Novel Symmetric Amphiphilic Dendritic Poly(L-lysine)-*b*-Poly(L-lactide)-*b*-Dendritic Poly(L-lysine) with High Plasmid DNA Binding Affinity as a Biodegradable Gene Carrier

Yang Li,[†] Liang Cui,[‡] Qiaobo Li,[†] Lin Jia,[†] Yuhong Xu,[‡] Qiang Fang,[†] and Amin Cao*[,†]

Laboratory for Polymer Materials, Shanghai Institute of Organic Chemistry, Chinese Academy of Sciences, 354 Fenglin Road, Shanghai 200032, China, and School of Pharmacy, Shanghai Jiaotong University, 800 Dongchuan Road, Shanghai 200240, China

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This study communicates the molecular design, preparation, and biological application of novel symmetric amphiphilic polycationic dendritic poly(L-lysine)-*b*-poly(L-lactide)-*b*-dendritic poly(L-lysine) **D_2-LLA_{15}-D_2** bearing two two-generation poly(L-lysine) **PLL** dendrons **D_2** and a central hydrophobic biodegradable poly(L-lactide) block **LLA_{15}**. First, an amino-protected precursor of **L_1-OH** was designed and synthesized and was further employed to prepare **L_1-LLA_{15}** with an organic 4-(dimethylamino)-pyridine-mediated living-ring-opening polymerization of L-lactide. Subsequently, the hydroxy end-capped **L_1-LLA_{15}** was coupled to synthesize a new triblock **L_1-LLA_{15}-L_1** with two one-generation amino-protected **PLL** dendrons **L_1**. Furthermore, with a repeated trifluoroacetic-acid-mediated amino deprotection−protection cycle, new amphiphilic triblock **D_2-LLA_{15}-D_2** was successfully prepared. By means of NMR, mass spectrometry, and gel permeation chromatography, these synthetic precursors and final amphiphilic product were characterized to bear well-defined triblock structures. In addition, this synthesized amphiphilic triblock polycationic macromolecule was applied as a new polycationic plasmid DNA carrier, and its DNA binding affinity was examined via an agarose electrophoresis and a fluorescence titration assay along with two important references of hydrophilic dendritic **D_2-HEX-D_2** and double-hydrophilic **D_2-PEG-4K-D_2** bearing the same two **D_2** dendrons; much enhanced DNA binding affinity was interestingly revealed for the new amphiphilic structural **D_2-LLA_{15}-D_2**. Moreover, the assembled polyplex microparticles of plasmid DNA/polycationic carrier were further analyzed by dynamic light scattering and transmission electron microscopy, indicating their averaged nanoparticle size around 150−200 nm. As for the cytotoxicity of the new **D_2-LLA_{15}-D_2**, MTT assays were conducted with a human hepatocellular carcinoma cell line (SMMC-7721), indicating a very low cytotoxicity as compared with commercial linear **PLL-23K** and **PEI-2K**, and a DNase I degradation of the assembled polyplex particles was also done in the HBS buffer solution to evaluate their stabilities. Finally, employing the new amphiphilic **D_2-LLA_{15}-D_2** as gene carrier, in vitro gene transfection experiments were conducted with the SMMC-7721 cell line, indicating a transfection efficiency increase of at least 10 times higher than that of the naked plasmid DNA under a N/P charge ratio of 10. Therefore, these interesting results may provide a new possible way to construct efficient polycationic macromolecular gene carriers with low toxicity and less expensive low-generation PLL dendrons.

Introduction

Recently, polycationic macromolecules have been extensively investigated as potential nonviral vectors for gene therapeutic applications.[1] To date, a series of synthetic polycationic molecules such as poly(ethylene imine) (PEI),[2] poly(2-dimethylaminoethyl methacrylate) (PDMAEMA),[3] and poly(L-lysine) (PLL)[4] have been developed with diverse structures and molecular weights and thus have been exploited as potential DNA carriers with respect to a broad area concerning DNA binding affinities, cell toxicities, and transfection efficiencies. It has also been known that dendrimers have perfectly well-defined structures with abundant end groups at the surfaces,[5] and this unique feature could further be employed to amplify weak interactions via supramolecular assembly between the host and guest molecules for specific applications.[5−8] Particularly, the structurally well-defined dendritic cationic polymers such as water-soluble poly(amido amine) (PAMAM),[9] poly(propylene imine) (PPI),[10] poly(L-lysine), and their series of functional derivatives[11,12] have been intensively studied for construction of new efficient DNA carriers with high binding ability, low cytotoxicity, and favorable transfection efficacy. However, a higher generation of the polycationic dendrimers was generally demanded for better DNA binding ability and ultimate transfection efficiency, and this will lead to practical problems of high cytotoxicity and too expensive preparation cost.

So far, among the currently well-studied polycationic macromolecules, PLL becomes more interesting because it can be synthesized from natural L-lysine, and this category of cationic polymers seems to be biocompatible. Dendritic PLL bearing diverse generations has also been known to be routinely synthesized stepwise with an amino group protection−deprotection cycle.[13] Recently Aoyagi et al.[14] employed a hexamethylene diamine as the initiator core and prepared a new series

* Author to whom correspondence should be addressed. Phone and Fax: +86-21-54925303. E-mail: acao@mail.sioc.ac.cn.

[†] Shanghai Institute of Organic Chemistry, Chinese Academy of Sciences.

[‡] Shanghai Jiaotong University.

