

PAPER



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Additive nanocomplexes of cationic lipopolymers for improved non-viral gene delivery to mesenchymal stem cells†

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It has been challenging to modify primary cells with non-viral gene delivery. Herein, we developed a ternary nano-formulation for gene delivery to umbilical cord blood and bone marrow derived mesenchymal stem cells (MSC) by using lipid-modified small (1.2 kDa) molecular weight polyethylenimine (PEI1.2). Linoleic acid (LA) was end-capped with carboxyl functionality by coupling with mercaptopropionic acid through thio-ester linkage, and then grafted onto PEI1.2 via *N*-acylation. The thio-ester LA grafted PEI1.2 (PEI-tLA) displayed a significantly lower (up to 6-fold) DNA binding capability and a higher propensity to dissociate upon polyanionic challenge. The dissociation ability of the complexes was further enhanced by incorporating hyaluronic acid (HA) into plasmid DNA (pDNA) complexes of PEI-tLA. The HA incorporation influenced the surface charge of complexes more so than the hydrodynamic size, but it clearly increased the propensity for dissociation upon a polyanionic challenge. The PEI-tLAs were less toxic on MSC and displayed significantly higher transgene expression in MSC than conventional PEI-LA. Ternary complexes of with HA (pDNA/HA = 2, w/w) further enhanced the efficiency of PEI-tLAs of low (~2 lipid/PEI) lipid substitution, which was comparable to or higher than commercial transfection reagents. We conclude that PEI-tLA of low lipid substitution can be employed as a gene carrier to design supersensitive nano-formulations.

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Introduction

Genetically modified mesenchymal stem cells (MSC) have emerged as the basis of potent therapies in various diseases, including cancers.^{1–5} This approach relies on genetic modification of patient cells with foreign genes and subsequent administration of modified cells back to a patient.⁶ The MSC has the extraordinary capacity to accumulate at disease sites (*e.g.*, tumors) and secrete a therapeutic agent *in situ* secretion that can lead to improved efficacy *via* local delivery, which reduces the adverse effects of systemic agents.⁷ The MSC are isolated from adult and fetal tissues including bone marrow, adipose tissue, amniotic fluid, menstrual blood, and umbilical cord blood,^{4,8} but MSC derived from umbilical cord blood (UCB-MSC) are attractive, since they display high proliferative capacity for *ex vivo* expansion, ability to differentiate into multiple lineages, and better immunogenicity as compared to adult cells.⁵

It has been a challenging task to transform UCB-MSC into therapeutically useful cells and most studies on modification of UCB-MSC were conducted with viral vectors, despite serious concerns on immunogenicity and genotoxicity of the viral approach.^{9–11} The limitations of viral vectors are stimulating development of alternative functional materials, namely bio-compatible polymers or lipids,¹² for modification of MSC. Cationic polymers display greater chemical diversity than lipids and accommodate versatile chemical schemes to incorporate functional moieties for better transfection.^{13–15} A promising candidate among cationic polymers for efficient transduction of MSC is polyethylenimine (PEI),¹⁶ in particular high (25 kDa) molecular weight (M_w) branched PEI (PEI25).^{17–21} However, severe cellular toxicity, caused by strong cationic charge density that leads the complex aggregation on cell surface and induction of necrosis, has limited the application spectrum of this polymer.^{22–24} The relatively non-toxic small M_w (0.6 to 2.0 kDa) PEIs were alternatively used as a starting template to prepare effective carriers by chemically grafting various hydrophobic/lipid groups.^{25,26} Hydrophobic modification generally increases the efficiency of the PEIs by virtue of improved cellular uptake.^{25,27–30} These substitutions generate relatively non-toxic PEI derivatives with superior efficacy in modifying cell lines, but modification of primary MSC is not as robust as the other cell lines.³¹

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Strong electrostatic interaction between cationic polymers and nucleic acids is beneficial for creating stable complexes to deliver DNA across cell membranes,^{32,33} but it was suggested to limit the efficacy of polymers due to insufficient unpacking of complexes in the cytoplasm.^{34,35} Complexes with improved sensitivity to dissociation might be more conducive for modification of primary cells. The hydrophobic modification of small M_w PEIs generally decreases the DNA binding efficiency.¹³ However, there was no clear evidence of the associated increase in dissociation of resultant plasmid DNA (pDNA)–polymer complexes. The grafted lipid moieties appeared to stabilize the complexes even though the pDNA binding capabilities of individual polymers were reduced.²⁵

This study was undertaken to create polymer–pDNA complexes more conducive for dissociation. Two approaches were taken for this purpose. First, we integrated an electronegative functionality along with hydrophobic tail to further decrease the binding strength between lipid-grafted polymers and pDNA. Second, we incorporated a polyanionic additive along with pDNA molecules into the complexes.³⁶ By using a combination of weakly DNA-binding polymers with polyanion additives in conventional pDNA/polymer formulations, we hypothesized that supersensitive formulations could be created for modification of primary cells. Here, we describe a facile synthesis of cationic lipopolymers by grafting carboxyl end-capped linoleoyl acid (LA) onto the 1.2 kDa PEIs (PEI1.2) *via* a thio-ester (–S–CO–) linkage. Formulations of a supersensitive nano-complex were derived from the synthesized polymers along with incorporating anionic hyaluronic acid (HA) into the pDNA–polymer complexes. The critical features of the binary complexes (*i.e.*, polymers + pDNA) were characterized against the additive complexes (*i.e.*, polymers + pDNA/HA), and their transfection efficiencies were assessed in MSC derived from human umbilical cord and bone marrow.

Results and discussion

The lipid-substituted PEIs were derived from the base polymer of PEI1.2, which had a lower M_w as compared to our previous carriers (2 kDa). This was expected to further improve the biocompatibility of the resultant polymers, given the inverse relationship between the PEI toxicities and the molecular weight.³⁷ The previous lipopolymers linked the lipid moieties to PEIs by using *N*-acylation, creating an amide linkage for lipid grafting. Here, we used a similar *N*-acylation for lipid attachment to polymers, but additionally incorporated a polar thio-ester linkage before the lipid moiety (Fig. 1A). A carboxyl-functionalized, thio-ester containing linoleic acid (tLA) was synthesized for this end by coupling mercaptopropionic acid (MPA) with LA, which was then grafted onto PEI1.2 *via* EDC/NHS activation. Grafting efficacy of tLA onto PEI1.2 was proportional to the feed ratio, where the highest efficacy observed was ~65% grafting at the feed ratio of 10 (Fig. 1B and C). Typical ¹H-NMR spectra (Fig. 1D) of tLA exhibited the characteristic resonance peaks of LA: CH_3 ($\delta \sim 0.8$ ppm), γCH_2 ($\delta \sim 1.27$, ~ 2.0 and ~ 2.52 ppm), βCH_2 ($\delta \sim 1.61$ ppm), αCH_2 ($\delta \sim 2.25$ ppm) and CH ($\delta \sim 5.35$ ppm).

