Enhanced gene delivery to the lung using biodegradable polyunsaturated cationic phosphatidylcholine-detergent conjugates

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ABSTRACT

Lung diseases are among the more representative causes of mortality and morbidity worldwide and gene therapy is considered as a promising therapeutic approach for their treatment. However the design of efficient nucleic acid carriers for airway administration still is a challenge and there is a pressing need for new developments in this field. Herein, new synthetic DNA carriers based on the conjugation of a phospholipid and C₁₂E₄, a nonionic detergent, are developed. DNA complexes with phosphatidylcholine-detergent conjugates are administered in mouse airways, and transgene expression and inflammatory activity as an index of toxicity are investigated as a function of time, DNA dose, and presence of helper and stealth lipids. Introduction of a biodegradable linker between the phosphatidylcholine and detergent moieties significantly attenuates the severity of inflammatory response that characterizes cationic lipid-mediated gene transfer. Concurrent introduction of polyunsaturated fatty acid chains in the carrier scaffold improves transgene expression and further reduces airway inflammation. Finally, the biodegradable phosphatidylcholine-detergent conjugates favorably compare to GL67A, the gold standard for DNA delivery to the airway that is currently under clinical evaluation. Our findings indicate that the lipid formulations described herein may have great potential as nucleic acid carriers for gene therapy.

Keywords

Polyunsaturated cationic lipid; *in vivo* gene delivery; lung transfection; nanoparticle; biodegradability; airway inflammation.

Abbreviations

BALFs, bronchoalveolar lavage fluids; br, broad; C₁₂E₄, tetraethyleneglycol monododecyl ether; CMV, cytomegalovirus; COPD, chronic obstructive pulmonary diseases; d, doublet; DMPE-mPEG₅₀₀₀, 1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[methoxy poly(ethylene glycol)-5000] (ammonium salt); DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; DOPE, 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine; EDTA, ethylenediaminetetraacetic acid; ELISA, enzyme-linked immunosorbent assay; GLuc, *Gaussia luciferase*; IL-6, interleukine-6; KC, keratinocyte-derived chemokine; mPEG, poly(ethylene glycol) monomethyl ether; PBS, phosphate-buffered saline; pDNA, plasmid DNA; PE, petroleum ether; PEG, poly(ethylene glycol); q, quartet; RLU, relative light units; s, singlet; SD, standard deviation; t, triplet; WHO, World Health Organization.

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1. Introduction

Lung disorders such as COPD, asthma, cystic fibrosis, acute lung injury, and bronchopulmonary cancer are among the more representative causes of mortality and morbidity worldwide (> 20 %) according to WHO (WHO, 2014). In 2012, COPD and asthma were responsible for 4 million deaths globally, and lung cancer killed 1.6 million people. Asthma is the most common noncommunicable disease among children and WHO estimates that 235 million people currently suffer from it.

Gene therapy is considered as a promising approach to treat lung diseases (Griesenbach and Alton, 2009). Gene transfer to the airway epithelium however has been more difficult than originally anticipated, largely because of significant extra- and intracellular barriers in the lung (Jeffery and Li, 1997). Although studies almost universally show that viral gene transfer to the airways is more efficient than nonviral gene transfer, most viral vectors do not efficiently penetrate human mucus secretions (Hida et al., 2011) and cause immune responses which interfere with gene expression on redosing, thus lacking good safety profile (Baum et al., 2003; Conese et al., 2011; Griesenbach et al., 2010; Sakurai et al., 2008; Trapani et al., 2014). Regarding the administration route of transfection nanoparticles, the relative accessibility of the pulmonary epithelium makes local delivery an attractive option as it is noninvasive, avoids first-pass metabolism, leads to a faster onset of therapeutic action, and offers a huge surface area (*ca.* 75-140 m²) (Gehr et al., 1978). Furthermore, it exclusively targets the lungs and thereby prevents that nanoparticles end-up in non-target tissues, minimizing potential risks associated with systemic delivery. However, the majority of viral vectors so far investigated cannot withstand the hydrodynamic shear forces generated during lung administration *via* aerosolization (Griesenbach et al., 2011a; Katkin et al., 1995). Finally, establishment of methods for large-scale virus production still is a bottleneck.

As an alternative to viral vectors, nonviral gene carriers offer potential for improved safety, ease of manufacturing and scale up, possibility of repeated dosing without generating a therapy-disabling immune response, and ability to accommodate larger pDNA compared to commonly tested viruses, such as adeno-associated virus. In addition, their complexes with nucleic acid much better resist aerosolization (Davies et al., 2014). For all these reasons, there is currently a general consensus that gene transfer to the lung with nonviral vectors is more suitable than with engineered viruses. The cationic lipid-based formulation GL67A developed by the UK Cystic Fibrosis Gene Therapy Consortium is thus under clinical evaluation in patients with cystic fibrosis and is currently the gold standard for pulmonary administration of gene therapy products (Alton et al., 2015; Alton et al., 1999; Davies et al., 2014; Griesenbach et al., 2011a; Sorscher et al., 1994). However its moderate efficacy can only highlight the pressing need for the development of more effective and safer nucleic acid carriers for airway administration.

Recently, conjugates between a membrane phospholipid and a detergent were proposed as synthetic carriers for *in vitro* DNA and siRNA delivery (Pierrat et al., 2012; Pierrat et al., 2013a; Pierrat et al., 2013b). 1,2-Dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), a major constituent of the cell membranes, was thus conjugated to various nonionic detergents (*e.g.* Triton X-100®, C₁₂E₄, and structural analogs) and the resulting cationic conjugates were evaluated for their capacity to mediate nucleic acid delivery into cultured airway epithelium cell lines. Two possible mechanisms of action were proposed to explain their transfection activity. The first one entails intrinsic fusogenicity of the conjugates (Pierrat et al., 2016b). The second one implies a Trojan horse mechanism, where potency of the carriers relies on their biodegradability (Pierrat et al., 2013a; Pierrat et al., 2013b) and on the membrane disrupting properties of the detergent molecules produced upon hydrolysis of the conjugates within the endosome compartment (Pierrat et al., 2016a). These two mechanisms rely on separate properties of the conjugates in the extracellular and intracellular compartments and are not mutually exclusive. It was shown that conjugation of DOPC with C₁₂E₄ resulted in

versatile carriers for intracellular delivery of both DNA and siRNA *in vitro*, with high efficiency and low cytotoxicity. On the one hand, structure-activity relationships suggested that some of these compounds may represent a reasonable compromise between the membrane activity of the released detergent and susceptibility of the conjugate to degradation enzymes. On the other hand, fusogenicity of various DOPC conjugates with *n*-alkyl oligo(ethylene glycol) ethers correlated well with transfection efficiency. It was thus shown that the latter was crucially depending on the length of the polyether moiety of the detergent species conjugated to the phospholipid. In particular, cationic lipid **1** (Fig. 1) was identified as a promising candidate for *in vivo* gene delivery. In the herein presented study, we have been focusing on the development of lipid formulations based on this compound for *in vivo* DNA delivery in the mouse lung through local administration. We investigated the influence of the structure of the PEGylated lipid introduced in the formulations on the transfection efficiency and safety of the resulting lipoplexes. We also introduced a series of five analogs of the lead compound **1** displaying a stimuli responsive linker on the phosphate head group, and incorporating various acyl chains in the *sn*-1 and *sn*-2 position on the glycerol backbone. In the following, we discuss the rationale of the study and describe the results obtained *in vivo* with the various lipid formulations, demonstrating their potential to deliver DNA in the mouse lung in a safe and effective way.

Fig. 1. Structure of the various cationic and PEGylated lipids (average number of ethylene glycol units indicated) introduced herein for the delivery of pDNA to the mouse lung.

2. Materials and methods

2.1. Materials

Unless otherwise stated, all chemical reagents were purchased from Alfa Aesar (Bischeim, France) and were used as received. DOPC was from Lipoid GmbH (Cham, Switzerland). DOPE and DMPE-mPEG₅₀₀₀ were from Avanti Polar Lipids (Alabaster, AL, U.S.A.). mPEG₂₀₀₀ and mPEG₅₀₀₀ were from Sigma-Aldrich (Saint-Quentin Fallavier, France). When required, solvents were dried by standard procedures just before use (Lebeau et al., 1992). Thin layer chromatography (TLC) was performed on precoated plates (0.25 mm Silica Gel 60, F₂₅₄, Merck, Darmstadt, Germany). Products were purified by chromatography over silica gel (Silica Gel 60, 40-63 μ m, Merck, Darmstadt, Germany). NMR spectra were recorded on Bruker 300 MHz Avance DPX and 400 MHz Avance III instruments. ¹H-, ¹³C-, and ³¹P-NMR chemical shifts δ are reported in ppm relative to their standard reference (¹H: CHCl₃ at 7.27 ppm, CD₂HOD at 3.31 ppm; ¹³C: CDCl₃ at 77.0 ppm, CD₃OD at 49.0 ppm; ³¹P: H₃PO₄ at 0.00 ppm). IR spectra were recorded on a FT-IR Nicolet 380 spectrometer in the ATR mode and absorptions values ν are in wave numbers (cm⁻¹). Mass Spectra (MS) were recorded on an Agilent technology 6520 Accurate Mass QToF, using electrospray ionization (ESI) mode. Mass data are reported in mass units (*m/z*).

Plasmid pCMV-GLuc (5.7 kbp, Nanolight Technology, Pinetop, Az, USA) was used as reporter gene to monitor *in vivo* DNA transfection activity (Griesenbach et al., 2011b). This plasmid encodes the *Gaussia luciferase* gene under the control of the human immediate/early CMV promoter/enhancer.