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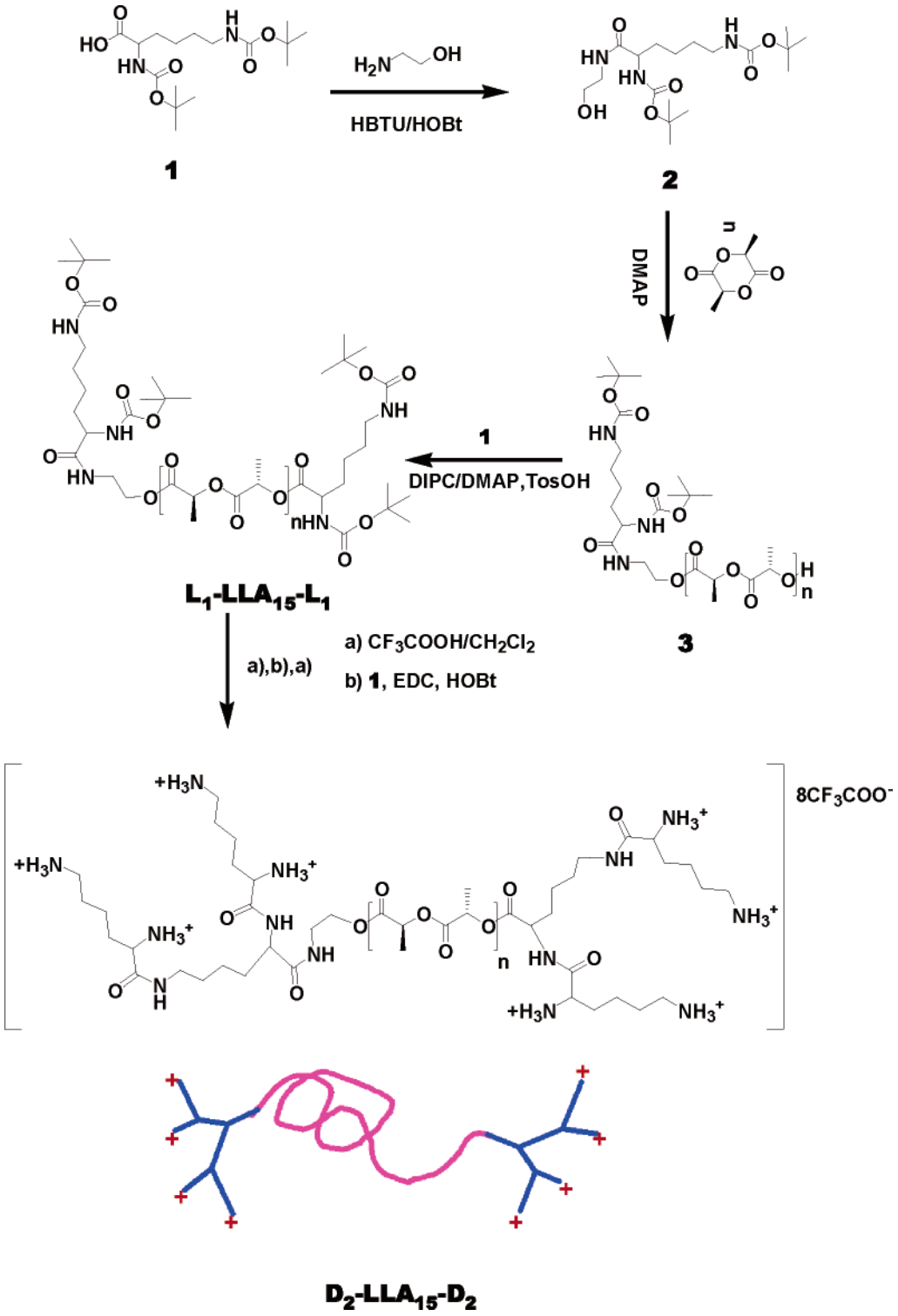
Scheme 1. Preparation of a New Amphiphilic Triblock $\mathbf{D_2\text{-}LLA_{15}\text{-}D_2}$ with Two Two-Generation Poly(L-lysine) Dendrons as Cationic Plasmid DNA Carrier



of symmetric structural dendritic PLL denoted as $\mathbf{D}_n$-**HEX**-$\mathbf{D}_n$ where **D** and *n* express the PLL dendrons and their generation, respectively. It was thereby revealed that only $\mathbf{D}_n$-**HEX**-$\mathbf{D}_n$ with very high *n* PLL dendrons could exhibit favorable DNA binding affinities and transfection efficiencies. To further enhance the gene transfection efficacy, Park et al.[15] alternatively prepared a new water-soluble poly(ethylene glycol) (PEG) bridged double-hydrophilic $\mathbf{D}_n$-**PEG**-$\mathbf{D}_n$ with decreased cytotoxicity; however, there was no remarkable improvement in transfection efficiency. As for the gene delivery, a high-affinity binding between different nanoscale objects is a "bottom-up" fabrication,[16] and a high number of positively charged amino groups of a polycationic carrier seems to be extremely important for realizing high DNA binding affinity.[17] However, it was also generally revealed that a high molecular weight or generation of a polycationic macromolecule carrier would finally lead to much increased cytotoxicity during cell transfection,[10,17,18] and new cationic systems comprised of low-molecular-mass dendrons[19,20] or lipidlike amphiphilic structures[12,19,21] have recently been designed for high DNA binding affinities.