Resonance peaks corresponding to thiol proton ($SH-CH_2$) of MPA was not resolved due to γ -protons of LA ($\delta \sim 1.27$ ppm), α and β -methylene proton ($-CH_2-CH_2-SH$) peak of MPA were appeared merging with γ -protons of LA at $\delta \sim 2.5$ to 3.0 ppm. The quantitative shifting of αCH_2 of LA from $\delta \sim 2.75$ to $\delta \sim 2.52$ ppm indicates the successful coupling between LA and MPA. The yield of tLA preparation was ~65% through this protocol. As expected, ¹H-NMR spectrum of PEI1.2-tLA exhibited the characteristic proton peaks of LA along with a distinct α -methylene proton ($-CH_2-CO-$) peak of MPA at $\delta \sim 4.12$ ppm shifted from $\delta \sim 2.9$ ppm, indicating the formation of amide bonding. Compared to *N*-acylated PEI-LA polymers, tLA-substituted polymers displayed better solubility in aqueous medium (not shown), possibly due to increased polarization of thio-ester linkage compared to the amide linkage.

Binding and dissociation properties of thio-ester bearing lipopolymers

Binding capacity of PEI-tLAs with pDNA was evaluated through an electrophoretic mobility assay (Fig. 2). Fraction of unbound pDNA was quantified to determine the binding capacity, which was based on BC_{50} ; *i.e.*, polymer/pDNA ratio required for 50% DNA binding. As expected, pDNA complexation was increased with polymer/pDNA ratio in all cases, indicating the predominance of cationic properties in binding. The BC_{50} of PEI-tLAs was increased from 0.2 to 1.28 as the grafted lipid molecules were increased from 0 to 6.5 (Fig. 2A). The decreased binding capacity was previously shown for several lipid substituents, ranging from C8 to C18, including the LA.¹³ The polymers prepared here, however, seemed to display lower binding as compared to the previously *N*-acylated PEI-LAs. Both the lower M_w of the polymer backbone (PEI1.2 *vs.* PEI2) as well as the presence of thio-ester moiety in current polymers could have contributed to this behavior. Using heparin as a complex unpacking agent, we further quantified the dissociation strength of PEI-tLAs–pDNA complexes, which was based on DC_{50} ; *i.e.*, heparin concentration required for 50% dissociation. The complexes prepared from native PEI1.2 (PEI1.2/pDN) had significantly higher DC_{50} (46.4 U mL^{-1}) as compared to PEI-tLAs–pDNA complexes, whose DC_{50} values ranged from 20 to 4.1 U mL^{-1} depending on the level of tLA substitution (Fig. 2B). Polymers with high tLA substitution ($>2/\text{PEI}$) seem weaker in protecting DNA, a critical issue when they are employed in biological application. This behavior was different from previous lipid-substituted PEIs, where the effect of grafted lipids on dissociation was ambiguous.¹³ The more weakly-binding thio-ester LA-functionalized PEI1.2 presumably allowed the effect of grafted lipids to be better revealed on the dissociation behaviour.

The size of PEI-tLAs–pDNA complexes (polymer/pDNA ratio of 5 (w/w) for all cases) is summarized in Fig. 2C. These complexes were smaller (105.2 ± 18.0 to 157.5 ± 1.6 nm, depending on tLA grafting levels) than the PEI1.2–pDNA complexes (241.2 ± 27.5 nm), but similar to PEI25–pDNA complexes (117.7 ± 9.5 nm). The ζ -potentials of PEI-tLA complexes (31 – 43 mV) were higher than the PEI25–pDNA (13.4 ± 2.5 mV) and PEI1.2–pDNA complexes (9.9 ± 1.5 mV) at this polymer/pDNA ratio. Lipid substitution on PEI1.2 had clearly shown a significant

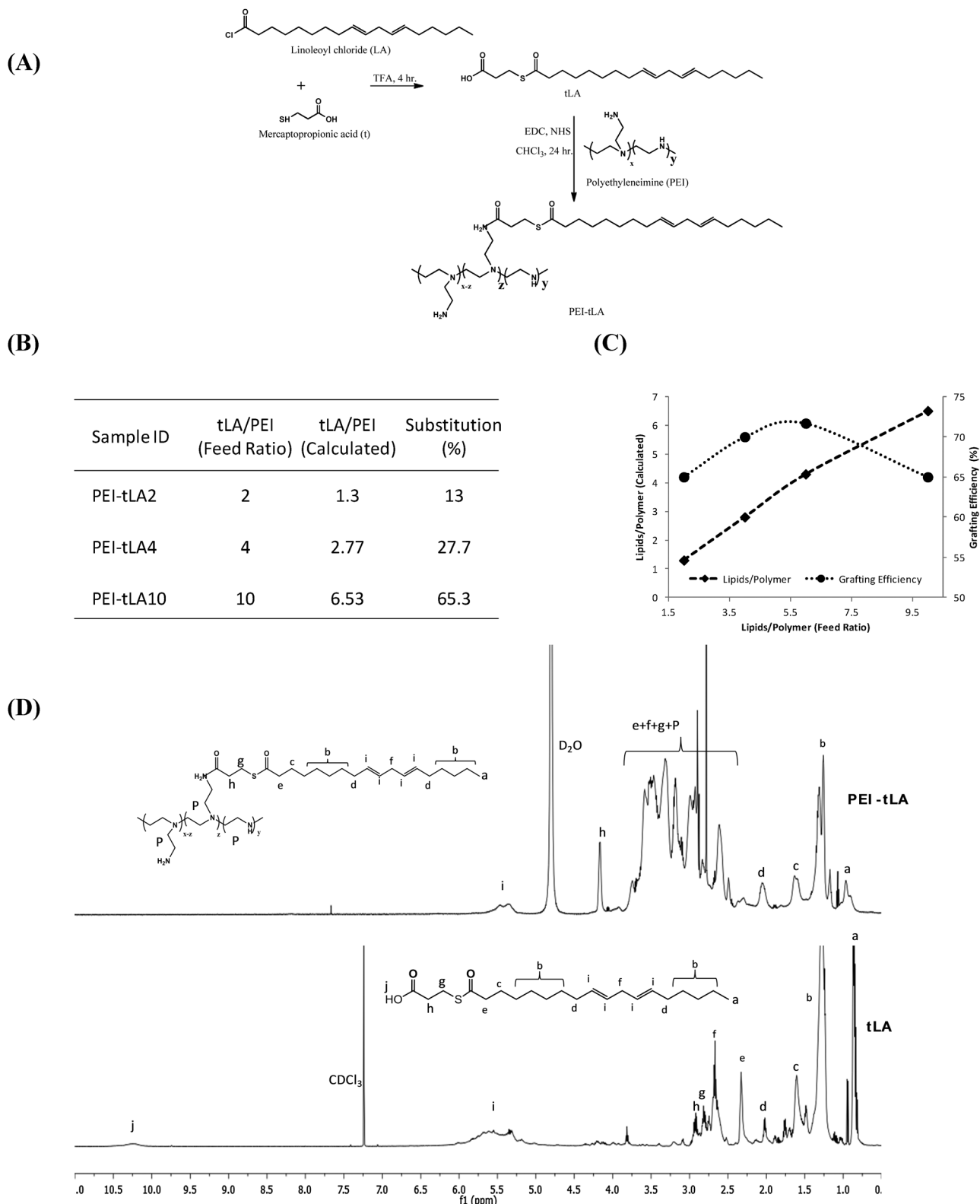


Fig. 1 Reaction scheme for the synthesis of carboxyl end-capped linoleic acid (tLA) and PEI-tLA (A). Summary of polymer synthesis and quantification of lipid substitution levels (B), (C). Typical ^1H -NMR spectroscopy of tLA and PEI-tLA in CDCl_3 and D_2O , respectively (D).

