Chapter 8
Summary and conclusion
8.1. Summary

Lung cancer is the most common cancer in developed and developing nations. India faces about 10% of the world lung cancer incidents. The most common etiological factor for the cause of lung cancer is smoking, which is on the rise in India. Lung cancer carried mostly by long term exposure to tobacco smoke accounts for more than 1.5 million deaths worldwide annually, with 80% mortality within a year of diagnosis. Lung cancer is currently treated with intravenous administration of chemotherapeutic agents but is non-selective as it cannot differentiate between host cells and cancer cells leading to normal cell toxicity. Further, the diagnostic tools available currently can inadequately detect the tumors and hence render the condition dejected. This provides impetus to pursue the research for effectively treating the lung cancer.

Lung cancer is well characterized by uninhibited cell growth in lung tissues leading to metastases, invasion to adjacent tissue and infiltration beyond the lungs. The two most common histological types of lung carcinoma include Non-small cell lung carcinoma (NSCLC) and small-cell lung carcinoma (SCLC). NSCLC generally leads to high mortality and hence proves to be very hostile. Although surgery is a preferred method of cancer removal, it cannot remove the tissue completely and is required to be supplemented by multi-drug chemotherapy and/or radiation as preferred treatment of choice. The chief chemotherapeutic agents used in the treatment of NSCLC are camptothecin, paclitaxel, carboplatin, cisplatin, docetaxel, topotecan, etoposide, gemcitabine etc., with their known reported toxicities. The medications are available as injections for systemic use and result in hazardous side effects due to their non-specificity on the dividing cells in the body. Intracellular transport of different biologically active molecules is one of the key problems in drug delivery in general. Currently the anticancer agents have poor intracellular concentration in the cancer cells. However, response to consequent systemic treatment is approximately 10% for single agents after the failure of initial therapy. Thus, resistance to systemic therapy does majorly rely on molecular characteristics of individual tumors rather than all-or-none phenomenon.

Ribonucleotide Reductase Subunit 1 (RRM1) gene encodes the regulatory subunit of ribonucleotide reductase, an essential enzyme that catalyses the reduction of ribonucleoside diphosphates to the corresponding deoxyribonucleotides. It is the molecular target of Gemcitabine (2', 2'-dfluorodeoxycytidine), an antimitabolite with
activity in several malignancies including NSCLC. Previously researchers had suggested that patients with low level of tumoral RRM1 expression had improved survival when treated with Gemcitabine-based chemotherapy as compared to high level of tumoral RRM1 expression. In addition, continuous exposure of lung cancer cell lines to increasing amounts of Gemcitabine resulted in increased RRM1 expression. There are proteins residing inside the cells responsible for the activation and metabolism, and thought to be a sole responsible for drug’s action. Gemcitabine is activated in cells by nucleoside kinases to Gemcitabine diphosphate (dFdCDP) and Gemcitabine triphosphate (dFdCTP). The cytotoxic effect of Gemcitabine has been attributed to the combination of two actions that lead to inhibition of DNA synthesis and subsequent apoptosis. First, dFdCDP inhibits ribonucleotide reductase which is required for augmenting the reactions that generate the deoxynucleotides for DNA synthesis and repair. Then, dFdCTP competes with dCTP for incorporation into DNA during replication, which results in a termination of chain elongation.

Recently, one study suggested that resistance to gemcitabine HCl is associated with Ribonucleotide reductase overexpression in several cancer cells. In particular, an increase in the expression of the RRM1 has been associated with gemcitabine resistance in NSCLC cell lines, while clinical studies demonstrated that NSCLC patients with low mRNA expression benefited from gemcitabine chemotherapy.