2.2. Synthesis

Preparation of the cationic conjugates **1** and **6** has been described elsewhere (Pierrat et al., 2016a; Pierrat et al., 2016b). GL67 and formulation GL67A were prepared as reported in the

literature (Chadwick et al., 1997; Griesenbach et al., 2011b). Compounds **2-5** and **7-9** were synthesized according to Scheme 1.

2.2.1. Synthesis of 1,2-dioleoyl-sn-glycero-3-(3-oxo-2,4,7,10,13,16-hexaoctacosyl)phosphocholine iodide (**2**)

To a solution of poly(ethylene glycol) monomethyl ether (average MW: 2000 Da, $n \approx 45$, 4.40 g, 2.20 mmol) and pyridine (390 μ L, 4.84 mmol) in freshly distilled CH_2Cl_2 was slowly added chloromethylchloroformate (387 μ L, 4.40 mmol) in CH_2Cl_2 (50 mL). The reaction mixture was stirred at room temperature under argon for 18 h and was then washed with 2% HCl and with water. The organic layer was dried over MgSO_4 , filtered, and volatiles were removed under reduced pressure. Analytically pure compound **10** (3.70 g, 78 %) was obtained as an off-white powder and was used in the next step without further purification. $^1\text{H-NMR}$ (400 MHz, CDCl_3) δ 5.71 (s, 2H), 4.35–4.33 (m, 2H), 3.80–3.43 (m, 174H), 3.35 (s, 3H). $^{13}\text{C-NMR}$ (100 MHz, CDCl_3) δ 153.2, 72.1, 71.7, 70.6, 70.5, 70.4, 68.5, 67.9, 58.9. FT-IR (film) ν_{max} 3504, 2857, 1766, 1456, 1348, 1248, 1097, 947, 845.

A mixture of chloromethylcarbonate **10** (1.00 g, 0.48 mmol), sodium iodide (71.2 mg, 0.48 mmol) and DOPC (47.0 mg, 59.9 μ mol) in anhydrous CHCl_3 (2 mL) was heated at 80 $^\circ\text{C}$ in a sealed reactor for 1 h under an argon atmosphere. The reaction mixture was cooled down at room temperature and directly purified, without concentration, by flash chromatography over silica gel ($\text{CH}_2\text{Cl}_2/\text{MeOH}$ 95:5 to 80:20) to afford compound **2** (67.0 mg, 37 %, mixture of two diastereomers) as a waxy solid. TLC R_f 0.34 ($\text{CH}_2\text{Cl}_2/\text{MeOH}$ 90:10). $^1\text{H-NMR}$ (400 MHz, $\text{CDCl}_3/\text{CD}_3\text{OD}$ 1:1) δ 5.72–5.67 (m, 2H), 5.34–5.27 (m, 5H), 4.59 (br, 2H), 4.37–4.13 (m, 6H), 3.91 (br, 2H), 3.82–3.47 (m, 178H), 3.37 (s, 3H), 3.29 (s, 9H), 2.34–2.31 (m, 4H), 1.98 (br, 8H), 1.59 (br, 4H), 1.39–1.18 (m, 40H), 0.85 (t, $^3J_{\text{HH}} = 6.3$ Hz, 6H). $^{13}\text{C-NMR}$ (100 MHz, $\text{CDCl}_3/\text{CD}_3\text{OD}$ 1:1) δ 174.3, 173.9, 154.3, 130.5, 130.3, 86.9, 77.0, 70.9, 70.8, 70.7 (2C), 70.2, 70.1, 70.0 (2C), 69.8, 69.3, 69.7, 67.4, 62.3, 61.0, 59.2, 54.8, 34.7, 34.6, 32.5, 30.3 (2C), 30.2 (2C), 30.1, 29.9, 29.9, 29.8 (2C), 29.7 (2C), 29.6, 27.7, 25.5, 23.2, 14.3. $^{31}\text{P-NMR}$ (162 MHz, $\text{CDCl}_3/\text{CD}_3\text{OD}$ 1:1) δ -3.43, -3.48. FT-IR (film) ν_{max} 2922, 2854, 1766, 1742, 1458, 1350, 1258, 1225, 1097, 1030, 985, 845, 730, 638.

Scheme 1. Synthetic routes employed for the preparation of PEG-lipids **2-5** and conjugates **7-9**.

2.2.2. Synthesis of 1,2-dioleoyl-sn-glycero-3-[methoxy(polyethylene glycol)-2000]phosphocholine trifluoromethanesulfonate (**3**)

Triflic anhydride (51 μ L, 0.30 mmol) was added dropwise to a solution of poly(ethylene glycol) monomethyl ether (average MW: 2000 Da, $n \approx 45$, 500 mg, 0.25 mmol) and pyridine (24 μ L, 0.30 mmol) in freshly distilled CH_2Cl_2 (10 mL), at -50 $^\circ\text{C}$. The reaction mixture was stirred under argon at -50 $^\circ\text{C}$ for 1 h before it was poured into ice-cold water (30 mL). The organic layer was separated and rapidly washed with cold water (2 x 10 mL), dried over anhydrous magnesium sulfate, filtered, and concentrated under reduced pressure at 10 $^\circ\text{C}$. The corresponding trifluoromethanesulfonyl ester **12** was obtained as a clear oil (530 mg, 98 %) and was used in the next step without further purification. Due to relative instability of the compound, $^1\text{H-NMR}$ spectrum was recorded immediately after isolation. $^1\text{H-NMR}$ (400 MHz, CDCl_3) δ 4.64 (t, $^3J_{\text{HH}} = 4.4$ Hz, 2H), 3.86–3.82 (m, 2H), 3.64–3.51 (m, 172H), 3.38 (s, 3H).

To a solution of DOPC (65 mg, 83 μ mol) in dry CHCl_3 (5 mL) was added a solution of the freshly prepared trifluoromethanesulfonyl ester **12** (530 mg, 0.25 mmol) in dry CHCl_3 (1 mL). The resulting reaction mixture was stirred at room temperature for 18 h under argon before it was concentrated under reduced pressure. The crude residue was purified by chromatography over silica gel ($\text{CH}_2\text{Cl}_2/\text{MeOH}$ 98:2 to 90:10) to provide compound **3** as a clear oil (mixture of two diastereomers, 95 mg, 40 %). TLC R_f 0.4 ($\text{CH}_2\text{Cl}_2/\text{MeOH}$ 88:12). $^1\text{H-NMR}$ (400 MHz,

CDCl₃) δ 5.34–5.29 (m, 4H), 5.24 (m, 1H), 4.55 (br, 2H), 4.31–4.16 (m, 6H), 3.89–3.85 (br, 2H), 3.81–3.45 (m, 178H), 3.37 (s, 3H), 3.30 (s, 9H), 2.34–2.28 (m, 4H), 2.00 (q, $^3J_{\text{HH}} = 5.9$ Hz, 8H), 1.59 (br, 4H), 1.32–1.23 (m, 40H), 0.87 (t, $^3J_{\text{HH}} = 6.8$ Hz, 6H). ¹³C-NMR (100 MHz, CDCl₃) δ 173.2, 172.8, 129.9, 129.6, 71.6, 70.2, 69.8, 69.3, 67.6, 65.6, 65.4, 61.6, 58.9, 54.3, 34.1, 33.9, 31.8, 29.7, 29.6, 29.5, 29.3, 29.2 (2C), 29.1, 29.0 (2C), 28.9, 27.2 (2C), 24.8, 22.6, 14.0. ³¹P-NMR (162 MHz, CDCl₃) δ -1.88, -1.89. FT-IR (film) ν 2922, 2854, 1742, 1458, 1350, 1258, 1225, 1097, 1030, 985, 847, 732, 638.

2.2.3. Synthesis of dioleoyl {2-oxa-3-oxo-3-[methoxy (polyethylene glycol)-5000]propyl} phosphate (**4**)

A solution of pyridine (4.0 mL, 50.0 mmol) and oleyl alcohol (12.7 g, 47.5 mmol) in anhydrous THF (10 mL) was added dropwise over 1 h to an ice-cooled solution of phosphorus oxychloride (2.4 mL, 25.0 mmol) in anhydrous THF (60 mL) under argon. The reaction mixture was stirred at 40 °C for 1 h, and then was cooled to room temperature. Pyridine (2.0 mL, 25 mmol) in anhydrous methanol (10 mL) was added and the resulting mixture was stirred at room temperature for 24 h. Volatile was removed under reduced pressure, and the crude residue was suspended in ethyl acetate (200 mL) and filtered to yield a clear organic layer. The filtrate was successively washed with 2 % HCl (2 x 100 mL), water (50 mL), and brine (100 mL). The organic layer was dried over MgSO₄, filtered and concentrated under reduced pressure. The residue was purified by chromatography over silica gel (PE/EtOAc 9:1 to 8:2) to provide compound **13** as a yellow oil (2.95 g, 20 %). TLC *R_f* 0.4 (PE/EtOAc 8:2). ¹H-NMR (400 MHz, CDCl₃) δ 5.38–5.31 (m, 4H), 4.06–4.01 (m, 4H), 3.76 (dd, $^3J_{\text{HH}} = 1.6$ and $^3J_{\text{PH}} = 11.1$ Hz, 3H), 2.05–1.95 (m, 8H), 1.72–1.64 (m, 4H), 1.40–1.22 (m, 44H), 0.89 (t, $^3J_{\text{HH}} = 6.8$ Hz, 6H). ¹³C-NMR (100 MHz, CDCl₃) δ 130.0, 129.7, 67.9, 67.8, 54.1, 54.0, 32.6, 31.9, 30.3, 30.2, 29.7 (2C), 29.6, 29.5, 29.4, 29.3, 29.2, 29.1, 27.2, 25.4, 22.7, 14.1. ³¹P-NMR (162 MHz, CDCl₃) δ 0.39. FT-IR (film) ν 3375, 2924, 2853, 2865, 1467, 1259, 1188, 1090, 715.

