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Aliphatic Lipid Substitution on 2 kDa Polyethylenimine Improves Plasmid Delivery and Transgene Expression

Artphop Neamnark,[†,‡] Orawan Suwantong,[†,‡] Remant Bahadur K. C.,[†] Charlie Y. M. Hsu,[§] Pitt Supaphol,[‡] and Hasan Uludağ*[,†,§,∥]

Department of Chemical & Material Engineering, Faculty of Engineering, University of Alberta, Edmonton, Alberta, Canada, The Petroleum and Petrochemical College and The Center for Petroleum, Petrochemicals and Advanced Materials, Chulalongkorn University, Bangkok, Thailand, Department of Biomedical Engineering, Faculty of Medicine, University of Alberta, Edmonton, Alberta, Canada, and Faculty of Pharmacy and Pharmaceutical Sciences, University of Alberta, Edmonton, Alberta, Canada

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Abstract: This study was conducted in order to develop amphiphilic, low molecular weight polymeric carriers for nonviral gene delivery. Caprylic, myristic, palmitic, stearic, oleic and linoleic acids were grafted onto the 2 kDa polyethylenimine (PEI) and properties critical for gene delivery were investigated using 293T and bone marrow stromal cells. The extent of lipid substitution on the polymers was controlled by the lipid:PEI feed ratio during the synthesis. The toxicity of the native and lipid-substituted 2 kDa PEI was relatively lower than the 25 kDa PEI, although lipid substitution generally increased the toxicity of the polymers *in vitro*. Lipid substitution reduced the ability of the polymers to complex DNA, as well as the stability of final complexes, as measured by heparin-induced dissociation. Once fully complexed to a plasmid DNA, however, the lipid-substituted polymers increased the plasmid DNA delivery to the cells. In 293T cells, the lipid-substituted polymers displayed a transfection ability that was equivalent to highly effective 25 kDa PEI, but without the toxic effect associated with the latter polymer. Among the lipids explored, no particular lipid emerged as the ideal substituent for transgene expression, although linoleic acid appeared to be superior to other lipid substituents. No correlation was evident between the level of substitution and DNA delivery efficiency of the polymers, and as little as 1 lipid substitution per PEI was effective in transforming the ineffective 2 kDa PEI into an effective carrier. The current structure−function studies are providing important clues about the properties critical for gene delivery and providing carriers effective for nonviral plasmid delivery.

Keywords: Polyethylenimine; lipid substitution; Green Fluorescent Protein (GFP); nonviral delivery; transfection; DNA binding

Introduction

Cationic polymers employed in nonviral approaches for gene delivery are capable of condensing stringlike plasmid DNA into spherical, nanosized particles via electrostatic interaction with the anionic phosphate groups of the DNA backbone.[1,2] The resulting net cationic charge on the DNA/polymer complex, typically assessed by ζ-potential measurements, allows particle binding to the anionic cell surfaces and facilitates cellular uptake of the particles.[3,4] A net

* Corresponding author. Mailing address: #830, Chemical & Materials Engineering Department, Faculty of Engineering, University of Alberta, Edmonton, Alberta, Canada T6G 2G6. Tel: (780) 492-0988. Fax: (780) 492-2881. E-mail: hasan.uludag@ualberta.ca.

† Faculty of Engineering, University of Alberta.

‡ Chulalongkorn University.

§ Faculty of Medicine, University of Alberta.

∥ Faculty of Pharmacy and Pharmaceutical Sciences, University of Alberta.

(1) Morille, M.; Passirani, C.; Vonarbourg, A.; Clavreul, A.; Benoit, J. P. Progress in developing cationic vectors for non-viral systemic gene therapy against cancer. *Biomaterials* **2008**, *29*, 3477–3496.

(2) Park, T. G.; Jeong, J. H.; Kim, S. W. Current status of polymeric gene delivery systems. *Adv. Drug Delivery Rev.* **2006**, *58*, 467–486.

(3) Paris, S.; Burlacu, A.; Durocher, Y. Opposing roles of syndecan-1 and syndecan-2 in polyethyleneimine-mediated gene delivery. *J. Biol. Chem.* **2008**, *283*, 7697–7704.

(4) Yu, L.; Nielsen, M.; Han, S. O.; Kim, S. W. TerplexDNA gene carrier system targeting artery wall cells. *J. Controlled Release* **2001**, *72*, 179–189.

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positive charge alone, however, is not sufficient for effective delivery of a DNA payload. The particles have to traverse though a lipid-based plasma membrane, where the DNA molecules are expected to remain bound to polymeric carriers and be effectively transported through a lipophilic milieu. Toward this end, cationic polymers have been substituted with short hydrophobic molecules to improve the polymer compatibility with the plasma membranes. Polyethylenimine (PEI), for example, has been modified with short hydrophobic substituents (C2 to C6 groups),[5−7] yielding more effective carriers after optimal modification conditions. Modification of other polymers, such as linear poly-L-lysine (PLL),[4,7] oligo(arginines),[9] and dendritic poly-L-Lysine and polyornithine,[10] with fatty acids, also resulted in an array of polymers with improved gene delivery. In the case of PLL[8] and oligo(arginines),[9] a direct relationship between the membrane permeability and transfection efficiency was noted. We recently reported modification of 22 kDa PLL with several aliphatic fatty acids.[11] Although lipid-substituted PEI and PLL were both effective in condensing plasmid DNA into nanoparticles, lipid substitution improved the DNA delivery capability of PLL,[11] but not PEI.[7,12−14] Upon lipid substitution on PLL, transgene expression was improved in line with improved delivery of the nucleic acid payload.

Unlike these high molecular weight (MW) polymers, the use of low MW polymers might be more desirable for gene delivery, due to better safety profiles. The cytotoxicity of the cationic polymers, such as PEI and PLL, is known to inversely depend on the polymer MWs,[15,16] due to the lower degree of interactions with plasma membranes at lower MWs.[17] In addition, small MW polymers can be readily eliminated from the systemic circulation when they are employed for direct administration into an organism, reducing their impact on an organism. However, transfection efficiency of PEI is also inversely dependent on the polymer MW. Although some studies noted improved transfection efficiency of branched PEI as the MW of the polymer was reduced from ∼25 to ∼10 kDa,[15,16] the lower MW PEIs (e.g., 2 kDa) are not effective for plasmid DNA delivery despite their acceptable cytotoxicity profiles.[18,19] Hydrophobic modification of the low MW polymers has also been attempted to improve their effectiveness. Kim and co-workers modified a 0.4 kDa linear PEI with cholesterol and obtained an improvement in transgene expression when the modified polymers were formulated as a liposome.[20] Cholesterol-substituted 1.8 kDa PEI was shown to be superior to its unmodified counterpart,[21] especially when the cholesterol moiety was attached onto secondary amines of the PEI.[18] The beneficial effect of cholesterol addition on 2 kDa PEI was also independently reported.[19] Dexamethasone, a molecule with structural similarity to cholesterol, has also been substituted on 2 kDa PEI, resulting in better nuclear transport of polyplexes and improved transgene expression.[22]

Unlike the multicyclic steroids, aliphatic lipids might be superior alternatives for PEI substitution, since they are amenable for better control during grafting reactions. Hy-

(5) Nimesh, S.; Aggarwal, A.; Kumar, P.; Singh, Y.; Gupta, K. C.; Chandra, R. Influence of acyl chain length on transfection mediated by acylated PEI nanoparticles. *Int. J. Pharm.* **2007**, *337*, 265–274.

(6) Doody, A. M.; Korley, J. N.; Dang, K. P.; Zawaneh, P. N.; Putnam, D. Characterizing the structure/function parameter space of hydrocarbon-conjugated branched polyethylenimine for DNA delivery in vitro. *J. Controlled Release* **2006**, *116*, 227–237.

(7) Forrest, M. L.; Meister, G. E.; Koerber, J. T.; Pack, D. W. Partial acetylation of polyethylenimine enhances in vitro gene delivery. *Pharm. Res.* **2004**, *21*, 365–371.

(8) Incani, V.; Tunis, E.; Clements, B. A.; Olson, C.; Kucharski, C.; Lavasanifar, A.; Uludag, H. Palmitic acid substitution on cationic polymers for effective delivery of plasmid DNA to bone marrow stromal cells. *J. Biomed Mater. Res.* **2007**, *81*, 493–504.

(9) Futaki, S.; Ohashi, W.; Suzuki, T.; Niwa, M.; Tanaka, S.; Ueda, K.; Harashima, H.; Sugiura, Y. Stearylated arginine-rich peptides: a new class of transfection systems. *Bioconjugate Chem.* **2001**, *12*, 1005–1011.

(10) Shah, D. S.; Sakthivel, T.; Toth, I.; Florence, A. T.; Wilderspin, A. F. DNA transfection and transfected cell viability using amphipathic asymmetric dendrimers. *Int. J. Pharm.* **2000**, *208*, 41–48.

(11) Abbasi, M.; Uludag, H.; Hsu, C.; Incani, V.; Jeffery, A. Further investigation of lipid-substituted poly(L-Lysine) polymers for transfection of human skin fibroblasts. *Biomacromolecules* **2008**, *9*, 1618–1630.

(12) Masotti, A.; Moretti, F.; Mancini, F.; Russo, G.; Di Lauro, N.; Checchia, P.; Marianecci, C.; Carafa, M.; Santucci, E.; Ortaggi, G. Comparison of different commercially available cationic liposome-DNA lipoplexes: Parameters influencing toxicity and transfection efficiency. *Bioorg. Med. Chem.* **2007**, *15*, 1504–1515.

(13) Yamazaki, Y.; Nango, M.; Matsuura, M.; Hasegawa, Y.; Hasegawa, M.; Oku, N. Polycation liposomes, a novel nonviral gene transfer system, constructed from cetylated polyethylenimine. *Gene Ther.* **2000**, *7*, 1148–1155.

(14) Brownlie, A.; Uchegbu, I. F.; Schatzlein, A. G. PEI-based vesicle-polymer hybrid gene delivery system with improved biocompatibility. *Int. J. Pharm.* **2004**, *274*, 41–52.

(15) Kunath, K.; von Harpe, A.; Fischer, D.; Petersen, H.; Bickel, U.; Voigt, K.; Kissel, T. Low-molecular-weight polyethylenimine as a non-viral vector for DNA delivery: comparison of physicochemical properties, transfection efficiency and in vivo distribution with high-molecular-weight polyethylenimine. *J. Controlled Release* **2003**, *89*, 113–125.

(16) Fischer, D.; Bieber, T.; Li, Y.; Elsasser, H. P.; Kissel, T. A novel non-viral vector for DNA delivery based on low molecular weight, branched polyethylenimine: effect of molecular weight on transfection efficiency and cytotoxicity. *Pharm. Res.* **1999**, *16*, 1273–1279.

(17) Ryser, H. J. A membrane effect of basic polymers dependent on molecular size. *Nature* **1967**, *215*, 934–936.

(18) Wang, D.; Narang, A. S.; Kotb, M.; Gaber, O. A.; Miller, D. D.; Kim, S. W.; Mahato, R. I. Novel branched poly(ethylenimine)-cholesterol water-soluble lipopolymers for gene delivery. *Biomacromolecules* **2002**, *3*, 1197–1207.

(19) Bajaj, A.; Kondaiah, P.; Bhattacharya, S. Synthesis and gene transfection efficacies of PEI-cholesterol-based lipopolymers. *Bioconjugate Chem.* **2008**, *19*, 1640–1651.

(20) Furgeson, D. Y.; Cohen, R. N.; Mahato, R. I.; Kim, S. W. Novel water insoluble lipoparticulates for gene delivery. *Pharm. Res.* **2002**, *19*, 382–390.

(21) Han, S.; Mahato, R. I.; Kim, S. W. Water-soluble lipopolymer for gene delivery. *Bioconjugate Chem.* **2001**, *12*, 337–345.

drophobic modification of low MW PEIs (0.6 and 1.8 kDa) with $-(CH_2)_{17}-CH_3$ (cetyl) moieties was explored, but the transfection efficiency of the modified polymers was investigated as a liposomal formulation and their efficiency on their own was not reported.[13,23] The 1.8 kDa PEI was also modified with $-(CH_2)_{11}-CH_3$ (dodecyl) and $-(CH_2)_{15}-CH_3$ (hexadecyl) moieties, significantly improving the gene delivery efficiency.[24] However, no systematic studies on structure−function relationships were conducted with these hydrophobically modified PEIs. The extent of substitutions was not even reported, and it was not known if these two hydrophobic moieties are the most effective substituents, or whether there are other, more-effective hydrophobic moieties for substitution. No studies were conducted to explore any relationship between the extent/nature of PEI modification and the polymer properties critical for DNA delivery.

