Evaluation of Tween 85 modified LPEI for pDNA delivery

in vitro and in *mdx* mice

Mingxing Wang[1]*, Bo Wu[1], Jay D. Tucker[1,2], Peijuan Lu[1], Sapana N Shah[1], Qilong Lu[1]

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

2. Department of Biology, University of North Carolina, Charlotte, 9201 University City Blvd., Charlotte, North Carolina 28223, USA

Tel.: 1-7043555588; Fax: 1-704-355-1679; Email: mingxing.wang@carolinashealthcare.org

Abstract:

Series of cationic amphiphlic copolymers constructed from Tween 85 and low molecular weight (Mw) polyethyleneimene (LPEI) were synthesized and evaluated in *in vitro* and *in vivo*. They could condense DNA efficiently with particles size below 200 nm at the weight ratio 5 of polymer/pDNA. The introduction of Tween 85 led to a significant increase in transfection efficiency in C2C12 cell line, but without increasing toxicity compared with the parent LPEI, and much lower toxicity than PEI 25k. The best formulation for pDNA delivery give 5, 15-fold compared with PEI 25k at the same dose *in vitro* and in *mdx* mice, respectively. There is no obvious muscle damage observed with these new copolymers. These results demonstrated that Tween 85 modified LPEI could be potentially safe and effective polymeric carriers for gene/drug delivery.

1. Introduction

Gene therapy has been a potential approach to treating heritable and acquired diseases, bue the delivery efficiency of gene alone have been limited due to low stability, immunogenicity and inability to cross cellular membranes. Successful delivery mostly depends on the effective and safe delivery vector. Among the numerous of synthetic vectors, polyethylenimine (PEI) is one of the upmost successful polymers and used widely for gene delivery because of the "proton sponge effect".[1] The transfection and toxicity is molecular size dependent, the higher molecular weight leading to higher transfection efficiency along with higher toxicity. [2]

In this study, a series of Tween 85 modified low molecular weight PEI (LPEI) were prepared by varying feed ratio and PEI size (Mw: 0.8k, 1.2k and 2.0k Da) for gene delivery. This study is based on the following considerations: 1) The use of LPEI is to reduce the potential toxicity of the copolymers, while remain its "proton sponge effect" and positive charges essential for effective binding of polymer vectors to negatively charged nucleic acid.[1] 2) Tween 85 is a sorbitan fatty acid ester ethoxylate and has been widely used in biochemical applications due to its amphiphilic nature and cytocompatibility.[3] 3) The amphiphilie has been demonstrate higher transfection compared with the hydrophilic ones because of better cell-uptaking and improved stability of polymer/DNA polyplex in circulation.[4-6] 4) Biodegradable carbamate linkages were introduced to reduce the cytotoxicity of the cationic amphiphile.[7] Their physicochemical properties, toxicities, vector effects for pDNA delivery in vitro and in vivo were examined. Results from the evaluation reveal a synergistic effect of PEIs and Tween 85 for the delivery of pDNA. Transfection efficiency of the copolymers is relative to molecular size, PEI content and HLB of copolymer.

2. Experimental Section

2.1. Materials

Tween 85, Polyethylenimine (PEI, branched, 0.8k, 1.2k 2.0k and 25k Da), 1,1'-carbonyldiimidazole (CDI), acetonitrile, and ethidium bromide (EB), ethanol were purchased from Sigma-Aldrich (St. Louis, MO, USA). Cell culture media, Dulbecco's Modified Eagle's Medium (DMEM), penicillin– streptomycin, fetal bovine serum, L-glutamine and HEPES were 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).

2.2. Synthesis and characterization

PEI-T85 copolymers were synthesized as our per report, and characteized by NMR.[8,9].

Table 1. Characteristics of Tween85-LPEI (Z polymers)

Code	Composition (Molar ratio) [a]	Mw(Da) [b]	PEI (%) [c]
Z0	**T85**		**0**
Z1	**T85-PEI 0.8k (1:1)**	**6981.4**	**13.28**
Z2	**T85-PEI 0.8k (1:3)**	**5534.8**	**15.61**
Z3	**T85-PEI 0.8k (3:1)**	**7223.6**	**12.36**
Z4	**T85-PEI 1.2k (1:1)**	**8370.2**	**15.45**
Z5	**T85-PEI 1.2k (1:3)**	**5796.3**	**18.72**
Z6	**T85-PEI 1.2k (3:1)**	**9289.4**	**13.95**
Z7	**T85-PEI 2.0k (1:1)**	**25863.7**	**8.55**
Z8	**T85-PEI 2.0k (1:3)**	**10808.3**	**20.91**
Z9	**T85-PEI 2.0k (3:1)**	**29485.6**	**6.78**

a. Feed ratio of starting materials; b. ^{1}H NMR analysis with 500 MHz Jeol ; c. Assume the N% is 33.33 wt% in each PEI.

