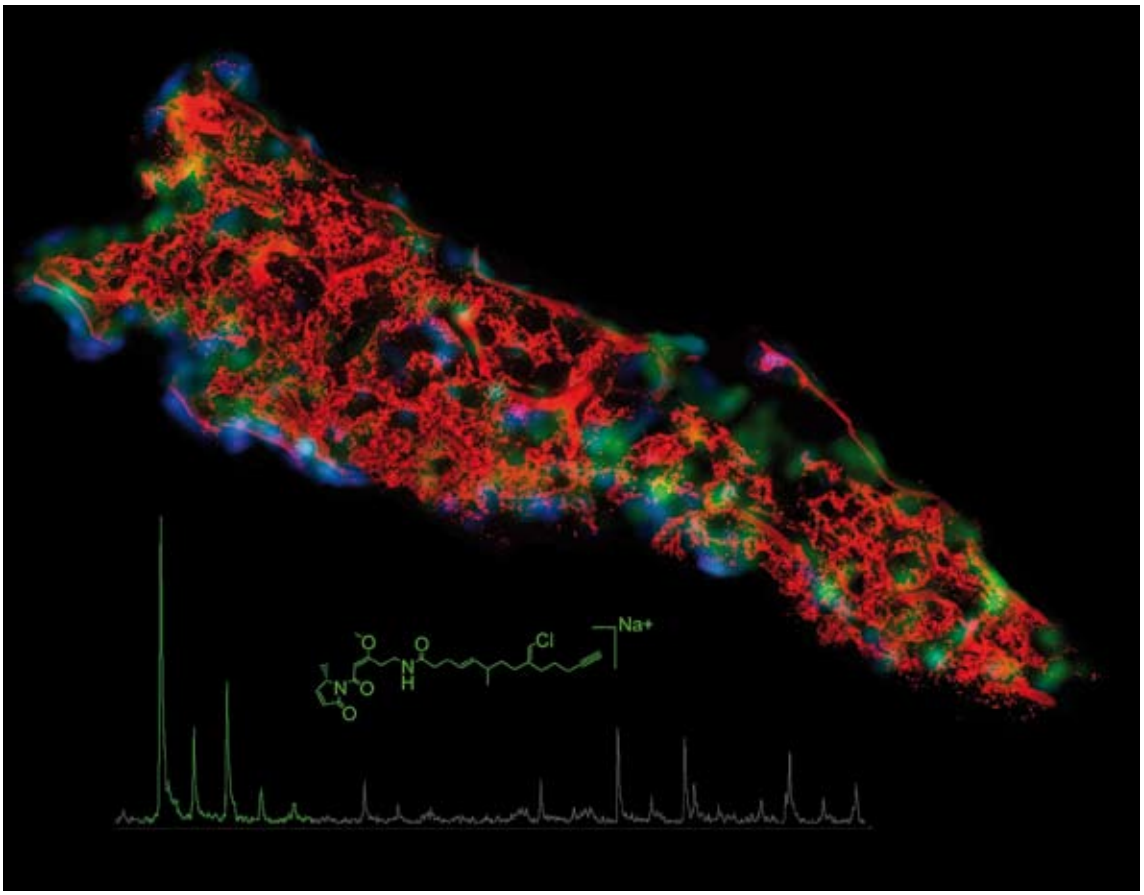
Molecular BioSystems

This article was published as part of the

2008 Emerging Investigators Issue

Highlighting the work of outstanding young scientists at the chemical- and systems-biology interfaces

Please take a look at the full table of contents to access the other papers in this issue.



Acid cleavable PEG-lipids for applications in a ternary gene delivery vector†‡

John B. Wong,[a] Stephanie Grosse,[b] Alethea B. Tabor,[a] Stephen L. Hart[b] and Helen C. Hailes*[a]

Received 3rd January 2008, Accepted 27th March 2008
First published as an Advance Article on the web 15th April 2008
DOI: 10.1039/b719782a

A novel class of pH-sensitive PEG lipids bearing acid-cleavable acetal linkages and short PEG chains have been synthesised and used in ternary vector formulations. The cleavage pH was influenced by structural components including the terminal PEG moiety and spacer length.

Introduction

Gene therapy has significant potential as treatment strategies for inherited or aquired diseases, however, an efficient vector system is required to deliver the gene of interest into target cells. Synthetic, non-viral, vectors offer advantages over viral systems in terms of their greater nucleic acid packaging capacity, lower immunogenicity and greater safety.[1] Several synthetic vectors have been described including polycationic polymers such as polyethylenimine (PEI), dendrimers, and cationic lipids (lipoplexes).[2–5] However, one major problem to date has been their poor transfection efficiency relative to viral vectors, particularly *in vivo*. To improve the efficacy and selectivity of synthetic vectors, targeting to cell-surface receptors using peptides, polysaccharides or antibodies has been carried out.[6] Ternary, three-component synthetic vectors (lipopolyplexes) have also recently been described by several groups[7] including Hart *et al*.[8] The targeted system was comprised of a lipid (L) (Lipofectin™ composed of a 1 : 1 mixture of DOTMA **1** and DOPE **2**)[9] and integrin-targeting peptide **3** (I) together with plasmid DNA (D) (Fig. 1) which combined electrostatically on mixing in solution to form the LID vector.[8] The peptide **3** was comprised of a Lys_{16} domain, which bound to the DNA, and a cyclic domain (CRRETAWAC) which specifically bound to a cell surface protein, the $\alpha_5\beta_1$ integrin.[8,10] The ternary formulation formed discrete particles and displayed high transfection efficiency and low toxicity *in vitro* and *in vivo*.[8,11–13] The stoichiometry and structure of the LID complex has also been studied which indicated that the peptide and plasmid DNA form a tightly condensed DNA–peptide inner core, surrounded by a disordered lipid layer, from which the integrin-targeting sequence of the peptide partially protrudes.[14]

Cationic lipids (cytofectins) in lipopolyplexes can be involved in DNA compaction, together with the peptide component, interact with anionic cell surface receptors, and enhance endosomal release. DOPE is believed to enhance liposome fusion with the endosomal membrane leading to endosomal escape.[2] Alteration of the chain length, linking moiety to the glycerol backbone and cationic head group in DOTMA can influence the transfection efficiency of both binary lipoplex and ternary lipopolyplex formulations such as LID. Indeed there have been several studies which have investigated different head groups or diether-linked chain length analogues in lipoplex[15–22] and lipopolyplex systems.[23]

Despite the recent developments in synthetic gene delivery vectors, one key problem is the stability of nonviral vector systems for systemic delivery where prolonged plasma circulation of the vector is essential.[24,25] Previous work has indicated that the tethering of poly(ethylene glycol) (PEG) moieties can provide a steric barrier, which shields the complexes from interactions with biological fluids, enhancing stabilisation *in vivo*.[25–27] PEGs are often used in the range 1000–5000 Da to give lipid conjugates (used as 2–10 mol% formulations) which have enhanced lipoplex stability in serum.[27–32] However, transfection efficiencies are frequently lower due to the PEG acting as a steric barrier.[27–32]

We have recently synthesised cationic lipids possessing short PEG-OH groups at the head group.[33] These cytofectins were shown to form compact vesicles, be more stable in the presence of serum than DOTMA, and give good gene delivery in lipopolyplexes. Notably, a cytofectin with a pendant PEG4-OH, rather than PEG6-OH, moiety was particularly affective

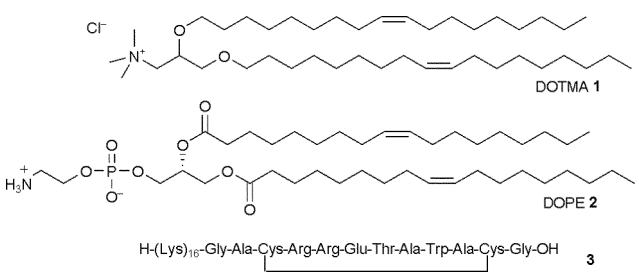


Fig. 1 LID vector components.

[a] *Department of Chemistry, University College London, 20 Gordon Street, London, UK WC1H 0AJ. E-mail: h.c.hailes@ucl.ac.uk; Fax: +44 20 7679 7463; Tel: +44 20 7679 4654*
[b] *Wolfson Centre for Gene Therapy of Childhood Disease, UCL Institute of Child Health, 30 Guilford Street, London, UK WC1N 1EH*

† This article is part of a *Molecular BioSystems* 'Emerging Investigators' issue highlighting the work of outstanding young scientists at the chemical- and systems-biology interfaces.

‡ Electronic supplementary information (ESI) available: Performance of lipids **19–25** compared to Lipofectin™ (**1** and **2**) in lipopolyplex formulations for 16HBE14o-, bEND.3 or PVSMCs cell lines. See DOI: 10.1039/b719782a

in lipopolyplexes, probably because it provided a balance between enhanced stability properties, but peptide targeting in the ternary complex was not affected when formulated with short PEG conjugates for steric reasons.[33]

The use of acid-labile linkers in synthetic lipids has also proven to be an attractive strategy for applications in lipoplexes to aid endosome destabilisation: the decrease in pH in the endosomal environment results in lipid cleavage.[34] This approach has been effective and acid-labile linkers reported include vinyl ethers,[35] ortho esters[36,37] and hydrazones.[38] When utilising PEG–lipid conjugates, they should remain stable until they reach the target site, and then release the payload from the endosome after cell internalisation. In a few lipoplex systems the incorporation of PEG stabilising groups into cytofectins *via* acid-cleavable linkers have been described. Examples include PEG-diplasmenyl lipids[35] and POD ortho esters.[39] Non-ionic acetal cleavable PEG-estradiol conjugates have also been described for use in drug delivery.[40] The rationale for the design of pH-sensitive PEG-lipids is to exploit the intrinsic low pH (pH 5.0–6.5) within endosomes, to induce hydrolysis of the acid-sensitive linkages and trigger shedding of the PEG moieties. Such lipids should be stable under neutral conditions but undergo destabilisation and become fusogenic under acidic conditions, thus leading to release of the encapsulated contents.

