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Bioresponsive Small Molecule Polyamines as Noncytotoxic Alternative to Polyethylenimine

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Abstract: Nonviral gene therapy continues to require novel synthetic vectors to deliver therapeutic nucleic acids effectively and safely. The majority of synthetic nonviral vectors employed in clinical trials to date have been cationic liposomes; however, cationic polymers are attracting increasing attention. One of the few cationic polymers to enter clinical trials has been polyethylenimine (PEI); however, doubts remain over its cytotoxicity, and in addition it displays lower levels of transfection than viral systems. Herein, we report on the development of a series of small molecule analogues of PEI that are bioresponsive to the presence of pDNA, forming poly(disulfide)s that are capable of efficacious transfection with no associated toxicity. The most effective small molecule developed, a cyclic disulfide based upon a spermine backbone, is shown to form very well-defined polyplexes (100−200 nm in diameter) that mediate murine lung transfection *in vivo* to within an order of magnitude of *in vivo jet*PEI, and at the same time display a much improved cytotoxicity profile.

Keywords: Nonviral; gene therapy; reducible; cyclic disulfide; polyamine synthesis

Introduction

Synthetic gene therapy vectors aim to match or surpass the ability of viruses to deliver therapeutic DNA without the associated health hazards that have been highlighted during clinical trials[1,2] with viral systems. Polycations,[3−5] including branched and linear poly(ethylenimine) (PEI), poly(L-lysine) (PL) and poly(amido amine) dendrimers, have attracted widespread attention within the field due to the stable complexes (termed polyplexes) they form with plasmid DNA (pDNA) which have proved able to transfect cells effectively both *in vitro* and *in vivo*. Linear PEI, currently widely accepted as the commercial "gold standard", has progressed

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into phase I clinical trials;[6] however, doubts over its toxicity combined with its lower efficacy compared to viruses[7] may limit its future use.

The root of the toxicity of PEI is the interactions this polycation establishes with cellular membranes, disrupting them and leading to severe damage to the cell.[8] There is considerable evidence that the extent of this damage is related to the molecular weight of PEI, with higher molecular weight species causing higher levels of toxicity, presumably due to the stronger nature of the interactions they form.[9−12] However, in order to affect efficacious pDNA delivery, PEI must also be of at least an intermediate molecular weight (2 kDa is generally accepted as the lower limit for this parameter), otherwise they are unable to form polyplexes of sufficient stability to protect the pDNA from premature degradation by nucleases.[9,13−16] This conflict of interests with respect to molecular weight has led to a plethora of biodegradable polycations, able to condense pDNA effectively while also improving upon the toxicity of stable polycations due to their subsequent breakdown into smaller, less toxic, units. In addition the destabilization of polyplexes that results from the degradation of the polymeric vector should also encourage pDNA release which, if it occurs in close enough proximity to a cell's nucleus, might also raise levels of transfection.

A number of different biodegradable bonds have been incorporated into polycationic vectors. Broadly speaking they can be grouped into three categories: hydrolyzable,[17] acid-triggerable[18−21] and reducible.[22−48] Reducible bonds are particularly advantageous as they will be stable within the body until they reach the cytoplasm of a cell, where the

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high concentrations of glutathione maintained there will cleave them.[49,50] This triggered degradation mechanism should ensure that the pDNA is not released until it has reached the vicinity of its site of action. Strategies involving disulfide bonds realized to date include the reversible cross-linking of low molecular weight PEI,[22,24,35] the reversible lateral stabilization of polyplexes,[23,25,30,32] linking amine containing pendant side chains to the polymer backbone via disulfide bonds,[36,39] bioreducible polypeptides,[26−29,33,34,37,38] bioreducible rotaxanes[31] and the synthesis of polyamines with disulfide linkages within their backbone,[40−48] including the recently published work of Park et al. on the synthesis of linear poly(ethylenimine sulfide)s.[42]

We were interested in designing and developing a bioreducible analogue of PEI that would retain or improve on its efficacy while remaining nontoxic. In order to achieve this we sought to incorporate regular disulfide linkages within a linear polyamine backbone. As described above this part of our design strategy was with significant precedent, therefore we decided to seek further innovation by constructing small molecule polyamines that were also either dithiols or cyclic disulfides in character. The molecular weight of any therapeutic polymer affects its biological and pharmacological profile, and even relatively small changes can have dramatic effects in a clinical setting.[51,52] Indeed, the molecular weight of PEI has been shown to affect both efficacy of pDNA delivery and cytotoxicity.[16] However, in spite of its clear importance, and the development of living polymerizations that allow for a degree of control to be exerted over it, this crucial variable often remains under weak synthetic control.[51] In contrast small molecules such as dithiols and cyclic disulfides have a clearly defined molecular weight which is under strict synthetic control, allowing for consistency of production across any number of different batches. How would such small molecules be able to condense pDNA into the stable polyplexes required for gene delivery? In both

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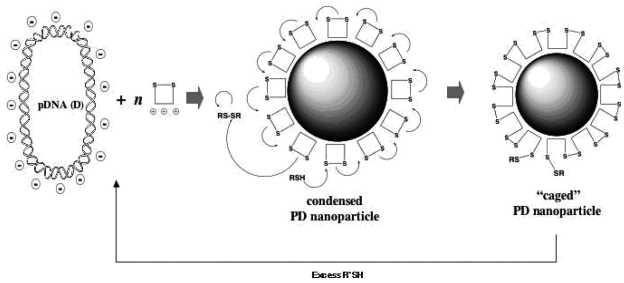

Figure 1. Schematic representation of the mechanisms of condensation for small molecule polyamine cyclic disulfides.

cases we envisaged that they would respond to the introduction of pDNA by forming high molecular weight poly(disulfide)s. In the case of dithiols, we anticipated that they might associate with pDNA through electrostatic interactions with phosphodiester backbone charges and then undergoing a sequence of controlled oxidations, utilizing the pDNA as a kinetic template (kinetically templated polymerization, KCP). This use of pDNA as a kinetic template has been described previously within the field of gene therapy, successfully resulting in the formation of polyplexes after polycondensations and vinylic polymerizations.[21,53] In the case of the cyclic disulfides (Figure 1), we anticipated that they should also interact electrostatically with pDNA and then undergo a sequence or series of thiol-disulfide exchange reactions to form a poly(disulfide) in a process that we hypothesized would be driven by entropic considerations (thermodynamically controlled templated polymerization, TCTP). This dynamic exchange should be catalyzed by the presence of small concentrations of thiols and has been widely used to great effect in dynamic combinatorial chemistry under biologically relevant conditions (i.e., pH 7.4).[54] From the outset, we appreciated that our combined approach would not solve the polymer molecular weight problem directly *per se*, in that the final condensing species were still likely to be polydisperse, but would remove any doubts about the influence of variations in the synthetic conditions employed on the biological properties of the vector in question.

Here we report the synthesis of small molecule polyamine dithiols and cyclic disulfides based upon a variety of di-, tri- and tetraamine starting materials. These novel compounds were then tested for their ability to complex and cage pDNA into condensed nanoparticles in a manner suitable for gene delivery, *in vitro* and *in vivo*. Studies involving efficacy of pDNA delivery and cytotoxicity were measured and studied throughout.

Materials and Methods

General Considerations. Dried CH_2Cl_2, MeOH and THF were obtained from a pure solv-MD standard design solvent purification system (Innovative Technologies, U.K.) using columns filled with 3 mm aluminum oxide balls (Sigma-Aldrich, U.K.). Dry TFA was obtained by distillation over CaH_2 at ambient pressure. All organic solvents used were dry, and reactions were performed under nitrogen unless stated. TLC was performed on Merck Kieselgel 60 F254 aluminum backed plates and visualized under UV light or stained with $KMnO_4$ solution. Purification over silica was achieved using Merck Kieselgel 60 (230−400 mesh). ^1H and ^{13}C NMR were recorded on a Bruker DRX400 spectrometer and processed using MestRe-C software (version 4.7.0.0, available from www.mestrec.com) using residual isotopic solvent as an internal reference for organic deuterated solvents. Samples run in D_2O were referenced using an external solution of dioxane in D_2O. The following abbreviations were used during assignment: s = singlet, d = doublet, t = triplet, q = quartet, quint = quintet, m = multiplet, br = broad. Chemical ionization (CI), fast atom bombardment (FAB) and electronic ionization (EI) mass spectra were recorded by Mr. John Barton using VG-70-250E and VG autospec Q instruments. Elemental analysis was performed by Stephen Boyer at London Metropolitan University. Melting points were measured on a Sanyo Gallenkamp hot stage. All chemicals were purchased from Sigma Aldrich, Lancaster or VWR unless otherwise stated and were used as received. 2-[(4-Methoxybenzyl)sulfanyl]ethanol[55] (**2**), 2-[(4-methoxybenzyl)sulfanyl]acetaldehyde[55] (**3**) and di-*tert*-butyl ethane-1,2-diylbis[(2-hydroxyethyl)carbamate][56] (**11**) were synthesized according to published procedures. Deacetylated linear PEI was synthesized according to the procedure of Thomas et al.[57]

pEGFP-Luc-DNA (1 mg/mL) was supplied by BD Biosciences, U.K. pUMV-C1-nt-beta gal was obtained from the University of Michigan Vector Core and was amplified by Plasmid Factory, Germany. Luciferase gene expression was measured with the Luciferase Assay System purchased from Promega, USA. Light output readings were done on a Berthold Lumal LB 9507 luminometer. Transfection efficiency, measured in relative light units (RLU), was normalized to the total mass of cellular protein using the BCA protein assay kit (Thermo Scientific, USA). LDH assay was

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carried out using the CytoTox 96 Non-Radioactive Cytotoxicity Assay (Promega, USA). PCS size measurements were carried out on a Coulter N4 dynamic light scattering instrument. SEM images were taken on a Leo-40 spectrometer, using samples gold plated *in vacuo* using standard techniques. All media and PBS were purchased from Invitrogen, U.K. Lysis buffer (5×) was obtained from Roche Diagnostics, U.K. Agarose, FCS, penicillin-streptomycin, HEPES free acid, glutaraldehyde, benzyl benzoate and benzyl alcohol were obtained from Sigma, U.K. OCT embedding matrix was purchased from Cellpath Ltd., and eosin was purchased from VWR. CHO-K1 and A549 cells were from the American Type Culture Collection and were incubated at 37 °C in 10% CO_2. F12 was used as the base medium for CHO-K1 cells and DMEM was used for A549 cells. 10% fetal calf serum and 1% penicillin-streptomycin were added to the base media to give serum containing media. Female BALB/c mice were purchased from Charles River Ltd., U.K., and housed under identical conditions. Murine experiments were performed in accordance with the UK Home Office regulations for the use of animals in research. Lung samples were homogenized using a Ultra-Turrax T25 homogenizer (Fisher Bioblock Scientific, U.K.), and β-gal expression was measured using a β-gal ELISA assay (Roche Diagnostics, U.K.). Histological images were taken using a Nikon Eclipse E600 microscope. Statistical analysis on the *in vitro* data was performed using Welch's *t* test (2-tailed) at a 0.05 significance level. *In vivo* data was analyzed by Imperial College Statistical Advisory Service using a Bonferroni corrected ANOVA test at a 0.05 significance level.

