Lipid-mediated DNA and siRNA Transfection Efficiency Depends on Peptide Headgroup

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Supporting Information

General Materials and Procedure for Synthesis

All chemicals were purchased from Aldrich. Solvents used during synthesis were dried and distilled prior to use. All reactions were done in dry conditions using nitrogen. NMR spectral analysis was performed using a Varion Mercury spectrometer operating at 400 MHz.
1,2-Di-tetradecanoyl-3-Gly-rac-glycerol (1), 1,2-Di-tetradecanoyl-rac-glycerol (2) and 1,2-Di-tetradecanoyl-3-Lys-Gly-Gly-rac-glycerol (6a, KGG) were synthesized as previously described (Prata, Carla A. H.; Zhang, Xiao-Xiang; Luo, Dan; McIntosh, Thomas J.; Barthelemy, Philippe; Grinstaff, Mark W. (2008) Lipophilic Peptides for Gene Delivery. *Bioconjugate Chem.* 19, 418–420).

**BocOrn(Boc)-GlyOEt (3b):** To a CH₂Cl₂ solution of BocOrn(Boc)OH (1.18 g, 3.55 mmol) and HGlyOEt·HCl (0.50 g, 3.55 mmol), DCC (0.81 g, 3.91 mmol) and DMAP (0.48 g, 3.91 mmol) were added. The solution was stirred at RT for 18 h before being filtered. The filtrate was purified through silica gel chromatography (30% to 50% EtOAc/Hexane) to afford the product as a powder (86%). 1H NMR (CDCl₃) δ ppm 1.23 (t, 3H), 1.39 (m, 18H), 1.53-1.61 (m, 3H), 1.79-1.84 (m, 1H), 3.02-3.07 (m, 1H), 3.22-3.25 (m, 1H), 3.87-4.04 (m, 2H), 4.12-4.17 (q, 2H), 4.27 (s, 1H), 4.80 (t, 1H), 5.28 (d, 1H), 7.09 (s, 1H); 13C NMR (CDCl₃) δ ppm 14.06, 24.88, 28.25, 30.07, 39.35, 41.17, 53.18, 61.34, 79.14, 79.86, 155.70, 156.37, 169.59, 172.61; HRMS calcd. for [C₁₉H₃₅N₃O₇+Na]⁺ 440.2382, found 440.2373.
**BocArg(Boc)₂-GlyOEt (3c):** The same procedure was used as that described for **BocOrn(Boc)-GlyOEt (3b)** to afford the product as a powder (86%). 1H NMR (CDCl₃) δ ppm 1.18 (t, 3H), 1.36-1.42 (m, 27H), 1.58 (s, 4H), 3.61-3.66 (m, 1H), 3.75-3.81 (m, 1H), 3.80-4.10 (m, 4H), 4.27 (d, 1H), 5.81 (d, 1H), 7.42 (s, 1H), 9.21-9.35 (d, 2H); 13C NMR (CDCl₃) δppm 13.92, 24.08, 27.82, 28.05, 28.24, 29.09, 41.20, 43.71, 53.15, 60.17, 61.04, 79.02, 79.40, 83.87, 154.71, 155.28, 160.84, 163.08, 169.25, 172.23; HRMS calcd. for [C₂₅H₄₅N₅O₉+H]⁺ 560.3296, found 560.3318.

**BocGly-GlyOEt (3d):** The same procedure was used as that described for **BocOrn(Boc)-GlyOEt (3b)** to afford the product as an oil (98%). 1H NMR (CDCl₃) δ ppm 1.18 (t, 3H), 1.35 (s, 9H), 3.76 (d, 2H), 3.93 (d, 2H), 4.11 (q, 4H), 5.59 (s, 1H), 7.08 (t, 1H); 13C NMR (CDCl₃) δppm 13.90, 28.01, 41.01, 43.83, 61.27, 79.89, 156.03, 169.70, 170.05; HRMS calcd. for [C₁₁H₂₀N₂O₅+H]⁺ 261.1450, found 261.1436.

**BocOrn(Boc)-GlyOH (4b):** KOH was added to a methanol solution of **BocOrn(Boc)-GlyOEt (3b)** (1.21 g, 2.90 mmol) until pH=12. The reaction was finished after stirring at RT for 3 h, as shown on TLC. Next, IRC-50 resin was added and the flask was shaken for 2 h. The resin was removed by filtration to afford the product as a powder (83%). 1H NMR (CD₃OD) δppm 1.41-1.43 (d, 18H), 1.52-1.60 (m, 2H), 1.80 (m, 2H), 3.04 (t, 2H), 3.78 (s, 2H), 4.04-4.09 (m, 1H); 13C NMR (CD₃OD) δppm 27.33, 28.70, 28.79, 30.71, 40.84, 79.24, 80.68, 155.83, 156.56, 169.76, 171.34; HRMS calcd. for [C₁₇H₃₁N₃O₇+Na]⁺ 412.2060, found 412.2059.

