Synthesis of Electroneutralized Amphiphilic Copolymers with Peptide Dendrons for Intramuscular Gene Delivery

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ABSTRACT: Intramuscular gene delivery materials are of great importance in plasmid-based gene therapy system, but there is limited information so far on how to design and synthesize them. Previous study showed that the peptide dendron-based triblock copolymer with its components arranged in a reversed biomembrane architecture could significantly increase intramuscular gene delivery and expression. Herein, we wonder whether copolymers with biomembrane-mimicking arrangement may have similar function on intramuscular gene delivery.
Meanwhile, it is of great significance to uncover the influence of electric charge and molecular structure on the function of the copolymers. To address the issues, amphiphilic triblock copolymers arranged in hydrophilic–hydrophobic–hydrophilic structure were constructed despite the paradoxical characteristics and difficulties in synthesizing such hydrophilic but electroneutral molecules. The as-prepared two copolymers, dendronG2(l-lysine-OH)-poly propylene glycol2k(PPG2k)-dendronG2(l-lysine-OH) (rL2PL2) and dendronG3(l-lysine-OH)-PPG2k-dendronG3(l-lysine-OH) (rL3PL3), were in similar structure but had different hydrophilic components and surface charges, thus leading to different capabilities in gene delivery and expression in skeletal muscle. rL2PL2 was more efficient than Pluronic L64 and rL3PL3 when mediating luciferase, β-galactosidase and fluorescent protein expressions. Furthermore, rL2PL2-mediated growth-hormone-releasing hormone expression could significantly induce mouse body weight increase in the first 21 days after injection. In addition, both rL2PL2 and rL3PL3 showed a good in vivo biosafety in intravenous and intramuscular administration. Altogether, rL2PL2-mediated gene expression in skeletal muscle exhibited applicable potential for gene therapy. The study revealed that the molecular structure and electric charge were critical factors governing the function of the copolymers for intramuscular gene delivery. It can be concluded that, combining previous study, both structural arrangements either reverse or similar to the biomembrane are effective in designing such copolymers. It also provides an innovative way in designing and synthesizing new electroneutralized triblock copolymers which could be used safely and efficiently for intramuscular gene delivery.

**KEYWORDS:** intramuscular gene transfer, gene expression, electroneutralization, amphiphilic copolymer, biomembrane, gene therapy

**INTRODUCTION**
Production of therapeutic molecules using skeletal myocytes is an appealing strategy for gene therapy due to its safety, low-cost and simplicity. However, it often fails to meet the therapeutic needs because the strategy employed for gene delivery is not efficient and, thus, the expression level is in general too low. Recently, a large amount of non-viral materials have been developed in order to enhance gene delivery efficiency. Cationic polymers and lipids are among the most widely-used materials that can be used for in vitro gene delivery, as they can efficiently deliver nucleic acids into culture cells. Unfortunately, they usually offered negative results when injected intramuscularly because they have strong interaction with muscle matrix which is negatively charged. It has been reported that some neutral polymers including poly(N-vinyl pyrrolidone), poly(vinyl alcohol) and Pluronics have the capacity to improve muscular gene delivery. We also constructed an electroneutralized triblock copolymer using peptide dendron and PEG\(_2k\) arranged as hydrophobic–hydrophilic–hydrophobic, a reversed biomembrane architecture. It presented much better performance on both reporter and function gene expressions in skeletal muscle than that of Pluronic L64, which was reported to give more homogeneous and stable gene expressions than other Pluronic derivatives. The components of the copolymer was considered to be arranged in a reverse architecture in comparison to that of biomembranes, whose bilayer structure usually consists of two outer hydrophilic units and one inner hydrophobic part, thus generating a hydrophilic–hydrophobic–hydrophilic architecture. Herein, we are interested in a further exploration of such copolymers about the effectiveness of intramuscular gene delivery while their components are reversely arranged, that is, a biomembrane-mimicking architecture in hydrophilic–hydrophobic–hydrophilic arrangement. In addition, we consider that the amount of electric charges and the architecture of the molecule
might exert determinant influence on the intramuscular gene delivery function of the copolymer. This point of view also needs further confirmation.

Pluronics have been considered to have hydrophilic–hydrophobic–hydrophilic architecture because their hydrophilic ethylene oxide (EO) and hydrophobic propylene oxide (PO) blocks are arranged as $EO_x$–PO$_y$–EO$_x$. Although several kinds of Pluronics, such as P85, P105 and L64, have attracted much attention for the intramuscular gene transfer ability, our recent study revealed that Pluronic L64 was able to generate pores across cell plasma membranes in a both time- and concentration-dependent way, which thereby inhibited the endocytosis of transfection complex. These effects would consequently compromise the viability of cells and, indeed, the biocompatibility of Pluronic L64 would be a concern. One approach to minimize the adverse effects may be the application of more suitable units to construct the triblock copolymers. Therefore, we chose peptide dendrons and (poly propylene glycol)$_{2k}$(PPG$_{2k}$) as components for assembling the new type of copolymers due to their authenticated biocompatibility both in vitro and in vivo. Meanwhile, polypeptide dendrons have supplementary advantages like their controllable surface functionalization and well-defined structures, which offers marvelous convenience for us to assemble copolymers with different molecular structures and surface charges using different generation of peptide dendrons.

In order to assess if the molecular structure and surface charge are critical factors in governing the gene delivery properties of these copolymers, two different copolymers were constructed: (i) dendronG2(1-lysine-OH)-PPG$_{2k}$-dendronG2(1-lysine-OH)(rL$_2$PL$_2$) and (ii) dendronG3(1-lysine-OH)-PPG$_{2k}$-dendronG3(1-lysine-OH)(rL$_3$PL$_3$). The two copolymers have the same hydrophilic–hydrophobic–hydrophilic architecture but different hydrophilic components as well as different surface charges. It is well-known that the hydrophilic terminal groups will interact with the
aqueous phase, thus the synthesis of such electroneutralized copolymers comes to be rather difficult. Therefore, it is of meaningful importance to set up a feasible and instructive way to synthesize such copolymers at first.

