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Research Article

## Etoposide delivery: mPEG-PCL based copolymeric micelles assessed by various *in-vitro* anti-cancer activity

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### ABSTRACT

Discerning the complexity of metastasis during primary tumour, Polymeric micelles has the potential to improve the tumour treatment with chemotherapy and known to that the amphiphilic block co polymer yields self - assemble polymeric micelles in aqueous milieu. The present study was engineered and optimized etoposide loaded polymeric micelles with mPEG/PCL di block copolymer thereby improving the dispersibility, prolonging the cellular uptake and enhance the bioavailability. Micelles were characterized by particle size, drug loading capacity, CMC and Surface density. The stealth micellar formulation were evaluated with various *in-vitro* cell line studies using highly metastatic B16F10 cell lines and compared with unmodified etoposide loaded micelles. The average particle size of etoposide-mPEG-PCL was approximately 72 nm and the polydispersity index was in the range between 1.2 and 1.3. The encapsulation efficiency of the etoposide was in the range between 82.78 and 92.06 % and the maximum drug loading capacity of 5.89 %. The resultant micelles demonstrated ~15 fold higher cellular uptake and After 24 h incubation period, the calculated IC<sub>50</sub> values of micellar formulations were up to 25 to 50 times higher as compared with unmodified etoposide. These findings illustrating that the engineered polymeric micelles may be a promising approach for the efficient treatment of tumour metastasis.

**Keywords:** Block co polymers; Etoposide; *In-vitro* studies – Cell viability and Cell uptake; Polymeric micelles.

### INTRODUCTION

Interest has been recently raised in exploiting the polymeric micelles as nanocarriers for hydrophobic molecules because the intrinsic nature of polymeric micelles provides a reservoir or cargo space to its hydrophobic core which are sterically stabilized by the hydrophilic core, thereby, poorly soluble drugs can be easily solubilized (Van Zuylen *et al.*, 2001). Block copolymers are defined as a combination of different polymer segments in a single polymer chain through various polymerization methods, combining the intrinsic properties of each individual block. Depending on how the monomer repeating units are distributed in the copolymer chains, these copolymers may present a variety of macromolecular architectures such as random, alternating, block, and graft copolymers. Block copolymers also can prevent the absorption of opsonins on the carrier, thereby reducing its capture by mononuclear phagocyte system, prolonging its circulation time and modifying its bio distribution (Jones and Leroux, 1999). The polymeric micelles can be used to target a drug either actively or passively. On one hand, the pas-

sive targeting takes advantages of the fact that the tumours present leaky vasculatures (where small micelles can spontaneously penetrate) and impaired lymphatic drainage. On the other hand, active target is achieved by modifying the micelle so as to favor its chemical recognition by a specific cell receptor or to induce a response to external stimuli such as pH or temperature variations.

Polymer micelles are nano-sized aggregates of amphiphilic polymers that can be used as a delivery agent for cancer chemotherapy. Micellar drug formulations have a number of advantages over conventional chemotherapy. Firstly, they increase the solubility of hydrophobic drugs, making them easier to administer intravenously (Torchilin V.P., 2004). Secondly, they have been shown to accumulate passively in tumors because of their leaky vasculatures, which is known as the enhanced permeation and retention (EPR) effect (Matsumara Y and Maeda H., 1986). Thirdly, they have considerable potential customization, in which additional functional groups can be attached to the surface and used to modulate micelle properties. Particularly, the addition of a targeting ligand can improve the specificity of drug delivery to tumors (Otsuka H. *et al.*, 2003).

The amphiphilic copolymer made by Methoxy-poly (ethylene glycol)-poly (Caprolactone) (mPEG-PCL) has got many distinguished characteristics for the development of micellar formulation for various delivery

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application. Micelles are made up of biocompatible and biodegradable either di or tri block copolymers which are widely used to formulate the nanoparticulate system, especially for poorly soluble drugs (Aliabadi *et al.*, 2007; Yang *et al.*, 2007).

Among Topoisomerase inhibitors, the etoposide is mainly used for the treatment of various cancers like lung cancer, testicular cancer, lymphoma and several type of leukemia. The topoisomerase II inhibitor that prevents re-ligation of unwinding DNA strands during DNA duplication causing double strand breaks, resulting in apoptotic cell death (Montecucco A. and Biamonti G., 2007).

Aside from pharmacokinetics, etoposide is poorly soluble in aqueous solutions necessitating slow infusion over 30–60 min and requires solubilizing agents such as surfactants and alcohols. The encapsulation of poorly soluble drugs in nanoparticles can enhance the therapeutic index and pharmacokinetics of cytotoxic treatments. These Nano therapeutics are made from organic or inorganic materials, typically 10–200 nm in diameter, and can be produced in a variety of formulations and configurations that improve the delivery or protection of the therapeutic agents (Joel SP *et al.*, 1994; Bertrand N *et al.*, 2014; Godin B *et al.*, 2011).

