# Chapter 10
# Summary and Conclusions

# SUMMARY

## Preamble

Cancer is one of the dreaded diseases that has thrown open a challenge to oncologists and scientists throughout the world. In particular, solid tumors have posed many challenges in systemic chemotherapy. According to World Health organization's (WHO) estimation, each year there will be around 20 million new cancer patients in the world, and more than 70 % of these patients will be in developing countries. Due to the high rate of mortality, cancer chemotherapy has been the imminent choice of research for scientist worldwide. The metastasizing consequence of cancer makes it more dreadful and throws open a lot of challenges in effective curing. Currently, the most exhaustively researched area in the pharmaceutical field deals with the carrier systems that selectively deliver drug molecules to the target site without disturbing the integrity of the healthy tissues in the body. Though effective, these anticancer drugs produce side effects due to the bio-distribution to other organs in addition to the target organ. This culminates in a narrow therapeutic index due to high level of toxicity in healthy tissues. Encapsulating the drugs in carrier systems such as liposomes will significantly reduce the volume of distribution and increase drug accumulation in the tumour; this in turn is bound to reduce the non-specific toxicity associated with the drug.

Encapsulating the drugs in liposomes and nanoparticles offers attractive possibilities for cancer therapy. This process of encapsulating pharmacologically active compounds in vesicular systems offers supplementary benefits such as protecting the drug from premature degradation and inactivation, in addition to facilitating easy excretion from the body. Liposomes are one of the preferred delivery systems for targeting the molecules as they are made up of natural biological lipids and have structural resemblance to cell membranes. The inherent advantages associated with liposomes are bio-degradability, reduced toxicity, low immunogenicity and ability to encapsulate hydrophobic as well as hydrophilic drug candidates. But the major disadvantage with the liposomal system is its rapid clearance from the blood stream by mononuclear phagocyte system (MPS), particularly in liver and spleen.

Significant advances have been made in overcoming many of the limitations associated with liposomal drug delivery. An elusive problem has been the difficulty in selectively increasing the bio-availability of the drug at the target tissue, while maintaining stability in the circulation. A recent investigation aimed at exploiting the pH of the tumor in order to release the contents into the cytoplasm. This approach relies on selective destabilization of liposomes following the acidification of the immediate vicinity of tumors in order to trigger destabilization. Such pH-Sensitive liposomes have been designed to release at least part of their contents into the cytosol prior to reaching the lysosomes. The pH sensitive liposomes can be targeted to areas of the body such as the sites of primary tumors and metastases or sites of inflammation and infection wherein leakage of liposomal "cargo" takes place due to low pH.

PEGylated or sterically stabilized liposomes have been proved to overcome MPS uptake. The polymeric coating of polyethylene glycol provides steric stability to the liposomes by creating a hydrophilic surface that limits the binding of serum opsonins and prevents direct interaction with the cells, most importantly with the reticuloendothelial system (RES).

**Key objective of the investigation:**

The prime objective of the present study was to formulate a stable intravenous preparation of pH sensitive, serum stable, long circulating liposomes containing anticancer drugs such as Paclitaxel (PCL) and Irinotecan Hydrochloride Trihydrate (IH) for effective treatment of cancer. The PEGylated pH sensitive liposomes encapsulating anticancer drugs are supposed to increase the blood circulation time due to avoidance of MPS uptake, and provide site specific targeting and tumor accumulation due to enhanced permeation and retention (EPR) effect. Thus, sterically stabilized pH sensitive liposomes injected into the bloodstream should release the drug into the tumour site after prolonged circulation in the body. This kind of drug delivery would not only minimizes the interactions of drugs with the healthy tissues of the body (thereby reducing the side effects associated with the system), but would also enable significant dose reduction. Hence, overall enhancement of therapeutic efficacy would be achieved.

**Investigation presented and discussed in this thesis:**

The present investigation deals with the preparation, characterization and *in-vivo* evaluation of conventional and pH sensitive liposomes containing anti-cancer drugs, namely Paclitaxel and Irinotecan.

