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Original Article

DUAL ANTICANCER DRUG LOADED METHOXY POLY (ETHYLENE GLYCOL)-POLY (ε-CAPROLACTONE) BLOCK COPOLYMERIC MICELLES AS NOVEL DRUG CARRIERS

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ABSTRACT

Objective: Curcumine (CUR) and rapamycin (RAPA) are two potent hydrophobic anticancer drugs. The clinical and preclinical applications of anticancer formulations are limited due to use of toxic excipients and poor bioavailability. In the present study, an approach has been made to develop CUR and RAPA loaded MePEG/PCL di-block copolymeric micelles keeping in the view to make excipient free formulation with slow release of drugs.

Methods: The CUR and RAPA loaded MePEG/PCL di-block copolymeric micelles were prepared. Physico-chemical characters like size, surface charge and encapsulation efficiency were measured. The *in vitro* release studies was carried out in pH 7.4 to evaluate the sustained release properties of micelles.

Results: MePEG/PCL di-block copolymeric micelles were efficiently encapsulate both the drugs, i. e. CUR (~ 64 %) and RAPA (~ 94 %) in the core and have loading capacity of ~ 12 % (CUR) and ~ 29 % (RAPA). The zetasizer measurement shows that particles have size range 128 nm to 176 nm with a negative zeta potential. SEM and AFM studies reveled that micelles have smooth exterior surface. The XRD and DSC studies explain that the drugs are uniformly distributed in the polymer matrix. The dual drug loaded micelles have sustained *in vitro* drug release activity as estimated in phosphate buffer (pH 7.4).

Conclusion: These MePEG/PCL di-block copolymeric micelles are capable of carrying both the hydrophobic anticancer drugs and the encouraging results suggest further studies to evaluate the bioavailability parameters as well as suitability of the formulation.

Keywords: Curcumin, Rapamycin, Poly (ethylene glycol)/ ε- caprolactone block copolymers, Micelles.

INTRODUCTION

Polymeric micelles are nanoscopic core shell structures formed by aqueous self assembly of amphiphilic block copolymers [1]. These micelles have hydrophobic core surrounded by hydrophilic outer shell. The inner core of the micelles is used for the encapsulation of poorly water soluble drugs and the outer core can be modified with heterogenous functionalities to facilitate drug loading and targeting. Over the past few years, block copolymeric micelles have been used as drug carriers for poorly water-soluble drugs that result in improved bioavailability, prolonged plasma circulation and decreased systemic toxicity of drugs. In addition to the rapid clearance by the reticuloendothelial system resolved the passive targeting of certain tissues through the enhanced permeation and retention (EPR) effect. These properties along with established safety record in humans has lead to a research encouraging it as an alternate to commercial anticancer formulations [2, 3]. Micelles characters can be influence by the monomers, chain architecture, composition, molecular weight and the method employed to disperse amphiphiles in to the desired media [4].

The poor water soluble drugs require safe vehicles for drug solubilization and intravenous infusion. The commonly used excipients (i. e. Cremophor EL, DMSO, phosphatidylcholine or ascorbyl palmitate etc.) For intravenous drug infusion are often toxic and obstruct the progress in therapy involving hydrophobic anticancer drugs. The excipients used in therapy involving more than one drug had run the risk of precipitation and additive toxicity caused by vehicles used for drug solubilization. For example, hypersensitivity reaction occurs in patients that receive Cremophor EL despite premedication with corticosteroids and histamine antagonists [5]. Other serious toxicities associated with Cremophor EL use include nephrotoxicity and neurotoxicity [6-8]. Thus, there is need to find alternate formulations and vehicles to deliver these agents without contributing to the side effects experienced by patients on chemotherapy. Various copolymeric micelles have shown monotherapeutic approach for delivery of anticancer agents like paclitaxel, docetaxel, rapamycin, doxorubicin, etoposide and curcumin [9-11]. The block copolymeric micelles of methoxy poly(ethylene glycol) (MePEG)/poly(ε-caprolactone) have high taxol loading capacity and can be used for anticancer activity *in vivo* male ICR mice model [12]. Due to the effectiveness of micellar system, PEG-b-PLA micelles loaded with paclitaxel (Genexol-PM) are approved in Korea for cancer treatment, and are also in phase II clinical trials in USA [13].

