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Deoxycholic acid-modified chitooligosaccharide/mPEG-PDLLA mixed micelles loaded with paclitaxel for enhanced antitumor efficacy

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ABSTRACT

Poly(ethylene glycol) (PEG) as a block in polymeric micelles can prolong circulation life and reduce systemic clearance but decrease the cellular uptake. To overcome this limitation, a mixed micelle composed of deoxycholic acid-modified chitooligosaccharide (COS-DOCA) and methoxy poly(ethylene glycol)-polylactide copolymer (mPEG-PDLLA) was designed to load paclitaxel (PTX). The PTX-loaded mixed micelles was prepared by nanoprecipitation method with high drug-loading efficiency of 8.03% and encapsulation efficiency of 97.09% as well as small size (~40 nm) and narrow size distribution. COS-DOCA/mPEG-PDLLA mixed micelles exhibited the sustained release property. Due to the positive charge and bioadhesive property of COS-DOCA, the cellular uptake of PTX in mixed micelles was higher in cancer cells but lower in macrophage cells compared to the mPEG-PDLLA micelles. The systemic toxicity of PTX in mixed micelles was much lower than Taxol using zebrafish as a toxicological model. Furthermore, the PTX-loaded COS-DOCA/mPEG-PDLLA mixed micelles can prolong the blood circulation time of PTX and enhance the antitumor efficacy in A549 lung xenograft model. Our findings indicate that COS-DOCA/mPEG-PDLLA mixed micelles could be a potential vehicle for enhanced delivery of anticancer drugs.

1. Introduction

Paclitaxel (PTX) is one of the most important first-line chemotherapeutic agents against a wide range of cancers (Rowinsky and Donehower, 1995). However, due to the poor aqueous solubility, its clinical application is limited. To improve the aqueous solubility of PTX, the commercial formulation Taxol® contains a high concentration of Cremophor EL, which leads to serious side effects, such as hypersensitivity, neurotoxicity and nephrotoxicity (Hennenfent and Govindan, 2006; Sharma et al., 1995; Weiss et al., 1990). With the goal of replacing Cremophor EL and improving delivery efficacy, various drug delivery systems have been developed, including nanoparticles (Wang et al., 2013; Win and Feng, 2006), liposomes (Koudelka and Turanek, 2012; Yoshizawa et al., 2011; Zhang et al., 2005), micelles (Gaucher et al., 2010; Kim et al., 2001a) and other formulations (Liu et al., 2010; Mora-Huertas et al., 2010).

Polymeric micelles formed by amphiphilic block copolymers have been extensively studied as an attractive nanocarrier system (Gong et al., 2012; Torchilin, 2007). Polymeric micelles may improve drug solubilization, control drug release, reduce non-specific uptake by reticuloendothelial system (RES), and increase tumor targeting by the enhanced permeability and retention (EPR) effect (Gaucher et al., 2010; Gong et al., 2012; Maeda, 2012). Because of their low critical micelle concentration, amphiphilic copolymer can self assemble to form nanosized aggregates that possess a core-shell structure. Poorly soluble drugs can be effectively incorporated into the hydrophobic core. Furthermore, the micelle corona formed by hydrophilic polymer blocks such as poly(ethylene glycol) (PEG) provides longevity in vivo by reducing their opsonization and clearance by RES (Chen et al., 2008; Fontana et al., 2001; Miller et al., 2012). Methoxy PEG-polylactide copolymer (mPEG-PDLLA) is a typical amphiphilic block copolymer, which is widely used to encapsulate anticancer drugs with a mesoscopic size range about 20–50 nm (Chen et al., 2008; Kim et al., 2001a). However, PEG can interfere with interactions between polymeric micellar nanocarrier systems and target cells, thus negatively influencing the therapeutic outcomes.

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Recently, a large number of studies have been focused on mixed polymeric micelles that can combine the prominent advantages of different types of single polymeric micelles (Kulthe et al., 2011; Mu et al., 2010; Saxena and Hussain, 2012; Zhang et al., 2011). The loading content and stability of drug in mixed micelles can be significantly improved compared with single copolymer micelles. More importantly, the release rate and function of micelles can be desirable to modify by forming mixed micelles (Harmon et al., 2011; Wang et al., 2005). For example, Wei et al. have reported the Pluronic mixed micelles composed of Pluronic P123 and F127 to encapsulate PTX. Compared to Taxol, the mixed polymeric micelles effectively enhanced the in vitro cytotoxicity of PTX and greatly increased the blood circulation time. It demonstrated the enhancement of the antitumor efficacy in A-549 lung tumor model (Zhang et al., 2011).

Chitooligosaccharide (COS) is an abundant natural polysaccharide composed of randomly distributed D-glucosamine and *N*-acetyl-D-glucosamine units (Garcia-Fuentes and Alonso, 2012; Park et al., 2010). The unique characteristics such as biodegradability, biocompatibility, hydrosolubility and positive charge make it ideal as nanometric drug delivery materials (Hu et al., 2002; Hyung Park et al., 2006; Kim et al., 2001b). Moreover, COS has widely been used as a coating material to tailor the surface charge and bioadhesive property in pharmaceutical and biomedical fields (Kim and Rajapakse, 2005). Coating of cationic water-soluble chitooligosaccharide onto poly (D,L lactide-co-glycolide) (PLGA) particle surface has been demonstrated the macrophage uptake reduction and the circulation half-life extension (Amoozgar et al., 2013; Sheng et al., 2009). The primary hydroxyl and amine groups of chitooligosaccharide allow for further chemical modification to control its physical properties. The hydrophobic conjugated chitooligosaccharide may form self-assembled nanoparticles that can encapsulate a large number of hydrophobic drugs such as PTX and a variety of hydrophobic moieties have been reported to develop amphiphilic chitooligosaccharide derivatives, including bile acids (e.g., cholic acid, deoxycholic acid and 5β-cholanic acid,) and fatty acids (e.g., stearic acid and oleic acid) (Garcia-Fuentes and Alonso, 2012; Hu et al., 2002; Hyung Park et al., 2006; Kim et al., 2001b; Park et al., 2010).

