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Oligonucleotide-cationic liposome interactions. A physicochemical study

Ilpo Jääskeläinen *, Jukka Mönkkönen, Arto Urtti

Department of Pharmaceutical Technology, University of Kuopio and AIV-institute, P.O. Box 1627, FIN-70211 Kuopio, Finland

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Abstract

Cationic liposomes are effective in delivering antisense oligonucleotides into cells in culture, but their interactions with the oligonucleotides are poorly understood. We studied the aggregation and fusion reactions during the formation of cationic lipid/oligonucleotide complexes in solution and their interactions with lipid bilayers. Phosphorothioate oligonucleotides (15-mer) were complexed with cationic liposomes composed of dimethyldioctadecylammonium bromide (DDAB) and dioleoylphosphatidylethanolamine (DOPE) at 8:15 molar ratio or of a commercial formulation DOTAP (*N*-(1-(2,3-dioleoyloxy)propyl)-*N*,*N*,*N*-trimethylammoniummethylsulfate), at different ratios with apparent −/+ charge ratios of 0.03–5.6. Mean size of the complexes increased with −/+ ratio so that at charge ratios 0.4–2.0 the size increased by at least an order magnitude due to the oligonucleotide induced aggregation. Resonance energy transfer experiments showed that in addition to aggregation oligonucleotides induced fusion of cationic liposomes, but the fusion was rate-controlled by the initial aggregation step. Rate constants for oligonucleotide induced aggregation were dependent on lipid concentration and were in the range of $(0.2–1)\cdot 10^7$ M^{-1} s^{-1} and $(1–10)\cdot 10^7$ M^{-1} s^{-1} for DDAB/DOPE and DOTAP, respectively. Increase in oligonucleotide concentration induced the aggregation and fusion until at high −/+ ratios electrostatic repulsion of negative surfaces inhibited further aggregation and fusion. DOTAP/oligonucleotide complexes did not induce leakage of calcein from neutral EPC liposomes, but did cause leakage at −/+ charge ratios of < 0.7 and > 2.0 from EPC/DOPE liposomes. Also at −/+ charge ratios below 0.8 DOTAP/oligonucleotide complexes induced leaking from negatively charged DPPC/DPPG liposomes. These results indicate that either phosphatidylethanolamine or negative charge are required in the cell membrane for fusion of cationic liposome-oligonucleotide complexes. The ratio of oligonucleotide to cationic lipid is critical in determining the physicochemical properties of the mixture.

Keywords: Cationic lipid; Oligonucleotide; Particle size; Calcein leakage; Fusion; Aggregation

1. Introduction

Antisense oligonucleotides can be used to inhibit the function of numerous proteins (e.g., enzymes) by inhibiting their biosynthesis. Complimentary oligonucleotides can be designed to bind to mRNA or pre-mRNA by base pairing and thus prevent the expression of the protein.

Cationic lipids, often with dioleoylphosphatidylethanolamine (DOPE) as an additional lipid component, form positively charged liposomes, which can bind negatively charged molecules (e.g., DNA, RNA, oligonucleotides) on the surface. Main mechanism of the cellular delivery is thought to be the fusion of cationic lipids with negatively charged cell membranes thus enabling enhanced cytoplasmic delivery [1]. In addition, endocytosis has been found to contribute in the entry of cationic liposomes into some cells [2]. Cationic liposomes could possibly be used to deliver antisense oligonucleotides into cells [3], like they have been used succesfully to deliver DNA and RNA [4,5]. Cationic lipids are theoretically an efficient system for cellular oligonucleotide delivery [3], and a few reports show

Abbreviations: DDAB, dimethyldioctadecylammonium bromide; DOPE, dioleoylphosphatidylethanolamine; PE, phosphatidylethanolamine; DOTAP, (*N*-(1-(2,3-dioleoyloxy)propyl)-*N*,*N*,*N*-trimethylammoniummethylsulfate); PC, phosphatidylcholine; EPC, egg phosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; DPPG, dipalmitoylphosphatidylglycerol; DOTMA, *N*-(1-(2,3-dioleoyloxy)propyl)-*N*,*N*,*N*-trimethylammonium chloride; QELS, quasi-elastic light scattering; RET, resonance energy transfer; *N*-Rh-PE, *N*-(lissamine rhodamine B sulfonyl)PE; *N*-NBD-PE, *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)DOPE.

* Corresponding author. Fax: +358 71 162456.

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that cationic lipids enhance the delivery and/or effect of antisense oligonucleotides [6–9]. Most of these experiments have been done with Lipofectin™ composed of *N*-(1-(2,3-dioleyloxy)propyl)-*N*,*N*,*N*-trimethylammonium chloride (DOTMA) and DOPE at 1:1 ratio (w/w).

As cationic liposomes are complexed with oligonucleotides the positive charges may be partly covered or reversed. The surface charge of the liposome-oligonucleotide complex should in theory be dependent on the concentrations of the lipid and oligonucleotide. Despite the potential usefulness of the cationic liposomes as a dosage form for oligonucleotides, their mutual physicochemical interactions have not been studied. In the present study some physicochemical properties of the cationic liposome/phosphorothioate oligonucleotide complexes at different oligo/lipid ratios are established. The studied properties were aggregation, lipid fusion, and interactions with different lipid bilayers. Cationic liposomes used were commercial transfection reagent DOTAP (1-(2,3-dioleoyloxy)propyl)-*N*,*N*,*N*-trimethylammoniummethylsulfate) and a self-made mixture of dimethyldioctadecylammoniumbromide (DDAB) and DOPE at 8:15 molar ratio used previously for transfection [10] and available commercially as LipofectACE™ (originally TransfectACE™).

