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Synthesis and Evaluation of Tween 85-LPEI Copolymers for Gene Transfection *In vitro* and *In vivo*

Mingxing Wang*, Bo Wu, Jay D Tucker, Peijuan Lu, Sapana N Shah, Shante Wade and Qilong Lu

Department of Neurology, McColl-Lockwood Laboratory for Muscular Dystrophy Research, Neuromuscular/ALS Center, Carolinas Medical Center, 1000 Blythe Blvd. Charlotte, NC 28231, USA

Abstract

A series of cationic amphiphlic copolymers constructed from Tween 85 and low molecular weight (Mw) polyethyleneimene (LPEI) were prepared in ambient conditions and characterized. The Mws of these copolymers ranged from around 5,000 to 25,000 Da and PEI content from 8.25% to 20.91%. The new copolymers condensed DNA efficiently with particles size below 200 nm at the weight ratio 5 of polymer/pDNA, and were stable in the presence of Serum or Heparin. The introduction of Tween 85 led to a significant increase in the cellular uptake of complexes with higher transfection efficiency in CHO, C2C12, and HSkM cell lines, but without increase in toxicity compared with the parent LPEI. The best formulation for pDNA delivery produced transgene expression efficiency 5, 20-fold of PEI 25k in vitro and in mdx mice in vivo, respectively. There is no obvious muscle damage with these new copolymers at the injection sites. These results indicated that the presence of more hydrophobic groups within the new polymers is associated with higher transfection efficiency (TE), and the higher PEI content and Mw of polymers show higher TE with relatively higher toxicity. The Tween 85 modified LPEI could be a potentially safe and effective polymeric carriers for gene/drug delivery.

Keywords: Nanoparticle; Low molecular weight polyethyleneimene; Gene transfection; Amphiphilic polymers; Non-viral vector; Muscular dystrophy

Introduction

Gene therapy holds great potential for treating heritable and acquired diseases such as HIV, cancer, cystic fibrosis, and others [1]. However, the challenge for successful gene therapy is to develop safe and efficient delivery vectors. Over the past decades, non-viral vectors, especially the synthetic polymers have attracted much attention because of the structural flexibility, larger capacity for therapeutic genes, ease in handling with greater safety and less expense than viral vectors [2-8]. Still, low transfection efficiency and toxicity are still major barriers for the progression of non-viral vectors to clinical applications [6-8]. Among the numerous synthetic vectors, polyethylenimine (PEI) is one of the most widely used and effective agents due to the so-called "proton sponge effect" [9]. However, transfection efficiency and toxicity are both molecular size dependent, with higher molecular weight leading to higher transfection efficiency together with higher toxicity [10].

In this study, a series of low molecular weight PEI (LPEI) modified with Tween 85 (T85) were prepared by varying feed ratio and PEI size (Mw: 0.8k, 1.2k and 2.0k Da) and examined for gene delivery potential. T85, an amphiphilic sorbitan fatty acid ester ethoxylate widely used in biochemical applications, contains an average of 20 units of ethylene oxide and three oleate chains, which have been reported to be able to encapsulate siRNA because of their flexibility [11]. We hypothesized that the conjugation of T85 and LPEI could improve DNA or oligonucleotide delivery through the combination of the beneficial properties of both polyoxyethylene and lipid components [12-14]. Our study aimed to test whether T85/LPEI could confer better transfection efficiency than PEI-containing hydrophilic polymers through improved cell-uptake and improved stability of polymer/DNA polyplex in circulation [9]. Biodegradable carbamate linkages were introduced as a means to reduce the cytotoxicity of the cationic amphiphile [15]. The copolymers code and ^{1}H-NMR are given in Figure 1. Their physicochemical properties, toxicities, effects for pDNA delivery *in vitro* and *in vivo* using dystrophic *mdx* mice were examined. Results from the evaluation reveal a synergistic effect of PEI and T85 components for the delivery of pDNA as T85 itself does not improve pDNA delivery. Transfection efficiency of these copolymers appears dependent on molecular size, PEI content and HLB of copolymer composition.

Experimental Section

Materials

Tween 85 (T85), Polyethylenimine (PEI, branched, 0.8k, 1.2k 2.0k and 25k Da), 1,1'-carbonyldiimidazole (CDI), acetonitrile, and ethidium bromide (EB), and ethanol were purchased from Sigma-Aldrich (St. Louis, MO, USA). Cell culture media, RPMI 1640, Dulbecco's Modified Eagle's Medium (DMEM), penicillin– streptomycin, fetal bovine serum, L-glutamine and HEPES were purchased from Invitrogen (Eugene, Oregon, USA). MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium]-based assay by Cell Titer 96®Aqueous One Solution Proliferation Kit was from Promega Corporation, (Madison, MI, USA). Lipofectamine-2000 (LF-2000) was from Life Technologies (Carlsbad, CA, USA). Trypsin-ethylenediaminetetraacetic acid (EDTA) (TE, 0.5% trypsin, 5.3 mM EDTA tetra-sodium) were obtained from Gibco BRL (Gaithersberg, MD, USA). All other chemicals were reagent grade without further treatment. Digital images were obtained using Olympus IX51 and IX71 fluorescent microscopes (Olympus America Inc, Milville, NY, USA). Digital images were taken using the Olympus DP Controller and DP Manager software (Olympus America Inc, Milville, NY, USA).

***Corresponding author:** Mingxing Wang, Department of Neurology, McColl-Lockwood Laboratory for Muscular Dystrophy Research, Neuromuscular/ALS Center, Carolinas Medical Center, 1000 Blythe Blvd. Charlotte, NC 28231, USA, Tel: 1-704-355-5588; Fax: 1-704-355-1679; Email: mingxing.wang@carolinashealthcare.org

