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Amphiphilic Oligoethyleneimine–β-Cyclodextrin "Click" Clusters for Enhanced DNA Delivery

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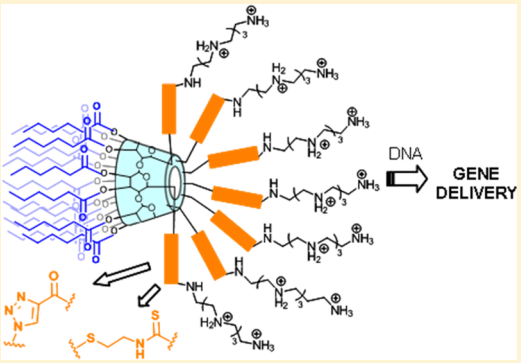
Supporting Information

ABSTRACT: Monodisperse amphiphilic oligoethyleneimine (OEI)–β-cyclodextrin (βCD) clusters have been prepared, and their potential as gene delivery systems has been evaluated in comparison with a nonamphiphilic congener. The general prototype incorporates tetraethyleneimine segments linked to the primary rim of βCD through either triazolyl or thioureidocysteaminyl connectors. Transfection efficiency data for the corresponding CD:pDNA nanocomplexes (CDplexes) in BNL-CL2 murine hepatocytes evidenced the strong beneficial effect of facial amphiphilicity.



The clinical success of gene therapy critically depends on the conception of efficient and safe delivery vehicles capable of reversibly complexing the relevant nucleic acid and enabling it to reach its target.[1] Although substantial advances toward safe viral vector-mediated gene therapy have been made, genotoxicity remains a serious concern nowadays.[2] The design of artificial (nonviral) carriers for gene delivery has emerged as a promising alternative[3] that has been boosted in the last decades by the advent of nanotechnology.[4] However, the delivery efficiency and selectivity of the current prototypes, despite few exceptions,[5] are far from that of their viral counterparts, and they are not fully devoid of cytotoxicity. Cyclodextrins (CDs), a family of macrocyclic oligosaccharides composed of six (αCD), seven (βCD) or eight (γCD) α-(1 → 4)-linked D-glucopyranoside units, have been long known as transfection enhancers and exploited to improve the gene delivery capabilities of first generation lipidic or polymeric nonviral vectors.[6−8] New functional materials for gene delivery have been designed by exploiting the inclusion capabilities of CDs to build up supramolecular architectures[9−13] as well as by inserting CD motifs in the backbone or as the central core in either linear[14,15] or star-like polycationic polymers.[16]

In the last years, monodisperse polycationic CDs (pCDs)[17−20] and polycationic amphiphilic CDs (paCDs),[21−26] obtained after regioselective single or dual face functionalization of the basket-shaped cyclooligosaccharide, respectively, have emerged as a new family of nonviral gene vectors. Both pCDs and paCDs self-assemble in the presence of plasmid DNA (pDNA) to form nanocomplexes (CDplexes) that promote in vitro transfection of several cell lines. The incorporation of multicharged oligoethyleneimine (OEI; tetraethyleneimine or higher)-triazolyl branches at the polycationic cluster was shown to be very convenient in pCD constructs (e.g., **1**),[20] whereas the combination of thioureidocysteaminyl and amine groups at the cationic domain and hexanoyl tails at the lipophilic region was optimal for paCD architectures (e.g., **2**).[26] Copper(I)-catalyzed azide–alkyne cycloaddition (CuAAC) and amine-isothiocyanate coupling were implemented for "click" multiconjugation, both ligation chemistries warranting full homogeneity.[27] In our ongoing efforts to develop CD-based artificial viruses for gene therapy applications,[28−30] contrasting the conclusions inferred from research on pCD versus paCD vectors in the same cell system was highly sought. Toward this end, we have now designed the new derivatives **3** and **4** as the first representatives of oligoethyleneimine-paCDs (OEI–paCDs) and conducted a parallel evaluation of their ability to condense pDNA into discrete cationic nanoparticles and on their in vitro transfection capabilities toward the BNL-CL2 murine hepatocyte cell line (Figure 1).

