CO-LIPIDS INFLUENCE THE CELLULAR UPTAKE AND MECHANISM OF CELL ENTRY OF CATIONIC LIPID DNA COMPLEX IN GENE DELIVERY
5.1 Introduction

Cationic liposomes are very promising vehicles for carrying DNA molecules into the cells \(^{49}\). Despite being a routine procedure in biology, transfection of cells using cationic lipids is subject to considerable variation \(^{50,90}\). The pathway of CLDC entry into a cell is broadly understood \(^6\). The primary effect of cationic lipids is occlusion of DNA into a particle due to self-assembling nature of the cationic lipid and DNA interactions \(^{47}\). These particles are taken up by the cell through endocytosis, subsequently escape from the endosomes and travel in the cytoplasm to ultimately localise into the nucleus. However, information about the influence of various individual steps on the overall transfection process is still unclear. Understanding the cellular events of lipofection is necessary to design more efficient lipofection agents.

Including helper lipids or co-lipids along with cationic lipids in transfection formulations resulted in remarkable enhancements in transfection efficiencies \(^{169}\). The choice of co-lipid is either DOPE or cholesterol. Several other lipids were tested viz. DOPC, DOPG etc. but were found to be inefficient \(^{170}\). Since DOPE has the tendency to form reverse micellar structures, it was proposed that DOPE containing liposomes have higher tendency to fuse with the endosomal membrane. Several \textit{in vitro} studies on DOPE have further supported its fusogenic role \(^{69,123}\). Cholesterol as a co-lipid has shown higher efficiencies with some cationic lipids \(^55\). Observed serum-stability of cholesterol containing formulations encouraged their use \textit{in vivo} transfection studies \(^{171}\). Structures of CLDC prepared with different co-lipids were investigated by SAXS \(^{47}\), transfection studies, zeta potentials and by size measurements \(^{146}\). Small CLDC in the size range of few hundred nanometers with positive surface charge are optimum structures for transfection \(^{172}\).

Since the demonstration of Zabner et.al., several reports have demonstrated that endocytosis is the predominant pathway for the entry of CLDC into cells \(^6,173\). Sensitivity of transfection efficiency to pretreatments of cells with NH\(_4\)Cl, chloroquine \(^{174}\), monensin \(^{175}\) or exposure to low temperature \(^6\) or depletion of
plasma membrane cholesterol \(^88\) indicated the involvement of endocytic organelles in CLDC uptake. It is well known that the endocytosis is mediated by clathrin or caveolae pathways, phagocytosis, macropinocytosis and constitutive non-clathrin uptake \(^{176}\). The participation of these processes in endocytosis of CLDC is largely unclear. Recently cholesterol-dependent clathrin mediated endocytosis was shown to be the predominant pathway for CLDC prepared from SAINT2:DOPE, based on the sensitivity of transfection to potassium depletion, plasma membrane cholesterol depletion and expression of dominant negative Eps15 \(^88\). The participation of caveolae-mediated pathways in transfection was found to be marginal. In another interesting study using FITC-labelled dextran beads of various sizes, it was demonstrated that mechanisms by which the beads were internalised and their subsequent intracellular routing, was strongly dependent on particle size \(^{177}\). Due to spontaneous nature of the formation of CLDC, the process of complexation is known to lead to heterogeneously sized particles and size of CLDC may influence their choice of endocytic pathway. It is important to understand whether uptake of CLDC involves single endocytic pathway or employs several pathways depending on the nature of the cationic lipid and co-lipid.

