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Lipid substitution on low molecular weight (0.6–2.0 kDa) polyethylenimine leads to a higher zeta potential of plasmid DNA and enhances transgene expression

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ABSTRACT

Cationic polymers are desirable gene carriers because of their better safety profiles than viral delivery systems. Low molecular weight (MW) polymers are particularly attractive, since they display little cytotoxicity, but they are also ineffective for gene delivery. To create effective carriers from low MW polymers palmitic acid (PA) was substituted on 0.6–2.0 kDa polyethylenimines (PEIs) and their efficiency for plasmid DNA (pDNA) delivery was evaluated. The extent of lipid substitution was dependent on the lipid/PEI feed ratio and the polymer MW. While the hydrodynamic size of the polymer/pDNA complexes (polyplexes) increased or decreased depending on the extent of lipid substitution, the ζ potential of the assembled complexes was consistently higher as a result of lipid substitution. Lipid substitution generally increased the in vitro toxicity of the PEIs, but it was significantly lower than that of the 25 kDa branched PEI. The in vitro transfection efficiency of the lipid-substituted polymers was higher than that of native PEIs, which were not at all effective. The delivery efficiency was proportional to the extent of lipid substitution as well as the polymer MW. This correlated with the increased uptake of lipid-substituted polyplexes, based on confocal microscopic investigations with FITC-labeled pDNA. The addition of chloroquine further increased the transfection efficiency of lipid-substituted PEIs, indicating that endosomal release was a limiting factor affecting the efficiency of these carriers. This study indicates that lipid substitution on low MW PEIs makes their assembly more effective, resulting in better delivery of pDNA into mammalian cells.

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1. Introduction

Gene therapy is a promising approach for the treatment of hereditary and acquired diseases [1]. Gene-based therapeutic agents relying on DNA molecules, however, cannot be used on their own and require effective carriers for successful delivery in a clinical setting. Non-viral gene carriers are receiving increasing attention for the delivery of gene-based therapeutics, because of their low cost, flexibility in chemical design and safety [2]. The key steps involved in non-viral gene delivery comprise complexation and condensation of DNA molecules into compact particles, uptake of complexes by the target cells, endosomal escape and dissociation of the complexes to release DNA, which is necessary for expression of the delivered genes [3]. Although several combinations of polyamines and cationic lipids have been employed for gene delivery, amphiphilic polymers that combine a cationic character with hydrophobic domains are more attractive, since they have the beneficial effects of cationic polymers in terms of nucleic acid condensation and compatibility with cellular membranes in single carriers [4–7]. To this end, high molecular weight (MW >20 kDa) polycations were modified with several hydrophobic substituents, including alanine and leucine, C2–C6 aliphatic acids and palmitic acid [8–10]. The performance of the resultant carriers was, however, only marginally increased, if at all, after such modifications. Modification of smaller polycations with hydrophobic moieties has been more successful, dependent on the specific polycation involved. Whereas lipid substitution on 4 kDa poly-L-lysine was not beneficial, substitution of 2 kDa polyethylenimine (PEI) with dodecyl/hexadecyl moieties, cholesterol and aliphatic lipids was shown to increase carrier efficiency, turning a non-functional polymer into an effective carrier of plasmid DNA (pDNA) [8,11–13]. The expected mechanism behind the beneficial effects of hydrophobic modification is improved cell delivery due to increased lipophilicity of the polymer/pDNA complexes [7,14]. However, hydrophobic modification of polymers is expected to change other critical

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properties of the resultant complexes, which were not investigated in previous studies [7,8]. It was not known, for example, whether the hydrophobic modification of polymeric carriers actually resulted in significant changes in hydrophobicity of the complexes or how the sizes and charges of the complexes were altered as a result of hydrophobic modification. These are important considerations not only to reveal the beneficial mechanism(s) behind hydrophobic modification, but also to design more effective carriers in the future.

This study explored the modification of low MW (0.6–2.0 kDa) branched PEIs with the endogenous lipid palmitic acid (PA) for pDNA delivery. Unlike their larger counterparts, these smaller cationic polymers are more attractive as gene carriers since they display minimal interaction with cellular membranes and manifest little cytotoxicity towards clinically relevant cells [15]. If administered in vivo they can be more safely eliminated from the systemic circulation due to their smaller polymeric size. PA was chosen as the substituent lipid due to its ability to modulate protein–membrane interactions and protein trafficking and the fact that it was found it to be a suitable substituent for pDNA delivery in previous studies [13,16]. The objective of this study was to elucidate changes in polymer–DNA complex properties as a result of lipid substitution and to assess the effectiveness of the resultant carriers. A better understanding of the role of hydrophobic substituents in gene carriers will pave the way for improved functional materials for gene delivery. The results of this study show an unexpected benefit of lipid substitution, namely an increased ζ potential of the assembled complexes. Functional pDNA carriers were obtained from lipid-substituted low MW PEI that were as effective as high MW (25 kDa) PEI without the cytotoxic effects of the latter carrier.