This study systematically investigated the effect of aliphatic lipid substitutions on 2 kDa branched PEI. Our goal was to convert this relatively nontoxic and DNA-binding, albeit ineffective polymeric carrier, into an effective delivery agent for plasmid DNA. The 2 kDa PEI was chosen due to its past history of use by several independent groups, its commercial availability and, more importantly, its low toxicity profile. Using several lipids, including caprylic (CA), myristic (MA), palmitic (PA), stearic (StA), oleic (OA), and linoleic acid (LA), we generated a library of lipid-substituted PEIs at different levels of substitutions and explored the properties of lipid-substituted polymers that influenced the gene delivery efficiency. Two different types of cells, relatively easy-to-transfect immortal 293T cells and primary bone marrow stromal cells (BMSC) derived from rats, were used to assess the gene delivery capability of the modified PEIs. The results indicated the feasibility of generating a range of lipid-substituted PEIs with controlled extent of substitutions. Although the binding efficiency was reduced as a result of modification, the lipid substitution transformed the ineffective 2 kDa PEI into a carrier as effective as the potent 25 kDa branched PEI.

Materials and Methods

Materials. The 2 kDa branched PEI (M_n, 1.8 kDa; M_w, 2.0 kDa), 25 kDa branched PEI (M_n, 10 kDa; M_w, 25 kDa), anhydrous dimethylsulfoxide (DMSO) and *N*,*N*-dimethylformamide (DMF), 5(6)-carboxyfluorescein-*N*-hydroxysuccinimide ester (FAM), linoleyl chloride (C18:2 9Z,12Z; 99%), Hanks' balanced salt solution (HBSS, with phenol red) and trypsin/EDTA were obtained from SIGMA (St. Louis, MO). Stearoyl chloride (C18; >98.5%) was obtained from Fluka. Caproyl chloride (C8; >99%), myristoyl chloride (C14; 97%), palmitoyl chloride (C16; 98%) and octanoyl chloride (C18:2 9Z,12Z; 99%) were purchased from Aldrich. Clear HBSS (phenol red free) was prepared in house. Dulbecco's modified Eagle medium (DMEM; high and low glucose with L-glutamine), penicillin (10000 U/mL), and streptomycin (10 mg/mL) were from Invitrogen (Grand Island, NY). Fetal bovine serum (FBS) was from PAA Laboratories (Etobicoke, Ontario). SYBR Green II was purchased from Cambrex BioScience (Rockland, ME). The sources of all PCR reagents were previously described.[11] DNA-free DNase Treatment and Removal Kit was purchased from Ambion Inc. (Austin, TX). The plasmids gWIZ (blank plasmid with CMV promoter) and gWIZ-GFP (AcGFP expressing plasmid with CMV promoter) were purchased from Aldevron (Fargo, ND). FAM-labeled plasmid was obtained by reacting 100 μg of gWIZ with 10 mM FAM (diluted from 50 mM in DMSO) in borate buffer (pH 8.9) for 24 h at 37 °C. The plasmid was precipitated with 95% ethanol, incubated at −20 °C for 15 min, centrifuged at 13800 rpm for 10 min, and washed with 95% ethanol (×2). The precipitated plasmid was dissolved in ddH_2O at 0.4 mg/mL concentration.

Synthesis and Characterization of Lipid-Substituted Polymers. The lipid-substituted polymers were synthesized by N-acylation of 2 kDa PEI with commercially available lipid chlorides (Scheme 1). The PEI was obtained as a 50% solution and purified by freeze-drying before use. The synthesis procedure was adopted from a previously described procedure.[6] Briefly, lipid chlorides were individually dissolved in 1 mL of DMF and added dropwise to 100 mg of PEI in 1 mL of DMSO. The lipid:PEI amine ratios were systematically varied during the synthesis procedure between 0.012 and 0.2, assuming one primary amine per PEI monomer unit as shown in Scheme 1 (a total of 14 primary amines in PEI). The mixture was allowed to react for 24 h at room temperature under argon. Excess ethyl ether was added to precipitate and wash (×3) the polymers, and then the polymers were dried under vacuum at ambient temperature overnight. The modified polymers were analyzed by ^{1}H NMR (Bruker 300 MHz; Billerica, MA) in D_2O. The characteristic proton shift of the lipids (δ ~0.8 ppm; $-CH_3$) and PEI (δ ~2.5−2.8 ppm; $-NH-CH_2-CH_2-NH-$) were integrated, normalized for the number of Hs in each peak, and used to obtain the extent of lipid substitutions on the modified polymers (Table 1). Where indicated, the number of lipid carbons (C) or methylenes substituted was also calculated by multiplying the number of lipids/PEI with the number of C's in each lipid.

Electrophoretic Mobility Shift Assay (EMSA). Electrophoretic mobility of carrier/DNA solutions was determined by loading the complexes (see preparation conditions below) into a 0.7−0.8% agarose gel containing 1 μg/mL of ethidium bromide in 1× Tris-Acetate/EDTA

(22) Mi Bae, Y.; Choi, H.; Lee, S.; Ho Kang, S.; Tae Kim, Y.; Nam, K.; Sang Park, J.; Lee, M.; Sig Choi, J. Dexamethasone-conjugated low molecular weight polyethylenimine as a nucleus-targeting lipopolymer gene carrier. *Bioconjugate Chem.* **2007**, *18*, 2029–2036.

(23) Sochanik, A.; Cichoń, T.; Makselon, M.; Strŏzyk, M.; Smolarczyk, R.; Jazowiecka-Rakus, J.; Szala, S. Antiangiogenic gene therapy in inhibition of metastasis. *Acta Biochim. Pol.* **2004**, *51*, 693–702.

(24) Thomas, M.; Klibanov, A. M. Enhancing polyethylenimine's delivery of plasmid DNA into mammalian cells. *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99*, 14640–14645.

Scheme 1

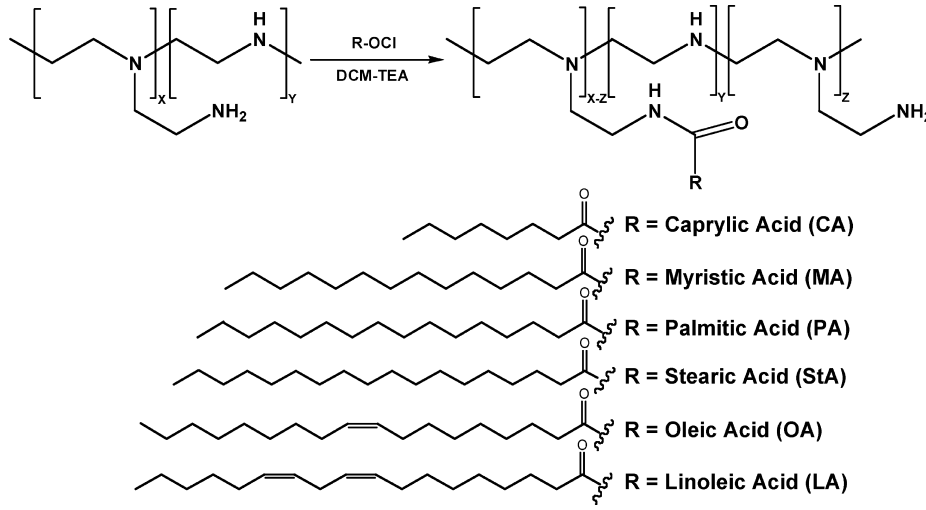

Table 1. Lipid-Substituted 2 kDa PEIs Prepared for This Study[a]

polymer designation	substituent	lipid:PEI mole ratio	lipid/PEI	lipid C/PEI	% substitution
PEI-CA-0.012	caprylic acid	0.012	0.5	3.7	2.9
PEI-MA-0.012	myristic acid	0.012	0.4	5.2	2.3
PEI-PA-0.012	palmitic acid	0.012	0.3	4.9	1.9
PEI-StA-0.012	stearic acid	0.012	0.2	3.2	1.1
PEI-OA-0.012	oleic acid	0.012	0.3	4.6	1.6
PEI-LA-0.012	linoleic acid	0.012	0.2	4.3	1.5
PEI-CA-0.066	caprylic acid	0.066	1.1	8.8	6.9
PEI-MA-0.066	myristic acid	0.066	0.6	8.3	3.7
PEI-PA-0.066	palmitic acid	0.066	0.6	9.5	3.7
PEI-StA-0.066	stearic acid	0.066	0.5	8.4	2.9
PEI-OA-0.066	oleic acid	0.066	1.0	18.1	6.3
PEI-LA-0.066	linoleic acid	0.066	1.0	17.3	6.0
PEI-CA-0.1	caprylic acid	0.1	2.4	19.0	14.8
PEI-MA-0.1	myristic acid	0.1	1.7	24.1	10.8
PEI-PA-0.1	palmitic acid	0.1	0.8	12.6	4.9
PEI-StA-0.1	stearic acid	0.1	3.6	66.6	22.8
PEI-OA-0.1	oleic acid	0.1	1.7	30.0	10.4
PEI-LA-0.1	linoleic acid	0.1	1.8	33.2	11.5
PEI-CA-0.2	caprylic acid	0.2	6.9	56.8	43.4
PEI-MA-0.2	myristic acid	0.2	1.5	20.8	9.3
PEI-PA-0.2	palmitic acid	0.2	1.1	18.0	7.0
PEI-StA-0.2	stearic acid	0.2	4.9	89.0	30.9
PEI-OA-0.2	oleic acid	0.2	2.5	44.1	15.3
PEI-LA-0.2	linoleic acid	0.2	3.2	57.7	20.0

[a] The table summarizes the lipid:PEI amine mole ratios used during the reaction for the designated polymers, as well as the actual number of lipids substituted per PEI calculated from ^{1}H NMR analysis. Lipid carbon (C) or methylene substitutions per PEI were calculated based on the number of C's present in each lipid and the number of lipids substituted per PEI. % substitution refers to the percentage of primary amines modified with the corresponding lipids.

buffer. The agarose gel was run at 130 V for ~20 min, and the DNA bands were visualized under UV light (Alpha Innotech; San Leandro, CA). The mean fluorescent density (in arbitrary units) of each band was measured by the manufacturer-supplied software. EMSA was used to investigate polymer-plasmid DNA binding as well as dissociation of complexes. For assessment of polymer binding to plasmid DNA, 200 ng of pEGFP-N2 was incubated with various concentrations of polymers in a final volume of 20 μL of 150 mM NaCl for 20 min and the complexes were analyzed by EMSA. A lane containing free plasmid DNA was run as a control (i.e., no binding), and the free plasmid DNA in complexes was quantitated at the site where the free plasmid DNA was expected to migrate. Percent binding (% binding) was calculated from the fluorescence density value (F) as $100\% \times \{[F(\text{DNA only}) - F(\text{specific polymer-DNA weight ratio})] \div [F(\text{DNA only}) - F(\text{background})]\}$. Percent binding was plotted as a function of polymer/plasmid weight ratio, and the polymer concentration required for 50% binding (BC_{50})

was estimated based on sigmoidal curve fits. An average BC_{50} value was obtained for each polymer from two independent runs.

For the dissociation studies, 350 ng of plasmid DNA in 150 mM NaCl was complexed with 1.4 μg of polymer in a final volume of 10 μL. After 30 min of incubation at room temperature, 10 μL of the basic medium (with 10% FBS) was added to the samples, and incubated at room temperature for an additional 1.5 h. Heparin sulfate was then added at a final concentration range of 0.01−1 μg/μL, and the samples were incubated for a further 30 min at 37 °C. The samples were then analyzed by EMSA. A lane containing an equivalent amount of free plasmid DNA served as the control, and the amount of free plasmid DNA after heparin treatment of complexes was quantitated at the site where plasmid DNA was expected to migrate. The percentage of plasmid DNA dissociated (% dissociation) was calculated from the fluorescence value (F) as: 100% {F(specific heparin concentration) − F(background)} ÷ {F(DNA only) − F(background)}. % dissociation was plotted as a function of the heparin concentration, and the heparin concentration required for 50% dissociation (DC_{50}) was estimated based on sigmoidal curve fits.

SYBR Green Dye Exclusion Assay. In addition to EMSA, plasmid DNA binding by the polymers was assessed by SYBR Green binding to plasmid DNA. Briefly, the gWIZ solutions (2 μg/mL) were prepared in 0.15 M NaCl in polypropylene tubes (in duplicate) and 0.5 to 32 μL of polymer solutions (5 μg/mL) was added to the tubes to give a final volume of ∼50 μL and polymer:plasmid mass ratios of up to 1:1. After 30 min of incubation at room temperature, 200 μL of 1 X SYBR Green II was added to the tubes and 200 μL of each sample was read on a 96-well plate (λ_{EX}, 485 nm; λ_{EM}, 527 nm) to quantify the amount of free DNA. The sigmoidal binding curves were generated by plotting % bound DNA vs polymer:plasmid ratio as described above, and the BC_{50} value was calculated when 50% of the plasmid binding was observed.

