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Anionic pH-sensitive pegylated lipoplexes to deliver DNA to tumors

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ABSTRACT

Anionic pegylated lipoplexes have been prepared from the combined formulation of cationic lipoplexes and pegylated anionic liposomes. To this end, two original (bis- and tetra-) carboxylated cholesterol derivatives have been synthesised. Titration of the particle surface charge was realised to determine the ratio between anionic and cationic lipids that would give pH-sensitive complexes. This ratio has been optimised to form particles sensitive to pH change in the range 5.5–6.5. Compaction of DNA into these newly formed anionic complexes was checked by DNA accessibility to picogreen and DNA electrophoresis on an agarose gel. Gene expression of the formulated gene was similar for the cationic formulation taken as a control and the anionic formulations prepared. The pH-sensitive properties of these formulations was shown in vitro using bafilomycin, a vacuolar H^+ATPase inhibitor. The efficiency of the new formulations to deliver DNA to the tumor was compared with cholesteryl hemisuccinate (CHEMS) formulations. The tetracarboxylated compound gave the most efficient formulations for tumor delivery in vivo.

1. Introduction

Non-viral gene therapy still suffers from limited efficacy in order to be involved in more clinical trials. Cationic particles have been the most widely studied, due to their strong interaction with DNA and increased intracellular delivery potency. However, this strong interaction is also a drawback for complete DNA intracellular delivery. Moreover, cationic charges induce a poor circulation time of these systems in the blood, mostly due to seric protein interaction. This has led to the use of a widely known polymer, the polyethylene glycol (PEG). Coating of the particles with PEG was shown to highly increase the circulation time of conventional liposomes (Zalipsky et al., 1994). Hence, this strategy was applied to cationic systems. Unexpectedly, conformation of the PEG on the particle surface did not highly increase the residential time of cationic particles in the blood, while it brought another drawback: inhibition of gene transfer. The question one can address is how to obtain long circulating particles able to carry DNA which would then lead to the expression of the protein it encodes for. For this purpose, neutral or slightly negative or positively charged particles should be privileged for increased circulation time. Moreover, these particles should be able to deliver their content upon a chosen trigger.

pH-sensitive particles represent a particular interest in cancer gene therapy. Hypoxia in the microenvironment of tumor induces a reduced pH, which could potentially trigger DNA release from acid-sensitive systems (Tannock and Rotin, 1989). DNA delivery vectors involving pH labile lipids (Guo and Szoka, 2003) or pH labile PEG lipids (Guo et al., 2003; Masson et al., 2004) have been shown to improve protein expression in the cells. This effect might be related to endosome acidification, even though this was indirectly shown by following degradation of the PEG lipids in acidic conditions.

Other interesting systems are acid-sensitive formulations. In this case, a mixture of well-chosen lipids would transmit the pH-sensitivity to the whole system. pH-sensitive liposomes have been prepared out of DOPE and CHEMS lipids. Stability of these liposomes is maintained while the lipid bearing carboxylates is charged, then upon a pH drop, protonation of this lipid lead to liposome destabilisation. pH-sensitive formulations were reported to release water soluble molecules such as calcein into the cytoplasm of cells. Ten years ago, Duzgunes et al. reported that it was possible to prolong circulation time of particles in vivo while maintaining calcein release into the cytoplasm of cells, and concluded at this time how useful it would be to deliver genes

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(Slepushkin et al., 1997). The point was that these particles were made of DOPE/CHEMS/PEG-PE, meaning negatively charged, and would not have been able to interact with genes without addition of cationic divalent ions. The concept was applied to antisense oligonucleotide delivery, however the stability of the complex in vivo was relatively poor (Fattal et al., 2004). A number of other combinations have been studied such as the phosphatidylglycerol/phosphatidylcholine mixture to deliver oligonucleotide and plasmid. The ionic forces of the buffers to increase nucleic acid to anionic liposomes association were pointed out and transgene expression in neurons was obtained (Lakkaraju et al., 2001).

The concept was also applied to targeted gene delivery. Simoes et al. (1998) associated the pH-sensitive peptide GALA, with cationic liposomes, under conditions where the liposome/DNA complex is negatively charged and found that it drastically increased the transgene expression. At the same time Lee and Huang (1996) developed the LPDII formulations, which are constituted of a mixture of DOPE/CHEMS/PEG-PE and polylysine. In that case, DNA/polylysine complexes were mixed with acid-sensitive CHEMS/DOPE/DOPE-Peg-folate liposomes to form acid-sensitive lipoplexes. Even though the concept works, a low amount of PEG lipid (0.1%) was used, far from the 5% usually used for systemic injections. Moreover, the pH change needed to destabilise the particles was not reported. Hafez et al. (2000) gave an advanced insight in these constructions, showing that it was possible to modulate the pH-sensitivity by controlling the number of cationic charges. Hence, by the association of CHEMS and the cationic lipid DODAC, the controlled amount of cationic lipid versus the anionic one could allow the control of the pH at which fusion occurred.

Particularly interested by these tunable systems, we ought to apply it to pegylated gene delivery systems. Indeed, such a pegylated formulation should be able to lead to DNA release upon a pH trigger, despite the presence of the PEG (Slepushkin et al., 1997). The cationic lipid we used exhibiting two primary amines, one secondary and one tertiary amine, we designed two original negatively charged cholesterol bearing two and four carboxylate moieties in order to limit the amount of cholesterol in the liposome. The mixture of the cationic lipid and the negatively charged cholesterol at appropriate ratio allowed condensing DNA in negatively charged particles. Thanks to the presence of the carboxylate moieties, the anionic particle charge was reversed to a cationic one at a determined appropriate pH.

2. Materials and methods

2.1. Abbreviations

PEG: polyethylene glycol; DOPE: dioleoylphosphatidylethanolamine; CHEMS: cholesteryl hemisuccinate; GALA: is a 30-amino acid synthetic peptide with a glutamic acid–alanine–leucine–alanine repeats; DODAC: dimethyldioctadecylammonium chloride; DPPC: dipalmytoylphosphatidylcholine.