Synthetic Chemistry. *General Procedure A: Bisalkylation of Small Molecule Polyamines with {[(4-Methoxybenzyl)sulfanyl]ethyl} Moieties.* The small molecule polyamine (1 equiv) and [(4-methoxybenzyl)sulfanyl]acetaldehyde (**3**) (2 equiv) were dissolved in dry DCM (100 mL). $MgSO_4$ (~1 g) was added, and the resulting suspension was stirred for 6 h. The suspension was filtered, and dry MeOH (100 mL) was added, followed by $NaBH_4$ (10 equiv), and the resulting suspension was stirred for 15 h. Concentrated HCl (20 mL) was added dropwise (effervescence observed), and the resulting suspension was refluxed for 6 h. The suspension was concentrated under reduced pressure, and the resulting slurry was filtered and then washed with EtOAc (100 mL) to give a white solid, which was recrystallized from H_2O to give the desired product as a white solid.

1,14-Bis[(4-methoxybenzyl)sulfanyl]-3,7,12-triazatetradecane (Tris-hydrochloride Salt) (5). Spermidine (0.82 mL, 5.2 mmol) was reacted according to general procedure A to give **5** as a white solid (1.63 g, 2.65 mmol, 53%). Mp > 220 °C. ^{1}H NMR (D_2O, 400 MHz): δ 1.76 (br, 4H, $NHCH_2CH_2$-CH_2CH_2NH), 2.12 (br quint, 2H, $NHCH_2CH_2CH_2NH$), 2.82 (t, J 7.0 Hz, 4H, $NHCH_2CH_2S$), 3.00−3.30 (m, 12H, $NHCH_2$), 3.82 (s, 6H, CH_3O), 3.88 (s, 4H, SCH_2Ar), 7.05 (d, 4H, J 8.5 Hz, Ar*H*), 7.40 (d, 4H, J 8.5 Hz, Ar*H*). ^{13}C NMR (D_2O, 100 MHz): δ = 22.4, 22.6, 22.8, 26.1, 26.2, 34.1, 43.9, 44.0, 45.8, 45.9, 46.0, 46.2, 55.0, 113.9, 129.6, 130.0, 158.3. HR-MS (*m*/*z*, +ve FAB): calculated ($C_{27}H_{43}N_3O_2S_2 \cdot H^+$) 506.2875; found 506.2897. Elemental anal.: calculated ($C_{27}H_{43}N_3O_2S_2 \cdot 3PF_6^-$) C 52.72% H 7.54% N 6.83%; found C 52.77% H 7.54% N 6.78%.

General Procedure B: Removal of 4-Methoxybenzyl Protecting Groups To Reveal Dithiol. Dry anisole (10 equiv) was dissolved in dry TFA (20 mL), and the resulting solution was degassed using the freeze−pump−thaw method (3 cycles). The bis[(4-methoxybenzyl)sulfanyl] (1 equiv) was added, and the resulting solution was heated at reflux for 3 days. The solution was then concentrated under reduced pressure, and the residue was washed with dry degassed Et_2O (50 mL). Degassed MeOH (20 mL) was added to the solid, and the resulting suspension was filtered. The solvent was then removed under reduced pressure and the solid redissolved into a minimum amount of degassed MeOH. Degassed Et_2O was added until the cloud point was reached, allowing the product to be purified via recrystallization to give the desired dithiol as a white powder.

3,7,12-Triazatetradecane-1,14-dithiol (Tris-trifluoroacetate Salt) (TriN-3,4-DT). 1,14-Bis[(4-methoxybenzyl)sulfanyl]-3,7,12-triazatetradecane (**5**) (400 mg, 0.650 mmol) was reacted according to general procedure B to give TriN-3,4-DT as a white powder (174 mg, 0.287 mmol, 44%). Mp > 220 °C. ^{1}H NMR (400 MHz, d_4-MeOH): δ 1.62 (br, 4H, $NHCH_2CH_2CH_2CH_2NH$), 1.94 (quint, 2H, $NHCH_2CH_2$-CH_2NH), 2.80 (m, 4H, 3.10, CH_2SH) 3.00−3.25 (m, 12H, $NHCH_2$). ^{13}C NMR (400 MHz d_4-MeOH): δ 20.9, 20.9, 24.0, 24.1, 24.2, 45.6, 45.8, 47.9, 48.2, 51.4, 51.5. HR-MS (*m*/*z*, +ve FAB): calculated ($C_{11}H_{27}N_3S_2 \cdot H^+$) 266.1725; found 266.1730. Elemental anal.: calculated ($C_{11}H_{27}N_3S_2 \cdot 3C_2HF_3O_2$) C 33.61% H 4.98% N 6.92%; found C 33.74% H 4.90% N 6.87%.

General Procedure C: Oxidation of Dithiols to Cyclic Disulfides. The dithiol was dissolved in concentrated HCl (10 mL). DMSO (0.1 mL) was added, and the resulting solution was stirred for 6 h, during which time a white precipitate developed. The solution was then concentrated under reduced pressure, and the product was precipitated with an excess of acetone (25 mL). The solid was filtered, washed with further portions of acetone (2 × 25 mL), dried under reduced pressure at 70 °C for 12 h and then redissolved in H_2O and lyopholized to give the desired cyclic disulfide as a white powder.

1,2-Dithia-5,9,14-triazacyclohexadecane (Tris-hydrochloride Salt) (TriN-3,4-CDS). 3,7,12-Triazatetradecane-1,14-dithiol (TriN-3,4-DT) (98 mg, 0.16 mmol) was reacted according to general procedure C to give TriN-3,4-CDS as a white powder (51 mg, 0.057 mmol, 87%). Mp > 220 °C. ^{1}H NMR (D_2O, 400 MHz): δ 1.83 (br, 4H, $NHCH_2CH_2$-CH_2CH_2NH), 2.19 (br quint, 2H, $NHCH_2CH_2CH_2NH$), 3.10−3.30 (m, 12H, $NHCH_2CH_2CH_2CH_2NH$ + $NHCH_2$-CH_2CH_2NH + CH_2S), 3.50 (m, 4H, $NHCH_2CH_2S$). ^{13}C NMR (D_2O, 100 MHz): δ 22.5, 22.5, 22.7, 31.7, 32.5, 44.3, 44.4, 45.6, 45.8, 46.8, 46.9. ES-MS (*m*/*z*): 263.6 (100%), 526.9 (24%). HR-MS (*m*/*z*, ES): calculated ($C_{11}H_{25}N_3S_2 \cdot H^+$)

264.1568; found 264.1577. Elemental anal.: calculated ($C_{11}H_{25}N_3S_2 \cdot 3HCl$) C 35.43% H 7.57% N 11.27%; found C 35.56% H 7.54% N 11.15%.

1,12-Bis[(4-methoxybenzyl)sulfanyl]-3,6,10-triazadodecane (Tris-hydrochloride Salt) (12). 1,6-Diamino-3-azahexane (0.32 mL, 2.6 mmol) was reacted according to general procedure A to give **12** as a white solid (703 mg, 1.28 mmol, 49%). Mp > 220 °C. ^{1}H NMR (D_2O, 400 MHz): δ 2.13 (br quint, 2H, $NHCH_2CH_2CH_2NH$), 2.81 (br t, 4H, $NHCH_2CH_2S$), 3.00−3.50 (m, 8H, $NHCH_2CH_2CH_2NH$ + $NHCH_2CH_2S$), 3.46 (s, 4H, $NHCH_2CH_2NH$), 3.70−3.85 (m, 10H, SCH_2Ar + CH_3O), 6.98 (br, 4H, Ar*H*), 7.33 (br, 4H, Ar*H*[3]). ^{13}C NMR (D_2O, 100 MHz): δ 23.0, 27.1, 27.2, 35.4, 43.6, 43.8, 44.9, 45.7, 47.3, 47.8, 56.4, 115.2, 130.8, 130.8, 159.0. HR-MS (*m*/*z*, +ve FAB): calculated ($C_{25}H_{39}N_3O_2S_2 \cdot H^+$) 478.2562; found 478.2576. Elemental anal.: calculated ($C_{25}H_{39}N_3O_2S_2 \cdot 3HCl$) C 49.41% H 6.85% N 7.52%; found C 49.47% H 6.94% N 7.43%.

3,6,10-Triazadodecane-1,12-dithiol (Tris-trifluoroacetate Salt) (TriN-2,3-DT). 1,12-Bis[(4-methoxybenzyl)sulfanyl]-3,6,10-triazadodecane (**12**) (550 mg, 0.984 mmol) was reacted according to general procedure B to give TriN-2,3-DT as a white powder (312 mg, 0.538 mmol, 55%). Mp > 220 °C. ^{1}H NMR (400 MHz, d_4-MeOH): δ 2.14 (quint, 2H, $NHCH_2CH_2CH_2NH$), 2.83 (m, 4H, CH_2SH) 3.10−3.35 (m, 8H, $NHCH_2CH_2CH_2NH$, $NHCH_2CH_2SH$), 3.47 (s, 4H, $NHCH_2CH_2NH$). ^{13}C NMR (400 MHz d_4-MeOH): δ 20.4, 20.4, 23.1, 43.5, 43.7, 44.7, 45.6, 50.5, 51.0. HR-MS (*m*/*z*, +ve FAB): calculated ($C_9H_{23}N_3S_2 \cdot H^+$) 238.1412; found 238.1418. Elemental anal.: calculated ($C_{11}H_{27}N_3S_2 \cdot 3C_2HF_3O_2$) C 31.09% H 4.52% N 7.25%; found C 30.97% H 4.60% N 7.42%.

1,2-Dithia-5,8,12-triazacyclobutadecane (Tris-hydrochloride Salt) (TriN-2,3-CDS). 3,6,10-Triazadodecane-1,12-dithiol (TriN-2,3-DT) (150 mg, 0.259 mmol) was reacted according to general procedure C to give TriN-2,3-CDS as a white powder (81 mg, 0.24 mmol, 92%). Mp > 220 °C. ^{1}H NMR (D_2O, 400 MHz): δ 2.17 (br quint, 2H, $NHCH_2CH_2CH_2NH$), 3.06 (br t, 4H, CH_2S), 3.25 (m, 4H $NHCH_2CH_2CH_2NH$), 3.45−3.55 (m, 8H, $NHCH_2CH_2S$ + $NHCH_2CH_2NH$). ^{13}C NMR (D_2O, 100 MHz): δ 23.3, 32.4, 43.7, 43.8, 45.1, 45.7, 46.6, 47.0. ES-MS (*m*/*z*): 236 (100%), 471 (58%). HR-MS (*m*/*z*, ES): calculated ($C_9H_{21}N_3S_2 \cdot H^+$) 236.1255; found 236.1261. Elemental anal.: calculated ($C_{11}H_{25}N_3S_2 \cdot 3HCl$) C 31.35% H 7.02% N 12.19%; found C 31.29% H 6.93% N 12.08%.

