Mono- and dicationic short PEG and methylene dioxyalkylglycerols for use in synthetic gene delivery systems†

Christopher A. Hurley,‡[a] John B. Wong,[a] Jimmy Ho,[a] Michele Writer,[b] Scott A. Irvine,[b] M. Jayne Lawrence,[c] Stephen L. Hart,[b] Alethea B. Tabor[a] and Helen C. Hailes*[a]

Received 20th December 2007, Accepted 14th May 2008
First published as an Advance Article on the web 30th May 2008
DOI: 10.1039/b719702k

A range of monocationic and dicationic dioxyalkylglycerol cytofectins have been synthesised possessing methylene and short n-ethylene glycol spacers. The monocationic compounds were found to be effective in transfections when formulated as lipopolyplexes with peptide and DNA components, in particular with shorter PEG head groups which may have less effect on peptide targeting in the ternary complex.

Introduction

Gene transfer technology has the potential to revolutionise many treatment strategies for inherited or acquired diseases; however, an efficient vector system is required to deliver the gene of interest into target cells. Synthetic vectors offer one solution and have several advantages over viral systems in terms of their greater nucleic acid packaging capacity, lower immunogenicity and greater safety.[1] Several different synthetic vector systems have been reported including complexes of polycationic polymers such as polyethylenimine (PEI), dendrimers, and cationic lipid delivery systems (lipoplexes).[2–5] However, the major limitation to date has been their poor transfection efficiency relative to viral vectors, and therefore the advantages of non-viral delivery systems will only be realised if gene transfer efficiencies can be improved *in vivo*. One approach has been to improve the efficacy and selectivity of synthetic vectors by targeting to cell-surface receptors using peptides, polysaccarides or antibodies.[6] Ternary synthetic vectors (lipopolyplexes) have recently been described by several groups[7] including Hart *et al.*[8] The targeted system was comprised of a mixture of lipids (**1** and **2**) (L), an integrin-targeting peptide **3** (I) and plasmid DNA (D) which combined electrostatically on mixing in solution to form LID vector particles (Fig. 1).[8] The lipid component (L) Lipofectin™, was a 1 : 1 mixture of the cationic lipid 2,3-dioleyloxypropyl-1-trimethylammonium chloride (DOTMA) (**1**) and neutral phospholipid dioleoyl L-α-phosphatidylethanolamine (DOPE) (**2**).[9] The peptide component contained a targeting motif, the cyclic $\alpha_5\beta_1$ integrin-specific sequence (I) CRRETAWAC,[10] and a sixteen-lysine motif to mediate DNA compaction separated *via* a linker GA (glycine-alanine).[8] The lipid/peptide/DNA ternary

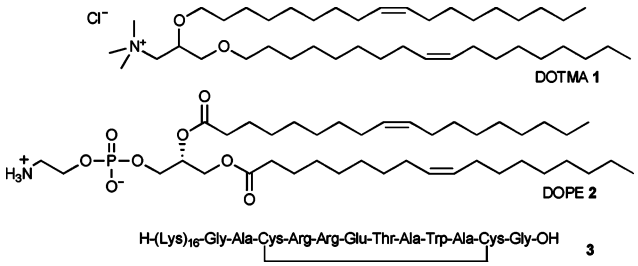


Fig. 1 LID vector components.

formulation reported was found to produce a synergistic increase in transfection efficiency compared to the corresponding binary vector formulations.[8,11] Indeed, the LID systems displayed high transfection efficiency and low toxicity *in vitro* and *in vivo*.[8,11–13] Particle sizing studies of this vector formulation indicated that discrete particles were formed upon combining the LID components.[8] The stoichiometry and structure of the LID complex has been studied using fluorescence correlation spectroscopy (FCS), fluorescence quenching experiments, and freeze-fracture electron microscopy. These indicated that the peptide (I) interacts with the plasmid DNA (D), resulting in a tightly condensed DNA–peptide inner core which is surrounded by a disordered lipid layer, from which the integrin-targeting sequence of the peptide partially protrudes.[14]

Cationic lipids (cytofectins) such as DOTMA have several roles in the gene delivery process including DNA compaction, together with the peptide component in LID, interaction with anionic cell surface receptors, and in enhancing endosomal release. The neutral lipid DOPE is also believed to help stabilise the liposome structure and enhance liposome fusion with the endosomal membrane leading to endosomal escape.[2] DOTMA contains a glycerol backbone, two hydrocarbon oleyl chains linked through ether moieties and a trimethylammonium cationic head group. Each of these components may influence the transfection efficiency of both binary lipoplex and ternary lipopolyplex formulations such as LID. Indeed there have been several studies which have investigated the use of different head groups or diether-linked chain length analogues in lipoplex systems including: *N*,*N*-dimethyl-*N*-ethanolamine headgroups (in for example DIMRIE and DORIE which possess C14:0 and C18:1 chain lengths respectively);[15] *N*,*N*,*N*-trialkylammonium head groups and C_{12} to C_{18} saturated chains used in sizing studies of cationic lipoplexes;[16]

[a]*Department of Chemistry, University College London, 20 Gordon Street, London, WC1H 0AJ, UK. 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, WC1N 1EH, UK*
[c]*Pharmaceutical Science Research Division, King's College London, Stamford Street, London, SE1 9NH, UK*

† Electronic supplementary information (ESI) available: Syntheses of the alkyl mesylates and **9**, **11–13**, **15**, **16**, **18–24**, **26**, **27**, **32**, **33**, **35**, **36**, **38**, **39**, **41** and **42** are described, together with formulation, sizing, transfection procedures and transfection data. See DOI: 10.1039/b719702k

‡ Current address: Argenta Discovery Ltd, 7–9 Spire Green Centre, Flex Meadow, Harlow, CM19 5TR, UK.