RNA interference (RNAi) is the process of mRNA degradation that is induced by double stranded RNA in a sequence-specific manner. RNA interference (RNAi) is a conserved cellular mechanism by which a small double stranded RNA (dsRNA) directs the degradation of complementary mRNA and therefore inhibits the expression of a specific gene. The ability to induce RNAi in mammalian cells using synthetic small interfering RNA (siRNA) has stimulated great interest in therapeutic applications of RNAi. In numerous studies, siRNAs have shown promise for treating a variety of diseases, including influenza and HIV infection, cancer and genetic defects. The double stranded RNA-based molecule, siRNA, has a high potential as therapeutic agent but efficient delivery into target cells is a key challenge in RNAi-based therapy. siRNA is generally having 21 nucleotides and highly charged surface with limited diffusion across the cell membrane. Further, siRNA is prone to degradation by nucleases in the circulation and interstitial space. The genetic consequences of cancer strongly support the rationale
behind the use of siRNA-mediated gene therapy in the cancer treatment. Numbers of siRNAs have been designed and investigated to target specific malfunctionally regulated oncogenes, or viral proteins involved in carcinogenesis. Furthermore, researchers have envisaged that therapeutic siRNAs can be utilized for silencing target molecules against tumor–host interactions and tumor resistance to chemotherapy and/or radiotherapy.

During the past few years, RGD peptides have become very popular agent for targeting of therapeutics and imaging agents to cancer tissue over expressing integrin. Various chemical modifications have been applied to attach RGD peptides and its modified forms to liposomes, polymers, peptides and radiotracers. RGD grafted liposomes have been investigated as an impending carrier for tumor targeting of chemotherapeutics. The present research work was aimed to develop stable siRNA nano-constructs using suitable lipid carrier in liposomal form and chemosensitization of the anticancer agent Gemcitabine HCl by pre exposure o'- siRNA encapsulated liposomes. RGD conjugated siRNA liposomes were used to target non-small cell lung cancer cells to achieve receptor based uptake of liposomal formulations and hence to avoid non selective distribution of siRNA in other tissues.

Spectrophotometric method was used for determination of siRNA content in the formulation. The determination was based on the zero order UV spectra of siRNA at the λ_max of 260 nm, developed in nuclease-free water to preserve integrity of siRNA against nucleases. Calibration plot showed a straight line expressed by the equation, y = 0.0193x + 0.0504 at 8-40 ppm concentration, with regression coefficient of 0.9994. UV-spectrophotometric method was evaluated for precision and accuracy by determining % recovery and relative standard deviation (%RSD) respectively. The % recovery was found to be between 99.5% to 100.5% and % RSD of interday and intraday measurements were below 1%, and hence, the method was found to comply with FDA and ICH guidelines on accuracy and precision of an analytical method validation.

Direct complexometric titration method was used for analysis of calcium content of liposomes. The determination was based on the formation of 1:1 complex between calcium and ethylene diamine tetraacetic acid (EDTA). Method was found to be linear having r² value of 0.9994 and had high accuracy and precision. Calibration plot showed a straight line expressed by the equation, y = 0.0214x + 0.0757.
Agarose gel electrophoresis was used for relative quantification of free siRNA migrated on the gel due to differences in the surface charge. Gel electrophoresis was carried out in TBE buffer at 100 and bands were visualized using ethidium bromide. was utilized for gel electrophoresis. The gel electrophoresis data showed that siRNA can be quantified at a minimum of 20 pmole concentration. At lower concentrations (below 20 pmole) the bands were not accurately quantifiable while at higher concentrations distinct bands were observed. To develop a calibration curve for the quantitation of siRNA, relative quantification was used i.e., the band density at highest siRNA concentration (50 pmole) being taken as 1 and evaluating band density of lower concentrations relative to the former. The results were found to be linear with regression coefficient of 0.9952 and the equation representing line was y = 0.1703x + 0.133. %recovery and %relative standard deviation of the method were found to be 102.7±2.6% and 2.55% concluding the adequacy of the analytical method for quantification of siRNA.

