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Assembly of nucleic acid-lipid nanoparticles from aqueous-organic monophases

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Abstract

Effective, reproducible, and scalable methods for DNA–lipid assembly are important for the success of non-viral vectors in in vivo gene therapy. We hypothesized DNA–lipid assembly would be optimal if started from a liquid monophase where both DNA and lipids separately form molecular or micellar solutions prior to mixing, without preexisting condensed lipid phases, thus allowing DNA–lipid assembly under conditions close to equilibrium. Previously, we found that mixing plasmid DNA, 1-palmitoyl-2-oleoyl-3-*sn*-phosphatidylcholine (POPC), cholesterol and a cationic lipid, 1, 2-dioleoyl-3-(trimethylammonio) propane (DOTAP) in 50% (v/v) aqueous ethanol spontaneously produced an optically transparent solution. Upon ethanol removal, DNA–lipid nanoparticles (Genospheres™) were formed. For comparison with well-known technologies, different DNA–lipid particles were prepared by interaction of plasmid DNA and stable or ethanol-destabilized lipid vesicles by combining the components in water or 30% (v/v) aqueous ethanol, respectively. Among the three studied DNA–lipid assembly methods, only Genospheres combined the properties of small size (less than or around 100 nm), high incorporation of both lipid and DNA, high degree of DNA protection (dye accessibility 5–12%), a narrow distribution of particle density and when immuno-targeted, the highest transfection efficiency in HER2-overexpressing cells in vitro. We conclude that the Genosphere assembly methodology offers advantages for the development of effective, scalable and targetable non-viral gene delivery vectors.

Keywords: Non-viral gene delivery; DNA–lipid complex; Nucleic acid encapsulation; HER2 targeting

1. Introduction

Gene therapy is a potential therapeutic strategy for the treatment and cure of inherited diseases such as cystic fibrosis and diseases that are acquired, such as cancer. Lipid based vectors are seen as a promising tool, overcoming many of the immunogenic side effects associated with viral carriers for gene delivery.

An ideal carrier should be small, have the ability to evade the RES system to reach remote diseased sites such as tumors and be able to deliver the intact genetic material intracellularly at the target cell. The carrier must also be easily modified in terms of altering composition and/or incorporation of targeting ligands to suit specific applications.

Presently, methodologies for DNA–lipid particle production include a liquid–liquid phase separation step, the presence of lipid solubilizing detergent, DNA interaction with pre-formed liposomes or a drying and rehydration step. We hypothesized that DNA–lipid assembly would be improved if occurring within a liquid monophase where both DNA and lipids were separately molecularly/micellarly soluble prior to mixing, without pre-existing condensed lipid phases, thus allowing

Abbreviations: DMSO, dimethyl sulfoxide; FRET, fluorescence resonance energy transfer; HEPES, 2-(4-[2-hydroxyethyl]piperazino)ethanesulfonic acid]); HBS, 5 mM HEPES, pH 7.4, 144 mM NaCl; PEDL, particles formed from ethanol-destabilized liposomes; PEG-DSPE, N-[ω-methoxy-(poly(oxyethylene)-α-oxycarbonyl]-DSPE (PEG mol. weight 2,000); PEG-DSG, 1,2-distearoyl-sn-glycerol 1-(methoxypoly(ethylene glycol) ether. (PEG Mol. weight 2,000)

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DNA–lipid assembly under conditions close to equilibrium. To affect the assembly, the organic solvent component should be removed without separation of the system into two liquid phases or to complete dryness.

Previously, we found that mixing plasmid DNA with neutral (POPC, Cholesterol) and cationic (DOTAP) lipids in an aqueous/organic solvent monophase where initially no lipid condensed phases were observed, spontaneously produced an optically transparent solution. Upon solvent removal, we observed that small DNA–lipid nanoparticles were formed (Genospheres™) [1]. In this study, we compared the DNA–lipid particles prepared by the monophase assembly (in particular, containing 50% (v/v) ethanol) with particles prepared by two well known methodologies; interaction of DNA with stable or 30% (v/v) ethanol-destabilized lipid vesicles as described in the literature by Felgner et al. [2] and Maurer et al. [3], respectively. The particles were characterized by: DNA compactness, lipid/DNA and cationic/neutral lipid proximity, DNA exposure; lipid lamellarity; compositional homogeneity; completeness of DNA and lipid incorporation; and in vitro transfection in a non-targeted or ligand-(antibody scFv fragment)-targeted variant. The data suggested that the Genospheres technology platform may offer advantages for the manufacture of pharmaceutical gene delivery formulations.

2. Materials and methods

2.1. Materials

1,2-Dioleoyl-3-(trimethylammonio)propane (DOTAP), 1-palmitoyl,2-oleoyl-3-*sn*-phosphatidylcholine (POPC), (*N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)dipalmitoyl-phosphatidylethanolamine) NBD-PE and N- CAP-carboxy-fluorescein- phosphatidylethanolamine (CFPE) were purchased from Avanti (Alabaster, AL) and used without further purification. Cholesterol (Chol) was purchased from Calbiochem (San Diego, CA). Stock solutions of lipids dissolved in ethanol (Gold Shield, Hayward, CA) were stored at −20 °C. *N*, *N*-di-[^{3}H] methyl-*N*,*N*-diocadecylammonium bromide ([^{3}H]-DDAB]), [1α, 2α (n)-^{3}H] cholesterol ([^{3}H]-Chol) (Amersham Pharmacia Biotech, Piscataway, NJ) and phosphatidylcholine, L-a-dipalmitoyl [dipalmitoyl-1-^{14}C], (American Radiolabeled Chemicals, Inc., St. Louis, MO) were stored at −80 °C prior to use.

Purified Firefly luciferase and D-(−) luciferin were obtained from Roche (Indianopolis, IN). PicoGreen®, OliGreen®, YOYO-1®, EtHD-2 (ethidium homodimer-2), 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine-5,5′-disulfonic acid (DiIC$_{18}$ (3)-DS), 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI(3)), bis-(3-propyl-5-oxoisoxazol-4-yl)pentamine oxonol (oxonol (VI)) and anti-fluorescein/Oregon Green rabbit polyclonal IgG Fab fragment were purchased from Molecular Probes (Eugene, OR). Lipofectamine 2000 was purchased from Invitrogen (Carlsbad, CA) while spermidine was obtained from Sigma (St. Louis, MO). Cell culture media was purchased from the UCSF cell culture facility (UCSF, San Francisco) and cells were obtained from ATCC (Rockland, MD). F5-PEG-DSPE conjugate was prepared from the purified anti-HER2 scFv, F5, by conjugation to the maleimide-activated PEG termini of the PEG-DSPE lipid anchor through an engineered c-terminal cysteine as described previously [4]. Other reagents of the highest possible grade were purchased and used without further purification.

2.2. Light scattering of lipid solutions of varying ethanol content

A DOTAP/POPC/Chol/PEG-DSPE (molar ratio 6/15/10/0.16) lipid solution was prepared from stock lipid solutions in ethanol. The mixture was filtered through a 0.2 μm polycarbonate membrane (Whatman, NJ) at 60 °C to remove any large particles that would interfere with the measurements. The filtrate was divided into four round-bottomed flasks and evaporated under vacuum; the resulting lipid films hydrated in the desired organic solvent/5% (w/w) dextrose solution followed by brief mixing. The lipid solutions were diluted to 2.1 mM phospholipid in their respective organic solvent containing dextrose solution, and the light scattering intensity was determined by photon correlation spectroscopy using a Nicomp C370 particle size analyzer (Particle Sizing Systems, Santa Barbara) at 632.8 nm at 23 °C and 55 °C by reading the scattering event frequency at constant gain. Alternatively, light scattering was measured a 90° angle using a fluorescence spectrometer (Fluorolog, Jobin Yvon Spex, NC) at $\lambda_{ex/em}$=633 nm with 1 nm slit widths, using a stirred, thermostated cell holder (±0.1 °C).

2.3. DNA–lipid particle preparation

A bacterial plasmid containing firefly luciferase gene under the control or early cytomegalovirus promoter (pCMV/luc$^+$) and a penicillin resistance gene was constructed as described previously [5]. *E. coli* transfected with pCMV/luc+ were selected on an ampicillin agar plate and grown in LB medium with ampicillin (50 μg/ml). The plasmid was extracted and purified using the EndoFree Plasmid Giga kit from Qiagen (Chatsworth, CA.) The plasmid concentration was determined by absorbance at 260 nm (ε=6600 l mol^{-1} cm^{-1}) and purity by calculating the ratio $A_{260\ nm}/A_{280\ nm}$ (Shimadzu, UV160U). DNA is considered to be sufficiently protein free with an $A_{260\ nm}/A_{280\ nm}$ ratio of at least 1.85. An siRNA duplex that was shown to have specific gene inactivation of an adaptor protein Shc A [6], which has been implicated in many types of receptor mediated cell signaling was obtained from Dharmacon (Layafette, CO) (AA-N19 mRNA Target 5′→3′; AACUACUUGGUUCGGUACAUGG). Phosphorothioate modified oligonucleotide was obtained from MWG-Biotech AG, NJ and had a sequence shown to have specific inhibition of up-regulated genes implicated in colon cancer (5′CACTGCCAATCTCATGGTCG 3′) (LC Chen and NM Lee, California Pacific Medical Center Research Institute, personal communication). Both siRNA and oligo were supplied in a lyophilized form and were rehydrated with deionized water with concentrations determined by absorbance at 260 nm using the expressions that 1O.D equates to 40 μg/ml siRNA and 33 μg/ml oligonucleotide.

From a plasmid DNA (pCMV/*luc*$^+$, 4.5 kbp) stock solution of 2 mg/ml in TE, pH 8.0 buffer, plasmid DNA, siRNA or oligonucleotide was diluted to 0.4 mg/ml in 5 mM HEPES–Na buffer, pH 7.4 and used in the preparation of complexes. For fluorescence resonance energy transfer (FRET) studies, intercalating dyes were added to aliquots of the plasmid DNA solution at a concentration of 1 dye per 200 bp and incubated at room temperature for 1 hour to allow adequate time for complete dye binding before further processing. A stock lipid solution of DOTAP, POPC, and Chol dissolved in ethanol at a molar ratio of 6:15:10 was used in the preparation of the complexes. The lipid mixture was added to the DNA as described below at a ratio of 6 nmol DOTAP per 1 μg of DNA. This corresponds to a 2-fold excess of cationic charge from DOTAP over the anionic phosphates of each nucleotide. Fluorescent lipid markers DiI(3), DiI(3)-DS and NBD-PE were added if required to the lipid mixtures prior to complex formation, at a molar ratio of 2:1 to the intercalating DNA dye in the final complex.

