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Cationic lipid saturation influences intracellular delivery of encapsulated nucleic acids

James Heyes [1], Lorne Palmer [1], Kaz Bremner, Ian MacLachlan *

Protiva Biotherapeutics Inc., 100-3480 Gilmore Way, Burnaby, B.C., Canada V5G-4Y1

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Abstract

An analogous series of cationic lipids (1,2-distearyloxy-*N,N*-dimethyl-3-aminopropane (DSDMA), 1,2-dioleyloxy-*N,N*-dimethyl-3-aminopropane (DODMA), 1,2-dilinoleyloxy-*N,N*-dimethyl-3-aminopropane (DLinDMA) and 1,2-dilinolenyloxy-*N,N*-dimethyl-3-aminopropane (DLenDMA)) possessing 0, 1, 2 or 3 double bonds per alkyl chain respectively, was synthesized to determine the correlation between lipid saturation, fusogenicity and efficiency of intracellular nucleic acid delivery. ^{31}P-NMR analysis suggests that as saturation increases, from 2 to 0 double bonds, lamellar (L_α) to reversed hexagonal (H_{II}) phase transition temperature increases, indicating decreasing fusogenicity. This trend is largely reflected by the efficiency of gene silencing observed in vitro when the lipids are formulated as Stable Nucleic Acid Lipid Particles (SNALPs) encapsulating small inhibitory RNA (siRNA). Uptake experiments suggest that despite their lower gene silencing efficiency, the less fusogenic particles are more readily internalized by cells. Microscopic visualization of fluorescently labelled siRNA uptake was supported by quantitative data acquired using radiolabelled preparations. Since electrostatic binding is a precursor to uptake, the pKa of each cationic lipid was determined. The results support a transfection model in which endosomal release, mediated by fusion with the endosomal membrane, results in cytoplasmic translocation of the nucleic acid payload.

Keywords: siRNA delivery; RNA interference; Cationic lipid; Phase transition temperature and SNALP

1. Introduction

RNA interference (RNAi) is a recently discovered gene-silencing tool. Small interfering RNA (siRNA) are short, double stranded RNA molecules that, in the presence of endogenous RNA-Induced Silencing Complex (RISC) unwind and bind to specific sequences of messenger RNA (mRNA) subsequently mediating the destruction of the target mRNA by endogenous cellular machinery. In this way, RNAi has the potential to selectively inhibit the expression of disease-associated genes in humans.

Stabilized Plasmid Lipid Particles (SPLP), consisting of a unilamellar lipid bi-layer encapsulating a single copy of plasmid DNA, have been reported

* Corresponding author. Tel.: +1 604 630 5064.
E-mail address: ian@protivabio.com (I. MacLachlan).
[1] The first two authors contributed equally to this work.

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as a novel method of systemic nucleic acid delivery [1–5]. Originally developed using a detergent dialysis technique, they are now prepared more quickly and uniformly using the spontaneous vesicle formation method of Jeffs et al [6]. This method has been adapted to encapsulate other nucleic acids such as duplex siRNA molecules. The resulting particles, referred to as Stabilized Nucleic Acid Lipid Particles (SNALP), exhibit the stability, small size (<200 nm) and low surface charge required for systemic delivery [7].

The SNALP bi-layer contains a mixture of cationic and fusogenic lipids that enable the cellular uptake and endosomal release of the particle's contents. SNALP also contain a diffusible poly (ethylene glycol)-lipid conjugate (PEG-lipid) that provides a neutral, hydrophilic coating to the particle's exterior. PEG-lipids both stabilize the particle during the formulation process and shield the cationic bi-layer, preventing rapid systemic clearance. Upon administration, the PEG-lipid conjugate dissociates from the SNALP at a rate determined by the chemistry of the PEG-lipid anchor, transforming the particle into a transfection-competent entity [1]. Cellular uptake of non-viral transfection reagents occurs primarily via endocytosis. Escape from the endosome is known to be a limiting step when using lipidic systems to deliver nucleic acids. Fusogenic systems readily overcome this barrier since they promote the breakdown of the endosomal membrane leading to cytoplasmic translocation of their nucleic acid payloads. Lipidic systems are most fusogenic when arranged in the reversed hexagonal phase (H_{II}), as opposed to the more stable bi-layer forming lamellar phase (L_α). A low phase transition temperature between the two states indicates a lower activation energy for the formation of the fusogenic H_{II} phase. Because of the highly defined nature of the SNALP particle, with its fully encapsulated nucleic acid payload and well-characterized mechanism of intracellular delivery, SNALP make an ideal system for examining the role of individual lipid components in the pharmacology of non-viral vector systems. In this work we explore the role of bi-layer fusogenicity in uptake, endosomal escape, and gene-silencing efficiency of SNALP.

