**Octadecyl chain-bearing PEGylated poly(propyleneimine)-based dendrimersomes:
physicochemical studies, redox-responsiveness, DNA condensation, cytotoxicity and
gene delivery to cancer cells**

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Abstract

Stimuli-responsive nanocarriers have become increasingly important for nucleic acid and drug delivery in cancer therapy. Here, we report the synthesis, characterization and evaluation of disulphide-linked, octadecyl (C18 alkyl) chain-bearing PEGylated generation 3-diaminobutyric polypropylenimine dendrimer-based vesicles (or dendrimersomes) for gene delivery. The lipid-bearing PEGylated dendrimer was successfully synthesized through *in situ* two-step reaction. It was able to spontaneously self-assemble into stable, cationic, nanosized vesicles, with low critical aggregation concentration value, and also showed redox-responsiveness in presence of a glutathione concentration similar to that of the cytosolic reducing environment. In addition, it was able to condense more than 70% of DNA at dendrimer: DNA weight ratios of 5:1 and higher. This dendriplex resulted in an enhanced cellular uptake of DNA at dendrimer: DNA weight ratios of 10:1 and 20:1, by up to 16-fold and by up to 28-fold compared with naked DNA in PC-3 and DU145 prostate cancer cell lines respectively. At a dendrimer: DNA weight ratio of 20:1, it led to an increase in gene expression in PC-3 and DU145 cells, compared with DAB dendriplex. These octadecyl chain-bearing, PEGylated dendrimer-based vesicles are therefore promising redox-sensitive drug and gene delivery systems for potential applications in combination cancer therapy.

Keywords: dendrimersome, PEG, octadecyl (C18 alkyl) chain, redox sensitivity, gene delivery, cancer

Graphical Abstract

1. Introduction

Novel “smart” delivery systems potentially able to carry therapeutic genes along with anti-cancer drugs for cancer treatment are urgently needed to overcome the resistance of cancer cells towards conventional chemotherapy.^{1,2} Despite recent advances in the field of cancer therapy, the possibility of using such a combination therapy is still hampered due to lack of safe delivery systems, which can simultaneously carry both therapeutic DNA and drugs and selectively deliver them to the tumors, without secondary effects to healthy tissues.^{1,2}

In order to address this challenge, we propose to conjugate octadecyl (C18 alkyl) lipid chain to the surface of PEGylated generation 3-diaminobutyric polypropylenimine (DAB) dendrimer, through a redox-sensitive disulphide linkage and evaluate the potential use of these lipid-modified PEGylated dendrimers as gene delivery systems.

Cationic polyamine dendrimers, which are highly branched three-dimensional macromolecules, are becoming promising non-viral vectors for the delivery of nucleic acids and anti-cancer drugs to cancer cells, through modification of their surface with targeting moieties, drugs or nucleic acids to achieve the desired therapeutic applications.³⁻⁷ The cationic charge of the polyamine dendrimers not only helps to form a stable ionic complex with negatively charged nucleic acids, but also increases cellular internalization of delivery vectors *via* endocytosis because of their electrostatic interactions with negatively charged cell surface.^{3,4} In addition to their high aqueous solubility, low toxicity, compact globular shape and ability to encapsulate various chemically sensitive anti-cancer drugs, these macromolecular dendritic entities can facilitate the passive targeting of anti-cancer drugs to tumor tissues due to the enhanced permeability and retention (EPR) effect which increases the permeability of tumor vasculature and reduces lymphatic drainage.⁸

In this work, we have chosen to use a generation 3-diaminobutyric polypropylenimine (DAB) dendrimer, as it has been reported to be safer and more efficacious than its higher generation counterparts for gene transfection.⁹⁻¹² Furthermore, tumor-targeted generation 3-DAB dendrimer complexed to therapeutic plasmid DNA led to successful delivery of therapeutic plasmid DNA to the tumors, resulting in gene expression and therapeutic efficacy in mice bearing subcutaneous tumors following intravenous injection.¹⁰⁻¹²

Heterobifunctional PEG of MW 2 kDa, namely orthopyridyl disulfide polyethylene glycol (PEG2000) succinimidyl carboxymethyl ester (OPSS-PEG-SCM), was used as cross-linker to conjugate the lipid on the surface of DAB dendrimer, as PEG is known to increase the water solubility as well as stealthiness of material by reducing non-specific interactions with circulatory proteins, thus leading to an enhanced circulatory life time of drug and gene

delivery systems.¹³⁻¹⁴ Recent reports from our laboratory also demonstrated that the conjugation of low-molecular weight PEG (2 kDa) to generation 3- and generation 4-DAB dendrimers significantly reduced the overall cytotoxicity and thereby resulted in an enhanced gene expression in various cancer cells lines compared to unmodified dendrimers.¹⁵

Lipidic long chain fatty acid is an essential structural and functional constituent of animal cell membrane.¹⁶⁻¹⁸ The conjugation of lipidic octadecyl or stearyl (C18 alkyl) chain to PEGylated cationic dendrimers would also increase the hydrophobicity of amphiphilic PEGylated dendrimers, facilitating their possible self-assembly, as hydrophobic interactions are one of the main driving forces for any amphiphilic molecules to self-aggregate.¹⁹⁻²⁵ This was evidenced by the recent development of various self-assembled nanostructures (such as micelles, vesicles or dendrimersomes) from amphiphilic surface-modified cationic dendrimers with diverse hydrophobic groups (such as steroid, aromatic, long hydrophobic chains, fluorinated groups).¹⁹⁻²⁵ In addition, the presence of lipids in the backbone of any amphiphilic polyamine dendritic molecule may help the dendrimer to form more compact, smaller-sized dendriplexes and enhance transfection efficacy due to the strong fusogenic activity of lipids.^{19,23,26-30} Furthermore, a recent study showed an improvement in serum-resistant transfection activity of the octadecyl lipid-modified generation 3-PAMAM dendrimer.²⁶

In this work, lipidic octadecyl chain was conjugated to PEGylated DAB dendrimer through disulphide linkage (-S-S-), so that their structural backbone could be cleaved into the corresponding thiols in presence of a reducing biothiol, such as glutathione (GSH), triggering the destruction of the delivery systems and the concomitant release of the guest at the desired site of action.^{20,31-32} The redox-sensitivity of any delivery system is particularly important in cancer therapy, as the concentration of GSH was found to be different in the intracellular (~10 mM) and extracellular (<10 μ M) compartments of living cells, and was even higher (by nearly 4-fold) in cancer cells than in normal tissues.^{20,31-32}

The objectives of this study were therefore 1) to synthesize and characterize disulphide-linked octadecyl chain-bearing PEGylated generation 3-DAB dendrimer, 2) to assess the self-assembly behavior of the modified dendrimer into redox-sensitive nanostructures, 3) to evaluate the DNA condensation ability of the lipid-modified dendrimer and 4) to assess *in vitro* the cellular uptake and transfection efficacy of the modified dendrimer complexed with DNA on various prostate cancer cell lines.

2. Experimental

2.1 Materials

Generation 3- diaminobutyric polypropylenimine dendrimer (DAB) was obtained from SyMO-Chem (Eindhoven, The Netherlands). Heterobifunctional PEG of MW 2 kDa (orthopyridyl disulfide polyethylene glycol (PEG2000) succinimidyl carboxymethyl ester (OPSS-PEG-SCM)) was purchased from JenKem Technology (Plano, TX). 1-Octadecanethiol (ODT), glutathione (GSH), trimethylamine (TEA), N-phenyl-1-naphthylamine (NPN), Nile red, 2,5-dihydroxybenzoic acid (DHB), and CDCl₃ and D₂O deuterated solvents were purchased from Sigma Aldrich (Poole, UK). Anhydrous tetrahydrofuran (THF) and methanol (MeOH) came from Fisher Scientific (Loughborough, UK) and used without further purification. The expression plasmid encoding β -galactosidase (pCMVsport β -galactosidase), obtained from Life Technologies (Paisley, UK), was purified using an Endotoxin-free Giga Plasmid Kit (Qiagen, Hilden, Germany). Minimum Essential Medium (MEM), tissue culture media and Quanti-iT[®] PicoGreen[®] dsDNA reagent were obtained from Life Technologies (Paisley, UK). Label IT[®] Fluorescein Nucleic Acid Labeling Kit was obtained from Cambridge Biosciences (Cambridge, UK). Vectashield[®] mounting medium containing 4', 6-diamidino-2-phenylindole (DAPI) was purchased from Vector Laboratories (Peterborough, UK). Passive lysis buffer (PLB) was obtained from Promega (Southampton, UK). Bioware androgen-irresponsive PC-3M-luc-C6 human prostate adenocarcinoma was obtained from Caliper Life Sciences (Hopkinton, MA), while androgen-irresponsive DU145 human prostate carcinoma was purchased from the European Collection of Cell Cultures (Salisbury, UK).

