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ARTICLE TYPE

Fluorinated Dendrimer for TRAIL Gene Therapy in Cancer Treatment

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The delivery of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) gene into cancer cells is a promising strategy for cancer treatment. However, the low transfection efficacy and/or the toxicity of vectors severely hamper the translation of TRAIL gene therapy to clinics. In this article, we employed our recently developed fluorinated dendrimer as a vector to deliver plasmid encoding TRAIL (pTRAIL) into cancer cells for cancer treatment, which holds the superiorities of both excellent transfection efficacy and low toxicity. Fluorinated poly (amidoamine) dendrimer (G4-$F7_{35}$) represented much higher TRAIL gene transfection efficacy than a series of transfection reagents including poly (ethylene imine) (PEI), SuperFect and Lipofectamine 2000, leading to a much higher cell apoptosis efficacy. G4-$F7_{35}$/pTRAIL complex, compared with PEI/pTRAIL, could more efficiently destroy three-dimensional multicellular spheroids consisted of MDA-MB-231 cells, and suppress the tumor growth in vivo. Furthermore, G4-$F7_{35}$ showed minimal toxicity in vitro and undetectable systematic toxicity in vivo. From this study, fluorinated dendrimer offers a promising prospect for TRAIL gene therapy.

Introduction

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-related apoptotic pathway is most promising for cancer treatment.[1-5] TRAIL induces significant apoptosis in cancer cells but not in normal ones except hepatocyte and keratinocyte,[6-8] since the death receptors are preferentially expressed in cancer cells, and the decoy ones in normal cells,[9, 10] which not only compete with the death receptor for binding to TRAIL but also suppress the death receptor-mediated apoptosis.[11] The preclinical studies suggest that the recombinant TRAIL (rTRAIL) could efficiently suppress TRAIL-sensitive tumors without lethal systematic toxicities.[1, 12-14] In the clinical trials, rTRAIL also shows excellent anti-tumor activity and synergetic effect with other therapeutic strategies such as chemotherapy[15] and radiotherapy,[16]and no major toxicities.[17]

Nevertheless, the systematic administration of TRAIL, typically via intravenous injection, is confronted with the disadvantages including short blood circulation time and inefficient TRAIL accumulation in tumor site.[18-20] Gene therapy is a promising alternative to overcome these obstacles, [21-25] since delivering TRAIL gene into cancer cells allows a continuous and sufficient expression of TRAIL in cancer cells and then efficiently killing of cancer cells. To date, a large number gene vectors of including viral ones such as adenovirus[26-28] and non-viral ones such as poly (amine-co-ester) terpolymers[29] and triazine-modified dendrimer[30] have been employed for TRAIL gene therapy. The viral vectors shows high transfection efficacy but are restricted with the safety issues related to immunogenicity and genetic toxicity,[31-34] while non-viral vectors have attracted much more attentions due to their limited immunogenicity, flexibility in synthesis and functionalization, degradable components and the additional capability for co-delivery with drugs.[35-38] However, the non-viral vector-mediated TRAIL gene therapy is hampered by the low transfection efficacy and the toxicity of vectors. [39-42] Therefore, to develop advanced non-viral vectors with high transfection efficacy and minimal toxicities for TRAIL gene therapy is urgent.

Recently, we developed a category of fluorinated dendrimers with high gene transfection efficacy and minimal toxicity, [30, 43-36] some of which surpass the transfection efficacy of commercial reagents including jetPEI, PolyFect and Lipofectamine 2000 (Lipo 2000). Fluorination on dendrimers allows gene transfection at extremely low nitrogen to phosphorus (N/P) ratios due to enhanced cell uptake of fluorinated dendrimer/DNA complexes and their escape from endosomes.[43] In this article, we synthesized a heptafluorobutyric acid modified generation 4 (G4) poly (amidoamine) (PAMAM) dendrimer (G4-$F7_{35}$) as a vector to deliver plasmid encoding TRAIL (pTRAIL) for cancer treatment. The DNA binding capability of G4-$F7_{35}$ was assessed and its transfection efficacy was primarily evaluated using plasmid encoding enhanced green fluorescent protein (pEGFP). The transfection efficacy and anti-proliferation efficacy of G4-$F7_{35}$/pTRAIL complex was first determined on MDA-MB-231 cells under an optimized transfection condition with minimal vector toxicity. Furthermore, G4-$F7_{35}$/TRAIL was tested on three-dimensional multicellular spheroids (3D-MCSs) consisted of MDA-MB-231 cells for their tumor-suppressing capability in vitro, and finally G4-$F7_{35}$/TRAIL was administrated in a MDA-MB-231 tumor-bearing model to evaluate its antitumor capability and systematic toxicity in vivo.

