"A GENE DELIVERY APPROACH FOR TREATMENT OF ATHEROSCLEROSIS"

Summary of the PhD thesis to be submitted to

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Summary and Conclusion

Atherosclerosis is a disease in which plaque builds up inside the arteries. Arteries are blood vessels that carry oxygen-rich blood to the heart and other parts of the body. Atherosclerosis is a disease in which inside of an artery narrows due to plaque building (1). Plaque is made up of fat, cholesterol, calcium, and other substances found in the blood. Over the time, plaque hardens and narrows the arteries (2). This limits the flow of oxygen-rich blood to the organs and other parts of the body. Initially there are generally no symptoms of atherosclerosis. However, when severe it can result in coronary artery disease, stroke, heart attack, peripheral artery disease, or kidney problems depending on the arteries which are affected (1). Figure 2.1 (a) shows a normal artery with normal blood flow and (b) shows an artery with plaque. The inset image shows a cross-section of both while (C) shows steps of formation of plaque.

Atherosclerosis can affect all large and medium-sized arteries, including the coronary, carotid, and cerebral arteries; the aorta; its branches; and major arteries of the extremities. It is the leading cause of morbidity and mortality in the US and in most developed countries. In recent years, age-related mortality attributable to atherosclerosis has been decreasing, but in 2015, cardiovascular disease (CVD), primarily coronary and cerebrovascular atherosclerosis still caused almost 15 million deaths worldwide (> 25% of all deaths (3). In the US, > 800,000 people died of CVD in 2014, corresponding to almost 1 in 3 of all deaths (4). Atherosclerosis is rapidly increasing in prevalence in developing countries, and as people in developed countries live longer, incidence will increase. Atherosclerosis is the leading cause of death worldwide."

Atherosclerosis management involves various facets. Lifestyle Changes focuses on weight management, physical activity and a healthy diet. Medications include administration of various lipid lowering drugs such as Satins (HMG CoA Reductase inhibitors), Fibrates, Bile acid binding resins and several others including nicotinic acid,

probucol etc. Severe cases of atherosclerosis may be treated by surgical procedures, such as angioplasty or coronary artery bypass grafting (CABG). However, the oral antihyperlipidemic therapy needs to be continued on a daily basis for maintenance of the blood cholesterol level. Although considerable progress has been made in the prevention and treatment of atherosclerotic cardiovascular disease, new therapeutic strategies are still needed. Atherosclerosis is a systemic disease and represents an attractive target for the development of somatic gene transfer intended to modulate systemic factors with the goal of inhibiting disease progression. Such gene delivery approaches act particularly by inducing or overexpressing receptors that lead to increase in LDL metabolism.

In the past decade various gene delivery approaches have been studied for the treatment of atherosclerosis. Such gene delivery approaches act particularly by inducing or overexpressing receptors that lead to increase in LDL metabolism. A hepatocellular-targeted, atheroprotective gene therapy as an approach to the prevention and treatment of atherosclerosis is being sought. Expression of these therapeutic gene is aimed at counteracting the fundamental processes that drive atherosclerosis, including lipid accumulation in the vascular intima and inflammatory cell recruitment. Apolipoprotein E (ApoE) is a critical ligand for the clearance of chylomicron and VLDL remnant lipoproteins. ApoE is synthesized by many tissues, but the ApoE in plasma is derived largely from the liver. Genetic deficiency of ApoE results in substantially elevated levels of lipoprotein remnants and is associated with an increased risk of premature atherosclerosis. ApoE appears to play a vital role in regulating the blood levels of these fats. Its primary purpose is to promote clearance of triglyceride-rich lipoproteins from the circulation. Systemic delivery of APOE along with pDNA to elevate ApoE levels in genetic deficiency or expression of gene at the site by use of vectors are promising approaches.

For delivery of gene therapeutics in atherosclerosis, few instances have been reported where non-viral gene delivery have been used in vivo preclinically. These vectors

include nanoparticles and lipoplexes. This opens a possibility to develop and use liposomal vectors among which lipoplexes offer inherited low toxicity characteristics of biocompatible bilayer structure. Further, lipoplexes can be modified in order to provide advantages such as ability to target various organs by modifying liposome surface by attaching appropriate ligands, reduced immunogenic response, differential release characteristics and protection of the complexed gene. However, delivery of lipoplexes containing therapeutic gene to the cell faces various challenges and thus focus of the research carried was also on overcoming toxicity, issues of loss of nucleic acid in systemic circulation, targeting to specific cell for preventing non-specific distribution and enhancing transfection efficiency for cellular uptake along with endosomal escape.