1,15-Bis[(4-methoxybenzyl)sulfanyl]-3,6,10,13-tetraazapentadecane (Tetra-hydrochloride Salt) (13). 3,7-Diazanonane-1,9-diamine (0.43 mL, 2.6 mmol) was reacted according to general procedure A to give **13** as a white solid (475 mg, 0.714 mmol, 23%). Mp > 220 °C. ^{1}H NMR (D_2O, 400 MHz): δ 2.18 (br quint, 2H, $NHCH_2CH_2CH_2NH$), 2.78 (t, 4H, J 6.5 Hz, $NHCH_2CH_2S$), 3.20−3.30 (m, 8H, $NHCH_2CH_2CH_2NH$ + $NHCH_2CH_2S$), 3.45 (s, 8H, $NHCH_2CH_2NH$), 3.79 (s, 4H, SCH_2Ar), 3.82 (s, 6H, OCH_3), 6.99 (d, 4H, J 8.5 Hz, Ar*H*), 7.35 (d, 4H, J 8.5 Hz, Ar*H*). ^{13}C NMR (D_2O, 100 MHz): δ 23.2, 27.2, 35.3, 43.6, 43.8, 45.7, 47.8, 56.4, 115.2, 130.8, 131.0, 159.0. HR-MS (*m*/*z*, ES$^+$): calculated ($C_{27}H_{44}N_4O_2S_2 \cdot H^+$) 521.2984; found 521.2981. Elemental anal.: calculated ($C_{27}H_{44}N_4O_2S_2 \cdot 4HCl$) C 48.65% H 7.26% N 8.40%; found C 48.51% H 7.16% N 8.49%.

3,6,10,13-Tetraazapentadecane-1,15-dithiol (Tetra-trifluoroacetate Salt) (TetraN-2,3,2-DT). 1,15-Bis[(4-methoxybenzyl)sulfanyl]-3,6,10,13-tetraazapentadecane (**13**) (300 mg, 0.450 mmol) was reacted according to general procedure B to give TetraN-2,3,2-DT as a white powder (77 mg, 0.10 mmol, 23%). Mp > 220 °C. ^{1}H NMR (400 MHz, d_4-MeOH): δ 2.21 (br quint, 2H, $NHCH_2CH_2CH_2$), 2.84 (t, J 7.0 Hz, CH_2SH), 3.10−3.40 (m, 8H, $NHCH_2CH_2CH_2NH$ + $NHCH_2CH_2SH$), 3.48 (s, 8H, $NHCH_2CH_2NH$). ^{13}C NMR (100 MHz, d_4-MeOH): δ 21.0, 24.4, 44.7, 44.9, 46.2, 51.9. HR-MS (*m*/*z*, ES): calculated ($C_{11}H_{28}N_4S_2 \cdot H^+$) 281.1834; found 281.1686. Elemental anal.: calculated ($C_{11}H_{28}N_4S_2 \cdot 4CF_3CO_2H$) C 30.98% H 4.38% N 7.61%; found C 30.90% H 4.33% N 7.56%.

1,2-Dithia-5,8,12,15-tetraazacycloheptadecane (Tetra-hydrochloride Salt) (TetraN-2,3,2-CDS). 3,6,10,13-Tetraazapentadecane-1,15-dithiol (TetraN-2,3,2-DT) (80 mg, 0.11 mmol) was reacted according to general procedure C to give TetraN-2,3,2-CDS as a white powder (38 mg, 0.090 mmol, 81%). Mp > 220 °C. ^{1}H NMR (D_2O, 400 MHz): δ 2.16 (br quint, 2H, $NHCH_2CH_2CH_2NH$), 3.06 (br t, 4H, CH_2S), 3.24 (m, 4H, $NHCH_2CH_2CH_2NH$), 3.40−3.60 (m, 8H, $NHCH_2CH_2NH$ + $NHCH_2CH_2S$). ^{13}C NMR (D_2O, 100 MHz): δ 23.6, 32.5, 43.90 (broad signal), 45.6, 47.0. HR-MS (*m*/*z*, ES): calculated ($C_{11}H_{26}N_4S_2 \cdot H^+$) 279.1677; found 279.1683. Elemental anal: ($C_{11}H_{26}N_4S_2 \cdot 4HCl$) C 31.14% H 7.13% N 13.20%; found 31.09% H 5.45% N 9.02%.

1,18-Bis[(4-methoxybenzyl)sulfanyl]-3,7,12,16-tetraazaoctadecane (Tetra-hydrochloride Salt) (14). Spermine (516 mg, 2.55 mmol) was reacted according to general procedure A to give **14** as a white solid (944 mg, 1.27 mmol, 50%). Mp > 220 °C. ^{1}H NMR (D_2O, 400 MHz): δ 1.79 (br, 4H, $NHCH_2CH_2CH_2CH_2NH$), 2.10 (br quint, 4H, $NHCH_2CH_2CH_2NH$), 2.79 (t, 4H, J 7.0 Hz, $NHCH_2CH_2S$), 3.00−3.20 (m, 16H, $NHCH_2$), 3.76 (s, 4H, SCH_2Ar), 3.79 (s, 6H, CH_3O), 6.95 (d, 4H, J 8.5 Hz, Ar*H*), 7.30 (d, 4H, J 8.5 Hz, Ar*H*). ^{13}C NMR (D_2O, 100 MHz): δ 23.0, 23.2, 27.2, 35.3, 45.0, 45.1, 47.3, 47.6, 56.4, 115.2, 130.7, 130.8, 159.0. HR-MS (*m*/*z*, ES): calculated ($C_{30}H_{50}N_4O_2S_2 \cdot H^+$) 563.3453; found 563.3438. Elemental anal.: calculated ($C_{30}H_{50}N_4O_2S_2 \cdot 4HCl$) C 48.35% H 7.44% N 7.52%; found C 48.55% H 7.61% N 7.35%.

Tetraazaoctadecane-1,18-dithiol (Tetra-trifluoroacetate Salt) (TetraN-3,4,3-DT). 1,18-Bis[(4-methoxybenzyl)sulfanyl]-3,7,12,16-tetraazaoctadecane (**14**) (750 mg, 1.01 mmol) was reacted according to general procedure B to give TetraN-3,4,3-DT as a white powder (507 mg, 0.651 mmol, 64%). Mp > 220 °C. ^{1}H NMR (400 MHz, D_2O): δ 1.74 (br, 4H, $NHCH_2CH_2CH_2CH_2NH$), 2.11 (br quint, 4H, $NHCH_2CH_2CH_2NH$) 2.83 (t, J 6.5 Hz, 4H, CH_2SH), 3.05−3.20 (m, 12H, $NHCH_2CH_2CH_2CH_2NH$ + $NHCH_2CH_2CH_2NH$), 3.26 (t, J 6.5 Hz, 4H, $NHCH_2CH_2SH$). ^{13}C NMR (100 MHz, D_2O): δ 20.4, 23.1, 23.3, 44.9, 45.0, 47.6, 50.5. HR-MS (*m*/*z*, +ve

FAB): calculated ($C_{14}H_{34}N_4S_2 \cdot H^+$) 323.2303; found 323.2311. Elemental anal.: calculated ($C_{14}H_{34}N_4S_2 \cdot 3C_2HF_3O_2$) C 33.93% H 4.92% N 7.20%; found C 34.06% H 5.02% N 7.16%.

1,2-Dithia-5,9,14,18-tetraazacycloeicosane (Tetra-hydrochloride Salt)] (TetraN-3,4,3-CDS). 3,7,12,16-Tetraazaoctadecane-1,18-dithiol (TetraN-3,4,3-DT) (150 mg, 0.193 mmol) was reacted according to general procedure C to give TetraN-3,4,3-CDS as a white powder (80 mg, 0.17 mmol, 89%). Mp > 220 °C. ^{1}H NMR (D_2O, 400 MHz): δ 1.77 (br, 4H, $NHCH_2CH_2CH_2CH_2NH$), 2.13 (br quint, 4H, $NHCH_2CH_2CH_2NH$), 3.00−3.20 (m, 16H, CH_2S + $NHCH_2$-$CH_2CH_2CH_2NH$ + $NHCH_2CH_2CH_2NH$), 3.47 (m, 4H, $NHCH_2CH_2S$).^{13}C NMR (D_2O, 100 MHz): δ 23.3, 23.4, 32.5, 45.1, 45.2, 46.5, 47.7. Each peak assigned in the ^{13}C NMR is 2 signals with almost identical chemical shifts. MS (*m*/*z*, ES): 321 (100%), 641 (14%). HR-MS (*m*/*z*, ES): calculated ($C_{14}H_{32}N_4S_2 \cdot H^+$) 321.2147; found 321.2151. Elemental anal.: calculated ($C_{11}H_{25}N_3S_2 \cdot 3HCl$) C 36.05% H 7.78% N 12.01%; found C 35.89% H 7.73% N 11.89%.

N^3,N^6-Di(tert-butoxycarbonyl)-1,8-dithioacetate-3,6-diazaoctane (11). PPh_3 (6.02 g, 22.1 mmol) was dissolved in dry THF (200 mL), and the resulting solution was cooled to 0 °C. Diisopropyl azodicarboxylate (4.52 mL, 22.1 mmol) was added to it, and the reaction mixture was stirred at 0 °C for a further 45 min, during which time a precipitate developed. N^3,N^6-Di(*tert*-butoxycarbonyl)-3,6-diazaoctane-1,8-diol (**10**) (1.75 g, 5.02 mmol) and thioacetic acid (1.64 mL, 22.1 mmol) were dissolved in dry THF (50 mL), and this solution was added to the previous suspension dropwise over 5 min. The resulting suspension was stirred at 0 °C for 1 h and then at ambient temperature for 15 h, during which time the suspension cleared to leave a yellow solution. This solution was concentrated under reduced pressure, and the crude product was then purified over silica (4:1, v:v, hexane: EtOAc) to give **11** as a white solid (1.53 g, 3.28 mmol, 65%). R_f = 0.29 (4:1, v:v, hexane:EtOAc). Mp = 76−78 °C. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 1.42 (s, 18H, $OCCH_3$), 2.28 (s, 6H, $SC(O)CH_3$), 2.90 − 3.10 (m, 4H, CH_2S), 3.20−3.35 (m, 8H, NCH_2). ^{13}C NMR ($CDCl_3$, 100 MHz): complex due to 4 possible rotamers present. HR-MS (*m*/*z*, CI^+): found 465.2097; calculated ($C_{20}H_{36}N_2O_6S_2 \cdot H^+$) 465.2093. Elemental anal.: found C 51.80% H 7.93% N 6.15%; calculated ($C_{20}H_{36}N_2O_6S_2$) C 51.70% H 7.81% N 6.03%.

3,6-Diazaoctane-1,8-dithiol (Bis-hydrochloride Salt) (DiN-2-DT). N^3,N^6-Di(*tert*-butoxycarbonyl)-1,8-dithioacetate-3,6-diazaoctane (**11**) (1.14 g, 2.54 mmol) was dissolved in concentrated HCl (50 mL), a process which caused effervescence to be observed. The resulting solution was heated at reflux for 1 h. The solvent was then removed under reduced pressure, and the resulting white powder was dried under reduced pressure to give DiN-2-DT as a white powder (543 mg, 2.14 mmol, 84%). Mp > 220 °C. ^{1}H NMR (D_2O, 400 MHz): δ 2.87 (t, J 6.5 Hz, 4H, CH_2SH), 3.34 (t, J 6.5 Hz, 4H, $NHCH_2CH_2SH$), 3.51 (s, 4H, $NHCH_2CH_2NH$). ^{13}C NMR (D_2O, 100 MHz): δ 20.5, 43.5, 51.0. HR-MS (*m*/*z*, ES): calculated ($C_6H_{16}N_2S_2 \cdot H^+$) 181.0833; found 181.0842. Elemental anal.: calculated ($C_6H_{16}N_2S_2 \cdot 2HCl$) C 28.46% H 7.16% N 11.06%; found C 28.55% H 7.05% N 10.95%.