2. Experimental procedures

2.1. Materials

DDAB was from Sigma (St. Louis, MO, USA). DOPE, *N*-Rh-PE and *N*-NBD-DOPE were from Avanti Polar Lipids (Pelham, AL, USA). Transfection-reagent DOTAP was from Boehringer Mannheim GmbH (Germany). DPPC and DPPG were from Orion Farmos Corp. (Turku, Finland). EPC was from Enzymatix – Lucas Meyer GmbH (Cambridge, UK).

2.2. Oligonucleotides

Phosphorothioate oligonucleotides (S-oligos) were 15 bases long and were synthesized with Applied Biosystems 381 A automatic DNA-synthesizer. Sequences for the 15-mer oligos were the same as antisense and random sequences (as O-oligos) previously used in studies against human c-myc in HL-60 cells [11].

2.3. Liposomes

DOTAP was a commercial aqueous dispersion of 1.29 mM (1 mg/ml). Dilutions of DOTAP were made in polystyrene tubes and oligonucleotides in eppendorf tubes in 20 mM Hepes buffer with 150 mM NaCl (pH 7.4). For *N*-Rh-PE and *N*-NBD-PE (1 mol% each) labelled liposomes, labeled lipids were evaporated to dryness. DOTAP, as an aqueous dispersion, was added and after 2 h the sample was sonicated for about 10 min. Mean diameter of the majority (> 85%) of liposomes was about 40 nm, similar to that of unlabeled DOTAP liposomes.

Cationic liposomes for size determinations, composed of DDAB and DOPE (8:15 by mol, 1.54 mg lipid in 1 ml) [10] were prepared in sterile water by the thin lipid hydration method followed by sonication until mean diameter of about 80 nm was achieved with a small portion having a mean diameter of about 200 nm. For *N*-Rh-PE and *N*-NBD-PE (1 mol% each) labelled liposomes, labeled lipids were evaporated to dryness together with DDAB and DOPE. Sterile H_2O was added and sonicated as above yielding a mean diameter of about 30 nm.

Neutral and negatively charged liposomes with entrapped calcein (60 mM calcein, 37.5 μmol of lipid in 1 ml of 20 mM Hepes buffer, pH 7.4) were prepared by the reverse phase evaporation (REV) method [12] and extruded through two stacked 0.2 μm polycarbonate membranes. Liposome diameters (mean ± S.D.) for different liposome compositions were 180 ± 69 nm (DPPC/DPPG, 4:1 by mol), 153 ± 56 nm (EPC/DOPE, 1:1 by mol), 159 ± 56 nm (DPPC/DOPE, 1:1 by mol) and 155 ± 57 nm (EPC). Unencapsulated calcein was separated from liposome-entrapped on a Sephadex G-50 column.

2.4. Size determinations

The sizes of oligonucleotide/lipid complexes were determined by quasi-elastic light scattering (Nicomp Submicron Particle Sizer, Model 370, Santa Barbara, CA, USA). Oligonucleotide solution was added to cationic lipid solution with consequent mild vortexing and the size determinations were performed after about 15 min. Cationic lipid concentration was 400 μM, if diluted 1:40 to make 10 μM with the medium is in the range normally used in transfection experiments. Ratios of S-oligo to cationic lipid were 0–0.4 and 0–0.56 (mol/mol ratio) or 0–7.9 and 0–5.6 (−/+ charge ratio) for DOTAP and DDAB, respectively. Charge ratio was calculated as 15 mer oligonucleotide having 14 negative charges and each cationic lipid one positive charge per molecule. Size distributions were determined as mean ± S.D. of diameter on the basis of vesicle number and volume (Fig. 1).

2.5. Lipid mixing

Lipid mixing in cationic liposomes induced by added oligonucleotides was monitored by resonance energy