To a solution of the previous compound (1.30 g, 2.12 mmol) in anhydrous DMF (5 mL) was added potassium cyanide (0.50 g, 7.64 mmol). The reaction mixture was heated at 120 °C for 18 h. DMF was removed under reduced pressure and the residue was purified by chromatography over silica gel (CH₂Cl₂/MeOH/H₂O 9:1:0 to 10:4:0.5) to provide compound **14** as a waxy solid (1.06 g, 79 %). TLC *R_f* 0.2 (CH₂Cl₂/MeOH/H₂O 10:3:0.2). ¹H-NMR (400 MHz, CDCl₃) δ 5.39–5.34 (m, 4H), 3.85 (br, 4H), 2.02–1.97 (m, 8H), 1.62–1.54 (br, 4H), 1.38–1.24 (m, 44H), 0.89 (t, $^3J_{\text{HH}} = 6.62$ Hz, 6H). ¹³C-NMR (100 MHz, CDCl₃) δ 129.9, 129.6, 66.0, 32.7, 32.6, 31.9, 30.9, 30.8, 29.9, 29.8 (2C), 29.7, 29.6, 29.3, 27.3, 27.2, 26.0, 22.7, 14.1. ³¹P-NMR (162 MHz, CDCl₃) δ -0.83. FT-IR (film) ν 3385, 3265, 2924, 2852, 2845, 1470, 1259, 1112, 705.

Compound **11** (9.90 g, 90 %) was obtained as an off-white powder from poly(ethylene glycol) monomethyl ether (average MW: 5000 Da, *n* $\approx$ 113, 11.0 g, 2.20 mmol), pyridine (532 μ L, 6.60 mmol) and chloromethylchloroformate (387 μ L, 4.4 mmol) following the same protocol as for **10** (*vide supra*). ¹H-NMR (400 MHz, CDCl₃) δ 5.71 (s, 2H), 4.36–4.34 (m, 2H), 3.81–3.51 (m, 450H), 3.36 (s, 3H). ¹³C-NMR (100 MHz, CDCl₃) δ 152.9, 71.9, 71.5, 70.1, 68.2, 67.6, 58.6. FT-IR (film) ν 3498, 2863, 2851, 1770, 1455, 1351, 1233, 1097, 812.

A mixture of compound **14** (188 mg, 290 μ mol), chloromethyl carbonate **11** (300 mg, 59 μ mol), and sodium iodide (9.0 mg, 59 μ mol) in anhydrous DMF (10 mL) was heated at 90 °C under vigorous stirring for 18 h under argon. After cooling to room temperature, the reaction mixture was concentrated to dryness under reduced pressure. The residue was triturated in diethyl ether and filtered to obtain a yellowish powdered solid that was extensively washed with diethyl ether. Next, the solid was triturated in ethyl acetate and vigorously vortexed. After centrifugation at 12,000 rpm for 10 minutes, the supernatant was collected and concentrated under reduced pressure to yield analytically pure compound **4** (30 mg, 9 %) as a

waxy solid. ¹H-NMR (500 MHz, CDCl₃) δ 5.63 (d, ³J_{HH} = 13.0 Hz, 2H), 5.38–5.30 (m, 4H), 4.34 (m, 2H), 4.07–4.05 (m, 4H), 3.79–3.49 (m, 450H), 3.38 (s, 3H), 2.01–1.99 (m, 8H), 1.70–1.64 (m, 4H), 1.35–1.21 (m, 44H), 0.88 (t, ³J_{HH} = 6.8 Hz, 6H). ¹³C-NMR (125 MHz, CDCl₃) δ 153.7, 129.9, 129.7, 85.5, 71.9, 70.8, 70.5, 70.3, 68.6, 68.3, 67.7, 59.0, 32.6, 31.9, 30.2, 30.1 (2C), 29.7 (2C), 29.6 (2C), 29.4 (2C), 29.3, 29.2, 29.1, 27.2, 25.3, 22.6, 14.1. ³¹P-NMR (162 MHz, CDCl₃) δ -2.62. FT-IR (film) ν 3265, 2924, 2915, 2852, 2845, 1765, 1470, 1455, 1351, 1233, 1261.

2.2.4. Synthesis of 2,3-bis(oleoyloxy)propyl ([methoxy (polyethylene glycol)-5000]) succinate (**5**)

A mixture of (S)-1,2-dioleoylglycerol (Lebeau et al., 1991) (3.0 g, 4.83 mmol), succinic anhydride (0.52 g, 5.17 mmol), triethylamine (0.67 mL, 4.83 mmol), and 4-DMAP (30 mg, 0.25 mmol) in anhydrous THF (40 mL) was stirred at room temperature for 2 d. The solvent was removed under vacuum, and the residue was dissolved in ethyl acetate and washed with HCl 5 % (2 x 50 mL), water (50 mL), and brine (50 mL). The organic layer was dried over MgSO₄, filtered and concentrated under reduced pressure. The residue was purified by flash chromatography over silica gel (PE/Et₂O 7:3 to 0:10) to provide compound **15** as a colorless oil (3.43 g, 98 %). TLC R_f 0.6 (Et₂O). ¹H-NMR (300 MHz, CDCl₃) δ 5.37–5.33 (m, 4H), 5.29–5.27 (m, 1H), 4.36–4.27 (m, 2H), 4.17 (dt, ³J_{HH} = 6.0 and 12.0 Hz, 2H), 2.68–2.65 (m, 4H), 3.31 (dt, ³J_{HH} = 2.1 and 7.5 Hz), 2.03–1.98 (m, 8H), 1.68–1.55 (m, 4H), 1.40–1.21 (m, 40H), 0.87 (t, ³J_{HH} = 6.0 Hz, 6H). ¹³C-NMR (100 MHz, CDCl₃) δ 171.2, 173.3, 172.9, 171.5, 130.0 (2C), 129.7 (2C), 68.7, 62.6, 34.1, 34.0, 31.9 (2C), 29.8 (2C), 29.7 (2C), 29.5 (2C), 29.3 (2C), 29.2 (2C), 29.1 (2C), 29.0 (2C), 28.7 (2C), 28.6 (2C), 2.2 (2C), 27.1 (2C), 24.8 (2C), 22.7 (2C), 14.1 (2C).

DIAD (180 μL, 0.91 mmol) was added dropwise to a mixture of carboxylic acid **15** (0.128 g, 0.18 mmol), poly(ethylene glycol) monomethyl ether (average MW: 5000 Da, n ≈ 113, 0.620 g, 0.12 mmol), and triphenylphosphine (0.238 g, 0.91 mmol) in anhydrous chloroform at room temperature. The reaction mixture was stirred for 4 h before the solvent was removed under vacuum. The residue was purified by chromatography over silica gel (CHCl₃/EtOH 9:1 to 7:3) to yield compound **5** (0.72 g, 91 %) as a white waxy solid. TLC R_f 0.5 (CHCl₃/EtOH 9:1). ¹H-NMR (400 MHz, CDCl₃) δ 5.3–5.27 (m, 4H), 5.23–5.21 (m, 1H), 4.28–4.07 (m, 6H), 3.65–3.55 (m, 454H), 3.34 (s, 3H), 2.61 (s, 4H), 2.30–2.24 (m, 4H), 1.98–1.95 (m, 8H), 1.60–1.50 (m, 4H), 1.32–1.20 (m, 40H), 0.84 (t, ³J_{HH} = 6.4 Hz, 6H). ¹³C-NMR (100 MHz, CDCl₃) δ 173.0, 172.6, 171.9, 171.6, 129.8 (2C), 129.5 (2C), 71.8, 70.4 (m), 68.9, 68.6, 63.7, 62.4, 61.8, 58.9, 33.9, 33.8, 31.7 (2C), 29.6–28.6 (m), 27.0 (4C), 24.6 (2C), 22.5 (2C), 13.9 (2C). FT-IR (film) ν 2881, 1740, 1466, 1359, 1342, 1279, 1146, 1102, 1060.

2.2.5. Synthesis of 1-oleoyl-2-linolenoyl-sn-glycero-3-(3-oxo-2,4,7,10,13,16-hexaoxaoctacosyl)phosphocholine chloride (**7**)

Chloromethylchloroformate (219 μL, 3.31 mmol) was slowly added to an ice-cold mixture of C₁₂E₄ (1.00 g, 2.76 mmol) and anhydrous pyridine (333 μL, 4.14 mmol) in anhydrous CH₂Cl₂, under argon. After stirring for 2 h at room temperature, the reaction mixture was washed with 2 % HCl (2 x 40 mL) and brine (2 x 40 mL). The organic layer was dried over MgSO₄, filtered and concentrated under vacuum to afford pure chloromethyl carbonate **16** as a colorless oil (1.07 g, 85 %). TLC R_f 0.4 (CH₂Cl₂/MeOH 98:2). ¹H-NMR (400 MHz, CDCl₃) δ 5.73 (s, 2H), 4.37 (td, ³J_{HH} = 3.7 and 6.0 Hz, 2H), 3.76–3.74 (m, 2H), 3.67–3.56 (m, 12H), 3.45 (t, ³J_{HH} = 6.79 Hz, 2H), 1.61–1.55 (m, 2H), 1.30–1.23 (m, 18H), 0.88 (t, ³J_{HH} = 6.9 Hz, 3H). ¹³C-NMR (100 MHz, CDCl₃) δ 153.3, 72.2, 71.5, 70.7, 70.6, 70.5 (2C), 70.0, 68.6, 68.0, 31.9, 29.6 (2C), 29.5, 29.4, 29.3, 26.1, 22.6, 14.1. FT-IR (film) ν 2924, 2858, 2380, 2335, 1766, 1250, 1110.

