Chapter 5: Ligand Attached *p*53 Lipoplexes for treatment of NSCLC

5.1 Introduction

Cancer, an acquired genetic disorder is caused because of genetic abnormality acquired in few cells leading to continuous cell growth along with activation of certain oncogenes and suppression or mutation of cell controller genes like tumor suppressor *p*53 gene [M. Saad et al (2008)]. NSCLC has been a prominent type of cancer leading to high mortality rate with poor diagnosis and treatment. The existing chemotherapy along with radiation and surgery has shown insufficient disease management and partial symptomatic cure [J. Aisner et al 1992] with significant side-effects. Use of ligand targeted liposomal systems and direct lung delivery by aerosol systems (as DPI, nebulizers, and pMDI) have enhanced the effect of chemotherapy to an extent, but still the actual genetic malfunctions causing cancer needs to be cured for complete treatment of cancer.

Gene therapy is a novel and promising approach for treatment acquired genetic disorders as lung cancer [I.M Verma & N. Somia (1997)]. The cancer predominantly caused because of chromosomal alterations and malfunction of cell growth controller genes as *p*53 has shown reduction in cell burden after restoration of *p*53 by viral or non-viral means. *p*53 has been most promising molecule for maintaince of normal cell function as it also plays a significant role in diverse cellular pathways activated in response to DNA damage, such as DNA repair, regulation of the cell cycle and programmed cell death (apoptosis) [R. Li et al (2003)] which when malfunctioned results in tumorogenesis. Statistically, mutation in *p*53 has been associated in 15-50 % of breast cancer, 25-75 % of lung cancer, 25-70 % prostate and bladder cancer, 33-100 % of head and neck cancer and various lymphomas and leukemias [K.F. Pirollo, et al (2000) & K. F. Pirollo, et al (1997)]. These abnormalities in *p*53 gene in a significant fraction of human cancers and its regulatory role for normal cell functioning make it one of the premiere candidates for cancer gene therapy.

Delivery of therapeutic *p*53 gene to the lung for the treatment of lung cancer has been widely reported. The various approaches as naked plasmid, viral delivery, cationic liposomal DNA delivery and polymeric complexation with DNA have all been well established [DY Logunov et al (2004), & S.H. Choi et al (2008)]. In the present work, we have tried to establish a stable DPI formulation of a ligand attached lipoplex formulation for treatment of lung cancer. Various methods as **Thin film hydration (TFH)), Ethanol injection (EI) and Supercritical Fluid Extraction (SCF)** were used to prepare blank liposomes, which when treated with *p*DNA

formed lipoplexes. The lipoplexes were developed with objective of maximum transfection and stability after lyophillization.

5.2 Development of Cationic Liposomes

5.2.1 Materials

1,2-dioleoyl-3-trimethylammonium– propane (DOTAP), 1,2-Dioleoyl-sn-Glycero-3-Phosphoethanolamine (DOPE) and 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) were obtained as gift sample from Genzyme Pharmaceuticals, Switzerland and Lipoid GmbH, Germany. Cholesterol was purchased from Sigma Aldrich, Bangalore, 5 ([4-(2-hydroxyethyl)-1-piperazine ethane sulphonic acid] (HEPES free acid) was purchased from Himedia. Nucleopore track etch polycarbonate membrane filter papers (0.45 and 0.22 μm) were purchased from Whatman Filter Paper and all other chemicals and solvents used were of analytical grade, and were confirmed for purity before use.

5.2.2 Methods

5.2.2.1 Thin Film Hydration

The TFH method was used to prepare cationic liposomes using DOTAP and DOPE in different molar ratios using the method described earlier [A.D. Bangham et al (1965)] Briefly, different molar ratios of DOTAP and DOPE (Total lipids of 50 micromoles) were weighed, transferred to 100 ml round bottom flask and dissolved in 10 ml of dry chloroform : methanol (2:1 v/v) mixture. The lipids were allowed to dissolve by vortexing. The organic solution was evaporated under vacuum using rota-evaporator at a temperature of 35 ± 5^0 C on a thermostatic bath at a rotation speed of 100 per minute of the rotor, theirby leaving behind the thin film of the lipid on the walls of the flask. The flask was kept in vacuum desiccator to remove any residual solvent in the flask. The thin film was hydrated with 1 or 2 ml of sterile water or sterile HEPES buffer, pH 8.0 at 35 ± 5^0 C with intermittent vortexing. The hydrated liposomes were formed after 90 – 120 min. of hydration and were confirmed for complete hydration to form multilamellar liposomes (MLV) under Olympus microscope (BX F-40, Tokyo, Japan). The liposomes were further size reduced by extrusion through 0.45 and 0.22 μm Nucleopore track etch polycarbonate membrane filter papers. The MLVs and size reduced liposomes were characterized for size and zeta potential and were used further for lipoplex formation.

5.2.2.2 Ethanol Injection

EI was used as an alternative method of cationic liposome formation. The method comprised of dissolving the weighed quantity of DOTAP and DOPE (Total lipids 100 micromole) in varying molar ratios in a small quantity of ethanol (1-2 ml) by gentle shaking. Ethanol was then injected at a fast speed using injection in warm HEPES buffer pH 8.0 at 40 $\pm$ 5^0 C under controlled stirring and was allowed to mix for 30 minutes. The milky white liposomal suspension was then dialyzed using a dialysis membrane tube with molecular weight cut of 12 kda against the aqueous hydration media for complete removal of ethanol by changing the external media every 2 hours. The formed liposomal suspension is then characterized for size, zeta potential and is further used for lipoplex formation.

5.2.2.3 Supercritical Fluid Extraction

The SCF extraction technology is a novel method to produce dry liposomes in a particulate form ready to get hydrated once they come in contact with water [S. Naik et al (2010)]. The technique helps to prepare ready to use sterile liposomes for preparing lipoplexes for transfection and thus increases the stability of the liposomes, a major obstacle in liposomal drug delivery. The technique employs dissolving the weighed quantity of lipids (DOTAP, DOPE, DPPC, Cholesterol) in Chloroform-Methanol (Varying ratios) mixture and pumping the solution through a peristaltic pump into a pressured chamber along with supercritical carbon dioxide. The supercritical carbon dioxide extracts the solvents preferentially to precipitate the lipid particles as discrete nanosized particles which are collected aseptically. The particle gets hydrated when they come in contact with sterile water or HEPES buffer to form nanosized multilamellar liposomes which are ready to complex with DNA because of the charge based electrostatic interactions. The formulations were optimized for particle size and % yield of the lipid particles by varying lipid concentration in organic solvent, CO_2 pressure and temperature in atomization pressure chamber, CO_2 flow rate, lipid solution flow rate and organic solvent composition. The optimized liposomes were characterized for particle size, zeta potential and were further used for lipoplex formation.

5.2.3 Characterization of Liposomes

5.2.3.1 Particle Size

The particle size (Z average and poly-dispersity index (PDI) of the liposomes was analyzed by photon correlation spectroscopy (PCS) using a Malvern Zetasizer Nano (Malvern Instruments;

UK). 0.2 ml of liposomal suspension was diluted to 1.0 ml with sterile distilled water and measured after an equilibration time of 2 minutes. The Zetasizer Nano operates with a 4 mW He-Ne-Laser at 633 nm and noninvasive back scatter technique at a constant temperature of 25 ^{0}C. The measurements were conducted in the manual mode using 20 sub runs of 10 seconds. The size distribution by intensity and volume was calculated from the correlation function using the multiple narrow mode of the Dispersion Technology Software; version 4.00 (Malvern, Herrenberg, Germany). Thereby, the resulting size distributions show the hydrodynamic diameter. The average particle size and PDI was calculated after performing the experiment in triplicate. PDI of 0.0 represents a homogenous particle population while 1.0 indicates a heterogeneous size distribution in the liposome.

5.2.3.2 Zeta potential analysis

The zeta potential of the various liposome suspension prepared was measured by micro electrophoresis using Malvern Zetasizer Nano ZS (Malvern Instruments, UK). Zeta potential of the undiluted liposome was measured to achieve highest sensitivity, accuracy and resolution of zeta potential. The instrument works on the principal of Brownian motion and measured the light by Phase Analysis Light Scattering (PALS). The determination of the zeta potential was measured at 25^{0}C after injecting 0.8 ml of sample into standard sample cell.

5.2.3.3 CryoTEM studies

Morphology, lamellarity and size of the liposomes was studied using Cryo-TEM (TECNAI G2 Spirit BioTWIN, FEI – Netherlands) operating at 200 kV with resolution of 0.27 nm and magnifications of the order of 750,000X. In order to perform Cryo-TEM observations, hydrophobic carbon grid was initially converted to hydrophilic nature by using Glow Discharge (Emitech K 100 X, Quoram Technologies, UK), on which 10 µl of liposomal suspension was evenly dispersed and the sample along with grid was cryo-freezed in Liquid Ethane at – 180° C. Cryo-freezed grid was transferred to cryo-holder maintained at -175 ° C using Liquid Nitrogen storage box. The cryo-holder was then inserted in the microscope for imaging the sample. Combination of bright field imaging at increasing magnification and of diffraction modes was used to reveal the form, lamellarity and globule size of the liposomes.

5.2.4 Results and Discussion

5.2.4.1 Thin Film Hydration and Ethanol Injection Method

The conventional methods as TFH and EI produce cationic liposomes after hydration in the aqueous phase. Both the lipids DOTAP and DOPE have Tg near 0^0C and are in semisolid stage at room temperature. Hence the hydration at room temperature is sufficient to achieve completely hydrated liposome formation. Further, the liposomes formed in HEPES media, pH 8.0 were better hydrated without precipitated lipids than their counterparts hydrated in aqueous water media. The improved hydration in HEPES media may have to do with incomplete hydration of DOPE in water, which generally has a pH of 5-6. DOPE has been reported to be completely hydrated in alkaline pH, hence HEPES buffer was used as hydration media [N. J. Zuidam et al (1997)]

The thin film hydration method formed multilamellar liposomes in the size range of 1-5 microns **(Figure 5.1)**. These liposomes on extrusion through 0.45 and 0.22 micron PC membrane filter were extruded to give large unilamellar vesicles (LUV) in the size range of 200-300 nm. The size was measured by Malvern Zetasizer Nano ZS and the morphology, lamellarity and size of LUVs were confirmed by cryoTEM studies. The cryoTEM studies of the liposomes as shown in **Figure 5.1**; showed LUVs with size near to 150 - 200 nm confirming the size distribution data. The LUVs showed the zeta potential of + 30 to + 60 mv because of presence of DOTAP as cationic agent.

