Published in final edited form as:

Biomaterials. 2013 January ; 34(4): 1289–1301. doi:10.1016/j.biomaterials.2012.10.024.

Lipid Modified Triblock PAMAM-Based Nanocarriers for siRNA Drug Co-Delivery

Swati Biswas, Pranali P. Deshpande, Gemma Navarro, Namita S. Dodwadkar, and Vladimir P. Torchilin*

Center for Pharmaceutical Biotechnology and Nanomedicine, 360 Huntington Avenue, 140 The Fenway, Northeastern University, Boston, Massachusetts 02115.

Abstract

RNA interference by short interfering RNA (siRNA) holds promise to attenuate production of specific target proteins but is challenging in practice owing to the barriers for its efficient intracellular delivery. We have synthesized a tri-block co-polymeric system, poly(amidoamine) dendrimer (generation 4)-poly(ethylene glycol)-1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (G(4)-D-PEG-2K-DOPE). G(4)-PAMAM dendrimer was utilized as a cationic source for efficient siRNA condensation; DOPE provided optimum hydrophobicity and compatible cellular interaction for enhanced cell penetration; PEG rendered flexibility to the G(4)-D for easy accessibility of siRNA for condensation; PEG-DOPE system provided stable micellization in a mixed micellar system. G(4)-D-PEG-2K-DOPE was incorporated into the self-assembled PEG-5K-PE micelles at a 1:1 molar ratio. Our results demonstrate that the modified dendrimer, G(4)-D-PEG-2K-DOPE and the micellar nanocarrier form stable polyplexes with siRNA, shows excellent serum stability and a significantly higher cellular uptake of siRNA that results in target protein down-regulation when compared to the G(4)-PAMAM-dendrimer. Moreover, the mixed micellar system showed efficient micellization and higher drug (doxorubicin) loading efficiency. The G(4)-D-PEG-2K-DOPE has the higher efficacy for siRNA delivery, whereas G(4)-D-PEG-2K-DOPE/PEG-5K-PE micelles appear to be a promising carrier for drug/siRNA co-delivery, especially useful for the treatment of multidrug resistant cancers.

Keywords

Dendrimer; siRNA; PEG-PE; co-delivery

1. Introduction

Small interfering RNA (siRNA), commonly known as post-transcriptional gene silencer, is a short, double-stranded nucleic acid molecule that holds great promise as a potent therapeutic tool due to its efficacy in suppression of the expression of specific disease-related genes by silencing specific complementary mRNA [1–3]. The effect of RNA interference is the down-regulation of the expression of any proteins. With the understanding of the biology of

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*Corresponding author. Vladimir P. Torchilin, Ph.D., D.Sc., Professor and Director, Center for Pharmaceutical Biotechnology and Nanomedicine, Northeastern University, 140 The Fenway, 360 Huntington Ave., Boston, Massachusetts 02115, v.torchilin@neu.edu, Phone. 617-373-3206, Fax. 617-373-7509.

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the sequence-specific gene silencing by the RNA-interference machinery and development of the methodology for the synthesis of siRNA, appropriately synthesized siRNA can silence nearly any gene, thereby, circumvents the limitations of small molecule drugs. However, therapeutic application of this advanced siRNA technology faces challenges due to the barriers associated with effective and non-toxic delivery of siRNA to the site of its action [4]. siRNA requires a delivery system for systemic circulation and transfection into the cells since naked siRNA is degraded by endogenous circulating enzymes and is too large (~13KDa) and negatively charged to cross the cell membrane by diffusion as with small molecule drugs [4–7]. Additionally, non-viral synthetic polymers are preferred as siRNA carriers over viral vectors since they are safer and less toxic [8].

Various non-viral polyamine polymers serving as siRNA carriers have been explored including low and high molecular weight polyethyleneimines (PEIs), poly-L-lysines (PLLs), polyarginines, chitosan, cationic PAMAM dendrimers, cationic liposomes and inorganic nanoparticles as a result of their ability to form stable siRNA-polymer complexes via electrostatic interaction under physiological conditions [4, 9–11]. These polymers not only protect siRNA from enzymatic degradation, they also facilitate endocytosis and endosomal disruption by a proton sponge effect [12]. Moreover, advances in nanotechnology have allowed development of various block copolymers that address some of the disadvantages of cationic polymers such as toxicity and immunogenicity [13].

Cationic dendrimers have found applications as non-viral delivery vectors for siRNA [14–18]. Minko, et al demonstrated efficient intracellular and intratumoral delivery of surface engineered poly(propyleneimine) dendrimers when the siRNA-dendrimer nanoparticles were caged with dithiol-containing cross-linker molecules followed by coating with PEG [14]. The pegylation and caging modifications led to extended systemic circulation. Attachment of targeting peptide at the distal end of the PEG-chain directed nanoparticles specifically to the cancer cells with a resultant improved gene-silencing. RGD-peptide modified PAMAM-G(5)-dendrimers delivered siRNA to glioma model system [16] Hayashi et al demonstrated hepatocyte-selective siRNA delivery by lactosylated dendrimer (G-3)/ α -cyclodextrin conjugates. In another study, PEG-modified PAMAM dendrimers were utilized for *in vitro* siRNA delivery and intramuscular gene silencing [18]. Among the cationic polymers, dendrimers as siRNA delivery system, have been largely unexplored especially for the treatment of cancer.

