Chapter 7

Summary and Conclusions

The research in localized delivery of anti cancer drugs directly to tumor sites has evoked a considerable interest recently due to obvious advantages over conventional routes. Localized drug delivery is a way to deliver the drug from a dosage form to a particular site in the biological system, where its entire pharmacological effect is desired. The localized delivery of chemotherapeutic agents has been explored to limit their indiscriminate toxicities on normal tissues. Localized delivery of paclitaxel will give sustained drug exposure to tumor cells and increase its tumor penetration and decrease the rate of replication of tumor cells. Local administration of chemotherapeutic agents at the tumor site is also thought to enhance the chemo responsiveness by exposing tumors and adjacent metastases to high drug concentration while reducing its systemic exposure. Two approaches have been widely studied for localized delivery of anti-cancer drugs. First is the dermal delivery and second is subcutaneous (SC) and intratumoral administration of hydrogel formulation. In the present study both approaches have been selected for localized delivery of paclitaxel.

In current clinical practice paclitaxel is administered by intravenous route. The i.v. administration of paclitaxel distributes the drug into some vital organs like heart, liver and kidney leading to very severe cardiotoxicity, myelosuppression and mucositis. In addition to this, very little amount of drug reaches the target tumor tissue like breast and skin cancers, resulting in very poor therapeutic efficacy. Paclitaxel is highly efficacious against many skin cancers, such as classical and HIV-associated forms of Kaposi’s sarcoma and basal cell carcinoma. However, the usefulness of paclitaxel for the therapy of skin cancer is limited due to the serious adverse effects associated with the i.v. administration. Such adverse effects may be circumvented by the development of topical formulation for localized delivery into viable skin layers. In spite of the obvious advantages associated with the cutaneous delivery of paclitaxel, there is no topical formulation commercially available. Breast cancer treatment also requires localized
delivery of paclitaxel. Localized delivery of paclitaxel can maintain desired tissue concentration by noninvasive zero order delivery, which would enhance its efficacy for the treatment of skin and breast cancers, with high patient compliance.

However, high molecular weight of paclitaxel makes it difficult for it to penetrate through a dense and hydrophobic stratum corneum at a rate sufficient to achieve therapeutic efficacy. One strategy to achieve the high local drug concentration at tumor vicinity and also sustain the drug release is to encapsulate paclitaxel in elastic liposomes. Elastic liposomes are basically modified liposomes that are of several orders of magnitude, more deformable than the conventional liposomes and thus well suited for the skin penetration and localized delivery. The elastic liposomes has advantages like high skin penetration and deposition potential, ability to accommodate drug molecules with a wide range of solubility, suitability for high as well as low molecular weight drugs, high drug encapsulation efficiency, biocompatibility and biodegradability. Elastic liposomes can deliver drugs in zero-order fashion for prolonged period of time and are easy to scale up as the method of preparation is simple.

In the present study an attempt was made to develop a paclitaxel formulation, which is free from Cremophor EL and enable the localization of high drug concentration at tumor site. To achieve these objectives two approaches were studied. In the first approach, elastic liposomal formulation was prepared and extensively characterized in-vitro, ex-vivo and in-vivo. The results obtained were compared against the marketed paclitaxel formulation. The safety profile of the elastic liposomal formulation was studied by conducting acute, sub-acute toxicity and histopathology studies. In the second approach, elastic liposomal in situ thermosensitive hydrogel formulation was studied.

The obtained gift sample of paclitaxel was identified and characterized for its purity. The absorption maximum of paclitaxel was obtained at 229 nm, which is comparable to the value reported in the literature. The drug purity was further confirmed by comparing IR spectra and melting point with the literature values.

