New unsymmetrical bolaamphiphiles: synthesis, assembly with DNA and application for gene delivery

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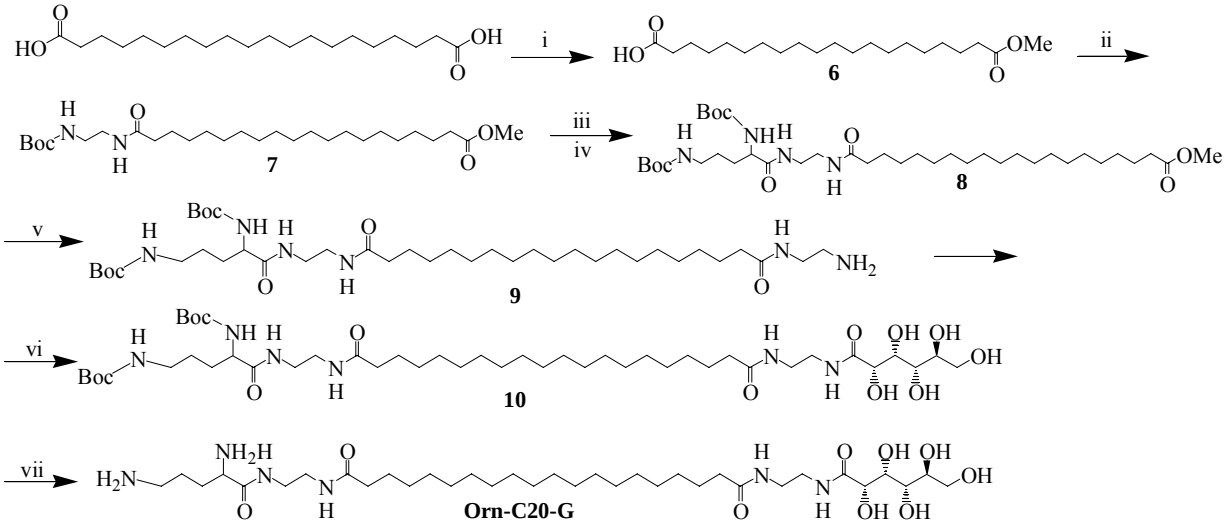
1. Synthesis of Orn-C12-G and Orn-C8-C12-G bolas.



Reagents: (i) MeOH, H_2SO_4, $C_2H_4Cl_2$, 85°C, 24h (25%) ; (ii) BOP, HOBt, DIEA, DMF, CH_2Cl_2, 24h (72%) ; (iii) TFA, H_2O, 1h; (iv) BOP, HOBt, DIEA, DMF, CH_2Cl_2, 40°C, 24h (70%) ; (v) Ethylene Diamine, 70°C, 72h (93%) ; (vi) Gluconic acid lactone, MeOH, DIEA ; (vii) TFA, H_2O, 1h (90%).

Scheme S1. Synthesis of **Orn-C20-G.**

Eicosanedioic acid monomethyl ester (6). The procedure was adapted from Ref. (1). Eicosanedioic acid (14.6 mmol, 5 g) was dissolved in 300 ml of ethylene chloride at 85°C. Methanol (7.4 mmol, 0.59 ml) was added to the clear solution, then H_2SO_4 (1.4 ml) was cautiously added, and the reaction was left for reflux overnight at 85°C. The solvent was concentrated and water was added. Three phases were obtained: aqueous, organic and slurry. To the organic phase, which contained mainly di- and mono-esters, carbon tetrachloride was added and placed in a separate funnel. The organic layer was washed with water, then dried over $MgSO_4$ and evaporated. Product was subjected to column chromatography, where di-ester was eluded with dichloromethane and the monoester **6** with ethyl acetate (25 % yield). ^{1}H NMR ($CDCl_3$, 300 MHz): 3.65 (s, 3H), 2.36-2.26 (m, 4H), 1.65-1.56 (m, 4H), 1.39-1.19 (m, 28H).