Fusogenicity is considered to contribute to cytoplasmic delivery of nucleic acids [8–11]. It has been shown, using gold-labeled particles, that upon uptake via endocytosis the majority of lipoplex remain localized in the endosome, failing to escape to the cytoplasm [12]. The incorporation of fusogenic lipids such as dioleoylphosphatidylethanolamine (DOPE) improves the efficiency of endosomal release by encouraging fusion events between the liposomal and endosomal bi-layers [12–14]. The resulting disruption to the endosomal bi-layer aids in the escape of the therapeutic nucleic acid to the cytoplasm. In the case of a fully encapsulated system, fusion is expected to result in cytoplasmic translocation of the nucleic acid payload.

The fusogenic nature of DOPE-containing bilayers is thought to be due to their polymorphic nature. Upon formulation, most lipids adopt the bilayer-forming lamellar phase (L_α). DOPE however, has a tendency to form the inverse hexagonal phase (H_{II}) [14]. Using video microscopy, Koltover et al. studied the interaction of lipoplexes with giant anionic vesicles (G-vesicles), a model for the endosomal membrane [15]. They demonstrated that while lipoplex comprised of lipids in the L_α phase would simply attach themselves stably to the surface of the G-vesicles, H_{II} phase forming lipoplex rapidly fused with model endosomes. Lipids that adopt the H_{II} phase are therefore regarded as 'fusogenic'.

Other researchers have noted that the degree of saturation of a lipid hydrophobic domain affects its ability to adopt the H_{II} phase [16–19]. All have reported a trend whereby an increasing number of double bonds corresponds with an increasing propensity to form the non-bi-layer phase. It might therefore be possible to increase the tendency of the SNALP bilayer to form the fusogenic H_{II} phase by decreasing the degree of saturation in the lipid hydrophobic domain of the cationic lipid component. Using the SNALP lipid DODMA (containing a single double bond per lipid chain) as a starting point, we synthesized a homologous series of lipids with 0, 1, 2 or 3 double bonds and studied the effect of these changes on physicochemical properties of bi-layers into which they were incorporated.

The lipids were incorporated in SNALP containing anti-luciferase siRNA and assessed for both uptake efficiency and their ability to inhibit luciferase expression in stably transfected Neuro 2A cells. The surface pKa was investigated using a toluene nitrosulphonic

acid (TNS) assay. The relative influence of each lipid on phase transition temperature (T_c) was also studied, using ^{31}P-NMR analysis.

2. Materials and methods

2.1. Materials

DPPS, 1,2-Distearoyl-*sn*-glycero-3-phosphocholine (DSPC) and cholesterol were purchased from Avanti Polar Lipids (Alabaster, AL). TNS was obtained from Sigma-Aldrich Canada (Oakville, ON). RiboGreen was obtained from Molecular Probes (Eugene, OR). The alkyl mesylates were purchased from Nu-Chek Prep, Inc. (Elysian, MN, USA). siRNA (anti-luciferase and mismatch control) was purchased from Dharmacon (Lafayette, CO, USA). The anti-luciferase sense sequence was 5′-G.A.U.U.A.U.G.U.C.C.G.-G.U.U.A.U.G.U.A.U.U-3′. The anti-luciferase antisense sequence was 5′-U.A.C.A.U.A.A.C.C.G.G.A.C.A.U.A.A.U.C.U.U-3′. All other chemicals were purchased from Sigma-Aldrich (Oakville, ON, Canada).

