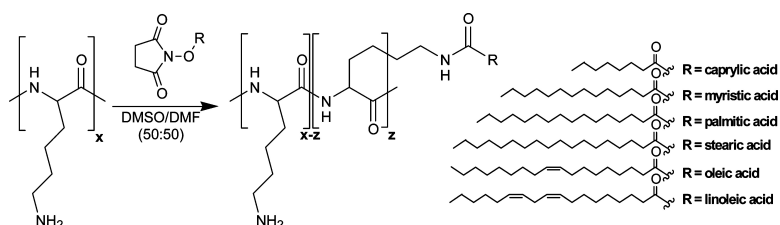Research Article

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Relationship between the Extent of Lipid Substitution on Poly(L-lysine) and the DNA Delivery Efficiency

Vanessa Incani,[†] Xiaoyue Lin,[‡] Afsaneh Lavasanifar,[†] and Hasan Uludağ*[,†,‡]

Faculty of Pharmacy and Pharmaceutical Sciences, University of Alberta, Edmonton, Alberta, Canada T6G 2N8, and Department of Chemical and Material Engineering, Faculty of Engineering, University of Alberta, Edmonton, Alberta, Canada T6G 2G6

ABSTRACT Poly(L-lysine) (PLL) is a commonly used polymer for nonviral gene delivery. However, the polymer exhibits significant toxicity and is not very effective for transgene expression. To enhance the gene delivery efficiency of the polymer, we imparted an amphiphilic property to PLL by substituting ~10% of ε-NH$_2$ with several endogenous lipids of variable chain lengths (lipid carbon chain ranging from 8 to 18). Lipid-modified PLL with high molecular weight (~25 vs 4 kDa) was found to be more effective in delivering plasmid DNA intracellularly in clinically relevant bone marrow stromal cells (BMSC). For lipid-substituted 25 kDa PLL, a correlation between the extent of lipid substitution and the plasmid DNA delivery efficiency was obtained. Additionally, transgene expression by BMSC significantly increased (20–25%) when amphiphilic PLLs were used for plasmid delivery as compared to native PLL and the commercial transfection agent Lipofectamine-2000. The transfection efficiency of the polymers was positively correlated with the extent of lipid substitution. The amphiphilic polymers were able to modify the cells up to 7 days after transfection, after which the expression was decreased to background levels within 1 week. We conclude that lipid-substituted PLL can be used effectively as a nonviral carrier for DNA, and the extent of lipid substitution was an important determinant of gene delivery.

KEYWORDS: bone marrow stromal cells • gene therapy • lipid • lipopolymer • nonviral • polycation

INTRODUCTION

Effective delivery of exogenous DNA into clinically relevant human cells, such as bone marrow stromal cells (BMSCs), is a major challenge in current gene therapy protocols. Viral and nonviral carriers are being pursued for this purpose. Because of the inherent mechanisms evolved to transfect mammalian cells, viral carriers have displayed more effective transgene delivery. Their clinical application, however, has been limited because of significant safety concerns, such as the host immune response mounted against them and the possibility of oncogenic transformations on target cells (1–4). On the other hand, nonviral carriers, in particular cationic polymers, offer several advantages including low immunogenicity, the capacity to deliver large DNA payloads, stability, and ease in scale-up. Cationic polymers facilitate the passage of DNA molecules through a cell membrane after condensing the DNA molecules into nanoparticles via cooperative electrostatic interactions. Poly(L-lysine) (PLL) and poly(ethylenimine) (PEI) have been initially used for DNA delivery (5, 6). Their general efficacy and utility, however, have been hampered by their relatively high toxicity (in the case of PEI) and low transfection efficiency (in the case of PLL), when compared to the viral carriers.

Because significant components of cellular membranes are lipid-derived, one approach to improving the performance of polymer-based nonviral carriers is to incorporate hydrophobic moieties into the carriers (7–11). These moieties are expected to function as membrane-anchoring moieties, enhancing the membrane compatibility of the polymeric carriers and facilitating the membrane crossing of genetic cargo into the cells (12). On the basis of this expectation, we recently designed (13) an amphiphilic lipopolymer by substituting the endogenous palmitic acid for PLL. Unlike unnatural hydrophobic molecules, endogenous lipids were preferred because they might ultimately be metabolized by mechanisms inherent in mammalian cells. The developed lipopolymer was shown to be biocompatible with the highly sensitive BMSC (13) and to be as effective as PEI, a relatively effective but toxic polymeric gene carrier. It is not known if palmitic acid was the ideal lipid for PLL substitution. This study was accordingly designed to explore the feasibility of substituting PLL with several endogenous lipids of variable chain lengths, ranging from 8 to 18 lipid carbon chains. It was our intent to identify most suitable lipid(s), if any, for gene delivery. A relationship between the gene delivery efficiency and the extent of lipid substitution on the carriers was additionally investigated. Our results did not identify a unique lipid for most effective DNA delivery but rather indicated a general relationship between the extent of lipid substitution and the intracellular plasmid delivery and transgene expression in BMSC.