***Chapter 1*** contains an introduction, present chemotherapy techniques available for cancer treatment and objectives of the present study.

***Chapter 2*** covers the literature related to cancer, lipids and polymers used for preparing pH sensitive delivery systems.

***Chapter 3*** narrates the profiles of the drugs used in the present study.

***Chapter 4*** deals with the development of the analytical methods for the estimation of the Paclitaxel and Irinotecan as the bulk drug in the formulation and in plasma/serum. Non-interference of the lipids used in the preparation (DOPE, HSPC, CHEMS, Cholesterol and mPEG-DSPE) with the analysis of drug has also been confirmed. The analytical methods were then evaluated for their accuracy and precision. These methods were subsequently subjected to statistical analysis and a range of statistical parameters were established. The results reveal that the analytical methods are accurate and highly precise. A simple spectrophotometric method was then developed for estimation of Paclitaxel and Irinotecan. For determination of Paclitaxel a HPLC method was developed to be used for estimating the sample in plasma. This was followed by the development of a sensitive, accurate and specific fluorimetric estimation method for the assessment of Irinotecan in serum/plasma. The developed method yields high relative fluorescence values for extracted Irinotecan in plasma/serum with good correlation coefficient values. This study reveals the high potential of fluorimetric estimation of Irinotecan in biological samples such as plasma.

***Chapter 5*** discusses the preparation, characterization and stability testing of liposomal formulations. Conventional liposomes of PCL and IH were prepared by lipid film hydration method while liposomes were formulated using a molar ratio [DMPG: HSPC: Chol] of 6: 2: 2.

In case of Paclitaxel, drug and all the lipids were dissolved in a mixture of chloroform and methanol (ratio 7:3 v/v) in a round bottom flask. The flask was rotated in a rotary flash evaporator at 100 rpm for 45 min in a thermostatically controlled water bath at 57 °C under vacuum (600mm of Hg) in order to remove traces of organic solvents. The thin dry lipid film formed was hydrated using 2 ml of PBS pH 7.4 and the flask was rotated once again at the speed of 80 rpm at the same temperature as above for 45 min to produce liposomal suspension. The liposomal suspension was then transferred to a suitable glass container and sonicated for 2 minutes and kept for annealing at room temperature for 2 h. The Multilamellar vesicles formed were sequentially extruded through stacked polycarbonate filters of 400-100nm for 4 times at 57 ± 2 °C. The unentrapped drug was removed by slow centrifugation at 5000rpm and collecting the settled free drug. Then the liposomal suspension was centrifuged at 25,000 rpm for 30 min and liposomal pellet was separated. This separated pellet was resuspended in fresh PBS pH 7.4 and freeze dried along with sucrose which was used as cryoprotectant (3 times by weight of total lipids) overnight at -20 °C, lyophilized for 24 h and stored in vials at 2-8 °C. In case of IH also sucrose was used as cryoprotectant (4 times by weight of total lipids).The lyophilized powder was rehydrated with water for injection before use.

Conventional liposomes of Irinotecan were also prepared in the similar manner. The only difference is that the drug was introduced into the system as a part of the hydration medium and not included along with the solution of lipids used for drying.

PCL and IH were determined using a UV-spectrometric method. The proportion of the system components like total lipids and the drug as well as the process parameters such as the volume of the solvent system, volume of hydration medium, hydration time, vacuum applied, speed of rotary flash evaporator and sonication time were optimized based on the percent drug entrapment. Drug: lipid ratio (1:10) for PCL and (1:15) for Irinotecan resulted in good encapsulation.

The major factors affecting drug encapsulation are drug: lipid ratio, total lipid: Chol ratio and volume of hydration medium. Accordingly, a $3^3$ factorial design was used to

investigate the combined influence of the above three variables in the liposomal preparation by thin film hydration method keeping the hydration time and vacuum applied constant. Based on the above factorial design, 27 batches were prepared for each drug. The liposome batches were evaluated for percent drug entrapment (PDE). By using factorial design the maximum drug entrapment that could be achieved was 84.56 % for PCL and 73.56 % for IH. Further, it has become possible to predict the PDE for various combinations of formulation variables using the contour plots.

