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Surface-Engineered Dendrimers with a Diaminododecane Core Achieve Efficient Gene Transfection and Low Cytotoxicity

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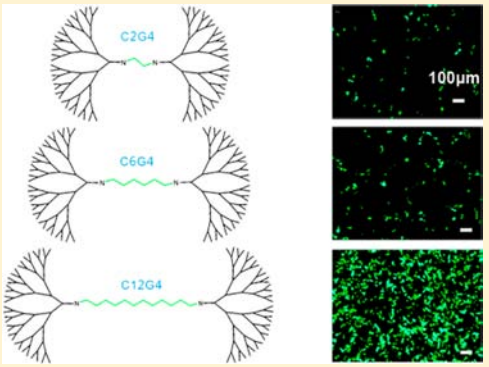
Supporting Information

ABSTRACT: Cationic dendrimers are widely used as gene vectors; however, these materials are usually associated with unsatisfied transfection efficiency and biocompatibility. In this study, we used an aliphatic hydrocarbon-cored polyamidoamine (PAMAM) dendrimer as an alternative to traditional cationic PAMAM dendrimers in the design of efficient gene vectors. Diaminododecane-cored generation 4 (C12G4) PAMAM dendrimer showed dramatically higher efficacy in luciferase and EGFP gene transfection than diaminoethane-cored generation 4 (C2G4) and diaminohexane-cored generation 4 (C6G4) PAMAM dendrimers. The viability of cells incubated with C12G4 at transfection concentrations is above 90%. The significantly improved gene transfection efficacy of C12G4 is attributed to the hydrophobic core of C12G4 which increases the cellular uptake of dendrimer/DNA polyplexes. Further modification of C12G4 with functional ligands such as arginine, 2,4-diamino-1,3,5-triazine, and fluorine compounds significantly increase its transfection efficiency on several cell lines. These results suggest that diaminododecane-cored dendrimers can be developed as a versatile scaffold in the design of efficient gene vectors.



INTRODUCTION

Gene therapy has attracted increasing interest in recent years due to its great promise in the treatment of diseases ranging from inherited disorders to acquired diseases.[1] However, the development of efficient and safe gene vectors remains the most significant barrier to clinical uses.[2] Viral vectors such as retroviruses and adenoviruses show dramatic efficacy in *in vitro* and *in vivo* gene delivery but have severe safety concerns such as immune response and insertional mutagenesis. Therefore, nonviral vectors including cationic liposomes and polymers are widely investigated as alternatives.[3] Polymeric gene vectors are particularly attractive among nonviral vectors due to the diversities in polymer structure, composition, and properties.[4−7]

Dendrimers are a new class of hyperbranched and nanoscale polymers of well-defined structure and molecular weight.[8−12] Among them, polyamidoamine (PAMAM) dendrimer is the most commonly investigated dendrimer architecture especially in gene delivery.[13−20] Though PAMAM-based gene transfection reagents such as SuperFect have already entered the market, these materials are usually criticized due to moderate transfection efficacy and serious toxicity on the transfected cells.[21] To solve this sticky situation, the surface of PAMAM dendrimers was modified with various ligands such as cyclodextrins,[22] lipids,[23] amino acids,[24−29] peptides,[30−32] sugars,[33] polyethylene glycol (PEG) chains,[34] and nanoparticles[35] to improve the cellular uptake and facilitate the intracellular trafficking of dendrimer/DNA polyplexes, or to reduce the cytotoxicity of dendrimers during gene transfection.

Lipids have fusogenic properties which prefer to interact with cell membranes as well as endosome membranes.[36] Therefore, several approaches were tested to improve the efficacy of dendrimer-based gene materials via lipid functionalization.[37−39] For example, Tomas et al. modified PAMAM dendrimers with aliphatic acids bearing different chain lengths, including dodecanoic acid (C12), tetradecanoic acid (C14), and hexadecanoic acid (C16).[37] Among the synthesized dendrimer−lipid conjugates, C12-modified dendrimers showed the highest transfection efficacy on mesenchymal stem cells (2-fold of SuperFect). Santoyo-Gonzalez and co-workers synthesized fatty acid and cholesterol modified PAMAM dendrimers and the conjugates showed superior transfection efficacy compared to commercial reagents such as Lipofectamine 2000 in CHO cells.[38] Kono and Peng et al. synthesized saturated and unsaturated fatty acid-cored PAMAM dendrons.[23,40−43] These lipid-based materials also showed promising applications in the delivery of plasmid DNA and siRNA into a list of cells. Despite continuous improvements in the transfection efficacies in these delivery systems, the applications of dendrimer−lipid conjugates are limited by several problems and disadvantages: (1) Lipid-based materials are associated with serious cytotoxicity due to the damage of lipid ligands on the cell membrane, which may lead to the leakage of cellular components.[44] (2) The dendrimer−lipid

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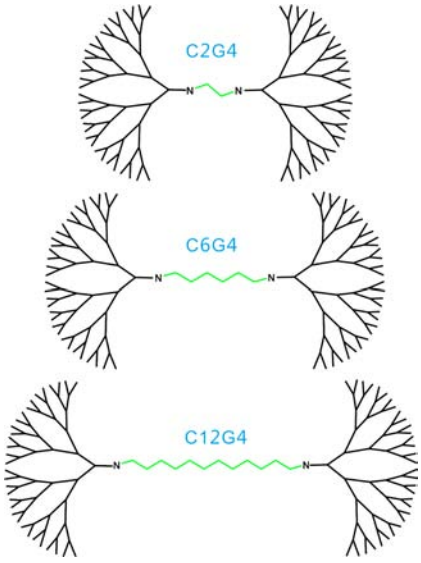
conjugate has a strong tendency to aggregate into micelles; thus, the lipids modified on the dendrimer surface may decrease the stability and solubility of dendrimers in aqueous solutions. (3) The fatty acid modified on the dendrimer surface has a long chain which will cause serious spatial hindrance for further dendrimer modification with other functional moieties such as targeting ligands.