N,*N*-dimethyl-3-aminopropyl head groups with C_{12} to C_{18} chains (in cytofectins such as GAP-DLRIE which proved effective in lipoplex *in vivo* and *in vitro* systems);[17–19] and spermine carboxyamido head groups (such as that in DOSPA).[20–22] We have also investigated the use of different chain length analogues in the LID ternary system.[23] The published data on these formulations indicated that the length of the alkyl chain and head group are important factors for achieving high transfection, but that transfection efficiencies are frequently system and cell-type dependent.

Despite the recent advances made in gene delivery, one key problem is the stability of nonviral vector systems in biological fluids, since for systemic delivery prolonged plasma circulation of the vector is essential. Positively charged particles are prone to non-specific interactions with plasma proteins leading to increased clearance by the reticuloendothelial system (RES).[24,25] In addition they are unstable in serum and buffers, leading to the formation of large aggregates.[24,25] Previous reports have indicated that the tethering of poly(ethylene glycol) (PEG) moieties can provide a steric barrier, thus shielding the complexes from interactions with biological fluids and enhancing stabilisation *in vivo*.[25–27] PEGs are typically used in the range 1000–5000 Da, and PEG-grafted lipids are added to the formulation at normally 2–10 mol%. This approach has proved to be effective and PEG–lipids have enhanced lipoplex stability in serum.[27–32] However, transfection efficiencies are frequently lower due to the PEG acting as a steric barrier, reducing particle and cell membrane contact.[27–32] The length of the hydrophobic chain in PEG–lipid conjugates has also been shown to be an important factor in particle stability and delivery properties.[27–32] One more recent strategy has been to introduce cleavable PEG groups to improve transfection efficiencies.[33]

We are interested in enhancing the LID particle stability in the presence of serum for *ex vivo* or *in vivo* applications. Since in the LID ternary vector targeting by the peptide component is essential, our strategy was to prepare modified cytofectins possessing PEG moieties, in particular shorter PEGs of defined length to ensure minimal disruption of peptide targeting, and moreover, to use these in formulations at high mol% to enhance shielding effects. Monocationic lipids **4** and dicationic lipids **5** were designed incorporating a glycerol skeleton, which has proven to be particularly effective in the LID formulation, with PEG directly attached to the head group and a pendant OH (**4**) or a second trimethylammonium cation (**5**) giving a dicationic cytofectin to enhance lipid-DNA interactions (Fig. 2). For comparison purposes, the dicationic species (**5**) were prepared with both a PEG and methylene spacer. Furthermore, in the most promising compounds the length of the hydrophobic chain was also varied because this has been shown to be important in long PEG–lipid conjugates and other lipid systems, and in a ternary system may also influence the availability of peptide targeting.[23,28–32]

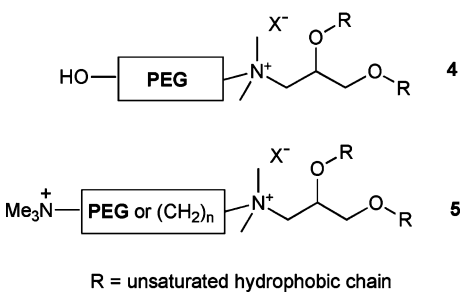
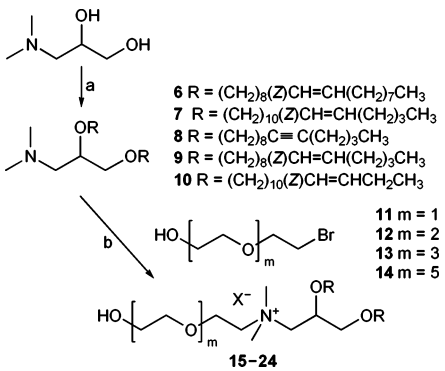


Fig. 2 Monocationic PEG–lipid conjugates **4** and dicationic lipids **5**.

