Gene Delivery

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Self-Assembled Fluorodendrimers Combine the Features of Lipid and Polymeric Vectors in Gene Delivery

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Abstract: *An ideal vector in gene therapy should exhibit high serum stability, excellent biocompatibility, a desired transfection efficacy and permeability into targeted tissues. Here, we describe a class of low-molecular-weight fluorodendrimers for efficient gene delivery. These materials self-assemble into uniform nanospheres and allow for efficient transfection at low charge ratios and very low DNA doses with minimal cytotoxicity. Our results demonstrate that these vectors combine the features of synthetic gene vectors such as liposomes and cationic polymers and present promising potential for clinical gene therapy.*

Gene therapy holds great promise in the treatment of various diseases.[1] Generally, the introduction of therapeutic genes into mammalian cells needs a vector.[2–4] Current vectors can be divided into two categories: recombinant viruses and synthetic vectors.[5] A recombinant virus allows efficient transfection with a very low DNA dose, but is associated with safety and production problems. To address these issues, a variety of synthetic vectors were developed as alternatives to recombinant viruses.[5–10]

Liposomes and cationic polymers are the mostly investigated synthetic vectors. Liposomes have a high affinity to cell membranes. They can easily enter cytoplasm by endocytosis or direct membrane fusion and release the bound DNA by lipid–phospholipid exchange.[11,12] As a result, these materials show high transfection efficacy on cells. However, liposomes have crucial limitations in gene delivery, including difficulties in reproducible liposome fabrication and low permeability in solid tissues as well as toxicity and instability especially for in vivo applications.[5] Cationic polymers have several unique features in gene delivery, for example, facile manufacturing, stable formulation, ease of modification, and high permeability.[13,14] These polymers form nanosized polyplexes with DNA through ionic interactions. However, the polyplexes can be easily destabilized by anionic biomolecules abundant in blood.[15] Although the use of polymers with high molecular weight or high charge density can make the polyplexes more stable, this results in increased cytotoxicity.[16] In addition, relatively low transfection efficacy of polymeric vectors limits their applications in clinical gene therapy. To date, a critical need still remains to develop efficient and safe gene vectors.[2,5]

A solution to this problem is combining the features of lipids and cationic polymers. A commonly used method is the modification of polymers with lipids to generate amphiphilic materials.[17,18] The lipid–polymer conjugates can assemble into micelles and show high affinity to cell membranes.[19–22] In addition, these conjugates can escape from endosomes through a combination of membrane fusion mechanism of lipid vectors and the proton sponge effect of polymers.[23] The materials show improved efficacy in gene delivery compared to unmodified polymers and liposomes. However, the presence of lipids on the materials may lead to increased cytotoxicity and low permeability in target tissues.[5,24] In addition, these lipid-modified polymers show poor serum stability.[25]

Unlike conventional lipids, fluorocarbon chains are both hydrophobic and lipophobic. These chemicals are inert and have a high phase-separation tendency in both water and the lipid bilayer.[26,27] These properties can solve the instability problems of liposomes in biological systems. Modification of polymers with fluorous compounds can improve their transfection efficacy.[28] More importantly, fluorocarbon chains can associate with each other through the fluorophilic effect.[26,29,30] Percec et al. found that replacement of lipids on a polymer with fluorous lipids causes drastic changes in the assembled structures.[31] Fluorine makes a difference in the self-assembly process.[32] These features motivate us to design self-assembled fluorous polymers as efficient gene vectors.

Dendrimers are a class of synthetic polymers which are widely used as gene vectors.[33–38] Here, we synthesized a series of low-molecular-weight fluorodendrimers with promising advantages in gene delivery, for example, drastically improved transfection efficacy at very low DNA doses, excellent serum stability, and minimal toxicity.