The heterogeneity of cancer cells and acquired drug resistance limits the single agent therapy and increases the possibility to use combinational chemotherapy and combating factors associated with multidrug resistance (MDR) [14]. Synergistic drug combinations produce an even greater response rate or survival time than is possible with each drug used alone at its optimum dose. For multidrug based cancer therapy, micelles provide a good platform to co-administer anticancer therapeutics for solubilization of hydrophobic drugs, sustained drug release activity, thereby allocating modulation of the pharmacokinetics properties and biodistribution of the drug [15]. Shin *et al.* Has developed PEG-b-PLA micellar system for combinational approach involving a chemotherapeutic drug and molecularly targeted agent, which provide a better approach for combination cancer therapy [16]. Therefore, in the present study, we prepared a combinational formulation of CUR and RAPA loaded in MePEG/PCL diblock copolymers and explored its physico-chemical properties [9].

MATERIALS AND METHODS

Materials

CUR-500, containing Curcumin (> 95 %) was purchased from UNICO Pharmaceuticals (Ludhiana, India). Rapamycin (97 % trans and 3 % cis enantiomer) was purchased from Fujian Kerui Pharmaceutical Co. Ltd., China. Methoxy poly (ethylene glycol) (MePEG, M_n= 5000 by supplier, M_n= 5541 by our GPC measurements), Caprolactone (ε-CL)

(M_w=114.14) was supplied by Fluka (Sigma-Aldrich, Saint Louis, MO) and used after proper purification. All other reagents were of analytical grades and used without further purification. Distilled-deionized water was prepared with Milli-Q plus System (Elix 10, Millipore corp. India).

Synthesis of MePEG/ ε-CL diblock copolymers

MePEG/PCL diblock copolymers with molar ratios (60:40) were synthesized as described in our previous paper [10]. In brief, the MePEG was activated by azeotropic distillation with toluene. MePEG/PCL diblock copolymers were synthesized by ring opening polymerization of PCL using MePEG homopolymer as micro initiator and $Sn(Oct)_2$ as a catalyst. The predetermined ratios of both the polymer were mixed in a round bottom flask, degassed and kept at 160 °C. When the polymerization was completed, the reaction product was cooled at an ambient temperature, and then dissolved in dichloromethane to remove MePEG homopolymers and any residual caprolactone monomers. The precipitate was collected by filtration and washed several time with diethyl ether and the resultant product was dried in a vacuum oven at 40 °C for 3 days. The final diblock copolymer synthesized by the above protocol was characterized by NMR and GPC and found to same as reported in our earlier publication [10].

Encapsulation of CUR and RAPA in MePEG/PCL micelles

Diblock copolymeric micelles containing either single drug (CUR or RAPA) or dual drugs (CUR+RAPA) were prepared by a modified dialysis method [11]. Briefly, 100 mg of MePEG/PCL diblock copolymers were dissolved in 10 ml of dimethylformamide, to that 10 mg of either CUR or RAPA for single drug formulation and a mixture of 5 mg of CUR and 5 mg of RAPA was added for dual drug loaded formulation and stirred at room temperature. The solution mixture was dialyzed for 24 h against 3 liters of ultrapure water using cellulose dialysis membranes (molecular weight cutoff: 12 kDa, average diameter 21 mm and average flat width 35 mm, as supplied, Sigma). The water was changed three-times during the dialysis process. The micellar solution from dialysis bag was collected and sonicated by using sonicator (VC 505, Vibracell Sonics, Newton) of energy output set at 55 W for 1 min in an ice bath. The obtained micellar solution was lyophilized by freeze drying method (-80 °C and <10 mm mercury pressure, Freezone 6lt, Labconco Corp., MO) to get lyophilized powder for further use.

Particle size analysis & zeta potential measurement

Hydrodynamic mean particle size distribution and surface potential of the drug loaded micelles were determined by Photon Correlation Spectroscopy and Laser Doppler Anemometry, respectively, using Nano ZS (Malvern Instruments, Worcestershire, UK). Size measurements were performed by taking the drug loaded micelles (~1 mg/ml) in Milli-Q water at 25 °C and sonicated for 30 s in an ice bath (VC 505, Vibracell Sonics, Newton, USA) and further diluted with MilliQ water (100 µl diluted to 1 ml). The same suspension was used for measuring the ζ potential of drug loaded micelles by using the same equipment.

