Evaluation of Tris[2-(Acryloyloxy)Ethyl]Isocyanurate Cross-Linked Polyethylenimine as Antisense Morpholino Oligomer Delivery Vehicle in Cell Culture and Dystrophic *mdx* Mice

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Abstract

Hyperbranched poly(ester amine)s (PEAs) based on tris[2-(acryloyloxy)ethyl]isocyanurate (TAEI) cross-linked low-molecular-weight polyethylenimine (Mw: 0.8k/1.2k/2.0k) have been evaluated for delivering antisense phosphorodiamidate morpholino oligomer (PMO) *in vitro* and *in vivo* in the dystrophic *mdx* mouse. The results show that the PEAs constructed with polyethylenimine (PEI) 2.0k (C series) improved PMO delivery more efficiently than those constructed with PEI 0.8k (A series) or 1.2k (B series) in a GFP reporter-based C2C12 mouse myoblast culture system. The highest efficiency of exon-skipping *in vitro* with the PMO oligonucleotide targeting human dystrophin exon 50 was obtained when the PEA C12 [TAEI-PEI 2.0k (1:2)] was used. Nearly all of the PEAs improved dystrophin expression in *mdx* mice by local injection with a 2–4-fold increase when compared with PMO alone. Improved transfection efficiency and lower toxicity indicate the potential of the biodegradable PEA polymers as safe and efficient PMO delivery vectors for *in vivo* applications.

Introduction

IN THE PAST FEW YEARS, antisense oligonucleotide (AO)-mediated exon-skipping has been demonstrated as a promising therapy to treat Duchenne muscular dystrophy by facilitating “skipping” of specific dystrophin gene exons to restore the reading frame of the mutated transcripts (Amanatana *et al.*, 2007; van Deutekom *et al.*, 2007; Wu *et al.*, 2008, 2009a,b, 2012; Yin *et al.*, 2008; Kinali *et al.*, 2009; Goemans *et al.*, 2011; Mendell *et al.*, 2013). AOs are short (typically 15–30 base pairs in length), single-stranded sequences of synthetic nucleic acids, or their chemically modified analogs, that have the ability to hybridize to specific targets by the base-pairing rules. Of the synthetic oligonucleotide chemistries, 2'-O-methyl-phosphorothioate RNA (2'-OMePS) and phosphorodiamidate morpholino oligomer (PMO) are the most widely used chemistries for exon skipping in the dystrophin gene and have recently been applied in clinical trials (Fletcher *et al.*, 2006; van Deutekom *et al.*, 2007; Kinali *et al.*, 2009; Cirak *et al.*, 2011; Goemans *et al.*, 2011; Malerba *et al.*, 2011; Mendell *et al.*, 2013). PMO, as a synthetic mimic of nucleic acid, has replaced the deoxyribose rings with morpholino rings linked through phosphorodiamidate inter-

subunits, resulting in little charge of the molecule. PMOs have exhibited excellent stability and lower toxicity compared with other counterparts such as 2'-OMePS or peptide nucleic acid (Summerton and Weller, 1997; Yano and Smyth, 2012). However, the relatively charge-neutral PMOs have low delivery efficiency *in vivo*. Studies in a number of animal models have demonstrated that a significant therapeutic effect can be achieved, but only with high doses, which could be cost-inhibitive and may pose potential risk of toxicity especially for long-term use (Wu *et al.*, 2009b). To increase delivery efficiency of PMOs, cell-penetrating peptides and cationic dendrimeric octaguanidines have been conjugated with PMO (Amanatana *et al.*, 2007; Wu *et al.*, 2008, 2009a, 2012; Yin *et al.*, 2008) and reported with significant improvement in targeting dystrophin exons, resulting in near-normal levels of dystrophin expression in muscles throughout the body by systemic delivery. However, the densely packed and highly positive charged peptide is associated with higher toxicity, with LD50 only near 100 mg/kg, making clinical applications risky (Wu *et al.*, 2008, 2012). In addition, peptide-related immune responses could damage targeted muscles and again prevent repeated administration (Amanatana *et al.*, 2007). Recently, we have developed a series of

