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A new liposomal formulation for antisense oligodeoxynucleotides with small size, high incorporation efficiency and good stability

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Abstract

Antisense oligodeoxynucleotides (asODN) are therapeutic agents that are designed to inhibit the expression of disease-related genes. However, their therapeutic use may be hindered due to their rapid clearance from blood and their inefficiency at crossing cell membranes. Cationic liposome complexes have been used to enhance the intracellular delivery of asODN in vitro; however, this type of carrier has unfavorable pharmacokinetics for most in vivo applications. Significant therapeutic activity of cationic liposomal asODN following systemic administration has not been demonstrated. In an effort to develop improved liposomal carriers for asODN for in vivo applications, we have evaluated the physical characteristics of two formulations which represent alternatives to cationic liposome–asODN complexes: asODN passively entrapped within neutral liposomes (PELA) and asODN formulated in a novel coated cationic liposomal formulation (CCL). Our results confirm that PELA can be extruded to small diameters that are suitable for intravenous administration. PELA are stable in human plasma; however, the incorporation efficiency is relatively low ($\sim$20%). The CCL formulation can also be extruded to small diameters ($<$200 nm), with significantly higher (80–100%) incorporation efficiency and are stable in 50% human plasma at 37°C. A liposomal carrier for asODN with these characteristics may provide a significant therapeutic advantage over free asODN for some therapeutic applications.

Keywords: Antisense oligodeoxynucleotides; Gene therapy; Liposomes; DOTAP; PEG

1. Introduction

Antisense oligodeoxynucleotides (asODN) can be used to decrease the expression of disease-related genes by inhibiting the translation of the mRNA of the target gene, through several possible mechanisms (reviewed in [1]). However, asODN must cross plasma membranes in order to reach their site of action in the cell and in vitro, liposomal carriers are often used to increase their intracellular delivery.

Different liposomal formulations have been used to deliver asODN to cells; however, the most widely used formulation at present consists of pre-formed cationic liposomes, with or without DOPE, mixed with asODN [2]. In vitro, these liposomes have been shown to increase the intracellular delivery of asODN, thus increasing the concentration and activity of asODN. The electrostatic interaction between the positively charged lipid and the negatively charged asODN results in the formation of lipid–asODN complexes or lipoplexes. Lipoplexes are usually formulated with excess positive charge in order to mediate interaction with cell membranes which carry a net negative charge. This type of formulation

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is very efficient at incorporating asODN and delivery to cells in vitro and have been shown in many systems to be necessary for an antisense activity [2,3]. In vivo, the utility of lipoplexes is less clear, and there are no examples that convincingly demonstrate the activity of asODN following intravenous administration. This is because lipoplexes have been optimized for in vitro applications, and in vivo their large size and excess positive charge result in unfavorable pharmacokinetics, mainly due to rapid uptake into tissues of the mononuclear phagocytic system (MPS).

Since asODN are hydrophilic, they can be passively encapsulated within the aqueous space of non-cationic liposomes, and this method has been used in several published reports [4–10]. The main advantage of this type of carrier is that the liposomes can be reduced to small diameters and there is no positive charge to trigger opsonization by serum proteins and MPS uptake. These properties make neutral liposomes attractive candidates for intravenous delivery of hydrophilic drugs like asODN. In addition, polyethylene glycol (PEG) can be grafted onto the liposome surface, which decreases the uptake of the liposomes into the MPS, leading to increased circulation times and enhanced localization of liposomal contents in diseased tissues [11–15].

An important limitation to passive encapsulation of asODN within the aqueous space of liposomes is the poor incorporation efficiency, especially within small (<200 nm) liposomes. For example, hydration of a dried lipid film (DPPC/CHOL) with a concentrated solution of a 15-mer phosphodiester asODN resulted in only 3% incorporation following extrusion through a 200 nm unipore filter [4]. In another study, liposomes encapsulating a 15-mer asODN were prepared using the reverse phase evaporation method and 10% incorporation efficiency was reported into liposomes 170 nm in diameter [7]. Other studies fail to present details describing the incorporation efficiency, the asODN to lipid ratio, or liposome size [8,10], and these are all important parameters to consider in the development and use of a liposomal carrier for asODN. Even when novel protocols are developed which increase the encapsulation efficiencies of oligonucleotides to 50–70%, the liposome size is not reported [5,16]. An additional criticism is that most of these studies made no effort to distinguish entrapped asODN from that passively associated with the outside of the liposomal membrane.

The purpose of the present study was twofold: first, we wanted to determine the encapsulation efficiency of asODN within the aqueous space of small (<200 nm) neutral liposomes containing PEG–DSPE. Our results indicate that the addition of 2–5 mol% PEG–DSPE into neutral liposomes approximately doubles the incorporation efficiency of asODN from less than 10% to 20%. The second purpose of the study was to develop and characterize a liposomal carrier for asODN containing cationic lipid, which would be suitable for intravenous administration. By using a method that optimizes the charge interaction between the asODN and the cationic lipid, combined with a method that provides an outer coating of neutral lipid, we have been able to produce a liposomal carrier which is very efficient at incorporating asODN (80–100%), has a small diameter (<200 nm), and is stable in human plasma. In this paper we provide a detailed description of PEG-containing liposomes passively entrapping asODN (PELA) and a novel formulation containing cationic lipid–asODN particles coated with PEG–DSPE and neutral lipids (CCL).

