

Enhanced antiproliferative and apoptosis effect of paclitaxel-loaded polymeric micelles against non-small cell lung cancers

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Abstract Amphiphilic copolymer monomethoxy poly(ethylene glycol)-poly(caprolactone)-D- α -tocopheryl polyethylene glycol 1000 succinate (MPEG-PCL-TPGS) was prepared. In the present study, MPEG-PCL-TPGS was used as a novel nanovehicle for the delivery of paclitaxel (PTX) in the treatment of resistant lung cancers. The PTX-loaded MPEG-PCL-TPGS (PTX/MPT) micelles exhibited sustained release profile (168 h) with accelerated drug release at acidic pH conditions. The blank polymeric micelles showed excellent biocompatibility with cell viability of >85 %, making it suitable for all in vivo applications. PTX/MPT micelles displayed superior cytotoxicity in A-549 lung cancer cells than that of free PTX. The selective delivery of PTX to cancer cells resulted in enhanced cancer cell death. The PTX/MPT micelles showed higher cellular uptake via endocytosis pathways. The PTX-bound micelles preferentially arrested the cells at G2/M phase and showed a marked increase in sub G1 cell population (~20 %). The pharmacokinetic study revealed a long blood circulation for PTX/MPT micelles. Finally, micellar formulation showed a remarkable tumor suppression effect in resistant A549/Taxol cells bearing xenograft nude mice along with no toxicity profile. The results indicate that the PTX-loaded biocompatible polymeric nanosystem could act as a potential delivery system for the treatment of lung carcinomas.

Keywords Lung cancer · Paclitaxel · Drug delivery · Drug resistance · Polymeric micelles

Introduction

Lung cancer is one of the leading causes of cancer-related death with high rate of morbidity and mortality worldwide [1]. The incidence of lung cancer constantly increases with each year, especially in developing countries like China. Nearly 80 % of lung cancer death was due to the metastatic process with an average survival rate of less than 20 % [2, 3]. Lung cancers can be broadly divided into non-small-cell lung cancer (NSCLC) and small-cell lung cancer (SCLC) [4]. Of two types, NSCLC is responsible for maximum lung cancer deaths. Present treatment options include surgical resection, radiation therapy, and chemotherapy [5]. Specifically, chemotherapy is widely practiced in clinics for the treatment of various cancers including lung cancer. However, conventional chemotherapeutic drugs diffuse into normal cells in an aggressive and nonspecific manner resulting in unwanted organ-related toxicity [6, 7].

Paclitaxel (PTX) is one of the important first-line chemotherapeutic agents used against wide range of malignancies [8]. It has proven efficacy against multiple cancers including breast, ovarian, prostate, and non-small cell and small cell lung cancer [9]. However, clinical application of PTX is limited by its poor aqueous solubility and nonspecific pharmacokinetics in systemic circulation. To improve the physicochemical property of PTX, a commercial formulation Taxol[®] was marketed. However, Cremophor-EL, an adjuvant used in Taxol, has been found to have serious side effects including hypersensitivity reactions, neurotoxicity, cumulative fluid retention, cardiotoxicity, and nephrotoxicity [10, 11].

In this regard, nanoscale carriers employing artificial or natural polymers are gaining importance in cancer research and treatment [12]. Such nanosized delivery system consisted

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of artificial or natural polymers are expected to increase the therapeutic efficacy of anticancer drugs while reducing its side effects [13]. The nanocarriers offers numerous benefits over conventional chemotherapy treatment such as high drug stability, protection of drug in systemic circulation, high drug-loading capacity, sustained drug release, avoidance of renal and reticuloendothelial systems (RES)-based clearance, and minimized drug loss during blood circulation [14]. Importantly, it will accumulate in the tumor in a preferential manner by virtue of enhanced permeability and retention (EPR) effect and facilitate the drug release within the tumor cells [15].

Biodegradable polymers constitute important part of novel nanocarriers system. In the present study, an amphiphilic copolymer monomethoxy poly(ethylene glycol)-poly(caprolactone) (MPEG-PCL) was used as carrier for targeting antitumor drugs [16]. MPEG-PCL was chemically conjugated with D- α -tocopheryl polyethylene glycol 1000 succinate (TPGS). The so-formed MPEG-PCL-TPGS is expected to offer ideal antitumor drug delivery. The hydrophobic drugs like PTX can be easily encapsulated in the PCL core, whereas PEG shell could effectively prevent the nanocarriers from adsorption of protein and recognition by the RES and could result in prolonged blood circulation. TPGS, a water-soluble derivative of the natural form of D- α -tocopherol, was employed to overcome multidrug resistance (MDR) and to increase the anticancer properties by inhibiting the p-glycoprotein efflux receptor (p-gp) via P-gp ATPase inhibition [17]. Conjugation of an effective p-gp inhibitor will induce a synergistic cell apoptosis in sensitive and resistance cancer cells [18]. Recently, Li et al. [19] reported a p-gp inhibitory property of TPGS and successfully delivered doxorubicin (DOX) cancer cells. Tsai et al. [20] conjugated TPGS to block polymer and showed enhanced cytotoxicity in MCF-7 cancer cells.

In the present study, we aimed to increase the chemotherapeutic efficacy of PTX by encapsulating in a novel monomethoxy poly(ethylene glycol)-poly(caprolactone)-D- α -tocopheryl polyethylene glycol 1000 succinate (PTX/MPT micelles)-based polymeric micelles. Amphiphilic MPEG-PCL-TPGS block copolymer is expected to form micelles by undergoing spontaneous self-assembly process. The three monomeric units of the copolymer (PCL, TPGS, and PEG) are expected to perform specified functions as mentioned above. Physical characterizations and release pattern of drug-loaded micelles were evaluated. Taxol-resistant subclones of the human lung adenocarcinoma cell line A549 were selected to evaluate the anticancer effect of PTX/MPT micelles. Cellular uptake, cell apoptosis, cell cytotoxicity, and cell cycle analysis were carried out to confirm the performance of PTX/MPT micelles. Antitumor efficacy study was performed in A-549 cancer cell bearing xenograft mice model.

