Chapter 8

Cell Line Studies

Chapter 8

8.1 Introduction

In vitro cell line studies are essential as they offer the methods for primary evaluation of direct effects of drugs and formulations on cells and tissues so as to form a basis for *in vivo* animal studies and clinical studies. These studies are important which can give ideas on clinical applicability in pathological conditions and understand molecular mechanisms as well as can screen the test samples for their efficacy and toxicity.

Mainly, culturing of cells is a technique in which a tissue or outgrowth of a primary explant is isolated, either mechanically or enzymatically and dispersed in culture medium to prepare a cell suspension. This cells suspension may form a solid substrate or an adherent monolayer or a cell suspension in culture medium. The cells undergo proliferation in cultures forming a monolayer or suspension, which constitute a passage. After a number of generations cells can transform into continuous cells having capability of high growth and uniformity of population. Adherent monolayer cell culture is widely used for *in vitro* experiments and the cells of this culture are generally anchorage dependent as for proliferation they require a substrate for attachment of cells. On the other side suspension cultures are rarely explored in experiments and are anchorage independent and can proliferate without attachment.

Primary cell cultures are sometimes not used for experimental studies due to their poor stability as they undergo constant adaptive alterations and it is challenging to select a period of when the total cell population is homogenous or stable. After confluence some cells may transform and become insensitive to contact inhibition and overgrow, therefore it is necessary to keep the cell density low to maintain the original phenotype. After first subculture or a passage, the culture is called cell line. In each subsequent subculture a population of cell having capacity to rapidly grow will predominate while slow growing cells dilute out. In most cases culture becomes stable after three passages.

The propagation and growth of cell line requires a culture media with distinct chemical composition to confirm consistent quality and reproducibility. Mostly all the cells grow efficiently well at pH 7.4 and in 5% CO_2 environment as CO_2 gas phase after dissolution into culture medium can establish an equilibrium with HCO_3^- ions present in the medium to maintain the pH. Besides HCO_3^- other ingredients such as pyruvate, high

concentration of amino acids are used as buffering agent in culture media. The cells also need oxygen, thus the depth of static culture should be kept within the range of 2-5 mm so as to maintain the rate of oxygen diffusion to the cells. The requirements of temperature rely on body temperature of animal from which cells were obtained and thus kept at 37 °C.

8.2 Materials and Instruments

Materials

Sr No	Chemicals/Materials	Source/Manufacturer
1.	HEPG2 cell line	NCCS, Pune, India.
2.	Thiazolyl Blue Tetrazolium Bromide (MTT)	HiMedia, Mumbai, India
3.	Dulbecco's Modified Eagle Medium (DMEM) (high glucose)	HiMedia, Mumbai, India
4.	Fetal Bovine Serum (FBS)	HiMedia, Mumbai, India
5.	Penicillin/streptomycin antibiotic	HiMedia, Mumbai, India
6.	Trypsin EDTA	HiMedia, Mumbai, India
7.	RNAse	HiMedia, Mumbai, India
8.	Trypan blue	HiMedia, Mumbai, India
9.	DAPI	HiMedia, Mumbai, India
10.	Dulbecco's Modified Eagle Medium	ATCC, USA

All other chemicals used were of analytical reagent grade and were used without any further purification.

Instruments

Sr No	Instruments	Company
1.	BOD Shaker Incubator	Orbitek, Scigenics
2.	Centrifuge (CPR-30)	Remi Elektrotechnik Ltd., India.
3.	UV Visible Spectrophotometer (1800)	Schimadzu, India
4.	Laminar air flow (HEPA filter)	Weiber vertical laminar air flow
5.	BD FACS AriaIII	BD Biosciences, USA
6.	Confocal laser scanning microscope	CarlZeiss LSM 710, Germany
7.	Jouan IGO150 CELL life CO2	Thermo Fisher Scientific, India
8.	Inverted microscope	Nikon Eclipse TS 100
9.	Deep Freeze ((-70 °C)	E.I.E Instrument Ltd., Ahmedabad
10.	ELISA micro plate Reader	Bio-Rad, Model 680 XR, Mumbai,
11.	Multichannel micropipette	Himedia, Mumbai, India
12.	96 well plates and culture flasks	Tarsons, India

8.3 Methods

General Methods and Preparations

- ***Preparation of complete media***

To prepare complete media, Dulbecco's Modified Eagle's Medium (DMEM) (incomplete medium) was first filtered through 0.2 μ membrane filter. Then, 1% v/v Antibiotic solution (Penicillin/streptomycin) and 10% v/v FBS (fetal bovine serum) were added in a filtered media. The procedure was carried out in vertical laminar air flow cabinet (Weiber Vertical Laminar Air Flow, India).

- ***Preparation of PBS (Phosphate Buffer Saline) pH 7.4***

8 gm of sodium chloride, 200 mg of potassium chloride, 1.44 gm 240 mg di-sodium hydrogen phosphate, potassium dihydrogen phosphate was added in 1 litre of distilled water and pH was checked. The buffer was autoclaved lastly.

- ***Preparation of FACS buffer***

0.5% w/v Bovine serum albumin and 0.5%v/v FBS were added in sterilized PBS pH 7.4 to prepare FACS buffer.

- ***Sub-culturing of cells***

The HEPG2 cells were grown and maintained in DMEM (Himedia). The medium of all the cell lines was supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin antibiotics. The cell cultures were incubated in a humidified atmosphere of 5% CO_2 (Jouan IGO150 CELL life CO_2 Incubator, Thermo Fisher Scientific, India) at 37 °C temperature. The cells were maintained as monolayer culture in T-75 cell culture flasks, and subcultured twice every week by taking 10^4 cells in T-75 flasks.

Following procedure was followed for the sub-culturing;

1. The culture flask was taken to the sterile area in laminar air flow unit and culture media was removed.

2. The cells were washed one time with sterile phosphate buffer saline pH 7.4 (PBS) to eliminate the traces of serum present in the media which may hinder the action of trypsin.
3. 2 mL of Trypsin-EDTA solution was added to flask and shaken gently to allow the detachment of the cells from each other and from surface. Then Trypsin-EDTA was removed to get residual film of cells and cells were kept in incubator for 2-3 minutes for rounding up.
4. Cells were observed under the inverted microscope until cell layer was detached (usually within 5 minutes).
5. Complete medium (2 ml) was added to disperse the cells, dispersion was made with pipetting as continuous cell line requires vigorous pipetting for complete disaggregation.
6. Cells count was performed on haemocytometer.
7. Then appropriate seeding concentration was added to the flask and 10 mL of complete medium was added to it. Passage number was marked on the T-75 culture flask.
8. The flask was closed and cells were incubated at 37 °C, 5% CO_2.

8.4 Cytotoxicity studies

Cytotoxicity studies are carried out for the delivery systems developed for delivery of nucleic acids. These studies establish their cytotoxic potential upon administration in the body and hence depend on the route of administration. For formulations to be administered through IV route, the first cellular structures the formulation encounters are the blood cells. Hence, the toxicity studies on erythrocytes serves to give primary toxicity of the formulations on the red blood cells. Additionally, this establishes the cytotoxic potential of the formulation on the other normal cells of the body due to its simplicity of execution as compared to other cytotoxicity assays. Additionally, the toxicity studies need to be done on the cells that are the targets for the delivery system. These in vitro cytotoxicity studies can be carried out using MTT/MTS/XTT/HDL assays which work based on the working enzymatic machinery of living cells.

8.4.1 Haemolysis Study

The properties of lipoplexes that provide compatibility of the formulation to the cells are the lipids having biocompatibility to the blood cells. Toxicity on the blood cells gives a primary idea of the effect of the liposomes and indirectly the lipid components of the liposomes on the all cells of the body apart from giving an apparent idea on the compatibility with blood cells. The use of cationic lipids along with fusogenic lipids can induce lipid mediated pore formation in the RBCs causing electrolyte loss as well as inducing immunological reactions inside the cell leading to RBC death which usually follows loss of haemoglobin from RBCs. Additionally, lipid components are prone to ester bond hydrolysis and generate fatty acids and lysophosphatidylcholine which may induce lysis of the erythrocyte by cell wall destruction leading to permeability defects. Such changes also induce changes in organization of liposomes causing transformation to micellar solutions (1). Thus, haemolysis potential of the liposomes is necessitated to be evaluated.

Haemolytic toxicity of formulated liposomes was checked by incubating the formulations with Red Blood Cells separated from Rat blood by centrifugation at low speed (2) and analysing the samples for haemoglobin release at 541 nm (3). The haemolysis with different formulations were compared with that obtained with Triton-X100 as a positive control (4).

8.4.1.1 Method

In vitro haemolysis test as described by Oku and Namba (3) was used with some modifications. Blood samples were collected in 2 mL Eppendorff tubes from the Sprague Dawley Rats by retro-orbital puncture. All blood samples were heparinised. The blood samples were washed with normal saline (0.9 % w/v Sodium Chloride in water) 3 times before use to remove plasma components. For washing, each mL of blood samples was treated with 1 mL normal saline and gently stirred up and then centrifuged on Remi Lab Centrifuge at low speed (3000 rpm) to separate the red blood cells (RBCs). The RBC pellet separated was resuspended in normal saline and washed the same way.

