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Amphiphilic polyether branched molecules to increase the circulation time of cationic particles

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Abstract—The preparation, physicochemical and biological properties of amphiphilic polyether branched molecules is described. These 'bunch shaped' molecules when inserted into cationic liposomes/DNA complexes have shown efficient surface charge shielding. As a consequence they efficiently inhibited the non specific interactions with blood components and significantly enhanced circulation time of the particles in the blood track. Formulations containing these molecules compared positively with those containing PEG lipids, providing a 5-fold increase in circulation time.

1. Introduction

Administration of therapeutic DNA to correct genetic diseases has received considerable attention these last 15 years.[1–4]

Cationic lipid complexes with DNA have been widely described;[5] these so-called cationic lipoplexes strongly interact by electrostatic neutralization, thus providing good protection and compaction of DNA. They present however severe drawbacks to their use in human therapy: the cationic lipids that enter into composition are toxic to the cell and the living body, as they generate strong inflammatory reactions.[6] They are also rapidly cleared from the blood track as they interact with cell membrane and circulating proteins.[7,8] Efforts to alleviate this problem have led to considerable amount of work. For instance, coating of conventional neutral liposomes with polyethylene glycol polymers (PEG) has been extensively described to increase their circulation time.[9–11] It is thought that this neutral, hydrophilic polymer provides a shielding of the particle surface that protects them from blood proteins and macrophages. These have been named 'stealth liposomes'.[12,13] Transposition of this technology to the cationic complexes was very tempting and has been proposed.[14] It has been found however that PEG coating of cationic liposomes did not completely inhibit the non-specific interactions mentioned above, and that despite this treatment, these particles were rapidly eliminated through the intervention of complement proteins and macrophages.[15] Furthermore, those PEG-coated cationic lipoplexes that could escape this event, and eventually make it through the target cell membranes, could not release their therapeutic contents into the cytoplasm.[16] We have recently described the synthesis and properties of new pH labile PEG lipids that are hydrolyzed in slightly acidic environment, such as that occurring in inflammatory or tumor tissues.[17] However, the problem of short circulation time was still not resolved. An alternative to the use of PEG lipids in the

Abbreviations: BOP, benzotriazolylhexamethylphosphoramide hexafluorophosphate; DODA, dioctadecylamine; DOPE, dioleylphosphatidylethanolamine; GFP, green fluorescent protein; PEG, polyethyleneglycol; TEA, triethylamine; TFA, trifluoroacetic acid; THF, tetrahydrofuran; TLC, thin layer chromatography.

Keywords: Liposome/DNA complexes; Neutral amphiphilic lipid; Surface charge shielding; Inhibition of nonspecific interactions; Blood circulation time enhancement; Synthesis; Physical chemistry; Biological properties.

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formulation of cationic lipoplexes, in view of their systemic administration, would then be of great interest.

We proposed the hypothesis that amphiphilic neutral structures, containing ramified polyether moieties and a lipid part, could advantageously replace the PEG. The bunch shaped structures could provide efficient shielding of the lipoplex surface charges, whereas their non-polymeric nature would not over-stabilize the complexes. These molecules would then protect cationic lipoplexes during blood circulation, thus increasing their plasmatic half-life. This would provide a better chance for the particle to reach the therapeutic target, either by passive targeting or through the interaction of a ligand specific of target cells' membrane receptors. Destabilization in the cytosol should be facilitated by the non-polymeric structure and the lower molecular weight of these molecules as compared to conventional PEG lipids.

We have prepared a family of molecules containing a bunch shaped hydrophilic structure attached to a lipidic moiety, with different hydrophilic densities. We describe here the synthesis, physicochemical and biological properties of these new molecules.

2. Results and discussion

2.1. Chemistry

The first step of the synthesis consisted in building the hydrophilic branched part. In the past few decades, dendritic architectures have been highly investigated because of their unique properties such as multivalent character, well-defined aspect, optimized interfaces, etc. Dendrimers consist of branched, wedge-like structures called dendrons that are attached to a multivalent core and emerge radially toward the periphery. The synthesis of such a structure requires successive reactions of activation and coupling. For our design, we chose a trivalent core with one function dedicated to attach the hydrophobic moiety, the other two allowing the growth of the polyether.

Divergent[18] and convergent syntheses[19] have been described for dendritic molecules: In the divergent route the molecule grows from the center core to the outside, the number of coupling reaction increasing with each generation a high reactivity of the terminal groups is required. As hydroxyl groups are not highly reactive, we favored the convergent route for the polyether part. Fréchet et al. have first described the convergent synthesis of a new family of dendrimers with an aliphatic polyether backbone.[20] We have adapted this early procedure to the synthesis of a branched polyether structure corresponding to the hydrophilic part of the final molecule, with a unique functionalized focal point (Fig. 1).

We took advantage of the two electrophilic sites of methallyl dichloride reagent to grow the polyether part. The alkylation reaction was performed according to a procedure developed earlier by us using potassium hydroxide and catalytic crown ether in tetrahydrofuran.[21] The yields varied from 74% (four benzyl terminal groups [**1**]) to 66% (eight benzyl terminal groups [**3**]) as a consequence of increasing sterical hindrance. Subsequent hydroxylation of the olefinic focal point was achieved by regioselective hydroboration using

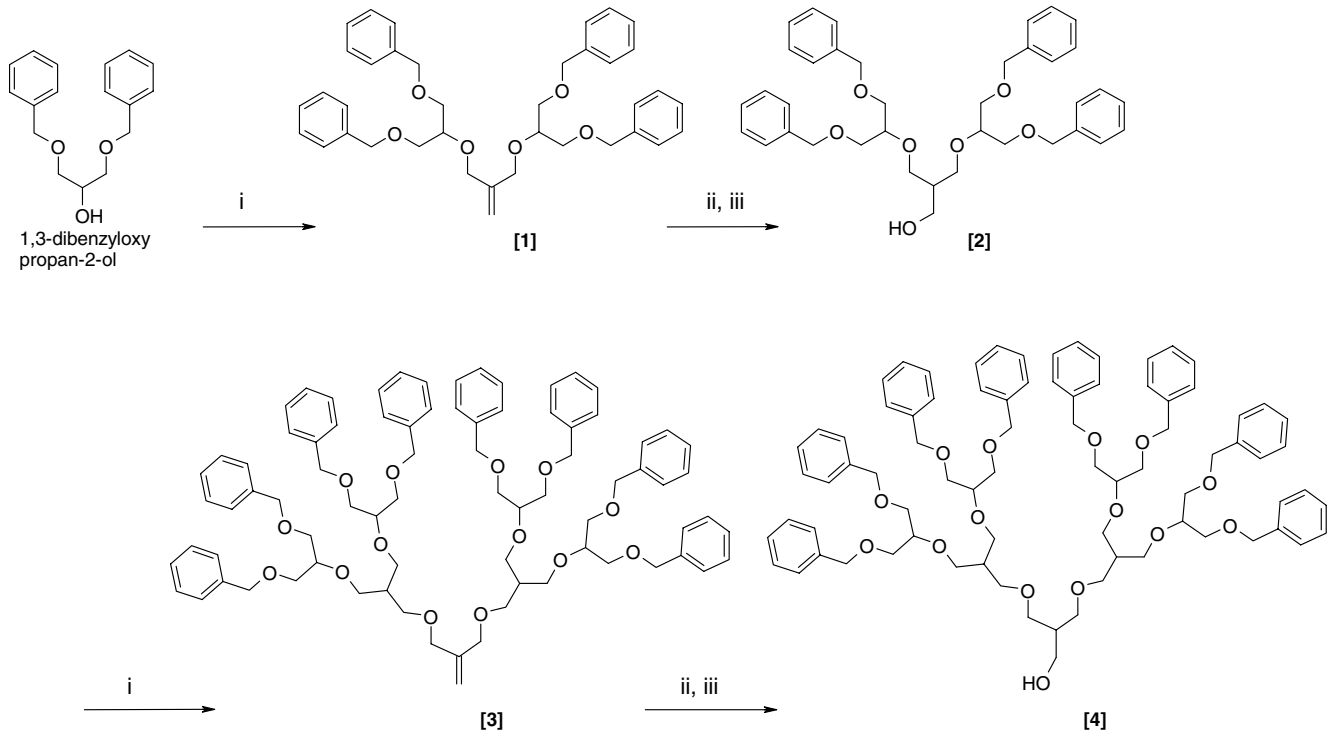

Figure 1. Synthesis of the branched polyether by the convergent method. (i) KOH, 18-Cr-6, dichloromethallyle, THF, 20 °C; (ii) BH_3/THF, 0 °C; (iii) 3 M NaOH, 30% H_2O_2, 20 °C.

borane:tetrahydrofuran complex followed by oxidation with hydrogen peroxide.