Experimental

Materials and Methods

G4 PAMAM dendrimer with ethylenediamine-core and amine-terminations was purchased from Dendritech, Inc. (Midland, MI).

Poly (ethylene imine) (PEI, branched, Mw~25000 Da) and heptafluorobutyric anhydride were purchased from Sigma Aldrich (St Louis, MO). Lipofectamine 2000 (Lipo 2000) was purchased from Invitrogen (Carlsbad, CA). jetPEI was obtained from Polyplus-Transfection (France). SuperFect and PolyFect were purchased from Qiagen (Germany). Methanol was obtained from Aladdin Industrial Co., Ltd (Shanghai, China). pTRAIL was provided by prof. Chen Jiang in Fudan University, China. Ethidium bromide and 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) was purchased from Sangon Biotech Co., Ltd (Shanghai, China).

Synthesis and characterization of G4-F7$_{35}$

G4-F7$_{35}$ was synthesized according to the method reported in our previous studies.[46] Briefly, heptafluorobutyric anhydride and G4 PAMAM dendrimer was mixed in methanol at a molar ratio of 32:1, and then was stirred at room temperature for 48 h. The reaction solution was dialyzed against PBS buffer and distilled water, and then was lyophilized at vacuum to obtain a gel-like product. The obtained product was characterized by matrix-assisted laser desorption ionization-time of flight mass spectroscopy (MALDI-TOF MS, Shimadzu), a ninhydrin assay and ^{19}F NMR (500.132 MHz).

Preparation and characterization of G4-F7$_{35}$/DNA complexes

G4-F7$_{35}$ and G4 PAMAM dendrimer were mixed with 0.8 μg pEGFP or pTRAIL at different N/P ratios (0:1, 0.5:1, 1:1, 2:1, 4:1 and 8:1) for 30 min, respectively. N denotes the number of residual primary amines on the dendrimer, while P represents that of phosphate anions in the DNA chains. The samples were run in 1% agarose gels at 90 V and then stained by ethidium bromide for 1 h. The sizes and zeta potentials of the as-prepared transfection reagent/DNA complexes were characterized at 25 °C by dynamic light scattering spectrometer (DLS, Zetasizer Nano ZS90, Malvern Instruments, UK).

Cell culture

MDA-MB-231 cells stably expressing luciferase (MDA-MB-231-luc) a human breast carcinoma cell line, ATCC) were cultured in MEM (GIBCO) containing 10% fetal bovine serum (FBS, GIBCO), 100 units/mL penicillin and 100 μg/mL streptomycin at 37 °C under 5% CO_2. HeLa cells (a human cervical carcinoma cell line, ATCC) were cultured in DMEM (GIBCO) containing 10% FBS, 100 units/mL penicillin and 100 μg/mL streptomycin at 37 °C under 5% CO_2. PC-9 cells (a human lung adenocarcinoma cell line, ATCC) were cultured in RPMI 1640 media (Invitrogen, 10% FBS, 100 μg/mL streptomycin) at 37 °C under 5% CO_2. 3D MCSs of MDA-MB-231 cells were cultured in MEM (10% FBS, 100 units/mL penicillin and 100 μg/mL streptomycin) in agarose coated 96-well plates with one spheroid per well at 37 °C under 5% CO_2.

Gene transfection of G4-F7$_{35}$

HeLa or MDA-MB-231-luc cells were seeded in 24-well plates with a density of 5×10^4 cells per well, and then were incubated at 37 °C for 24 h. G4-F7$_{35}$/pEGFP (N/P = 2, 0.8 μg pEGFP) in 250 μL culture medium was incubated with the cells for 6 h, and then was replaced by 500 μL fresh medium. 48 h later, the expression of EGFP in the cells was observed by using fluorescence microscope (Olympus, Japan) and quantitatively analyzed by flow cytometer (BD FACSCalibur, San Jose). G4 PAMAM (N/P = 8:1), PEI (N/P = 8:1) and commercial transfection reagents including Lipo 2000 (1.5 μL), SuperFect (0.8 μL), PloyFect (0.8 μL) and jetPEI (0.8 μL) were used as controls. The optimal transfection conditions for these reagents were screened before comparing with G4-F7$_{35}$. Three repeats were conducted for each reagent.

In vitro cancer-cell-killing by G4-F7$_{35}$/pTRAIL

MDA-MB-231-luc cells were seeded in 96-well plates with a density of 10^4 cells per well and then incubated at 37 °C for 24 h. The cells were then treated with G4-F7$_{35}$/pTRAIL (N/P = 2, 0.2 μg pTRAIL) in 100 μL MEM for 48 h. The viability of MDA-MB-231-luc cells was determined by the standard MTT assay. The naked pTRAIL (0.2 μg) and G4 PAMAM, PEI, Lipo 2000, SuperFect, PolyFect, and jetPEI, and their complexes with pTRAIL were used as controls. The optimal transfection conditions for these reagents were screened before comparing with G4-F7$_{35}$. Five repeats were conducted for each treatment. This assay was further conducted on HeLa and PC-9 cells.