A simple, reliable, reproducible and validated analytical method is required during various stages of experimental work in vitro, ex-vivo and in-vivo. In order to
estimate paclitaxel, calibration curves were prepared at $\lambda_{\text{max}}$ 229 in methanol, 1% SLS: PBS (pH 7.4) and PBS (pH 7.4): methanol (1:1). The estimation procedure was found to be fairly reproducible and linear in the concentration range of 2-20 $\mu$g/mL. Correlation coefficient value was about 0.99 in all the cases. For measurement of paclitaxel in biological matrices like skin, plasma, tissue homogenates and cell culture, etc., more sensitive HPLC method at a concentration range of 0.2-2.0 $\mu$g/mL was developed and validated. Solubility of paclitaxel in phosphate buffer saline (PBS) pH 7.4, 1% w/v SLS in PBS (7.4), 2% w/v SLS in PBS (7.4) and PEG 400: PBS (7.4) was determined for selection of receptor fluid to provide the sink condition for in vitro drug release, skin permeation, deposition and drug encapsulation efficiency measurement studies. Paclitaxel was found to have the maximum solubility in PBS (pH 7.4) containing 1% w/v SLS and it was selected as receptor fluid.

The elastic liposomes were prepared by conventional rotary evaporation sonication method. Different batches of elastic liposomes were prepared using surfactant and phospholipid in different ratio. The major ingredients of elastic liposomes are phospholipid (vesicle forming component) and surfactant as edge activator (for providing the flexibility to vesicle membrane) in the optimum ratio. In this study, Span 80 (Formulation code EL-SP1-6) and sodium deoxycholate (Formulation code EL-SD1-6) were used as surfactant at different concentrations (0.25, 0.5, 0.75, 1.0, 1.25 and 1.5% w/v of total lipid concentration 5% w/v). Span 80 was selected as the edge activator due to its biocompatible and lipophilic nature. Sodium deoxycholate was also selected as the edge activator due to its biocompatible nature and structural feature. This bile salt molecule exhibits facial polarity, with its hydrophilic group on the concave face of the molecule and lipophilic steroidal skeleton on the convex face of the molecule. Due to this structural feature, it is easy to assimilate sodium deoxycholate with lipid bilayer of vesicle membrane. Conventional liposomes consisting of phospholipid and cholesterol (7:3) was used as control for comparison purpose. Formulation and process variables such as amount of drug loaded in formulation, surfactant type and concentration, hydration media and sonication time were optimized. Formulation optimization was
carried out on the basis of drug encapsulation efficiency, size, shape, elasticity, vesicles population, drug loading and turbidity measurement studies.

Elastic liposomal formulations were found to have a spherical shape and vesicular structure. Morphological characterization by Transmission Electron Microscopy (TEM) showed that there was no change in vesicle structure upon increasing the concentration of surfactant from 0.25 to 0.75% w/v and further increase in concentration from 0.75 to 1.5% w/v lead to decrease in vesicle population. The drug entrapment efficiency of elastic liposomes was also found to depend on the surfactant concentration. After a threshold level (0.75% w/v), further increase in the surfactant concentration lead to decrease in drug entrapment efficiency. This is possibly because at a lower concentration (up to 0.75% w/v), surfactant molecules get associated with the phospholipid bilayer resulting in better partitioning of drug. Above 0.75% w/v, surfactant molecules start aggregating and forming micelles in bilayer leading to depletion in lipidal cavity of vesicle membrane. These mixed micelles are reported to possess lower drug carrying capacity and poor skin permeation due to absence of lipid bilayer cavity for drug encapsulation. Above hypothesis was further supported by results of vesicle population, turbidity and elasticity measurement studies. These studies showed the maximum values of these characteristic parameters at 0.75% w/v concentration of surfactant.

Vesicle size and zeta potential of elastic liposomal formulations were found between 100-200 nm and -7.0 to -19.1 mV, respectively. Drug content of optimized elastic liposomal formulations EL-SP3 and EL-SD3 was found 96.2±4.2% and 95.2±4.7%, respectively. The maximum amount of drug loaded in the elastic liposomal formulations EL-SP3 and EL-SD3 was found to be 6.0 mg/mL with drug entrapment efficiency of 73.5±3.8% and 8.0 mg/mL with drug entrapment efficiency of 82.7±3.8%, respectively. The strength of paclitaxel in the formulation available commercially is 6 mg/mL. In comparison, the maximum amount of drug that could be loaded into the conventional liposomal formulation was found to be 2.0 mg/mL with drug entrapment efficiency of 48.1±1.9%. Elastic liposomes consist of phospholipids and a sub-lytic concentration of surfactant. Surfactant causes an increase in fluidity of the vesicle bilayer and is responsible for better retention of paclitaxel.
Skin permeation of paclitaxel through the abdominal skin of rat was studied for the elastic liposomal formulations, conventional liposomes and drug solution, using franz diffusion cell. Flux of paclitaxel from the elastic liposomal formulations ranged between 7.9±0.6 to 46.8±3.7 μg/cm²/h. The enhancement ratio (ER) of steady state transdermal flux for the elastic liposomal formulation was found to be 13.4 fold higher than that of the drug solution prepared in Propylene glycol (PG): Ethanol (7:3). The flux of paclitaxel in the conventional liposome formulation was found to be 7.3±0.6 μg/cm²/h.