2.2. Materials

CHEMS was purchased from Sigma, L-α-dioleoyl phosphatidylethanolamine (DOPE), and 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) were purchased from Avanti Polar Lipids. The solvents were from Carlo Erba-SDS (analytical grade). The pCAT-Control Vector was purchased from Promega.

The chol-PEG_{110} was obtained in one step from the reaction of cholesteryl chloroformate and α-amino-ω-methoxy-PEG. The names of the different lipids were generated with AutoNom 2000 software which is based on IUPAC rules. The cationic lipid whose name according to the nomenclature is 2-{3-[bis-(3-amino-propyl)-amino]-propylamino}-*N*-ditetradecylcarbamoyl methyl-acetamide or RPR209120 that we called DMAPAP was previously described in the supporting information of Thompson et al. (2005). The dicarboxylated cholesterol compound, (*cholesteryloxycarbonyl-carboxymethyl-amino*)-*acetic acid*, will be named CCDC and the tetracarboxylated derivative, [(*2*-{*cholesteryloxycarbonyl*-[*2*-(*bis-carboxymethyl-carbamoyloxy*)-*ethyl*]-*amino*}-*ethoxycarbonyl*)-*carboxymethyl-amino*]-*acetic acid*, will be named CCTC.

pK_a were calculated using Marvin Sketch 3.4.3 from ChemAxon software (http://www.chemaxon.com/marvin).

Size and zeta potentials measurements were performed on a Zeta Sizer NanoSeries from Malvern Instruments, France.

2.3. Synthesis

2.3.1. (Cholesteryloxycarbonyl-carboxymethyl-amino)-acetic acid (CCDC)

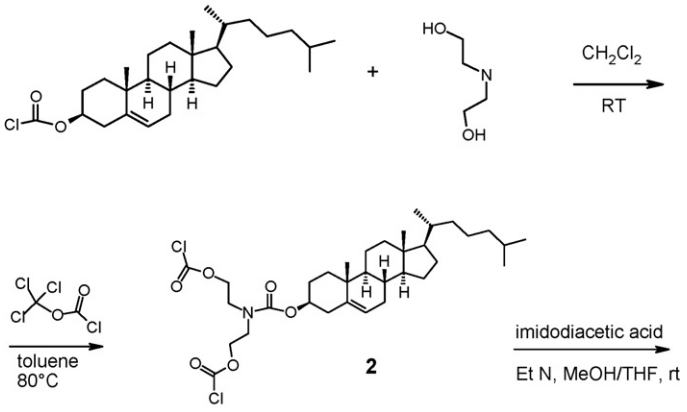
Iminodiacetic acid (0.15 g; 1.1 mmol) was suspended in anhydrous methanol (5 mL) with triethylamine (0.48 mL; 3 equiv.) and the mixture was stirred until dissolution. Cholesteryl chloroformate (0.45 g; 1 mmol) was added and the mixture was left 1 h at ambient temperature.

The solvent was then evaporated, the residue taken up in diethylether (20 mL) and washed with 1N HCl (1× 5 mL), H_2O (2× 5 mL) and 36 g/L NaCl (1× 5 mL). The organic layer was dried over sodium sulfate, filtered and concentrated to a white solid (0.5 g; 91%)

^1H NMR (400 MHz, CDCl3): δ (ppm) 0.66 (s, 3H: CH_3); 0.86 (d, J = 8 Hz, 6H: CH_3); 0.90 (d, J = 8 Hz, 3H: CH_3); 0.98 (s, 3H: CH_3); 1.64–1.03 (m, 22H: CH_2); 1.82 (m, 2H: CH_2); 1.97 (m, 2H: CH_2); 2.31 (m, 2H: CH_2); 3.94 (s, 2H: CH_2); 3.99 (s, 2H: CH_2); 4.5 (m, 1H: CH); 5.32 (m, 1H: =CH); 10.92 (bs, 2H: COOH).

^{13}C NMR (100 MHz, acetone D6): δ (ppm) 172.839; 172.560; 157.156; 141.683; 124.088; 77.210; 58.531; 58.014; 51.962; 51.146; 44.062; 41.583; 41.227; 40.083; 38.739; 38.279; 37.945; 37.597; 33.667; 33.580; 30.242; 29.909; 29.661; 25.908; 25.537; 24.082; 23.843; 22.736; 20.664; 20.148; 13.229.

2.3.2. [(2-{Cholesteryloxycarbonyl-[2-(bis-carboxymethyl-carbamoyloxy)-ethyl]-amino}-ethoxycarbonyl)-carboxymethyl-amino]-acetic acid (CCTC)



2.3.2.1. Synthesis (3 steps). Bis-(2-hydroxy-ethyl)-carbamic acid cholesteryl ester (*1*): a solution of cholesteryl chloroformate (1 g; 2.2 mmol) in dichloromethane (10 mL) was added dropwise to a solution of diethanolamine (0.42 mL; 4.4 mmol) in dichloromethane (10 mL). After 2 h the starting material had disappeared and the reaction medium was washed with 36 g/L NaCl (2× 5 mL), dried over $MgSO_4$, filtered and concentrated. The residue was crystallised in methanol (0.78 g, 67%).

^{1}H NMR (*400 MHz, CDCl3*): δ (ppm) 0.66 (s, 3H: CH_3); 0.86 (d, J = 8 Hz, 6H: CH_3); 0.90(d, J = 8 Hz, 3H: CH_3); 0.98 (s, 3H: CH_3); 2.03–1.03 (m, 27H: CH_2, CH); 2.32 (m, 2H: CH_2); 3.47 (m, 4H: CH_2); 3.81 (m, 4H: CH_2); 4.52 (m, 1H: CH); 5.37 (m, 1H: =CH).