**EXPERIMENTAL SECTION**

**Materials and Characterization.**

Propargylamine was purchased from Acros Organics (Geel, Belgium). \(N_\alpha,N_\varepsilon\)-di-t-butyloxycarboryl-l-lysine (Boc-Lys(Boc)-OH), 1-hydroxybenzotriazole (HOBT), \(N,N\)-Diisopropylethylamine(DIPEA), \(N,N,N',N'-\)tetramethyl-O-(1\(H\)-benzotriazol-1-yl)uroniumhexafluorophosphate (HBTU) and trifluoroacetic acid (TFA) were from Gil Biochem. Co. Ltd. (Shanghai, China). Pluronic L64 (EO\(_\text{13}PO_{\text{30}}EO_{\text{13}}\); \(M_w = 2900\)), Sodium ascorbate, sodium azide (NaN\(_3\)), polyetheramine (NH\(_2\)-PPG-NH\(_2\), \(M_w \approx 2000\)), and other chemicals were from Sigma-Aldrich (St Louis, MO, USA). \textbf{G2 (G2-4NHBoC-alkynyl)} was synthesized according to previous protocol.\(^{20}\)

Characterization and structure confirmation of the intermediates and products were measured by electrospray ionization time-of-flight mass spectrometry (ESI-TOF MS, Waters Q-TOF Premier), \(^1\)HNMR (400 MHz Bruker Advanced 600 Spectrometer) and matrix assisted laser desorption ionization time-of-flight (MALDI-TOF, Autoflex MALDI-TOF/TOF) mass spectrometry. Zeta potentials of \textbf{rL\(_2\)PL\(_2\)}, \textbf{rL\(_3\)PL\(_3\)} and L64 dissolved in deionized water were measured by a Malvern Zetasizer Nano ZS (Malvern Instruments).

Plasmids of pSC-LacZ, pSC-Luc, pSC-E2 and pSC-GHRH, which carry genes of \(\beta\)-d-galactosidase, luciferase, far-red fluorescent protein and growth-hormone-releasing hormone respectively, were constructed in our laboratory and prepared as described.\(^{32}\)

**Synthesis of G3 (G3-8NHBoC-alkynyl).**
Under a nitrogen atmosphere, the peptide G2 (4.0 g, 476 mmol) was dissolved in anhydrous dichloromethane (15 mL), cooled to 0 °C and trifluoroacetic acid (15 mL, 197 mmol) was added. After a 8-hour stirring at room temperature, trifluoroacetic acid and solvents were cleared by rotary steam. The collected solid was dissolved in 15 mL anhydrous DMF and the mixture of HOBT (6.2 g, 46 mmol), Boc-Lys (Boc)-OH (12.6 g, 364 mmol), anhydrous DMF (10 mL) and HBTU (12.0 g, 36 mmol) were added in. The as-prepared solution was cooled down to 0 °C, and 139 mmol DIPEA (23.0 mL) was added in under nitrogen. After a 72-hour stirring at room temperature, the mixture was diluted with ethyl acetate and washed by 3×15 mL saturated sodium bicarbonate solution, 3×15 mL 1M hydrochloric acid and 3×15 mL saturated sodium chloride solution, respectively. The organic layer was dried with anhydrous magnesium sulfate, followed by concentrating, recrystallizing and vacuum drying, generating a white solid. $^1$H NMR (400 MHz, CDCl$_3$) $\delta$: 4.45–4.33 (d, 7H), 4.04 (s, 2H), 3.32–2.91 (m, 14H), 2.20 (s, 1H), 1.87–1.75 (m, 28H), 1.46–1.43 (m, 86H).

**Synthesis of 2-Azidoethanol (AE).**

3.5 g sodium azide was dissolved in 15 mL water, and then 2.8 mL 2-Chloroethanol was slowly added at room temperature. After stirring for 24 h at 80 °C, the mixture was cooled down to room temperature, saturated with sodium chloride and extracted with 3×10 mL dichloromethane. The organic layer was dried using anhydrous sodium sulfate and the solvent was removed under reduced pressure to obtain the colorless liquid AE (2.8 g, 74%). $^{31}$H NMR (400 MHz, CDCl$_3$) $\delta$: 3.81–3.75 (m, 2H), 3.48–3.41 (m, 2H), 2.42 (dd, 1H).

**Synthesis of 2-Azidoethyl 1H-imidazole-1-carboxylate (AE-CDI).**

AE-CDI was synthesized from the obtained AE. Briefly, a dry round-bottomed flask was charged with 4.8 g carbonyldiimidazole (CDI) and 40 mL dichloromethane, yielding a
suspension. At room temperature, 1.9 g AE with 20 mL dichloromethane was added dropwise and vigorously stirred to generate a clear solution, which was then washed with water for three times. After drying with anhydrous magnesium sulfate, the organic layer was filtered and evaporated, yielding 3.5 g colorless liquid AE-CDI. 1H NMR (400 MHz, CDCl3) δ: 8.17 (s, 1H), 7.45 (t, 1H), 7.10–7.06 (m, 1H), 4.60–4.55 (m, 2H), 3.68–3.63 (m, 2H).

**Synthesis of Azido-terminated PPG (N3-PPG-N3).**

In a dry flask with round-bottom, 3.4 g AE-CDI was dissolved in 20 mL dichloromethane and dropped slowly (about 1.5 h) into a solution of NH2-PPG-NH2 (2000 Da, 16.7 g, 8 mmol) in 25 mL dichloromethane at room temperature. Next, the reaction mixture was heated to 35 °C using an oil bath until no primary amine groups were detected in the solution (detection by 0.5% ninhydrin solution). After removal of dichloromethane under reduced pressure by evaporation, a yellow liquid was obtained and dissolved in 30 mL chloroform, followed with washing using 3×15 mL deionized water. The organic layer was dried with anhydrous sodium sulfate and the solvent was removed under reduced pressure to obtain N3-PPG-N3. The product, a light yellow liquid was dissolved in CDCl3 and characterized by 1H NMR. No unreacted AE-CDI was detected. The relative molecular weight (Mn) was calculated to be 2170 dalton or g/mol, as analyzed later. 1H NMR (400 MHz, CDCl3) δ: 4.2(s, 4H), 3.6–3.7 (m, 8H), 3.4 (m, 89H), 1.14(m, 93H).

