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Modeling cytoplasmic release of encapsulated oligonucleotides from cationic liposomes

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Abstract

Transfection activity of antisense oligodeoxynucleotides (ODN)-loaded cationic liposomes is mainly restricted by uptake and ODN release into cytoplasm, which is difficult to evaluate in cell culture studies. Well-designed models of cellular membranes, aim of the present study, might facilitate investigation of such processes. In this investigation, a phosphorothioate ODN was actively encapsulated in a DODAP-containing cationic liposome by ethanol injection with 73% efficiency. ODN release was determined by fluorescence dequenching of FITC-ODN upon incubation of liposomes with early endosomal (EE), late endosomal (LE) and plasma membranes (PM) models. LE provided the highest release (up to 76%) in a temperature-dependent manner. Release by EE (<16%), total PM (<11%) and PM external layer (≈0) were not temperature sensitive. These differences are attributed to lipid charge, chain mobility, critical packing parameter and cholesterol content of the models. Intracellular distribution of FITC-ODN, determined by fluorescence microscopy and flowcytometry in the presence and absence of sodium azide, confirmed that liposomes were internalized mainly via endocytosis; hence inability of our PL models to simulate such active processes. Instead, release of ODN from endosomes into cytoplasm was pH-sensitive and in good agreement with model membrane studies in terms of amount and mechanism.

Keywords: Gene delivery; Cationic liposome; Antisense oligodeoxynucleotide; Encapsulation; Model membrane; Cytoplasmic release; Cellular uptake

1. Introduction

The use of antisense oligodeoxynucleotides (AsODNs) as both research tools and therapeutic molecules has emerged as 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 (Crooke, 1998; Dias and Stein, 2002).

The major limiting step in oligodeoxynucleotide (ODN) application includes the inefficient delivery of ODN to cells and the poor bioavailability of ODN to intracellular targets. The polyanionic nature and the large molecule of ODN render them practically impermeable to cell membrane and consequently their biological activity is significantly compromised. To overcome this obstacle, rationally designed carriers are required (Akhtar et al., 2000). 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 entrapment efficiency has limited their use. Instead, cationic liposomes entrap nucleic acids efficiently through the formation of complexes which are called lipoplexes (Templeton et al., 1997). Despite of the enhanced cellular association of lipoplexes, their serum instability and rapid clearance, within a few minutes of their administration into blood stream, has restricted their pharmaceutical application (Pastorino et al., 2001).

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 (Radler et al., 1997). This geometry, which is a kind of ODN encapsulation rather than complexation with cationic liposomes, is of special interest because it protects ODN almost completely and functions more efficiently as a transfecting agent (Lasic et al., 1997). Accordingly, some liposome specialists studied the above-mentioned approach of ODN encapsulation in cationic liposomes. Efficient encapsulation has been achieved by calcium chloride or ethanol

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assisted destabilization of liposomes (Bailey and Sullivan, 2000; Maurer et al., 2001; Semple et al., 2001).

Lipid-based carriers (e.g. lipoplexes) are usually internalized into the cells via endocytosis (Friend et al., 1996; Xu and Szoka, 1996). Subsequently, ODN is released from endosomal compartment into cytoplasm through membrane destabilization or fusion mechanisms, nevertheless the lysosomal degradation of ODN occurs as pH falls in endosomal compartment (Monkkonen and Urtti, 1998; Harashima et al., 2001). The destabilization reactions that lead to ODN release from liposomes into cytoplasm are the most significant steps in defining transfection efficiency (Bally et al., 1999).

ODN delivery to cells and their release into cytoplasm is usually evaluated through cell culture studies using fluorescence techniques such as fluorescence microscopy and flow cytometry of fluorophore-conjugated ODN. Such studies are time-consuming and expensive. They require special facilities, techniques and containments. In order to facilitate such evaluation, it was decided here to study the release of AsODNs from cationic liposomes using models of cellular membranes. To perform this investigation, the vesicular models of plasma membrane, and early and late endosomal membranes, with which liposomes may interact during internalization into cells, were developed. The models were designed based on physicochemical properties of the above-mentioned membranes. Subsequently, fluorophore-conjugated ODN release from cationic liposomes was studied upon interaction with the model membranes by fluorimetry. To evaluate the predictability of the models, the cytoplasmic release of FITC-ODN from the liposomes was studied in cell culture using fluorescence microscopy and flow cytometry. To the best of our knowledge, not such kind of modeling has been reported previously in the literature.

2. Materials and methods

2.1. Lipids

1,2-Dioleoyl-3-dimethylammoniumpropane (DODAP), 1-*o*-(2′-(ω-methoxypolyethylene glycol) succinoyl)-2-*N*-dodecanoylsphingosine (PEG-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 was purchased from Sigma Chemical Company (St. Louis, MO). Metafectene® was received as a gift from Biontex Company (Germany).

