Chapter 4

ENDOCYTIC ACTIVITY OF A CELL-TYPE INFLUENCE THE EFFICACY OF LIPOSOME MEDIATED GENE DELIVERY
4.1 Introduction

Efficiency of cationic liposome mediated DNA delivery for a given cell-type depends on several parameters, which includes biology of the cell-type, size of the plasmid DNA and quality of the DNA sample, promoter and enhancer combination, chemical nature of the cationic lipids, composition of liposomes, and buffer conditions of complex formation. Significant variation in transfection efficiency is also seen between different cell-types when tested with a single transfection reagent.

For transcription to occur, the DNA present in CLDC must cross the plasma membrane, escape from the endosomal membrane and eventually cross the nuclear membrane. Observed differences in transfection efficiency among different cell-types could be due to both qualitative and quantitative differences in the processes mentioned above. Stability of the DNA in the cytoplasm or transcriptional efficiency in the nucleus between different cell-types does not significantly contribute to the differences in transfection efficiencies observed.

Nuclear import of the plasmid from the cytoplasm is considered to be another rate limiting factor in overall transfection efficiency. However, the uptake rates determined by combination of cytoplasmic microinjection and in situ hybridisation indicated that the process is slow and is comparable between different cell-types. Which cellular process has a critical bearing on the transfection efficiency?

Uptake of CLDC predominantly occurs through endocytosis in cells. Many lines of evidence demonstrate vesicular uptake of CLDC, including dependence of transfection efficiency on endocytic inhibitors, colocalisation of CLDC with vesicular markers and localisation of CLDC in vesicles by EM. Endocytosis is known to depend on the cell-type and also cell cycle stage of a cell. How robustly the endocytic rate determines the transfection efficiency in various cell-types?

For example, in airway epithelia, poor uptake of CLDC and slow rate of cell division were found to be responsible for low transfection efficiency. Few reports based on the study of two cell-types suggested that the endocytic activity...
of a cell-type might play a role in determining the transfection efficiency. Besides these reports, there have not been many studies that specifically addressed the dependence of transfection efficiency on endocytic activity in different cell-types. Is there any correlation between level of endocytic activity and transfection efficiency?

We estimated the relative endocytic activity by horseradish peroxidase (HRP) uptake, a well-known fluid-phase endocytic marker in 11 different cell-types, which differ in their transfection ability significantly. Our results provide the first direct evidence for the uptake of CLDC by endocytic activity has important bearing on the transfection efficiency. Evaluation of the relationship between the endocytic activity of cell-type and transfection efficiency showed a good correlation between transfection efficiency and endocytic activity. We have used another model system to demonstrate the dependence of transfection process on the endocytic activity of a cell-type. During mitotic phase it has been clearly demonstrated that the endocytic activity of a cell is considerably reduced compared to interphase cells. Here we show that the mitotic cells show poor uptake of CLDC and also show reduced transfection. These results clearly demonstrate that the initial CLDC uptake by endocytosis is the first molecular barrier in the transfection process.

4.2 Materials and methods

4.2.1 Chemicals:
DOTAP and DOPE were purchased from Avanti Polar Lipids Inc. pCMV.SPORT-β-gal plasmid was from Invitrogen. Horseradish Peroxidase was purchased from Bangalore Genei, Bangalore, India. Texas Red-transferrin, FITC-dextran (70kDa), Rh-DHPE and Hoechst 33258 were from Molecular Probes. Dulbecco's Modified Eagles Medium (DMEM), colcemid and wortmannin were from Sigma Chemicals. All other reagents used were of high quality grade.
4.2.2 Cationic liposome preparation:
DOTAP:DOPE (mol:mol) liposomes were prepared as described in chapter 2. In case of rhodamine labelling, above liposomes were prepared with 5 mol % Rh-DHPE.