Two prototypes of formulations were developed for siRNA delivery i.e. cationic siRNA liposomes and calcium phosphate encapsulated siRNA liposomes.

siRNA containing cationic liposomes were prepared by incubating siRNA with preformed liposomes. Preformed liposomes were prepared by thin film hydration method containing HSPC, cholesterol, DOTAP, DOPE and mPEG2000-DSPE as lipid components and nuclease free water as hydration media. The size of liposomes was the reduced using successive extrusion through 1, 0.4, 0.2 and 0.1 μm polycarbonate membrane filter. Optimized liposomes were grafted with 1, 2 and 3 mole% of RGD by incorporation RGD-mPEG2000-DSPE into the liposome during initial stage of film formation. Liposomes were characterised for particle size and zeta potential, assay, entrapment of siRNA and surface morphology. Process parameters such as organic solvent composition, solvent evaporation time, speed of rotation, hydration time and vacuum applied were optimized to obtain desired formulation characteristics. N/P ratio was optimized to achieve complete complexation of free siRNA with cationic preformed liposomes. Liposomes were developed by incorporating different types of lipids in varying concentrations and finally DD liposomes (containing DOTAP and DOPE), DDH liposomes (containing DOTAP, DOPE and HSPC), DDC liposomes (containing DOTAP, DOPE and cholesterol), DDHC liposomes (containing DOTAP, DOPE, HSPC and cholesterol) and RGD-DDHC liposomes (containing DOTAP, DOPE, HSPC, cholesterol and RGD) were screened on
the basis of optimized N/P ratio for further studies. Here said all screened liposomes were sterically stabilized using mPEG$_{2000}$-DSPE. Further optimization was performed on the basis of particle size of liposomes after complexation with siRNA. Optimized liposomes showed complete complexation of siRNA above N/P=2.0. However, D liposomes and DD liposomes showed loose complexation at this N/P level and hence were found to be inferior. Particle size of D liposomes, DD liposomes and DDH were found to increase more than 200 nm after complexation with siRNA, while DDC liposomes, DDHC liposomes showed particle size of 174.3±6.7 nm and 145.9±8.7 nm, respectively. RGD grafting on the liposomal surface (1, 2, and 3 mole %) did not affect the size of liposomes significantly as compared to DDHC liposomes and showed particle size below 150 nm. Zeta potential of liposomes were found to decrease after complexation with siRNA. Mean zeta potential values for D liposomes (15.84±0.64 mV), DD liposomes (16.24±0.76 mV), DDH liposomes (13.39±0.87 mV), DDC liposomes (13.52±0.68 mV), DDHC liposomes (12.90±0.68 mV) and RGD-DDHC liposomes were found to lie between 11 to 17 mV as compared to initial values, which were lying between 33-39 mV. Cationic liposomes were lyophilized using sucrose as a cryoprotectant in a concentration of 50 mg/mL.

Assay was determined by Phenol/Chloroform extraction method. Extracted siRNA was collected in aqueous layer and quantified using gel densitometry and UV spectroscopy. All formulations showed no degradation of siRNA during processing and in all formulations, detected siRNA was within limit (95-105%).

All formulations were subjected to study entrapment of siRNA, either encapsulated within liposomes or complexed to the surface. Gel retardation assay method provided amount of free siRNA migration and hence, entrapped siRNA was calculated by deducting the free siRNA from initially added siRNA. Optimized formulations were also subjected to ultracentrifuge method to determine siRNA entrapment by direct analysis of liposomal fraction only, because free siRNA was removed from the supernatant after centrifugation. DDC liposomes and DDHC liposomes showed entrapment efficiency of 97.5±3.60 % and 98.2±1.89 %, respectively, as detected by gel retardation assay. More than 95% of entrapment was achieved in all the optimized formulations and that can be considered as complete complexation of siRNA with pre-formed liposomes. RGD grafting did not affect the entrapment efficacy and difference between with and without RGD grafting was insignificant.
Images obtained by Transmission Electron Microscopy revealed that prepared liposomes were spherical in shape. All vesicles are unilamellar in structure and having particle size below 200 nm. Bilayer thickness was also measured and found to be in-between 5-10 nm in size.