In our previous work we have improved lipopolyplex particle stability for *ex vivo* or *in vivo* applications *via* attachment of short PEG chains to the lipid head group, to ensure minimal disruption of peptide targeting. Our aim was now to develop a series of pH-sensitive PEG-lipids for use in lipopolyplexes that are readily synthesised and undergo hydrolysis under distinct physiological conditions. To aid endosome destabilisation whilst maintaining peptide targeting, we have prepared a novel class of lipids based on structure **4** (Fig. 2) that possess acetal linkages between the cationic headgroups and short PEG moieties. The effect of varying the PEG unit and spacer length, lipid chain length, as well as the nature of the PEG units has also been evaluated.

Results and discussion

Several acid labile moieties have been described, however we were interested in incorporating an acetal functionality which will enable two PEG chains to be attached to enhance shielding capacity. Acetals are readily cleavable, and the rate of hydrolysis normally increases ten-fold with every unit of pH decrease.[41] Formation of the acid-labile linker, incorporating a spacer with a pendant alkyl halide for facile coupling to the lipid anchor, was initially investigated *via* oxidation of haloalcohols to the aldehyde followed by acetal formation with PEG chains (Scheme 1).

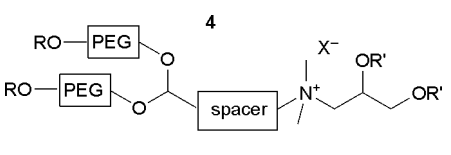
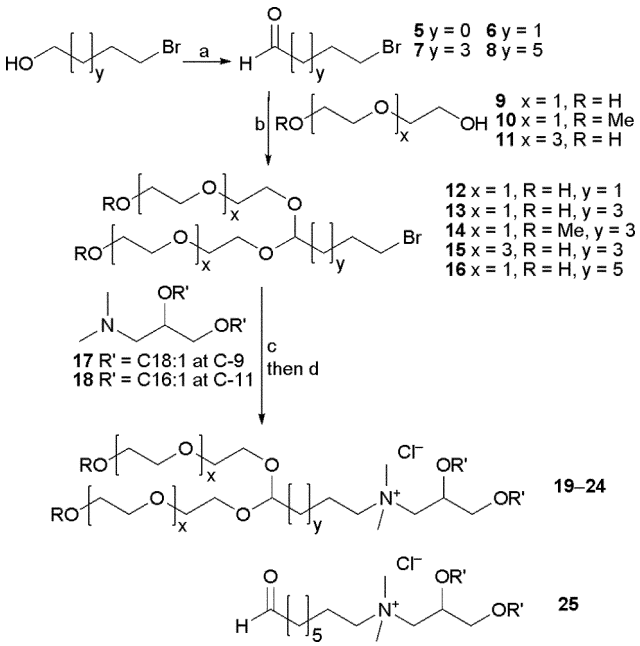


Fig. 2 Acid-labile PEG-lipids **4**.



Scheme 1 Synthesis of acid-cleavable cytofectins **19–24**. *Reagents and conditions*: (a) TPAP, NMO; (b) *p*-TsOH, PEGOH; (c) tertiary amine, acetone, 40 °C; (d) Amberlite® IRA-400 (Cl). Structure of lipid **25**.

The synthesis of 3-bromopropan-1-al **5** was initially explored *via* the oxidation of 3-bromopropan-1-ol using Swern conditions,[42] Dess–Martin periodinane,[43] and pyridinium dichromate (PDC), however the reactions were either low yielding or overoxidation occurred. An alternative route using acrolein and hydrogen bromide gas[44] led to the formation of a dibrominated addition product. In view of this, and the tendency for the C-3 aldehyde to undergo elimination reactions, C-4, C-6 and C-8 spacers were then used. To ensure a facile work-up procedure and avoid side-product formation, oxidations of the primary alcohols 4-bromobutan-1-ol, 6-bromohexan-1-ol and 8-bromohexan-1-ol were carried out using tetra-*n*-propylammonium perruthenate (TPAP) and *N*-methylmorphine-*N*-oxide (NMO).[45] TLC analysis indicated that aldehydes **6–8** were formed after 10 min, and the products were isolated by filtering the crude mixture through a short pad of silica. The crude product was then directly used with short commercially available *n*-ethylene glycols (**9–11**) in acetal formation.

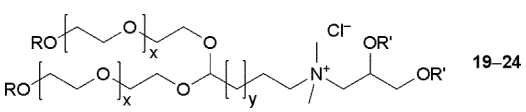
The conversion of **6** into acetal **12** was first attempted using an excess of diethylene glycol (**9**), with *p*-TsOH as catalyst (Scheme 1) with activated molecular sieves. Compound **9** was also pre-dried over molecular sieves to reduce the water content. The reaction was monitored by ESMS and after 48 h **12** was isolated and purified by neutral alumina chromatography in 40% yield over the two steps. Although the yield of the reaction was modest, the synthetic route was used with the other aldehydes and short PEGs because only two steps were required and the reagents were relatively inexpensive. The procedure was therefore repeated using **7** and **9** to generate **13** in 12% yield over two steps. The lower yield reflected both a moderate conversion to the acetal and problems encountered during the isolation of **13** using silica chromatography due to very similar R_f values for **9** and **13**.

The reaction time for acetal formation was varied from 24 to 72 h but this failed to increase the yield of **13**. Despite this, due to the straightforward procedure used, acetal **14** with a pendant methoxy group was then prepared in the same way from diethyleneglycol methyl ether (**10**) and **7**, in 37% over the two steps. We have previously observed that variation in the short PEG length can have significant effects on the transfection activity of PEG–lipid conjugates and therefore **15** was synthesised from **7** and **11** incorporating tetraethylene glycol units. Finally, a longer spacer was used to synthesise **16** from **8** and **9** in 39% yield.

The C18:1 (*cis* at C-9) tertiary amine **17** and C16:1 (*cis* at C-11) amine **18** were synthesised as previously described.[46] Synthesis of the pH-sensitive lipids **19–23** was then carried out *via* quaternisation of **17** with acetal linkers **12–16** at 40 °C to avoid degradation of the acetal. The crude mixtures were passed through an Amberlite® IRA-400 (Cl) ion exchange resin column, to furnish the corresponding PEG-acetal lipids as the chloride salts in moderate yields (21–48%) (Scheme 1, Table 1). An analogous route to a selected C16:1 lipid **24** using **13** and **18** was carried out using the same method. In order to determine the effect of the PEG units an aldehyde lipid **25** was also prepared. Amine **17** was quaternised with 8-bromooctanol, followed by ion exchange using an Amberlite® IRA-400 (Cl) ion exchange resin column, in 47% overall yield. Finally, oxidation of the hydroxyl moiety with TPAP and NMO as oxidant, afforded the aldehyde lipid **25** (Scheme 1) in 53% yield.

To evaluate the lipid stability at acidic pH, a TLC assay, in conjunction with ESMS, was employed to monitor the lipid hydrolysis. In a typical procedure, lipids in HMA buffers ranging from pH 3 to 7.5 were incubated at 37 °C for 30 min to 3 h, after which small aliquots were removed, neutralised and analysed. For example, lipid **19** was examined between pH 3 and 7.5 after 30 min of incubation at 37 °C. TLC analysis of the aliquots demonstrated that degradation was detectable at pH 5.5, increasing at even lower pHs (Fig. 3). This was confirmed by ESMS with hydrolysis products such as the corresponding aldehyde detected by ESMS (*m*/*z* 691), whereas **19** alone gave rise to a single peak at *m*/*z* 885 ($M^+ - Cl$). Hydrolyses of all the PEG-acetal lipids **19–24** were monitored in similar fashion and the results highlighting

Table 1 Short PEG-lipid conjugates synthesised and degradation pH



Compound	*x*	R	*y*	R′	pH[a]
19	1	H	1	C_{18} (*cis* C-9)	5.5
20	1	H	3	C_{18} (*cis* C-9)	3.5
21	1	Me	3	C_{18} (*cis* C-9)	5.0
22	3	H	3	C_{18} (*cis* C-9)	3.0
23	1	H	5	C_{18} (*cis* C-9)	4.0
24	1	H	3	C_{16} (*cis* C-11)	3.0

[a] pH at which degradation is initiated.

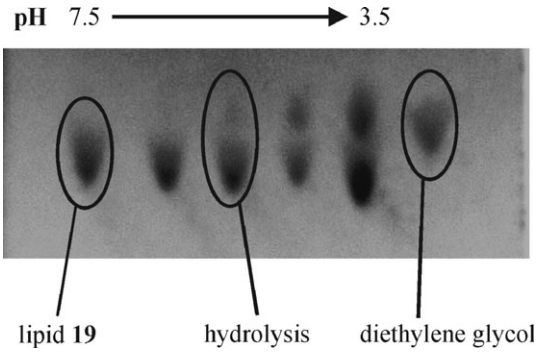


Fig. 3 Monitoring degradation of lipid by TLC analysis.

the pHs at which degradation is initiated are summarised in Table 1.