2.3.1. Lipoplex

The complexes made from mixing cationic liposomes and DNA together are commonly referred to as lipoplexes [7]. An aliquot of the lipid stock solution containing 3.1 μmol (total lipid) lipid was brought to dryness under vacuum and re-hydrated with 0.5 ml 5 mM HEPES, pH 7.4. The lipid suspension was vortexed and sonicated to clarity (5 min) in a bath sonicator (Laboratory Supplies Company, Hicksville Inc., NY) to produce liposomes [5]. Stock DNA solution was diluted twice to a final volume of 0.5 ml in 5 mM HEPES (pH 7.4) and added to the liposome solution rapidly at room temperature. These lipoplexes were used within 1–2 h after preparation.

To validate the use of DiIC$_{18}$(3) and DiIC$_{18}$(3)-DS as appropriate FRET markers for cationic and neutral lipids, respectively, sonicated liposomes were prepared containing each dye separately in a 1:1 molar mixture with DOPE as described above. Lipoplex complexes were prepared by mixing liposomes with DNA in a ratio of two dyes per DNA phosphate. After incubation at room temperature for 30 min, the suspensions were briefly centrifuged (13,000 rpm for 5 min). An estimate of the amount of fluorescence lipid remaining in solution

was made by comparison of the fluorescence intensity of the solution before and after centrifugation, by dissolving known aliquots in 0.1% Triton X-100 and measuring intensities using (λ_{ex} 549 nm/λ_{em} 566 nm).

2.3.2. Particles prepared from water/organic solvent mixtures

Aliquots (3.1 μmol) of the lipid mixture stock solution were diluted with appropriate volumes of ethanol and 5 mM HEPES, pH 7.4 buffer and ethanol to bring the concentrations of ethanol to 30% (v/v) or 50% (v/v), in a final volume of 0.5 ml. Alternatively, the lipid was dried and re-dissolved in the appropriate organic solvent/aqueous mixture. In separate tubes, aliquots of the nucleic acid stock solution (containing 100 μg) were mixed with appropriate volumes of 5 mM HEPES, pH 7.4 buffer and organic solvent to obtain 0.5 ml of a solution containing the desired water miscible solvent concentration. The nucleic acid and lipid solutions were pre-warmed at 60 °C, and the nucleic acid solution was added rapidly to the lipid solution of corresponding solvent concentration, pipetting up and down 4–5 times to ensure complete mixing. The mixtures were further incubated at 60 °C for 5 min, then removed from the water bath and allowed to reach room temperature. The mixture was dialyzed exhaustively against 5 mM HEPES, pH 7.4 buffer with or without 0.15 M NaCl (as indicated below) at room temperature, using dialysis tubing with a molecular weight cut-off of 6000–8000 Da.

2.3.3. HER2 targeted particles

HER2-targeted lipid–DNA particles were prepared using the method previously described for liposomes [4]. HER2-specific, highly internalizable single chain Fv was conjugated via a c-terminal cysteine group to a thiol-reactive amphiphilic lipid anchor maleimido-PEG-DSPE, and the micelle-forming conjugate (F5-PEG-DSPE) was purified by size exclusion chromatography. F5-PEG-DSPE was incubated with the DNA–lipid particles in HEPES–saline buffer (5 mM HEPES–Na, 144 mM NaCl, pH 7.4) at 37 °C overnight with typically the amount of conjugate equal to 5 μg antibody added per μmole of POPC in the sample. The antibody conjugate is presented in a solution also containing a proportion of cysteine quenched maleimide-PEG-DSPE [8,9] and therefore non-targeted control samples were heated in the same manner using an identical amount of additional PEG-DSPE (0.73 nmol PEG-DSPE per μmol PC in sample). Attachment of the antibody is achieved by spontaneous incorporation of the lipophilic (DSPE) portion of the conjugate into the outer lipid layer of the particles during the incubation. Allen and coworkers describe this technique in detail [10]. Genosphere samples were stored at 4 °C until use. To maintain sterility of the lipoplexes, DNA, sonicated liposomes, and F5-PEG-DSPE solutions were filter-sterilized and combined under aseptic conditions.

2.4. Particle size analysis

The volume-weighted mean and standard deviation (SD) of the particle diameter distribution was determined in 5 mM HEPES–saline pH 7.4 at 23 °C using quasielastic light scattering (Nicomp C370 Particle Size Analyzer (Nicomp Particle Sizing Systems, Santa Barbara, CA) using the solid particle mode and Gaussian analysis for chi of 3.0 or less, or SDP analysis for chi over 3.0. The measurement of particle sizes for the studies reported in (Fig. 3A, B) was made by a Beckman PCS Particle Sizer, using the volume weighted size and standard deviation analysis function.

2.5. PicoGreen dye accessibility and particle DNA quantification assay

Aliquots (100 μl) of the particle suspensions containing approximately 700 ng/ml of DNA were placed in a 96-well mictotiter plate, diluted with 100 μl of HEPES–saline, pH 7.4, and mixed with 2.5 μl of the PicoGreen DNA intercalating dye stock solution (Molecular Probes, Inc., OR). DNA concentration standards in the range of 50–1000 ng/ml were prepared in the same manner using pCMV/luc$^+$ plasmid solutions instead of the particle suspensions. To provide for 100% DNA-accessibility reference, a 2× dissociation solution (10% (v/v) DMSO, 1% (w/v) Zwittergent 3–14 in HEPES–saline) was used as a particle suspension diluent instead of HEPES–saline, and the samples were incubated at 60 °C for 30 min to solubilize the lipid components of the particles. The samples were cooled down to the room temperature, mixed with PicoGreen dye stock and. incubated for 20 min in the dark. The fluorescence intensity of the samples was determined in quadruplicate using microplate fluorometer (FL-600A, Bio-Tek Instruments, Inc.) equipped with a 485/20 nm bandpass excitation filter and a 530/25 nm bandpass emission filter. The percent of dye-accessible DNA was determined as the fluorescence intensity ratio between non-dissociated particle samples and dissociated (solubilized) particle samples times 100%, and the total amount of DNA was quantified from the fluorescence intensity of the dissociated particle samples using the concurrently run standard curve. Picogreen was used for plasmid DNA and siRNA quantification, whereas Oligreen was used for oligonucleotide determination in a similar manner as described above.

2.6. FRET studies

2.6.1. DNA compactness

The FRET efficiency between the DNA-intercalating fluorophores YOYO-1 (λ_{ex} 470 nm/λ_{em} 509 nm) and EthD-2 (λ_{ex} 530 nm/λ_{em} 620 nm) forming a donor–acceptor pair was used to characterize the compactness of the complexed DNA. Each dye was added to the DNA at the ratio of 1 dye per 200 bp. A parameter E reflecting the energy transfer efficiency was calculated from the measured fluorescence intensities (Fluorolog, Jobin Yvon Spex, NC) (I) as follows:

$$E = \frac{F}{F_0} - 1 \tag{1}$$

$$F_0 = \frac{\left(I^{\lambda 620\ \mathrm{nm}}_{\mathrm{EthD-2}} + I^{\lambda 620\ \mathrm{nm}}_{\mathrm{YOYO-1}}\right)}{\left(I^{\lambda 509\ \mathrm{nm}}_{\mathrm{EthD-2}} + I^{\lambda 509\ \mathrm{nm}}_{\mathrm{YOYO-1}}\right)} \tag{2}$$

$$F = \frac{I^{\lambda 620\ \mathrm{nm}}_{\mathrm{YOYO-1+EthD-2}}}{I^{\lambda 509\ \mathrm{nm}}_{\mathrm{YOYO-1+EthD-2}}} \tag{3}$$

i.e., F_0 was determined from the measurements when only one of the probes was present, and therefore, was indicative of the spectral intensities at zero FRET efficiency, and F was determined when both probes were present, and FRET may have occurred. The measurements were taken at an excitation wavelength of 470 nm; the superscripts and subscripts in formulas (2) and (3) refer to the emission wavelengths and the particular dye(s) measured, respectively.

2.6.2. Relative distances between DNA and cationic lipid or neutral lipid

YOYO-1 labeled DNA and either DiI(3) (cationic) or DiI(3)-DS (neutral) lipid probes were used. The FRET efficiency parameter (E) was determined as above, using the fluorescence intensities of the emission maxima of the respective probes (DiI(3), DiI(3)-DS at 566 nm).

2.6.3. Relative proximity of cationic lipid and neutral lipid within the complexes

The lipid probes NBD-PE (λ_{ex} 465 nm/λ_{em} 530 nm) and DiI(3) (λ_{ex} 549 nm/λ_{em} 566 nm) were used as a FRET donor/acceptor pair to characterize the proximity of cationic lipid and neutral dialkyl-lipid in the DNA–lipid particles and lipoplexes. The lipid dyes were added at 0.1 mol% total lipid and the parameter E was calculated as above.

In the FRET studies outlined above, the samples can become diluted during dialysis. To account for the fluorescence intensity variations due to particle dilution, the fluorescence intensity was normalized to the total amount of phosphate-containing compounds in the particle preparation [11]. Complexes prepared without fluorescent probes were used to correct for background scattering.