Final pellet was used to prepare 2 % v/v dispersion of RBCs based on the final volume. Specific volumes of different liposomal formulations were sampled in these centrifuge tubes and the volume was made up to 1mL with normal saline. A semi-log

increase in concentration range from 0.001 mM to 0.1 mM based on the cationic lipid content of the liposomes was chosen to cover a large concentration range which covered range from on the basis of cationic lipid content (i.e. other lipids used in formulation were not considered in calculation). The specified concentration was achieved in the sample by adequate addition of volumes of different liposomes/formulation.

Positive Control was prepared by getting 100 % haemolysis of RBCs by using 0.5 % Triton-X100 (20 µL in 5 mL). Negative Control was prepared by using the dilutions without any formulation treatment (Dilution only with 0.9% w/v normal saline). Hemolysis potentials of PEGylated and non-PEGylated liposomes, DOTAP liposomes, and liposomes of all synthesized lipids were determined and impact of pDNA complexation on hemolysis was determined by evaluating the hemolysis potential of lipoplexes. Formulations were added to the RBC dispersion and gently mixed. The dispersion was then incubated at 37°C for 30 min in incubator (Jouan CO_2 incubator). Post incubation the dispersion was centrifuged at 3000 rpm for 5 min to separate the pellet. The supernatant was analysed for absorbance at 541 nm in UV spectrophotometer against normal saline as blank. Percentage of haemolysis was determined for different samples considering the absorbance value of sample treated with 0.5% Triton-X100 to represent 100 % haemolysis and normal saline treated samples to serve as negative control. % relative hemolysis was determined by following expression.

$$\%\text{relative hemolysis} = (A_{sample} - A_{negative\ control}) \times 100 / (A_{positive\ control} - A_{negative\ control})$$

where A_{sample}, $A_{negative\ control}$ and $A_{positive\ control}$ are the absorbance values obtained with formulation, triton X100 and normal saline.

8.4.1.2 Results and Discussion

Cationic lipids and DOPE can cause hemolysis by pore formation in the cell membranes of erythrocytes. Hemoglobin released from erythrocytes shows a typical UV absorption spectrum (**Figure 8.1**). Hemoglobin leaked out can be used for spectrophotometric estimation of haemolytic potential of liposomes prepared with cationic lipids by determination of absorption at 541 nm.

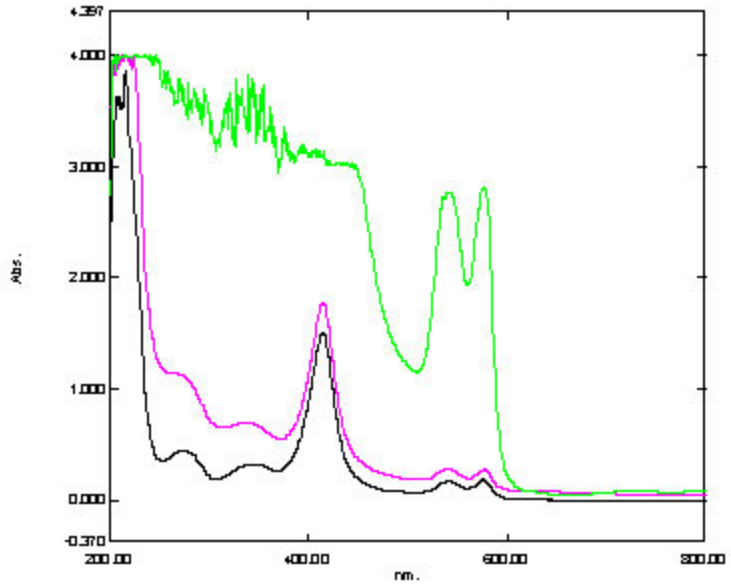

Figure 8.1 Hemoglobin absorption spectra obtained with 0.1 mM SA-DSPE liposomes (pink), negative control (black) and positive control (green)

The haemolysis observed with different formulations as compared to that with Triton-X100 is shown graphically in **Figure 8.1** and **Figure 8.2.**

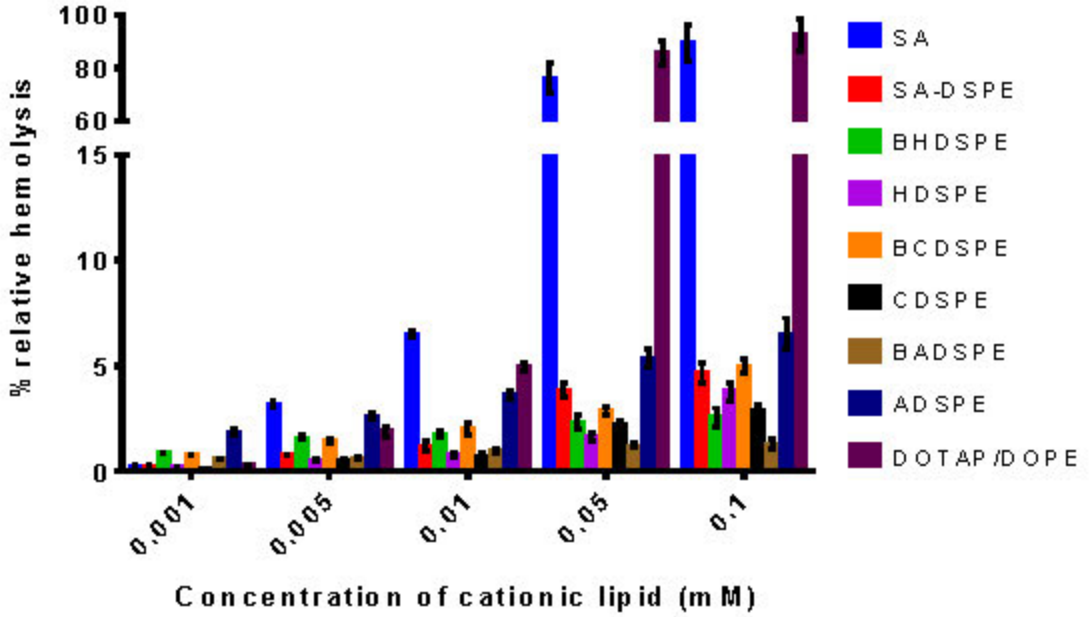

Figure 8.2 Haemolytic Potential of Lipoplexes

As it can be observed that DOTAP/DOPE liposomes showed highest haemolytic potential ranging from 85.65% at 0.05 mM DOTAP concentration to 92.36 % at 0.10 mM lipid concentration. Similar hemolysis potential was also observed with the SA:DOPE (1:1 molar ratio, designated as SA) lipoplexes which demonstrates the haemolytic potential of stearyl amine as a cationic lipid i.e. ~65% relative haemolysis at 0.05 mM SA concentration and ~78% at 0.1 mM SA concentration. Higher haemolytic potential of these lipoplexes can be due to the cationic lipid employed and the lipid composition dependent. DOTAP along with DOPE is shown to cause pore formation and thus lead to hemolysis. In case of SA liposomes, stearyl amine may cause damage to the cell and was in a fashion similar to DOTAP acting as a cationic lipid and causing pore formation in erythrocytes. However, the non-PEGylated lipoplexes prepared with other helper lipids showed very low haemolysis as compared to SA lipoplexes and DOTAP/DOPE lipoplexes even at all concentrations signifying the effect of lipid composition of lipoplexes on cytotoxicity. Additionally, the cytotoxicity was of even lower intensity with lipoplexes of stearyl amines and DSPE modified with Boc-His, Boc-carnosine, Boc-arginine, histidine, carnosine and arginine. All these lipoplexes showed relative haemolytic potential less than 10%. This indicates the structure dependent cytotoxicity of the lipids present in the lipoplexes i.e. single chain of stearyl amine vs. double acyl chain of phospholipids. Thus, from the observations, it can be concluded that the optimized lipoplexed formulations have low potential to cause haemolysis at therapeutic concentration of the lipids employed and its exposure to the body.

8.4.2 MTT assay - on HEPG2 cells

In vitro toxicity testing is the scientific analysis of the effects of toxic chemical substances on cultured bacteria or mammalian cells. *In vitro* testing methods are used either to check potentially toxic effects of chemicals or to confirm the lack of toxic properties of chemicals, which might be useful in the development of new therapeutic agents or their delivery systems, agricultural chemicals, or food additives.

Different methods used for cell counting and cytotoxicity assays along with the dyes or probes used, the detection method, sensitivity, and features (5). Of all methods the most popular ones are the MTT and XTT dye (tetrazole dyes) based assay methods due to their ease, sensitivity and scalability. Here in present investigation, MTT based

cytotoxicity assay was used to evaluate cytotoxicity of the lipoplex formulations developed for delivery of APOE3 gene on HEPG2 cells.

MTT assay is a colorimetric assay for determining the viable cell count depending on the mitochondrial dehydrogenase activity measurement. The MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a yellow tetrazole dye) is taken up and reduced inside the living cells which have mitochondrial dehydrogenase (reductase) enzyme activity intact. This reduction produces a purple coloured formazan compound that gives a direct estimate of number of viable cells when measured spectrophotometrically (**Figure 8.3**).