**BocArg(Boc)₂-GlyOH (4c):** The same procedure was used as that described for **BocOrn(Boc)-GlyOH (4b)** to afford the product as a powder (90%). 1H NMR (CDCl₃) δppm 1.25-1.48 (m,
27H), 1.63-1.91 (m, 4H), 3.27 (s, 2H), 3.83 (d, 2H), 4.18 (s, 2H), 9.21-9.30 (m, 1H); 13C NMR (CDCl₃) δ ppm 27.86, 27.89, 28.18, 28.28, 43.73, 44.12, 54.11, 78.73, 79.43, 83.73, 154.82, 155.59, 155.67, 160.54, 163.52; HRMS calcd. for [C₂₃H₄₁N₅O₉+Na]⁺ 554.2802, found 554.2803.

BocGly-GlyOH (4d): The same procedure was used as that described for BocOrn(Boc)-GlyOH (4b) to afford the product as a powder (76%). 1H NMR (CD₃OD) δ ppm 1.42 (s, 9H), 3.27 (t, 1H), 3.71 (s, 2H), 3.79 (s, 2H); 13C NMR (CD₃OD) δ ppm 28.67, 43.42, 44.66, 80.76, 158.38, 172.51, 175.37; HRMS calcd. for [C₉H₁₆N₂O₅+Na]⁺ 255.0957, found 255.0967.

1,2-Di-tetradecanoyl-3-BocOrn(Boc)-Gly-Gly-rac-glycerol (5b): To a CH₂Cl₂ solution of BocOrn(Boc)-GlyOH (4b) (0.13 g, 0.33 mmol), 1,2-Di-tetradecanoyl-3-Gly-rac-glycerol (1) (0.21 g, 0.33 mmol) was added along with EDCI (0.07 g, 0.37 mmol) and DMAP (cat.). The reaction was stirred at RT for 16 h. Next, the reaction mixture was filtered and purified via silica gel chromatography (20% to 33% EtOAc/Hexanes) to afford the product as a powder (74%). 1H NMR (CDCl₃) δ ppm 0.84-0.87 (m, 6H), 1.25 (m, 40H) 1.41 (s, 18H), 1.57-1.64 (m, 8H), 1.72 (s, 2H), 1.80 (m, 2H), 2.30 (t, 4H), 3.06-3.09 (m, 2H), 2.27 (m, 1H), 3.88-4.06 (m, 4H), 4.12-4.17 (m, 2H), 4.28-4.31 (m, 2H), 4.73 (s, 1H), 5.18-5.23 (m, 2H), 7.00 (s, 1H), 7.13 (s, 1H); 13C NMR (CDCl₃) δ ppm 14.07, 22.64, 24.77, 26.31, 28.26, 28.37, 29.07, 29.21, 29.31, 29.43, 29.56, 29.60, 29.63, 31.87, 33.93, 39.35, 41.06, 43.01, 54.12, 61.61, 70.44, 79.33, 80.25, 156.11, 156.41, 168.93, 169.26, 173.13, 173.32; HRMS calcd. for [C₅₀H₉₂N₄O₁₂+H]⁺ 941.6790, found 941.6815.

1,2-Di-tetradecanoyl-3-BocArg(Boc)₂-Gly-Gly-rac-glycerol (5c): The same procedure was used as that described for 1,2-Di-tetradecanoyl-3-BocOrn(Boc)-Gly-Gly-rac-glycerol (5b) to
afford the product as an oil (52%). 1H NMR (CDCl₃) δ ppm 0.85 (t, 6H), 1.25 (m, 40H), 1.42 (s, 9H), 1.47-1.49 (d, 18H), 1.56-1.59 (t, 4H), 1.68 (m, 6H), 1.82 (m, 2H), 2.27-2.31 (m, 4H), 3.67 (m, 1H), 3.86-3.97 (m, 4H), 4.04-4.14 (m, 3H), 4.24-4.30 (m, 3H), 5.20 (m, 2H), 6.00-6.02 (m, 1H), 7.12 (s, 1H), 7.45 (s, 1H), 9.27-9.40 (d, 2H); 13C NMR (CDCl₃) δ ppm 14.11, 22.67, 24.23, 24.80, 27.64, 28.20, 28.39, 29.10, 29.25, 29.34, 29.46, 29.60, 29.63, 29.78, 31.90, 33.95, 40.94, 43.30, 43.86, 54.10, 61.69, 70.33, 79.65, 80.19, 84.21, 85.11, 154.77, 155.95, 161.10, 161.93, 163.06, 168.76, 169.26, 173.15, 173.30, 174.26; HRMS calcd. for [C56H102N6O14+H]⁺ 1083.7532, found 1083.7567.