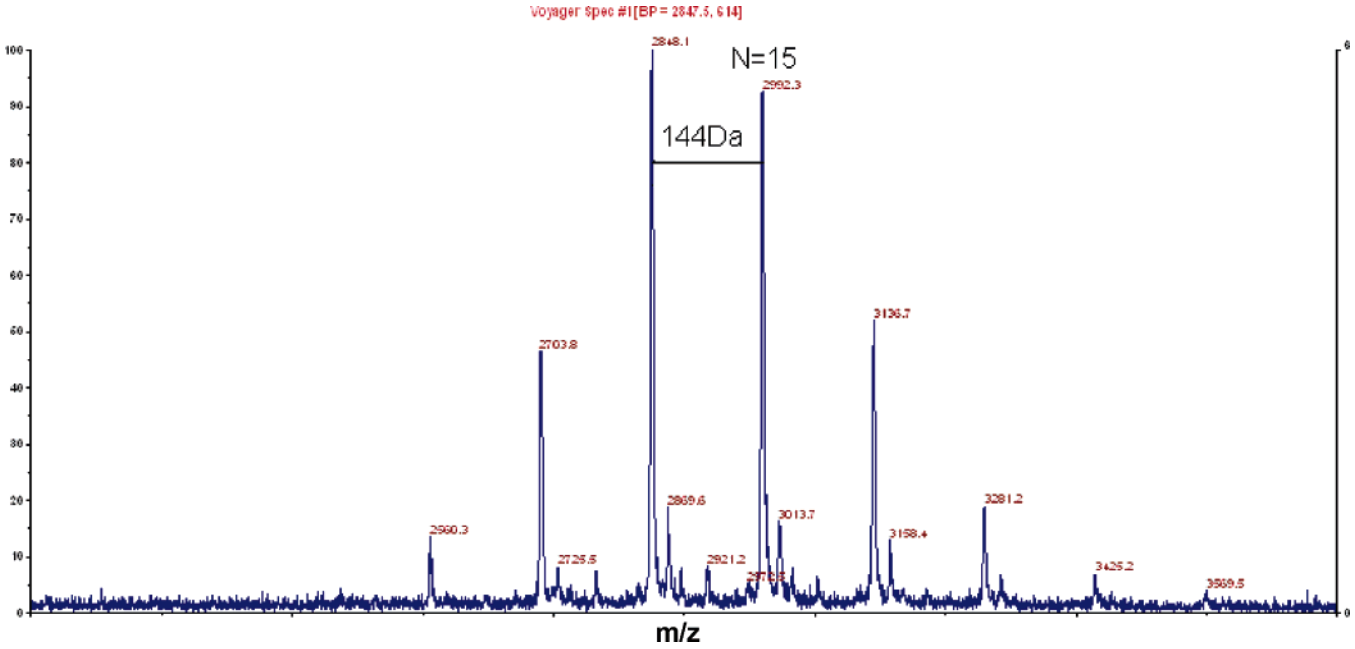


Figure 1. MALDI-FTMS spectrum for new amphiphilic triblock dendritic poly(L-lysine)-*b*-poly(L-lactide)-*b*-dendritic poly(L-lysine) **D_2-LLA_{15}-D_2**.

For new construction of highly efficient DNA carriers, we will communicate molecular design and preparation of a novel well-defined amphiphilic polycationic macromolecule of dendritic poly(L-lysine)-*b*-poly(L-lactide)-*b*-dendritic poly(L-lysine) **D_2-LLA_{15}-D_2** comprised of two two-generation hydrophilic PLL dendrons and a hydrophobic biodegradable PLLA block. Upon incorporating the hydrophobic PLLA block, new amphiphilic polycationic **D_2-LLA_{15}-D_2** bearing two low-generation PLL dendrons and total of eight amino groups could complex plasmid DNA with a much enhanced plasmid DNA binding affinity and low cytotoxicity. Employing the new amphiphilic macromolecule as the polycationic carrier, gene transfection efficiency was also studied.

Experimental Section

Materials. N^{α},N^{ϵ}-Di-Boc-L-lysine was prepared as partially referred to in the literature.[22] The solvent DMF was dehydrated over molecular sieves (4 Å), and chloroform of analytical grade was in turn washed by sulfuric acid and distilled water, dried over CaH_2 for 24 h, and then distilled before use. Monomers of L-lactide from TCI (Tokyo, Japan) were purified via recrystallization in ethyl acetate. 4-(Dimethylamino) pyridine (DMAP) was purchased from TCI, and reagents of linear structural **PEI-2K** (50% in water) and **PLL-23K** (M_w = 23 000 Da) were supplied by Aldrich Chemical. All other solvents and starting chemicals were purchased from commercial suppliers and used as received.

Preparation of Novel Structural Amphiphilic Polycationic D_2-LLA_{15}-D_2. In this study, new structural amphiphilic polycationic macromolecule **D_2-LLA_{15}-D_2** was molecularly designed and synthesized as shown in Scheme 1.

Synthesis of ***L_1OH***. Exactly 3.46 g (10 mmol) of N^{α},N^{ϵ}-di-Boc-L-lysine **1** and 1.80 g (30 mmol) of 2-amino-ethanol were first dissolved in 50 mL of DMF. Then 2.0 g (15 mmol) of hydroxyl benzotriazole (HOBt) and 5.68 g (15 mmol) of *O*-benzotriazole-*N*,*N*,*N*′,*N*′-tetramethyl uromium hexafluoro phosphate (HBTU) were placed into the reaction system as solid mixtures, and the reaction mixture kept stirring at the room temperature for 16 h. Afterward, most of the DMF solvent was evaporated under reduced pressure, and the residue was allowed to drop into 0.5 M HCl aqueous solution. The achieved crude product was further extracted with ethyl acetate and then purified by column chromatography with a mixed solvent (silica, ethyl acetate/hexane = 1:1) to give the final white solid **L_1OH** product **2** (3.35 g, step yield 88%).

^{1}H NMR($CDCl_3$, δ in ppm): 6.84 (brs, 1H, -CONH), 5.44 (brs, 1H, −CONH), 4.78 (brs, 1H, −CONH), 4.06 (brm, 1H, COCH(R)-NH), 3.72 (m, 2H, $HOCH_2$), 3.43 (m, 2H, CH_2CH_2NHCO), 3.12 (m, 2H, $OCCH_2NH(CH_2)$), 1.48−1.99 (m, 6H, CH_2), 1.44 (s, 18H, $C(CH_3)_3$)

Mass spectrum (ESI, *m*/*z*): $[\mathbf{L_1OH} + Na]^+$ $C_{18}H_{35}N_3O_6Na$; 412.3

HRMS (ESI, *m*/*z*): ($C_{18}H_{35}N_3O_6Na$) $[\mathbf{L_1OH} + Na]^+$; found, 412.2418; calcd, 412.2418.

Preparation of ***L_1-LLA_{15}***. Continuously, 0.38 g (1 mmol) of the above-prepared **L_1OH 2**, 3.60 g (25 mmol) of cyclic L-lactide monomer and 0.24 g (2 mmol) of DMAP were dissolved in 10 mL of chloroform, and then the mixture kept stirring at 55 °C under argon atmosphere for 36 h. Finally, the reaction mixture was precipitated in cold methanol, and the collected solid product **L_1-LLA_{15} 3** was dried under vacuum with a step yield of 3.26 g (82%).

^{1}H NMR ($CDCl_3$, δ in ppm): 5.18 (q, nH, $-(O(C{=}O)CH(CH_3)_n-$), 4.35 (q, 1H, $-O(C{=}O)CH(CH_3)OH$), 3.10(m, 2H, $OCCH_2NH(CH_2)$), 1.65−1.99(m, 6H, CH_2), 1.55(d, 3nH, $-(O(C{=}O)CH(CH_3))_n-$), 1.43-(s, 18H, $C(CH_3)_3$)

GPC ($CHCl_3$): PDI (M_w/M_n) = 1.11.