2.2. Oligonucleotides

A 20-mer phosphorothioate modified AsODN against protein kinase C-α, 5′-TsCsCs AsTsGs AsCsGs AsAsGs TsAsCs AsGsCs CsGs-3′ (Song et al., 2003), was synthesized by TIB (Berlin, Germany). 5′-Fluorescein isothiocyanate-conjugated phosphorothioate ODN (FITC-ODN) was synthesized and purified by Synthegen (Houston, USA).

2.3. Other materials

Newborn calf serum, Dulbecco's modified Eagle's medium, trypan blue stain 0.4% and trypsin–EDTA solution were purchased from Gibco BRL (Gaithersburg, MD). Polycarbonate filters (100 and 200 nm pore size) and Sephadex G-25 were supplied by Northern lipids (Vancouver, Canada) and Amersham (Sweden), respectively. Triton X-100, puromycin, sodium azide, 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI), paraformaldehyde and monensin (sodium salt) were obtained from Sigma Chemical Company (St. Louis, USA). All other reagents were of analytical grade.

2.4. Preparation of ODN encapsulated liposome

Liposome was prepared by a well-designed ethanol injection method (Maurer et al., 2001; Semple et al., 2001). Lipid solution in absolute ethanol was injected gently under vigorous vortexing into a mixture of ODN/FITC-ODN (9:1) in citrate buffer (pH 4). Lipid was composed of DSPC/Cholesterol/PEG-Cer_{20}/DODAP (20:45:10:25 mol%). DODAP, a cationic lipid with a tertiary amine functional group, was incorporated in cationic liposome to entrap ODN actively at low pH. Dialysis was then performed first against citrate buffer (pH 4) at room temperature and subsequently against HEPES buffered saline (HBS, pH 7.5) at 4 °C to ensure the complete removal of ethanol. At pH 7.5, DODAP becomes charge-neutral and the bound ODNs to the external surface of liposome dissociate and are removed during dialysis. Residual free ODNs were removed by Sephadex G-25 gel filtration chromatography through a previously conditioned column (25 cm × 1 cm) with HBS (pH 7.5). Liposomes were kept at 4 °C until use within 1 month.

2.5. Particle size and zeta-potential determination

Particle size and zeta-potential of liposome were determined by Malvern Zetasizer (UK) after 20-fold dilution with HBS. Measurements were performed at 25 °C, viscosity of 0.88 cP and reflex index of 1.33.

2.6. ODN encapsulation efficiency in cationic liposome

ODN encapsulation efficiency was expressed as recovered ODN/recovered lipid weight ratio. ODN and lipid recoveries were determined separately as follows.

ODN content was determined by UV-spectrophotometry (Shimadzu, Japan) at 260 nm after liposome solubilization in chloroform/methanol (1:2.1) using a calibration curve in the range of 0–50 μg/ml. The percent of recovered ODN was calculated accordingly and was expressed as mean ± S.E. ($n = 6$).

Phospholipid content was determined after lipid extraction (Bligh and Dyer, 1959). 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 1700 × *g* for 5 min. Chloroform phase was assayed for phospholipid calculated from inorganic phosphorus content using a calibration curve in the

range of 0–50 μg of EPC (Ames, 1966). Accordingly, 30 μl of 10% Mg $(No_3)_2$ solution in absolute ethanol was added to samples. The mixtures were boiled until the brown fumes disappeared. 300 μl HCl solution (0.5 M) was added to the samples and boiled for another 15 min. Ammonium molybdate (600 μl, 0.42%) was mixed with 100 μl of freshly prepared ascorbic acid solution (10%) and added to the samples and incubated at 45 °C for 20 min. Absorbances were measured at 680 nm by ELISA reader (Tecan-Spectra Rainbow, Austria). The percent of lipid recovery was determined based on the phospholipid content and was expressed as mean ± S.E. ($n = 5$).

2.7. ODN localization in cationic liposome

ODN localization in cationic liposome was investigated in comparison with Metafectene®/ODN lipoplex and free ODN. Lipoplexes were prepared according to the manufacturer's instruction. Briefly, 60 μl of 2% Metafectene® solution and 25 μl of 50 μM ODN:FITC-ODN solution (9:1) were diluted separately with distilled water to 100 μl in polystyrene microtubes. Diluted ODN solution was admixed with the diluted Metafectene® and incubated for 15 min at ambient temperature. FITC-ODN/Metafectene® complexes were then diluted with 800 μl HBS to 1 ml.

To differentiate ODN encapsulation from complexation with cationic liposome, KI solution was employed as an aqueous quencher to study the quenching of FITC-ODN fluorescence (Guilbault, 1990). KI at different concentrations was added to 50 nM FITC-ODN either alone, condensed through complex formation with Metafectene® or in our liposome preparation. Sufficient amount of KCl was added to keep the ionic strength of the medium constant and equal to 1 M KI. Fluorescence intensity was measured with a Jenway 6200 fluorimeter (UK) at $\lambda_{excit} = 495$ nm and $\lambda_{emit} = 540$ nm. Data were analyzed according to the Stern–Volmer equation (Eq. (1)):

$$K_{SV} = \frac{(F/F_0) - 1}{Q} \quad (1)$$

where F_0 and F are the fluorescence intensity of sample in the absence and the presence of KI, respectively, Q, KI concentration and K_{SV}, the Stern–Volmer quenching constant.

