Chapter 9

Development of Targeted Lipoplexes

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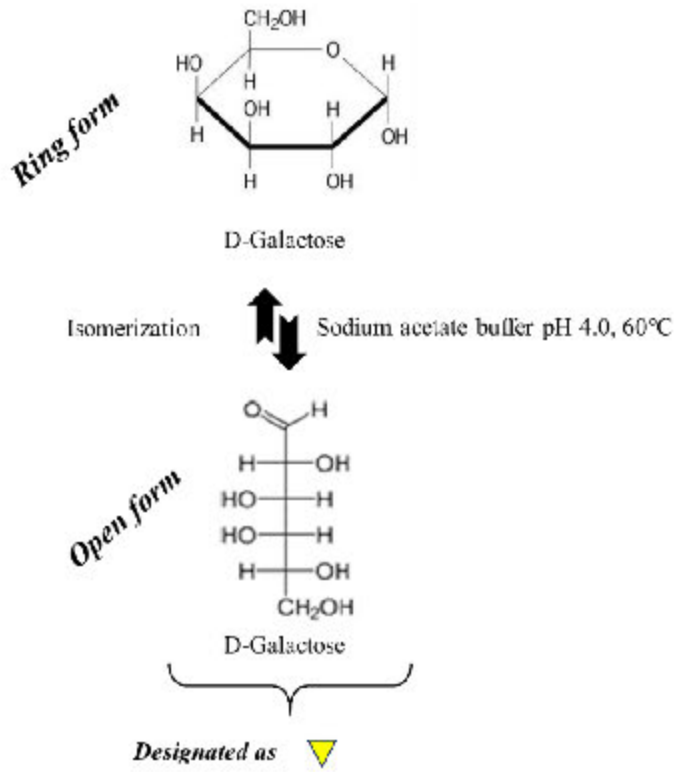
9.1 Development and evaluation of targeted lipoplexes

9.1.1 Preparation of ligand anchored cationic liposomes containing modified lipids

Ligand conjugated liposomes were prepared by post insertion method, by incubating suspension of lipoplexes with varying molar ratio (in comparison to stearyl amine present in liposomes) of activated galactose (G) solution in saline. This method facilitates the activation of ligand by ring opening at acidic pH (4.0) followed by the reaction of aldehyde group of activated ligands with the free amino group present at the outward of prepared liposomes. Galactose was first dissolved in 0.1 M sodium acetate buffer (pH 4.0) and then mixed with lipoplexed dispersion. The mixture was allowed to continuously agitate on a magnetic stirrer maintained at an 30°C temperature for 4 hr to ensure completion of the reaction. Ligand-anchored lipoplexes were subjected to dialysis against double distilled water in a dialysis tube (MWCO 12KDa) for 1 hr to remove free ligand and other impurities followed by lyophilization. Targeted lipoplexes were characterized for various parameters.

9.1.2 Preparation of ligand anchored cationic liposomes



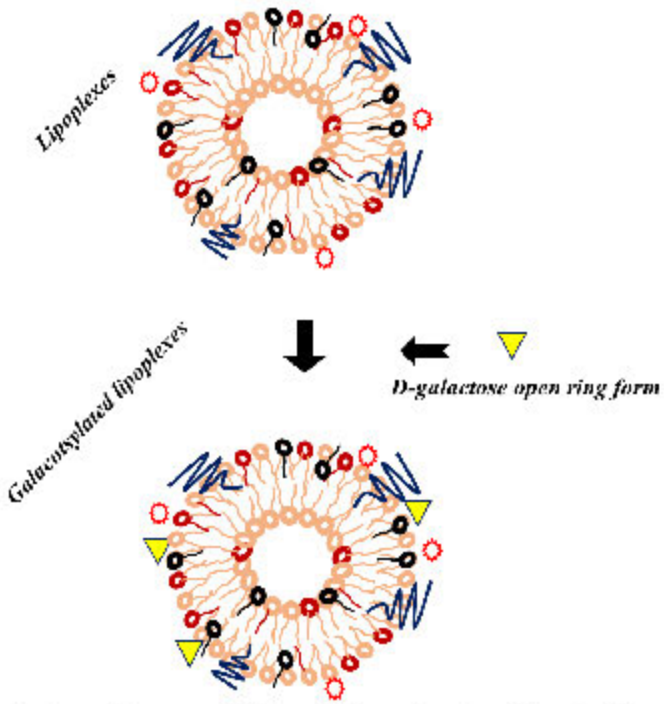


Figure: Schematic presentation of ligand conjugation to lipoplexes

Lipoplexes containing modified DSPE lipids along with stearyl amine were surface conjugated with sugar moieties for targeting it to liver cells. Estimation of percentage conjugation was based on the amount of free amines remaining before and after the reaction (TNBS reaction).

