

CHAPTER 7: SUMMARY AND CONCLUSION

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 nonselective 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.

Liposomes are microscopic or sub-microscopic bilayer vesicles with size ranging from 10 nm to 20 μ m. They are composed of one or several bilayers enclosing aqueous compartments. When phospholipids are hydrated, they spontaneously form lipid spheres (liposomes) enclosing the aqueous medium and the solute. Kulkarni et al. (1995) have explained the mechanism of liposome formation upon hydration of phospholipids. Phospholipids are amphipathic molecules containing a hydrophobic tail and a hydrophilic or polar head.

Because of this amphipathic nature, phospholipids form closed bilayers in the presence of water. When phospholipids are exposed to water the fatty acid tails align towards each other, excluding water from this hydrophobic domain in that process. Conversely, the polar head groups orient themselves towards the bulk aqueous phase, leading to a bilayer configuration. The large free energy difference between the aqueous and the hydrophobic environment promotes the formation of bilayer structures in order to achieve the lowest free energy level. Bilayer structures do not exist in the absence of water, because it is the water that provides the driving force for lipid molecules to assume a bilayer. Depending on the number of bilayers formed and diameter of the vesicles, liposomes are broadly classified into small unilamellar vesicles (SUVs; single bilayer, size 10 to 100 nm), large unilamellar vesicles (LUVs; single bilayer, size 100 to 1000 nm), and multilamellar vesicles (MLVs; several bilayers, size 100 nm to 20 μ m). Liposomes have a wide range of therapeutic application ranging from topical cosmetics to the intracellular delivery of genetic materials. An array of compounds can be encapsulated in liposomes, including small molecules, proteins, and nucleic acids. The therapeutic applications of liposomes have been extensively reviewed in the literature. A major advantage of liposome carriers is their ability to alter the pharmacokinetics of the free drug. The particulate nature of the liposomes causes them to be distributed within the body in a pattern significantly different from that of the free drug. Drugs with varying lipophilicities can be encapsulated in liposomes, either in the phospholipid bilayer, in the entrapped aqueous volume or at the bilayer interface. Numerous procedures have been developed to prepare liposomes. There are at least fourteen major published methods for making liposomes. The seven, most commonly employed methods are, Lipid film hydration method, Ethanol injection method, Ether infusion method, Detergent dialysis method, French press method, Rehydration-dehydration techniques and Reverse phase evaporation method.

The use of liposomes as drug delivery agents has evolved from a line of research originating over 40 years ago, based on the ability of these unilamellar vesicles to entrap material in an aqueous compartment. It was then known that most amphipathic membrane lipids form multilamellar vesicles (MLV) consisting of concentric bilayers when they are dispersed in aqueous media. MLV are relatively large (micron) sized structures, however they can be extruded through 100 nm pore size polycarbonate filters to produce unilamellar vesicles with a homogeneous size distribution. Typically, the resulting liposomes are 100 nm in diameter and each particle contains numerous lipid molecules. Liposomes have been widely used as models of biological membranes to study membrane permeability and transport across the bilayer. In addition to their utility as model membranes, drugs may be

encapsulated within their interior aqueous compartment. The ability of liposomes to deliver drugs preferentially to disease sites, such as solid tumors, can result in considerable improvements in efficacy; therefore, liposomes are widely studied for use in therapeutic applications.

The concept of site specific drug delivery for treatment of localized disease in the body to improve therapeutic index of the drug is considered as perennial challenge to the formulator in modern formulation design. Constant efforts have been pursued in designing such an ideal drug delivery system which can effectively overcome dose related toxicity and adverse side effects and thus improve patient compliance. One such area which has attracted ever growing attention of pharmaceutical scientist and has shown tremendous potential and promise is colloidal drug carrier system. The idea of drug carrier with targeted specificity has fascinated scientists for number of years and in the last decade successful efforts have been made to achieve this goal. The ultimate form of targeted drug delivery system should be realization of Paul Ehrlich's "magic bullet concept" which documents the delivery of drug exclusively to a preselected targeted cell type.

Amongst all targeted drug delivery systems, Liposomes are recently gaining popularity because of their biological inert nature, freedom from antigenic, pyrogenic or allergic reaction and their enhanced stability. Vastly improved technology in terms of drug capture, vesicle stability on storage, scale-up production and the design of formulations for special tasks has facilitated the application of a wide range of drugs in the treatment and prevention of diseases in experimental animals and clinically. Liposomes are micro-particulate or colloidal carriers which form spontaneously when certain lipids are hydrated in aqueous media. Liposomes are composed of relatively biocompatible and biodegradable material and they consist of aqueous volume trapped by one or more bilayers of natural or synthetic lipids. Generally hydrophobic molecules are incorporated into the lipid bilayers whereas hydrophilic compounds are entrapped in the internal aqueous volume.