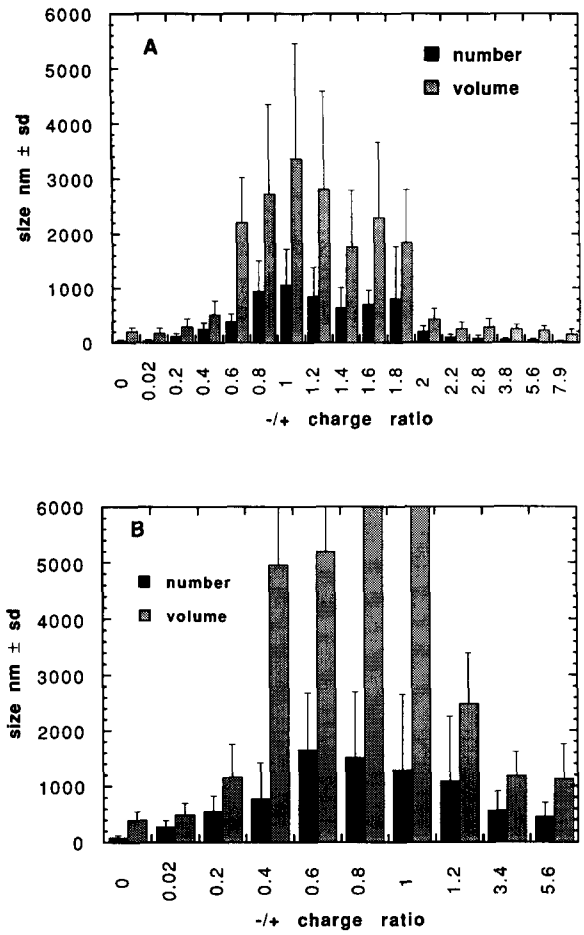


Fig. 1. Vesicle sizes (nm, mean ± S.D.) after incubation of increasing amounts of a 15-mer S-oligonucleotide with cationic liposomes (increase in −/+ charge ratio) composed solely of DOTAP (A) or DDAB/DOPE 8:15 by mol (B). In case of DDAB/DOPE liposomes, at charge ratios 0.4–1.0 the results are indicative, since the particles are too large to be reliably measured by QELS (up to 10–20 μm based on % volume).

transfer (RET) at 460 nm (ex) and 530 nm (em) in a cuvette with magnetic stirring. The method is based on having acceptor (*N*-Rh-PE) and donor (*N*-NBD-PE) probes in the same vesicle at high enough concentration. In this case the acceptor will fluoresce when the donor is excited. Dilution of the probes during fusion with unlabeled vesicles will increase the donor (*N*-NBD-PE) fluorescence that can be monitored continuosly. Into 10 μl of *N*-Rh-PE and *N*-NBD-PE (1 mol% each) labeled and 40 μl of unlabeled DOTAP or DDAB/DOPE liposomes in a cuvette, 1.9 ml Hepes buffer was added and after 20 s (time point 0), 50 μl oligonucleotides (0, 0.04, 0.29, 1.14, 1.71 or 8.00 μM final concentration) were added. Cationic lipid concentrations were 2 μM and 20 μM for DDAB and 4 μM and 20 μM for DOTAP, respectively. Lipid mixing, as increase of the donor (*N*-NBD-PE) fluorescence (ex 460 nm, em 530 nm) was followed for 3 min on a Perkin Elmer LS 50B Luminescence Spectrometer (UK). 50 μl of liposomes containing 0.2 mol% of both fluorescent probes were used to assess maximal 100% lipid mixing. This simulates the theoretical situation in which all labeled liposomes in the cuvette have fused with unlabeled liposomes [13].

2.6. Kinetic analysis

In general, fusion of liposomal lipids takes place in two steps: aggregation and fusion. We carried out the kinetic analysis of fusion using principles of Nir et al. [14,15]. Aggregation of the vesicles is assumed to take place according to second order kinetics (rate constant: $(\text{concentration})^{-1} \times (\text{time})^{-1}$), the fractional rate of aggregation being dependent on aggregating vesicle concentrations. After aggregation, fusion takes place obeying the first order kinetics with the rate constant, $(\text{time})^{-1}$. During fusion labeled (L) and unlabeled (V) vesicles aggregate and fuse 1:1 according to following scheme:

$$L + V \underset{D}{\overset{C}{\rightleftharpoons}} A(1, 1) \xrightarrow{f} F(1, 1)$$

C is the rate constant of aggregation and the aggregation rate is $C \times L \times V$, when the dissociation constant is assumed to be negligible. Aggregate (A) fuses irreversibly at the rate $f \times A$ to form fused lipoidal structures (F). This scheme describes adequately the initial fusion resulting in fused particles of two liposomes. Formation of larger aggregates composed of several liposomes at further stages of fusion is too complex phenomena to be accurately described by this scheme.

The lipid concentrations were converted to approximate molar concentrations of liposomes as described previously [15,17]. Liposomes of 30 nm and 40 nm were calculated to contain approx. 7000 and 15000 lipid molecules, respectively. For DDAB/DOPE liposomes with mean diameter of 30 nm concentrations in the RET study were for lipids 5.75 μM (2 μM DDAB) and 57.5 μM (20 μM DDAB) and the corresponding liposome concentrations are 0.82 nM and 8.2 nM, respectively. For DOTAP (4 and 20 μM of lipid; mean diameter 40 nm) vesicle concentrations are 0.26 nM and 1.3 nM. In all RET experiments 20% of the vesicles were fluorescently labeled (L_0) and 80% unlabeled (V_0) initially.

Initial aggregation rate constants after addition of the oligonucleotides to the cuvette were calculated assuming $I(t) = CV_0t$, where $I(t)$ is the fraction of the fluorescence of the maximal lipid mixing, C is the aggregation rate constant ($M^{-1}\ s^{-1}$), V_0 is the initial concentration of the unlabeled vesicles (M) and t is time (s). This approach is applicable at early times when $CL_0t << 1$ [14], which was the case in our study.

Slope of measured $I(t)$ vs. t was divided by V_0 to obtain C.

A kinetic model was constructed using STELLA 2.2 software (High Performance Systems, Hanover, NH). Initial vesicle concentrations in the cuvette (L_0, V_0), instead of lipid concentrations, were used as the starting point. They were obtained as described above. In the model, concentration dependent second-order aggregation of the vesicles ($C \times L \times V$) is followed by the first-order fusion step ($f \times A$). Non-detectable fusion (e.g., among unlabeled vesicles) is distinguished from the fusion that produces the fluorescent signal. This was done by taking into account the initial fractions of labeled and unlabeled liposomes and the approximate number of fusing liposomes (size factor). The fraction of fluorescent signal of the total fusion taking place is estimated by Nir [14] as $I = 2 \times (V/(L+V))((n-1)/n)$. In the equation n is the number of fusing particles that was estimated from the size distributions by quasi-elastic light scattering (QELS). At $n = 2$ I is 0.4 and with increasing values of n it approaches 0.8.