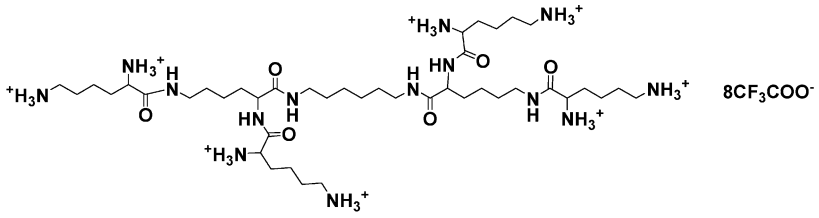
Synthesis of ***L_1-LLA_{15}-L_1***. Exactly 2.50 g of **L_1-LLA_{15} 3** and 1.73 g (5 mmol) of N^{α},N^{ϵ}-di-Boc-L-lysine **1** were first dissolved in 30 mL of chloroform. Subsequently, 244 mg (2 mmol) of DMAP and 346 mg (2 mmol) of *p*-toluene sulfonic acid were further added as a solid mixture. Afterward, the reaction mixture was cooled down in an ice bath and stirred for 10 min, and then 0.63 g (5 mmol) of *N*,*N*′-diisopropylcarbodiimide (DIPC) was added. The reaction mixture was further kept stirring at room temperature for 24 h under argon atmosphere, the solvent was evaporated under reduced pressure, and the product was then precipitated with cold methanol. The collected precipitates were washed with cold methanol and dried under reduced pressure to give the white solid product **L_1-LLA_{15}-L_1** (2.05 g, step yield 82%).

^{1}H NMR ($CDCl_3$, δ in ppm): 5.16 (q, nH, $-(O(C{=}O)CH(CH_3)_n-$), 3.10 (m, 4H, $OCCH_2NH(CH_2)$), 1.65−1.99 (m, 12H, CH_2), 1.56 (d, 3nH, $-(O(C{=}O)CH(CH_3))_n-$), 1.43 (s, 36H, $C(CH_3)_3$)

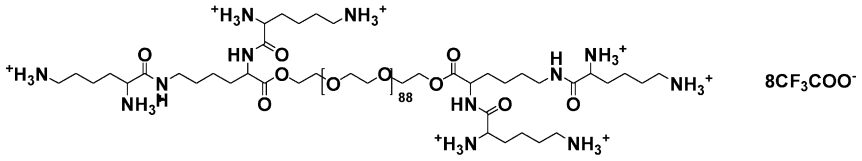
GPC ($CHCl_3$): PDI (M_w/M_n) = 1.09.

Preparation of New Amphiphilic Triblock Polycationic Macromolecular D_2-LLA_{15}-D_2. With the above-prepared **L_1-LLA_{15}-L_1**, the amino group deprotection was first conducted with trifluoroacetic acid

Scheme 2. Molecular Structures of Polycationic Dendritic **D_2-HEX-D_2** and Double-Hydrophilic **D_2-PEG4K-D_2**



D_2-HEX-D_2



D_2-PEG4k-D_2

(TFA). After the mixture was stirred for 1 h at room temperature, the mixture was evaporated and then precipitated with diethyl ether to give the amino group deprotected **D_1-LLA$_{15}$-D_1** with two PLL **D_1** dendrons. Subsequently, second generation of the PLL dendrons was further prepared with a repeated liquid-phase peptide synthetic strategy. Typically, 3 equiv (compared to the amino group of **D_1-LLA$_{15}$-D_1**) of N^{α},N^{ϵ}-di-Boc-L-lysine was first dissolved in DMF, and then the same equivalent of DCC and HOBt were added as a solid mixture. The reaction system kept stirring for 1 h at room temperature, the synthesized **D_1-LLA$_{15}$-D_1** was added, then 1.5 equiv of *N*,*N*-diisopropylethylamine (DIPEA) and 0.5 equiv of DMAP were further added into the reaction mixture, and the reaction was kept at room temperature for 1 day. After complete coupling, the reaction mixture was filtered and evaporated under reduced pressure to remove most of DMF solvent, and the residue was further precipitated with 10% citrate aqueous solution. The collected precipitates were washed with water, then redissolved in chloroform, and dried over anhydrous $MgSO_4$, and the concentrated solution was further precipitated with cold ether. The achieved precipitates were dried under vacuum to obtain the **L_2-LLA$_{15}$-L_2** product with a step yield of 85%. Finally, with the TFA-mediated amino group deprotection of **L_2-LLA$_{15}$-L_2**, a new amphiphilic polycationic **D_2-LLA$_{15}$-D_2** bearing two two-generation PLL dendrons was achieved.

MALDI-FTMS: average degree of polymerization of LLA = 15.

In addition, two important references of hydrophilic **D_2-HEX-D_2** and double-hydrophilic triblock **D_2-PEG4K-D_2** with the same two two-generation PLL dendrons were concurrently prepared as partially referred to refs. 14 and 15, and were further characterized.

Characterization Procedures. *GPC Characterization.* Molecular weights of the synthesized Boc-protected samples were measured at 40 °C on a Perkin-Elmer 200 series of gel permeation chromatograph equipped with a refractive index (RI) detector and a network chromatograph interface NCI 900. Two PL gel 5 μm mixed D-type of 300 mm × 7.5 mm columns (Polymer Laboratories Ltd., U.K.) were set in series with chloroform as the eluent at 1.0 mL/min, and a commercial polystyrene standard kit from Showa Denko Ltd, Japan was employed for elution trace calibration. Thus, molecular weights (M_w, M_n) and their polydispersity indexes (M_w/M_n) were evaluated.

NMR Measurement. ^{1}H NMR spectra were characterized under ambient temperature on a Varian VXR 300 Fourier transform NMR spectrometer, operating at 300.0 for the ^{1}H nuclei, and tetramethylsilane (TMS) was applied as the internal chemical shift reference.

Mass Spectra. Mass spectra (ESI, MALDI-FTMS) were measured on a Varian SATURN 2000 or FTMS-7 spectrometer.

Agarose Gel Electrophoresis Assay. Polycationic macromolecules and plasmid DNA (pGL3 plasmid DNA (Promega) encoding the luciferase reporter gene) were first complexed under various negative/positive (N/P) charge ratios in pure water. After an incubation of 30 min, each complexed sample was analyzed by electrophoresis on an agarose gel (0.5 w/v%) containing ethidium bromide (0.5 μg/mL in the gel) in pH = 8 TAE buffer solution and then illuminated under UV light to record the plasmid DNA molecular migration.