2.2. Synthesis and characterization of DAB-PEG-SS-ODT

OPSS PEG succinimidyl carboxymethyl ester (OPSS-PEG-SCM) and ODT were conjugated to the same arm of amine-terminated, generation 3-diaminobutyric poly(propyleneimine) dendrimer (DAB) through an *in situ* two-step reaction using MeOH-THF (5:1) as solvent (Figure 1).

Figure 1. General synthetic scheme for lipid-modified PEGylated dendrimer (DAB-PEG-SS-ODT), with $m=45$.

Briefly, TEA (~20 equivalents, 40 μ L) was added to DAB solution (1 equivalent, 20 mg, 1.5 mL) and left for stirring at 20 $^{\circ}$ C for 30 minutes. A freshly prepared methanolic solution of OPSS-PEG-SCM (2 equivalents, 54.39 mg in 1.5 mL methanol) was then added dropwise to the reaction mixture over 10 minutes and the reaction mixture was then stirred at 37 $^{\circ}$ C, protected from light. After 6 h, 1-octadecanethiol (ODT) (2 equivalents, 6.79 mg in 0.6 mL THF) was then added as a whole to the reaction mixture containing PEGylated DAB. The colorless reaction mixture was left for stirring at 37 $^{\circ}$ C, protected from light, and gradually became yellow over time. After 15 h, 5 mL distilled water was added to the reaction mixture before being transferred into a dialysis bag. The water-soluble modified dendrimer was dialyzed using a dialysis tubing with a molecular weight cut-off of 3.5 kDa against distilled water (2 L) to remove organic solvents and excess water-soluble reagents. During the dialysis, the water was changed twice per day for 3 days at 25 $^{\circ}$ C. The dialyzed solution was then filtrated with a Whatman cellulose filter paper before being freeze-dried using a Christ Epsilon 2-4 LSC[®] freeze dryer (Osterode am Harz, Germany). The final product DAB-PEG-SS-ODT, a white-colored compound, was stored at 4 $^{\circ}$ C for long-term storage.

The infrared spectra of DAB-PEG-SS-ODT dendrimer, unmodified DAB dendrimer and OPSS-PEG-SCM, were measured using an IRSpirit[®] QATR-S Fourier-transform infrared (FTIR) spectrophotometer with attenuated total reflectance (ATR) probe (Shimadzu, Kyoto, Japan), where transmittance (4000–400 cm^{-1}) was recorded at a 4 cm^{-1} resolution. The collected spectra were processed with the LabSolutions IR[®] software (Shimadzu, Kyoto, Japan).

The ¹H-NMR spectrum of DAB-PEG-SS-ODT dendrimer (4-5 mg in 0.5 mL deuterated solvents) were recorded using the residual proton resonance of the solvent as the internal standard on a Bruker Avance[®] III-HD500 NMR spectrometer (Billerica, MA).

The molecular weights of DAB-PEG-SS-ODT dendrimer, DAB and OPSS-PEG-SCM were determined by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry using an Axima-CFR[®] mass spectrometer (Kratos Analytical, Shimadzu, Kyoto, Japan), where a N₂ laser operating at 337 nm was used to generate the ions before being accelerated to 20 kV. 2,5-Dihydroxybenzoic acid (DHB) was used as the matrix, which was prepared at a concentration of 10 mg/mL in methanol. The sample was prepared by thoroughly mixing 10 μ L DAB-PEG-SS-ODT solution in ultrapure water with the 10 μ L matrix solution in 1/1 (v/v) ratio. One μ L of these sample mixtures was then placed to the MALDI sample plate and left for air-drying before measurement using linear mode with 4-

point calibration. Mass spectra of DAB-PEG-SS-ODT and its starting materials mainly exhibited singly charged molecular ions $[M]^+$ with their fragmented products.

2.3. Characterization of DAB-PEG-SS-ODT self-aggregates

2.3.1. Entrapment of N-phenyl-1-naphthylamine (NPN)

The self-assembly formation by DAB-PEG-SS-ODT dendrimer was assessed by steady-state fluorescence technique using a hydrophobic fluorescent probe molecule, N-Phenyl-1-naphthylamine (NPN). An aliquot (1.5 μ L) of NPN stock solution (1.32×10^{-3} M, 2.89 mg in 10 mL methanol) was added to 5 mL plastic vials and was left in the dark for 5-6 h to completely evaporate methanol. One mL DAB-PEG-SS-ODT dendrimer solutions at various concentrations, ranging from 0 to 400 μ g/mL in PBS (pH 7.4), were prepared in these plastic vials from a stock dendrimer solution (2 mg/mL), making the final probe concentration 2×10^{-6} M. Each sample was vigorously vortexed for 5 minutes and kept in the dark at 20 °C. After nearly 16 h of incubation, fluorescence spectra of NPN were then recorded for each sample with a Varian Cary Eclipse[®] Fluorescence spectrophotometer (Palo Alto, CA) (λ_{exc} : 340 nm, λ_{em} : 360-600 nm, excitation and emission slits at 10 nm and 20 nm respectively).

2.3.2. Hydrodynamic size and zeta potential

The hydrodynamic diameter (d_H) and zeta potential of DAB-PEG-SS-ODT dendrimer-based self-assembled nanostructures at various concentrations in aqueous medium (PBS, pH 7.4) at 37 °C were respectively measured by dynamic light scattering (DLS) and laser Doppler electrophoresis using a Malvern Nano ZS[®] instrument (Malvern Instruments, Malvern, UK) with a 4 mW He-Ne laser ($\lambda = 632.8$ nm) source. The scattering photons were collected at 173° scattering angle.

2.3.3. Transmission electron microscopy (TEM) imaging

The morphology of the DAB-PEG-SS-ODT dendrimer-based nanostructures was observed using transmission electron microscope (TEM) operating at an accelerating voltage of 80 kV, JEOL JEM-1200EX[®] (Jeol, Tokyo, Japan). One drop (3 μ L) of dendrimer solution (400 μ g/mL in PBS pH 7.4) was drop cast on a carbon-coated copper grid (400 mesh size) and was dried in a desiccator overnight. The sample was stained with uranyl acetate (2%, v/v) before imaging.

2.3.4. Entrapment of coumarin-153 (C153)

Coumarin-153 (C153), a hydrophobic fluorescent probe, was used to measure the micropolarity of DAB-PEG-SS-ODT dendrimersomes. Two μL of C153 stock solution (1×10^{-3} M, 3.19 mg in 10 mL methanol) was added to 5 mL plastic vials. Following the complete evaporation of the methanol protected from light, after 5–6 h, 1 mL DAB-PEG-SS-ODT solution (0, 1, 50, 100, 300, 500 and 1000 $\mu\text{g/mL}$ in PBS pH 7.4) was added to the vials containing C153 probe to make the final probe concentration 2×10^{-6} M. Each sample solution was vigorously vortexed for 5 minutes and then incubated for 24 h at 25 °C, protected from light. The fluorescence emission spectrum of C153 probe in aqueous phosphate buffer (pH 7.4) was then recorded in presence or absence of DAB-PEG-SS-ODT at various concentrations (λ_{exc} : 420 nm, λ_{em} : 440–800 nm, excitation and emission slits at 10 nm), using a Varian Cary Eclipse[®] fluorescence spectrophotometer (Palo Alto, CA). The polarity of the microenvironment of the self-aggregate was calculated by π^* -polarity scale using the frequency of C153 fluorescence emission, as follows:

$$\bar{\nu}_{\text{em}} = 21.217 - 3.505\pi^*$$

where $\bar{\nu}_{\text{em}}$ (in 10^3 cm^{-1}) is the wavenumber corresponding to the emission maximum of C153.

2.3.5. Phase transition behavior by differential scanning calorimetry (DSC)

The phase transition behavior of the self-aggregated DAB-PEG-SS-ODT solutions (1 and 2 mg/mL in PBS pH 7.4) was measured by differential scanning calorimetry (DSC), using a Netzsch DSC214 Polyma[®] differential scanning calorimeter (Netzsch-Gerätebau GmbH, Selb, Germany). To do so, 2.7 (± 0.1) mg of sample solutions (PBS, pH 7.4) were added to 40 μL aluminium pans by micro-pipetting, followed by sealing with aluminium lids hermetically. Similarly, blank and reference pans were prepared with 2.7 (± 0.1) mg of PBS solution (pH 7.4) as negative controls. Sample solutions and references were cooled to -15 °C at a rate of 2 °C/min and held for 5 min at -15 °C, prior to heating to 50 °C at a rate of 5 °C/min. Under the same measurement conditions, blank correction was carried out with PBS solution prior to sample analysis to remove any interference from the solvent. A repeat cycle was carried out on each sample to check the reproducibility. Samples were analyzed in duplicate. The onset temperature, peak temperature and enthalpy of measured peaks were determined with Netzsch Proteus Analysis[®] Software v7.0.1 (Netzsch-Gerätebau GmbH, Selb, Germany).