effects in both hydrodynamic sizes and surface charge of the complexes. This observation was contradictory to previous studies in which lipid grafting was seen to increase the complex

size significantly (to as much as ~ 600 nm). The increased size was previously attributed to particle aggregation under aqueous conditions, due to exposed hydrophobic surfaces.³⁸ The polar

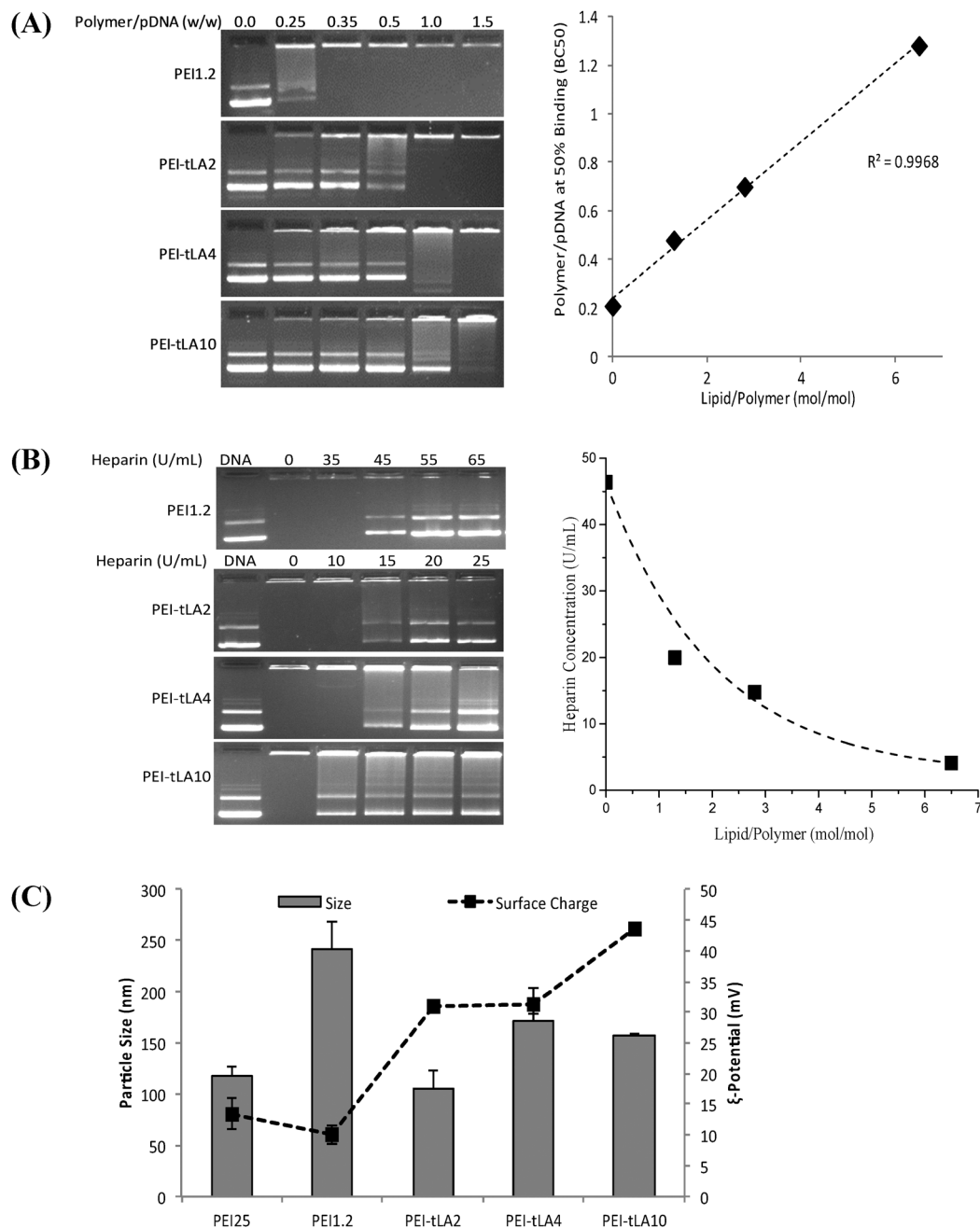


Fig. 2 (A) Electrophoretic gel mobility assay for binding of PEI-tLAs with pDNA. (B) Electrophoretic gel mobility assay for dissociation of PEI-tLAs–pDNA complexes with heparin. The binding in A was quantitated based on BC_{50} (i.e., polymer/pDNA ratio at 50% binding) and dissociation in B based on DC_{50} (i.e., heparin concentration ($U\ mL^{-1}$) required for 50% pDNA release), and summarized as a function of substitution level. (C) Hydrodynamic diameter (Z-average) and ζ -potential of complexes (polymer/pDNA = 5, w/w) determined from DLS and ELS.

thiol-ester moiety, therefore, appeared to prevent particle aggregation as compared to PEIs lipid grafted by simple *N*-acylation. The size of these complexes was still considered small enough for efficient uptake.³⁹ The enhancing effect of tLA substitution on the surface charge of complexes was in line with the observations in our previous study.³⁸

Ternary complexes with HA

We next explored the possibility of formulating additive complexes by using PEI-tLA polymers and a mixture of HA and pDNA.