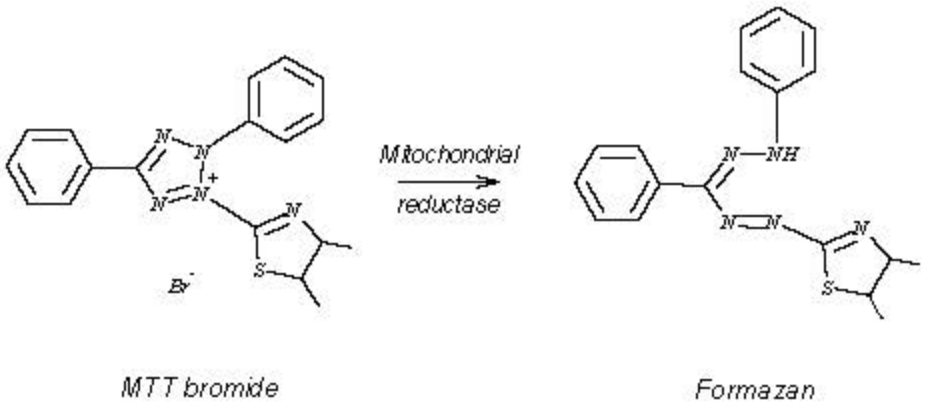

Figure 8.3 MTT Dye Reduction by Mitochondrial Reductase Enzyme of Viable Cells

Formazan formed so is water insoluble, so solubilization of formazan precipitates is done by using chemicals like dimethyl sulfoxide (DMSO), an acidified ethanol solution or a surfactant (Sodium lauryl sulphate) solution in diluted hydrochloric acid. Formazan derivative absorbs UV radiation which can be used for estimation of quantity of formazan formed. This assay can be of use to determine the viable cell count of any culture. This in turn can be useful in determining the cytotoxicity of any chemical of medical interest or any delivery system developed for delivery of drug or therapeutic genes.

8.4.2.2 Methods

a) Media Preparation

Complete media was prepared by mixing Dulbecco's modified Eagle's medium (DMEM) filtered through sterile 0.2 μ membrane filter with antibiotic solution (1% v/v) and 10%

v/v fetal bovine serum (FBS). Media was stored in a sterile screw capped bottle. The bottle was then sealed with parafilm and wrapped with aluminum foil. The whole process carried out in vertical laminar air flow cabinet (Weiber Vertical Laminar Air Flow, India).

b) Subculturing of cell line (6)

HEPG2 cell line was obtained in NCCS, Pune. The cells were maintained as monolayer culture in T-75 cell culture flasks, and subcultured twice every week by taking 10^4 cells in T-75 flasks. Cell lines obtained from NCCS, Pune were subcultured at 37°C in a humidified atmosphere at 95% air and 5% CO_2 (Jouan IGO150 CELLlife CO_2 Incubator, Thermo Fisher Scientific, India) in complete media. Fresh complete medium was replaced every 3 days.

Following procedure was followed for the subculturing.

1. Culture medium was removed from the Tissue culture T-75 flask containing cells.
2. 2 mL of Trypsin-EDTA solution was added to flask and shaken gently to allow the detachment of the cells from each other. Then Trypsin-EDTA was removed to get residual film of cells and cells were kept in incubator for 2-3 minutes for rounding up.
3. Cells were observed under the inverted microscope until cell layer was detached (usually within 5 minutes).
4. 10 mL of complete growth medium was added to flask and cells were aspirated gently by pipette. Passage number was marked on the T-75 culture flask.
5. Subcultures were incubated at 37 ^{0}C with 5% CO_2 level.

c) Cell Counting Using Haemocytometer

Preparing Haemocytometer:

1. Haemocytometer was cleaned properly using 70% ethanol.
2. The shoulders of the haemocytometer were moistened and the coverslip was affixed firmly using gentle pressure and small circular motions.

Preparing Cell Suspension:

3. The cell suspension to be counted was mixed properly by gentle agitation of the flask containing the cells.
4. Before the cells started settling down about 1 mL of cell suspension was sampled using a serological pipette and placed in microcentrifuge tube.

5. Using a 100 µl pipette, cells in this sample were mixed again (gently to avoid cell lysis). And then 100 µL was taken out and placed into a new microcentrifuge tube which was then treated with 100 µl trypan blue and mixed with it gently.

Counting:

6. Using the micro pipette, some cell suspension containing trypan blue was drawn out and carefully filled in the haemocytometer by gently resting the end of the tip at the edge of the chamber taking care to avoid overfilling of chamber.
7. The grid lines of the haemocytometer were focused using the 10X objective of the microscope. One set of 16 corner squares as indicated by the circle in the **Figure 8.4.**
8. 16 squares were focused at a time.

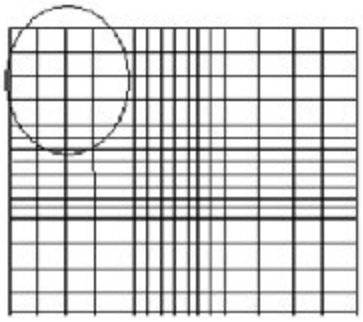

Figure 8.4 Haemocytometer diagram indicating the 16 corners squares which should be used for counting

9. Using a hand tally counter, the number of cells in this area of 16 squares was counted. When counting, only live cells that look unstained by trypan blue were counted. Cells that are within the square and any positioned on the right hand or bottom boundary line were counted.
10. Counting of cells was continued for all other remaining set of 16 corner squares.
11. The haemocytometer is designed so that the number of cells in one set of 16 corner squares is equivalent to the number of cells x 10^4 / mL.
12. Calculation of the average no. of cells in 4 sets of 16 corners is as follows: The total count from 4 sets of 16 corners = (Average no. of cells/mL) x 10^4 x 2
    Where 10^4 is conversion factor (Conversion of 0.1 mm^3 to mL) and 2 is dilution factor.

d) Preparation of formulation for treatment

Stock solutions of formulations were sterilized by filtering through 0.2 μ membrane filter. All the dilutions and filtration were carried out in Laminar Air Flow Hood and all the materials and equipment used were sterilized appropriately before use. Toxicity studies were carried out with the liposomes and lipoplexes thereof.

e) MTT Assay Protocol (6)

Plating out cells:

1. Subconfluent monolayer culture in one T-25 flask was trypsinized and 5 mL of complete growth medium was added. The medium was removed to remove trypsin and again 5 mL of complete growth medium containing serum was replenished. Cells were aspirated gently with pipette. Tips of pipette were discarded after single use.
2. Cells were counted and diluted to 25×10^3 cells/mL
3. Then cell suspension was transferred to 96-well plates (except for the column 1 which is to be used as a control) with a multichannel pipette to produce cell concentration of 5×10^3 cells per well. Then 200 μL of complete medium was added in each well with a multichannel pipette. Lid was placed over the plate. 3 plates were prepared similarly for each study period of 24 hr, 48 hr and 72 hr.
4. Cells were incubated in an incubator at 37°C and 5% CO_2 exposure for 24 hr before exposure to the lipoplexes.

Formulation treatment:

5. Medium from the wells was removed using multichannel pipette and discarded.
6. Cells were treated with formulation diluted in sterile filtered incomplete media (DMEM) at different cationic lipid concentrations and different N/P ratios. Cells treated with 200 μL of fresh incomplete medium (DMEM) were used as negative control (100% viability will be assumed from the absorbance of wells containing these cells) while those treated with carrier 0.5% Triton X100 were kept as a positive control. Commercially available non-viral lipid transfecting Lipofectamine-2000 (Invitrogen, USA) was used as a reference control to compare the toxicity of the developed formulations. Treatment with each formulation was performed in triplicate.

7. Lid was placed on the plates and the plate was returned to incubator (37°C and 5% CO_2 exposure). The cells were incubated for 6 hr exposure period.

Growth period:

8. At the end of the exposure period, medium was removed from all the wells and wells were fed with 200 µL of fresh complete medium.
9. The plates were replenished with fresh complete medium after 24 hr for 48 hr viability study.

Estimation of surviving cell numbers:

10. Plates were fed with 200 µL of fresh complete medium after washing with Phosphate Buffer Saline pH 7.4 at the end of the growth period (24 hr, 48 hr and 72 hr) and 100 µL of MTT solution (1 mg/mL MTT solution was filtered through 0.2 µ membrane filter and stored at 2-8°C in dark.) was added to all of the wells in plate.
11. Each plate was wrapped in aluminium foil, and incubated for 4 hr at in the incubator at 37°C at 5% CO_2.
12. Medium along with MTT were removed from the wells. Cell lysis and solubilization of formazan crystals was done by adding 200 µL of DMSO to all of the wells.
13. Absorbance was recorded at 570 nm with a reference filter of 655 nm on microplate reader (ELISA microplate reader, BioRad, USA) immediately, because the product is unstable.

Analysis of MTT assay:

14. Determination of the cell viability was done by using the following equation.

$$\% \text{ Viability} = \frac{\text{Mean Absorbance of Sample}}{\text{Mean Absorbance of Control}} \times 100$$

where absorbance of sample and control cells represent the amount of formazan determined for cells treated with the different formulations and for control cells (nontreated), respectively.

15. Viability plots were plotted by plotting % viable cells (y-axis) against the concentration of cationic lipids (x-axis).