Here, we propose a new strategy to solve these issues of dendrimer−lipid conjugates. The aliphatic hydrocarbon moiety was moved to the core of a PAMAM dendrimer rather than modified on the dendrimer surface. The lipid moiety embedded in the dendrimer decreases the damage of lipid to cell membrane as well as the associations between aliphatic moieties on different dendrimers. Tomalia synthesized a list of aliphatic hydrocarbon-cored PAMAM dendrimers which are now commercially available.[45] These dendrimers were reported with distinct physicochemical properties compared with traditional dendrimers with an ethylenediamine or ammonia core.[45] In this study, diaminododecane-cored generation 4 (C12G4) was used as a versatile scaffold to develop a new class of efficient gene vectors. The transfection efficacy and cytotoxicity of C12G4 were compared with diaminohexane-cored generation 4 (C6G4) and diaminoethane-cored generation 4 (C2G4) PAMAM dendrimers which possess the same number of surface charges but much shorter hydrocarbon chain lengths in the core (Scheme 1). Luciferase and EGFP plasmids

Scheme 1. Chemical Structures of C2G4, C6G4, and C12G4 PAMAM Dendrimers



were used as model genes. The C12G4 was further modified with functional groups such as arginine (ARG), 2,4-diamino-1,3,5-triazine (DAT), and fluorine compounds without involving sophisticated synthesis to achieve efficient gene transfection. This study provides a versatile scaffold in the design of high efficient and low cytotoxic gene carriers.

EXPERIMENTAL PROCEDURES

Materials. C2G4 with a molecular weight 14215 Da, C6G4 with a molecular weight 14271 Da, C12G4 with a molecular weight 14355 Da, and branched polyethylenimine with a molecular weight of 25 kD (bPEI 25 kD) were purchased from Sigma-Aldrich (St. Louis, MO). Diaminoethane-cored generation 6 (G6) with a molecular weight 58048 Da was purchased from Dendritech, Inc. (Midland, MI). G2G4, C6G4, and C12G4 PAMAM dendrimers have a theoretical number of 64 surface amine groups, while G6 PAMAM dendrimer has a theoretical number of 256 surface amine groups. Commercial gene reagents SuperFect and Lipofectamine 2000 were purchased from Qiagen and Invitrogen (Carlsbad, CA), respectively. The DNA labeling dye YOYO-1 was obtained from Invitrogen. LAMP-2 antibody conjugated with Alexa Fluor 647 was purchased from Santa Cruz Biotechnology (Santa Cruz) and used to label the endosomes and lysosomes. If not specialized, other chemicals used in this study were obtained from Sigma-Aldrich and were used without further purification.

Synthesis of Surface-Engineered C12G4 PAMAM Dendrimers. ARG-modified C12G4 was synthesized according to the method described by Park et al.[46] Generally, amine-protected ARG (Boc-Arg(Pbf)-OH) was activated by dicyclohexylcarbodiimide (DCC) and *N*-hydroxysuccinimide (NHS) in *N*,*N*-dimethyl formamide (DMF) for 6 h at room temperature, followed by the addition of C12G4 dissolved in dimethyl sulfoxide (DMSO). The molar ratio of Boc-Arg(Pbf)-OH to C12G4 is 96:1. The reaction was continued for 7 d at room temperature. The Boc protecting group on each modified arginine was removed using trifluoroacetic acid. The product was then purified by extensive dialysis (MWCO ~3500 Da) against DMSO, PBS buffer, and double-distilled water, respectively. The purified C12G4-ARG conjugate was lyophilized to obtain white powders. The yielding product was characterized by ^{1}H NMR (699.804 MHz, D_2O).

DAT-modified C12G4 was synthesized as described below. 2-Chloro-4,6-diamino-1,3,5-triazine was reacted with C12G4 in a water and ethanol mixture (50/50, v/v) at 80 °C for 24 h. The molar ratio of 2-chloro-4,6-diamino-1,3,5-triazine to C12G4 is 32:1. $NaHCO_3$ was added to remove the yielding acid. The product was purified by extensive dialysis (MWCO ~3500 Da) and lyophilized as white powders. The yielding C12G4-DAT conjugate was characterized by ^{1}H NMR (699.804 MHz, D_2O).

Heptafluorobutyric acid (HFA) modified C12G4 was synthesized according to the following method. C12G4 and heptafluorobutyric anhydride were dissolved in methanol. The molar ratio of heptafluorobutyric anhydride to C12G4 is 64:1. The mixtures were stirred at room temperature for 48 h and dialyzed against PBS buffer and distilled water, followed by lyophilization under vacuum to obtain the fluorinated C12G4. Due to the lack of protons on the modified HFA on C12G4 surface, the number of HFA modified on each C12G4 was measured by a well-established ninhydrin assay, in which the residual amine groups on C12G4 was analyzed.

Formation of Dendrimer/DNA Polyplexes. Dendrimer/DNA polyplexes were prepared by mixing 1.6 μg plasmid DNA (EGFP or luciferase plasmid) with dendrimers or surface-engineered dendrimers at different N/P ratios for 30 min at room temperature. N here represents the number of cationic groups on the dendrimer surface, while P represents the number of phosphate anions in the DNA chains. For C2G4, G6G4, C12G4, and G6 PAMAM dendrimers, N number equals the number of primary amine groups on the dendrimer surface. For C12G4-DAT and C12G4-HFA conjugates, N numbers equal the number of residual amine groups (the two amine groups on the DAT moiety have a pK_a value around 5 and are not protonated at physiological condition of pH 7.4, thus DAT

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was not considered when calculating the N numbers). For C12G4-ARG conjugate, the N number is a sum of 64 and the number of ARG conjugated on C12G4 since the conjugation of each ARG group on dendrimer surface brings an extra cationic guanidyl group. For SuperFect and Lipofectamine 2000, the complexes were prepared according to the manufacturer's protocols. The polyplexes were diluted with PBS buffer or Dulbecoo's modified Eagles medium (DMEM) before characterization or gene transfection experiments.