Cytotoxicity Evaluation by MTT Assay. The cytotoxicity of the polymers was tested on rat BMSC and 293T cells in 48-well flat-bottomed multiwell plates. The cells were seeded with 500 μL of tissue culture medium and allowed to attach overnight. The medium for BMSC was high-glucose DMEM with 100 U/mL penicillin, 100 μg/mL streptomycin, 50 μg/mL ascorbic acid and 10% FBS, whereas the medium for 293T cells was low-glucose DMEM with 100 U/mL penicillin, 100 μg/mL streptomycin, and 10% FBS. A 1 mg/mL polymer solution was then added to the wells (in triplicate) to give polymer concentrations of 5, 10, and 20 μg/mL. After 24 h of incubation at 37 °C in a humidified 95/5% air/CO_2 atmosphere, 100 μL of MTT solution (5 mg/mL in HBSS) was added to each well. After a further 1.5 h incubation, the medium was removed and 500 μL of DMSO was added to dissolve the MTT crystals formed. The optical density in each well was measured at 570 nm. The absorbance of the polymer-treated samples was normalized with the absorbance of untreated cells, which was used as a reference control (i.e., 100% cell viability), in order to calculate the % cell viability.

Plasmid Uptake by Flow Cytometry. Cells were grown in 12-well plates in the basic medium to quantitate plasmid uptake by using FAM-labeled gWIZ plasmid. Different volumes of FAM-labeled gWIZ solution (0.4 mg/mL in ddH_2O) were mixed with the desired volumes of polymer solutions (1 mg/mL in ddH_2O) in 150 mM NaCl to give plasmid DNA:polymer ratios of 1:4 (μg:μg). After incubation for 30 min, the complexes were added to the cells grown in 0.5 mL of basic medium/well (in triplicate). After 24 h incubation, the medium was removed and the cells were washed with clear HBSS, trypsinized and suspended in HBSS with 3.7% formaldehyde. The plasmid uptake was quantified by a Beckman Coulter QUANTA SC flow cytometer using FL1 channel (3000−5000 events/sample). The instrument was calibrated so that the negative control sample (i.e., FAM-labeled gWIZ without any polymeric carrier) gave 1−2% positive cells as the background. The percentage of cells exhibiting FAM-fluorescence and the mean fluorescence in the total cell population were determined. As an additional control, cells were incubated with the complexes prepared from the unlabeled gWIZ to investigate any induction of cellular fluorescence as a result of the exposure to the complexes.

Analysis of GFP Expression by Flow Cytometry. Rat BMSC and 293T cells were grown in 12-well plates to assess GFP expression by flow cytometry. Desired volumes of the individual polymer solutions (1 mg/mL in ddH_2O) were complexed with either gWIZ or gWIZ-GFP plasmids (0.4 mg/mL in ddH_2O) in 150 mM NaCl (see figure legends for the exact concentrations) and 20 μL/well of the complexes were typically added to the wells (in triplicate). Cells were incubated for 24 h with the complexes, after which the medium was replaced. Cells were trypsinized at desired time points and fixed with 3.5% formaldehyde in clear HBSS (300 μL). The GFP expression was quantified by Beckman Coulter QUANTA SC flow cytometer using FL1 channel (3000−5000 events/sample). Instrument settings were calibrated for each run so as to obtain a background level of the GFP expression of 1−2% for the control samples (i.e., untreated cells). The mean fluorescence/cell for the total population or GFP-positive cells was also determined. An aliquot of the cell suspension used for flow cytometry was manually counted with a hemacytometer to obtain the total number of cells recovered from the wells. Changes in the cell number as a function of time were used as a measure of treatment toxicity.

Analysis of GFP Expression by RT-PCR. RT-PCR was carried out to determine GFP expression at the mRNA level. After 24 h of transfection of the cells in 6-well plates with complexes as above, the cells were treated with Trizol on days 3 and 7, and the recovered solutions were frozen at −20 °C until further processing. Harvested BMSC were thawed on ice, and 450 μL of chloroform:isoamyl alcohol (24:1) was added to the samples. The samples were vortexed and centrifuged at 12000g for 15 min to separate the aqueous

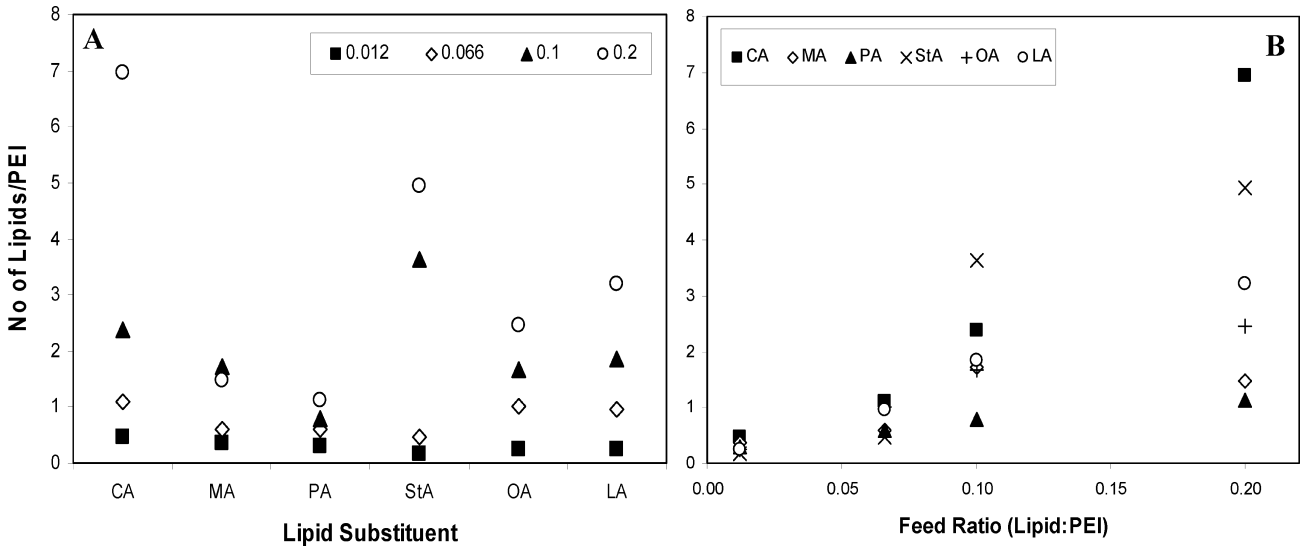

Figure 1. (A) Extent of lipid substitution on PEI (i.e., no of lipids/PEI) for different lipid substituents. The substituents were ranked in the order of increasing chain length, but no clear effect of lipid chin length on the extent of lipid substitution was apparent. (B) Extent of lipid substitution on PEI as a function of lipid:PEI amine feed ratio during the reaction. The feed ratio effectively controlled the extent of substitution for each lipid.

phase. The samples were then treated with 250 μL of ethanol (70%), and the solutions were vortexed. The total RNA was subsequently isolated by using the RNeasy Mini Kit. The samples were additionally treated with the DNA-free DNase Treatment and Removal Kit according to the manufacturer's protocol. The obtained RNA yield was quantified by using SYBR green and the samples were frozen at −20 °C until reverse transcriptase reaction. The GFP cDNA synthesis and amplication were carried out essentially as described in ref 11, and a housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), was amplified as a control.

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

Results

Synthesis of Lipid-Substituted Polymers. A procedure previously optimized for lipid substitution on 25 kDa PEI[8] was utilized for the modification of 2 kDa PEI. The reaction conditions and the resultant polymers were summarized in Table 1. Four series of lipid substitutions were attempted based on four different lipid:PEI amine ratios employed. At the lipid:PEI amine ratios of 0.012, 0.066, 0.01 and 0.2, the extent of lipid substitutions ranged between 0.2 and 0.5, 0.5 and 1.1, 0.8 and 3.6, and 1.1 and 6.9 lipids per PEI, respectively. For all lipids, a general trend of increasing lipid substitutions with increasing lipid:PEI amine ratio was evident (Figure 1A,B). Among the chosen lipids, MA and PA generally gave lower substitutions. The highest amine substitution occurred for CA at the lipid:PEI ratio of 0.2, which amounted to ~43% of primary amines, assuming all substitutions occurring on primary amines. The remaining polymers had substitutions less than ~31%, leaving sufficient amine groups for nucleic acid binding. All of the obtained polymers were found to be soluble in pure water. The only exception was the stearic acid-substituted PEI at the highest lipid:PEI amine ratio of 0.2 (see Figure 1A). This insoluble polymer was not used in this study.

Plasmid DNA Binding. The semiquantitative EMSA was first used to assess the binding of the polymers with a plasmid DNA. The fraction of unbounded plasmid was determined in EMSA, which was used to calculate the amount of plasmid participating in polyplex formation. As expected, increasing the polymer:plasmid ratio resulted in an increase in DNA complexation for all polymers (Figure 2). The binding curves typically followed a sigmoidal curve for most polymers, except the linear curves obtained for PEI-StA-0.012 (Figure 2A) and PEI-LA-0.2 (Figure 2D). Based on the generated curve fits, BC_{50} values were determined as a relative measure of the plasmid binding efficiency of polymers. The native PEIs had the lowest BC_{50} values among the polymers (0.05−0.06 for 2 and 25 kDa PEI), and all lipid-substituted polymers displayed higher BC_{50} values (Figure 3). For some lipids, a general trend of increasing BC_{50} with increasing lipid substitution was clearly evident (e.g., PA and LA), but not all lipids gave such a clear trend. A general relationship between the extent of lipid substitution and BC_{50} values was explored for all polymers; the obtained linear regression coefficient ($r^2 \sim 0.4462$; dashed line in Figure 3A) indicated a significant correlation ($p < 0.001$) between the two variables. Since each lipid contained a variable number of C's, we also explored a correlation between BC_{50} and the extent of lipid C substitution (see Table 1 for exact values of lipid Cs substituted). The regression coefficient was similar

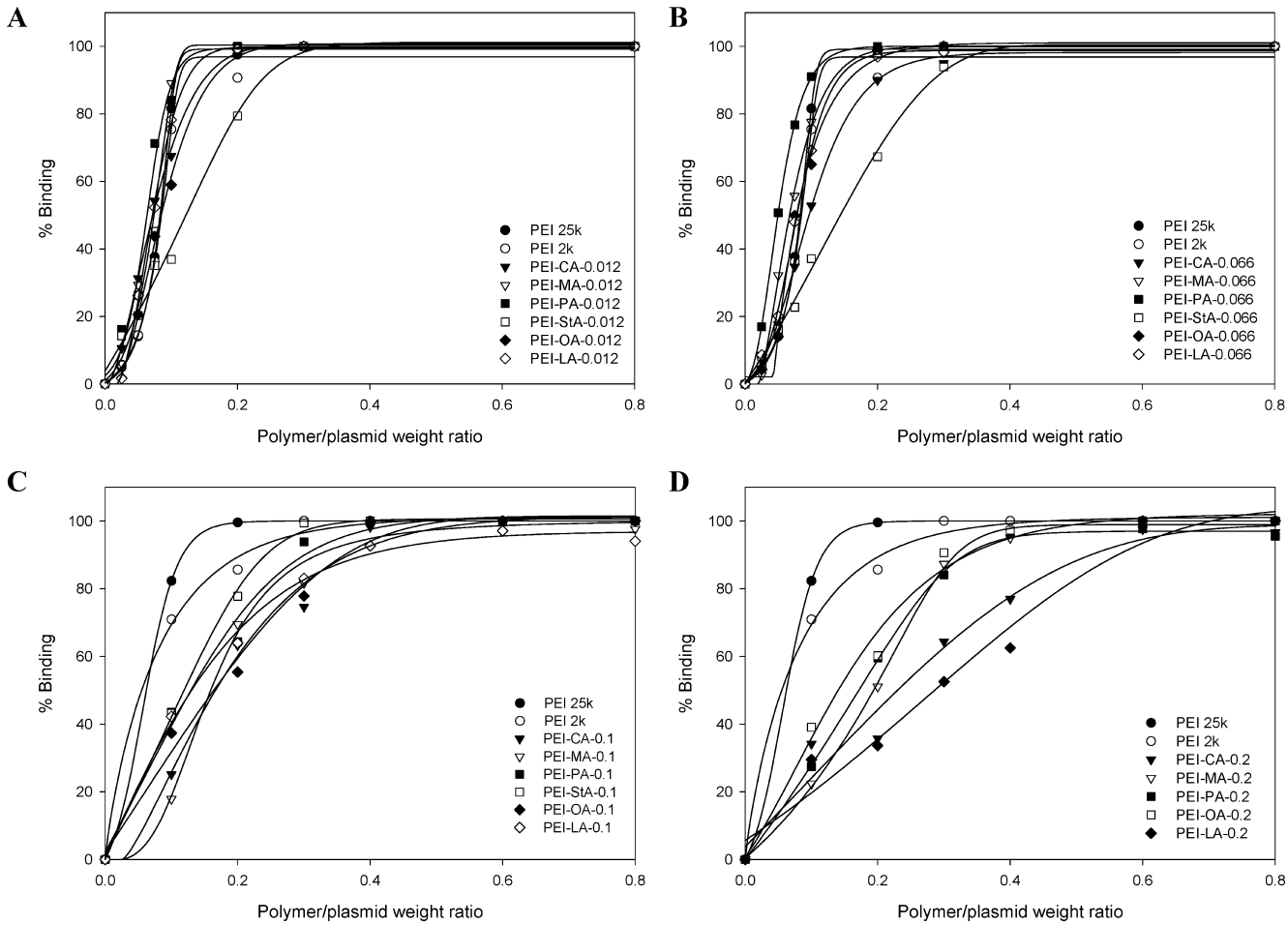

Figure 2. Binding curves obtained after complexing plasmid DNA with the polymers derived from the lipid:PEI amine feed ratios of 0.012 (A), 0.066 (B), 0.1 (C) and 0.2 (D). % binding values obtained from EMSA were plotted as a function of polymer:plasmid mass ratio (w/w). Note that no major differences in % binding were observed for polymers at low substitutions (A and B), but polymers with higher substitutions gave % binding curves that were shifted leftward (in particular polymers obtained from the feed ratio of 0.2, D).

to the previous case ($r^2 \sim 0.4509$; dashed line in Figure 3B), indicating a significant correlation ($p < 0.001$) between BC_{50} and the extent of lipid methylene substitution.