2.2. Synthesis of DSDMA and DODMA

DSDMA and DODMA were synthesized using the respective alkyl bromides with methodology derived from that of a DOTMA precursor [20]. 3-(Dimethylamino)-1,2-propanediol (714 mg, 6 mmol) and 95% sodium hydride (NaH, 1.26 g, 50 mmol) were stirred in benzene (30 mL) under argon for 30 min. The correct (either oleyl or stearyl) alkyl bromide (5.0 g, 15 mmol) was added and the reaction refluxed under argon for 18 h. The reaction mixture was then cooled in an ice bath while quenching via the slow addition of ethanol. Following dilution with a further 150 mL of benzene, the mixture was washed with distilled water (2×150 mL) and brine (150 mL), using ethanol (~20 mL) to aid phase separation if necessary. The organic phase was dried over magnesium sulphate and evaporated. The crude product was purified on a silica gel (Kiesel Gel 60) column eluted with chloroform containing 0–5% methanol. Column fractions were analyzed by thin layer chromatography (TLC) (silica gel, chloroform/methanol 9:1 v/v, visualized with molybdate) and fractions containing pure product (R_f=0.5) were pooled and concentrated. The product was decolorized by stirring for 30 min in a suspension of activated charcoal (1 g) in ethanol (75 mL) at 60 °C. The charcoal was removed by filtration through Celite, and the ethanol solution concentrated to typically yield 2.4 g (65%) of pure product. ^{1}H-NMR (DSDMA): δ_H 3.65 – 3.32 (m, 7H, OCH, $3 \times OCH_2$), 2.45 – 2.31 (m, 2H, NCH_2), 2.27 (s, 6H, $2 \times NCH_3$), 1.61 – 1.45 (m, 4H, OCH_2CH_2), 1.40 – 1.17 (m, 60H, $H_{stearyl}$), 0.86 (t, 6H, CH_2CH_3). ^{1}H-NMR (DODMA): δ_H 5.4 – 5.27 (m, 4H, $2 \times CH=CH$), 3.65 – 3.35 (m, 7H, OCH, $3 \times OCH_2$), 2.47 – 2.33 (m, 2H, NCH_2), 2.28 (s, 6H, $2 \times NCH_3$), 2.06 – 1.94 (m, 8H, $4 \times CH_2CH=CH$), 1.61 – 1.50 (m, 4H, OCH_2CH_2), 1.38 – 1.20 (m, 48H, H_{oleyl}), 0.88 (t, 6H, CH_2CH_3).

2.3. Synthesis of DLinDMA and DLenDMA

The DLinDMA and DLenDMA were synthesized similarly to the DSDMA and DODMA, but used the alkyl mesylates instead of alky bromides. The general synthetic protocol was identical for those of DSDMA and DODMA, substituting the alkyl mesylates for the bromides in the same molar ratios. The activated charcoal decolorization step was omitted, since the products here contain conjugated double bonds and activated charcoal is expected to adsorb compounds containing such features. Yields were typically 2.0 g (55%). ^{1}H-NMR (DLinDMA): δ_H 5.43 – 5.27 (m, 8H, $4 \times CH=CH$), 3.65 – 3.35 (m, 7H, OCH, $3 \times OCH_2$), 2.77 (t, 4H, $=CHCH_2CH=$), 2.47 – 2.33 (m, 2H, NCH_2), 2.28 (s, 6H, $2 \times NCH_3$), 2.05 (q, 8H, $4 \times CH_2CH_2CH=$), 1.62 – 1.50(m, 4H, OCH_2CH_2), 1.40 – 1.22 (m, 32H, $H_{linoleyl}$), 0.89 (t, 6H, CH_2CH_3). ^{1}H-NMR (DLenDMA): δ_H 5.44 – 5.27 (m, 8H, $4 \times CH=CH$), 3.62 – 3.48 (m, 7H, OCH, $3 \times OCH_2$), 2.80 (t, 4H, $=CHCH_2CH=$), 2.43 – 2.32 (m, 2H, NCH_2), 2.26 (s, 6H, $2 \times NCH_3$), 2.12 – 1.99 (m, 8H, $4 \times CH_{2/3}CH_2CH=$), 1.61 – 1.51 (m, 4H, OCH_2CH_2), 1.40 – 1.22 (m, 20H, $H_{linolenyl}$), 0.98 (t, 6H, CH_2CH_3).