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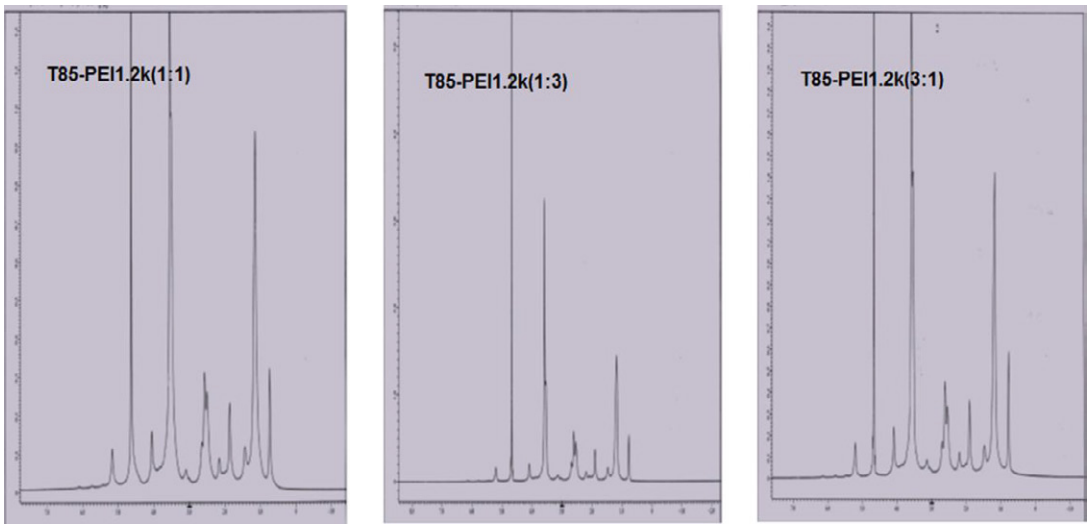
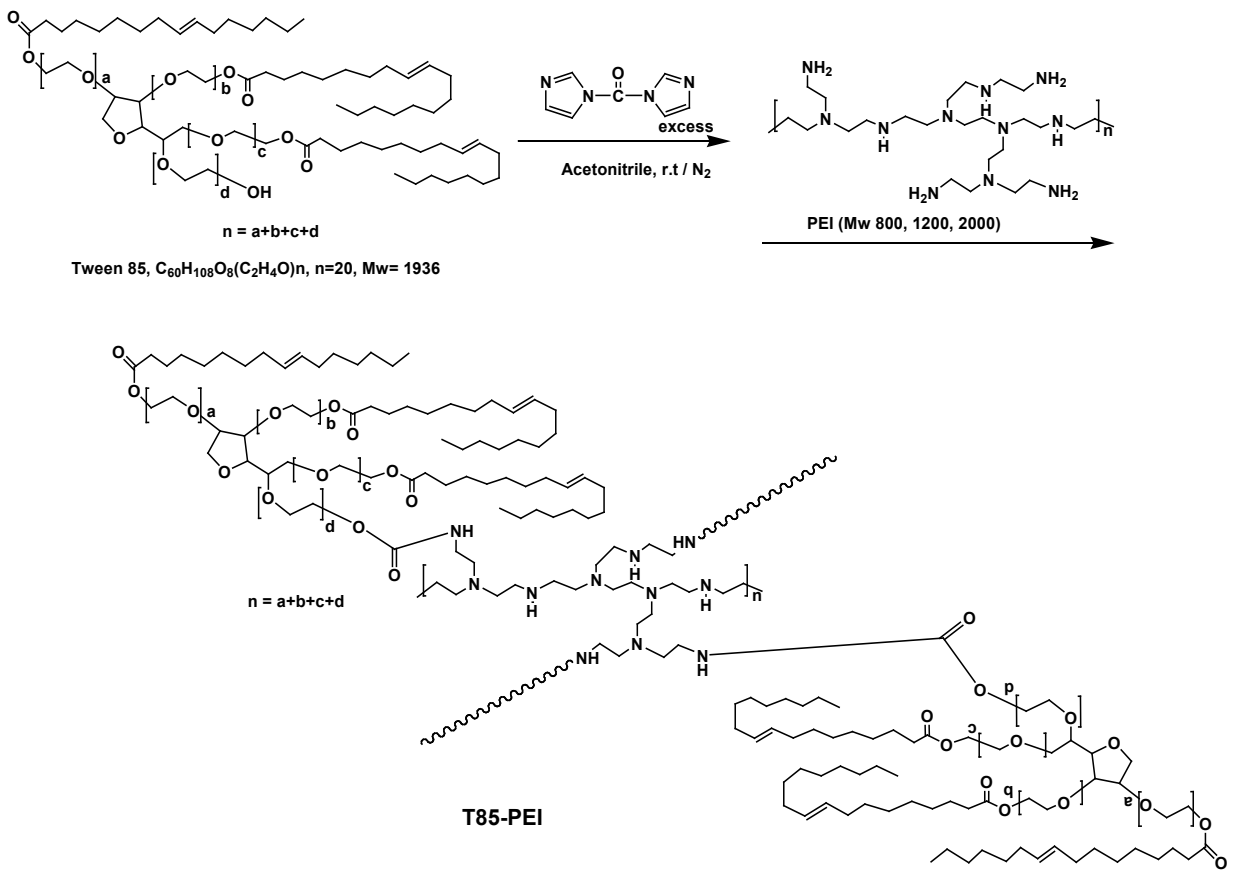


Figure 1: ^{1}H NMR of T85-LPEI (Z polymers) in D_2O with 500 MHz JEOL.



Scheme 1: Synthesis of T85-LPEI (Z polymers) copolymers.

Synthesis and characterization

T85-PEI copolymers were synthesized as shown in Scheme 1 [16,17]. Briefly, the T85 was dried overnight in vacuum at 40 °C prior to modification, and then activated with excess of 1, 1'-carbonyldiimidazole (CDI) in 10 mL of anhydrous acetonitrile. After stirring for 3 hours at room temperature, the reaction mixture was diluted with water and dialyzed in spectrapore membranes with MWCO 500 Da against 10% ethanol, and lyophilized to the imidzolyl-

carbonyl-activated T85. Excess of PEI was added dropwise into 20% aqueous ethanol of the activated T85 under N_2 at room temperature under stirring overnigh. To the reaction mixture, an equivalent volume of pure water was added and filtered using 0.45 mm micropore film to remove insoluble impurities. The product was then dialysed against pure water using dialysis membrane (MWCO 500 for PEI 0.8k, MWCO 1000 for PEI 1.2k and 2.0k) for 48 hours, and the dialysis medium was refreshed every 12 hours to remove residual small Mw reagents including PEI. The conjugates were then processed with cation exchange chromatography for the separation of unconjugated T85 from the conjugated form. The final products were then lyophilized for characterization by Nuclear Magnetic Resonance (JEOL ECA-500 NMR) and elemental microanalysis to determine the composition.

Amplification and purification of pDNA

One Shot® TOP10 chemically competent Escherichia coli (Invitrogen, Eugene, Oregon) was transformed with pZsGreen1-N1 Vector as reporter gene (Clontech, Mountain View, CA) per the manufacturer's instructions. The transformed bacteria were grown on LB agar overnight at 37°C with tetracycline. Single clones were selected and grown for 8 hours in LB broth media. Plasmid was extracted from the bacterial cultures using the Miniprep protocol, digested with Eco R1 restriction enzyme and electrophoresed on 1% agarose gel to confirm the right size. The pDNA was purified using the Qiafilter plasmid purification kit from Qiagen, dissolved in purified water, and stored at -80°C until later use. The purity and concentration of DNA was determined by NANODROP-2000c Spetrophotometer (Thermo Scientifitic, USA) measuring ultraviolet (UV) absorbance at 260/280 nm and correct size was further confirmed by 1% agarose gel electrophoresis.

Complexation study of polymer/DNA

DNA binding: All polymer/DNA complexes were prepared immediately before use by gently vortexing a mixture of DNA and polymer solution at various polymer/DNA weight ratios. The complexes were incubated at room temperature for 30 minutes in 24 µl medium, and then the samples were electrophoresed on a 1% (w/v) agarose gel stained with 0.1 µg/ml ethidium bromide in TAE buffer at 100 V for 40 min, and analyzed by a UV illuminator to visualize the location of pDNA bands.

Resistance to serum and heparin: To determine the stability of the polymer/DNA complexes in the presence of serum, 10 µl of 5:1 ratio of polymer/ DNA complexes were added to 0.5 ml Eppendorf tubes. Fetal Bovine Serum (FBS) was then added by volume to achieve final concentrations up to 50%, mixed, and incubated for 30 minutes at 37°C. These samples were then electrophoresed in 1% agarose gel to determine the stability of the complexes. For the analysis of their resistance to heparin, varying amounts of 1 µg/µl heparin sodium was added to polymer/DNA complexes of 5:1 ratio to achieve final concentrations of 0, 10, 25, 50 µg/ml. Samples were then incubated at 37°C for 20 minutes and electrophoresed in a 1% agarose gel to determine stability [17].