EI method used dilution of dissolved lipids in hydration media to precipitate lipids in nanosized range as a principle to formulate the cationic liposomes. The liposomes were formed in the nanosized range and required no extrusion cycles for sizing. The size of the liposomes varied with change in ethanol and aqueous medium (HEPES buffer) volume, stirring speed and needle used for ethanol injection. The optimization of the process and formulation parameters is depicted in the table 5.1 as below:

The liposomes showed lower size and milky dispersion when prepared in HEPES buffer as hydration media. As compared to water, HEPES buffer was used as hydration media, because of its tendency to hydrate DOPE better than water. Further, use of finer needle helped to achieve better particle size (reduced) with lower PDI. Further, when quantity of ethanol was increased to dissolve the lipids, particle size of the liposomes reduced. Also, when quantity of aqueous media was increased, particle size of liposomes was reduced to a certain extent. Overall, lowest particle

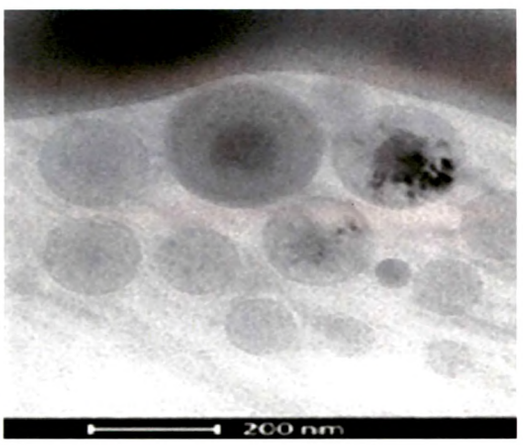

Figure 5 (a)

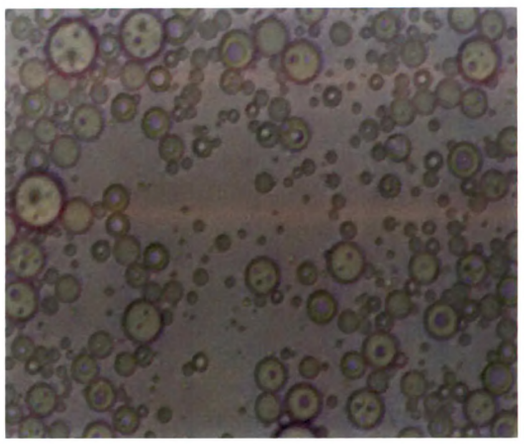

Figure 5 (b)

Figure 5.1: Morphology of the nanosized extruded liposomes as studied by CryoTEM (Fig 5(a)) and of the hydrated multi-lamellar liposomes prepared by Thin Film Hydration Technique by phase contrast microscopy (Fig 5(b))

size was obtained when, 100 micromoles of lipids was dissolved in 2 ml of ethanol and was injected in 10 ml of HEPES buffer using 26 gauge needle. The liposomes showed particle size of 113.9 $\pm$ 12.1 nm. Further, all the liposomes showed zeta potential in the range of 25-51 mv because of presence of cationic DOTAP lipid. When ratio of DOTAP : DOPE was changed, keeping the process parameters constant, there was non-significant effect on particle size of liposomes, however, the liposomes showed altered zeta potential according to DOTAP content.

Table 5.1: Optimization of process and formulation parameters for optimized size of liposomes by Ethanol injection technique

Qty of lipids (micromoles)	Qty of Ethanol (ml)	Qty of HEPES buffer pH 8.0 (ml)	Stirring Speed (RPM)	Needle Used (gauge)	Size of the liposomes (nm)
100	1	5	300	24	899.3 $\pm$ 31.8
100	1	5	300	26	581.3 $\pm$ 29.9
100	2	5	300	26	329.1 $\pm$ 16.5
100	2	10	300	26	172.0 $\pm$ 22.1
100	2	10	500	26	113.9 $\pm$ 12.1
100	2	15	500	26	115.9 $\pm$ 14.1
100	2	10	1000	26	173.2 $\pm$ 21.9

The liposomes formed by EI method were unilamellar in nature. The morphology and lamellarity of the liposomes was confirmed by CryoTEM images as shown in **figure 5.2**.

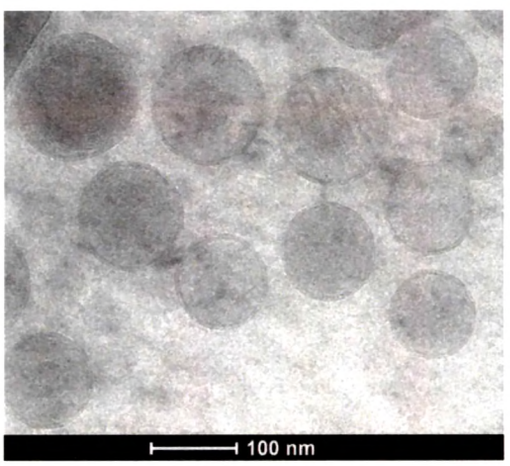

Figure 5.2: CryoTEM image of liposomes formed by Ethanol Injection method.

The formulated liposomes were dialyzed against 1 Ltr of HEPES buffer pH 8.0 to remove the ethanol present in the formulation for stabilizing the liposomes. The extra-compartmental buffer was changed every 2 hrs (3 times) to ensure complete removal of ethanol. The optimization of these formed liposomes was performed on the basis of transfection studies performed with varying liposomal compositions with the optimized process parameters and hydration medium and volume.

5.2.4.2 Supercritical Fluid Technology:

The liposomes prepared by TFH and EI methods were nanosized in range, however, these liposomes were unstable and aggregated in 48-72 hrs at 2-8^{0}C. These liposomes were required to be freeze dried to keep them stable. In comparison, the SCF technology was used to develop liposomes in nano and micron size which are stable in nature because of their dry nature and were ready to form liposomes when they come in contact with aqueous media.

In general, the liposomes were formed by precipitation of lipids from organic solvent in the extraction chamber. The solvents were driven away by the supercritical CO_2 because of their preferential miscibility over lipids at supercritical condition. Further, various process and formulation parameters as CO_2 automated back pressure, CO_2 flow, temperature of extraction chamber, flow of solvent containing dissolved lipids, % lipid solid content after dissolving in

solvents, solvent composition, were studied for formation of liposomes with minimum particle size and maximum yield. The summary of effect of these parameters has been depicted in the table 5.2 as below:

Table 5.2 Effect of process and formulation parameters in liposome development by SCF technique:

Lipid Composition (DOTAP:DOPE:DPPC:CHOL) (mg)	Solvent Composition (Chloroform:Methanol)	Lipid Concentration (mg lipid / ml of solvent)	CO_2 Automated Back Pressure (ABP)	CO_2 Flow Rate (gm/min)	Solvent flow rate (ml/min)	Particle Size	% Yield
1:1:0:0	1:0	1000/10	80	30	1	Sticky mass observed	
2:1:0:0	1:0	1000/10	80	30	1	Sticky mass observed	
1:1:10:5	1:0	1000/10	80	30	1	Sticky mass observed	
0.5:0.5:14:5	1:0	1000/10	80	30	1	1231.6	11.9
0.5:0.5:14:5	1:0	1000/5	80	30	1	1587.2	34.2
0.5:0.5:14:5	2:1	1000/5	120	40	0.5	677	41.2
0.5:0.5:14:5	1:1	1000/5	120	40	0.5	Solvent was not completely evaporated	
0.5:0.5:14:5	2:1	1000/5	120	50	0.25	321.9	35.2
0.5:0.5:14:5	2:1	1000/5	150	50	0.25	341.2	5.0

From the table, it was clear that, alone DOTAP and DOPE were unable to form the lipid particles in the SCF process because of their lower Tg than the atmospheric temperature and formed sticky mass in the process chamber. Hence, DPPC and cholesterol were added in the lipid composition. DPPC because of its lung compatability and transfection suitability and cholesterol because of its membrane fusogenecity theirby helping the pDNA transfection, were preferentially incorporated in the lipid composition. With increase in DPPC and cholesterol content, liposomes were formed as discrete lipid particles and when dispersed in aqueous hydration media, resulted into liposomes. At DOTAP:DOPE:DPPC:Cholesterol ratio of 0.5:0.5:14:5, lipid particles were observed. However, the temperature of the extraction chamber

was of prime importance to get lipid particles. Above temperature of 38-40^{0}C, even this composition provided sticky mass, because of Tg of DPPC, in the range of 42-45^0 C. Hence, a controlled chamber temperature of 32-35^0 C was maintained throughout the experiment.

Further, process parameters as solvent composition and lipid concentration in solvent was studied to maximize yield and reduce particle size. As methanol was incorporated in part with chloroform, it avoided immediate evaporation of lipid containing solvent droplets and helped to reduce the liquid droplet particle size theirby reducing the particle size in part. However, when solvent ratio was changed to 1:1 of chloroform and methanol, the methanol was partially un-evaporated at the evaporating temperature of 35^0 C theirby optimizing the chloroform : methanol ratio of 2:1 v/v. Further, lipid concentration played an important role in maximizing the yield of lipid particles and the particle size. As lipid concentration was increased, the liposomal recovery increased significantly and was maximum at lipid concentration of 1000 mg / 5 ml of solvent. The yield of 35-40 % was observed as compared to earlier yield of 15-25 % at lipid concentration of 1000 mg/ 10 ml solvent. The higher yield may have to because of lower processing time, which helps to reduce the quantity of lipids getting solublized along with solvent through the supercritical fluid, theirby minimizing the lipid precipitation.

Further, the liposomes showed enhancement in yield as well as reduced particle size with increase in automated back pressure and increase in CO_2 flow rate. The higher ABP helps to enhance the lipid precipitation by enhanced solvent solubility in CO_2 and the higher CO_2 flow rate automizes the lipid – solvent solution into finer mist thus enhancing the yield with reduced particle size. The lipid solution at ABP of 120 bar and CO_2 flow rate of 40 gm/min resulted in particle size of 677 nm and yield of 41.2 % w/w.

