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Synthesis and Characterisation of Polyamine–Poly(ethylene glycol) Constructs for DNA Binding and Gene Delivery

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Abstract—Improved non-viral vector systems are needed for efficient delivery of DNA to target cell nuclei in gene therapy. A series of linear polyamine–poly(ethylene glycol) (PEG) constructs has been synthesised by reaction of appropriately Boc-protected thermine derivatives with ω-methoxyPEG oxiranylmethyl ethers. Constructs carrying 1–3 MeOPEG units and 0, 2 or 4 *N*-methyl groups have been prepared by this method. $H_2N(CH_2)_3NBoc(CH_2)_3NBoc(CH_2)_3NHBoc$ was prepared efficiently by mono-trifluoroacetylation of thermine, attachment of Boc and removal of the trifluoroacetyl group in one pot. A similar process gave $H_2N(CH_2)_3NBoc(CH_2)_3NBoc(CH_2)_3NH_2$. $BocMeN(CH_2)_3NHMe$ was alkylated by 1,3-dibromopropane to give $BocMeN(CH_2)_3NMe(CH_2)_3NMe(CH_2)_3NMeBoc$. A cyanoethylation/reduction sequence extended $H_2N(CH_2)_3NBoc(CH_2)_3NBoc(CH_2)_3NH_2$ to give $H_2N(CH_2)_3NBoc(CH_2)_3NBoc(CH_2)_3NBoc(CH_2)_3NBoc(CH_2)_3NH_2$, which was converted to its mono- and di-MeOPEG550 derivatives. Deprotection gave the linear polyamine–MeOPEG constructs. A branched triamine–poly(ethylene glycol) construct was prepared by acylation of $(BocHN(CH_2)_3)_2N(CH_2)_3NH_2$ with ω-methoxyPEG 550 chloroformate, followed by deprotection. A cyanoethylation/reduction/protection sequence from $(H_2N(CH_2)_3)_2$ $N(CH_2)_3NHBoc$ gave a protected pentamine. Alkylation with $Br(CH_2)_5CONH(CH_2)_2NHBoc$, deprotection, acylation with MeOPEG chloroformate and deprotection gave a pentamine–MeOPEG construct in which the MeOPEG is attached through a linker to the central amine. The linear hexamine construct carrying MeOPEG550 at only one terminus was the most effective DNA-interactive member of the two series in an ethidium displacement assay and was effective in delivering a reporter gene to RIF-1 tumours.

Introduction

Gene therapy is of growing interest in the therapy of several diseases. In some diseases, there is a defect in a specific gene and the therapeutic strategy is to supply a gene that will lead to biosynthesis of the appropriate functional protein. In some other cases, the strategy is to deliver a gene that encodes a protein that is not missing or dysfunctional in the patient but which would provide a different therapeutic benefit. For example, genes encoding toxic proteins may be introduced into tumour cells. The Gene Directed Enzyme Prodrug Therapy (GDEPT) approach is also being developed[1] for cancer therapy; in GDEPT, a gene that encodes a prodrug-activating enzyme is introduced (e.g., herpes simplex virus thymidine kinase, which activates ganciclovir by phosphorylation). Genes that render malignant cells more sensitive to cytotoxic therapy or which protect normal cells against drug toxicity have also been proposed.[2] Stimulation of anti-tumour immune responses through gene therapy has been investigated.[3,4]

One of the major limiting factors in the development of gene therapy strategies is the unavailability of efficient and selective delivery of DNA to the nucleus of the target cells. Current delivery vectors (systems for delivery of DNA) are broadly divided into two types: viral and non-viral. Approximately 70% of current clinical trials use viral gene delivery and the majority of these use retroviruses. Although, at present, viral vectors appear to be more efficient than non-viral systems, there are a number of problems associated with their use, including immunogenicity, transient duration of gene expression, low capacity for foreign genes and difficulties in generation of sufficiently high viral titres.[5–7] Non-viral vectors fall broadly into two classes: cationic lipids and cationic polymers (such as poly-L-Lys (PLL) and polyethyleneimine (PEI)). Both bind electrostatically to the polyanionic DNA, provide charge neutralisation and cause condensation of DNA. Several cationic lipids and lipid–polyamine constructs are available for use as transfection agents to

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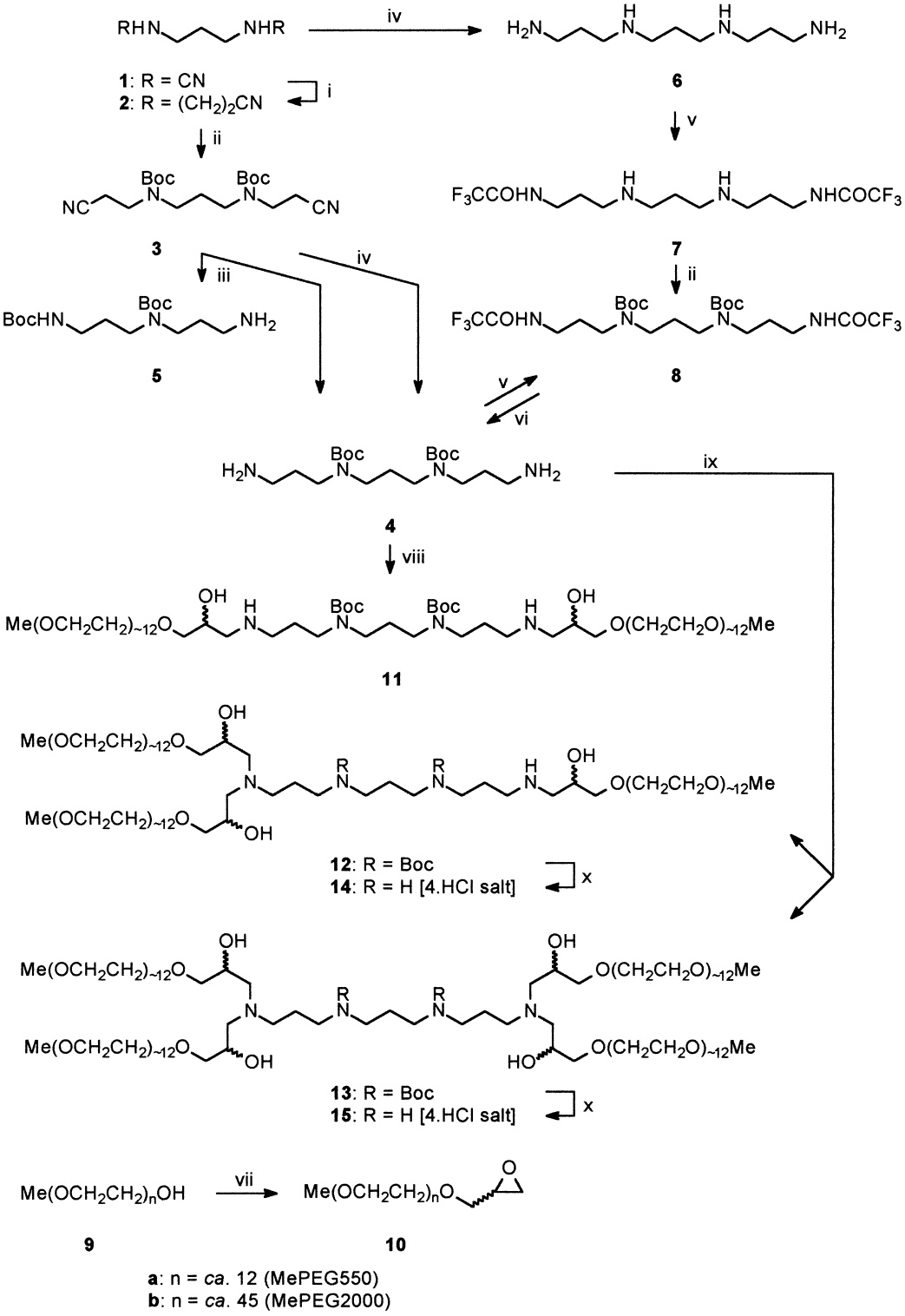
carry DNA across cell membranes in vitro. These include DOTMA,[8] DOTAP, DOGS, DOSPA, DC-Chol[9] and CTAP.[10] However, there are a number of problems with lipidic systems. Firstly, complexes of cationic lipids and DNA tend to aggregate in aqueous solutions, particularly in the presence of salts or proteins,[11] thus only small amounts of DNA can be formulated in this way. After intravenous injection these hydrophobic particles typically activate complement;[12] they are oposonised and subsequently rapidly cleared by the liver and spleen.[13,14] PLL–DNA complexes also activate complement; however, surface modification of preformed PLL–DNA



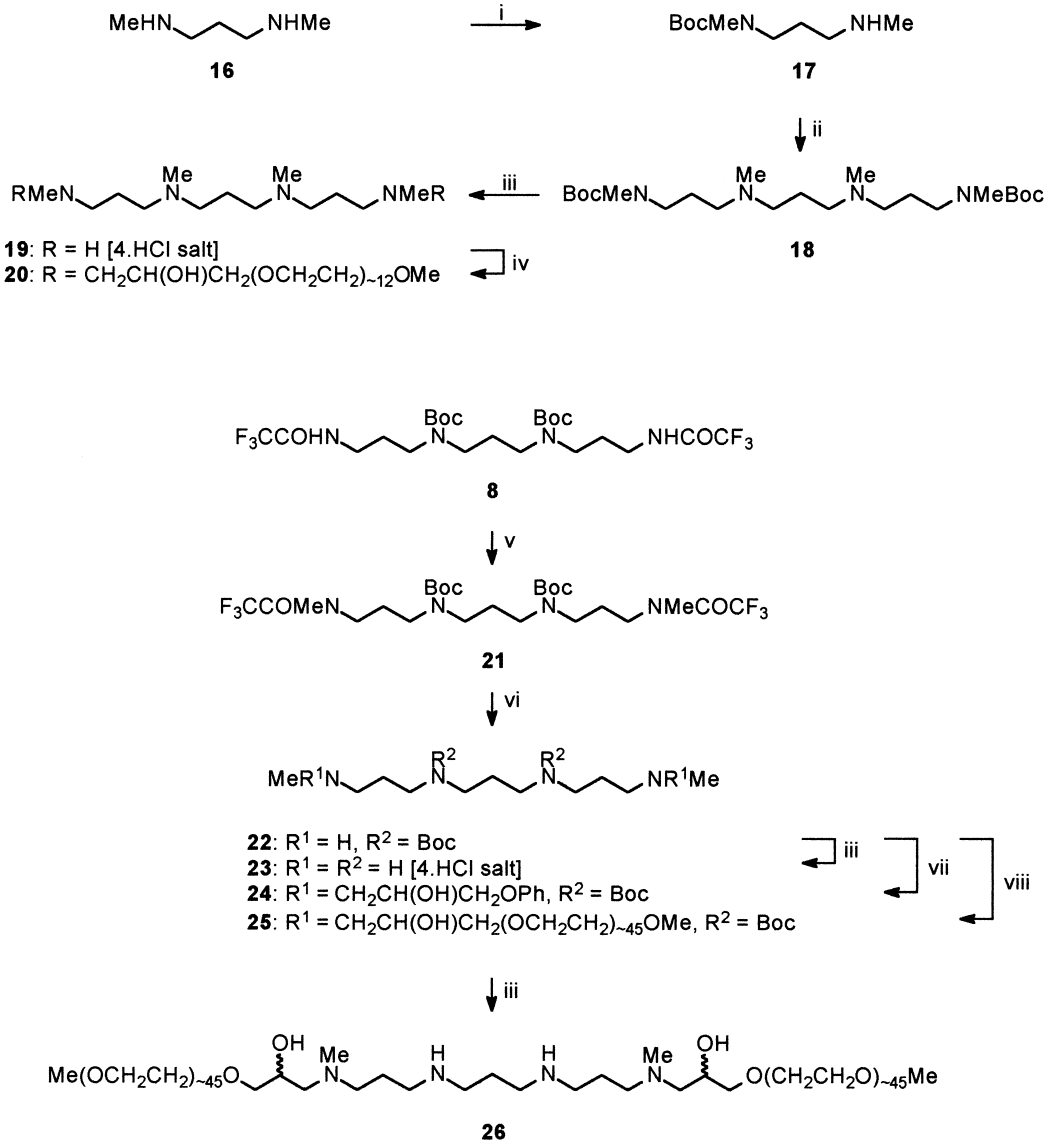
Scheme 1. Synthesis of tetramine–MeOPEG constructs **14** and **15**. Reagents: (i) propenenitrile, MeOH; (ii) Boc_2O, CH_2Cl_2; (iii) $LiAlH_4$, Et_2O, THF; (iv) H_2, Raney Ni, MeOH, NH_3; (v) CF_3CO_2Et, MeOH; (vi) NH_3, MeOH; (vii) chloromethyloxirane, NaOH, H_2O; (viii) **10a**, Pr^iOH, Δ, 24 h; (ix) **10a**, Pr^iOH, Δ, 24 h; (x) HCl, CH_2Cl_2.

complexes with PEG considerably reduces complement activation.[12] Secondly, lipidic systems are unsuitable for injection into solid tissues such as tumour and muscle where dispersion of the formulation is thought to be important;[13] the transfection efficiency of these 'lipoplexes' is low when injected directly into tumours, in comparison to that of naked DNA.[15] Thirdly, lipidic complexes are relatively unstable.[13,14]

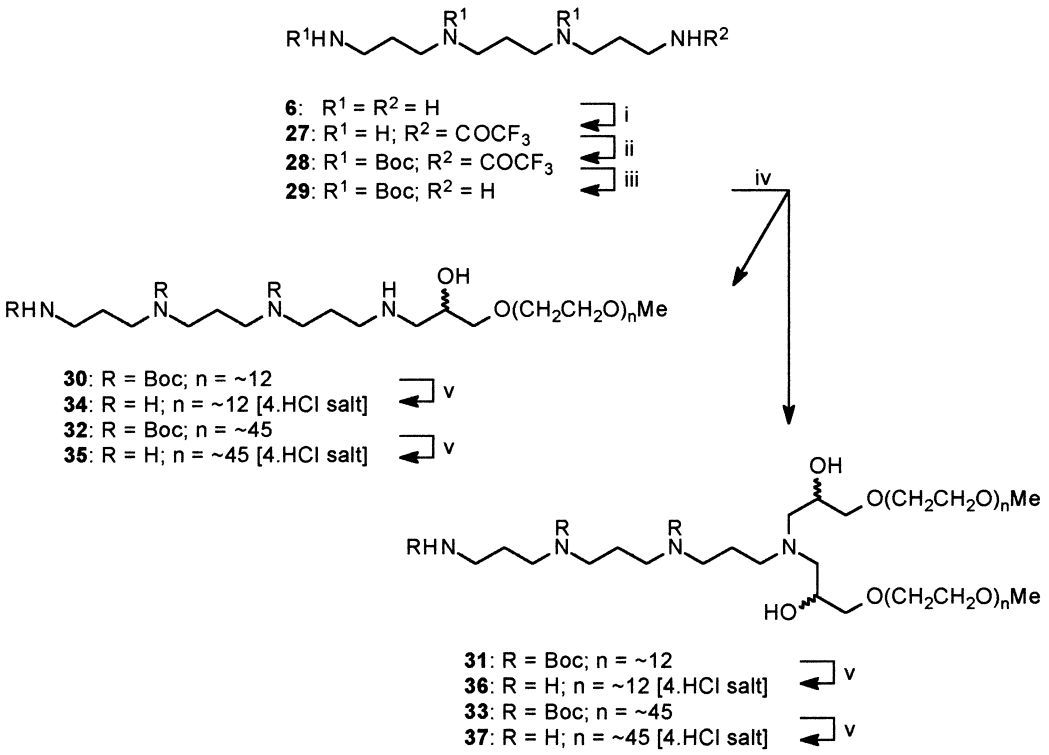
Thus, there has been increasing interest in water-soluble complexes, as opposed to lipidic systems.[16–22] These systems typically consist of a polycation linked to a hydrophilic polymer. For delivery to solid tissues (muscle and solid tumours), complete charge neutralisation and condensation of DNA does not appear to be a requirement, as the neutral hydrophilic polymers poly(1-vinylpyrrolidin-2-one) (PVP) or poly(vinyl alcohol) (PVA)[20,21] appear to be active as gene transfer agents in these tissues.[3,20–22] These are the so-called Protective Interactive Non-Condensing (PINC) polymers. The aim of the work presented in this paper was to synthesise a family of polyamine–poly(ethylene glycol) constructs which would be capable of forming hydrophilic complexes with DNA, coating the DNA with a sheath of poly(ethylene glycol) (PEG) chains. PEG is also non-immunogenic and relatively non-toxic.[23] Results of our study on the binding of the constructs to DNA are also presented, along with those of our preliminary study on the effectiveness of two constructs for gene delivery in vivo.