19-(2-tert-Butoxycarbonylamino-ethylcarbamoyl)-nonadecanoic acid methyl ester (7). *N*-Boc-ethylenediamine (1 mmol, 0.162 g) was coupled with mono-methyl ester **6** (0.84 mmol, 0.3 g) using BOP (0.93 mmol, 0.41 g), HOBt (1.2 mmol, 0.16 g) and DIEA (3.4 mmol, 0.6 ml) in DMF (5 ml) and dichloromethane (20 ml). The mixture was stirred overnight at room temperature. Then the solvent was evaporated and the product was crystallized from acetonitrile to give product **7** (0.3 g,

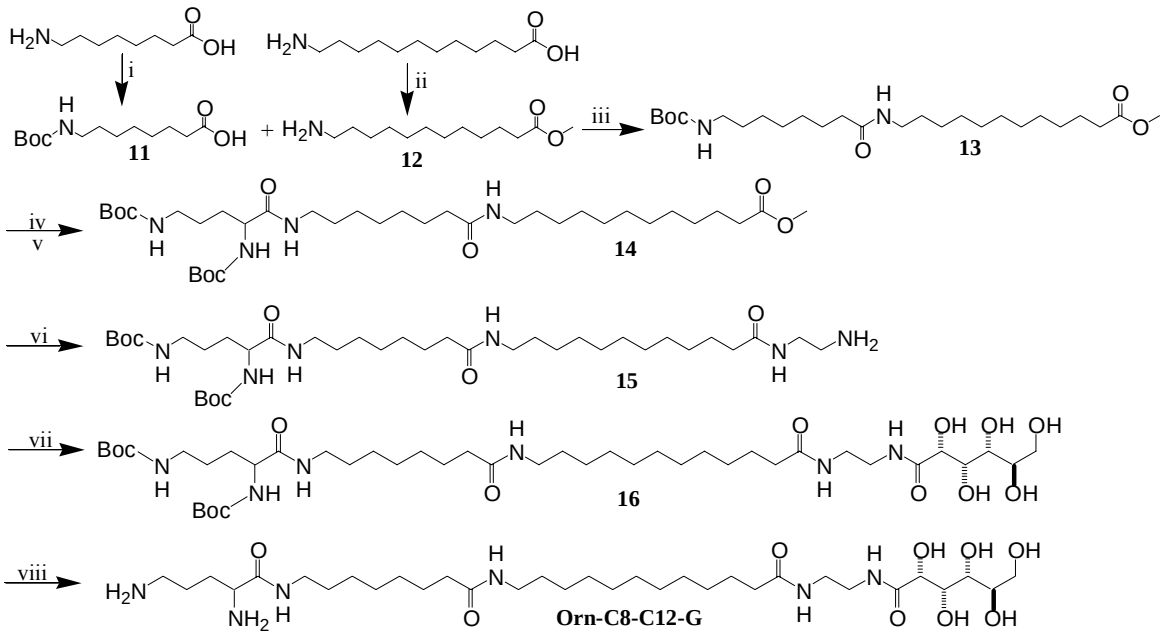
72 %). ^{1}H NMR (DMSO, 300 MHz): 7.62 (s, 1H), 6.61 (s, 1H), 3.58 (s, 3H), 3.05 (t, 2H), 2.97 (t, 2H), 2.27 (t, 2H), 2.03 (t, 2H), 1.57-1.42 (m, 4H), 1.4-1.33 (m, 9H), 1.32-1.17 (m, 28H). LC-MS: (m/z) Found $[M+1]^+ = 399.2$ (calcd for $C_{28}H_{54}N_2O_5^+ - C_5H_9O_2$ (Boc) = 399.3).