2.3.6. Atomic force microscopy (AFM) imaging

DAB-PEG-SS-ODT vesicles were visualized by atomic force microscopy (AFM) to assess their size and shape using Dimension FastScan[®] AFM (Bruker, Santa Barbara, CA), where a PeakForce Tapping[®] scanning mode was used at 20 °C with a ScanAsyst-Air[®] probe (Bruker, Billerica, MA) with nominal spring constant $k = 0.4$ N/m and nominal tip radius of 2 nm. One drop (5 μ L) of dendrimer solution (200 μ g/mL and 500 μ g/mL in PBS pH 7.4) was placed on a freshly cleaved mica surface and was kept in a desiccator at 20 °C overnight in the dark for drying before measurements. Images were analyzed using NanoScope Analysis[®] 1.5 software (Bruker, Santa Barbara, CA). Height images were corrected by first-order flattening.

2.3.7. Evaluation of redox sensitivity

The redox-responsive change in hydrodynamic diameter of DAB-PEG-SS-ODT vesicles (1.5 mL, 100 μ g/mL in PBS pH 7.4) was measured by dynamic light scattering at various time intervals, in presence and absence of glutathione (10 μ M and 10 mM) at 37 °C. Sample solutions in presence and absence of glutathione were prepared by mixing 75 μ L of stock dendrimer solutions (2 mg/mL) with 150 μ L glutathione solution (100 mM or 100 μ M) and 1275 μ L PBS (pH 7.4), or 1425 μ L PBS solution (pH 7.4) for control measurement. Each sample was then incubated in the dark under stirring at 37 °C for 48 h throughout the measurement.

2.4. Characterization of DAB-PEG-SS-ODT-DNA dendriplex

2.4.1. Gel retardation assay

Gel retardation assay was used to qualitatively assess the DNA condensation ability of DAB-PEG-SS-ODT dendrimer at dendrimer: DNA weight ratios 20:1, 10:1, 5:1, 2:1, 1:1, 0.5:1 and 0:1, in TE buffer (10 mM Tris, 1 mM EDTA, pH 7.5), where DNA concentration was fixed at 20 μ g/mL for each sample. Each sample solution (10 μ L) was mixed with loading buffer (2 μ L, Bioline, UK) and then loaded on a 1 $\times$ Tris-Borate-EDTA (TBE) (89 mM Tris base, 89 mM boric acid, 2 mM Na₂-EDTA, pH 8.3) buffered 0.8% (w/v) agarose gel containing ethidium bromide (0.4 μ g/mL), with 1 $\times$ TBE as a running buffer. As a DNA size marker, HyperLadder 1Kb (Bioline, UK) was used. The gel was run at 50 V for 1 h before being photographed under UV light with 40 ms exposure.

2.4.2. PicoGreen[®] assay

PicoGreen[®] assay was performed according to the supplier's protocol to quantitatively assess the ability of DAB-PEG-SS-ODT dendrimer to condense plasmid DNA encoding β -galactosidase. One mL of dendrimer: DNA complexes at various dendrimer: DNA weight ratios (20:1, 10:1, 5:1, 2:1, 1:1, 0.5:1 and 0:1) were prepared by adding DNA solution (500 μ L, 10 μ g/mL in TE buffer) to each sample of dendrimer solution (500 μ L in TE buffer) and vortexed to make the solution homogeneous. PicoGreen[®] reagent was diluted to 200-fold in Tris–EDTA (TE) buffer (10 mM Tris, 1 mM EDTA, pH 7.5) on the day of the experiment. One mL PicoGreen[®] solution was then added to 1 mL dendriplexes and vortexed before measurement. The dendriplex solutions with PicoGreen[®] were then kept in the dark for the rest of the measurements at various time intervals. Varian Cary Eclipse[®] Fluorescence spectrophotometer (Palo Alto, CA) was used to measure the fluorescence intensity of PicoGreen[®] (λ_{exc} : 480 nm, λ_{em} : 520 nm, excitation and emission slits: 5 nm each) at various times. Results were represented as percentage of DNA condensation.

2.4.3. Size and zeta potential measurement

The hydrodynamic diameter and zeta potential of dendriplexes in 5% (w/v) glucose solution and PBS (pH 7.4) were measured by using a Malvern Nano ZS[®] instrument (Malvern Instruments, Malvern, UK), immediately after mixing a fixed amount of DNA (50 μ g) to DAB-PEG-SS-ODT dendrimer at various concentrations (dendrimer: DNA weight ratios: 0.5:1, 1:1, 2:1, 5:1, 10:1 and 20:1).

2.4.4. Transmission electron microscopy (TEM) imaging

The morphology of the DAB-PEG-SS-ODT-DNA dendriplexes was visualized using a JEOL JEM-1200EX[®] transmission electron microscope (TEM) (Jeol, Tokyo, Japan) operating at an accelerating voltage of 80 kV. One drop (3 μ L) of each dendriplex solution in PBS (pH 7.4) was drop cast on a carbon-coated copper grid (400 mesh size) immediately after mixing a fixed amount of DNA (50 μ g) to the dendrimer at various concentrations (dendrimer: DNA weight ratios: 10:1 and 20:1). The prepared samples were then kept in a desiccator at 20 °C overnight in the dark for drying before imaging.

2.4.5. Atomic force microscopy (AFM) imaging

DAB-PEG-SS-ODT-DNA dendriplexes were characterized by atomic force microscopy to assess their size and shape using the same machine, scanning mode and probe as mentioned

in Section 2.3.6. One drop (5 μ L) of each dendriplex solution in Tris–EDTA (TE) buffer (10 mM Tris, 1 mM EDTA, pH 7.5) was placed on a freshly cleaved mica surface immediately after mixing a fixed amount of DNA (50 μ g) to various dendrimer concentrations (dendrimer: DNA weight ratios: 10:1 and 20:1) and was kept in a desiccator at 20 °C overnight in the dark for drying before measurements. Images were analyzed using the software mentioned in Section 2.3.6. Height images were corrected by second-order flattening.

2.5. *In vitro* analysis

2.5.1. Cell culture

PC-3M-luc-C6 and DU145 cells were grown as monolayers in 1% (v/v) L-glutamine containing Minimum Essential Medium (MEM), supplemented with 10% (v/v) fetal bovine serum, and 0.5% (v/v) penicillin–streptomycin. Cells were cultured at 37 °C in a 5% carbon dioxide, humid atmosphere.

2.5.2. Cell viability

A standard MTT assay was used to evaluate the cell viability following treatment with DAB-PEG-SS-ODT dendrimer at various concentrations. PC-3 and DU145 cells were seeded at a density of 5 000 cells per well in 96-well plates and left to grow at 37 °C in a 5% carbon dioxide, humid atmosphere. After 24 h, the medium was removed and the cells were then treated with DAB-PEG-SS-ODT solutions at various concentrations (ranging from 5 to 50 μ g/mL). After 72 h of treatment, 50 μ L of MTT solution (0.5% w/v in PBS pH 7.4) was added in each well. Following 4-h incubation at 37 °C, the medium was removed and the formazan dye produced during the reduction of MTT by the living cells was solubilized in 200 μ L DMSO, before being quantified by spectrophotometry at 540 nm with a Multiskan Ascent[®] plate reader (Thermo Scientific, Waltham, MA).

2.5.3. Cellular uptake of the dendriplexes

The cellular uptake of DAB-PEG-SS-ODT dendrimer-based vesicles complexed to fluorescein- labelled DNA by PC-3 and DU145 prostate cancer cells was qualitatively assessed using confocal microscopy. Plasmid DNA was labelled with the fluorescent probe fluorescein using a Label IT[®] Fluorescein Nucleic Acid Labeling kit, as described by the manufacturer. The cells were seeded at a concentration of 2×10^5 cells per well on coverslips placed in 6-well plates and left to grow at 37 °C in 5% carbon dioxide, humid atmosphere. The old medium was removed after 24 h and the cells were washed with 2 mL phosphate

buffered saline (PBS) once to remove any dead cells. The cells were then treated with DAB-PEG-SS-ODT vesicles complexed to fluorescein-labelled DNA (2.5 µg/well), at dendrimer: DNA weight ratios of 10:1 and 20:1. Cells treated with DAB dendrimer complexed to fluorescein-labelled DNA (2.5 µg/well), at a dendrimer: DNA weight ratio of 5:1, naked DNA or left untreated were also used as controls.