Design and Chemical Synthesis of the Constructs

The simplest design of a PEG–polyamine construct would be to allow a PEG-derived electrophile to react with the nucleophilic terminal amino groups of a linear polyamine. N^1,N^3-Bis(3-aminopropyl)propane-1,3-diamine ('thermine', **6**, Scheme 1) was selected as the



Scheme 2. Synthesis of tetramethyltetramine–MeOPEG construct **20** and dimethyltetramine–MeOPEG construct **26**. Reagents: (i) Boc_2O, THF; (ii) $Br(CH_2)_3Br$, K_2CO_3, DMF; (iii) HCl, CH_2Cl_2; (iv) NaOH, **10a**, Pr^{i}OH; (v) KOBu^t, MeI, THF; (vi) NH_3, MeOH; (vii) phenoxymethyloxirane, Pr^{i}OH; (viii) **10b**, Pr^{i}OH.



Scheme 3. Synthesis of "one ended" tetramine–MeOPEG constructs **34**–**37**. Reagents: (i) CF_3CO_2Et, EtOH; (ii) Boc_2O, EtOH; (iii) NH_3, MeOH; (iv) **10a**,**b**, Pr^{i}OH, Δ; (v) HCl, CH_2Cl_2.

initial polyamine, as it has the potential to carry up to four positive charges at physiological pH to provide good interaction with the polyanionic DNA. Furthermore, alkane-α,ω-diamines having three or five CH_2 units between the amines are reported[24] to be considerably more effective in inducing DNA compaction than were other alkane-α,ω-diamines. Boc protection of the secondary amines was necessary to ensure that only the terminal primary amines reacted with the PEG-derived electrophile; this protection should also be readily removable under conditions which do not destroy the PEG, giving co-products which are easily separable from the oligomeric highly polar target constructs. The required protected polyamine **4** was accessed through two routes (Scheme 1). In the first route, treatment of propane-1,3-diamine **1** with propenenitrile gave the dinitrile **2** quantitatively.[25] The secondary amines were then protected as their Boc derivatives in **3**, in the usual way. McCormick et al.[26] report that selective reduction of the nitrile groups of **3** can be achieved using $LiAlH_4$ at 0 °C to give the diBoc-tetramine **4** in 50% yield. In our laboratory, these conditions gave a poor yield (22%) of the desired diBoc-tetramine **4**, the major product (37%) being the diBoc-triamine **5**. Formation of **5** can be rationalised in terms of a retro-Michael reaction of the substrate **4** or an intermediate, initiated by highly basic AlH_4^-. However, reduction of the nitriles was achieved by Raney-Ni-catalysed hydrogenation under forcing conditions in ammonia-saturated methanol to afford **6**. In the second route, reduction of the nitriles (of **2**) was carried out before selective protection of the secondary amines. Catalytic hydrogenation under similar conditions gave thermine **6**. Xu et al.[27] developed conditions for selective trifluoroacetylation of primary amines in the presence of secondary amines using ethyl trifluoroacetate in THF at low temperature, the selectivity arising from steric factors. Application of a modified procedure gave the di-TFA-protected tetramine **7**. This material was difficult to purify; immediate reaction with di-*t*-butyl dicarbonate afforded the orthogonally protected tetramine **8** in high yield. Removal of the trifluoroacetyl groups with ammonia gave the desired diBoc-tetramine **4** quantitatively. The sequence **1**→**2**→**6**→**7**→**8**→**4** thus represents the most efficient route to provide **4** which is now available for reaction with a suitable PEG-derived electrophile.

The PEG unit for most of the constructs was PEG550 monomethyl ether (MeOPEG550, **9a**, a mixture of oligomers of mean MW 550 Da). A specific requirement in converting the alcohol of **9a** into a suitable electrophile for reaction with **4** is that the basicity of all four nitrogen atoms in the construct should be uncompromised, to permit formation of a tetracation for efficient binding to DNA under physiological conditions. Oxiranes react readily with primary amines to form 2-hydroxyalkyl secondary amines; reaction with secondary amines gives 2-hydroxyalkyl tertiary amines. We recently used[28,29] α,ω-bis(oxiranylmethyl)PEGs as electrophiles in the assembly of cathepsin B-degradable polymers for delivery of drugs and imaging agents. MeOPEG550 **9a** was converted to its oxiranylmethyl ether **10a** by treatment with epichlorohydrin; MeOPEG2000 **9b** was converted[‡]

[‡]Experiment performed by Dr. S. E. Matthews, Institute of Macromolecular Chemistry, Czech Academy of Sciences, Prague, Czech Republic. Present address: Inorganic Chemistry Laboratory, Department of Chemistry, University of Oxford, South Parks Road, Oxford OX1 3QR, UK. Compound **10b** was a kind gift from Dr. Matthews.

similarly to **10b**. In a preliminary experiment, **4** reacted with two equivalents of **10a** in boiling propan-2-ol to give crude bis-adduct **11**. Longer reaction times led to formation of the tris-adduct **12** (11%) and the tetrakis-adduct **13** (24%), which were isolable by chromatography; both were deprotected, giving the constructs **14** and **15**, respectively. Clearly, the addition of the bis-primary amine to the oxirane is not a clean reaction and the secondary β-hydroxyalkylamine of the intermediate **11** is capable of further nucleophilic addition to oxirane.

Our solution to this problem was to arrange the polyamine to have secondary amines at the termini, so that the addition reaction could proceed once only at each site (Scheme 2). The tetramines **19** and **22** were therefore designed as appropriate polyamines for reaction with MeOPEG oxiranylmethyl ethers. The former will give rise to a PEG–polyamine construct in which all four amines are methylated, whereas the construct derived from the latter will have less sterically demanding secondary amines in the 'inner' positions. Treatment of excess N^1,N^3-dimethylpropane-1,3-diamine **16** with di-*t*-butyl dicarbonate[30] gave the mono-Boc derivative **17**. The tetramine was then assembled by alkylation of **17** with 1,3-dibromopropane, giving **18** in good yield. Deprotection (giving salt **19**) and reaction with **10a** cleanly afforded the desired bis-PEG construct **20** after chromatographic purification. As tertiary amines, the 'inner' amines were unreactive towards the oxirane and thus did not require protection/deprotection. In contrast, the ''inner'' (secondary) amines did require protection in polyamine derivative **22**, prior to reaction with oxiranes. The dianion generated from the trifluoroacetamide termini of **8** reacted with iodomethane to afford the protected α,ω-dimethyltetramine **21**, from which the trifluoroacetyl groups were readily removed. The resulting diprotected dimethyltetramine **22** was treated with phenoxymethyloxirane as a model, giving the bis-adduct **24**. Using the same conditions, the required bis-MeOPEG adduct **24** was prepared and deprotected, giving pure **26** in high yield, uncontaminated by polyamines bearing more or less MeOPEG units. Compound **22** was deprotected to provide **23** as a specimen of this parent polyamine for comparative DNA-binding studies.

The above constructs carry MeOPEG units at both ends of the polyamine. To investigate whether enhanced binding to DNA may be achieved by having one end of the polyamine sterically unimpeded, a series of constructs was assembled in which MeOPEG was attached to only one end of the tetramine. The tetramine for this part of the study was **6**, to minimise possible steric crowding in binding. This requires the tetramine to be Boc protected at the two secondary amines and at one of the terminal primary amines (as in **29**, Scheme 3), prior to reaction with MeOPEG oxiranylmethyl ethers. McCormick et al.[26] have prepared **29** in moderate yield by careful treatment of the diBoc-tetramine **4** with di-*t*-butyl dicarbonate. However, it was more efficient to adapt a one-pot synthesis[31] of tri-Boc-spermine to reaction of **6** with one equivalent of ethyl trifluoroacetate at low temperature, giving the crude mono-trifluoroacetamide **27**, which was treated in situ with di-*t*-butyl dicarbonate to introduce the three Boc groups in **28**. Selective deprotection gave the tri-Boc-thermine **29**. The sole exposed amine in **29** is primary and thus may add twice to MeOPEG-derived oxiranes. Indeed, treatments of **29** with single equivalents of **10a** and **10b** gave a separable mixture of the mono-adducts **30** (21%) and **32** (35%), respectively, and of the bis-adducts **31** (9%) and **33** (28%), respectively. Reaction with two equivalents of **10a** increased the isolated yield of **31** to 61%. Figure 1 shows the MALDI-TOF mass spectrum of **33**; the peaks correspond to $[M + Na]^+$ for the PEG oligomers. Deprotection afforded the adduct salts **34**–**37**.

Constructs **11**, **14**, **15**, **20**, **26** and **34**–**37** represent variations in the number, size and location of the MeOPEG units and introduction of various numbers of N-Me groups while retaining the core linear tetramine. Constructs **42** and **45** (Scheme 4) are analogous to **34** and **11**, respectively, but have a hexamine core. This core, in which the basic nitrogens are still linked by $(CH_2)_3$ units, has the potential to exist as a hexacation and thus bind more strongly to the polyanionic DNA. Hexamines of this type are commercially unavailable; thus a suitably tetra-Boc-protected hexamine **40** had to be assembled by extension from a tetramine. Regioselective cyanoethylation of **6** and Boc protection of intermediate **38** gave **39**. Hydrogenation of the nitriles afforded the tetra-Boc-hexamine **40**. Prolonged reaction with one equivalent of **10a** gave a moderate yield of the mono-adduct **41**, whereas the lower reactivity of this larger protected polyamine was evident in the poor yields of the bis-adduct **43** (12%) and the tris-adduct **44** (6%) on treatment with two equivalents of the oxirane. Deprotection gave quantitatively the corresponding mono- and bis-MeOPEG–hexamine constructs **42**, **45**. Thus the set of linear PEG–polyamine constructs with variation in length and number of PEG chains, number of cationic sites and sterically demanding *N*-methylation was complete.

In an alternative design strategy, the PEG unit is attached to a point in the centre of the polyamine, rather than at a terminus as in **45**. This has the potential advantage of leaving the terminal primary amines of the

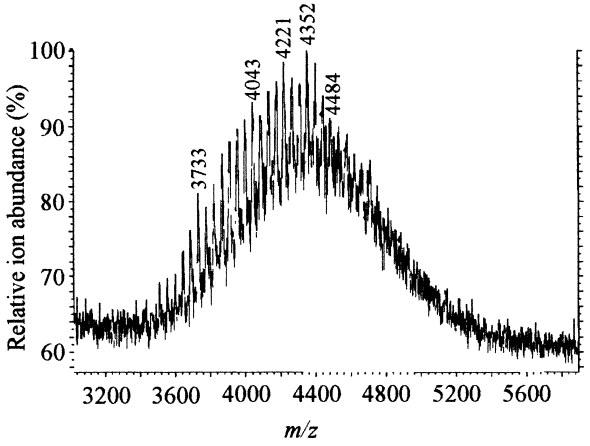
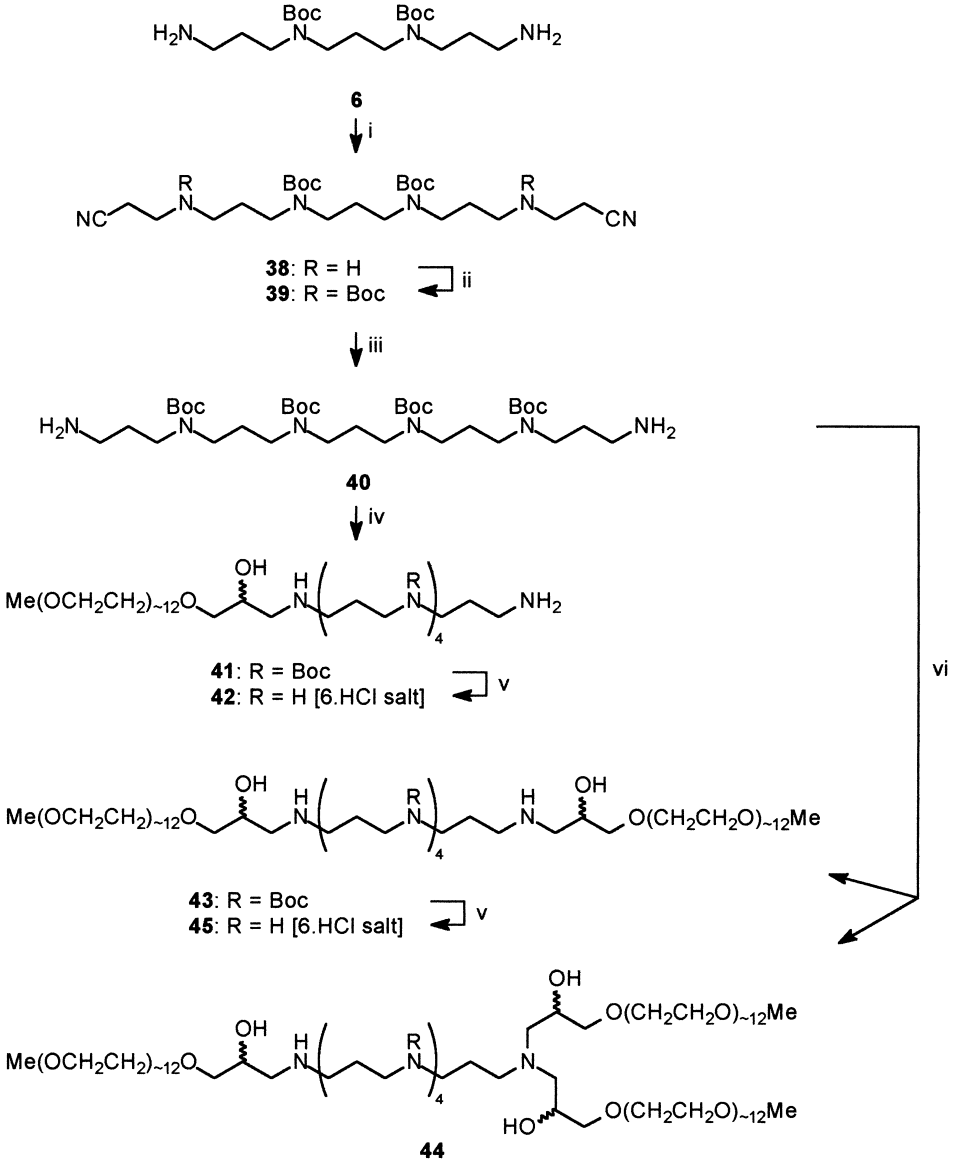


Figure 1. MALDI-TOF mass spectrum of protected construct **33**. Peaks correspond to $[M + Na]^+$ for the PEG oligomers.

polyamine sterically unencumbered. It was considered essential to preserve molecular symmetry by attaching the PEG unit to the central nitrogen of the polyamine and to retain the basicity of this tertiary amine; thus the target branched polyamine–PEG constructs **53** and **67** (Scheme 5) were based on a linear triamine and a linear pentamine, respectively.

