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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.

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

100 μg ml^{-1} streptomycin under a humidified atmosphere of 95% air, 5% CO_2 at 37 °C. Cell confluence was typically 80–90% at the time of testing. The polymer solution (0.5 μg μl^{-1}) was directly added to each well (in triplicate) to give final concentrations of 1.25, 2.5, 5, 10 and 20 μg ml^{-1}, and the cells were incubated for an additional 24 h. One hundred microliters of MTT solution (5 mg ml^{-1} in HBSS) was added to the cells and incubated for ~2 h. The medium was then replaced with 500 μl of DMSO to dissolve the MTT crystals formed. The absorbance was measured at 570 nm using a microplate reader (ELX 800, Bio-Tek Instruments) as a measure of cell activity. Relative cell viability (%) was calculated by normalizing the absorbance of the polymer-treated cells with the absorbance of untreated cells, which was used as a reference control (i.e. 100% cell viability).

This procedure was also adopted to measure the cytotoxicity of the complexes. The polymer/pDNA complexes were prepared in 150 mM NaCl as above with a mass ratio of 10.0 and added to the cells (in triplicate) for a period of 24 h. The final polymer concentrations were 10 and 20 μg ml^{-1}. The MTT assay was then performed on the cells immediately after 24 h incubation with the complexes (day 1) or on days 3 or 5. In the latter cases the cell culture medium was replaced with fresh medium after the initial 24 h incubation period.

2.5. Polyplex uptake determined by confocal microscopy

The uptake of pDNA was assessed in 293T cells with FITC-labeled gWIZ. For plasmid labeling 20 μl of gWIZ (100 μg μl^{-1}) was incubated with 30 μl of FITC (100 mM in DMSO) in 300 μl of aqueous DMSO (50:50 vol.%) for 3 h at 37 °C. The labeled plasmid was precipitated with 825 μl of ethanol (95%) in the presence of 30 μl of sodium acetate (3 M). The plasmid suspension was cooled to −20 °C for 15 min, centrifuged for 10 min at 13,800 rpm, and washed twice with 95% ethanol. The plasmid collected as a palate was dissolved in ddH_2O to give a 0.4 mg ml^{-1} gWIZ-FITC solution. To investigate pDNA uptake cells were seeded on glass coverslips (Catalog No. 12-545-83, Fischer Scientific) fixed to the bottom of wells and allowed to grow to ~50% confluence before the addition of FITC-labeled complexes. The complexes were prepared in 0.15 M NaCl as described earlier and added (10 μl) to the cells in 12-well multiwell plates in duplicate. After 24 h incubation the cells were washed with HBSS solution and fixed with formaldehyde (3.5% in HBSS) for 30 min. Then the cells were washed with HBSS solution, stained with Hoechst 33258 (500 ng ml^{-1}) for 45 min, mounted on slides using Fluoromount-G and analyzed under a confocal microscope (Leica TCS-SP2 multiphoton confocal laser scanning microscope).

2.6. Analysis of GFP expression by flow cytometry

Transfection efficiency of the polymers was measured as GFP expression in 293T cells using flow cytometry. The plasmids used for transfections were gWIZ without any functional expression system and gWIZ–GFP with a GFP expression system under the CMV promotor. The cells treated with PEI25 complexes and no complexes were used as positive and negative controls, respectively. Complexes with different mass ratios were prepared in 0.15 M NaCl by incubating polymers with pDNA for 30 min at room temperature and directly added (in triplicate) to 293T cells in 24-well plates. The cells were seeded in the multiwell plates 24 h prior to the addition of complexes and typically reached ~50% confluence before complex addition. The cell were incubated with the complexes for 24 h in DMEM supplemented with 10% FBS, 100 U ml^{-1} penicillin and 100 μg ml^{-1} streptomycin in a humidified atmosphere of 95% air, 5% CO_2 at 37 °C and the culture medium replaced with fresh medium containing 10% FBS. The cells were then trypsinized at the desired time points (see figure legends) and fixed using 300 μl of formaldehyde (3.5% in HBSS). GFP expression was quantified using a Beckman Coulter Quanta™ SC flow cytometer using the FL1 channel (3000–5000 events per sample). The instrument settings were calibrated before each run so as to obtain a background level of GFP gene expression of 1–2% for control samples (i.e. untreated cells). The mean fluorescence per cell and the percentage GFP-positive cells were 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 after different post-transfection periods.

In one set of experiments the transfection efficiency of the polymers was investigated in the presence of the endosomolytic agent chloroquine. The cells were treated with chloroquine (50 μM) for 20 min prior to addition of the complexes and the complexes (mass ratio 5.0) were directly added to the cells in triplicate. The chosen chloroquine concentration was based on prior dose–response studies which indicated no apparent effect on cell growth. The cell were incubated with the complexes for 24 h and the culture medium was replaced with fresh medium containing 10% FBS and cultured for 48 h. GFP expression in the cells was studied by flow cytometry as described earlier.

2.7. Analysis of bFGF expression and secretion

As an independent verification of transfection efficiency the polymers were used to deliver pDNA containing a dual expression system for GFP and basic fibroblast growth factor (bFGF–IRES–AcGFP). The bFGF–IRES–AcGFP complexes were prepared with the desired polymers with mass ratios of 5 and 10, and added to the cells of 24-well plates (in triplicate). The complexes were removed after 24 h and the cells incubated with fresh medium for 6 days. The supernatant were recovered for bFGF analysis and the cells for GFP expression as described earlier. For assessment of the bFGF concentration 96-well plates were coated with a capture antibody and the remaining sites blocked with 1% BSA solution. The coated wells were washed with phosphate-buffered saline containing Triton X-100 (0.5%). The supernatants (100 μl) of the transfected cells were added to the wells and incubated for 2 h at room temperature. The wells were then washed and incubated with a biotinylated detection antibody (100 μl) for 2 h, followed by the addition of horseradish peroxidase-conjugated streptavidin solution (100 μl) for 30 min in the dark. After washing, 100 μl of substrate solution was added to the wells and the reaction was stopped after 20 min with 50 μl of 2 N H_2SO_4. The absorbance in individual wells was measured at 450 nm using a microplate reader and converted into a bFGF concentration using a calibration curve generated using the manufacturer supplied bFGF standard.

