Supplementary Information

Polyglycerol-Based Amphiphilic Dendrons as Potential siRNA Carriers for In Vivo Applications

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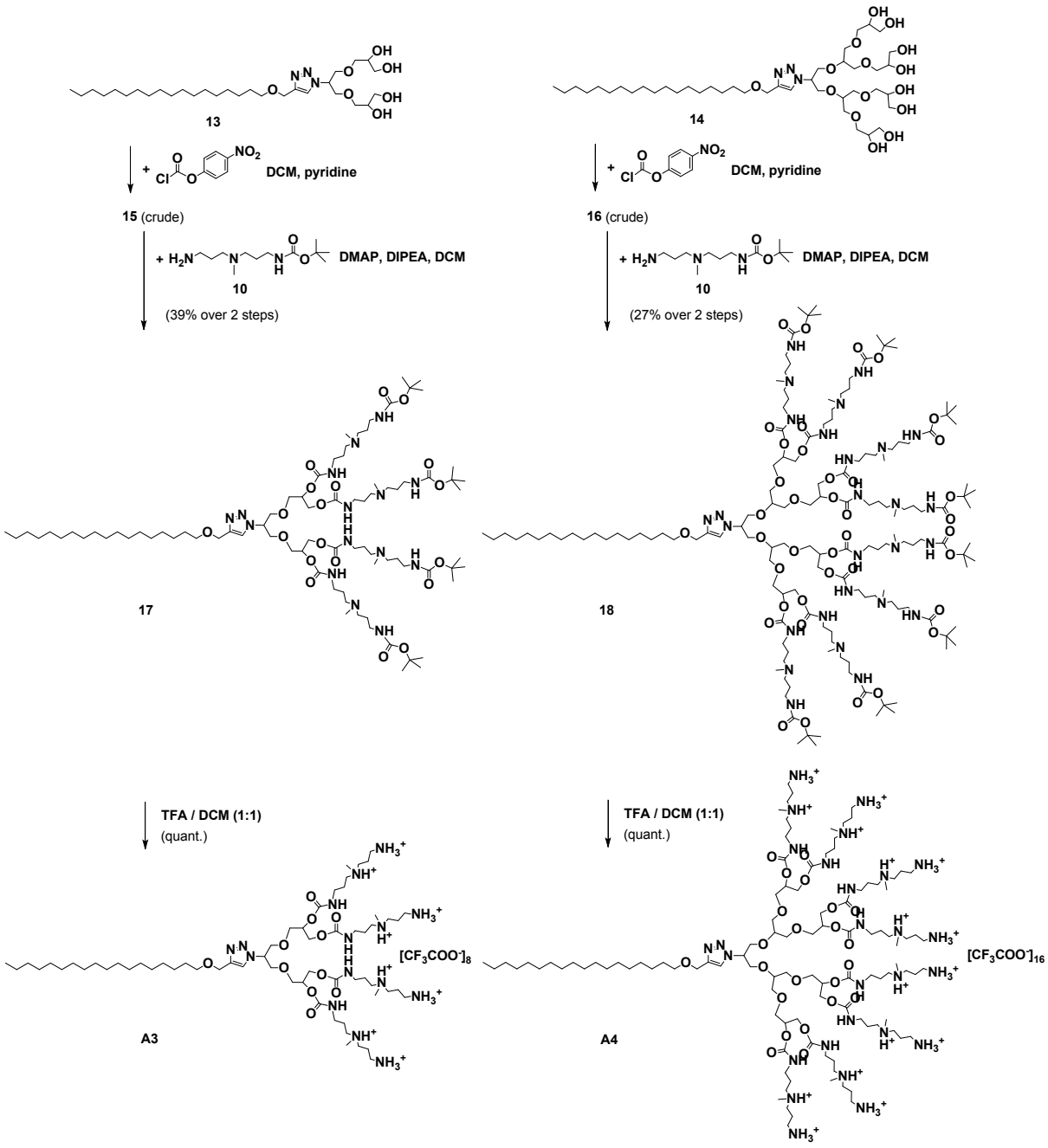
Synthesis of G1-Trz-DAPMA (A3) and G2-Trz-DAPMA (A4)



Scheme S1. Synthesis of G1-Trz-DAPMA (**A3**) and G2-Trz-DAPMA (**A4**).

