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Interaction of oligonucleotides with cationic lipids: the relationship between electrostatics, hydration and state of aggregation[1]

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Abstract

Lipoplexes, which are spontaneously formed complexes between oligonucleotide (ODN) and cationic lipid, can be used to deliver ODNs into cells, both in vitro and in vivo. The present study was aimed at characterizing the interactions associated with the formation of lipoplexes, specifically in terms of electrostatics, hydration and particle size. Large unilamellar vesicles (~100 nm diameter), composed of either DOTAP, DOTAP/cholesterol (mole ratio 1:1) or DOTAP/DOPE (mole ratio 1:1) were employed as a model of cationic liposomes. Neutral vesicles (~100 nm diameter), composed of DOPC/DOPE (mole ratio 1:1), were employed as control liposomes. After ODN addition to vesicles, at different mole ratios, changes in pH and electrical surface potential at the lipid–water interface were analyzed by using the fluorophore heptadecyl-7-hydroxycoumarin. In separate 'mirror image' experiments, liposomes were added at different mole ratios to fluorescein isothiocyanate-labeled ODNs, thus yielding data about changes in the pH near the ODN molecules induced by the complexation with the cationic lipid. Particle size distribution and turbidity fluctuations were analyzed by the use of photon correlation spectroscopy and static light-scattering, respectively. In additional fluorescent probe studies, TMADPH was used to quantify membrane defects while laurdan was used to measure the level of hydration at the water–lipid interface. The results indicate that mutual neutralization of cationic lipids by ODNs and vice versa is a spontaneous reaction and that this neutralization is the main driving force for lipoplex generation. When lipid neutralization is partial, induced membrane defects cause the lipoplexes to exhibit increased size instability.

Keywords: Cationic liposome; Oligonucleotide; 4-Heptadecyl-7-hydroxycoumarin; Fluorescence; Static light-scattering

Abbreviations: DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphatidylcholine; DOPE, 1,2-dioleoyl-*sn*-glycero-3-phosphatidylethanolamine; DOTAP, *N*-(1-(2,3-dioleoyloxy)propyl)-*N,N,N*-trimethylammonium chloride; FITC, fluorescein isothiocyanate; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid); HC, heptadecyl-7-hydroxycoumarin; laurdan, 6-dodecanoyl-2-dimethylaminonaphthalene; LUV, large unilamellar vesicle; MLV, multilamellar vesicle; ODN, oligonucleotide; PCS, photon correlation spectroscopy; S-ODN, phosphorothioate ODN; TMADPH, 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene; Zwittergent 3-14, *N*-tetradecyl-*N,N*-dimethyl-3-ammonio-1-propane sulfonate

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1. Introduction

Antisense oligonucleotides (ODNs) are now widely employed in elucidating genetic control as well as in therapeutic applications [1,2]. However, a major obstacle to the clinical application of ODNs is their inefficient cellular uptake [3]. One promising clinical delivery strategy involves the use of ODN–cationic lipid complexes, known as lipoplexes, in which the negatively charged nucleotides bind electrostatically to a cationic lipid such as DOTAP [4,5]. Such lipoplexes spontaneously form when cationic liposomes, frequently containing a neutral co-lipid (also referred to as a helper lipid) such as DOPE at 1:1 mole ratio, are mixed with ODNs. Although the ODN to cationic lipid mole ratio appears to be a critical parameter for determining lipoplex stability and delivery [6], the precise nature of the interactions of cationic liposomes with ODNs and the type of complexes formed are not fully characterized [7]. In fact, optimization of lipoplex delivery has so far been largely based on a trial and error approach.

In order to enhance our understanding in this field, the present study was aimed at investigating the electrostatics, hydration and particle size changes associated with the formation of ODN–cationic lipid complexes in dispersion and their relationship to defects in lipid packing. Our investigations focused on DOTAP, which is one of the most commonly used cationic lipids, in the presence or absence of helper lipid (either DOPE or cholesterol). Neutral liposomes (DOPC/DOPE 1:1 mole ratio) were employed as control vesicles. We used S-ODNs throughout since these are preferable to phosphodiesters due to their greater chemical and biological stability [1,8].

2. Materials and methods

2.1. Materials

DOTAP, DOPE and DOPC were purchased from Avanti Polar Lipids (Albaster, AL, USA). Both HC and TMADPH were purchased from Molecular Probes (Eugene, OR, USA) while laurdan was purchased from Lambda (Graz, Austria). Hepes sodium salt and cholesterol were obtained from Sigma Chemical Co. (St Louis, MO, USA). Acetic acid was obtained from Frutarom (Haifa, Israel). Sodium acetate trihydrate was obtained from BDH Chemicals (Poole, UK). 5′-FITC was purchased from Glen Research Inc. (Sterling, VA, USA). All these chemicals were of analytical grade or better. All solutions were prepared in water that had been deionized, double-distilled, and filtered in a WaterPro PS system (Labconco Corp., Kansas City, MO, USA).