The hydrophobic part was composed of dioctadecylamine (DODA) with or without a three-glycine spacer, previously used in our laboratory to anchor polyethylene glycol moiety to liposomes.[22] The triglycine spacer is thought to allow a better presentation of the polyether part on the lipoplex surface and a higher lipid insertion in the hydrophobic lipid bilayer.

The molecules with the peptidic spacer were obtained by coupling the Boc-protected tripeptide (Boc-NH-Gly-Gly-Gly-COOH) with the DODA using BOP reagent and triethylamine to give compound [**5**]. Subsequent deprotection by trifluoroacetic acid (TFA) gave [**6**] (Fig. 2).

The coupling reaction between, respectively, the two hydrophobic parts (DODA and [**6**]) and the two branched molecules ([**2**] and [**4**]) was achieved by first reacting the hydroxyl core of the dendritic molecule with trichloromethyl chloroformate.[23] The chloroformate thus formed reacted spontaneously in the presence of base with the amino group of the hydrophobic anchors to give the corresponding carbamate derivatives. Products [**7**], [**8**], [**9**], and [**10**] were obtained in yields varying between 35% and 70%. Deprotection of the peripheric hydroxyls was performed by catalytic hydrogenolysis (Pd/C) of the benzyl ethers as the last step to give [**11**], [**12**], [**13**], and [**14**] (Fig. 3).

2.2. Physico-chemical characterization

2.2.1. DNA compaction. The newly synthesized lipids were incorporated in a formulation as a 1:1 molar ratio with the lipopolyamine RPR209120[24] (Fig. 4) and compared to RPR209120/DOPE 1:1 previously described.[25]

In the absence of DNA, the cationic particles formed by the thin film method exhibited a diameter in the 200 nm range (Table 1). Formulation of the particles including the branched polyether lipids with a $(gly)_3$ spacer (compounds [**12**] and [**14**]) was relatively straight, only few minutes of ultrasonication were necessary to obtain an average size around 200 nm. The same size range was obtained with the lipid tetraol [**11**] without drastic treatment, whereas it needed 75 min of sonication to form the particles including the DODA-octaol [**13**]. The diameter of these particles still remained larger than those of the other formulations, this might be due to the large hydrophilic octaol moiety.

When associated with DNA, at a charge ratio (*N*/*P*) equal to 4, colloidal stabilization was achieved for all the formulations, leading to suitable particles for systemic injection.

Association of DNA to the four original formulations was checked and compared to the RPR209120/DOPE. Using the fluorescence of PicoGreen® associated with non complexed DNA as an index of DNA accessibility (i.e. characterizing non-compacted DNA), we observed no differences between the four formulations containing compounds [**11**]–[**14**] when half of the fluorescence was measured. This showed that the affinity of RPR209120 to DNA was not affected by the tetraol or octaol compounds (Fig. 5). A slight difference was obtained at *N*/*P* charge ratios from 1 to 3, which might be related to the modified accessibility of DNA due to the large hydrophilic head of the four branched lipids, but this might also be due to the unstability of the colloids (aggregation due to the low lipid/DNA ratio).

When cationic charges were used in excess (ratio $\geqslant 4$), DNA was not accessible to the PicoGreen® probe in any of the formulations, thus confirming its efficient compaction.

2.2.2. Surface charges. After showing that incorporation of polyether branched lipids in cationic particles did not

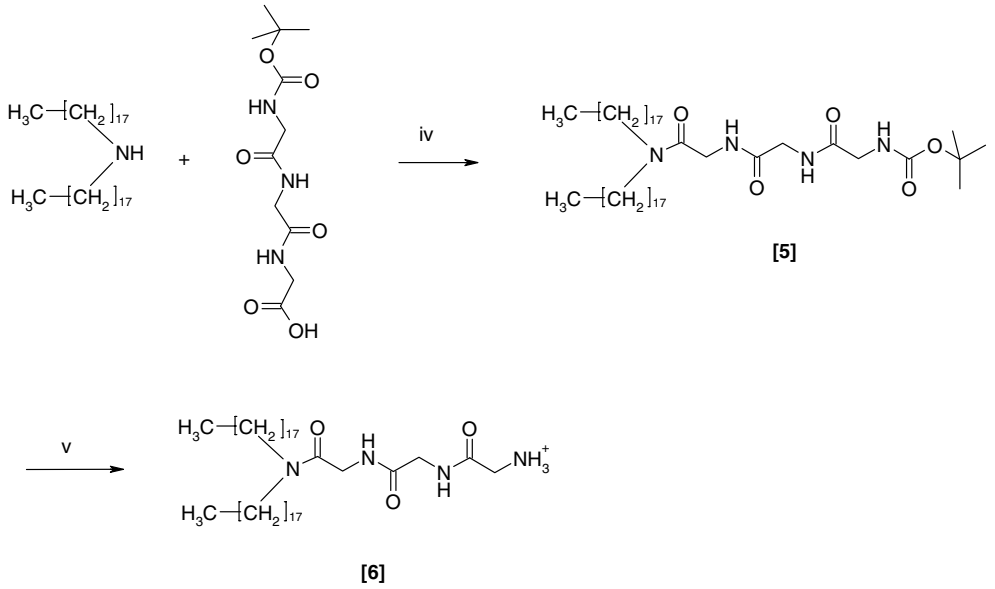

Figure 2. Synthesis of the hydrophobic part with the triglycine spacer. (iv) BOP, Et_3N, $CHCl_3$, 20 °C; (v) TFA, 20 °C.

