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Efficient *in vitro* siRNA delivery and Intramuscular Gene Silencing using PEG-modified PAMAM Dendrimers

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Abstract

Although siRNA techniques have been broadly applied as a tool for gene knockdown, substantial challenges remain in achieving efficient delivery and *in vivo* efficacy. In particular, the low efficiency of target gene silencing *in vivo* is a critical limiting step to the clinical application of siRNA therapies. Poly(amidoamine) (PAMAM) dendrimers are widely used as carriers for drug and gene delivery; however, *in vivo* siRNA delivery by PAMAM dendrimers remains to be carefully investigated. In this study, the effectiveness of G5 and G6 PAMAM dendrimers with 8% of their surface amines conjugated to MPEG-5000 was studied for siRNA delivery *in vitro* and for intramuscular *in vivo* delivery in mice. The results from the PEG-modified dendrimers were compared to the parent dendrimers as well as Lipofectamine 2000 and INTERFERin. Both PEG-modifed dendrimers protect the siRNA from being digested by RNase and gave high transfection efficiency for FITC-labeled siRNA in the primary vascular smooth muscle cells (VSMC) and mouse peritoneal macrophages. The PEG-modified dendrimers achieved knockdown of both plasmid (293A cells) and adenovirus-mediated green fluorescence protein (GFP) expression (Cos7 cells) *in vitro* with efficiency similar to that shown for Lipofectamine 2000. We further demonstrated *in vivo* that intramuscular delivery of GFP-siRNA using PEG-modified dendrimer significantly suppressed GFP expression in both transiently adenovirus infected C57BL/6 mice and in GFP transgenic mice.

Keywords

PEG-conjugated PAMAM dendrimer; cytotoxicity; RNase stability; in vitro and intramuscular siRNA delivery; gene silencing efficiency

Introduction

Since the discovery of RNA interference (RNAi), thousands of researchers have applied this technique to investigate the functions of genes, produce transgenic animal models, and design new therapeutics. Small interfering RNA (siRNA), a class of double-stranded RNA molecules with a length of 20–25 nucleotides, can bind and mediate the degradation of

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target mRNA[1]. Now, the external synthesis and transfection of siRNAs into cells has been broadly accepted for target gene knockdown[2].

Since siRNA acts by interference in the cytoplasm and does not interact with chromosomal DNA, siRNA delivery does not require transport into the nucleus or protection from ribozyme degradation, which are substantial challenges in DNA delivery. Therefore, siRNA transfection and gene knockdown usually has higher efficiency than that of DNA transfection and expression. These are substantial advantages for future therapeutic applications in the clinic[3].

Despite the great promise of siRNA as therapeutic, substantial challenges remain for effective *in vivo* siRNA delivery including stabilization in the bio-environment during the delivery process and the reduction of off-target effect[4,5]. Degradation by serum ribonucleases and clearance by the kidney result in a blood plasma half-life shorter than 10 minutes for naked siRNA[6]. The negative surface charge of siRNA serves to increase clearance from the circulation system and retards crossing the anionic cell membrane[7]. All of these aspects make siRNA-mediated *in vivo* gene silencing transient and inefficient[8].

Improving the efficiency of siRNA delivery using non-viral vectors strategies has been of great interest since they have the potential to be safer and less toxic than viral vectors[9,10]. Cationic polymers, lipid carriers, supramolecular nanocarriers and other non-viral vectors have proven to be effective for intracellular delivery of siRNA *in vitro*; however, development of clinically ready *in vivo* agents remains an unmet challenge[11–14]. Although Lipofectamine 2000 (L-2000) is a successful *in vitro* transfection reagent, it is inefficient for *in vivo* transfection and expression of DNA[15]. Polyethylenimine (PEI), a widely investigated polycation for nucleic acid delivering, has been studied for the delivery of siRNA targeting the HER-2 receptor in a subcutaneous mouse tumor model[16]. Intraperitoneal injection of PEI/siRNA polyplex succeeded in reducing the rate of tumor growth and provided an important proof-of-concept for the application of non-viral vectors to siRNA delivery *in vivo*[16,17]; however, there are no commercial reagents that can mediate the efficient delivery of siRNA intramuscularly.

In the clinic, diseases such as Duchenne muscular dystrophy (DMD) and Facioscapulohumeral muscular dystrophy (FSHD) are reported to be associated with the mutations of specific genes[18]. Clinical trials are aiming to find a treatment through the intramuscular injection of siRNA or antisense oligoribonucleotides designed to skip mutated exons of the mRNA splicing machinery[19,20]. Developing a carrier that could intramuscularly deliver siRNA with high efficiency is an important and meaningful goal to accomplish gene therapy for these diseases.

A highly branched structure combined with an easily functionalized amine-terminated surface and a narrow molecular weight distribution have made poly(amidoamine) (PAMAM) dendrimers a widely explored drug-delivery polymer. PAMAM dendrimers have also been applied as DNA and siRNA delivery carriers, since the positive charges born on their terminal amine groups in aqueous media serve to compact these biomolecules into nanoparticles and facilitate delivery of oligonucleotides and plasmids into cells[21,22]. Research into the effectiveness of dendrimer-based siRNA delivery is quite limited with most studies to date addressing *in vitro* applications[23–26]. A notable exception is the recent study exploring the use triazine dendrimers for both *in vitro* and *in vivo* siRNA delivery[27]. In this study, the cationic dendrimers show good knockdown efficiency *in vitro*, successfully protected the siRNA from degradation in the blood plasma, and the polyplexes were passively targeted to the lungs of mice. This work indicates that dendritic systems have great promise for *in vivo* siRNA delivery.

Our previous study showed that PEG-conjugated generation 5 (G5) and 6 (G6) PAMAM dendrimers facilitate efficient intramuscular DNA delivery in neonatal mice[28]. These results, combined with the promising results shown by others for dendrimeric structures in siRNA delivery[29,30], prompted us to investigate the intramuscular delivery of siRNA by dendrimer carriers and their gene knock-down efficacy.

In the current study, we tested G5 and G6 PAMAM dendrimers that had an average of 8% of their surface groups conjugated with MPEG-5000 (G5-PEG, G6-PEG) for their effectiveness in delivering siRNA *in vitro* and *in vivo*. The delivery of GFP-siRNA using these dendrimers resulted in the effective *in vitro* gene silencing of both plasmid and adenovirus-expressed GFP genes. In addition, GFP-siRNA delivered intramuscularly by G5-PEG showed significant gene silencing effects for both transiently (adenovirus) and permanently (transgenic) expressed GFP gene in C57BL/6 and GFP transgenic mice, respectively.