As shown in Fig. 3A, binding capacity of PEI-tLAs was decreased with HA integration into the complexes as a function of HA/pDNA ratio. The BC_{50} values of the PEI-tLA2, PEI-tLA4 and PEI-tLA10 were decreased from 0.48 to 0.87, 0.61 to 1.03 and 1.2 to 1.7 with increasing amount of HA/pDNA ratio from 0 to 3, respectively. Based on BC_{50} of HA integrated complexes, PEI-tLA2 preserved its binding strength more effectively than the other polymers. Particle formation was evident in the zetasizer analysis when the complexes were formed with HA and pDNA mixtures. The effect of HA on the size and ζ -potential

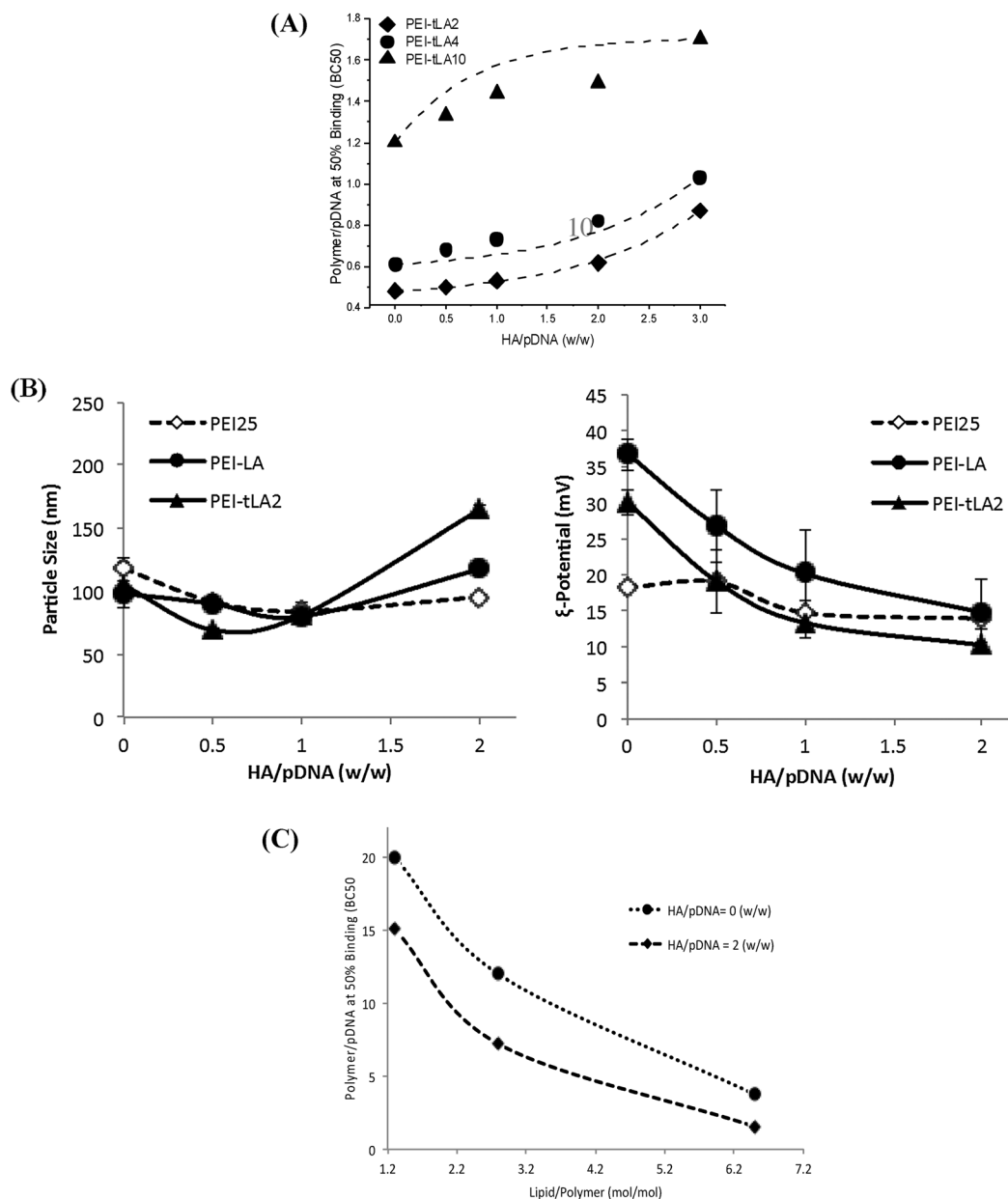


Fig. 3 (A) BC₅₀ values for binding of PEI-tLAs with pDNA–HA mixtures (BC₅₀) as a function of HA/pDNA ratio added to the complexes. The complexes were prepared with polymers at 3 levels of tLA substitutions. (B) Hydrodynamic diameter (left) and ζ -potential (right) of complexes (polymer/pDNA = 5, w/w) as a function of HA/pDNA ratio added to the complexes. (C) DC₅₀ values for dissociation of PEI-tLAs–pDNA + HA complexes with heparin as a function of level of tLA substitution (HA/pDNA ratio of 2).

of PEI25/pDNA was negligible, unlike the complexes prepared with PEI-LA and PEI1.2-tLA2. Size of PEI-tLA2/pDNA was increased from 105.2 ± 18.0 to 165.2 ± 3.3 nm and ζ -potential was significantly ($p < 0.003$) decreased from 30.1 ± 1.7 to 10.2 ± 0.5 mV upon addition of HA (HA/pDNA ratio of 2; Fig. 3B). Despite reduction, the obtained cationic surface charge density was considered sufficient for complex internalization *via* adsorptive endocytosis.^{40,41} Finally, the dissociation of HA incorporating complexes was assessed with heparin. The HA incorporation imparted a significant effect in unpacking of PEI-tLA complexes; at HA/pDNA ratio of 2, DC₅₀ values were decreased from 20 to 15.7 (U mL⁻¹), 11.97 to 7.2 (U mL⁻¹) and 3.74 to 1.54 (U mL⁻¹)

for polymers with tLA substitutions of 1.3, 2.8, and 6.5 LA per PEI1.2, respectively. HA integration enhanced the dissociation of complexes (lower DC₅₀ values) with higher tLA substitutions (> 2 /PEI). This indicates lower stability of HA integrated complexes with higher tLA substitutions (> 2 /polymer), which might not be suitable for biological application.

These observations indicated that HA incorporation was not that influential to control the hydrodynamic size, but surface charge of PEI-tLA complexes was most affected with HA incorporation. Our HA incorporation method could be an alternative to commonly used ‘coating’ approach to control the ζ -potential of ternary complexes.⁴² Ternary complexes previously described

in the literature were intended to neutralize the cationic charge of complexes by coating the binary complexes with polyanionic molecules, including HA.^{42–44} However, the complex size could significantly increase after this process due to particle aggregation,^{42,43} which in itself significantly decreased the surface charges of complexes. The approach described here, *i.e.*, incorporating polyanion additives along with pDNA during complex formation, exhibits marginal impact on the size, while controlling the surface charge in a controlled way, and improving the dissociation efficiency.

Cytotoxicity and cellular uptake

We next investigated the toxicity and cellular uptake of PEI-tLA complexes with/without HA incorporation. The toxicity of complexes was increased with the polymer content irrespective of the type of cell (UCB-MSC or BM-MSC) or the polymer used in the assay (Fig. 4A and B). Lipid substitution generally increased the toxicity of the polymers, but the toxicity of PEI-tLAs remained lower than the commercial reagents Lipo2 and PEI25. The toxicity in UCB-MSC appeared to be slightly less as compared to the toxicity manifested on BM-MSC. The toxicity profiles of complexes prepared with HA additive were also evaluated (Fig. 4C and D). The effect of HA addition was negligible on cytotoxicity even though surface charge (ζ -potential) of complexes

was significantly decreased with the HA additive. Cellular toxicity of PEI-like cationic polymers is usually attributed to strong cationic surface charge, but it seems that this was not a factor with the complexes prepared with the HA. It is likely that the HA-induced decrease in ζ -potential was not sufficient to alter cellular interactions, since the formulated complexes were still cationic within our experimental range.