Results and discussion

The syntheses of monocationic PEG-lipids were carried out as outlined in Scheme 1. The tertiary amines **6–10** were prepared from 3-(dimethylamino)-1,2-propandiol and the corresponding alkyl mesylate as previously described.[34] Synthesis of the unsaturated C_{14} (at C-11) C_{16} and C_{18} mesylates have been reported:[34] the C_{14} (at C-9) alkyne and alkene mesylates were readily prepared from the alcohols which were synthesised *via* a route similar to that recently reported by Basita-Pereira *et al.*[35] Mono-brominations of commercially available PEG diols were achieved using hydrobromic acid[36] or thionyl bromide to give **11–14** in 36–58% yield. Quaternisation of amines **6–10** with PEG bromoalcohols **11–14** in methanol using a sealed tube readily gave the PEG–lipid conjugates **15–24** (Scheme 1, Table 1). These were purified by low temperature recrystallisation where possible to avoid complexation of the lipids to SiO_2 which was observed when using flash silica chromatography. When this was used silica was removed by dissolving the lipid in chloroform, centrifugation and filtration.



Scheme 1 *Reagents and conditions*: (a) ROMs, NaH, 61–85%; (b) **11–14**, MeOH, 90 °C, 34–73%.

It has previously been observed that multiply charged cationic lipids can enhance transfection efficiencies compared to conventional mono-cationic lipids.[17,37] Typically the charged species is formed within the acidic environment of the endosome from a lipid possessing primary or secondary amine moieties. Dicationic quaternary ammonium lipids have also previously been reported

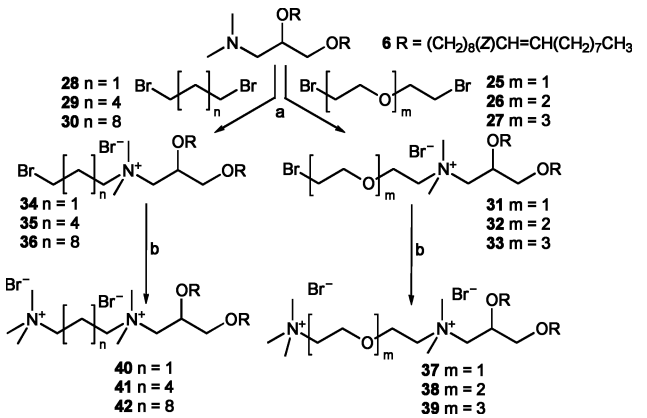
Table 1 Short PEG–lipid conjugates synthesised

Compound	Chain	R	*m*	X
15	C_{18}	$(CH_2)_8(Z)CH{=}CH(CH_2)_7CH_3$	1	Br
16	C_{18}	$(CH_2)_8(Z)CH{=}CH(CH_2)_7CH_3$	2	Br
17a	C_{18}	$(CH_2)_8(Z)CH{=}CH(CH_2)_7CH_3$	3	Br
17b	C_{18}	$(CH_2)_8(Z)CH{=}CH(CH_2)_7CH_3$	3	Cl[a]
18	C_{18}	$(CH_2)_8(Z)CH{=}CH(CH_2)_7CH_3$	5	Br
19	C_{16}	$(CH_2)_{10}(Z)CH{=}CH(CH_2)_3CH_3$	3	Br
20	C_{16}	$(CH_2)_{10}(Z)CH{=}CH(CH_2)_3CH_3$	5	Br
21	C_{14}	$(CH_2)_8C{\equiv}C(CH_2)_3CH_3$	1	Br
22	C_{14}	$(CH_2)_8(Z)CH{=}CH(CH_2)_3CH_3$	1	Br
23	C_{14}	$(CH_2)_{10}(Z)CH{=}CHCH_2CH_3$	1	Br
24	C_{14}	$(CH_2)_{10}(Z)CH{=}CHCH_2CH_3$	3	Br

[a] Chloride prepared from the bromide *via* ion-exchange.

for use in gene delivery, including PropEce[38] and a pentaerythritol-derived tetraester,[39] and demonstrated good activities. The presence of a second quaternary amine group could increase the electrostatic interaction between the lipid and DNA, or less lipid may be required to maintain the charge ratio, and may also increase interactions with the endogenous negatively charged lipids in the endosome, enhancing endosomal escape. The diquaternary ammonium lipids were designed with a lipophilic methylene or hydrophilic PEG spacer between the cationic moieties (**4**, Fig. 2). In addition, the length of the spacer was also varied to obtain information regarding the optimal charge separation; however, in general shorter linkers were used to ensure the lipids did not interfere with peptide targeting in the ternary lipopolyplex vector.

The dicationic lipids were synthesised as shown in Scheme 2 using spacers **25–30**. The PEG dibromides **26** and **27** were readily prepared from tri- and tetraethylene glycol and triphenylphosphine and carbon tetrabromide in 90% and 68% yield respectively.[40] The quaternisations of **6**, using an excess of the dibromides to avoid a double quaternisation, were achieved in reasonable yields to give **31–36**. We noted that yields of products using the lipophilic spacers were generally higher (46–87%) than for the PEG dibromides (22–48%) and no di-quaternised products were detected. Introduction of the second quaternary ammonium centre to give **37–42** was then readily achieved using trimethylamine and heating in a sealed tube, where again the higher product yields were for the lipophilic series.



Scheme 2 *Reagents and conditions*: (a) **24–29**, solvent, 90 °C, 22–87%; (b) NMe_3 (45 wt% in H_2O), MeOH, 90 °C, 32–93%.

