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ORIGINAL ARTICLES

Investigation on design of stable etoposide-loaded PEG-PCL micelles: effect of molecular weight of PEG-PCL diblock copolymer on the *in vitro* and *in vivo* performance of micelles

Mukesh Ukawala[1], Tushar Rajyaguru[1], Kiran Chaudhari[1], A. S. Manjappa[1], Smita Pimple[1], A. K. Babbar[2], Rashi Mathur[2], A. K. Mishra[2], and R. S. R. Murthy[1,3]

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Abstract

In the present study, six different molecular weight diblock copolymer of methoxy poly (ethylene glycol)-b-poly (ε-caprolactone) (MPEG-PCL) were synthesized and characterized and was used for fabrication of etoposide-loaded micelles by nanoprecipitation technique. The particle size and percentage drug entrapment of prepared micelles were found to be dependent on the molecular weight of PCL block and drug to polymer ratio. The maximum drug loading of 5.32% was found in micellar formulation MPEG5000-PCL10000, while MPEG2000-PCL2000 exhibited 2.73% of maximum drug loading. A variation in the fixed aqueous layer thickness and PEG surface density of micellar formulations was attributed to difference in MPEG molecular weight and interaction of PEG and PCL block of copolymer. The MPEG2000-PCL2000 micelles demonstrated poor *in vitro* stability among other micellar formulations, due to its interaction with bovine serum albumin and immediate release of drug from micelles. Furthermore, plain etoposide and MPEG2000-PCL2000 micelles exhibited greater extent of hemolysis, due to presence of surfactants and faster release of drug from micelles, respectively. The biodistribution studies carried out on Ehrlich ascites tumor-bearing Balb/C mice confirmed higher accumulation of etoposide-loaded micellar formulation at tumor site compared to plain etoposide due to enhanced permeability and retention effect.

Keywords: Micelles, etoposide, polymeric drug delivery system, nanoprecipitation, PEG surface density, hemolysis, fixed aqueous layer thickness

Introduction

Recently, polymeric micelles have been emerged as a novel nanomedicine platform for drug delivery, due to their smaller size, stability, versatility, and biocompatibility (Shen et al., 2007; Sutton et al., 2007). These micelles have demonstrated their ability to efficiently solubilize lipophilic agents with altered drug pharmacokinetics and in several cases have been reached to clinical trials (Allen et al., 2000; Matsumura et al., 2004; Zhang et al., 2004; Forrest et al., 2008; Lee et al., 2008; Xiong et al., 2008) Micelles made up of core-shell structure are normally fabricated by a self-assembly approach using amphiphilic block copolymers in a selective solvent in which the hydrophobic core part provides a cargo space for solubilization of various lipophilic drugs (Aliabadi et al., 2007; Molavi et al., 2008). The core region surrounded by hydrophilic corona part, that is, of higher molecular weight poly (ethylene glycol) (PEG) provides stabilization of micelles with no need of additional stabilizers. Due to smaller size (<150 nm) and presence of PEG chains on the surface of micelles, these carriers can have a longer blood circulation and ability to passively target tumor tissues by preferentially accumulating into the "leaky" tumor vasculature via the enhanced

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permeation and retention (EPR) effect (Forrest et al., 2008; Yanez et al., 2008). Till date, micelles made up of biocompatible and biodegradable amphiphilic block copolymer, such as PEG-PLGA, PEG-PLA, PEG-PCL, and triblock copolymers PCL–PEG–PCL, have been extensively studied as a nanoparticulate drug delivery system, especially for hydrophobic drugs (Aliabadi et al., 2007; Yang et al., 2007; Lee et al., 2008; Wei et al., 2009).

Despite, significant therapeutic outcome shown by micellar drug delivery, still it remains to be fully evaluated and understood for various aspects which help to design a micellar drug delivery system which indeed act as a real carrier *in vivo*. Critical micelles concentration (CMC) is considered as one of the important aspect for stability of micelles and release of drug *in vivo*, which is usually determined by fluorescent hydrophobic probe, such as pyrene (Shi et al., 2005; Letchford et al., 2008). It is reported that lower the CMC value, higher will be the stability of micelles, which can be achieved by increasing the molecular weight of hydrophobic core part (Letchford et al., 2008). However, it was reported that paclitaxel dissociated quickly from MePEG-b-PDLLA micelles to blood despite lower CMC values, which implies some interaction between blood components and micelles (Burt et al., 1999). In addition, the use of pyrene as probe for determination of CMC has raised some unanswered questions regarding the difference in the solubility of drug and pyrene, method of preparation of micellar formulation, interactions between the micelles and biological components, and the dissociation kinetics of micelles (Bae and Yin 2008). If micelles dissociate into unimers immediately after intravenous injection due to poor *in vivo* stability, the loaded hydrophobic drug would be immediately released out and precipitated. Hence, the purpose of higher drug accumulation to tumor by EPR effect will be badly affected and will create suspicion about stability of micelles (Savic et al., 2006).

Therefore, in addition to CMC determination, it is necessary to investigate the *in vitro* performance of prepared micellar formulation thoroughly before it goes to preclinical or clinical stage. It is well known that besides particle size and zeta potential, the PEG surface density and fixed aqueous layer thickness (FALT) of micelles play important role in the *in vitro* and *in vivo* behavior of nanoparticles/micelles (Shi et al., 2005). Blood persistent drug delivery system with potential applications for intravenous drug administration can be achieved by PEG-grafted amphiphilic block copolymer, which can reduce protein adsorption and opsonization of micelles. This dysopsonic effect is a function of PEG surface density and PEG chain length (Gref et al., 2000). The *in vitro* stability of micelles in presence of bovine serum albumin (BSA) could also provide some indication about the *in vivo* fate of micelles. Majority of hydrophobic drugs have a high plasma protein affinity, and in this situation, the micellar carrier should function as true carrier rather than mere solubilizers because the incorporated drug have tendency to quickly dissociate from micelles after intravenous injection (Liu et al., 2005, 2007). Determination of hemolytic property is one of the most common tools that the formulation scientist may utilize for assessment of safety of parenteral formulation. Kevin and his colleague have suggested that the stable core with well-hydrated corona of micelles would result in intact micelles with few unimers available for penetration into the cell membrane, which reduce membrane perturbation and subsequent hemolysis (Letchford et al., 2009). Therefore, based on the various studies mentioned above, it is requisite to evaluate the micellar carries in terms of their *in vitro* stability and drug release performance before the *in vivo* evaluations are carried out.

The aim of present study was to investigate the effect of MPEG-PCL diblock copolymer molecular weight on *in vitro* stability and *in vivo* performance of etoposide (ETO)-loaded micelles. The micellar formulations prepared using various molecular weight of PEG-PCL copolymer were evaluated for CMC, FALT, *in vitro* stability, hemolysis, and PEG surface density. Finally, the biodistribution study of selected micellar formulations were carried out using Ehrlich ascites tumor-bearing Balb/C mice.

Materials and methods

Materials

Monomethoxy poly(ethylene glycol) (CH_3O-PEG-OH) of molecular weight 2000 g and 5000 g, ε-caprolactone (CL), HCl in diethyl ether (HCl-Et_2O), and pyrene were purchased from Sigma Aldrich, (Mumbai, India). The ETO was a generous gift from Cipla Laboratory (Mumbai, India). Acetone, dry toluene, diethylether, acetonitrile, triethylamine, and potassium iodide of AR grade were acquired from S.D. Fine Chem. Ltd. (Mumbai, India). Drabkin's reagent was bought from Monozyme India Ltd. (Secunderabad, India). The BSA was procured from Himedia Laboratory (Mumbai, India). Dichloromethane (Spectrochem Lab, Mumbai, India) was dried and distilled using calcium hydride before use. The CL was dried over calcium hydride for 48 h and distilled under reduced pressure, prior to use. All other chemicals were of analytical grade and used without further purification.