EXPERIMENTAL SECTION

Materials. Poly(L-lysine) hydrobromides (PLL-HBr; 25 500 and 4000 Da), triethylamine, anhydrous dimethyl sulfoxide (DMSO), anhydrous N,N-dimethylformamide, caprylic acid (C8; 98 %), myristic acid (C14; 99−100 %), palmitic acid N-hydroxysuccinimide ester (C16; 98 %), stearic acid (C18; 95 %), oleic acid (C18:1 9Z; 99 %), linoleic acid (C18:2 9Z,12Z; 99 %), N-hydroxysuccinimide (NHS) ester, dicyclohexylurea (DCC), Hanks' Balanced Salt Solution (HBSS), 5 % (w/v) 2,4,6-trinitrobenzosulfonic acid (TNBS), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and trypsin/ethylenediaminetetraacetic acid were purchased from Sigma (St. Louis, MO). Deuterated chloroform (CDCl₃) and water (D₂O) used as ^{1}H NMR solvents were purchased from Cambridge Isotope Laboratories (Andover, MA). Anhydrous ethyl ether and dry ethyl acetate were purchased from Fisher Scientific (Fairlawn, NJ). Dulbecco's Modified Eagle Medium (DMEM; high glucose with L-glutamine), penicillin (10 000 U/mL), streptomycin (10 000 μg/mL), and Lipofectamine-2000 were purchased from Invitrogen (Carlsbad, CA). Fetal bovine serum (FBS) was purchased from Atlanta Biologicals (Lawrenceville, GA). A succinimide ester of Cy5.5 (Cy5.5-NHS) used for labeling of plasmid DNA was purchased from Amersham (St. Laurence, Quebec, Canada). A 4.7 kb plasmid incorporating an enhanced green fluorescent protein (pEGFP-N₂) and a kanamycin resistance gene was obtained from BD Biosciences. The plasmid was replicated in a kanamycin-resistant DH5-α *Escherichia coli* strain grown in a Luria-Bertani medium. After purification with a Qiagen Plasmid Giga Kit (Mississauga, Ontario, Canada), the plasmid concentration and purity were determined by UV spectroscopy. The plasmid was dissolved in ddH₂O at 0.4 mg/mL for use in this study.

Synthesis and Characterization of the Lipopolymers. The lipopolymers were synthesized by N-acylation of PLLs with NHS esters of the chosen lipid. The NHS esters (NHS-X, where X = caprylic, myristic, palmitic, stearic, oleic, and linoleic acid) were synthesized in-house by mixing equal molar amounts of lipids, NHS, and DCC in dry ethyl acetate. Briefly, the desired amounts of lipids and NHS were dissolved in 3 mL of ethyl acetate, and then DCC in 1 mL of ethyl acetate was added dropwise to the lipid/NHS mixtures. The reaction was allowed to proceed overnight at room temperature under N₂, the urea byproduct was then filtered, and the remaining NHS esters were concentrated with a rotary evaporator. The desired products were recrystallized from absolute ethanol at 4 °C, and the purity of the obtained product was confirmed by thin-later chromatography (14).

The lipid/lysine ratios during the PLL substitution reactions were 0.16 and 0.66, and experimental details were according to a published method from our laboratory (13). The lipid substitution ratios were determined by ^{1}H NMR (Bruker 300 AM) after dissolution of the polymers in D₂O and using the characteristic hydrogen shift of the lipids ($\delta \sim$0.8 ppm; −CH₃) and PLL ($\delta \sim$4.3 ppm; −NHCHCO). Where indicated, the lipid methylene content was determined by multiplying the number of lipids substituted on the polymer (value determined by ^{1}H NMR) and the number of carbons present in each lipid chain. Further characterization of the modified PLL was attempted by mass spectroscopy, but this was impeded by aggregate formation of the polymers. After dissolution of the polymers in H₂O, TNBS assay (15) was used to determine the free amine content of the polymers for subsequent studies.

BMSC Isolation and Expansion. BMSCs were isolated from the femurs of Sprague−Dawley rats as described in ref 16. The cells were suspended in a basic medium (DMEM supplemented with 10 % FBS, 50 mg/mL of ascorbic acid, 100 U/mL of penicillin, and 100 μg/mL of streptomycin), seeded in a single 75 cm² flask (Falcon) for initial expansion, and incubated in a humidified atmosphere of 95/5 % air/CO₂ at 37 °C. After a medium change on day 3, the cells were either expanded on 75 cm² flasks (1:4 dilution) or seeded in multiwell plates (6-, 12-, or 24-well plates from Corning) for the specific studies conducted (see figure legends for specific configurations). The cells from passages 2−4 were only used for this study.

Cellular Uptake of Plasmid DNA. pEGFP-N₂ was labeled with Cy5.5-NHS as previously described (13). For the uptake study, BMSCs were seeded in 12-well plates and allowed to attach for 24 h before the uptake study. The labeled DNA was complexed with the desired polymer concentrations in the presence of 150 mM NaCl. After 30 min of incubation, the polyplexes were added to BMSC grown in DMEM with (10 %) or without (0 %) FBS. The final concentration of plasmid was 1.2 μg/mL, and the polymers were 10, 3, and 1 μg/mL. After incubation for 24 h at 37 °C, the cells were washed with HBSS and trypsinized, and cellular uptake was detected by BD Dickenson FAIScalibur flow cytometry (FL4 channel, 635 nm laser, 5000 events/sample). The flow cytometer was calibrated for each run to obtain a background level of ∼1 % for control samples (i.e., untreated cells).