Assessment of BC_{50} values with SYBR Green assay was also carried out, and the results indicated a good correlation ($r^2 \sim 0.8276$; $p < 0.001$) with the values obtained from the EMSA assessment (Figure 4A). Similar to the outcome obtained with the EMSA (see Figure 3), the BC_{50} obtained from the SYBR Green binding was dependent on the extent of lipid substitution ($r^2 \sim 0.5882$; Figure 4B) as well as lipid C substitution ($r^2 \sim 0.6002$; not shown), indicating an adverse effect of lipid substitution on BC_{50} values.

Dissociation of Polymer/Plasmid DNA Complexes with Heparin. Stability of the polymer/plasmid DNA complexes was investigated with EMSA by incubating the complexes with heparin. The anionic heparin is well-known to destabilize polyplexes and commonly used to assess polyplex dissociation.[25] The dissociation was investigated in the presence of cell culture medium (50% by volume, giving 5% serum), so as to better mimic the conditions to which the polyplexes were exposed. No apparent dissociation was evident in the absence of heparin, indicating the polyplex stability in the presence of 5% serum. However, the polyplex dissociation was readily obtained as a function of heparin concentration for all polymers (not shown). The DC_{50} values were calculated as a quantitative measure of the polyplex propensity to dissociate. The DC_{50} values for the native PEIs were similar for both 2 and 25 kDa polymers, suggesting an equivalent stability of these polyplexes (Figure 5). Most DC_{50} values obtained for the lipid-substituted PEIs were lower than the DC_{50} values of the native 2 kDa PEI, with a few exceptions (Figure 5). No apparent correlation was evident between the obtained DC_{50} values and (i) the number of lipid substitutions (Figure 5A) or (ii) the number of lipid C substitutions (Figure 5B).

Polymer Cytotoxicity. Cytotoxicity of the polymers was evaluated by using two different cells, human kidney 293T cells and primary rat BMSC. Whereas the former is an immortalized cell line commonly used in transfection studies,

(25) Moret, I.; Esteban Peris, J.; Guillem, V. M.; Benet, M.; Revert, F.; Dasí, F.; Crespo, A.; Aliño, S. F. Stability of PEI-DNA and DOTAP-DNA complexes: effect of alkaline pH, heparin and serum. *J. Controlled Release* **2001**, *76*, 169–181.

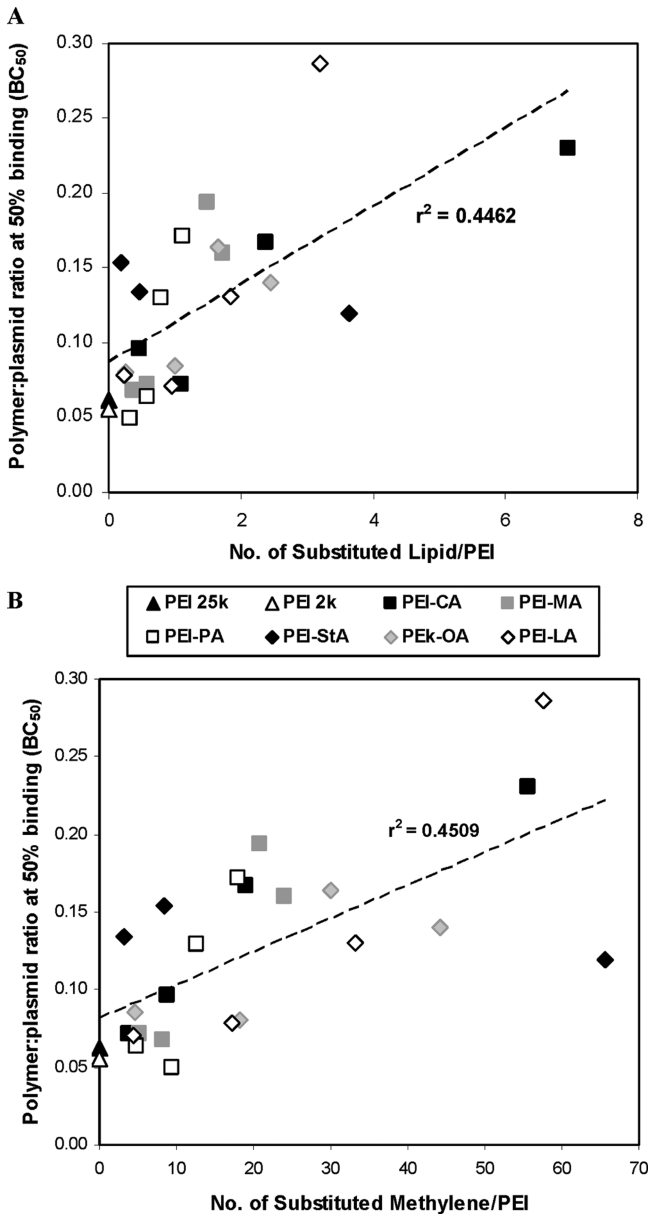

Figure 3. BC_{50} values (i.e., polymer:plasmid mass ratio at 50% binding) as a function of number of substituted lipids per PEI (A) and the number of substituted lipid C per PEI (B). BC_{50} values were derived from Figure 2 using sigmoidal curve fits. A positive correlation was obtained between the BC_{50} values and the extent of substitutions in both A and B, indicating reduced binding tendency of the polymers as a function of lipid substitution.

the latter is of primary origin that has significant clinical potential. The viability of the cells was investigated after 24 h treatment with the polymers, the typical transfection period in this study. Cells not treated with any reagent were used as a control (i.e., 100% viability). At 5 μg/mL polymer concentration, 293T cells (Figure 6A, black bars) displayed cytotoxicity with 25 kDa PEI and two lipid-substituted PEIs (6.9 CA/PEI and 0.3 OA/PEI). None of the other polymers, including the native 2 kDa PEI, displayed any cytotoxic effect at this concentration. The cells displayed a similar pattern of cytotoxicity at 10 μg/mL (Figure 6A, red bars), where the 25 kDa PEI and PEIs highly substituted with CA (2.4 and 6.9 CA/PEI) displayed cytotoxicity, unlike the other polymers. At 20 μg/mL (Figure 6A, blue bars), most cytotoxicity was observed with the 25 kDa PEI, whereas a marginal cytotoxicity was observed with the 2 kDa PEI. Significant cytotoxicity was observed for all other polymers at this concentration, indicating an undesirable effect of the lipid substitution on polymer toxicity. A correlation between the extent of lipid substitution and the toxicity observed at this concentration was probed with 293T cells (see Figure 1S in the Supporting Information). No obvious correlation was evident with the extent of lipid substitution ($r^2 \sim 0.063$; $p > 0.2$) or the lipid methylene substitution ($r^2 \sim 0.029$; $p > 0.2$).

The results for BMSC are summarized in Figure 6B. At 5 μg/mL (Figure 6B, black bars), the cells displayed cytotoxicity with the 25 kDa PEI, and 2 kDa PEIs substituted with LA and OA (1.8 LA/PEI and 1.7 OA/PEI, respectively). No other polymers, including the native 2 kDa PEI, displayed any cytotoxic effect. In fact, the MTT absorbance obtained from the cells was slightly increased for all lipid-substituted polymers at this concentration. This effect was previously observed with rat BMSC,[7] and was indicative of activation of cellular metabolic pathways (i.e., succinate dehydrogenase in the TCA cycle) without significant damage to the cellular metabolism. At 10 μg/mL (Figure 6B, red bars), rat BMSC displayed a significant cytotoxicity when incubated with the 25 kDa PEI, as well as 2 kDa PEI substituted with 1.7 and 2.5 OA/PEI. At 20 μg/mL (Figure 6B, blue bars), the most significant cytotoxicity was again observed with the 25 kDa PEI and some of the lipid-substituted polymers that were equivalent to the 25 kDa PEI toxicity. The cytotoxic polymers were from the highest substitution reactions (lipid:PEI feed ratios of 0.1 and 0.2), and contained all lipids. This was again indicative of an undesirable effect of the lipid substitution on cell compatibility. Polymers at low substitutions (i.e., derived from the feed ratios of 0.016 and 0.066) typically displayed cytotoxicity that was equivalent to the native 2 kDa PEI. A correlation between the extent of lipid substitution and the toxicity observed was probed with rat BMSC (see Figure 2S in the Supporting Information). A significant correlation was present with the extent of lipid substitution ($r^2 \sim 0.1917$; $p < 0.05$), but not with the lipid methylene substitution ($r^2 \sim 0.094$; $p > 0.1$).

We then probed a correlation between the toxicity effects on 293T cells and BMSC (Figure 7). As expected, no significant correlation was evident for the 5 and 10 μg/mL concentrations, since little toxicity was noted at these concentrations. A significant correlation was obtained at the relatively toxic 20 μg/mL concentrations ($r^2 \sim 0.2291$; $p < 0.05$), suggesting a general similarity in the adverse influence of polymers on these two widely different cell types.

Plasmid DNA Delivery into Cells. A select group of polymers (from the lipid:PEI amine feed ratio of 0.066) was first evaluated for plasmid DNA delivery to the cells. Using 1.3, 4, and 12 μg/mL polymer concentrations, the plasmid delivery was found to be ineffective at the lowest concentra-

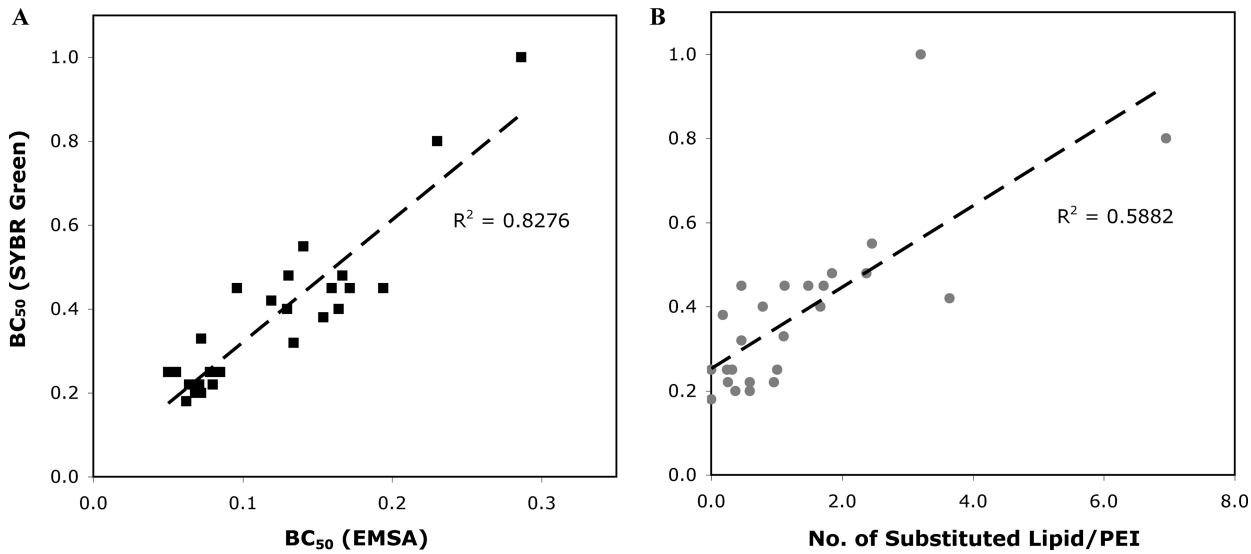

Figure 4. (A) Correlation between the BC_{50} values (i.e., polymer:plasmid mass ratio at 50% binding) obtained from the EMSA and the SYBR Green binding assay. The data was summarized for all polymers synthesized for this study. There was a significant correlation ($r^2 \sim 0.8276$; $p < 0.001$) between the independent BC_{50} values obtained from the two methods. (B) Correlation between the BC_{50} values from SYBR Green binding assay and the number of substituted lipids per PEI. A positive correlation ($r^2 \sim 0.5882$) was obtained between the BC_{50} values and the extent of lipid substitutions, indicating reduced binding tendency of polymers as a function of lipid substitution.

tion investigated, but a significant uptake was noted at the two higher concentrations in both cell types (not shown). All polymers were then evaluated for the delivery of FAM-labeled gWIZ at 4 μg/mL. With 293T cells (Figure 8A), the uptake of FAM-gWIZ alone in the absence of the polymers was relatively low (<5% of cells), and the exposure of the unlabeled gWIZ to the cells did not give any autofluorescence (not shown). The plasmid delivery by the 25 kDa PEI was superior to the 2 kDa counterpart. A beneficial effect of the lipid substitution was noted with CA, MA, PA and LA, but only for the polymers with relatively high substitutions. A correlation between the plasmid uptake and the extent of lipid substitution was evident in the case of CA and LA substitutions (Figure 3S in the Supporting Information).

