www.ijrps.pharmascope.org

ISSN: 0975-7538
Research Article

Effects of pentapeptide conjugation on etoposide loaded polymeric micelles for the treatment of Cancer cells

Aramudan S*, Senthil kumar K.L

Padmavathi college of Pharmacy, Periyanahalli, Dharmapuri Dist,Tamil Nadu India

ABSTRACT

To getting the desirable therapeutic treatment for cancer, the efficacy and the reduction of toxicity of anticancer drugs are to be improvised. Although many of the drugs are in hydrophobic characteristics, the polymeric miceller delivery system would be the potential way for the targeted delivery and the effective treatment of metastasis. The present study was documented that the hydrophobic drug, etoposide was encapsuled with the biodegradable block co polymer mPEG-PCL which further conjugated with the cell adhesion peptides YIGSR and EILDV peptides for targeting the laminin and fibronectin receptors respectively. The results revealed that the conjugated micelles having the affinity towards the tumour cells, thereby increase in cytotoxicity as well as anti-metastatic effects observed from the cell line studies using B16F10 melanoma cells. Interestingly, the study confirmed that the YIGSR peptide conjugated etoposide has more cytotoxic effects than the other Pentapeptide EIDLV conjugated etoposide. Also, to understand the predictable drug release in physiological conditions, the in-vitro release study was carried out with PBS at pH 7.4, observed the significant difference between the peptide conjugated micelles and the non-conjugated micelles. Further, lyophilization studies were carried out with different cryoprotectants / different ratios and the redispersibility index was calculated, observed that the single cryoprotectant was not retained the original particle size than the combinations. Overall, etoposide loaded-conjugated miceller formulations proved higher efficacy and reduced the toxicity in the treatment of cancer cells than the non-conjugated miceller formulations.

Keywords: Polymeric micelles; YIGSR & EILDV Pentapeptides; mPEG-PCL block co polymer; etoposide; tumour metastasis; lyophilization; In-vitro release; In-vitro cell line studies.

INTRODUCTION

One of the challenging task of the tumor treatment is to annihilate the tumor cells effectively without causing harmful effect of normal cell at the best extent. Apparently, most of the anti-cancer drugs has the severe toxic effects due its non-specificity with the cells. Therefore the tumor targeted delivery system plays an important role in chemotherapy (Shuai et al., 2004). To attain the objective, the polymeric micelles has evidenced the potential nanocarrier for the delivery of hydrophobic anticancer drugs for minimizing the toxicity, enhancing the efficacy and the targeted delivery due its versatile features like particle size and biocompatibility (Forrest et al, 2006) also prolonging its circulation time and modifying its biodistribution (Jones and Leroux 1999).

The polymeric micelles has been fabricated through the self-assembly of amphiphilic block copolymers which can be synthesized by altering the polar and non-polar moiety without affecting one another (Xiong et al 2007). These changes may be required to design the polymeric micelles for higher drug loading and for targeted sustained/controlled delivery (Letchford et al 2007). Drugs to reach the specific cells the polymeric micelles has been delivered either actively or passively, the passive targeting system takes the advantages that the cancer cells present in the leaky vasculatures are impaired (Torchilin 2007) and on the other side the active targeting is achieved by inducing the specific cell receptor and responding to the external stimuli. The bioavailability of the nanoparticles are extremely limited by P-gp, efflux mechanism (Zhao and young 2008) and this can be overcome by cellular internalization, that is the nanocarriers are to be bound with the specific ligands/ receptors on the cell surface. For achieving the activing tumor targeting many of peptide (c RGD), carbohydrate moiety (galactose), antibodies and aptamers have been widely used and reported in various publications. The cell surface receptors binds with ECM proteins and involves the recognition of the specific target sequence of the ligands (Singleton and Menino 2005). The peptides like YIGSR , cRGD, EIDLV peptides have been used as targeting moieties for

* Corresponding Author
Email: aramudans@gmail.com
Contact: +91-7093401852
Received on: 23-05-2016
Revised on: 07-06-2016
Accepted on: 12-06-2016

nanocarriers to target the laminin receptors αvβ5 and α4β1 respectively (lopez-barcons et al 2004 : Nasongkla et al 2004).

In this present study, we have made an attempt to conjugate the etoposide loaded polymeric micelles with cell adhesion peptides YIGSR and EILDV on the surface of the micelles. The chosen formulations were lyophilized with different cryoprotectant like sucrose / trehalose and poloxamers 188, to understand the redispersibility/impact of cryoprotectants of the micelles. Different in-vitro cell line studies were carried out using the highly metastatic B16F10 cell lines to determine the effectiveness of the conjugated peptides-micceller formulations and compared with non-conjugated etoposide micelles.

MATERIALS AND METHODS

Materials

Active pharmaceutical ingredients, Etoposide (EPD) were gifted from Jiangsu Hengrui Medicine Co., Ltd, China. Poly ($\mathcal{E}$-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 (I2), 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. YIGSR-NH_2 and EILDV-NH_2 were procured from Bio concept Lab. Pvt. Ltd., Haryana. 1-ethyl-3(3-dimethylaminopropyl) carbodiimide (EDC) and dialysis tube (Mw 12000), N-hydroxysuccinimide (NHS) were procured from Sigma Aldrich, Mumbai.