Materials and methods

Materials

Poly(ethylene glycol) methyl ether (MPEG, Mn=550 and 2,000, Aldrich, USA), ϵ -caprolactone, stannous octoate (Sn[Oct]₂), and 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (methyl thiazolyl tetrazolium, MTT) were purchased from Sigma-Aldrich (China). D- α -tocopheryl polyethylene glycol 1000 succinate (TPGS) was procured from Shanghai Yare Biotech (Shanghai, China). PTX was purchased from Meilian Pharm (Chongqing, China). All other chemicals were reagent grade and used without further modifications.

Preparation of MPEG-PCL-*b*-TPGS block amphiphilic copolymer

MPEG-PCL-*b*-TPGS was synthesized in the presence of Sn(Oct)₂ using a ring-opening polymerization method. In brief, weighed amounts of MPEG-PCL polymer and TPGS_{2k} and Sn(Oct)₂ were added in a glass tube which is, in turn, connected to a vacuum system. The tube was connected with nitrogen process and repeated three times. The tube containing mixture was sealed and heated at 150 °C in oil bath for 12 h. The reaction tube was cooled for 2 h, and the product formed was dissolved in dichloromethane and precipitated in excess cold methanol. The purified products were collected by filtration and vacuum-dried at 45 °C for 48 h. The percentage yield was calculated to be 94.5 %.

Preparation of PTX-loaded MPEG-PCL-*b*-TPGS micelles

PTX-loaded MPEG-PCL-*b*-TPGS (PTX/MPT) micelles were prepared by a solvent extraction and evaporation method. Briefly, a measured amount of PTX along with 100 mg of MPEG-PCL-*b*-TPGS copolymer was dissolved in 10 ml of dichloromethane. This organic solution was poured in an aqueous polyvinyl alcohol solution under constant stirring. The mixture was immediately sonicated for 5 min to form an oil/water emulsion system. The emulsion was stirred overnight to evaporate the organic solvent and was followed by treatment under reduced pressure. The resultant polymeric micelles suspension was centrifuged at 18,000 rpm for 15 min and removed the untrapped drug and polyvinyl alcohol. The nanoparticles (NPs) were freeze-dried and stored until further use.

Particle size and zeta potential analysis

The particle size and zeta potential were analyzed using dynamic light-scattering (DLS) technique. Zetasizer (Nano-ZS 90, Malvern, Worcestershire, UK), which works on the DLS

principle, was used to measure size and charge. The samples were measured at 25 °C at a fixed angle of 90 °C. Each sample was measured in triplicate.

Morphology analysis

The morphology of nanoparticles was analyzed using transmission electron microscope (TEM; JEM-2010, JEOL, Japan). For this, a drop of nanoparticle dispersion was placed on the carbon-coated copper grid and negatively stained with 0.1 % (w/v) phosphotungstic acids.

Drug-loading and encapsulation efficiency

The drug-loading efficiency and entrapment efficiency of PTX in nanoparticles were calculated by high-performance liquid chromatography (HPLC) technique (LC 1200; Agilent Technologies, Santa Clara, CA, USA). The freeze-dried nanoparticles were dissolved in 1 ml of dichloromethane and sonicated vigorously for 10 min. This solution was centrifuged (20,000 rpm), and supernatant was collected and injected into HPLC column. A reverse-phase C18 column (250×4.6 mm; GL Science, Tokyo, Japan) was used. The mobile phase was run at 1 ml/min and detected at 227 nm. The drug encapsulation efficiency was calculated as the ratio between the amount of PTX encapsulated in the NPs and that feeding into the process.

$$DL\% = \frac{\text{Drug}}{\text{Polymer} + \text{Drug}} \times 100\%$$

$$EE\% = \frac{\text{Actual drug loading}}{\text{Theoretical drug loading}} \times 100\%$$

In vitro release study

In vitro release study of PTX from MPEG-PCL-*b*-TPGS micelles was carried out by dialysis method. Briefly, 1 ml of PTX/MPT micelles (containing 500 µg) solution was placed in a dialysis tube (molecular mass cutoff was 8.0 to 10 kDa) and incubated in a 25 ml of phosphate-buffered saline (PBS; pH 7.4) and acetate-buffered saline (pH 5.0) at 37 °C. The dialysis medium was prepared with 1 % Tween 80. The dialysis tube was maintained at 37 °C with 100 rpm. At specific time period, 1 ml of release media was removed and replaced with fresh media. The release media was determined using HPLC technique.

Cellular uptake analysis

The cellular uptake of free PTX and PTX/MPT was evaluated in A-549 cells. The cells were seeded in a 12-well plate at a

density of 1×10^5 cells/well and allowed to attach for 18 h. The free PTX and PTX/MPT were incubated to cell at a concentration of 20 µg/ml for designated time period. The cells were washed thrice with cold PBS and lysed with the help of lysis buffer. The drug was extracted with centrifugation and estimated using HPLC as mentioned before. The cellular uptake in cells was further visualized using confocal scanning light microscopy. The cells were incubated with red dye-loaded nanoparticles along with Hoechst 33342 as nuclear staining. After washing with PBS, cells were fixed with 4 % paraformaldehyde and visualized using laser confocal scanning microscopy (Olympus, Japan).

In vitro cytotoxicity assay

The cytotoxicity of blank polymer, free PTX, and PTX/MPT was analyzed using MTT assay. For this, A-549 cells were plated at a seeding density of 1×10^4 in a 96-well plate. The growth medium was prepared in RPMI-1640 containing 10 % FBS and 1 % penicillin streptomycin mixture. After 24 h, cells were exposed to blank polymer, free PTX, and PTX/MPT at different dosing levels. The cells were incubated for 24, 48, and 72 h accordingly. At each time point, plate was removed and treated with 100 µl of MTT solution (5 mg/ml) to each 96-well plate and incubated for 4 h. The formed formazan crystals were extracted by adding dimethyl sulfoxide and incubated for additional 30 min. The absorbance of each plate was read at 570 nm using a microplate reader (Thermo-Fisher, USA). All experiments were repeated four times.