2.8. Preparation of cytoplasmic and endosomal model membranes

Cytoplasmic and endosomal models were developed based on the lipid composition of the cellular membranes and the pH of surrounding media (Table 1). It is well known that, opposite to endosomal membranes, plasma 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 (Baszkin et al., 2000). Therefore, in addition to the whole plasma membrane model, a model for the outer monolayer of plasma membrane was also prepared to exhibit the asymmetry of lipid localization (Table 1). PH of early endosome and plasma membrane is almost neutral, but it decreases during endosome maturation to about 5 within late endosomes. Therefore, the models of early endosomal and cytoplasmic membrane were prepared at pH 7.5 and that of late endosome at pH of 5.5 while its composition was kept the same as early endosomal membrane.

Table 1
Lipid composition and pH of the models of cellular membranes

Type of model	Lipid composition (wt%)			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

Models were prepared by the method of ether injection (Deamer and Bangham, 1976). Lipid solution in diethyl ether (0.2 g/ml) was injected rapidly into HBS (pH 7.5) or citrate buffer (pH 5.5) at 60 °C and under homogenization (Heidolph, Germany) at 20,000 rpm for 30 min. Residual ether was removed in a Heidolph VV2000 rotary-evaporator (Germany) under vacuum for 2 h. Liposomes were extruded (Northern Lipids, Canada) five times through double-stacked polycarbonate filters (Northern Lipids, Vancouver, Canada) with the pore size of 100 and 200 nm. The size of the prepared vesicles was kept constant and the size variation between plasma and endosomal membranes was disregarded to minimize the number of interfering factors. Lipid recoveries of the models were determined based on inorganic phosphorus content (Ames, 1966) as described earlier.

2.9. ODN release from cationic liposomes

The amount of released ODN was determined based on fluorescence resonance energy transfer (FRET), the self-quenching of FITC-ODN fluorescence inside the liposomes especially at acidic pH and the dequenching if encapsulated FITC-ODN was released and diluted in the surrounding media (Struck et al., 1981). Accordingly, 30 μl of FITC-ODN encapsulated liposomes were diluted in HBS (pH 7.5) or citrate buffer (pH 5.5) to the final lipid concentration of 0.1 mM. Increasing amounts of the model membranes were then admixed with the liposomes and incubated at 4, 25, and 37 °C for the different times of 5, 30, and 60 min. Fluorescence intensity of FITC-ODN was measured at $\lambda_{excit} = 495$ nm and $\lambda_{emit} = 540$ nm and ODN efflux was determined according to Eq. (2):

$$\%\ \text{released ODN} = \frac{F - F_0}{F_{max} - F_0} \times 100 \quad (2)$$

In this equation, F is the sample fluorescence, F_0 the background fluorescence corresponding to the liposomes alone, and F_{max} is maximum dequenching after addition of 0.5% Triton X-100 to the liposomes. The percent of released ODN was expressed as mean ± S.E. ($n = 6$).

2.10. Cell culture and transfection

A stably transfected murine fibroblast NIH-3T3 with EWS/fli-1 fusion gene was used as a model of Ewing cell. Puromycin-resistant transformed cells (Ewing cells) were grown selectively in Dulbecco's modified Eagle's medium and 10% newborn calf serum supplemented with 2.5 μg/ml puromycin. Cells were maintained at 37 °C in a humidified atmosphere containing 5% CO_2 incubator (Heraeus, Germany). Viable cells were counted by 0.4% trypan blue stain under light-inverted microscope (Leica, Germany).

In a six-well plate (NUNC, Denmark), 300,000 cells/well were seeded. After 1 day incubation at 37 °C/4%CO_2, the cells were treated with 300 nM free or liposomal FITC-ODN either at 4 °C or 37 °C/4%CO_2 for 4 h. Meanwhile, some cells were co-treated with sodium azide (10 mM). After incubation, the culture medium was removed and the cells were washed twice with cold Hank's buffered saline solution and processed as follows.

2.11. Flow cytometry and fluorescence microscopy

Cellular uptake and intracellular distribution of liposome encapsulating FITC-ODN were evaluated quantitatively and qualitatively by flow cytometry and fluorescence microscopy, respectively. To perform flow cytometry, the cells were detached from the plates with 500 μl trypsin–EDTA/well. Subsequently, trypsin was neutralized and the cells were re-suspended in 5 ml of serum-containing culture medium. Cell suspensions were centrifuged at 70 × *g* for 5 min to sediment the cells. Cell pellets were rinsed with cold PBS and reconstituted in 500 μl of either cold PBS or monensin solution (20 μM) in PBS. Cells were kept on ice until the measurement of the cell-associated fluorescence by Facscalibur dual laser flowcytometer (Becton Dickinson, USA). Cell Quest software (Becton Dickinson, USA) was used for data acquisition through FL1 (530 nm) and analysis for 10,000 gated events. Cell-associated fluorescence intensities were expressed as the average of these measurements ± S.E. ($n = 3$).