1,2-Di-tetradecanoyl-3-BocGly-Gly-Gly-rac-glycerol (5d): The same procedure was used as that described for 1,2-Di-tetradecanoyl-3-BocOrn(Boc)-Gly-Gly-rac-glycerol (5b) to afford the product as an oil (91%). 1H NMR (CDCl₃) δ ppm 0.81 (t, 6H), 2.12 (m, 40H), 1.38 (s, 9H), 1.52-1.55 (m, 4H), 2.25 (t, 4H), 2.77-2.78 (d, 2H), 3.95-4.00 (m, 4H), 4.08-4.13 (m, 2H), 4.23-4.27 (m, 2H), 5.17 (m, 1H), 6.65 (s, 1H), 7.31-7.34 (m, 2H); 13C NMR (CDCl₃) δ ppm 13.98, 22.55, 24.69, 28.17, 28.99, 29.14, 29.23, 29.35, 29.52, 31.79, 33.84, 40.99, 42.68, 44.14, 61.44, 68.44, 70.33, 80.14, 156.34, 169.01, 169.43, 170.48, 173.25; HRMS calcd. for [C42H77N3O10+H]⁺ 784.5687, found 784.5682.

1,2-Di-tetradecanoyl-3-Orn-Gly-Gly-rac-glycerol (6b, OGG): 1,2-Di-tetradecanoyl-3-BocOrn(Boc)-Gly-Gly-rac-glycerol (5b) (0.19 g, 0.20 mmol) was dissolved in 1 mL of CH₂Cl₂, and 1 mL TFA was added slowly to this ice cold solution. The reaction was allowed to run at RT for 1 h. The solvent and TFA were then removed under reduced pressure. The residue was washed with cold hexanes twice to afford the product as a solid (77%). 1H NMR (CDCl₃) δ ppm 0.83-0.90 (m, 6H), 1.23-1.27 (m, 40H), 1.56 (s, 4H), 1.79-1.93 (m, 4H), 2.28 (s, 4H), 2.96 (s, 2H), 3.92-4.26 (m, 9H), 5.10-5.20 (d, 1H), 7.86-8.87 (m, 6H); 13C NMR (CDCl₃) δ ppm 14.03,
14.06, 22.63, 22.67, 24.79, 27.83, 29.12, 29.33, 29.54, 29.68, 31.88, 31.91, 33.93, 38.83, 40.98, 42.69, 52.92, 61.15, 70.32, 162.02, 162.45, 169.88, 170.24, 173.87; HRMS calcd. for [C40H76N4O8+H]^+ 741.5741, found 741.5750.

1,2-Di-tetradecanoyl-3-Arg-Gly-Gly-rac-glycerol (6c, RGG): The same procedure was used as that described for 1,2-Di-tetradecanoyl-3-Orn-Gly-Gly-rac-glycerol (6b) to afford the product as a powder (80%). 1H NMR (CDCl₃) δ ppm 0.83-0.87 (m, 6H), 1.23 (m, 40H), 1.45-1.56 (m, 6H), 1.82-1.85 (m, 2H), 2.28 (s, 4H), 3.12 (s, 2H), 3.50-4.28 (m, 9H), 5.11 (m, 1H), 6.68 (m, 2H), 8.29 (m, 4H); 13C NMR (CDCl₃) δ ppm 14.08, 22.68, 24.78, 27.64, 29.11, 29.28, 29.37, 29.47, 29.53, 29.61, 29.68, 29.72, 31.92, 33.94, 34.08, 40.83, 52.50, 61.15, 70.34, 157.21, 162.36, 170.14, 173.90; HRMS calcd. for [C41H78N6O8+H]^+ 783.5959, found 783.5934.

