Cholic Acid Modified 2 kDa Polyethylenimine As Efficient Transfection Agent

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New lipopolymers were synthesized by conjugating cholic acid (ChA) to polyethylenimines (PEI; 2 and 25 kDa) and a polyallylamine (PAA; 15 kDa) via N*-acylation to develop effective gene delivery systems. The extent of ChA substitution linearly varied with the feed ratio during synthesis, indicating good control over grafting ratio. While ChA did not affect binding to plasmid DNA (pDNA) for higher molecular weight (MW) polymers, ChA substitution to 2 kDa PEI significantly affected the pDNA binding. Toxicity of the 2 kDa PEI was unaffected by ChA substitution, but it was improved for the higher MW polymers. Using immortal 293T cells and primary cord blood-derived mesenchymal stem cells, low MW (2 kDa) PEI was shown to display much better transfection efficiency as a result of ChA substitution, unlike the higher MW polymers. We conclude that ChA could be a suitable substituent for non-toxic (low MW) PEIs in order to improve their transfection efficiency.* © 2013 American Institute of Chemical Engineers *Biotechnol. Prog.*, 29:1337–1341, 2013
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Efficient gene delivery holds the key for therapy of genetic disorders and other conditions that are unresponsive to current drug therapies. Non-viral gene carriers are attractive for clinical gene therapy due to their safety, but they display low delivery efficiencies. High molecular weight (MW) polyethylenimine (PEI) is an effective gene carrier, since it can deliver plasmid DNA (pDNA) into cytoplasm via endosomes due to its proton-sponge effect.[1] However, this PEI cannot be clinically employed due to its excessive cytotoxicity. On the other hand, low MW PEIs (<5 kDa) are much less cytotoxic, but they are not efficient carriers for pDNA delivery into cells. To improve the transfection efficiency of PEIs, water-soluble lipopolymers were synthetized by combining lipidic components with low MW PEI.[2] Attempts to identify the appropriate lipid substituent suggested that steroid receptors could be beneficial in facilitating pDNA trafficking to cell nucleus.[3] Dexamethasone was conjugated to polyamidoamine dendrimers (PAMAM-Dex), whose transfection efficiency was higher than that of PAMAM or PEI.[4] Another sterol, cholesterol, was also shown to enhance the PEI's transfection efficiency.[2] Bile acids could represent better lipid substituents since, unlike the previous sterols, they are water-soluble, biocompatible with cells, can efficiently destabilize cell membranes owing to their amphiphilic characters, and are natural ligands for steroid receptors found on nuclear membrane.[3,5]

In this study, we explored the suitability of the bile acid cholic acid (ChA) to manipulate the lipophilicity of cationic polymers for use as gene carriers. The ChA with three —OHs axially placed on one side represents a planar amphiphile as opposed to head-to-tail amphiphiles.[6] The polymers used were 2 kDa (PEI2) and 25 kDa (PEI25) branched PEIs, and 15 kDa polyallylamine (PAA). The conjugates were synthesized by coupling primary $-NH_2$ of polymers with the —COOH group of ChA using carbodiimide chemistry (Scheme 1, Supporting Information). Reaction solvents had to be adjusted to account for solubility differences of the polymers (PEI2 and PEI25 in $CHCl_3$ with DCC as coupling agent and PAA in 1:1 water:methanol mixture with EDC as coupling agent). While PEI conjugates were purified with ether precipitation, dialysis was used for PAA conjugates since precipitated product contained unacceptable levels of impurities.

Additional Supporting Information may be found in the online version of this article.