**Synthesis of G2-terminated PPG (G2-PPG-G2) and G3-terminated PPG (G3-PPG-G3).**

The peptide G2 (0.6 g, 0.7 mmol) or G3 (1.6 g, 0.9 mmol) and the azide N3-PPG-N3 (0.7 g, 0.3 mmol) were dissolved in 44 mL of anhydrous N,N-dimethyl-formamide (DMF) and 14 mL of water to form a solution mixture. Then, 0.2 equiv CuSO4·5H2O and 1.0 equiv sodium ascorbate were added to the solution,34 stirred for 36 h (G2) or 48 h (G3) at room temperature
under N₂. Finally, the rubber septum was removed in about 2 h under stirring, generating oxidation of Cu (I) catalyst into Cu (II). After the removal of the solvents, the crude product was dissolved in chloroform (30 mL) and washed with deionized water (3×15 mL), followed by drying with anhydrous sodium sulfate. A yellow solid was obtained after the solvent was removed under reduced pressure. The excess G2 or G3 was removed by dialysis against chloroform/methanol (3:1 or 2:1, V/V) in a cellulose ester membrane (Spectra/Por Biotech MWCO 2000; Spectrum Laboratories) for 2 days and evaporated to obtain a fluffy, yellow solid.

G2: ¹H NMR (400 MHz, CDCl₃) δ: 7.63 (s, 2H), 4.83 (s, 4H), 4.61–4.04 (m, 14H), 3.94 (s, 2H), 3.79–3.21 (m, 97H), 3.01 (s, 12H), 1.79–1.53 (m, 12H), 1.49–1.18 (m, 102H), 1.07 (dd, 93H).

G3: ¹H NMR (400 MHz, CDCl₃) δ: 7.66 (s, 2H), 5.01–4.82 (m, 4H), 4.55–4.27 (m, 22H), 3.95 (s, 2H), 3.83–3.12 (m, 97H), 3.02 (s, 28H), 1.66 (s, 28H), 1.52–1.25 (m, 203H), 1.21–0.90 (m, 93H).

Synthesis of Isopropylidene-2,2-bis(oxymethyl)propionic Acid (DHPA).

DHPA was synthesized according to previous procedure.³⁵ Briefly, 2, 2-bis (hydroxymethyl) propionic acid (13.4 g, 0.1 mol) was dissolved in 90 mL of anhydrous acetone at room temperature. Then 2,2-dimethoxypropane (18.4 mL, 0.2 mol) and p-toluenesulfonic acid (1.7 g, 10mmol) were added to the solution. After stirring for 6 h, the catalyst in the reaction mixture was neutralized by adding 5 mL NH₄OH/EtOH solution (1:1, V/V) and the solvent was evaporated at room temperature. The product was dissolved in 60 mL CH₂Cl₂ and washed with deionized water (3×15 mL). The organic layer was dried with anhydrous sodium sulfate and the solvent was removed under reduced pressure to obtain the white solid DHPA (16.5 g, 94%). ¹H NMR (400 MHz, CDCl₃) δ: 4.18 (s, 1H), 4.15 (s, 1H), 3.72 (s, 1H), 3.69 (s, 1H), 1.46 (s, 3H), 1.44–1.42 (m, 3H), 1.20 (s, 3H).
Synthesis of N-hydroxysuccinimidoisopropylidene-2,2-bis(oxy methyl)propionate (DHPA-NHS).

DHPA (13.1 g, 75 mmol) and N-hydroxysuccinimide (NHS) (9.2 g, 80 mmol) were dissolved in 200 mL of dichloromethane at room temperature. Subsequently, N,N'-dicyclohexylcarbodi-imide (DCC) (16.5 g, 80 mmol) in 60 mL dichloromethane was slowly added dropwise to the solution at 0 °C around 1 h. The reaction mixture was then stirred for 24 h at room temperature. The white precipitate of dicyclohexylurea (DCU) was removed by suction filtration, and the filtrate was concentrated under reduced pressure. The waxy solid was recrystallized from ethyl acetate and dried to obtain DHPA-NHS as a white crystals (17.5 g, 86%). $^1$H NMR (400 MHz, DMSO-d$_6$) δ: 4.09 (d, 2H), 3.79 (d, 2H), 2.81 (s, 4H), 1.40 (s, 3H), 1.28 (s, 3H), 1.21 (s, 3H). Good agreement was found with the literature.\textsuperscript{36}

Synthesis of rL$_2$PL$_2$ and rL$_3$PL$_3$.