After 2-h incubation at 37 °C, the medium was removed and cells were then washed three times with 2 mL phosphate buffered saline (PBS). The cells were then fixed with 2 mL methanol for 10 min at 20 °C. Methanol was then removed and the cells were air-dried for 10 min before being stained with Vectashield® mounting medium containing the nuclear stain DAPI. The cells were visualized using a Leica TCS SP8® confocal microscope (Wetzlar, Germany). DAPI (which stained the cell nuclei) was excited with the 405 nm laser line (emission bandwidth: 415-491 nm) and fluorescein (which labelled the DNA) was excited with the 514 nm laser line (emission bandwidth: 550-620 nm).

The cellular uptake of the DAB-PEG-SS-ODT dendrimer-based vesicles complexed to DNA by PC-3 and DU145 cells was quantitatively carried out by flow cytometry using a FACSCanto® flow cytometer (BD, Franklin Lakes, NJ). Both cell lines were seeded at a density of 10⁵ cells per well in 6-well plates and left to grow at 37 °C in a 5% carbon dioxide, humid atmosphere. The medium was discarded after 72 h and cells were washed with 2 mL phosphate buffered saline (PBS) once to remove any dead cells. The cells were then treated with DAB-PEG-SS-ODT vesicles complexed to fluorescein-labelled DNA (2.5 µg/well), at a dendrimer: DNA weight ratios of 10:1 and 20:1 for 2 hours at 37 °C. Cells treated with DAB dendriplex at dendrimer: DNA weight ratio of 5:1 or DNA solution were used as controls. After a 2-h incubation with the treatments, treatment was removed and the cells were washed 3 times with 2 mL PBS. Then, 250 µL trypsin was added to each well, followed by incubation at 37 °C, and 500 µL fresh medium was then added after 5 min to each well once the cells have detached to prepare single cell suspensions. The mean fluorescence intensity of these samples was analyzed with FACSDiva® software (BD, Franklin Lakes, NJ), counting ten thousand cells (gated events) for each sample.

2.5.4. Gene expression of the dendriplexes

The transfection efficacy of the dendriplexes formed between DAB-PEG-SS-ODT cationic dendrimer and DNA was assessed on PC-3 and DU145 cells using plasmid DNA encoding β-galactosidase (pCMV β-gal). Both cell lines were seeded at a concentration of 2 000

cells/well in 96-well plates and left to grow at 37 °C in a 5% carbon dioxide, humid atmosphere. After 72 h of incubation, the old medium was removed and cells were then treated with the dendriplexes in quintuplicate in each 96-well plate at various dendrimer: DNA weight ratios (20:1, 10:1, 5:1, 2:1, 1:1 and 0.5:1), where DNA concentration was fixed at 1 µg/well throughout the experiment. Naked DNA and DAB-DNA (dendrimer: DNA weight ratio 5:1) were used respectively as negative and positive controls. To measure the transfection efficacy at various time intervals, PC-3 cells were treated for 24 h, 48 h and 72 h before analysis. For comparative analysis of highest transfection efficiency, PC-3 and DU145 cells were treated for 72 h before analysis. Following treatment, the wells were emptied and the cells were then lysed with 1 X passive lysis buffer (PLB) (50 µL/well) for 20 min at 37 °C, before being tested for β-galactosidase expression. Briefly, 50 µL of the assay buffer (2 mM magnesium chloride, 100 mM mercaptoethanol, 1.33 mg/mL O-nitrophenyl- β-D-galactosidase, 200 mM sodium phosphate buffer, pH 7.3) was added to the each well and the cell lysates were incubated for 2 h at 37 °C. Multiskan Ascent[®] plate reader (MTX Lab Systems, Brandenton, FL) was used to measure the absorbance of the samples at 405 nm.

2.6. Statistical analysis

Results were expressed as means ± standard error of the mean. Statistical significance was assessed by one-way analysis of variance and Tukey multiple comparison post-test (Minitab[®] software, State College, PE). Differences were considered statistically significant for P values lower than 0.05.

3. Results and discussion

3.1. Synthesis and characterization of the disulfide-linked lipid-bearing PEGylated dendrimer (DAB-PEG-SS-ODT)

The disulfide-linked octadecyl chain modified PEGylated (DAB-PEG-SS-ODT) dendrimer was synthesized using *in situ* two-step reaction. The synthesis of DAB-PEG-SS-ODT dendrimer was confirmed by FTIR, ^1H -NMR and MALDI-TOF mass spectrometry. Infrared (ATR) spectra of DAB-PEG-SS-ODT dendrimers (Figure S1A) showed the appearance of a new peak at 1647 cm^{-1} (corresponding to an amide bond) and the absence of a peak at $1719\text{--}1740\text{ cm}^{-1}$ (corresponding to the succinimidyl carboxymethyl ester group of the OPSS-PEG-SCM), in comparison to starting materials (Figure S1B and S1C), therefore demonstrating the successful conjugation of PEG to DAB through amide linkage.

The successful conjugation of octadecyl chain and PEG to DAB dendrimer was also confirmed by ^1H -NMR, as follows: ^1H -NMR (500 MHz, CDCl_3): δ 2.36 ppm, DAB ($\text{N-CH}_2\text{-CH}_2\text{-CH}_2\text{-N}$) (a); δ 3.91 ppm, PEG ($\text{NH-CO-CH}_2\text{-OCH}_2\text{CH}_2$) (b); δ 0.83-0.80 ppm, ODT ($\text{CH}_2\text{-CH}_3$) (c) (Figure S2).

The molecular weight of DAB-PEG-SS-ODT dendrimer was assigned by analyzing MALDI-TOF mass spectrum (Figure S3), which mainly exhibited singly charged molecular ions $[\text{M}]^+$ with its fragmented products. The average molecular weight of DAB-PEG-SS-ODT was 4522.73 Da, indicating that the ratios of the conjugated PEG as well as lipid (octadecyl chain) per dendrimer were nearly 1. MALDI-TOF mass spectrometry data showed that 6.33% lipid was conjugated to DAB-PEG-SS-ODT dendrimer (Section S1). Conjugation of PEG and lipid chain on one amine group of dendrimer helped to retain more primary amine groups on the surface of the dendrimer, leaving them free for complexation of nucleic acids. Furthermore, the successful synthesis of lipid-bearing dendrimers with lower hydrophobe (octadecyl) content than that in DAB-PEG-SS-ODT was found to be difficult, as they became insoluble in water after lyophilization due to the well-known problem of freeze-thaw evidenced for various cationic lipids and emulsion-based foods consisting of fat droplets dispersed within an aqueous medium.³³⁻³⁴

The ^1H NMR spectrum of DAB-PEG-SS-ODT dendrimer in D_2O only showed peaks corresponding to hydrophilic DAB and PEG, (Figure S4), unlike the ^1H NMR spectrum of the dendrimer in CDCl_3 (Figure S2), which displayed peaks for the hydrophobic lipid and hydrophilic DAB and PEG. The appearance and disappearance of ^1H NMR peaks (δ 0.83-0.80 ppm and δ 1.19 ppm) of conjugated octadecyl chain (ODT) to PEGylated dendrimer respectively in CDCl_3 and in D_2O indicated the amphiphilic nature of DAB-PEG-SS-ODT

dendrimer, tending to possible formation of DAB-PEG-SS-ODT-based self-assembled nanostructure in aqueous medium, where lipid chains may form the hydrophobic domain.³⁵

3.2. Self-assembly formation of DAB-PEG-SS-ODT self-aggregates

The self-assembly formation of the amphiphilic dendrimer DAB-PEG-SS-ODT aggregates was studied using steady-state fluorescence technique measuring fluorescent intensity of NPN. NPN is weakly fluorescent in water because of its hydrophobic nature, but its fluorescence intensity increases when it is solubilized in a non-polar medium or within the hydrophobic core of any self-assembled aggregates.³⁶⁻³⁷ The self-assembly formation of amphiphilic dendrimer DAB-PEG-SS-ODT aggregates was demonstrated by the gradual rise of relative fluorescence intensities (I/I_0 , where I and I_0 are respectively the fluorescence intensities in the presence and absence of dendrimer) of NPN with increasing concentrations of dendrimer in PBS (pH 7.4) (Figure 2A), due to the solubilization of the probe in the hydrophobic domain of self-assembled dendrimer-based aggregates. The critical aggregation concentration (CAC) value ($\sim 50 \mu\text{g/mL}$) was calculated from the inflection point of the plot of relative fluorescence intensities (I/I_0) of NPN at various dendrimer concentrations (Figure 2A).

