View metadata, citation and similar papers at core.ac.uk
brought to you by CORE
provided by Elsevier - Publisher Connector

FEBS 29519 FEBS Letters 579 (2005) 2635–2642

Quantitative aspects of endocytic activity in lipid-mediated transfections

Tekkatte Krishnamurthy Prasad*, Nandini Rangaraj, Nalam Madhusudhana Rao

Centre for Cellular and Molecular Biology, Uppal Road, Hyderabad 500 007, India

Received 26 January 2005; revised 14 March 2005; accepted 30 March 2005

Available online 9 April 2005

Edited by Sandro Sonnino

Abstract Variation in transfection efficiency observed in different cell-types is poorly understood. To investigate the influence of endocytic activity on lipid-mediated transfections, we have monitored both the processes in 12 different cell-types. The endocytic activity shows a strong positive correlation ($P < 0.01$), with transfection efficiency. Treatment with wortmannin resulted in cell-type-dependent inhibition of transfection. Studies on M-phase cells by confocal microscopy show that compared to interphase cells, uptake of cationic liposomes was substantially reduced. In addition, transfection efficiency of cells in mitotic phase was inhibited by >70% compared to controls. Our study based on several cell-types demonstrates for the first time that quantitative aspects of endocytosis have decisive influence on the overall process of lipid-mediated transgene expression.
© 2005 Federation of European Biochemical Societies. Published by Elsevier B.V. All rights reserved.

Keywords: Cationic lipid; Gene delivery; Endocytosis; Mitosis

1. Introduction

Cationic lipid-mediated gene delivery is procedurally simple and widely used for in vitro transfection of cells. A variety of cationic lipids have been synthesized and successfully tested for their transfection ability in several cell-types [1–6]. It is well documented that the transfection efficiency significantly depends on cell-type and physico-chemical properties of the cationic lipid DNA complexes (CLDC). Quality of the DNA sample, additives in the liposomes and conditions employed during complex formation and transfection protocols also influence the levels of transgene expression [7–10]. Significant variations in transfection efficiency are reported between different cell-types when tested with a single transfection protocol involving a liposomal formulation [11]. Observed variations were attributed to the cellular differences in CLDC adsorption and uptake processes that are cell-type specific. Cell-type specific differences were also ascribed to differences in endocytosis, variable half-lives of DNA in the cytoplasm and promoter-/enhancer-dependent nuclear import and gene expressions [12–15].

After the report by Zabner et al. using electron microscopy, several lines of evidence were presented to show the involvement of endocytosis in the uptake of CLDC. Several inhibitors of endocytosis such as chloroquine [16], low temperature [2], cholesterol depletion, chlorpromazine, potassium depletion [17], cytochalasin [18], etc. were shown to reduce the transgene expression. Fluorescently labeled CLDC were shown to colocalize with the well-known markers of endosomes [17]. Dominant negative mutants that interfere with endocytosis were shown reduce the cellular levels of CLDC [17]. The enhancing effects of endosomolytic moieties present in the CLDC on transgene expression further confirmed localization of CLDC within the endosomes [19]. These reports establish the role of endocytosis in uptake of CLDC unambiguously, however it is also of interest to understand the quantitative dependence of level of transgene expression in a given cell line on the endocytic activity observed in that cell-type. We have quantitated fluid phase endocytosis in 12 different cell-types and transgene expression in these cell-types to understand the correlation of first event of transfection, i.e., endocytosis with the final event of transfection, i.e., transgene expression.

Besides its dependence on cell-type, endocytic activity also depends on the cell cycle [20]. Temporary downregulation of endocytosis during prophase to telophase of cell cycle was demonstrated in several cell-types. The depressed endocytic activity measured by uptake of horseradish peroxidase (HRP) [21], fluorescein-labeled dextran particles [22] or recycling of transferring receptors [22] recovers fully once M-phase is completed. We have estimated the internalized fluorescently labeled CLDC in M-phase and interphase cells. Transfection efficiency of M-phase enriched cells is severely depressed. These studies affirm that in several cell-types quantitative aspects of endocytic activity have strong bearing on overall transfection efficiency.

2. Materials and methods

2.1. Chemicals

1,2-Dioleoyl-3-trimethylammonium-propane (DOTAP) and L-α-Dioleoyl phosphatidylethanolamine (DOPE) were purchased from Avanti Polar Lipids Inc. pCMV.SPORT-β-gal plasmid and Lipofectamine 2000™ were from Invitrogen. Horseradish peroxidase was purchased from Bangalore Genei, Bangalore, India. Texas Red-transferrin, fluorescein isothiocyanate (FITC)-dextran (70 kDa), rhodamine B 1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine (Rh-DHPE), *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-1,2-dihexadecanoyl-*sn*-glycerol-3-phosphaethanolamine (NBD-PE) and Hoechst 33258 were from Molecular Probes. Dulbecco's modified Eagle's medium (DMEM),

*Corresponding author. Fax: +91 40 2716 0591/0311.
E-mail address: tkprasad1975@gmail.com (T.K. Prasad).

Abbreviations: CLDC, cationic lipid DNA complexes; DOPE, L-α-dioleoyl phosphatidylethanolamine; DOTAP, 1,2-dioleoyl-3-trimethylammonium-propane; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; FITC, fluorescein isothiocyanate; HRP, horseradish peroxidase; NBD-PE, *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-1,2-dihexadecanoyl-*sn*-glycerol-3-phosphaethanolamine; PBS, phosphate buffered saline; Rh-DHPE, rhodamine B 1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine

0014-5793/$30.00 © 2005 Federation of European Biochemical Societies. Published by Elsevier B.V. All rights reserved.
doi:10.1016/j.febslet.2005.03.083

colcemid and wortmannin were from Sigma Chemicals. TNF-α was purchased from Calbiochem. All other reagents used were of high quality grade.