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hyperbranched poly(ester amine)s (PEAs), composed of tris[2-(acryloyloxy)ethyl]isocyanurate (TAEI) and low-molecular-weight polyethylenimine (LPEI; Mw: 0.8k/1.2k/2.0k), and evaluated their effect on plasmid DNA delivery *in vitro* and *in vivo* (Wang *et al.*, 2012b). The results showed that PEAs have significantly lower cytotoxicity when compared with higher molecular weight PEI 25k in several cell lines. The PEAs composed of PEI 2.0k (C series) showed higher transgene expression compared with PEAs of PEI 0.8k (A series) or 1.2k (B series). Among them, PEA C12 [TAEI-PEI 2.0k (1:2)] produced the highest gene transfection efficiency in CHO, C2C12 myoblast and human skeletal muscle cells and up to eightfold higher than PEI 25k in muscle *in vivo* by local injection. No obvious muscle damage was observed with the new polymers.

In this study, we further investigated these polymers for antisense PMO delivery. The results demonstrate that the PEA polymers increased PMO-induced exon-skipping efficiency compared with PEIs *in vitro* and *in vivo*. The PEAs composed of PEI 2.0k (C series) produced highest delivery efficiency, comparable to Endo-porter (a commercially available delivery reagent for PMO from GeneTools, Philomath, OR), with lower toxicity than PEI 25k. The higher efficiency and lower toxicity indicate the potential of these polymers as gene/AO delivery enhancing agents for treating Duchenne muscular dystrophy or other diseases.

Materials and Methods

Materials

The TAEI cross-linked LPEI polymers (PEAs) were synthesized as reported previously (Wang *et al.*, 2012b). Cell culture medium Dulbecco's modified Eagle's medium (DMEM), penicillin–streptomycin, fetal bovine serum (FBS), L-glutamine, and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer solution (1 M) were purchased from Gibco–Invitrogen Corp. (Carlsbad, CA). All other chemicals were of reagent grade. PMO targeting human dystrophin gene exon 50 (PMOE50, 5'-AACTTCCTCTTTAACAGAAAAGCATAC-3') (Sazani *et al.*, 2001; Hu *et al.*, 2010) and PMO targeting mouse dystrophin gene exon 23 (PMOE23, 5'-GGCCAAA CCTCGGCTTACCTGAAAT-3') (Gebbski, *et al.*, 2003) were purchased from GeneTools, and the specific sequences were selected as per reported (Wu *et al.*, 2008, 2009b, 2012; Hu *et al.*, 2010; Wang *et al.*, 2013).

Cell viability assay

Cytotoxicity was evaluated in the C2C12E50 cell line using 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS)-based assay by Cell Titer 96 Aqueous One Solution Proliferation Kit (Promega Corporation, Madison, MI) 24 hr after the treatment with different doses of polymers. Cells were seeded in a 96-well tissue culture plate at 10^4 cell per well in 200 μ l medium containing 10% FBS. Cells achieving 70–80% confluence were exposed to polymer at different doses for 24 hr followed by addition of 20 μ l of Cell Titer 96 Aqueous One Solution Reagent. After further incubation for 4 hr, the absorbance was measured at 570 nm using Tecan 500 Plate reader (Tecan US Inc., Morrisville, NC) to obtain the metabolic activity of the cell. Viability of untreated cells was taken as 100% and wells

without cells were used as blanks. The relative cell viability was calculated as follows: $(A_{\text{treated}} - A_{\text{background}}) \times 100 / (A_{\text{control}} - A_{\text{background}})$. All viability assays were carried out in triplicate.