A suspension of glycerophosphoryl choline (4.00 g, 15.6 mmol) and dibutyltin oxide (4.08 g, 16.4 mmol) in *iso*-propanol (200 mL) was refluxed for 6 h. The resulting suspension was cooled to 0 °C in an ice bath. Anhydrous triethylamine (2.32 mL, 17.2 mmol) and oleoyl

chloride (5.67 mL, 3.56 mmol) were added. The reaction was stirred for 18 h at room temperature. A mixture of $\text{CHCl}_3/\text{MeOH}$ (2:1, 200 mL) was added and the precipitate was filtered off. After concentration under reduced pressure, the residue was purified by chromatography over silica gel ($\text{CH}_2\text{Cl}_2/\text{MeOH}/\text{H}_2\text{O}$ 9:1:0 to 10:8:2) to provide lysophosphocholine **17** as a white powder (7.40 g, 91 %). TLC R_f 0.2 ($\text{CH}_2\text{Cl}_2/\text{MeOH}/\text{H}_2\text{O}$ 10:4:0.5). $^1\text{H-NMR}$ (400 MHz, CDCl_3) δ 5.34–5.32 (m, 2H), 4.33 (m, 2H), 4.06–3.83 (m, 6H), 3.35 (s, 9H), 2.29 (dd, $^3J_{\text{HH}} = 7.1$ and 8.2 Hz, 2H), 2.0 (q, $^3J_{\text{HH}} = 5.9$ Hz, 4H), 1.58–1.55 (m, 2H), 1.31–1.22 (m, 20H), 0.87 (t, $^3J_{\text{HH}} = 6.7$ Hz, 3H). $^{13}\text{C-NMR}$ (100 MHz, CDCl_3) δ 173.7, 129.9, 129.6, 68.8, 67.0, 66.0, 65.1, 59.3, 54.1, 34.1, 31.8, 29.7, 29.4, 29.2, 29.1, 27.1, 24.8, 22.6, 14.0. $^{31}\text{P-NMR}$ (162 MHz, CDCl_3) δ 0.13. FT-IR (film) ν 3358, 2924, 2854, 2360, 2342, 1732, 1466, 1233, 1086, 1058, 969.

To a stirred solution of α -linolenic acid (53 mg, 0.19 mmol) in anhydrous CHCl_3 (10 mL) was added CDI (0.4 M in CH_2Cl_2 , 625 μL , 0.25 mmol) under argon, at room temperature. In the mean time, DBU (30 μL , 0.19 mmol) was added to a solution of compound **17** (50 mg, 96 μmol) in anhydrous DMSO (10 mL). After stirring for 2 h, the CHCl_3 solution was transferred to the DMSO solution *via* a cannula and the resulting mixture was stirred for 24 h at room temperature. The crude reaction mixture was decomposed with 0.1 M acetic acid (10 mL), and extracted with $\text{CHCl}_3/\text{MeOH}$ 2:1 (2 x 30 mL). The organic layers were combined and concentrated under reduced pressure. The residual orange-brown viscous oil was purified by chromatography over silica gel ($\text{CH}_2\text{Cl}_2/\text{MeOH}/\text{H}_2\text{O}$ 9:1:0 to 10:4:0.5) to yield compound **18** as an off-white waxy solid (49 mg, 65 %). TLC R_f 0.2 ($\text{CH}_2\text{Cl}_2/\text{MeOH}/\text{H}_2\text{O}$ 10:4:0.5). $^1\text{H-NMR}$ (400 MHz, CDCl_3) δ 5.41–5.31 (m, 8H), 5.23–5.21 (m, 1H), 4.43–4.37 (m, 3H), 4.14 (dd, $^3J_{\text{HH}} = 7.1$ and 12.1 Hz, 1H), 4.02–3.94 (m, 4H), 3.42 (s, 9H), 2.83–2.80 (m, 4H), 2.34–2.27 (m, 4H), 2.09–2.01 (m, 8H), 1.63–1.55 (m, 4H), 1.36–1.25 (m, 28H), 1.06–0.89 (m, 6H). $^{13}\text{C-NMR}$ (100 MHz, CDCl_3) δ 173.5, 173.1, 131.9, 130.2, 129.9, 129.6, 128.3, 128.2, 127.7, 127.1, 70.5, 70.4, 66.4, 63.4, 62.9, 59.2, 54.5, 50.4, 34.3, 34.1, 31.9, 31.5, 29.7, 29.6, 29.5, 29.3 (2C), 29.2 (2C), 29.1 (2C), 29.0, 27.2, 27.1, 25.6, 25.5, 24.9, 24.8, 22.6, 22.5, 20.5, 14.2, 14.1. $^{31}\text{P-NMR}$ (162 MHz, CDCl_3) δ -1.67. FT-IR (film) ν 2922, 2853, 2360, 2341, 1738, 1457, 1436, 1416, 1258, 1235, 1213, 1154, 1065, 1022, 967.

To a solution of 1-oleoyl-2- α -linolenoyl-*sn*-glycero-3-phosphatidylcholine **18** (275 mg, 0.35 mmol) in anhydrous CHCl_3 (2 mL) was added chloromethyl carbonate **16** (1.27 g, 2.80 mmol) in CHCl_3 (1 mL). The mixture was refluxed for 18 h under an argon atmosphere. Then, it was cooled down at room temperature and concentrated under reduced pressure. Purification by chromatography over silica gel ($\text{CH}_2\text{Cl}_2/\text{MeOH}$ 98:2 to 90:10) provided compound **7** (mixture of two diastereomers, 130 mg, 30 %) as a waxy solid. TLC R_f 0.2 ($\text{CH}_2\text{Cl}_2/\text{MeOH}$ 88:12). $^1\text{H-NMR}$ (400 MHz, CDCl_3) δ 5.71–5.66 (m, 2H), 5.41–5.31 (m, 8H), 5.26–5.25 (m, 1H), 4.61 (br, 2H), 4.40–4.12 (m, 8H), 3.77–3.57 (m, 16H), 3.45 (s, 9H), 2.83–2.77 (m, 4H), 2.35–2.33 (m, 4H), 2.1–2.0 (m, 8H), 1.62–1.56 (m, 6H), 1.31–1.26 (m, 46H), 0.98–0.86 (m, 12H). $^{13}\text{C-NMR}$ (100 MHz, CDCl_3) δ 173.2, 172.8, 153.2, 131.9, 130.2, 130.0, 129.7, 128.3, 128.2, 127.7, 127.1, 86.0, 71.5, 70.7, 70.6 (2C), 70.5, 70.1, 70.0, 69.2, 68.9, 68.5, 68.1, 68.0, 67.1, 66.6, 66.5, 65.3, 65.2, 62.4, 62.3, 62.2, 61.4, 61.3, 54.6, 50.8, 34.1, 33.9, 31.9, 31.8, 29.7 (2C), 29.6 (2C), 29.5 (2C), 29.3 (2C), 29.2 (2C), 29.1, 29.0 (2C), 28.9, 27.2 (2C), 27.1, 26.1, 25.6, 25.5, 24.8, 22.7, 22.5, 20.5, 14.2, 14.1, 14.0. $^{31}\text{P-NMR}$ (162 MHz, CDCl_3) δ -3.13, -3.30. ESI-MS (m/z): calc. for $\text{C}_{66}\text{H}_{123}\text{NO}_{15}\text{P}$ 1200.86, found 1200.8. FT-IR (film) ν 2922, 2852, 2846, 2362, 1742, 1270, 1234.

2.2.6. Synthesis of 1,2-dilinolenoyl-*sn*-glycero-3-(3-oxo-2,4,7,10,13,16-hexaoxaoctacosyl)phosphocholine chloride (**8**)

To a stirred solution of α -linolenic acid (1.43 mL, 4.68 mmol) in anhydrous CH_2Cl_2 (25 mL) was added CDI (0.4 M in CH_2Cl_2 , 15.2 mL, 6.08 mmol) under an argon atmosphere, at room

temperature. In the mean time, DBU (700 μ l, 4.68 mmol) was added to a solution of glycerophosphoryl choline (401 mg, 1.56 mmol) in anhydrous DMSO (25 mL). After stirring for 3 h, the CH_2Cl_2 solution was transferred to the DMSO solution *via* a cannula. After stirring for 24 h at room temperature, the crude reaction mixture was decomposed with 0.1 M acetic acid (60 mL), and extracted with $\text{CHCl}_3/\text{MeOH}$ 2:1 (5 x 50 mL). The organic layers were washed with a $\text{H}_2\text{O}/\text{MeOH}$ 1:1 (2 x 100 mL), dried over MgSO_4 , filtered and reduced under vacuum. The residual brown viscous oil was purified by chromatography over silica gel ($\text{CH}_2\text{Cl}_2/\text{MeOH}/\text{H}_2\text{O}$ 10:4:0 to 10:4:0.5) to provide compound **19** as an off-white, waxy solid (808 mg, 67 %). TLC R_f 0.25 ($\text{CH}_2\text{Cl}_2/\text{MeOH}/\text{H}_2\text{O}$ 10:4:0.5). ^1H -NMR (400 MHz, CDCl_3) δ 5.42–5.28 (m, 12H), 5.22–5.17 (m, 1H), 4.42–4.27 (m, 3H), 4.16–4.09 (m, 1H), 3.99–3.89 (m, 2H), 3.81 (br, 2H), 3.36 (s, 9H), 2.82–2.79 (m, 8H), 2.31–2.26 (m, 4H), 2.11–2.02 (m, 8H), 1.61–1.54 (m, 4H), 1.35–1.27 (m, 20H), 0.99–0.87 (m, 6H). ^{13}C -NMR (100 MHz, CDCl_3) δ 173.5, 173.1, 131.9, 130.2, 128.3, 128.2, 127.7, 127.1, 70.6, 70.5, 66.5, 66.4, 63.4, 63.3, 63.0, 59.3, 59.2, 54.5, 34.3, 34.1, 31.5, 29.6 (2C), 29.3, 29.2 (2C), 29.1 (3C), 27.2, 25.6, 25.5, 24.9, 24.8, 22.6, 20.5, 14.3. ^{31}P -NMR (162 MHz, CDCl_3) δ -1.05. FT-IR (film) ν 3403, 2923, 2853, 1740, 1465, 1377, 1243, 1152, 1070, 969.

