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Electroneutralized Amphiphilic Triblock Copolymer with a Peptide Dendron for Efficient Muscular Gene Delivery

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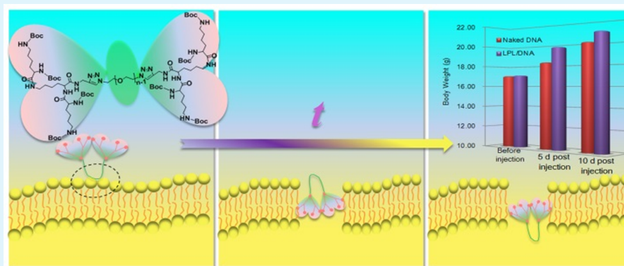
Ⓢ *Supporting Information*

ABSTRACT: Hydrophilic−hydrophobic−hydrophilic triblock copolymers, such as Pluronic L64, P85, and P105, have attracted more attention due to their enhancement in muscular gene delivery. In the present study, a new kind of electroneutralized triblock copolymer, LPL, dendron G2(L-lysine-Boc)-PEG_{2k}-dendron G2(L-lysine-Boc), was designed and investigated. This hydrophobic−hydrophilic−hydrophobic copolymer is composed of a structure reverse to that of L64, one of the most effective materials for intramuscular gene delivery so far. Our results showed that LPL exhibited good *in vivo* biocompatibility after intramuscular and intravenous administration. LPL mediated higher reporter gene expression than L64 in assays of β-galactosidase (LacZ), luciferase, and fluorescent protein E2-Crimson. Furthermore, LPL-mediated mouse growth hormone expression significantly accelerated mouse growth within the first 10 days. Altogether, LPL-mediated gene expression in skeletal muscle exhibits the potential of successful gene therapy. The current study also presented an innovative way to design and construct new electroneutralized triblock copolymers for safe and effective intramuscular gene delivery.



KEYWORDS: *gene delivery, amphiphilic, electroneutralization, triblock copolymer, pluronics*

INTRODUCTION

In gene therapy trials, the skeletal muscle has been an attractive target for the delivery of functional genes because myocytes can act as the "factory" to express and secrete proteins for therapeutic purpose.[1] Compared with a viral vector-based method, intramuscular injection of plasmid DNA (pDNA) is a safer, cheaper, and easier alternative for gene therapy. However, it usually failed to give satisfying therapeutic results due to the low gene delivery efficiency and expression level. The bottleneck in the pDNA-based gene therapy system is still the gene transfer efficiency governed by gene delivery materials.

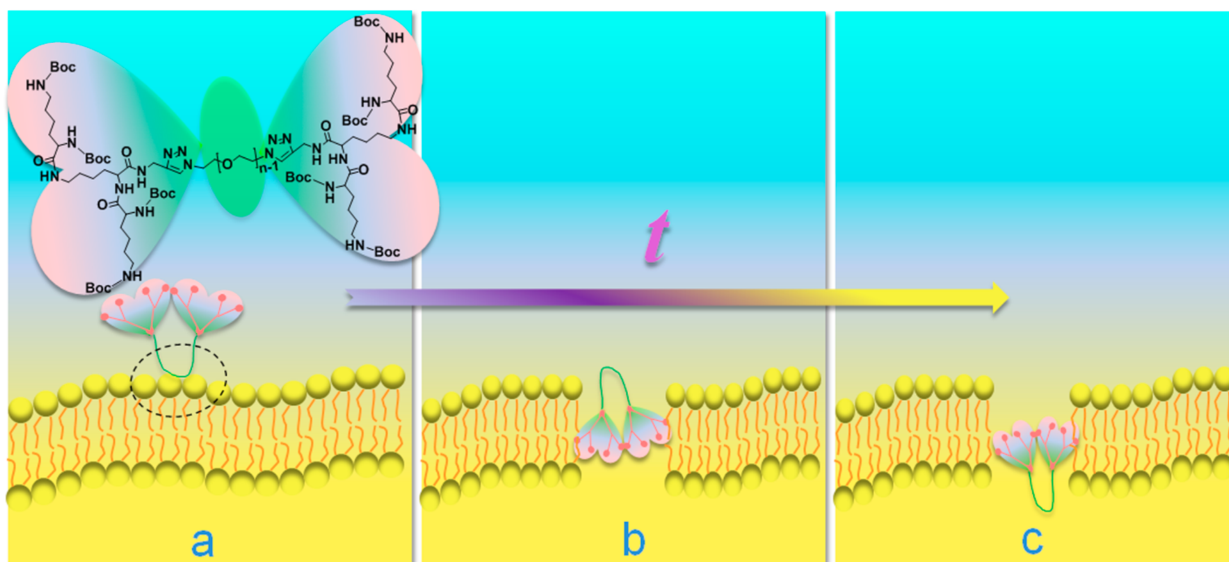
It is well known that many gene delivery materials, such as cationic polymers and lipids, can efficiently deliver nucleic acids into cultured cells.[2−4] The cationic materials, however, usually had worse results than naked pDNA in intramuscular gene delivery trials.[5−8] This may be due to their interaction with the negatively charged extracellular matrix in the muscles.[9] It was found that some neutral polymers such as poly(*N*-vinylpyrrolidone), poly(vinyl alcohol), and Pluronics are able to enhance muscular gene delivery. Several kinds of Pluronics, such as L64, P85, and P105, have attracted more attention in muscular gene transfer due to their better performance than other polymers.[10−16] Among them, L64 was reported to have more stable performance than its companions.[16] Pluronics are amphiphilic neutral copolymers with hydrophobic propylene oxide (PO) and hydrophilic ethylene oxide (EO) blocks arranged in a basic structure: EO_x-PO_y-EO_x. The numbers of EO and PO units are variable.[17] Different Pluronics have different structural parameters and physicochemical properties. The cell membrane is basically constituted by phospholipid pairs arranged in a "hydrophilic−hydrophobic−hydrophilic" sandwich-like structure because the exterior polar heads are hydrophilic, while the inner nonpolar fatty acid chains are hydrophobic. Therefore, Pluronic molecules share the analogous structure with the phospholipid pairs in the cell membrane. It has been considered that, on the basis of the structural similarity, Pluronics were able to interact with the cell membrane and disturb its integrity, leading to increased permeability on the membrane which would facilitate the entrance of nucleotides into cells.[18] This may be the reason why Pluronics can promote gene delivery efficiency into the skeletal muscle.

