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Polycationic Pillar[5]arene Derivatives: Interaction with DNA and Biological Applications

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Dedicated to Professor Dirk M. Guldi on the occasion of his 50th birthday

Abstract: Dendritic pillar[5]arene derivatives have been efficiently prepared by grafting dendrons with peripheral Boc-protected amine subunits onto a preconstructed pillar[5]arene scaffold. Upon cleavage of the Boc-protected groups, water-soluble pillar[5]arene derivatives with 20 (**13**) and 40 (**14**) peripheral ammonium groups have been obtained. The capability of these compounds to form stable nanoparticles with plasmid DNA has been demonstrated by gel electrophoresis, transmission electron microscopy (TEM), and dynamic light scattering (DLS) investigations. Transfection efficiencies of the self-assembled **13**/pCMV-Luc and **14**/pCMV-Luc polyplexes have been evaluated in vitro with HeLa cells. The transfection efficiencies found for both compounds are good, and pillar[5]arenes **13** and **14** show very low toxicity if any.

Keywords: click chemistry · dendrimers · gene technology · pillar[5]arenes · polycations

Introduction

Whereas cyclotriveratrylenes (CTV) and calix[*n*]arenes have been known for decades, their paracyclophane analogues, namely pillar[*n*]arenes, have been only recently discovered.[1,2] These macrocyclic compounds are composed of 1,4-disubstituted hydroquinone subunits linked by methylene bridges in their 2,5-positions.[2] In contrast to CTV and calix[*n*]arenes, which generally adopt cone-shaped conformations, pillar[*n*]arenes are tubular-shaped compounds with a D_n symmetry. Moreover, both rims of the pillar[*n*]arene macrocycle are equivalent and functionalized with *n* alkoxy substituents. Pillar[*n*]arenes are therefore appealing compact cores for the preparation of unique nanomaterials with a controlled distribution of functional subunits.[3–5] Examples include water-soluble polycationic or polyanionic systems for host-guest chemistry,[3] multichromophoric assemblies with peculiar photophysical properties,[4] and liquid-crystalline materials.[5] Pillar[*n*]arenes have also started to be used as building blocks for the preparation of new molecules for bio-oriented applications. For example, artificial transmembrane channels obtained from pillar[5]arene monomeric and dimeric derivatives have been reported by Hou and co-workers.[6] Single-channel conductance measurements and isotope effect experiments under acidic conditions showed selective proton transport through the channels, which were mediated by water wires formed in the pillar[5]arene backbones. The same group has also reported hydrazide-appended pillar[5]arene derivatives.[7] These tubular derivatives have been inserted into the lipid membranes of vesicles, leading to the transport of water through the channels produced by single molecules.[7] Huang and co-workers have shown that the formation of supramolecular complexes between percarboxylated pillar[6]arenes and paraquat leads to a reduction of the toxicity of paraquat.[8] Indeed, the formation of stable host-guest complexes reduces the interaction of paraquat with reducing agents in the cell and the generation of its radical cation is thus more difficult, resulting in efficient reduction of paraquat toxicity. Pillar[5]arene has also been used as a multivalent core unit to prepare glycoconjugates, and a mannosylated pillar[5]arene derivative has been assayed as an inhibitor of the adhesion of an uropathogenic *Esherichia coli* strain to red blood cells.[9] Following this first report on glycopillar[5]arenes, another approach was reported by Huang and co-workers.[10] They prepared an amphiphilic pillar[5]arene containing galactose subunits as the hydrophilic part and alkyl chains as the hydrophobic part.[10] This compound self assembles in water to produce nanotubular structures that have been utilized as cell glues to agglutinate *Esherichia coli*.

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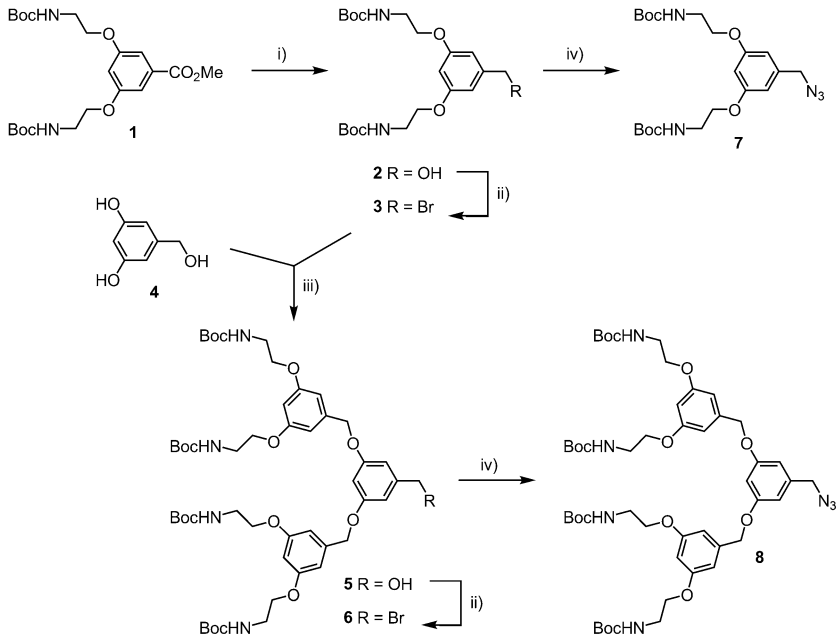
As part of this research on biologically active pillar[*n*]arene derivatives, we now report the preparation of polycationic dendritic pillar[5]arene derivatives and demonstrate their capacity to interact with DNA. Owing to their efficient ability to compact DNA, these compounds can be used in gene transfer experiments,[11,12] thus opening new research avenues in the field of biological applications with pillar[*n*]arene derivatives. The design of new vectors combining high efficiency and low toxicity remains a major challenge, and synthetic chemistry is still at the center of this research.