siRNA containing CPE liposomes were prepared in two steps; Step-1: Preparation of calcium phosphate liposomes and Step-2: Loading of siRNA in calcium phosphate entrapped liposomes. Both steps were optimized individually. However, liposomes prepared in step-1 were consequently used in step-2 and hence, step-2 relied on the product quality obtained from step-1. Calcium phosphate liposomes were prepared by the selected TFH method using DPPC, cholesterol, DOPE and mPEG\textsubscript{2000}-DSPE. Liposomes were hydrated using calcium chloride solution and Particle size of liposomes was reduced using successively passing through 1, 0.4, 0.2 and 0.1 μm polycarbonate membranes. Unentrapped calcium was removed by passing through sephadex column. Calcium entrapped liposomes were made permeable by addition of ethanol and incubated with disodium hydrogen phosphate solution. This addition allowed formation of calcium phosphate precipitates inside as well as outside of the liposomes. Outer precipitates were removed by centrifugation and unreacted phosphate was removed by passing through sephadex column. These liposomes were added with siRNA in presence of ethanol and incubated at 48°C for 20 minutes. Optimal formulation was further improved by incorporation of cyclic RGD peptide for its capability to target tumor cells. Prepared CPE liposomes were characterised for calcium entrapment by complexometric titration, Assay, entrapment of siRNA, particle size and zeta potential and surface morphology. The size of Liposomes was measured by dynamic light scattering with a Malvern Zetasizer. Assay and entrapment of siRNA were detected as mentioned in above summarised cationic liposomal section.

Liposomes were optimized to maximize entrapment and minimize particle size. Calcium phosphate liposomes (step-1) and siRNA loading (step-2) was optimized using $3^3$ factorial design by varying lipid:calcium molar ratio (0.1, 0.3 and 0.5), DPPC: cholesterol molar ratio (1.0, 5.0 and 9.0), and concentration of calcium (75, 100 and 125 mg/mL) for step-1 and by varying Calcium:siRNA molar ratio (5.0, 7.0 and 9.0), Lipid:Ethanol molar ratio (1.0, 1.5 and 2.0), and siRNA concentration (10.0, 12.0 and 14.0 μg/mL) for step-2 at 3
different levels as low (-1), medium (0) and high (1), by keeping all other process and formulation parameter invariant.

RSM was applied to fit second order polynomial equations, obtained by multiple linear regression analysis (MLRA) approach. Two dimensional contour plots and three dimensional response surface plots were established by varying levels of two factors and keeping the third factor at fixed levels at a time. Optimized formulation was derived by specifying goal and importance to the formulation variables and response parameters.

For calcium phosphate liposomes (obtained from step-1), the optimized batch (lipid:calcium molar ratio= 0.29, DPPC:cholesterol molar ratio = 1.0, and concentration of calcium = 87.72 mg/mL) showed calcium entrapment of 23.470 ± 1.173 and particle size of 114.745 ± 3.101. Final formulation i.e. calcium phosphate encapsulated siRNA liposomes (step-2) with optimized batch (Calcium:siRNA molar ratio = 6.83, Lipid:Ethanol molar ratio = 1.51, and siRNA concentration = 10.82) showed siRNA entrapment of 83.86±2.19 and particle size 117.85±2.15.

P-value > 0.05 indicates the differences between predicted and experimental values are statistically insignificant. In checkpoint analysis higher r² values (0.9947 and 0.9995 for PDE and PR respectively) of the linear correlation plots suggest excellent goodness of fit and high predictive capability of RSM.

Assay values for CPE and RGD-CPE liposomes were found to lie within the limit i.e. 95-105%. Incorporation of RGD at 1, 2 and 3 mole% did not affect the calcium and siRNA entrapment efficiencies. Calcium entrapment for 1. 2 and 3 mole % RGD incorporated liposomes was found to be 24.37±1.62 %, 23.78±1.09 % and 22.81±1.36 % respectively, while siRNA entrapment was 81.72±3.02 %, 84.19±2.89 % and 82.93±3.45 % respectively. Zeta potential values for CPE liposomes, RGD-CPE liposomes(1%), RGD-CPE liposomes(2%), RGD-CPE liposomes(3%) were found to be 11.90±0.52, 12.21±0.13, 11.45±0.44 and 11.23±0.31 respectively. Clear and distinct precipitates were seen during electron microscopy and same can be seen in the images taken from microscopy. Bilayer thickness was also measured and found to be in-between 5-10 nm in size.