One of the essential feature that confers interaction of liposomal delivery system with cells is the ionic character. For efficient interaction and internalization, the liposomes are prepared with cationic lipids. However, many of these cationic lipids have the disadvantage of being high cytotoxicity leading to cell death. Hence, these cationic lipids are modified to decrease their cytotoxicity and improving biocompatibility. Herein, cationic lipid has been incorporated in the liposomes at fixed level, so as to minimize cytotoxic potential and a neutral lipid (DSPE) was modified to impart the system with endosomal escape capacity. For targeting the system for hepatocyte specificity, galactose was used as ligand for conjugation to the liposomes. As the liver express asialoglycoprotein receptor, use of galactose as a ligand is well suited for targeting purpose.

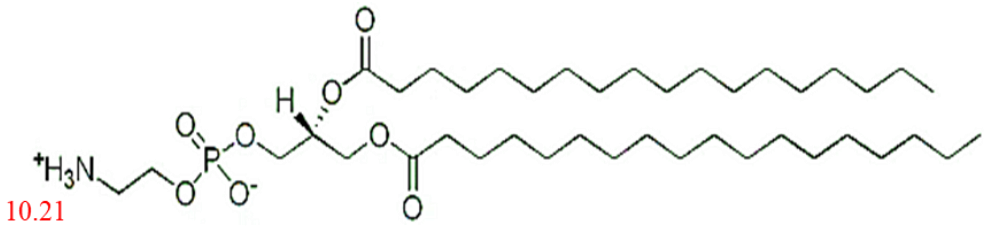
Analytical methods required in successful development of lipoplex formulations were developed and partially validated for suitability of analysis. Three methods were used for estimation of purity of the isolated plasmid and for quantification of pDNA namely, UV spectrophotometric method, Spectrofluorometric method and Gel electrophoresis. From UV spectroscopic analysis, the purity of the DNA isolated was confirmed to be high along with determination of linearity in concentration range of 2 – 200 ng/µL and further quantitation of the band was performed from 10 – 200 ng pDNA concentration relative to

200 ng band. Electrophoresis study of agarose gel showed that ≥ 10 ng concentrations of pDNA were detectable. Analytical methods used in the synthesis of lipids were TNBS assay (which was used to detect free unconjugated lipids) and Sakaguchi assay (which was used to determine quantitate guanidine groups) in order to determine the conjugation efficiency of the synthesis method employed for conjugation of lipid with Boc-amino acid derivatives. Accuracy and precision was evaluated for both the methods.

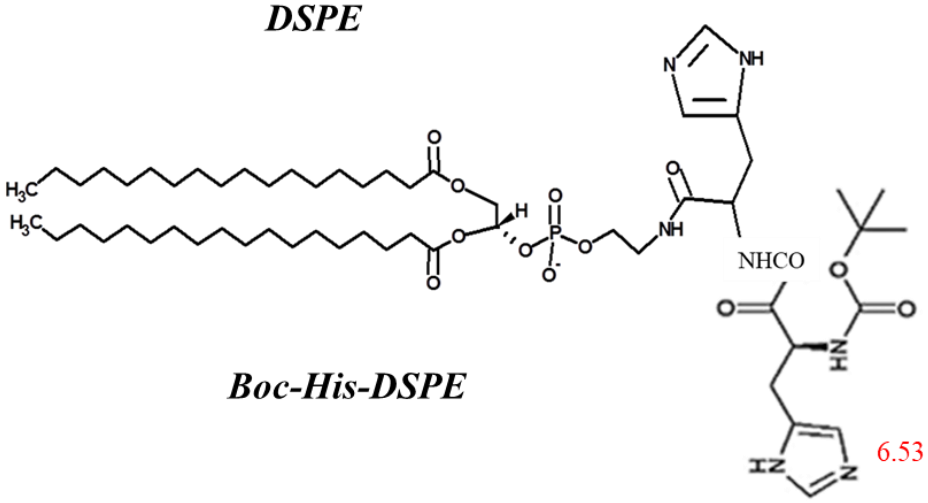
In context of development of gene delivery systems, green fluorescent proteins (GFP) have become essential tools in order to evaluate the intracellular delivery and expression of genes through easy fluorescence detection systems and the pDNA for GFP was used to develop lipoplex systems and was used for in vitro cell line studies in order to evaluate the transfection potential of lipoplex based gene delivery system. The plasmid DNA was isolated from the working culture of the E. Coli transformed strains using the alkaline lysis method. The digestion of the isolated plasmid was carried out by restriction endonuclease enzyme for confirming the pDNA with the transformed DNA. The isolated pDNA after linearizing with restriction digestion showed a single strong band on the agarose gel while undigested intact pDNA showed typical two band characteristics. The ApoE3 plasmid showed a linear band corresponding to molecular weight of 5954 bp and eGFP plasmid showed the linear band corresponding to its molecular weight of 5446 bp there by confirming the plasmid DNA.