Previous studies have shown that the neutral and amphiphilic copolymers were hopeful materials for intramuscular gene delivery. We supposed that copolymers with the structure reverse to phospholipid pairs in the cell membrane (abbreviated as R-membrane), i.e., "hydrophobic−hydrophilic−hydrophobic" structure, should have different methods of interaction with the cell membrane as compared with that of Pluronics. The interaction between the copolymers and cell membrane was mainly driven by the hydrophilic−hydrophobic effect. In a Pluronic molecule, the hydrophobic PO fragment could insert

Received: June 17, 2014
Accepted: August 15, 2014
Published: August 15, 2014

 © 2014 American Chemical Society dx.doi.org/10.1021/am503808b | *ACS Appl. Mater. Interfaces* 2014, 6, 15344−15351

Scheme 1. Hypothetical Model of the Interaction between the R-Membrane Copolymer Molecule and Cell Membrane[a]



[a]The hydrophilic region interacted first with the external hydrophilic side of the cell membrane (a). Thereafter, the flowable membrane components were squeezed by the R-membrane copolymer, and the inner hydrophobic fatty acid chains were exposed, followed by the entrance of the hydrophobic parts of the copolymer (b). When the hydrophilic region of the copolymer was pushed by the hydrophobic fatty acid chains, the copolymer molecule might be turned over to make its hydrophilic component close to the inner side of plasma membrane (c). t: time.

into the interior hydrophobic region, while the hydrophilic EOs interact with both sides of the cell membrane so that a part of the Pluronic molecules could stably integrate into the membrane. In the R-membrane copolymer, the hydrophilic region was thought to interact first with the external hydrophilic side of the cell membrane (Scheme 1a). Thereafter, the flowable membrane components were squeezed by the R-membrane copolymer, and the inner hydrophobic fatty acid chains were exposed, followed by the entrance of the hydrophobic parts of the copolymer (Scheme 1b). When the hydrophilic region of the copolymer was pushed by the hydrophobic fatty acid chains, the copolymer molecule might be turned over to make its hydrophilic component close to the inner side of plasma membrane (Scheme 1c). The hypothetical interaction model showed that R-membrane copolymers had more active and dynamic influence on the cell membrane than Pluronics. For this reason, we are interested in knowing if the R-membrane triblock copolymers could give excellent performance in intramuscular gene delivery.

To address this point, the R-membrane copolymer molecules should be rationally designed with regard to the components, structure, molecular weight, etc. Polypeptide dendrimers have been confirmed to be excellent gene delivery materials due to their superior characteristics, such as well-defined structures, controllable surface functionalization, and good biocompatibility.[19,20] Poly(ethylene glycol) (PEG) has been widely used as the component of drug carriers and gene delivery materials because of its high solubility in water and good biocompatibility.[21,22] Here, we designed a new amphiphilic triblock copolymer, dendron G2(L-lysine-Boc)-PEG$_{2k}$-dendron G2(L-lysine-Boc) (LPL). The propylene oxide units used in Pluronics were replaced by the dendron G2(L-lysine-Boc) because the latter was safer for *in vivo* applications. This LPL molecule had an R-membrane structure and was neutralized by protecting the terminal amino groups. We hope the LPL copolymer could give satisfied results on gene delivery into skeletal muscles through an optimized gene transfer procedure.

EXPERIMENTAL SECTION

Materials and Characterization. Boc-Lys(Boc)-OH, *N*,*N*-diisopropylethylamine (DIPEA), 1-hydroxybenzotriazole (HOBT), trifluoroacetic acid (TFA), *N*,*N*,*N*′,*N*′-tetramethyl-*O*-(1*H*-benzotriazol-1-yl) uronium hexafluorophosphate (HBTU) were purchased from Gil Biochem. Co. Ltd. (Shanghai, China). Propargylamine were purchased from Acros Organics (Geel, Belgium) and used without further purification. Sodium azide (NaN_3), sodium ascorbate, PEG (M_w = 2000), Pluronic L64 ($EO_{13}PO_{30}EO_{13}$; M_w = 2900), branched polyethylenimine (PEI; M_w = 25 kDa), and other chemicals were purchased from Sigma-Aldrich (St Louis, MO, USA) and used without further purification.