Long circulating PEGylated pH sensitive liposomes were prepared in the same method except changing the combination of lipids keeping all other parameters same. The lipids (DOPE: HSPC: CHEMS: Chol: mPEG-DSPE) were dissolved in Chloroform and methanol mixture along with PCL and thin film was prepared using rotary flash evaporator. The dried film was hydrated with HEPES buffer pH 8.2 for 45 min. The liposomal suspension was then sonicated for 2 min and kept passed through polycarbonate filters 400-100 nm and extruded 4 times to get unilamellar vesicles. The free drug was separated using centrifugation at 5000 rpm and the unentrapped drug settled down was analysed. The supernatant was separated and centrifuged at 25,000rpm for 30 min and liposomal pellet was separated from which the encapsulated drug was analysed. The liposomal suspension which is free from unentrapped drug was lyophilized using sucrose as cryoprotectant (3 times by weight of total lipid) in case of PCL. In case of IH also sucrose was used as cryoprotectant (4 times by weight of total lipids).

The encapsulated drug was analyzed by developed UV-spectrometric method. Drug: lipid ratio (1:10) for PCL and (1:15) for Irinotecan gave good encapsulation.

The major factors effecting drug encapsulation were Drug: lipid, total lipid: Chol and volume of hydration medium. So, $3^3$ factorial design was used to investigate the combined influence of three variables in the liposomal preparation by thin film hydration method keeping the hydration time and vacuum applied constant throughout the study. Based on factorial design 27 batches were prepared for each drug. The liposome batches were evaluated for percent drug entrapment (PDE). By using factorial design the

maximum drug entrapment could achieved was 87.13 % for PCL and 75.18 % for IH and could predict the PDE for various combinations of formulation variables using contour plots.

The amount of $mPEG_{2000}$-DSPE required for steric stabilization was optimized using electrolyte induced flocculation test. Here, a fixed volume of liposomal suspension was diluted to a predetermined volume using different molar concentrations of an electrolyte, sodium sulphate. The absorbance of the resulting suspension was spectrometrically measured. The concentration of the steric stabilizing agent, which did not allow any significant change in the absorbance when different molar concentrations of the electrolyte were added to liposomes was selected as the optimum concentration and used for further studies. 5 mol% of the total lipids $mPEG_{2000}$-DSPE was found adequate for steric stabilization of the liposomal suspension in both cases of PCL and IH. It was observed that the presence of combination of lipids leads to better entrapment of PCL and IH as compared to conventional liposomes. The entrapment was not affected by the presence of the steric stabilizing agent. This was followed by characterization of conventional and PEGylated pH sensitive liposomes containing PCL and IH for various aspects.

The particle size distribution and zeta potential of the conventional as well as pH sensitive liposomes have been estimated by Malvern Nano-ZS particle size analyzer which uses New Dynamic Light Scattering Technique or Photon Correlation spectroscopy. All the liposomal preparations (conventional as well as PEGylated pH sensitive liposomes) were found to have a mean particle size ranging from 130nm to 200nm after repeated extrusion through polycarbonate filters. However, PEGylated pH sensitive liposomes exhibited narrower size distribution and lower uniformity value compared to conventional liposomes.

The morphology and lamellarity of the liposomes prior to extrusion were analysed through photomicrographs of the suspension taken using Olympus microscope which revealed spherical structure of liposomes. The photomicrographs revealed the spherical

shape of the liposomes. It was observed that majority of the liposomes formed in each case were multilamellar before extrusion. Some unilamellar vesicles were also found along with the multilamellar vesicles due to the sonication process adopted.

DSC thermograms were obtained by differential scanning calorimetry of the pellets of liposomal formulations on a Mettler, star SW 8.10 differential scanning calorimeter. Powder X-ray diffraction patterns were obtained using an X-ray diffractometer (Philips PW 1710). These studies revealed the status of the crystal structure of the drug inside the liposome. The DSC thermograms and X-ray diffraction patterns indicated the amorphous nature of the drug encapsulated inside the liposomes.

