Chapter 8
Cell Line Studies

Sometimes, the things we are afraid of, are the things we want the most.

-From "Redline"

8.1 Cytotoxicity studies

Cytotoxicity studies are carried out for the delivery systems developed for delivery of nucleic acids due to their cationic nature i.e. cationic lipids or cationic polymers. 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 i.e. MTT/XTT/HDL assays. Additionally, the toxicity studies needs 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.

8.1.1 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].

8.1.1.1 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 SA liposomes, DOTAP liposomes, and liposomes of all synthesized lipids were determined. 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.

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

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

8.1.1.2 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.

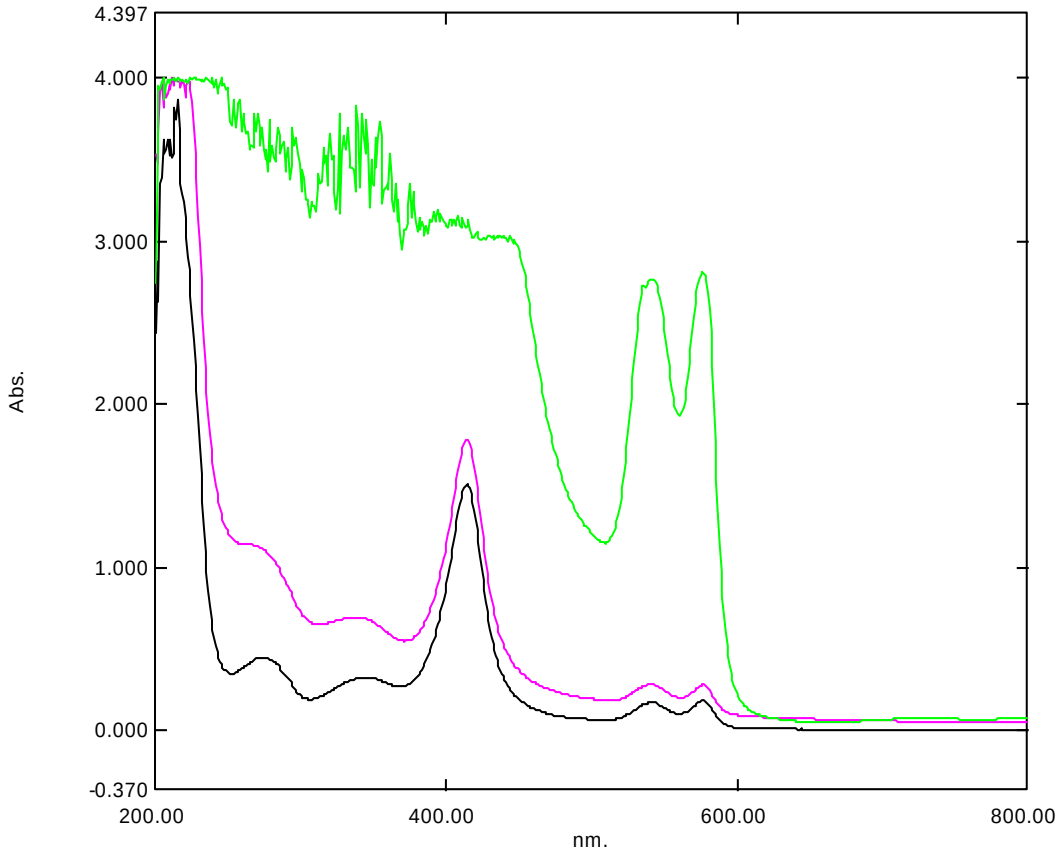

Figure 8.1 Hemoglobin absorption spectra obtained with 0.1 mM SA liposomes (pink), negative control (black) and positive control (green)

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

Figure 8.2 and

Figure 8.3.

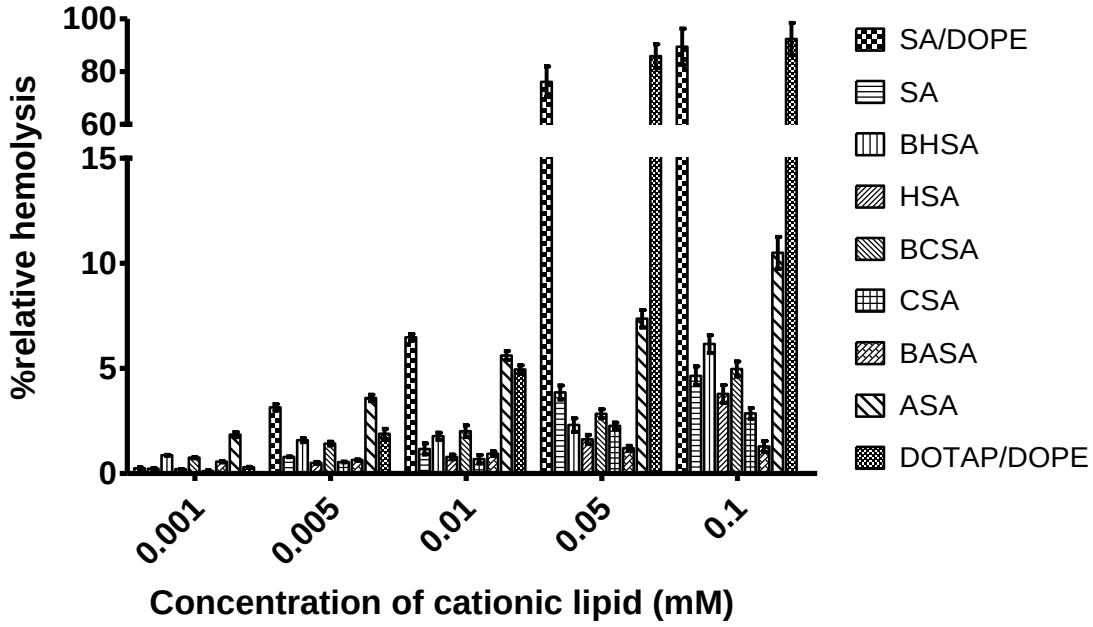

Figure 8.2 Haemolytic Potential of SA based Cationic liposomess

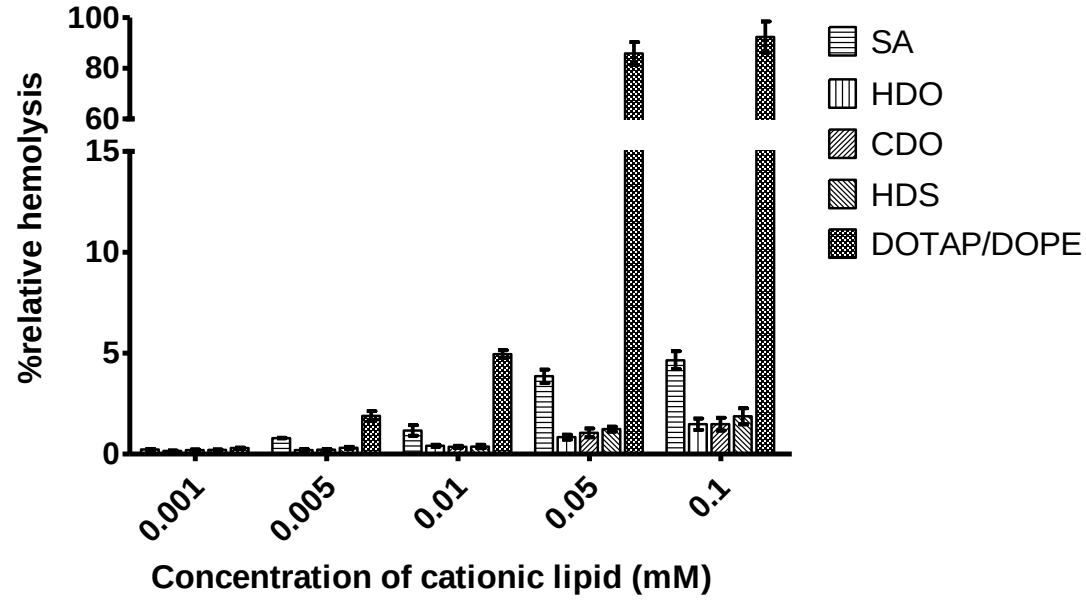

Figure 8.3 Haemolytic Potential of HDO, CDO and CDS liposomes

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) liposomes which demonstrates the haemolytic potential of stearyl amine as acationic lipid i.e. ~66% relative haemolysis at 0.05 mM SA concentration and ~79% at 0.1 mM SA concentration. Higher haemolytic potential of these liposomes 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:DOPE liposomes, stearyl amine may cause damage to the cell was in a fashion similar to DOTAP acting as a cationic lipid nd causing pore formation in erythrocytes. However, the non-PEGylated SA liposomes prepared with other helper lipids showed very low haemolysis as compared to SA:DOPE liposomes and DOTAP/DOPE liposomes even at all concentrations signifying the effect of lipid composition of liposomes on cytotoxicity. Additionally, the cytotoxicity was of even lower intensity with liposomes of stearyl amines modified with Boc-His, Boc-carnosine, Boc-arginine, histidine, carnosine and arginine. All these liposomes showed relative haemolytic potential less than 10%. With liposomes made up of HDO, CDO and HDS, the hemolysis was even lesser (<5%) than that observed with their corresponding stearyl amine containing lipoplexes. This indicates the structure dependent cytotoxicity of the lipids present in the liposomes i.e. single chain of stearyl amine vs. double acyl chain of phospholipids. Thus, from the observations, it can be concluded that the optimized liposomal formulationshave low potential to cause haemolysis at therapeutic concentration of the lipids employed and its exposure to the body.

8.1.2 MTT assay - toxicity on the C2C12 cells

With the advent of various new drug delivery systems like micelles, nanoparticles and liposomes in therapeutics, there's been rise in the toxicological evaluation of such formulations. This toxicological evaluation has also been revolutionized from *in vivo* to *in vitro* evaluation which provides better and rapid results on potential of new chemicals before they can be tested and used in animals or humans. *In vitro* cell culture is a potential tool that provides an insight about the clinical relevance of chemicals or formulations in specific diseases and studying cell toxicity mechanisms. And that's why *in vitro*

evaluation has been used for many years as an alternative to *in-vivo* evaluation as there has been found a close correlation between them.

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. *In vitro* cytotoxicity study data are now being considered by various regulatory agencies like Environment Protection Agency (EPA), National Institute of Health (NIH), National Institute of Cancer (NIC), Food and Drugs Administration (FDA). This necessitates the evaluation of cytotoxicity study of any formulation of any new drug molecule.

Different methods used for cell counting and cytotoxicity assays along with the dyes or probes used, the detection method, sensitivity, and features are tabulated in **Table 8.1** [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 BMP-9 gene on C2C12 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 8.4**).