8.4.2.3 Results and Discussion

Cytotoxicity study of the liposomes and lipoplexes was carried out to evaluate the effect of cationic lipids and pDNA complexation on the cytotoxicity. The results of the cytotoxicity study of liposomes are depicted in

Figure 8.5, **Figure 8.6** and **Figure 8.7** and that of lipoplexes are depicted in **Figure 8.8**, **Figure 8.9**, **Figure 8.10** *(Note: For lipofectamine-2000 lipoplexes x-axis represent, instead of N/P ratio, lipofectamine-2000 reagent volume range as per standard transfection protocol i.e. 0.4 µL, 0.8 µL and 1.0 µL. SA/DOPE lipoplexes were composed of 1:1 molar ratio of SA and DOPE*). Cytotoxicity of liposomes was evaluated by considering the concentration of cationic lipids on mM basis. Concentration range of 0.05, 0.5, 2.0 and 5.0 mM of cationic lipid was evaluated for cytotoxicity of liposomes and N/P ratio range of 2, 4 and 8 was considered for evaluation of cytotoxicity of lipoplexes

As it can be seen from the cytotoxicity study results it was observed that SA/DOPE formulation was most toxic among all formulations including DOTAP/DOPE liposomes. This is due to the single chain character of stearyl amine which might cause cell membrane damage more considerably than double chain lipids. Additionally, the preparation is a mixture of micellar and lamellar structures of SA with DOPE of which former would be in high levels due to single chain character of stearyl amine preferring the micellar formulation as compared to lamellar formulation in higher concentrations (7-10). SA liposomes prepared with HSPC/Chol were second most toxic liposomes. The reduced toxicity is due to the ability of bilayered structure to hold stearyl amine in the bilayer structure. Inclusion of DOPE in the formulation i.e. SA liposomes prepared with HSPC/DOPE/Chol led to reduction in the cytotoxicity. The effect might be due to the reduced cationic charge as depicted by the lowered zeta potential of the liposomes with DOPE. Also, the structure of DOPE can also be considered an important factor. Tilted acyl chains of the DOPE allow higher interaction between the hydrophobic chain of stearyl amine holding stearyl amine strongly inside the liposomal structure. Additionally, presence of lipid bilayer forming lipids i.e. HSPC and Chol reduces the influence of

DOPE on cytotoxicity of lipoplexes through formation of strong bilayer structure. Further, incorporation of the 3 mole% DSPE-mPEG2000 almost significantly reduced the cytotoxicity of the liposomes. This might be due to the masked surface charge and due to reduced direct interaction of the cationic lipid i.e. SA with the cell membrane. Additionally, presence of PEG chains extending over liposomal surface helps prevent SA molecules from taking off of the liposomal bilayer. Thus, subsequent improvement in cell toxicity behaviour of liposomes of SA prepared with DOPE, HSPC/Chol, HSPC/DOPE/Chol and HSPC/DOPE/Chol/DSPE-mPEG2000 is due to improved strength of bilayer structure to hold SA preventing dislodging of SA molecules from bilayer. PEGylated liposomes even showed improved cell viability than DOTAP/DOPE liposomes which are used as standard reagent by several scientists.

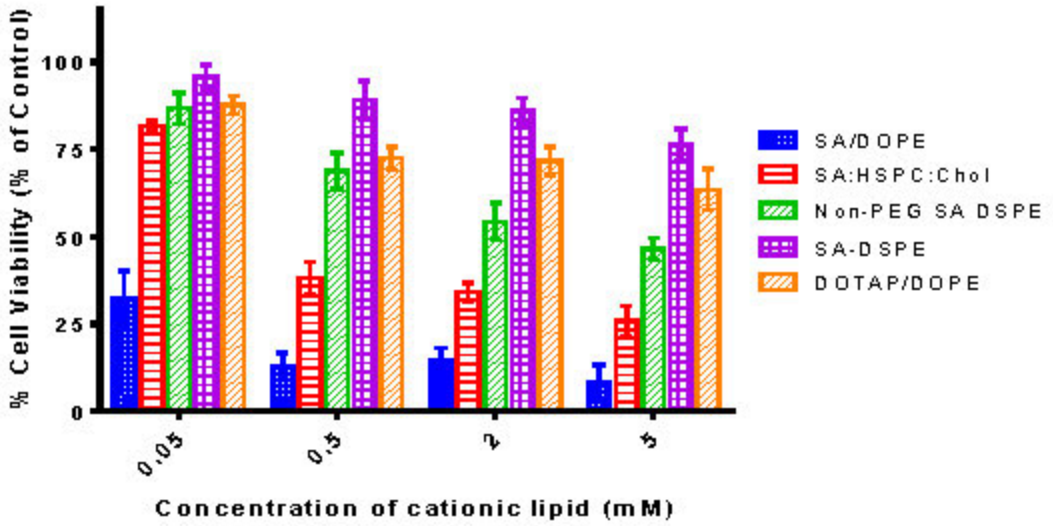

Figure 8.5 Cytotoxicity of liposomes prepared without and with DOPE (SA/DOPE, SA:HSPC:Chol lioposomes and SA DSPE-nonPEGylated liposomes respectively) and PEGylated liposomes against DOTAP/DOPE liposomes.

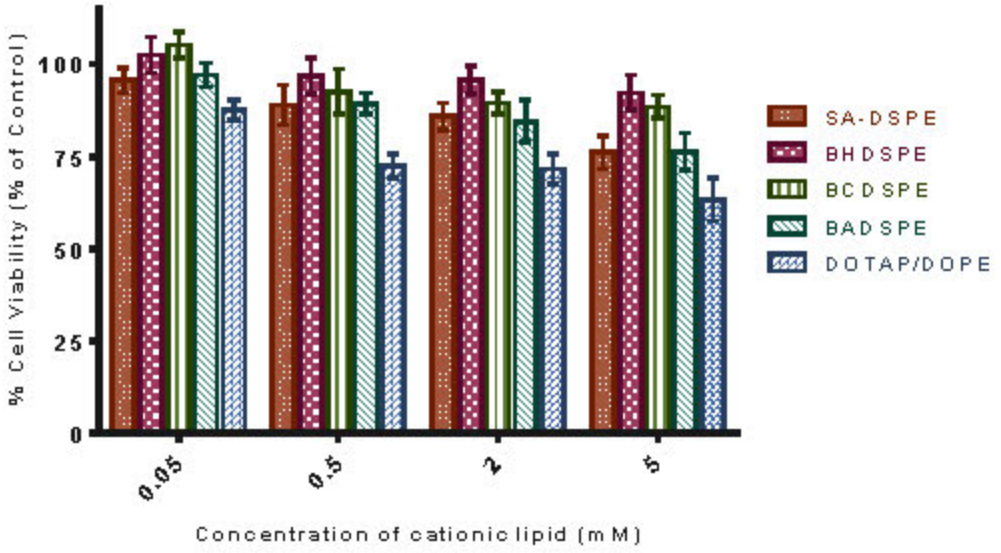

Figure 8.6 Cytotoxicity of BHDSPE, BCDSPE and BADSPE liposomes against SA-DSPE liposomes and DOTAP/DOPE liposomes

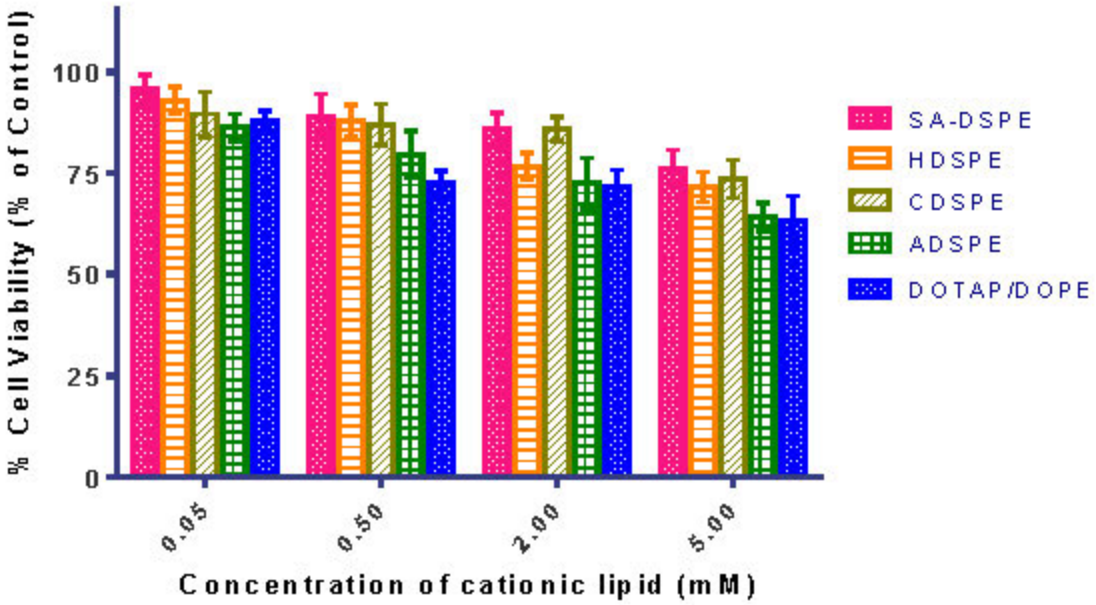

Figure 8.7 Cytotoxicity of HDSPE, CDSPE and ADSPE liposomes against SA-DSPE liposomes and DOTAP/DOPE liposomes

Among liposomes of different version of the synthesized lipids, cytotoxicity was higher for Boc-deprotected lipids. The toxicity order for any modified lipid followed following order:

HDSPE>BHDSPE, CDSPE>BCDSPE and ADSPE>BADSPE

This order of cytotoxicity can be justified by the cationic valency of the lipids i.e. 2+ charge on the Boc-deprotected lipids vs. 1+ charge of Boc-protected lipids.