To a solution of compound **19** (234 mg, 0.30 mmol) in anhydrous CHCl_3 (4 mL) was added **16** (1.09 g, 2.41 mmol) in CHCl_3 (1 mL). The resulting reaction mixture was refluxed for 18 h under an argon atmosphere. Then, it was cooled down at room temperature and concentrated under reduced pressure. The residue was purified by chromatography over silica gel ($\text{CH}_2\text{Cl}_2/\text{MeOH}$ 98:2 to 90:10) to provide **8** as a waxy solid (mixture of two diastereomers, 102 mg, 28 %). TLC R_f 0.25 ($\text{CH}_2\text{Cl}_2/\text{MeOH}$ 88:12). ^1H -NMR (400 MHz, $\text{CDCl}_3/\text{CD}_3\text{OD}$ 1:1) δ 5.72–5.64 (m, 2H), 5.39–5.32 (m, 12H), 5.27–5.22 (m, 1H), 4.60 (br, 2H), 4.37–4.11 (m, 8H), 3.78–3.42 (m, 25H), 2.81–2.75 (m, 8H), 2.36–2.30 (m, 4H), 2.11–2.03 (m, 8H), 1.68–1.54 (m, 6H), 1.37–1.23 (m, 34H), 0.99–0.87 (m, 9H). ^{13}C -NMR (100 MHz, $\text{CDCl}_3/\text{CD}_3\text{OD}$ 1:1) δ 173.2, 172.8, 153.2, 131.9, 130.2, 130.1, 128.3, 128.2, 127.7, 127.1, 85.9, 71.5, 70.6, 70.5, 70.0, 69.9, 69.2, 69.1, 69.0, 68.5, 68.1, 68.0, 67.0, 66.6, 66.5, 66.4, 65.2, 65.1, 62.3, 62.2, 62.1, 61.4, 61.3, 54.6, 34.1, 33.9, 31.2, 31.5, 29.6 (2C), 29.4, 29.3, 29.2 (2C), 29.1, 29.0 (2C), 27.2, 26.0, 25.6, 25.4, 24.8, 22.7, 22.5, 20.5, 14.3, 14.1, 14.0. ^{31}P -NMR (162 MHz, $\text{CDCl}_3/\text{CD}_3\text{OD}$ 1:1) δ -3.13, -3.28. ESI-MS (m/z): calc. for $\text{C}_{66}\text{H}_{119}\text{NO}_{15}\text{P}$ 1196.83, found 1196.8. FT-IR (film) ν 2931, 2857, 2375, 2330, 1765, 1250, 1115.

2.2.7. Synthesis of 1-oleoyl-2-[cholesteryloxy(oxomethyl)]-sn-glycero-3-(3-oxo-2,4,7,10,13,16-hexaoxa octacosyl)phosphocholine chloride (**9**)

A solution of cholesteryl chloroformate (1.12 g, 2.25 mmol) in anhydrous CHCl_3 (5 mL) was added drop-wise to an ice-cold solution of compound **17** (500 mg, 0.98 mmol) and 4-DMAP (482 mg, 3.94 mmol) in anhydrous CHCl_3 (25 mL), under argon. The resulting reaction mixture was stirred for 16 h at room temperature. After concentration under reduced pressure, the residue was purified by chromatography over silica gel ($\text{CH}_2\text{Cl}_2/\text{MeOH}/\text{H}_2\text{O}$ 95:5:0 to 10:3:0.5) to provide compound **20** as an off-white, waxy solid (684 mg, 76 %). TLC R_f 0.6 ($\text{CH}_2\text{Cl}_2/\text{MeOH}/\text{H}_2\text{O}$ 10:3:0.2). ^1H -NMR (400 MHz, $\text{CDCl}_3/\text{CD}_3\text{OD}$ 1:1) δ 5.38–5.37 (m, 1H), 5.32–5.29 (m, 2H), 5.07–5.02 (m, 1H), 4.45–4.38 (m, 2H), 4.26–4.16 (m, 3H), 4.04–4.01 (m, 2H), 3.60–3.57 (m, 2H), 3.19 (s, 9H), 2.36–2.28 (m, 4H), 2.03–1.77 (m, 9H), 1.66–0.83 (m, 56H), 0.67 (s, 3H). ^{13}C -NMR (100 MHz, $\text{CDCl}_3/\text{CD}_3\text{OD}$ 1:1) δ 174.5, 154.6, 139.8, 130.5, 130.2, 123.7, 79.1, 74.9, 71.2, 70.6, 67.1, 63.1, 59.7, 59.6, 57.4, 56.8, 54.6, 54.5 (2C), 50.7, 49.9, 42.9, 40.4, 40.1, 38.6, 37.5, 37.1, 36.8, 36.2, 34.6, 32.5 (2C), 30.3, 30.1, 29.9 (2C), 29.8, 29.7, 29.6 (2C), 28.7, 28.5, 28.3, 27.7 (2C), 25.4, 24.8, 24.3, 23.2, 23.0, 22.8, 21.6, 19.6, 19.1, 14.3, 12.2. ^{31}P -NMR (162 MHz, $\text{CDCl}_3/\text{CD}_3\text{OD}$ 1:1) δ -0.73. FT-IR (film) ν 2922, 2853, 2165, 2138, 2055, 2046, 2023, 1998, 1982, 1740, 1070.

Compound **9** (mixture of two diastereomers, 35 mg, 8 %) was obtained as a waxy solid from **20** (275 mg, 0.35 mmol), following the same procedure as for **8**. TLC R_f 0.2 ($\text{CH}_2\text{Cl}_2/\text{MeOH}$

88:12). ¹H-NMR (400 MHz, CDCl₃/CD₃OD 1:1) δ 5.73–5.67 (m, 2H), 5.39–5.31 (m, 3H), 5.09–5.07 (m, 1H), 4.57–4.56 (br, 2H), 4.39–4.27 (m, 7H), 3.78–3.73 (m, 4H), 3.64–3.56 (m, 12H), 3.44 (t, ³J_{HH} = 6.8 Hz, 2H), 3.24 (s, 9H), 2.38–2.31 (m, 4H), 2.03–1.78 (m, 8H), 1.68–1.43 (m, 12H), 1.34–1.24 (m, 42H), 1.16–0.86 (m, 26H), 0.67 (s, 3H). ¹³C-NMR (100 MHz, CDCl₃/CD₃OD 1:1) δ 174.3, 154.3, 154.3, 139.7, 139.6, 130.5, 130.2, 123.8, 86.9, 79.6, 78.4, 72.1, 71.0, 70.9 (2C), 70.5, 69.1, 68.6, 57.3, 56.8, 42.9, 40.4, 40.1, 38.5, 37.4, 37.1, 36.8, 36.1, 34.5, 32.5, 30.3 (2C), 30.2 (2C), 30.1, 30.0 (2C), 29.9 (2C), 29.8, 29.6 (2C), 28.8, 28.5, 28.3, 28.2, 27.7, 27.6, 26.6, 25.4, 24.8, 24.4, 23.2, 23.0, 22.8, 21.6, 19.6, 19.1, 14.3, 12.2. ³¹P-NMR (162 MHz, CDCl₃/CD₃OD 1:1) δ -3.32, -3.51. ESI-MS (*m/z*): calc. for C₇₅H₁₃₇NO₁₆P 1338.96, found 1338.9. FT-IR (film) ν_{max} 2924, 2854, 2361, 2341, 2184, 2172, 1749, 1261, 984.

2.3. Animals

Nine-week-old male BALB/c mice were purchased from Charles River Laboratories (Saint-Germain-sur-l'Arbresle, France). They were housed in polycarbonate exhaust ventilated cages (M.I.C.E.[®] cages, Animal Care Systems) at a number of 4 mice per cage, with bedding made from spruce wood chips (Safe, Villemoisson, France). Ventilation in the cages was set to 10-12 changes per hour, according to the manufacturer's recommendations. The animal room was maintained under controlled environmental conditions, with a temperature of 20 ± 2 °C, a relative humidity of 50 ± 10 % and a 12 h/12 h light/dark cycle. Food (standard diet 4RF21, Mucedola) and tap water were available *ad libitum*. The animals were acclimated for 1 week before the initiation of the study. Animal experimentation was conducted with the approval of the government body that regulates animal research in France (Agreement number: AL/23/30/02/13).

2.4. Lipoplex preparation

Typically, the lipid components of the desired formulation were first mixed at the appropriate molar ratio in CHCl₃/MeOH (2/1, v/v) in a glass sample vial. The solvent was removed under vacuum for at least 3 h. The dried lipid film was then hydrated for 10 min in pure water and vigorously vortexed for 2 min to generate liposomes. Lipid-DNA complexes were prepared by gently mixing equal volumes of liposomes and pDNA solutions. The complexes were allowed to form for 15 min at room temperature without handling. Finally, the mixture was homogenized by pipetting up and down and subsequently used for *in vivo* gene delivery experiments. GL67A-pDNA complexes were prepared according to the literature (Griesenbach et al., 2011b).

2.5. DNA retardation assay

Freshly prepared lipoplexes at the desired N/P ratio were analyzed by electrophoresis through a 1 % agarose gel. The gel was run in a 40 mM Tris-acetate-EDTA buffer, pH 8.0 (TAE) and the DNA was stained using an ethidium bromide solution (EB, 0.5 μg/mL).