Transfection Studies. The polymer/pEGFP polyplexes were prepared by mixing the desired concentration of pEGFP and polymers in 150 mM NaCl. After 30 min, the polyplexes were added to BMSC in 6-well plates containing 1 and 10 % FBS to give a final concentration of 1 μg/mL plasmid and 10 and 3 μg/mL polymers or Lipofectamine-2000. After 24 h at 37 °C, either the cells were trypsinized for assessment of the EGFP expression by BD Dickenson FAIScalibur flow cytometry (FL1 channel, 488 nm argon laser, 5000 events/sample) or the medium was replaced with a fresh basic medium (DMEM with 10 % FBS) for longer time cultures. The flow cytometer was calibrated for each run to obtain a background level of the EGFP expression of ∼1 % for control samples (i.e., untreated cells).

Cytotoxicity. Evaluation of the cell viability was performed by MTT assay. BMSCs were seeded on 48-well flat-bottomed microplates. The cells were incubated with 20 μL of the desired polymer concentration and 500 μL of a basic medium (10 % FBS) for 22 h at 37 °C. The cell culture medium was then replaced (400 μL), and 100 μL of MTT (5 mg/mL) was added per well. After 2 h, the medium was removed, 500 μL of DMSO was added to each well, and the plate was incubated in the dark for 5 min at room temperature so the crystals formed can be dissolved. The absorbance was read at 570 nm using a microplate reader. The negative control was assigned to 100 % cell viability, and the remaining samples were normalized to this value (17).

Data Analysis. Where indicated, all results are summarized as mean ± standard deviation of the indicated number of replicates. Variations between the group means were analyzed by the Student's t test ($p < 0.05$). The significance ($p < 0.05$) of correlations between the variables was tested by calculating the Pearson product−moment correlation coefficient ($\mathbf{r}$) for sample size N and relating the correlation coefficient $\mathbf{r}$ to the t distribution by $t = \mathbf{r}/[(1 - \mathbf{r}^2)/(N - 2)]^{1/2}$.

RESULTS

Characterization of Lipopolymers. Cationic lipopolymers were synthesized by modifying low (4 kDa) and high (25 kDa) molecular weight (MW) PLLs with NHS esters of several lipids (Scheme 1).

The average degrees of substitution (^{1}H NMR) obtained on PLLs after the reaction are summarized in Table 1 and Figure 1. With 4 kDa PLL, the number of lipids per PLL ranged from 0.9 to 4.1, whereas with 25 kDa PLL, the number of lipids per PLL ranged from 2.3 to 10.0. No obvious correlations were evident between the chain length of the lipid and the number of lipids substituted per PLL. A higher lipid substitution was expected at higher lipid/lysine

Scheme 1. Synthesis of Lipid-Substituted PLL by N-Acylation with NHS-Activated Lipids[a]
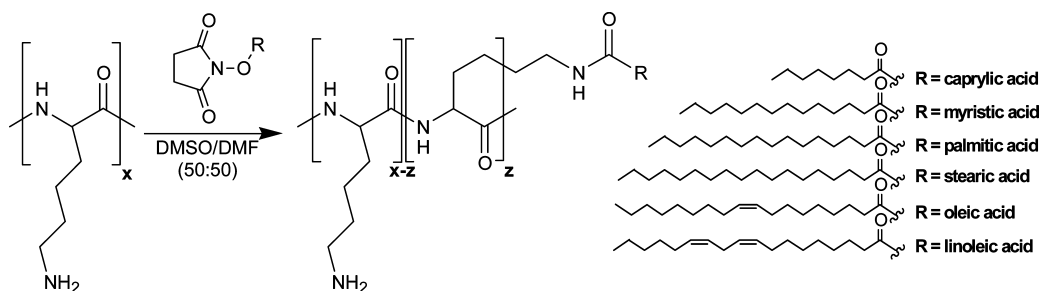
[a] R = caprylic acid, myristic acid, palmitic acid, stearic acid, oleic acid, or linoleic acid.

Table 1. Characteristics of the Polymer Library Prepared from 25 and 4 kDa PLL at Lipid/Lysine Feed Ratios of 0.16 and 0.66

polymer MW [kDa]	lipid	no. of carbons in the lipid chain	feed ratios (lipid/lysine)	Z^b	lipid methylene content[c]
25	caprylic acid	8	0.16	3.9	31.2
			0.66	6.9	55.2
25	myristic acid	14	0.16	8.7	121.8
			0.66	9.9	138.6
25	palmitic acid	16	0.16	7.4	118.4
			0.66	9.8	156.8
25	stearic acid	18	0.16	4.9	88.2
			0.66	10	180
25	oleic acid	18:1[a]	0.16	6.7	120.6
			0.66	6.2	111.6
25	linoleic acid	18:2[a]	0.16	2.3	41.4
			0.66	4.5	81
4	caprylic acid	8	0.16	3	24
			0.66	1.9	15.2
4	myristic acid	14	0.16	3.2	44.8
			0.66	3.6	50.4
4	palmitic acid	16	0.16	4.1	65.6
			0.66	3.8	60.8
4	stearic acid	18	0.16	2.4	43.2
			0.66	2.4	43.2
4	oleic acid	18:1[a]	0.16	2.3	41.4
			0.66	1.9	34.2
4	linoleic acid	18:2[a]	0.16	2.1	37.8
			0.66	0.9	16.2

[a] Number of carbons in the lipid chain:number of unsaturation.
[b] Substitution (number of lipids per PLL) determined by ^1H NMR.
[c] Lipid methylene content = Z × number of carbons in the lipid chain.

feed ratios; however, this was not the case for the lipopolymers prepared from the 4 kDa PLL (Figure 1). This expectation was realized for most lipids in the case of 25 kDa PLL, except oleic acid (C18:1 in Figure 1).