General procedure for the synthesis of compounds 15 and 16:

The starting material (**13**, **14**) was synthesized according to our recent report.[1] The reaction was performed under inert gas atmosphere and exclusion of water. A solution of *p*-nitrophenyl chloroformate (9.44 g, 46.81 mmol, 12 eq) in 20 mL dry DCM was added dropwise to dry DCM (20 mL) and dry pyridine (3.77 mL, 46.81 mmol, 12 eq), while stirring at 0°C in an ice bath. On addition a white precipitate was formed. Subsequently, a solution of compounds **13** or **14** (3.90 mmol, 1 eq), dissolved in dry DCM (60 mL) and dry pyridine (0.37 mL, 4.68 mmol, 1.2 eq) was added at 0°C *via* a dropping funnel over a period of 2 h. The mixture was stirred in the thawing ice bath for 14 h. The reaction mixture was then diluted with DCM (50 mL) and washed with $NaHSO_4$ (2 x 50 ml, 1.33 M) and sat. brine (50 ml). The organic phase was dried over $MgSO_4$, filtered, and the filtrate evaporated *in vacuo*. The reaction mixture was roughly purified by HPLC (Silica column, DCM/MeOH 98:2, 64 mL/min). Due to the relative instability and since thorough purification was not required at this stage, the intermediates **15** and **16** were used in their crude form for further synthesis.

General procedure for the synthesis of compounds 17 and 18:

Each solution of the crude compounds **15** or **16** (0.68 mmol), which were dissolved in dry DCM (120 mL), were added dropwise over 2 h at 0°C into a solution of mono-Boc-DAPMA (2.00 g, 8.15 mmol, 12 eq, dissolved in 50 mL dry DCM) employing dry reaction conditions. Immediately, the solution turned yellow due to the displacement of *p*-nitrophenol. A solution of DMAP (0.17 g, 1.36 mmol, ~0.5 eq per *p*-nitrophenyl branch) and DIPEA (0.47 mL, 2.72 mmol, 1.0 eq per *p*-nitrophenyl branch) in dry DCM (30 mL) was added and the reaction mixture was stirred at room temperature for 72 h. The solvent was then removed under reduced pressure.

Purification was performed both by column chromatography ($CHCl_3$/MeOH/NH_4OH 90:9:1) and size exclusion chromatography (SEC) using Sephadex™ LH-20 ($CHCl_3$/MeOH 1:1). Drying under high vacuum yielded the products **17** and **18** as yellowish oils.

Compound 17

Obtained as a yellowish viscous oil (0.44 g, 39% over two steps). ^{1}H NMR (700 MHz, methanol-d_4) δ 8.02 (t, *J* = 12.6 Hz, 1 H, trz), 5.05–4.92 (m, 3 H, dendron), 4.59 (m_c, 2 H, O-C$\underline{H}_2$-trz), 4.22–4.00 (m, 4 H, dendron), 3.93 (m_c, 4 H, dendron), 3.68–3.55 (m, 4 H, dendron), 3.51 (m_c, 2 H, CH_2-C$\underline{H}_2$-O), 3.12 (t, *J* = 6.7 Hz, 8 H, C$\underline{H}_2$-NH), 3.07 (t, *J* = 6.8 Hz, 8 H, C$\underline{H}_2$-NH), 2.41 (m_c, 16 H, C$\underline{H}_2$-N-CH_3), 2.23 (br s, 12 H, N-C$\underline{H}_3$), 1.73–1.61 (m, 16 H, NH-CH_2-C$\underline{H}_2$), 1.58 (m_c, 2 H, C$\underline{H}_2$-CH_2-O), 1.43 (br s, 36 H, Boc CH_3), 1.29 (br s, 30 H, alkyl C$\underline{H}_2$), 0.90 ppm (t, *J* = 7.1 Hz, 3 H, alkyl C$\underline{H}_3$); ^{13}C NMR (176 MHz, methanol-d_4) δ 158.5, 158.0, 145.8, 125.0, 79.9, 72.3, 71.7, 71.1, 64.7, 64.4, 62.2, 42.3, 40.2, 39.7, 33.1, 30.8, 30.7, 30.6, 30.5, 28.9, 28.2, 27.2, 23.7, 14.4 ppm. HRMS: *m*/*z* Calcd for $C_{82}H_{159}N_{15}O_{19}Na$ $[M+Na]^+$: 1681.1829. Found: 1681.2035.

Compound 18

Obtained as a yellowish viscous oil (0.56 g, 27% over two steps). ^{1}H NMR (400 MHz, methanol-d_4) δ 8.09 (t, *J* = 12.6 Hz, 1 H, trz), 4.94-5.00 (m, 5 H, dendron), 4.60 (m_c, 2 H, O-C$\underline{H}_2$-trz), 4.20-4.27 (m, 4 H, dendron), 4.05-4.11 (m, 6 H, dendron), 3.47-3.65 (m, 26 H, dendron + CH_2-C$\underline{H}_2$-O), 3.05-3.16 (m, 32 H, C$\underline{H}_2$-NH), 2.42 (t, *J* = 6.8 Hz, 32 H, NH-CH_2-CH_2-C$\underline{H}_2$), 2.24 (br s, 24 H, N-C$\underline{H}_3$), 1.62-1.72 (m, 32 H, NH-CH_2-C$\underline{H}_2$), 1.43 (br s, 74 H, Boc CH_3 + C$\underline{H}_2$-CH_2-O), 1.29 (br s, 30 H, alkyl C$\underline{H}_2$), 0.90 ppm (t, *J* = 7.1 Hz, 3 H, alkyl C$\underline{H}_3$); ^{13}C NMR (176 MHz, methanol-d_4) δ 158.4, 125.1, 79.9, 79.4, 72.6, 71.7, 71.2, 64.7, 56.2, 42.3, 42.2, 40.0, 39.6, 30.6,

30.4, 29.0, 27.9, 27.2, 23.5, 14.3 ppm. HRMS: *m/z* Calcd for $C_{146}H_{284}N_{27}O_{39}$ $[M+H]^+$: 3040.1064. Found: 3041.1092.