2.6. Dynamic light scattering measurements

The average particle size of lipoplexes was measured by photon correlation spectroscopy using a Zetasizer nanoZS apparatus (Malvern Instruments, Paris, France). All measurements were performed at 25 °C and in triplicate. Data were analyzed using the multimodal number distribution software supplied with the instrument and expressed as mean (± SD).

2.7. Airway gene delivery in mice

The lipoplexes (containing 40 μ g pCMV-GLuc in 50 μ L pure water, unless otherwise stated) were administered as a single dose into the lung of mice by intranasal instillation carried out under light anesthesia with 50 mg/kg ketamine (Imalgen®, Merial, Lyon, France) and 3.33 mg/kg xylazine (Rompun®, Bayer, Puteaux, France) given intraperitoneally. Control animals received instillation of the same volume of water. Mice were kept in a 60° inclined supine position until recovery from anesthesia to promote airway delivery of the solution through breathing. The experiment was terminated at 24 h (unless otherwise stated) by intraperitoneal injection of an overdose of ketamine and xylazine. The trachea was cannulated to perform bronchoalveolar lavages by 2 instillations of 0.5 mL ice-cold saline supplemented with 2.6 mM EDTA (saline-EDTA). BALFs were centrifuged (200 g for 5 min at 4 °C), and the resulting supernatant was stored at –20 °C until cytokine measurements. After thoracotomy, lungs were perfused *in situ* through the pulmonary artery with 10 mL of ice-cold PBS, collected, frozen in liquid nitrogen, and stored at –80 °C until assessment of the *Gaussia luciferase* gene expression.

2.8. *Gaussia luciferase* (GLuc) assay

Gaussia luciferase gene expression was assessed by measuring activity of the enzyme in mouse lung homogenates prepared in PBS containing a protease inhibitor cocktail (Complete EDTA-free tablets, Roche, Germany), using an Ultra-Turrax® homogenizer (T25, Ika, Staufen, Germany). After a 20-min incubation period on ice, the homogenates were transferred into Qias shredder columns (Qiagen, Courtabœuf, France) and centrifuged at 13,000 g for 1 min. Columns were then removed, the homogenates were centrifuged for an additional 5-min period and the supernatants were collected. *Gaussia luciferase* activity was measured by monitoring light production on an aliquot of sample for 1 sec upon addition of the coelenterazine substrate. Protein content was determined using a bicinchoninic acid assay. Luciferase activity in RLU was normalized to protein content. Value for each sample is the mean of a triplicate determination ($\pm$ SD).

2.9. Cytokine assays

Interleukine-6 and keratinocyte-derived chemokine were measured in BALFs by ELISA according to the manufacturer's instructions (BD Biosciences, Le Pont de Claix, France).

2.10. Statistics

Data are presented as means $\pm$ SD. Statistical differences between groups were determined by one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test, using the Kaleidagraph 4.5 software (Synergy Software, Reading, PA, USA). Data were considered as significantly different when $p < 0.05$ (*).

3. Results and discussion

3.1. From *in vitro* to *in vivo* transfection

While a wide variety of lipid formulations are used in cell culture experiments, the number of formulations investigated *in vivo* or in clinical research for gene therapy is more limited (Yin et al., 2014). The barriers to transfection *in vivo* are very different to those in cultured cells and not all formulations are compatible with use in live animals. One of the challenges is the technical problem associated with the preparation of monodispersed transfection particles at the concentration required for *in vivo* testing. Whereas *in vitro* experiments are typically conducted in the 1-10 μ M range for DNA phosphate content, *in vivo* transfection requires administration of the transfection reagents in the mM range, *i.e.* a thousand times higher concentration. This high

concentration most often introduces major alterations in the physical properties of the formulations which translate into the loss of transfection efficiency in animals and total inability for extrapolating *in vitro* results to *in vivo*, going so far as to question the relevance of *in vitro* experiments (Grosse et al., 2008). On that basis, *in vivo* evaluation of the title compounds just had to be open, even if that meant reversing previously obtained *in vitro* results. Optimum conditions for *in vitro* DNA delivery with cationic lipid **1** to various cell lines were obtained at charge neutrality (N/P = 1) and without the need for addition of helper lipid (e.g. 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine, DOPE) to improve transfection efficiency or decrease cytotoxicity (Pierrat et al., 2016a). Furthermore, the introduction of PEGylated lipids in the formulations led to significant loss in transfection efficiency *in vitro* (Fig. S1). This is a common observation as it was shown that the steric shielding due to PEGylation not only reduces undesired interactions with components of the cell culture medium, but also suppresses the electrostatic interactions with the plasma membrane that are conducive to cellular uptake (Mislick and Baldeschwieler, 1996; Song et al., 2002). Furthermore, it interferes with endosomal escape of the complexes by inhibiting fusion of the membranes of the lipoplexes with those of the endosomes, thus favoring DNA degradation upon endosome maturation and fusion with lysosomes (Chan et al., 2012; Mishra et al., 2004; Shin et al., 2003). Nevertheless, the distances to be crossed by transfection particles *in vivo* to reach target cells are much longer than in wells of cell culture plates, and the environment is harsher as well. Consequently, efficient *in vivo* transfection requires the use of transfection particles with extended half-life so they can safely transport nucleic acid over longer distances. In the absence of any more appropriate alternative to date, this is invariably achieved through stealth coating of the particles with PEG (Amoozgar and Yeo, 2012), and various biodegradable PEG-lipid conjugates have been developed to get the most of this strategy in lipofection (Chan et al., 2012; Kim et al., 2012; Li et al., 2005; Shin et al., 2003).

Starting from the results formerly obtained *in vitro* (Pierrat et al., 2016a), we first examined the *in vivo* transfection efficiency of DNA formulations with cationic lipid **1** incorporating increasing amounts of DOPE and PEG-lipid **2** (Fig. 1). PEG-lipid **2** displays a phosphoacetal moiety that is expected to provide the conjugate with biodegradability, as was demonstrated in our previous work for other DOPC derivatives (Pierrat et al., 2013a; Pierrat et al., 2013b). This first screening was realized using one equivalent of cationic lipid **1** with respect to the DNA phosphate content, as suggested by results obtained *in vitro*. The electrophoretic pattern obtained in the agarose gel retardation assay for the lipoplexes prepared under these conditions indicated that DNA was fully retarded (data not shown). Lipoplexes were prepared so as 40 μ g of pDNA (pCMV-GLuc) were administered in 50 μ L of water to anaesthetized mice by intranasal instillation. This route of administration was used, as it is noninvasive, effective in delivering particles into the lung of mice all throughout the airways, and reproducible (Ronzani et al., 2012). Larger administration volumes were not considered due to the low mouse tidal volume (ca. 120 μ L). Animals were sacrificed 24 h post-administration for measuring the level of reporter gene expression in the lungs. As might have been anticipated, the conditions previously optimized for transfection of cultured cells fully failed to promote any significant expression of the exogenous *Gaussia luciferase* (Fig. 2A). Contrary to our observations *in vitro*, addition of DOPE in the formulation revealed highly beneficial and essential for effective lung transfection. The level of transgene expression was maximized with **1**/DOPE/**3** 1:2:0.3 (molar ratio with respect to phosphate content of DNA), resulting in 10-fold higher gene expression than with naked DNA. As PEG-lipid **2** brings a cationic charge to the complex, this raised the question of the influence of the "extra" charge on the transfection efficiency. We thus investigated formulations with increasing N/P values and observed a quasi-linear fit with the transgene expression level in the range examined (Fig. 2B). With the **1**/DOPE/**3** 2:4:0.6 formulation (N/P = 2.6), gene expression was thus improved by a 20-fold factor when compared to naked DNA. The *in vivo* transfection efficiency of lipoplexes with charge ratios higher than 2.6 however have not been

investigated because the resulting formulations rapidly became highly viscous and would have triggered acute respiratory distress syndrome in mice.

Fig. 2. *In vivo* transfection efficiency of cationic lipid **1**. (A) Lipoplexes were prepared with 1 equiv. of cationic lipid **1** (with respect to the phosphate content of DNA), in the presence of DOPE (0, 1 and 2 equiv.) and PEGylated lipid **2** (0.02 to 1 equiv.). (B) Effect of charge ratio N/P on the *in vivo* transfection efficiency of cationic lipid **1**. pDNA was complexed with **1**/DOPE/**2** at the indicated molar ratio. The lipoplexes (containing 40 μ g DNA) were administered to animals as a single dose in pure water (50 μ L) as described in the Materials and methods section. Control (C) refers to mice that received naked DNA (40 μ g) in pure water (50 μ L). Statistically significant differences at $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***) when compared to control (n = 4).

3.2. GLuc expression profile in mouse lung

In order to assess the time course of transgene expression, mice were administered with pCMV-GLuc complexes prepared from **1**/DOPE/**2** in the 2:4:0.6 molar ratio and sacrificed at different time points. The gene expression profile was transient which follows the expected profile for the short-acting CMV promoter/enhancer (Fig. 3). The highest level was observed at day 1 and was by two orders of magnitude higher than background GLuc expression (*i.e.* at time zero). At day 3 after transfection, residual transgene expression was ten times higher than background. Longer time has not been investigated. This expression profile is consistent with a comparatively short half-life of GLuc *in vivo* (approximately 20 min (Tannous, 2009)). Though the protein was shown to be rapidly removed from the systemic circulation by excretion *via* the kidneys, it is less clear how it is cleared from the lung. It may be however hypothesized that the secreted protein may be degraded by proteases which are abundant in the airway mucus (Andersen and Sarangapani, 2001; Garcia-Verdugo et al., 2010; Goeminne et al., 2014; Kurnatowska, 1998).