2.7. Calcein release

Calcein release from neutral and negatively charged liposomes was used to study the destabilizing effect of cationic liposome/oligonucleotide complexes on lipid bilayers. The release of calcein was determined as the increase of fluorescence due to dilution of self-quenching calcein concentration from DPPC/DPPG (4:1 by mol), EPC/DOPE (1:1 by mol), DPPC/DOPE (1:1 by mol) and EPC liposomes. Sizes of the liposomes are shown above. The effect of cationic lipid/S-oligonucleotide mixtures on calcein release was monitored in a cuvette with magnetic stirrer (set at high position) with ex 494 nm and em 515 nm. Fluorescence was followed for 5 min after adding DOTAP/oligonucleotide mixtures to liposomes in 20 mM Hepes buffer (pH 7.4) using different charge ratios. 50 μM lipid concentration in 2 ml of Hepes buffer for both DOTAP and target liposome lipids was used. Triton X-100 at 0.2% by volume was used to assess total liposomal fluorescence after correcting for the quenching effect of Triton X-100 [18].

3. Results

3.1. Size distributions

Fig. 1A shows the mean sizes of 15-mer S-oligo/DOTAP complexes, with increasing negative to positive (−/+) charge ratios. The size distributions for ratios 0, 0.02 and 5.6 did not show Gaussian distribution and had double peak distributions. For these ratios only the major peaks (usually 80–98% of total based on diameter) are shown. The mean size of DOTAP liposomes in the experiment with no added oligo was about 40 nm with small fraction of vesicles having a diameter of about 150 nm. After addition of oligonucleotides the sizes increased gradually up to the −/+ ratio of about 1. Complexes with −/+ ratio of 1.0 to 1.6 were not always measurable due to formation of visible solid particles too large for adequate size determination with QELS. Additional increase of the ratio caused a gradual decrease of particle sizes down to a diameter of about 30 nm. At higher ratios very fast complexation, aggregation and fusion occurs and possibly the coverage of the surface with negative charges blocks further aggregation yielding small particle sizes.

Similar behaviour was found with oligonucleotide complexes with DDAB/DOPE liposomes (Fig. 1B), but the increase in size started at lower charge ratios (−/+ 0.02) and at high charge ratios mean particle size remained larger than in the case of DOTAP.

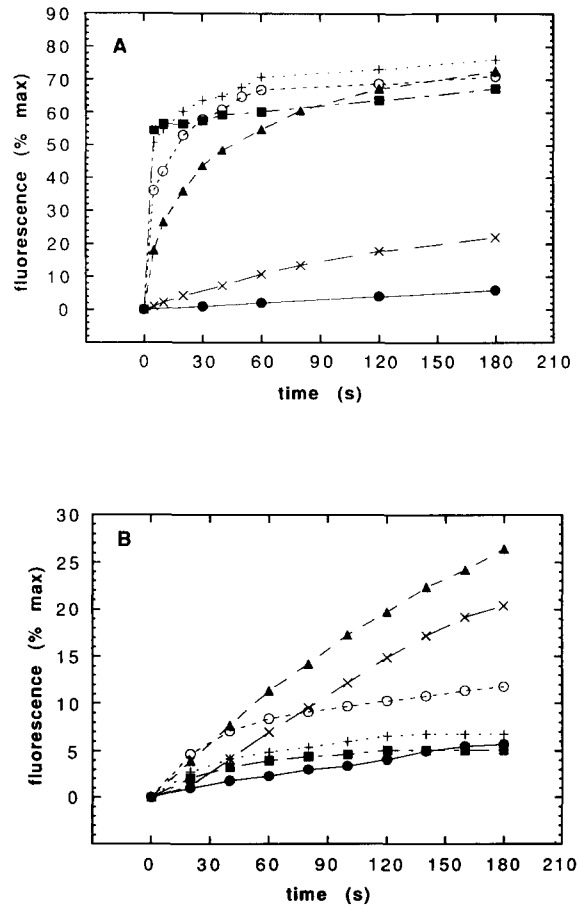


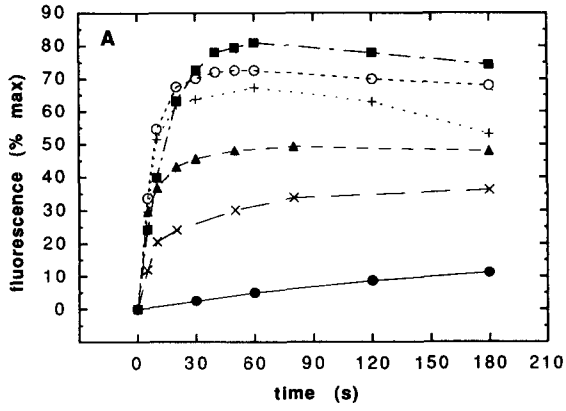
Fig. 2. Time course of lipid mixing after addition of oligonucleotides to liposomes containing 16 μM and 4 μM (A) or 3.2 μM and 0.8 μM of DOTAP (B) in fluorescently unlabeled and labeled liposomes, respectively. Charge ratios in the mixtures after the addition of oligonucleotides were 0 (●), 0.03 (×), 0.2 (▲), 0.8 (○), 1.2 (+), 5.6 (■).