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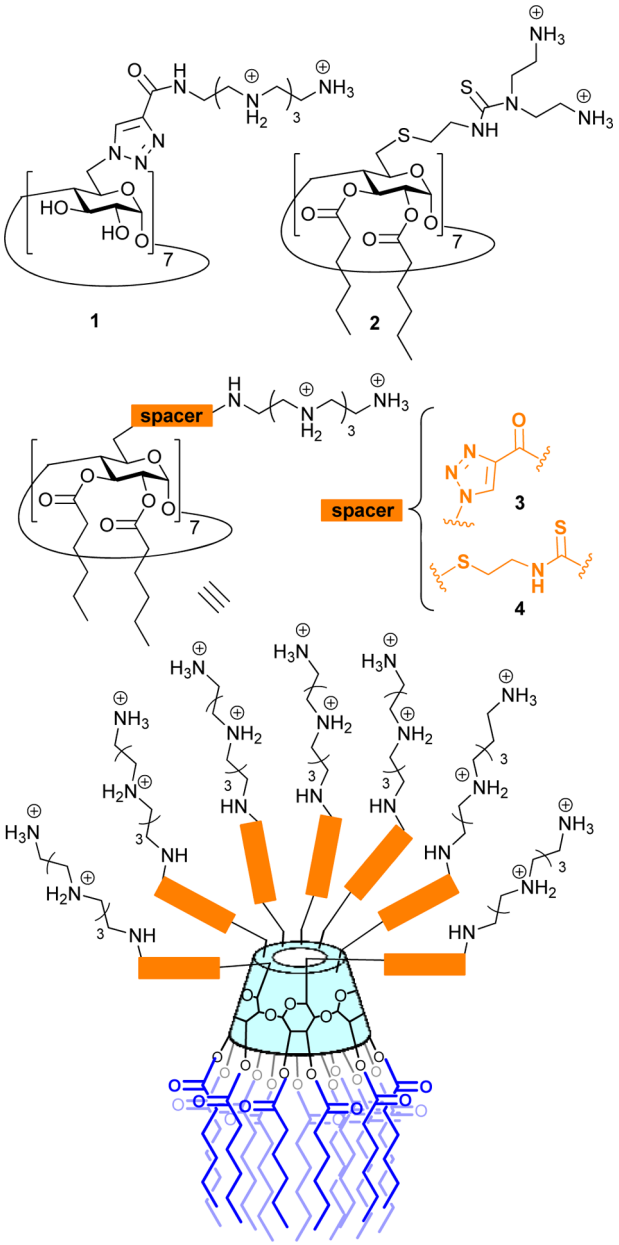
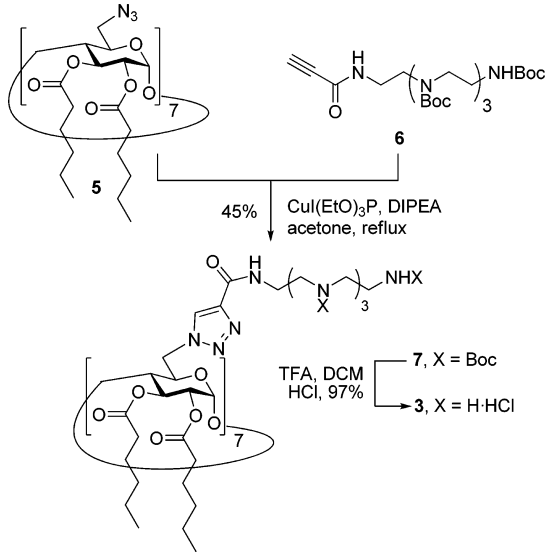


Figure 1. Structures of the oligoethyleneimine (OEI)−βCD "click" clusters **1**,[20] **2**,[26] **3**, and **4**. A schematic representation evidencing the facial amphiphilic character of the latter is presented.

Both **3** and **4** incorporate tetraethyleneimine segments attached to the primary positions of the CD core through either triazole or thioureidocysteaminyl bridges, which should give us precise information about the influence of the linking group in DNA complexation and transfection. By including the nonamphiphilic OEI−pCD "click" cluster **1**[20] in our study, we intend to specifically assess the effect of facial amphiphilicity,[31] a characteristic of paCDs and other molecular vector families, such as polycationic calixarenes,[32] alleged to improve self-assembling and biological membrane-crossing abilities. As positive controls we have used the dendritic paCD derivative **2**, already shown to exhibit good hepatocyte transfection properties in vitro[26] as well as in vivo in mice,[33,34] and the commercial linear polyethyleneimine vector Jet-PEI, considered the gold standard in nonviral gene delivery.[35,36]

The synthesis of **3** was accomplished in just two steps from the known per-(C-6)-azido per-(*O*-2,*O*-3)-hexanoyl-βCD precursor **5**[37] by 7-fold CuAAC with the "clickable" tetra-Boc-protected-tetraethyleneimine-*N*⁵-propynamide derivative **6**.[20,38] The reaction was conducted under homogeneous conditions in acetone using copper(I) iodide:triethylphosphite as the catalyst, affording the heptakis(1,2,3-triazol) adduct **7** in 45% yield. Subsequent trifluoroacetic acid-promoted hydrolysis of the 28 carbamate groups provided, after exchange of the counterions by chloride, the target "bouquet"-type OEI−paCD **3** in almost quantitative yield (Scheme 1).

Scheme 1. Synthesis of the Amphiphilic OEI−βCD Triazolyl Cluster 3[a]



[a]The final compound was found to contain 21 protonated amine centers in average as inferred from microanalytical data.

