Preparation of oligodeoxynucleotide encapsulated cationic liposomes and release study with models of cellular membranes

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ABSTRACT

Cationic liposomes are used for cellular delivery of antisense oligodeoxynucleotide (AsODN), where release of encapsulated AsODN is mainly controlled by endocytosis and fusion mechanisms. In this investigation, it was tried to model such a release process that is difficult to evaluate in cell culture. For this purpose, an AsODN model (against protein kinase C-α) was encapsulated in a DODAP-containing cationic liposome and evaluated for size, zeta-potential, encapsulation and ODN stability. Vesicular models of outer layer and total plasma membranes and early and late endosomal membranes were developed, based on lipid content and pH, using ether injection method. ODN release was determined by the fluorescence dequenching of encapsulated FITC-ODN.

Zeta potential, size and ODN encapsulation efficiency of the prepared liposomes were -2.49 ± 7.15 mV, 108.4 nm and 73% respectively. ODN protection was 3-4 times more than that of conventional liposome/ODN complexation method.

There was a correlation between model concentration and percent of ODN release. At 7.5 μM, the percent of released ODN was 76% for the cholesterol-free model of the late endosome and 16% for the early endosomal membrane; while the release was less than 11% for the models of plasma membrane. ODN release increased with temperature in the range of 4-37°C for the late endosomal model, but not for others, possibly due to their high cholesterol contents or acidic pH. The interaction was fast and completed within 5 minutes and didn't change in the range of 5-60 minutes. Our data are in agreement with published cell culture studies and reveal that cell-liposomes interaction can be modeled by lamellar membranes.

Keywords: Gene delivery, Antisense oligonucleotide, Cationic liposomes, Model membrane, Release

INTRODUCTION

The use of antisense oligodeoxynucleotides (AsODNs) both in research and therapy has emerged as a powerful alternative or complement to small molecule inhibitors employing traditional drug design strategies. AsODNs are short pieces of synthetic and chemically modified DNA designed to hybridize to specific mRNA sequences. They inhibit gene expression mainly through RNase-H activation or hybrid arrest, steric blockage of translation (1, 2). They are being explored as potential therapeutic tools against viral infections, cardiovascular, inflammatory and hematological diseases and cancer (3).

The major limiting step in oligodeoxynucleotide (ODN) application is the inefficient delivery of ODN to cells and the poor bioavailability to intracellular targets. The polyanionic nature and the large size of ODN render them practically impermeable to cell membranes and consequently their biological activity is significantly compromised. To overcome this obstacle, rationally-designed carriers are required (4). Liposomes are the most wide-spread non-viral carriers for nucleic acid delivery. Although anionic and neutral liposomes have been studied for ODN delivery, their poor nucleic acid entrapment efficiency has limited their uses. Instead, cationic liposomes entrap nucleic acids efficiently through formation of complexes which are called lipoplexes (5).

Despite enhanced cellular association of lipoplexes, their serum instability and rapid clearance, a few minutes after administration, has restricted their pharmaceutical applications (6). This is especially true when non-bilayer forming helper lipids (e.g. dioleoyl phosphatidyl-ethanolamine) are incorported in cationic liposomes (7, 8).

Since 1997, it has been known that DNA could be ordered in smectic layers sandwiched between the bilayer lamellae of multilamellar membranes of cationic liposome (9). This geometry, which is called ODN encapsulation rather than complexation with cationic liposomes, is of

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special interest because it protects ODN almost completely and functions more efficiently as a transfecting agent (10). Accordingly, some liposome specialists have studied ODN encapsulation in cationic liposomes. Efficient encapsulation has been achieved by calcium chloride or ethanol assisted destabilization of liposomes (11-13).

Lipid-based carriers, whether lipoplexes or ODN encapsulated liposomes, are mostly internalized into the cells via endocytosis (14, 15). Subsequently, ODN is released from endosomal compartment into cytoplasm through membrane destabilization or fusion mechanisms. The lysosomal degradation of ODN occurs as pH of endosomal compartment falls (16, 17). The destabilization reactions that lead to ODN release into cytoplasm are the most significant steps in transfection efficiency (18).