Further, the solvent flow rate also affects the particle size of the lipid particles. The particle size of 0.25 ml/min lead to enhanced process time, but this particle size along with enhanced CO_2 flow rate resulted in lowest particle size of 321.9 nm and yield of 35.2 %. With increase in process time, some lipid also tends to get dissolved along with organic solvent thus reducing the yield. Hence, further increasing the CO_2 ABP reduces the yield drastically. At ABP of 150 bar, keeping other parameters constant, the yield of liposomes was reduced to 5%. Hence, ABP of 120 bar was optimized with CO_2 flow rate of 50 gm/min and solvent rate of 0.25 ml/min. At this process parameters, lipid ratio was changed as DOTAP : DOPE molar ratio of 1:1, 2:1 and 3:2

keeping DPPC and cholesterol concentration constant. The optimization of these liposomes was carried out after transfection studies with *β galactosidase* *p*DNA.

The optimized liposomes with minimum particle size and significant yield were checked for particle size after distribution in water and bath sonicating for 10 seconds to break the aggregates. Further these liposomes after hydration were studied for cyoTEM studies and revealed size near 300-1000 nm (higher PDI) and multilamellar structure as shown figure 5.3.

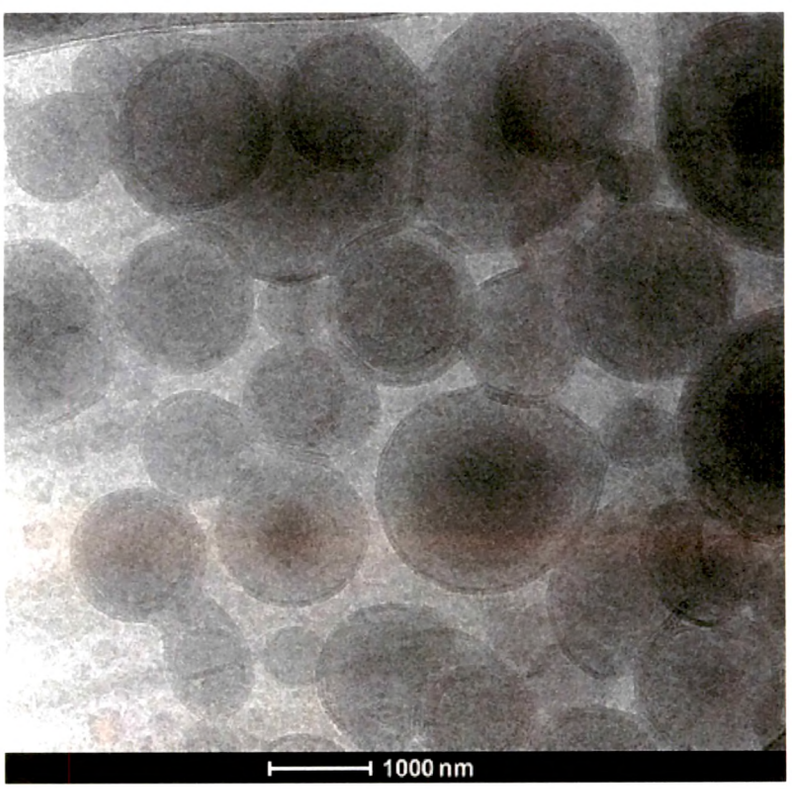

Figure 5.3 CryoTEM study of liposomes prepared by SCF technique

5.3 Lipoplex Development and Characterization:

5.3.1 Materials: The varying liposomal compositions prepared using TFH, EI, and SCF method with varying molar ratios of DOTAP, DOPE and other lipids were used to prepare lipoplexes with β gal and p53 pDNA. The *p*53 and *β galactosidase* *p*DNA were isolated and purified as discussed earlier (260 / 280 ratio of 1.9) and used. The HEPES buffer and Complete Doulbecco's Modified Eagle Medium (DMEM), Fetal Bovine serum (FBS) were purchased from Himedia.

DNAseI enzyme was purchased from Sigma Aldrich (2000 units~ 0.5 mg/vial). All other chemicals were purchased of analytical grade and were checked for purity before use.

5.3.2 Methods:

5.3.2.1 Lipoplex Development:

The lipoplex were developed at varying N/P ratio keeping quantity of pDNA constant and varying liposomes quantity. The liposome quantity was decided on basis of molar ratio of DOTAP as positive charge against molar ratio of pDNA as negative charge. The solutions of liposomes and pDNA were diluted to a constant volume and were mixed while vortexing. These oppositely charged moieties when mixed interact with each other to form tightly condensed lipoplexes. These lipoplexes were studied for particle size, zeta potential and agarose retardation assay for studying the pDNA condensation on cationic liposomes. Further, the lipoplexes were studied for pDNA stabilization against extacellular DNAse I enzyme which degrades the pDNA. The lipoplex development was carried out in HEPES buffer as well as DMEM medium. For particle size analysis, zeta potential and agarose retardation assay, lipoplexes were developed in HEPES buffer, whereas for cell line studies, lipoplexes were freshly prepared in DMEM medium.

5.3.2.2. Particle Size and Zeta Potential of Lioplexes:

The particle size and zeta potential of lipoplexes was determined as previously described. For size determination, diluted sample was used, whereas for zeta potential determination, concentrated undiluted solution is used.

5.3.2.3 Agarose Gel Retardation of pDNA and DNAse I Protection Assay:

The complexation of pDNA with the cationic liposomes was studied with agarose gel retardation assay. The basic principle of electrostatic interaction of oppositely charged pDNA and cationic liposomes resulting in tightly condensed pDNA theirby reducing its movement against current is used in the process. The developed lipoplex formulations with varying N: P ratios ranging from 1: 1 to 3:1 were used for agarose gel retardation assay. After mixing with gel loading dye, the DNA alone and the complexes were run on 1 % agarose gel containing ethidium bromide and were visualized under UV trans-illuminator for gel retardation of the pDNA describing complete pDNA complexation with the liposomes. Further, the integrity of these lipoplexes and its ability to protect complexed pDNA against DNAse I enzyme was studied by treatment of naked DNA and complexed pDNA with DNAse I enzyme. The DNAseI endonuclease enzyme acts by

degrading the double stranded pDNA to 3'-OH oligonucleotide small chains. The DNAseI stock solution prepared by dissolving the purified enzyme (2000 units ~ 0.5 mg of enzyme) in equal ratio of 10 mM Tris-HCl (pH 7.5) containing 50 mM Sodium Chloride and 1 mM Magnesium Chloride and Glycerol in the concentration of 1 mg/ml. The stock was stored at -20^0 C in aliquots until used. The working solution was prepared in the strength of 1 µg/µl by diluting with the dilution buffer of 10 mM Tris HCl(pH 7.5) containing 150 mM sodium chloride and 1 mM magnesium chloride. The stability of complexed pDNA was ascertained by treatment of 2 µg pDNA and lipoplex containing 2 µg pDNA with 1 unit of enzyme in 100 µl of reaction mixture (dilution buffer) and was allowed to treat for 30 minutes. The reaction was stopped by addition of 5 µl of 0.5 M EDTA solution. The pDNA when treated as such was loaded directly with loading buffer in a well. When pDNA was complexed with liposomes, the complexed and treated pDNA was back extracted with phenol chloroform followed by ethanol precipitation and dissolving in Tris – HCl buffer. This dissolved pDNA was loaded along with loading buffer and was studied for pDNA extraction in ethidium bromide prestained gel.

5.3.3 Result and Discussion:

The lipoplexes were developed by mixing the liposomes prepared by EI, TFH and SCF technique with purified pDNA by vortexing and allowing stabilization for 15 minutes.

5.3.3.1 Particle Size and Zeta Potential:

These lipoplexes on development showed significantly different particle sizes and zeta potential in HEPES buffer / DMEM without serum and complete DMEM containing serum. The anionic serum proteins interacted with the cationic liposomes to form complexes with increased particle size (500 nm – 1 micron in size) as compared to lipoplexes in HEPES buffer and incomplete DMEM (200-500 nm). The particle size of the lipoplexes at varying DOTAP:DOPE ratio at different N/P ratios was nearly similar because of the similar initial particle size and same N/P ratio causing electrostatic interaction to form lipoplex. The zeta potential of the lipoplexes was in the positive side because of presence of cationic lipid DOTAP in the lipoplexes. The size and zeta potential of the lipoplexes at varying N/P ratios with varying DOTAP:DOPE molar ratios and varying methods in HEPES buffer is as tabulated in table 5.3.

In general, liposomes prepared by EI method displayed smallest particle size and SCF method showed highest particle size. The lipoplexes prepared by these methods showed particle size according to the liposomal particle size i.e. in the ratio of SCF>TFH>EI method. Further, the

lipoplexes showed higher particle size at N/P ratio near neutrality (1/1) because of strong interaction of cationic liposome and anionic pDNA at similar molar concentration. As N/P ratio increased from 1/1 to 5/1, the size of lipoplexes comparatively decreased in all methods. The EI injection method showed maximum zeta potential upto 44 mv. However, all other lipoplexes also showed comparable zeta potential in the range of 20-40 mv.

Table 5.3 Size and Zeta Potential of the Lipoplexes in HEPES buffer pH 8.0 at varying N/P ratio: (n=3) (Mean± SEM)

