Safe and efficient *in vitro* and *in vivo* gene delivery: tripodal cationic lipids with programmed biodegradability†

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The therapeutic use of nucleic acids has long been heralded as a panacea of medicinal opportunity, a vision enhanced by the introduction of RNA interference technology. The Achilles heel of such an approach is the *in vivo* delivery of the desired nucleic acid into cells, a practice that lacks selectivity, safety and/or efficiency. Herein we report the safe and efficacious *in vitro* and *in vivo* delivery of nucleic acids using tripodal biodegradable cationic lipids. Toxicity reduction and transfection potency of these novel amphiphiles were addressed by designing the compounds to undergo complete intracellular degradation thereby enhancing cargo release while minimising toxicity and potential tissue accumulation. Compounds demonstrated high-efficiency in transfecting DNA into cells both *in vitro* and *in vivo* with no signs of toxicity, thus potentially offering a safer alternative to viral transfection for gene therapy application.

Introduction

Delivery of nucleic acids into cells has an ever-increasing number of applications with outstanding advances in gene and antisense therapy[1] and, for example, in the reprogramming of somatic cells to induced pluripotent stem cells.[2] While viruses are currently the most efficient transfection vectors, they suffer from numerous innate disadvantages (antigenicity, potential mutagenesis, *etc.*) that limit their appeal.[3] As such non-viral delivery systems[4] (predominantly cationic lipids[4a–e] and polymers[4d–f]) are an attractive alternative, particularly because of their procedural simplicity and tuneable design.

Over the past two decades, a wide range of cationic lipids with the ability to complex and deliver nucleic acids into cells has been developed.[4a–e] The general structure of these materials is characterised by two constituent domains of contrasting chemical polarity: the hydrophobic part/s and the cationic headgroup/s. While the structural diversity investigated to date is relatively broad, the chemical nature of these domains is typically invariant, consisting of one or more nitrogen-based cationic motifs (mainly guanidinium[5] or mono/polyalkyl ammonium groups[6]) and a lipophilic domain (typically composed of a steroidal moiety[5a,6c–f] or two hydrophobic chains[5b,6a,b,7]). Cationic lipids containing a single hydrocarbon chain have been explored much less intensely[8] due to their anticipated toxicity.[9] However, it has recently been demonstrated that tripodal single-tailed cationic lipids have excellent transfection efficiencies while displaying low toxicity.[10]

The lipophilic domains and the cationic headgroups are covalently connected by a backbone or linking moiety. The chemical nature of the linking moiety has a critical role in the gene transfer abilities of the cationic lipid since it influences fundamental characteristics of the structure such as the conformational flexibility and the degree of stability.[11] With this in mind, many researchers have investigated the incorporation of stimuli-responsive bonds[12] in the linking moiety (*e.g.* enzyme,[13] pH[14] or redox[15] susceptible chemical groups) to improve the transfection efficiency and reduce the toxicity of cationic lipids. While quite a few of these clever designs have demonstrated remarkable *in vitro* transfection properties, cationic lipid-based carriers have not yet met the criteria for gene therapy uses since the *in vivo* efficacy of these carriers needs to be increased and their cytotoxicity substantially reduced for both research purposes and clinical applications.[16]

One of the critical factors to be addressed in any eventual gene therapy treatment is the cytotoxicity associated with the continued cytoplasmic residence and tissue accumulation of the delivery system. To overcome this issue some researchers have previously investigated the use of natural polymers which metabolize into known degradation products.[17] By application of an analogous strategy to cationic lipids, a family of esterase-sensitive tripodal cationic lipids were designed to generate lipoplexes able to enter cells by endocytosis and, subsequently, facilitate intraendosomal destabilization and undergo complete intracellular metabolization into amino acids, fatty acids and tris,[18] **1**, a gamut of benign materials (Scheme 1a). In addition to a reduction of toxicity, it was hypothesized that, upon cell uptake, ester cleavage would trigger endosomal DNA–lipid dissociation. The enzymatic disassembling of the lipoplexes[12a,19] would facilitate the free lipids to partition into the endosome