To further elucidate cellular interactions of complexes, uptake of complexes was determined in UCB-MSC using Cy3-labeled pDNA (Fig. 5A and B). Based on flow cytometry analysis, no apparent differences were noted in the uptake of Lipo2-pDNA complexes with and without HA additive (*i.e.*, ~70% Cy3-pDNA positive cells in both cases). The uptake of polymer-pDNA complexes with HA was generally higher than the complexes without HA (for PEI25 as well as PEI-tLAs). Uptake efficacy of PEI-tLA-pDNA complexes was significantly higher than Lipo2-pDNA, and it was comparable to PEI25-pDNA complexes, which gave the most HA-induced increase based on mean Cy3-pDNA levels in cells. Confocal microscopy also indicated distinct fluorescent (red) particles around the nucleus of all cells treated with the complexes (Fig. 5C), indicating internalization of complexes. The intensity and numbers of red fluorescent particles in cells treated with PEI-tLA-pDNA complexes appeared to be higher than PEI25/pDNA treated cells.

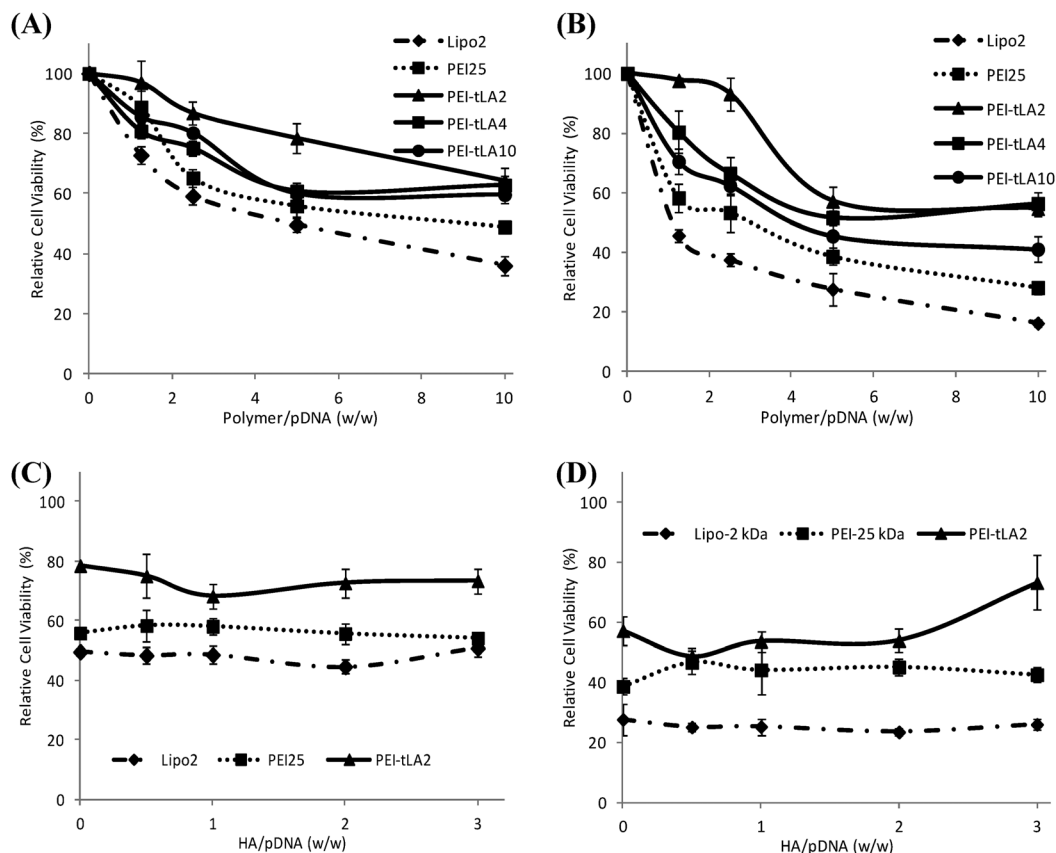


Fig. 4 Relative viabilities of UCB-MSC (A) and hBM-MSC (B) incubated with polymer-pDNA complexes at different polymer/pDNA ratios. Relative viabilities of UCB-MSC (C) and hBM-MSC (D) cells based as a function of the amount of HA added into complexes. In all cases, the viabilities of the complex treated cells were expressed as a percentage of un-treated cells.

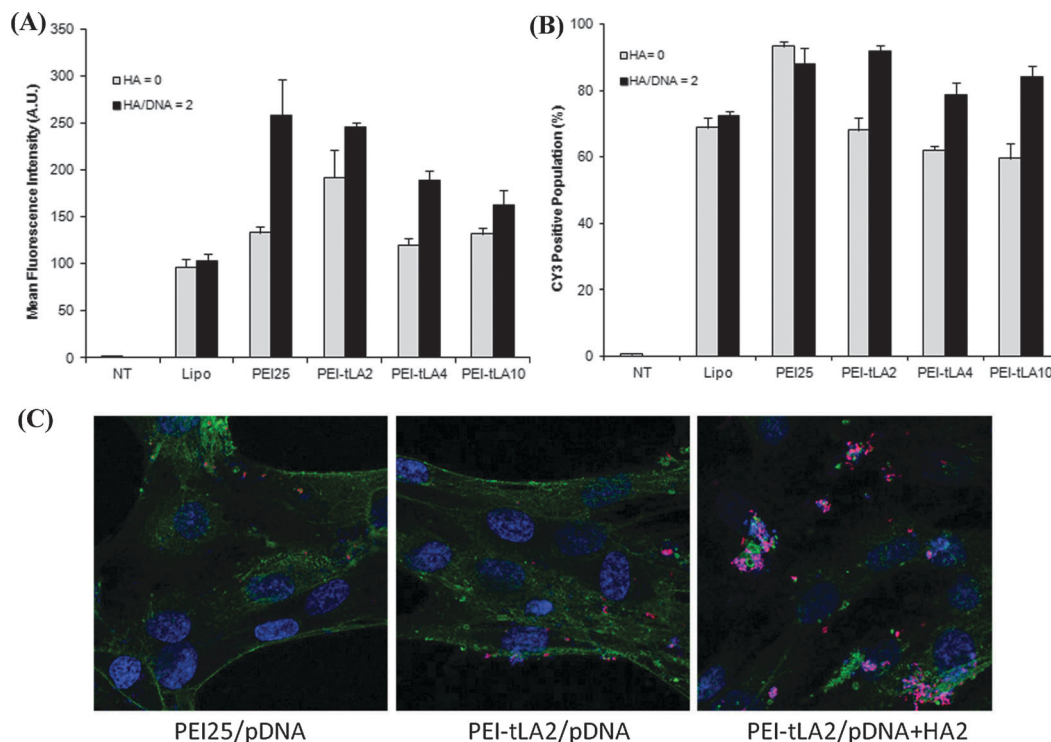


Fig. 5 Cellular uptake of the complexes in UCB-MSC as determined by flow cytometry (A, B) and confocal microscopy (C). Complexes (polymer/pDNA = 5, w/w) were prepared by incubating polymers with Cy3-labeled pDNA and exposed to the cells for 24 h. The flow cytometry analysis was summarized as the mean fluorescence intensity per cell (A) and Cy3-labeled pDNA positive cell population (B).