The delivery of liposomes at the appropriate site, however, is still not achieved. For this purpose, both active targeting and passive targeting are considered. Conventional liposomes, however, tend to be trapped by the reticuloendothelial system (RES) such as liver and spleen before encountering the target. On the contrary, passive targeting, especially targeting to tumor tissues, could be achieved by reducing the RES trapping, since the vasculature in the tumor tissues is leaky enough to extravasate liposomes and circulating liposomes may accumulate passively in tumor tissues. The development of liposomes containing lipid derivatives of PEG or saturated phospholipids such as DSPC with cholesterol

has made targeted liposomal therapy more feasible by reducing the uptake by the RES system and thereby prolonging the circulation time. Particularly, PEG is useful because of its ease of preparation, relatively low cost, controllability of molecular weight and linking ability to lipids or peptide including RGD peptide by a variety of methods. Active targeting of liposomes to tumor cells is generally attempted by conjugating ligands to the liposomal surface which allow a specific interaction with the tumor cells. Several types of ligands have been used for this purpose, including antibodies or antibody fragments, vitamins, glycoproteins, peptides (RGD-sequences), and oligonucleotide aptamers. Among the different approaches of active targeting, RGD grafted liposomes using RGD motif as a targeting ligand and a lipid vesicle as a carrier for both hydrophilic and hydrophobic drugs, is a fascinating prospect in cancer therapy. Targeting using small peptides like RGD has certain advantages over the use of conventional protein macromolecules. These include ease of preparation, lower antigenicity, and increased stability. RGD peptides have reportedly been used to deliver cytotoxic molecules to the tumor. Tumor vasculature is a suitable target for targeted cancer therapy because it is composed of nonmalignant endothelial cells that are genetically stable and therefore unlikely to mutate into drug-resistant variants. In an attempt to reduce macromolecular ligands to small recognition sequence, the tri-peptide motif arginine-glycine-aspartic acid (RGD) was identified as the minimal essential cell adhesion peptide sequence in fibrinectin. Since then, cell adhesive RGD sites were identified in many other extracellular matrix proteins, including vitronectin, fibrinogen, Van Willebrand factor, collagen, laminin, osteopontin, tenascin and bone sialoprotein. It has also been identified in membrane proteins of viral and bacterial origin, and in snake venoms. The conformations of the RGD containing loop and its flanking amino acids in the respective proteins are mainly responsible for their different integrin affinity. RGD sequence constrained in a cyclic conformation was reported to bind $\alpha_v\beta_3$ integrins with high affinity. Using small peptides, such as RGD, as targeting ligand has several advantages over large macromolecular ligands, such as antibodies. Production of proteins is costly and time consuming. A large quantity of protein must be used because of its high molecular weight. On the other hand, small peptides, can be easily synthesized and small amounts of peptides are enough for effective targeting. 2) Proteins may elicit an undesirable immune response such allergic reactions to certain antibodies derived from murine origin. 3) Conformation and orientation of the protein effects receptor binding. 4) Small peptide motifs exhibit higher stability toward sterilization conditions, heat treatment and pH variation, storage, and conformational shifting as well as easier characterization and cost effectiveness. 5) Because of lower space requirements peptides can be packed with a higher density on to surfaces. Rapidly proliferating angiogenic endothelial cells in tumor vasculature can be selectively

targeted using RGD peptides that bind to $\alpha v\beta 3$ integrin on these endothelial cells. In the light of the available literature to achieve success rate in cure of lung cancer having second highest incidence and mortality rate. The current cure chemotherapy for lung cancer has limitation being non-selective and manifests in toxicity. This project aims to develop a liposomal targeted delivery system for the chemotherapeutic agent gemcitabine HCl, to selectively take this drug to the tumor site. The overall hypothesis is that liposome drug carriers bearing appropriate ligands can be targeted to tumor cells via up-regulated adhesion molecules. Spectrophotometric method was used for determination of gemcitabine content in the formulation. The determination was based on the zero order UV spectra of GEM at the λ_{\max} of 268 nm, developed in methanol. Calibration plot showed a straight line expressed by the equation, $Y=0.0342x - 0.0362$ at 2-30 ppm concentration, with regression coefficient of 0.9993. The % RSD of interday and intraday measurements were below 2%, and hence, the method was found to comply with FDA and ICH guidelines on accuracy and precision of an analytical method validation.

