CHAPTER - VII

SUMMARY

Polymeric micelles have been widely studied for targeted drug delivery and other biomedical application, especially for anticancer drugs. These carriers are able to provide a series of unbeatable advantages- they can solubilize poorly soluble drugs by hydrophobic core resulting in the increase of drug stability and bioavailability. They can stay in the body long enough providing gradual accumulation in the required area. Their size permits them to accumulate in the body regions with leaky vasculature. The drugs loaded in the polymeric micelles can be well protected from possible inactivation under the effect of biological surroundings and their bioavailability is increased (Allen et al., 1999).

Camptothecin (CPT), a plant alkaloid extracted from Camptotheca acuminata, acts as a potent antitumour agent by inhibiting the nuclear enzyme topoisomerase I. It inhibits the growth of a wide range of tumours. However, the major drawbacks of the drug have always been water insolubility and lactone instability. The lactone ring in CPT plays an important role in the drug's biological activity but it exists in a pH dependent equilibrium with an open ring carboxylate form. The lactone ring opens at physiological pH or above, making this drug much less active and highly toxic (such as myelosuppression, hemorrhagic cystitis and diarrhoea) and precludes its clinical use (Hatefi et al., 2002).

Hence the aim of this work was to use amphiphilic block copolymer based micelles to develop a novel delivery system. Camptothecin is chosen as a model drug and the polymeric micelle formulation is developed and evaluated. The Me-PEG-b-PCL block copolymers can successfully form micelles in water with the hydrophobic domains (PCL) as the core of the micelles.

Thin film hydration technique was used to prepare micelles from Me-PEG-b-PCL copolymers with varying the weight ratios of camptothecin to the block
copolymers, following hydration of the copolymer film in phosphate buffer saline; sonication was employed as a means to reduce the size of the micelles.

Various formulation parameters were optimized by trial and error method to achieve uniform and smaller size of polymeric micelles, higher encapsulation efficiency with improved in vivo circulation and antitumour activity. To achieve this two different molecular weight copolymers (Me PEG<sub>5000</sub> -b-PCL<sub>13,000</sub> and Me PEG<sub>5000</sub> -b-PCL<sub>5000</sub>), a sonication time of 2 min, a temperature in the RFE of 40 °C at 100 rpm, and centrifugation at 13,200 rpm for 1 hr at 4 °C were used in the study. Hydration medium used was PBS pH 7.4. These parameters were kept constant for all the formulations for reproducibility.

Since polymeric micelles size has a crucial impact on the in vivo fate of a particulate drug delivery system, control over the micellar size is of great importance for drug carriers. Optical microscopy results revealed that the PMs prepared with tetrahydrofuran showed higher average diameter with some aggregation and higher polydispersity when compared with PMs containing chloroform: acetonitrile. The polydispersity index for the micelles containing chloroform: acetonitrile found to be relatively low which usually leads to a more stable micelle system in vivo. It can be seen that higher the copolymer concentration in organic phase, the smaller the size of the polymeric micelle (PI- F4, F8 and PII- F11, F14, drug: polymer ratio 1:25).

The morphology of the polymeric micelles formed from block copolymer was also investigated by SEM and TEM. The scanning electron microscopic analysis revealed a spherical morphology of the micelles containing F4 (PI, 1:25) and F8 (PII, 1:25). The TEM showed smaller size (195.4 nm and 170.5 nm for F4 and F8 respectively) due to the shrinking of the thick PEG shell in the drying process. The shrinkage and collapse during the drying process in TEM analysis, resulted in an irregular shape of PMs.
The CPT PMs containing F4 (Me PEG_{5000-b-PCL_{13000}}, 1:25 ratio) showed smaller particle size and narrow size distribution when compared with other formulations. From the zeta potential measurement, it could be found that the zeta potential of micelles containing F4 (PI, 1:25) and F8 (PII, 1:25) was in the range of -6.8 and -7.05 respectively. The increase of zeta potential might be related to the shielding of ion charge by PEG shell because of its non-ionic nature (Lin et al., 2003).

The results obtained here imply that particle size adjustment could be achieved by employing different copolymers via variation of the copolymer concentration in organic phase.

The loading efficiency increased as the content of hydrophobic PCL block in the copolymer increased (Hamidreza Montazeri Aliabadi et al., 2007). When chloroform: acetonitrile was used as solvent, higher encapsulation efficiency was obtained (F1-F8) than those obtained using THF (F9-F14) for all the block copolymer ratios. Therefore, chloroform: acetonitrile is a more favorable solvent for CPT than THF when emulsification solvent evaporation method was used (Sang Cheon Lee et al., 2003).

The PMs prepared with a 1:25 ratio of block copolymer I (F4) showed higher encapsulation efficiency than PMs prepared with 1:5 and 1:12.5 ratios of the same polymer. Higher copolymer concentrations would accelerate the solidification of PMs, which could in turn retain the drug in the polymer matrix more effectively. However, it was found that a drug /copolymer feeding ratio higher than 1:25 caused a large amount of particle aggregation and very low polymeric micelle yield.