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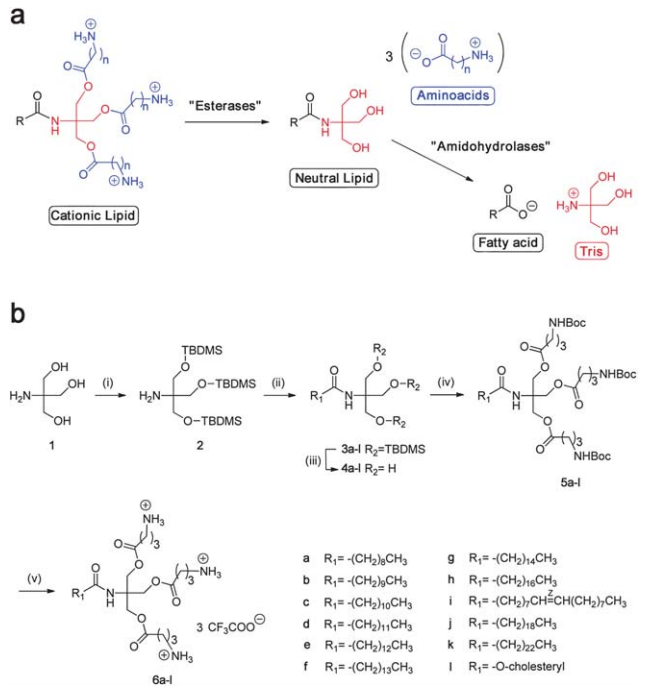

Scheme 1 (a) A biodegradable cationic lipid and its programmed enzymatic processing. Metabolisation affords amino acids, fatty acids and tris, an FDA-approved material[18] widely used as a constituent of biological buffers. (b) Synthesis of biodegradable compounds **6a–l**. *Reagents and conditions*: (i) TBDMS-Cl (3 equiv.), imidazole, DMF, 4 h, 95%; (ii) fatty acid (1.1 equiv.), DCC (1.1 equiv.), DMAP (0.1 equiv), DCM, 2 h, 82–95%; or cholesteryl chloroformate (1.1 equiv.), Pyr (1.1 equiv.), DMAP (0.1 equiv.), DCM, 2 h, 87%; (iii) TBAF (3 equiv.), THF, 1 h, 71–88%; (iv) *N*-'Boc-GABA (3.3 equiv.), EDC (3.3 equiv.), DMAP (0.1 equiv.), DMF : DCM (1 : 1), μW, 60 °C, 30 min, 71–88%; (v) DCM : TFA (1 : 1), 30 min, 100%.

lipid bilayer,[20] where lipid mixing would sterically destabilise endosomal integrity and facilitate DNA escape.

Experimental

Synthesis of compounds

The cationic lipids were synthesized as shown in Scheme 1b. The hydroxyl groups on tris, **1**, were selectively protected using *tert*-butyldimethylsilyl chloride[21] (TBDMS-Cl) and the amine subsequently coupled to a variety of fatty acids or cholesteryl chloroformate giving intermediates **3a–l**. Removal of the silyl groups using tetrabutylammonium fluoride (TBAF) and coupling with Boc-protected γ-aminobutyric acid (*N*-'Boc-GABA) to the free hydroxyl groups using EDC/DMAP (with microwave irradiation) gave compounds **5a–l**. Removal of the *N*-Boc groups under acidic conditions (DCM : TFA (1 : 1)) gave **6a–l** as the trifluoroacetic salts.[22] The identities and purities of compounds **6a–l** were established by HRMS, ^{1}H and ^{13}C NMR, and Elemental Analysis (see Section 1 of ESI†).

Lipoplex preparation protocol

The corresponding cationic lipids **6a–l** (1 mM in methanol) with or without DOPE (1 mM in methanol) were mixed and the organic solvent removed by evaporation (37 °C, overnight). The resulting films were then hydrated with PBS. The mixtures were pipetted up and down (20×) or sonicated for 5 min and then incubated at room temperature for 30 min. DNA (0.04 mg mL^{-1} of pEGFP-C1 in PBS) was subsequently added to the formulation and mixed by pipetting up and down (20×). Cationic lipids and DNA were mixed as a function of their charges (N/P ratio[10]). The lipoplexes were incubated at room temperature for 30 min before being used.