1,2-Di-tetradecanoyl-3-Gly-Gly-Gly-rac-glycerol (6d, GGG): The same procedure was used as that described for 1,2-Di-tetradecanoyl-3-Orn-Gly-Gly-rac-glycerol (6b) to afford the product as a powder (85%). 1H NMR (CD3OD) δ ppm 0.89 (t, 6H), 1.28 (m, 40H), 1.58-1.61 (m, 4H), 2.33 (t, 4H), 3.71 (s, 2H), 3.97-3.98 (d, 4H), 4.17-4.21 (m, 2H), 4.30-4.34 (m, 2H) 5.27 (m, 1H); 13C NMR (CDCl₃) δ ppm 14.05, 22.65, 24.79, 29.10, 29.26, 29.33, 29.50, 29.63, 29.65, 29.67, 31.89, 33.91, 41.20, 42.65, 61.18, 70.29, 167.72, 169.73, 170.11, 170.44, 173.68; HRMS calcd. for [C37H69N3O8+H]^+ 684.5163, found 684.5175.
1,2-Di-tetradecanoyl-3-BocLys(Boc)-Gly-rac-glycerol (7a): To a CH$_2$Cl$_2$ solution of BocLys(Boc)-OH (0.24 g, 0.60 mmol), 1,2-Di-tetradecanoyl-rac-glycerol (2) (0.46 g, 0.60 mmol) was added along with EDCI (0.13 g, 0.65 mmol) and DMAP (cat.). The reaction was stirred at RT for 16 h. Next the reaction mixture was filtered and purified via silica gel chromatography (20 to 33% EtOAc/Hexanes) to afford the product as an oil (59%). 1H NMR (CDCl$_3$) $\delta$ ppm 0.85 (t, 6H), 1.23 (m, 40H), 1.35-1.42 (m, 20H), 1.46-1.50 (m, 2H), 1.54-1.65 (m, 5H), 1.81-1.88 (m, 1H), 2.26-2.29 (m, 1H), 3.08 (m, 2H), 4.00-4.04 (m, 2H), 4.07-4.17 (m, 2H), 4.20-4.22 (m, 1H), 4.25-4.35 (m, 2H), 4.62 (s, 1H), 5.14 (s, 1H), 5.23-5.27 (m, 1H), 6.64 (m, 1H); 13C NMR (CDCl$_3$) $\delta$ppm 14.11, 22.48, 22.67, 24.80, 24.83, 28.30, 28.43, 29.07, 29.10, 29.24, 29.26, 29.34, 29.46, 29.64, 31.91, 33.96, 34.01, 34.15, 39.81, 41.12, 54.26, 61.70, 61.86, 63.27, 68.51, 79.02, 79.13, 80.23, 156.17, 168.98, 169.26, 172.38, 172.95, 173.27, 173.33; HRMS calcd. for [C$_{49}$H$_{91}$N$_3$O$_{11}$+Na]$^+$ 920.6551, found 920.6566.

1,2-Di-tetradecanoyl-3-BocOrn(Boc)-Gly-rac-glycerol (7b): The same procedure was used as that described for 1,2-Di-tetradecanoyl-3-BocLys(Boc)-Gly-rac-glycerol (7a) to afford the product as an oil (50%). 1H NMR (CDCl$_3$) $\delta$ ppm 0.86 (t, 6H), 1.26 (m, 40H), 1.40-1.42 (d, 18H), 1.50-1.62 (m, 6H), 1.82-1.85 (m, 2H), 2.27-2.31 (m, 4H), 3.04-3.08 (m, 1H), 3.31 (m, 1H), 3.97-4.04 (m, 2H), 4.07-4.21 (m, 2H), 4.25-4.34 (m, 3H), 4.68 (m, 1H), 5.13 (m, 1H), 5.25 (m,
1H), 6.93 (s, 1H); 13C NMR (CDCl₃) δ ppm 14.11, 22.68, 24.80, 24.83, 26.32, 28.29, 28.38, 29.07, 29.10, 29.24, 29.35, 29.47, 29.60, 29.64, 29.67, 30.00, 31.91, 33.95, 34.01, 34.14, 39.24, 41.03, 41.09, 53.01, 61.70, 61.89, 63.21, 68.53, 70.42, 79.29, 80.01, 155.75, 156.51, 168.89, 169.18, 172.59, 172.92, 172.94, 173.28, 173.32; HRMS calcd. for [C₄₈H₈₉N₃O₁₁+Na]⁺ 906.6395, found 906.6364.