One of the major adverse effects associated with long-term chemotherapy is multi-drug resistance, mainly due to the over-expression of drug transporter proteins such as P-glycoprotein (P-gp) encoded by the *mdr-1* gene [19, 20]. A nanoparticulate drug delivery system that can simultaneously deliver a chemotherapeutic drug and siRNA to the tumor is emerging as a promising treatment strategy for cancer treatment [21–26]. Wang et al observed the simultaneous delivery of siRNA and paclitaxel via micellar nanoparticles of a biodegradable triblock copolymer PEG-b-poly(ϵ -caprolactone)-b-poly(2-aminoethyl ethylene phosphate) (PEG-b-PCL-b-PPEEA) [25]. In another study, a micellar system was constructed from functionalizable, biodegradable poly(ethylene oxide)-b-PCL (PEO-b-PCL) [26]. The end groups in PCL were functionalized with short polyamines for siRNA condensation, whereas the end group of PEO was functionalized for cancer cell targeting. This multifunctional polymeric system delivered doxorubicin (DOX) and siRNA to the intracellular targets.

For this study, we developed a poly(ethylene glycol)-dioleoylphosphatidyl ethanolamine (PEG-DOPE) modified G(4)-PAMAM nanocarrier for the purpose of drug-siRNA co-delivery. Lipid modification of cationic polymers with fatty acids and cholesterol has been reported to promote efficient transfection [27–30]. In our previous studies, we showed the

efficient delivery of siRNA with phosphatidyl choline and DOPE-modified PEIs compared to unmodified PEI [31, 32]. In addition, the PEG chain was used to impart flexibility to the dendrimers for interaction with siRNA for effective condensation [33–35]. We also developed a unique micellar-dendrimer system, where a newly synthesized construct, G(4)-PAMAM-PEG-2K-DOPE was utilized to prepare a mixed micellar formulation with PEG-5K-PE. This mixed micellar system was expected to (i) have higher micellization efficiency with low CMC and higher drug loading, a fundamental requirement for drug-siRNA co-delivery, (ii) impart higher stability and protection of the condensed siRNA against enzymatic degradation, (iii) have enhanced cell penetration resulting in efficient transfection, (iv) have less cytotoxicity due to PEGylation and less immunogenicity in the systemic circulation. The goal of this report was to demonstrate effective intracellular accumulation of siRNA and drug co-delivered by these systems.

2. MATERIALS AND METHODS

2.1. Materials

PAMAM Dendrimer, ethylenediamine core, generation 4, 10 weight % solution in methanol (G(4)-D) was purchased from Sigma-Aldrich. 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-5000](ammonium salt) (PEG-5K-DOPE), 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE) were purchased from Avanti Polar Lipids (AL, USA). NPC-PEG-2K-NPC was purchased from Laysan Bio (AL, USA). Geneticin (G-418), FAM-labeled negative control siRNA and silencer Cy-3-labeled GAPDH siRNA were purchased from Ambion Life Technologies Invitrogen (NY, USA). siRNA, targeting green fluorescence proteins (siGFP): 5'-AUGAACUUCAGGGUCAGCUdTdT-3' (sense), (36) Non-targeting control siRNA (siNegative): 5'-AGUACUGCUUACGAUACGGdTdT-3' (sense). Nuclease-free water was purchased from Qiagen (MD, USA). Doxorubicin HCl (DOX) was purchased from Sigma-Aldrich. The cellTiter-Blue® Cell Viability Assay was purchased from Promega (WI, USA). Human alveolar adenocarcinoma cell line, A549, mouse yolk sac embryo cells, c-166, stably transfected with a plasmid reporter vector, pEGFP-N1, encoding for the enhanced GFP, were purchased from the American Type Culture Collection (ATCC). Dulbecco's modified Eagle's media (DMEM) and fetal bovine serum (FBS) was obtained from Gibco (Carlsbad, CA). Penicillin-streptomycin solution was obtained from CellGro (VA, USA). Mitotracker deep red FM and Hoechst 33342 were purchased from Molecular Probes Inc. (Eugene, OR). *Para*-formaldehyde was from Electron Microscopy Sciences (Hatfield, PA). Fluoromount-G was from Southern Biotech (Birmingham, AL). The CellTiter 96® Aqueous One Solution Cell Proliferation Assay kit was purchased from Promega (Madison, WI). The Trypan blue solution was obtained from Hyclone (Logan, UT).

2.2. Polymers Synthesis and Characterization

The triblock co-polymer G(4)-D-PEG-2K-DOPE (modified dendrimer, MD) was synthesized following scheme 1. The starting polymer NPC-PEG2K-DOPE was synthesized following the previously reported procedure [34, 37]. Briefly, into the solution of NPC-PEG_{2K}-NPC (1 g, 0.5 mmol) in chloroform, DOPE (37.2 mg, 0.05 mmol) solution in chloroform mixed with 20 µL of triethylamine was added drop wise. The reaction mixture was stirred overnight at room temperature. Following day, the reaction mixture was evaporated using rotary evaporator and freeze dried to remove traces of solvent. The dry crude reaction mixture was dissolved in HCl solution (0.01 M) and purified by gel filtration chromatography using Cl₄B column.

For the synthesis of G(4)-D-PEG_{2K}-DOPE, a methanol solution of G(4)-D was evaporated in a pre-weighed vial and freeze-dried to remove traces of methanol. Into the solution of

G(4)-D in DMF (42.9 mg, 3.02 μMol), and triethylamine (10 μL) was added NPC-PEG-2K-DOPE (7.7 mg, 4.9 μMol) in DMF. The reaction mixture was stirred overnight at room temperature. The DMF was removed by rotary evaporator. The resulting crude reaction mixture was dissolved in water and dialyzed against water using a cellulose ester membrane (MWCO. 12–14,000 KDa) and freeze-dried. G(4)-D-PEG-2K-DOPE was characterized by ^1H nuclear magnetic resonance (NMR) spectroscopy using Varian 400 MHz spectroscope. The starting materials, G(4)-D and NPC-PEG_{2K}-DOPE and the polymer was dissolved in *d*-methanol at 5–10 mg/mL for NMR spectroscopy analysis.