2. Materials and methods

2.1. Materials

Partially hydrogenated egg phosphatidylcholine iodine number 40 (PC40) and poly(ethyleneglycol) (molecular mass 2000) covalently attached to distearoylphosphatidylethanolamine (PEG–DSPE) were generous gifts from SEQUUS Pharmaceuticals (Menlo Park, CA) and have been described elsewhere [17,18]. Cholesterol (CHOL) and 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) were purchased from Avanti Polar Lipids (Alabaster, AL). Sepharose CL-4B, Na-^{125}I (560–625 MBq/μg iodine), and scintillation fluor/aqueous counting scintillant (ACS) were purchased from Amersham Pharmacia Biotech (Piscataway, NJ). Iodogen was purchased from Pierce Chemical Co. (Rockford, IL). Bio-Spin 6 chromatography columns were purchased from Bio-Rad (Hercules, CA). Human plasma, containing sodium citrate as an anticoagulant, was obtained

from Canadian Blood Services (Edmonton, AB). Metrizamide (Grade 1) was purchased from Sigma Chemical Co. (St. Louis, MO). Cholesteryl-[1,2-^{3}H-(*N*)]-hexadecyl ether ([^{3}H]CHE), 1.48–1.22 TBq/mmol, was purchased from New England Nuclear (Mississauga, ON). [γ-^{32}P]Adenosine-5′-triphosphate ([γ-^{32}P]ATP) (110–167 TBq/mmol) was purchased from ICN Pharmaceuticals (Irvine, CA) or the Department of Biochemistry DNA Core Services Laboratory at the University of Alberta (Edmonton, AB). T4 polynucleotide kinase was purchased from Gibco BRL (Burlington, ON). A phosphorothioate asODN complementary to the *MDR1* initiation codon (5′-GTCCCCTTCAAGATCCAT-3′) was synthesized by the University Core DNA Services Laboratory at the University of Calgary (Calgary, AB). For most experiments PEI-cellulose chromatography sheets were purchased from J.T. Baker (Phillipsburg, NJ). Nuclepore polycarbonate filters for extrusion were purchased from Corning Costar (Kennebunk, ME). All other chemicals were of analytical grade quality.

2.2. Preparation of radiolabeled oligodeoxynucleotides

[^{32}P]asODN and [^{125}I]asODN were prepared and used as tracers in order to follow the incorporation of asODN within the different liposomal formulations. Phosphorothioate asODNs were labeled with ^{125}I using Iodogen similar to the method described by Piatyszek et al. [19]. A 1-ml reaction vial was coated with a thin film of Iodogen by dissolving approximately 1 mg of Iodogen in chloroform and then drying the solution under a stream of nitrogen. Next, 100–500 μg asODN in 50 μl along with 300 μl of 0.35 M sodium acetate (pH 4.0) and 185 MBq (2300 pmol) of ^{125}I in a volume of 50 μl were added to the vial and incubated at 40°C for 45 min. Free ^{125}I was removed on a Sephadex G-15 column. Dialysis or precipitation of the asODN, followed by rehydration, resulted in less than 5% free ^{125}I. The specific activity of the [^{125}I]asODN was approximately 4 MBq/nmol. This protocol results in the ^{125}I label being covalently attached at the C-5 position of cytidine bases [19,20].

In some experiments, [^{32}P]asODNs were also used in order to determine incorporation efficiency. T4 polynucleotide kinase and forward labeling buffer were used as outlined by the manufacturer. Free [γ-^{32}P]ATP was separated using a Bio-Spin 6 column. The specific activity of labeled phosphorothioate asODNs was approximately 9 MBq/nmol. Contamination of the sample by [γ-^{32}P]ATP was negligible as determined by thin-layer chromatography.

2.3. Preparation of liposomes passively entrapping asODN

Passively entrapped liposomal asODN (PELA) were prepared by a method similar to that described by Thierry et al. [5,16]. PC40/CHOL/PEG–DSPE were mixed at a 2:1:0, 2:1:0.04, 2:1:0.08 or 2:1:0.1 molar ratio (3 μmol total phospholipid) in $CHCl_3$ (along with a trace of [^{3}H]CHE) and dried to a thin film by rotary evaporation. Nine μl of a 10 mg/ml solution of asODN, determined by absorbance at 260 nm (A_{260}), in distilled deionized water (ddH_2O), plus trace [^{125}I]asODN, was added, and the film was hydrated overnight at 4°C. The following day an additional 9 μl of 10 mg/ml asODN (including trace [^{125}I]asODN) was added and the tube was vortexed vigorously for 30 s. Next, 18 μl of Hepes buffer (25 mM Hepes, 140 mM NaCl, pH 7.4) was added and the sample was vortexed vigorously again. After a 2-h incubation at room temperature the tubes were sonicated for 3 min in a bath sonicator. An additional 450 μl of Hepes buffer was added and the liposomes were extruded with a syringe-tip extruder through progressively smaller polycarbonate filters (800–200 nm). In some experiments, prior to the extrusion step, liposomes were subjected to 10 freeze (liquid N_2) and thaw (40°C) cycles following sonication. Liposome size was determined by dynamic light scattering using a Brookhaven B190 submicron particle analyzer (Brookhaven Instruments, Holtsville, NY). Free asODN was separated from encapsulated asODN by passing the mixture down a Sepharose CL-4B column (1×20 cm).