Experimental Sections

Materials

G5 (Mn=27726, end groups=110, PDI=1.036) and G6 (Mn=55725, end groups=218, PDI=1.069) PAMAM dendrimers, 4-Nitrophenyl chloroformate, RNaseA and RNase inhibitor were purchased from Aldrich-Sigma Chemical Company (Milwaukee, WI, USA) or Dendritech, Inc (Midland, MI, USA). The PAMAM dendrimers were purified and characterized before use. Polyethylene glycol monomethylether of molecular weight 5000 (MPEG-5000) was purchased from Fluka Chemical Company (Buchs SG, Switzerland). Transfection reagents INTERFERin™ and *In-vivo* JetPEI™ were purchased from Polyplus Co. (Illkirch, France), and L2000 was purchased from Invitrogen (CA, USA). Adenovirus Containing Green Fluorescent Protein (Ad-GFP) was constructed, proliferated and purified by our lab. Dulbeco's modified Eagle's medium (DMEM) and RPMI Media 1640 were purchased from Gibco Corporation (CA, USA). All other reagents were provided from local suppliers, unless otherwise mentioned.

Synthesis of PEG-Conjugated PAMAM dendrimers

PEG-conjugated PAMAM dendrimers (PEG-PAMAM) were synthesized according to the procedure described in our previous publication[28]. Briefly, MPEG-5000 was mixed with 4-nitrophenyl chloroformate to synthesize MPEG 4-nitrophenyl carbonate. MPEG 4-nitrophenyl carbonate was then conjugated to G5 or G6 PAMAM dendrimer at a theoretical PEG conjugation ratio of 10 % (molar ratio of PEG to surface amine per dendrimer). The products of the PEG-PAMAM dendrimers were purified by dialysis and obtained by lyophilization. The final products were analyzed by ^{1}H NMR and HPLC and the conjugation-ratio of PEG to dendrimers was determined by ^{1}H integration. The number of terminal amine groups of dendrimers were determined by GPC and potentiometric titration[31].

Formation and Physical Characteristics of the Dendrimer/siRNA Dendriplexes

The dendriplexes of PEG-PAMAM dendrimer and siRNA were formed by using a 21 bp double-stranded RNA. Briefly, 1OD siRNA was dissolved in 150 μL of Diethylpyrocarbonate (DEPC) treated water to achieve a concentration of 20 μM (267 ng/μL). G5-PEG and G6-PEG were dissolved in Tris-HCl buffer to a concentration of 2 μg/μL, respectively. To create the dendriplexes at different N/P ratios (the molar ratio of the terminal amine groups in the dendrimer to phosphorus atoms in siRNA) ranging from 1/10 to 10/1, different volumes of PEG-PAMAM dendrimers (for example, 6 μL for the N/P ratio of 10) were taken from the 2 μg/μL solution and diluted with Tris-HCl buffer to 10 μL. This

solution was mixed with the same volume of siRNA, which had been obtained by the dilution of 2 μL of 20 μM solution of siRNA to achieve the required N/P ratio. The resulting mixtures were incubated at room temperature for 30 min to form dendriplexes. The dendriplexes of the parent G5 or G6 PAMAM dendrimer with siRNA were made according to the same procedure. For example, to make the dendriplexes at the N/P ratio of 10, 2.1μL of 2 μg/μL G5 or G6 was mixed with 2 μL of 20 μM siRNA at the same volume of 10 μL. To analyze the size and zeta potential of dendriplexes (Zetasizer Nano-ZS Nanoseries, Malvern, USA), the dendriplexes were formed in 1 ml distilled water at N/P ratios of 10 and 20, and the final concentration of siRNA was 2.4 μg/ml.

Gel retardation of PEG-PAMAM dendrimers to siRNA

The dendriplexes were formed at different N/P ratios following the procedure described above. Using siRNA as a control, gel retardation was performed to assess the stability and stoichiometry of the PEG-PAMAM/siRNA dendriplexes as compared to PAMAM/siRNA dendriplexes. Electrophoresis was carried out on 3.5% (w/v in 1×TBE) agarose gel containing 0.5 μg/mL ethidium bromide. The gel was run in a TBE buffer at a constant 100V for 50 min. The gel pictures of the electrophoresis were taken under a UV illuminator to reveal the location of siRNA.

RNaseA stability experiments

To study the ability of PEG-PAMAM dendrimers in protection of siRNA from being degradated by RNaseA, dendriplexes of G5-PEG/siRNA and G6-PEG/siRNA were formed at an N/P ratio of 10 following the procedure described above. Next, 1 μL of 30 ng/μL RNaseA was added to the dendriplexes and the resulting mixtures incubated at 37°C for 0, 15, 30, 60, 120 or 240 min. After incubation, 1 μL of 50 U/μL RNaseA inhibitor was added to stop the degradation-reaction, and then 3 μL of 1% SDS was added to dissociate the siRNA from the dendriplexes. A parallel experiment was conducted as a control using the same amount of siRNA (2 μL of 20 μM) mixed with 5 μL water according to the same procedure. Electrophoresis was carried out on 3.5% (w/v in 1×TBE) agarose gel according to the same procedure described in the gel retardation experiment. The gel pictures of the electrophoresis were taken under a UV illuminator, and the results were analyzed to identify the remains of siRNA after the degradation of RNaseA.

Cytotoxicity assay

The cytotoxicity of G5 and G6 dendrimers before and after 8% PEGylation was assessed by using CCK-8 kits (cell counting kit-8, Dojindo Laboratories, Japan)[28]. Briefly, Cos-7 cells were seeded in 96-well plates at 1×10^4 cells per well in 95μL DMEM medium containing 10% FBS. After 24-hour incubation, the cells were exposed to 5μL of the PEGylated or non-PEGylated dendrimer solutions with concentrations from 8 to 256 ng/μL for 4 hours, 10μL of CCK-8 reagent was then added to each well. Absorbance of each well was measured at 450nm by a microplate reader (Bio-Rad, CA, U.S.A.) and compared with untreated cells.

In vitro siRNA transfection

1. Delivery of FITC-labelled siRNA into primary smooth muscle and macrophage cells—Vascular smooth muscle cells (VSMC) and macrophages were chosen as primary cell lines to evaluate the effectiveness of siRNA delivered by PEG-PAMAM dendrimers. Rat VSMC and mouse peritoneal macrophage cells were harvested and seeded in 24-well plates at a cell density of 5×10^4 per well and were grown to 70–80% confluence in complete DMEM and RPMI 1640 medium, respectively (medium contained 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin). With L2000 as a positive

control, RNA delivery was performed by using FITC-labeled siRNA (FITC-siRNA). Briefly, 3 μL of 20 μM FITC-siRNA was diluted with a serum-free medium to 25μL, and 9 μL of 2 μg/μL G5-PEG or G6-PEG were diluted to the same volume of 25μL and mixed with the diluted siRNA to give an N/P ratio of 10. The resulting mixtures were incubated at room temperature for 30 min to form dendriplexes. The dendriplexes were then added to the cells and incubated for 3 h in serum-free medium. The cells were rinsed three times with serum-free medium and incubated in 500 μL per well of fresh complete medium for 12h. The fluorescence intensity of FITC-siRNA inside the cells was determined by flow cytometry (n=3).