For preparation of lipoplexes, syntheses of Boc-histidinylated DSPE (BHDSPE) and Histidinylated DSPE (HDSPE) were carried out by EDC/NHS coupling method. For synthesis of carnosine (dipeptide of alanine and histidine) modified lipids, free amino group of carnosine was first protected using Boc anhydride (dibutylpyrocarbonate). Subsequently Boc-carnosine and carnosine modified DSPE was synthesized. Further, Boc-Arginine DSPE (BADSPE) and Arginine DSPE (ADSPE) was also synthesized using the same chemistry (Figure 1). Efficiency of conjugation of the amino acid derivatives to the lipids was determined by carrying out the TNBS assay or Sakaguchi assay. Further,

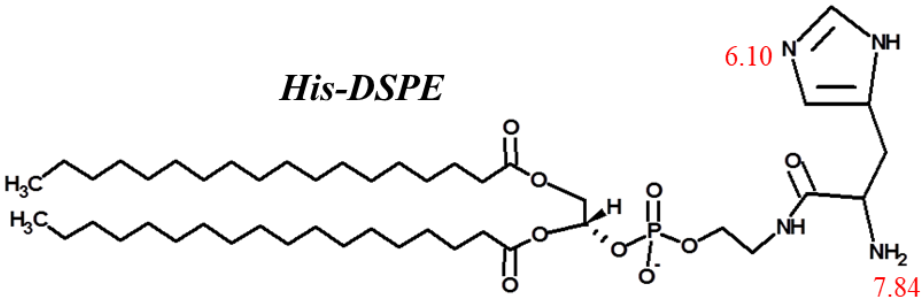
synthesized lipids were evaluated for their buffering capacity and for pKa determination and identification by Mass, FTIR, NMR and UV spectroscopy. pH titration study was performed on the lipids to evaluate the buffering activity of the lipids.