2.7. Particle lamellarity determination

The proportion of the carboxyfluorescein-labeled phospholipid headgroups (CFPE) exposed on the particle outer surface, i.e., whose fluorescence was quenched by a membrane-impermeable quencher, anti-fluorescein Fab′ fragment was considered indicative of the particle lamellarity. This allows one to obtain structural information on the number of enclosed bilayers contained within the particle. DNA–lipid particles and unilamellar liposomes (control)

were prepared using an ethanolic solution of DOTAP/POPC/Chol (6:15:10 molar ratio) with CFPE (0.5 mol% of POPC content) as described above. The particle samples were diluted to approximately 5 μM POPC in HBS, pH 7.4 and fluorescence intensity was measured at (λ_{ex} 497 nm/λ_{em} 528 nm) before and after the addition of 12 μl of 0.5 mg/ml anti-fluorescein/Oregon Green IgG stock solution per 0.6 ml of sample. No further quenching was seen above this ratio of anti-fluorescein IgG to CFPE. Quenching was expressed as the percentage drop in CFPE emission after addition of antibody.

2.8. Sucrose density gradient centrifugation

Sucrose gradients (0–13% v/w) in HEPES-buffered saline were reproducibly prepared using a precision dispensing system that consisted of two P-500 high precision programmable pumps, a GM-1 gradient mixer, a MV-8 valve, and an LCC-500 FPLC system controller (Amersham Pharmacia Biotech). The controller was programmed to dispense sequentially six linear 0–100% gradients of solvent B (13% sucrose in 5 mM HEPES–saline, pH 7.4) in solvent A (5 mM HEPES–saline, pH 7.4) into six Beckman Ultra-Clear 13×51 mm ultracentrifuge tubes at the total dispensed volume of 4 ml per tube. Sample aliquots (200 μl) were carefully applied onto the top of the gradient solution, and the tubes were centrifuged at 192,000×*g* for 10 h at 22 °C (Ultracentrifuge L8-70M, Beckman) using an SW50.1 rotor. After centrifugation, the tubes were illuminated from the top and photographed against a black non-reflective background to detect the location of particle bands by light scattering. The gradients were divided into 150 μl fractions, starting from the top, and the fractions were analyzed for lipid content by ^{14}C and ^{3}H radioactivity measurements (for neutral and cationic lipid, respectively), particle size, and nucleic acid content (by PicoGreen or Oligreen). The sucrose solution densities of the fractions were calculated from the measurements of sucrose concentrations using a refractometer (Leica AR200, Leica Microsystems, IL) and conversion to density by using tables found in [12].

2.9. In vitro transfection

HER2-overexpressing human breast adenocarcinoma cells SK-BR-3 cells were cultured in McCoy's 5A medium supplemented with 10% (v/v) fetal bovine serum, 100 μg/ml of streptomycin sulfate and 100 U/ml of penicillin G at 37 °C, 5% CO_2. The cells were plated at a density of 150,000 cells per well in 12 well plates (Corning) and acclimated overnight in the above media without serum or antibiotic supplements. The final media volume in each well was 1 ml and each well received 1 μg of pCMV/luc^{+} as either Lipofectamine 2000-DNA complex (using transfection conditions recommended by the Lipofectamine manufacturer), free DNA, or a DNA–lipid preparation. The cells were exposed to the DNA–lipid samples for 12 h, and Lipofectamine 2000 complexes for 6 h,

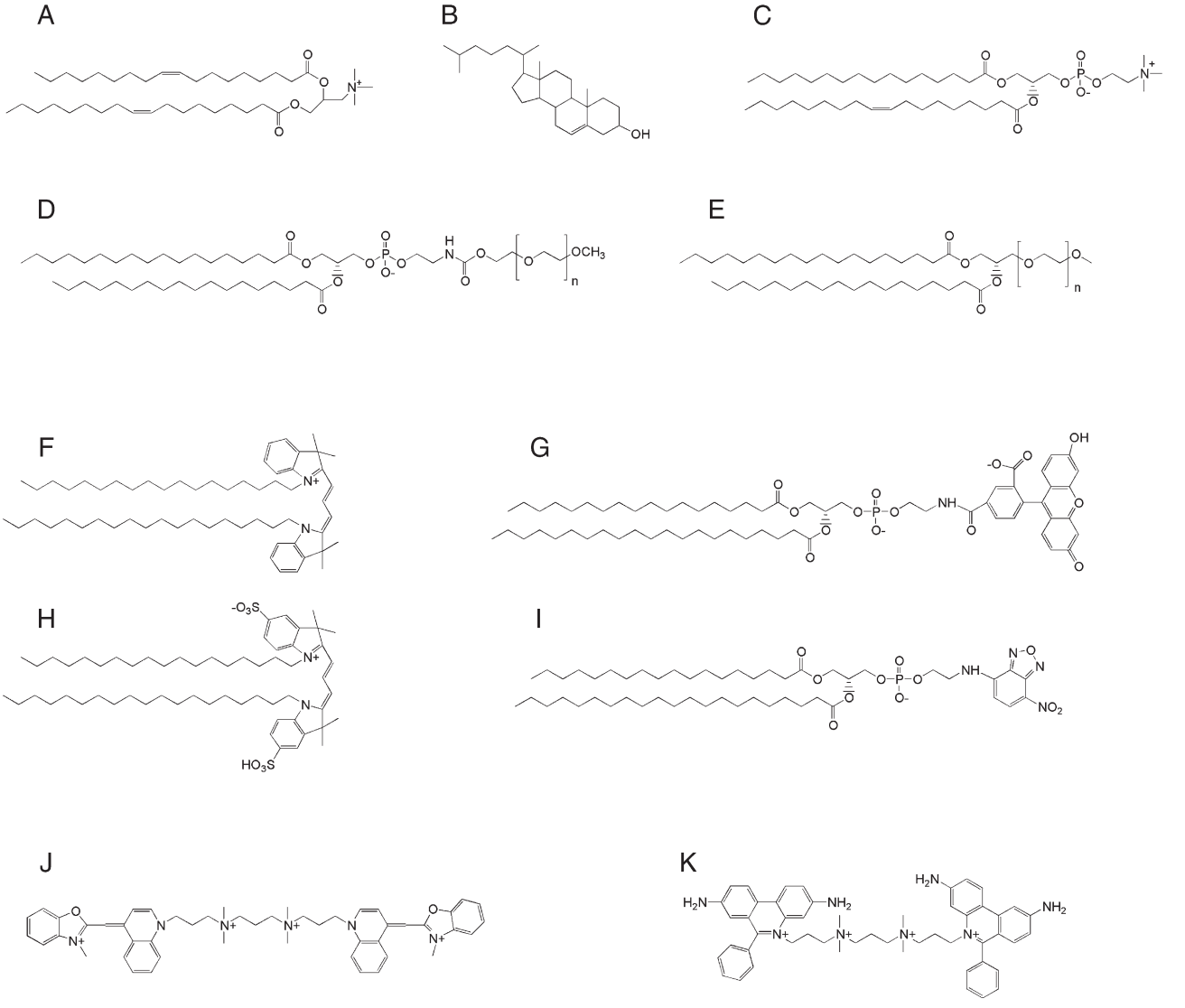

Fig. 1. The structures of fluorescent, non-fluorescent lipid components and DNA intercalators used in this study. Molecular structures denoted as follows; (A) DOTAP; (B) Chol; (C) POPC; (D) PEG-DSPE; (E) PEG-DSG; (F) $DiIC_{18}(3)$; (G) CFPE; (H) $DiIC_{18}(3)$-DS; (I) NBD-PE; (J) YOYO-1 and (K) EthD-2.

after which time the media was aspirated, cells washed twice with PBS, and the supplemented growth medium added to a final volume of 1 ml per well. After further incubation for 24 h, the cells were washed with PBS twice, and then 1 ml PBS (with 3 mM EDTA) added to each well to detach cells. After 5 min cell suspension was thoroughly mixed and transferred to a clean Eppendorf tube and centrifuged at 14,500×*g* for 2 min. The supernatant was removed, and the pellet washed with PBS. After removal of PBS by centrifugation as above, 0.1 ml sodium phosphate 0.1 M was added and tubes quickly frozen at −80 °C. Cellular disruption was achieved by 2 freeze–thaw cycles, and a clear cell lysate was obtained following centrifugation at 14,500×*g* for 30 s.

Luciferase in the supernatants was quantified by luminometry (Monolight 3010, Analytical Luminescence Laboratory). An aliquot (100 µl) of freshly prepared substrate solution containing 0.5 mM D(−)-luciferin, 30 mM Tris–HCl pH 7.8, 3 mM ATP, 15 mM $MgSO_4$, 10 mM DTT, and 0.2% Triton X-100 was injected into the luminometer cuvette containing 20 µl of the cell lysate, and after a 2-s delay, the luminescence was read for 10 s. The luciferase standards were prepared by 10-fold serial dilutions of the 0.5 mg/ml firefly luciferase stock made with the cell lysis buffer to a typical concentration range of 5.10^{-9} to 5.10^{-5} mg/ml, and read in a similar manner. Protein concentration in the cell lysates was measured using a Micro BCA protein assay (Pierce, Rockford, IL) using bovine serum albumin as the standard. The amount of luciferase was expressed in ng luciferase per mg of cell lysate protein.

3. Results

3.1. Determination of suitable aqueous/organic solvent mixtures to prepare Genospheres

The structures of the fluorescent and non-fluorescent lipid components used in the present study as well as the fluorescent DNA binding molecules are shown in Fig. 1. Condensed lipid phases in solution tend to form particles whose presence may be detected by increased light scattering. The light scattering intensities of a lipid mixture, DOTAP/POPC/Chol/PEG-DSPE (6:15:10:0.16), dispersed in water/water-soluble organic solvent mixtures of varying organic solvent concentrations was measured (Fig. 2). The mixtures were measured at two temperatures, 23 °C and 55 °C. For each solvent tested, there was appreciable scattering intensity at lower organic solvent concentrations (less than 30% (v/v)) at both temperatures. However, at 55 °C, in the case of isopropanol, ethanol and ethylene glycol dimethyl ether, higher organic solvent concentrations caused the scattering intensity to drop to the level of the background solvent, indicating the disappearance of condensed lipid phases and the formation of a molecular (true) or micellar solution.