Synthesis of mPEG-PCL Block Copolymers

The pioneering Ring opening polymerization method was used for the synthesis of Methoxy PEG-PCL diblock copolymers (Aliabadi H M et al., 2007). The different molecular weight of mPEG (5k)-PCL (7k) were chosen for the synthesis of di block copolymer. Two grams of mPEG (Mw 5000) was azeodistilled with 100ml of dried toluene to remove the water completely. To this, added 20 ml of dried dichloromethane and 2grams PCL (Mw 7000) was added slowly to the reaction mixture at 20-25 °C. Under vigorous stirring, slowly 3ml of HCl-Et2O (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 discorded 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. Aramudan et al 2016 described the selection of different molecular weight of carboxyl and methoxy polyethylene glycol and the hydrophobic moiety of PCL. Among the various molecular weight of block copolymers, ABCP 57 selected further for drug entrapment.

Preparation of Etoposide (EPD) Micelles

The reported method of nanoprecipitation technique was employed for the preparation of Etoposide loaded micelles (Zhang et al., 2004). Concisely, 30 mg of diblock copolymer mPEG 5k-PCL 7k (ABCP 57) and the required quantity of etoposide (2mg) were solubilized in 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 unentrapped drug particles. The characterization of the micceller formulations has been described in Table 1, further EPM 57 formulation was chosen for conjugation.

Assembly of Peptide Conjugation and Characterization

Peptides YIGSR was attached to the prepared micelles (EPM 57) by EDC and NHS, dissolving in phosphate buffer, pH 4.5. To this the prepared etoposide micelles were added (1: 4 ratio) and stirred well at 25 ^{0}C for 10 minutes and the final pH were adjusted at 7.5 with base solution NaOH (0.1 N). The calculated quantity of peptides YIGSR dissolved in PBS, pH 7.4 and added to the prepared micceller solutions (PEG-5k: PCL 7k) and stirred well at 25 ^{0}C for 3 hours and the final pH were adjusted at 7.5 with base solution NaOH (0.1 N). The micceller formulations were dialyzed (Mw 12000) to remove the free peptides using distilled water, finally filtered through 0.45 micron membrane PVDF filter to remove the aggregates of the polymers, their characteristics were studied and values are described in Table-1a. The same procedure was employed to conjugate the EILDV–NH_2 peptides also.

Lyophilization

To discern the long-term product stability Lyophilization technique was employed. Lyophilization of formulations was performed using cryoprotectant sucrose and trehalose at different weight ratio to the total solid content of formulation. Poloxamer-188 was also used at different ratio along with sucrose to check the redispersibility of lyophilized formulation. Briefly, definite

amount of cryoprotectant with or without poloxamer-188 was dissolved to micellar solution and frozen at -40 °C. Samples were then lyophilized for 24 h using virtis lyophilizer. The resulting lyophilized products were sealed immediately and stored at 2-8 °C. After lyophilization, samples were reconstituted with distilled water and vortexed for 3 min to uniformly redisperse the solid content. Particle size of samples was analyzed using Zetasizer, Nano-ZS (Malvern Inst., U.K.).

***In-Vitro* Release Studies**

In-vitro release studies were performed using dialysis bag, PBS medium at pH 7.5. The required quantity of conjugated miceller formulations (EPM 57, CPY 57 and CPE-57) were filled into the dialysis bag and kept in the container contains PBS. Also, ensured the continuous stirring at 50 rpm and the temperature of the solution maintained at 37±2^{0}C. Periodic samples were withdrawn and analyzed by UV-Visible spectrophotometer at 284nm.

***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 ºC with 5 % CO2. 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 µl MTT in 80 µl of PBS and incubated for 4 h at 37 ºC. Plates were then centrifuged at 500 rpm for 20 min. To dissolve the Formazan crystals, further 100 µl 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 X 10^3 cells/100 µl/well & 1.5 X 10^3 cells/100 µl/well respectively and cells were incubated in presence of drug for respective time point. Percent cell viability was plotted against concentration and IC_{50} 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 ± 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 X 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 ºC. Cells were washed with ice cold PBS twice and harvested with saline EDTA. The final count of cell was made to 2 X 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).

$$Drug\ uptake\ percentage\ (\%) = \frac{C/_M}{Ci/_{Mi}} \times 100$$

C = Intera cellular concentration of EPD measured by HPTLC.

M = unit weight (mg) of cellular protein after incubation.

Ci = Intial concentration of EPD.

Mi = the intial unit weight (milligram) of cellular protein.

RESULTS AND DISCUSSION

Characterization of conjugated YIGSR / EILDV peptides in Etoposide polymeric micelles.

Several methods has been reported for the preparation of mPEG-PCL block copolymer using different catalyst like stannous octoate, aluminum hydroxide or HCl-Et2O (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-Et2O (an activator) and obtained the colorless di-block copolymers with the maximum quantitative yield (>90%) after separation by precipitation in cold diethyl ether.

