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Small hydrophobe substitution on polyethylenimine for plasmid DNA delivery: Optimal substitution is critical for effective delivery

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ABSTRACT

Cationic polymers have been turned into effective gene delivery agents by functionalizing with long-chain aliphatic lipids, but little information exists if small hydrophobic moieties can serve as effective substituents for this purpose. To explore this issue, we modified small molecular weight (1.2 kDa) polyethylenimine (1.2PEI) by a small hydrophobe, propionic acid (PrA), through *N*-acylation and investigated the efficacy of resultant polymers to deliver plasmid DNA (pDNA) to breast cancer cells MDA-231 and MCF-7. A significant impact of PrA grafting was observed on physicochemical features of polymers and resultant pDNA complexes. pDNA binding capacity, as measured by BC_{50} (weight ratio for 50% binding), was decreased from 0.25 to 0.64 with PrA substitution. Hydrodynamic size of polymer/pDNA complexes was not altered, but the surface charge (ξ-potential) was increased with low PrA substitution and decreased at higher PrA substitutions. Similarly, *in vitro* pDNA transfection efficacy in MDA-231 and MCF-7 cells was significantly increased with PrA grafting and optimum efficacy was observed in polymers with modest substitution, 0.25–1.0 PrAs/PEI (mol/mol), but higher substitutions was detrimental to transfection. The transfection efficiency of PEI-PrAs was higher than aliphatic lipid (linoleic acid) substituted PEI and more stable than 25 kDa branched PEI. However, unlike studies reported elsewhere, siRNA had no effect on transfection efficacy of pDNA/PEI-PrA complexes when used as an additive. We conclude that small hydrophobe substitution on low MW PEI converts it into effective pDNA delivery agent in breast cancer cells up to an optimal ratio, indicating that balancing hydrophobicity of polymer is critical for pDNA transfection.

Statement of Significance

This manuscript investigated the influence of small hydrophobe (propionic acid, PrA, 3 carbon) grafted onto small molecular weight polyethylenimine (1.2PEI) in pDNA delivery. We have explored this approach as an alternative of common strategies to graft long chain and/or bulky lipids [linoleic acid (18 carbon), cholesterol]. At optimal substitution, transfection efficiency of these polymers was significantly higher than long chain lipid substituted 1.2PEI, emphasizing a proper hydrophobic/hydrophilic balance for optimum gene delivery. The overall results establish the feasibility of using small hydrophobes to create functional carriers, as long as the polymers are engineered with optimal ratio of substituent. The reported studies should facilitate the efforts of biomaterials scientists and engineers to design new carriers for gene therapy.

1. Introduction

Improved delivery systems are needed for intracellular delivery of difficult-to-deliver biologics such as polynucleotides. Cationic polymers have been developed as non-viral gene delivery agents due to their advantage of being safe, reproducible, and allowing

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facile chemistry for functionalization, as compared to their viral counterparts [1–3]. Cationic polymers can complex with anionic plasmid DNA (pDNA) via electrostatic interactions, and condense them into compact and nano-sized polyplexes, which provide sufficient protection from extracellular nucleases and enable effective cellular uptake [4]. To date, the clinical application of cationic polymers has been hampered by their low transfection efficiency and undesirable toxicity. In order to address this issue, cationic polymers have been modified with various substituents to improve gene delivery efficacy and compatibility [5–7]. Hydrophobic modification of cationic polymers with long lipid chains has been a common approach since grafted hydrophobic lipids improve the compatibility of condensed DNA polyplexes with cellular membranes and facilitate the endocytosis of the polyplexes [8]. Palmitic acid substitution on poly-L-Lysine (PLL), for example, has shown higher binding efficiency to pDNA and resulted in enhanced gene transfection as compared to unmodified PLL [9].

Among the cationic polymers, polyethylenimine (PEI) based polymers have become well-established for both *in vitro* and *in vivo* applications [10,11]. PEIs display strong buffering property at acidic pH to escape endosomes via a process called 'proton sponge' activity [12]. Presence of abundant primary, secondary and tertiary amine groups makes it also possible to undertake hydrophobic modifications, while preserving its buffering capacity. While high molecular weight (MW) PEIs, and in particular branched 25 kDa PEI (25PEI), have emerged as broadly active gene delivery agents, they can cause severe cellular and systemic toxicities due to high cationic charge density that destabilizes plasma and mitochondrial membranes [13]. Employing lower MW forms of PEI for gene delivery, on the other hand, is advantageous because of their low toxicities; these polymers display less interaction with plasma membranes and can be readily eliminated from the circulation *in vivo* [5,14,15]. Unfortunately, low MW PEIs are not also effective for gene delivery [5,16], but it is possible to chemically functionalize them with hydrophobic groups to improve their efficiency [5,17,18]. Modification of 0.4 kDa linear PEI with cholesterol improved transgene expression when the modified polymer were formulated as a liposome [19]. Similarly, cholesterol-substituted 1.8 kDa PEI was a superior gene delivery agent than the native polymer [20]. Besides this multicyclic steroid, 1.8 kDa PEI were modified with long-chain dodecyl and hexadecyl moieties with significantly improved gene delivery results [21]. We also reported hydrophobic modification of low MW PEIs with aliphatic lipids of variable chain lengths and demonstrated improved gene delivery efficacy in different cell lines including primary cells [5,22]. Among the lipids, unsaturated linoleic (C18) acid was found to be a superior lipid substituent for pDNA delivery [5]. However, hydrophobic modification of PEI and PEI-like polymers with long aliphatic chains and bulky multicyclic groups do not allow good control over grafting efficiency. Beyond a critical grafting amount, polymers become insoluble in aqueous systems and grafting efficiency is not well controlled due to bulky lipid substituents. Undertaking modification with smaller hydrophobic moieties may be advantageous in these aspects, but no information exists about using small hydrophobic moieties to convert low MW PEI into an effective transfection agent.

This study was designed to investigate the potential of a particular short chain hydrophobe, namely propionic acid (PrA), for grafting onto 1.2 kDa PEI (1.2PEI) in order to improve its pDNA delivery efficiency. Delivery of pDNA was explored in two models of breast cancer cells (MDA-231 and MCF-7 cells), due to urgent need to explore alternative treatments for cancer therapy. Since an optimal balance between the cationic charge (i.e., buffering capacity and pDNA binding) and hydrophobicity (i.e., cell membrane compatibility) is critical for enhanced cellular uptake and unpacking of complexes, extent of PrA substitution on PEI was varied, and physiochemical characteristics and transfection efficiency of the resultant polyplexes were investigated. Our goal was to convert the relatively non-toxic but ineffective polymer onto an effective pDNA delivery agent.

2. Materials and methods

2.1. Materials

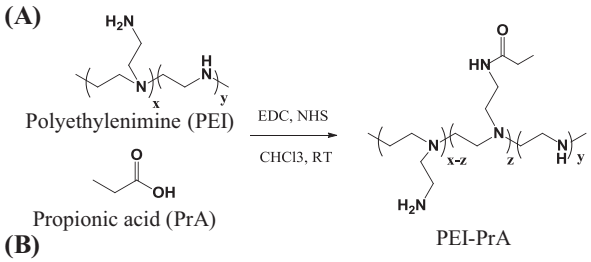
The 1.2PEI (M_n:1.1 kDa, M_w:1.2 kDa), 2PEI (2PEI; M_n: 1.8 kDa, M_w:2 kDa), 25PEI (M_n: 10 kDa, M_w: 25 kDa), dimethyl sulfoxide (DMSO), PrA, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), N–hydroxysuccinimide (NHS), N,N-dimethyl-formamide (DMF), linoleyl chloride, chloroform ($CHCl_3$), methanol (MeOH) and diethylether, trypsin/EDTA solution, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were obtained from Sigma–Aldrich (St. Louis, MO). Hank's balanced salt solution (HBSS with phenol red) was purchased from Lonza (Walkersville, MD). Dulbecco's Modified Eagle's Medium (DMEM; low glucose with L-glutamine) and penicillin/streptomycin (10,000 U/ml and 10,000 μg/ml) solution were purchased from Life Technologies (Grand Island, NY). Fetal bovine serum (FBS) was from VWR (PAA, Ontario, Canada). Clear HBSS (phenol red free) was prepared in–house. UltraPure™ agarose was purchased from Invitrogen (Carlsbad, CA). The gWIZ and gWIZ-GFP plasmids were purchased from Aldevron (Fargo, ND). The scrambled control siRNA (CsiRNA) and FAM-siRNA was obtained from Ambion (Austin, TX).

