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# The Characterization of Cationic Fusogenic Liposomes Mediated Antisense Oligonucleotides into HeLa Cells

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## ABSTRACT

Antisense oligonucleotides (ODNs) are potential therapeutic agents, but their development is still limited due to poor cellular uptake and high degradation rate in biological media. To resolve these problems, we propose to attach the Sendai virus to cationic liposomes. Cationic–fusogenic liposomes (CFLs) were prepared by reverse-phase evaporation and fused with the Sendai virus. The mean diameter was about 186 nm, determined by photon correlation laser light scattering method. The cytotoxicity of CFLs and the ODN loading efficiency depended on the +/− charge ratio. The fluorescence intensity in cytoplasm was enhanced with the increasing of DC-Chol content and +/− charge ratio. We also investigated the mechanism of cellular uptake using temperature shifts and lysosomotropic agent. The results indicated that the vector was introduced into the cells, not via endocytosis but membrane fusion. The preliminary experiment showed that CFLs are a promising formulation for ODN delivery with high levels of transfection and minimal cytotoxicity.

*Key Words:* Cationic fusogenic liposomes; Antisense oligonucleotides; Sendai virus; Transfection efficiency.

## INTRODUCTION

Synthetic oligonucleotides (ODNs) are becoming popular as a tool to control the expression of specific genes. The use of ODNs to down-regulate specific gene expression requires ODNs to enter cells and hybridize with the target mRNA.[1,2] However, a major problem of delivering ODNs into cells is poor permeation into

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membranes and rapid degradation by nuclease.[3,4] In the past few years, many attempts have been performed to overcome these drawbacks, mainly including chemical modification of ODNs and the use of carrier systems.[5,6] One of the most efficient methods is encapsulating ODNs into liposomes, especially synthetic cationic lipids such as DOTMA or DOTAP. Nevertheless, most of these cationic liposomes and ODNs complexes are mainly taken up into cells via endocytosis, consequently, arriving in lysosomes where the conditions of low pH and various lysosomal enzymes inactivate the enclosed material. Degradation of ODNs in lysosomes is known to be one of the limiting factors in gene transfer mediated by liposomes. Furthermore, cytotoxicity of cationic lipids at high dosage constrains their application.[7,8]

Previously, Nakanishi et al.[9] developed fusogenic liposomes (FLs) by fusing the Sendai virus (hemagglutinating virus of Japan, HVJ) with neutral multilamellar liposomes at 37° C. Reconstituted Sendai virus envelopes containing two glycoproteins, F (fusion protein) and HN (hemagglutinin–neuraminidase), which fuse the plasma membrane of target cells efficiently, are excellent carriers for fusion-mediated microinjection of biologically active macromolecules in vitro. The peculiar characteristics of introducing macromolecules into cells through FLs were the active and rapid absorption onto the cell membrane by HN glycoprotein and the succeeding efficient membrane fusion triggered by F glycoprotein.[10]

Since ODNs are hydrophilic, they are passively encapsulated within the aqueous space of neutral liposomes with poor drug loading efficiency.[11] Cationic lipids possess physical characteristics that show high drug loading efficiency by means of electrostatic interaction between the positively charged lipids and negatively charged ODNs. In these experiments, we adopted new fusogenic liposomes with cationic lipids called cationic fusogenic liposomes (CFLs) that have a positive zeta potential. They have been proved to have a higher transfection efficiency than negative fusogenic liposomes.[12,13] However, there is still little information on protection from the nuclease and the mechanism of delivering ODNs using CFLs, because this vector possessed characteristics of both cationic and fusogenic liposomes. In this experiment, we first compared the cytotoxicity of CFLs with that of cationic liposomes (CLs). Secondly, we evaluated the ability of CFLs to prevent ODN degradation. Finally, we used flow cytometry to study the cellular uptake of fluorescence-labeled ODNs mediated by CFLs, and the mechanism of CFL/ODN complexes entering cells was also investigated.