Indeed, the therapeutic application of micellar delivery system has to be completely understood and evaluate the various parameters during the design of micellar drug delivery system. Critical Micellar Concentration is one of the important parameter to be considered for stability and release of the micelles determined by pyrene. Shai *et al.*, 2005; Letchford *et al.*, 2008, reported that increasing the molecular weight of hydrophobic moiety in block copolymer yields the lower CMC values, thereby increasing the stability of the micelles. Besides the understanding various characteristics like particle size, PEG surface density and zeta potential of the polymeric micelles plays an important role in the *in-vitro* and *in-vivo* (Shai *et al.*, 2005). The non-ionic hydrophilic PEG shell can intimidate the protein absorption and subsequent clearance by mononuclear phagocyte system, as a result, micelles circulation gets prolonged which increases the biodistribution of the loaded drug.

In this present study, we have made an attempt to formulate the biodegradable, mPEG-PCL polymeric micellar system as a nanocarriers for etoposide encapsulation enhances the aqueous solubility, stability and controlled delivery of etoposide for Cancer cells. First, synthesized the mPEG-PCL di block co polymer with various molecular weights of hydrophobic moiety (PCL) and their characterizations were analyzed. Second, the incorporation of topoisomerase inhibitor, etoposide was fabricated and characterized. Finally, *in-vitro* cell line studies were carried out with the selected Etoposide-loaded micelles formulation using highly metastat-

ic B16F10 cells lines and compared with unmodified etoposide.

## MATERIALS & METHODS

Active pharmaceutical ingredients, Etoposide (EPD) were gifted from Jiangsu Hengrui Medicine Co., Ltd, China. Poly ( $\epsilon$ -Caprolactone) with different lengths were procured from Merck, India. Methoxy poly (ethylene glycol) (mPEG) with different lengths and Trinitro Benzene Sulfonic acid (TNBS), Acetonitrile (ACN), Hydrochloric acid (HCL) and Dichloromethane were procured from S.D. Fine Chem. Ltd., Mumbai., Potassium dihydrogen phosphate Disodium hydrogen phosphate, Iodine (I<sub>2</sub>), Sodium chloride Potassium iodide (KI) were procured from Spectrochem Ltd, Mumbai. Methanol (AR grade), Glacial acetic acid and water of HPLC grade were purchased from Merck, India. A highly metastatic B16F10 melanoma Cells were purchased from National Centre for Cell Science, Pune, India. From Hi-media Lab, Fetal bovine serum (FBS), MTT A.R. (3-(4, 5-dimethyl-2-yl)-2, 5-diphenyl tetrazolium bromide) and Iscove's Minimum Dulbecco's Medium (IMDM) were procured.

### Synthesis of mPEG-PCL block co polymers

The pioneering Ring opening polymerization method was used for the synthesis of Methoxy PEG-PCL di-block copolymers (Aliabadi H M *et al.*, 2007). The different molecular weight of mPEG-PCL were chosen for the synthesis of di block copolymer, details are shown in Table-1. Two grams of mPEG (Mw 5000) was azeo-distilled with 100ml of dried toluene to remove the water completely. To this, added 20 ml of dried dichloromethane and 2grams PCL (Mw 5000) was added slowly to the reaction mixture at 20-25 °C. Under vigorous stirring, slowly 3ml of HCl-Et<sub>2</sub>O (1M solution) was added, the polymerization reaction was allowed and maintained at 25 °C for 24 h under nitrogen atmosphere. The reaction was ceased by quenching with 0.1ml of triethylamine (5%) and the precipitated salt (triethylamine-HCl) was discarded by filtration. The copolymer precipitation is obtained by adding cold diethyl ether (-20 °C) and washed with cold methanol thrice to remove residual monomer. The final product obtained was kept in desiccator for 48 h. The same procedure has been used for the synthesis of different molecular weight of PEG-PCL di block co polymers by varying the molecular weight of PEG and PCL. The synthetic scheme of polymerization of mPEG-PCL copolymer is outlined in Figure 1.

### Characterization of mPEG-PCL di block copolymer

#### <sup>1</sup>H NMR Spectrum Copolymer

The synthesized block co polymers spectra were measured in FT-NMR (400MHz Bruker, Advance II model, Germany) at 25°C using Deuterated Chloroform (CDCl<sub>3</sub>) as solvent.

### FTIR Analysis

The FTIR spectrum of mPEG-PCL was carried out using FTIR (Bruker, Alpha-T, Germany). Required quantity of di-block co polymer was mixed with potassium bromide (KBr) and Pelletized under the pressure of 10 tons for 2 min and scanned over a range between 4000 and 400  $\text{cm}^{-1}$  at room temperature.

### GPC Analysis

Gel permeation chromatography used to determine the synthesized, different molecular weights of mPEG-PCL di-block copolymers. The instrument is equipped with PE series 200 RI detector, mixed column PLGel >5  $\mu$ , series 200 (Perkin Elmer). Samples were prepared by dissolving Tetrahydrofuran (THF) and injected to column at the rate of 1ml/min. The synthesized polymer molecular weight and its distribution was calculated by using turbosize size exclusion software. Different molecular weight of polystyrene standards were used for calibration of GPC.