For fluorescence microscopy, cells were fixed with paraformaldehyde (3.7%) in PBS at 4 °C for 20 min, followed by washing with PBS. Cells were permeabilized with 1 ml Triton X-100 (0.4%) for 5 min and washed again with PBS. Subsequently, the nucleus was stained with DAPI (50 μM) for 20 min. DAPI solution was decanted and replaced with either cold PBS or monensin solution in PBS. Cellular internalization of liposomal FITC-ODN was studied by Axiovert fluorescence microscope (Zeiss, Germany) using Axiovision software (Zeiss, Germany).

2.12. Statistical analysis

Means of Stern–Volmer quenching constants, the percent of released ODN, and the cell-associated fluorescence intensities were compared by one-way analysis of variance (ANOVA) and followed up by Tukey test. Data were analyzed with Graphpad Prism software (San Diego, CA) and the differences were considered significant when $P < 0.05$.

3. Results

3.1. Physicochemical properties of cationic liposomes

The zeta-potential of liposomes at physiologic pH was found to be −2.49 ± 7.15 mV (mean ± S.D., $n = 6$) that did not differ from zero significantly ($P > 0.05$); therefore, the liposomes can be considered neutral. However, they are still called cationic based on their composition and as it is usual in the field. Size measurement revealed that the ethanol injection method has provided particles with a mean diameter of 108.4 nm and a polydispersity index of 0.118 without performing any sizing procedure (e.g. extrusion).

It was calculated that 69 ± 2.2 (wt%) and 86 ± 4.8 (wt%) of initial ODN and lipid contents were recovered from the liposomes, respectively. ODN encapsulation efficiency was calculated from the above-mentioned recovery data to be 73% that equals to 0.18 ODN/lipid (w/w).

Stern–Volmer equation (Eq. (1)) was applied to correlate the quantum yields of quenching of FITC-ODN fluorescence and the concentrations of the quencher (KI). The pattern of ODN localization was studied by comparing the quenching constants (K_{sv}) of FITC-ODN fluorescence at different conditions (Fig. 1). It was found that the complexation of free FITC-ODN with Metafectene® did not affect K_{sv}, as the corresponding calculated values (3.74 ± 0.11 for free FITC-ODN and 3.49 ± 0.07 for FITC-ODN/Metafectene® complex) were not significantly different ($P > 0.05$). On the contrary, K_{sv} dramatically decreased to 0.52 ± 0.05 for our cationic liposomes, but increased significantly to 3.62 ± 0.02 as FITC-ODN was de-shielded after disruption of liposomes with Triton X-100.

3.2. ODN release from the liposomes (model membrane study)

The release of encapsulated ODN was studied upon incubation of the liposomes with the models of cellular membranes.

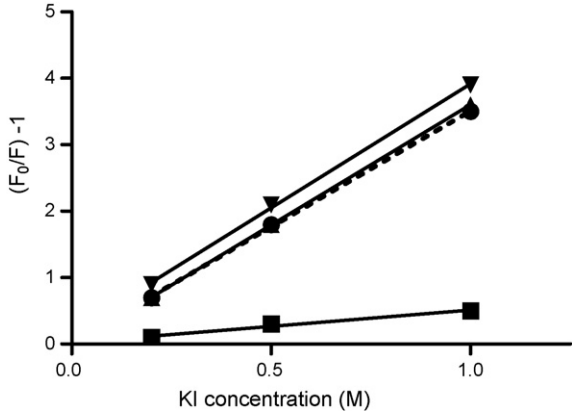

Fig. 1. Potassium iodide quenching of fluorescein isothiocyanate-conjugated oligodeoxynucleotide (FITC-ODN). Potassium iodide was added to free FITC-ODN (▼); FITC-ODN containing liposomes (■); Triton X-100 solubilized liposomes (●); Metafectene®/FITC-ODN complex (▲). The slopes provide Stern–Volmer quenching constants.

The percent of released ODN was determined based on our observations that FITC-ODN fluorescence was quenched as the fluorescence resonance energy of FITC-ODN could be transferred from one molecule to another in its close proximity especially at the acidic pH of liposome inner compartment. FITC-ODN fluorescence was fully recovered after disrupting the liposomes with 0.5% Triton X-100 (F_{max}). The relationship between the fluorescence intensity (F) and the concentration of FITC-ODN encapsulated liposomes was linear and the ratio of F/F_{max} remained statistically constant in the range of 3–30 μl of the liposomes. However, the slope increased significantly with the medium pH in a range of 5–8. Therefore, different standard curves, based on the medium pH, were used for determining the percent of released ODN according to Eq. (2). This method does not require isolation of free ODN from that still remained inside the liposomes.