DSPE



Boc-His-DSPE



His-DSPE

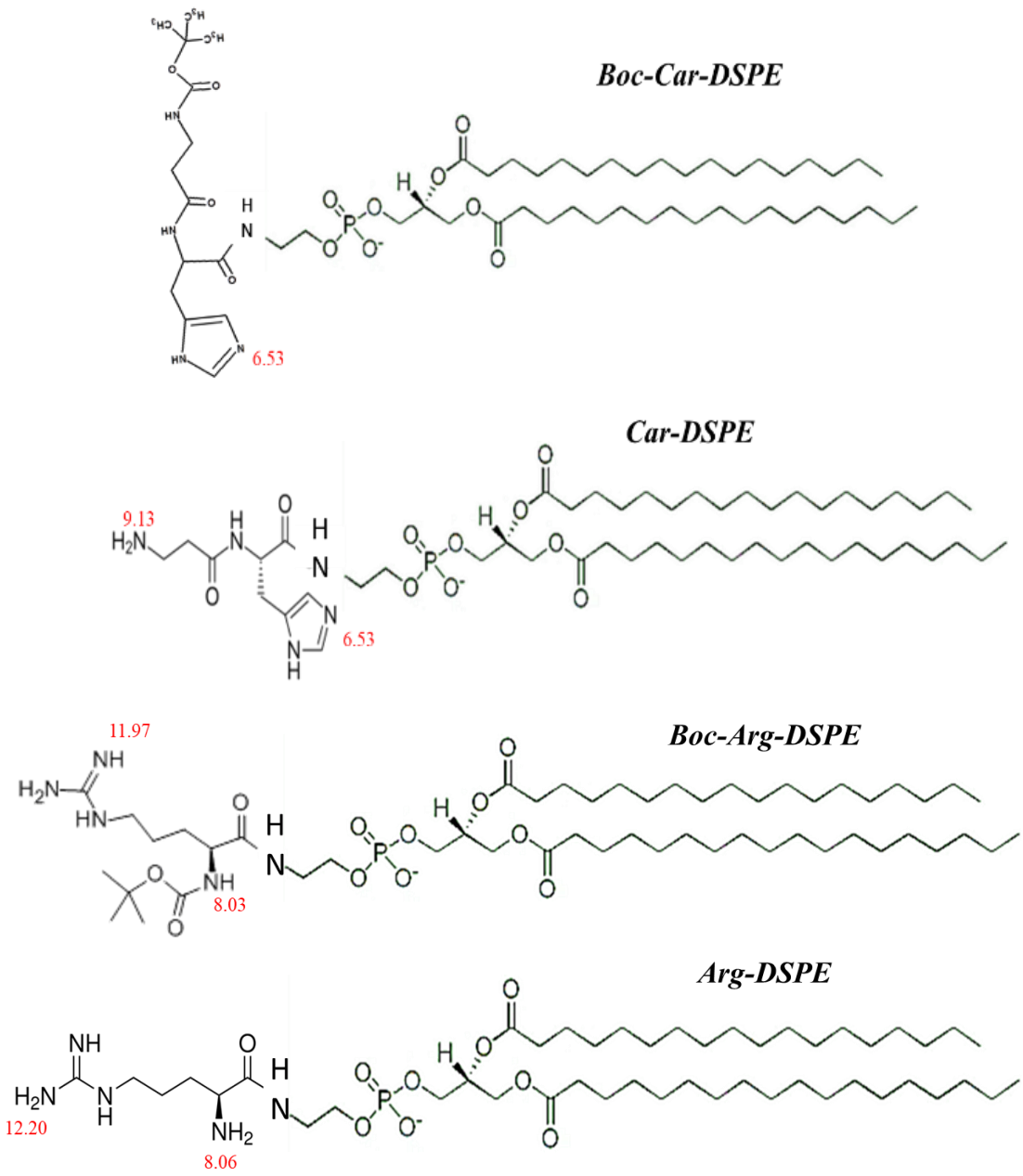


Figure 1: Synthesized lipids by modification of DSPE

Initially, liposomes were developed using lipids i.e. stearyl amine (cationic) and HSPC and optimization of the processing condition for formation of liposomes was carried

out for parameters like solvent evaporation time, vacuum applied, rotation speed of flask for film formation, hydration time and temperature etc. to optimize a level of cationic stearyl amine having low cytotoxicity. However, the formed liposomes were having higher cytotoxicity. Thus, for decreasing the toxicity, strategic modification of helper/other lipid was carried out. Stearyl amine-DSPE liposomes were developed using different other lipids i.e. HSPC, DSPC, DMPC, DPPC, Egg PC, DOPE, DSPE, and Cholesterol. Different molar ratios of lipids were tried to develop DSPE liposomes. All liposomes were developed using 3 mole% of mPEG2000-DSPE to develop PEGylated liposomes. Thin film hydration method was used for liposome preparation. The prepared liposomes were characterized for particle size and size distribution wherein the target was minimum particle size with lowest PDI. The prepared liposomes batches that exhibited monomodal distribution with low PDI and a positive zeta potential were selected. For further optimization of SA-DSPE liposomes, DOE was employed using D-optimal design by taking HSPC, EPC and cholesterol as variables and Particle size and PDI as response. The optimized batch parameters based on desirability plot was obtained as HSPC:EPC:Chol::35:28:14 and exhibited particle size of 106.7±5.2 nm and PDI of 0.206±0.011 with 0.892 desirability. The predicted response was confirmed experimentally. Optimized batch obtained through DoE was further used for preparation of the liposomes of modified DSPE wherein equimolar amounts of DSPE was replaced with BHDSPE/HDSPE; BCDSPE/CDSPE or BADSPE/ADSPE to prepare DSPE liposomes.