## MATERIALS AND METHODS

### Materials

Eighteen Mer phosphorothioate oligodeoxynucleotide designed to be complementary to the template in the telomerase RNA with the sequence of 5′-CTC, AGT, TAG, GGT, TAG, ACA-3′ was synthesized by Biocolor Biological Science and Technology Co., Ltd., Shanghai, China. For experiments of cellular uptake, N-(3-fluoranthyl) maleimide (FAM) was labeled at the 5′-end of the ODN. Soybean phosphatidylcholine (SPC), cholesterol (Chol), Triton X-100, chloroquine, 3β-[N-(N′,N′-dimethylamineoethane) carbamoyl]cholesterol (DC-Chol) were obtained from Sigma (St. Louis, MO). Fetal bovine serum (FBS) and RMPI-1640 were purchased from Gibco BRL (Gra Island, NY). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazol-iumbromide (MTT) was purchased from Amresco (Solon, OH). All other reagents and solvents were of analytical grade.

### Cell and Virus

HeLa cells were provided by the Institute of Infectious Disease, Zhejiang University. They were cultured in RPMI 1640 supplemented with 10% FBS at 37° C and 5% $CO_2$ humidified atmosphere.

Sendai virus (HVJ), Z strain, was kindly gifted by Professor Mayumi, Osaka University, Japan. The virus was grown in the allantoic cavity of 10-day old embryonated chicken eggs, harvested and purified according to published protocols.[14] Purified HVJ was inactivated by ultraviolet irradiation (110 erg/mm$^2$/sec 15,000 hemagglutinating units) for 3 min before using.[15]

### Preparation of Liposomes and Complexes

Cationic liposomes were prepared by a reverse-phase evaporation method. Briefly, 25 mg SPC, 5 mg Chol, and appropriate DC-Chol were mixed and dissolved in chloroform. The lipid mixture was transferred into a round-bottom flask and dried with a rotary evaporator (Yarong Biochemical Co. Ltd., Shanghai, P.R. China). Dried lipid was redissolved in chloroform, and then added to a balanced salt solution (BSS) consisting of 150 mM Sodium chloride, 1 mM ethylenediaminetetraacetic acid (EDTA), 10 mM Tris hydrochloric acid (HCl), pH 7.3. This binary system was sonicated to form a w/o emulsion. The aqueous suspension was obtained by evaporating the organic solvent. The final volume of blank cationic liposomes

(CLs) was diluted to 10 mL. These CLs were then extruded through a 0.2-μm polycarbonate membrane (Millipore, Cork, Ireland) three times. The CFLs were obtained by fusing CLs with inactive HVJ at 4° C for 10 min, then gently shaking for 60 min at 37° C.

To prepare the complexes, liposomes and ODNs were first individually diluted with appropriate medium. The CL/ODN complexes were achieved by mixing the appropriate ODN solution with CLs and allowing to stand at room temperature for 20 min, while the formation of CFL/ODN complexes were based on fusing CL/ODN complexes with inactive HVJ as mentioned above. To purify the CFL/ODN complexes, discontinuous density gradient centrifugation with sucrose was used to remove free HVJ. The preparation of CL/ODN or CFL/ODN complexes was adjusted by the desired +/− charge ratio. In this study, the final +/− charge ratio of cationic lipid to ODNs was defined according to cationic DC-Chol lipid molar concentration to that of the ODN phosphate group.[16]

## Characterization of CFLs

### Size and Morphology

The diameter of CFL/ODN complexes was measured by photon correlation spectroscopy (PCS) (Coulter N4MD submicron particle analyzer, United Kingdom). The morphology of CLs and CFLs was observed with transmission electron microscopy (JEM-1200EX TEM , JEOL, Japan)

### Loading Efficiency

A series of different +/− charge ratios (from 0.5:1 to 8:1) of CFL/ODN complexes were prepared as described above. The total amount of ODNs was kept at the fixed concentration of 33 μg/mL. The unbound ODNs were separated by centrifuging (15,000 g× 15 min, Hereaus Instruments) using Nanosep® OD030C33 centrifuge tube ( Pall Biosciences Lab) and measured by UV spectrophotometer (Pharmacia Biotech, Gene Quaut II) at 260 nm. The amount of unbound ODNs was calculated with a calibration curve. The ODN loading efficiency was calculated from the unbound ODNs with respect to the initial ODNs.