With the mono- and dicationic lipids in hand, ternary lipopolyplexes were formulated using these lipids, plasmid DNA and peptide **3**. A preliminary assessment of the transfection properties in human airway epithelial HAE cells (1HAEo−) was performed in the ternary lipopolplex vector using peptide **3**. In general, the dicationic lipids possessing the lipophilic spacers (**40–42**), when formulated with or without DOPE (**2**), transfected at a lower level than Lipofectin™, although the transfection efficiency of **40** was significantly higher than **42**, suggesting less charge separation in the lipid was preferable (data in ESI†). The diquaternary ammonium hydrophilic spacer lipids **37–39** generally displayed activities lower than for **40–42** when formulated with or without DOPE. Although the activities were low, **37** with the shorter spacer was the most active of the three analogues **37–39**, highlighting again that a shorter linker between the cationic charges is preferred.

The monocationic PEG conjugates **15–24** displayed the best transfection activities, and therefore these were investigated in more detail. Sizing and zeta potential measurements were carried out with and without DOPE (**2**) on a subset of compounds, **21–23**. After sonication and equilibration for 24 h the vesicle sizes for **21–23** were approximately 160–170 nm without DOPE (**2**) and 70–80 nm with **2**. Zeta potentials were in the range 60–73 mV without **2** and 46–57 mV with **2**. The size of liposomes comprised of trimethylammonium head group lipids such as DOTMA (**1**) together with DOPE (**2**) are reported as 100–200 nm depending on the formulation method,[41,42] and we measured the size of liposomes of **1** + **2** as approximately 140 nm. This indicated that the short PEG lipids with **2** formed comparably smaller vesicles.

Some representative transfection data is shown in Fig. 3–5, for compounds **17a**, **18**, **19** and **20** highlighting our key observations. The PEG chain conjugate cytofectins, containing PEG4-OH moieties and C_{16} or C_{18} alkyl chains, compounds **17a** and **19**, were found to be particularly effective transfection reagents in HAE cells (1HAEo−)[43] (Fig. 3), and SCFTE29o− cells[44] (Fig 4) (see ESI for preliminary transfection data on other PEGylated lipids†). Attachment of a PEG6-OH to the cytofectin head group (compounds **18** and **20**) led to decreased levels of transfection,

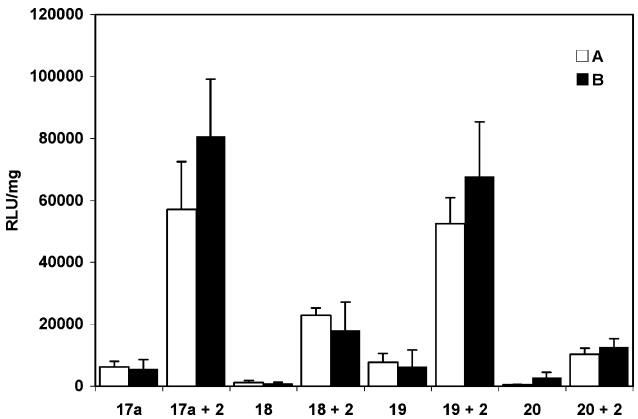


Fig. 3 Transfection of 1HAEo− cells with PEG lipids **17a–20** in LID ternary complexes with and without DOPE (**2**). **A** is a 2 : 1 mixture (by weight) of total lipid and DNA. **B** is a 4 : 1 ratio (by weight) of total lipid and DNA (error bars represent the mean and SD from 6 experiments).

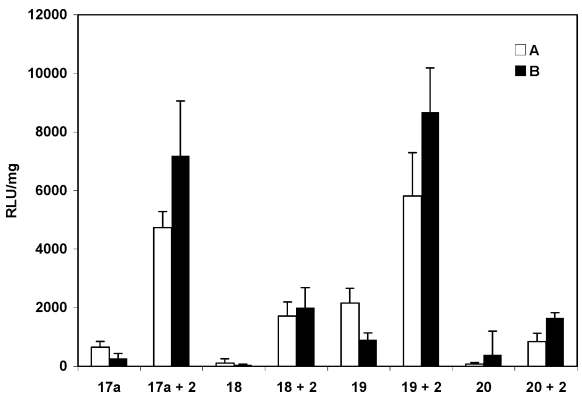


Fig. 4 Transfection of SCFTE29o− cells with PEG lipids **17a–20** in LID ternary complexes with and without DOPE (**2**). **A** is a 2 : 1 ratio (by weight) of total lipid and DNA. **B** is a 4 : 1 ratio (by weight) of total lipid and DNA (error bars represent the mean and SD from 6 experiments).