The preparation of the heptakis(thioureidocysteaminyl) congener **4** involved multinucleophilic amine-isothiocyanate addition[39,40] of the tetra-Boc-protected tetraethyleneamine **9**[20,41] to the hepta-(*C*-6)-isothiocyanatoethylthio tetradeca-(*O*-2,*O*-3)-hexanoyl βCD **8**[26] in DMF (→ **10**, 74%), followed by Boc-cleavage (Scheme 2). The homogeneity and purity of all compounds was confirmed by ^{1}H and ^{13}C NMR, mass spectrometry and combustion analysis. Most importantly, the spectroscopic data were consistent with the expected C_7 symmetry for homogeneously substituted βCD-centered clusters.

The capability of the OEI−paCDs **3** and **4** and the nonamphiphilic control **1** to compact and protect pDNA (luciferase encoding plasmid of 5739 base pairs) and the size and surface potential of the resulting nanocomplexes were examined at variable nitrogen/phosphate (N/P) ratios (N/P from 0 to 10). Agarose gel electrophoresis retardation experiments (Figure 2) demonstrated that **3** and **4** were both able to fully complex pDNA at N/P ≥ 5, as indicated by the absence of free mobile plasmid in the corresponding lanes. In contrast, in the CDplexes formulated with the nonamphiphilic OEI−pCD **1**, the pDNA cargo remained accessible to ethidium bromide (EB) intercalation even at N/P 10. The results for **3** and **4** are very similar to those obtained for CDplexes formulated with the reference paCD **2** and clearly indicate that endowing the system with facial amphiphilic character

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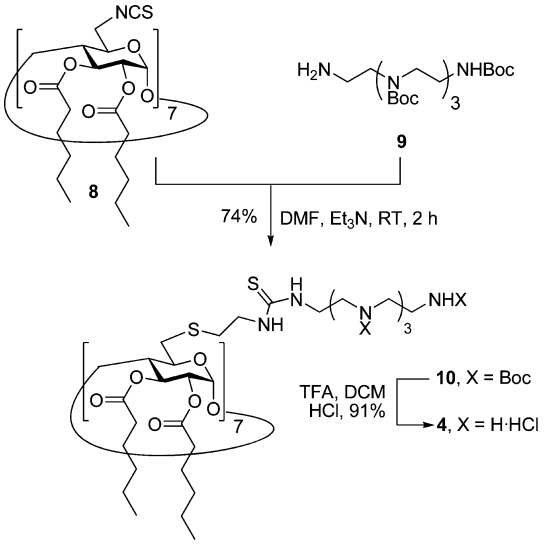
Scheme 2. Synthesis of the Amphiphilic OEI–βCD Thioureidocysteaminyl Cluster 4



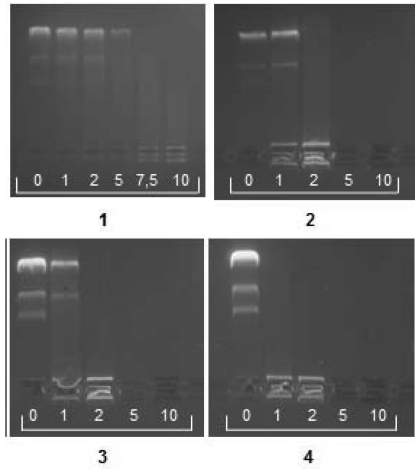


Figure 2. Agarose gel electrophoresis retardation experiments for pDNA complexes (20 μL, 0.4 μg of plasmid; see refs 26 and 27 for details) formulated with pCD **1**, the reference paCD **2**, and the new paCDs **3** and **4** at N/P ratios between 0 (naked pDNA) and 10. Ethidium bromide was used as staining reagent.

dramatically improves self-assembling of the polycationic CD clusters onto the DNA matrix.

The pDNA-CD nanoparticle hydrodynamic diameters and ζ-potentials were determined for CDplexes formulated with **1**, **3** and **4** at N/P 5 and 10 (Figure 3). The **3**:pDNA and **4**:pDNA complexes consisted in cationic nanoparticles (ζ-potential from +40 to +55 mV) of quasi-monodisperse size distribution with average hydrodynamic diameters in the 40–60 nm range, which is much smaller than the sizes measured for the JetPEI polyplexes (150–200 nm). The nonamphiphilic CD derivative **1** led to much larger and polydisperse aggregates (up to 250 nm at N/P 10). Moreover, these CDplexes presented ζ-potential values from negative (−47 mV) at N/P 5 to close to neutrality at N/P 10. The data are consistent with the gel retardation experiments and highlight the potential of adjusting the hydrophilic/hydrophobic balance in the CD vector to tailor the properties of the resulting vector:pDNA self-assembled nanoparticles.