2.4. Synthesis of PEG2000-C-DMA

PEG-C-DMA was synthesized as follows. In brief, a C_{14} lipid anchor was prepared by first alkylating the hydroxyl groups of 3-allyloxypro-

pane-1,2-diol with myristyl bromide. The allyl group was subsequently removed via palladium catalysis, resulting in the C_{14} hydroxyl lipid. The hydroxyl group was converted to the primary amine by mesylation and amination to yield 1,2-dimyristyloxypropyl-3-amine, the lipid anchor. Conjugation with PEG was effected by treating monomethoxy poly(ethylene glycol) (average molecular weight 2000) with an excess of diphosgene to form the chloroformate. Addition of the C_{14} amine lipid anchor and stirring overnight yielded PEG_{2000}-C-DMA, referred to here as PEG-C-DMA.

2.5. SNALP Preparation

SNALP with a lipid composition of DSPC:Chol:-PEG-C-DMA:Cationic Lipid (20:48:2:30 molar percent) were prepared using the spontaneous vesicle formation by ethanol dilution method [6]. The samples were diafiltered against 100 mL of PBS (20 wash volumes) using a cross flow ultrafiltration cartridge (Amersham Biosciences, Piscataway, NJ) and sterile filtered through Acrodisc 0.8/0.2 μm syringe filters (Pall Corp., Ann Arbor, MI). The siRNA concentration of final samples was determined using the RiboGreen assay and a siRNA standard curve. Particle size and polydispersity was determined using a Malvern Instruments Zetasizer 3000HSA (Malvern, UK). Nucleic acid encapsulation was determined using a RiboGreen assay, comparing fluorescence in the presence and absence of Triton X-100. RiboGreen fluorescence was measured using a Varian Eclipse Spectrofluorometer (Varian Inc) with λ_{ex}=500 nm, λ_{em}=525 nm.

2.6. TNS Assay

20 μM of SNALP lipid and 6 μM of TNS were mixed in a fluorescence cuvette in 2mL of 20 mM sodium phosphate, 25 mM citrate, 20 mM ammonium acetate and 150 mM NaCl, at a pH that was varied from 4.5 to 9.5. Fluorescence was determined at each pH using a Varian Eclipse Spectrofluorometer (Varian Inc) with settings of λ_{ex}=322 nm, λ_{em}=431 nm. Fluorescence for each system at the various pH values was then normalized to the value at pH 4.5. The pKa values are the point at which 50% of the molecules present are charged. By assuming that minimum fluorescence represents zero charge, and maximum fluorescence represents 100% charge, pKa can be estimated by measuring the pH at the point exactly half way between the values of minimum and maximum charge.

2.7. ^{31}P Nuclear magnetic resonance spectroscopy

Multilamellar vesicles (MLV) were prepared comprising DPPS and cationic lipid at a molar ratio of 1:1. This was accomplished by drying the lipids from chloroform solution, transferring to 10 mm NMR tubes, and hydrating in 1.5 mL of 10 mM sodium citrate, pH 4. Free induction decays (FIDs) corresponding to 1000 scans were obtained with a 3.0 μs, 60° pulse with a 1 s interpulse delay and a spectral width of 25 000 Hz. A gated two-level proton decoupling was used to ensure sufficient decoupling with minimum sample heating. An exponential multiplication corresponding to 50 Hz of line broadening was applied to the FIDs prior to Fourier transformation. The sample temperature (+/− 1 °C) was regulated using a Bruker B-VT1000 variable temperature unit. Chemical shifts were referenced to 85% phosphoric acid as an external standard.

2.8. In Vitro Transfection

Cells were cultured in MEM (Invitrogen) containing 10% fetal bovine serum (FBS) (CanSera) and 0.25 mg/mL G418 (Invitrogen). Neuro2A-G cells (Neuro2A cells stably transfected to express luciferase [21]) were plated at a concentration of 4×10^4 cells per well in 24-well plates and grown overnight. Cells were treated with SNALP at doses of 0.0625 – 1.0 μg/mL nucleic acid (AntiLuc Active or Mismatch Control) and incubated for 48 h at 37 °C and 5% CO_2. Cells were then washed with PBS and lysed with 200 μL 250mM sodium phosphate containing 0.1% Triton X-100. The luciferase activity for each well was determined using Luciferase Reagent (Promega) and a standard luciferase protein (Roche). The luminescence for each was measured using a Berthold MicroLumat-Plus LB96V plate luminometer. The resulting luciferase activity was then normalized for the amount of protein using the Micro BCA assay kit (Pierce). Luciferase knockdown relative to a control was then determined for each system.