2.2. Cationic liposome preparation

DOTAP:DOPE (mol:mol) liposomes were prepared by drying appropriate amount from their chloroform stock solutions to get thin film at the bottom of a glass tube. Tubes were then dried in vacuum for 2 h. Dried lipids were hydrated overnight with sterile Milli-Q water. Liposome solution was then sonicated to get clear solution. In case of fluorescent labeling, above liposomes were prepared either with 5 mol% Rh-DHPE or 5 mol% NBD-PE.

2.3. Transient transfections

We used 12 cell-types for transfection experiments. Table 1 gives the list of the cell-types used along with the tissue and organism from which they are derived. Primary rat skin fibroblasts (RSF) were isolated as previously reported [23]. All the cell-types were maintained in DMEM containing 10% fetal calf serum and 500 μg/ml penicillin, 600 μg/ml streptomycin, 1 mg/ml kanamycin except C2C12, which was maintained in DMEM with 20% fetal calf serum containing antibiotics at 37 °C and 5% CO_2. 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 mol/mol) complexed with pCMV β-gal at 1:1 N/P charge ratio. Complexes were incubated with cells for about 3 h, 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 24 h before estimating the β-galactosidase activity. After 24 h medium was removed and cells were washed with phosphate buffered saline (PBS) and lysed with 50 μl lysis buffer (250 mM Tris–Cl, pH 8.0, containing 0.5% NP40) for 10 min at room temperature. 5 μl of cell lysate was used for protein estimation by modified Lowry's method [24] and to the remaining cell lysate 50 μl of β-galactosidase assay mix (200 mM sodium phosphate, pH 7.4, 2 mM $MgCl_2$, and 1.33 mg/ml *ortho*-nitrophenyl β-galactoside) was added. Plates were incubated at 37 °C and the color 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 normalized against milligram of cell protein. To see the effect of wortmannin on transfection, cells were treated with 250 ng/ml concentration of wortmannin for 30 min at 37 °C before adding the CLDC. Control cells were treated with only dimethyl sulfoxide (DMSO) at the same concentration used in case of wortmannin. Transient transfection and β-galactosidase activity assay was carried out as described before. Data are presented as wotmannin treated/untreated (+/−) ratio of relative β-galactosidase activity.

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 25 cm^2 flasks overnight. One flask was treated with the drug colcemid at 2 μg/ml concentration to block cells in mitotic phase and another flask was treated with only DMSO for 24 h. DMSO treated cells were trypsinized 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 3 h at 37 °C and 5% CO_2. After 3 h, DMEM with 10% fetal calf serum was added. Cells were assayed for β-galactosidase activity after 24 h of transfection as described before. β-galactosidase activity was normalized with milligram of protein.

Table 1

Cell-type	Tissue	Organism
COS-1	Kidney, SV40 transformed	African green monkey
CV-1	Kidney, normal	African green monkey
HeLa	Cervix	Human
CHO	Ovary	Hamster
Hep G2	Liver	Human
NIH 3T3	Embryo	Mouse
F111	Embryonic fibroblast	Rat
MCF-7	Mammary glad	Human
BRL	Liver	Rat
L929	Areolar and adipose	Mouse
C2C12	Muscle	Mouse
RSF	Skin fibroblast (primary)	Rat

2.5. Reporter assays for determining NF-κB activity

To measure the levels of NF-κB in the cells after colcemid treatment, we transiently transfected COS-1 and MCF-7 cells using pHIVLuc plasmid (gift from Wallach D., Weissman Institute, Israel). This plasmid has NF-κB responsive element in the promoter region and luciferase expression is sensitive to the levels of NF-κB in the nucleus [25]. Luciferase activity from pHIVLuc was normalized by β-galactosidase activity from cotransfected pCMV β-gal plasmid. Transient transfections were carried out using Lipofectamine 2000™ according to the manufacturer's protocol. After about 24 h of transfection, cells were treated with DMSO (control) or colcemid (2 μg /ml) or TNF-α (20 ng/ml) for 6 h. At the end of 6 h, cells were washed once with PBS and lysed with lysis buffer (250 mM Tris–Cl, pH 8.0, containing 0.5% NP40) for 10 min at room temperature. Lysate was used to measure luciferase activity and β-galactosidase activity. Luciferase activity was normalized with β-galactosidase activity and the data are presented as fold activation of NF-κB.

2.6. 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, HRP. HRP uptake was determined by following a previously described method [26] with slight modifications. Cells grown in 24-well plates at high density were washed once with DMEM. HRP uptake was initiated by adding 100 μg/ml concentration of HRP in DMEM to cells. Cells were incubated at 37 °C. After 2 h 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 HRP activity by adding 2× HRP substrate solution (1.5 mg/ml of *o*-phenylenediamine in 1 N sodium acetate, pH 5.2, containing 0.12% H_2O_2) and incubating at 37 °C for 10 min. By adding an equal volume of 0.1 N H_2SO_4, reaction was stopped and color developed was read at 490 nm. Protein content of cell lysate was estimated by modified Lowry's method [24]. Activity of HRP was expressed as absorbance at 490 nm/mg of protein. Initially we have assured ourselves that the HRP uptake was linear for more than 2 h.

2.7. Transferrin, dextran and CLDC uptake by interphase and mitotic cells

For assessing transferrin and dextran uptake by interphase and mitotic cells, unsynchronized COS-1 and MCF-7 cells were grown on coverslip. Cells were washed once with DMEM and incubated with either 80 μg/ml of Texas Red-transferrin for 5 min or 5 mg/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 10 μg/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 labeled 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% CO_2. Cells were washed twice with DMEM and stained with Hoechst 33258 (10 μg/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 100× 1.4 NA oil immersion objective, whereas CLDC uptake was imaged with 63× 1.2 NA water immersion objective. 488 nm laser line was used for exciting FITC and 543 nm was used for exciting Rhodamine and Texas-Red. Emission was collected using appropriate emission filters. About 0.75 μm thick slices were collected using 150 μm

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.