Figure 2. (A) Relative fluorescence intensity (I/I_0) of N-Phenyl-1-naphthylamine in function of the concentration of dendrimer in PBS buffer (pH 7.4) (error bars smaller than the symbol size at low dendrimer concentrations). (B) Size (■) and zeta potential (○) of dendrimer-based self-assembled nanostructures in PBS buffer (pH 7.4) at 37 °C (C) Stained TEM images of dendrimer-based vesicles (400 $\mu\text{g/mL}$ in PBS, pH 7.4) (bar size: 100 nm). (D) Fluorescence spectra of Coumarin-153 (λ_{exc} : 420 nm, λ_{em} : 440-800 nm in presence or absence of dendrimer solutions in PBS buffer (pH 7.4).

The CAC value for the octadecyl chain-bearing PEGylated DAB dendrimer was found to be higher than that of vesicle formation by 9.5% and 12.5% cholesterol-bearing PEGylated DAB dendrimers.¹⁹ This comparatively higher CAC value for DAB- PEG-SS-ODT self-aggregates than for cholesterol bearing dendrimer-based aggregates might be attributed to the overall lower hydrophobe content and lower hydrophobicity of alkyl chain than steroidal molecule forming less compact aggregates, as the self-assembled aggregate formation occurs mainly through the hydrophobic interactions among the lipid molecules. In addition, the CAC value of this lipid-bearing dendrimer was found to be well below those of dendrimersomes and glycodendrimersomes prepared from self-assembling amphiphilic Janus dendrimers (typically between 0.5 mg/mL and 1 mg/mL), indicating comparatively enhanced hydrophobic interactions among DAB-PEG-SS-ODT molecules due to the presence of lipids in its backbone.³⁸ The initial concentration-independent plateau of the fluorescence intensity curve at dendrimer concentrations below ~ 50 $\mu\text{g/mL}$ reveals that the self-association of the dendrimer starts through the inter-chain aggregation above the CAC, without formation of unimer aggregates (Figure 2A).

3.3. Hydrodynamic size and zeta potential of DAB-PEG-SS-ODT self-aggregates

The hydrodynamic diameter (d_H) of DAB-PEG-SS-ODT dendrimer-based self-assembled nanostructures displayed a gradual decrease in average size from low to high concentrations (Figure 2B). This decrease in size suggested the formation of more compact and stable nanostructures with smaller size deviation at high dendrimer concentrations, due to increased hydrophobic interactions among lipid molecules with increasing concentration. The DAB-PEG-SS-ODT-based nanostructures showed a consistent size of $260.8 \text{ nm} \pm 36.5 \text{ nm}$ at $100 \mu\text{g/mL}$ and $221.4 \text{ nm} \pm 39.4 \text{ nm}$ at $200 \mu\text{g/mL}$ respectively with nearly no statistically difference and reached the smallest size of $162.1 \text{ nm} \pm 8.1 \text{ nm}$ at $400 \mu\text{g/mL}$ concentration. These sizes were below the cut-off size (400 nm) for extravasation to most tumor cells³⁹ and displayed a similar trend as some of the reported spontaneous or stimuli-sensitive vesicular structure formation of nearly 200-500 nm size from C17 and C18 alkyl chain-bearing polyamine amphiphilic dendrimers.⁴⁰⁻⁴¹

DAB-PEG-SS-ODT-based self-assembled nanostructures were bearing a positive zeta potential at all the tested concentrations. They displayed an increase in zeta potential with increasing concentrations, to reach a maximum value of $2.43 \text{ mV} \pm 0.54 \text{ mV}$ at $400 \text{ }\mu\text{g/mL}$ concentration. The positive zeta potential of these nanostructures was found to be lower than that obtained with disulfide cross-linked hyperbranched PAMAM-based vesicles ($\sim 5 \text{ mV}$) and cholesterol-bearing PEGylated DAB dendrimersomes ($\sim 4\text{-}7 \text{ mV}$).^{19,42} The positively charged DAB-PEG-SS-ODT-based self-assembled nanostructures should therefore enhance the non-specific uptake of the nanostructures by the cells because of favorable electrostatic interactions between the negatively charged cell membrane and positively charged nanoparticles.⁴³

3.4. Observation of the morphology of the self-assembled DAB-PEG-SS-ODT

TEM images of the DAB-PEG-SS-ODT dendrimer-based nanostructures revealed that the lipid-modified dendrimer assembled into spherical, unilamellar vesicles (dendrimersomes) with a size about 100 nm (Figure 2C). The size of the vesicles obtained from the TEM images was found to be smaller than that observed with DLS, which might be due to the drying of the samples before TEM imaging, which is not the case for the measurement of hydrodynamic sizes in DLS. The vesicular nanostructure was comparatively smaller than C17 and C18 alkyl chain modified-polyamine amphiphilic dendrimers-based vesicles of nearly $200\text{-}500 \text{ nm}$ sizes,^{26,41} but larger in comparison to the vesicles generated from cholesterol and camptothecin-bearing PEGylated DAB dendrimers, which were more compacted.^{19,20}

3.5. Micropolarity of DAB-PEG-SS-ODT-based dendrimersomes

An increase in steady-state fluorescence intensity of emission maximum of coumarin-153 (C153) with increasing concentrations of DAB-PEG-SS-ODT dendrimersomes (Figure 2D) suggested an increasing hydrophobicity of the vesicular bilayer membrane with concentration. The rise in fluorescence intensity was associated with only a small blue shift ($\sim 4 \text{ nm}$) of the emission maximum of C153 in presence of DAB-PEG-SS-ODT than in its absence, indicating a comparatively polar microenvironment of this vesicular bilayer. The micropolarity (π^* -value = 0.83) of the hydrophobic microenvironment of bilayer, predicted from the blue shift of maximum

emission ($\lambda_{\text{max}} = 545.94 \text{ nm}$) of C153 solubilized within DAB-PEG-SS-ODT ($1000 \mu\text{g mL}^{-1}$) solution, is similar to the polarity of the polar aprotic solvent propylene carbonate (0.83).⁴⁴ The more polar nature of the hydrophobic bilayer of DAB-PEG-SS-ODT vesicles made them less favorable for encapsulation of a substantial amount of hydrophobic drugs.

3.6. Thermal analysis of phase transition behavior of DAB-PEG-SS-ODT-based dendrimersomes

Differential scanning calorimetry was performed to investigate the impact of the lipidic alkyl chain on the backbone structure of the PEGylated dendrimersomes at two concentrations (1 and 2 mg/mL) of DAB-PEG-SS-ODT solution. A crystallization exotherm was visible for both DAB-PEG-SS-ODT concentrations at nearly 0 °C during the heating phase, and also appeared during the second heating cycle (Figure 3). It indicated the transition of the lipid-bearing dendrimer from “amorphous” state to “crystalline” or “semi-crystalline” state, which is very similar to that observed for 20% dodecyl-modified generation 5-hydroxyl-terminated PAMAM dendrimer, which displayed a crystallization exotherm at $-15.1 \text{ }^{\circ}\text{C}$.⁴⁵ Although dendrimer-based vesicles showed nearly the same melting peak at both concentrations, the melting enthalpy (area under the exothermic curve) decreased to a much lower value (average 2.93 J/g) at lower DAB-PEG-SS-ODT concentration (1 mg/mL) compared with the higher value observed at higher DAB-PEG-SS-ODT concentration (average 330.95 J/g at 2 mg/mL) (Figure 3). This was due to a progressive “dilution” of the lipid in the vesicular bilayer membrane from high to low dendrimer concentrations.⁴⁶ The narrow transition peaks indicated the presence of a pure crystalline structure, as an increased broadening of this peak is associated with the presence of impurities.⁴⁷

Figure 3. DSC spectra of DAB-PEG-SS-ODT (1 and 2 mg/mL) for the first heating and second heating cycles (both at 5 °C min⁻¹).