We then prepared plasmid DNA/lipid complexes in aqueous/organic solvent mixtures using the above lipid formulation at such organic solvent concentrations, where condensed phases were absent, to determine if the organic solvent component in the aqueous/organic solvent mixture affected the properties of the resultant plasmid DNA–lipid particles. Subjecting the DNA–lipid particles to sucrose density gradient centrifugation assessed one such property, particle homogeneity. This technique allowed separation of lipid-rich (light) and DNA-rich (heavy) fractions, as well as the removal of free DNA, if any was present. After centrifugation, all preparations formed distinct light-scattering bands (Fig. 3A). The particles produced from 40% (v/v) isopropanol and 50% (v/v) ethanol had a similar density (1.016–1.02) and almost identical lipid/DNA ratios as the starting material indicating the particles were homogeneous with respect to density and composition (Table 1). However, those particles made in 50% dioxane and 60% (v/v) ethylene glycol dimethyl ether, produced distinct bands that were lipid rich and/or heavier DNA rich bands signifying that these particular solvents may not be optimal for producing homogenous complexes.

DNA-binding hydrophilic dyes, such as PicoGreen® greatly increase their fluorescence upon binding to DNA exposed to the solution. The increase in the dye fluorescence in the presence of DNA–lipid particles is proportional to the amount of "dye-accessible" DNA, i.e., the DNA that is incompletely encapsulated, free, or adsorbed on the particle surface. After the release

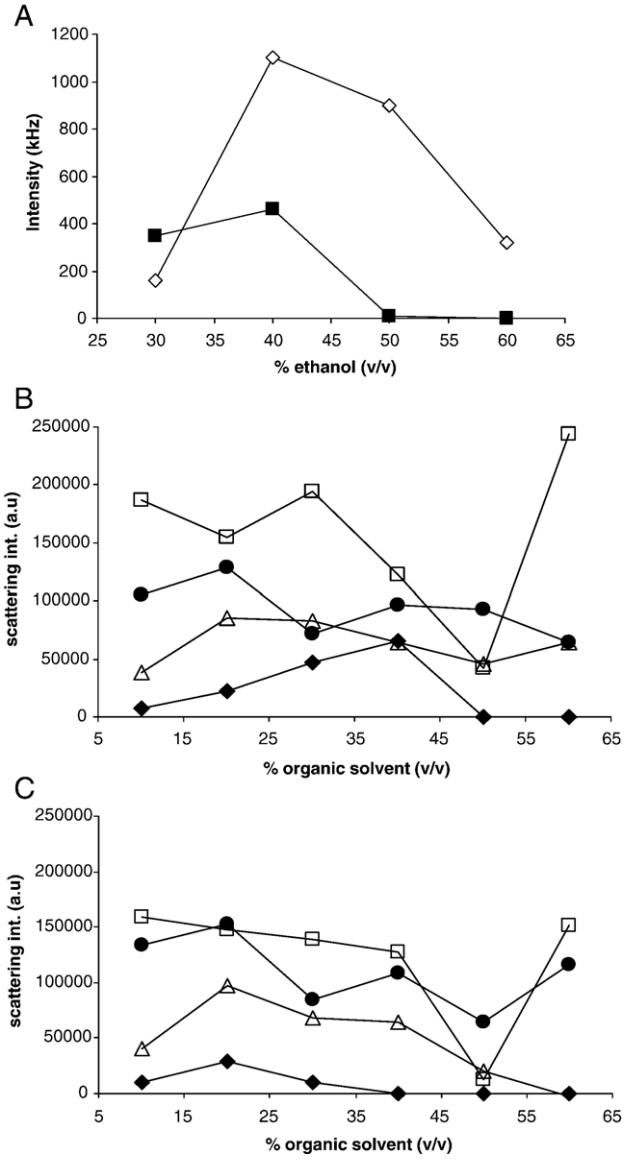

Fig. 2. The light scattering intensities of DOTAP/POPC/Chol/PEG-DSPE (molar ratio 6/15/10/0.16) lipid mixtures in 5% (w/w) dextrose with varying organic solvent concentrations. All measurements were made at a phospholipid concentration of 2.1 mM in (A) ethanol containing solutions at 23 °C (open diamond) and 55 °C (filled square) or at 23 °C (B) and 55 °C (C) using the following solvents, dioxane (open square), isopropanol (filled diamond), dimethylformamide (filled circles) and ethyleneglycol dimethyl ether (open triangle).

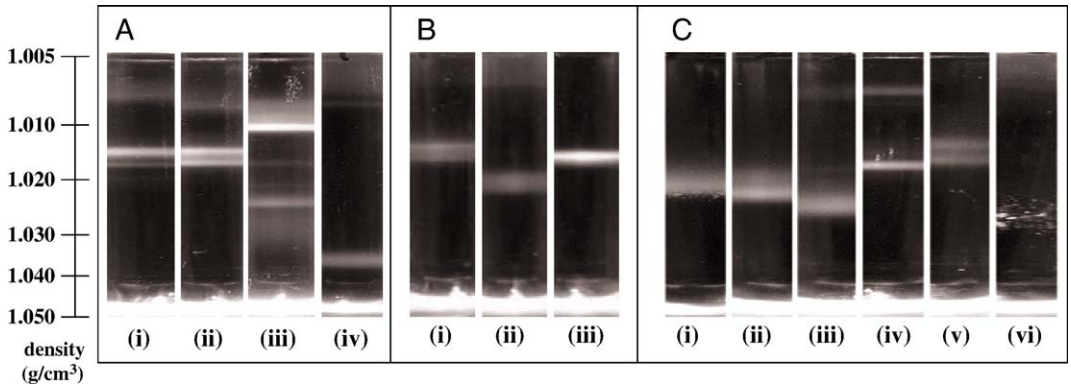

Fig. 3. Analysis of nucleic acid-lipid particles produced from various aqueous/organic-solvent mixtures using sucrose density centrifugation (0–13% (w/w) sucrose). (A) Complexes of formulation DOTAP/POPC/Chol/PEG-DSPE (molar ratio 6/15/10/0.16 nmol/nmol/nmol/nmol per μg plasmid DNA) prepared from 40% (v/v) isopropanol, 50% (v/v) ethanol, 50% (v/v) dioxane, and 60% (v/v) ethylene glycol dimethyl ether were applied to the top of each column (i-iv respectively) and tubes centrifuged at 192,000 g for 10 h at 22 °C. (B) Genospheres containing DOTAP/POPC/Chol with the molar ratio of 6/15/10 nmol/nmol/nmol of lipid per μg plasmid DNA, siRNA or oligonucleotide (i-iii) respectively were prepared and centrifuged as described above. (C) DNA-lipid complexes (DOTAP/POPC/Chol (6/15/10 nmol/nmol/nmol lipid per μg plasmid DNA) prepared from 0, 20, 30, 40, 50 and 60% (v/v) ethanol were applied to the sucrose gradients (tubes (i-vi) respectively).

of DNA from the complexes, e.g., by solubilization with a detergent, the fluorescence further increases, now being proportional to the total amount of DNA contained in the sample. The ratio of DNA-binding dye fluorescence between the native and solubilized particles is known as the dye accessibility and serves as the measure of completeness of the DNA encapsulation within the lipid [13]. Using this technique, we observed that the particles produced in either 40% (v/v) isopropanol and 50% (v/v) ethanol had relatively low dye accessibilities and when purified on the sucrose gradient the values were found to be close to the starting material (Table 1). However, with 50% (v/v) dioxane and 60% (v/v) ethylene glycol dimethyl ether we observed initially high dye accessibilities, which was reduced by purification on the gradient as a consequence of non-encapsulated DNA removal. In both cases the majority of DNA resided in the bottom fraction.

In order to determine if the use of an aqueous/organic solvent monophase was suitable for encapsulation of other nucleic acid types, we used one such monophase, a 50% (v/v) ethanol/water mixture with the above lipid formulation to separately entrap small double stranded RNA with 22 bp (siRNA) and short single stranded DNA with 20 bases (phosphorothioate modified antisense oligonucleotide). We then compared these Genosphere complexes with Genosphere particles that contained plasmid DNA (4.5 kbp) in a similar manner as above (Fig. 3B). We observed the main light scattering bands for the plasmid (i) and oligo (iii) entrapped complexes to have similar densities (ρ=1.014–1.017 and 1.016–1.018, respectively), while the siRNA (ii) sample was slightly more dense (ρ=1.019–1.022). Some free lipid was observed on the gradient in the case of the siRNA. The lipid/plasmid DNA and lipid/oligo ratios for their respective main bands were almost identical to the starting components (Table 2).

This technique facilitated DNA–lipid interaction in a medium that initially does not contain lipid condensed phases. We then compared the particles made from the Genosphere method, to particles produced by interaction of plasmid DNA and stable (lipoplex) or ethanol-destabilized lipid vesicles by combining the components in water or 30% (v/v) aqueous ethanol, respectively, using the same lipid formulation. The particles made from ethanol-destabilized liposomes will be designated as PEDL.

3.2. DNA compaction within the complexes

As an increase in DNA compactness brings distant nucleotide pairs closer to each other, the extent of FRET between the dyes (YOYO-1 and EthD-2), sparsely and randomly distributed along the DNA molecule would serve as a direct measure of the closeness of DNA base pairs and therefore of the DNA compactness [14]. Indeed, in the absence of DNA compacting agents, the FRET efficiency parameter (E) values of YOYO-1

Table 1
Characterization of DNA–lipid particles made from various aqueous/organic-solvent mixtures before and after purification by centrifugation on a sucrose density gradient

Solvent, % (v/v)	Size (nm±S.D. nm)	Initial PC/DNA	Initial % Dye Accessibility	PC/DNA main band	% Dye Accessibility of main band	Density (g/cm^3)
Isopropanol, 40%	115.8±46.9	14.6±0.5	11.6±0.4	13.3±0.6	8.9±0.3	1.016–1.017
Ethanol, 50%	116.2±49.4	13.4±0.5	9.5±2.7	13.0±0.8	8.5±0.5	1.016–1.017
Dioxane, 50%	192.9±58.2	14.1±1.2	32.5±2.6	8.8±0.3	12.8±0.8	1.016–1.017
Ethylene glycol dimethyl ether, 60%	552.5±223.7	66.2±2.7	44.2±1.6	22.4±1.4	38.7±1.8	1.043–1.047

The formulation used was DOTAP/POPC/Chol/PEG-DSPE with the molar ratio of 6/15/10/0.16 nmol of lipid, respectively, per μg DNA. The refractive index of each fraction was measured and sucrose density (g/cm^3) calculated as described above.