Because polymers with variable numbers of lipids/PLL were obtained, the extent of lipid substitution was expressed by calculating the lipid methylene content in each polymer (=number of lipids/PLL obtained from ^1H NMR multiplied by the number of carbons present in corresponding lipid chains; Table 1 and Figure 1B). Polymers substituted with myristic, palmitic, and stearic acids generally gave the highest lipid methylene content among the lipopolymers.

The ability of the synthesized polymers to condense DNA molecules was evaluated by agarose gel electrophoresis (13). By using pEGFP, all polymers were able to effectively bind and condense the plasmid DNA in a concentration-dependent manner (data not shown). The concentration for effective condensation, typically given by IC_{50} (i.e., polymer/plasmid mass ratio to obtain 50% complex formation as assessed by agarose gel electrophoresis) (13), ranged between 0.3 and 0.5, and no clear effect of lipid substitution was evident on this particular parameter.

The cytotoxicity of the polymers was evaluated on BMSCs at polymer concentrations of 10 and 3 μg/mL (Figure 2). These concentrations were chosen based on our previous studies that determined concentrations for an effective plasmid delivery. The results indicated that the cytotoxicity of the lipopolymers derived from 4 kDa PLL was negligible in the chosen concentration range (Figure 2A). Most lipopolymers derived from 25 kDa PLL did not display cytotoxicity as well, except two polymers obtained from myristic and palmitic acid substitution at a lipid/lysine ratio of 0.16 (Figure 2C).

Note that polymers derived from 4 kDa PLL did not display any toxicity. Two lipopolymers derived from 25 kDa PLL (myristic and palmitic acids derived from a lipid/lysine ratio of 0.16) displayed toxicity at 10 μg/mL, but there was no obvious correlation between the lipid methylene content and cytotoxicity. The MTT absorbance from untreated cells is indicated with a gray triangle (Figure 2C). It was not clear why these two polymers displayed a cytotoxic effect because (i) similar polymers with higher myristic and palmitic acid substitution (from a feed ratio of 0.6) did not give such a cytotoxicity and (ii) no clear relationship between the extent of the lipid methylene content and cytotoxicity was evident (Figure 2C). At this point, we are unable to account for the observed cytotoxicity.

Cellular Delivery of Plasmid DNA. All native and lipopolymers were complexed with Cy5.5-labeled pEGFP at polymer/pEGFP ratios of 0.83, 2.5, and 8.33, all in excess of IC_{50} values determined from gel electrophoresis. The final polymer concentrations in contact with cells were 1, 3, and 10 mg/mL, in line with our previous studies that identified concentrations necessary for effective plasmid delivery. The cellular delivery was assessed in a medium rich in FBS (10%) by using flow cytometry. With a relatively high concentration of polymers (10 μg/mL), the lipopolymers derived from 25
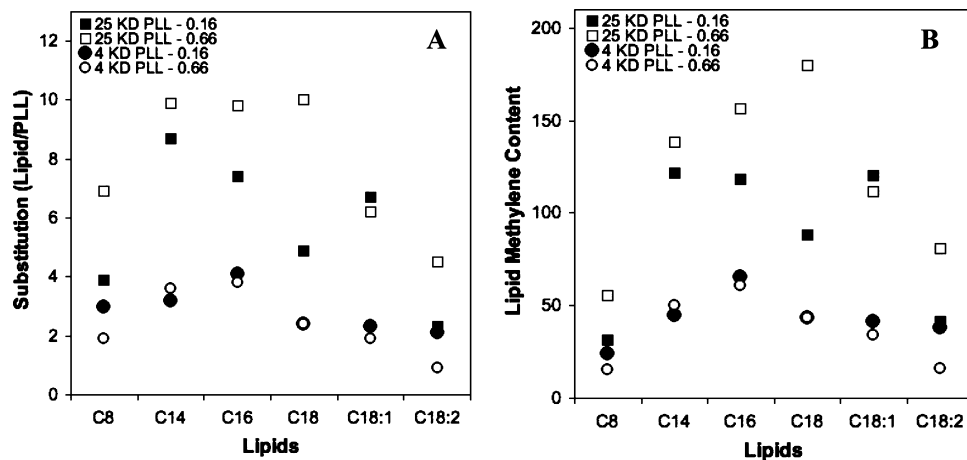
FIGURE 1. Number of lipids (A) and lipid methylene content (B) per PLL after substitution reactions. The data are summarized for PLLs of 25 kDa (squares) and 4 kDa (circles). The lipid/lysine feed ratio was either 0.16 (closed symbols) or 0.66 (open symbols). Note that the larger PLL had generally higher substitutions, but no clear relationship between the lipid chain length and the extent of substitution was evident.

kDa PLL gave significantly higher uptake as compared to the polymers derived from 4 kDa PLL: >70% of the cells displayed plasmid uptake in the case of 25 kDa polymers, whereas <30% of cells displayed plasmid uptake with smaller PLL (Figure 3A). Given this significant difference in plasmid delivery, we decided not to pursue smaller PLLs and to focus only on the polymers derived from 25 kDa PLL in further studies.