2.9. Cellular uptake

SNALP were prepared incorporating the non-exchangeable tritium-labeled lipid cholesteryl hexadecyl ether (^{3}H-CHE) (11.1 μCi/ μmol total lipid) [22]. Neuro2A cells (ATCC, VA, USA) were plated in 12 well plates at 1.6×10^5 cells per well in minimal essential media. The following day, media was removed and replaced with media containing radiolabelled SNALP at 0.5 μg/mL nucleic acid. After 24 h, the media and unincorporated SNALP were removed, adherent cells gently washed 4 times with PBS, and then lysed with 600 μL Lysis Buffer (250 mM phosphate with 0.1% Triton X-100). The resulting cell lysate (500 μL) was added to glass scintillation vials containing 5 mL Picofluor 40 (Perkin Elmer) and ^{3}H-CHE was determined using a Beckman LS6500 scintillation counter (Beckman Instruments). The protein content of cell lysates was determined using the Micro BCA assay (Pierce). Uptake was expressed as a percentage of the total amount of activity applied to the cells per mg of cellular protein.

2.10. Uptake of SNALP containing Cy3-labeled siRNA

SNALP were formulated as previously described, but using siRNA labelled with the fluorophore Cy3 (Cy3-siRNA was a gift of Sirna Therapeutics Inc, Boulder, CO). The encapsulation, siRNA concentration, and particle size were determined as described.

For the uptake study, 8×10^4 Neuro2A-G cells were grown overnight on 4-well chamber slides (BD Falcon, Mississauga, ON) in MEM containing 0.25 mg/mL G418. DSDMA, DODMA, DLinDMA, and DLenDMA SNALP containing Cy3-siRNA, as well as naked Cy3-siRNA and unlabeled DSDMA SNALP were placed on the cells at 0.5 μg/mL siRNA. After a 4 h incubation with the transfection media, the cells were washed with PBS, then with MEM containing G418 and finally with PBS once more. The cells were then fixed in a 4% paraformaldehyde solution in PBS for 10 min at room temperature. The cells were washed with PBS and stained with 300 nM DAPI (Molecular Probes, Eugene, OR) in PBS for 5 min. The cells were washed with PBS, the mounting media ProLong Gold Antifade Reagent (Molecular Probes, Eugene, OR) applied and a cover slip added. The cells were viewed using an Olympus BX60 Microscope modified for fluorescence capabilities. Cy3 fluorescence within the cells was visualized using a rhodamine cube set (Microgen Optics, Redding, CA) and the DAPI fluorescence was visualized using a DAPI cube set (Carsen Group, Markham, ON). Digital pictures were captured using an Olympus DP70 camera system. Pictures of the cells were taken at exposure times of 1/4 s when examining Cy3 fluorescence and 1/80 s when examining DAPI fluorescence.

3. Results

3.1. Formulation characteristics of unsaturated lipids are uniform and reproducible

SNALP containing the various cationic lipids were prepared as described and encapsulated RNA and particle size assessed (Table 1). The three unsaturated cationic lipids resulted in formulations that were approximately the same size (132 – 140 nm). Polydispersity of all formulations was low, indicating a narrow distribution of particle size. RNA encapsulation in the final particles was 84– 85% of the total. Attempts to encapsulate siRNA in SNALP using the saturated lipid DSDMA resulted in the formation of slightly larger particles (180 nm) with encapsulation of 67%.

3.2. pKa of cationic lipids is influenced by saturation

The apparent pKa of the cationic lipids was determined as described in Materials and Methods. Lipid pK_a correlated with degree of saturation with

Table 1
Physical properties of SNALP formulations

Cationic lipid	Percentage Encapsulation	Diameter (nm)	Polydispersity
DSDMA	67 ± 3	182 ± 11	0.15 ± 0.03
DODMA	84 ± 1	137 ± 4	0.12 ± 0.01
DLinDMA	84 ± 3	140 ± 6	0.11 ± 0.02
DLenDMA	85 ± 1	132 ± 7	0.13 ± 0.03

'Percentage Encapsulation' is determined using the RiboGreen fluorescence assay to measure the amount of encapsulated nucleic acid relative to the total nucleic acid present. Particle diameter and polydispersity was measured using a Malvern Zetasizer. Values are the mean of 3 separate experiments, the error is standard deviation.

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