Generation 1 (G1) and G2 polyamidoamine (PAMAM) dendrimers were reacted with heptafluorobutyric anhydride to generate fluorodendrimers (Scheme 1). The obtained materials are termed G*n*-F7$_x$, in which "*n*" represents the dendrimer generation and "*x*" denotes the average number of conjugated heptafluorobutyric acid (HFA). According to the ^{1}H NMR spectrum and a ninhydrin assay (Figure S1 and Table S1),[39] the materials are termed G1-F7$_{4.9}$, G1-F7$_{5.5}$, G1-F7$_{6.2}$, G2-F7$_{8.8}$, G2-F7$_{9.3}$, and G2-F7$_{11.3}$. The synthesized

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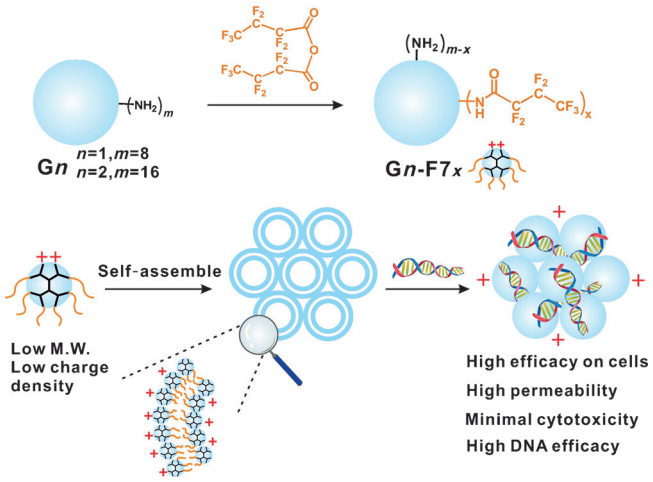

Scheme 1. Self-assembly of fluorodendrimers for efficient gene delivery.

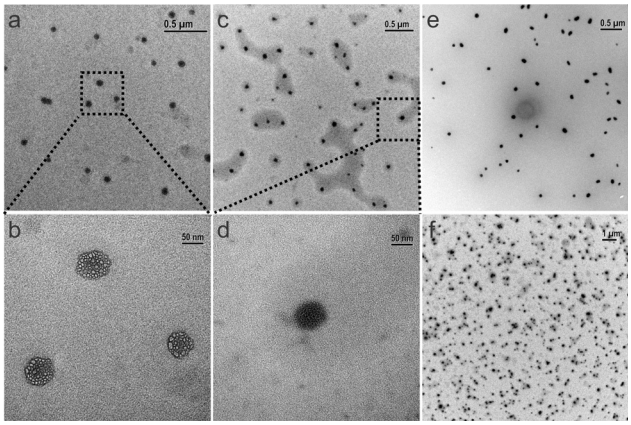

Figure 1. Self-assembly of fluorodendrimers. TEM images of nanostructures assembled from a,b) G1-F7$_{4.9}$, c,d) G1-F7$_{6.2}$, e) G2-F7$_{8.8}$, and f) G2-F7$_{11.3}$. (b) and (d) are magnified images of the marked area in (a) and (c), respectively. Scale bars: 0.5 μm (a, c, e), 50 nm (b and d), 1 μm (f).

fluorodendrimers were also characterized by mass spectrometry (Figure S2).

We further characterized the self-assembled structures of fluorodendrimers by TEM. As shown in Figures 1 and S3, the fluorodendrimers self-assemble into nanospheres in aqueous solutions. The average size of these aggregate structures is listed in Table S2. In comparison, unmodified dendrimers fail to form such nanostructures. These results suggest that HFA modified on the dendrimers contributes to this self-assembly behavior. An increase in HFA number on the G1 dendrimer causes a morphology transition from a porous nanostructure (G1-F7$_{4.9}$, Figure 1b) to a relatively solid one (G1-F7$_{6.2}$, Figure 1d).