2.3. 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 volume, 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.

2.4. In vitro Test

Cytotoxicity Assay: The cytotoxicity was evaluated with 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.[9]

Transfection in vitro: GFP reporter C2C12 myoblasts (ATCC) were grown in DMEM, and maintained at 37 ^{o}C and 10% CO_2 in a humidified incubator. 5 x 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 serum containing media prior to addition of polymer/DNA polyplexes formulated with varying ratio of polymer/DNA. Formulation of 1µg GFP vector and appropriate amount of polymers was added into the wells 20 minutes after combination. PEI 25k was used as control for delivery.

2.5. In vivo transfection

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. 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 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.

3. Results and discussion

The interaction between Z polymers and pDNA was assessed by agarose gel electrophoresis. As illustrated in Fig. 1, the parent polymer Tween 85 (Z0) was unable to bind and condense pDNA. All modified polymers (Z1-Z9), however, showed high capacity to condense pDNA as demonstrated by the shifting of the ethidium bromide (EB) stained pDNA towards significantly higher molecular size in the gel. Some complexes migrated even slightly toward the anode when the ratio of the PCM/pDNA increased to 1 or higher suggesting that the complex became positive charged. Some polymers prepared with feed ratio of T85/PEI =1:1 or 1:3 (such as Z4, Z5 and Z8) showed very strong binding ability with

pDNA even at low concentration of 1 µg with majority of DNA being retained within the loading wells.

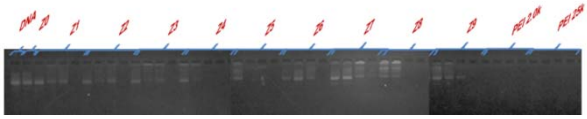

Fig. 1. Electrophoretic mobility of gene mixed with polymers at various weight ratios (all polymer/pDNA = 0.2, 0.5, 1.0, 2.0, except Z0 only at the ratio of 2).

DNA/polymer complex size is important for effective gene transfer, and the nano-sized particles are considered preferable. The morphology of polymers/pDNA complexes was confirmed by TEM analysis. These nanoparticles were well defined and uniformly distributed with sizes below 100 nm at a representative w/w ratio of 5. The physical mixture of same proportion of PEI/pDNA produced again aggregates of various sizes, characteristics of interaction between free PEI and DNA as reported previously.[9,10]

The cytotoxicity of the Z copolymers was determined in C2C12 cell lines with MTS-based cell viability assay (Fig. 2). Most Z polymers showed remarkably lower cytotoxicity than PEI 25k. Cell death rates with all Z polymers even at the dose of 20 µg/ml were much lower than that with PEI 25k at 4 µg/ml. This improved cell viability is undoubtedly contributed to the formation of low-toxic building blocks, and the steric shielding of T85 reduced density of the positively charged PEI. Toxicity was also associated with PEI content and Mw of copolymers.

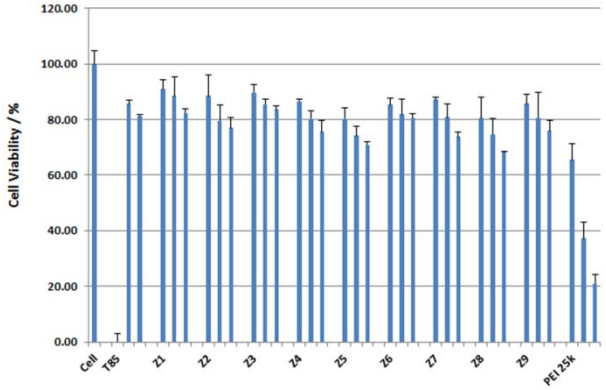

Fig. 2. Cell viability of C2C12 cell line after treatment with polymers at various doses. Cells were seeded in 96 well plates at an initial density $1x10^4$ cells /well. Cell viability was determined by MTS assay (The percentage is the average of triplicate experiments. The concentration of polymers are 4, 10, 20 µg/ml from left to right. (error bar represents standard deviation, n = 3).