Animals

Female Balb/C mice of age group between 6 and 8 weeks were used for biodistribution studies. The animals were maintained in a room (23 ± 2°C and 60 ± 10% humidity) under a 12 h light/dark cycle. Food and water were given *ad libitum*. All animal studies were carried out under the guidelines compiled by Committee for the Purpose of Control and Supervision of Experiments on Animals, Ministry of Culture, Government of India (CPCSEA), and all the study protocols were approved by the Animal Ethics Committee of the INMAS, New Delhi.

Synthesis and characterization of MPEG-PCL diblock copolymer

The MPEG-PCL diblock copolymers were synthesized using metal-free cationic ring opening polymerization method with slight modification as reported earlier (Kim et al., 2005). The typical process for the polymerization to give MPEG-PCL of molecular weight 2000–2000is as described. Briefly, 1 g of CH_3O-PEG-OH (0.5 mmol, Mn = 2000) was azeodistilled twice using dry toluene (50 mL) to remove water and toluene was then distilled completely. To CH_3O-PEG-OH the dried dichloromethane was added (10 mL), followed by addition of CL (1.0 g, 8.75 mmol) using syringe. The polymerization was initiated by the addition of 1.0 M solution of HCl-Et_2O (1.5 mL, 1.5 mmol) and the reaction was maintained at 25°C for 24 h with vigorous stirring under nitrogen atmosphere. After completion of reaction, it was terminated with the help of 0.1 mL of triethylamine and the precipitated triethylamine-HCl salt was removed by filtration. The copolymer was collected by precipitation of filtered solution in cold diethyl ether and washed with cold methanol thrice to remove residual monomer. The final product was kept in desiccator at room temperature for 48 h and used for further study. Similarly, different molecular weight MPEG-PCL diblock copolymers were synthesized by changing the feed ratio of CL to PEG of molecular weight 2000 or 5000 g.

The ^{1}H-NMR spectra of the diblock copolymers were recorded using a Bruker spectrometer operating at 400 MHz using $CDCl_3$ as solvent. Chemical shifts (d) were given in ppm using tetramethylsilane (TMS) as internal reference. The integration ratio of peak at δ 4.05 ppm due to PCL blocks to peak at δ of 3.65 ppm due to the PEG blocks was used to calculate the average molecular weight. Average molecular weight and its distributions was measured by gel permeation chromatography (GPC) (Perkin Elmer Totalchrom) using tetrahydrofuran (THF) as elution solvent and medium molecular weight (1×10^3 to 3×10^5) polystyrene as a standards (Polysciences Inc.).

Preparation of micelles

The MPEG-PCL copolymeric micelles loaded with ETO were prepared by nanoprecipitation method (Aliabadi et al., 2005). Briefly, a 50 mg of MPEG2000-PCL2000 and 2 mg of ETO were dissolved in 3 mL of acetone and added dropwise (1 mL/min) to distilled water under stirring at 1000 rpm and stirring was continued till complete evaporation of acetone. Residual amount of acetone was removed using rotary vacuum evaporator. The resulted bluish micellar formulation was filtered through 0.45 micron filter membrane to remove nonincorporated drug and copolymer aggregates. Mean particle size and polydispersity index (PDI) of micelles were measured by dynamic light scattering (DLS) method using Zetasizer, NanoZS, (Malvern Inst., UK). The percentage drug entrapment or loading of ETO was determined by UV-visible spectrophotometer (UV 1700, PharmaSpec, Shimadzu, Japan) at 284 nm by dissolving an aliquot of micellar dispersion in acetonitrile. Effects of amount of polymer and ratio of aqueous phase to organic phase on particle size and percentage drug entrapment were assessed by varying formulation parameters.

In vitro evaluation of MPEG-PCL micelles

Critical micelles concentration

The CMC of MPEG-PCL micelles were determined by fluorescence spectrophotometer (RF-540, Shimadzu Corporation, Japan) using pyrene as fluorescent probe at room temperature (Choi et al., 2006). A known amount of pyrene was dissolved in acetone and added to a series of vials and acetone was evaporated under nitrogen. The final concentration of pyrene in MPEG-PCL micellar solution was kept 6×10^{-7} M. A 10 mL of various concentration of MPEG-PCL micellar solution was added to each vial and kept at 65°C for 2 h and cooled to room temperature with overnight equilibrium. The excitation spectra were recorded between 300 and 400 nm with an emission wavelength of 390 nm. The intensity ratios of I_{338} to I_{335} were plotted as a function of logarithm of polymer concentration. The CMC value was taken from the intersection of the tangent to the curve at the inflection with the horizontal tangent through the points at low concentration.

FALT

The FALT of the micelles was calculated using Gouy–Chapmann theory (Shi et al., 2005). According to this theory, zeta potential Ψ[L] as the electrostatic potential at the position of the slipping plane L (nm) is expressed as

$$\mathrm{In}\,\psi[\mathrm{L}] = \mathrm{InA} - \mathrm{KL}$$

where A is regarded as a constant, k is the Debye–Huckel parameter, equal to $\sqrt{c/03}$ (c is the molality of electrolytes) for universal salts, and L gives the position of the slipping plane or thickness of the fixed aqueous layer in nanometer units. Briefly, to 1 mL of ETO-loaded MPEG-PCL micelles, required amount of NaCl was added in micellar solution to make final concentration of NaCl to 25 mM, 50 mM, 75 mM, and 100 mM, and after 30 min their zeta potential were measured using Zetasizer, NanoZS (Malvern Inst., UK). The natural logarithm of zeta potential were plotted against k as per above equation and the slope obtained represents the FALT of micelles.

In vitro *stability study*

The *in vitro* stability of ETO-loaded MPEG-PCL micelles was evaluated in both the absence and presence of physiologically relevant concentrations of BSA (Liu et al., 2005). Specifically, the micelle solutions were mixed with equal volumes of phosphate buffer saline pH 7.4 (PBS) in the absence and presence of BSA (45 g/L) and incubated at 37°C. At various time points, 1 mL of samples were removed and analyzed by DLS for size measurement using Zetasizer, NanoZS (Malvern Inst., UK).

Hemolysis study

The ETO-loaded MPEG-PCL micelles were investigated to find out the percentage hemolytic effect on erythrocytes with minor modifications (Letchford et al., 2009). One Sprague–Dawley rat was sacrificed by isoflurane and blood was obtained by cardiac puncture. The blood collected in EDTA sodium containing tubes was centrifuged at 1500 rpm for 15 min at 4°C. The supernatant was discarded and the pellet containing erythrocytes was washed three times with cold PBS to remove debris and serum proteins by centrifugation at 1500 rpm for 15 min at 4°C. After washing, erythrocyte stock dispersion with fixed concentration of hemoglobin was prepared with buffer (three parts centrifuged erythrocytes plus 11 parts of PBS). The stock dispersion was stored at 2°C–8°C for maximum 24 h and its stability was checked by photometric monitoring (100 µL in 0.9 mL of buffer solution) to ensure 0% hemolysis.