Although the G1 dendrimer is positively charged in aqueous solution, the zeta-potential value of its solution (1 mg mL^{-1}) is found to be -9.9 ± 0.8 mV. This is because G1 has a small size ($\approx$1 nm) and is surrounded by counterions such as OH^- in solution. Upon HFA modification, the dendrimer self-assembles into nanospheres and the zeta-potential value turns from negative to around +50 mV for G1-F7$_{4.9}$, G1-F7$_{5.5}$, and G1-F7$_{6.2}$ (Figure S4). The assembled G1-F7$_{6.2}$ nanospheres are more stable than those consisting of G1-F7$_{4.9}$ and G1-F7$_{5.5}$. These molecular characteristics and self-assembly features are important in forming polyplexes with favorable biophysical properties (Figures S5–S7). For example, G2-F7$_{11.3}$ is able to form small uniform polyplexes below 200 nm at a low nitrogen-to-phosphorus ratio (N/P, the nitrogen number equals the number of residual primary amine groups on the materials) of 3.6 (Figure S6a) and the formed polyplexes have low charge densities (around +10 mV, Figure S6b). Uniform polyplexes ensures reproducible gene transfection and a low charge density indicates low cytotoxicity.[11]

In vitro transfection efficacies of fluorodendrimers are tested on HEK293 and HeLa cells. The optimal N/P ratio for each material is screened on both cell lines. As shown in Figures S8 and S9, the optimal N/P ratio for fluorodendrimers significantly decreases with a minimal increase in the number of conjugated HFA. For example, the optimal N/P ratio for G1-F7$_{4.9}$ on HEK293 cells is 63, whereas those for G1-F7$_{5.5}$ and G1-F7$_{6.2}$ are 42 and 9.5, respectively (Figure S8). This phenomenon can be explained by a more stable assembled nanostructure of the fluorodendrimer with a higher fluorine content (Figure S4). Similar results are obtained for G2-F7$_{8.8}$, G2-F7$_{9.3}$, and G2-F7$_{11.3}$, the optimal N/P ratio for G2-F7$_{11.3}$ is as low as 3.6 (Figure S9). A low N/P ratio indicates that a low material concentration is needed for transfection and low charge density on the polyplexes. This feature is very important for efficient gene delivery with low cytotoxicity. After determining the optimal N/P ratio for each material, their efficacies were compared with unmodified G1, G2, and G5 PAMAM dendrimers as well as commercial transfection reagents such as SuperFect, jetPEI, and Lipofectamine 2000 (Lipo2000). As shown in Figures S10 and S11, G1 and G2 dendrimers show a significantly increased EGFP transfection efficacy after HFA modification. The efficacies of G1-F7$_{4.9}$, G1-F7$_{5.5}$, G1-F7$_{6.2}$, and G2-F7$_{11.3}$ are superior to SuperFect and jetPEI, and comparable to Lipo2000. When delivering a luciferase plasmid, the efficacies of the fluorodendrimers are three orders of magnitude higher than those of unmodified G1 and G2 dendrimers. Fluorodendrimers such as G1-F7$_{4.9}$ show high efficacies when delivering both EGFP and luciferase plasmids, in comparison, Lipo2000 shows high EGFP transfection efficacy but a relatively low luciferase efficacy. These results suggest that the fluorodendrimers have promising efficacy in gene delivery.

It is worth noting that the transfection mechanism of low-molecular-weight fluorodendrimers is distinct from fluorinated high-generation dendrimers such as G5-F7$_{68}$. For example, fluorination significantly increases the cellular uptake of G5 dendrimer in G5-F7$_{68}$,[28] but a reverse trend is observed for fluorodendrimers in this study (Figure S12). The fluorodendrimers behave like lipid vectors rather than polymeric vectors, for example, they show weak DNA/vector association (Figures S13 and S14) and low cellular uptake (Figure S15). Relatively weak DNA association is beneficial for efficient DNA unpacking in the cytoplasm, which is a big challenge for polymers with a high charge