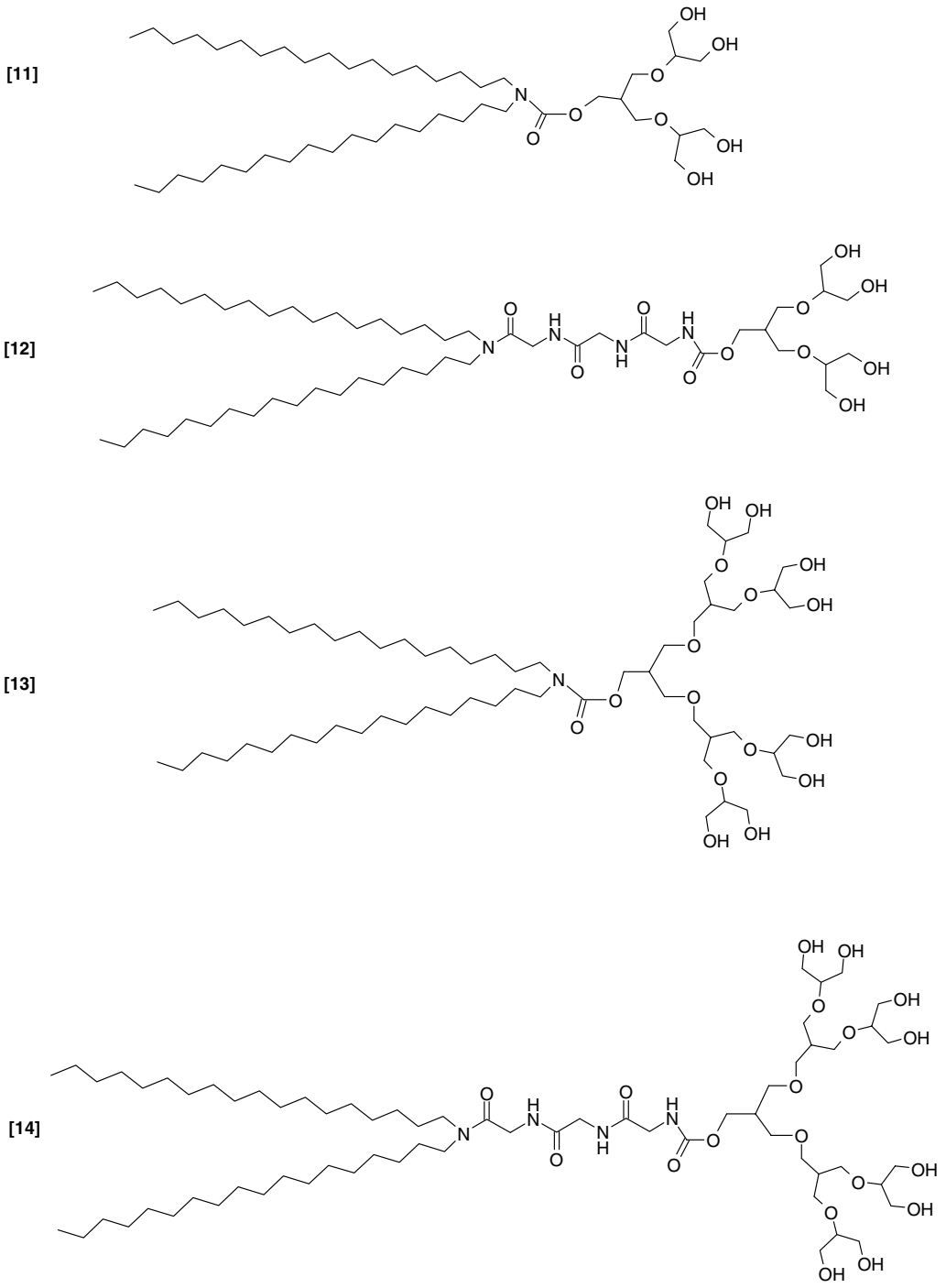

Figure 3. Amphiphilic branched molecules synthesized and studied for their physicochemical and biological ability to stabilize DNA/cationic lipid complexes.

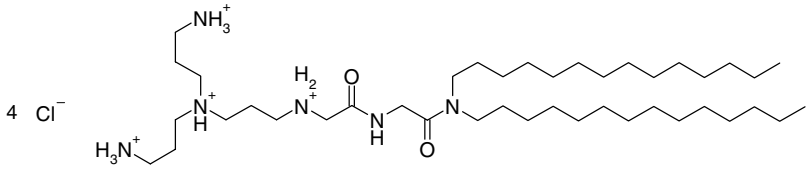

Figure 4. Cationic lipid (RPR209120) used in lipoplex preparations.

interfer with their ability to interact and associate with DNA, we focused on their potential charge masking effect (Table 1). First, the effect of the spacer between the lipidic chain and the hydrophilic head was evaluated.

Table 1. Particle diameter (nm) and zeta potential (mV) of formulations containing the branched polyether lipids [**11**]–[**14**] and the formulation RPR209120/DOPE, in the absence or presence of DNA (CR = charge ± ratio of 4) in NaCl 150 mM (mean ± SE of 3 determinations)

Formulation	−DNA		+DNA (CR = 4)	
	Size (nm)	Zeta (mV)	Size (nm)	Zeta (mV)
RPR209120/DOPE	169 ± 3.78	+74.1 ± 11	155 ± 10.2	+41.6 ± 2.5
RPR209120/DODA-tetraol [**11**]	126 ± 2.33	+77.1 ± 4.4	189 ± 9.53	+49 ± 5.1
RPR209120/DODA-octaol [**13**]	393 ± 73.8	+48.9 ± 8.3	334 ± 46.3	+30.7 ± 12
RPR209120/DODA-$(gly)_3$-tetraol [**12**]	179 ± 2.04	+56 ± 2.8	212 ± 3.46	+38.8 ± 16.9
RPR209120/DODA-$(gly)_3$-octaol [**14**]	237 ± 7.26	+55.4 ± 3.5	203 ± 26.4	+23.5 ± 4.9

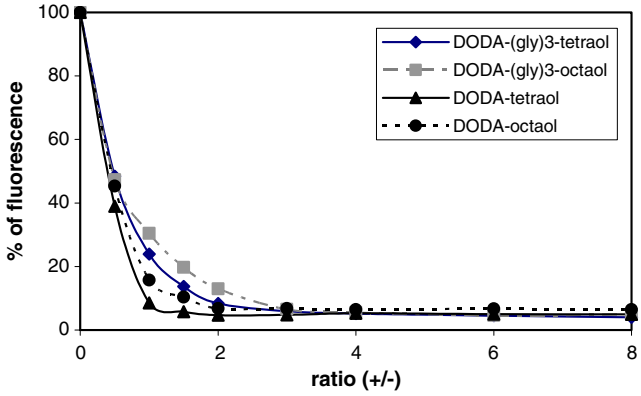

Figure 5. Fluorescence of DNA associated PicoGreen for formulations including RPR209120 and the polyether branched lipids or DOPE, and DNA at different charge ratios.

When comparing formulations containing the two lipids bearing the smallest polyether part (DODA-tetraol [**11**] and DODA-$(gly)_3$-tetraol [**13**]), we observed that the presence of the triglycine spacer in [**13**] led to a significant zeta potential decrease (Δ = 30 mV) compared to the cationic reference RPR209120/DOPE.

Then, the effect of the hydrophilic head of the polyether branched lipid on the particle zeta potential was evaluated ([**11**] vs [**13**] and [**12**] vs [**14**]). Comparison of the zeta potential of the cationic formulations containing DOPE or the two lipids without spacer indicated that the octaol moiety was more efficient in reducing the zeta potential than the tetraol part. This effect was equally observed with the two lipids bearing the triglycine spacer [**12**] and [**14**].

From these data, one might assume that the polyether branched lipid bearing the larger head is the most efficient to mask the cationic charges of the particles and should be able to reduce the non-specific interactions with circulating proteins. Also, the presence of a spacer between the lipid and the head might help exposing the hydrophilic part on the surface of the particles, and thus more efficiently mask the cationic charges.

2.2.3. Particle stability in biological medium. Based on the above results we incubated in serum containing medium the cationic particles composed of the spacer containing polyether branched lipids (compounds [**12**] and [**14**]), as compared to RPR209120/DOPE formulation.

As reported in Table 2, the cationic formulation taken as a reference as well as the formulation containing the polyether with the smallest hydrophilic head group [**12**] aggregated rapidly in the presence of serum, thus suggesting seric proteins induced colloidal destabilization. On the opposite, the larger polyether branched lipid [**14**] increased significantly the stability of the particles. Colloidal destabilization started only after 2 h of incubation at 37 °C, while the two other formulations already aggregated after 15 min. This again suggests a better masking effect of the large polyether derivative at the particle surface, which would in turn prevent non-specific interactions with seric proteins. Moreover this result is in correlation with the zeta potential decrease observed for this formulation. From these data, we could expect that formulations bearing the octaol derivative [**14**] might present an increased circulation time, as compared to RPR209120/DOPE particles.

2.3. In vitro results

2.3.1. Transfection efficiency. In order to evaluate the structure/activity relationship, we were interested in the transfection efficiency of the formulations bearing polyether branched lipids containing a triglycine spacer and bearing a small or large head group (compounds [**12**] and [**14**]). For this purpose, plasmid encoding the luciferase reporter gene was associated, at different cationic lipid/DNA ratios, to the cationic formulations containing the polyether lipids and compared to the cationic RPR209120/DOPE.