A 100 µL of aliquot of erythrocytes dispersion was added to 900 µL of diluted MPEG-PCL micelles (prepared in PBS) with different concentration of ETO. The ETO and ETO-free injectable solutions (contains citric acid, benzyl alcohol, polysorbate-80, PEG-300, and ethanol) were also prepared according to marketed formulation composition and evaluated to find out hemolytic potential. Samples were incubated at 37°C for various time periods in shaking water bath at low speed. After incubation, debris and intact erythrocytes were removed by centrifugation at 1500 rpm for 10 min. A 100 µL of supernatant was added to 3 mL of Drabkin's reagent at room temperature and kept for 5 min. Absorbance of samples were measured at 540 nm by UV-visible spectrophotometer (UV 1700, PharmaSpec, Shimadzu, Japan), using Drabkin's reagent as blank. Positive control value was achieved by treating erythrocyte dispersion with 2% Triton X-100, while PBS-treated erythrocyte dispersion yielded negative control value. Finally, the percentage hemolysis was calculated using following equation.

$$\%\,\text{Hemolysis} = \frac{\text{Abs sample} - \text{Abs negative control}}{\text{Abs Positive control} - \text{Abs negative control}} \times 100$$

PEG surface density

The PEG surface density of MPEG-PCL micelles of various block length was determined as reported earlier (Peracchia et al., 1997; Shi et al., 2005). The ETO-loaded MPEG-PCL micelles were diluted with distilled water, and to 5 mL of diluted dispersion, 2 mL of 2 N NaOH were added and kept for 5 days at 50°C. Samples were neutralized with 1 N HCl to pH 7.0 and to this samples 250 µL of I_2/KI solution (I_2 1 g/100 mL and KI 2 g/100 mL) was added. Samples were mixed well and absorbance was measured at 525 nm by UV-visible spectrophotometer (UV-1700, PharmaSpec, Shimadzu, Japan). Fraction of PEG was calculated based on the amount of PEG divided by total amount of MPEG-PCL copolymer used in formulation. Finally, PEG surface density was calculated based on equation

$$\delta = \frac{N \times d \times \alpha \times r}{3\text{MwPEG}}$$

where δ is the surface density of PEG chains (PEG/nm^2), N is the Avogadro number 6.021×10^{23}/mole, r is the particle radius neglecting the PEG layer thickness (error < 10%), d is the density of micelles, α is the fraction of PEG content after micelles degradation, and MwPEG stands for molecular weight of PEG. From the data of surface density of PEG chains, the average distance D between two neighboring PEG chain was calculated based on the following equation:

$$D = \sqrt{1/\delta}$$

X-ray diffraction study

The X-ray diffraction study of plain ETO powder and lyophilized micellar formulations (i.e., MPEG2000-PCL3500 and MPEG5000-PCL7000) was performed using Bruker AXS D8 Advance X-ray diffractometer with DIFFRAC plus software, version 3.2 (Bruker Inc., Madison, WI, USA) by Cu-Kα radiation. Plain ETO or lyophilized samples of micelles was placed on a zero background quartz plate and continuously scanned at a rate of 0.75° 2θ/min over a range of 5° to 50° 2θ.

In vitro *drug release study*

In vitro release study of ETO-loaded formulations (MPEG2000-PCL3500 and MPEG5000-PCL7000) and ETO injection was carried out using dialysis bag diffusion techniques, as reported previously (Lin et al., 2005). The micellar dispersion equivalent to 1 mg of ETO was filled in cellulose dialysis tube and sealed at both ends. The bag containing formulation was dipped in container having 50 mL of PBS pH 7.4, stirred at 100 ± 10 rpm and maintained at 37 ± 2°C. Samples were withdrawn at specified time points and the release medium was replaced by the same volume of fresh medium. The release of drug was quantified by UV-visible spectrophotometer (UV 1700, PharmaSpec, Shimadzu, Japan) at wavelength of 284 nm. The cumulative amount of drug released at each sampling point was corrected with the volume of the release medium and a graph of percentage cumulative release versus time was plotted.

In vivo studies

Determination of radiolabeling efficiency and in vitro *stability*

The ETO and selected ETO-loaded micellar formulations (i.e., MPEG2000-PCL3500 and MPEG5000-PCL7000) were radiolabeled with Technetium-99m (^{99m}Tc) by direct labeling method using stannous chloride ($SnCl_2$) as the reducing agents, as reported earlier (Snehalatha et al., 2008). The final radioactivity present in the formulation was checked using dose calibrator (Gamma ray

scintillation counter, Capintec, CAPRAC-R). The labeling efficiency of ETO and ETO-loaded micellar formulations was evaluated by similar method as reported earlier using instant thin-layer chromatography strips (ITLC) coated with silica gel (Babbar et al., 1991; Mishra et al., 1991) The percentage free pertechnetate and radiolabeled colloids (reduced/hydrolyzed technetium) were calculated using acetone and pyridine: acetic acid: water (3:5:1.5 v/v), respectively, as mobile phase. By subtracting the migrated activity with the solvent front using acetone from that using pyridine: acetic acid: water mixture, the net amount of ^{99m}Tc-ETO or ^{99m}Tc-ETO-loaded micelles were calculated.

The stability of the ^{99m}Tc-labeled complexes of ETO and ETO-loaded micellar formulations was determined *in vitro* in normal saline by ascending TLC technique. The labeled complex (0.1 mL) was incubated with normal saline (0.4 mL) at 37°C up to 24 h. The samples were withdrawn at regular intervals up to 24 h and spotted on ITLCSG paper and TLC was performed as reported above. These strips were counted for radioactivity in gamma ray spectrometer and percentage labeling efficiency at different time interval was calculated for ETO and ETO-loaded micellar formulations.

Biodistribution study

Biodistribution study of ^{99m}Tc-labeled ETO and ETO-loaded micellar formulations (0.2 mg (ETO)/0.1 mL) was studied in Ehrlich ascites tumor (EAT)-bearing mice (Yadav et al., 2008). The EAT cells grown in the ascites fluid of mice were harvested and 1.8×10^6 cells per mouse was injected subcutaneously in thigh of the right hind leg of female Balb/c mice. A palpable tumor in the volume range of 0.9 ± 0.1 cm^3 was observed after 10 days. The mice were divided into three groups of 20 animals each (total 60 mice; five mice per formulation per time point). All animals were fasted overnight before the experiment but allowed free access to water *ad libitum*. Each mouse injected a 100 μCi (100 μL) dose of the labeled formulations (plain ETO and ETO-loaded micelles) by intravenous route via tail vein. After certain time interval, the mice were killed by cervical dislocation and the blood as well major organs like heart, lung, liver, kidney, spleen, stomach, intestine, and tumor were isolated. The radioactivity present in the organs/tissue was measured using well-type gamma scintillation counter (Gamma ray scintillation counter, Capintec, CAPRAC-R) along with three aliquots of the diluted standard representing 100% of injected radioactivity. The results were expressed as percentage of injected dose (ID) per gram of an organ.

Results

Synthesis and characterization

The MPEG-PCL diblock copolymers of different molecular weight were prepared by varying feed ratio of CL to CH_3-PEG-OH by ring opening polymerization method in the presence of monomer activator (HCl-Et_2O). Table 1 represents the results of polymerization. The molecular weight of polymer by ^{1}H-NMR was calculated using integration ratio of peak at δ 4.05 ppm due to PCL blocks to peak at δ of 3.65 ppm due to the PEG blocks (Shen et al., 2007). Moreover, ^{1}H-NMR spectra of block copolymer (Figure 1) showed the characteristic chemical shifts corresponding to both PCL (1.39, 1.63, 2.31, and 4.05 ppm) and MPEG (3.38 and 3.65 ppm). The molecular weights obtained by GPC are comparable to that calculated by ^{1}H-NMR data. A unimodal distribution peak in the GPC chromatograms (Figure 2) was observed, which indicates that all impurities were removed after purification. These copolymers have different PCL or PEG lengths, and thus allowed investigation of effect of copolymer compositions on the micelle properties.

Table 1. Characteristics of synthesized MPEG-PCL diblock copolymer.

Polymer code	Mw of PEG	[CL]/ [PEG][a]	Mn cal[b]	Mn NMR[c]	Mn GPC[d]	Mn/Mw (GPC)
MPEG2000-PCL2000	2000	17.50	4000	3957	4364	1.27
MPEG2000-PCL3500		30.62	5500	5349	5870	1.21
MPEG2000-PCL5000		43.80	7000	6843	7291	1.39
MPEG5000-PCL5000	5000	43.80	10000	9477	10784	1.30
MPEG5000-PCL7000		61.32	12000	11592	12836	1.43
MPEG5000-PCL10000		87.61	15000	14687	16190	1.23

Note: Condition: [HCl- Et_2O]/ PEG (molar) = 3.
[a] [CL]/[PEG] is the molar ratio of CL to PEG used in synthesis;
[b]Mncal = MnPEG + MnPCL; MnPCL is the calculated molecular weight of the PCL block based on the feed ratio of caprolactone to MPEG;
[c]MnNMR = MnPEG + MnPCL, calculated from ^{1}H-NMR spectrum;
[d]Mn GPC is the relative molecular weight determined by GPC with respect to poly(styrene) standards.

