Anionic Nucleotide–Lipids for In Vitro DNA Transfection

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A family of new anionic nucleotide based lipids featuring thymidine-3’-monophosphate as nucleotide and 1,2-diacyl-sn-glycerol as lipid moiety for in vitro delivery of nucleic acids is described. The nucleotide lipids were prepared in three steps starting from 1,2-diacyl-sn-glycerols and 2’-deoxymethylene-3’-phosphoramidate. Gel electrophoresis experiments show that nucleotide-based lipid–DNA complexes are observed at Ca2+ concentration higher than 1 mM. The transfection experiments carried out on mammalian Hek cell lines clearly demonstrate that the nucleotide moiety enhances the transfection efficacy of the natural anionic DPPA and DPPG lipids. SAXS studies indicate that the enhancement in transfection for nucleotide-based lipid formulations compared to those of the abasic natural derivative (DPPA) is likely due to the presence of the 2D columnar inverted hexagonal phase (HII) with a unit cell parameter a = 69.1 Å in the nucleotide lipid formulations. The cytotoxicity studies of lipoplexes, evaluated against Hek cells using an MTS assay, revealed that palmitoyl nucleotide derivative complexes were not toxic even after 4 h of incubation, thus indicating that the anionic nucleotide lipids presented in this work offer an alternative to cationic transfection reagents.

INTRODUCTION

Therapies ± using natural or synthetic nucleic acids have emerged as promising strategies for developing cures against inherited and acquired disorders or diseases, including cancer (1, 2). One of the significant advantages of nucleic acid based drugs over low molecular weight pharmaceutical drugs is their selectivity for their biological target and their specificity of ± action (3). To be effective, nucleic acid based therapies require the use of vector platforms for delivering various genetic materials into cells. The earliest synthetic DNA delivery systems, first introduced in the 1980s (4, 5), were based on cationic lipids. Today, cationic lipids are still important transfecting tools for research, likely because of the researchers’ ability to control their chemical and physical properties (6). However, most of these cationic systems exhibit specific problems outlined by Burgess et al. (7), including cytotoxicity, inactivation in the presence of serum, and inefficiency in vivo, that scientists are attempting to address (8–12). To overcome these limitations, alternative cationic synthetic vectors ± are currently under investigation by a number of research groups (13–16). In ± recent years, there has also been intense research activity in the development of various innovative lipid systems, including pH-sensitive fusiogenic polymer-modified liposomes (17), pH-sensitive pegylated lipoplexes (18), nucleolipoplexes (19–21), imidazole (22) and charge reversal lipids (23, 24), lipopolythioureia (25), lipophilic peptides (26), zwitterionic lipids (27), and glycosylated lipids (28). Additionally, amphiphiles possessing a nucleoside as a lipid headgroup and lipophilic alkyl chains have been identified as promising tools for gene delivery into cells (19–21) (28–30) and for self-assembling nucleic acid–nucleolipid supramolecular systems (31–40).

Examples of anionic amphiphiles for gene delivery are rare. Recently, an anionic amphiphile (1,2-dioleoyl-sn-glycerol-3-[phospho-rac-(1-glycerol] sodium salt), zwitterionic amphiphile (DOPE), and Ca2+ system have been reported that exhibited transfection efficiencies similar to the cationic lipid lipofectamine (7) (41). To our knowledge, no reports of anionic nucleotide based amphiphiles for gene transfection have been reported.

Within this context, we hypothesized that an anionic amphiphile possessing a nucleotide polar head for DNA–lipid association would enable formation of lipoplexes in the presence of Ca2+ and afford in vitro tranfection. For this purpose, we synthesized a series of anionic nucleotide–lipids with a thymidine nucleotide polar head and various 1,2-diacyl-sn-glycerol of C12, C14, and C16 chain length as hydrophobic species. The potential of these novel anionic nucleotide based lipids for DNA delivery and expression was investigated. Herein, we report the synthesis, physicochemical studies, cytotoxicity, and in vitro transection results of these new anionic nucleotide based lipids (Scheme 1).