3. Results and discussion

The lipid-substituted polymers were prepared by N-acylation of PEI with palmitoyl chloride (Fig. 1A) [8]. The relative ratios of PEI to palmitoyl chloride were controlled to give lipid:PEI feed ratios of 1, 2 and 4 during synthesis. ^{1}H NMR analysis showed the expected palmitoyl protons, –CH_3 (δ ~0.8 ppm), γ-CH_2 (δ ~1.26 ppm), β-CH_2 (δ ~1.6 ppm), and α-CH_2 (δ ~2.16 ppm), in the obtained polymers (Fig. 1B). The characteristic resonance shifts corresponding to PA, –CH_3 (δ ~0.8 ppm), and PEI, –NH–C_2H_4– (δ ~2.5–3.0 ppm), were used to obtain the extent of lipid substitution (Table S1). The number of grafted lipids generally increased with increasing feed ratio and PEI MW. At the highest feed ratio of 4.0 the lipid substitutions were 0.3, 2.0 and 3.0 lipids per PEI in PEI0.6, PEI1.2 and PEI2.0, respectively.

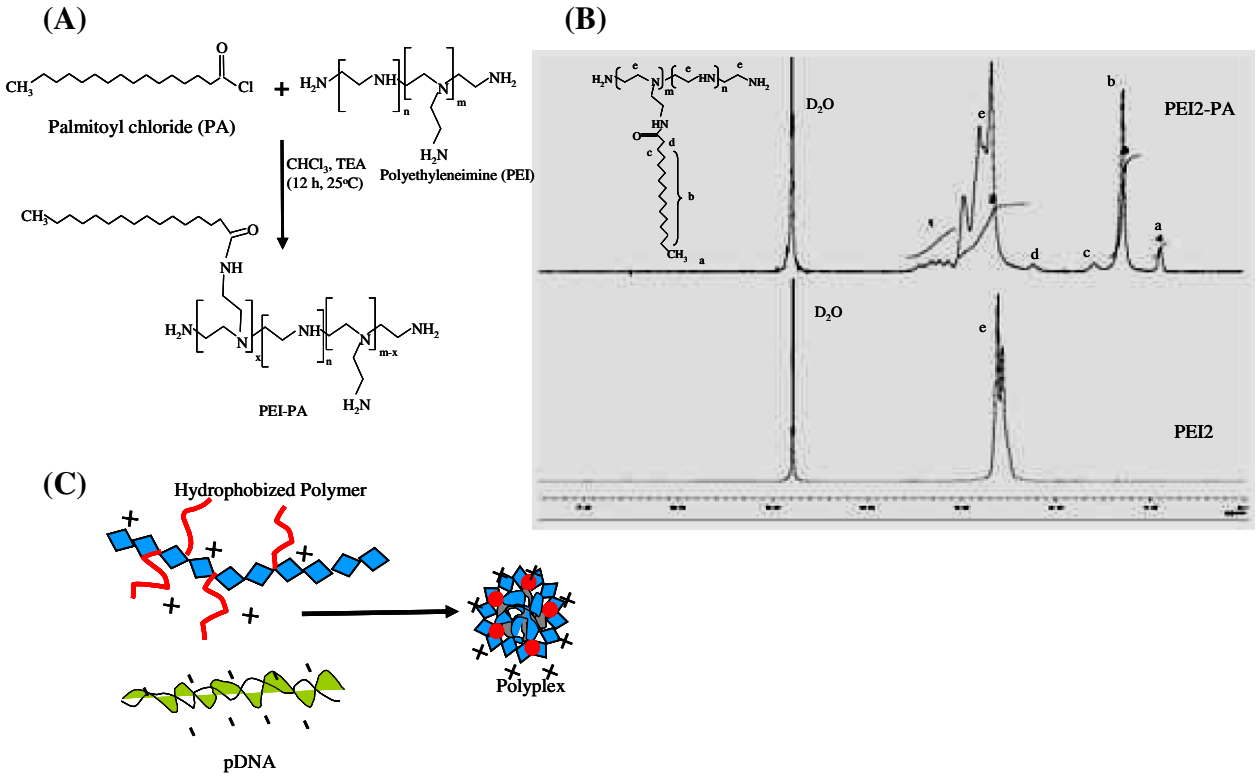

Fig. 1. (A) Schematic illustration of the chemistry of lipid modification of polyethyleneimine (PEI) using palmitoyl chloride (PA). (B) Typical ^{1}H NMR spectra of PEI (PEI2) and lipid substituted PEI (PEI2-PA) in deuterated water (D_2O) and a sketch illustrating polyplex formation between hydrophobized polymers and pDNA at room temperature. The resonances a–d correspond to palmitoyl protons, $-CH_3$ (δ ~0.8 ppm), γ-CH_2 (δ ~1.26 ppm), β-CH_2 (δ ~1.6 ppm), and α-CH_2 (δ ~2.16 ppm), respectively, while the resonance corresponds to PEI $-NH-C_2H_4-$ (δ ~2.5–3.0 ppm).