It is well-known that the positive charge can usually enhance the endocytosis of various nanoparticles by cells (Amoozgar et al., 2013; Harmon et al., 2011; He et al., 2010; Wang et al., 2005; Yim et al., 2013). In this work, the objective was to develop a new mixed polymeric micellar formulation comprised of PEG-PDLLA and COS for enhanced antitumor efficacy of PTX. COS was first modified by coupling with deoxycholic acid (DOCA). The DOCA modified chitooligosaccharide(COS-DOCA) was amphiphilic and desired to form the mixed micells with mPEG-PDLLA. The PTX-loaded mixed micelles (PTX-M) were prepared by nanoprecipitation method. The particle size, morphology, in vitro release profile and cytotoxicity were investigated. Compared with Taxol® and single mPEG-PDLLA micelle, pharmacokinetics, toxic effects, and the antitumor efficacy of PTX loaded mixed micelles were evaluated.

2. Materials and methods

2.1. Materials

Paclitaxel was purchased from Knowshine Pharmachemicals Inc. (Shanghai, China). Cremophor-based paclitaxel injection (Taxol) was ordered from Yangtze River Pharmaceutical (Group) Co., Ltd. (Jiangsu, China). Deoxycholic acid (DOCA), chitooligosaccharide (COS) (Mn = 5000), *N*-hydroxysuccinimide (NHS), 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC) and 3-(4, 5-dimethyl-thiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) were purchased from Sigma–Aldrich (Shanghai local agent, China). Monomethoxy poly(ethylene glycol)-block-poly(D,L-lactide) (PEG-PDLLA) (PEG Mw = 5000 Da, PDLLA Mw = 10,000 Da) was purchased from Advanced Polymer Materials Inc. (Montreal, Canada). Penicillin-streptomycin, RPMI1640, fetal bovine serum (FBS) and 0.25% (w/v) trypsine 0.03% (w/v) EDTA solution were purchased from Hycolon (USA). All other reagents and buffer solution components were of analytical grade.

HeLa cell line, A549 cell line, MGC-803 cell line and J774A.1 cell line were obtained from the Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences (Shanghai, China). Culture plates and dishes were purchased from Corning Inc. (NY, USA).

Male Sprague-Dawley (SD) rats (250 ± 20 g) and female BALB/c nude mice (20 ± 2 g), supplied by Shanghai SLAC laboratory animal Co., Ltd. (Shanghai, China) and kept under SPF conditions.

2.2. Synthesis of COS-DOCA

COS-DOCA was synthesized by a conjugation reaction between amino-groups of COS and carboxyl groups of DOCA using EDC as a coupling agent (Hyung Park et al., 2006; Kim et al., 2001b). In brief, COS-DOCA was obtained as follows: 160 mg COS (Mn = 5000), 80 mg DOCA 194.7 mg EDC and 116.3 mg NHS were added to 100 mL round-bottomed flask and dissolved with 40 mL methanol/H_2O (1:1, v/v). Under stirring, the reaction mixture was maintained at 30 °C in a water bath. After 30 h, the solution was transferred into a dialysis bag (MWCO 3500) and dialyzed against doubled distilled water for 24 h to remove the unreacted substances. The final product was frozen and dried in a vacuum. The products of synthesized were determine by Fourier Transform Infrared spectroscopy (FT-IR). These dried products were mixed with KBr and pressed to the plate for measurements. FT-IR spectra were recorded on an FT-IR spectrometer (Bio-Rad FTS-6000). The conjugation of DOCA moiety to COS chain was further confirmed by ^{1}H NMR and gel permeation chromatography (GPC) measurements.

2.3. Preparation of PTX-loaded mixed micelles

PTX-loaded mixed micelles were prepared by a nanoprecipitation method. 2 mg of PTX powder and 20 mg mPEG-PDLLA were dissolved into 5 mL acetone. 2 mg freeze-dried COS-DOCA powder was also weighed into 25 mL beaker and dissolved in 11 mL H_2O. Under magnetic stirring, 5 mL acetone solution was dropped into 5 mL COS-DOCA solution at the speed of 60 mL/h. The available mixture was evaporated by rotary vacuum evaporation to remove the organic solvents at 37 °C. After evaporation, the PTX-loaded mixed micelles were obtained.

2.4. Characterization of PTX-loaded mixed micelles

Micelles were diluted 3-fold with water. The particle sizes of PTX-loaded mixed micelles were measured by dynamic light scattering (DLS) using Zetasizer (Malvern, UK) at 25 °C. The detection range was from 2 to 5000 nm. Each sample was analyzed

in triplicate. The morphology of samples was observed with transmission electron microscope (TEM) (JEM-2010; JEOL, Japan). A drop of sample after dilution was placed onto a carbon-coated copper grid to form a thin liquid film. The films on the grid were negatively stained with 0.1% (w/v) phosphotungstic acids. After excess solution was removed, the sample was air-dried at room temperature.

Drug-loading (DL%) and encapsulation efficiency (EE%) were calculated by the following equations.

$$DL\% = \frac{\text{Weight of the drug in micelles}}{\text{Weight of the feeding polymer and drug}} \times 100\%$$

$$EE\% = \frac{\text{Weight of the drug in micelles}}{\text{Weight of the feeding drug}} \times 100\%$$

2.5. In vitro release of PTX from Mixed Micelles

The in vitro release properties of PTX from mixed micelles were investigated in a phosphate buttered saline (PBS) (pH 7.4) medium containing 0.2% Tween 80 by dialysis method. 1 mL of PTX-loaded mixed micelles in PBS solution (1 mg/mL, PTX equivalent) was diluted to 10 mL. Take 1.0 mL of PTX-loaded mixed micelles solution to calculate the initial concentration and then the rest of 9.0 mL was introduced into a dialysis bag (MWCO = 14 kDa). The end-sealed dialysis bag was immersed into 50 mL PBS (pH 7.4) containing 0.2% Tween 80 at 37 °C and shaken at the speed of 100 rpm for 48 h. Sample of 1.0 mL were withdrawn at different time intervals (0, 1, 2, 4, 8, 12, 24, 36, 48 h) and replaced with an equal volume of fresh release medium. The concentration of PTX in samples was determined by the HPLC method described below.