19-[2-(2,5-Bis-tert-butoxycarbonylamino-pentanoylamino)-ethylcarbamoyl]-nonadecanoic acid methyl ester (8). Boc-group was removed from **7** (0.54 mmol, 0.27 g) by treating with 1 ml TFA and 3% H_2O, for 1 h at RT. The solvent was evaporated and obtained de-protected amine was coupled with 2,5-Bis-tert-butoxycarbonyl-aminopentanoic acid (0.62 mmol, 0.207 g) using BOP (0.93 mmol), HOBt (1.2 mmol) and DIEA (3.4 mmol, 0.6 ml) in DMF (10 ml) and dichloromethane (10 ml). The mixture was stirred overnight at 40 °C. Then the solvent was evaporated and the product was crystallized from ethyl acetate. The precipitate was filtered off and dried to give solid crystals of compound **8** (0.34 g, 70 %). ^{1}H NMR ($CDCl_3$, 300 MHz): 6.95 (s, 1H), 6.39 (s, 1H), 5.18 (s, 1H), 4.76 (s, 1H), 4.17 (s, 1H), 3.65 (t, 3H), 3.4-3.2 (m, 5H), 3.11-2.99 (m, 1H), 2.28 (t, 2H), 2.15 (t, 2H), 1.87-1.7 (m, 2H), 1.67-1.5 (m, 8H), 1.46-1.38 (m, 18H), 1.34-1.17 (m, 30H). LC-MS: (m/z) Found $[M+1]^+ = 613.4$ (calcd for $C_{38}H_{72}N_4O_5^+ - C_5H_9O_2$ (Boc) = 613.4).

(1-{2-[19-[2-(2-Amino-ethylcarbamoyl)-nonadecanoylamino]-ethylcarbamoyl}-4-tert-butoxycarbonylamino-butyl)-carbamic acid tert-butyl ester (9). Methyl ester **8** (0.4 mmol, 0.3 g) was reacted with ethylenediamine (150 mmol 10 ml) for 72 h at 70°C. Then the excess of ethylenediamine was evaporated and water was poured into the reaction flask. Formed precipitate was filtered off to give compound **9** (0.29 g, 93 %). ^{1}H NMR (CD_3OD, 300 MHz): 3.96 (s, 1H), 3.05 (t, 2H), 2.73 (t, 2H), 2.23-2.16 (m, 4H), 1.67-1.5 (m, 8H), 1.46-1.44 (m, 18H), 1.39-1.23 (m, 30H). LC-MS: (m/z) Found $[M+1]^+ = 741.4$ (calcd for $C_{39}H_{76}N_6O_7^+ = 741.5$).

[4-tert-Butoxycabonylamino-4-(2-{19-[2-(2,3,4,5,6-pentahydroxy-hexanoylamino)-ethylcarbamoyl]-nonadecanoylamino}-ethylcabamoyl)-butyl]-carbamic acid tert-butyl ester (10). δ-Gulonic-γ-Lactone (0.23 mmol, 0.04 g) was added to solution of **9** (0.13 mmol, 0.1 g) in 10 ml of methanol. Then, DIEA (1.4 mmol, 0.24 ml) was added and reaction mixture was stirred at 70°C for about 24 h. Solvents were evaporated in vacuo. The obtained compound was crystallized from methanol to give product **10** (0.095 g, 95 %). ^{1}H NMR (CD_3OD, 300 MHz): 4.21 (s,1H), 4.1 (s, 1H), 3.98 (s, 1H), 3.84-3.59 (m, 5H), 3.07 (t, 2H), 2.25-2.12 (m, 4H), 1.68-1.49 (m, 8H), 1.49-1.4 (m, 18H), 1.4-1.21 (m, 30H). LC-MS: (m/z) Found $[M+1]^+ = 919.6$ (calcd for $C_{45}H_{86}N_6O_{13}^+ = 919.6$).

Eicosanedioic acid [2-(2,5-diamino-pentnoylamino)-ethyl]-amide[2-(2,3,4,5,6-pentahydroxy-hexanoylamino)-ethyl]-amide (Orn-C20-G). 40 mg of the Boc-protected product **10** was treated with 0.4 ml TFA and 3 % H2O for clean removal of Boc group to get final compound **Orn-C20-G** (27 mg, 90 %). LC-MS: (m/z) Found $[M+1]^+ = 719.5$ (calcd for $C_{35}H_{71}N_6O_9^+ = 719.5$).



Reagents: (i) Boc_2O, DMF, CH_2Cl_2, 20h (92%); (ii) $SOCl_2$, MeOH, 20h (94%); (iii) BOP, HOBt, DIEA, DMF, CH_2Cl_2, 24h (81%); (iv) TFA, CH_2Cl_2, 1h; (v) BOP, HOBt, DIEA, DMF, CH_2Cl_2, 24h (92%); (vi) $C_2H_8N_2$, 70°C, 72h (96%); (vii) MeOH, DIEA, 70°C, 24h; (viii) TFA, H_2O, 1h (92%).