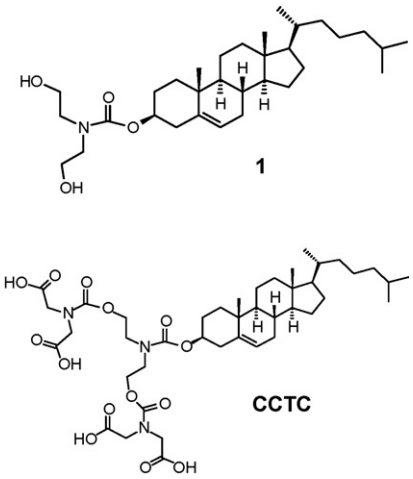
^{13}C NMR (*100 MHz, $CDCl_3$*): δ (ppm) 156.675; 139.590; 122.604; 75.253; 61.790; 56.618; 56.100; 52.508; 51.997; 49.932; 42.261; 39.678; 39.464; 38.572; 36.948; 36.511; 36.133; 35.771; 31.858; 31.808; 28.199; 27.972; 24.241; 23.821; 22.785; 22.524; 21.004; 19.324; 18.679; 11.812.

Bis-(2-chlorocarbonyloxy-ethyl)-carbamic acid cholesteryl ester (*2*): to a solution of **1** (0.5 g; 0.1 mmol) in toluene (15 mL), trichloromethyl chloroformate (0.7 mL; 0.6 mmol) was added and the mixture was heated at 80 °C during 2 h. The solution was evaporated to dryness then coevaporated with petroleum ether (3× 5 mL) and the gummy residue was used in the next step without further purification.

^{1}H NMR (*400 MHz, CDCl3*): δ (ppm) 0.68 (s, 3H: CH_3); 0.86 (d, J = 8 Hz, 6H: CH_3); 0.91(d, J = 8 Hz, 3H: CH_3); 1.02 (s, 3H: CH_3); 2.06–1.06 (m, 25H: CH_2, CH); 2.37 (m, 2H: CH_2); 3.63 (m, 4H: CH_2); 4.50–4.37 (m, 4H: CH_2); 4.55 (m, 1H: CH); 5.39 (m, 1H: =CH).

^{13}C NMR (*100 MHz, $CDCl_3$*): δ (ppm) 155.259; 155.244; 139.428; 139.416; 139.393; 136.244; 122.831; 75.973; 75.923; 69.753; 69.588; 56.617; 56.072; 49.926; 42.266; 42.266; 39.662; 39.473; 38.432; 38.412; 36.891; 36.514; 36.136; 35.757; 31.856; 31.800; 28.191; 27.981; 24.242; 23.784; 22.794; 22.534; 20.997; 18.679; 11.819.

[(*2-{Cholesteryloxycarbonyl-[2-(bis-carboxymethyl-carbamoyloxy)-ethyl]-amino}-ethoxycarbonyl)-carboxymethyl-amino*]-*acetic acid* (*CCTC*): iminodiacetic acid (1 g; 7.5 mmol; 4 equiv.) was suspended in methanol (20 mL), triethylamine (3.15 mL; 12 equiv.) was added and the suspension stirred at room temperature until dissolution. It was then added to a solution of **2** (1.28 g; 1.9 mmol) in THF (5 mL). The mixture was left 1 h at ambient temperature. It was evaporated, redissolved in diethyl ether (50 mL) and washed with 1N HCl (3× 10 mL), 36 g/L NaCl (10 mL), dried over Na_2SO_4, filtered and evaporated. The residue was suspended in petroleum ether and filtered (1.4 g; 90%).



^{1}H NMR (*400 MHz, acetone D6*): δ (ppm) 0.73 (s, 3H: CH_3); 0.87 (d, J = 8 Hz, 6H: CH_3); 0.95(d, J = 8 Hz, 3H: CH_3); 1.05 (s, 3H: CH_3); 1.76–1.07 (m, 13H: CH_2, CH); 2.37 (m, 2H: CH_2); 3.56 (m, 4H: CH_2); 4.17 (m, 8H: CH_2); 4.43 (m, 1H: CH); 5.39 (m, 1H: =CH).

^{13}C NMR (*100 MHz, acetone D6*): δ (ppm) 172.549; 157.548; 157.075; 141.981; 123.884; 76.605; 65.638; 58.551; 58.001; 52.013; 51.185; 50.950; 48.971; 48.550; 44.062; 41.596; 41.220; 40.186; 38.864; 38.315; 37.939; 37.589; 33.691; 33.595; 30.249; 30.103; 29.907; 29.763; 29.661; 25.909; 25.509; 24.066; 23.828; 22.743; 20.705; 20.133; 13.222.

2.4. Physico-chemistry and biology

2.4.1. Plasmid preparation

Luciferase encoding gene: pVax2 is a derivative of the commercial plasmid pVax1 (InVitrogen). pVax1 was digested with the restriction enzymes HincII and BamHI to excise the promoter. The plasmid was then blunted with the Klenow fragment and dephosphorylated with alkaline phosphatase. pCMVbeta (Clontech) was digested with EcoR1 and BamHI to excise the CMV promoter. The CMV promoter was blunted with the Klenow enzyme and ligated into the blunted pVax1 to give pVax2. The plasmid pXL3031 was digested with EcoRI and BamHI, and then treated with the Klenow fragment to produce a blunted fragment containing luciferase cDNA. This fragment was ligated into pVax2 after EcoR1 digestion and phosphatase alkaline dephosphorylation to give pVax2-Luc. A maxiprep Qiagen was used to produce the plasmid which was controlled as the right length: 4626 bp.