## Cytotoxicity Assay

The quantitative MTT colorimetric assay was used to determine the toxicity of the vector towards HeLa cells. HeLa cells were seeded into 24-well plates at a density of $2\times10^5$ cells per well and cultured in RPMI 1640 supplemented with 10% FBS for 24 h at 37° C. Then, cells were washed three times with phosphate buffered saline (PBS, pH 7.4) and replaced with 650 μL of serum-free RPMI 1640 medium. One hundred μL of special concentration of CFLs or CLs was added to each well. The relative amount of vectors was based on the various +/− charge ratios, while the concentration of ODNs was fixed at 4 μg per well. After incubation for 4 or 12 h, the medium was then removed and 80 μL of 5 mg/mL MTT solution was added. The cells were further incubated for 4 h, then the supernatants were removed after centrifugation at $400\times g$ for 5 min. Cells were lysed by treatment with a solution of 25% sodium dodecylsulfate (SDS). The quantification of the resulting blue dye was performed by measuring the absorbance with a spectrophotometer at a wavelength of 490 nm and a reference wavelength of 630 nm.

## DNase I Protection Assay

In order to evaluate whether CFLs have the role of protecting ODNs against DNase I, free ODN or CFL/ODN complexes (4 μg ODNs for each sample) were incubated at 37° C in the presence of $2.2\times10^{-2}$ U of DNase I. At various times, the reaction was stopped by a 10-min incubation of samples at 65° C and stored at 4° C. In some instances, samples were incubated with 1% Triton X-100 to dissolve the lipid vesicles after 65° C incubation. Equal volumes of each sample were then analyzed using 20% denaturing polyacryamide gel electrophoresis (PAGE) containing 7 M urea. Electrophoresis was carried out at 40 V for 2 h. Visualization was facilitated using 0.5 μg/mL ethidium bromide under ultraviolet light (UV).

## Cellular Uptake

Cellular uptake was carried out using HeLa cells as a model. The cells were seeded in 24-well plates at the density of $2\times10^5$ cells per well and cultured in RPMI 1640 medium containing 10% FBS prior to the experiment. After 80% confluence, the cells were washed three times with PBS and to each well was added 650 μL of serum-free RPMI 1640 medium and 100 μL of specified concentration of CFL/ODN complexes. For all experiments, FAM-ODNs were used at the fixed concentration of 4 μg per well. At the indicated time, cells were collected by trypsinizing and centrifuging (2000 rpm·min$^{-1}$, 10 min), rinsing twice with PBS and resuspended in PBS. Cells were then analyzed by flow cytometry (Coulter) at 488 nm

excitation and with 530-nm emission filters. The percentage of positive cells (α) and mean fluorescence intensity (MFI) were measured, and total fluorescence intensity (TFI) was calculated as follows:

$$TFI = 10^4 \times \alpha \times MFI$$

Data was acquired on $10^4$ random, viable cells.

We investigated both the effect of +/− charge ratio and the content of DC-Chol in CFL preparation on cellular uptake. Next, in order to definite the optimal transfection time, we investigated the time course of cellular uptake. To study the mechanism of delivering ODNs mediated by CFLs, we compared the change of TFI in cytoplasm at 37° C with or without 0.1 mM chloroquine and at 4° C for various incubation times.

## RESULTS AND DISCUSSIONS

### Size and Loading Efficiency

The morphology of CLs and CFLs was round shaped and integrated, as observed under the transmission electron microscope. The particle sizes of CFLs and CFL/ODN complexes were 186 ± 25 nm and 280 ± 40 nm, respectively.

Our initial goal was to select an ideal +/− charge ratio of CFL/ODN complexes that would incorporate ODNs efficiently and stably. The loading efficiency of anionic ODNs was only 5–10% when ODNs were passively encapsulated in the aqueous space of conventional lipid vesicles.[17] Thus, to achieve modest loading efficiency, extreme concentrations of lipid and ODNs must be employed for a population of large multilamellar vesicles with a mean diameter of 100 nm. This makes formulation difficult, time consuming, and wasteful. Cationic lipids improve the association of anionic ODNs with vesicles through electrostatic interactions. But when these systems are administered intravenously, they are rapidly eliminated from the circulation[18] and exhibit poor accumulation at disease sites, such as tumors and sites of inflammation.[19] In this study, we investigated the ODNs loading efficiency based on the 33.3% DC-Chol content in CFLs preparation at different +/− charge ratios. The loading efficiencies were found to depend on the +/− charge ratios. When +/− charge ratios were 0.5:1, 2:1, 4:1, and 8:1, the ODNs loading efficiency was 34.8 ± 2.23%, 74.9 ± 2.46%, 89.3 ± 3.89%, and 94.7 ± 3.22%, respectively (n = 3). We attributed the increased loading efficiency to electrostatic interactions. As described above, passive entrapment of ODNs within neutral liposomes results in poor incorporation efficiency, so we incorporated cationic lipids into fusogenic liposomes to enhance the loading efficiency.