Chemical modification of PEIs may alter the pDNA binding capacity of polymers due to steric hindrance by the substituents and/or consumption of primary amines on the polymer backbone. Previous studies showed that pDNA binding of hydrophobically modified polymers may decrease after substitution due to the decreased number of protonated amines [9,13,14]. pDNA binding efficiency of the synthesized polymers was studied by the SYBR® Green exclusion assay (Fig. S1). There was no significant difference in pDNA binding by PEI1.2 and PEI2.0 after lipid substitution, and pDNA binding by PEI0.6 was even slightly improved by lipid substitution. Given the low level of modification of the polymers (<20% of primary amines, Table S1), it was not surprising that pDNA binding was unaltered.

The hydrophobicity of the polymers was measured by a pyrene extraction assay, where the partitioning of pyrene into polymer solutions (in 150 mM NaCl) was used as a measure of polymer hydrophobicity [17]. The absorption intensity of pyrene in PEI–PA solutions linearly increased with increasing polymer concentration (Fig. 2A), indicating the hydrophobic nature of the synthesized PEI–PA complexes. This was unlike the PEI solutions (Fig. 2A, only PEI2.0 shown), which gave pyrene absorbances similar to aqueous solutions, indicating minimal hydrophobicity of the native PEIs. The hydrophobicity of the polymer/pDNA complexes (using plasmid gWIZ) was also determined in this assay. The complexes with native PEIs showed no hydrophobicity (Fig. 2B, only PEI2.0 shown), whereas the PEI–PA complexes displayed significant hydrophobicity. The pyrene absorbance of PEI–PA complexes appeared to be lower than the corresponding polymers, indicating an increased hydrophilicity due to complexation of anionic pDNA with the polymers.

The hydrodynamic size and ζ potential of polymer/pDNA complexes (using plasmid gWIZ) were measured in 150 mM NaCl. Significant changes in the hydrodynamic size of complexes were evident as a result of lipid substitution on the polymers (Fig. 3A). The polymer:pDNA mass ratio used to form complexes was a significant determinant of the hydrodynamic size, more so than the extent of lipid substitution on the polymers (Fig. 3B). At lower polymer:pDNA ratios (2.5 and 5) the hydrodynamic sizes of the lipid-substituted complexes were generally larger than the complexes formed with the corresponding native PEIs (Fig. 3B). At higher polymer:pDNA ratios (10) the size of all complexes (~150 nm) was uniformly smaller than the native PEIs (400–600 nm, Fig. 3B). The size of the PEI25 complexes (~100 nm) was slightly lower than the complexes formed with lipid-substituted PEIs at the polymer:pDNA mass ratio of 10. The increase in hydrodynamic size likely reflects the consequences of a lower polymer amine content necessary for pDNA condensation. The decrease in complex size was presumably driven by the high lipid content in the complexes, facilitating stronger hydrophobic associations among the lipids and resulting in more compact particles. In some studies hydrophobic substituents were shown to lead to aggregation of particles, increasing the particle size significantly [14,18,19]. This did not seem to be the case even for the highly substituted PEIs in our hands, which yielded smaller, stable particles with pDNA.

The influence of lipid substitution was clearly evident on the ζ potential of complexes (Fig. 3C). The complexes of native PEIs gave either negative (with PEI0.6) or weakly positive ζ potentials (with PEI1.2 and PEI2.0). After lipid substitution the ζ potentials of the complexes were strongly positive at all mass ratios (25–40 mV, Fig. 3D), and higher than that of PEI25 complexes (~10 mV). The mass ratio also influenced the ζ potential of complexes for PEI–PA from 0.6 kDa PEI (i.e. increasing ζ potential with increasing mass ratio), but this was not the case for 1.2 and 2.0 kDa PEI–PA

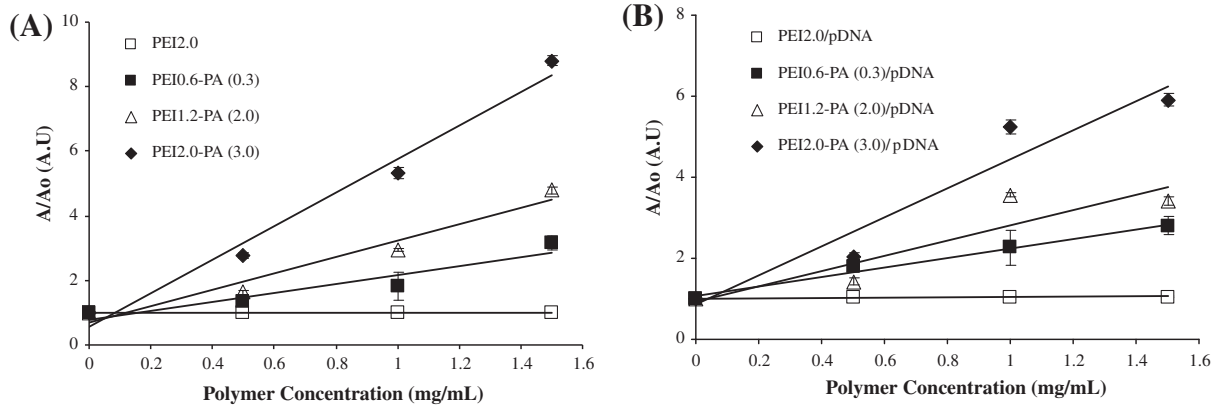

Fig. 2. Pyrene solubility in polymer (A) and complex solutions (B). The PEI-PAs with the highest PA substitutions (0.3, 2.0 and 3.0 PA per PEI for PEI0.6, PEI1.2 and PEI2.0, respectively) were used to assess pyrene solubility. The pyrene solubility was expressed as an absorbance ratio (A/A_0, i.e. absorbance in polymer solution/absorbance in water at 335 nm) and used as an indication of polymer or complex hydrophobicity.