Quantification of entrapment efficiency by RP-HPLC method

The entrapment efficiency of different drugs in the micellar formulation (either single or dual) was determined by Reverse Phase High Performance Liquid Chromatography (RP-HPLC) method [10]. Briefly, 10 mg of the lyophilized drug loaded micelles were dissolved in 5 ml of acetonitrile, sonicated for 2 min in an ice bath (VC 505, Vibracell Sonics, Newton, USA) centrifuged at 13,800 rpm for 10 min at 25 °C (Sigma 1-15K, Osterode, Germany) to get a clear supernatant, which was then analyzed by using a isocratic mode of RP-HPLC system of Agilent-1100 (Agilent Tech. Waldbronn Analytical Division, Germany) with C18 column (Zorbax Eclipse XDB-C18, 150× 4.6 mm ID) having internal standard of dimethylphthalate. Using the mobile phase of acetonitrile and water in 80:20, v/v ratio, both the drugs were quantified with their respective wavelengths. For this, 20 µl of the sample was injected manually in the injection port and analyzed at a flow rate of 1 ml/min using a quaternary pump (Model-G1311A) at 25 °C with Thermostat (Model-G1316A). The estimation of CUR level was quantified at 420 nm whereas RAPA was quantified at 278 nm with absorbance detector (DAD, Model-G 1315A). The amounts of CUR and RAPA in drug loaded micelles were determined from the obtained area under curve. The standard curve of the single drug and in combination was prepared separately under identical conditions. The samples (in triplicate) were analyzed and the drug encapsulation efficiency (EE) was calculated [11].

Scanning electron microscopy (SEM)

The surface morphology of the (CUR+RAPA) loaded micelles were characterized by SEM (ZEISS EVO18, Carl Zeiss SMT GmbH, Germany) operating at an accelerating voltage of 10-30 kV. The micelles were gold coated and placed on a copper stub prior to the acquisition of SEM images.

Atomic force microscopy (AFM)

The morphology of the (CUR+RAPA) loaded micelles were visualized by AFM (JPK nanowizard II, JPK instrument, Berlin, Germany), after over night air drying of the micellar solution on a clean mica surface, using a pyramidal cantilevers with silicon probes having force constants of 0.2 Nm^{-1}. The shape was observed and imaged using JPK data processing software in non contact mode with frequency of 312 kHz and scan speed of 2 Hz.

X-ray diffraction (XRD)

X-ray diffraction studies were carried out to know the crystallographic state of the native drugs and to confirm its physical state in the micellar formulation. The XRD patterns of the samples were obtained by using XRD diffractometry (D8 ADVANCE, BrukerAXS Inc, Madison, WI). A monochromator features high flux K-α-1 radiation at 40 kV and 100 mA was used. Diffractograms were performed with 2θ ranges from 0° to 50° with a step of 0.02°, at a scanning speed of 4°/min (2θ).

Differential scanning calorimetric (DSC)

The physical states of the dual drug loaded micelles were characterized using a differential scanning calorimetric (DSC) thermogram analyser (STA 6000 Simultaneous Thermal Analyser, Perkin Elmer, Waltham, MA). Briefly, 8 mg of each sample (native CUR, native RAPA and (CUR+RAPA) loaded micelles) were sealed separately in a standard aluminum pan, and purged with pure dry nitrogen gas set at a flow rate of 10 ml/min, the temperature variation was set at 10 °C/min, and the heat flow was recorded from 0 to 350 °C.

***In vitro* drug release kinetics study for drug loaded micelles**

In vitro release kinetics of either curcumin or rapamycin from the (CUR+RAPA) loaded micellar system was performed by dissolving 10 mg of the sample in 3 ml of PBS (0.1 M, pH 7.4, containing 0.1 % v/v of Tween 80) [11]. Tween 80 was used to maintain the sink condition [10]. The micellar suspension was mixed properly by vortexing, and equally divided in three tubes containing 1 ml each and kept in orbit shaker rotating at 150 rpm in 37 °C (Wadegati Lab equip, India). At pre-determined time intervals these tubes were taken out from shaker and centrifuged at 13,800 rpm, 4 °C for 10 min (SIGMA 1-15K, Germany), supernatants were collected, the leftover pellets were re-suspended with 1 ml of fresh PBS (0.1 M, pH 7.4) containing 0.1 % v/v tween 80 solutions and replaced in shaker for further samplings. The collected supernatants were lyophilized for 48 h and dissolved in 1 ml of acetonitrile, centrifuged at 13,800 rpm for 10 min at 10 °C, (Sigma 1-15K, Germany) to collect the drug in supernatant. The amount of drug released with respect to different time intervals was analysed by RP-HPLC system and all measurements were performed in triplicates [10].