EXPERIMENTAL PROCEDURES

Materials. General Experiments and Analytical Conditions. Unless noted otherwise, all starting materials and solvents were obtained from Aldrich, Bachem, Genzyme, and Glen Research and were used without further purification. All compounds were characterized using standard analytical and spectroscopic techniques such as 1H, 13C, and 31P spectroscopy (apparatus Bruker Avance DPX-300, 1H at 300.13 MHz, 13C at 75.46 MHz, and 31P at 121.49 MHz) and mass spectrometry (Instrument JEOL SX 102, NBA matrix). The NMR chemical shifts are reported in ppm relative to tetramethylsilane using the deuterium signal of the solvent (CDCl3) as a heteronuclear reference for 1H. The 1H NMR coupling constants, Js, are reported in Hz. TEM microscopy experiments were performed...
Scheme 1. Chemical Structure of an Anionic Nucleotide–Lipid Used in This Study, the Thymidine 3′-(1,2-dipalmitoyl-sn-glycero-3-phosphate) (diC₁₂-dT) and Non-Nucleotide–Lipids DPPA and DPPG.

with a Hitachi H 7650 (negative staining with uranyl acetate 1% in water, nickel carbon coated grids). Fluorescence microscopy experiments were performed on a Zeiss axioplan 200. Silica gel 60 (particle size: 45 μm) was used for flash chromatography (Biotage). Thin layer chromatograms were performed with aluminum plates coated with silica gel 60 F₂₅₄ (Merck). The revealing solution for TLC is an anisaldehyde solution (85 mL ethanol, 10 mL acetic acid, 5 mL sulfuric acid, and 0.5 mL p-anisaldehyde).

Synthesis. Thymidine 3′-(1,2-Dilauroyl-sn-glycero-3-phosphate), diC₁₂-3′-dT. Compound 1. 5′-O-(4,4′-Dimethoxytrityl)-2′-deoxythymidine-3′-[(2-cyanoethyl)-N,N-diisopropyl]phosphoramidite (0.500 g, 1 equiv, 0.67 mmol), 1,2-dilauroyl-sn-glycerol (0.398 g, 1.3 equiv, 1.87 mmol) dissolved in 3 mL of THF) and a tetrazole solution in acetonitrile (0.45 M, 2 mL, 0.87 mmol) were dissolved in dry acetonitrile (4 mL, 1.75 mmol), and a tetrazole solution 0.45 M in THF/Pyr/H₂O). After 12 h at room temperature followed by oxidation with 40 mL of a solution of I₂ (0.02 M in THF/Pyr/H₂O). The reaction mixture was stirred for 7 h at room temperature followed by oxidation with 40 mL of a solution of I₂ (0.02 M in THF/Pyr/H₂O). After 12 h at room temperature, the solvent was evaporated under high vacuum to yield intermediate products. The contents of the reaction flask were dissolved in 10 mL of methylene chloride and then washed first with 3 × 10 mL of HCl (0.1 N) and second with 3 × 10 mL of a saturated solution of Na₂SO₄. Product 1 (160 mg) was isolated after purification on silica gel (DCM/MeOH/TEA from 98:2:1 to 50:49:1). Yield: 47% Rₛ = 0.3 (DCM/MeOH 8:2).

1H NMR (300 MHz, CDCl₃): δ in ppm 0.88 (t, 6H, J = 6.9 Hz, 2CH₃), 1.25 (m, 48H, 16CH₂), 1.42 (dd, 4H, J₁ = 6.5 Hz, H₁), J₂ = 2.5 Hz, H₂), 2.25 (m, 4H, 2CH₂), 3.04 (t, 2H, 3.7–4.4 ppm (m, 7H, 2CH₂(glycerol), H₄’, H₅’), 5.28 (m, 1H, CH glycerol), 6.21 (t, 1H, J = 6.5 Hz, H’₁), 7.61 (s, 1H, H base).