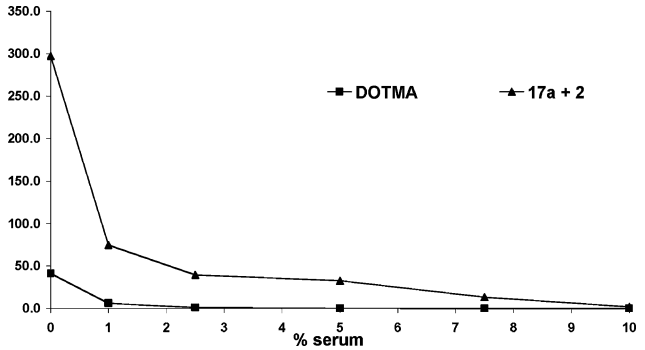


Fig. 5 Comparison of transfection performance in 1HAEo− cells (2 : 1 ratio by weight of total lipid:DNA) reflecting serum stabilities of the lipopolyplex complex without PEG–lipid and with PEG–lipid **17a** (RLU/10^4 cells are shown).

most likely due to unfavourable steric interactions, with the PEG chain on the ternary particle surface blocking access of the targeting peptide to the cell surface. Conjugates **17a** and **19** PEG4-OH cytofectins formulated into lipopolyplexes were also observed to be more stable in the presence of foetal calf serum (FCS) than compounds with shorter n-ethylene glycol units (compounds **15**, **16**, **21–23**), and higher levels of transfection were observed for **17a** compared to DOTMA (Fig. 5). Thus the PEG4-OH conjugates may provide an optimal balance between enhanced stability properties, and accessibility of the targeting peptide appears to be unaffected. Since the addition of PEG6-OH groups to **6** appeared to have a detrimental effect on the levels of transfection achieved using LID, we did not synthesise conjugates possessing longer PEGs, which could reduce the peptide targeting efficiency further.

Conclusions

In summary, routes to cytofectins possessing dicationic quaternary amine groups separated by hydrophilic and lipophilic linkers have been described. Dicationic lipids **37–39** and **40–42** generally gave lower transfection levels than that for Lipofectin™ when formulated with or without DOPE. Cationic lipids have also been prepared possessing short PEG-OH groups on the head group. These lipids were shown to form compact vesicles, and give efficient gene delivery vectors when formulated as lipopolyplexes. Lipids containing PEG4-OH rather than PEG6-OH moieties were shown to be particularly effective when used in the ternary lipopolyplex formulations. This is probably because peptide targeting in the ternary complex is not affected when formulated with short PEG conjugates for steric reasons. There are an increasing number of delivery systems utilising targeting moieties where shielding facilities can enhance *in vivo* properties. It is however crucial that strategies to ensure minimal steric obstruction of the targeting group by the shielding functionality are used. This issue of the accessibility of a peptide ligand to the surface of cells and length of PEG chains has also been highlighted by Tirrell and co-workers.[45] Our approach has been to incorporate short PEG groups used exclusively as the cationic cytofectin in the formulation, and these results are important for the design of other delivery systems possessing cell-targeting groups. Detailed *in vitro* and *in vivo* studies are now underway and will be reported elsewhere.

Experimental

General methods

Unless otherwise noted, solvents and reagents were reagent grade from commercial suppliers and used without further purification. THF was dried by distillation from a sodium/benzophenone suspension under a dry N_2 atmosphere. CH_2Cl_2 was dried by distillation from CaH_2 under a dry N_2 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 with detection by UV, or permanganate and phosphomolybdic acid stains. Flash column chromatography was carried out using silica gel (particle size 40–63 μm). Melting points are uncorrected. ^{1}H NMR and ^{13}C NMR spectra were recorded in $CDCl_3$ at the field indicated. *J* values are given in Hz. Representative procedures are shown below for the synthesis of mono- and dicationic lipids.

Syntheses

2,3-Di-(tetradec-9-ynyloxy)propyl-*N*,*N*-dimethylamine (8). To a stirring solution of sodium hydride (60% in mineral oil; 0.275 g, 6.88 mmol) in anhydrous toluene (35 mL) at rt was added 3-(dimethylamino)propane-1,2-diol (0.27 mL, 2.28 mmol). The mixture was heated at 50 °C for 20 min, and tetradec-9-ynyl mesylate (1.98 g, 6.88 mmol) was added. The reaction was then heated at reflux for 72 h. On cooling, water (100 mL) was added and the product extracted with ethyl acetate (3 × 50 mL). The combined organic extracts were washed with a solution of saturated sodium hydrogencarbonate (50 mL), saturated sodium chloride (50 mL) and dried ($MgSO_4$). The solvent was removed *in vacuo* to give the crude product which was purified by silica gel flash chromatography (5% MeOH in CH_2Cl_2) to afford ***8*** (0.760 g, 66%) as a pale yellow oil. $R_f = 0.36$ (5% MeOH in CH_2Cl_2); ν_{max} (film)/cm^{-1} 2932, 2855, 2361, 1458; δ_H (300 MHz; $CDCl_3$) 0.88 (6H, t, *J* 7.2, 2 × CH_2CH_3), 1.28–1.46 (32H, m), 2.12 (8H, m, 2 × $H_2CC{\equiv}CCH_2$), 2.28 (6H, s, $N(CH_3)_2$), 2.41 (2H, m, NCH_2CH), 3.39–3.60 (7H, m, $CHOCH_2$, CH_2OCH_2); δ_C (75.4 MHz; $CDCl_3$) 13.6 (2 × CH_3CH_2), 18.4, 18.7, 21.9, 26.0, 28.8, 29.1 (signal overlap), 29.4, 29.6, 30.2, 31.3, 46.2 ($N(CH_3)_2$), 61.0 (NCH_2CH), 70.1 ($CHCH_2O$), 71.6 and 71.9 (2 × OCH_2CH_2), 76.6 ($CHOCH_2$), 80.2 ($C{\equiv}C$, signal overlap); *m*/*z* (+ES) 504.5 (MH^+, 100%); Found (+HRES) MH^+ 504.47930. $C_{33}H_{62}NO_2$ requires 504.47806.