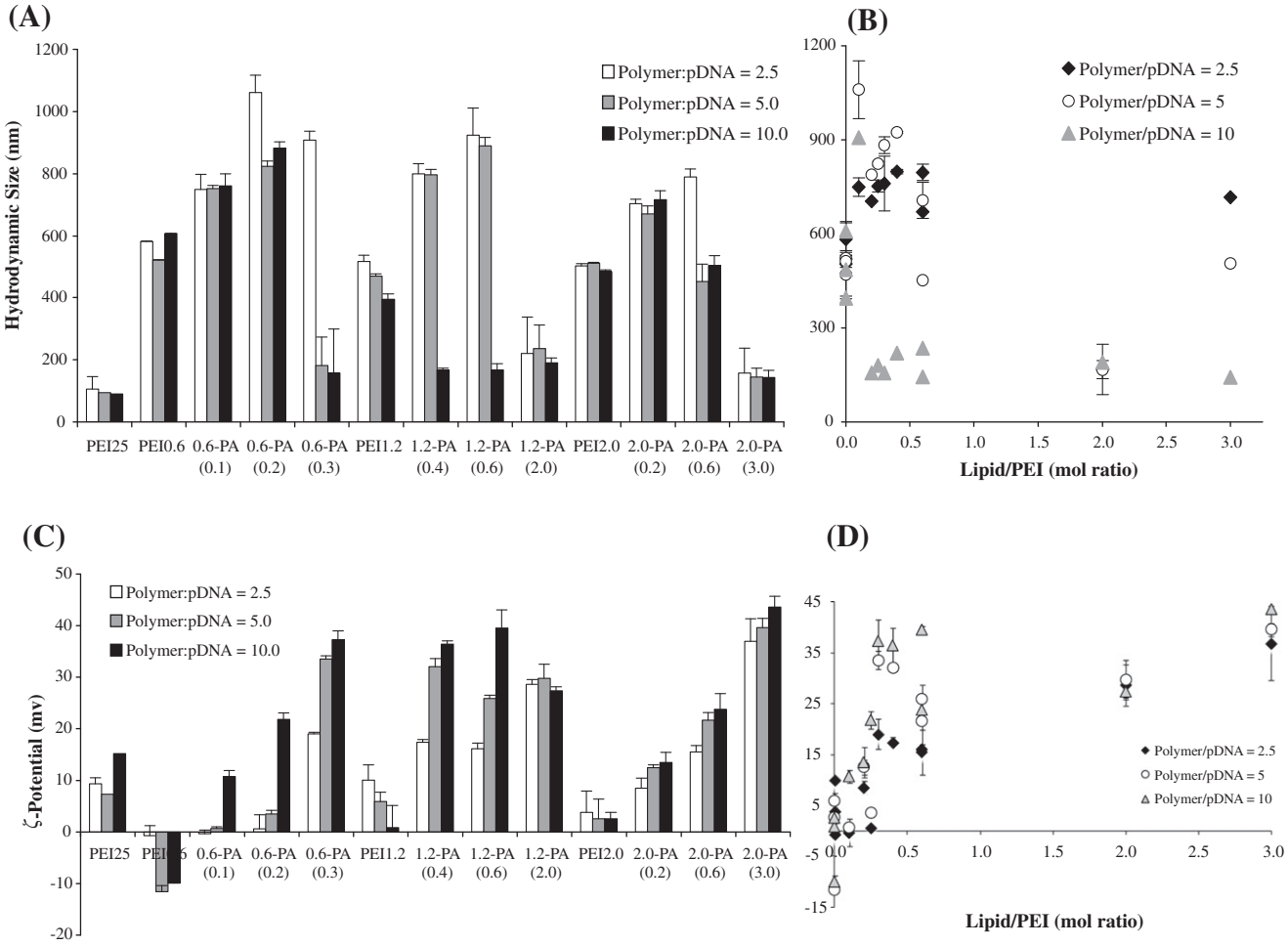

Fig. 3. Hydrodynamic size (A and B) and ζ potential (C and D) of the complexes (polymer:pDNA mass ratios of 2.5, 5 and 10) prepared by incubating the polymers with pDNA in 0.15 M NaCl ($n = 3$). The extent of lipid substitution is indicated by the numbers in brackets next to the polymer used. The data in (B) was obtained from (A), with the complexes of different PEIs pooled for each polymer:pDNA mass ratio used. The complex size increased or decreased depending on the polymer:pDNA mass ratio used to form the complexes. No obvious correlation between the extent of lipid substitution and hydrodynamic size was evident (B). The ζ potential data in (D) was obtained from (C), with the complexes of different PEIs pooled for each polymer:pDNA mass ratio used. The ζ potential increased significantly with the extent of lipid substitution for all polymers. The polymer:pDNA mass ratio did not have a substantial effect on the ζ potential.

complexes, whose complexes gave a relatively constant ζ potential (Fig. 3C). Whereas one may expect a lower ζ potential on using polymers with fewer amine groups, our data indicated an opposite trend in this respect. Lipid–lipid interactions in complexes are likely to facilitate more stable assembly of polymers on soluble pDNA, hence increasing the overall ζ potential. The presence of li-

pid moieties may also shield the cationic charges in complexes, preventing their repulsive interactions and thereby stabilizing the complex.