3.2. Resonance energy transfer

Results of RET measurements after adding oligonucleotides into DOTAP and DDAB/DOPE liposome suspensions are shown in Figs. 2 and 3, respectively. With increasing −/+ ratio at 20 μM cationic lipid concentrations for both liposomes (Figs. 2A and 3A), there was a substantial increase in the achieved fluorescence and in the initial rate of fluorescence increase.

Fluorescence, as % of the maximum, increased much slower when the measurements were done with 2 μM DDAB or 4 μM DOTAP instead of 20 μM, but using the same oligonucleotide/lipid charge ratios. Charge ratios of 0.8–1.2 in DDAB/DOPE and 0.03–0.2 in DOTAP experiment showed most extensive lipid mixing.

The initial rate of lipid mixing (%/s) in 20 μM mixture of cationic lipids was increased with added oligonucleotide concentration until a plateau was



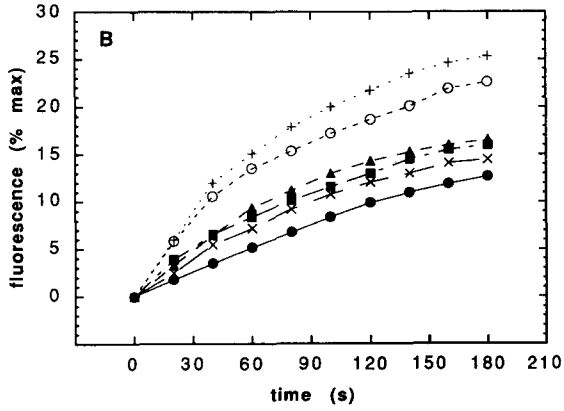


Fig. 3. Time course of lipid mixing after addition of oligonucleotides to DDAB/DOPE (8:15 by mol) liposomes containing 16 μM and 4 μM (A) or 1.6 μM and 0.4 μM of DDAB (B) in fluorescently unlabeled and labeled liposomes, respectively. Charge ratios in the mixtures after the addition of oligonucleotides were 0 (●), 0.03 (×), 0.2 (▲), 0.8 (○), 1.2 (+), 5.6 (■).

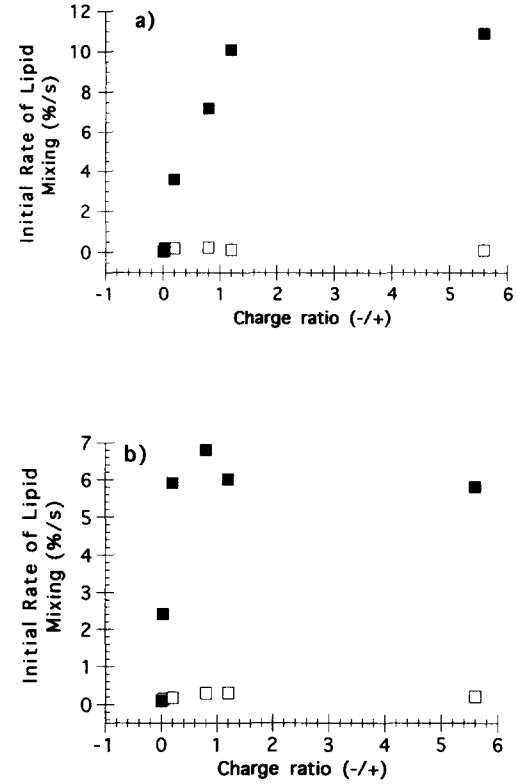


Fig. 4. Initial rate of lipid mixing (%/s) in 1:4 mixture of fluorescently labeled and unlabeled cationic vesicles in resonance energy transfer experiment. Oligonucleotides were added to the mixture to yield apparent −/+ charge ratios from 0 to 5.6. Total concentrations of cationic lipid were 4 μM (□) and 20 μM (■) in DOTAP experiment (a) and 2 μM (□) and 20 μM (■) in DDAB/DOPE (8:15 by mol) experiment (b).

achieved (Fig. 4). The plateau was achieved at smaller −/+ ratios in the case of DDAB (0.2) than in DOTAP experiments (1.2). Fusion appears to be controlled by the aggregation rate as evidenced by the difference in the fractional initial rates of fluorescence increase at different vesicle concentrations (Fig. 4). Therefore, aggregation rate constants were calculated. From the −/+ ratio of zero the added oligonucleotide increased the aggregation rate constant in DOTAP suspension about 5-times and 300-times at cationic lipid concentrations 4 μM and 20 μM, respectively (Fig. 5a). In the case of DDAB/DOPE the corresponding increases were 3- and 80-fold at DDAB concentrations of 2 μM and 20 μM, respectively (Fig. 5b). At high −/+ ratios the aggregation rate constant seemed to decrease with increasing oligonucleotide concentration at low lipid concentrations (2 μM DDAB and 4 μM DOTAP). At 20 μM of cationic lipid aggregation rate constant did not vary at high −/+ ratios.