2.2. Polymer synthesis and characterization

Hydrophobic modification of 1.2PEI and 2PEI using PrA was performed via *N*-acylation (Fig. 1A). Briefly, PrA (3.34 mM in $CHCl_3$) was activated with EDC (5 mM in $CHCl_3$) for 30 min and then with NHS (5 mM in MeOH) at room temperature. The activated PrA solution was added dropwise to 1.2PEI or 2PEI solutions (3.34 mM in $CHCl_3$) under stirring and left stirring over night at room temperature. The crude product of PrA-grafted PEI (PEI-PrA) was precipitated (3×) in ice-cold diethylether and dried under vacuum for 48 h. To prepare a lipid-substituted 1.2PEI, *N*-acylation of 1.2PEI was performed with the linoleyl chloride according to previously described procedure [5]. Briefly, linoleyl chloride was dissolved in DMF and added to 100 mg of PEI solution in 1 ml of DMSO. This mixture was allowed to react for 24 h at room temperature under nitrogen. Polymer was recovered by precipitating with excess of ethyl ether, and freeze dried. The composition of PEI-PrA and PEI-LAs (i.e., number of PrA/LA groups per 1.2PEI) was elucidated through ^{1}H NMR spectroscopy (Bruker 300 MHz, Billerica, MA) using TMS as an internal standard in D_2O. Buffering capacity of PEI-PrAs was determined by acid-base titration as described earlier [23]. The polymer solution (1 mg/mL) was set at pH 10.0 and titrated with HCl (0.1 M) up to pH 2.0. As a control 25PEI and 1.2PEI were titrated. Here buffering capacity of the polymers was defined as percentage of amines protonated from pH 7.4–5.1 and it was quantified with the protocol described earlier [24].

2.3. DNA binding by polymers

pDNA binding capacity of the polymers was elucidated by agarose gel retardation assay using 0.8% of agarose gel containing ethidium bromide (1 μg/mL). The stock polymer solution (1 μg/μL) was diluted in ddH_2O in polypropylene tubes to give final concentrations between 0 and 0.05 μg/μL (final volume of 22 μL). Subsequently, 2 μL of pDNA solution (gWIZ at 0.275 μg/μL) was added to each tube and gently vortexed to get complexes from 0 to 2.0 polymer:pDNA (w/w) ratios. The complexes were incubated for



(B)

Polymer ID	Feed Ratio (mol/mol)	Calculated, NMR (mol/mol)
PEI-PrA0.5	0.5	0.28
PEI-PrA1	1.0	0.76
PEI-PrA2	2.0	1.10
PEI-PrA4	4.0	1.6

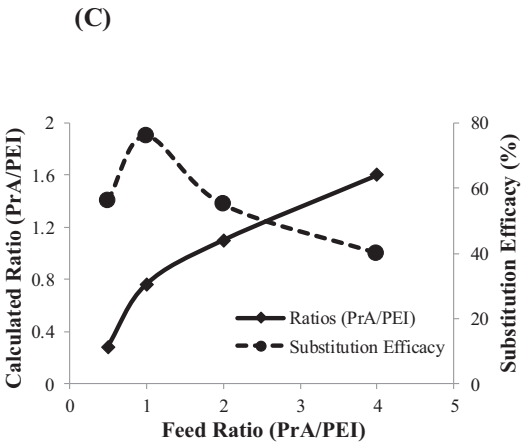


Fig. 1. Scheme for synthesis of PEI-PrA polymers (A), the obtained PrA substitutions (B and C) and substitution efficiency (C) as a function of PrA:polymer feed ratio. The number of PrA substituted was increased with feed ratio, but the substitution efficiency peaked at the feed ratios of 1.0.

30 min at room temperature and mixed with loading buffer (4 μL), loaded to agarose gel, electrophoresed for 45 min at 120 mV and pDNA bands were visualized under UV illumination (Alpha Imager EC). Binding capacity of the polymers was quantified and expressed as BC_{50} (polymer required for 50% pDNA binding) by the quantification of free pDNA in lanes.

2.4. Size and ξ-potential of pDNA/polymer complexes

Hydrodynamic diameter (Z-average) and surface charge (ξ-potential) of polymer/pDNA complexes was studied in ddH_2O through dynamic light scattering (DLS) and electrophoretic light scattering (ELS) using Zetasizer Nano-ZS (Malvern, UK) equipped with He-Ne laser and operated at 10 mW. Freshly prepared complexes (polymer:pDNA = 2.5, 5.0, 10.0 w/w) were diluted to 1 mL ddH_2O for each measurement.

2.5. Cell culture

Human breast cancer cells MDA-231 and MCF-7 used as model cell lines were obtained from Dr. Michael Weinfeld (Department of Oncology, U. of Alberta) and Dr. Afsaneh Lavasanifar (Faculty of Pharmacy & Pharmaceutical Sciences, U. of Alberta), respectively. Cells were maintained in DMEM supplemented with 10% FBS, 100 unit/mL Penicillin, 100 μg/mL Streptomycin under a humidified atmosphere (95/5% air/CO_2) at 37 °C. Cells were typically sub-cultured once a week using 1:10 dilution.

2.6. Cytotoxicity assay

In vitro cytotoxicity of PEI-PrAs/pDNA complexes (2.5, 5.0, 7.5, 10.0, 15.0 and 20.0, w/w) was evaluated in MDA-231 and MCF-7 cells using the MTT assay. Cells without any treatment were used as negative controls. Cells were seeded in 48-well plates at a density of 50,000 cells/well and allowed to attach for 24 h (250 μL medium/well). The complexes were prepared in serum free DMEM and directly added to each well and incubated for 24 h with complete culture medium under a humidified atmosphere (95/5% air/CO_2) at 37 °C. The culture medium of each well was replaced with 250 μL of fresh medium after 24 h and the cells were incubated for another 24 h. The MTT reagent (5 mg/mL) was added to each well to give final concentration of 1 mg/mL and incubated for 1 h. The medium was replaced with DMSO (200 μL) to dissolve formazan crystals and the optical density of the solution was measured in universal microplate reader (ELx; Bio-Tech Instrument, Inc.) at λ = 570 nm. The cell viabilities were expressed as a percentage of non-treated cells.

2.7. In vitro uptake of PEI-PrA/pDNA complexes

The uptake of PEI-PrA/pDNA complexes was assessed in MDA-231 and MCF-7 cells through flow cytometry and confocal microscopy using Cy^{TM3}-labelled pDNA (labeling following the protocol of the manufacturer). The cells were seeded in 24 well plate and grown overnight. The complexes with polymers and Cy^{TM3}-labelled pDNA of different composition, and with or without CsiRNA (see Section 3) were prepared. Then, cells were treated with these complexes. After 24 h of treatment, cells were washed (3×) with HBSS, trypsinized and fixed with formaldehyde (300 μL, 3.7% in HBSS) and analyzed by flow cytometer. For confocal microscopy study, MDA-231 cells were seeded on cover slips (15 mm diameter) inserted into 6 well-plates and grown overnight (~50% confluences). Complexes were prepared as described above and were directly added to cells and incubated for 24 h under a humidified atmosphere (95/5% air/CO_2) at 37 °C. Cells were then washed (3×) with HBSS (pH 7.4) and fixed with 1 mL formaldehyde (3.7% in HBSS) for 30 min and washed with ddH_2O. The cells nuclei were stained with 4,6-diamino-2-phenylindole (DAPI) and cytoplasm with wheat germ agglutinin (WGA), Oregon Green® 488 conjugate. Finally, the cover slips were mounted onto the slides and then it was observed under 60 × 1.3 oil plan-apochromat lenses in Laser Scanning Confocal Microscopy (LSM710, Carl Zeiss AG, Oberkochen, Germany).