As observed in Figure 6, the three different ratios tested showed a decrease in the transfection efficiency for the formulations containing the polyether branched lipids [**12**] or [**14**] as compared to the cationic control. Even

Table 2. Particle size (nm) after incubation in cell medium containing 10% fetal bovine serum

	RPR209120/DOPE	RPR209120/DODA-$(gly)_3$-tetraol	RPR209120/DODA-$(gly)_3$-octaol
0	169	179	237
15 min	896.3	955.3	334.6
1 h	2000	2000	370.1
2 h	2000	2000	749.1

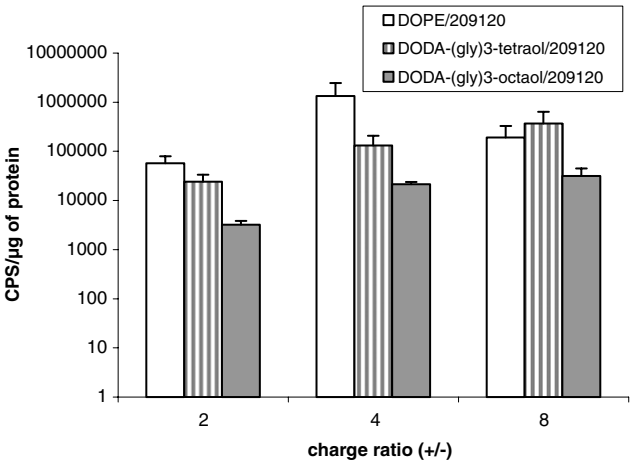

Figure 6. Transfection of B16 cells performed with the formulations DOPE/RPR209120, DODA-$(gly)_3$-tetraol [**12**]/RPR209120, and DODA-$(gly)_3$-octaol [**14**]/RPR209120 at charge ratios 2, 4, and 8.

though this effect was more pronounced with the octaol as compared to the tetraol, these particles still induced a good transfection level. This result was also in agreement with the zeta potential and serum resistance data as already mentioned. Indeed, increasing the hydrophilic moiety of the lipid from tetraol [**12**] to octaol [**14**] reduced the zeta potential of the particles and increased their stability in serum. These data led us to conclude that the larger bunch-shaped structure in the case of [**14**] would insure better shielding of the particle surface, thus reducing particle to cell membrane interactions.

2.3.2. Correlation between particle internalization and protein expression. To demonstrate that particle surface shielding was the reason for transfection efficiency decrease, we performed cell internalization experiments. To this aim DOPE-Rhodamine (0.2%) was incorporated in the different formulations as a fluorescent marker.

Comparison after 24 h, of internalizations of the cationic lipid/DOPE versus cationic lipid/tetraol [**12**] versus cationic lipid/octaol [**14**], was straightforward as can be seen in Figure 7. The particles without branched lipid (DOPE/RPR209120) were internalized into B16 cells as large aggregates (upper left side). A large amount of lipid was incorporated by the cells and GFP encoding plasmid was expressed. The use of tetraol [**12**] into the formulation did not significantly modify the pattern of particle internalization. Aggregates as well as punctuations were observed inside the cells, and approximately the same amount of cells expressed GFP (Fig. 7), which was in correlation with luciferase expression quantification (Fig. 6).

In opposite, a significant difference in particle internalization was observed when the octaol [**14**] was incorporated into the formulation. Even though we did not quantify it, the amount of internalized particle was clearly reduced by the presence of the octaol lipid [**14**]. Scarce isolated punctuations were observed at the cellular level, confirming the stability of this formulation in

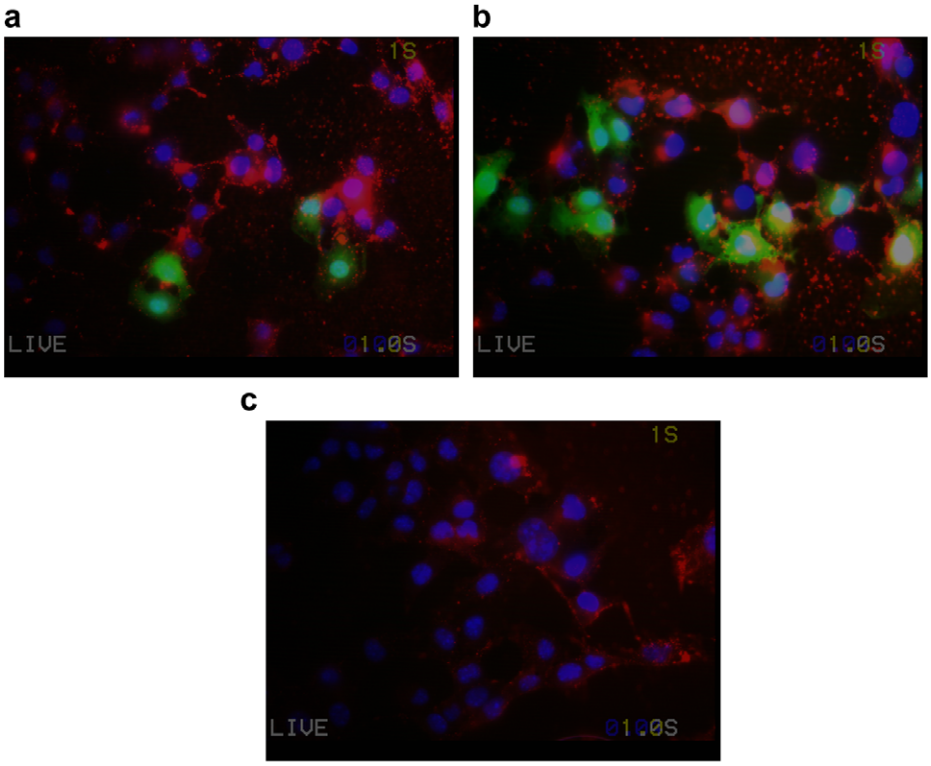

Figure 7. Internalization experiments on B16 cells. Fluorescence microscopy was performed 24 h after the transfection. (a) DOPE/RPR209120/DOPE-Rh, 1:1:0.2%; pCMU-GFP plasmid was mixed at a charge ratio (+/−) of 8 (b) DODA-$(gly)_3$-tetraol [**12**]/RPR209120/DOPE-Rh, 1:1:0.2% ; pCMU-GFP plasmid was mixed at a charge ratio (+/−) of 8 (c) DODA-$(gly)_3$-octaol [**14**]/RPR209120/DOPE-Rh, 1:1:0.2% ; pCMU-GFP plasmid was mixed at a charge ratio (+/−) of 8.

the cell medium. Moreover, GFP expression was observed in a very small amount of cells. Hence, low transgene expression was well correlated with poor particle internalization, which tended to confirm our primary hypothesis of the efficient shielding provided by the octaol lipid compared to the tetraol derivative.

2.4. Biodistribution studies

To further evaluate the efficacy of cationic particle shielding by the octaol lipid [**14**], we performed biodistribution studies on mice. The lipopolyamine formulations containing the DODA-$(gly)_3$-tetraol and DODA-$(gly)_3$-octaol lipids were compared to the cationic control RPR209120/DOPE or to the same formulation containing 5% DODA-$(gly)_3$-PEG_{5000}. The different cationic particles were injected to the mice tail vein, and blood was collected 30 min after injection. The data expressed as the percentage of the injected dose indicated significant differences between the formulations (Fig. 8). Indeed, 8% and 22% were recovered when, respectively, tetraol and octaol polyether lipids were incorporated in the formulation, while only 2.7% of the cationic particles were recovered in total blood. A 3-fold enhancement in blood circulation was thus obtained with the adjunction of the DODA-$(gly)_3$-tetraol lipid, whereas an 8-fold factor was obtained with the DODA-$(gly)_3$-octaol lipid. It is noteworthy that only 10% of the injected dose was recovered in the blood when DODA-$(gly)_3$-PEG_{5000} was added to the formulation, which is comparable to the level found with the RPR209120/DODA-$(gly)_3$-tetraol particles, and below the 22% obtained with the octaol lipid. It is also interesting to note that blood cells interaction was significantly reduced in the case of branched lipids, corroborating the shielding effect of these lipids. However, no passive accumulation could be observed in the 3LL tumor implanted in mice flank.