3.7. AFM imaging of DAB-PEG-SS-ODT-based dendrimersomes

At a lower concentration (200 µg/mL) of DAB-PEG-SS-ODT, the cross-section analysis of AFM images showed a slight deformation in the shape of the dendrimer-based vesicles, which changed from spheres to spherical caps with an average typical height of about 25-30 nm (Figure 4A). The strong adsorption of lipid-bearing DAB-PEG-SS-ODT dendrimer onto the freshly cleaved mica surface is a probable reason for this deformation, leading to the broadening of the vesicle diameter. The effect of adsorption of the dendrimer-based vesicles onto the mica surface was even more pronounced at a higher concentration of dendrimer solution (500 µg/mL). Because of the presence of a larger amount of lipids at higher concentration of dendrimer, a more pronounced deformation due to an enhanced spreading or fusion of these vesicles on the mica surface was observed, leading to the formation of a thick layer of dendrimer on mica, with an average typical height of about 5 nm (Figure 4B). This is in accordance with previous reports of the deformation of various vesicular structures (i.e. liposomes, dendrimersomes, polymersomes) on mica surface, leading to fusion.^{19,48-49}

Figure 4. AFM peak force error and height sensor images of 5 x 5 μm surfaces of (A) 200 $\mu\text{g/mL}$ and (B) 500 $\mu\text{g/mL}$ dendrimer-based vesicles adsorbed on a mica (silicon) surface. The average heights were measured by cross section analysis.

3.8. Redox sensitivity of the DAB-PEG-SS-ODT dendrimersomes

The hydrophobic bilayer of the DAB-PEG-SS-ODT-based dendrimersomes is made of octadecyl chains. This hydrophobic group was conjugated to the PEGylated DAB dendrimer via a disulfide linkage, which is sensitive to any reducing agent or conditions such as glutathione, a reducing biothiol. To assess the redox-responsiveness of the vesicles, the hydrodynamic size of the DAB-PEG-SS-ODT-based vesicles was measured in presence and absence of glutathione at various time intervals and at various concentrations of glutathione (10 μM and 10 mM, corresponding to reducing environments in the extracellular and intracellular compartments respectively) (Figure 5). As the thiol–disulfide exchange reaction was reported to show quantitative or near-quantitative yields only under mild or neutral conditions rather in acidic conditions, the redox-

sensitive nature of DAB-PEG-SS-ODT vesicles was assessed at pH 7.4.⁵⁰ DAB-PEG-SS-ODT-based vesicles displayed similar sizes (~250-300 nm) in presence of 10 μ M or without glutathione at all the tested durations, implying that the extracellular reducing environment (10 μ M GSH concentration) has little effect on the integrity of the vesicles. However, at higher concentration (10 mM) of glutathione, corresponding to the reducing environment in the intracellular compartment, a pronounced increase in size of aggregates was observed from 6 h (229.4 ± 60.1 nm) to 24 h (816.9 ± 375.5 nm) and 48 h (1259.0 ± 151.0 nm) (Figure 5). This can be attributed to the cleavage of the disulfide bonds and partial disintegration of the vesicles, forming larger, unstable and less compact aggregates, suggesting strong redox sensitivity in the intracellular reducing environment like previously reported redox-responsive cholesterol-bearing PEGylated DAB dendrimers.¹⁹ This increase in size of aggregates for redox-responsive lipid-bearing PEGylated DAB dendrimer, DAB-PEG-SS-ODT was observed to be more pronounced than the effects observed for redox-responsive PEGylated PAMAM dendrimers that showed minute difference in hydrodynamic size distribution (~4 nm) in 10 mM GSH concentration.⁵¹ The strong redox-responsive nature of DAB-PEG-SS-ODT in the intracellular or cytosolic reducing environment (corresponding to 10 mM glutathione) could be useful for these octadecyl-bearing dendrimersomes as potential redox-responsive drug carriers for cancer treatment, as cancer cells have even further elevated intracellular glutathione concentration.

Figure 5. Evaluation of GSH sensitivity of dendrimer-based vesicles: Hydrodynamic size distribution of DAB-PEG-SS-ODT dendrimer-based vesicles (100 μ g/mL in PBS (pH 7.4)) at various time intervals in the presence or absence of GSH (10 μ M and 10 mM) at 37 $^{\circ}$ C (n = 3).

3.9. Characterization of dendriplex formation

3.9.1. Evaluation of DNA condensation

The protonated primary amines of polyamine dendrimers, such as DAB dendrimer, can efficiently form dendrimer-DNA complexes (or dendriplexes) with the negatively charged phosphodiester groups of the DNA through electrostatic interactions. The DNA condensing ability of DAB-PEG-SS-ODT dendrimer was qualitatively confirmed through gel retardation assay (Figure 6A). The partial DNA condensation by DAB-PEG-SS-ODT dendrimer was observed at dendrimer: DNA weight ratios of 0.5:1 to 2:1, as free DNA was visible at these ratios. DNA was fully condensed by DAB-PEG-SS-ODT at dendrimer: DNA weight ratios of 5:1 and above.

DNA condensing ability of DAB-PEG-SS-ODT dendrimer was also quantitatively assessed by PicoGreen[®] assay (Figure 6B). PicoGreen[®] shows a significant increase in its fluorescence intensity due to its intercalation within the double strand of free DNA, but its fluorescence intensity decreases with the formation of stable dendriplex as a result of electrostatic interactions between the negatively charged DNA and the positively charged dendrimer. The DAB-PEG-SS-ODT dendrimer was able to condense ~70% of the DNA at dendrimer: DNA weight ratios of 5:1 and higher, because of the presence of a smaller number of primary amines of the modified DAB dendrimer at its periphery available for complexation (Figure 6B). DAB-PEG-SS-ODT dendrimer can condense DNA instantly and the formed dendriplexes were found to be stable for at least 24 h. The overall DNA condensing ability of DAB-PEG-SS-ODT dendrimer was comparatively higher than that of two C18 alkyl chain-bearing generation 1-PAMAM dendrimer, but lower than that with two C18 alkyl chain-bearing generations 2- and 3-PAMAM dendrimers, two dodecyl chain-bearing generation 3-PAMAM dendrimers, and cholesterol-modified generations 1- and 3-PAMAM dendrimers and generation 3-DAB dendrimer.^{19,26,29,52-53} The presence of more octadecyl chains on PEGylated generation 3-DAB dendrimer surface may therefore increase the DNA condensation.

Figure 6. Evaluation of the DNA complexation of the DAB-PEG-SS-ODT dendrimer: (A) Gel retardation assay of DAB-PEG-SS-ODT-DNA complexes at various DAB-PEG-SS-ODT: DNA weight ratios (0.5:1, 1:1, 2:1, 5:1, 10:1 and 20:1) and DNA only, where the amount of DNA was fixed at 20 μ g for each complex. (B) DNA condensation of dendriplexes using PicoGreen[®] reagent at various durations and dendrimer: DNA weight ratios: 20:1 (■, black), 10:1 (●, red), 5:1 (▲, blue), 2:1 (▼, magenta), 1:1 (◆, green) and 0.5:1 (◄, violet), where the amount of DNA was fixed at 10 μ g for each complex. Results are expressed as mean $\pm$ SEM (n=4).

3.9.2. Size and zeta potential measurement

DAB-PEG-SS-ODT dendriplex displayed an average size less than ~100-150 nm at all tested dendrimer: DNA weight ratios (Figure 7A). The size of the dendriplexes initially slightly increased with increasing dendrimer: DNA weight ratios ranging from 0.5:1 to 2:1 and then decreased to ~100 nm or less for higher dendrimer: DNA weight ratios, for which DAB-PEG-SS-ODT showed maximum condensation ability (Figure 6B). The smaller hydrodynamic size of these dendriplexes might be due to the presence of the highly hydrophobic alkyl chain in the dendrimer backbone, leading to the formation of compact aggregates, which was comparable to the size of two C18 alkyl chain-modified generation 3-PAMAM dendrimer-based complexes, but well below that of two C12 alkyl chain modified generation 3-PAMAM dendrimer-based complexes.²⁶ Furthermore, the dendriplexes have shown an overall average size lower than the average size (~196 nm) of unmodified DAB dendriplex.¹⁰

Figure 7. Size (A) and zeta potential (B) of DAB-PEG-SS-ODT-DNA dendriplex at various dendrimer: DNA weight ratios. Unstained TEM images of DAB-PEG-SS-ODT-DNA dendriplex at dendrimer: DNA weight ratios of 10:1 (C) and 20:1 (D) (bar: 100 nm). The amount of DNA was fixed at 50 μ g for each complex.