The analysis of PTX levels in vitro was carried out using a reversed phase HPLC method on a system equipped with LC-20AT pumps and a SPD-20A UV–vis detector (Shimadzu, Kyoto, Japan) operated at 227 nm. A reversed-phase column (Hypersil ODS2, 5 μm, 4.6 mm × 250 mm, Elite, Dalian, China) was used at 30 °C. The mobile phase consisted of acetonitrile and water (50:50, v/v). 20 μL samples were injected into HPLC column for all the analysis. The retention time of PTX was approximately 8.7 min with a flow rate of 1.0 mL/min for the mobile phase.

2.6. In vitro cytotoxicity

Human cervical cancer HeLa cells and human gastric cancer MGC-803 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) heat-inactivated fetal bovine serum, 100 IU/mL penicillin G, 0.25 μg/mL amphotericin B, and 100 μg/mL streptomycin at 37 °C with 5% CO_2. Human lung cancer A549 cells were cultured in RPMI 1640 medium and other conditions the same as above.

The in vitro anticancer effects of drug-loaded micelles were evaluated using the MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide] method. Briefly, cells were seeded at the density of 5×10^3 cells per well in 96-well plates. After 24 h of incubation at 37 °C with 5% CO_2, the growth medium was replaced with 200 μL medium containing PTX loaded mixed micelles and Taxol with concentration ranging from 0.005 to 100 μg/mL. After 48 h incubation, cell survival was then measured using tetrazolium salt MTT assay. 30 μL of MTT (5 mg/mL) solution was added to each well. The plate was incubated for an additional 4 h, and then medium was removed and 200 μL of DMSO was added to each well to dissolve any purple formazan crystals formed. The plates were vigorously shaken before measuring the relative color intensity. The cytotoxicity was measured following the absorbance of the degraded MTT (formazan) at 492 nm using a microplate reader.

2.7. In vitro cellular uptake

A549 human lung cells and J774A.1 murine macrophage cells were cultured in RPMI-1640 medium supplemented with 10% (v/v) FBS or 20% (v/v) FBS, 100 U/mL of penicilin and 100 U/mL of streptomycin at 37 °C, 5% CO_2.

For the cellular uptake study, A549 and J774A.1 cells were seeded separately on two 6-well cell culture plates at the density of 1×10^6 cells per well and incubated at 37 °C to allow cell attachment. When the cells were grown to 90% confluence, the cell culture medium was removed and replaced with fresh medium containing PTX-loaded mixed micelles and Taxol injection (100 μg/mL). The incubation was continued at 37 °C for various time intervals (1, 2, 4, 8 and 12 h).

At the time points, the cells were rinsed with PBS (pH 7.4) softly to eliminate excess particles which were not entrapped by the cells. After that, 0.2 mL 0.25% trypsin was added, and then the cells were harvested by centrifuged at 2000 rpm for 2 min. In order to expose the internalized nanoparticles, 100 μL 10% SDS was added, followed by sonication for 15 min, and then 300 μL acetonitrile was added and vortexed to extract paclitaxel. The cell lysate was centrifuged at 15,000 rpm for 10 min. The drug content in the supernatant after centrifugation was measured by HPLC method. The results were expressed as the dose/10^6cells. The experiments were performed in triplicate.

2.8. Toxic effects PTX-loaded mixed micelles on zebrafish

The breed of zebrafish embryos in the way of natural mating pairs. Four to five pairs of zebrafish were set up for natural mating every time. On average, 200–300 embryos were generated. Embryos were maintained at 28 °C in fish water (0.2% Instant Ocean Salt in deionized water, pH 6.9–7.2, conductivity 480–510 μS/cm and hardness 53.7–71.6 mg/L ($CaCO_3$). The embryos were washed and staged at 6 and 24 h postfertilization (hpf). Hunter Biotechnology, Inc. is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAA LAC) International.

The experiment is divided into 10 groups, containing the control group, solvent group, Taxol 50 ng, 15 ng, and 5 ng group, PTX-loaded mixed micelles 50 ng, 15 ng, and 5 ng groups. 35 zebrafish in each group. Drugs were dissolved in water and diluted to proper concentrations for yolk sac microinjection. Before microinjection, zebrafish at 2 days postfertilization (dpf) were anesthetized with 0.03% tricaine and loaded on a customized microplate designed specifically for zebrafish microinjection. The drug at designated concentrations was loaded into a pulled glass capillary that was drawn on an electrode puller and then trimmed to form a needle with a resulting internal diameter of approximately 15 micron and the outer diameter of approximately 18 micron. The microneedle was attached to an air driven Cell Tram. The tip of the needle was inserted into the yolk sac of a 2 dpf zebrafish under a dissecting microscope and the pulse time was controlled to deliver 10 nL of the drug solution into the yolk sac through the glass capillary. Injected zebrafish were transferred to six-well microplates, 35 zebrafish per well with 3 mL of fish water for a treatment period of 24 h. Zebrafish injected with 0.9% NaCl or ultrapure water served as a vehicle control and untreated zebrafish were used to confirm that the vehicle solvent did not have an adverse effect on the zebrafish.

2.9. In vivo pharmacokinetic studies

Twelve male Sprague-Dawley rats (250 ± 20 g) were used to investigate the effect of formulation on the pharmacokinetics of PTX after intravenous administration. Rat divided into 3 group at random ($n = 4$), and given a single 8 mg/kg dose of Taxol injection or PTX-loaded mixed micelles by tail vein injection. Blood samples (0.5 mL) were collected into heparinized tubes from the femoral artery at 5, 15, 30 min, 1, 2, 4, 8, 12, 24 h after intravenous administration. Blood was immediately processed for plasma by centrifugation at 4000 rpm for 10 min. The plasma was stored at −80° C prior to analysis by HPLC.