1,2-Dithia-5,8-diazacyclodecane (Bis-hydrochloride Salt) (DiN-2-CDS). 1,8-Dithio-3,6-diazaoctane (DiN-2-DT) (150 mg, 0.592 mmol) was reacted according to general procedure C to give DiN-2-CDS as a white powder (136 mg, 0.541 mmol, 91%). Mp > 220 °C. ^{1}H NMR (D_2O, 400 MHz): δ 3.06 (t, J 6.5 Hz, 4H, CH_2S), 3.53 (m, 8H, $NHCH_2$). ^{13}C NMR (D_2O, 100 MHz): δ 32.4, 43.8, 47.0. HR-MS (*m*/*z*, ES): calculated ($C_6H_{16}N_2S_2 \cdot H^+$) 179.0677; found 179.0673. Elemental anal.: calculated ($C_6H_{14}N_2S_2 \cdot 2HCl$) C 28.68% H 6.42% N 11.15%; found C 28.80% H 6.36% N 11.00%.

Biological Assays. *Formation of Polyplexes.* A solution of pDNA (10 mg mL^{-1}) in the appropriate buffer was further diluted with the buffer before the appropriate amount of the cyclic disulfide solution (either 1 mg mL^{-1} or 0.1 mg mL^{-1} in the same buffer) was added. The resulting solution was shaken, centrifuged at 13,200 rpm for 5 s and then allowed to stand for 30 min before use.

Agarose Gel Electrophoresis. Standard Conditions. Polyplex solutions of various N/P ratios containing 500 ng of pDNA in 8 μL of 4 mM HEPES (pH = 7.4) were made up using the procedure described previously. These solutions were diluted with 8 μL of HEPES and then 4 μL of loading buffer and run for 1 h at 110 V through a 0.8% agarose gel plate containing 20 ng μL^{-1} of ethidium bromide. The resulting plate was visualized under UV light.

Reductive Conditions. Polyplex solutions of various N/P ratios containing 500 ng of pDNA in 8 μL of 4 mM HEPES (pH = 7.4) were made up using the procedure described previously. These solutions were diluted with 8 μL of a 200 mM DTT solution (in HEPES) and then 4 μL of loading buffer and run for 1 h at 110 V through a 0.8% agarose gel plate containing 20 ng μL^{-1} of ethidium bromide. The resulting plate was visualized under UV light.

PCS Measurements. Polyplex solutions of various N/P ratios containing 10 μg of DNA in 200 μL of 4 mM HEPES (pH = 7.4) were made up using the procedure described previously. Three readings were taken for each sample, and the results were expressed as the mean and standard deviation of these three readings.

Scanning Electron Microscopy. A polyplex solution containing 10 μg of pDNA was formed with TetraN-3,4,3 at an N/P ratio of 10 in 30 μL of 4 mM HEPES (pH = 7.4). This solution was applied to a standard SEM stub, and the solvent was evaporated off under ambient conditions. The stub was then gold plated using standard techniques and analyzed via SEM.

In Vitro Transfections. 50,000 cells were seeded in a 24-well plate and allowed to grow in 500 μL of serum containing medium until they were 90% confluent (usually 48 h). The medium was then removed, and each well was washed with 500 μL of serum free medium. 200 μL of Opti-MEM was then added to each well, followed by a 150 μL polyplex solution, each containing 2 μg of plasmid DNA at various N/P ratios, in 4 mM HEPES (pH = 7.4). The cells were

incubated at 37 °C for 6 h, after which time the polyplex solutions were removed and replaced with 500 μL of serum containing medium. The cells were incubated for a further 40 h. The medium was then removed from the cells, which were washed with 500 μL of PBS before 300 μL of 1× lysate buffer was added. After being shaken at ambient temperature for 30 min the lysate was removed, using vigorous means, from the wells and centrifuged at 13,200 rpm for 2.5 min. 50 μL of the lysate supernatant was analyzed for luciferase content, and 25 μL was analyzed for protein content using standard assays. Results were presented as relative light units (RLU) per mg of protein. *jet*PEI was used in ratios in accordance with the manufacturer's instructions in identical procedures to those described above using HEPES as the buffer. Each sample was analyzed in triplicate, allowing for the calculation of a mean and standard deviation for each one.

Cytotoxicity (LDH Assay). 20,000 cells were seeded in a 96-well plate and allowed to grow in 200 μL of serum containing medium until they were 90% confluent (usually 48 h). The medium was then removed, and each well was washed with 200 μL of serum free medium. 75 μL of Opti-MEM was then added to each well, followed by a 75 μL polyplex solution, each containing 1 μg of plasmid DNA at various N/P ratios, in 4 mM HEPES (pH = 7.4). In addition a blank sample, consisting of 75 μL of Opti-MEM and 75 μL of 4 mM HEPES, was also added to one set of cells to allow the background level of LDH released to be measured. The cells were incubated at 37 °C for 24 h, after which time the medium was removed, centrifuged at 13,200 rpm for 2.5 min and kept at 4 °C. The cells were then washed with 200 μL of PBS before 75 μL of 1× lysate buffer was added. After being shaken at ambient temperature for 30 min the lysate was removed, using vigorous means, from the wells, diluted with 75 μL of Opti-MEM and centrifuged at 13,200 rpm for 2.5 min. 50 μL from each of the medium samples and lysates was analyzed for LDH content according to the manufacturer's instructions. Prior to performing the colorimetric measurement for LDH content the spectrometer was zeroed using a mixture of ddH_2O and Optim-MEM (1:1, v:v). The % cell death was calculated as the amount of LDH released divided by total LDH content from the well. *jet*PEI was used in ratios in accordance with the manufacturer's instructions in identical procedures to those described above. Each sample was analyzed in triplicate, allowing for the calculation of a mean and standard deviation for each one.

Cytotoxicity (MTT Assay). 20,000 cells were seeded in a 96-well plate and allowed to grow in 200 μL of serum containing medium until they were 90% confluent (usually 48 h). The medium was then removed and each well was washed with 100 μL of PBS. 50 μL of DMEM was then added to each well, followed by a 50 μL polyplex solution, each containing 1 μg of plasmid DNA at various N/P ratios, in 4 mM HEPES (pH = 7.4). In addition cells treated with just DMEM (100 μL) were used as the positive control. The cells were incubated at 37 °C for 4 h before the polyplex solutions were removed and the cells washed with 100 μL of PBS before being incubated in serum-containing medium for a further 19 h. 20 μL of a 5 mg/mL solution of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was then added to each of the samples, which were then incubated at 37 °C for 5 h. The medium was removed, and 200 μL of DMSO was added to each well. The absorbance of each well at 550 nm was then measured and the % cell viability calculated as a percentage of the positive control. *jet*PEI was used in ratios in accordance with the manufacturer's instructions in identical procedures to those described above. Each sample was analyzed in triplicate, allowing for the calculation of a mean and standard deviation for each one.

Gene Delivery to Mouse Airways and β Gal Expression *in Vivo*. *Transfection Protocol.* The mice were anesthetized, using isoflurane, and polyplex solutions, containing 25 μg of pDNA in 100 μL of 5% glucose, were applied intranasally in one dose. After 24 h the mice were humanely sacrificed with an intraperitoneal injection of pentobarbital and their lungs removed. Those mice that were pretreated with *N*-acetylcysteine (NAC) had 100 μL of a 50 mM NAC solution in 5% glucose applied intranasally 30 min prior to the instillation of the polyplexes.

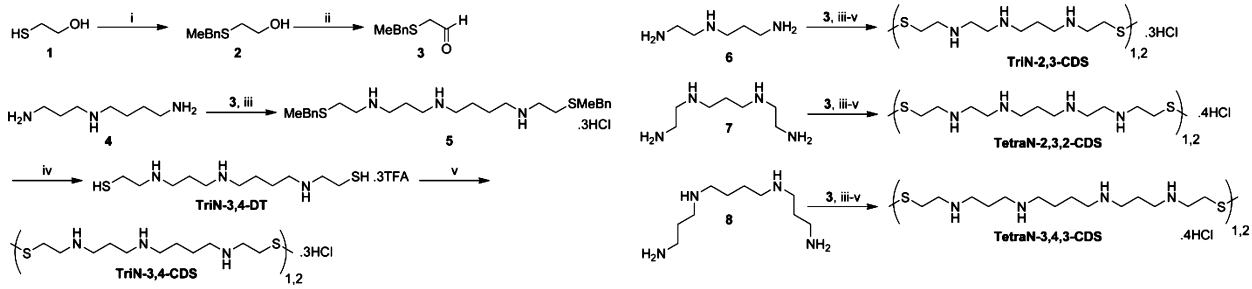
ELISA Assay. The lung tissue from above was placed in lysis buffer (1×) and homogenized on ice for 30 s before being lysed using three freeze−thaw cycles. The lysate was then centrifuged and the supernatant was analyzed for both β-gal and total protein content using standard assays.

Histology. The lung tissue originating from the left lobe was fixed by placing it into a 0.5% glutaraldehyde solution for 10 min. It was then washed repeatedly with PBS before being incubated for 24 h at 37 °C in an X-gal solution in order to stain it for β-gal. The tissue was then washed with PBS and dehydrated by sequential immersion in 70%, 95% and finally absolute ethanol. It was then cleared by soaking it in a mixture of benzyl benzoate and benzyl alcohol (2:1, v:v) and examined under a light microscope. Two separate histochemical analyses were then performed: OCT embedding after cryosectioning or paraffin embedding. For the former the samples were decleared via sequential immersion in 100% MeOH, MeOH in PBS and finally PBS. They were then infiltrated with 20% sucrose in PBS before being embedded in a mixture of OCT and 20% sucrose in PBS and frozen via immersion in isopentane, previously precooled with liquid N_2. Cryostat sections 9 μm thick were counterstained with eosin to provide the final sample to be analyzed. Other samples were embedded in paraffin and then cut into 4 μm sections, which were then counterstained with eosin. Both groups of samples were then analyzed for β-gal staining by direct light microscopy.

Results

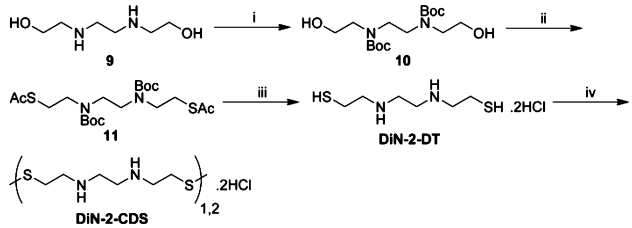
Synthesis of Dithiols and Cyclic Disulfides. The synthetic methodology developed to selectively difunctionalize terminal primary amines of tri- and tetraamines with 2-mercaptoethyl groups and their subsequent conversion to dis-

Scheme 1[a]



[a] (i) 4-Methoxybenzyl chloride, K_2CO_3, MeOH; 2 h; 96%. (ii) SO_3-pyridine, DMSO, DIPEA, DCM; 30 min; 52%. (iii) (a) $MgSO_4$, DCM, 8 h; (b) $NaBH_4$, DCM:EtOH (1:1, v:v); 15 h; (c) HCl, DCM:EtOH (1:1, v:v); 60 °C; 6 h; 49−52%. (iv) TFA; reflux; 3 days; 44−64%. (v) Concentrated HCl, DMSO; 6 h; 38−92%. Abbreviations: MeBn = 4-methoxybenzyl.