Among all the lipids, toxicity order can be given as

ADSPE > CDSPE > HDSPE > BADSPE > BCDSPE ≥ BHDSPE

The higher toxicity of the BADSPE among other Boc protected lipids and ADSPE among other Boc-deprotected lipids can be explained by the higher charge density of the arginine modified lipids i.e. completely ionized primary amine and guanidine groups vs. partially ionized imidazole groups.

Cytotoxicity of lipoplexes prepared with liposomes of different cationic lipids at different N/P ratios i.e. 2, 4 and 8 are shown in **Figure 8.8**, **Figure 8.9** and **Figure 8.10**.

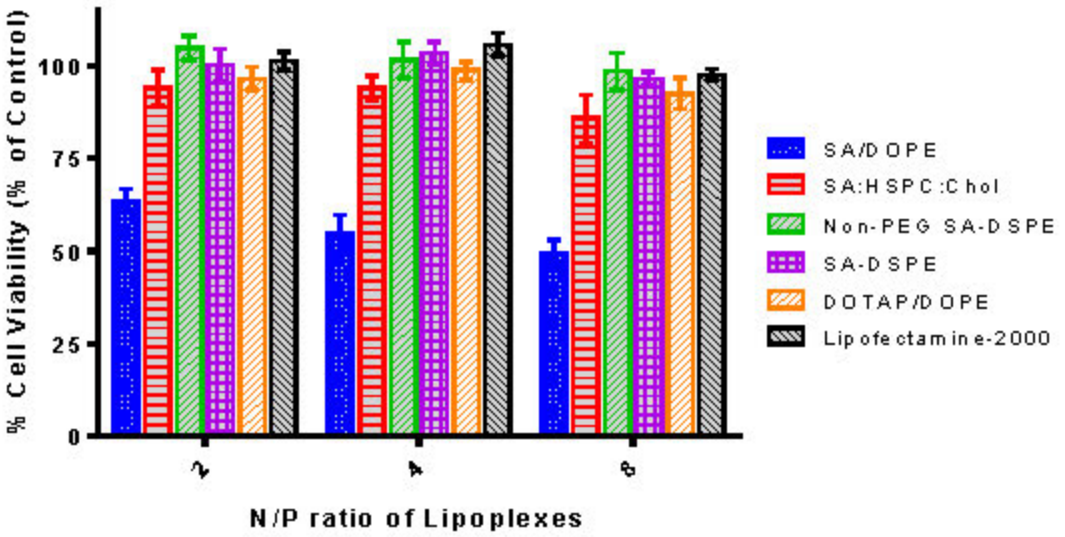

Figure 8.8 Cytotoxicity of lipoplexes prepared with liposomes without and with DOPE (SA/DOPE, SA:HSPC:Chol lioposomes and SA-DSPE nonPEGylated liposomes respectively) and PEGylated SA-DSPE liposomes against DOTAP/DOPE and lipofectamine-2000 lipoplexes.

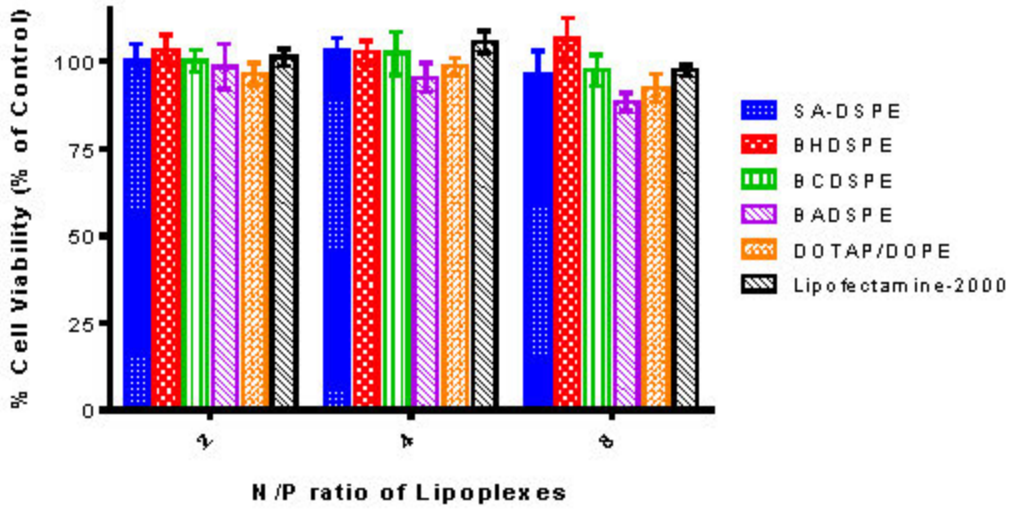

Figure 8.9 Cytotoxicity of BHDSPE, BCDSPE and BADSPE lipoplexes against SA, DOTAP/DOPE and lipofectamine-2000 lipoplexes.

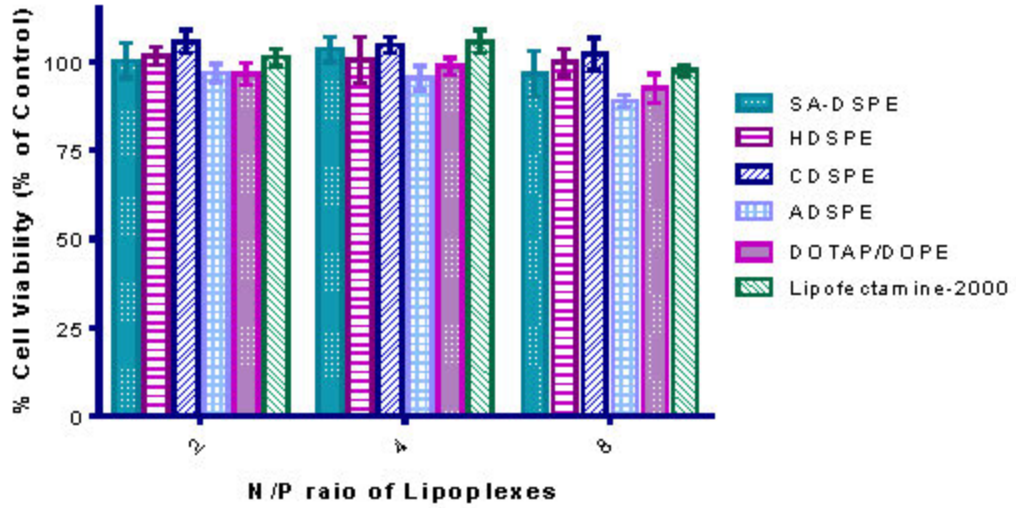

Figure 8.10 Cytotoxicity of HDSPE, CDSPE and ADSPE lipoplexes against SA, DOTAP/DOPE and lipofectamine-2000 lipoplexes.

Considering the cytotoxicity of the lipoplexes, even though the liposomes of Boc-deprotected lipids showed higher cytotoxicity as compared to Boc-protected lipids, the cytotoxicity of the lipoplexes prepared with Boc-deprotected liposomes at all N/P ratios were lower or similar to that observed with Boc-protected lipids. This is due to the requirement of lower concentration (half on mole basis) of Boc-deprotected lipids for complexation of same amount of pDNA against the Boc-protected lipids.

8.5 GFP expression studies

Green fluorescence protein (GFP) is expressed by GFP gene. The expressed protein, as the name indicates, gives out a green fluorescence upon exposure of light. This property of the protein can be used to see the efficiency of the developed gene delivery vector through carryout out expression studies using GFP gene to enhance the uptake and expression of the gene inside the target cells. Quantitative and qualitative estimations can be made of gene expression after transfection with suitable delivery systems by using flow cytometry (fluorescence activated cell sorting) and fluorescence or confocal microscopy respectively.

8.5.1 Flow cytometry

Flow-cytometry is a powerful technique for characterizing cells in clinical diagnosis and biomedical research for quantifying aspects about their size, internal complexity and surface markers. In a flow cytometer, the suspension of cells is hydro-dynamically focused in a single cell wide stream of fluid containing a fast-moving sheath fluid around the slow-moving cell suspension emerging through a 70 µm nozzle. This is achieved by with air or gas pressure and the differential pressure between the streams controls the samples introduction rate. This laminar steam stream of particles is subsequently interrogated by one or more laser beams placed perpendicular to it and only illuminate single cell at a time. At this point the laser is scattered at the same wavelength at different directions. The light scattered in forward direction (FSC) is proportional to the size of the cells. While the light scattered in perpendicular direction (SSC) correlates with intracellular granularity or complexity. Thus scattering itself gives information about the size and composition of the cells (11). The second technique of detection relies on use of fluorescent probes attached to cells, which fluorescence after interaction with laser at interrogation point and emit light at longer wavelengths. Here, the non-fluorescent cells will be counted as negative while the fluorescent cells will be called as positive cells. Further, the intensity of emission gives information about the number of fluorescent probes. Downstream the interrogation points, the particle stream is broken into discrete droplets which can be selectively charged and deflected using an electric field into a collector and the remainder are disposed. The results of fluorescence and scatter are displayed as histogram. Before starting the experiment, appropriate controls are needed to enable interpretation of the results in the context of the purpose of experiment. At least

three controls are essential in nay experiment which are: set up control (instrument), specificity control (gating) and biological comparison control (12). 'Setup controls' are required to ensure that instrument is properly set up with respect to photomultiplier voltage gains and compensation; 'Specificity or gating controls' are used to set location of gates or graphical regions to classify the cells as required for the purpose of the experiment. A 'biological comparison control', consisting of unstained/unstimulated cells in biologically relevant conditions, is required to set up positive/negative boundaries.