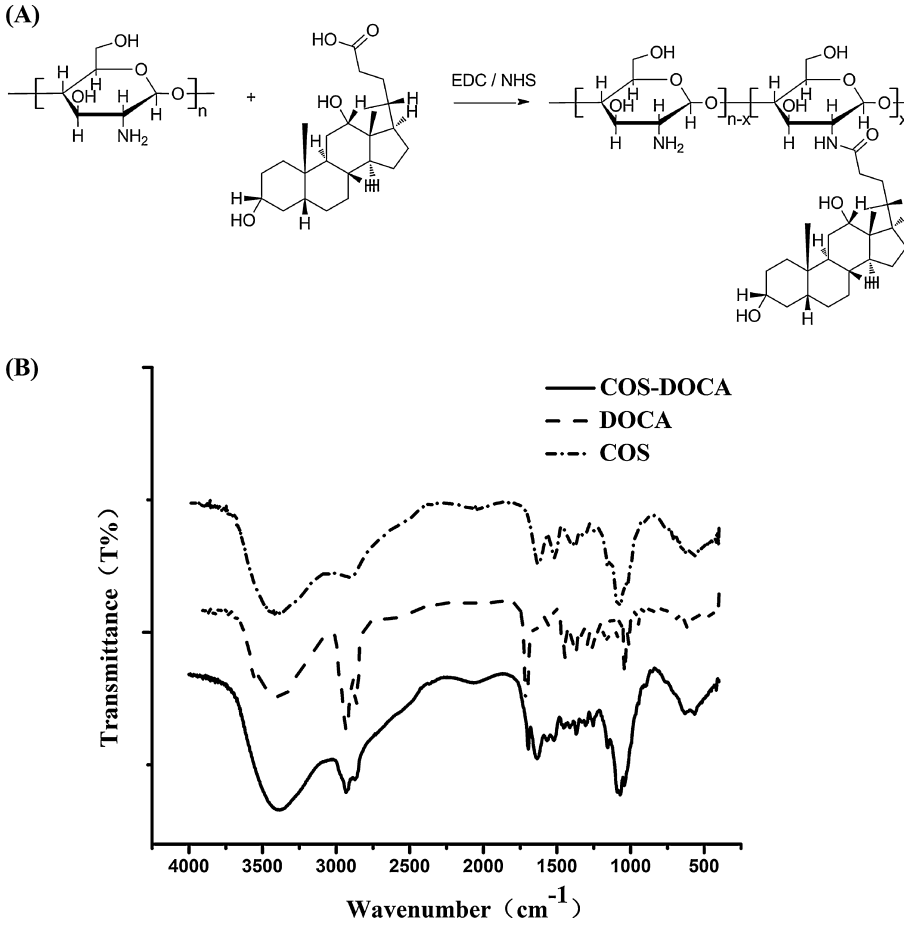


Fig. 1. (A) Schematic illustration of the synthetic step for COS-DOCA. (B) FT-IR spectrum of the resultant COS-DOCA.

Liquid–liquid extraction was performed prior to analysis. Briefly, 200 μL of plasma sample was added to 2 mL centrifuge tubes, 50 μL of the internal standard diazepam (10 μg/mL acetonitrile) was added and vortexed, and then 350 μL of acetonitrile was added. After vigorous vortex mixing for 2 min, the mixture was centrifuged at 15,000 rpm for 10 min, and then 400 μL of the organic layer was transferred to a glass tube and 20 μL was analyzed using HPLC system. HPLC condition is the same as in vitro release samples, except the mobile phase change to PBS (pH 3.0) and acetonitrile (50:50, v/v).

2.10. In vivo antitumor activity

The in vivo antitumor efficacy of PTX-loaded COS-DOCA/mPEG-PDLLA mixed micelles was evaluated in A549 cell-bearing BALB/c nude mice. A549 cells were injected subcutaneously into 1×10^7 cells from the right flanks of nude mice to establish human lung cancer xenografts. The in vivo antitumor studies were started when the tumor volumes reached about 100 mm^3 (designated as Day 0). Mice were randomly assigned to four groups ($n = 7$): group 1 for PBS, group 2 for Taxol injection (10 mg/kg), and group 3 for mPEG-PDLLA micelles, group 4 for PTX-loaded COS-DOCA/mPEG-PDLLA mixed micelles (10 mg/kg), respectively. Mice were administered according to their group protocol, through the tail vein three times a day every 3 days. Tumor volume and mouse weight were monitored at the predetermined time points. 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 harvested, weighed, and photographed. The tumor inhibition rate was calculated using the equation,

$$\text{Inhibitionration}(\%) = \frac{Wc - Wt}{Wc}$$

where Wc is the weight of the tumor in the control group and Wt is the weight of the tumor in a test formulation group.

3. Results and discussion

3.1. Synthesis and characterization of COS-DOCA

Chitooligosaccharide (COS) is often used as the hydrophilic shell for many biomedical applications including drug and gene delivery (Garcia-Fuentes and Alonso, 2012; Park et al., 2010). COS is usually modified by some hydrophobic moieties to improve its hydrophobicity. In our design, DOCA was selected as the hydrophobic segment to obtain the amphiphilic DOCA grafted COS (COS-DOCA). Fig. 1A illustrates the synthetic scheme of COS-DOCA. The FT-IR spectra of COS-DOCA was showed in Fig. 1B. The increase of the amide I band at 1653 cm^{-1} in the IR spectrum of the product confirms the formation of an amide linkage between amino groups of COS and carboxyl groups of DOCA. In ^1H NMR spectrum, the characteristic peaks corresponding to DOCA and COS mainly occurred at 1–2 ppm and 2–5 ppm, respectively. The GPC peak of COS-DOCA appeared at an earlier elution volume count position than that of COS, indicating the formation of COS-DOCA with higher molecular-weight (data not shown).

COS-DOCA can form self-assembled micelles in an aqueous solution via hydrophobic interactions between the DOCA parts,

Fig. 2. Schematic illustration of formation of self-assembled COS-DOCA/mPEG-PDLLA mixed micelles with encapsulation of paclitaxel.

primarily to minimize interfacial free energy. These DOCA hydrophobic groups may help to stabilize the micelle structure. Poorly soluble drugs such as PTX may be incorporated into the hydrophobic core and protected them from the biological environment.