4.2.3 Transient transfections:
We used eleven cell-types for transfection experiments. Table 4.1 gives the list of the cell-types used along with the tissue and organism from which they are derived. All the cell-types were maintained in DMEM containing 10% foetal calf serum and 500 µg/ml penicillin, 600µg/ml streptomycin, 1mg/ml kanamycin except C2C12, which was maintained in DMEM with 20% foetal calf serum containing antibiotics at 37°C and 5% CO₂. All the cell-types were plated in 96-well plate and allowed to reach a confluency of 60% - 70%. Transfection was carried out using DOTAP: DOPE (1:1 mole/mole) complexed with pCMV β-gal at 1:1 N/P charge ratio. Complexes were incubated with cells for about 3h, after which complex containing medium was replaced with 10% serum containing DMEM medium (20% serum containing medium in case of C2C12 cells). Cells were incubated further for about 24h before estimating the β-galactosidase activity. After 24h medium was removed and cells were washed with PBS and lysed with 50µl lysis buffer (250mM Tris-Cl pH 8.0 containing 0.5% NP40) for 10 min. at room temperature. 5µl of cell lysate was used for protein estimation by modified Lowry's method and to the remaining cell lysate 50µl of β-galactosidase assay mix (200mM sodium phosphate pH 7.4, 2mM MgCl₂, 1.33 mg/ml ortho-nitrophenyl β-galactoside) was added. Plates were incubated at 37°C and the colour developed was read in SPECTRA Max (109) ELISA plate reader. β-galactosidase activity was calculated from a standard graph constructed from commercial β-galactosidase enzyme. β-galactosidase activity was normalised against milligram of cell protein. To see the effect of wortmannin on transfection, cells were treated with 250ng/ml concentration of wortmannin for 30 min. at 37° C before adding the CLDC. Control cells were treated with only DMSO at the same concentration used in case of wortmannin. Transient
transfection and β-gal activity assay was carried out as described before. Data is presented as wortmannin treated /untreated (+/-) ratio of relative β-gal activity.

4.2.4 Transient transfections in interphase and mitotic cells:

For determining the transfection efficiency differences between interphase and mitotic cells, we grew the cells in 25cm² flasks overnight. One flask was treated with the drug colcemid at 2μg/ml concentration to block cells in mitotic phase and another flask was treated with only DMSO for 24h. DMSO treated cells were trypsinised and collected. Colcemid treated cells were collected by tapping the flask few times (mitotic shake-off). Cells were washed with PBS once and were finally resuspended in DMEM without serum. Cells were plated in 96-well plate. CLDC were prepared as described before, mixed with these cells and incubated for 3h at 37° C and 5% CO₂. After 3h, DMEM with 10% foetal calf serum was added. Cells were assayed for β-gal activity after 24h of transfection as described before. β-gal activity was normalised with milligram of protein

4.2.5 Measurement of endocytic activity of cells:

In order to measure the endocytic activity of different cell-types, we used a well-known fluid phase marker, HAP. HAP uptake was determined by following a previously described method with slight modifications. Cells grown in 24-well plates at high density were washed once with DMEM. HAP uptake was initiated by adding 250μg/ml concentration of HAP in DMEM to cells. Cells were incubated at 37° C. After 2h of incubation, medium was removed and cells were washed five times with ice cold PBS containing 1% BSA followed by three times with PBS. Cells were then lysed with lysis buffer (250 mM Tris-Cl pH 8 containing 0.5% NP40) for 10 min. at room temperature. An aliquot of cell lysate was used for measuring HAP activity by adding 2X HAP substrate solution (1.5 mg/ml of o-phenylenediamine in 1N sodium acetate pH 5.2 containing 0.12% H₂O₂) and incubating at 37° C for 10 min. By adding an equal volume of 0.1N H₂SO₄, reaction was stopped and colour developed was read at 490nm. Protein content
of cell lysate was estimated by modified Lowry’s method. Activity of HRP was expressed as absorbance at 490nm/mg of protein.

4.2.6 Transferrin, dextran and CLDC uptake by interphase and mitotic cells:
For assessing transferrin and dextran uptake by interphase and mitotic cells, unsynchronised COS-1 and MCF-7 cells were grown on coverslip. Cells were washed once with DMEM and incubated with either 80µg/ml of Texas Red - transferrin for 5 min. or 5mg/ml of FITC-dextran (70 kDa) for 10 min. at 37° C. Cells were then washed twice with DMEM, fixed with 3.7% formaldehyde for 15 min. at room temperature followed by extensive washing with PBS. Cells were stained for nucleus with Hoechst 33258 at 10µg/ml concentration for 10 min. at room temperature. Cells were again washed with PBS extensively and mounted onto glass slides with mountant (Vectashield). For CLDC uptake, cells were grown in 2-chambered coverglass (Lab-Tek, Nalge Nunc International Corp.). Cells were washed with DMEM. Rhodamine labelled liposomes was used for preparing CLDC. CLDC were prepared as described for transient transfection and added onto the cells. Cells were incubated for 30 min. at 37° C and 5% CO₂. Cells were washed twice with DMEM and stained with Hoechst 33258 (10µg/ml in HEPES buffered DMEM) for 30 min. on ice. Cells were washed twice with HEPES buffered DMEM and chamber was filled with the same buffer. Confocal imaging was done with LSM 510 META, Carl Zeiss. Mitotic cells were selected by observing nuclear staining using DAPI filter. Transferrin and dextran uptake was imaged with 100X 1.4 NA oil immersion objective, whereas CLDC uptake was imaged with 63X 1.2 NA water immersion objective. 488nm laser line was used for exciting FITC and 543nm was used for exciting Rhodamine and Texas-Red. Emission was collected using appropriate emission filters. About 0.75µm thick slices were collected using 150µm pinhole. In case of CLDC uptake, optical sections were collected both in the fluorescence and transmission channels simultaneously. Optical sections passing through the cells were merged.
4.3 Results

