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Research Paper

Dendrimeric Alkylated Polyethylenimine Nano-carriers with Acid-Cleavable Outer Cationic Shells Mediate Improved Transfection Efficiency Without Increasing Toxicity

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Purpose. Improved polycation-based non-viral DNA vectors were sought by preparing dendrimers with polyethylenimine cores surrounded by various shells incorporating structural features intended to facilitate steps in transfection mechanisms. Dendrimeric vectors were designed with (a) an outer oligocation shell, intended to facilitate DNA release inside cells, (b) a hydrophobic C-16 alkyl shell, and (c) a polycationic core, the latter two intended to combine lipid-depletion and osmotic burst endosome escape mechanisms, respectively, and were (d) attached through an a acid-cleavable linker reported to hydrolyze at endosomal pH values.
Methods. Vectors and DNA complexes were characterized by dynamic and static light scattering. Flow cytometry was used to quantitate transfection activity and cytotoxicity in CHO–K1 cells.
Results. About 5-fold increased transfection activity was obtained for a vector constructed with an outer shell of oligocations attached through acid-cleavable linkers, relative to a control dendrimer with an acid-stable linker. The most effective oligocation component of outer shells tested was spermine. Neither modification was associated with increased cytotoxicity. This vector design did not permit an evaluation of the benefit of combining endosome release mechanisms.
Conclusion. Using acid-cleavable linkers to attach an outer shell of short, highly-charged oligocations to a PEI-based dendrimeric vector substantially increased transfection efficiency without increasing cytotoxicity.

KEY WORDS: acid-cleavable; dendrimers; gene therapy; polyethylenimine; transfection.

INTRODUCTION

Gene therapy, the use of therapeutic DNA for the treatment of disease, has not achieved its anticipated therapeutic potential due to severe drug delivery problems resulting from the chemical and physical properties of DNA. DNA is too big, too polar and too charged (1) to allow effective delivery to its intended site of action inside the cell without the use of a suitable vector. Viral vectors have had problems with immunogenicity (2,3) and carcinogenicity resulting from insertional mutagenicity (4,5). Non-viral vectors are being investigated (6) as alternatives with potential advantages of being non-immunogenic, simple to produce, stable, non-carcinogenic and able to give transient gene expression with little or no insertion in host DNA.

A wide variety of strategies have been investigated to increase the level and duration of gene expression and decrease the toxicity obtained with non-viral DNA vectors. The strategies include novel approaches, such as Sleeping Beauty transposons (7), and simpler structural variations on the standard polycation vector, polyethylenimine (PEI) (8,9) aimed at increasing transfection efficiency and/or decreasing cytotxicity (10). Simply adding ligands to PEI has yet to achieve major increases in transfection efficiency. Slow progress is at least partly due to incomplete understanding of the mechanisms by which PEI-DNA complexes are taken into cells, or by which the DNA is released from the complex in a functional form. When hydrophobic moieties are attached to PEI, an appropriate balance between the lipophilicity and hydrophilicity appears to be necessary for efficient transfection of DNA in polyplexes, presumably because lipophilicity of a PEI-based vector may influence both its interactions with DNA and interactions of the DNA-PEI complexes with cell membranes (11–14). An additional concern is that many of the types of reactions used to attach modifying groups to branched PEI involve preferential coupling to and loss of primary amines. The branched form of PEI contains primary, secondary, and tertiary amines, all of which typically become protonated in the pH range 9–11 when they are in an isolated form (15). However, it is

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buffering capacity in the pH range through which Na^+,K^+-ATPases lower endosomal pH (pH 5.2–7.0) that is the basis of the osmotic burst mechanism for escaping endosomes before they fuse with lysosomes (9). The 25 kDa form of bPEI exhibits substantial buffering capacity in this critical pH range, possibly associated with internal amines affected by undetermined environmental effects.

Recent efforts have aimed to reduce cytotoxicity through use of biodegradable cationic polymers. Polymers were synthesized incorporating chemistries known to be labile within the physiological milieu. These include disulfide (16,17), diacrylate (18), and acid-labile poly-orthoester (19) and ketal (20,21) bonds. Another commonly used strategy has been to attach different types of ligands to branched or linear PEI, including attaching various hydrophobic moieties (11,22,23), the hydrophilic polymer poly(ethyleneglycol) (24,25), carbohydrates such as dextran (26), and ligands which bind to cell surfaces with a greater degree of specificity than polycations do (27,28).

One potentially useful strategy for making polycation derivatives with improved transfection activity is to alter them in ways that should facilitate known transfection mechanisms of cationic liposomes and cationic polymers (29–31). In this study, we report the preparation, partial characterization and measurement of DNA transfection and cytotoxic activities of dendrimeric vectors designed to improve non-viral gene delivery by incorporating into various shells structures intended to facilitate steps in the transfection mechanism. As presented in Scheme 1, these vectors consist of a polycationic core, a hydrophobic shell, a linker shell expected to hydrolyze at pH values near the low end of the endosomal pH range, and an outer oligocation shell. These vectors were designed to test two mechanism-based hypotheses. The first hypothesis is that transfection efficiency can be increased by incorporating into the vector a mechanism for releasing the DNA from the vector when inside the cell. This hypothesis has been investigated by preparing a series of dendrimeric vectors in which the outer shell is composed of oligocations held together in place by acid-labile linkers reported to be stable at pH values found outside cells, but to hydrolyze rapidly at pH values found in endosomes. Linker hydrolysis should release small oligocations, intended to bind DNA weakly enough to not interfere with its expression inside the cell.

The second hypothesis is that transfection efficiency can be increased by incorporating into the same dendrimeric vector two endosome release mechanisms. Some studies on the transfection mechanisms of polycation vectors have suggested that endosome release is a rate-limiting factor in transfection efficiency (31,32). This hypothesis has been investigated by preparing dendrimeric vectors containing (a) a branched polyethyleimine (bPEI) core, which along with the outer oligocationic shell provides buffering capacity for a 'proton sponge' or osmotic release mechanism, and (b) a hydrophobic shell of C-16 alkyl chains designed to provide a "lipid sink" to absorb structural lipids from endosome membranes causing lysis by membrane depletion.

MATERIALS AND METHODS

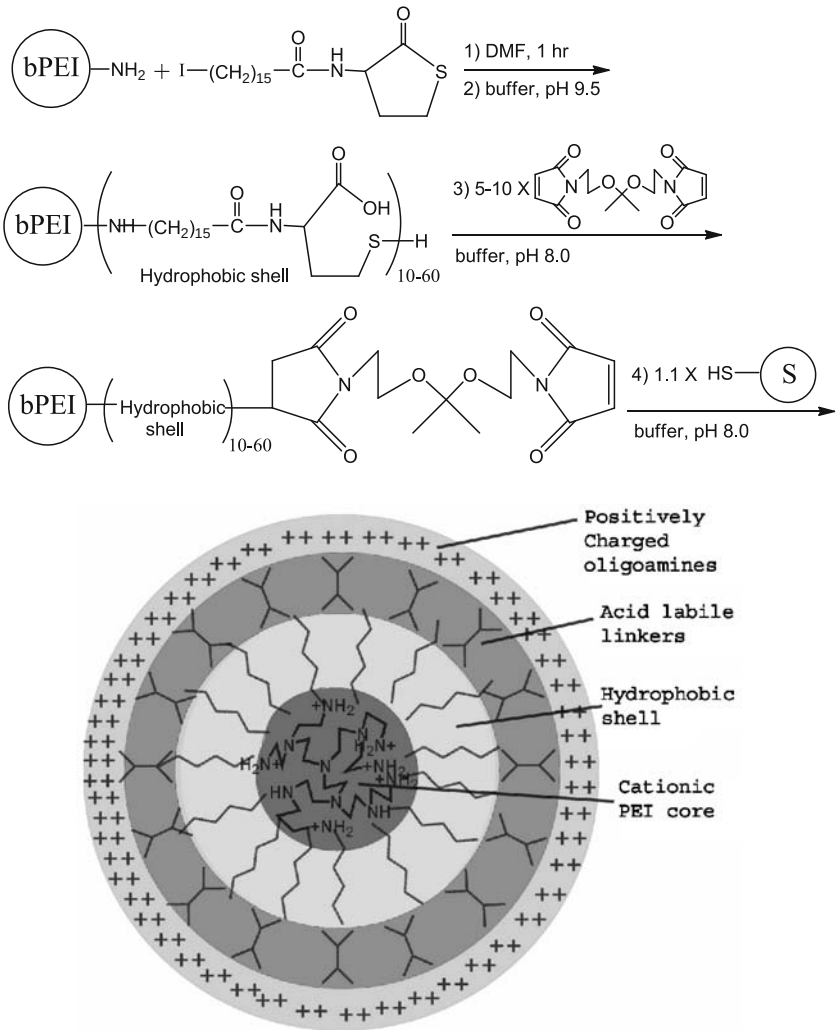
Materials

Unless otherwise indicated, all biochemicals were purchased from Sigma–Aldrich (St. Louis, MO). Polyethyleneimine (manufacturer's designated molecular weights of 600, 1,800 and 25,000 Da) was purchased from Polysciences Inc. (Warrington, PA) and used without further purification. Polyethyleneimine with the manufacturer's designated molecular weight of 25,000 Da was determined by size-exclusion chromatography to have an average molecular weight of 32,000 Da. The experimentally determined value has been used throughout this presentation. 1,4-Bis-maleimidobutane was purchased from Pierce Chemical (Rockford, IL). 3,9-Diethylidene-2,4,8,10-tetraoxaspiro[5.5]undecane was the gift of Dr. Jorge Heller, Advanced Polymer Systems, Inc., Redwood City CA. CHO–K1 cells were purchased from American Type Culture Collection (Manassas, VA). Bovine calf serum was purchased from Hyclone Laboratories, Inc. (Logan, UT). pEGFP plasmid was purchased from Clontech Laboratories, Inc. (Mountain View, CA). Membrane filters (0.02, 0.2 and 0.45 μm) were obtained from Fisher Scientific (Pittsburgh, PA). Deuterated solvents ($CDCl_3$, D_2O, and DMF-D_7) were obtained from Cambridge Isotope Laboratories (Andover, MA).