2.8. In vitro transfection

Transfection efficiency of PEI-PrAs was investigated in MDA-231 and MCF-7 cells through flowcytometry using gWIZ-GFP with a Green Fluorescent Protein (GFP) expression system under the CMV promoter. The 25PEI served as positive control and blank medium as the negative control during the transfections. Prior to each study, cells were seeded in 24 well-plates (50,000 cells/well)

and allowed to attach overnight. The complexes of variable composition (mass ratios) with/without CsiRNAs was prepared in serum free DMEM (see Section 3), as described above. The complexes were directly added to each well and incubated for 24 h under a humidified atmosphere (95/5% air/CO_2) at 37 °C. The culture medium was replaced with fresh medium after 24 h and then incubated for designed time period. The cells were then processed for flow cytometry; cells were washed (3×) with HBSS, trypsinized and fixed with formaldehyde (300 μL, 3.7% in HBSS). The GFP-positive population was quantified by Beckman Coulter QUANTA™ SC Flow Cytometer using FL1 channel (3000 events/sample). The setting of the instrument was calibrated for each run to obtain GFP expression of 1–2% for control samples (i.e., untreated cells). The mean fluorescence and the percentage of GFP positive cells were determined.

2.9. Statistics

The data were presented as mean ± standard deviation of three different replicates and analyzed for statistical significance by Student's two-tailed *t*-test (assuming equal variance).

3. Results and discussion

3.1. Polymer synthesis and characterization

We designed a series of PEI-PrAs by grafting propionate onto 1.2PEI and explored their efficacy to deliver pDNA to MDA-231 and MCF-7 breast cancer cells. Hydrophobic modification of PEIs via *N*-acylation is a straightforward method for synthesis of amphiphilic polymers, which can generate effective non-viral vectors [5]. The reaction conditions and resultant PEI-PrA polymers are summarized in Fig. 1. As expected, PrA substitution onto 1.2PEI was increased with PrA:PEI feed ratio (Fig. 1B and C). The ^{1}H NMR spectrum of PEI-PrAs showed the characteristic proton resonance peaks of 1.2PEI (2.4–3.5 ppm) and PrA (1.05 and 2.15 ppm), indicating the desired modification (Fig. S1A). The substitution efficacy was generally increased (up to ~70%) and then gradually decreased (~40%) with the feed ratios. The highest grafting was 1.6 PrA/polymer obtained from the feed ratio of 4.0 (PrA/PEI), which corresponded to 15.9% primary amine consumption, hence, leaving sufficient amines for nucleic acid binding. When long lipids were used as a substituent, amount of substitution ranged from 0.2 to 6.9 lipids per PEI with lipid:PEI amine feed ratios of 0.016–0.2 and as usual substitution per PEI increased with increase in the feed ratio [5,25]. Higher substitutions of lipid per PEI might be result of the using PEI of 2 kDa, which contains more amines for reaction than the 1.2PEI. Among these lipids, stearic acid substitution at highest the lipid:PEI amine ratio (0.2) was insoluble in water [25]. However, all polymers obtained from PrA conjugation were readily soluble in water in our hand.

3.2. Physicochemical characterization of polymers and their complexes

While chemical modification is intended to add beneficial features for gene delivery, *N*-acylation of PEIs could adversely affect critical features associated with successful gene delivery, such as buffer and DNA binding capacity. As expected, buffer capacity of the polymers was decreased from 31.7% (1.2PEI) to 24.8% (PEI-PrA1) upon PrA substitution as a function of modification (Fig. S1), consistent with consumption of primary amines through *N*-acylation. The impact of PrA grafting was also observed in pDNA binding profiles (Fig. 2).

The binding capacity of modified 1.2PEI was generally decreased with PrA substitution in proportion with the substitution amount (Fig. 2A); the BC_{50} of 1.2PEI was increased from 0.25 (polymer/pDNA, w/w) with PrA substitution, reaching a maximal value of 0.59 (polymer/pDNA, w/w) with the highest substituted PEI-PrA (1.6 PrA/PEI). This was likely due to direct consequence of both primary amine consumption and steric hindrance arising from the PrA chains. This is a common phenomenon that we have been observing in aliphatic lipids substituted PEIs through *N*-acylation [5,22,26], and usually required polymer to nucleic acid ratios of >1.0 for complete binding. Although PrA substitution increased the BC_{50} values for pDNA, one can still use excess polymers for pDNA binding in order to realize the desired improved intracellular delivery.

Hydrodynamic diameters and surface charges of polymer/pDNA complexes are additional parameters that may affect gene delivery efficacy [27,28], as it is important to condense pDNA into cationic, nano-sized particles. All PEI-PrA polymers were able to condense pDNA into nanoparticles of 150–200 nm (Fig. 3). The sizes of PEI-PrA/pDNA complexes at various polymer:pDNA ratios (2.5, 5.0 and 10, w/w) were similar irrespective of the PrA substitution level (Fig. 3). These sizes were comparable to the size of native 1.2PEI/pDNA complexes. It is interesting to note that long lipid, linoleic acid (C16) substitutions on PEI significantly change the hydrodynamic size of complexes and importantly, the polymer:pDNA mass ratio used to form complexes had an impact on sizes as compared to extent of lipid substitution on polymer [22]. Unlike long lipids, modification with small hydrophobe may have less steric hindrances allowing the backbone of native 1.2PEI to undertake the necessary condensation process. Some studies showed that hydrophobic substitutions results in significant increase in particles size of complexes due to aggregation of particles [29], presumably due to hydrophobic interactions under aqueous conditions. However, in another study, longer lipid-substitution on 2 kDa PEIs did not alter the size of complexes in a specific way [25]. The small hydrophobe PrA substitution also did not appear to affect the size of complexes indicating availability of enough amine content to condense pDNA. On the other hand, PrA grafting significantly increased the cationic charge of the complexes at the indicated polymer:pDNA ratios; the highest ς-potential of PEI-PrA/pDNA complexes was +45 mV that was significantly higher than the 1.2PEI/pDNA complexes (+20 mV) (Fig. 3). Interestingly, surface charge of polyplexes increased with low extent of PrA substitution and again decreased to levels consistent with the native 1.2PEI complexes with excess substitution. It is likely that while low PrA substitution enhanced the assembly of the complexes, the higher PrA substitution led to excessive consumption of amines and/or displayed steric hindrance to assembly.

3.3. Cytotoxicity of modified polymers and their polyplexes

The MW dependent cytotoxicity of PEIs has been well recognized, which encouraged the use small MW PEIs as a template for gene carriers [30]. Complexes with pDNA and each polymer were prepared at different polymer to pDNA weight ratio and cytotoxicity was measured by the MTT assay. Modification of small MW PEIs with aliphatic lipids generally increases cellular toxicity of the resultant polymers due to enhanced polymer interaction with cells, as we have been observing in our studies [5,22]. Consistent with this expectation, cytotoxicity of complexes with polymers PEI-PrA0.5 and PEI-PrA1 in MDA-231 and MCF-7 cells was higher than 1.2PEI/pDNA complexes. However, these complexes still displayed better cell compatibility compared to 25PEI/pDNA complexes (Fig. 4). Cholesterol, caprylic, myristic, palmitic, stearic, oleic and linoleic acid substituted 2PEI also showed lower cytotoxicity than 25PEI [5,16]. As expected, increasing the polymer to pDNA weight ratio during complex preparations increased the cytotoxicity. At higher polymer to pDNA ratio, a significant amount

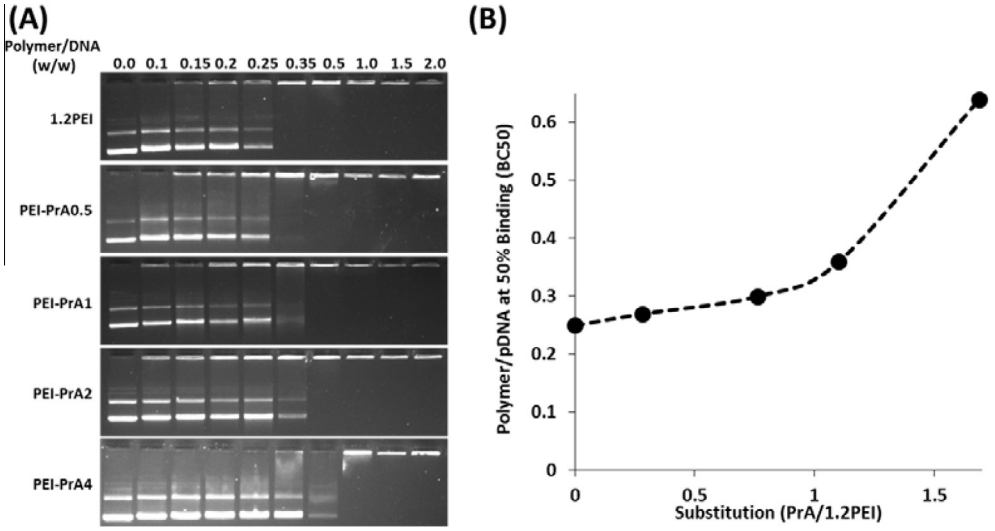


Fig. 2. Agarose gel retardation assay of complexes of pDNA with native and PEI-PrAs at varying weight by weight ratios (A) and BC_{50} values (polymer:pDNA mass ratio to get 50% of pDNA binding) as a function of PrA substitution per 1.2PEI (B). BC_{50} values were calculated using sigmoidal curve fits where% of pDNA binding with polymer obtained from gel electrophoresis, and plotted as a function of polymer:pDNA mass ratio. BC_{50} values increases as a function of PrA substitution, indicating reduced binding tendency of polymers with PrA substitution.