2. Materials and methods

2.1. Materials

The PEI with average MWs of 0.6 (PEI0.6) and 1.2 kDa (PEI1.2) were purchased from Polysciences (Warrington, PA). PEI with average MWs of 2.0 (PEI2.0) and 25.0 kDa (PEI25), palmitoyl chloride (PA), triethylamine (TEA), deuterated chloroform ($CDCl_3$), deuterated water (D_2O), 2,4,6-trinitrobenzene sulfonic acid solution (TNBS), fluorescein isothiocyanate isomer I (FITC), Hank's balanced salt solution (HBSS with phenol red), trypsin/EDTA and methylthiazolyldiphenyl tetrazolium bromide (MTT) were obtained from Sigma–Aldrich (St. Louis, MO). Anhydrous chloroform ($CHCl_3$) and diethyl ether were purchased from Fisher Scientific (Fairlawn, NJ). SYBR® Green II was purchased from Cambrex Bio Science (Rockland, MD). Dulbecco's modified Eagle's medium (DMEM), glutaMAX-1 and penicillin/streptomycin (10,000 U ml^{-1} and 10,000 $\mu g\ ml^{-1}$) solution were purchased from Invitrogen (Carlsbad, CA). Fetal bovine serum (FBS) was from VWR (PAA, Ontario, Canada). The plasmids gWIZ and gWIZ-GFP used in the transfection studies were purchased from Aldevron (Fargo, ND). Construction of the plasmid expressing the bFGF and GFP genes (bFGF-IRES-AcGFP) was described previously [18]. A commercially available bFGF ELISA was obtained from R&D Systems (Minneapolis, MN) and used to quantitate bFGF concentrations in the culture supernatants.

2.2. Polymer synthesis and characterization

PEI2.0 (50% aqueous solution) was dehydrated by lyophilization and used for lipid substitution, whereas PEI0.6 and PEI1.2 were used directly. The substituted product was prepared according to a previously published process with slight modifications (Table S1) [14]. Briefly, the starting polymers (0.1 mmol, 60 mg PEI0.6, 120 mg PEI1.2 and 200 mg PEI2.0) were dissolved in chloroform (200 ml) at room temperature by stirring for 30 min and triethylamine (1.15 mmol, 160 μl) was added to the solution and stirred for an additional 30 min period. The mixture was then cooled to ~4 °C and different amounts of PA (27.45, 55.0 and 110.0 mg, corresponding to 0.1, 0.2 and 0.4 mmol in $CHCl_3$) were added dropwise and the mixture stirred at room temperature for 12 h. The final product was collected by precipitation in excess ether.

The structural composition of the lipid-substituted polymers was analyzed by ^1H NMR (Bruker 300 MHz, Billerica, MA) in D_2O using tetramethyl silane (TMS) as an internal reference. The integrated values of the characteristic resonance shifts corresponding to PA (δ ~0.8 ppm, $-CH_3$) and PEI (δ ~2.5–3.0 ppm, $-NH-C_2H_4-$) were used to obtain the extent of lipid substitution (Table S1).

The hydrophobicity of the polymers after lipid substitution was measured by a pyrene extraction assay [17]. Briefly, 25 μl of pyrene solution (4 $\mu g\ \mu l^{-1}$ in ethanol) was added to glass tubes and dried overnight under vacuum. One milliliter of polymer solution of different concentrations (0.5, 1.0, 1.5 and 2.0 mg ml^{-1}) was then added to the tubes and stirred for 24 h at room temperature. The polymer solution was centrifuged for 15 min at 15,000 rpm to remove any insoluble components and the absorbance (A) was measured at 335 nm. As a reference, pyrene absorbance in water (A_0) was determined, and the value of A/A_0 was used as a measure of polymer hydrophobicity.

2.3. Complex formation and characterization

pDNA binding efficiency of the polymers was studied by a dye exclusion assay. The polymers (1 mg ml^{-1}) were diluted with 0.15 M NaCl in polypropylene tubes to give final concentrations of 0–4 $\mu g\ ml^{-1}$. Subsequently, 4 μl of pDNA (25 $\mu g\ ml^{-1}$) was added to each tube (in triplicate) and gently vortexed to form complexes with mass ratios of 0–1 (polymer/pDNA). After 30 min incubation 1.0 ml of 1 SYBR Green II (1×) was added to the tube and 250 μl of each sample was read in black 96-well plates at λ_{EX} = 485 and λ_{EM} = 527 nm to calculate the amount of free pDNA. The sample without polymer was used as a reference standard and pDNA binding efficiency of the polymers was expressed relative to the standard.

Hydrodynamic size and ζ potential of the complexes were measured in aqueous medium. Briefly, the polymers (2 mg ml^{-1}) were diluted in 600 μl of 0.15 M NaCl to give a final concentration of 4.16, 8.33 and 16.66 $\mu g\ ml^{-1}$. Forty microliters of pDNA (25 $\mu g\ ml^{-1}$) was added to the polymer solution to give complexes with mass ratios of 2.5, 5.0 and 10.0 (polymer/pDNA). After 30 min incubation at room temperature the complexes were diluted to 1 ml with 0.15 M NaCl and the size and ζ potential of complexes were measured using a Zetasizer 3000 HS instrument (Malvern Instruments, Malvern, UK) equipped with a He–Ne laser and operated at 10 mW. As a control, polymer solutions were prepared without plasmid DNA and analysed by the Zetasizer. There was no detectable particles in this case, indicating that polymer in solution did not form micelles.

The hydrophobicity of the complexes was also determined by pyrene extraction assay. For this polymer complexes with a mass ratio of 5.0 were prepared in 0.15 M NaCl and subjected to pyrene extraction as described above and A/A_0 for each type of complex determined.

2.4. Cytotoxicity evaluation by MTT assay

The in vitro cytotoxicity of the polymers and the complexes were tested in 293T cells. Cells were seeded in 48-well plates and allowed to grow for 24 h prior to the study in 0.25 ml of DMEM supplemented with 10% FBS, 100 U ml^{-1} penicillin and

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