Table 9.1 % molar conjugation in the reaction mixture after dialysis post reaction period

Liposomes	Molar ratio of lipid to sugar	% molar conjugation	Size (nm)	Zeta
Galactosylated				
gBHDSPE	1:4	95.5±4.2	129.2±2.8	1.2±3.1
	1:2	92.8±5.9	130.5±5.6	1.5±2.2
	1:1	82.1±3.3	126.1±4.2	2.3±2.1
	1:0.5	68.6±4.6	121.2±4.6	12.6±2.6
	1:0.25	66±5.0	124.9±4.5	22.1±1.9
gBCDSPE	1:0.25	62.1±3.2	135.2±2.9	21.1±3.8
gHDSPE	1:0.25	66.3±2.6	120.4±3.9	18.1±2.5
gDSPE	1:0.25	65.5±3.0	119.6±3.2	16.6±1.8

For the development of the liver targeted gene delivery system, based on the above review, galactose has been chosen for targeting. Based on the cytotoxicity studies and the

cellular uptake studies of different lipoplexes developed, HDSPE and CDSPE were chosen for targeting.

Complexation efficiency of the lipoplexes were determined by gel electrophoresis to see if the conjugation and/or incubation with the ligand leads to detrimental effect on the lipoplex stability. It was observed that the complexation efficiency of lipoplexes was not affected by the conjugation with the ligand (**Figure 9.1**and **Table 9.2**).

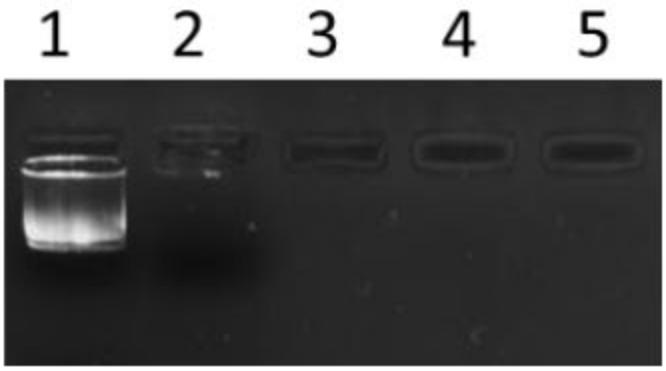


Figure 9.1 Complexation efficiency of lipoplexes before and after ligand conjugation (200 ng pDNA/well, N/P ratio of 2: lane 2-HDSPE lipoplexes before conjugation, lane 3-HDSPE lipoplexes after conjugation, lane 4-CDSPE lipoplexes before conjugation, lane 5-CDSPE lipoplexes after conjugation)

Table 9.2 Physicochemical characteristics of the lipoplexes

Physicochemical property	**HDSPE lipoplexes**		**CDSPE lipoplexes**	
Before/after conjugation	Before	After	Before	After
%Complexation efficiency (Gel electrophoresis)	98.64±1.58	99.55±2.15	99.08±1.81	100.65±2.15
%Complexation efficiency (UV spectrophotometry)	98.52±2.21	102.15±1.59	100.51±0.93	99.48±2.53
%Complexation efficiency (QuantiFluor® Assay)	98.75±2.10	101.45±2.15	99.45±1.36	100.36±2.96
Particle size (nm)	132.6±6.5	138.4±5.9	142.4±9.1	150.1±4.4
Zeta potential (mV)	20.5±3.1	18.2±0.9	19.9±3.5	16.3±1.4

