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Effect of chain length on transfection properties of spermine-based gemini surfactants

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The synthesis and associated structure–activity relationships for gene transfection of a series of spermine-derived cationic gemini surfactants incorporating diamino acid headgroups and either identical (symmetrical) or different (unsymmetrical) lipophilic tailgroups is described. Transfection activity is found to depend critically upon the structural elements present.

Introduction

The development of safe, non-toxic vectors that are able to mediate the efficient transfer of polynucleotides into cells is currently an intense area of research.[1] Although, in contrast to viral vectors, synthetic non-viral vectors, such as cationic lipid surfactants, are typically less efficient, a wide variety of formulated surfactants are available that can lead to high levels of gene transfection, particularly in immortal cell lines. Such delivery agents are therefore extremely valuable tools that facilitate cell experimentation *in vitro* (for example in biological target validation studies). Moreover, non-viral transfection agents largely avoid the complications and safety issues associated with viral vector transfection and therefore, subject to the identification of improved agents, have considerable potential for *in vivo* antisense oligonucleotide therapy and corrective gene therapy.[2]

In the early 1990s a new class of 'twinned' gemini surfactants, charactarised by the presence of two polar head groups and two lipophilic chains separated by a linking group were introduced (Fig. 1).[3] Cationic gemini surfactants have been demonstrated to be efficient, non-toxic transfection agents in a variety of cell types. In contrast to their monomeric counterparts, these amphiphiles have significantly enhanced interfacial properties and gemini surfactants, in particular, have been shown to readily bind DNA and form "lipoplexes" that are able to cross outer cell membranes. Ultimately, likely following pH-mediated changes in aggregation state of the resulting liposome within the cell, this can lead to efficient delivery of the DNA cargo to the cell nucleus.[4]

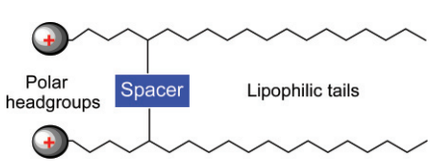
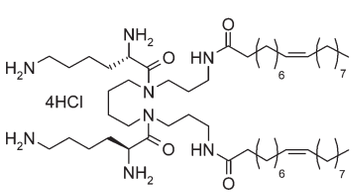


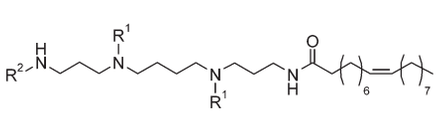
Fig. 1 General structural representation of cationic gemini surfactants.

Although gene transfection is a complex multistep process governed by both biological variables and structural elements of the cationic lipid, some underlying structure–activity relationships (SAR) are beginning to emerge, and it is likely that the development of a better understanding of the factors governing the transfection abilities of cationic surfactants will assist in the rational design of improved non-viral vectors of clinical significance.[5] Toward this end, it is interesting to note that although many lipids with symmetrical lipophilic domains have been prepared, comparatively few examples of surfactants incorporating tailgroups of different lengths have been reported.[6]

Herein, we describe the preparation of a series of gemini surfactants based upon GSC103[7] (Fig. 2) incorporating different diamino acid headgroups (L-Lys, D-Lys, L-Orn, L-Dab, L-Dap). In addition, we describe the preparation of an alternative novel unsymmetrical series in which one lipophilic remains invariant (oleyl) and the other is selected from a set of lipophilic acids of varying chain lengths and degrees of unsaturation (C_6–C_{18}; Table 1). The ability of these surfactants to mediate transfection in a selection of immortal cell lines (HEK-293, Ishikawa and MCF-7) was determined and the resulting structure–activity relationships (SAR) are presented.



GSC103



1{*x*,*y*}

R^1OH = **5**{*x*}: L-Lys **5**{*1*}, D-Lys **5**{*2*}, L-Orn **5**{*3*}, L-Dab **5**{*4*}, L-Dap **5**{*5*}

R^2OH = **8**{*y*}: see Table 1.

Fig. 2 Symmetrical and unsymmetrical derivatives of GSC103.

Results and discussion

Chemistry

A series of symmetrical spermine-derived geminis were prepared as shown in Scheme 1. A pair of oleyl lipophilic tail groups, as are found in GSC103, were incorporated into each gemini. Bis-acylation of the starting diamine **2**[8] with oleic acid *N*-hydroxysuccinimide ester afforded the intermediate carbamate **3** which was Boc-deprotected by treatment with trifluoroacetic acid and then coupled with a set of bis-Boc diamino acids **5**{*x*} in the presence of 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TBTU) and 1-hydroxybenzotriazole (1-HOBt) to afford the amides **6**{*x*}. The Boc protecting groups were removed with a solution of hydrogen chloride in ethyl acetate and the products purified by mass-directed preparative HPLC to afford, after counter ion exchange, the geminis **1**{*x*,*4*} as their hydrochloride salts in high purity.

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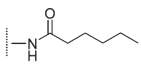
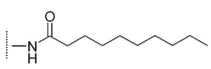
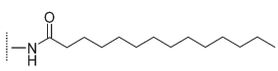
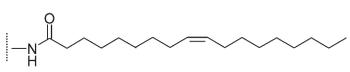
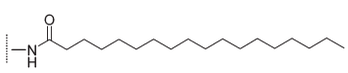
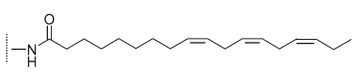
Table 1 Set of lipophilic tail groups R^2CO_2H **8**{*y*}

8{*1*}; C_6	
8{*2*}; C_{10}	
8{*3*}; C_{14}	
8{*4*}; $C_{18:1}^{\Delta 9}$	
8{*5*}; C_{18}	
8{*6*}; $C_{18:3}^{\Delta 9,12,15}$	