2,3-Di-((9*Z*)-octadecenyloxy)propyl-*N*-(2-{2-[2-(2-hydroxyethoxy)ethoxy]ethoxy}ethyl)-*N*,*N*-dimethylammonium bromide (17a). Compound **13** (137 mg, 0.533 mmol) and the amine **6** (300 mg, 0.485 mmol) in methanol (2 mL) were stirred at 90 °C in a sealed tube for 24 h. The solvent was removed *in vacuo* and the product purified by low temperature recrystallisation (ethyl acetate) to yield ***17a*** as a pale yellow oil (245 mg, 58%). $R_f = 0.23$ (10% MeOH in CH_2Cl_2); ν_{max} (film)/cm^{-1} 3404, 2920, 2858, 1634, 1466; δ_H (300 MHz; $CDCl_3$) 0.85 (6H, t, *J* 7.0, 2 × CH_2CH_3), 1.25 (44H, m), 1.54 (4H, m, 2 × OCH_2CH_2), 2.01 (8H, m, 2 × $CH_2CH{=}CHCH_2$), 2.58 (1H, br s, *OH*), 3.43 (6H, s, 2 × N^+CH_3), 3.48–4.20 (25H, m, 6 × CH_2O (PEG), $CHOCH_2$, $CHOCH_2$, $CH_2OC_{18}H_{35}$, $CH_2OCH_2CH_2$, 2 × N^+CH_2, CH_2OH), 5.35 (4H, m, 2 × $CH{=}CH$); δ_C (75.4 MHz; $CDCl_3$) 14.0 (2 × CH_2CH_3), 22.6, 26.0, 26.2, 27.2, 29.1, 29.3, 29.5, 29.6, 29.7 (signal overlap),

30.4, 32.3, 33.0, 53.1 and 53.6 (2 × N^+CH_3), 61.2 (CH_2OH), 65.1, 66.7, 68.7, 69.3, 70.1 (signal overlap), 70.5, 72.0, 72.6, 73.5 ($CHOCH_2$), 129.8 (2 × CH=*C*H), 130.0 (2 × *C*H=CH); *m*/*z* (+ES) 797 (M^+ − Br, 100%); Found (+HRFAB) (M^+ − Br), 796.7399. $C_{49}H_{98}NO_6$ requires 796.7394; Found C, 66.19; H, 10.99; N, 1.50. $C_{49}H_{98}NO_6Br{\cdot}H_2O$ requires C, 65.74; H, 11.26; N, 1.56%.

2,3-Di-((9*Z*)-octadecenyloxy)propyl-*N*-(2-{2-[2-(2-hydroxyethoxy)ethoxy]ethoxy}ethyl)-*N*,*N*-dimethylammonium chloride (17b). Crude **17a** (approximately 250 mg) (generated as described above) in methanol (1 mL) was passed through an Amberlite® IRA-400 (Cl) ion exchange column eluting with dichloromethane–methanol (1 : 1). The solvents were removed *in vacuo* and the crude product purified by low temperature recrystallisation (ethyl acetate) to yield **17b** as a pale yellow oil (~90% from crude **17a**).

2,3-Di-((9*Z*)-octadecenyloxy)propyl-*N*-[2-(2-bromoethoxy)ethyl]-*N*,*N*-dimethylammonium bromide (31). 2-Bromoethyl ether (**25**) (278 mg, 1.20 mmol) and the amine **6** (300 mg, 0.483 mmol) were stirred in methanol (2 mL) at 90 °C in a sealed tube for 24 h. The solvent was removed *in vacuo* and the product purified by low temperature recrystallisation (ethyl acetate) to yield ***31*** as a pale yellow oil (197 mg, 48%). ν_{max} (film)/cm^{-1} 2927, 2854, 2344, 1642, 1465; δ_H (300 MHz; $CDCl_3$) 0.84 (6H, t, *J* 7.0, 2 × CH_2CH_3), 1.25 (44H, m), 1.52 (4H, m, 2 × $OCH_2CH_2CH_2$), 1.98 (8H, m, 2 × CH_2CH=CHCH_2), 3.41 (4H, m, $CH_2OCH_2CH_2$, $CHOCH_2$), 3.48–4.10 (19H, m, 2 × CH_2O (PEG), $CHOCH_2$, $CH_2OC_{18}H_{35}$, 2 × N^+CH_2, CH_2Br, 2 × N^+CH_3), 5.31 (4H, m, 2 × *C*H=*C*H); δ_C (75 MHz; $CDCl_3$) 14.0 (2 × CH_2CH_3), 22.6, 26.0, 26.2, 27.2, 29.1, 29.2–29.4 (signal overlap), 29.5, 29.6, 29.7, 30.0, 30.8, 31.8, 32.5, 53.3 and 53.5 (2 × N^+CH_3), 64.9, 66.8, 68.6, 69.3, 71.0, 72.0 (signal overlap), 73.4 ($CHOCH_2$), 129.7 (2 × CH=*C*H), 129.9 (2 × *C*H=CH); *m*/*z* (+ES) 773 (M^+ − Br (^{81}Br), 100%), 771 (M^+ − Br (^{79}Br), 88); Found (+HRFAB) (M^+ − Br), 770.6039. $C_{45}H_{89}O_3NBr$ requires 770.6020.