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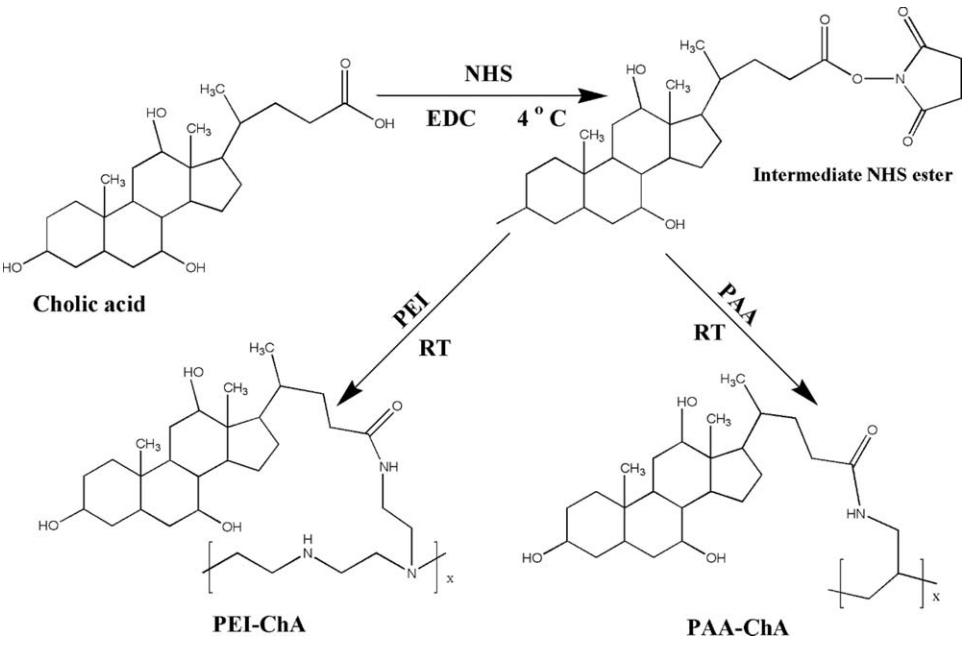

Sch 1. Coupling of polymeric amines with *N*-hydroxysuccinimide (NHS)-activated ChA.

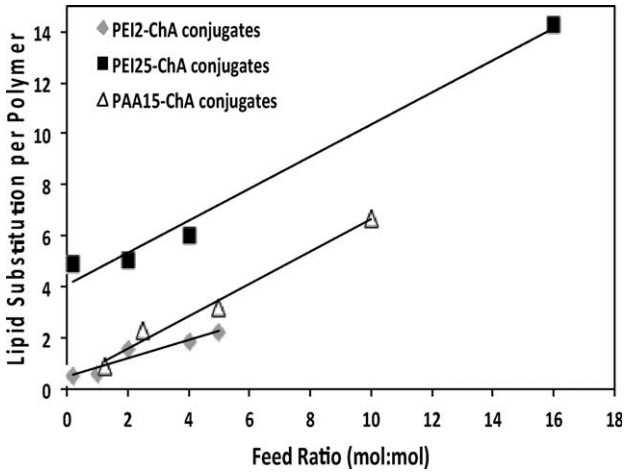

Figure 1. Correlation between the lipid/polymer feed ratio (mol/mol lipid/polymer) and substitution levels in ChA conjugates.

The characteristic resonance shifts in ^{1}H-NMR spectra corresponding to ChA (—C$\underline{H}_3$, $\delta = 0.95$ ppm), PEI (—NH—C$\underline{H}_2$—C$\underline{H}_2$—, $\delta = 2.5$–2.8 ppm) and PAA (NH_2—C$\underline{H}_2$—CH, δ = 1.3–1.7 ppm) were used to calculate substitution levels (Supporting Information Figure 1S and Table 1S). A clear correlation was obtained between the lipid/polymer molar feed ratios during synthesis and ChA substitution levels (Figure 1). The lipid substitution was also increased with increasing MW of the PEI polymer; 0.5–1.90 ChA/PEI2 was obtained for feed ratio of 0.2–4, while 4.9–14.3 ChA/PEI25 was obtained for feed ratio 0.2–16. The lipid substitution for PAA was increased from 0.9 to 6.7 as the lipid/PAA feed ratio was increased from 1.25 to 10. All conjugates were soluble in water, except the highest substituted PEI25 (14.3 ChA/PEI25; not used for further studies). Controlling lipid substitution level is paramount for preparation of reproducible polymers, and our synthesis scheme was able to achieve this. In studies involving Dex conjugation to PAMAM, no such relationship was explored; one study reported a conjugate with 4 Dex/PAMAM,[4] while another reported 1.3 Dex/PAMAM.[7] Reaction conditions leading to such a difference in substitution levels were not articulated. Even with a linear PEI, ample substitutions were reported for Dex (~23 Dex/polymer), but whether the substitution level was controlled by reaction conditions was not evident.[8] Similarly, Gusachenko et al.[9] reported cholesterol substitution to PEI25 using a preactivated cholesteryl chloroformate, but the authors did not confirm the extent of modification even though several polymer:cholesteryl chloroformate ratios were used for synthesis. Although Bajaj et al.[10] could control the extent of substitution, they used a four-step reaction to prepare PEI-cholesterol conjugates. The reaction scheme described here is simpler and provides the needed control over the lipid substitution ratios.