13C NMR (75 MHz, CDCl₃): δ in ppm 12.4 (CH3 base), 14.1 (CH base), 17.9 (CH₂), 22.6 (CH₂), 24.8 (CH₂), 29.0–29.5 (CH₂), 31.9 (CH₂), 33.9 (CH₂), 34.1 (CH₂), 61.5 (CH₂), 61.7 (CH₂), 62.5 (CH₂), 62.6 (CH₂), 66.1 (CH₂), 66.2 (CH₂), 69.1 (CH), 78.8 (CH), 85.5 (CH), 86.1 (CH), 110.7 (C base), 136.2 (CH base), 150.5 (C=O base), 164.0 (C=O base), 173.0 (C=O chain), 173.5 (C=O chain).

31P NMR (121 MHz, CDCl₃): δ 2 ppm. High-resolution ESI MS⁺ [M−H]+, theoretical ml/z = 871.5449, observed ml/z = 871.5473.

Preparation of Liposomes. Liposomes were prepared from binary mixtures of the negatively charged lipid n-dodecyl phosphatidyl ethanolamine (NL) and neutral lipid DOPE. Stock solutions of DOPE and NL were mixed at the desired ratio and then sonicated with an ultrasonic bath. The resultant liposomes were dried under dry N₂ and placed in glass tubes. The resultant liposomes were dissolved in 5 mL of HCl (0.1 N) and sonicated using an ultrasonic bath. The resultant liposomes were dried under dry N₂ and then desiccated under vacuum overnight. Milli-Q Water was added to the dried lipids to obtain liposome solutions (1 mg/mL). This was followed by five thaw–sonication cycles using a water bath at 55 °C and an ultrasonic bath. The resultant solution was extruded at room temperature through a 50 nm pore-size nucleopore polycarbonate filter.

Preparation of Plasmid DNA. Plasmid containing a GFP reporter gene under the cytomegalovirus promoter, pE-GFP, was propagated in transformed Escherichia coli cells. E. coli cells were grown in standard Luria Bertani medium at pH 7.0. Cells were harvested by centrifugation, and the plasmid DNA was
extracted and purified using a Qiagen Plasmid Maxi Kit (Qiagen, Santa Clarita, CA) as per the manufacturer’s recommended protocols.

**Preparation of Anionic Lipoplexes.** DNA (250 µg/mL), liposomes (1 mg/mL), and divalent salt (CaCl2 2H2O, 200 mM) samples were mixed to yield anionic lipoplexes at different global salt concentrations. The DNA to lipid charge stoichiometry (D/L ratio) was defined as the total charge of DNA divided by the total charge of anionic nucleolipides.

**Particle Size Determination.** Anionic liposomes and lipoplexes were prepared as described above. Particle size was determined using Zetasizer 3000 HAS Malvern. All measurements were conducted at 25 °C.

**Electrophoresis Studies.** Electrophoresis studies were conducted in 0.8% agarose gels containing ethidium bromide in 0.5 Tris-borate without EDTA buffer. Anionic lipoplexes were prepared as described above. For this purpose, 20 µL of each sample was mixed with 4 µL loading buffer (glycerol 30% (v/v), bromophenol blue 0.25% (w/v), and xylene cyanol 0.25% (w/v)) and subjected to agarose gel electrophoresis for 35 min at 100 V. The electrophoresis gel was visualized and digitally photographed using a G.BOX camera.

**Transmission Electronic Microscopy (TEM).** Anionic liposomes and lipoplexes were prepared as described above and were visualized by negative staining microscopy. Ten microliters of liposomes (1 mg/mL) was transferred to a carbon-coated grid for 10 min. The sample was then dried and stained with 1% (W/W) of uranyl acetate for 30 s. The specimens were observed with a Hitachi H 7650 electron microscope.

**Transfection Assays.** Cell lines (Hek 293 human embryo kidney) were cultured in Dulbecco’s Modified Eagle’s Medium (D-MEM, Invitrogen) and supplemented with 10% fetal bovine serum (FBS, invitrogen) and 1% L-glutamine at 37 °C in a 5% CO2 atmosphere. Cells were split every 3–4 days to maintain monolayer coverage.