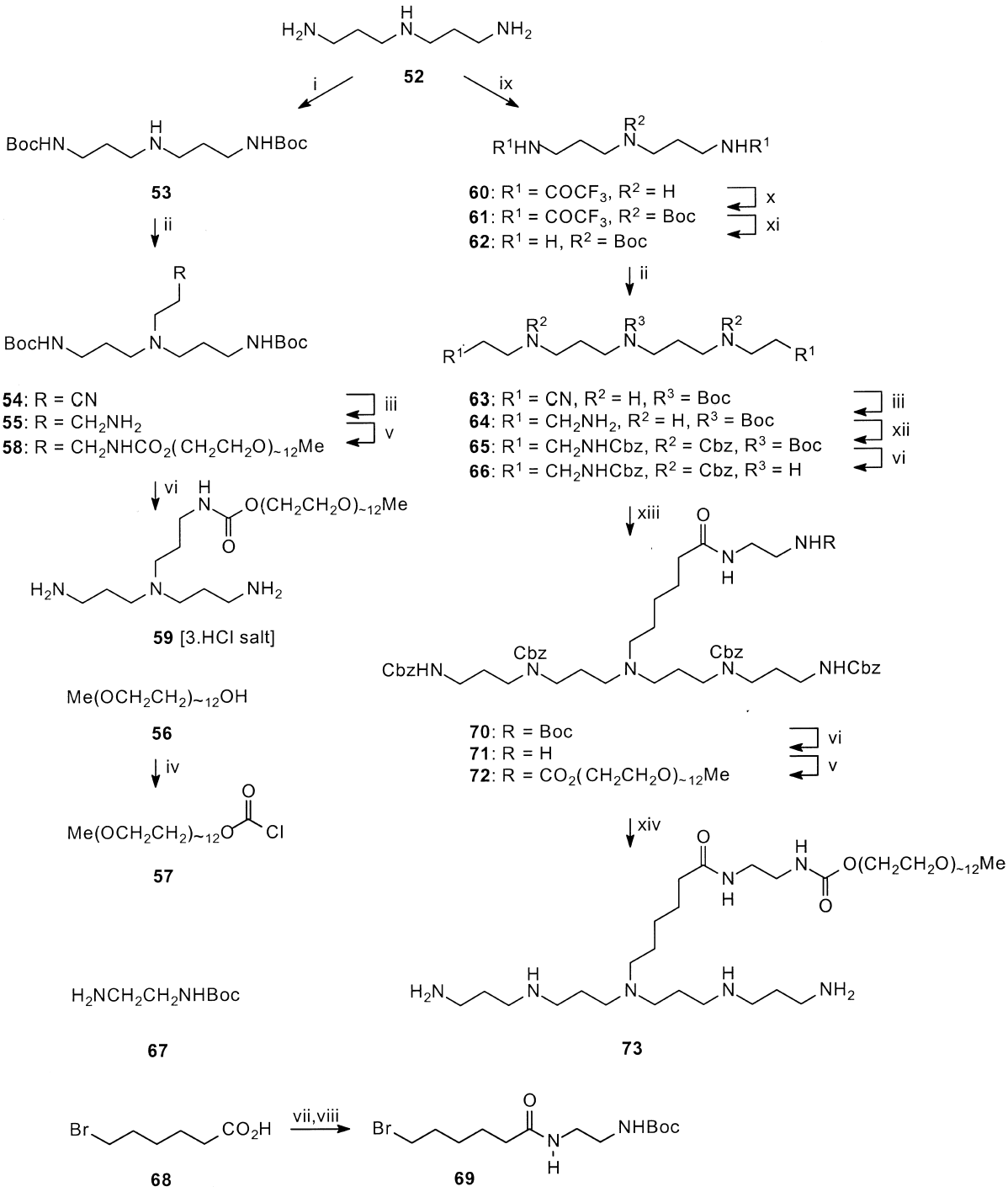
The simple triamine **46** was protected at the termini with Boc, giving **47**. Cyanoethylation of the crowded secondary amine required forcing conditions to give a good yield of the nitrile **48**, which was reduced to the amine **49**. In these branched constructs, the MeOPEG is not attached directly to one of the polyamine nitrogens, as it is in **42**. Rather, it is attached through the 3-aminopropyl linker in **53**. This has the benefit that the nitrogen to which the MeOPEG unit is attached does not have to be basic, as it is not required for electrostatic interaction with the DNA; thus a more reactive MeOPEG electrophile can be used. MeOPEG550 **50** was converted to its chloroformate **51** with excess phosgene. Reaction of **51** with the polyamine derivative **49** was very rapid at 20 °C in forming the protected construct **52**; deprotection gave the branched MeOPEG550 triamine construct as its trihydrochloride salt **53**.

The pentamine construct **67** (Scheme 1) not only has the potential for formation of a pentacation for increased electrostatic interaction with DNA but also has a longer linker between the central tertiary amine and the MeOPEG. To assemble this linker, 6-bromohexanoic acid **62** was coupled with the mono-Boc-protected ethane-1,2-diamine **61**.[32] The amide **63** then contains a Boc-masked amine (for later reaction with the PEG-derived electrophile) and a bromoalkyl group for alkylation of a suitably protected pentamine. Protection orthogonal to Boc of all but the central amine of the pentamine was required. By the general procedure of Xu et al.,[27] **46** was treated with



Scheme 4. Synthesis of hexamine–MeOPEG constructs **42** and **45**. Reagents: (i) propenenitrile, MeOH; (ii) Boc_2O, CH_2Cl_2; (iii) H_2, Raney Ni, MeOH, NH_3; (iv) **10a** (1 equiv), Pr^iOH, Δ; (v) HCl, CH_2Cl_2; (vi) **10a** (2 equiv), Pr^iOH, Δ.

ethyl trifluoroacetate at low temperature to give the crystalline α,ω-diamide **54** in good yield. Boc protection of the central amine was followed by removal of the terminal trifluoroacetyl groups in the usual way, affording **56**. Cyanoethylation (giving **57**) and hydrogenation lead to the mono-protected pentamine **58**, carrying Boc at the central nitrogen only. The other four amines were converted to their benzyl carbamates (in **59**) and the Boc group was removed, giving the pentamine derivative **60**, which has only the central secondary amine exposed. This amine was alkylated efficiently with **63** to provide the protected pentamine **64** carrying the long functionalised side chain at the central nitrogen. Deprotection of the linker and reaction of the exposed primary amine with the chloroformate **51** gave the protected construct **66** in good yield. Hydrogenolysis of the Cbz groups afforded the target branched MeOPEG550 pentamine construct **67**.



Scheme 5. Synthesis of branched triamine–MeOPEG constructs **53** and **67**. Reagents: (i) BocON, THF; (ii) propenenitrile, THF; (iii) H_2, Raney Ni, MeOH, NH_3; (iv) $COCl_2$, PhMe, CH_2Cl_2; (v) **51**, Et_3N, CH_2Cl_2; (vi) HCl, CH_2Cl_2; (vii) $(COCl)_2$, DMF, CH_2Cl_2; (viii) **61**, Et_3N, CH_2Cl_2; (ix) CF_3CO_2Et, EtOH; (x) Boc_2O, CH_2Cl_2; (xi) NH_3, MeOH; (xii) $(BnO_2C)_2O$, THF; (xiii) **63**, K_2CO_3, DMF; (xiv) H_2, Pearlman's catalyst, MeOH.

DNA Binding

The DNA-binding affinity of the constructs was measured by an ethidium-displacement assay. Compounds that bind to DNA can displace the intercalator ethidium from ethidium–DNA complexes. This displacement can be effected by intercalating compounds and by non-intercalating agents, such as polycations, which bind as

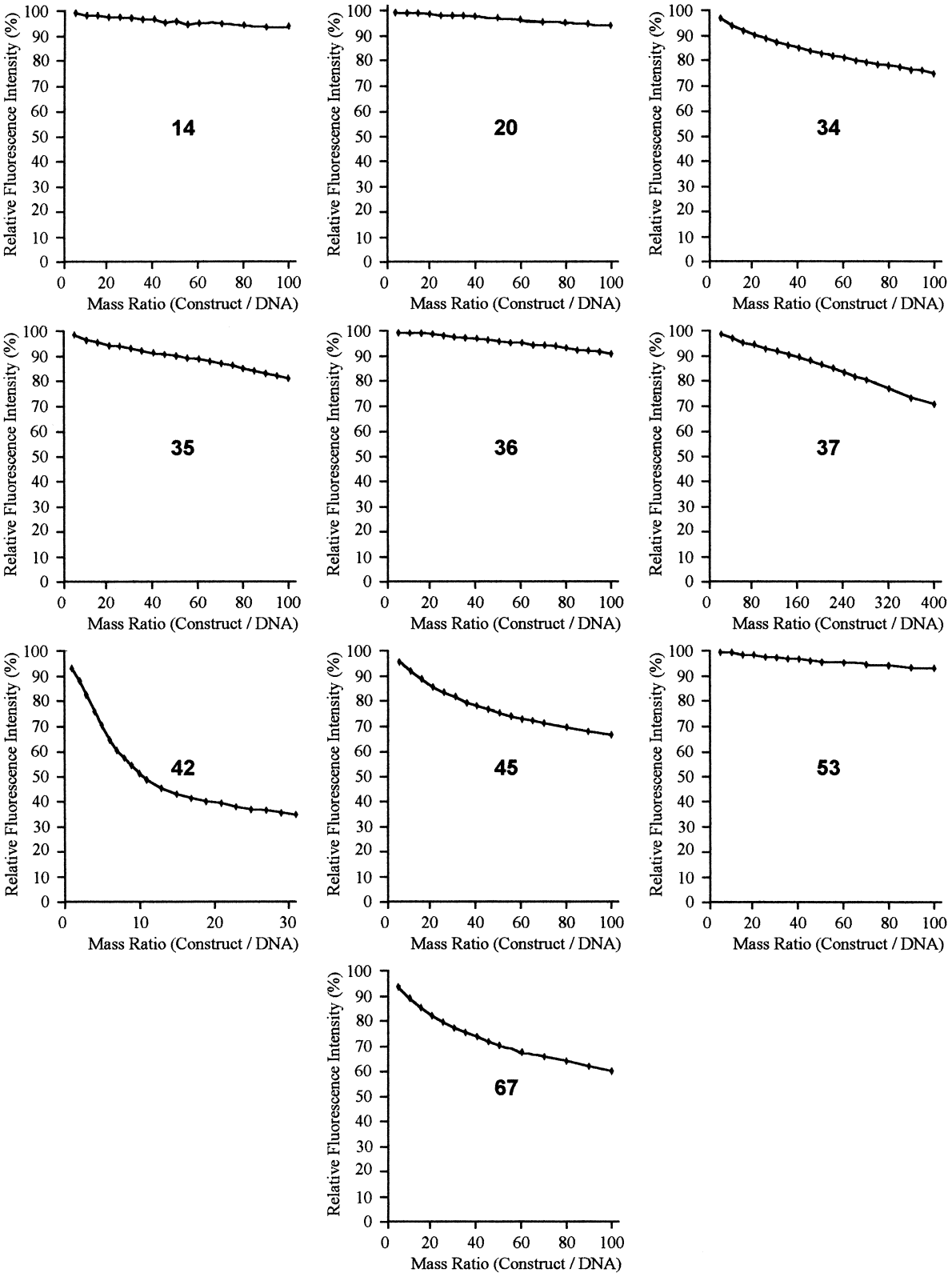


Figure 2. Graphs of the effects of addition of the linear polyamine–MePEG constructs **14**, **20**, **34**–**37**, **42** and **45** on the fluorescence of a DNA–ethidium complex. Decrease in fluorescence is a measure of the binding of the constructs to DNA.

a result of electrostatic interactions with the polyanionic DNA. There is correlation between the affinity of a binding agent for DNA and the efficiency of displacement of ethidium.[33] When ethidium binds to DNA, there is a large increase in the fluorescence of the ethidium; this fluorescence is quenched when higher affinity compounds displace the ethidium. Simple spectrofluorimetric methods can be used to study the interaction of a range of compounds with DNA; the technique has been widely described.[33–37]

Preliminary experiments showed that the ethidium exclusion assay developed by Gershon et al.[34] gave irreproducible results when applied to the MeOPEG–polyamine constructs. Thus the alternative ethidium displacement assay[35] was adopted to measure the binding of the constructs to DNA. The constructs were added in small aliquots to a pre-formed ethidium–DNA complex; decrease in fluorescence is a measure of displacement of ethidium and thus of binding of the constructs (Fig. 2). The mechanism by which ethidium is displaced by agents which do not intercalate, such as polyamines, may be non-competitive and may involve changes in DNA conformation.[36] Displacement could result from polyamine binding in the major groove, minor groove or along the phosphate backbone;[37] the exact mechanism is not known.

Figure 2 shows the diminution of fluorescence of the ethidium–DNA complex caused by addition of the constructs. Results are expressed as percentage of the original fluorescence versus mass ratio of construct/DNA. Only constructs **34**, **42**, **45** and **66** show any significant and ratio-dependent interaction with the DNA. The simplest bis-MeOPEG550–tetramine construct **14** causes no diminution of the fluorescence at mass ratios up to 100. Introduction of four N-Me groups on the polyamine in construct **20** has no positive effect on the apparent binding. Similarly, construct **26**, which carries N-Me groups at the termini of the polyamine but has longer PEG chains, does not appear to interact with DNA at mass ratios up to 100 (data not shown). The parent dimethyltetramine **23** (lacking MeOPEG units) binds strongly to DNA and causes condensation and, thus, displacement of ethidium at low mass ratios; a mass ratio of ca. 2.5 causes 50% decrease in fluorescence. This shows that *N*-methylation per se does not have a strongly deleterious effect on the binding of polyamines to DNA. Interactions between PEG and DNA may be thermodynamically unfavourable;[38,39] this may be associated with the observed collapse of DNA structure with increasing concentrations of PEG in aqueous solution. However, addition of MeOPEG2000 **9b** (two equivalents per equivalent of **23**) does not measurably affect the binding of **23** to DNA (Fig. 3) and the concentration of PEG used does not itself induce DNA coil collapse.[38,40] Constructs **34**–**37** have MeOPEG units attached to only one end of the tetramine and lack N-Me groups. Of these, the most potent in displacing ethidium from its DNA complex is the mono-MeOPEG550–tetramine construct **34**; even this only achieves a 25% diminution of fluorescence at a mass ratio of 100 (Fig. 2). A second MeOPEG unit (in **35**) or longer MeOPEG

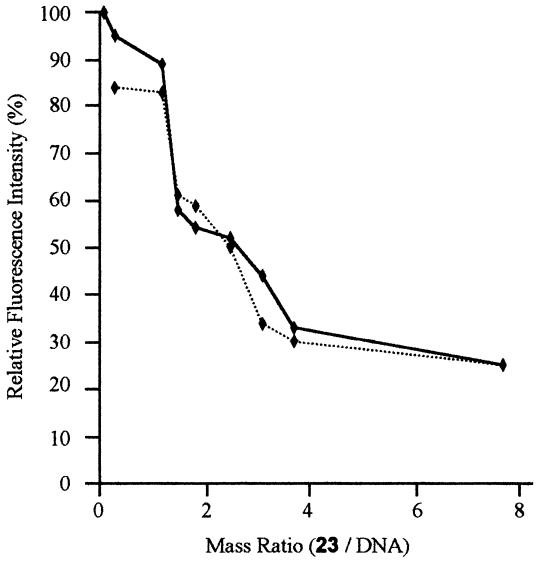


Figure 3. Graphs of the effects of addition of the polyamine **23** on the fluorescence of a DNA–ethidium complex in the absence —◆— and presence ····◆···· of MeOPEG2000. Decrease in fluorescence is a measure of the binding of the polyamine to DNA.

chains (**36** and **37**) reduce binding. These effects suggest that (i) there is steric obstruction to binding caused either by the PEG chain attached to the polyamine attempting to bind or the PEG chain of a different construct molecule that is already bound, or (ii) the mode of attachment of the MeOPEG lowers the pK_a of one nitrogen such that it is not protonated at pH 7.4, the pH of the assay. To test this latter hypothesis, the binding of **34** to DNA was also measured at pH 5.5. The results shown in Figure 4 indicate that **34** displaces ethidium more effectively at pH 5.5 than it does at pH 7.4. This is consistent with the amine to which the MeOPEG is attached having a low pK_a, owing to the β-OH group which can lower pK_a by ca. 1.3 units.[41]

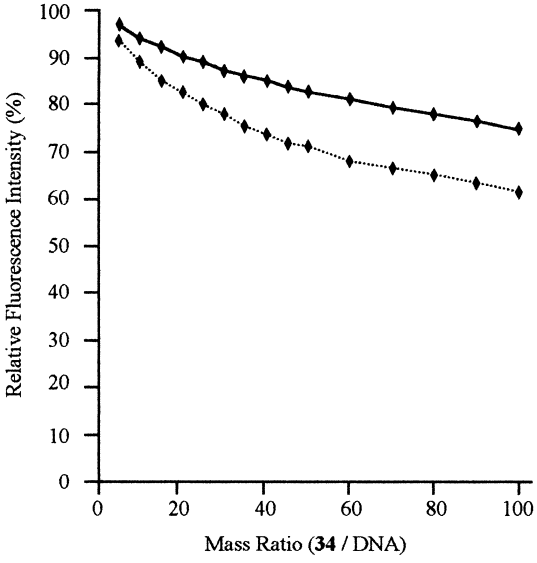


Figure 4. Graphs of the effect of pH on the change in fluorescence caused by addition of the polyamine–MePEG construct **34** to a DNA–ethidium complex. Experiments were performed at pH 7.4 —◆— and pH 5.5 ····◆····. Decrease in fluorescence is a measure of the binding of the constructs to DNA.

Extending the polyamine to be a hexamine should ameliorate this effect, in that the central tetramine should now be fully protonated at pH 7.4; furthermore, there should also be some protonation of the terminal amines, leading to enhanced binding to the DNA. Hence the mono-MeOPEG550–hexamine construct **42** and the bis-MeOPEG550–hexamine construct **45** were designed and evaluated. Construct **42** is relatively efficient at expelling ethidium from the complex (Fig. 2), indicating that it has good affinity for DNA. The fluorescence of the ethidium–DNA complex is diminished by ca. 50% at a mass ratio of 10 **42**/DNA, which compares very favourably with the effect of the analogous tetramine construct **34** (ca. 5% diminution of fluorescence at mass ratio 10). Similarly, **45** appears to bind weakly but significantly at mass ratio 100.