2,3-Di-((9*Z*)-octadecenyloxy)propyl-*N*-(3-bromopropyl)-*N*,*N*-dimethylammonium bromide (34). Amine **6** (0.500 g, 0.806 mmol) and 1,3-dibromopropane (**28**) (0.82 mL, 8.10 mmol) were stirred in hexane (2 mL) in a sealed tube at 80 °C for 18 h. The solvent was removed *in vacuo* and the product purified by flash silica gel chromatography (gradient: CH_2Cl_2 to 10% MeOH in CH_2Cl_2) to yield ***34*** as a pale yellow oil (0.410 g, 62%). R_f = 0.17 (5% MeOH in CH_2Cl_2); ν_{max} (film)/cm^{-1} 2924, 2853, 1634, 1464; δ_H (300 MHz; $CDCl_3$) 0.85 (6H, t, *J* 6.7, 2 × CH_2CH_3), 1.27 (44H, m), 1.53 (4H, m, 2 × $OCH_2CH_2CH_2$), 1.97 (8H, m, 2 × CH_2CH=CHCH_2), 2.42 (2H, m, CH_2CH_2Br), 3.40 (4H, m, $CH_2OCH_2CH_2$, $CHOCH_2$), 3.45–3.92 (14H, m, $CH_2OC_{18}H_{35}$, 2 × N^+CH_2, CH_2Br, 2 × N^+CH_3), 4.06 (1H, m, $CHOCH_2$), 5.32 (4H, m, 2 × *C*H=*C*H); δ_C (75 MHz; $CDCl_3$) 14.0 (2 × CH_2CH_3), 22.7, 26.1, 26.3, 27.3, 28.7, 29.2, 29.3–29.8 (signal overlap), 30.1, 31.9, 32.6, 52.8 and 53.0 (2 × N^+CH_3), 64.5, 65.8, 68.4, 69.4, 72.1, 73.3 ($CHOCH_2$), 129.7 (2 × CH=*C*H), 129.8 (2 × *C*H=CH); *m*/*z* (+ES) 743 (M^+ − Br (^{81}Br), 100%), 741 (M^+ − Br (^{79}Br), 88); Found (+HRFAB) (M^+ − ^{79}Br), 740.5939. $C_{44}H_{87}BrNO_2$ requires 740.5915.

2,3-Di-((9*Z*)-octadecenyloxy)propyl-*N*-[2-(2-(*N*,*N*,*N*-trimethylammonium)ethoxy)ethyl]-*N*,*N*-dimethylammonium dibromide (37). Bromide **31** (100 mg, 0.118 mmol) and trimethylamine (45 wt% in H_2O; 0.36 mL, 2.35 mmol) were stirred in methanol (3 mL) in a sealed tube at 90 °C for 24 h. The solvent was removed *in vacuo* and the residue triturated at low temperature (diethyl ether) to obtain ***37*** (73 mg, 67%). ν_{max} (film)/cm^{-1} 2922, 2853, 1462; δ_H (300 MHz; $CDCl_3$) 0.85 (6H, t, *J* 6.6, 2 × CH_2CH_3), 1.24 (44H, m), 1.51 (4H, m, 2 × $OCH_2CH_2CH_2$), 1.98 (8H, m, 2 × CH_2CH=CHCH_2), 3.40–4.14 (32H, m, 2 × CH_2O (PEG), $CH_2OC_{18}H_{35}$, $CH_2OCH_2CH_2$, $CHOCH_2$, $CHOCH_2$, 3 × N^+CH_2, 5 × N^+CH_3), 5.35 (4H, m, 2 × *C*H=*C*H); δ_C (75 MHz; $CDCl_3$) 14.1 (2 × CH_2CH_3), 22.7, 26.1, 26.2, 27.2, 29.2–29.8 (signal overlap), 30.1, 31.9, 32.6, 52.9 (N^+CH_3), 53.8 (N^+CH_3), 54.7 (3 × N^+CH_3), 64.8, 65.0, 65.5, 65.9, 66.6, 69.0, 69.4, 72.1, 73.4, 129.8 (2 × CH=*C*H), 130.0 (2 × *C*H=CH); *m*/*z* (+ES) 375.6 ($\frac{1}{2}$[M − 2Br]$^+$, 100%); Found (+HRFAB) (M^+ − ^{79}Br), 829.6729. $C_{48}H_{98}BrN_2O_3$ requires 829.6755.