Instrumentation

^{1}H NMR spectra were obtained on an Oxford 300 MHZ-Mercury spectrometer (Varian, Inc., Palo Alto, CA) in 5 mm o.d. NMR tubes in deuterated solvents. ^{13}C NMR spectra were obtained on an Oxford 600 MHZ–Inova spectrometer (Varian, Inc., Palo Alto, CA) in 8 mm o.d. NMR tubes using D_2O solvent. Fluorescence was measured on a Varian Cary Eclipse fluorescence spectrophotometer (Varian, Inc., Palo Alto, CA). Dynamic laser light scattering was carried out on a custom-assembled modular instrument consisting of a Lexel Model 95 Ion 488 nm adjustable intensity laser (Lexel Laser, Inc., Fremont, CA) directed into a Malvern PCS100 goniometer and photomultiplier tube (Malvern Instruments, Inc., Southborough, MA). Photomultipler signals were analyzed using a Brookhaven Instruments BI-9000AT autocorrelation card and processed with the Windows version of Brookhaven Instruments DLS software version 3.24 (Brookhaven Instruments Corp., Holtsville, NY). Fluorescence polarization measurements were carried out on fluorescein isothiocyanate derivatives using a 96-well spectrofluorometer (Tecan Corp. model Genios Pro, Durham, NC) with excitation at 480 nm and a 530 nm filter block.

The primary method for analyzing dendrimeric polycation vectors and synthetic intermediates was size-exclusion chromatography with multi-angle laser light scattering detection on a custom-assembled modular HPLC instrument with multiple detectors in series. The instrument included a Beckman (Beckman Coulter, Inc., Fullerton, CA) HPLC system with a Rheodyne (PerkinElmer Life and Analytical Sciences, Inc., Waltham, MA) manual injector fitted with a 100 μL sample loop and Beckman 125 pumps controlled by Beckman 32 karat software, and equipped with PSS Novema 8×300 mm 300 Å and 1,000 Å columns (Polymer Standards Service-USA, Inc., Warwick, RI) in series. The primary detection method for molecular weight analysis was static light scattering performed on a Wyatt Dawn EOS multi-angle laser light scattering detector (Wyatt Technology Corporation Santa Barbara, CA) controlled by Wyatt Astra software



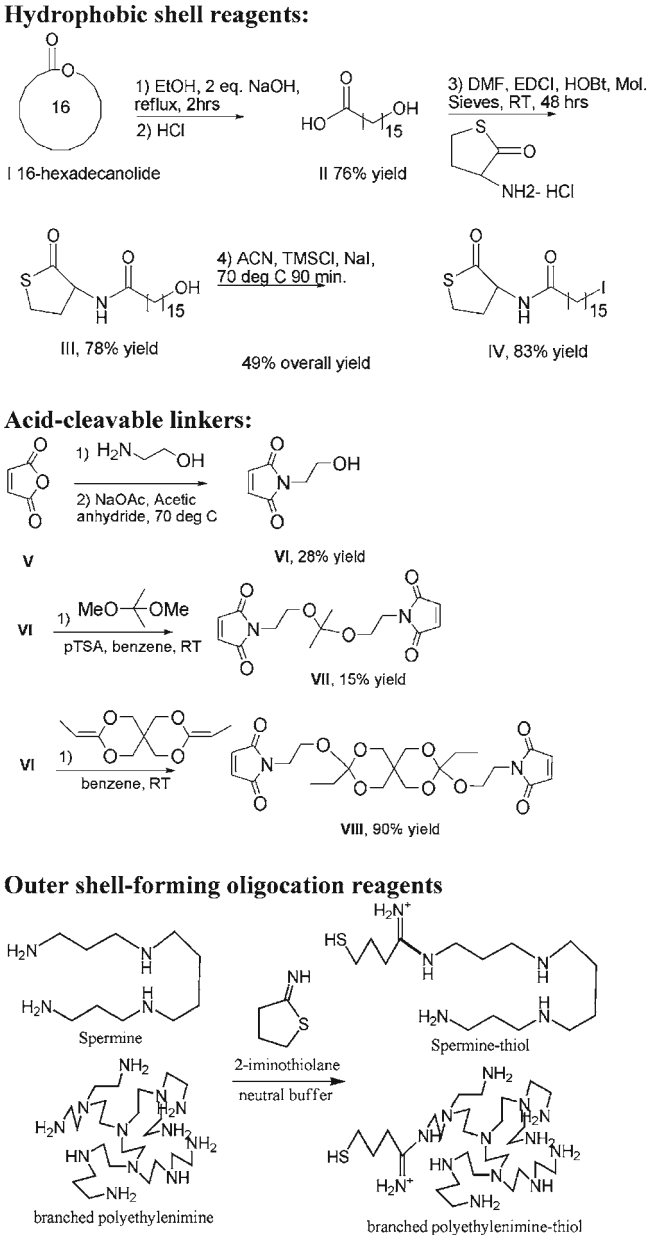
Scheme 1. Synthesis of dendrimeric alkylated polyethylenimine nano-carriers with acid-cleavable outer cationic shells. *Top line*: The central core consisting of 32,000 Da branched polyethylenimine (bPEI) was alkylated with 16-iodo-hexadecanoic acid homocysteine thiolactone amide to add a hydrophobic shell, and the thiolactone ring opened under alkaline conditions. *Second line*: The addition of an acid-cleavable linker, or a non-cleavable control linker by a Michael addition reaction with one end of a dimaleimido derivative of the linker present in excess. *Third line*: The addition of the outer oligocation shell by reaction of thiolated spermine (S) with terminal unreacted maleimide residues to attach the outer oligocation shell and complete the assembly (bottom). Abbreviation: *DMF* N,N′-dimethylformamide.

version 5.1 (available at ftp.wyatt.com). Additional detectors in series included an Altex 156 Refractive Index detector (Beckman Coulter, Inc., Fullerton, CA) calibrated with Wyatt RICAL software (available at www.wyatt.com), a Beckman 166 UV detector, and a JASCO FP-1520 programmable fluorometer (JASCO Inc., Easton, MD). Flow cytometry was carried out on a Becton–Dickinson FACScaliber with cell counts analyzed with CellQuest Pro software (BD Biosciences, San Jose, CA). Zeta-potentials were calculated using a ZM-80 zeta–meter (Zeta–Meter Inc., Staunton, VA) with a type GT-2 electrophoresis cell using a molybdenum cylinder anode and a platinum rod cathode.

Synthesis of Dendrimer Shell-Forming Reagents

*Synthesis of the Hydrophobic Shell-Forming Reagent: 16-iodo-hexadecanoyl-homocysteine Thiolactone Amide (***IV***) (Scheme 2)*

A solution of 16-hexadecanolide (24.5 g, 96 mmol) in 2 L ethanol was mixed with 40 mL of 200 mM aqueous sodium hydroxide and heated under reflux for 1 h. The solution was acidified with concentrated hydrochloric acid and crystals allowed to form overnight at 4°C. The crystals were collected by filtration and the filtrate concentrated on a rotary



Scheme 2. Synthesis of reagents used to add shells to branched polyethylenimine cores to construct dendrimeric alkylated polyethylenimine nano-carriers with acid-cleavable outer cationic shells. The alkylating reagent used for the addition of the hydrophobic shell was 16-iodo-hexadecanoic acid homocysteine thiolactone amide, **IV**. Maleimide coupling units were added to each end of acid-cleavable linkers. Outer shell-forming oligocation reagents were prepared by thiolation of spermine and low molecular weight (600 Da and 1,800 Da) polyethylenimines with Traut's reagent, 2-iminothiolane. Abbreviations: *ETOH* ethanol; *eq.* equivalent, *DMF* N,N′-dimethylformamide; *EDCI* 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride; *HOBt* 1-hydroxybenzotriazole; *Mol.* molecular; *RT* room temperature; *TMSCl* chlorotrimethylsilane; *deg* degrees; *ACN* acetonitrile; *NaOAc* sodium acetate; *pTSA* *p*-toluenesulfonic acid; *RT* room temperature.

evaporator to ~800 mL, and a second crop of crystals was obtained at 4°C. The two crops of crystals were combined, dissolved in benzene and heated under reflux to remove water azeotropically using a Dean–Stark trap. The solution was cooled to room temperature yielding 20 g of 16-hydroxy-hexadecanoic acid, **II**, crystals, which were collected by filtration and dried in vacuum (76% yield). ^{1}H NMR (300 MHZ, CD_3OD, δ) 1.29 (br s, 22H, CH_2), 1.55 (m, 4H, CH_2), 2.65 (t, 2H, CH_2), 3.53 (t, 2H, CH_2), ^{13}C NMR (CD_3OD, δ) 25.07 (s, 1C), 25.93 (s, 1C), 29.22 (s, 1C), 29.5 (br s, 11C), 32.5 (s, 1C), 33.92 (s, 1C), 61.91 (s, 1C), 176.42 (s, 1C).

16-Hydroxy-hexadecanoic acid, **II** (1.36 g, 5 mmol), was placed into a 500 mL one-necked flame-dried round-bottom flask along with homocysteine thiolactone (785 mg, 5.1 mmol), hydroxybenzotriazole (690 mg, 5.1 mmol), 1-(3-dimethlyaminopropyl)-3-ethylcarbodiimide (978 mg, 5.1 mmol), and 4 Å heat-activated molecular sieves (3 g), mixed with 150 mL of dry dimethylformamide in a flame-dried flask and stirred for 72 h at room temperature. The reaction mixture was filtered to remove molecular sieves. The filtrate was poured into a separatory funnel containing 500 mL distilled water and extracted three times with 100 mL aliquots of ethyl acetate. The combined organic phases were washed with water, 1 N hydrochloric acid, 5% aqueous sodium hydroxide, and saturated sodium chloride solution. The organic phase was dried over anhydrous magnesium sulfate, and evaporated on a rotary evaporator to yield a white powder. The white powder was crystallized from acetone to yield 1.45 g (78%) of 16-hydroxy-hexadecanoyl-homocysteine thiolactone amide, **III**. ^{1}H NMR (300 MHZ, $CDCl_3$, δ) 1.25 (br s, J=2.97, $OCCH_2CH_2CH_2(CH_2)_9CH_2CH_2CH_2OH$, 18H), 1.61 (m, J=1.89, 6.07, 7.3, $OCCH_2CH_2CH_2(CH_2)_9CH_2CH_2CH_2OH$, 4H), 2.25 (m, J=1.89, 2.96, $OCCH_2CH_2CH_2(CH_2)_9CH_2CH_2CH_2OH$, 4H), 2.45 (br s, OH, 1H), 2.94 (m, J=1.22, 4,71 $COSCH_2CH_2CH$, 2H), 3.36 (m, J=1.22, $COSCH_2CH_2CH$, 2H), 3.65 (t, J=6.07, $OCCH_2CH_2CH_2(CH_2)_9CH_2CH_2CH_2OH$, 2H), 4.05 (t, J=7.3, $OCCH_2CH_2CH_2(CH_2)_9CH_2CH_2CH_2OH$, 2H), 4.50 (m, J=4.58, 4.71, $CH_2CH_2CH(NH)(CO)$, 1H), 6.05 (br s, J=4.58, $CHNHCO$, 1H), ^{13}C ($CDCl_3$, δ) 25.9–36.8 (s, 13C), 59.8 (s, 1C), 63.38 (s, 1C), 173.84 (s, 1C), 205.64 (s, 1C).