ODN delivery to cells and their release into cytoplasm is usually evaluated by the confocal fluorescence microscopy and the flow cytometry of fluorophore-conjugated ODN in cell culture studies. Such studies are time-consuming and expensive. They require special facilities, techniques and containments. Besides, our preliminary study confirms that passive diffusion of encapsulated ODN through liposomal membrane in acidic and neutral pH media is negligible (19). Therefore, it was decided to develop models of plasma, early and late endosomal membranes in such a way that their interaction with liposomes during the internalization into cells is probable. The models were designed based on the physicochemical properties of the mentioned membranes. Subsequently, FITC-conjugated ODN release was studied upon interaction with the model membranes by fluorimetry. To the best of our knowledge, such kind of comprehensive modeling has not been reported previously in the literature. Of few reports in this area, Jaaskelainen, *et al.* (20) studied the release of FITC-ODN from its complex (not encapsulated) with cationic liposomes. They found that fluorescence of FITC-ODN was dequenched upon interaction with an anionic model of endosomal membrane. Besides, ODN release was triggered by the model membrane through displacement of adsorbed ODN on the surface of cationic liposomes or lipid mixing mechanisms (20). However, such mechanisms couldn't explain well the release of encapsulated ODN from the cationic liposomes especially if ODN is entrapped between lipid bilayers inside the liposomes. Therefore, it was decided to study the release behavior of encapsulated ODN from the cationic liposomes.

MATERIALS AND METHOD

Lipids

1,2-Dioleoyl-3-dimethylammoniumpropane (DODAP), 1-o-(2'-(ω-methoxypolyethylene glycol) succinoyl)-2-N-dodecanoylsphingosine ($PEG\text{-}Cer_{20}$) and 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC) were purchased from Northern Lipids (Vancouver, Canada). 1,2-Dimyristoyl-sn-glycero-3-phosphoglycerol (DMPG) and egg phosphatidylcholine (EPC) were obtained from Lipoid GMBH (Ludwigshafen, Germany). Cholesterol (Chol) was purchased from Sigma Chemical Company (St. Louis, USA).

Oligonucleotides

A 20-mer phosphorothioate modified AsODN 5'-TsCsCs AsTsGs AsCsGs AsAsGs TsAsCs AsGsCs CsGs-3', directed against protein kinase C-α mRNA, was synthesized by TIB (Berlin, Germany) and used as a previously-validated model of antisense therapy in non-small cell lung cancer (21). 5'- Fluorescein isothiocyanate–conjugated phosphorothioate ODN (FITC-ODN) was synthesized and purified by Synthegen (Houston, Texas).

Other materials

Bromophenol blue, Triton x-100, TEMED and ethidium bromide were obtained from Sigma Chemical Company (St. Louis, USA). Acrylamide:bis-acrylamide (29:1) and ammonium persulfate were purchased from Merck (Germany). Polycarbonate filters and Sephadex G-25 were supplied by Northern lipids (Vancouver, Canada) and Amersham (Sweden), respectively. Newborn calf serum was form Gibco BRL. Metafectene was received as a gift from Biontex (Germany). All other reagents were of analytical grade.

Preparation of ODN encapsulating liposome

Cationic liposome was prepared by the method of ethanol injection (11, 13). Briefly, lipid solution in absolute ethanol was injected gently under vigorous vortexing into ODN solution in citrate buffer (pH=4). Lipid was composed of DSPC / Cholesterol / $PEG\text{-}Cer_{20}$ / DODAP (20:45:10:25 mol%). DODAP, a tertiary amine containing lipid which becomes cationic at acidic pH of the medium, was incorporated in the liposome to entrap ODN actively at low pH and to expel non-entrapped ODN at neutral pH. Cholesterol and DSPC were employed as neutral lipids to stabilize the liposome. $PEG\text{-}Cer_{20}$ not only renders the liposome sterically-stabilized for in-vivo application but also allows the control of liposome size (11, 13).

Liposomes were then dialysed and citrate buffer was replaced with HEPES buffered saline (HBS, pH=7.5). At pH 7.5, DODAP charge becomes neutral and the bound ODNs to the external surface of liposome dissociate and are removed during dialysis. Residual free ODN were subsequently removed by Sephadex G-25 gel filtration chromatography through a previously conditioned column with HBS (pH=7.5). Liposomes were physicochemically stable at 4°C within 1 month as the turbidity of the liposomes and ODN encapsulation efficiency did not change significantly during this time.

Preparation of ODN/Metafectene® complex

ODN/Metafectene® complex (lipoplex) was prepared according to the manufacturer's instruction. Briefly, 60µl of 2% Metafectene® (Biontex, Germany) and 25µl of ODN solution (100µM) were diluted separately with distilled water to 100µl in polystyrene microtubes. Diluted ODN solution was admixed with diluted Metafectene® and incubated for 15 min at room temperature. ODN/ Metafectene® complexes were diluted with HBS to 1ml.