For the labeling of G(4)-D with FITC, into a solution of G(4)-D (42.9 mg, 3.0 μM) in DMF was added FITC-NHS (1.4 mg, 3.0 μM) in DMF. The reaction mixture was stirred at room temperature overnight and the solvent was removed by rotary evaporator, dialyzed (MWCO. 10 KDa) and freeze-dried. The FITC-G(4)-D was used to prepare FITC-MD following the above mentioned procedure. Solid products were dissolved in BHG buffer (5 % glucose in 20 mM HEPES in RNase-free water, pH 7.4) at concentration of 5 mg/mL for all experiments using siRNA.

2.3. Particle Size

Measurement of size and size distribution analysis of G(4)-D and MD were performed by dynamic light scattering (DLS) using a Coulter® N4-Plus Submicron Particle Sizer (Coulter Corporation, Miami, FL). G(4)-D and MD were dissolved in water at 5 mg/mL for analysis of particle size. Size distribution was also confirmed by using a transmission electron microscopy (TEM) (Jeol, JEM-1010, Tokyo, Japan).

2.4. Preparation of Pegylated and DOX-Loaded Nanoparticles

To prepare a PEG-PE micellar system with the triblock co-polymer, MD was mixed with PEG-5K-DOPE at molar ratio of 1:1. This modified dendrimer-micelle (MDM) solution was mixed gently and the volume was adjusted with BHG buffer to obtain the desired concentration. To prepare DOX-loaded nanoparticles D-Dox and MD-Dox, a pre-mixed solution of 1 mg of Dox (1.72 μMol) and 10 μL of triethylamine in methanol was added to the methanol solution of D, MD or a chloroform solution of PEG-PE (3.45 μMol). For the preparation of MDM-Dox, 1 mg of Dox was mixed with a mixture of MD and PEG-PE at an equimolar ratio (1.73 μMol). The organic solvent was evaporated and freeze-dried to remove the traces of solvent. The dry lipid film was hydrated in 500 μL of HBS to obtain a solution of Dox at a concentration of 2 mg/mL. The solutions were passed through a 0.2 μm syringe filter (Nalgene, NY) and dialyzed in HBS in a cellulose ester membrane (MWCO. 12–14,000 KDa) to remove unincorporated Dox.

2.5. Preparation of Dendriplexes, Gel Retardation and Ethidium Bromide Exclusion Assay

G(4)-D and MD were diluted with BHG buffer to 10 μL at varying concentrations (at different N/P ratios) and incubated with 750 ng of siRNA (10 μL) for 20 min at room temperature for the formation of dendriplexes. For N/P calculations, the reported molecular weight and the surface charge of the G(4)-D were taken into consideration ruling out the possibility of incomplete branching. The dendriplexes were subjected to electrophoresis on a 0.8 % agarose gel containing Ethidium Bromide (EtBr), using an E-Gel electrophoresis system (Invitrogen Life Technologies) and visualized under UV light.

The complex-forming ability of polymers with siRNA was examined by a quenching method using EtBr. Free or siRNA complexed with polymers at different N/P ratios was incubated with 12 $\mu\text{g/mL}$ of EtBr (ICN Biochemical, Aurora, OH) and the fluorescence intensity was measured at excitation/emission wavelengths of 540 and 580 nm respectively using 96-well plate reader (Synergy HT, Biotek). Fluorescence of EtBr in the absence of

siRNA was considered background and was subtracted from the readings. The fluorescence of the siRNA-EtBr complex in the absence of polycations was considered as 100 and the % relative fluorescence was determined for different N/P ratios. Finally, the recovery of fluorescence after dissociation of complexes following the addition of heparin sulfate (10 units/ μg of siRNA) was measured.

2.6. Cell Culture

A549 cells and C166-GFP cells were grown in DMEM, supplemented with 10 % fetal bovine serum and antibiotics (For C166-GFP cells, 0.2 mg/mL of Geneticin and for A549 cells, 100 IU/mL of penicillin, streptomycin and 250 ng/mL amphotericin-B) at 37 °C with 5 % CO_2 .

2.7. Flow Cytometry

After the initial passage in T-75 cm^2 tissue culture flasks (Corning Inc., NY), for cellular uptake study of FITC-labeled nanoparticles, A549 cells (0.2×10^6 /well) were seeded in 6-well tissue culture plates. The following day, the cells were incubated with FITC-labeled G(4)-D, MD and MDM ($\sim 1.0 \mu\text{M}$) in 2 mL of serum-free media for 1 h and 4 h incubation periods (The fluorescence was normalized before each experiment). The media were removed, the cells washed several times, trypsinized, suspended in 1 mL PBS and then centrifuged at 1000 RPM for 5 min. The cell pellet was suspended in PBS, pH 7.4 before analysis using a BD FACS Caliber flow cytometer. The cells were gated using forward (FSC-H)-versus side-scatter (SSC-H) to exclude debris and dead cells before analysis of 10,000 cell counts. For the analysis of the quenching of fluorescence by Trypan blue, 10 μL of 0.4 % trypan blue solution was added to 400 μL of cell suspension and analyzed again by FACS.

For determination of siRNA-delivery efficiency, Cy-3 labeled siRNA was mixed with siNegative at a 1:1 mole ratio and used for complexation with G(4)-D, MD and MDM at a N/P ratio of 10. After incubation of dendriplexes for 20 min, the complexes were added to wells seeded with A549 cells in serum-free media. After 2 h incubation, the above mentioned post-incubation steps were performed before FACS analysis. For assessment of Dox-delivery efficiency, the A549 cells were incubated with Dox-loaded nanoparticles at a Dox concentration of 4 $\mu\text{g/mL}$ for 2 h before analysis of the cellular Dox fluorescence by FACS. For assessment of co-delivery efficiency, A549 cells were treated with Dox-loaded G(4)-D, MD and MDM at a Dox concentration of 4 $\mu\text{g/mL}$, complexed with 100 nM of FAM-siRNA in serum-free media. The incubation period was 2 h before analysis of cell associated fluorescence by FACS in FL1 and FL2 channels. The interference of Dox signal in the green channel was compensated by running the samples with Dox-loaded nanoparticles without FAM-siRNA. The representative histogram, the statistics to obtain the geometric mean of fluorescence and quadrant statistics for each cell sample were performed using BD Cell Quest Pro Software for all FACS experiments. For quadrant statistics, the axis, FL1-H and FL-2H, represent the FAM-siRNA and Dox labeling, respectively.