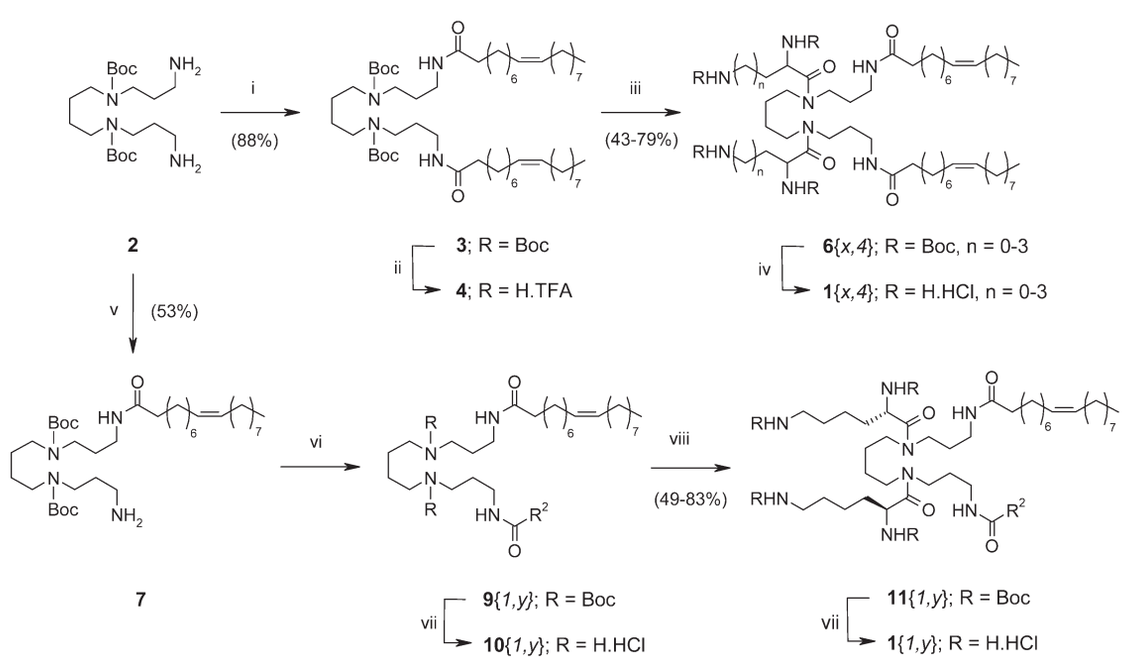
A related series of unsymmetrical gemini surfactants **1**{*1*,*y*} was also prepared starting from the same starting amine **2**. Efficient mono-acylation of this diamine was achieved by slow addition (3 h) of a solution of pentafluorophenyl oleate ester[9] in dichloromethane at low temperature (−78 °C), and then allowing the resulting mixture to slowly warm to room temperature over several hours. In this way, after separation from the concomitantly formed bis-adduct **3**, the mono-adduct **7** was routinely obtained in greater than 50% isolated yield. The amine **7** was further homologated by condensation with a set of lipophilic carboxylic acids (C_6–C_{18}) **8**{*y*} to afford the unsymmetrical adducts **9**{*y*}. These intermediates were deprotected upon exposure to 2 M ethereal hydrogen chloride to afford the corresponding intermediate bis-hydrochlorides **10**{*y*} and coupled with bis-Boc-(L)-lysine in the presence of TBTU/1-HOBt. The resulting Boc-protected geminis **11**{*1*,*y*} were treated with a solution of hydrogen chloride in ethyl acetate and purified by mass-directed HPLC to afford the geminis **1**{*1*,*y*} incorporating a combination of different lipophilic tail groups.

The inherent amphiphilic nature of gemini surfactants and their associated synthetic intermediates typically introduces difficulties in both the handling and purification of these compounds. Indeed, this may provide a rationalisation for the inter-batch variation in activity that has been encountered with amphiphilic transfection agents.[10] In order to establish a robust SAR, it is clearly important to deal with pure materials. Toward this end, we found that in the absence of strong UV absorbing chromophoric groups, HPLC purification was best performed using mass detection to control fractionation. Compound purities were subsequently confirmed using either an evaporative light-scattering (ELSD) or a chemiluminescent nitrogen detector (CLND).

Biology

In order to systematically investigate the effects of structural modifications on transfection ability, the geminis **1**{*x*,*y*} were screened over a range of concentrations (typically 2.5–20 μg mL^{-1}) in duplicate experiments in representative cell lines (HEK-293, Ishikawa, MCF-7) for their ability to deliver a plasmid expressing green fluorescent protein (pCMV-GFP). GFP expression was corrected against protein levels at the end of the transfection period (24 hours), in order to account for any well to well variation between replicates and experiments. Cytotoxicity was quantified by measuring lactate dehydrogenase (LDH) release into the cell supernatant, again at the end of the transfection period. Standard assays were used to measure these end-points and are described in the Experimental section.

Considering the series of geminis with different polar head groups but identical oleyl lipophilic tails **1**{*1–5*,*4*}, it is apparent that GFP expression in HEK-293 cells following transfection with pCMV-GFP follows a clear structure related trend (Fig. 3a). At the lowest concentration of surfactant (2.5 μg mL^{-1}), as the chain connecting the amino substituents within each polar headgroup decreases in length, transfection ability is seen to decrease accordingly. However, at the highest concentration of surfactant (20 μg mL^{-1}) the opposite is true, and transfection ability is observed to increase as the linking chain decreases in length. This apparent paradox is rationalised by a consideration of the cytotoxicity data for these compounds (Fig. 3b) from which it is apparent that the efficacy of the most effective transfection agents **1**{*1*,*4*} (L-Lys) and **1**{*2*,*4*} (D-Lys) is moderated at higher concentrations by the impact of cytotoxicity. Conversely, inherently less cytotoxic geminis such as **1**{*5*,*4*} although less potent may be utilised at a 10-fold higher concentration (20 μg mL^{-1}) to produce a similar level of GFP expression. Ultimately, transfection efficacy represents a



Scheme 1 *Reagents and conditions*: (i). Oleic acid *N*-OSuc, K_2CO_3, THF, H_2O, rt, 18 h; (ii). TFA, CH_2Cl_2, rt, 1.5 h; (iii). Boc.Aa(Boc)·OH **4**{*x*}, TBTU, HOBt, $^iPr_2NEt$, CH_2Cl_2, rt, 18 h; (iv). 4.5 M HCl–EtOAc, rt, 2 h; (v). Oleic acid pentafluorophenyl ester, Et_3N, CH_2Cl_2, −78 °C–rt, 3 h; (vi). R^2CO_2H **8**{*y*}, TBTU, HOBt, $^iPr_2NEt$, CH_2Cl_2, rt, 18 h; (vii). 2 M HCl–Et_2O, rt, 2 h; (viii). Boc-L-(Boc)Lys·OH, TBTU, 1-HOBt, $^iPr_2NEt$, CH_2Cl_2.

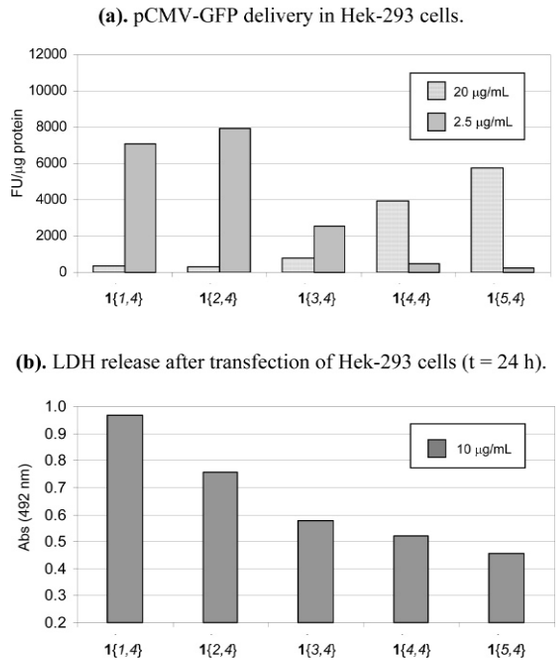


Fig. 3 Relationship beween chain length and toxicity for gemini surfactants **1**{*x*,*4*} in HEK-293 cells: (a). pCMV-GFP delivery in HEK-293 cells; (b). LDH release after transfection of HEK-293 cells (t = 24 h).

balance between the potency of the transfection agent and its associated cytotoxicity.