Prepared liposomal formulations were characterised for in vitro cell line studies. The cytotoxicity of siRNA carriers were determined using 3-(4, 5-dimethylthiazole-2-yl)-2,5-di-phenyl tetrazolium bromide (MTT) assay. cells were treated separately with DD
liposomes, DDC liposomes, DDHC liposomes and RGD-DDHC liposomes at varying N/P ratio ranging from 2.5 to 12.5 in DMEM media containing 10% FBS and antibiotics. In case of CPE liposomes and RGD-CPE liposomes cytotoxicity was carried out using increasing amount of CPE liposomes by varying Ca:siRNA ratio (5.0 to 15.0). Cells treated with PBS were considered as negative control and commercially available non-viral lipid transfecting carrier Lipofectamine 2000 was kept as positive control. It was seen that at N/P of 2.5 DDHC (composed of DOTAP, DOPE, HSPC and Cholesterol) and RGD grafted DDHC liposomes were significantly less toxic than lipofectamine 2000 in both cell lines i.e A549 and H1299. Even at higher N/P ratio of 12.5 at 100 nM siRNA concentrations these liposomes were non-significant in toxicity as compared to Lipofectamine 2000. At all ratios, CPE liposomes and RGD-CPE liposomes showed significantly higher cell viability than positive control lipofectamine 2000. Further, there was no significant difference in cell viability by RGD incorporation (1%, 2% and 3%).

For cellular uptake studies, FAM labeled negative control siRNA (FAM-NC-siRNA) was used. Flow cytometry was utilized for quantitative cell uptake to determine the mean fluorescent intensity while qualitative intracellular accumulation was determined using confocal microscopy. Liposomal formulations containing FAM-NC-siRNA at a final concentration of 100 nM were exposed to A549 and H1299 cells and analysed for mean fluorescence activity using fluorescence activated cell sorter. Naked FAM-NC-siRNA and Lipofectamine 2000 complexed siRNA were used as negative and positive control respectively. 2 mole% of RGD was found to be optimal for both types of liposomal formulations in A549 as well as H1299 cells. Fluorescence intensity in A549 cells after treatment with various siRNA formulations was as follow: Naked siRNA < DDC liposomes < CPE liposomes < Lipofectaine 2000 < RGD-CPE liposomes < DDHC liposomes < RGD-DDHC liposomes. In H1299 cells the order of fluorescence intensity in cells after treatment with various siRNA formulations was as follow: Naked siRNA < DDC liposomes < CPE liposomes < Lipofectaine 2000 < DDHC liposomes < RGD-CPE liposomes < RGD-DDHC liposomes. Maximum MFI in A549 cells were found to be 88.67±1.02 and 82.40±1.47 for RGD-DDHC liposomes (2%) and RGD-CPE liposomes (2%) respectively while, Maximum MFI in H1299 cells were found to be 80.30±0.70 and 75.83±1.05 for RGD-DDHC liposomes (2%) and RGD-CPE liposomes (2%), respectively. MFI values for Lipofectamine 2000 in A549 and H1299 were 74.63±1.39 and 71.83±1.19 respectively.
Cellular internalization of FAM labelled siRNA in A549 and H1299 cells was monitored by confocal microscopy. Cells were transfected with liposomal formulations containing 100nM of FAM labelled siRNA. Cells were also stained with nucleus staining dye DAPI and proceeded for confocal microscopy using confocal laser scanning microscope. Further, Live imaging was performed using confocal microscopy to access the potential of RGD grafting on the liposomal surface. After 6 h incubation, FAM-NC-siRNA was mainly observed in cytoplasm with a relative uniform distribution. Confocal microscopy also showed that RGD grafting helps to enhance the cellular localization in both cell lines. Live images revealed that naked siRNA only bound to the surface or lie outside of the cells. DDHC liposomes and CPE liposomes get accumulated inside the liposomes soon after transfection but RGD-DDHC and RGD-CPE liposomes showed different pattern for uptake. They initially bound to the cell surface and surface bound liposomes further taken up inside the cells. These results suggest the receptor based translocation of liposomal siRNA inside the cell.