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 results are shown in Table 1(Aramudan et al 2016). Conjugation of peptide YIGSR-NH_2 or EILDV-NH_2 to carboxyl functionalized PEG-PCL micelles was carried out using NHS and EDC through amide bond linkage. EDC is a dehydrating agent used to activate carboxylate groups to reactive o-acylisourea (Staros et al., 1986)

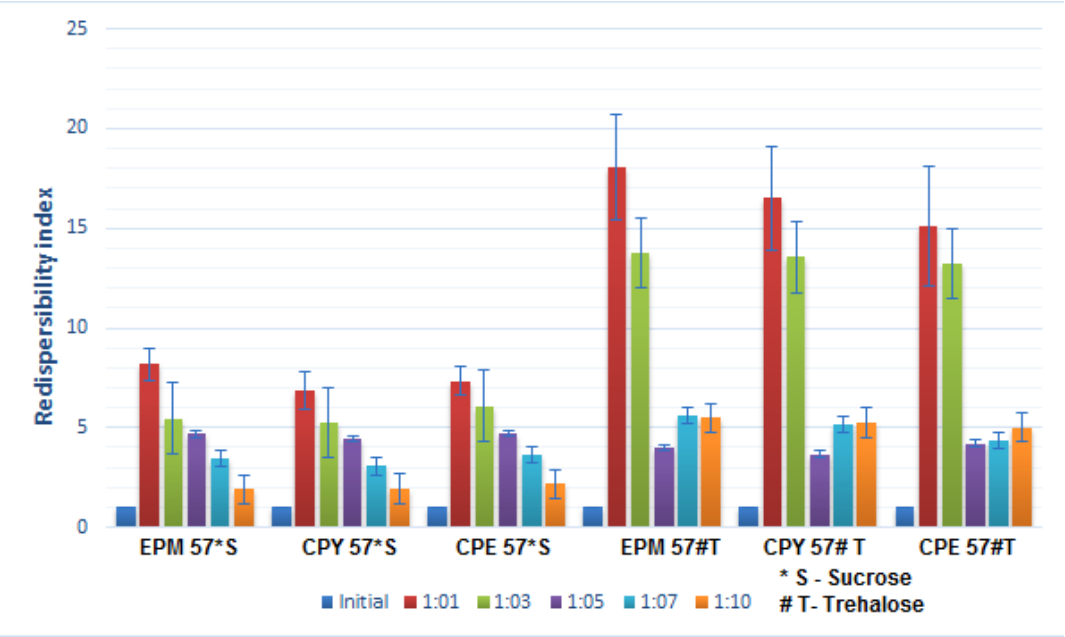

Figure 1: Redispersibility index of micellar formulations at various weight ratios of total solid content to sucrose and trehalose (n=3)

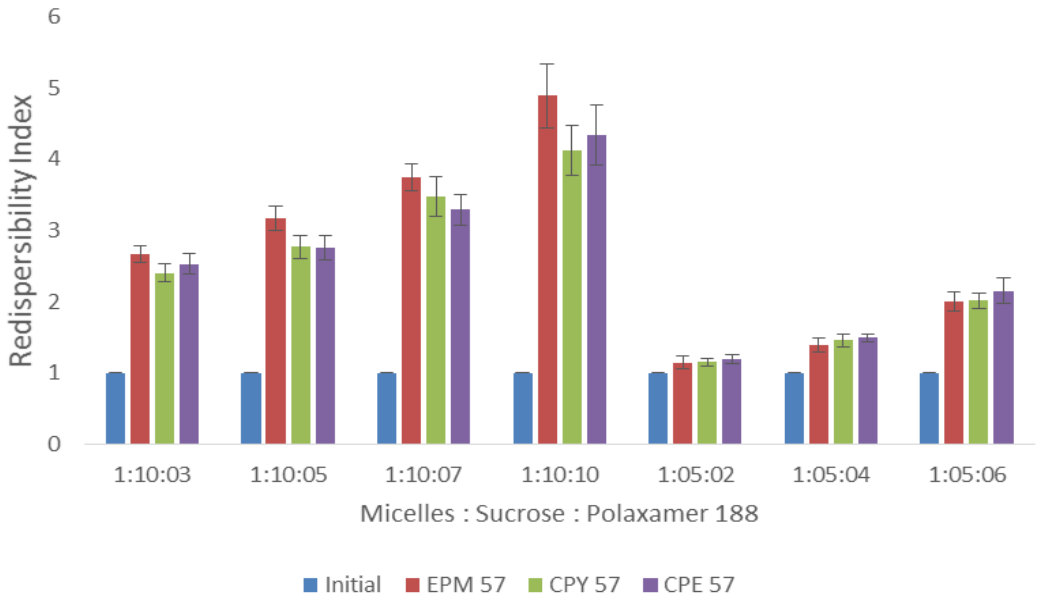

Figure 2: Redispersibility index of micellar formulations using various ratio of total solid content to sucrose and poloxamer-188 (n=3)