2.8. Quantitation of CLDC uptake by interphase and mitotic cells

Cells were grown in chambered coverglass (Lab-Tek, Nalge Nunc International Corp.) to a confluency of about 60%. CLDC were prepared as described for transient transfections using 5 mol% NBD-PE labeled liposomes and added to the cells. They were incubated at 37 °C and 5% CO_2 for 30 min. Subsequently, cells were washed thrice with ice-cold HEPES–Hank's balanced salt solution (HEPES–HBSS) and stained with Hoechst 33258 (10 μg/ml in HEPES–HBSS) for 30 min on ice to stain the nuclear DNA. This was followed by quenching of fluorescence from externally bound CLDC by adding 0.4% trypan blue containing HEPES–HBSS. Addition of trypan blue has been shown to efficiently quench the external fluorescence of CLDC [17]. Cells were later washed twice with ice-cold HEPES–HBSS and imaged. Images were collected using LSM 510 META, Carl Ziess, confocal microscope with 40×/0.95 NA objective at 0.7 zoom and open pinhole (1000 μm). 488 nm laser line was used for exciting NBD. Emission was collected using appropriate emission filters. Mitotic cells were selected by observing nuclear staining using DAPI filter. The total mean fluorescence intensity per cell was determined for interphase and mitotic cells using the LSM 510 META software. Fluorescence was averaged over at least 40 interphase cells and 20 mitotic cells in each cell-type.

3. Results

3.1. Relationship between transfection and endocytic activity

We performed transient transfections in 12 different cell-types as described in Section 2. Table 1 gives the details of all the cell-types used. These 12 cell-types were selected based on their known variability in transfection efficiency and their different tissue (or organism) origin. Transfections were performed using DOTAP:DOPE and quantitated using β-galactosidase as the reporter gene. As can be seen from Fig. 1A, transfection efficiency varies between different cell-types by nearly 80-fold. Highest transfection was seen with CHO cells and least transfectable cells are RSF, C2C12, L929 and NIH 3T3. Transfection efficiencies of rest of the cell-types were distributed between these extremes. On the same batch of cells we measured endocytic activities, using HRP as a marker. Fig. 1B shows endocytic activities of all the cell-types. Amount of HRP present within the cells after 2 h of incubation varied over 15-fold among these cell-types. Strongly transfected cell-types also demonstrate increased uptake of HRP. When plotted, transfection efficiency shows significant correlation with HRP uptake efficiency (Fig. 1C). We observed a correlation coefficient of 0.85, which is significant ($P < 0.01$) for the sample size. Two cell-types, L929 and MCF-7, were observed outliers in the relation.

3.2. Effect of wortmannin on the CLDC cell entry

Endocytosis constitutes several processes, where each process was characterized by the nature of the opsonized ligand or sensitivity to inhibitors. To probe whether similar endocytic pathways were involved in different cell-types we have determined the effect of wortmannin, a PI 3-kinase inhibitor on transfection efficiency in different cell-types. Wortmannin treatment does not inhibit transfection to the same extent in different cell-types tested (Fig. 2). 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. The mixed response of various cell-types to wortmannin treatment suggests involvement of PI 3-kinase dependent and independent CLDC uptake pathways.

3.3. Transferrin, dextran and CLDC uptake in interphase and mitotic cells

During mitosis the cell significantly reduces the endocytic activity. We employed this relation to demonstrate the essential role of endocytosis in CLDC uptake. Mitotic cells have significantly reduced endocytic activity compared to interphase cells [20–22]. Uptake of fluorescently labeled transferrin and dextran (70 kDa) was studied in COS-1 and MCF-7 cells. Transferrin is a marker for receptor-mediated endocytosis and dextran is a marker for fluid phase endocytosis. Confocal microscopy was used to study the uptake as described in Section 2. We specifically looked at the relative uptake of these

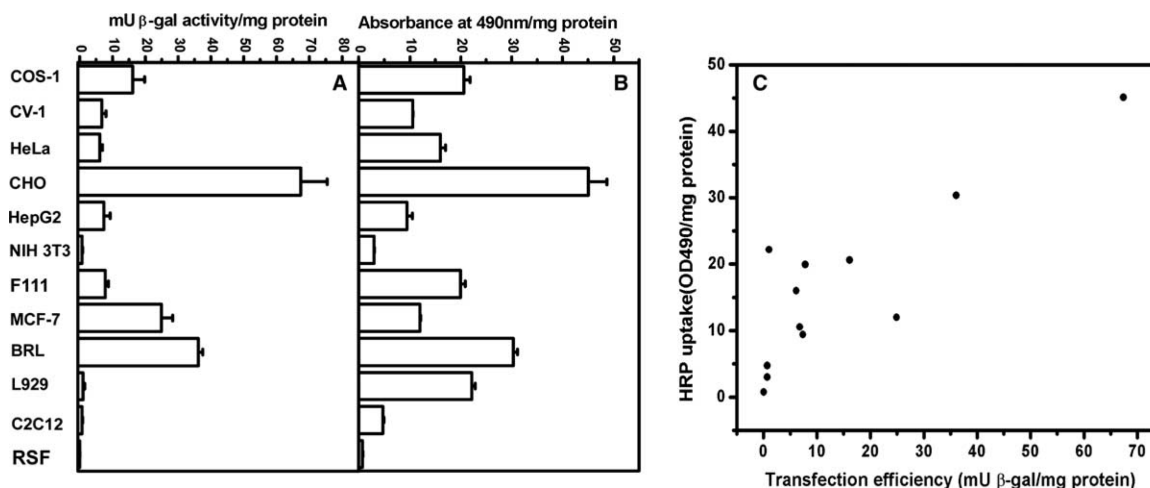

Fig. 1. Relationship between transfection efficiency and endocytic activity. (A) Transient transfection was carried out in 12 cell-types as described in Section 2. Data are presented as milliunits of β-galactosidase activity normalized to protein content of the cell lysate. (B) Horseradish peroxidase was used as a marker to measure the endocytic activity of different cell-types as described in Section 2. Data are presented as absorbance at 490 nm normalized to protein content. (C) Correlation plot drawn between transfection efficiency and endocytic activity.