4.3.1 Relationship between transfection and endocytic activity

We performed transient transfections in several different cell-types as described in the experimental section. Table 4.1, gives the details of all the cell-types used. Following criteria were used in the selection of the cell-types: (a) they should show large difference in transfection efficiency, (b) should represent different tissues or organs and (c) should be from different organisms.

Table 4.1

<table>
<thead>
<tr>
<th>Cell-type</th>
<th>Tissue</th>
<th>Organism</th>
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<tbody>
<tr>
<td>COS-1</td>
<td>Kidney, SV40 transformed</td>
<td>African green monkey</td>
</tr>
<tr>
<td>CV-1</td>
<td>Kidney, normal</td>
<td>African green monkey</td>
</tr>
<tr>
<td>HeLa</td>
<td>Cervix</td>
<td>Human</td>
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<tr>
<td>CHO</td>
<td>Ovary</td>
<td>Hamster</td>
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<tr>
<td>Hep G2</td>
<td>Liver</td>
<td>Human</td>
</tr>
<tr>
<td>NIH 3T3</td>
<td>Embryo</td>
<td>Mouse</td>
</tr>
<tr>
<td>F111</td>
<td>Embryonic fibroblast</td>
<td>Rat</td>
</tr>
<tr>
<td>MCF-7</td>
<td>Mammary glad</td>
<td>Human</td>
</tr>
<tr>
<td>BRL</td>
<td>Liver</td>
<td>Rat</td>
</tr>
<tr>
<td>L929</td>
<td>Areolar and adipose</td>
<td>Mouse</td>
</tr>
<tr>
<td>C2C12</td>
<td>Muscle</td>
<td>Mouse</td>
</tr>
</tbody>
</table>

As can be seen from fig. 4.1A, transfection efficiency varies between different cell-types by nearly 80-fold. Highest transfection was seen with CHO cells and lowest with C2C12, L929 and NIH 3T3. Transfection efficiencies of rest of the cell-types were distributed between these extremes. On the same batch of cells and on the same day we also measured relative endocytic activities, using HRP as a marker as described in the experimental section. Fig. 4.1B shows relative endocytic activities of all the cell-types. It is clear from the fig. 4.1B for many of the cell-types, the level endocytic activity is in good agreement with their transfectability. Fig. 4.1C show there is significantly high correlation ($r = 0.85$)
Fig. 4.1: Relationship between transfection efficiency and endocytic activity.

(A): Transient transfection was carried out in eleven cell-types as described in the experimental section. Data is presented as milliunits of β-galactosidase activity normalised to protein content of the cell lysate.

(B): Horseradish peroxidase was used as a marker to measure the endocytic activity of different cell-types as described in the experimental section. Data is presented as absorbance at 490 nm normalised to protein content.

(C): Correlation plot drawn between transfection efficiency and endocytic activity.
between endocytic activity and transfection efficiency. Significant positive ‘r’ value indicates that the endocytic activity of the cell-type has strong bearing on the transfection efficiency.

4.3.2 Effect of wortmannin on the CLDC cell entry: Further we determined the effect of wortmannin, a PI 3-kinase inhibitor on transfection efficiency in different cell-types. Fig. 4.2 show, wortmannin, does not inhibit transfection to the same extent in all the cell-types. A maximum inhibition of about 50%-55% was seen with CV-1, CHO and HepG2 cell-types. COS-1, NIH 3T3, HeLa and F111 did not show appreciable inhibition after treating the cells with wortmannin. This suggests the involvement of PI 3-kinase dependent and independent pathway of CLDC entry is cell-type dependent.