THF Method						
N:P ratio	DOTAP:DOPE (1:1)		DOTAP:DOPE (2:1)		DOTAP:DOPE (3:2)	
	Size	Zeta	Size	Zeta	Size	Zeta
1:0	205.3 ± 12.9	35.2 ± 2.0	212.3 ± 9.9	39.1 ± 1.9	211.9 ± 11.1	36.1 ± 1.0
1:1	511.6 ±14.6	19.3 ± 1.3	481.6 ±8.6	25.3 ± 1.4	501.1 ± 11.6	23.3 ± 2.3
3:2	412.1 ± 15.7	24.2 ± 2.3	402.5 ± 11.7	28.2 ± 3.3	416.3 ± 11.7	26.2 ± 3.3
2:1	312.3 ± 12.1	24.4 ± 4.1	321.1 ± 10.1	30.1 ± 3.1	309.2 ± 9.1	28.4 ± 2.1
3:1	288.4 ±14.3	26.9 ± 1.4	268.1 ±11.3	31.9 ± 2.4	279.1 ±10.8	29.9 ± 2.4
5:1	278.3 ± 9.1	27.9 ± 2.9	251.1 ± 13.1	34.1 ± 1.9	250.0 ± 7.5	34.9 ± 3.9
EI Method						
N:P ratio	DOTAP:DOPE (1:1)		DOTAP:DOPE (2:1)		DOTAP:DOPE (3:2)	
	Size	Zeta	Size	Zeta	Size	Zeta
1:0	113.9 ± 12.1	37.9 ± 2.3	123.3 ± 13.2	44.6 ± 3.3	121.2 ± 11.1	43.1 ± 3.8
1:1	289.6 ±11.6	24.2 ± 2.5	312.2 ±12.6	31.2 ± 3.1	318.1 ± 24.9	30.9 ± 2.5
3:2	261.1 ± 7.7	24.9 ± 1.9	300.2 ±11.4	34.3 ± 2.2	281.6 ±22.8	32.9 ± 2.4
2:1	229.1 ± 10.1	26.8 ± 3.2	266.6 ±14.9	37.8 ± 3.9	252.0 ±17.5	35.2 ± 2.5
3:1	161.2 ±13.1	28.1 ± 3.2	195.7 ± 12.1	37.9 ± 4.1	210.3 ± 21.4	38.1 ± 2.9
5:1	141.3 ± 11.3	32.9 ± 2.9	149.8 ± 12.8	41.8 ± 2.9	151.2 ±11.4	41.9 ± 2.1
SCF Method [DPPC and CHOL concentration kept constant]						
N:P ratio	DOTAP:DOPE (1:1)		DOTAP:DOPE (2:1)		DOTAP:DOPE (3:2)	
	Size	Zeta	Size	Zeta	Size	Zeta
1:0	321.9 ± 23.1	31.9 ± 3.2	341.7 ± 19.2	34.6 ± 3.3	347.9 ±23.1	32.9 ± 3.1
1:1	563.2 ± 31.6	17.3 ± 3.2	602.1 ± 29.3	24.0 ± 2.1	578.1 ±29.9	25.2 ± 3.1
3:2	492.1 ± 15.2	21.2 ± 3.6	469.5 ± 26.1	25.3 ± 2.2	486.6 ±25.8	27.4 ± 2.9
2:1	449.3 ± 11.1	21.9 ± 3.2	454.6 ±22.9	26.4 ± 2.1	452.2 ±20.1	28.1 ±4.0
3:1	400.3 ±14.5	25.3 ± 1.9	391.2 ±21.5	28.3 ± 3.0	417.9 ±21.0	30.0 ± 2.1
5:1	389.6 ± 12.1	28.2 ± 2.9	379.1 ±11.9	32.2 ± 3.1	397.1 ±15.4	30.8 ± 2.9

5.3.3.2 Gel Retardation Assay and DNAse I Protection Assay:

The gel retardation assay as demonstrated in **figure 5.4** showed capability of developed lipoplexes to effectively condensate and protect *p*DNA. The liposomes were able to bind with

the DNA efficiently because of opposite charge and electrostatic attraction resulting in retardation of DNA on agarose gel. With increasing the liposomal concentration, more retardation of the DNA was observed.

All the methods showed enhanced retardation with increasing N/P ration. **Figure 5.4(A-1)** displayed complete gel retardation of the *p*DNA mobility as observed in lane 5 indicating complete complexation of *p*DNA and liposomes at the N/P ratio of 2:1. At all further combinations with increasing N/P ratio, complete *p*DNA retardation was observed. Further, complete protection of the DNA against extracellular DNAse offered by the complexation with liposomes was confirmed by treatment of DNAse-I. The naked DNA on treatment with DNAse I showed complete degradation (lane 2). However, the DNA after complexation with liposomes showed no degradation as observed in lane 4 (**figure 5.4 A-2**). The results displayed development of a stable lipoplex system which has a capability of intracellularly delivering the stable non-degraded *p*DNA against extracellular DNAse in cellular environment

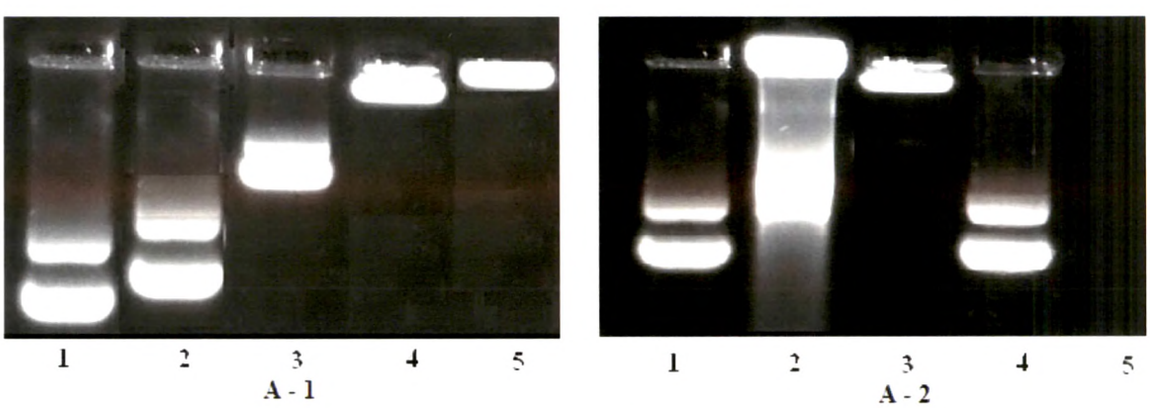

Figure 5.4 (A) Agarose gel retardation of Plasmid alone and after complexation with liposomes at different N / P ratios prepared by TFH method [A1: Lane 1: Plasmid alone, Lane 2 – Lipoplex (N / P – 1/2), Lane 3 - Lipoplex (N / P – 1/1), Lane 4 - Lipoplex (N / P - 1.5 / 1) , Lane 5 : Lipoplex (N / P – 2 / 1)] and stability of DNA after formation of lipoplex against DNAse-I [A2-– Lane 1 : Plasmid Alone, Lane 2: Degraded Plasmid after treatment with DNAse I, Lane 3: Lipoplex at N / P ratio 2/1 , Lane 4 : Stable DNA after treatment of lipoplex with DNAse I followed by DNA purification]

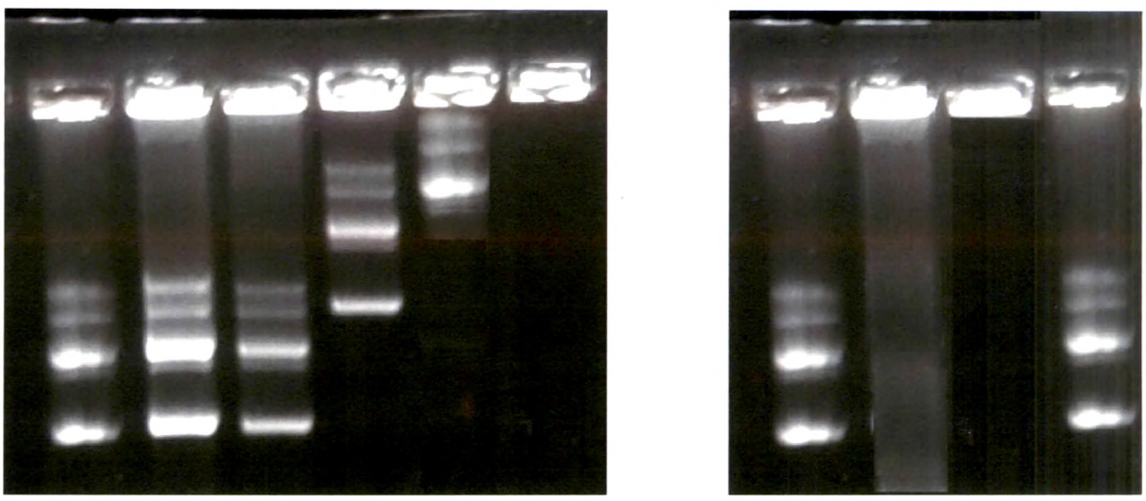

Figure 5.4 (B) Agarose gel retardation of Plasmid alone and after complexation with liposomes at different N / P ratios prepared by SCF method [B1: Lane 1: Plasmid alone, Lane 2 – Lipoplex (N / P – 1/2), Lane 3 - Lipoplex (N / P – 1/1), Lane 4 - Lipoplex (N / P – 3/2) , Lane 5 : Lipoplex (N / P – 2/1), Lane 6 : Lipoplex (N / P – 3/1)] and stability of DNA after formation of lipoplex against DNAse-I [A2-– Lane 1 : Plasmid Alone, Lane 2: Degraded Plasmid after treatment with DNAse I, Lane 3: Lipoplex at N / P ratio 3/1 , Lane 4 : Stable DNA after treatment of lipoplex with DNAse I followed by DNA purification]

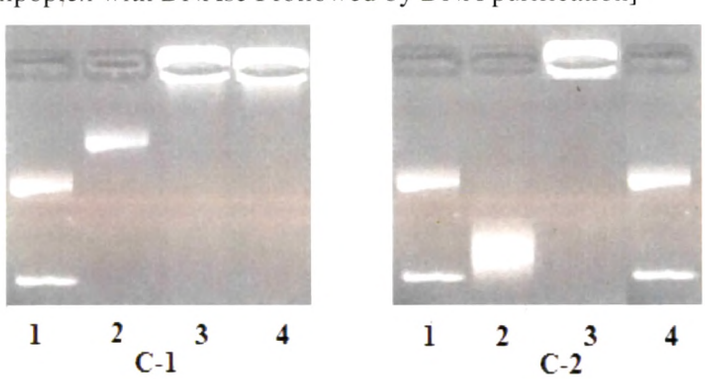

Figure 5.4 (C) **Agarose gel retardation of Plasmid alone and after complexation with liposomes at different N / P ratios prepared by EI method** [C1: Lane 1: Plasmid alone, Lane 2 – Lipoplex (N / P – 1/1), Lane 3 - Lipoplex (N / P – 3/2)], and stability of DNA after formation of lipoplex against DNAse-I [D2-– Lane 1 : Plasmid Alone, Lane 2: Degraded Plasmid after

treatment with DNAse I, Lane 3: Lipoplex at N / P ratio 3/2 , Lane 4 : Stable DNA after treatment of lipoplex with DNAse I followed by DNA purification]

Similar results of agarose gel retardation assay and DNAse I protection assay were observed with liposomes prepared by EI and SCF technique. The SCF technique showed stable lipoplex formation at N/P ratio of 3/1 and EI technique showed stable lipoplex formation at N/P ratio of 3/2. Both these complexes showed complete pDNA retardation and showed protection against DNAseI enzyme.

