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Biodegradable polymeric micelles of camptothecin: formulation and characterization

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Abstract

The purpose of this study was to formulate and characterize biodegradable polymeric micelles (PMs) of diblock copolymers for delivering the anticancer drug camptothecin (CPT) and maintaining its biological activity for a prolonged time. To achieve this, PMs were prepared by emulsification solvent evaporation (ESE) using a rotary flash evaporator (RFE) with the diblock copolymers, methoxy polyethylene glycol-b polycaprolactone (Me PEG-b-PCL) of different molecular weights and loaded with CPT as a model drug. The PMs were evaluated morphologically, and their particle size and critical micelle concentration were determined. *In vitro* release and *in vivo* pharmacokinetic studies were also done. The results show that formulating PMs using a long polycaprolactone (PCL) chain increases the entrapment efficiency, loading and stability of CPT and results in slower drug release. This study demonstrates that polyethylene glycol (PEG) and PCL chain length play a crucial role in the *in vitro* properties of PMs.

Keywords: methoxy-polyethylene glycol-b-polycaprolactone, camptothecin, polymeric micelles, diblock copolymers, critical micellar concentration

Introduction

Camptothecin (CPT) is a naturally occurring quinolone alkaloid found in the bark of the camptotheca tree and the Asian nothapodytes tree. CPT prevents the activity of DNA topoisomerase I by binding non-covalently to the enzyme, and this inhibits the growth of a wide range of tumours. It has been reported that the lactone ring is essential for its tumouricidal activity (Kumi Kawano et al. 2006). There are two major drawbacks to the effective use of CPT, however. One is its insolubility in water and the other is the instability of its lactone moiety. In the body, the lactone of CPT is converted to carboxylate in a pH-dependent reaction making the drug less active and highly toxic. Several approaches have been tried to increase the drug's solubility and stability. Polymeric microspheres, liposomes, micro-emulsions, formation of inclusion complexes with cyclodextrins all have been explored with some success. Polymeric micelles (PMs) with a PEG shell for injectable delivery of CPT have recently shown promise.

Micelles are spherical colloidal nanoparticles, spontaneously formed by many amphiphilic

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molecules in aqueous media. Encapsulation within micelles increases the stability, solubility and bioavailability of poorly soluble drugs *in vivo*, improves their pharmacokinetics and biodistribution and decreases their toxicity. PMs which are prepared from block copolymers possessing both hydrophilic and hydrophobic chains have received much attention in drug delivery research because of their small particle size, high structural stability, extended drug release and prevention of rapid clearance by the reticuloendothelial system (RES) (Kwon 2003).

A number of amphiphilic copolymers, such as poly-(methoxy polyethylene glycol cyanoacrylate-co-hexadecyl cyanoacrylate) (Me PEG-PHDCA), poly (N isoprolylacrylamide)-b-polyethylene glycol, poly (D, L-lactide)-b-methoxy-polyethylene glycol have been reported to form PMs. These copolymers are degraded into biocompatible and nontoxic monomers that are cleared from the body. Recently biodegradable polyethylene glycol-coated PMs using polycaprolactone-b-polyethylene glycol (PEG-b-PCL) exhibited 'stealth' properties in *in vitro* and in *vivo* experiments, which means they avoid being taken up by the RES. PMs with a PEG shell are reported to stay in the blood circulation longer than non-coated PMs, which prolongs exposure of tumour cells to antitumor drugs and enhances passive targeting by increasing permeability and retention (Maeda 2000).

Considering all these factors, we chose PCL as the hydrophobic segment of the block copolymer since it provides good encapsulation ability and favourable biodegradability. The PEG segment generates a hydrophilic shell that stabilizes these particles through stearic repulsion, avoiding the use of additional surfactants. In this study, we prepared biodegradable PEG-b-PCL PMs encapsulating CPT to improve aqueous solubility and stability in biological fluids. Particles capable of sustained release were produced by emulsification solvent evaporation using a rotary flash evaporator with different drug: polymer ratios. The CPT16 loaded PMs were viewed by scanning and transmission electron microscopy and characterized by dynamic light scattering. *In vitro* release profiles, encapsulation efficiency and critical micellar concentration of the PMs were also determined.

Materials and Methods

Materials

S-(+) camptothecin (>95% pure by HPLC) was obtained from Sigma-Aldrich, Germany. Me PEG-b-PCL (molecular weight 5000-5000 & 5000-13,000), pyrene and all other chemicals of analytical grade were purchased from Sigma-Aldrich, USA.

Preparation of CPT loaded PMs using chloroform: acetonitrile

Weighed amounts of diblock copolymers and CPT at various CPT to copolymer ratios (1:2.5, 1:5, 1:12.5 and 1:25, w/w) were dissolved in organic solvents (chloroform:acetonitrile, 3:2, v/v). The resulting solutions were stirred at 40°C under reduced pressure in a rotary flash evaporator (Double Coiled Condenser, Superfit Rotary Vacuum) (Masato Watanabe et al. 2006) until the solvent was completely evaporated. Water (10 mL) was added to the residue and the solution sonicated in a bath sonicator (PCI 400078, 3.5 L 100) for 2 min (5 sec sonication with 1 sec standby). The resulting solutions were centrifuged at 13200 rpm for 1 h at 4°C in a refrigerated centrifuge (Eppendorf, model 5415, Germany) to obtain CPT-loaded PMs (Table 1).