The pDNA was complexed with the liposomes to form lipoplexes and characterization was carried out for % entrapment of the complexed DNA by gel electrophoresis, UV spectroscopy and spectrofluorimetry. Further, each synthesized lipoplexes were characterized for particle size, zeta potential, morphology by Cryo-TEM, assay and residual water content. Optimization parameter for effective complexation involved selection of optimum incubation time (30 - 60 min) and incubation temperature (25°C, 37°C, 45°C) for non-PEGylated and PEGylated lipoplexes and was confirmed by

gel electrophoresis. Optimization of the formulation parameters was done by determining the complexation efficiency in terms of ratio of moles of modified lipid to moles of phosphate of pDNA (L/P ratio). Complexation efficiencies of liposomes prepared with different lipids synthesized from DSPE were determined at different L/P ratios. In case of DOTAP/DOPE lipoplexes, complete retardation required L/P ratio of 1.50 and higher. L/P ratio of 2 for BHDSPE/BCDSPE lipoplexes and L/P ratio of 1 for HDSPE/CDSPE lipoplexes was used. Also, complete complexation of pDNA at L/P ratio of 1 for BADSPE lipoplexes and L/P ratio of 0.5 for ADSPE lipoplexes was observed. All the prepared lipoplexes showed pDNA content ranging from 99.5 to 101.7%. Complexation efficiencies of lyophilized lipoplexes were determined by UV spectrophotometry and spectrofluorometry after ultracentrifugation of the samples. Complexation efficiencies of lipoplexes of DSPE based liposomes prepared at L/P ratio of 2.00 and showed no significant change in the complexation efficiencies of lipoplexes after lyophilization indicating that the cryoprotectant-bulking agent (sucrose), the freezing process and the drying cycles did not affect the electrostatic interaction between the liposomes and pDNA. L/P rations were chosen to attain the N/P ratio of 2.00 which helped in making comparisons of the physicochemical properties of the lipoplexes as well as in vitro and in vivo outcomes of the lipoplexes.

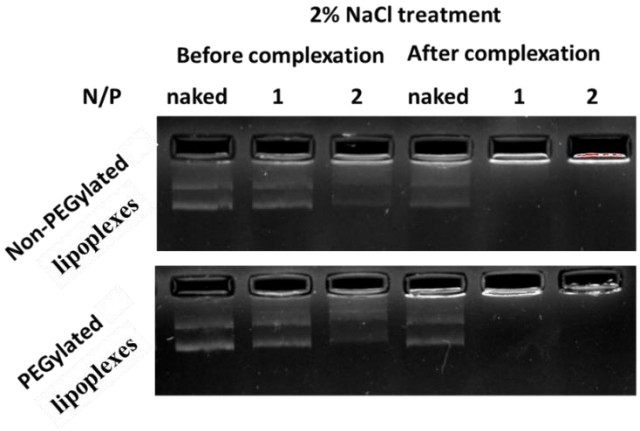


Figure 2: Effect of electrolyte (2% NaCl) on complexation on efficiency of non-PEGylated and PEGylated lipoplexes at N/P ratio of 1.0 and 2.0 (200 ng pDNA/well)

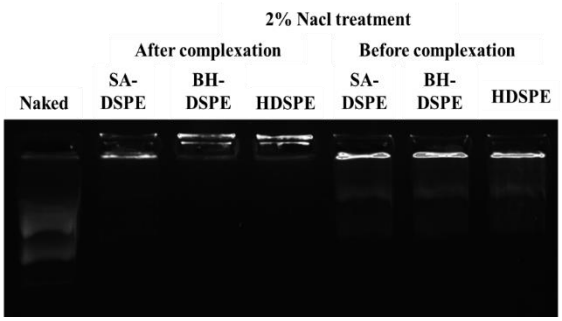


Figure 3: Effect of electrolyte (2% NaCl) on complexation on efficiency of SA-DSPE, BHDSPE and HDSPE lipoplexes at N/P ratio of 2.0 (200 ng pDNA/well)

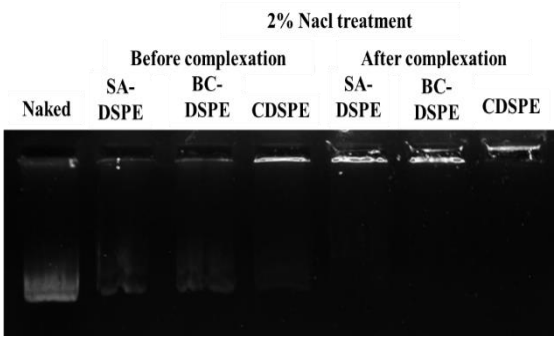


Figure 4: Effect of electrolyte (2% NaCl) on complexation on efficiency of SA-DSPE, BCDSPE and CDSPE lipoplexes at N/P ratio of 2.0 (200 ng pDNA/well)