2. Co-transfection to silence plasmid-expressed GFP genes in 293A cells—To study the gene silencing efficacy of siRNA delivered by PEG-PAMAM to plasmid expressed genes, enhanced-GFP plasmid and GFP-siRNA were co-transfected into 293A cells. Briefly, 0.8 μg pEGFP and 3 μL of 20 μM GFP-siRNA were diluted to 25 μL and mixed with the same volume of diluted G5-PEG or G6-PEG dendrimers (17 μL of 2 μg/μL solution) in serum-free medium to give an N/P ratio of 10 and then incubated at room temperature for 30 min to form PEG-PAMAM/DNA and PEG-PAMAM/siRNA dendriplexes. For the positive control, L2000 was mixed with 0.8 μg pEGFP and 3 μL of 20 μM GFP-siRNA according to the standard transfection procedure. The resulting dendriplexes were added to 293A cells, which were cultured in complete DMEM to 70–80% confluence in 24-well plates before use. After being incubated with the DNA and siRNA dendriplexes for 3 h in 500 μL per well of serum-free DMEM, the cells were rinsed three times with serum-free DMEM and then incubated with 500 μL per well of freshly completed DMEM for 48h. The transfections of L2000, G5-PEG or G6-PEG dendrimers with pEGFP alone, as well as pEGFP with a non-specific siRNA (non-siRNA), were completed in parallel to evaluate the initial GFP expression and the influence of non-siRNA on the GFP expression, respectively (n=3). The fluorescence intensity of GFP was determined by flow cytometry and compared to the results of non-PEGylated G5 and G6 dendriplexes (6 μL of 2 μg/μL solution) from parallel experiments (n=3).

3. Gene silencing of adenovirus-expressed GFP gene in Cos-7 cells—To study the silencing of adenovirus-expressed genes by PEG-PAMAM/siRNA dendriplexes, Cos-7 cells were seeded 24 h ahead of time in 24-well plates with a cell density of 2×10^4 per well and infected with Ad-GFP at a dose of MOI 25 (MOI: multiplicity of infection) in 100 μL of serum-free DMEM for 90 min. Next, 400μL complete DMEM were added in each well and the cells were incubated overnight.

With L2000 and INTERFERin™ (INTERFERin) as positive controls, the RNA interference experiments were carried out on the Ad-GFP-infected Cos-7 cells. First, the dendriplexes were made by mixing 3 μL of 20 μM GFP-siRNA and 9 μL of 2 μg/μL G5-PEG or G6-PEG at an N/P ratio of 10 in serum-free medium as described above, and incubated at room temperature for 30 min to form PEG-PAMAM/siRNA dendriplexes. The resulting dendriplexes were then added to the Ad-GFP-infected Cos-7 cells, and incubated in 500 μL per well of serum-free medium for 3h. The cells were then rinsed three times with serum-free medium, and incubated with freshly completed DMEM for 24 h. Parallel experiments were performed using dendriplexes made by non-siRNA and PEG-PAMAM. The fluorescence intensity of GFP was determined by flow cytometry and compared with that of the cells only infected with Ad-GFP, which was defined as 100%, to calculate the decreased percentage of fluorescence intensity (n=3).

Intramuscular gene silencing in mice

C57BL/6 and GFP neonatal mice were selected to carry out the intramuscular gene-silencing of siRNA delivered by G5-PEG and G6-PEG. Neonatal mice with their mothers were purchased from the animal department of Peking University Health Science Center (Beijing, China). The Laboratory Animal Care Principles (NIH publication no. 85-23, revised 1996) were followed, and the experimental protocol was approved by the Animal Care Committee, Peking University Health Science Center.

1. GFP gene silencing in Ad-GFP infected C57 BL/6 mice—Intramuscular gene silencing was carried out in 1-week-old C57BL/6 mice that had an approximate body weight of 2.5–3 g. A single dose of 1×10^7 pfu of Ad-GFP was injected into the quadriceps of the mice to express the GFP gene. Two hours after injection, the dendriplexes, which consisted of 18 μL of 4 μg/μL G5/G6-PEG and 6μL of 20 μM GFP-siRNA (120 pmol), or 36 μL of 4 μg/μL G5/G6-PEG and 12 μL of 20 μM GFP-siRNA (240 pmol) in 50 μL 5% glucose injection at an N/P ratio of 20, were injected into the mouse quadriceps. All treated mice were raised under a 12-hour light/dark cycle with free access to food and water. After 48 h or 72 h from the time of the injection, the mice were sacrificed and the quadriceps muscle around the injection points was cryosectioned (7 μm, 300 slices) to analyze the average fluorescence intensity. Control experiments employing Ad-GFP alone and PEG-PAMAM/non-siRNA dendriplexes were also performed (n=5 in each group).

2. GFP gene silencing in GFP transgenic mice—Intramuscular gene silencing was carried out in GFP transgenic mice so as to study the interference efficacy of GFP-siRNA delivered by G5-PEG or G6-PEG to a permanently expressed GFP gene. The dendriplexes were formed by mixing 36 μL of 4 μg/μL G5-PEG or G6-PEG with 240 pmol GFP-siRNA in 50 μL 5% glucose injections at an N/P ratio of 20, and were injected to the quadriceps of GFP transgenic mice. The treated mice were raised under normal conditions for 48 h or 72 h from the time of the injection. The quadriceps muscle of the mice was cryosectioned and the average fluorescence intensity of the muscular sections was analyzed as described above. Control experiments were completed using *in vivo* JET-PEI™ (by the standard *in vivo* transfection procedure) and PEG-PAMAM/non-siRNA dendriplexes at the same dose and delivered by the same route. These controls were compared to the active dendriplexes and to the fluorescence of untreated muscles of GFP transgenic mice (n=5 in each group).

Results

Conjugation of G5 and G6 PEG-PAMAM dendrimers

After the conjugation and purification process, the PEG-conjugated dendrimers were analyzed by ^{1}H NMR spectroscopy and HPLC. The conjugation-ratios of MPEG-5000 to G5 and G6 dendrimers were both 8% as determined by ^{1}H NMR integration. These two products were used to form the dendriplexes in the following experiments. The parent G5 and G6 PAMAM dendrimers were used to form the control PAMAM/siRNA dendriplexes.

Physical Characteristics of the Dendrimer/siRNA Dendriplexes

The size and ζ potential of all dendriplexes used in the cell and animal studies were determined and shown in Table 1. The size of siRNA, which was around 250 nm initially, was condensed dramatically after interaction with the dendrimers, and resulted in a significantly decreased size after the formation of the dendriplexes. Both G5-PEG and G6-PEG more strongly compacted the siRNA and generated a smaller size of the dendriplexes than their parent G5 or G6. The ζ potential of the siRNA was negative because of the negatively-charged phosphate side groups in its chains; however, the overall charge became

positive after the formation of the dendriplexes with positive-charged dendrimers at the N/P ratios of 10 or 20.