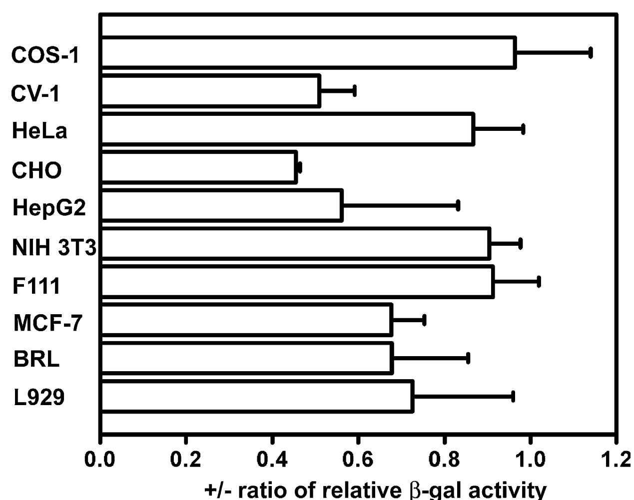

Fig. 2. Effect of wortmannin treatment on transient transfection. Transient transfection was carried out after wortmannin treatment in several different cell-types as described in Section 2. Data are presented as wortmannin treated/untreated (+/−) ratio of relative β-gal activity.

markers in interphase cells and mitotic cells. Asynchronous populations used in this approach remove ambiguity associated with studies on synchronized cells prepared using chemical treatments. Equal number of optical sections passing through the cells was merged and is shown in Fig. 3. For each area imaged by confocal microscopy, images were also collected for nuclear stain (Hoechst 33258) using UV emission filter. Cells with condensed nuclei were scored as cells in mitosis, which are indicated by arrowheads in Fig. 3b, d, f and h. Internalization of both the markers was significantly reduced in the mitotic cells compared to interphase cells in both MCF-7 and COS-1 cells (indicated by arrowheads). Next we carried out CLDC uptake again in COS-1 and MCF-7 cells. Rhodamine labeled liposomes was used to prepare CLDC as described in Section 2. These liposomes were equally efficient in transfection as that of unlabeled liposomes (data not shown), indicating that, the behavior of labeled liposome is similar to that of unlabeled liposome during transfection. Imaging of internalized fluorescent CLDC 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. 4A shows CLDC uptake is highly reduced in mitotic cells (indicated by arrowheads) when compared to surrounding interphase cells in both MCF-7 and COS-1. We further estimated CLDC uptake in interphase and mitotic cells of MCF-7, COS-1, HeLa and CHO. NBD-PE labeled liposomes were used to prepare CLDC and the fluorescence contributed by the external cell surface associated CLDC was quenched by adding trypan blue [17]. This allowed us to quantitate only internalized CLDC. Images were collected from several areas for both interphase and mitotic cells for analysis. Fig. 4B shows mean fluorescence intensity due to CLDC uptake in four cell-types. It is very clear that in all the cell-types tested, there is more than 90% decrease in CLDC uptake by mitotic cells compared to interphase cells. Decrease in the uptake of ligands of endocytosis, dextran and transferrin, along with CLDC in mitotic cells indicates that all the three ligands pathways of endocytosis were depressed.

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 unsynchronized cells, which predominantly represent interphase cells, and in population of cells enriched with mitotic cells by treatment with colcemid as described in Section 2. Colcemid is a microtubule depolymerizing agent and blocks cells

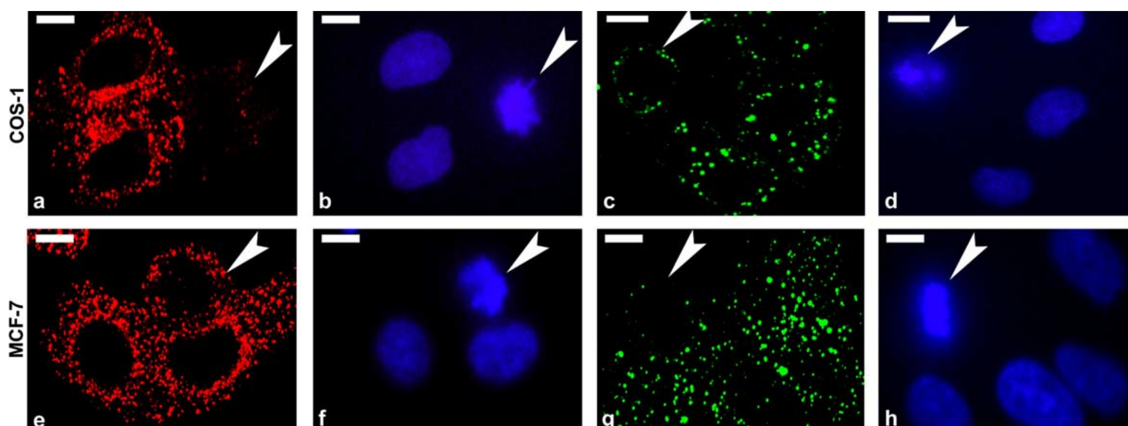

Fig. 3. Endocytic activity of interphase and mitotic cells. Texas-Red labeled transferrin and FITC labeled dextran (70 kDa) uptake was carried out in COS-1 and MCF-7 cells as described in Section 2. Equal number of optical sections passing through the cells, collected by confocal microscope was merged 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 10 μm length.