Table 2
Characterization of Genospheres made using different nucleic acid types, before and after purification by sucrose density gradient centrifugation

Nucleic acid	Initial PC/ nucleic acid	% dye accessibility (initial)	Size (initial)[a]	PC/nucleic acid (main band)	% dye accessibility (main band)	Size (main band)[a]	Density (g/cm^3)
Plasmid 5.5 kbp	13.3±0.4	3.5±0.2	123±5	13.4±0.5	6.1±0.3	122.5±47	1.014–1.017
siRNA 21 bp	13.9±0.2	18.6±1.1	148.8±57.6	8.4±0.4	17.5±0.9	128.7±62.1	1.019–1.022
Oligonucleotide 20 bases	14.0±1.0	13.8±0.6	105.7±43.4	20.4±1.9	14.5±0.2	117.4±54.5	1.016–1.018

The formulation used was DOTAP/POPC/Chol in the molar ratio of 6/15/10 nmol of lipid, respectively, per μg nucleic acid.
[a] Size (nm±S.D. nm).

and EthD-2 dual-labeled plasmid DNA did not exceed 0.6 in the various water and water/ethanol mixtures tested, while in the presence of spermidine, a well-known DNA condensing agent [15], *E* values increased to 6–8 (Table 3). When the lipid mixture described above was added to DNA in the form of liposomes, the value was close to 2. Lower values were obtained when lipid is added in 30% (v/v) and 50% (v/v) ethanol, i.e., close to 1 in both cases indicating slightly less DNA compaction. After dialysis, the Genospheres and PEDL particles showed *E* values of 1 and 2.1, respectively. When the salt concentration was adjusted to a physiological concentration, all particles exhibited approximately equal and a rather modest degree of DNA compaction.

3.3. Validation of the use of cationic and neutral FRET lipid pairs

The structures of fluorescent lipid dyes DiI(3) and DiI(3)-DS (Fig. 1) suggested them as good position markers for cationic and neutral dialkyl lipids, respectively, when used as either a FRET-donor or acceptor in a multicomponent lipid assembly. In order to validate this suggestion, we studied the ability of these dyes to form liposomes and further form complexes with DNA in a manner characteristic for conventional cationic liposome. Small sonicated liposomes were prepared with each dye in combination with DOPE (1:1), and mixed with DNA at a ratio of 2 dyes per DNA phosphate, which is a common procedure for making lipoplexes (1). Upon mixing, turbidity developed in the DiI(3)/DOPE–DNA liposome sample, and no apparent turbidity developed in the DiI(3)-DS/DOPE–DNA preparation. After centrifugation approximately 50% of the DiI precipitated from solution, indicating the formation of charge stoichiometric complex between the dye liposomes and the DNA, while none of the DiI-DS liposomes precipitated, as quantified by fluorescence measurements of the supernatants.

Table 3
The FRET parameter *E* of dual labeled uncomplexed DNA, DNA–spermidine complexes and DNA incorporated into various lipid complexes in different aqueous media

Sample	aqueous ethanol	5 mM HEPES, pH 7.4	HBS, pH 7.4
DNA	0.3	0.6	N/A
DNA+Spermidine	8.4	6.2	N/A
Lipoplex	N/A	2.0	1.4
Genosphere	1.2	1.0	0.8
PEDL	1.0	2.1	1.3

EthD-2 and YOYO-1 were added to DNA (1:200 dyes per base pair for both fluorescent probes) and formulated into various cationic lipid-containing complexes as described above. *E* was measured in the various media and for comparison, also measured for uncomplexed and condensed DNA (with spermidine). The values shown for Genospheres and PEDL in ethanol/aqueous mixtures are for 50% and 30% (v/v), respectively. The *E* values for DNA and DNA with spermidine are those measured in 50% (v/v) ethanol and are similar to values obtained in 30% (v/v) ethanol. Spermidine and sodium chloride were added from concentrated solutions to give final concentrations of 10 mM and 144 mM, respectively. All measurements were taken at 23 °C.

3.4. Determination of closeness of DNA to cationic and neutral dialkyl lipid in the particles

Similarly, we used the FRET efficiency parameter *E* between the DNA bound donor dye YOYO-1 and the fluorescently labeled DiI(3) (cationic) and DiI(3)-DS (neutral) lipid acceptor labels to characterize the proximity of the cationic or neutral dialkyl–lipid components of the particles to the DNA molecule (Table 4). In a lipoplex, both cationic and neutral lipid labels were at similar distances from DNA as indicated by the similar *E* values. When the lipids were combined with DNA in a 50% (v/v) ethanol monophase at 60 °C. The cationic lipid attained a significantly closer proximity to DNA ($E=1.6$) than did the neutral lipid ($E=0.2$), indicating that cationic lipid component assembled on the DNA molecule first. After the temperature decreased to ambient, neutral and cationic lipids showed the

Table 4
The FRET parameter *E* as measured using YOYO-1 bound DNA as the donor molecule and either $DiIC_{18}(3)$ (cationic) or $DiIC_{18}(3)$-DS (neutral) as the acceptor molecules

Sample	60 °C in aqueous ethanol	22 °C in aqueous ethanol	22 °C in 5 mM HEPES, pH 7.4	22 °C in HBS, pH 7.4
Lipoplex				
Cationic	N/A	N/A	1.9	1.1
Neutral	N/A	N/A	1.7	1.3
Genosphere				
Cationic	1.6	1.5	2.3	1.7
Neutral	0.2	2.0	2.3	2.1
PEDL				
Cationic	0.7	2.0	3.2	2.3
Neutral	0.6	1.6	1.5	1.6

FRET measurements were taken at various stages during the preparation procedure of the complexes and after removal of the organic solvent by dialysis against 5 mM HEPES, pH 7.4. Sodium chloride was added from a stock solution to give a final concentration of 144 mM.

same proximity to DNA (E=2.0) suggesting that the neutral lipid components joined the cationic lipid in a DNA-enclosing lipid shell. After dialysis, E increased only slightly for both probes, suggesting that no major redistribution of the lipid components in relation to DNA occurred during the removal of ethanol. In contrast, when the lipids were combined with DNA at 60 °C in 30% (v/v) ethanol, cationic and neutral lipids were equidistant from DNA (E=0.6–0.7), suggesting the DNA first bound to the pre-formed cationic-neutral lipid condensed phase as a whole. Upon cooling to room temperature, the FRET efficiency between DNA label and both neutral and lipid component equally increased (E=1.6–2.0), indicating that structural rearrangements took place bringing the lipids closer to DNA; and upon removal of ethanol, the cationic lipid–DNA proximity further increased (E=3.2). This sequence of events is consistent with the proposed mechanism of "small multilamellar liposome" formation through DNA-induced rearrangements of ethanol-destabilized lipid vesicles [3]. Increasing the sodium chloride concentration to 144 mM caused the E parameter to become smaller, especially between cationic lipid and DNA in all cases. There was relatively little change in E with respect to DNA and neutral lipid. This indicates some further structural rearrangements occur upon exposure to physiological salt concentrations where the distance from cationic lipid and DNA become greater. The smallest changes in E upon adding salt are seen for Genospheres indicating they may be less susceptible structural changes induced by the ionic strength of the surrounding media.

3.5. Relative lipid distribution within the particles

To understand how cationic and neutral lipids were distributed relative to each other, we measured the efficiency of FRET between two lipid labels, a headgroup-labeled "neutral" lipid label NBD-CAP-PE serving as a donor, and a cationic lipid acceptor $DiIC_{18}(3)$ (Table 5). In the absence of DNA, liposomes prepared by various routes (extrusion of an aqueous lipid dispersion, or ethanol removal from 30% (v/v) or 50% (v/v) ethanol mixtures) showed fairly efficient FRET between neutral and cationic lipid labels (E=1.1–2.2), and so did DNA–lipid particles prepared by different routes, suggesting that all these structures contained mixed lipid phases in which cationic and neutral components were not clearly separated. In the presence of 30% (v/v) ethanol, even at 60 °C , there was relatively efficient FRET between neutral and cationic lipid components (E=0.8), indicating the presence of a mixed lipid condensed phase, which is consistent with the light scattering measurements. In the presence of DNA a further increase in FRET (E=1.7) was observed, pointing to the compaction of the lipid phase upon interaction with DNA, possibly resulting from the exclusion of interdigitating ethanol molecules. In 50% (v/v) ethanol without DNA at 60 °C, as expected from the light scattering data, no detectable FRET was observed between the neutral and cationic lipid until the solvent was dialyzed away, and liposomes formed. In the presence of DNA at 60 °C and 50% (v/v) ethanol, the neutral-to-cationic lipid FRET was also undetectable, consistent with the observation that only cationic lipid was bound to DNA under these conditions. Upon cooling to ambient temperature, the neutral lipid joined the condensed lipid phase around the DNA molecule, and the neutral-to-cationic lipid FRET became efficient (E=1.7). Exposure to saline caused a small increase in E, roughly to the same extent for all lipid assemblies. We did, however, observe the presence of scattering material in 50% (v/v) at 22 °C ethanol (Fig. 2) but did not measure any detectable FRET, and this suggests that the particles responsible for the scattering in this case did not contain both of the probes; for example it is possible that cholesterol is less soluble at 22 °C in this solvent mixture.

Table 5
FRET parameter (E) between cationic and neutral lipids within the various lipid assemblies measured in aqueous ethanol and buffer at 22 °C and 60 °C

Sample	60 °C in aqueous ethanol	22 °C in aqueous ethanol	22 °C in 5 mM HEPES, pH 7.4	22 °C in HBS, pH 7.4
Lipoplex	N/A	N/A	1.8	2.2
Lipoplex 'minus' DNA	N/A	N/A	1.2	1.6
Genosphere	0	1.7	2.4	2.7
Genosphere 'minus' DNA	0	0	1.1	1.4
PEDL	1.3	2.3	2.2	2.5
PEDL 'minus' DNA	0.8	1.7	2.2	2.5

The neutral donor NBD-PE and the cationic acceptor $DiIC_{18}(3)$ molecules were incorporated into the formulations as previously described. For each of the particle-forming methods, replicas were made without DNA (i.e., 'minus' DNA) to provide information on how the presence of DNA affects the relative lipid distributions.