1,2-Di-tetradecanoyl-3-BocArg(Boc)-Gly-rac-glycerol (7c): Same procedure was used as that described for 1,2-Di-tetradecanoyl-3-BocLys(Boc)-Gly-rac-glycerol (7a) to afford the product as an oil (50%). 1H NMR (CDCl₃) δ ppm 0.83 (t, 6H), 1.21 (m, 40H), 1.40-1.47 (m, 27H), 1.74 (s, 4H), 2.24-2.27 (m, 4H), 3.68 (m, 1H), 3.87 (m, 1H), 4.05-4.16 (4H), 4.20-4.31 (m, 3H), 5.21 (s, 1H), 5.85 (s, 1H), 7.51 (s, 1H), 9.40 (m, 2H); 13C NMR (CDCl₃) δ ppm 14.05, 22.61, 24.11, 24.71, 24.76, 27.92, 28.15, 28.34, 29.00, 29.04, 29.12, 29.19, 29.28, 29.40, 29.58, 29.61, 30.85, 31.85, 33.85, 33.94, 34.04, 41.08, 41.13, 43.77, 53.11, 61.69, 61.84, 62.98, 63.03, 68.49, 68.52, 70.08, 79.34, 79.57, 84.12, 154.76, 155.40, 162.91, 168.71, 169.02, 169.04, 172.35, 172.40, 172.79, 173.15, 173.18, 173.20; HRMS calcd. for [C₅₄H₉₉N₅O₁₃+H]⁺ 1026.7318, found 1026.7325.

1,2-Di-tetradecanoyl-3-BocGly-Gly-rac-glycerol (7d): The same procedure was used as that described for 1,2-Di-tetradecanoyl-3-BocLys(Boc)-Gly-rac-glycerol (7a) to afford the product as an oil (68%). 1H NMR (CDCl₃) δ ppm 0.85 (t, 6H), 1.23 (m, 40H), 1.44 (s, 9H), 1.58 (m, 4H), 2.30 (m, 4H), 3.83-3.84 (d, 2H), 4.05-4.06 (d, 2H), 4.11-4.16 (m, 2H), 4.30-4.33 (m, 2H), 5.14 (s, 1H), 5.26 (m, 1H), 6.57 (s, 1H); 13C NMR (CDCl₃) δ ppm 14.10, 22.67, 24.80, 28.27, 29.09, 29.23, 29.34, 29.45, 29.59, 29.62, 29.66, 31.90, 33.96, 41.10, 44.23, 61.67, 70.68, 80.48, 155.99, 168.96, 169.64, 173.32; HRMS calcd. for [C₄₀H₇₄N₂O₉+Na]⁺ 749.5292, found 749.5274.
1,2-Di-tetradecanoyl-3-Lys-Gly-rac-glycerol (8a, KG): 1,2-Di-tetradecanoyl-3-BocLys(Boc)-Gly-rac-glycerol (7a) (0.29 g, 0.32 mmol) was dissolved in 1 mL of CH$_2$Cl$_2$, and 1 mL TFA was added slowly to this ice cold solution. The reaction was allowed to run at RT for 1 h. The solvent and TFA were then removed under reduced pressure. The residue was washed with cold hexanes twice to afford the product as a powder (87%). 1H NMR (CDCl$_3$) $\delta$ ppm 0.85 (t, 6H), 1.22 (m, 40H), 1.56 (m, 6H), 1.65 (m, 2H), 1.85 (m, 2H), 2.28 (m, 4H), 2.91 (s, 2H), 3.94 (m, 2H), 4.13 (m, 3H), 4.25 (d, 2H), 5.11-5.21 (d, 1H), 7.91 (m, 4H), 8.75 (s, 1H); 13C NMR (CDCl$_3$) $\delta$ppm 14.07, 20.91, 22.67, 24.08, 24.85, 26.07, 28.28, 29.12, 29.15, 29.28, 29.32, 29.36, 29.53, 29.55, 29.66, 29.70, 30.89, 31.91, 33.98, 34.14, 39.06, 40.89, 52.94, 61.19, 62.16, 70.38, 169.01, 169.47, 170.22, 173.45, 173.61, 173.64, 173.73, 173.79; HRMS calcd. for [C$_{39}$H$_{75}$N$_3$O$_7$+H]$^+$ 698.5683, found 698.5674.

1,2-Di-tetradecanoyl-3-Orn-Gly-rac-glycerol (8b, OG): The same procedure was used as that described for 1,2-Di-tetradecanoyl-3-Lys-Gly-rac-glycerol (8a) to afford the product as a powder (85%). 1H NMR (CDCl$_3$) $\delta$ ppm 0.84 (m, 6H), 1.23 (m, 40H), 1.56 (s, 4H), 1.84 (s, 2H), 1.98 (s, 2H), 2.28 (m, 4H), 2.98 (m, 2H), 3.93 (s, 2H), 4.11 (s, 2H), 4.27 (s, 2H), 5.11-5.21 (d, 1H), 7.94 (s, 2H), 8.37 (s, 2H), 8.85 (s, 1H); 13C NMR (CDCl$_3$) $\delta$ppm 14.10, 22.34, 22.68, 24.80, 24.86, 27.93, 29.10, 29.12, 29.15, 29.29, 29.33, 29.38, 29.54, 29.57, 29.68, 29.70, 29.72, 30.93, 31.92, 33.97, 34.15, 38.81, 41.04, 52.48, 61.18, 62.23, 63.21, 68.63, 70.38, 168.94, 169.42, 169.76, 173.74, 173.88; HRMS calcd. for [C$_{38}$H$_{73}$N$_3$O$_7$+H]$^+$ 683.5449, found 684.5526.