2.8. Cytotoxicity

Cytotoxicity of the dendrimers and modified dendrimers were determined with a CellTiter 96[®] Aqueous One Solution Cell Proliferation Assay kit following the manufacturer's protocol. C166-GFP and A549 cells were seeded in 96-well tissue culture plates at 5×10^3 /well. The next day, the cells were incubated with G(4)-D, MD and MDM at varying concentrations (0–1000 nM) for 24 h. At the end of the incubation period, the cells were washed with PBS and supplemented with 100 μL of the serum-free DMEM followed by the addition of 20 μL of Cell Titer Blue Assay Reagent. The cells were incubated with the reagent for 2 h. The fluorescence intensity was measured with a multi-detection microplate

reader (Biotek, Winooski, VT) using 530/590 ex/em wavelengths. Cells treated with medium only were considered 100 % viable.

2.9. Critical Micelles Concentrations (CMC)

The CMC was determined by the pyrene method[38–39]. Briefly, 0.5 mg of pyrene crystals in chloroform were added to each tube. The solvent was evaporated with nitrogen and product freeze-dried. Each tube containing pyrene crystals was shaken with polymers and polymer-micelles at a concentration range of 0–0.8 mM in darkness for 24 h at room temperature. The following day, the unincorporated pyrene was filtered through 0.2 µm syringe filters. The filtrate was transferred to a 96-well plate as 100 µL in triplicates and the fluorescence intensity was measured with a multi-detection microplate reader (Biotek, Winooski, VT) using 339/390 ex/em wavelengths.

2.10. Dox-Loading Efficiency

For assessment of the Dox-loading efficiency, a calibration curve was plotted by measuring the fluorescence intensities of Dox over a concentration range of 0–30 µg/mL using a fluorescence microplate reader. An aliquot of Dox loaded nanoparticles was diluted 100 times in water containing 1 % Triton-X. The loading was determined as follows. The % of Dox loading polymers = (Dox concentration in Dox-loaded nanoparticles obtained from the calibration curve)×100/Theoretical Dox concentration considering no loss during the Dox loading procedure.

2.11. Fluorescence Imaging by Confocal Microscopy

The following method was used for experiments involving the visualization of cells under the confocal microscope. After the initial passage in tissue culture flasks, A549 cells (40,000 cells on cover-slips) were grown on circular cover glasses placed in 12-well tissue culture plates in complete media. On the following day, for cellular uptake study using FITC-labeled nanocarriers, the cells were incubated with FITC-labeled dendrimer solutions (~1 µM) for 1 h in serum-free media, after normalization of FITC-fluorescence in each sample before addition. After the incubation, the cells were washed with PBS, added hoechst 33342 (Molecular Probes, Eugene, Oregon, USA) at 5 µg/mL for 5 min, fixed with 4 % *para*-formaldehyde for 10 min at room temperature and the cover-slips were mounted cell-side down on superfrost microscope slides with fluorescence-free glycerol-based mounting medium (Fluoromount-G; Southern Biotechnology Associates) and viewed with a Zeiss Confocal Laser Scanning Microscope (Zeiss LSM 700) equipped with FITC filter (ex. 450–505 nm, em. 515–545 nm) for *in vitro* imaging. The *z*-Stacked images (*z* 1–15, slice thickness. 0.75 µm) were obtained by capturing serial images of the *xy* planes by varying the focal length of the same to image consecutive *z*-axis. For siRNA delivery, cells on cover-slips were incubated with the polymer-Cy-3-labeled siRNA complexes for 2 h, following the incubation procedure used for siRNA delivery in the flow-cytometry section. The LSM picture files were analyzed using Image *J* software.

2.12. GFP-Silencing

C166-GFP cells were seeded in 12-well plates at a density of 5×10^4 /well, 24 h prior to the transfection. The complete media was replaced by serum-free DMEM. Anti-GFP-siRNA (siGFP) complexed with G(4)-D, MD and MDM at N/P ratio of 10, were added to cells at final siRNA concentration of 200 nM. A non-targeting control duplex (siGFP-ve), used as a control siRNA, was used corresponding to each siGFP treatment at same concentration. After 4 h of incubation, the media was removed and the cells were incubated in fresh complete media for additional 48 h. The cells were washed, detached by trypsinization and GFP down-regulation was analyzed by flow-cytometry. To assess the GFP-silencing by

fluorescence microscopy, C166-GFP cells were grown on microscope cover-slips (22 mm circle), placed in 12-well tissue culture plates at cell density, 40,000 on cover glass. The following day, the plates were washed with serum-free media and incubated with dendriplex and micellar dendriplexes of siGFP (200 nM) at a N/P ratio of 10 for 4 h in 1 mL serum-free media. After 4 h of incubation, the media was removed and the cells were incubated in fresh complete media for an additional 48 h. The next steps followed were as written in the section of fluorescence imaging by confocal microscopy and studied with a Nikon Eclipse E400 microscope with a UV filter (ex. 330–380 nm) for Hoechst staining and with the FITC filter (ex. 465–495) for the GFP signal.