4.3.3 Transferrin, dextran and CLDC uptake in interphase and mitotic cells: In order to further validate our observation of correlation of endocytic activity on the transfection efficiency, we chose to compare the CLDC uptake and transfection efficiencies in interphase and mitotic cells. Mitotic cells have significantly reduced endocytic activity compared to interphase cells 158-160. Initially uptake of transferrin and dextran (70 kDa) was carried out in COS-1 and MCF-7 cells. Transferrin is a marker for receptor mediated endocytosis and dextran is a marker for fluid phase endocytosis. We specifically looked at the relative uptake of these markers in interphase cells and mitotic cells. Confocal microscopy was used to study the uptake as described in the experimental section. Equal number of optical sections passing through the cells was merged and is shown in fig. 4.3. For each area imaged by confocal microscopy, images were also collected for nuclear stain (Hoechst 33258) using UV emission filter. Nuclei of mitotic cells show condensed DNA staining compared to interphase cells, which are indicated by arrowheads in fig. 4.3B, 4.3D, 4.3F and 4.3H. Fig. 4.3A, shows the uptake of transferrin and fig. 4.3C, shows the uptake of dextran in COS-1. Fig. 4.3E shows the uptake of transferrin and fig. 4.3G, shows the uptake of dextran in MCF-7 cells. Internalisation of both the markers is significantly reduced in the mitotic cells compared to interphase cells. (indicated
Fig. 4.2: Effect of wortmannin treatment on transient transfection. Transient transfection was carried out after wortmannin treatment in several different cell-types as described in the experimental section. Data is presented as wortmannin treated / untreated (+/-) ratio of relative β-gal activity.
Fig. 4.3: Endocytic activity of interphase and mitotic cells. Texas-Red labeled transferrin and FITC labeled dextran (70kDa) uptake was carried out in COS-1 and MCF-7 cells as described in the experimental section. Equal number of optical sections passing through the cells, collected by confocal microscope was merged in all the panels. 
(A) and (B) show transferrin uptake and nuclear staining in COS-1 cells respectively. (C) and (D) show dextran uptake and nuclear staining in COS-1 cells respectively. 
(E) and (F) show transferrin uptake and nuclear staining in MCF-7 cells respectively. (G) and (H) show dextran uptake and nuclear staining in MCF-7 cells respectively. In each panel arrowheads indicate mitotic cells. Scale bar indicates 10μm length.
by arrowheads). Next we carried out CLDC uptake again in COS-1 and MCF-7 cells. Rhodamine labelled liposomes was used to prepare CLDC as described in experimental section. These liposomes were equally efficient in transfection as that of unlabelled liposomes (data not shown), indicating that, the behaviour of labelled liposome is similar to that of unlabelled liposome during transfection. Imaging was done with live COS-1 and MCF-7 cells in case of CLDC uptake study using confocal microscope. Mitotic cells were selected by looking at the nuclear staining (Hoechst 33258) after exciting with UV excitation filter and optical sections were collected simultaneously both in rhodamine channel and transmission channel. Since there was increased photo bleaching of nuclear staining in case of live cells, while using mercury arc lamp of the microscope to excite Hoechst dye, we could not collect nuclear stained images for each area imaged, instead we collected images in transmission channel. Fig. 4.4 shows CLDC uptake is highly reduced in mitotic cells (indicated by arrowheads) when compared to interphase cells. Fig. 4.4, upper panel shows image of COS-1 cells and lower panel shows image of MCF-7 cells. In both the cell-types, CLDC fluorescence was considerably lower in mitotic cells compared to interphase cells. These results together indicate that the general endocytic activity is reduced in mitotic cells and this results in reduced uptake of CLDC.

4.3.4 Relative transfection efficiencies in interphase and mitotic cells:

Our CLDC uptake study in interphase and mitotic cells using confocal microscopy indicated poor CLDC uptake in mitotic cells as compared to interphase cells. To determine the relative transfection efficiencies in interphase and mitotic cells, we carried out transient transfection experiments with unsynchronised cells, which predominantly represent interphase cells and mitotic cells as described in the experimental section. Mitotic cells were obtained by treating the cells with colcemid. Colcemid is a microtubule depolymerising agent and blocks cells in metaphase. Unsynchronised cells had more than 85% interphase cells and cells enriched for mitotic cells had more than 90% cells in mitotic phase as determined by FACS analysis (data not shown). COS-1 and MCF-7 cells were again used for these transient transfections. Fig. 4.5 shows the
**Figure 4.4**