Particle size, ζ -potential measurements and transmission electron microscopy (TEM): The size and ζ -potential measurements of polyplexes were performed at 25°C using Zetaplus Zeta Potential Analyzer (Brookhaven Instrument Co. NY, USA) equipped with a 15 mV solid-state laser operated at a wavelength of 635 nm. Effective hydrodynamic diameter was measured by photon correlation spectroscopy using the same instrument at 25°C with the angle of 90°. The polyplexes were prepared at a final concentration of 5 µg/ml pDNA at various weight ratios for size measurements and in HBSS (25 mM HEPES, 150 mM NaCl, pH 7.4) for ζ -potential experiments, respectively. The polyplexes were then incubated at room temperature for 30 minutes. The size and ζ -potential values are presented as the average values of three assays. T85-PEI 2.0k/pDNA polyplexes were prepared at an optimal weight ratio, as determined from previous experimental assays. The morphologies of resulting polyplexes were observed using TEM (Phillips CM-10, PhilipsElectronics North America corp., Andover, MA, USA). The samples were prepared using negative staining with 1% phosphotungstic acid. Briefly, one drop of polymer/pDNA complex solution was placed on a formvar and carbon coated grid (Electron Microscopy Sciences, Hatfield, PA, USA) for 1 hour, and the grid was blotted dry. Samples were then stained for 3 minutes, before the grids were blotted dry again. Samples were analyzed at 60 kV. Digital images were captured with a digital camera system from 4pi Analysis (Durham, NC, USA).

In vitro tests

Cell line: Chinese Hamster Ovary (CHO), mouse myoblasts cell (C2C12) and Human Skeletal Muscle (HSkM) cell lines were used in this study.

Cytotoxicity assay: The cytotoxicity was evaluated with CHO and C2C12 cells using the MTS-based assay by Cell Titer 96® Aqueous One Solution Proliferation Kit (Promega Corporation, Madison, MI, USA) 24 hours after the treatment with different doses of polymers. MTS assay by Cell Titer 96® Aqueous One Solution Proliferation Kit (Promega, Madison, WI, USA) 24 hours after the treatment with polymers. Briefly, the CHO and C2C12 cells were seeded in 96-well plates at a density of $1x10^4$ cell per well respectively, and then cells were incubated in 100 µl RPMI-1640 or DMEM respectively, containing 10% fetal bovine serum (FBS) for 24 hours prior to adding the polymers, after the polymers were added for 2 days, the medium was replaced with 200 µl of fresh medium. Then 20 µl MTS was added to each well and further incubated for 4 hours. The absorbance was measured at 570 nm using Tecan 500 Plate reader (Tecan US, Inc, Morrisville, NC, USA) to obtain the metabolic activity of the cell. Viability of untreated cells were taken as 100% and wells without cells as blanks. The relative cell viability was calculated by: $(\mathbf{A}_{treated}\text{-}\mathbf{A}_{background}) \times 100/(\mathbf{A}_{control}\text{-}\mathbf{A}_{background})$. All viability assays was carried out in triplicate.

Transfection in vitro: C2C12, HSKM muscle cells and CHO cells were grown in DMEM or RPMI-1640, respectively, and incubated at 37°C in a humidified atmosphere containing 10% CO_2. 5×10^4 cells per well were seeded in a 24 well plate in 500 µl medium containing 10% FBS and grown to reach 70-80% confluence prior to transfection. Cell culture medium was replaced with the growth media prior to addition of polymer/DNA (1 µg plasmid DNA) polyplexes with varying ratio of polymer to pDNA PEI 25k was used as positive control. Transfection efficiencies were determined quantitatively using flow cytometry (BD FACS calibur, BD), the percentages of transfected cells expressing GFP were quantified from a gated viable population of 10,000 cells. And relative efficiency was also recorded using Olympus IX71 inverted microscopy.

Transfection *in vivo*

Ten *mdx* mice aged 4 to 6 weeks were used for each experimental group. 10 µg plasmid DNA with or without 2 µg polymer in 40 µl saline was used for each tibialis anterior (TA) muscle via local injection. The muscles were examined 5 days after injection by Olympus IX71 inverted fluorescent microscope for the expression of GFP. The number of GFP expressing muscle fibers was counted from a minimum of 6 sections spanning at least half the length of the muscles. Maximum

number of GFP positive fibers in one section for each TA muscle was used for comparison in transfection efficiency. Experimental protocols were approved by the Institutional Animal Care and Use Committee (IACUC), Carolinas Medical Center.

Statistical analysis

For statistical analysis, triplicate data were analyzed using Student's t-test. $p<0.05$ was considered statistically significant. Data are presented as the mean ± SD.

Results and Discussion

Synthesis and characterization of T85-PEI copolymers (Z series)

T85 was first activated by CDI followed by reaction with different molar ratio of the low molecular weight PEI (LPEI). The final products were purified by dialysis to remove unreacted PEI and other small molecules, followed by cation exchange chromatography to separate unconjugated T85. The composition of the final purified products was determined by the ^{1}H-NMR spectra using integral values obtained for the $-CH_2CH_2O-$ or CH_3- protons of T85 and - CH_2CH_2NH- protons of PEI, and the nitrogen content analyzed by microanalysis. The two techniques confirmed the results coincide well with each other. The molecualr weight (Mw) of the copolymers was found to depend on the LPEI size and feed ratio of T85/LPEI: (1) copolymers were obtained with lower Mw when the PEI was in excess (the feed ratio of T85/PEI = 1/3) as compared to equal ratio (T85/PEI=1/1) or T85 in excess (T85/PEI=3/1) as tested. This indicates that the primary amines of PEI are not completely grafted, as the PEI content should be higher than the other two feed ratios; (2) when T85 is in excess or equal to PEI, there is no obvious Mw difference determined by microanalysis and ^{1}H NMR, indicating that the equal ratio result in the PEI grafted with T85 up to a saturated state; (3) the Mw of copolymers constructed with PEI 0.8k and PEI 1.2k showed little difference from one another, while PEI 2.0k-based coplymers showed significantly increased Mw where equal or excess of T85/PEI 2k (23391.9 Da, 24242.5 Da, respectively) was used. This finding suggests that the PEI 2.0k higher reaction activity also lead to the highest PEI content (20.91%) with the feed ratio T85/PEI 2.0k at 1/3 among all copolymers. The measurement of Mw with ^{1}H-NMR analysis showed slightly higher values than that determined by element analysis with the exception of Z5 (T85/PEI 1.2k=1/3). The nomenclature & characteristics of the cationic amphiphilic polymers can be referenced in Table 1.

Characterization of polymer/pDNA polyplexes

The interaction between Z polymers and pDNA was assessed by agarose gel electrophoresis. As illustrated in Figure 2, the parent polymer T85 (Z0) was unable to bind and condense the pDNA. In contrast, all of the new polymers (Z1-Z9) showed high capacity to bind and condense pDNA as demonstrated by the shifting of the ethidium bromide (EB) stained pDNA towards significantly higher molecular size on gel electrophoresis. Some complexes migrated even slightly toward the anode when the ratio of the Z polymer/pDNA was increased to 2 or higher suggesting that the polyplex had a net positive charge. The polymers tested such as Z4, Z5 and Z8, owning to relatively high Mw (7766.7, 6410.3, 9564.8, respectively) or higher PEI content (15.45%, 18.72% and 20.91%, respectively), showed very strong binding ability with pDNA even at the ratio of the Z polymer/pDNA = 1/1 with the majority of DNA being retained within the loading wells. These resulting characteristics indicate a near complete neutralization of the negatively charged pDNA by the polymers or the formation of larger complexes. The Z polymers such as Z1, Z3, Z6, Z7 and Z9 with lower PEI content only showed weak binding when used with the same ratio of polymer to pDNA, as shown in Figure 2.