For the apoptosis detection assay, MDA-MB-231-luc cells were seeded in 12-well plates with a density of 10^5 and then cultured at 37 °C for 24 h. The cells were incubated with naked pTRAIL, G4-F7$_{35}$/pTRAIL, Lipo 2000/pTRAIL, and PEI/pTRAIL for 24 h. The cells were then stained by Annexin V-FITC for 15 min and prodium iodide (PI) for 5 min according to the standard protocol of apoptosis kit (BD Biosciences), and then quantitatively analyzed by flow cytometer.

Real-time reverse transcription quantitative PCR (RT-qPCR)

The expression of TRAIL mRNA in the MDA-MB-231 and HeLa cells were analyzed by RT-qPCR using TRAIL specific primers (TRAIL-forward: 5'-GACAAGGTGTACGTGAACACG -3'; TRAIL-reverse: 5'-CCACACTGTGTCGCCGTAG -3'). Total RNA was isolated from the transfected cells and reverse-transcribed into cDNA using a cDNA Synthesis Kit (TOYOBO, Osaka, Japan). The cDNA was subjected to RT-qPCR analysis targeting TRAIL and 18S using an SYBR Green Real time PCR Master Mix (TOYOBO, Osaka, Japan). The data were normalized to 18S as the endogenous reference (18S-forward: 5'-GGACACGGACAGGATTGACA-3'; 18S-reverse: 5'-GACATCTAAGGGCATCACAG-3'), and relative to that of untreated cells.

Western-blot analysis

MDA-MB-231 cells were seeded at a density of 2×10^5 cells per well in a 6-well plate. After incubation for 24 h, the cells were treated with naked pTRAIL, G4-F7$_{35}$/pTRAIL, Lipo 2000/ pTRAIL and PEI/pTRAIL at their optimal transfection conditions for 24 h (0.8 μg DNA in each well; N/P = 2 for G4-F7$_{35}$/pTRAIL, 1.5 μL for Lipo 2000/pTRAIL and N/P = 8 for PEI/pTRAIL, respectively). Cells without treatment served as negative control. The cells were then collected for Western-blot analysis. Generally, 50 mg protein per lane was separated by SDS–PAGE under reducing conditions, transferred onto a PVDF membrane and blocked with 7% milk. The membrane was probed with

antibodies of TRAIL (Santa Cruz), PARP (Cell Signaling Technology) and cleaved PARP (Cell Signaling Technology), and with anti-mouse-HRP secondary antibody. The target proteins were visualized using an ECL western-blotting detection system (Upstate Temecula, USA). β-Actin was probed as a loading control.

In vitro tumor suppression on 3D MCSs by G4-F7$_{35}$/pTRAIL

MDA-MB-231-luc 3D MCSs were prepared according to the well-developed hanging-drop method.[47] 3D MCSs with a diameter size of 300 μm were transferred into agarose coated 96-well plates with one spheroid per well and divided into four groups with ten MCSs in each one. 3D MCSs were then treated with fresh medium, naked pTRAIL, G4-F7$_{35}$/pTRAIL (N/P = 2) and PEI/pTRAIL (N/P = 8), respectively. 0.8 μg pTRAIL was used for each MCS. The culture media were replaced by the same treatment every 48 h for 3 times. The spheroid diameters were measured according the optical images every day, and the spheroid volumes were determined according to the formula: volume = long diameter × short diameter^2 / 2.

In vivo tumor suppression by G4-F7$_{35}$/pTRAIL

4-week-old male BALB/c nude mice with an average body weight of 20 g were purchased from SLAC Laboratory Animal Co. Ltd. (Shanghai, China). The animals were housed under specific pathogen-free condition. All the animal experiments were carried out according to the National Institutes of Health guidelines for care and use of laboratory animals and approved by the ethics committee of East China Normal University. The MDA-MB-231-luc tumor-bearing model was established by the subcutaneously injecting 10^6 MDA-MB-231-luc cells in 200 μL PBS into the back of mice. After the tumors grew up to a size of 100 mm^3, the mice were randomly divided into four groups (seven mice in each group), and then were injected with 5% glucose in distilled water, and pTRAIL, PEI/pTRAIL (N/P = 8) and G4-F7$_{35}$/pTRAIL (N/P = 2) suspended in 5% glucose solution, respectively into the tumor. 10 μg pTRAIL was used for each mouse. The treatments were performed every 48 h for 3 times. The tumor sizes, the body weights of mice, and the numbers of survival mice were recorded every day. The observation and measurements were continued for 30 days. The tumor sizes were measured using a digital caliper and the tumor volumes were calculated according to the formula: tumor volume = long diameter × short diameter^2 / 2.