Characterization and structural confirmation of dendritic intermediates and products were measured by ^{1}HNMR (400 MHz Bruker Advanced 600 Spectrometer), electrospray ionization time-of-flight mass spectrometry (ESI-TOF MS, Waters Q-TOF Premier) and matrix assisted laser desorption ionization time-of-flight (MALDI-TOF, Autoflex MALDI-TOF/TOF) mass spectrometry. The Zeta potentials of LPL copolymers and L64 were measured using a Malvern Zeta sizer Nano ZS (Malvern Instruments).

Plasmids encoding the luciferase (pSC-Luc), *β*-D-galactosidase (pSC-LacZ), far-red fluorescent protein (pSC-E2), and mouse growth hormone (pSC-mGH) were previously constructed in our lab,[23] amplified in *Escherichia coli* DH5*α*, and extracted using the EndoFree Plasmid Kit (Invitrogen, Carlsbad, CA,USA).

Synthesis of Comp-1 (G1-2NHBoc-alkynyl). Boc-Lys (Boc)-OH (1.47 g, 4.2 mmol), HOBT (0.76 g, 4.2 mmol), and HBTU (1.84 g, 4.2 mmol) were dissolved in anhydrous DMF (30 mL). The above solution was cooled to 0 °C in an ice bath, and DIPEA (4.0 mL, 4.2 mmol) was added under nitrogen. The mixture was stirred for 0.5 h at 0 °C, and then, propargylamine (0.4 mL) was added, followed by stirring for an additional 24 h at room temperature. After the reaction, the mixture was diluted with a large quantity of ethyl acetate and then sequentially washed with saturated sodium bicarbonate solution (3×), 1 M hydrochloric acid (3×), and saturated sodium chloride solution. The organic layer was collected and dried with anhydrous magnesium sulfate. After concentration and recrystallization with *n*-hexane, and

drying under vacuum, a white solid was obtained. ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.44 (s, 1H, N*H*CO), 5.09 (s, 1H, bocN*H*), 4.59 (s, 1H, bocN*H*), 4.04 (s, 3H, CHCC*H*$_2$, NHC*H*CO), 3.11 (s, 2H, NHC*H*$_2$), 2.22 (s, 1H, C*H*C), 1.91−1.53 (m, 4H, NHCH$_2$C*H*$_2$, CHC*H*$_2$), 1.44 (s, 18H, boc-C*H*$_3$), 1.38 (d, 2H, CH$_2$C*H*$_2$CH$_2$). ESI-MS (*m/z*): 384.23, calcd for [M + H$^+$] (*m/z*), 383.24, Anal. Calcd for $C_{19}H_{33}N_3O_5$.

Synthesis of Comp-2 (G2-4NHBoc-alkynyl). Under a nitrogen atmosphere, G1-2NHBoc-alkynyl (0.8 g, 2.1 mmol) was dissolved in anhydrous dichloromethane (3.2 mL). The above solution was cooled to 0 °C in an ice bath, and trifluoroacetic acid (3.2 mL, 42 mmol) was added, followed by stirring for 8 h at room temperature. After the reaction, solvents and trifluoroacetic acid were removed by rotary steam. Then, the solid was dissolved in anhydrous DMF (15 mL). Boc-Lys (Boc)-OH (1.73 g, 5 mmol). HOBT (0.95 g, 7 mmol), and HBTU (2.31 g, 7 mmol) were mixed, and anhydrous DMF (10 mL) was added. The above solution was cooled to 0 °C in an ice bath, and DIPEA (5.0 mL, 30 mmol) was added under nitrogen. The mixture was then stirred for 24 h at room temperature. After the reaction, the mixture was diluted with a large quantity of ethyl acetate and then washed with saturated sodium bicarbonate solution (3×), 1 M hydrochloric acid (3×), and saturated sodium chloride solution in proper sequence. The organic layer was collected and dried with anhydrous magnesium sulfate. After concentration and recrystallization with *n*-hexane, and then drying under vacuum, a white solid was obtained. ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.93 (s, 1H N*H*CO), 5.91 (s, 1H, N*H*CO), 5.56 (s, 1H, N*H*CO), 4.55 (d, 7H, bocN*H*, NHC*H*CO), 4.02 (s, 2H, CHCC*H*$_2$), 3.10 (t, 6H, NHC*H*$_2$), 2.23 (s, 1H, C*H*C), 1.51 (s, 12H, NHCH$_2$C*H*$_2$, CHC*H*$_2$), 1.43 (d, 36H, boc-C*H*$_3$), 1.33 (d, 6H, CH$_2$C*H*$_2$CH$_2$). ESI-MS (*m/z*): 840.56, calcd for [M + H$^+$] (*m/z*), 839.54, Anal. Calcd for $C_{41}H_{73}N_7O_{11}$.

Synthesis of Comp-3 (MsO-PEG-OMs). PEG2000 (2.76 g, 2.76 mmol of OH) was dissolved in anhydrous dichloromethane (30 mL) containing triethylamine (1.9 mL, 13.8 mmol). The flask was cooled to 0 °C in an ice bath, and 1.1 mL (13.8 mmol) of methanesulfonyl chloride was added dropwise over 10 min under nitrogen.[24] The mixture was stirred under nitrogen in the ice bath for about 1 h and then stirred for an additional 3.5 h at room temperature. After the reaction, the mixture was washed twice with saturated sodium bicarbonate solution. The organic layer was collected, dried with anhydrous magnesium sulfate, precipitated twice into cold diethyl ether, and dried under vacuum, and then a pale yellow solid was obtained. ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.48 (4H, m, C*H*$_2$OMs), 3.87 (m,C*H*$_2$CH$_2$OMs), 3.65−3.75 (176H, m, C*H*$_2$C*H*$_2$O), 3.25 (6H, s,C*H*$_3$SO$_2$O).