Statistical analysis

Statistical analyses were performed using a Student's t test. The values of $p < 0.05$ (*) were indicative of significant difference and very significant difference if $p < 0.005$ (**).

RESULTS

The dual drugs CUR and RAPA were successfully loaded in the MePEG/PCL diblock copolymeric micelles by modified dialysis method [10]. The solubility of the drugs in the micelles was significantly increased as compared to their intrinsic solubility in water. The drug(s) to polymer weight percentage ratio was kept constant (10 %) in all formulations. For all micelle formulations approximately 100 % of the initial amount of the drug(s) and polymer was recovered as drug loaded MePEG/PCL diblock copolymeric micelles. The encapsulation efficiency of (CUR+RAPA) loaded micelles as determined by RP-HPLC was found to be ~ 64 % (CUR) and ~ 94 % (RAPA), which were not have much significant difference from the single drug loaded micelles. Similarly, loading capacities of these micelles were nearly equal to single drug loaded micelles, as given in **Tab. 1**.

Particle size analysis & zeta potential measurement

The particle size and ζ potential measurement of the micelles by dynamic laser light scattering technique accomplished that the micelles were having a diameter range ~ 128 to 176 nm along with low polydispersity index, implying a narrow size distribution (**Fig. 1a**). The hydrodynamic diameters of drug loaded micelles were slightly higher than that of void micelle due to incorporation of drugs either single or in combination (**Tab. 1**). PDIs of all formulations were below 0.2, indicating narrow particle size distribution. The loaded micelles illustrated negative zeta potential (~ -11 mV to -14 mV) in all the formulations (**Fig. 1b**) [10].

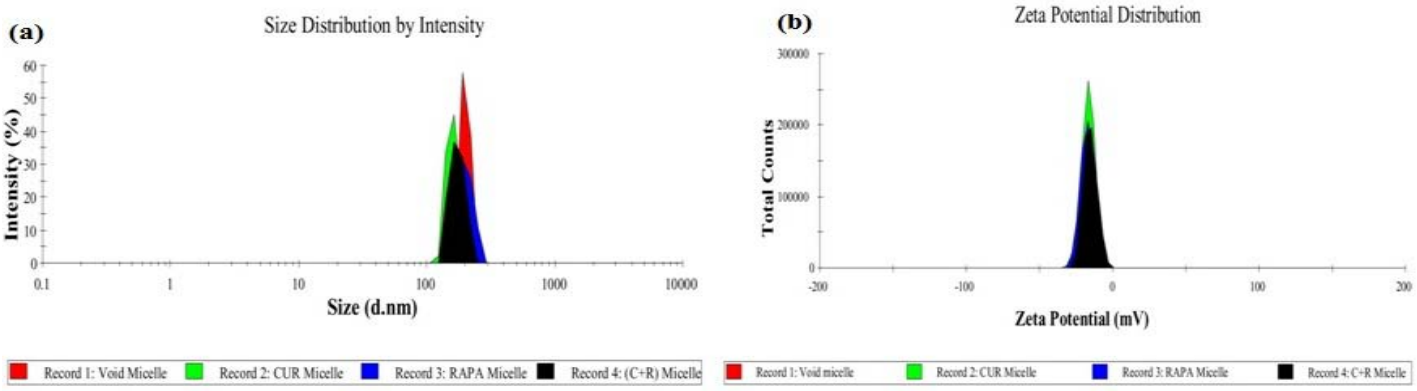

Fig. 1: (a) Mean particle size of Void micelles, CUR loaded micelles, RAPA loaded micelles and (CUR+RAPA) loaded micelles (data as mean ± S. E. M; n = 6), (b) Zeta potential of Void micelles, CUR loaded micelles, RAPA loaded micelles and (CUR+RAPA) loaded micelles (data as mean ± S. E. M; n = 6).