The cells treated with the HA-containing complexes displayed higher numbers/intensity of fluorescent particles compared to complexes without the HA, which was consistent with flow cytometry based uptake data.

Besides the practical impact of cytotoxicity and cell uptake, the obtained results could be indicative of cellular interactions of the formulated complexes. Since PEI1.2 did not give any pDNA uptake (and HA incorporation did not affect pDNA delivery; not shown), better delivery by PEI-tLAs was presumably due to higher surface hydrophobicity of polymer-pDNA complexes.³⁸ The effect of HA incorporation on cellular toxicity and uptake of complexes was consistent; no major changes in toxicity was evident with HA incorporation into the complexes, while the uptake was increased modestly. We attributed the increased uptake of HA formulations to preferential localization of hydrophobic groups on complex surfaces, since increased polyanionic charge in the complex core will discourage penetration of hydrophobic groups into the core of complexes.

Transfection efficiency

In vitro transfection efficiency was studied in UCB-MSC and BM-MSC using two compositions of complexes (polymer/pDNA = 5 and 10, w/w). A GFP-expressing pDNA was used as a reporter gene, while PEI25 and Lipo2 served as reference reagents for transfections. The transfection efficiency of PEI25 was significantly higher than Lipo2 in UCB-MSC (Fig. 6A and B). The transfection efficiencies of PEI-tLAs complexes were generally increased at the higher polymer/pDNA ratio of 10. The PEI-tLAs

demonstrated significantly higher efficiencies than the Lipo2, while PEI-tLA2 and PEI-tLA4 displayed comparable or higher efficacy at the higher ratio (10). The performance of PEI-tLAs was also better than PEI25 in BM-MSC, where the pattern of transfection efficiencies was similar to the UCB-MSC (Fig. 6C and D), except the extent of transgene expression was higher in BM-MSC (based on mean GFP levels; compare Fig. 6A vs. 6C). Transfection of UCB-MSC was also investigated at different time points (Fig. S1, ESI[†]); the efficiency of Lipo2, PEI25 and PEI-tLAs were highest initially (after 2 days of transfection) and then decreased to levels of negative controls (un-treated cells) on days 5 and 8. The obtained transfection efficiencies generally matched the cell uptake results, where (i) the carrier with lowest delivery (Lipo2) also gave the lowest transfection efficiency, and (ii) PEI25 and PEI-tLAs gave similar pDNA delivery and transfection efficiencies. The difference between the latter two polymers was the better cell compatibility in the case of PEI-tLAs.

We next evaluated the efficacy of HA formulations (HA/pDNA = 1, 2 and 3) in the primary cells. The impact of HA was clearly observed for all complexes (Fig. 7). At the HA/pDNA ratios of 1 and 2, the mean GFP levels obtained with Lipo2 in UCB-MSC was increased with HA addition, but it was dramatically decreased at the higher HA/pDNA ratio of 3, indicating an interference by the HA at relative high additions. Interestingly, the impact of HA in PEI25 was completely negative and transfection efficiency was almost abolished in these formulations. The impact of HA was more beneficial in PEI-tLA2 complexes than the higher lipid-grafted PEI-tLA4 and

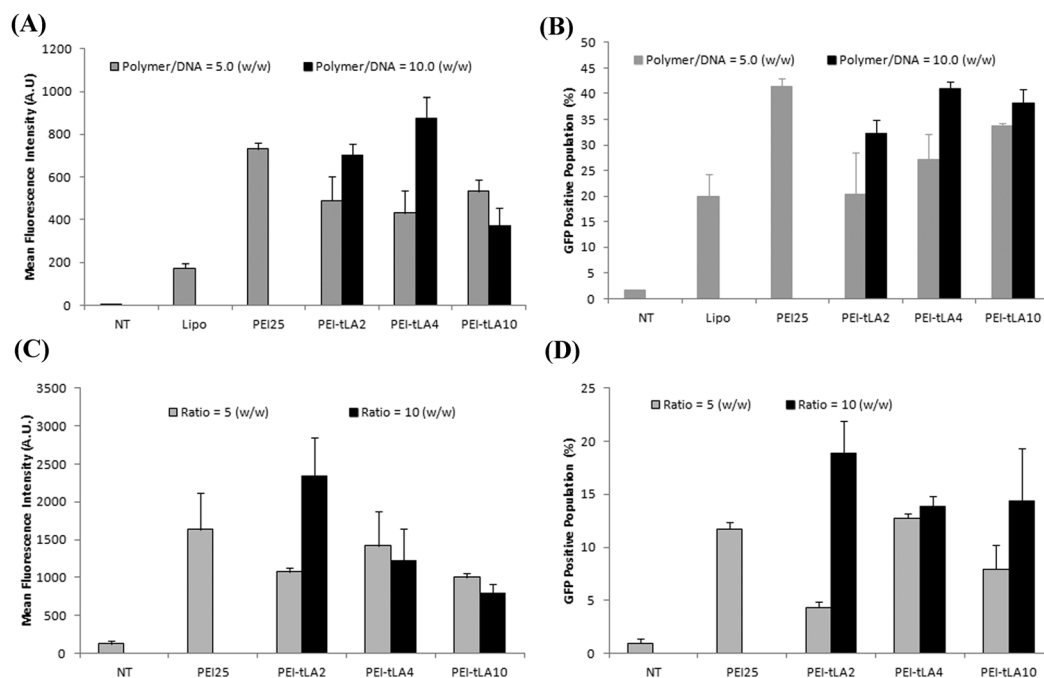


Fig. 6 The GFP expression in UCB-MSC (A, B) and hBM-MSC (C, D) as analyzed by flow cytometry after 48 h incubation with the polymer–pDNA complexes. The polymer/pDNA ratios used for complex formation were 5 and 10. The GFP expression in cells was expressed as the mean fluorescence intensity per cell (A, C) and GFP-positive population (B, D).

PEI-tLA10 complexes. With PEI-tLA2, transfection efficiency in UCB-MSC was the highest (optimal) at the HA/pDNA ratio of 2. The PEI-tLA2 with the HA/pDNA ratio of 2 also displayed higher efficiency in BM-MSC compared to other formulations (Fig. S2, ESI†), indicating the need to properly balance the composition of additive complexes.