The series of unsymmetrical geminis **1**{*1*,*1–6*} in which the polar head group remains invariant (L-Lys) and different combinations of lipophilic tail groups are incorporated, was evaluated in the three different cell lines (HEK-293, Ishikawa, and MCF-7) (Fig. 4). In those cases where significant transfection of pCMV-GFP and expression of GFP was obtained, these geminis were found to be most effective at lower concentrations. At higher concentrations (20 μg mL^{-1}), this efficacy was again mitigated by increased cytotoxicity (data not shown) resulting generally in lower levels of expression. This trend is particularly clear in HEK-293 cells (Fig. 4a). However, in all three cell lines, another trend is apparent (Fig. 4a–c). To produce a high level of GFP expression, a structure-related dependence emerges consistent with the need for a contribution from two lipophilic tails, optimally with a chain length of C_{18}, in each surfactant molecule. One degree of unsaturation is tolerated, but the presence of two polar head groups alone is not sufficient to ensure high levels of plasmid transfection in the cell lines studied. Significantly, although the absolute levels of transfection across the different cell lines varies, the underlying structure-related trend remains the same in all three cell lines. This provides a compelling illustration of the contribution made by the presence of 'twinned' lipophilic tail groups present in the basic gemini structural motif in mediating gene transfecting ability.

Finally, as a control experiment, we compared GSC103, **1**{*1*,*4*} with the commercial transfection agent Lipofectamine 2000™ (LP2K). In HEK-293 cells, both of these transfection agents were found to give rise to comparable levels of GFP expression (Fig. 5). However, unlike GSC103, LP2K is supplied as a co-formulation. Co-formulation of surfactants with helper lipids is a well known method to enhance transfection ability and the neutral lipid DOPE (1,2-dioleyl-3-phospatidyl-ethanolamine) is often used for this purpose. Consequently, for comparison, GSC103 was co-formulated with DOPE (1 : 1). In this way, it was possible to enhance transfection such that the GSC103 : DOPE (1 : 1) co-formulation performed better than LP2K giving higher levels of GFP expression in the HEK-293 cells, thereby demonstrating the scope for further optimisation of these transfection agents.

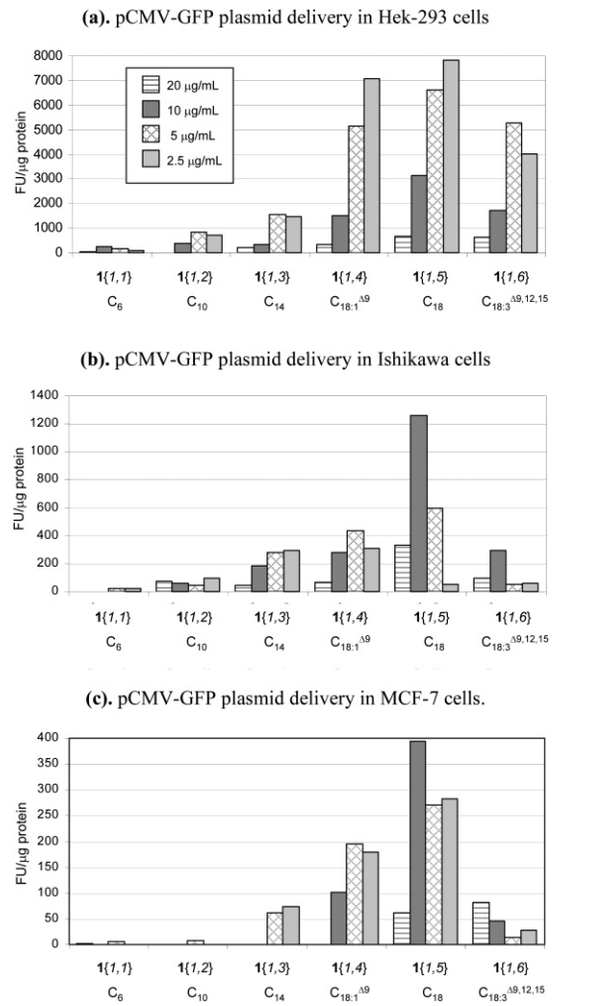


Fig. 4 Comparison of transfection efficiences of unsymmetrical geminis **1**{*1*,*y*} measured in fluorescence units per μg of protein in mammalian cell lines: (a). pCMV-GFP plasmid delivery in HEK-293 cells; (b). pCMV-GFP plasmid delivery in Ishikawa cells; (c). pCMV-GFP plasmid delivery in MCF-7 cells.

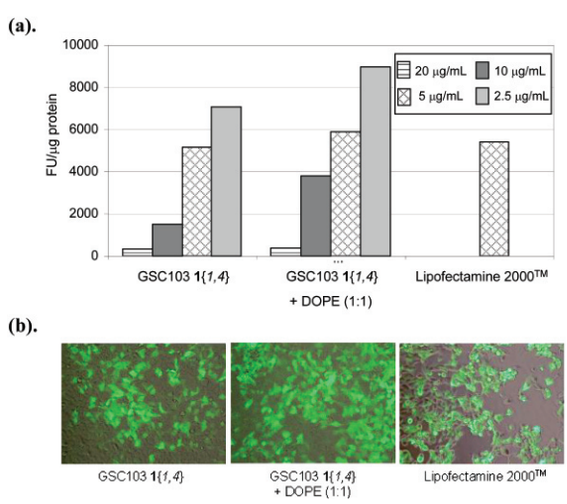


Fig. 5 Comparison of pCMV-GFP plasmid delivery in HEK-293 cells using GSC103, GSC103 + DOPE, and the commercial transfection reagent Lipofectamine 2000™: (a). Quantitative analysis of GFP expression; (b). Representative fluorescence/phase overlay micrographs.

Conclusions

Alterations to the gemini template have provided important insights into the structure–activity relationships that govern their transfection ability. In particular, from the study of a series of unsymmetrical gemini surfactants **1**{*1*,*1–6*} in a variety of cell lines, we find that the presence of two lipophilic tail

groups with an optimal chain length of C_{18} is required for high transfection activity.

Variations in the cationic polar head group to incorporate diamino acid residues that have different length linking chains connecting the amino groups were found to be tolerated in HEK-293 cells. However, a study of the series of geminis **1**{*1–5,4*} revealed an interesting inter-relationship between potency and toxicity in determining overall efficacy. Although, at low concentrations (approx. 2.5 μg mL^{-1}), the incorporation of either L-Lys or D-Lys proved to give rise to the most potent transfection agents, at higher concentrations, increased cytotoxicity led to significantly reduced levels of GFP expression. In contrast, the gemini surfactant bearing L-Dap head groups, though less potent was tolerated at 10-fold higher concentrations without significant toxic effects (20 μg mL^{-1}) and, under these conditions, was found to give rise to similar levels of GFP expression.

Finally, co-formulation of **1**{*1,4*} with DOPE (1 : 1) was shown to significantly improve transfection ability in HEK-293 cells to a level above that observed with the commercial transfection agent LP2K when used at a similar concentration.