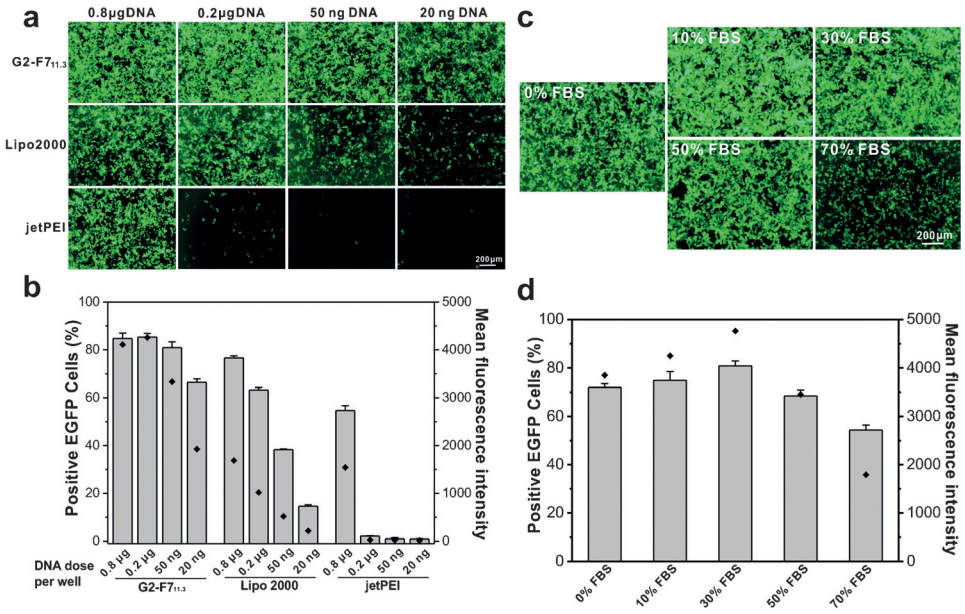

Figure 2. EGFP transfection efficacies of G2-F7$_{11.3}$ in HEK293 cells at a,b) different DNA doses and c,d) different serum concentrations. The G2-F7$_{11.3}$ dose is fixed at 1.8 nmol, Lipo 2000 and jetPEI doses are fixed at 2 μL and 3 μL, respectively. The DNA dose in (c) and (d) was 0.2 μg.

density.[16] Further discussions on the transfection mechanism are available in the Supporting Information.

We also tested the transfection efficacies of fluorodendrimers at low DNA doses. The polymer G2-F7$_{11.3}$, for example, achieves a very high efficacy at extremely low doses of 50 and 20 ng, which is much lower than the dose (0.5–1.0 μg) used in traditional gene transfection (Figure 2 a,b). The mean fluorescence intensity of cells transfected with 50 ng EGFP plasmid approaches that of cells transfected with 0.8 μg plasmid. In comparison, the polymeric vector jetPEI and lipid vector Lipo 2000 show significantly decreased efficacy with decreasing DNA doses. These self-assembled fluorodendrimers behave like viral vectors which have uniform nanostructures and achieve high efficacy at an extremely low DNA dose.

Serum stability is an important parameter for in vivo gene transfection. We further tested the transfection efficacy of fluorodendrimers in medium containing 10–100 % serum. As shown in Figure 2 c,d, G2-F7$_{11.3}$ maintains dramatic efficacy in the presence of 10–50 % serum. Even in the medium containing 70 % serum, this material still shows relatively high transfection efficacy (> 50 % positive EGFP cells, 59.4 % of EGFP fluorescence intensity in the transfected cells relative to 0 % serum). However, in medium containing 90 % and 100 % serum, the cell growth is significantly inhibited, and therefore the transfection results in these systems are not shown. The high serum stability of G2-F7$_{11.3}$ suggests that it is a promising vector for in vivo gene delivery.