A subsequent plasmid delivery study was performed with 25 kDa PLL-derived lipopolymers at different polymer concentrations (10, 3, and 1 μg/mL). The results showed a positive correlation ($p < 0.002$, 0.05, and 0.008 for 10, 3, and 1 μg/mL, respectively) between the lipid methylene content and the plasmid delivery (Figure 3B). A 3–8-fold increase in plasmid delivery was obtained when compared to the unmodified 25 kDa PLL, which gave plasmid delivery to <10% of the cells under the experimental conditions. Polymers with the highest substitution of myristic, palmitic, and stearic acids (~10 lipids/PLL) gave the most effective pEGFP delivery. Incubating the cells with pEGFP in the absence of any carriers yielded <2% Cy5.5-positive cells for all experiments, indicating the percentage of cells capable of plasmid uptake without the need of a carrier.

Additionally, we studied the effect on pEGFP delivery in the presence and absence of serum proteins (i.e., with 0 and 10% FBS) in the cell culture medium. Polymers from the feed ratio of 0.66 and concentrations of 10 and 3 μg/mL were tested in this study. Serum proteins were found not to interfere with plasmid delivery by the lipopolymers (Figure 3C).

Transfection Efficiency. Transfection studies with lipopolymers were conducted along with the most commonly used commercial lipid formulation, Lipofectamine-2000. After 24 h of exposure of cells to complexes in a FBS-containing medium (10% FBS), we observed a correlation ($p < 0.01$ and 0.008 for 10 and 3 μg/mL 10% FBS, respectively, and $p < 0.002$ for 10 μg/mL 1% FBS) between the transfection efficiency and the extent of lipid methylene content at the two polymer concentrations tested (Figure 4A). As much as 20–25% of the cells were modified under

these conditions with the most substituted PLL (~10 myristic, palmitic, and stearic acids per PLL). Unmodified PLL gave ~4% modification under the same conditions. The transfection of the cells was also investigated in a medium with a reduced serum content (oMEM with 1% FBS). A correlation between the transfection efficiency and the lipid methylene content was again evident (not shown). The Lipofectamine-2000 formulation gave 2–3% transfection after 1 day of exposure (data not shown).

Using a select set of lipid-substituted polymers (polymers from a feed ratio of 0.66), the transfection efficiency was evaluated on days 7 and 14 (Figure 4B). The correlation ($p < 0.0002$ and 0.02 for days 7 and 14, respectively) between the extent of lipid substitution and the transfection efficiency was retained even after 7 days; however, a significant drop in the transfection efficiency was noted after 14 days. The EGFP expression with commonly used commercial transfection reagent Lipofectamine-2000 was ~1% on days 7 and 14 (data not shown).
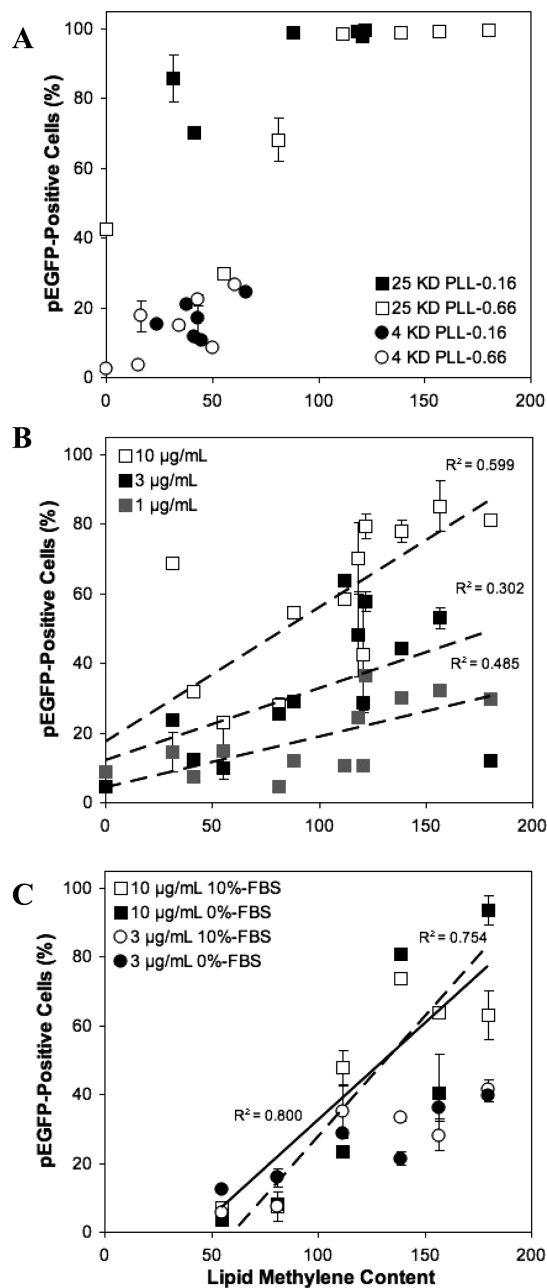
DISCUSSION