2.4. Preparation of coated cationic liposomes

Bligh and Dyer extractions [21], using the cationic lipid 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) and an 18-mer phosphorothioate asODN, were carried out in a similar method to that used for plasmid DNA [22,23]. DOTAP was diluted in 0.25 ml

$CHCl_3$, and 0.52 ml of MeOH was added followed by 0.25 ml of asODN (the amount was measured by A_{260}) diluted in ddH_2O (unless otherwise specified). Following 30 min at room temperature, 0.25 ml of $CHCl_3$ and 0.25 ml of ddH_2O were added and the tubes were centrifuged for 7 min at $830 \times g$. Following centrifugation, the phases were separated and radioactivity measured, or the A_{260} was measured in the aqueous phase, to determine the amount of asODN extracted.

Following extraction and removal of the aqueous phase, PC40, CHOL, and PEG–DSPE were added to the organic phase at the indicated ratios. ddH_2O was then added (to give 10–30 mM lipid concentration in the water volume), and the tube was vortexed vigorously (20 s) and then sonicated for 1 min to produce a stable emulsion. $CHCl_3$ was then evaporated under vacuum ($\sim$500 mmHg) on a rotary evaporator until a gel phase was reached. Subsequent evaporation lead to the inversion of the system from a gel to a liquid. During this procedure we hypothesize that the DOTAP–asODN particles have been coated with non-cationic lipids. The following experiments describe the characterization of coated cationic liposomes (CCL) prepared in this way.

Some CCL formulations (those lacking PEG–DSPE) would not migrate on a Sepharose CL-4B column, possibly due to aggregation, and therefore incorporation efficiency was determined by separation on a discontinuous metrizamide gradient. Twenty percent and 10% metrizamide were made up in ddH_2O and gradients were set up with 20% in the bottom of the tube (2.5 ml), 10% in the middle (7 ml) and 0% (2.5 ml) on top. Liposomes were mixed with the 20% layer prior to set-up and then tubes were centrifuged for 6–12 h at approximately $200\,000 \times g$. Liposomes localized primarily at the 10%/0% interface.

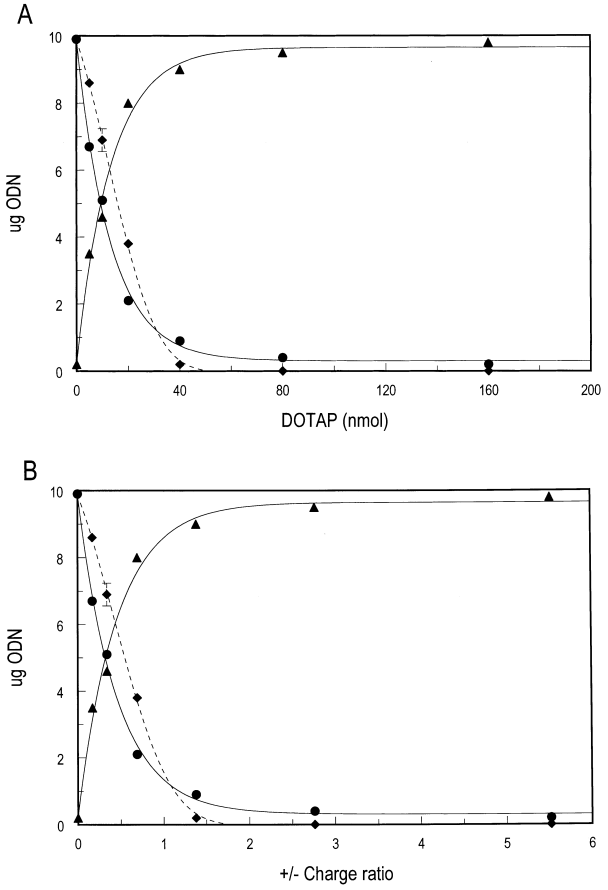

Fig. 1. Bligh and Dyer extractions of 10 μg 18-mer phosphorothioate asODN using the cationic lipid DOTAP. Triangles represent asODN in the organic phase and circles represent asODN remaining in the aqueous phase as determined by using a radiotracer ([^{32}P]asODN). Diamonds represent asODN remaining in the aqueous phase as determined by spectrophotometric assay (mean ± standard deviation of three experiments). (A) Amount of DOTAP expressed as nmol. (B) Amount of DOTAP expressed as +/− charge ratio.

3. Results

3.1. Effect of PEG–DSPE on passive entrapment of asODN

The first set of experiments were performed in order to determine the effect of PEG–DSPE on the incorporation efficiency of asODN within the aqueous space of neutral liposomes (PELA). Passive incorporation has been used in the past to entrap DNA or asODN in liposomes; however, the size of liposomes produced and the incorporation efficiency are not often described in detail. Therefore, an effort was made to accurately describe the conditions, the final diameter, the incorporation efficiency, the asODN/lipid ratio and also the trapped volume of liposomes produced.