The branched triamine construct **53** does not interact strongly with DNA, up to mass ratio (construct/DNA) = 100. However, a significant ratio-dependent diminution in fluorescence is caused by the pentamine construct **67**, with > 35% diminution at mass ratio = 100. In **53** and **67**, the MeOPEG unit is attached in such a way that the pK_a of this central amine should be unaffected. Nevertheless, the electrostatic interaction of the potentially tricationic polyamine in **53** may not be sufficient to hold the MeOPEG unit close to the DNA. In **67**, several structural modifications may allow binding: (i) it is now a potential pentacation, which will allow more electrostatic attraction and hydrogen-bonding with the DNA phosphate backbone; (ii) the linker is less sterically demanding in the region which may lie in the DNA groove.

It is interesting to compare these results with those reported using PEG–poly-L-lysine (PLL–PEG), PEG–poly(ethyleneimine) (PEI–PEG) and PEG–polyspermine (PEG–PS) constructs. PLL–PEG has been produced in two variants, one a block co-polymer involving the N-terminal α-NH_2[18] and the other involving the Lys ε-NH_2 groups,[19] both using long amine chains (in one case 120 Lys). Both variants condensed DNA to levels close to those achieved using free poly-L-Lys, irrespective of whether the Lys ε-NH_2 polymer had a PEG coverage of 5, 10 or 25 mol%. PEG–PEI and PEG–PS also condensed DNA, producing very small particles (12–32 nm). It is unlikely that the constructs described in this paper will produce particles, although the ethidium exclusion assays for **42** and for histone H1 are similar; since histone H1 produces particles,[42] it is conceivable that **42** may also form particles with DNA. A major problem with PLL–PEG, PEI–PEG and PEG–PS polymers is that, on addition to DNA, complexes form which are poorly soluble and aggregate. Thus condensed DNA (particulate) delivery systems cannot be produced in sufficiently high concentrations for intramuscular or intratumoural injection.[43] The PEG–polyamine constructs in the present study, including **42**, allow formulation of DNA at 2500 μg mL^{-1}, which is ca. 50× the maximum concentration that can be formulated using poly-L-Lys.[44]

Although 'naked' plasmid DNA has little or no activity in a typical cell culture transfection experiment, naked DNA can be active in vivo after direct injection into muscle[45–48] or tumours.[15] This activity may be equal or greater than that produced by lipoplexes,[15] despite cell culture models that predict that lipoplexes are several orders of magnitude more active. Gene expression from naked plasmid DNA in vivo after direct injection into solid tumours can be enhanced by formulation of the DNA in a hydrophilic polymer solution. This phenomenon has been reported after direct injection of a reporter plasmid into mouse muscle using either PVP or PVA.[20,21,49] There are very little data relating to the injection in vivo of plasmid DNA formulations in which the DNA is only partially condensed.

Having no eukaryotic equivalent, the chloramphenicol acetyltransferase (CAT) gene has become one of the standard markers used in transfection studies both in vitro and in vivo. It also benefits from a relatively long half-life (50 h in vivo), making it particularly useful in animal experiments. Formulations of pCMVCAT (a plasmid containing the CAT gene) with saline, PVP, **34** or **42** were injected into RIF-1 tumours and the expression was measured after 48 h (Fig. 5). All three plasmid formulations produced enhanced expression of CAT (3- to 4-fold) in comparison to pCMVCAT injected in saline. There was no statistically significant difference in tumour expression between PVP, **34** and **42** formulations. The enhancement of expression of CAT in the tumours was comparable to that reported by Mumper et al.,[20] who used the CAT reporter gene in muscle. The expression levels obtained following direct injection of

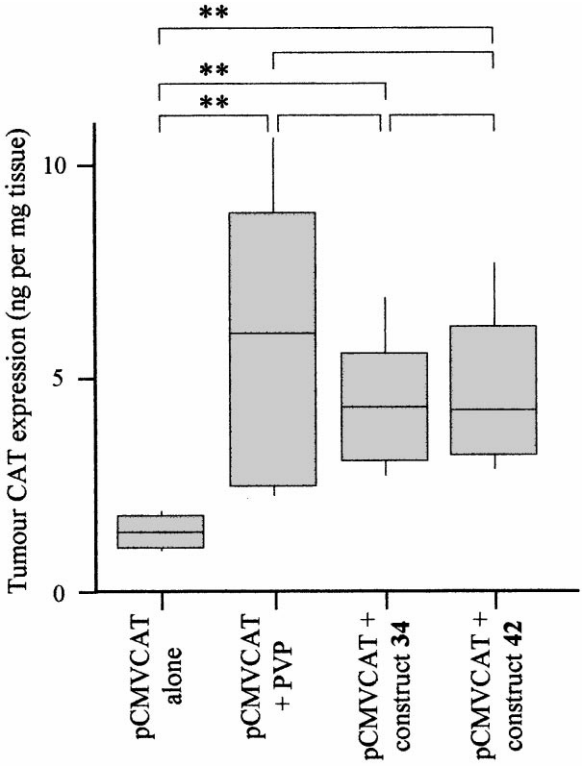


Figure 5. Effect of polymer solution (poly(1-vinylpyrrolidin-2-one), SG166 or **42**) on expression of CAT in RIF-1 tumour tissue. Plasmid pCMVCAT (50 μg) was injected directly into each tissue in either isotonic saline (20 μL), 5% PVP, 5% **34** or 5% **42** ($n = 8$). [**denotes 99% significance.]

plasmid were variable, as reported for mouse skeletal muscle.[48,50] Constructs **34** and **42** also enhanced CAT expression in the tumour tissue, with respect to formulation in saline, but were no more effective than PVP. These data suggest a generic effect of soluble interacting agents on gene expression that is not dependent on high molecular weight. Such an enhancement could be valuable for formulation of DNA vaccines; further studies will probe the mechanism of this enhancement.

Conclusions

Versatile, efficient syntheses of protected tetramines, suitable for coupling to electrophilic MeOPEGs, have been developed; N-methylation was achieved in high yield by alkylation of trifluoroacetamides. A high yielding synthesis of a protected hexamine has also been devised. Attachment of MeOPEG (via its oxiranylmethyl ether) gave the corresponding linear MeOPEG–polyamine constructs. This oxirane-addition reaction led to multiple reaction with some primary amines but chromatographic separation of constructs with differing numbers of MeOPEG units was facile. A novel triamine and a novel pentamine have also been synthesised and were linked to MeOPEG550 via the central amine while retaining its basicity, giving branched constructs.

The constructs show a range of interactions with DNA, as demonstrated by ethidium exclusion. The more and larger the MeOPEG units are, the weaker is the interaction of the construct with DNA. Increasing the number of basic nitrogens in the polyamine enhances DNA interaction, as demonstrated by the hexamine construct **42**. The novel PEG–polyamine constructs all interacted with DNA to a greater extent than did PVP (data not shown). However, unlike poly-L-Lys,[51] none of the constructs were able to condense DNA, with the possible exception of **42**, which caused a diminution in ethidium–DNA complex fluorescence of ca. 65% (at mass ratio 100), suggesting a significant conformational change in the DNA. Poly-L-Lys and many other polycations show a threshold effect with increasing concentration in the ethidium-displacement assay. This threshold effect is consistent with the theory that DNA condensation induced by multivalent cations occurs when 89–90% of its negative charge is neutralised.[52] Interestingly, the MeOPEG–polyamine constructs **34**, **35**, **42** and **45** show a progressive displacement of ethidium with increasing concentration, which phenomenon is unprecedented in the literature. Given the nature of the DNA-complex as determined by the ethidium assays, these novel constructs may have potential as PINC systems, exploiting Rolland and Mumper's[22] "Interactive Window of Opportunity".

The successful delivery of pCMVCAT in preliminary studies in vivo points to the potential utility of these MeOPEG–polyamine constructs. Further studies are in progress to determine the nature of the complex between DNA and these constructs. The results of fuller studies in vivo on the use of these linear polyamine–MeOPEG constructs as gene delivery agents will be presented elsewhere.[53]

Experimental

General methods

NMR spectra were recorded on samples in $CDCl_3$, unless otherwise stated. Mass spectra were obtained by fast atom bombardment (FAB) in the positive ion mode, unless otherwise stated. The stationary phase for chromatography was silica gel. Melting points are uncorrected. Solutions in organic solvents were dried with $MgSO_4$. Solvents were evaporated under reduced pressure. The brine was saturated.

***N*¹,*N*³-Bis(2-cyanoethyl)propane-1,3-diamine (2).** Propenenitrile (14.3 g, 270 mmol) was added to propane-1,3-diamine (10.0 g, 130 mmol) in MeOH (15 mL) at 0 °C during 20 min. The mixture was stirred at 0 °C for 20 min and at 20 °C for 16 h. Evaporation gave **2** (24.3 g, 100%) as a colourless liquid: $bp_{0.3}$ (Kugelrohr) 235 °C (lit.[25] $bp_{0.5}$ 176 °C). Found C, 60.0; H, 9.0, N, 31.2. $C_9H_{16}N_4$ requires C, 59.96; H, 8.94, N, 31.08%; ^{1}H NMR δ 1.49 (2H, s, 2×NH), 1.67 (2H, qn, J = 6.7 Hz, $CH_2CH_2CH_2$), 2.53 (4H, t, J = 6.7 Hz, CH_2CN), 2.73 (4H, t, J = 6.7 Hz, $CH_2CH_2CH_2$), 2.91 (4H, t, J = 6.7 Hz, CH_2CH_2CN); ^{13}C NMR δ 18.67, 29.77, 45.08, 47.55, 118.98.

***N*¹,*N*³-Bis(2-cyanoethyl)-*N*¹,*N*³-bis(1,1-dimethylethoxycarbonyl)propane-1,3-diamine (3).** Compound **2** (2.50 g, 13.9 mmol) was stirred with Boc_2O (6.06 g, 27.8 mmol) in CH_2Cl_2 (10 mL) at 0 °C for 20 min and at 20 °C for 5 h. Evaporation gave **3** (5.3 g, 100%) as a colourless oil (lit.[26] oil): ^{1}H NMR δ 1.48 (18H, s, 2×Bu^t), 1.81 (2H, qn, J = 7.3 Hz, $CH_2CH_2CH_2$), 2.62 (4H, m, CH_2CN), 3.29 (4H, t, J = 7.3 Hz, $CH_2CH_2CH_2$), 3.48 (4H, t, J = 6.5 Hz, CH_2CH_2CN). This material was used without further purification or characterisation.

***N*¹,*N*³-Bis(3-aminopropyl)-*N*¹,*N*³-bis(1,1-dimethylethoxycarbonyl)propane-1,3-diamine (4). Method A.** Compound **3** (3.58 g, 9.4 mmol) in dry THF (40 mL) was added dropwise to $LiAlH_4$ (2.60 g, 69 mmol) in dry Et_2O (200 mL) under N_2 at 0 °C during 10 min; the mixture was stirred at 0 °C for 2 h. Aqueous NaOH (1 M) was added cautiously until effervescence ceased. The supernatant was decanted and the residue was extracted with Et_2O (3×50 mL). Evaporation and chromatography (CH_2Cl_2:MeOH:35% aq NH_3, 20:10:1) gave **4** (790 mg, 22%) as a colourless oil (lit.[26] oil): ^{1}H NMR δ ($(CD_3)_2SO$) 1.39 (18H, s, 2×Bu^t), 1.46–1.68 (10H, m, 3×CH_2CH_2 CH_2 + 2×NH_2), 2.50 (4H, t, J = 7.0 Hz, 2×CH_2NH_2), 3.10 (4H, t, J = 7.0 Hz, 2×CH_2NBoc), 3.17 (4H, t, J = 6.8 Hz, 2×CH_2NBoc); MS (FAB) m/z 389 (M + H), 189 (M + H−2×Boc). Further elution gave **5** (1.15 g, 37%) as a colourless oil (lit.[54] oil): ^{1}H NMR δ 1.44 (9H, s, Bu^t), 1.46 (9H, s, Bu^t), 1.67 (4H, m, 2×$CH_2CH_2CH_2$), 2.74 (2H, m, CH_2NH_2), 3.13–3.35 (8H, m, 3×CH_2NBoc + NH_2), 3.67 (1H, m, NH); MS (FAB) m/z 332 (M + H), 232 (M + H−Boc), 132 (M + H−2×Boc).

***N*¹,*N*³-Bis(3-aminopropyl)-*N*¹,*N*³-bis(1,1-dimethylethoxycarbonyl)propane-1,3-diamine (4). Method B.** The dinitrile **3** (2.74 g, 7.2 mmol) in MeOH (10 mL) was saturated with NH_3 at 0 °C and was treated with H_2 (3000 Torr) in the presence of W-2 Raney Ni for 72 h. Filtration

(Celite®), evaporation and chromatography (CH_2Cl_2:MeOH:35% aq NH_3, 20:10:1) gave **4** (1.93 g, 69%) as colourless oil as above.

N^1,N^3-Bis(3-aminopropyl)-N^1,N^3-bis(1,1-dimethylethoxycarbonyl)propane-1,3-diamine (4). Method C. The diamide **8** (540 mg, 930 μmol) was heated with 35% aq NH_3 (1 mL) in MeOH (8 mL) at 60 °C in a sealed vessel for 5 h. Evaporation and chromatography (CH_2Cl_2:MeOH:35% aq NH_3, 20:10:1) gave **4** (360 mg. 100%) as a colourless oil as above.

N^1,N^3-Bis(3-aminopropyl)propane-1,3-diamine (6). Compound **2** (2.00 g, 11.1 mmol) in MeOH (20 mL) was saturated with NH_3 for 30 min and treated with H_2 at 2700 Torr in the presence of W-2 Raney Ni (1.0 g) for 65 h. Filtration (Celite®), evaporation and distillation (Kugelrohr) gave **6** (1.40 g, 67%) as a colourless liquid: $bp_{0.08}$ (Kugelrohr) 285 °C (lit.[25] $bp_{0.07}$ 97–100 °C); ^{1}H NMR ($(CD_3)_2SO$) δ 1.40–1.53 (6H, m, 3 $CH_2CH_2CH_2$), 1.67 (6H, br, 2×NH + 2×NH_2), 2.47–2.56 (12H, m, 3×$CH_2CH_2CH_2$); MS *m*/*z* 189 (M + H).

N^1,N^3-Bis(1,1-dimethylethoxycarbonyl)-N^1,N^3-bis(3-trifluoroacetamidopropyl)propane-1,3-diamine (8). Method A. EtO_2CCF_3 (7.9 g, 56 mmol) was added to **6** (5.1 g, 27 mmol) in MeOH (10 mL) at 0 °C during 5 min. The mixture was stirred at 20 °C for 2.5 h. Evaporation gave crude **7** (10.4 g, 100%) as a colourless oil: ^{1}H NMR δ 1.67 (2H, qn, *J* = 7.0 Hz, central $CH_2CH_2CH_2$), 1.73 (4H, qn, *J* = 6.1 Hz, 2×$NCH_2CH_2CH_2NCOCF_3$), 2.66 (4H, t, *J* = 7 Hz, central $CH_2CH_2CH_2$), 2.79 (4H, t, *J* = 5.9 Hz, 2×$NCH_2CH_2CH_2NCOCF_3$), 3.44 (4H, t, *J* = 6.1 Hz, 2×CH_2NCOCF_3), 7–10 (4H, br, 4 NH); ^{13}C NMR δ 27.1, 30.0, 40.2, 48.0, 48.8, 116.2 (q, $J_{C\text{-}F}$ = 288 Hz, 2×CF_3), 157.2 (q, $J_{C\text{-}F}$ = 37 Hz, 2×$COCF_3$). This material (180 mg, 470 μmol) was stirred with Boc_2O for 16 h. Evaporation, chromatography (EtOAc:hexane, 1:1) and recrystallisation (EtOAc–hexane) gave **8** (254 mg, 93%) as a white solid: mp 70–71 °C. Found C, 47.7; H, 6.7, N, 9.6. $C_{23}H_{38}F_6N_4O_6$ requires C, 47.58; H, 6.60, N, 9.65%; ^{1}H NMR δ 1.47 (18H, s, 2×Bu^t), 1.74–1.80 (6H, m, 3×$CH_2CH_2CH_2$), 3.11–3.19 (4H, m, 2×CH_2NCOCF_3), 3.21–3.40 (8H, m, 2×$CH_2NBocCH_2$), 8.29 (2H, br, 2×NH); ^{13}C NMR δ 27.1, 27.7, 28.4, 35.9, 43.1, 44.9, 80.6, 116.0 (q, $J_{C\text{-}F}$ = 287 Hz, 2×CF_3), 156.8 (q, $J_{C\text{-}F}$ = 37 Hz, 2×COCF_3$); MS (FAB + ve ion) *m*/*z* 603 (M + Na), 581 (M + H), 481 (M + H−Boc), 381 (M + H−2×Boc); MS (FAB −ve ion) *m*/*z* 579 (M−H).