A vector for gene therapy should have both desirable transfection efficacy and high permeability in targeted tissues. We further compare the efficacies of fluorodendrimers and Lipo 2000 on 3D spheroids. Unlike conventional cells cultured in a monolayer, the 3D cell culture preserves specific biochemical and morphological features of corresponding tissues in vivo. The use of 3D cell culture can predict in vivo gene transfection efficacy of a material.[40] It represents a powerful tool for a better understanding of the influence of the cellular microenvironment on tumor biology. As shown in Figure S16, G1-F7$_{4.9}$ and G2-F7$_{11.3}$ exhibit much higher efficacies on HEK293 3D spheroids than Lipo 2000. Even at a low dose of 0.3 μg DNA, the fluorodendrimers are more efficient than Lipo 2000 at 0.8 μg DNA. Besides the transfection efficacy, the fluorodendrimers show a higher ability to penetrate the interior of spheroids than Lipo 2000 (Figures 3 a and S17). Lipid vectors are considered to fuse with cell membranes during gene delivery. These liposomes have a high ability to transfect cells on the surface of 3D spheroids, but fail to transfect interior cells due to the disassembly of liposome/DNA complexes when fusing with cell membranes. Therefore, lipid gene vectors show low permeability in 3D spheroids. On the contrary, fluorodendrimers maintain the

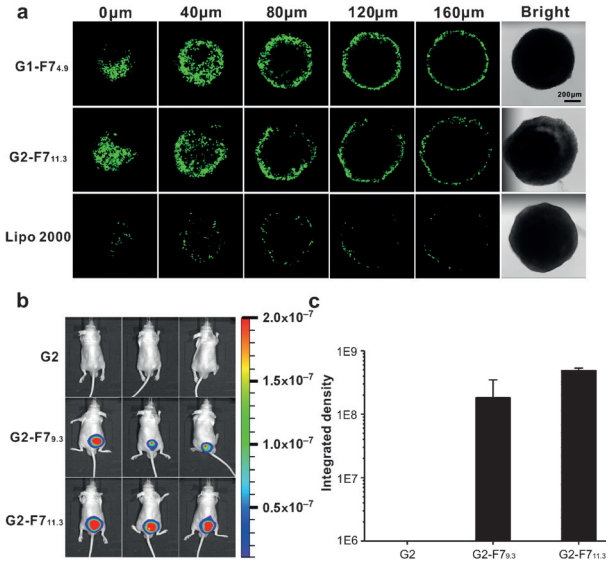

Figure 3. Transfection efficacies of fluorodendrimers on 3D cell cultures and tumor-bearing mice. a) EGFP expressions on the HEK293 3D spheroids are observed by confocal microscopy. N/P = 63 for G1-F7$_{4.9}$ and 3.6 for G2-F7$_{11.3}$. Lipo 2000 is used as a control (2.5 μL per 1 μg DNA). DNA dose was 0.5 μg. Scale bar: 200 μm. b) Bioluminescence images of HeLa tumor-bearing mice transfected with G2/DNA (N/P = 40), G2-F7$_{9.3}$/DNA (N/P = 18), and G2-F7$_{11.3}$/DNA (N/P = 3.6) complexes containing 10 μg luciferase plasmids. c) Photon flux in (b) (n = 3).