The effect of PEG–DSPE on asODN entrapment efficiency was examined for PC40/CHOL liposomes, approximately 200 nm in diameter (Table 1). In order to determine the percentage of asODN that may

Table 1
Encapsulation of oligodeoxynucleotides within neutral liposomes containing increasing concentrations of PEG–DSPE

mol% PEG–DSPE	Size (nm)	Trapped volume (μl/μmol PL)	Incorporation efficiency (%)	nmol asODN/μmol PL
0	230 ± 10	1.0 ± 0.35	8 ± 3.2	0.6 ± 0.35
2	190 ± 12	2.5 ± 0.84	15 ± 7.7	1.6 ± 0.80
4	190 ± 15	2.3 ± 0.30	19 ± 3.6	1.4 ± 0.36

Each value represents the mean of three experiments ± S.D.

be associated with the liposome exterior, liposomes were made by the method described, except asODN was not included in the hydration buffer. AsODN was added to the liposomal suspension just before extrusion and then the mixture was separated on a Sepharose CL4B column. Less than 1% of the added asODN eluted with the lipid for all three groups (0, 2, 4 mol% PEG–DSPE) (data not shown). Therefore, values for incorporation efficiency presented in Table 1 can be taken to represent asODN mainly encapsulated within the aqueous space of liposomes.

The results in Table 1 indicate that by adding 2 mol% or 4 mol% PEG–DSPE to PC40/CHOL liposomes, the trapping efficiency of asODN approximately doubled compared to liposomes lacking PEG–DSPE. The incorporation data are also expressed as asODN to phospholipid ratios and as trapped volumes to facilitate comparisons with other published data. The trapped volumes were calculated using radiolabeled asODN as the aqueous phase solute, and the results are consistent with the expected values for liposomes of this size, prepared in this way and suggest that the liposomes lacking PEG–DSPE are multilamellar [24]. Subjecting the liposomes to freezing and thawing did not increase the incorporation efficiency of asODN within the aqueous space of these liposomes.

3.2. Formation of hydrophobic asODN/DOTAP particles

Reimer et al. described the formation of hydrophobic plasmid DNA–cationic lipid particles through an organic extraction procedure [22]. They demonstrated that cationic lipids could be used to extract plasmid DNA from an aqueous phase, into an organic phase through a Bligh and Dyer monophase [21]. The extraction was shown to be mediated by the electrostatic interaction between the positively charged lipid and the negatively charged DNA. This procedure should also be applicable to negatively charged asODN, and we hypothesized that the hydrophobic particles in the organic phase could be coated with neutral lipids through a reverse evaporation step. We predicted that this procedure would result in liposomes having a high incorporation efficiency for asODN. Furthermore, the addition of neutral lipids, including PEG–DSPE, would serve to coat the cationic lipid–asODN particles, adding stability, decreasing the non-specific adsorption of serum proteins and increasing their circulation half-lives.

The first step was to confirm that the extraction procedure is applicable to asODN, and to determine the amount of cationic lipid required for efficient extraction of the asODN into the organic phase. Ten μg of 18-mer phosphorothioate asODN (including trace [^{32}P]asODN) was extracted using 0–160 nmol DOTAP through a Bligh and Dyer monophase as described in Section 2. Fig. 1 illustrates the results from such an experiment and demonstrates that increasing the amount of DOTAP increases the asODN extracted from the aqueous phase into the organic phase. When 40 nmol or more of DOTAP was used, almost 100% of the asODN could be extracted (Fig. 1A). These data can be expressed in terms of the +/− charge ratio of DOTAP to asODN phosphate as shown in Fig. 1B. Thirty nmol DOTAP corresponds approximately to a 1:1 +/− charge ratio with 10 μg asODN and at this ratio approximately 90% of the asODN was extracted into the organic phase. When several replicates of the extraction were done at a 1:1 charge ratio, 90–95% of the asODN was extracted into the organic phase.

The assumption in this extraction procedure is that it is the electrostatic interaction between the positively charged DOTAP and the negatively charged asODN which drives the extraction. This is sup-

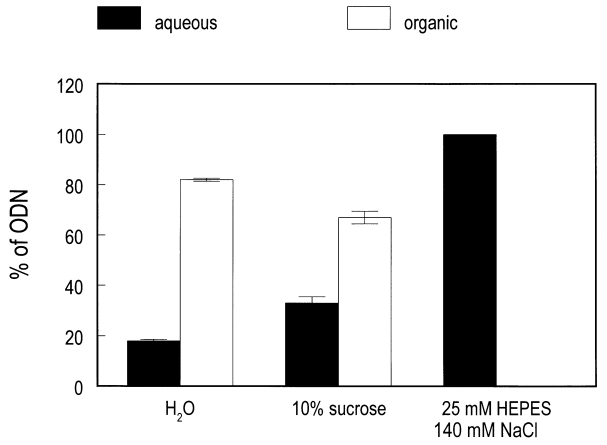

Fig. 2. Extraction by DOTAP (0.15 μmol) of 60 μg 18-mer phoshorothioate asODN dissolved in different aqueous phases (0.88 +/− charge ratio). Extraction was determined by spectrophotometric assay of the aqueous phase. 100% asODN in the aqueous phase was determined by carrying out the extraction in the absence of DOTAP. Values for asODN in the organic phase were determined as the difference between asODN in the aqueous phase and total asODN added (100%). Each value represents the mean ± S.D. of three experiments.