16-Hydroxy-hexadecanoyl-homocysteine thiolactone amide (372 mg, 1 mmol) and sodium iodide (600 mg, 4.0 mmol) were dissolved in 10 mL dry acetonitrile under an argon atmosphere. The mixture was heated to 70°C, chloro-trimethylsilane (326 mg, 3.0 mmol) was added by syringe, and the yellow solution was allowed to react for 90 min. The reaction mixture was cooled to room temperature and poured into 100 mL of 10% aqueous sodium thiosulfate. The mixture was extracted twice with 50 mL aliquots of ethyl acetate, the extracts combined, washed with 10% aqueous sodium thiosulfate and with saturated sodium chloride solution and dried over anhydrous magnesium sulfate. The solution was evaporated on a rotary evaporator, and the tan residue was crystallized from methanol to yield 16-iodo-hexadecanoyl-homocysteine thiolactone amide, **IV**, (400 mg, 83% yield): ^{1}H NMR (300 MHZ, $CDCl_3$, δ) 1.27 (br s, J=3.01, $OCCH_2CH_2CH_2(CH_2)_9CH_2CH_2CH_2I$, 18H), 1.64 (m, J=1.87, 4.65, 7.22, $OCCH_2CH_2CH_2(CH_2)_9CH_2CH_2CH_2I$, 4H), 2.25 (m, J=1.87, 3.01, $OCCH_2CH_2CH_2(CH_2)_9CH_2CH_2CH_2I$, 4H), 2.94 (m, J=1.27, 4,77 $COSCH_2CH_2CH$, 2H), 3.20 (t, J=4.65, $OCCH_2CH_2CH_2(CH_2)_9CH_2CH_2CH_2I$, 2H), 3.36 (m, J=1.27, $COSCH_2CH_2CH$, 2H), 4.05 (t, J=7.22, $OCCH_2CH_2CH_2(CH_2)_9CH_2CH_2CH_2I$, 2H), 4.50 (m, J=4.77, 4.97, $CH_2CH_2CH(NH)(CO)$, 1H), 6.05 (br s,

J=4.97, CHN*H*CO, 1H), ^{13}C ($CDCl_3$, δ) 7.86 (s, 1C), 25.8–36.8 (s, 13C), 59.8 (s, 1C), 63.38 (s, 1C), 173.97 (s, 1C), 205.76 (s, 1C).

*Synthesis of Acid-Cleavable Linker-Forming Reagents (Scheme 2). Synthesis of 2-Hydroxyethyl-maleimide (***VI***)*

Maleic anhydride (**V**, 15.4 g, 157 mmol) and freshly-distilled ethanolamine (9.59 g, 157 mmol) were each dissolved in 50 mL acetone and slowly added to 50 mL acetone in a round-bottom flask in an ice bath. After addition was complete, the ice bath was removed and the mixture allowed to react overnight at room temperature. Approximately 75% of the acetone was removed on a rotary evaporator and the solution placed at −20°C until crystallization was complete, yielding 25.3 g (81% yield) 2-[(3-carboxy-1-oxo-2-propenyl)amino]-ethanol (33): mp=73°C, ^{1}H NMR (300 MHZ, CD_3OD, δ) 3.07 (m, 1H, NH) 3.42 (t, 2H, CH_2), 3.66 (t, 2H, CH_2), 5.56 (br s, 1H, OH), 6.26 (d, 1H, CH), 6.46 (d, 1H, CH), ^{13}C NMR (CD_3OD, δ) 42.48 (s, 1C), 59.94 (s, 1C), 132.38 (s, 1C), 134.06 (s, 1C), 166.81 (s, 1C), 167.44 (s, 1C)

2-[(3-Carboxy-1-oxo-2-propenyl)amino]-ethanol (1.59 g, 10 mmol) was dissolved in 10 mL acetic anhydride. Freshly fused and crushed sodium acetate (0.82 g, 10 mmol) was added and the solution heated at 70°C overnight. The resulting black solution was poured onto 50 mL crushed ice plus 50 mL 1 N hydrochloric acid in a separatory funnel and mixed until the ice melted. The mixture was extracted with 50 mL ethyl acetate and the aqueous layer discarded. The organic layer was washed twice with water, once with saturated sodium chloride solution, dried over anhydrous magnesium sulfate and concentrated on a rotary evaporator to few mL of liquid. The dark brown liquid was fractionated using 1:1 ethyl acetate:hexane on a silica gel column to yield 0.5 g of 2-hydroxyethylmaleimide, **VI** (35% yield): mp=70–72°C, ^{1}H NMR (300 MHZ, $CDCl_3$, δ) 3.8 (t, 2H, CH_2), 4.2 (t, 2H, CH_2), 6.74 (s, 2H, CH), ^{13}C NMR ($CDCl_3$, δ) 36.61 (s, 1C), 61.12 (s, 1C), 134.48 (s, 2C), 205.5 (s, 2C).

Synthesis of Acid-Cleavable Linker 2,2-bis(2-maleimidoethoxy)-propane (VII)

2-Hydroxyethylmaleimide (**VI**, 140 mg, 1 mmol), 2,2-dimethoxypropane (75 mg, 0.07 mmol), *p*-toluenesulfonic acid (2 mg), and crushed heat-activated 4 Å molecular sieves (100 mg) were added to 10 mL dry benzene in a 50 mL round-bottom flask under an argon atmosphere. The mixture was stirred for 2 h and evaporated to dryness on a rotary evaporator. An additional 10 mL benzene and 50 mg 2,2-dimethoxypropane were added, and the solution stirred another 2 h, filtered, mixed with two drops of pyridine and evaporated on a rotary evaporator. The clear oil remaining was crystallized from acetone: hexane at −20°C to yield 2,2-bis(2-maleimidoethoxy)-propane (**VII**, 50 mg, 15% yield) (34): mp. 123–125°C (lit. rep. 124–126), (300 MHZ, CD_3CN, δ) 1,21 (s, 6H, CH_3), 3.46 (t, 24, CH_2), 3.56 (t, 4H, CH_2), 6.77 (s, 4H, CH), ^{13}C NMR (CD_3CN, δ) 24.4 (s, 2C), 37.96 (s, 2C), 58.1 (s, 2C), 117.58 (s, 2C), 134.45 (s, 2C), 171.0 (s, 1C).

Synthesis of acid-cleavable linker 3,9-bis(2-maleimidoethoxy)-3,9-ethane-2,4,8,10-tetraoxaspiro[5.5]undecane (**VIII**), 2-Hydroxyethylmaleimide (**VI**, 262 mg, 2 mmol) and 3,9-diethylidene-2,4,8,10-tetraoxaspiro[5.5]undecane (290 mg, 1.04 mmol) were added anaerobically to 50 mL anhydrous ether in a septum-sealed round-bottom flask under argon, and stirred at room temperature for 2 h until the solution turned turbid. The mixture was filtered and the filtrate concentrated on a rotary evaporator to a clear oil that crystallized on standing at −20°C to yield 3,9-bis(maleimdioethoxy)-3,9-ethane-2,4,8,10-tetraoxaspiro[5.5]undecane (33) (**VIII**, 450 mg, 90% yield): ^{1}H NMR (300 MHZ, $CDCl_3$, δ) 0.83 (m, CH_2, 6H), 1.10 (m, CH_2, 4H), 1.66 (m, CH_2, 4H). 3.53 (m, CH, 2H), 3.69 (m, CH, 2H), 3.93 (m, CH_2, 4H), 6.82 (s, CH, 4H) ^{13}C NMR ($CDCl_3$, δ) 7.20 (s, 2C), 28.38 (s, 2C), 30.43 (s, 2C), 37.66 (s, 2C), 59.85 (s, 2C), 62.65 (s, 4C), 117.59 (s, 1C), 134.64 (s, 4H), 171.07 (s, 4C).

Synthesis of Outer Shell-Forming Oligocation Reagents (Scheme 2)

Three oligocations that span a range of relatively low molecular weights were used: spermine and two low molecular weight PEIs (600 and 1,800 Da). An oligocation (100 mg, containing 1 mmol of primary amine) was dissolved in 50 mL of 50 mM sodium borate, 1 mM EDTA, pH 8.0 buffer. 2-Iminothiolane (Traut's reagent, 68 mg, 0.5 mmol) was added as a 427.4 mM stock solution in small aliquots to a vigorously stirred solution of the oligocation until a 1:1 molar ratio was reached. The reaction was allowed to proceed to completion for 30 min at room temperature. To remove large aggregates, thiol-derivatized oligoamines were loaded into Amicon-15 ultrafiltration centrifuge tubes, 10,000 MWCO, and centrifuged for 30 min at 2000×g. Comparison of the free thiol concentration before and after ultrafiltration using the fluorogenic thiol quantitation assay described below indicated a 6.5, 9.2, and 15.9% free thiol concentration decrease for spermine, 600 Da PEI, and 1,800 Da PEI, respectively. No further purification was employed.

Dendrimeric Nanoparticle Assembly (Scheme 1)

The dendrimeric nanoparticle was assembled using a divergent strategy in the initial steps, beginning with a core consisting of commercially available bPEI (32,000 Da measured molecular weight). The hydrophobic second shell was added to the core by diluting 32,000 Da bPEI (1.0 g, about 7.8 mmol primary amines) in 10 mL of a solution of the hydrophobic shell reagent, 16-iodo-hexadecanoyl-homocysteine thiolactone amide in anhydrous DMF. One mL aliquots of the solution (100 mg bPEI) were diluted 1:10, and sufficient hydrophobic shell reagent was added to give hydrophobic shell: bPEI molar ratios of 10, 19, 48 and 96 per mole of bPEI, which corresponds to alkylation of about 5%, 10%, 25% and 50% of primary amines in bPEI, respectively, assuming a 1:2:1 ratio of primary: secondary: tertiary amines in bPEI and complete reaction with primary amines. A brief yellow coloration of the reaction mixture was observed initially, which then turned colorless. The solution was vigorously mixed on a Buchler Evapo-mix vortex mixer at room temperature for 1 h.

The reaction mixtures containing the alkylated bPEI cores were directly submitted to homocysteine thiolactone

ring opening conditions by adding them drop-wise to a vigorously mixed solution of 45 mL of 100 mM sodium borate plus 1 mM EDTA pH 9.5 or 100 mM ammonium hydroxide plus 1 mM EDTA pH 9.5 and allowed to react overnight at room temperature. Alternatively, the solution of alkylated bPEI cores was added drop-wise to 45 mL of vigorously mixed 100 mM ammonium hydroxide plus 1 mM EDTA at pH 9.5 and heated under reflux for 30 min for faster homocysteine thiolactone ring opening. Ten mL of the solution of ring-opened alkylated bPEI cores was loaded into a 10,000 Da molecular weight cutoff ultrafiltration tube (Amicon Ultra-15) and centrifuged at 1000×g for 30 min. The retentate was dissolved in 10 mL of 50 mM sodium borate, 1 mM EDTA, 1 mM tris-(2-carboxyethyl)-phosphine, pH 8.0, and centrifuged again. The procedure was repeated. The number of ring-opened alkyl chains per bPEI core was determined by measuring the amount of free thiol with a fluorogenic reagent (see below).