G$_2$-PPG-G$_2$ (0.65 g, 0.18 mmol) or G$_3$-PPG-G$_3$ (1.0 g, 0.2 mmol) was dissolved in 15 mL or 20 mL anhydrous trifluoroacetic acid/dichloromethane (1:1, V/V) and the solution was stirred for 4 h or 8 h respectively under N$_2$ at room temperature. After the removal of the solvent by rotary evaporation, the precipitates were ultrasonic washed by saturated sodium carbonate solution and then collected by suction filtration. The sample was dried for 12 h under high vacuum (with phosphorus pentoxide). The deprotected product with 8 (G$_2$-PPG-G$_2$) or 16 (G$_3$-PPG-G$_3$) primary amine groups was dissolved in 20 mL anhydrous dichloromethane/Et$_3$N (1:1, V/V) at room temperature. DHPA-NHS (0.8 g, 3 mmol for G$_2$-PPG-G$_2$; or 1.6 g, 6 mmol for G$_3$-PPG-G$_3$) was added to the solution and then stirred for 24 h or 36 h at room temperature. The solvents were removed after work-up by rotary evaporation. The product was purified by dialysis against chloroform/methanol (1:1, V/V) in a cellulose ester membrane (Spectra/Por Biotech.
MWCO 2000) for 48 h and evaporated to obtain a pale yellow solid. This product, DHPA-terminated **G2-PPG-G2** or **G3-PPG-G3**, was dissolved in 60 or 90 mL of methanol and concentrated sulfuric acid (21 or 25 drops) was added. The solution was stirred at room temperature for 24 h and then neutralized with NH₄OH/EtOH solution. By filtration, insoluble NH₄SO₄ was removed and the filtrate was condensed. The **rL₂PL₂** or **rL₃PL₃** residue was dissolved in 20 or 15 mL deionized water and transferred into a Spectra/Por Biotech MWCO 2000 cellulose ester membrane for 48 or 72 h. The retained solution was filtered through a 1-μm Acrodisc and lyophilized to a pale yellow liquid. **rL₂PL₂**: ¹H NMR (400 MHz, DMSO-d₆) δ: 7.62 (s, 2H), 4.55 (s, 4H), 4.29 (d, 6H), 4.20 (s, 4H), 3.84 (s, 2H), 3.55–3.47 (m, 97H), 3.47–3.25 (m, 32H), 3.01 (d, 12H), 1.57 (d, 12H), 1.36 (s, 12H), 1.22 (d, 12H), 1.12–0.90 (m, 121H).**rL₃PL₃**: ¹H NMR (400 MHz, DMSO-d₆) δ: 7.42 (s, 2H), 4.55 (s, 4H), 4.29–4.19 (m, 22H), 3.84 (s, 2H), 3.85–3.47 (m, 97H), 3.47–3.30 (m, 64H), 3.00 (s, 28H), 1.77–1.11 (m, 112H), 1.10–0.91 (m, 147H).

**Micelle Characterization**

The formation of micellar structures were studied by steady-state fluorescence of pyrene. The fluorescence emission spectra were scanned by a Spectrophotometer (F-7000 FL, Hitachi) upon excitation at 390 nm.

**DNA Retardation Assay.**

pDNA (1 µg) and different amounts of **rL₂PL₂** or **rL₃PL₃** were diluted respectively in 10µL of 0.9% saline, incubated for 30 min at room temperature after mixing. The mixtures were then run on 1% (w/v) agarose gel at 80 V for 1 h. The DNA bands were photographed and visualized in the imager (Bio-Rad ChemiDoc XRS+, USA) after ethidium bromide (EB) staining.

**In Vivo Gene Delivery Assay.**
Animals were handled and operated as previously described with some modifications. Briefly, pSC-GHRH (50 μg in 80 μL solution) instead of pSC-GH was used to evaluate the mouse growth rate. Mice were sacrificed 4 days after administration and the TA muscles were separated and processed for different experimental tests. *In vivo* reporter gene assays were operated according to the manufacturers’ protocols of different Kits, such as *In Situ* β-Galactosidase Staining Kit (Beyotime, Shanghai, China) for the β-galactosidase activity assay, the Reporter Assay Kit (Promega, Madison, WI, USA) and BCA Protein Assay Kit (Pierce, Rockford, IL, USA) for luciferase activity analysis. The expression of the fluorescent protein E2-Crimson was detected on the days 7 and 14.

**Histological Section Analysis.**

Histological analysis of the TA muscles were performed 24 h after intramuscular injection of 40 μL 0.9% saline or 0.005% rL₂PL₂ or 0.02% rL₃PL₃. The separated TA muscles were fixed for 24 h in 4% fresh formaldehyde solution, dehydrated with gradient concentrations of ethanol, embedded in paraffin and then sliced into 10-μm sections in cross and longitudinal directions. The sections were immersed for 3 min in hematoxylin, dipped in eosin and mounted in mineral oil under a coverslip. Figures of the sections were taken with a microscope (Leica DMI 4000B; Wetzlar, Germany) for histopathological analysis.

**Serum Biochemical Test.**

Mice were injected with 200 μL of saline or 0.005% rL₂PL₂ or 0.02% rL₃PL₃ through tail veins. The blood was collected for sera separation on day 4 by centrifugation at 3000 rpm for 10 min at 4°C. The sera were biochemically analyzed using the 7020 Automatic Analyzer (Hitachi, Japan).

**Statistical Analysis.**
Data were provided as mean ± standard deviation (SD.). Comparisons were analyzed by t-test among groups. The value of P < 0.05 was considered to be significantly different, while P < 0.01 was highly significant.

RESULTS AND DISCUSSION


Some key characteristics of Pluronics might exert influence on their local gene transfer capability, such as the polymerization level of EO and PO units, hydrophilic/lipophilic balance (HLB), structure of the whole molecule, critical micelle temperature (CMT) and critical micelle concentration (CMC). However, there is not any reliable theory relevant to the molecular design of the electroneutralized amphiphilic copolymers. It is also difficult to predict the intramuscular gene delivery capability of a synthesized molecule. Theoretically, if the terminal groups in a molecule are hydrophilic, there should be an interaction between them and water phase, which will make the molecule positively charged. So it is difficult to construct an uncharged molecule with hydrophilic terminal groups. Herein, the first challenge in synthesizing such amphiphilic triblock copolymers is to neutralize their electric charges. With trials and errors, we finally synthesized rL2PL2 and rL3PL3 copolymers. In these molecules, NH2-terminated PPG was used as the middle hydrophobic unit to connect both ends of hydroxylated hydrophilic dendron G2 or G3. The whole structure was symmetrical and presented with a “butterfly” architecture.
Scheme 1 Synthetic route of G2-PPG-G2 and G3-PPG-G3 by efficient and fast azide-alkyne click reaction.