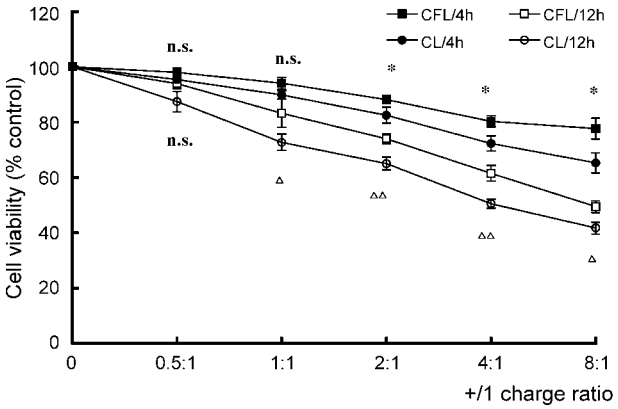


***Figure 1.*** Cytotoxic effect of the CLs and CFLs at different +/− charge ratios on HeLa cells. Data represent the % of cell growth compared with untreated cells. Values shown are the means ± standard deviations of triplicate experiments. Statistical tests were evaluated between the CL and CFL preparations at various charge ratios after 4 h incubation (asterisk) or 12 h incubation (triangle) using Student's t-test. n.s.; no significant difference; *; $p<0.05$; △; $p<0.05$; △△; $p<0.005$.

### Cytotoxicity Assay

The potential toxicity of CFLs in vivo was estimated on the basis of cytotoxicity studies in vitro. In the present work, the integrity of mitochondrial enzyme activity was assessed by the MTT tetrazolium salt assay. Metabolic active cells have the capacity to transform MTT tetrazolium salt into MTT formazan. The measured disappearance of the blue dye obtained by the MTT metabolism after lysis in basic SDS solution correlated to cell viability. The control for untreated cells was set to 100%. Figure 1 shows the cytotoxicity of CLs and CFLs at different +/− charge ratios and incubation times. The cytotoxicity of CFLs was less than that of CLs under the same conditions of +/− charge ratio or incubation time. The significance difference was to be higher than the 2:1 charge ratio between two vectors.

### DNase I Protection Assay

We then determined if CFLs can protect ODNs against DNase I. Figure 2 shows that naked ODNs rapidly degradated in the presence of DNase I within