The next shell to be added consisted of the acid-cleavable linker. Three types of maleimido-containing linker reagents were used: 2,2-bis(2-maleimidoethoxy)-propane (**VII**), 3,9-bis(2-maleimidoethoxy)-3,9-ethane-2,4,8,10-tetraoxaspiro[5.5]undecane (**VIII**) and 1,4-bis-maleimidobutane, which is a non-cleavable structural analog of VII included in the study as a negative control. Each maleimido-containing linker reagent was dissolved in anhydrous DMF at 5 mg/mL. Ring-opened alkylated bPEI cores (100 mg) were dissolved in 10 mL of 50 mM sodium borate, 1 mM EDTA, pH 8.0, and added dropwise to sufficient vigorously stirred maleimido-containing linker reagent solution to give a 5- to 10-fold molar equivalent excess of maleimido moiety to the amount of free thiol groups measured on the ring-opened alkylated bPEI cores. The reaction mixture was stirred an additional 10 min, loaded into a 10,000 Da molecular weight cutoff ultrafiltration centrifuge tube (Amicon Ultra-15), and centrifuged at 2000×g for 15 min. The retentate was dissolved in a 10 mL aliquot of buffer (50 mM sodium borate, 1 mM EDTA, 1 mM tris-(2-carboxyethyl)-phosphine, pH 8.0) and centrifuged again. This procedure was repeated. The amount of thiol groups remaining unreacted on the dendrimer preparations were measured with a fluorogenic reagent (see below).

Sufficient outer shell-forming oligocation reagent was dissolved in 3–10 mL of buffer (50 mM sodium borate, 1 mM EDTA, 1 mM tris-(2-carboxyethyl)-phosphine, pH 8.0) to provide a 1.1-fold molar ratio of free thiol moieties to the maleimide moieties on 10 mg of a dendrimer preparation with an acid-cleavable linker in its outer shell. The dendrimer preparation dissolved in 5 mL of the same buffer was added drop-wise to the solution of outer cationic shell reagent with vigorous vortex mixing. The mixture was allowed to react for 10 min, then loaded into a 5,000 or 10,000 Da molecular weight cutoff ultrafiltration tube (Amicon Ultra-15) and centrifuged at 2000×g for 15 min. The retentate was dissolved in a 4–15 mL aliquot of buffer (50 mM sodium borate, 1 mM EDTA, 1 mM tris-(2-carboxyethyl)-phosphine, pH 8.0) and centrifuged again. The procedure was repeated once to give the completely assembled nanoparticle vector. The nanoparticle vectors assembled with the non-cleavable 1,4-bis-maleimidobutane linker were characterized by size-exclusion chromatography with multiangle laser light scattering detection. The nanoparticle vectors assembled with acid-labile linkers were complexed with DNA and used in transfection studies without further characterization.

Preparation of pDNA Complexed with Dendimeric Nanoparticles

Preparation of pEGFP

The pEGFP plasmid was transformed into DH5α competent cells for amplification. The DNA was purified using both cesium chloride-ethidium bromide gradients (35) and Qiagen Plasmid Maxi kits (Qiagen Inc. Valencia, CA USA).

Preparation of pDNA-Dendrimer Complexes

Dendrimer samples (10 μg) were dissolved in 1 mL of deionized water or saline and filtered through a 0.2 μm membrane filter. Stock solutions of pEGFP were prepared by diluting 3.20 μg of pDNA into deionized water or saline and filtering through a 0.45 μm membrane filter. The pDNA solution was pipetted into dendrimer-containing solutions in an amount calculated to achieve a 10:1 molar ratio of vector nitrogen to DNA phosphate. The pDNA and dendrimer were allowed to complex for 30 min at room temperature before being analyzed by laser light scattering.

Characterization of Constructs

Quantitation of Thiols Using a Fluorogenic Reagent

The fluorogenic reagent solution was 1 mM ammonium 7-fluorobenzo-2-oxa-1,3-diazole-4-sulfonate (36) (SBD-F) in buffer (100 mM sodium borate, 1 mM EDTA, pH 8.5). Thiol groups were measured by dissolving a sample or standard in 0.495 mL of the same buffer and mixing with 0.5 mL of the fluorogenic reagent solution plus 0.005 mL 100 mM tris-(2-carboxyethyl)-phosphine in a 1.5 mL Eppendorf tube then heated at 60°C for 1 h in an aluminum heating block. The reaction was cooled to room temperature, and fluorescence was immediately read on a fluorescence spectrophotometer in a 1 mL quartz cuvette at 385 nm excitation wavelength and 524 nm emission wavelength using a 5 nm slit for 100 μs. The photomultiplier tube voltage was typically set so that the highest standard was 10–20% below saturation.

Spectrophometric Quantitation of bPEI by Copper Complexation (37)

A 2 mL aliquot of copper reagent solution (0.145 mg/mL copper sulfate, 100 mM acetic acid, adjusted to pH 5.4 with sodium hydroxide) was added to 800 μL of standard bPEI solution or a sample containing 5.0–50.0 μg/mL bPEI. A blue color developed immediately. Absorbance was measured at 285 nm. Unknown samples were compared to a bPEI standard curve prepared with bPEI of the same molecular weight.

Size-Exclusion Chromatography with Detection by Static Light Scattering

The size-exclusion HPLC system with a multiangle laser light scattering detector described above was used to provide

relatively accurate and precise molecular weight data. To allow mass measurements, the Altex 156 refractive index detector was calibrated using five sodium chloride concentrations known to the fourth significant digit (*e.g.*, 0.1847 mg/mL) spanning the range of 0.0000–2.000 mg/mL (NaCl specific refractive index increment, dn/dc=0.172 mL/g) (38). The Wyatt Dawn EOS 18-angle detectors were normalized using spectrophotometric grade toluene. The UV detector, Wyatt Dawn EOS, and refractive index detector were aligned using an injection of 0.50 mg bovine serum albumin monomer fractionated by a Superdex 200 10/300 GL gel permeation column. Alkylated bPEI and 1,4-bis-maleimidobutane-containing dendrimer constructs were analyzed using three PSS Novema columns in series: a guard column, a 300 Å 8×300 mm column, and a 1,000 Å 8×300 mm column (38). All samples were dissolved in 1% aqueous formic acid filtered through a 0.2 μm pore size filter and eluted with the same solvent. All samples were passed through a 0.45 μm syringe filter directly into the injector.

Dynamic Light Scattering

Dendrimeric vector/pEGFP complexes were prepared by mixing vector with 3.2 μg enhanced green fluorescent protein plasmid DNA in 1 mL of 0.02 μm filtered phosphate buffered saline at a N:P ratio of 10:1 and allowed to complex for 15–30 min before readings were taken. Laser power was adjusted between 200 and 700 kcounts per second for dendrimer/pEGFP complexes. Maximum laser intensity was used for dendrimer size measurements without DNA complexation. Each sample was analyzed for 1 min after the first and last delay times were experimentally determined on the Brookhaven Instruments DLS software for Windows. Particle size distributions were derived according to the non-negative least squares (NNLS) algorithm included in the Brookhaven Instruments DLS software for Windows.

Zeta-Potential Analysis

Suspensions of dendrimer/pDNA complexes (10 μg) prepared as described above for dynamic light scattering measurements were diluted into 15 mL of filtered (0.2 μm) deionized water and the specific conductance measured in a Zeta-meter ZM-80. Voltage was set according to specific conductance measurements and was typically 200 V, as specified by the manufacturer. Between six to ten particles were timed with a stopwatch for the distance of one micrometer. Direction was noted for polarity, and the time-averaged values were compared with an electrophoretic mobility/zeta potential conversion chart to yield the zeta potential of each dendrimer/pDNA complex.

Measurement of Transfection Efficiency of Dendrimeric Vectors

Transfection experiments were conducted in 96-well plates. Dendrimer stock solutions (1 mg/mL in distilled water) were pipetted into 20 μL of Dulbecco-modified Eagle's medium. pEFGP (320 ng) in 25 μL of the same medium was subsequently added. The plates were placed on an orbital shaker and shaken for 15 min at room temperature to form the vector-DNA complexes. Dendrimer-DNA complexes were tested in triplicate at four bPEI nitrogen to DNA phosphate ratios (N:P ratio) from 5:1 to 25:1. CHO–K1 cells (100 μL of 500 cells/μL) in 5% (v/v) calf serum in medium were added to each well and cultured for 24 h and 48 h at 37° C in a 5% CO_2 atmosphere. The medium was drawn off, and 100 μL of 1% (v/v) trypsin in buffered saline pH 7.2 was added and incubated for 1 min. The trypsin solution was drawn off and immediately replaced with 200 μL culture medium containing 5% calf serum and 2 μg/mL propidium iodide. The cells were removed from the substratum by flushing with a pipet and transferred to pre-chilled test tubes kept on crushed ice until the expressed green fluorescent protein (GFP) was quantified by flow cytometry.

Flow Cytometric Analysis of Transfection Efficiency

Plasmid DNA-transfected CHO–K1 cells were measured for GFP expression and propidium iodide fluorescence 48 h after transfection using flow cytometry analysis on a FACSCalibur analytical flow cytometer (Becton Dickinson, Erembodegem, Belgium). For each sample, 2,000 cells were quantitated, and the intensity of FL1 (GFP) and FL3 (propidium iodide) was recorded. Flow cytometry parameters were set using untransfected CHO–K1 cells (FL1 negative, FL3 negative), freeze-thawed CHO–K1 cells (FL1 negative, FL3 positive), and fluorescein diacetate (10 μL, 1 mg/mL acetone solution)-treated CHO–K1 cells (FL1 positive, FL3 negative). In this context, negative denotes cells with no measurable fluorescence, and positive with measurable fluorescence.

Statistics

Significance of differences between means was calculated using unpaired Student's *t*-test.

RESULTS

Construction of Dendrimeric Vectors

Dendrimeric vectors were constructed using primarily a divergent strategy (*i.e.*, sequential addition of shells, one at a time, proceeding outward from a central core), followed by limited use of a convergent strategy (*i.e.*, addition of multiple shells in one step as pre-assembled segments) for the final assembly. The central core consisted of commercially available 32,000 Da bPEI, which was selected for its established effectiveness in the 'proton sponge' endosomal escape mechanism (6,8,9,29–31,39).