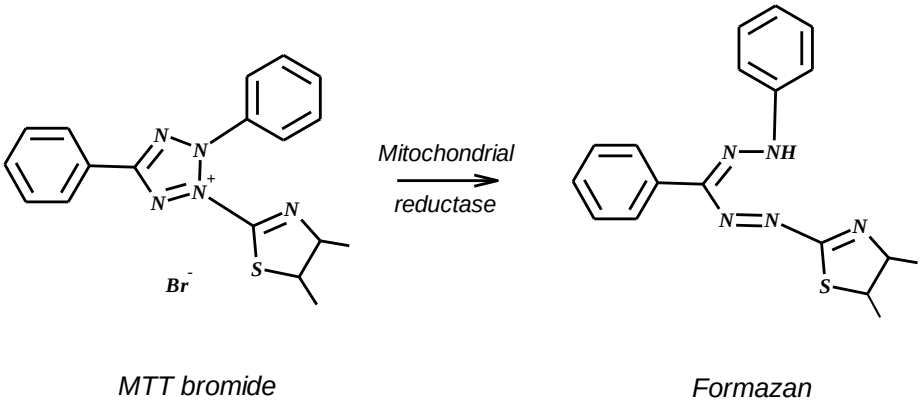

Figure 8.4 MTT Dye Reduction by Mitochondrial Reductase Enzyme of Viable Cells

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.

Table 8.1 Different Cell Counting and Cytotoxicity Assay Methods

Probe	Principle	Detection Method	Dead	Viable	Proliferating	Features/Advantages - Drawbacks
Trypan blue	Membrane exclusion	Colorimetric Microscopy	++	++	++	Cheap, but time consuming, not scalable. Do not state on viability.
Hoechst	DNA probe exclusion	Fluorimetric	++	++	+++	Cheap, Scalable, Non toxic. Do not state on viability. More rapid than MTT/XTT ; unfixed or fixed samples.
MTT	Formazan dye, orange precipitate.	Colorimetric	-	++	+++	Popular method. Sensitive, Scalable. Non toxic Increased solubility and performance from MTT to XTT and WST.
XTT	Same as MTT but more soluble.	Colorimetric	-	++	+++	
WST	Formazan dye, soluble & not toxic		-	++	+++	
UptiBlue	ratiometric blue probe for cell redox	Colorimetric Fluorimetric	-	+++	+++	No solubilization step (unlike MTT). Applyalso to adherent cells. Sensitivity similar to MTT/XTT, but easier to use Fluorimetry/Superior sensitivity to MTT / XTT.
Calcein-AM	Calcein accumulation in cytoplasm	Fluorimetric	-	+++	++	No solubilization step (unlike MTT/XTT). Adaptable to a wide variety of techniques, including : microplate assays, in vivo cell tracing. Do not work for bacteria. May alter some cell functions.
GAPDH	Release of GAPDH coupled to ATP assay	Bioluminescence	-	+++	+	Measurement of Cell-Mediated (T Cells, ADCC, NK) or Complement-Mediated Cytolsis.
CFSE	Fluorescein protein labeling	Fluorimetric	++	++	++	Useful when other method do not work properly. Do not state on viability.
AnnexinV	AnnexinV/PhosphoSerine	Fluorimetric	+	+++	+	Useful for Apoptosis study.
LDH	convertion in colored product		-	++	+	Recommended for cytotoxicity assays Serum Interference.
Luciferin Syst.	ATP measure	Luminescence	-	+	+++	Pros : sensitivity / linearity. Cons : signal depends on each cell line, on temperature
-3H Thymidine	DNA incorporation of radioactivity	Radioactivity	-	+	+++	Cons : hazardous (radioelements).
BRDU	DNA incorporation	Immunoassay	-	+	+++	
^{51}Cr release EU^{3+}	Release of radioactivity by cytoplasm	Radioactivity	-	-	+++	Recommended for cytotoxicity assays. Cons : hazardous (radioelements).
Propidium Iodide, AAD	7-Membrane permeability	Fluorimetric	+++	-	-	Used in combinaison of green fluorescence dye like. Annexin V-FP488 to discriminate dead cells from alive cells.

8.1.2.1 Materials

C2C12 cell line (mouse myoblast cell line) and NIH 3T3 mouse fibroblast cell line were procured from National Centre for Cell Science (NCCS), Pune, Maharashtra.

Dulbacco's Modifies Eagle's Medium (DMEM), Fetal Bovine Serum, Trypsin-EDTA solution, antibiotic solution (Penicillin G 100 units/mL, Streptomycine 100 μg/mL& Amphotericin B 50 μg/mL), MTT dye, Trypan blue dye, and DMSO (biological grade) were purchased from Himedia, Mumbai, India. Instruments and equipment used are listed in **Table 8.2**.

Table 8.2 List of Instruments and Their Sources

Instruments	**Sources**
Multichannel micropipette	Himedia, Mumbai.
Laminar air flow (HEPA filter)	Weiber Vertical Laminar Air Flow,
96 well plates	Tarsons, India
Culture Flasks	Tarsons, India
Olympus CKX 41 Inverted Microscope	Olympus, India.
Deep Freezer	E.I.E Instruments Ltd., Ahmedabad.
Jouan IGO150 CELLlife CO2 Incubator	Thermo Fisher Scientific, India.

8.1.2.2 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]

C2C12 cell lines were established 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[3]

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 8.5.**
8. 16 squares were focused at a time.

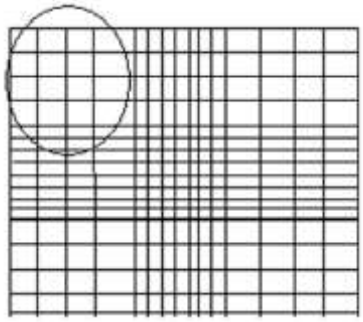

Figure 8.5 Haemocytometer diagram indicating the 16 corners squares which should be used for counting

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 $\times 10^3$ 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 $\times$ 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).

8.1.2.3 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 8.6,

Figure 8.7,

Figure 8.8 and

Figure 8.9 and that of lipoplexes are depicted in
Figure 8.10, **Figure 8.11**,

Figure 8.12and

Figure 8.13. Cytotoxicity of liposomes was evaluated by considering the concentration of cationic lipids on mM basis. Concentration range of 0.02, 0.05, 0.2, 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, 8 and 10 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 2 mole% DSPE-mPEG$_{2000}$ 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-mPEG$_{2000}$ 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.

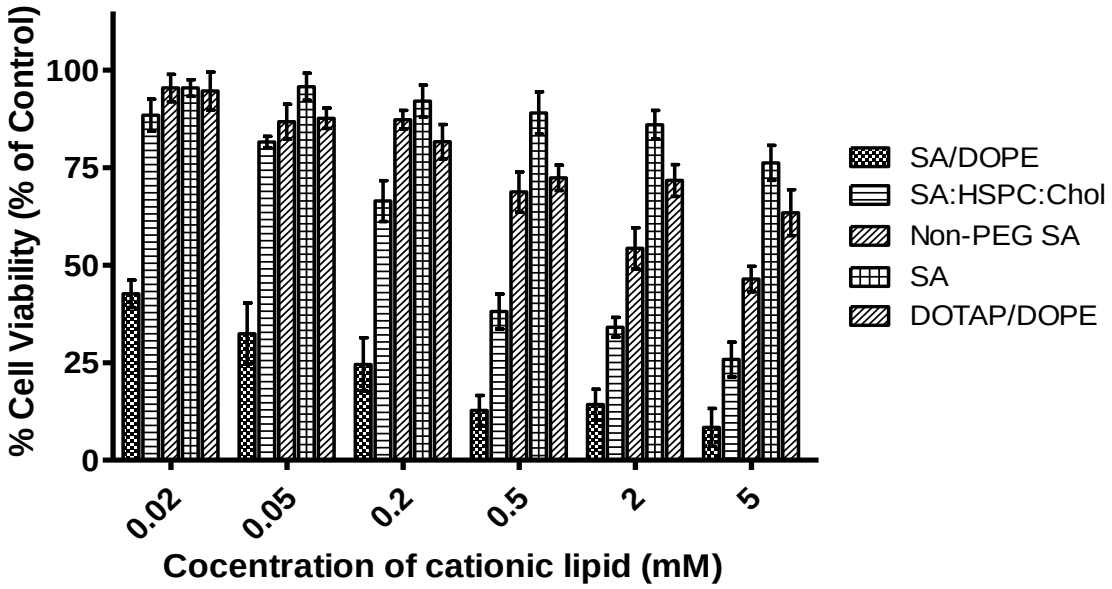

Figure 8.6 Cytotoxicity of SA liposomes prepared without and with DOPE (SA/DOPE, SA:HSPC:Chol lioposomes and SA-nonPEGylated liposomes respectively) and PEGylated SA liposomes against DOTAP/DOPE liposomes. SA/DOPE liposomes are composed of 1:1 molar ratio of SA and DOPE.

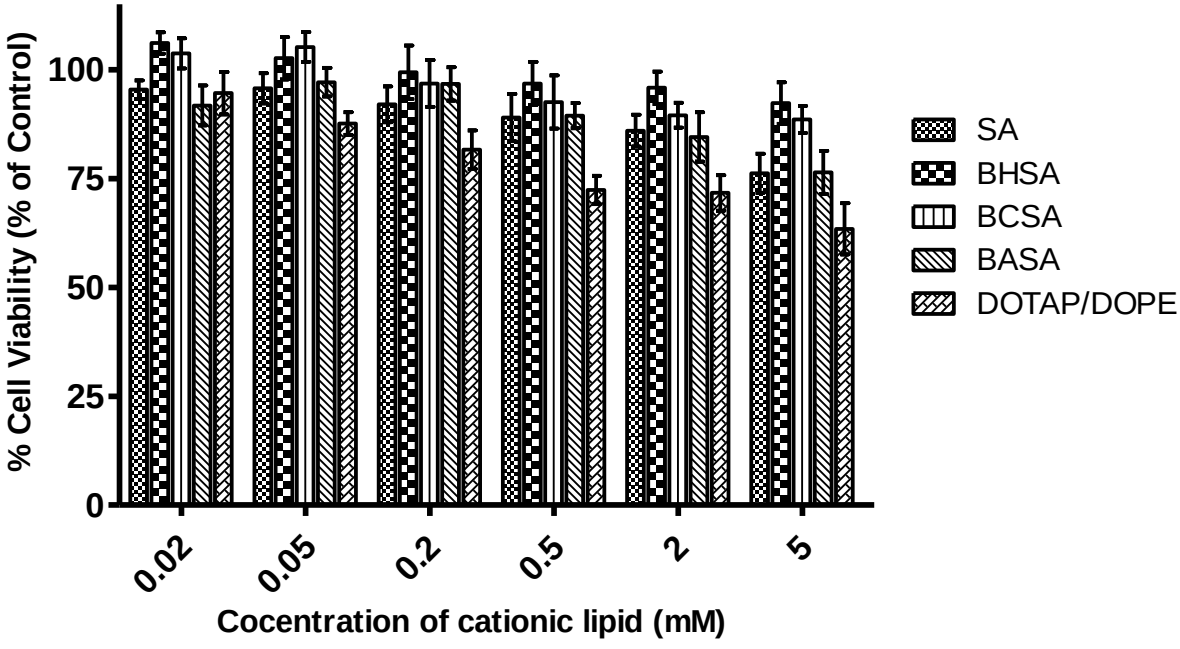

Figure 8.7 Cytotoxicity of BHSA, BCSA and BASA liposomes against SA liposomes and DOTAP/DOPE liposomes