Table 1: Effect of peptide conjugation on particle size, zeta potential and percent drug entrapment (Results are mean ± S. D. and n=3)

Conjugated Micelles code	Particle size (nm)		Zeta Potential (mV) at pH 7.4		Percent Drug Entrapment	
	Before	After	Before	After	Before	After
CPY 57 (YIGSR-NH_2)	73.21 ± 4.09	83.80 ± 4.22	-21.3 ± 3.03	-5.16 ± 2.01	91.06 ± 3.48	85.50 ± 3.70
CPE 57 (EILDV-NH_2)	72.57 ± 3.90	80.32 ± 4.89	-19.8 ± 3.19	-5.39 ± 2.24	90.70 ± 4.81	83.98 ± 4.90

Table 1a: Characterization of miceller formulation

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

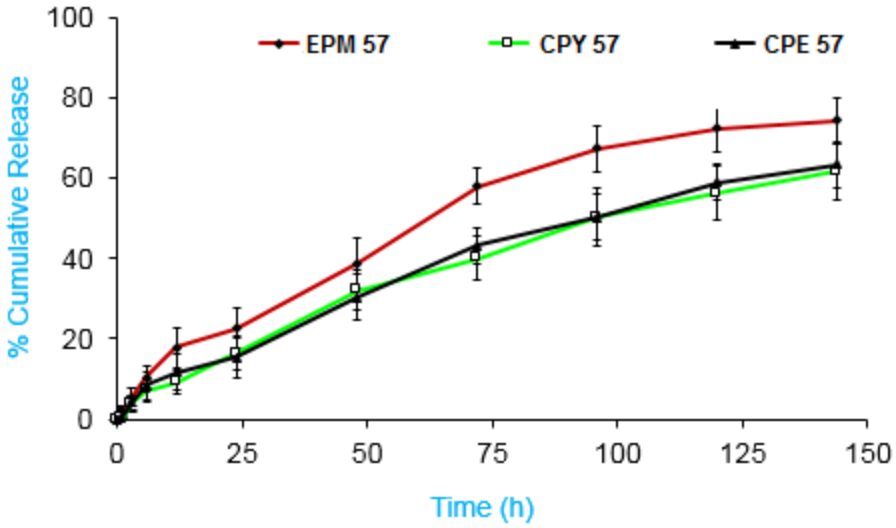

Figure 3: In-vitro release of miceller formulation vs conjugated peptide miceller formulations (mean ± S.D, n=3 individual experiments)

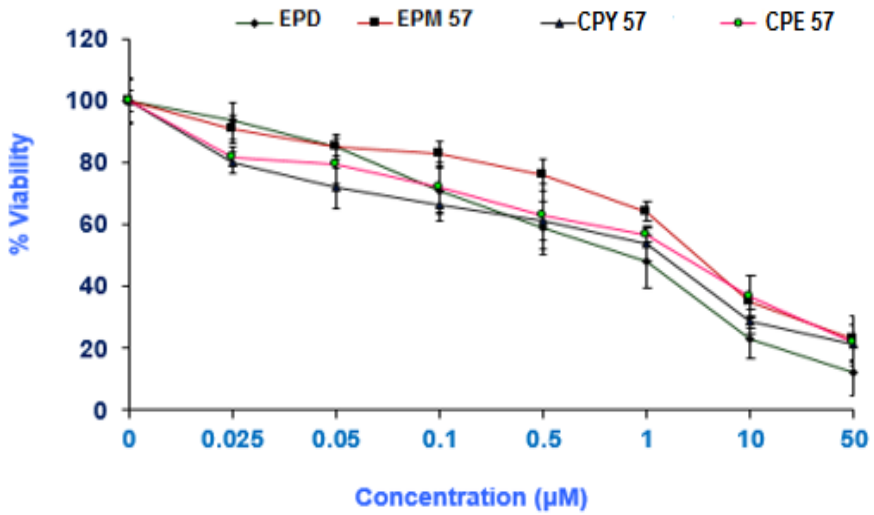

Figure 4: Cell viability of B16F10 cells after 24 h incubation period

but the formed group is unstable and short-lived in aqueous solution. It is because of the hydrolysis of oacylisourea, regenerating the initial carboxyl group and hence it is necessary to add NHS, which reacts with the o-acylisourea to yield a semi-stable amine reactive NHS-ester with half-life of 4-5 h at pH 7.0 (Grabarek et al., 1990). The final reaction with amines is greatly favored with a stable amine bond formation. This method permits two-step crosslinking procedures, which allows the carboxyl groups eventually present on molecule to remain unaltered. This method has an advantage that it maintains intrinsic properties of ligand attached.