Gel retardation assay

All of the PEGylated and non-PEGylated G5 and G6 dendrimers are positively charged in physiological buffer and can retard the mobility of siRNA when the N/P ratio is sufficiently high. PEGylation did not influence the compaction of G5(Fig. 1A) or G6(Fig. 1B) to siRNA, since the mobility of siRNA could be fully bound by all of the G5-PEG, G6-PEG or the parent G5 or G6 dendrimers when N/P ratio equals to 10. Therefore, The N/P ratio 10 was used to form dendriplexes for the following *in vitro* experiments.

RNase stability

When incubated with RNase A without the presence of PEGylated G5 or G6 dendrimer, siRNA was completely degraded within 5 minutes (Fig. 2A). After the formation of dendriplexes with G5- or G6-PEG dendrimer at an N/P ratio of 10, siRNA bound within the dendriplex was stable to RNase A over a 4h incubation at 37 °C (Fig. 2B, C).

Cytotoxicity in Cos-7 cells

Similar to the previous results in 293A cells[28], PEGylation dramatically reduces the cytotoxicities of G5 and G6 PAMAM dendrimers. Conjugation of 8% MPEG 5000 to G5 dendrimer increased the viability of Cos-7 cells to 68% (47% for G5, $P<0.05$), and 37% (10% for G5, $P<0.005$) at polymer concentrations of 64 ng/μL and 256 ng/μL, respectively (Fig. 3A). A similar trend was observed for G6-PEG PAMAM dendrimer (Fig. 3B). At 16 ng/μL, G6-PEG PAMAM dendrimer increased cell viability from 67% to 80% ($P<0.05$) as compared to the non-PEGylated dendrimer. At the highest concentration of 256 ng/μL, cell viability increased from 19% to 51% for G6 and G6-PEG, respectively ($P<0.005$).

In vitro siRNA transfection into primary cell lines

1. Transfection of FITC-siRNA into primary VSMC and macrophage cells— Compared with L2000, a commercial transfection reagent, G5- and G6-PEG PAMAM dendrimers achieved a significantly higher transfection of FITC-labelled siRNA in primary VSMC (5 to 7 fold higher, Fig. 4A) and macrophage cells (26–27 fold higher, Fig. 4B). In addition, G6-PEG was more efficient in siRNA transfection than G5-PEG in VSMC ($P<0.005$, Fig. 4C). However, non-PEGylated G5 and G6 PAMAM dendrimers were less efficient for transfection than L2000 (data not shown).

2. Co-transfection to test silencing of plasmid-expressed GFP gene—Compared with non-PEGylated G5 or G6, 8% PEG conjugation of the PAMAM dendrimer significantly improved the transfection efficiency of pEGFP, which is consistent with our previous results[28]. The GFP expression was dramatically inhibited by GFP-siRNA delivered by PAMAM dendrimers ($P<0.005$, Fig. 5A & B) regardless of the PEGylation. Of the four dendrimer materials, dendriplexes consisting of GFP-siRNA/G5-PEG presented the most efficient inhibition to the expression of GFP gene (only 34% of the initial pEGFP expression, $P<0.005$). This reduction percentage was equal to the GFP-siRNA delivered by L2000, which is an efficient commercial DNA and siRNA co-transfection reagent (Fig. 5B).

3. Gene silencing adenovirus-expressed GFP gene—The GFP gene was highly and evenly expressed in Cos-7 cells by Ad-GFP. GFP-siRNA delivered either by commercial siRNA-transfection reagents (L2000 and INTERFERin) or by G5-PEG or G6-PEG could significantly inhibit GFP expression (Fig. 6A). Compared with L2000 and INTERFERin, which could achieve 56–65% reduction ($P<0.005$) of adenovirus-expressed GFP

expresssion, the G5-PEG and G6-PEG dendriplexes gave an inhibition efficacies of 63% and 62% reduction, respectively($P<0.005$), to GFP expression (Fig. 6B).

Intramuscular gene silencing in mice

The *in vivo* gene silencing effects of G5 and G6 dendriplexes to a transiently and permanently expressed GFP gene was investigated in C57BL/6 and GFP transgenic mice, respectively.

1. Knockdown of Ad-GFP expression in C57BL/6 mice—The GFP gene was highly expressed in the quadriceps of C57BL/6 mice 48h after they received an injection of Ad-GFP (1×10^7 pfu) intramuscularly (Fig. 7A). At the high dose of 240 pmol, GFP-siRNA delivered either by G5-PEG or G6-PEG could markedly knock down the GFP expression (55% reduction, $P<0.01$) in the quadriceps of mice 48h after the treatment. G5-PEG mediated intramuscular GFP-siRNA delivery employing an siRNA dose of 120 pmol also showed significant silencing of GFP expression (about 40% reduction, $P<0.05$) at both 48h and 72h after the injection; however, GFP-siRNA delivered by G6-PEG at the same dose of siRNA did not show significant inhibition to the GFP expression at either time point (Fig. 7B).

2. Down-regulation of GFP expression in GFP transgenic mice—GFP was universally expressed over the whole body of GFP transgenic mice. A high dose of GFP-siRNA (240 pmol) was delivered by G5-PEG or G6-PEG dendriplexes intramuscularly into the quadriceps of the mice to down-regulate the permanently expressed GFP gene (Fig. 8A). At 48h after the injection, G5-PEG/GFP-siRNA dendriplexes dramatically inhibited the GFP expression (44% reduction, $P<0.005$) around the injection sites, but the inhibitory effects dissipated by 72h. G6-PEG/GFP-siRNA dendriplexes did not inhibit GFP expression at either time point (Fig. 8B). By way of comparison, *in vivo* JET-PEI™ did not significantly down-regulation GFP expression at the two time points chosen (Data not shown).

Discussion

Our results indicate that PEG-PAMAM dendrimers compact and protect siRNA molecules against degradation (Fig. 2), which is needed to increase the stability of siRNA *in vivo*. In addition, the net positive charge of G5-PEG/siRNA or G6-PEG/siRNA dendriplexes formed at an N/P ratio above 10 (Fig. 1) will enhance the uptake of siRNA into target cells. Moreover, previous work has shown that cationic polymers as a non-viral vector provide a sustained release of siRNA over a period of time[23], which may extend the knockdown effects of siRNA to the target genes.

Toxicity is one of the major concerns in the application of all cationic polymers, including PAMAM dendrimers as gene delivery vehicles[32,33]. Our previous results[28] indicated that PEG conjugation can dramatically decrease the cytotoxicity and hemolysis of G5 and G6 PAMAM dendrimers. We reached a similar conclusion in this study that G5-PEG and G6-PEG showed lower cytotoxicities than non-PEGylated dendrimers in Cos-7 cells (Fig. 3). The *in vivo* studies in C57BL/6 and GFP transgenic mice also did not show any visible adverse effects or morphological changes of the muscles around the injection points after 48 h *via* intramuscular injection of G5-PEG/siRNA or G6-PEG/siRNA dendriplexes (data not shown). The lower toxicities and hemolysis are highly beneficial for *in vivo* application of 8% PEG-conjugated PAMAM dendrimers.