The Transverse Electron Microscopy (TEM) photographs of the pH sensitive liposomes reveal that the liposomes were spherical in shape and the particle sizes were well below 200nm after extrusion.

pH induced flocculation study in buffers with varied pH demonstrated that as the buffer pH shifted towards acidic side the particle size of the liposomes increased and flocculation of the liposomes.

Serum stability study of the pH sensitive liposomal preparations revealed the aggregation process of colloidal system on storage in serum due to the adherence of serum proteins and a consequent increase in size and zeta potential values over a period of time.

Drug release kinetic studies from liposomes were carried up to 72 hours in phosphate buffered saline, pH 7.4 and for 1 hour in pH 5.0 as dissolution media, subjecting the liposomes to dialysis using sigma dialysis membrane under controlled temperature and agitation. The *in-vitro* release studies revealed that the long circulation property of Paclitaxel and Irinotecan loaded pH-sensitive liposomes in PBS pH 7.4 compared to the plain drug solution and conventional liposomes of the respective drugs, where as complete destabilization at cytosolic acidic pH of 5.0. Different dissolution models were applied to drug release data of pH sensitive liposomal formulations in order to evaluate

release mechanisms and kinetics. The drug release data fit well to zero order kinetics but fairly good fit into the Higuchi and Peppas expression (in PBS pH 7.4) from pH sensitive Paclitaxel liposomes whereas, it demonstrated Peppas and Higuchi expression in acidic buffer pH 5.0. In case of Irinotecan pH sensitive liposomes the drug release data fit well to Peppas but fairly good fit to zero order expression (in PBS 7.4) whereas Peppas and Higuchi expression in acidic buffer pH 5.0.

Stability of the PEGylated pH sensitive liposomal suspension and lyophilized cake containing PCL or IH was investigated. The samples were subjected to stability studies, in triplicate, at conditions according to ICH guidelines. At predetermined time intervals, samples were removed and studied for parameters such as size and size distribution, uniformity value, zeta potential, assay and drug retention. Change in particle size, uniformity value and zeta potentials were determined by Malvern particle size analyzer. Percent drug leached from the liposomes was determined by centrifuging the liposomal suspension and analyzing the supernatant for the drug content in case of IH. In case of PCL, the unentrapped drug from the suspension was separated first by slow centrifugation. Then the liposomal pellet was separated by ultracentrifugation and drug content was analyzed. Assay was carried out as per the methods developed and explained in analytical development section.

The Lyophilization of liposomal suspensions was carried out using sucrose, trehalose and maltose but due to high drug retention capacity in liposomes, sucrose was finalized and used in all the formulations. The liposomal suspensions were freeze dried along with sucrose -3 times by weight of total lipids for PCL and 4 times by weight of total lipids for IH overnight at -20 °C, lyophilized for 24h. The lyophilized liposomal products were also tested for its stability at two temperatures mentioned above. At predetermined time intervals, samples were removed, rehydrated and studied for different parameters.

Results of the stability studies indicate that the liposomal suspensions are not suitable for commercialization. The liposomal suspensions are relatively more stable at refrigerated condition in comparison to samples kept at 30 ± 2 °C/65 % RH. But, the samples which

were lyophilized exhibited very good stability on storage at different temperatures selected for stability study, up to 6 months for both the drugs.

***Chapter 6*** reports the *in-vitro* cell line studies of liposomal formulations in B16F1 (low metastatic) and B16F10 (highly metastatic) melanoma cell lines. The *in-vitro* cytotoxicity of the drugs (PCL and IH) and their liposomal formulations were evaluated using MTT [3-(4, 5-dimethyl thiazole-2yl)-2, 5-diphenyl tetrazolium bromide] assay. MTT cell proliferation assay offers a quantitative and convenient method for evaluating a cell population's response to external treatment, whether it is increase in cell growth, no effect or a decrease in growth due to necrosis or apoptosis. The yellow tetrazolium salt is reduced in metabolically active cells to form insoluble purple crystals of formazan which is solubilized using solvents like DMSO or propanol. The colour intensity can be quantified using spectrophotometric means. For each cell type, a linear relationship between cell number and absorbance has been established, which enables direct quantification of changes in cell proliferation.