The delivery performance *in vitro* was tested in C2C12 myoblasts for GFP gene delivery. The result of qualitative and quantitative analyses by fluorescent microscopy and FACS. The transgene efficacy (TE) significantly improved with the dose increasing from 0-10 µg with 1 µg pDNA in 500 µl medium, the TE reached up to over 85% when the dose is 10 µg, even at the dose of 20 µg without necrosis cell observed, the optimum dose looks between 5-10 µg for most copolymers. Therefore we further study the TE and cytotoxicity. TE remained much higher in the cells treated with polymers Z2, Z4, Z5 Z7 Z8 and Z9 at the dose of 10 µg compared to the rest of polymers and the PEI 25k (5 µg). The simple formulation (mixture) of Tween 85 and LPEI with DNA at the ratio corresponding to those contained in the Z polymers gave no GFP expression as pDNA only. The results indicate a synergistic effect between PEI and Tween 85 in copolymers microstructure.

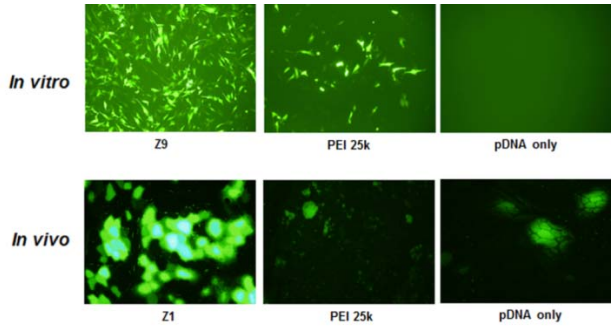

Fig. 3. GFP expressed in C2C12 cell line and in *mdx* mice transfected with polymer/pDNA complexes. *In vitro*, with the formulation of 10 µg polymer and 1µg pDNA after 48hrs incubation (PEI 25k 2 µg used here due to high toxicity); *In vivo*, 2 µg polymer formulated with 10 µg pDNA in TA muscles of *mdx* mice (age 4-6 weeks) 5 days after i.m. injection.

The *in vivo* evaluation of these polymers were examined in *mdx* mice - an animal model of Duchenne muscular dystrophy (DMD), DMD is caused by mutations in the dystrophin gene and fundamental treatment must be aimed primarily to correct the mutated genes or provide a functional product to replace the mutated gene.[11-13] Based on transfection efficiency and in the cell culture systems, we further examine their potentials for gene delivery in muscle by intramuscular injection. GFP expression vector at the dose of 10 µg together with 2µg polymers was injected into the TA muscles of the mdx mice aged 4-6 weeks. GFP expression was examined 5 days after the injection. The numbers of GFP expressing muscle fibers were 161 ± 86, 124 ± 27, 163 ± 26, 78 ± 22 and 100 ± 18 for Z1, Z3, Z7 and Z9, respectively. As a control, Tween 85 (Z0), PEI 25k at the same dose induced 17 ± 3, 6 ± 5 positive muscle fibers, respectively. Histologically, there was no clearly observable muscle damage in the muscles treated with these Z copolymers when compared to the muscles injected with saline only. In contrast, 2 µg PEI 25k induced significant muscle damage with large areas

of necrotic fibers and focal infiltrations. These results therefore indicate that the Tween 85 modified LPEI copolymers could potentially be developed as a vehicle for gene or oligonucleotides delivery in muscle in vivo for treating muscular dystrophy and other diseases.

4. Conclusions

A series of cationic amphiphilic polymers based on the low molecular weight PEI (Mw 0.8, 1.2k, and 2.0k Da) modification with Tween 85 can nano-sized particles with pDNA at a polymer/pDNA weight ratio of above 2, and reveal the synergistic effect of PEIs and Tween 85 for the delivery of pDNA in vitro and in vivo. The introduction of Tween 85 led to a significant increase in transfection efficiency in C2C12 cell line, but without increasing toxicity compared with the parent LPEI, and much lower toxicity than PEI 25k. The best formulation for pDNA delivery give 5, 15-fold compared with PEI 25k at the same dose in vitro and in mdx mice, respectively. These results demonstrated that Tween 85 modified LPEI could be potentially safe and effective polymeric carriers for gene/drug delivery.

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