In terms of *in vitro* transfection, primary cells are usually more difficult to transfect than immortalized cancer cell lines. Even L2000, a popular and efficient *in vitro* transfection reagent, could not transfect FITC-labeled siRNA in primary VSMC and macrophages with a

satisfactory efficiency. G5-PEG and G6-PEG unexpectedly accomplished much better transfection efficiency to FITC-labeled siRNA for these two primary cells (Fig. 4). The transfection efficiency in other primary cells is under exploration. The higher transfection efficiency of 8% PEG-conjugated PAMAM dendrimers for siRNA in primary cells will also be an advantage for *in vivo* siRNA delivery.

Although GFP-siRNA delivered either by G5-PEG or G6-PEG didn't show any difference in terms of down-regulation of the Ad-expressed GFP in Cos-7 cells, it might be the greatest down-regulation rate (62%) they could obtain at the experimental conditions, since the GFP-siRNA delivered by L2000 also gave a similar down-regulation (65%) to GFP expression (Fig. 6). G5-PEG achieved higher transfection efficiency to pEGFP than G6-PEG did (Fig. 5) and a better down-regulation rate (66%) to GFP gene expression than G6-PEG did (50%). The results suggest that dendriplexes made with G5-PEG have higher transfection efficiency and achieve better gene silence effects than those made with G6-PEG. The reason could be that G5 has a more flexible structure than G6, which contributes an enhanced compaction of siRNA and increased the fusion of the dendriplexes with the cell membrane[34]. In addition, the smaller size of the G5-PEG/siRNA may increase uptake as well as enhance release of the siRNA in the cells as compared to G6-PEG/siRNA[35].

The *in vivo* results indicated that the effects of intramuscular GFP gene knock-down by GFP-siRNA were correlated with the dosages of siRNA and the time after delivery of siRNA, which is consistent with previous results of intravenous administration of siRNA[36]. Although GFP-siRNA delivered intramuscularly by PEG-PAMAM dendrimers showed better down-regulation effects to a transient gene expression mediated by Ad-GFP in C57 mice than a permanent GFP gene expression in the GFP transgenic mice, G5-PEG/GFP-siRNA dendriplexes formed with a siRNA dose of 240 pmol accomplished significant down-regulation to both of a transient (55% decrease) and a permanent (44% decrease) GFP expression 48h after the intramuscular injection. Although the down-regulation effects to GFP expression were not long-lasting, G5-PEG shows as an efficient siRNA *in vivo* delivery vehicle.

Conclusion

This study shows that G5-PEG PAMAM dendrimer facilitated intramuscular siRNA delivery can significantly silence both transiently and the permanently expressed GFP gene in C57BL/6 and GFP transgenic mice, respectively. These data are very promising and efforts to further develop these materials for the intramuscular knock-down of target genes in the clinic are in progress.

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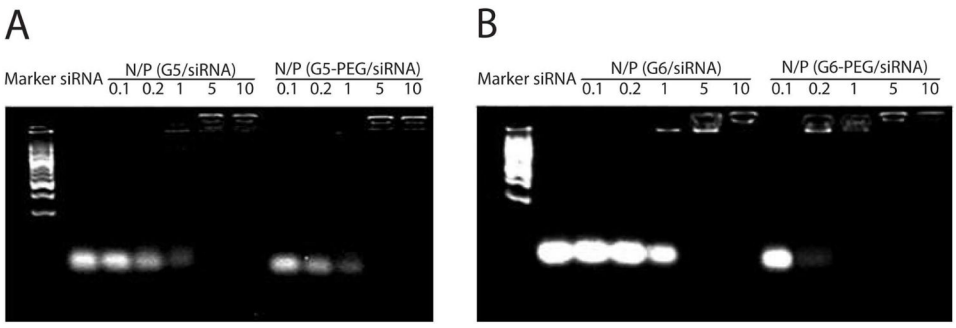

Fig. 1.

siRNA mobility retardation assays by 3.5% agarose gels. A (from left to right): marker, siRNA alone, G5/siRNA dendriplexes, G5-PEG/siRNA dendriplexes; B (from left to right): marker, siRNA alone, G6/siRNA dendriplexes, G6-PEG/siRNA dendriplexes. The dendriplexes were formed at different N/P ratios from 0.1 to 10. Note that PEGylation does not influence the compacting capability of G5 and G6 dendrimers to siRNA.

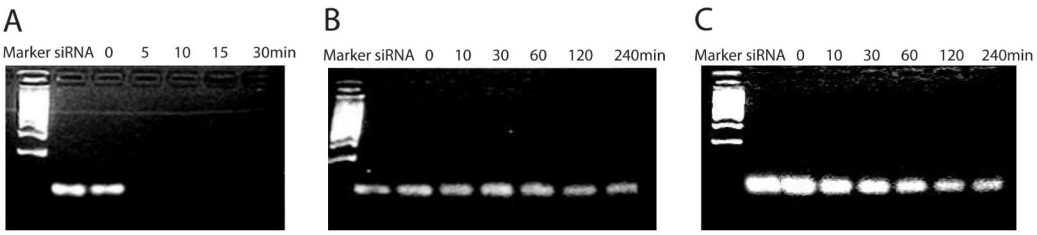

Fig. 2.

The protection effect of PEGylated-dendrimers on siRNA in the presence of RNase A. A: siRNA only (control); B: G5-PEG/siRNA dendriplexes formed at an N/P ratio of 10; C: G6-PEG/siRNA dendriplexes formed at an N/P ratio of 10. Note that both G5-PEG and G6-PEG can protect siRNA from being degraded by RNase A at 37 °C for 4h (B & C). However, siRNA alone can be degraded by RNase A within 5 minutes under the same experimental conditions.

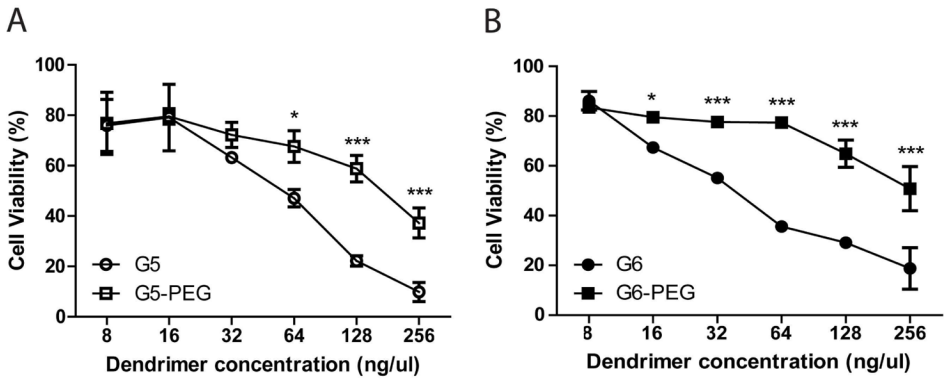

Fig. 3.