Chloramphenicolacetyltransferase encoding gene: the pMP6-CAT plasmid expressing the chloramphenicol acetyltransferase (*CAT*) gene was kindly provided by RPR-GenCell (Santa Clara, CA). The plasmid contains a mammalian expression cassette consisting of the cytomegalovirus (CMV) immediate early promoter and enhancer followed by a hybrid intron consisting of an adenovirus major late intervening sequence, a mouse immunoglobulin intervening sequence, and a simian virus (SV)40 polyadenylation sequence. The 5′ heterologous intron and SV40 polyadenylation signal sequence are designed to increase the stability of the transgene mRNA and to direct the mRNA out of the nucleus into the cytoplasm where it can be effectively translated. The expression cassette containing the *CAT* reporter gene is flanked by AAV left and right terminal repeats. The AAV terminal repeats are intended to

enhance transgene expression in primary and slowly dividing cells and to serve as origins of replication. Plasmid DNA was isolated from bacterial cultures using alkaline lysis followed by anion-exchange column chromatography (Qiagen, Valencia, CA). Endotoxin levels were measured to be below 0.05 endotoxin units (EU)/μg DNA, by using a kit (E-Toxate; Sigma, St. Louis, MO).

2.4.2. Preparation of cationic liposomes

The cationic lipid DMAPAP (10 μmol, 10 mg) and DOPE (10 μmol, 7.3 mg) were dissolved in chloroform and the solvent was removed under reduced pressure on a rotary evaporator during 1 h. The resulting thin film was further dried on the rotary evaporator at 5 mbar for 2 h in order to remove any remaining solvent. The film was rehydrated in 1 mL H_2O gentle rotation overnight at room temperature to afford a final concentration of 20 mM. The particles were sonicated during 5 min to afford a rather homogenous size distribution of approximately 150–200 nm as measured by dynamic light scattering on a nanoZS (Malvern Instruments).

2.4.3. Preparation of cationic lipoplexes

Lipoplexes were prepared in tris-maleate 25 mM, glucose 2.5% with a charge ratio cationic lipid/anionic lipid = 6, which corresponds to a ratio total lipid to DNA = 12. As an example for in vitro experiments, plasmid DNA (6 μg in 100 μL tris-maleate 25 mM, glucose 2.5%) was added in few seconds to DMAPAP/DOPE (15 μL, 5 mM in 100 μL tris-maleate 25 mM, glucose 2.5%) dropwise with constant vortexing. The samples were left 2 h at room temperature to incubate before being added to the anionic liposomes.

2.4.4. Preparation of anionic pegylated liposomes

The anionic liposomes were prepared by the film method. An example of preparation is given with the cholesterol derivative CCTC, all other anionic liposomes were prepared according to the same protocol.

DPPC (5 μmol, 3.7 mg), CCTC (15 μmol, 7.4 mg) and Chol-PEG_{110} (0.5 μmol, 2.5 mg) were dissolved in chloroform and the solvent was removed under reduced pressure on a rotary evaporator. The resulting thin film was further dried on the rotary evaporator at 5 mbar for 2 h in order to remove any remaining solvent. The film was rehydrated in 1 mL H_2O gentle rotation overnight at room temperature to afford a final concentration of 20.5 mM. The particles were successively filtered on 0.45 and 0.22 μm polyethylsulfonate filters.

2.4.5. Preparation of anionic pegylated lipoplexes

The preformed cationic lipoplexes were added to the anionic liposomes according to the charge lipid ratio (±) = 4, 2.5 and 1.3 for CHEMS, CCDC and CCTC, respectively.

As an example, the preformed lipoplexes described above should be added to a suspension of DPPC/CCTC/Chol-PEG_{110} (100 μL of the liposomes at 0.48 mM in Hépès 20 mM, glucose 10%).

For in vivo experiments, the volumes were reduced in order to obtain the following DNA concentration: 50 μg in 200 μL final volume, but all the ratio and buffers were maintained as described.

2.4.6. Titration experiments

Titration experiments were performed with a Zeta Sizer NanoSeries from Malvern Instruments equipped with a MPT2 autotitrator. The cationic and the three anionic formulations were submitted to pH changes. The buffer used was HCl 0.1 M. The initial pH point was taken at pH 8.25 and pH was decreased to reach pH 3.7. Measurement of the size and electrophoretic mobility was taken every half pH after pH stabilisation. Electrophoretic mobility was converted to the ζ potential according to the Smoluchowski equation.

2.4.7. Gel retardation experiments

The complexes were loaded on an agarose gel (0.8% in TBE: 1 M Tris, 0.9 M boric acid, 0.01 M EDTA) and submitted to an electrophoresis at 80 V/cm. DNA was revealed with ethidium bromide and visualised under UV light.

2.4.8. Fluorescence picogreen

Free DNA or complexed DNA (40 ng) was diluted in a solution of Picogreen® (Molecular Probes) diluted as described by the provider (1/200 in tris-EDTA buffer). Samples were loaded as tripliquets in 96-well plate. Lecture of the emission at 450 ± 10 nm under an excitation at 350 ± 10 nm was performed on a Wallac Victor2 1420 Multilabel Counter (PerkinElmer). Picogreen alone was taken as the negative control and its background was removed from the sample data. Free DNA was taken as the positive control and taken as 100% fluorescence level.

2.4.9. In vitro experiments

B16 murine cells were grown into DMEM supplemented with L-glutamin (29.2 mg/mL), penicillin (50 U/mL), streptomycin (50 U/mL), and 10% fetal bovine serum.

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 for 24 h.

One hour before transfection, cells were washed once with fresh medium with or without bafilomycin. Then, 100 μL of complexes containing 0.5 μg DNA were loaded onto each well, and the plates were incubated at 37 °C for 6 h in the presence of 5% CO_2, then replaced by fresh medium for 18 h.

The cells were washed twice with PBS and treated with 200 μL of a passive lysis buffer (Promega). After 15 min, the cells were centrifuged for 5 min at 12,000 r/min. Supernatant (10 μL) and iodoacetamide (10 μL) were added to a 96-well plate, which was incubated at 37 °C for 1 h. Protein quantification was performed with the BCA protein assay KIT (PIERCE) and reported to BSA taken as a reference curve. Luciferase activity was quantified using a commercial kit Luciferase assay system (PROMEGA). On 10 μL of the lysed cells, 50 μL of the luciferase substrate was injected via an injector, and the absorbance was read immediately at 563 nm on a Wallac Victor2 1420 Multilabel Counter (PerkinElmer). Background of the untreated cells, taken as negative controls, was removed from the sample data. The cationic formulation was taken as the positive reference formulation.