Table 1: Summary of physico-chemical characterization of MePEG/PCL block copolymeric micelles. (Data represents mean ± SD)

Sample	Particle size (nm)	Poly dispersity index	Zeta potential (mV)	Encapsulation Efficiency[a] (%)	Loading Capacity[b] (%)
Void Micelle	128 ± 2.6	0.13 ± 0.008	-11.50 ± 2.77	--	--
CUR Micelle	140 ± 2.56	0.28 ± 0.004	-14.8 ± 1.96	60.3 ± 1.73	14 ± 1.7
RAPA Micelle	145 ± 2.86	0.18 ± 0.004	-15.8 ± 1.26	98.3 ± 1.43	31 ± 1.5
(CUR+RAPA) Micelle	176 ± 3.06	0.19 ± 0.006	-14.0 ± 2.60	64 ± 2.6 (CUR) 94 ± 3.4 (RAPA)	12 ± 2.1 (CUR) 29 ± 1.8 (RAPA)

[a, b] Encapsulation efficiency and Loading capacity (expressed as %) were estimated by HPLC.

$$\text{EE}\left(\%\frac{\text{w}}{\text{w}}\right) = \frac{\text{Weight of the drug in micelle}}{\text{Weight of the drug added}} \times 100$$

$$\text{LC}\left(\%\frac{\text{w}}{\text{w}}\right) = \frac{\text{Weight of the drug in micelle}}{\text{Weight of the polymer and drug added}} \times 100$$

Surface morphology studies

The surface morphology of the (CUR+RAPA) loaded micelle was determined using scanning electron microscopy [17]. The SEM image of nanoparticles revealed spherical shape with smooth surface as shown in **Fig. 2a**. The atomic force microscopy (AFM) investigations showed spherical structure of the particles with slight aggregation due to freeze drying of the product as shown in **Fig. 2b**. The microscopic studies also reveal that the micelles were surrounded by polymeric soft layer [17].

X-ray diffraction (XRD)

XRD analysis was conducted to confirm the physical state of CUR and RAPA in (CUR+RAPA) loaded micelles, which suggested that the CUR and RAPA were in amorphous form or in the solid state solubilized form in the micellar formulation as shown in **Fig. 3a** [18].

Differential scanning calorimetric (DSC)

The DSC analysis was performed for native CUR, native RAPA, and (CUR+RAPA) loaded micelles to found out the physical state of the drug inside the micellar formulation as illustrated in **Fig. 3b**.

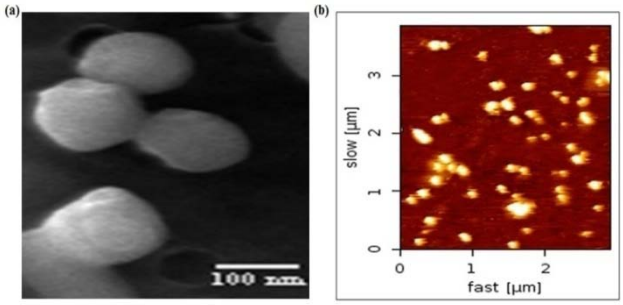

Fig. 2: (a) Scanning electron micrograph of (CUR+RAPA) loaded micelles (bar = 100 nm), (b) Atomic Force microscopy (AFM) images of (CUR+RAPA) loaded micelles.

It was observed that the native CUR and native RAPA had an endothermic peak of melting point at ~176 °C and ~190 °C respectively [19]. These characteristic peaks were absent in the (CUR+RAPA) loaded micelles. The absence of the detectable crystalline endothermic peak of the CUR and RAPA in the micellar formulation clearly indicated that CUR and RAPA encapsulated in the micelles were in the form of amorphous or in solid-state solubilized form in the polymeric matrix [10].

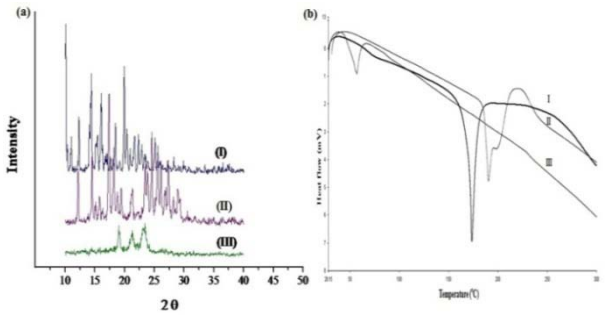

Fig. 3: (a) XRD analysis of (I) Native RAPA, (II) Native CUR, (III) (CUR+RAPA) loaded micelles, (b) DSC thermogram of (I) Native CUR, (II) Native RAPA, (III) (CUR+RAPA) loaded micelles.

In vitro drug release kinetics study

The release kinetics study was carried out to estimate the amount of drug released from the micellar system under *in vitro* conditions. The *in vitro* release profile of CUR and RAPA from (CUR+RAPA) loaded polymeric micelles showed a biphasic release pattern as shown in **Fig. 4**. The observed initial rapid release of drugs might be due to diffusion of drugs present at the surface of the micelles and the encapsulated drugs inside the core of the micelles showed the sustained release [20]. The result was correlated with the CUR and RAPA release from the CUR loaded micelles and RAPA loaded micelles respectively.