Table 6
Size and % dye accessibility of DNA–lipid complexes prepared using lipoplex, PEDL or Genosphere preparation procedures

Sample	Size (nm)	% Dye Accessibility
Lipoplex	216.9±100.6	61.4±1.3
Genosphere	66.4±32.8	4.6±0.8
PEDL	86.6±40.5	38.1±0.6

Complexes were prepared as described above using the lipid composition of DOTAP/POPC/Chol (6/15/10 nmol per μg DNA) with ethanol removal affected by dialysis against unbuffered saline.

3.6. Size and dye accessibility of particles

We compared the average particle size and dye accessibilities of lipoplexes, PEDL particles, and Genospheres having the same composition as those used for FRET proximity studies (Table 6). The average size of the particles produced using the lipoplex methodology was over 200 nm, consistent with a multilamellar structure [16]. The dye accessibility is high (>60%), suggesting that a substantial amount of DNA is either adsorbed on the surface of the lipid structure or is incompletely covered with lipid. Both Genospheres (50% (v/v) ethanol) and PEDL (30% (v/v) ethanol) preparations consisted of small particles (100 nm) resembling unilamellar liposomes by size, but differed in the amount of dye-accessible DNA. This indicates the particles assembled from the DNA and lipid components in solution provided for somewhat more complete

entrapment of DNA within the lipid than the particles formed by deformation of pre-existing vesicles.

3.7. Particle homogeneity of particles made from water/ethanol mixtures of varying ethanol concentrations

To assess particle homogeneity, DNA–lipid particles prepared from a range of ethanol concentrations (20, 30, 40, 50 and 60% (v/v)) were subjected to sucrose density gradient as described before (Fig. 3C). Particles prepared at 0% ethanol (lipoplex) and 20% (v/v) ethanol formed a single band with $\rho = 1.021–1.022$. In a 30% (v/v) ethanol preparation, the band with $\rho = 1.025$ was accompanied by another band at $\rho = 1.007$ which was found to contain practically no DNA ("empty" liposomes). In a 40% (v/v) ethanol preparation, the main band with $\rho = 1.019$ was still accompanied by the "empty liposomes" band, while at 50% (v/v) ethanol, there was no "empty liposomes" band, and two closely spaced bands with $\rho = 1.014$ and $\rho = 1.019$ were detected. The preparation made at 60% (v/v) ethanol showed a diffuse light-scattering area at the top of the gradient, and a band of highly aggregated material having $\rho = 1.028–1.030$.

To determine if the mode of particle assembly affected the particle size and composition, the gradients were fractioned and analyzed for total DNA (by PicoGreen assay), and for two lipid components, cationic (DOTAP) and neutral (POPC), using multiple radioactive labels, [^{3}H]-DDAB (cationic) and [^{14}C]-DPPC (neutral phospholipid), respectively. In addition, the main bands were analyzed for particle size and DNA dye accessibility (Table 7). The low density bands ($\rho = 1.007$ or less) were confirmed as having practically no DNA, while various small amounts of free DNA were found in the bottom fractions, consistent with the higher buoyant density of free DNA ($\rho > 1.050$). In the isolated main bands, the smallest particles (65–68 nm on average) with the most extensively encapsulated DNA (dye accessibility 11–12%) were observed in the preparations made in 40% (v/v) and 50% (v/v) ethanol. However, the highest level of incorporation of both DNA and lipid in the particles, reaching about 87–88% of the lipid and practically quantitative for DNA, was observed in the preparation made at 50% (v/v) ethanol. This finding was consistent with the absence of the "empty liposome" band on the gradient, and also with the fact that the particle composition (lipid/DNA ratios) closely approximated the theoretical value of 6.0 (DOTAP/DNA) and 15.0 (POPC/DNA) (Table 7). We did not find any differences in the composition or other properties between the particles in the closely spaced bands recovered from the 50% (v/v) ethanol preparation. In the preparation at 40% (v/v) ethanol only about 55–60% of the lipid was incorporated, resulting in heavier (more DNA-rich) particles and substantial amount of free lipid. In the preparation produced at 60% (v/v) ethanol, the particles were grossly aggregated, but DNA was well encapsulated (dye accessibility 16%) compared to lipoplexes (71%). These particles were also poor in lipid, containing only about one fifth of the added lipid material. This finding is consistent with the hypothesis that in the 60% (v/v) ethanol preparation, the DNA prior to encapsulation has partially aggregated due to the higher ethanol concentration resulting in lower surface area available for lipid assembly. The PEDL material (30% (v/v) ethanol) after centrifugation showed an increase in size (from 113 nm to 180 nm average), a moderate degree of DNA encapsulation (dye accessibility 46%), and substantial amount (approx. 60%) of lipid present in the non-DNA bound form. Thus, the amount of ethanol in the monophase used to assemble the DNA–lipid particles had a profound effect on the particle composition and properties of the particles assembled under the mutual solubility conditions. Preparation of complexes with a monophase consisting of 50% (v/v) ethanol showed the highest encapsulation of DNA and lipid, small size, and low level of DNA exposure to the environment.

Table 7
Analysis of size, dye accessibility, lipid and DNA content of purified complexes from a sucrose gradient

Tube ID	% Ethanol	% D.A.	Size (nm)	% DOTAP	% POPC	% DNA
A	0	71.3±4.4	450.4±210.6	79.3	77.8	82.7
B	20	42.6±3.4	104.6±46.3	49.1	44.1	90.8
C	30	46.1±3.9	177.6±71.4	39.5	34.5	92.2
D	40	11.1±1.6	67.8±31.9	58.2	54.1	90.2
E	50	11.8±0.7	64.9±28.4	88.3	86.8	99.5
F	60	16.2±0.8	N/A	21.6	16.8	91.2

Examination of the main bands taken from the sucrose gradients of the complexes prepared from 0 to 60% vol. ethanol. Size was measured by quasielastic light scattering; lipid was quantified by scintillation counting and DNA dye accessibility (% D.A.). Total DNA content was measured by PicoGreen analysis.

Table 8
The fluorescence quenching of CFPE molecules incorporated into the DNA–lipid assemblies by an anti-fluorescein IgG

Sample	% Quenched
Unilamellar liposomes	57.7
Lipoplexes	18.2
Prepared in 20% (v/v) ethanol	53.3
Prepared in 30% (v/v) ethanol	63.7
Prepared in 40% (v/v) ethanol	59.7
Prepared in 50% (v/v) ethanol	49.3
Prepared in 60% (v/v) ethanol	41.2

DNA–lipid complexes comprising of DOTAP/POPC/Chol (6:15:10 molar ratio) per 1 μg DNA were prepared which included CFPE (0.5 mol% of POPC content). Fluorescence was measured before and after the addition of anti-fluorescein/Oregon Green IgG.

3.8. Lamellarity of particles

Using a non-membrane penetrating antibody that recognizes and selectively quenches the carboxyfluorescein fluorophore (anti-fluorescein IgG) to approximately 7% of its fluorescence intensity [17], we estimated the proportion of EPE with a carboxyfluorescein headgroup exposed on the surface of the particles and used this as an indication of the degree of lamellarity of the particles (Table 8). We found that all particles prepared from aqueous ethanol solutions, except those formed at 60% (v/v) ethanol, had 49–64% of the CF-labeled lipid

exposed on the surface. This is in contrast with the lipoplex particles with contained less than 20% of the lipid on the outside of the particles. The particles formed at 60% (v/v) ethanol had somewhat increased amount of lipid exposed on the surface. This finding, along with the lower particle aggregation stability and higher buoyant density (Fig. 3C) suggested that, at this ethanol concentration, the particles assembled differently, perhaps due to the partial loss of molecular solubility of the DNA. As a control, 0.05 μm extruded liposomes of the same formulation were tested and gave a quenching value (58%) consistent with a unilamellar vesicle with a slight transmembrane lipid density asymmetry originating from the bilayer curvature.

3.9. In vitro transfection activity of particles made in 0, 30 and 50% (v/v) ethanol/water mixtures

To correlate the mode of particle assembly with their capacity to deliver functional DNA into living cells, we studied the levels of transgene (luciferase) expression by cultured cells exposed to the particles containing a luciferase-encoding plasmid. A human HER2-specific scFv antibody–lipid conjugate, F5-PEG-DSPE, was added to a portion of each set of particles. This ligand can induce internalization and has been used previously with anti-HER2 immunoliposomes for the targeted drug delivery to HER2 (c-erbB-2,neu) over-expressing cancers [4,18]. Lipofectamine 2000 was used as positive control to confirm the activity of the luciferase encoded plasmid (Table 9). All three DNA–lipid preparations (lipoplexes, PEDL and Genospheres) had the same lipid and DNA composition and were incubated with the cells for 12 h. The addition of an internalizable targeting ligand F5-PEG-DSPE conferred an increase of transfection activity in all cases, the highest activity enhancement occurring for Genospheres (over 20-fold). Thus, DNA packaging into lipid particles using Genosphere process did not adversely affect the transfection properties of the plasmid and in fact enhanced it over the other DNA–lipid complexes tested.

Table 9
Luciferase expression in HER2 overexpressing human breast cancer cells SKBR3, transfected with DNA–lipid complexes prepared in 0, 30 and 50% (v/v) ethanol/water mixtures

Sample	Luc (ng/mg protein)	T/NT
F5-DSPE Genospheres	21.04±4.1	15.8
Non-targeted Genospheres	1.33±0.5	
F5-DSPE PEDL	5.3±0.64	2.7
Non-targeted PEDL	1.95±0.5	
F5-DSPE Lipoplex	1.8±0.4	11.3
Non-targeted Lipoplex	0.16±0.07	
Lipofectamine 2000	162.8±49.1	
Plasmid DNA	0.003±0.005	
Untreated cells	0.003±0.001	

Particles that were made target specific by the addition of the highly internalizable human anti-HER2 scFv antibody F5 (F5-PEG-DSPE) conjugate were tested in parallel with non-targeted ones. The lipid formulation was DOTAP/POPC/Chol in the molar ratios 6/15/10 nmol, respectively, per μg DNA. Improved recovery of particles following sterilization by microfiltration, could be achieved by the addition of 2 mol% of a neutral PEG-lipid derivative (PEG-DSG) to the initial lipid formulations. PEG-DSG was included into the lipoplex formulation post DNA–lipid complexation under aseptic conditions by a procedure adapted from Hong et al. [5].