Code	Composition (Molar ratio) [a]	N (%) [b]	Mw(Da)		PEI (%) [d]	Grafted T85 /PEI [b]
			EA [b]	^{1}H NMR [c]		
Z0	T85	0			0	
Z1	T85-PEI 0.8k (1:1)	4.43	6019.6	6981.4	13.28	6.53
Z2	T85-PEI 0.8k (1:3)	5.20	5123.5	5534.8	15.61	5.40
Z3	T85-PEI 0.8k (3:1)	4.12	6422.5	7223.6	12.46	7.02
Z4	T85-PEI 1.2k (1:1)	5.17	7766.7	8370.2	15.45	5.47
Z5	T85-PEI 1.2k (1:3)	6.24	6410.3	5796.3	18.72	4.34
Z6	T85-PEI 1.2k (3:1)	4.65	8602.2	9289.4	13.95	6.17
Z7	T85-PEI 2.0k (1:1)	2.85	23391.8	25863.7	8.55	10.70
Z8	T85-PEI 2.0k (1:3)	6.97	9564.8	10808.3	20.91	3.78
Z9	T85-PEI 2.0k (3:1)	2.75	24242.5	29485.6	8.25	11.12

[a] Feed ratio of starting materials; [b] Microanalysis of nitrogen; [c] ^{1}H NMR analysis with 500 MHz Jeol ; [d] Assumed N% is 33.33 wt% in each PEI.

Table 1: Characteristics of T85-LPEI (Z polymers).

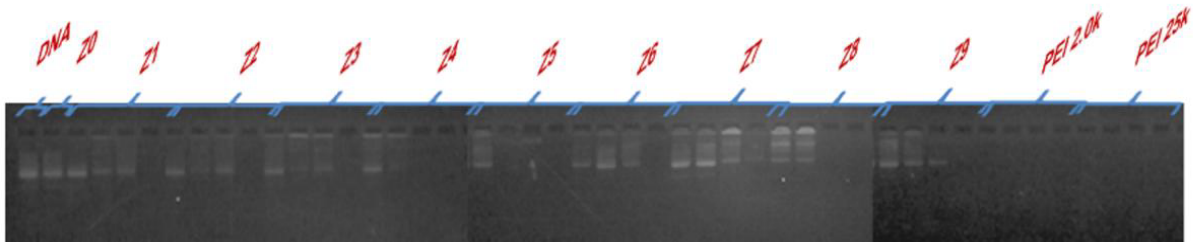


Figure 2: Electrophoretic mobility of gene mixed with polymers at various weight ratios (all polymer/pDNA = 0.2, 0.5, 1.0, 2.0, except T85 (Z0) only at the ratio of 2).

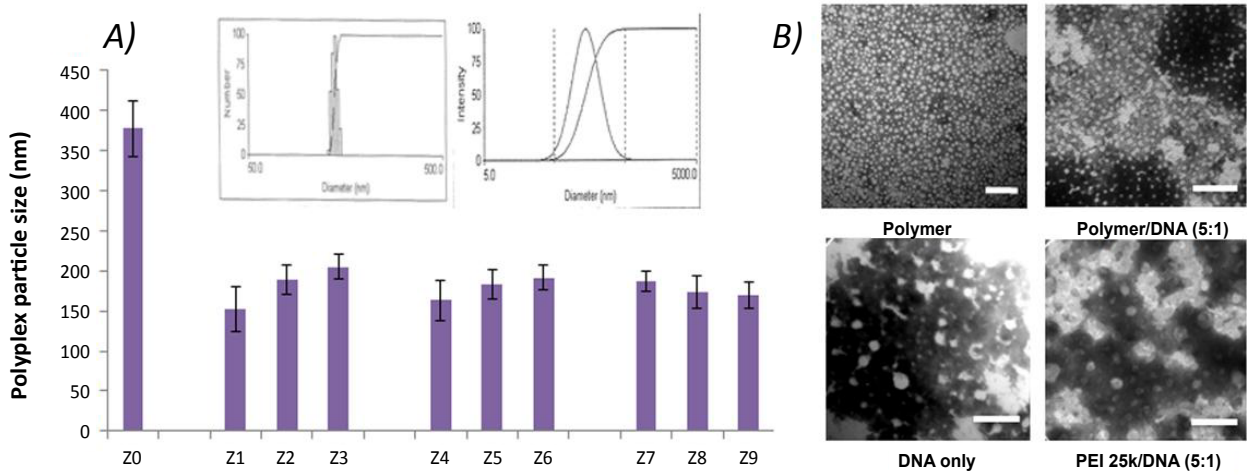


Figure 3: Biophysical properties of polymer/pDNA complex. A) Particle sizes of polymer/DNA=5:1 (weight ratio); Inserted is the distribution of size; B) Negative staining TEM images of polymer/pDNA (5:1) complexes (scale bar 200 nm).

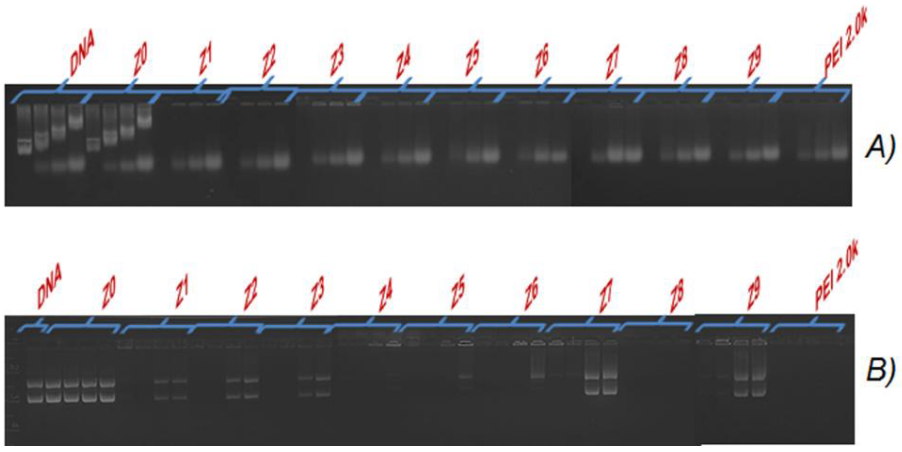


Figure 4: Stability of complexes [Polymer/pDNA=2/1(weight ratio)] to serum or heparin. A) In the presence of serum. 4 lanes for each polymer with FBS concentration of 0, 10, 25, 50% from left to right; The lowest bands are nonspecific binding of EB to serum components. B) Sodium heparin (0, 10, 25, 50 μg/ml) from left to right.

DNA/polymer complex size is an important characteristic for effective gene transfection with nano-sized particles generally considered as preferable. We therefore examined the complex of Z polymers and pDNA by dynamic light scattering (DLS). All of the modified polymers condensed DNA into nano-sized particles at the polymer/pDNA ratio of 2 and above, with highly homogenous hydrodynamic diameter with a narrow size distribution (Figure 3A). The particle size of PEI/pDNA varied, depending closely upon the size of PEIs. LPEI/pDNA complex formed aggregated particles of >500 nm, whereas PEI 25k formed very tight particles of around 130 nm [17]. To gain further insight into the polymers/pDNA complexes, we performed TEM analysis using the optimized polymer/pDNA formulation. The nanoparticles were well defined and uniformly distributed with sizes below 100 nm at a representative w/w ratio of 5 (Figure 3B). The physical mixture of the same proportion of PEI/pDNA again produced aggregates of various sizes, characteristics of interaction between free PEI and DNA as has been reported previously [17,18]. The noticeably smaller particle size observed under TEM in comparison with that from DLS analysis was most likely an artifact result of TEM processing which required the samples to be dried, with the evaporation causing slightly diminished particle size observation. The results therefore suggest that the T85 component in the Z polymers was able to form desirable particles by preventing PEI from aggregation with DNA [18-20].