It is essential to have an optimal peptide density on the surface of micelles. The optimal density was defined as number of molecules of peptide on the surface of nanoparticle/ micelles to confer maximum targeted cellular uptake with avoidance of unnecessary masking of

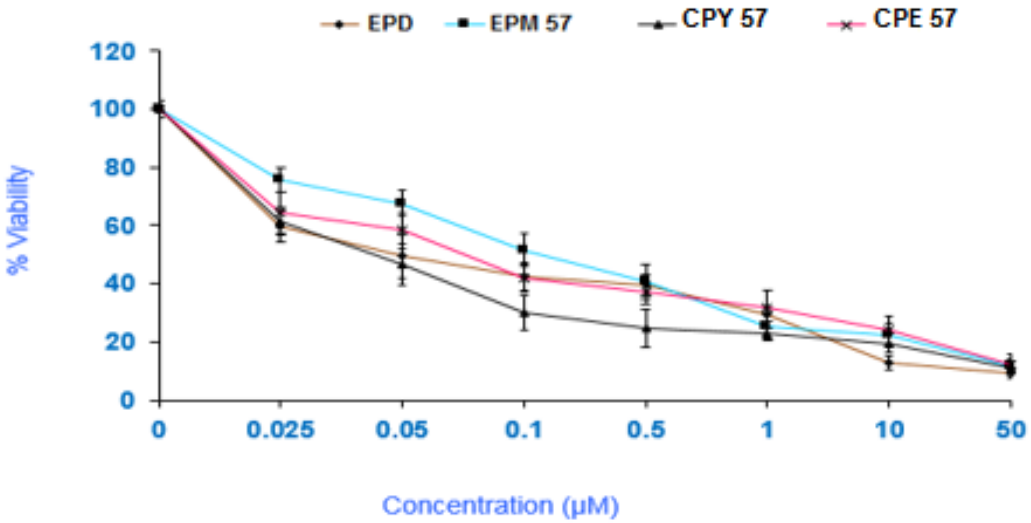

Figure 5: Cell viability of B16F10 cells after 48 h incubation period

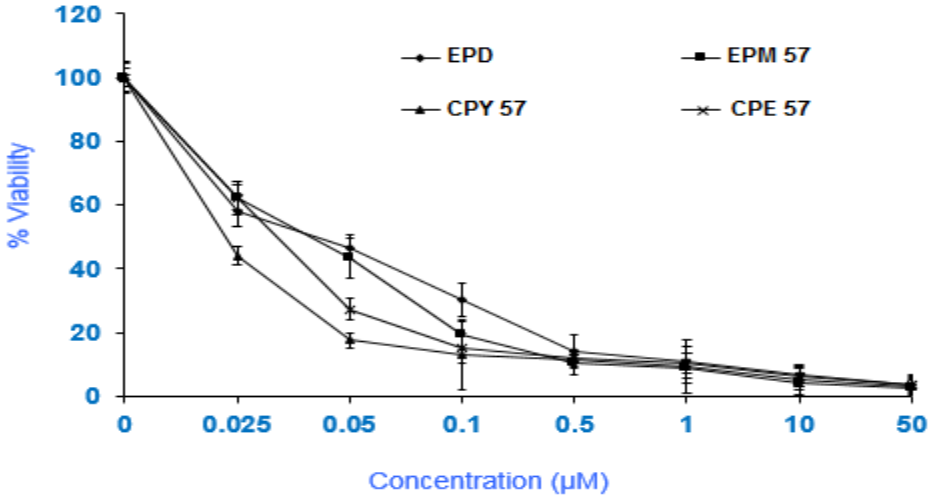

Figure 6: Cell viability of B16F10 cells after 72 h incubation period

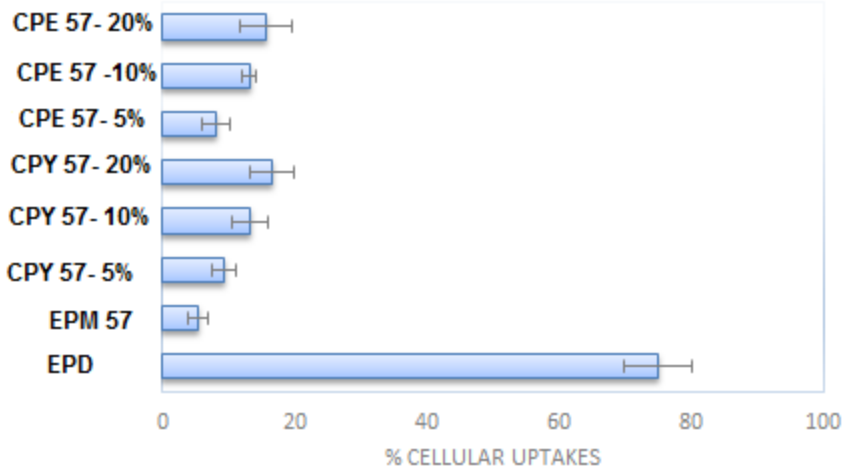

Figure 7: Percent cellular uptake of peptide conjugated micelles with different surface density of peptides

PEG on nanoparticle/micelles by excess peptide that will not give additional targeting benefit (Gu et al., 2008). Adjusting the density of peptide on the surface, the antibiofouling properties of PEG can be retained which is required for its long circulation. In this experiment, peptide conjugation was optimized with peptide density of 10 % using a blend of functional polymer and non-functional polymer. Micellar formulations with surface density of 5 and 20 percent were also prepared

after optimization of process and formulation parameters to assess the maximum cellular uptake of micelles.