4. Discussion

Non-viral vectors are generally prepared by the assembly of nucleic acids with carrier components, such as lipids. Lipids because of their amphiphilic nature have a propensity to self-assemble into more or less stable structures. Interactions of nucleic acids with self-assembled lipid structures depend in large part on the ability of the lipid molecules in the structures to undergo rearrangements as a result of the interaction. This ability is hindered by strong attraction between the assembled lipid molecules. Therefore, the lipid–nucleic acid complexes obtained from lipids self-assembled into bilayers are heterogeneous [19] and with time undergo slow changes known as "maturation" [20]. Reproducible assembly of particles can be aided by weakening the intermolecular interactions between the lipid molecules leading to a reduction or elimination of the kinetically limited component in the nucleic acid–lipid interactions. This has been accomplished by solubilizing the lipid with a dialyzable detergent [21] or through destabilizing preformed lipid bilayers with 30%–40% ethanol [3]. Alternatively, DNA in the form of its cationic lipid salt was extracted into organic phases, in which DNA would not be ordinarily soluble [22,23].

We explored the possibility of assembling the DNA–lipid particle in the environment where particle-forming lipids would not form a condensed phase beyond a micelle, and DNA would still be soluble by itself. We found that such conditions could be satisfied in a number of different organic solvent/water mixtures. For the purpose of establishing the feasibility of this approach, we initially chose to use plasmid DNA, a common vector for carrying an expressible transgene, and a combination of lipids containing a DNA-binding, transfection-enhancing cationic lipid DOTAP [24] a bilayer-forming neutral lipid, POPC, and a membrane-rigidifying lipid, cholesterol in the amount of 6/15/10 nmol/nmol/nmol per μg DNA. The average molecular weight of a DNA base is 330 g mol^{-1}, so one microgram of DNA corresponds to approximately 3 nmol of bases, i.e., 3 nmol of DNA phosphate. Genosphere particles were formed with 6 nmol cationic lipid per μg DNA, giving a N $(CH_3)_3^+/PO_4^-$ ratio of 2. This was enough cationic lipid to completely neutralize the DNA phosphate charge and ensure stability during formation of the particle. Therefore, Genospheres were constructed with a minimum of excess cationic lipid, which is known to endow lipid–DNA complexes with tropism toward the elements of capillary wall [25] and which may interfere with the ability of the complexes to reach the intended anatomical target.

Of the solvent mixtures tested, we found that 40% isopropanol and 50% (v/v) ethanol solutions were ideal in this respect, as they produced optically transparent lipid solutions (Fig. 2) and upon combining DNA and lipid in such mixtures, subsequently produced small, homogenous DNA–lipid particles (Fig. 3A, Table 1). We then sought to see if this methodology would entrap nucleic acids of varying size and chemical

composition and found that it was relatively efficient at entrapping both small double and single stranded nucleic acid molecules (Fig. 3B, Table 2). In addition, entrapment by this method was also relatively insensitive to the chemical composition of the backbone linkages (phosphodiester of plasmid and siRNA versus phosphorothioate of oligonucleotide) and base composition (RNA vs. DNA) of the nucleic acid used. Also, the fact that the resultant particles had similar sizes, while the nucleic acid component size ranged from a single stranded 20 nucleotide oligo to 4.5 kbp plasmid indicates that the nucleic acid molecular size does not dictate the overall particle size.

Taking one such monophase composition, i.e., 50% (v/v) ethanol, the particles ("Genospheres") were then compared to particles produced using stable liposomes and from ethanol-destabilized liposomes (PEDL) by the methods described by Felgner et al. [2] and Maurer et al. [3], respectively, using identical plasmid DNA and lipid compositions. While the components of these three types of formulations were identical, the resulting particles differed by their size, the degree of DNA exposure, homogeneity of the preparations, and the completeness of DNA and/or lipid incorporation.

It was of interest to assess the state of DNA condensation in the DNA–lipid particles obtained by different methods. DNA condensation has been observed in solution in many instances using cationic multivalent salts such as spermidine^{3+} and cobalt hexamine^{3+} [26]. Cationic polymers such as poly-L-lysine, [27], cationic peptides [28], polyethylenimine [29] and dimerizable surfactants [30] have been used to condense DNA in the formation of DNA–lipid particles. It has been suggested that charge neutralization of DNA by monovalent cationic lipids, also results in compaction of DNA within these types of complexes [31]. To measure the extent of DNA compaction within the different particles, we used a simple approach, measuring the FRET between an intercalating donor/acceptor fluorescent DNA label pair YOYO-1 and EthD-2 (Table 3), respectively. These bis-intercalating probes bind to DNA with sufficient affinity and YOYO-1 has been used previously as probe for DNA condensation [32], where it was shown that electronic interactions among YOYO-1 molecules at high dye/nucleotide ratios (1:50) led to a decrease in the probe quantum yield during condensation. We used a lower YOYO-1 dye/nucleotide ratio (1:200), where the fluorescence was unaffected by condensation. Also, it was shown that DNA bound YOYO-1 was not displaced by the binding of PEI or CTAB [32]. As the DNA is compacted, the sparsely distributed DNA-bound YOYO-1 and EthD-2 molecules become closer to each other, and the magnitude of FRET increases. Indeed, when both probes were bound to plasmid DNA the FRET parameter was 0.6 or less, compared to the simple sum of DNA-bound YOYO-1 or EthD-2 spectra recorded at the same concentrations. However, in the presence of spermidine, a known DNA-condensing agent, the FRET value increased to 6.0–8.0. The increase in EthD-2 emission intensity due to FRET after the addition of spermidine suggests that EthD-2 is also not displaced from DNA either in aqueous or aqueous ethanol mixtures.

Assuming that E values greater than 6 (DNA with large excess of spermidine) correspond to highly condensed plasmid, and values less than 0.6 reflect an uncondensed state, it appears that DNA inside all of the studied lipid complexes was far from being highly condensed, but was compacted slightly with E values ranging from 0.8 to 2.1.

Differences in the mechanism of particle formation between Genospheres and PEDL were apparent when the proximity of cationic and neutral dialkyl-lipids to the DNA was studied using FRET between a DNA-bound probe and a fluorescent cationic lipid probe (DiI) or neutral lipid probe (DiI-DS) (Table 4). As a control, we demonstrated that when mixed with so called "helper" lipid (DOPE) in a liposome formulation DiI(3) behaved as a cationic lipid, by electrostatically interacting with DNA, forming large complexes and that DiI(3)-DS behaved as a neutral lipid by not reacting with the polyanionic DNA. This functional similarity produced further evidence for fitness of these the cationic and neutral DiI lipid dyes as probes for the position of cationic or the neutral dialkyl lipids used in the present FRET study in the structure of DNA–lipid complex assemblies.

The data suggests that during Genosphere formation, cationic lipid is first to assemble around the DNA, while neutral lipid remains in solution. When the solution is cooled, neutral lipid joins the lipid moiety around the DNA, and after ethanol removal both types of lipid are at equal distance from DNA in the resulting particle. During the formation of PEDL at 60 °C, both lipids are equidistant from DNA from the beginning, indicating that the lipid vesicle interacts with the DNA as a whole. When the temperature drops, the cationic lipid becomes closer to DNA. When DNA interacted with preformed stable liposomes, the relative distances of cationic and dialkyl-neutral lipids to DNA in the resulting complex were also very similar throughout the procedure. Thus, unlike Genospheres, the PEDL and liposome–DNA complexes are likely to be formed through deformation of the mixed condensed lipid phases without their dissociation into lipid components.

These conclusions were in agreement with the results of a FRET study measuring relative proximities of cationic (DiIC$_{18}$ (3)) and dialkyl-neutral lipid (NBD-PE) in aqueous ethanol solutions in the presence or absence of DNA (Table 5). In 30% (v/v) ethanol at 60 °C (DNA absent), there was appreciable amount of FRET measured, indicating the presence of a mixed condensed lipid phase. In contrast, in 50% (v/v) ethanol at the same temperature (DNA absent) practically no FRET was detectable, indicating the cationic and neutral lipids were at significant distances from each other. This was consistent with the light scattering data indicating the absence of lipid condensed phases in 50% (v/v) ethanol. However, when DNA is added in the 50% (v/v) ethanol case (Genosphere), FRET was only measured when the ethanolic solution was cooled. This suggests that cationic and dialkyl-neutral lipids are spaced far from each other when DNA is present at 60 °C, but assume a closer proximity when cooled to 22 °C. Together with the results demonstrating at 60 °C in 50% (v/v) ethanol, cationic lipid is close to DNA and neutral lipid is not, we can see that Genosphere formation is a multi-step process with the cooling step importantly allowing the dialkyl-neutral lipid to condense on the DNA–cationic lipid complex in such a way that both types of lipid are equidistant from DNA. Removal of ethanol

caused both lipids to become even closer to DNA (Table 4) and to each other (Table 5).

Alternatively, the case of PEDL, FRET was measurable initially in the presence of DNA and increased upon cooling. This implies that both type of lipids are close to DNA during the initial mixing step and during cooling and subsequent ethanol removal, cationic lipid moves closer to DNA. This is similar to the lipoplex situation, where both lipids are equally spaced from DNA (Table 4) and are relatively close to each other in the final complex (Table 5). At the present, we can only account for the neutral dialkyl-lipid (POPC) distribution within the complexes as this was reported to us by the structurally similar DiI(3)-DS. However, the neutral lipid pool also contains cholesterol and its distribution is at this time unclear. This will be the subject of a future study. Also, we must point out that the described studies give information on relative DNA and lipid molecular organization at defined points during the particle assembly process, and as yet we have no data regarding rates of assembly for individual components of the complexes.