Chemosensitization is well governed at sub inhibitory concentration and hence, cell cycle analysis was used to determine the DNA content of cells at varying concentration of RRM1 siRNA i.e. 50 pM, 100 pM, 500 pM and 2.5 nM. Cells were transfected with RRM1 siRNA containing RGD-DDHC liposomes at varying siRNA concentrations. Cells were fixed by ethanol, stained with propidium iodide and analysed using FACS. Results suggested that 50 pM of RRM1 siRNA concentration is sub-inhibitory concentration and can be taken for chemosensitization.

siRNA mediated transfection was studied by quantifying mRNA knock-down of RRM1 gene by the mean of silencing potential of RRM1 siRNA containing formulations. Real time PCR (RT-PCR) was utilized to quantify the amount of mRNA present in the transfected cells of both cell lines (A549 & H1299). RGD grafted formulations showed significantly higher mRNA knock down (p < 0.05) than non-grafted liposomes in both cell lines and also more transfection was achieved as compared to lipofectamine 2000 at 5 nM concentrations. At 5 nM concentration RGD-DDHC liposomes and RGD-CPE liposomes showed 23.2±2.6% and 24.2±3.4% gene expression, while naked siRNA exhibited 83.50±2.5% gene expression in A549 cells. In H1299 cells, gene expression was found to be 23.05±2.85% for RGD-DDHC liposomes, 23.95±3.55% for RGD-CPE liposomes and 85.9±2.5% for naked siRNA. At lower concentrations, 500 pM and 50 pM, inhibition was
markedly decreased with both liposomal formulations and Lipofectamine 2000. RT-PCR also suggested that 50 pM is the sub-growth inhibitory concentration which was utilized for further Chemosensitization studies.

Chemosensitization effect was evaluated by studying the cytotoxic effect of Gemcitabine HCl in RRM1 siRNA pre-exposed lung cancer cells. In vitro cytotoxicity of anticancer drug Gemcitabine HCl at sequential concentrations was assessed with pre-treatment of RGD grafted siRNA nano-constructs (RGD-DDHC liposomes and RGD-CPE liposomes) in A549 and H1299 cells. Gemcitabine HCl solution (Gem.sol.) and Gemcitabine HCl liposomes (Gem. lipo.) were used as chemotherapeutic agents. IC₅₀ values for these different sets of cells were used to compare the chemosensitization efficacy. Chemosensitization effect was described by the fold change in IC₅₀ values when siRNA liposomes were pre-exposed in different sets of cells. The order of IC₅₀ value for Gemcitabine HCl in both A549 and H1299 cells were as follow: Gemcitabine solution < Gemcitabine liposomes < RGD-CPE liposomes (2%) + Gemcitabine solution < Lipofectamine 2000 + Gemcitabine solution < RGD-DDHC liposomes (9%) + Gemcitabine solution < Lipofectamine 2000 + Gemcitabine liposomes < RGD-CPE liposomes (2%) + Gemcitabine liposomes < RGD-DDHC liposomes (2%) + Gemcitabine liposomes.

Highest chemosensitization for cationic liposomes (fold change=5.11 in A549 and 4.94 in H1299) was observed in cells pre-treated with RGD-DDHC liposomes (2%) followed by treatment with Gemcitabine liposomes as compared to treatment with Gemcitabine solution alone while, RGD-CPE liposomes (2%) showed 4.94 and 4.20 fold change in IC₅₀ value in A549 and H1299 cells respectively in same sets of cells. The order of fold change in IC₅₀ values for RGD-DDHC liposomes was more as compared lipofectamine 2000 and no significant difference was observed between RGD-CPE-liposomes and lipofectamine 2000. Results suggest the efficacy of developed formulation for chemosensitization of lung cancer cells against Gemcitabine HCl by pre-exposure of RRM1 siRNA in liposomal form.