General procedure for the Boc-deprotection of compounds 17 and 18:

TFA (6.0 mL, excess) was slowly added to a solution of compounds **17** or **18** (0.012 mmol) in DCM (6.0 mL) and stirred overnight at room temperature. The solvent was removed *in vacuo* and the residue washed alternately with hexane and diethyl ether. Purification was accomplished *via* SEC (Sephadex^{TM} LH20, MeOH) to remove any trace amounts of impurities. Freeze drying yielded the compounds **A3** and **A4** as white foams.

Compound A3

Obtained as a white foam (24 mg, quant.). ^{1}H NMR (700 MHz, methanol-d_4) δ 8.03 (t, J = 10.0 Hz, 1 H, trz), 5.16–4.91 (m, 5 H, dendron), 4.58 (m_c, 2 H, O-C$\underline{H}_2$-trz), 4.41–3.40 (m, 16 H, 10 × dendron, 2 × CH_2-C$\underline{H}_2$-O, 4 × C$\underline{H}_2$-NH), 3.24–3.02 (m, 28 H, 12 × C$\underline{H}_2$-NH, 16 × C$\underline{H}_2$-N-CH_3), 2.82 (m_c, 12 H, N-C$\underline{H}_3$), 2.22–1.82 (m, 16 H, NH-CH_2-C$\underline{H}_2$), 1.58 (m_c, 2 H, C$\underline{H}_2$-CH_2-O), 1.28 (br s, 30 H, alkyl C$\underline{H}_2$), 0.89 ppm (t, J = 7.1 Hz, 3 H, alkyl C$\underline{H}_3$); ^{13}C NMR (176 MHz, methanol-d_4) δ 163.1 (q, J = 34.5 Hz, $CF_3\underline{C}OOH$), 158.7, 158.3, 145.8, 124.9, 118.2 (q, J = 293.1 Hz, $\underline{C}F_3COOH$), 72.5, 72.4, 71.9, 71.2, 70.9, 64.6, 64.4, 62.3, 55.4, 54.5, 40.5, 38.8, 38.1, 33.0, 30.8, 30.7, 30.6, 30.4, 27.2, 26.0, 23.7, 14.4 ppm. HRMS: *m/z* Calcd for $C_{62}H_{128}N_{15}O_{11}$ $[M+H]^+$: 1258.9912. Found: 1259.0009.