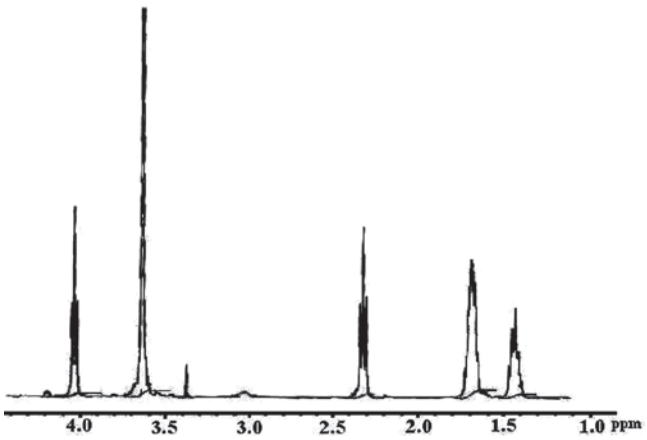

Figure 1. 1H-NMR spectrum of MPEG2000-PCL3500 diblock copolymer.

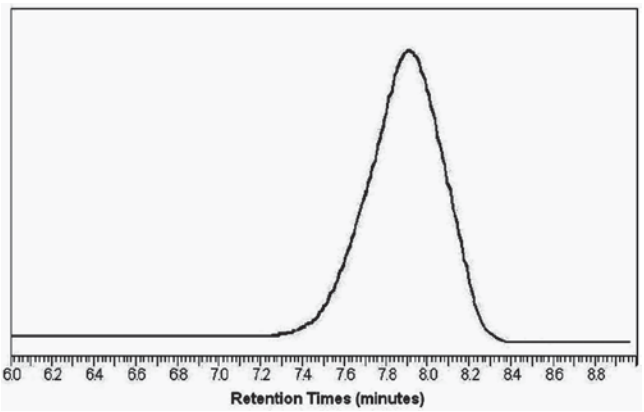

Figure 2. Typical GPC chromatogram of MPEG2000-PCL3500 diblock copolymer.

Preparation of micelles

Nanoprecipitation technique was employed for fabrication of MPEG-PCL micelles using synthesized block copolymers without use of surfactant. Effects of ratio of drug to polymer and aqueous to organic phase on particle size and percentage drug entrapment were evaluated, and results obtained are represented in Tables 2 and 3. The average particle size of all micellar formulation was observed between 38 and 80 nm and was dependent on molecular weight of copolymer. An increase in particle size and percentage drug entrapment was observed with increase in the ratio of drug to polymer and increase in the molecular weight of hydrophobic block PCL. Increase in the ratio of aqueous phase to organic phase from 1:0.6 to 1:0.8 resulted in no significant change in percentage drug entrapment but particle size and PDI were found increased in all micellar formulations except MPEG5000-PCL7000 and MPEG5000-PCL10000, which exhibited reduction in particle size and PDI.

Percentage drug loading was evaluated by altering the drug amount, keeping other parameters constant for all micellar system (data not shown). The maximum practical percentage drug loading obtained in all micellar formulations is represented in Figure 3. It was observed that upon increase in molecular weight of hydrophobic core part, percentage drug loading was increased. The maximum drug loading found with MPEG2000-PCL2000 and MPEG5000-PCL10000 micelles was 2.73% and 5.32 %, respectively.

Critical micelles concentration

The CMC values were obtained from the self aggregation behaviors of the MPEG-PCL micelles in an aqueous phase using fluorescence emission spectra of the copolymer solutions of various concentrations in the presence of pyrene. Plot of the fluorescent emission intensity ratio (I_{338}/I_{335}) as a function of copolymer concentration illustrated that the intensity values remained almost constant below the CMC, while a substantial increase in fluorescence after certain concentration called CMC reflected incorporation of pyrene in the hydrophobic

Table 2. Effect of ratio of drug to polymer on particle size, PDI and percentage drug entrapment of MPEG-PCL micelles (results are mean ± SD, $n=3$).

Formulation code	Ratio of drug to polymer (mg)	Particle size (nm)	PDI	% drug entrapment
MPEG2000-PCL2000	1:20	36.5 ± 3.1	0.268 ± 0.02	61.4 ± 5.1
	1:25	39.0 ± 4.2	0.245 ± 0.06	71.9 ± 3.1
MPEG2000-PCL3500	1:20	39.5 ± 3.1	0.156 ± 0.02	82.5 ± 4.8
	1:25	43.6 ± 2.7	0.162 ± 0.02	86.4 ± 3.5
MPEG2000-PCL5000	1:20	61.2 ± 1.9	0.183 ± 0.02	89.0 ± 3.8
	1:25	62.1 ± 3.3	0.189 ± 0.02	91.0 ± 4.2
MPEG5000-PCL5000	1:20	68.3 ± 4.1	0.180 ± 0.02	88.7 ± 4.3
	1:25	68.9 ± 3.1	0.194 ± 0.02	91.7 ± 3.8
MPEG5000-PCL7000	1:20	78.3 ± 2.1	0.182 ± 0.03	91.0 ± 3.1
	1:25	80.8 ± 4.7	0.189 ± 0.02	93.9 ± 2.7
MPEG5000-PCL10000	1:20	86.9 ± 3.0	0.185 ± 0.03	95.9 ± 3.7
	1:25	90.2 ± 4.4	0.196 ± 0.02	96.8 ± 4.0

Table 3. Effect of ratio of aqueous to organic phase on particle size, PDI and percentage drug entrapment of MPEG-PCL micelles (results are mean ± SD, $n=3$).

Formulations	Ratio of aqueous phase to organic phase	Particle size (nm)	PDI	% drug entrapment
MPEG2000-PCL2000	1:0.6	38.2 ± 4.7	0.213 ± 0.05	71.3 ± 3.8
	1:0.8	53.1 ± 4.2	0.308 ± 0.05	72.6 ± 5.1
MPEG2000-PCL3500	1:0.6	38.5 ± 2.5	0.152 ± 0.02	81.1 ± 2.0
	1:0.8	42.7 ± 3.0	0.182 ± 0.02	80.8 ± 3.6
MPEG2000-PCL5000	1:0.6	52.9 ± 2.2	0.164 ± 0.03	88.7 ± 2.1
	1:0.8	56.2 ± 2.3	0.191 ± 0.02	86.9 ± 4.7
MPEG5000-PCL5000	1:0.6	67.8 ± 3.0	0.159 ± 0.02	88.5 ± 3.2
	1:0.8	72.8 ± 4.3	0.174 ± 0.03	86.8 ± 5.0
MPEG5000-PCL7000	1:0.6	75.7 ± 2.8	0.164 ± 0.02	91.3 ± 2.6
	1:0.8	73.3 ± 4.0	0.143 ± 0.01	92.0 ± 3.4
MPEG5000-PCL10000	1:0.6	79.9 ± 3.8	0.157 ± 0.02	83.8 ± 1.7
	1:0.8	76.2 ± 2.6	0.136 ± 0.03	82.7 ± 3.6

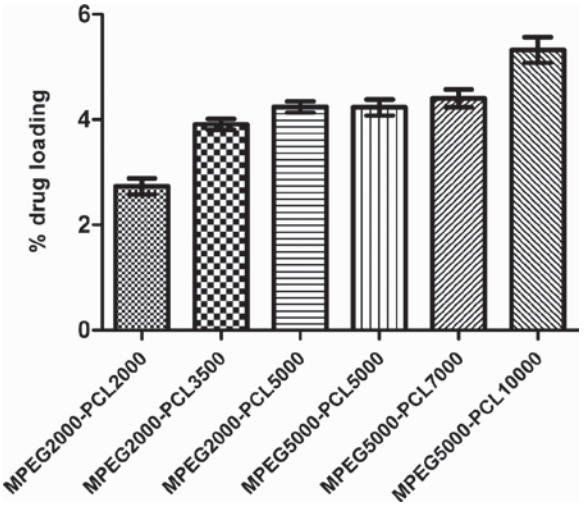

Figure 3. Maximum practical % drug loading achieved in micelles of different molecular weight (Results are mean ± S.D., $n=3$).

core of the polymeric micelles. Based on the intensity versus concentration data, the CMC values of MPEG-PCL micelles prepared from different molecular weight diblock copolymer were calculated by the crossover point at low concentration ranges and represented in Table 4. By comparing CMC values between copolymers, it was evident that there was an inverse correlation between the CMC and the PCL block length.