The day before transfection, cells were seeded in 24 well plates (50 000 cells/well). On the day of transfection, the medium containing serum was removed from the well plates and replaced with 200 µL of transfection medium without serum.

Lipoplexes were prepared by adding 4 µL of plasmid (250 ng/µL) in 50 µL of medium without serum and 42 µL of anionic liposome dispersions (1 mg/mL). Anionic lipoplex formation was achieved by the addition of various amounts of a calcium chloride solution (200 mM). These complexes were left at room temperature for 15 min before being added to the cells. In the case of fluorescence microscopy samples, a similar procedure was followed. In that case, an oligonucleotide fluorescein (random sequence, 18mers) was mixed with the DNA fraction and added to the sample. 50 µL of medium without serum, 42 µL of liposomes (1 mg/mL), and 3 µL DNA (250 µg/µL) + 1 µL oligonucleotide–fluorescein (250 µg/µL) were mixed. The complex formation was achieved by adding Ca2+ (final concentration 15 mM).

The cells were incubated with lipoplexes in a media without serum for 2 or 4 h to permit transient transfection. Afterward, the complex-containing suspension was removed and replaced by 200 µL of the complete growth medium. GFP expression was analyzed after 48 h and measured with flow cytometry. Lipofectamine transfections were performed, as per the manufacturer’s instructions, using 2 µL of lipofectamine and an identical amount of plasmid 4 µL (250 µg/µL).

**Flow Cytometry Studies.** Cells were assayed for GFP expression 48 h after being exposed to the transfection agents. Cells in the well plates were rinsed with PBS, treated using 100 µL trypsin EDTA, and incubated for 5 min (37 °C) in 5% CO2 incubator. The cell suspension was then diluted to 200 µL using PBS, pH 7.4. The cells were then assayed for expression of GFP. GFP expression was analyzed using a FACS Canto dual laser flow cytometry.

**Toxicity Assay.** The toxicity of the thymidine-3′-(1,2-diacyl-sn-glycero-3-phosphate) (compounds 1, 2, 3) for the cells was determined 48 h after exposure to the transfection agents using the MTS tetrazolium. Cells in the well plates were treated using 50 µL of MTS tetrazolium and incubated for 2–4 h (37 °C) in a 5% CO2 incubator. Once coloring developed, the absorbance of 200 µL of each sample was analyzed at 490 nm.

**X-ray Diffraction.** SAXS measurements were carried out on the Bruker Nanostar apparatus at the Centre de Recherche Paul Pascal (Pessac, France). The wavelength of the incident beam was λ = 1.54 Å, and the sample-to-detector distance was 0.25 m (q range investigated from 0.05 Å−1 to 0.6 Å−1). The diffraction patterns were recorded by a 2D multiwire gas-filled detector. Anionic membranes formulated with the negatively charged nucleolipids (NL) and the neutral lipid (DOPE) were mixed with deionized water to obtain solutions with a concentration of 10 mg/mL. A plasmid–DNA solution in water was prepared (300 mg/mL) and added to the membrane solution in order to get the molar ratio NL/DNA = 2. The complex formation was achieved by adding divalent salt (CaCl2 2H2O, 200 mM) with a final concentration [Ca2+] = 15 mM. The suspension was held in a 1.5 mm size glass capillary and then centrifuged for a few minutes in order to locally concentrate the complex on the bottom of the capillary. The capillary was sealed and inserted in a sample holder at room temperature (25 °C). The collected 2D diffraction spectra were angularly integrated to get a 1D intensity vs q pattern.