The physicochemical properties of the lipoplexes were evaluated for the ligand conjugated lipoplexes. It was observed that the particle size of the lipoplexes did not change after conjugation; however, zeta potential of the lipoplexes was changed

significantly for the lipoplexes. This is due to the consumption of free amine group of stearyl amine that was conferring higher positive charge to the lipoplexes. The amine group reacts with the hydroxyl group forming carboxyl bond. However, due to higher concentration of cationic charge, the lipoplexes still were showing positive zeta potential (~15 mV). From the various molar conjugation ratio tested, the lowest was thus selected (i.e. 1:0.25) wherein the zeta potential is highest among the screened ratios, thus minimizing the decrease in transfection efficiency that may occur due to neutralization of surface potential. Further, it was projected that ligand conjugation will favor specific uptake by the cells due to receptor mediated interaction and thus a slight sacrifice in zeta potential would not adversely affect the performance of the delivery system. Though zeta potential of the lipoplexes was changed significantly, the complexation efficiency of the lipoplexes was not affected by the complexation. This is due to very low concentration of the ligand selected for reaction. Additionally, conjugation of the ligand would not hinder the already complexed pDNA's interaction with the cationic charges; rather this might help prevent loss of the pDNA due to stresses by trapping the pDNA between the attractive forces from the cationic lipids and repelling forces of the negatively charged ligand.

Cytotoxicity studies of the targeted lipoplexes were carried out to compare their cytotoxicity against the non-targeted lipoplexes prepared without targeting ligand. It was observed that there was no significant difference in the cytotoxicity of the lipoplexes before and after conjugation to the targeting ligand (**Figure 9.2**).

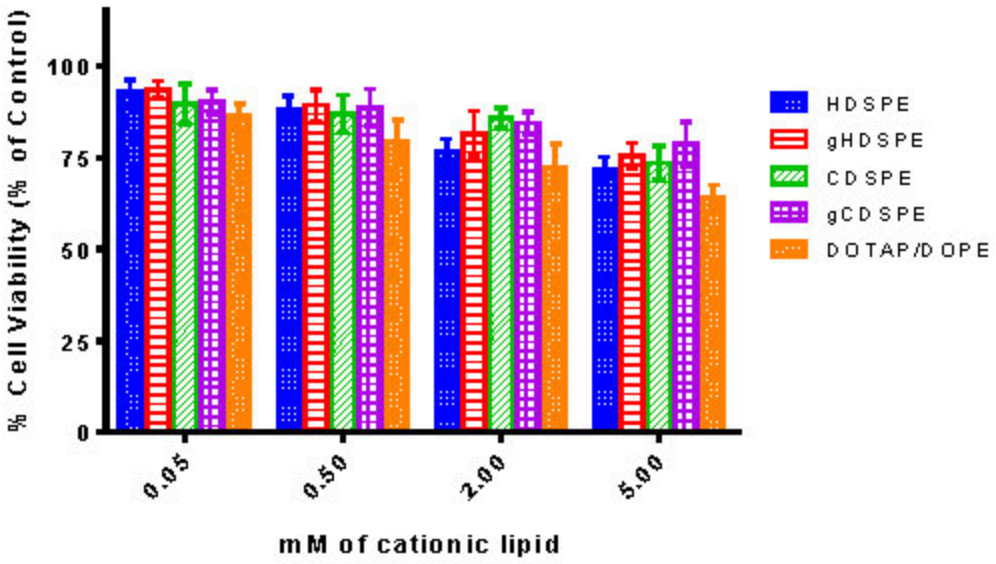


Figure 9.2 Effect of ligand conjugation on the cytotoxicity of the lipoplexes. Cytotoxicity of targeted and non-targeted lipoplexes are shown against DOTAP/DOPE lipoplexes.

Quantitative and qualitative cellular gene expression studies were carried out using flow cytometry and confocal microscopy respectively in order to see the effect of the ligand conjugation on the cellular internalization of lipoplexes and gene expression. It was observed that the cellular expression of the lipoplexes improved post ligand conjugation (**Table 9.3** and **Figure 9.3**). There was increase in transfection efficiency as well as uptake for the targeted lipoplexes as compared to the non-targeted ones as evident from the FACS data and confocal microscopy images. To confirm whether receptor mediated uptake is the reason for this improved efficacy, the lipoplexed solution were mixed with galactose solution (20 mM) and then the cellular uptake protocols were carried out (1) (2). It was observed that, there was a drastic decrease in uptake in cases where lipoplexes were treated with galactose solution indicating the receptor will have been occupied by free galactose which is the site for interaction of targeted lipoplexes with the cells and thus adversely affected the transfection potential of the lipoplexes (**Figure 9.4**). As the transfection potential of the histidine formulation was highest, it was further selected for pre-clinical testing. It can be anticipated that ligand conjugation would similarly show superior results in in-vivo performance compared to non-targeted once and hence only the targeted ones were screened in in-vivo studies.