Table 1. Formulation of polymeric micelles using chloroform:acetonitrile

Polymer	Formulation code	Drug: polymer ratio	Organic solvent used	Volume of organic solvent
Me PEG $_{5000}$ -b-PCL $_{13000}$ (PI)	F1	1:2.5	Chloroform: acetonitrile	(3:2 v/v), 10 mL
	F2	1:5		
	F3	1:12.5		
	F4	1:25		
Me PEG $_{5000}$ -b-PCL $_{5000}$ (PII)	F5	1:2.5		
	F6	1:5		
	F7	1:12.5		
	F8	1:25		

Preparation of CPT-loaded PMs using tetrahydrofuran (THF)

Weighed quantities of CPT and diblock copolymers were dissolved in THF at various CPT to copolymer ratios. The solutions were added drop-wise with vigorous stirring to distilled water and stirred over night. The slow evaporation of the THF produces uniform micelles. These were transferred to the rotary flash evaporator to remove residual THF, and the resulting preparations were centrifuged to remove polymer residues and free CPT aggregates (Table 2).

Table 2. Formulation of polymeric micelles using tetrahydrofuran

Polymer	Formulation code	Drug: Polymer ratio	Organic solvent used	Volume of organic solvent
Me PEG $_{5000}$ -b-PCL $_{13000}$ (PI)	F9	1:5	Tetrahydrofuran	10 mL
	F10	1:12.5		
	F11	1:25		
Me PEG $_{5000}$ -b-PCL $_{5000}$ (PII)	F12	1:5		
	F13	1:12.5		
	F14	1:25		

Morphological examination of PMs

The structural features of the PMs were visualized by scanning electron microscopy (JEOL 6701 field-emission SEM) and transmission electron microscopy (TEM) (Cristiana Lima Dora et al. 2006). For SEM, the PMs were coated with gold under vacuum and dispersed in water. For TEM, a drop of PM suspension was placed on a carbon-coated grid and left to form a thin film. Before the film dried on the grid, a drop of 1% phosphotungstic acid negative staining solution was pipette onto the film and the excess removed with filter paper. The grid was allowed to air dry thoroughly and sample morphology was visualized using an accelerating voltage of 80 kV.

Particle size determination

The mean diameter of the vesicles was determined using a dynamic light scattering particle size analyzer (Zetasizer Nano ZS 90, Malvern Instruments, UK). The PM formulation (100 µL) was diluted with an appropriate volume of water and the vesicle diameter was determined.

Drug loading and encapsulation efficiency determination

The amount of CPT incorporated into the PMs was determined by UV detection at 352 nm. CPT-loaded PMs were centrifuged at 13,200 RPM for 1hr at 4°C to separate the free CPT. The supernatant was collected, diluted with DMSO: water (9:1 ratio) and analyzed by UV detection at 352 nm (Koo et al.

2004). The analyses were carried out in triplicate. CPT loading and encapsulation efficiency were calculated by the following formulas:

$$\text{CPT loading (\%)} = \frac{\text{Weight of CPT in PMs}}{\text{Weight of PMs}} \times 100$$

$$\text{Encapsulation efficiency (\% w/w)} = \frac{\text{CPT in PMs - CPT in supernatant}}{\text{Initial CPT}} \times 100$$

Determination of critical micellar concentration (CMC) of Me PEG-b-PCL copolymer by fluorescence spectroscopy

To estimate the CMC of Me-PEG-PCL copolymer, pyrene was used as a hydrophobic probe. A known amount of pyrene in acetone was added to the PM solution and the acetone was evaporated by stirring the solution overnight at room temperature. The concentration of pyrene was 5×10^{-1} M. The emission wavelength was fixed at 383 nm (5 nm bandwidth of both excitation and emission wavelengths) and excitation spectra were recorded at micelle concentrations of 100 to 500 µg/mL using a Spectrofluorometer (Jasco, FP 750, UK). A plot of the ratios of the fluorescent intensities at excitation wavelengths of 339 and 334 nm (I339/I334) was used to determine the CMC (Shi et al. 2005).

In vitro release of CPT-loaded PMs

The *in vitro* release of CPT from PMs was measured using a dialysis bag diffusion technique. The micellar solutions were introduced into pre-swelled dialysis membrane bags with a molecular weight cut off of 12,000-14,000 daltons (Himedia Laboratories, India). The bags were immersed in 100 mL of phosphate-buffered saline release medium and the samples were maintained at 37°C with gentle stirring (Magnetic stirrer, Remi Equipment Ltd., India) (Zhang et al. 2007). At predetermined time intervals, 5 mL aliquots of the aqueous solution were withdrawn from the release medium, replaced with fresh medium, and analyzed by UV spectrophotometer at 370 nm (Shimadzu, Japan). The samples were compared with a standard solution of CPT analyzed under the same experimental conditions, and the percentage of CPT released *vs.* time (h) was plotted.