Histopathological examination

The mice with different treatments were sacrificed after experiment, and their major organs including heart, liver, spleen, lung and kidney were harvested and fixed in 4% formalin solution at room temperature for 48 h. The organ tissues were then embedded in paraffin blocks and sectioned into slices with a thickness of 4 μm, and then were mounted on glass slides and stained with hematoxylin and eosin (H&E), respectively. The staining tissue sections were examined by using optical microscope. The tumor tissues of mice in the four groups were also harvested and processed with TUNEL staining. Tumor tissues were fixed in 4% formalin solution at room temperature for 48 h, and then were embedded in paraffin and sectioned into 4 μm slices. The tumor sections were incubated with proteinase K,

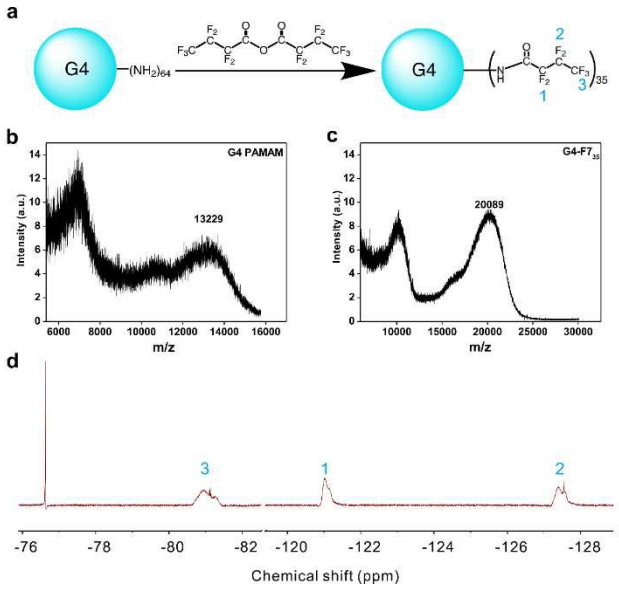

Fig.1 Synthesis and characterization of G4-F7$_{35}$. (a) The synthesis procedure of G4-F7$_{35}$. (b) and (c) MALDI-TOF-MS spectra of G4 PAMAM (b) and G4-F7$_{35}$ (c). (d) ^{19}F NMR spectrum of G4-F7$_{35}$. The marked peaks in (d) corresponding to the different fluorine atoms shown in (a).

TUNEL reaction solution and Hoechest 33342 according to the standard protocol of in situ apoptosis detection kit (Roche, Mannheim Germany). The apoptotic cells in the sections were detected by using a fluorescence microscope.

Results and discussion

Characterizations of G4-F7$_{35}$ and its complex with DNA

G4-F7$_{35}$ was synthesized according to the method reported in our previous study (Fig. 1a).[46] The accurate number of fluorous chains on G4 PAMAM dendrimer was determined by MALDI-TOF-MS and ninhydrin assay. As shown in Fig. 1b and c, the average molecular weight for unmodified G4 PAMAM dendrimer and fluorinated G4 dendrimer was measured to be 13229 Da and 20089 Da, respectively, which suggests an average number of 35 heptafluorobutyric acids were modified on the surface of each G4 PAMAM dendrimer. The conjugate number calculated from ninhydrin assay is 36, which is consistent with the MS result. The product was termed G4-F7$_{35}$ according to the number calculated from MS. The ^{19}F NMR spectrum further protect DNA from digestion and facilitate the cell uptake of transfection reagent/DNA complexes. The agarose gel electrophoresis suggests that G4-F7$_{35}$ completely retained pEGFP and pTRAIL at N/P ≥ 4, while G4 PAMAM dendrimer did that above 1 (Fig. 2a and S3). Moreover, DLS analysis confirmed the successful conjugation of heptafluorobutyric acid on G4 PAMAM dendrimer (Fig. 1d).

The transfection efficacy for polymeric reagents is determined by a series of aspects related to DNA condensation and protection, serum stability, cellular uptake efficiency, endosome escape, DNA unpacking, and nuclear entry.[47] The precondition for polymeric reagents is to condense DNA into a reasonable size to protect DNA from digestion and facilitate the cell uptake of transfection reagent/DNA complexes. The agarose gel