1,2-Di-tetradecanoyl-3-Arg-Gly-rac-glycerol (8c, RG): Same procedure was used as that described for 1,2-Di-tetradecanoyl-3-Lys-Gly-rac-glycerol (8a) to afford the product as a powder (91%). 1H NMR (CDCl$_3$) $\delta$ ppm 0.85 (t, 6H), 1.22 (m, 40H), 1.45 (m, 2H), 1.55 (m, 4H),
1.66-1.91 (m, 4H), 2.28 (m, 4H), 3.12 (m, 2H), 3.92 (m, 2H), 4.10 (m, 2H), 4.26-4.31 (m, 2H), 5.10-5.21 (d, 1H), 6.79 (s, 2H), 8.38 (s, 2H), 8.98 (s, 1H); 13C NMR (CDCl₃) δ ppm 14.07, 22.67, 24.79, 27.63, 29.10, 29.26, 29.29, 29.36, 29.51, 29.66, 29.70, 30.90, 31.91, 33.98, 41.34, 52.18, 61.23, 68.42, 70.05, 157.29, 162.75, 167.02, 170.24, 173.67, 174.00; HRMS calcd. for [C39H75N5O7+H]+ 726.5745, found 726.5734.

1,2-Di-tetradecanoyl-3-Gly-Gly-rac-glycerol (8d, GG): The same procedure was used as that described for 1,2-Di-tetradecanoyl-3-Lys-Gly-rac-glycerol (8a) to afford the product as a powder (92%). 1H NMR (CDCl₃) δ ppm 0.86 (t, 6H), 1.23 (m, 40H), 1.57 (m, 4H), 2.26-2.31 (m, 4H), 3.88 (s, 2H), 4.00 (s, 2H), 4.07-4.19 (m, 2H), 4.27-4.31 (m, 2H), 5.16-5.24 (m, 1H), 8.25 (m, 2H); 13C NMR (CDCl₃) δ ppm 14.10, 22.68, 24.82, 24.86, 29.10, 29.12, 29.15, 29.28, 29.31, 29.37, 29.52, 29.53, 29.55, 29.67, 29.70, 31.92, 33.97, 34.00, 34.16, 40.93, 41.12, 61.29, 62.12, 63.30, 68.58, 70.54, 167.09, 169.19, 169.55, 173.35, 173.61, 173.71; HRMS calcd. for [C35H67N2O7+H]+ 627.4948, found 627.4955.

Liposome Preparation

A chloroform solution of the lipid (~2 mg) was added to a pear shaped flask. The solvent was removed first with a stream of N₂, and then dried under a vacuum, leaving a thin film deposited onto the flask wall. A total of 1 mL nuclease-free water was then added and the film was peeled off by vortexing, hydrated by sonicating at 45 °C for 15 min, and annealed for 15 min. The solution was extruded through a polycarbonate membrane (100 nm) using an Avanti polar lipids mini-extruder until a homogeneous liposome solution was obtained (typically 21 extrusions).

Lipoplex Preparation
Lipoplexes were formed by first diluting liposomes in serum-free cell medium at 37 °C. The nucleic acid solution (either DNA or RNA) was then added to the liposome solution, vortexed briefly, and incubated at room temperature for 15 min to allow time for complexation. The ratio of amphiphile:nucleic acid was determined based on the amount of nucleic used in each experiment.

**Dynamic Light Scattering**

The diameter of the liposomes and lipid/DNA assemblies was measured at a 90° angle using a Brookhaven Instruments Model 90 Plus. All solutions used in the study were prepared and filtered through 0.02 μm pore size filter (Whatman) into the scattering cell. The diameter reported corresponds to the average of three experiments in serum-free F-12K cell medium. The 90 Plus particle sizing software was used for data acquisition and analysis.

**Zeta Potential**

The zeta potentials of the lipoplexes were measure at a 15° angle using a Brookhaven Instruments Model 90 Plus. Sample preparations were the same as in the dynamic light scattering experiments. Zeta potentials were measured both in pure water and serum-free F-12K cell medium.