In vitro transfection

The C2C12E50 cell has a human dystrophin exon sequence 50 (hDysE50) placed inside the coding sequence of a GFP gene under the control of an actin promoter (Hu *et al.*, 2010). Upon specific antisense oligonucleotide delivery, the flanking intron sequences and the dystrophin exon are spliced out, resulting in the restoration of an in-frame GFP transcript. C2C12E50 cells were grown in DMEM and maintained at 37°C and 10% CO₂ in a humidified incubator. About 5×10^4 cells per well were seeded in a 24-well plate in 500 μ l medium containing 10% FBS and grown to reach 70–80% confluence before transfection. Cell culture medium was replaced with serum-free medium before addition of polymer-formulated 5 μ g PMO with a varying ratio of polymer/PMOE50. The formulation of polymer/PMO was mixed in total of 50 μ l serum-free medium at room temperature 20 min before being added into the wells. The cells were then incubated for 4 hr at 37°C. The serum-free DMEM was then replaced with fresh DMEM containing 10% FBS and the cells were further incubated for 2 days. PEIs were used as control for delivery. Transfection efficiencies were measured using an Olympus IX71 fluorescent microscope (Olympus America Inc., Melville, NY). Digital images were taken using the Olympus DP Controller and DP Manager software (Olympus America Inc.). C2C12E50 cells treated with PMOE50 were also examined by flow cytometry to determine the percentage of GFP-expressing populations. Two days after PMOE50 treatment, cells were briefly washed with PBS (1 $\times$), digested with 0.05% Trypsin–EDTA, neutralized by FBS, and then pelleted by centrifugation. Cell pellets were then re-suspended in 1 ml PBS. Samples were run on a FACS Calibur flow cytometer (BD, Franklin Lakes, NJ). In total, 5×10^3 cells were counted and analyzed with CellQuest Pro (BD) software package.

Cellular uptake and intracellular localization

For cellular uptake and intracellular localization study, fluorescein-labeled PMO (GeneTools) was combined with polymers at predetermined ratios, followed by imaging under confocal microscopy. C2C12 cells were seeded onto 8-well glass Lab-Tek II chamber slides (Scientific, Ocala, FL) at 5×10^3 cells/well, and cultured to 70% confluence before the addition of polymer/PMO formulation for testing. About 24 hr after addition of the samples, cells were washed with warm PBS to remove any residual polymer/PMO formulations not taken up by cells and incubated with media containing LysoTracker Red DND-99 (Life Technologies, Carlsbad, CA) per manufacturer's recommendation to label lysosomes. Cells were also counterstained with Hoechst 33258 (Life Technologies) to label cellular nuclei. Confocal microscopy was performed on a Zeiss LSM-710 inverted confocal microscope (Carl Zeiss Microscopy LLC, Thornwood, NY), and the resulting images were analyzed for uptake and localization by single-channel images. Colocalization of polymer/PMO to the lysosome was visualized by merged channel images.

FIG. 1. Synthesis of poly(ester amine)s based on TAEI cross-linked LPEI. LPEI, low-molecular-weight poly-ethylenimine; TAEI, tris[2-(acryloyloxy)ethyl]isocyanurate.

Transmission electron microscopy

The polymer/PMO polyplex solution containing 5 μg of PMO was prepared at a weight ratio of 10/5 (polymer/PMO) in 200 μl medium and analyzed using transmission electron microscopy (TEM; Philips CM-10; Philips Electronic North America Corp., Andover, MA). The corresponding polymer and PMO only were used as comparison. The samples were prepared using negative staining with 1% phosphotungstic acid. Briefly, one drop of sample solution was placed on a formvar and carbon-coated carbon grid (Electron Microscopy Sciences, Hatfield, PA) for 1 hr, and the grid was blotted dry, followed by staining for 3 min. The grids were blotted dry again. Samples were analyzed at 60 kV. Digital images were captured with a digital camera system from 4 pi Analysis (Durham, NC).

In vivo delivery and reverse transcription polymerase chain reaction

Mdx mice aged 4–5 weeks were used with four samples for each experimental group. Experimental protocols were approved by the Institutional Animal Care and Use Committee (IACUC), Carolinas Medical Center. For intramuscular (i.m.) injections, 2 μg PMOE23 in total of 40 μl saline, with or without polymer, was used for each tibialis anterior (TA) muscle. The muscles were examined 2 weeks later. Total RNA was extracted after dissection, and 100 ng of RNA template was used for a 50 μl reverse transcription polymerase chain reaction (RT-PCR) with the Stratascript One-Tube RT-PCR System (Stratagene, Santa Clara, CA). The primer sequences for the RT-PCR were Ex20Fo 5'-CAGAATTCTGCCAATTGCTGAG-3' and Ex26Ro 5'-TTCTTCAGCTTGTCATCC-3' for amplification of mRNA from exons 20 to 26. The intensity of the bands of PCR-amplified products obtained from the treated muscles was measured by National Institutes of Health (NIH) ImageJ software. The bands of the two PCR products representing normal mRNA and mRNA with exon 23 skipped were examined by sequencing.