The influence of lipid substitution on cytotoxicity was tested in 293T cells using the MTT assay [13]. Cell viability in the presence of the parent polymers PEI0.6, PEI1.2 and PEI2.0 was unaffected at all concentrations tested (up to 20 μg ml^{-1}) (not shown), indicating minimal interaction of these polymers with the cells. Polymers with the highest lipid substitution imparted some toxicity depending on the MW of the PEI; lipid substitution on PEI0.6 did not result in any additional toxicity, whereas significant toxicity was evident with PEI1.2-PA and PEI-2.0-PA for the highest substituted polymers (not shown). The relative cell viability was also evaluated after treating the cells with pDNA complexes (using plasmid gWIZ). At 10 μg ml^{-1} (equivalent polymer concentration, Fig. 4) some toxicity was evident for higher MW PEIs (1.2 and 2.0 kDa) at the highest lipid substitution. A similar result was obtained after treating the cells with 20 μg ml^{-1} complexes (equivalent polymer concentration), but the polymers with higher MW PEIs (1.2 and 2.0 kDa) at the highest lipid substitution (PEI1.2-PA (2.0), PEI2.0-PA (0.6) and PEI2.0-PA (3.0)) showed toxicity until days 1 and 3, however, cell viability had recovered after 5 days culture (Fig. 4C). The viability of the cells treated with PEI25 complexes was low after 24 h, but unlike the lipid-substituted PEIs, cell viability did not recover after PEI25 treatment, even on day 5 (Fig. 4C). The increased lipophilicity of the complexes is expected to improve the interaction of complexes with the cells. The observed toxicity is likely to be an inevitable outcome of the increased association with cellular membranes, which can lead to their disruption and a loss of cell viability [20]. However, the observed toxicity of PEI–PAs seems to be transient and the cells were able to recover from the initial effects of the polymers. The cytotoxicity observed in this study is unlikely to be due to the specific lipid grafted (i.e. palmitic acid), since the 2 kDa PEI modified with other lipids (e.g. myristic acid, stearic acid) also displayed increased toxicity [13].

The cellular uptake of pDNA was assessed by confocal microscopy after exposing 293T cells to complexes formed by the FITC-labeled plasmid gWIZ. Distinct uptake profiles of the complexes were evident (Fig. 5). There was no cellular uptake of FITC–gWIZ plasmid alone without any polymer. The cells exposed to the complexes of native PEIs gave some cell-associated complexes that were dependent on the MW of PEI. Whereas PEI0.6 complexes were not associated with the cells, some PEI1.2 and PEI2.0 complexes were associated with the cells. This observation was in line with previous studies that described better uptake of complexes as the MW of the PEI was increased [21]. The cellular uptake of FITC–gWIZ complexes was significantly increased when the complexes were formed with lipid-substituted PEIs. In this case no distinct differences between the three lipid-substituted PEIs of different MWs were evident based on qualitative microscopic observations (Fig. 5). The complexes appeared as distinct particles, mostly around the nucleus, after 24 h uptake. The uptake of complexes with the lipid-substituted PEIs was similar to that of PEI25/pDNA complexes.

Transfection efficiency of the polymers was measured in 293T cells by flow cytometry. The plasmids used for transfections were the control plasmid gWIZ (i.e. without a functional expression system) and plasmid gWIZ–GFP with a green fluorescent protein (GFP) expression system. With other cells (e.g. skin fibroblasts [21] and bone marrow stromal cells [22]) cellular exposure to control complexes yielded GFP-like fluorescence at times, indicating the induction of autofluorescence as a result of complex exposure per se, rather than functional GFP expression. However, there was no GFP fluorescence detected in 293T cells treated with gWIZ complexes in this study (not shown), indicating negligible autofluorescence of cells after treatment with the lipid-substituted PEIs. All

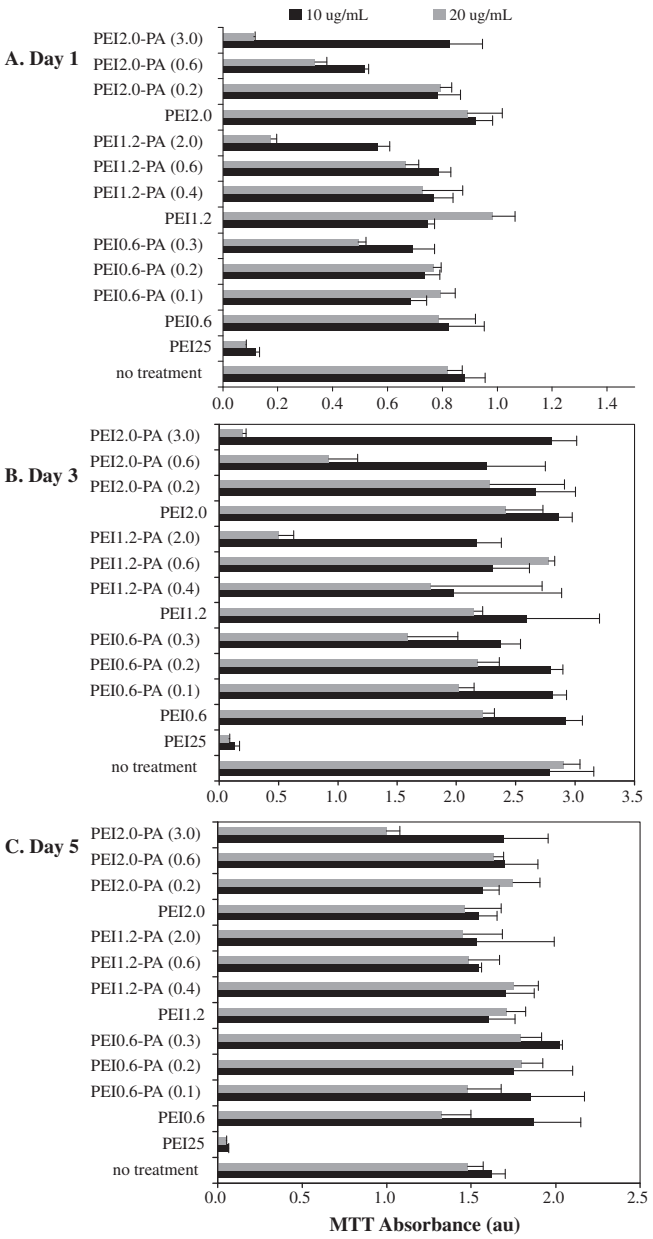

Fig. 4. The viability of cells treated with complexes (polymer:pDNA mass ratio = 10.0) at polymer concentrations of 10 and 20 μg ml^{-1} after 1 (A), 3 (B) and 5 (C) days. The cell viabilities are based the formazan absorbance in the MTT assay. The samples on day 5 were diluted (×2) with DMSO to maintain the absorbance within the readable range of the plate reader. Cell viability generally decreased at higher lipid substitutions (indicated by the numbers in brackets next to the polymer used), but had recovered by the end of day 5. This was unlike cells treated with PEI25 complexes, the viability of which did not recover.