The development of effective nonviral gene delivery systems is crucial for successful gene therapy. To achieve this goal, we imparted lipophilic character to a relatively ineffective DNA carrier, PLL, by grafting hydrophobic moieties onto the polymer backbone, and evaluated its DNA delivery capability in BMSCs. Two different PLLs (4 and 25 kDa) were used to assess the influence of the polymer MW on DNA delivery (17–19). These PLL sizes were selected because they combine a relatively good toxicological profile with some membrane activity and efficient condensation of DNA into stable particles (19). Additionally, several endogenous lipids (myristic, palmitic, stearic, oleic, and linoleic acids) were chosen for PLL substitution to avoid unnatural moieties in the carriers (except caprylic acid). Because PLL enables plasmid DNA condensation via interaction of its primary amines with anionic phosphate groups of DNA, the reaction conditions were controlled to substitute <10% of the PLL lysine, leaving sufficient free ε-NH$_2$ to condense DNA while maintaining polymer aqueous solubility. By using two
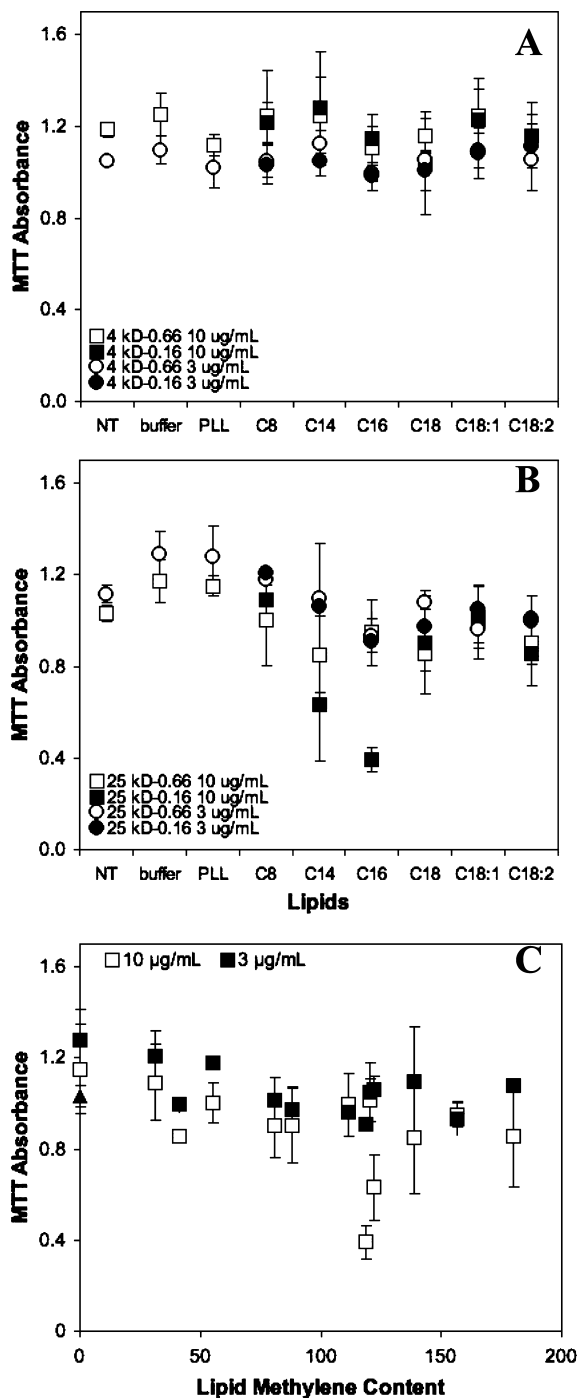
FIGURE 2. Cytotoxicity of polymers on BMSCs at polymer concentrations of 10 and 3 μg/mL. The results are summarized for polymers derived from 4 kDa PLL (A) and 25 kDa PLL (B) at lipid/lysine ratios of 0.16 and 0.66. (C) Cytotoxicity of polymers derived from 25 kDa as a function of the lipid methylene content.

FIGURE 3. pEGFP delivery to BMSCs as a function of the lipid methylene content of PLLs. The cellular uptake was assessed after 24 h of incubation of complexes with cells. (A) Comparison of polymers derived from 4 and 25 kDa PLL. (B) pEGFP delivery by lipid-substituted 25 kDa PLL at 10, 3, and 1 μg/mL (in DMEM with 10% FBS). (C) Effect of serum (0 vs 10% FBS in DMEM) on the pEGFP delivery to BMSCs by selected lipid-substituted PLL (polymer:pEGFP = 10:1.2 and 3:1.2 μg/mL; polymers from a feed ratio of 0.66). pEGFP without any carrier yielded ~2% pEGFP-positive cells for all experiments. Linear regressions in Figure 1C are for the high concentration of polymers.

lipid/lysine feed ratios (0.16 and 0.66), we were able to synthesize a series of modified polymers with different degrees of lipid substitution. The obtained lipopolymers did not always follow a predictable pattern of lipid substitution, and it is likely that improved reaction conditions need to be identified for better control of lipid substitution. Other solvents that can better solvate the reactants with widely differing properties (i.e., cationic polymer and hydrophobic lipids), or a scaled-up reaction (our current reaction called