### Preparation of Etoposide (EPD) micelles

The reported method of nanoprecipitation technique was used for the preparation of Etoposide loaded micelles (Zhang *et al.*, 2004). Concisely, 30 mg of di -block copolymer mPEG 5k-PCL 5k and 2 mg of etoposide were dissolved in organic solvent, (acetone 5ml). To this, added 5 ml of distilled water slowly with stirring using magnetic stirrer at room temperature. The stirring was allowed till the complete evaporation of organic solvent. The collected bluish aqueous dispersion was filtered through PVDF 0.45 micron membrane filter membrane to remove un-entrapped drug particles. Similarly the synthesized other molecular weight of block co polymers were loaded with etoposide as shown in Table 2.

### Evaluation of EPD micelles

#### Particle size and zeta potential

Particle size and zeta potential of micelle samples were determined by dynamic light scattering method, using Zetasizer, Nano-ZS (Malvern Inst., U.K.). Sample was filled in the cuvette and the average volume-mean particle size and zeta potential was recorded after performing the experiments in triplicate.

#### Estimation of percent entrapment efficiency (EE) and percent drug loading (DL)

The entrapment efficiency of EPD micelles was determined by UV-visible spectrophotometer (UV 1700, PharmaSpec, Shimadzu, Japan). An aliquot of micellar formulation was dissolved in acetonitrile and the amount of EPD was determined at  $\lambda$  max 284 nm by UV spectroscopy. The percent drug entrapment and percent drug loading were calculated using following equations.

$$\% \text{ Drug entrapment} = \frac{\text{Amount of EPD in micelles}}{\text{Amount of EPD added}} \times 100$$

$$\% \text{ Drug loading} = \frac{\text{Amount of EPD in micelles}}{\text{Amount of EPD added}} \times 100$$

$$\% \text{ Drug loading} = \frac{\text{EPD}}{(\text{EPD} + \text{Polymer})} \times 100$$

### Critical Micelle Concentration (CMC)

The CMC values of synthesized block copolymers were determined by fluorescence spectroscopy using pyrene as fluorescent probe as reported earlier (Choi *et al.*, 2006; Kim *et al.*, 2005). Fluorescence spectra were recorded by spectrofluorophotometer (RF-540, Shimadzu Corporation Japan) at room temperature. The calculated quantity of pyrene was dissolved in acetone. The dissolved pyrene was distributed to a series of 5 ml vials and allowed to evaporate the dispersant, acetone under nitrogen atmosphere. The diluent, water was used to achieve the final concentration of EPD micellar solution was made and added into the mixture. To equilibrate, the mixture were kept in shaking incubator at 150 rpm at 25°C for 12 hours. The resultant mixture was filtered for the removal of free pyrene by using membrane filters (0.45  $\mu$ ). The observed emission wave length of 390nm while running the spectra between the range 300 and 400 nm and the graph was plotted against as the function of logarithm of polymer concentration and Intensity.

### PEG surface density

The surface density of the polymeric micelles plays the vital role in the in-vitro and the in-vivo performance of nanoparticles. The synthesized block copolymers and the EPD loaded micelles surface density were determined by the reported method (Shai. *Et al.*, 2005). Placebo and active EPD loaded micelles were dispersed with distilled water. 2N Sodium hydroxide (2 ml) was added to the 5ml of the dispersion and kept aside for 3 days at 40°C. Then the samples were brought into pH 7.4 using 1N HCl and finally the volume was adjusted up to 10 ml. Iodine and Potassium iodide solution (500  $\mu$ l) was added into the above solution and absorbance was measured at 525 nm by UV spectrophotometer (UV, Shimadzu, Japan). The total amount of PEG after micelle degradation was calculated.

$$D = \sqrt{\frac{N \cdot d \cdot \rho \cdot r}{3M_w \text{PEG}}} \quad \text{Where } \delta = \frac{N \cdot d \cdot \rho \cdot r}{3M_w \text{PEG}} \quad \delta \text{ is the surface density of PEG chains (PEG/nm}^2\text{)}$$

N is the Avogadro number  $6.021 \times 10^{23}$ /mole.

r is the particle radius neglecting the PEG layer thickness (error <10%).

d is the density of micelles.

g is the fraction of PEG content after micelles degradation

$M_w$ PEG stands for molecular weight of PEG.

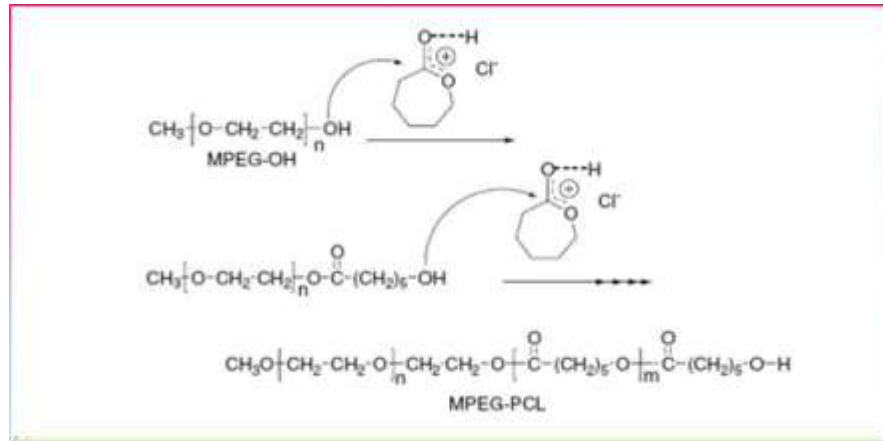


Figure 1: Synthetic scheme of mPEG-PCL synthesis by ring-polymerization method.