Pharmacokinetics of CPT-PMs in rabbits

Three groups of New Zealand rabbits were used in this experiment. Group 1 was treated with CPT solution, Group 2 with F4 and Group 3 with F8. For administration, micelles were dispersed in sodium chloride (0.9%) at the required concentration. Simultaneously, a solution of CPT in the same vehicle at the same concentration was prepared by diluting CPT injection. Each animal was injected intravenously with 2.5 mg CPT / kg. After administration, blood was collected at various time intervals from the ear vein, centrifuged at 13,500 rpm for 4 min to obtain the plasma and CPT was extracted with chloroform: methanol (4:1 v/v). After extraction, 25µl of the chloroform: methanol layer was directly injected into the HPLC system (Shimadzu LC-20AT) to determine the concentration of CPT. The separation was done using a Luna 5 micron phenyl-hexyl column (Phenomenex, USA) with UV detection at 352 nm (Koo et al. 2004). The analyses were carried out in triplicate. The mobile phase was 40:60 (v/v) acetonitrile and ammonium acetate buffer with 2% triethylamine (pH adjusted to 5 with 5% glacial acetic acid). The flow rate was set at 1 mL/min and the ratio of solvent used was 40:60. (The study was approved by Institutional Animal Ethical Committee- PSG Institute of Medical Sciences and Research, Coimbatore Registration number – 158/1999/CPCSEA).

Statistical analysis

The results were analyzed by analysis of variance (ANOVA) and the post hoc Dunnett's test. When comparisons between groups yielded a value for p of less than 0.05, the difference between those groups was considered significant.

Results

Optimization of the formulation parameters

CPT as a model drug was loaded into PMs by emulsification solvent evaporation (ESE) at various CPT: polymer ratios. The CPT-loaded Me PEG-b-PCL micelles were characterized by measuring encapsulation efficiency and *in vitro* drug release. The results show that the addition of PEG and the PCL chain length may play very important roles in determining the *in vitro* properties of micelles (Miura et al. 2004).

The ESE method has several advantages over other methods including easier scale-up and less chance for drug loss during encapsulation (Hamidreza Montazeri et al. 2006). In order to achieve uniform particle shape and size and to improve drug loading and encapsulation efficiency, various CPT-PM formulation parameters were optimized in this study. PMs were prepared using two different molecular weight diblock copolymers to see whether their length influenced drug loading. It has been reported that, if the length of a hydrophilic block is too high, the copolymers exist in water as unimers, while molecules with very long hydrophobic blocks form non-micellar structures such as rods and lamellae.

Chloroform - acetonitrile or THF, were used as the organic co-solvents to dissolve the block copolymers. The selection of organic solvent was based on its miscibility with water, ability to dissolve both CPT and Me PEG-b-PCL and a low boiling point, to facilitate its evaporation and complete removal (Hamidreza Montazeri et al. 2006).

Optimization of solvent evaporation using a rotary flash evaporator (RFE)

In order to evaporate the solvents more quickly and ensure stability of the formulation, the RFE was attached to a vacuum pump and the temperature was optimized to 40°C in a water bath to evaporate the solvents. Under these conditions the formulations were stable with formation of only a small amount of aggregated micelles. Various rotation speeds and temperatures were tested, and at higher temperatures, we found that fusion of micelles occurred and their shapes were not discrete (data not shown).

Optimization of sonication

Sonication was done using a bath-type sonicator at various times from 2 to 10 min. At 10 min and 5 min, the PMs were found to be unstable, and at the end of sonication there were no micelles seen. When the sonication time was reduced to 2 min, however, the micelles were stable and of discrete shape, had little particle aggregation, and the size had also been reduced. Hence the sonication time was fixed at 2 min (data not shown).

Optimization of centrifugation

Refrigerated centrifugation was selected in our study to separate the PMs from free CPT and other particles. At the beginning, the preparations were centrifuged at 6000 and 10000 rpm at 4°C for 1/2 h, but there was no separation of PMs. At 13200 rpm at 4°C for 1 h, however, the PMs separated cleanly from free CPT and other particles, and were collected and stored for the other studies (data not shown).

For our final optimized procedure to prepare PMs with block copolymers, we used two different molecular weight copolymers, a sonication time of 2 min, a temperature in the RFE of 40°C at 100 rpm, and centrifugation at 13200 rpm for 1 h at 4 °C. These parameters were kept constant for all the formulations for reproducibility.

Morphological examination and particle size determination

Since particle size has a crucial impact on the *in vivo* fate of a particulate drug delivery system, control over the particle size is of great importance for all drug carriers. Particles smaller than 200 nm and with a slight negative zeta potential will produce stealth effects. To determine the morphology of our PMs, we examined them by scanning electron microscopy (Fig. 1) and transmission electron microscopy (Fig. 2).