for 25 mg of PLL, which is a relatively small amount), might be possible solutions. Because the resultant lipopolymers displayed a variable lipid substitution, we preferred to normalize the extent of lipid substitution based on the lipid methylene content in each polymer. In this way, we explored correlations between the DNA delivery efficiency and the extent of modifications based on a common variable (i.e., lipid methylene content).
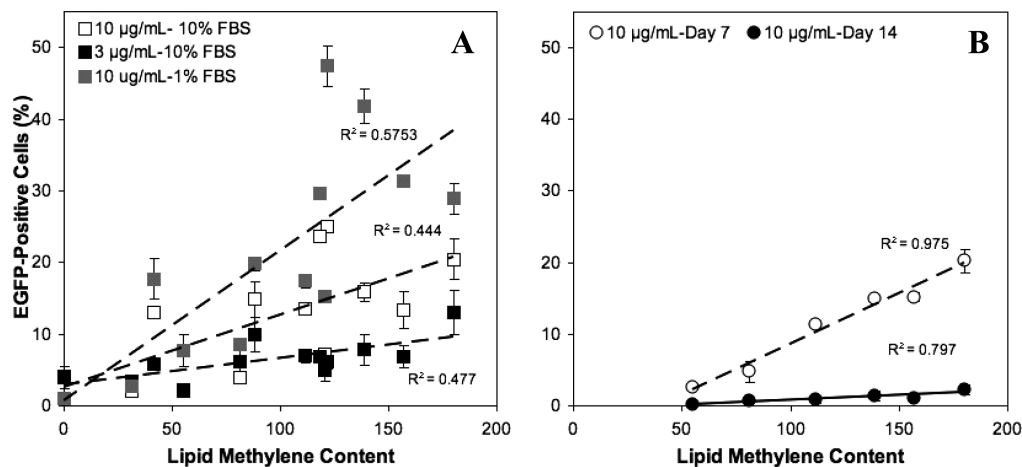
FIGURE 4. (A) Effect of the lipid methylene content of polymers on the EGFP expression on day 1. The plasmid concentration was 1 μg/mL, whereas the polymer concentrations were 3 and 10 μg/mL. (B) EGFP expression on days 7 and 14 at polymer/pEGFP concentrations of 10 and 1 μg/mL, respectively. Only polymers from a feed ratio of 0.66 were used for this study.

The condensation of DNA molecules for cellular internalization is a prerequisite for efficient gene delivery. In our case, all polymers were able to effectively bind and condense pEGFP even at the lowest polymer/pEGFP ratio used (1:1). Once plasmid DNA complexation was confirmed, the capability of lipid-substituted carriers for intracellular delivery of plasmid DNA was evaluated. Comparisons between carriers generated from PLLs of different MWs showed a greater uptake when high-MW lipopolymers were used. This difference was previously noted by others and was likely due to a higher density of ε-NH$_2$ present on high-MW PLL, increasing the number of DNA–polymer interactions necessary for a stable complex formation (19), which, in turn, increased the amount of genetic payload delivered through the plasma membrane. We also investigated the effect of the polymer concentration on cellular uptake with lipopolymers derived from 25 kDa PLL; the plasmid delivery into the cells was proportional to the polymer concentration and, more importantly, a correlation between the lipid methylene content and plasmid delivery was evident at all concentrations tested. This indicates that there is a greater complex–membrane interaction when hydrophobic moieties are involved in complex formation, particularly for polymers with the highest content and carbon lengths of 14–18 (~10 lipids/PLL), which gave the most effective pEGFP delivery. These findings are in agreement with our previous publication and other research groups that coupled PLL to hydrophobic agents to enhance cellular uptake and transcription (7, 9, 13). Although full pEGFP complexation was obtained even at the lowest polymer concentration (1 μg/mL) under our experiment conditions, an excess of polymer was still needed for more effective plasmid delivery because increasing the polymer concentration from 1 to 10 μg/mL continued to improve the intracellular delivery, whereas unmodified PLL yielded <10 % Cy5.5-positive cells under the experimental conditions.

Positively charged complexes are necessary for interaction with anionic cell surfaces. However, the cationic nature of the complexes may lead to additional unspecific interactions with negatively charged serum proteins. This could decrease cellular association with complexes, thus reducing or inhibiting endocytosis (19–21). Cationic liposomes, for example, may display low DNA delivery efficiency because of strong interactions with serum proteins. Avenues to overcome serum inhibition involve increasing the charge ratio of lipid to DNA and/or allowing sufficient maturation time for complexes before exposure to a serum-containing medium for transfection (22, 23). Interactions of poly(amino acid)-based complexes with a biological environment are often problematic, and PLL polyplexes are well-known to interact with body fluids and rapidly cleared from the systemic circulation (24, 25). Consequently, several strategies to address this problem have been used. One common strategy is based on the use of hydrophilic polymers, such as PEG (25–27) or HPMA (28), to create a hydrophilic coating that reduces interactions with serum proteins. More recently, Brown et al. (10) designed a neutral gene delivery system based on palmitoyl–PEG–PLL that contained cholesterol, and the authors showed that this system augmented the half-lives of the complexes in the bloodstream as compared to complexes formed with PLL alone. Kim et al. (7) also observed a beneficial effect of low-density lipoprotein (LDL) in complexes formed with hydrophobized PLL (stearyl-PLL), and this beneficial effect was lost in a serum-free medium. Given the importance of serum interactions, we investigated the effect of serum on pEGFP delivery. Our experiments showed no apparent interference of serum proteins in cellular delivery of DNA by the lipid-substituted polymers. As was the case in the presence of FBS, plasmid delivery was proportional to the lipid methylene content in the absence of FBS (Figure 3C). This result suggests that the lipid-substituted PLLs could efficiently deliver DNA without the need for further modification of the carrier and/or polyplexes (e.g., lipid helpers, lipoproteins, or hydrophilic coating).