5.4 Cell Uptake Studies:

5.4.1 Materials:

*β-galactosidase p*DNA were isolated and purified as discussed earlier (260 / 280 ratio of 1.9) and used. *p*53 null Human lung adenocarcinoma H 1299 cell line was obtained from Dr. Samit Chattopadhyay, Scientist, National Centre for Cell Sciences, Pune, India and was grown in high glucose DMEM medium (HiMedia, Mumbai, India) supplemented with 10 % fetal bovine serum (HiMedia, Mumbai, India) and 2mM L-glutamine, $100Uml^{-1}$ Penicillin, 100 μgml^{-1} Streptomycin and 50 μgml^{-1} of Amphotericin B. The cells were grown in a humidified atmosphere of 5% CO_2 at 37°C. All experiments were performed on cells in the exponential growth phase in between the passage no. 91-101. 6-Coumarin was purchased from Sigma Aldrich and was tested for fluoroscence as per specifications. Lipids and all other chemicals were used as discussed earlier and were confirmed for purity before use.

5.4.2 Development of 6 – Coumarin Liposomes and Lipoplexes:

The fluorescent lipoplexes for estimating cell uptake was formulated by TFH, EI and SCF technique for encapsulating 6-coumarin. The 6-Coumarin liposomes were formulated by dissolving 6- Coumarin along with lipids at a concentration of 1 mg of 6-Coumarin per 100 mg of lipids and keeping other formulation and process parameters same as for optimized batches. The batches were prepared in dark considering fluoroscence sensitivity of 6-Coumarin in light. The lipoplexes were prepared with N/P ratio of 2/1 for TFH, 3/2 for EI and 3/1 for SCF respectively with *β-galactosidase p*DNA by the procedure discussed earlier and were confirmed for agarose gel retardation and DNAseI protection assay. The particle size and zeta potential of these lipoplexes was similar to the lipoplexes prepared earlier with same formulation and process variables and are mentioned in the table below:

Table 5.4 Particle Size and Zeta Potential of 6-Coumarin loaded Lipoplexes prepared by EI, TFH and SCF technique (n=3) (Mean± SEM)

Technique	N/P ratio for lipoplex development	Lipoplex Particle Size	Lipoplex Zeta Potential
TFH	2/1	321.9 ± 21.2	26.2 ± 2.1
EI	3/2	265.1 ± 10.9	32.9 ± 2.8
SCF	3/1	423.9 ± 23.1	27.9± 11.2

These developed lipoplexes were used for cell uptake studies in H 1299 cell line.

5.4.3 Qualitative Cell Uptake of 6 – Coumarin Lipoplexes prepared by TFH, EI and SCF technique:

Cell uptake of liposomes and lipoplexes were studied qualitatively in H-1299 cell line using 6 Coumarin encapsulated lipoplexes as described above. The qualitative cell uptake was studied using an independent set of experiments by fluoroscence imaging of the cells. The cells were first seeded on a sterile cover slip at a seeding density of 0.5 X 10^6 cells/well in 6 well plates and grown overnight in DMEM growth medium supplemented with 10 % FBS and 1% Penicillin–Streptomycin- Amphotericin B solution. On next day, cells were treated with 6-Coumarin lipoplexes prepared using different methods (EI, SCF, TFH) in DMEM medium at a concentration of 0.25 mg / ml. After 3 h of incubation, the cells were washed thoroughly with PBS, and imaged under fluoroscent microscope (Nikon) with measurements at excitation and emission wavelengths of 430 nm and 485 nm respectively [K.Y. Win & S.S. Feng (2005)]. The images were created with the help of cell quest software and were compared for qualitative uptake.

5.4.4. Results and Discussion:

6-Coumarin, a hydrophobic fluorescent marker molecule was internalized inside the liposomes by TFH, EI and SCF with optimized parameters and allows for the investigation of coumarin containing lipoplex internalization inside the cells using fluoroscence microscopy. **Figure 5.5** shows qualitative analysis of cellular uptake of coumarin lipoplexes prepared by all the three methods in H 1299 cell line using a fluoroscent microscopy.

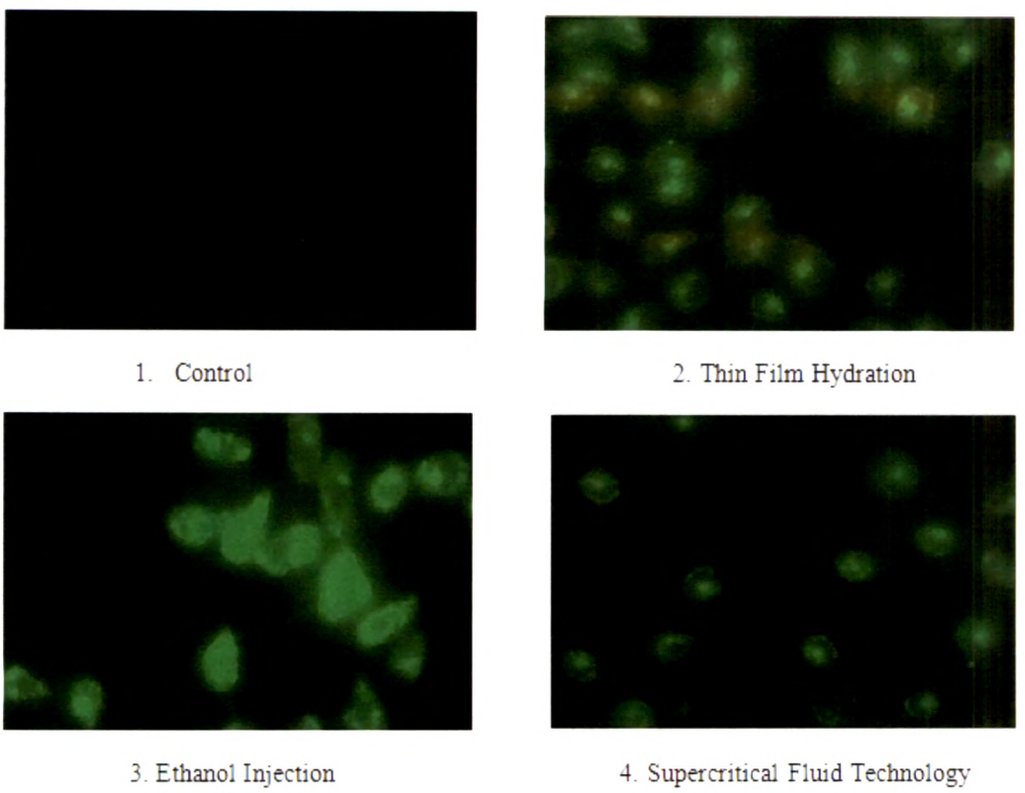

Figure 5.5 : Intracellular uptake of 6-coumarin lipoplexes prepared by TFH, EI and SCF techniques in H 1299

Intense fluorescent signals were observed in the cells treated with coumarin loaded lipoplexes as compared to control cells. Comparatively, more intense signals were observed after treatment of lipoplexes prepared by EI method as compared with SCF and THF lipoplexes. Further, more intracellular deposition and dispersed fluoroscence was observed intracellularly after EI lipoplexes. The higher uptake may be because of the higher zeta potential and lower particle size of lipoplexes in EI method than SCF and THF. The dispersed fluoroscence at higher intensity inside cells was because of possible disruption of DOPE containing lipoplexes at lower pH of endosome indicating efficient release of coumarin inside cells which can be efficiently diffused inside the nucleus. The lipoplexes prepared by TFH and SCF method also showed significant fluoroscence in the cells. Comparatively, the lipoplexes prepared by TFH showed little higher fluoroscence possibly because of their lower particle size resulting in better cell uptake.

5.5 Transfection Studies:

5.5.1 Materials:

*β-galactosidase p*DNA were isolated and purified as discussed earlier (260 / 280 ratio of 1.9) and was used to study transfection in *p*53 null Human lung adenocarcinoma H 1299 cell line which was maintained in high glucose DMEM medium supplemented with 10 % fetal bovine serum and 2mM L-glutamine, 100Uml^{-1} Penicillin, 100 µgml^{-1} Streptomycin and 50 µgml^{-1} of Amphotericin B as described earlier. Ortho-nitrophenyl-β-D-galactopyranoside (ONPG) was purchased from Sigma Aldrich. Lipids and all other chemicals were used as discussed earlier and were confirmed for purity before use.

5.5.2 Methods:

Efficiency of the developed cationic liposomes by all the methods viz. TFH, EI and SCF technique, to induce gene expression in H 1299 cells was determined using *β gal* reporter *p*DNA. The expression is studied at various DOTAP : DOPE molar ratios and N/P ratios at which complete *p*DNA condensation is achieved. The cells were seeded in 96 well plate at a density of 5000 cells / well in 100 µl of DMEM growth medium supplemented with 10 % FBS and 1% Penicillin–Streptomycin- Amphotericin B solution. After 24 Hrs, the culture medium was replaced with fresh complete DMEM medium containing DNA/Liposome complexes prepared at different N/P ratios keeping the quantity of *p*DNA constant (300 ng). After 4 hrs of incubation of the lipoplexes with cells, the culture media was removed; the cells were washed with PBS pH 7.4 and were replaced with complete media. After 48 hrs, the culture media was removed, cells were washed with PBS pH 7.4 and were lysed using 50 µl lysis buffer (0.5 % Nonidet P 40 in Tris buffer pH 8.0) and were treated with 50 µl of 2 X ONPG solution, a substrate for β galactosidase protein. After 15 min of incubation at 37°C, the intensity of yellow color was measured in ELISA micro well plate reader (Biorad, Model 680 XR, Mumbai, India) at 405 nm as a function of quantity of β galactosidase protein expressed in the cells which converts the ONPG into O- Nitro phenol yielding the yellow color. In all the experiments, naked *p*DNA transfected cells were used as negative control and the Lipofectamine (Invitrogen) transfected cells were used as a positive control.