Table 9.3 GFP expression after transfection with ligand conjugated and non-conjugated lipoplexes against naked pDNA and Lipofectamine-2000 lipoplexes*

Formulation	% GFP expressing cells
Naked pDNA	7.26±2.18
HDSPE	64.42±2.89
HDSPE + 20mM galactose	62.05±3.9
gHDSPE	76.98±2.45
gHDSPE + 20mM galactose	19.17±3.1
CDSPE	61.33±3.41
gCDSPE	68.94±2.74
Lipofectamine-2000	43.65±2.16

*Experiments were performed in triplicate.

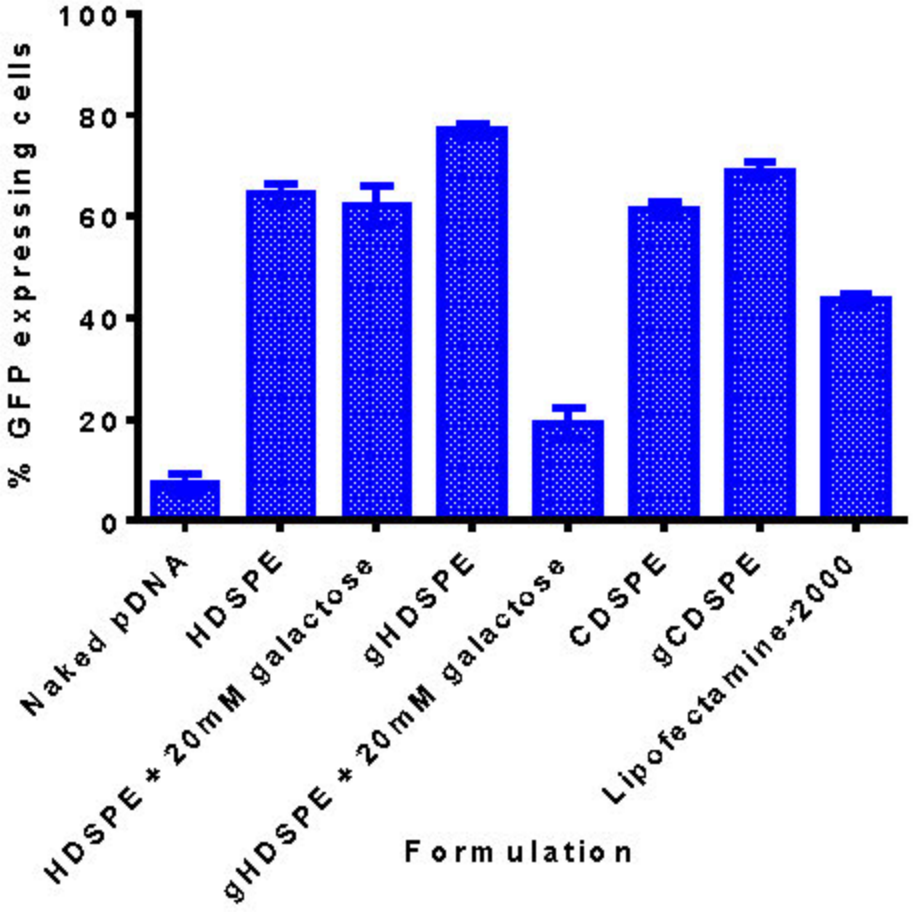


Figure 9.3 %GFP expression observed after transfection with ligand conjugated and non-conjugated lipoplexes. Untreated cells were taken as negative control while cells treated with naked pDNA and lipoplexes of lipofectamine-2000 were taken as a reference control for comparison.