Effect of PEGylation on the cytotoxicities of G5 (A) and G6 (B) PAMAM dendrimers in Cos-7 cells. Cell viability was determined by CCK-8 assay after incubating the cells with different concentration of dendrimers for 4 hours. Note that the cell viability of Cos-7 cells was significantly increased after PEG-conjugation to G5 and G6 dendrimers. Statistical analysis was done with two-tailed unpaired Student's t-test (n=3, * $p<0.05$, *** $p<0.005$, compared with the parent G5 or G6 at the same concentration).

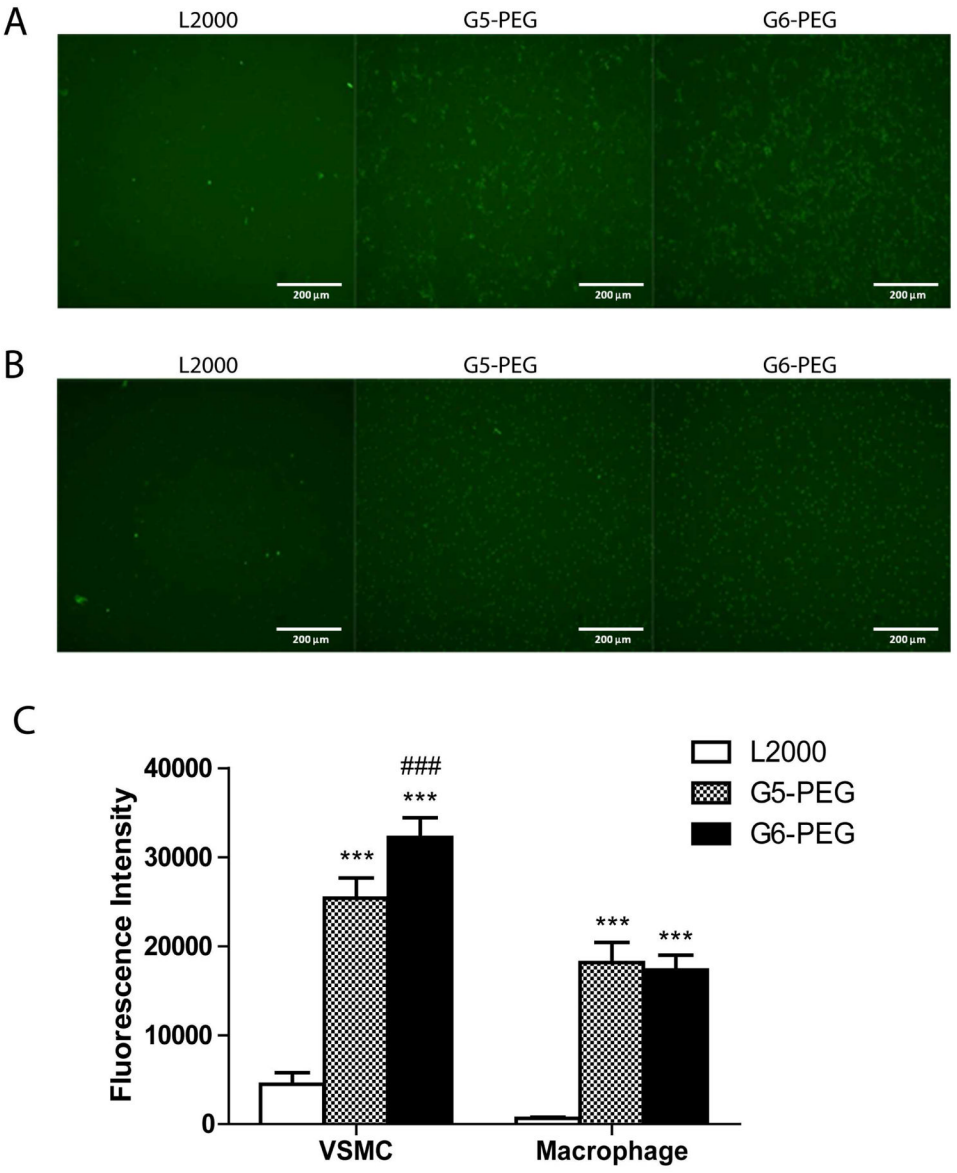

Fig. 4.

The transfection efficacy of G5- and G6- PEG PAMAM dendrimers to FITC-labeled siRNA in primary VSMC and peritoneal macrophages. Fluorescence images were observed in VSMC (A) and macrophages (B) (left to right: L2000, G5-PEG, G6-PEG.), and fluorescence intensity (C) was measured by flow cytometry. Statistical analysis was done with one-way ANOVA test (n=3, *** $p<0.005$ *vs* L2000, ### $p<0.005$ *vs* G5-PEG). Note that G5-PEG and G6-PEG have better transfection to FITC-siRNA than L2000 in these two primary cells.

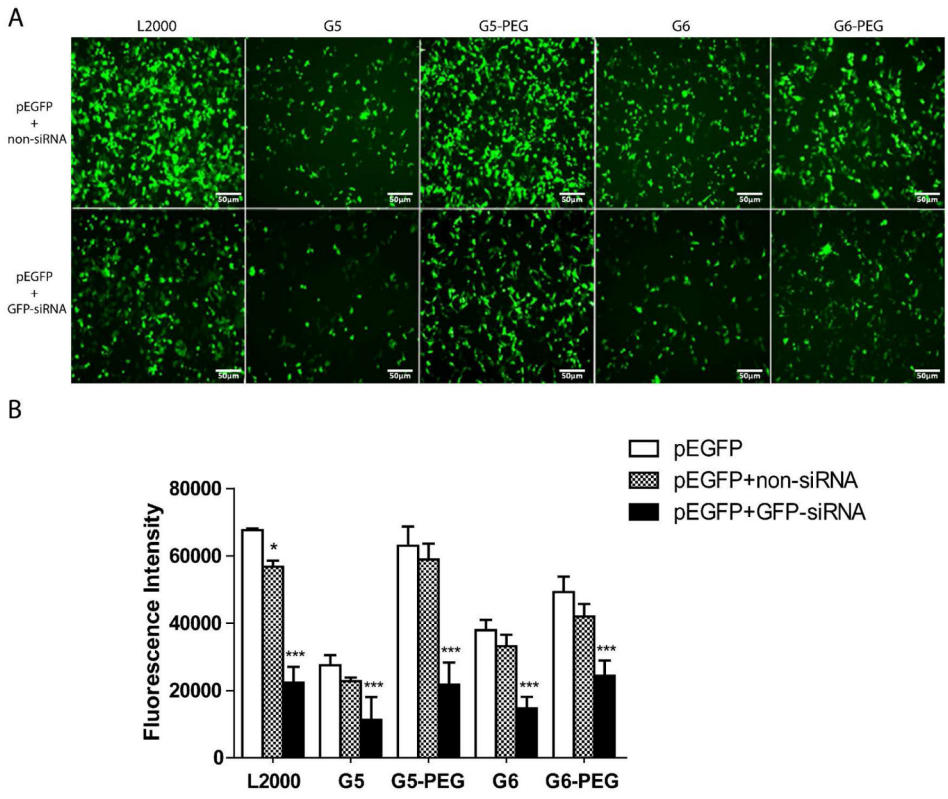

Fig. 5.