Similar results were obtained with the BMSC when CA-, MA-, PA- and LA-substituted PEIs were tested at 4 μg/mL concentration (Figure 8B). As in Figure 8A, the uptake of FAM-gWIZ alone in the absence of the polymers was relatively low (<5% of cells), and the exposure of the unlabeled gWIZ to BMSC did not give any autofluorescence (not shown). Both the 2 and 25 kDa PEIs gave equivalent plasmid delivery in BMSC. For the lipid-substituted polymers, the beneficial effect of the lipid substitution was discernible with CA and LA, where the higher substitution gave higher plasmid delivery, but no clear relationship was evident in the case of MA and PA substitutions (Figure 3S in the Supporting Information).

Transfection Efficiency. *293T Cells.* Using a select group of polymers, transfection efficiency was initially investigated by using two plasmids: blank gWIZ and GFP-coding gWIZ-GFP. We recently noted a significant autofluorescence from cells solely due to the polyplex treatment (i.e., without active GFP transcription),[11] so that the former plasmid was utilized to discriminate a possible "background" due to cellular autofluorescence. The transfection results are summarized in Figure 9 over a period of 10 days. As expected, incubating plasmid alone did not give any fluorescence distinct from the untreated cells (Figure 9A). With the 25 kDa PEI, a strong GFP expression was obtained with gWIZ-GFP, which peaked at >90% GFP-positive cells on day 3 after which a gradual decline in transfection was noted. Incubating the cells with the 25 kDa PEI/gWIZ polyplexes also gave some autofluorescence, which was <15% of cells during the testing period. The 2 kDa PEI was not effective for GFP expression when used to deliver gWIZ-GFP plasmid, and did not give autofluorescence when used to deliver the blank gWIZ plasmid. PEI-PA (0.6 PA/PEI) and PEI-LA (1.0 LA/PEI) gave significant GFP expression with the gWIZ-GFP plasmid, but some autofluorescence was also seen on day 1, after which it gradually decreased. The transfection efficiency as well as the autofluorescence obtained with the LA-substituted PEI was comparable to the 25 kDa PEI (Figure 9A). The extent of the transfection was gradually reduced with time, in line with the transient nature of nonviral transfections. The polymers were also compared based on the amount of GFP fluorescence in the GFP-positive cells (Figure 9B). A more clear difference between the gWIZ- and the gWIZ-GFP-treated cells was evident in this population of cells. The 25 kDa PEI and the PEI-LA were again the most effective polymers based on this analysis. Exposure of cells to the plasmid alone did not alter the growth pattern significantly when compared to the untreated cells (Figure 9C). The 293T cells treated with the 25 kDa PEI displayed a retarded growth during the first 7 days where there were no changes in the cell numbers. This was the case with both gWIZ and the gWIZ-GFP polyplexes, suggesting no effect of GFP-expres-

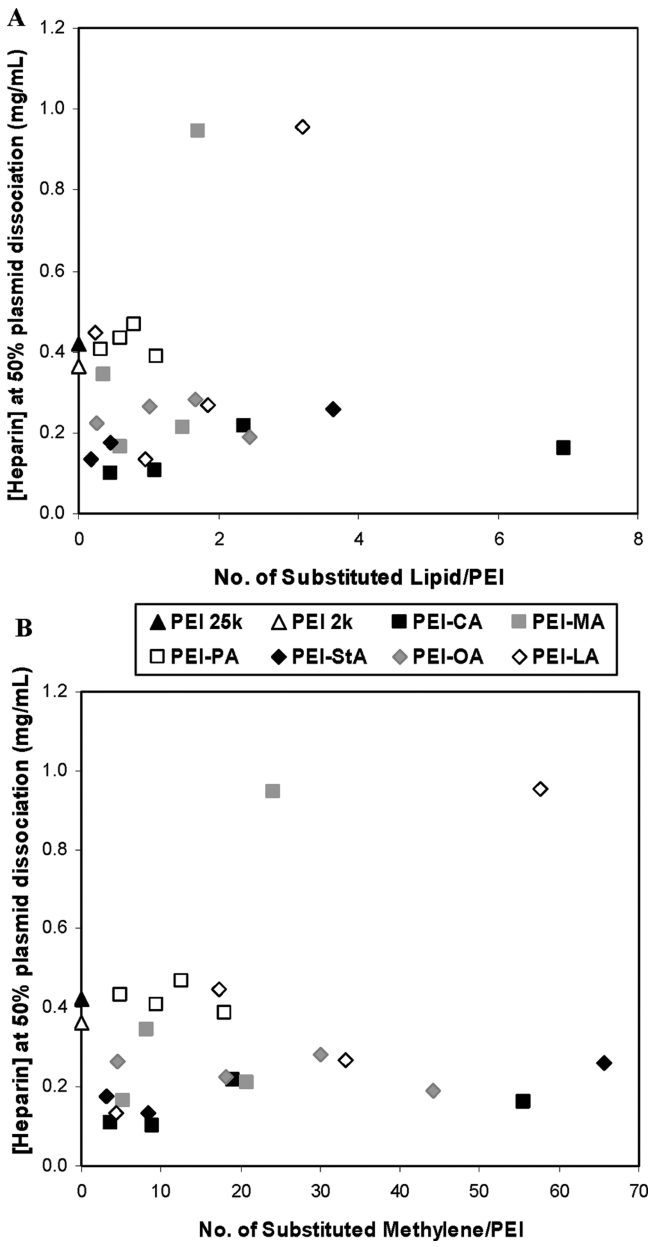

Figure 5. DC_{50} values (i.e., heparin concentration for 50% dissociation of polymer/plasmid DNA complexes) as a function of number of substituted lipids per PEI (A) and number of substituted lipid C per PEI (B). The complexes were prepared at a polymer:plasmid ratio of 4 and incubated with increasing concentrations of heparin to obtain DC_{50} values after EMSA. The sigmoidal curve fits for % binding vs heparin concentration was used to calculate the DC_{50} values. The DC_{50} values for lipid substituted polymers were generally lower than the unsubstituted PEIs, but there were no correlations between the DC_{50} values and the extent of substitutions.

sion *per se* on the growth pattern. All other polymers gave a relatively robust cell growth during the study period, but the number of cells at the end of the study period was significantly lower than that of the untreated cells. Utilizing either gWIZ or gWIZ-GFP plasmid did not alter the cell growth outcome.

Given the successful results form the LA-substituted PEI, the relative efficiency of an LA-substituted PEI (1.8 LA/PEI) to that of 25 kDa native PEI was further compared. Different polymer:plasmid ratios were employed for this purpose, and the cells with treated with the polyplexes for 4 and 24 h (Figure 10). There was an increase in the extent of GFP-positive cells and GFP fluorescence with increasing polymer:plasmid ratios for the 25 kDa PEI and the PEI-LA. The LA-substituted PEI again gave equivalent transgene expression to that of 25 kDa PEI, but at higher polymer:plasmid ratios. There was no evidence of transgene expression by the 2 kDa (unsubstituted) PEI at the polymer:plasmid ratios employed.

BMSC. As expected, incubating the cells with the plasmid alone did not give any fluorescence distinct from the untreated cells (Figure 11A). With the 25 kDa PEI, incubating the cells with both of the gWIZ and the gWIZ-GFP polyplexes gave similar populations of GFP-positive cells, indicating relatively strong autofluorescence in BMSC. Only on day 7, a significant difference between the two types of polyplexes was evident. A similar observation was noted for the native and the lipid-modified 2 kDa PEIs as well; the autofluorescence values obtained with the gWIZ polyplexes were equivalent to that of the gWIZ-GFP polyplexes (Figure 11A). Based on the amount of GFP fluorescence in the GFP-positive cells, only LA-modified PEI gave a clear difference between the gWIZ and the gWIZ-GFP treated cells (Figure 11B). Even the 25 kDa PEI did not indicate in the level of GFP-expression for the GFP-positive cells.

The exposure of BMSC to the plasmid alone did not alter the growth pattern when compared to the untreated cells (Figure 11C). As in 293T cells, BMSC treated with the 25 kDa PEI displayed a retarded growth during the 10-day study period where no significant changes in the cell numbers were observed. Whereas the 2 kDa PEI gave a relatively robust growth throughout the study period, the PA- and LA-substituted PEIs did not allow an observable cell growth during the first 3 days and the growth resumed after this time point for these polymers. The cell numbers on day 10 were significantly lower for the polyplex-treated cells as compared to those of untreated cells.

Given the lack of the clear evidence for GFP expression by flow cytometry, a parallel study assessed GFP expression in BMSC based on the semiquantitative and more-sensitive RT-PCR. On day 3, RT-PCR indicated a detectable level of GFP transcript for all polymers used for the gWIZ-GFP delivery (25 and 2 kDa PEI, PEI-PA and PEI-LA; Figure 4S in the Supporting Information). The level of the GFP transcript was lower for the 2 kDa PEI, but equivalent for the other polymers tested. As expected, the delivery of gWIZ by the polymers did not yield any GFP transcript. By day 7, no GFP transcript was detectable in BMSC incubated with the gWIZ-GFP/polymer complexes, confirming the transient nature of the nonviral transgene expression.