Until now, the main strategy developed in non viral gene delivery with lipoplexes to avoid toxicity and non specific interactions was by coating the particles with PEG. As we mentioned before, despite an increased blood circulation time, PEG does not fulfill the needs. In the present paper, we have described the synthesis and the biological study of four different molecules, composed of a double alkyl chain (insertion in the formulation), with or without a triglycine spacer and a bunch shaped polyether structure varying in size. We have incorporated these molecules into cationic formulations and have obtained stable DNA compacting complexes. We have assumed that the presence of a spacer and a large polyether hydrophilic part would improve the shielding of the surface charges of the particles, thus decreasing the cationic complexes non specific interactions. According to these data, internalization and transfection was considerably decreased with particles bearing the largest triglycine derivative. The tripeptide spacer supposedly facilitated the organization of the branched structure at the complex surface. In addition, the larger the hydrophilic part was, the more efficient was the charge shielding, leading to a large decrease of non specific interactions. Moreover, these particles were still able to transfect as shown with in vitro transfection experiments.

With an octaol moiety branched to a lipid moiety through a triglycine linker, we have presently obtained an original formulation with increased blood circulation. Indeed, as compared to PEG, the insertion of the triglycine octaol [**14**] in the formulation limits the cellular internalization and results in a longer blood circulation time. Active targeting could then be achieved either by coupling a ligand on the terminal hydroxyls or by using a small amount of PEG-lipid-ligand. Work is in progress to this aim.

3. Experimental

3.1. Materials and methods

The chemicals were obtained from Sigma–Aldrich. The solvents were purchased from SDS and were of synthesis grade. Analytical TLC was run on Merck silica-precoated aluminum plates and silica for preparative

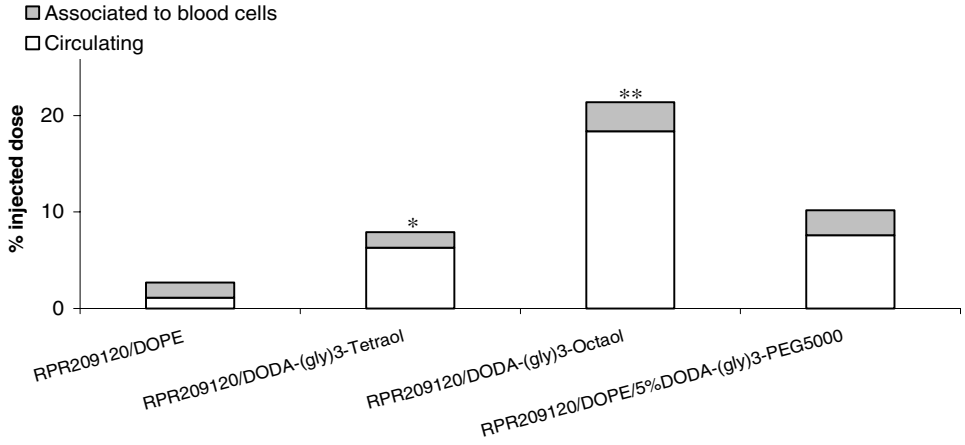

Figure 8. Distribution of the cationic particles in the blood 30 min after injection. Percentage of the injected dose was evaluated by extraction of rhodamine-lipid in the blood after systemic injection. Statistics have been performed with Kruskal–Wallis test on two separate experiments including three mice each. Significant differences were found between RPR209120/DOPE and RPR209120/DODA-$(gly)_3$-tetraol, $^{*}P < 0.05$, RPR209120/DOPE and RPR209120/DODA-$(gly)_3$-octaol, $^{**}P < 0.01$.

chromatography was also purchased from Merck. Products were revealed by a 50/50 solution of 1 M iodine and 1 M H_2SO_4 spray. RPR209120 was synthesized as described before.[26] Dioleylphosphatidylethanolamine (DOPE) was purchased from Avanti Polar, Picogreen® from Molecular Probes. Compounds were named using MDL AutoNom software according to IUPAC rules, for the sake of clarity trivial names have been defined and appear between inverted commas. NMR spectra were obtained from a Bruker Avance instrument.

3.1.1. Chemistry

3.1.1.1. 3-(2-Benzyloxy-1-benzyloxymethyl-ethoxy)-2-(2-benzyloxy-1-benzyloxymethyl-ethoxymethyl) propene. 'Tetrabenzyl allyl' [1]. To a stirred solution of 1,3-dibenzyloxypropan-2-ol (1 g, 3.67 mmol) in tetrahydrofuran (4 mL), fine potassium hydroxide powder (2 g, 36.7 mmol), 18-crown-6 (97 mg, 0.367 mmol), and methallyl dichloride (193.2 μL, 1.67 mmol) were added. The mixture was stirred at room temperature for 2 h. A large volume of dichloromethane was added and the mixture was washed three times with brine. The organic phase was then dried over Na_2SO_4, filtered, and evaporated to dryness. Chromatography of the residue on silica gel eluted with cyclohexane/ethyl acetate (7:3, v/v) gave a viscous colorless oil (737.6 mg, 1.24 mmol, yield 74%) corresponding to the expected product. ^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) 3.52–3.62 (m, 8H: CH_2—O); 4.69–4.75 (m, 2H: CH—O); 4.15 (s, 4H: CH_2—O); 4.5 (s, 8H: CH_2—Bz); 5.2 (s, 2H: CH_2=C); 7.2–7.4 (m, 20H: CH aromatic). ^{13}C NMR (75 MHz, $CDCl_3$): δ (ppm) 70.2 (CH_2—O), 71 (CH_2—O), 73 (CH_2—Bz), 77 (CH—O), 106 (CH=), 128 (CH), 129 (CH), 132.5 (CH), 138 (C).

3.1.1.2. 3-(2-Benzyloxy-1-benzyloxymethyl-ethoxy)-2-(2-benzyloxy-1-benzyloxymethyl-ethoxymethyl) propane. 'Tetrabenzyl alcohol' [2]. The olefin [**1**] (500 mg, 0.84 mmol) was stirred in anhydrous tetrahydrofuran in a round-bottomed flask under argon and maintained at 0 °C. A solution of boran/THF complex (1 M, 922 μL, 0.922 mmol) was then slowly added and the mixture was stirred at 0 °C for 5 h. A 3 M solution of sodium hydroxide (1.26 mL, 3.77 mmol) and 30% hydrogen peroxide (285 μL, 2.51 mmol) were then cautiously added. The mixture was stirred at room temperature for 30 min, saturated with K_2CO_3, and extracted with ether. The organic layer was dried over anhydrous Na_2SO_4 and the solvent was removed under vacuum. Purification of the residue by chromatography on silica gel with cyclohexane/ethyl acetate (7:3 v/v) gave a viscous colorless oil (288 mg, 0.47 mmol, yield 66%) corresponding to the expected product. ^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) 2.11 (m, 1H: CH); 3.51 (m, 8H: CH_2—O); 3.6–3.8 (m, 8H: CH—O, CH_2—O, CH_2—OH); 4.52 (s, 8H: CH_2—Bz); 7.2–7.4 (m, 20H: CH aromatic). ^{13}C NMR (75 MHz, $CDCl_3$): δ (ppm) 41.3 (CH), 63 (CH_2—O), 69.5 (CH_2—O), 70 (CH_2—OH) 73 (CH_2—Bz), 78 (CH—O), 126.5–128.8 (CH), 137.8 (C).

IR: υ 1100 cm^{-1} (C—O—C), 2858 cm^{-1} (CH_2—Bz), 3460 cm_{-1} (OH). MS (DCI): m/z = 632 MNH_4^+, m/z = 615 MH^+, m/z = 290 $(C_{17}H_{20}O_3 + NH_4)^+$.