2.13. Serum stability

Serum stability of naked siRNA and siRNA complexed with MD and MDM were investigated by incubating the siRNA-dendrimer complexes in 50 % FBS at 37 °C. Eight samples were prepared by mixing 750 ng of siRNA and polymers at a N/P ratio of 10 in RNase-free water and incubated for 20 min. Equal volumes of FBS were added and the solutions were incubated for 1 h, 6 h and 24 h. In samples, without both FBS and naked siRNA, water was added to make up the volume. The complex was dissociated by addition of heparin sulfate (10 units/ μ g of siRNA) and analyzed by gel electrophoresis using 0.8 % agarose gel containing EtBr.

2.14. Statistical analysis

All numerical *in vitro* data are expressed as mean $\pm$ SD, $n=3$. The data were analyzed for statistical significance using the paired Student's t-test using GraphPad prism 5 (GraphPad Software, Inc.; San Diego, CA). Any p value less than 0.05 was considered statistically significant. Any p -Value less than 0.05, 0.01 and 0.001 was denoted as *, ** and *** respectively.

3. RESULTS

3.1. Synthesis, Purification and Characterization of modified dendrimer

The primary amine end-groups of dendrimers reacted readily with a activated acid group (p-nitrophenylcarbonyl-) functionalized PEG-DOPE copolymer. The reaction was high yielding, rapid and spontaneous. The dialysis of the reaction mixture using a cellulose ester membrane (MWCO 10 KDa) removed small molecule impurities. The G(4)-D-anchored PEG_{2K}-DOPE copolymer was characterized by ¹H NMR spectroscopy (Figure 1). The characteristic peaks noted at different ppm values are as follows (where singlet, doublet, triplet, multiplet are noted as s, d, t, m respectively): For starting material NPC-PEG_{2K}-DOPE, ¹H-NMR in CDCl₃. δ ppm 0.90 (t, 6H), 1.30–1.33 (d), 1.58–1.62 (m), 2.01–2.06 (m), 2.31–2.37 (m), 3.30–3.50 (m), 3.58–3.68 (m), 3.78–3.81 (m), 3.99–4.00 (m), 4.11–4.18 (m), 5.33–5.36 (m), 7.50 (d), 8.35 (d). The most shielded peak at δ ppm 0.90 (t, 6H) was from the two terminal -CH₃ groups of the lipid chains. The sharp peak at 3.6 was from (-CH₂-CH₂-)_n group of PEG. The peak at δ ppm 5.33–5.36 (m) was characteristic of -CH=CH- proton signals. The two most deshielded doublets are from the 4-protons of *p*-nitrophenyl (NO₂-C₆H₄-) group.

For G(4)-D, ¹H-NMR in CDCl₃. δ ppm 2.32–2.39 (m), 2.56–2.61 (m), 2.67–2.69 (t), 2.77–2.85 (m), 3.19–3.28 (m).

For G(4)-D-PEG_{2K}-DOPE, ¹H-NMR in CD₃OD. δ ppm 0.90 (t, 6H), 1.31–1.33 (d), 1.58–1.62 (m), 2.01–2.05 (m), 2.36–2.39 (m), 2.58–2.60 (m), 2.78–2.82 (m), 3.26–3.32 (m), 3.64 (s), 5.30–5.34 (m). The characteristic peaks of both the starting materials were present in G(4)-D-PEG_{2K}-DOPE.

3.2. Particle Size

Particle size of MD, obtained by DLS was 56 ± 2.5 nm was higher than the particle size of the parent G(4)-D (20 ± 5.2 nm). The TEM pictures of the polymers demonstrated similar size range as analyzed by DLS (Figure 1).

3.3. siRNA Binding

To assess the siRNA-binding ability of the synthesized polymers, gel retardation and ethidium bromide exclusion assays were performed. In gel retardation assay, MD and MDM condensed siRNA as effectively as parent G(4)-D. Complete binding of siRNA with polymer began at a molar ratio of nitrogen in the carrier/phosphate in the siRNA (N/P) of 2/1 (Figure 2A). The ethidium bromide exclusion assay demonstrated the complete condensation or quenching of the siRNA at a N/P ratio of 2/1 (Figure 2B). The fluorescence intensity of the naked siRNA in the presence of ethidium bromide was considered as non-quenching and an emission of maximum fluorescence. The amount of naked siRNA in the system decreased resulting in a decreased fluorescence with the gradual increase in the concentration of the polymers (N/P=0–2) (Figure 2B). Upon the addition of heparin, the fluorescence reached its maximum level (Figure 2C).

3.4. Cytotoxicity Studies

Cytotoxicity studies were performed to investigate whether the nanocarriers have inherent toxicity over the dose range used for the experiments performed (Figure 2D). There was no apparent toxicity observed for the polymers at the tested dose range of 0–1 μ M for 24 h with two tested cell lines, C166 and A-549.

3.5. Micelles Properties

Determination of critical micelle concentration (CMC) confirmed the potential of the developed polymeric systems for a drug delivery application (Figure 3A). The CMC of the polymers was compared to that of stable PEG_{2K}-PE micelles that demonstrated the CMC value of 2.5×10^{-5} M. Mixed micellar system (MDM) and the polymeric dendrimer (MD) had CMC values of 5×10^{-5} M and approximately 10×10^{-5} M, respectively. The synthesized polymer MD, mixed with PEG-PE (MDM), can form a stable mixed micellar system with a low CMC, but failed to efficiently self-assemble alone in the system, resulting in a very slow rise in the solution fluorescence. G(4)-D showed no pyrene incorporation.

Dox-loading efficiency of the nanocarriers was dependent on the CMC (Figure 3B). The PEG_{2K}-PE had the lowest CMC value and the Dox-loading efficiency was *ca.* 65 %. The MDM's loading efficiency was *ca.* 42 % and MD had the lowest loading efficiency (*ca.* 20 %).