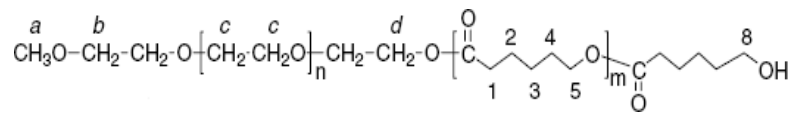


Figure 2: Chemical structure of PEG-PCL polymer

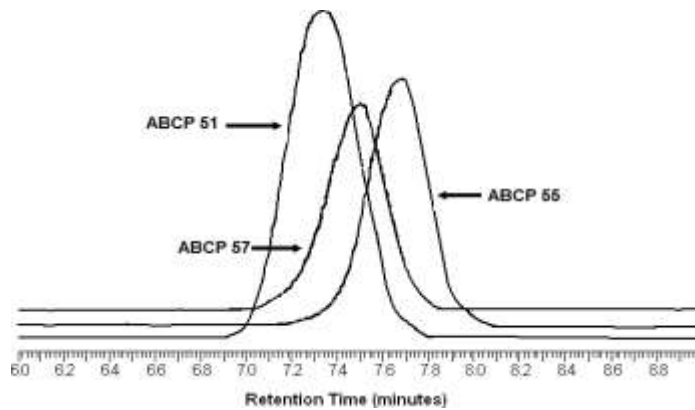


Figure 3: GPC Chromatogram of Synthesized polymer ABCP 51, ABCP 57 and ABCP 55

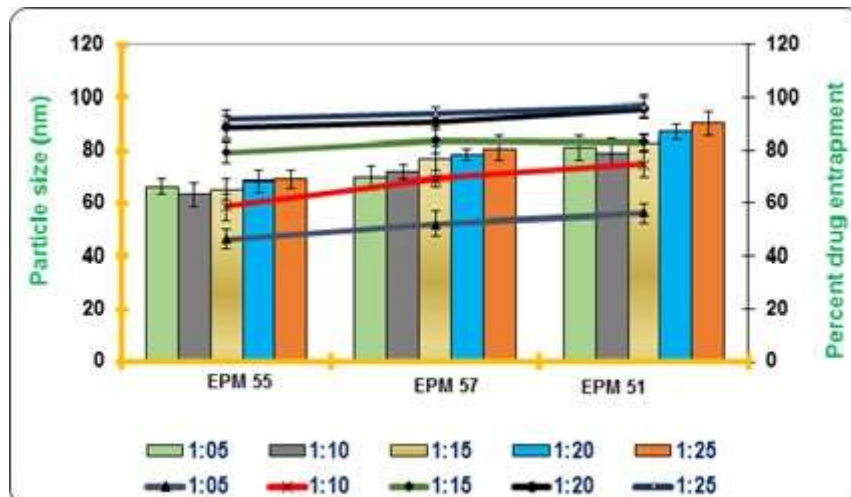


Figure 4: Influence of ratio of drug to polymer on particle size and percent drug entrapment of micelles (Results are mean  $\pm$  S. D. and n=3)

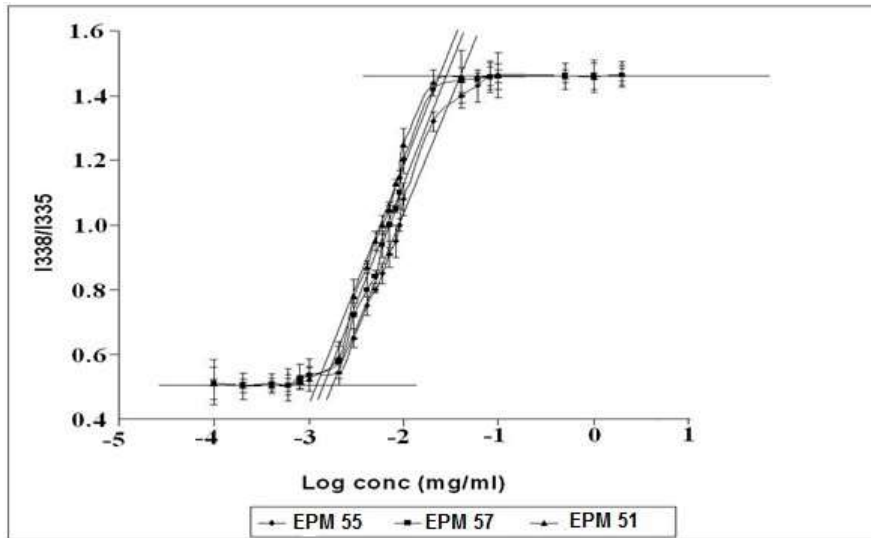


Figure 5: Plot of I338/I335 (from pyrene excitation spectra) vs. log C of EPM micelles