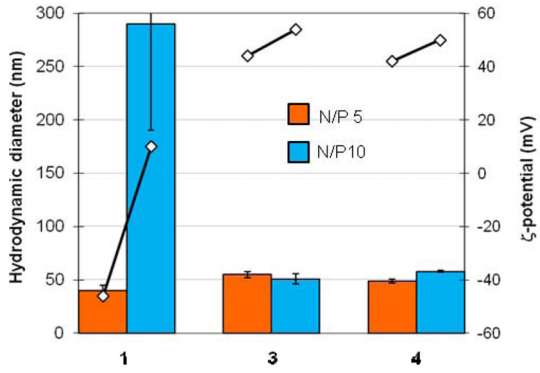


Figure 3. Hydrodynamic diameter (left axis; bars) and ζ-potential (right axis: ◇ and lines) of the CDplexes obtained from **1**, **3** and **4** at N/P ratios 5 and 10.

The transfection efficiency and cell viability of the CDplexes formulated with the pCD **1** and the paCDs **3** and **4** at N/P 5 and 10 were evaluated in vitro in 10% serum-containing medium using a luciferase-encoding reporter gene (pTG11236, pCMV-SV40-luciferase-SV40pA; 5739 base pairs) on adherent BNL-CL2 cells. JetPEI (22 kDa) N/P 10 polyplexes and naked pDNA were used as positive and negative controls, respectively. For comparative purposes, we have also included in our study CDplexes formulated with paCD **2**, which is one of the most efficient CD vectors reported up to date (Figure 4).

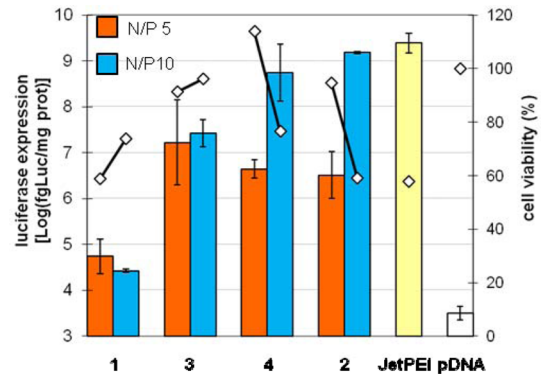


Figure 4. In vitro gene transfection efficiency (bars) and cell viability (◇ and lines) in BNL-CL.2 cells mediated with pDNA CDplexes formulated with pCD **1** and the new paCDs **3** and **4** at N/P 5 or 10, in comparison with CDplexes obtained from the reference paCD **2**, naked pDNA and JetPEI polyplexes (N/P 10) in the presence of serum (10%).

CDplexes formulated from the nonamphiphilic polycationic CD **1** exhibited very poor transfection efficiency and significant cell toxicity (50–60% cell viability), which is most probably related to its reduced pDNA condensing and protecting abilities and the low stability of the corresponding CDplexes in serum-containing medium. Compound **1** was previously found to efficiently promote transfection in serum-free medium in human cervix cancer HeLa cells,[20] which is in apparent contradiction with the present results. It must be emphasized, however, that the presence of serum can drastically modify the properties of the nanocomplexes by virtue of aggregation with serum proteins and, possibly, proteolytic degradation.[42] Another important point is that different cell lines can internalize the CDplexes through different mechanisms and, thus, different efficiencies.[43] In particular, HeLa cells express

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high proportions of heparansulphate proteoglycans at its surface,[43,44] which probably facilitate cell uptake of cationic **1**:pDNA CDplexes formulated at high N/P ratios in the absence of serum.

Incorporation of the hydrophobic hexanoyl chains at the secondary rim onto the polycationic triazole-linked CD "click" cluster resulted in a substantial increase in luciferase expression. Indeed, paCD **3** was found to mediate gene transfer and expression in BNL-CL2 cells with much higher efficiencies (more than 2 orders of magnitude) as compared with the nonamphihilic pCD homologue **1**. Replacing the triazol linking group into a thioureidoethylthio segment, i.e., going from **3** to **4**, led to a further improvement at N/P 10, the optimal formulation ratio for paCD-based CDplexes. It is worth noting that such improvement is not associated with differences in size or ζ-potential, the N/P 5 and 10 formulations, whether from paCD **3** or **4**, displaying indeed similar sizes and ζ-potentials (Figure 3). Actually, luciferase expression data for **4**:pDNA CDplexes compared well with data for CDplexes formulated with the control paCD **2** and with JetPEI-based polyplexes, but with a more favorable toxicity profile (75% cell viability for **4** as compared to 60% for **2** or JetPEI).

The ensemble of results indicate that the thiourea-linked amphiphilic OEI–βCD cluster **4** is a promising new gene vector prototype and warrants further research in vivo. It must be highlighted that given the presence of 28 protonable amino groups in the structure of **4**, as compared to 14 groups in **2**, half molar proportion of **4** is required to achieve an identical N/P ratio upon pDNA nanocomplexation, which might account for the observed lower toxicity of CDplexes formulated with this paCD. The fact that the thiourea groups in **4** are *N*,*N*′-disubstituted whereas in **2** they are *N*,*N*′,*N*′-trisubstituted may also have an effect. The favorable influence of thiourea groups in transfection has been previously ascribed to the participation of thioureas in reversible complexation of the phosphate groups in the oligonucleotide backbone through hydrogen bonding interactions.[26,27] Indeed, lipopolythioureas have been reported to form transfectious aggregates with pDNA.[45] In any case, our results firmly support the concept that dual-face CD functionalization strategies, in combination with efficient "click" multiconjugation chemistries, provide powerful tools for the design and optimization of molecular, self-assembling gene delivery systems.