8.5.1.1 Method

1. Media preparation, sub-culturing of cells, cell counting and preparation of formulations were followed as described in the MTT assay earlier.

2. Cells were seeded at a density of 5×10^4 cells/well in a 24 well plates and allowed to grow for 24 hr in complete media.

3. After 24 hr, cells were transfected with lipoplexes prepared using optimized liposomal formulations using GFP pDNA at N/P ratio of 2 (**Table 8.1**) at a final concentration of 500 ng GFP pDNA per well.

4. Treatment was carried out for 6 hr and post-treatment, cells were washed with phosphate buffered saline.

5. Fresh media was replaced and cells were incubated for 24 hr for expression of GFP.

6. Cells were then trypsinized and resuspended in the phosphate buffered saline and analysed using fluorescence activated cell sorter (FACS-BD-AriaIII, BD, USA) at 10000 events per second. Cells without any treatment were used as negative control. Naked pDNA and Lipofectamine-2000 lipoplexes were used as reference controls for comparison.

Table 8.1 Cell treatment parameters for FACS

Formulations	Cells and cell density	pDNA concentration	Treatment conditions	Expression Conditions
Naked GFP pDNA	HEPG2 cells (50000 cells/well)	500 ng eGFP pDNA per well	Incubation time= 6 h Temperature =	48 hr after transfection
Formulations (SA-DSPE, BHDSPE,				

HDSPE, BCDSPE, CDSPE, BADSPE, and ADSPE)			37°C (5% CO_2)	Temperature = 37°C (5% CO_2)
Lipoplexes of Lipofectamine-2000 (LL2)				

8.5.1.2 Results and discussion:

FACS histograms of cellular uptake of developed lipoplexes are shown in **Figure 8.11 Table 8.2**, **Figure 8.12** show the quantitative representation of the cellular expression of the GFP after transfection with different formulations. It can be seen that the naked DNA showed very marginal transfection efficiency which is obvious from the already reported properties of the pDNA i.e. higher molecular weight, negative charge and hydrophilicity, all of which negatively affect the cellular uptake. All lipoplex formulations showed significantly higher expression of GFP which is due to the cationic nature of the lipoplexes. SA-DSPE lipoplexes showed similar transfection efficiency as that of Lipofectamine-2000. The transfection efficiencies of the lipoplexes of the modified lipids were even higher than the SA-DSPE and lipofectamine-2000 lipoplexes.

Examining the histograms gives the idea about the expression GFP after transfection with the lipoplexes. There is a rightward shift of the histograms of naked pDNA as well as lipoplexes with lipoplexes it is extensively higher. Successful transfection of the cells using lipoplexes depict that the lipoplexes are easily uptaken by the cells through endocytosis due to their cationic characteristic. The cellular release of the lipoplexes is offered by either fusion of the lipoplexes with the endosomal membrane under acidic conditions due to presence of DOPE in lipoplexes leading to the cytosolic release of the pDNA. This hypothesis supports the cellular transfection by the lipofectamine as well as other lipoplexes. Additionally, the higher transfection efficiency of other lipoplexes which has lower DOPE content as compared to lipofectamine might be due to the role played by the modified lipid. Along with DOPE, stearyl amine and other modified DSPE lipids can afford the endosomal membrane damage under acidic conditions due to simultaneous activity of the SA-DSPE/modified DSPE lipids along with DOPE on the endosomal membranes. Additionally, in case of modified DSPE lipids, the

higher head-group size would provide higher endosomal escape as compared to SA-DSPE.

The shift depicts the level of GFP expression which helps identify the differences among different lipids in terms of their transfection efficiency. There is marginal expression of GFP by the naked pDNA. In case of the lipoplexes, SA-DSPE lipoplexes show GFP expression similar to the lipofectamine-2000 lipoplexes. The rightward shift depicts the higher expression of GFP which turns out to be giving higher fluorescent intensity inside the cells. Hence, the rightward shift follows the order

CDSPE=HDSPE≥BCDSPE=BHDSPE=ADSPE=BADSPE>Lipofectamine=SA-DSPE>naked pDNA

This indicates role of the structural differences among the lipids. Imidazole containing lipids i.e. BHDSPE, HDSPE, BCDSPE and CDSPE show higher expression of the lipid which might be due to the larger head-group of these lipids as compared to other lipids as well as the buffering capacity rendered by the imidazole moiety of the lipids which protect the pDNA from harsh acidic environment of the endosomes after uptake. Additionally, this would provide the proton sponge effect classical to the polymers such as PEI used in gene delivery by inducing the consequent accumulation of chlorides and water inside the vesicles leading to bursting of the endosomes.

Table 8.2 GFP expression after transfection with different lipoplexes against naked pDNA and Lipofectamine-2000 lipoplexes*

Formulations	% GFP expressing cells
Naked pDNA	7.26±2.18
SA-DSPE	42.56±4.22
BHDSPE	54.67±2.21
HDSPE	64.42±1.89
BCDSPE	56.01±1.75
CDSPE	61.33±1.41
BADSPE	50.91±2.46
ADSPE	52.87±2.69
Lipofectamine-2000	43.65±1.16

*Experiments were performed in triplicate.

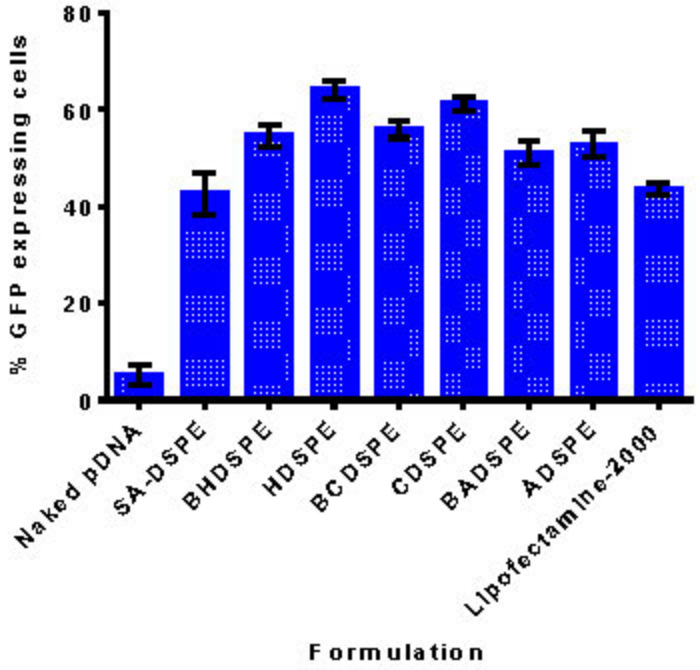

Figure 8.11 %GFP expression observed after transfection with naked pDNA, lipoplexes of SA-DSPE and Modified DSPE lipoplexes. Untreated cells were taken as negative control while cells treated with lipoplexes of lipofectamine-2000 were taken as a reference control for comparison.