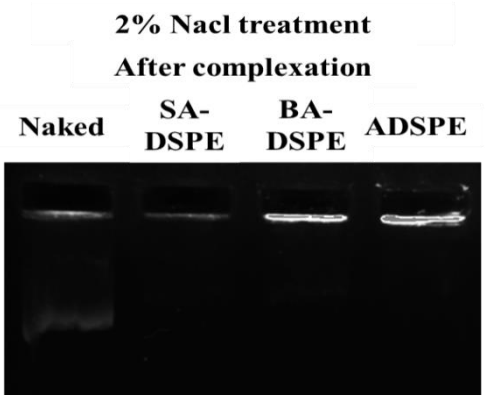


Figure 5: Effect of electrolyte (2% NaCl) on complexation on efficiency of SA-DSPE, BADSPE and ADSPE lipoplexes at N/P ratio of 2.0 (200 ng pDNA/well)

Formulation was evaluated in vitro for physichochemical characteristics in presence of simulated conditions i.e. presence of electrolytes and serum. The formulation was exposed to different percentage of electrolytes and it was demonstrated that the developed lipoplexes maintained the particle size well below 160 nm for the lipoplexes prepared with synthesized lipids. Additionally, it was observed that the effect of electrolytes on complexation was predominantly dependent on the presence of electrolyte

before complexation process or after complexation process and on the PEGylation characteristics of the lipoplexes. Results demonstrated that there was no effect of 2% sodium chloride on the complexation efficiency of lipoplexes when exposure was made after the lipoplexes are formed (Figure 2-5). However, presence of electrolytes before complexation with pDNA significantly affected the pDNA complexation efficiency of lipids.

On the next stage the lipoplexes were evaluated for the cell line studies in which cytotoxicity of the formulations were evaluated through hemolysis study by exposing the formulation with RBCs in vitro and through exposing the adherent hepatic HEPG2 cell line (Figure 6). Thorough cytotoxicity studies evaluated that the developed lipids were less toxic as compared to the used cationic lipids. Also, the toxicity of the developed lipoplexes were less than the lipoplexes prepared with the DOTAP and stearyl amine indicating the potential use of amino acid derivatives to conjugate on the DSPE lipid. Fluorescent microscopy and FACS analyses (Figure 7) confirmed qualitatively and quantitatively the cellular uptake and subsequent expressed on the complexed pDNA. It was observed that developed series of formulations significantly enhanced the cellular expression of the gene even more than the commercially available standard lipid formulation Lipofectamine 2000. This is attributed to the structural features of lipids and on the formulation compositions. All developed lipoplex formulations demonstrated better in vitro performance over Lipofectamine 2000 as well as common cationic agents such as DOTAP and stearyl amine (Figure 7). As a next step to cell line studies, formulations were developed using the same composition to target the hepatic cells using galactose as targeting ligand to effectively deliver the therapeutic gene in the site of action. The lipoplexes containing Histidine and Carnosine modified DSPE were selected for ligand conjugation and their targeting potential was evaluated using confocal (Figure 8) and FACS. Both the results suggested higher uptake of the formulation due to receptor mediated uptake.

Acute toxicity studies in mice demonstrated safety attribute of the selected lipid based lipoplex formulations which enabled high dosage of cationic lipids. *In vivo* studies in atherosclerotic mice model were performed to determine the *in vivo* performance. Plasma lipid profile were monitored for 3 months following treatment and aortic sections were evaluated for decrease in lesion area after treatment. Results indicate that selected formulations were able to reduce the elevated lipid levels along with a decrease in the lesion area for the treated group. However, targeted lipoplexes were more effective in the recovery of the lesion than the non-targeted lipoplexes as evidenced from the plaque area measured (Figure 9). The results conclude the effectiveness of the developed formulation for management of atherosclerosis through intravenous administration. It was demonstrated that the targeted formulations were more effective for in vivo therapeutic purpose.