EXPERIMENTAL SECTION

I. General Methods. Reagents and solvents were purchased from commercial sources and used without further purification, with the following exception: dichloromethane was distilled under an Ar stream over CaH_2. Optical rotations were measured at 20 °C in 1-cm or 1-dm tubes. IR spectra were recorded using an FTIR spectrometer. ^{1}H (and ^{13}C NMR) spectra were recorded at 500 (125.7), 400 (100.6) and 300 (75.5) MHz. 2D COSY, 1D TOCSY, and HMQC experiments were used to assist with the NMR spectroscopy assignments. NMR spectra and a guide to the notation used for the assignments can be found in the Supporting Information. Thin-layer chromatography (TLC) was carried out on aluminum sheets coated with Kieselgel 60 F245, with visualization by UV light and by charring with 10% H_2SO_4 or 0.1% ninhydrin in EtOH. Column chromatography was carried out on silica gel 60 (E. Merck, 230–400 mesh). Electrospray mass spectra (ESIMS) were obtained for samples dissolved in MeCN, MeOH, or H_2O–MeOH mixtures at low μM concentrations. For the final paCDs **3** and **4**, the absence of TFA in the samples was confirmed by ^{19}F NMR (recorded at 282.4 MHz), the absence of line broadening in the ^{1}H NMR spectra, the absence of the characteristic carbon resonances in the ^{13}C NMR spectra and by microanalytical data.

II. Synthesis of Oligoethyleneimine-Cyclodextrin Conjugates. *Heptakis[6-deoxy-6-(4-(triethylenetetraaminoethylcarbamoyl)-1H-1,2,3-triazol-1-yl)]cyclomaltoheptaose (**1**).* Compound **1** was prepared following the previously reported procedure.[13]

*Heptakis[6-deoxy-2,3-di-O-hexanoyl-6-(4-(triethylenetetra(tert-butoxycarbonylamino)ethylcarbamoyl)-1H-1,2,3-triazol-1-yl)]-cyclomaltoheptaose (**7**).* To a solution of heptakis(6-azido-6-deoxy-2,3-di-*O*-hexanoyl)cyclomaltoheptaose[33] (**5**; 100 mg, 0.037 mmol) and N^1,N^2,N^3,N^4-[triethylenetetra((*tert*-butoxycarbonyl)amino)]-ethylene-N^5-propynamide[20] (**6**; 204 mg, 0.318 mmol) in acetone (7 mL), $CuI(EtO)_3P$ (18 mg, 0.052 mmol) and DIPEA (0.044 mL, 0.26 mmol) were added, and the reaction mixture was stirred and refluxed for 24 h. Then 100 mg of silica-N_3 were added to remove the excess of **6**, and the mixture was stirred and refluxed for 9 h. The solution was filtered and concentrated. The residue was purified by column chromatography (20:1 → 15:1 DCM–MeOH → 1:2 acetone–cyclohexane) to give **7** as an amorphous solid. Yield: 120 mg (45%); R_f = 0.37 (9:1 DCM–MeOH); $[\alpha]_D$ = +17.0 (*c* 1.0 in DCM); UV (DCM) 214 nm (ε_{mM} 82.1); IR (NaCl) 3354, 2966, 2932, 2104, 1754, 1696, 1572, 1366, 1246, 1166, 1042 cm^{-1}; ^{1}H NMR (500 MHz, CD_3OD, 333 K) δ 8.41 (bs, 7 H, = CH), 5.54 (m, 14 H, H-1, H-3), 4.88 (bs, 7H, H-6a), 4.74 (bs, 14 H, H-2, H-6b), 4.57 (bs, 7 H, H-5), 3.64 (bs, 14 H, CH_2), 3.63 (bs, 7 H, H-4), 3.54–3.28 (m, 84 H, CH_2), 3.21 (t, 14 H, $^3J_{H,H}$ = 6.0 Hz, CH_2NHBoc), 2.34 (bs, 14 H, CH_aCO), 2.21 (m, 14 H, CH_bCO), 1.57 (m, 28 H, CH_2CH$_2$CO), 1.44 (s, 63 H, CMe_3),1.30 (m, 56 H, CH_2CH$_3$, CH_2CH$_2$CH$_3$), 0.89 (m, 42 H, CH_3); ^{13}C NMR (125.7 MHz, CD_3OD, 333 K) δ 172.8, 171.9 (CO ester), 160.9 (CO amide), 156.8, 155.9, 155.7 (CO carbamate), 142.9 (C-4 triazole), 128.1 (C-5 triazole), 96.8 (C-1), 80.0 (CMe_3), 78.8 (C-4), 69.9 (C-2, C-3, C-5), 50.3 (C-6), 45.3 (CH_2), 38.8 (CH_2NHBoc), 38.1, 37.1 (CH_2), 33.8, 33.5 (CH_2CO), 31.2, 31.0 (CH_2CH$_2$CH$_3$), 27.5 (CMe_3), 24.1 (CH$_2$CH_2CO), 22.0 (CH_2CH$_3$), 13.0, 12.9 (CH_3); ESIMS *m*/*z* = 3610.4 $[M + 2Na]^{2+}$ (Calcd. *m*/*z* = 3609.11). Anal. Calcd. for $C_{343}H_{588}N_{56}O_{105}$: C, 57.40; H, 8.26; N, 10.93. Found: C, 57.19; H, 8.09; N, 10.61.