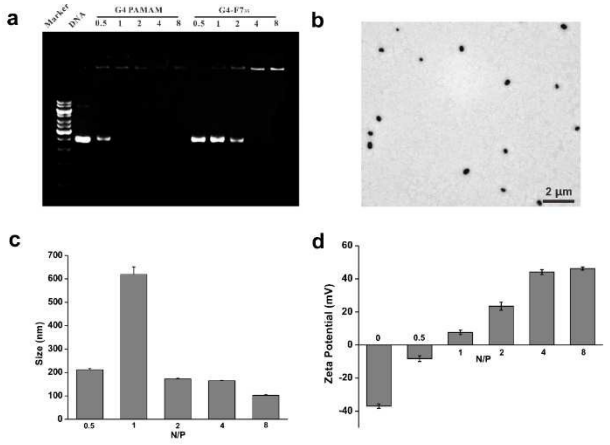

Fig.2 (a) Gel retardation assay to determine pTRAIL binding capacity of G4 PAMAM and G4-F7$_{35}$. (b) TEM images of G4-F7$_{35}$/pTRAIL. (c) Hydrodynamic diameter and (d) zeta potential of G4-F7$_{35}$/pTRAIL prepared at different N/P ratios. Error bars represent the s.e. (n = 3).

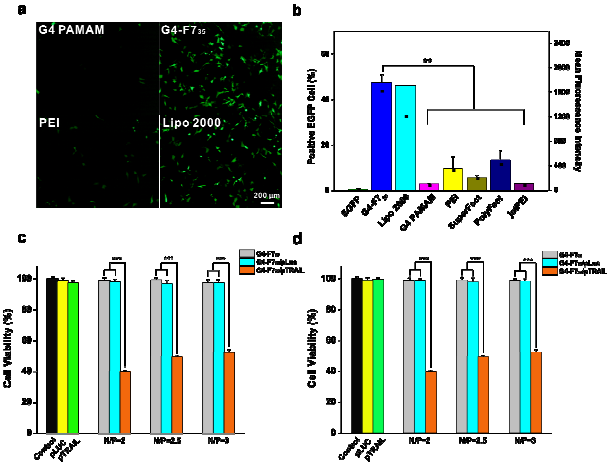

Fig.3 (a) EGFP expressions in MDA-MB-231 cells transfected by G4 PAMAM, G4-F7$_{35}$, PEI and Lipo 2000. (b) Quantitatively analysis of EGFP transfection efficacy of G4-F7$_{35}$ in MDA-MB-231-luc cells by flow cytometry. pEGFP, G4 PAMAM, PEI, Lipo 2000, SuperFect, PolyFect and jetPEI were used as controls. Square in (b) represents the mean fluorescence intensity. Three repeats were conducted for each transfection. Error bars represent the s.e. (n = 3). **p < 0.01 analyzed by student's t-test. Cell viability of HeLa (c) and PC-9 (d) cells transfected with pLUC or pTRAIL by G4-F7$_{35}$ at different N/P ratios. Error bars represent the s.e. (n = 5). ***p < 0.001 analyzed by student's t-test.

electrophoresis suggests G4-F7$_{35}$ completely retained pEGFP and pTRAIL at N/P ratio above 4, while G4 PAMAM dendrimer did that above 1 (Fig. 2a and S3). Moreover, DLS analysis indicates that G4-F7$_{35}$ efficiently condensed DNA (pEGFP or pTRAIL) into small nanoparticles with diameter size below 200 nm at N/P > 2 (Fig. 2c and S2b), while G4 PAMAM dendrimer did that at N/P > 1 (Fig. S1a and S2a). The TEM image taken for G4-F7$_{35}$/pTRAIL confirms the formation of G4-F7$_{35}$/pTRAIL complex with a diameter size of 160 ± 2 nm (Fig. 2b). The decreased DNA-condensing capability for G4-F7$_{35}$ compared with G4 PAMAM dendrimer is mainly due to the reduced positive charges on their surface (Fig. 2d, S1b, S2c and S2d), which however might facilitate the DNA unpacking.

In vitro gene transfection efficacy of G4-F7$_{35}$

The in vitro transfection efficacies of G4-F7$_{35}$ were initially assessed to transfect pEGFP on MDA-MB-231 and HeLa cells. The result shows that G4-F7$_{35}$ had comparable gene transfection efficacy with Lipo 2000, but was much more efficient than G4 PAMAM dendrimer and PEI, and other commercial transfection reagents including SuperFect, PolyFect, and jetPEI (Fig. 3 and S4). Our previous studies have demonstrated that fluorination enhanced cellular uptake, endosome escape, and serum resistance of dendrimer/DNA polyplexes, and moreover, the easier DNA unpacking capability of fluorinated dendrimer compared with the unmodified one might facilitate the intracellular release of DNA from the polyplexes.[43-46] All these superiorities contributed to the significantly enhanced transfection efficacy for G4-F7$_{35}$.