3.1.1.3. 3-[3-(2-Benzyloxy-1-benzyloxymethyl-ethoxy)-2-(2-benzyloxy-1-benzyloxymethyl-ethoxymethyl)-propoxy]-2-[3-(2-benzyloxy-1-benzyloxymethyl-ethoxy)-2-(2-benzyloxy-1-benzyloxymethylethoxymethyl)-propoxymethyl]-propene. 'Octabenzyl allyl' [3]. The title compound was prepared from compound [**2**] as described for product [**1**] (yield 66%). ^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) 2.15 (m, 2H: CH); 3.3–3.6 (m, 36H: CH_2—O); 3.82 (s, 4H: CH_2—C=); 4.5 (s, 16H: CH_2—Bz); 5.05 (s, 2H: CH_2=C); 7.2–7.4 (m, 40H: CH aromatic). ^{13}C NMR (75 MHz, $CDCl_3$): δ (ppm) 41 (CH), 68.8 (CH_2—O), 70 (CH_2—O), 72 (CH_2—O), 73.5 (CH_2—Bz), 78.5 (CH—O), 106 (CH=), 128 (CH), 129 (CH), 132.5 (CH), 138 (C).

3.1.1.4. 3-[3-(2-Benzyloxy-1-benzyloxymethyl-ethoxy)-2-(2-benzyloxy-1- benzyloxymethyl-ethoxymethyl)-propoxy]-2-[3-(2-benzyloxy-1-benzyloxymethyl-ethoxy)-2-(2-benzyloxy-1-benzyloxymethylethoxymethyl)-propoxymethyl]-propanol. 'Octabenzyl alcohol' [4]. The title compound was prepared from compound [**3**] as described for product [**2**] (yield 50%). ^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) 2.05 (m, 1H: CH); 2.15 (m, 2H: CH); 3.3–3.7 (m, 38H: CH—O, CH_2—O, CH_2—OH); 4.5 (s, 16H: CH_2—Bz); 7.2–7.4 (m, 40H: CH aromatic). ^{13}C NMR (75 MHz, $CDCl_3$): δ (ppm) 41 (CH), 42 (CH), 64 (CH_2—OH), 68.8 (CH_2—O), 70 (CH_2—O), 70.3 (CH_2—O), 72 (CH_2—O), 73,4 (CH_2—Bz), 78 (CH—O), 127.6–128.4 (CH), 139 (C).

3.1.1.5. ({[(Dioctadecylcarbamoylmethyl-carbamoyl)-methyl]-carbamoyl}-methyl)-carbamic acid *tert*-butyl ester. 'DODA-(gly)3-Boc' [5]. To a stirred solution of Boc-*N*-gly-gly-gly-OH (1 g, 3.5 mmol) and TEA (1.44 mL, 10.4 mmol) in chloroform (70 mL), dioctadecylamine (1.8 g, 3.5 mmol) was added and the mixture was stirred until solubilization. BOP reagent (1.7 g, 3.8 mmol) was then added and the mixture was stirred at room temperature for 4 h. It was then successively washed with 0.5 M $KHSO_4$ (4 × 120 mL), saturated $NaHCO_3$ (4 × 20 mL), and finally with brine. The organic layer was dried over $MgSO_4$, filtered, and evaporated to dryness. The residue was washed with acetonitrile, centrifuged, and dried to obtain the expected product as a white powder (2.18 g, 2.75 mmol, yield 78%). ^{1}H NMR (300 MHz, $CDCl_3$): δ (ppm) 0.9 (t, J = 6.5 Hz, 6H: CH_3); 1.28 (s, 64H: CH_2); 1.47 (s, 9H: CH_3); 3.16 (t, J = 8 Hz, 2H, CH_2); 3.32 (t, J = 8 Hz, 2H: CH_2); 3.89 (d, J = 7.6 Hz, 2H: CH_2); 4.03 (dd, J_1 = 5.2 Hz, J_2 = 8.4 Hz, 4H: CH_2); 5.15 (s broad, 1H: N*H*); 6.76 (t, J = 5.2 Hz, 1H: N*H*); 7.96 (t, J = 4.8 Hz, 1H: N*H*). ^{13}C NMR (75 MHz, $CDCl_3$): δ (ppm) 14 (CH_3—CH_2), 23 (CH_2—CH_3), 27 (CH_2—CH_2), 28.5 (CH_3—C), 29.5 (CH_2), 30 (CH_2—CH_2), 32 (CH_2), 41.2 (CH_2—NH), 43 (CH_2—NH), 46.5 (CH_2—NH), 47 (CH_2—N), 167 (C=O), 168.5 (C=O), 170 (C=O).

3.1.1.6. 2-Amino-*N*-[(dioctadecylcarbamoylmethyl-carbamoyl)-methyl]-acetamide; TFA salt. 'DODA-(gly)$_3$; TFA salt' [6]. The Boc-protected product [**5**] (2 g, 2.52 mmol) was deprotected at room temperature in trifluoroacetic acid (10 mL) for 2 h. TFA was evaporated under vacuum. The expected product was obtained

(1.87 g, 2.32 mmol, yield 92%). ^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) 0.9 (t, J = 6.5 Hz, 6H: CH_3); 1.28 (s, 64H: CH_2); 3.14 (t broad, 4H, CH_2); 3.5–3.6 (q, J = 5.2 Hz, 2H: CH_2); 3.8 (d, J = 4.8 Hz, 2H: CH_2); 3.87 (d, J = 5.6 Hz, 2H: CH_2); 7.93 (s, 3H: N*H*); 8.02 (t, J = 5.2 Hz, 1H: N*H*); 8.56 (t, J = 5.2 Hz, 1H: N*H*). MS (DCI): m/z = 693 MH^+.

3.1.1.7. Dioctadecyl-carbamic acid 3-(2-benzyloxy-1-benzyloxymethyl-ethoxy)-2-(1-benzyloxymethyl-2-benzyloxy-ethoxymethyl)-propyl ester. 'DODA-tetrabenzyl' [7]. To a stirred solution of compound [**2**] (150 mg, 0.24 mmol) in toluene (15 mL), trichloromethylchloroformate (295 μL, 2.44 mmol) was added. The mixture was heated to 65 °C and stirred for 4 h. Successive evaporations under reduced pressure with toluene (3 × 5 mL) and diethyl ether (3 × 5 mL) gave a colorless oil. The residue was dissolved in THF (2 mL) and TEA (342 μL, 2.4 mmol) was added. Dioctadecylamine (116 mg, 0.22 mmol) and TEA (342 μL, 2.4 mmol) were solubilized in THF (2 mL) and added to the previous mixture. The solution was stirred at room temperature for 3 days and solvents were evaporated under vacuum. The residue was dissolved in ethyl acetate and washed with brine. The organic layer was dried over Na_2SO_4, filtered, and concentrated. Chromatography of the crude residue on silica gel in cyclohexane/ethyl acetate (8:2, v/v) gave a colorless oil (93 mg, 0.08 mmol, yield 37%) of the expected product. ^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) 0.89 (t, J = 6.4 Hz, 6H: CH_3); 1.26 (s, 64H: CH_2); 2.25 (m, 1H: CH); 3.1 (s broad, 2H: CH_2); 3.2 (s broad, 2H: CH_2); 3.5–3.7 (m, 14H: CH, CH_2); 4.16 (d, J = 6 Hz, 2H: CH_2); 4.5 (s, 8H: CH_2); 7.24–7.34 (m, 20H, CH).