Zeta potential experiments demonstrated a gradual change in charges from negative values (for dendriplexes at dendrimer: DNA weight ratios 0.5:1 (-37.1 ± 1.88 mV), 1:1 (-22.3 ± 3.73 mV) and 2:1 (-5.44 ± 0.81 mV)) to positive values (for dendriplexes at dendrimer: DNA weight ratios 5:1 (9.46 ± 0.58 mV), 10:1 (12.7 ± 0.90 mV) and 20:1 (14.4 ± 0.75 mV)) (Figure 7B). The complete reversal from a negative to positive zeta potential for these dendriplexes from 5:1 weight ratio could be justified by the increasing DNA condensation ability of DAB-PEG-SS-ODT from that particular weight ratio. The positively charged dendriplexes would facilitate interactions with the negatively charged cancer cell surface, resulting in an enhanced cellular uptake of these dendriplexes. The positive zeta potential of DAB-PEG-SS-ODT dendriplexes at 5:1 to 20:1 ratios was comparable with the positive charges of PEGylated lipidic alkyl chain-bearing poly(amidoamine) dendron-based lipoplexes ($\sim 4\text{--}10$ mV),²⁷ but was lower than that of dendriplexes formed with the more hydrophobic cholesterol-modified generations 1- and 3- PAMAM dendrimers ($\sim 18\text{--}23$ mV) and generation 3-DAB dendrimer (~ 40 mV).^{19,52-}

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3.10. Morphology of the dendriplexes

3.10.1 Transmission electron microscopy imaging

Transmission electron microscopy images showed that DAB-PEG-SS-ODT dendrimer has retained its ability to form vesicles even after complexation with DNA, with sizes lower than 100 nm, at dendrimer: DNA weight ratios 10:1 and 20:1 (Figure 7C & 7D). At higher weight ratio (20:1), vesicles appeared to be smaller than that formed at lower weight ratio (10:1), which is well supported by their size distribution in light scattering studies (Figure 7A). The capability of C18 alkyl chain-bearing PEGylated DAB dendrimer to form stable vesicles with size lower than 100 nm in presence of DNA, was found to be advantageous over C17 alkyl chain-modified polyamine-based dendrimersomes of ~ 200 nm diameter that transformed, in presence of siRNA, from vesicles to nanosized siRNA-dendrimer complexes composed of many highly ordered spherical micellar substructures of 6–8 nm diameter.⁴¹

3.10.2 Atomic Force Microscopy imaging

AFM images were recorded for DAB-PEG-SS-ODT-based dendriplexes at dendrimer: DNA weight ratios 10:1 and 20:1. At both weight ratios, the presence of smaller-sized particles with some larger-sized particles were observed on mica surface during scanning (Figure 8). The larger-sized particles might be formed by aggregation of smaller particles of the DAB-PEG-SS-ODT-DNA complexes. The smaller-sized particles are about 150-200 nm in size, measured through cross-sectional analysis, which is well corroborated with light scattering and TEM studies of dendriplexes. Their size is also comparable with that of two C18 alkyl chain-modified generation 3-PAMAM dendrimer-based complexes.²⁶ The cross-sectional analysis of these dendriplexes showed the presence of spherical caps of aggregates, rather than spheres, with an average typical height of about 5-10 nm (Figure 8), suggesting an adsorption and thereby broadening of the diameter of these lipid-bearing dendriplexes onto the freshly cleaved mica surface in comparison with that in TEM images. Unlike native DAB-PEG-SS-ODT dendrimer-based vesicles, DAB-PEG-SS-ODT-DNA dendriplex displayed less deformation, leading to a diminishing tendency of adsorption on mica surface after complexation with DNA.

Figure 8. AFM peak force error and height sensor images of 5 x 5 μm surfaces of dendriplex-based vesicles at (A) 20:1 and (B) 10:1 DAB-PEG-SS-ODT: DNA weight ratios adsorbed on a mica (silicon) surface. The average heights were measured by cross section analysis.

3.11. *In vitro* analysis

3.11.1. Cell viability

Cell viability experiments demonstrated that PC-3 cells remained highly viable after 72 h of incubation with various DAB-PEG-SS-ODT dendrimer solutions (0-50 $\mu\text{g/mL}$) (Figure 9). However, this was not the case for DU145 cells: only about 50% of the cells were still viable following 72 h of incubation with the dendrimer, over the whole concentration range (Figure 9). The difference of cell viability observed in PC-3-Luc and DU145 cells

following treatment with the DAB-PEG-SS-ODT dendrimer might be due to the various expression of apoptosis-related proteins in these cancer cell lines. Although both cell lines are androgen-irresponsive human prostate adenocarcinomas, PC-3 cells are P53-null, unlike DU145 cells that express a large amount of P53 protein.⁵⁴

Figure 9. Cell viability of DAB-PEG-SS-ODT on PC-3 (light grey) and DU145 (dark grey) human prostate cancer cells at various concentrations after 72-h incubation (n = 15).

3.11.2. Cellular uptake of the dendriplexes

3.11.2.1. Qualitative analysis

The confocal microscopy images qualitatively confirmed the successful cellular uptake of the dendriplexes formed between DAB-PEG-SS-ODT dendrimer and fluorescein-labeled DNA by

PC-3 and DU145 cells (Figures 10 and 12). Treatment with DAB-PEG-SS-ODT dendriplexes at both 10:1 and 20:1 weight ratios for 2 h showed the dissemination of fluorescein-labeled DNA in the cytoplasm of the both prostate cancer cell lines. In PC-3 cell line, fluorescence intensity appeared to be comparable for the samples treated with the DAB-PEG-SS-ODT-based complexes and DAB dendriplex. In DU145 cell line, fluorescence intensity appeared to be more pronounced for the samples treated with the DAB-PEG-SS-ODT-based complexes than with DAB dendriplex. No co-localization of DNA in the nuclei was visible after 2 h incubation in both cell lines for DAB-PEG-SS-ODT and DAB dendriplexes.

Figure 10. Confocal microscopy images of the cellular uptake of FITC-labeled DNA (2.5 µg/well), complexed with DAB-PEG-SS-ODT at various weight ratios (control: DAB: DNA, only DNA) by PC-3-Luc prostate cancer cells after 2 h incubation. Blue: nuclei stained with DAPI (excitation: 405 nm laser line; bandwidth: 415–491 nm), green: fluorescein-labelled DNA (excitation: 453 nm laser line; bandwidth: 550–620 nm) (magnification: x20, bar: 50 µm).

Figure 11. Confocal microscopy images of the cellular uptake of FITC-labeled DNA (2.5 µg/well), complexed with DAB-PEG-SS-ODT at various weight ratios (control: DAB: DNA, only DNA) by DU145 prostate cancer cells after 2 h incubation. Blue: nuclei stained with DAPI (excitation: 405 nm laser line; bandwidth: 415–491 nm), green: fluorescein-labelled DNA (excitation: 453 nm laser line; bandwidth: 550–620 nm) (magnification: x20, bar: 50 µm).

3.11.2.2. Quantitative analysis

The cellular uptake of fluorescein-labeled DNA complexed with DAB-PEG-SS-ODT dendrimer, was quantitatively confirmed by flow cytometry. Treatment of PC-3 cells with the DAB-PEG-SS-ODT dendriplexes resulted in very high cellular fluorescence at dendrimer: DNA ratios of 10:1 (2158 ± 64.58 a.u.) and 20:1 (2293.33 ± 37.89 a.u.), which were respectively 1.3-fold and 1.4-fold higher than that observed after treatment with DAB dendriplex (1601.33 ± 31.34 a.u.) and were respectively 15.3-fold and 16.3-fold higher than that observed after treatment with DNA solution (140.33 ± 2.08 a.u.) (Figure 12A). Treatment of PC-3 cells with the DAB-PEG-SS-ODT dendriplexes also resulted in very high FITC positive cells at dendrimer: DNA ratios of 10:1 ($93.5 \pm 0.26\%$) and 20:1 ($95.6 \pm 0.17\%$), which were both 1.2-fold higher than that observed after treatment with DAB dendriplex ($74.36 \pm 0.11\%$). They were respectively 35.5-fold and 36.3-fold higher than that observed after treatment with DNA solution ($2.63 \pm 0.23\%$) (Figure 12A).

Increased cellular fluorescence was also observed following treatment of DU145 cells with the DAB-PEG-SS-ODT dendriplexes at a dendrimer: DNA ratio of 10:1 (1823.33 ± 178.83 a.u.) and 20:1 (1758.33 ± 38.52 a.u.), which were respectively 3.3-fold and 3.1-fold higher than that observed after treatment with DAB dendriplex (550.33 ± 14.50 a.u.) and were respectively 28.6-fold and 27.6-fold higher than that observed after treatment with DNA solution (63.66 ± 1.52 a.u.) (Figure 12B). Treatment of DU145 cells with the DAB-PEG-SS-ODT dendriplexes also resulted comparatively in high FITC positive cells at a dendrimer: DNA ratio of 10:1 ($58.33 \pm 1.45\%$) and 20:1 ($56.16 \pm 0.51\%$), which were respectively both 2.4-fold higher than that observed after treatment with DAB dendriplex ($23.56 \pm 0.20\%$) and were respectively 291.6-fold and 280.8-fold higher than that observed after treatment with DNA solution ($0.2 \pm 3.39.10^{-17}\%$) (Figure 12B).