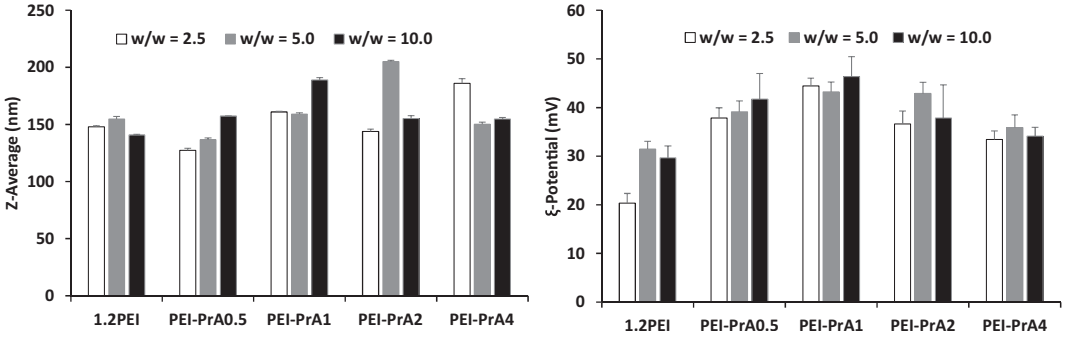


Fig. 3. Hydrodynamic size (Z-average) and ξ-potential of polymer/pDNA complexes of polymer:nucleic acid ratios of 2.5, 5.0 and 10.0 (w/w). The PrA substitution did not significantly impact the hydrodynamic size but the ξ-potential was significantly altered as a function of PrA substitution. ξ-potential initially increased with PrA substitution and then decreased with high PrA substitution.

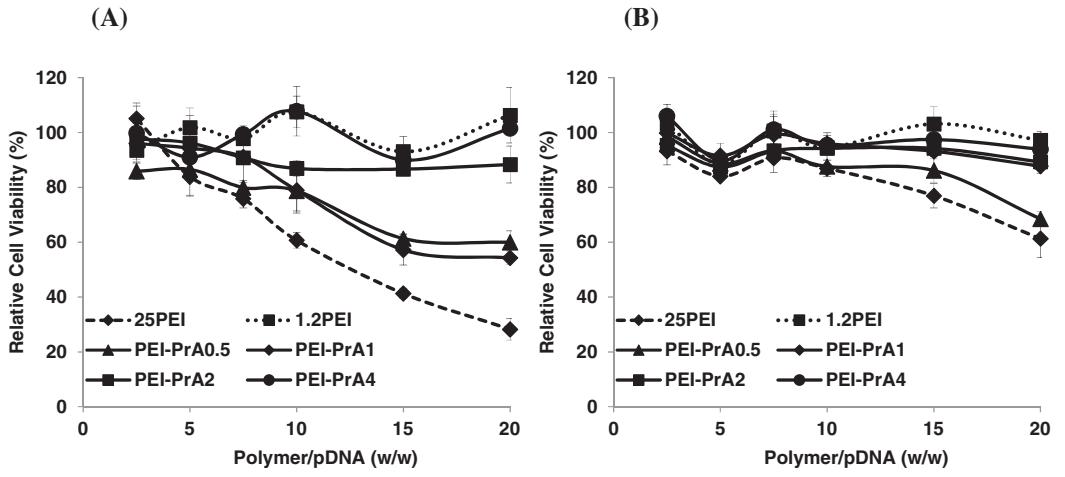


Fig. 4. Cellular toxicity of polymer/pDNA complexes in MDA-231 cells (A) and MCF-7 cells (B), as assessed by the MTT assay. Complexes with PEI-PrAs at lower substitution (PEI-PrA0.5 and PEI-PrA1) were more cytotoxic than complexes with 1.2PEI, but complexes with higher substitution (PEI-PrA4 and PEI-PrA2) gave similar toxicities to that of 1.2PEI.

of free polymer is expected to be present which represents the most cytotoxic component of the complexes. It is interesting to note that the complexes with high PrA substituted polymers, PEI-PrA2 and PEI-PrA4, did not induce any toxicity and this is in line with the surface charge of complexes from these polymers. PEI-PrA0.5/pDNA and PEI-PrA1/pDNA complexes displayed high ς-potential than other polymers, which was presumably responsible for the observed cytotoxicity. These are likely more interactive complexes with the cells, leading to non-specific cytotoxicity. The toxicity similarities with these polymers in 2 different cell lines (MDA-231 and MCF-7 cells) suggested a common, nonspecific mechanism such as cell membrane disruption. Cytotoxicity of polymers alone were tested in both cell lines in which concentration of polymers were equivalent to polyplexes of polymer to pDNA weight ratio of 2.5–20. In contrast to toxic 25PEI, all PEI-PrA polymers were nontoxic even at high concentration (Fig. S2).

3.4. Cellular uptake of pDNA complexes

One of the first key factors for effective non-viral gene delivery systems is the uptake of complexes. To further elucidate gene delivery efficacy, uptake of the complexes was determined using flow cytometry in MDA-231 and MCF-7 cells using Cy^{TN3}-pDNA. Polymer complexes with Cy^{TN3}-pDNA were prepared at the ratio of 5 and 10 for flow cytometry analysis. Uptake of 25PEI complexes in MDA-231 was equivalent to PEI-PrA1 complexes and more than other PEI-PrAs (Fig. 5A). In case of MCF-7 cells, uptake of 25PEI complexes was higher than 1.2PEI complexes and its derivatives (Fig. 5C). Interestingly, PEI-LA gave higher cellular uptake of complexes than the 25PEI in MDA-231 cells, but the same did not apply for MCF-7 cells. Our previous study showed beneficial effect of long aliphatic lipid (LA-linoleic acid, CA-caprylic acid, MA-myristic acid) substitutions on 2 kDa PEI but only at relatively high substitutions. pDNA uptake was increased with increase in lipid substitution and correlation between pDNA uptake and lipid substitution was evident [5]. In this study, short hydrophobe substitution on 1.2PEI showed beneficial effect on the pDNA uptake but non-monotonic relation was obtained between pDNA uptake and PrA substitution: an optimal substitution was evident (with PEI-PrA1 having 0.76 PrA/PEI), after which a decrease in pDNA uptake was evident in both cell lines. PEI-PrA0.5 (0.28 PrA/PEI) and PEI-PrA2 (1.1 PrA/PEI) showed significantly higher uptake than parent 1.2PEI but the polymer with highest PrA substitution (PEI-PrA4: 1.6 PrA/PEI) was unable to deliver pDNA, reminiscent of 1.2PEI. The results were similar in the MCF-7 cells as well. pDNA delivery with PEI-PrA polymers were in line with the surface charge of the polyplexes, where PEI-PrA1 complexes had the highest ς-potential and the 1.2PEI with highest PrA substitution (PEI-PrA4) had the lowest ς-potential complexes.