Degradation of compounds **19–24** was observed between pH 3.0 and 5.5 after a 30 min incubation. The acid sensitivity was in the order: **19** $\geq$ **21** $>$ **23** $\geq$ **20** $\geq$ **22** and **24**. From this trend there were several points to note. Firstly, from the degradation data on compounds **20** and **22** (at pH 3.5 and 3.0), variation of the short PEG chain length had little influence on lipid stability under acidic conditions. Secondly, the methoxyPEG (mPEG) lipid derivative **21** was hydrolysed at pH 5.0, and was more acid-labile than the hydroxy-PEG analogue **20**. This may be due to some shielding effects by the terminal hydroxyl group *via* hydrogen-bonding interactions with water. The length of the methylene spacer group between the acetal moiety and the cationic headgroup was also shown to affect lipid stability under mildly acidic conditions. With the same lipid anchor and PEG chain length, the shorter C-4 spacer lipid **19** was more acid labile than the C-6 (**21**) and C-8 (**23**) analogues. Indeed, the hydrolysis of **19** was initiated at pH 5.5, the pH within the endosome.[34] Finally, similar pHs for cleavage were observed for the C18:1 lipid **20** and C16:1 analogue **24** (pH 3.0–3.5), indicating that varying the lipid chain length had little influence on the acid sensitivity of the acetal linkages. TLC analysis also indicated that lipids **19–24** were stable for over 3 h at pH 7.5, and that over the pH range of 3.0 to 7.5 used to monitor the stability of compounds **19–24**, full degradation required more than 3 h.

Encouraged by these results, preliminary studies were performed to investigate the *in vitro* activities of lipids **19–25**. They were mixed with the helper lipid DOPE **2** (1 : 1 ratio) and then formulated as LID ternary complexes with peptide **3** or peptide **26**[47] or as LD complexes. Initial experiments established that the addition of DOPE **2** to the pH sensitive lipids enhanced the levels of transfections significantly (data not shown). The enhancement in activity may be as a result of the ability of **2** to promote H_{II} lipid phase formation and facilitate endosomal escape.[48]

Gene transfer efficiencies of the pH-sensitive lipids were studied in different cell types using a plasmid encoding the luciferase gene. In all cell lines lipids **19–25** required complexation to a peptide, and formulation as a ternary vector to achieve transfection. Data for LD systems is shown in Fig. 4(a) and (b) (16HBE14o- and Neuro-2A cells, respectively, third column per compound) as examples.

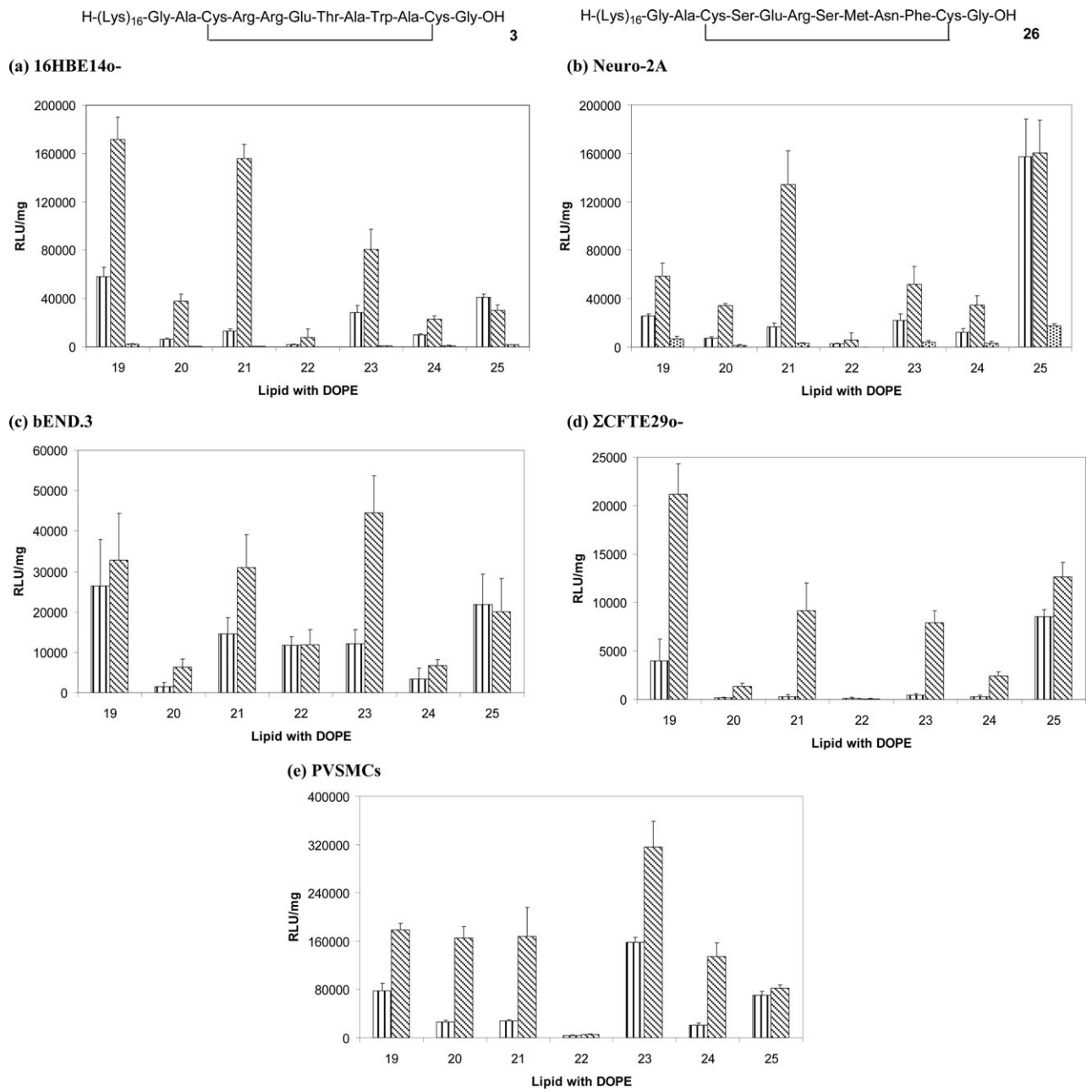


Fig. 4 Structure of peptides **3** and **26**. Transfections with LID or LD complexes were performed in (a) human bronchial epithelial cells, 16HBE14o-; (b) mouse neuroblastoma cells, Neuro-2A; (c) mouse endothelial cells, bEND.3; (d) cystic fibrosis tracheal epithelial cells, ΣCFTE29o-; (e) primary porcine vascular smooth muscle cells, PVSMCs. Complexes were prepared with a pH-sensitive lipid/DOPE : peptide **3** (vertical stripes) or **26** (diagonal lines) or none (dotted, (a) and (b) only) : pCI-Luc, with a weight ratio of 2 : 4 : 1. Transfection incubation was performed for 4 h and luciferase activity was measured 24 h later. The relative light units (RLU) measured for 10 s are expressed as means ± s.e.m. per mg of protein.

In all the cells, when the pH-sensitive lipids were complexed to peptide **3** or **26** (Fig. 4), the best lipids for transfections were **19**, **21** and **23** which were hydrolysed at pH 5.5, 5.0 and 4.0, respectively. The least efficient lipids for transfection were **20**, **22** and **24** which hydrolysed at pH 3.5, 3.0 and 3.0, respectively. This may reflect their ability to cleave less readily within the endosome. Moreover, in all cells apart from Neuro-2A, the pH-sensitive lipid giving rise to the best transfection efficiency out-performed the representative cleaved aldehyde lipid, **25**. Indeed, in 16HBE14o- (Fig. 4(a)) and ΣCFTE29o- cells (Fig. 4(d)), **19** was 6 and 2 times better than **25**, respectively, when they were complexed to peptide **26** ($p < 0.05$). In bEND.3 (Fig. 4(c)) and PVSMCs cells (Fig. 4(e)), **23** was 2 and 4 times better than **25**, respectively, when they were complexed to peptide **26** ($p < 0.05$). Also in Neuro-2A cells, **21** was as efficient as **25** when they were complexed to peptide **26** ($p > 0.05$; Fig. 4(b)).

In all the cell lines, complexes formulated with peptide **26** had transfection efficiencies at least equal to, and frequently much greater than, complexes formulated with peptide **3**. This

arises because of the differing integrin-targeting sequences used in the two peptides. The targeting sequence of peptide **3**, RRETAWA, binds to $\alpha_5\beta_1$ integrins and was used in the original ternary vectors.[8] However, as these receptors are inaccessible in confluent epithelium, panning experiments were previously carried out to identify sequences that would bind to the apical surfaces of airway epithelial cells.[47] The sequence SERSMNF (in peptide **26**) was identified as binding to accessible receptors, accounting for the higher transfection efficiencies observed in 16HB14o- and ΣCFTE29o- cells with complexes formulated with peptide **26**. In Neuro-2A and bEND.3 cells α_v integrins are upregulated, and the transfection results indicate that the SERSMNF sequence binds more tightly to this receptor than the RRETAWA sequence.

With the acetal lipids there appears to be a correlation between the transfection efficiency and sensitivity of the lipids to acidic conditions which initiates their degradation, enabling the rapid dissociation of lipopolyplexes after their uptake by endocytosis. One of the limiting steps for the gene transfer with synthetic vectors is the dissociation of complexes within the cell, that will allow the entry of DNA into the nucleus and transcription to occur.[49] Using pH-sensitive lipids for gene transfer allows the cleavage of these lipids after localization in endosomal vessels where the pH is acidic. This leads to endosomal membrane destabilization which increases the amount of plasmid DNA released into the cytoplasm and so, the transfection efficiency.[50] Indeed, the destabilisation of LD complexes containing pH-sensitive PEG lipids has been described as a result of dePEGylation on the surface of the lipid/DNA complex under mildly acidic conditions.[35] This caused a disruption of the liposomal assembly and promoted the $L \rightarrow H_{II}$ phase transition of liposomes, thus triggering the release of encapsulated contents. The PEG-acetal lipids when internalised may behave in a similar fashion, and the incorporation of the fusogenic lipid DOPE (**2**) may also facilitate the transition.

Finally, the performance of lipids **19–25** were also noted compared to Lipofectin™ (**1** and **2**) in lipopolyplex formulations for several cell-lines.‡ The results were again highly dependent on cell type, with the Lipofectin™ containing complexes being slightly more effective in 16HBE14o- cells, but not in bEND.3 or PVSMCs.