Scheme 2[a]



[a] (i) $(Boc)_2O$, $CHCl_3$; 5 h; 48%. (ii) PPh_3, DIAD, AcSH, THF; 0 °C−rt; 15 h; 65%. (iii) Concentrated HCl; reflux; 1 h; 84%. (iv) Concentrated HCl, DMSO; 6 h; 91%. Abbreviations: Boc = *tert*-butoxy carbonyl, Ac = acetyl.

ulfides is outlined in Scheme 1. After an extensive search of suitable reactions, reductive amination of spermidine with aldehyde **3** (synthesized according to previous literature example),[55] followed by extended reflux in trifluoroacetic acid, gave the desired dithiol, TriN-3,4-DT, in good yield. Facile oxidation of this species under high dilution, using dimethyl sulfoxide (DMSO) as the oxidant, furnished the corresponding cyclic disulfide, which was labeled TriN-3,4-CDS. In this nomenclature the prefix refers to the number of secondary amine functionalities between each disulfide bond and the numbers refer to the methylene spacings between them. The code finishing the label identifies the compound as either the dithiol (DT) or cyclic disulfide (CDS). Three further amines (**6**−**8**) were also successfully treated via the same methodology to give the corresponding dithiols and cyclic disulfides. ES-MS analysis of all four cyclic disulfides showed peaks corresponding to mono- and bicyclic species. No evidence for any higher order molecular weight cyclic oligomers or polymers was obtained. No further purification of these cyclic compounds was found to be necessary other than heating under reduced pressure to remove excess DMSO. This was undertaken to ensure that a small concentration of free thiols remained to assist the anticipated thiol−disulfide exchange reaction during pDNA condensation.

For the functionalization of diamines, an alternative route (Scheme 2) was sought due to our inability to successfully functionalize 1,2-ethylenediamine with aldehyde **3**. After Boc-protection of amine functional groups on diol **9**,[56] Mitsunobu displacement of the alcohols with thioacetate was performed according to a previously published procedure,[58] followed by universal deprotection in refluxing concentrated HCl giving DiN-2-DT as the dihydrochloride salt. Oxidation with DMSO resulted in the expected cyclic disulfide DiN-2-CDS. With all these compounds in hand, a series of pDNA condensation experiments were performed to assess whether either the dithiols or cyclic disulfides formed polyplexes suitable for gene delivery.

Polyplex Formation and Characterization. Initially, the pDNA condensing capacities of two spermidine derivatives, TriN-3,4-DT and TriN-3,4-CDS, were investigated by agarose gel electrophoresis and photon correlation spectroscopy (PCS). Polyplexes of varying N/P ratios were assembled by the addition of the cationic species to pDNA in 4 mM HEPES buffer (pH 7.4). Both compounds were found to fully condense pDNA at an N/P ratio of 2.5 (Figure 2). Addition of dithiothreitol (DTT) (100 mM, 1 h) was found to enable clean release of pDNA as illustrated by the reappearance of pDNA bands on the agarose gel for all N/P ratios where pDNA condensation had previously been observed.

Particle sizing of the polyplexes was determined by means of PCS (Figure 3a). The condensation of pDNA with TriN-3,4-DT resulted in the formation of large aggregates of well over 1000 nm in diameter that were not considered suitable for gene delivery. Pleasingly, however, polyplexes of between 150 and 250 nm were obtained when pDNA was condensed with TriN-3,4-CDS at an N/P ratio >2.5. Scanning electron microscopy (SEM) images of polyplexes formed with TetraN-3,4,3-CDS at an N/P ratio of 10 confirm the presence of such nanometric structures, circular in shape with diameters of around 200 nm (Figure 3b). Therefore, given these data, we came to the conclusion that the cyclic disulfide approach should be a more effective strategy for pDNA condensation than dithiols. Accordingly DiN-2-CDS, TriN-2,3-CDS, TriN-3,4-CDS, TetraN-2,3,2-CDS and TetraN-3,4,3-CDS were taken forward for further *in vitro* evaluation.

(58) Oiry, J.; Pue, J. Y.; Laval, J. D.; Fatome, M.; Imbach, J. L. Synthesis and Radioprotective Activity of WR-1065 Derivatives: N-(2-acetylthioethyl)-1,3-propanediamine and N, N′-bis(2-acetylthioethyl)-1,3-propanediamine. *Eur. J. Med. Chem.* **1995**, *30* (1), 47–52.

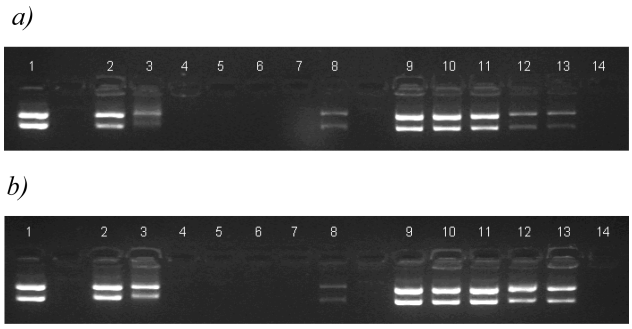


Figure 2. Agarose gels showing condensation of pDNA and its release under reductive conditions (100 mM DTT) for (a) TriN-3,4-DT and (b) TriN-3,4-CDS. Polyplex solutions containing 500 ng of pDNA were made up in 10 μL of 4 mM HEPES at various N/P ratios. After 30 min one set of samples was further diluted with 10 μL of 4 mM HEPES while the other set was diluted with 10 μL of 200 mM DTT. Following a further 30 min incubation the samples were run on a 0.8% agarose plate a 110 V for 1 h. Key: 1, naked pDNA; 2, N/P = 0.5; 3, N/P = 1; 4, N/P = 2.5; 5, N/P = 5; 6, N/P = 12.5; 7, deacetylated PEI N/P = 5; 8, spermine N/P = 25; 9, N/P = 0.5 (100 mM DTT); 10, N/P = 1 (100 mM DTT); 11, N/P = 2.5 (100 mM DTT); 12, N/P = 5 (100 mM DTT); 13, N/P = 12.5 (100 mM DTT); 14, deacetylated PEI N/P = 5 (100 mM DTT).

Gratifyingly, all four analogues based on tri- and tetraamine structures were able to condense pDNA into polyplexes with diameters of 150−250 nm (Table 1). Agarose gel electrophoresis showed that DiN-2-CDS was unable to condense pDNA even at an N/P ratio of 12.5 (data not shown), indicating that it was unlikely to be an effective vector; however, it was still tested *in vitro* to confirm this assumption.

In Vitro **Transfection.** The ability of the four cyclic disulfides to transfect both a CHO-K1 and an A549 cell line with a luciferase expressing pDNA was assessed in comparison with *jet*PEI, which was used in ratios in accordance with the manufacturer's guidelines[6] for each specific cell line. Polyplexes containing 2 μg of pEGFP-Luc-DNA were prepared at various N/P ratios in 4 mM HEPES and applied to cells for a period of 6 h at 37 °C. Serum-free conditions were used during this incubation as it has been reported previously that serum can lower the transfection efficacies of polyamine vectors such as PEI. This is probably due to its destabilizing effect on polyplexes,[13,59] although its effect on the cyclic disulfide's polyplexes was not tested and hence it is not known whether they would be similarly affected. The polyplex solutions were then removed and replaced with media, and the levels of luciferase expression were assessed after a further 40 h of cell incubation using standard assays (Figure 4).

(59) Lim, Y.-B.; Kim, S.-M.; Suh, H.; Park, J.-S. Biodegradable, Endosome Disruptive, and Cationic Network-Type Polymer as a Highly Efficient and Nontoxic Gene Delivery Carrier. *Bioconjugate Chem.* **2002**, *13* (5), 952–957.

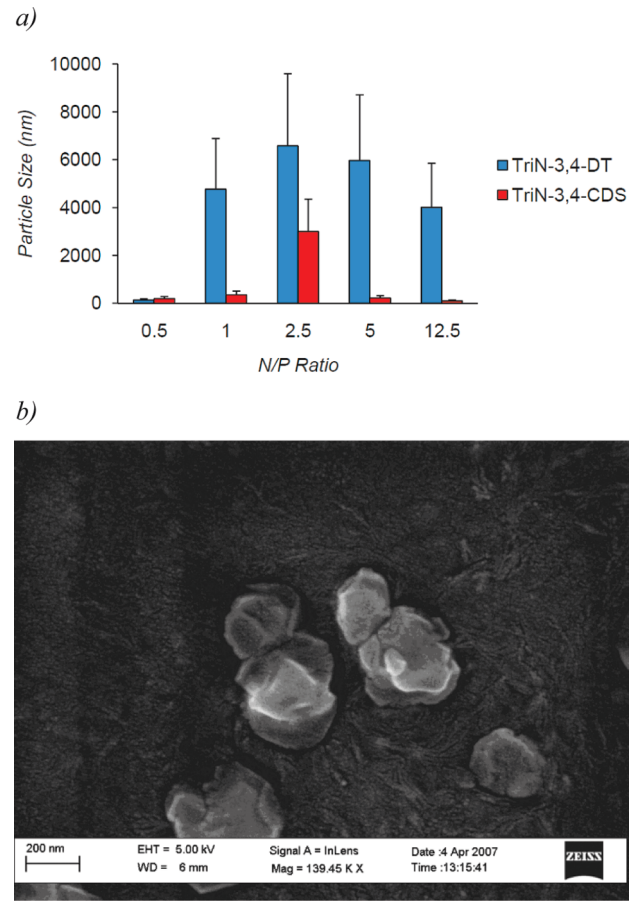


Figure 3. (a) PCS measurements of polyplexes formed from TriN-3,4-DT and TriN-3,4-CDS. Polyplexes containing 10 μg of pDNA in 200 μL of 4 mM HEPES buffer (pH = 7.4) were made up with TriN-3,4-DT and TriN-3,4-CDS at various N/P ratios and measured by PCS. (b) SEM images of polyplexes formed in 4 mM HEPES from pDNA and TetraN-3,4,3-CDS at N/P ratio of 10.