The prototypes of formulation was developed for gemcitabine delivery. GEM containing liposomes were prepared by prepared by thin film hydration method containing DPPC, DSPG, cholesterol and mPEG₂₀₀₀-DSPE as lipid components and GEM solution in 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, 3 and 5 mole% of RGD by incorporation RGDmPEG₂₀₀₀-DSPE into the liposome during initial stage of film formation. Liposomes were characterized for particle size and zeta potential, assay, entrapment of GEM 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.

The liposomes composed of various lipids were used to encapsulate drug. The lipids such as HSPC, DPPC and DMPC were primary lipid for liposomes preparation and gemcitabine HCl encapsulation. Combinations of lipids were tried to encapsulate gemcitabine HCl. Optimization of the lipid composition for Gemcitabine HCl loaded liposome were done on the basis of drug entrapment. Different lipid compositions were tried and optimized for maximum drug entrapment within minimum amount of lipid. The drug entrapment were found to be highest for DPPC based liposomes followed by HSPC and DMPC based liposomes. In addition, the drug entrapment for the liposomes composed of combination of DSPG with HSPC, DPPC and DMPC based liposomes were also higher than the combination of DPPG with HSPC, DPPC and DMPC based lipid structures. Overall, amongst the various lipid combination tried the PDE of the liposomes composed of DPPC and DSPG were found to be the highest.

GEM liposomal formulations were optimized using 3^3 full factorial design by varying the Drug: lipid molar ratio (1:5, 1:10 and 1:15), DSPG molar % (1, 2 and 3) and hydration volume (1.5mL, 3mL and 5mL) at three different levels such as low (-1) middle (0) and high (1) for higher drug content and lower particle size by keeping all other process. The increase in drug: lipid ratio shows increase in drug loading and mean particle size. At 1:10 drug to lipid ratio we observed maximum GEM loading and minimum particle size. With increase in drug: lipid ratio beyond this level (i.e. 1:15) we observed similar amount of drug loading but increased mean particle size than 1:10. Hence, we considered drug to lipid ratio of 1:10 as optimal condition. The DSPG level was optimized (2 mol %) for maximum drug content by preparing liposomes at different levels of DSPG by varying the DPPC level while keeping cholesterol concentration as constant. The decrease in GEM loading was observed when the DSPG ratio was 1 mol % as compared to 2 and 3 mol %. There was no significant improvement in the drug content was observed at 2 and 3 mol % DSPG levels. The hydration volume of 3-5mL was considered as optimal. We observed increase in GEM loading and decrease in mean particle size with increase in hydration volume from 1.5mL to 5mL and in between 3-5mL we observed similar results. The optimized formulation showed % GEM loading of $62.06 \pm 1.52\%$ and mean particle size of $126 \pm 3\text{nm}$ (PDI: 0.242 ± 0.022) was observed at drug to lipid ration of 1:10, DSPG level of 2 mol% and hydration volume of 3mL. Different drug loading levels at optimized drug : lipid ratio (1:10) were tried and optimized. The drug loading levels from 5mg/ml to 10mg/ml were tried and optimized for maximum drug entrapment. The drug level was optimized (5 mg/ml) for maximum drug content by preparing liposomes at different levels of drug loading at optimized drug: lipid ratio. There was no significant improvement was observed in the drug content at 5mg/ml - 10mg/ml drug loading.

At optimized formulation and process variables the liposomes containing different concentrations of cholesterol were prepared to determine the effect of cholesterol concentration on mean particle size and % drug content and loaded drug retention character. GEM liposomes containing different concentrations of cholesterol were prepared and analysed for % GEM loading, mean particle size. The maximum % GEM loading ($62.06 \pm 1.52\%$) and minimum mean particle size ($126 \pm 3\text{nm}$) was observed at cholesterol concentration of 1 mol%. As the cholesterol concentration was increased from 1 mol% to 4 mol% the decrease in % GEM loading and increase in mean particle size was observed. After 2 weeks of storage at $2-8^\circ\text{C}$ the drug content of this formulation was found to be maximum ($56.81 \pm 2.20\%$) and mean particle size was minimum ($150 \pm 5\text{nm}$) as compared to other

formulations. Hence, the cholesterol concentration of 2 mol% was considered optimal because at this cholesterol concentration the formulation showed minimum drug leakage ($10 \pm 2\%$) as compared to other concentrations.