FALT

The FALT of various micellar formulations was calculated based on Goy Chapman theory using NaCl concentration ranging from 25 to 100 mM. A rapid reduction in charge of micelles was observed with increase in the concentration of NaCl; moreover, this effect was more prominent in case of micelles made up of PEG with molecular weight of 5000 g (data not shown). The initial zeta potential and calculated FALT of micellar formulations are represented in Table 4.

In vitro stability study

In vitro stability study of MPEG-PCL micelles carried out in presence and absence of BSA at 37°C showed that all micellar formulations remained stable over a period of 24 h, except MPEG2000-PCL2000 micelles (Figure 4). The particle size of MPEG2000-PCL2000 micelles increased from 36.1 nm to 267.2 nm and to 301.7 nm in absence and presence of BSA after 48 h storage at 37°C, respectively. The maximum increase in particle size up to 15 nm was observed for rest of all micellar formulations after 48 h of incubation (Figure 4).

Table 4. CMC, zeta potential, and FALT of MPEG-PCL micelles ($n=3$).

Formulations	CMC (mg/mL) × 10^{-3}	Initial zeta potential (mV)	FALT (nm)
MPEG2000-PCL2000	2.00	−4.76 ± 0.65	3.25
MPEG2000-PCL3500	1.86	−4.25 ± 0.28	3.05
MPEG2000-PCL5000	1.73	−4.71 ± 0.38	2.94
MPEG5000-PCL5000	1.65	−4.66 ± 0.51	4.69
MPEG5000-PCL7000	1.38	−4.81 ± 0.40	4.56
MPEG5000-PCL10000	1.14	−5.02 ± 0.61	4.40

Hemolysis study

The *in vitro* hemolysis study was performed at different concentrations of ETO-loaded MPEG-PCL micelles and ETO injection. The ETO-free injectable solution was used in the study to check the hemolytic effect of surfactant. It was seen that all MPEG-PCL micelles exhibited very low or negligible hemolysis, compared to ETO and ETO-free injectable solution after 30 min of incubation (Figures 5 and 6). The ETO injectable solution showed hemolysis of 29.8 ± 4.3% at concentration of 25 µg/mL, while ETO-free injectable solution (equivalent to similar concentration) showed percentage hemolysis of 5.8 ± 2.6 (Figure 5). This implies that marketed product of ETO injection have substantial hemolytic properties due to drug and surfactant, such as Tween-80. The MPEG-PCL micellar formulations except MPEG2000-PCL2000 showed very less or negligible hemolytic effect, that is, 1% to 5 % at a 200 µg/mL drug concentration after 24 h of incubation.

PEG surface density

Increase in molecular weight of PCL core part in MPEG-PCL micelles resulted into reduction in PEG surface density with increase in distance between two PEG chains (Table 5). The MPEG-PCL micelles with PEG of molecular weight of 5000 g exhibited lower PEG surface density and larger distance of between two neighboring PEG chains in comparison to MPEG-PCL micelles with PEG of molecular weight of 2000 g.

X-ray Diffraction

Figure 7 shows the X-ray diffraction pattern of pure ETO, MPEG2000-PCL3500, and MPEG5000-PCL7000

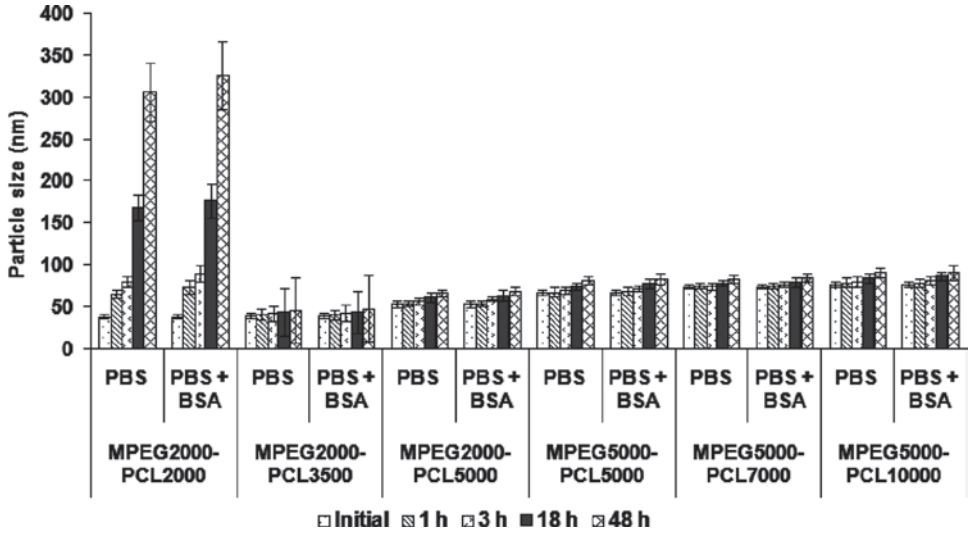

Figure 4. *In vitro* stability of MPEG-PCL micelles with respect to their particle size after incubation with and without BSA at various time points (Results are mean ± S.D., $n=3$).

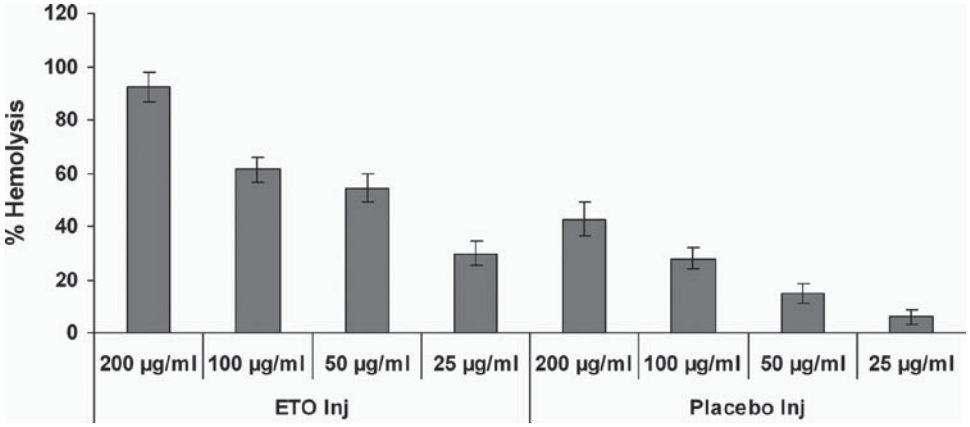

Figure 5. Percentage hemolysis of ETO and ETO-free injectable solution at various concentrations after 30 min of incubation with erythrocytes (results are mean ± SD, $n = 3$).