Gel electrophoresis assays

DNA retardation assay. Lipoplexes of pDNA and compounds **6a–l** (N/P ratios of 1.5, 3 and 6) were prepared in the absence of DOPE as described before. Subsequently, complexes were mixed with GelPilot DNA Loading Dye (Qiagen) and loaded into an agarose gel (1% agarose, 1 μg mL^{-1} ethidium bromide). The gel was run in TBE buffer at 100 V for 1 h and then imaged under UV light.

Esterase-mediated DNA liberation assay. Compounds were complexed as described before with pDNA at N/P ratio 6 and then incubated at 37 °C with porcine liver esterase (final concentration 10 μM) for 0, 3 or 6 h before being loaded into an agarose gel. The gel was run and imaged as above.

Cell culture

Cells were grown in RPMI or DMEM (Supelco) supplemented with 4 mM glutamine, 10% FCS and 100 units mL^{-1} penicillin/streptomycin (CM) at 37 °C and 5% CO_2 until 70–80% cell confluency. Cells were detached using trypsin/EDTA, diluted in their corresponding supplemented media and counted. 2×10^4 cells in 100 μL of media per well were seeded in 96 well plates and incubated overnight before transfection.

DNA transfection assay

Lipoplex formulations (0.2 μg per 100 μL of pEGFP-C1 mixed with the corresponding amounts of compounds and DOPE) were added in triplicate. Culture media was not changed during the transfection experiments. After incubation for two days, the GFP expression was observed by microscopy (Leica) and measured using a BioTek microplate reader FLx800 (485/20 excitation, 530/25 emission). Hits were analyzed by flow cytometry. *Procedure:* cells were washed twice with PBS, detached with trypsin/EDTA, harvested with 2% FCS in PBS, centrifuged and resuspended with 2% FCS in PBS. Cell fluorescence was analyzed using a BD FACSAria flow cytometer. Data were expressed as a percentage of transfected cells and total mean fluorescence. Effectene® (Qiagen) and Lipofectamine™ 2000 (Invitrogen) were used as positive controls and untreated cells as a negative control. Ester-free tripod-like cationic lipids[10] **7a** and **7b** (see Fig. S2† of the ESI†) were used with DOPE (1 : 1.5 molar ratio) with an N/P ratio of 6.

Cell viability assay

Twenty-four hours after the addition of the lipoplexes, cell death was measured using an MTT cell proliferation assay (Promega). Absorbance was measured at 570 nm using a spectrophotometer.

Particle size and ζ potential analysis

6i : DOPE aqueous dispersions were analyzed by dynamic light scattering and laser doppler electrophoresis using a Zetasizer ZS (Malvern Ltd). *Procedure:* lipoplexes (2 μg of pEGFP-C1 and the corresponding amount of compound **6i** and DOPE, in a total volume of 100 μL of PBS) were prepared as described before and then diluted with 0.9 mL of PBS or RPMI-CM. Diluted formulations were incubated at room temperature for 30 min prior to analysis. Folded capillary cells (Malvern Ltd) were loaded with each formulation (1 mL) following the procedure described by the supplier and analyzed in triplicate.

In vivo experiments

Mice ($n = 3$, per experiment) were anaesthetized and intubated following standard protocols. 16 μg of a luciferase-reporter plasmid (pLux, 4–6 mg mL^{-1} in PBS) was complexed with compound **6i** (N/P 12, 1 : 1.5 mol mixture with DOPE) following the protocol described above. Lipoplex formulation (total volume = 50 μL) and naked plasmid (16 μg of plasmid in 50 μL of PBS) were then administrated into the lung by direct intratracheal instillation through peroral intubation. Mice were monitored and analyzed on a daily basis, and all of them behaved normally and appeared healthy throughout the study. Firefly luciferin (15 mg kg^{-1}) was intraperitoneally administrated to the anesthetized mice 15 min before scanning for luminescence. Imaging was performed on the IVIS Spectrum (Caliper LS) with large binning, open filter for 10 min.