In vitro cancer-cell-killing effect of G4-F7$_{35}$/pTRAIL

TRAIL-mediated extrinsic apoptotic pathway is promising for cancer treatment, since it could selectively induce significant apoptosis in most of cancer cells but not in normal cells. Therefore, G4-F7$_{35}$/pTRAIL was tested for the cancer-cell-killing effect in vitro. A series of transfection reagents including PEI, SuperFect, Lipo 2000, PolyFect, and jetPEI were used as controls, and their optimal transfection conditions were screened before the experiment (Fig. S5), on which the transfection reagents were non-toxic but had the highest transfection efficacy. As shown in Fig. 4a, G4-F7$_{35}$/pTRAIL represented a significant reduction of the cell viability, while the naked pTRAIL, and the control transfection reagents/pTRAIL showed minimal cell toxicity, which suggests that G4-F7$_{35}$ could more efficiently transfect pTRAIL in cancer cells compared with the other transfection reagents. We further conducted the transfection assay on HeLa and PC-9 cells by using G4-F7$_{35}$/pTRAIL. The data show that the viability of cells after the treatment of G4-F7$_{35}$/pTRAIL was significantly reduced, while the cells treated with G4-F7$_{35}$/luciferase plasmid (pLuc) had a comparable viability with untreated cells (Fig. 3c and d). This result confirms that the transfection of pTRAIL by G4-F7$_{35}$ could efficiently reduce the cell viability in different cancer cell lines and also suggests that the cytotoxicity was from the expression of TRAIL but not the transfect reagent, G4-F7$_{35}$, and the transfection process. The cancer-cell-killing effect was further evaluated via Annexin V/PI staining. As shown in Fig. 4b, G4-F7$_{35}$/pTRAIL induced cell death was indeed through the apoptotic pathway. G4-F7$_{35}$/pTRAIL could induce substantial apoptosis of 58.21% in MDA-MB-231 cells, while Lipo 2000/pTRAIL and PEI/pTRAIL could only induce a minimal apoptosis of 10.01% and 6.16%, respectively. This result confirms that G4-F7$_{35}$ could more efficiently transfect pTRAIL in cancer cells.

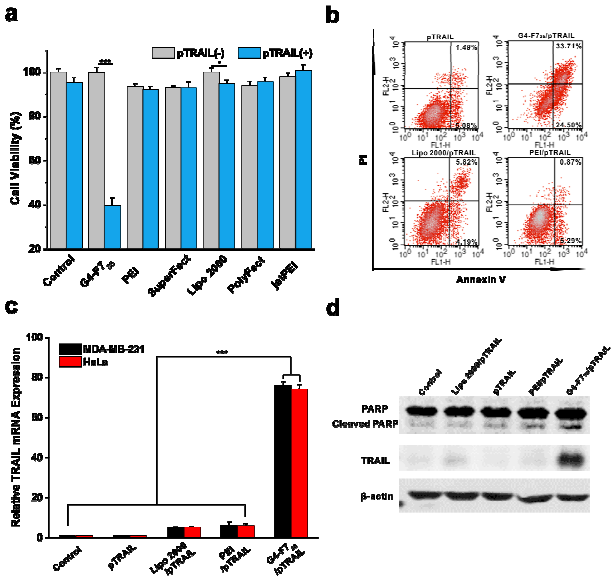

Fig.4 Cancer-cell-killing efficacy of G4-F7$_{35}$/pTRAIL on MDA-MB-231-luc cells. (a) Cell viability of MDA-MB-231-luc cells treated with pTRAIL, G4-F7$_{35}$/pTRAIL, PEI/pTRAIL, Lipo 2000/pTRAIL, SuperFect/pTRAIL, PolyFect/pTRAIL and jetPEI/pTRAIL for 48 h. Equal amounts of gene vector were tested to exclude the material cytotoxicity. Error bars represent the s.e. (n = 5). *p < 0.05, ***p < 0.001 analyzed by student's t-test. (b) Apoptosis analysis of the cells transfected with pTRAIL, G4-F7$_{35}$/pTRAIL, Lipo 2000/pTRAIL, and PEI/pTRAIL for 24 h. The cells were stained by Annexin V/PI and analyzed by flow cytometer. (c) TRAIL mRNA expression in MDA-MB-231 and HeLa cells 24 h after the transfection of pTRAIL by G4-F7$_{35}$, Lipo 2000 and PEI revealed by RT-qPCR. Error bars represent the s.e. (n = 4). ***p < 0.001 analyzed by student's t-test. (d) TRAIL and cleaved PARP expression in MDA-MB-231 cells 24 h after the treatment of pTRAIL, Lipo 2000/pTRAIL, PEI/pTRAIL and G4-F7$_{35}$/pTRAIL revealed by western blot analysis.