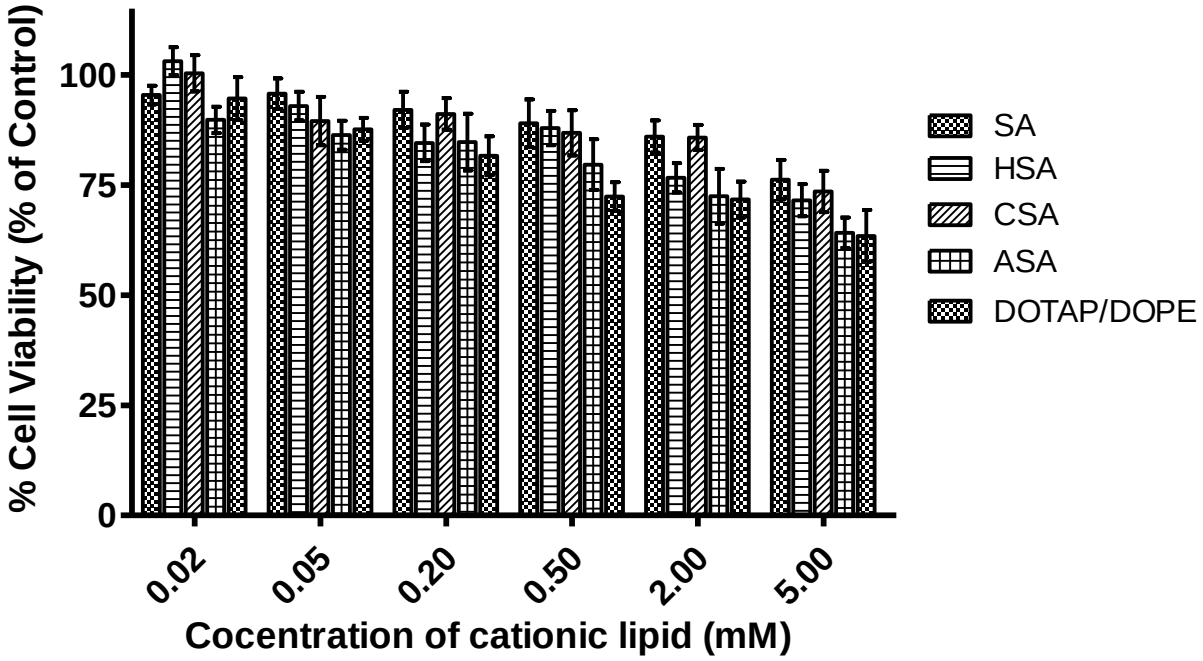

Figure 8.8 Cytotoxicity of HSA, CSA and ASA liposomes against SA liposomes and DOTAP/DOPE liposomes

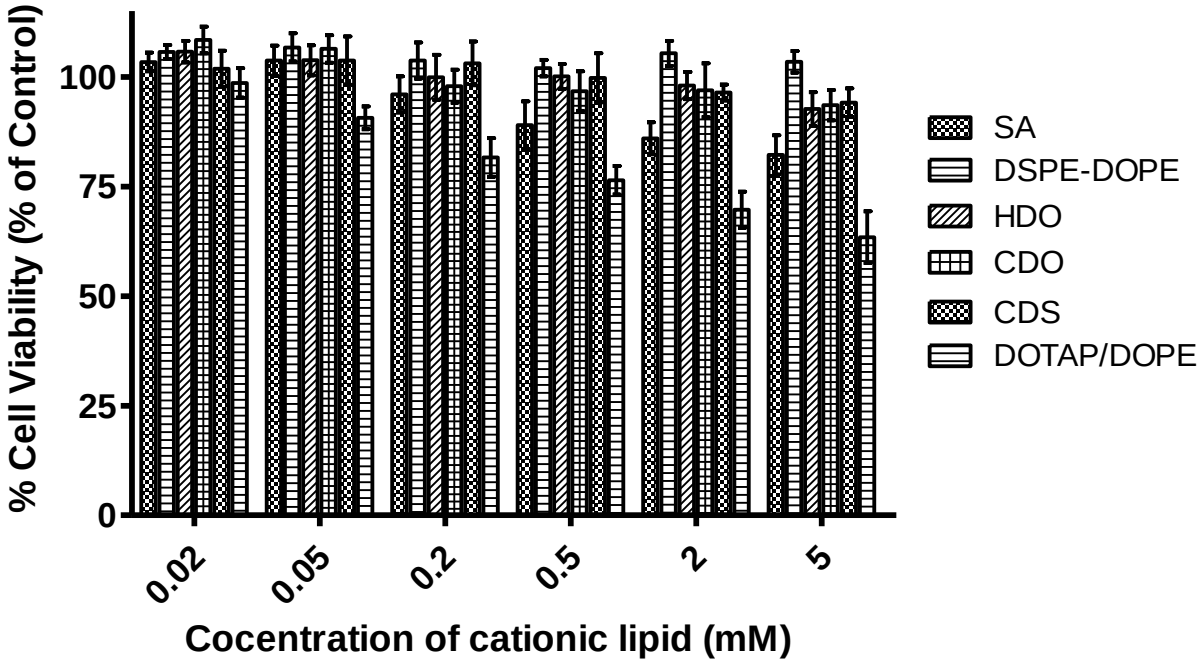

Figure 8.9 Cytotoxicity of HDO, CDO and CDS liposomes against SA liposomes and DOTAP/DOPE liposomes. For comparison, control batch prepared with DSPE and DOPE without any modification was also evaluated at the same concentration range.

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

HSA>BHSA, CSA>BCSA and ASA>BASA

This order of cytotoxicity can be justified by the cationic valency of the lipids i.e. 2+ charge on the Boc-deprotected lipids (HSA, CSA and ASA) vs. 1+ charge of Boc-protected lipids.

Among all the lipids, toxicity order can be given as

ASA > CSA > HSA > BASA > BCSA ≥ BHSA

The higher toxicity of the BASA among other Boc protected lipids and ASA 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.

Among all the liposomes, cytotoxicity was least for the liposomes prepared with HDO, CDO and CDS liposomes. The cell viability was almost similar to the control liposomes prepared with DOPE and DSPE indicating that the structural features also play role in the cytotoxicity of the liposomes with lipids comprising two aliphatic chains being less cytotoxic as compared to the lipids comprising single acyl chain. This indicates the cationic lipids with two carbon chains are hold firmly in the lipid bilayer of liposomes as compared to the single carbon chain as in case of stearyl amine and modified stearyl amine liposomes. Among the HDO, CDO and CDS liposomes, there was no significant difference in the cytotoxicity profile, however, the cationic nature imparted significant cytotoxicity as compared to control liposomes. Though, the cytotoxicity even at highest concentration of 5 mM cationic lipids was less 10% indicating the safety of the liposomes.

Cytotoxicity of lipoplexes prepared with liposomes of different cationic lipids at different N/P ratios i.e. 2, 4, 8, and 10 are shown in **Figure 8.10**, **Figure 8.11**, **Figure 8.12** and

Figure 8.13.

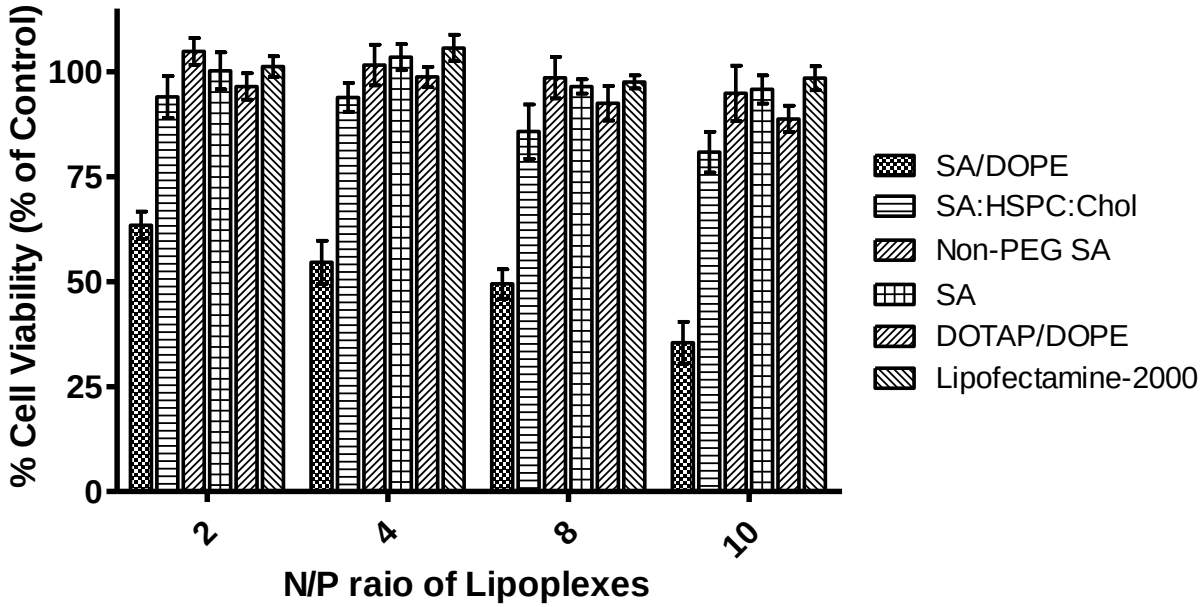

Figure 8.10 Cytotoxicity of lipoplexes prepared with SA liposomes without and with DOPE (SA/DOPE, SA:HSPC:Chol lioposomes and SA-nonPEGylated liposomes respectively) and PEGylated SA liposomes against DOTAP/DOPE and lipofectamine-2000 lipoplexes. For lipofectamine-2000 lipoplexes x-axis represent, instead of N/P ratio, lipofectamine-2000 reagent volume range as per standard transfection protocol i.e. 0.4 µL, 0.6 µL, 0.8 µL and 1.0 µL. SA/DOPE lipoplexes are composed of 1:1 molar ratio of SA and DOPE.