*Heptakis[6-deoxy-2,3-di-O-hexanoyl-6-(4-(triethylenetetraamino)ethylcarbamoyl)-1H-1,2,3-triazol-1-yl)]-cyclomaltoheptaose Unicosahydrochloride (**3**).* Compound **7** (48 mg, 0,0067) was treated with TFA–DCM (1:1, 2 mL) at rt for 2 h, followed by evaporation of the solvents and freeze-drying from a solution of 0.1 N HCl to give **7** as a white foam. Yield: 35 mg, 97%; $[\alpha]_D$ = +161.4 (*c* 1.05 in MeOH); UV (MeOH) 218 nm (ε_{mM} 66.4); IR (KBr) 3443, 2928, 2107, 1747, 1679, 1383, 1180, 1044 cm^{-1}; ^{1}H NMR (500 MHz, 5:1 CD_3OD-D_2O, 323 K) δ 8.55 (bs, 7 H, = CH), 5.55 (t, 7 H, $J_{2,3}$ = $J_{3,4}$ = 9.5 Hz, H-3), 5.47 (d, 7 H, $J_{1,2}$ = 3.0 Hz, H-1), 4.90 (bs, 7H, H-6a), 4.74 (dd, 7 H, H-2), 4.70 (bs, 7H, H-6b), 4.60 (bs, 7 H, H-5), 3.80 (m, 14 H, CH_2), 3.66 (t, 7 H, $J_{4,5}$ = 9.5 Hz, H-4), 3.55–3.42 (m, 98 H, CH_2), 2.44 (m, 14 H, CH_aCO), 2.31, 2.22 (m, 14 H, CH_bCO), 1.62 (m, 28 H, CH_2CH$_2$CO), 1.33 (m, 56 H, CH_2CH$_3$, CH_2CH$_2$CH$_3$), 0.90 (m, 42 H, CH_3); ^{13}C NMR (125.7 MHz, 5:1 CD_3OD-D_2O, 323 K) δ 173.8, 172.3 (CO ester), 162.1 (CO amide), 141.9 (C-4 triazole), 128.9 (C-5 triazole), 96.8 (C-1), 77.4 (C-4), 70.3 (C-2), 69.8 (C-5), 69.5 (C-3), 50.7 (C-6), 45.0, 44.8, 44.7, 43.8, 36.3, 35.8 (CH_2), 33.7 (CH_2CO), 31.2, 31.0 (CH_2CH$_2$CH$_3$), 24.2, 24.1 (CH$_2$CH$_2$CO), 22.1, 22.0 (CH_2CH$_3$), 13.0 (CH_3); ESIMS *m*/*z* = 1458.7 $[M + 3H]^{3+}$ (Calcd. *m*/*z* = 1458.57), 1094.1 $[M + 4H]^{4+}$. Anal. Calcd. for $C_{203}H_{385}N_{56}O_{49}Cl_{21}$: C, 47.44; H, 7.55; N, 15.26. Found: C, 47.65; H, 7.39; N, 14.88.

*Heptakis[2,3-di-O-hexanoyl-6-(2-(N′-(triethylenetetra(tert-butoxycarbonylamino)ethylthioureido)ethyl)thio)]-cyclomaltoheptaose (**10**).* To a solution of heptakis[6-(2-isothiocyanatoethylthio)-2,3-di-*O*-hexanoyl]-cyclomaltoheptaose[26] (**8**; 100 mg, 0.031 mmol) and N^1,N^2,N^3,N^4-tetra(*tert*-butoxycarbonyl)-tetraethylenepentamine[20,41] (**9**; 138 mg, 0.235 mmol, 1.08 equiv) in DMF (5 mL), Et_3N (0.030 mL, 0.217 mmol) was added, and the reaction mixture was stirred at rt for 2 h. The solvent was removed, and the residue was purified by column chromatography (25:1 → 20:1 DCM–MeOH) to give **10** as an amorphous solid. Yield: 168 mg (74%); R_f = 0.45 (9:1 DCM–MeOH); $[\alpha]_D$ = +40.7 (*c* 0.97 in DCM); UV (DCM) 249 nm (ε_{mM} 109.5); IR (NaCl) 3342, 2971, 2931, 1752, 1697, 1366, 1247, 1164 cm^{-1}; ^{1}H NMR (500 MHz, CD_3OD, 333 K) δ