A post-insertion method consisting of two step reaction was performed to form amide bond between free terminal amine group of peptide (YIGSR-NH_2 and EILDV-NH_2) and carboxyl group present on surface of PEG-PCL micelles. The conjugation efficiency of peptide YIGSR-NH_2 and EILDV-NH_2 was assessed by changing various parameters like incubation temperature, incubation time and ratio of functional polymer to peptide. The molar concentration of EDC and NHS used was four times higher to that of functional polymer used. After conjugation, the micellar solution was dialyzed against distilled water and the unconjugated peptide found in dialyzed medium was estimated by developed TNBS method.

Optimized batch of peptide conjugated micelles were evaluated for particle size, zeta potential and percent drug entrapment before and after peptide conjugation. It was observed as shown in Table 1a, particle size increased up to 10 nm in all formulation after conjugation, which showed the evidence of peptide conjugation. Moreover, a decrease in zeta potential after conjugation was also observed at pH 7.5, which indicates the surface free carboxyl groups on micelles were utilized for formation of amide bond to amine groups of peptide. A reduction in percent drug entrapment up to 6 percent was also observed, which might be due to initial burst release of drug occurred during incubation and removed from micelles in dialysis step.

Lyophilization of micellar formulations was carried out using cryoprotectant sucrose and trehalose which was reported to have good cryoprotectant properties (Saez et al., 2000). Initially, lyophilization was carried out using sucrose or trehalose in the weight ratio of total solid content to cryoprotectant of 1:1, 1:3, 1:5, 1:7 & 1:10. Lyophilized formulations were reconstituted with distilled water and vortexed well to redisperse the suspension followed by particle size measurement. The redispersibility index was calculated based on the ratio of particle size obtained after reconstitution to that of original or initial particle size. Figure 1 shows the effect different amount of cryoprotectant (sucrose or trehalose) on particle size of micelles after reconstitution i.e. redispersibility index. It was found that both sucrose and trehalose were unable to preserve the original particle size. Sucrose used at maximum ratio (1:10) of total solid content to sucrose showed redispersibility index from 1.9 to 3.4. This implies that sucrose is not capable to preserve the original particle size up to ratio of 1:10. Trehalose which is considered as a best cryoprotectant for nanocarrier during lyophilization gave unsatisfactory results and the redispersibility index obtained was very much high compared to sucrose. At 1:7 ratio of total solid content to trehalose, EPM 57, CPY 57 and CPE 57 at same ratio showed redispersibility index between 5 and 6. Moreover, there was no significant difference in particle size was observed after reconstitution was observed 1:7 and 1:10 weight ratio which might be due to saturation of lyoprotective activity of trehalose (Jaeghere et al., 1999).

A complete redispersion with RI near to 1 was not achieved at different ratio of total solid content to trehalose, as reported by Jaeghere et al. (1999) in PLA-PEO nanoparticles. The probable reason behind this could be due to high PEG concentration, presence of drug and molecular weight of di-block copolymer used. The results obtained proved that trehalose is not a suitable candidate as cryoprotectant for micelles made of PEG and PCL blocks. PEG which is having inherent properties of steric stabilization in aqueous colloidal dispersion was unable to maintain the original particle size in absence of cryoprotectants (Jaeghere et al., 1999). The reason for this differential behavior has been attributed to the tendency of PEG to crystallize during freeze drying (Izutsu et al., 1996).

Layre et al. (2006) studied the effect of four sugars (glucose, saccharose, maltose, and trehalose) and one surfactant (Poloxamer-188), on freeze-drying of PIBCA and PCL-PEG nanoparticles. It was found that in absence of Poloxamer-188, a significant increase in particle size after freeze drying took place and all four sugars failed to protect the nanoparticles against aggregation during freeze drying. In contrast to this, nanoparticles freeze dried in presence of poloxamer-188 showed tyndall effect upon redispersion and concluded that poloxamer-188 is required to maintain the original particle size after freeze drying. Based on above finding (Layre et al., 2006), poloxamer-188 was tried with sucrose at different ratio as shown in Figure 2

Promising results were obtained with the use of surfactant poloxamer-188 along with sucrose (Figure 2). Although, when particle size was measured after reconstitution, it was observed that weight ratio still affected much on particle size. At 1:10:5 ratios, redispersibility index obtained was in between 2.75 to 3.65. EPM 57, CPY 57 and CPE 57 exhibited refractive index near to 1 at 1: 5: 2 ratios. A different ratio required for both groups to achieve redispersibility index near to one might be due to changes in PEG content in micelles.

The ***in-vitro*** drug release from micelles is quite complex mechanism and there are many factors are affected polymer degradation, molecular weight, crystallinity, glass transition temperature and binding affinity of drug to polymer (Shin. et al 1998). *In vitro* release studies of formulation were performed in phosphate buffer saline at pH 7.4 to simulate the drug release in physiological conditions. The cumulative % release profile was depicted in Figure 3 for EPM 57, CPY 57 and CPE 57. After 12 h, EPM 57, CPY 57 and CPE 57 at the same time exhibited drug release of 17.98 %, 9.08 % and 11.69 % and after 144 h the drug release was found 74.32 %, 61.91% and 63.33 % respectively. The significant different was observed between conjugated and

non-conjugated miceller formulations as shown graphically in figure no.3

In-vitro cell line studies

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 4-6 also calculated the IC_{50} values for plain EPD, EPD micelles and conjugated peptide formulations CPY 57 and CPE 57. 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_{50} values of 0.077, 0.050 and 0.043 µM respectively. After 24 h incubation period, the calculated IC_{50} 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).