Fluorescence Titration Assay. Ethidium bromide (5.0 μg) and 5.0 μg of pGL3 plasmid DNA were mixed in 1 mL of 0.1 M PBS buffer solution containing 150 mM NaCl (pH = 7.4). After 10 min of incubation at room temperature, polycationic macromolecules under various N/P charge ratios ranging from 0.1 to 40 were individually added into the plasmid DNA and ethidium bromide (EB) mixture solution and further incubated for 3 min. Then, their fluorescence spectra were recorded on a Hitachi F-3010 fluorescence spectrometer with excitation (λ_{ex}) and emission (λ_{em}) wavelengths of 510 and 590 nm, respectively.

Particle Size Analysis. The polycationic macromolecule/plasmid DNA polyplexes were prepared by mixing 20 μg of pCMV_S plasmid DNA encoding the preS2-S gene in the pcDNA 3.1 vector with various amounts of new amphiphilic **D_2-LLA$_{15}$-D_2** in 1 mL of PBS buffer solution (pH = 7.4) and were then analyzed on a dynamic light scattering (DLS) instrument (Zetasizer 3000HS, U. K.). The DLS measurements were conducted at 25 °C with a laser wavelength (λ) of 633 nm and a scattering angle of 90°.

TEM Characterization. First, the amphiphilic **D_2-LLA$_{15}$-D_2** and pGL3 plasmid DNA under a N/P charge ratio equal to 5.0 were complexed in pure water, and its final DNA concentration was set to be 10^{-3} mg/mL. For the transmission electron microscopy (TEM) measurements, a drop of the polyplex solution was deposited onto the carbon-coated copper grid, and then the water was evaporated at ambient temperature prior to the measurement. TEM images were recorded by a Philips CM 120 transmission electron microscope, operating at 80 kV.

DNase I Degradation Assay. The polycationic macromolecules and pCMV_S plasmid DNA were first complexed by mixing equivalent volumes of pCMV_S plasmid DNA solution (40 μg/mL in water) and the polycationic macromolecules in the HBS buffer solution. Subsequently, into 0.5 mL of the organized polyplex solution under an appropriate N/P charge ratio, 2 μL (20 units) of DNase I (Sigma, St. Louis, MO) was added and further mixed. Finally, UV absorbance at 260 nm was recorded to quantitatively assay the enzymatic degradation of plasmid DNA molecules for the characterization of the DNA molecule protection ability of each polyplex nanoparticle system.

Cytotoxicity Assay. Evaluation of cytotoxicity was conducted by the MTT method. First, a human hepatocellular carcinoma cell line (SMMC-7721) was seeded into 96-well microplates (10 000 cells/well in 0.1 mL of DMEM growth medium containing 10% FBS). Each

polycationic macromolecule was individually introduced to the wells and incubated for 48 h, and then the old medium was replaced with fresh growth medium containing 3-(4,5-dimethylthiazol-2-yl)−2,5-diphenyltetrazolium bromide) MTT. After a continuous incubation at 37 °C for 4 h, the medium was removed, and dimethyl sulfoxide was further added to dissolve the formazan formed by proliferating cells. As the result, UV absorbance at 570 nm was measured using a microplate reader (Molecular Devices Co., Menlo Park, CA) to evaluate the cell viabilities as the percentages relative to the control.

Cell Transfection Assay. At first, the SMMC-7721 cells in 24-well plate were washed with 1 mL of serum-free medium, and then 10 μL of the amphiphilic **D_2-LLA_{15}-D_2** solution under a predetermined N/P charge ratio was added into 240 μL of serum-free medium containing pGL3 plasmid DNA. After 15 min standing at room temperature, the **D_2-LLA_{15}-D_2**/DNA mixture solution was gently poured into the wells and incubated at 37 °C for 4 h. Subsequently, 1 mL of FBS medium was added and further incubated for 24 h. Finally, the cells were harvested, and the luciferase assays were accordingly conducted as referred to in the protocol of the PicaGene lumin escence kit (Toyo Ink, Tokyo, Japan). Consequently, the luciferase relative light unit (RLU) was analyzed by a luminometer (Maltibiolumat LB9505, Berthold, Germany) to evaluate the cell transfection efficiency.

Results and Discussion

Scheme 1 illustrates the preparation of a new amphiphilic triblock **D_2-LLA_{15}-D_2** with two two-generation poly(L-lysine) dendron blocks. At first, an amphiphilic diblock dendritic poly(L-lysine)-*b*-poly(L-lactide) **L_1-LLA_{15}** was synthesized via a metal-free organic DMAP-mediated living-ring-opening polymerization of L-lactide as we recently reported.[22] Then its terminal hydroxy functional group was allowed to couple with the carboxylic acid group of N^{α},N^{ϵ}-di-Boc-L-lysine **1** to give a triblock **L_1-LLA_{15}-L_1** with two amino-group-protected one-generation PLL dendrons **L1**. Finally, new amphiphilic triblock **D_2-LLA_{15}-D_2** bearing two two-generation PLL dendrons was further synthesized via sequential amino deprotection of **L_1-LLA_{15}-L_1** with TFA, coupling of the deprotected amino groups of the dendron **L1** with N^{α},N^{ϵ}-di-Boc-L-lysine **1**, and a successive TFA-mediated surface amino group deprotection. Here, MALDI-FTMS (Figure 1) was employed to elucidate the structure of new amphiphilic triblock polycationic **D_2-LLA_{15}-D_2**. The main series of signals occurring at 2560 *m*/*z*, 2703 *m*/*z*, 2848*m*/*z*, 2992 *m*/*z*, 3136 *m*/*z*, and 3281 *m*/*z* could be assigned to the protonated amphiphilic **D_2-LLA_{15}-D_2** while another series of MS signals detected at 2725 *m*/*z*, 2869 *m*/*z*, 3013 *m*/*z*, and 3158 *m*/*z* could also be attributed to their Na^+ ionized counterparts. Meanwhile, the mass difference between two main neighboring MS signals was observed to be regularly equal to 144 Da, which is just equal to the mass of a lactide repeating unit, and this confirmed that no appreciable intermolecular transesterification happened during the ring-opening polymerization of L-lactide as previously reported.[22] On the basis of the MALDI-FTMS evidence, an averaged degree of polymerization (DP) for the central hydrophobic poly(L-lactide) block could thus be calculated to be approximately 15, which was well consistent with the result evaluated by ^{1}H NMR. Therefore, the MALDI-FTMS and GPC evidence (see Supporting Information) substantiated successful preparation of new structurally well-defined amphiphilic **D_2-LLA_{15}-D_2** with two two-generation PLL dendrons and a hydrophobic biodegradable PLLA block. In addition, two important references of hydrophilic dendritic **D_2-HEX-D_2**[14] and double-hydrophilic **D_2-PEG-4k-D_2**[15] as illustrated in Scheme 2 were concurrently synthesized with the same two two-generation PLL dendrons.