2.2. ODN and plasmid DNA preparation

The S-ODNs, namely, AM-TIS [S-d-5′(GGG AAG GAT GGC GCA CGC TG)], Bcl2-TIAS [S-d-5′(CAG CGT GCG CCA TCC TTC CC)], AM-TIS-FITC [FITC-S-d-5′(GGG AAG GAT GGC GCA CGC TG)] and Bcl2-TIAS-FITC [FITC-S-d-5′(CAG CGT GCG CCA TCC TTC CC)], were synthesized by using the phosphoramidite method on an ABI 2-column synthesizer (model #392), as previously described [8]. The sulfurization agent was [^{3}H]1,2-benzodithiol-3-one [9]. The FITC-linked ODNs were prepared using the 5′-FITC reagent. All ODNs were purified by precipitation twice from ethanol. Purified ODN was then dissolved in either a small volume of 20 mM Hepes buffer (pH 7.4) or 0.2 M acetate buffer (pH 5.7). If any precipitate was present, it was removed by centrifugation and isolation of the ODN-containing supernatant. The final ODN concentration was determined by conducting an organic phosphate assay [10,11].

An *Escherichia coli* containing the plasmid S16 hGH was kindly given by Dr. O. Meyuhas of our department [12]. By employing the techniques previously reported [10], the plasmid DNA was isolated and its purity was validated by agarose gel electrophoresis. Purified DNA was then dissolved in a small volume of 20 mM Hepes buffer (pH 7.4) and its concentration was quantified by organic phosphate assay [10,11].

2.3. Liposome preparation

LUVs, composed of either DOTAP, DOTAP/DOPE, DOTAP/cholesterol or DOPC/DOPE, were prepared in the manner previously described [13]. The appropriate fluorescent probe, dissolved in or-

ganic solvent, was added to the lipid mixture prior to vesicle preparation. For experiments requiring HC-labeled vesicles, HC was added in tertiary butanol such that the mole ratio of total lipid to probe was 400 to 1. For TMADPH-labeling, the probe was dissolved in tetrahydrofuran/ethanol (1:1 v/v) and added at a mole ratio of total lipid to probe of 200 to 1. Laurdan-labeled vesicles were prepared by adding laurdan in *N*,*N*-dimethylformamide at a ratio of 1000 moles of total lipid per mole of probe. In all cases, the lipid mixture was vortexed in tertiary butanol and subsequently freeze-dried overnight under vacuum. The lyophilized cake thus formed was hydrated with either 20 mM Hepes buffer (pH 7.4) or 0.2 M acetate buffer (pH 5.7), and vortexed for several minutes to produce a dispersion exhibiting a total lipid concentration of 31 mM. The resultant MLVs were converted to LUVs and downsized by use of a 'Liposofast' extrusion system (Avestin, Ottawa, Canada) [14]. This involved extruding the dispersion an odd number of times (usually 17) through 0.4-μm pore-size and 0.1-μm pore-size filters (Poretics, Livermore, CA, USA) successively. The particle size distribution of each liposome dispersion was determined at 25°C by photon correlation spectroscopy (PCS) with a Coulter model N4 SD (Coulter Electronics, Hialeah, FL, USA) [10].

2.4. Fluorescence characterization of the FITC-labeled ODN

The aim of this study was to characterize the electrostatics of the ODN before, and as result of, ODN–cationic liposome interaction by measuring the effect of bulk pH on FITC fluorescence intensity. Initially, it was necessary to select optimal spectroscopic parameters and cuvette volumes. To this end, a 5-μl aliquot (12.63 nmol) of FITC-labeled AM-TIS was diluted in 3 ml of 20 mM Hepes buffer (pH 7.4) and fluorescence characterization was performed with stirring, at ambient temperature on an LS50B luminescence spectrometer (Perkin Elmer, Norwalk, CT, USA). The fluorescence of the FITC-labeled ODN was measured by scanning the excitation wavelength between 400 and 510 nm at an emission wavelength of 521 nm (bandwidth 5 nm). The excitation peak was identified at 493 nm and with the excitation wavelength subsequently set at this value, an emission scan was run over the 450 to 550 nm range (bandwidth 5 nm). By reducing the volume of FITC-labeled ODN in the cuvette to 3.5 μl (representing 2.95 μM), and enlarging the emission bandwidth to 10 nm, it was possible to obtain a fluorescence intensity signal of suitable magnitude.

In order to study the effect of pH on the fluorescence intensity of FITC-labeled ODN, 3.5-μl aliquots, each containing 8.84 nmol of FITC-labeled AM-TIS, were diluted in test tubes with 3 ml of 20 mM Hepes buffer (pH 7.4). The pH of each medium was adjusted to a desired value in the pH 4–11 range by the addition of an appropriate amount of concentrated hydrochloric acid or sodium hydroxide. To abolish any pH gradient, the samples were sonicated for about 5 s in a water bath (Elma transonic 450/H). The fluorescence intensity of each sample solution was then measured with the excitation wavelength fixed at 493 nm and the emission wavelength set at 515 nm (excitation bandwidth at 5 nm; emission bandwidth at 10 nm).

2.5. Fluorescence and static light-scattering (turbidity) experiments with HC-labeled vesicles

An aliquot of HC-labeled liposome dispersion, pre-prepared in 20 mM Hepes buffer (pH 7.4), was diluted in a cuvette with 3 ml of the same buffer, to a final DOTAP concentration of 4×10^{-5} M. An appropriate volume of AM-TIS (or Bcl2-TIAS in the case of DOTAP/cholesterol vesicles) was added such that the ODN/cationic lipid mole ratio reached either 0, 0.1, 0.2, 0.5, 1, 1.5 or 2. Both the fluorescence and turbidity of each ODN–lipid sample were recorded just before the addition of ODN as well as 17 min after ODN addition. Fluorescence of HC was measured at excitation wavelengths of 330 nm and 380 nm using a constant emission wavelength of 450 nm (excitation and emission bandwidths at 5 nm) and employing an emission filter of 430 nm [13]. Turbidity was measured concurrently on the same instrument as static light-scattering at 90°, with both the excitation and emission wavelength set at 600 nm. All the above measurements were performed using magnetic stirring at ambient temperature. Each individual experiment was performed at least twice and generally in triplicate, employing liposomes from different batches.