native PEIs showed a negligible (<4%) population of GFP-expressing cells after treatment with gWIZ–GFP complexes, whereas lipid-substituted PEIs yielded a significant proportion of GFP-positive cells (Fig. 6A). The extent of GFP expression was relatively lower with the smaller PEI0.6-PAs and higher GFP expression was obtained with more substituted PEIs, although the latter relationship was not always linear. The increased GFP expression was in line with improved cellular delivery of pDNA with lipid-substituted polymers, as observed by confocal microscopy. For each different MW PEI the polymer with the highest lipid substitutions was selected for further study.

The effect of polymer:pDNA mass ratio on transfection efficiency was investigated by using complexes prepared at mass ratios of 2.5,

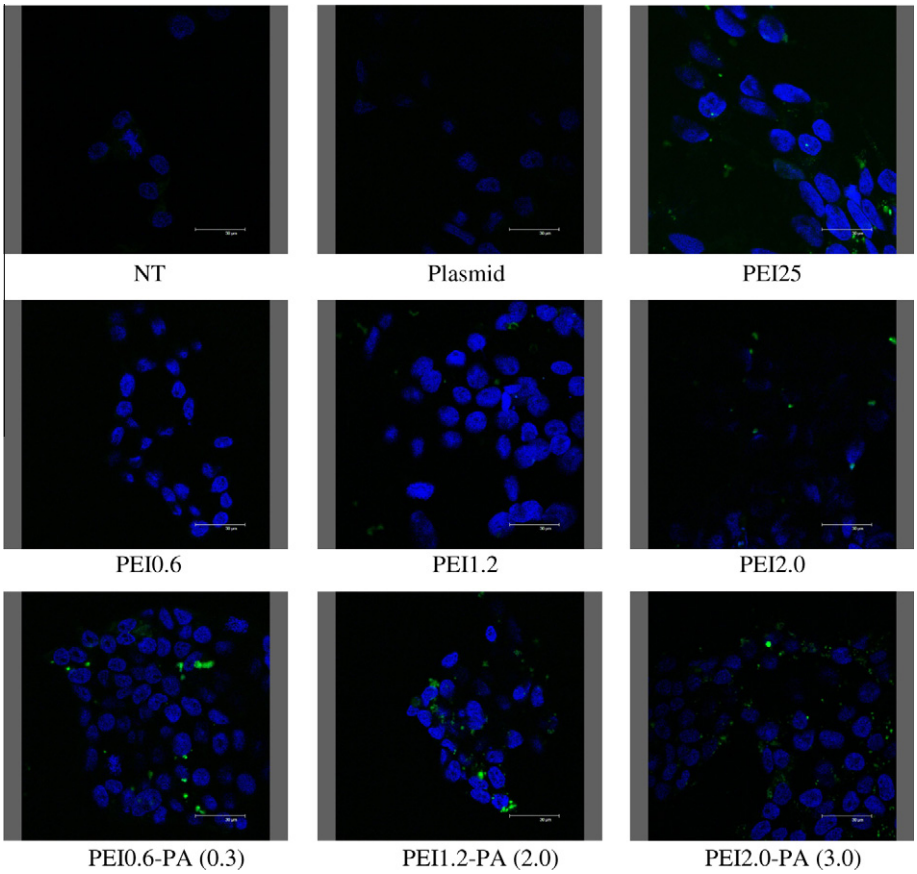

Fig. 5. Representative confocal images of 293T cells treated with the complexes (polymer:pDNA mass ratio of 5.0) for 24 h. Fluorescently labeled pDNA (FITC–gWIZ, green) was used in complexes to visualize uptake, while the cell nucleus was stained with Hoechst 33258 (blue). Note (i) the lack of uptake in the absence of a polymeric carrier (gWIZ alone) and (ii) the more pronounced complex uptake with the lipid-substituted PEIs compared with complexes of native PEIs.

5.0 and 10.0. Based on the percentage of cells positive for GFP (Fig. 6B), there was an increase in transfection efficiency as the mass ratio of PEI–PA complexes increased. The native PEIs were ineffective at all polymer:pDNA ratios and the higher MW PEIs were generally more effective than the low MW PEI (PEI0.6–PA). No obvious differences were observed for the PEI–PAs derived from the 1.2 and 2.0 kDa PEI (Fig. 6B). Given the dependence of hydrodynamic size on the mass ratio, efficient transfection was correlated with the smaller complex sizes. Although larger complexes are sometimes known to sediment [24] and give higher cellular uptake, sedimentation did not appear to be a significant factor, since the smaller complexes were most effective in our hands.