Optimal formulation containing DPPC, DSPG and cholesterol was further improved by incorporation of (1mol%, 3mol% and 5mol %) mPEG2000-DSPE (PEGylated) and tumor cells targeting cyclic RGD peptide-polymer conjugate; (1mol%, 3mol% and 5mol %) RGD-mPEG2000-DSPE (Functionalised) was also added along with above listed lipids in the initial phase during thin film formation to incorporate mPEG2000-DSPE and RGD into the liposomes. The mean particle size and % GEM content of formulation were analysed and it was noted that upon increasing the DSPE-mPEG₂₀₀₀ and DSPE-mPEG₂₀₀₀-RGD concentration the GEM loading increases slightly but not significantly. Although the mean particle size remains unchanged at 1 and 3 mol%, we observed increased in mean particle size at 5mol%. The zeta potential of prepared PEGylated and functionalized liposomes were found to be slightly increased (-55.2 ± 5.2 mV) as compared to conventional liposomes (-43.6 ± 4.9 mV). Prepared liposomes were lyophilized to impart physical stability to the liposomes. Various types of cryoprotectants are used at different ratio to optimize the lyophilization and to preserve particle size during freeze drying.

The lyophilized formulations were tested for particle size, zeta potential and physical appearance. Lactose, mannitol and sucrose were used at three different concentrations i.e. 20 g/mL, 40 mg/mL, 60 mg/mL in the final formulation (RGD- liposomes (3%). Lactose and mannitol did not preserve the particle size of liposomes. At all concentration these two sugars failed to maintain the particle size below 200 nm. Sucrose did perform the task by maintaining the size of liposomes at 40 mg/mL and 60 mg/mL concentrations. At all concentrations the maintenance of particle size by these cryoprotectants followed below given order: Lactose < Mannitol < Sucrose. At all concentrations of used cryoprotectants, the zeta potential value did not change significantly. This result suggest the liposomal dispersion stability and hence preservation particle aggregation after lyophilization. Lower concentration of cryoprotectant i.e. 20 mg/mL did not form physical good cake. Lyophilized material was not in an intact form and poor quality of cake was formed. Less than 40 sec were required to reconstitute the lyophilized formulations with all types of cryoprotectant. However, at higher concentration 60 mg/mL, more than 60 sec were required for the reconstitution. Water content at 20 mg/mL and 40 mg/mL concentrations were found below 2% w/w. As sucrose preserved the particle size within narrow range as compared to non-lyophilized liposomes, the concentration having minimum particle size, good cake property

and good redispersion property was selected. Taking collectively these results, 40 mg/mL of sucrose as a cryoprotectant was chosen.

Particle size and zeta potential of the optimized RGD PLs liposome was found to be 135 ± 4 nm and -55.52 ± 0.55 respectively. Water content of all lyophilized samples such as conventional, peylated and RGD-grafted liposomes were found to contain below 2% of moisture after lyophilization. Images obtained by TEM revealed that prepared liposomes are spherical in shape and all vesicles are unilamellar in structure and having particle size below 200 nm.

Prepared liposomal formulations were characterised for *in vitro* cell line studies. The cytotoxicity of GEM carriers were determined using 3-(4, 5-dimethylthiazole-2-yl)-2,5- diphenyl tetrazolium bromide (MTT) assay. cells were treated separately with free drug, CL liposomes, PLs liposomes and RGD-grafted liposomes at varying different concentrations of gemcitabine (0.01, 0.1, 1 and 10 μ M) in DMEM media containing 10% FBS and antibiotics. It was seen that all formulations resulted in concentration dependent inhibition of the proliferation of A549. The lowest cell viability, i.e. the highest cell mortality, appeared at the highest concentration of the liposomal formulations, which proves the controlled and sustained efficacy of the liposomal formulation. Furthermore, the liposomal formulations prevent the toxic effect of the drug applied at high concentration of drugs and thus can increase the maximum tolerance dose (MTD). It is clear from the results that the PLs and RGD grafted liposomes demonstrated higher cytotoxicity than the free drug formulation at the same drug concentration and exposure time, which means that for the same therapeutic effect, the drug needed for the PLs and RGD grafted liposomal formulation could be much less than that for the free drug. Therefore, the development of the PLs and RGD grafted liposomes thus can enhance the therapeutic effect of gemcitabine.

For cellular uptake studies, rodhamine encapsulated liposomes 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 rodhamine at a final concentration of 10 μ M were exposed to A549 cells and analysed for mean fluorescence activity using fluorescence activated cell sorter. 3 mole% of RGD was found to be optimal for liposomal formulations in A549. Fluorescence intensity in A549 cells after treatment with various siRNA formulations was as follow: CLs liposomes < RGD-grafted liposomes. Maximum MFI in A549 cells were found to be 85.15 ± 1.22 for RGD-grafted liposomes (3%) and 66.08 ± 1.34 for CLs.