Synthesis of Comp-4 (N_3-PEG-N_3). MsO-PEG-OMs (2.15 g, 1 mmol) were dissolved in anhydrous DMF (40 mL), and sodium azide (0.65 g, 10 mmol) was added. The mixture was stirred under nitrogen at 40 °C for 48 h. The product was precipitated twice in cold diethyl ether, with redissolution in dichloromethane after each precipitation. The solid precipitate was collected by vacuum filtration and dried in a vacuum oven. ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.65−3.75 (168H, m, C*H*$_2$C*H*$_2$O), 3.50 (4H, m, C*H*$_2$N$_3$).

Synthesis of LPL. The peptide G2-4NHBoc-alkynyl (0.3 g, 0.36 mmol) and the azide N_3-PEG-N_3 (0.3 g, 0.15 mmol) were dissolved in 22 mL of DMF and 7 mL of water to form a solution mixture. $CuSO_4\cdot 5H_2O$ (0.2 equiv) and sodium ascorbate (1.0 equiv) were added to the solution.[25] The resulting solution was stirred for 36 h at room temperature under argon. At the end of the reaction, the rubber septum was removed under stirring, leading to oxidation of Cu (I) catalyst into Cu (II). Then, the catalyst was removed by dialysis against water in a Spectra/Por Biotech MWCO 1000 cellulose ester membrane (Spectrum Laboratories) for 4 days, frozen at −80 °C, and lyophilized to obtain a fluffy, pale yellow solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.87 (s, 1H N*H*CO), 6.76 (s, 1H N*H*CO), 5.91 (s, 2H, N*H*CO), 5.51 (s, 2H, N*H*CO), 5.21−4.14 (m, 14H, bocN*H*, NHC*H*CO), 4.02 (s, 4H, CHCC*H*$_2$), 3.70−3.58 (m, 76H, C*H*$_2$C*H*$_2$O), 3.47−2.77 (m, 12H, NHC*H*$_2$), 2.22 (t, 2H, C*H*C), 1.92−1.64 (m, 24H, NHCH$_2$C*H*$_2$, CHC*H*$_2$), 1.63−1.32 (m, 72H, boc-C*H*$_3$), 1.30 (d, *J* = 40.0 Hz, 12H, CH$_2$C*H*$_2$CH$_2$).

DNA Retardation Assay. One microgram of pDNA and different amounts of LPLs were diluted in 10 μL of 0.9% saline, mixed, and incubated for 30 min at room temperature. The LPL/pDNA mixtures with various mass ratios were run on 1% (w/v) agarose gel at 80 V for 1 h. The gel was stained with ethidium bromide (EB), and the mobility of pDNA bands was visualized and photographed in an imager (Bio-Rad ChemiDoc XRS+, USA).

Animal Treatment and Intramuscular Gene Transfer. Animals were cared in compliance with the national and local animal welfare rules, and experiments were approved by the local ethics committee (Animal Care and Use Committee of Sichuan University). BALB/c mice (6-week-old males; from Experimental Animal Centre of Sichuan Province) were used to evaluate the *in vivo* gene delivery ability of LPL. For intramuscular injection, mice hindlimbs were shaved and depilated with hair removal cream. Ten micrograms of pDNA (pSC-Luc, pSC-LacZ, or pSC-E2) in 40 μL of solution with or without material was injected into the middle of a tibialis anterior (TA) muscle in 2−3 s using a 29-gauge BD Ultra-Fine insulin syringe (BD, Franklin Lakes, NJ, USA). For the pSC-mGH plasmid, 50 μg of pDNA in 80 μL of solution was averagely divided into two parts and injected into both TA muscles of a mouse.

***In Vivo* Assays of Reporter Gene Expressions.** Mice were sacrificed, and the TA muscles were separated 4 days after treatment. For the β-galactosidase activity assay, muscles were fixed in the freshly prepared fixative (2% paraformaldehyde and 0.2% glutaraldehyde in PBS) for 1 h on ice and rinsed 3 times with PBS at room temperature. The fixed samples were then stained using an *In Situ* β-Galactosidase Staining Kit (Beyotime, Shanghai, China) and photographed with a digital camera.

For the luciferase activity assay, each TA muscle was weighed and homogenized in 1 mL of ice-cold reporter lysis buffer (Promega, Madison, WI, USA) using a T10 ULTRA-TURRAX homogenizer (IKA-werke, Baden Wuerttemberg, Germany). The homogenized samples were frozen at −80 °C overnight, thawed, and centrifuged at 12,000*g* for 5 min at 4 °C. Twenty microliters of the supernatant of each sample was added in a 96-well plate, mixed with 100 μL of luciferase substrate (Promega, Madison, WI, USA), and the luciferase activity was detected by measuring the light emission for 10 s in a spectral scanning multimode reader (Varioskan Flash; Thermo Fisher). The luciferase activity was normalized to total protein concentration in the lysate supernatant measured with a BCA Protein Assay Kit (Pierce, Rockford, IL, USA) and presented as the Relative Light Unit (RLU) per muscle.