N^1,N^3-Bis(1,1-dimethylethoxycarbonyl)-N^1,N^3-bis(3-trifluoroacetamidopropyl)propane-1,3-diamine (8). Method B. The diamine **4** (620 mg, 1.6 mmol) was stirred with EtO_2CCF_3 (1.1 g, 8 mmol) in MeOH (10 mL) for 16 h. Evaporation and chromatography (EtOAc:hexane, 1:1) gave **8** (890 mg, 96%) as a white solid as above.

(ω-MeOPEG550oxymethyl)oxirane (10a). MeOPEG550 OH **9a** (10.0 g, 18 mmol) was dried by azeotropic removal of water with toluene. NaOH (powder, 2.18 g, 54 mmol), water (300 μL) and chloromethyloxirane (20.35 g, 220 mmol) were added and the mixture was stirred vigorously at 60 °C for 3 h. CH_2Cl_2 (100 mL) was added and the suspension was filtered. The combined filtrate and CH_2Cl_2 washings were dried and the solvent and excess reagent were evaporated to give **10a** (10.8 g, 98%) as a colourless oil: ^{1}H NMR ($(CD_3)_2SO$) δ 2.53 (1H, dd, *J* = 5.0, 2.5 Hz, oxirane 3-H), 2.72 (1H, t, *J* = 5.0 Hz, oxirane 3-H), 3.09 (1H, m, oxirane 2-H), 3.27 (2H, m, oxirane-CH_2), 3.24 (3H, s, Me), 3.41–3.73 (ca. 45H, m, *n*×OCH_2CH_2O); MS (electrospray) *m*/*z* 771 (20%), 727 (36%), 683 (56%), 639 (74%), 595 (92%), 551 (100%), 597 (92%), 463 (80%), 419 (58%), 375 (46%) (all M + Na).

N^1,N^3-Bis(1,1-dimethylethoxycarbonyl)-N^1,N^3-bis(3-(*N*-(2-hydroxy-3-(ω-methoxyPEG550oxy)propyl)amino)propyl)propane-1,3-diamine (11). The diamine **4** (82 mg, 210 μmol) was boiled under reflux with the oxirane **10a** (253 mg, 420 μmol) in Pr^iOH (3.0 mL) for 24 h. Evaporation gave crude **11** (330 mg, 100%) as a pale-yellow oil: ^{1}H NMR δ 1.41 (18 H, s, 2×Bu^t), 1.62–1.95 (6H, m, 3×$CH_2CH_2CH_2$), 2.51–2.59 (8H, m, 2×CH_2NHCH_2), 3.15–3.25 (8H, m, 4×CH_2NBoc), 3.35 (6H, s, 2×OMe), 3.41–3.53 (12H, m, 6×OCH_2), 3.60–3.78 (ca. 106 H, m, *n*×OCH_2), 3.80–3.83 (4H, m, OCH_2 + 2×$CHOH$); MS ^{13}C/^{12}C isotope clusters centred at *m*/*z* 1490, 1446, 1402, 1358, 1314 (all M + H).

N^1,N^3-Bis(1,1-dimethylethoxycarbonyl)-N^3-(3-(*N*-(2-hydroxy-3-(ω-methoxyPEG550oxy)propyl)amino)propyl)-N^1-(3-(*N*,*N*-bis(2-hydroxy-3-(ω-methoxyPEG550oxy)propyl)amino)propyl)propane-1,3-diamine (12) and N^1,N^3-bis(1,1-dimethylethoxycarbonyl)-N^1,N^3-bis(3-(*N*,*N*-bis(2-hydroxy-3-(ω-methoxyPEG550oxy)propyl)amino)propyl)propane-1,3-diamine (13). The diamine **4** (1.75 g, 4.5 mmol) was stirred at 80 °C with the oxirane **10a** (5.4 g, 9.0 mmol) in Pr^iOH (20 mL) for 38 h. Evaporation and chromatography (CH_2Cl_2:MeOH, 6:1) gave **13** (1.40 g, 24%) as a pale-yellow oil: ^{1}H NMR δ 1.45 (18H, s, 2×Bu^t), 1.60–1.70 (6H, m, 3×$CH_2CH_2CH_2$), 2.50–2.55 (12H, m, 2×$N(CH_2)_3$), 3.15–3.26 (8H, m, 4×CH_2NBoc), 3.38 (12H, s, 4×OMe), 3.41–3.49 (6H, m, 3×OCH_2), 3.51–3.56 (8H, m, 4×OCH_2), 3.56–3.68 (ca. 180H, m, *n*×OCH_2), 3.80–3.84 (4×$CHOH$); MS (electrospray) ^{13}C/^{12}C isotope clusters centred at *m*/*z* 1339, 1318, 1296, 1274, 1252, 1230, 1208 (all $[M + H]^{2+}$). Further elution gave **12** (560 g, 11%) as a pale-yellow oil: ^{1}H NMR δ 1.47 (18H, s, 2×Bu^t), 1.60–1.70 (4H, m, 2×$CH_2CH_2CH_2$), 1.95–2.05 (2H, m, $CH_2CH_2CH_2$), 2.48–2.60 (6H, m, 3×NCH_2), 2.95–3.05 (6H, m, 3×NCH_2), 3.15–3.25 (8H, m, 4×CH_2NBoc), 3.41 (9H, s, 3×OMe), 3.44–3.50 (4H, m, 2×OCH_2), 3.54–3.58 (6H, m, 3×OCH_2), 3.58–3.80 (ca. 130H, m, *n*×OCH_2), 3.80–3.94 (3H, m, 3×$CHOH$); MS (electrospray) ^{13}C/^{12}C isotope clusters centred at *m*/*z* 1098, 1076, 1054, 1031, 1009, 988, 966 (all $[M + H]^{2+}$).

N^3-(3-(*N*-(2-Hydroxy-3-(ω-methoxyPEG550oxy)propyl)amino)propyl)-N^1-(3-(*N*,*N*-bis(2-hydroxy-3-(ω-methoxyPEG550oxy)propyl)amino)propyl)propane-1,3-diamine (14). HCl was passed through **12** (2.27 g, 1.1 mmol) in CH_2Cl_2 (40 mL) for 30 min. Evaporation and freeze-drying gave **14** (2.07 g, 100%) as an off-white glass: ^{1}H NMR (D_2O) δ 2.13 (6H, m, 3×$CH_2CH_2CH_2$), 3.11–3.27 (18H, m, 9×NCH_2), 3.39 (9H, s, 3×OMe), 3.53–3.64 (16H, m, 8×OCH_2), 3.68–3.88 (ca. 120H, m, *n*×OCH_2), 4.14 (3H, 3×$CHOD$).

N^1,N^3-Bis(3-(N,N-bis(2-hydroxy-3-(ω-methoxyPEG 550oxy)propyl)amino)propyl)propane-1,3-diamine (15). Compound **13** was treated with HCl, as for the synthesis of **14**, to give **15** (100%) as an off-white glass: ^{1}H NMR (D_2O) δ 2.10 (6H, m, 3×$CH_2CH_2CH_2$), 2.97–3.22 (20H, m, 10×NCH_2), 3.39 (12H, s, 4×OMe), 3.53–3.64 (20H, m, 10×OCH_2), 3.66–3.72 (ca. 164H, m, n×OCH_2), 4.17 (4 H, 4×CHOD).

1,1-Dimethylethyl N-methyl-N-(3-methylaminopropyl)-carbamate (17). Boc_2O (1.10 g, 5.0 mmol) in dry THF (10 mL) was added dropwise during 40 min to **16** (1.53 g, 15 mmol) in dry THF (10 mL) at 0 °C. Stirring continued at 0 °C for 1 h and at 20 °C for 20 h. Evaporation and chromatography (CH_2Cl_2:MeOH:35% aq NH_3, 60:20:1) gave **17** (723 mg, 71%) as a colourless oil (lit.[30] oil): ^{1}H NMR δ 1.46 (9H, s, Bu^t), 1.69–1.82 (2H, m, $CH_2CH_2CH_2$), 2.46 (3H, s, $NHCH_3$), 2.61 (2H, t, J = 7.0 Hz, CH_2NH), 2.85 (3H, s, $BocNCH_3$), 3.17 (1H, brs, NH), 3.3 (2H, m, CH_2NBoc); MS (FAB) m/z 204.1795 (M + H) ($^{13}C^{12}C_9H_{23}N_2O_2$ requires 204.1793); 203.1763 (M + H) ($^{12}C_{10}H_{23}N_2O_2$ requires 203.1760).

N^1,N^3-Bis(3-(N-(1,1-dimethylethoxycarbonyl)-N-methylamino)propyl)-N^1,N^3-dimethylpropane-1,3-diamine (18). The carbamate **17** (277 mg, 1.4 mmol) was stirred with 1,3-dibromopropane (138 mg, 680 μmol) and K_2CO_3 (200 mg, 1.45 mmol) in DMF (4 mL) at 80 °C for 8 h. The solvent was evaporated and the residue was extracted with $CHCl_3$ (4×30 mL). Evaporation and chromatography (CH_2Cl_2:MeOH:35% aq NH_3, 70:10:1) gave **18** (160 mg, 52%) as a pale-yellow oil: ^{1}H NMR δ 1.45 (18H, s, 2×Bu^t), 1.63–1.71 (6H, m, 3×$CH_2CH_2CH_2$), 2.21 (6H, s, 2×amine NMe), 2.30–2.37 (8H, m, 2×CH_2NMeCH_2), 2.85 (6H, s, 2×$BocNCH_3$), 3.22 (4H, t, J = 7.0 Hz, 2×CH_2NBoc). This material was used without further characterisation.

N^1,N^3-Bis(3-methylaminopropyl)-N^1,N^3-dimethylpropane-1,3-diamine tetrahydrochloride (19). The bis-carbamate **18** (115 mg, 260 μmol) was stirred with hydrochloric acid (5 M, 5 mL) for 16 h. Evaporation and trituration (CH_2Cl_2) gave **19** (100 mg, 100%) as a pale-buff solid: mp 217–220 °C; ^{1}H NMR (D_2O) δ 2.15–2.30 (6H, m, 3×$CH_2CH_2CH_2$), 2.75 (6H, s, 2×NMe), 2.94 (6H, s, 2×NMe), 3.15 (4H, t, J = 7.0 Hz, 2×CH_2N), 3.27–3.35 (8H, m, 4×CH_2N); MS (FAB) m/z 321 (M + 3 H + 2 ^{37}Cl), 317 (M + 3 H + ^{37}Cl + ^{35}Cl), 317 (M + 3 H + 2 ^{35}Cl), 283 (M + 2 H + ^{37}Cl), 281 (M + 2 H + ^{35}Cl), 246.2729 (M + H) ($^{13}C^{12}C_{12}H_{33}N_4$ requires 246.2739), 245.2709 (M + H) ($^{12}C_{13}H_{33}N_4$ requires 245.2705).

N^1,N^3-Bis(3-(N-(2-hydroxy-3-(ω-methoxyPEG550oxy)-propyl)-N-methylamino)propyl)-N^1,N^3-dimethylpropane-1,3-diamine tetrahydrochloride (20). The tetramine **19** (83 mg, 210 μmol) was stirred with aq NaOH (5 M, 213 μL, 1.0 mmol) in Pr^{i}OH (1.0 mL) for 5 min. The oxirane **10a** (255 mg, 420 μmol) in Pr^{i}OH (1.0 mL) was added and the mixture was stirred at 75 °C for 24 h. Evaporation and chromatography (CH_2Cl_2:MeOH:35% aq NH_3, 40:10:1) gave a pale-yellow oil. Aqueous HCl (9 M, 2.0 mL) was added. Freeze-drying gave **20** (105 mg, 32%) as an off-white wax: ^{1}H NMR (free base) δ 1.80–1.90 (6H, m, 3×$CH_2CH_2CH_2$), 2.40–2.49 (12H, m, 4×NMe), 2.57–2.70 (16H, m, 8×$NHCH_2$), 3.38 (6H, s, 2×OMe), 3.49–3.51 (4H, m, 2×OCH_2), 3.54–3.56 (4H, m, 2×OCH_2), 3.64–3.66 (ca. 96H, m, n×OCH_2), 4.00 (2H, m, 2×CHOH); MS ^{13}C/^{12}C isotope clusters centred at m/z 1390, 1346, 1302, 1258, 1214, 1170, 1126 (all M + H).

N^1,N^3-Bis(1,1-dimethylethoxycarbonyl)-N^1,N^3-bis(3-(N-methyltrifluoroacetamido)propyl)propane-1,3-diamine (21). KOBu^t in THF (1.0 M, 2.6 mL, 2.6 mmol) was added to **8** (712 mg, 1.2 mmol) in dry THF (25 mL) under N_2 and the mixture was stirred for 5 min. Iodomethane (426 mg, 3.0 mmol) was added and the mixture was stirred for 20 min under N_2. The solvent and excess reagent were evaporated and the residue, in EtOAc, was washed with water and with brine and was dried. Evaporation of the solvent gave **21** (730 mg, 98%) as a colourless oil: ^{1}H NMR δ ($(CD_3)_2SO$, 22 °C) 1.37 (18H, s, 2×Bu^t), 1.65–1.77 (6H, m, 3×$CH_2CH_2CH_2$), 2.95 (2H, s, 0.67×NMe), 3.09–3.15 (11H, m, 1.33×NMe + 3.5×NCH_2), 3.32–3.44 (5H, m, 2.5×NCH_2); ^{1}H NMR δ ($(CD_3)_2SO$, 100 °C) 1.42 (18H, s, 2×Bu^t), 1.68–1.80 (6H, m, 3×$CH_2CH_2CH_2$), 2.96 (6H, s, 2×NMe), 3.10–3.20 (8H, m, 4×NCH_2), 3.41 (4H, t, J = 7.6 Hz, 2×NCH_2); ^{19}F NMR ($(CD_3)_2SO$, 22 ° C) −69.11 (4F, s), −68.17 (2F, s); MS (FAB) m/z 610.3122 (M + H) ($^{13}C^{12}C_{24}H_{43}F_6N_4O_6$ requires 610.3120), 609.3091 (M + H) ($^{12}C_{25}H_{43}F_6N_4O_6$ requires 609.3087).

N^1,N^3-Bis(1,1-dimethylethoxycarbonyl)-N^1,N^3-bis(3-methylaminopropyl)propane-1,3-diamine (22). Compound **21** (144 mg, 240 μmol) was stirred with 35% aq NH_3 (2.0 mL) in MeOH (8 mL) at 55 °C in a sealed vessel for 3.5 h. Evaporation and chromatography (CH_2Cl_2:MeOH:35% aq NH_3, 16:8:1) gave **22** (78 mg, 79%) as a colourless oil: ^{1}H NMR δ 1.45 (18H, s, 2×Bu^t), 1.72–1.74 (6H, m, 3×$CH_2CH_2CH_2$), 2.39 (6H, s, 2×NMe), 2.58 (4H, t, J = 7.0 Hz, 2×CH_2NMe), 2.76 (2H, s, 2×NH), 3.12–3.20 (4H, m, 2×NCH_2), 3.22–3.28 (4H, m, 2×NCH_2); MS (FAB) m/z 418.3473 (M + H) ($^{13}C^{12}C_{20}H_{45}N_4O_4$ requires 418.3474), 417.3443 (M + H) ($^{12}C_{21}H_{45}N_4O_4$ requires 417.3441), 317 (M + H − Boc), 217 (M + H − 2×Boc).

N^1,N^3-Bis(3-methylaminopropyl)propane-1,3-diamine tetrahydrochloride (23). Compound **22** was treated with HCl, as for the synthesis of **14**, to give **23** (96%) as a highly hygroscopic white solid (lit.[55] hygroscopic solid): ^{1}H NMR (D_2O) δ 2.08–2.20 (6H, m, 3×$CH_2CH_2CH_2$), 2.75 (6H, s, 2×Me), 3.12–3.24 (12H, m, 6×CH_2N); MS m/z 217 (M + H).