Fig. 3. Time course of GLuc expression in mouse lung after transfection. Mice were administered with pCMV-GLuc DNA plasmid (40 μ g) complexed with **1**/DOPE/**2** in the 2:4:0.6 molar ratio (with respect to the phosphate content of DNA). Animals were euthanized 24, 48, and 72 h post-administration for determination of luciferase expression in lung homogenate. Values at time zero correspond to mice treated with the vehicle alone (pure water, 50 μ L) and euthanized at 72 h. Statistically significant differences at $p < 0.01$ (**), and $p < 0.001$ (***) when compared to negative control (T = 0) (n = 4).

3.3. Effect of the DNA dose

Dose escalation was investigated. The lipoplexes were prepared with **1**/DOPE/**2** in the 2:4:0.6 molar ratio. All mice were administered with the same volume of aqueous preparation and sample viscosity thus increased with increasing dose. Animals received up to 60 μ g of DNA (*i.e.* nucleic acid concentration of 1.2 mg/mL). Higher DNA doses were not evaluated because of heterogeneity and viscosity of the resulting preparations. Transgene expression was analyzed 24 h post-administration. The data collected revealed a linear fit of luciferase expression with the DNA dose administered within the range examined ($R^2 = 0.986$) (Fig. 4A). In order to evaluate the toxicity of the formulations, we measured the level of two cytokines involved in acute inflammation, IL-6 and KC, in BALFs. Toxicity of cationic lipids may result from inflammatory effects *in vivo* and subsequent immunological response of cells (Chadwick et al., 1997; Li et al., 1999; Loney et al., 2012; Scheule et al., 1997; Yew et al., 1999). As they participate in the communication networks that form the foundations of immunity, polymorphonuclear neutrophils (PMNs) play an essential role in lung immunopathogenesis. They are one of the first cell types to arrive at sites of infection and secrete cytokines and chemokines, of which IL-6 and KC, that are critical in the unfolding of the inflammatory response and in establishing the correct environmental conditions for launch of the adaptive immune response (Amulic et al., 2012). Monitoring of IL-6 and KC revealed a quasi-linear fit of the data with the DNA dose (IL-6: $R^2 = 0.965$; KC: $R^2 = 0.927$) (Fig. 4B).

This was most probably indicating that acute inflammation was linked to the efficacy of the gene delivery process rather than to inadequate rheological properties of the samples administered, namely in terms of viscosity and particle size.

Fig. 4. Transfection efficiency in the lung (**A**) and cytokine concentration in BALFs (**B**, IL-6: blue bar; KC: red bar) from mice treated with an escalating DNA dose. pDNA was complexed with **1**/DOPE/**2** in the 2:4:0.6 molar ratio. Control (C) refers to the administration of the vehicle alone (pure water, 50 μ L). Statistically significant differences at $p < 0.01$ (**), and $p < 0.001$ (***) when compared to control (n = 4).

3.4. Effect of the PEG-lipid biodegradability

As was mentioned above, biodegradability of a PEG-lipid used to stabilize lipoplexes may have crucial impact on the *in vivo* transfection efficiency. In order to further characterize our nucleic acid delivery platform, we made comparisons between the biolabile PEG-lipid **2** and its more robust phosphotriester analog **3** (Fig. 1). Lipoplexes were thus prepared with **1**/DOPE/**2** and **1**/DOPE/**3** in the 2:4:0.6 molar ratio and administered to mice. GLuc expression was found similar in both series of treated animals and the PEG-lipid biodegradability did not make significant difference in terms of transfection efficiency of the resulting lipoplexes (Fig. 5A). Increased interindividual variability was however observed in animals treated with the DNA formulation containing the more stable PEG-lipid. With regard to the inflammatory response, the level of cytokine production clearly appeared under the control of the biodegradability of the PEG-lipid (Fig. 5B). Indeed, production of IL-6 in BALFs was reduced by 71% when improved biodegradability was introduced in the PEG-lipid (*i.e.* compound **2**). The same applied, albeit to a lesser extent, to KC production that was decreased by 15%. These results indicate that a higher biodegradability of the formulation, but also possibly a modification of the site where degradation takes place (Zhao et al., 2011), is/are beneficial to the transfection efficiency of the PEGylated lipoplexes. Furthermore, although pro-inflammatory activity of the DNA formulation might be reduced depending on the nature of the PEG-lipid incorporated, the fact remained that the induced inflammatory response still was highly significant. Average IL-6 and KC production in BALFs established at 68.1 and 33.6 pg/mL (resp.) for untreated mice whereas it was about ten times higher (673.1 and 344.1 pg/mL, resp.) for mice that received pDNA formulated with **1**/DOPE/**2** 2:4:0.6. These conclusions prompted us to carry out additional experiments with other PEG-lipids specifically developed in our group. Compounds **4** and **5** thus differ from the initial PEG-lipids **2** and **3** as i-they incorporate a longer polymer chain (mPEG₅₀₀₀ vs. mPEG₂₀₀₀, resp.), and ii-they display a different backbone thus hopefully offering various degradation rates and profiles. Moreover, as inflammatory response most probably resulted from the high concentration of the cationic lipids in cells, the decision was made to decrease the amount of the latter in the formulations so as to lower their toxicity, even if this meant losing some transfection efficiency. Consequently we conducted the subsequent investigations at a charge ratio N/P of 1, with 1 molar equivalent of DOPE and 0.05 equivalent of PEG-lipid (Fig. 6). For comparison, DMPE-mPEG₅₀₀₀ was introduced in the series as it is used in a number of DNA delivery platforms, including GL67A. As can be seen, the three lipids conjugated with mPEG₅₀₀₀ revealed significantly more effective to improve DNA delivery than the mPEG₂₀₀₀-based lipid **2**, allowing a 12- to 25-fold increase in transfection efficiency. It is also worth noting that lipid **4** that was designed for improved biodegradability (through the insertion of an acetal linker between the lipid anchor and the PEG moiety) appeared twice as effective as DMPE-mPEG₅₀₀₀ and lipid **5** that do not incorporate a specifically engineered cleavage site. As the phosphoacetal motif was present in both PEG-lipids **2** and **4**, we then wanted to figure out whether superiority of the mPEG₅₀₀₀ derivative not just resulted from a higher surface coverage of the transfection particles by the polymer, *i.e.* moving from a mushroom to a brush regime for the grafted polymer chains (Allen et al., 2002). Mice were

thus administered with lipoplexes prepared with increasing amounts of the two PEG-lipids and both transfection efficiency and inflammation were measured 24 h post-administration (Fig. 7). It clearly appeared that the lesser length of the polymer chain in **2** could not be compensated by an increase of the lipid amount in the formulation. Therefore, lipoplexes prepared with 0.05 molar equivalent of PEG-lipid **2** displayed the same ratio of lipid to PEG monomer units as those prepared with 0.02 equivalent of PEG-lipid **4**, and yet showed transfection efficiency that was fifteen times less. This is consistent with recent results published by Moribe *et al.* who demonstrated that the PEG chain length rather than the PEG-lipid ratio contributes to the PEG chain flexibility which is responsible for the extended lifetime of PEGylated particles in biological media (Abe *et al.*, 2015). Besides, this experiment also demonstrated that 2-5 % of PEG-lipid **4** in the formulation was a good compromise between transfection efficiency and inflammatory response (Fig. 7B). Indeed higher dose had adverse effects on the transfection efficiency whereas lung inflammation was induced in a dose-dependent manner through the whole concentration range investigated. Additional efforts to refine the **1**/DOPE/**4** formulation did not allow further improvement of the transfection efficiency reached with these particles (Fig. S2). Thus by decreasing the charge ratio and selecting an adequate PEG-lipid, we could significantly reduce the inflammatory response in mice (-30 % for IL-6 release, -59 % for KC release). This however was accompanied with significant reduction of the transgene expression level (-43 %).

Fig. 5. Transfection efficiency (A) and inflammatory response (B; IL-6: blue bar; KC: red bar) in mice upon administration of lipoplexes (40 μ g of pDNA per animal) prepared from **1**/DOPE/**2** and **1**/DOPE/**3** in the 2:4:0.6 molar ratio. Control (C) stands for the administration of the vehicle alone (pure water, 50 μ L). Statistically significant differences at $p < 0.01$ (two symbols) and $p < 0.001$ (three symbols) when compared to control (*) or indicated formulation (#) (n = 4).

Fig. 6. Transfection efficiency of the association of cationic lipid **1** and DOPE 1:1, in the presence of 5 molar % of the PEGylated lipids **2**, **4**, **5**, or DMPE-mPEG₅₀₀₀ (n = 4).

Fig. 7. Transfection efficiency (A) and inflammatory response (B; IL-6: blue bar; KC: red bar) after administration of lipoplexes prepared from **1**/DOPE/**2** and **1**/DOPE/**4** in the 1:1:0.02-0.2 molar ratio. Control (C) stands for the administration of the vehicle alone (pure water, 50 μ L). Statistically significant differences at $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***) when compared to control (n = 4).