GFP expression by transfected cells as a function of time is summarized in Fig. 6C. The percentage of GFP-positive cells exposed to the PEI25 complexes gradually decreased (from ~80% to ~40%) from days 2 to 7, while remaining constant with the lipid-substituted PEIs (Fig. 6C). After 7 days the levels of gene expression were equivalent for PEI25 and the higher MW PEI–PA complexes. A unique feature of the lipid-substituted PEIs is the steady transfection for up to 1 week. The declining efficiency of PEI25 complexes was attributed to the treatment toxicity, where the transfected cells were gradually eliminated from the population due to excess cytotoxicity in this cell population. More cell compatible PEI–PAs presumably did not result in such selective cell depletion. Although the initial transfection efficiency of PEI25 seemed to be higher in these studies, this might be misleading, since such a high percentage was obtained but with a significant loss of cell viability (>50%) (from Fig. 4). The fact that cells treated with PEI–PA complexes displayed higher viabilities than the PEI25-treated cells, as well as displaying a rapid recovery of cell mass on prolonged cell culture, compensates for the relatively lower efficiency of the PEI–PAs during the initial stages of transfection.

For an independent verification of relative transfection efficiency, the lipid-substituted PEIs were also used to deliver a plasmid designed for bFGF and GFP co-expression (plasmid bFGF–IRES–AcGFP with an internal ribosomal entry site) [22]. The results indicated that, unlike the cells treated with plasmid alone, the cells treated with PEI–PA complexes of bFGF–IRES–AcGFP gave significant bFGF secretion (Fig. S2). Similar to previous results, (i) the PEI–PAs derived from the higher MW PEIs (1.2 and 2.0 kDa) were more effective than the 0.6 kDa PEI derived polymer, (ii) increasing the polymer:pDNA mass ratio from 5.0 to 10.0 improved bFGF secretion for the effective polymers, and (iii) the native PEIs were ineffective in bFGF expression and secretion. There was a good correlation between GFP expression and the bFGF secretion from this plasmid (Fig. S2).

Finally, the efficiency of polymers was investigated in the presence of the endosomolytic agent chloroquine to determine endosomal escape of internalized complexes [23]. Chloroquine did not improve the transfection efficiency of native PEIs (Fig. 7). It is likely that the lack of cellular plasmid delivery was the reason for the lack of GFP expression with these polymers, rather than the inability of internalized complexes to escape the endosomal compartment. No chloroquine effect was obvious for PEI25, with some transfection parameters being improved (e.g. per cent GFP-positive cells at a mass ratio of 5.0) but others reduced (e.g. mean GFP expression at a mass ratio of 10.0). This result is consistent with the well-known ability of PEI25 to escape the endosomal compartment on its own [24]. However, chloroquine consistently improved the transfection efficiency of all PEI–PAs (Fig. 7), indicating incom-