2.6. Fluorescence and static light-scattering (turbidity) experiments with FITC-labeled ODN

For these 'mirror image' experiments, a 5.5-μl aliquot containing 8.85 nmol of Bcl2-TIAS-FITC, pre-prepared in 0.2 M acetate buffer (pH 5.7), was diluted with a further 3 ml of the same buffer. An appropriate volume of liposome dispersion, pre-prepared in the same acetate buffer, was then added in order to adjust the ODN/cationic lipid mole ratio to either 0.1, 0.2, 0.5, 1, 2, 5 or infinity. Both the fluorescence and turbidity of each ODN–lipid sample were recorded just before liposome addition as well as 17 min after liposome addition. Using the spectrofluorometer described above, the fluorescence emitted by FITC was measured at 515 nm with the excitation wavelength at 493 nm. Simultaneous static light-scattering measurements were undertaken with the excitation and emission wavelength at 600 nm (excitation bandwidth at 5 nm; emission bandwidth at 10 nm). No emission filter was required. The static light-scattering values produced by simple liposome-buffer solutions (no ODN present) of varying mole ratios were also measured. Hence, the static light-scattering due to lipoplex formation could be quantified by subtracting the liposome-buffer values from the corresponding ODN–liposome-buffer values. All these measurements were conducted under stirring conditions at ambient temperature. Each individual experiment was performed at least twice and generally in triplicate, employing liposomes from different batches.

2.7. Fluorescence experiments with laurdan-labeled and TMADPH-labeled vesicles

For studies involving both laurdan-labeled and TMADPH-labeled vesicles, an aliquot of the selected liposome dispersion, pre-prepared in 20 mM Hepes buffer (pH 7.4), was diluted in a cuvette with 1 ml of the same buffer to the desired concentration. The cationic lipid concentrations in the liposome dispersions were 4.65×10^{-5} M and 4.65×10^{-6} M at $ODN^-/L^+<1$ and $ODN^-/L^+>1$, respectively. An appropriate volume of AM-TIS (at a nucleotide concentration of 0.9 mM), as well as plasmid DNA in the case of the laurdan studies, was added such that the ODN/cationic lipid mole ratio reached a specific value within the 0–2 range. Fluorescence measurements were performed using a 1-cm light path cell, using the spectrofluorometer described above.

For studies with TMADPH-labeled liposomes, Fluorescence was measured using an excitation wavelength of 360 nm and an emission wavelength of 430 nm. For experiments conducted with $ODN^-/L^+<1$, both the excitation and emission bandwidths were set at 5 nm while for experiments conducted with $ODN^-/L^+>1$, both the excitation and emission bandwidths were set at 10 nm.

For studies involving laurdan-labeled liposomes, fluorescence was measured at excitation wavelengths of 340 nm and 410 nm using emission wavelengths of 440 nm and 490 nm, respectively. For experiments conducted with $ODN^-/L^+<1$, both the excitation and emission bandwidths were set at 10 nm while for experiments conducted with $ODN^-/L^+>1$, both the excitation and emission bandwidths were set at 15 nm. Information about hydration changes at the water–lipid interface was determined by calculating the excitation generalized polarization (GP_{340}) value for each ODN^-/L^+ mole ratio investigated. The GP_{340} was calculated as follows:

$$GP_{340} = (I_{440} - I_{490})/(I_{440} + I_{490})$$

where I_{440} and I_{490} are the intensities of emission at wavelengths of 440 nm and of 490 nm at the excitation wavelengths of 340 nm or 410 nm, respectively [15]. Each value of GP_{340} was transformed into a ΔGP_{340} value according to the equation:

$$\Delta GP_{340} =$$

$$GP_{340}(\text{at } [ODN^-]<1) - GP_{340}(\text{at } [ODN^-] = 0)$$

For both types of probes, the fluorescence of each ODN–lipid sample was recorded just before the addition of ODN as well as 5 min after ODN addition. The measurements were always performed with the temperature maintained at 25°C using a water-circulating bath. All data were corrected for light-scattering as described by Borenstain and Barenholz [16].

3. Results

3.1. Addition of ODN to HC-labeled liposomes

PCS showed that all the liposomes, prepared from

either DOTAP, DOTAP/DOPE (1:1), DOTAP/cholesterol (1:1) or DOPC/DOPE (1:1), exhibited a unimodal size distribution with a similar mean diameter (~100 nm). Previous research employing cryo-transmission electron microscopy has demonstrated that virtually all these vesicles are unilamellar [17].