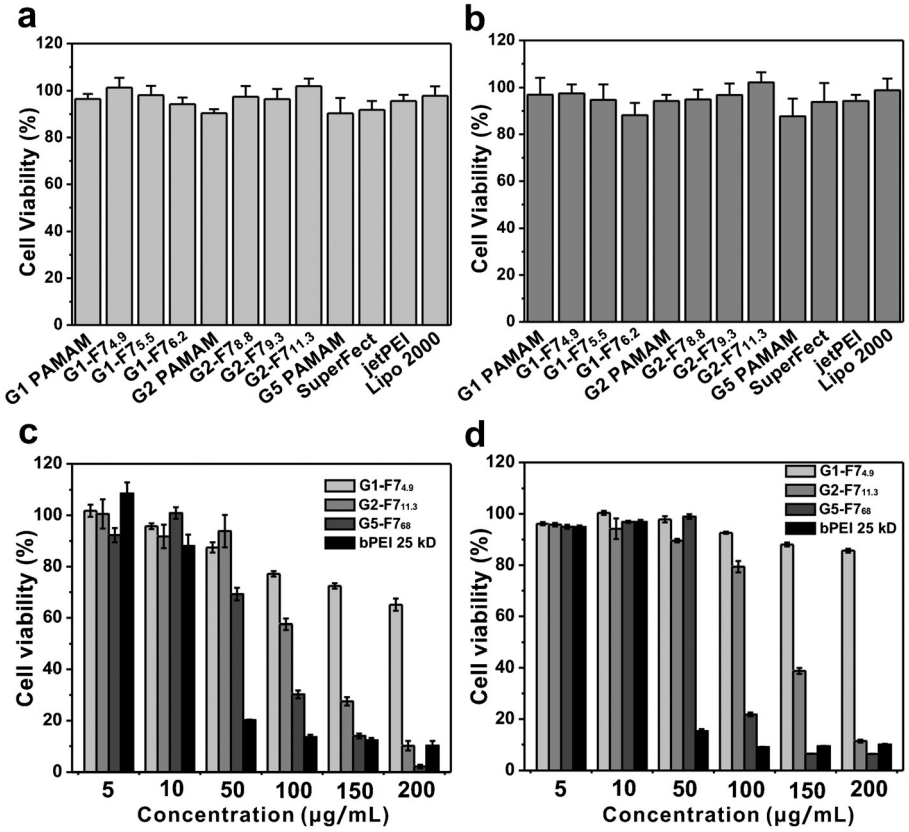

Figure 4. Cytotoxicity of fluorodendrimers. Cell viability of fluorodendrimers and control materials on a) HEK293 and b) HeLa cells at concentrations equal to those in optimized transfection experiments (HEK293 cells: N/P = 63, 42, 9.5, 24, 18, and 3.6 for G1-F7$_{4.9}$, G1-F7$_{5.5}$, G1-F7$_{6.2}$, G2-F7$_{8.8}$, G2-F7$_{9.3}$, and G2-F7$_{11.3}$, respectively; HeLa cells: N/P = 83, 36, 11, 28, 18, and 2.4 for G1-F7$_{4.9}$, G1-F7$_{5.5}$, G1-F7$_{6.2}$, G2-F7$_{8.8}$, G2-F7$_{9.3}$, and G2-F7$_{11.3}$, respectively). Comparison of fluorodendrimers with bPEI 25kD and G5-F7$_{68}$ with regard to cytotoxicity on c) HEK293 and d) HeLa cells.

high permeability of cationic dendrimers. The fluorodendrimers also show high in vivo transfection efficacy. The HeLa tumor xenograft mice were administered with polymer/DNA polyplexes containing 10 μg luciferase plasmids by injection into the tumor. As shown in Figure 3b and 3c, G2-F7$_{9.3}$ and G2-F7$_{11.3}$ successfully express luciferase in the tumor, whereas the G2 dendrimer failed to transfect the gene in the same model. These fluorodendrimers have potential applications in cancer gene therapy.

We further investigated the cytotoxicity of the fluorodendrimers by an MTT assay. As shown in Figure 4a,b, the fluorodendrimers show minimal cytotoxicity. In addition, these fluorodendrimers show much lower cytotoxicity compared to high-molecular-weight polymers such as branched polyethylenimine (bPEI 25kD) as well as G5-F7$_{68}$ (Figure 4c,d). The safe range is essentially extended from 10 to at least 50 μg mL^{-1} using the new low-molecular-weight systems. There is usually a transfection efficacy–cytotoxicity correlation associated with polymeric vectors: high-molecular-weight polymers possess a relatively high transfection efficacy but exhibit serious cytotoxicity, whereas low-molecular-weight polymers show low efficacy and minimal cytotoxicity. In addition, surface modification on the dendrimer reduces its surface charges and diminishes its toxicity. The synthesized fluorodendrimers here are based on low-molecular-weight dendrimers and have a low surface-charge density and thus show minimal toxicity on the transfected cells.

In summary, we synthesized a series of fluorodendrimers and used them as non-viral gene vectors. These materials are able to self-assemble into uniform nanospheres, and have low molecular weight, low charge densities, and relatively weak DNA associations. They show high transfection efficacy in vitro and in vivo, high permeability in 3D spheroids, minimal toxicity, and high serum stability. In addition, the fluorodendrimers combine the features of lipid and polymeric gene vectors. This study provides a new strategy to design efficient gene vectors with low cytotoxicity.

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