5.5.3 Result and Discussion:

Lipid based non-viral gene delivery system has attracted more attentions in recent years due to their non-immungenic properties as opposed to viral ones, but their relatively lower transfection

efficiency usually limits their application. In order to evaluate the transfection capability of the developed lipoplexes, *in vitro* transfection experiment was performed on H 1299cell line. Transfection efficiency of liposomes complexed with marker *β-gal p*DNA was measured by comparative determination of yellow color developed by ONPG hydrolysis to Ortho Nitro Phenol, which is directly proportional to the quantity of β galactosidase protein formed after *p*DNA transfection. The transfection in terms of β gal activity has been tabulated in **table 5.5.** Transfection with naked uncomplexed *β-gal p*DNA was kept as negative control which showed no transfection and transfection with Lipofectamine was considered to be positive control, which showed maximum transfection. Transfection achieved at different N/P ratios with differing DOTAP:DOPE compositions of liposomes prepared by all the three methods, in H 1299 cell line has been tabulated in table 5.5. The results showed higher transfection of H 1299 cells by the lipoplexes prepared by EI method followed by TFH method followed by SCF method. The transfection was observed to also varied by lipoplex N/P ratio, size and zeta potential. [S. Prabha et al (2002)]

In general, the *β-gal p*DNA expression varied with the size and zeta potential of complexed lipoplex in the medium and cellular uptake. The lipoplexes prepared by EI showed lowest particle size and correspondingly highest cell uptake. Further, the presence of DOPE helped to enhance the lipid fusion with the endosome cell wall promoting the transfection. Correspondingly, highest quantity of DOPE resulted in highest cell transfection as observed in DOTAP:DOPE 1:1 ratio in EI method. Highest transfection was achieved with DOTAP:DOPE ratio 1:1 at N/P 3/2, which showed stable pDNA retardation and stability against DNAse I. At higher N/P ratio, the zeta potential of the system increases and particle size decreases. Higher cationic charge attracts serum proteins to for unstable lipoplexes and reduces their cell uptake, however, lower particle size enhances the cell uptake. These two factors in together determines the cell uptake and the transfection.

In comparison to EI, TFH method showed, lower transfection at all N/P and DOTAP:DOPE ratio. This may have to do with higher particle size of EI prepared liposomes and lipoplexes. Further, the N/P ratio 1:1 and 3:2 showed lower transfection may be because of the DNAseI mediated partial DNA degradation. It was observed that, the lipoplexes showed complete DNA retardation and safety against DNAse I at N/P ratio 2/1. The complete complexation was responsible for enhanced transfection resulting in β-galactosidase expression.

Table 5.5 Transfection of β galactosidase pDNA at different N/P ratios of liposomes prepared by TFH, EI and SCF technique at different DOTAP:DOPE ratio (n=3) (Mean± SEM)

Transfection Agent		β galactosidase expression in mU at different N/P ratios (With Serum)					
Control (No treatment)		0.00 ± 0.00					
Lipofectamine (As per protocol)		1.89 ± 0.21					
Blank Liposome		0.00 ± 0.00					
β galactosidase pDNA		0.06 ± 0.04					
N/P Ratio		1/1	3/2	2/1	3/1	4/1	5/1
Thin Film Hydration DOTAP:DOPE	1:1	0.71 ± 0.12	1.02 ± 0.19	1.41 ± 0.11	1.30 ± 0.13	1.09 ± 0.11	0.80 ± 0.21
	2:1	0.89 ± 0.10	1.07 ± 0.13	1.60 ± 0.09	1.29 ± 0.18	1.11 ± 0.15	0.91 ± 0.12
	3:2	0.61 ± 0.09	0.72 ± 0.17	0.91 ± 0.11	0.81 ± 0.07	0.91 ± 0.09	0.92 ± 0.07
Ethanol Injection DOTAP:DOPE	1:1	0.49 ± 0.12	1.82 ± 0.21	1.69 ± 0.11	1.12 ± 0.13	0.88 ± 0.09	0.71 ± 0.09
	2:1	0.18 ± 0.09	1.59 ± 0.11	1.37 ± 0.23	0.98 ± 0.21	0.71 ± 0.08	0.59 ± 0.12
	3:2	0.38 ± 0.12	1.50 ± 0.24	1.28 ± 0.11	0.87 ± 0.18	0.79 ± 0.09	0.49 ± 0.11
Super Critical Fluid Technology DOTAP:DOPE:DPPC:Cholesterol	0.5:0.5:7:2 (1/1)	0.12 ± 0.09	0.23 ± 0.11	0.45 ± 0.09	1.09 ± 0.31	0.78 ± 0.22	0.82 ± 0.12
	0.66:0.33:7:2(2/1)	0.19 ± 0.10	0.28 ± 0.09	0.61 ± 0.13	1.41 ± 0.18	1.21 ± 0.19	1.31 ± 0.19
	0.6:0.4:7:2(3/2)	0.17 ± 0.08	0.23 ± 0.12	0.43 ± 0.11	1.01 ± 0.11	0.63 ± 0.21	0.59 ± 0.12

The SCF technique required use of DPPC and cholesterol for stabilizing the system. However, these ingredients lead to reduced transfection as compared with EI and SCF technique. Further, higher particle size and requirement of higher lipid (mg) for complexation of same quantity of pDNA also lead to lower transfection. The cellular uptake of these lipoplexes was also on comparatively lower side and less fluoroscence was observed in the cells, resulting in lower cell transfection. At all N/P ratios with different DOTAP:DOPE composition along with DPPC and cholesterol, highest transfection was observed at N/P ratio of 3/1 at DOTAP:DOPE ratio of 2:1. The β gal expression of 1.41 was observed to highest, but still lower than the EI and TFH method. However, still the method showed unique formulation technique for developing dry liposomes ready to be complexed with pDNA with higher stability.

Compared to Lipofectamine, a standard transfection agent, EI method showed nearly similar transfection (non-significant difference) ($p>0.05$), however, TFH and SCF technique showed significantly lower transfection than the Lipofectamine. SCF has been a novel technology to prepare liposomes and these liposomes were stable in dry condition for a long period of time upto 3 M at 2-8^0 C and retained its transfection ability, which was advantageous over other two methods.

5.6 Development of Ligand Attached Lipoplexes:

Attachment of ligands as Folate, Transferrin, Wheat germ agglutinin, Peptides as RGD, Sigma receptor antagonists as Haloperidol, Anisamide have shown to over-express on the cancer cell surface and have been playing a vital role to enhance the cell uptake of the nanoconstructs conjugated with these ligands. Further, these ligands also enhance the site specificity and therapeutic index of the encapsulated drug. Hence, ligand attached liposomes have been a prime target of modern chemotherapy and many formulations are currently in research and clinical trials. Transferrin has shown to be over-expressed in NSCLC and has been a target ligand for improving the treatment of lung cancer. p53 attached lipoplexes in presence of transferrin are expected to show enhanced chemotherapeutic effect with added cytotoxicity.

5.6.1 Materials:

*β-galactosidase p*DNA were isolated and purified as discussed earlier (260 / 280 ratio of 1.9) and was used to study transfection in *p*53 null Human lung adenocarcinoma H 1299 cell line which was maintained in high glucose DMEM medium supplemented with 10 % fetal bovine serum and 2mM L-glutamine, 100Uml^{-1} Penicillin, 100 µgml^{-1} Streptomycin and 50 µgml^{-1} of Amphotericin B as described earlier. Ortho-nitrophenyl-β-D-galactopyranoside (ONPG) was purchased from Sigma Aldrich. Holo-Transferrin was purchased from Merck, India. Lipids and all other chemicals were used as discussed earlier and were confirmed for purity before use.

5.6.2 Methods:

The liposomes were prepared by Ethanol Injection method as described above with the optimal DOTAP:DOPE ratio showing maximum transfection. At highest transfection N/P ratio, lipoplexes were developed conjugated with Transferrin (Tf) as per protocol provided by L. Xu et al (1997) with slight modifications. [L. Xu et al (1997)]

Briefly, the hydrated liposomes diluted in HEPES buffer at desired lipid concentration were mixed with Transferrin, previously dissolved in HEPES buffer, pH 8.0 followed by activation

with Ferric Chloride; at a ratio of 5, 10 and 15 µg of Transferrin per µmol of DOTAP. The solution was incubated for 10 minutes and then allowed to complex with pDNA as per standard protocol and N/P ratio. Varying quantities of Tf was used to assess optimum Tf concentration for better uptake. These lipoplexes were studied for agarose gel retardation and DNAse I stability. For cell line studies, the lipoplexes containing Tf were diluted with DMEM medium and were used immediately within 1 Hr after preparation.

Similarly, 6-Coumarin containing lipoplexes were prepared with varying Tf content and 1 mg of coumarin per 100 mg of lipid concentration and was used for cell uptake studies within 1 Hr of formulation development. These ligand attached lipoplexes were also studied for β galactosidase pDNA expression in H 1299 cell line, as per the protocol described previously, which also shows over-expressed receptors for Transferrin protein.

5.6.3 Result and Discussion

The Tf-p53-Liposome complex was developed in a sequential manner as described by L. Xu et al (1997) and L. Xu et al (1999) except that, the lipoplexes were initially made in HEPES buffer and then diluted with medium prior to use. These lipoplexes were stable for 24 Hrs in HEPES buffer, latter on showed increase in size and reduction in zeta potential. The Tf containing lipoplexes showed comparatively higher particle size and reduced zeta potential, as shown in Table 5.6, because of anionic nature of Transferrin. Hence the sequence of addition of Tf becomes critical for uniform binding of Tf with lipids. However, the lipoplexes with 15 µg of Transferrin per µmol of DOTAP showed non-uniform complexation probably because of higher concentration of anionic Tf against cationic lipids. Further, the lipoplexes with 5 and 10 µg of

Table 5.6 Size and Zeta Potential of Tf attached Lipoplexes: (n=3) (Mean± SEM)

Batch No.	Composition	Size ± SEM (nm)	Zeta Potential ± SEM (mv)
EIL	EI Liposome + pDNA (3/2) at DOTAP/DOPE 1:1	261.1 ± 7.7	24.9 ± 1.9
EI – Tf 5	EI Liposome + Tf 5µg per µmol of DOTAP + pDNA (3/2) at DOTAP/DOPE 1:1	302.9 ± 11.8	17.9 ± 2.4
EI – Tf 10	EI Liposome + Tf 10µg per µmol of DOTAP + pDNA (3/2) at DOTAP/DOPE 1:1	334.1 ± 21.1	15.9 ± 1.9
EI – Tf 15	EI Liposome + Tf 15µg per µmol of DOTAP + pDNA (3/2) at DOTAP/DOPE 1:1	1284.1 ± 124.9	6..9 ± 3.1

Transferrin per µmol of DOTAP showed complete pDNA retardation and pDNA stability against DNAseI enzyme thus supporting the stability of the pDNA during complexation, but lipoplexes with 15µg of Transferrin showed partial pDNA degradation indicating incomplete complexation.