The negative influence of HA in PEI25 complexes was presumably due to compacting effect between bigger size PEI25 and pDNA.³⁶ It was not due to an inhibition of pDNA uptake since the uptake studies with complexes indicated better internalization of the ternary complexes. It is also possible that the HA interfered with the ‘proton sponge’ effect of PEI25 in endosomes, which facilitates endosomal escape of complexes.⁴⁵ The exact reasons for this observation were considered beyond the scope of this study and were not investigated. Since HA was also not beneficial for PEI-tLAs with high degree of tLA substitution, it appears that optimization for both HA:pDNA ratio as well as extent of lipid substitution were needed for most effective complex formulations; while increasing dissociation sensitivity might be beneficial up to a point, excessive dissociation sensitivity caused by excess lipid substitution or HA incorporation might be detrimental on transgene expression. We additionally explored conventional PEI-LA polymers for transfection (using PEI1.2 as the base polymers with 1.4 to 2.3 grafted LAs per polymer), but neither the polymers on their own or as formulated with HA as ternary complexes led to effective transfection in UCB-MSC (Fig. S3, ESI†). This result further confirms the unique feature of newly described thiol-grafted lipophilic PEIs and their potential for modification of primary cells.

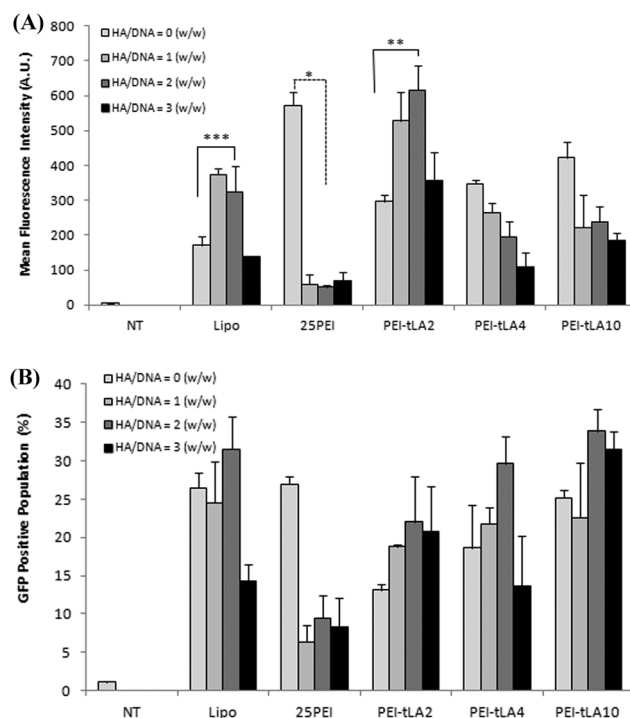


Fig. 7 The GFP expression in UCB-MSC as analyzed by flow cytometry after 48 h incubation with the polymer–pDNA + HA complexes (polymer/pDNA = 5, w/w). The transfection efficiencies were assessed at different HA/pDNA ratios (1, 2 and 3) and summarized as mean fluorescence intensity per cell (A) and GFP-positive population (B) (* p < 0.003, ** p < 0.02 and *** p < 0.11).

Conclusions

We successfully synthesized a class of cationic lipopolymers by grafting carboxyl-capped linoleic acid *via* thio-ester linkage onto PEI1.2 through *N*-acylation. Due to reduced binding and increased dissociation propensity, these polymers were intended to serve as candidates in designing supersensitive nano-formulations using the polyanion “HA” as an additive to the conventional binary complexes (*i.e.*, polymer + pDNA). These complexes displayed exceptionally higher dissociation efficacy, while the hydrodynamic diameter of the complexes remained <200 nm and surface charge ~ 15 mV. The complexes were less toxic in UCB-MSC and BM-MSC as compared to commercial transfection reagents, PEI25 and Lipofectamine™ 2000. Transfection efficiency of PEI-tLAs in UCB-MSC and BM-MSC was higher or comparable to commercial transfection reagents, PEI25 and Lipofectamine™ 2000. Among the prepared PEI-tLAs, transfection efficiency of PEI-tLA2 was further increased by adding “HA” which was beneficial up to HA/pDNA = 2 (w/w). Thus, (i) integration of electronegative functionality (thio-ester) into lipids grafted PEIs and (ii) formulation with a polyanionic additive with polymers of minimal (~ 2 /PEIs) tLA grafting can be a powerful approach for designing complexes for transfection of primary mesenchymal stem cells.

Experimental section

Polymer synthesis and characterization

Hydrophobically-modified PEIs were synthesized *via* *N*-acylation using carboxyl end-capped aliphatic lipids (Fig. 1A). Prior to *N*-acylation, carboxyl end-capping of aliphatic lipids was prepared by coupling linoleoyl chloride (LA) with mercaptopropionic acid (MPA) through thio-ester ($-S-CO-$) bonding. Briefly, LA (332 μ L, 1.0 mmol) and MPA (332 μ L, 2.5 mmol) were dissolved separately in trifluoroacetic acid (600 μ L). MPA solution was added dropwise to LA solution and the reaction mixture was stirred for 3 h at room temperature in a dark environment. The carboxyl end-capped LA (hereafter tLA) was collected by precipitation ($3\times$) in ice cold hexane and dried under vacuum for 48 h. The tLA was then grafted onto branched PEI1.2 (hereafter, PEI-tLA) through EDC/NHS activation (Fig. 1B). In typical reaction, tLA (0.2 mmol in 20 mL $CHCl_3$) was mixed with EDC (0.4 mmol in 1 mL $CHCl_3$) and stirred for 1 h at room temperature. Then, NHS (0.4 mmol in 1 mL methanol) was added dropwise and stirred for another 1 h. The activated tLA solution was then added to PEI1.2 solution (0.1 mmol in 100 mL $CHCl_3$) and the mixture was stirred overnight at room temperature. Reaction solution was concentrated by removing $CHCl_3$ through rotary evaporator. The crude product was precipitated ($3\times$) in ice cold diethyl ether and dried under vacuum for 48 h. As a control group, PEI1.2 modified with LA *via* amide bonding was prepared according to an already reported protocol.²⁵ Structural composition of tLA and PEI-tLA were elucidated through 1H -NMR spectroscopy (Bruker 300 MHz, Billerica, MA) using $CDCl_3$ and D_2O as solvents, respectively (Fig. 1C). Substitution content of lipid molecules onto PEI1.2 was additionally quantified by the TNBS assay.⁴⁶