ported by the work of Reimer et al. [22], and experiments performed in our laboratory indicate that neutral lipids do not result in the extraction of asODN (data not shown). Experiments have also indicated that the aqueous phase in which the asODN is dissolved plays an important role in the extraction efficiency. Fig. 2 illustrates the results from a series of extractions carried out using 60 μg asODN and 0.15 μmol DOTAP with the asODN diluted in ddH_2O, 10% sucrose, or 25 mM Hepes buffer containing 140 mM NaCl (pH 7.4). At the DOTAP/asODN ratio in this experiment (+/− = 0.88), more than 80% of the asODN is extracted from the ddH_2O aqueous phase. When the asODN was dissolved in 10% sucrose approximately 70% of the asODN was extracted from the aqueous phase, and the extraction was completely inhibited when 25 mM Hepes, 140 mM NaCl (pH 7.4) was used as the aqueous phase. This is likely caused by a charge-shielding effect of the Na^+ and Cl^- ions preventing the efficient interaction between the DOTAP and the asODN, since a non-ionic solute such as sucrose had less of an effect on the extraction efficiency.

3.3. Coated cationic liposome formation by reverse phase evaporation

The size, and incorporation efficiency were determined for CCL at different lipid ratios (Table 2). Most CCL preparations were formed by extraction of asODN into the organic phase at a 3.5:1 +/− charge ratio, which ensured that nearly 100% of the asODN would be extracted into the organic phase. Following extraction of 50 μg asODN with 0.5 μmol DOTAP, the organic phase was isolated and different amounts of coating lipid were added (Table 2). ddH_2O was then added and the reverse evaporation was carried out as described. After reversion into the aqueous phase, no visible aggregates or precipitates were present and particle diameter was in the 400–500 nm range. Incorporation efficiencies were in excess of 70%. The amount of coating lipid had no effect on the incorporation efficiency, and very little effect on the liposome diameter for non-extruded liposomes (Table 2). Changes in the asODN/PL ratios in Table 2 simply reflect differences in the amount of added coating lipid.

For many in vivo applications smaller diameter liposomes would be advantageous, so the CCL

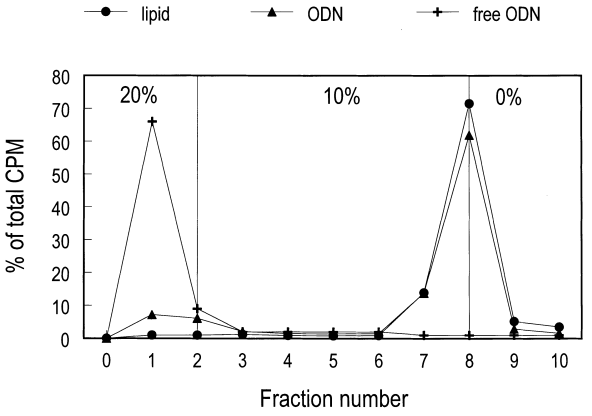

Fig. 3. Metrizamide gradient profile for CCL containing [^{3}H]CHE as a lipid marker and [^{125}I]asODN as a tracer. CCL were composed of PC40/CHOL/DOTAP/PEG–DSPE (3.0:1.75:0.5:0.175 μmol). Gradients were fractionated by making a small hole in the bottom of the plastic tube and collecting fractions of equal drops. The first two fractions contained the majority of the 20% metrizamide layer, while fractions 3–8 contained the majority of the 10% layer. Closed circles represent lipid and closed triangles represent asODN formulated in CCL. Plus-marks are for a control showing the lack of migration of free asODN on the gradient.

were extruded to reduce their size. We observed that the amount of coating lipid added effected our ability to extrude CCL. When the PC40/DOTAP molar ratio was less than 4:1, extrusion through 200-nm filters led to immediate aggregation and destabilization (data not shown). However, at a PC40/DOTAP molar ratio of 3:1, CCL could be extruded through 200-nm filters when 5 mol% PEG–DSPE was included in the coating lipids to prevent aggregation (Table 2). Following extrusion through 200-nm polycarbonate filters, the average diameter of CCL was 173 nm (Table 2). Incorporation efficiencies were determined on a discontinuous metrizamide gradient (Fig. 3). Free [^{125}I]asODN remained at the bottom of the gradient (20% metrizamide), while liposomes, along with the loaded asODN, migrated to the 10%/0% interface. For the experiment represented in Fig. 3, approximately 86% of the asODN migrated through the gradient with the lipid fraction and the average of three experiments is presented in Table 2.

Separation on a metrizamide gradient does not discriminate between spurious association of asODN with the liposome exterior and that in the aqueous liposome interior. Therefore the amount of asODN which could associate with the liposome exterior was determined by making empty (without asODN) CCL by the procedure outlined above, and then adding asODN before separation on metrizamide gradients. When formulated in this manner, approximately 50% of the asODN bound to the liposome exterior and migrated through the gradient with the lipid. However, when the liposomes are made in the absence of asODN, a significant amount of DOTAP (i.e., positive charge) may be present in the outer phospholipid monolayer of the liposome and available for binding to asODN added to the liposome exterior. When CCLs are made from extracted DOTAP/asODN particles, we propose that the DOTAP is sequestered primarily in the liposome interior, associated with asODN.