Imaging of Fluorescent Protein Expression in Mice. The plasmid pSC-E2 was injected into the TA muscles in both hindlimbs of a mouse. At days 7 and 14 after the injection, mice were anesthetized with 300 mg/kg chloral hydrate, and their TA muscles were completely depilated. The locally expressed far-red fluorescent protein E2-Crimson was detected in EX In-Vivo Imaging System (CRI Maestro, Boston, USA). Mice were placed on a 37 °C platform, illuminated by yellow light, and scanned ranging from 600 to 700 nm in the 25 °C environment. Image acquirement and picture treatment were performed using the built-in software system with unified parameters throughout the experiment.

Histological Section Preparation and Analysis. Mice were sacrificed 24 h after an intramuscular injection of a 40 μL solution of 0.9% saline or 0.0075% LPL. The TA muscles were separated, fixed in 4% fresh formaldehyde solution for 24 h, dehydrated with gradient concentrations of ethanol, and embedded in paraffin. Both cross- and longitudinal-sections in the same TA muscle were sliced with 10 μm thickness and stained with hematoxylin and eosin. Tissue sections were photographed (Leica DMI 4000B; Wetzlar, Germany) for histopathological analysis.

Serum Biochemical Test. Mice were injected with 200 μL of solution of saline or 0.0075% LPL through tail veins. Four days later, blood was collected, and sera were separated by centrifugation at 3000 rpm for 10 min at 4 °C. Sera biochemical tests were analyzed using the Analyzer Medical System AUTOLAB (AMS, Italy).

Statistical Analysis. Data were presented as the mean ± standard deviation (SD). Comparisons among groups were analyzed by the *t*

Scheme 2. Synthesis Routes for (A) Comp-1 and Comp-2, (B) Comp-3 and Comp-4, and (C) LPL

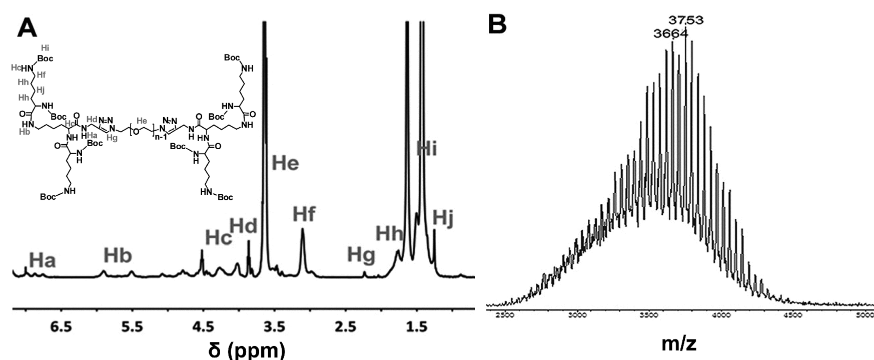


Figure 1. ^{1}H NMR spectrum (A) and MALDI-TOF mass spectrum (B) of LPL in $CDCl_3$.

test. The value of $P < 0.05$ was considered as significantly different, while $P < 0.01$ was highly significant.

■ RESULTS AND DISCUSSION

Molecular Design and Synthesis of LPL. Previous studies on Pluronics showed that some key characteristics might influence the local gene transfer ability, such as the hydrophilic/lipophilic balance (HLB), polymerization degree of the EO and PO units, length of PEO, PPO, and the whole molecule, architecture of the whole molecule, critical micelle concentration (CMC), and critical micelle temperature (CMT). However, it is still unable to establish the theory to guide the molecular design. It is also difficult to predict the ability of a synthesized molecule. Recently, a copolymer with structure reverse to Pluronics, 25R2, was reported to have the ability to transfer genes into mouse TA muscle. Its gene transfer activity was similar to that of Pluronic P105[26] and L64,[16] and its biocompatibility was not displayed. However, this single

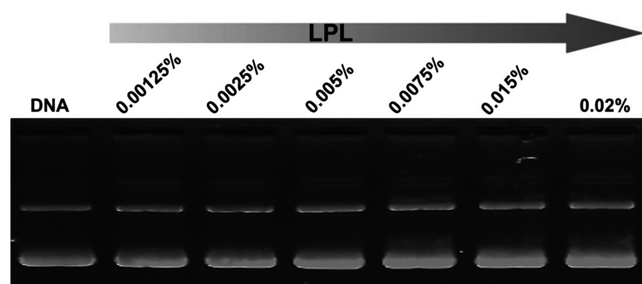


Figure 2. DNA retardation assay. Lane 1, naked pDNA; lanes 2−7, the LPL/pDNA mixtures with increasing mass ratios (m/m %).

successful case still gave us confidence to synthesize the new R-membrane copolymers for efficient local gene delivery. We considered that the length, shape, and size of the whole copolymer molecule and its components should match the architecture of the cell membrane to a certain extent. The oversized copolymer molecules should have a difficult time entering the cell membrane, whereas the undersized ones have limited action on the cell membrane. Here, the Boc-protected dendron G2(L-lysine) was used as the hydrophobic units and PEG2000 as the hydrophilic units due to the suitable size, good biocompatibility, and simplicity for synthesis. The LPL molecule was expected to work well in gene delivery trials.