The release experiments were performed at two different concentrations of model membranes (3.75 and 7.5 μM). Incubation of the liposomes with the cellular membrane-modeling vesicles resulted in a concentration-dependent fluorescence recovery of FITC-ODN (ODN efflux from the liposomes, Fig. 2a). At 7.5 μM model membrane concentration, the amount of released ODN was the highest for the cholesterol-free model of late endosome (76 ± 2.2%) followed by the anionic model of early endosome (16 ± 4.0%) and total cytoplasmic membrane model (11 ± 3.1%). The percent of ODN release from the model of the external monolayer of plasma membrane (−1 ± 4.2%) was the least and not significant ($P > 0.05$). At 3.75 μM model membrane concentration, ODN release was less than that of 7.5 μM, but followed the same order (late endosomal > early endosomal > plasma membranes), however, it was only found significant for the models of late endosomal membrane (Fig. 2a).

The effect of incubation time was also studied. Results showed that the calculated fluorescence recovery did not change significantly with incubation time in the range of 5–60 min for all the model membranes (Fig. 2b). ODN release was insignificant in the absence of the vesicular models at both acidic (Fig. 2b) and neutral pH (negative controls) over the studied time period.

Fig. 2c shows that ODN release increases with temperature in the range of 4 to 37 °C significantly ($P < 0.0001$) upon incubation with the late endosomal-modeling vesicles. However, ODN release did not change significantly ($P > 0.05$) with the incubation temperature for the models of early endosome and plasma membranes in the range studied.

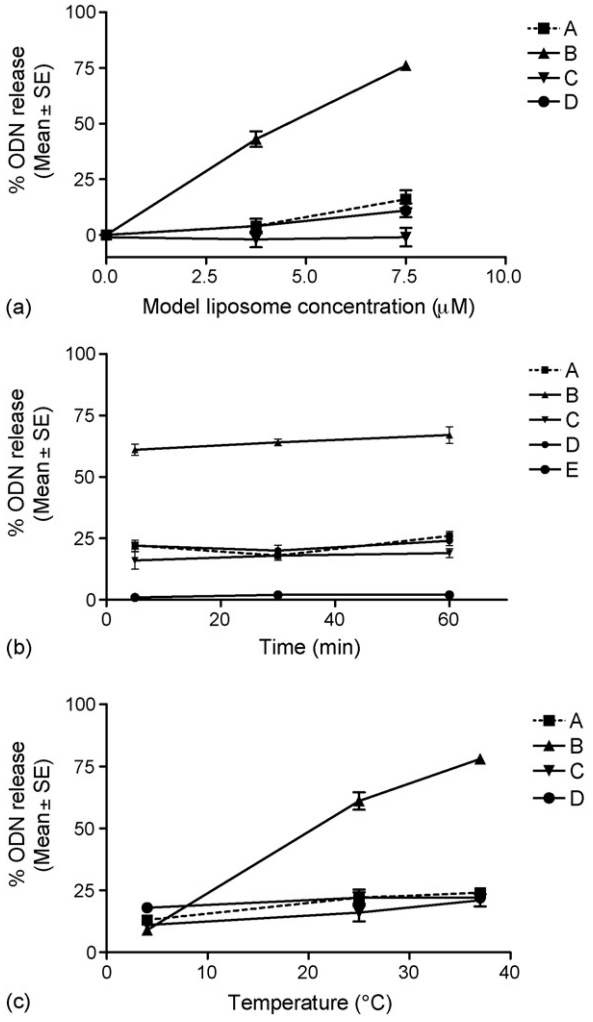

Fig. 2. Release of fluorescein isothiocyanate-conjugated oligodeoxynucleotide from the liposomes upon interaction with the model membranes. 0.1 mM oligodeoxynucleotide encapsulated liposomes were added to an increasing amount of the model membranes and incubated for 5 min at 37 °C (a) or mixed with the model membranes (3 μM) and incubated at 37 °C for different incubation durations (b) or at an increasing temperature for 5 min (c). A–D correspond to the models of cellular membranes and E is the negative control in the absence of the models at acidic pH ($n = 6$). A: early endosome; B: late endosome; C: external monolayer of plasma membrane; D: total plasma membrane.

3.3. Flow cytometry and fluorescence microscopy

Flow cytometric analysis of the cellular uptake of liposomal FITC-ODN revealed that ODN uptake increased several times after encapsulation of free ODN in cationic liposomes. Cell-associated fluorescence intensity was observed to be 9.0 ± 0.5 and 84.3 ± 3.9 for free and liposomal FITC-ODN, respectively (Fig. 3). Cellular uptake of liposomal ODN was extensively inhibited at 4 °C as the cell-associated fluorescence intensity decreased to 22.82 ± 1.98 significantly ($P < 0.0001$). In addition, co-incubation with sodium azide, an inhibitor of endocytosis, decreased the fluorescence intensity of cells to 29.01 ± 4.39 as well. The fluorescence intensity of the internalized FITC-ODN, which is known to be quenched in an acidic environment, was enhanced significantly to 112.46 ± 5.24 after post-incubation of the transfected cells with monensin-mediated pH equilibration (Fig. 3). Co-incubation of the cells with chloroquine, a lysosomotropic agent known to interfere with endocytosis by neutralizing endosomal and lysosomal pH, decreased the cell-associated fluorescence intensity to 46.2 ± 3.9 significantly (Fig. 3). Therefore, an acidic medium inside the endosomal compartment was required for the release of ODN.