Western blot analysis

Whole-cell extracts were prepared using radioimmunoprecipitation assay (RIPA) buffer containing protease and phosphatase inhibitors and were clarified by a low-speed centrifugation. The protein extracts were quantitated by BCA assay, were resolved by SDS-PAGE on 4–12 % Bis-Tris gels, and were transferred to polyvinylidene difluoride (PVDF) membranes. Primary antibodies against cleaved caspase (1:1,000 dilutions) were purchased from Cell Signaling Technology (Beverly, MA, USA). β-actin antibody (1:1,000 dilution; Sigma-Aldrich) was used for detecting β-actin expression for ensuring equal protein loading that served as internal loading control. Membranes were blotted with 1:1,000 dilution of primary antibody, were detected using horseradish peroxidase-conjugated secondary antibodies, and were visualized by enhanced chemiluminescence per protocol (Pierce).

Cell apoptosis assay

Apoptosis assay was carried out using Annexin V and propidium iodide (PI) kit (China KeyGEN Biosciences, China) as per manufacturer's instruction. Briefly, A-549 cells

were seeded in a 12-well plate and allowed to attach for 24 h. The cells were treated with free PTX and PTX/MPT (1 µg/ml) for 24 h. After which, cells were washed twice with PBS and trypsinized. The trypsinized cells were centrifuged, and pellets were resuspended in a binding buffer. 1 µl of PI and Annexin V-FITC was added to cells and incubated for 15 min. The cell apoptosis was analyzed using a FACScan cytometer equipped with Cell Quest software (BD Biosciences, USA).

Cell cycle analysis

Cell cycle analysis was carried out using flow cytometer. Briefly, A-549 cells were seeded in a 12-well plate and allowed to attach for 24 h. The cells were treated with free PTX and PTX/MPT (1 µg/ml) for 24 h. The cells were washed twice with PBS, were trypsinized and fixed in 70 % ethanol solution, and were stored at 4 °C for 1 h. The cells were centrifuged at 1,200 rpm for 4 min and were then incubated with 0.2 mg/ml of RNAase at 37 °C. The cells were then stained with PI and analyzed using a flow cytometer as mentioned previously. The proportion of cells in each phase or stage of the cell cycle was determined using Cell Quest software.

In vivo pharmacokinetic study

Animal studies were approved by Institutional Animal Care Committee, Xiangya Hospital Central South University, China. Eight male Sprague–Dawley rats (220±15 g) were equally divided into two groups and were used to investigate the pharmacokinetic properties. Each group was administered with free PTX and PTX/MPT (10 mg/kg) via tail vein injection. Femoral artery was cannulated to collect the blood samples. The samples were collected at 0.25, 0.5, 1, 2, 4, 8, 12, and 24 h postintravenous administration of samples. Blood was added with heparin and was immediately centrifuged at 4,000 rpm for 10 min. The plasma was stored at –80 °C until further analysis. Liquid–liquid extraction process was performed to analyze the plasma concentration. In brief, 100 µl of plasma was mixed with 50 µl of internal standard solution (docetaxel) and vortexed. Additional 350 µl of acetonitrile was added and vortexed for 1 h. The mixture was centrifuged at 13,000 rpm for 10 min; supernatant was separated, and 20 µl of this was injected into HPLC system. The mobile phase was PBS:acetonitrile (55:45, v/v) at pH 3.0.

In vivo antitumor efficacy study

The antitumor efficacy of free PTX and PTX/MPT micelles were investigated in A-549 cell bearing BALB/c xenograft nude mice. The mice were subcutaneously injected with A-549 cells (1×10^6) on the right flank of nude mice. The antitumor studies were started when the tumor volume

reached 100 mm³. Mice were equally divided into three groups with 8 mice in each group. Group I received free PTX and Group II received PTX/MPT micelles at a dose of 10 mg/kg, respectively. Group III received saline solution and served as control. All formulations were administered via tail vein injection three times during the first 2 weeks (every 3 days) of study period. The study was carried out for 20 days. At specific time period, tumor volume and body weight of the mice were noted. Tumor volume was determined using Vernier caliper. Tumor volume was calculated by the equation $V=(L \times W^2)/2$, where L represents the longest diameter, and W represents the shortest diameter perpendicular to length. At the end of the experiment, the animals were sacrificed, and the tumor masses were harvested and weighed.

Statistical analysis

Differences between groups were examined for statistical significance with the Student's t test, and P values <0.05 were considered statistically significant. The experiments were carried out in triplicates, and values were expressed as mean± standard deviation (SD).