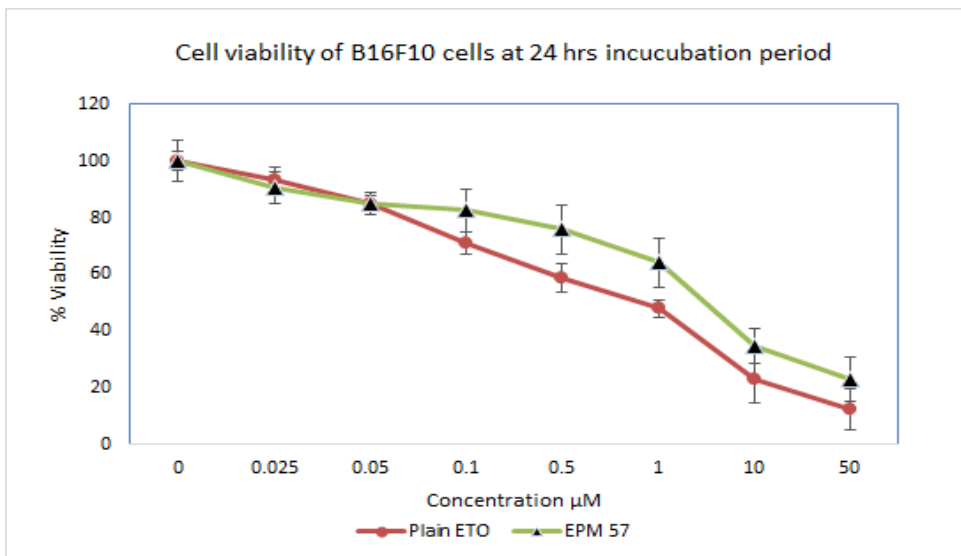


Figure 6: Plot of Concentration (µM) Vs % cell viability after 24 hours incubation

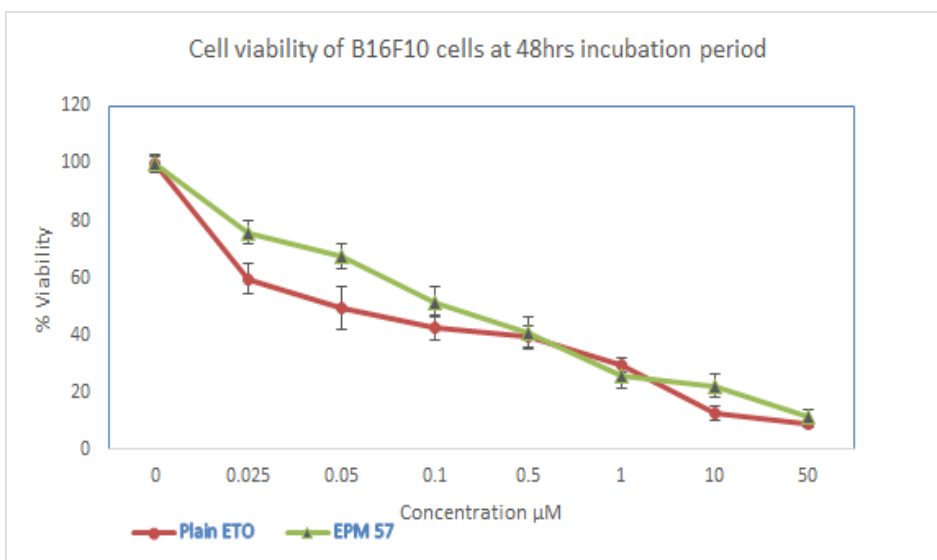


Figure 7: Plot of Concentration (µM) Vs % cell viability after 48 hours incubation

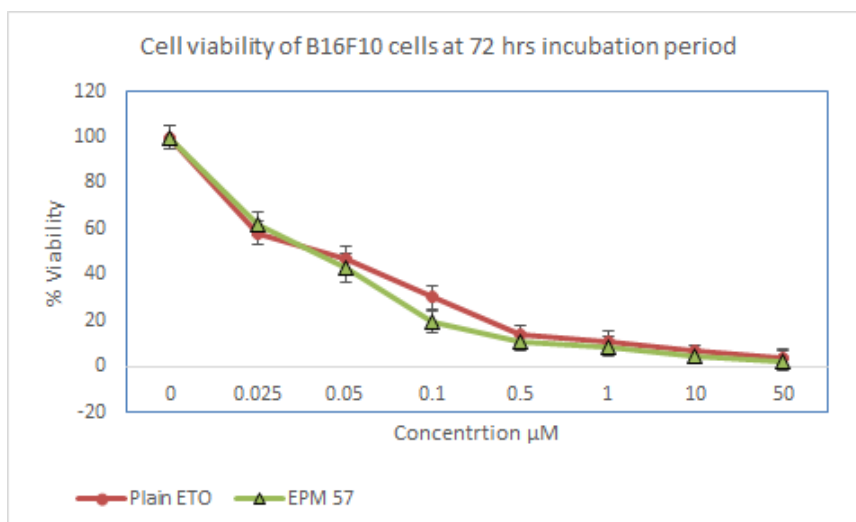


Figure 8: Plot of Concentration ( $\mu\text{M}$ ) Vs % cell viability after 48 hours incubation Percent (Results are mean  $\pm$  S.D. of three individual experiments)