Table 1. Particle Sizes of Polyplexes Formed with TriN-2,3-CDS, TriN-3,4-CDS, TetraN-2,3,2-CDS and TetraN-3,4,3-CDS as Measured by PCS

	0.5	1	2.5	5	12.5
TriN-2,3	312 ± 126	282 ± 119	195 ± 77	201 ± 78	128 ± 51
TriN-3,4	193 ± 86	351 ± 160	3000 ± 1350	221 ± 92	101 ± 38
TetraN-2,3,2	291 ± 129	260 ± 112	2163 ± 966	204 ± 76	197 ± 79
TetraN-3,4,3	388 ± 163	2191 ± 984	118 ± 46	200 ± 80	149 ± 60

When tested on CHO-K1 cells (Figure 4a), at N/P ratios of 5 and above, the four analogues based upon tri- and tetraamine backbones were able to mediate levels of transfection to within an order of magnitude of *jet*PEI. In contrast, DiN-2-CDS was unable to mediate transfection above background levels. For each of the four efficient analogues the levels of transfection rose to a maximum before falling again as the N/P ratio was increased further, presumably due to the increasing cytotoxicity of the vector. There is very

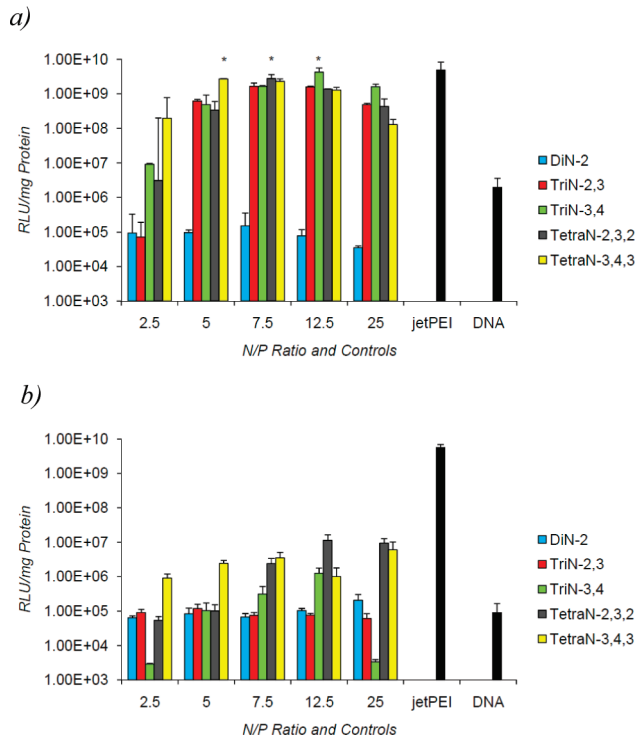


Figure 4. Transfection of (a) CHO-K1 and (b) A549 cells with luciferase pDNA using cyclic disulfides. Naked pDNA and *jet*PEI were used as negative and positive controls respectively. Polyplex solutions, made up in a mixture of 4 mM pH 7.4 HEPES and serum-free medium, containing 2 μg of luciferase pDNA were incubated with the cells for 6 h before being replaced with serum-containing medium for a further 40 h. The cells were then lysed, and the luciferase and protein content of the lysates was measured (CHO-K1: for cyclic disulfides $n = 3 \pm$ standard deviation; *jet*PEI $n = 12 \pm$ standard deviation; DNA $n = 12 \pm$ standard deviation. A549: for cyclic disulfides $n = 4 \pm$ standard deviation; *jet*PEI $n = 12 \pm$ standard deviation; DNA $n = 20 \pm$ standard deviation).The optimal N/P ratio for each cyclic disulfide was compared to *jet*PEI using Welch's *t* test ($p = 0.05$), and those cyclic disulfides able to transfect at levels statistically indistinguishable from *jet*PEI are marked with *.

little to choose between the optimal performance of any of the tri- and tetraamine analogues in this cell line, in contrast to the results obtained when A549 cells were used (Figure 4b). Here the two tetraamine analogues TetraN-2,3,2-CDS and TetraN-3,4,3-CDS mediated optimal levels of transfection at least half an order of magnitude higher than the tri- and diamines. Noticeably this cell line proved harder to transfect with the cyclic disulfides, as their performance remained orders of magnitude beneath that of *jet*PEI. These results demonstrate that small molecule cyclic disulfides with tri- and tetraamine backbones can successfully transfect both A549 and CHO-K1 cells significantly above background levels and, in the case of CHO-K1 cells, to within half an order of magnitude of *jet*PEI, currently considered the most effective commercial cationic polymeric vector.

Cytotoxicity. The cytotoxicity of the five cyclic disulfides, along with *jet*PEI, was measured using a standard lactate dehydrogenase (LDH) release assay. LDH is a cytoplasmic enzyme that is released into medium due to cell death, hence the LDH assay provides a sensitive method to assess cytotoxicity. The cytotoxicity of one analogue (TetraN-3,4,3-CDS) was also measured using the MTT assay, however this assay was judged to be less sensitive than the LDH one and hence was not used for all analogues (see Supporting Information). Polyplexes containing 1 μg of pEGFP-Luc-DNA were prepared at various N/P ratios in 4 mM HEPES and applied to cells, previously grown to 90% confluence, for a period of 24 h at 37 °C. It is conceivable that the high confluence of the cells might reduce the cytotoxic effect of the polyplexes, however such an effect should apply equally to all experiments and hence any differences observed should be the direct result of the characteristics of the polyplexes themselves. Covering media were then removed from the cells in each well, which were then independently lysed. Analysis of the LDH content of both the media and lysate was performed using a standard kit, and the level of cell death was calculated by taking the LDH content of the media as a percentage of the total LDH content measured in a corresponding untreated population of cells in a well (Figure 5).

When tested on CHO-K1 cells both analogues based upon a propylene/butylene inter-nitrogen spacing (i.e., TriN-3,4-CDS and TetraN-3,4,3-CDS) showed no cytotoxicity levels above background at N/P ratios of 5 and 7.5, at which ratios optimal transfection was also mediated. This compares favorably with *jet*PEI, which caused the release of 53 ± 14% of the LDH, significantly above background levels (21 ± 7%) thereby demonstrating that the bioreducible vectors employed here are able to alleviate some of the cytotoxicity associated with PEI. When higher N/P ratios were tested, it was noted that TetraN-3,4,3-CDS proved to be marginally more toxic than TriN-3,4-CDS, perhaps indicating a negative consequence of raising the number of intradisulfide amines. In comparison, two of the cyclic disulfides based upon ethylene/propylene inter-nitrogen spacings (DiN-2-CDS and TriN-2,3-CDS) exhibited high cytotoxicity even at the relatively low N/P ratios (5 and above). This cytotoxicity could be obviated by preparing the polyplexes in a stronger buffer solution (40 mM HEPES, data shown in Supporting Information) to buffer the greater acidity of these polymers compared with TriN-3,4-CDS and TetraN-3,4,3-CDS. Interestingly TetraN-2,3,2-CDS, despite its ethylene/propylene spacings, exhibited no such extreme toxicity for reasons which are currently unclear. Similar overall trends were observed with A549 cells although these were far less susceptible to polyamine induced damage. Indeed even *jet*PEI did not provoke any LDH release significantly above background levels.

The cytotoxicity of TetraN-3,4,3-CDS, one of our most successful compounds in terms of mediating high levels of transfection at low N/P ratios with no associated cytotoxicity, was also assessed as the free material in direct comparison

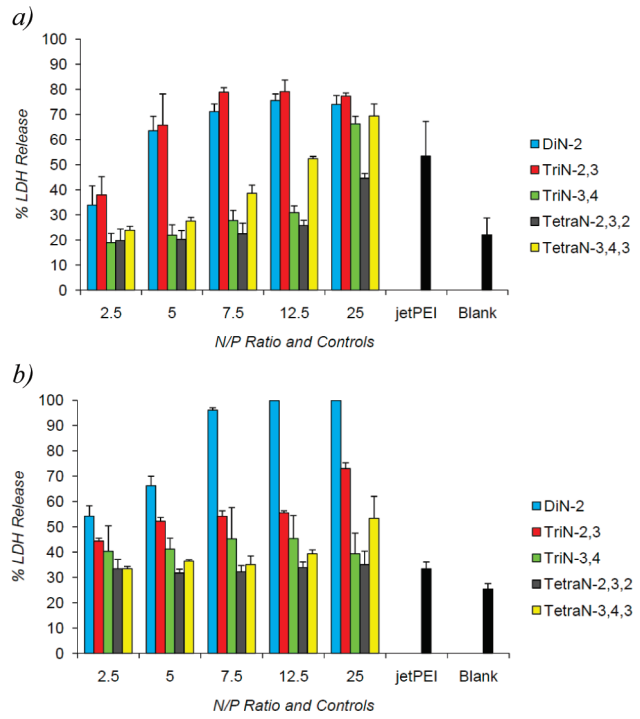


Figure 5. Cytotoxicity caused by polyplexes containing cyclic disulfides to (a) CHO-K1 and (b) A549 cells assessed via LDH release assay. Blank solutions of buffer and medium (1:1, v:v) and *jet*PEI were used as negative and positive controls respectively. Polyplex solutions, made up in a mixture of 4 mM pH 7.4 HEPES and serum-free medium (1:1, v:v), containing 1 μg of luciferase pDNA were incubated with the cells for 24 h prior to measurement of LDH content of both media and cell lysate ($n = 3$, $\pm$ standard deviation). (CHO-K1: for cyclic disulfides $n = 3 \pm$ standard deviation; *jet*PEI $n = 24 \pm$ standard deviation; blank $n = 24 \pm$ standard deviation. A549: for cyclic disulfides $n = 4 \pm$ standard deviation; *jet*PEI $n = 12 \pm$ standard deviation; DNA $n = 24 \pm$ standard deviation).

to *jet*PEI by means of the same LDH assay (Figure 6). Happily TetraN-3,4,3-CDS exhibited a markedly better cytotoxicity profile than *jet*PEI, causing minimum cell death without toxicity when equivalent levels of *jet*PEI resulted in near 90% cell death (0.02 mg mL^{-1}). In order to ensure that this effect was not the result of TetraN-3,4,3-CDS's lower density of amines, the results were also converted to a function of amine concentration (Figure 6b) and, although less pronounced, the less cytotoxic nature of TetraN-3,4,3-CDS was still evident.

Gene Transfection into the Mouse Airways *in Vivo*. The two most promising candidates identified during the *in vitro* tests, TetraN-3,4,3-CDS and TetraN-2,3,2-CDS, were taken forward for *in vivo* transfection analysis. PEI has proved to be a successful vector for transfection of the lung when instillated intranasally,[60] marking it out as having great potential for the treatment of lung disorders such as cystic fibrosis. Hence we decided to compare our two lead compounds to *in vivo* *jet*PEI in this situation. Polyplexes were formulated initially with TetraN-3,4,3-CDS and *in vivo*

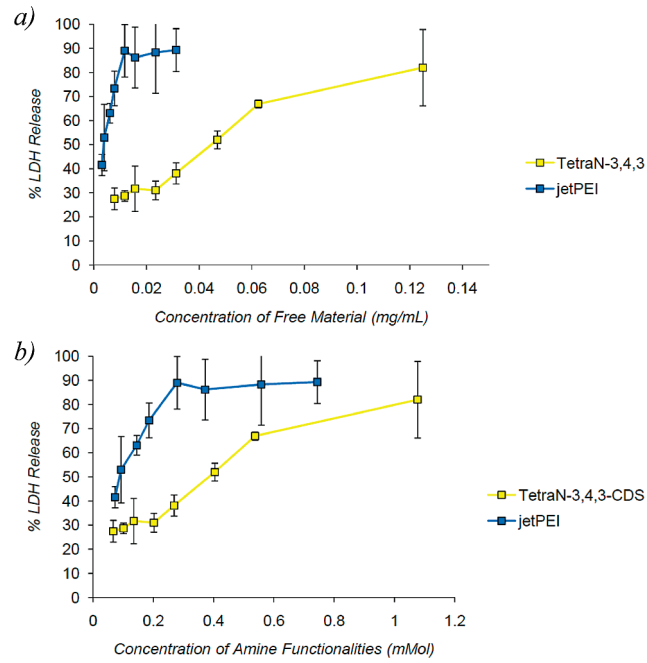


Figure 6. Cytotoxicity of *jet*PEITM and TetraN-3,4,3-CDS to CHO-K1 cells as free materials measured by LDH release assay. Free material was diluted to the appropriate final concentrations with a mixture of 4 mM HEPES and Opti-MEM (150 μL, 1:1, v:v) and then incubated with the cells for 24 h prior to the measurement of LDH content of both media and cell lysate ($n = 3$, $\pm$ standard deviation). (a) Results presented as a function of concentration of free material (mg/mL). (b) Results presented as a function of the concentration of amine groups (mmol).