Antibodies, immunohistochemistry, and Western blots

Serial sections were cut from the muscles and the sections were stained with a rabbit polyclonal antibody P7 for the dystrophin protein and detected by goat antirabbit Igs Alexa 594 (Invitrogen). Protein extraction and Western blot were

done as described previously (Wu *et al.*, 2008, 2012). Briefly, the membrane was probed with NCL-DYS1 monoclonal antibody against dystrophin rod domain (Vector Laboratories, Burlingame, CA). The bound primary antibody was detected by HRP-conjugated goat anti-mouse IgG (Santa Cruz Biotechnology, Santa Cruz, CA) and the ECL Western Blotting Analysis System (Perkin-Elmer, Waltham, MA). The intensity of the bands with appropriate size was measured and compared with that from normal muscles of C57BL mice with NIH ImageJ software. α -Actin was detected by rabbit antiactin antibody (Sigma, St. Louis, MO).

Statistical analysis

The statistical analysis of experimental data was evaluated using Student's *t*-test and results were reported as mean $\pm$ SEM. Statistical significance was accepted when $p \leq 0.05$.

Results and Discussion

Synthesis and characterization of PEAs

The PEA polymers were successfully synthesized by Michael addition reaction as reported previously (Wang

TABLE 1. CHARACTERISTICS OF SYNTHESIZED POLY(ESTER AMINE)S

Series	Code	Mole ratio of TAEI/PEI	Mw PEI (kDa)	Mv ^a	PEI mol (%) ^b	Yield of copolymer (%)
A	A11	1:1	0.8	4980	63.2	28.1
	A12	1:2	0.8	4570	72.5	32.4
	A14	1:4	0.8	4390	53.4	40.5
B	B11	1:1	1.2	7160	51.7	33.6
	B12	1:2	1.2	5940	70.3	37.8
	B14	1:4	1.2	5580	63.5	43.3
C	C11	1:1	2.0	9870	54.8	38.5
	C12	1:2	2.0	8430	65.6	41.3
	C14	1:4	2.0	7650	71.4	53.2

PEI, polyethylenimine; TAEI, tris[2-(acryloyloxy)ethyl]isocyanurate.
^aDetermined by viscosity measurements in 0.9% NaCl solution at 25°C.

^bDetermined by ¹H NMR in CDCl₃.

FIG. 2. Cell viability of C2C12E50 cell line after polymer treatment at various doses. Cells were seeded in 96-well plates at an initial density of 1×10^4 cells/well in 200 μ l growth media. Cell viability was determined by MTS assay. The concentration of polymers are 4, 10, and 20 μ g/ml from left to right for each sample. $n = 3$, two-tailed t -test, $*p \leq 0.05$ compared with untreated cell as control. PEI, polyethylenimine.

et al., 2012b). The syntheses and characteristics of the cationic amphiphilic polymers are illustrated in Fig. 1 and Table 1.

PMO delivery in C2C12 myoblast cell lines expressing GFP/hDysE50

In this study, a C2C12 myoblast cell line stably expressing a GFP reporter that is bifurcated by the insertion of the hDysE50, referred to as C2C12E50 cells, was used to test the efficacy of PEAs for the delivery of PMO (Sazani *et al.*, 2001; Hu *et al.*, 2010). The expression of GFP relies on the targeted removal of exon 50 by AOs. We first examined the cytotoxicity of the PEAs with the C2C12E50 cells using an MTS-based cell viability assay as shown in Fig. 2. Toxicity of PEI was clearly size-dependent, with higher molecular weight PEI resulting in higher toxicity and lower molecular weight PEI having lower toxicity. Viability dropped to less than 32%, 47%, and 76% for the cells treated with PEI 25k at a concentration of 20, 10, and 4 μ g/ml, respectively. In contrast, all PEAs at a dose of 20 μ g/ml, except C11 [TAEI-PEI 2.0k (1:1), the ratio of the two components used in synthesis], showed cell viability over 80%, which is higher than PEI 25k at 4 μ g/ml. This is consistent with earlier reports demonstrating a correlation of lower toxicity to the lower density of positively charged PEI modifications (Nguyen *et al.*, 2000; Cho *et al.*, 2006; Wang *et al.*, 2012a,b). C11 had the highest toxicity among all PEAs, but maintained over 67% cell viability at a dose of 20 μ g/ml.