Particle size and zeta-potential determination

Particle size and zeta-potential of liposome were determined by Malvern Zetasizer (UK). Liposomes were diluted 20-fold with HBS. Measurements were carried out under conditions of: temperature, 25°C; viscosity, 0.88 cP; reflex index, 1.33.

ODN encapsulation efficiency in cationic liposome

ODN encapsulation efficiency was expressed as recovered ODN/lipid weight ratio. The recovery of ODN and lipid were determined separately as follows:

ODN content was determined by UV-spectrophotometery (Shimadzu, Japan) at 260 nm after liposome solubilization in chloroform/methanol (1:2.1). Absorbance was set to zero with a blank solution containing the mixture of distilled water/ chloroform/ methanol (1:1:2.1). Subsequently, the measurement was carried out for ODN standard solutions in the concentration range of 0-50 µg/ml (n =6). Data were analyzed using linear regression. The corresponding slope, Y-intercept and P value were determined 0.028±0.01 $(\mu g/ml)^{-1}$, 0.026±0.036 and less than 0.01, respectively. The concentrations of standard solutions were predicted inversely from the calibration curves and accordingly ODN recoveries and relative standard deviations were calculated which were in the range of %100±15 and less than 6%, respectively. Therefore, the assay was valid for ODN concentration in the range of 1.8 (limit of quantification) to 50 µg/ml.

Phospholipid content was determined after lipid extraction by Bligh & Dyer method (22). Briefly, aqueous phase (sample or HBS as a blank) was mixed with chloroform/methanol (1:2.1). After vortexing, chloroform/HBS (1:1) was added to the above solution. Subsequently, the mixture was centrifuged at 1700g for 5 min. Chloroform phase was assayed for phospholipid by calculation of inorganic phosphorus content according to Ames method (23) using ELISA reader (Tecan-Spectra Rainbow, Austria) for 5 replicates.

ODN accessibility

To determine the amount of accessible ODN in liposomes, ethidium-bromide (Et-Br) exclusion assay was performed (24). Briefly, 4µg of Et-Br was added to 3ml HBS (pH=7.5) containing 2.5µM of cationic liposomal dispersion prepared in this study ODN/Metafectene® complex. After incubation for 5min at room temperature, the fluorescence intensity (F) was measured at λ_{excit} = 540nm and λ_{emit} = 610nm before and after the solubilization with Triton X-100. Fluorimeter was calibrated with HBS containing Et-Br alone (F_0) and Et-Br/free ODN complex (F_{max}). The percentage of accessible ODN was calculated from the Equation1:

$$\%\text{Accessible ODN}=((F-F_0)/(F_{max}-F_0))\times 100 \qquad \text{(Eq. 1)}$$

The percentages of accessible ODN before and after solubilization were compared by paired t-test while unpaired t-test was performed to compare the fluorescence intensities for ODN encapsulated liposome and ODN/liposome complex.

ODN localization in liposome

To investigate the localization of ODN in liposomes and differentiate between ODN complexation and encapsulation, KI solution, as an aqueous quencher, was employed to study the quenching of FITC-ODN fluorescence (25). Then, KI at different concentrations was added to 0.1mM FITC-ODN containing liposome or Metafectene®/ODN complex. Sufficient amount of KCl was added to keep the ionic strength of the medium constant and equal to 1M KI. Fluorescence intensity was measured with a Jenway 6200 fluorimeter (UK) at λ_{excit}=495nm and λ_{emit}=540nm. Data were analyzed according to the Stern-Volmer equation (Equation 2):

$$Ksv = ((F/ F_0)-1)/ Q) \qquad \text{(Eq. 2)}$$

F_0 and F are the fluorescence intensities of samples in the absence or the presence of KI,

respectively. Q is KI concentration and K_{SV}, the Stern-Volmer quenching constant. K_{sv} was compared for free ODN, liposome encapsulating ODN, Triton X-100 solubilized ODN and Metafectene/ODN complex by one-way analysis of variance (ANOVA).

Serum stability of ODN

The biological stability of liposomal ODN was compared with free ODN. Liposome encapsulating or free ODN was incubated with newborn calf serum at 37°C for 6h. Serum enzymes were inactivated at 70°C for 15 min. Triton X-100 was added to the samples to disrupt the liposomes completely. The stability of ODN was assessed by urea denatured 20% polyacrylamide gel electrophoresis (Urea-PAGE) according to the Qiagen instruction for the analysis of oligonucleotides with some modifications. The intensities of the resulted bands were analyzed with UN-SCAN-IT software (Silk Scientific Corporation) after capturing the image of the gel with a HP Scanjet 2400.