3.6. Association with the cells

Flow cytometry analysis and visualization with confocal microscopy were performed to quantify the association of FITC-labeled nanocarriers with the cells. Confocal microscopy images showed significantly higher cellular accumulation of MD and MDM after 1 h incubation compared to G(4)-D (Figure 4A), with MD showing a higher cellular association than MDM. However, no significant difference in cell association was observed between the samples after 4 h. A time-dependent increase in the cellular association of nanocarriers was observed using FACS analysis (Figure 4B). The analysis of the internalization of nanocarriers was performed by quenching the surface-associated FITC-fluorescence with Trypan blue (Figure 4C). The data showed decreased fluorescence after the Trypan blue treatment, indicating that a higher portion of the cell-associated fluorescence was from

surface association. However, it has been observed that the cellular internalization also significantly increases over time.

3.7. Cellular internalization

To assess the cellular internalization, Z-stacked images of the cells in the XY plane were obtained allowing different regions of the cells to be viewed along the Z-axis (Figure 4D). The center Z-slices out of 12 slices of uniform thickness showed that the FITC-labeled MD and MDM had significantly higher cellular internalization compared to parent G(4)-D.

3.8. siRNA delivery

The ability of the nanocarriers to deliver Cy-3-labeled GAPDH siRNA was investigated by FACS analysis (Figure 5). There was a time-dependent increase in siRNA delivery observed within 2 h. Both MD and MDM delivered significantly higher amounts of siRNA than parent G(4)-D (geo mean of fluorescence. 3.56 ± 0.06 for G(4)-D, 8.94 ± 0.33 for MD, 6.68 ± 0.22 for MDM and 3.39 ± 0.37 for free siRNA), resulting in higher labeling of the cells with Cy-3-siRNA. Histogram and quadrant statistics (Figure 5B and C) showed that the MD-siRNA complex had a significantly higher cellular uptake than the MDM-siRNA complex. Quadrant statistics demonstrated that the G(4)-D-Cy3-siRNA complex shifted 7 % of the total cell population to the lower right quadrant, whereas for the MD and MDM-Cy3-siRNA complexes, the cell population increased to 59.7 and 43.5 %, respectively. Compared to free siRNA treatment, G(4)-D showed no improvement in siRNA delivery. Visualization of Cy3-siRNA:D, MD and MDM complex- dosed A549 cells supported the results of the FACS analysis that MD delivered higher amount of siRNA compared to G(4)-D and micellar dendrimer, MDM. However, both MD and MDM showed comparable siRNA-delivery efficiency compared to G(4)-D.

3.9. siRNA-mediated gene silencing

In vitro gene-silencing of dendrimer-based siRNA-complexes was investigated using GFP-specific siRNA (siGFP) and non-specific siRNA (siNegative) in C166 cells-stably expressing GFP. The gene silencing efficacy was *ca.* 10 % for the G(4)-D, *ca.* 22 % for MD and 18 % for MDM (Figure 6A). High GFP expression down-regulation was not observed with any dendriplexes. However, silencing efficacy was significantly higher for the modified dendrimer systems, MD and MDM compared to G(4)-D. The fluorescence microscopic visualization of the C166-GFP cells indicated that the siGFP, delivered by MD and MDM systems, had decreased GFP expression.

3.10. Drug delivery

Apart from the ability of the nanocarrier systems to deliver siRNA, drug delivery efficiency of the nanocarriers was also investigated (Figure 7). The ability of the nanocarriers to deliver a fixed Dox dose of 4 $\mu\text{g/mL}$, irrespective of their loading was assessed by FACS analysis. It was observed that the MDM delivered a higher amount of Dox to the cells (geo mean of cellular fluorescence. 184.7 ± 4.4) than D (142.3 ± 1.0), MD (116.1 ± 9.3) and PEG-PE (54.9 ± 0.04) (Figure 7B).

3.11. siRNA/Drug co-delivery

For the purpose of drug-siRNA co-delivery, Dox-loaded nanocarriers were complexed with FAM-labeled siRNA. Simultaneous labeling of cells with green and red fluorescence due to the co-delivery of FAM-siRNA and Dox was analyzed by flow cytometry. Higher labeling of cell population was observed with FAM-siRNA in the MD-Dox:siRNA complex (Geo mean of fluorescence. 13.7 ± 0.3) than the D.Dox:siRNA (6.5 ± 0.0) and MDM.Dox:siRNA (8.3 ± 0.1) complex-treated cell populations, whereas higher Dox-labeling of cells was

observed with MDM.Dox:siRNA (77.3 ± 4.9) than with the other nanocarriers (22.4 ± 2.2 for D.Dox:siRNA and 52.4 ± 1.6 for MD.Dox:siRNA). The representative quadrant statistics in Figure 8B showed the shift in the cell population due to increased labeling with the nanocarriers in both FL1 and FL2 axis. Shift of the cell population in upper right quadrant, indicative of siRNA labeling was highest in MD (30.2 % compared to 4.4 % for D and 7.7 % for MDM), whereas in lower right quadrant, indicative of Dox labeling was highest in MDM (92 % compared to 52.3 for D and 69 % for MD).

3.12. Serum Stability

To check on whether the prepared nanocarriers are suitable for *in vivo* application, the serum stability of the siRNA in dendriplexes was assessed (Supplementary Figure-1). The result showed that the free siRNA showed partial instability at 1 h and complete enzymatic digestion within 6 h, whereas the siRNA, complexed with dendrimers, showed complete protection against enzymatic degradation.