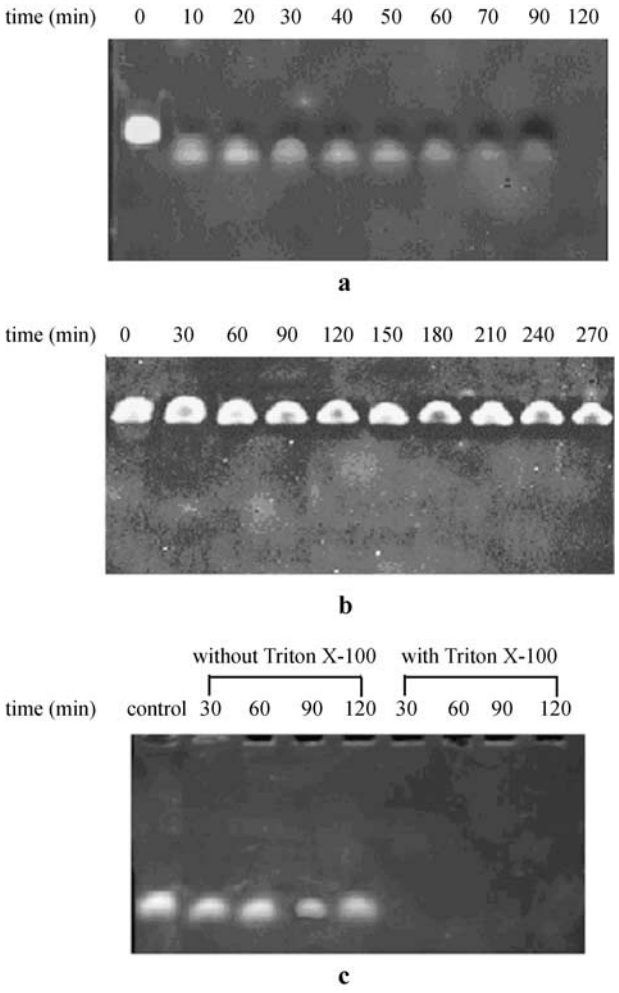


***Figure 2.*** Role of CFLs in ODNs protection against DNase I. (a) Free ODNs in the presence of DNase I during various incubating times. (b) CFL/ODN complexes in the presence of DNase I during various incubating times. (c) Degradation of CFL/ODN complexes in the presence or absence of 1% Triton X-100 during various incubating times. Triton X-100 was added after incubating CFL/ODN complexes at 65° C for 10 min.

10 min (Fig. 2a), while the ODNs incorporated into CFLs were completely protected (Fig. 2b). To determine if this protection is the result of an inhibition of DNase I, 1% Triton X-100 was added to solubilize the lipid bilayer. Under this condition, the degree of degradation was the same as that of naked ODNs (Fig. 2c). The role of protection disappeared with the addition of detergent. These results indicate that the ODNs in CFLs are indeed encapsulated within the lipid vesicles and are not simply adsorbed to the vesicle surface. It may be speculated from this experiment that CFLs have the role of protecting ODNs against nucleases. The mechanisms of CFL/ODN complexes and ODNs entrapment are not fully understood. In this regard, Huebner et al. recently observed the formation of compact multilamellar structures when fragmented, linear genomic DNA was added to unilamellar cationic vesicles composed of DMPC/DC-Chol.[20] The author explain that vesicles coated with an absorbed layer of DNA are forced into close contact with neighboring vesicles through electrostatic interactions. The vesicles become flattened and deformed in the contact region with the DNA sandwiched between the membranes. Although not the same structure as described by Huebner et al., CFL/ODN complexes may be similar, even the vesicles coated and fused by HVJ.

## Cellular Uptake

We performed flow cytometry experiments with fluorescent ODNs at using different +/− charge ratios of CFL/ODN complexes and various DC-Chol content in CFLs preparation. When naked FAM-ODNs were applied to HeLa cells, there exhibited little or no fluorescence signal. In contrast, transfered FAM-ODNs mediated by CFLs showed significantly improved fluorescence signals. The level of fold increase was influenced by both +/− charge ratio and DC-Chol content in CFLs (Fig. 3). In the case of the specified FL's composition, the TFI in cytoplasm was

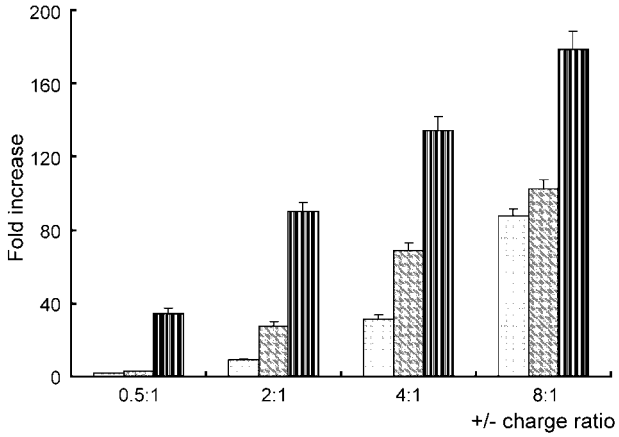


***Figure 3.*** Effect of DC-Chol content on transfection efficiency in HeLa cells mediated by CFLs. Three CFL preparations contained 17.5% (dotted), 20.0% (diagonal lines), and 33.3% (vertical lines) DC-Chol, respectively. Data was represented as a fold increase compared to cytoplasmic fluorescence intensity of free ODNs. Values shown are the means ± standard deviations of three experiments.