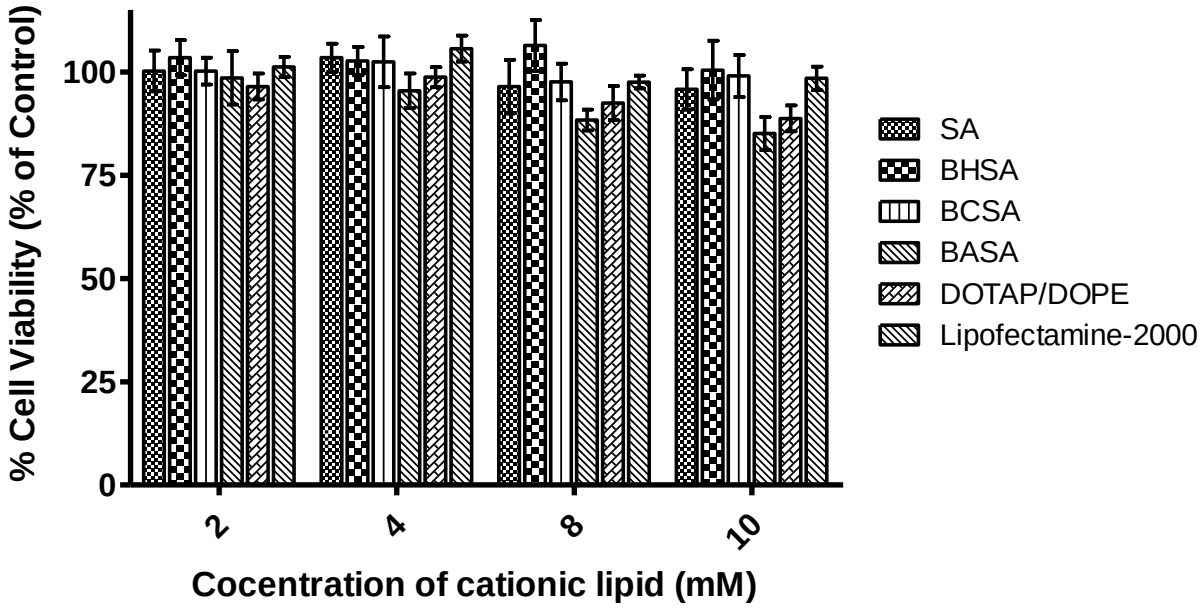

Figure 8.11 Cytotoxicity of BHSA, BCSA and BASA lipoplexes against SA, DOTAP/DOPE and lipofectamine-2000 lipoplexes. For lipofectamine-2000 lipoplexes x-axis represent, instead of N/P ratio, lipofectamine-2000 reagent volume range as per standard transfection protocol i.e. 0.4 µL, 0.6 µL, 0.8 µL and 1.0 µL.

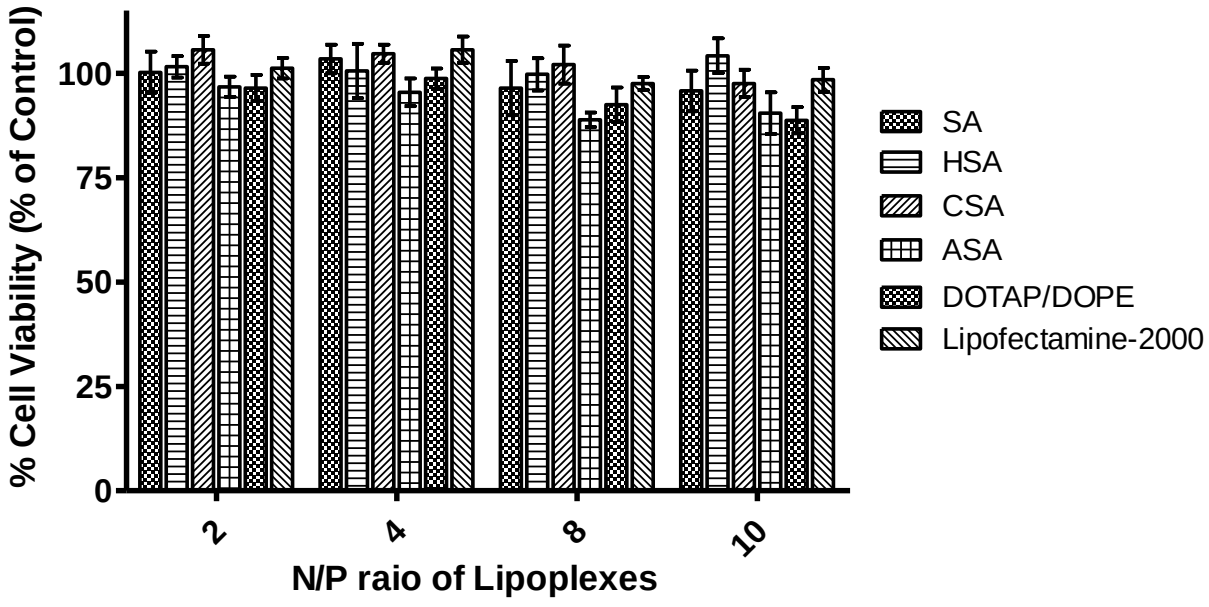

Figure 8.12 Cytotoxicity of HSA, CSA and ASA lipoplexes against SA, DOTAP/DOPE and lipofectamine-2000 lipoplexes. For lipofectamine-2000 lipoplexes x-axis represent, instead of N/P ratio, lipofectamine-2000 reagent volume range as per standard transfection protocol i.e. 0.4 µL, 0.6 µL, 0.8 µL and 1.0 µL.

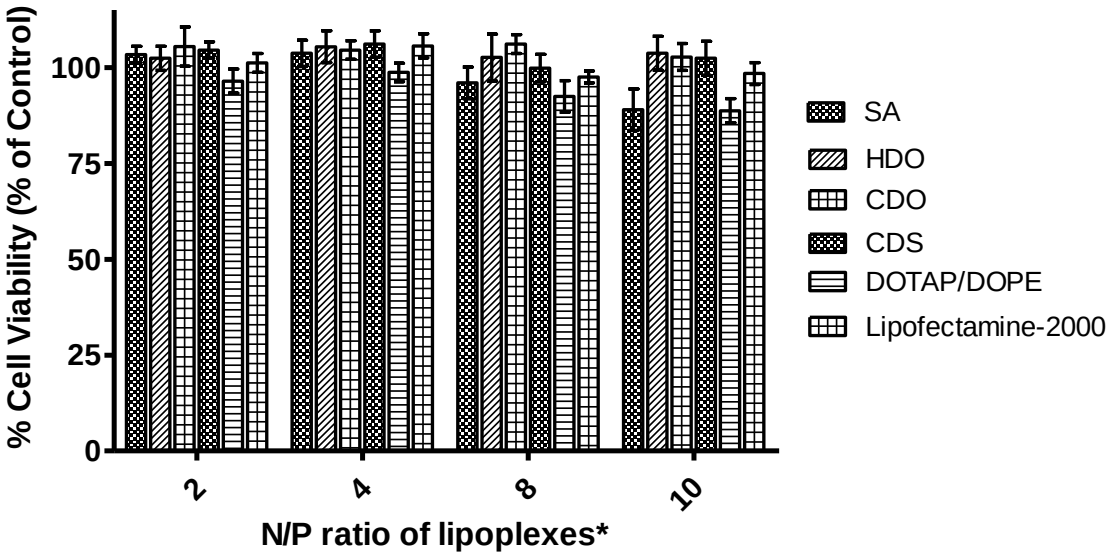

Figure 8.13 Cytotoxicity of HDO, CDO and HDS lipoplexes against SA, DOTAP/DOPE and lipofectamine-2000 lipoplexes. For lipofectamine-2000 lipoplexes x-axis represent, instead of N/P ratio, lipofectamine-2000 reagent volume range as per standard transfection protocol i.e. 0.4 µL, 0.6 µL, 0.8 µL and 1.0 µL.

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. This indicates that the exposure level of the stearyl amine/ modified stearyl amines is also a factor apart from the cationic charge of the lipid that plays a role in cytotoxicity. Considering the lipoplexes of these liposomes, cytotoxicity was even lower for the lipoplexes of modified DSPE and DOPE lipoplexes. This is due to double chain character of the lipids which are hold firm in the bilayer structure.

8.2 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 8.delivery systems by using

flow cytometry (fluorescence activated cell sorting) and fluorescence or confocal microscopy respectively.

8.2.1 Flow cytometry

Since the first application of flow cytometry (FCy) in the 1970s [11], the machines have become widely popular in research and clinical diagnostics. In principle, FCy can be combined with nearly any staining procedure, assay or biotechnological process. Whenever fluorescence is introduced into a microorganism or a cell it can be exploited in flow cytometry for assessing information about the specimen. To a low extent the technology is applied for other objects than microorganisms and cells [12]. But with the combination of fluidics and laser triggered fluorescence detection it is the ideal tool to detect nanoparticulate systems in cells. Subtle changes in scattering and emission of a cell can be observed – which are directly linked to the cellular uptake of fluorescing particles.

In flow cytometry, a fluidics system is coupled with the detection of fluorescence and of light scattering in small and wide angle position. For this application the objects of interest must be prepared as a diluted dispersion commonly not exceeding a concentration of several thousand objects per µl. In the machine, a sample stream is injected into the core of a flowing stream of so called sheath liquid (water or physiological buffer) and a laminar flow is established. The two streams do not mix and the sample flow is surrounded by a layer of sheath liquid flow in a concentric setup. This is termed hydrodynamic focusing. This stream of two concentric layers is directed through the measurement chamber, a narrow glass capillary. In the measurement chamber, the sample stream is hit orthogonally by a laser beam. It is important to note that the objects, e.g. cells, pass this laser beam single-filed. Placed behind an array of filters and mirrors, several detectors successively detect the properties of each cell passing the laser beam. This includes fluorescence signals but also of wide angle (sideward scatter, SSC) and small angle (forward scatter, FSC) scattering. Flow cytometers thus allow for the rapid measuring of individual objects in dispersion. Another obvious advantage is the short exposure of each object to the laser (µs scale), unlike e.g. in microscopy where exposure lasts seconds to minutes. Extremely light sensitive objects can be analyzed by flow cytometry. Within one second, several thousand objects can be measured separately and their number per volume can be counted. But only when one object passes through the beam of the reference laser, data acquisition is triggered. In this instant, a digital event is

created and the acquired data from every active channel is assigned to this event i.e. assigned to this particular object. Each event now represents a comprehensive data set, including fluorescence intensities in various channels and scattering intensities at two fixed positions (small and large angle i.e. FSC and SSC). This collected raw data consists of up to hundred thousands of events which represent background (e.g. pieces of cell debris) and wanted objects (cells) alike. Before final data interpretation the signals must be sorted from the background events. Fluorescence, granularity (SSC) or the presumable size (FSC or SSC) are features which can be applied to identify the wanted objects. Commonly a threshold condition is set on one of the detection channels so that unwanted signals are excluded from detection. In nearly every system a 488 nm laser is present as standard reference, but often additional lasers (e.g. 640 nm, 561 nm, 375 nm) are available.

8.2.1.1 Method

Media preparation, subculturing of cells, cell counting and preparation of formulations were followed as described in the MTT assay earlier. 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. 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 200 ng GFP pDNA per well. Treatement was carried out for 6 hr and post-treatment, cells were washed with phosphate buffered saline. Fresh media was replaced and cells were incubated for 24 hr for expression of GFP. 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 treatement were used as negative control. Naked pDNA and Lipofectamine-2000 lipoplexes were used as reference controls for comparison.