3.5. Modulation of the cationic lipid scaffold

En route for a further optimization of our formulations, we also investigated some modifications of the scaffold of the cationic phospholipid-detergent conjugates. What makes cationic lipids effective to mediate nucleic acid intracellular delivery probably also is what makes their basic shortcomings. Indeed, the toxicity of cationic lipids is mainly determined by their cationic nature, though it must be considered that the effect of hydrophobic chain on toxicity has not been adequately addressed to date (Lv *et al.*, 2006). Cationic DNA/lipid nanoparticles interact with the cell membrane, and the high density of positive charges close to the cell surface is likely to be recognized as a danger signal by cells and contributes to activate various intracellular signaling pathways like pro-apoptotic and pro-inflammatory cascades (Lonez *et al.*, 2012). It has also been proposed that lipoplexes may mechanically trigger membrane disruption provoking Ca²⁺ influx and, by the way, cytotoxicity and cell death (Hwang *et al.*, 2015). Consequently, vector candidates displaying a transient cationic charge might help reducing the toxicity of the resulting lipoplexes. With this in mind, we designed a series of analogs of cationic lipid **1** (compounds **6-9**, Fig. 1). In these compounds, the stability of the phosphotriester backbone was compromised through the installation of an acetal motif, as already performed in the two PEG-lipids **2** and **4**. Improved biodegradability should accelerate the decomposition of these compounds into neutral phosphocholine

derivatives with lower impact on cell viability. We already evidenced advantageous effects of such a transformation *in vitro* (Pierrat et al., 2013a) but validation still was awaited *in vivo*. Structure of compound **6** was thus directly extrapolated from cationic lipid **1**. Besides, additional modifications with regards to the fatty acid chains on the glycerophosphocholine backbone were introduced as they might as well provide ameliorations in the transfection efficiency and safety of the resulting reagents. Endogenous and naturally occurring fatty acids or alcohols were considered because no major toxicity or inflammation was expected to result from compounds incorporating the latter or from their potential degradation products. This is all the more true for polyunsaturated fatty acids for which anti-inflammatory and anti-oxidant activities were reported (Yuan et al., 2014). Anti-oxidant properties of α -linolenic acid and esters may thus present special interest in the course of the transfection process. Endocytosis of particles is often accompanied by oxidative stress as cells have to manage/dispose a non natural perturbing material. The anti-oxidant activity of α -linolenic derivatives may help cells to re-achieve oxidative equilibrium, thus improving cell survival and therapeutic index. Furthermore, recent studies suggest that the high degree of unsaturation in hydrocarbon chains is able to facilitate nucleic acid delivery (Heyes et al., 2005; Le Gall et al., 2010). This is related to the increased ability of such lipids to form H_{II} phase, thus increasing the fusogenicity of the system (Epand et al., 1991; Sankaram et al., 1989; Szule et al., 2002). We thus designed cationic lipids **7** and **8** where the α -linolenoyl moiety was used as a surrogate for one or both oleoyl chains in the original DOPC scaffold. Last, in order to check whether some destabilization of the transfection particles upon interaction with the mucus components could be such as to partially reduce the transfection efficiency, cationic lipid **9** was prepared that displayed a cholesteryl substituent at the *sn*-2 position on the glycerol backbone. This modification was based on a previous work realized in Szoka's group revealing improved biomembrane properties for sterol modified phospholipids (Huang and Szoka, 2008). Compounds **6-9** were evaluated *in vivo*, using the above determined conditions for DNA delivery to the mouse lung, *i.e.* at the charge ratio N/P of 1, with a stoichiometric amount of DOPE and 5 molar % of PEG-lipid **4**. Regarding to transgene expression, significant improvements were obtained with this series of biodegradable compounds (Fig. 8). First, all of the compounds except compound **9** revealed superior to the parent lipid **1**. This is a strong indication that biodegradability of the cationic species is indeed a key for higher transfection efficiency. Second, the substitution of the monounsaturated oleoyl chain for a polyunsaturated α -linolenoyl one was clearly conducive to higher transgene expression, and replacement of both chains in the original DOPC backbone was especially fruitful. At the opposite, introduction of the cholesterol moiety did not translate into improved transfection properties. The best result thus were obtained with formulation **8**/DOPE/**4** 1:1:0.05 that achieved transfection in the mouse lung at a rate that was three times higher than that obtained with GL67A, the current benchmark for DNA delivery to the airway. Regarding the inflammatory response, results were even more pronounced (Fig. 9). The only introduction of an acetal linker in the lipid structure was responsible for a decrease in IL-6 and KC release by 68 and 90 %, respectively. When this modification was combined with replacement of one or two oleoyl chains by α -linolenoyl ones, no inflammation could be detected, cytokine release being not statistically different from that measured in control mice treated with the vehicle alone. Treatment with the GL67A-based lipoplexes provoked significant increase in IL-6 production (147.3 pg/mL *vs.* 42.5 pg/mL for control mice) and even more in KC production (168.6 pg/mL *vs.* 15.7 pg/mL for control mice). This is consistent with a previous report in the literature describing acute alveolar inflammation for GL67A (Scheule et al., 1997). Hence, the formulation of DNA with **8**/DOPE/**4** 1:1:0.05 revealed especially effective for promoting transgene expression in the mouse lung with minimal toxicity.

Fig. 8. Transfection efficiency of the cationic lipids **1** and **6-9** and comparison with formulation GL67A. Lipoplexes were prepared at charge ratio N/P = 1, with DOPE (1.0 equiv.) and PEGylated lipid **4** (0.02 equiv., except for compound **1**: 0.05 equiv.). Control (C) stands for the administration of the vehicle alone (pure water, 50 μ L). Naked DNA refers to the administration of DNA in water without any carrier. Statistically significant differences at $p < 0.05$ (one symbol), $p < 0.01$ (two symbols) and $p < 0.001$ (three symbols) when compared to control (*) or to the GL67A formulation (#) (n = 4-8).

Fig. 9. Airway inflammation induced by carriers **1** and **6-9** and comparison with formulation GL67A, as determined by measuring IL-6: (blue bar) and KC (red bar) in BALFs. Complexes were prepared at charge ratio N/P = 1, with DOPE (1.0 equiv.) and PEGylated lipid **4** (0.02 equiv., except for compound **1**: 0.05 equiv.). Control (C) stands for the administration of pure water. Naked DNA refers to the administration of DNA in water without any carrier. Statistically significant differences at $p < 0.05$ (one symbol), $p < 0.01$ (two symbols) and $p < 0.001$ (three symbols) when compared to control (*) or to the GL67A formulation (#) (n = 4-8).

3.6. Size and ζ potential of the transfection particles

The results obtained in the course of this work may be tentatively analyzed with regard to the intrinsic properties of the transfection particles, namely their size and zeta potential. Indeed, the latter are recognized parameters that govern to a great extent the *in vitro* transfection efficiency of nonviral vectors. *In vivo* however, things appear much less clear and depend, furthermore, very much on the administration route. A characteristic feature of the administration of gene therapy products through the airway results from the specific properties of lung epithelium which is covered by a thin mucus layer (5–55 μ m) (Lai et al., 2009; Roy and Vij, 2010). This viscous colloid composed of a dense mesh of glycoprotein (mucin) fibers poses a diffusional barrier to protect epithelial cells and traps particles, including nucleic acid containing nanoparticles, thus preventing their access to the underlying mucosa, while favoring their elimination *via* mucociliary transport or coughing. The mesh-pore size in mucus is in the range of 20-200 nm (Olmsted et al., 2001; Saltzman et al., 1994), and large particles are sought to undergo slower diffusional transport through mucus barriers or not even are they capable of crossing mucosal barriers. Our results however did not support this general view, since the size of the particles in the most powerful formulations were in the range 373–625 nm (Table SI-1, entry 16, 17, 25-27). A possible explanation for this may come from the very nature of mucus that does function like a size-exclusion chromatography matrix (Lai et al., 2007). In a network with heterogeneous pore size, small particles can access a greater number of small pores or pockets in the gel, resulting in an overall reduced transport rate over long distance. In the lung, to efficiently reach the target cells, transfection particles have to diffuse through the mucus to the periciliary layer faster than the viscous biopolymer is swept by mucociliary activity (Schuster et al., 2013). Consequently, retardation of the small particles getting lost in the smaller pores and pockets in the mucus gel favors their elimination by mucociliary clearance whereas larger particles move more rapidly towards the periciliary layer and may escape the elimination process. That being so, not every large particles described herein proved effective in delivering transgene to the lung cells (Table SI-1, entry 4, 7, and 9). Considering the charge of the transfection particles, no general trend did appear. Indeed, most transfection particles investigated in this work were negatively charged and, however, some of them revealed more effective for delivering the pCMV-GLuc transgene than the positively charged ones (Table SI-1, entry 25-27 vs. entry 16 and 17). It is generally invoked that transfection particles have to be positively charged to establish electrostatic interactions with the negatively charged plasma membrane of the cells so as to trigger cellular uptake through an endocytosis process. This ideal picture however most likely does not match *in vivo* reality as transfection particles interact with a number of negatively charged proteins before they can reach the target cells. As a consequence, a protein corona forms within seconds or minutes around the charged particles thus preventing direct contact between the nanoparticles and the membrane of cells (Caracciolo, 2015; Deng et al., 2013; Milani et al.,

2012; Mortensen et al., 2013; Walkey and Chan, 2012). Even when PEG shielding is protecting the particles, formation of a protein corona cannot be fully avoided and the biological identity of the nanoparticles is altered (Kim et al., 2007; Pozzi et al., 2014).

4. Conclusion

In summary, safe and effective synthetic carriers have been developed for gene delivery to the lung. These carriers result from the conjugation between a phospholipid and a nonionic detergent, C₁₂E₄. Conjugation through a biodegradable linker significantly attenuated the severity of inflammatory response that characterizes cationic lipid-mediated gene transfer. Concurrent introduction of polyunsaturated fatty acid chains within the carrier scaffold allowed significant improvement of transgene expression in the mouse lung as well as additional reduction of airway inflammation. The resulting phosphatidylcholine-detergent conjugates thus favorably compare to GL67A, the gold standard for DNA delivery to the airway that is currently under clinical evaluation. The biodegradable nucleic acid carriers investigated in this work could thus have great potential in gene therapy clinical applications. To this end, future research will focus on determination of the cell types and tissues (tracheal, bronchial/bronchiolar or alveolar) that are transfected in order to possibly get a hint on which respiratory disease could be a privileged target. As well, lipoplex viability under the more suitable but more challenging delivery conditions via aerosolization will be investigated.

Conflict of interest

The authors report no conflict of interest in this work.

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Appendix A. Supplementary data

In vitro transfection data, *in vivo* transfection efficiency and toxicity of additional formulations, size and ζ potential of the transfection particles.

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