2,3-Di-((9*Z*)-octadecenyloxy)propyl-*N*-3-(*N*,*N*,*N*-trimethylammoniumpropyl)-*N*,*N*-dimethylammonium dibromide (40). Bromide **34** (100 mg, 0.12 mmol) and trimethylamine (45 wt% in H_2O; 0.094 mL, 0.61 mmol) were stirred in methanol (2 mL) in a sealed tube at 90 °C for 24 h. The solvent was removed *in vacuo* and the product purified by low temperature recrystallisation (ethyl acetate) to give ***40*** (87 mg, 82%). ν_{max} ($CHCl_3$)/cm^{-1} 2922, 2852, 1656, 1456; δ_H (300 MHz; $CDCl_3$) 0.86 (6H, t, *J* 6.5, 2 × CH_2CH_3), 1.25 (46H, m), 1.53 (4H, m, 2 × OCH_2CH_2), 2.00 (8H, m, 2 × CH_2CH=CHCH_2), 3.38–4.01 (27H, m, $CH_2OC_{18}H_{35}$, $CH_2OCH_2CH_2$, $CHOCH_2$, 3 × N^+CH_2, 5 × N^+CH_3), 4.11 (1H, m, $CHOCH_2$), 5.33 (4H, m, 2 × *C*H=*C*H); δ_C (75 MHz; $CDCl_3$) 14.1 (2 × CH_2CH_3), 22.6, 25.8, 26.0, 27.1, 29.0–29.6 (signal overlap), 29.9, 31.7, 32.4, 51.5 (N^+CH_3), 53.6 (N^+CH_3), 54.0 (3 × N^+CH_3), 62.6, 63.1, 67.2, 68.8, 69.2, 71.9, 73.0 ($CHOCH_2$), 129.6 (2 × CH=*C*H), 129.7 (2 × *C*H=CH); *m*/*z* (+ES) 361 ($\frac{1}{2}$[M − 2Br]$^+$, 100%); Found (+HRFAB) (MNa − 2Br)$^+$, 743.7350. $C_{47}H_{96}O_2N_2Na$ requires 743.7346.

Acknowledgements

The EPSRC are thanked for a DTA studentship to C.A.H., J.B.W., and an EPSRC-LSI grant to J.H. (GR/S05878/01). M.W was supported by a grant from the BBSRC. The authors also thanks Dieter Gruenert, California Pacific Medical Center Research Insitiute, San Francisco, CA, for kindly providing the cell lines 1HAEo− and SCFTE29o−.

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 For exampleT. 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*, 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.*, 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*, 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*, 2005, **1669**, 155–163.
33 X. Guo, J. A. MacKay and F. C. Szoka, *Biophys. J.*, 2003, **84**, 1784–1795.
34 C. A. Hurley, J. B. Wong, H. C. Hailes and A. B. Tabor, *J. Org. Chem.*, 2004, **69**, 980–983.
35 L. G. Bastista-Pereira, K. Stein, A. F. de Paula, J. A. Moreira, I. Cruz, M. L. C. Figueiredo, J. Perri and A. G. Corrêa, *J. Chem. Ecol.*, 2006, **32**, 1085–1099.
36 G. Herve, D. Uwe Hahn, H. C. Hailes, A-C. Herve, K. J. Goodworth and A. M. Hill, *Org. Biomol. Chem.*, 2003, **1**, 427–435.
37 J.-P. Behr, B. Demeneix, J.-P. Loeffler and J. Perez-Mutul, *Proc. Natl. Acad. Sci. U. S. A.*, 1989, **86**, 6982–6986.
38 H. S. Rosenzweig, V. A. Rakhmanova and R. C. MacDonald, *Bioconjugate Chem.*, 2001, **12**, 258–263.
39 A. M. Aberle, F. Tablin, J. Zhu, N. J. Walker, D. C. Bruenert and M. H. Nantz, *Biochemistry*, 1998, **37**, 6533–6540.
40 R. Appel, *Angew. Chem., Int. Ed. Engl.*, 1975, **14**, 801–811.
41 F. Sakurai, R. Inoue, Y. Nishino, A. Okuda, O. Matsumoto, T. Taga, F. Yamashita, Y. Takakura and M. Hashida, *J. Controlled Release*, 2000, **66**, 255–269.
42 S. Kawakami, A. Sato, M. Nishikawa, F. Yamashita and M. Hashida, *Gene Ther.*, 2000, **7**, 292–299.
43 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.*, 1992, **28A**, 735–744.
44 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.
45 Y. Dori, H. Bianco-Peled, J. B. McCarthy and M. Tirrell, *J. Biomed. Mater. Res.*, 2000, **50**, 75–81.