**Modulated Differential Scanning Calorimetry**

The thermal properties of the lipid were measured on a DSC (TA Q100). 2 mg of the lipid in 15 μL of water was hermetically sealed in an aluminum pan. The modulation was set to ±1.00 °C every 60 s, and the pan was equilibrated at −50 °C for 15 min. The temperature was increased at 0.5 °C/min to 90 °C where it has held for 15 min. The temperature was then reduced to −50 °C.
and held at this temperature for 15 min. This heating-cooling cycle was repeated two more times. The data collected on the third cycle were analyzed to obtain the Tₘ.

**DNA Binding Affinities**

A competitive displacement fluorescence quenching assay using ethidium bromide was used for the DNA binding studies. This assay involves the addition of aliquots of the compound to a 3 mL solution of EtBr (1.3 µM) and calf thymus DNA (3 µM) in buffer (100 mM NaCl, 100 mM Tris, pH 7.4) with the decrease of fluorescence (λₑₓ=546 nm, λₑₘ=600 nm; 1 cm path length glass cuvette, slit width 6 nm) recorded after 5 minutes of equilibrium time following each addition.

**X-ray Diffraction**

X-ray diffraction was performed on both fully hydrated liposomes and oriented multilayers by methods detailed in previous publications (references 25 and 22). In the case of the fully hydrated preparations, the liposomes were pelleted by centrifugation. The pellet was then transferred to a sealed quartz-glass X-ray capillary and analyzed with a point collimated X-ray beam. For the oriented multilayers, liposomes were partially dried onto a curved glass surface under a controlled relative humidity atmosphere. These oriented multilayers were transferred to a humidity chamber and X-rayed with a line-focused beam oriented at a grazing angle to the glass surface. Typically, for both unoriented and oriented preparations, the distances of specimen to film were 10 cm and the exposure times were 2-6 h. A Rigaku rotating anode X-ray generator was used to produce Cu Kα X-radiation and diffraction patterns were obtained using a flat plate film cassette loaded with Kodak DEF X-ray film.

**Cell Culture and In Vitro Transfection**
Chinese hamster ovarian cells (CHO, ATCC, Manassas VA) were cultured in complete F-12K media (ATCC) containing 10% fetal calf serum (Sigma) and 1% penicillin and streptomycin (500 IU/mL and 5000 µg/ml, respectively, Mediatech, Herndon, VA) at 37 °C in 5% CO₂ with humidity. When the CHO cells reached about 90% confluency, the cells were split into 24-well plates with a 1:4 ratio using a standard trypsin-based technique. Transfections were performed 24 h later by modification of previously published methods. Briefly, plasmid DNA coding for a reporter gene, β-galactosidase (β-gal, pVax-LacZ1, Invitrogen) was first mixed with lipids in serum-free cell medium at room temperature. Depending on the experimental design, the ratio of DNA and amphiphile was varied. The mixture was incubated for 15 minutes at room temperature before adding to the cells. The amount of DNA used (0.5 µg/well) was the same as used in naked DNA control and positive control (commercially available transfection reagents). After incubation at 37 °C and 5% CO₂ for 3 h, fresh growth medium was added. Transfection efficiencies were assessed at 48 h post transfection. Negative controls were constructed with 0.2 mL of serum-free cell medium and naked DNA controls were using 0.2 mL of serum-free F-12K medium with 0.5 µL (0.5 µg) of reporter gene. The positive control was the commercial cationic lipid Lipofectamine 2000 (Invitrogen).

**Reporter Gene Transfection Efficiency Assay**

Reporter gene (β-gal) assay was performed with a β-galactosidase enzyme assay system (Promega, Madison, WI) following the manufacturer’s protocol. Briefly, cells were first lysed and enzymatic activities were determined. A standard curve was constructed for each experiment using dilutions of purified β-gal protein. The β-gal activities from experimental samples were determined by comparison to the standard curve (enzyme activity vs. enzyme concentration).
Efficiency of each transfection was calculated as β-gal activity normalized to total protein. Total protein was determined using a Coomassie Blue protein kit (Pierce) as described below.

**Cytotoxicity**

Cytotoxicity was assessed using a formazan-based proliferation assay (CellTiter 96 AQueous One Solution Cell Proliferation Assay kit, Promega) and a total protein-based assay (Pierce). Briefly CHO (or NIH 3T3) cells were seeded onto a 24-well microliter plate with an appropriate density of 1 x 10^5 cells per well. After 48 h the MTS substrate was added to each well and the plate was incubated for 1 h at 37 °C in a humidified, 5% CO₂ incubator. The amount of soluble formazan produced by cellular reduction of the substrates MTS was recorded at 490 nm using a multi-well plate reader. For the total protein-based proliferation assay, cells were lysed at the same time as the transfection efficiency assay. 5 μL of each lysate was transferred to a separate multi-well plate. Total protein contents were assessed using the Coomassie Blue protein kit (Pierce, Rockford, IL) following the manufacturer protocol. Negative and positive controls were non-treated cells and commercial lipid treated cells, respectively. The proliferation results were expressed as percentages of non-treated cells.