Although the majority of the cells in the culture can internalize the plasmid/carrier complexes, only a small fraction of the cells actually express the transgene. Generally, after internalization of the complex, plasmid/carrier complexes are transported through the endosomal/lysosomal

pathway, leading to vesicle acidification to pH 5–6 by the action of an ATPase. A fraction of the complexes are degraded, but also some complexes are released from these compartments into the cytosol, so that it can be transported to the nucleus. In a previous publication (29), we were able to locate pEGFP/palmitic-PLL in the cytoplasm as well as in the nucleus on BMSCs after 24 h. This showed that the designed amphiphilic carriers not only protected the DNA from enzymatic degradation but also delivered the genetic payload into the nucleus. We expect that the use of other lipids on PLL, i.e., the ones described in this paper, might have a similar response. Because of the large numbers of carriers, however, this issue was not explored in this study, and additional studies are planned to explore the details of complex trafficking as a function of substituted lipid on the carriers.

Consistent with the cellular uptake results, yjr EGFP expression was proportional to the extent of lipid methylene content of the polymers. Lipid-substituted polymers with a lipid length between 16 and 18 gave the highest transfection levels; 20–25% of the cells were modified under these conditions, while native PLL only gave ∼4% and Lipofectamine-2000 gave 1–3% modification under the same conditions, indicating the significant potential of the designed polymers for plasmid delivery. Interestingly, cells under reduced serum conditions gave up to 2-fold higher extent of modification than the cells under normal serum conditions (10% FBS). Unlike plasmid internalization, it is possible that serum proteins might interfere with intracellular complex dissociation necessary for the EGFP expression, leading to a lower level of EGFP expression. Future studies are planned to explore the reasons of better gene expression under reduced serum conditions. The transgene expression was maintained up to 7 days, and correlations between the extent of lipid substitution and the transfection efficiency were observed during this time period. However, a significant drop in the transgene expression was noted after 14 days, which is consistent with the transient nature of nonviral delivery in general (30).

Minimal or no cytotoxicity of a synthetic carrier is an important requirement for gene delivery systems. The use of gene carries that mimic naturally occurring molecules (e.g., lipoproteins) could significantly reduce the toxicity of delivery systems. PLL has been known to be toxic on mammalian cells (31); however, attaching hydrophobic molecules to the polymeric backbone seems to decrease the cytotoxicity, when compared to unmodified polymers (13, 32, 33). The high positive charge density of gene carriers is generally considered to be the reason for cytotoxicity (31, 34), and shielding these charges with hydrophilic polymers or lipoproteins has been a productive strategy. Kim et al. (7) observed a reduction of the toxicity of stearyl-PLL when LDL was combined with this carrier, but the beneficial effect of stearic acid substitution, if any, was not studied in this polymer. Brown et al. (10) and Pan et al. (35) also used lipid-modified PLLs (palmitoyl-PLL-PEG and palmitoyl-PLL in a "nanoemulsion" formulation, respectively) and showed a

decrease of the toxicity as compared to unmodified PLL. The toxicity of these carriers without PEG grafting or without the nanoemulsion formulation was not evaluated, so that the benefit of lipid modification in reducing toxicity could not be assessed. In our study, the cytotoxicity of the polymers at concentrations effective for intracellular plasmid delivery to BMSCs was independent of the hydrophobic moiety used as well as, for the majority of the cases, to the extent of lipid substitution. These results suggest the possibility of improving plasmid delivery by the lipopolymers without adversely affecting the cellular physiology. We recognize that the cytotoxicity of polycationic gene carriers is dependent on the cell system chosen. For example, our studies (36) using a different clinically relevant cellular system, skin fibroblasts, showed some toxicity with the polymers, in particular for those that gave effective pEGFP delivery (e.g., myristic acid-substituted PLL). Similarly, Choksakulnimitr et al. (34) studied the degree of toxicity of polycationic carriers in brain microvessel endothelial cells, macrophages, and hepatocytes. They observed that macrophages had a higher sensitivity to polymers than the hepatocytes and endothelial cells. We focus on highly sensitive BMSCs because they are clinically useful, unlike other commonly used immortal cells. The BMSCs might be more tolerant to our gene carriers than other cell types (e.g., skin fibroblasts), and this observation highlights the importance of evaluating the toxicity of gene carriers in desired target cell phenotypes.

CONCLUSIONS

The results of this study indicated that several endogenous lipids are suitable for incorporation into PLL to serve as effective DNA carriers. Carriers with high MW and high lipid methylene content increased cellular uptake, generating significant gene expression with relatively low toxicity on clinically relevant BMSCs. Myristic, palmitic, and stearic acid-substituted polymers gave the most effective DNA delivery, but this was likely because of the high substitution ratios obtained with these lipids (∼10 lipids/PLL). We noted for the first time a general relationship between the extent of lipid substitution and gene delivery efficiency. Even though other lipids were not effective, we speculate that they could be as effective as the myristic, palmitic, and stearic acids if they are sufficiently substituted on PLL, given that the established correlations were equally valid based on both the number of lipids and the number of lipid methylenes substituted per polymer. We conclude that the described amphiphilic polymers should be effective for ex vivo modification of clinically relevant cells and may be further engineered with targeting moieties for the modification of cells in vivo.

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