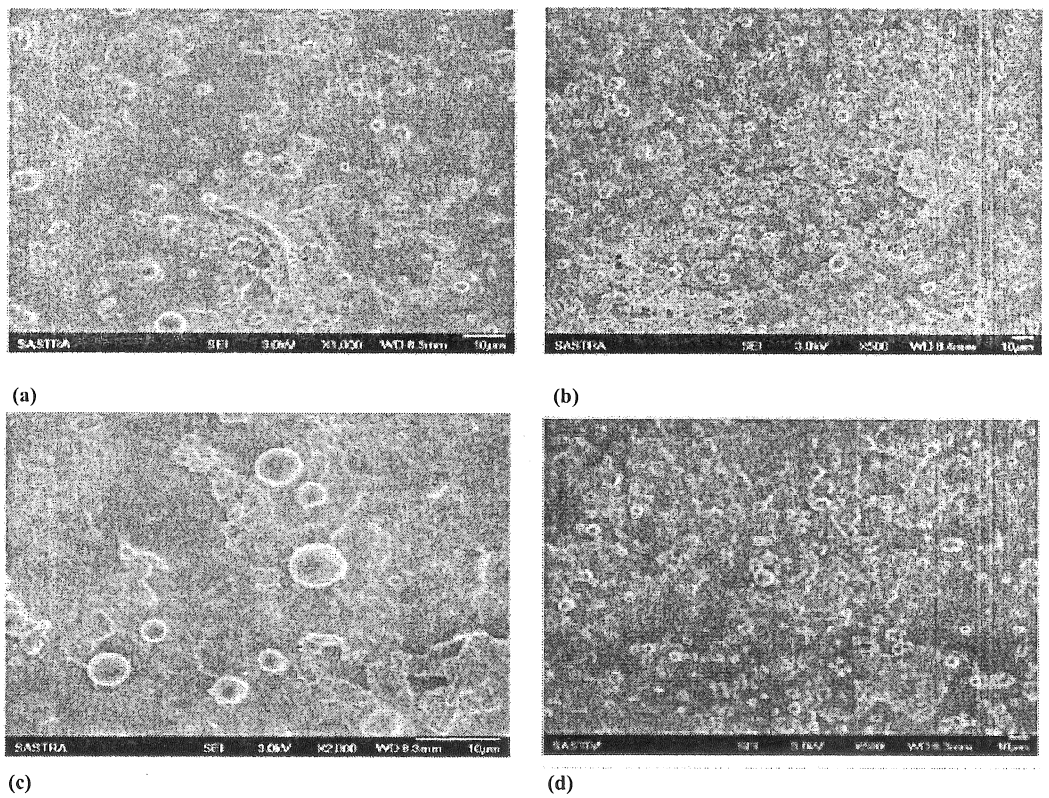

Figure 1. SEM photograph of CPT PMs containing *(a)* 1:12.5 w/w drug polymer ratio of F3, *(b)* 1:25 w/w drug polymer ratio of F4, *(c)* 1:12.5 w/w drug polymer ratio of F7 and *(d)* 1:25 w/w drug polymer ratio of F8

The SEM and TEM images of Me PEG- b-PCL PMs showed that the micelles were discrete and round in shape.

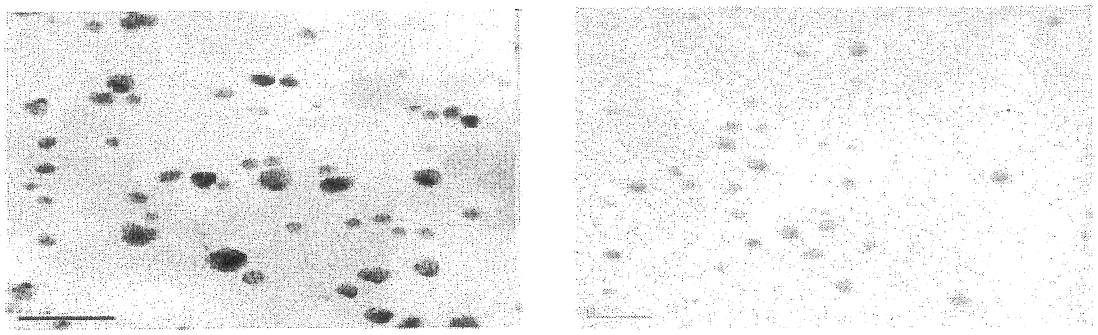

Figure 2. TEM photograph of CPT PMs containing *(a)* 1:25 w/w drug polymer ratio of F4, *(b)* 1:25 w/w drug polymer ratio of F8

PM particle size was measured using a Malvern Zetasizer and ranged from 0.437 μm to 0.178 μm (F1-F8) and for formulations containing F9-F14, it ranged from 0.921 μm to 0.615 μm (shown in Tables 3).