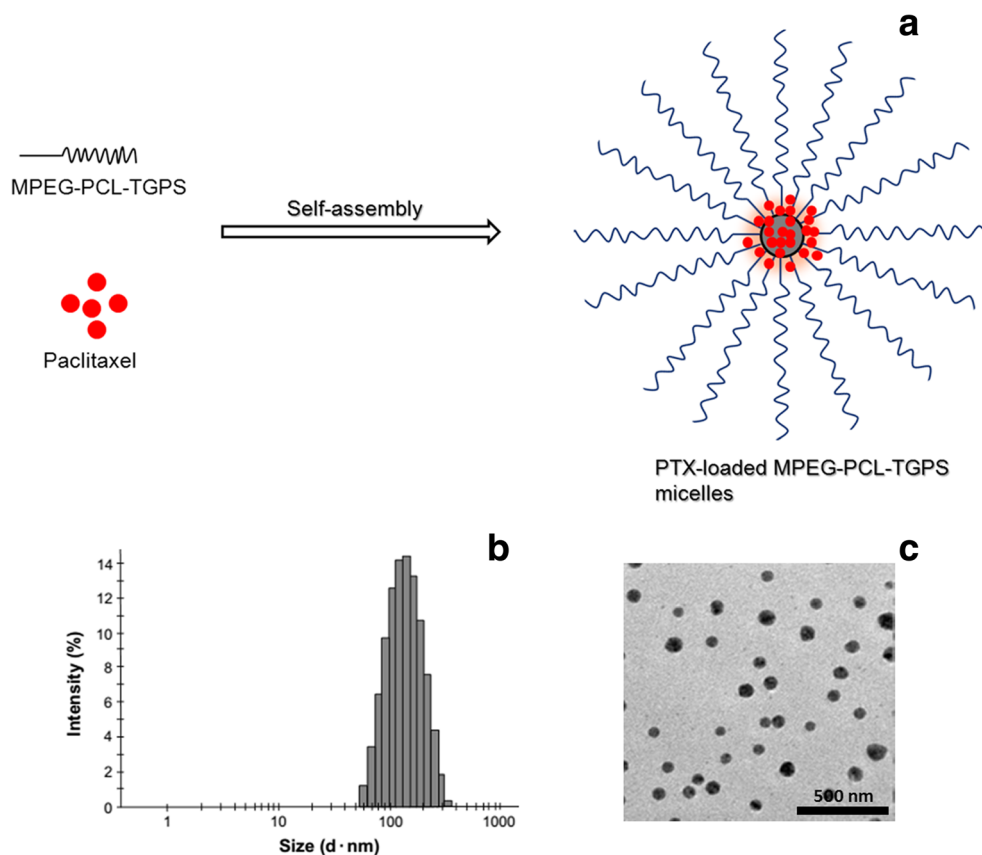
Results and discussion

Lung cancer is one of the leading causes of cancer-related death with high rate of morbidity and mortality worldwide. Non-small cell lung cancer accounts for nearly 85 % of all lung cancers with poor survival rate. In this regard, chemotherapy became main treatment option for the treatment of lung cancers along with surgical resection of tumor tissue. Nevertheless, conventional chemotherapeutic methods fail to achieve therapeutic concentrations in the tumor tissue and far from satisfactory [2, 3]. Besides, conventional treatment results in serious organ-related toxicity. Paclitaxel (PTX) is one of the most important first-line chemotherapeutic agents against a wide range of malignancies. It has proven efficacy against multiple cancers including breast, ovarian, prostate, and non-small cell and small cell lung cancer. However, clinical application of PTX is limited by poor aqueous solubility and nonspecific pharmacokinetics in systemic circulation. In this study, therefore, we have designed a novel amphiphilic MPEG-PCL-TPGS block copolymer-based polymeric micelles (Fig. 1a). The “stealth” PEG coating provides a steric barrier to the nanoparticles that protects them from opsonization and clearance by RES.

Preparation of PTX-loaded micelles

The particle size of PTX/MPT micelles was around 110 nm with a uniform size distribution (polydispersity index, PDI~0.150; Fig. 1b). It has been reported that particle size of around

Fig. 1 **a** Schematic illustration of preparation of paclitaxel-loaded polymeric micelles (PTX/MPT), **b** size distribution of PTX/MPT, and **c** TEM image of PTX/MPT. The size distribution of polymeric micelles was determined by dynamic light-scattering technique and morphological examination as carried out using transmission electron microscope



200 nm is ideal for tumor targeting. The smallest particle size with lowest PDI might be attributed to the excellent self-assembly process of MPEG-PCL-TPGS copolymer. The small size of particles is expected to reduce the chances of RES uptake and to prolong the systemic circulation that could facilitate extravasation from leaky capillaries. Furthermore, the zeta potential of nanoparticle is of utmost importance from a stability perspective as well as from cellular interaction point of view. In the present study, PTX/MPT micelles showed about 26 mV potential, which is in the excellent range of dispersion stability and in vivo circulation.

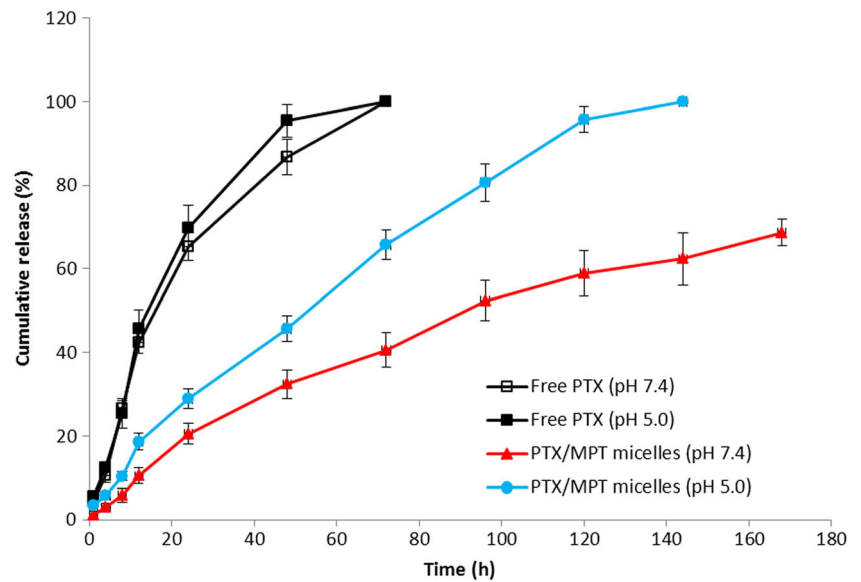
Morphological analysis

The surface morphology and particle size was, in turn, confirmed by transmission emission microscopy (TEM). The TEM showed a spherical-shaped particle of PTX/MPT micelles, which were evenly distributed in the carbon-coated copper grid (Fig. 1c). The TEM size was smaller than that of DLS size because TEM measurements are done in dry state while DLS in aqueous state. The amphiphilic block polymers form a loose structure in water, while they form rigid and solid particles in dry state.

In vitro release study

The release profile of PTX from drug-loaded nanoparticles was studied in phosphate-buffered saline (pH 7.4) and acetate-buffered saline (pH 5.0) at 37 °C. As seen in Fig. 2, free drug is readily dispersed in the release media and could freely diffuse through the dialysis membrane with more than 80 % of drug released within 24 h of study period. As expected, encapsulation of PTX in polymeric nanoparticles significantly prolonged the release of the drug in both pH conditions. Specifically, MPEG-PCL-TPGS-based nanoparticles exhibited a pH-sensitive drug release. Nearly 50 % of drug released in pH 5 medium versus only approximately 36 % in pH 7.4 medium were seen within 48 h of study period. The trend continued up to the end of the release study. Almost 100 % of drug released from micelles in pH 5 medium, while only 60 % of drug released in pH 7.4 medium were seen after 140 h study period. Generally, PTX is encapsulated in the hydrophobic core of the PCL that effectively controlled the release. Overall, nanoparticles showed a sustained release of drug, indicating their potential applicability as a drug delivery system that minimizes the exposure of healthy tissues and increases the accumulation of chemotherapeutic drugs in the tumor region.