The relatively higher toxicity of C11 is likely because of its higher molecular weight when compared with other PEAs. The fact that increased units of LPEI within the PEAs did not significantly increase toxicity as compared with parent LPEI alone suggests that the density of the positive charge, rather than the overall number of charge groups, mainly determines toxicity of the PEAs. This result was consistent with our previous report in other cell lines, indicating a much lower toxicity of the PEAs in cell culture compared with PEI 25k (Wang *et al.*, 2012b).

We next examined whether the PEA polymers might enhance the exon-skipping effect of PMO. A PMO sequence, PMOE50 (5'-AACTTCCTCTTTAACAGAAAAGCATAC-3'), with previously confirmed efficacy of targeted removal of human dystrophin exon 50 was used (Sazani *et al.*, 2001; Hu *et al.*, 2010). C2C12E50 GFP reporter cells were treated with a fixed amount (5 μ g) of PMOE50 formulated with each polymer at four different doses (2, 5, 10, and 20 μ g). Transfection efficiency was examined by fluorescence microscopy analysis. The results showed that almost all PEA polymers at 5 μ g increased GFP expression compared with the PMOE50 only. The highest levels of GFP expression were achieved at the dose of 10 μ g with most PEAs, reaching up to 80% with C12 (Fig. 3). In contrast, less than 5% of the cells were GFP positive when treated with PMOE50 alone. The exon-skipping efficiency remained higher at the dose of 20 μ g of PEAs, but some toxicity was observed with C11. The exon-skipping efficiency and toxicity of PEAs at the dose of 10 μ g were then examined by FACS analysis. A lower dose of 5 μ g Endo-porter and 2 μ g PEI 25k

FIG. 3. Dose-dependent PMO delivery with PEA C12 in C2C12E50 cells. C12 was used at the doses of 2, 5, 10, and 20 μ g together with 5 μ g PMOE50 in 500 μ l medium. Original magnification, $\times 100$. Images were taken 48 hr after treatment. PEA, poly(ester amine); PMO, phosphorodiamidate morpholino oligomer.

FIG. 4. Determination of exon-skipping efficiency and toxicity of PEA-mediated PMOE50 delivery in C2C12E50 cells by fluorescence microscopy (a) and FACS analysis (b and c). In the tests, 5 μ g PMOE50 was formulated with 10 μ g PEAs, 5 μ g Endo-porter, or 2 μ g PEI-25 in 500 μ l medium, respectively. (a) Representative fluorescence images of the cells 48 hr after PMOE50 treatment with or without polymers. Original magnification, $\times 100$. (b) Percentage of cells expressing GFP (indicating exon-skipping efficiency) determined by FACS analysis with a total 5,000 cells counted. Control, cells without PMO treatment ($n = 3$, two-tailed t -test, $*p \leq 0.05$ compared with PMO only). (c) Cell viability ($n = 3$, two-tailed t -test, $*p \leq 0.05$ compared with the control). Color images available online at www.liebertpub.com/hum

were used as controls, because of their high toxicity (Fig. 4). PMO formulated with the C series of PEAs produced higher exon-skipping efficiency indicated by 50–80% of cells expressing GFP. This was especially seen with C12, which demonstrated GFP expression comparable to Endo-porter-mediated delivery and a much higher expression than cells with PEAs of either A or B series and all PEIs alone. This expression was also noted to be up to 20-fold higher when compared with PMO alone (Fig. 4). The C series has relatively bigger PEI size; thus, more positive charges distributed within the molecules when compared with B or A series. This result, together with the C series showing only marginal increase in toxicity, indicates the importance of charge balance in vector microstructure design for effective gene/AO delivery. Specifically, the size of TAEI cross-linked LPEIs and the structural arrangement of positive charges could contribute to both delivery efficiency and toxicity of the polymers.