Characterization of the Dendrimer/DNA Polyplexes. The DNA binding capacities of C2G4, C6G4, and C12G4 were evaluated by agarose gel electrophoresis. Generally, 10 μL polyplex solutions containing 0.8 μg DNA and dendrimers at different N/P ratios (0.5:1, 1:1, 2:1, 4:1, 6:1, and 8:1) were incubated for 30 min at room temperature, followed by the addition of 2 μL DNA loading buffers. The samples were run on a 1% (w/v) agarose gel at 90 V for 40 min. The size and zeta potential of the prepared dendrimer/DNA polyplexes were measured by dynamic light scattering (DLS) using Malvern Zetasizer Nano ZS 90 (Malvern, UK) at 25 °C. Generally, 1.6 μg DNA were complexed with dendrimers at N/P ratios of 0.5:1, 1:1, 2:1, 4:1, 6:1, and 8:1, and the complex solutions were incubated for 30 min and diluted to 1 mL before measurement.

***In Vitro* Gene Transfection.** HEK293 (ATCC, a human embryonic kidney cell line) and HeLa (ATCC, a human cervical carcinoma cell line) were grown in DMEM (Invitrogen) containing 100 U/mL penicillin sulfate, 100 μg/mL streptomycin, and 10% (v/v) heat-inactived fetal bovine serum (FBS, Invitrogen) at 37 °C under a humidified atmosphere containing 5% CO_2. The cells were cultured in 24-well plates overnight before *in vitro* gene transfection. Generally, 1.6 μg luciferase or EGFP plasmids were complexed with dendrimers or surface-engineered dendrimers at different N/P ratios for 30 min. The polyplex solutions were diluted with DMEM (containing 10% FBS) and the cells were incubated with the polyplexes for 6 h. After that, the medium in each well was replaced with 500 μL fresh DMEM containing 10% FBS. The cells were further cultured for 24 h (HEK293 cells) or 48 h (HeLa cells). G6 PAMAM dendrimer, bPEI 25 kD, SuperFect, and Lipofectamine 2000 were used as positive controls. EGFP expressions in the cells were observed by a fluorescent microscopy or quantitatively analyzed using flow cytometry. Luciferase expressions were analyzed according to the manufacturer's protocols (Promega).

To investigate the pH buffering capacity of C12G4, bafilomycin A1 (300 nM) and sucrose (5 mM) were incubated with HeLa cells for 1 h before the addition of dendrimer/luciferase polyplexes. Luciferase expressions in the cells treated with and without bafilomycin A1 and sucrose were measured according to the manufacturer's protocol.

Cellular Uptake of the Dendrimer/DNA Polyplexes. EGFP plasmids were labeled with YOYO-1 for 10 min according to the manufacturer's protocols (Invitrogen) and followed by complexation with the gene transfection materials at their optimal N/P ratios. HeLa cells were cultured in 24-well plates and added with the YOYO-1 labeled polyplexes for 30 min. After that, the medium in the wells was removed and the cells were washed with PBS for three times. The cells were then digested using trypsin and the cellular uptake (YOYO-1 positive cells as well as the mean fluorescence intensity) of the polyplexes was analyzed by flow cytometry.

To investigate the endocytosis pathway of C12G4/DNA polyplexes, the polyplexes were prepared at the optimal N/P ratio of C12G4. Before the addition of dendrimer/DNA polyplexes, the cells were incubated with endocytosis inhibitors including genistein (350 μM and 700 μM), chlorpromazine (10 μM and 20 μM) and cytochalasin D (5 μM and 10 μM) for 1 h. The inhibitors are nontoxic at the mentioned concentrations. The untreated cells were used as controls. The medium of the cells was then replaced by DMEM containing YOYO-1 labeled C12G4/DNA polyplexes. After incubation for 2 h, the medium was removed and the cells were washed with PBS. The cellular uptake of YOYO-1 labeled polyplexes in the presence and absence of endocytosis inhibitors was analyzed by flow cytometry.

Cytosolic Nuclease Activity Assay. We employed an oligonucleotide molecular beacon (MB) assay to analyze the cytosolic nuclease activity in cells transfected with C2G4/DNA, C6G4/DNA, and C12G4/DNA polyplexes.[47] Generally, bland plasmid (0.8 μg) and MB (0.8 μg) were mixed and followed by complexation with C2G4, C6G4, C12G4 at an N/P ratio of 8:1. The formed polyplex solution was added with DMEM and incubated at room temperature for 30 min before transfection. HeLa cells in a 24-well plate were incubated with the polyplexes for 3 h and the medium in each well was replaced with 500 μL fresh medium. After 9 h, the cells were rinsed with PBS, trypsinized, centrifuged, and suspended in 300 μL PBS. The MB fluorescence in the transfected cells was analyzed by flow cytometry.[47]

Confocal Microscopy. To monitor intracellular trafficking behaviors of C12G4/DNA polyplexes, the cells were incubated with the polyplexes for 2 or 4 h. DNA was labeled with YOYO-1 before complexation with C12G4, while the acidic vesicles and the nuclei of HeLa cells were stained with LAMP-2 antibody conjugated with Alexa Fluor 647 and DAPI, respectively. Co-localizations of the YOYO-1 labeled polyplexes with acidic vesicles in HeLa cells were observed by a laser scanning confocal microscopy.

Cytotoxicity Assay. The cytotoxicities of C2G4, C6G4 and C12G4 were measured by a well-established MTT assay. Generally, HeLa or HEK293 cells were seeded in 96-well plates at 10^4 cells per well. The cells were incubated with dendrimers at different concentrations for 48 h (HeLa cells) or 24 h (HEK293 cells). The dendrimer concentrations were chosen at those used in gene transfection experiments. After that, the cells were incubated with MTT and the purple crystals yielded were dissolved using DMSO. A microplate reader (MQX200R, BioTek Inc.) was used to measure the absorbance in each well of the plate at 578 nm. Five repeats were conducted for each sample and data were given as means ± SEM and analyzed by Students'*t* test.