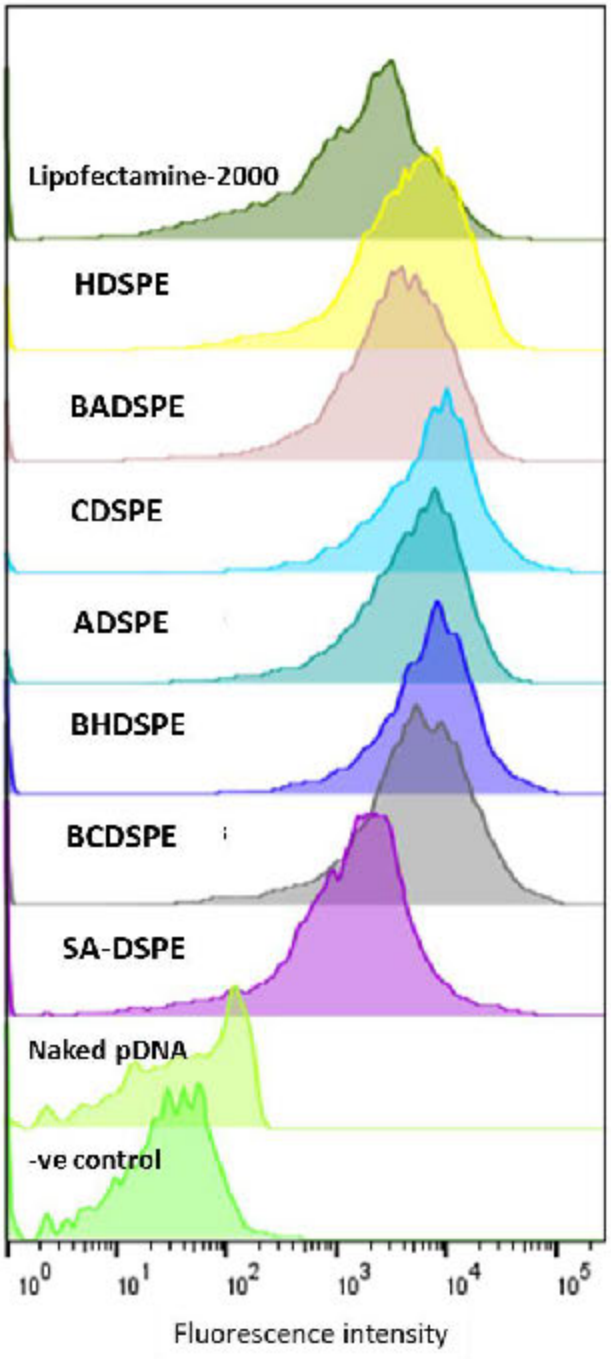

Figure 8.12 Overlay histograms of FACS analyses of cellular expression of eGFP by naked pDNA, lipoplexes of SA-DSPE and Modified DSPE lipoplexes. Untreated cells were taken as negative control while cells treated with lipoplexes of lipofectamine-2000 were taken as a reference control for comparison.

8.5.2 Confocal microscopy

The laser scanning confocal microscope (LSCM) is an essential component of modern day biomedical research applications. In a conventional microscopy, the entire specimen is illuminated from a mercury or xenon source. However, in confocal microscopy the illumination is achieved by scanning one or more laser beams across the specimen to create an optical section of specimen in a non-invasive manner. It uses confocal pinholes that allow light coming only from the plane of focus to reach the photomultiplier tube detector and excludes the 'out of focus' light coming to the detector. This enables imaging of the living specimens and generation of 3-dimensional data in the form of Z-stacks. It uses laser as light source, a sensitive photomultiplier tube detector and a computer to control the scanning mirrors and build images. The optical path used in confocal microscopy is based on conventional reflected light wide-filed epi-fluorescence microscope with a point light source and a pinhole in front of detector which are confocal with each other. The specimens are labelled with one or more fluorescent probes. The confocal microscopy also offers the advantage greater resolution due to use of highly sensitive photomultiplier tube detectors. The series of time-lapse run can be converted into a 3-D image from the obtained data with time as the z-axis. This can be useful for observing physiologic changes during development. Further a 4-dimension data set can be produced consisting of three spatial dimensions X, Y, Z and time as fourth dimension. In cellular biology, confocal microscopy has been used for visualizing intracellular organelles, cellular uptake, intracellular localization of drugs and drug delivery systems using fluorescent probes.

8.5.2.1 Method

1. Media preparation, subculturing of cells, cell counting and preparation of formulations were followed as described in the MTT assay earlier.

2. Cells were seeded onto 6-well plates with a glass cover slip at the bottom. Cells were seeded at a density of 5 x 10^4 cells/well on flame sterilized 0.17 mm square glass cover slips in a 6 well plates and allowed to grow for 24 hr in complete media.

3. After 24 hr, cells were transfected with lipoplexes prepared using optimized liposomal formulations using GFP pDNA at N/P ratio of 2 (**Table 8.3**) at a final concentration of 500 ng eGFP pDNA per well.

4. Treatment was carried out for 6 hr and post-treatment, cells were washed with phosphate buffered saline.

5. Fresh media was replaced and cells were incubated for 24 hr for expression of GFP.

6. Cells were washed with PBS followed by treatment with nucleus stain DAPI for 10 minutes followed by washing again with sterile phosphate buffered saline to remove excess DAPI from milieu.

7. Coverslips were mounted on sterile glass slides and confocal microscopy was performed on confocal laser scanning microscope (CLSM 710, Carl-Zeiss Inc., USA). Cells treated with naked eGFP pDNA and lipoplexes prepared with Lipofectamine-2000 were used as controls.

Table 8.3 Cell treatment parameters for confocal microscopy

Formulations	Cells and cell density	pDNA concentration	Treatment conditions	Expression Conditions
Naked GFP pDNA	HEPG2 cells (10000 cells/well)	500 ng eGFP pDNA per well	Incubation time= 6 h Temperature = 37°C (5% CO_2)	48 hr after transfection Temperature = 37°C (5% CO_2)
Formulations (SA-DSPE, BHDSPE, HDSPE, BCDSPE, CDSPE, BADSPE, and ADSPE)				
Lipoplexes of Lipofectamine-2000 (LL2)				

8.5.2.2 Results and discussion:

Confocal microscopy images of the cell uptake studies are shown in **Figure 8.13**. **Figure 8.14** and **Figure 8.15**. Each image depicts the cellular uptake of developed lipoplexes against the naked DNA, DOTAP/DOPE lipoplexes and Lipofectamine-2000 lipoplexes for comparison.

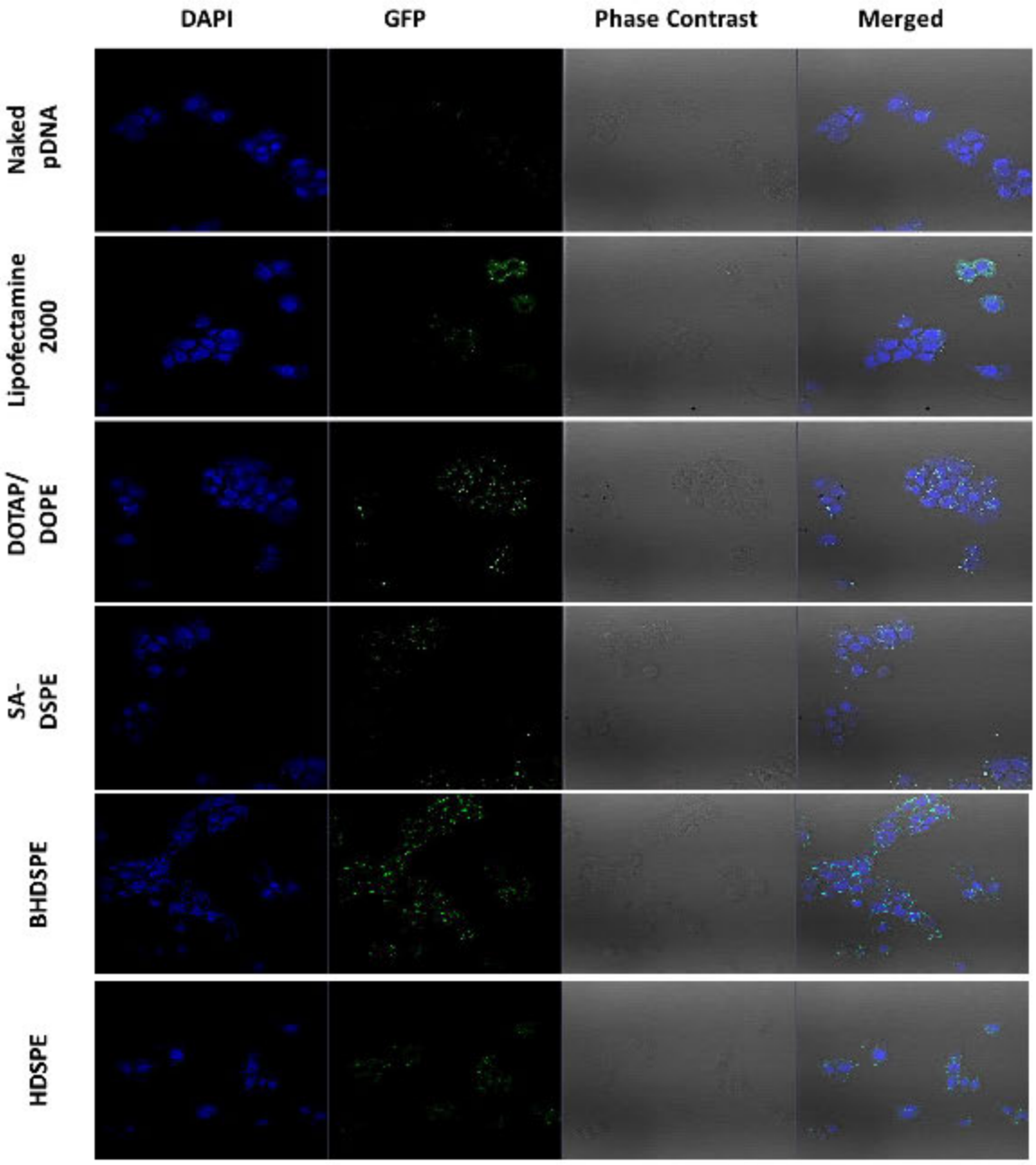

Figure 8.13 Confocal images of SA-DSPE, BHDSPE and HDSPE lipoplexes. Cellular expression is shown in comparison to that after transfection done with naked eGFP pDNA, lipoplexes of DOTAP/DOPE and Lipofectamine-2000.