Table 1: Characterization of synthesized mPEG-PCL di block co polymer

Polymer Ratio (mPEG : PCL) and Code	Mn <sup>a</sup>	Mn NMR <sup>b</sup>	Mn GPC <sup>c</sup>	Polydispersity Index (Mn/Mw)	Percentage yield (%)
ABCP 55 (5K <sup>#</sup> – 5K)	10000	9366	10584	1.201	92.98
ABCP 57 (5K – 7K)	12000	11396	12636	1.331	94.65
ABCP 51 (5K- 10K)	15000	14879	15190	1.235	92.09

# represents x1000

<sup>a</sup> Sum of theoretical molecular weight of Polymers (mPEG + PCL)

<sup>b</sup> Molecular weight calculated from <sup>1</sup>H NMR spectrum

<sup>c</sup> Relative molecular weight determined by GPC

Table 2: Characterization of micellar formulations

Micelle Formulations code	CMC (mg/ml) x 10 <sup>-3</sup>	Particle size (nm)	Percentage drug loading (%)	Percent drug entrapment	Zeta potential (mV) $\pm$ S.D
EPM 55	1.42	67.89 $\pm$ 3.08	5.23 $\pm$ 0.15	88.51 $\pm$ 3.20	-4.16 $\pm$ 0.37
EPM 57	1.28	73.39 $\pm$ 4.08	5.40 $\pm$ 0.17	92.06 $\pm$ 3.48	-4.16 $\pm$ 0.39
EPM 51	1.34	76.25 $\pm$ 2.69	5.89 $\pm$ 0.24	82.78 $\pm$ 3.60	-5.12 $\pm$ 0.41

### In-vitro Cytotoxicity study

Cytotoxicity assay were performed as reported earlier (Dua and Gude, 2006; Liu *et al.*, 2008). Concisely, B16F10 cells were allowed to grow in exponential phase and stabilized for 24 h in incubator at 37 °C with 5 % CO<sub>2</sub>. The cells were treated with plain EPD and micellar formulations (Serial concentration) and incubated for 24 h. Plain EPD used for study, was dissolved in DMSO and final concentration of DMSO was kept at 0.1 %. Post treatment cells were washed with PBS and treated with MTT in PBS with the ratio of 1:4 (20  $\mu\text{l}$  MTT in 80  $\mu\text{l}$  of PBS and incubated for 4 h at 37 °C. Plates were then centrifuged at 500 rpm for 20 min. To dissolve the Formazan crystals, further 100  $\mu\text{l}$  of DMSO was added to each well and kept on shaker for 5 min. The optical density was measured in an Enzyme Linked Immuno Sorbent Assay (ELISA) at 540 nm with a reference wavelength of 690 nm.

For 48 and 72 h drug incubation study, B16F10 cells were seeded at concentration of  $2 \times 10^3$  cells/100  $\mu\text{l}$ /well &  $1.5 \times 10^3$  cells/100  $\mu\text{l}$ /well respectively and cells were incubated in presence of drug for respective time point. Percent cell viability was plotted against concentration and IC<sub>50</sub> values were calculated from the dose effect curve at the drug concentration that decreased the cell viability to 50 % and results were expressed as mean  $\pm$  S.D.

### Cell uptake Study

Cell uptake studies were carried out to determine the intracellular uptake of EPD by B16F10 cells after incubation period of 2 h. Plain EPD, EPD - mPEG - PCL micellar formulations, i.e. 5 %, 10 % and 20 % were also evaluated for their maximum receptor mediated cellular uptake (Zhao & Yung, 2008). The cellular content of EPD was determined by developed HPLC method using diazepam as internal standard. Briefly,  $4 \times 10^4$  B16F10 cells/ml were added to 90 mm tissue culture plates and

allowed to grow to 90 % confluency. The grown cells were processed with plain EPD and micellar formulations at 20 µg/ml drug concentration for 2 h at 37 °C. Cells were washed with ice cold PBS twice and harvested with saline EDTA. The final count of cell was made to  $2 \times 10^6$  cells/ml and centrifuged at 1000 rpm for 20 min, followed by cell lysis was done using probe sonicator for 3 min at 80 % duty cycle. Bovine serum albumin used as standard for the determination of protein content in the cell lysate. The percentage drug uptake in the cells were calculated using following formula as reported earlier (Yuan *et al.*, 2008).

$$\text{Drug uptake percentage (\%)} = \frac{C/M}{C_i/M_i} \times 100$$

C – Intracellular concentration of EPD measured by HPLC

M – Unit weight (mg) of cellular protein after incubation

$C_i$  – initial concentration of EPD

$M_i$  – the initial unit weight (milligram) of cellular protein.