*jet*PEI in 5% glucose, the recommended solvent for *in vivo* *jet*PEI, at various N/P ratios with a β-galactosidase (β-gal) expressing pDNA and delivered intranasally to the lungs of female BALB/c mice. At 24 h postinstillation, the murine lungs were removed and analyzed for β-gal expression using standard assays. The amount of β-gal expressed (ng of β-gal protein/100 mg of protein) was then calculated, and the results obtained are shown in Figure 7 (see Supporting Information for the individual results for each mouse). While no significant β-gal expression levels were observed in nontreated mice, a significant level was clearly measurable in the lung homogenates of mice treated with polyplexes formed with TetraN-3,4,3-CDS at both N/P ratios of 5 and 8 (respectively 1.663 ± 0.3976, $n = 10$, and 1.608 ± 0.3180, $n = 5$), with the highest activity being observed at N/P of 5. Nevertheless, these β-gal levels were still lower than those obtained with *in vivo* *jet*PEI (3.059 ± 0.2644, $n = 15$), hence we sought an adjuvant that might improve the performance of the cyclic disulfide. *N*-Acetylcysteine (NAC) is a major component of Nacystelyn, an agent widely used in the clinic to alter mucus properties.[61] It is also known to be a precursor

(60) Wiseman, J. W.; Goddard, C. A.; McLelland, D.; Colledge, W. H. A Comparison of Linear and Branched Polyethylenimine (PEI) with DCChol/DOPE Liposomes for Gene Delivery to Epithelial Cells *In Vitro* and *In Vivo*. *Gene Ther.* **2003**, *10*, 1654–1662.

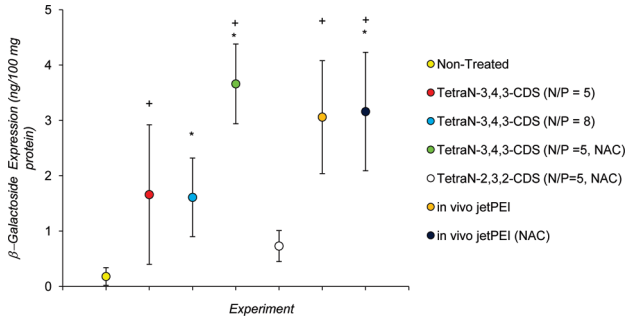


Figure 7. Summary of β-Gal expression from murine lung following intranasal instillation of polyplexes constructed with TetraN-3,4,3-CDS and *jet*PEI. Polyplexes containing 25 μg of pDNA in 100 μL of 5% glucose were introduced intranasally into female BALB/c mice. Twenty-four hours later the mice were sacrificed and the β-Gal levels in their lungs measured via a β-Gal ELISA assay. The groups of mice which were pretreated with *N*-acetylcysteine (marked as NAC) were perfused intranasally with 100 μL of a 50 mM NAC solution 30 min prior to the instillation of the polyplex solutions. Those groups marked with + achieved levels of transfection significantly higher than the nontreated mice (ANOVA, Bonferroni corrected, $p < 0.05$) and those marked with * were statistically indistinguishable from *jet*PEI (ANOVA, Bonferroni corrected, $p > 0.05$).

of glutathione,[49] hence we postulated that it might be able to aid a reductively active vector such as TetraN-3,4,3-CDS. Mice were perfused with NAC (100 μL, 50 mM in 5% glucose) intranasally 30 min prior treatment with the polyplexes, significantly increasing β-gal expression with TetraN-3,4,3-CDS (3.662 ± 0.3228, $n = 5$) to the levels exhibited by *in vivo jet*PEI, which itself was unaffected by pretreatment with NAC. TetraN-2,3,2-CDS was subsequently tested, using the NAC pretreatment, however it was unable to match TetraN-3,4,3-CDS, mediating levels of transfection that were significantly lower (0.733 ± 0.279, $n = 5$).

Histological analysis of lung tissues transfected with β-gal using either TetraN-3,4,3-CDS or *jet*PEI as a vector was then performed to precisely localize the transfected areas. Figure 8a shows an *in toto* x-Gal staining performed on the whole left lobes extracted from untreated mice and those treated with either TetraN-3,4,3-CDS or *jet*PEI. A strong blue staining is observed in both TetraN-3,4,3-CDS- and *jet*PEI-treated tissues, while only a light staining is seen in untreated mice. Subsequently histological analysis of the murine lung tissues was performed using two separate staining protocols, namely OCT embedding after cryosectioning and paraffin embedding. In both cases eosin counterstaining was used to aid the identification of the blue β-gal expressing areas

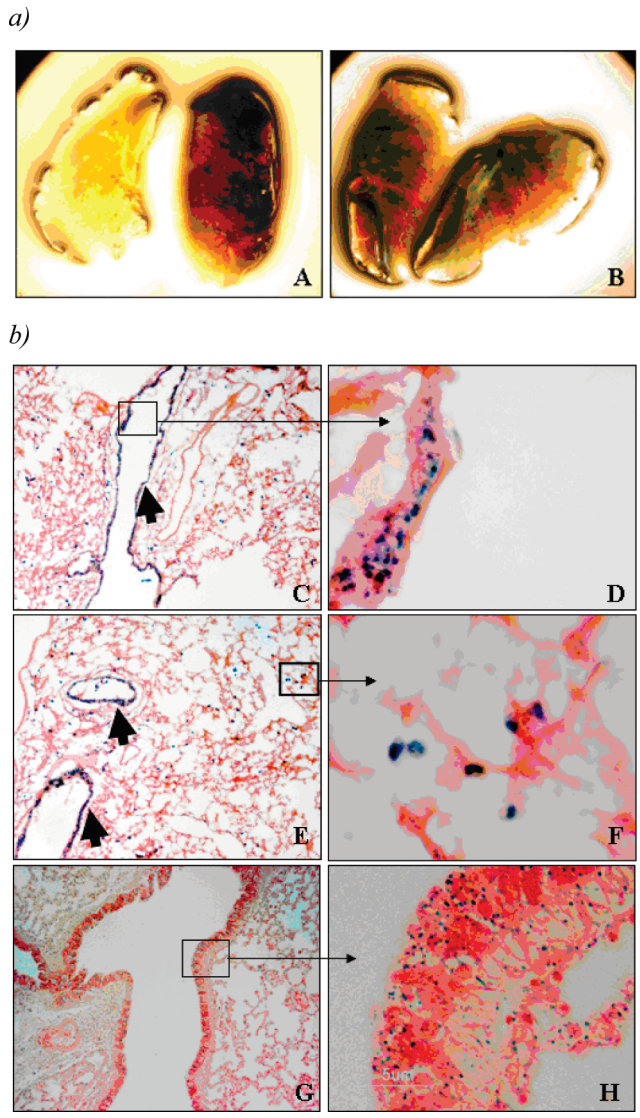


Figure 8. Histological analysis of lung tissue transfected with β-gal using either TetraN-3,4,3-CDS or *jet*PEI as a vector. (a) β-Gal expression in the mouse lungs after an *in toto* x-Gal staining of the whole left lobe: A-left, nontreated;A-right,TetraN-3,4,3-CDS;B-left,TetraN-3,4,3-CDS + NAC; B-right, *jet*PEI. (b) Localization of blue-stained cells using lung tissues produced either by cryosectioning followed by OCT-embedding procedure (C to F) or via paraffin treatment (G to H). Scale bars: 5 μm.

(Figure 8b). Both methods highlighted the successful transfection achieved with TetraN-3,4,3-CDS, with close examination revealing that the major sites of transfection, marked with arrowheads in Figure 8b, were airway epithelia from the bronchioles and bronchioli (C, D). Evidence was also observed for the transfection of macrophages and possibly pneumocytes in the parenchymal area (E, F), however the significant levels of background expression found in these cells do not allow for firm conclusions to be made on this point.

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Discussion

The success of gene therapy is dependent on the development of safe, efficient vectors. Cationic polyamines, especially PEI, have long been considered as promising, however to date their progress toward the clinic has been hampered by concerns over their toxicity and a lack of efficacy when compared to viruses. This paper has described the synthesis and biological characterization of several small molecule cyclic disulfide vectors that were designed to respond to the presence of pDNA by forming bioreducible poly(disulfide)s via a TCTP. The most effective analogues were able to effect high levels of transfection, statistically indistinguishable from *jet*PEI, currently one of the leading commercially available synthetic vectors, under certain conditions both *in vitro* and *in vivo*. Crucially they also exhibited a markedly improved cytotoxicity profile compared to *jet*PEI, potentially making them more viable for future use in the clinic.

The cyclic disulfides were synthesized from the analogous dithiols under high dilution in order to ensure that they remained small molecules, with ES-MS showing the presence of only mono- and bicyclics. Such species should be ineffectual vectors due to their low molecular weight, however we postulated that the introduction of pDNA would initiate a TCTP resulting in the formation of suitable poly(disulfide)s which would subsequently be able to effect pDNA condensation. It was considered that the addition of pDNA to a solution of a cyclic disulfide, buffered at a pH of 7.4, would act in the same way as the introduction of a template molecule to a dynamic combinatorial library in that it creates a new energy minimum for the system to access. It has been established that the process of pDNA condensation by polycationic species is entropically driven, with the entropic term being a combination of both the favorable release of small molecule counterions and the putative effect of bimolecular combination.[62,63] Considering a range of molecular weights from small molecule cyclic disulfides to high molecular weight polymers, it can be seen that the favorable release of counterions is constant, however the bimolecular combination penalty paid is greater the lower the molecular weight of the polycation. In response to this entropic driving force the system will strive to reduce this penalty via the rearrangement of the small molecule cyclics into high molecular weight polymers. All the biological data obtained for the cyclic disulfides points to the presence of high molecular weight poly(disulfide)s upon the addition of pDNA as both pDNA condensation and successful transfection were mediated at low N/P ratios (2.5 and 5 respectively for TriN-3,4-CDS). When exposed to strongly reductive conditions (100 mM DTT) complete pDNA release was observed for all the cyclic disulfides, demonstrating clearly that the analogous monomeric species were unable to effect effective pDNA condensation. Taken together the performance of these vectors provides excellent evidence for the proposed TCTP, although to date no direct evidence of its occurrence has been obtained. Dithiols were also assessed as potential gene delivery vectors, however despite proving able to condense pDNA at low N/P ratios via a kinetically controlled polymerization (KCP) the resultant polyplexes were large aggregates of the order of thousands of nanometers in diameter, in contrast to the discrete polyplexes of between 100 and 250 nanometers formed with the cyclic disulfides, indicating that they were not suitable vectors.