To study the intracellular localization of PEA/PMO polyplex, PEAs were complexed with 3'-carboxyfluorescein-labeled PMO at a weight ratio of 10/2. The presence of PEA apparently affected the pathway of the PMO uptake as demonstrated by confocal microscopy analysis (Fig. 5). PMO alone distributed evenly within the cytoplasm of the cells, in agreement with a passive diffusion model reported (Summerton and Weller, 1997). Signals for PMO were considerably stronger in the cells treated with PEA-formulated PMO. Furthermore, concentrated signals as punctuates appeared within the cytosol and especially around the nuclear areas and codistributed with the signals for endo-lysosomes labeled by the LysoTracker DND-99 dye for acidic organelles. This result validates the effect of PEA on PMO delivery and is consistent with the expected effect of PEI-mediated nucleic acid delivery through enhanced endocytosis (Boussif *et al.*, 1995; Dominska and Dykxhoorn, 2010).

FIG. 5. Confocal microscopic images of C2C12 cells treated with PMO without (upper panel) and with (lower panel) PEA A12. PMO (green), lysosomes (red), and nuclei (blue) were demonstrated with 3'-carboxyfluorescein-labeled PMO, LysoTracker Red, and Hoechst, respectively. Original magnification, $\times 200$.

Interaction between PEA and PMO

The affinity between polymer and oligonucleotide is an important parameter for their efficient delivery into cells. Here, we chose the most effective C12 polymer for PEA/PMOE50 polyplex examination under TEM. As illustrated in Fig. 6, the polymer C12 alone formed different-sized particles likely because of aggregation, whereas the PMO oligonucleotides alone formed particles with the size below 50 nm. This is most likely a result of hydrophobic interactions among PMO molecules. The polyplex of C12/PMO at a weight ratio of 10/5 formed spherical particles with an average diameter around 100 nm. The mechanisms of interaction between the PMO and the PEA molecules are not clear, but the chemical nature of PMO likely creates a hydrophobic interaction with the PEA polymers, possibly forming hydrogen bonds between the base groups of PMO and free amines of the PEA. Although positively charged groups within the PEAs unlikely play a key role for the interaction with PMO, the groups within or on the surface of

the polyplex may stabilize the particles in a biological environment for a longer period than PMO alone. This may lead to a higher serum level and more effective delivery of PMO into the vicinity of muscles and improvement in the uptake of PMO through the vasculature and cell membrane. The exact pathway remains to be explored.

Delivery of PMO with PEAs *in vivo*

We next evaluated the effect of the PEA polymers for PMO delivery *in vivo* by i.m. injection. PMOE23 targeting mouse dystrophin exon 23 was injected to each TA muscle of *mdx* mice aged 4–5 weeks. The mouse contains a nonsense mutation in the exon 23, preventing the production of the functional dystrophin protein. Targeted removal of the mutated exon 23 is able to restore the reading frame of dystrophin transcripts, and thus the expression of the dystrophin protein. All PEA polymers were examined at the dose of 5 μ g premixed with 2 μ g of PMOE23 in 40 μ l saline. The treated TA muscles were harvested 2 weeks later.

FIG. 6. Negatively stained transmission electron microscopy images of C12, C12+PMO (10/5) complexes and PMO only (scale bar = 100 nm).