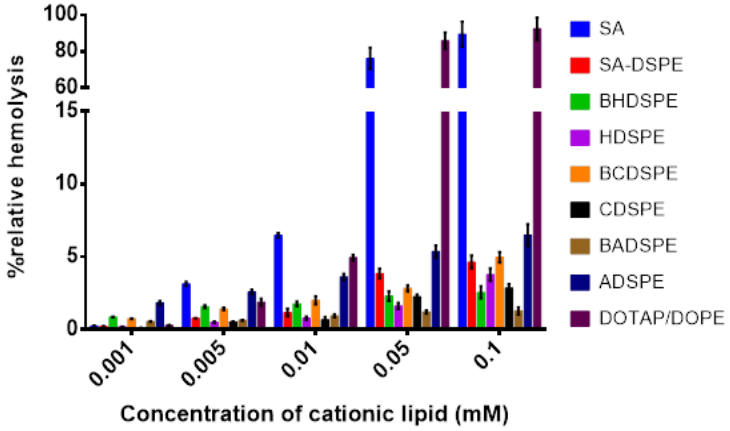


Figure 6: Haemolytic potential of lipoplexes

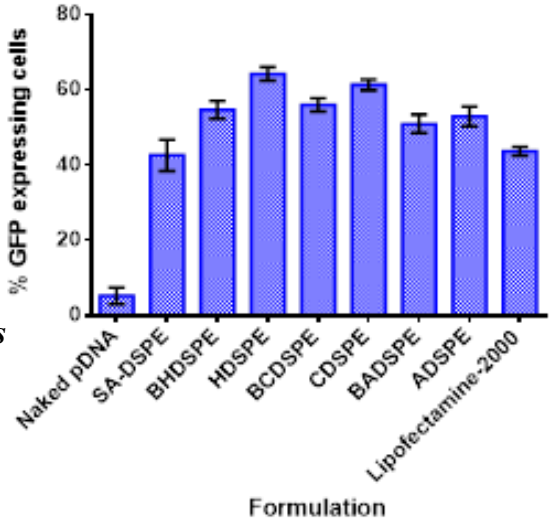


Figure 7: %GFP expression observed after transfection with naked pDNA, lipoplexes of SA-DSPE and Modified DSPE lipoplexes