The high cellular uptake of these dendriplexes might be due to their stable, smaller-sized, positively charged complex formation, and the presence of lipid molecules in the dendrimer's backbone, as these parameters not only hold stability of the dendriplex by binding/protecting DNA but also help to cross the cell membrane in comparison with unmodified DAB. The cellular internalization of nanocarriers has previously been reported to increase with increasing

tumorigenic potential of the cell line.⁵⁵ The higher cellular uptake observed in PC-3-Luc cells could therefore be attributed to PC-3-Luc's higher metabolic and energy demands required to meet its aggressive and highly proliferative nature, compared to DU145 cells.

Figure 12. Quantification of the cellular uptake of fluorescein-labelled DNA (2.5 $\mu\text{g}/\text{well}$) complexed with DAB-PEG-SS-ODT, by (A) PC-3-Luc cells and (B) DU145 cells (controls: DAB-DNA, DNA solution) (n=3) (*: $P < 0.05$ compared with DAB-DNA).

3.11.3. Gene expression of the dendriplexes

The treatment of PC-3 cells with DAB-PEG-SS-ODT dendrimer and DAB dendrimer (control) complexed to DNA at various time intervals (24, 48 and 72 h) led to the highest gene expression after 72 h at dendrimer: DNA weight ratios of 20:1 and 10:1 (Figure 13A). The comparative study of transfection in PC-3 and DU145 prostate cancer cell lines was therefore only done at

this duration. The DAB-PEG-SS-ODT dendriplex led to the highest gene expression (3.81 ± 0.41 U/mL) at dendrimer: DNA weight ratio of 20:1, by 1.2-fold compared with DAB dendriplex in PC-3 cells (Figure 13B).

In DU145 cells, gene expression following treatment with the DAB-PEG-SS-ODT dendriplex was lower than in PC-3 cells at ratios 20:1 and 10:1, but similar for the other tested ratios. As for PC-3 cells, the highest transfection (2.31 ± 0.23 U/mL) was obtained as a result of treatment with DAB-PEG-SS-ODT dendriplex at a dendrimer: DNA weight ratio of 20:1, and was 1.7-fold higher than that of DAB dendriplex (Figure 13B). The comparatively higher transfection efficiency of these dendriplexes in PC-3 cells might result from the higher cellular uptake of the dendriplexes in PC-3 cells compared with that observed in DU145 cells. The enhanced transfection resulting from treatment with octadecyl-modified PEGylated DAB dendrimer compared with that previously reported for cholesterol-modified PEGylated DAB dendrimers might be explained by the prevention of DNA release from the cholesterol-modified dendrimers.¹⁹

Figure 13. Transfection efficacy of DAB-PEG-SS-ODT complexed with DNA encoding β -galactosidase (1 μ g per well) on (A) PC-3 cells after 24, 48 and 72 h, and (B) PC-3 and DU145 cells after 72 h (controls: DAB-DNA (5: 1 ratio), DNA solution) (n = 15) (*: P < 0.05 compared with DAB-DNA).

To the best of our knowledge, this is the first report on the octadecyl lipid chain-bearing PEGylated DAB dendrimer forming spontaneous, cationic, stable vesicles, which showed redox-sensitive disintegration at intracellular reductive condition. A lower CAC value of this lipid-dendrimer than those of dendrimersomes and glycodendrimersomes prepared from self-assembling amphiphilic Janus dendrimers, suggested enhanced hydrophobic interactions among DAB-PEG-SS-ODT molecules due to the presence of lipid in its backbone.³⁸ The spontaneous dendrimersome formation made this fatty acid chain-modified dendrimer a potentially better choice over temperature-responsive vesicle formation by two C18 alkyl chain-bearing generations 2- and 3-PAMAM-based amphiphilic dendrimers.⁴⁰ In addition to vesicular structure formation, this lipid-dendrimer can also condense DNA towards successful gene transfection which made it superior than lipid-bearing cationic dendrimers as gene transfecting agents.²⁶⁻³⁰ The DAB-PEG-SS-ODT dendrimersome was also able to maintain its vesicular structure in presence of DNA, unlike C17 lipid chain-modified amphiphilic dendrimer-based vesicle which turned into a structural rearrangement of smaller, spherical micelles to interact with siRNA.⁴¹ The DAB-PEG-SS-ODT dendrimersome showed better transfection ability at some polymer: DNA weight ratios than generation 3-DAB dendrimer, unlike its analogous cholesterol-modified PEGylated DAB dendrimers due to the additional influence of steroidal molecules towards binding and release of genetic materials.¹⁹

4. Conclusion

In this study, a disulphide-linked octadecyl lipid chain-bearing PEGylated cationic dendrimer has been successfully synthesized through *in situ* two-step reactions. The lipid-dendrimer was able to spontaneously self-assemble into stable vesicles in PBS (pH 7.4) above a comparatively lower critical aggregation concentration value. The cationic, nano-sized vesicles, or dendrimersomes, were able to entrap guest molecules in their nanostructured environment, and also showed a redox-responsive disassembly at higher glutathione concentration corresponding to a reducing environment equivalent to intracellular compartment. The lipid-bearing dendrimersomes were found to have a crystallization exotherm at 0.2-0.3 °C, along with an increased adsorption tendency on mica surface, with both properties more pronounced with increasing concentration. Further, the vesicles were able to condense nearly 70% of the DNA at dendrimer: DNA weight ratios of 5:1 and higher, forming positively charged and ~100-150 nm sized, vesicular-shaped dendriplexes. These dendriplexes led to an enhanced cellular uptake of DNA both at dendrimer: DNA weight ratios of 10:1 and 20:1, by up to 15-fold and 16-fold compared with naked DNA on PC-3 prostate cancer cell line respectively, and by up to 28-fold and 27-fold compared with naked DNA on DU145 prostate cancer cell line. Consequently, this resulted in an increase in gene expression in PC-3 and DU145 cells at dendrimer: DNA weight ratio of 20:1 compared with DAB dendrimer. Furthermore, the incubation of the cells with DAB-PEG-SS-ODT resulted in higher cell viability, enhanced cellular uptake and gene expression in PC-3 cells than in DU145 cells. These novel octadecyl lipid-bearing PEGylated DAB dendrimer-based cationic vesicles are therefore very promising as redox-sensitive, non-viral gene delivery systems for future applications in cancer combination therapy.

Conflicts of interest

There are no conflicts to declare.

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Supporting information

Figure S1. FTIR spectra of lipid-bearing PEGylated DAB dendrimer (DAB-PEG-SS-ODT) (A), DAB (B), and OPSS-PEG-SCM (C).

Figure S2. ^1H -NMR spectrum of DAB-PEG-SS-ODT dendrimer (in CDCl_3 , 500 MHz).

Figure S3. MALDI-TOF MS spectra of DAB-PEG-SS-ODT (A), DAB (B) and OPSS-PEG-SCM (C).

Section S1. Amount of conjugated lipid to dendrimer

The amount of lipid loading to modified dendrimer (DAB-PEG-SS-ODT) is calculated as the weight of conjugated ODT as a percentage of the total average molecular weight:

$$\text{Lipid-loading (\%)} = \{(n \times \text{MW}_{\text{ODT}})/[\text{M}]^+\} \times 100$$

Where, n is the number of ODT conjugated to modified dendrimer, MW_{ODT} is the molecular weight of ODT and $[\text{M}]^+$ is the average molecular weight of the modified dendrimer (DAB-PEG-SS-ODT) analyzed from MALDI-TOF MS.

$$\text{Amount of ODT in DAB-PEG-SS-ODT (\%)} = \{(1 \times 286.56)/4522.73\} \times 100 = 6.33\%$$

Figure S4. ^1H -NMR spectrum of DAB-PEG-SS-ODT dendrimer (in D_2O , 500 MHz).

Figure S5. Flow cytometry histograms of PC3-Luc cells following 2-h incubation with dendriplexes (DAB-PEG-SS-ODT dendrimer: fluorescein-labeled DNA weight ratios: 20:1 (A), 10:1 (B)) (controls:

DAB dendriplex (dendrimer: fluorescein-labeled DNA weight ratio 5:1 (C)) and fluorescein-labeled DNA solution (D)).

Figure S6. Flow cytometry histograms of DU145 cells following 2-h incubation with dendriplexes (DAB-PEG-SS-ODT dendrimer: fluorescein-labeled DNA weight ratios: 20:1 (A), 10:1 (B)) (controls:

DAB dendriplex (dendrimer: fluorescein-labeled DNA weight ratio 5:1 (C)) and fluorescein-labeled DNA solution (D)).