Results and discussions

DNA retardation assays

To assess the ability of compounds **6a–l** to complex DNA, lipoplexes were prepared at different N/P ratios (1.5, 3 and 6) and analysed by gel electrophoresis. As observed in Fig. 1a, compounds **6d–l** completely inhibited the electrophoretic mobility of DNA when used at N/P ratio ≥ 6, indicating the formation of stable lipoplexes. To illustrate the enzyme-responsive properties of these lipoplexes, complexes were incubated with porcine esterase and analysed by electrophoresis, demonstrating the liberation of the DNA from the complex after 3 h (Fig. 1b). This relatively slow esterase mediated release rate highlighted the stability of the complex, an aspect that is critical for *in vivo* transfection. Electrophoresis analysis of the lipoplexes incubated with serum-containing medium for two days showed no DNA liberation, which confirmed the high stability of the lipoplexes towards serum.

DNA transfection *in vitro*

The gene transfer abilities of compounds **6a–l** were first evaluated with HeLa cells employing pEGFP-C1 as a reporter vector. Lipoplexes were formulated using DOPE as co-lipid (in molar ratios 1 : 1 and 1 : 2) and DNA at a variety of N/P ratios (6, 12 and 24, in accordance with the electrophoretic observations) with the cytotoxicity examined in parallel (MTT assay). Compounds were tested in triplicate using Lipofectamine™ 2000 (Invitrogen) and Effectene (Qiagen) as positive controls and untreated cells as a negative control. Compounds **7a** and **7b** (ester-free tripodal cationic lipids containing an oleoyl and lignoceryl chain, respectively[10]) were also tested to investigate if the presence of ester bonds in the tripodal cationic lipids had a positive role on the delivery process. Analysis demonstrated that transfection ability was strongly dependent on the lipid moiety (see Sections 2 and 3 of the ESI†), with none or little transfection detected with compounds **6a–c**, in accordance with their poor ability to complex DNA (Fig. 1b). In general, the lipid : DOPE ratio was critical for high transfection efficiency, with a 1 : 2 ratio being optimal for most of the library members. At this ratio, EGFP expression was very high with compounds **6d**, **6e**, **6f**, **6i** (N/P 12) and **6k** (N/P 6) (see Fig. 1c), indicating that the incorporation of esters clearly has a positive influence on the transfection abilities of the novel amphiphiles, and that this effect operates synergistically with the DOPE ability to promote the endosomal disruption.[23] Flow cytometry analysis (Fig. 1d) showed that cationic lipid **6i** (the oleoyl derivative) had the highest levels of