We also examined the surface charge of the pDNA/polymer nanoparticles by DLS measurement. As expected, all of the Z/pDNA complexes showed significantly lower zeta potentials when compared to PEI/DNA complex. The average zeta potentials of the Z polymers were in the range of -17.3 to 12.5 mV using the polymer/pDNA weight ratio from 0.5-20. The reduced positive charge observed is consistent with the grafting of T85 onto the primary amines of PEI within the new polymers.

Stability of Z/pDNA polyplexes in the presence of serum and heparin

Serum, which contains a number of negatively charged molecules from proteins to glycosaminoglycans (GAG), has a significant effect on gene transfection efficiency in vitro and especially in vivo, when

positively charged polymers are used as delivery carriers. Negatively charged serum components can compete with negatively charged DNA for the binding of positively charged polymers, leading to the dissociation of DNA from the carrier and modulation of gene delivery effect. The presence of DNAase in the serum as well, can degrade accessible pDNA thus reducing effective concentration or constitution of transgenes. The stability of the pDNA/polymer complex in the presence of serum is therefore an important factor for both in vitro and in vivo applications of gene carriers. To determine the stability of polymer/pDNA polyplex, the complexes were prepared at different ratios and FBS was added to a final concentration of 0, 10, 25, and 50% followed by the mixtures being analyzed by gel electrophoresis (Figure 4A). These results were in agreement with previous reports, with pDNA reacting with serum components to form complexes indicated by an increase in the size of the pDNA bands as increasing amounts of FBS were applied [16,17]. When pDNA was bound to the Z polymers at polymer/DNA ratio of 2, however, serum had no apparent effect on the polymer-DNA particles, a result similar to the pDNA when complexed with PEI 2.0k. This result suggests that LPEIs within the copolymers maintain their binding affinity and strength, thereby preventing the bound pDNA cargo within the particles from being replaced by serum components.

Sodium heparin also has strong negative charges and has been used to further evaluate binding strength between polymer and pDNA [21,22]. When the complex of polymer/pDNA at the weight ratio of 2 and in the presence of 10 μg/ml sodium heparin, EB signals for DNA remained in the loading well in all samples with polymers. Increasing the concentration of sodium heparin to 25 μg/ml led to near complete dissociation of DNA from the polymers including Z1, Z2, Z3, Z7 and Z9. However, the majority of DNA within Z4, Z5, Z6 and Z8 complexes remained bound, likely due to both their higher PEI composition and/or higher Mw as compared with the others tested. Almost all pDNA was dissociated from the Z polymer/DNA complexes when the concentration of sodium heparin was increased to 50 μg/ml except for Z8 with higher Mw (9,564.8) and highest PEI content (20.91%). The complexes of PEI/pDNA remained intact in the presence of sodium heparin even at 50 μg/ml (Figure 4B). These results further suggest that the increasing affinity of Z polymers is associated with increasing Mw, and the presence of T85 in the polymers weakens the binding of LPEI to DNA to some degree. The concentration-dependent release of DNA from Z polymer/DNA complexes in the presence of highly negative charged heparin, the abundance of which varies considerably amongst tissues, can aid in the design of polymers for controlled release of cargo DNA with greater tissue specificity.

Cell viability

The cytotoxicity of the Z polymers was determined in CHO and C2C12 cell lines with MTS-based cell viability assay. PEI toxicity to C2C12 cells was clearly size-dependent with higher molecular weight PEI demonstrating higher toxicity. The LPEI (0.8k, 1.2k and 2.0k) showed limited cytotoxicity with no significant cell death up to 20 μg/ml, while the cell viability dropped to around 20% when treated with PEI 25k at the same dose [17]. Cell viability with all of the Z polymers at the dose of 20 μg/ml were similar to or lower than that of their parental LPEIs, and much lower than observed with PEI 25k at 4 μg/ml. The polymers showed similar trend in both cell lines, with a slightly higher cell viability observed in CHO (data not shown) than in C2C12 (Figure 5). This improved cyto-compatibility is undoubtedly contributed to the low-toxicity of the building blocks, and the steric shielding of T85 reducing density of the positively charged PEI, thereby resulting in a decrease of the average protonation constant of the polymer [23]. Toxicity was also found to be associated with PEI content and Mw of polymers. The polymers with higher PEI content such as Z5 and Z8 has relatively elevated toxicity at higher doses tested.

Transfection efficiency of PCM polymers in vitro

Transfection efficiency (TE) was assessed in CHO, C2C12 and

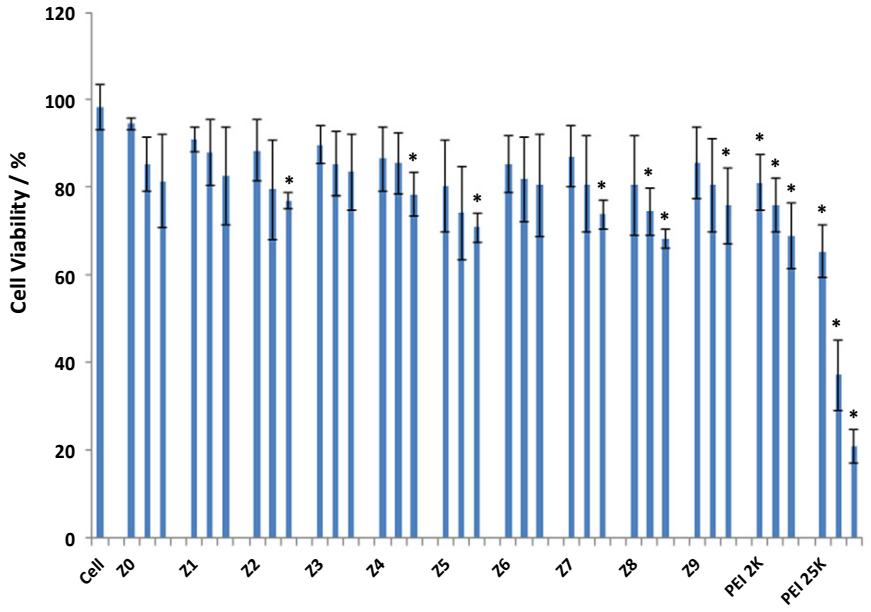


Figure 5: Cell viability of C2C12 cell line after treatment with polymers at various doses. Cells were seeded in 96 well plates at an initial density 1×10^4 cells /well. Cell viability was determined by MTS assay (The percentage is the average of triplicate experiments. The concentration of polymers are 4, 10, 20 μg/ml from left to right. (*p<0.05 compared with untreated cell as control, two-tailed Student's t-test, error bar represents standard deviation, n=3).

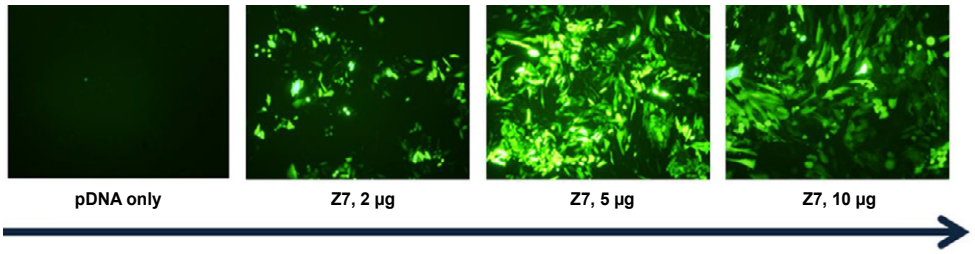


Figure 6: Dose dependent GFP expression for pDNA delivery of Z7 in CHO cell (polymer used 2,5,10 µg with pDNA 1 µg in 500 µl 10% FBS medium).