Results and Discussion

Synthesis: The synthesis of polycationic pillar[5]arene derivatives relies upon the copper-catalyzed alkyne–azide cycloaddition (CuAAC) reaction[13] to introduce dendrons with peripheral benzyloxycarbonyl (Boc)-protected amine subunits on both rims of the macrocyclic core. This methodology has proven to be a powerful procedure for the functionalization of pillar[5]arene derivatives due to its versatility and the ready availability of starting materials.[4b,5,14] The preparation of the Boc-protected amine terminated poly(aryl ether) dendritic branches is depicted in Scheme 1. They have been obtained by a convergent synthesis by using the classical methodology developed by Hawker and Fréchet.[15] Compound **1** was prepared as previously described.[16] Lithium aluminum hydride (LAH) mediated reduction of **1** gave benzylic alcohol **2** in 99% yield. Subsequent bromination with *N*-bromosuccinimide (NBS) and triphenylphosphine (PPh_3) gave benzylic bromide **3** in 82% yield. Reaction of **3** with 3,5-dihydroxybenzyl alcohol (**4**) in the presence of K_2CO_3 in acetone at reflux afforded the next generation alcohol **5**, which was converted into the corresponding benzylic bromide **6** by treatment with NBS/PPh_3. Finally, azide derivatives **7** and **8**, which are required for conjugation on the pillar[5]arene core, were obtained by reaction of the corresponding benzylic bromides with sodium azide (NaN_3) in *N*,*N*-dimethylformamide (DMF) at room temperature.

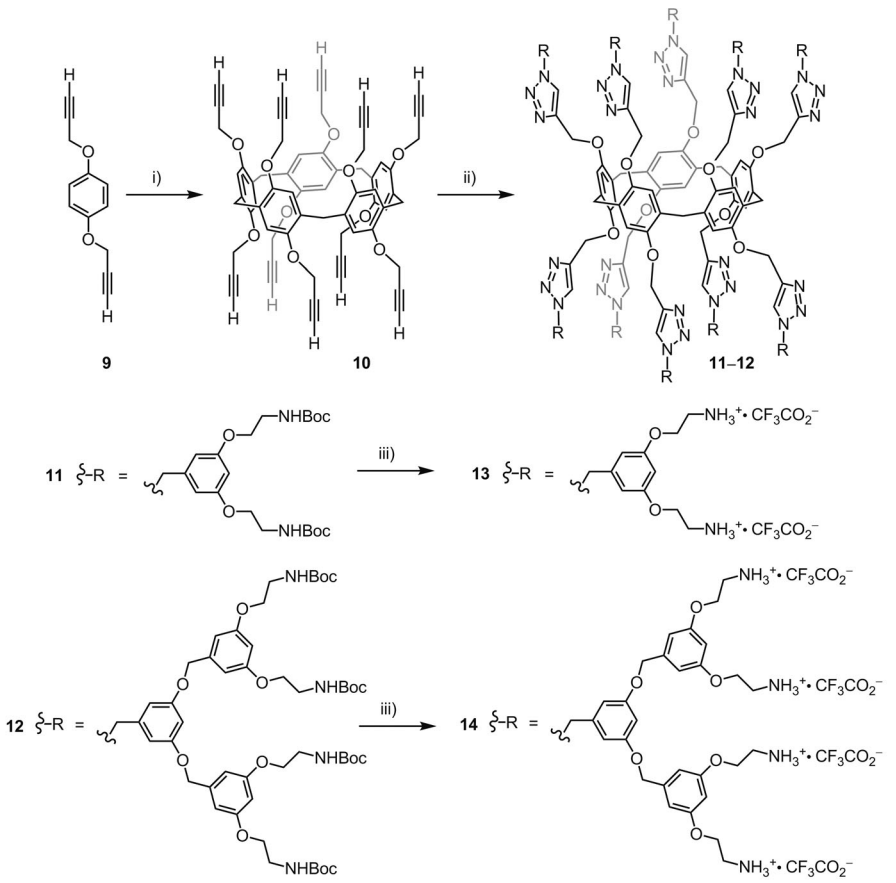
As shown in Scheme 2, the complementary pillar[5]arene building block **10** was prepared from compound **9**[17] under the classical conditions developed by Ogoshi and co-workers.[18] Treatment of **9** and paraformaldehyde with $BF_3{\cdot}Et_2O$ in 1,2-dichloroethane afforded cyclopentamer **10** in 40% yield.[19] The terminal alkyne function is therefore compatible with these conditions. It should, however, be noted that only traces of pillar[5]arene **10** were obtained when the reaction was performed with $AlCl_3$ or $FeCl_3$ as the Lewis acid catalyst. Under these conditions, mainly polymers were obtained. Grafting of dendrons **7** and **8** onto pillar[5]arene **10** was achieved under the CuAAC conditions we have previously developed for the functionalization of multi-alkyne cores.[20] A mixture of **10** (1 equiv), **7** (11 equiv), $CuSO_4{\cdot}5H_2O$ (0.1 equiv), and sodium ascorbate (0.3 equiv) in CH_2Cl_2/H_2O was stirred for two days. After work-up and purification, compound **11** was thus obtained in 88% yield. Similarly, reaction of azide **8** with **10** gave dendronized pillar[5]arene **12** in 81% yield.