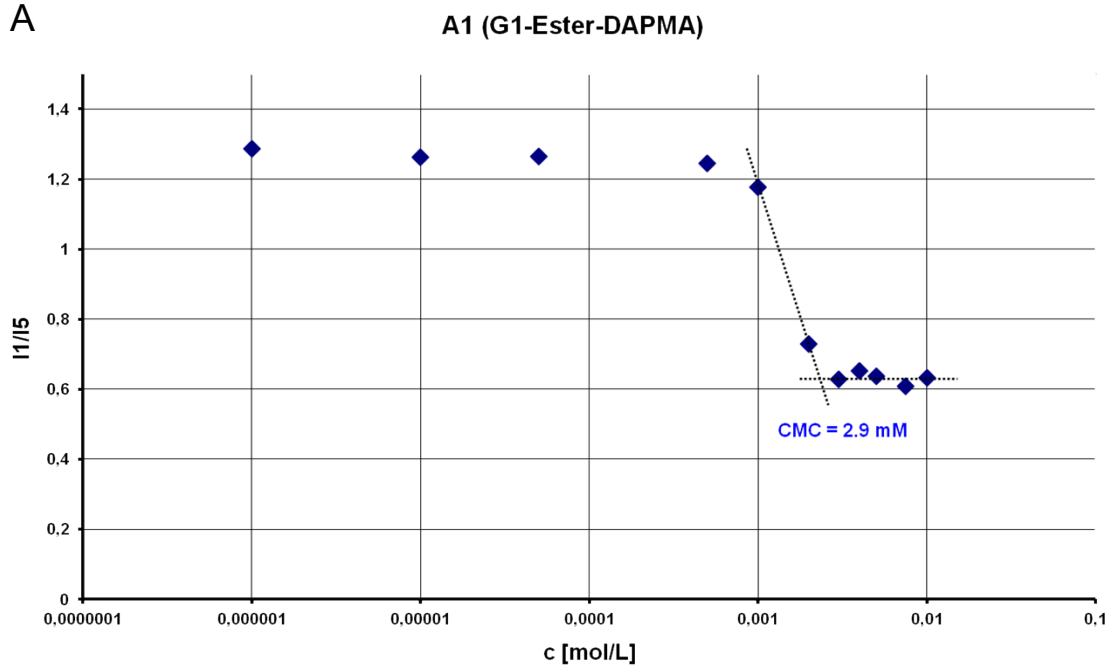
Compound A4

Obtained as a white foam (45 mg, quant.). ^{1}H NMR (700 MHz, methanol-d_4) δ 8.11 (t, J = 10.0 Hz, 1 H, trz), 4.98 (m_c, 5 H, dendron), 4.62 (br s, 2 H, O-C$\underline{H}_2$-trz), 4.32 (m_c, 4 H, dendron), 4.06

(m_c, 6 H,dendron), 3.43-3.67 (m, 26 H, dendron + CH_2-C<u>H</u>$_2$-O), 3.23-3.30 (m, 48 H, C<u>H</u>$_2$-N-CH_3 + NH_2-C<u>H</u>$_2$), 3.08 (m_c, 16 H, NH-C<u>H</u>$_2$), 2.92 (br s, 24 H, N-C<u>H</u>$_3$), 2.16 (m_c, 16 H, NH_2-CH_2-C<u>H</u>$_2$), 1.97 (m_c, 16 H, NH-CH_2-C<u>H</u>$_2$), 1.62 (m_c, 2 H, C<u>H</u>$_2$-CH_2-O), 1.31 (br s, 30 H, alkyl C<u>H</u>$_2$), 0.92 ppm (t, J = 7.1 Hz, 3 H, alkyl C<u>H</u>$_3$); ^{13}C NMR (176 MHz, methanol-d_4) δ 163.1, 158.7, 158.4, 119.1, 117.4, 79.9, 72.7, 71.1, 64.7, 55.4, 54.2, 40.4, 38.8, 37.8, 33.1, 30.8, 30.5, 27.3, 25.8, 23.7, 23.5, 14.4 ppm. HRMS: *m*/*z* Calcd for $C_{106}H_{220}N_{27}O_{23}$ $[M+H]^+$: 2239.6870. Found: 2239.6881.

Determined CMC values of A1, A2, A3, and A4 by fluorescence method

A



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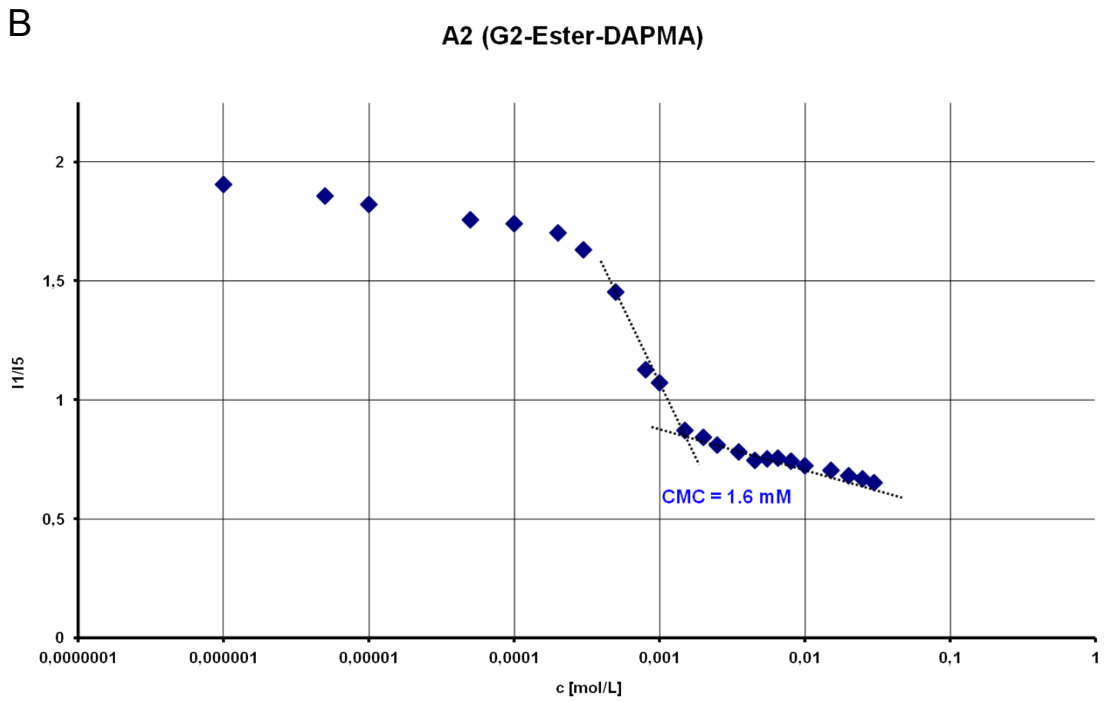