Preparation of cytoplasmic models and endosomal membranes

Two parameters were considered in the development of model membranes: lipid composition of the cellular membranes and the pH of the media in which the interaction of liposome with the membranes may occur. Models of early and late endosomal, external and total cytoplasmic membranes were prepared by the method of ether injection (26). Lipid composition for the above mentioned models are shown in Table1. 500µl lipid solution in diethyl ether (0.2 g/ml) was injected rapidly into 10ml HBS (pH=7.5) or citrate buffer (pH=5.5) at 60°C and under homogenization (Heidolph, Germany) at 20000 rpm for 30 min. Residual ether was removed in a Heidolph VV2000 rotary-evaporator (Germany) under vacuum for 2 hours. Liposomes were extruded (Northern Lipids, Vancouver, Canada) 5 times by passing through double-stacked polycarbonate filters (Northern Lipids, Vancouver, Canada) with the pore size of 100 and 200 nm. Lipid recoveries of the models were determined by the phosphate assay of phospholipids.

ODN release from cationic liposome

The amount of released ODN was determined based on fluorescence resonance energy transfer (FRET), self-quenching of FITC-ODN fluorescence while inside the liposomes and de-quenching if encapsulated FITC-ODN was released and diluted in the surrounding media (27). 30µl of FITC-ODN encapsulated liposomes were diluted in HBS (pH=7.5) or citrate buffer (pH=5.5) to the final lipid concentration of 0.1mM. Increasing amounts of the model membranes were admixed with liposome and incubated at 4°C, 25°C,and 37°C for 5, 30, and 60 min. Fluorescence intensity of FITC-ODN was measured at λ_{excit} = 495nm and λ_{emit} = 540nm. FITC-ODN fluorescence correlated linearly with the concentration in the range of the study (data are not shown). Therefore, ODN efflux was determined according to the Equation 1 where F was the fluorescence of sample, F_0 was the background fluorescence corresponding to the liposome alone, and F_{max} was the maximum dequenching after addition of 0.5% Triton X-100 to the liposome. One-way analysis of variance (ANOVA) was performed for point-to-point comparison of ODN efflux among the model membranes.

RESULT AND DISCUSSION

Size and Zeta-potential of cationic liposomes

Ethanol injection method facilitates the interaction of lipids and oligonucleotide due to destabilization of liposomal membrane and finally leads to the formation of heterogeneous oligolamellar vesicles in which ODN is sandwiched between bilayers (11, 13). Liposomes were neutral at physiologic pH with the zeta-potential of -2.49 ± 7.15 mV (mean ± SD, n = 3, Figure1) that didn't differ from zero significantly (P>0.05).

Measurement of liposome size revealed that the particles with the mean diameter and the polydispersity index of 108.4nm and 0.118 were obtained without extrusion.

ODN encapsulation efficiency of cationic liposomes

ODN recovery in final liposomes was calculated to be 69 ± 2.2 (wt%) of initial ODN. During the removal of free ODN through the column, lipids especially PEG-Cer_{20} are not completely recovered. It seems that they are displaced from the liposome upon ODN entrapment (11, 13). Lipid recovery was estimated from the phospholipid contents of liposomes to be 86 ± 4.8 (wt%). The encapsulation efficiency was calculated from the above mentioned recovery data to be 73 % which is equal to 0.18 ODN/lipid (w/w). The encapsulation efficiency appeared to be almost 3 times higher than what obtained by passive encapsulation based on the trapped volume (11, 13).

ODN accessibility

To investigate how ODN was localized in the liposomes, first, the level of accessible ODN was determined by Et-Br intercalation assay and

Table 1. Lipid composition and pH of model cellular membranes. DMPG: dimyristoyl phosphatidylglycerol, EPC: egg phosphatidylcholine.