As controls for the FRET studies, "minus one" principle was used, where the matching liposomes, lipoplexes, or complexes having just one of the FRET pair members were used to assess a zero-FRET intensities, matching the actual environment of these probes in the samples containing both FRET members. This is because the fluorescent intensity of a probe may depend on the environment of the probe, including the aggregate state of the lipids surrounding the probe, and on the chemical composition of the probe environment. Therefore, by using the zero-FRET controls with identical lipid/DNA composition and method of preparation, the effects of lipid environment on the FRET probes were accounted for. In addition, we included the correction for light scattering/autofluorescence control, using similarly prepared complexes without any of the FRET pair labels.

Therefore, despite differences in the manner of the lipid attachment to DNA among the three complexation methodologies, the extent of DNA compaction and the relative proximity of the lipids to DNA and of the cationic lipids to neutral lipids in the resulting particles were similar. However, the particles differed by their size, lamellarity, and DNA dye accessibility. Genospheres and PEDL were relatively small with average particle diameter of approximately 66 nm and 87 nm, respectively, lipoplexes were considerably larger (greater than 200 nm) (Table 6). Accessibility of encapsulated DNA to small molecules, such as enzymes (e.g., DNase) is to be avoided if functionally intact nucleic acids are to be delivered. Therefore, the degree of DNA exposure to its environment is an important characteristic of a synthetic delivery vector and can be measured by a DNA-binding dye accessibility assay. Of all three types of DNA–lipid particles tested, Genospheres had the lowest dye accessibility (Table 6). Differences in the observed DNA accessibility may result from differences in the DNA encapsulation mechanisms leading to DNA exposure, or from the non-homogeneity of particle preparation, such as the presence of particle species with incomplete encapsulation and/or surface-bound DNA molecules.

Differences in the relative DNA/lipid content in the particles would result in different buoyant densities. Therefore, we employed ultracentrifugation on a sucrose density gradient to analyze the homogeneity of the particles made by plasmid DNA–lipid complexation in aqueous solutions containing no ethanol or 20–60% (v/v) ethanol, with subsequent ethanol removal by dialysis (Fig. 3C and Table 7). The preparations showed at least one distinct, primary light-scattering band, and sometimes a second distinct band, corresponding to different particle densities. The lipid and DNA amounts in the main bands varied among the preparations made at different ethanol concentrations. While the amount of DNA incorporated into the particles of the main band was consistently above 80% (v/v), the amount of incorporated lipid in the particles produced in the presence of 20–40% (v/v) ethanol and at 60% (v/v) ethanol was quite incomplete (16–58%) resulting in "heavy" bands with higher DNA content, accompanied (in 30 and 40% (v/v) ethanol preparations), with a light band of "empty" liposomes that did not contain any significant amount of DNA. Unexpectedly, in the particles produced at 50% (v/v) ethanol, i.e., using the Genosphere method, the incorporation of both DNA and the lipid was practically complete (86–99%). The particles showed two closely spaced bands whose lipid and DNA compositions were the same. The narrow distribution of particle density and closeness of the composition of purified complexes to the relative composition of the original starting materials pointed to the homogenous nature of the Genosphere preparation.

Measurements of the proportion of the lipids exposed on the surface of the particles produced in aqueous ethanol solutions are consistent with the oligolamellar structure (Table 8). This is in contrast to the lipoplex particles with contain less than 20% of the lipid on the outside of the particles, thus indicating numerous bilayers, as described previously [16,33,34]. Particles produced using the Genosphere, PEDL and lipoplex methodologies had different levels of transfection activity in cell culture (Table 9). While the absolute levels of activity were lower than the positive control prepared using Lipofectamine 2000, optimized for in vitro gene transfer, these data show that small DNA–lipid particles containing lipid-protected DNA, can give similar levels of transfection activity to the large lipoplex complexes of a similar composition. In fact, Genospheres gave the highest activity, possibly due to the well-protected nature of the DNA encapsulation. Also, the particles of all three types could be rendered targetable by non-covalent association with an amphipathic ligand–lipopolymer conjugate in a manner similar to production of antibody-targeted immunoliposomes [4,10] and more than an order of magnitude enhancement of activity was observed for Genospheres. In light of the results from previous studies suggesting that larger non-targeted complexes are more active at delivering DNA in vitro [35], it was important to demonstrate that small particles made from a monophase did not in some way render the resulting targeted particles inactive. The fact that the same lipid formulation when processed in such a way that produced small uniform particles (Genosphere), elicited higher transfection activity that that obtained from the sonicated liposome/DNA complex was surprising. As Genospheres are designed ultimately for systemic administration as an injectable therapeutic gene delivery vehicle, this finding is advantageous and important in light of the poor pharmacokinetic characteristics often associated with lipoplexes [36,37].

The lipid–DNA particles obtained by complexation in the presence of 50% (v/v) aqueous ethanol showed a number of beneficial properties (small, uniform size; uniform density; high degree of incorporation of both DNA and the lipid; and low DNA exposure), without the need for a large excess of cationic lipid. We hypothesize that these properties are a result of conducting the DNA–lipid complexation under the conditions where both lipids and DNA independently form molecular (true) or micellar solutions, therefore the assembly of the particles does not require rearrangements of pre-formed condensed phases (e.g., bilayers) that introduce a high activation energy, kinetically slow reaction step. Instead, we speculate that high efficiency of DNA entrapment is due to the high degree of utilization of the cationic lipid to augment the hydrophobic character of the DNA molecule, as a result of freer movement of molecularly-or micellarly-dissolved cationic lipid component at the early stage of Genosphere assembly. This is supported by our observations on the cationic versus neutral lipid proximity to DNA during the Genosphere assembly process. The existence of areas of mutual independent solubility of DNA and a cationic surfactant in alcohol–water solutions has been reported by Sergeyev et al [38]. The apparent long axis length of T4 phage DNA within a DNA-CTAB complex measured by fluorescence microscopy revealed a globule–coil–globule transition of DNA at elevated alcohol concentration. At intermediate ethanol concentrations 40–60% (v/v) the complex was solubilized due to the transfer of CTAB into solution, and DNA relaxed from a globule into a random coil conformation. At higher ethanol contents, the DNA compacted into a globule because of the loss of solubility, and at lower ethanol concentrations, the DNA was compacted by the assembling detergent molecules. This window of mutually independent solubility of DNA and CTAB is remarkably similar to the ethanol concentration window that produces Genospheres. We proposed that using 50% (v/v) ethanol to separately solubilize the particle components is ideal in this respect. Furthermore, while ethanol has an advantage of being quite acceptable in the pharmaceutical manufacturing process, it is very likely that other water-miscible organic solvents, such as isopropanol can be used instead of ethanol at concentrations providing for independent solubility of both nucleic acid and lipid components. Perhaps more important is the requirement that the organic solvent maintains complete water miscibility during its removal from the DNA–lipid mixture, leaving water behind. The systems that separate phases at increasing water content [23], even if at first homogenous, eventually lead to extraction of the DNA–lipid complex in the organic-rich phase, thus preventing efficient particle assembly.

By varying the concentration of ethanol in a water-organic monophase, we were able to compare the assembly of DNA–lipid nanoparticles in the systems with identical DNA and lipid compositions in three different modes: DNA association with stable cationic liposomes (lipoplex process, no ethanol); DNA association with ethanol-destabilized liposomes (PEDL process, 30% (v/v) ethanol), and DNA association with molecularly or micellarly dissolved lipids (Genosphere process, 50% (v/v) ethanol). All three processes resulted in DNA–lipid particles with nearly identical DNA compaction, lipid–DNA proximity. However, it was only the Genosphere methodology that produced particles that also were small, homogenous with respect to composition, contained well-protected DNA, and resulted in 90% or better incorporation of both DNA and the lipids. The underlying mechanism of this unexpected finding is not yet clear. A two-step assembly may be envisioned as a viable hypothesis. At the first (germination) step, upon mixing of DNA and the lipids in 50% (v/v) ethanol, uncoiled DNA molecules interact with at least a portion of the cationic lipid component to form partially compacted cationic lipid–DNA complexes, while neutral lipids are still in solution. At the second (deposition) step, upon cooling, the solubility of neutral and/or excess cationic lipid components decreases and causes them to self-assemble into neutral or mixed neutral-cationic lipid bilayers which are deposited around pre-formed cationic lipid–DNA complexes acting as colloidal germini for the assembly. The deposition continues until the lipids are exhausted from solution, thus leading to uniform and nearly quantitative inclusion of both lipid and DNA into the particles. The fact that Genospheres gave the highest in vitro transfection activity with the most targeting enhancement of activity may be attributable to these features of high DNA protection and particle uniformity.

The Genosphere process also offers advantages in manufacturing, which is an important consideration for the clinical translation of any pharmaceutical technology. It is a one-pot process that takes place under near equilibrium conditions, without the need for mechanical shearing steps such as dispersion, membrane extrusion or microfluidization, to pre-formulate the lipids into liposomes or post-formulate the lipid–DNA complexes into a nano-sized format. It involves essentially two well-scalable operations, temperature-controlled solution mixing and ethanol removal; the latter can be conveniently scaled up using tangential flow ultrafiltration or hollow fiber dialysis, the procedures quite common in pharmaceutical manufacturing. The process does not use toxic solvents, such as chloroform or methanol, and resulting nanoparticle preparations are sterilizable by microfiltration.

Uniquely, DNA and lipid were separately solubilized in an identical aqueous-solvent monophase, thereby avoiding areas of variable solvent concentrations during the mixing step. The mixing of two different solvents in such a process could place more critical importance on the rate of mixing so that non-uniformities of the resulting particles do not occur.

In this study, we focused on the general features and physicochemical characterization of Genospheres and the Genosphere process. The studies of particle stability upon storage and in the presence of blood plasma, as well as of the effect of particle lipid composition on the surface charge, transfection properties, and antibody-directed targeting of Genospheres have been performed with encouraging results, have been reported separately [1]. In conclusion, we hope that the described Genosphere methodology for the assembly of nano-sized, targetable nucleic acid delivery systems will help to overcome current barriers in bringing nucleic acid-based therapeutics into medical practice.

Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.bbamem.2006.03.020.

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