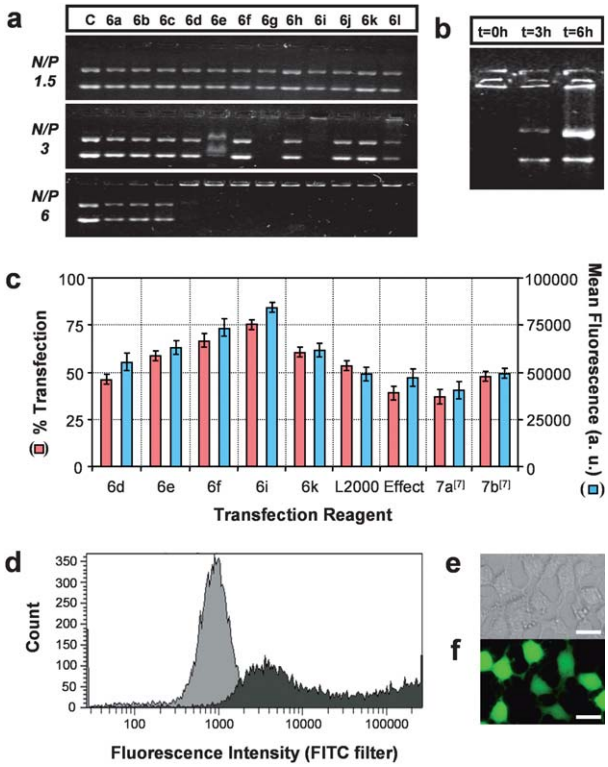

Fig. 1 (a) Electrophoretic DNA retardation assays. C = naked pEGFP-C1 (showing both supercoiled and nicked circular forms). Compounds **6a–l** were complexed with pEGFP-C1 with N/P ratios of 1.5, 3 and 6 before being loaded onto a 1% agarose gel (with 1 μg mL^{-1} ethidium bromide). (b) Esterase-mediated lipoplex cleavage. Compound **6i** was complexed with pEGFP-C1 at N/P 6 and incubated with porcine liver esterase (10 μM) for 0–6 h before electrophoretic analysis. (c) Flow cytometry analysis of HeLa cells 48 h after transfection with pEGFP-C1 (0.2 μg/100 μL) by compounds **6d**, **6e**, **6f**, **6i**, **6k**, Lipofectamine™ 2000 (L2000), Effectene (Effect) and compounds **7a** and **7b**. Results are expressed as a percentage of transfected cells and mean fluorescence (arbitrary units). (d) Flow cytometry analysis of untransfected HeLa cells (light grey) and cells transfected with lipid **6i** (dark grey). (e and f) Brightfield and fluorescent images of HeLa cells after transfection with lipid **6i** (scale bar = 30 μm).