The triblock copolymer was synthesized using the liquid-phase peptide synthesis method according to Scheme 2. Each synthesis step was monitored by the ninhydrin test, ^{1}H NMR, and MALDI-TOF-MS. The data analyses of ^{1}H NMR and mass spectra are listed in Experimental Section. The mass spectra of Comp-1 (Figure S2A, Supporting Information) and Comp-2 (Figure S2B, Supporting Information) were equal to the theoretically obtained molecular weights. Good correspondences were found between the theoretical and experimental values for all the synthesized components. From the results of ^{1}H NMR (Figure S1 (Supporting Information) and Figure 1A) and mass spectra (Figure S2 (Supporting Information) and Figure 1B), it was confirmed that the synthesis was completed. In the MALDI-TOF mass spectrum of LPL, due to the presence of PEG, each peak appeared discretely according to its degree of polymerization. The detected and theoretical values of the molecular weight of LPL were well matched (Figure 1B).

DNA Retardation Assay and Zeta Potential. Previous studies already confirmed that cationic gene delivery materials usually presented negative effects on local gene transfer into skeletal muscles, whereas electroneutral materials, such as Pluronics (L64, P85, P105, etc.) and PVP, were able to facilitate *in situ* gene delivery. Therefore, it should be investigated whether the synthesized LPL molecules were neutralized as designed. The DNA retardation assay has been commonly used to detect if the molecules can interact with DNA, as their combination, either through a physical or chemical manner, will inhibit DNA migration in agarose gel electrophoresis. Theoretically, once terminal NH_2 groups in the dendron G2 L-lysine were completely protected by the Boc groups, the whole LPL molecule should be neutralized so that it will not interact with negatively charged DNA molecules. As shown in Figure 2, DNA bands in the gel were completely identical in migration and strength, indicating that LPL does not bind with DNA molecules, even at the highest concentration. The zeta potentials also confirmed that L64 and LPL molecules were almost electroneutral, regardless of the concentrations of LPL solutions (Table 1).

***In Vivo* Biocompatibility Assays of LPL.** To study the biocompatibility of our copolymer, cross- and longitudinal-sections from the injected TA muscles were prepared for histopathological analysis. The sections showed no difference between 0.9% saline (Figure 3a and c) and 0.0075% LPL (Figure 3b and d) groups. There were no pathological changes

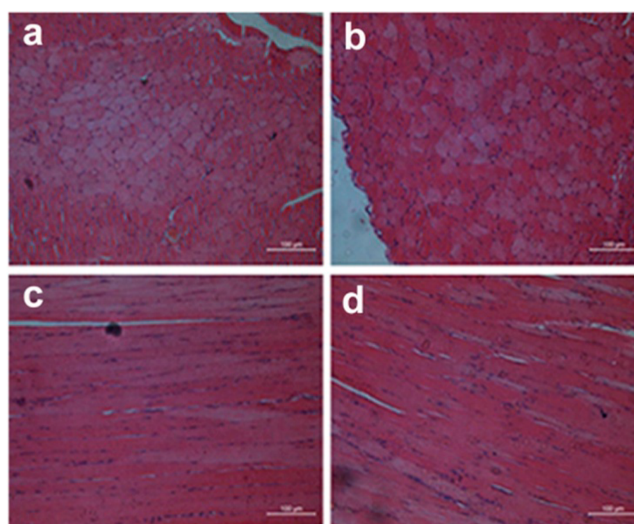


Figure 3. Representative HE-stained tissue sections in TA muscles injected with saline (a and c) and 0.0075% LPL (b and d). a and b, cross-sections; c and d, longitudinal sections.

Table 2. Serum Biochemical Test of Mice Injected with Saline and LPL[a]

items		saline	LPL
liver function	ALT (U/L)	36.01 ± 3.46	36.67 ± 2.53
	AST (U/L)	111.67 ± 11.94	102.02 ± 11.39
	ALP (U/L)	184.01 ± 13.65	192.67 ± 15.63
	GGT (U/L)	2.67 ± 0.58	3.05 ± 0.52
	TP (g/L)	36.55 ± 2.77	41.47 ± 3.23
	ALB (g/L)	24.77 ± 0.95	24.9 ± 1.11
	TBIL (μmol/L)	22.9 ± 1.34	30.03 ± 1.88
	DBIL (μmol/L)	3.66 ± 0.35	3.24 ± 0.26
renal function	BUN (mmol/L)	6.61 ± 0.47	7.17 ± 0.36
	CRE (μmol/L)	43.33 ± 2.15	50.13 ± 3.57
blood glucose	GLU (mmol/L)	6.89 ± 0.57	6.16 ± 0.33

[a]Abbreviations: ALT, alanine amino transferase; AST, aspartate amino transferase; ALP, alkaline phosphatase; GGT, γ-glutamyl transpeptidase; TP, total protein; ALB, albumin; TBIL, total bilirubin; DBIL, direct bilirubin; BUN, blood urea nitrogen; CRE, creatinine; GLU, glucose.