Appropriate amounts of AM-TIS (or Bcl2-TIAS in the case of the DOTAP/cholesterol system) were added to HC-loaded cationic LUVs. At both time 0 and 17 min after ODN addition, the fraction of ionized HC to total HC within the lipid bilayer was determined from the ratio of fluorescence intensity at the excitation wavelength of 380 nm to the fluorescence intensity at the excitation wavelength of 330 nm (the pH-independent isosbestic point). Fig. 1 presents a plot of the fluorescence ratio of HC-containing vesicles as a function of ODN to cationic lipid mole ratio. The initial fluorescence ratio value for DOTAP vesicles was almost twice that for DOTAP/DOPE vesicles and this reflects the greater charge density and hence greater initial HC dissociation in the 100% DOTAP system. The DOTAP/cholesterol vesicles exhibited an intermediate initial fluorescence intensity ratio. The addition of ODN to all three types of cationic vesicle systems caused a decrease in the dissociation degree of HC in the lipid assemblies, thus indicating that S-ODN neutralized the cationic lipid. In all three systems, the extent of neutralization is a function of ODN to cationic lipid ratio. For 100% DOTAP, DOTAP/cholesterol and

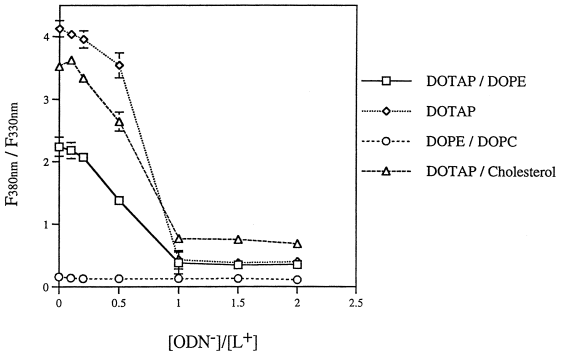

Fig. 1. Change in the ratio of the excitation fluorescence intensities at 380 and 330 nm of HC in the membranes upon addition of S-ODN to cationic (or neutral control) liposomes as a function of the mole ratio of ODN to cationic lipid ($n=3$). The S-ODN was AM-TIS except in the DOTAP/cholesterol experiments, where Bcl2-TIAS was employed.

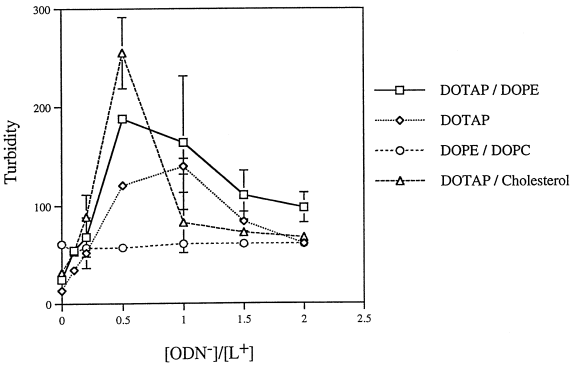

Fig. 2. Static light-scattering at 600 nm of S-ODN/lipid complexes upon addition of ODN to cationic (or neutral control) liposomes as a function of the mole ratio of ODN to cationic lipid ($n=3$). The S-ODN was AM-TIS except in the DOTAP/cholesterol experiments, where Bcl2-TIAS was employed.

DOTAP/DOPE liposomes, the HC dissociation curve against ODN to cationic lipid ratio resembles an inverted sigmoid with saturation occurring at ODN/cationic lipid mole ratio of 1. The degree of sigmoidicity was in the order: 100%DOTAP > DOTAP/cholesterol > DOTAP/DOPE. The addition of ODN to 100% DOTAP and DOTAP/DOPE at a mole ratio = 1 induced close to, but less than, 100% protonation of assembly-incorporated HC. Across the mole ratio range, the extent of assembly-incorporated HC protonation was also in the same order in the DOTAP/cholesterol system except at $[ODN^-]/[L^+] > 1$, for which the HC 380 nm/330 nm fluorescence ratio was somewhat higher than the 100% DOTAP and DOTAP/DOPE systems. This suggests that the maximal level of DOTAP neutralization in DOTAP/cholesterol is lower than for the other two cationic vesicle systems and the lipoplex will be somewhat more positively charged. In contrast to cationic vesicles, neutral DOPE/DOPC vesicles showed a lack of change in their 380/330 fluorescence intensity ratio, suggesting no interaction between the ODN and the lipids.

Fig. 2 shows the static light-scattering data obtained 17 min after ODN addition. For 100% DOTAP and DOTAP/DOPE liposomes, the maximal light-scattering was observed when the mole ratio of ODN/cationic lipid was between 0.5 and 1. The mean maximal values were greater for the DOTAP/DOPE system than the 100% DOTAP system. The large error in measured light-scattering observed for DOTAP/DOPE vesicles when the ODN/cationic lipid

ratio was 1 suggests that the complexes formed under these conditions were unstable. The DOTAP/cholesterol system behaved differently in that light-scattering peaked at an ODN/cationic lipid mole ratio of 0.5 and was diminished dramatically at an ODN/cationic lipid ratio of 1. No change in static light-scattering occurred upon mixing of DOPC/DOPE LUVs with ODN. This supports the HC data described above indicating that without the presence of cationic lipids in the vesicle, there was no interaction between LUVs and ODN.

3.2. Titration curve for FITC-labeled ODN

Fig. 3 presents the pH titration curve for AM-TIS-FITC. By applying a sigmoid curve regression analysis, the titration curve was determined to deviate little from an ideal sigmoid ($R = 0.9862$). The fitted equation also showed that the pK_a of the probe, attached to ODN, was 7.34. Importantly, the curve can be employed to relate fluorescence intensity of FITC-labeled ODN to the pH in the immediate vicinity of the molecule. As the pK_a is very close to the pH (7.4) used in the lipid neutralization by ODN, we decided to perform the mirror image experiments at a lower pH of 5.7 so complexation-induced changes in the local pH of the ODN-attached FITC could be better followed. At pH 5.7, FITC fluorescence intensity is low, so relatively small increases in the pH sensed by the probe would induce relatively dramatic increases in probe fluorescence intensity.