Model	Lipid composition (%)			pH
	DMPG	EPC	Cholesterol	
Plasma membrane (total bilayer)	16.6	66.8	16.6	7.5
Plasma membrane (external monolayer)	-	83.4	16.6	7.5
Endosomal membrane (early)	16.6	83.4	-	7.5
Endosomal membrane (late)	16.6	83.4	-	5.5

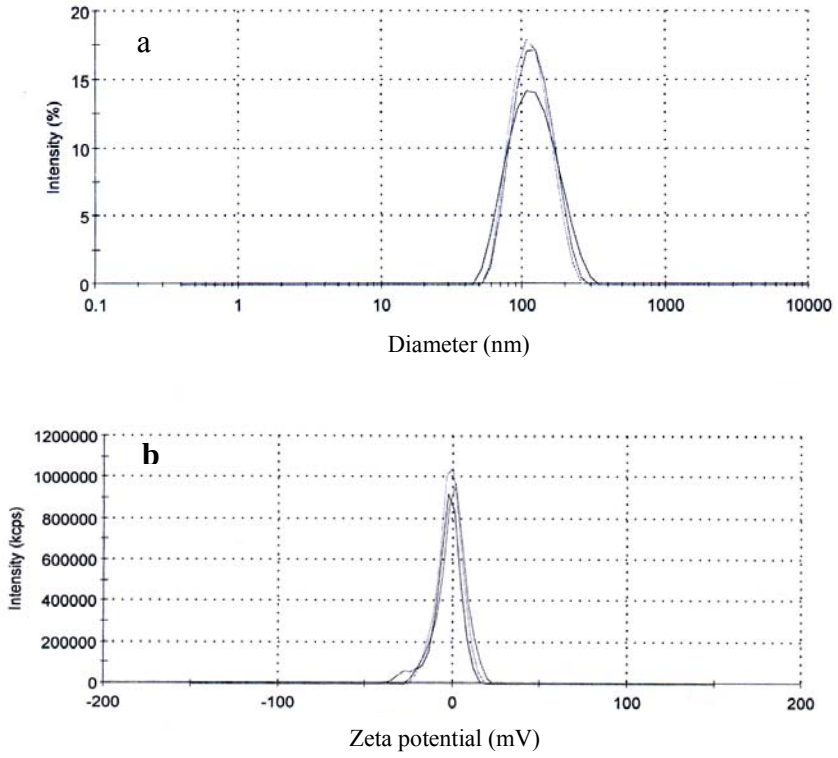

Figure 1. Size (a) and zeta-potential (b) distribution of the antisense oligodeoxynucleotide encapsulating liposome (n=3).

compared with that of Metafectene®/ODN complex. Metafectene® is a commercially-available cationic liposome that is designed to condense ODN to a compact structure which are called lipoplex and to promote the entry of ODN into cells through the mechanism of repulsive membrane acidolysis (RMA) technology; the osmotic destruction of the endosomal membrane (according to the manufacturer's instruction).

Intercalated Et-Br exhibits a strong fluorescence augmentation while fluorescence is quenched. According to the model of Sobell *et al,* Et-Br exists in equilibrium between the ODN intercalated sites and free form in solution (28). Free Et-Br is dramatically quenched by the aqueous solvent. When Et-Br intercalates with ODN, it displays a bathochromic shift and strong enhancement of the fluorescence intensity, as the hydrophobic surrounding of Et-Br allows slow proton transfer to water molecule and leads to a longer life-time for the excited state (28). Inversely, the loss of flexibility in ODN structure upon condensation with cationic liposome (e.g. Metafectene®) changes the bound Et-Br into the free form with the resultant loss in the fluorescence intensity (28).

It was found that 13.6±5.7% and 95.8±2.8% of ODN was accessible in our liposomal preparation before and after solubilization with Triton X-100; while these percentages were 47±2% and 101.3±3% for Metafectence®/ODN complex, respectively. Triton X-100 was able to recover the fluorescence almost completely as the fluorescence intensity did not differ from 100% significantly for both liposome and Metafectence®/ODN complex (P>0.05). It was found that there was 3-4 times more condensation or protection of ODN in our liposomal preparation

in comparison to its complexation with transfectants such as Metafectene®. The underlying mechanism is remained to be elucidated.

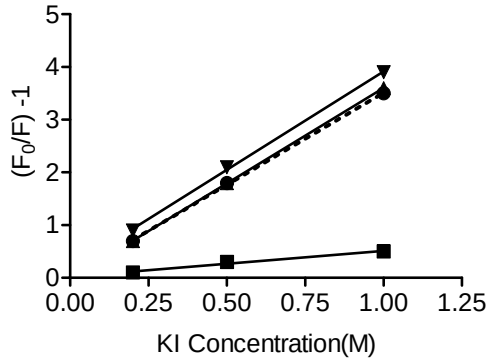

Figure 2. Potassium iodide Stern-Volmer quenching of fluorescein isothiocyanate-conjugated oligodeoxynucleotide (FITC-ODN). Potassium iodide was added to free FITC-ODN (▼), FITC-ODN containing liposome (■), Triton X-100 solubilized liposomes (●) and Metafectene®/oligodeoxynucleotide complex (▲). (See the text for more details.)