3.2. Preparation and characterization of PTX-loaded mixed micelles

For systemic application, PEG is always the hydrophilic coating on nanocarrier surface (Chen et al., 2008; Fontana et al., 2001; Miller et al., 2012). The "stealth" PEG coating provides nanocarriers with a steric barrier that protects them from opsonization and clearance by RES. On the other hand, the use of PEG may decrease the interactions between nanocarriers and cancer cells, negatively affecting the final antitumor efficacy (Amoozgar et al., 2013). To overcome this challenge, we propose COS-DOCA as an alternative surface coating, which can protect the nanocarriers and meanwhile maintain the strong cellular interactions.

To prove our hypothesis, we designed a degradable cationic mixed micelle system for delivery of PTX. The PEG-PDLLA is primarily used to encapsulate the poorly water soluable PTX. COS-DOCA provides a strong cationic charge on the surface of the mixed micelles. The single mPEG-PDLLA micelle was used as a control. We adopted the nanoprecipitation method to prepare PTX loaded polymeric micelles with minor modifications. The scheme for the preparation of the COS-DOCA/mPEG-PDLLA mixed micelles is displayed in Fig. 2. The physicochemical characterization and drug-loading parameters of PTX-loaded polymeric micelles such as particle size, zeta potential and PTX encapsulation were summarized in Table 1.

The mean diameter of PTX-loaded COS-DOCA/mPEG-PDLLA mixed micelles was close to 40 nm (Fig. 3A and B). The small particle sizes could reduce the RES uptake and prolong the circulation time in the blood, and could facilitate extravasation from leaky capillaries (Maeda, 2012). Hence, the size of the COS-DOCA/mPEG-PDLLA mixed micelles was suitable for tumor specific accumulation via the EPR effect.

The COS-DOCA/mPEG-PDLLA-PTX exhibited the positive surface charge. In contrast, single micelle mPEG-PDLLA loading PTX exhibited weakly negative surface charge. It can be seen from Table 1 that the introduction of COS-DOCA could slightly improve DL% and EE%.

The TEM images of the PTX loaded micelles were shown in Fig. 3C and D, which reveals that the PTX micelles are spherical and homogeneous in aqueous solution. The particle size observed by TEM correlated well with the results of the DLS (Fig. 3A and B), indicating the polymeric micelles well-dispersed in aqueous solution.

3.3. In vitro release of PTX

The accumulative drug release profiles of the PTX loaded micelles in vitro are shown in Fig. 4. Both the PEG-PDLLA micelle and the mixed micelle had a similar release profile of PTX. Approximately 50% PTX was released at a constant faster rate at first 10 h. After that, a slower release rate was observed. The result shows that release profile of mixed micelles is better than single micelle. The mixed micellar nanocarrier can not only solubilize the poorly soluble drugs, but also sustain PTX release.

The PTX release rate of mixed micelles in this medium was slower than that of mPEG-PDLLA micelles. The inclusion of COS-DOCA in mixed release may improve the hydrophobic interaction between PTX and hydrophobic core in presence of DOCA segments. The controlled release property of PTX from the COS-DOCA/mPEG-PDLLA micelles indicates their potential applicability as a drug delivery system to minimize the exposure of healthy tissues and increase the tumor accumulation of chemtherapeutic drugs.

3.4. In vitro cytotoxicity

The in vitro cytotoxicity of PTX-loaded COS-DOCA/mPEG-PDLLA mixed micelles were investigated and compared with Taxol on MGC-803, HeLa and A-549 cells. Cells were exposed to a series of equivalent concentrations of PTX micelles or Taxol for 48 h. Then the viability of the cells was quantified using the MTT assay. This study was carried out in order to estimate the effectiveness of COS-DOCA/mPEG-PDLLA mixed micelles in inhibiting tumor cell growth in vitro. Fig. 5 shows the cytotoxicity of the different drug concentrations (0.005–100 μg/mL) in vitro for MGC-803, HeLa and A549 cells. As shown in Fig. 5 PTX-loaded COS-DOCA/mPEG-PDLLA mixed micelles achieved better cytotoxic effect than single mPEG-PDLLA micelle. Moreover, a dose-dependent cytotoxicity was observed. The results indicate that the loading of PTX in COS-DOCA/mPEG-PDLLA micelles enhances the cytotoxic activity of PTX.

3.5. In vitro cellular uptake of PTX-loaded mixed micelles by cancer and macrophage cells

The cellular uptake studies were carried out to demonstrate if the inclusion of COS-DOCA in mixed micelles can facilitate uptake of PTX by cancer cells but reduce uptake by macrophage. Cells were

Table 1
The physicochemical characterization of PTX-loaded micelles ($n = 3$).

Formulation	Size (nm)	PDI	Zeta (mv)	DL (%)	EE (%)
COS-DOCA/mPEG$_{5K}$-PDLLA$_{10K}$-PTX	38.5 ± 2.2	0.275 ± 0.018	17.67 ± 0.98	7.53 ± 0.02	90.37 ± 0.73
mPEG$_{5K}$-PDLLA$_{10K}$-PTX	32.3 ± 1.8	0.237 ± 0.014	-3.96 ± 0.11	7.12 ± 0.02	88.58 ± 0.33