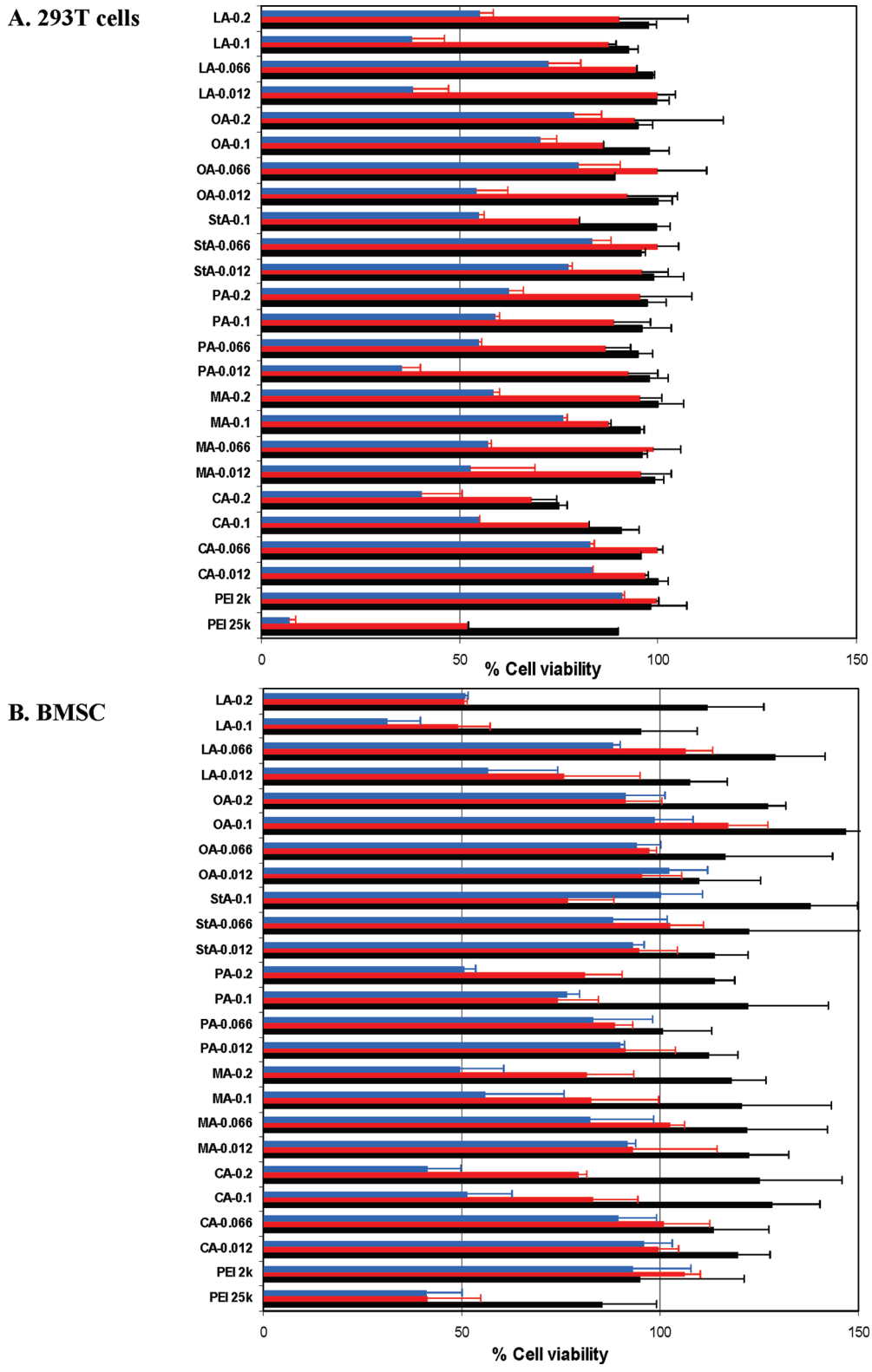

Figure 6. (A) The toxicity of unmodified PEI (25 and 2 kDa) and lipid-substituted PEIs on 293T cells. Cell viability was determined by the MTT assay after treating the cells with 5, 10, and 20 μg/mL polymers for 24 h, and expressed as a percentage of untreated cells (mean + SD of triplicate wells). Note that 25 kDa PEI displayed the highest toxicity among the polymers, but lipid substitution on the 2 kDa PEI increased the toxicity of the polymer as compared to the nontoxic 2 kDa PEI. (B) The toxicity of unmodified PEI (25 and 2 kDa) and lipid-substituted PEIs on BMSC (mean + SD of triplicate wells). Note that the 25 kDa PEI displayed high toxicity among the polymers, but lipid substitution on the 2 kDa PEI increased the toxicity of the polymer as compared to the nontoxic 2 kDa PEI. Some of the lipid-substituted polymers, especially the highest substituted polymers, displayed toxicity similar to that of 25 kDa PEI.

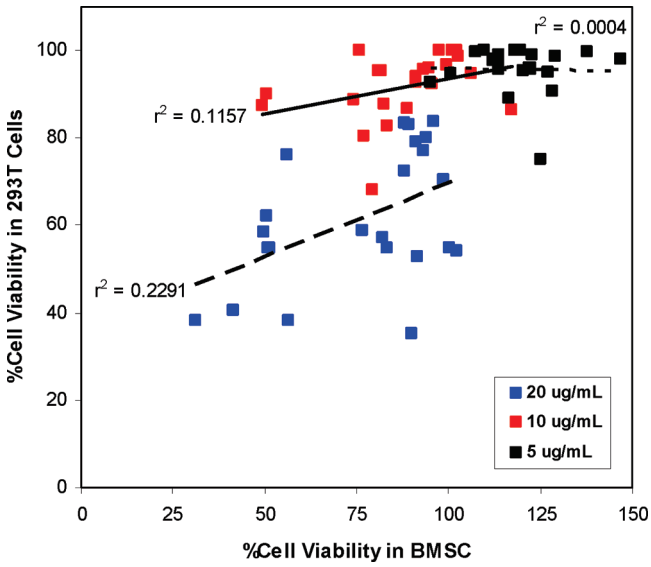

Figure 7. Correlation between the toxicity observed in 293T cells and BMSC. No correlation was evident for polymers tested at 5 and 10 μg/mL since little toxicity was observed under these conditions, but a correlation was evident at the 20 μg/mL polymer concentration, where significant toxicities were observed.

Correlation between Lipid Substitution and Transfection. Since a robust transfection was obtained in 293T cells, a correlation between the transfection efficiency and the extent of lipid substitution was explored in these cells. Polymers from the lipid:PEI ratios of 0.66, 0.1, and 0.2 were used for this study since the range of the substitutions in these polymers was sufficiently diverse. The results are summarized in Figure 12 in terms of mean GFP fluorescence and % GFP-positive cells obtained. The transfection levels obtained with the CA-substituted polymers were generally lower than those obtained with the polymers substituted with longer lipids. No obvious correlation between the transfection efficiency and the extent of lipid substitution (nor the lipid methylene substitution, not shown) was evident. The LA appeared to be the most effective lipid substituent, considering the best outcomes obtained based on both parameters of transfection. As little as 1 lipid substitution per PEI appeared to be effective with no apparent benefit of further substitutions.

Discussion and Conclusions

This study prepared a series of 2 kDa PEIs substituted with a range of caprylic, myristic, palmitic, stearic, oleic and linoleic acids. Similar to our experience with the 25 kDa PLL,[7] the lipid:PEI amine feed ratio during the synthesis generally controlled the extent of lipid substitutions. Whereas the substitution of the shortest lipid CA was higher than the other lipids, no clear relationship emerged between the length of the lipid chain and the extent of substitution for the remaining lipids. We intended to keep the extent of substitutions relatively low (<20% of amines), in order not to compromise the primary amines of PEI necessary for DNA complexation. The reduced aqueous solubility of the modified PEIs was another consideration for minimizing the extent of substitutions. The 2 kDa PEI, however, has been substituted to a greater extent with cholesterol, where 20−100% of amines were modified.[19] The cholesterol in this case was tethered with a relatively hydrophilic oxyethylene linkage (−O−CH_2−CH_2−), which might be critical to obtain water-soluble polymers despite complete substitution of all amines with the bulky cholesterol moiety.[19]

The lipid substitution on PEI led to a reduced tendency of the polymers to form complexes with the plasmid DNA (i.e., based on higher BC_{50} values from two independent methodologies), irrespective of the lipid. Reduced availability of the free −NH_2 groups and/or the stearic hindrance due to lengthy aliphatic chains were likely reason(s) for this observation. The plasmid affinities of cholesterol- and dodecyl/hexadecyl-substituted 2 kDa PEIs were not reported,[19,24] but dexamethasone substitution resulted in an increased tendency to condense plasmid DNA, contrary to our results.[22] The reduced tendency to complex plasmid DNA, however, was not detrimental to delivery; the lipopolymers yielded improved plasmid delivery once the plasmid DNA was fully complexed with excess polymer. We anticipated an inverse correlation between the propensity of the complexes to dissociate (given by DC_{50}) and the extent of lipid substitution, based on our experience with the lipid-substituted PLLs.[11] The evidence in this study, however, did not support this conclusion with the 2 kDa PEIs. Other studies with the modified 2 kDa PEI did not explore this issue either.[20−24]

The increased cytotoxicity of the polymers as a result of lipid substitution was indicative of enhanced polymer interactions with the cells. Free polymers were utilized in our cytotoxicity experiments (rather than complexes) since a significant fraction of polymers in the complex formulations were expected to be in free form. For example, complete binding was usually achieved at a polymer:plasmid DNA ratio of 0.6 (Figure 2), whereas the complexes prepared for transfections (Figures 9 to 12) typically had 5−10-fold excess polymer. Although some fraction of the polymers was still expected to be associated with the complexes even after the full binding was reached (i.e., polymer:plasmid DNA ratio >0.6), it is likely that most of the polymer remained free, and this was expected to represent the most cytotoxic component of our formulations. The increased cytotoxicity is expected to be an inevitable result of designing polymers with improved cell interactions, and cautions one to utilize the lipopolymers at a concentration where the undesired effect is minimized. An exception to this relationship (i.e., higher cellular affinity leading to higher toxicity) was reported recently,[26] but this was valid for polymers that were intracellularly cleavable.[26] The cytotoxicity of the lipid-substituted polymers, however, was less than the branched 25 kDa PEI. This was the case with the cholesterol-substituted 2 kDa PEI as well;[19] the cholesterol-substituted

(26) Breunig, M.; Lungwitz, U.; Liebl, R.; Goepferich, A. Breaking up the correlation between efficacy and toxicity for nonviral gene delivery. *Proc. Natl. Acad. Sci. U.S.A.* **2007**, *104*, 14454–14459.

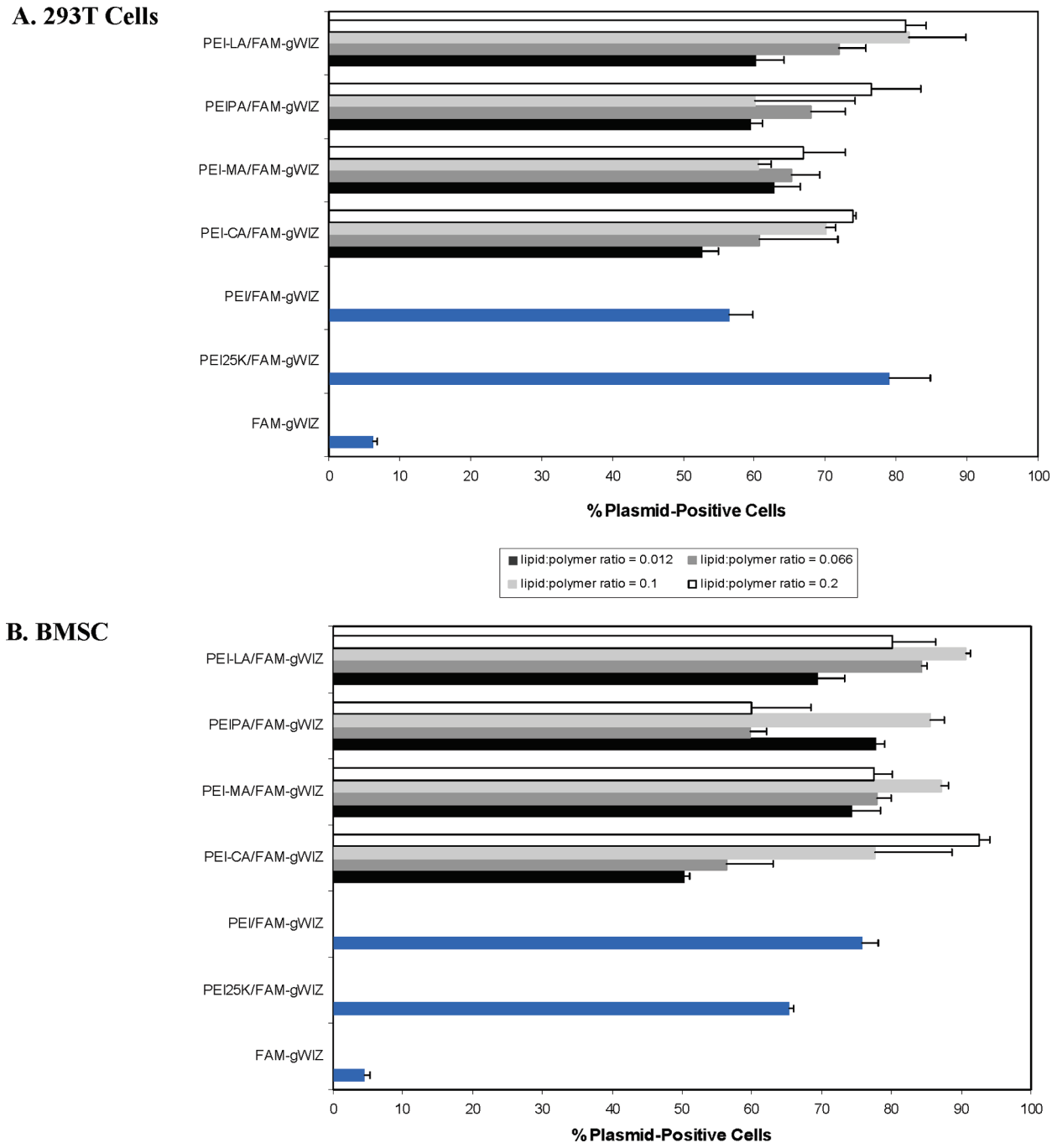

Figure 8. (A) Uptake of FAM-labeled gWIZ by 293T cells at plasmid DNA:polymer concentration of 1:4 μg/mL for polymers derived from CA, MA, PA and LA substitutions. Polymers with the highest lipid substitution generally displayed a higher plasmid delivery to the cells, which was significantly higher than the unmodified 2 kDa PEI. The 25 kDa PEI gave the highest delivery of the plasmid. (B) Uptake of FAM-labeled gWIZ by BMSC at plasmid DNA:polymer concentration of 1:4 μg/mL for polymers derived from CA, MA, PA and LA substitutions. Apart from CA-substituted polymers, no clear effect of lipid substitution on plasmid delivery was evident. Note that some lipid-substituted PEIs gave lower plasmid uptake than the unmodified PEI in BMSC.