N^1,N^3-Bis(1,1-dimethylethoxycarbonyl)-N^1,N^3-bis(3-(N-(2-hydroxy-3-phenoxypropyl)-N-methylamino)propyl)-propane-1,3-diamine (24). The diamine **22** (45 mg, 110 μmol) was heated at reflux with phenoxymethyloxirane (33 mg, 220 μmol) in Pr^{i}OH (1.0 mL) for 10 h. Evaporation and preparative layer chromatography (CH_2Cl_2:MeOH:35% aq NH_3, 140:20:1) gave **24** (20 mg, 26%) as a colourless oil: ^{1}H NMR δ 1.45 (18H, s, 2×Bu^t), 1.65–1.80 (6H, m, 3×$CH_2CH_2CH_2$), 2.32 (6H, s, 2×NMe), 2.44–2.60 (8H, m, 2×CH_2NCH_2), 3.10–3.35 (10H, m, 4×$BocNCH_2$ + 2×OH), 3.97 (4H, m, 2×OCH_2), 4.08 (2H, m, 2×CHOH), 6.94 (4H, d, J = 8.0 Hz, 2×Ph 2,6-H_2), 7.25–7.31 (6H, m, 2×Ph 3,4 5-H_3); MS (FAB) m/z

718.4853 (M + H) ($^{13}C^{12}C_{38}H_{65}N_4O_8$ requires 718.4836), 717.4811 (M + H) ($^{12}C_{39}H_{65}N_4O_8$ requires 717.4802).

N^1,N^3-Bis(1,1-dimethylethoxycarbonyl)-N^1,N^3-bis(3-(*N*-(2-hydroxy-3-(ω-MeOPEG2000oxy)propyl)-*N*-methylamino)propyl)propane-1,3-diamine (25). The diamine **22** (43 mg, 103 μmol) was heated at reflux with ω-MeO-PEG2000 oxiranylmethyl ether **10b** (410 mg, 210 μmol) in Pr^{i}OH (3.0 mL) for 27 h. Evaporation gave **25** (450 mg, 100%) as a pale-yellow wax: ^{1}H NMR δ 1.45 (18H, s, 2×Bu^t), 1.66–1.75 (6H, m, 3×$CH_2CH_2CH_2$), 2.25 (6H, s, 2×NMe), 2.31–2.45 (8H, m, 2×CH_2NCH_2), 3.15–3.20 (8H, m, 4×BocNCH_2), 3.38 (6H, s, 2×OMe), 3.42–3.60 (12H m) and 3.60–3.77 (ca. 360 H, m) (*n*×OCH_2CH_2O), 3.81–3.88 (6H, m, 2×OCH_2 + 2×C*H*OH).

N^1,N^3-Bis(3-(*N*-(2-hydroxy-3-(ω-MeOPEG2000oxy)propyl)-*N*-methylamino)propyl)propane-1,3-diamine tetrahydrochloride (26). Compound **25** was treated with HCl, as for the synthesis of **14**, to give **26** (100%) as a white wax: ^{1}H NMR (D_2O) δ 2.43 (6H, m, 3×$CH_2CH_2CH_2$), 2.98 (6H, m, 2×NMe), 3.31–3.36 (16H, m, 8×$NHCH_2$), 3.38 (6H, s, 2×OMe), 3.48–3.83 (ca. 370H, m, *n*×OCH_2), 4.33 (2H, m, 2×C*H*OD).

N^1-(3-Aminopropyl)-N^3-(3-(1,1-dimethylethoxycarbonylamino)propyl)-N^1,N^3-bis(1,1-dimethylethoxycarbonyl)-propane-1,3-diamine (29). EtO_2CCF_3 (1.74 g, 12.3 mmol) in EtOH (50 mL) was added dropwise during 40 min to **6** (2.09 g, 11.1 mmol) in EtOH (50 mL) at −78 °C. The mixture was stirred at 0 °C for 30 min. Boc_2O (9.7 g, 44.5 mmol) in EtOH (20 mL) was added dropwise during 10 min and the mixture was stirred at 20 °C for 16 h. The solvent was evaporated. The residue, in MeOH (100 mL), was stirred with 35% aq NH_3 (70 mL) in a sealed vessel for 20 h. Evaporation and chromatography (CH_2Cl_2:MeOH:35% aq NH_3, 70:10:1→30:10:1) gave **29** (2.76 g, 51%) as a colourless oil (lit.[26] oil): ^{1}H NMR δ 1.44 (9H, s, Bu^t), 1.46 (9H, s, Bu^t), 1.47 (9H, s, Bu^t), 1.63 (2H, m, $CH_2CH_2NH_2$), 1.75 (4H, m, 2×BocNCH_2CH_2 CH_2NBoc), 1.97 (2H, brs, NH_2), 2.71 (2H, t, J = 6.7 Hz, CH_2NH_2), 3.10–3.28 (10H, m, 5×BocNCH_2), 4.85 (0.5H, brs, 0.5×NH), 5.30 (0.5H, brs, 0.5×NH); MS (FAB) *m*/*z* 490.3678 (M + H) ($^{13}C^{12}C_{23}H_{49}N_4O_6$ requires 490.3686), 489.3651 (M + H) ($^{12}C_{24}H_{49}N_4O_6$ requires 489.3652).

N^1,N^3-Bis(1,1-dimethylethoxycarbonyl)-N^3-(3-(1,1-dimethylethoxycarbonylamino)propyl)-N^1-(3-(*N*-(2-hydroxy-3-(ω-MeOPEG550oxy)propyl)amino)propyl)propane-1,3-diamine (30) and N^1,N^3-bis(1,1-dimethylethoxycarbonyl)-N^1,N^3-bis(3-(*N*-(2-hydroxy-3-(ω-MeOPEG550oxy)propyl)amino)propyl)-N^3-(3-(1,1-dimethylethoxycarbonylamino)-propyl)propane-1,3-diamine (31). The amine **29** (959 mg, 1.97 mmol) was heated at reflux with ω-MeOPEG550 oxiranylmethyl ether **10a** (1.19 g, 1.97 mmol) in Pr^{i}OH (10 mL) for 48 h. Evaporation and chromatography (CH_2Cl_2:MeOH, 7:1) gave **31** (315 mg, 9%) as a colourless oil: ^{1}H NMR δ 1.44 (9H, s, Bu^t), 1.45 (9H, s, Bu^t), 1.46 (9H, s, Bu^t), 1.62–1.74 (6H, m, 3×CH_2CH_2 CH_2), 2.48–2.60 (6H, m, 3×NCH_2), 3.15–3.24 (10H, m, 5×NCH_2), 3.38 (6H, s, 2×OMe), 3.50–3.57 (12H, m) and 3.60–3.73 (ca. 90 H, m) (*n*×OCH_2CH_2O), 3.83 (2H, m, 2×C*H*OH); MS (FAB) $^{13}C/^{12}C$ ion clusters centred at *m*/*z* 1590, 1546, 1501, 1457, 1413, 1369 (M + H). Further elution gave **30** (460 mg, 21%) as a colourless oil: ^{1}H NMR δ 1.44 (9H, s, Bu^t), 1.45 (9H, s, Bu^t), 1.46 (9H, s, Bu^t), 1.65–1.78 (6H, m, 3×$CH_2CH_2CH_2$), 2.90–2.98 (4H, m, 2×NCH_2), 3.14–3.27 (10H, m, 5×NCH_2), 3.38 (3H, s, OMe), 3.54–3.58 (6H, m) and 3.62–3.66 (ca. 44H, m) (*n*×OCH_2CH_2O), 3.82 (1H, m, C*H*OH); MS (FAB) 1149 (M + H), 1105 (M + H), 1061.7005 (M + H) ($^{12}C_{50}H_{101}N_4O_{19}$ requires 1061.7060), 1017.6772 (M + H) ($^{12}C_{48}H_{97}N_4O_{18}$ requires 1017.6798), 973.6511 (M + H) ($^{12}C_{46}H_{93}N_4O_{17}$ requires 973.6536).

N^1,N^3-Bis(1,1-dimethylethoxycarbonyl)-N^3-(3-(1,1-dimethylethoxycarbonylamino)propyl)-N^1-(3-(*N*-(2-hydroxy-3-(ω-MeOPEG2000oxy)propyl)amino)propyl)propane-1,3-diamine (32) and N^1,N^3-bis(1,1-dimethylethoxycarbonyl)-N^1,N^3-bis(3-(*N*-(2-hydroxy-3-(ω-MeOPEG2000oxy)propyl)amino)propyl)-N^3-(3-(1,1-dimethylethoxy carbonylamino)propyl)propane-1,3-diamine (33). The amine **29** (986 mg, 2.02 mmol) was heated at reflux with **10b** (4.15 g, 2.02 mmol) in Pr^{i}OH (21 mL) for 100 h. Evaporation and chromatography (CH_2Cl_2:MeOH, 7:1) gave **33** (2.53 g, 28%) as a pale-buff waxy solid: ^{1}H NMR δ 1.44 (9H, s, Bu^t), 1.45 (9H, s, Bu^t), 1.47 (9H, s, Bu^t), 1.65–1.75 (6H, m, 3×$CH_2CH_2CH_2$), 2.48–2.59 (6H, m, 3×NCH_2), 3.08–3.26 (10H, m, 5×NCH_2), 3.39 (6H, s, 2×OMe), 3.45–3.49 (8H, m) and 3.55–3.72 (ca. 360 H, m) (*n*×OCH_2CH_2O), 3.81 (2H, m, 2×C*H*OH). Further elution gave **32** (1.80 g, 35%) as a pale-buff waxy solid: ^{1}H NMR δ 1.44 (9H, s, Bu^t), 1.45 (9H, s, Bu^t), 1.46 (9H, s, Bu^t), 1.66–1.75 (6H, m, 3×$CH_2CH_2CH_2$), 2.85–2.91 (4H, m, 2×NCH_2), 3.10–3.25 (10H, m, 5×NCH_2), 3.38 (3H, s, OMe), 3.54–3.56 (8H m) and 3.64–3.70 (ca. 186 H, m) (*n*×OCH_2CH_2O), 3.82 (1H, m, C*H*OH).

N^3-(3-Aminopropyl)-N^1-(3-(*N*-(2-hydroxy-3-(ω-MeOPEG550oxy)propyl)amino)propyl)propane-1,3-diamine tetrahydrochloride (34). Compound **30** was treated with HCl, as for the synthesis of **14**, to give **34** (100%) as a white wax: ^{1}H NMR (D_2O) δ 2.12 (6H, m, 3×$CH_2CH_2CH_2$), 3.10–3.20 (14H, m, 7×NCH_2), 3.38 (3H, s, OMe), 3.64–3.72 (ca. 45H, m, *n*×OCH_2CH_2O), 4.01 (1H, m, C*H*OD); MS $^{13}C/^{12}C$ isotope clusters centred at *m*/*z* 849, 805, 761, 717, 673, 630 (all M + H).

N^3-(3-Aminopropyl)-N^1-(3-(*N*-(2-hydroxy-3-(ω-MeOPEG2000oxy)propyl)amino)propyl)propane-1,3-diamine tetrahydrochloride (35). Compound **32** was treated with HCl, as for the synthesis of **14**, to give **35** (100%) as a white wax: ^{1}H NMR (D_2O) δ 2.15 (6H, m, 3×$CH_2CH_2CH_2$), 3.15–3.28 (14H, m, 7×NCH_2), 3.39 (3H, s, OMe), 3.65–3.76 (ca. 185H, m, *n*×OCH_2CH_2O), 4.10 (1H, m, C*H*OD).

N^3-(3-Aminopropyl)-N^1-(3-(*N*-(2-hydroxy-3-(ω-MeOPEG550oxy)propyl)amino)propyl)propane-1,3-diamine tetrahydrochloride (36). Compound **31** was treated with HCl, as for the synthesis of **14**, to give **36** (100%) as a pale-yellow wax: ^{1}H NMR (D_2O) δ 2.14–2.23 (6H, m, 3×$CH_2CH_2CH_2$), 3.10–3.25 (10H, m, 5×NCH_2), 3.35–3.46 (12H, m, 3×NCH_2 + 2×OMe), 3.62–3.85 (ca. 100H, m, *n*×OCH_2CH_2O), 4.23 (2H, m, 2×C*H*OD); MS $^{13}C/^{12}C$ isotope clusters centred at *m*/*z* 1334, 1290, 1246, 1202, 1158, 1114 (all M + H).

N^3-(3-Aminopropyl)-N^1-(3-(*N*-(2-hydroxy-3-(ω-MeOPEG 550oxy)propyl)amino)propyl)propane-1,3-diamine tetrahydrochloride (37). Compound **33** was treated with HCl, as for the synthesis of **14**, to give **37** (100%) as a white wax: ^{1}H NMR (D_2O) δ 1.97–2.13 (6H, m, 3×$CH_2C\mathit{H}_2CH_2$), 3.07–3.33 (12H, m, 6×NCH_2), 3.35–3.46 (4H, m, 3×NCH_2), 3.37 (6H, s, 2×OMe), 3.61–3.73 (ca. 370H, m, *n*×OCH_2CH_2O), 4.23 (2H, m, 2×C*H*OD).

N^1,N^3-Bis(1,1-dimethylethoxycarbonyl)-N^1,N^3-bis(3-(2-cyanoethylamino)propyl)propane-1,3-diamine (38). The diamine **6** was treated with propenenitrile, as for the synthesis of **2**, to give **38** (3.03 g, 98%) as a colourless oil: ^{1}H NMR δ 1.45 (18H, s, 2×Bu^t), 1.70 (4H, qn, *J* = 6.7 Hz, 2×$CH_2C\mathit{H}_2CH_2$), 1.76 (4H, m, $BocNCH_2C\mathit{H}_2CH_2N$-Boc + 2×NH), 2.61 (4H, t, *J* = 6.7 Hz, 2×CH_2CN), 2.62 (4H, t, *J* = 6.7 Hz, 2×CH_2N), 2.91 (4H, t, *J* = 6.7 Hz, 2×CH_2N), 3.16–3.43 (8H, m, 4×$BocNCH_2$); MS (FAB) *m*/*z* 496.3704 (M + H) ($^{13}C^{12}C_{24}H_{47}N_6O_4$ requires 496.3692), 495.3676 (M + H) ($^{12}C_{25}H_{47}N_6O_4$ requires 495.3659).

N^1,N^3-Bis(3-(*N*-(3-aminopropyl)-*N*-(1,1-dimethylethoxycarbonyl)amino)propyl)-N^1,N^3-bis(1,1-dimethylethoxycarbonyl)propane-1,3-diamine (40). The dinitrile **38** (2.92 g, 5.9 mmol) was stirred with Boc_2O (2.64 g, 12 mmol) in CH_2Cl_2 (40 mL) at 0 °C for 1.5 h and at 20 °C for 16 h. Evaporation gave crude **39** (4.1 g, 100%) as a colourless oil: ^{1}H NMR δ 1.46 (18H, s, 2×Bu^t), 1.47 (18H, s, 2×Bu^t), 1.69–1.88 (6H, m, 3×$CH_2C\mathit{H}_2CH_2$), 2.55–2.70 (4H, m, 2×CH_2CN), 3.17–3.29 (12H, m, 6×CH_2NBoc), 3.48 (4H, t, *J* = 6.9 Hz, 2×$C\mathit{H}_2CH_2CN$). This material (2.58 g, 3.7 mmol) in MeOH (30 mL) was saturated with NH_3 and was treated with H_2 (3500 Torr) in the presence of W-2 Raney Ni (2.0 g) for 65 h. The suspension was filtered (Celite®) and the solvent was evaporated from the combined filtrate and MeOH washings. Chromatography (CH_2Cl_2:MeOH:35% aq NH_3, 60:10:1) gave **40** (2.29 g, 88%) as a colourless oil: ^{1}H NMR δ 1.45 (36H, s, 4×Bu^t), 1.66 (4H, qn, *J* = 6.7 Hz, 2×$C\mathit{H}_2CH_2NH_2$), 1.71–1.78 (6H, m, 3×$BocNCH_2C\mathit{H}_2CH_2N$Boc), 1.88 (4H, brs, 2×$NH_2$), 2.70 (4H, t, *J* = 6.7 Hz, 2×$C\mathit{H}_2NH_2$), 3.17–3.43 (16H, m, 8×CH_2NBoc); MS (FAB) *m*/*z* 704.5381 (M + H) ($^{13}C^{12}C_{34}H_{71}N_6O_8$ requires 704.5367), 703.5348 (M + H) ($^{12}C_{35}H_{71}N_6O_8$ requires 703.5333).