To investigate the cell biological basis of co-lipid dependency on transfection, we have used two popular cationic lipids DOTAP and DDAB, whose transfection is critically dependent on the nature of the co-lipid - DOPE or cholesterol, present in the formulations. Studies with such defined formulations would help us to establish the helper role of the co-lipids at various stages of transfection. Electron microscopy and confocal microscopy were used to investigate the structures and uptake of the four CLDC in CHO and correlated with their transfection efficiencies to understand the role of co-lipid in transfection. In addition, investigations employing various inhibitors of endocytosis revealed a co-lipid-dependent sensitivity of the CLDC uptake pathways in cells.
5.2 Materials and Methods

5.2.1 Chemicals: DOTAP, DDAB and DOPE were purchased from Avanti Polar Lipids Inc. Cholesterol, bafilomycin A1, wortmannin, cytochalasin D, DMEM and MβCD were obtained from Sigma Chemicals. Horseradish peroxidase was from Bangalore Genei, Bangalore. Rh-DHPE was purchased from Molecular Probes. Plasmid DNA pCMV.SPORT-β-gal was from Invitrogen. All other chemicals used were of highest purity.

5.2.2 Liposome preparation: Cationic liposomes were prepared by drying appropriate amount from their chloroform stock solutions as describe in the materials and methods section of chapter 2. All the four liposomes used in this study were prepared at 1:1 mole ratio. In case of rhodamine labelling, above liposomes were prepared with 5 mol % Rh-DHPE.

5.2.3 Transient transfections and determination of percent transfection: CHO cells were used for all the experiments. CHO cells were maintained as described in the experimental section of chapter 4. For transfection, cells were plated in 96-well plate and grown overnight. Transfection was done when the confluency had reached about 80%. Transient transfections with four different cationic lipid:co-lipid formulations were carried out at various N/P charge ratios (9:1, 3:1, 1:1 and 0.3:1) and transfection efficiency was determined by measuring β-gal activity as described in chapter 4. As transfection efficiency at 1:1 N/P charge ratio was found to be maximum for all four-liposome formulation, we used 1:1 N/P charge ratio in all other experiments. For determining percent-transfected cells, transfection was carried out as described before. After 24h of transfection, cells were fixed with 0.5% glutaraldehyde in PBS for 15 min. at room temperature. Cells were then washed extensively with PBS and incubated with β-gal substrate buffer (5mM potassium ferricyanide, 5mM potassium ferrocyanide, 1mM magnesium sulphate and 1mg/ml X-Gal) at 37° C for about 3h. About 500 cells were counted in each sample for X-Gal positive cells and percent transfection was determined.
5.2.4 *In vitro DNA binding and CLDC stability:* For determining the extent of DNA binding by four different cationic liposomes, *in vitro* DNA binding was carried out as described in the experimental section of chapter 2. To measure the relative stability of each of the four CLDC, we prepared CLDC and incubated with 4% foetal calf serum for 15 min. at 37°C. Lipids were then extracted twice with phenol:chloroform followed by chloroform once and the DNA samples were run on 1% agarose gel. Gel was stained with ethidium bromide and relative extent of DNA degradation was determined by quantitating (Gene Tools, Syngene) the intact DNA bands. Extent of DNA degradation in case of free DNA was considered as 1.

5.2.5 *Confocal microscope imaging of CHO cells incubated with various CLDC:* For observing the extent of CLDC entry into the cells, liposomes were prepared with Rh-DHPE at 5 mol %. CHO cells were grown in two-chambered coverglass (Lab-Tek, Nalge Nunc International Corp.) to a confluency of about 60%. CLDC were prepared as described for transient transfections. CLDC were incubated with cells for about 1 h at 37°C and 5% CO₂. After 1 h, cells were extensively washed with medium and imaged with confocal microscope (LSM 510 META, Carl Zeiss). Optical sections of cells were collected using 63X 1.2NA water-immersion objective at about 0.5μm interval. 543nm laser line was used to excite rhodamine label and images were collected simultaneously both in rhodamine and transmission channels for each optical section. Images of several cells were analysed and equal number of optical sections passing through the cells was merged for each sample. Rh-DHPE labelled liposomes did not show any loss in transfection efficiency compared to their respective unlabelled liposomes (data not shown). To quantitate the total fluorescence pixel intensity associated with single cells, background fluorescence from each image was removed. Using LSM 510 META software the fluorescence intensities were quantitated. Relative total pixel intensity associated with single cells in case of DOTAP:Chol and DDAB:Chol was calculated by considering the pixel intensity...
associated with single cells in case of DOTAP:DOPE and DDAB:DOPE as 1 respectively.