Various concentrations of free drugs and their liposomal formulations were incubated with a specific number of cells for various time periods at 37 °C at 5% $CO_2$ atmosphere. The results of the MTT assay showed that there was a concentration-dependent cytotoxic effect for both the anticancer drugs used. In particular, the pH sensitive liposomal formulations and conventional liposomal formulations showed similar cytotoxic effects as that of free drug after 48 hours, in both the cell lines used for the study. Consequently, all further studies were carried out keeping the incubation period as 48 h.

As the concentration of the drug (in free form as well as in liposomal formulation) increases, there was a gradual reduction in the % cell viability. This explains the concentration dependent cytotoxic effect of PCL / IH and their liposomal formulations. The cytotoxicity pattern produced by PCL / IH and their liposomal formulations were almost similar and not much significant difference was observed between them in both the cell lines. $IC_{50}$ values for plain drug solution and its liposomal formulations were arrived at by plotting the graph of % viability against log concentration. Subsequent

studies were carried out using two sub-toxic concentrations of the respective $IC_{50}$ values of particular treatment.

Haematoxyline and Eosin stained cover slips containing B16F1 and B16F10 cells showed significant changes in the morphology of cells for both the drugs and their liposomal formulations compared to untreated cells in Leighton tube assay. Cells showed destruction of the cell wall, nucleus and cell protrusions which resulted in the formation of a large number of dendrites. Cells appeared spindle-shaped with some amount of hypertrophy as compared to control cells.

Confocal microscopy revealed *in-vitro* cellular uptake and intracellular distribution of pH sensitive liposome encapsulating FITC-Dextran qualitatively. The cytosolic delivery of the pH sensitive liposomes loaded with fluorescent material in both B16F1 and B16F10 cell lines was clearly observed. Within 1 hour the fluorescent material was inside the cell, indicating rapid internalization of pH sensitive liposomes. This trend increased with increase in incubation time, confirming the time dependant accumulation.

The colony inhibition effect of one of the sub-toxic (below $IC_{50}$ value) concentrations of different treatments showed that pH sensitive liposomes inhibited the colony formation to the maximum by reducing the growth in a number of colonies compared to plain drug solution and conventional liposomes of both the drugs in B16F1 and B16F10 cell lines. The percentage colony inhibition was highest for pH sensitive liposomes compared to all other treatments, even at sub-toxic concentrations.

Wound assay, one of the simple methods to analyze migration of cancer cells, was made use of in both B16F1 and B16F10 melanoma cell lines. The percent migration and relative wound widths for wounds treated with different formulation treatments were calculated after 48 hours of treatment. In both the cell lines, pH sensitive liposomal formulations showed larger wound width which depicts the less migration of cells and thereby increased anti-migratory activity compared to other treatments. The relative wound width was more in the case of pH sensitive liposomes. It was therefore concluded

that even at sub-toxic concentration, pH sensitive liposomal formulations exhibit excellent anti-migration activity.

The pH sensitive liposomal formulation of PCL arrested B16F1 and B16F10 melanoma cells in the $G_2$-M phase of cell cycle similar to that of plain PCL, where as pH sensitive liposomal formulation of IH arrested the S-phase of the cell cycle at the sub-toxic levels. Analysis of DNA content of Propidium Iodide (PI) stained B16F1 and B16F10 melanoma cells showed more significant dose-dependent inhibition in the $G_2$-M phase for pH sensitive liposomes of PCL and S-phase for IH as compared to the control cells and placebos which are in $G_0$-$G_1$ phase in both the cell lines.