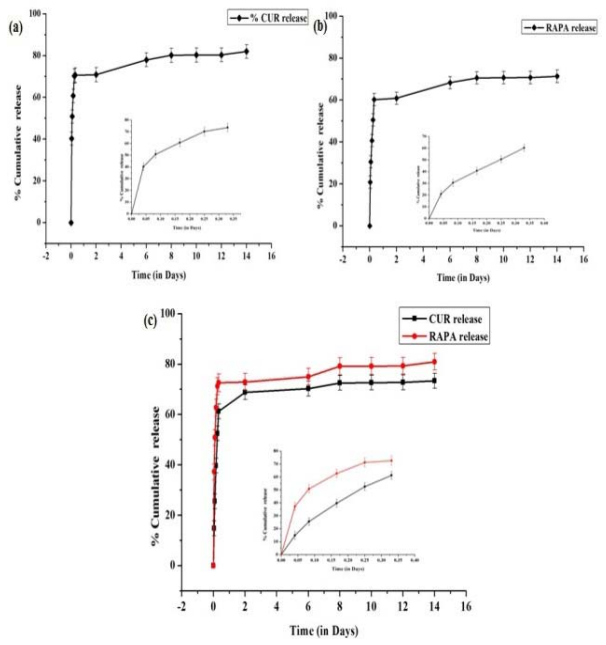

Fig. 4: *In vitro* release kinetics of (a) CUR from the CUR loaded micelle, (b) RAPA from RAPA loaded micelle, (c) CUR and RAPA from (CUR+RAPA) loaded micelles. The inserts shows the percentage cumulative drug release from 1hr to 8 hrs. Data as mean ± S. E. M (n = 3).

DISCUSSION

GBM is the most common primary malignant brain tumor, having a low survival rate despite of available treatments such as surgery, radiotherapy and non-invasive chemotherapy. Utilization of nanotechnology based drug delivery system are exceptionally helpful for the treatment of brain tumors as constant dose of anticancer drugs can be delivered directly to cancer tissues [21]. Moreover, cancer therapy relying on single therapeutic agent remains suboptimal. Therefore, combination of two or more therapeutic agents with different working mechanism can cooperatively prohibit cancer development, has become one of the promising approach for effective cancer treatment [22, 23]. These combinations of drugs promote synergism than that of single drug against cancer cells and it suppresses drug resistance and increases the therapeutic index through distinct mechanisms of action [24].

In the current study an attempt was taken to explore the micellar system to deliver simultaneously two anticancer drugs and characterized their physico-chemical properties. Here, two potent anticancer drugs, CUR and RAPA were encapsulated successfully in MePEG/PCL diblock copolymeric micellar system. The resulting (CUR+RAPA) loaded micelles showed high drug encapsulation efficiency, uniform smooth particle topology, negative zeta potential and sustained release of ~ 85 % of the drug in two weeks from the micellar system, which is an indispensable requirement for cancer therapy [25]. The small size of the (CUR+RAPA) loaded micelles helps in the passive targeting to tumor tissue by enhanced permeability and retention (EPR) effect [26]. The amorphous state of the entrapped drug inside the micellar formulation as illustrated from XRD and DSC analysis further attributed to the sustained release activity of both the drugs. However, the *in vitro* drug release kinetics showed that both CUR and RAPA were released from (CUR+RAPA) loaded micelles simultaneously, without affecting each other's release profile. These properties of the drug loaded micelles would enhance the bioavailability and circulation half-life upon *in vivo* administration [27].

CONCLUSION

The present study demonstrated that encapsulation of the dual drugs CUR and RAPA within the MePEG/PCL diblock copolymeric micellar system can increase the therapeutic efficacy of the drug. The harsh excipients related severe side effects and the stability concerns related to administering combination chemotherapy can be circumvented. MePEG/PCL diblock copolymeric micellar system offers a novel alternative to the current commercial formulations. The formulation was able to improve the physicochemical characteristics of both the hydrophobic anticancer drugs and it shows slow release of both curcumin and rapamycin simultaneously. These physical characteristics and release behavior shows that the MePEG/PCL diblock copolymeric micelles were compatible for the encapsulation of both the drugs and the formulation may be used for further *in vitro* and *in vivo* studies.

CONFLICT OF INTERESTS

Declared None

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