5.2.6 Transmission electron microscopy of CLDC: CLDC were prepared as described for transient transfections. Samples were then placed on 200mesh formvar coated copper grids and stained with 0.1% uranyl acetate. These grids were examined in JEOL 100CX at 80 KV using 20μm objective aperture.

5.2.7 Effect of cholesterol depletion on transfection efficiency: Cholesterol depletion was done by standard protocol using 10mM MβCD. Transfection was done with normal CHO cells or cholesterol depleted CHO cells. β-gal activity and protein content were determined after 24h of transfection as described in the experimental section of chapter 4.

5.2.8 Effect of wortmannin and cytochalasin D treatments: In case of wortmannin and cytochalasin D treatments, cells were treated with 250ng/ml of wortmannin or 5μM cytochalasin D or only DMSO (controls) for 30 min. before CLDC addition and kept along with CLDC during transfection period. β-gal activity and protein content was determined after 24h of transfection as described previously.

5.2.9 Effect of bafilomycin A1 on transfection efficiency: Transfection was done as described previously. During CLDC incubation, cells were either treated with 20ng/ml concentration of bafilomycin A1 (dissolved in DMSO) or with only DMSO at the same concentration as used in bafilomycin A1 treated samples. After about 24h of transfection, cells were processed for β-gal activity and protein content determination as described previously.
5.3 Results

5.3.1 Transient transfections: CHO cells were transfected by preparing CLDC with pCMV β-gal and each of the four liposomes as described in the experimental procedures. The four formulations (DOTAP and DDAB with DOPE or cholesterol) were tested initially at various N/P charge ratios to determine the optimum charge ratio at which maximum transfection was observed. With each of the formulations the maximum transfection was observed at 1:1 N/P charge ratio. As shown in fig. 5.1A, both DOTAP and DDAB with DOPE showed about 2-3-fold increase in transfection efficiency at 1:1 N/P charge ratio compared to their respective cholesterol formulations. For all the subsequent experiments 1:1 N/P charge ratio was used. To determine the percentage of transfected cells, cells were stained with X-Gal after transfection as described in the experimental section. Fig. 5.1B shows that CLDC containing DOPE transfected three times more number of cells compared to their respective CLDC containing cholesterol.