Further, skin deposition study of different elastic liposomal formulations in comparison to conventional liposomes and drug solution was carried out with the objective to determine the depot forming ability, using rat skin. For the elastic liposomal formulation, the amount of drug deposited in the skin (Enhancement ratio of drug deposition) was found to be 16.3 fold higher than that of the drug solution, after 24 h of application.

After optimizing the different formulation and process variables and, performing \textit{in vitro} characterization, two paclitaxel elastic liposomal formulations were selected for further \textit{ex-vivo} and \textit{in-vivo} studies. One formulation containing Span 80 (EL-SP3) and another contain sodium deoxycholate (EL-SD3) in optimized concentration. These two formulations were found to have the maximum value of characteristic parameters like drug entrapment efficiency, drug loading, vesicle population, vesicle size, elasticity of vesicle membrane, steady state transdermal flux and amount of drug deposited into skin.

Results of skin permeation and deposition study indicated higher skin permeation and deposition potential of elastic liposomes in comparison to drug solution. The mechanism of better skin permeation of elastic liposomes is less known to the scientific world. To evaluate the mechanism for percutaneous absorption of elastic liposomes, vesicle-skin interaction studies were conducted by using biophysical techniques, Fourier transform infrared spectroscopy (FTIR) and Fourier transform infrared attenuated total reflectance spectroscopy (FTIR-ATR); microscopic techniques, TEM and scanning electron microscopy (SEM) and quantitative estimation of cholesterol and triglyceride contents of skin lipids.
The main objective of SEM of treated skin was to study the effect of elastic liposomes on surface morphology of skin in terms of lipid perturbation, surface irregularities and interlamellar distance. SEM photomicrograph of rat skin treated with paclitaxel solution showed no lipid perturbation and surface irregularities demonstrated that paclitaxel itself does not act as permeation enhancer. In comparison, increase in interlamellar distance of stratum corneum lipid bilayers and surface disordering were observed in rat skin treated with elastic liposomal formulations. This may be due to the interaction of elastic liposomal formulation with stratum corneum lipids bilayers, which impaired barrier function of these layers. Rat skin treated with conventional liposomal formulation showed significantly less surface disordering.

Further TEM study of treated skin was performed to verify the results of SEM. The main objective of TEM study was to obtain detailed information about the interaction of elastic liposomes with skin lipids and to demonstrate its lipid fluidization and perturbation effect. TEM photomicrograph of rat skin treated with elastic liposomes showed that elastic liposomal formulation affected the ultra structure of stratum corneum lipids. Disorganization and partial extraction of skin lipid bilayers were observed with elastic liposomes treatment. Areas of lipids with electron dense material and lamellar stacks that are known to be fixed by osmium tetraoxide (OsO$_4$) were visualized deeper down in the stratum corneum with elastic liposomes treatment. Since stratum corneum lipids lamellae cannot be fixed by OsO$_4$ as evidenced by TEM photomicrograph of control (untreated) skin, the observed stained material can be hypothesized to have originated from the vesicles. The lamella stacks appeared squeezed in between the bilayers of the intercellular spaces thereby, disrupting the highly organized landmann units and creating dislocation. This can be attributed to the deformable nature of elastic liposomes and formation of depot by mixing and fluidization of skin lipid bilayers. These OsO$_4$ fixed lipid areas containing electron dense material were not observed in rat skin treated with PBS and conventional liposome.