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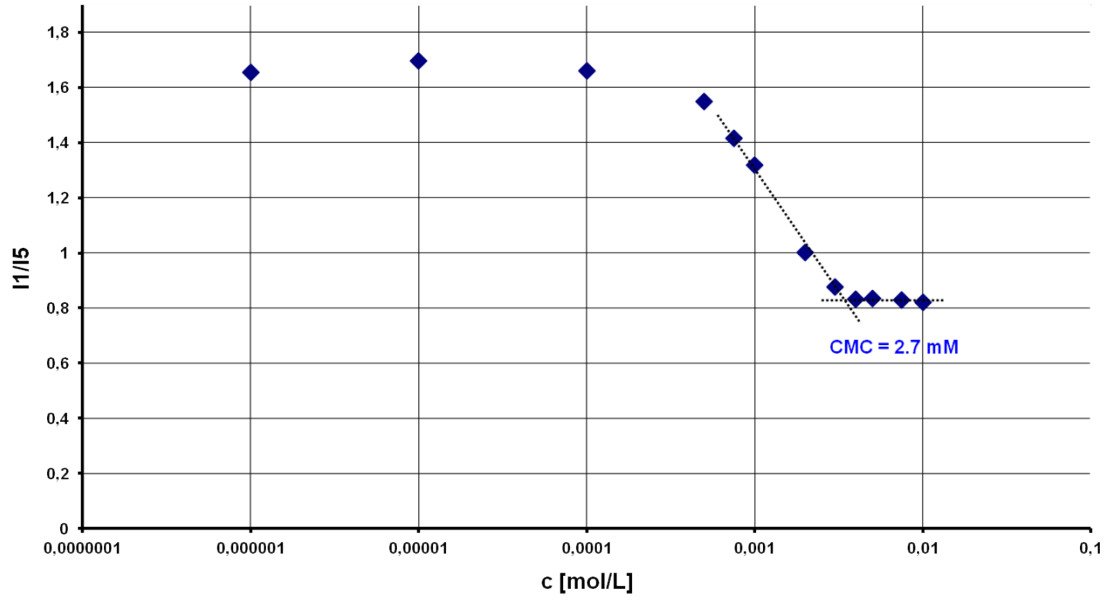

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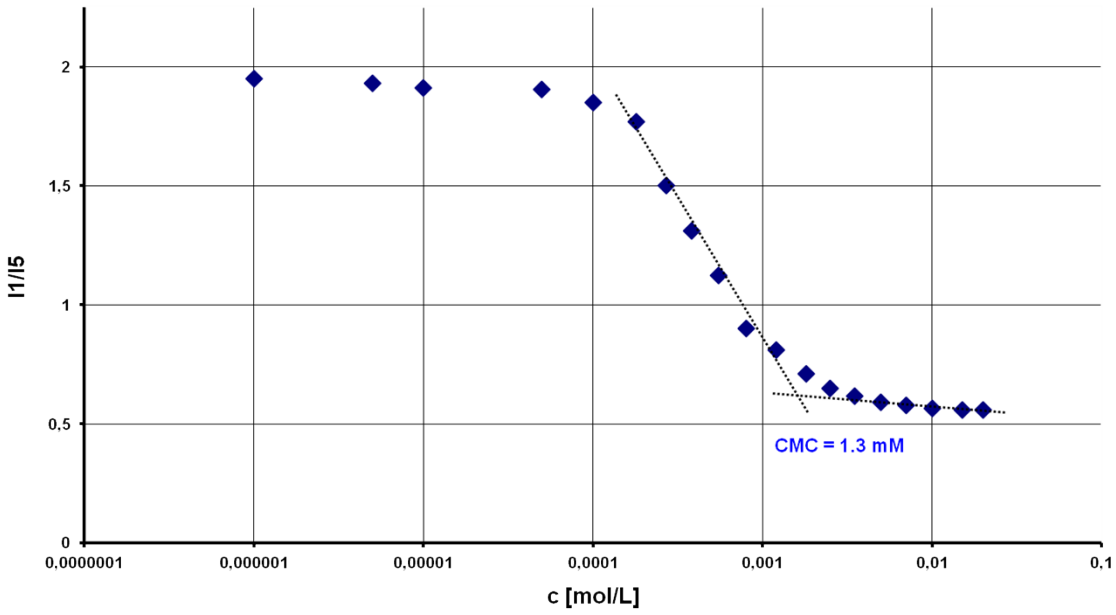

Figure S1. CMC values of **A1** (A), **A2** (B), **A3** (C), and **A4** (D) determined by means of fluorescence spectroscopy in 0.5 µM pyrene aqueous HEPES saline buffer (pH 7.2, 9.4 mM NaCl).