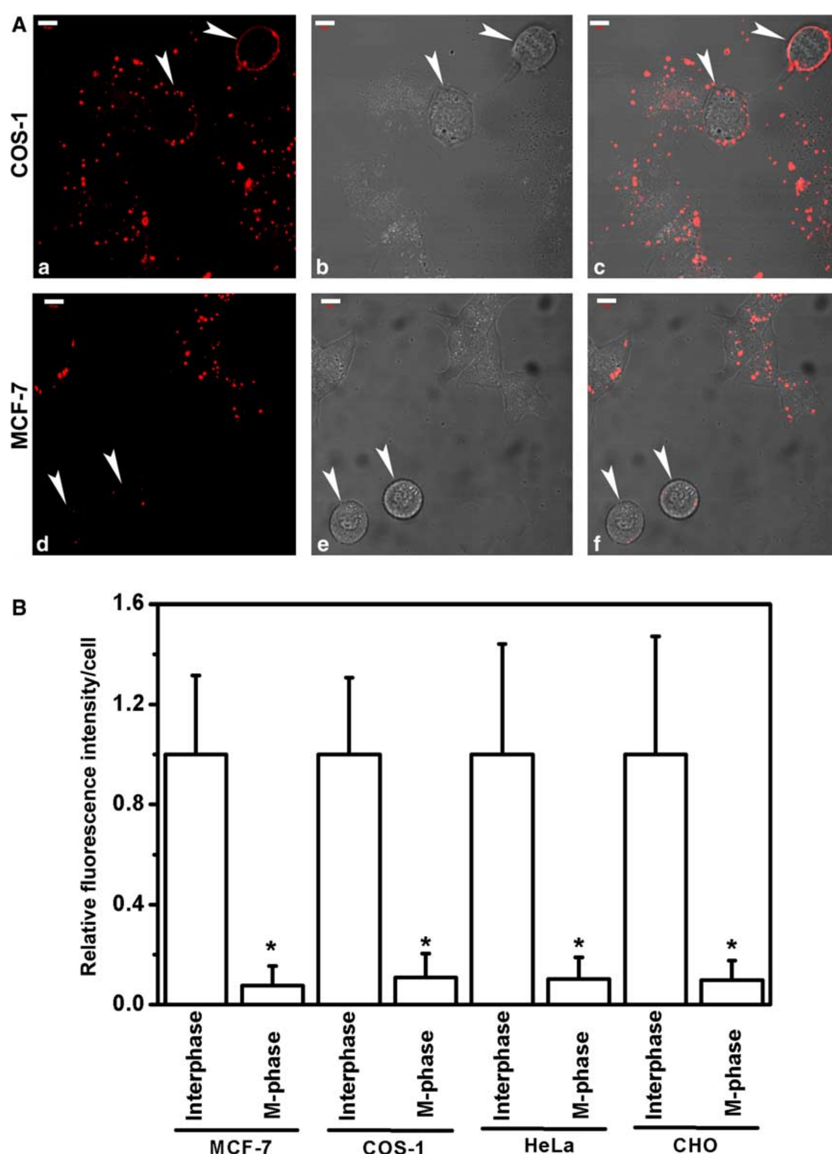

Fig. 4. CLDC uptake in interphase and mitotic cells. (A) Representative confocal images of CLDC uptake. CLDC was prepared using rhodamine labeled liposomes and CLDC uptake experiment was carried out as described in Section 2. Upper panel shows CLDC uptake in COS-1 cells: (a) fluorescence channel image, (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 10 μm length. (B) Quantitation of CLDC uptake in interphase and mitotic cells. CLDC uptake was quantitated in interphase and M-phase cells of multiple cell-types by determining the total mean fluorescence intensity per cell as described in Section 2. Data are presented as normalized total mean fluorescence intensity per cell. Statistical analysis of the data indicates the difference in CLDC uptake between interphase and mitotic cells was significant at $P < 0.001$ (*).

in metaphase [27]. Unsychronized 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. 5A shows the relative transfection efficiencies between interphase and mitotic cells of COS-1 and MCF-7 cells. In both the cell-types, transfection efficiency was significantly reduced in colcemid treated cells. 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.

It is known that microtubule depolymerization could result in change in the activity of NF-κB [28–30]. Since the plasmid used in our reporter gene activities contains NF-κB binding sites, we wanted to evaluate the contribution of altered activity of NF-κB in the observed reporter gene activities. To measure the activity of NF-κB in the above cell-types after colcemid treatment, we employed pHIVLuc plasmid, containing a NF-κB responsive element. Fig. 5B shows that there no significant change in the NF-κB activation after colcemid treatment in both the cell types, whereas treatment with TNF-α, a known inducer of NF-κB [31–34] showed nearly a 3-fold activation of NF-κB in both the cell-types.