Addition of a Hydrophobic Shell to Polycationic Cores

Hydrophobic shells were added to bPEI cores by alkylating outer primary amines at four molar ratios (10, 19, 48 and 96) of alkylating agent IV to bPEI. Alkylation of bPEI with a number of different hydrophobic moieties has been reported as an approach to enhancing catalytic properties (40), attaching gold colloids (41), and enhancing bactericidal activity (42). The most commonly used reaction has been nucleophilic substitution with alkyl halides. The alkyl chain

reagent developed for the present study, 16-iodo-hexadecanoyl-homocysteine thiolactone amide (**IV**), was designed to react readily with a bPEI amino group at one end, attaching the hydrophobic alkyl domain with a protected thiol at the opposite end. The C16 chain was selected to provide a shell with hydrophobic character to act as a sink for released endosomal lipids and to prevent pDNA released from outer oligocation shell from binding electrostatically to the bPEI core. The thiol moiety was included to enable subsequent coupling to a maleimide moiety attached to an acid-labile linker. It was initially protected as a thiolactone ring to prevent polymerization by reaction of free thiols with iodo-alkane moieties. The thiolactone ring was opened by alkaline hydrolysis using slow addition to a basic buffer (Scheme 1) to yield a free thiol (an efficient nucleophile) and a carboxylate ion, the negative charge of which was expected to both aid in solubilization during construction of the vector and later in preventing electrostatic collapse of pDNA onto the polycationic core after decomposition of vector-pDNA complexes at low pH.

Addition of Acid-Labile Linkers

Acid-cleavable linkers were selected for their reported ability to maintain moderate stability above pH 7.0, but hydrolyze at pH 5.5, a pH value achieved inside the endocytotic vesicle (33). The following three types of linker reagents were prepared for attaching outer shells to inner shells, and maleimide moieties were added at each end: (a) 1,4-bis maleimidobutane, (b) 2,2-bis(2-maleimidoethoxy)-propane, **VII**, and (c) 3,9-bis(2-maleimidoethoxy)-3,9-ethane-2,4,8,10-tetraoxaspiro[5.5]undecane, **VIII** (see Scheme 2). 1,4-Bis-maleimidobutane is a non-cleavable linker included as a negative control to assess whether acid-cleavable linkers would improve transfection efficiency, cytotoxicity, or both. The other two linkers, **VII** and **VIII**, have reported cleavage half-times of 42 and 18 min, respectively, at pH 5.5 (33). Maleimide moieties undergo facile, essentially irreversible Michael addition to strong nucleophiles such as thiol groups. An acid-cleavable linker was attached to the free thiol groups at the ends of each alkyl chain by reacting with excess bis-maleimido-linker construct, which attached the linker using one maleimido moiety, while leaving an unreacted maleimido moiety at the new terminus of each chain. Maleimide-containing dendrimers were handled with extreme care, due to their inherent lability. Aqueous solutions of **VII** and **VIII** were made on the day they were used, and never stored. **VII** and **VIII** could only be stored neat; degradation was observed during storage in all organic solvents tested. To prevent aggregation of dendrimers due to crosslinking, the solutions of alkylated bPEI were added slowly to a solution containing 5- to 10-fold molar excess of linker reagent. Reactions were monitored for completion by determining if free thiols were present using a fluorogenic reagent (36). Unreacted excess linker reagents were removed (i.e., reduced to <0.05 equivalents) by repeated cycles of dilution with buffer and concentration in ultrafiltration tubes.

Addition of the Outer Oligocationic Shell

Effective dendrimeric transfection agents must include an outer polycationic shell to condense the plasmid DNA and to bind the particle electrostatically to the surface of the mammalian cell. In this study, outer polycationic shells were constructed of oligoamines, which were expected to bind pDNA effectively when held together as an aggregate in the outer shell, but which would release bound pDNA more effectively than a larger polycation in the cytoplasm after dispersion of the vector-pDNA complex at low pH. Three oligoamines were examined in the present study: spermine, which gives a terminal oligocation with a maximum of 4 positive charges; 600 Da PEI, with a maximum of about 14 positive charges; and 1,800 Da PEI, with a maximum of about 42 positive charges. The range in MWs allowed differences in cationic surface charge and electrostatic binding ability to be compared. To facilitate coupling to the unreacted maleimido moiety of acid-labile linkers, the oligoamines were derivatized with Traut's reagent (2-iminothiolane), which reacts with an amino group attaching a free thiol group. The numbers of free thiol groups attached were quantified using a fluorogenic reagent, SBD-F. A reducing agent, 1 mM tris-(2-carboxyethyl)-phosphine, was included in assay and reaction buffers to keep the free thiols in reduced form. Thiolated oligoamines were allowed to react in borate buffer at pH 8.0 with the maleimido moieties on acid-labile linkers bound to alkylated bPEI. Coupling reactions with maleimide derivatives were carried out in the shortest possible time due to the instability of the linker moiety. Excess unreacted thiolated oligoamines were removed using repeated cycles of dilution with buffer and concentrating in ultrafiltration tubes. Fully assembled vectors were dissolved in 5% DMF/50 mM sodium borate buffer, pH 8.0, flash frozen and stored in liquid nitrogen.

Characterization of Assembled Dendrimeric Vectors

Various approaches were used to characterize the four intermediates at the two-shell stages and the nine fully assembled vectors prepared from each by attaching one of three different linkers and to each one of three different outer shell oligocations, except for alkylation at the 96 molar ration for which only spermine and 600 Da PEI oligocations were used (43). The characterization methods included (a) ratios of alkyl chains to bPEI cores by integrating proton NMR signals for methylene groups in core bPEI and hydrophobic shell alkyl chains, (b) quantitation of bPEI by copper complexation (37) and quantitation of thiol groups by fluorescent derivatization (36), (c) molecular weight determination by static light scattering, and (d) particle size determination by dynamic light scattering.

Characterization Studies on Two-Shell Intermediates

Characterization studies were carried out on the two-shell dendrimeric intermediates consisting of a bPEI core and a hydrophobic shell with terminal free thiol groups by integration of methylene protons in the NMR spectra in deuterated water. Methylene protons on alkyl chains could be accurately integrated for reaction products with 10 and 19 mol of alkyating agent IV per mole of bPEI core, indicating the presence of 7.7 and 18.0 alkyl chains per bPEI core, respectively. The thiol contents of these intermediates were consistent with most, if not all, of the thiolactone moieties having reacted with aqueous base to generate the

free thiols needed for subsequent coupling to an acid-labile linker. Molar ratios of alkylating agent IV to bPEI core >20 yielded products which gave proton NMR spectra unsuitable for quantitating methylene proton due to micelle formation, aggregation, or both (43). The molar ratios obtained by measuring thiols by fluorescence after derivatization with a fluorogenic reagent (36) and bPEI cores by copper complexation (37) were similar (7.6 and 15.3 alkyl chains per bPEI core for alkylation with 10 and 19 mol of alkyating agent IV per mole of bPEI core, respectively). Molar ratios of thiol to bPEI cores measured by chemical analysis indicated 42.7 and 60.6 alkyl chains per bPEI core for alkylation with 48 and 96 mol of alkyating agent IV per mole of bPEI core, respectively. Solubility of the dendrimeric intermediate with 60.6 alkyl chains per bPEI core was limited, and it could not be subjected to a freeze-thaw cycle without precipitation.

Molecular weights of alkylated bPEI intermediates were also determined by dynamic and static light scattering (43) and by size exclusion chromatography with multi-angle laser light scattering detection (Table I). The observed molecular weights of intermediates prepared with 48:1 and 96:1 molar ratios of alkylating agent to bPEI core deviated widely from values predicted on the basis of complete reactions. The deviations may be the result of aggregation of the dendrimers due to high hydrophobicity. Elution of highly alkylated bPEI polymers from Novema size exclusion chromatography columns required harsh acidic conditions (1% formic acid); none of a wide variety of neutral solvent mixtures (triethylamine acetate, phosphate, and borate buffers, with or without methanol or acetonitrile) were effective.

Two-shell intermediates were complexed with pEGFP DNA and examined for transfection activity and cytotoxicity (43). Cytotoxicity increased dramatically with N:P ratio, typically from <5% cytotoxicity at N:P=5:1 to over 50% at N:P=50:1. Modest transfection activity was observed only at the lowest alkylation level (calculated 10 alkyl chains per bPEI core).

Characterization Studies on Completed Vectors

A variety of strategies were used in the characterization of fully assembled dendrimeric vectors. The bPEI content of each was determined using copper complexation (37). It was not possible to use the unique NMR absorption bands of the acetal moieties for characterization, due to excessive resonance band broadening because the large size and rigidity of the dendrimers prevent molecular tumbling in the NMR time scale. Fully assembled vectors were analyzed by size exclusion chromatography with 1% formic acid as mobile phase and multi-angle laser light scattering detection (Table II). A wide variety of solvent systems were tested as mobile phases for elution of PEI derivatives, including 450 mM ammonium hydroxide, pH 8.5; 250 mM triethylamine acetate, pH 8.0; 500 mM acetate buffer, pH 5.0; with a range of column settings from room temperature to 60°C, but among them only 1% formic acid successfully eluted the polymers. For size exclusion chromatography of vectors containing the uncleavable 1,4-bis-maleimidobutane linker in 1% formic acid as mobile phase, higher numbers of alkyl chains conjugated to the bPEI core (calculated 48 and 96 alkyl chains per core, representing about 25% and 50% of primary amines alkylated, respectively) resulted in larger deviations of the measured molecular weight from the calculated value. At the highest alkylation level, anomalously high molecular weight values were measured, consistent with aggregation. Attempts to study vectors containing acid-labile linkers (**VII** and **VIII**) by size exclusion chromatography yielded M_w values well below calculated, consistent with cleavage of the acid-labile linker in the low pH mobile phase and loss of the outer cationic shell.