For in vivo applications, a carrier system lacking excess positive charge would be desirable to reduce MPS uptake. At a 1:1 +/− charge ratio of DOTAP to asODN phosphate, 90–95% of asODN was extracted into the organic; however, when neutral lip-

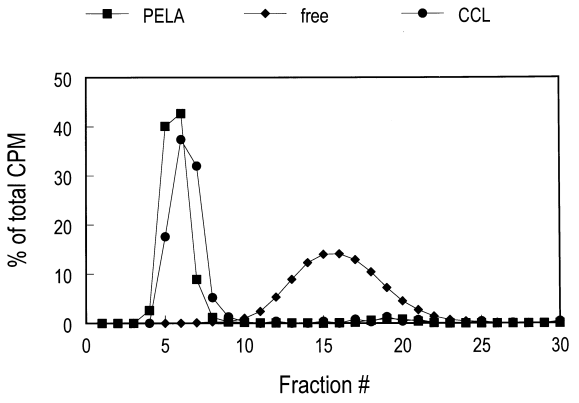

Fig. 4. Sepharose CL-4B column profile of PELA and CCL incubated at 37°C for 24 h in 50% human plasma. [^{32}P]asODN was used as a tracer. Fractions 4–8 represent the lipid fractions as determined by visual observation and by using [^{3}H]CHE as a lipid tracer (counts not shown). Closed squares represent asODN passively entrapped within PC40/CHOL (2:1 molar) liposomes containing 5 mol% PEG–DSPE, while closed circles represent asODN formulated in CCL with the composition PC40/CHOL/DOTAP/PEG–DSPE (3:2:1:0.2 molar). Closed diamonds represent free asODN (including [^{32}P]asODN tracer) incubated with 50% human plasma.

Table 2
Size and incorporation efficiency of liposomes formed by the extraction–reverse evaporation procedure

PC40/CHOL/DOTAP/PEG–DSPE (μmol)	+/− charge ratio	Size (nm)	Incorporation efficiency (%)	nmol asODN/μmol PL
Not extruded				
0.5:0.5:0.5:0	3.5	360 ± 60	72 ± 10	7.9 ± 2.1
1.0:0.75:0.5:0	3.5	530 ± 170	74 ± 4	5.4 ± 0.4
2.0:1.25:0.5:0	3.5	510 ± 200	73 ± 10	3.4 ± 0.5
Extruded				
3.0:1.75:0.5:0.175	3.5	173 ± 4.2	86 ± 5	2.1 ± 0.1
3:2:1:0.2	1	219 ± 28	90 ± 9	17

Each value represents the mean ± S.D. of three experiments, except for the asODN/PL ratio for the last row, which is the result from one experiment.