The formation of core shell type structures of Me PEG-b-PCL was further confirmed by a fluorescence probe technique applying pyrene as a hydrophobic probe. In very low concentrations of Me PEG-b-PCL below CMC, the marker of pyrene was dissolved in a polar environment of water and fluorescence intensity
in I 339/ I 334 was very low. However in presence of micelles, a hydrophobic micelle core solubilizing pyrene resulted in an increase of fluorescence intensity.

In this study the CMC of F4 (PI, 1:25, chloroform: acetonitrile) and F8 (Pll, 1:25, chloroform: acetonitrile) were found to be 5 and 12 µg/ml respectively, whereas for F11 (PI, 1:25, THF) and F14 (Pll, 1:25, THF) it was found to be 26 and 38 µg/ml respectively. The polymeric micelles prepared using chloroform: acetonitrile showed decrease in CMC than the micelles containing THF. Also the CMC decrease with an increase in the length of PCL core block (F1-F4 and F9-F11), in which it has been shown that a larger lipophilic area facilitates and stabilizes micellar formation (Yang et al., 2002).

The lower the critical micellar concentration value of a given amphiphilic polymer, the more stable micelles are even at a low net concentration of amphiphile in the medium. This is especially important, since upon dilution with a large volume of blood, micelles with a high critical micellar concentration value may dissociate in to unimers and their content may precipitate in the blood.

The in vitro dissolution reports showed that all samples exhibited a burst release of CPT at the initial stage. This may be due to a portion of the drug deposited at the region near or within the PEG shell and could gain access to aqueous medium without the need of a long diffusion time (Yuan-Chia Chang et al., 2008). After the initial burst, the CPT release rate slowed down and became steady in a sustained-release manner.

These results also show that the longer the PCL chain length, the slower the drug release. The longer chain length of PCL could result in larger particle size, smaller surface area and greater diffusion layer thickness which slow down the drug release. The slow steady release of CPT observed with all these preparations demonstrates that the drug is more effectively encapsulated by ESE method. In addition, the presence of PCL can increase the interaction between
the polymer matrix and the loaded drug and further slow the release rate. Similar results were reported by Lin et al., 2006.

All block copolymer chains prepared by the emulsification solvent evaporation method containing higher molecular weight PCL with chloroform: acetonitrile as a solvent showed slow release for a prolonged period of time when compared to PMs containing THF.

In order to sufficiently acquire the EPR effect, the antitumour activity of CPT loaded polymeric micelles were evaluated.

Since in vitro studies of the formulations containing chloroform:acetonitrile showed smaller particle size, low polydispersity index, higher entrapment efficiency and slower release, further in vivo studies were carried out for the formulations F4 (PI, 1:25, chloroform:acetonitrile) and F8 (PII, 1:25, chloroform:acetonitrile).

The in vivo antitumour activity results of the present study clearly demonstrate the tumour inhibitory activity of camptothecin polymeric micelles (F4 and F8) against DLA and EAC cell strain. The reliable criteria for judging the value of anticancer drug is the prolongation of life span of animal. In this study, an increase in life span was observed among the CPT PMs treated groups. The DLA bearing mice administered with camptothecin polymeric micelles formulation at different doses (2 mg, 4 mg, 8 mg/ kg) showed significant increase (p<0.01) in average life span when compared with CPT drug solution.

In DLA tumour bearing mice, a regular rapid increase in ascitic tumour cell volume was observed. However the percentage increase in body weight and number of viable tumour cells were found to be significantly less (p<0.01) in camptothecin polymeric micelle formulations treated mice than the animals treated with CPT solution, indicating the anticancer nature of camptothecin polymeric micelles. When CPT-PMs were used, the reason of increased
survivability might be due to the enhanced vascular permeability and retention effect of polymeric micelles. It was reported that higher molecular weight copolymers showed more effectiveness in the tumour accumulation of CPT (Thomas et al., 2006).

In cancer chemotherapy, major problems are myelosuppression and anemia (Maseki et al., 1981). The anemia encountered in tumour bearing mice is mainly due to reduction in RBC, hemoglobin percentage, platelet count and this may occur either due to iron deficiency or due to hemolytic or myelopathic conditions. Treatment with camptothecin polymeric micelle formulations (F4 and F8) restored the hemoglobin content and the RBC. WBC cell counts were near to normal values. This indicates that the camptothecin formulations (F4, PI and F8, PII) have protective action on the haematopoietic system. Moreover, formulation F4 showed the highest protection at 4mg and 8 mg/kg dose than the formulation F8.