FTIR study reveals the molecular conformations of fatty acyl chains of lipids and proteins in the skin and thus, provides an insight into the biophysical status of skin. FTIR spectra of control (untreated) skin showed characteristic peaks of asymmetric and
symmetric C-H vibrations at 2950 and 2850 cm\(^{-1}\), respectively. These can be ascribed to hydrocarbon chains of SC. The height, area and displacement of these two peaks show the lipid perturbation and extraction of skin lipids. The treated skin with the elastic liposomal formulation significantly broadened the area of these peaks indicating its lipid perturbation effect. Other characteristic peaks found near 1637 and 1550 cm\(^{-1}\) represented amide 1 (C\(\equiv\)O stretching) and amide 2 (C–N stretching) linkages of the helical secondary structure found in epidermal keratin. Similarly, significant reduction in intensity of these peaks was observed with elastic liposomes treatment in comparison to conventional liposomes and drug solution treatments. Results of FTIR study demonstrated the skin lipid extraction and fluidization effects as the mechanism of percutaneous absorption of drug by elastic liposomes. Similarly, results of skin lipid extraction and fluidization effects of elastic liposomes treatment found by FTIR-ATR and quantitative estimation of skin lipids studies.

The results of vesicle-skin interaction studies showed that high skin permeation and deposition ability of paclitaxel from the elastic liposomal formulation could possibly be due to a combination of one or more of the following mechanisms (1) increased skin-vehicle partitioning of the drug (2) improved elasticity of the vesicle membrane (3) alteration of barrier properties of skin.

The skin localization potential of lipophilic fluorescence marker Rhodamine 123 loaded elastic liposomal formulations was studied by using two techniques i.e. confocal laser scanning microscopy (CLSM) and fluorescence microscopy. The fluorescence marker Rhodamine 123 was not found to penetrate and deposit into deeper layers of skin, when applied in the form of solution. However, this fluorescence marker reached the deeper layer of skin in significantly higher quantities when applied as elastic liposomal formulations. The result of fluorescence microscopy was further confirmed by CLSM of treated skin. CLSM graph of elastic liposomal formulation treated skin showed the 5 fold deeper skin deposition of fluorescence marker in comparison to the skin treated with conventional liposomal formulation.
In recent years, \textit{in situ} thermosensitive hydrogels based on chitosan have gained considerable interest for sustained and localized delivery of drugs. In the present study, attempt has also been made for preparation of \textit{in situ} thermosensitive hydrogel formulations of optimized elastic liposomal formulations EL-SP3 and EL-SD3. \textit{In situ} forming systems are liquid aqueous solution before administration, but gel under physiological conditions. The gelling chitosan solution was prepared by supplementing an aqueous solution of chitosan with a polylol salt dibasic sodium phosphate (DSP). The concentration of DSP was optimized on the basis of gelation temperature, gelation time and viscosity. Morphology of optimized formulation was studied by using SEM. Elastic liposomal formulations, EL-SP3 and EL-SD3, were incorporated into optimized hydrogel formulation and characterized for various parameters. Drug release was sustained to 24 h (88.7±2.3\%) with elastic liposomal formulation, as compared to drug solution, which released > 95\% drug within 2 h. Further, addition of hydrogel system to elastic liposomal formulations was found to decrease the release significantly (p < 0.05). Only 51±4.8\% of drug was released in 24 h with elastic liposomal thermosensitive hydrogel formulation. The sustained release observed with hydrogel system is due to the dual barrier nature of this system ‘drug-in vesicles-in-hydrogel’.

Cytotoxicity of elastic liposomes in comparison to Cremophor EL was evaluated by assessing A549 cell viability using MTT assay. No cytotoxicity of dummy elastic liposomes (without drug) was observed on A549 cells at the highest concentration of 5000 \(\mu\)g/ml (100±2.5\% cell viability). In comparison, Cremophor EL showed the significant (p<0.05) reduction in cell viability even at 100 fold less concentration of 50 \(\mu\)g/ml (69±2.2\% cell viability).