2.4.10. In vivo experiments

Six-week-old female (≈20 g) C57Bl/6J mice (Janvier) were anesthetised by intraperitoneal injection of a mix of ketamine (85.8 mg/kg) and xylazine (3.1 mg/kg) diluted in 150 mM NaCl. A 200 μL volume of lipoplexes containing 50 μg plasmid was injected into the mouse tail vein. Mice (7 per group) were sacrificed at 24 h postinjection, and the liver, spleen, lungs and tumor were removed, weighed and homogenised in pH 7.4 phosphate-buffered saline(PBS, 5 mL/g tissue) using an Ultra Thurax (Diax 600, Heidolph, Fisher). The samples were incubated (30 min, 20 °C), and the amount of chloramphenicol acetyl transferase (CAT) transgene expressed was determined using a standard enzymelinked immunosorbent assay (ELISA) kit (Roche Diagnostics). The cationic formulation was taken as the reference. The background level of the different organs was removed from each individual data. Statistics were made using the Mann–Whitney test.

Experiments were conducted following NIH recommendations for animal experimentation and regional ethic committee on animal care and experimentation.

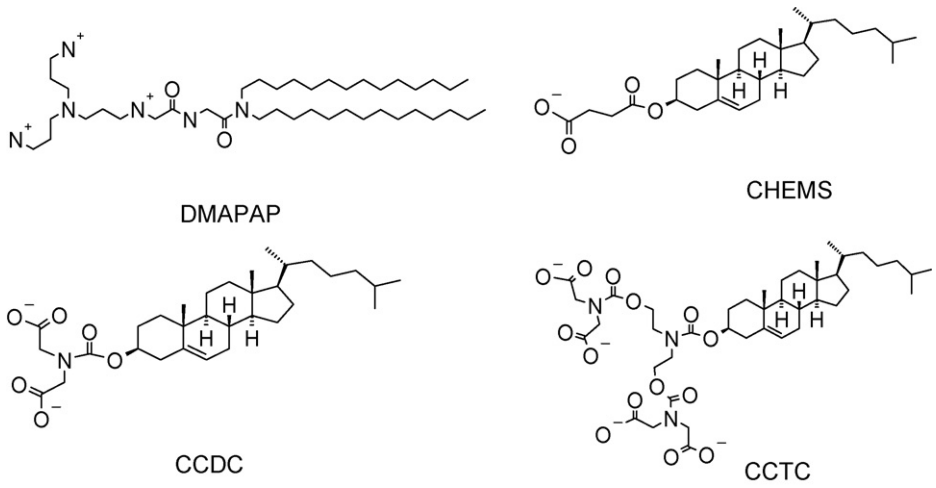


Fig. 1. Compounds used in the tunable formulations: DMAPAP, CHEMS, CCDC and CCTC.

3. Results

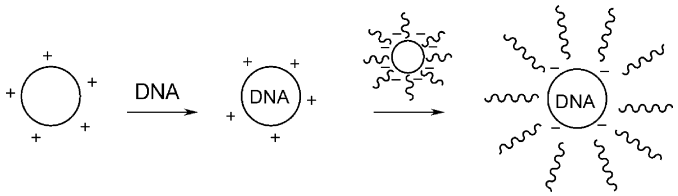
3.1. Design of the compounds and synthesis

We previously screened cationic lipopolyamines for gene delivery and chose one particular lipid for its reduced toxicity and high reproducibility as an in vitro transfecting agent (Byk et al., 1998). This cationic lipid, whose name in the IUPAC nomenclature is 2-{3-[bis-(3-amino-propyl)-amino]-propylamino}-*N*-ditetradecylcarbamoylmethyl-acetamide; hydrochloride salt, and that we related as RPR209120 in previous articles (Thompson et al., 2005; Tranchant et al., 2004), will be simplified here as DMAPAP for DiMyristylAmidoCarbamoylMethylAminoPropyl-diAminoPropylAmine. This cationic lipid exhibits two primary amines, one secondary and one tertiary amine. Cholesterol content in liposome does not usually exceed 30% of the total lipid amount. Hence, addition of an anionic cholesterol in a formulation involving this cationic lipid, would require more than one negative charge per cholesterol for the system to be tunable at the right pH, without exceeding the 30% amount above which the particle stability could be reduced. In this context, we designed and synthesised two anionic cholesterol: a cholesterol derivative bearing two carboxylates that we called CCDC for cholesteryl carbonyl dicarboxylate and a cholesterol bearing four carboxylate moieties that we named CCTC for cholesteryl carbonyl tetra carboxylate, presented in Fig. 1. The commercially available cholesterylhemisuccinate (CHEMS) is also presented on this figure as it was used for comparison in the different formulations.

The dicarboxylate cholesterol (CCDC) was obtained from the reaction of commercially available cholesteryl chloroformate and iminodiacetic acid. The tetracarboxylate was synthesised in three simple steps: cholesteryl chloroformate was reacted with diethanolamine and then with trichloromethylchloroformate to give the corresponding bis-chloroformate derivative. This latter compound was subsequently condensed with iminodiacetic acid to give CCTC. Synthesis and characterisations of the compounds are fully described in Section 2.

3.2. Formulation

To control more efficiently each step, formulation was performed in two steps that are: first, DNA condensation in cationic particles, second, fusion of the lipoplexes to anionic pegylated liposomes to form anionic pegylated lipoplexes (Scheme 1).



Scheme 1. Schematised representation of the sequential process used for the formation of anionic lipoplexes. It does not take into account the structure of the complexes neither the position of DNA into the particle lipidic bilayer.

The advantages of the first step are the control of DNA condensation and particle size, which obviously become the difficulties of the second step. Upon anionic to cationic liposome mixture, the size of the particles might significantly increase. As under submicron particle size is preferable for systemic injection, particle size is a primary issue. Particles should fuse but not aggregate. This might be controlled in several ways. First, we added PEG to the formulation to maintain steric stabilisation, second, we used a strong base to buffer the particles and avoid charge changes over the process, then, we kept a low salt concentration to be able to maintain the system non-aggregating, still keeping an osmolar system for further in vivo application.