Uptake of pDNA/polymer complexes was further studied by confocal microscopy. The confocal micrographs of MDA-231 cells indicated distinct red fluorescent particles (i.e., Cy^{TN3}-labeled pDNA) around the nucleus of all cells, indicating internalization of complexes (Fig. 5). The intensity and numbers of fluorescent particles in cells varied with the type of polymer used. Both 25PEI and PEI-PrA1 were able to deliver pDNA into MDA-231 cells as revealed by red particles next to the nuclear membrane. Confocal microscopy also showed that pDNA complexes with PEI-PrA4 (highest PrA substitution) were not internalized as confocal microscopy image did not indicate any particles inside cells when using polymer indicated. While the hydrophobicity of PEI-PrA4 should be higher compared to PEI-PrA1 (hence displaying better membrane compatibility), the lower ς-potential (equivalent to non-effective 1.2PEI) was likely the reason for reduced intracellular delivery of these complexes.

Finally, the effect of siRNA addition to pDNA/polymer complexes was investigated, since such siRNA addition may further enhance uptake of complexes [31]. For this purpose, complexes were prepared with or without control siRNA (CsiRNA) using PEI-PrA1 and uptake was assessed in MDA-231 and MCF-7 cells by flow cytometer. Complexes with CsiRNA were prepared at different CsiRNA:pDNA ratio (from 0 to 2) using a polymer:nucleic acid (CsiRNA + pDNA) ratio of 5, which resulted in polymer:pDNA ratio from 5 to 15 (see Fig. 6). As a control, complexes without CsiRNA but having an equivalent polymer:pDNA ratio were prepared. In MDA-231 cells, addition CsiRNA at pDNA complexes with PEI-PrA1 was beneficial at polymer:pDNA ratio of 15 (where CsiRNA:pDNA ratio was 2), but no clear relation was evident in between CsiRNA amount added and uptake efficiency (Fig. 6A and Fig. S3A). Complexes with equivalent amounts of pDNA and PEI-PrA1 polymer (but without CsiRNA) also gave similar pDNA uptake, indicating that beneficial effect of CsiRNA addition could be duplicated by adjusting the polymer additive (Fig. 6B and Fig. S3B). While CsiRNA addition had detrimental effect on the uptake of pDNA complexes with 25PEI and same effect was observed with equal amount of pDNA and 25PEI. These results clearly indicated that CsiRNA had no specific effect on the uptake of pDNA complexes in MDA-231 cells. In MCF-7 cells, the CsiRNA additive to pDNA complexes with PEI-PrA1 also enhanced the uptake of complexes which was most prominent at the polymer:pDNA ratio of 15 (where CsiRNA:pDNA was 2:1); however, same results could be obtained with increasing polymer amount to match the equivalent complexes in the absence of CsiRNA (Fig. 6C and D). Similar to the MDA-231 cells, effect of CsiRNA on uptake of complexes with 25PEI was not evident in MCF-7 cells. Addition of CsiRNA to the 1.2PEI complexes did not either improve the uptake of pDNA complexes (data not shown).

Beneficial effect of siRNA addition on uptake was shown in an independent study where the pDNA/siRNA/polymer (1/1/4 w/w/w) complexes showed higher uptake than pDNA/siRNA/polymer (1/0/2 w/w/w) [31]. However, the higher amount of polymer in the former complexes might have been the reason for higher uptake, since the appropriate control (i.e., complexes with equivalent amount of DNA and polymer) was missing. When the cationic polymers are used for complex formation, size of siRNA complexes is usually higher than with pDNA complexes [32]. Another study showed that the long winding pDNA and short rigid siRNA complexes with PEI with different hierarchical mechanisms [33]. It is possible that combined siRNA and pDNA polyplexes might have different polyplexes properties such as size and ς-potential that might affect transfections [31]. In our hands, the addition is CsiRNA did not alter the sizes and ς-potential of pDNA/PEI-PrA1 complexes (Fig. S4), which might be the reason for the negligible effect of CsiRNA on pDNA complexes uptake.

3.5. Transfection efficiency of polymers

Modification of small MW PEIs with aliphatic lipids generally increases pDNA transfection efficacy due to improved cellular uptake [5,22]. As expected, pDNA transfection efficacy (using GFP as a reporter gene) in breast cancer MDA-231 and MCF-7 cells was significantly increased with PrA grafting on 1.2PEI while unmodified 1.2PEI was not effective at all (Fig. 7 and Fig. S5). Interestingly, the relation between PrA substitution and transfection efficiency is not monotone which is in line with the surface charge and uptake of polyplexes with the respective polymers. The transfection efficiency was dependent on the cell type, where MDA-231 cells displayed up to ~70% GFP-positive cells whereas MCF-7 cells gave only ~25% GFP-positive cells (Fig. 7B and D). As expected, transgene expression in both cell lines was dependent on

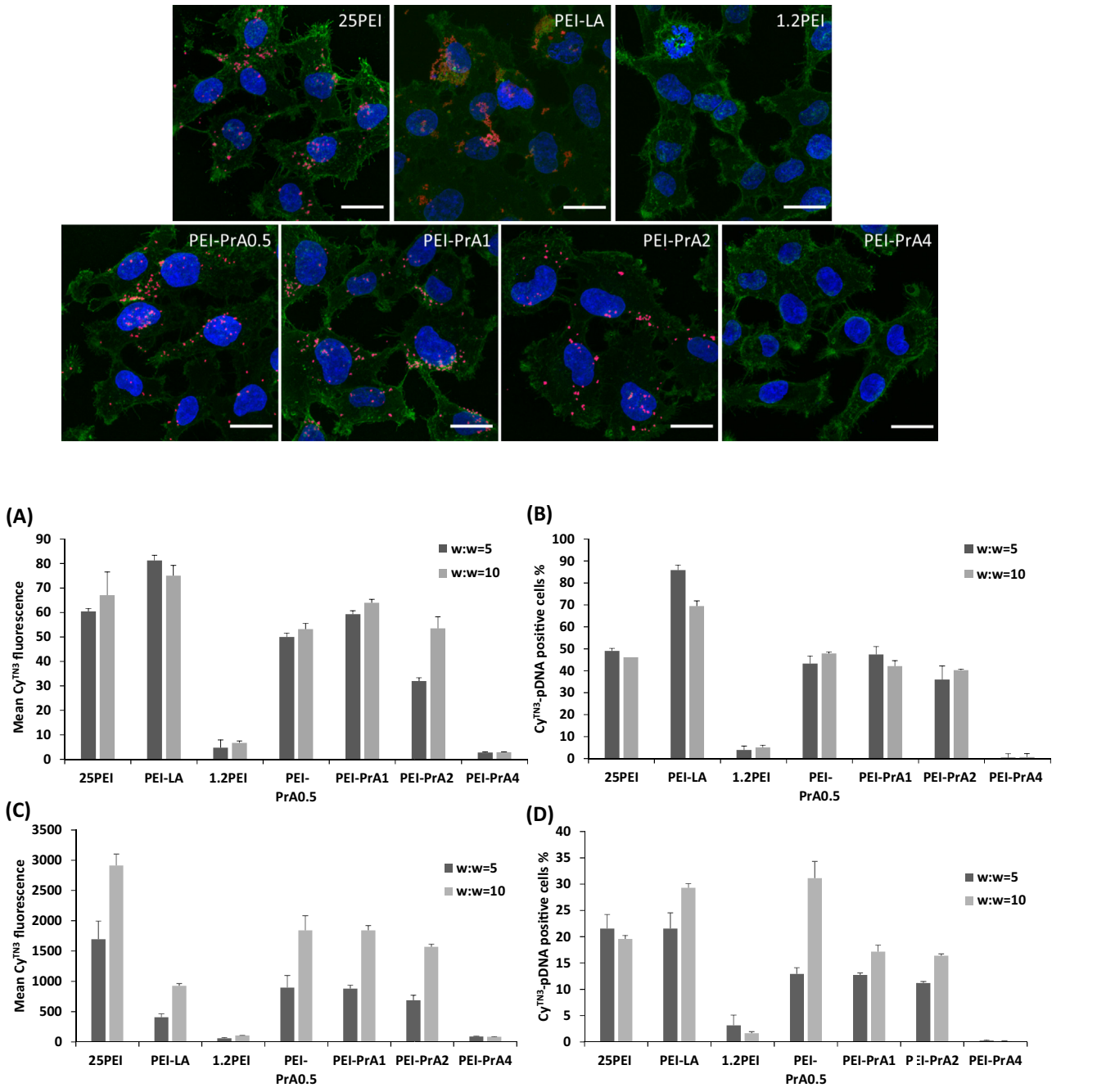


Fig. 5. Confocal micrographs of MDA-231 cells after 24 h treatment with complexes of polymer and pDNA-Cy^{TM3} (red), w/w = 10. Note that lack of uptake with PEI-PrA4 polymer, unlike PEI-PrA1 that gave robust uptake. Cells were stained with WGA oregon green® 488 conjugate and nuclei are stained with DAPI (blue). Scale bar is 20 μm. pDNA uptake to MDA-231 cells (A and B) and MCF-7 cells (**C** and **D**) with PEI-PrAs; measured by flowcytometry. The uptake was determined by Cy^{TN3}-labeled pDNA, and expressed as either mean pDNA uptake per cells (A and C) or as percentage of cells positive for pDNA (B and D). While small amount of PrA substitution helped pDNA delivery, excess PrA was detrimental for pDNA delivery.