Conclusions

We have designed and prepared a novel class of pH-sensitive PEG lipids bearing acid-cleavable acetal linkages and short PEG chains. It has proven possible to alter the degradation pH of these lipids by varying lipid structural properties including the terminal PEG moiety, the spacing of the linkage, and to a smaller extent the lipid chain length. Hydrolysis studies demonstrated that these lipids are hydrolysed over a pH range of 3.0 to 5.5 after incubation at 37 °C for 30 min. In addition, lipopolyplexes formulated with PEG-acetal lipids and DOPE have been utilised in preliminary studies to transfect different cell types *in vitro*, and displayed cell-type dependent activity. Generally, the transfection results indicated a good correlation between the transfection efficiency and lipid stability under mildly acidic conditions. The best candidates in LID formulations were lipids that undergo hydrolysis at physiologically accessible pHs. These observations suggest that pH-sensitive PEG lipids are promising candidates for lipopolyplex formulations that may protect the LID complexes before cell internalisation and induce endosomal fusion, thus enhancing the transfection efficacy. Future work will investigate the use of these lipids for *in vivo* applications.

Experimental

General synthetic methods

Unless otherwise noted, solvents and reagents were reagent grade from commercial suppliers and used without further purification. Anhydrous solvents were HPLC grade and distilled over calcium hydride (dichloromethane), and 4Å activated molecular sieves (acetone), under a nitrogen atmosphere. All moisture-sensitive reactions were performed under a nitrogen atmosphere using oven-dried glassware. Reactions were monitored by TLC on Kieselgel 60 F_{254} plates for normal phase TLC and Merck 60 F_{254} neutral alumina plates for neutral alumina TLC, with detection by UV, or permanganate, and phosphomolybdic acid stains. Flash column chromatography was carried out using silica gel (particle size 40–63 μm) and on neutral alumina using aluminium oxides (activated, neutral, Brockmann I) purchased from Sigma-Aldrich Co. Ltd.

IR spectra were recorded using a FT-IR Shimadzu 8700 instrument. Only selected peaks were reported (cm^{-1}). ^1H and ^{13}C NMR spectra were recorded on Bruker® AMX300 MHz, AVANCE500 MHz machines. Deuterated chloroform ($CDCl_3$) solution used to record NMR spectra of acid-sensitive compounds was filtered through basic alumina prior to use. Mass spectra (+ES) were recorded on a Micromass Quatto LC spectrospray (+ES), a UG70FE (FAB), and a MAT 900XP spectrometer (+HRFAB and +HRES). For liposome preparations, water (sterile-filtered, cell culture tested) was purchased from Sigma-Aldrich Co. Ltd. Glass vials were sterilised by soaking in ethanol for 5 min and subsequently heated at 140 °C for 2 h. A Kerry sonicator was used to produce the lipid vesicles.

Syntheses

General procedure for acetal linker synthesis, compounds 12–16, *via* 6–8. To a solution of the bromo alcohol (1 eq.), *N*-methylmorpholine-*N*-oxide (NMO; ~1.1 eq.) and activated molecular sieves (4 Å, powdered; 300 mg per mmol) in anhydrous dichloromethane, was added tetra-*n*-propylammonium perruthenate (TPAP; ~0.1 eq.). After stirring at rt for 10 min, the suspension was filtered through a small plug of silica gel (~2 cm depth) and the residue washed with dichloromethane (3 × 20 mL). The combined organic filtrates were concentrated *in vacuo* to yield the corresponding aldehyde, which was subsequently used without further purification.

A solution of the crude aldehyde (~1 eq.), PEG-alcohol (~4 eq.), pTsOH·H_2O (~0.3 eq.) and activated powdered molecular sieves (4 Å; 400 mg per mmol of aldehyde) in anhydrous dichloromethane was stirred at rt for 48 h. The suspension was filtered through a small plug of silica gel

(~2 cm depth) and the residue washed with dichloromethane (3 × 20 mL). The combined organic filtrates were washed with saturated sodium hydrogencarbonate solution (30 mL), water (30 mL) and brine (30 mL), dried (sodium sulfate), then concentrated *in vacuo* and purified.

7-(3-Bromopropyl)-3,6,8,11-tetraoxatridecane-1,13-diol (12). The above procedure was carried out using the following quantities: 1-bromobutan-4-ol (**6**) (1.70 g, 11.1 mmol), NMO (1.47 g, 12.5 mmol), molecular sieves (3.3 g), dichloromethane (45 mL) and TPAP (0.390 g, 1.11 mmol); then diethylene glycol (**9**) (3.99 mL, 42.1 mmol), *p*TsOH·H_2O (0.637 g, 3.35 mmol), molecular sieves (4.4 g) and dichloromethane (50 mL). The product was purified by neutral alumina chromatography (gradient; dichloromethane to 2% methanol in dichloromethane) to give **12** as a pale yellow oil (1.53 g, 40%). R_f 0.35 (5% MeOH in CH_2Cl_2); ν_{max}(film)/cm^{-1} 3358, 2929, 1128; δ_H (300 MHz; $CDCl_3$) 1.73 (2H, m), 1.87 (2H, m), 3.37 (2H, t, *J* 6.6, CH_2Br), 3.43 (2H, m, 2 × O*H*), 3.50–3.81 (16H, m, 8 × OCH_2), 4.58 (1H, t, *J* 5.6, OC*H*O); δ_C (75.4 MHz; $CDCl_3$) 28.0 (*C*H_2CH$_2$Br), 31.7 (*C*H_2Br), 33.5 (CH-*C*H_2), 61.8 (*C*H_2OH), 64.6, 70.6, 72.8, 102.2 (O*C*HO); *m*/*z* (+ES) 369 ([M(^{81}Br) + Na]$^+$, 97%), 367 ([M(^{79}Br) + Na]$^+$, 100); Found (+HRES) [M(^{79}Br) + Na]$^+$, 367.07233. $C_{12}H_{25}BrO_6Na$ requires 367.07267.

7-(5-Bromopentyl)-3,6,8,11-tetraoxatridecane-1,13-diol (13). The above procedure was carried out using the following quantities: 1-bromohexan-6-ol (**7**) (1.76 g, 9.72 mmol), NMO (1.33 g, 11.3 mmol), molecular sieves (3.0 g), dichloromethane (40 mL), TPAP (0.370 g, 1.05 mmol); diethylene glycol (**9**) (4.63 mL, 48.8 mmol), *p*TsOH·H_2O (0.590 g, 3.11 mmol), molecular sieves (4.0 g) and dichloromethane (50 mL). Purification by flash chromatography on silica gel (gradient; dichloromethane to 5% methanol in dichloromethane) yielded **13** as a pale yellow oil (0.450 g, 12%). R_f 0.38 (5% MeOH in CH_2Cl_2); ν_{max}(film)/cm^{-1} 3362, 2924, 1124; δ_H (300 MHz; $CDCl_3$) 1.34 (4H, m, 2 × CH_2), 1.58 (2H, dt, *J* 8.2 and 5.7, CHCH_2), 1.78 (2H, tt, *J* 7.1 and 6.7, CH_2CH$_2$Br), 3.26 (2H, t, *J* 5.7, 2 × O*H*), 3.35 (2H, t, *J* 6.7, CH_2Br), 3.50–3.81 (16H, m, 8 × OCH_2), 4.53 (1H, t, *J* 5.7, OC*H*O); δ_C (75.4 MHz; $CDCl_3$) 23.8, 27.8, 32.6, 32.8, 33.8, 61.6 (*C*H_2OH), 64.3, 70.5, 72.7, 102.8 (O*C*HO); *m*/*z* (+ES) 398 ([M(^{81}Br) + Na]$^+$, 95%), 396 ([M(^{79}Br) + Na]$^+$, 100); Found (+HRES) [M(^{79}Br) + Na]$^+$, 395.10388. $C_{14}H_{29}BrO_6Na$ requires 395.10397.

9-(5-Bromopentyl)-2,5,8,10,13,16-hexaoxaheptadecane (14). The above procedure was carried out using the following quantities: 1-bromohexan-6-ol (**7**) (1.76 g, 9.72 mmol), NMO (1.33 g, 11.3 mmol), molecular sieves (3.0 g), dichloromethane (40 mL), TPAP (0.370 g, 1.05 mmol); diethylene glycol methyl ether (**10**) (3.63 mL, 29.6 mmol), *p*TsOH·H_2O (0.590 g, 3.11 mmol), molecular sieves (4.0 g) and dichloromethane (50 mL). The product was purified by neutral alumina chromatography (gradient; dichloromethane to 2% methanol in dichloromethane) and yielded **14** as a pale yellow oil (1.45 g, 37%). R_f 0.43 (2% MeOH in CH_2Cl_2); ν_{max}(film)/cm^{-1} 2930, 1113; δ_H (300 MHz; $CDCl_3$) 1.38 (4H, m, 2 × CH_2), 1.59 (2H, dt, *J* 8.3 and 5.7, CHCH_2), 1.81 (2H, tt, *J* 7.0 and 6.8, CH_2CH$_2$Br), 3.35 (6H, s, 2 × CH_3), 3.24–3.49 (2H, m, CH_2Br), 3.51–3.82 (16H, m, 8 × OCH_2), 4.56 (1H, t, *J* 5.7, OC*H*O); δ_C (75.4 MHz; $CDCl_3$) 23.7, 27.8, 32.6, 32.9, 33.6, 58.9 (CH_3), 64.3, 70.4, 70.5, 71.9, 103.0 (O*C*HO); *m*/*z* (+ES) 425 ([M(^{81}Br) + Na]$^+$, 94%), 423 ([M(^{79}Br) + Na]$^+$, 100); Found (+HRES) [M(^{79}Br) + Na]$^+$, 423.13514. $C_{16}H_{33}BrO_6Na$ requires 423.13527.