Hence, as shown in Scheme 1, we preformed cationic lipoplexes and added the anionic pegylated liposomes to the lipoplexes in order to obtain anionic pegylated lipoplexes at physiological pH. Four different lipoplexes were prepared: a formulation which did not contain an anionic cholesterol derivative as a cationic control, and three anionic formulations containing cholesterol derivatives bearing 1, 2 or 4 negative charges (CHEMS, CCDC and CCTC, respectively). Formulations and hydrodynamic diameters of the particles formed are given in Table 1.

All particles containing DPPC obtained gave a similar size average. However, the more homogeneous particle trend was obtained when using the modified tetracarboxylate cholesterol CCTC as given by the polydispersity index (PDI). We checked this point by visualising the particles by transmission electronic microscopy. TEM observations confirmed the size obtained by light scattering and that the more homogeneous particles were obtained for the CCTC containing formulation (unshown data).

To obtain anionic particles containing DNA at pH 7 that would dissociate or fuse in the endosomes at pH 5.5–6.5, we had to choose the correct ratio between the cationic lipid and the anionic cholesterol. For this purpose, we performed titrations by dynamic light

Table 1
Hydrodynamic diameters (nm) and polydispersity index of the complexes

	Hydrodynamic diameter (nm)	Polydispersity index
DMAPAP/DOPE/DNA	149 ± 6	0.25
DMAPAP/DOPE/DNA/DPPC/Chol-PEG_{110}	279 ± 15	0.27
DMAPAP/DOPE/DNA/DPPC/CHEMS/Chol-PEG_{110}	289 ± 15	0.29
DMAPAP/DOPE/DNA/DPPC/CCDC/Chol-PEG_{110}	314 ± 3	0.25
DMAPAP/DOPE/DNA/DPPC/CCTC/Chol-PEG_{110}	192 ± 18	0.19

Anionic lipoplexes prepared in Hepes/glucose were added to cationic lipoplexes prepared in tris-maleate/glucose as described in the experimental part.

scattering. Hence, we measured the charge variations at the surface of the particles according to the pH, as well as the particle size variation. We had previously calculated roughly the ratio that would be required to obtain a destabilisation at pH 5.5–6.5, and we screened the preparations by titration. The cationic/anionic lipid ratio was clearly defined by a zeta potential change within the desired pH range. The curves obtained for the chosen particles are presented in Fig. 2A. It should be noticed, that the full particulate system was submitted to pH changes, which means lipids, DNA and buffers were present as they are part of the particle formation. The transition from anionic to cationic in these particular conditions, should be obviously different from the one the particles will encounter in the biological media in vitro or in vivo. It still gave us a means to determine a lipidic ratio to start with. The particle size according to the pH was also studied (Fig. 2B) and indicated that size of the particles made of CHEMS and CCTC had a tendency to increase. This would tend to indicate a possible disorganisation by aggregation. This phenomenon was not observed for the cationic particles or the particles made of the cholesterol derivative CCDC. This does not indicate if DNA is released during the disorganisation process but one could think that this destabilisation could help the interaction with other lipidic membranes.

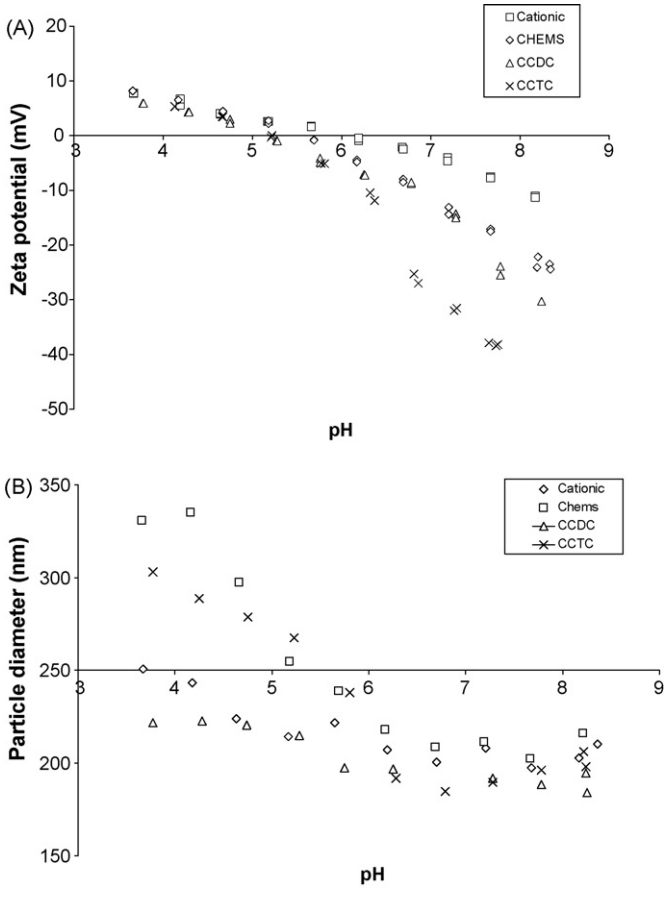


Fig. 2. (A) Zeta potential of the particles as a function of the pH and (B) diameter of the particles as a function of the pH. The pH was initially measured at pH 8.25 and decreased by addition of a solution of HCl at 0.1 M, in order to obtain steps with a pH decrease of 0.5. A measure of the electrophoretic mobility and dynamic diameter was measured every variation of 0.5 in pH after the pH was stabilised. The measures were taken at room temperature.