Molecular weight is known to be a key determinant of a polymer's characteristics and has been demonstrated to impact both PEI's efficacy and its cytotoxicity. But, despite its prominence in determining the success of a polymeric vector, it is a factor that remains under poor synthetic control with difficulties remaining in achieving consistency between separate batches of the same compound. These issues are resolved by the use of a small molecule such as the cyclic disulfides, which can be consistently and reproducibly produced due the high dilutions employed during their synthesis. In addition structure−activity relationships can be proposed without concerns that they might be influenced by differing molecular weights. This is not to say that the final molecular weight distributions of the condensing species are identical as it is likely that each cyclic disulfide will respond to the introduction of pDNA uniquely, however such differences are inherent properties of the cyclic disulfides themselves rather than the consequence of synthetic variations.

The range of cyclic disulfides accessible using the synthetic methodology described allowed for various structure−activity relationships to be probed. Two variables were of particular interest: first the number of amines between each disulfide and second the buffering capacity of the amines. This second factor was influenced by varying the inter-nitrogen spacing, which has a direct effect of the acidity of the protonated amines[64] with shorter spacings resulting in more acidic protons. The effect of employing more acidic protons should be an increased buffering capacity at physiological pH, a factor that has been suggested to be crucial to PEI's ability to affect endosomal escape via the so-called "proton sponge hypothesis"[65,66] and hence mediate high levels of transfection.

The transfection data from the five analogues synthesized allows us to draw several conclusions. The most obvious is that at least three secondary amines between each disulfide

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bond are required to mediate any transfection above that shown by naked pDNA. DiN-2-CDS, based upon an ethylenediamine structure, proved unable to transfect either cell line investigated, a result that was expected due to its inability to condense pDNA even at an N/P ratio of 12.5. However those structures based upon a tri- and tetraamine structure successfully mediated transfection at N/P ratios of 5 and above. There was little to choose between the performances of these vectors in CHO-K1 cells, with the optimal levels of transfection being similar across all four analogues. At the highest N/P ratio tested, 25, falls in efficacy were noted for each vector, which was ascribed to their toxicity beginning to overwhelm the cells. Pleasingly the optimal levels of transfection achieved were within an order of magnitude of *jet*PEI for each of these four analogues, demonstrating their efficacy for this cell line. When examined using Welch's *t* test (2-tailed) no significant difference was found between *jet*PEI and either TriN-3,4-CDS, TetraN-2,3,2-CDS or TetraN-3,4,3-CDS ($p > 0.05$), showing that we had succeeded in matching the efficacy of the current industrial gold standard. In contrast when tested on A549 cells the cyclic disulfides mediated levels of transfection that were orders of magnitude beneath those of *jet*PEI. However this cell line, possibly as it proved to be harder to transfect, did highlight some interesting differences between the vectors. Both tetraamine derivatives proved to be more efficacious vectors than their triamine analogues, mediating optimal levels of transfection that were significantly higher. Lengthening the number of amine functionalities between each disulfide should allow for the formation of more stable polyplexes, one possible explanation for the improved transfections observed. In addition it is also conceivable that lowering the density of disulfides renders the polyplex less susceptible to reductive degradation, allowing it to persist in the cytoplasm for a longer period of time and hence perhaps allowing it to deliver its cargo closer to the cell's nucleus. While this may be beneficial for the transfection, the LDH assays on the CHO-K1 reveal that this might be hazardous in terms of cytotoxicity with TetraN-3,4,3-CDS provoking higher levels of LDH release than TriN-3,4-CDS at N/P ratios of 7.5 and 12.5. Again such a relationship might be expected as a higher number of amines would result in increased interactions with cellular membranes, potentially leading to greater damage and hence the observed rise in cytotoxicity.

In asking whether the buffering capacity can influence the efficacy of the cyclic disulfide for gene delivery it is instructive to examine any differences between TriN-2,3-CDS and TriN-3,4-CDS and between TetraN-2,3,2-CDS and TetraN-3,4,3-CDS. When considering their optimal levels of transfection across the two cell lines the only discernible difference is TriN-3,4-CDS's greater efficacy in A549 cells. It seems reasonable to assign the relatively poor performance of TriN-2,3-CDS in this situation to the fact that it will be less charged than TriN-3,4-CDS, potentially rendering it unable to form polyplexes of sufficient stability to transfect this cell line. The only other trend of note is that TetraN-3,4,3-CDS mediates its highest levels of transfection at lower N/P ratios compared to TetraN-2,3,2-CDS, an observation that again can be explained by its greater charge at physiological pH. The lack of any evidence for an advantage to shorter amine spacings leads to the conclusion that buffering capacity is not a dominant factor in the performance of these vectors. However when considering the cytotoxicity of the polyplexes, it is striking that both DiN-2-CDS and TriN-2,3-CDS are far more cytotoxic than the other analogues. The fact that this relatively extreme cytotoxicity can be improved by raising the strength of the buffer used to form the polyplexes to 40 mM HEPES shows that this difference is simply due to incomplete buffering of the more acidic amines from these compounds rather than any inherent feature of the polyamine structure itself, however as we would prefer to employ lower strength buffers *in vivo* this would be a considerable disadvantage for these analogues. Precisely why such an effect was not observed for TetraN-2,3,2-CDS is not clear at present, although the clear implication is that the pK_a values for its amine are higher than those of DiN-2-CDS and TriN-2,3-CDS.

Many of the structure−activity trends described above correlate well with those already reported in the literature. Of particular interest is the work of Reineke and co-workers on glycopolyamines, wherein the number of secondary amines between the sugar groups and their basicity is varied.[67,68] They noted a distinct increase in transfection upon raising the number of amines between the glycol units, an observation in line with our data. Lee et al. noted a similar trend when they tested various poly(ethylene sulfide)s, with those polymers with six or eight inter-disulfide nitrogens showed higher transfection than those with either two or four.[42] Other groups have also investigated this parameter, however their results are clouded somewhat by the varying degrees of branching produced by their synthetic routes, which introduces another factor which must be taken into account.[40,41] Reineke et al. also found no correlation between buffering capacity and the ability to transfect cells,[68] indeed their analogues based upon spermine demonstrated far higher transfection than similar analogues incorporating ethylene spacings despite their lower buffering capacities. Similar results were also reported by Zhang et al. during their work on poly(phosphoramidate)s.[69] Forrest et al. varied the degree of acetylation of PEI in order to control its buffering capacity, observing an increase in transfection upon lowering the

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ability of PEI to absorb protons at physiological pH.[70] However, while these results demonstrate that buffering capacity is not always a crucial factor, it should be noted that when the buffering capacity of PEI is completed ablated, as shown by Thomas et al. when they per-alkylated PEI, transfection does indeed suffer significantly.[71] It is also instructive to note that PL's transfection efficiency can be raised via the incorporation of histidine residues able to buffer at physiological pH.[72] Collectively these results, along with our own, strongly suggest that while it is crucial for a vector to possess some buffering capacity, it is frequently not the dominant factor that determines efficacy. Indeed recent research has cast considerable doubt upon the validity of the proton sponge hypothesis by suggesting that those polyplexes that become transfectionally active are taken up into the cell via neutral caveosomes,[73,74] rendering the endolytic ability of PEI irrelevant to its efficacy.

The improvement in cytotoxicity for the cyclic disulfides compared to *jet*PEI was highlighted for both the polyplexes and the free materials. When tested in CHO-K1 cells *jet*PEI provoked the release of around 50% of their LDH, significantly higher than the background levels. In contrast, at N/P ratios at which they were able to achieve statistically indistinguishable levels of transfection (Welch's t test, $p = 0.05$), neither TetraN-2,3,2-CDS nor TetraN-3,4,3-CDS showed any evidence of cytotoxicity despite the long incubation times used (24 h). This improvement was also evident when the *jet*PEI and TetraN-3,4,3-CDS were compared as free materials, with far higher concentrations of the cyclic disulfide being tolerated before any LDH release above background was observed. This effect could not be ascribed solely to the lower density of amine functionalities found in TetraN-3,4,3-CDS as when the results were presented as a function of this variable its cytotoxicity remained markedly lower implying that its bioreducible nature was central to its improved performance.

Having established that cyclic disulfides were able to mediate transfection *in vitro* we progressed onto testing their efficacy *in vivo*, specifically for transfection of murine lungs with a β-gal containing plasmid after intranasal administration. Pleasingly TetraN-3,4,3-CDS, used at an N/P ratio of 5, proved able to effect levels of transfection that were significantly above nontreated mice (ANOVA analysis, Bonferroni corrected, $p = 0.12$), although still below those exhibited by *in vivo jet*PEI. However when the mice were pretreated with NAC, the levels of β-gal expression observed with TetraN-3,4,3-CDS rose significantly to levels that were statistically indistinguishable from our positive control (ANOVA analysis, Bonferroni corrected, $p = 1.0$). Interestingly no such response was seen with *in vivo jet*PEI, indicating that the effect may have been the result of the temporary raising of glutathione levels in the lung rather than the mucolytic properties of NAC. TetraN-2,3,2-CDS was also tested, however even after pretreatment with NAC it did not perform as well as TetraN-3,4,3-CDS or *jet*PEI. Histological analysis of the transfected tissue showed that the major sites of transfection were airway epithelial cells in the bronchioles and bronchioli. These cells are the target for the treatment of cystic fibrosis, highlighting the potential of TetraN-3,4,3-CDS to be used for this purpose.

In conclusion we were able to develop synthetic methodology to synthesize small molecule cyclic disulfides, able to form stable, biologically active polyplexes via a TCTP on pDNA, from a range of di-, tri- and tetraamines. Those analogues based upon tri- and tetraamine structures were able mediate successful transfection, at the same level as *jet*PEI in CHO-K1 cells, while remaining essentially nontoxic. While they were not as effective when tested in A549 cells, our lead compound, TetraN-3,4,3-CDS, was able to match the ability of *jet*PEI to transfect murine lungs if used after pretreatment with NAC. Initial structure−activity investigations suggested that the buffering capacity of these species is unlikely to have a profound effect of their ability to transfect cells, however the number of amines between the disulfides may prove to be crucial. Further investigations are needed though before any precise relationships can be settled upon. In addition the ability of the cyclic disulfides to transfect cells effectively in the presence of serum, which has been reported to reduce the efficacy of polyamine vectors such as PEI, might be tested to ascertain whether they are able to retain their performance under such conditions. The efficacy shown by TetraN-3,4,3-CDS in transfecting the airway epithelia of the murine lungs paves the way for it to be examined in more detail as a potential vector for the treatment of cystic fibrosis, one of the major targets for the gene therapy community.

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Supporting Information Available: Additional data on LDH, MTT and β-Gal expression assays. This material is available free of charge via the Internet at http://pubs.acs.org.

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