When mixed with pDNA in ultrapure water, the conjugates spontaneously formed nanoparticles, as investigated by transmission electron microscopy (Supporting Information Figure 2S). The particles were compact and spherical, and size measurements from micrographs indicated the particles to be <200 nm for all conjugates. While PEI formed distinct spherical particles, PAA-based polymers formed aggregated particles. Quantitative binding of polymers to pDNA was investigated by SYBR Green I dye. The binding curves as a function of polymer/pDNA weight ratios for all ChA conjugates were depicted in Figure 2**.** The pDNA binding capacity of PEI25 (Figure 2B) and PAA (Figure 2C) conjugates remained relatively unaltered, while binding capacity of PEI2 conjugates was significantly decreased with increasing ChA substitution (Figure 2A). This decreased binding capacity may be attributed to consumption of primary amines, which are responsible for pDNA condensation. However, we note that the highest substituted PEI2 had 1.9 ChA/PEI2, corresponding to ~13.6% reduction in amines (1.9 out of ~14 primary amines). Considering that the binding was decreased by ~5-fold for this polymer (based on concentration needed for 50% pDNA binding), steric effect of ChA might be more significant on binding given the

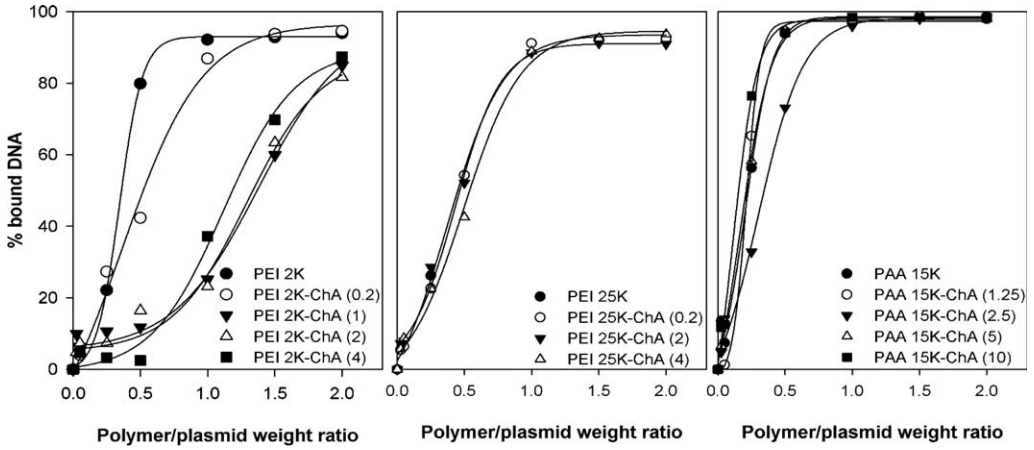

Figure 2. Binding curves of PEI2 (A), PEI25 (B), and PAA (C) conjugates. Binding was determined at different polymer/pDNA weight ratios by using SYBR Green I binding for free pDNA.

The ChA feed ratios used in the preparation of polymers are indicated in parenthesis in the legend (Supporting Information Table S1). The polymer/plasmid weight ratio of 2.0 corresponds to N/P ratio of ~15.0 for unmodified PEI2 and PEI25, while it corresponds to N/P ratio of ~11.3 for unmodified PAA.