enhanced by increasing the +/− charge ratio. Meanwhile, the higher percentage content of DC-Chol in CFLs, the more fold increase would be at the same +/− charge ratio. Many studies have demonstrated that the +/− charge ratio directly affects the size and zeta potency of lipoplexes, which were two important parameters determining the transfection efficiency.[21] Our experiments were consistent with these conclusions. It is reasonably believed that the high +/− charge ratio not only adsorbs ODNs efficiently, but also facilitates the interaction of negatively charged cell membranes and positively charged liposomes.[22] However, our experiments also found that the damage to cell membranes apparently occurred at the higher +/− charge ratio. It is also a key factor that has considerable influence on transfection yields.

To analyze the mechanism involved in the uptake and trafficking of CFL/ODN complexes similar experiments were carried out at 4° C and in the presence of a lysomotripic agent—chloroquine. No significant difference was found in three cultured conditions at all incubated times mediated by CFLs (Fig. 4). This result did not comply with other ODNs carriers such as conventional cationic liposomes or nanoparticles. These carriers are supposed to promote the cellular uptake via endocytosis. It is generally believed that energy-dependence is essential for cellular uptake mediated via endocytosis. At 4° C, the low energy condition, the endocytosis pathway is markedly blocked, so that fusion becomes the major pathway for cellular uptake.[23,24] We then used chloroquine, which prevents the fusion of endosomes and lysosomes, to study the intracellular fate of the CFL/ODN complexes. Chloroquine is known to increase the pH of endocytotic vesicles and the endocytotic pathway. When the CFL/ODN complexes entered the acid lysosomal compartment (pH=4.7∼4.8), the intracellular TFI showed an increase in the presence of chloroquine. Our results demonstrate that the intracellular fluorescence signal does not increase in the presence of chloroquine at 37° C, suggesting that CFL/ODN complexes do not locate in the lysosomes. These results indicate that the mechanism of delivering ODNs mediated by CFLs is not via endocytosis, but via fusion. Although both cationic and fusogenic liposomes can deliver ODNs efficiently, the Sendai virus imparts its fusogenic function to CFLs, playing a key role in increasing the cellular uptake of ODNs, while the DC-Chol was mainly used to incorporate ODNs. The ODNs uptaken by endocytosis enter the lysosomes and subsequently are degraded by lysosomal enzymes. Thus, any ODNs carrier that efficiently drives ODNs through cell membranes and then escapes from lysosomal compartments may improve performance.

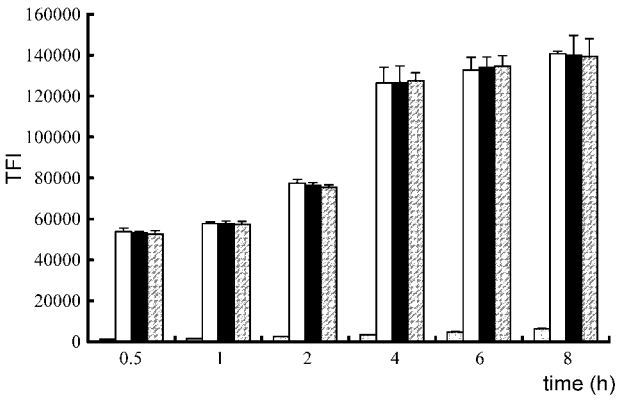


***Figure 4.*** Comparison of cellular uptake of FAM-labeled ODNs in HeLa cells under different cultured conditions at various times. Bars represent free ODNs at 37° C (dotted), 2:1 CFL/ODN complexes at 37° C (white), 2:1 CFL/ODN complexes at 37° C in the presence of chloroquine (black), and 2:1 CFL/ASON complexes at 4° C (diagonal lines). The experiment was performed three times.

## CONCLUSION

In summary, our results showed that HVJ–cationic liposomes appear as efficient carriers for delivering ODNs. Cationic–fusogenic liposomes can protect ODNs from DNase I and enhance cellular uptake by membrane fusion. Moreover, the vector showed low cytotoxity to HeLa cells. We are currently engaged in studies on the efficiency of CFLs for therapeutic antisense oligonucleotides.

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