## RESULTS AND DISCUSSIONS

Most of the hydrophobic drugs encapsulation including anti-cancer agents have been widely studied with the biodegradable and the USFDA approved polymers of PEG and PCL because of its micelles forming properties and the stability. Several methods are reported for the preparation of PEG-PCL block co polymer using different catalyst like stannous octoate, aluminum hydroxide or HCl-Et<sub>2</sub>O (Zhou *et al.*, 2003; Hsu *et al.*, 2004; Kim *et al.*, 2005). By understanding the removal of organometallic catalyst like stannous octoate during synthesis is a controversy for the use of mPEG-PCL di block co polymers in biological use. The ring –opening polymerization method used to control the unfavorable reaction (Kim *et al.*, 2005). The block lengths of mPEG and PCL were adjusted by changing the molecular weight of mPEG and the molar ratio of Caprolactone and mPEG. The polymerization of PEG-PCL was performed varying different feed ratios of PCL with respect to the molecular weight of PEG (5000) in the presence of HCl -Et<sub>2</sub>O (an activator) and obtained the colorless di -block copolymers with the maximum quantitative yield (>90%) after separation by precipitation in cold diethyl ether.

<sup>1</sup>H NMR spectrum was recorded to determine the molecular weight and the composition of the synthesized polymer. The interpretation of NMR spectra revealed that the signals of terminal methoxy protons (a) and methylene protons (c) of mPEG at  $\delta = 3.29$  and 3.47 ppm (NMR chromatogram not shown), respectively. Also the PCL moiety's peaks were observed that the assignable peak (1-5) to the sequence of  $\alpha$ ,  $\beta$ ,  $\delta$ ,  $\gamma$  and  $\epsilon$ -methylene protons of the ester carbonyl at 2.21, 1.57, 1.33 and 3.98 ppm, respectively (Figure 2). Apart, PCL moiety's polymer chain end proton (8) was observed at 3.88 ppm and terminal  $\omega$ -methylene protons

(d) of the mPEG blocked by PCL was observed at around 4.12 ppm.

In Gel permeation chromatography analysis, the unimolar peak appeared in GPC chromatograms as shown in Figure 3, which confirmed that the maximum purity of the copolymer during purification process. Furthermore, the polydispersity (M<sub>w</sub>/M<sub>n</sub>) of the copolymer (defined as the ratio of weight average molecular weight to the number average molecular weight) was found in narrow range with less than 1.33. In addition, decrease in retention time in GPC chromatograms was observed with increase in molecular weight. The calculated NMR values were confirmed by the number averaged molecular weight obtained from the GPC chromatogram and polydispersity index was calculated as shown in Table 1. The peaks in NMR spectra and GPC chromatograms are accordance with the reported results earlier (Hsu *et al.*, 2004; Kim *et al.*, 2005).

In FTIR spectra, at 1726 cm<sup>-1</sup>, observed strong absorption band which (FTIR Spectra not Shown) confirms the presence of carbonyl group and the two bands at 1110 and 1244 cm<sup>-1</sup> indicates the presence of ester group of copolymer. The band at 2945 cm<sup>-1</sup> confirms the CH<sub>2</sub> in PCL block, whereas the band at 2868 cm<sup>-1</sup> corresponds to the C-H stretching vibration of PEG. In addition, broad absorption band at 3439 cm<sup>-1</sup> confirmed the terminal hydroxyl groups in the block copolymer and carboxyl group in case of HOOC-PEG-PCL di-block copolymer.

Nano precipitation method was opted because of lower aqueous solubility of synthesized polymer of mPEG-PCL to be dissolved in organic solvent first which enhance the drug binding into the block co polymer, there by obtaining maximum percent drug entrapment (Aliabadi *et al.*, 2005). The low boiling point solvent, acetone was used as water miscible organic solvent. The formation of smaller particles observed upon the addition of organic solution in water by drop wise. Micellar formulations EPM 55, EPM 57 and EPM 51 exhibited the particle size of  $67.89 \pm 3.08$ ,  $73.39 \pm 4.08$  &  $76.25 \pm 2.69$  nm with  $88.51 \pm 3.20$ ,  $92.06 \pm 3.48$  &  $82.78 \pm 3.60$  percent drug entrapment at drug to polymer ratio of 1:20 and obtained 5.23, 5.40 and 5.89 percent drug loading.

Observation has been made that increasing the particle size and drug entrapment by increasing the ratio of drug molecule to polymer, also the maximum practical drug loading achieved by various micellar formulations. A practical drug loading obtained with different micellar formulations was solely dependent on the molecular weight of block copolymer especially the hydrophobic core part and increased with increase in hydrophobic core part molecular weight as shown in Table 2 and Figure 4. Moreover, a high negative or positive zeta potential is required interaction among nano particles / micelles in dispersion resulting into high stability. From the prepared EPM micelles zeta potential were ob-

served near to neutral which indicated that the excellent stability of micelles in aqueous solutions. The possible reason could be the PEG segments which capped the carboxyl acid end groups of the PCL chains leads in to low negative zeta potential as reported earlier (Hu *et al.*, 2007).