Scheme S2. Synthesis of **Orn-C8-C12-G.**

8-tert-Butoxycabonylamino-octanoic acid (11). Amino group of 8-Aminooctanoic acid (31.4 mmol, 5 g) was protected by Boc using Di-tert-butyl dicarbonate (34.6 mmol, 7.54 g) in solution of DMF (30 ml) and dichloromethane (40 ml).Then, DIEA (94.2 mmol, 16.38 ml) was added and the reaction mixture was stirred for 20 h at RT. The solvent was evaporated and product was crystallized from dichloromethane. The precipitate was filtered off and dried to give compound **11** (7.5 g, 92 %). ^{1}H NMR ($CDCl_3$, 300 MHz): δ 4.55 (s, 1H), 3.07 (t, 2H), 2.3 (t, 2H), 1.67-1.5 (t, 4H), 1.56-1.37 (t, 9H), 1.37-1.21 (m, 6H).

12-Amino-dodecanoic acid methyl ester (12). Thionyl chloride (103.4 mmol, 7.5 ml) was added drop-wise to 100 ml of methanol at 0 °C and the mixture was stirred for another 20 min. Then, 10 g of 12-aminododecanoic acid (46.5 mmol) was added and reaction mixture was stirred for 20 h at RT. The solvent was evaporated and product was crystallized from heptane to give compound **12** (10 g, 94 %). ^{1}H NMR (DMSO, 300 MHz): δ 7.89 (s, 2H), 3.68 (s, 3H), 2.75 (t, 2H), 2.28 (t, 2H), 1.58-1.15 (m, 18H).

12-(8-tert-Butoxycarbonylamino-octanoylamino)-dodecanoic acid methyl ester (13). Compound **12** (6.5 mmol, 1.5 g) was coupled with **11** (7.2 mmol, 1.87 g) using BOP (7.2 mmol, 3.18 g), HOBt (8.9 mmol, 1.2 g) and DIEA (25.9 mmol, 4.5 ml) in DMF (15 ml) and dichloromethane (30 ml). The reaction mixture was stirred 24 h at RT. Then the solvents were evaporated and product was crystallized from ethyl acetate : heptane (8:2). The precipitate was filtered off and dried to give compound **13** (2.5 g, 81 %). ^{1}H NMR ($CDCl_3$, 300 MHz): δ 5.41 (s, 1H), 4.48 (s, 1H), 3.65 (s, 3H), 3.21 (t, 2H), 3.08 (t, 2H), 2.29 (t, 2H), 2.13 (t, 2H), 1.69-1.15 (m, 39H). LC-MS: (m/z) Found $[M+1]^+ = 371.2$ (calcd for $C_{26}H_{50}N_2O_5^+$ - $C_{10}H_{18}O_4$ (2Boc) = 371.2).

12-[8-(2,5-Bis-tert-butoxycarbonylamino-pentanoylamino)-octanoylamino]-dodecanoic acid methyl ester (14). Boc group of **13** (2.13 mmol, 1 g) was removed in dichloromethane (3 ml) in the presence of 2 ml of TFA for 1 h at RT. The solvent was evaporated and 1 g (2.1 mmol) of the obtained deprotected amine was coupled with 2,5-Bis-tert-butoxycarbonyl amino-pentanoic acid (2.2 mmol, 0.72 g) using BOP (2.4 mmol, 1.04 g), HOBt (2.96 mmol, 0.4 g) and DIEA (8.63 mmol, 1.5 ml) in DMF (5 ml) and dichloromethane (15 ml). The mixture was stirred overnight at RT. Then solvent was evaporated and product **14** (1.3 g, 92 %) was crystallized from acetonitrile. ^{1}H NMR ($CDCl_3$, 300 MHz): δ 6.46 (s, 1H), 5.56 (s, 1H), 5.2 (s, 1H), 4.73 (s, 1H), 4.17 (s, 1H), 3.65 (s, 3H), 3.28-3.04 (m, 6H), 2.28 (t, 2H), 2.13 (t, 2H), 1.66-1.19 (m, 50H). LC-MS: (m/z) Found $[M+1]^+$ = 585.4 (calcd for $C_{36}H_{68}N_4O_8^+$ - $C_{10}H_{18}O_4$ (2Boc) = 585.4).