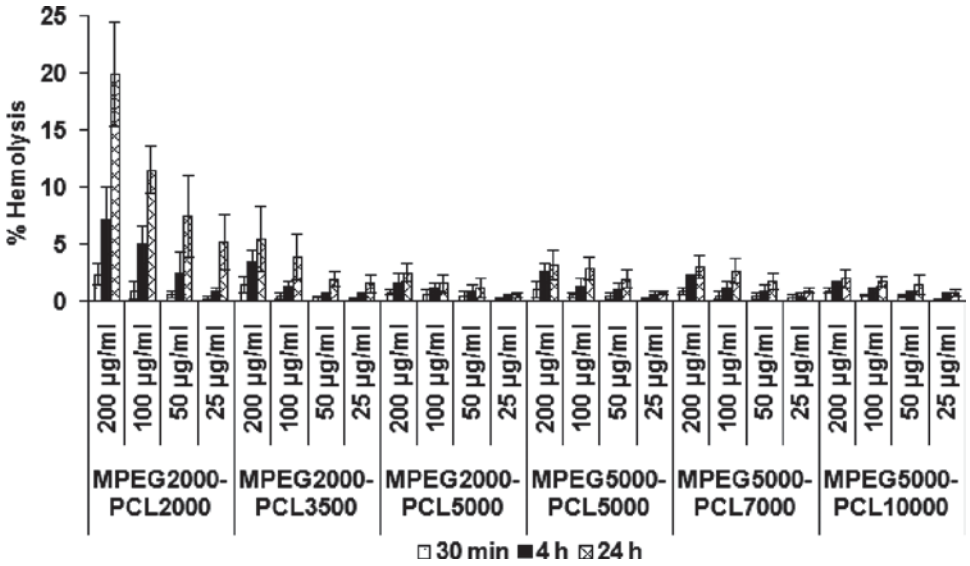

Figure 6. Percentage hemolysis of MPEG-PCL micelles at various drug concentrations and different time of incubation with erythrocyte (results are mean ± SD, $n = 3$).

Table 5. PEG surface density and average distance (nm) between two neighboring chain of MPEG-PCL micelles (results are mean ± SD, $n = 3$).

Formulation code	Fraction of PEG (α)	PEG surface density/ nm^2 (δ)	Average distance (D) nm
MPEG2000-PCL2000	0.442 ± 0.030	0.823	1.21
MPEG2000-PCL3500	0.405 ± 0.044	0.791	1.26
MPEG2000-PCL5000	0.278 ± 0.023	0.751	1.32
MPEG5000-PCL5000	0.459 ± 0.063	0.631	1.58
MPEG5000-PCL7000	0.385 ± 0.038	0.572	1.74
MPEG5000-PCL10000	0.320 ± 0.050	0.493	2.02

Note: D is the average distance between two neighboring PEG chains.

micelles. The ETO showed its characteristic peaks at 2 θ values of 13.3°, 17.3°, 19.4°, 22.3°, 26.9°, 31.2°, and 33.6°. It was observed that after lyophilization, both MPEG2000-PCL3500 and MPEG5000-PCL7000 micelles were crystalline in nature with sharp peaks observed at 2 θ angle of 19.1 and 21.3 corresponding to PEG and PCL blocks, respectively. A third broad peak at 23.7 observed was due to overlap of secondary PEG and PCL peaks, although, there were no characteristics peaks of ETO found in X-ray diffractogram of MPEG-PCL micelles.

In vitro drug release studies

In vitro release study of selected micellar formulations, that is, MPEG2000-PCL3500 and MPEG5000-PCL7000 micelles and ETO injection were performed in PBS pH 7.4 to correlate the predictable drug release *in vivo*. The percentage cumulative release profile of ETO injection, MPEG2000-PCL3500, and MPEG5000-PCL7000 micelles is represented in Figure 8. A drug release of more than 85% was observed with ETO injection after 1 h, while MPEG2000-PCL3500 and MPEG5000-PCL7000 micelles showed an initial burst release of 9.56 ± 2.8% and 5.50 ± 2.0%, respectively, after 3 h. As time lengthens, the release profiles of drug were found

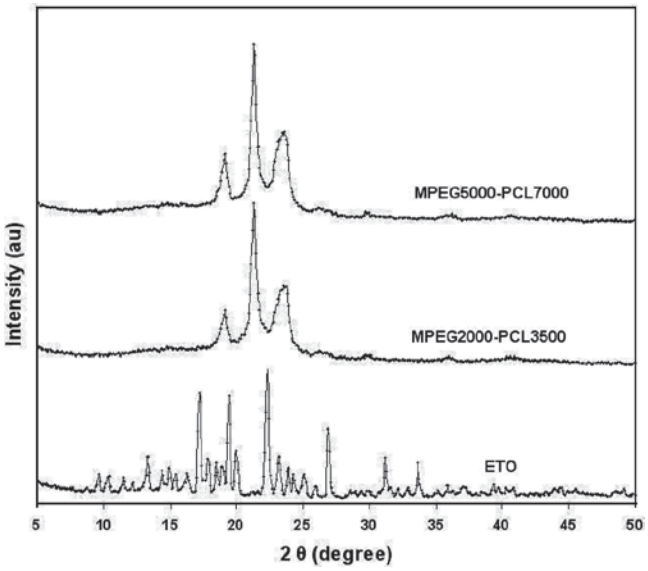

Figure 7. Powder X-ray diffraction patterns of ETO and MPEG2000-PCL3500 and MPEG5000-PCL7000 micelles.

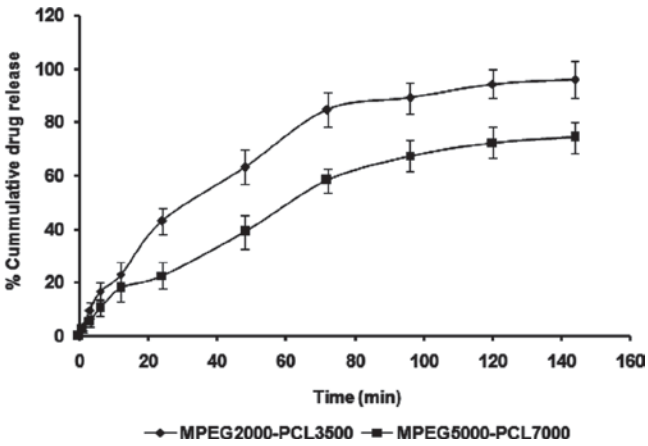

Figure 8. *In vitro* release study of ETO injection, MPEG2000-PCL3500, and MPEG5000-PCL7000 micelles in PBS pH 7.4.

totally different between two micellar formulations and were more dependent on the molecular weight of PCL block. After 144 h, MPEG2000-PCL3500 micelles showed 95.8 ± 6.9% drug release, while at the same time point, MPEG5000-PCL7000 micelles exhibited 74.3 ± 5.7% drug release.