Table 8.3 Cell treatment parameters for FACS

Formulations	Cells and cell density	pDNA concentration	Treatment conditions	Expression Conditions
Naked GFP pDNA	C2C12 cells (50000 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, BHSA, HSA, BCSA, CSA, BASA, ASA, HDO, CDO and HDS				
Lipoplexes of Lipofectamine-2000 (LL2)				

8.2.1.2 Results and discussion:

FACS histograms of cellular uptake of developed lipoplexes are shown in **Figure 8.15** and **Figure 8.17**. **Table 8.4**, **Table 8.5**,

Figure 8.14 and **Figure 8.16** 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 lipoplexes showed similar transfection efficiency as that of Lipofectamine. The transfection efficiencies of the lipoplexes of the modified lipids were even higher than the SA and lipofectamine-2000 lipoplexes which describes potential of the single chain lipids in the transfection efficiency which would enhance the endosomal escape of the pDNA.

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 cationic lipid. Along with DOPE, stearyl amine and other modified SA lipids can afford the endosomal membrane damage under acidic conditions due to simultaneous activity of the SA/modified SA lipids along with DOPE on the endosomal membranes. Additionally, in case of modified stearyl amine lipids, the higher head-group size would provide higher endosomal escape as compared to stearyl amine.

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 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

CSA=HSA≥BCSA=BHSA=ASA=BASA>Lipofectamine=SA>naked pDNA

This indicates role of the structural differences among the lipids. Imidazole containing lipids i.e. BHSA, HSA,BCSA and CSA 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 8.4 GFP expression after transfection with different lipoplexes against naked pDNA and Lipofectamine-2000 lipoplexes*

Formulations	% GFP expressing cells
Naked pDNA	8.24±2.15
SA	72.73±4.25
BHSA	84.45±2.29
HSA	94.42±1.85
BCSA	85.45±1.78
CSA	93.67±1.45
BASA	81.53±2.48
ASA	84.88±2.63
Lipofectamine-2000	73.48±1.19

*****Experiments were performed in triplicate.

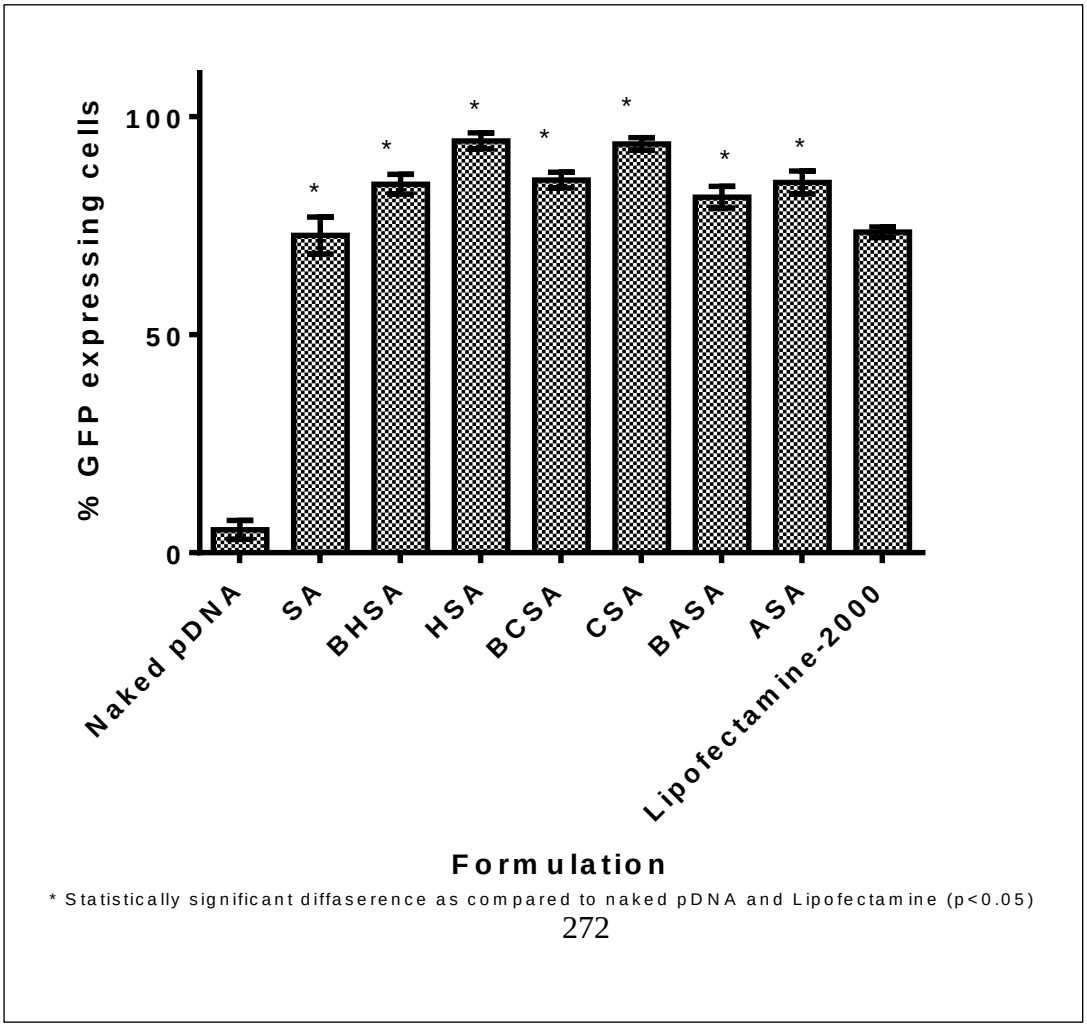

Figure 8.14 %GFP expression observed after transfection with naked pDNA, lipoplexes of SA and Modified SA lipoplexes. Untreated cells were taken as negative control while cells treated with lipoplexes of lipofectamine-2000 were taken as a reference control for comparison.

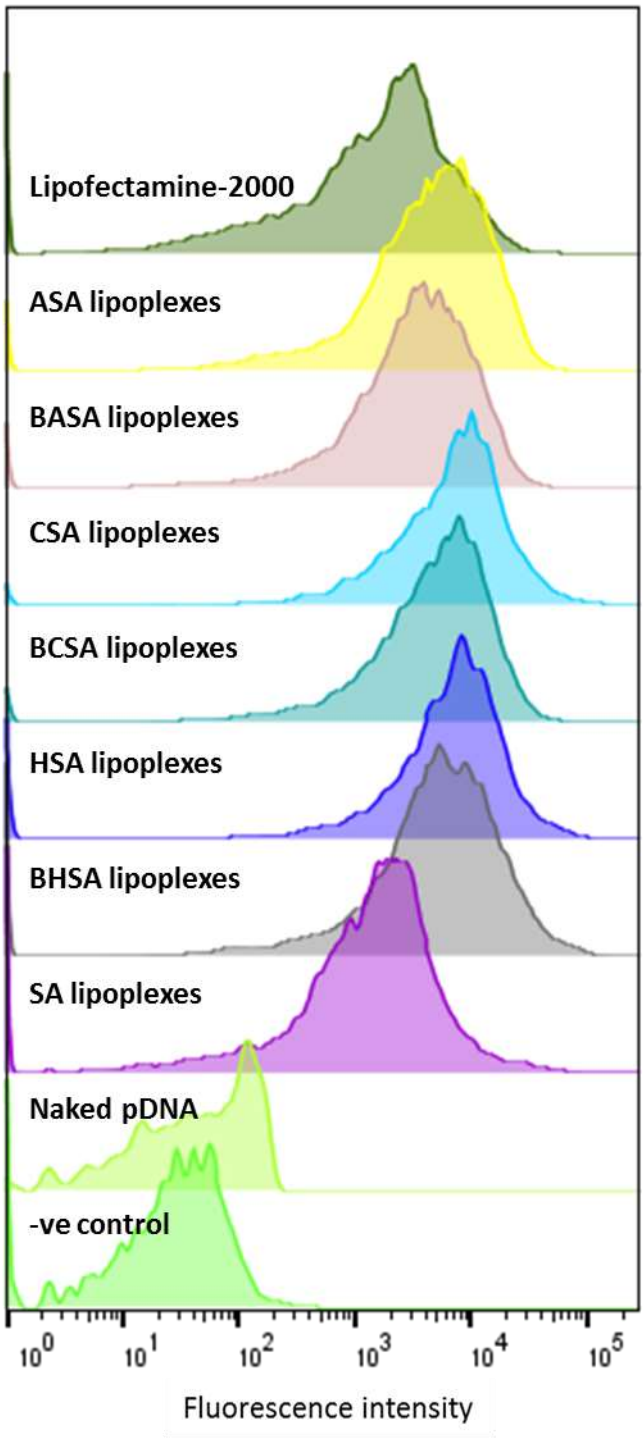

Figure 8.15 Overlay histograms of FACS analyses of cellular expression of eGFP by naked pDNA, lipoplexes of SA and Modified SA lipoplexes. Untreated cells were taken as negative control while cells treated with lipoplexes of lipofectamine-2000 were taken as a reference control for comparison.

In case of modified DOPE and DSPE, it was seen that the higher transfection efficiency was observed in case of the HDS lipoplexes than HDO and CDO lipoplexes. This might be due to the reduced DOPE content of the lipoplexes i.e. lipoomes of HDO,

CDO and HDS were prepared by replacing the respective mole% of DOPE and DSPE in the control liposomes. This causes reduced DOPE content of the lipoplexes prepared using HDO and CDO. Eventhough , the transfection efficiencies were higher for HDO, CDO and HDS lipoplexes than lipofectamine-2000 and naked pDNA.