IC\textsubscript{50} value for paclitaxel elastic liposomal formulations (0.9±0.05 to 1.24±0.12 \(\mu\)g/mL) was found to be lesser than the marketed formulation (3.81±0.29 \(\mu\)g/mL). It was also observed that IC\textsubscript{50} value for paclitaxel elastic liposomal formulations decreased after 48 h treatment. In comparison, the IC\textsubscript{50} of marketed formulation increased. This is probably due to the sustained release and better intracellular uptake of paclitaxel from elastic liposomal formulation, this leads to the sustained exposure of the drug and thus increase the duration of chemotherapeutic effect.
The intracellular uptake of fluorescence dye acridine orange (AO) loaded elastic liposomal formulation in tumor cell line (A549) was evaluated using fluorescence microscopy and fluorescence activated cell sorting assay (FACS). Results demonstrated intracellular uptake of 85.4% to 95.8±2.5% for acridine orange (AO) dye loaded elastic liposomal formulations. In comparison, AO dye solution in Cremophor EL vehicle showed only 19.8±1.1% intracellular uptake. Fluorescence microscopy of treated cells was conducted to support the FACS data. Similarly, higher intracellular uptake of marker loaded elastic liposomal formulations was observed in fluorescence microscopy study. The intracellular concentration of paclitaxel is important for its anti-cancer activity. Several authors correlated the development of drug resistance with the reduced intracellular uptake. Result of FACS assay demonstrated 4-fold increase in intracellular uptake of AO with elastic liposomes. This could overcome the problem of poor intracellular uptake and development of resistance associated with the conventional formulation. The significantly higher intracellular uptake of elastic liposomes is probably due to its nanometric size, elastic nature, endocytosis-mediated intracellular uptake and intracellular retention.

Paclitaxel injectable formulation has been reported to cause hemolytic toxicity due to the presence of high concentration of ethanol and Cremophor EL. Encapsulation of paclitaxel in elastic liposomes found to reduce the hemolytic toxicity in comparison to marketed formulation. The paclitaxel elastic liposomal formulation showed 7.9±0.3% hemolysis at the concentration of 6000 µg/mL. In comparison, marketed formulation showed 42.1±3.0% hemolysis at the same concentration. The major reason for significantly less hemolytic toxicity of elastic liposomal formulations is absence of Cremophor EL : ethanol vehicle.

To ensure the bio-safety of paclitaxel elastic liposomal formulations, in vivo single dose acute and repeated dose 28 days sub-acute toxicity studies were conducted. Acute toxicity study was carried in the dose range of 10 mg/kg to 200 mg/kg and sub-acute toxicity was conducted at maximum tolerated dose (MTD) found in acute toxicity study. MTD for elastic liposomal and marketed formulation was found to be 160 mg/kg and 40 mg/kg, respectively. Based on these result, LD50 was calculated and found to be
141.6 mg/kg and 16.7 mg/kg, respectively for paclitaxel elastic liposomal formulation and marketed formulation. \(LD_{50}\) of elastic liposomal formulation was 8-fold higher than commercial paclitaxel formulation.

In repeated dose toxicity study, there were no treatment related mortality and toxic symptoms in animals treated with the elastic liposomal formulation at doses upto 120 mg/kg and in the marketed formulation upto 20 mg/kg. However, at the higher dose of elastic liposomal (160 mg/kg) and marketed (80 mg/kg) formulation clinical signs of toxicity such as aggressive behavior, skin pallor, rough fur, hair loss and decrease in body weight were observed. Food consumption was also found to decrease at a higher dose of treatment.

Hematological and biochemical parameters were evaluated at 0 and day 28\(^{th}\) after administration of paclitaxel elastic liposomal formulations and marketed formulation at different doses. There was no significant \((p < 0.05)\) change in hematological and biochemical parameters upto dose 120 mg/kg and 20 mg/kg with paclitaxel elastic liposomal formulations and marketed formulation, respectively. However significant \((p < 0.05)\) change in the day 0 and day 28 values were observed at 160 mg/kg and 40 mg/kg doses of these formulations. Further the results were supported with histopathology of liver, kidney, lung, heart and spleen. No sign of toxicity was seen in liver, kidney and lung of animals treated with elastic liposomal formulations. The marketed formulation treated animals showed chronic inflammatory infiltrate in hepatic tissue, mild acute tubulointerstitial nephritis in kidney and significant inflammation in lung. Photomicrographs of heart and spleen of paclitaxel elastic liposomal formulation and marketed formulation treated groups were comparable and no evidence of toxicity was observed.