To confirm the reduced cell viability caused by the expression of TRAIL, we detected the expression of TRAIL mRNA in MDA-MB-231 and HeLa cells after different transfection treatments by using RT-qPCR. The result shows that both the two cells after the treatment of G4-F7$_{35}$/pTRAIL represented a much higher expression of TRAIL mRNA compared with the ones treated with naked pTRAIL, Lipo 2000/pTRAIL and PEI/pTRAIL (Fig. 4c). Furthermore, we determined the TRAIL expression in MDA-MB-231 cells after the different transfection treatments, which reveals that a much higher TRAIL expression in the cells treated with G4-F7$_{35}$/pTRAIL compared with the ones treated with naked pTRAIL, Lipo 2000/pTRAIL and PEI/pTRAIL (Fig. 4d). A cleaved poly (ADP-ribose) polymerase (PARP) was also observed in the cells treated with G4-F7$_{35}$/pTRAIL (Fig. 4d). These results confirm that G4-F7$_{35}$ could more efficiently transfect pTRAIL in cancer cells, leading to the expression of TRAIL and then cell apoptosis.

In vitro tumor suppression of G4-F7$_{35}$/pTRAIL on 3D MCSs

We further assessed the tumor-suppressing effect of G4-F7$_{35}$/pTRAIL on 3D MCSs. 3D MCSs are widely used to mimic the pathological feature of tumor in vitro. Their unique 3D

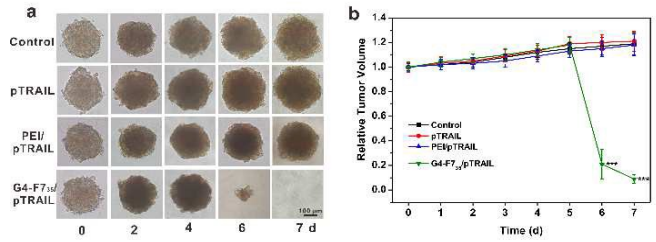

Fig.5 Tumor-suppressing capability of G4-F7$_{35}$/pTRAIL on 3D MCSs. (a) Optical photographs taken for MDA-MB-231-luc MCSs treated with fresh medium, naked pTRAIL, PEI/pTRAIL and G4-F7$_{35}$/pTRAIL at different time points. (b) The size evolution of 3D MCSs shown in (a). Error bars represent the s.e. (n = 10). ***p < 0.001 by analyzed by student's t-test.

structure restricts both of the oxygen and nutrient diffusion into the interior of MCSs, leading to cancer cells inside of MCSs are majorly dormant and necrotic. The dormant cells are generally arrested in the G1 phase,[48] which are difficult to be transfected. Moreover, the limited intercellular gap in MCSs also hampers the diffusion of gene complex into their core region. In this case, MDA-MB-231-luc MCSs with an initial diameter size of 300 μm were divided into four groups and then treated with culture

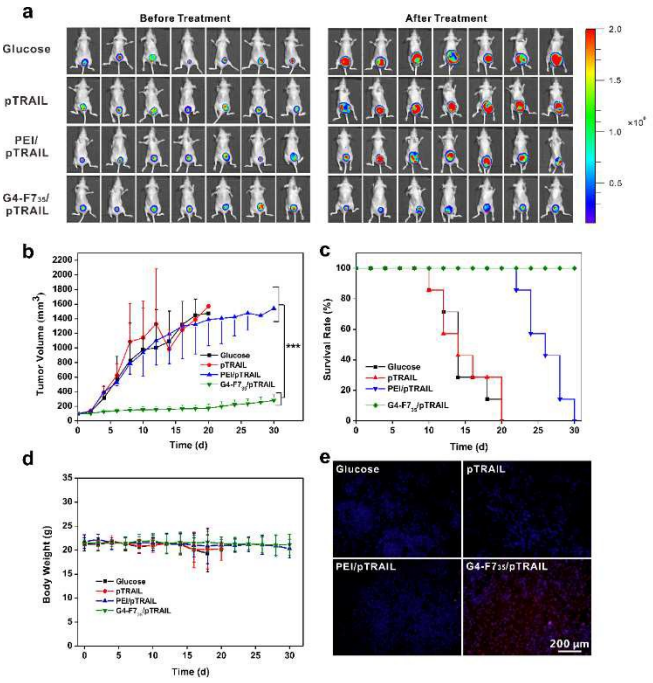

Fig.6 Antitumor capability of G4-F7$_{35}$/pTRAIL in vivo. (a) Luminescence images of MDA-MB-231-luci tumor-bearing mice before and after the dissimilar gene therapeutic treatments. (b-d) Time-elapsed evolution of tumor sizes (b), the survival rate of mice (c), and the body weight changes of mice (d) during the therapeutic period. Error bars in (b and d) represent the s.e. (n = 7). ***p < 0.001 by analyzed by student's t-test. (e) Apoptosis in the tumor tissues with different treatments. The apoptotic cells (red) were detected via TUNEL method. The cell nuclei were labelled with Hoechst 33342 (blue).