The chemical structures of compounds **11** and **12** were confirmed by NMR and IR spectroscopic analysis as well as by mass spectrometry and elemental analysis (see the Supporting Information). Their ^1H and ^{13}C NMR spectra, showing one set of signals for all the ten equivalent peripheral subunits, were fully consistent with their D_5-symetrical structures. The expected molecular ion peaks were also observed in the MALDI-TOF mass spectra of both **11** and **12**, despite a high level of fragmentation resulting from the cleavage of terminal Boc-protecting groups as well as from the cleavage of ether O–CH_2–triazole bonds. The monodispersity of both **11** and **12** was further supported by their chromatograms recorded with a HPLC instrument equipped with a PLgel size-exclusion column (Figure 1). The difference in retention time was in perfect agreement with the increasing size of the compounds when going from **11** to **12**.



Scheme 1. Reagents and conditions: i) LAH, THF (99%); ii) NBS, PPh_3, THF (**3**: 82%, **6**: 73%); iii) K_2CO_3, acetone (97%); iv) NaN_3, DMF (**7**: 93%, **8**: 90%).

Treatment of **11** and **12** with a large excess of trifluoroacetic acid (TFA) gave the corresponding deprotected derivatives as their trifluoroacetate



Scheme 2. Reagents and conditions: i) $BF_3{\cdot}Et_2O$, $(CH_2O)_n$, $ClCH_2CH_2Cl$ (40%); ii) $CuSO_4{\cdot}5H_2O$, sodium ascorbate, CH_2Cl_2/H_2O (**11**: 88%, **12**: 81%); iii) TFA (**13**: quantitative, **14**: quantitative).

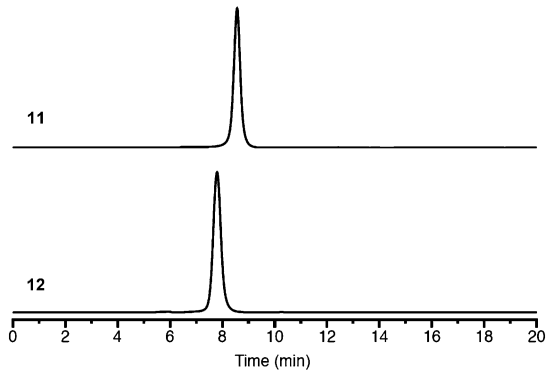


Figure 1. HPLC traces on a size exclusion column (PLgel, CH_2Cl_2, UV detector at $\lambda = 254$ nm) obtained for compounds **11** and **12**.

salts in quantitative yields. The ^1H and ^{13}C NMR spectra of **13** and **14** clearly show the disappearance of the Boc-protecting groups. The broadening observed in the ^1H NMR spectra recorded in D_2O is ascribed to aggregation (see the Supporting Information). This view was supported by dynamic light scattering (DLS) measurements. At a concentration higher than 3 nM, large aggregates were evidenced for both compounds. The distribution in size was rather large (from ca. 100 to 1000 nm in diameter), suggesting that the self-assembled nanostructures are not well-defined. Compounds **13** and **14** are indeed bolaamphiphilic compounds and aggregation results most probably from hydrophobic interactions between the central cores of the molecules.[21] At lower concentration (<1.5 nM), compound **14** no longer aggregates, whereas compound **13** still forms nanoparticles with an average diameter of ca. 70 nm. This difference in behavior between **13** and **14** results from differences in the size of their polar subunits. When the generation number of the peripheral dendrons is increased, intermolecular contacts become more difficult for steric reasons and less favorable due to increased electrostatic repulsion.

Interaction with DNA: The ability of compounds **13** and **14** to bind plasmid DNA (pCMV-Luc) was first assessed through the use of gel electrophoresis. The polyplexes[22] were prepared from pCMV-Luc and **13** or **14** with increasing N/P (vector amine per DNA phosphate) ratios in 5% aqueous glucose solutions. Ethidium bromide fluorescence revealed full DNA condensation at and above N/P = 2 for both pillar[5]arenes (Figure 2). Effectively, the condensed plasmids remain in the well as soon as N/P ≥ 2. Furthermore, starting from N/P = 3 and above, the polyplexes are not visible in the gel because the staining agent (ethidium bromide) can no longer bind the highly condensed DNA. Polyplexes were also prepared from pCMV-Luc and **13** or **14** at different N/P ratios in aqueous 150 mM NaCl solutions. Agarose gel electrophoresis (gel shift) of these polyplexes also revealed full DNA condensation from N/P = 2 for both **13** and **14** (see the Supporting Information).

Polyplexes obtained from pCMV-Luc and **13** or **14** in either isotonic 150 mM NaCl or iso-osmotic 5% glucose solutions in water were further characterized by transmission electron microscopy (TEM). As typical examples, TEM images observed for polyplexes prepared at N/P = 5 in 5% glucose aqueous solutions are shown in Figure 2. Discrete spherical nanostructures were observed in both cases and the size distribution was quite narrow. These results were also confirmed by DLS measurements made on these polyplexes (see the Supporting Information). Interestingly, the polyplexes obtained from both **13** and **14** were smaller in size irrespective of the N/P ratios when compared with the