Co-transfection of pEGFP and GFP-siRNA to knock down the GFP expression in 293A cells. pEGFP and GFP-siRNA were co-transfected by PEGylated or non- PEGylated G5 or G6 PAMAM dendrimers, and the inhibition efficacy to GFP expression was compared with that of L2000. A: Fluorescence images of co-transfection of pEGFP with non-siRNA (top row) or GFP-siRNA (bottom row). From left to right: L2000, G5, G5-PEG, G6, G6-PEG; B: Fluorescence intensity. Statistical analysis was done with one-way ANOVA test (n=3, * $p<0.05$, *** $p<0.005$, compared with the initial GFP expression). Note that G5-PEG has higher co-transfection efficiency and achieves better down-regulation to GFP expression than G6-PEG.

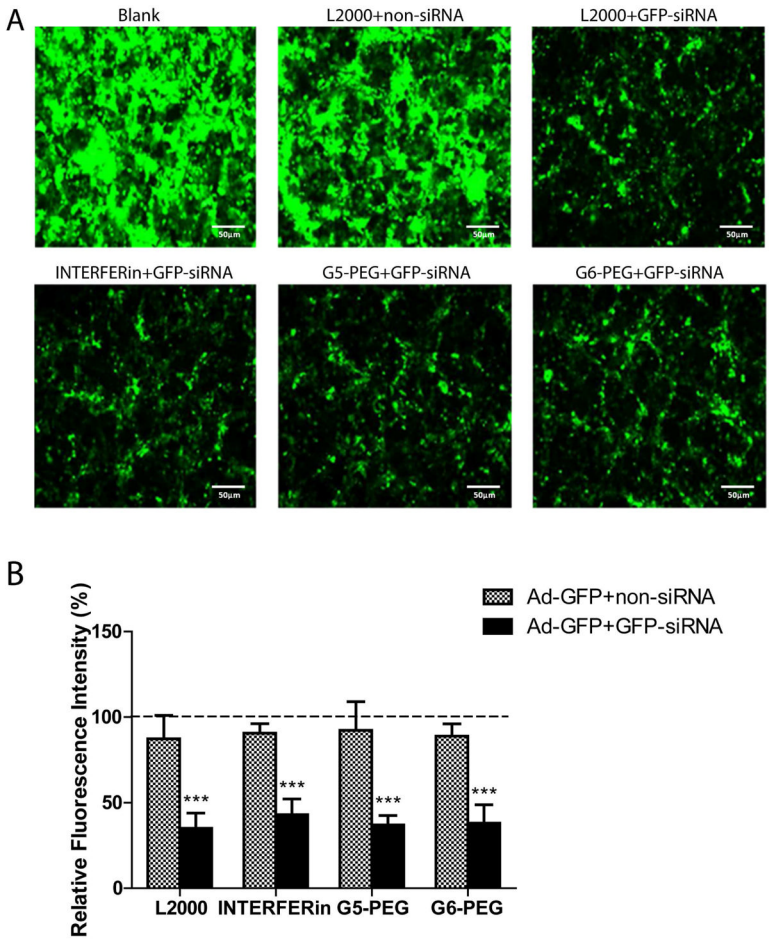

Fig. 6.

Delivery of GFP-siRNA in Cos-7 cells to block down the GFP expression mediated by Ad-GFP. Cos-7 cells were firstly infected by Ad-GFP to express GFP gene, and GFP-siRNA was then transfected by G5-PEG or G6-PEG to block down the GFP expression. A: Fluorescence images of GFP expression in Cos-7 cells. From left to right: top row: Ad-GFP only, Ad-GFP with non-siRNA delivered by L2000, Ad-GFP with GFP-siRNA delivered by L2000; bottom row: Ad-GFP with GFP-siRNA delivered by INTERFERin, Ad-GFP with GFP-siRNA delivered by G5-PEG, Ad-GFP with GFP-siRNA delivered by G6-PEG; B: Relative fluorescence intensity against Ad-GFP. Statistical analysis was done with one-way ANOVA test (n=3, *** $p<0.005$, compared with non-siRNA transfected by each mediator at the same experimental conditions). Note that compared with L2000 and INTERFERin, G5-PEG and G6-PEG delivered GFP-siRNA achieved the similar down-regulation effects to GFP expression.

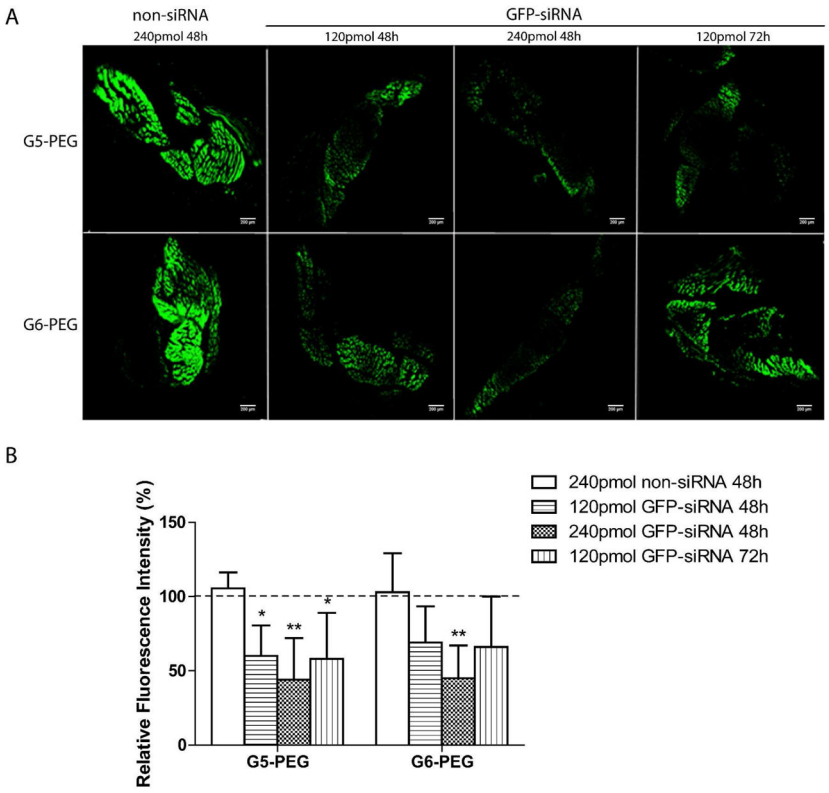

Fig. 7.