Finally, anti-cancer activity of paclitaxel elastic liposomal formulation in comparison to the marketed formulation was evaluated by using Cytoselect 96-well cell transformation assay (Soft agar colony formation) and Enlirch ascetic cell induced tumor in mice. In Cytoselect 96-well based assay, cancer cells are grown on agar matrix to form the colony and this assay measures the morphological transformation of cell colonies induced by anti-cancer substances. Colony formation was found to reduce
86±3.5% and 63±2.8% with paclitaxel elastic liposomal and marketed formulation, respectively in comparison to control (untreated) group. Quantitative measurement of % viability of cell also depicted significantly higher (p<0.05) anti-cancer activity of paclitaxel elastic liposomal formulation in comparison to the marketed formulation.

In Enlirch ascetic cell model, tumor was generated by subcutaneously inoculated 15x10^6 ascetic cells into the right hind limb (thigh) of the mice. After 6 days of tumor implantation treatment was started. Tumor volume was measured on 7\textsuperscript{th} day and measurement was carried out every 7\textsuperscript{th} day for a period of 21 days. Tumor volume was found to reduce 88.4±2.9% and 63.7±1.9% with paclitaxel elastic liposomal and marketed formulation, respectively in comparison to control (untreated) group. Histopathology of tumor tissue treated with elastic liposomal formulations had shown notably less inflammation in comparison to the marketed formulation. Tumor cell and mass cell population were also found to be significantly less with elastic liposomal formulation treatment in comparison to marketed formulation.

Pharmacokinetic parameters and biodistribution behavior of paclitaxel in tumor bearing mice treated with different formulations were determined. The values of MRT, AUC and \( t_{1/2} \) were found significantly higher for paclitaxel loaded elastic liposomal formulation than those of marketed formulation. The higher concentration of paclitaxel in tumor tissues of mice treated with elastic liposomal formulation indicates that elastic liposomal formulation was notably localized in tumor tissue. These results suggest that sustained release and depot effect of elastic liposomal formulation might offer enough chance for paclitaxel to maintain the effective therapeutic concentrations for a long period of time for localized delivery.

The stability of elastic liposomal formulations was tested at 4±2 °C and 25±2°C for 90 days. Appearance, particle size distribution, zeta potential and drug encapsulation efficiency were evaluated after 15, 30, 60 and 90 days. There was no significant change in the particle size of elastic liposomal formulations stored at 4±2°C for 90 days. But a significant increase in the particle size from 136±11nm to 270±9.0 nm was observed when the elastic liposomal formulations were stored at 25±2°C upto 90 days. Decrease in drug encapsulation efficiency of paclitaxel in elastic liposomes was found at 25±2°C after
90 days, but no significant change was found at 4±2°C. Result of stability study showed that recommended storage condition for elastic liposomal formulation is at 4±2°C.

The results of present study demonstrated that Cremophor EL free paclitaxel elastic liposomal formulation is an attractive approach for localized delivery. Maximum amount of drug loading was found to be 6 mg/mL, similar to the strength of commercial formulation, but without using Cremophor EL. Amount of paclitaxel deposited in skin was found to be 16.5-fold higher with elastic liposomal formulation in comparison to the drug solution, showing the depot forming ability of elastic liposomes for localized delivery. Toxicity screening of developed formulation showed a better ex vivo and in vivo tolerance with 6-fold increase in MTD and 8-fold increase in LD50 in comparison to the marketed formulation. Elastic liposomal formulation showed a higher intracellular uptake and favorable pharmacokinetic and biodistribution profile with localization of drug at tumor. In terms of efficacy, this formulation also showed significantly higher anti-cancer activity in comparison to marketed formulation. Elastic liposome based formulations showed a great advantage to circumvent the use of Cremophor EL as solubilizer for paclitaxel. Finally, the results of this work suggested that the elastic liposomal formulation can be considered as a promising alternative in comparison to Cremophor EL based marketed formulation for delivery of paclitaxel with enhanced anti-cancer activity and reduced toxicity. However, it would be worthy to evaluate the safety and efficacy of paclitaxel elastic liposomal formulation in clinical trial.