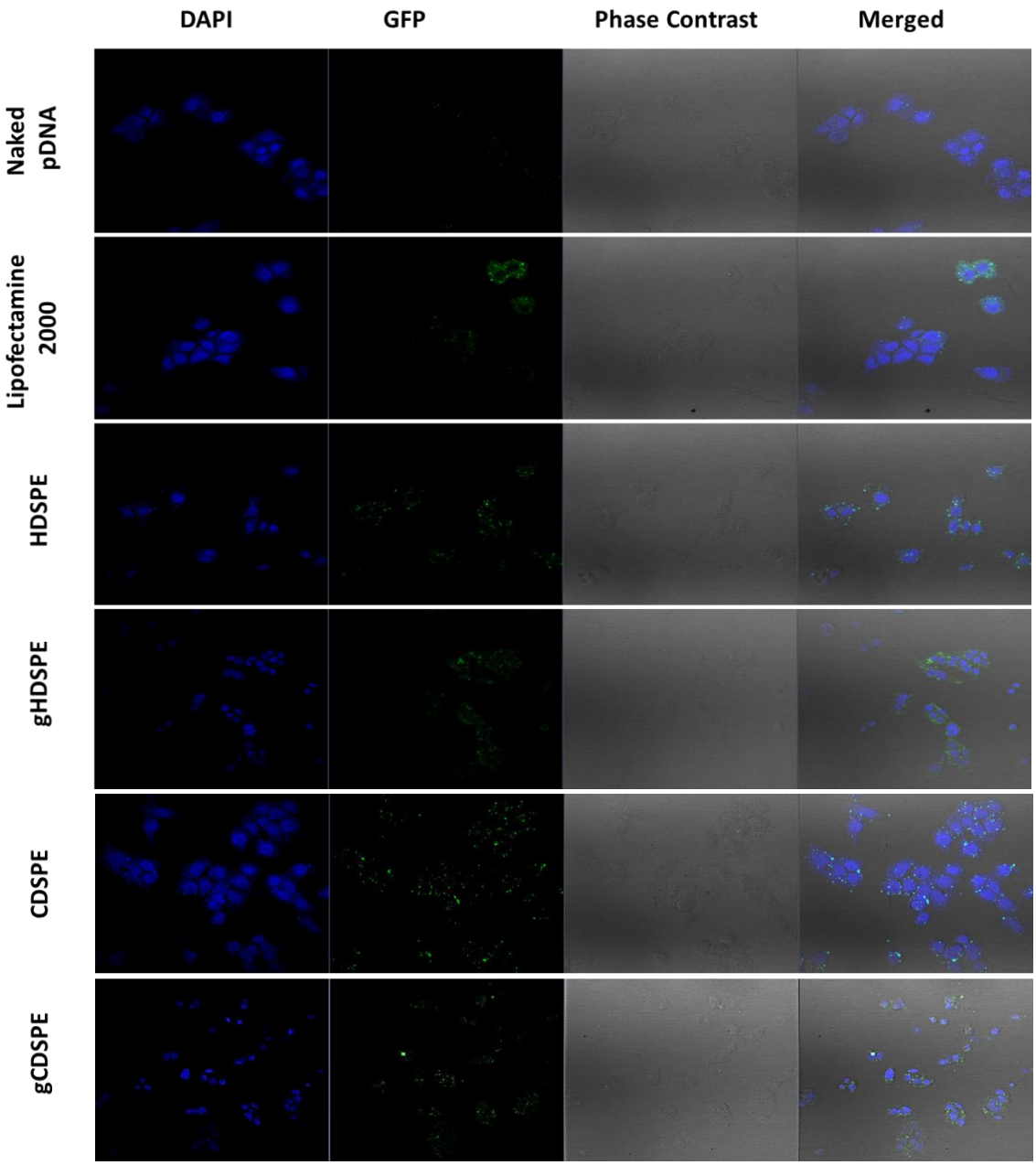


Figure 8: Confocal images of GFP expression after transfection with ligand conjugated vs non-conjugated lipoplexes.

Among developed targeted formulations, lipoplexes that were ligand conjugated and which represented each group of synthesized lipids (amino acid derivative modified DSPE) were evaluated for stability studies i.e. gHSDSPE lipoplexes. Due to similar compositions of the lipoplexes except for the modified lipid, the stability data can be extended to have an idea on the stability of the other lipoplexes as well. Formulations in lyophilized form exhibited stable characteristics over the tested period of 3 months.

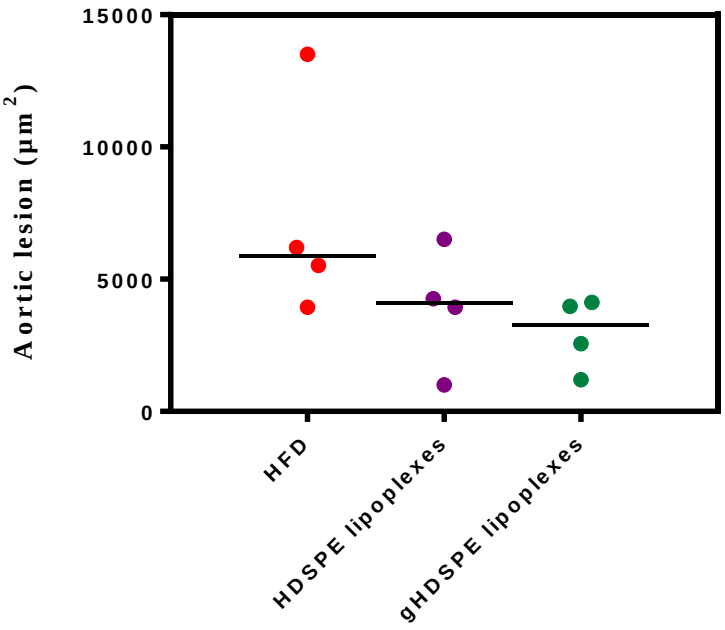


Figure 9: The areas of atherosclerotic lesions in untreated (HFD) and treated (HDSPE lipoplexes and gHDSPE lipoplexes) animals (n = 4 in each group) are shown in the graph (overlapping dots are not shown) at end of 2 months. The line indicates the mean value.

Conclusively, it can be stated that synthesized lipids can be used as gene delivery vectors for management of atherosclerosis due to their advantages of buffering effect, effective cellular expression, over current lipid systems. Thorough further animal studies need to be performed for evaluation of the synthesized lipids for the other toxicological

parameters, developed lipids can be established in the treatment of genetic diseases including atherosclerosis. Established performance and stability profiles also demonstrate that the lipids developed in these processes can also be used in gene delivery approaches for other diseases also.