5.3.2 In vitro DNA binding efficiency and CLDC stability: We have investigated the binding efficiency of these four formulations with the plasmid using agarose gel electrophoresis and quantitated the free plasmid by densitometry. Fig. 5.2A shows variations in the extent of binding of these four lipid formulations to the plasmid. DOTAP:DOPE binding to the plasmid was strongest followed by DDAB:Chol and DDAB:DOPE. DOTAP:Chol binds inefficiently to the plasmid, where more than 70% of plasmid was uncomplexed in the formulation. To determine the relative stability of four CLDC we incubated CLDC in 4% serum as described in the experimental section and measured relative extent of DNA degradation. Fig. 5.2B shows DOTAP:Chol is least stable resulting in 80% DNA degradation followed by DDAB:DOPE (60%), DDAB:Chol (40%) and DOTAP:DOPE (25%). Neither the extent of DNA binding nor stability of CLDC correlated with their transfection behaviour.
Fig. 5.1: Transient transfections of CHO cells with four CLDC:
(A): CLDC were prepared at various N/P charge ratios and transfection was carried out on CHO cells as described in the experimental section. After 24h of transfection β-galactosidase activity and protein content of cell lysates were measured as described in the experimental section. Milliunits (mU) of β-galactosidase activity was expressed after normalising with protein content. DOTAP:DOPE (■); DOTAP:Chol (●); DDAB:DOPE (▲); DDAB:Chol (▼). (B): CHO cells were transfected with different CLDC and after 24h cells were stained with X-Gal as described in the experimental section. Results were expressed as percent-transfected cells.
Fig. 5.2: In vitro DNA binding and CLDC stability: (A): CLDC were prepared in 10mM sodium phosphate buffer and were run on 1% agarose gel. DNA band intensities were quantitated after staining with ethidium bromide and data was expressed as relative free DNA. (B): CLDC were prepared in 10mM sodium phosphate buffer at 1:1 N/P charge ratio and incubated with 4% serum at 37°C for 15min. Lipids were extracted as described in the experimental section and DNA samples were run 1% agarose gel. DNA band intensities of ethidium bromide stained gels were quantitated and data was expressed as relative extent of DNA degradation by considering the extent of DNA degradation of free DNA as 1. These experiments were repeated and standard error was found to be within 20%.
5.3.3 **Confocal microscope imaging of CHO cells:** We used confocal microscopy to estimate the internalised CLDC. CHO cells were incubated with fluorescently labelled liposomes complexed with DNA for 1h as described in the experimental section and observed under confocal microscope. Equal number of optical sections only passing through the cells were merged for each sample and used for analysis. Images of several cells were analysed. Fig. 5.3A shows representative image for each type of CLDC. DOTAP:Chol and DDAB:Chol show less amount of cell associated fluorescence compared to DOTAP:DOPE and DDAB:DOPE. Particulate fluorescence observed was localised in the cytoplasm. We quantitated the total fluorescence pixel intensities associated with single cells in each case as described in the experimental section. As can be seen from fig. 5.3B, DOTAP:Chol shows about 70% less amount of CLDC associated with single cells compared to DOTAP:DOPE. DDAB:Chol shows about 90% decrease in the amount of CLDC associated with single cells as compared to DDAB:DOPE. Thus, the poor efficiency of CLDC containing cholesterol to transfect cells compared to their DOPE containing counterparts, seems to be due to less amount of internalised CLDC.