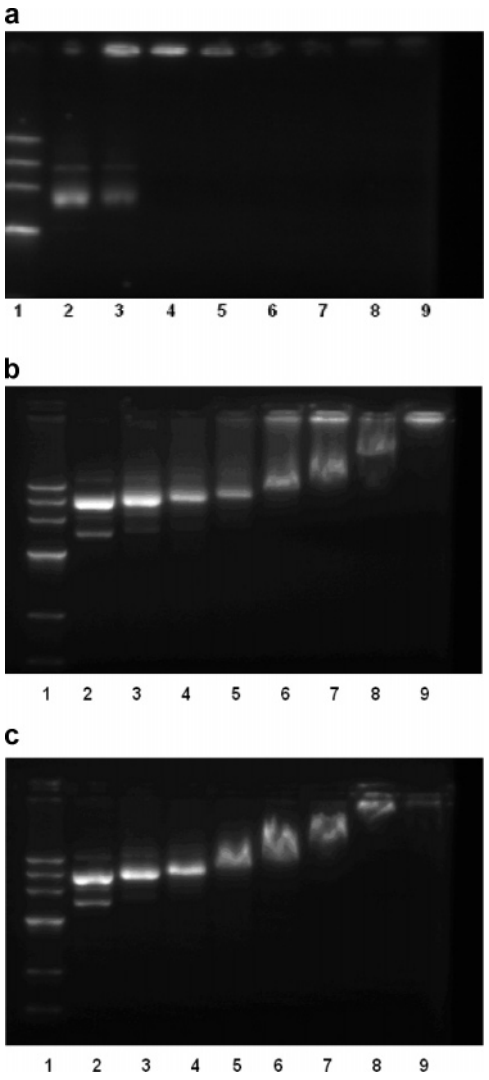


Figure 2. Agarose gel electrophoresis for the polyplexes of plasmid DNA complexed with different polycationic carriers under various N/P ratios: (a) **D_2-LLA_{15}-D_2**; (b) **D_2-HEX-D_2**; (c) **D_2-PEG-4K-D_2**. Lanes: (1) marker; (2) naked DNA; (3) 1:1; (4) 2:1; (5) 4:1; (6) 8:1; (7) 15:1; (8) 30:1; (9) 50:1.

As for the DNA binding affinity of new amphiphilic polycationic **D_2-LLA_{15}-D_2**, here a pGL3 plasmid DNA was employed to characterize its DNA binding ability with an agarose gel electrophoresis assay (0.5%), and a series of predetermined amounts of new polycationic **D_2-LLA_{15}-D_2** under various N/P charge ratios were individually mixed together with 0.50 μg of the pGL3 plasmid DNA before the agarose gel electrophoresis experiments. Figure 2 depicts the electrophoresis assay for the new amphiphilic **D_2-LLA_{15}-D_2** (a) as well as two hydrophilic references of **D_2-HEX-D_2** (b) and **D_2-PEG-4K-D_2** (c) under various N/P charge ratios. In Figure 2a, it was obvious that hardly any DNA molecule migration under a N/P charge ratio equal to 2.0 could be detected as compared to the naked DNA control (lane no. 2). This indicated that DNA molecules were entirely bound with new **D_2-LLA_{15}-D_2**, thus leading to be charge-neutralized or positively charged to form stable polyplexes. In contrast, the hydrophilic **D_2-HEX-D_2** and double-hydrophilic **D_2-PEG-4K-D_2** with the same two two-generation PLL dendrons were observed to complex with the same DNA molecules with much lower binding affinities, and even at a N/P charge ratio higher than 30, the DNA molecule migration could also be detected for either hydrophilic **D_2-HEX-D_2** or double-hydrophilic **D_2-PEG-4K-D_2**. Intriguingly, the extremely

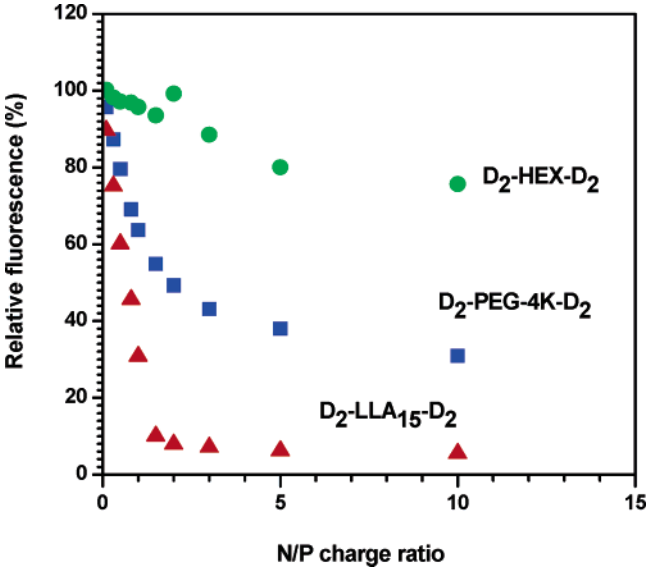


Figure 3. Fluorescence titration assay of the plasmid DNA binding affinities for the new amphiphilic **D_2-LLA_{15}-D_2** as well as hydrophilic **D_2-HEX-D_2** and double-hydrophilic **D_2-PEG-4K-D_2**.