Given that dendrimers prepared with acid-labile linkers could not be characterized by size exclusion chromatography, the dendrimers prepared with the acid-stable control linker, 1,4-bis maleimidobutane, were extensively characterized using the technique, and it was assumed that, because similar bis-maleimide chemistry was used with all the linkers, dendrimers prepared with acid-labile **VII** and **VIII** would have analogous structures. Additional information about the dendrimers was obtained by preparing model vectors in which the inner polycationic core was fluorescently labeled with fluorescamine (FLA) (44) and the outer oligocationic shells were fluorescently labeled with fluorescein isothiocyanate (FITC) (45). Vectors prepared in this manner retained transfection and cytotoxic activities (43). The spectral properties of FLA and FITC are sufficiently different to allow facile simultaneous analysis of both. The FITC-labeled outer cationic shell and the FLA-labeled alkylated bPEI core elute from size exclusion chromatography (Fig. 1) at the same time

Table I. Estimation of the Average Molecular Weight of Partially Assembled (Two-Domain) Dendrimeric Vector Intermediates

Molar ratio of alkylating agent to bPEI polymer	Calculated molecular weight[a] (Da)	Observed molecular weight[b]	
		Weight average, M_w (Da)	Number average, M_n (Da)
0	3.2×10^4	3.2×10^4	1.7×10^4
10	3.6×10^4	5.4×10^4	3.5×10^4
19	3.9×10^4	6.7×10^4	3.7×10^4
48	5.0×10^4	1.1×10^5	8.8×10^4
96	6.8×10^4	1.8×10^6	1.4×10^6

bPEI branched polyethylenimine

[a] Assuming complete reaction of bPEI (32,000 Da measured molecular weight) with 16-iodo-hexadecanoyl-homocysteine thiolactone amide adding a 371 Da unit

[b] Size exclusion chromatography was carried out on custom-assembled modular HPLC instrumentation in 1% formic acid with Novema 8×300 mm 300 Å and 1,000 Å columns in series and multi-angle laser light scattering detection. Dn/dc values used to calculate the mass and molecular weight were based on the mass ratio of the polyethylenimine core (dn/dc=0.253[16]) and n-eicosane (dn/dc=0.169[17]).

Table II. Average Molecular Weights of Selected Fully-Assembled Dendrimeric Vectors Using Static Light Scattering in 1% Formic Acid

Alkylation ratio	Linker	Outer shell oligocation	Calculated MW	Weight average (Mw)[a]	Number average (Mn)[a]
10	BMB	spermine	3.8×10^4	6.3×10^4	5.5×10^4
10	BMB	PEI-600	4.2×10^4	1.1×10^5	9.6×10^4
10	BMB	PEI-1800	5.6×10^4	9.1×10^4	7.5×10^4
10	VIII	spermine	3.8×10^4	4.8×10^4	3.8×10^4
19	BMB	spermine	4.3×10^4	1.3×10^5	1.8×10^5
19	BMB	PEI-600	5.0×10^4	1.6×10^5	1.4×10^5
19	BMB	PEI-1800	7.8×10^4	1.2×10^5	1.1×10^5
48	BMB	spermine	6.0×10^4	3.6×10^5	3.0×10^5
48	BMB	PEI-600	7.9×10^4	5.8×10^5	4.5×10^5
48	VII	PEI-600	7.9×10^4	3.7×10^4	3.6×10^4
48	BMB	PEI-1800	1.5×10^5	4.0×10^5	2.9×10^5
96	BMB	spermine	1.0×10^5	4.2×10^6	3.5×10^6
96	BMB	PEI-600	1.3×10^5	5.6×10^6	5.0×10^6

MW molecular weight; *BMB* 1,4-bis-maleimidobutane; *VII* 2,2-bis(2-maleimidoethoxy)-propane; *VIII* 3,9-bis(2-maleimidoethoxy)-3,9-ethane-2,4,8,10-tetraoxaspiro[5.5]undecane, *PEI* branched polyethylenimine with the manufacturer's designated molecular weight in Da

[a] Mass and molecular weight were calculated with a mass ratio for polycationic cores of dn/dc=0.253[16] and for n-eicosane of dn/dc=0.169[18]. A 5% error was assumed for M_w and M_n data.

as total sample measured by the 90 degree light scattering signal, providing evidence that the two cationic shells are coupled as one molecule by the 1,4-bis-maleimidobutane linker. FLA and FITC contents were quantitated at their absorbance maxima (44,45) for vectors prepared with calculated 19 and 48 C16 alkyl chains per bPEI core, the acid-stable linker, and outer oligocation shells of 600 and 1,800 Da PEI (Table III). For 19 alkyl chains per bPEI core, the results indicate nearly 100% coverage of alkyl chains with an oligocation, whereas with 48 alkyl chains per bPEI core, there was only ~65% coverage of alkyl chains with an oligocation. The size of the outer shell oligocation did not affect the extent of alkyl chain coverage, because the molar ratios of outer oligocation component to cores were the same within error despite the size differences between the FITC-labeled PEIs with 600 and 1,800 Da molecular weights.

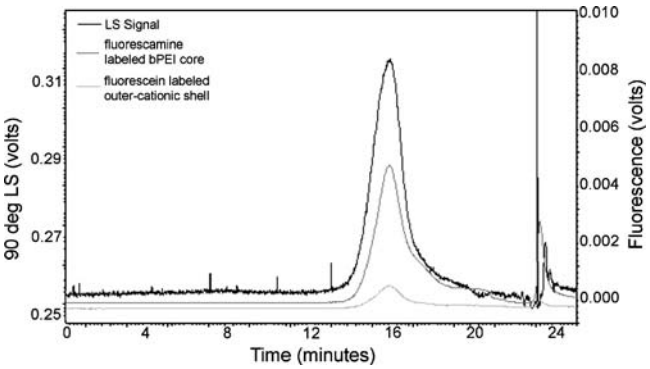


Fig. 1. Size-exclusion chromatography of a completed dendrimeric vector prepared with a fluorescamine-labeled (44) 32,000 Da branched polyethylenimine core, alkylated with a calculated average of 19 C-16 alkyl chains coupled through the non-cleavable linker 1,4-bis-maleimidobutane to a fluorescein-labeled (45) 600 Da polyethylenimine oligocation outer shell. Size-exclusion chromatography used formic acid (1%) as eluent on a custom-assembled modular HPLC instrument with multiple detectors in series. Matter, measured by static light scattering (black line), fluorescamine fluorescence (dark grey line), and fluorescein fluorescence (light grey line) all co-eluted. Similar results were obtained for alkylation of the polyethyleimine core with a calculated average of 48 C-16 alkyl chains (43).

Preparation and Characterization Studies on Polyplexes

The DNA condensation/complexation capacity of dendrimeric vectors with pEGFP DNA was evaluated by dynamic light scattering. The overall stability of the vector/pEGFP complexes was investigated by determining the zeta-potential, a measure of flocculation potential and colloidal stability. Table IV presents examples of values obtained for a series of completely assembled vectors containing 32,000 Da bPEI cores alkylated with an amount of alkylating agent calculated to attach 10 alkyl chains per core, and containing one of the three outer shell oligocations attached through either an acid-cleavable or the non-cleavable control linker. Fully and partially completed vectors with all levels of alkylation formed complexes with pEGFP plasmid DNA, except the most highly alkylated intermediate (96 molar ratio of alkylating agent to bPEI core) lacking an outer oligocation shell (43). The simplest explanation for the failure of the highly alkylated core to complex with DNA is the presence of a net negative surface charge due to large numbers of terminal carboxylate moieties. Table IV presents an example of data for a fully assembled vector series prepared from 32,000 Da bPEI cores alkylated at a level calculated to give 10 alkyl chains per bPEI core and linked to one of three oligocations by an acid-cleavable or non-cleavable linker. The vectors complexed pEGFP plasmid DNA tightly with sizes ranging from 74 to 110 nm. Similar sizes were seen for pEGFP plasmid DNA complexed with the vector series alkylated at a level calculated to give 19 alkyl chains per bPEI core and linked to one of three oligocations by an acid-cleavable or non-cleavable linker. However, particle sizes were much larger, typically >500 nm, for complexes formed between DNA and vectors of the corresponding vector series alkylated at a level calculated to give 48 and 96 alkyl chains per bPEI core, and the complexes had negative zeta-potentials (43).

Evaluation of the Ability of Acid-Labile Linkers to Increase Transfection Activity of Dendrimeric Vectors with Oligocation Outer Shells

Dendrimeric vectors alkylated at a level calculated to give 10 alkyl chains per bPEI core and linked through a non-

Table III. Composition of Fully-Assembled Dendrimeric Vectors Prepared with Fluorescamine-labeled 32,000 Da Branched Polyethylenimine Cores, Fluorescein Isothiocyanate-Labeled Outer Oligocationic Shells and the Acid-Stable Linker, 1,4-bis-maleimidobutane

Vector components				
Calculated alkylation ratio	Outer shell oligocation molecular weight	Measured[a] mass/moles of polycation cores (μg/nmol)	Measured[a] mass/moles of oligocation outer shells (μg/nmol)	Ratio of measured moles of outer of shell oligocations per mole of polycationic core
19	600 Da	23/0.34	4.1/6.8	20
48	600 Da	12/0.11	2.1/3.5	32
19	1,800 Da	30/0.45	14/7.0	15
48	1,800 Da	33/0.31	19/9.6	31

[a] Based on fluorescence measurements following size exclusion chromatography

cleavable linker (1,4-bis-maleimidobutane) to any of the outer cationic shell components tested exhibited only very low levels (1 and 2%) of transfection efficiency. In contrast, the corresponding vectors prepared with the acid-labile linkers, **VII** and **VIII**, and with spermine as the cationic shell component exhibited an optimal transfection efficiency about five times higher (11±1% with 21±5% cytotoxicity at a N:P ratio of 10:1, Fig. 2A). This represents a significant (Student's *t*-test, $P<0.005$) increase in transfection efficiency relative to that of the corresponding vector with the non-cleavable linker, 1,4-bis-maleimidobutane. Significantly increased transfection activity was also observed for vectors with acid-cleavable linkers and alkylation at 19 chains per bPEI core and N:P ratios ≤10, but not at higher N:P or higher alkylation ratios (43). The observed increased transfection activity with vectors incorporating an acid-cleavable linker provides proof-of-concept evidence that a low pH releasable outer oligocation shell can increase transfection efficiency. However, the optimal transfection activity observed with acid-cleavable linkers was still about half that of unaltered 25 kDa bPEI (Fig. 2A).