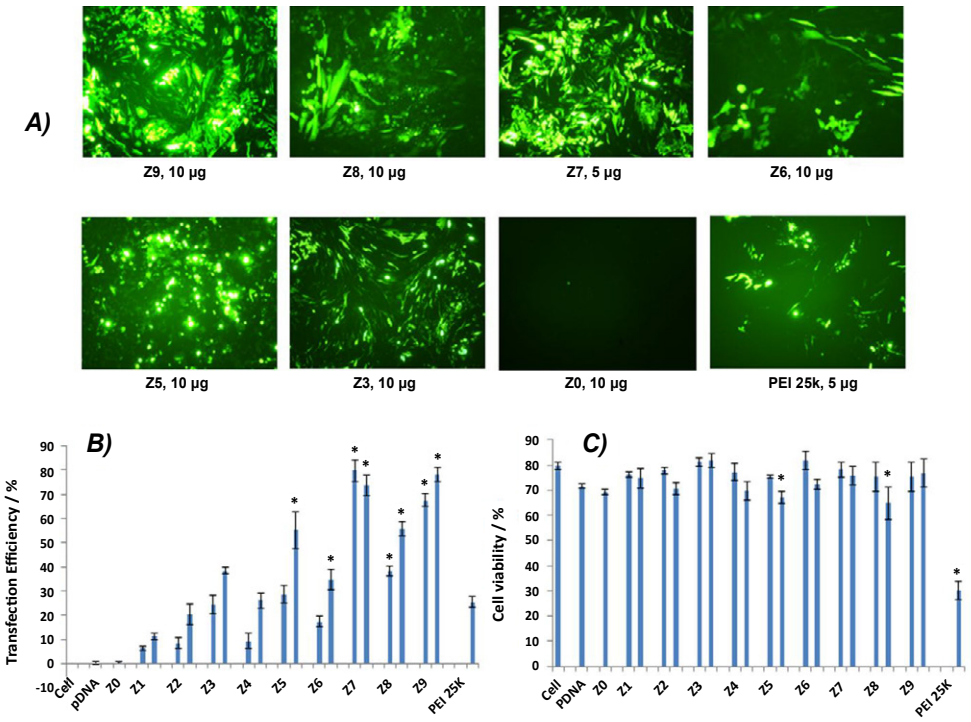


Figure 7: pDNA delivery in CHO cell line transfected with polymer/pDNA complexes at weight ratios of 5 &10 after 48 hrs incubation. [Z0 (10 µg), Z1-Z9 (5/10 µg), PEI 25k (5 µg); pDNA 1 µg in 500 µl medium in CHO cell line, (PEI 25k was tested with the dose of 5 µg, since 10 µg caused nearly 100% cell death). A) GFP expression illustrated under fluorescent microscopy. B) Transfection efficiency analyzed with FACS (*p<0.05 compared with PEI 25k, two-tailed Student's t-test, error bar represents standard deviation, n=3). C) Cell viability (*p<0.05 compared with untreated cell as control, two-tailed Student's t-test, error bar represents standard deviation, n=3).

HSkM cell lines with plasmid DNA encoding green fluorescent protein (GFP) transgene as the reporter vector at 1 µg /500 µl culture medium, complexed with designated amount of polymer in 24 well plates.

Transfection efficiency in CHO cell: CHO cells are commonly used in studies of genetics, toxicity screening, nutrition and gene expression because of their rapid growth. These Z polymers were initially evaluated in CHO cell line for GFP gene delivery and expression. The results of qualitative and quantitative analyses by fluorescent microscopy and FACS are illustrated in the Figures 6 and 7. We initially investigated the dose effect on transfection efficiency (TE), finding the TE significantly improved with increasing doses from 0 to 10 µg with 1 µg pDNA in 500 µl medium. The TE reached over 80% when the dose is 5 µg for some polymers, without apparent improvement in GFP expression when the dose of the Z polymers increased to 10 µg, which means the optimum dose was between 5-10 µg for most polymers (Figure 6). Therefore we further examined TE and cytotoxicity using the dose 5 & 10 µg of Z polymers under the same condition as above (Figure 7). Polymers such as Z7, Z9 and Z8 achieved a TE of around 80%, 80% and 60% respectively at the dose of 5 or 10 µg, much higher than the other Z polymers tested at the same dose, most likely due to the higher Mw. The relatively higher TE with Z7 and Z9 when compared to Z8 suggests that higher T85 content provides better plasmid transfection. Similarly, polymer Z3 and Z6 of higher Mw demonstrated higher TE than other polymers of the same size LPEI construction. Again, higher Mw of the Z polymer containing the same LPEI would indicate that more primary amines were likely replaced by the T85. These results further support the notion that higher number of amphiphilic T85 groups confer an advantage for gene delivery. As controls, PEI 25k produced about 25% TE and this was associated with clearly high toxicity as indicated by reduced cell density and cell viability down to 30% when used at the ratio of 5. The cell viability of pDNA formulated polymer is quite consistent with the polymer only. Moreover, it is clear that the more hydrophobic Z polymers result in higher TE as compared to more

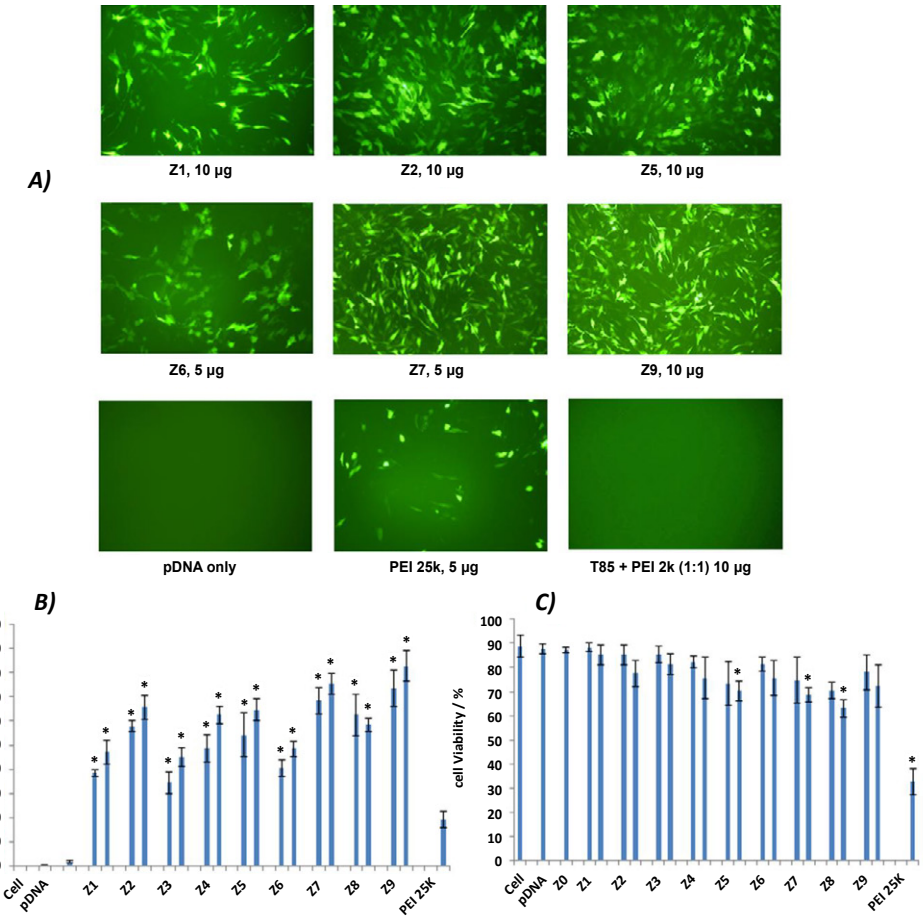


Figure 8: pDNA delivery in C2C12 cell line transfected with polymer/pDNA complexes at weight ratios of 5 &10 after 48 hrs incubation. [Z0 (10 µg), Z1-Z9 (5/10 µg), PEI 25k (5 µg); pDNA 1 µg in 500 µl medium in C2C12 cell line. A) GFP expressed illustrated under fluorescent microscopy. B) Transfection efficiency analyzed with FACS (*p<0.05 compared with PEI 25k, two-tailed Student's t-test, error bar represents standard deviation, n=3). C) Cell viability (*p<0.05 compared with untreated cell as control, two-tailed Student's t-test, error bar represents standard deviation, n=3).

hydrophilic counterparts. One likely reason is that stronger hydrophobic components enhance cell penetrating effects. These results suggest that appropriate positive charge, distribution of polymer/DNA complexes and resulting molecular size, as well as HLB within copolymers are critical for effective gene delivery.