Table 3. Particle size and polydispersity index of polymeric micelles containing camptothecin

Formulation code	Mean particle diameter (μm)	Polydispersity index
F1	0.437 ± 0.10	0.31 ± 0.02
F2	0.408 ± 0.09	0.34 ± 0.04
F3	0.369 ± 0.17	0.41 ± 0.09
F4	0.197 ± 0.16	0.29 ± 0.03
F5	0.332 ± 0.05	0.44 ± 0.08
F6	0.296 ± 0.11	0.46 ± 0.06
F7	0.241 ± 0.13	0.52 ± 0.07
F8	0.178 ± 0.09	0.47 ± 0.06
F9	0.921 ± 0.15	0.86 ± 0.11
F10	0.853 ± 0.21	0.75 ± 0.03
F11	0.743 ± 0.23	0.71 ± 0.12
F12	0.799 ± 0.19	0.85 ± 0.09
F13	0.648 ± 0.25	0.70 ± 0.05
F14	0.615 ± 0.11	0.66 ± 0.13

It was observed that increasing the molecular weight of Me PEG-b-PCL increased the particle size; however, increasing the PEG chain length with similar PCL chain length produced a smaller particle size. This is because PEG was able to moderate the association of the copolymer molecules during the formation of the particles (Watanabe et al. 2006). It can be seen that the higher the copolymer concentration in organic phase, the larger the polymeric micelle. The higher copolymer concentration leads to slower diffusion of the organic phase into the aqueous phase, which may result in larger PMs. (Zhang et al. 2007). PMs prepared with THF showed higher average diameter (0.9-0.6μm) and some aggregation with higher polydispersity.

The CPT PMs containing F4 (Me PEG 5000-b-PCL 13,000, 1:25 ratio) and F8 (Me PEG 5000 - b- PCL 5000, 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 (Zhang et al. 2007).

Drug loading and encapsulation efficiency

Several factors may affect drug loading and encapsulation efficiency of the core shell structured PMs prepared by ESE:

a) The affinity of the loaded drug for the core-forming polymer

b) The volume of the hydrophobic core

c) Drug-drug interaction (i.e., its ability to self-aggregate) (Opanasopit et al. 2004).

Among these three, the compatibility between the drug and the core-forming block is said to be the main factor.

In order to obtain PMs with high drug loading, formulations containing 14 different ratios of drug and polymer were prepared. For CPT-PMs prepared using chloroform: acetonitrile, the results indicated that the encapsulation efficiency of camptothecin was around 82.7 ± 0.5% for PMs containing a 1:12.5 ratio of polymer - I and 79.5 ± 0.5% for PMs containing a 1:12.5 ratio of polymer-II.

The encapsulation efficiency was 83.8 ± 0.8% for PMs containing a 1:25 ratio of polymer-I and 82.0 ± 0.4% for PMs containing a 1:25 ratio of polymer-II, analyzed by HPLC. For PMs containing 1:5 ratio of block copolymer, the encapsulation efficiency was around 78.02 ± 0.5% for polymer-I and 77.89 ± 0.7% for polymer-II. For PMs prepared using THF, the encapsulation efficiency was very low and ranged from 41.53 ± 1.2% to 48.19 ± 0.5% (Table 4).

Table 4. Encapsulation efficiency of camptothecin polymeric micelles (n=3)

Formulation code	Drug:polymer ratio	Drug entrapped % ± SD
F1	1:2.5	60.30 ± 0.6
F2	1:5	78.02 ± 0.5
F3	1:12.5	82.75 ± 0.5
F4	1:25	83.80 ± 0.8
F5	1:2.5	56.21 ± 0.8
F6	1:5	77.89 ± 0.7
F7	1:12.5	79.50 ± 0.5
F8	1:25	82.00 ± 0.4
F9	1:5	41.53 ± 1.2
F10	1:12.5	45.61 ± 0.9
F11	1:25	51.03 ± 0.7
F12	1:5	38.64 ± 1.1
F13	1:12.5	44.54 ± 0.6
F14	1:25	48.19 ± 0.5

It can be seen that PMs prepared with higher copolymer concentrations in the organic phase yielded higher encapsulation efficiencies (Bin Shi et al. 2005). Higher copolymer concentrations

would accelerate the solidification of PMs, which could in turn retain the drug in the polymer matrix more effectively. In our study, the PMs prepared with a 1:25 ratio of block copolymer showed higher encapsulation efficiency than PMs prepared with 1:5 and 1:12.5 ratios of the same polymer. However, it was found that a drug /copolymer feeding ratio higher than 1:25 caused a large amount of particle aggregation and very low PM yield (data not shown). This is important because the state of the incorporated drug in the PMs is the primary factor that determines the release profile of a drug delivery system.

In vitro release behaviour

Camptothecin has very poor solubility in water and most organic solvents, which limits its application as an anticancer drug. In this study CPT was loaded in PMs by ESE. CPT-loaded PMs preserved their spherical shape and retarded CPT release (Zhang et al. 2006). The release behaviour of a lipophilic compound from the core shell of structured PMs is largely dependent on the hydrophobic property of the inner core. The other factors influencing rate of drug release include:

a) release medium used

b) characteristics of the copolymer

c) internal structure of the micelles

In this test, we used phosphate-buffered saline, pH 7.4, as the release medium in order to investigate the other factors concerned. All the PM formulations exhibited an initial burst release of CPT from the dialysis bag, indicating that the release lag time induced by the dialysis bag could essentially be neglected (Chang et al. 2008). *In vitro* release profiles of all the formulation were compared with a CPT solution. The cumulative percentage of CPT released within 6 hrs was found to be 96.6 ± 0.6% at 37°C in PBS. All samples exhibited a burst release of CPT at the initial stage. This may be because a portion of the drug was 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. After the initial burst, the CPT release rate slowed down and became steady in a controlled release manner.