Fig. 2 In vitro drug release behavior of PTX suspension and PTX/MPT in phosphate-buffered saline (pH 7.4) and acetate-buffered saline (pH 5.0) at 37 °C. The respective release media was prepared with 0.5 % Tween 80. Data are expressed as the mean \pm SD ($n=3$)



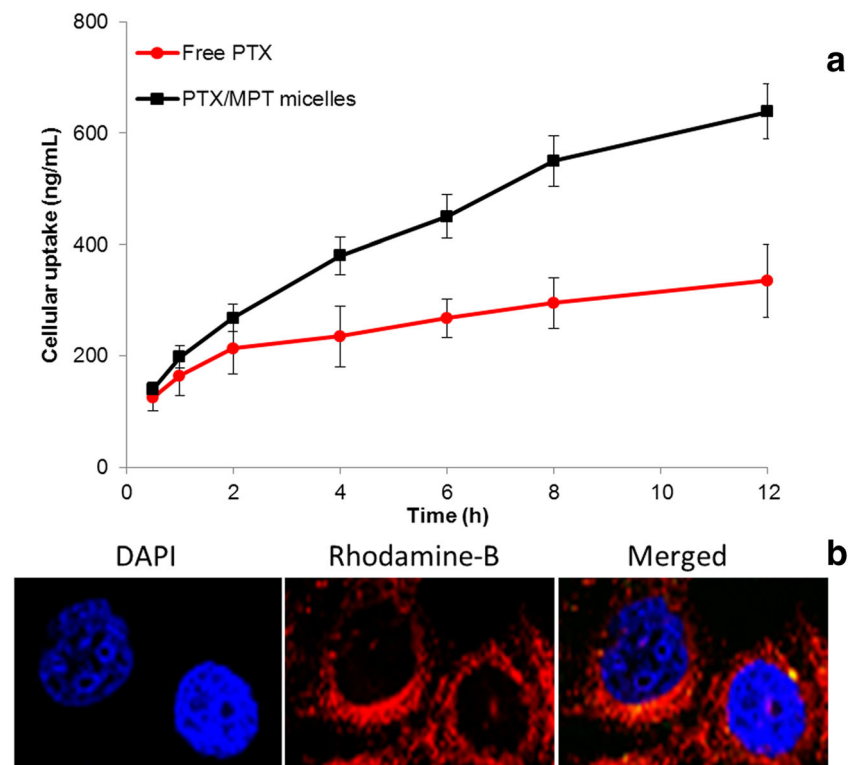
Cellular uptake analysis

The cellular uptake of free PTX and PTX/MPT micelles was studied in A-549 cells in a time-dependent manner. The cells were incubated with respective formulations, and uptake was determined using HPLC technique. As seen, both formulations showed definite uptake at 30 min and kept increasing with time. The uptake pattern of two formulations was significantly different (Fig. 3a). PTX/MPT micelles showed remarkable uptake with twofold higher uptake than free PTX.

It should be noted that micelles were internalized rapidly during first 4 h of the study period, while after that, it showed slower uptake up to 12 h. It could be expected that free drug diffuse freely into the cancer cell membrane, while micelles were internalized in a specific manner (might be endocytosis uptake). Moreover, PTX from micelles might be released in a controlled manner without being expelled out of the cells that contributed to its high cellular uptake.

We then evaluated the mechanism of cellular uptake and intracellular distribution of Rhodamine-B-loaded micelles

Fig. 3 a In vitro cellular uptake of free PTX and PTX/MPT micelles in A549 cancer cells. The cells were incubated with formulations for various time points, and cellular uptake was analyzed using HPLC technique. **b** Qualitative cellular uptake analysis by confocal microscopy. DAPI was used to stain the nucleus, and rhodamine B was used to analyze the cellular uptake



(Fig. 3b). The nuclei were stained with Hoechst 33382 to contrast between cytoplasm and nucleus core. It can be clearly seen that micelles were internalized via endocytosis process with dark red fluorescence in the cytoplasm region (endosome and lysosome regions). The drug from the nanoparticle is expected to be released in the intracellular compartments and to travel to nucleus and surrounding organelles (microtubule). This could further support that the MPT micelles can be an effective drug delivery system.

In vitro cytotoxicity assay

The in vitro cytotoxicity of MPEG-PCL-TPGS-based micelles was studied in A-549 adenocarcinoma cells. The cytotoxic potential of MPEG-PCL-TPGS was tested up to 500 $\mu\text{g/ml}$ concentration range. The results showed that copolymer was complete safe up to the tested concentration (Fig. 4a). At maximum concentration of the polymer, the cell viability was >85 %, indicating its excellent biocompatibility and low toxicity, making it suitable for all in vivo applications.

In vitro anticancer activity of free PTX and PTX/MPT micelles was also studied in A-549 cells. The cells were incubated with the formulations for 24, 48, and 72 h in order to observe the time-dependent cytotoxicity. As shown in Fig. 4b–c, both free PTX and PTX/MPT showed a time-dependent and concentration-dependent cytotoxicity. The

results further showed a superior antitumor effect of PTX/MPT micelles compared to that of free PTX in the same concentrations. IC₅₀ value was calculated to quantitate the difference between these two formulations. The IC₅₀ value of PTX/MPT micelles was determined to be 1.25, 0.68, and 0.26 $\mu\text{g/ml}$ after 24, 48, and 72 h incubation, respectively. This was significantly smaller than free PTX (5.84, 1.35, and 0.85 $\mu\text{g/ml}$) for the same incubation time. The higher antitumor effect of PTX/MPT micelles was mainly attributed to its higher cellular uptake and sustained release of drug within the cell membrane. Secondly, NSCLC cells are generally less sensitive to the free anticancer drugs [21]. These results clearly suggested that nanoparticles maintained the pharmacological activity of PTX and were efficiently delivered to cells. One more important factor could be the P-gp-inhibiting activity of TPGS, which is a constituent of the nanocarriers [19].