4. Discussion

Silencing gene expression to down-regulate the production of aberrant proteins is considered a most powerful technique to combat diseases with multiple hallmarks such as cancer. Sequence-specific knockdown of the target gene expression can overcome some of the limitations of conventional medicinal chemistry. Our understanding about the process of RNA interference and the methodologies to prepare specific post-transcriptional gene silencers has expanded considerably over the last decade. Nonetheless, several hurdles remain regarding delivery of the siRNA to the target site. For the present work, we designed a dendrimer-based siRNA delivery system. The modification was performed on the G(4)-D by the conjugation of polyethylene glycol-dioleoyl phosphatidylethanolamine (G(4)-D-PEG-_{2K}-DOPE, modified dendrimer or MD). In addition, a mixed dendrimer-micellar system (MDM) constituting of 1:1 mixture of MD and longer chain (5K) PEG-DOPE was developed for the purpose of both drug and siRNA delivery into cancer cells to overcome multi-drug resistance, a serious problem that limits the effectiveness of conventional chemotherapy. Figure 1A shows the scheme of the formation of triblock co-polymer, MD and the mixed micellar system (MDM). The chemical structure and the reaction scheme is given in Figure 1B. In general, combining multiple functions in a single delivery system is synthetically challenging, but highly effective therapeutically [40]. In our attempt to develop a highly potent multifunctional nanocarrier, we developed a triblock PEG-PE-modified dendrimer system. Dendrimers serve as the cation source for siRNA condensation. PAMAM dendrimers are relatively unexplored for their ability to promote siRNA delivery compared to other polycations such as PEI and PLL. We expected that the conjugation of PEG-PE to G(4)-D would assist in its incorporation into the micellar system, while still allowing the poly-cationic G(4)-D to electrostatically interact with siRNA in the micelles, lipid modification would enhance the intracellular delivery and the use of long PEG chains in the micelles should impart a shielding effect to protect the bound siRNA from enzymatic degradation.

The triblock polymer, MD showed a nearly spherical morphology with a mean diameter of 60 nm, as shown by the transmission electron microscopic images (Figure 2A). The size was bigger compared to parent G(4)-D with a much smaller size range (15–20 nm). The structure of the synthesized MD was confirmed by ¹H-NMR, using 400 MHz spectrophotometer (Varian). Six-proton signal as triplet at 0.9 ppm from the terminal methyl groups of the fatty acid chains was one of the characteristics peaks of DOPE, which was present in both pNP-PEG-_{2K}-DOPE and G(4)-D-PEG-_{2K}-DOPE. The cumulative ethylene (-CH₂-CH₂-) signal from PEG chain were present at 3.68 ppm in G(4)-D-PEG-_{2K}-DOPE. The aliphatic signals of G(4)-D were overlapped with the aliphatic signals of DOPE in G(4)-D-PEG-_{2K}-DOPE.

The signals of G(4)-D at 2.37, 2.58, 2.71, 2.77, 3.25 ppm were also observed in G(4)-D-PEG-_{2K}-DOPE at 2.38, 2.58, 2.79, 3.30 ppm as multiplet respectively. Therefore, the appearance of characteristic peaks from lipid moiety, PEG and G(4)-D in the final product indicated the successful conjugation (Figure 2A).

In both the assays (gel retardation and ethidium bromide exclusion assays) to evaluate the siRNA binding ability of the polymers, MD and MDM condensed siRNA as effectively as parent G(4)-D. Heparin treatment following ethidium bromide exclusion assay confirmed that the decrease in the ethidium bromide fluorescence was due to the efficient condensation of the siRNA with the polymers. The cytotoxicity studies demonstrated non-toxicity of the polymers at the tested dose range of 0–1 μ M for 24 h. All the polymer treatments on cells were in this dose range, indicating no probable incidence of polymer mediated cellular toxicity. It is obvious that cationic polymers show toxicity due to their non-specific interaction with the cell membrane and intracellular compartments, which limits their application as a nucleic acid delivery system. Based on current literature, various methods have been adopted to improve the cell viability in presence of dendrimers, among which the acetylation to block the primary amines was one of the more effective approaches [41]. Adopting this modification could increase the therapeutic window. However, for dendrimers to be used as a siRNA delivery system, special attention has to be given not to block all the primary amines for binding of siRNA via electrostatic interaction.

Determination of the critical micelle concentration (CMC) and Dox-loading efficiency were important experiments which confirmed the potential of the developed polymeric system for a drug delivery application. The reason behind a poor micelles forming ability of MD could be due to the fact that the bulky, highly cationic spherical dendrimer structure which, when attached on stable micelle-forming PEG-PE, hindered the system self-assembly due to electrostatic repulsion. This instability was overcome in the mixed micellar system. Dox-loading efficiency was dependant on the CMC of the nanocarriers. These studies identified MDM as a promising nanocarrier for drug delivery applications. At this point, siRNA delivery efficiency had to be judged for the purpose of drug-siRNA co-delivery.

Flow cytometry analysis and visualization with confocal microscopy demonstrated that MD and MDM have higher cellular association/internalization than G(4)-D. MD demonstrated highest cellular association. The main reason for the enhanced cellular interaction of MD and MDM is due to the lipid modification that facilitates cell membrane penetration.[27–30] The nanocarriers with higher cellular internalization efficiency had improved siRNA delivery and siRNA-mediated gene silencing ability. High GFP expression down-regulation was not observed with any dendriplexes. However, silencing efficacy was higher for the modified dendrimer systems, MD and MDM compared to G(4)-D. The fluorescence microscopic visualization of the C166-GFP cells indicated that the siGFP, delivered by MD and MDM systems, had increased gene-silencing efficiency, resulting in down-regulation of GFP expression with a subsequent decrease in fluorescence. The experiment investigating drug delivery efficiency using Dox demonstrated that MDM delivered a higher amount of Dox to the cells than other nanocarriers at a fixed Dox dose. The micellar dendrimer system, MDM had higher drug loading efficiency with higher CMC value than MD. Even though, PEG-PE showed higher loading of Dox than MDM, the cationic surface of MDM allowed better cellular association and Dox delivery compared to PEG-PE. The experiment investigating siRNA/drug co-delivery efficiency corroborated the previous finding that polymeric dendrimer (MD) delivered higher amount of siRNA compared to G(4)-D and MDM, whereas MDM delivered higher amount of Dox. Assessment of serum stability confirms the stability of the siRNA-dendrimer complexes in the systemic circulation.