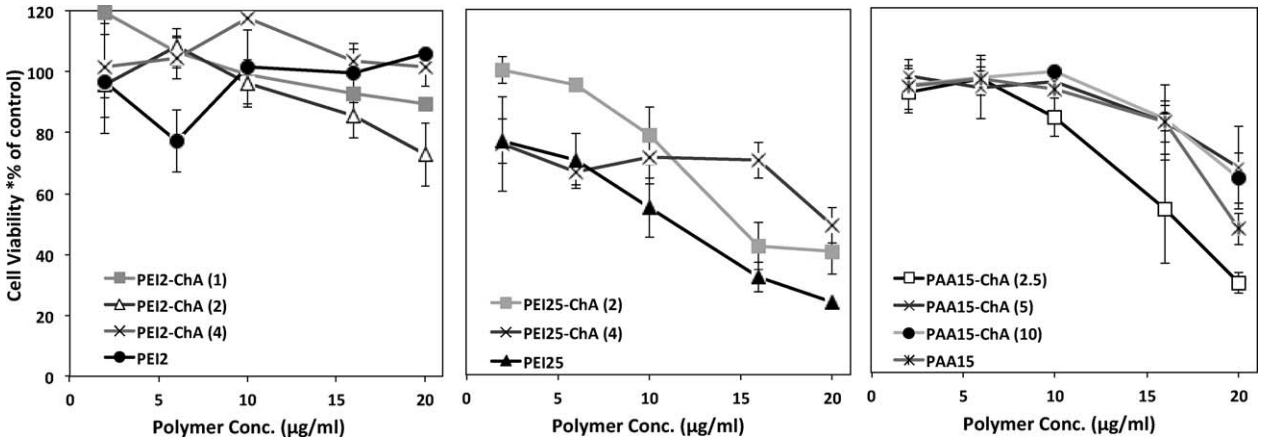

Figure 3. Cytotoxicity of PEI2 (A), PEI25 (B), and PAA (C) and their ChA conjugates on 293T cells.

The feed ratios for the samples are shown in the Legend. The cell viability was determined by the MTT assay and expressed as a percentage of control (i.e., untreated) cells.

relatively small amount of amines consumed. With PEI25 and PAA, <10% of amines were modified with ChA and this did not appear to cause a substantial effect on pDNA binding. Since ChA conjugates with PEI were not reported before, we are unable to directly compare our results to the literature. However, unlike our results, Huh et al.[11] noted that chitosan-ChA conjugates better self-assembled under aqueous conditions due to strong hydrophobic interactions among the ChA moieties. Similarly, Dex-polymer complexes (PEI25 and PEI2) were reported to have increased pDNA binding as compared to native PEI.[7,8] Enhanced hydrophobic associations in the case of Dex might explain this difference from our results.[12] Therefore, it could be concluded that whether a lipophilic substituent enhances or impedes pDNA binding might depend on substituent's nature.

The cytotoxicity of ChA conjugates was investigated next in human 293T fibroblasts. PEI2 and its ChA conjugates did not show a clear dose-dependent toxicity (Figure 3A). This is consistent with known compatibility of PEI2 with mammalian cells and a lack of effect by ChA conjugation on the toxicity. The toxicity of PEI25 was evident (Figure 3B). The ChA substitution on PEI25 generally decreased the toxicity of PEI25. The consumption of some amines is one of the likely reasons for the decreased toxicity. A similar result was obtained with cholesterol-substituted PEIs, where the substitution and amine reduction improved the toxicity of such polymers.[10] The PAA similarly displayed a dose-dependent toxicity (Figure 3C), albeit to a lower extent than PEI25. In this case, ChA conjugation appeared to increase the cytotoxicity for some of the samples, but a clear relationship between the level of substitution and final cytotoxicity was not immediately evident.

Transfection efficiency of the conjugates was evaluated in 293T cells using a Green Fluorescent Protein (GFP) plasmid.[13] Based on microscopic analysis, PEI2 was not effective in inducing GFP expression, but ChA-substituted PEI2 showed readily detectable GFP expression (Figure 4A). The PEI25 also induced GFP expression, and ChA substitution sustained the transfection activity of PEI25 (Figure 4A). Whether ChA-substitution improved GFP expression was not clear from the microscopic analysis. However, PAA was not able to induce transfection and ChA substitution did not impart a transfection efficiency to this polymer (not shown). The PAA lacks secondary and tertiary amines, which may be