polymer:pDNA ratio, as higher ratio gave better efficacy compared to lower ratio (10 vs. 5). The transfection levels were higher on day 2 and decreased after day 7, which is common with non-viral transfections. In MDA-231 cells, the maximal transfection efficacy was obtained with PEI-PrA1 (0.76 PrA/PEI), whereas in MCF-7 cells, a wider range of PrA substitutions (0.3–1.1 PrA/PEI) displayed significant effect, which is supported by the cellular uptake of pDNA using respective polymers. The highest PrA substituted PEI (PEI-PrA4), consistent with cellular uptake results, did not yield any transfection efficiency. Such a drop in transfection efficiency was not observed with previous lipid-substituted PEIs, which typically displayed high transfection efficiency with higher lipid substitutions. Lower ς-potential was one indication that increased PrA substitution might be detrimental for the overall charge of complexes by consuming cationic residues.

We prepared an equivalent line of polymers from 2 kDa PEI (2PEI) in order to confirm the observed PrA substitution effect with an independent series of polymers. Our results (summarized in Fig. S6) with the 2PEI polymers indicated a similar trend; the ineffective 2PEI became an effective gene delivery agent after some PrA substitution, but high PrA substitution was again detrimental on transfection efficiency. Therefore, these two sets of results clearly emphasized that appropriate ratio of hydrophobic substituent to be critical for PEI-mediated transfection.

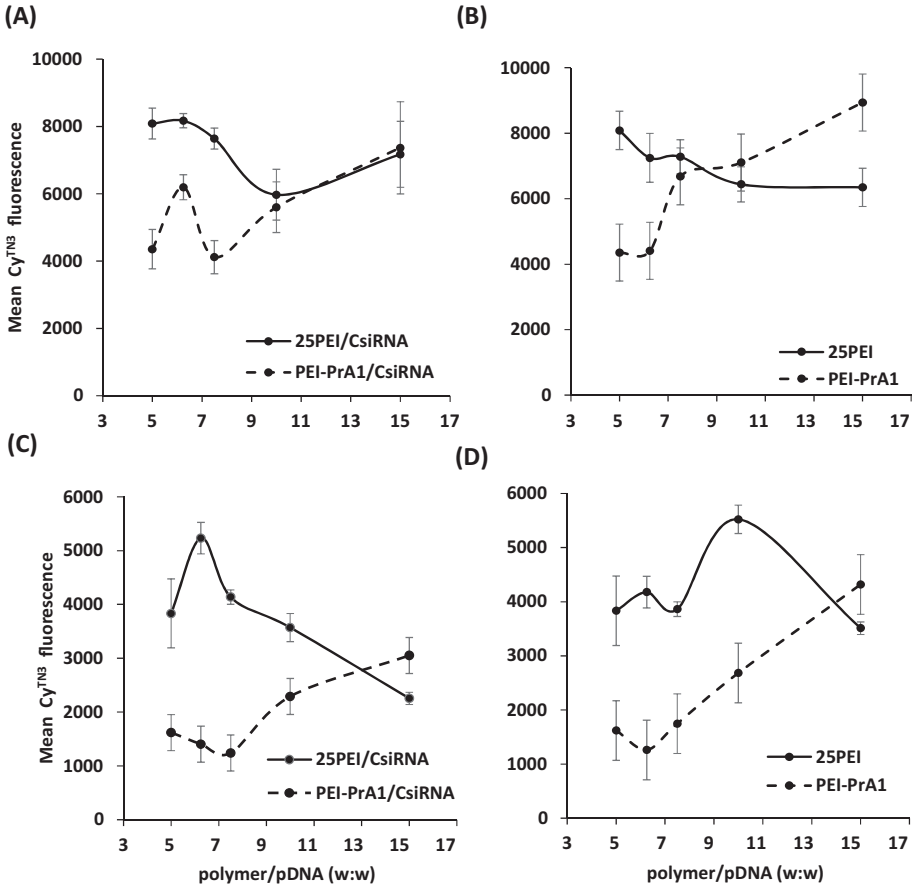


Fig. 6. pDNA uptake in MDA-231 cells (A and B) and MCF-7 (C and D) cells in the presence (A and C) and absence (B and D) of CsiRNA. Cy^{TN3}-labaled pDNA was complexed with the indicated polymers in the presence and absence of CsiRNA and uptake was determined after 24 h as mean fluorescence of Cy^{TN3}-pDNA. Note that CsiRNA was not beneficial for increasing uptake, as increased polymer to pDNA ratio was sufficient to give similar uptake in the absence of CsiRNA.

Improved transfection with hydrophobic modification may be due to increased lipophilicity resulting in better membrane compatibility and/or weaker interactions with pDNA, resulting in increased dissociation of complexes in the cytoplasm. An independent study also explored substitution of small hydropbobic moieties (acetate, butanoate and hexanoate) on 25PEI. Transfection efficiency was increased at low degree of substitution, where optimal modification was seen at ~25% acetylation, after which a reduction in transfection efficiency was noted [7]. In contrast, other investigators had shown that complete deacylation of linear PEIs (25, 22, 87 and 217 kDa) enhanced its transfection efficiency in A549 (human lung carcinoma) cells, supposedly by increasing the number of protonatable Ns, thereby increasing the binding affinity to pDNA [34]. However, branched 25PEI itself is a relatively effective transfection agent and it is not clear if the toxicity of this agent could be overcome by such a modification. Another group modified 2PEI with alkyl chains dodecyl and hexadecyl, which dramatically increased the transfection efficiency [21], but there was no modification with small hydrophobes was reported. Here we were able to modify 1.2PEI with small hydrophobe and find optimum degree of substitution for effective gene delivery. Our result suggested that using EDC/NHS conjugation method to substitute small hydrophobe where only primary amines of PEI are modified, substitution at the range of 0.3–1.1 small hydrophobe per PEI molecules enhances transfection, but the beneficial effects of PrA substitution reversed above these levels.

We then compared the transfection capacity of the most effective PEI-PrA1 and the broadly effective 25PEI. As before, unmodified 1.2PEI showed negligible efficacy in both cell lines (data not shown) whereas the efficacies of 25PEI and PEI-PrA1 were comparable at day 2 (Fig. 8). While the transfection efficacy was decreased with time (Fig. 8A, Fig. S5), the efficiency of PEI-PrA1 in MDA-231 cells was higher than the 25PEI at day 7 and day 14, indicating more stable transfection with PEI-PrA1. The transfection efficiency of PEI-PrA1 was comparable to 25PEI in MCF-7 cells at Day 2 and Day 7 (Fig. 8B). It should be noted that complexes with PEI-PrA1 polymer were less toxic than the complexes with 25PEI in both cell lines.