13-(5-Bromopentyl)-3,6,9,12,14,17,20,23-octaoxapentacosane-1,25-diol (15). The above procedure was carried out using the following quantities: 1-bromohexan-6-ol (**7**) (1.76 g, 9.72 mmol), NMO (1.33 g, 11.3 mmol), molecular sieves (3.0 g), dichloromethane (40 mL), TPAP (0.370 g, 1.05 mmol); tetraethylene glycol (**11**) (8.88 mL, 51.5 mmol), *p*TsOH·H_2O (0.590 g, 3.11 mmol), molecular sieves (2.7 g) and dichloromethane (50 mL). Purification by flash chromatography on silica gel (gradient; dichloromethane to 5% methanol in dichloromethane) yielded **15** as a pale yellow oil (0.49 g, 9%). R_f 0.33 (5% MeOH in CH_2Cl_2); ν_{max}(film)/cm^{-1} 3418, 2923, 1185; δ_H (300 MHz; $CDCl_3$) 1.33 (4H, m, 2 × CH_2), 1.55 (2H, dt, *J* 8.0 and 5.7, CHCH_2), 1.79 (2H, tt, *J* 7.3 and 6.7, CH_2CH$_2$Br), 3.00 (2H, t, *J* 6.3, 2 × O*H*), 3.36 (2H, t, *J* 6.7, CH_2Br), 3.50–3.75 (32H, m, 16 × OCH_2), 4.51 (1H, t, *J* 5.7, OC*H*O); δ_C (75 MHz; $CDCl_3$) 23.6, 27.7, 32.4, 32.7, 33.6, 61.4 (*C*H_2OH), 64.1, 70.1, 70.3–70.4 (signal overlap), 72.3, 102.8 (O*C*HO); *m*/*z* (+ES) 573 ([M(^{81}Br) + Na]$^+$, 96%), 572 ([M(^{79}Br) + Na]$^+$, 100); Found (+HRES) [M(^{79}Br) + Na]$^+$, 571.20805. $C_{22}H_{45}BrO_{10}Na$ requires 571.20883.

7-(7-Bromoheptyl)-3,6,8,11-tetraoxatridecane-1,13-diol (16). The above procedure was carried out using the following quantities: 1-bromooctan-8-ol (**8**) (2.00 g, 9.57 mmol), NMO (1.12 g, 9.57 mmol), molecular sieves (3.0 g), dichloromethane (40 mL), TPAP (0.340 g, 0.967 mmol); diethylene glycol (**9**) (5.18 mL, 54.6 mmol), *p*TsOH·H_2O (0.55 g, 2.90 mmol), molecular sieves (4.0 g) and dichloromethane (40 mL). Purification by flash chromatography on silica gel (gradient; dichloromethane to 3% methanol in dichloromethane) yielded **16** as a colourless oil (1.50 g, 39%). R_f 0.35 (5% MeOH in CH_2Cl_2); ν_{max}(film)/cm^{-1} 3434, 2930, 1128; δ_H (300 MHz; $CDCl_3$) 1.22–1.47 (8H, m, 4 × CH_2), 1.60 (2H, m, CHCH_2), 1.83 (2H, tt, *J* 7.1 and 6.8, CH_2CH$_2$Br), 3.22 (2H, t, *J* 6.2, 2 × O*H*), 3.37 (2H, t, *J* 6.8, CH_2Br), 3.56–3.76 (16H, m, 8 × OCH_2), 4.58 (1H, t, *J* 6.0, OC*H*O); δ_C (75.4 MHz; $CDCl_3$) 24.6, 28.0, 28.6, 29.2, 32.7, 33.0, 34.0, 61.7 (*C*H_2OH), 64.3, 70.6, 72.7, 102.9 (O*C*HO); *m*/*z* (+ES) 425 ([M(^{81}Br) + Na]$^+$, 100%), 423 ([M(^{79}Br) + Na]$^+$, 83); Found (+HRES) [M(^{79}Br) + Na]$^+$, 423.1361. $C_{16}H_{33}BrO_6Na$ requires 423.1353.

General procedure for acetal lipid formation 19–24

The acetal bromide (1 eq.) and tertiary amine (~1.1–1.3 eq.) were heated in a sealed tube at 40 °C for 48 h. The mixture was dissolved in a 1 : 1 mixture of chloroform–methanol, passed through an Amberlite® IRA-400 (Cl) ion exchange column eluting with chloroform–methanol (1 : 1), and then concentrated *in vacuo*.

***N*-2,3-Bis[(*Z*)-octadec-9-enyloxy]propyl-4,4-bis[2-(2-hydroxyethoxy)ethoxy]-*N*,*N*-dimethylbutan-1-ammonium chloride (19).** The above procedure was carried out using the

following quantities: amine **17** (0.150 g, 0.242 mmol) and bromide **12** (0.083 g, 0.240 mmol). Purification by flash chromatography on silica gel (5% methanol in dichloromethane) yielded **19** as a pale yellow oil (81 mg, 36%). R_f 0.32 (5% MeOH in CH_2Cl_2); ν_{max}(film)/cm^{-1} 3383, 2924, 1634; δ_H (300 MHz; $CDCl_3$) 0.83 (6H, t, *J* 6.6, 2 × CH_2CH_3), 1.23 (44H, m), 1.50 (4H, m, 2 × $OCH_2CH_2CH_2$), 1.74 (2H, m, $OCHCH_2CH_2$), 1.95 (10H, m, 4 × $CH_2CH{=}CH$, $CH_2CH_2N^+$), 3.28 (3H, s, N^+CH_3), 3.31 (3H, s, N^+CH_3), 3.39 (4H, t, *J* 6.9, 2 × OCH_2), 3.48–3.94 (22H, m), 4.00 (1H, m, $OCHCH_2$), 4.79 (1H, m, OC*H*O), 5.33 (4H, m, 2 × $CH{=}CH$); δ_C (75 MHz; $CDCl_3$) 14.1 (2 × CH_2CH_3), 17.9, 22.7, 26.0, 26.2, 27.2, 29.2–30.2 (signal overlap), 31.9, 32.6, 51.9 (N^+CH_3), 52.2 (N^+CH_3), 61.2 (CH_2OH), 65.2, 65.9, 66.0, 68.8, 69.3, 70.6, 72.0, 72.7, 73.3, 103.0 (O*C*HO), 129.8 (2 × *C*H═CH), 129.9 (2 × CH═*C*H); *m*/*z* (+ES) 885 (M^+ − Cl, 100%); Found (+HRES) (MH^+ − Cl), 885.7988. $C_{53}H_{107}NO_8$ requires 885.7991.

***N*-2,3-Bis[(*Z*)-octadec-9-enyloxy]propyl-6,6-bis[2-(2-hydroxyethoxy)ethoxy]-*N*,*N*-dimethylhexan-1-ammonium chloride (20).** The above procedure was carried out using the following quantities: amine **17** (0.300 g, 0.484 mmol) and bromide **13** (0.160 g, 0.430 mmol). Purification by flash chromatography on silica gel (5% methanol in dichloromethane) yielded **20** as a yellow oil (0.120 g, 29%). R_f 0.34 (5% MeOH in CH_2Cl_2); ν_{max}(film)/cm^{-1} 3357, 2924, 1622; δ_H (300 MHz; $CDCl_3$) 0.86 (6H, t, *J* 6.7, 2 × CH_2CH_3), 1.23 (46H, m), 1.42 (2H, m), 1.51 (4H, m, 2 × $OCH_2CH_2CH_2$), 1.65 (2H, dt, *J* 6.2 and 6.0, $OCHCH_2CH_2$), 1.79 (2H, m, $CH_2CH_2N^+$), 1.98 (8H, m, 4 × $CH_2CH{=}CH$), 2.46 (2H, s, 2 × O*H*), 3.29 (3H, s, N^+CH_3), 3.33 (3H, s, N^+CH_3), 3.38 (8H, m, 2 × OCH_2, 2 × CH_2N^+), 3.52–3.91 (18H, m, 9 × OCH_2), 3.97 (1H, m, $OCHCH_2$), 4.65 (1H, t, *J* 5.6, OC*H*O), 5.34 (4H, m, 2 × $CH{=}CH$); δ_C (75 MHz; $CDCl_3$) 14.1 (2 × CH_2CH_3), 22.6, 22.7, 23.9, 25.7, 26.0, 26.2, 27.2, 29.3–30.0 (signal overlap), 31.9, 32.6, 32.8, 51.9 (N^+CH_3), 52.3 (N^+CH_3), 61.5 (CH_2OH), 65.0, 65.2, 68.5, 69.4, 70.5, 72.0, 72.7, 73.3 ($CHOC_{18}H_{35}$), 103.0 (O*C*HO), 129.8 (2 × *C*H═CH), 130.0 (2 × CH═*C*H); *m*/*z* (+ES) 914 (MH^+ − Cl, 100%). Found (+HRES) (M^+ − Cl), 912.82347. $C_{55}H_{110}NO_8$ requires 912.82260.