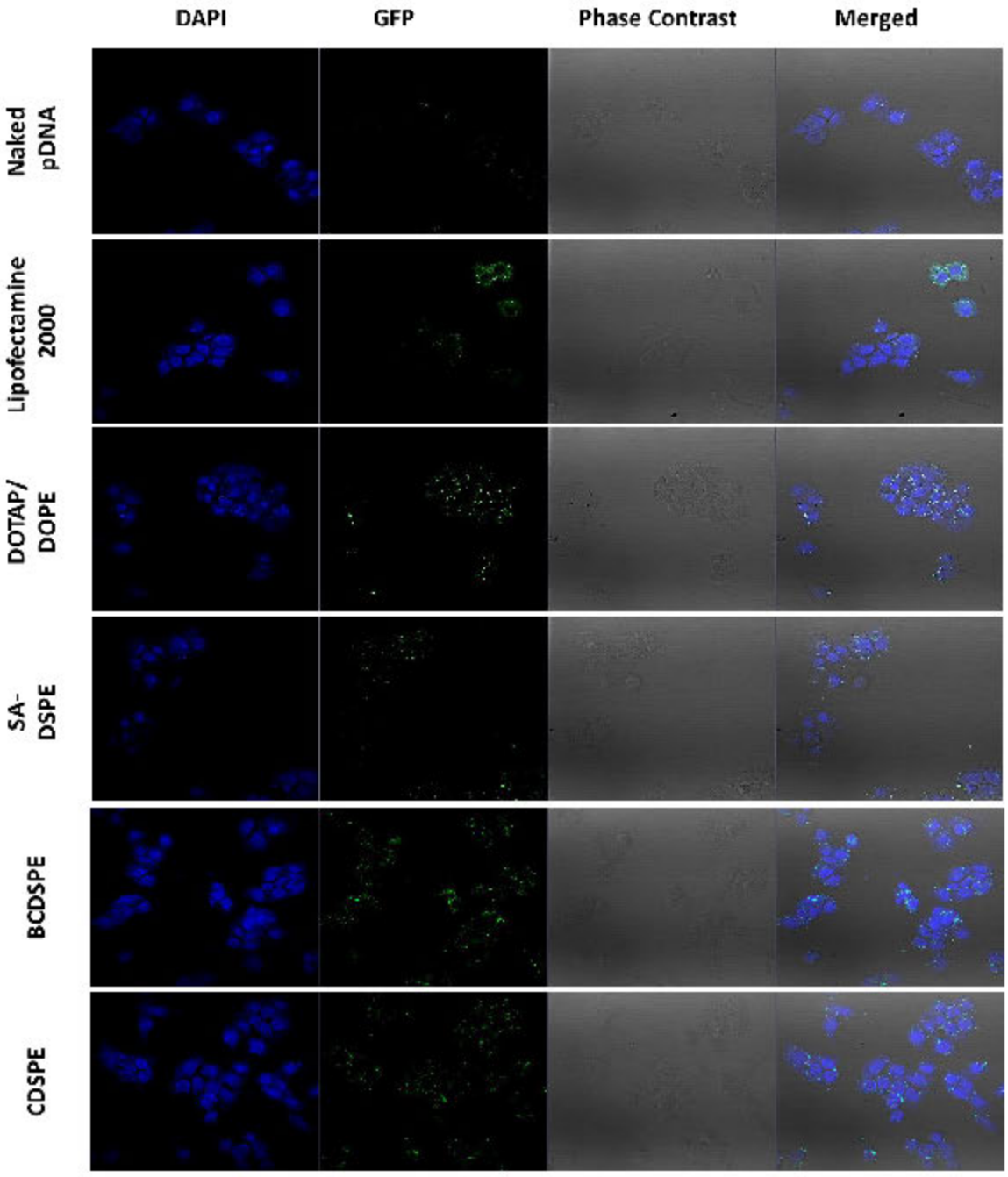

Figure 8.14 Confocal images of GFP expression after transfection with BCDSPE and CDSPE lipoplexes. Cellular expression is shown in comparison to that with naked eGFP pDNA and lipoplexes of SA, DOTAP/DOPE and Lipofectamine-2000.

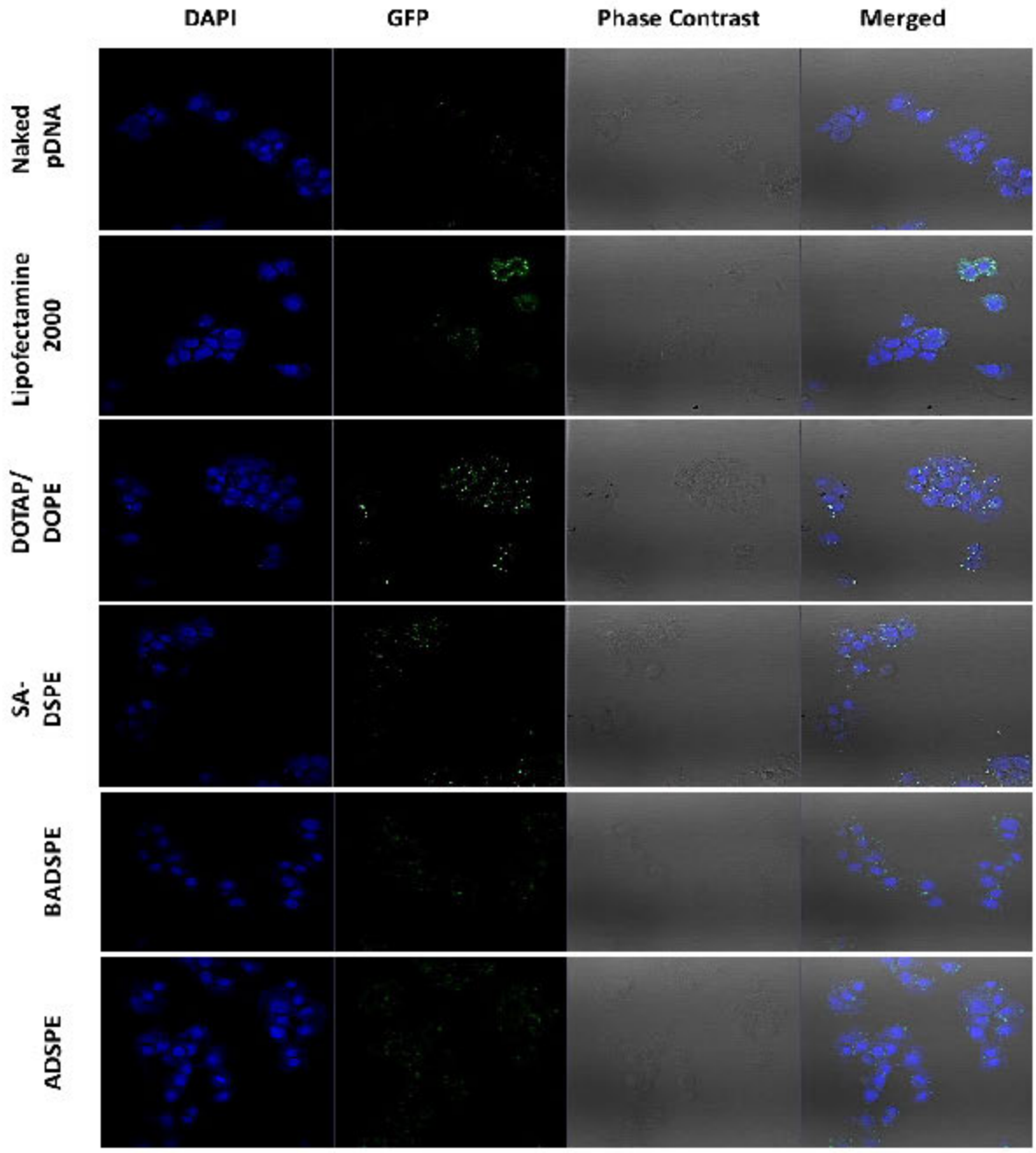

Figure 8.15 Confocal images of GFP expression after transfection with BADSPE and ADSPE lipoplexes. Cellular expression is shown in comparison to that with naked eGFP pDNA and lipoplexes of SA, DOTAP/DOPE and Lipofectamine-2000.

As it can be seen, there is no expression seen in case of the naked eGFP while all other formulations showed extensive eGFP expression as indicated by intense green

fluorescence from the cells. Highest fluorescence is seen with modified DSPE containing formulations as compared to that obtained with SA-DSPE lipoplexes and lipofectamine-2000. The increased transfection efficiency of the modified DSPE containing liposomes can be considered due to the multiple effects playing role. Increased uptake in case of BHDSPE, HDSPE, BCDSPE and CDSPE containing liposomes might be due to buffering effect of the lipids as well as the effective escape of the liposomes which would be provided by the DOPE as well as the modified head-group structures of the lipids i.e. increased head-group size that would, upon complete ionization of the lipids, will provide better membrane destabilization leading to release of pDNA cargo inside the cytosol. Similar explanation can be extended to the BADSPE and ADSPE except that there will be no buffering effect of the lipid, however, the strong charge density of the lipid will help destabilize the lipid membrane of endosomes releasing pDNA inside cytosol. However, the enhanced transfection effect of the ADSPE might also be compensated by the higher cytotoxicity as seen in the cytotoxicity studies.

8.6 References

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