**siRNA Knockdown Assay**

The adherent cells were trypsinized and diluted in normal growth medium to 1 x 10^5 cells per mL. The transfection reagent (i.e., peptidic lipids) was diluted in serum free medium and incubated at room temperature for 10 min. *Silencer* GAPDH siRNA (Ambion) was also diluted in serum-free medium. Next, both solutions were combined, incubated for 10 min at RT, and then dispensed into a 96-well culture plate (concentration 50 nM siRNA per well). Depending on the experimental design, the ratio of lipid to siRNA was varied. The cell suspension was then
overlayed onto the transfection mixture and incubated at 37 °C and 5% CO2. Gene knockdown was assessed after 48 h.

Specifically, the gene knockdown assay performed was the KDalert™ GAPDH Assay (Ambion) following the manufacturer’s protocol, which includes the use of Silencer GAPDH siRNA. Briefly, 48 h after siRNA transfection, the culture medium was aspirated from transfected cells. The KDalert Lysis Buffer (200 μL) was added to each sample well. The cells were then incubated at 4 °C for 20 min to lyse the cells. The cell lysate was pipetted up and down 4-5 times to homogenize the lysate. Next, 10 μL of each lysate or GAPDH enzyme dilution (including the GAPDH working stock; control solution) was transferred to another 96-well plate. Finally, 90 μL of KDalert Master Mix was added to each sample using a multi-channel pipettor and the increase of fluorescence at RT in a 4 min time period was measured. The percentage of siRNA knockdown was expressed as the increase of fluorescence signal compared to the cells transfected with negative control siRNA (Negative Control #1 siRNA; Ambion), which has no homology to mouse or human genes. The delivery agent used as the positive control was the commercial reagent NeoFX (Ambion).

**Cellular Uptake and Flow Cytometry**

The adherent CHO cells were cultured in complete F-12K media (ATCC) containing 10% fetal calf serum (Sigma) and 1% penicillin and streptomycin (500 IU/mL and 5000 μg/ml, respectively, Mediatech, Herndon, VA) at 37 °C in 5% CO2 with humidity. Cells were split into 12-well plates at a seeding density of 500,000 cells per well and allowed to grow in complete F-12K media for 36 h. Media was then removed and cells were washed with 1x Dulbecco’s Phosphate Buffered Solution (1x DPBS w/o Ca and Mg, Mediatech) prior to incubation with
lipoplexes. DNA (β-gal control vector, Invitrogen) and siRNA (GAPDH negative control vector, Ambion) samples were labeled with Label IT Cx-Rhodamine Labeling Kit (Mirus) following the manufacturer’s protocol. Lipoplexes were formed prior to use by mixing liposomes in 1x DPBS (prepared as described above) with the appropriate concentration of either rho-DNA or rho-siRNA and the volume was increased to 500 uL/well with serum-free F-12K media. Liposomes and nucleic acid were left to complex for 15 min at RT. Each well received 2.5 µg of nucleic acid and the appropriate amount of liposome solution based on desired molar ratio (lipid/nucleic acid). Each sample was then added to the CHO cells and incubated for the appropriate time (30 min, 1 h, 2 h, etc.). The positive (Lipofectamine 2000) and negative (cells alone) controls were incubated with cells for the longest time period (12 h). After incubation, cells were washed (including numerous washes using both 1xDPBS with Ca and Mg and 1xDBPBS without, as well as an incubation with CellScrub™ Buffer from Genlantis) and prepared for flow cytometry analysis. An LSRII flow cytometer (BD Biosciences) at the Boston University Flow Cytometry Laboratory was used to analyze the fluorescence signal intensity of 10,000 cells from each sample. Cells were gated based on forward and side scattering properties and fluorescent signal was measured using the 561 nm laser and 610/20 filter set. Positive controls (Lipofectamine 2000) for the flow cytometry experiments using rho-DNA and rho-siRNA are shown in Figure S1.
Figure S1: Positive controls for cell uptake experiments using CHO cells. (Top) rho-DNA delivered by Lipofectamine 2000, lipoplexes incubated with cells for 12 h; (bottom) rho-siRNA delivered by Lipofectamine 2000, lipoplexes incubated with cells for 12 h.