The triblock copolymers were firstly synthesized according to the liquid-phase peptide synthesis method as shown in Scheme 1. G2 was synthesized in our previous work. Here, the third-generation of l-lysine G3 was synthesized from G2. In order to connect PPG with G2 or G3 through organic azide and alkyne click chemistry, NH2-ended PPG should be turned into N3-ended PPG firstly, as shown in Scheme 1. In particular, N,N-carbonyldiimidazole (CDI) was an potent activating agent for the connection of 2-azidoethanol and NH2-PPG-NH2. The amidation
by CDI showed advantages of mild reaction condition and near-quantitative yield.\textsuperscript{37, 38} As showed in scheme 1, \textbf{G2-PPG-G2} and \textbf{G3-PPG-G3} with designed chemical structure and relative molecular weight were obtained by high-efficiency click reaction of different generation of L-lysine with active alkyl group in \textit{N\textsubscript{3}}-\textit{PPG-N\textsubscript{3}}.

The structure and purity of G3 was confirmed in Figure S1. Figure 1 (A–H) showed \textsuperscript{1}H NMR spectra and MALDI-TOF-mass spectra of N\textsubscript{3}-PPG-N\textsubscript{3}, \textbf{G2-PPG-G2} and \textbf{G3-PPG-G3}. The bifunctional PPG with two amine terminal groups (NH\textsubscript{2}-PPG-NH\textsubscript{2}) is commercially available and also named as poly(propyleneglycol) bis(2-aminopropyl ether). In general, PPG showed hydrophobicity when its molecular weight is lower than 740 g/mol.\textsuperscript{39} Compared with N\textsubscript{3}-PEG-N\textsubscript{3} in our previous work,\textsuperscript{20} PPG caused different synthetic methods (Scheme 1). Particularly, NH\textsubscript{2}-PPG-NH\textsubscript{2} was used as the initial raw material for N\textsubscript{3}-PPG-N\textsubscript{3} preparation to avoid the interference with terminal groups, such as monohydroxy PPG.\textsuperscript{40} AE was simply translated into AE-CDI, and CDI was obviously demonstrated to be linked to AE due to the appearance of chemical shifts of number c, d and e of hydrogen (Figure 1A and S2). \textsuperscript{1}H NMR analysis of the acquired product (Figure 1B) showed that the starting compound AE-CDI was absent (the NMR spectra of AE-CDI vs NH\textsubscript{2}-PPG-NH\textsubscript{2} in Figure 1A and C, respectively). The azide-functionalization of PPG was confirmed by the absence of the peaks at low field (higher than 7.0 ppm) for N\textsubscript{3}-PPG-N\textsubscript{3} as well as the disappearance of the protons of primary amine and chemical shift of protons neighbored to amide bonds. The intermediate of N\textsubscript{3}-PPG-N\textsubscript{3} was then clicked with the propargyl focal point \textbf{G2} or \textbf{G3}-typed dendrons to produce \textbf{G2-PPG-G2} or \textbf{G3-PPG-G3} by Cu\textsuperscript{1} -catalyzed cycloaddition (CuAAC) (Scheme 1). The signal of new proton at about 7.54 ppm, the typical peak of methine in the triazole ring, appeared within the \textsuperscript{1}H NMR spectra of \textbf{G2-PPG-G2} (Figure 1D or \textbf{G3-PPG-G3} in Figure S3), whereas the original peak of azide-
methylene (CH$_2$–N$_3$) for N$_3$-PPG-N$_3$ completely disappeared. The results demonstrated that the intermediate of N$_3$-PPG-N$_3$ was quantitatively transformed into G$_2$-PPG-G$_2$ or G$_3$-PPG-G$_3$.

**Figure 1.** Characterization of the intermediates. $^1$H NMR spectra of AE-CDI (A), N$_3$-PPG-N$_3$ (B), NH$_2$-PPG-NH$_2$ (C) and G$_2$-PPG-G$_2$ (D). MALDI-TOF mass spectra of G$_2$-PPG-G$_2$ (E) and G$_3$-PPG-G$_3$ (H). Partial enlarged details of G$_2$-PPG-G$_2$ with relative molecular weights around m/z 1813.4 (F) and m/z 3735.9 (G).

Furthermore, the mass spectrometry MALDI-TOF analysis of G$_2$-PPG-G$_2$ showed an almost symmetrical main distribution of peaks centered around m/z 3735.9 and a series of peaks lower in mass corresponding to a small amount of schizolytic compound of G$_2$-PPG-G$_2$ (Figure 1E–G).
Peaks centered on m/z 1813.4 were assigned to $\text{N}_3$-$\text{PPG}$-$\text{N}_3$. Peak of m/z 2706.0 was assigned to the single substitution product of G2. The repetitive unit of PG with m/z 58 was clearly presented in the mass spectra (Figure 1F and 1G). In Figure 1H, the mass spectra of G3-$\text{PPG}$-G3 were shown to match well with the theoretical molecular weights.

Scheme 2 Structures and Synthesis of rL$_2$PL$_2$ and rL$_3$PL$_3$.

The last step was the hydrophilization of G2-$\text{PPG}$-G2 and G3-$\text{PPG}$-G3, as shown in Scheme 2. In our previous studies, peptidedendrimers were synthesized using azide-alkyne click reaction followed by the deprotection of the protecting groups (Boc groups), so as to generate the final water-soluble dendrimers due to the hydrophilic amine and their hydrochloride or trifluoroacetic acid salt. One major challenge to develop effective intramuscular gene delivery
materials is to make the material electroneutralized or with non-easily ionized terminal group, and alcoholic hydroxyl is one of the very few choices.\textsuperscript{35, 42} Here, the novel cyclic carbonate monomer, DHPA-NHS, was easily prepared by a two-step reaction starting from 2,2-bis(hydroxymethyl)propionic acid (Scheme 2). The 2,2-dimethoxypropane was selected to protect the diol against the inter- and intramolecular side reactions of 2,2-bis(hydroxymethyl)propionic acid. In addition, NHS has the ability to protect the carboxylic group due to the formed active ester, which can react with primary amines under mild conditions in the following steps. The acetonide-protecting group can be removed easily at room temperature by stirring the methanol solution of DHPA-capped polymer with Dowex 50W cation resin, or directly adding a few drops of concentrated sulfuric acid.\textsuperscript{35, 42, 43}
Figure 2. Characterization of rL2PL2 and rL3PL3. 1H NMR spectra of rL2PL2 (A) and hydrophilization of G2-PPG-G2 (B). MALDI-TOF mass spectra of rL2PL2 (C) and rL3PL3 (D).