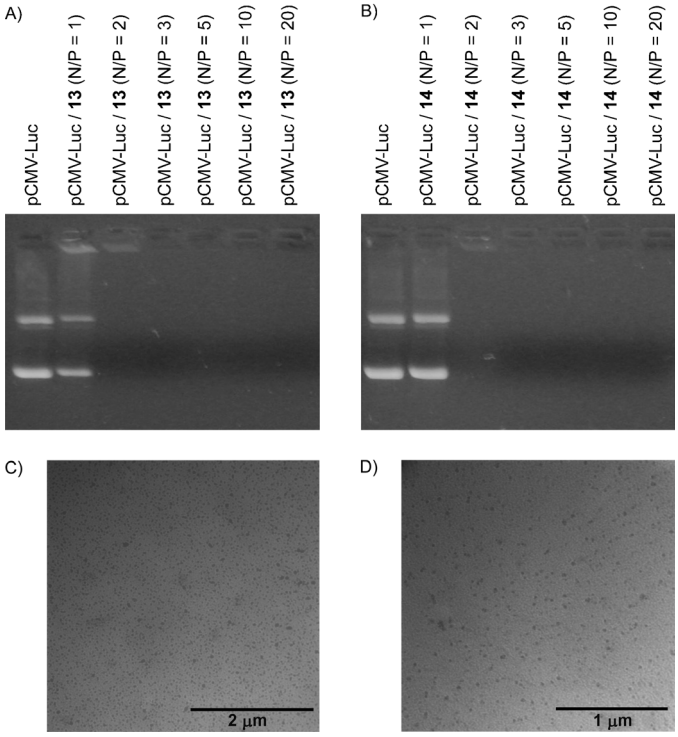


Figure 2. Top: Electrophoresis mobility shift assays performed with polyplexes prepared in 5% glucose solutions in water with plasmid DNA (pCMV-Luc) (0.4 μg) and A) **13** or B) **14** at increasing N/P (indicated above the gel); the control is plasmid pCMV-Luc without vector. Bottom: TEM images of C) **13**/pCMVLuc (scale bar: 2 μm) and D) **14**/pCMVLuc polyplexes (scale bar: 1 μm) at N/P 5 in water with 5% glucose.

aggregates observed for **13** and **14** alone under the same conditions. It appears that the electrostatic interactions of the negatively charged pCMV-Luc plasmid with the aggregates of **13** or **14** is capable of disrupting/remodeling, at least in part, the hydrophobic interactions of the bolaamphiphilic pillar[5]arene derivatives, thus showing that they must be rather weak. However, the addition of electrostatic and van der Waals interactions leads to strong complexation of plasmid DNA. Nevertheless, the bolaamphiphilic character of **13** and **14** plays an important role in the transfection experiments (see below). Zêta (ζ) potential measurements showed positively charged polyplexes from N/P = 2 and above, in agreement with the transfection experiments.

The polyplexes prepared from both **13** and **14** in 150 mM aqueous NaCl are larger in size than those obtained in 5% glucose aqueous solutions. At N/P ratios higher than 5, the polyplexes are clumped and highly irregular in shape, which are actually made of aggregated spheres (see the Supporting Information). A similar behavior has been also reported for polyplexes prepared from DNA and polyethylenimine (PEI),[23] and results from ionic strengths effects (i.e., a decrease in the electrostatic repulsions between particles). The results of DLS measurements on the particles formulated in NaCl were consistent with the TEM observations for both **13** and **14**. It should be noted that the ζ potentials could not be determined under the saline conditions.

Transfection experiments: The transfection efficiency of the self-assembled **13**/pCMV-Luc and **14**/pCMV-Luc polyplexes prepared either in 5% glucose or 150 mM NaCl aqueous solutions was evaluated in vitro on HeLa cells. A commercially available gene delivery system (Jet-PEI, Polyplus-Transfection, Illkirch France) and naked pCMV-Luc were used as positive and negative controls, respectively, for comparison purposes. The transfection capabilities were higher for polyplexes prepared under iso-osmotic conditions than those formulated under isotonic conditions (see the Supporting Information). This is consistent with the TEM and DLS results; the polyplexes prepared in glucose have the appropriate size for an optimum cellular uptake and should be of interest for in vivo experiments.[23]

For polyplexes prepared under iso-osmotic conditions, **13** and **14** had practically the same level of luciferase expression at their optimal N/P ratio, that is, between 10^9 and 10^{10} RLU per mg of protein (Figure 3). Although being less

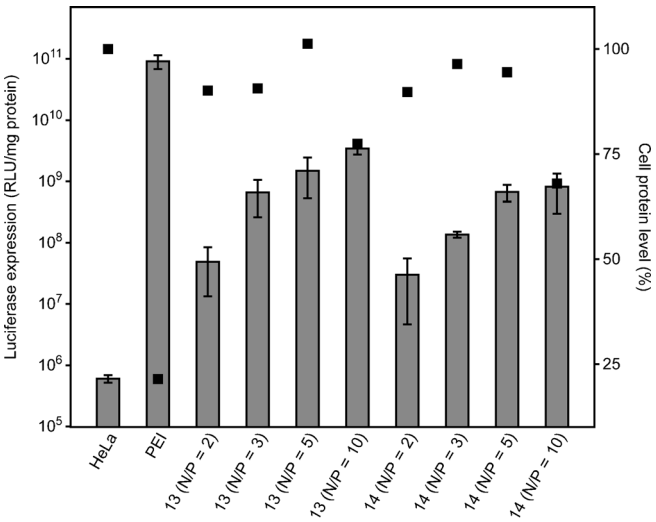


Figure 3. In vitro gene delivery experiments on HeLa cells of pCMVLuc with pillar[5]arenes **13** and **14** at various N/P (polyplexes prepared in 5% glucose solutions). Luciferase expression (bars) and percentage of total cellular proteins (squares) are given for negative control (untreated HeLa cells), positive control (PEI), **13**, and **14**. Means and standard deviation of separate triplicates are given.