It was observed by fluorescence microscopy that fluorescein fluorescence was diffusively scattered in the cytoplasm and less in the nucleus (Fig. 4). The monensin-mediated fluorescence enhancement was recognized here as a punctured distribution in cytoplasm next to the nucleus membrane (Fig. 4). This vesic-

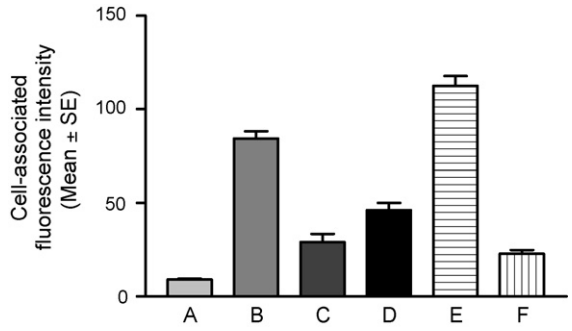

Fig. 3. Flow cytometric analysis of the cellular uptake of fluorescein isothiocyanate-conjugated oligodeoxynucleotide (ODN). Cells were incubated for 4 h with 300 nM free ODN (A) and liposomal ODN at 37 °C alone (B) or with either 10 mM sodium azide (C) or 200 μM chloroquine (D). Some transfected cells were treated again with 20 μM monensin for 20 min (E). Incubation with liposomal ODN at 4 °C was considered as a negative control (F). Experiment was carried out immediately after sample preparation ($n = 3$).

ular distribution is most likely due to an endocytotic uptake of liposomal ODN into the cells.

4. Discussion

Ethanol injection method of liposome preparation facilitates the interaction of lipids and ODNs due to the destabilization of liposomal membrane and finally leads to the formation of heterogeneous vesicles in which ODN is sandwiched between bilayers (Semple et al., 2001). This mechanism explains high ODN encapsulation efficiency of 73% (ODN/lipid = 0.18) in our cationic liposomes that appears to be almost three times higher than what is obtained by passive encapsulation based on the trapped volume (Maurer et al., 2001).

K_{sv} values were used to study the pattern of ODN localization, whether encapsulated inside or adsorbed on the surface of the liposomes. Results revealed that complexation of ODN with Metafectene® did not keep FITC moiety away from the quencher (KI), and therefore, no difference between K_{sv} of free FITC-ODN and FITC-ODN/Metafectene® complex