Experimental

Chemistry

All moisture-sensitive reactions were carried out under a nitrogen atmosphere in oven-dried glassware. All solvents and reagents were used as supplied unless otherwise stated. Analytical thin layer chromatography (TLC) was carried out on Merck Silica Gel F_{254} plates. Analytical high pressure liquid chromatography (HPLC) was performed using an Agilent 1100 instrument. *Column*: Phenomenex Luna™ C18(2) 15 cm × 2.1 mm, 3 μm. *Eluent* A: water, 0.05% TFA, B: acetonitrile 0.05% TFA. *Flow rate*: 400 μL min^{-1}. *Detection*: UV (diode array), ELSD and ESI-MS. *Method*: gradient 40–100% B in A over 30 min. A Polymer Laboratories PL-ELS 2100 evaporative light-scattering detector was used: nebuliser: 50 °C, evaporator: 70 °C. Infrared spectra were collected on a Perkin-Elmer Spectrum One ATR FT-IR. Liquid chromatography/mass spectra (LC/MS) were recorded on an Agilent 1100/Micromass ZQ under electrospray positive conditions. *Column*: Phenomenex Luna C18(2) 100 × 2.1 mm, 3 μm. *Eluent* A: 0.1% (v/v) formic acid, water, B: 0.1% formic acid, acetonitrile. *Flow rate*: 0.5 mL min^{-1}. *Detection*: UV (diode array: 215, 230, 254 nm). *Method*: gradient 0–100% B in A over 5.0 min. Chemiluminescent nitrogen detection (CLND) data was recorded using an Antek Model 8060c Nitrogen Specific HPLC Detector connected to an Agilent Series 1100 HPLC. *Eluent A*: water (+ 0.1% v/v formic acid), B: methanol (+ 0.1% v/v formic acid). *Flow rate*: 0.5 mL min^{-1}. *Gradient*: 0–100% B in A over 10 min. Mass-directed autopreparative HPLC was performed using a system based upon a Waters 600 gradient pump attached to a Micromass ZMD mass spectrometer and utilised the methods described. Accurate mass spectra were recorded on a Micromass Q-Tof 2 hybrid quadrupole time-of-flight mass spectrometer, equipped with a Z-spray interface, over a mass range of 80–1200 Da. NMR spectra were recorded on Bruker instruments at the frequency specified in the indicated solvent. Chemical shifts (δ) are reported in parts per million (ppm) relative to tetramethylsilane as an internal standard; the following abbreviations are used for multiplicities: s = singlet; d = doublet; t = triplet; q = quartet; m = multiplet; dd = doublet of doublets; br = broad; and coupling constant *J*-values are quoted in Hz.

N^1,N^{12}-Dioleyl-N^4,N^9-bis(*tert*-butyloxycarbonyl)-1,12-diamino-4,9-diazadodecane (3). Solutions of oleic acid *N*-hydroxysuccinimide ester (2.50 g, 6.60 mmol) in THF (50 mL) and potassium carbonate (1.03 g, 7.50 mmol) in water (15 mL) were added to a solution of the diamine **2** (1.20 g, 3.00 mmol) in THF (180 mL). The resulting mixture was stirred at room temperature for 16 h, and then concentrated *in vacuo*. The residue was dissolved in ethyl acetate (300 mL) and washed with 5% aq. citric acid (75 mL) and brine (125 mL). The organic phase was dried (Na_2SO_4), and concentrated *in vacuo* to leave an oil which was purified by column chromatography (Biotage, 25+S, silica) eluting first with (75 : 25), then with (25 : 75) hexanes in ethyl acetate, to give **3** as a pale brown oil. (2.46 g, 88%). TLC (SiO_2): R_f = 0.42 (EtOAc); ν_{max}(neat)/cm^{-1} 3302, 2925, 2854, 1694, 1165; ^{1}H-NMR (400 MHz, $CDCl_3$): δ_H 0.87(3H, t, *J* = 6.7), 1.38–1.18(20H, m), 1.46(22H, m), 1.62(8H, m), 1.95(8H, q, *J* = 6.0), 2.15(4H, t, *J* = 6.5), 3.34–3.05(12H, m), 5.32(4H, m), 6.65(2H, brs); ^{13}C-NMR (100 MHz, $CDCl_3$): δ_C 173.6, 156.8, 130.3, 130.1, 80.1, 47.1, 43.7, 37.3, 35.8, 34.4, 32.3, 30.7, 30.1(2C), 29.9, 29.7, 29.6, 28.2, 28.1, 27.6(2C), 26.4, 26.2, 26.0, 25.3, 23.1 and 14.5; HRMS (ESI +ve) *m/z* calcd ($C_{56}H_{106}N_4O_6Na$) 953.8010, found 953.7974 $[M + Na]^+$.

N^1,N^{12}-Dioleyl-1,12-diamino-4,9-diazadodecane bis(trifluoroacetate) salt (4). Trifluoroacetic acid (11 mL) was added with stirring to a cooled solution of the bis-carbamate **3** (5.03 g, 5.4 mmol) in dichloromethane (11 mL) maintained at 0 °C. The resulting mixture was slowly allowed to warm to room temperature and stirred for a further 1.5 h. The solvent was evaporated *in vacuo*, and the residue was triturated with ether to give **3**, as a white solid (4.93 g, 95%). TLC (SiO_2): R_f = 0.58 (MeOH–0.88NH_3; 9 : 1); ν_{max}(neat)/cm^{-1} 2923, 2854, 1666, 1146, 799; ^{1}H-NMR (400 MHz, d^6-DMSO): δ_H 0.82(3H, t, *J* = 6.8), 1.32–1.12(20H, m), 1.45(4H, m), 1.59(4H, brs), 1.68(4H, quin, *J* = 7.2), 1.95(8H, q, *J* = 6.0), 2.05(4H, t, *J* = 7.5), 2.95–2.78(8H, m), 3.07(4H, q), 5.29(4H, m), 7.94(2H, t, *J* = 6.0), 8.55(4H, brs); ^{13}C-NMR (100 MHz, d^6-DMSO): δ_C 172.9, 159.0, 158.7, 158.4, 158.1, 129.8(2C), 46.2, 44.8, 35.7, 35.5, 31.9, 31.4, 29.3(2C), 29,0, 28.9, 28.8, 26.8, 26.7, 26.3, 25.4, 22.8, 22.2 and 14.1; LC/MS (ESI +ve): t_R = 17.06 min (*m/z* 731.7 $[M + H]^+$ (100%)); HRMS(ESI +ve) *m/z* calcd ($C_{46}H_{91}N_4O_2$) 731.7142, found 731.7145 $[M + H]^+$.