Cell apoptosis analysis

In order to evaluate the apoptosis potential of each formulation, Annexin V–PI staining assay was performed to quantitate the amount of cells in early and late apoptosis stage. The scatter plot has four quadrants: lower left quadrant (Q3) indicates viable cells (Annexin –ve, PI –ve), lower right quadrant (Q4) indicates early apoptotic cells (Annexin +ve, PI –ve),

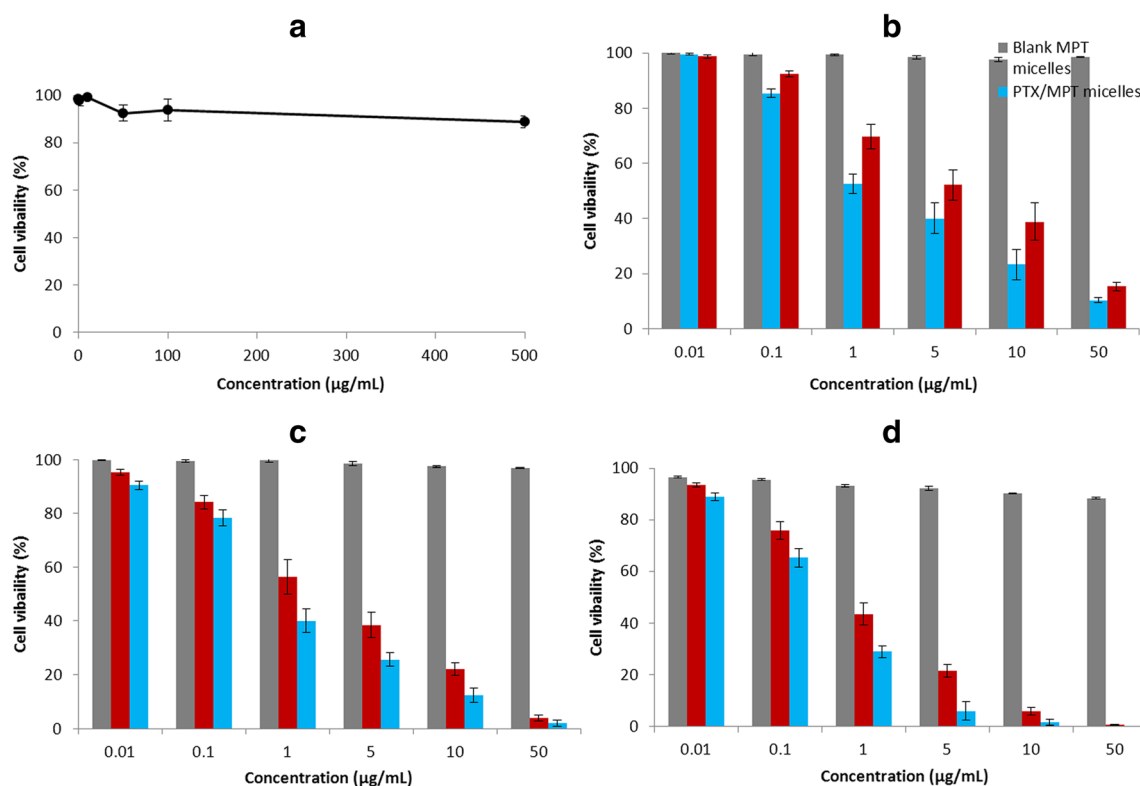


Fig. 4 a Cytotoxicity analysis of blank polymeric micelles in A549 cancer cells in a concentration-dependent manner. b–d Cytotoxicity study of free PTX and PTX/MPT micelles on A549 cells after 24, 48,

and 72 h incubation, respectively. The cytotoxicity profile of individual formulation was evaluated by means of MTT assay

upper right quadrant (Q2) indicates late apoptotic cells (Annexin +ve, PI +ve), and upper left quadrant (Q1) indicates necrotic cells (PI +ve). As shown in Fig. 5a, free drug and drug-loaded formulation induced the cell apoptosis in different proportions. Specifically, PTX/MPT micelles (28.5 %) showed significantly higher apoptosis than free drug (11.3 %). The result was consistent with the cytotoxicity assay and lower IC₅₀ value of PTX/MPT micelles.

Cell cycle analysis

We also studied the cell cycle analysis of A-549 cells post-treatment with different formulations. To be specific, we have studied the G2/M and sub G1 phase analysis of cancer cells. As shown in Fig. 5b,c, cells treated with PTX/MPT micelles showed significantly higher percentage of cells in G2/M phase (~55 %) compared to those treated with free PTX (~30 %). The result is consistent with the general observation that PTX arrests the cell at G2/M phase of the cell cycle. Along with G2/M phase arrest, PTX/MPT micelles showed a marked increase in sub G1 cell population (~20 %). The results indicate that PTX released from micelles induced a mitotic arrest that destabilized the microtubules which, in turn, interfered with the mitotic spindle function and cell arrest in G2/M phase. The results clearly highlight the importance of PTX/MPT micelles as a drug delivery carrier for lung cancer targeting.

Caspase assay

Finally, Western blot analysis was performed to analyze the apoptosis marker, such as caspase-3, which is an indicator of the apoptosis (Fig. 5d). Consistent with the higher apoptosis for PTX/MPT micelles, it showed a higher expression for cleaved caspase-3 as compared to that of free PTX and untreated group.

In vivo pharmacokinetic study

The mean plasma concentration–time profiles of free PTX and PTX/MPT following intravenous administration is presented in Fig. 6. The individual formulation was administered at 10 mg/kg of animals as a single dose. The Sprague–Dawley rats were divided into two groups with four rats in each group. As clearly seen, free PTX was rapidly eliminated from blood circulation within 6 h of administration. In contrast, PTX/MPT micelles revealed a significantly prolonged blood circulation of up to 24 h. Throughout all time points, PTX/MPT micelles maintained higher concentration than free drug. Even at the end of 24 h, PTX/MPT micelles maintained about 100 ng/ml of plasma concentration.