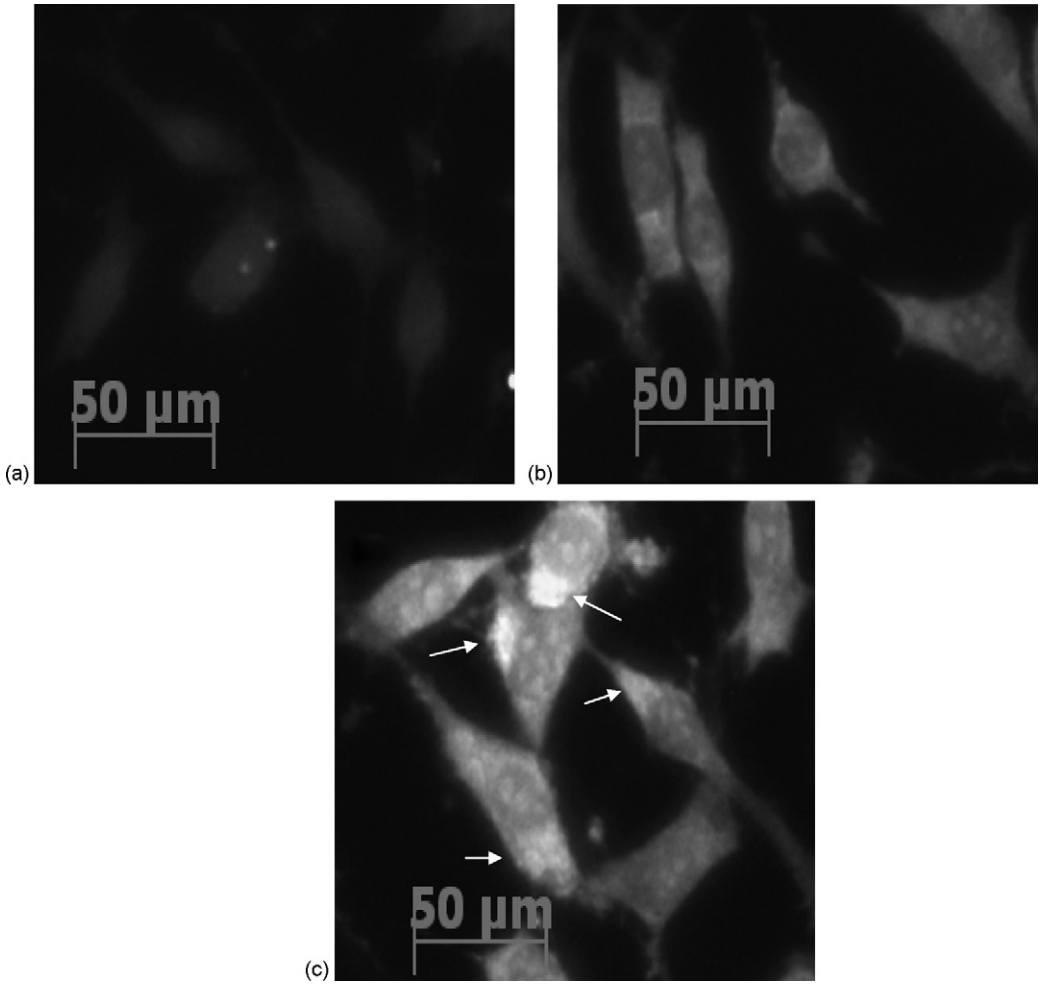

Fig. 4. Cellular distribution of fluorescein isothiocyanate-conjugated oligodeoxynucleotide (FITC-ODN) by fluorescence microscopy. Cytoplasmic distribution of fluorescein fluorescence was demonstrated after 4 h incubation with 300 nM liposomal FITC-ODN at 4 °C (a) or 37 °C (b). To avoid underestimating FITC-ODN fluorescence, the cells were treated with liposomal FITC-ODN followed by 20 μM monensin (c). The unreleased FITC-ODN inside late endosomes is indicated by arrows.

was observed (Fig. 1). These data show that ODN is mainly adsorbed on the surface and available to the quencher in FITC-ODN/Metafectene® system. But, K_{sv} dramatically decreased for our cationic liposomes. This quenching effect was not related to the liposome composition as it did not change with liposome dilution. These findings imply that FITC-ODN must be shielded in our liposomal preparation, located inside the liposomes and kept out of the reach of the quencher in the aqueous phase. This was confirmed as K_{sv} increased significantly to that of free FITC-ODN after disruption of the liposomes with Triton X-100 (Fig. 1).

Our results showed that ODN release from the cationic liposomes was insignificant in the absence of the vesicular models (Fig. 2b) over the studied time period, with a half-life of more than 10 days at ambient temperature, evaluated roughly from our preliminary studies. This might be due to ODN immobilization between lipid bilayers through electrostatic binding to cationic lipids at acidic pH. In agreement to this finding, it has been shown that the efflux of ODN out of neutral and anionic liposomes is very slow with a half-life of more than 1 week (Akhtar et al., 1991). This behavior shows that passive diffusion through lipid bilayers plays a minor role in the release of encapsulated ODN from liposomes and destabilization reactions with the cellular membranes are required for the cytoplasmic release of ODN as discussed by Bally et al. (1999). This argument supports our hypothesis in using vesicular models to evaluate intracellular release of ODN from the liposomes.

It was revealed that, at both concentrations of model membranes, incubation of the liposomes with the late endosomal model provided the highest amount of ODN release (up to 76%) that could be mainly due to its acidic pH as discussed below. Induced positive charge on the liposomal membrane at the acidic pH of late endosome, through the protonation of the pH-sensitive lipid (DODAP), might facilitate the electrostatic interaction of the liposomes with the anionic face of endosomal membrane as discussed by Bally et al. (1999). Besides, negative charge density of DMPG and its head group volume decrease at acidic pH and, accordingly, its critical packing parameter (CPP) increases. This makes the vesicles more susceptible to change from liposomal to reverse hexagonal phase (Burger, 2000) that might affect liposome–model interaction. The early endosomal membrane also caused higher release than those of plasma membrane models, though the difference was not significant (Fig. 2a). This might show that higher rigidity induced by cholesterol in the plasma membrane-modeling vesicles also plays role to lower the interaction of these vesicles in comparison to endosomal-modeling systems.

Regardless of the amount of released ODN, the process was completed in less than 5 min and did not show any difference with those incubated for 60 min, which indicate that the process requires a trigger (Fig. 2b). On the other hand, the amount of released ODN was increased in all systems with an increase in the model membrane/liposome ratio (Fig. 2a). Both of these observations confirm the above-mentioned argument about the importance of membrane–liposome interaction (trigger) in the release of ODN from the liposomes.

Parallel to the model membrane study, and to evaluate the applicability of the model membranes, ODN release was determined in cell culture by flow cytometry and fluorescence microscopy as well. It was shown that the enhancement of ODN uptake was reversed extensively with sodium azide. This finding emphasizes endocytosis as the main mechanism of ODN entry into the cells. Different factors including incorporation of PEG-conjugated lipids (e.g. PEG-Cer_{20}) prevent the fusion of the liposomes with cytoplasmic membranes (Song et al., 2002). Considering the cell-associated fluorescent intensities, our calculations indicate that the level of inhibition by sodium azide at 37 °C is about 80–90%. These results show that such active processes cannot be modeled by simple liposome–vesicle interaction. On the other hand, the low interaction of the models of plasma membrane and the liposomes might explain the minor role of passive processes like fusion in the cellular uptake of the liposomes.