efficient than the 'golden standard' Jet-PEI, both **13** and **14** are by far less toxic, as attested by much higher levels of total cellular proteins. Importantly, in contrast to classical dendritic vectors, for which high efficiency requires generally high generation numbers,[24] the first-generation dendrimer (**13**) already has optimum gene delivery capabilities. This is certainly associated with the bolaamphiphilic character of compound **13** and its ability to aggregate, thus increasing the stability of the polyplexes. Indeed, globular systems with an isotropic repartition of charges requires sufficient numbers of peripheral amino groups to ensure DNA compaction into stable and positively charged polyplexes.[25,26] For example, an analogous hexasubstituted fullerene deriva-

tive substituted with 24 peripheral ammonium groups has shown low gene transfer capabilities whereas the corresponding next generation compound with 48 peripheral ammonium groups is highly efficient.[26]

Conclusion

Dendritic pillar[5]arene derivatives **11** and **12** have been efficiently prepared by grafting dendrons with peripheral Boc-protected amine subunits onto a preconstructed pillar[5]arene scaffold. Upon cleavage of the Boc-protected groups, water-soluble pillar[5]arene derivatives **13** and **14** with 20 and 40 peripheral ammonium groups, respectively, have been obtained. The capacity of these compounds to form stable nanoparticles with plasmid DNA has been demonstrated by gel electrophoresis, TEM, and DLS investigations. Finally, the capability of both **13** and **14** to efficiently condense plasmid DNA into stable and positively charged polyplexes has been exploited in gene delivery experiments. The transfection efficiencies found for both **13** and **14** are good, albeit slightly lower than that obtained for PEI. What is more, these pillar[5]arene derivatives exhibit good efficiency, while maintaining low toxicity. Both **13** and **14** are by far less toxic than PEI. Owing to the possibility of preparing efficiently rotaxanes from pillar[5]arene derivatives,[14b,27] additional functional groups (e.g., specific sugars for cell targeting or fluorescent moieties for monitoring cell uptake) should be easily incorporated onto the molecular thread of rotaxane derivatives incorporating **13** or **14** as their macrocyclic component. Therefore, the results reported herein pave the way towards the development of a new generation of vectors capable of carrying out several tasks. Work in this direction is underway in our laboratories.

Experimental Section

General: All reagents were used as purchased from commercial sources without further purification. Compounds **1**[16] and **9**[17] were prepared according to previously reported procedures. Evaporation and concentration were performed at water aspirator pressure and drying in vacuo at 10^{-2} Torr. Column chromatography: silica gel 60 (230–400 mesh, 0.040–0.063 mm) was purchased from E. Merck. Thin-layer chromatography (TLC) was performed on glass sheets coated with silica gel 60 F_{254} purchased from E. Merck, visualization by UV light. NMR spectra were recorded with a Bruker AC 300 or AC 400, with solvent peaks as reference. IR spectra were recorded with a Spectrum Two PerkinElmer FTIR spectrometer. Elemental analyses were performed by the analytical service of the Faculty of Chemistry (University of Strasbourg). MALDI-TOF mass spectra were recorded by the analytical service of the School of Chemistry (Strasbourg, France).

Compound 2: LAH (1 M in THF, 8.2 mL, 8.23 mmol) was added dropwise to a solution of **1** (3.74 g, 8.23 mmol) in anhydrous THF (100 mL) at 0 °C under argon. After 3 h, MeOH (1 mL) was slowly added, followed by H_2O (several drops). The resulting mixture was filtered through Celite and evaporated. Column chromatography (SiO_2; CH_2Cl_2/EtOAc 1:1) gave **2** (3.48 g, 99 %) as a colorless solid. ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 6.53$ (d, $J = 2$ Hz, 2H), 6.37 (t, $J = 2$ Hz, 1H), 4.95 (br. s, 2H), 4.63 (s, 2H), 4.01 (t, $J = 5$ Hz, 4H), 3.52 (m, 4H), 1.45 ppm (s, 18H); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta = 160.0$, 156.0, 143.9, 105.5, 100.6, 79.7, 67.3, 65.0, 40.1, 28.5 ppm; IR (neat): $\tilde{\nu} = 3347$ (O–H), 3360 (N–H), 1689 cm^{-1} (C=O); elemental analysis calcd (%) for $C_{21}H_{34}N_2O_7$ (426.50): C 59.14, H 8.03, N 6.57; found: C 58.56, H 8.04, N 6.31.

Compound 3: A solution of **2** (3.52 g, 8.25 mmol), PPh_3 (2.81 g, 10.73 mmol), and *N*-bromosuccinimide (1.91 g, 10.73 mmol) in THF (50 mL) was stirred at RT. After 15 min, H_2O was added and the resulting aqueous layer was extracted with CH_2Cl_2 (3×). The combined organic layers were dried ($MgSO_4$), filtered, and evaporated. Column chromatography (SiO_2; cyclohexane/EtOAc 8:2) gave **3** (3.31 g, 82 %) as a colorless solid. ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 6.54$ (d, $J = 2$ Hz, 2H), 6.38 (t, $J = 2$ Hz, 1H), 4.94 (br. s, 2H), 4.39 (s, 2H), 4.00 (t, $J = 5$ Hz, 4H), 3.52 (m, 4H), 1.45 ppm(s, 18H); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta = 159.8$, 155.5, 139.9, 107.8, 101.4, 79.8, 67.3, 40.0, 33.3, 28.4 ppm; IR (neat): $\tilde{\nu} = 3366$ (N–H), 1677 cm^{-1} (C=O); elemental analysis calcd (%) for $C_{21}H_{33}BrN_2O_6$ (489.40): C 51.54, H 6.80, N 5.72; found: C 51.27, H 6.59, N 5.57.