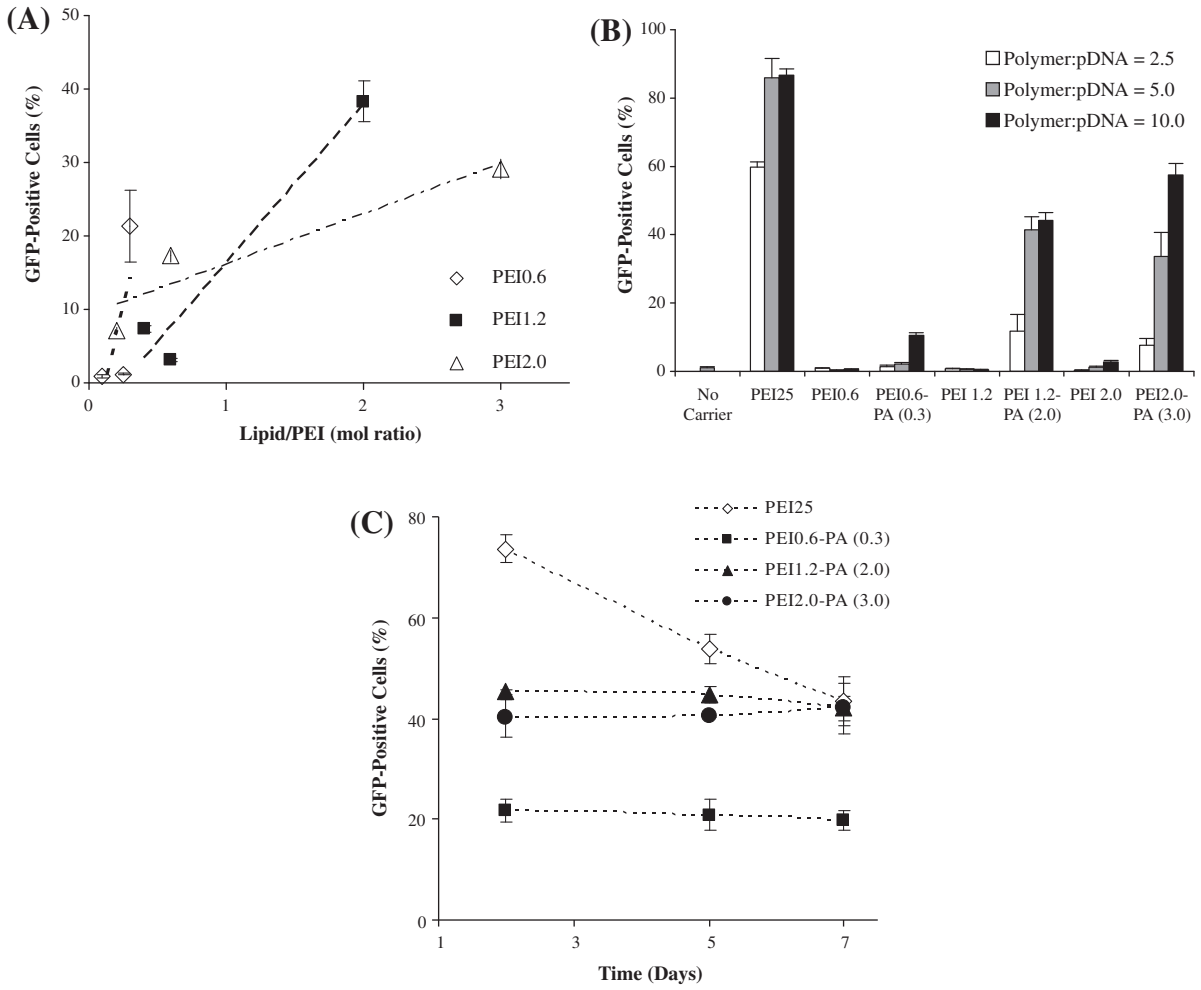

Fig. 6. (A) Transfection efficiency as a function of lipid substitution (polymer:pDNA mass ratio of 5). The results are summarized as per cent GFP-positive cells (means ± SD of triplicate wells). The transfection efficiency of the polymers linearly increased with the extent of lipid substitution (dashed lines to guide the eye). (B) Transfection efficiency with complexes formed at polymer:pDNA mass ratios of 2.5, 5.0, and 10.0. 0.6, 1.2 and 2.0 kDa PEIs with the highest lipid substitution were selected and the results are summarized as per cent GFP-positive cells (means ± SD of triplicate wells). Note the increased transfection efficiency with increasing mass ratio used for pDNA complexation. (C) Changes in transfection efficiency (complexes at a mass ratio of 10.0) with time. The results are summarized as per cent GFP-positive cells (means ± SD of triplicate wells). The efficiency of PEI25 decreased significantly with time, while the extent of transfection remained constant for PEI–PA polymers.

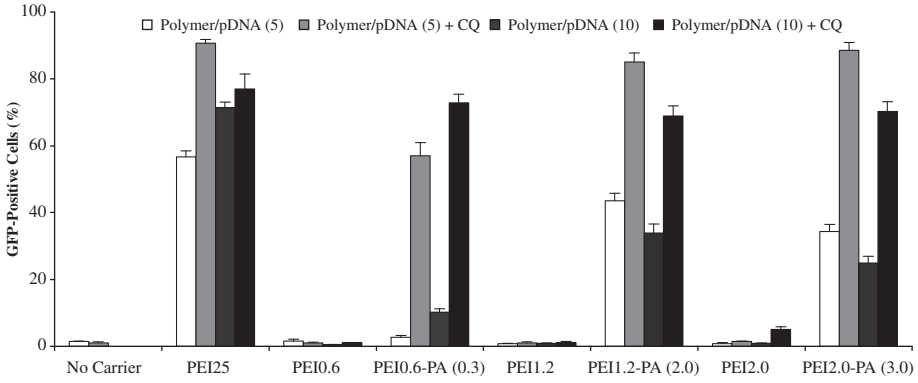

Fig. 7. Transfection efficiency in the absence and presence of chloroquine for cells treated with complexes at mass ratios of 5 and 10. The results are summarized as per cent GFP-positive cells (means ± SD of triplicate wells). A beneficial effect of chloroquine was observed with all lipid-substituted PEIs, but not with native PEIs or PEI25 complexes at a mass ratio of 10.

plete release of the internalized complexes from the endosomal compartment. The lower efficiency of PEI–PAs observed initially compared with PEI25 might be attributed to this. One would expect PA substitution to impart fusogenic properties and facilitate endosomal escape, because of its structural analogy to cationic lipids. However, the structural geometry of the complexes with PA-substituted polymers may not be a regular micelle, like that of cationic lipids, and may not assist the endosomal release

through a flip-flap mechanism [1]. Cationic (i.e. lysine-rich) peptides grafted with PA were also shown to be affected by chloroquine treatment, so that grafting lipids onto polymeric carriers might not always translate into fusogenic activity [25]. Additional effectors such as peptides might be necessary in order to facilitate the release of cationic polymers.

It should be stated that the transfection experiments in this study were successfully performed in the presence of serum. Lipid modification of the polymers and, hence, the polyplexes might be expected to increase serum protein opsonization (due to a higher charge and/or hydrophobicity), but this issue did not seem to be a significant impediment for in vitro transfection in our hands. Serum proteins are known to influence (either increase or decrease) transfection efficiencies [26,27], and this issue will be explored in future studies. It will be especially critical for in vivo applications of the polymers, where increased hydrophobicity may affect opsonization (and subsequent immune recognition) and particle–particle aggregation, influencing the pharmacokinetics and biodistribution of the particles.

4. Conclusions

PA substitution on low MW (0.6–2.0 kDa) PEIs through N-acylation is an effective approach to increase the lipophilicity of polymers and the resulting complexes after pDNA binding. Although the size of the complexes formed with PA-substituted polymers was influenced by factors other than lipid substitution (e.g. polymer:pDNA mass ratio), the assembled complexes consistently displayed enhanced ζ potentials (+35–40 mV) and increased cellular uptake after PA substitution on PEI. The transfection efficiency of the polymers was accordingly enhanced after lipid substitution; a 1.2 kDa PEI with $\sim$2 PA moieties per PEI was an effective carrier for transgene expression. An undesirable feature of the lipid substitution was increased cytotoxicity of the PEIs, but this effect was transient and the cells rapidly proliferated to overcome this, unlike the high MW (25 kDa) PEI. The efficiency of the PA-substituted PEIs was further increased in the presence of chloroquine, indicating that the endosomal barrier was still a factor limiting the efficiency of the internalized complexes and providing further possibilities to enhance the transfection efficiency of these lipid-substituted PEIs.

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Appendix A. Figures with essential colour discrimination

Certain figures in this article, particularly Figures 1 and 5, are difficult to interpret in black and white. The full colour images can be found in the on-line version, at doi:10.1016/j.actbio.2011.01.027.

Appendix B. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.actbio.2011.01.027.

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