3.1.1.8. ({[(Dioctadecylcarbamoylmethyl-carbamoyl)-methyl]-carbamoyl}-methyl)-carbamic acid 3-(2-benzyloxy-1-benzyloxymethyl-ethoxy)-2-(1-enzyloxymethyl-2-benzyloxy-ethoxymethyl)-propylester. 'DODA-(gly)$_3$-tetrabenzyl' [8]. To a stirred solution of product [**2**] (580 mg, 0.94 mmol) in toluene (15 mL), trichloromethylchloroformate (456 μL, 3.77 mmol) was added. The mixture was heated to 65 °C and stirred for 4 h. Successive evaporation under reduced pressure with toluene and diethyl ether gave a colorless oil. The residue was immediately dissolved in THF (10 mL). The product [**6**] (758.7, 0.94 mmol) and diisopropylamine (1.64 mg, 9.4 mmol) were dissolved in THF (10 mL) and added to the previous mixture. The solution was stirred at room temperature for 24 h. Solvents were evaporated under vacuum, the residue was dissolved in ethyl acetate and washed with brine. The organic layer was dried over Na_2SO_4, filtered, and concentrated to a yellow oil. Chromatography of the crude residue on silica gel in cyclohexane/ethyl acetate (2:8, v/v) gave a white gum (350 mg, 0.26 mmol, yield 28%) of the expected product. ^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) 0.88 (t, J = 7 Hz, 6H: CH_3); 1.26 (s, 64H: CH_2); 2.2 (m, 1H: CH); 3.15 (t, J = 7.6 Hz, 2H: CH_2); 3.3 (t, J = 7 Hz, 2H: CH_2); 3.5–3.7 (m, 14H: CH, CH_2); 3.8 (d, J = 4.6 Hz, 2H, CH_2); 3.97 (d, J = 4.3 Hz, 2H, CH_2); 4 (d, J = 2.8 Hz, 2H: CH_2); 4.2 (d, J = 5.6 Hz, 2H: CH_2); 4.51 (s, 8H: CH_2); 5.3 (t broad, 1H: N*H*); 6.77 (t, J = 1.96 Hz, 1H: N*H*); 6.99 (t broad, 1H: N*H*); 7.25 to 7.35 (m, 20H, CH). ^{13}C NMR (75 MHz, $CDCl_3$): δ (ppm) 14.3 (CH_3—CH_2), 23 (CH_2—CH_3), 27 (CH_2—CH_2), 30 (CH_2—CH_2), 32 (CH_2), 40.6 (CH_2), 42 (CH_2—N), 44.3 (CH_2—NH), 46.2 (CH_2—NH), 47 (CH_2—NH), 62 (CH_2—O), 67 (CH_2—O), 69 (CH_2—O), 72 (CH_2—Bz), 77 (CH—O), 126–128 (CH aromatic), 166 (C═O), 168 (C═O), 170 (C═O).

3.1.1.9. Dioctadecyl-carbamic acid 3-[3-(2-benzyloxy-1-benzyloxymethyl-ethoxy)-2-(1-benzyloxymethyl-2-hydroxy-ethoxymethyl)-propoxy]-2-[3-(2-benzyloxy-1-benzyloxymethyl-ethoxy)-2-(1-benzyloxymethyl-2-hydroxy-ethoxymethyl)-propoxymethyl]-propyl ester. 'DODA-octabenzyl' [9]. The title compound was prepared similarly to product [**7**] starting from compound [**4**] (68 mg, 0.036 mmol, yield 74%). ^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) 0.89 (t, J = 6.4 Hz, 6H: CH_3); 1.26 (s, 64H: CH_2); 2.25 (m, 3H: CH); 3.1 (s broad, 2H: CH_2); 3.2 (s broad, 2H: CH_2); 3.5–3.7 (m, 35H: CH, CH_2); 4.16 (d, J = 6 Hz, 2H: CH_2); 4.5 (s, 16H: CH_2); 7.24–7.34 (m, 40H, CH).

3.1.1.10. ({[(Dioctadecylcarbamoylmethyl-carbamoyl)-methyl]-carbamoyl}-methyl)-carbamic acid 3-[3-(2-benzyloxy-1-benzyloxymethyl-ethoxy)-2-(1-benzyloxymethyl-2-hydroxy-ethoxymethyl)-propoxy]-2-[3-(2-benzyloxy-1-benzyloxymethyl-ethoxy)-2-(1-benzyloxymethyl-2-hydroxy-ethoxymethyl)-propoxymethyl]-propyl ester. 'DODA-(gly)3-octabenzyl' [10]. The title compound was prepared similarly to product [**8**] but using [**4**] (52 mg, 25.7 μmol, yield 40%). ^{1}H NMR (600 MHz, $CDCl_3$): δ (ppm) 0.89 (t, J = 6.4 Hz, 6H: CH_3); 1.26 (s, 64H: CH_2); 2.25 (m, 3H: CH); 3.1 (s broad, 2H: CH_2); 3.2 (s broad, 2H: CH_2); 3.5–3.6 (m, 36H: CH, CH_2); 3.85 (s broad, 2H, CH_2); 3.98 (s broad, 2H, CH_2); 4.16 (m, 4H: CH_2); 4.5 (s, 16H: CH_2); 5.3 (t broad, 1H: N*H*); 6.77 (t, J = 1.96 Hz, 1H: N*H*); 6.99 (t broad, 1H: N*H*); 7.24–7.34 (m, 40H, CH).

3.1.1.11. Dioctadecyl-carbamic acid 3-(2-hydroxy-1-hydroxymethyl-ethoxy)-2-(2-hydroxy-1-hydroxymethyl-ethoxymethyl)-propyl ester. 'DODA-tetraol" [11]. The product [**7**] (90 mg, 77.4 μmol) was dissolved in ethyl acetate (4 mL) and Pd on activated carbon (10%) (20 mg, 19 μmol) was added. The mixture was placed under H_2 at atmospheric pressure and stirred at room temperature for 1 h. The solution was then filtered and concentrated. The expected product (50 mg, 62 μmol, yield 81%) was obtained as a colorless viscous oil. ^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) 0.9 (t, J = 6.4 Hz, 6H: CH_3); 1.26 (s, 64H: CH_2); 2.26 (m, 1H: CH); 3.2 (s broad, 4H: CH_2); 3.45–3.48 (m, 2H: CH); 3.63–3.78 (m, 12H: CH_2); 4.24 (d, J = 6 Hz, 2H: CH_2).

3.1.1.12. ({[(Dioctadecylcarbamoylmethyl-carbamoyl)-methyl]-carbamoyl}-methyl)-carbamic acid 3-(2-hydroxy-1-hydroxymethyl-ethoxy)-2-(2-hydroxy-1-hydroxymethyl-ethoxymethyl)-propyl ester. 'DODA-(gly)$_3$-tetraol' [12]. The product [**8**] (100 mg, 75 μmol) was dissolved in methanol (8 mL) and Pd on activated carbon (10%) (18 mg, 17 μmol) was added. The mixture was placed under H_2 at atmospheric pressure and stirred at room

temperature overnight. The solution was then filtered and concentrated. The expected product (45 mg, 46.2 μmol, yield 62%) was obtained as a white powder. ^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) 0.88 (t, J = 7 Hz, 6H: CH_3); 1.26 (s, 64H: CH_2); 2.2 (m, 1H: CH); 3.15 (t, J = 7.6 Hz, 2H: CH_2); 3.3 (t, J = 7 Hz, 2H: CH_2); 3.5–3.7 (m, 14H: CH, CH_2); 3.8 (d, J = 4.6 Hz, 2H, CH_2); 3.97 (d, J = 4.3 Hz, 2H, CH_2); 4 (d, J = 2.8 Hz, 2H: CH_2); 4.2 (d, J = 5.6 Hz, 2H: CH_2); 5.3 (t broad, 1H: N*H*); 6.77 (t, J = 1.96 Hz, 1H: N*H*); 6.99 (t broad, 1H: N*H*).

3.1.1.13. Dioctadecyl-carbamic acid 3-[3-(2-hydroxy-1-hydroxymethyl-ethoxy)-2-(2-hydroxy-1-hydroxymethyl-ethoxymethyl)-propoxy]-2-[3-(2-hydroxy-1-hydroxymethyl-ethoxy)-2-(2-hydroxy-1-hydroxymethyl-ethoxymethyl)-propoxymethyl]-propyl ester. 'DODA-octaol' [13]. The title compound was prepared similarly to product [**11**] but using [**9**] (yield 64%). ^{1}H NMR (600 MHz, $CDCl_3$): δ (ppm) 0.89 (t, J = 6.4 Hz, 6H: CH_3); 1.26 (s, 64H: CH_2); 2.15 (m, 3H: CH); 3.23 (s broad, 4H: CH_2); 3.4 to 3.7 (m, 36H: CH, CH_2); 4.02 (s broad, 2H: CH_2).