Consequently, plasma concentration was fitted into two-compartment model to observe the pharmacokinetic parameter. Importantly, PTX/MPT micelles (~4,000 µg/l/h) showed a

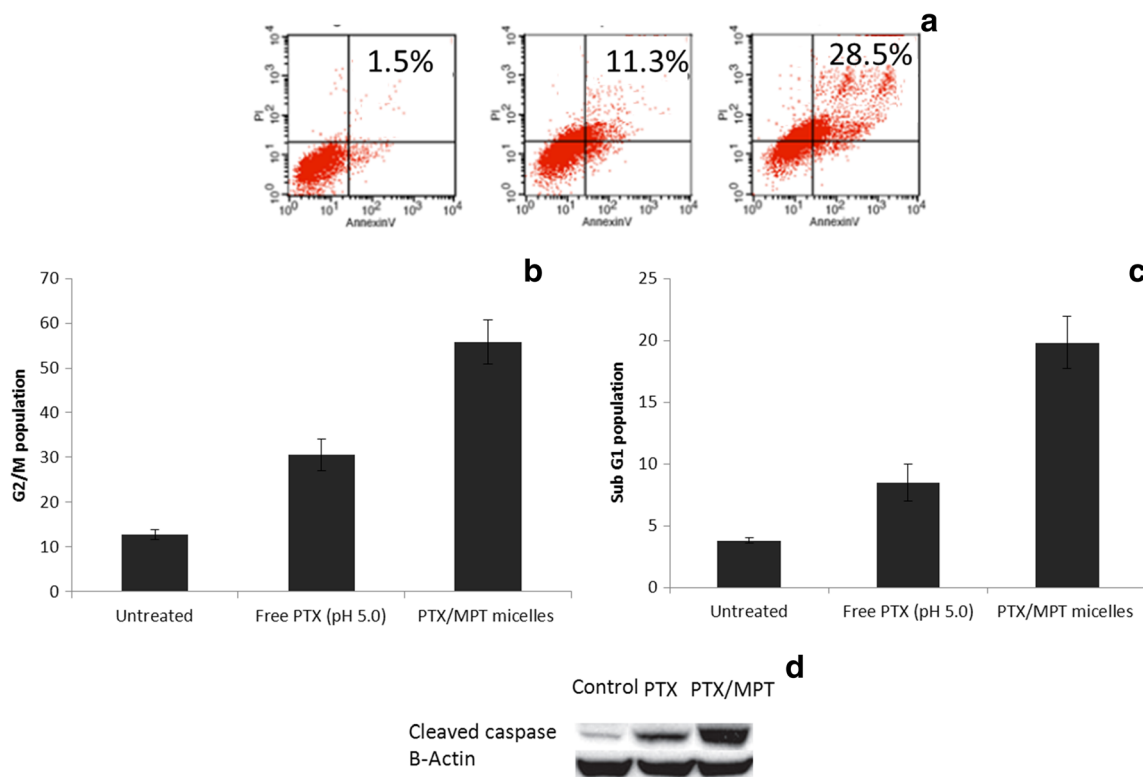


Fig. 5 **a** Apoptosis analysis of free PTX and PTX/MPT micelles in A549 cells by flow cytometer using Annexin V-FITC and PI staining. Cell cycle progression of A549 cells when treated with free PTX and PTX/MPT

micelles, **b** G2/M phase, **c** sub G1 phase, and **d** cleaved caspase-3 analysis by Western blot assay

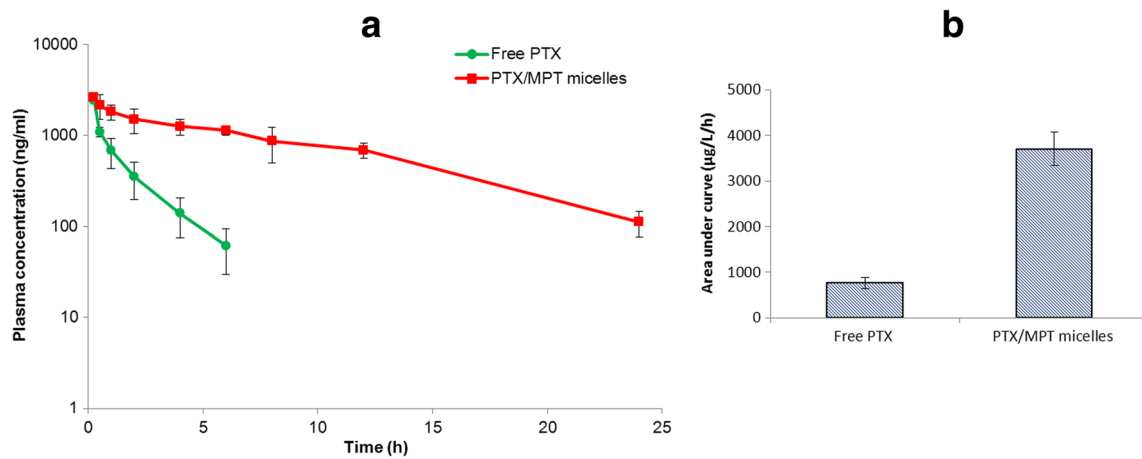


Fig. 6 a Plasma concentration–time profile of free PTX and PTX/MPT micelles upon single-dose intravenous administration via tail vein. The drugs were administered at a fixed dose of 10 mg/kg (b) area under the curve (AUC) analysis by two-compartment modeling

fourfold higher area under the curve (AUC) than free drug (~900 µg/L/h). The micellar formulation could extend the elimination half-life ($t_{1/2}$) of PTX from nearly 1.5 to 2.99 h. The superior performance (long blood circulation) of PTX/MPT micelles in blood circulation was mainly attributed to the presence of hydrophilic PEG shell on the surface of the

nanocarriers. Such stealth nanocarriers remain invisible to macrophages and can create clouds at the surface that will, in turn, repel the plasma proteins and prolong the systemic circulation. In addition, small particle size and sustained release of drug further improve its in vivo performance [22].