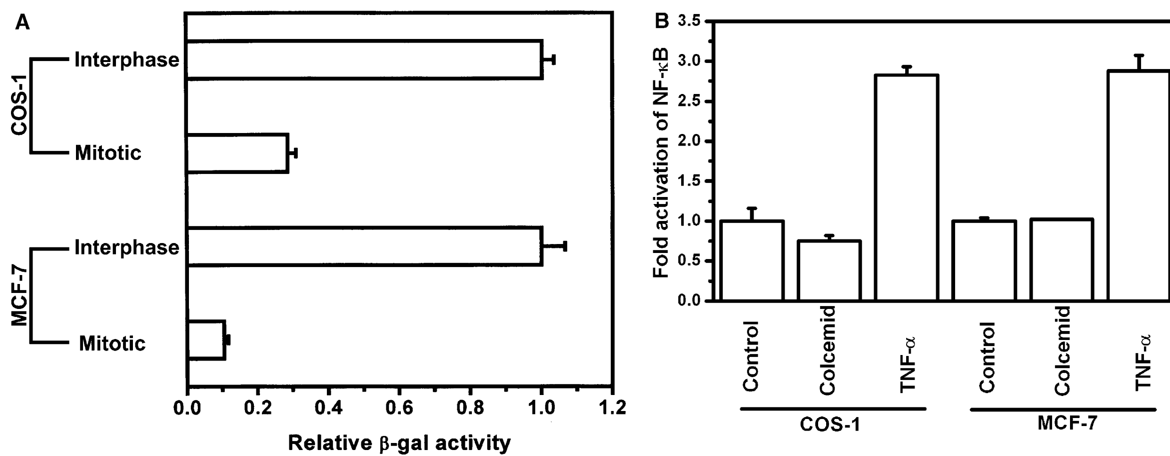

Fig. 5. (A) Transient transfection in interphase and mitotic cells. Unsynchronized cells were used as a source of interphase cells and cells treated with colcemid were used as a source of mitotic cells. Transient transfection experiment was performed as described in Section 2. Data are presented as relative β-gal activity. (B) Luciferase activities from pHIVLuc in COS-1 and MCF-7 Transient transfections were done with pHIVLuc plasmid using Lipofectamine 2000™. 24 h after of transfection, cells were treated for a duration of 6 h with DMSO or colcemid (2 μg/ml) or TNF-α (20 ng/ml). Luciferase expression levels were determined at the end of 6 h. Data are presented as fold activation of NF-κB.

4. Discussion

Transfection is a multistep process [2]. 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, a process made nucleotropic with the involvement of several viral protein, cationic lipid-mediated gene delivery is understood to be largely a stochastic 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 [35]. 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 [2,36]. A strong dependency of transfection efficiency on the endocytic activity seen in our study suggests that the amount of CLDC that enter the cells might primarily determine the internal concentration and thereby the output of the process, i.e., reporter gene expression.

In addition to endocytosis, half-life of plasmid DNA in the cytoplasm and subsequent rate of nuclear import are also considered to be rate limiting. Half-life of plasmid DNA in the cytoplasm is critical for transgene expression, however cell-type differences in plasmid half-lives are not well documented. Some studies reported presence of plasmid specific nucleases in cytoplasm [13]. The significance of nuclear import in transgene expression is well studied using the method of microinjection of plasmid into the cytoplasm and nucleus followed by in situ hybridization. Though considered to be slow, compared to the nuclear import of viral DNA, rate of import of lipid-mediated DNA shows cell-type specificity. In situ hybridization studies on five different cell-types showed significant differences in early time points and the differences were reduced at later time points (>8 h) [14]. Based on these observation strong correlation between transfection efficiency and endocytic activity on widely different cell lines argues for the importance of quantitative aspects of endocytosis in transgene expression.

Route of receptor-mediated endocytosis was specifically exploited to increase the uptake of CLDC by chemical linking of a ligand of known receptor to the cationic lipids [37,38]. Undecorated cationic lipids predominantly use fluid-phase endocytosis to gain entry into the cells. It would be of interest to see the effect of stimulation of fluid phase endocytosis on lipid-mediated transfections. Endocytosis involves several sub-pathways. By determining the sensitivity of CLDC uptake to an inhibitor, which blocks a specific regulatory protein involved in endocytosis, it is possible to ascertain the pathway encountered by the CLDC. PI 3-kinases play crucial role in the vesicular trafficking during endocytosis [39]. It has been demonstrated that there may be PI 3-kinase dependent and independent endocytic trafficking in cells [40,41]. Variable sensitivity of transfection to wortmannin treatment in different cell-types indicates that in addition to flux of CLDC via endocytosis, a chemically identical CLDC may access different pathways depending on the cell-type. CHO shows maximum uptake of HRP with 60% inhibition of transfection by wortmannin, whereas NIH 3T3 shows only 10% of HRP uptake compared to CHO but was only marginal sensitive to wortmannin treatment. Using a set of inhibitors a recent report suggested the involvement of clathrin-mediated endocytosis in CLDC uptake in COS-7 [17]. Understanding relative abundance of different endocytic pathways in cells is important to appreciate the variance in transfection efficiencies in cell-types.

Profound suppression of endocytosis as the cell enters the M-phase is well documented from several lines of evidence [20–22]. Endocytosis is rapidly (<30 s) inhibited during prophase of mitosis and rapidly regains its endocytic activity during telophase [20]. Inhibition of internalization of markers of receptor-mediated endocytosis (transferrin) and fluid phase markers of endocytosis (dextran) along with CLDC in mitotic cells suggests that the endocytic activity is significantly suppressed in M-phase cells. Consequently, internalized CLDC in mitotic cells was significantly lower compared to interphase cells in asynchronous population of MCF-7, COS-1, HeLa and CHO cells. In COS-1 and MCF-7 transfection efficiency in col-

cemid treated cells (more than 90% cells in M-phase) was substantially reduced compared to cells present in asynchronous cells emphasizing the need for active endocytosis for CLDC uptake. The observed decrease in transfection efficiency upon colcemid was truly due to depressed endocytosis in M-phase cells and not due to any changes in the NF-κB levels upon tubulin depolymerization. Our reporter plasmid assays using plasmid construct pHIVLuc (responsive to the NF-κB nuclear levels) clearly shows that the activity of NF-κB in both COS-1 and MCF-7 cells do not change significantly after treating cells with colcemid (Fig. 5B). TNF-α, well-known inducer of NF-κB [31–34], results in about 3-fold increase in luciferase gene expression. This ruled out the possibility of decrease in the activity of NF-κB playing a role in the decreased gene expression observed in M-phase cells. Easy enumeration of adjacent mitotic and interphase cells based on nuclear staining combined with comparable rates of M-phase progression and the rates of endocytosis makes this system very convenient to investigate the role of endocytosis in cellular uptake of ligands. Methods to demonstrate the role of endocytosis in CLDC uptake such as exposure to 4 °C, cholesterol depletion may have pleiotropic effects. Use of M-phase cells in asynchronous population is free of any treatment and hence more reliable.