3.1.1.14. ({[(Dioctadecylcarbamoylmethyl-carbamoyl)-methyl]-carbamoyl}-methyl)-carbamic acid 3-[3-(2-hydroxy-1-hydroxymethyl-ethoxy)-2-(2-hydroxy-1-hydroxymethyl-ethoxymethyl)-propoxy]-2-[3-(2-hydroxy-1-hydroxymethyl-ethoxy)-2-(2- hydroxy-1-hydroxymethyl-ethoxymethyl)-propoxymethyl]-propyl ester. 'DODA-(gly)$_3$-octaol' [14]. The title compound was prepared similarly to product [**12**] but using [**10**] (yield 50%). ^{1}H NMR (300 MHz, $CDCl_3$): δ (ppm) 0.89 (t, J = 6.4 Hz, 6H: CH_3); 1.26 (s, 64H: CH_2); 2.25 (m, 3H: CH); 3.1 (s broad, 2H: CH_2); 3.2 (s broad, 2H: CH_2); 3.5–3.6 (m, 36H: CH, CH_2); 3.8 (s broad, 2H, CH_2); 3.98 (s broad, 2H, CH_2); 4.1 (m, 4H: CH_2); 4.16 (s large, 2H: CH_2).

3.1.2. Physicochemistry and biology

3.1.2.1. Preparation of liposomes bearing the branched polyether lipids or DOPE. The cationic lipid RPR209120 (2.10 mg, 2.5 μmol) and DOPE (1.86 mg, 2.5 μmol) were dissolved with chloroform in a round-bottomed flask (5 mL) and dried under reduced pressure until 5 mbar to form a thin film in the flask. After 2 h at this pressure, the film was hydrated overnight with distilled and filtered (0.22 μm) water (500 μL) to obtain a final concentration of 10 mmol/L. The liposomes bearing the polyether branched lipids [**11**]–[**14**] were formulated according to the same procedure replacing DOPE by molecules [**11**]–[**14**]. Liposomes were then sonicated to obtain a diameter in the 200 nm range. The size was determined by dynamic light scattering with a Malvern Zetasizer Nano Series.

3.1.2.2. Preparation of lipoplexes. Lipoplexes were prepared in NaCl (150 mL) at different charge ratios of cationic lipid/DNA. Plasmid DNA used contained either the luciferase or the GFP reporter gene under the cytomegalovirus (CMV) promoter and was prepared as described.[27] To prepare 500 μL of complexes at charge ratio equal to 8, DNA (250 μL, 2 μg/mL, NaCl 300 mM) was added dropwise to liposomes (250 μL, 32 mM of total lipids, H_2O) with constant vortexing. The charge ratio was calculated stoichiometrically as mole ratio of cationic lipid RPR209120 (3 positive charges per molecule) to DNA nucleotide residue (M 330, 3 nmol of phosphate in 1 μg of DNA).

3.1.2.3. Light scattering experiments. Diameter and zeta potential values of the complexes RPR209120/DOPE or lipids [**11**]–[**14**] ± DNA were determined with dynamic light scattering instruments (respectively, Malvern Zetasizer Nano Series and Malvern Zetasizer 3000HSA). For size measurement, 5 μL of complexes was diluted in 900 μL of milliQ water filtered with 0.2 μm syringe filter and for the zeta potential 200 μL of complexes was diluted in 2 mL NaCl 20 mM.

3.1.2.4. Evaluation of plasmid DNA compaction with PicoGreen®. Compaction of DNA in the liposomes containing the polyether derivatives was verified by addition of Picogreen® (100 μL of Picogreen® diluted 200-fold in trisborate-EDTA 1 N for 40 ng of DNA) and measurement of the fluorescence (λ_{exc} = 450 ± 50 nm ; λ_{em} = 530 ± 25 nm) which decreases when DNA is compacted.

3.1.2.5. Stability in fetal bovine serum. A solution of cationic liposomes RPR209120/DOPE or branched lipids [**11**]–[**14**] (120 nmol) was incubated at 37 °C in 400 μL of serum (MEM Gibco, L-glutamine 2 mM, penicillin 50 U/mL, streptomycin 50 U/mL, and 10% of fetal bovine serum). At different times (15 min, 1 h, and 2 h) 50 μL aliquots of this solution were diluted in 900 μL of filtered water before light scattering analysis.

3.1.2.6. Cell culture. B16 cells were grown in Minimum Essential Medium (MEM Gibco) supplemented with 10% (v/v) fetal bovine serum (FBS), L-glutamine 2 mM, penicillin 50 U/mL, streptomycin 50 U/mL, and a solution of non essential amino acids. Cultures were maintained at 37 °C in a 5% CO_2/air incubator.

3.1.2.7. Lipofection. The day before the experiment, B16 cells were seeded into 24-well culture plates at a density of 50,000 cells per well. They were incubated at 37 °C, under 5% CO_2 in MEM for 24 h. Just before transfection, cells were washed twice with fresh medium with or without FBS. The lipoplex solutions prepared as described above at least 30 min before lipofection were added to the cells (0.5 μg of plasmid per well). 10% (v/v) FBS was added to the culture wells containing serum-free lipofection medium 2 h later at 37 °C. The cells were incubated for 24 h at 37 °C in the presence of 5% CO_2.

3.1.2.8. Luciferase assay. Cells were washed with PBS and lysed with 200 μL of cell culture lysis reagent (Promega). Luciferase expression was quantified on 5 μL of centrifuged lysate supernatant using a luciferase assay kit (Promega). Light emission was measured using a luminometer (Multilabel counter 1420 Victor2; EG&G Wallac), equipped with a co-injector that delivered 80 μL of luciferase substrate into 40 μL of cell extracts. Relative light units (RLU) were calculated versus back-

ground activity. Light emission was normalized to the protein concentration of cell extracts, determined using the Bradford protein assay kit (Bio-Rad).

3.1.2.9. Internalization experiments. The day before the experiment, B16 cells were seeded into 12-well culture plates containing lamella at a density of 80,000 cells per well. They were incubated at 37 °C, under 5% CO_2 in MEM supplemented with 10% (v/v) fetal bovine serum (FBS), L-glutamine 2 mM, penicillin 50 U/mL, streptomycin 50 U/mL, and a solution of non essential amino acids, for 24 h. Wells were washed and MEM was added (transfection with serum). The lipoplexes (0.5 μg of GFP plasmid/well) were added to the cells. 10% (v/v) FBS was added to the culture wells containing serum-free lipofection medium after 2 h at 37 °C. The cells were incubated in the darkness, for 24 h at 37 °C in the presence of 5% CO_2. Cells were washed with 500 μL PBS and then fixed with paraformaldehyde (500 μL 3%). After 30-min incubation in the darkness, cells were washed with 500 μL PBS and DAPI solution (500 μL 0.1 μg/mL) was added. Cells were incubated for 30 min in the dark, then washed with PBS, water and mounted in mowiol prior examination. Slides were then analyzed with a Zeiss Axiophot microscope equipped with a Zeiss Neofluor 100× objective lens.

3.1.2.10. In vivo distribution in tumor-bearing mice. Experiments were performed on five-week-old female C57Bl/6 mice. Fifteen days before the experiment, 100 μL (10^5 cells/100 μL) of 3LL cellular solution (Lewis lung carcinoma tumors) was implanted subcutaneously in the right flank of the mice. The mice were anesthetized by intraperitoneal injection with a mix of ketamine (85.8 mg/kg; Centravet) and xylazine (3.1 mg/kg; Bayer) diluted in 150 mM NaCl. A 200 μL volume of rhodamine-labeled complexes (100 nmol total lipids in 5% glucose) was injected into the mouse tail vein. Blood was collected by cardiac puncture 30 min after injection.

Rhodamine-labeled lipids were extracted according to Takeuchi et al.[28] from 100 μL of blood with 3 mL chloroform/methanol (1:1, v/v) by vigorous mixing during 30 min followed by centrifugation (3000 rpm, 10 min). The fluorescence intensity was assayed on 100 μL supernatant with a luminometer Wallac Victor2 (1420 Multilabel Coulter, 570 nm). The amount of complexes in the blood was evaluated with a calibration curve and expressed as the remaining percentage of injected dose.

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