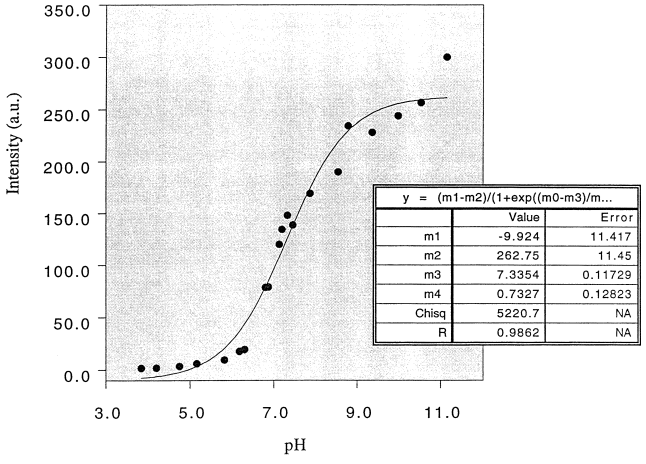

Fig. 3. Fluorescence intensity of AM-TIS-FITC as a function of pH. The excitation wavelength was 493 nm and emission wavelength was 515 nm.

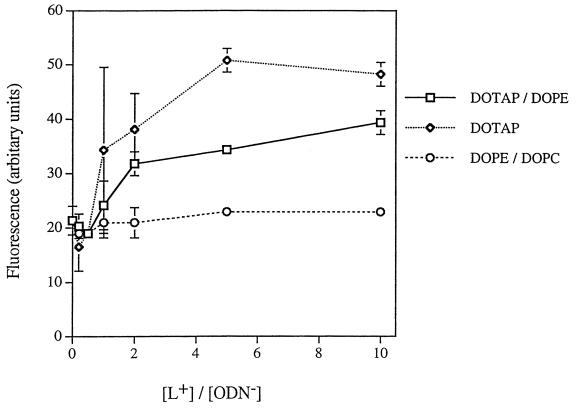

Fig. 4. Change in the ratio of the excitation fluorescence intensities at 380 and 330 nm of HC in the membranes upon addition of cationic (or neutral control) liposomes to Bcl2-TIAS-FITC as a function of the mole ratio of cationic lipid to ODN ($n = 3$).

3.3. The addition of liposomes to FITC-labeled ODN

In 'mirror image' experiments, appropriate amounts of cationic LUVs were added to Bcl2-TIAS-FITC in acidic buffer (pH 5.7). At both time 0 and 17 min after liposome addition, the emitted fluorescence was determined with the excitation wavelength set at 493 nm and the emission wavelength at 515 nm. The derived data are presented in Fig. 4. Note that fluorescence here is plotted as a function of cationic lipid/ODN mole ratio, which is the reciprocal of the ratio used in the previous experiments. In interpreting Fig. 4, fluorescence intensity values can be related to pH values using Fig. 3. Regarding DOTAP, at an excess of ODN, with the mole ratio of ODN to cationic lipid above 2, the pH in the immediate vicinity of the labeled ODN remained largely unchanged. The addition of more lipid such that the ODN to cationic lipid ratio was between 0.5 and 2 produced a progressive elevation in local probe pH so that at [ODN]/[cationic lipid] = 0.5, the ODN observed an upward shift of approximately 0.45 pH units (pH 5.7 to pH 6.15). The addition of more DOTAP liposomes, such that [ODN]/[cationic lipid] = 0.2, elevated the local probe pH by a further 0.25 pH units (pH 6.4). The addition of DOTAP/DOPE liposomes to the ODN produced a similar pattern of pH shifts though their magnitude was smaller. For instance, at [ODN]/[cationic lipid] = 0.2, the ODN observed a pH of 6.12 compared to a pH of 6.4 for DOTAP alone. In contrast, the

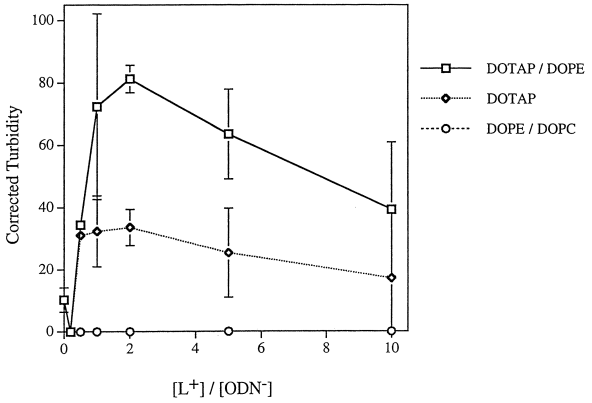

Fig. 5. Static light-scattering at 600 nm of S-ODN/lipid complexes upon addition of cationic (or neutral control) liposomes to Bcl2-TIAS-FITC as a function of the mole ratio of cationic lipid to ODN ($n=3$).

addition of neutral DOPC/DOPE liposomes to the ODN did not affect the local probe pH, irrespective of liposome concentration.