**RESULTS AND DISCUSSION.**

In this study, we hypothesized that anionic lipid–nucleic acid complexes could take advantage of lipid polar head groups featuring nonionic interactions such as hydrogen-bonding and base π-π stacking. These additional interactions would likely influence the lipid–DNA associations and enhance the transfection efficacy of the natural anionic lipids previously reported (7). To demonstrate this hypothesis, the following constituting natural building blocks were selected to prepare the anionic nucleotide based lipids: thymidine (nucleotide polar head) and 1,2-diacyl-sn-glycerol (lauryl, myristoyl, and palmitoyl acyl chains). Both moieties, the diacyl-sn-glycerol motif and the thymidine as a nucleotide polar head, were selected on the basis of their natural occurrence in biological systems. In this contribution, we report the synthesis of a family of thymidine-3′-(1,2-diacyl-sn-glycero-3-phosphate), the formation of lipoplexes between DNA and nucleotide based lipids in the presence of Ca2+, SAXS studies, cytotoxicity, and successful in vitro gene delivery.

**Synthesis.** In order to evaluate the impact of the lipid structure on the transfection properties, a series of nucleotide derivatives composed of thymidine 3′-phosphates bearing natural 1,2-diacyl-sn-glycerol as hydrophobic building blocks was prepared. Although, to the best of our knowledge, nucleoside-5′-monophosphate lipids structures have been reported by Baglioni, Berti, and co-workers (31, 32), no double-chain nucleoside-5′-monophosphate amphiphile has been described so far. The double-chain nucleoside-3′-monophosphate lipids were synthesized by using a recently published (37) synthetic approach for single-chain nucleolipids (Scheme 2). Coupling reactions between 5′-DMT protected commercial phosphoramidite (5′-dimethoxytrityl-2′-deoxythymidine-3′-[(2-cyanoethyl)-N,N-disisopropyl]-phosphoramidite) and lipophilic 1,2-diacyl-sn-glycerol were undertaken in acidic conditions (tetrazole) similar to those found in oligonucleotide synthesis. The resulting phosphate intermediates were then oxidized with iodine in a THF/pyridine/water mixture at room temperature under argon to provide the
cyanoethyl-protected phosphates. Removal of the DMT group led to the production of compounds 1, 2, and 3, which were fully characterized by 1H, 13C, 31P NMR, and HRMS. The cyanoethyl protecting group was removed via an elimination reaction under basic conditions (TEA). Importantly, the synthetic strategy developed allowed easy access to the expected thymidine-3′-(1,2-diacyl-sn-glycero-3-phosphate) in four steps from the commercially available starting materials.

**Complexation Studies.** Next, to determine whether or not the nucleotide based lipids 1, 2, and 3 complex nucleic acids, we performed a standard electrophoresis gel shift assay with plasmid DNA for the assessment of DNA complexation. Since the lipid polar head bears an anionic charge, just as was the case for the previous anionic delivery vectors (7), the formation of lipid–DNA complexes was evaluated in the presence of different concentrations of divalent calcium. Standard agarose gel electrophoresis revealed two bands for naked DNA (supercoil, nonsupercoil forms, high and low mobility, respectively) (Figure 1A, lane 2). As a control, the addition of Ca

\[\text{Ca}^{2+}\]

+ DNA complexes at Ca

\[\text{Ca}^{2+}\]

concentrations of 10 mM (3), 30 mM (4), and 50 mM (5). (B) The lanes represent control DNA ladder (1); plasmid DNA (2); plasmid DNA and empty anionic liposomes (3); anionic lipoplexes at Ca

\[\text{Ca}^{2+}\]

concentrations of 1, 50, and 100 µM, and 1, 5, 10, 15, 30, and 60 mM (7–12).

**Nucleolipid – Ca

\[\text{Ca}^{2+}\]

– DNA Lipoplexes.** The hydrated mixtures of DOPE/diC

\[\text{C}_{16}-3\prime\text{-d}T\]

in aqueous solution were investigated without plasmid–DNA by transmission electronic microscopy (TEM) and zetasizer (3000 HAS Malvern instrument). As was expected, the extruded mixtures were found to self-assemble at room temperature into liposome-like structures in aqueous solutions. Under these conditions, at room temperature, the DOPE/diC

\[\text{C}_{16}-3\prime\text{-d}T\]

were in a “fluid” state. As seen in TEM images (Figure 3), in the absence of both nucleic acids and Ca

\[\text{Ca}^{2+}\]

and after extrusion with a 50 nm filter, different sizes of liposomes ranging from 40 to 80 nm were observed. These sizes are consistent with the average sizes of the same samples measured by dynamic light scattering (hydrodynamic diameter: 84 nm polydispersity index 0.38). Interestingly, in the presence of DNA and Ca

\[\text{Ca}^{2+}\]