RESULTS AND DISCUSSION

Comparisons of C2G4, C6G4, and C12G4 on DNA Binding, Transfection Efficacy, and Cytotoxicity. As shown in Figure 1, C2G4, C6G4, and C12G4 form stable polyplexes with DNA at N/P ratios above 1:1, suggesting that the three dendrimers have strong tendency to binding DNA and the introduction of aliphatic hydrocarbons to the core of PAMAM dendrimer does not decrease its DNA binding capacity. On the contrary, DNA binding abilities of C6G4 and C12G4 were even stronger than that of C2G4 (Figure S1), which is due to the decreased spatial hindrance on the charged surface and increased flexibility of C6G4 and C12G4. This result is further confirmed by DLS studies in Figure 2. C6G4 and C12G4 are able to condense the plasmid DNA into

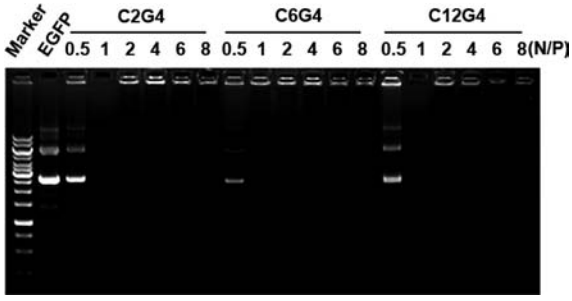

Figure 1. Agarose gel electrophoresis analysis of C2G4, C6G4, and C12G4 polyplexes with EGFP plasmid. Lane 1 and lane 2 represent marker and naked plasmid DNA, respectively. Lanes 3−8 represent C2G4/DNA polyplexes at N/P ratios of 0.5:1, 1:1, 2:1, 4:1, 6:1, and 8:1, respectively. Lanes 9−14 represent C6G4/DNA polyplexes at N/P ratios of 0.5:1, 1:1, 2:1, 4:1, and 8:1, respectively. Lanes 15−20 represent C12G4/DNA polyplexes at N/P ratios of 0.5:1, 1:1, 2:1, 4:1, 6:1, and 8:1, respectively.

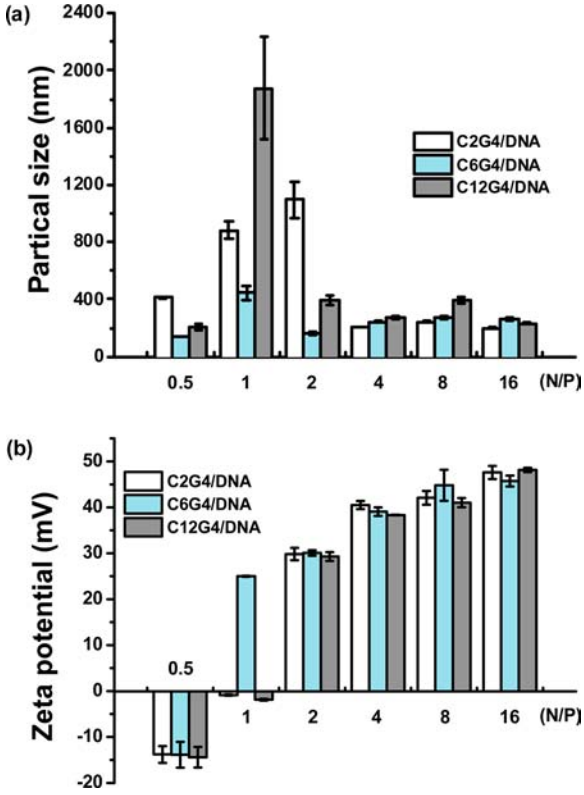

Figure 2. Size (a) and zeta potential (b) analysis of C2G4/DNA, C6G4/DNA, and C12G4/DNA polyplexes at different N/P ratios.

nanoparticles at N/P ratio of 2:1, while C2G4 archives DNA compaction at N/P ratio of 4:1. The polyplexes turn from negatively charged to positively charged at N/P ratios above 1:1. The sizes of the C2G4, C6G4, and C12G4 polyplexes are below 400 nm at N/P ratios equal to or above 4:1. This particle size range was proved to be suitable for polymer-based gene delivery.

In vitro gene transfection efficacy of C2G4, C6G4, and C12G4 was measured on HEK293 and HeLa cells using luciferase plasmid (Figure 3, Figure S2, and Figure S3). As shown in Figure 3, C12G4 shows dramatically higher transfection efficacy than C2G4 and C6G4 on the cells. For example, the luciferase expression (RLU/mg protein) by

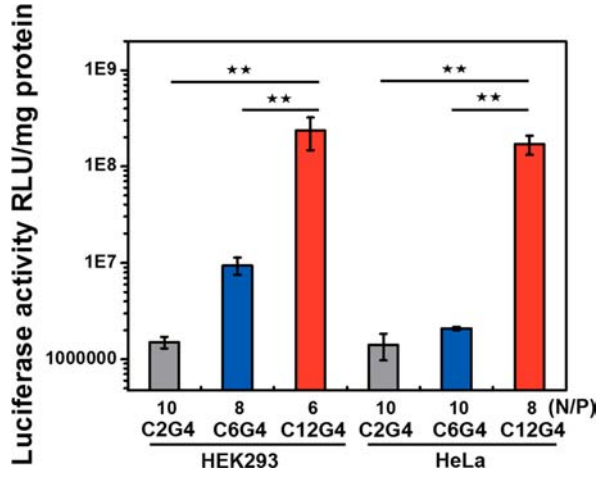

Figure 3. Luciferase gene expressions of C2G4/DNA, C6G4/DNA, and C12G4/DNA polyplexes in HEK293 and HeLa cells at optimal N/P ratios. The luciferase gene expression level is expressed as relative luciferase light units (RLU)/mg of protein. Statistically significant differences are denoted by $^{**}p < 0.05$.