Fig. 5 shows the static light-scattering data obtained 17 min after liposome addition. For both the DOTAP and DOTAP/DOPE systems, the maximal light-scattering was observed when the mole ratio of $L^+/ODN^- = 2$ ($ODN^-/L^+ = 0.5$). At either side of this value, when the mole ratios of L^+/ODN^- were 10, 5 or 1, large standard deviations in light-scattering were observed. This indicates that the complexes formed at these mole ratios were probably unstable. When the mole ratio of L^+/ODN^- ranged between 0.5 and 10, the lipoplexes formed from DOTAP/DOPE exhibited greater light-scattering than the 100% DOTAP vesicles. In contrast, the static light-scattering produced by neutral DOPC/DOPE liposomes was unaffected by ODN, suggesting a lack of interactions between ODN and liposomes.

3.4. Addition of ODN to TMADPH-labeled liposomes

Appropriate amounts of AM-TIS were added to TMADPH-labeled LUVs and fluorescence measurements were undertaken at both time 0 and 5 min after ODN addition. The derived data were transformed into values of relative fluorescence intensity (F/F_0) which represent the ratio of fluorescence 5 min after S-ODN addition to fluorescence at time zero. Fig. 6A shows the changes in relative fluorescence intensity as a function of the ODN to cationic lipid mole ratio over the 0 to 0.4 range. The data relate to experiments conducted with a cationic lipid concentration of 4.65×10^{-5} M. Fig. 6B shows the changes in relative fluorescence intensity as a function of ODN to cationic lipid mole ratio over the 0 to 2 range. The data here relate to experiments conducted with a cationic lipid concentration of 4.65×10^{-6}. While at an ODN^-/L^+ mole ratio ≤ 0.4, ODN induced TMADPH quenching in DOTAP/DOPE vesicles, at an ODN^-/L^+ mole ratio ≥ 1, the fluorescence intensity of TMADPH, in the same vesicles, was enhanced. Probe quenching at an ODN^-/L^+ mole ratio ≤ 0.4 was also observed in experiments with 100% DOTAP vesicles. We have previously shown that changes in TMADPH fluorescence are related to exposure of the fluorophore to water [17]. The fluorescence quenching at low ODN^-/L^+ mole ratio values indicates an increase in the fluorophore exposure to water, while the enhanced fluorescence occurring at higher ODN^-/L^+ mole ratio values indicates a reduction in the exposure to water

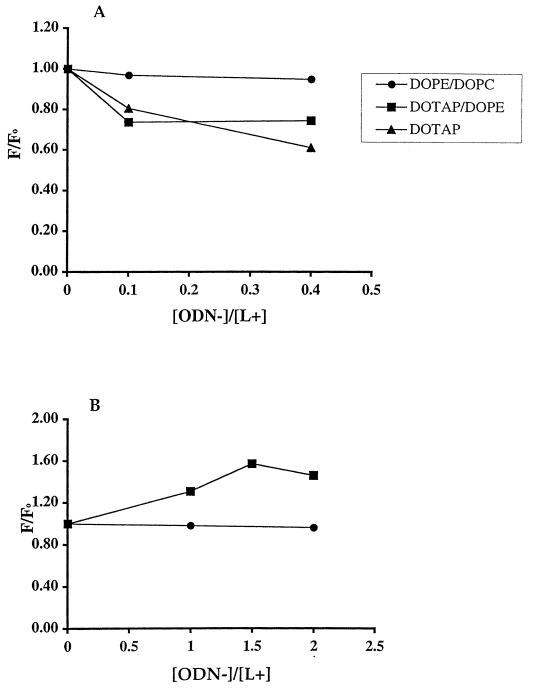

Fig. 6. Effect of AM-TIS on relative fluorescence intensity (F/F_0) of cationic (or neutral control) liposomes containing the fluorescent probe TMADPH. (A) Initial cationic lipid concentration of 4.65×10^{-5} M. (B) Initial cationic lipid concentration of 4.65×10^{-6} M.

molecules [17]. In contrast to cationic vesicles, when TMADPH-labeled DOPC/DOPE vesicles were tested, the relative fluorescence intensity remained constant throughout the entire ODN^-/L^+ mole ratio range. This indicates that S-ODN induces fluctuations in hydration levels near the water–lipid interface only when cationic lipid is present in the liposomes.

3.5. Addition of polynucleotides to laurdan-labeled liposomes

Appropriate amounts of AM-TIS or plasmid DNA were added to laurdan-labeled liposomes and fluorescence measurements were undertaken at both time 0 and 5 min after polynucleotide addition. The results showed that before ODN addition, the mean GP_{340} values for DOTAP/DOPE liposomes were -0.07 and -0.08 at cationic lipid concentrations of 4.65×10^{-5} M and 4.65×10^{-6} M, respectively. The corresponding values for DOPE/DOPC liposomes were 0.028 and 0.03. Thus, the cationic liposomes were more hydrated than the neutral liposomes [18]. Fig. 7 presents a plot of ΔGP_{340} as a function of ODN^-/L^+ mole ratio for the three systems investigated. Positive values of ΔGP_{340} indicate that interaction with S-ODN reduced hydration at the water–lipid interface [15]. It can be seen that as S-ODN was added to DOTAP/DOPE LUVs, the water–lipid interface became progressively less hydrated. When plasmid DNA was added to the same vesicles the extent of dehydration was smaller than for equivalent mole phosphate of ODN. In contrast, when S-ODN was added to neutral DOPC/DOPE vesicles, the extent of dehydration was negligible, thus suggesting that the presence of cationic lipid is required for an interaction to take place.