ODN localization in liposome

Contrary to intercalated Et-Br, the fluorescence of FITC-conjugated ODN was independent of ODN flexibility. Stern-Volmer equation was applied to correlate the quanta yields of quenching of FITC-ODN and the concentrations of a quencher (KI). The plot is shown in Figure 2 from which the quenching constant (K_{sv}) of the FITC-ODN autofluorescence by KI in different conditions can be calculated. The fluorescence of free FITC-ODN did not change after complexation with Metafectene® (Figure 2) since calculated K_{sv} (3.74±0.11 for free ODN versus 3.49±0.07 for ODN/Metafectene® complex) wasn't significantly different ($P>0.05$). However, K_{sv} dramatically deceased to 0.52±0.05 for our cationic liposome. This quenching effect was not related to the liposome composition as it didn't change with dilution.

These findings imply that FITC-ODN was shielded in our liposome preparation, located inside the liposome and kept out of the reach of the quencher in the aqueous phase. This was confirmed as K_{sv} increased to 3.62±0.02 when FITC-ODN was deshielded through disruption of liposomes with Triton X-100. Conclusively, ODN was encapsulated in our liposomal preparation. ODN encapsulation was differentiated from the complexation of ODN with cationic liposome. Since the fluorescence intensity of FITC-ODN was independent of the flexibility of ODN that decreases through complexation.

Serum stability of ODN

Free ODN was degraded significantly in serum as the intensity of ODN band decreased in Urea-PAGE (Figure 3). Our liposomal preparation was able to protect ODN from nuclease degradation completely (Figure 3). This result complies with the finding that ODN was inaccessible to the nuclease containing medium as it was entrapped inside the liposome and couldn't passively release into the medium. As there was not found any discrete band of degradation products, it was concluded that ODN is fully protected.

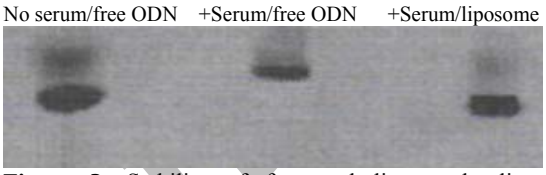

Figure 3. Stability of free and liposomal oligodeoxynucleotide upon incubation with newborn calf serum at 37°C for 6h. Stability was analyzed by urea-denatured 20% polyacrylamide gel electrophoresis.

ODN release from cationic liposome

It has been demonstrated that the efflux of ODN across the membrane of neutral and anionic liposomes is very slow with a half-life of more than 1 week (19). The results of the preliminary study exhibited that the half-life of ODN efflux increased several times as ODN was entrapped between lipid bilayers and immobilized by cationic lipids through electrostatic binding at acidic pH of liposome inner compartment. These data and other supporting results show that the main mechanism of ODN delivery is the interaction of cationic liposome with the cellular membranes.

The interaction of FITC-ODN encapsulated liposome with different models of cellular membranes were compared in this study. The models were developed based on the lipid composition and pH of the cellular membranes. It is known that plasma against endosomal membrane is stuffed with cholesterol. Cholesterol along with neutral lipids constitutes the outer monolayer of plasma membrane while phosphatidylserine (an anionic phospholipid) is mainly located in the inner monolayer of the membrane (29, 19). Therefore, the model of the outer monolayer of plasma membrane was prepared additionally to exhibit the asymmetry of lipid localization within plasma membrane. PH of early endosomes is almost neutral as well as the plasma membrane, but it decreases during maturation of endosome to about 5 within late endosomes (20). Other interfering variables were kept fixed in this study. For example, the models were prepared with a homogeneous and narrow