***Chapter 7*** deals with Radiolabeling of conventional and PEGylated pH sensitive liposomes along with the plain drugs. Liposomes were labeled with high efficiency by the direct labeling technique using reduced $^{99m}$Tc. The pertechnetate used for the study was reduced to its lower valence state using stannous chloride dihydrate and then the pH was adjusted to neutral before mixing with drug or liposomal suspension. The radiolabeling was optimized by taking three factors into account. Viz., pH of the complex, incubation time and concentration of stannous chloride dihydrate ($SnCl_2.2H_20$). The optimum conditions required for maximum labeling efficiency were established. The radiolabeling of PCL and IH and their liposomal formulations were radiolabeled after reducing the technetium with $SnCl_2.2H_20$ at pH 7, incubated for 30 minutes yielded maximum labeling efficiency of more than 98 %.

Stability of the $^{99m}$Tc-labeled complexes (PCL, IH and their liposomal formulations) with time was studied with saline and serum at 37 °C for different time intervals upto 24hours. The experimental data revealed that there was minimal detachment of the radioisotope from the complex even after a period of 24 hour incubation. Only 3-5 % decrease in labeled product signifies the high stability of the radiolabeled complex and its suitability for *in-vivo* use. DTPA and Cysteine challenge tests demonstrated the high binding capacity of $^{99m}$Tc with PCL, IH and their liposomal formulations.

***Chapter 8*** presents the *in-vivo* bio-distribution and scintigraphy study in Balb/c mice by radiolabeling technique. The tumor targeting studies were also carried out in Ehrlich Ascites Tumour (EAT) bearing mice. The biodistribution of liposomes administered in vascular and extra vascular spaces was studied by the administration of radiolabeled liposomes, followed by scintigraphic imaging.

The blood kinetic study of PCL, IH and their liposomal formulations were carried out in Balb/c mice at different time intervals after i.v. administration of the radiolabeled complex through the tail vein. The plain drug was eliminated from the body much faster compared to conventional liposomes. The PEGylated pH sensitive liposomes showed enhanced circulation compared to conventional liposomes. Even after 48 hours, PEGylated pH sensitive liposomes showed higher blood concentration compared to conventional liposomes.

Bio-distribution studies were performed for the plain drugs PCL, IH and their liposomal formulations in 2-3 months old EAT bearing Balb/c mice. Fixed amount of $^{99m}$Tc-drug / $^{99m}$Tc-liposome complex was administered through the tail vein of the mouse. The mice were sacrificed at different time intervals and blood was obtained by direct cardiac puncture. The blood was weighed and the radioactivity present in the whole blood was calculated, considering 7.3% of the body weight as total blood weight. Subsequently, tissues (heart, lung, liver, spleen, kidney, stomach, intestine and tumor) were dissected, washed with normal saline, made free from adhering tissues, weighed and their radioactivity was measured in a shielded well gamma scintillation counter. Results of the *in-vivo* biodistribution studies indicated that mPEG-DSPE containing pH sensitive liposomes produced steric stabilization compared to conventional liposomes. Plain drug and conventional liposomes were found to be cleared from the body quickly compared to PEGylated pH sensitive liposomes. These liposomes showed enhanced circulation in the body and increased tumor accumulation compared to conventional liposomes and plain drugs. The pH sensitive liposomes accumulated more than 9 folds (in case of Paclitaxel) and 8 folds (in case of Irinotecan) in tumour compared to normal muscle.

Conventional liposomes showed significant increase in accumulation in liver and spleen compared to PEGylated pH sensitive liposomes. It could be described as the combined action of the circulating blood passing through the organ as well as that due to particle uptake by cells of reticuloendothelial system (RES). Even after 24 hours, the very low amount of radioactivity in stomach confirmed that radiolabeled complexes were stable *in-vivo*.

Scintigraphy studies were carried out in EAT bearing mice after administration of fixed amount of $^{99m}$Tc labeled complexes through the tail vein. The mice were fixed on the board and imaging was done using a Single Photon Emission Computerized Tomography (SPECT) gamma camera. The scintigraphy images revealed that conventional liposomes and pH sensitive liposomes clearly accumulated in various organs including liver, spleen and tumour, whereas very less accumulation in tumour occurred by plain drug. The conventional liposomes showed increased liver and spleen accumulation in comparison to PEGylated pH sensitive liposomes because of increased opsonisation. pH sensitive liposomes accumulated much more in tumour at all the time points as compared to conventional liposomes and plain drug.