+, the mean aggregates size was increased from 84 to 128 nm, indicating the formation of larger aggregates. Dried samples of these aggregates investigated by TEM revealed objects possessing a nonvesicular morphology (data not shown).

It is noteworthy that no modification of size was noticed for the samples containing liposomes in the presence of DNA without Ca

\[\text{Ca}^{2+}\]

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**SAXS Studies.** Next, to further characterize the molecular organization within the DOPE–NL–Ca

\[\text{Ca}^{2+}\]

– DNA lipoplexes, small-angle X-ray scattering (SAXS) experiments were performed. Figure 4 shows the diffraction patterns of the DOPE–DPPA samples with (curve 2) and without (curve 1)
the plasmid DNA. For the system without plasmid DNA, two distinct sets of peaks are identified. A 2D hexagonal lattice ($q_0$) with a unit cell spacing of $a = 72.6$ Å can be indexed. This pattern corresponds to the well-known 2D columnar inverted hexagonal phase (HII). The second set of Bragg peaks can be attributed to a lamellar structure with a stacking period $d = 54$ Å. Bragg peaks up to the fifth and third order are observed for the hexagonal and lamellar phases, respectively. The lamellar structure unambiguously corresponds to the lamellar phase observed (data not shown) for the pure DPPA system. On the other hand, the 2D hexagonal lattice seems to be very similar to that noticed for the pure DOPE system ($42$). These results suggest a very weak miscibility of the DPPA lipid in the DOPE membrane. The sample with plasmid DNA exhibits a more complicated pattern (empty circles) with a three-phase coexistence domain. We can identify the two phases previously observed for the free plasmid DNA system with a 2D hexagonal lattice ($q_0$) and a stacking period $d = 54$ Å. Interestingly, in the presence of plasmid DNA, a new lamellar phase ($q''_0$) can be indexed with a lamellar periodicity $d = 48$ Å. This new lamellar phase can be identified as lamellar complex made from DNA Ca$^{2+}$ and DOPE (data not shown).

Figure 2. Electrophoretic mobility of different formulations and Ca$^{2+}$ concentrations. The lanes represent control DNA ladder (1); plasmid DNA with different concentrations of Ca$^{2+}$ (2–4); lipoplexes (anionic nucleotide lipid–Ca$^{2+}$–DNA) diC16T/DOPE mol ratios of 10/90 (5,6), 25/75 (7,8), and 50/50 (9,10) with [Ca$^{2+}$] = 15 and 30 mM, respectively.

Figure 3. Transmission electron microscopic (TEM) images of anionic DOPE/diC16-3′dT liposomes (mol ratio of 10/90) in water with uranyl acetate (1%) as negative stainer. Scale bars, 200 nm.

Figure 4. SAXS patterns of DOPE–DPPA–Ca$^{2+}$ system without plasmid DNA (curve 1) and with plasmid DNA (curve 2).

Figure 5. SAXS patterns of DOPE–diC16-3′dT–Ca$^{2+}$ system without plasmid DNA (curve 1) and with plasmid DNA (curve 2).
differences for the structural parameters observed for the samples with and without plasmid DNA strongly suggest the presence of DNA inside the two phases H2 and Lc, and a good miscibility of DOPE with the nucleotide lipid (diC16-3′-dT). These results prove that the structure of the various complexes is formulation-dependent. In particular, the presence of the nucleotide lipid enhances the existence of a 2D inverse hexagonal phase (a = 69.1 Å) which does not occur in the nucleotide free lipid system (DOPE−DPPA−plasmid−DNA−Ca2+), while the lamellar phase with a stacking period d = 48 Å is present in both systems.