The chemical structure of DHPA and DHPA-NHS were confirmed by 1H NMR spectroscopy (Figure S5). Each synthesis step of rL2PL2 or rL3PL3 was detected by the 1H NMR, ninhydin test and MALDI-TOF-MS. It was confirmed that the syntheses of rL2PL2 and rL3PL3 were completed (Figure 2A and Figure S6). Linkage of DHPA to Boc-deprotected G2-PPG-G2 was obviously demonstrated by the disappearance of chemical shifts of Boc-methyl-protons (Figure 2B). Each theoretically predicted peak of the chemical shifts of rL2PL2 and rL3PL3 in DMSO-d6 solution could be found, which meant that the designed polymers were obtained. And even the MALDI-TOF-mass spectra of rL2PL2 and rL3PL3 have proved to be in accordance with the designed (Figure 2C and 2D). The results confirmed that the detected value of the molecular weight well matched the theoretical one.

Due to the amphiphilicity of the two copolymers, the onset of the formation of rL2PL2 or rL3PL3 micelles was measured using pyrene as a hydrophobic probe. The intensity ratios of the pyrene excitation bands at 338 and 334 nm (I338/I334) to the logarithms of the concentrations of rL2PL2 or rL3PL3 were shown in Figure S7. The CMC of rL2PL2 or rL3PL3 was 0.056 mg/mL or 0.251 mg/mL, obtained from the intersection of the two straight lines.

**DNA Retardation Assay and Zeta Potential.**

Cationic gene delivery materials usually decreased the intramuscular gene delivery and expression level, whereas electroneutral materials increased the efficiency. Therefore, electric charges of rL2PL2 and rL3PL3 molecules should be detected at first. DNA retardation assay has
been widely used for the detection of the interaction between molecules and DNAs, because DNAs are negatively charged and may interact with positively charged molecules. Upon combination, either in chemical or physical manner, DNA migration will be retarded in agarose gel electrophoresis.\textsuperscript{44} Theoretically, when alcoholic hydroxyl groups are arranged as the ending groups, there would be an interaction between the copolymer and the aqueous phase, resulting in the positively charged copolymer. Therefore, the charge of the copolymer is uncertain. It depends on the number of hydroxyl groups in the copolymer. When the number is small enough, the interaction will be extremely weak and can be ignored. However, if there are too many hydroxyl groups in one molecule, the interaction will be superimposed to be strong. Here we can see that in the agarose gel, the DNA bands were completely identical in both migration and strength even at the highest concentration, indicating that neither \textit{r}L\textsubscript{2}PL\textsubscript{2} nor \textit{r}L\textsubscript{3}PL\textsubscript{3} bind with DNA molecules (Figure3A). Zeta potentials were further tested to quantitatively analyze the surface charges of the copolymers. The charge of \textit{r}L\textsubscript{2}PL\textsubscript{2} was 3.39 mV while \textit{r}L\textsubscript{3}PL\textsubscript{3}, 8.54 mV (Figure3B). As the charges were not strong enough to compress DNAs at each concentration (Figure3A), the two copolymers might be considered to be approximately electroneutralized.

\textbf{Figure 3.} DNA retardation assay (A) and Zeta potentials (B). Lane 1: naked pDNA; Lane 2-17: the copolymer/pDNA mixtures with increasing mass ratios (m/m%).

\textit{In Vivo} Biocompatibility Assays of \textit{r}L\textsubscript{2}PL\textsubscript{2} and \textit{r}L\textsubscript{3}PL\textsubscript{3}.
To evaluate the biocompatibility of the copolymers, we sectioned the injected TA muscles crossly and longitudinally for histopathological analysis. The sections didn’t show any pathological changes including inflammatory reaction and necrosis focus. In addition, there was no visible difference among 0.9% saline (Figure 4, A & D), 0.005% rL2PL2 (Figure 4, B & E) and 0.02% rL3PL3 (Figure 4, C & F) groups. These results clearly indicated that rL2PL2 and rL3PL3 could be used for local intramuscular administration safely. Furthermore, the serum biochemical tests were used to evaluate the biosafety of rL2PL2 and rL3PL3 when 200 μL of 0.005% rL2PL2 or 0.02% rL3PL3 solution was intravenously injected into a mouse, respectively. The widely accepted clinical indexes to value liver and renal function and blood glucose were examined. The data showed no obvious difference among 0.9% saline, 0.005% rL2PL2 and 0.02% rL3PL3 treatments (Table 1). It further confirmed that rL2PL2 and rL3PL3 copolymers had good biocompatibility.