Table 8.5 GFP expression after transfection with different lipoplexes against naked pDNA and Lipofectamine-2000 lipoplexes*

Formulations	% GFP expressing cells
Naked pDNA	5.24±2.15
SA	72.73±4.25
HDO	78.15±2.21
CDO	81.74±2.45
HDS	85.94±1.74
Lipofectamine-2000	73.48±1.19

*****Experiments were performed in triplicate.

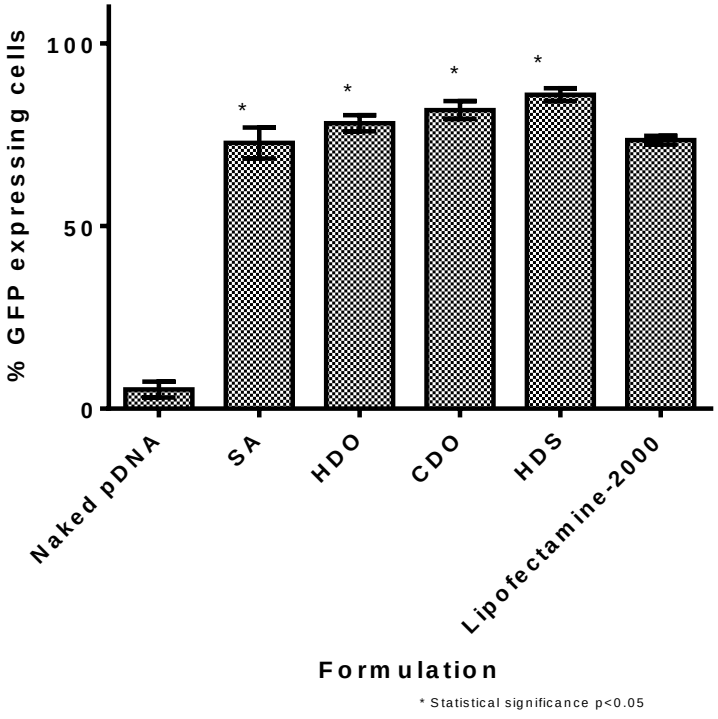

Figure 8.16 %GFP expression observed after transfection with naked pDNA, HDO, CDO and HDS lipoplexes. Untreated cells were taken as negative control while cells treated with lipoplexes of lipofectamine-2000 were taken as a reference control for comparison. eGFP expression by SA lipoplexes are also included for comparison.

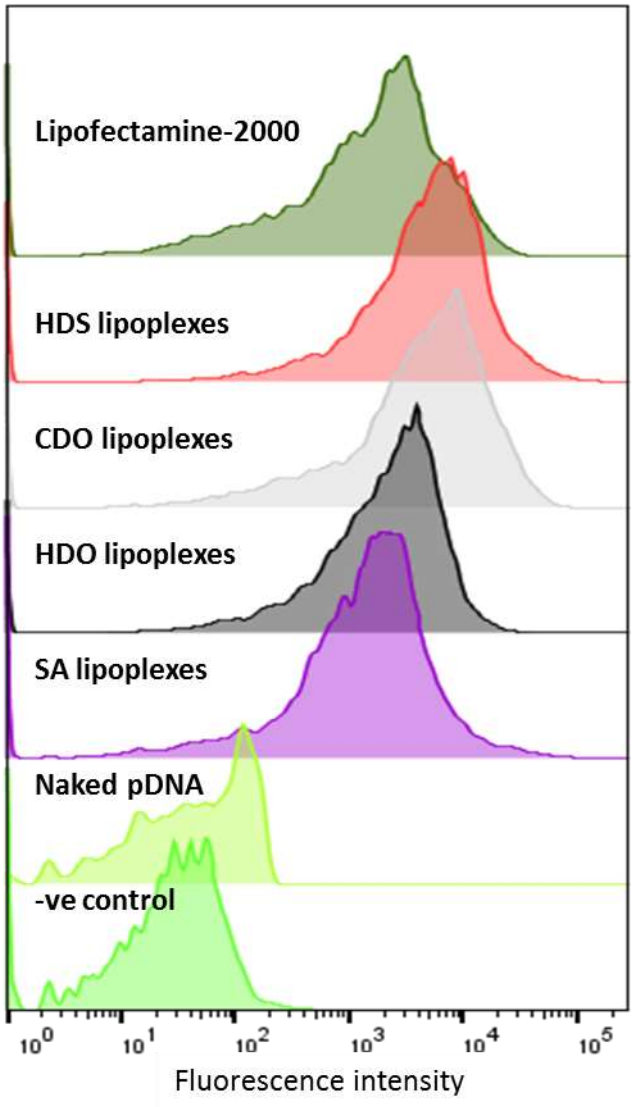

Figure 8.17 Overlay histograms of FACS analyses of cellular expression of eGFP by naked pDNA, HDO, CDO and HDS lipoplexes. Untreated cells were taken as negative control while cells treated with lipoplexes of lipofectamine-2000 were taken as a reference control for comparison. Histogram of eGFP expression by SA lipoplexes are also included for comparison.

8.2.2 Confocal microscopy

Cellular uptake and expression studies can be performed using confocal microscopy which allows imaging of cells at a specific focal point creating sharper images reducing the noises from scattering [13, 14]. Fluorophores are added to the samples to illuminate the specific structures of the cells i.e. cytoplasm, nuclei, mitochondria etc. depending on the purpose of the experimentation [15]. Fluorophores improve the imaging through sharper detection of the targets with enhanced sensitivity and specificity [14]. More than one fluorophores can be used simultaneously to illuminate cells and by switching laser wavelength to excite a specific fluorophore, one can observe different parts of specimen. Excitation beam of laser hits the target at a defined focal point generating high intensity fluorescence. Both incident excitation beam and emitted fluorescence beam pass through a dichroic mirror that functions to reflect the incoming, high energy (short wavelength) laser light while allowing the low energy (short wavelength) fluorescent light to pass through which can be detected by light detector. Additionally, a pinhole is used in fluorescence confocal microscopy to eliminate the scattered light, and thus, resulting in collection of fluorescent light from highly focused point. This is helpful in detecting the fluorescence generated at different parts of specimen by changing the focal point. For example, in case of cellular uptake studies of a fluorescent dye loaded targeted drug delivery system, the fluorescence concentrated on the cell surface and cytosol can be imaged separately to give insight on the actual uptake and the surface localization of the formulation.

8.2.2.1 Method

Media preparation, subculturing of cells, cell counting and preparation of formulations were followed as described in the MTT assay earlier. 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. After 24 hr, cells were transfected with lipoplexes prepared using optimized liposomal formulations using GFP pDNA at N/P ratio of 2 (**Table 8.6**) at a final concentration of 500 ng eGFP pDNA per well. Treatement was carried out for 6 hr and post-treatment, cells were washed with phosphate buffered saline. Fresh media was replaced and cells were incubated for 24 hr for expression of GFP. Cells were washed with PBS followed by treatment with nucleus stain DAPI for 10 minute followed by washing again with sterile phosphate buffered saline to remove excess DAPI

from milieu. 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 8.6 Cell treatment parameters for confocal microscopy

Formulations	**Cells and cell density**	**pDNA concentration**	**Treatment conditions**	**Expression Conditions**
Naked GFP pDNA	C2C12 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, BHSA, HSA, BCSA, CSA, BASA, ASA, HDO, CDO and HDS				
Lipoplexes of Lipofectamine-2000 (LL2)				

8.2.2.2 Results and discussion:

Confocal microscopy images of the cell uptake studies are shown in **Figure 8.18**, **Figure 8.19**, **Figure 8.20** and **Figure 8.21**. Each image depicts the cellular uptake of developed lipoplexes against the naked DNA, DOTAP/DOPE lipoplexes and Lipofectamine-2000 lipoplexes for comparison.

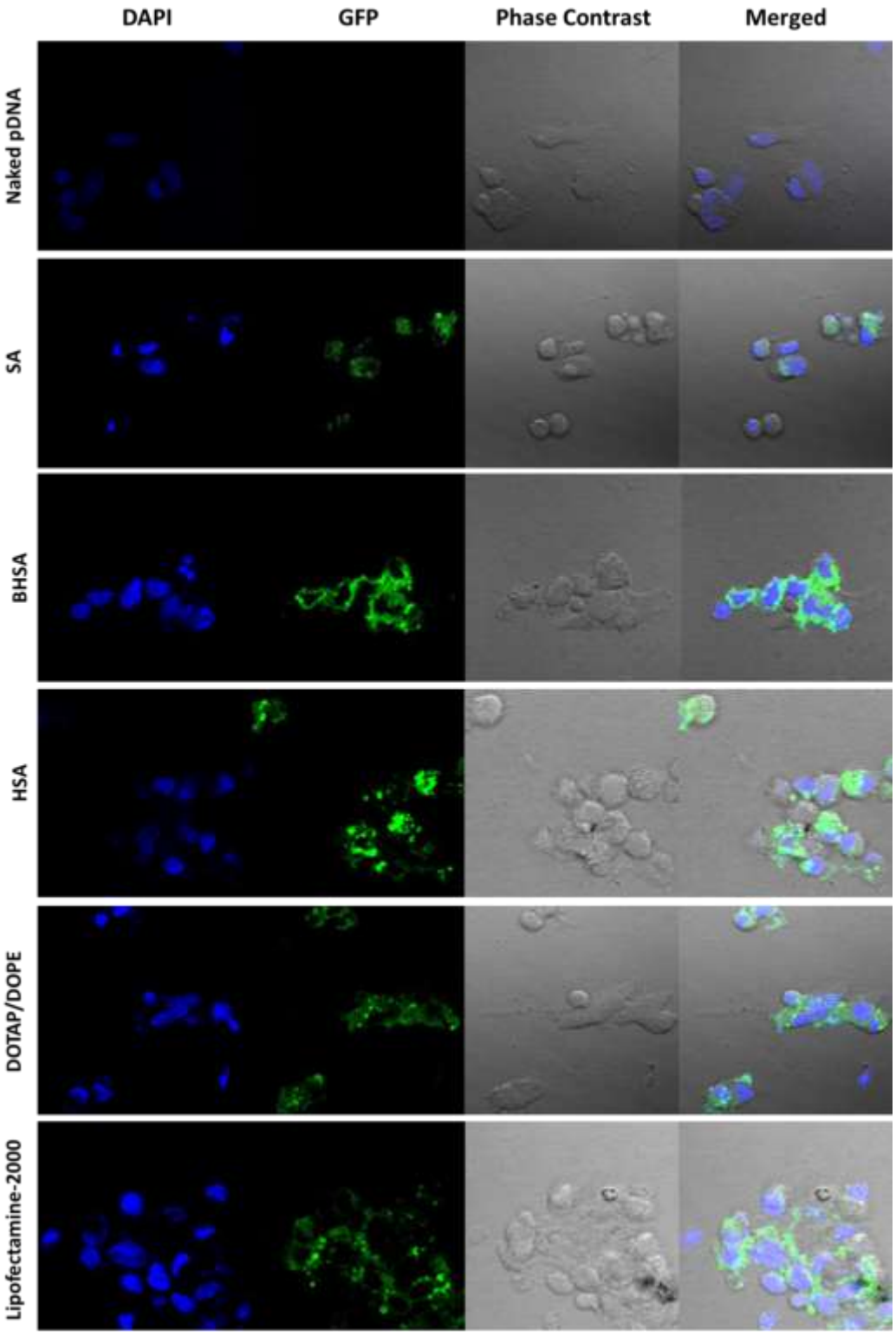

Figure 8.18 Confocal images of SA, BHSA and HSA lipoplexes. Cellular expression of GFP is shown for SA, BHSA and HSA lipoplexes in comparison to that after transfection done with naked eGFP pDNA, lipoplexes of DOTAP/DOPE and Lipofectamine-2000.