General procedure for the preparation of symmetrical geminis (1{*x,4*})

Example: N^1,N^{12}-dioleyl-N^4,N^9-bis(L-diaminopropyl)-1,12-diamino-4,9-diazadodecane·4 HCl (1{*5,4*}). These preparations were performed on a 0.1–1.0 mmol scale as follows: The bis-Boc protected diamino acid **5**{*5*} (70 mg, 230 μmol), 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TBTU) (74 mg, 230 μmol), 1-hydroxybenzotriazole (HOBt) (31 mg, 230 μmol) and diisopropylethylamine (125 μL, 710 μmol) were added to a solution of the bis(trifluoroacetate) **4** (100 mg, 104 μmol) in dichloromethane (1.5 mL). The mixture was stirred at room temperature for 18 h and then the mixture was concentrated *in vacuo*. The crude product was dissolved in ethyl acetate (20 mL) and washed with 5% aq. $KHSO_4$ solution (3 × 8 mL), brine (10 mL), 5% aq. K_2CO_3 solution (3 × 8 mL), and brine (10 mL), dried (Na_2SO_4), and the organic phase was concentrated *in vacuo*. The residue was purified by reverse phase column chromatography (C-18) eluting with a 50–100% mixture of methanol and water to afford the intermediate carbamate **6**{*5,4*} as a colourless gum. The gum was dissolved in ethyl acetate (14 mL) and diluted with a solution of hydrogen chloride in ethyl acetate (10 mL × 4.5 M). The resulting mixture was stirred at room temperature for 2 h, and the precipitated solid was collected and washed with diethyl ether (10 mL). Purification was performed by mass-directed preparative HPLC (column: 7 μm Kromasil C8; 25 cm × 5 cm id) eluting with a 0–100% linear gradient of MeCN (+0.5% TFA) in water (+0.5% TFA) at a flow rate of 80 mL min^{-1} over 40 min to afford the trifluoroacetate salt. The salt was adsorbed onto a Isolute Env+ column and the column was washed first with water and then with methanol to elute the free base. The free base was treated with a solution of ethereal hydrogen chloride (1 mL × 2.0 M) and the resulting precipitate was collected and

lyophilised to afford the gemini hydrochloride salt **1**{*5*,*4*} as a white powder (16 mg, 7%). TLC (SiO_2): R_f = 0.44 (MeOH–0.88NH_3; 95 : 5); ν_{max}(neat)/cm^{-1} 2924, 2856, 1643, 1456, 1055, 721; ^{1}H-NMR (500 MHz, d^6-DMSO): δ_H 0.83(6H, t, J = 6.7), 1.18–1.33(40H, m), 1.40–1.79(12H, m), 1.97(8H, q, J = 6.2), 2.09(4H, m), 3.03(2H, m), 3.05–3.45(12H, m), 3.61(2H, m), 4.60(2H, m), 5.32(4H, m), 7.89(1H, m), 8.08(1H, m), 8.82(12H, m); ^{13}C-NMR (125 MHz, d^6-DMSO): δ_C 172.4, 172.2, 165.4, 165.2, 129.7, 48.6, 48.4, 45.1, 36.2, 35.5, 35.4, 31.4, 29.3, 29.2, 28.9, 28.8, 28.7(2C), 28.4, 26.7, 26.6, 25.4, 22.2 and 14.0; LC/MS (ESI +ve): t_R = 13.14 min (*m*/*z* 903.8 $[M + H]^+$ (50%), 452.4 $[M + 2H]^{2+}$ (100%)); HRMS (ESI +ve) *m*/*z* calcd ($C_{52}H_{103}N_8O_4$) 903.8102, found 903.8101 $[M + H]^+$.

The following compounds were formed by the same general procedure on a 0.1–1.0 mmol scale:

***N*^{1},*N*^{12}-Dioleyl-*N*^{4},*N*^{9}-bis(L-lysyl)-1,12-diamino-4,9-diazadodecane·4 HCl (1{*1*,*4*}).** White solid (38 mg, 6%). TLC (SiO_2): R_f = 0.38 (MeOH–0.88NH_3; 80 : 20); IR: ν_{max}(neat)/cm^{-1} 2923, 2853, 1641, 1467, 722; ^{1}H-NMR (400 MHz, d^6-DMSO): δ_H 0.83 (6H, t, J = 6.8), 1.14–1.31(40H, m), 1.32–1.75(24H, m), 1.95(8H, q, J = 6.0), 2.03(4H, m), 2.73(4H, m), 2.92–3.53 (12H, m), 4.20(2H, m), 5.29(4H, m), 7.89(1H, m), 8.12(7H, m), 8.35(6H, m); ^{13}C-NMR (100 MHz, d^6-DMSO): δ_C 174.4, 174.2, 172.9, 170.3, 131.7, 51.2, 46.9, 40.3, 38.3, 38.0, 37.5, 33.4, 32.4, 31.3, 31.2, 30.9(2C), 30.8, 30.7, 29.5, 28.7(2C), 28.5, 27.5, 25.5, 24.2, 23.0, 16.1 and 13.8; LC/MS (ESI +ve): t_R = 12.28 min (*m*/*z* 987.9 (70%) $[M + H]^+$, 494.5 (100%) $[M + 2H]^{2+}$); ELSD: 100%; CLND: 100%; HRMS (ESI +ve) *m*/*z* calcd ($C_{58}H_{115}N_8O_4$) 987.9041, found 987.9034 $[M + H]^+$.

***N*^{1},*N*^{12}-Dioleyl-*N*^{4},*N*^{9}-bis(D-lysyl)-1,12-diamino-4,9-diazadodecane·4 HCl (1{*2*,*4*}).** White solid (14 mg, 7%). TLC (SiO_2): R_f = 0.21 (MeOH–0.88NH_3; 85 : 15); ν_{max}(neat)/cm^{-1} 2921, 2853, 1640, 1467, 721; ^{1}H-NMR (400 MHz, CD_3OD): δ_H 0.88(6H, t, J = 6.8), 1.20–1.40(40H, m), 1.45–1.95(24H, m), 2.03(8H, m), 2.19(4H, dt, J = 7.0 and 12.0), 2.97(4H, brs), 3.07–3.80(12H, m), 4.36(2H, m), 5.33(4H, m); ^{13}C-NMR (125 MHz, CD_3OD): δ_C 176.6, 176.3, 170.1(2C), 130.9, 130.7, 51.5, 49.5, 49.4, 49.3, 49.2, 49.1, 49.1, 49.0(3C), 48.9(3C), 48.8, 48.6, 48.4, 33.0, 30.8(2C), 30.6, 30.4(2C), 30.3, 28.1, 27.1, 23.7, 22.6, 19.3, 14.4; LC/MS (ESI +ve): t_R = 11.79 min (*m*/*z* 987.9 (45%) $[M + H]^+$, 494.5 (100%) $[M + 2H]^{2+}$); ELSD: 100%; CLND: 100%; HRMS (ESI +ve) *m*/*z* calcd ($C_{58}H_{115}N_8O_4$) 987.9041, found 987.9054 $[M + H]^+$.