Figure 4. Representative HE-stained tissue sections in TA muscles injected with saline (A&D), 0.005% rL2PL2 (B&E) and 0.02% rL3PL3 (C&F). A, B & C: cross sections; D, E & F: longitudinal sections. Scale bar = 100 μm.
**Table 1.** Serum biochemical tests of mice injected with saline, rL\textsubscript{2}PL\textsubscript{2} and rL\textsubscript{3}PL\textsubscript{3}

<table>
<thead>
<tr>
<th>Items</th>
<th>Saline</th>
<th>rL\textsubscript{2}PL\textsubscript{2}</th>
<th>rL\textsubscript{3}PL\textsubscript{3}</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALT (U/L)</td>
<td>51.50±14.79</td>
<td>44.25±6.23</td>
<td>51.25±10.04</td>
</tr>
<tr>
<td>AST (U/L)</td>
<td>107.75±24.39</td>
<td>107.75±21.75</td>
<td>111.00±19.91</td>
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<tr>
<td>Liver function</td>
<td>ALP (U/L)</td>
<td>236.25±24.58</td>
<td>237.00±22.45</td>
</tr>
<tr>
<td></td>
<td>TP (g/L)</td>
<td>55.48±2.71</td>
<td>54.58±2.01</td>
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<tr>
<td></td>
<td>ALB (g/L)</td>
<td>27.03±1.32</td>
<td>26.40±0.91</td>
</tr>
<tr>
<td>Renal function</td>
<td>BUN (mmol/L)</td>
<td>18.63±1.25</td>
<td>16.28±0.88</td>
</tr>
<tr>
<td>Blood glucose</td>
<td>GLU (mmol/L)</td>
<td>9.34±1.99</td>
<td>10.27±0.57</td>
</tr>
</tbody>
</table>

*Abbreviations*: ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALP, alkaline phosphatase; TP, total protein; ALB, albumin; BUN, blood urea nitrogen; GLU, glucose. Data were presented as mean ± SD (n = 6/group).

**Reporter Gene Delivery and Expression via Intramuscular Injection.**

Herein, rL\textsubscript{2}PL\textsubscript{2}-and rL\textsubscript{3}PL\textsubscript{3}-mediated reporter gene expressions through intramuscular administration were tested both qualitatively and quantitatively. The expression of β-galactosidase (LacZ) in mice TA muscles could offer a visible and fast evaluation of the transgene expression level as well as its spatial distribution. In this study, 0.1% L64 was used as the positive control, as its local gene transfer activity was similar to and also more stable than that of the other Pluronics (0.05% P104, 0.05% P123, 0.1% P105 and 0.05% F127). As shown in Figure 5A, L64(0.1%)/pDNA and most of rL\textsubscript{2}PL\textsubscript{2}/pDNA mixtures with different mass ratios from 0.001% to 0.05% as well as some rL\textsubscript{3}PL\textsubscript{3}/pDNA mixtures showed higher LacZ expression level than that of the naked pDNA group, which was evident from the color and distribution patterns shown on the surfaces of stained muscles. For further analysis, luciferase expression...
level indicated by its activity was examined and gave quantitative results in different groups (Figure 5B). In \textbf{rL}_2\textbf{PL}_2/p\text{DNA} groups, the luciferase activity was not as good as \text{pDNA} when the concentration of \textbf{rL}_2\textbf{PL}_2 was 0.001%. However, the activity sharply rose when the mass ratio of \textbf{rL}_2\textbf{PL}_2 was elevated. It reached the peak at 0.005% and then gradually decreased along with the increase of \textbf{rL}_2\textbf{PL}_2 concentration from 0.01% to 0.05%. The luciferase activity of the 0.05% group was dropped to a similar level as that in 0.001% and \text{pDNA} groups. Quantitatively, 0.005% \textbf{rL}_2\textbf{PL}_2 increased transgene expression level up to 15-fold compared to that of \text{ naked DNA} and 2.7-fold as for 0.1% L64. For \textbf{rL}_3\textbf{PL}_3, although the luciferase expression was improved at the concentration of 0.02%, the results were significantly fluctuated, indicating that \textbf{rL}_3\textbf{PL}_3-mediated transgene expression was unstable.

\textbf{Figure 5.} \textit{In vivo} transgene expression assays. \(n = 6/\text{group.} \) (A). Representative figures of \( \beta \)-galactosidase expression in mice TA muscles. a) Naked \text{pDNA}; b) L64(0.1%)/\text{pDNA}; c-g) \textbf{rL}_2\textbf{PL}_2/\text{pDNA} mixtures with various mass ratios (c: 0.001%; d: 0.005%; e: 0.01%; f: 0.02%; g: 0.05%); h-l) \textbf{rL}_3\textbf{PL}_3/\text{pDNA} mixtures with various mass ratios (h: 0.001%; i: 0.005%; j: 0.01%; k: 0.02%; l: 0.05%). (B) Luciferase activity assay in mice TA muscles. a) Naked \text{pDNA}; b)
L64(0.1%)/pDNA; c-g) rL_{2}PL_{2}/pDNA mixtures with various mass ratios (c: 0.001%; d: 0.005%; e: 0.01%; f: 0.02%; g: 0.05%); h-l) rL_{3}PL_{3}/pDNA mixtures with various mass ratios (h: 0.001%; i: 0.005%; j: 0.01%; k: 0.02%; l: 0.05%). **: P < 0.01 vs group a; ##: P < 0.01 vs group b.

Expression of transgenes encoding visible products in living animal has been considered as more convenient method in the evaluation of expression intensity and duration. To address this issue, the far-red fluorescent protein E2-Crimson expressed by the plasmid pSC-E2 was detected in a certain period of time using an in vivo imaging system to monitor the transgene expression level in TA muscles. After a single injection, expression levels of the E2-Crimson were examined at Day 7 and 14 (Figure 6). By comparing the intensity and stability of the fluorescent signals in the 4 groups, the tendency about their effects can be summarized as follow: pDNA/rL_{2}PL_{2}(0.005%) > pDNA/L64(0.1%) > pDNA/rL_{3}PL_{3}(0.02%) > naked DNA. Signals in pDNA/rL_{2}PL_{2}(0.005%) group were strong and lasted for at least 14 days without visible attenuation. It indicated the potential of rL_{2}PL_{2} as an efficient material capable of delivering gene (pDNA) into skeletal muscle for therapeutic application.
**Figure 6.** In vivo imaging of the far-red fluorescent protein expression at Day 7 (left) and 14 (right) after administration. pDNA: naked pDNA; L64: pDNA/L64(0.1%); rL₂PL₂: pDNA/rL₂PL₂(0.005%); rL₃PL₃: pDNA/rL₃PL₃(0.02%).