In our study, CPT-PMs prepared using chloroform: acetonitrile containing a 1:5 ratio of polymer-I exhibited a burst release of 17.124 ± 0.5% in the first h. For PMs containing a 1:12.5 ratio of polymer-I it was 19.547 ± 0.1%, and for PMs containing a 1:25 ratio of block copolymer it was 10.939 ± 0.2%. For polymer-II PMs containing 1:5, 1:12.5 and 1:25 ratios of block copolymer, the burst release was 21.062 ± 0.4%, 19.595 1 ± 0.5 % and 15.183 ± 0.9% respectively. The maximum percentage of drug (96.6 ± 0.6%) was released within 6 h for a solution of pure CPT. For the PM formulations containing CPT, however, the release was found to be 32.078 ± 0.9% (F2), 42.671 ± 0.9% (F3), and 25.395 ± 0.1% (F4) at 6 h. For the formulations containing P-II (F6, F7, F8), the percent released within 6 h was 45.241 ± 0.7%, 43.321 ± 0.8%, and 35.294 ± 0.5% respectively. About 88.31 ± 0.1% of the total loaded drug was released from PMs containing 1:5 ratio of block copolymer I in 36 h. For the 1:12.5 ratio of block copolymer I, it was found to be 82.613 ± 0.1% and 78.58 ± 0.1% for PMs containing a 1:25 ratio of block copolymer I. For PMs containing 1:5, 1:12.5 and 1:25 ratios of polymer-II,

the total CPT released in 36 h was 94.015 ± 0.3, 90.315 ± 0.5 and 89.36 ± 0.7% respectively ($p<0.05$) (Fig. 3 and 4).

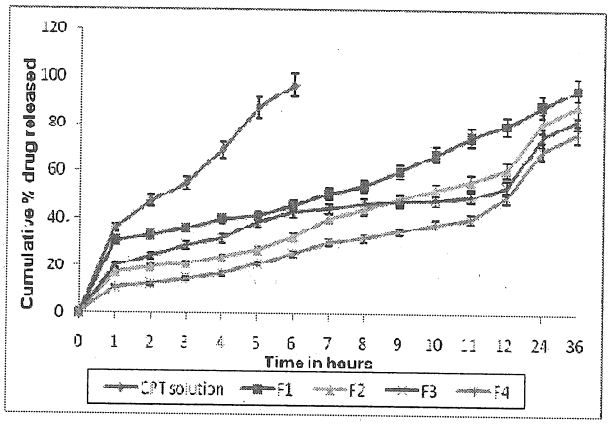

Figure 3. *In vitro* release profiles of CPT from free drug and CPT loaded polymeric micelles (F1-F4) in pH 7.4 PBS solution at 37°C.

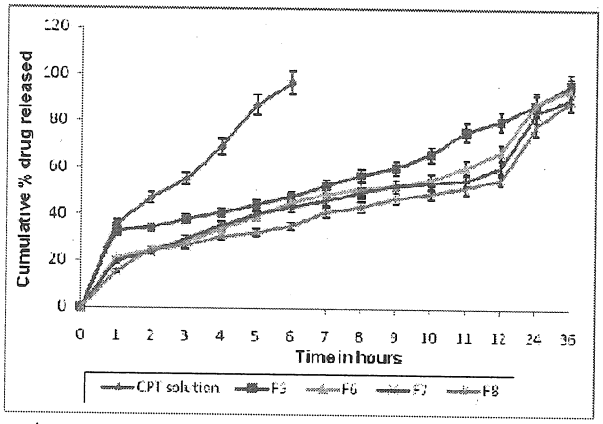

Figure 4. *In vitro* release profiles of CPT from free drug and CPT loaded polymeric micelles (F5-F8) in pH 7.4 PBS solution at 37°C.

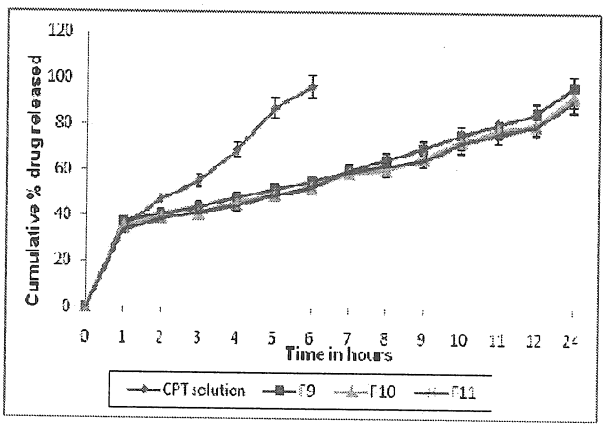

Figure 5. *In vitro* release profiles of CPT from free drug and CPT loaded polymeric micelles (F9-F11) in pH 7.4 PBS solution at 37°C.

For formulations prepared using THF as a solvent, the release rate was faster than formulations prepared using chloroform:acetonitrile. For formulations F9, F10 and F11 the release was found to be 96.66 ± 0.5%, 92.62 ± 0.8% and 90.44 ± 0.8% respectively within 24 h. For formulations F12, F13 and F14 the release was 96.34 ± 0.2%, 94.91 ± 0.9% and 93.31 ± 0.7% respectively within 24 h (Fig. 5 and 6).