Cell Proliferation. The effect on the proliferation of mammalian cell lines of the nucleolipid−Ca2+−DNA lipoplexes was compared to a commercially available transfectant, lipofectamine and Ca2+−DNA binary mixtures. The in vitro lipoplex cytotoxicity was evaluated against Hek cells using an MTS assay. The proliferation of cells is expressed as percentages of nontreated controls (nontreated Hek cells). Note, furthermore, that no toxicity was observed even after 4 h of incubation for the palmitoyl derivative. In contrast, Hek cell viability decreased in the presence of lipofectamine, DOPE/DPPG−Ca2+−DNA, and Ca2+−DNA mixtures for both 2 and 4 h of incubation. The cytotoxic effect was much stronger for ternary mixtures featuring non-nucleotide lipid (DOPE/DPPA−Ca2+−DNA) than for di-palmitoyl nucleolipid (diC16−3′-dT), because the cell viability was reduced by 30% and 40% for 2 and 4 h, respectively. A similar cytotoxicity effect was observed with DOPE/diC12−3′-dT−Ca2+−DNA samples, indicating that an optimal chain length of C16 is required to avoid toxicity. Toxic effects of dilauroyl derivatives were previously reported for cationic lipids bearing 1,3-diamino-2-propanol backbone.

Fluorescence Microscopy Studies. We next used fluorescence microscopy to determine whether the DOPE/diC16−3′-dT−Ca2+−DNA complexes penetrated the cultured eukaryotic cells. For this purpose, DOPE/diC16−3′-dT liposomes and a mixture of nucleic acids containing plasmid DNA (0.75 µg) and oligonucleotide−fluorescein (0.25 µg) were mixed. The complex formation was realized by adding Ca2+ (final concentration 15 mM). Hek cells were incubated later with the fluorescein-labeled complexes for 2 h at 37 °C. As shown in Figure 7 (left), the fluorescein-labeled complexes were internalized at 37 °C, indicating that anionic lipoplexes can transfer DNA into cells.

Figure 6. Cytotoxicities of anionic lipoplexes composed of nucleolipids (diC12,14,16T), DPPA or DPPG and DOPE (10:90 mol ratio)−Ca2+ (15 mM)−DNA compared to lipofectamine and DNA with 15 mM Ca2+ and untreated cells.

Figure 7. Fluorescence microscopy images of anionic lipoplexes ([Ca2+] = 15 mM) carrying DNA fluorescein labeled (left) and transfected Hek cells (GFP expression) (right).

Figure 8. Transfection efficiencies of anionic lipoplexes composed of nucleolipids/DOPE (10:90 mol ratio) (diC12,14,16T), DPPA/DOPE (10:90 mol ratio)−Ca2+ (15 mM)−DNA, DPPG/DOPE (10:90 mol ratio)−Ca2+ (15 mM)−DNA, compared with lipofectamine and DNA with 15 mM Ca2+.

It is important to note that Hek cells incubated in the presence of anionic lipoplexes−prepared without fluorescein-labeled oligonucleotides−(DOPE/diC16−3′-dT−Ca2+−DNA), where the DNA is an expression vector (pEGFP) encoding GFP, confirm the release and the expression of the anionic lipoplexes from the GFP-plasmid DNA. (Figure 7 right).