Hydrodynamic diameters of A2 and A4 determined by DLS

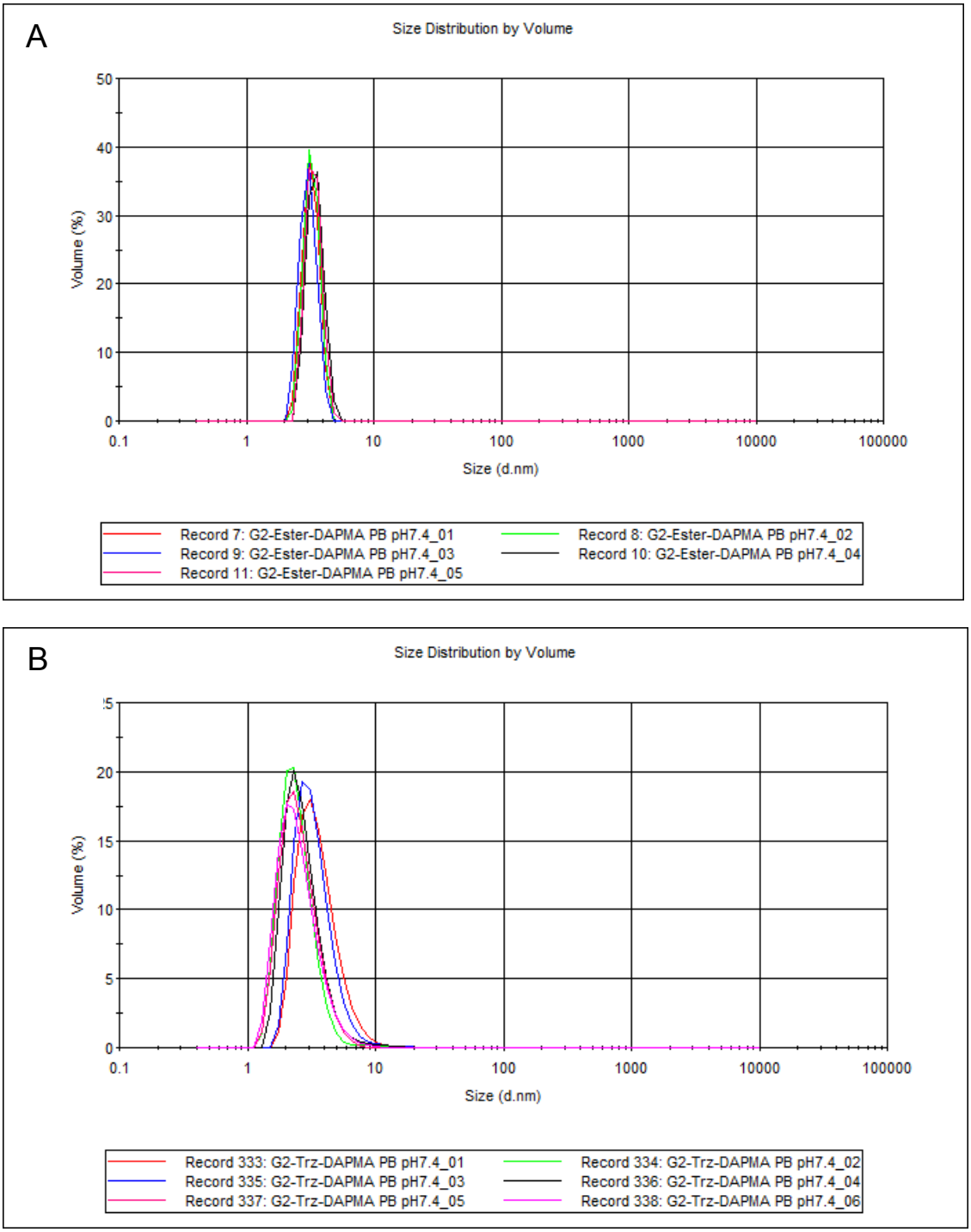

Figure S2. Hydrodynamic diameters of **A2** (G2-Ester-DAPMA) (A) and **A4** (G2-Trz-DAPMA) (B) determined by DLS in aqueous phosphate buffer (pH 7.4, 10 mM). Size distribution by volume.

Results and discussion of conducted EthBr assay

The EthBr assay makes use of the fact that ethidium bromide intercalates with DNA and can be replaced by stronger binding agents, which results in the reduction of its fluorescence intensity (FI). For comparative analysis the data can be presented in terms of CE_{50} values which represent the necessary "charge excess" required to cause 50% EthBr displacement. The C_{50} values report the corresponding concentration of gene carrier required to achieve the same 50% reduction in fluorescence. Thus, lower CE_{50} and C_{50} values characterize a more effective binding event, since a smaller amount of positive charge is required to effectively bind the negative charge associated with the DNA. In this study, double-stranded 21-mer DNA oligonucleotides were employed, which serve as a representative model for siRNA in order to evaluate the general gene binding affinity of the amphiphiles.