Additional characterization of the acid lability of dendrimeric vectors prepared with linkers VII and VIII was undertaken in an attempt to better understand why they did not exhibit higher transfection activity than the unaltered bPEI core. Degradation of the acid-cleavable acetal linkers in dendrimeric vectors was studied by three different methods: (a) fluorescence polarization, (b) ultrafiltration, and (c) dynamic light scattering. Initial studies used fluorescence polarization, a technique which estimates the molecular weight of molecules based on their rotational mobility after excitation of a fluorescent label with polarized light. In dendrimeric vectors prepared with FITC-labeled outer shell oligocations, the fluorescent label experiences a large drop in molecular weight when linker hydrolysis releases oligocations from the alkylated bPEI core. A decrease in fluorescence polarization at lower pH was expected for vectors prepared with acid-labile linkers (**VII**, and **VIII**), but not expected for vectors synthesized with the 1,4-bis-maleimidobutane linker. However, increased fluorescence polarization was observed, particularly at low pH with acid-labile linkers. Fig. 3 contains an example obtained with the series of three linkers in vectors prepared from 32,000 Da bPEI cores alkylated at a level calculated to give 19 alkyl chains per bPEI core and containing 1,800 Da FITC-labeled PEI oligocationic outer shell components. The largest change was seen using the **VIII** linker at pH 4.0. The increased fluorescence polarization suggests that the predominant reaction occurring under the

Table IV. Plasmid DNA Condensation/Complexation Facility with Branched Polyethylenimine Cores, Partially Assembled (Two-Domain) Dendrimer Intermediates and Completely Assembled Dendrimeric Vectors

Vector shell components				Size[a] of complex with pEGFP DNA (nm)	ζ-potential of complex with pEGFP DNA (mV)
Dendrimer stage	Alkylation ratio	Linker type	Outer shell oligocation		
Core only	–	–	–	430±20	28
" + Alkyl shell	10	–	–	540±30	19
" + Alkyl shell	19	–	–	150±10	20
" + Alkyl shell	48	–	–	160±10	15
" + Alkyl shell	96	–	–	1,600±100	16
" " + Oligocation	10	BMB	PEI-600	70±10	22
" " + Oligocation	10	BMB	PEI-1800	110±30	9
" " + Oligocation	10	BMB	Spermine	80±10	14
" " + Oligocation	10	VII	PEI-600	90±10	14
" " + Oligocation	10	VII	PEI-1800	80±10	13
" " + Oligocation	10	VII	Spermine	80±10	18
" " + Oligocation	10	VIII	PEI-600	110±10	9
" " + Oligocation	10	VIII	PEI-1800	80±10	12
" " + Oligocation	10	VIII	Spermine	80±10	15

– not present in the complex; *BMB* 1,4-bis-maleimidobutane; *VII* 2,2-bis(2-maleimidoethoxy)-propane; *VIII* 3,9-bis(2-maleimidoethoxy)-3,9-ethane-2,4,8,10-tetraoxaspiro[5.5]undecane, *PEI* branched polyethylenimine with the indicated molecular weight in Da

[a] Hydrodynamic size determined by dynamic light scattering

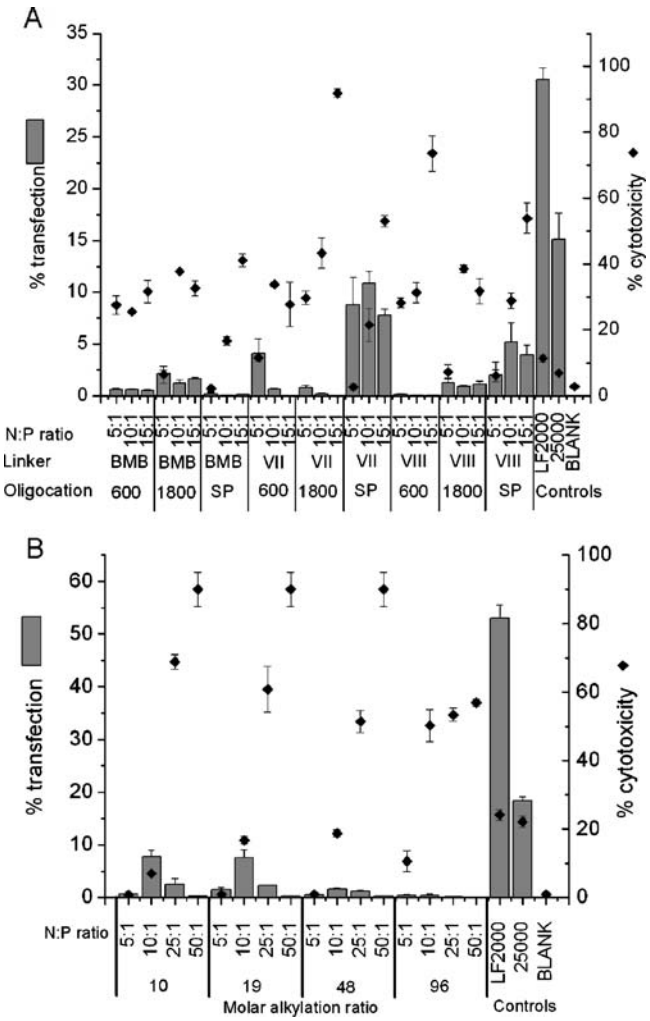


Fig. 2. Transfection efficiency and cytotoxicity in CHO–K1 cells with green fluorescent protein plasmid DNA complexed with (**A**) fully-assembled dendrimeric vectors prepared with acid-labile and stable control linkers or (**B**) incomplete dendrimeric vector intermediates lacking linkers and an outer oligocation shell. Assays were carried out in triplicate with the indicated N:P ratio (estimated moles of vector amines to DNA phosphate) of vectors consisting of branched polyethylenimine cores alkylated with the indicated calculated average of C-16 alkyl chains (10, 19, 48 and 96). The positive controls LF2000 and unaltered 2,500 Da bPEI were tested at an N:P ratio of 10:1. In (**A**) 10 C-16 alkyl chains were coupled through one of three linkers (acid-cleavable linkers, VII or VIII, or the non-cleavable control linker, BMB) to one of three outer shell oligocations (600 Da branched polyethylenimine, 1,800 Da branched polyethylenimine or spermine). Transfection and cytotoxicity activities were measured simultaneously on a fluorescence-activated cell sorter as described in "Materials and Methods." Abbreviations: *BMB* 1,4-bis-maleimidobutane; *VII* 2,2-bis(2-maleimidoethoxy)-propane; *VIII* 3,9-bis(2-maleimidoethoxy)-3,9-ethane-2,4,8,10-tetraoxaspiro[5.5]undecane; *600* 600 Da bPEI; *1,800* 1,800 Da bPEI; *25,000* 25,000 Da (manufacturer's specification) bPEI; *SP* spermine; *LF2000* Lipofectamine 2000®.

conditions used was aggregation, not hydrolytic release of oligocation outer shell components. Aggregation could result from release of outer shell oligocations or from altered dendrimer conformations which have lost the shell structure and assumed a form which allows the previously internal hydrophobic regions of different dendrimers to interact hydrophobically. Failure to observe release of fluorescently labeled oligocations (see below) is consistent with the latter mechanism.

The second approach to characterizing the acid lability of dendrimeric vectors prepared with linkers VII and VIII used ultrafiltration to monitor release of 1,800 Da FITC-labeled PEI oligocationic outer shell components from the vector series prepared from 32,000 Da bPEI cores alkylated at a level calculated to give 19 alkyl chains per bPEI core. Over the course of 2 h, no fluorescently labeled outer cationic shell components were detected crossing the 10,000 Da molecular weight cutoff ultrafiltration membrane at pH 4.0, 5.5, or 8.0 (43). It was also observed that dilution by pH 4.0 and pH 5.5 buffers caused the vector solutions to turn opalescent or cloudy.

Dynamic light scattering was the third method used to investigate the degradation/aggregation of vectors at lower pH. Table V presents an example obtained with the series of three linkers in vectors alkylated at a level calculated to give ten alkyl chains per bPEI core and containing 1,800 PEI oligocationic outer shell components. Similar results were obtained with vectors alkylated at a level calculated to give 19 alkyl chains per bPEI core, whereas vectors alkylated at a level calculated to give 48 and 96 alkyl chains per bPEI core exhibited increased aggregation (43). The hydrodynamic radii for vectors alkylated at a level calculated to give ten alkyl chains per bPEI core and completed with the three linkers differed significantly only at pH 2.0, at which the vector with the acid-stable linker was significantly larger (Student's *t*-test, $P<0.05$), consistent with loss of the outer oligocationic shells from vectors containing the acid-labile linkers **VII** and **VIII** (43). At pH values of 4.0 and 5.5, aggregates of 300–500 nms were formed, which provides an explanation for the opalescence observed during the ultrafiltration experiments.

Evaluation of Combining Endosomal Escape Mechanisms as a Strategy for Increasing Transfection Activity of Dendrimeric Vectors

bPEI cores alkylated at levels calculated to give averages of 10–96 alkyl chains per bPEI core and lactone rings opened were complexed with pEGFP plasmid DNA and transfected into cells to determine the latent transfection efficiency and cytotoxicity not associated with the presence of a releasable outer cationic shell. bPEI cores alkylated at levels calculated to give 10 and 19 alkyl chains per bPEI core had ~8% transfection efficiency and 10–12% cytotoxicity at a 10:1N:P ratio (see Fig. 2B). This represents about half the transfection efficiency of unalkylated bPEI cores complexed with pEGFP plasmid DNA under the same transfection conditions. bPEI cores alkylated at levels calculated to give 48 and 96 alkyl chains per bPEI core exhibited no observable transfection efficiency or increased cytotoxicity (43).

Increased alkylation of bPEI cores beyond the level calculated to give ten alkyl chains per bPEI core did not increase the transfection efficiency or lessen the cytotoxicity in the fully assembled dendrimeric vectors. No dendrimeric vector prepared with alkylation at levels calculated to give 19 and 48 alkyl chains per bPEI core exhibited transfection efficiencies of over 5% (43) even if constructed with an acid-cleavable linker and spermine as the outer shell oligocation.