**Functional Gene Expression Assay.**

It has been a promising strategy to express therapeutic molecules for the treatment of many diseases by using intramuscular administration of pDNA, even though most of the trials have been focused only on the reporter genes so far. When it is for therapeutic purposes, the absolute amount of the expressed molecules have to reach a high level that can meet the required concentration either in a local tissue or the circulatory system in order to keep their therapeutic functions. Gene delivery material has to be given prime attention, considering the role it plays in this process as one of the most important determinants. As the expression of functional gene has been mostly evaluated by its biological or therapeutic effects, and unlike reporter gene whose expression could be based on simply data comparison, it would be more convincible if we
use functional gene to investigate the applicable potential of these materials. Growth-hormone-releasing hormone (GHRH) was used as the functional gene in this study as it has a significant physiological effect on the progress of mouse growth. A single injection of 50 μg plasmid pSC-GHRH had been administrated, and then the body weights of mice in different groups were recorded. Compared to mice in the saline group, which were considered as natural growth, mice from the pSC-GHRH/rL2PL2(0.005%) group showed an accelerated growth rate for 21 days after the intramuscular injection (Figure 7). When compared with the naked DNA and pDNA/L64(0.1%) groups, the pDNA/rL2PL2 treatment also contributed a significant increase to mouse growth. But such an increase was not obtained in the pDNA/rL3PL3(0.02%) group. This means the expression amount of GHRH upon a pDNA/rL2PL2(0.005%) injection was high enough for physiological effects. It is also conceivable that some therapeutic effects might be acquired if the suitable therapeutic gene(s) instead of GHRH had been administrated here.

Figure 7. Average animal body weights after the injection of saline, naked pDNA, pDNA/L64(0.1%), pDNA/rL2PL2(0.005%) and pDNA/rL3PL3(0.02%), respectively. n =
6/group. 50 μg pSC-GHRH plasmid per mouse. *: P < 0.05 vs pDNA; #: P < 0.05 vs pDNA/L64(0.1%).

From the acquired data, it can be found that the different capabilities of rL2PL2 and rL3PL3 on both reporter and functional gene expressions mainly derive from their different surface charges (3.39 mV vs 8.54 mV) and molecular architectures. Firstly, the higher surface charge of rL3PL3 (8.54 mV) will result in easier and stronger interactions between rL3PL3 and negatively charged molecules in the muscles. These interactions may consequently interfere and weaken the interaction of the copolymer with cell membranes. We have revealed that Pluronic L64 could exert a membrane-disturbing effect on and thus facilitate the gene transfer through the cell membrane, so we would like to consider that rL2PL2 and rL3PL3 may have an analogous mechanism because they share the same amphiphilic structure with L64 and biomembranes. Additional perfect examples about the influence of surface charge on gene delivery were previous findings that intramuscular gene delivery and expression trials were usually greatly depressed by cationic polymers and lipids. Secondly, the different capabilities of rL2PL2 and rL3PL3 may also derive from their different molecular architectures. As the optimal concentrations of the two copolymers were below their CMCs, they were unimers instead of micelles upon injection into the muscles. The initial parts of the copolymer unimers interacting with cell membranes should be their outer hydrophilic parts, that is, G3 for rL3PL3 or G2 for rL2PL2. Because the dendron G3 has a more complicated structure than G2, we might image that it should be more difficult for rL3PL3 to disturb and even penetrate cell membranes. That means the architecture of rL3PL3 may be beyond the fitness for biomembranes whereas rL2PL2 has a more suitable one. In addition, rL2PL2 had better performance than L64. This may be mainly due to their difference in the outer hydrophilic units. The dendron G2 wings in
rL_{2}PL_{2} are bigger than the linear PEOs in L64, which may give an explanation of why rL_{2}PL_{2} could have more active and dynamical function on the cell membrane than PEOs. These potential mechanisms are still under investigation.

**CONCLUSIONS**

Neutral polymers had much better performance than cationic polymers and lipids in intramuscular gene delivery and expression trials. In this study, two amphiphilic triblock copolymers rL_{2}PL_{2} and rL_{3}PL_{3} were designed, synthesized and electroneutralized. Although they shared the same hydrophilic–hydrophobic–hydrophilic structure with Pluronic L64, the three copolymers had different intramuscular gene delivery capabilities. rL_{2}PL_{2} had best performance in both reporter and functional gene delivery and expression assays among the three copolymers. In comparison, it seems that rL_{2}PL_{2} possessed more suitable parameters for intramuscular gene delivery, such as the surface charge, molecular weight and structure of the outer hydrophilic unit, and the whole architecture of the copolymer. In addition, the dendritic peptides that made rL_{2}PL_{2} and rL_{3}PL_{3} have good biocompatibility in local injection and systemic administration. The most exciting result was that functional gene expression mediated by rL_{2}PL_{2} had exhibited significant biological effects on mice, which indicates an applicable potential of rL_{2}PL_{2} on plasmid-based gene therapy. In combination with previous study,^{24} we concluded that both types of copolymers with their architectures either reverse or similar to the biomembrane are effective in intramuscular gene delivery and expression. Our exploratory work has introduced an appealing way to design molecules that are safe, efficient and have potent intramuscular gene delivery capability. It also displayed the importance of structure and charge of the molecule for its intramuscular gene delivery function.

**ASSOCIATED CONTENT**
Supporting Information.

$^1$H NMR spectra of G3(DMSO-$_d_6$), AE (CDCl$_3$), AE-CDI (CDCl$_3$), G3-PPG-G3 (DMSO-$_d_6$), NH$_2$-PPG-NH$_2$(CDCl$_3$),DHPA (CDCl$_3$), DHPA-NHS (CDCl$_3$) and rL$_3$PL$_3$(CDCl$_3$), and mass spectrum of G3.

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The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.‡These authors contributed equally.

Notes

The authors declare no competing financial interest.

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