C12G4 on HeLa cells is 120-fold of C2G4 and 81-fold of C6G4 at their optimized transfection conditions (optimized N/P ratios of 8:1, 10:1, and 10:1 for C12G4, C6G4, and C2G4, respectively, on HeLa cells. The ratios are 6:1, 8:1, and 10:1 for the three materials on HEK293 cells). The high transfection efficacy of C12G4 is further confirmed using EGFP plasmid on the two cell lines. As shown in Figure 4, EGFP gene expressions

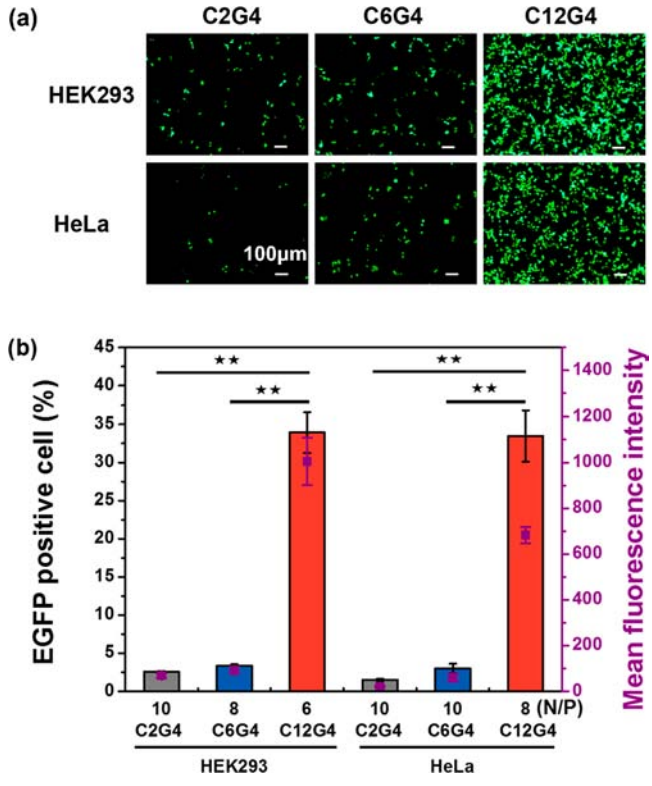

Figure 4. Fluorescent microscopy images of HEK293 cells and HeLa cells transfected by C2G4, C6G4, and C12G4 at their optimal N/P ratios (a). EGFP gene transfection efficiency (%) of the materials measured by flow cytometry (b). Statistically significant differences are denoted by $^{**}p < 0.05$.

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of C12G4/EGFP polyplexes are much higher than that of C2G4/EGFP and C6G4/EGFP polyplexes at optimal N/P ratios. C12G4 transfected 33.5% HeLa cells and 33.9% HEK293 cells, while C2G4 and C6G4 only transfected less than 5% HeLa and HEK293 cells. In addition, the cells transfected with C12G4 show much stronger fluorescent intensities than those transfected with C2G4 and C6G4. These results clearly demonstrate that the incorporation of a single aliphatic C12 chain into the dendrimer core dramatically improves the gene transfection efficacy of the dendrimers.

We further evaluated the cytotoxicities of C2G4, C6G4, and C12G4 on HeLa and HEK293 cells using an MTT assay. As shown in Figure 5, the increase of aliphatic chain length from

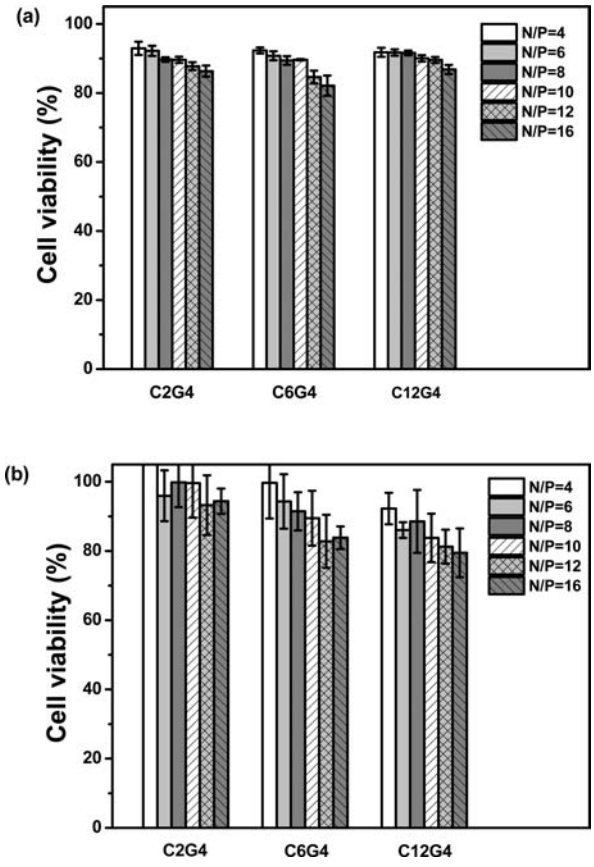

Figure 5. Comparison of C2G4, C6G4, and C12G4 cytotoxicity on HeLa (a) and HEK293 cells (b). The dendrimer concentrations equal to those in polyplexes at different N/P ratios.

C2 to C12 in the core of PAMAM dendrimer scarcely influences its cytotoxicity. The viabilities of cells are higher than 80% when the cells were incubated with C12G4 at N/P ratios up to 16:1. Since the optimized N/P ratios of C12G4 are within the range from 6:1 to 8:1 on HeLa and HEK293 cells, C12G4 can achieve efficient gene transfection with low cytotoxicity.