***Chapter 9*** deals with antimetastatic activity of the PCL, IH and their liposomal formulations in C57BL/6 mice. The most dreaded aspect of cancer is its ability to spread or metastasize very rapidly. To assess the antimetastatic potential of drugs and their liposomal formulations, pretreated B16F10 melanoma cells were injected through tail vein of the mice and after 20 days the animals were sacrificed, lungs were resected and analysed for metastasis. The lung colonies or metastatic nodules formed on the surface were manually counted for each animal. The average number of colonies for different formulation treatment and percentage inhibition in lung nodule formation was calculated. pH sensitive Paclitaxel liposome inhibited lung nodule formation by 95.45 % whereas 72.83 % by pH sensitive liposomes of Irinotecan which is much better than their respective conventional liposomes and plain drug. It became evident from the data that even at sub-toxic level, pH sensitive liposome treated B16F10 melanoma cells inhibited lung homing and thereby enhanced antimetastatic activity. Haematoxyline and Eosin stained sections of the lungs were microscopically observed. The tissue sections were

systematically observed for area of metastasis, melanocytes with pigments, macrophage with pigment, mitotic figures, pleomorphism, epitheloid cells and necrotic foci. Scoring of individual animals showing microscopic changes in their lung was graded accordingly. Very few tumor islands were observed in the slides of lungs which were treated by pH sensitive liposomes compared to untreated cells. Hence it is confirmed that pH sensitive liposomes containing anticancer drugs offer a positive and effective approach to the antimetastatic therapy of a variety of tumors.

## CONCLUSIONS

The present study has brought out findings which may be effectively utilized in improving the therapeutic efficacy of anticancer agents through the use of pH sensitive liposomal system.

The results of this work have revealed the following:

1. pH sensitive liposomes encapsulating Paclitaxel or Irinotecan can be prepared by lipid film hydration technique using DOPE as pH sensitive lipid with high encapsulation efficiency. The combination of lipids used in the system helped in improved encapsulation of anticancer drugs and did not alter the pH sensitivity of the system to any appreciable extent.
2. Freeze dried pH sensitive liposomes containing Paclitaxel and Irinotecan showed excellent stability at refrigerated temperature and at 30 ± 2°C/ 65 % RH compared to liposomal suspension.
3. At sub-toxic level, pH sensitive liposomes containing Paclitaxel or Irinotecan showed excellent *in-vitro* cytotoxicity, cell colony inhibition and anti-migratory activity judged against plain drugs and conventional liposomes.
4. Increased cellular uptake and intracellular distribution was observed with pH sensitive liposomal formulations prepared with combination of lipids.
5. pH sensitive liposomes were labeled with high efficiency by the direct labeling technique using reduced $^{99m}Tc$ with consequent high level of *in-vivo* stability.

6. PEGylated pH sensitive liposomes containing Paclitaxel or Irinotecan showed enhanced serum stability and long circulatory effect compared to conventional liposomes.
7. Sterically stabilized liposomes containing Paclitaxel or Irinotecan confirmed target specific drug delivery and increased accumulation of anticancer drugs at the tumor site when treated in Balb/c mice model.
8. PEGylated pH sensitive liposomes containing Paclitaxel or Irinotecan showed excellent *in-vivo* antimetastatic activity and inhibition of lung nodule formation when treated in C57BL/6 mice model.

Based on the above findings, it can be postulated that pH sensitive liposomal system has the potential to improve the chemotherapy of certain cancers. The effective use of this system will help in reducing the dose of neoplastic agents for a given degree of therapeutic response. This system may also provide excellent openings for drugs which are resistant to certain cancers due to their inherent cell uptake phenomenon. Their effectual handling will not only increase patient compliance but will also reduce the systemic toxicity and side effects associated with the drug. These novel drug delivery systems can play an imperative role in overcoming the limitations associated with the current cancer chemotherapy.