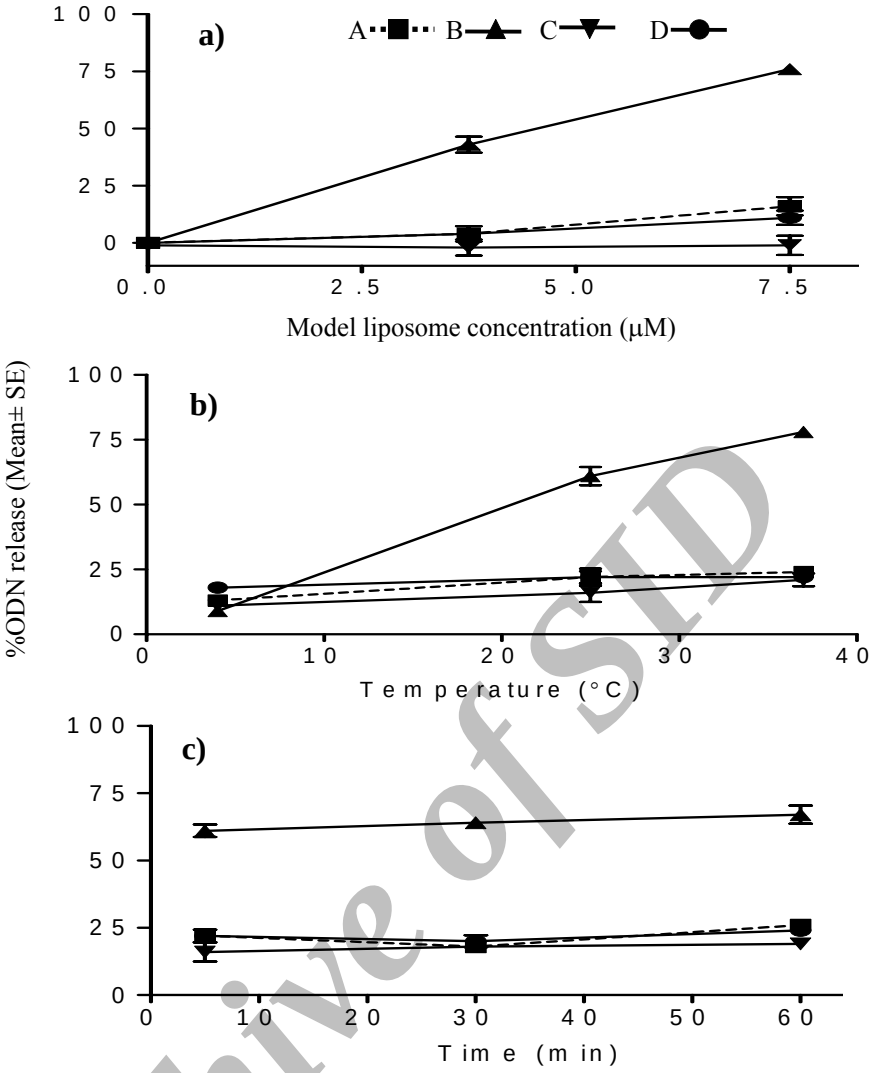

Figure 4. Release of fluorescein isothiocyanate-conjugated oligodeoxynucleotide (FITC-ODN) from liposome upon interaction with model membranes. 0.1 mM oligodeoxynucleotide encapsulated liposome was added to an increasing amount of the models and incubated for 5min at 37°C (**a**) or mixed with 3μM of the model membranes and incubated at an increasing temperature for 5min (**b**) or at 37°C for different incubation times (**c**). A: early endosome, B: late endosome, C: external monolayer of plasma membrane, D: total plasma membrane. n= 6.

size distribution, i.e., the natural curvature variation between plasma and endosomal membranes (difference between cell size and the size of endosomes) was not considered. Our model of endosomal membrane was different from Jaaskelainen's model (20) as the later comprises phosphatidylserine (an anionic lipid) along with neutral lipids and cholesterol, while no cholesterol was incorporated in our model of endosomal membrane.

The presence of a variable amount of free ODN co-existed with the lipoplexes makes it difficult to quantify exactly ODN release through the fluorescence dequenching of FITC-ODN (20). However, the free ODN was isolated thoroughly by gel filteration chromatography in the present study. To validate the method of determination of ODN release, linear regression analysis was performed between the liposome concentration and the fluorescence intensity of FITC-ODN. The slope increased significantly with the medium pH. The fluorescence was fully recovered after solubilization of liposome with 0.5% Triton X-100 (F_{max}). The ratio of F/F_{max} remained statistically constant in the range of 3-30 μl/ml. The fluorescence of FITC-ODN was self-quenched in the acidic pH of the liposome inner compartment or as the fluorescence resonance energy of FITC-ODN could be transferred from one molecule to another if they were situated in a close proximity inside the liposomes.