***N*-2,3-Bis[(*Z*)-octadec-9-enyloxy]propyl-6,6-bis[2-(2-methoxyethoxy)ethoxy]-*N*,*N*-dimethylhexan-1-ammonium chloride (21).** The above procedure was carried out using the following quantities: amine **17** (0.300 g, 0.484 mmol) and bromide **14** (0.180 g, 0.450 mmol). Purification by flash chromatography on silica gel (2% methanol in dichloromethane) yielded **21** as a pale yellow oil (0.210 g, 48%). R_f 0.41 (2% MeOH in CH_2Cl_2); ν_{max}(film)/cm^{-1} 2924, 1633, 1123; δ_H (300 MHz; $CDCl_3$) 0.80 (6H, t, *J* 6.6, 2 × CH_2CH_3), 1.15–1.41 (48H, m), 1.47 (4H, m, 2 × $OCH_2CH_2CH_2$), 1.55 (2H, m, $OCHCH_2CH_2$), 1.67 (2H, m, $CH_2CH_2N^+$), 1.92 (8H, m, 4 × $CH_2CH{=}CH$), 3.30 (6H, s, 2 × N^+CH_3), 3.30–3.43 (14H, m, 2 × OCH_3, 2 × OCH_2, 2 × CH_2N^+), 3.46–3.91 (18H, m, 9 × OCH_2), 3.99 (1H, m, $OCHCH_2$), 4.50 (1H, t, *J* 5.6, OC*H*O), 5.28 (4H, m, 2 × $CH{=}CH$); δ_C (125 MHz; $CDCl_3$) 14.0 (2 × CH_2CH_3), 22.5, 22.6, 24.1, 25.9, 26.1, 27.1, 29.1–30.0 (signal overlap), 31.8, 32.7, 51.8 (N^+CH_3), 52.1 (N^+CH_3), 59.0 (2 × OCH_3), 64.5, 64.8, 66.1, 68.4, 69.2, 70.3, 70.5, 71.8, 73.3 ($CHOC_{18}H_{35}$), 102.8 (O*C*HO), 129.6 (2 × *C*H═CH), 130.0 (2 × CH═*C*H); *m*/*z* (+FAB) 941 (M^+ − Cl, 100%). Found (+HRES) (MH^+ − Cl), 941.8602. $C_{57}H_{115}NO_8$ requires 941.8622.

***N*-2,3-Bis[(*Z*)-octadec-9-enyloxy]propyl-6,6-bisethoxy]ethoxy-*N*,*N*-dimethylhexan-1-ammonium chloride (22).** The above procedure was carried out using the following quantities: amine **17** (0.300 g, 0.484 mmol) and bromide **15** (0.240 g, 0.440 mmol). Purification by flash chromatography on silica gel (5% methanol in dichloromethane) yielded **22** as a pale yellow oil (0.180 g, 36%). R_f 0.31 (5% MeOH in CH_2Cl_2); ν_{max}(film)/cm^{-1} 3362, 2924, 1643; δ_H (300 MHz; $CDCl_3$) 0.85 (6H, t, *J* 6.5, 2 × CH_2CH_3), 1.15–1.41 (48H, m), 1.52 (4H, m, 2 × $OCH_2CH_2CH_2$), 1.62 (2H, m, $OCHCH_2CH_2$), 1.74 (2H, m, $CH_2CH_2N^+$), 1.97 (8H, m, 4 × $CH_2CH{=}CH$), 2.78 (2H, s, 2 × O*H*), 3.31 (3H, s, N^+CH_3), 3.34 (3H, s, N^+CH_3), 3.37–3.50 (8H, m, 2 × OCH_2, 2 × CH_2N^+), 3.51–3.93 (34H, m, 17 × OCH_2), 4.03 (1H, m, $OCHCH_2$), 4.58 (1H, t, *J* 5.4, OC*H*O), 5.34 (4H, m, 2 × $CH{=}CH$); δ_C (75 MHz; $CDCl_3$) 14.1 (2 × CH_2CH_3), 22.5, 22.7, 23.9, 25.8, 26.0, 26.2, 27.2, 29.3–30.0 (signal overlap), 31.9, 32.6, 52.0 (2 × N^+CH_3), 61.4 (CH_2OH), 64.6, 65.1, 68.5, 69.3, 70.1, 70.4, 70.5–70.6 (signal overlap), 72.0, 72.8, 73.4 ($CHOC_{18}H_{35}$), 103.0 (O*C*HO), 129.8 (2 × *C*H═CH), 130.0 ((2 × CH═*C*H); *m*/*z* (+ES) 1090 (MH^+ − Cl, 100%); Found (+HRES) (M^+ − Cl), 1088.92801. $C_{63}H_{126}NO_{12}$ requires 1088.92745.

***N*-2,3-Bis[(*Z*)-octadec-9-enyloxy]propyl-8,8-bis[2-(2-hydroxyethoxy)ethoxy]-*N*,*N*-dimethyloctan-1-ammonium chloride (23).** The above procedure was carried out using the following quantities: amine **17** (0.270 g, 0.436 mmol) and bromide **16** (0.160 g, 0.400 mmol). Purification by flash chromatography on silica gel (5% methanol in dichloromethane) yielded **23** as a pale yellow oil (81 mg, 21%). R_f 0.32 (5% MeOH in CH_2Cl_2); ν_{max}(film)/cm^{-1} 3331, 2924, 1661; δ_H (300 MHz; $CDCl_3$) 0.85 (6H, t, *J* 6.7, 2 × CH_2CH_3), 1.15–1.45 (52H, m), 1.52 (4H, m, 2 × $OCH_2CH_2CH_2$), 1.62 (2H, m, $OCHCH_2CH_2$), 1.73 (2H, m, $CH_2CH_2N^+$), 1.97 (8H, m, 4 × $CH_2CH{=}CH$), 3.36 (3H, s, N^+CH_3), 3.38 (3H, s, N^+CH_3), 3.31–3.50 (8H, m, 2 × OCH_2, 2 × CH_2N^+), 3.52–3.80 (18H, m, 9 × OCH_2), 4.01 (1H, m, $OCHCH_2$), 4.60 (1H, t, *J* 5.8, OC*H*O), 5.34 (4H, m, 2 × $CH{=}CH$); δ_C (75 MHz; $CDCl_3$) 14.1 (2 × CH_2CH_3), 22.7, 22.8, 24.3, 26.0, 26.2, 27.2, 28.9, 29.0, 29.3–30.0 (signal overlap), 31.9, 32.6, 33.0, 52.1 (2 × N^+CH_3), 61.6 (CH_2OH), 64.6, 64.9, 68.4, 69.3, 70.6, 72.0, 72.7, 73.4 ($CHOC_{18}H_{35}$), 103.0 (O*C*HO), 129.8 (2 × *C*H═CH), 130.0 (2 × CH═*C*H); *m*/*z* (+ES) 941 (M^+ − Cl, 100%). Found (+HRES) (MH^+ − Cl), 941.8659. $C_{57}H_{115}NO_8$ requires 941.8622.

***N*-2,3-Bis[(*Z*)-hexadec-11-enyloxy]propyl-6,6-bis[2-(2-hydroxyethoxy)ethoxy]-*N*,*N*-dimethylhexan-1-ammonium chloride (24).** The above procedure was carried out using the following quantities: amine **18** (0.270 g, 0.480 mmol) and bromide **13** (0.160 g, 0.430 mmol). Purification by flash chromatography on silica (5% methanol in dichloromethane) yielded **24** as a pale yellow oil (0.130 g, 34%). R_f 0.33 (5% MeOH in CH_2Cl_2); ν_{max}(film)/cm^{-1} 3358, 2924, 1661; δ_H (300 MHz; $CDCl_3$) 0.86 (6H, t, *J* 6.9, 2 × CH_2CH_3), 1.15–1.40 (40H, m), 1.50 (4H, m, 2 × $OCH_2CH_2CH_2$), 1.61 (2H, m,

OCHCH_2CH$_2$), 1.79 (2H, m, CH_2CH$_2$N$^+$), 1.92 (8H, m, 4 × CH_2CH=CH), 2.64 (2H, s, 2 × O*H*), 3.30–3.46 (14H, m, 2 × N$^+$CH_3, 2 × OCH_2, 2 × CH_2N$^+$), 3.50–3.86 (18H, m, 9 × OCH_2), 4.00 (1H, m, OC*H*CH$_2$), 4.62 (1H, t, *J* 5.5, OCHO), 5.35 (4H, m, 2 × C*H*=C*H*); δ_C (75 MHz; CDCl$_3$) 13.9 (2 × CH$_2$*C*H$_3$), 22.2, 22.7, 24.1, 25.8, 26.0, 26.2, 29.2–30.0 (signal overlap), 31.8, 32.2, 32.6, 32.9, 52.1 (2 × N$^+$*C*H$_3$), 61.5 (*C*H$_2$OH), 64.9, 66.0 68.5, 69.3, 70.6, 72.0, 72.7, 73.4 (*C*HOC$_{18}$H$_{35}$), 103.0 (O*C*HO), 130.3 (2 × *C*H=*C*H, overlap); *m*/*z* (+ES) 858 (MH$^+$ − Cl, 100%); Found (+HRES) (MH$^+$ − Cl), 857.7660. $C_{51}H_{103}NO_8$ requires 857.7683.

***N*-2,3-Bis[(*Z*)-octadec-9-enyloxy]propyl-8-hydroxy-*N*,*N*-dimethyloctan-1-ammonium chloride.** 8-Bromooctan-1-ol (0.150 g, 0.718 mmol) and amine **17** (0.400 g, 0.646 mmol) were heated in a sealed tube at 40 °C for 48 h. The mixture was dissolved in a 1 : 1 mixture of chloroform–methanol, passed through an Amberlite® IRA-400 (Cl) ion exchange column eluting with chloroform–methanol (1 : 1), and then concentrated *in vacuo*. Purification by flash chromatography on silica gel (5% methanol in dichloromethane) yielded *the titled compound* as a colourless oil (0.240 g, 47%). R_f 0.28 (5% MeOH in CH$_2$Cl$_2$); δ_H (300 MHz; CDCl$_3$) 0.85 (6H, t, *J* 6.4, 2 × CH$_2$CH_3), 1.16–1.45 (50H, m), 1.52 (8H, m), 1.75 (2H, m, CH_2CH$_2$N$^+$), 1.98 (8H, m, 4 × CH_2CH=CH), 3.33–3.50 (12H, m, 2 × N$^+$CH_3, 3 × OCH_2), 3.52–4.06 (7H, m, 2 × CH_2N$^+$, OC*H*CH$_2$, OCH_2), 5.34 (4H, m, 2 × C*H*=C*H*); δ_C (75 MHz; CDCl$_3$) 14.1 (2 × CH$_2$*C*H$_3$), 22.7, 25.4, 25.9, 26.1, 26.3, 27.2, 28.8, 29.3–30.0 (signal overlap), 31.9, 32.4, 32.6, 52.2 (N$^+$*C*H$_3$), 52.4 (N$^+$*C*H$_3$), 62.5 (*C*H$_2$OH), 64.9, 66.3, 68.4, 69.3, 72.0, 73.4 (*C*HOC$_{18}$H$_{35}$), 129.8 (2 × *C*H=CH), 130.0 ((2 × CH=*C*H); *m*/*z* (+FAB) 749 (M$^+$ − Cl, 100%); Found (+HRES) (MH$^+$ − Cl), 749.7592. $C_{49}H_{99}NO_3$ requires 749.7624.