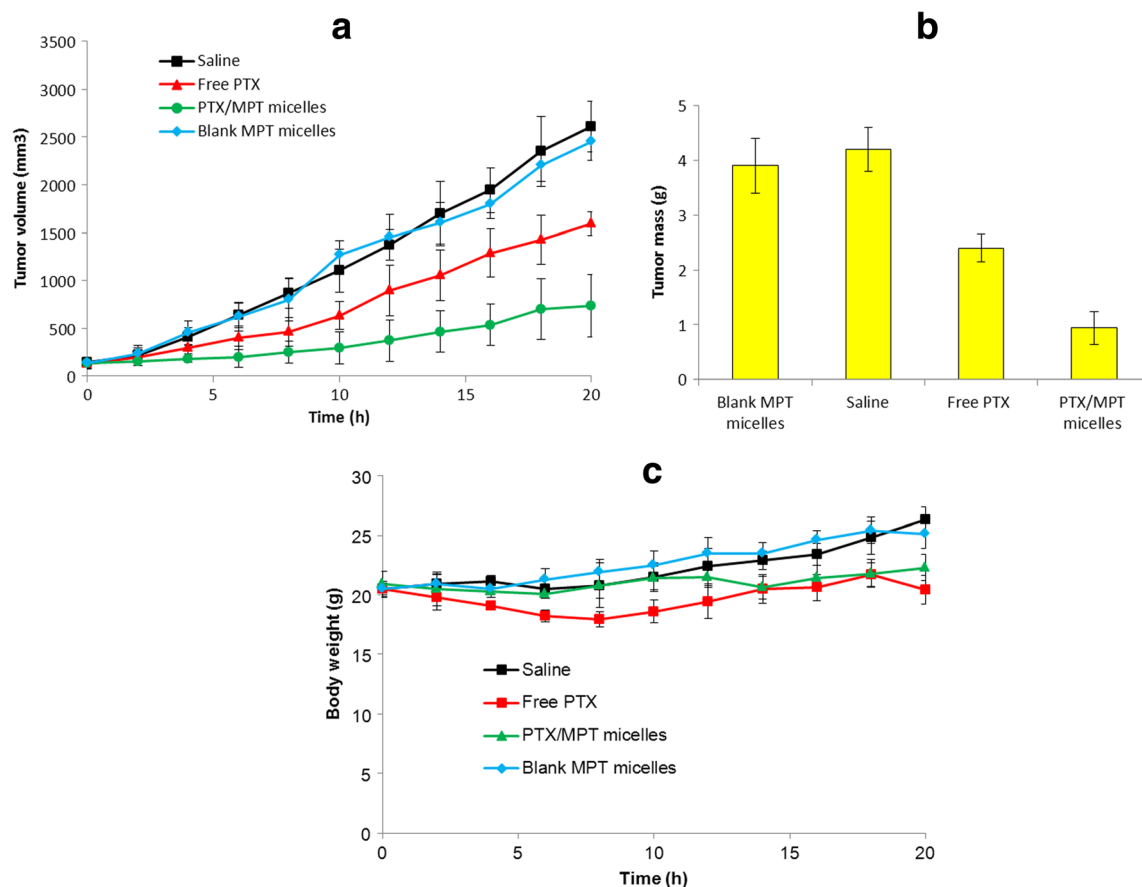


Fig. 7 In vivo antitumor efficacy study of a changes in tumor volume, b tumor mass, and c changes in mice body weight. The antitumor study was carried out in A549/Taxol cell bearing xenograft model and administered

with 10 mg/kg of respective formulation three times during first 2 weeks of study period

In vivo antitumor activity evaluation

The antitumor efficacy of free PTX and PTX/MPT micelles was studied in resistant A549/Taxol cells bearing xenograft nude mice (Fig. 7a). The formulations were injected at a dose of 10 mg/kg thrice during the first 2 weeks of study period. As shown in Fig. 3, PTX/MPT micelles significantly reduced the tumor progression and presented a relatively smaller tumor volume than the group treated with free PTX. The tumor volume almost remained the same until day 4; however, after that, significant differences were observed between different groups. Especially, by day 20, tumor volume increased to about 2,700 mm³ in mice group treated with saline while administration with free PTX controlled the tumor growth to certain extent (~1,600 mm³). Of all, PTX/MPT micelles effectively controlled the tumor cell proliferation and exhibited nearly twofold and fourfold smaller tumor volume as compared to that of free PTX and untreated group. Similar observations were made when the tumor mass was extracted and weighed (Fig. 7b). It can be concluded that antitumor efficacy of PTX/MPT micelles was highly superior to that free drug in A549/Taxol MDR tumor model. The result was consistent with the enhanced cell cytotoxicity and cell apoptosis for this group. A combined effect of EPR effect and enhanced sensitization of MDR cells by TPGS moiety could be the primary reason behind its superior performance and tumor suppression [23]. Besides, small particle size, core shell architecture, hydrophilic PEG shell, and prolonged systemic circulation led to an increased accumulation of drug in the tumor cells.

Additionally, body weight changes have been observed to monitor the toxicity profile of individual formulations. As seen in Fig. 7c, free PTX reduced the body weight of mice around 10 % of original body weight, indicating its toxic properties at 10 mg/kg. It has been reported than body weight reduction is associated with serious systemic side effects. As expected, PTX encapsulation in carrier system did not show any body weight reduction, indicating that drug remained in the core of the micelles and released in a controlled manner. All this results advocate the fact that PTX/MPT micelles could effectively inhibit the tumor progression without causing any systemic side effects.

Conclusion

In conclusion, we have synthesized and designed novel MPEG-PCL-TPGS-based polymeric micelles to entrap anti-cancer drug PTX. The micelles exhibited a sustained release pattern for up to 168 h with accelerated release pattern at acidic pH conditions. The blank polymeric micelles showed cell viability of >85 %, indicating its excellent biocompatibility and low toxicity, making it suitable for all in vivo applications. PTX/MPT micelles displayed superior antitumor efficacy in A-549 lung cancer cells as compared to that of free PTX.

The cytotoxicity assay clearly showed the enhanced cancer cell death due to the selective delivery of PTX by micelles. The PTX/MPT micelles showed higher cellular uptake via endocytosis pathways. The PTX-bound micelles preferentially arrested the cells at G2/M phase and showed a marked increase in sub G1 cell population (~20 %). The pharmacokinetic study revealed a long blood circulation for PTX/MPT micelles. The micellar formulation showed a remarkable tumor suppression effect in resistant A549/Taxol cells bearing xenograft nude mice along with no toxicity profile. We believe that this novel block copolymer-based delivery system will make progress in the treatment of MDR-resistant lung cancers. We intend to carry out further studies to ascertain the superior effect of PTX/MPT micelles.

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Conflicts of interest None

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