FIG. 7. Dystrophin exon-skipping and protein expression after i.m. administration of PMOE23 without and with PEAs in TA muscle of *mdx* mice (aged 4–5 weeks) 2 weeks after treatment. **(a)** The dystrophin protein in TA muscles was detected by immunohistochemistry with rabbit polyclonal antibody P7 against dystrophin. Blue nuclear staining with DAPI. Muscles treated with PMOE23 (2 μ g) only and PEI 0.8k/1.2k/2k/25k were used as controls. All polymers were used at the dose of 5 μ g except for PEI 25k (2 μ g). Original magnification, $\times 100$. **(b)** Percentage of dystrophin-positive fibers. The maximum number of dystrophin-positive fibers was counted in a single cross section ($n = 5$, two-tailed t -test, $*p \leq 0.05$ compared with 2 μ g PMO). **(c)** Detection of exon 23 skipping by reverse transcription polymerase chain reaction. Total RNA of 100 ng from each sample was used for amplification of dystrophin mRNA from exon 20 to exon 26. The upper bands (indicated by E20–E26) correspond to the normal mRNA, and the lower bands (indicated by E23 skipped) correspond to the mRNA with exon E23 skipped confirmed by sequencing (data not shown). **(d)** Western blots demonstrate the expression of the dystrophin protein from treated *mdx* mice compared with C57BL/6 and untreated *mdx* mice. Dys, dystrophin detected with monoclonal antibody Dys 1. α -Actin was used as the loading control. In total, 20 μ g protein was loaded. i.m., intramuscular. Color images available online at www.liebertpub.com/hum

FIG. 8. Dystrophin expression in TA muscles of *mdx* mice after i.m. injections of 2 μ g PMOE23 only and formulated with 5 μ g of A12 and C12. Immunostaining shows dystrophin-positive fibers covering most area of the entire cross section of the muscle when A12 and C12 were used. Color images available online at www.liebertpub.com/hum

Immunohistochemistry showed that the PMOE23 alone induced up to 12% maximum dystrophin-positive fibers in one cross section of the TA muscle. Dystrophin-positive fibers dramatically increased in the muscles treated with PEA-formulated PMOE23, reaching over 30% with all PEAs except for B11. In particular, the use of A12, A14, B12, B14, C11, and C12 increased the dystrophin-positive fibers up to 41%, 37%, 36%, 35%, 37%, and 48%, respectively (Fig. 7). As controls, PEI 0.8k, PEI 1.2k, and PEI 2.0k achieved about 22%, 19%, and 25% dystrophin-positive fibers, respectively. Dystrophin expression and levels of exon-skipping were also examined by Western blot and RT-PCR. The levels of exon-skipping were 25%, 23%, 29%, 22%, 24%, and 19% for A14, B12, C11, C12, C14, and PEI 25k, respectively. Dystrophin protein expression levels were found to be 45%, 57%, 39%, 27%, 37%, and 28% of normal levels for A12, A14, B11, B12, C11, and PEI 25k, respectively. Figure 8 illustrates the difference in dystrophin expression between A12, C12, and controls. These results suggest that PEAs with higher molecular size and/or higher PEI content are more effective for PMO delivery. It should be noted that although this enhancement in exon-skipping with the PEAs by local injection is only 2–4-fold higher when compared with PMO only, this improvement indicates the potential for systemic delivery, since the highly effective peptide–PMO conjugate was only able to improve local delivery efficiency by 5-fold over PMO alone (Wu *et al.*, 2008).

Histologically, the muscles treated with PEA copolymers were similar to the controls of saline-treated samples, indicating no obvious local toxicity at the test dose. Similarly, no toxicity was seen with the LPEIs. However, 5 μ g PEI 25k induced large areas of muscle damage indicated by the presence of necrotic fibers and focal infiltrations. Collectively, these data further confirm the importance of charge balance and molecular size in vector microstructure for effective gene/AO delivery with reduced toxicity (Wang *et al.*, 2012b, 2013).

In summary, the hyperbranched PEAs based on TAEI cross-linked LPEI (Mw: 0.8k/1.2k/2.0k) have been evaluated for the first time for their ability to deliver antisense PMO *in vitro* and in dystrophic *mdx* mice. The results show that PEAs improve the delivery efficiency of PMO while maintaining low toxicity, even though they are larger in size than their corresponding parent LPEIs. These data suggest that optimization of polymer size and density of the positively charged PEI group, in combination with consideration of biodegradability, can achieve enhanced gene/AO delivery. PEAs are potential vehicles for antisense oligomer delivery to diseases in which delivery efficiency is the key to achieving therapeutic value.

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Author Disclosure Statement

The authors declare no conflicts of interest in relation to this article.

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