Depletion of muscle proteins, glycogen and lipids leads to an elevation of free cholesterol and a decrease in neutral fat. The loss of major nutrients, particularly excessive catabolism of lipids, leads to alterations in the activity of host tissue enzymes, changes in endocrine homeostasis and immunologic mechanisms, chronic and acute cathetic conditions, finally leading to the death of animals (Theologides et al., 1979). Hypercholesterolemia has been reported in various disease conditions (Ellefson et al., 1976). Hypercholesterolemia associated with the DLA inoculated animals may be due to the mobilization of fat from the stores to meet the increased demand of the tumour bearing host. The lipidemia subsides gradually as the fat stores are emptied to meet the caloric requirements of the host during acute cancer condition. In the current study, serum triglyceride content decreased in camptothecin polymeric micelles formulations (F4 & F8) and significant changes (p<0.01) were observed in CPT polymeric micelles containing F4 and F8 when compared with CPT solution.
The solid tumour volume has been measured in mice inoculated with the EAC cells. This study indicated that compared with free CPT, CPT loaded polymeric micelles showed significant changes in tumour volume (p<0.01). This may be because of the sustained release characteristic of the CPT loaded polymeric micelles which may result in prolonged exposure of the tumour cells to the attack of the antitumour drug, which is essential for S-phase specific drugs like CPT.

Fast growing tumour tissues need a tremendous amount of oxygen and nutrients supplied by blood vessels. They release special growth factors including vascular endothelial cell growth factor (VEGF) to facilitate neo-vascularization. As a result, many new vessels are formed, but their cell junctions are not as tight as those of normal tissues. CPT-PMs were likely to freely pass through the endothelial junctions of the capillaries in tumour tissue, but not in normal tissue. This study showed that a combined effect of the passive targeting and enhanced cellular uptake would be the main reason for the suppression of tumour growth (Hyuk Sang Yoo et al., 2004).

All these findings enable to conclude that camptothecin and its CPT loaded PMs formulations at various doses possess a protective effect against DAL and EAC induced tumour in vivo.

Tissue distribution studies were carried out in mice bearing EAC cells since tumour bearing animals may be different from normal animals due to some physiological changes brought about by tumour development.

For both the formulations, rapid accumulation of CPT in the various organs was observed. Blood plasma levels of CPT loaded micelles containing F4 (PI, 1:25, chloroform: acetonitrile) and F8 (PII, 1:25, chloroform: acetonitrile) were relatively higher (7.8% and 6.60% respectively) than CPT solution (1.72%). Liver showed significantly decreased uptake of CPT polymeric micelles containing F4 and F8 (1.52% and 1.88% respectively) when compared to CPT solution (11.8%). Tumour accumulation of CPT loaded micelles of F4 and F8 was
higher (21.0% and 15.8% respectively) than CPT solution (1.98%). These results showed that the PMs possess the ability to deliver large amounts of CPT, in its most active form, to the tumour site by passive targeting with a long circulating carrier.

The CPT concentration in lung (4.02% and 5.75% for F4 and F8 respectively) was also high due to the filtration effect of the lung capillary bed that removed some large particles or their aggregates. Concentration in kidney, one of the major elimination pathways of the original CPT, was low and did not show any significant difference between groups treated with CPT loaded polymeric micelles. However the CPT concentration in liver, a major RES organ, was significantly (p<0.01) lower, which might be attributed to the steric stabilization provided by the PEG shell and could be viewed as a proof of the RES evading ability of polymeric micelles. When compared with camptothecin polymeric micelles containing F8 (PII, 1:25), F4 (PI, 1:25) showed highly significant results due to its longer lipophilic chain length (PCL 13,000).

The Me PEG-b-PCL micelles showed initial higher blood circulating levels compared with free CPT. After 4 hrs, the free CPT had been removed from the circulation and could not be detected. On the contrary, Me-PEG-b-PCL micelles exhibited a remarkably delayed blood clearance and the concentration of CPT of Me PEG5000-b-PCL13,000 (F4, 1:25) and Me PEG5000-b-PCL5000 (F8, 1:25) in blood at 4 hrs after i.v.administration was about 35.4 ng/ml and 7.2 ng/ml respectively.

Since Me-PEG5000-b-PCL13,000 (F4, 1:25) micelles had longer PCL chain length and more rigid structure, they possessed higher drug loading efficiency and they would release drug more slowly and have a relatively longer half-life. Significant increase (p<0.01) in AUC, MRT and V_d was observed in F4 (PI, 1:25, chloroform: acetonitrile) when compared to CPT solution and clearance was found to be significantly low (p<0.01) when compared with CPT in solution with the same dose. The MRT of PMs increased in plasma compared with the same dose of CPT solution which may be due to the coating of PEG on the surface of PMs and sustained release of CPT from CPT-PMs.
When compared with F4 (PI, 1:25), formulation F8 (PII, 1:25) found to be less significant (p<0.05) because of its shorter lipophilic (PCL) chain length.

Stability studies were carried out at room temperature and refrigeration temperature. The CPT loaded micelle formulations did not show any noticeable change in the amount of drug entrapped after 3 months at 2-8°C. Around 10-13% of drug was leaked out from the polymeric micelles when they were stored at room temperature. Lowered entrapment efficiency observed when stored under room temperature may be due to drug expulsion from the polymeric micelles. No change in colour of stored formulations were also observed during the study period.