Compound 5: A mixture of **3** (3.24 g, 6.62 mmol), **4** (0.40 g, 2.88 mmol), and K_2CO_3 (1.59 g, 11.54 mmol) in acetone (55 mL) was heated to reflux. After 2 days, the mixture was filtered and evaporated. Column chromatography (SiO_2; CH_2Cl_2/EtOAc 7:3) gave **5** (2.67 g, 97 %) as a colorless solid. ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 6.61$ (d, $J = 2$ Hz, 2H), 6.56 (d, $J = 2$ Hz, 4H), 6.50 (t, $J = 2$ Hz, 1H), 6.39 (t, $J = 2$ Hz, 2H), 5.00 (br. s, 4H), 4.97 (s, 4H), 4.64 (d, $J = 4$ Hz, 2H), 4.00 (t, $J = 5$ Hz, 8H), 3.51 (m, 8H), 1.45 ppm (s, 36H); IR (neat): $\tilde{\nu} = 3346$ (O–H/N–H), 1686 cm^{-1} (C=O); elemental analysis calcd (%) for $C_{49}H_{72}N_4O_{15}$ (957.12): C 61.49, H 7.58, N 5.85; found: C 61.60, H 7.66, N 5.70.

Compound 6: A solution of **5** (2.68 g, 2.80 mmol), PPh_3 (0.95 g, 3.63 mmol), and *N*-bromosuccinimide (0.65 g, 3.64 mmol) in THF (25 mL) was stirred at RT. After 20 min, H_2O was added and the resulting aqueous layer was extracted with CH_2Cl_2 (3×). The combined organic layers were dried ($MgSO_4$), filtered, and evaporated. Column chromatography (SiO_2; cyclohexane/EtOAc 7:3) gave **6** (2.08 g, 73 %) as a colorless solid. ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 6.63$ (d, $J = 2$ Hz, 2H), 6.56 (d, $J = 2$ Hz, 4H), 6.52 (t, $J = 2$ Hz, 1H), 6.40 (t, $J = 2$ Hz, 2H), 4.98 (br. s, 4H), 4.96 (s, 4H), 4.41 (s, 2H), 4.01 (t, $J = 5$ Hz, 8H), 3.52 (m, 8H), 1.45 ppm (s, 36H); IR (neat): $\tilde{\nu} = 3347$ (N–H), 1691 cm^{-1} (C=O); elemental analysis calcd (%) for $C_{49}H_{71}BrN_4O_{14}\cdot 0.6\ CH_2Cl_2$: C 55.69, H 6.81, N 5.24; found: C 55.76, H 6.86, N 5.25.

Compound 7: A solution of **3** (0.92 g, 1.88 mmol) and NaN_3 (0.247 g, 3.8 mmol) in DMF (20 mL) was stirred at RT. After 12 h, H_2O was added and the resulting aqueous layer was extracted with Et_2O (3×). The combined organic layers were washed with H_2O, dried ($MgSO_4$), filtered, and evaporated. Column chromatography (SiO_2; cyclohexane/EtOAc 7:3) gave **7** (0.79 g, 93 %) as a colorless solid. ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 6.46$ (d, $J = 2$ Hz, 2H), 6.41 (t, $J = 2$ Hz, 1H), 4.96 (br. s, 2H), 4.26 (s, 2H), 4.01 (t, $J = 5$ Hz, 4H), 3.52 (m, 4H), 1.45 ppm (s, 18H); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta = 160.2$, 156.0, 138.0, 107.0, 101.3, 79.7, 67.5, 54.9, 40.2, 28.5 ppm; IR (neat): $\tilde{\nu} = 3345$ (N–H), 2098 (N_3), 1680 cm^{-1} (C=O); elemental analysis calcd (%) for $C_{21}H_{33}N_5O_6$ (451.52): C 55.86, H 7.37, N 15.51; found: C 55.50, H 7.09, N 15.11.

Compound 8: This compound was prepared as described for **7** starting from **6** (600 mg, 0.59 mmol) and NaN_3 (76 mg, 1.17 mmol) in DMF (10 mL). After work-up, column chromatography (SiO_2; cyclohexane/EtOAc 7:3) gave **8** (521 mg, 90 %) as a colorless solid. ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 6.55$–6.57 (m,7H), 6.40 (t, $J = 2$ Hz, 2H), 4.99 (br. s, 4H), 4.97 (s, 4H), 4.27 (s, 2H), 4.01 (t, $J = 5$ Hz, 8H), 3.52 (m, 8H), 1.45 ppm (s, 36H); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta = 160.2$, 160.1, 156.0, 139.3, 137.8, 107.4, 106.1, 101.9, 101.0, 79.6, 70.0, 67.4, 54.9, 40.2, 28.5 ppm; IR (neat): $\tilde{\nu} = 3347$ (N–H), 2100 (N_3), 1694 cm^{-1} (C=O); elemental analysis calcd (%) for $C_{49}H_{71}N_7O_{14}$ (982.13): C 59.92, H 7.29, N 9.98; found: C 59.93, H 7.34, N 9.68.