Table 1. Zeta Potentials of L64 and LPL

	L64	LPL					
materials	0.1%	0.00125%	0.0025%	0.005%	0.0075%	0.015%	0.02%
ζ-potential (mV)	−1.07	−2.16	−2.06	−0.236	−0.918	−1.43	−1.54

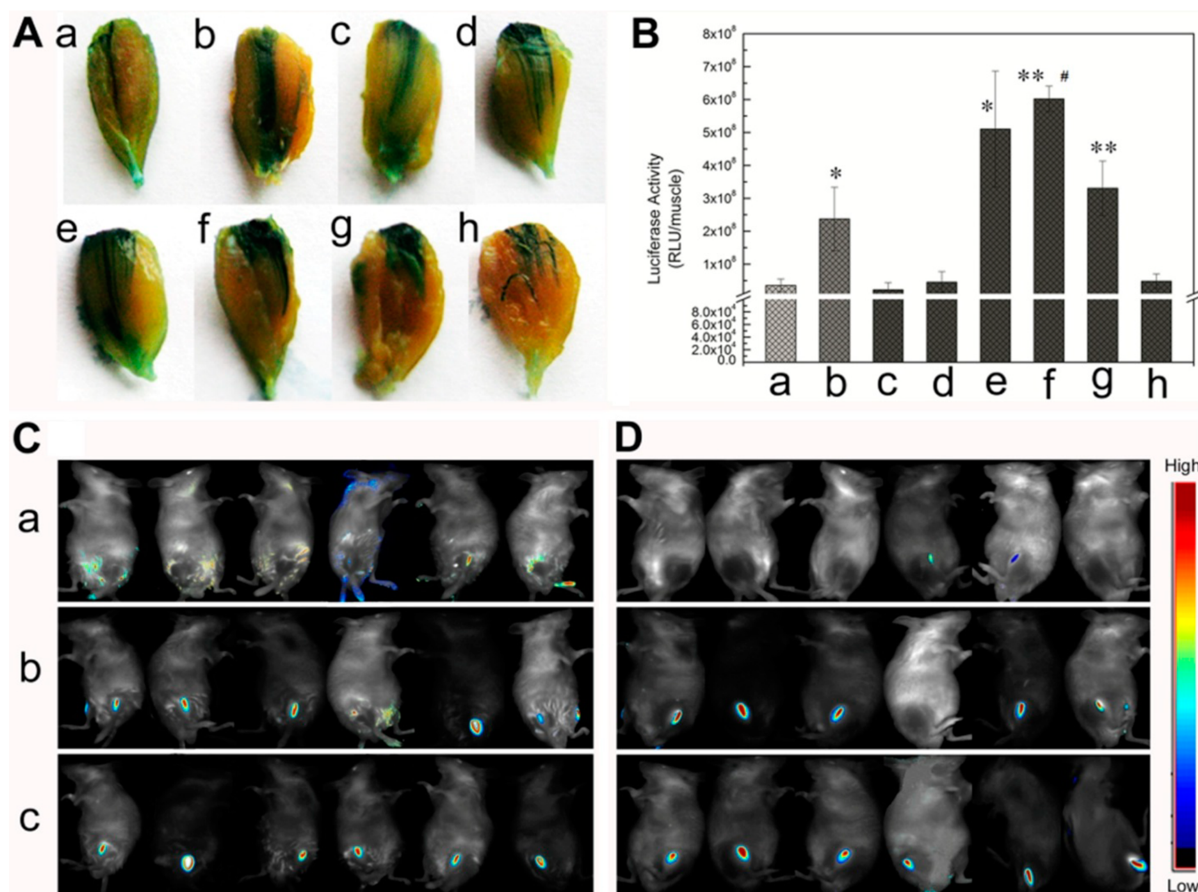


Figure 4. *In vivo* transgene expression assays. $n = 6$/group. A and B: (a) naked pDNA; (b) L64 (0.1%)/pDNA; (c−h) LPL/pDNA mixtures with various mass ratios (c, 0.00125%; d, 0.0025%; e, 0.005%; f, 0.0075%; g, 0.015%; and h, 0.02%). (A) Representative figures of β-galactosidase expression in mouse TA muscles. (B) Luciferase activity assay in mouse TA muscles. *, $P < 0.05$ vs group a; **, $P < 0.01$ vs group a; #, $P < 0.05$ vs group b. C and D: *In vivo* imaging of the far-red fluorescent protein expression at days 7 (C) and 14 (D) after administration. Row a, naked pDNA; b, pDNA/L64(0.1%); and c, pDNA/LPL(0.0075%).

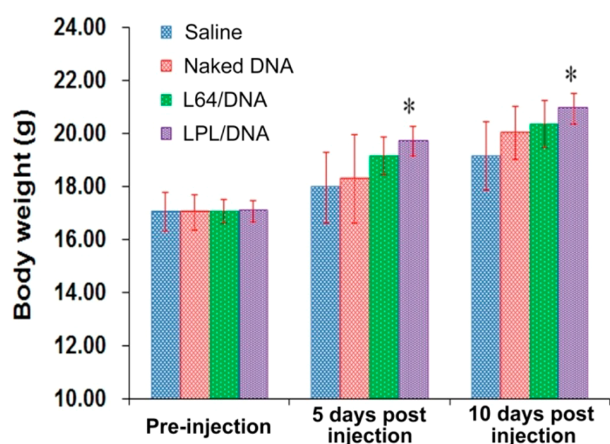


Figure 5. Average animal body weights after an injection of saline, naked pSC-mGH, pSC-mGH/L64(0.1%), or pSC-mGH/LPL(0.0075%). $n = 6$/group; 50 μg of pSC-mGH plasmid per mouse. *: $P < 0.05$ vs the saline group.

such as inflammatory reaction or necrosis focus. It indicated that our LPL copolymer was safe for local administration into skeletal muscles. Furthermore, the sera biochemical tests were performed to evaluate the biosafety of the LPL when 200 μL of 0.0075% LPL solution was systematically administrated through intravenous injection into a mouse. The commonly used clinical indexes for liver function, renal function, and blood glucose were detected. The data did not show any obvious difference between 0.9% saline and 0.0075% LPL groups (Table 2). It further confirmed the good biocompatibility of the LPL copolymer.