Gene Transfection Mechanism of C12G4 PAMAM Dendrimer. The efficacy of a gene vector depends on its ability on condensation/release of DNA, cellular uptake, as well as its ability to escape from the endosomes after endocytosis.[48] As demonstrated in Figure 1, C12G4 shows a similar behavior to C2G4 and C6G4 on DNA condensation (stronger than C2G4 but weaker than C6G4), in both agarose gel electrophoresis and DLS experiments. To explain why C12G4 dendrimer has a much higher transfection efficacy than C2G4 and C6G4 dendrimers, we further investigated the cellular uptake and endosomal escape behaviors of C12G4/DNA polyplexes. As shown in Figure 6, C12G4/YOYO-1-labeled

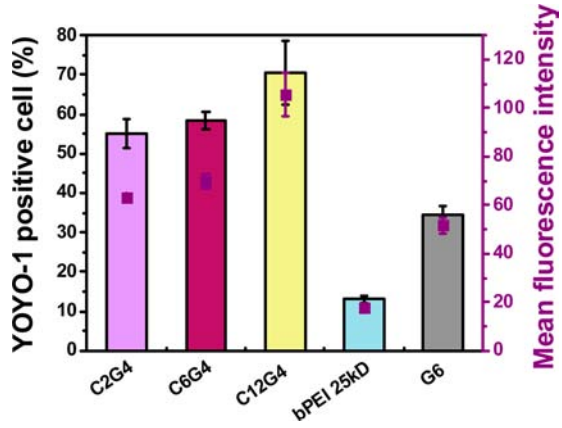

Figure 6. Polyplexes uptake by HeLa cells after 2 h of incubation. Cellular uptake efficiency of YOYO-1-labeled DNA with C2G4, C6G4, C12G4, bPEI 25 kD, and G6 was determined by flow cytometry.

DNA polyplexes show a much higher cellular uptake (both on YOYO-1 positive cells and mean fluorescence intensity) than C2G4/DNA, C6G4/DNA, as well as G6/DNA and PEI/DNA polyplexes, suggesting that the presence of a C12 aliphatic chain in the core of PAMAM dendrimer significantly improves the cellular uptake of the dendrimer/DNA polyplexes.

It is believed that dendrimer/DNA polyplexes enter into the cell cytoplasm by endocytosis.[49] To investigate the endocytosis pathway of C12G4/DNA polyplexes, three uptake inhibitors were incubated with HeLa cells before the addition of YOYO-1 labeled C12G4/DNA polyplexes. Cytochalasin D, genistein, and chlorpromazine can inhibit macropinocytosis, caveolae-mediated endocytosis, and clathrin-mediated endocytosis, respectively.[49] As shown in Figure 7, the internalization of C12G4/DNA polyplexes is slightly changed in the presence of genistein and chlorpromazine; however, cytochalasin D, which is known as an inhibitor of macropinicytosis and phagocytosis, significantly inhibited the cellular uptake of the polyplexes (below 20% for C12G4). Because HeLa cells are considered as

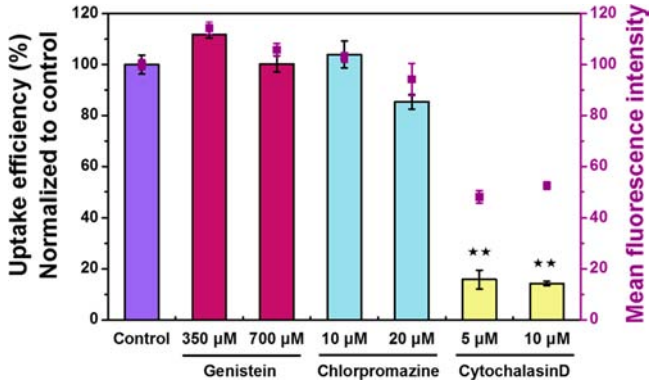

Figure 7. Cellular internalization pathways of C12G4/DNA polyplexes. HeLa cells were pretreated with genistein (350 and 700 μM), chlorpromazine (10 and 20 μM), and cytochalasin D (5 and 10 μM) for 1 h before the addition of polyplexes. Uptake efficacy of the polyplexes was evaluated by flow cytometry. Statistically significant differences are denoted by $^{\star\star}p < 0.05$.

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non-phagocytosing cells, the inhibition of polyplex internalization by cytochalasin D suggests that endocytosis of C12G4/DNA polyplexes is mainly mediated by macropinocytosis.[50]

Degradation of DNA by cytosolic nuclease presents a barrier to efficient gene delivery. We can test the cytosolic nuclease activity in the cells transfected by C2G4/DNA, C6G4/DNA, and C12G4/DNA polyplexes using MB as an indicator. MB is a single-stranded DNA with two fluorescent probes. The intact oligonucleotide leads to quenched fluorescence and cleavage of the MB by cytosolic nuclease leads to increased fluorescent intensity. As shown in Figure 8, the fluorescence intensities of

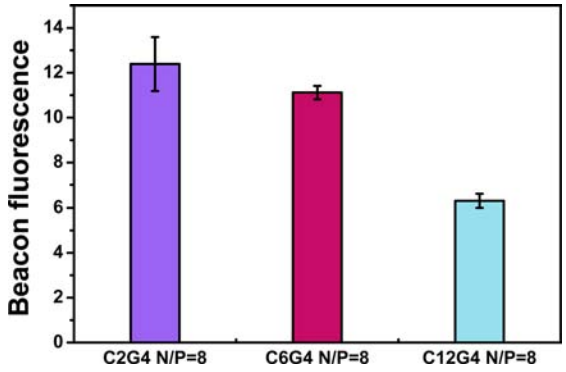

Figure 8. Cytosolic nuclease activity of cells transfected by C2G4/DNA, C6G4/DNA, and C12G4/DNA polyplexes analyzed by an MB probe. The polyplexes are prepared at an N/P ratio of 8:1.

cells transfected with C12G4/DNA polyplexes are much lower than C2G4/DNA and C6G4/DNA polyplexes, suggesting that reduced cytosolic nuclease activation is one of the reasons that C12G4 shows much higher gene transfection efficacy than C2G4 and C6G4.