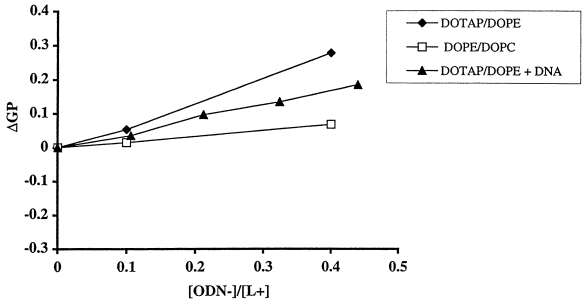

Fig. 7. Effect of AM-TIS or plasmid DNA on ΔGP_{340} of laurdan-labeled DOTAP/DOPE (or neutral control) liposomes.

4. Discussion

4.1. Characteristics of HC, TMADPH and laurdan in cationic liposomes

When incorporated into liposomes, the fluorescent probe HC locates in the lipid bilayer, with its long-chain tail parallel to the lipid acyl chains and its pH and electrical surface potential-sensitive fluorophore present at the water–lipid interface (Fig. 3 in [22]). Crucially, the probe practically does not dissolve in aqueous solution in a monomeric form while HC aggregates in the aqueous phase produce fully-quenched assemblies which do not contribute to fluorescence intensity. HC also exhibits the key property that its fluorescence lifetime is unperturbed by temperature or the physical state of the lipids and that its fluorescence is evenly distributed in the membrane plane, irrespective of lateral phase separation [16,19]. Consequently, upon addition of ODN to liposomes, variations in the extent of HC dissociation in the lipid will mainly reflect changes occurring at the water–lipid interface, which is the plane of interaction between the negatively-charged ODN and the cationic lipid assemblies.The fluorescence properties of TMADPH are notably sensitive to the polarity of the environment, the degree of its exposure to water as well as solvent relaxation effects [17,20]. When TMADPH is incorporated into liposomes, its positively charged quaternary amine residues reside close to the water–lipid interface, while the fluorophore is aligned parallel to the upper part of the acyl chain region (Fig. 3 in [22]). Consequently, TMADPH senses variations in the organization of the lipid assembly in the region that is close to the water–lipid interface. We have previously shown that any agent that affects the level of hydration at the location of the fluorophore, will induce changes in the total fluorescence intensity and therefore will affect the value of F/F_0 [17]. Lowering the exposure of the fluorophore to water increases intensity, while increasing the exposure, decreases intensity. Thus, upon addition of ODN to liposomes, fluctuations in TMADPH fluorescence intensity will mainly re-

flect changes in hydration levels occurring in the acyl chain region.

Laurdan, a naphthalene-based amphiphilic molecule, exhibits fluorescence properties that are highly sensitive to the polarity and to the dipolar dynamics of its environment. When incorporated into liposomes, laurdan resides at the water–lipid interface of the bilayer [21], with the lauric acid moiety aligned parallel to the acyl chain region and the fluorescent naphthalene residue located at the level of the glycerol backbone [22]. When it is within a lipid membrane, laurdan exhibits a 50-nm red shift of the emission spectrum as the membrane changes from a gel into a liquid crystalline state [15]. The magnitude of the spectral shift is related to the number of water molecules around the fluorescent moiety of laurdan. The spectroscopic parameter, GP_{340}, can be used to measure the red shift, thus permitting quantification of the hydration level at the water–lipid interface where the laurdan fluorophore is anchored.

4.2. Electrostatics of lipoplex formation

The electrostatics of lipoplex formation was followed through changes in surface potential and surface pH occurring in both the lipid (HC-labeled) and the ODN (FITC-labeled) upon complexation. Our data concerning the addition of ODN to cationic liposomes (see Fig. 1) showed that the curves describing HC dissociation were sigmoid. At an ODN^-/L^+ mole ratio of 1, the 100% DOTAP and DOTAP/DOPE systems underwent approximately 90% neutralization of cationic lipid, while the DOTAP/cholesterol system underwent slightly less neutralization of cationic lipid. Neutralization was quantified by referring to our previously obtained data relating both HC dissociation degree and fluorescence ratio to bulk pH ([13] and unpublished data). However, the validity of comparing the DOTAP/cholesterol systems to the other two is, as yet, uncertain, as a different S-ODN was employed in the DOTAP/cholesterol complexation experiments and our preliminary data suggest some dependence on ODN sequence and/or composition.

When cationic liposomes were added to ODN, a sigmoid relationship was again obtained for the pH observed by the fluorescent ODN (see Fig. 4). However, the maximal increase in pH of ODN-attached FITC occurred at a lower ODN^-/L^+ mole ratio with the actual mole ratio values somewhat different for 100% DOTAP and DOTAP/DOPE 1:1 ($ODN^-/L^+ = 0.2$ and $ODN^-/L^+ = 0.1$, respectively). Also, throughout the entire range of titration of the ODN^- by the L^+, the pH increase near the FITC-labeled ODN was higher for the 100% DOTAP system than for the DOTAP/DOPE 1:1 system. This titration of ODN by the cationic lipids can be explained either as a measure of the distribution of ODN between solutions and lipoplexes and/or changes in the structure of ODN in the lipoplexes. The contribution of each of these factors is now under investigation. However, for both explanations, the difference between DOTAP and DOTAP/DOPE (1:1) lipoplexes is expected, bearing in mind our previous finding that 100% DOTAP liposomes are more positively charged than DOTAP/DOPE (1:1) [13].