In vivo studies

The radiolabeling efficiency of ETO and ETO-loaded micellar formulations was observed greater than 95% and all formulations were found stable up to 24 h in normal saline without significant reduction in the radioactivity (data not shown). The biodistribution profile of plain ETO and micellar formulations are represented in Figure 9 and expressed as the percentage ID per gram of tissue or organ (% ID/gm tissue/organ). Compared to plain ETO, micellar formulations exhibited totally different distribution pattern. Moreover, the percentage uptake and distribution was found different for different organs/tissues. In plain ETO, after 1 h post injection, the blood

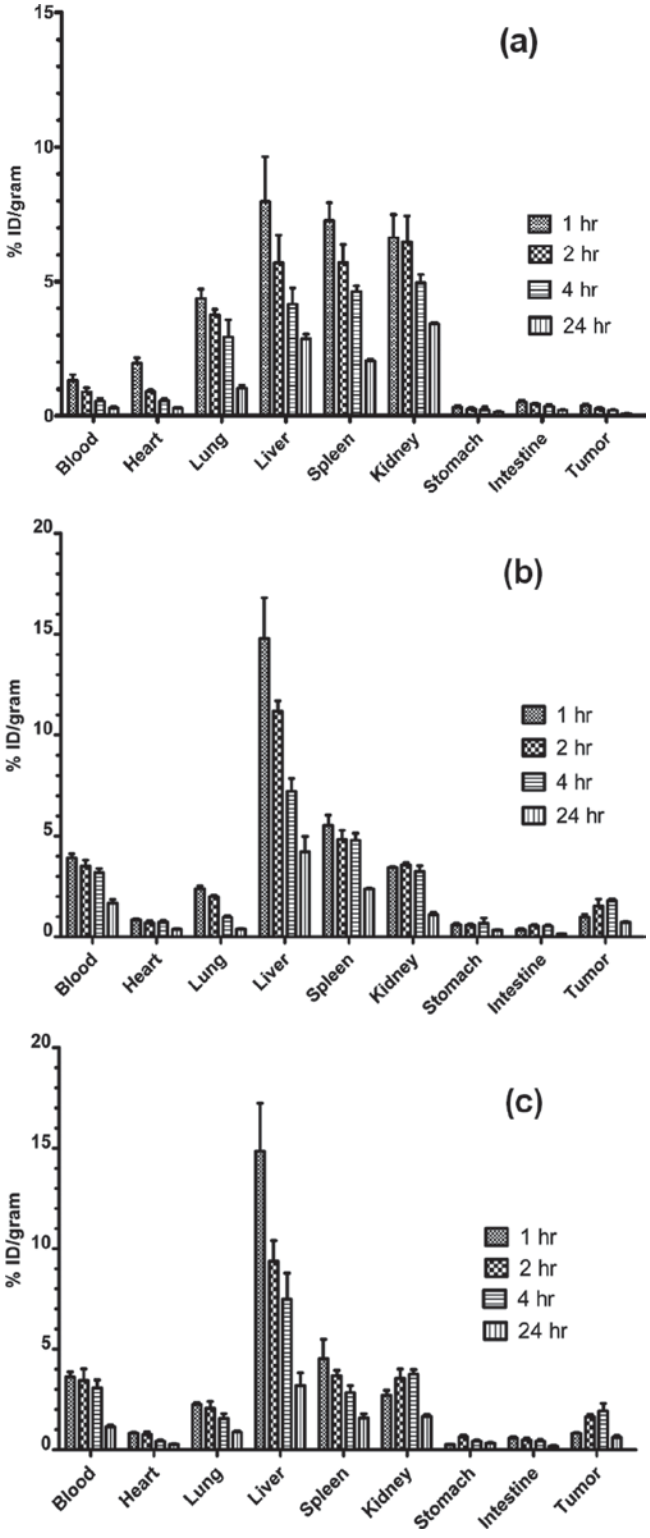

Figure 9. (a) Biodistribution of 99mTc-labeled ETO, (b) MPEG2000-PCL3500, and (c) MPEG5000-PCL7000 after intravenous injection in EAT-bearing Balb/c mice. Radioactivity was counted in each organ and expressed as percentage ID per gram of organ/tissue (results are mean ± SD, $n = 5$).

concentration observed was almost three-fold lower with $p < 0.001$, compared to micellar formulations and was reduced quickly to 0.29% ID after 24 h. The MPEG2000-PCL3500 and MPEG5000-PCL7000 micelles showed

5.75- and 3.93-fold greater concentrations in blood after 24 h compared to plain ETO ($p < 0.001$), respectively. Liver showed 7.96 ± 1.68% ID of the plain ETO after 1 h post injection and later reduced more quickly with 2.87 ± 0.17% ID after 24 h post injection. Both the micellar formulations, after 1 h post injection exhibited near to two-fold increase in liver uptake compared to plain ETO, in addition at all time point they demonstrated comparable liver uptake (Figure 9). Spleen is the second organ, where higher uptake of plain ETO and micellar formulation was observed. However, the overall uptake of micellar formulations by spleen was found in lesser extent than plain ETO (Figure 9). The spleen uptake after 1 h post injection of MPEG2000-PCL3500 and MPEG5000-PCL7000 was found to be 5.52 ± 0.51% and 4.52 ± 0.97% with $p < 0.001$ compared to plain ETO, respectively.

The lung uptake of plain ETO was found 4.36 ± 0.35% at 1 h post injection, which was nearly to two-fold higher ($P < 0.001$), compared to both the micellar formulations. A higher radioactivity in kidney was exhibited by plain ETO compared to that of micellar formulation (Figure 9). After 1 h post injection, the kidney uptake of plain ETO was 6.63 ± 2.36%, while MPEG2000-PCL3500 and MPEG5000-PCL7000 exhibited 1.93- and 2.46-fold lower accumulations ($p < 0.001$) compared to plain ETO at same time point. Radioactivity of micellar formulation in heart was lower compared to plain ETO after 1 h post injection, while stomach showed less than 1%. A significant difference ($p < 0.001$) in tumor uptake of micellar formulation was observed compared to plain ETO (Figure 9). Plain ETO showed very poor tumor penetration and hence lower uptake was observed at all the time point compared to both the micellar formulations. Plain ETO after 1 h post injection showed 0.38% tumor uptake and it reduced to 0.08% after 24 h post injection, while tumor uptake of micellar formulations increased up to certain time point and the level started to decline. The MPEG2000-PCL3500 and MPEG5000-PCL7000 micelles showed a 2.57- and 2.15-fold higher tumor uptake to plain ETO at 1 h post injection and exhibited maximum tumor uptake of 1.78 ± 0.10% and 1.92 ± 0.38%, respectively after 4 h post injection.

Discussion

The MPEG-PCL is one of the most widely studied polymer for encapsulation of various hydrophobic drugs, including anticancer agents due to its micelles-forming properties, stability as well as US-FDA approval of both PEG and PCL polymer. However, extensive studies related to the drug-loaded assembly of these micelles are required to design a ideal carrier with suitable molecular weight to combat against the possible *in vivo* interaction resulting into failure of such drug delivery system. In the present study, synthesis of MPEG-PCL diblock copolymer was carried out using CH_3-PEG-OH and CL in presence of HCl-Et_2O as activator. It is reported that HCl-Et_2O initiate reaction by activated monomer cationic polymerization method which inhibits unfavorable reactions, such as back-biting and disproportionation (Kim et al., 2004). The experimental molecular weight obtained by GPC and NMR were identical with the calculated molecular weight. The peaks obtained in NMR spectra are in accordance with results obtained earlier, while GPC analysis exhibited a narrow and symmetrical peak of the copolymer (Kim et al., 2005; Hu et al., 2007).

Nanoparticles were formed by the solvent displacement method also referred to as nanoprecipitation. This method was chosen due to the low aqueous solubility of the synthesized MPEG-PCL copolymer requiring that the copolymer first be dissolved in an organic solvent (Aliabadi et al., 2005). Moreover, the solvent displacement method bears advantage over dialysis method including more feasibility for scale up and less chance for drug loss during dialysis in the encapsulation process (Vangeyte et al., 2004). Acetone was selected as water miscible organic solvent due to its low boiling point which may help in terms of scale up of formulations. A minor difference in particle size of MPEG2000-PCL2000 and MPEG2000-PCL3500 micelles was observed, which might be attributed to limitation of cosolvent method employed in case of MPEG2000-PCL2000 (Soppimath et al., 2001). It was observed that both particle size and percentage drug entrapment increased with increase in the ratio of drug to polymer. Moreover, an increase in particle size and drug entrapment was also observed with increase in molecular weight of hydrophobic part of all different block length copolymer. The reason is that as the PCL block length increases, the aggregation number of the micelle increases, resulting in a larger core, which allows a higher loading efficiency of drug. The results obtained are in accordance with earlier reports (Gadelle et al., 1995). An increase in particle size and PDI with increase in the ratio of aqueous to organic phase was exhibited, which might be due to rapid diffusion of organic phase into aqueous phase (Vangeyte et al., 2004). Moreover, these effects were seen in lower molecular weight block copolymers, while MPEG5000-PCL7000 and MPEG5000-PCL10000 exhibited lower particle size upon increase in the aqueous to organic phase ratio. The obtained result signifies that particle size adjustment could be attained deliberately using different copolymers or simply via variation of the copolymer concentration in organic phase. An enhancement in drug loading was achieved with increase in the molecular weight of PCL block but at the same time particle size also increased, implying that the drug loading can be achieved to desired level using suitable molecular weight copolymer. A drop in percentage drug loading with increase in particle size and PDI in all micellar formulation was observed after saturation limit was attained during optimization of drug loading process. The probable reason behind this might be attributed to the fact that higher drug concentration in the organic phase leads to a higher diffusion of ETO into water and hence a larger portion of the drug might move out from the organic phase without being encapsulated before the formation of the micelles and hampering the overall stability of micelles (Hu et al., 2007).