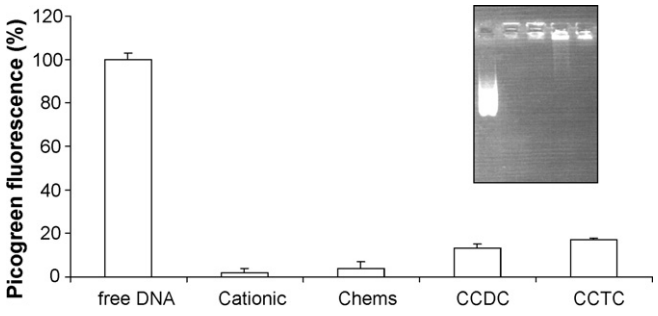


Fig. 3. Fluorescence measurements of DNA accessibility using Picogreen® dye. The level of the Picogreen® fluorescence with free DNA was taken as 100% fluorescence. Mean values and standard deviation from three individual points are represented. Background level of the picogreen in the same buffer has been removed from the sample data. Onset: agarose gel electrophoresis of the lipid/DNA complexes in the same order as in the graph abscisses.

pH-sensitive particle formation should only be valuable if DNA is still condensed within the particles. As anionic liposomes are added to cationic lipoplexes, a legitimate concern was the release of DNA during this process. Thus, DNA condensation has been controlled after anionic liposome addition by agarose gel electrophoresis and fluorescence measurements (Fig. 3). Fluorescence measurement using picogreen allowed controlling DNA accessibility into the complexes. A slight DNA release was evidenced for the complexes bearing CCDC and CCTC as 13 and 17% fluorescence was measured as referred to free DNA taken as 100% fluorescence. However, this release was not observed on agarose gel as shown in the onset (Fig. 3).

3.3. In vitro studies

Preparing complexes using a plasmid encoding for the luciferase reporter gene, the different formulations were assayed in vitro on B16 cell line, by measuring the luciferase expression level. Results are indicated in Fig. 4. It is noteworthy that the four formulations allowed a similar gene expression level while three formulations are anionic particles as indicated by the zeta potential taken at room temperature, at pH 6.8 (Fig. 4, right scale). Formulations containing CCDC and CCTC exhibit the highest negatively charged surface, which supposes that they will not enter the cells as efficiently as the cationic formulation. Moreover, the picogreen data indicated that a slight part of the DNA might have been released, which we did not take into account for in vitro testing. The slight reduction obtained with anionic particles might be explained by their increasing zeta potential from −5 to −20 mV. Despite this strong zeta potential difference, the in vitro expression obtained is not significantly reduced from the CHEMS to the CCTC formulation, suggesting that they might benefit from a trigger in the cells, most probably a pH-sensitive pH trigger.

To test if the luciferase level obtained with the anionic formulations was indeed due to their pH-sensitive nature, we used bafilomycin known as being a specific inhibitor of the vacuo-

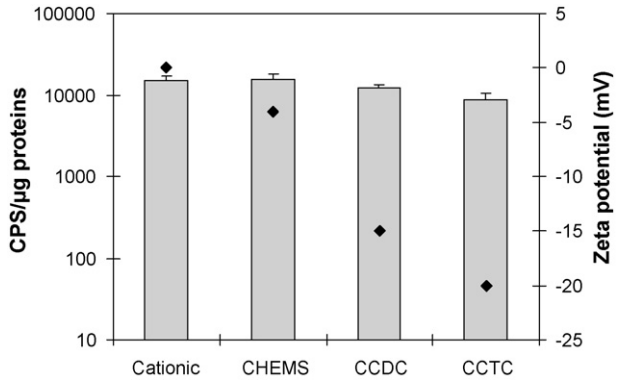


Fig. 4. Transfection of the different formulations using a plasmid encoding for the luciferase reporter gene. The left axis represents the level of luciferase expression per protein (μg). The right axis represents the zeta potential in millivolts. The expression level (mean values + S.D.) corresponding to each formulation is represented by the column, the zeta potential for each formulation is represented by the lozenges. The background level of the untreated cells was removed for each samples.

lar H^+ ATPase. Inhibiting endosome acidification should reduce the release due to pH trigger thus reducing the level of gene expression. Transfection results are reported in Fig. 5. As can be seen, the transfection efficacy of all formulations is strongly influenced by the presence of bafilomycin. Moreover, the transfection inhibition increases with the anionic charges, the more important being obtained for the CCTC containing formulation. Anionic formulations are sensitive to the bafilomycin effect indicating a pH-sensitivity of these formulations.

3.4. In vivo results

Based on the previous results, we evaluated the efficacy of the different formulation as gene carriers in mice bearing 3LL tumors. The plasmid used for these experiments encoded for chloramphenicolacetyltransferase, which was the protein we dosed because of a higher sensitivity of the dosage as compared to luciferase. We searched for gene expression in the liver, spleen, lung, and tumor as presented in Fig. 6. We could see that the presence of anionic lipids had a strong influence on the gene expression. In the liver and spleen, there was no increase in gene expression. In the lung, no expression could be found which showed that we did not loose complexes in the lung due to non-specific interactions as we often observed with cationic complexes. Quite interesting was the fact that we obtained a gene expression in the tumor, rather low, but with a slightly higher level for the CCTC containing formulation, the difference between CHEMS and CCDC and also between CCDC and CCTC formulations was statistically significant as calculated by the Mann–Whitney test.

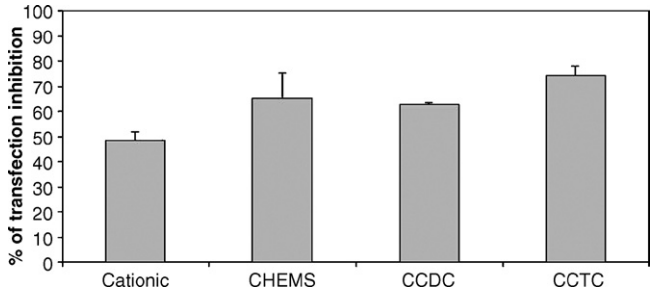


Fig. 5. Percentage of transfection inhibition for the different formulations using a plasmid encoding for the luciferase reporter gene in presence of bafilomycin. The percentage of inhibition was obtained by dividing the transfection level obtained with bafilomycin by the transfection level obtained without bafilomycin. Indicated values represent a mean (+S.D.) of two individual experiments performed in triplicate.