3.3. Interaction with lipid bilayers

In Hepes buffer highly charged hydrophilic calcein did not leak from the liposomes. The calcein release

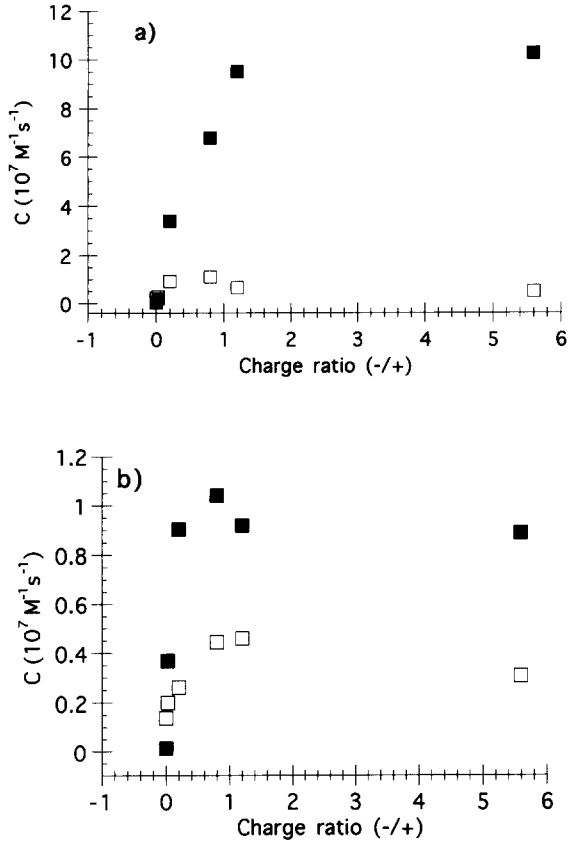


Fig. 5. Apparent initial binding rate constants (C, M^{-1} s^{-1}) of liposomes in resonance energy transfer experiment with 1:4 ratios of fluorescently labeled and unlabeled vesicles. Oligonucleotides were added to the mixture to yield apparent $-/+$ charge ratios from 0 to 5.6. Total concentrations of cationic lipid were 4 μM (□) and 20 μM (■) in DOTAP experiment (5a) and 2 μM (□) and 20 μM (■) in DDAB/DOPE (8:15) experiment (5b), respectively.

from negative DPPC/DPPG and from neutral EPC/DOPE and EPC liposomes after incubation with S-oligo/DOTAP mixtures for 5 min is shown in Fig. 6. DPPC/DPPG and EPC/DOPE liposomes showed

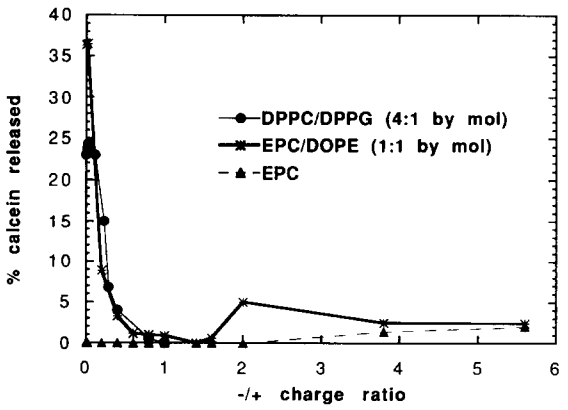


Fig. 6. The effect of a 15-mer S-oligonucleotide/cationic liposome DOTAP (50 μM) complexes with increasing $-/+$ ratio on calcein release from DPPC/DPPG (4:1 by mol), EPC/DOPE (1:1 by mol) and EPC liposomes after incubation of 5 min.

rapid calcein release up to S-oligo/DOTAP $-/+$ charge ratio of about 0.3 and slower release up to a charge ratio 1. Unlike negative DPPC/DPPG liposomes, EPC/DOPE liposomes showed calcein release also at higher (> 1.6) charge ratios. DPPC/DOPE liposomes were used to check the possible impurities of EPC affect the leakage, but the results were identical (data not shown). Pure EPC liposomes did not show any significant release of calcein.

4. Discussion

Cationic liposomes attract anionic molecules on their surface with electrostatic interactions. In this way they are used to complex DNA [1]. Fusion of the cationic liposome on the plasma membrane of the cells is considered to be the main mechanism of the DNA delivery into the cells with the cationic liposomes although endocytosis of the liposomes may also play a role [2]. Mechanistic cellular delivery studies with cationic liposomes and anti-sense oligonucleotides have not been carried out.

Aggregation of the liposomes can take place only when the energetic barrier at the liposomal surface is overcome [14]. Further, lipid fusion can only take place when the liposomes are first aggregated. Aggregation and fusion controlled systems can be distinguished by carrying out the resonance energy transfer experiments at two different vesicle concentrations [14,15]. At low concentrations aggregation is rate limiting, because it is slowed down due to the longer distances between the vesicles. Since lipid mixing rate was highly dependent on the vesicle concentration (Fig. 4), it seems that the vesicle aggregation, and not fusion step, is controlling the rate. This means that the oligonucleotides increase fusion of the cationic liposomes by enabling their aggregation in the first place.