It is well understood that the biological stability of micelles depends upon the CMC values of the micelles. The lower the CMC values, better the stability of micelles. Upon dilution to CMC, the drug starts releasing slowly because of dissociation of micelles into unimers. To investigate the behavior of the mPEG-PCL polymers and its self-arrangements in an aqueous medium, pyrene was used as a fluorescence probe which is sensitive to change the micro environment. The determined CMC values are shown in the Table-2 and observed that the intensity values almost remains same below the CMC, even though the substantial increase in fluorescence after certain concentration. In another way, a low concentration of polymeric micelles below CMC, the marker of pyrene was dissolved in an aqueous medium and fluorescence intensity was very low when in the presence of micelles. The graph was plotted between the intensity and concentration and the CMC values of various micellar formulations were calculated. The EPM micelles CMC values were found the range between  $1.28 \times 10^{-3}$  to  $1.42 \times 10^{-3}$  mg/ml. Based on the results, hydrophobic moiety of polymer is directly proportional of the CMC values, means, increasing the hydrophobic part in the polymer chain decrease the CMC values. Moreover, the CMC values obtained are in agreement with the values reported earlier for mPEG-PCL micelles (Choi *et al.*, 2006).

It is required that the injected polymeric nanocarriers should not be recognized as foreign particles in the body by RES system. PEG coating in polymeric nanoparticles may reduce either the protein adsorption or complementing activation as a function of PEG density. It was observed that PEG surface density/nm<sup>2</sup> for EPM 55, EPM 57 and EPM 51 micelles exhibited PEG surface density between 0.640 and 0.487 with distance (D) from 1.56 to 1.70 nm. From the results, it was found that when the hydrophobic moiety's molecular weight increased, the surface density of PEG was reduced, therefore the distance between the two PEG chains were increased. The possible reason could be the distance between the two PCL moieties occupied less molecular number of di block copolymer to form the unimolar micelles. These values could be very helpful to understand the in vivo behavior of the EPM micelles. Based on the characterization of EPM micelles like particle size, percent drug entrapment, CMC, PEG surface density were taken into the consideration for the selection of micelles, thereby EPM 57 was selected further for in-vitro cell line studies.

In-vitro cell line studies

Discerning the complexity for lung cancer and to simulate the in-vivo cancer cell treatment in in-vitro, the artificial murine melanoma cell B16F10 cells were selected to assess the cell viability and cellular uptake with new developed EPD –loaded polymeric micelles. The *in vitro* cytotoxic activity of plain EPD and EPD loaded micellar formulation on B16F10 cells were expressed as % cell viability with various drug incubation time i.e. 24, 48 and 72 hours as shown in Figure 6 -8 also calculated the IC<sub>50</sub> values for plain EPD and EPD loaded micellar formulation from cytotoxicity profile. Both EPD and EPD loaded micellar formulations displayed resembling concentration and time dependent cytotoxicity. Plain EPD at 24, 48 and 72 h incubation time exhibited IC<sub>50</sub> values of 0.087, 0.050 and 0.043  $\mu$ M respectively. After 24 h incubation period, the calculated IC<sub>50</sub> values of micellar formulations were up to 25 to 50 times higher compare to plain EPD. When incubated with the cells, the drug concentration of EPD loaded micelles slowly increased due to its sustained release profile. The higher toxic level observed in plain EPD at 24 h incubation time compared to micellar formulation, since it is in soluble form and can diffuse in to cells quickly. Earlier findings with plain drug doxorubicin and cisplatin also showed similar cytotoxicity profile compared to nanoparticulate system at initial incubation time points (Zhao & Yung, 2008; Li *et al.*, 2008). In cellular uptake study, it was observed that plain EPD after incubation of 2 h resulted into  $75.02 \pm 5.23$  percent cellular drug uptake. The higher uptake of plain EPD was attributed due to its solubilized state of drug which resulted into rapid diffusivity of drug through cell membrane inside cells. The results obtained, supports the higher cytotoxicity exhibited by EPD at 24 h incubation. The non-conjugated micelles EPM 57 showed a percent cellular drug uptake of  $5.30 \pm 1.51$ . A very low cellular uptake was observed with EPM 57 compared to plain EPD due to its different route cell internalization.

## CONCLUSION

The USFDA approved and biocompatible polymers were selected for the synthesis of novel, stable di block copolymers (mPEG-PCL) and prepared polymeric micellar formulation consisting of a as a carrier for poorly soluble drug, etoposide has been characterized and optimized with small particle size, high encapsulation efficiency, high drug loading, CMC and surface density while comparing with unmodified etoposide preparation. The present study also documented that the encapsulated micelles delivered etoposide completely in aqueous medium, thus overcoming the poor water solubility of etoposide. Finally, the prepared polymeric micelles nano sized formulation (EPD-mPEG-PCL) were taken up further to understand the cancer cell lysis using the murine melanoma carcinogenic cell B16F10 and observed that the maximum inhibitory effect on growth of metastatic cell and compared with unmodified etoposide Thus, together with the present findings



proved that the polymeric micelles are the effective carrier for etoposide and holds greater promise for effective anticancer drug delivery.

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