***N*^{1},*N*^{12}-Dioleyl-*N*^{4},*N*^{9}-bis(L-ornityl)-1,12-diamino-4,9-diazadodecane·4 HCl (1{*3*,*4*}).** White solid (36 mg, 12%). TLC (SiO_2): R_f = 0.15 (MeOH–0.88NH_3; 85 : 15); ν_{max}(neat)/cm^{-1} 2924, 2855, 1638, 1467; ^{1}H-NMR (400 MHz, CD_3OD): δ_H 0.88 (6H, t, J = 6.9) 1.20–1.40(40H, m), 1.48–1.95(28H, m), 2.19(4H, m), 3.00(4H, brs), 3.08–3.80(12H, m), 4.42(2H, m), 5.32(4H, m); ^{13}C-NMR (125 MHz, CD_3OD): δ_C 177.7, 176.6, 176.3, 169.6(2C), 169.4, 135.9, 131.9, 131.3, 131.2, 130.7, 87.5, 79.4, 79.2, 51.3, 49.5, 49.3, 49.1, 49.0, 48.8(3C), 48.7(2C), 48.6(2C), 48.4, 33.0, 30.8, 30.6, 30.4, 30.3(2C), 28.1, 23.7, 23.6, 19.3, 14.4; LC/MS (ESI +ve): t_R = 11.96 min (*m*/*z* 959.9 $[M + H]^+$ (55%), 480.4 $[M + 2H]^{2+}$ (100%)); ELSD: 100%; CLND: 100%; HRMS (ESI +ve) *m*/*z* calcd ($C_{56}H_{111}N_8O_4$) 959.8728, found 959.8721 $[M + H]^+$.

***N*^{1},*N*^{12}-Dioleyl-*N*^{4},*N*^{9}-bis(L-diaminobutyl)-1,12-diamino-4,9-diazadodecane·4 HCl (1{*4*,*4*}).** White solid (16 mg, 5%). TLC (SiO_2): R_f = 0.43 (MeOH–0.88NH_3; 9 : 1); ν_{max}(neat)/cm^{-1} 2922, 2853, 1641, 1464, 722; ^{1}H-NMR (700 MHz, d^6-DMSO): δ_H 0.84(6H, t, J = 6.9), 1.18–1.32(40H, m), 1.40–1.79(12H, m), 1.97(8H, m), 2.02–2.18(8H, m), 2.87–3.55 (16H, m), 4.42(2H, m), 5.32(4H, m), 7.93(1H, m), 8.12(1H, m), 8.25–8.58(12H, m); ^{13}C-NMR (176 MHz, d^6-DMSO): δ_C 172.4, 172.2(2C), 168.1, 167.8, 129.7(2C), 47.6, 47.5, 43.4, 35.5, 31.4, 29.3, 29.2, 28.9, 28.8, 28.7(2C), 28.6, 26.7, 26.6(2C), 25.4, 22.2 and 14.0; LC/MS (ESI +ve): t_R = 12.45 min (*m*/*z* 931.8 $[M + H]^+$ (50%), 466.4 $[M + 2H]^{2+}$ (100%)); ELSD: 100%; HRMS (ESI +ve) *m*/*z* calcd ($C_{54}H_{107}N_8O_4$) 931.8415, found 931.8423 $[M + H]^+$.

***N*′-Oleyl-*N*^{4},*N*^{9}-bis(*tert*-butyloxycarbonyl)-1,12-diamino-4,9-diazadodecane (7).** A solution of pentafluorophenyl oleate ester[10] (3.89 g, 8.69 mmol) in dry dichloromethane (100 mL) was added dropwise with stirring to a cooled (−78 °C) solution of triethylamine (2.26 mL, 48.0 mmol) and the diamine **2** (3.50 g, 8.69 mmol) in dry dichloromethane (150 mL) over 3 h. The resulting mixture was slowly allowed to warm to room temperature and then stirred for 18 h. The mixture was concentrated *in vacuo* and the residual gum was purified by column chromatography (Biotage 40 + M, Si) eluting with a mixture of ethyl acetate–methanol–0.88NH_3 (96 : 2 : 2) to give the amine **7** as a viscous gum (3.08 g, 53%). TLC (SiO_2): R_f = 0.2 (MeOH–0.88NH_3; 85 : 15). IR: ν_{max}/cm^{-1} 3310, 2925, 1672, 1543, 1496.0, 1365. ^{1}H-NMR (400 MHz, $CDCl_3$): δ_H 5.32(2H, m), 3.10–3.33(10H, m), 2.69(2H, t, J = 6.8), 2.16(2H, t, J = 7.3), 2.00(8H, m), 1.36–1.55(20H, m), 1.21–1.35(18H, m), 0.88(3H, t, J = 6.9); ^{13}C-NMR (100 MHz, $CDCl_3$): δ_C 130.9, 130.7, 78.3, 78.0, 77.6, 47.6, 37.9, 32.8, 30.7, 30.5, 30.2, 30.1, 29.4, 28.1, 26.7, 23.6, 15.0; LC/MS (ESI +ve): t_R = 15.54 min; HRMS *m*/*z* calcd ($C_{38}H_{75}N_4O_5$) 667.5737, found 667.5730 $[M + H]^+$.

General procedure for the preparation of amides (9{*1*,*1–6*})

The amine **7** (2.50 g, 3.95 mmol) was dissolved in dry CH_2Cl_2 (100 mL) and this solution was divided equally into five aliquots. To each aliquot was added with stirring TBTU (288 mg, 0.899 mmol), HOBt (121 mg, 0.899 mmol), a fatty acid **8**{*y*} (0.948 mmol) and finally diisopropylethylamine (0.78 mL, 4.49 mmol). The resulting mixtures were stirred at room temperature for 18 h and then concentrated *in vacuo.* The residual gums were each dissolved in dichloromethane (10 mL) and washed successively with 5% aq. citric acid (2 × 5 mL), 2 M aq. sodium carbonate (2 × 5 mL) and water (2 × 10 mL). The organic solutions were dried (Na_2SO_4), and the solvent was evaporated *in vacuo* to leave the amides **9**{*1*,*1–6*} as colourless gums, which were were used without further purification.

General procedure for the preparation of trifluoroacetate salts (10{*1*,*1–6*})

The amides **9**{*1*,*1–6*} were each dissolved in anhydrous diethyl ether (10 mL) and treated with 2 M ethereal hydrogen chloride (5 mL) at room temperature with stirring under a N_2 atmosphere for 2 h. The resulting precipitates were collected by filtration and washed with anhydrous diethyl ether under N_2 (2 × 10 mL) to afford the hydrochloride salts **10**{*1*,*1–6*} as white powders, which were used without further purification.

General procedure for the preparation of carbamates (11{*1*,*1–6*})

The hydrochloride salts of **10**{*1*,*1–6*} were added to a stirring solution of Boc-L-Lys(Boc)–OH (650 mg, 0.187 mmol) containing TBTU (60.0 mg, 0.187 mmol), HOBt (25.0 mg, 0.187 mmol) and DIPEA (0.086 mL, 0.479 mmol) in anhydrous dichloromethane (10 mL). The reactions were stirred at room temperature under a N_2 atmosphere for 18 h and then concentrated *in vacuo.* The residual solids were dissolved in dichloromethane (10 mL) and washed with 5% aq. citric acid (2 × 5 mL), 2 M aq. sodium carbonate (2 × 5 mL) and water (2 × 10 mL). The organic solutions were dried (Na_2SO_4), and the solvent was evaporated *in vacuo* to leave the carbamates **11**{*1*,*1–6*} as colourless gums, which were used without further purification.