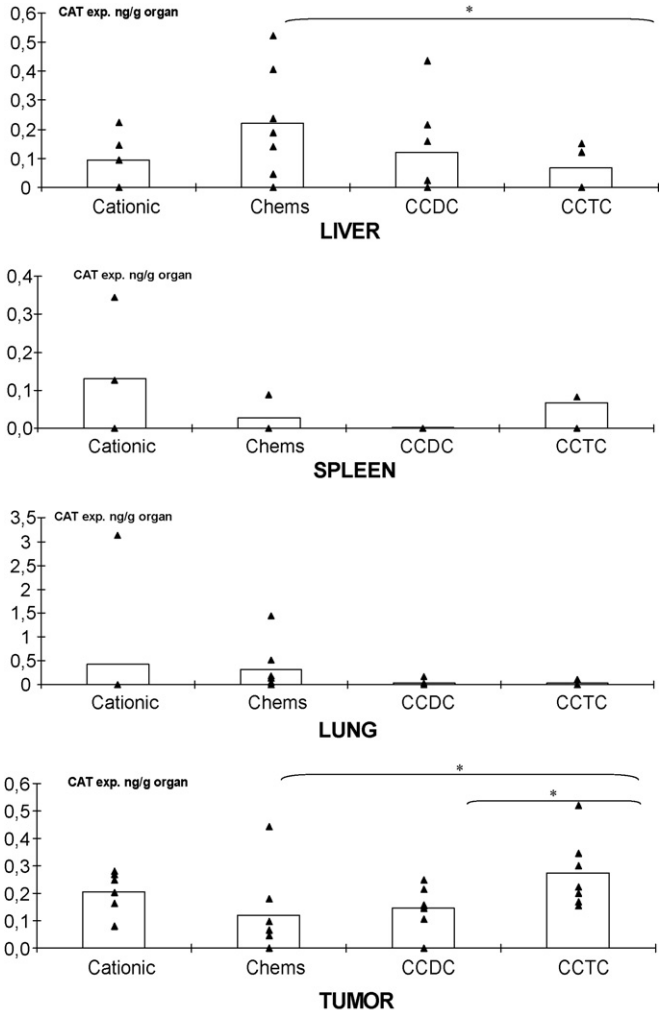


Fig. 6. Chloramphenicolacetyltransferase expression in tumor, liver, spleen and lung after i.v. injection of the formulations to C57Bl/6 mices implanted subcutaneously with 3LL tumors. The columns represent the mean value, the points represent the individual data. Statistical significance is represented by the symbol * for $p < 0.05$ as calculated by the Mann–Whitney test.

4. Discussion and conclusion

The field of nucleic acid vectorisation still needs efficient nucleic acid release into the cells. The question received new interest in the context of siRNA delivery. There are many ways of improving nucleic acid release from particles, acid-sensitive trigger remains a way to achieve it. pH-sensitive linkages might be beared by the lipid such as sugar-based Gemini surfactants (Wasungu et al., 2006), orthoester lipid (Huang et al., 2006), histidinylated cholesterol (Singh et al., 2004), or by the PEG-lipid (Garinot et al., 2007). Addition of helper lipid can also induce pH-sensitivity to the formulation as was shown for the DOPE lipid (Hirsch-Lerner et al., 2005).

One can also form combination of anionic and cationic systems to form pH-sensitive formulations. However, maintaining DNA complexation in anionic particles is not obvious as it was shown that the interaction of lipoplexes with anionic membrane lipids might lead to DNA release (Caracciolo et al., 2007; Tarahovsky et al., 2004). Some authors have investigated the mixtures of cationic

and anionic lipid entities to form anionic lipoplexes in a controlled manner (Bonincontro et al., 2007), which might require the presence of Ca^{2+} (Patil et al., 2005). The work performed by Cullis et al. showed that conceiving the right anionic to cationic ratio could allow obtaining a lipidic fusion at the desired pH (Hafez et al., 2000). Moreover addition of DOPE but also CHEMS in these formulations did contribute to the pH-sensitivity of the formulations (Hafez and Cullis, 2000). This type of formulation could become then interesting for DNA delivery if the right anionic to cationic ratio was used for pH-sensitivity, but also to maintain DNA compaction. Formulation conditions had to be worked out for this purpose.

Hence, we developed original cholesterol derivatives to be able to tune the system to the pH-sensitivity of interest (pH 5.5–6.5). These cholesterol lipids exhibit two (CCDC) or four (CCTC) acidic functions with pK_a between 2.8 and 4.1 (4.07 for CHEMS) particularly adapted to the cationic lipid that we are using bearing 4 amines (including one tertiary, one secondary and two primary amines with respective pK_a: 4.57, 8.70, 9.79 and 10.58). The lower pK_a of the cholesterol derivatives allow using a reduced amount of these cholesterol in the complexes and thus prevent destabilisation that could occur by insertion of these molecules into the lipidic bilayer. We thus determined that the ratios between cationic and anionic lipid were 4, 2.5 and 1.3 for CHEMS, CCDC and CCTC, respectively.

From the formulation tested, the one bearing the CCTC lipid appeared to be the more interesting one in terms of formulation ease, size homogeneity and particle stability. This anionic cholesterol presented interesting properties in terms of solubility and suspension ability, and it would be very interesting to go further in determining its own characteristics, such as phase formation. Moreover, the formulation containing the CCTC lipid did induce the highest expression level in vitro and in vivo. We could show in vitro that the CCTC formulation was the most pH-sensitive and that part of its transfection efficiency was due to this sensitivity, even though it might not be the only mechanism involved in the DNA release.

A slight but significant improvement of transfection in tumor has also been observed with lipoplex including this lipid (CCTC), with low expression in the lung and other organs compared to the other formulations tested in this report.

As anionic lipoplexes are formed in this study, targeting these particles would be appropriate to increase the amount of DNA delivered to the tumor and hopefully increase gene expression.

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