N^1,N^3-Bis(1,1-dimethylethoxycarbonyl)-N^1-(3-(N-(1,1-dimethylethoxycarbonyl)-*N*-(3-(*N*-(2-hydroxy-3-(ω-MeOPEG550oxy)propyl)amino)propyl)amino)propyl)-N^3-(3-(*N*-(3-aminopropyl)-*N*-(1,1-dimethylethoxycarbonyl)amino)propyl)propane-1,3-diamine (41). The diamine **40** (537 mg, 760 μmol) was heated at reflux with **10a** (465 mg, 750 μmol) in Pr^{i}OH (10 mL) for 48 h. Evaporation and chromatography (CH_2Cl_2:MeOH:35% aq NH_3, 80:10:1) gave **41** (302 mg, 32%) as a pale-yellow oil: ^{1}H NMR δ 1.45 (36H, s, 4×Bu^t), 1.72–1.81 (10H, m, 5×$CH_2C\mathit{H}_2CH_2$), 2.67–2.72 (6H, m, 3×NCH_2), 3.14–3.30 (16H, m, 8×$BocNCH_2$), 3.38 (3H, s, OMe), 3.45–3.58 (8H, m) and 3.62–3.70 (ca. 50H, m) (*n*×OCH_2CH_2O + C*H*OH); MS (FAB) 1320 (M + H), 1276 (M + H), 1232 (M + H), 1188 (M + H), 1144 (M + H).

N^1-(3-(*N*-(3-(*N*-(2-Hydroxy-3-(ω-MeOPEG550oxy) propyl)amino)propyl)amino)propyl)-N^3-(3-(3-aminopropyl amino)propyl)propane-1,3-diamine hexahydrochloride (42). Compound **41** was treated with HCl, as for the synthesis of **14**, to give **42** (86%) as a white wax: ^{1}H NMR (D_2O) δ 1.93–2.08 (10H, m, 5×$CH_2C\mathit{H}_2CH_2$), 3.01–3.10 (22H, m, 11×NCH_2), 3.24 (3H, s, OMe), 3.44–3.52 (8H, m) and 3.52–3.60 (ca. 49H, m) (*n*×OCH_2CH_2O), 3.73 (1H, m, C*H*OD).

N^1,N^3-Bis(1,1-dimethylethoxycarbonyl)-N^1,N^3-bis(3-(*N*-(1,1-dimethylethoxycarbonyl)-*N*-(3-(*N*-(2-hydroxy-3-(ω-MeOPEG550oxy)propyl)amino)propyl)amino)propyl)propane-1,3-diamine (43) and N^1,N^3-bis(1,1-dimethylethoxycarbonyl)-N^3-(3-(*N*-(1,1-dimethylethoxycarbonyl)-*N*-(3-(*N*-(2-hydroxy-3-(ω-MeOPEG550oxy)propyl)amino)-propyl)amino)propyl)-N^1-(3-(*N*-(1,1-dimethylethoxycarbonyl)-*N*-(3-(*N*,*N*-bis(2-hydroxy-3-(ω-MeOPEG-550oxy)propyl)amino)propyl)amino)propyl)propane-1,3-diamine (44). The diamine **40** (845 mg, 1.2 mmol) was heated at 65 °C with **10a** (1.45 g, 2.4 mmol) in Pr^{i}OH (25 mL) for 44 h. Evaporation and chromatography (CH_2Cl_2:MeOH, 6:1) gave **44** (167 mg, 6%) as a colourless oil: ^{1}H NMR δ 1.45 (36H, s, 4×Bu^t), 1.73 (10H, m, 5×$CH_2C\mathit{H}_2CH_2$), 2.54 (10H, m, 5×NCH_2), 3.15–3.19 (16H, m, 8×CH_2NBoc), 3.38 (9H, 3×OMe), 3.54–3.60 (20H, m, 10×OCH_2), 3.60–3.70 (ca. 140H, m, *n*×OCH_2CH_2O), 3.83 (3H, m, 3×C*H*OH); MS (MALDI-TOF) *m*/*z* 2685, 2640, 2596, 2551, 2507, 2463, 2419 (all M + H). Further elution gave **43** (262 mg, 12%) as a colourless oil: ^{1}H NMR δ 1.45 (36H, s, 4×Bu^t), 1.73 (10H, m, 5×$CH_2C\mathit{H}_2CH_2$), 2.48 (8H, m, 4×NCH_2), 3.10–3.20 (16H, m, 8×CH_2NBoc), 3.38 (6H, 2×OMe), 3.45–3.60 (10H, m, 5×OCH_2), 3.61–3.70 (ca. 100H, m, *n*×OCH_2CH_2O), 3.85 (2H, m, 2×C*H*OH).

N^1,N^3-Bis(3-(*N*-(3-(*N*-(2-hydroxy-3-(ω-MeOPEG550oxy) propyl)amino)propyl)amino)propyl)propane-1,3-diamine hexahydrochloride (45). Compound **43** was treated with HCl, as for the synthesis of **14**, to give **45** (100%) as a white glass: ^{1}H NMR (D_2O) δ 1.95–2.03 (10H, m, 5×$CH_2C\mathit{H}_2CH_2$), 3.04 (24H, ca. t, *J* = 7.6 Hz, 12×NCH_2), 3.22 (6H, 2×OMe), 3.43–3.48 (ca. 14H, m, 7×OCH_2), 3.53–3.63 (ca. 120H, m, *n*×OCH_2CH_2O), 3.99 (2H, m, 2×C*H*OH).

Bis(3-(1,1-dimethylethoxycarbonylamino)propyl)amine (47). 2-(*t*-Butoxycarbonyloxyimino)-2-phenylacetonitrile (BocON) (5.78 g, 23.5 mmol) in THF (50 mL) was added during 1.5 h to $(H_2N(CH_2)_3)_2NH$ **46** (1.40 g, 10.7 mmol) in THF (20 mL) at 0 °C. The mixture was then stirred at 20 °C for 40 min. Evaporation and chromatography (EtOAc:MeOH, 4:1) gave **47** (1.87 g, 54%) as a white solid: mp 70–71 °C (lit.[56] mp 70–72 °C); ^{1}H NMR δ 1.44 (18H, s, 2×Bu^t), 1.64 (4H, qn, *J* = 6.4 Hz, 2×$CH_2C\mathit{H}_2CH_2$), 1.65 (1H, br, NH), 2.65 (4H, t, *J* = 6.4 Hz, 2×$C\mathit{H}_2NH$), 3.22 (4H, m, 2×CH_2NBoc), 5.28 (2H, m, 2×NHBoc); MS (FAB) *m*/*z* 333.2580 (M + H), ($^{13}C^{12}C_{15}H_{34}N_3O_4$ requires 333.2583), 332.23548 (M + H), ($^{12}C_{16}H_{34}N_3O_4$ requires 332.2549).

***N*,*N*-Bis(3-(1,1-dimethylethoxycarbonylamino)propyl)-2-cyanoethylamine (48).** The amine **47** (1.11 g, 3.4 mmol)

was heated at reflux with propenenitrile (1.5 g, 21 mmol) in THF (10 mL) for 60 h. Evaporation and chromatography (EtOAc) gave **48** (1.04 g, 80%) as a pale-yellow oil (lit.[57] oil): ^{1}H NMR δ 1.44 (18H, s, 2×Bu^t), 1.64 (4H, qn, J = 6.6 Hz, 2×$CH_2CH_2CH_2$), 2.45 (2H, t, J = 6.9 Hz, CH_2CN), 2.48 (4H, t, J = 6.7 Hz, 2×CH_2NH), 2.76 (2H, t, J = 7.0 Hz, CH_2CH_2CN), 3.18 (4H, m, 2×CH_2NBoc), 5.12 (2H, m, 2×NHBoc); MS (FAB) m/z 386.2853 (M + H) ($^{13}C^{12}C_{18}H_{37}N_4O_4$ requires 386.2848), 385.2818 (M + H) ($^{12}C_{19}H_{37}N_4O_4$ requires 385.2815).

3-Amino-*N*,*N*-bis(3-*t*-butoxycarbonylaminopropyl)propylamine (49). W-2 Raney Ni (1.0 g) was added to **48** (971 mg, 2.5 mmol) in MeOH (35 mL). NH_3 was passed through the suspension for 30 min at 0 °C. The mixture was treated with H_2 (3000 Torr) for 72 h. The suspension was filtered (Celite®). Evaporation of the solvent from the combined filtrate and MeOH washings gave crude **49** (1.0 g, 99%) as a pale-blue oil, which was used immediately: ^{1}H NMR δ 1.43 (20H, s + br, 2×Bu^t + NH_2), 1.63–1.66 (6H, m, 3×$CH_2CH_2CH_2$), 2.44–2.75 (8H, m, 4×CH_2N), 3.12–3.20 (4H, m, 2×CH_2NBoc), 5.28 (2H, br, 2×NH); MS m/z 390.3160 (M + H) ($^{13}C_1C_{18}H_{41}N_4O_4$ requires 390.3161), 389.3126 (M + H) ($C_{19}H_{41}N_4O_4$ requires 389.3128).

ω-MethoxyPEG 550 chloroformate (51). MeOPEG550 **50** (10.0 g, 18 mmol) was stirred with phosgene (CAUTION, 20% solution in toluene, 100 mL) in CH_2Cl_2 (150 mL) for 48 h. This solution was stored until required. The solvents and excess reagents were evaporated from required aliquots to give **51** as a colourless oil: ^{1}H NMR δ 3.38 (3H, s, Me), 3.54 (2H, m, CH_2), 3.60–3.70 (ca. 45H, m, $(CH_2)_n$), 3.78 (2H, m, CH_2), 4.46 (2H, m, CH_2OCO); MS m/z 777 (8%) (M + Na), 733 (10%) (M + Na), 689 (12%) (M + Na), 645 (14%) (M + Na), 601 (14%) (M + Na), 557 (12%) (M + Na), 513 (8%) (M + Na), 755 (7%) (M + H), 711 (8%) (M + H), 667 (10%) (M + H), 623 (12%) (M + H), 579 (13%) (M + H), 535 (12%) (M + H).

***N*,*N*-Bis(3-(1,1-dimethylethoxycarbonylamino)propyl)-3-(ω - MeOPEG550oxycarbonylamino)propylamine (52).** The crude amine **49** (94 mg, 240 μmol) was stirred with ω-MeOPEG550 chloroformate **51** (192 mg, 300 μmol) and Et_3N (50 mg, 500 μmol) in CH_2Cl_2 (5 mL) for 16 h. Evaporation and chromatography (CH_2Cl_2:MeOH, 7:1) gave **52** (65 mg, 28%) as a colourless oil: ^{1}H NMR δ 1.44 (18H, s, 2×Bu^t), 1.63–1.66 (6H, m, 3×$CH_2CH_2CH_2$), 2.44–2.50 (6H, m, 3×CH_2N), 3.12–3.25 (6H, m, 2×CH_2NCO_2R), 3.38 (3H, s, OMe), 3.54–3.56 (2 H, m) and 3.62–3.70 (ca. 40H, m) (n×OCH_2CH_2O), 4.20 (2H, m, CH_2O_2CN), 5.27 (2H, br, 2×NHBoc), 5.64 (1H, br, NH); MS (FAB) m/z 1019 (25%) (M + H), 975 (40%) (M + H), 931 (46%) (M + H), 887 (55%) (M + H), 843 (57%) (M + H), 799 (50%) (M + H), 755 (44%) (M + H), 711 (30%) (M + H).

ω-MeOPEG550 *N*-(3-(*N*,*N*-di(3-aminopropyl)amino)-propyl)carbamate trihydrochloride (53). Compound **52** was treated with HCl, as for the synthesis of **14**, to give **53** (100%) as a colourless glass: ^{1}H NMR (D_2O) δ 1.75–1.81 (2H, m, $CH_2CH_2CH_2$), 1.92–2.02 (4H, m, 2×$CH_2CH_2CH_2$), 2.94 (4H, t, J = 7.7 Hz, 2×CH_2N), 3.07–3.16 (8H, m, 4×CH_2N), 3.22 (3H, s, OMe), 3.46–3.48 (2H, m) and 3.54–3.61 (ca. 40H, m) (n×OCH_2CH_2O), 4.06 (2 H, m, CH_2O_2CN).

Bis(3-trifluoroacetamidopropyl)amine (54). $(H_2N(CH_2)_3)_2$NH **46** (15.0 g, 115 mmol) was stirred with EtO_2CCF_3 (33.4 g, 235 mmol) in EtOH (100 mL) for 60 h. The solvent was evaporated. The residue, in EtOAc, was washed with water and with brine and was dried. Evaporation gave **54** (37.0 g, 100%) as a pale-yellow solid: mp 168–171 °C; ^{1}H NMR δ 1.74 (4H, qn, J = 6.6 Hz, 2×$CH_2CH_2CH_2$), 2.00 (1H, br, NH), 2.73 (4H, t, J = 6.3 Hz, 2×CH_2NH), 3.44 (4H, t, J = 6.3 Hz, 2×CH_2NCOCF_3), 8.67 (2H, br, 2×$NHCOCF_3$); ^{13}C NMR δ 27.8, 39.3, 47.9, 116.1 (q, $J_{C\text{-}F}$ = 288 Hz, CF_3), 157.3 (q, $J_{C\text{-}F}$ = 37 Hz, $COCF_3$); MS (FAB) m/z 325.1192 (M + H) ($^{13}C^{12}C_9H_{16}F_6N_3O_2$ requires 325.1180), 324.1162 (M + H) ($^{12}C_{10}H_{16}F_6N_3O_2$ requires 324.1146).

1,1-Dimethylethyl *N*,*N*-di(3-aminopropyl)carbamate (56). Compound **54** (270 mg 840 μmol) was stirred with Boc_2O (182 mg, 840 μmol) in CH_2Cl_2 (5 mL) for 2 days. Evaporation gave crude **55** (360 mg, 100%) as a colourless oil: ^{1}H NMR δ (23 °C) 1.47 (9H, s, Bu^t), 1.77 (4H, m, 2×$CH_2CH_2CH_2$), 3.25 (8H, m, 4×CH_2N), 6.90 (1H, br, NH), 8.24 (1H, br, NH); ^{1}H NMR δ (−40 °C) 1.47 (9H, s, Bu^t), 1.61 (2H, m, 2×$CH_2CH_2CH_2$), 1.83 (2H, qn, J = 7.4 Hz, $CH_2CH_2CH_2$), 3.22 (2H, t, J = 7.0 Hz, CH_2N), 3.28–3.35 (4H, m, 2×CH_2N), 3.37 (2H, td, J = 7.0, 5.4 Hz, CH_2NCOCF_3), 7.55 (1H, m, NH), 8.69 (1H, t, J = 5.4 Hz, NH); MS (FAB +ve ion) m/z 446 (M + Na), 424 (M + H); MS (FAB −ve ion) m/z 422 (M−H). This material **55** (310 mg, 730 μmol) in MeOH (6 mL) was heated with 35% aq NH_3 (3 mL) at 60 °C in a sealed vessel for 5 h. Evaporation and chromatography (CH_2Cl_2:MeOH:35% aq NH_3, 10:5:1) gave **56** (155 mg, 91%) as a colourless oil (lit.[58] oil): ^{1}H NMR ($(CD_3)_2SO$) δ 1.41 (9H, s, Bu^t), 1.75 (4H, tt, J = 7.3, 6.8 Hz, 2×$CH_2CH_2CH_2$), 2.76 (4H, t, J = 7.3 Hz, 2×CH_2NH_2), 3.18 (4H, t, J = 6.8 Hz, 2×CH_2NBoc), 3.38 (4H, brs, 2×NH_2); MS (FAB) m/z 254 (M + Na), 232 (M + H), 132 (M + H−Boc).

1,1-Dimethylethyl *N*,*N*-di(3-(2-cyanoethylamino)propyl)-carbamate (57). The diamine **56** (2.4 g, 10.5 mmol) was stirred with propenenitrile (1.5 g, 28 mmol) in MeOH (60 mL) for 40 h. The solvent was evaporated. The residue, in CH_2Cl_2, was washed with water and with brine and was dried (fraction A). The water was evaporated from the combined aqueous phases. The residue was extracted with MeOH (30 mL). Propenenitrile (1.8 g, 34 mmol) was added and the mixture was stirred for 4 days. K_2CO_3 (1.5 g, 11 mmol) was added and the mixture was stirred for 14 h. The solvent was evaporated. The residue, in CH_2Cl_2, was washed with water and with brine and was dried. This solution was combined with fraction A. Evaporation and chromatography (CH_2Cl_2:MeOH, 4:1→3:1) gave **57** (1.04 g, 30%) as a pale-yellow oil: ^{1}H NMR δ 1.46 (9H, s, Bu^t), 1.72 (6H, m, 2×$CH_2CH_2CH_2$ + 2×NH), 2.50 (4H, t, J = 6.5 Hz, 2×CH_2CN), 2.63 (4H, t, J = 6.6 Hz, 2×CH_2CH_2CN), 2.89 (4H, t, J = 6.6 Hz, 2×CH_2N), 3.22 (4H, m, 2×CH_2NBoc); MS (FAB) m/z 339.2588 (M + H) ($^{13}C^{12}C_{16}H_{32}N_5O_2$

requires 339.2589), 338.2566 (M + H) ($^{12}C_{17}H_{32}N_5O_2$ requires 338.2556), 238 (M + H−Boc).