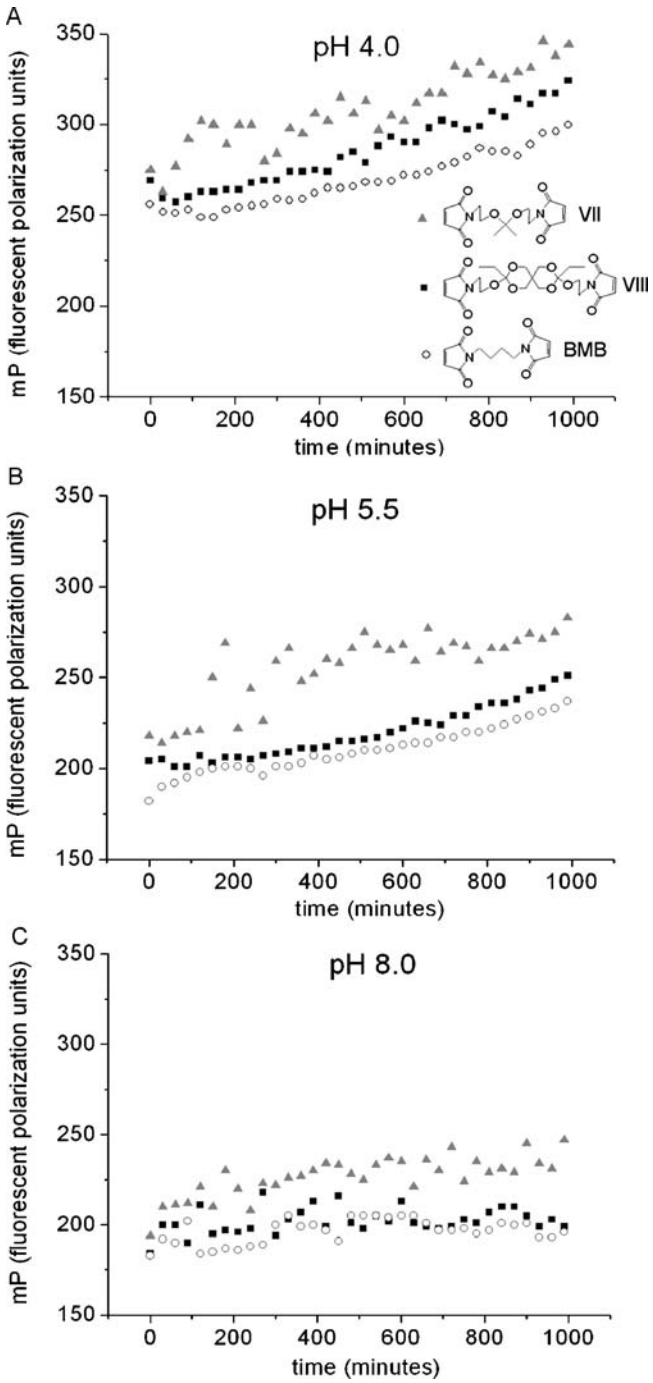


Fig. 3. Hydrolysis of fully-assembled dendrimeric vectors with acid-cleavable and stable, control linkers at various pH values as monitored by fluorescence polarization. Fully-assembled dendrimeric vectors prepared with 32,000 Da branched polyethylenimine cores were alkylated with a calculated average of 19 C-16 alkyl chains and linked to fluorescein-labeled 1,800 Da branched polyethylenimine outer shell oligocations through one of two acid-cleavable linkers, VII or VIII, or the non-cleavable control linker, 1,4-bis-maleimidobutane. Vector solutions (5 mg/mL) were diluted 1:20 in one of the following three buffers: pH 4.0: 50 mM sodium citrate; pH 5.5: 50 mM sodium citrate; or pH 8.0: 50 mM sodium borate. Fluorescence polarization was measured every 10 min for 17 h at room temperature on a 96-well spectrofluorometer. Fluorescence polarization decreases when small fluorescein-labeled oligocations are released from large dendrimers by linker hydrolysis, and it increases when intact vectors aggregate. Abbreviations: *BMB* 1,4-bis-maleimidobutane; *VII* 2,2-bis(2-maleimidoethoxy)-propane; *VIII* 3,9-bis(2-maleimidoethoxy)-3,9-ethane-2,4,8,10-tetraoxaspiro[5.5]undecane.

No completed dendrimeric vector prepared with alkylation at levels calculated to give 96 alkyl chains per bPEI core exhibited transfection efficiencies of over 1% (43). The underivatized bPEI core was a more efficient vector for transfection with generally comparable or lower cytotoxicity than any of the multi-shell dendrimers (Fig. 2A, B and reference 43).

Other Structural Features of Dendrimeric Vectors Associated with Increased Transfection Activity

Of the three outer shell oligocations tested (spermine, 600 Da PEI and 1,800 Da PEI), spermine generally gave the highest transfection activities at all levels of alkylation examined except at a calculated average of 96 alkyl chains per bPEI core (43). Cytotoxicity was heavily dependent on N:P ratios with little effect of alkylation level even at a calculated average of 96 alkyl chains per bPEI core (Fig. 2A, B and reference 43). Thus, optimal transfection to cytotoxicity ratios were observed at low alkylation levels with spermine as the outer cation.

DISCUSSION

DNA Release with Acid-Labile Linkers as a Strategy for Increased Transfection Efficiency

Studies were carried out to investigate the hypothesis that transfection efficiency can be increased by incorporating into the structure of a dendrimeric vector a mechanism that facilitates release of the DNA from the vector after the complex has been taken into the target cell. The approach used was to prepare a dendrimeric vector in which the outer shell (which binds the DNA being transfected) consists of oligocations held in place by linkers reported to be stable at neutral pH, but to hydrolyze rapidly at pH values found inside endosomes (pH 4.5–5.5). During the initial electrostatic binding of DNA to the fully-assembled vector, and the binding of the vector-DNA complex to the negatively charged outer surface of the cell at neutral pH, the acid-labile linkers should remain intact. However, following endocytosis Na, K-ATPases pump protons into endocytic vesicles, lowering the pH in preparation for fusion with a primary lysosome (30). When exposed to lower pH inside endosomes, acid-labile linkers should hydrolyze, releasing the outer shell and its bound DNA. Small oligocations should bind electrostatically to DNA weakly enough not to interfere with its expression inside the cell. Consistent with this hypothesis, the transfection efficiency of vectors containing acid-cleavable linkers was observed to be up to five-fold greater than for vectors containing the non-cleavable control linker, 1,4-bis-maleimidobutane (Fig. 2A). However, optimal transfection efficiency with vectors containing acid-labile vectors was less than that observed in control experiments with the unaltered bPEI core. There are various possible explanations for these observations including (a)

Table V. Hydrodynamic Radius (R_H) of Dendrimeric Vectors Following Exposure to a Range of pH Values

Linker	Hydrodynamic radius[a] (R_H, nm) at indicated buffer pH			
	pH 2.0[b]	pH 4.0[b]	pH 5.5[b]	pH 8.0[c]
1,4-bis-maleimidobutane (BMB)	16±2	300±10	490±20	10±2
2,2-bis(2-maleimidoethoxy)-propane (VII)	12±2	270±10	480±20	11±2
3,9-bis(2-maleimidoethoxy)-3,9-ethane-2,4,8,10-tetraoxaspiro-[5.5]undecane (VIII)	12±2	300±10	530±30	9±2

[a] Particle sizes were continuously measured by dynamic light scattering for 60 min and the final value noted, except for the pH 8.0 value, which was read after 1 min.
[b] Measurements at pH 2.0, 4.0, and 5.5 used a 50 mM sodium borate citrate buffer
[c] Measurements at pH 8.0 was 50 mM sodium borate, 1 mM EDTA buffer

other structural features built into the dendrimeric vectors reduced transfection efficiency more than the improvement provided by acid-labile linkers, (b) very little acid hydrolysis of linkers occurred under the conditions encountered, and (c) DNA release from the vector is not a rate-limiting step in transfection.

Additional studies conducted on degradation of pure vectors in low-pH buffers indicated that hydrolysis and release of outer oligocations did not occur as expected. Hydrolysis of acid-labile linkers with release of the outer cationic shell was not observed at pH 4.0 and 5.5 using several experimental approaches (Table V), whereas hydrolysis did occur at pH 2.0 in the buffer used for size exclusion chromatography (Fig. 1). Spontaneous aggregation of vectors was also observed at pH 4.0 and 5.5 by both fluorescence polarization and dynamic light scattering measurements (Fig. 3 and Table V), but not at pH 2.0, when hydrolysis did occur. The observations are consistent with stabilization of acid-labile bonds in the environment found in the completely assembled dendrimer, slowing or preventing release of outer shell oligocations, and with vector aggregation that occurs at least until hydrolysis has taken place. Suh *et al.* (46) showed that for bPEI alkylated at a 10:1 molar ratio, less than 2% of the amino groups are protonated at pH 8, 19% at pH 5, 34% at pH 4 and 53% at pH 3. The low percent protonation at pH 4.0 and 5.5 may permit aggregation, which may result in reduced transfection efficiency. However, the relevance of studies on linker stability in buffer to stability in endosomes has not been established. The presence of cellular proteins in the endosome may prevent aggregation of alkylated bPEI cores, and they may also prevent re-binding of the pEGFP to the aggregated alkylated bPEI cores.

Combining Endosome Disruption Mechanisms as a Strategy for Increasing Transfection Efficiency

In the second hypothesis being tested in these studies, structural components required for two endosome release mechanisms were incorporated into the same dendrimeric vector to determine if they would be additive or synergistic in facilitating DNA transfection. The hypothesis was tested with two sets of dendrimeric vectors, the alkylated bPEI cores and the completed dendrimers including acid-releasable outer oligocationic shells. The reduction in transfection efficiency of bPEI was associated with alkylation by 16-iodo-hexadecanoyl-homocysteine thiolactone amide (see Fig. 2B), and more extensive alkylation of the bPEI core resulted in further reductions in transfection efficiency. Similar reductions in transfection efficiency with increased alkylation were observed in completed dendrimeric vectors containing an acid-releasable outer oligocationic shell (see Fig. 2A). One explanation for reduced transfection efficiency resulting from addition of an alkyl shell is provided by the observations of Suh *et al.* (46), who showed that alkylating bPEI shifted pK_a values two pH units lower so that buffering capacity in the pH range found in endosomes (pH 5 and 6), which is believed responsible for the osmotic burst/proton sponge mechanism proposed by Behr (30), was shifted to lower pH values (pH 3 and 4). Alkylation of the less toxic 10 kDa bPEI resulted in improved transfection efficiency when the primary amine content was maintained (47), whereas alkylcarboxylate grafting to 10 kDa bPEI resulted in lowered cytotoxicity, but increased transfection efficiency only when shorter alkyl chains (C-6, C-10) were used at low (<20%) percent alkylation of the calculated amount of primary amines (48). Thus, the dendrimeric vectors prepared in this study did not provide an adequate experimental system to fully test the advantage of combining osmotic burst and lipid depletion mechanisms, because the structural alterations used to add the lipid depletion mechanism reduced the osmotic burst mechanism too much. Studies on alkylcarboxylate PEI derivatives (47,48) suggest that greater transfection efficiency, and presumably better lipid depletion, is achieved when hydrophobic shells are formed with shorter alkyl chains (C-6 to C-10) than used in this study (C-16).

Other Structural Features Associated with Increased Transfection Efficiency

The transfection efficiency was greatest when the outer shell oligocation was spermine. One explanation may be that spermine derivatives, which have only four charges with high charge density, may be more easily displaced from DNA by salts and proteins in the cytoplasm or nucleus than outer shell components prepared with more heavily charged 600 or 1,800 Da bPEI. Additional studies will be needed to determine if more efficient displacement of spermine moieties from pEGFP DNA is involved in the improved transfection efficiencies with spermine-containing dendrimers.

CONCLUSIONS

A series of dendrimeric non-viral vectors were prepared from commercial 25 kDa branched PEI as an approach to

identifying structural features associated with increased transfection activity without increased cytotoxicity. When a vector was constructed with an outer shell of oligocations attached through acid-cleavable linkers designed to be cleaved inside target cells when the endosomal pH is lowered, about five-fold increased transfection activity was obtained relative to an analogous control dendrimer with an acid-stable linker. Of the oligocations examined as outer shell components of the dendrimeric vectors prepared in the study, spermine, with four possible positive charges, exhibited the highest transfection efficiencies. The highest transfection efficiencies were observed at the lowest levels of alkylation of bPEI cores tested. The dendrimer design used in this study did not permit an adequate evaluation of the benefit of combining two endosome release mechanisms, osmotic burst and lipid depletion, into a single dendrimeric non-viral vector.

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