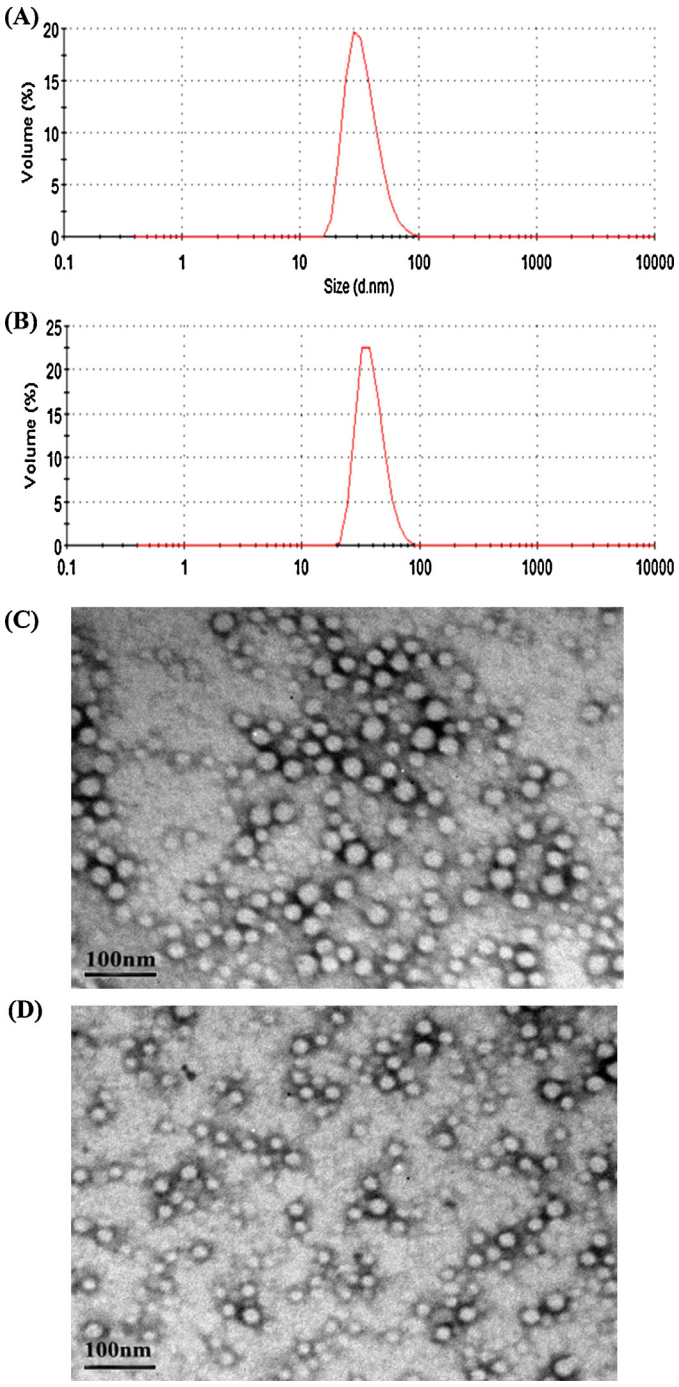


Fig. 3. The physicochemical characteristics of PTX-loaded micelles. The particle size and size distribution of COS-DOCA/mPEG-PDLLA-PTX (A) and mPEG-PDLLA-PTX, (B) were obtained from dynamic light scattering (DLS). TEM images of COS-DOCA/mPEG-PDLLA-PTX (C) and mPEG-PDLLA-PTX (D). The scale bar represents 100 nm.

incubated with PTX-loaded micelles a concentration of 100 mM of PTX for indicated time, then washed, and quantified by HPLC (Fig. 6). In contrast to mPEG-PDLLA micelle, COS-DOCA/mPEG-PDLLA mixed micelles slightly facilitated A549 cells uptake at 12 h of treatment (Fig. 6A). In parallel, for macrophage J774A.1 cells, COS-DOCA/mPEG-PDLLA mixed micelles showed the slower uptake rate compared to the mPEG-PDLLA micelle (Fig. 6B).

Cationic nanoparticles can allow for the strong interaction with the membrane of the cancer cells, thus facilitating endocytosis (Amoozgar et al., 2013; Harmon et al., 2011; He et al., 2010; Wang et al., 2005; Yim et al., 2013). In our case, the introduction of

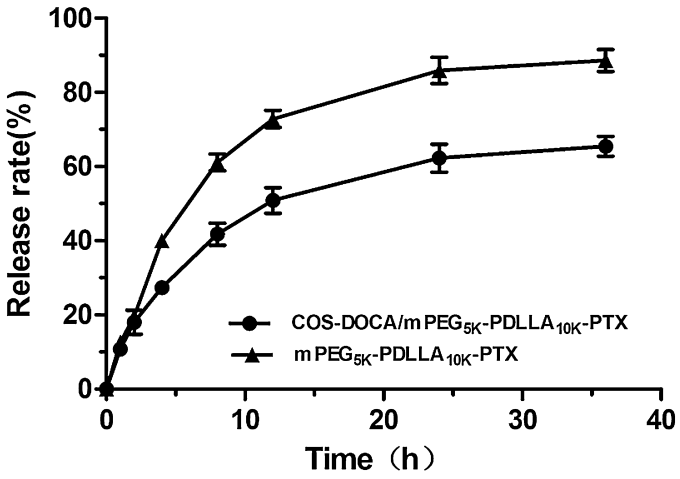


Fig. 4. Release profiles of PTX from PTX-loaded micelles in PBS (pH 7.4) medium containing 0.2% Tween 80 medium at 37 °C. Data expressed as the mean ± SD ($n = 3$).

COS-DOCA in mixed micelles may greatly enhance the capacity of crossing cell membrane. The hydrophilic COS has been confirmed to induce preferential cellular uptake of NPs and reduce opsonization and phagocytic uptake (Amoozgar et al., 2013). Our findings were in good agreement with it. Overall, these results indicated that the addition of COS-DOCA can avoid the phagocytolysis of macrophages and facilitate the uptake of tumor cells. COS-DOCA may be useful for both protecting micelles during circulation and enhancing their cellular uptake in a tumor site.