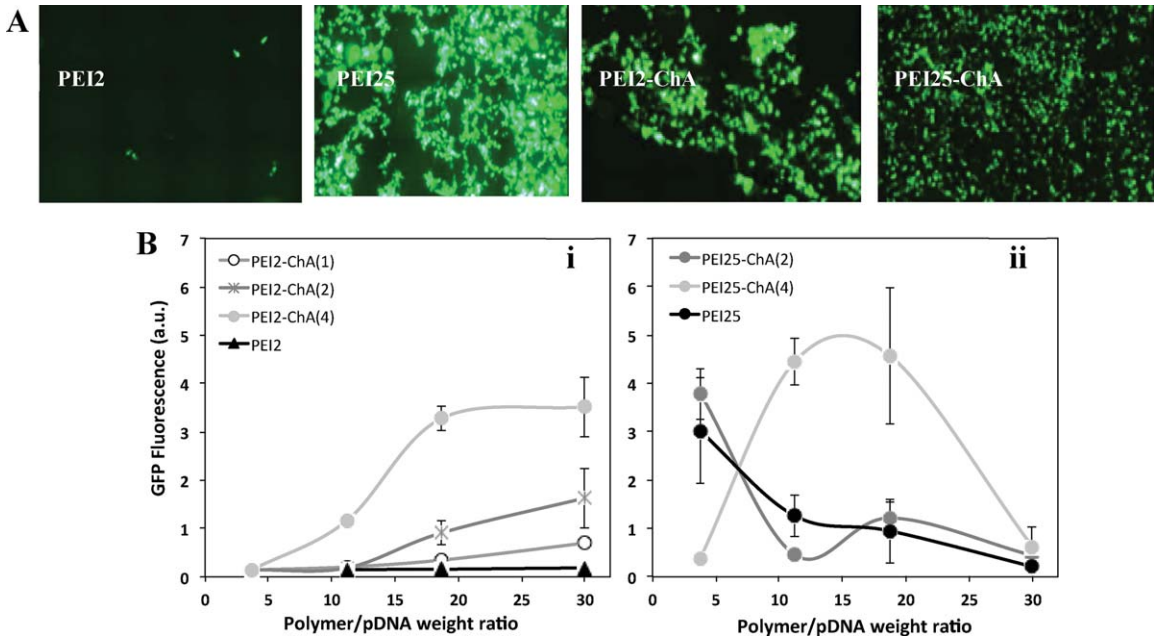

Figure 4. A. Representative images of 293T cells after treatment with polyplexes of gWIZ-GFP (polymer/pDNA ratio of 10 for PEI25 and 15 for PEI2). B. Transfection efficiencies of PEI2 (i) and PEI25 (ii) conjugates as a function of polymer/pDNA ratios (results are in arbitrary units).

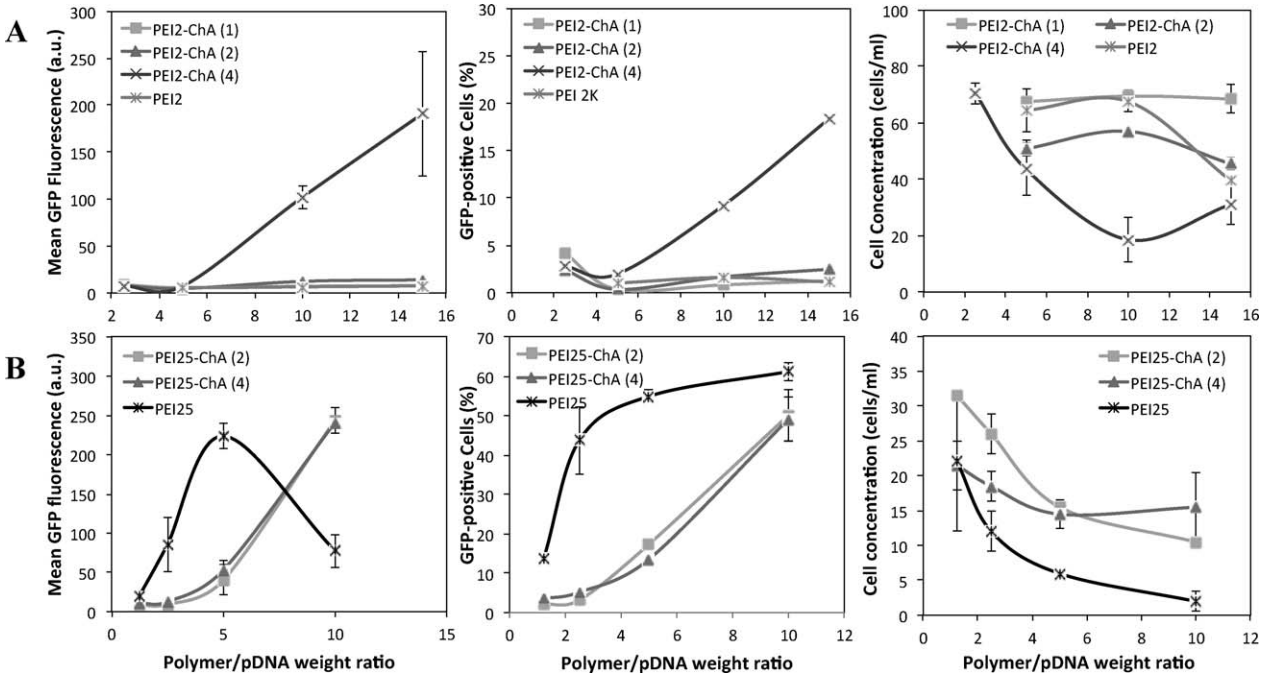

Figure 5. Transfection efficiency of PEI2 (A) and PEI25 (B) conjugates in CB-MSC. The results are summarized as mean GFP fluorescence (in arbitrary units; left), percentage of GFP-positive cells (right) and cell concentration from flow cytometry.