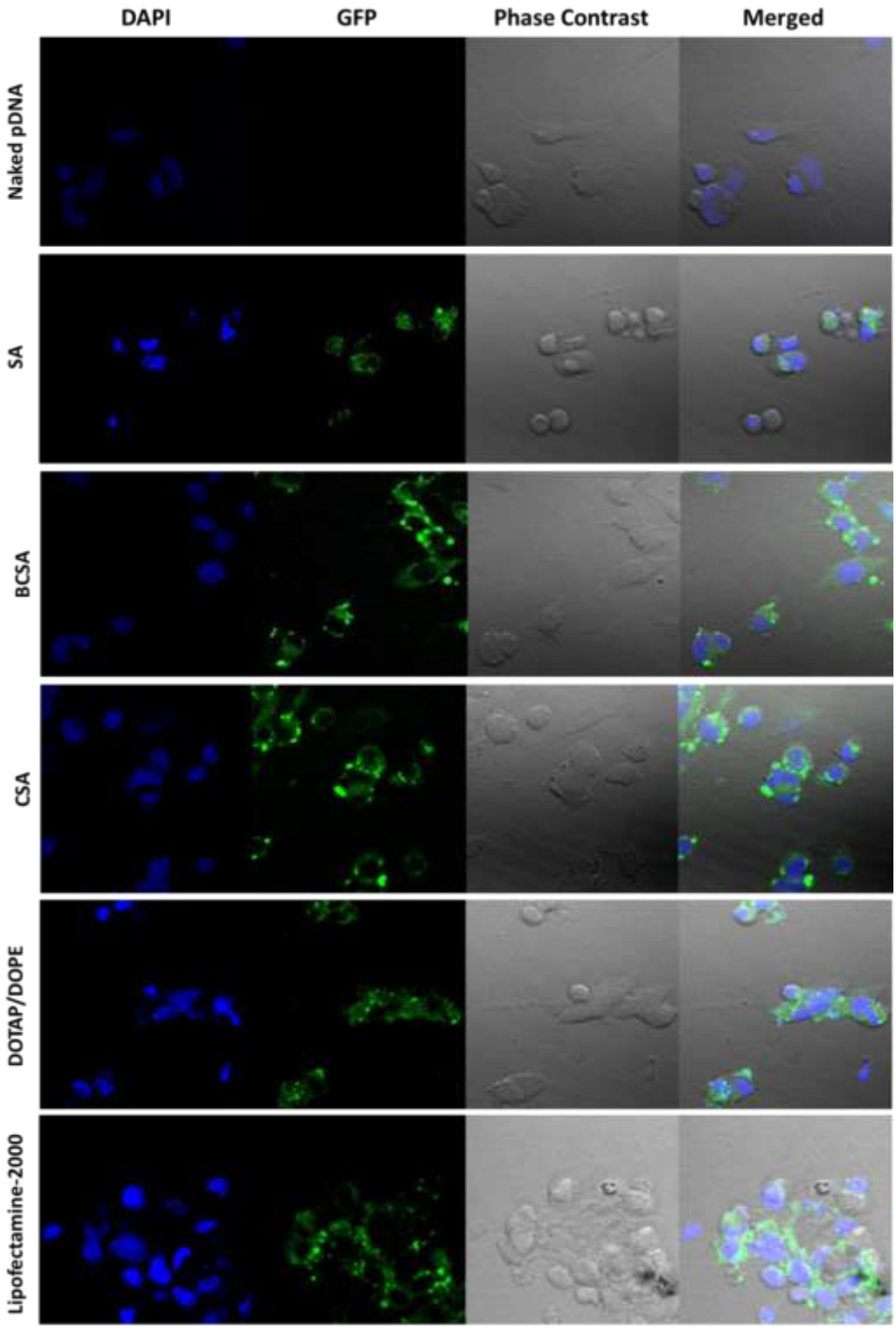

Figure 8.19 Confocal images of GFP expression after transfection with BCSA and CSA lipoplexes. Cellular expression of GFP for BCSA and CSA lipoplexes is shown in comparison to that with naked eGFP pDNA and lipoplexes of SA, DOTAP/DOPE and Lipofectamine-2000.

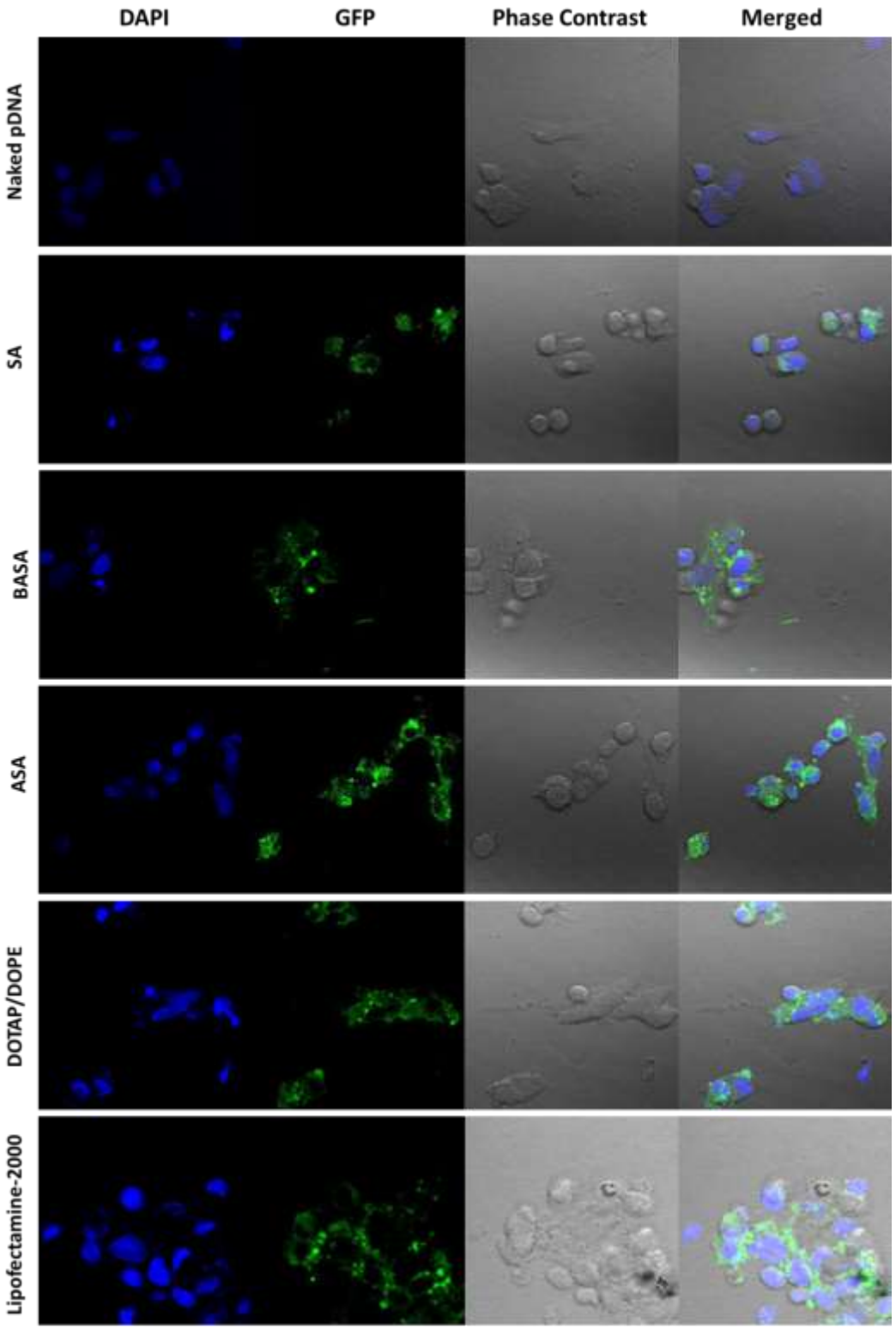

Figure 8.20 Confocal images of GFP expression after transfection with BASA and ASA lipoplexes. Cellular expression of GFP with BASA and ASA lipoplexes is shown in comparison to that with naked eGFP pDNA and lipoplexes of SA, DOTAP/DOPE and Lipofectamine-2000.

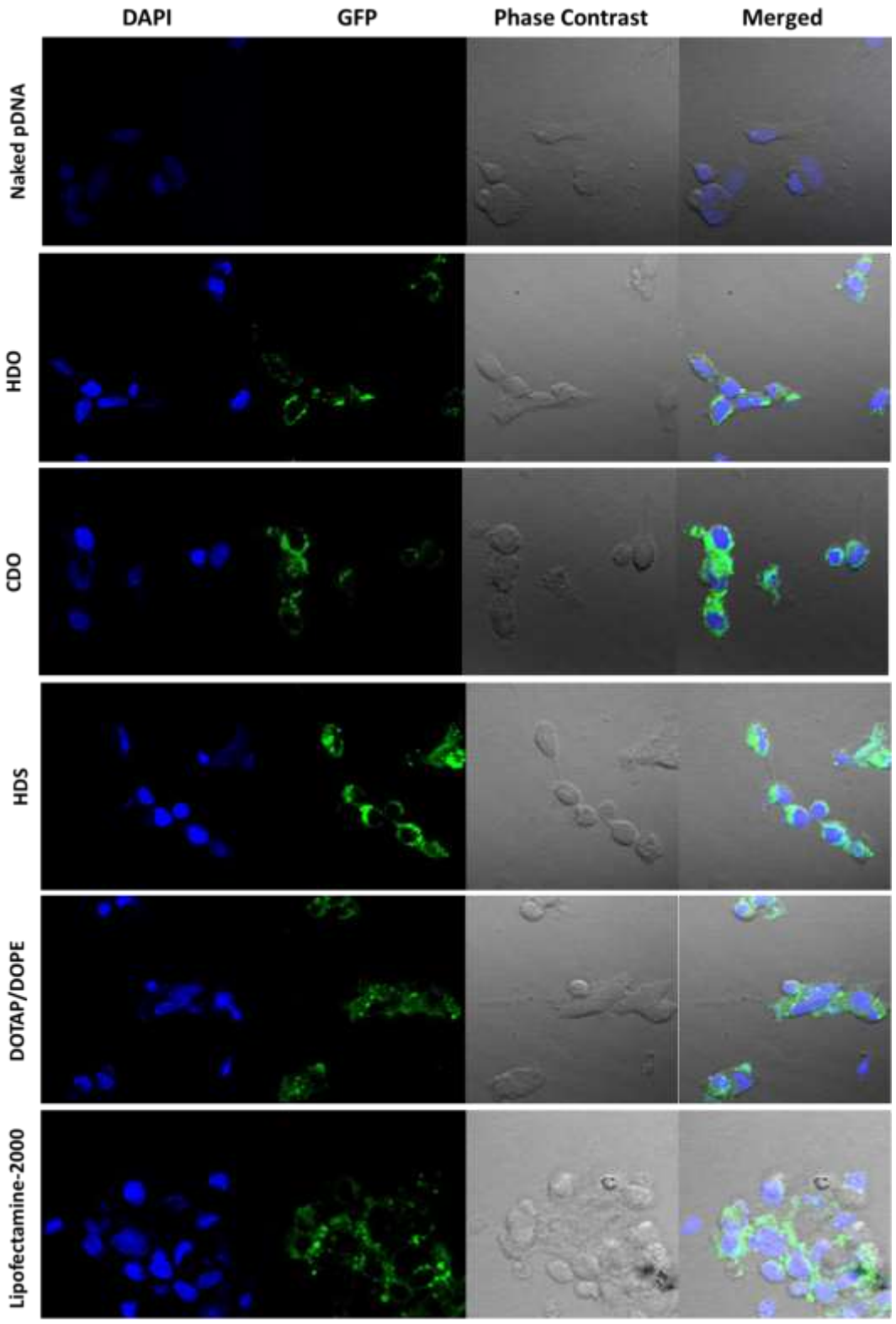

Figure 8.21 Confocal images of GFP expression after transfection with HDO, CDO and HDS lipoplexes. Cellular expression of GFP with HDO, CDO and CDS lipoplexes is shown in comparison to that with naked eGFP pDNA and lipoplexes of SA, DOTAP/DOPE and Lipofectamine-2000.