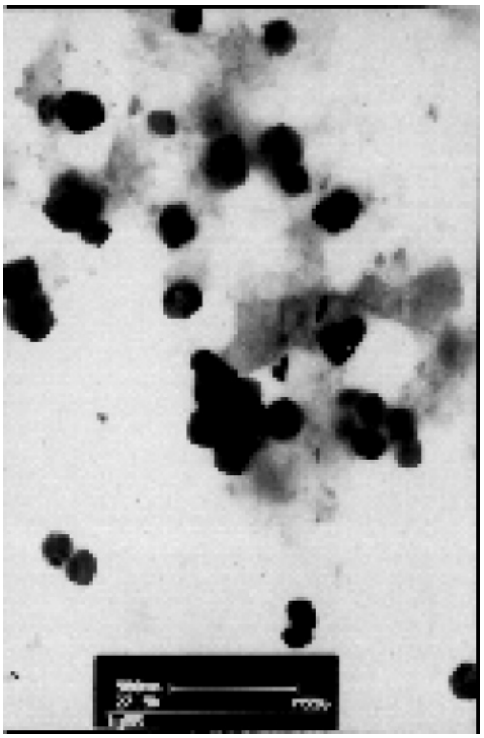


Figure 4. TEM image of the polyplex nanoparticles for the plasmid DNA complexed with new amphiphilic triblock **D_2-LLA_{15}-D_2** under a N/P charge ratio equal to 5.0. (The scale bar is 500 nm.)

high DNA binding affinity of new **D_2-LLA_{15}-D_2** was found to be comparable to that of symmetric PLL dendrimers with at least four-generation[14] or double-hydrophilic **D_n-PEG-D_n** with two four-generation PLL dendrons.[15] This result indicated a new interesting hydrophobic block associated enhancement (**HBAE**) in plasmid DNA binding affinity for the new structurally well-defined amphiphilic polycationic **D_2-LLA_{15}-D_2**.

Alternatively, a fluorescence titration assay was also employed to characterize the DNA binding affinity of new amphiphilic **D_2-LLA_{15}-D_2** along with the above two hydrophilic **D_2-HEX-D_2** and **D_2-PEG-4K-D_2**. For this purpose, 2.0 μg of EB and 5.0 μg of the pGL3 plasmid DNA were allowed to mix together in 1 mL PBS buffer solution containing 150 mM NaCl

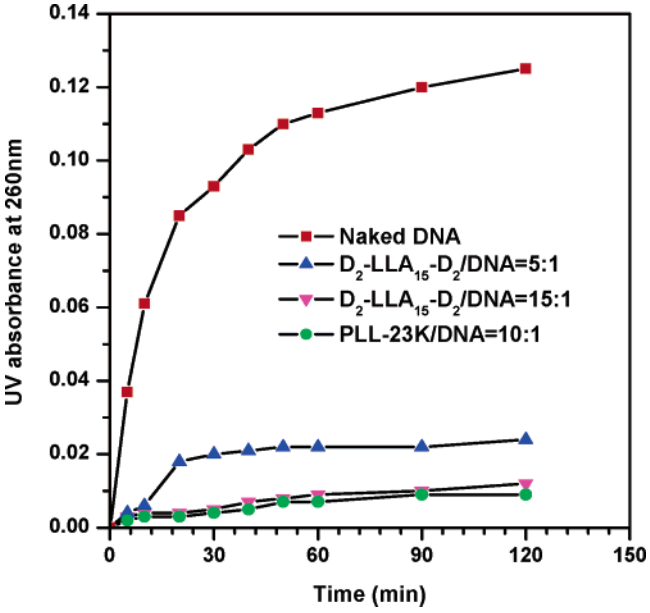


Figure 5. DNase I degradation time dependence of the complexed plasmid DNA molecules in the assembled polyplex nanoparticles.

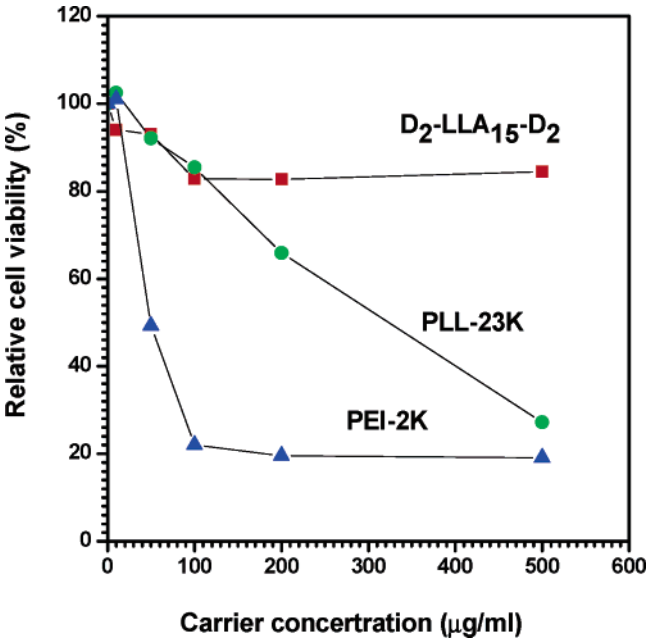


Figure 6. SMMC-7721 cell toxicity assay for the new amphiphilic triblock **D_2-LLA_{15}-D_2** as well as the linear **PLL-23K** and **PEI-2K**.

(pH = 7.4) and then incubated at ambient temperature for 10 min. Subsequently, various amounts of the new amphiphilic polycationic **D_2-LLA_{15}-D_2** under a series of N/P charge ratios spanning a range from 0.1 to 40 were individually placed into each plasmid DNA/EB mixture solution and were further incubated for 3 min. Taking the relative fluorescence intensities of the DNA/EB mixture solution and the DNA-free EB solution as the 100% and 0% references, relative fluorescence intensities were hereby measured for the new **D_2-LLA_{15}-D_2** and two above-mentioned hydrophilic references as shown in Figure 3. It could be seen that the DNA/EB mixture solution containing new amphiphilic **D_2-LLA_{15}-D_2** exhibited a much stronger N/P charge ratio dependence of the relative fluorescence intensities than those of two hydrophilic **D_2-HEX-D_2** and **D_2-PEG-4K-D_2**. Even under a very low N/P charge ratio of 1.5 with the addition of a small amount of new amphiphilic **D_2-LLA_{15}-D_2**, the relative

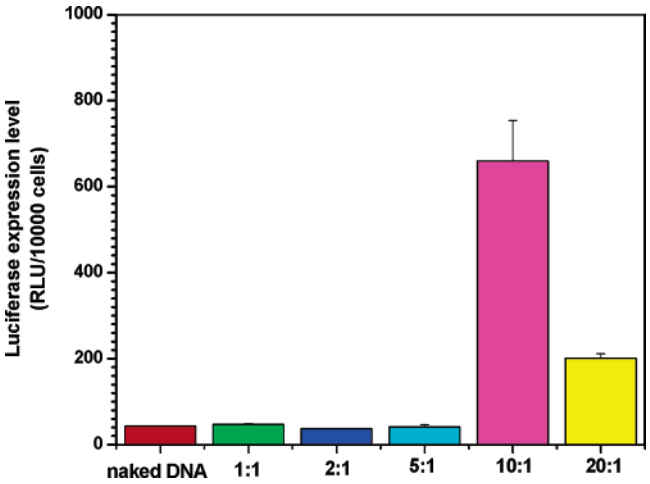


Figure 7. SMMC-7721 cell gene transfection efficiencies for the new amphiphilic triblock **D_2-LLA_{15}-D_2** carrier under various N/P charge ratios.