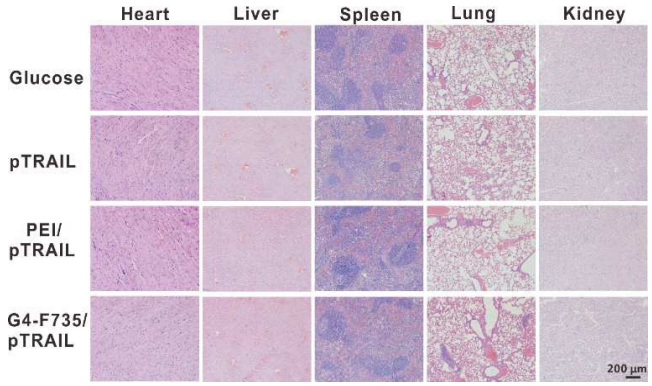

Fig.7 Histological examination of the main organs of MDA-MB-231-luci tumor-bearing mice after treatment with 5% glucose, naked pTRAIL, PEI/pTRAIL, and G4-F7$_{35}$/pTRAIL by H&E staining.

medium, naked pTRAIL, G4-F7$_{35}$/pTRAIL, and PEI/pTRAIL every 48 h for 3 times. MCSs in G4-F7$_{35}$/pTRAIL group became loose and broken down at 6th day and completely degraded at 7th day, while the ones in culture medium group grew up to 1.2 time of their initial average volume without any structure rupture, and the ones in naked pTRAIL and PEI/pTRAIL group grew up with the similar size and density with the ones in culture medium group. These results suggest that G4-F7$_{35}$ could efficiently transfect pTRAIL in 3D MCSs and plus suppress their growth.

In vivo tumor suppression by G4-F7$_{35}$/pTRAIL

The in vivo antitumor efficacy of G4-F7$_{35}$/pTRAIL was determined on a breast-cancer-bearing model. MDA-MB-231-luc tumor-bearing mice with an average tumor size of 100 mm^3 were randomly divided into four groups, and were intratumorally administrated with 5% glucose, and naked pTRAIL, PEI/pTRAIL, and G4-F7$_{35}$/pTRAIL in 5% glucose every 48 h for 3 times, respectively. The luminescence images of mice in four groups were recorded before and after treatments. As shown in Fig. 6a, the tumor-site luminescence intensities in G4-F7$_{35}$/pTRAIL group were significantly reduced compared with that in the glucose, naked pTRAIL, and PEI/pTRAIL groups, suggesting G4-F7$_{35}$/pTRAIL more efficiently suppressed the tumor growth than naked pTRAIL and PEI/pTRAIL. The tumor volumes were recorded every two days during therapeutic period, and mice were euthanized with carbon dioxide gas when the tumor grew up to a size of 1500 mm^3. The time-elapsed evolution of tumor sizes confirms that G4-F7$_{35}$/pTRAIL could more capably suppress the tumor growth than naked pTRAIL and PEI/pTRAIL (Fig. 6b). Furthermore, mice treated with G4-F7$_{35}$/pTRAIL showed prolonged survival time compared to the ones treated with glucose, naked pTRAIL and PEI/pTRAIL (Fig. 6c). The TUNEL assay confirms that a significant apoptosis existed in the tumor tissues treated with G4-F7$_{35}$/pTRAIL, while negligible apoptosis in that treated with glucose, naked pTRAIL, and PEI/pTRAIL. There was no obvious body weight lost for the mice treated with G4-F7$_{35}$/pTRAIL during the therapeutic period (Fig. 6d), and non-detectable pathological changes observed in the H&E staining of main organ tissues of mice after treatment with G4-F7$_{35}$/pTRAIL (Fig. 7), suggesting that G4-F7$_{35}$ was minimally toxic in the systematic treatment. Taken together, fluorinated dendrimer, G4-F7$_{35}$, could efficiently transfect pTRAIL in tumor tissues and consequently suppress the tumor growth.

Conclusions

In summary, we firstly used the fluorinated dendrimer as gene vector to transfect pTRAIL for cancer treatment. We demonstrated that G4-F7$_{35}$, compared with other transfection reagents, could more efficiently transfect pTRAIL in 2D cell culture and 3D MCSs in vitro, and thus G4-F7$_{35}$/pTRAIL could more efficiently kill cancer cells and eradicate 3D MCSs. We further proved that G4-F7$_{35}$/pTRAIL could more efficiently suppress the tumor growth than the naked pTRAIL and PEI/pTRAIL in vivo. The major apoptosis in the tumor tissues treated with G4-F7$_{35}$/pTRAIL confirms that the substantial expression of TRAIL in tumor tissue. It is also demonstrated that G4-F7$_{35}$ showed minimal toxicity in vitro and undetectable systematic toxicity in vivo. This investigation suggests that fluorinated dendrimers are a promising vector for gene cancer therapy.

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