Our study focuses on the endocytosis during the M-phase. Absence of nuclear membrane, a kinetic barrier for transgene expression, during M-phase was shown to aid in increasing the transgene expression [42]. These studies discuss the advantage of addition of CLDC to cells before they reach M-phase, i.e., during G1 phase or G2/M phase and allowed to go through the M-phase after removing the block at various time points [43]. To take advantage of the dissolution of nuclear membrane in M-phase, the CLDC must enter the cells in pre M-phase when endocytosis is not down regulated. Barrier property of nuclear membrane was unambiguously demonstrated by FISH (fluorescence in situ hybridization) of microinjected plasmid into the nucleus or into the cytoplasm [14]. These observations emphasize the requirement to add CLDC before M-phase is entered.

Our results provide evidence for the strong influence of endocytosis on the overall process of transgene expression. Sample size of 12 involving cell-types of large differences in transfectability substantiates the underlying relation. Confocal microscopy based simultaneous quantitation of endocytosed particles in mitotic and interphase cells offers convenience to investigate the role of endocytosis in lipofection. Unlike receptor-mediated endocytosis, where the process is specifically exploited by synthetic molecular tags [37,38], similar efforts could also be directed at increasing the fluid phase endocytosis to increase the transgene expression.

Acknowledgments: Authors thank Mubarak Ali for helping with isolation of primary rat skin fibroblast cells. T.K.P. thanks Council of Scientific and Industrial Research for senior research fellowship.

References

[1] Miller, A.D. (1998) Cationic liposomes for gene therapy. Angew. Chem. Int. Ed. 37, 1768–1785.

[2] Zabner, J., Fasbender, A.J., Moninger, T., Poellinger, K.A. and Welsh, M.J. (1995) Cellular and molecular barriers to gene transfer by a cationic lipid. J. Biol. Chem. 270, 18997–19007.

[3] Gao, X. and Huang, L. (1991) A novel cationic liposome reagent for efficient transfection of mammalian cells. Biochem. Biophys. Res. Commun. 179, 280–285.

[4] Felgner, J.H., Kumar, R., Sridhar, C.N., Wheeler, C.J., Tsai, Y.J., Border, R., Ramsey, P., Martin, M. and Felgner, P.L. (1994) Enhanced gene delivery and mechanism studies with a novel series of cationic lipid formulations. J. Biol. Chem. 269, 2550–2561.

[5] Banerjee, R., Das, P.K., Srilakshmi, G.V., Chaudhuri, A. and Rao, N.M. (1999) Novel series of non-glycerol-based cationic transfection lipids for use in liposomal gene delivery. J. Med. Chem. 42, 4292–4299.

[6] Ren, T., Zhang, G., Song, Y.K. and Liu, D. (1999) Synthesis and characterization of aromatic ring-based cationic lipids for gene delivery in vitro and in vivo. J. Drug Target 7, 285–292.

[7] Kreiss, P., Cameron, B., Rangara, R., Mailhe, P., Aguerre-Charriol, O., Airiau, M., Scherman, D., Crouzet, J. and Pitard, B. (1999) Plasmid DNA size does not affect the physicochemical properties of lipoplexes but modulates gene transfer efficiency. Nucleic Acids Res. 27, 3792–3798.

[8] Kerner, M., Meyuhas, O., Hirsch-Lerner, D., Rosen, L.J., Min, Z. and Barenholz, Y. (2001) Interplay in lipoplexes between type of pDNA promoter and lipid composition determines transfection efficiency of human growth hormone in NIH3T3 cells in culture. Biochim. Biophys. Acta 1532, 128–136.

[9] Banerjee, R., Mahidhar, Y.V., Chaudhuri, A., Gopal, V. and Rao, N.M. (2001) Design, synthesis, and transfection biology of novel cationic glycolipids for use in liposomal gene delivery. J. Med. Chem. 44, 4176–4185.

[10] Ferrari, M.E., Rusalov, D., Enas, J. and Wheeler, C.J. (2002) Synergy between cationic lipid and co-lipid determines the macroscopic structure and transfection activity of lipoplexes. Nucleic Acids Res. 30, 1808–1816.

[11] Boussif, O., Zanta, M.A. and Behr, J.P. (1996) Optimized galenics improve in vitro gene transfer with cationic molecules up to 1000-fold. Gene Ther. 3, 1074–1080.

[12] Bally, M.B., Harvie, P., Wong, F.M., Kong, S., Wasan, E.K. and Reimer, D.L. (1999) Biological barriers to cellular delivery of lipid-based DNA carriers. Adv. Drug Deliv. Rev. 38, 291–315.

[13] Pollard, H., Toumaniantz, G., Amos, J.L., Avet-Loiseau, H., Guihard, G., Behr, J.P. and Escande, D. (2001) Ca^{2+}-sensitive cytosolic nucleases prevent efficient delivery to the nucleus of injected plasmids. J. Gene Med. 3, 153–164.

[14] Dean, D.A., Dean, B.S., Muller, S. and Smith, L.C. (1999) Sequence requirements for plasmid nuclear import. Exp. Cell Res. 253, 713–722.

[15] Dean, D.A. (1997) Import of plasmid DNA into the nucleus is sequence specific. Exp. Cell Res. 230, 293–302.

[16] Farhood, H., Serbina, N. and Huang, L. (1995) The role of dioleoyl phosphatidylethanolamine in cationic liposome mediated gene transfer. Biochim. Biophys. Acta 1235, 289–295.

[17] Zuhorn, I.S., Kalicharan, R. and Hoekstra, D. (2002) Lipoplex-mediated transfection of mammalian cells occurs through the cholesterol-dependent clathrin-mediated pathway of endocytosis. J. Biol. Chem. 277, 18021–18028.

[18] Hui, S.W., Langner, M., Zhao, Y.L., Ross, P., Hurley, E. and Chan, K. (1996) The role of helper lipids in cationic liposome-mediated gene transfer. Biophys. J. 71, 590–599.