The lipoplexes when studied for cell uptake after encapsulating coumarin, showed a vast difference in cell uptake as shown in the figure 5.6 below. The conjugation of Tf with the liposomes enhanced the cell uptake vastly as evident with 6 –Coumarin fluoroscence inside cells. The enhanced uptake has to do with over-expressed Tf receptors which allows for higher endocytosis of the lipoplexes. Further, the endosomes fuses with the lipoplex walls in presence of anioninc Tf in a better way so as to enhance the pDNA release and better transfection [N. Sakaguchi et al (2008)].

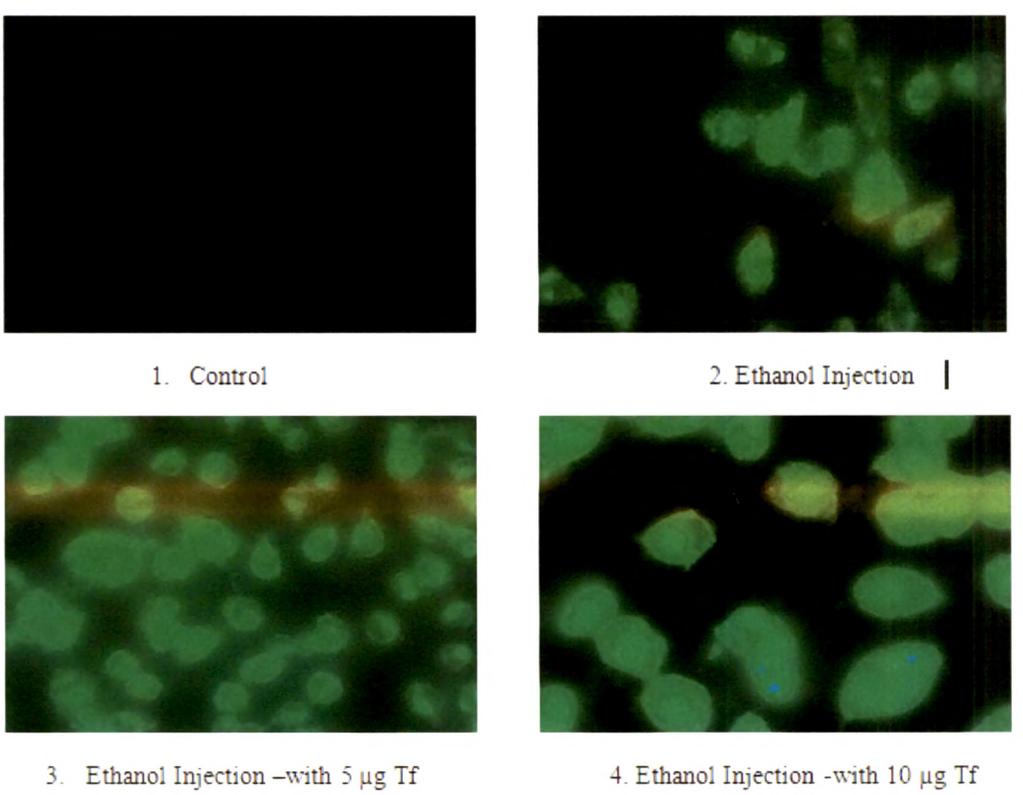

Figure 5.6: Intracellular uptake of 6-coumarin lipoplexes prepared by EI after Tf attachment in H 1299 cell line

The conventional lipoplexes containing pH-sensitive fusogenic lipids as DOPE have been able to achieve efficient transfection through membrane fusion with intracellular acidic compartments such as endosomes. Because transferrin receptor is known to be overexpressed in cancer cells, in this study, we investigated the effect of transferrin as a ligand for transfection of various cancer-derived cell lines mediated by the liposome-lipoplex hybrid complexes [N. Sakaguchi et al (2008)]. Results showed that these hybrid complexes with transferrin exhibited higher transfection efficiency toward these cells than complexes without transferrin, but the extent of the transferrin induced enhancement was dependent on the quantity of Tf and correlate with the activity of internalization of transferrin receptor into the cells. The transfection of β-galactosidase pDNA in H 1299 cell line in absence and in presence of Tf has been tabulated in table 5.7 as below:

Table 5.7 β-galactosidase pDNA expression of Tf attached Lipoplexes in H 1299 cell line (n=3) (Mean± SEM)

Batch No.	Composition	β-galactosidase pDNA expression (mU)
EIL	EI Liposome + pDNA (3/2) at DOTAP/DOPE 1:1	1.82 ± 0.21
EI – Tf 5	EI Liposome + Tf 5μg per μmol of DOTAP + pDNA (3/2) at DOTAP/DOPE 1:1	3.01 ± 0.19
EI – Tf 10	EI Liposome + Tf 10μg per μmol of DOTAP + pDNA (3/2) at DOTAP/DOPE 1:1	3.98± 0.35
Tf solution pretreatment followed by Ei-Tf 10	Tf Solution pretreatment for 3 Hrs followed by EI Liposome + Tf 10μg per μmol of DOTAP + pDNA (3/2) at DOTAP/DOPE 1:1	0.92 ± 0.18

As observed in cell uptake studies, EI-Tf 10 lipoplexes showed significantly higher transfection ($p<0.001$) when compared with lipoplexes without Tf. The EI-Tf-5 lipoplexes also showed significantly higher transfection compared to EIL. The transfection values supports role of Tf in enhancing the endocytosis of the lipoplexes inside the cells also the fact, higher the quantity of Tf conjugated, higher the endocytosis [N. Joshee et al (2002)].

Further, transferrin solution pretreatment followed by Tf attached lipoplexes showed very low transfection, ever lower than the Tf unattached lipoplexes. These results suggested saturation of Tf receptors after Tf solution treatment and thus enabling lower cell uptake by Tf- mediated endocytosis. The results confirms role of Tf surface receptors over-expressed on H 1299 cell line.

5.7 Cytotoxicity of p53 Based Lipoplexes:

5.7.1 Materials: *p*53 and *β galactosidase p*DNA were isolated and purified as discussed earlier (260 / 280 ratio of 1.9) and used. *p*53 null H 1299 cell line were used to study Cytotoxicity. The cells were grown in high glucose DMEM medium as described previously. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Dimethyl Sulphoxide (DMSO), were purchased from himedia. Lipids and all other chemicals were used as discussed earlier and were confirmed for purity before use. All experiments were performed on cells in the exponential growth phase in between the passage no. 91-101 for H 1299

5.7.2 Method:

*p*53, a cell growth regulator, is actively involved in inducing apoptosis and exerting cytotoxic effect on cancer cell lines. *p*53 has also shown enhancement in the cytotoxicity of anticancer agents when given along with them. In this investigation, we have studied the cytotoxicity of p53 based lipoplexes prepared by different methods and the effect of attachment of Tf in terms of decreased cell viability when compared with Tf unattached lipoplexes.

The lipoplexes with *p*53 were prepared in a similar manner at the same conditions with optimized DOTAP : DOPE and N/P ratio which resulted in maximum *β gal p*DNA expression as studied earlier and the cytotoxicity assay was performed. Various formulations for studying the effect of *p*53 in cell transfection and associated cytotoxicity are as mentioned below:

1. Control group treated only with medium
2. Blank cationic liposomes
3. *p*53 solution as Negative control
4. Cationic Lipid- Non therapeutic *β gal p*DNA at N/P ratio 3/2 prepared by EI
5. EI -*p*53 lipoplexes (N/P ratio 3/2)
6. TFH-p53 lipoplexes (N/P ratio 2/1)
7. SCF-p53 lipoplex (N/P ratio 3/1)
8. EI –Tf 5-*p*53 lipoplexes (N/P ratio 3/2)

9. EI –Tf 10-*p*53 lipoplexes (N/P ratio 3/2)

Briefly, cytotoxic effect of all these formulations was estimated by MTT cell viability assay. The cells H 1299 were seeded in 96 well plates at cell density of 5000 cells/ well and grown overnight. On the next day the cells were incubated with all the formulations in complete medium for 4 Hrs, rinsed well with PBS and the cells were allowed to grow for 48 hrs. After 48 hrs, the cells were rinsed with PBS and then treated with 100 µl of MTT solution in DMEM (without serum) at concentration of 1mg/ml and incubated for 4 hrs. After the incubation, the medium was replaced with 200µl DMSO to dissolve the formazan crystals formed after internalization of MTT by live cells. The samples were assayed with Microplate reader (Biorad) at 570 nm keeping reference absorbance at 630 nm as blank. The absorbance of cells without any chemical treatment is considered as positive control and viability of cells after formulation treatment is calculated accordingly.

The percentage cell viability (%) is determined as

$$\text{Cell Viability (\%)} = \frac{\text{Sample absorbance at 570 nm}}{\text{Control absorbance at 570 nm}} \times 100$$

The cytotoxicity tests were repeated three times, and the data is expressed as mean and standard deviation of 3 replicates. The results were compared statistically with single factor ANOVA.

5.7.3 Result and Discussion:

The cytotoxicity assay of *p*53 based formulations was performed to estimate lowering of % cell viability after the treatment and the results have been tabulated in **table 5.8** as below. Cytotoxicity studies were performed in p53 null H 1299 (p53 null) cell line which mimics the deleted and inactive p53 character in majority of lung cancers *in vivo* [D. Sidransky &, M. Hollstein, (1996) , L. Xua et al (2001)].

The cells treated with medium only were considered as control with 100 % cell viability and viability of other formulations was calculated against control. Cationic liposomes and *p*53 solution and cationic lipoplexes of *β gal p*DNA showed non-significant toxicity. The lipoplexes prepared with highest *β gal p*DNA expression showed highest cytotoxicity indicating significant p53 expression inside cells, which results in apoptosis restoration of the cells and theirby retards the cell multiplication and imparts cell death.

The *p*53 mediated cell cytotoxicity has been observed because of normalization of apoptosis function, with suppression of BCL-2 protein. The BCL-2 protein is postulated to block the

release of cytochrome C after the initiation of apoptosis, which prevents the downstream propagation of the death signal, thereby promoting cell survival [S. D. Li, and L.Huang (2006)].