(1-{7-[11-(2-Amino-ethylcarbamoyl)-undecylcarbamoyl]-heptylcarbamoyl}-4-tert-butoxycarbonylamino-butyl)-carbamic acid tert-butyl ester (15). The methyl ester of **14** (1.75 mmol, 1.2 g) was reacted with ethylenediamine (150 mmol, 10 ml) for 72 h at 70°C. Then the excess of ethylenediamine was evaporated, the water was added and the product **15** was filtered (1.2 g, 96 %). ^{1}H NMR (CD_3OD, 300 MHz): δ 3.99 (s, 1H), 3.28-3.13 (m, 6H), 3.06 (t, 2H), 2.73 (t, 2H), 2.24-2.15 (m, 4H), 1.67-1.28 (m, 50H). LC-MS: (m/z) Found $[M+1]^+$ = 713.4 (calcd for $C_{37}H_{72}N_6O_7^+$ = 713.4).

[4-tert-Butoxycabonylamino-4-(7-{11-[2-(2,3,4,5,6-pentahydroxy-hexanoylamino)-ethylcarbamoyl]-undecylcarbamoyl}-heptylcabamoyl)-butyl]-carbamic acid tert-butyl ester (16). δ-Gulonic-γ-Lactone (0.95 mmol, 0.17 g) was added to solution of **15** (0.56 mmol, 0.4 g) in 30 ml of methanol. Then, DIEA (5.64 mmol, 0.98 ml) was added and the reaction mixture was stirred at 70°C for about 24 h. Solvents were evaporated in vacuo. The obtained compound was crystallized from methanol to get product **16** (0.15 g, 88 %). ^{1}H NMR (DMSO, 300 MHz): δ 7.83-7.61 (m, 4H), 6.8-6.64 (m, 2H), 5.35 (d, 1H), 4.57-4.26 (m, 5H), 4.0-3.75 (m, 3H), 3.61-3.38 (m, 6H), 3.22-2.81 (m, 11H), 2.06-1.95 (m, 4H), 1.54-1.37 (m, 10H), 1.36-1.32 (m, 18H), 1.31-1.11 (m, 24H). LC-MS: (m/z) Found $[M+1]^+$ = 891.6 (calcd for $C_{43}H_{82}N_6O_{13}^+$ = 891.6).

12-[8-(2,5-Diamino-pentanoylamino)-octanoylamino]-dodecanoic acid[2-(2,3,4,5,6-pentahydroxy-hexanoylamino)-ethyl]-amide (Orn-C8-C12-G). Boc group of **16** was removed from 0.1 g (0.11 mmol) of the compound using 1 ml TFA and 3% of H2O to afford the final product **Orn-C8-C12-G** (0.095 g, 92 %). MS: (m/z) Found $[M+1]^+$ = 691.5 (calcd for $C_{33}H_{67}N_6O_9^+$ = 691.5). MS: (m/z) Found $[M+1]^+$ = 691.5 (calcd for $C_{33}H_{67}N_6O_9^+$ = 691.5).

2. 1,8-ANS fluorescence data.

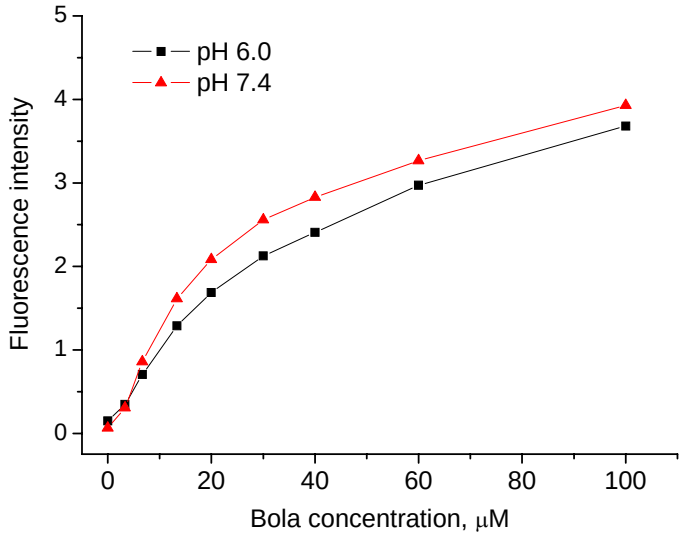


Fig. S1. Fluorescence intensity of 1,8-ANS (50 nM) as a function of Orn-C20-G bola concentration at two different pH.