[19] Reddy, J.A. and Low, P.S. (2000) Enhanced folate receptor mediated gene therapy using a novel pH-sensitive lipid formulation. J. Contr. Release 64, 27–37.

[20] Berlin, R.D. and Oliver, J.M. (1980) Surface functions during mitosis. II. Quantitation of pinocytosis and kinetic characterization of the mitotic cycle with a new fluorescence technique. J. Cell Biol. 85, 660–671.

[21] Berlin, R.D., Oliver, J.M. and Walter, R.J. (1978) Surface functions during Mitosis I: phagocytosis, pinocytosis and mobility of surface-bound Con A. Cell 15, 327–341.

[22] Warren, G., Davoust, J. and Cockcroft, A. (1984) Recycling of transferrin receptors in A431 cells is inhibited during mitosis. EMBO J. 3, 2217–2225.

[23] Vaughan, F.L. and Bernstam, L.I. (2005) Isolation, purification, and cultivation of murine and human keratinocytes. Methods Mol. Biol. 290, 187–206.

[24] Markwell, M.A., Haas, S.M., Tolbert, N.E. and Bieber, L.L. (1981) Protein determination in membrane and lipoprotein samples: manual and automated procedures. Methods Enzymol. 72, 296–303.
[25] Zhang, S.Q., Kovalenko, A., Cantarella, G. and Wallach, D. (2000) Recruitment of the IKK signalosome to the p55 TNF receptor: RIP and A20 bind to NEMO (IKKgamma) upon receptor stimulation. Immunity 12, 301–311.
[26] Li, G. and Stahl, P.D. (1993) Structure–function relationship of the small GTPase rab5. J. Biol. Chem. 268, 24475–24480.
[27] Rieder, C.L. and Cole, R. (2000) Microtubule disassembly delays the G2-M transition in vertebrates. Curr. Biol. 10, 1067–1070.
[28] Wang, L. and MacDonald, R.C. (2004) Effects of microtubule-depolymerizing agents on the transfection of cultured vascular smooth muscle cells: enhanced expression with free drug and especially with drug-gene lipoplexes. Mol. Ther. 9, 729–737.
[29] Rosette, C. and Karin, M. (1995) Cytoskeletal control of gene expression: depolymerization of microtubules activates NF-kappa B. J. Cell Biol. 128, 1111–1119.
[30] Mistry, P., Deacon, K., Mistry, S., Blank, J. and Patel, R. (2004) NF-kappaB promotes survival during mitotic cell cycle arrest. J. Biol. Chem. 279, 1482–1490.
[31] Han, Y.P., Tuan, T.L., Wu, H., Hughes, M. and Garner, W.L. (2001) TNF-alpha stimulates activation of pro-MMP2 in human skin through NF-(kappa)B mediated induction of MT1-MMP. J. Cell Sci. 114, 131–139.
[32] Ozes, O.N., Mayo, L.D., Gustin, J.A., Pfeffer, S.R., Pfeffer, L.M. and Donner, D.B. (1999) NF-kappaB activation by tumour necrosis factor requires the Akt serine-threonine kinase. Nature 401, 82–85.
[33] Soler, A.P., Marano, C.W., Bryans, M., Miller, R.D., Garulacan, L.A., Mauldin, S.K., Stamato, T.D. and Mullin, J.M. (1999) Activation of NF-kappaB is necessary for the restoration of the barrier function of an epithelium undergoing TNF-alpha-induced apoptosis. Eur. J. Cell Biol. 78, 56–66.
[34] Rath, P.C. and Aggarwal, B.B. (1999) TNF-induced signaling in apoptosis. J. Clin. Immunol. 19, 350–364.
[35] Mislick, K.A. and Baldeschwieler, J.D. (1996) Evidence for the role of proteoglycans in cation-mediated gene transfer. Proc. Natl. Acad. Sci. USA 93, 12349–12354.
[36] Friend, D.S., Papahadjopoulos, D. and Debs, R.J. (1996) Endocytosis and intracellular processing accompanying transfection mediated by cationic liposomes. Biochim. Biophys. Acta 1278, 41–50.
[37] Reddy, J.A., Dean, D., Kennedy, M.D. and Low, P.S. (1999) Optimization of folate-conjugated liposomal vectors for folate receptor-mediated gene therapy. J. Pharm. Sci. 88, 1112–1118.
[38] Lee, H., Kim, T.H. and Park, T.G. (2002) A receptor-mediated gene delivery system using streptavidin and biotin-derivatized, pegylated epidermal growth factor. J. Contr. Release 83, 109–119.
[39] Spiro, D.J., Boll, W., Kirchhausen, T. and Wessling-Resnick, M. (1996) Wortmannin alters the transferrin receptor endocytic pathway in vivo and in vitro. Mol. Biol. Cell 7, 355–367.
[40] Araki, N., Johnson, M.T. and Swanson, J.A. (1996) A role for phosphoinositide 3-kinase in the completion of macropinocytosis and phagocytosis by macrophages. J. Cell Biol. 135, 1249–1260.
[41] Kanagasundaram, V., Christy, E., Hamilton, J.A. and Jaworowski, A. (1998) Different pathways of colony-stimulating factor 1 degradation in macrophage populations revealed by wortmannin sensitivity. Biochem. J. 330, 197–202.
[42] Mortimer, I., Tam, P., MacLachlan, I., Graham, R.W., Saravolac, E.G. and Joshi, P.B. (1999) Cationic lipid-mediated transfection of cells in culture requires mitotic activity. Gene Ther. 6, 403–411.
[43] Brunner, S., Sauer, T., Carotta, S., Cotten, M., Saltik, M. and Wagner, E. (2000) Cell cycle dependence of gene transfer by lipoplex, polyplex and recombinant adenovirus. Gene Ther. 7, 401–407.