Transfection. To investigate the efficiency of the anionic nucleotide based lipids to deliver DNA into cells, we performed a transient transfection assay of mammalian cell lines (Hek 293 human embryo kidney). The transfected DNA is an expression vector (pEGFP) encoding GFP, which enables the detection of fluorescence by FACS. Different formulations of anionic nucleolipids were mixed with 1 µg of pEGFP, and then lipoplexes were incubated with Hek cells. Single-cell suspensions were analyzed by FACS Canto dual laser flow cytometry. The GFP negative and positive gates were defined using the control experiment carried out in the absence of lipoplex. In each experiment, 50 000 cells were sorted, and the results are shown in Figure 8. Anionic lipoplexes are efficient for transfection at a concentration of liposomes (42 µg, DOPE/diC16−3′-dT−Ca2+−DNA) per well. As control experiments, the transfection capability of lipofectamine−DNA and calcium−DNA mixtures were evaluated. As expected for this cell line, lipofectamine appeared to be the most efficient transfecting reagent, whereas calcium salts alone remained less effective in transfecting EGFP. Likewise, with only 18% and 24% of transfection after 2 h of incubation, the DPPA−Ca2+−DNA and DPPG−Ca2+−DNA lipoplexes, respectively, exhibited weak transfecting activity. On the contrary, the nucleolipids exhibited higher transfecting efficacies. With a level of transfection higher...
than 40%, the DOPE/diC\textsubscript{16}-3′-dT-Ca\textsuperscript{2+}-DNA lipoplex shows the best efficacy among the anionic systems. Note that this level was increased to 55% after incubating the Hek cells in the presence of this complex for 4 h (data not shown). An explanation for the lower activity of dilauroyl derivatives could be found in the early work of Minsky and co-workers (44). They have suggested that fatty acid chains of 12 carbons are not long enough to create the hydrophobic environment required for efficient DNA compaction. Therefore, it appears that the anionic nucleotide lipids are more efficient compared to both DPPA-Ca\textsuperscript{2+}-DNA and Ca\textsuperscript{2+}-DNA transfecting systems, demonstrating that the nucleotide moiety influences the lipid–DNA associations and enhances the transfection efficacy of the natural anionic DPPA lipid.

CONCLUSION

In this study, we synthesized a new series of nucleotide-based lipids featuring thymidine-3′-monophosphate as nucleotide and 1,2-diacyl-sn-glycerol as hydrophobic moieties for in vitro delivery of nucleic acids. The amphiphilic structures were prepared in three steps from commercially available starting materials: 1,2-diacyl-sn-glycerols and 2′-deoxothyminidine-3′phosphoramidite. Gel electrophoresis experiments indicate that nucleotide-based lipid–DNA complexes are observed above a concentration of 1 mM, which is higher than the intracellular calcium concentration ([Ca\textsuperscript{2+}] 1 µM) indicating that DNA can be released inside the cells. Electronic microscopy and dynamic light scattering investigations reveal that extrusion of DOPE–diC\textsubscript{16}-3′-dT mixtures provide homogenous liposomes, whereas larger aggregates are observed in the presence of DNA and Ca\textsuperscript{2+} confirming the formation of complexes.

SAXS studies indicate that the structure of the complexes is strongly dependent on the formulation. Different multiphase systems can be obtained for the DOPE–diC\textsubscript{16}-3′-dT and DOPE–DPPA samples with and without plasmid DNA. The transfection experiments carried out on mammalian Hek cell lines clearly demonstrate that the nucleotide moiety enhances the transfection efficacy of the natural anionic DPPA and DPPG lipids. The SAXS studies also indicate that the enhancement in transfection is likely due to the presence of the 2D columnar inverted hexagonal phase (H\textsubscript{2}) with a unit cell parameter a = 69.1 Å, which does not occur in the nucleotide free lipid system (DOPE–DPPA). These results confirm the interest of using anionic species (45) such as nucleotide lipids in formulations to optimize transfection efficacy.

The issue of transfection reagent cytotoxicity is particularly important in protocols that attempt to modulate gene function in vitro and/or in vivo. As nontoxic and efficient transfection reagents, the anionic nucleotide lipids presented in this work offer an alternative to cationic species, which can be, in some instances, especially toxic (46). These investigations further support the need for the synthesis and evaluation of nucleotide-based lipids as nucleotide acids delivery vehicles. In vivo, nucleotide-based lipids further extend the possibilities of modulating the interactions between nucleic acids and lipids. Depending upon the nature of both partners, nucleotide lipids may offer new alternatives to lipids, and could be exploited in the case of small therapeutic oligonucleotides, for example.

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LITERATURE CITED