Table S1. DNA binding data obtained *via* ethidium bromide displacement assay.[a]

Sample	**Nominal charge**	**CE_{50} value**[b]	**C_{50} value**[c] [μM]
G1-Ester-DAPMA (**A1**)	8	0.33	0.039
G2-Ester-DAPMA (**A2**)	16	0.31	0.019
G1-Trz-DAPMA (**A3**)	8	0.29	0.018
G2-Trz-DAPMA (**A4**)	16	0.28	0.018
DAPMA-Boc (**10**)	2	(23)[d]	(2.718)[d]

[a] Sample solutions in HEPES saline buffer (pH 7.4, 9.4 mM NaCl). [b] CE_{50} represents the charge excess (N/P ratio) required to decrease EthBr fluorescence by 50%. [c] C_{50} represents the concentration of amphiphile needed to displace 50% of EthBr. [d] Interpolated value.

The obtained data (Table S1) demonstrate that all four amphiphiles efficiently bind to DNA exhibiting CE_{50} values between 0.28 - 0.33. Indeed, only minor differences between amphiphiles **A1**-**A4** are noticeable. A small dissimilarity can be detected between the different dendron generations of one amphiphilic pair so that the G1 derivative of the ester-linked amphiphiles (**A1**, **A2**) as well as the G1 version of the triazole amphiphiles (**A3**, **A4**) possesses marginally higher CE_{50} values than their G2 analogs. Overall, amphiphile G2-Trz-DAPMA (**A4**) displaying a CE_{50} value of 0.28 is just the most efficient binder. This finding is in accordance with theoretical expectations, as dendritic structures exposing a multivalent array typically amplify the strength of a weak binding process in comparison to their lower generation counterparts - known as the multivalency effect.[2] As anticipated, this result is further supported by the control measurement of the Boc-protected monovalent amine group DAPMA-Boc (**10**), which only gives an interpolated, very high CE_{50} value of ~ 23. Indeed, the univalent amine moiety is not capable of displacing more than 45% of the ethidium bromide from the DNA even at an N/P ratio of 20 (data not shown), thereby demonstrating that the strategy of organizing amine units into a well-defined multivalent array has a significant impact.