Dox and siRNA co-delivery have a potential for the treatment of multi-drug resistance (MDR). The intracellular drug concentration is reduced by pumping out of the drug by membrane transporters, such as P-glycoprotein (P-gp), which are over-expressed in multi-drug resistant (MDR) tumors. To circumvent the problem of MDR, the effective treatment strategy is the co-delivery of drug and siRNA, where siRNA silences the genes for over expressed transporters, such as P-gp or other proteins important for MDR so that the drug delivery system would maintain an effective cytotoxic drug concentration. In general, nanocarrier-mediated drug delivery has found potential applicability in cancer treatment, since nanoparticles can specifically target cancer cells, avoid rapid renal clearance, minimize non-specific toxicity and address the poor solubility issues of some otherwise potent anti-cancer drugs.[42] We propose that this newly developed nanocarrier has the unique combination of functionalities to qualify as a truly multifunctional nanomedicine.

5. CONCLUSION

In summary, we designed, synthesized and evaluated a triblock copolymer G(4)-D-PEG-DOPE, which demonstrated efficient siRNA binding and delivery with maximal suppression of the expression of the target gene. We also engineered a mixed micellar system, combining G(4)-D-PEG-DOPE and PEG-DOPE (1:1) with superior properties in terms of drug loading and siRNA-drug co-delivery. The combination of the properties of dendrimer and polymeric micelles in a single nanocarrier resulted in a truly multifunctional nanomedicine, which could address the challenges of drug and siRNA co-delivery for therapeutic purposes, especially in multidrug resistant cancers.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The work was supported in part by NIH grants RO1 CA121838 and RO1 CA128486 to Vladimir P. Torchilin.

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A **B** **Figure 1.**

(A) TEM images of G(4)-PAMAM-D and G(4)-D-PAMAM-PEG-DOPE. (B). ^1H -NMR spectra of PEG-DOPE, G(4)-D-PEG-DOPE and two starting materials, G(4)-PAMAM-D and NPC-PEG-DOPE recorded in methanol.

Figure 2.

Binding ability of tested nanoparticles to siRNA. Different ratios of nitrogen in G(4)-D to phosphate in siRNA (N/P) of the nanoparticles were tested by gel retardation assay (A) and ethidium bromide exclusion assay ((B) and (C)). (D) Evaluation of cytotoxicity of the nanoparticles in C166 and A549 cells after 24 h of incubation.

Figure 3. Evaluation of loading efficiencies of polymers and mixed micellar system. (A) Determination of critical micelles concentrations by pyrene incorporation method. (B) Determination of doxorubicin-loading efficiency of the nanoparticles.

Figure 4.

Cellular uptake of nanoparticles (FITC-labeled) in A549 cells. Cells were incubated with 1.5 μ M of polymers for 2 h for visualization by confocal microscopy (A) and for 1h and 4h time period for analysis by flow cytometry (B and C). Trypan blue was added for the purpose of quenching the surface associated fluorescence (C). (D) Selected images of the cells in the XY plain at consecutive Z-axis (Z-2,4,6,8).

Figure 5. Evaluation of siRNA delivery efficiency of the polymers and the mixed micellar system. (A) Representative histogram plot, obtained from fluorescence-activated cell sorting analysis, showing the uptake of Cy5 labeled siRNA, condensed with polymers and micellar system at

N/P= 10 after 2 h of incubation with A549 cells. (B) Cellular uptake/delivery of Cy5-labeled siRNA-dendriplxes, measured by the geometric mean of fluorescence, obtained from FACS analysis at 1 h and 4 h time points. (C) Representative dot plot, obtained from FACS analysis, showing the cells labeled with Cy5-siRNA, delivered by various siRNA-dendriplexes. (D) The confocal laser scanning microscope (CLSM) images of siRNA-dendriplexes-dosed A549 cells, after incubation for 2 h. Cell nuclei were stained with Hoechst 33342. Both FACS and microscopy studies were performed at siRNA concentration 100 nM (1:1 mixture of Cy5-labeled siRNA and scramble siRNA) at N/P ratio of 10.

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Figure 6.

Efficiency of down-regulating the target protein by siRNA-polymer dendriplexes. Green fluorescence protein-silencing siRNA (siGFP) (200 nM) was delivered to the C166-GFP cells (stably expressing GFP) via dendriplexes in polymer systems (at N/P ratio=10). (A) Geometric mean of fluorescence of the siGFP-treated cells (obtained from the histogram statistics in FACS analysis) was plotted compared to the siNegative treated cells. (B) Fluorescence micrograph, demonstrating the GFP-protein downregulation effect of siGFP treatment. The nuclei was stained with Hoechst 33342.

Figure 7.

Dox-delivery efficiency of the nanoparticles analyzed by flow cytometry. (A) Representative histogram plot, demonstrating the differences in the dox-labeling of the A549 cells treated with a fixed dose of dox (4 $\mu\text{g/mL}$) for 1 h, loaded in the nanoparticles. (B) Quantitative comparison of dox-loaded nanocarrier mediated dox-delivery. Mean fluorescence of the treated cells (obtained by analyzing the histogram statistics) was plotted compared to control.

Figure 8.

Assessment of co-delivery efficiency of nanoparticles by flow cytometry. (A) Quantitative comparison of geometric mean of fluorescence from Dox and FAM-labeled siRNA, delivered to the A549 cells via Dox-loaded dendriplex systems. (B) Representative dot plot, obtained by FACS analysis, showing the difference in the Dox- and FAM-siRNA-labeling in the cell populations.

B

Scheme 1.

(A) Schematic representation of the formation of mixed micellar system with G(4)-PAMAM-D-PEG-DOPE/PEG-DOPE. (B) Synthetic scheme of G(4)-PAMAM-D-PEG-DOPE.