General procedure for the preparation of hydrochloride salts (1{*1*,*1–6*})

The carbamates **11**{*1*,*1–6*} were each dissolved in anhydrous diethyl ether (10 mL) and treated with 2 M ethereal hydrogen chloride (5 mL) at room temperature with stirring under a N_2 atmosphere for 2 h. The resulting precipitates were collected

by filtration and washed with anhydrous diethyl ether under N_2 (2 × 10 mL) to afford the crude hydrochloride salts as white powders. Purification was performed by mass-directed preparative HPLC (column: 5 μm Supelcosil ABZ$^+$Plus; 10 cm × 2 cm id) eluting with a 15–55% linear gradient of MeCN–water (95 : 5 + 0.05% formic acid) in water (+ 0.05% formic acid) at a flow rate of 20 mL min^{-1} over 10 min to afford the formate salts. The acetonitrile was evaporated *in vacuo* to leave an aqueous solution which was treated with concentrated hydrochloric acid (~15% v/v) and then lyophilised to afford the hydrochloride salts **1**{*1*,*1–6*} as white powders.

***N*^{1}-Oleyl,*N*^{12}-caproyl-*N*^{4},*N*^{9}-bis(L-lysyl)-1,12-diamino-4,9-diazadodecane·4 HCl (1{*1*,*1*}).** White solid (27 mg; 35%). TLC (SiO_2): R_f = 0.40 (MeOH–0.88NH_3; 80 : 20); IR: ν_{max}/cm^{-1} 3365, 2925, 1638, 1500, 1465; ^{1}H-NMR (400 MHz, CD_3OD): δ_H 0.89(6H, t, *J* = 6.9), 1.33–1.28(24H, m), 1.40–1.99(24H, m), 2.03(4H, m), 2.20(4H, m), 2.97(4H, m), 3.11–3.57(10H, m), 3.73(2H, m), 4.37(1H, t, *J* = 5.9), 4.50(1H, m), 5.33(2H, m)); ^{13}C-NMR (125 MHz, CD_3OD): δ_C 14.3, 22.9, 23.5, 26.8, 28.2, 28.3, 30.3, 32.6(2C), 37.2(2C), 40.3, 48.5, 48.7, 48.8, 49.0, 49.1(5C), 49.2(3C), 49.3(2C), 49.5, 51.6, 51.7(2C), 51.8(2C), 69.1, 130.7, 130.9, 131.3(2C), 169.7, 169.9, 176.3, 176.6, 177.7; LC/MS (ESI +ve): t_R = 4.33 min (*m*/*z* 821.7 (60%) [M + H]$^+$, 411.4 (100%) [M + H]$^{2+}$); CLND: 100%; HRMS (ESI +ve) *m*/*z* calcd ($C_{46}H_{93}N_8O_4$) 821.7320, found 821.7310 (64%) [M + H]$^+$, 411.36 (100%) [M + 2H]$^{2+}$.

***N*^{1}-Oleyl, *N*^{12}-capryl-*N*^{4},*N*^{9}-bis(L-lysyl)-1,12-diamino-4,9-diazadodecane·4 HCl (1{*1*,*2*}).** White solid (27 mg; 23%). TLC (SiO_2): R_f = 0.40 (MeOH–0.88NH_3; 80 : 20); IR: ν_{max}(neat)/cm^{-1} 3372, 2922, 1638, 1466, 722; ^{1}H-NMR (500 MHz, CD_3OD): δ_H 5.32 (2H, m), 4.53 (1H, m), 4.37 (1H, t, *J* = 6.4 Hz), 3.67(2H, m), 3.62–3.09(10H, m), 2.96(4H, dt, *J* = 2.7, 7.7 Hz), 2.19(4H, q, *J* = 7.4 Hz), 2.03(4 H, m), 1.99–1.52(24H, m), 1.40–1.28(32H, m), 0.89(6H, t, *J* = 6.8 Hz,); ^{13}C-NMR (125 MHz, CD_3OD): δ_C 176.6(2C), 176.4, 176.3, 170.0(2C), 169.7, 130.9, 130.7, 51.8, 51.7(2C), 51.6, 46.7(2C), 46.6, 44.9, 44.5, 40.3(2C), 38.0, 37.9, 37.8, 37.3, 37.2, 33.1(2C), 32.1, 32.0, 31.9, 30.9, 30.8, 30.6(2C), 30.5, 30.4, 30.3(2C), 30.2, 29.9(2C), 28.5(2C), 28.3, 28.2, 28.1(2C), 27.3, 27.1, 26.9, 25.5, 23.7, 22.9, 22.8, 22.7, 22.6, 14.5; LC/MS (ESI +ve): t_R = 7.99 min (*m*/*z* 877.8 (50%) [M + H]$^+$, 439.4 (100%) [M + 2H]$^{2+}$); ELSD: 100%; CLND: 100%; HRMS (ESI +ve) *m*/*z* calcd ($C_{50}H_{101}N_8O_4$) 877.7946, found 877.7940 (45%) [M + H]$^+$, 439.39 (100%) [M + 2H]$^{2+}$.

***N*^{1}-Oleyl,*N*^{12}-myristyl-*N*^{4},*N*^{9}-bis(L-lysyl)-1,12-diamino-4,9-diazadodecane·4 HCl (1{*1*,*3*}).** White solid (29 mg, 33%). TLC (SiO_2): R_f = 0.40 (MeOH–0.88NH_3; 80 : 20); IR: ν_{max}(neat)/cm^{-1} 3379, 2922, 1641, 1462, 721; ^{1}H-NMR (500 MHz, CD_3OD): δ_H 5.32 (2H, m), 4.52(1H, m), 4.37(1H, t, *J* = 6.4 Hz), 3.68(2H, m), 3.65–3.07(10H, m), 2.96(4H, dt, *J* = 2.7, 7.7 Hz), 2.21(4H, m), 2.03(4H, m), 1.95–1.48(24H, m), 1.38–1.22(40H, m), 0.88(6H, t, *J* = 6.9 Hz); ^{13}C-NMR (125 MHz, CD_3OD): δ_C 176.6(2C), 176.4, 176.3(2C), 170.0(2C), 169.7, 130.9, 130.7, 51.7, 51.6, 46.8, 46.7(2C), 44.7, 44.5, 40.3(2C), 38.0, 37.9, 37.8, 37.3, 37.2, 33.1(2C), 32.1, 32.0, 31.9, 30.9, 30.8(4C), 30.7, 30.6, 30.5, 30.4(2C), 30.3(2C), 28.6, 28.5, 28.3, 28.2(2C), 28.1, 27.3, 27.1, 26.9, 25.8, 25.5, 23.7, 22.9(2C), 22.7, 22.6, 14.5(2C); LC/MS (ESI +ve): t_R = 10.19 min (*m*/*z* 933.9 (50%) [M + H]$^+$, 467.4 (100%) [M + 2H]$^{2+}$); ELSD: 100%; CLND: 100%; HRMS (ESI +ve) *m*/*z* calcd ($C_{54}H_{109}N_8O_4$) 933.8572, found 933.8571 (40%) [M + H]$^+$, 467.4 (100%) [M + 2H]$^{2+}$.

***N*^{1},*N*^{12}-Dioleyl-*N*^{4},*N*^{9}-bis(L-lysyl)-1,12-diamino-4,9-diazadodecane·4 HCl (1{*1*,*4*}).** Data as reported above.