Reporter Gene Delivery and Expression via Intramuscular Injection. Herein, the reporter gene expressions via LPL-mediated intramuscular injection were tested qualitatively and quantitatively. The β-galactosidase (LacZ) expression offered visible and fast evaluation of the level and spatial distribution of transgene expression in mice TA muscles. As the local gene transfer activity of 0.1% L64 was similar to but more stable than those of other Pluronics (0.05% P104, 0.05% P123, 0.1% P105, and 0.05% F127), we used 0.1% L64 as the positive control in this study. As shown in Figure 4A, L64(0.1%)/pDNA and LPL/pDNA mixtures with mass ratios from 0.00125% to 0.015% showed stronger LacZ expression than the naked pDNA group, evident from the color and distribution on the surfaces of stained muscles. Further analysis of the luciferase activity showed quantitative results in different groups (Figure 4B). In LPL/pDNA groups, the luciferase activities sharply rose along with the increase of mass ratios (from 0.00125% to 0.0075%) and then gradually decreased. Quantitatively, the RLU values were 3.55×10^7 in the naked pDNA group, 2.37×10^8 in the L64/pDNA group, and 6.02×10^8 in the LPL/pDNA (0.0075%) group. It indicated that

0.0075% LPL increased transgene expression to 17-fold that of naked DNA and 2.5-fold that of 0.1% L64. Considering that Pluronic L64 is among the most effective nonviral materials for local gene delivery, the R-membrane material LPL is well worth further exploration.

Visible transgene expression in living animals was considered to be more convenient for evaluating the expression intensity and duration. The plasmid pSC-E2, which expresses the far-red fluorescent protein E2-Crimson driven by an enhanced hybrid promoter and the *in vivo* imaging system were used to address the issue. Expressions of E2-Crimson were observed at days 7 (Figure 4C) and 14 (Figure 4D) after a single injection. There was no obvious difference of fluorescence intensity between the pDNA/L64(0.1%) and pDNA/LPL(0.0075%) groups, but 0.0075% LPL had more stable results than 0.1% L64 because all the 6 sites in the pDNA/LPL(0.0075%) group still showed obvious fluorescence at day 14, whereas 1 site lost a detectable signal in the pDNA/L64(0.1%) group. In the naked pDNA group, only 2 of 6 sites showed visible but weaker fluorescent signals compared with those in the other 2 groups. Meanwhile, the duration of fluorescent signals in the pDNA/LPL(0.0075%) group were sustained for at least 14 days without any attenuation.

Functional Gene Expression Assay. Intramuscular administration of pDNA to express therapeutic molecules has been an attractive strategy for the treatment of many diseases; however, most trials so far still focused on the expression of reporter genes. For therapeutic purposes, the absolute amount of expressed molecules should be high enough to meet the required concentration in local tissue or the circulatory system. To address this question, gene delivery material is one of the most important determinants. Unlike reporter gene expression trials that are based on data comparison, expression of a functional gene was usually evaluated by the biological or therapeutic results. Therefore, it is more convincing to investigate functional gene expression. It can evaluate the applicable potential of the LPL-mediated gene therapy system. Mouse growth hormone (mGH) has physiological function on mouse growth, so it was used as the functional gene. After a single administration of 50 μg of plasmid, mice in the pSC-mGH/LPL(0.0075%) group showed accelerated growth rate in the first 10 days, as indicated by body weights compared with those in the saline group (considered as natural growth) (Figure 5). This means that the expression amount of mGH upon pSC-mGH/LPL(0.0075%) treatment is high enough for physiological function. It can also be concluded that if the suitable therapeutic gene(s) instead of the mGH were used here, the therapeutic effects may be acquired.

■ CONCLUSIONS

In this study, an electroneutralized amphiphilic triblock copolymer LPL, dendron G2(L-lysine-Boc)-PEG-dendron G2(L-lysine-Boc), was designed and synthesized. It had a structure reverse that of the phospholipid pairs in the cell membrane and was considered to have more activities and dynamic influence on the cell membrane than the traditional Pluronic molecules. LPL had good biocompatibility in local muscles and whole bodies of mice. In local gene delivery trials, LPL showed better performance on reporter gene expressions than Pluronic L64. Moreover, LPL-mediated functional gene expression exhibited significant biological effects on mice, indicating the applicable potential of LPL. Our exploratory work presented an attractive way to design safe and efficient molecules with potent intramuscular gene delivery ability. However, this study is just the beginning, and many questions still remain with regard to the molecule design and biological/chemical mechanisms underlying LPL's performance. We are trying to explore the rules for molecule design through the synthesis of a series of LPL molecules containing different hydrophilic and hydrophobic units, and to establish more powerful LPL-mediated gene therapy systems capable of going through clinical trials.

■ ASSOCIATED CONTENT

Supporting Information

^{1}H NMR spectra of Comp-1, Comp-2, Comp-3, and Comp-4 in $CDCl_3$; and mass spectra of Comp-1 and Comp-2. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This work was supported by the National Basic Research Program of China (973 program, No. 2011CB606206), National Natural Science Foundation of China (No. 31370972, 51133004, and 81361140343), and National Support Program of Science and Technology (No. 2012BAI17B06).

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