The transfection efficiency of PEI-PrA1 was also compared with linoleic acid (LA)-substituted 1.2PEI (PEI-LA), since LA was previously found to be the best performing lipid substituent on 2PEI [5]. In that study, unlike the PEI-PrAs here, no correlation was evident between transfection efficiency and degree of LA substitution, although transfection efficiency increased with high substitution level. The PEI-LA we used had 2.3LA per 1.2PEI and compared its transfection efficiency to PEI-PrA1 (Fig. 8C). The transfection efficiency of PEI-PrA1 was higher than PEI-LA, suggesting that small hydrophobe PrA can effectively impart higher transfection efficiency than the longer lipid chains. Although cellular uptake with PEI-LA complexes was higher than PEI-PrA1 complexes (see Fig. 5A), lower transfection efficiency was observed in the former case. Increased lipophilicity (i.e., membrane compatibility) may be the reason for high uptake of particles with PEI-LA. At the same time, long lipids may facilitate stronger hydrophobic associations among lipids and form more compact particles [35], which may hinder dissociation of complexes in cytoplasm. This may not be the case for the short hydrophobes, which may explain their superior effectiveness. This observation suggested that balance of

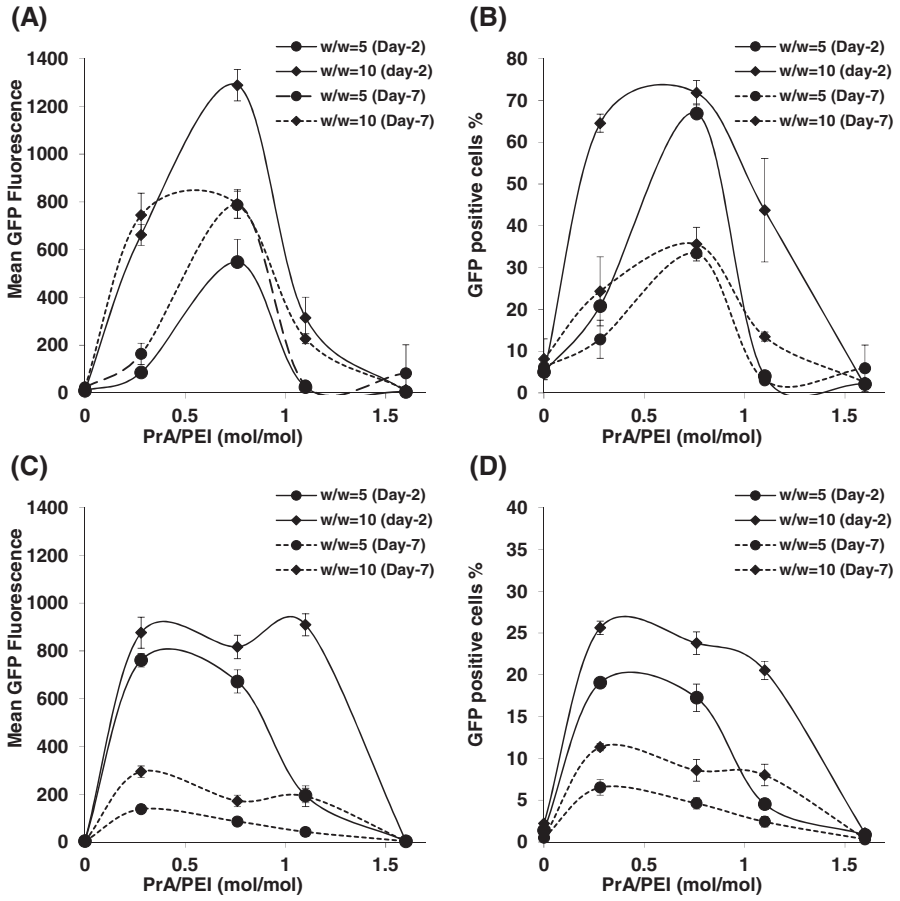


Fig. 7. GFP expression in MDA-231 (A and B) and MCF-7 (C and D) cells after treatment with PEI-PrA/pDNA complexes on day 2 and day 7. Cells were analyzed through flow cytometry for GFP expression and the data was expressed in mean fluorescence intensity (A and C) and percentage of GFP-positive population (B and D).

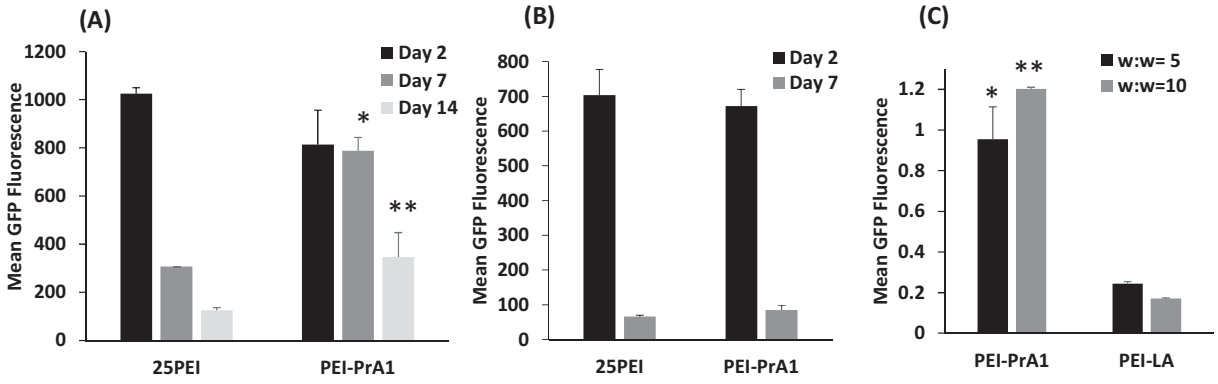


Fig. 8. GFP expression in MDA-231 (A) and MCF-7 (B) cells at different time point after the treatment with polymer/pDNA (w/w = 5) complexes. PrA grafting significantly increased the transgene expression in both cells lines and the efficacy was comparable with 25PEI ($^{*}P < 0.001$, $^{**}P < 0.05$ Vs 25PEI (Day 7 and Day 14 respectively). (C) Comparison of GFP expression in MDA-231 with PEI-PrA1 and PEI-LA. The complexes were formed at polymer:pDNA ratio of 5 and 10, and transfection efficiency was assessed after 2 days ($^{*}P < 0.05$ $^{**}P < 0.001$ Vs PEI-LA (w:w = 5 and 10 respectively).

hydrophobicity is critical for enhanced transfection. Therefore, the chosen small hydrophobe could be a better option than the long aliphatic lipids, in addition convenience and better control during the substitution reactions.

We noted that an independent study performed with 25PEI showed a more beneficial effect of smaller hydrophobes; using β-galactosidase transfection in COS-7 cells where branched 25PEI modified with alanine gave superior performance than leucine (1C vs. 4C side chain) substitution [21]. Modification with amino acids can maintain total number of protonable Ns similar to unmodified 25PEI, which can maintain the DNA binding capacity. Teo et al. recently, reported hydrophobic modification of 1.8PEI with hydrophobic group of variable (and longer) chain lengths; methyl carboxytrimethylene carbonate (MTC)-ethyl, MTC-octyl and MTC-deodecyl [36]. Among the substituents, the shortest MTC-ethyl was found to be more effective than the longer chains, reportedly due to lower cellular uptake with the longer hydrophobic groups. Unfortunately, it is difficult to directly compare these

results with PrA and LA substitutions on 1.2PEI reported here, since MTC incorporated between the 1.8PEI and alkyl chains might impact the physical properties of latter polymers unpredictably. In addition, modification with longer alkyls resulted less cellular uptake of complexes, which was not in line with our results (PEI-LA gave superior uptake than PEI-PrA1 in MDA-231). Unlike the conjugates created with the MTC intermediate, Doody et al. modified 25PEI with hydrophobic acetyl (C2) butyl (C4) and hexyl (C6) moieties and suggested no clear correlation in between hydrocarbon length and transfection efficiency [7]. Because of the solubility problems, hydrocarbon lengths was limited in that study, so that longer lipids (i.e., LA) could not be substituted onto their PEIs and a comparison between very short substituents (e.g., acetyl) and aliphatic lipid substituents could not be compared in that study.