Decrease in CMC value, with increase in PCL block molecular weight implies that the hydrophobic PCL blocks mainly affect the CMC (Choi et al., 2006). The FALT was demonstrated to be one of the physical factors that defined the pharmacokinetics of the PEG-modified liposomes. An increase in FALT with increase in PEG molecular weight led to enhancement in escape from reticuloendothelial system and augmentation in tumor uptake of liposomes (Sadzuka et al., 2002, 2006). Similar kind of results were observed, and the copolymer with PEG of molecular weight 5000 g showed higher FALT compared to micelles prepared with PEG of molecular weight 2000 g. A reduction in FALT was also seen with increase in the molecular weight of PCL block; these may be attributed due to decrease in PEG surface coverage (Riley et al., 1999). Interactions of nanocarrier with serum proteins present in blood after intravenous administration decide the *in vivo* fate of nanocarrier by two possible mechanisms (Liu et al., 2005). In the first case, after intravenous administration, opsonins protein may adsorb on the surface of nanocarrier immediately which lead to opsonization and rapid clearance by RES system particularly if the surface is charged or hydrophobic. Second, if drug has high protein affinity, drug might leach out rapidly due to attraction by proteins. The ETO is reported to have protein affinity near to 95% (Aita et al., 1999). Hence, in MPEG2000-PCL2000 micelles, ETO might have leached immediately and hampered the stability of micelles in presence of BSA. Moreover, increase in particle size of MPEG2000-PCL2000 micelles in absence of BSA indicated poor *in vitro* stability. The enhancement of stability of micelles, except MPEG2000-PCL2000, even after 48 h of incubation implies that stronger interaction exists between drug and core-forming block which prevented the drug–protein interactions (Liu et al., 2007). It is well known that surfactants at high enough concentrations are capable of disrupting cell membranes, such as those of erythrocytes, by penetration and saturation of the membrane with unimers followed by solubilization of the membrane lipids and proteins (Letchford et al., 2009). Based on the daily dose of ETO injection of 50 to 100 mg/M^2 body surface area, the average dose of ETO for adult is 130 mg/day and accordingly the concentration of ETO in blood on single dose will remains around 20 μg/mL. In present study, the hemolytic activity was done up to the concentration range of 200 μg/mL to study the immediate effect ETO concentration on hemolysis, when it comes in contact with erythrocytes. In the present study, both plain ETO and ETO-free injectable solutions exerted hemolytic effect due to presence of free drug and surfactant in formulations. Further compared to other micellar formulations, a significant hemolysis effect was observed with MPEG2000-PCL2000 micelles due to rapid release of drug from micelles due to its lower molecular weight (Zastre et al., 2007).

The increase in the molecular weight of hydrophobic core resulted into reduction in the surface density with increase in distance between two PEG chains because of strengthened hydrophobic interaction between PCL chain lengths, which occupied less molecular number of diblock copolymer to shape a single micelle (Shi et al., 2005). A decrease in surface density with increased in distance (D) in MPEG-PCL micelles carrying PEG of molecular weight 5000 g was seen compared to MPEG-PCL micelles carrying PEG of molecular weight 2000 g. The PEG was reported to change the association of the copolymer molecules during the formation of the particles (Riley et al., 1999). Thus, it could be predictable that longer PEG chain requires more space to retain their flexibility, which made the surface PEG sparser. Furthermore, it was suggested that a distance of 1.2–1.4 nm between two grafted PEG (2000) chains is required for avoiding complement consumption to avoid the adsorption of small proteins (approximated as spheres with a radius of 2 nm) and around 1.5 nm for larger proteins (6–8 nm) (Bazile et al., 1995; Jeon et al., 1991). From the X-ray diffraction peaks of ETO-loaded MPEG2000-PCL3500 and MPEG5000-PCL7000 micelles, it was deduced that ETO was either molecularly dispersed or distributed in an amorphous state in the PCL core of micelles (Forrest et al., 2006). The initial burst of drug release during *in vitro* drug release study was attributed to the presence of drug deposited on the surface or in the microchannels probably existing in micelles (Ge et al., 2002). The extent of drug release was found in controlled manner and dependent on the molecular weight of hydrophobic block. In addition, MPEG5000-PCL7000 showed lower drug release profile compared to MPEG2000-PCL35000 due to higher microviscosity and larger diameter of the hydrophobic core, as these factors will decrease the diffusion rate of the drug through the PCL (Forrest et al., 2006).

The organ biodistribution studies carried out using EAT-bearing Balb/C mice showed promising outputs of developed formulations. The long circulating effect of micelles was attributed due to presence of PEG chain on the surface of micelles, which provided stealth effect to the micelles (Forrest et al., 2008). Compared to MPEG2000-PCL3500, MPEG5000-PCL7000 micelles showed lower blood levels, which might be attributed to their larger particle size of MPEG5000-PCL7000. Furthermore, a higher liver uptake of micellar formulations compared to plain ETO, suggests RES uptake of the micelles. Nevertheless, the liver uptake of micellar formulations was two- to three-fold lower than the liver uptake of PLGA and PCL nanoparticles reported (Halder et al., 2008; Snehalatha et al., 2008). This was due to lower particle size of micellar formulations. It is assumed that micelles were distributed mainly to parenchymal cells of the liver after intravenous injection and stayed longer time compared to plain drug (Stolnik et al., 2001). According to Stolnik et al., (1995), when particle size was greater than 200 nm, the accumulation of micelles in liver was higher, and when the size was below 200 nm, the spleen was more powerful to catch the micelles because of its tighter structure. However, in present study, contrary results were observed and spleen showed lower uptake compared to liver. These could be

due to smaller size of micelles, below 100 nm, which can penetrate more in liver compared to particle size of nanocarrier above 100 nm (Avgoustakis et al., 2003). A higher tumoral uptake of micellar formulations due to the EPR effect was observed due to presence of PEG chains on the surface of micelles, which made the micelles to remain in circulation for longer duration for passive targeting (Yadav et al., 2008).

Conclusion

The present study was aimed to design stable MPEG-PCL micelles based on their *in vitro* and *in vivo* performance. It is found that the composition of the copolymers influences the particle size, encapsulation efficiency as well as *in vitro* release characteristics. *In vitro* stability studies in presence of BSA revealed that hydrophobic core part with lower molecular weight are more prone to interact with protein, suggesting poor *in vivo* performance. Hemolysis study showed that micellar formulations are more beneficial in terms of prevention of hemolysis and anemic conditions. The biodistribution studies further suggested that micelles with different molecular weight of polymer exhibit different behavior *in vivo*. Thus, together with the current findings, this work established a platform for further development of the PEG-PCL micelles for effective anticancer drug delivery.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper. This work was financially supported by Indian Council of Medical Research, New Delhi, India, awarded to Mukesh Ukawala.

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