Delivery of GFP-siRNA to silence the GFP expression mediated by Ad-GFP in C57BL/6 mice. Ad-GFP (1×10^7 pfu) was injected intramuscularly to the quadriceps of C57BL/6 mice to express GFP gene, and GFP-siRNA with the two doses of 120 and 240 pmol was then delivered respectively to the quadriceps of the mice by G5-PEG or G6-PEG. The gene silence efficacy was investigated at the two time points (48 and 72h) after the GFP-siRNA delivery. Fluorescence images (A) of the muscle's cryosections of the C57BL/6 mice near the injection point were observed after GFP-siRNA being delivered by G5-PEG (top row) or G6-PEG (bottom row). From left to right: 240 pmol non-siRNA 48h, 120 pmol GFP-siRNA 48h, 240 pmol GFP-siRNA 48h, 120 pmol GFP-siRNA 72h. Relative fluorescence intensity (B) against Ad-GFP infected mice quadriceps was calculated. Statistical analysis was done with one-way ANOVA test (n=5, * $p<0.05$, ** $p<0.01$, compared with non-siRNA transfected by each mediator at the same experimental conditions). Note that not only at a high dose (240 pmol) but also at a low dose (120 pmol) of siRNA, G5-PEG delivered GFP-siRNA could accomplish significant down-regulation effect to adenovirus-expressed GFP.

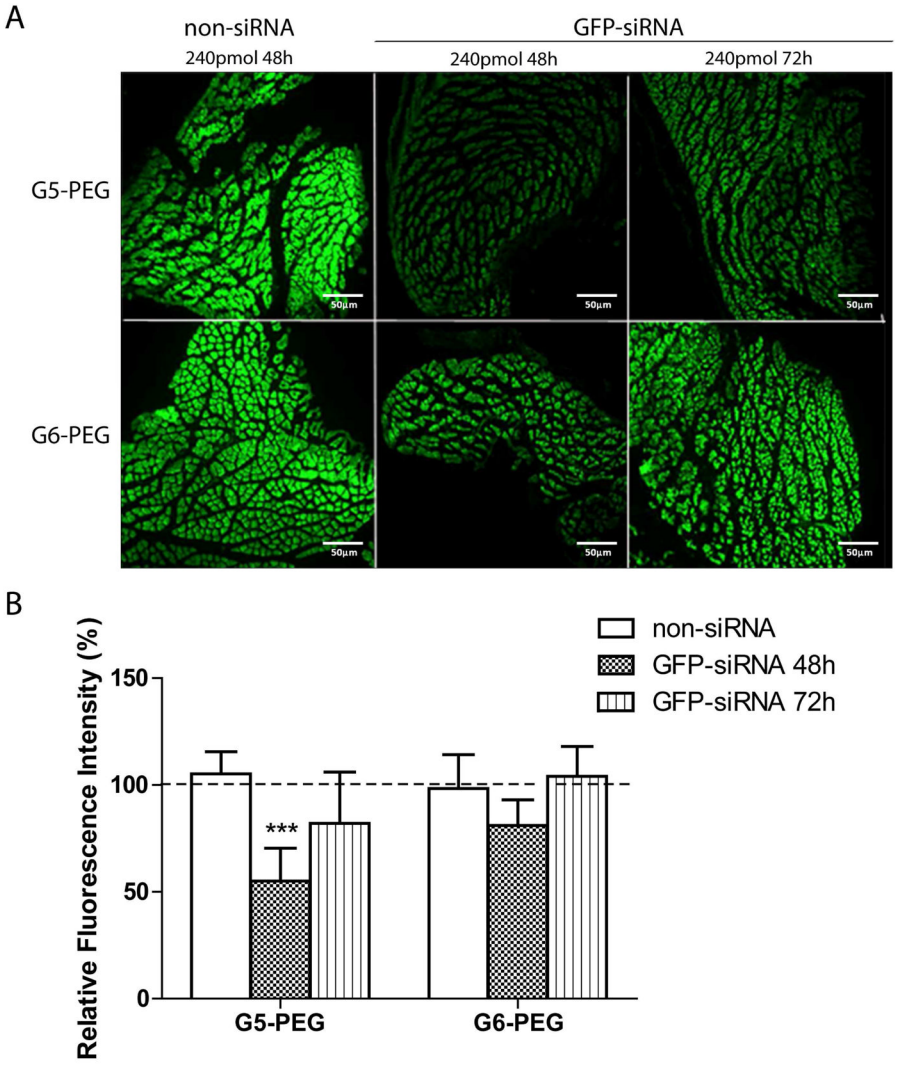

Fig. 8.

Delivery of GFP-siRNA to silence the expression of GFP gene in GFP transgenic mice. GFP-siRNA with the dose of 240 pmol was delivered by G5-PEG or G6-PEG to the quadriceps of the GFP transgenic mice, and the inhibition efficacy to a permanently expressed GFP gene around the injective point was evaluated at the 48 and 72h after the GFP-siRNA delivery. Fluorescence images of the muscle's cryosections (A) of the GFP transgenic mice near the injection points were observed after GFP-siRNA being delivered by G5-PEG (top row) or G6-PEG (bottom row). From left to right: 240 pmol non-siRNA 48h, 240 pmol GFP-siRNA 48h, 240 pmol GFP-siRNA 72h. Relative fluorescence intensity (B) against the original fluorescence intensity of the GFP mice was calculated. Statistical analysis was done with one-way ANOVA test (n=5, *** $p<0.005$, compared with non-siRNA transfected by each mediator). Note that G5-PEG delivered GFP-siRNA has significant down-regulation effect to a permanently expressed GFP gene at 48h after siRNA delivery.

Table 1

Average size and ζ potential of the dendrimer/siRNA dendriplexes.

Dendriplex	N/P ratio	PDI	Size (nm)	ζ potential
siRNA		0.396	241.9±48.1	−23.6±1.2
G5/siRNA	10/1	0.216	146.8±22.2[a]	34.5±2.6
G6/siRNA	10/1	0.167	134.9±24.1[a]	33.6±1.5
G5-PEG/siRNA	10/1	0.245	109.8±16.3[b]	26.9±3.7
	20/1	0.236	102.9±30.8[b]	30.7±3.6
G6-PEG/siRNA	10/1	0.193	129.4±12.0[b]	27.9±2.8
	20/1	0.237	114.8±39.8[b]	31.2±1.4

The dendriplexes were formed in 1 ml distilled water at various N/P ratios and the final concentration of siRNA was 2.4μg/ml.

[a] $p<0.05$, compared with naked siRNA;

[b] $p<0.01$, compared with naked siRNA.