To investigate the endosomal escape behaviors of C12G4/DNA polyplexes, the cells were treated with sucrose and bafilomycin A1 before gene transfection. Sucrose is a lysosomotropic agent which facilitates the escape of particles from acidic vesicles; on the contrary, bafilomycin A1 inhibits the acidification in endosome and lysosome, thus blocking the endosome escape process.[51] As shown in Figure 9, the addition of sucrose failed to further improve the transfection efficacy of C12G4 but the presence of 300 nM bafilomycin A1 significantly decreased the efficacy of C12G4, suggesting that C12G4 has excellent pH-buffering capacity, which is essential

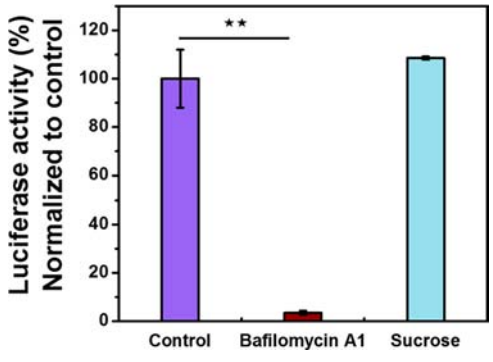

Figure 9. Endosomal escape abilities of C12G4/DNA polyplexes. HeLa cells were pretreated with bafilomycin A1 or sucrose, followed by the incubation of C12G4/DNA polyplexes for 48 h. Statistically significant differences are denoted by $^{\star\star}p < 0.05$.

for efficient gene transfection. The pH-buffering capacity of C12G4/DNA polyplexes is similar to that of C2G4/DNA polyplexes (Figure S4). The efficient endosomal escape of C12G4/DNA polyplexes is further confirmed by confocal microscopy studies. As shown in Figure 10, YOYO-1 labeled

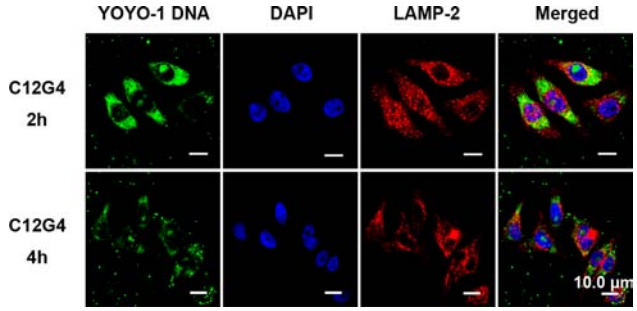

Figure 10. Confocal microscopic images of HeLa cells treated with YOYO-1 labeled C12G4/DNA polyplexes for 2 and 4 h. The lysosomes were stained with LAMP-2 conjugated with a red dye, and the nuclei were stained with DAPI.

C12G4/DNA polyplexes exhibited efficient cellular uptake (~100%) in HeLa cells after 2 h and most of the DNA escaped from the acidic vesicles stained by LAMP-2 antibody conjugated with Alexa Fluor 647 after 4 h. Taken together, these results may explain why C12G4 exhibits efficient gene transfection on the cells.

Surface-Engineered C12G4 Dendrimer for Efficient Gene Transfection. In a previous study, Tomas et al. conjugated 4 or 8 C12 aliphatic chains to the surface of a G5 PAMAM dendrimer;[37] the lipid functionalized dendrimer showed a maximum of 4.5-fold higher than native G5 dendrimer in transfection efficacy. Due to the presence of lipids on the dendrimer surface, the dendrimers are poorly soluble in aqueous solutions and difficult to modify with other functional groups for more efficient gene transfection. Here, the unmodified C12G4 already shows promising properties in gene transfection, and its surface amine groups remain available for modification with functional ligands to modulate DNA condensation, cellular uptake, intracellular trafficking, and release; to reduce dendrimer cytotoxicity; and to allow selective cell targeting.[48] C12G4 was modified with three types of functional ligands including arginine, DAT, and fluorine compounds using facile strategies (Scheme 2). The role of arginine in improving the gene transfection efficacy of biomaterials was already demonstrated by Park et al. DAT- and fluorine-containing polymers also showed great promise in the design of efficient and biocompatible gene vectors.[52,53] After modification, average numbers of 44 ARG, 23 DAT, and 43 HFA groups were conjugated on each C12G4 and the products were termed C12G4-ARG44, C12G4-DAT23, and C12G4-HFA43, respectively (Figure S5, Figure S6, and Note S1).

As shown in Figure 11, C12G4-ARG44, C12G4-DAT23, and C12G4-HFA43 showed much higher transfection efficacy than unmodified C12G4 at their optimal transfection conditions (Figures S7, S8, and S9). All the three conjugates showed transfection efficacies higher than 50% on HeLa cells, which are much higher than bPEI 25 kD, G6 PAMAM dendrimer. The synthesized C12G4 conjugates are even more efficient than commercial gene transfection reagents such as SuperFect and Lipofectamine 2000. C12G4-HFA43 achieves excellent trans-