Gene delivery in muscle cells: Our primary objective is to improve gene delivery for the treatment of muscular dystrophies. Muscular dystrophies are characterized by progressive muscle weakness largely caused by gene mutations with defects in muscle proteins [24,25]. First, these polymers were applied for pDNA delivery in C2C12 cells (mouse myoblasts) for the expression of a pDNA encoded GFP reporter. The dose effect on the transgene delivery and cytotoxicity in C2C12 cells were examined at the dose of 5 and 10 µg of polymer with 1 µg pDNA in 500 µl medium. The results of qualitative and quantitative analyses by fluorescent microscopy and FACS are illustrated in Figure 8. TE was found to be higher in the cells treated with the polymers Z2, Z4, Z5, Z7, Z9 at the dose of 10 µg compared to the remaining polymers and the PEI 25k (5 µg); Z8 reached the maximum GFP expression at 5 µg, which is most likely resulting from the higher PEI content as compared to others. In contrast, pDNA alone and a simple formulation (mixture) of T85 and LPEI with pDNA at the same ratio corresponding to the Z polymers resulted in very limited GFP expression. These results indicate that chemical conjugation of the two basic components is essential for effective delivery, probably due to improved stability upon administration in physiological conditions. The cell viability assays showed a similar trend to that observed in the CHO cell line (Figure 7) or without pDNA in formulation (Figure 5), which further confirmed these Z polymers retain low cytotoxicity of LPEI after modification with T85, although the molecular size increased.

In order to examine the transgene delivery efficiency as dependent on cell type, we also further studied them in Human Skeletal Muscle (HSkM) cells using the same conditions as tested in the C2C12 cell line. HSkM cells are human primary cells which are isolated from the skeletal muscle of limbs from adult or fetal donors. These cells can undergo differentiation to exhibit actin and myosin myofilaments, and also have the potential to act as a cardiac graft, mending damage to the heart [26]. It provides a useful model system to study many aspects of muscular function and disease, but efficient gene transfer in primary cells remains limited [27]. We further tested our polymers in HSkM cell lines as they are biologically more relevant and resemble our targets in vivo for the treatment of muscular dystrophy. The results showed that the polymers had similar effect in the HSkM as in C2C12 cell line. Nearly all of the polymers produced TE over 60%, such as Z1, Z2, Z3 Z4, Z6, Z7, Z8 at the dose of 5µg, and Z9 at the dose of 5 or 10 µg, with

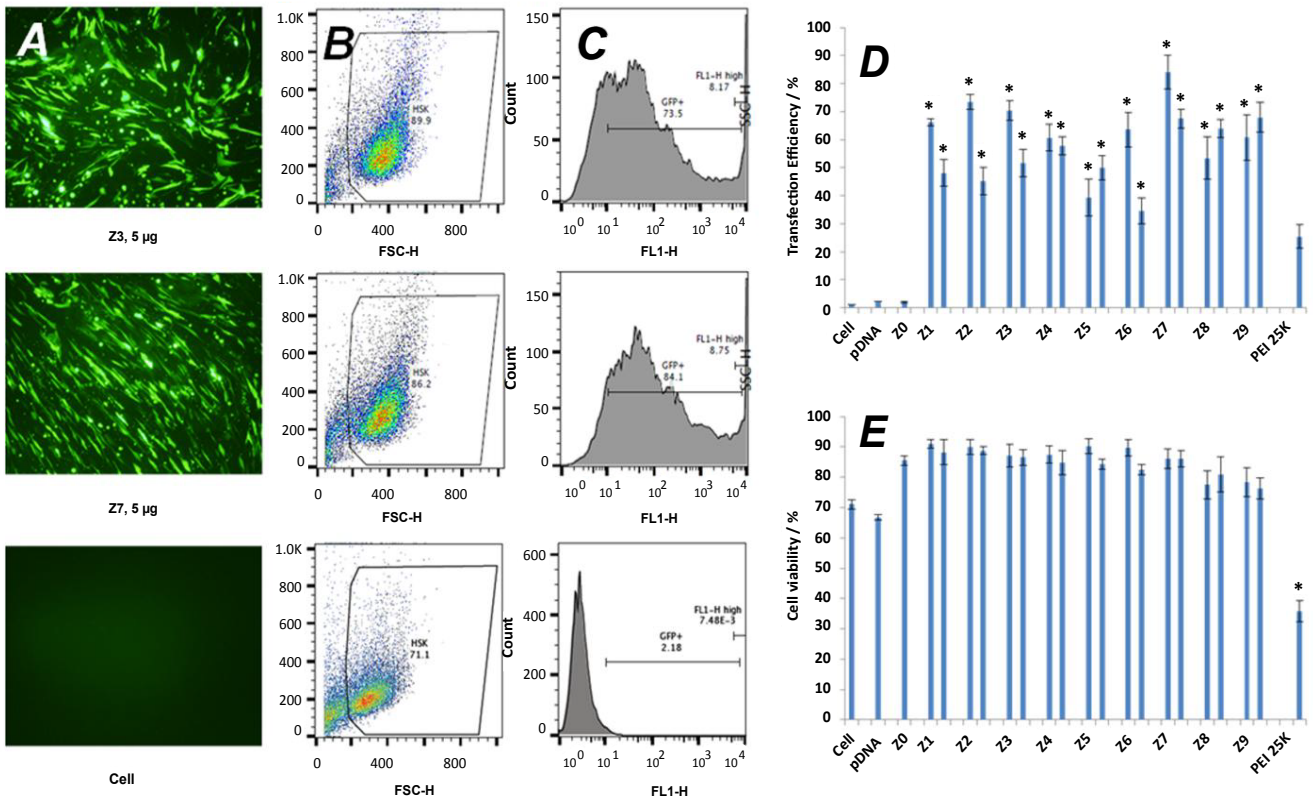


Figure 9: pDNA delivery in HSkM cell line transfected with polymer/pDNA complexes at weight ratios of 5 &10 after 48 hrs incubation. [T85 (10 µg), Z1-Z9 (5/10 µg), PEI 25k (5 µg); pDNA 1 µg in 500 µl medium in HSkM cell line. Column A: GFP expressed illustrated under fluorescent microscopy; Column B and C illustrate living cell percent of collected cells and GFP expression, respectively. D) Transfection efficiency analyzed with FACS (*p<0.05 compared with PEI 25k, two-tailed Student's t-test, error bar represents standard deviation, n=3). E) Cell viability (*p<0.05 compared with untreated cell as control, two-tailed Student's t-test, error bar represents standard deviation, n=3).

the TE enhanced reaching nearly 80% for polymer Z7. The TE of this polymer series formulated with pDNA in this cell line is higher than that achieved in C2C12 cells, while the cytotoxicity still remains low even at the elevated dose of 20 µg (Figure 9). The results indicate that the TE of pDNA delivery is cell line dependent for this polymer series, though the mechanism is not determined. Overall, the polymer's Mw, PEI %, and hydrophilic-lipophilic balance (HLB) are important factors and have intricate relations to one another. The PEI 2.0k-constructed polymers Z7/Z9 showed higher transgene efficiency and lower cytotoxicity as compared to Z8 in our tested 3 cell lines. These results demonstrated the more T85 grafted onto PEI to higher Mw, and the more lipophilic nature within the polymer retains, leads to a more effective transgene delivery.