5.3.4 **Transmission electron microscopy of CLDC:** In order to observe the macroscopic structures formed by these CLDC we used transmission electron microscopy. CLDC containing DOPE, form compact and discrete particles, unlike CLDC containing cholesterol (fig. 5.4), which mostly, form network-like structures connecting CLDC particles, with very few discrete CLDC particles. CLDC particles connected to one another results in the increase in the overall size of CLDC, which may hinder the ability of the CLDC containing cholesterol to enter the cells as efficiently as CLDC containing DOPE. Though DDAB and DOTAP are chemically different both in acyl chain saturation and linker groups, it is interesting to note that they form similar aggregates with a given co-lipid. Presence of cholesterol leads to network-like structure, whereas DOPE facilitates the formation of more discrete particles as seen in fig. 5.4.
Fig. 5.3: Confocal microscope imaging of CHO cells:
Confocal microscope imaging of live CHO cells and quantitation of cell associated CLDC. Representative images of each sample obtained 1h after the treatment of cell with CLDC. Rhodamine (lipid) fluorescence and transmission images were merged. (A): Confocal images of CHO cells treated with DOTAP:DOPE, DOTAP:Chol, DDAB:DOPE and DDAB:Chol. Scale bar represents 10μm. Optical sections of about 0.5μm thickness each spanning the entire thickness of the cell were collected. (B): Confocal images collected from several cells were used for quantitating the total cell associated pixel intensity for each of the four CLDC in CHO cells as described in the experimental section. Total single cell associated pixel intensity for DOTAP:DOPE and DDAB:DOPE was considered as 1 to calculate the relative total single cell associated pixel intensity for DOTAP:Chol and DDAB:Chol respectively. Data is presented as relative total pixel intensity.
**Fig. 5.4:** Transmission electron microscopy of CLDC: CLDC were prepared and stained with uranyl acetate as described in experimental procedures to visualise CLDC by transmission electron microscopy. Scale bar represents 100nm. DOTAP: DOPE and DDAB: DOPE show compact and discrete structures, whereas DOTAP: Chol and DDAB: Chol show highly interlinked, disorganised structures. Arrows in case of DOTAP: Chol and DDAB: Chol indicate the possible separated particles, which could be transfection competent.
5.3.5 Effect of inhibitors on CLDC cell entry: It is well established that the uptake of CLDC into the cells is mediated by endocytosis, however endocytosis involves a number of processes, whose involvement could be intervened by using inhibitors. We investigated the sensitivity of reporter gene activity elicited by these four CLDC to various inhibitors of endocytosis in CHO cells. Depletion of plasma membrane cholesterol was shown to inhibit endocytic pathways in several cell-types including CHO. Cholesterol content of the plasma membrane of the cells was depleted using MβCD as described in the experimental section to see its effect on the reporter gene activity. As shown in fig. 5.5A, upon cholesterol depletion transfection from CLDC containing DOPE was significantly inhibited, where 75% and 70% inhibition was observed with DOTAP:DOPE and DDAB:DOPE respectively. Interestingly, cholesterol depletion did not inhibit the transfection from CLDC containing cholesterol. In fact, CLDC of DOTAP:Chol showed increased transfection (about 2.5 fold) after cholesterol depletion (fig. 5.5A). When cells were treated with wortmannin, an inhibitor of PI 3-kinase involved in vesicle trafficking in endocytosis, CLDC containing DOPE showed significant inhibition in reporter gene activity compared to CLDC containing cholesterol (fig. 5.5B). DOTAP:DOPE showed about 60% inhibition and DDAB:DOPE showed about 70% inhibition compared to the controls (fig. 5.5B). Transfections with CLDC containing cholesterol were either unaffected or marginally inhibited (<15%). Actin plays a vital role in facilitating endocytosis. Cytochalasin D, an inhibitor of actin polymerisation, inhibits the actin dependent endocytosis. Cytochalasin D treatment again inhibited DOTAP:DOPE mediated transfection by more than 90% and DDAB:DOPE transfection by 40% (fig. 5.5C). Cytochalasin D treatment has nearly doubled the DDAB:Chol mediated transfection and marginally increased the reporter gene activity of DOTAP:Chol (fig. 5.5C) implicating differential sensitivity of the pathways of these two co-lipid based formulations. Since the effects of these inhibitors on cells are reversible and these treatments were carried out only during the initial CLDC uptake period, we believe that the steps affected by these treatments are the steps of initial entry and may not involve events related to transcription and translation.
Fig. 5.5: Treatment of CHO cells with various inhibitors: (A) MβCD treatment, (B) Wortmannin treatment, (C) Cytochalasin D treatment, (D) Bafilomycin A1 treatment. Relative activity of β-galactosidase expressed with each of the CLDC. DOTAP:DOPE (1), DOTAP:Chol (2), DDAB: DOPE (3) and DDAB:Chol (4). β-galactosidase activity in the absence of treatment was considered to be one. '-' and ' +' below each bar indicates without and with treatment respectively. The differences observed between control and treatment were significant at $P = 0.010$ in each of the cases.
Bafilomycin A1 is a specific inhibitor of vacuolar ATPase, which is involved in maintaining the acidic pH of lysosomes and is known to prevent acidification of lysosomes. Lysosome neutralising agents such as chloroquine has been shown to enhance the transfection efficiency. In order to see the extent of detrimental effect of lysosomal acidic pH on the transfection efficiency of four different CLDC, we treated cells with bafilomycin A1. As shown in fig. 5.5D, all the CLDC seem to show increase in transfection efficiency after bafilomycin A1 treatment. A remarkable increase in transfection was seen only with DOTAP:Chol, which showed about 8-fold increase in transfection efficiency after bafilomycin A1 treatment. All other CLDC showed about 2-fold increase in transfection efficiency after bafilomycin A1 treatment, indicating that the pathways of all four CLDC encountered lysosomes and the lysosomal acidic pH is responsible at least in part for their reduced transfection efficiency.