The data show that the release rate decreased as the diblock copolymer ratio increased. PMs with larger size have a slower release rate. Larger polymeric micelles have bigger hydrophobic cores and smaller surface area, so it takes longer for the incorporated drug to diffuse across the polymer matrix to the particle surface and finally into the aqueous medium, resulting in slower release. 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. All block copolymer chains prepared by the emulsification solvent evaporation method existed as uniform micelles without aggregation and showed slow release for a prolonged period. Therefore, these micelles could be useful as injectable, circulating, slow-release drug carriers.

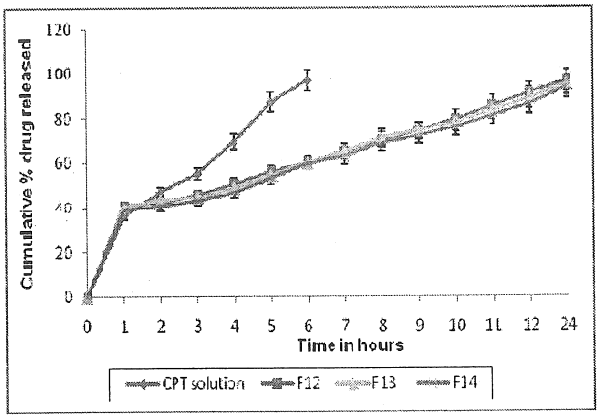

Figure 6. *In vitro* release profiles of CPT from free drug and CPT loaded polymeric micelles (F12-F14) in pH 7.4 PBS solution at 37°C.

The slow steady release of CPT observed with all these preparations demonstrates that the drug is more effectively encapsulated by our 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.

Critical micellar concentration (CMC)

The stability of micelles *in vitro* depends on the CMC values. The stability is a very important parameter in the drug delivery application of polymeric micelles because intravenous injections of micellar solutions are associated with extreme dilutions by circulating blood. If the concentration of a micelle forming polymer in the circulation drops below the CMC, the micelles may be prematurely destroyed resulting in the release of encapsulated drug into the circulation before it reaches its target; this could be dangerous because a poorly soluble drug may precipitate inside blood vessels. Upon dilution to CMC, micelles begin to dissociate into

unimers and thus release drugs. The lower the CMC value, the better the micelle stability (Yamamoto et al. 2007).

CMCs of block copolymers were analyzed using pyrene as a fluorescent probe. Pyrene is known to exhibit a peak shift in its excitation spectrum upon incorporation into a hydrophobic inner core. At very low concentrations of Me PEG-b-PCL, the fluorescence intensity was very low, but in the presence of PMs, there was an increase in the fluorescence intensity ratio.

The CMC was found to be 5-18 μg/mL for the PMs prepared using Me PEG-b-PCL, 5000-13000. For the PMs containing Me PEG-b-PCL, 5000-5000, it was 12-32 μg/mL using chloroform: acetonitrile as a solvent. For the PMs prepared using THF it ranges from 26-42 μg/mL (shown in Table 5). It is known that the stability of micelles both *in vitro* and *in vivo* depends on the CMC values. Upon dilution to CMC, micelles begin to dissociate into unimers and thus release drugs. In general, the lower the CMC value, the better the micelle stability (Yamamoto et al. 2007). Also these results on experimental CMC values vs hydrophobic block length coincide well with those reports given by Astafieva *et al.* (1996).They reported that the onset of micellization was determined mainly by the nature and the length of the hydrophobic block, where the nature of the hydrophilic block had only a slight dependence on the onset of micellization.

Table 5. Critical micellar concentration of CPT-PMs (n=3)

Formulation code	Drug:polymer ratio	CMC (μg/mL)
F1	1:2.5	18
F2	1:5	10
F3	1:12.5	13
F4	1:25	5
F5	1:2.5	32
F6	1:5	28
F7	1:12.5	20
F8	1:25	12
F9	1:5	34
F10	1:12.5	31
F11	1:25	26
F12	1:5	42
F13	1:12.5	40
F14	1:25	38

Drug release kinetics

Camptothecin polymeric micelles were prepared by ESE using diblock copolymers to retard release and achieve the required release profile. To study the release kinetics, data obtained from *in vitro* release studies were plotted in various kinetic models viz., zero order, first order, Higuchi's and Korsmeyer-Peppas plots (Ju et al. 1995).

It was found that the *in vitro* release of CPT from PMs was best explained by Higuchi's equation, as the plot showed the highest linearity followed by first order, followed by zero order for all the formulations. The log-cumulative percentage drug release vs. time plot for the Korsmeyer-Peppas equation indicated a good linearity. The release exponent *n* was between

0.52-0.78, which appears to indicate a diffusion and erosion mechanism for all the formulations containing P-I and P-II.