The incubation of liposome with the vesicular models of the cell membranes resulted in a very significant fluorescence recovery of FITC-ODN

and ODN efflux from liposomes (Figure 4a). The ODN efflux was more pronounced in acidic than neutral medium ($P<0.05$). At the model membrane concentration of 7.5 μM, the percent of ODN release was the highest for the cholesterol-free model of late endosome (76% ± 4.0%) followed by to the anionic model of early endosome (16% ± 2.2%). These findings were in agreement with the cellular study that the antisense activity of ODN was compromised as the pH of endosome was neutralized with chloroquine (7) which emphasizes the pH-sensitivity of ODN release from the liposomes.

Besides, no significant ODN release was found with the models of cytoplasmic membranes; 11 ± 3.1 and -1 ± 4.2 for the total bilayer and the external monolayer of plasma membranes respectively (Figure 4a). The low direct interaction between the cationic liposomes and the model of plasma membrane is possibly due to the cholestrol content of the model of plasma membrane and the incorporation of PEG-Cer_{20} in the liposomes which compromise the interaction of the liposome with the cellular membranes via the extensive inhibition of membrane fusion (7). It is shown in the cellular studies that liposomes were internalized actively into the cells through endocytosis (7, 16) which is in agreement with our model membrane study in which the role of plasma membrane is mainly restricted to the cellular internalization of liposomes. This non-reactivity of the model of plasma membrane with the cationic liposomes guarantees the stability of the liposomes in the cell culture medium.

Figure 4b shows that the ODN release increases with temperature in the range of 4°C to 37°C upon interaction with the model of late endosome significantly ($P<0.0001$), but not the rest of the model membranes ($P>0.05$). For the plasma membranes, it is possibly due to their high cholesterol contents that render the bilayers more rigid with transition temperatures above 40°C. On the contrary, the fluorescence recovery increased at the higher incubation temperatures (25°C and 37°C) for the model of late endosome. The model of late endosome is not only a cholesterol free model, but also geometrically more unstable at acidic pH as determined by turbidimetry; hence, not such a release was observed for early endosome at pH = 7.5 (Figure 4b). This experiment might imply the role of temperature-sensitive passive mechanism in ODN release from the liposomes upon interaction with the model of endosomal membrane. Our finding is confirmed by the cellular studies in which the efficiency of ODN was diminished extensively as the cells were incubated at 4°C (6).

The fluorescence recovery didn't change with the incubation time in the range of 5-60 minutes ($P>0.05$) (Figure 4c). It implies that the interaction was almost fast and was completed within 5 minutes. This result is in agreement with Szoka who investigated the release of ODN from its complex with cationic liposomes by ethidium bromide intercalation assay of accessible ODN. A significant amount of ethidium bromide fluorescence was recovered upon interaction of lipoplex with anionic liposome. This interaction was almost fast and completed within 50 seconds (15). This rapid interaction may explain why time-dependent release mechanism such as diffusion was not determinant for ODN release from the cationic liposomes.

To the best of our knowledge, there is not such a modeling of cellular membranes published in the literature. Similarly, Jaaskelainen, *et al.* studied the release of FITC-ODN from its complex with cationic liposome. They found that fluorescence of FITC-ODN was dequenched upon interaction with an endosomal model membrane especially at acidic pH of 5-6, for DOPE-containing cationic liposomes and the negative/positive charge ratio of less than 1.5 (29). Our results are consistent with the above mentioned study, though different endosomal membranes were employed. Therefore, it seems the pattern of release could be the same for the ODN encapsulated liposomes and lipoplexes; while the underlying mechanism may differ.

Conclusively, our data implies that ODNs could be released from cationic liposomes upon interaction with the endosomal membranes at acidic pH while the liposomes are stable and do not interact with plasma membrane. This is in agreement with data from cell culture studies (16, 17) and shows that interaction of liposomes with cellular membranes can be modeled by lamellar structures. Such models, when optimized, can be used as excellent alternatives or compliments for cell culture studies. These models also provide opportunities for studying the individual variables of liposome-cell interactions.

CONCLUSION

Our data showed that it is possible to encapsulate efficiently and protect ODN thoroughly by the presented method of liposome preparation. These properties along with the small, neutral and sterically-stabilized characters of the liposomes are promising for an in-vivo delivery of antisense drugs. Besides, it was understood from the model membrane study that ODN release is triggered by cholesterol-free model of anionic endosomal membrane especially at acidic pH. Our observations were in agreement with the previously published cellular study that the antisense activity of ODN was compromised as the pH inside the endosomal compartment was

neutralized by chloroquine. Therefore, we believe that this model could serve as a screening tool to predict the probable release behaviors of such liposomes in cellular studies. However, further evaluation of the intracellular delivery of ODN in cell culture is required.

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