***N*^{1}-Oleyl,*N*^{12}-stearyl-*N*^{4},*N*^{9}-bis(L-lysyl)-1,12-diamino-4,9-diazadodecane·4 HCl (1{*1*,*5*}).** White solid (10 mg, 9%). TLC (SiO_2): R_f = 0.32 (MeOH–0.88NH_3; 80 : 20); IR: ν_{max}(neat)/cm^{-1} 3372, 2923, 2852, 1639, 1466, 720; ^{1}H-NMR (400 MHz, CD_3OD): δ_H 5.32(2H, m), 4.51(1H, m), 4.35(1H, m), 3.68 (2H, m), 3.58–3.08(10H, m), 2.96(4H, t, *J* = 7.4 Hz), 2.19(4H, dt, *J* = 7.0, 12.0 Hz), 2.03(4H, m), 1.95–1.48(24H, m), 1.38–1.23(48H, m), 0.88(6H, t, *J* = 6.9 Hz); ^{13}C-NMR (125 MHz, CD_3OD): δ_C 176.6(2C), 176.4, 170.0(2C), 169.7, 130.9, 130.7, 51.8, 51.7(2C), 51.6, 46.8, 46.7, 46.5, 40.3, 38.0, 37.9, 37.8, 37.2, 33.1(2C), 32.1, 32.0, 31.9, 30.9, 30.8(3C), 30.7, 30.6, 30.5(3C), 30.4(2C), 30.3(2C), 29.9, 28.6, 28.5, 28.3, 28.2, 28.1, 27.3, 27.1, 26.9, 25.8, 25.5, 23.7, 22.9(2C), 22.7, 22.6, 14.5, 14.4; LC/MS (ESI +ve): t_R = 12.79 min (*m*/*z* 989.9 (50%) [M + H]$^+$, 495.5 (100%) [M + 2H]$^{2+}$); ELSD: 100%; CLND: 100%; HRMS (ESI +ve) *m*/*z* calcd ($C_{58}H_{117}N_8O_4$) 989.9198, found 989.9162 (50%) [M + H]$^+$, 495.5 (100%) [M + 2H]$^{2+}$.

***N*^{1}-Oleyl,*N*^{12}-linolenyl-*N*^{4},*N*^{9}-bis(L-lysyl)-1,12-diamino-4,9-diazadodecane·4 HCl (1{*1*,*6*}).** White solid (28 mg, 3%). TLC (SiO_2): R_f = 0.45 (MeOH–0.88NH_3; 80 : 20); IR: ν_{max}(neat)/cm^{-1} 3255, 2924, 2854, 1638, 1460, 721; ^{1}H-NMR (400 MHz, CD_3OD): δ_H 5.32(2H, m), 4.51(1H, m), 4.35(1H, t, *J* = 6.0 Hz), 3.68(2H, m), 3.58–3.08(10H, m), 2.96(4H, m), 2.79(4H, m), 2.20(4H, m), 2.12–1.95(8H, m), 1.94–1.44(24H, m), 1.38–1.15(28H, m), 0.95(3H, t, *J* = 7.5 Hz), 0.88(3H, t, *J* = 6.8 Hz); ^{13}C-NMR (125 MHz, CD_3OD): δ_C 176.7, 176.3, 170.0, 169.7, 131.0, 130.9, 130.8, 51.7, 51.6, 46.8, 44.9, 44.5, 40.3, 37.9(2C), 37.8, 37.2, 33.1, 32.1, 31.9, 30.9, 30.8, 30.7, 30.6, 30.5, 30.4(2C), 30.3(2C), 30.2, 29.9, 28.6, 28.5, 28.3, 28.2, 28.1, 27.3, 27.1, 26.9, 26.7, 26.5, 26.4, 26.0, 25.7, 25.5, 23.7, 22.9, 22.8, 22.7, 22.6, 14.4; LC/MS (ESI +ve): t_R = 10.40 min (*m*/*z* 987.9 (100%), 494.4 (100%) [M + H]$^{2+}$); ELSD: 100%; CLND: 100%; HRMS (ESI +ve) *m*/*z* calcd ($C_{58}H_{111}N_8O_4$) 983.8728, found 983.8711 (30%) [M + H]$^+$, 492.4 (100%) [M + 2H]$^{2+}$.

Biology

Gemini surfactant preparation. All gemini surfactants were stored as solid, in a dessicator at 4 °C. Solutions were prepared in nuclease free water at 1 mg mL^{-1} and filter sterilised through a 0.2 μm filter after which they were stored at 4 °C until use. Co-formulation with DOPE was carried out as follows: both **1**{*1*,*4*} and DOPE were separately dissolved in chloroform at 1 mg mL^{-1}. These were then mixed in a (1 : 1) ratio and the resulting mixture dried under a stream of nitrogen. The dried formulation was reconstituted at 1 mg mL^{-1} in nuclease free water, vortexed, sonicated for 1 min, and filter sterilised through a 0.2 μm filter.

***In vitro* transfection.** The cell lines HEK-293, MCF-7 and Ishikawa were obtained from the American Type Culture Collection (ATCC for HEK-293 and MCF-7) and the European Collection of Cell Culture (ECACC for Ishikawa) and cultured according to the recommended protocols. On the day before transfection, cells were sub-cultured by trypsinisation and plated into 96 well tissue culture treated microplates, at 20 000 cells per well, and allowed to adhere overnight. Gemini-DNA complexes were prepared by mixing 10 μL of a 10× concentrated gemini solution (diluted from a 1 mg mL^{-1} stock solution made up in nuclease free water) in optimem (Gibco-BRL), to 10 μL of optimen containing 0.25 μg of pCMV-eGFP-C1 (Clontech). This mixture was incubated at room temperature for 15 min prior to the addition of an additional 80 μL of optimem. This complex was added to cells which had been washed once in optimem, and incubated for 6 hours at 37 °C, 5% CO_2 in a humidified incubator. At this time the complex was removed and replaced with normal growth media. The cells were incubated for an additional 18 h prior to assaying.

Fluorescence microscopy. Cells were observed by fluorescence microscopy at the end of the transfection period. Both fluorescent and phase contrast images were captured and overlayed using a Nikon TE300 inverted microscope in conjunction with Lucia software.

LDH measurement. At the end of the incubation period, 50 μL of cell supernatant was collected and assayed for release of LDH. This was carried out using the CytoTox 96® Non-Radioactive Cytotoxicity Assay kit (Promega), according to the manufacturers instructions.

GFP expression and protein assay. Cells were washed once in phosphate buffered saline pH 7.4, prior to the addition of 50 μL cell lysis buffer (5× stock solution: 10 mM EDTA, 0.25% triton X-100, 10 mM DTT, 250 mM HEPES pH 7.5) and a subsequent freeze-thaw cycle at −80 °C. Cell lysates were allowed to reach room temperature prior to measurement of GFP expression using a Tecan Ultra plate reader (Tecan). An aliquot of lysate (typically 10 μL) was then used to quantitate the total protein levels per well. This was performed using the Coomassie® Plus Protein Assay Reagent Kit (Pierce) according to the manufacturers protocol. Fluorescence units were then expressed as relative to per microgram of protein.

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