Table 5.8: % Cell viability of H 1299 cells after p53 formulations treatment: (n=3, mean ± SEM)

Formulation	% Cell Viability
Control group treated only with medium	100%
Blank cationic liposomes	99.82± 0.09
*p*53 solution as Negative control	98.92 ± 0.11
Cationic Liposome-*β gal p*DNA lipoplex at N/P ratio 3/2 prepared by EI	99.11±0.14
EI -*p*53 lipoplexes (N/P ratio 3/2)	56.95 ± 3.21
TFH-p53 lipoplexes (N/P ratio 2/1)	61.21 ± 2.92
SCF-p53 lipoplex (N/P ratio 3/1)	69.10 ± 3.03
EI –Tf 5-*p*53 lipoplexes (N/P ratio 3/2)	39.25 ± 2.8
EI –Tf 10-*p*53 lipoplexes (N/P ratio 3/2)	29.22 ± 3.11

Further, the bystandard action of *p*53 protein after expression in lung cancer cell lines has also reported to play a significant role in enhancing the action of *p*53 throughout the complete cells even after possibly getting transected in fewer cells thereby enhancing the cytotoxic action of the p53 [D K Frank et al (1998)]. This bystandard effect nullifies the disadvantage possible lower transfection of *p*53 in vivo and results in superior therapy.

The effect of p53 in terms of cytotoxicity was similar to the β galactosidase expression. The ethanol injection method produced lipoplexes showed maximum cell death indicating maximum p53 expression and maximum apoptosis. Further, Addition of Tf enhanced the cell death significantly ($p<0.01$) and the quantity of Tf also affected the cell death. The liposome-Tf10-p53 lipoplex showed maximum cell death of 29 %, 200 % higher than the p53-lipoplexes without Tf ($p<0.001$). The results demonstrate development of a excellent carrier with superior anticancer activity for treatment of NSCLC.

5.8 Lyophillization of Lipoplexes:

5.8.1 Materials:

Optimized lipoplexes of p53 as developed previously were used. Sugars as sucrose and trehalose were purchased from Himedia. All other chemicals were purchased of analytical grade and were used as discussed earlier.

5.8.2 Methods:

The optimized lipoplexes were lyophilized for enhanced stability of the formulations and DPI development for direct lung deposition. The lyophillization was optimized with sugars as sucrose and trehalose, to achieve same physical properties of the lipoplexes when they come in contact with aqueous medium to release nanosized lipoplexes. Lyophillization was carried out by freezing the lipoplex + sugar suspension at -20^0 C for 8 Hrs followed by freeze drying by applying the vacuum for 24 Hrs. Previously, sugar molecules as isomaltose and sucrose have shown capacity to protect the liposomes and lipoplexes from aggregation and maintain their transfection ability for 3 months at $2\text{-}8^0$ C [Y. Maitani et al (2008)]. Our lipoplexes were lyophilized with main objective of maintaining same particle size of lipoplexes which may result in similar transfection efficiency.

5.8.3 Results and Discussion

The lipoplexes were lyophilized with sugars as sucrose and trehalose at a weight ratio of 1:1, 1: 3 and 1:5 of lipid: sugar and were checked for particle size after resuspension in HEPES buffer. The results of particle size observed after lyophillization and resuspension are tabulated in Table 5.9 as below.

After lyophillization at different lipid : cryoprotectant ratios, particle size of the liposomes in the cake was determined after resuspension in 2 ml of HEPES buffer followed by vortexing and bath sonicating for 10 seconds to break the lumps. The lipoplexes showed comparatively lower size with Trehalose than sucrose and at ratio 1:3, the redispersed cake showed nonsignificant difference in particle size. No further improvement was observed on increasing the ratio of trehalose hence, lipid : trehalose ratio of 1:3 was optimized and used for all batches and the formulations were developed for DPI formulation.

The lyophilized lipoplexes were tested for pDNA stability on agarose gel retardation and cytotoxicity after rehydration in HEPES buffer and no significant change was observed in the lipoplexes in terms of pDNA stability and cytotoxicity indicating retained transfection efficiency

of lipoplexes in terms of trehalose. The cytotoxicity of lipoplexes after lyophillization and rehydration has been tabulated in Table 5.10 as below:

Table 5.9: Particle size of Lipoplexes before and after lyophillization: (n=3) (Mean± SEM)

Lipoplexes + Cryoprotectant used	Lipid : Cryoprotectant Ratio (w/w)	Particle Size Before Lyophillization (nm)	Particle Size after resuspension in HEPES buffer pH 8.0 (nm)
EI -*p*53 lipoplexes (N/P ratio 3/2) + Sucrose	1:1	268 ± 13.7	611.2 ± 19.3
	1:3		455.2 ± 31.2
	1:5		430.5 ± 16.3
EI -*p*53 lipoplexes (N/P ratio 3/2) + Trehalose	1:1		411.2 ± 19.1
	1:3		281.2 ± 12.1
	1:5		279.2 ± 10.7
TFH-p53 lipoplexes (N/P ratio 2/1) + Trehalose	1:3	324.1 ± 11.3	369.1 ± 22.1
SCF-p53 lipoplex (N/P ratio 3/1) + Trehalose	1:3	420.2 ± 13.9	511.0 ± 21.2
EI –Tf 5-*p*53 lipoplexes (N/P ratio 3/2) + Trehalose	1:3	302.9 ± 11.8	361.1 ± 12.9
EI –Tf 10-*p*53 lipoplexes (N/P ratio 3/2) + Trehalose	1:3	334.1 ± 21.1	401.8 ± 20.1

Table 5.10: Cytotoxicity of Formulations before and after lyophillization: (n=3) (Mean± SEM)

Formulation	**% Cell Viability**	
	Before Lyophillization	**After Lyophillization**
EI -*p*53 lipoplexes (N/P ratio 3/2)	56.95 ± 3.21	54.45 ± 3.11
TFH-p53 lipoplexes (N/P ratio 2/1)	61.22 ± 2.92	59.20 ± 2.91
SCF-p53 lipoplex (N/P ratio 3/1)	69.10 ± 3.03	73.11 ± 4.10
EI –Tf 5-*p*53 lipoplexes (N/P ratio 3/2)	39.25 ± 2.80	42.11 ± 3.12
EI –Tf 10-*p*53 lipoplexes (N/P ratio 3/2)	29.22 ± 3.11	33.11 ± 3.12

The developed trehalose based lyophilized formulations on testing for cytotoxicity showed statistically non-significant changes in results as compared to the freshly prepared formulations

theirby offering the more stable formulations for DPI development and *in vivo* drug delivery. These lyophilized formulations were kept for stability at 2-8^0 C and showed nonsignificant differences in particle size and cytotoxicity after 3 Months.

5.9. *In Vitro* lung deposition of developed lipoplexes by Anderson cascade impactor:

5.9.1 Materials:

Optimized lyophillzed lipoplex formulations, Size 2 Capsules.

5.9.2 Methods:

The efficacy of *p*53 complexed lipoplexes in the lung environment actually depends on their deep lung deposition and the dose delivered. *In Vitro* lung deposition pattern of the lyophillized *p*53 complexed lipoplexes in the alveolar region was determined by the Anderson cascade impactor as per USP (Vol.1 Edition 34.). The capsules containing 25 mg of dry lyophilized powders (after passing through 100 #)loaded with therapeutic lipoplexes and trehalose demonstrating maximum cytotoxicity in the cell lines were aerosolized using Rotahaler (Cipla) after applying vacuum at 30 LPM for 10 seconds for determining their lung deposition pattern. 12 Capsules were aerosolized through rotahaler and the powder deposited on each plate was estimated for p53 content by phenol-chloroform extraction, followed by spectroscopy at 260 nm. The stability of the *p*DNA in the lipoplex during aerosolization was also estimated by phenol-chloroform extraction of the lipoplex followed by agarose gel electrophoresis of isolated and purified *p*DNA. Fine particle fraction and mean mass aerodynamic diameter of the formulation was determined using the calculations as per USP and were correlated with *in vitro* lung deposition of the formulations.

5.9.3 Result and Discussion:

In Vitro lung deposition of the formulation is a characteristic of the particle size of the formulation and the density of the formulation. The in vitro lung deposition in terms of mean mass aerodynamic diameter indicates determines the site of formulation penetration and the fine particle fraction determines the actual dose delivered to the lung.

All the formulations when tested on Anderson Cascade Impactor under the standard conditions of USP, showed significant in vitro lung deposition. The FPF and MMAD of the tested formulations has been tabulated in Table 5.11 below:

Table 5.11 FPF and MMAD of the Lyophillized Liposomal and Lipoplex formulations: (n=3) (Mean± SEM)

Formulation	FPF (%)	MMAD (μm)
EI -*p*53 lipoplexes (N/P ratio 3/2)	41.12 ± 3.31	3.13 ± 0.32
TFH-p53 lipoplexes (N/P ratio 2/1)	36.92 ± 2.19	3.63 ± 0.11
SCF-p53 lipoplex (N/P ratio 3/1)	39.12 ± 3.12	3.41 ± 0.28
EI –Tf 5-*p*53 lipoplexes (N/P ratio 3/2)	35.39 ± 2.17	3.79 ± 0.21
EI –Tf 10-*p*53 lipoplexes (N/P ratio 3/2)	38.35 ± 2.49	3.43 ± 0.13

All the formulations with their similar nanosized particle size in range of 200-500 nm and same lipid:trehalose ratio showed similar deposition pattern with FPF in range of 35-42 % and MMAD of 3-4 μm. The MMAD of 3-4 μm indicates lung distribution in trachea, upper respiratory area and upper alveoli. When the powder comes in contact with lung fluids, the liposomes get hydrated and after getting detached from sugar moiety. These liposomes then can be internalized to give intracellular cytotoxic effect as well as pDNA transfection. The powder on impactor plates when collected and purified for DNA extraction showed supercoiled DNA on agarose gel indicating pure and stable cDNA with efficient transfection capacity.

5.10 Conclusion:

The chapter described development of β galactosidase and p53 lipoplexes by Ethanol Injection, Thin Film Hydration and Supercritical Fluid Technology. The lipoplexes were characterized for size, zeta potential, agarose retardation assay and DNAse I protection assay. In general, the liposomes and lipoplexes developed using ethanol injection method showed lowest particle size and consequently higher cellular uptake and transfection. Further, use of Transferrin as a cancer cell over-expressing receptor ligand enhanced the transfection upto 200 % and showed significantly higher cell uptake as evidenced with cell uptake studies. The lipoplex with Transferrin showed superior cytotoxicity (cell viability of 29%) than the other formulations and

showed good promise as anticancer agent for lung cancer treatment. The formulations after lyophillization was formulated as a dry powder inhaler and showed significant in vitro lung deposition pattern. Overall, the developed formulations were suitable for direct lung deposition of the anticancer lipoplexes for treatment of lung cancer.

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