Extending the incubation time to 48 h, it was observed lower cell viability at each dose compared to that of 24 h for plain ETO and micellar formulations. The calculated IC_{50} values for EPM, CPY and CPE 57, the IC_{50} values obtained were 0.120, 0.045 & 0.075µM respectively. Peptide conjugated micelles CPY 57and CPE 57 showed higher cytotoxicity compared to non-conjugated micelles but the effect was still comparable to plain EPD. After 72 h incubation period, plain EPD showed no further significant increase in cytotoxicity compared to 48 h, while a major reduction in cell viability were observed with all micellar formulations. The calculated IC_{50} values of all micellar formulations were lower than plain ETO. Peptide YIGSR-NH_2 conjugated micelles CPY 57 showed 2.1 fold increases in toxicity respectively compared to plain EPD. EILDV-NH_2 conjugated micelles CPE 57 showed increase in cytotoxic effect of 1.4 fold compared to plain EPD.

In cellular uptake study, it was observed that plain EPD after incubation of 2 h resulted into 75.02 ± 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 ± 1.51. A very low cellular uptake was observed with EPM 57 compared to plain EPD due to its different route cell internalization. Peptide YIGSR-NH_2 and EILDV-NH_2 was conjugated on the surface of micelles at different surface density i.e. 5 %, 10 % and 20 %. As shown in Figure 7, YIGSR at 5 % surface density increased the cell internalization of micelles (CPY 57) near to two fold compared to EPM 57 micelles. CPE 57 at 5 % surface density showed increase in cell uptake with one and half fold compared to non-conjugated micelles. With surface density of 10 % of both the peptides, CPY 57 showed percent cellular uptake of 13.30 ± 2.62 while CPE 57 showed 13.28 ± 1.06 percent cellular uptakes. There was no much increase in cellular uptake observed when surface density of peptide doubled to 20 % from 10 %. The result implies that after critical surface density of ligand, there was no effect of increase in ligand concentration effect on the cellular uptake enhancement.

CONCLUSION

The prepared polymeric micelles nano sized formulation (EPD-mPEG-PCL) was conjugated with YIGSR and EILDV Pentapeptides and evaluated by in-vitro release studies to understand the drug release of polymeric micelles at the physiological pH 7.4, confirmed the significant difference of release between conjugated and non-conjugated miceller formulations. Further, the study extended to find out the cytotoxicity using murine melanoma carcinogenic cell B16F10 and observed that the maximum inhibitory effect on growth of metastatic cell and compared with non-conjugated etoposide. Thus, the present study confirms that the conjugated etoposide miceller formulations has more efficacy for the treatment of tumor cells than the plain etoposide and non-conjugated miceller formulations

ACKNOWLEDGEMENT

The author is grateful to Dr. Mukesh Ukkawala who supported for this work and declares no conflicts of interest.

REFERENCES

Aliabadi HM, Elhasi S, Mahmud A, Gulamhusein R, Mahdipoor P, Lavasanifar A. Encapsulation of hydrophobic drugs in polymeric micelles through co-solvent evaporation: The effect of solvent composition on micellar properties and drug loading. International Journal of Pharmaceutics, 2007, 329 (1-2), 158-165.

Aramudan S and SenthilKumar K L, Etoposide delivery: mPEG-PCL based copolymeric micelles assessed by various in-vitro anti-cancer activity, 2016, 7(2), 122-131.

Dua P, Gude RP. Antiproliferative and antiproteolytic activity of pentoxifylline in cultures of B16F10 melanoma cells. Cancer Chemotherapy and Pharmacology, 2006, 58, 195-202.

Forrest LM, Won CY, Malick AW, Kwon GS. In vitro release of the mTOR inhibitor rapamycin from poly (ethylene glycol)-b-poly((-caprolactone) micelles. Journal of Controlled Release 2006a, 110, 370-377.

Grabarek Z, Gergely J. Zero-length crosslinking procedure with the use of active esters. Analytical Biochemistry, 1990, 185, 131-135.

Gu F, Zhang L, Teply BA, Nina Mann N, Wang A, Radovic-Moreno AF, Langer R,Farokhzad OC. Precise engineering of targeted nanoparticles by using self-assembled biointegrated block copolymers. Proceeding of the National Academic of Sciences of the USA 2008; 105: 2586-2591.

Hsu SH, Tang CM, Lin CC., Biocompatibility of poly ($\mathcal{E}$ -caprolactone)/poly (ethylene glycol) diblock copolymers with nanophase separation, Biomaterials, 2004, 25, 5593-5601.