3.6. Effect of siRNA additive on transfection

We further explored the transfection efficiency of PEI-PrA1 by formulating additive polyplexes using CsiRNAs. Electrostatic interaction strength of pDNA and siRNA with cationic polymers is expected to be different due to difference in characteristics of the nucleic acids, including size, morphology, rigidity and charge [31]. Unlike long and flexible pDNA, siRNA is short and rigid, which may result in much lower strength of association with cationic polymers [28]. Hence addition of CsiRNA during complexation is believed to enhance the dissociation kinetics of complexes, which could facilitate non-viral transfection [26,37]. To investigate effects of CsiRNA on transfection efficiency, we formulated 2 types of additive complexes: (i) complexes where polymer:pDNA ratio was fixed and CsiRNA amount was serially increased, and (ii) complexes where polymer:nucleic acid (pDNA + siRNA) ratio was fixed by increasing the polymer amount in proportion with CsiRNA, while keeping pDNA constant in both cases. Addition of CsiRNA in the former complexes where the polymer:pDNA ratio was fixed while increasing CsiRNA amount, was not beneficial in both cell lines. Transfection efficiency by both 25PEI and PEI-PrA1 decreased with increasing CsiRNA amount in these complexes (data not shown). Addition of CsiRNA without increasing polymer amount decreases cation:anion (polymer:pDNA + CsiRNA) ratio and was expected to reduce the transfection efficiency due to insufficient polymer to condense the available pDNA/CsiRNA. We then increased the polymer amount to ensure the same polymer: polynucleotide ratio (w/w = 5) and assessed pDNA transfection (Fig. 9 and Fig. S7). In MDA-231 cells, addition of CsiRNA to the complexes did not alter transfection efficiency as long as polymer:polynucleotide ratio was retained (Fig. 9A). With complexes bearing an equivalent amount of polymer and pDNA (but without CsiRNA), transfection efficiency of PEI-PrA1 increased at increasing polymer/pDNA ratio but it dropped significantly at high polymer/pDNA ratio of 15 (Fig. 9B). This may be due to toxicity of excessive polymer or decreases complex dissociation since uptake of these complexes were still high. Complexes with the same amount and pDNA and polymer (polymer/pDNA = 15) but with CsiRNA

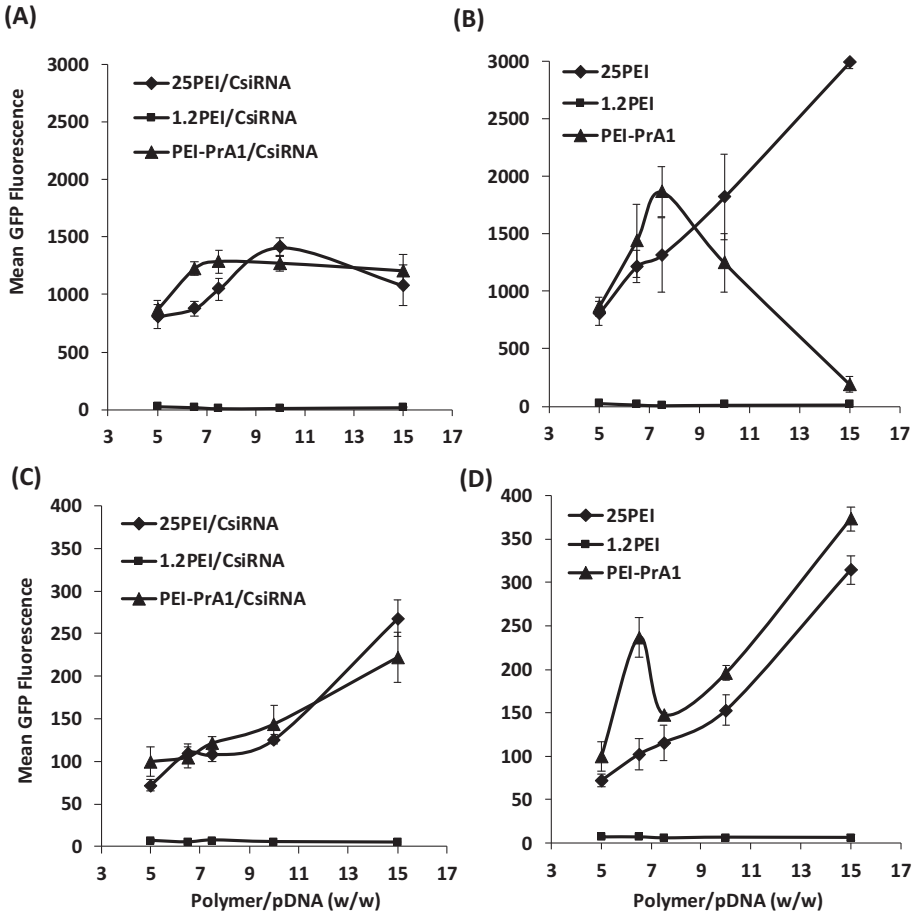


Fig. 9. Transgene expression in MDA231 (A and B) and MCF-7 (C and D) cells with (A and C) and without (B and D) CsiRNA additive (polymer:pDNA + siRNA ratio of 5) after 48 h of transfection. In MDA-231 cells, adding CsiRNA did not alter the transfection efficiency (as long as polymer:polynucleotide ratio was constant, see A), while in MCF-7 cells, adding CsiRNA helped with transfection efficiency (see C) but the same effect was obtained with addition of equivalent amount of polymer in the absence of CsiRNA (see D).

(siRNA/pDNA = 2) retained same level of transfection indicating beneficial role of CsiRNA in the complexes with high polymer/pDNA ratio. With MCF-7 cells, CsiRNA bearing complexes had increased transfection efficiency (Fig. 9C), but the same response was obtained with an equivalent of polymer and pDNA complexes without the added CsiRNA (Fig. 9D). Hence, addition of CsiRNA in pDNA transfection was not detrimental in both cell lines as long as polymer:polynucleotide ratio was maintained. Similar results were observed after day 7 of transfection wherein transfection efficiency of PEI-PrA1 was higher than the 25PEI (Fig. S7). Transfection efficiency in terms of percentage of GFP positive cells also showed a similar trend (Figs. S8 and S9). In addition, we found no significant change in the size and surface charge of polyplexes after addition of siRNA, as long as polymer:polynucleotide ratio was kept constant (Fig. S4).

The beneficial effect of siRNA in transgene expression was reported elsewhere [31]; DNA transfection efficiencies of both 25PEI and arginine-rich oligopeptide-grafted 25PEI modified with polyethlylene glycol with or without siRNA were studied. Beneficial effects of siRNA addition were noted in MCF-7 and MCF-7/Adr cells. In both cell lines, complexes DNA/siRNA/polymer (D/S/P) of weight ratio 1/2/3 and 1/1/2 resulted in higher transfection that by complexes without siRNA i.e. D/S/P of weight ratio 1/0/1 [31]. Yet, the polymer amount in former complexes, which showed high transfection, was higher than the latter complexes, and this effect was not specifically probed in that study. Our study showed equivalent amount of transfection can be obtained with simply increasing the polymer amount without CsiRNA. Similarly, another group studied effects of siRNA on GFP expression by PLL in HEK293 (kidney fibroblast) cells. GFP expression was higher with siRNA addition (PLL/pGFP/siRNA at cation:anion ratio of 2) vs. complexes without siRNA (PLL/pGFP of equivalent cation:anion ratio) [38]. It should be noted that to maintain the same cation:anion ratio, these two complexes had different amounts of polymer. In these studies, control experiments (transfection with equivalent polymer and DNA amounts in the absence of siRNA) were missing and enhanced transfection efficiency might have been simply due to this factor, as demonstrated in our study.

4. Conclusions

We successfully synthesized a small hydrophobe (PrA) modified 1.2 kDa PEI through *N*-acylation and validated the efficacy of resultant polymers for pDNA delivery in breast cancer cells. PrA grafting decreased pDNA binding and buffering capacity of the polymers, as well as increasing the toxicity to some extent. *In vitro* gene delivery efficacy of PEI-PrA to MDA-231 and MCF-7 cells increased with degree of PrA substitution, but excess PrA (>1.2 PrA/PEI) was detrimental suggesting that an optimum ratio between the substituent and polyethylenimine backbone was critical. The transfection efficiency after PrA substitution was more effective than long chain lipid (linoleic acid) substitution on 1.2PEI, emphasizing importance of balancing hydrophobicity of polymer for optimum gene delivery. However, siRNA supplementation, unlike literature reports, did not have specific effect on the pDNA transfection efficiency, as long as polymer amounts are adjusted in the formulations. Thus, integration of small hydrophobic groups into cationic PEIs is an effective approach for designing polymers for pDNA delivery and could prove useful in gene therapy approach for cancer.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.actbio.2016.01.025.

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