***N*-2,3-Bis[(*Z*)-octadec-9-enyloxy]propyl-*N*,*N*-dimethyl-8-oxooctan-1-ammonium chloride (25).** To a solution of alcohol (0.231 g, 0.293 mmol), *N*-methylmorpholine-*N*-oxide (41 mg, 0.35 mmol) and activated molecular sieves (4 Å, powdered; 100 mg) in anhydrous dichloromethane (5 mL), was added tetra-*n*-propylammonium perruthenate (10 mg, 0.028 mmol). After stirring at rt for 10 min, the suspension was filtered through a small plug of silica gel (~2 cm), and the residue washed with dichloromethane (3 × 10 mL), and then concentrated *in vacuo*. Purification by flash chromatography on silica gel (3% methanol in dichloromethane) yielded **25** as a yellow oil (0.121 g, 53%). R_f 0.30 (3% MeOH in CH$_2$Cl$_2$); ν_{max}(film)/cm^{-1} 2924, 1722, 1634, 1121; δ_H (300 MHz; CDCl$_3$) 0.87 (6H, t, *J* 6.6, 2 × CH$_2$CH_3), 1.17–1.43 (50H, m), 1.54 (8H, m), 1.75 (2H, m, CH_2CH$_2$N$^+$), 1.97 (8H, m, 4 × CH_2CH=CH), 2.44 (2H, dt, *J* 7.2 and 1.5, CH_2CHO), 3.32–3.50 (10H, m, 2 × OCH_2, 2 × N$^+$CH_3), 3.52–4.06 (7H, m, 2 × CH_2N$^+$, OC*H*-CH$_2$, OCH_2), 5.35 (4H, m, 2 × C*H*=C*H*), 9.76 (1H, t, *J* 1.5, C*H*O); δ_C (125 MHz; CDCl$_3$) 14.5 (2 × CH$_2$*C*H$_3$), 22.1, 23.1, 26.5, 27.6, 29.2, 29.7–30.4 (signal overlap), 32.3, 44.1 (*C*H$_2$CHO), 52.5 (N$^+$*C*H$_3$), 52.8 ((N$^+$*C*H$_3$), 65.4, 66.4, 68.8, 69.7, 72.4, 73.8 (*C*HOC$_{18}$H$_{35}$), 130.1 (2 × *C*H=*C*H, overlap), 202.9 (C=O); *m*/*z* (+FAB) 747 (M$^+$ − Cl, 100%); Found (+HRES) (M$^+$-Cl), 746.7408. $C_{49}H_{96}NO_3$ requires 746.7385.

Methods for assessing the stability of lipids 19–24 under acidic conditions. To evaluate the lipid stability over a pH range, a TLC assay, in conjunction with ESMS, was employed to monitor the hydrolysis and degradation of the lipids. In a typical procedure, 0.15 mg of lipid was diluted in HMA buffers at pHs ranging from 3 to 7.5, to achieve a final lipid concentration between 0.19–0.23 mM. The resulting lipid solutions were incubated at 37 °C for 30 min to 3 h, after which small amounts were removed and neutralised. The degradation of lipids was monitored by TLC and ESMS analysis.

Lipid formulation. Cationic lipids were either formulated alone or with DOPE (weight ratio, 1 : 1). Lipid (10 mg mL^{-1}; 100 μL [1 mg of lipid]) in chloroform was placed in a sterile glass vial. The solvents were removed *in vacuo* and further traces of chloroform removed on the high vacuum for 24 h. Sterile water (1 mL) was added to the lipid film, to generate a 1 mg mL^{-1} (total lipid) lipid suspension in water. The suspension was allowed to hydrate at 4 °C for 24 h. After warming to 40 °C the mixture was sonicated for approximately 5 min to generate a clear solution.

Transfection experiments

Cell cultures. The human bronchial epithelial (16HBE14o-)[51] and the human cystic fibrosis tracheal epithelial (ΣCFTE29o-)[52] cell lines, were maintained in Eagle's minimum essential medium (MEM) HEPES modification supplemented with 10% foetal bovine serum (FBS), 2 mM L-glutamine, 100 U mL^{-1} penicillin and 100 μg mL^{-1} streptomycin. The mouse neuroblastoma (Neuro 2A) and the mouse endothelial (bEND.3) cell lines, obtained from ATCC (Manassas, VA, USA), were maintained in Dulbecco's modified Eagle's medium (DMEM) with Glutamax-1 supplemented with 10% FBS, 1% non-essential amino acids, 1% sodium pyruvate, 100 U mL^{-1} penicillin and 100 μg mL^{-1} streptomycin. The primary porcine vascular smooth muscle cells (PVSMCs) were prepared using the explant method described previously[53] and maintained in DMEM with Glutamax-1 supplemented with 20% FBS and 100 U mL^{-1} penicillin and 100 μg mL^{-1} streptomycin. All cells were adherent and grew in Falcon 75-cm^2 plastic tissue culture flasks and were maintained in a humidified atmosphere of 95% air and 5% CO$_2$ at 37 °C.

Peptides. The peptides used in these studies were peptide **3**[8] and **26**.[47] They were synthesized by Alta Bioscience (Birmingham, UK) and were more than 80% pure and the sequence of each was confirmed by mass spectrometry and HPLC analysis. They were dissolved in water (Sigma, Poole, UK) at a concentration of 10 mg mL^{-1} and stored at −20 °C.

Plasmid DNA. The plasmid pCI-Luc (5.7 kb) consists of pCI (Promega, Madison, WI, USA) containing the luciferase gene driven by the cytomegalovirus (CMV) immediate/early promoter-enhancer. Plasmid DNA was grown in *E. coli* DH5α and purified, after bacterial alkaline lysis, on resin columns (Qiagen Ltd., Crawley, UK). Isopropanol-precipitated DNA pellets were washed with 70% ethanol, then dissolved in water at 1 mg mL^{-1}.

Gene transfer procedure. Cells were seeded into 96-well plates at 2.5×10^3 cells per well for bEND.3 cells and at 2×10^4 cells per well for all the other cells, then incubated overnight at 37 °C in complete growth medium. The following day, lipopolyplex formulations were prepared essentially as described previously,[8] by mixing the components in the following order: 50 μL of lipid (L) at 80 μg mL^{-1} in OptiMEM, 70 μL of peptide at 110 μg mL^{-1} in OptiMEM and 50 μL of plasmid DNA at 40 μg mL^{-1} in OptiMEM corresponding to a weight ratio of 2 : 4 : 1, respectively. The LD complexes were prepared in the same way but without peptide. All the complexes were mixed by pipetting briefly, kept for 2 h at room temperature and then diluted in OptiMEM to a final volume of 1.57 mL. Two hundred microlitres of complexes corresponding at 0.25 μg of plasmid DNA were added to each culture well after removal of the complete growth medium. All the transfections were carried out in 6 wells each. The cells were incubated with the complexes for 4 h before replacing with fresh media for 24 h, after which reporter gene expression was analysed by luciferase assay (Promega, Madison, WI, USA). For bEND.3 cells, 5 mM sodium butyrate was added to the transfection medium and to the fresh media to achieve optimal expression levels.

Luciferase and protein assays. Cells were washed twice with PBS before the addition of 20 μL of 1X Reporter Lysis Buffer (Promega, Madison, WI, USA) to the cells for 20 min at 4 °C before freezing at −80 °C for at least 30 min, followed by thawing at rt. Then the luciferase activity was measured using the Luciferase Assay System (Promega, Madison, WI, USA) and a Lucy-1 Luminometer (Anthos Ltd., Salzburg, Austria). The amount of protein present in each transfection lysate was determined with the Bio-Rad (Hercules, CA, USA) protein assay reagent by the manufacturer's instructions, adding 20 μL from the luciferase test to 180 μL of the reagent diluted 1 in 5 and incubating at room temperature for 10 min before comparing the OD590 to a range of BSA standards. Luciferase activity was expressed as Relative Light Units (RLU) per milligram of protein (RLU mg^{-1}). Student's t test was performed to assess the statistical significance between different experimental groups with probabilities less than 0.05 ($p < 0.05$) taken to be significant.

Acknowledgements

The EPSRC are thanked for a DTA studentship to J. B. W., and the BBSRC for a grant to S. G. (BBS/B/03645). The authors also thank Dieter Gruenert, California Pacific Medical Center Research Institute, San Francisco, CA, for kindly providing the cell lines 16HBE14o- and ΣCFTE29o-.