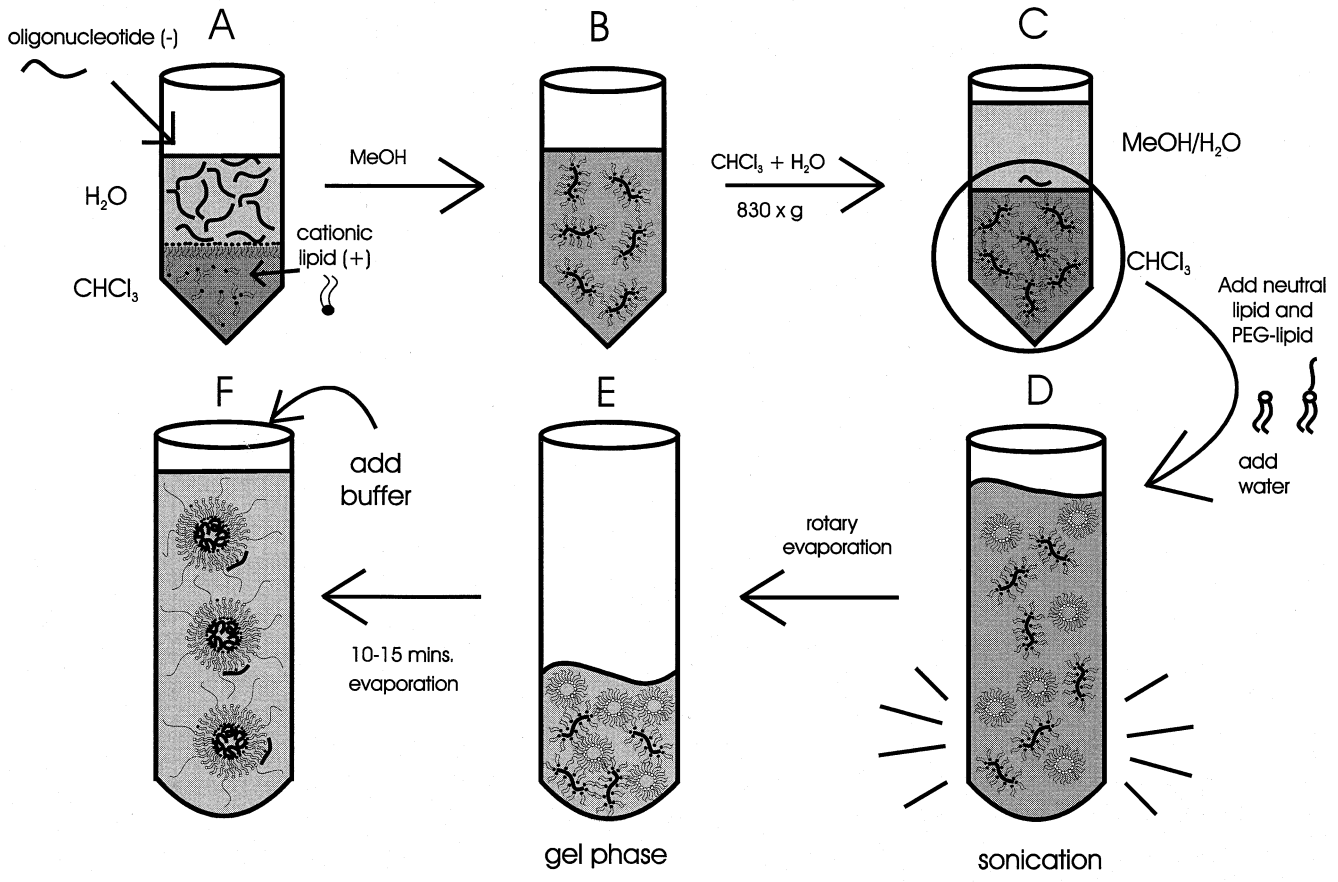

Fig. 5. Schematic demonstrating the steps and hypothesized intermediate structures involved in making coated cationic liposomes (CCL). (A) A mixture of chloroform and water exists as a biphase, with the upper aqueous phase (water) containing the asODN and the lower organic phase (chloroform) containing the cationic lipid. (B) When methanol is added, a Bligh–Dyer monophase [21] is created, allowing electrostatic interactions between the positively charged cationic lipid (black headgroups) and the negatively charged asODN, which form micelle-like structures with the hydrophobic phospholipid tails facing the organic phase and the asODN sequestered inside. (C) The system reverts back into a biphase following the addition of chloroform and water with the hydrophobic asODN-cationic lipid particles now concentrated in the organic phase. (D) The hydrophobic particles are used as intermediates in the formation of liposomes through a reverse evaporation procedure [30]. The addition of neutral 'coating lipids' (white headgroups), PEG and water followed by brief sonication will produce a 'water-in-oil emulsion' containing inverse micelles of the neutral lipids. (E) Following evaporation of the chloroform, an intermediate gel phase is reached and further evaporation causes inversion of the micelles, which will coat the cationic lipid–asODN-particles (F) leaving CCL in an aqueous solution, sterically stabilized with PEG.

ids were added without PEG–DSPE, lipid aggregates formed during the reverse evaporation procedure. It has been observed in other systems that significant aggregation occurs as the +/− charge ratio approaches 1 [25] and addition of PEG has been shown to prevent aggregation and precipitation in these electrostatic systems [26]. In the presence of 5 mol% PEG–DSPE no aggregation was observed, and sequential extrusion through 200 nm filters resulted in an average particle diameter of 219 nm and an incorporation efficiency of 90% (Table 2).

3.4. *Effect of plasma on stability and dissociation of asODN*

The stability of CCL and PELA in plasma-containing medium at 37°C was evaluated. CCL and PELA were prepared at a 1:1 charge ratio DOTAP/asODN, and contained 5 mol% PEG–DSPE. PELA had an average diameter of 185 ± 4 nm and a 21% trapping efficiency while the CCL had an average diameter of 190 ± 2 nm and a 80% trapping efficiency. An aliquot from each group was diluted in an

equal volume of human plasma and the samples were incubated at 37°C for 24 h. No increase in diameter was observed for either preparation. Following fractionation of each preparation on Sepharose CL-4B columns, the asODN eluted almost exclusively with the liposomes in the void volume (Fig. 4). These results demonstrate that both formulations were able to maintain their size and stability with minimal leakage of asODN in plasma-containing media.

4. Discussion

In this study we examined the physical characteristics (diameter, stability) and incorporation efficiency of two different liposomal formulations of asODN which may be suitable for in vivo applications. Cationic lipoplexes are not suitable for systemic administration due to their large size and excess positive charge which leads to their rapid removal from circulation, primarily into liver and lung. Pharmacokinetic studies have demonstrated that cationic lipoplexes are unable to deliver asODN to tissues other than the MPS and lung [27,28].

Passive encapsulation within neutral liposomes is an alternative to using cationic lipoplexes; however, our results confirm that this is an inefficient process and only a fraction of the added asODN is loaded. An unexpected finding was that the incorporation efficiency of an 18-mer phosphorothioate asODN was increased by the addition of 2–5 mol% PEG–DSPE. This may be a result of an increased trapped volume due to decreased lamellarity of the liposomes and while it is an interesting observation, the incorporation efficiency obtained was still quite low (20%).

It is difficult to compare the incorporation efficiencies observed in our study with previously published results. As mentioned in the Introduction, few studies give sufficient details to properly characterize the encapsulation of asODN within neutral liposomes. For example, Thierry et al. report incorporation efficiencies of 50–70% of added asODN. However, the size of the liposomes is not reported and small size is an important consideration for in vivo applications [5,16].

Neutral PEG-containing liposomes have longer circulation half-lives in vivo than cationic lipoplexes lacking PEG [11,18,27–29]. However, as described above, these liposomes have poor incorporation efficiencies. Therefore, we developed a CCL formulation that combines the benefits of neutral liposomes, such as stability and long circulation times, with the high incorporation efficiency of asODN obtained with cationic lipoplexes. CCL are produced in a two step process in which asODN are first complexed with cationic lipid at a 1:1 charge ratio, followed by a coating procedure in which neutral lipids (with or without PEG–DSPE) are added to produce coated cationic liposomes. CCL can be extruded to diameters below 200 nm, they are stable in plasma and have incorporation efficiencies of around 90%. Our results indicate that asODN can be extracted through a Bligh and Dyer monophase into an organic phase using cationic lipid, similar to the extraction of plasmid DNA as described by Reimer et al. [22]. These cationic lipid–asODN hydrophobic complexes serve as useful intermediates in the formation of CCL through a reverse evaporation procedure.