**Fig. 4.4:** CLDC uptake in interphase and mitotic cells. CLDC was prepared using rhodamine labelled liposomes and CLDC uptake experiment was carried out as described in experimental section. Upper panel shows CLDC uptake in COS-1 cells. (A) Fluorescence channel image, (B) Transmission channel image and (C) Merged image. Lower panel shows CLDC uptake in MCF-7 cells. (D) Fluorescence channel image, (E) Transmission channel image and (F) Merged image. Arrowheads indicate mitotic cells. Scale bar indicates 10μm length.
Fig. 4.5: Transient transfection in interphase and mitotic cells. Unsynchronised cells were used as a source of interphase cells and cells treated with colcemid were used as a source of mitotic cells. Transient transfection experiment was performed as described in the experimental section. Data is presented as relative β-gal activity.
relative transfection efficiencies between interphase and mitotic cells of COS-1 and MCF-7 cells. In both the cell-types, mitotic cells have dramatically reduced transfection efficiency. In case of COS-1 cells, mitotic cells have about 70% reduced transfection efficiency compared to interphase cells. Whereas, in case of MCF-7 cells, mitotic cells have about 90% reduced transfection efficiency compared to interphase cells.

4.4 Discussion
Transfection is a multistep process. Though cationic lipid mediated DNA delivery is a promising non-viral approach, it is not as efficient as viral mediated gene delivery. Unlike viral mediated gene delivery, cationic lipid mediated gene delivery is non-vectorial process. CLDC association or binding with the cell plasma membrane is believed to be a non-specific association. CLDC cell surface binding is mostly driven by electrostatic interactions between CLDC and proteoglycans present on the cell surface. Because of this, the extent of cell surface binding of CLDC depends on the surface charge of CLDC and abundance of cell surface proteoglycans. CLDC enter cells predominantly by endocytosis, but it has also been proposed that these may also enter the cells by plasma membrane fusion. A strong dependency of transfection process on the endocytic activity seen in our study (fig. 4.1C) suggests, that the amount of CLDC that enter the cells might primarily determine the internal concentration and thereby the transfection efficiency. Endocytosis involves several sub-pathways. Each of these sub-pathways is regulated by a set of proteins. By determining the sensitivity of ligand uptake to a particular inhibitor, which inhibits a specific regulatory protein involved in endocytosis, it is possible to ascertain the possible route that the ligand is following to enter the cell. PI 3-kinases play crucial role in the vesicular trafficking during endocytosis. It has been demonstrated that there may be endocytic trafficking dependent and independent of PI 3-kinase action in cells. We determined the effect of wortmannin, a PI 3-kinase inhibitor on the transfection efficiency in several cell-types. Results obtained from this experiment clearly suggest that depending on the cell-type,
CLDC uptake follows either PI 3-kinase dependent or independent pathway. But we did not find any significant correlation between the transfection efficiency and sensitivity to wortmannin treatment.

In order to substantiate our first observation of relationship between relative endocytic activity and transfection efficiency, we used interphase and mitotic cells as a model system for differential endocytic activities. It is well established that during mitosis, many of the mammalian cells show highly suppressed endocytic activity compared to interphase cells. Endocytosis is inhibited during prophase of mitosis and starts to regain its endocytic activity only during telophase. We used two cell-types - COS-1 and MCF-7, to study the differences in the CLDC uptake and transfection efficiency in interphase and mitotic cells. First we established that the general endocytosis is highly inhibited in mitotic cells of both COS-1 and MCF-7 cells. There was poor uptake of transferrin and dextran compared to their interphase cells. These mitotic cells also showed poor uptake of CLDC compared to surrounding interphase cells. It is thus clear that the endocytic activity determines the amount of CLDC that enter the cells. Interestingly, even the transfection efficiency in mitotic cells of both COS-1 and MCF-7 cells was greatly reduced compared to their interphase cells. Therefore, gene delivery efficiency is highly influenced by the amount of CLDC that enter the cells, which in turn is dependent on the endocytic activity of the cells.

In conclusion, our results provide evidence for the strong influence of endocytosis on the process of lipid mediated gene delivery. We propose CLDC entry into the cells by endocytosis is the first rate-limiting step in gene delivery process. The results presented here also suggest different pathway of entry of CLDC depending on the cell-type. The data reported here is significant in understanding the gene delivery process better and should also help to improve the process.