Compound 10: $BF_3\cdot Et_2O$ (2.82 g, 19.87 mmol) was added to a stirred solution of **9** (3.70 g, 19.87 mmol) and paraformaldehyde (1.79 g, 59.61 mmol) in 1,2-dichloroethane (200 mL). The reaction mixture was concentrated after being heated at 30 °C for 3 h. Column chromatography (SiO_2; cyclohexane/CH_2Cl_2, 1:1) gave **10** (1.59 g, 40 %) as a colorless

solid. The analytical data of **10** were in complete agreement with literature data.[4b,19]

Compound 11: A mixture of **10** (200 mg, 0.20 mmol), **7** (1.0 g, 2.22 mmol), $CuSO_4 \cdot 5H_2O$ (5 mg, 0.02 mmol), and sodium ascorbate (12 mg, 0.06 mmol) in CH_2Cl_2/H_2O (1:1, 16 mL) was vigorously stirred at RT under Ar. After two days, H_2O was added and the aqueous layer was extracted with CH_2Cl_2 (3×) and the combined organic layers were dried ($MgSO_4$), filtered, and concentrated. Column chromatography (SiO_2; CH_2Cl_2 containing 3% methanol) followed by gel permeation chromatography (Biobeads SX-1, CH_2Cl_2) gave **11** (973 mg, 88%) as a colorless glassy product. ^{1}H NMR (CD_2Cl_2, 300 MHz): $\delta = 7.88$ (s, 10H), 6.96 (s, 10H), 6.36 (br. s, 20H), 6.35 (d, $J = 2$ Hz, 10H), 5.46 (br. s, 20H), 5.36 (m, 20H), 4.80 (AB, $J = 12$ Hz, 20H), 3.90 (t, $J = 5$ Hz, 40H), 3.73 (s, 10H), 3.42 (m, 40H), 1.44 ppm (s, 180H); ^{13}C NMR ($CDCl_3$, 75 MHz): $\delta =$ 160.1, 156.0, 149.7, 144.6, 137.3, 128.7, 123.6, 115.8, 106.9, 100.9, 79.4, 67.3, 62.5, 53.9, 53.2, 39.9, 28.4 ppm; IR (neat): $\tilde{\nu} = 3348$ (N–H), 1692 cm^{-1} (C=O); MALDI-TOF-MS: *m/z* calcd for $C_{275}H_{381}N_{50}O_{70}$: 5507.27 $[M+H]^+$; found: 5507; elemental analysis calcd (%) for $C_{275}H_{380}N_{50}O_{70} \cdot 2CH_2Cl_2$ (5676.13): C 58.61, H 6.82, N 12.34; found: C 58.91, H 6.55, N 12.34.

Compound 12: A mixture of **10** (100 mg, 0.10 mmol), **8** (1.09 g, 1.11 mmol), $CuSO_4 \cdot 5H_2O$ (2.5 mg, 0.01 mmol), and sodium ascorbate (6 mg, 0.03 mmol) in CH_2Cl_2/H_2O (1:1, 6 mL) was vigorously stirred at RT under Ar. After 6 days, H_2O was added and the aqueous layer was extracted with CH_2Cl_2 (3×) and the combined organic layers were dried ($MgSO_4$), filtered, and concentrated. Column chromatography (SiO_2; CH_2Cl_2 containing 2% methanol) followed by gel permeation chromatography (Biobeads SX-1, CH_2Cl_2) gave **12** (930 mg, 81%) as a colorless glassy product. ^{1}H NMR (CD_2Cl_2, 300 MHz): $\delta = 7.85$ (s, 10H), 6.93 (s, 10H), 6.40 (s, 60H), 6.30 (s, 30H), 5.39 (br. s, 40H), 5.33 (s, 20H), 4.70 (m, 60H), 3.86 (s, 80H), 3.66 (s, 10H), 3.38 (d, $J = 3$ Hz, 80H), 1.38 ppm (s, 360H); ^{13}C NMR (CD_2Cl_2, 75 MHz): $\delta = 160.5$, 160.3, 156.3, 149.8, 145.0, 139.5, 137.9, 129.1, 124.0, 115.3, 107.4, 106.4, 102.2, 100.9, 79.5, 70.1, 67.6, 62.5, 54.3, 54.1, 40.4, 28.6 ppm; IR (neat): $\tilde{\nu} = 3348$ (N–H), 1692 cm^{-1} (C=O); MALDI-TOF-MS: *m/z* calcd for $C_{555}H_{761}N_{70}O_{150}$: 10812.43 $[M+H]^+$; found: 10812; elemental analysis calcd (%) for $C_{555}H_{760}N_{70}O_{150} \cdot 4CH_2Cl_2$ (11152.10): C 60.20, H 6.94, N 8.79; found: C 60.28, H 6.59, N 8.75.