The exact structure of the hydrophobic particles produced by the extraction procedure is unknown, but we can make a prediction (Fig. 5). In order for the asODN to exist in a hydrophobic environment such as $CHCl_3$, it must be shielded by the lipid, possibly in the form of an inverted micelle (Fig. 5C). This structure serves as a useful intermediate in the formation of liposomes through a reverse phase evaporation method similar to that described by Szoka and Papahadjopoulos [30]. The outline for such a procedure is described in Fig. 5C–F. By formulating the asODN and cationic lipid in this way, we are attempting to sequester a majority of the cationic lipids and the asODN within a coating of neutral and PEG-modified lipids. In this way, the cationic lipid serves to efficiently load the asODN, but is not available for interaction with cells or serum proteins. This model for CCL formation is supported by our results demonstrating a lack of aggregation or release of asODN in the presence of human plasma (Fig. 4). Similarly, PELA did not aggregate or leak; however, this is not surprising since these liposomes contain 5 mol% PEG–DSPE and no cationic lipid.

Very recently, other formulations have been developed which attempt to incorporate some of these characteristics into a liposomal carrier for asODN, for example, the LPDII particles described by Li et

al. [31]. LPDII particles are 150–200 nm in diameter and entrap approximately 60% of added asODN; however, their stability or activity in the presence of plasma have not been demonstrated. Meyer et al. mixed asODN with pre-formed liposomes composed of DOPE, PEG–PE and a cationic lipid [32]. The authors report very good incorporation with a high asODN/lipid ratio and suggest that the complexes are stable in plasma. However, roughly 40% of the asODN dissociated from liposomes upon incubation in 50% plasma, and pharmacokinetic experiments indicated that even more dissociation occurs upon intravenous injection (D. Kirpotin, personal communication). This is probably a result of asODN, associated with the outside of the liposomes, dissociating in plasma. Our experiments indicate that up to 50% of asODN added to the outside of CCL, formed in the absence of asODN, would bind to the CCL and could dissociate. However, when CCL were formed with asODN present during the extraction we observed no dissociation of the asODN in plasma, suggesting that the asODN is sequestered in the interior of the liposomes in association with the cationic lipid (Fig. 5F).

In the CCL formulation we used a charge neutral ratio of DOTAP/asODN and added 5 mol% PEG–DSPE in order to prevent aggregation and decrease adsorption of serum proteins and interaction with non-target cells. This is in contrast to typical cationic lipid–asODN complexes which carry an excess positive charge and results in non-specific interactions with most cell types. Experiments in our laboratory indicate that cells treated with LipofectAMINE–asODN complexes take-up more asODN than cells treated with asODN formulated in CCL. However, when CCL are targeted by coupling a monoclonal antibody to the surface, the levels of cell associated asODN are increased significantly, and approach levels observed following treatment delivery using LipofectAMINE. In a mixed cell population, for example in vivo, it would be an advantage to use an asODN carrier which has selectivity for the target cell population, rather than one which interacts non-specifically (albeit efficiently) with all cell types encountered.

Over the past decade, it has become apparent that liposomes which are able to circulate in the bloodstream, without rapid uptake into the liver or spleen, may be able to passively target sites of disease such as solid tumors and sites of infection [12,33–38]. A separate manuscript (submitted for publication) demonstrates that the CCL formulation has long-circulating pharmacokinetics similar to PELA and therefore may be able to take advantage of this passive targeting effect. Other studies have demonstrated that enhanced circulation times are required for ligand-mediated targeting of liposomes [39] and this targeting has been shown to result in enhanced antitumor activity when the liposomes are loaded with doxorubicin [13,15]. The CCL formulation can also be targeted to a specific cell type by adding a coupling lipid (e.g., 4-(*p*-maleimidophenyl)butyrate–PEG–DSPE) to the coating lipids and attaching a targeting ligand following CCL formation. Another paper (submitted for publication) demonstrates that antibody-targeted CCL (and to a lesser extent non-targeted CCL) are effective at delivering a c-*myb* asODN resulting in an antiproliferative effect in a glioblastoma cell line.

Significant progress has been made in recent years in bringing asODN into the clinical setting and there are several early to mid-phase clinical trials using asODN to treat diseases ranging from cancer, to human immunodeficiency virus infection (HIV) to inflammatory conditions. However, only one asODN has received approval for marketing in the United States and that is a phosphorothioate anti-cytomegalovirus–asODN used to treat CMV retinitis in AIDS patients [40]. The anti-CMV–asODN is administered via local (intravitreal) injection and is not subject to significant redistribution via the bloodstream. However, the intravenous route of administration will be most convenient for asODN targeted to other diseases such as cancer or HIV infection and in these situations, the activity of the asODN may be significantly improved by the use of a well-designed liposomal carrier. The CCL formulation that we have described meets many of the requirements of a useful drug carrier and could significantly increase the therapeutic activity of asODN.

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