fluorescence intensity was significantly decreased to a value below 10%, indicating its extremely high DNA binding affinity. Furthermore, an important parameter of CE_{50},[15,19−20] which expresses the N/P charge ratio to achieve 50% relative fluorescence intensit,y was calculated, and a value of CE_{50} close to 0.6 was evaluated for this new amphiphilic **D_2-LLA_{15}-D_2**, indicating its extremely high DNA binding affinity in the PBS buffer solution.[20]

With regard to the particle sizes and morphologies of the organized plasmid DNA/new **D_2-LLA_{15}-D_2** polyplexes, a series of predetermined amounts of the **D_2-LLA_{15}-D_2** were allowed to mix with 20 μg of another pCMV_S plasmid DNA in 1 mL of PBS buffer solution (pH = 7.4) and continuously incubated at room temperature for 15 min, and then the organized polyplexes were analyzed by DLS and TEM. Under various N/P charge ratios in aqueous solution, averaged sizes of the plasmid DNA/**D_2-LLA_{15}-D_2** polyplexes were found to range from 150 to 180 nm, slightly depending on their different N/P charge ratios (see Supporting Information), and this result was very close to that measured by TEM as shown in Figure 4. Under a N/P charge ratio of 5.0, the plasmid DNA/**D_2-LLA_{15}-D_2** polyplexes showed spherelike nanoparticle morphologies with an averaged particle size close to 170 nm. In contrast, either hydrophilic **D_2-HEX-D_2** or double-hydrophilic **D_2-PEG-4K-D_2** could not efficently complex with the same plasmid DNA to form stable polyplexes.[14,15]

In addition, the degradation of plasmid DNA by serum enzymes or nucleases seems to be an important barrier for successful administration of genetic materials as the scaffolds.[23,24] Here, according to the method proposed by Park et al.,[25] the DNase I was further employed for the DNA molecule protection ability assay, and the experiments were conducted for the organized polyplexes of new **D_2-LLA_{15}-D_2**/plasmid DNA and linear structural **PLL-23K**/plasmid DNA and the naked plasmid DNA. As seen in Figure 5, the DNA molecule degradation of the complexed polyplexes seems much slower than that of the naked DNA control. When increasing the N/P charge ratio from 5 to 15, the DNA molecule degradation remarkably decreased and could keep a very low level for at least 2 h. This implied that the organized stable polyplexes by new amphiphilic **D_2-LLA_{15}-D_2** and plasmid DNA could efficiently protect the bound plasmid DNA molecules against the DNase I degradation in the HEPES buffer solution.

Cytotoxicity is also another important factor that has a strong impact on gene transfection. Here, cell toxicities were evaluated with the human hepatocellular carcinoma cell line SMMC-7721 for the new amphiphilic **D_2-LLA_{15}-D_2** as well as commercial linear structural **PEI-2K** and **PLL-23K** as references. As seen in Figure 6, the SMMC-7721 cell viabilities significantly decreased with increasing the concentrations of **PEI-2K**, indicating its relatively high cytotoxicity, and much higher cell toxicity was also observed for the linear **PLL-23K** when its concentration approached 500 μg/mL. Notably, even at a high concentration equal to 500 μg/mL, only a slight decrease in the SMMC-7721 cell viabilities could be detected, indicating the much lower cytotoxicity of the new amphiphilic polycationic **D_2-LLA_{15}-D_2**. Moreover, employing the synthesized new amphiphilic **D_2-LLA_{15}-D_2** as the polycationic plasmid DNA carrier, preliminarily studies on gene transfection efficiency were done with the SMMC-7721 cell line and the naked DNA as the control. It was seen that the gene transfection efficiency by the new amphiphilic **D_2-LLA_{15}-D_2** carrier remarkably increased at least 10 times higher than that of the naked DNA under a N/P charge ratio equal to 10 as seen in Figure 7.

Conclusions

In this work, a new symmetric amphiphilic triblock polycationic **D_2-LLA_{15}-D_2** with two hydrophilic two-generation PLL dendrons **D_2** and a biodegradable hydrophobic poly(L-lactide) block **LLA_{15}** was for the first time designed and synthesized with well-defined structure. Agarose gel electrophoresis assay and EB replacement assay clearly indicated that this new amphiphilic polycationic macromolecule could show much increased plasmid DNA binding affinity compared to those of hydrophilic dendritic **D_2-HEX-D_2** and double-hydrophilic **D_2-PEG-4K-D_2** via a possible hydrophobic block associated enhancement of plasmid DNA binding, and its low cell toxicity and high efficiency of DNA molecule protection were also notably revealed. With the new amphiphilic polycationic **D_2-LLA_{15}-D_2** as a plasmid DNA carrier, in vitro SMMC-7721 cell gene transfection experiments indicated an efficiency increase for at least 10 times higher than that of the naked DNA under a N/P charge ratio equal to 10. Therefore, these results would provide a new way to utilize low toxicity and less expensive low-generation polycationic dendrons to molecularly design and construct new efficient biodegradable amphiphilic structural gene carriers with extremely high DNA binding affinities, low cytotoxicity, and favorable protection of DNA molecules during cell transfection, and further studies on the polycationic **D_n-LLA_{15}-D_n** macromolecules and new star-shaped amphiphilic polycationic macromolecules with surface PLL dendrons **D_n** are now under investigation in this lab and will soon be reported in a forthcoming paper.

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Supporting Information Available. ^{1}H NMR spectrum for the synthetic precursor **L_2-LLA_{15}-L_2,** GPC elution traces for the synthetic precursors **L_1-LLA_{15}**, **L_1-LLA_{15}-L_1,** and **L_2-LLA_{15}-L_2**, and averaged polyplex particle sizes measured by DLS for the plasmid DNA complexed with the new amphiphilic triblock **D_2-LLA_{15}-D_2** under various N/P charge ratios. This material is available free of charge via the Internet at http://pubs.acs.org.

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