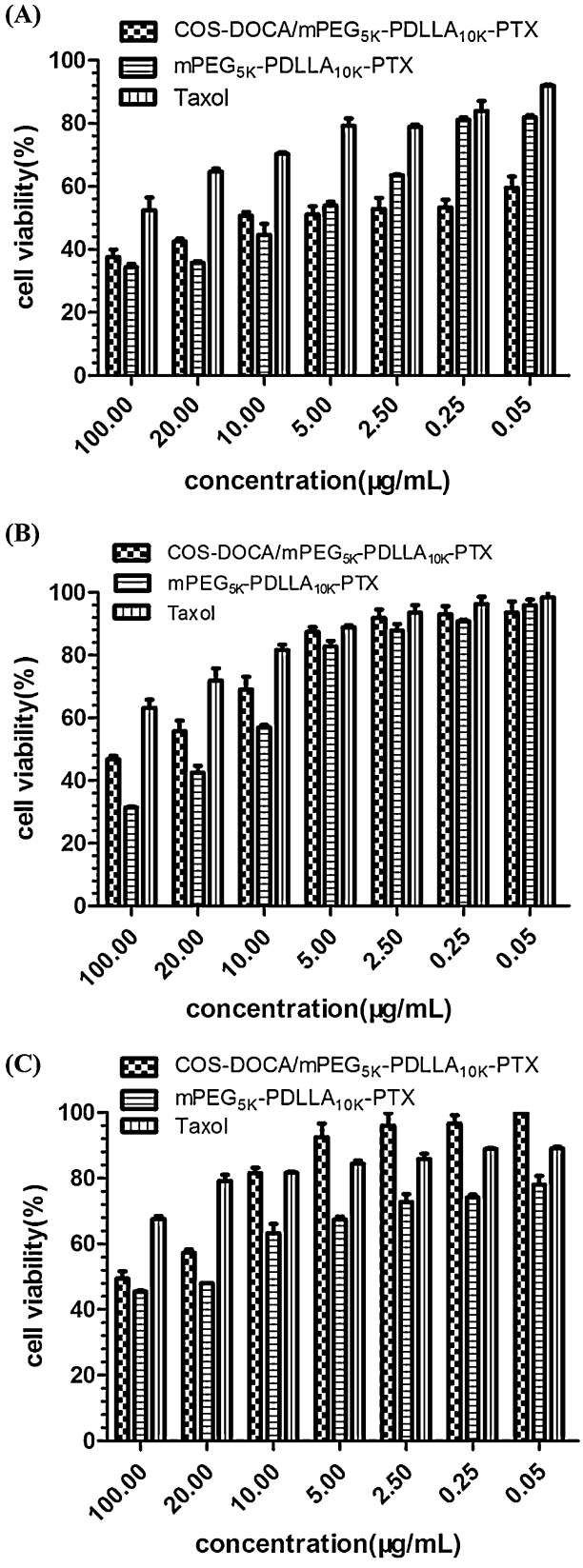
3.6. Toxic effects on zebrafish

Zebrafish has been a prominent model vertebrate in a variety of biological disciplines. Their values in toxicology as well as drug discovery has been recognized (Asharani et al., 2008). There are numerous advantages for the use of zebrafish as a toxicological model (McGrath and Li, 2008; Zon and Peterson, 2005) The zebrafish (2 dpf) treated by PTX-loaded mixed micelles and Taxol showed dose-dependent toxicity under laboratory conditions. Fig. 7A shows the survival rate of zebrafish treated by PTX formulations. The control groups (Blank and Solvent) and PTX-loaded mixed micelles without death, but Taxol treated group at 5 ng and 15 ng appeared death, the mortality was 3% and 34%, respectively. The emergence of pericardial edema was recorded in Fig. 7B. The Blank and Solvent groups did not appear pericardial edema. The PTX-loaded mixed micelles treated group 14% and 57% appeared pericardial edema at 5 ng and 15 ng. The Taxol treated group 3%, 32% and 69% appeared pericardial edema at 5 ng and 15 ng. The result shows that the toxicity of PTX-loaded mixed micelles is much lower than Taxol.

3.7. Pharmacokinetics of PTX-loaded mixed micelles

In order to investigate the pharmacokinetic profile of COS-DOCA/mPEG-PDLLA -PTX mixed micelles, we have performed pharmacokinetic studies of different PTX formulations after intravenous injection (8.0 mg/kg) using male Sprague-Dawley rats. PTX loaded mPEG-PDLLA micelle and Taxol were used as control. PTX in plasma was completely separated under analytical conditions, and standard curves ranging from 0.005 to 100 μg/mL were linear ($r = 0.9998$). The plasma concentration of PTX versus time profile was illustrated in Fig. 8.

As shown in Fig. 8, the difference between maximum plasma concentration (C_{max}) of COS-DOCA/mPEG-PDLLA-PTX and Taxol was not significant, but much higher than mPEG-PDLLA-PTX. The



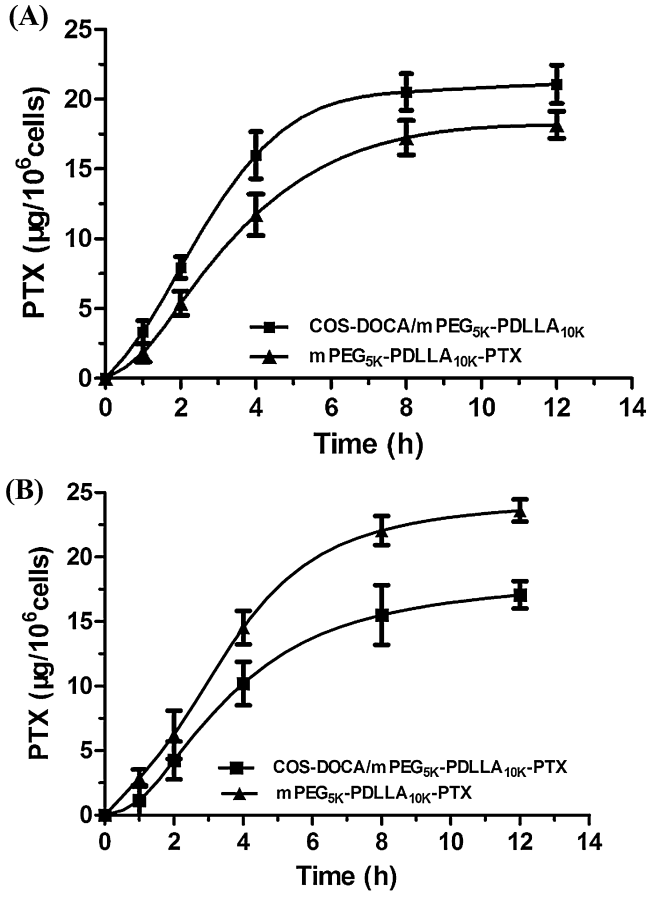


Fig. 6. PTX-loaded mixed micelles uptake by A549 (A) and J774A.1 (B). To confirm intracellular drug levels, cell lysates of cultured A549 treated with 100 μg/mL of PTX-loaded mixed were collected at specified times and assayed by HPLC.