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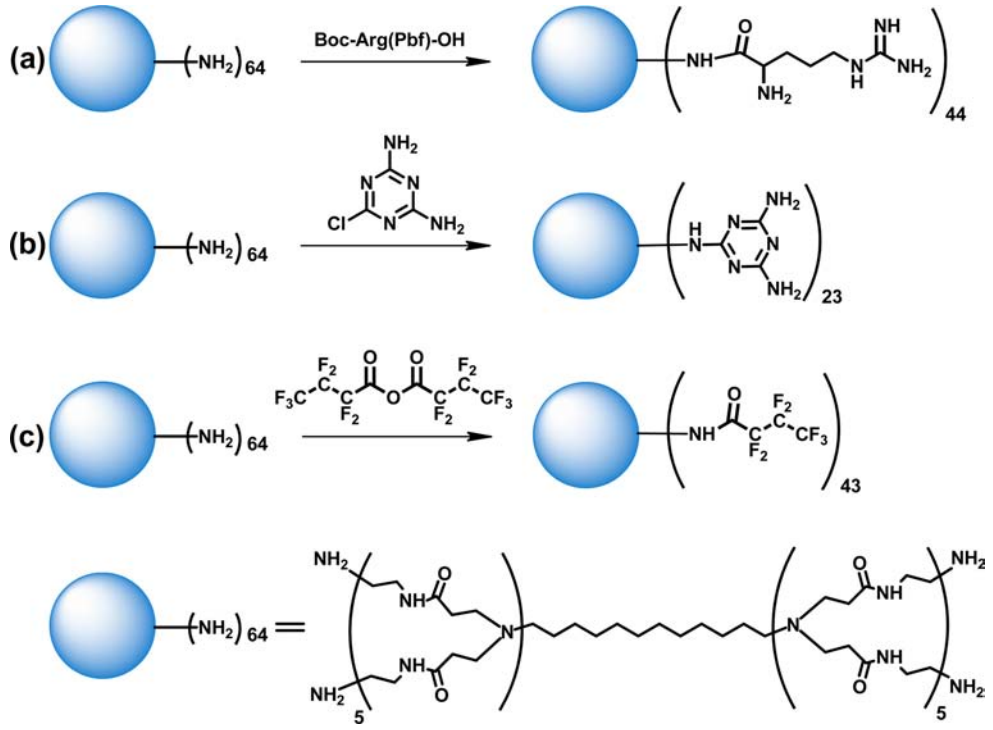
Scheme 2. Synthesis of C12G4-ARG44 (a), C12G4-DAT23 (b), and C12G4-HFA43 (c) Using Facile Strategies



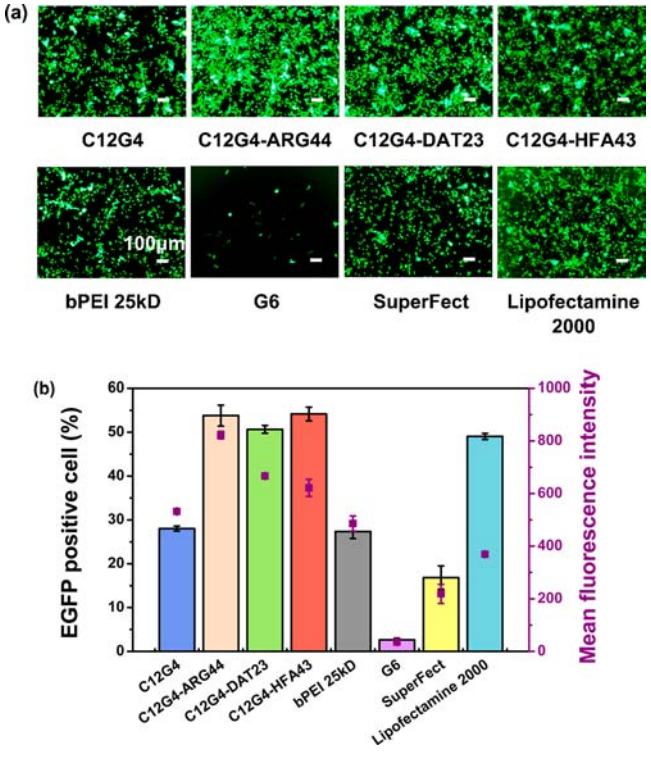


Figure 11. Fluorescent microscopy images of HeLa cells transfected by C12G4, C12G4-ARG44, C12G4DAT23, C12G4HFA43, bPEI 25 kD, G6, SuperFect, and Lipofectamine 2000 for 48 h (a). EGFP plasmid expressions in HeLa cells were observed in green fluorescence. Gene transfection efficiencies of the materials in HeLa cells determined by flow cytometry (b). The N/P ratios for C12G4/DNA, C12G4-ARG44/DNA, C12G4-DAT23/DNA, C12G4-HFA43/DNA, bPEI 25 kD/DNA, and G6/DNA polyplexes were 8:1, 16:1, 24:1, 1:1, 8:1, and 10:1, respectively. SuperFect and Lipofectamine 2000 were used according to the manufacturers' protocols.

fection efficacy at an extremely low N/P ratio of 1:1, which is in accordance with our recent results on fluorinated dendrimers.[53] In addition, these materials exhibited low cytotoxicity at their transfection concentrations (Figure S10). It is worth noting that all the gene transfection experiments in this study are conducted in medium containing 10% FBS, suggesting the excellent serum-resistance of C12G4 and its surface-engineered products in gene delivery. These results clearly demonstrated that surface-engineered C12G4 have potential applications for in vitro and in vivo gene delivery.

CONCLUSIONS

Diaminododecane-cored PAMAM dendrimer C12G4 shows much higher transfection efficacies than C2G4 and C6G4 dendrimers. The diaminododecane core facilitates the internalization of dendrimer/DNA polyplexes while maintaining the DNA condensation, biocompatibility, and endosomal escape capacities of traditional PAMAM dendrimers with an EDA (C2) core. The introduction of the diaminododecane chain to the dendrimer core allows further modification on the dendrimer surface. Functionalization of C12G4 dendrimer with ligands such as ARG, DAT, and HFA further enhances the transfection efficacy of C12G4. These materials show higher transfection efficacies than commercial gene transfection reagents such as SuperFect and Lipofectamine 2000. The diaminododecane-cored dendrimers can be developed as a versatile scaffold in the design of efficient gene vectors.

ASSOCIATED CONTENT

Supporting Information

Further data on characterization of the synthesized materials, polyplexes, and *in vitro* gene transfection results. 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.

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