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5.35 (t, 7 H, $J_{2,3} = J_{3,4}$ = 8.0 Hz, H-3), 5.18 (d, 7 H, $J_{1,2}$ = 3.5 Hz, H-1), 4.85 (dd, 7 H, H-2), 4.20 (m, 7 H, H-5), 3.95 (t, 7 H, $J_{3,4}$ = 8.0 Hz, H-4), 3.77, 3.68 (2 bt, 28 H, CH_2NHCS), 3.46−3.32 (m, 70 H, CH_2), 3.28 (bd, 7 H, $J_{6a,6b}$ = 12.0 Hz, H-6a), 3.22 (t, 14 H, $^3J_{H,H}$ = 6.0 Hz, CH_2NHBoc), 3.19 (dd, 7 H, $J_{5,6b}$ = 5.2 Hz, H-6b), 2.94 (m, 14H, CH_2S), 2.46 (m, 14 H, CH_aCO), 2.31 (m, 14 H, CH_bCO), 1.66 (m, 28 H, CH_2CH_2CO), 1.51, 1.50, 1.46 (s, 63 H, CMe_3), 1.41 (m, 28 H, $CH_2CH_2CH_3$), 1.37−1.31 (m, 28 H, CH_2CH_3), 0.95 (m, 42 H, CH_3); ^{13}C NMR (100.6 MHz, CD_3OD, 323 K) δ 183.0 (CS), 173.2, 172.0 (CO ester), 156.8, 155.9, 155.7 (CO carbamate), 96.8 (C-1), 80.0 (CMe_3), 78.6 (C-4), 71.9 (C-5), 70.6 (C-3), 70.3 (C-2), 45.2 (CH_2), 43.8, 42.4 (CH_2NHCS), 38.6 (CH_2NHBoc), 33.9 (C-6), 33.8, 33.7 (CH_2CO), 32.8 (CH_2S), 31.2, 31.1 ($CH_2CH_2CH_3$), 27.7, 27.6, 27.5 (CMe_3), 24.2 (CH_2CH_2CO), 22.1 (CH_2CH_3), 13.1, 12.9 (CH_3); ESIMS m/z = 3694.3 $[M + 2Na]^{2+}$ (Calcd. m/z = 3694.5). Anal. Calcd. For $C_{343}H_{616}N_{42}O_{98}S_{14}$: C, 56.08; H, 8.45; N, 8.09; S, 6.11. Found: C, 56.17; H, 8.35; N, 7.84, S, 6.02.

Heptakis[2,3-di-O-hexanoyl-6-(2-(N′-(triethylenetetraamino)-ethylthioureido)-ethyl)thio)]cyclomaltoheptaose Octaicosahydrochloride (4). Compound **10** (138 mg, 0.019 mmol) was treated with TFA−DCM (1:1, 4 mL) at rt for 2 h, followed by evaporation of the solvents and freeze-drying from a solution of 0.1 N HCl to give **4** as a white foam. Yield: 104 mg, 91%; R_f = 0.10 (5:3:5 CH_3CN-H_2O-NH_4OH); $[\alpha]_D$ = +34.3 (c 1.05 in MeOH). UV (MeOH) 244 nm (ε_{mM} 26.42); ^{1}H NMR (500 MHz, 5:1 CD_3OD−D_2O, 333 K) δ 5.32 (t, 7 H, $J_{2,3} = J_{3,4}$ = 8.5 Hz, H-3), 5.17 (d, 7H, $J_{1,2}$ = 3.5 Hz, H-1), 4.85 (dd, 7 H, H-2), 4.16 (bs, 7 H, H-5), 3.95 (t, 14 H, $^3J_{H,H}$ = 6 Hz, CH_2NHCS), 3.92 (t, 7 H, $J_{3,4}$ = 8.5 Hz, H-4), 3.74 (bs, 14 H, CH_2NHCS), 3.49−3.32 (m, 84 H, CH_2), 3.25 (bd, 7 H, $J_{6a,6b}$ = 13.0 Hz, H-6a), 3.15 (dd, 7 H, $J_{5,6b}$ = 5.5 Hz, H-6b), 2.92 (m, 14H, CH_2S), 2.45 (m, 14 H, CH_aCO), 2.31 (m, 14 H, CH_bCO), 1.62 (m, 28 H, CH_2CH_2CO), 1.41 (m, 28 H, $CH_2CH_2CH_3$), 1.36 (m, 28 H, CH_2CH_3), 0.93 (m, 42 H, CH_3); ^{13}C NMR (125.7 MHz, 5:1 CD_3OD-D_2O, 333 K) δ 183.0 (CS), 173.9, 172.3 (CO ester), 96.9 (C-1), 78.8 (C-4), 71.9 (C-5), 70.7 (C-3), 70.3 (C-2), 45.7, 45.3, 44.8 (CH_2), 44.2 (CH_2NHCS), 43.9 (CH_2), 40.2 (CH_2NHCS), 34.0(C-6), 33.8, 33.7 (CH_2CO), 32.6 (CH_2S), 31.1, 31.0($CH_2CH_2CH_3$), 24.2, 24.1 (CH_2CH_2CO), 22.0 (CH_2CH_3), 13.2, 13.0 (CH_3); ESIMS m/z = 1515.1 $[M + 3H]^{3+}$ (Calcd. m/z = 1514.87). Anal. Calcd. for $C_{203}H_{420}N_{42}O_{42}S_{14}Cl_{28}\cdot 10\ H_2O$: C, 42.45; H, 7.72; N, 10.24; S, 7.82. Found: C, 42.28; H, 7.60; N, 10.33; S, 7.75.