3. Gel electrophoresis data.

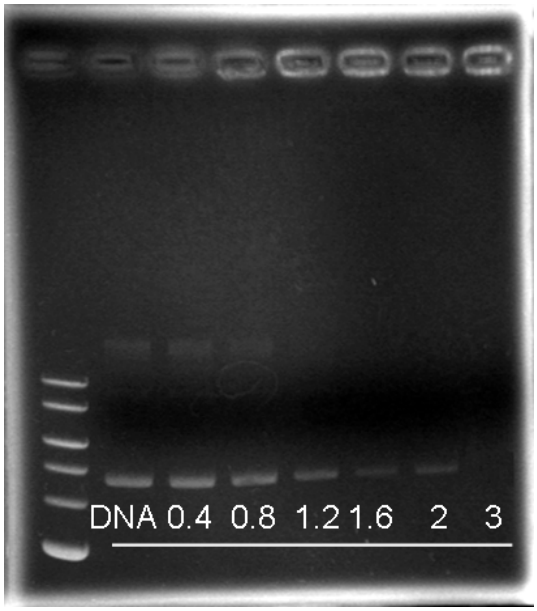


Fig. S2. Agarose gel electrophoresis (0.9%) of Orn-C16-G/DOPE (1:1) mixture complexed with pDNA at different N/P ratios. Bands at the left of the gel correspond to 10 kbp DNA ladder.

4. DLS data

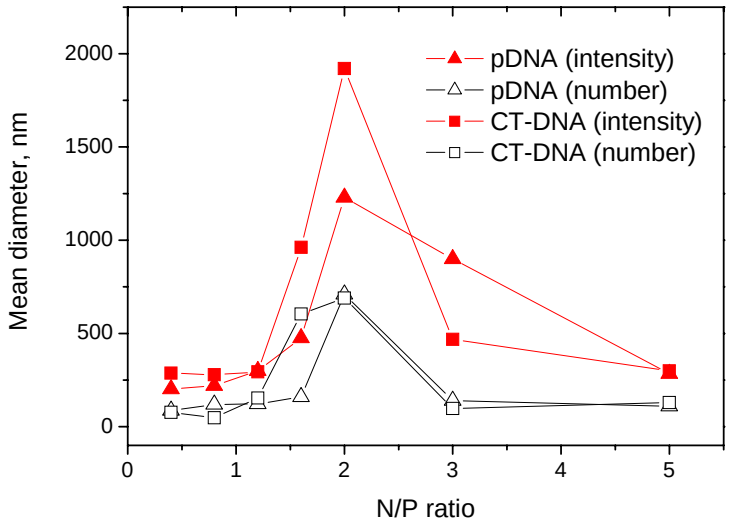
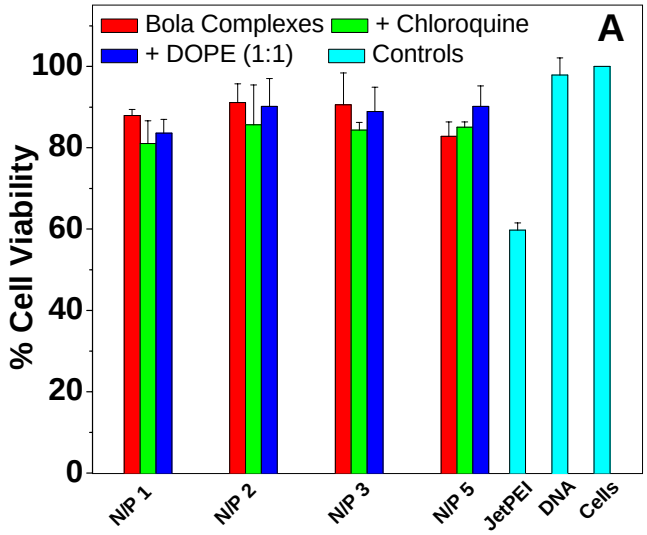