In contrast to the other cases, cationic liposomes without oligonucleotides fused at the same rate both at low and high liposome concentrations both in DOTAP and DDAB/DOPE experiments (Figs. 2 and 3). This could suggest a completely fusion controlled system. Assuming that fusion step controls the lipid mixing in cationic liposomes we calculated the apparent fusion rate constant using following equation of Nir [14].

$$I(t)/A_0 = 1 - e^{-ft}$$

For DDAB/DOPE the best fit was obtained at f values 0.0007–0.0008 s^{-1} ($r > 0.98$) and for DOTAP at 0.00033–0.00035 s^{-1} ($r > 0.98$). Due to the size factor 40% is the theoretical maximum of lipid fusion (see Methods). This was taken into account by dividing the first order rate constants above by 0.4 to obtain true rate constants for the simulations with the model. The obtained values were $(1.7–2.0) \cdot 10^{-3}$ s^{-1} (DDAB/

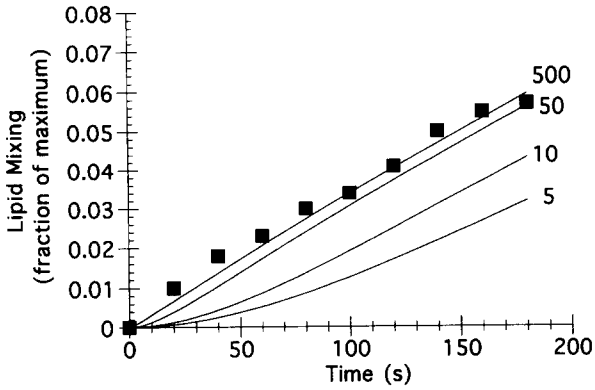


Fig. 7. Simulation of the effect of binding rate constant ((5–500)·10^7 M^{-1} s^{-1}) on lipid mixing in RET experiment. Fusion rate constant was set at $8.9 \cdot 10^{-4}$ s^{-1}, the fusion products were limited to two fusing vesicles and DOTAP concentration of the liposomes 4 μM. Symbols (■) are the experimental data points.

DOPE) and (8.3–8.9)· 10^{-4} s^{-1} (DOTAP). These values were fixed in the kinetic model while aggregation rate constant was varied. The aggregation rate constants should be $> 5 \cdot 10^7$ M^{-1} s^{-1} for DDAB/DOPE (data not shown) and $> 5 \cdot 10^8$ M^{-1} s^{-1} for DOTAP (Fig. 7) in order to give the observed fusion rates in fusion controlled system at all experimental lipid concentrations of our study. Compared to the initial aggregation rate constants (Fig. 5) these values seem unrealistic for binding of cationic liposomes without oligonucleotides. Charge repulsion should make the aggregation of the liposomes improbable and, indeed, we did not see aggregation in size distributions of the liposomes at $-/+$ ratio of 0 (Fig. 1). Thus, the concentration independent slow lipid mixing among the cationic liposomes without oligonucleotides is not mediated by aggregation.

Further evidence for a process not mediated by aggregation was seen in simulations, where aggregation rate constant (C) was kept constant at realistic level (DDAB/DOPE: $0.1 \cdot 10^7$ M^{-1} s^{-1} and DOTAP: $0.5 \cdot 10^7$ M^{-1} s^{-1}) and f was varied from 0.001 s^{-1} to 1.0 s^{-1}. For 4 μM DOTAP the observed lipid mixing was achieved only at $f \geq 0.5$ s^{-1} while at 20 μM the experimental rate was seen in simulation at $0.002 < f < 0.003$ s^{-1}. However, in all cases the simulated lipid mixing rate (%/s) was several times higher at 20 μM than at 4 μM suggesting aggregation rate controlled system. In the case of DDAB/DOPE simulated fusion rate was also substantially higher at 20 μM than at 2 μM. These simulations suggest that at $-/+$ ratio of zero an aggregation initiated fusion rate controlled system is not a valid explanation for the observed concentration independent mixing of the lipids.

Rather, the fluorescent lipid dilution without oligonucleotides may be due to exchange of the fluorescent lipids via the medium. Earlier the fluorophores used in this study were found to be virtually non-exhangeable between phospholipid vesicles [19,20]. Later, similar extent of spontaneous lipid mixing as reported here has been found for DOPE containing liposomes [21,22] and for cationic DOTMA/PE liposomes [13] in buffer. In this study the cationic liposomes were SUVs from which fluorophores may detach easier due to the relative instability of the highly curved bilayer and also the magnetic stirring of the cuvette may accelerate the lipid mixing.

Our studies based on oligonucleotide-cationic lipid complex size determinations (Fig. 1) and RET (Figs. 2 and 3) indicate that the negative oligonucleotides induce aggregation and, subsequently, fusion of the cationic liposomes. Likewise, the aggregation and fusion of didodecyldimethylammonium bromide is induced by dianions of dipicolinic acid [23,24]. When the oligonucleotide concentration is increased relative to the cationic liposomes the probability for formation of larger aggregates increases, but at very high $-/+$ ratios (> 2 for DOTAP and > 1.2 for DDAB/DOPE) the mean size decreases again. This is most probably due to efficient shielding of the cationic charges by the oligonucleotides. Consequently, repulsion by the negative charges prevents formation of the larger aggregates (Fig. 1). The increase of the complex size of DDAB/DOPE liposomes took place at lower charge ratios ($-/+ 0.02$) than in case of DOTAP. Due to the larger initial size and smaller surface area of DDAB/DOPE liposomes less oligonucleotide is needed to decrease the electrostatic repulsion. Also the cationic charge density is smaller in DDAB/DOPE than in DOTAP due to the neutral lipid DOPE.