PEI was less toxic than the 25 kDa PEI, but increased substitution led to higher toxicities on HeLa cells. The similarities of polymer toxicities on both BMSC and 293T cells indicated that the exhibited cytotoxicity was not cell-specific, and likely involved common mechanisms such as the disruption to the plasma membrane.

The beneficial effect of the lipid substitution was manifested in higher DNA delivery as well as higher transgene (GFP) expression. FAM-labeled plasmid DNA was used for uptake studies, and degradation of this plasmid DNA (either fluorophore cleavage from plasmid DNA or plasmid backbone cleavage) was not expected to occur in the 24 h study

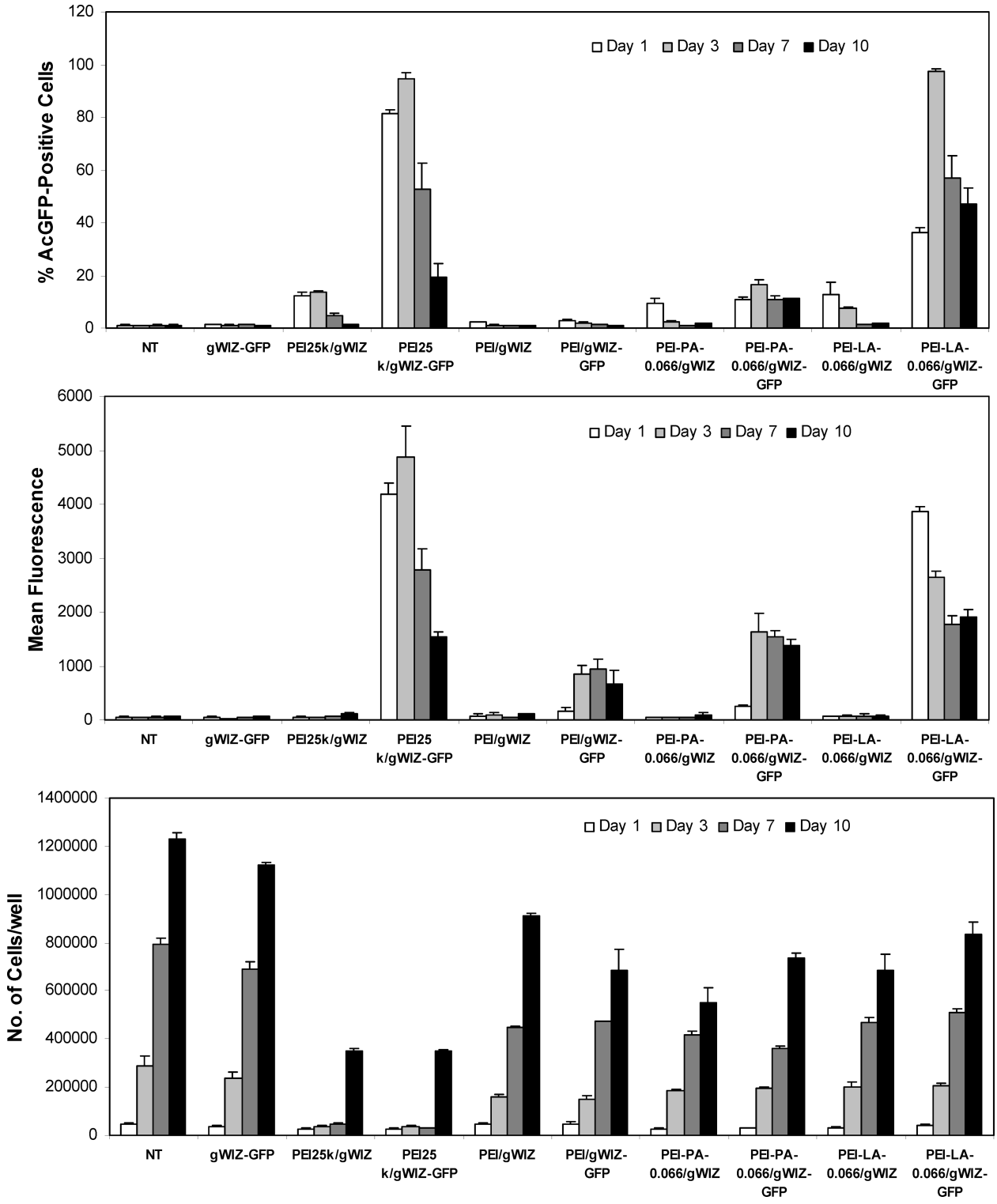

Figure 9. Transfection efficiency of lipid-substituted PEIs in 293T cells. The cells were treated with plasmid DNA/polymer complexes (2/10 μg/mL in medium) for 24 h, after which they were washed and cultured for 1, 3, 7, and 10 days. The polymers were obtained from the feed ratio of 0.066. The cells were detached with trypsin and analyzed by flow cytometry for the percentage of GFP-positive cells (A) and mean fluorescence of the GFP-positive population (B). The number of cells in each sample was counted by a hemocytometer (C). Among the polymers tested, 25 kDa PEI and PEI-LA yielded significant population GFP positive cells, which peaked at day 3 and decreased gradually afterward. Cell proliferation was robust for nontreated cells and cells exposed to gWIZ-GFP alone, whereas the cells treated with 25 kDa PEI complexes displayed retarded proliferation. The cells treated with the 2 kDa PEI (native as well as lipid-substituted PEIs) displayed the expected growth pattern.

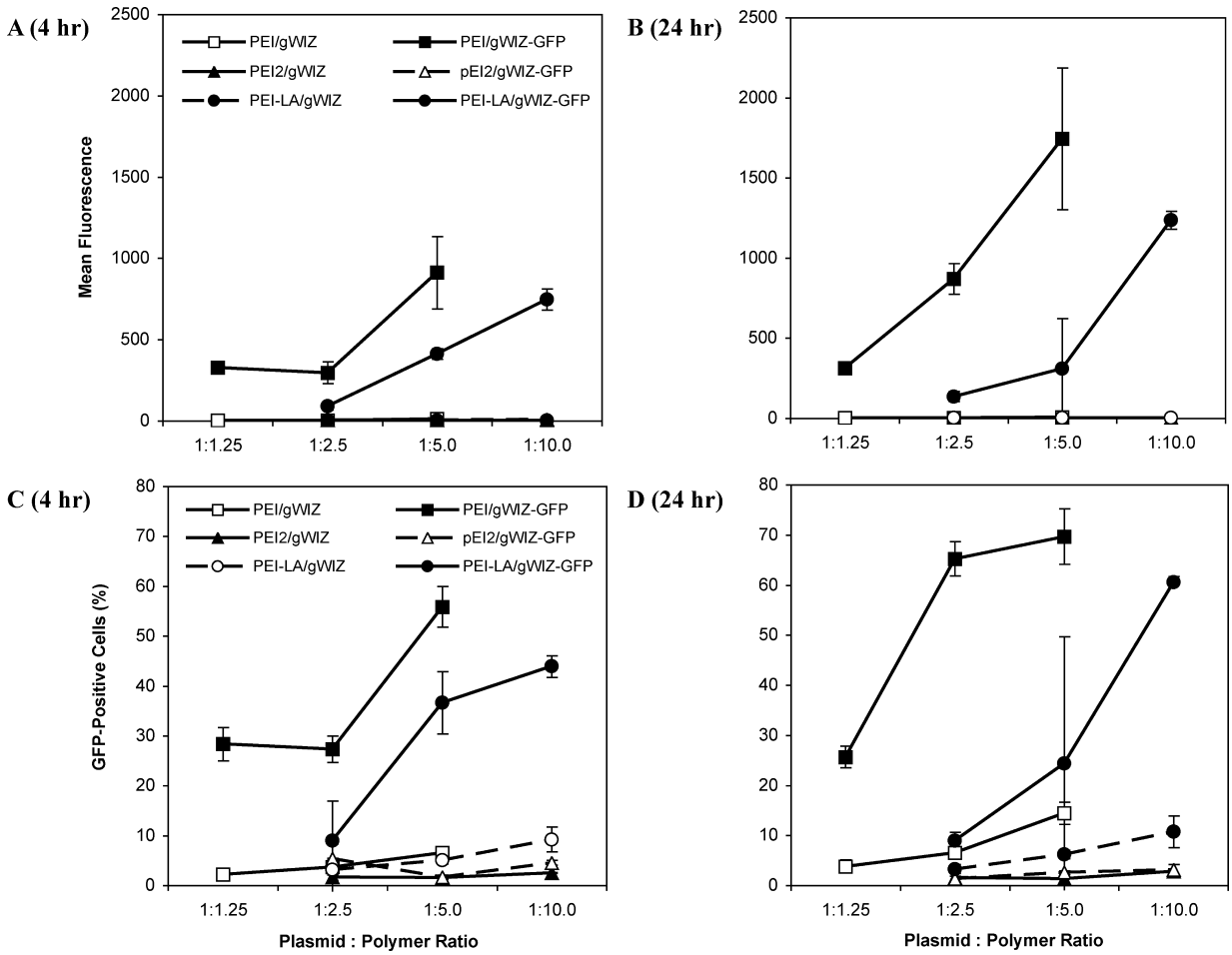

Figure 10. Transfection efficiency of 25 and 2 kDa native PEI and LA-substituted (1.8 LA/PEI) 2 kDa PEI at different polymer:plasmid ratios. The plasmid concentration was 1 μg/mL whereas the polymer concentrations were varied as indicated in the horizontal axis. The 293T cells were treated with the plasmid/polymer complexes for 4 (A and C) or 24 h (B and D). After 24 h, the cells were detached with trypsin and analyzed by flow cytometry for the mean fluorescence of GFP-positive population (A and B) and the percentage of GFP-positive cells (C and D). An equivalent level of GFP expression was noted between the 25 kDa PEI and PEI-LA, albeit a higher polymer:plasmid ratio was needed for the latter polymer. No GFP expression was evident for cells incubated with the gWIZ complexes or both plasmids delivered with the 2 kDa PEI.

uptake period, based on previous results obtained with similarly labeled plasmids in human fibroblasts.[11] We are assuming that (i) no significant exocytosis of the internalized plasmid took place during the 24 h uptake period in this study, or (ii) the FAM label on the plasmid DNA was not differentially quenched once the complexes were internalized. Based on these assumptions, no clear differences between the two cell types were noted for the DNA uptake studies. Both cell types yielded similar extents of plasmid uptake, where a high proportion of cells (>50%) was positive for the plasmid uptake. Not all of the plasmid is expected to be internalized by the cells, and it is possible that a fraction of the cell-associated plasmid remains bound to the surface of the cells during this time, which cannot be distinguishable by flow cytometry. This may explain (i) why the lipid substitution only gave a relatively small increase (20% over the background of 50−70%) in the plasmid-positive cells, and (ii) why the 2 kDa PEI was ineffective in transgene expression despite a relatively high proportion of the plasmid-positive cells with this carrier. The relatively high plasmid uptake in 293T cells translated into a strong GFP expression, where >50% of the cells expressed GFP routinely. This was not the case with BMSC where only a small fraction of the cells (i.e., insignificant in most cases) was positive for the GFP expression. The autofluorescence observed in the gWIZ-treated BMSC was significant and equivalent in some cases to the gWIZ-GFP treated cells. Obviously this may lead to erroneous results if a control plasmid (i.e., plasmid without a GFP vector) is not utilized in transfection studies. It must be emphasized that our initial studies[8] and the previous studies on the hydrophobically modified PEIs[13,18,19,22,24] were conducted without this control. Such an approach will overestimate the transfection efficiency of the carriers, especially in hard-to-transfect cells such as the BMSC. Nevertheless, some GFP expression was present in BMSC in this study, which was evident from the RT-PCR results