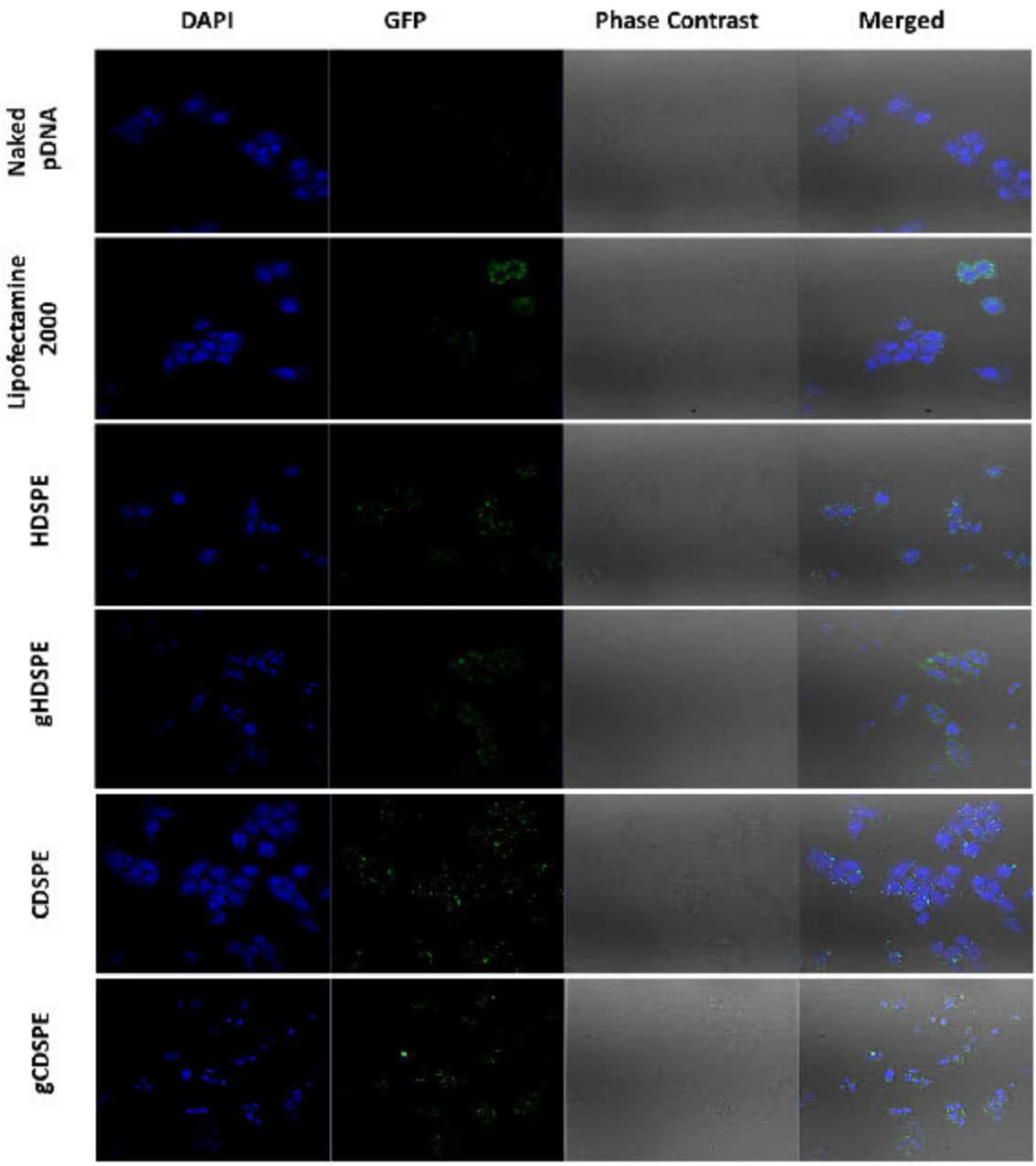


Figure 9.4 Confocal images of GFP expression after transfection with ligand conjugated and non-conjugated lipoplexes.

Results show that some characteristics of the lipoplexes were changed for the targeted lipoplexes after ligand conjugation; however, the changes were not detrimental to the performance of the lipoplexes. As expected, ligand conjugation enhanced the cellular uptake of the lipoplexes. The results indicated that the therapeutic potential of the lipoplexes. Thus, the targeted lipoplexes were successfully developed which showed promising results for further *in vivo* evaluation.

9.2 References

1. Sonoke S, Ueda T, Fujiwara K, Kuwabara K, Yano J. Galactose-modified cationic liposomes as a liver-targeting delivery system for small interfering RNA. Biological and Pharmaceutical Bulletin. 2011;34(8):1338-42.
2. D'souza AA, Devarajan PV. Asialoglycoprotein receptor mediated hepatocyte targeting—strategies and applications. Journal of Controlled Release. 2015;203:126-39.