5.4 Discussion
Addition of co-lipid enhances the transfection efficiency of all cationic lipid formulations compared to the cationic lipid alone. The enhancements observed in transfection efficiency is also known to be specific to the co-lipid and cationic lipid combinations and also on the charge ratios employed in making the formulations. The cell biological role of co-lipid in transfection was speculated based on their biophysical properties. Transfection efficiency of a CLDC depends on the proportion of CLDC that enter the cell and subsequent events undergone by CLDC containing organelles within the cell. Cholesterol was shown to be a poor co-lipid, compared to DOPE, with both DOTAP and DDAB at all the charge ratios tested in vivo transfection studies. Cationic lipid, DMRIE was also shown to give best results with DOPE compared to cholesterol. Transfection efficiencies reported here with four CLDC showed that DOPE is a better co-lipid than cholesterol. The transfection efficiencies showed clear correlation with the internalised CLDC estimated from confocal microscopy. Evidently, presence of cholesterol has reduced the uptake efficiency of CLDC. Interestingly, we also observed a correlation between the macroscopic structures
formed by these CLDC and their cellular internalisation efficiency. Transmission electron microscopic images of CLDC prepared with cholesterol showed CLDC particles connected with each other forming network-like structures with few discrete particles. DOPE containing CLDC showed predominantly discrete CLDC particles. Such morphological differences may influence the interaction of CLDC with the cell surface and subsequent uptake.

Several studies established the role of endocytosis in cationic lipid mediated transfections. Incubation at 4°C reduced the transfection

and chloroquine treatments enhanced transfection suggesting that endocytosed CLDC encounter lysosomes⁶³. Using specific inhibitors, transfection by a cationic lipid formulation SAINT2:DOPE, was shown to be mediated by clathrin-mediated endocytosis⁸⁸.

Studies reported here demonstrate differential susceptibility of CLDC containing DOPE and cholesterol to various inhibitors of endocytosis. The nature of the cationic lipid did not influence the inhibitor sensitivity. More importantly, the co-lipid apparently determined inhibitor sensitivity. Transfection by CLDC containing DOPE was inhibited significantly by plasma membrane cholesterol-depletion, wortmannin and cytochalasin D treatments indicating the dependency of plasma membrane cholesterol, actin and functional PI 3-kinases on the uptake process of these CLDC. In contrast, the lack of inhibition and enhancement of transfection in some cases with CLDC containing cholesterol indicates that the pathway encountered by these CLDC was different from CLDC containing DOPE. It is reported that inhibition of one pathway of endocytosis led to the up regulation of other or alternate pathway(s)¹⁸⁴, which could be the possible reason for the observed enhancements in transfection efficiencies with CLDC containing cholesterol upon various treatments. The inhibitors used in the present study may not be exclusive to one particular pathway and could affect more than one pathway. For example, both clathrin-dependent and caveolae-dependent pathways were affected by cholesterol depletion by MβCD¹⁷⁸,¹⁸⁵. In addition inhibition of a pathway by an inhibitor or a treatment, could either be incomplete
or ligand specific, requiring additional evidence. Enhancement of transfection efficiencies of all four CLDC on bafilomycin A1 treatment suggest that lysosomes were encountered during their cell fate.

Taken our experimental data together, it is clear that co-lipids influence the macroscopic structures, cell association/internalisation of CLDC and the pathway of CLDC entry into the cells. Finally the data presented here indicates that the difference in transfection efficiencies between CLDC containing DOPE and cholesterol is due to a combination of both the amount of CLDC that enters the cell and also the type of endocytic route they take to enter cells.