Pharmacokinetic studies

Since *in vitro* studies of the formulations containing chloroform:acetonitrile showed smaller particle size, low polydispersity index, higher entrapment efficiency and slower release, pharmacokinetic studies were carried out in rabbits for the formulations F4 and F8. The pharmacokinetic parameters of CPT loaded in micelles after intravenous injection are shown in Table 6. The Me-PEG-PCL micelles showed higher initial circulating levels in blood compared with free CPT.

Table 6. Pharmacokinetic parameters of camptothecin solution and camptothecin polymeric micelles

Parameters	Drug in solution (i.v)	F4 (PI, 1:25) (i.v)	F8 (PII, 1:25) (i.v)
$AUC_{0\text{-last}}$ (ng/mL.h)	33.6	3519.3**	68.71*
$t_{1/2}$ (h)	0.85	3.8**	3.3*
MRT(h)	0.9	5.5**	4.82*
Total clearance (mL/min/kg)	59.1	9.47**	37.3*
V_d (mL/kg)	692.5	1923.9**	1092.5*
K_{el} (min^{-1})	0.85	0.18*	0.21*

Data are given as mean ± SEM (n=3) Pharmacokinetic parameters of CPT PMs and CPT solution following i.v. administration in rabbits. **denotes $p<0.01$, *denotes $p<0.05$ when compared with CPT solution (ANOVA followed by post hoc Dunnett's test)

The concentration of F4 and F8 in blood at 5 h was about 19.5 ng/mL and 4.5 ng/mL respectively. By 4 h, the free CPT had been removed from the circulation and could not be detected (data not shown). On the contrary, Me PEG-b-PCL micelles exhibited a remarkably delayed blood clearance. It could be seen that the Me PEG-b-PCL (F4 and F8) micelles remained in the blood for 7 h. Me PEG-b-PCL micelles with PEG molecular weight of 5000 could extend the half-life of CPT to 3.8 h and 3.3 h respectively (F4 and F8). The AUC for CPT-PMs also increased compared to free CPT.

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 it was understandable that they would release the 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 also found to be significantly low ($p<0.01$) when compared with CPT in solution. Increase in MRT in plasma compared with the same dose of CPT solution may be due to the coating of PEG on the surface of polymeric micelles and sustained the release of CPT from CPTPMs. The high value for V_d confirms broad tissue distribution for the copolymeric micelles to the various tissues and organs within a short period of time. In this way, the penetration of the copolymeric micelles into tissues may be enhanced.

The *in vivo* characteristics of Me-PEG-PCL micelles were consistent with the micelles' *in vitro* physicochemical characteristics. Since Me PEG-b-PCL (5000-13000) micelles had longer PCL chain length and more rigid structure, they possessed higher drug loading efficiency and it was understandable that they would release drug more slowly and have a relatively longer half-life.

These data confirm that Me PEG-b-PCL micelles provide enhanced drug stability in the blood and prevent the adsorption of various blood components onto their surface. These findings suggest that the stable incorporation of CPT into PMs by the hydrophobic interaction of intact CPT with inner core of PM may be important in maintaining drug stability in the circulation.

Discussion

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 solubilise 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 anti-tumour 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 and Amsten 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 copolymer, 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 anti-tumour activity. To achieve this two different molecular weight copolymers (Me PEG5000-b-PCL13,000 and Me PEG5000-b-PCL5000), a sonication time of 2 min, at temperature in the RFE of 40 °C at 100 rpm, and centrifugation at 13,200 rpm for 1 h 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 PEG5000-b-PCL13,000, 1:25 1 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 et al. 2006). 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 co polymer ratios. Therefore, chloroform: acetonitrile is a more favourable solvent for CPT than THF when emulsification solvent evaporation method was used (Sang, C.L 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 solubilising pyrene resulted in an increase of fluorescence intensity.

In this study the CMC of F4 (PI, 1:25, chloroform: acetonitrile) and F8 (PII, 1:25, chloroform: acetonitrile) were found to be 5 and 12 μg/mL respectively, whereas for F11 (PI, 1:25, THF) and F14 (PII, 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 (Chang and Chu 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.

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.

Conclusion

The ultimate goal for controlled drug release is to maximize therapeutic activity while minimizing the negative side effects of the drug. In this regard, polymeric micelles have emerged as a novel drug carrier system for hydrophobic anticancer drugs. We have shown that camptothecin in polymeric micelles is a promising nanomedicine with improved drug solubility and stability.

CPT-PMs containing two different molecular weight diblock copolymers were formulated and their entrapment efficiency and *in vitro* release behaviour evaluated. In addition, a fluorescent probe was used to measure the CMC. It was found that the composition of the copolymers and the preparation conditions of PMs influenced the particle size, encapsulation efficiency and *in vitro* release, which render this drug delivery system highly flexible and adjustable. These characteristics suggest that PMs possess the ability to deliver larger doses of CPT in its more active lactone form with long persistence in the circulation.

It is anticipated that, because of their size and steric stability, CPT-PMs will be passively targeted to solid tumours *in vivo,* resulting in high drug concentration in tumours and reduced

drug toxicity to the normal tissues. In the years to come, it is expected that knowledge gained in cancer biology and polymer chemistry will catalyze the further development of novel multifunctional micellar systems with greater customization to achieve more efficacious antitumor responses.

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