References

1 N. Somia and I. M. Verma, *Nat. Rev. Genet.*, 2000, **1**, 91–99.
2 A. D. Miller, *Angew. Chem., Int. Ed.*, 1998, **37**, 1768–1785.
3 M. E. Davis, *Curr. Opin. Biotechnol.*, 2002, **13**, 128–131.
4 K. Kostarelos and A. D. Miller, *Chem. Soc. Rev.*, 2005, **34**, 970–994.
5 D. Putnam, *Nat. Mater.*, 2006, **5**, 439–451.
6 C. M. Varga, T. J. Wickham and D. A. Lauffenburger, *Biotechnol. Bioeng.*, 2000, **70**, 593–605.
7 T. Tagawa, M. Manvell, N. Brown, M. Keller, E. Perouzel, K. D. Murray, R. P. Harbottle, M. Tecle, F. Booy, M. C. Brahimi-Horn, C. Coutelle, N. R. Lemoine, E. W. F. W. Alton and A. D. Miller, *Gene Ther.*, 2002, **9**, 564–576.
8 S. L. Hart, C. V. Arancibia-Cárcamo, M. A. Wolfert, C. Mailhos, N. J. O'Reilly, R. R. Ali, C. Coutelle, A. J. T. George, R. P. Harbottle, A. M. Knight, D. F. P. Larkin, R. J. Levinsky, L. W. Seymour, A. J. Thrasher and C. Kinnon, *Hum. Gene Ther.*, 1998, **9**, 575–585.
9 P. L. Felgner, T. R. Gadek, M. Holm, R. Roman, H. S. Chan, M. Wenz, J. P. Northrop, G. M. Ringold and H. Danielsen, *Proc. Natl. Acad. Sci. U. S. A.*, 1987, **84**, 7413–7417.
10 E. Koivunen, B. Wang and E. Ruoslahti, *J. Cell Biol.*, 1994, **124**, 373–380.
11 R. Parkes, Q.-H. Meng, K. E. Siapati, J. R. McEwan and S. L. Hart, *J. Gene Med.*, 2002, **4**, 292–299.
12 R. G. Jenkins, Q.-H. Meng, R. J. Hodges, L. K. Lee, S. E. W. Bottoms, G. J. Laurent, D. Willis, P. Ayazi Shamlou, R. J. McAnulty and S. L. Hart, *Gene Ther.*, 2003, **10**, 1026–1034.
13 R. G. Jenkins, S. E. Herrick, Q.-H. Meng, C. Kinnon, G. J. Laurant, R. J. McAnulty and S. L. Hart, *Gene Ther.*, 2000, **7**, 393–400.
14 M. F. M. Mustapa, P. C. Bell, C. A. Hurley, A. Nicol, E. Guénin, S. Sarkar, M. J. Writer, S. E. Barker, J. B. Wong, M. A. Pilkington-Miksa, B. Papahadjopoulos-Sternberg, P. Ayazi Shamlou, H. C. Hailes, S. L. Hart, D. Zicha and A. B. Tabor, *Biochemistry*, 2007, **46**, 12930–12944.
15 J. H. Felgner, R. Kumar, C. N. Sridhar, C. J. Wheeler, Y. J. Tsai, R. Border, P. Ramsay, M. Martin and P. L. Felgner, *J. Biol. Chem.*, 1994, **269**, 2550–2561.
16 D. Liu, W. Qiao, Z. Li, S. Zhang, L. Cheng and K. Jin, *Chem. Biol. Drug Des.*, 2006, **67**, 248–251.
17 C. J. Wheeler, P. L. Felgner, Y. J. Tsai, J. Marshall, L. Sukhu, S. G. Doh, J. Hartikka, J. Nietupski, M. Manthorpe, M. Nichols, M. Plewe, X. Liang, J. Norman, A. Smith and S. H. Cheng, *Proc. Natl. Acad. Sci. U. S. A.*, 1996, **93**, 11454–11459.
18 L. Baccaglini, A. T. M. S. Hoque, R. B. Wellner, C. M. Goldsmith, R. S. Redman, V. Sankar, A. Kingman, K. M. Barnhart, C. J. Wheeler and B. J. Baum, *J. Gene Med.*, 2001, **3**, 82–90.
19 M. E. Ferrari, D. Rusalov, J. Enas and C. J. Wheeler, *Nucleic Acids Res.*, 2002, **30**, 1808–1816.
20 G. Byk, C. Dubertret, V. Escriou. M. Frederic, G. Jaslin, R. Rangara, B. Pitard, J. Crouzet, P. Wils, B. Schwartz and D. Scherman, *J. Med. Chem.*, 1998, **41**, 224–235.
21 J. G. Lewis, K.-Y. Lin, A. Kothavale, W. M. Flanagan, M. D. Matteucci, R. B. DePrince, R. A. Mook Jr, R. W. Hendren and R. W. Wagner, *Proc. Natl. Acad. Sci. U. S. A.*, 1996, **93**, 3176–3181.
22 S. Bhattacharya and S. De, *Chem.–Eur. J.*, 1999, **5**, 2335–2347.
23 M. Writer, C. A. Hurley, S. Sarkar, J. B. Wong, S. Irvine, F. Afzal, J. Ho, M. Odlyha, J. R. McEwan, M. J. Lawrence, A. B. Tabor, P. Ayazi Shamlou, H. C. Hailes and S. L. Hart, *J. Liposome Res.*, 2006, **16**, 373–389.
24 O. Zelphati, L. S. Uyechi, L. G. Barron and F. C. Szoka, *Biochim. Biophys. Acta: Biomembranes*, 1998, **1390**, 119–113.
25 A. Chonn and P. R. Cullis, *Adv. Drug Delivery Rev.*, 1998, **30**, 73–83.
26 J. M. Harris and R. B. Chess, *Nat. Rev. Drug Discovery*, 2003, **2**, 214–221.
27 A. D. Miller, *Curr. Med. Chem.*, 2003, **10**, 1195–1211.
28 K. W. C. Mok, A. M. I. Lam and P. R. Cullis, *Biochim. Biophys. Acta*, 1999, **1419**, 137–150.
29 P. Harvie, F. M. P. Wong and M. B. Bally, *J. Pharm. Sci.*, 2000, **89**, 652–663.
30 L. Y. Song, Q. F. Ahkong, Q. Rong, Z. Wang, S. Ansell, M. J. Hope and B. Mui, *Biochim. Biophys. Acta: Biomembranes*, 2002, **1588**, 1–13.
31 F. Shi, L. Wasungu, A. Nomden, M. C. A. Stuart, E. Polushkin, J. B. F. N. Engberts and D. Hoekstra, *Biochem. J.*, 2002, **366**, 333–341.
32 E. Ambegia, S. Ansell, P. Cullis, J. Heyes, L. Palmer and I. MacLachlan, *Biochim. Biophys. Acta: Biomembranes*, 2005, **1669**, 155–163.
33 C. A. Hurley, J. B. Wong, J. Ho, M. Writer, M. J. Lawrence, S. L. Hart, A. B. Tabor and H. C. Hailes, *Org. Biomol. Chem.*, submitted.

34 X. Guo and F. C. Szoka, *Acc. Chem. Res.*, 2003, **36**, 335–341.
35 J. A. Boomer and D. H. Thompson, *Chem. Phys. Lipids*, 1999, **99**, 145–153.
36 K. By and M. H. Nantz, *Angew. Chem., Int. Ed.*, 2004, **43**, 1117–1120.
37 H. Chen, H. Zhang, C. M. McCallum, F. C. Szoka and X. Guo, *J. Med. Chem.*, 2007, **50**, 4269–4278.
38 A. Aissaoui, B. Martin, E. Kan, N. Oudrhiri, M. Hauchecorne, J. P. Vigneron, J. M. Lehn and P. Lehn, *J. Med. Chem.*, 2004, **47**, 5210–5223.
39 X. Guo and F. C. Szoka, *Bioconjugate Chem.*, 2001, **12**, 291–300.
40 E. R. Gillies, A. P. Goodwin and J. M. J. Fréchet, *Bioconjugate Chem.*, 2004, **15**, 1254–1263.
41 T. H. Fife and L. K. Jao, *J. Org. Chem.*, 1965, **30**, 1492–1495.
42 R. A. Bunce and J. C. Allison, *Synth. Commun.*, 1999, **29**, 2175–2186.
43 D. B. Dess and J. C. Martin, *J. Am. Chem. Soc.*, 1987, **100**, 2226–2227.
44 J. C. Stowell and D. R. Keith, *Synthesis*, 1979, 132–133.
45 S. V. Ley, J. Norman, W. P. Griffith and S. P. Marsden, *Synthesis*, 1994, 639–666.
46 C. A. Hurley, J. B. Wong, H. C. Hailes and A. B. Tabor, *J. Org. Chem.*, 2004, **69**, 980–983.
47 M. J. Writer, B. Marshall, M. A. Pilkington-Miksa, S. E. Barker, M. Jacobsen, P. C. Bell, D. H. Lester, A. B. Tabor, H. C. Hailes, N. Klein and S. L. Hart, *J. Drug Targeting*, 2004, **12** 185–194.
48 I. Koltover, T. Salditt, J. O. Radler and C. R. Sadinya, *Science*, 1998, **281**, 78–81.
49 D. Lechardeur, A. S. Verkman and G. L. Lukacs, *Adv. Drug Delivery Rev.*, 2005, **57**, 755–767.
50 I. M. Hafez, N. Maurer and P. R. Cullis, *Gene Ther.*, 2001, **8**, 1188–1196.
51 A. L. Cozens, M. J. Yezzi, M. Yamaya, J. A. Wagner, D. Steiger, S. S. Garber, L. Chin, E. M. Simon, G. R. Cutting, P. Gardner, D. S. Friend, C. B. Basbaum and D. C. Gruenert, *In Vitro Cell Dev. Biol.: Anim.*, 1992, **28**, 735–744.
52 A. L. Cozens, M. J. Yezzi, K. Kunzelmann, T. Ohrui, L. Chin, K. Eng, W. E. Finkbeiner, J. H. Widdicombe and D. C. Gruenert, *Am. J. Respir. Cell Mol. Biol.*, 1994, **10**, 38–47.
53 R. Ross, *J. Cell Biol.*, 1971, **50**, 172–186.