At the highest $-/+$ ratio (5.6) of oligonucleotide/DOTAP (20 μM) the lipid mixing was initiatially very fast, but stopped abruptly at lower fluorescence level (about 60%) than at charge ratios 0.8 and 1.2 (Fig. 2A). At lower lipid concentration (4 μM) the same phenomenon is more clear: at high (> 0.8) charge ratios there is a decrease in the fusion rate (Figs. 2B and 4a) and this is seen also as a decrease of the initial aggregation rate constant at high $-/+$ ratios (Fig. 5a). Similar decrease in the fusion rate is seen also at low DDAB concentration (Figs. 3B and 4b). Probably, at higher $-/+$ ratios and later incubation times the oligonucleotides have more chances to reverse the liposomal surface charge before the liposome binds and fuses with other liposomes. Accordingly, the initial fusion rates (Fig. 4) and aggregation rate constants (Fig. 5) are much higher at high vesicle concentration compared to the low concentrations. At high vesicle concentration oligo-induced binding and fusion take place rapidly before the negative electrostatic repulsion becomes predominant. At low vesicle concentration oligonucleotides have more time to bind on the liposomes and to reverse the charge before the liposomes contact each other. DDAB/DOPE liposomes

had a lower lipid mixing threshold possibly because DOPE is prone to undergo phase transition and fusion [25].

The kinetic model was tested also for simulation of the time courses of oligonucleotide induced lipid mixing at the experimental conditions. In most cases the model provided good predictions, but often the simulations deviated clearly from the data (simulations not shown). This is not surprising because at later times larger aggregates and very complex mixtures of fused and aggregated particles are formed. In order to take into account all the possibilities in the fusing system of liposomes the model should be exceedingly complicated.

As the mechanism of action for cationic liposomes is thought to be the fusion of cationic lipids with negatively charged membranes we studied the release of calcein from neutral (with and without DOPE) and negatively charged liposomes at different $-/+$ charge ratios.

For DPPC/DPPG liposomes the mechanism of calcein release seems to be simply the electrostatic attraction of opposite charges. With excess negative charges of oligo/DOTAP mixtures no calcein release was seen. This was probably due to electrostatic repulsions, fewer collisions of the larger aggregates and the lack of DOPE in the liposomes.

Similar calcein release profile up to a charge ratio of about 1 was seen in the case of EPC/DOPE liposomes. Again, positive net charge was essential for interaction. At ratios of about 0.6–1.7 there was very low calcein release possibly due to the increased particle sizes leading to decreased collision rate. At higher ratios, there was slow leakage at the charge ratios above 2.

Interestingly, DOTAP did not cause significant calcein release from EPC liposomes. The result is different from the EPC/DOPE liposomes and demonstrates that PE of the cell membrane may be essential for the capability of DOTAP to fuse with cell membranes. In contrast, in the case of negatively charged membrane PE is not required (Fig. 6). Likewise, studies made on interactions of PE and/or PC liposomes with low molar DOTAP fractions have shown aggregation with negative liposomes at physiological or lower ionic strengths and the enhancement of aggregation with increased PE/PC ratio [26].

Due to the presence of phosphatidylethanolamine and negatively charged phospholipids in the cell membranes DOTAP containing liposomes have the ability of fusing to lipid vesicles or to cell membranes and ability to transfer DNA [26,27], RNA [28] and oligonucleotides [9] into the cells. DDAB/DOPE liposomes have similar properties and have been used successfully for transfections [10].

Effects of the charge ratio on the cellular delivery of oligonucleotides has not been studied. Also, in the case of DNA delivery it is difficult to make firm conclusions on the importance of the charge ratio. DOTAP has been used for transfection of functional capped CAT RNA containing about 800 nucleotides [28] at molar ratio of RNA/DOTAP about 0.005 (apparent $-/+$ charge about 3.7). Recently, as high as $-/+$ ratio of about 14 (both DOTAP and Lipofectin were used) has been used succesfully to enhance cellular uptake and increase the stability of oligonucleotides against nucleases [9]. Interestingly, no enhancement of cell uptake was seen at about 0.14 charge ratio (0.1 μM oligonucleotide, 13 μM DOTAP). Successful experiments with oligonucleotides using Lipofectin have had optimal $-/+$ charge ratios of about 0.3 and 0.5 [6,7]. Negative to positive charge ratio in the range of 0.4–0.9 has been found optimal for DNA transfections with Lipofectin [29] and the complex diameter has been shown to be about 520 nm with an optimal 0.5 $-/+$ ratio and 0.1 weight ratio of DNA to lipofection reagent with the plasmid and cell lines used [2,30].

Studies on DNA-cationic liposome (DOTMA) complexes have demonstrated, that DNA molecules have collapsed leading to structures, where DNA is supposedly completely covered by lipid bilayers [31]. Similar mechanism (endocytosis) has been postulated earlier for liposomes containing quaternary ammonium detergent as an alternative model for the delivery of virus RNA into plant protoplasts [32]. Recently, it has been postulated that the uptake mechanism of DNA is mainly endocytosis with subsequent destabilization of the endosomal membrane [33]. These results are contradictory to the fusion mechanism postulated earlier [1], where DNA/DOTMA complexes were hypothesized to consist of four liposomes associated with one plasmid and fusion being the mechanism for the cellular delivery.

Direct comparison between liposomal delivery of oligonucleotides and DNA should be avoided, because oligonucleotides are much smaller molecules and their possibilities of having tertiary structures and liposome induced structural changes is different from DNA. In any case, we have established the liposome – liposome interactions and the importance of the charge ratio on the behavior of the oligonucleotide – cationic lipid complexes in solutions. These interactions in the solutions may also complicate the introduction of cationic liposomes as a pharmaceutical dosage form for antisense oligonucletides.

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