The average pH shift induced by the interaction of ODN^- with L^+ is smaller for the FITC associated with ODN than for HC present at the water–lipid interface. This suggests that either less of the FITC than the HC moieties are affected by the interaction and/or by the fact that the activity of water and the dielectric constant near the FITC groups is higher than for the HC.

The fact that neutral unilamellar vesicles (DOPC/DOPE 1:1) were not affected by ODN and that FITC-labeled ODN did not interact with these neutral liposomes supports the claim that charge neutralization, associated with a lowering of the electrostatic free energy of the system, drives lipoplex formation. The sigmoid relationships obtained can be predicted and calculated from theory by the Gouy–Chapman approximation [13,23]. Importantly, when an excess of ODN was added, the dissociation degree of HC in cationic lipid assemblies never reached the value of zero. This may indicate that not all the positive charges of the cationic lipids are reached and/or fully neutralized by the negatively charged phosphate groups of the ODN. An alternative explanation is that HC was located so close to the cationic lipid that it sensed more the positive charge of the cationic lipid than the negative charge of ODN. A similar phenomenon has been identified for the zwitterionic detergent Zwittergent 3-14 [24]. Similar trends have been previously found to occur for DNA–cationic lipid complex formation [25]. It is noteworthy that

the cooperative nature of the interaction of ODN with L^+ resembled the interaction previously identified for DNA^-/L^+ interaction [25].

4.3. Particle size changes associated with lipoplex formation

The specific turbidity (static light-scattering) reflects the sum of all the changes in number of particles, size of particles and variations in the refractive index. In both the ODN-addition studies and in the mirror-image liposome-addition studies, turbidity was maximal when the ODN to cationic lipid mole ratio ranged between 0.5 and 1 or when the ratio was exactly 0.5 in the case of Bcl2-TIAS addition to DOTAP/cholesterol vesicles (Figs. 2 and 5). In contrast, neutral unilamellar vesicles composed of DOPC and DOPE (at 1:1 mole ratio) showed a lack of change in particle size when mixed with ODNs. This supports the claim that the presence of cationic lipid induces complexation via the process of electrostatic neutralization [26]. The change in specific turbidity can therefore be used as one indication of lipoplex formation. The variations in specific turbidity obtained in our study confirm the results described in many other studies relating neutralization and size changes, such as Jaaskelainen et al. [27]. Previous work [28] established that turbidity fluctuations are due to the fact that when there is insufficient ODN to interact with all the cationic lipid, lateral phase separation leads to membrane defects at the upper part of the bilayer acyl chains. These defects permit water penetration through the membrane into this region of the lipid bilayer. In order to overcome these defects, thermodynamic factors cause aggregation and probably fusion of the lipoplexes to form larger particles. In contrast, when the mole ratio of ODN to cationic lipid was greater than 1, there were less defects or no defects and the turbidity increase was smaller. This is due to the fact that, at this mole ratio, all cationic lipids interact with the nucleotide homogeneously. Thus, there is no phase separation between ordered condensed domains containing ODN and less condensed domains lacking ODN. Such a mechanism has been shown to develop also when cationic lipids interact with linear and plasmid DNA [17]. These processes fit well with the model of Dan [29], which suggests that at low DNA/cationic lipid mole ratio, ordered domains in less ordered bilayers are formed.

4.4. Hydration fluctuations associated with lipoplex formation

Membrane defects in the upper part of the acyl chains region were followed, during lipoplex formation, by using TMADPH. For lipoplexes forming at $[ODN^-]/[L^+] \leq 0.4$, there was a decrease in TMADPH fluorescence intensity, brought about by the process of solvent relaxation. This indicates that at this mole ratio range, the cationic lipid surface interacts heterogeneously with S-ODN, leading to defect formation at the contact between condensed lipid domains and the uncondensed continuum. Therefore, there is greater exposure of the hydrophobic regions to water. In contrast, lipoplexes forming at $[ODN^-]/[L^+] \geq 1$ showed increased TMADPH fluorescence intensity. This indicates that, at this mole ratio range, there is a reduction in the exposure of the hydrophobic regions to water as the cationic lipid surface is interacting homogeneously with S-ODN. These findings confirm our previous results, derived from studying DNA–cationic lipid interactions [17].

Laurdan was employed in order to follow hydration fluctuations at the water–lipid interface which occur during complexation over the mole ratio range $0 \leq ODN^-/L \leq 0.4$. The results showed that hydration was lowered as the amount of S-ODN increased over the mole ratio range. Furthermore, the magnitude of dehydration was proportional to the ODN^-/L^+ mole ratio. This supports the hypothesis that water is excluded from the lipid head group region as heterogeneous condensation of cationic lipids by S-ODN takes place.

5. Conclusions

The data presented indicate that mutual neutralization of cationic lipids by ODN and vice versa is a spontaneous reaction and that this neutralization is the main driving force for lipoplex generation. When lipid neutralization is partial, induced membrane defects cause the lipoplexes to exhibit increased size instability. The degree of size instability is dependent

on the ODN/cationic lipid mole ratio and it reaches a maximum at a ratio of 0.5. We have now initiated studies to determine how the electrostatics and macrostructure of ODN–cationic lipid complexes affect antisense efficiency in vitro and in vivo.

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