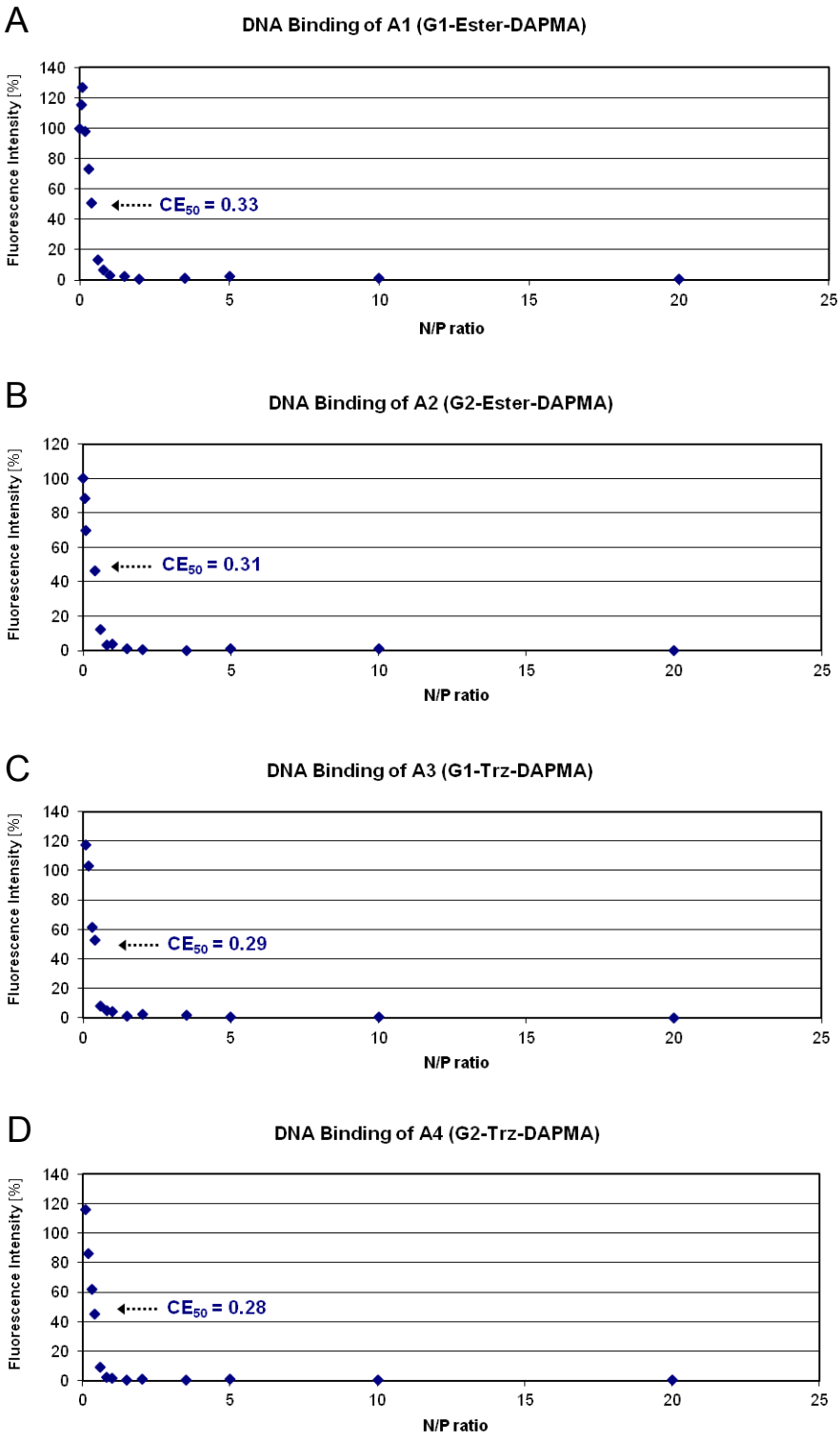

Figure S3. DNA binding affinities (CE_{50} values) of amphiphiles **A1** (A), **A2** (B), **A3** (C), and **A4** (D) determined by means of ethidium bromide displacement assay.

In vitro transfection and cell viability studies with A3 and A4

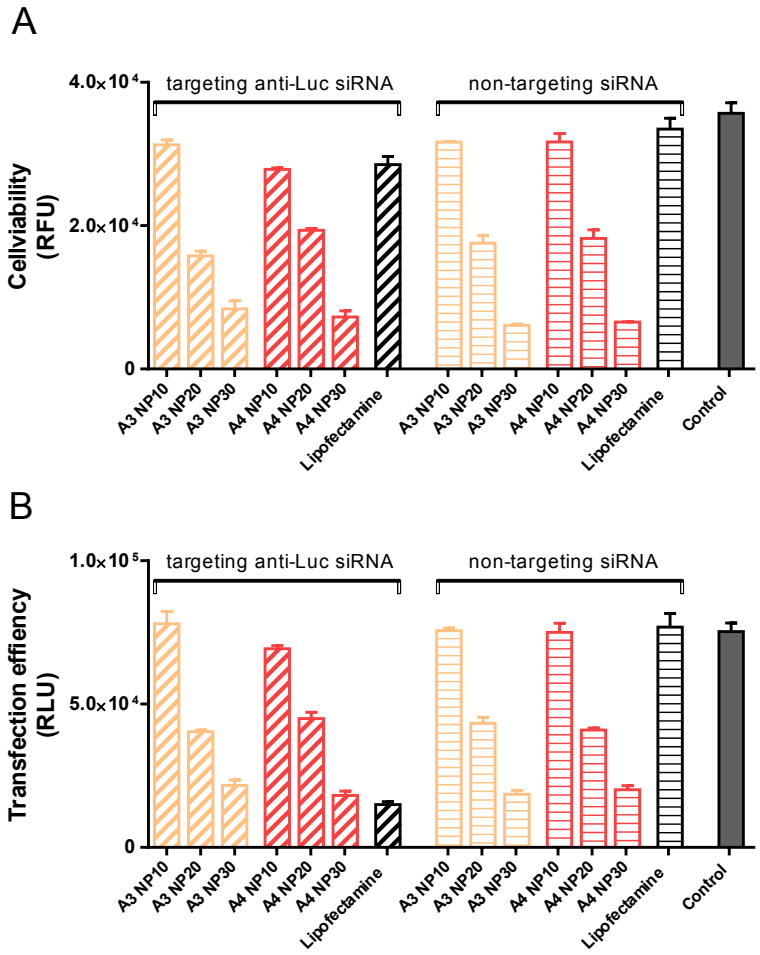

Figure S4. 786-O Luc transgenic cells were transfected with luciferase specific and non-targeting siRNA (ON-TARGETplus Non-targeting siRNA, Dharmacon) complexed with nanocarriers **A3** and **A4** at N/P ratios of 10, 20 and 30 for 48 h. Lipofectamine was used as positive control and untreated cells as negative control. Cell viability (A) and transfection efficacy (B) was measured by using the ONE-Glo + Tox Luciferase Reporter and Cell Viability Assay (Promega). Results are shown as mean±SD of triplicates.

1. S. Malhotra, H. Bauer, A. Tschiche, A. M. Staedtler, A. Mohr, M. Calderón, V. S. Parmar, L. Hoeke, S. Sharbati, R. Einspanier and R. Haag, *Biomacromolecules*, 2012, **13**, 3087-3098.
2. A. Barnard and D. K. Smith, *Angewandte Chemie International Edition*, 2012, **51**, 6572-6581.