The siRNA liposomes were studied for the integrity of incorporated siRNA in presence of serum at pH 7.4 for possible in vivo degradation because of degradation during circulation, and degradation due to extracellular and intracellular RNase. Naked siRNA, RGD-DDHC liposomes (2%) and RGD-CPE liposomes (2%) were incubated with non-heat inactivated FBS at 37°C for various time periods. At 0.5 h more than 10% of siRNA was degraded,
which reached up to 60% within 3 h. siRNA release at blood pH was also ascertain to maintain the siRNA within liposomes and hence to achieve the intracellular localization. After 6 h less than 20% of siRNA was remained as compared to initially loaded siRNA. RGD-DDHC liposomes and RGD-CPE liposomes encapsulated siRNA was stable even after 24 h and more than 75% of siRNA was preserved. Upto 8 hrs > 95% of the siRNA was preserved in both types of liposomes. It is generally observed that most of the liposomal formulations are distributed to different tissue within 6 hrs of injection and hence, prepared cationic formulation will be able to face in vivo fate.

Haemolytic toxicity of formulated liposomes was checked by incubating the formulation with erythrocyte separated from rat blood by centrifugation at low speed and analysing the supernatant at 541 nm. The haemolysis with different formulations was compared with that obtained with Triton-X100 as a positive control. RGD-DDHC liposomes (2%) showed 8.90±0.40% haemolysis while RGD-CPE liposomes (2%) showed only 2.645±0.05% haemolysis. Maximum haemolysis was observed with DD placebo liposomes i.e. 27.6±13.0%.

Prepared liposomal formulations in three different categories, i.e. without PEGylation, with PEGylation and with RGD grafting, were studied for electrolyte induced flocculation test. This test confirms the stability of liposomal formulations in presence of electrolyte in vivo. siRNA containing liposomal formulations at (100 nM) were incubated at varying concentration of sodium chloride i.e. 1, 2, 3, 4 and 5%. After 1 hr of incubation at 37°C particle size was determined. Particle size of non-pegylated liposomes was found to increase significantly at all concentration of added salt in both, cationic and CPE, types of liposomal formulations. However, again in both cases, incorporation of 5 mol% of mPEG2000-DSPE did help in maintaining the particle size. Upto 2% NaCl addition was found to maintain particle size of DDHC and RGD DDHC liposomes below 150 nm. Addition of 3% and above concentration of salt increased the particle size upto 300 nm. Particle size of the CPE and RGD-CPE liposomes was maintained below 150 nm upto 3% electrolyte addition. Even 5% salt did not able to cross particle size beyond 200 nm.

In vivo toxicity of developed liposomal formulations were evaluated in female swiss albino mice. For determination of maximum tolerated dose (MTD) of siRNA loaded liposomes, Fixed Dose Procedure of OECD-Organization for Economic Cooperation and Development was used. Typical protocol includes administration of a drug/drug product in
escalating doses through intravenous route and observing animals for any signs of toxicity. Fixed dose test substances (siRNA loaded liposomes) were administered in a constant dose volume of 20 mL/kg. All doses were prepared prior to administration. Above maximum therapeutic doses of siRNA loaded liposomes (0.75 mg/kg of siRNA), only liposomal carrier (without siRNA loading) was tested to ascertain the safety profile of developed liposomal carrier systems. MTD values for RGD-DDHC liposomes and RGD-CPE liposomes were found to be > 0.75 mg/kg of siRNA, whereas RGD-DDHC liposomes (placebo) and RGD-CPE liposomes (placebo) showed MTD values of >300 mg/kg and >600 mg/kg of total lipids respectively.

The stability testing of prepared liposomal formulations, RGD-DDHC liposomes and RGD-DDHC liposomes was performed at accelerated condition (25°C ± 2°C, 60% RH ± 5% RH) for six months and at long-term conditions (2-8°C) up to six months. Various parameters, i.e. assay, siRNA entrapment, particle size and zeta potential, were evaluated after each predetermined time points (1, 2, 3 and 6 month). Apart from these, water content was also determined for RGD-DDHC liposomes. In case of RGD-DDHC liposomes both accelerated and refrigerated conditions, Assay and siRNA entrapment values were found within range (95-105% of initial) and change was non-significant. There was no significant increase in particle size and zeta potential after six month at both conditions. Water content was increased to a significant extent (3.79 ± 0.19% w/w at 6 month) at accelerated condition while refrigerated condition maintained the water content value < 3% w/w even after six months of storage. While in case of RGD-CPE liposomes, accelerated condition showed that Assay value (94.62 ± 2.90 at 6 month) and siRNA entrapment (75.80 ± 1.87 at 6 month) were found to decrease significantly as compared to initial values. Particle size was also increased significantly from first month only. At 2-8°C condition, Assay and siRNA entrapment values were found to lie within range (95-105% of initial value) and particle size was also maintained below 150 nm.