Cell studies showed that decreasing the temperature from 37 to 4 °C causes a dramatic inhibition of cell-associated fluorescence, comparable to that of sodium azide (Fig. 3). This might show that a temperature-sensitive passive mechanisms (e.g. fusion) does not play an important role in the cellular entry of liposomal ODN. Model membrane data also support such an argument as ODN release upon liposome incubation with the plasma membrane models did not change over 4–37 °C (Fig. 2c). It is possibly due to the high cholesterol contents of plasma membrane-modeling vesicles that render the bilayers more rigid with transition temperatures of above 40 °C.

ODN release was temperature-dependent for the model of late endosome, though it did not change with temperature for the early endosome-modeling vesicles. Mobility of lipid hydrocarbon chains increases upon heating and subsequently the models favor reversed hexagonal phase (Larsson and Lundstrom, 1976) due to its higher CPP (Burger, 2000). Acidic pH of late endosome-modeling vesicles further increases CPP upon decreasing DMPG head group negative charge density (volume) that might facilitate the phase transition and the interaction of the late endosomal model with the liposomes; hence temperature-sensitivity of late endosomal model membrane-induced ODN release.

Comparing the average of cell-associated fluorescence intensities for the liposomal ODN incubated cells with or without monensin post-incubation exhibited that more than one-third of internalized FITC-ODNs were not detected normally as they resided in a deep acidic compartment and did not release into cytoplasm after 4 h transfection. This finding complies with the extent of ODN release with the model of late endosome.

To show the importance of intra-endosomal pH in the cytoplasmic release of encapsulated ODN, we conducted flow cytometric studies in the cells co-incubated with chloroquine and found that it prevents cytoplasmic ODN release (Fig. 3). Weak bases such as chloroquine are generally used to elevate the pH inside endosomes (Ciftci and Levy, 2001). The possible role of acidic pH inside the endosome could be explained according to DODAP, an ionizable amino-lipid, which is neutral at physiologic pH, but becomes cationic at acidic pH of late endosome (Maurer et al., 2001). The positive charge facilitates

the electrostatic interaction of the liposomes with anionic lipids of endosomal membrane and subsequently the release of ODN into cytoplasm from the late endosomal compartment, a process that could be diminished by chloroquine-induced pH augmentation. Besides, chloroquine can also act as calmodulin antagonists and subsequently inhibits late endosome–lysosome interaction (Pryor et al., 2000). Contrary to what was found here, these mechanisms are generally used to enhance ODN transfection efficiency by liposomal carriers (e.g. LipofectAMINE®) through inhibition of endosome maturation and lysosomal degradation of ODNs (Ciftci and Levy, 2001). This dissimilarity may be raised from that LipofectAMINE® is naturally a poly-cationic liposome and the elevation of pH by chloroquine does not change the positive charge density of LipofectAMINE® profoundly. Instead, it decreases the lysosomal degradation of ODN at the elevated pH of endosome and enhances LipofectAMINE® transfection efficiency. Other mechanisms might also be involved that requires further investigation. Higher ODN release at lower pH values was also demonstrated in our model membrane experiments as the ODN efflux was significantly higher (by about five times) in the acidic medium of late endosomal membrane than that of the neutral system of early endosomal membrane. This shows that vesicular membranes might be able to model ODN release from endosomes. It has been shown that chloroquine does not affect cell-free system on endosomal model interaction (Lenhard et al., 1992); therefore, its effect on models was not studied here.

To the best of our knowledge, the release characteristics of encapsulated ODN from the cationic liposomes has not been reported yet; however, there are some related publications about the release of ODN from its complex (not encapsulated) with cationic liposomes which is discussed here. Xu and Szoka studied the release of ODN from its complex with Lipofectin® by ethidium bromide intercalation assay of accessible ODN. They found that a significant amount of ethidium bromide fluorescence was recovered upon interaction of lipoplexes with anionic liposomes and that the interaction was completed within 50 s (Xu and Szoka, 1996), in agreement with our finding that the process is completed in less than 5 min, the lowest studied time. Jaaskelainen et al. similarly studied the release of FITC-ODN from its complex with Lipofectin®. The fluorescence of FITC-ODN was dequenched upon incubation with an endosomal model membrane especially at acidic pH of 5–6 (Jaaskelainen et al., 1998). Our results are consistent with the above-mentioned study, though different models of endosomal membrane have been employed.

Conclusively, it was understood from both the model membrane and the cellular studies that the direct interaction of DODAP-containing cationic liposomes with plasma membrane was not determinant of the cytoplasmic release of encapsulated ODN, though it exhibits the physicochemical stability of ODN encapsulating liposomes upon incubation with cells during transfection. Liposomes were taken up into the cells mainly via endocytosis; however, the release of ODN from engulfed liposomes, which does not involve active mechanisms, is mainly governed by the bilayer interactions and can be modeled by simple vesicular membranes. This process depends on pH, temperature, model composition and possibly other variables that may require further studies.

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