Compound 13: Compound **11** (297 mg, 0.055 mmol) was dissolved in TFA (5 mL). After 5 h, the mixture was evaporated and dried under vacuum to afford **13** as its trifluoroacetate salt (317 mg, quantitative) as a colorless solid. ^{1}H NMR (300 MHz, D_2O): $\delta = 7.71$ (s, 10H), 6.39 (s, 30H), 6.29 (s, 10H), 5.31 (s, 20H), 4.17 (d, $J = 12$ Hz, 10H), 3.98 (m, 50H), 3.48 (s, 10H), 3.17 ppm (s, 40H); ^{13}C NMR (75 MHz, D_2O): $\delta =$ 162.6 (q, $J = 35$ Hz), 159.2, 149.8, 143.7, 137.4, 129.3, 124.6, 116.4 (q, $J =$ 290 Hz), 116.3, 107.5, 101.5, 64.0, 62.0, 53.6, 48.9, 38.7 ppm; IR (neat): $\tilde{\nu} =$ 1672 cm^{-1} (C=O); elemental analysis calcd (%) for $C_{175}H_{240}N_{50}O_{30} \cdot (CF_3CO_2)^-_{20} \cdot 15H_2O$ (5964.56): C 42.65, H 4.49, N 11.57; found: C 42.60, H 4.47, N 11.48.

Compound 14: Compound **12** (200 mg, 0.019 mmol) was dissolved in TFA (6 mL). After 5 h, the mixture was evaporated and dried under vacuum to afford **14** as its trifluoroacetate salt (215 mg, quantitative) as a colorless solid. ^{1}H NMR (300 MHz, D_2O): $\delta = 7.67$ (br. s, 10H), 6.35 (m, 100H), 5.09 (br. s, 20H), 4.45 (br. s, 40H), 3.91 (m, 100H), 3.42 (br. s, 10H), 3.17 ppm (br. s, 80H); ^{13}C NMR (100 MHz, D_2O): $\delta = 162.5$ (q, $J =$ 35 Hz), 159.7, 158.9, 149.3, 143.9, 139.1, 137.2, 130.2, 128.9, 124.2, 116.4 (q, $J = 291$ Hz), 107.3, 106.5, 101.8, 101.2, 69.3, 63.9, 61.5, 53.6, 51.2, 38.8 ppm; IR (neat): $\tilde{\nu} = 1672$ cm^{-1} (C=O); elemental analysis cacld (%) for $C_{355}H_{480}N_{70}O_{70} \cdot (CF_3CO_2)^-_{40} \cdot 60H_2O$ (12449.59): C41.97, H 4.85, N 7.87; found: C 41.45, H 4.23, N 7.45.

Agarose gel electrophoresis analysis: Water (30 μL) containing glucose (5%) or NaCl (150 mM), pCMVLuc (0.4 μg) and increasing amounts of cationic vectors **13** and **14** were subjected (30 min of complexation time) to electrophoresis in a 1% agarose gel containing 1 mM EDTA and 40 mM Tris acetate buffer and 0.5 μg mL^{-1} ethidium bromide, for 90 min at 100 V. DNA was visualized with an UV transilluminator at 254 nm.

Size of particles, ζ potential: The hydrodynamic radii were determined on the basis of dynamic light scattering measurements with a Malvern nanoZS apparatus with the following specifications: sampling time = 120 s; refractive index of medium (water with 5% glucose) = 1.340; refractive index of particles = 1.43; medium viscosity = 1.0140 cP; temperature = 25 °C. Data were analyzed by using the multimodal number distribution software included with the instrument. ζ potentials were measured with the same apparatus and with the following specifications: 20 measurements per sample; dielectric constant = 80; temperature = 25 °C; beam mode F(Ka) = 1.5 (Smoluchowski model). Polyplexes (1 mL volume) were prepared as described for delivery experiment.

Electron microscopy analysis: Images were taken with a TEM Delong LVEM5 Instrument (Cordouan Technologies, Pessac, France). Polyplexes were transferred onto 300 mesh ultrathin carbon film copper grids (EMS, Washington, USA) by placing the grid on top of 10 μL drop for 1 min. Grid with adherent particles was wicked from one side and air dried before imaging.

Cell culture: HeLa cells (ATCC, Eurobio, Courtabœuf, France) were grown in Eagle's MEM supplemented with 10% FBS, L-glutamine (2 mM), penicillin (100 units per mL), and streptomycin (100 μg mL^{-1}). Cells were maintained at 37 °C in a 5% CO_2 humidified atmosphere and all experiments were performed in triplicate. The day before experiment, cells were seeded in 24-multiwell plates at 50.10^3 cells/well in fresh complete medium (1 mL).

Polyplexe formation for pCMVLuc delivery: The procedure is for a 24-multiwell plate (ref. 3524, Corning, NY, USA) experiment. Typically, an aqueous solution of **13** or **14** (volume depending on the desired N/P ratio) was diluted to 50 μL in water containing 5% glucose or 150 mM NaCl. The solution was vortexed and left for 10 min. Separately, an aqueous solution of pCMVLuc (corresponding to 2 μg pCMVLuc) was diluted to 50 μL in water containing 5% glucose or 150 mM NaCl. The solution was then vortexed and left for 10 min, after which time the **13** or **14** solution was added to the pCMVLuc solution, and vigorously mixed (15 s). Finally, the polyplexes were incubated for 30 min at RT and added to each well by dilution with the cell medium without serum (1 mL). After 4 h, each well was completed with serum (0.1 mL). The gene expression profiles were analyzed 24 h after addition of polyplexes.

Quantification of luciferase gene expression: Luciferase gene expression was determined 24 h after delivery with a commercial kit, using the manufacturer's protocol (Luciferase Assay System, Promega, Charbonnières, France). The luminescence was measured from 2 μL of lysate during 1 s with a luminometer (Centro LB960 XS; Berthold, Thoiry, France). Luciferase activity is expressed as the mean of light units integrated over 10 s (RLU) and normalized per mg of cell protein by using the BCA assay (Pierce, Brebières, France). The errors bars represent standard deviation derived from triplicate experiments.

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