8.2. Conclusions
To conclude, RRM1 siRNA encapsulated nanoconstructs in liposomal forms were successfully prepared. Two types of liposomal formulations were developed, i.e. cationic liposomes containing DOTAP as a key cationic lipid and calcium phosphate encapsulated liposomes containing calcium phosphate complexed siRNA inside the liposomes. Both formulations were optimized to achieve maximum siRNA encapsulation. Particle size of
the liposomes was also chosen as one of the optimization parameters as particle size was found to increase in case of cationic liposomes due to complexation between cationic lipid and siRNA. Developed formulations were well characterised using Cryo-TEM and proved the bilayer structure of liposomes with uni-lamallarity. Presence of calcium phosphate precipitates inside the RGD-CPE liposomes was clearly observed. Both the formulations showed less cell cytotoxicity at therapeutic and higher concentrations. RGD grafting on liposomal surface was found to increase the cell uptake of siRNA. Optimal RGD concentration was found to be 2 mole%. RGD grafted liposomes significantly increased intracellular localization of siRNA as compared to non RGD grafted liposomes. Further, RGD also governed the cell uptake via receptor mediated pathway, which was shown by live uptake studies. Sub-inhibitory concentration (50 pM) was obtained by cell cycle analysis and transfection studies. mRNA quantification after 2.5 nM concentration of siRNA showed transfection efficacy of developed liposomal formulations. More than 75% of gene silencing was obtained with both liposomal formulations and hence transfection efficacy was proven. Pre-exposure of siRNA liposomes at 50 pM concentration sensitized the lung cancer cells against Gemcitabine HCl up to 5 fold in both the lung cancer cells. Haemolysis study showed that PEGylation markedly decreased the haemolysis of erythrocyte RGD-DDHC liposomes(2%) and RGD-CPE liposomes(2%) at very high lipid concentration (5 mM) showed less than 10% of haemolysis as compared to >25% haemolysis with DOTAP:DOPE placebo liposomes. This high percentage of haemolysis might be due to amount of free DOTAP lipid, which is carrying very high positive surface charge. RGD-DDHC and RGD-CPE both liposomes preserved the siRNA in the liposomal forms against serum nucleases and less than 5% of siRNA was released within 8 hrs in presence of serum at pH 7.4. Effect of addition of electrolyte to govern flocculation was well studied and PEGylation was found to maintain the particle size against added electrolyte for maintaining the particle size. Both formulations showed non-significant change in particle size (below 150 nm) upto 2% addition of electrolyte. However, RGD-CPE liposomes maintained the particle size (below 200 nm) even upto 5% of electrolyte concentration. In vivo toxicity studies revealed that developed RGD grafted formulations are safe to administer even at much higher concentrations than required for achieving therapeutic effect. Stability studies indicated that both formulations should be stored at 2-8°C/refrigerated condition. At 25°C±2°C / 60±5RH% condition, RGD-DDHC liposomes in
lyophilized form showed increase in water content and RGD-CPE liposomes showed decrease in assay and entrapment values. Hence, refrigerated condition was recommended. Present investigation shows a promising way to treat lung cancer using genomic approach with enhanced margin of safety and reduced dose dependent toxicity of the Gemcitabine HCl. Pre-exposure of RGD grafted siRNA liposomes targeting RRM1 protein caused sensitization of cancer cells. Developed liposomes are stable in presence of serum and delivered the siRNA inside the cells along with efficient transfection. Sub-inhibitory concentration of siRNA will avoid toxicity related to localization of siRNA in unwanted sites. Taken collectively, suggested approach will definitely open a vista in the era of cancer treatment with reduced dosing profile.