In vivo transfection

The effect of Z polymers on transfection efficiency were examined in vivo with mdx mice - an animal model of Duchenne muscular dystrophy (DMD). DMD is caused by mutations in the dystrophin gene resulting in lack of dystrophin protein expression. Restoration of dystrophin by gene therapy has been demonstrated with long term therapeutic effect [28-30]. However, successful gene therapy remains an enormous challenge, especially for muscular dystrophy which requires delivery of transgenes into body-wide muscles. Based on transfection efficiency in the cell culture systems, we examined the Z polymers for gene delivery in muscle by intramuscular (i.m.) injection. pDNA at the dose of 10 µg together with 2 µg polymers was injected into the TA muscles of mdx mice aged 4-6 weeks. GFP expression was examined 5 days after the injection. The numbers of GFP expressing muscle fibers were 161 ± 50, 35 ± 5, 124 ± 27, 55 ± 34, 34 ± 6, 37 ± 9, 163 ± 26, 78 ± 22 and 100 ± 18 for Z1, Z2, Z3, Z4, Z5, Z6, Z7, Z8 and Z9, respectively. As a control, T85 (Z0), PEI 1.2k and PEI 25k at the same dose induced 17 ± 3, 2 ± 1, 6 ± 5 positive muscle fibers, respectively (Figure 10). The highest efficiency observed with the polymers Z1, Z3, Z7 and Z9 reached 27 and 80 fold improvement compared to PEI 25k and LPEI only, respectively. Histologically, there was no clear increase in damage in the muscles treated with any Z polymer as compared to the muscles injected with saline only. In contrast, 5 µg PEI 25k induced significant muscle damage with large areas of necrotic fibers and focal infiltrations. The improved TE without tissue damage of the Z polymers are likely related to small size PEI, homogenous stable particles of the complex and rational HLB due to the presence of the T85. The in vivo results therefore, indicate that the T85 modified LPEI polymers could potentially be developed as a vehicle for gene or oligonucleotide delivery in muscle for treating muscular dystrophy and other diseases.

Conclusions

A series of cationic amphiphilic polymers were prepared based on the low molecular weight PEI (Mw 0.8, 1.2k, and 2.0k Da) modified with T85, and susequently characterized. The percentage of PEI in the conjugates ranged from 8.25% to 20.91%. The bigger LPEI produced

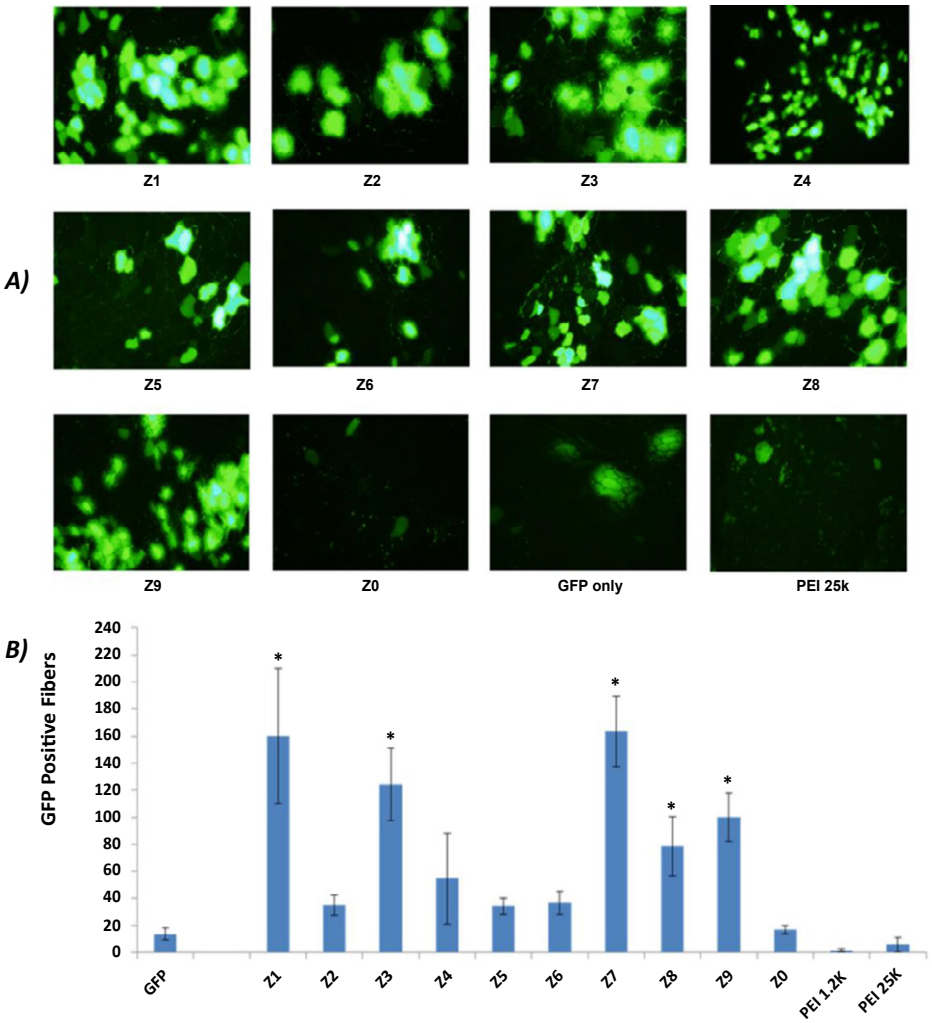


Figure 10: Restoration of dystrophin in TA muscles of mdx mice (aged 4-6 weeks) 5 days after i.m. injection. A) GFP expression illustrated under fluorescent microscopy. B) Numbers of GFP-positive fibers after treatment of 10 μg pDNA with 2 μg polymers. The maximum numbers of GFP-positive fibers were counted in a single cross-section (n=5, two tailed t-test, * $p < 0.05$ compared with GFP only).

larger molecule size of polymer, with nano-sized polyplexes being formed at a polymer/pDNA weight ratio of 2 or above. Results from our evaluation in CHO, C2C12 and HSkM cell lines in vitro as well as in vivo of the dystrophic mdx mouse reveal a synergistic effect of LPEIs and T85 for the delivery of pDNA. Transfection efficiency of these polymers depends on molecular size, PEI content and HLB of polymer composition. Highest TE was achieved with Z7 and Z9 in all cell lines, and with Z1, Z3, Z7, Z8 and Z9 in vivo. The most effective Z polymers for in vivo delivery are Z1 and Z7, up to 80 folds of the control PEI 25k. The PEI 1.2k-based copolymers Z4, Z5, Z6 and the PEI 2.0k-based Z8 are more hydrophilic as compared to other counterparts constructed from PEI 0.8K/2.0k. These results suggest that appropriate positive charge, distribution of polymer/DNA particles and molecular size, HLB within copolymers are critical for effective gene delivery. Transfection efficiency is higher with polymers of more hydrophobic, larger size, but with relatively higher toxicity as well. These properties present these polymers to be potential vehicles for further investigation of local and systemic effect on transgene and antisense oligonucleotide delivery.

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