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 stearyl amine containing formulations as compared to that obtained with SA lipoplexes, lipofectamine-2000 lipoplexes modified DOPE and modified DSPE containing lipoplexes. The increased transfection efficiency of the modified stearlyl amine containing liposomes can be considered due to the multiple effects playing role. Increased uptake in case of BHSA, HSA, BCSA and CSA 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-gorup 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 BASA and ASA 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 ASA might also be compensated by the higher cytotoxicity as seen in the cytotoxicity studies.

Similar trends as observed in the flow cytometry were observed with the HDO, CDO and HDS lipoplexes as well. The higher cellular uptake and subsequent cytosolic unloading of pDNA as well as protection provided by the buffering effect rendered by the modified lipids against the Lipofectamine-2000 demonstrate their potential use as gene delivery carriers.

8.2.3 Calcium deposition studies – Therapeutic activity *in vitro*

In vitro demonstration of therapeutic activity of the gene delivery system is an essential tool for predicting its in vivo success. There are several assays that can be used for confirming the therapeutic activity of the gene delivery system. Some examples are measuring quantitatively the expression in terms of the therapeutic protein estimation through tests like western blotting and other antibody based assays. However, these assays are indirect and do not give idea of the therapeutic activity of the expressed protein. The direct estimation of the therapeutic activity of the expressed protein confirms the success of the gene delivery system.

In the present investigation, C2C12 cell line (mouse myoblast cell line) is used. The cell line bears the capability to cross-differentiation to the bone forming cells and hence serves as an appropriate choice to demonstrate the therapeutic activity of the BMP-9 gene delivery system. If the gene delivery system is able to express the protein that enables its differentiation to the bone forming cells, the cells become capable of depositing the inorganic soluble calcium to the calcium deposits as seen with the osteoblast cells. Calcium, deposited so, can be detected by specialized stains i.e. Von Kossa stain. Hence, in the present investigation the calcium deposition assay has been performed in order to demonstrate the therapeutic activity of the developed gene delivery system in vitro.

8.2.3.1 Method

Media preparation, subculturing of cells, cell counting and preparation of formulations were followed as described in the MTT assay earlier. Cells were seeded onto 24 well-plates at a density of 10^4 cells/well and incubated for 24 hr in complete media. After 24 hr, cells were transfected with lipoplexes prepared using optimized liposomal formulations using BMP-9 pDNA at N/P ratio of 2 at a concentration of 200 ng BMP-9 pDNA per well (**Table 8.7**). After transfection period of 6 hr, cells were washed with phosphate buffered saline. Cells were incubated for 7 days in complete media supplemented with disodium hydrogen phosphate solution (pH 7.4 set with 0.1N HCl) at a concentration of 10 mg/mL of dihydrogen phosphate. Cells were washed with phosphate buffered saline post-incubation and Von Kossa staining was performed on the cells. Cells were fixed in 4% paraformaldehyde solution in 0.1M phosphate buffer for 10 min at room temperature and then washed with phosphate buffered saline followed by sterile distilled water. Fixed cells were treated with 5% silver nitrate solution and illuminated with UV light for 20 min. Unreacted silver nitrate is removed by washing with 5% sodium thiosulfate solution for 2 minutes. Cells were then counterstained with nuclear fast red (Sigma-Aldrich, USA) solution (0.1%) for 5 minutes. Microscopy was performed on inverted microscope (Nikon 2000 inverted Fluorescence Microscope, Nikon, Japan) Calcium mass deposits are visible as black or brown-black deposits while intracellular dispersed calcium is visible as orange-brown granules against the pink background. Untreated cells were used as a negative control. Cells transfected with naked BMP-9 pDNA and Lipofectamine-2000 lipoplexes were uses as reference controls.

Table 8.7 Cell treatment parameters for in vitro calcium deposition studies

Formulations	Cells and cell density	pDNA concentration	Treatment conditions	Growth period post-transfection
Naked GFP pDNA	C2C12 cells (10000 cells/well)	200 ng eGFP pDNA per well	Incubation time= 6 h Temperature = 37°C (5% CO_2)	7 days after transfection Temperature = 37°C (5% CO_2)
Formulations (SA, BHSA, HSA, BCSA, CSA, BASA, ASA, HDO, CDO and HDS				
Lipoplexes of Lipofectamine-2000 (LL2)				

8.2.3.2 Results and discussion:

Microscopic images of cells after Von Kossa staining are shown in the **Figure 8.22** and **Figure 8.23**. It has been shown that treatment of the C2C12 myoblast cells undergo osteoblastic differentiation pathway on treatment with bone morphogenetic proteins [16-18]. Such differentiation can be estimated by the deposition of calcium in the form of calcium phosphate which can be detected by special stains such as Von Kossa stain which help identify the mass calcium deposits as brown-black colour and dispersed calcium as brown to orange colour and background and cytoplasm as pink colour. Negative control (untreated cells) did not show any mass deposits of calcium except for marginal dispersed calcium inside the cells after 7 days of incubation. Same was the case with the cells transfected with naked pDNA. All lipoplex formulations showed positive staining confirming the intracellular dispersed calcium deposits as well as calcium phosphate mass deposits. As it can be seen the calcium deposition was highest with HSA, CSA and ASAS lipoplexes as compared to their corresponding BOC-Protected lipids. Notably, all SA based lipoplexes showed higher calcium deposition as compared to DOTAP/DOPE and Lipofectamine-2000 lipoplexes.

Calcium deposition was lower in case of the HDO, CDO and HDS lipoplexes as compared to their corresponding SA based lipoplexes. Among them, HDS showed the higher calcium deposition as compared to HDO and CDO. The cellular expression studies done with GFP corroborate the results obtained here.

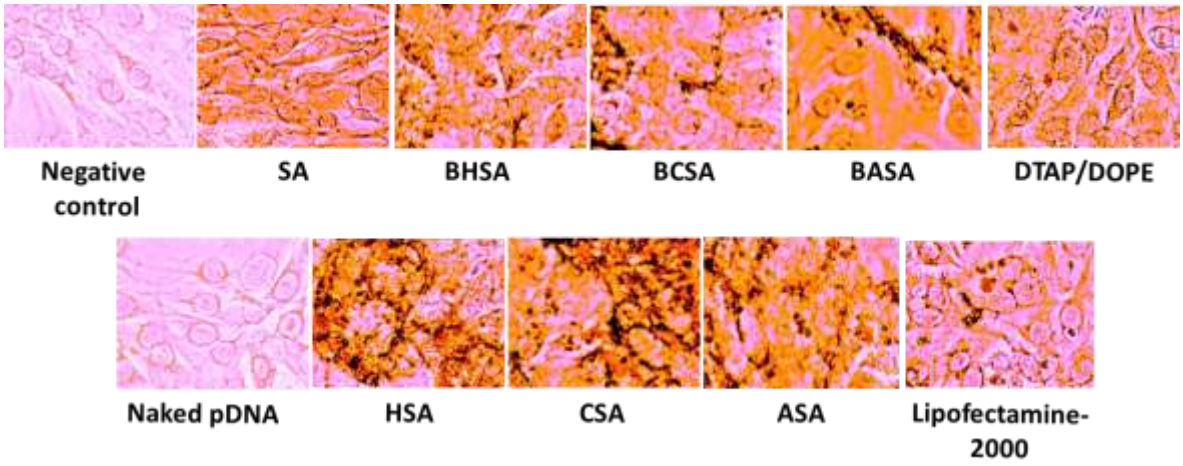

Figure 8.22 Calcium deposition study: Von Kossa staining of cells after transfection with different lipoplex formulations. Von Kossa stained cells 7 days after transfection with SA, BHSA, HSA, BCSA, CSA, BASA and ASA lipoplexes are shown in comparison to that with untreated cells (negative control), naked pDNA and lipoplexes of DOTAP/DOPE and Lipofectamine-2000.

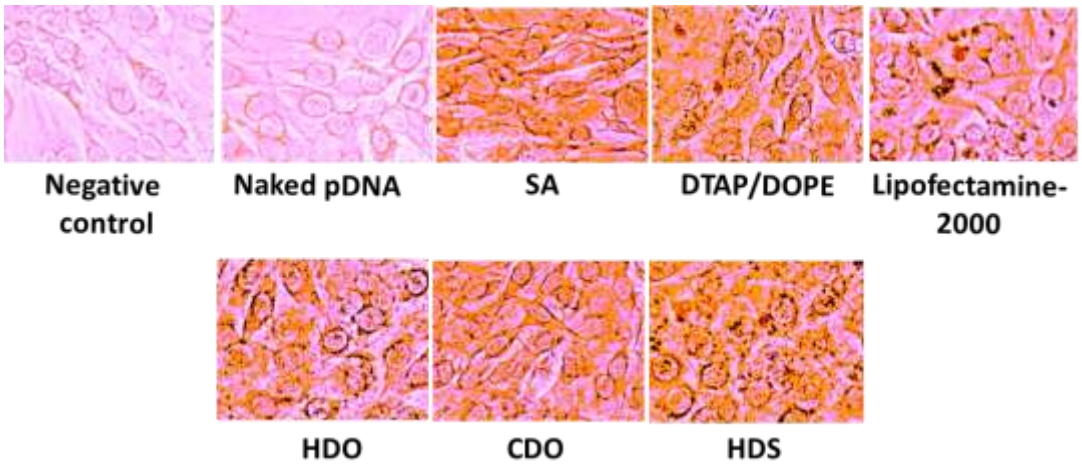

Figure 8.23 Calcium deposition study: Von Kossa staining of cells after transfection with different lipoplex formulations. Von Kossa stained cells 7 days after transfection with SA, BHSA, HSA, BCSA, CSA, BASA and ASA lipoplexes are shown in comparison to that with untreated cells (negative control), naked pDNA and lipoplexes of DOTAP/DOPE and Lipofectamine-2000.

8.3 References

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