Fig. S3. Mean diameters as measured by DLS of Orn-C16-G complexes with pDNA (triangle) and CT-DNA (square) at different N/P ratios. The experiments were performed in MES buffer (pH 7.4) by addition of increasing quantities of bola stock solution (in DMF/water) to pDNA solution. Each measurement was proceeded 5 min after each addition of bola aliquot. Mean diameters are presented based on statistics of scattered intensity (filled symbols) and particle number (open symbols).

5. Cytotoxicity and total protein assays.



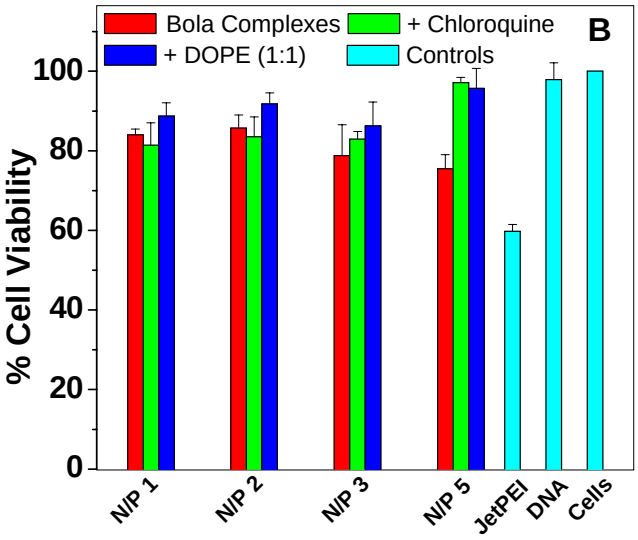


Fig. S4. Total protein concentration from the transfection experiments in COS-7 cells for **Orn-C16-G** (A) and **Orn-C20-G** (B) bolas. The data are normalized to 100% for the control non-treated cells. Cells were incubated in serum-free Opti-MEM with a bolaplex composed of plasmid DNA (1 μg per well), bola and DOPE (when indicated) at pH 7.4. Different N/P ratios were tested. After 3 h, the transfection medium was replaced with fresh complete culture medium, and cells were cultured for an additional 45 h. When indicated the medium contained 100 μM chloroquine. Then cells were lysed and the total protein was estimated using BC assay.

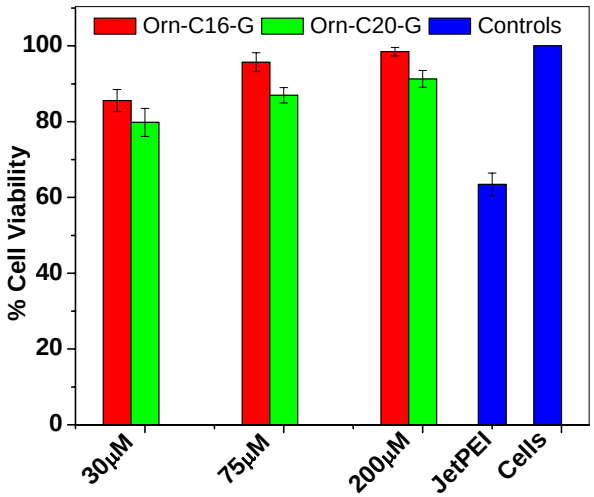


Fig. S5. Cytotoxicity of bolas based on MTT assay. COS-7 cells were incubated for 48 h with the bolas (as described in Fig. S1) at the mentioned concentrations or with JetPEI (150 μM, expressed as concentration of nitrogen residues).

References

(1) Buller, R., Cohen, H., Jensen, T. R., Kjaer, K., Lahav, M.; Leiserowitz, L. (2001) Self-Assembly of Bolaamphiphiles Forming Alternating Layer Arrangements with Lead and Copper Divalent Ions. *J. Phys. Chem. B 105*, 11447–11455.