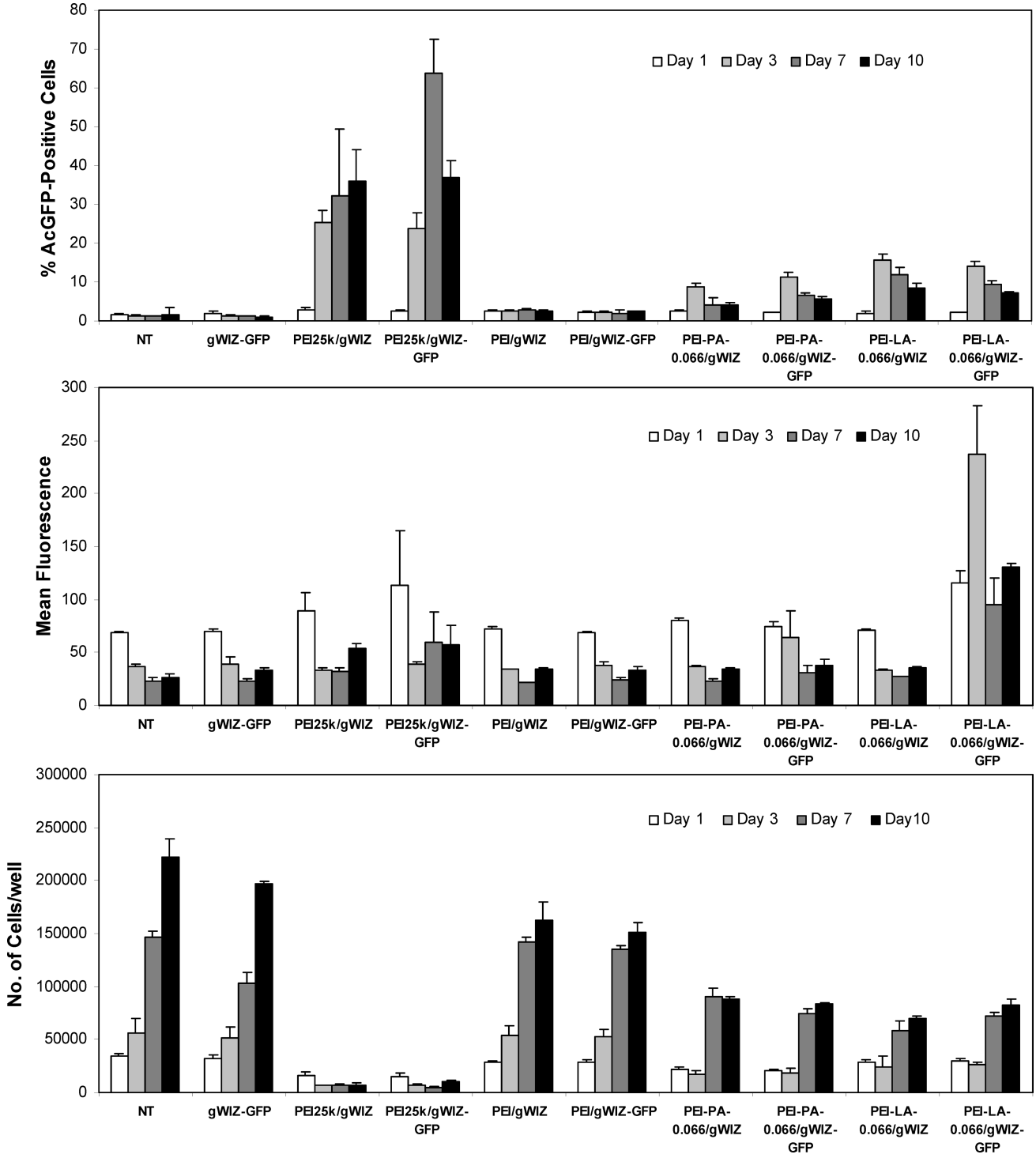

Figure 11. Transfection efficiency of lipid-substituted PEIs on BMSC. The cells were treated with plasmid DNA/polymer complexes (2/10 μg/mL in medium) for 24 h, after which they were washed and cultured for 1, 3, 7, and 10 days. The polymers were obtained from the feed ratio of 0.066. The cells were detached with trypsin and analyzed by flow cytometry for the percentage of GFP-positive cells (A) and mean fluorescence of the GFP-positive population (B). The number of cells in each sample was counted by a hemocytometer (C). Among the polymers tested, only 25 kDa PEI on day 7 gave significant population GFP-positive cells (i.e., different from gWIZ complexes). Only the cells treated with PEI-LA complexes displayed a significant difference in the mean fluorescence of GFP positive cells, indicative of some GFP expression. Cell proliferation was robust for nontreated cells and cells exposed to gWIZ-GFP alone, whereas the cells treated with 25 kDa PEI complexes displayed no proliferation. The cells treated with the lipid-substituted PEIs gave slower cell growth as compared to the native 2 kDa PEI.

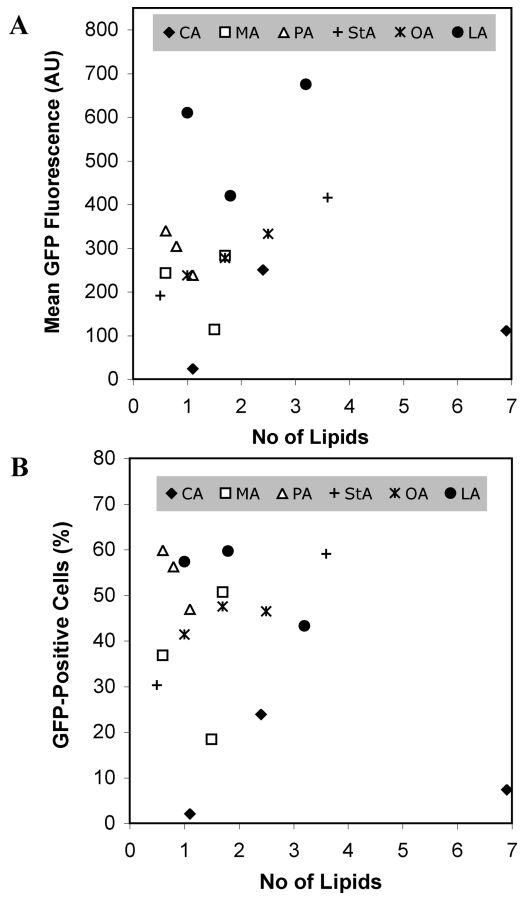

Figure 12. Transfection efficiency of lipid-substituted PEIs in 293T cells. The polymers were derived form the lipid:PEI amine feed ratios of 0.06, 0.1 and 0.2, and the polymer/plasmid complexes were added to the cells at 10/2 μg/mL concentrations. The transfection efficiency was assessed after 5 days, and the results are summarized as percentage of GFP-positive cells (A) and mean GFP fluorescence for the total cell population (B). Note that there was no obvious relationship between the number of lipid substitution and the transfection efficiency in A or B. Whereas PA- and LA-substituted PEIs generally gave higher transfection efficiencies, CA-substituted PEIs gave relatively lower transfection efficiencies. All modifications, however, resulted in higher transfection efficiency than the unmodified PEI (<2% under the experimental conditions).

and the higher levels of the mean fluorescence in the cells designated to be GFP-positive. It will be important to identify the reason(s) for the robust transgene expression in 293T cells, or conversely, the reason(s) for minimal transgene expression in BMSC. Among the polymers evaluated in this study, the linoleic acid-substituted PEI in particular was found to be as effective as the 25 kDa PEI for transgene expression in 293T cells. The transgene expression with this lipopolymer, however, was achieved with relatively less cytotoxicity on cells as compared to the 25 kDa PEI. A clear correlation between the extent/nature of the lipid substituent and the gene expression was not readily obvious and as little as 1 lipid per PEI was usually sufficient to significantly improve the delivery capability of the native 2.0 kDa PEI. However, several lipids apart from linoleic acid were also effective substituents on PEI, which turned this ineffective carrier into an effective plasmid DNA delivery agent (at least with 293T cells). Using an approach similar to the one reported in this study, we previously substituted similar lipids onto a low MW (~4 kDa) PLL without success;[27] i.e., the polymers obtained did not yield an appreciable improvement in plasmid delivery efficiency. A combination of lipid and ethyleneimine subunits appears to be critical for overall functioning of the carriers, which cannot be replaced with the lipid-lysine combination. Our future studies will further explore this issue. This observation also suggested that the lipid substitution approach might be suitable for some, but not all, polymeric carriers.

Our results indicate that the chosen aliphatic lipids may serve as alternatives to cholesterol, making hydrophobic modification of PEI more convenient and possibly better controlled as a result of employing aliphatic lipids in the reactions. Previous studies have typically utilized shorter alkyl chains for PEI modification. For example, a very high molecular weight (750 kDa) PEI was acylated with C2 to C4 chains and substitution with C3 (propionic) moieties, in particular, was found to enhance the transfection capacity of this PEI.[5] Although the exact reasons for the improved efficiency were not always determined, increased lipophilicity (i.e., better membrane compatibility) and/or weaker interactions with plasmid DNA (i.e., increased dissociation in cytoplasm[28]) were attributed to beneficial effects of the hydrophobic modifications. An independent study, using the branched 25 kDa PEI, confirmed the beneficial effect of the acylation (with C2, C4 and C6) up to ~25 mol %,[6] but noted an ambiguous dependence of the transfection efficiency on (i) the length of the substituted carbon and (ii) the extent of lipid substitution. Using a similar analysis to our work, where the transfection efficiency was correlated to the number of substituted C's, an optimal substitution ratio was noted for the most effective transfection efficiency after which a significant reduction was obtained.[6] Our data indicated that the relatively shorter substituent CA was not as effective as the longer lipids, so that effective modification with hydrophobic moieties may call for increased lipid chain lengths. In particular, the role of the lipid substituent LA (the leading candidate for PEI substitution) needs to be better elucidated; this lipid differs from the others used in this study by the presence of two double bonds in the chain backbone. This substituent, when added to a lipid membrane on its own, is known to reduce membrane crystallinity and lower the

(27) Incani, V.; Lin, X.; Lavasanifar, A.; Uludağ, H. Relationship between the extent of lipid substitution on poly(L-lysine) and the DNA delivery efficiency. *ACS Appl. Mater. Interfaces* **2009**, 841–848.

(28) Gabrielson, N. P.; Pack, D. W. Acetylation of polyethylenimine enhances gene delivery via weakened polymer/DNA interactions. *Biomacromolecules* **2006**, *7*, 2427–2435.

phase transition temperature of membranes.[29,30] Increased motility for membrane-traversing molecules is the result after such a change, and this may also occur with polyplexes bearing LA substituents. An assessment of the membrane flux of polyplexes should elucidate this issue, and it may help to reveal if this substituent helps solely with increased uptake or whether additional intracellular events (e.g., endosomal escape or nuclear leakage) are facilitated after LA-substitution.

In conclusion, we have shown that aliphatic lipids can be used to modify low MW (2 kDa) PEI, resulting in improved carriers as compared to the native polymer. The transfection efficiency obtained with some lipophilic low MW (2 kDa) PEIs was equivalent to the more effective, high MW (25 kDa) PEI, but with less of the toxic effects associated with the latter. Such lipophilic PEIs can be used on their own as gene delivery carriers, or in combination with lipid-based reagents for improved delivery.[31] Although lipid substitution was shown to reduce the plasmid DNA binding ability of the PEIs, it was beneficial in delivering a higher amount of plasmid DNA into cells. Further studies will be needed to better elucidate the role of lipid substitution on polyplex dissociation and its impact on transgene expression. It is likely that a larger library of lipid-substituted polymers with a wider range of substitutions will be needed for this purpose. Nevertheless, the beneficial effect of lipid substitutions on transgene expression was evident and the reported carriers should serve as effective reagents for DNA delivery.

(29) Hochgraf, E.; Mokady, S.; Cogan, U. Dietary oxidized linoleic acid modifies lipid composition of rat liver microsomes and increases their fluidity. *J. Nutr.* **1997**, *127*, 681–686.

(30) Roach, C.; Feller, S. E.; Ward, J. A.; Shaikh, S. R.; Zerouga, M.; Stillwell, W. Comparison of cis and trans fatty acid containing phosphatidylcholines on membrane properties. *Biochemistry* **2004**, *43*, 6344–6351.

(31) Lampela, P.; Soininen, P.; Urtti, A.; Männistö, P. T.; Raasmaja, A. Synergism in gene delivery by small PEIs and three different nonviral vectors. *Int. J. Pharm.* **2004**, *270*, 175–184.

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Supporting Information Available: Figures depicting the correlation between the obtained cell viability (293T cells and BMSC) and the extent of lipid substitution on 2 kDa PEI, plasmid delivery to 293T cells and BMSC, and RT-PCR analysis of gene expression on days 3 and 7. This material is available free of charge via the Internet at http://pubs.acs.org.

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