Note that the GFP fluorescence in CB-MSC was below the detection limit of spectrofluorometry used for 293T cells, so that flow cytometry was used for CB-MSC.

the reason for the lack of transfection activity, since these features are critical for "proton sponge" effect needed for endosomal escape.[14] We recently noted that endosomal entrapment was still significant for lipopolymers[15] and it appears that ChA substitution did not overcome this limitation for the PAA. Hence, PAA conjugates were not tested any further.

Based on quantitative GFP expression by spectrofluorometry, the three highest substituted PEI2s showed an increasing GFP transfection with increasing polymer/pDNA ratio from 3.75 to 30 (Figure 4Bi and ii), unlike PEI2, which did not show transfection at all ratios. An increased transfection efficiency was also observed with increasing lipid substitution for PEI2 conjugates, where >20-fold increase in mean GFP fluorescence was observed for the highest substituted PEI2-ChA (1.9 ChA/PEI2). The PEI25 conjugates followed a different pattern than the PEI2 conjugates. The PEI25 polyplexes gave highest transfection efficiency at a relatively low polymer/pDNA ratio (~4), after which the transfection efficiency

gradually decreased. The polymer from the feed ratio of 2 (PEI25-ChA-2) also gave a similar result, but the polymer PEI25-ChA-4 displayed an optimal transfection at higher polymer/pDNA ratio, which was quantitatively similar to maximal transfection observed with PEI25. The reduction in transfection efficiency at high polymer/pDNA ratios was likely due to toxicity of PEI25 on the cells, since we microscopically observed a gradual cell death with increasing ratios. Since PEI2 and PEI2-ChA conjugates did not exhibit such a toxicity, increasing polymer/pDNA ratio resulted in GFP expression unhindered by the complications of cell death.

Finally, the synthesized polymers were evaluated in primary cord-blood mesenchymal stem cells (CB-MSC), a promising cell type for clinical applications of gene therapy protocols. A quantitative analysis of CB-MSC transfection was conducted with flow cytometry. The transfection efficiency of low-substituted PEI2 conjugates was not significantly different from native PEI2 (Figure 5A). Only at the highest ChA (1.9 lipids/PEI2) substitution, a better transfection was evident in terms of mean GFP fluorescence (expressed per cell) and %GFP-positive cells. Under best conditions, ~20% of CB-MSC were found to be expressing GFP. Although this may constitute a relatively low level of modification (compared to immortalized cells), the required level of transfection is likely going to depend on specific application and this level of modification might be suitable for applications requiring low level of transgene expression. In the case of PEI25 conjugates, polyplexes of native PEI25 gave a maximal transfection efficiency at an optimal polymer/pDNA ratio of ~5 (Figure 5B), after which a reduction was evident. Similar to 293T cells, this was attributed to the toxicity at this polymer. The PEI25-ChA conjugates, however, gave an increasing transfection with increasing polymer/pDNA ratio; conjugates with higher substitution level gave better transfection efficiency. While ChA substitution on PEI25 improved cell numbers recovered for flow cytometry, the most effective PEI2-ChA conjugate was also more toxic as compared to other PEI2s (as expected).

We conclude that ChA substitution on low MW PEI2 enhanced the transfection efficiency of the polymer. This was evident in immortalized cells as well as in primary cells useful for clinical applications. Conjugation of ChA alone to a polymer, however, was not sufficient to impart a transfection capability (as in the case with PAA) and that polymer features also contributed to the transfection capability. The binding results showed a reduced binding to pDNA as a result of ChA substitution on PEI2, but this was not an impediment to transfection as long as excess polymer was used to complex the pDNA to a full extent (polymer/pDNA ratio > 5). The ChA conjugation to PEI2 did not alter the compatibility of this polymer with cells, a critical consideration for future clinical use of this conjugate.

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