pDNA binding and unpacking assay

DNA binding capacity of the polymers and unpacking of resultant complexes was elucidated by an agarose gel retardation assay. Briefly, a polymer solution ($0.045\text{ }\mu\text{g }\mu\text{L}^{-1}$) was diluted with 0.15 M NaCl in polypropylene tubes to give final concentrations from 0 to $0.045\text{ }\mu\text{g }\mu\text{L}^{-1}$. Subsequently, 2 μ L of pDNA ($0.3\text{ }\mu\text{g }\mu\text{L}^{-1}$) was added to each tube and gently vortexed to get complexes between 0 to 1.5 ratios of polymer/pDNA (w/w). In parallel, 0.8% of agarose gel containing EtBr ($1\text{ }\mu\text{g mL}^{-1}$) was prepared in TAE buffer ($1\times$). After 30 min of incubation, complexes were mixed with the loading buffer (4 μ L, $6\times$) and then loaded onto the agarose gel. The gel was electrophoresed for 45 min at 120 mV and the pDNA bands were visualized under UV (Alpha Imager EC). Binding capacity of PEI-tLA2 was also evaluated for complexes prepared with HA. These complexes were prepared using the same protocol as mentioned above except pDNA was mixed with HA for complex formation. Agarose gel retardation assay was also carried out to investigate the stability of the complexes in anionic environment using heparin, a common complexes unpacking agent.⁴² Heparin solution was added to the complexes (polymer/pDNA = 5, w/w) to get final concentration of 10 to 25 U mL^{-1} and incubated for 1 h at room temperature, followed by electrophoresis as mentioned above. Similarly, unpacking of the complexes of PEI1.2 and PEI-LA was also performed under identical conditions.

Physicochemical characterization

Hydrodynamic size (Z-average) and surface charge (ζ -potential) of the complexes was assayed in ddH₂O using 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 = 5) were diluted to 1 mL ddH₂O for measurements at room temperature. To elucidate the effect of HA in hydrodynamic size and surface charge, complexes (polymer/pDNA = 5) were prepared by incubating polymers with a mixture of HA and pDNA in three different HA/pDNA ratios (0.5, 1.0 and 2.0). Then, the complexes were diluted to 1 mL ddH₂O for measurements at room temperature.

Cell culture

Human umbilical cord blood mesenchymal stem cells (UCB-MSC) and bone marrow mesenchymal stem cells (BM-MSC) were used for transfection studies. UCB-MSC was collected with the mother's informed consent in accordance with the guidelines of the University of Alberta Health Research Ethics Board, as described previously.⁴⁷ The BM-MSC was from bone marrow specimens obtained from femoral reaming during total hip arthroplasty procedures (15–48 year-old patients), following informed consent and approval by the institutional Health Research Ethics Board, as described in ref. 48. Cells were routinely maintained in 75 cm^2 tissue culture flask under a humidified atmosphere (95/5% air/ CO_2) at 37 $^\circ\text{C}$ in different medium. For UCB-MSC, the medium was α -MEM supplemented with 10% FBS, 100 Unit per mL penicillin, 100 $\mu\text{g mL}^{-1}$

streptomycin and $1 \times$ per mL Gibco MEM nonessential amino acid and for BM-MSC, the medium was DMEM supplemented with 10% FBS, 1 mg mL^{-1} glucose, bFGF, L-glutamine, NaHCO_3 and pyridoxine HCL. Cells were sub-cultured (1:4 dilution typically) as they reached $\sim 75\%$ confluence.

Cytotoxicity assay

In vitro cytotoxicity of the complexes was studied in UCB-MSC and BM-MSC cells by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide) assay. Cells were plated at density of 50 000 cells per well in 48-well plates 12 h prior to each experiment. Complexes of ratios from 2.5 to 10.0 (w/w) were prepared in serum free α -MEM, directly added to each well and centrifuged for 5 min at 1130 rpm. After 4 h of incubation, cell culture medium was replaced with fresh medium (250 μL) and incubated for another 20 h. The MTT reagent (50 μL , 6 mg mL^{-1} in HBSS) was added to each well to get final concentration 1 mg mL^{-1} and incubated for 3 h. The medium was replaced with DMSO (200 μL) to dissolve the deposited formazan crystals. Finally, optical density was measured in universal microplate reader (ELx; Bio-Tech Instrument, Inc.) at $\lambda = 570 \text{ nm}$. The cells without any treatment were used as reference and the cell viability was expressed as a percentage of the non-treated cells.

In vitro uptake of pDNA complexes

The uptake of the complexes was assessed in UCB-MSC by flow cytometry and confocal microscopy. Cells were seeded either in 24 wells plates (for flow cytometry) or on cover slips (15 mm diameter) inserted into 6 well-plates (for confocal study) and grown until $\sim 50\%$ confluences. Complexes (polymer/pDNA = 5) were prepared using pDNA labeled with Cy3 fluorescence probe. Labeling was achieved according to the protocol provided by the manufacturer. Complexes were directly added to cells and centrifuged for 5 min at 1130 rpm. After 4 h of incubation, medium was replaced with fresh medium and cells were incubated for an additional 20 h. The cells were then processed for flow cytometry; cells were washed ($3 \times$) with HBSS, trypsinized and fixed with formaldehyde (300 μL , 3.5% in HBSS). The Cy3-positive population was quantified by Beckman Coulter QUANTA™ SC Flow Cytometer using FL2 channel (3000 events per sample). The setting of the instrument was calibrated for each run to obtain $\text{Cy}^{\text{TM}3}$ expression of 1–2% for control samples (*i.e.*, untreated cells). The mean fluorescence/cell and the percentage of $\text{Cy}^{\text{TM}3}$ positive cells were determined. For the microscopic study, cells were washed ($3 \times$) with HBSS (pH 7.4) and fixed with 1 mL formaldehyde (3.75% in HBSS). Cells nuclei were stained with 4,6-diamino-2-phenylindole (DAPI) and cytoplasm with wheat germ agglutinin (WGA)-Alexa Fluor®. Cells were washed ($3 \times$) with HBSS (pH 7.4) to remove unbounded dye and the cover slips were mounted onto slides and then observed under 40×1.3 oil plan-apochromat lens in Laser Scanning Confocal Microscopy (LSM710, Carl Zeiss AG, Oberkochen, Germany).

Transgene expression

In vitro transfection efficiency of the polymers was investigated in UCB-MSC and BM-MSC through flowcytometry using

gWIZ-GFP with a Green Fluorescent Protein (GFP) expression system under the CMV promotor. Commercially available transfecting agents PEI25 and Lipo2 were used as positive controls and blank medium as negative controls. Prior to transfection studies, cells were seeded in 24 well-plates and grown till $\sim 50\%$ confluences. The complexes of different mass ratios were prepared in serum free α -MEM. Then the complexes were directly added to each well and centrifuged for 5 min at 1130 rpm. After 4 h of incubation, cell culture medium was replaced with fresh medium and incubated for designed time period. Transfection efficiency was quantified based on GFP positive population and mean fluorescence intensity of the cells using flow cytometry, as described above.

Statistical analysis

The results were reported as mean \pm standard deviation of three different replicates. Student's two-tailed *t*-test assuming equal variance was calculated to determine statistical significance ($p < 0.05$) of the experimental data.

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