III. Preparation of DNA:CD Complexes (CDplexes). The preparation of the DNA complexes from the CD derivatives **1**−**4** and JetPEI has been performed according to a procedure that has been detailed elsewhere.[26,27] The plasmid pTG11236 (pCMV-SV40-luciferase-SV40pA) used for the preparation of the DNA complexes and for the transfection assay is a plasmid of 5739 bp (base pairs). The quantities of compounds used were calculated according to the desired DNA concentration of 0.02 or 0.07 mg mL^{-1} (i.e., 60 or 200 μM phosphate, respectively), the N/P ratio (1, 2, 5, 7.5 or 10), the molar weight, and the number of protonable nitrogens in the selected CD derivative or cationic polymer (JetPEI).

IV. Characterization of CDplexes. The average sizes and ζ-potentials of the CDplexes were measured according to procedures that have been detailed elsewhere.[26,27] Sizes were determined by DLS and analyzed using the multimodal number distribution software included in the instrument. ζ-Potentials were determined using the "mixed-mode measurement" phase analysis light scattering (M3-PALS). Before each series of experiments, the performance of the instruments was checked with either 90 nm monodisperse latex beads (Coulter) for DLS or with DTS 50 standard solution (Malvern) for ζ-potentials. The electrophoretic mobility of the DNA complexes was analyzed following the procedure previously described.[26,27]

V. In Vitro Transfection. Twenty-four hours before transfection, BNL-CL.2 cells were grown at a density of 2×10^4 cells per well in 96-well plates in Dulbelcco modified Eagle culture medium (DMEM) containing 10% fetal calf serum (FCS), glucose (4.5 g/L), glutamine (2 mM), penicillin (100 units/mL), and 10 mg mL^{-1} of gentamycin in a wet (37 °C) and 5% CO_2/95% air atmosphere. The above-described CD:pDNA (= pTG11236) complexes and JetPEI:pDNA polyplexes were diluted to 100 μL in DMEM supplemented with 10% FCS so as to have 0.5 μg of pDNA in the well (15 μM phosphate). The culture medium was removed and replaced by these 100 μL of the complexes. After 4 and 24 h, DMEM (50 and 100 μL) supplemented 10% FCS, respectively, were added. After 48 h, the transfection was stopped, the culture medium was discarded, and the cells were washed twice with PBS (100 μL) and lysed with lysis buffer (50 μL). The lysates were frozen at −32 °C before the analysis of luciferase activity. This measurement was performed using a luminometer in dynamic mode for 10 s on the lysis mixture (20 mL) and using the "luciferase" determination system in 96-well plates. The total protein concentration per well was determined by the BCA test. Luciferase activity was calculated as femtograms (fg) of luciferase per mg of protein. The percentage of cell viability was calculated as the ratio of the total protein amount per well of the transfected cells relative to that measured for untreated cells × 100. The data were calculated from three or four repetitions in two fully independent experiments (formulation and transfection).

VI. Statistical Analysis. Statistical tests were performed with STATGRAPHICS Plus 5.0 software. Analysis of variance (ANOVA) was run on the logarithmic transformation of transfection levels (log 10(fg of luciferase per mg of protein)) and on the cell viability to fit normal distributions of the data. Two factors, that is, the nature of the complexing agent (CD derivative and PEI) and the N/P ratio, were analyzed as the source of the variation of logarithmic transformation of the transfection levels and of cell variability percentages using a multiple comparison procedure. Tukey's honestly significant difference (HSD) method was used to discriminate among the means of cell viability percentages and the logarithmic transformation of luciferase expression levels.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Copies of the ^{1}H and ^{13}C NMR spectra for all new compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

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Notes

The authors declare no competing financial interest.

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