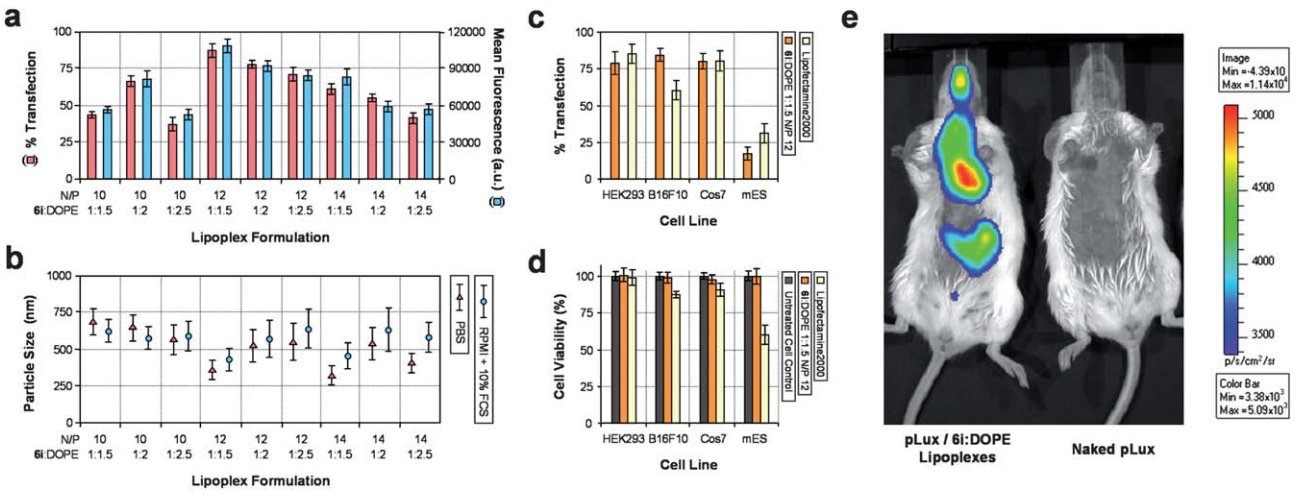

Fig. 2 (a) Optimization of oleoyl derivative **6i**. Compound **6i** was complexed with pEGFP-C1 at various N/P and DOPE ratios, with cellular fluorescence determined by flow cytometry after 48 h. Results are expressed as transfection efficiency (%) and mean fluorescence (arbitrary units). (b) Size of the lipoplexes as determined by dynamic light scattering using a Zetasizer Nano ZS (Malvern Ltd) with the particle dispersity represented by the error bars. (c) Flow cytometry analysis of cells transfected with pEGFP-C1 using an optimised formulation of compound **6i** and Lipofectamine™ 2000. (d) Viability assay: cell control, optimised formulation of compound **6i**, and Lipofectamine™ 2000. (e) Representative luminescence imaging of anaesthetized mice transfected, by instillation, with 16 μg of pLux complexed with a formulation **6i** : DOPE 1 : 1.5 with an N/P ratio of 12 (left mouse) and the naked plasmid (right mouse) after 48 h. Mice were imaged 15 min after intraperitoneal administration of firefly luciferin.

transfection, with no apparent toxicity for any of the formulations tested (see Section 3.3 of the ESI†).

To study the influence of DOPE and the reagent : DNA charge ratio (N/P ratio) on **6i** transfection abilities, the compound was formulated with DOPE and pEGFP-C1 at a variety of molar (1 : 1.5, 1 : 2 and 1 : 2.5) and N/P ratios (10, 12 and 14) respectively, and screened against HeLa cells (Fig. 2a), with formulation **6i** : DOPE 1 : 1.5 with an N/P ratio of 12 observed to be optimal. Attempting to correlate the lipoplex transfection abilities with the aggregation properties of the formulations, particle size and ζ potential were analysed. Prior to the addition of DNA, liposome sizes ranged between 460 and 700 nm and a ζ potential of 31–34 mV, with the formulation of **6i** : DOPE 1 : 1.5 having the smallest sizes (459 ± 42 nm). Upon the addition of DNA, lipoplex potentials were slightly reduced for most formulations (25–30 mV) in PBS and dramatically reduced (<−9 mV) in media containing serum (see Section 4 of the ESI†). As observed in Fig. 2b, lipoplex size was highly modulated by the formulation and the dilution medium, with **6i** : DOPE 1 : 1.5 formulations at N/P 12 (the optimal for transfecting HeLa) and N/P 14 being the smallest and most homogeneous particle size distribution in both the presence (<500 nm) and absence of serum (<400 nm).

To investigate this optimal reagent, experiments were performed on a variety of cell lines (HEK293T, B16F10 and COS-7) with the optimized formulation of **6i**, demonstrating significant transfection efficacy (>75%) while being non-toxic (Fig. 2c and d). Transfection of E14 mouse embryonic stem (mES) cells was 18%, which although less than Lipofectamine™ 2000 (29%), showed no indication of cellular toxicity (Fig. 2d) or altered cellular morphology unlike the latter (see Section 3.5 of ESI†).

DNA transfection *in vivo*

Given the potential use of gene therapy to treat various pulmonary diseases (*e.g.* cystic fibrosis,[24] pulmonary adenocarcinoma,[25] pulmonary arterial hypertension,[26] pulmonary metastatic tumor,[27] *etc.*), the optimal formulation of compound **6i** was used to perform *in vivo* local transfection of a luciferase-reporter plasmid (pLux) into the lungs of mice,[28] with the naked plasmid employed as a control (pLux was used to allow real-time *in vivo* analysis of gene expression). Two days after instillation, firefly luciferin was administrated intraperitoneally and the mice imaged using an IVIS Spectrum. Positive luminescence was detected in mice transfected with derivative **6i** while no luminescence was observed in the control (see Fig. 2e). Luciferase activity signal was detected not only in the lungs but also in the mouth, trachea and liver, which highlights the potent *in vivo* transfecting ability of derivative **6i**.

Conclusions

A series of tripodal cationic lipids were designed to be readily metabolised once inside cells into benign materials, thus rendering them safe for repetitive dosing. The best-performing compound of the library (the oleoyl derivative) showed remarkable transfection efficiency along with no toxicity in a variety of immortalised cells and mES cells. Moreover, preliminary *in vivo* studies in murine model underlined the potential applicability of this reagent for the delivery of DNA to the respiratory tract.

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28 Animal experimentation was approved by UK regulatory authorities (Project licence 60/3545 from UK Home Office).