Izutsu KI, Yoshioka S, Kojima S, Randolphy TW, Carpenter JF. Effects of sugar and polymers on crystallization of poly (ethylene glycol) in frozen solutions: phase separation between incompatible polymers. Pharmaceutical Research, 1996, 13, 1393-1400.

Jaeghere D, Allemann F, Leroux E, Stevels JC, Feijen W, Doelker J, Gurny R. Formulation and lyoprotection of poly(lactic acid-co-ethylene oxide) nanoparticles: influence on physical stability and in vitro cell uptake. Pharmaceutical Research, 1999, 16, 859-866.

Jones M.C., and J.C Leroux, Polymeric micelles - a new generations of colloidal drug carriers, European Journal of Pharmaceutics and Biopharmaceutics, 1999, 48 (2), 101-111.

Kim MS, Hyun H, Cho YH, Seo KS, Jang WY, Kim SK, Khang G, Lee HB. Preparation of methoxy poly (ethylene glycol)-block poly (caprolactone) via activated monomer mechanism and examination of micellar characterization, Polymer Bulletin, 2005, 55, 149–156.

Layre AM, Couvreur P, Richard J, Requier D, Eddine G, Gref R. Freeze-drying of composite core-shell nanoparticles. Drug Development and Industrial Pharmacy, 2006, 32, 839-846.

Letchford K, Burt H., A review of the formation and classification of amphiphilic block copolymer nanoparticulate structures: micelles, nanospheres, nanocapsules and polymersomes, European Journal of Pharmaceutics and Biopharmaceutics, 2007, 65, 259-269.

Liu B, Yang M, Li R, Ding Y, Qian X, Yu L, Jiang X.The antitumor effect of novel docetaxel-loaded thermosensitive micelles. European Journal of Pharmaceutics and Biopharmaceutics, 2008, 69, 527-534.

Lopez BA, Polo D, Reig Z, Fabra A. Pentapeptide YIGSR-mediated HT-1080 fibrosarcoma cells targeting of adriamycin encapsulated in sterically stabilized liposomes. Journal of Biomedical Materials Research Part-A, 2004, 69A, 155–163.

Nasongkla N, Shuai X, Ai H, Weinberg BD, Pink J, Boothman DA, Gao J. cRGD-functionalized polymer micelles for targeted doxorubicin delivery. Angewandte Chemie International Edition, 2004, 116, 6483-6487.

Saez A, Guzman M, Molpeceres J, Aberturas MR. Freeze drying of polycaprolactone and poly (D,L-lactic-glycolic) nanoparticles induce minor particle size changes affecting the oral pharmacokinetics of loaded drugs. European Journal of Pharmaceutics and Biopharmaceutics, 2000, 50, 379-387.

Shuai X, Merdan T, Schaper AK, Xi F, Kissel T.. Core-cross-linked polymeric micelles as paclitaxel carriers. Bioconjugate Chemistry, 2004b; 15: 441–448.

Shuai, X, Ai H, Nasongkla N, Kim S, Gao J.. Micellar carriers based on block copolymers of poly(epsilon-caprolactone) and poly(ethylene glycol) for doxorubicin delivery. J Control Release 2004a; 98: 415–426.

Singleton C, Menino AR. Effects of inhibitor of integrin binding on cellular outgrowth from bovine inner cell masses in vitro. In Vitro Cellular & Developmental Biology-Animal, 2005, 40, 29-37.

Staros JV, Wright RW, Swingle DM. Enhancement by N-hydroxysulfosuccinimide of water-soluble carbodiimide mediated coupling reactions. Analytical Biochemistry, 1986, 156, 220-222.

Torchilin V.P, Targeted polymeric micelles for delivery of poorly soluble drugs, Cellular and Molecular life sciences, 2004, 61(19), 2549 – 2559.

Xiong XB, Mahmud A, Uludag H, Lavasanifar A. Conjugation of arginine-glycine-aspartic acid peptides to Poly(ethylene oxide)-*b*-poly(ε-caprolactone) micelles for enhanced intracellular drug delivery to metastatic tumor cells. Biomacromolecules, 2007, 8, 874–884.

Yuan H, Miao J, Du YZ, You J, Hu FQ, Zeng S. Cellular uptake of solid lipid nanoparticles and cytotoxicity of encapsulated paclitaxel in A549 cancer cells. International Journal of Pharmaceutics, 2008,348, 137-145.

Zhang L, Hu Y, Jiang X, Yang C, Lu W, Yang YH. Camptothecin derivative-loaded poly (caprolactone-co-lactide) b-PEG-b-poly (caprolactone-co-lactide) nanoparticles and their biodistribution in mice, Journal of Controlled Release, 2004, 96, 135-148.

Zhao H, Yung LY. Selectivity of folate conjugated polymer micelles against different tumor cells. International Journal of Pharmaceutics, 2008, 349, 256–268.

Zhou S, Deng X, Yang H., Biodegradable poly (ε-caprolactone)-poly (ethylene glycol) block copolymers: characterization and their use as drug carriers

for a controlled delivery system, Biomaterials, 2003, 24, 3563-3570.