Cellular internalization of rhodamine loaded liposomes in A549 cells was monitored by confocal microscopy. Cells were transfected with liposomal formulations containing 10 μ M of rhodamine. 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, rhodamine liposomal 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. The CLs liposomes get accumulated inside the liposomes soon after transfection but RGD grafted 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 GEM 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.

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 (2%)+ 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.

Hemolysis study was performed to investigate the potential toxicity after the intravenous injection of PLs, CL and RGD grafted liposomes *in vivo*. 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- liposomes (3%) showed 4.68±0.65% haemolysis. Maximum haemolysis was observed with CLs liposomes i.e. 14.95±0.550%.

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*. GEM containing liposomal formulations (CLs, PLs and RDG grafted liposomes) 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 liposomal formulations. However, again in both cases, incorporation of 5 mol% of mPEG₂₀₀₀-DSPE did help in maintaining the particle size. Upto 3% NaCl addition was found to maintain particle size of PLs and RGD grafted liposomes below 200 nm. Addition of 4% and above concentration of salt increased the particle size upto 300 nm.

In vivo toxicity of developed liposomal formulations were evaluated in female swiss albino mice. For determination of maximum tolerated dose (MTD) of GEM 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 (GEM 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 GEM loaded liposomes (100mg/kg of GEM), only liposomal carrier (without GEM loading) was tested to ascertain the safety profile of developed liposomal carrier systems. MTD values for RGD-grafted liposomes were found to be >100mg/kg of GEM, whereas RGD-grafted liposomes (placebo) showed MTD values of >3000mg/kg of total lipids respectively.

The stability testing of prepared liposomal formulations, RGD-grafted liposomes was performed at accelerated condition ($25^{\circ}\text{C} \pm 2^{\circ}\text{C}$, $60\% \text{ RH} \pm 5\% \text{ RH}$) for three months and at long-term conditions ($2-8^{\circ}\text{C}$) up to three months. Various parameters, i.e. assay, entrapment, particle size and zeta potential, were evaluated after each predetermined time points (1, 2 and 3 months). Apart from these, water content was also determined for RGD-grafted liposomes. In case of RGD-grafted liposomes both accelerated and refrigerated conditions, Assay and GEM entrapment values were found within range (90-115% of initial) and change was non-significant. There was no significant increase in particle size and zeta potential after three month at both conditions. Water content was increased to a significant extent ($3.19 \pm 0.54\% \text{ w/w}$ at 3 month) at accelerated condition while refrigerated condition maintained the water content value $< 3\% \text{ w/w}$ even after three months of storage..

8.2. Conclusions

To conclude, GEM encapsulated nanoconstructs in liposomal forms were successfully prepared. The liposomal formulations were developed, containing DPPC, DSPG and CH as lipid. The formulations were optimized to achieve maximum GEM encapsulation. Particle size of the liposomes was also chosen as one of the optimization parameters as particle size was important for the entry of nannoconstructs to tumor vasculature. Developed formulations were well characterised using Cryo-TEM and proved the bilayer structure of liposomes with uni-lamallarity. Presence the size of the liposomal formulation was ariun 150 nm and the liposomes were uni-laminar in nature. The formulations showed less cell cytotoxicity at therapeutic and higher concentrations. RGD grafting on liposomal surface was found to increase the cell uptake of GEM. Optimal RGD concentration was found to be 3mole%. RGD grafted liposomes significantly increased intracellular localization of GEM 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. 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. The conventional liposomes without PEG showed much higher extents of hemolysis rate than the PEGylated liposomes at all the concentration ($p < 0.05$). Without PEG, CL induced mild hemolysis (~15%). On the contrary, the hemolysis rates in all PEGylated liposomes were less than 5%. It is known that, PEG is a highly hydrated polymer and has a high degree of segmental flexibility in aqueous solution. Further RGD grafted liposomes (3%) showed $4.68 \pm 0.65\%$ haemolysis. Maximum haemolysis was observed with CLs liposomes i.e. $14.95 \pm 0.550\%$. 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 200 nm) upto 3% addition of electrolyte. *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 $25 \pm 2^\circ\text{C}$ / $60 \pm 5\text{RH}\%$ condition, RGD-grafted liposomes in lyophilized form showed increase in water content. Hence, refrigerated condition was recommended. Present investigation shows a promising way to treat lung cancer using targeted drug delivery 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. 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.