1,1-Dimethylethyl *N*,*N*-di(3-(*N*-phenylmethoxycarbonyl)-*N*-(3-(phenylmethoxycarbonylamino)propyl)amino)propyl)carbamate (59). The dinitrile **57** (1.00 g, 3.0 mmol) in MeOH (20 mL) was saturated with NH_3 and was then treated with H_2 (3500 Torr) in the presence of W-2 Raney Ni (1.0 g) for 60 h. The suspension was filtered. The solvent was evaporated from the combined filtrate and MeOH washings to give crude **58**, which was stirred with Cbz_2O (4.23 g, 15 mmol) in THF (30 mL) for 36 h. Evaporation and chromatography (EtOAc/hexane, 1:1 →2:1) gave **59** (1.57 g, 65%) as a colourless oil: ^{1}H NMR δ 1.40 (9H, s, Bu^t), 1.61–1.78 (8H, m, 4×$CH_2CH_2CH_2$), 3.05–3.38 (16H, m, 8×CH_2N), 5.09 (8H, s, 4×$PhCH_2$), 5.68 (2H, br, 2×NH), 7.33 (20H, m, 4×Ph-H_5); MS (FAB) *m*/*z* 904 (M + Na), 883.4661 (M + H) ($^{13}C^{12}C_{48}H_{64}N_5O_{10}$ requires 883.4687), 882.4646 (M + H) ($^{12}C_{49}H_{64}N_5O_{10}$ requires 882.4653), 782 (M + H−Boc).

Di(3-(*N*-phenylmethoxycarbonyl)-*N*-(3-(phenylmethoxycarbonylamino)propyl)amino)propyl)amine (60). HCl was passed through **59** (1.33 g, 1.5 mmol) in CH_2Cl_2 (40 mL) for 30 min. The solvent and excess reagent were evaporated. The residue, in MeOH (10 mL), was treated with aq NaOH (5 M, 900 μL, 4.5 mmol). The solvents were evaporated. The residue, in CH_2Cl_2, was washed with water and with brine and was dried. Evaporation gave **60** (1.07 g, 91%) as a colourless oil: ^{1}H NMR δ 1.55–1.77 (8H, m, 4×$CH_2CH_2CH_2$), 2.10 (1H, br, NH), 2.40–2.58 (8H, m, 4×CH_2N), 3.06–3.39 (8H, m, 4×CH_2NCbz), 5.10 (8H, s, 4×$PhCH_2$), 5.69 (2H, br, 2×NH), 7.33 (20H, m, 4×Ph-H_5); MS (FAB) *m*/*z* 783.4168 (M + H) ($^{13}C^{12}C_{43}H_{56}N_5O_8$ requires 783.4162), 782.4139 (M + H) ($^{12}C_{44}H_{56}N_5O_8$ requires 782.4129).

6-Bromo-*N*-(2-(1,1-dimethylethoxycarbonylamino)ethyl)-hexanamide (63). 6-Bromohexanoic acid **62** (1.53 g, 7.9 mmol) was stirred with oxalyl chloride (5.0 g, 39 mmol) and DMF (70 μL) in CH_2Cl_2 (15 mL) at 0 °C for 30 min and at 20 °C for 100 min. The solvent and excess reagent were evaporated. CH_2Cl_2 (5 mL) was added and evaporated. The residue was stirred with $BocNH(CH_2)_2NH_2$ **61**[32] (2.04 g, 12.5 mmol) and Et_3N (2.02 g, 10 mmol) in CH_2Cl_2 (20 mL) at 0 °C for 30 min and at 20 °C for 90 min. Washing (water, brine), drying, evaporation and recrystallisation (EtOAc) gave **63** (1.79 g, 68%) as white crystals: mp 104–106 °C; ^{1}H NMR δ 1.44 (9H, m, Bu^t), 1.47 (2H, m, 4-H_2), 1.66 (2H, qn, *J* = 7.2 Hz, 3-H_2), 1.87 (2H, qn, *J* = 7.2 Hz, 5-H_2), 2.20 (2H, qn, *J* = 7.3 Hz, 2-H_2), 3.27–3.37 (4H, m, NCH_2CH_2N), 3.41 (2H, qn, *J* = 6.8 Hz, 6-H_2), 5.04 (1H, br, NH), 6.38 (1H, br, NH); MS (FAB) *m*/*z* 340.1118 (M + H) ($^{13}C^{12}C_{12}H_{26}{}^{81}BrN_2O_3$ requires 340.1139), 339.1097 (M + H) ($^{12}C_{13}H_{26}{}^{81}BrN_2O_3$ requires 339.1106), 338.1113 (M + H) ($^{13}C^{12}C_{12}H_{26}{}^{79}BrN_2O_3$ requires 338.1160), 337.1121 (M + H) ($^{12}C_{13}H_{26}{}^{79}BrN_2O_3$ requires 337.1127).

***N*-(2-(1,1-Dimethylethoxycarbonylamino)ethyl-6-(di(3-(*N*-phenylmethoxycarbonyl)-*N*-(3-(phenylmethoxycarbonylamino)propyl)amino)propyl)amino)hexanamide (64).** Compound **60** (1.07 g, 1.4 mmol) was stirred with **63** (693 mg, 2.1 mmol) and K_2CO_3 (285 mg, 2.0 mmol) in DMF (4.0 mL) at 85 °C for 20 h. The solvent was evaporated. The residue, in CH_2Cl_2, was washed with water and with brine and was dried. Evaporation and chromatography (EtOAc:MeOH, 6:1→4:1) gave **64** (965 mg, 67%) as a colourless oil: ^{1}H NMR δ 1.24–1.42 (4H, m, 3,4-H_4), 1.45 (9H, s, Bu^t), 1.59–1.68 (10H, m, 5-H_2 + 4×$NCH_2CH_2CH_2N$), 2.14 (2H, m, 2-H_2), 2.25–2.40 (6H, m, 3×CH_2N), 3.15–3.29 (16H, m, 8×CH_2NCO), 5.10 (8H, s, 4×$PhCH_2$), 5.74 (1H, m, NH), 6.43 (1H, m, NH), 7.32 (20H, m, 4×Ph-H_5); ^{13}C NMR δ 25.2, 25.6, 25.8, 26.1, 26.4, 27.0, 28.1, 28.4, 28.9, 36.4, 37.7, 38.3, 40.4, 44.4, 45.2, 46.0, 51.2, 51.3, 53.4, 66.5, 67.1, 79.5, 127.8, 128.0, 128.3, 128.5, 136.0, 136.5, 136.7, 156.0, 156.6, 156.9, 173.7; MS (FAB) *m*/*z* 1040.5960 (M + H) ($^{13}C_2{}^{12}C_{55}H_{80}N_7O_{11}$ requires 1040.5983), 1039.5912 (M + H) ($^{13}C^{12}C_{56}H_{80}N_7O_{11}$ requires 1039.5949), 1038.5893 (M + H) ($^{12}C_{57}H_{80}N_7O_{11}$ requires 1038.5915).

***N*-(2-Aminoethyl)-6-(di(3-(*N*-phenylmethoxycarbonyl)-*N*-(3-(phenylmethoxycarbonylamino)propyl)amino)propyl)amino)hexanamide (65).** HCl was passed through **64** (860 mg, 830 μmol) in CH_2Cl_2 (25 mL) for 30 min. The solvent and excess reagent were evaporated. The residue, in MeOH (20 mL), was treated with aq NaOH (5 M, 660 μL, 3.3 mmol). The solvents were evaporated. The residue, in CH_2Cl_2, was washed with water and with brine and was dried. Evaporation gave **65** (676 mg, 67%) as a colourless oil: ^{1}H NMR δ 1.25–1.33 (4H, m, 3,4-H_4), 1.61–1.68 (10H, m, 5-H_2 + 4×$NCH_2CH_2CH_2N$), 2.03–2.16 (4H, m, 2-H_2 + CH_2NH_2), 2.25–2.35 (6H, m, 3×CH_2N), 2.78 (2H, br, NH_2), 3.15–3.26 (14H, m, 7×CH_2NCO), 5.10 (8H, s, 4×$PhCH_2$), 5.75 (1H, m, NH), 6.27 (1H, m, NH), 7.32 (20H, m, 4×Ph-H_5); MS (FAB) *m*/*z* 939.5412 (M + H) ($^{13}C^{12}C_{51}H_{72}N_7O_9$ requires 939.5425), 938.5383 (M + H) ($^{12}C_{52}H_{72}N_7O_9$ requires 938.5391).

6-(Di(3-(*N*-phenylmethoxycarbonyl)-*N*-(3-(phenylmethoxycarbonylamino)propyl)amino)propyl)amino)-N-(2-(ω-MeOPEG550oxycarbonylamino)ethyl)hexanamide (66). The amine **65** (408 mg, 440 μmol) was stirred with **51** (320 mg, 500 μmol) and Et_3N (120 mg, 1.2 mmol) in CH_2Cl_2 (6.0 mL) for 3 h. Further **51** (45 mg, 70 μmol) in CH_2Cl_2 (1.0 mL) was added and the mixture was stirred for 4 h. Evaporation and chromatography (CH_2Cl_2:MeOH, 20:1) gave **66** (274 mg, 42%) as a colourless oil: ^{1}H NMR δ 1.25–1.38 (4H, m, 3,4-H_4), 1.60–1.70 (10H, m, 5-H_2 + 4×$NCH_2CH_2CH_2N$), 2.14 (2H, m, 2-H_2), 2.24–2.38 (6H, m, 3×CH_2N), 3.15–3.31 (16H, m, 8×CH_2N), 3.38 (3H, s, OMe), 3.53–3.56 (2H, m, OCH_2), 3.62–3.82 (ca. 36H, m, *n*×OCH_2CH_2O), 4.18 (2H, m, CH_2O_2C), 5.10 (8H, s, 4×$PhCH_2$), 5.52 (1H, m, NH), 5.71 (1H, m, NH), 7.33 (20H, m, 4×Ph-H_5); MS (FAB) ^{13}C/^{12}C isotope clusters centred at *m*/*z* 1481 (3%) (M + H), 1437 (4%) (M + H), 1391 (5%) (M + H), 1349 (6%) (M + H), 1305 (6%) (M + H), 1261 (5%) (M + H), 1217 (4%) (M + H).

6-(*N*,*N*-Di(3-(3-aminopropylamino)propyl)amino)-*N*-(2-(ω-MeOPEG550oxycarbonylamino)ethyl)hexanamide (67). The protected construct **66** (75 mg, 50 μmol) was

treated with H_2 (3500 Torr) in MeOH (5 mL) in the presence of Pearlman's catalyst (50 mg) for 40 h. The mixture was diluted with MeOH (10 mL) and filtered (Celite®). Aqueous HCl (5 M, 2.0 mL, 10 mmol) was added. Evaporation and freeze-drying gave **67** (57 mg, 100%) as a colourless glass: ^{1}H NMR (D_2O) δ 1.60–1.71 (4H, m, 3,4-H_4), 2.06–2.15 (10H, m, 4×NCH_2CH_2-CH_2N + 5-H_2), 2.27 (2H, t, J = 7.0 Hz, 2-H_2), 3.09–3.30 (22H, m, 11×CH_2N), 3.38 (3H, s, OMe), 3.62–3.66 (2H, m, OCH_2), 3.70–3.75 (ca. 36H, m, n×OCH_2CH_2O), 4.22 (2H, m, CH_2O_2C); MS (FAB) $^{13}C/^{12}C$ isotope clusters centred at m/z 944 (15%) (M + H), 900 (22%) (M + H), 856 (32%) (M + H), 812 (35%) (M + H), 768 (30%) (M + H), 724 (30%) (M + H), 680 (22%) (M + H).

DNA binding studies

Plasmid DNA (pCMVcat) was a generous gift from GeneMedicine, The Woodlands, Texas, USA. The experiments were performed in HEPES-buffered saline (HBS), containing 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES) (20 mM) and NaCl (150 mM); the pH was adjusted to 7.4 by addition of aq NaOH (1.0 M). The experiment at pH 5.5 was performed in MES-buffered saline, containing morpholine-4-ethanesulfonic acid (MES) (20 mM) and NaCl (150 mM); the pH was adjusted to 5.5 by addition of aq HCl (1.0 M). Fluorescence was measured on a Perkin Elmer LS-50B luminescence spectrometer with excitation wavelength 260 nm and emission wavelength 600 nm.

For each assay, aqueous ethidium bromide (3.0 μL, 0.5 mg mL^{-1}) was added to DNA (6 μg) in HBS (3 mL) and the mixture was stirred in a fluorimeter cuvette until no change in fluorescence occurred (usually 2–3 min). The test construct in HBS (4.0 μL) was added with continuous stirring. The new fluorescence reading was taken 60 s later. An additional aliquot of test construct solution was added. This sequence was repeated until no further change in fluorescence is observed or when the total volume of construct solution added is 2.5% of the total volume of the system. The percent relative fluorescence intensity was determined using

$$F_r = (F_{obs} - F_e)/(F_0 - F_e) \quad (1)$$

F_r is the relative fluorescence, F_{obs} is the measured fluorescence, F_e is the fluorescence of ethidium bromide without DNA and F_0 is the initial fluorescence of the ethidium–DNA complex before any construct is added.

DNA delivery studies in vivo

Female C3H mice were obtained from Charles River at 6–7 weeks of age and then allowed to acclimatise for a further week prior to experiments. Before implanting murine fibrosarcoma RIF-1 cells[59] the fur from the lower half of the mouse back was removed using clippers. The RIF-1 cells were previously cultured in RPMI 1640 medium supplemented with 15% fetal calf serum, penicillin and streptomycin, and were not passaged more than 7 times prior to trypsinisation for implantation. On the day of implantation the mice were lightly anaesthetised using intraperitoneal Hypnorm (mix of fentanyl citrate (0.315 mg mL^{-1}) and fluanisone (10 mg mL^{-1}), 0.05 mL per mouse) and an injection of 0.05 mL PBS containing 2×10^5 RIF-1 cells made intradermally midway along the back of the mouse (approximately 2 cm from the base of the tail). Tumours were visible after approximately 7 days and were ready for gene delivery experiments 12–14 days post-implantation. One day before treatment with DNA, the mice were weighed and the dimensions of the tumour were recorded. The average tumour was approximately 6–7 mm in diameter and 250 mg in weight at this stage. The mice were then randomly assigned to the different treatment cages.

The plasmid pCMVCAT was supplied in bulk as a gift from GeneMedicine Inc. (now Valentis Inc.). Injections were made of plasmid DNA (50 μg) in an isotonic solution (20 μL), either alone or with the test polyamine–PEG construct. After 48 h, the animals were killed and the excised tumours were weighed, then homogenised for 30 s in a screw cap sterile tube containing lysis buffer (1.5 mL, potassium phosphate buffer (0.1 M, pH 7.8), 0.2% Triton X-100) using an Ultraturax homogeniser. The tissue suspension was then spun at 12,000×g for 15 min at 4 °C to remove cellular debris. This was followed by two freeze–thaw cycles, freezing at −70 °C and then thawing at room temperature. The protein content of this supernatant was analysed using a Bio-Rad D_c Protein assay kit. Supernatant containing 10–100 μg of protein was assayed for the bacterial enzyme chloramphenicol acetyltransferase type I (CAT), using a commercial ELISA colourimetric enzyme immunoassay from Boehringer-Mannheim (Mannheim, Germany). Expression was normalised with respect to either total tissue weight.

Statistical comparisons were performed using non-parametric Kruskal–Wallis and post-hoc Mann–Whitney tests (Minitab for Windows version 11). These rank order tests make no assumptions regarding the type of distribution of values around the mean (e.g., normal, log-normal, etc.), and are valid for use in comparing two independent groups that contain the same or different numbers of values. Experimental group medians were considered significantly different from each other or control group medians if the p value was less than 0.05. The data are presented in Figure 5 as box plots. The box represents the data between the lower and upper quartiles, that is, 50% of data, and lines (whiskers) are drawn to represent the lower and upper extremes. The median is represented by a horizontal line.

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