pharmacokinetic behaviors for PTX in COS-DOCA/mPEG-PDLLA-PTX, mPEG-PDLLA micelle and Taxol were found to be similar at the first 4 h. However, after 4 h, the plasma concentration of PTX delivered by the mixed micelles was slightly higher than mPEG-PDLLA-PTX and Taxol, which might be attributed to the relatively slow release property and reduced macrophage cell uptake observed in Figs. 4 and 6.

3.8. In vivo antitumor activity evaluation

The antitumor effects of the different formulations were studied in A549-bearing nude mice. Fig. 9A shows the tumor growth observed for 27 days in the mice injected with PBS, Taxol, COS-DOCA/mPEG-PDLLA-PTX and mPEG-PDLLA-PTX. It was found that the tumor volumes of COS-DOCA/mPEG-PDLLA-PTX group were smaller than those of PBS, Taxol and mPEG- PDLLA-PTX groups in the whole measuring period. As a result, the COS-DOCA/mPEG-PDLLA mixed micelles can be used to greatly enhance antitumor efficacy of PTX

The enhanced anticancer efficacy of PTX loaded micelles could be explained via the EPR effect (Acharya and Sk, 2011; Maeda, 2012; Taurin et al., 2012; Wang et al., 2013). Additionally, cationic nanoparticles can potentially penetrate into tumour tissues. For example, a paclitaxel (PTX)-loaded degradable cationic nanogel consisted of acetylated pullulan and low molecular weight polyethyleneimine was found their deeper penetration in heterogeneous tumours (Yim et al., 2013).

Fig. 9B shows the body weight changes of the tumor-bearing mice during the study of antitumor efficacy. There was not significant loss in body weight after giving PTX formulations. The result shows that PTX-loaded mixed micelles has not systemic toxicity.

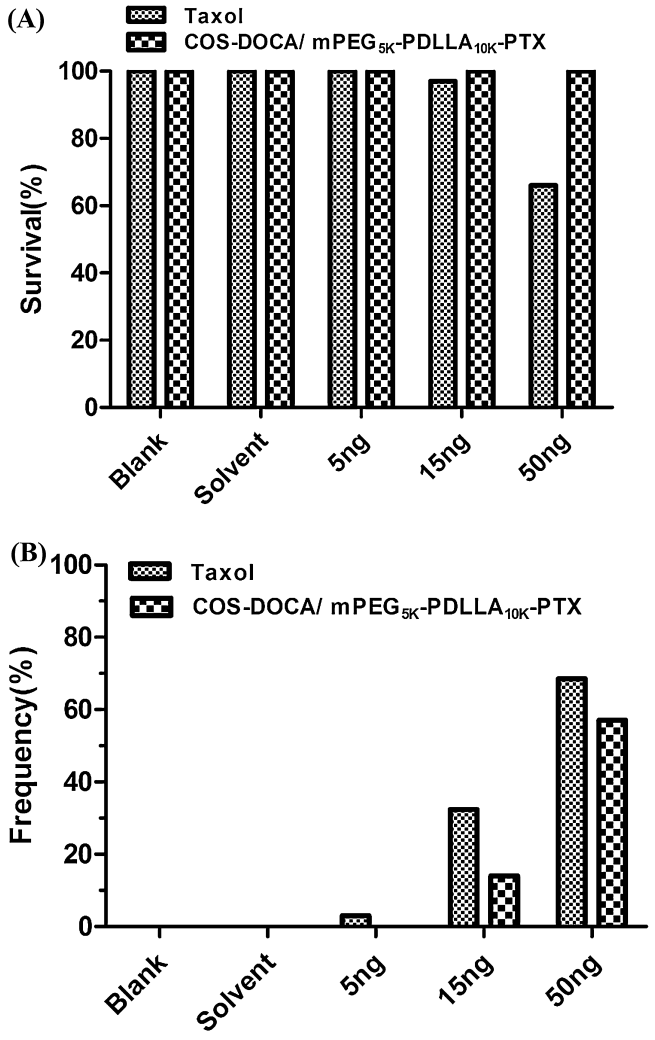


Fig. 7. Graphs representing the toxicity of PTX-loaded mixed micelles and Taxol in terms of mortality (A) and pericardial edema (B).

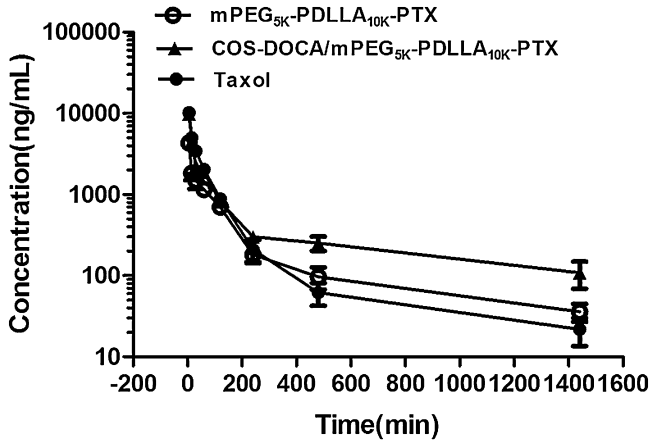


Fig. 8. Plasma concentration – time profiles of PTX after intravenous injection of PTX-loaded mixed micelles, mPEG-PDLLA micelle and Taxol at 8.0 mg/kg dose in SD rats, ($n = 4$)

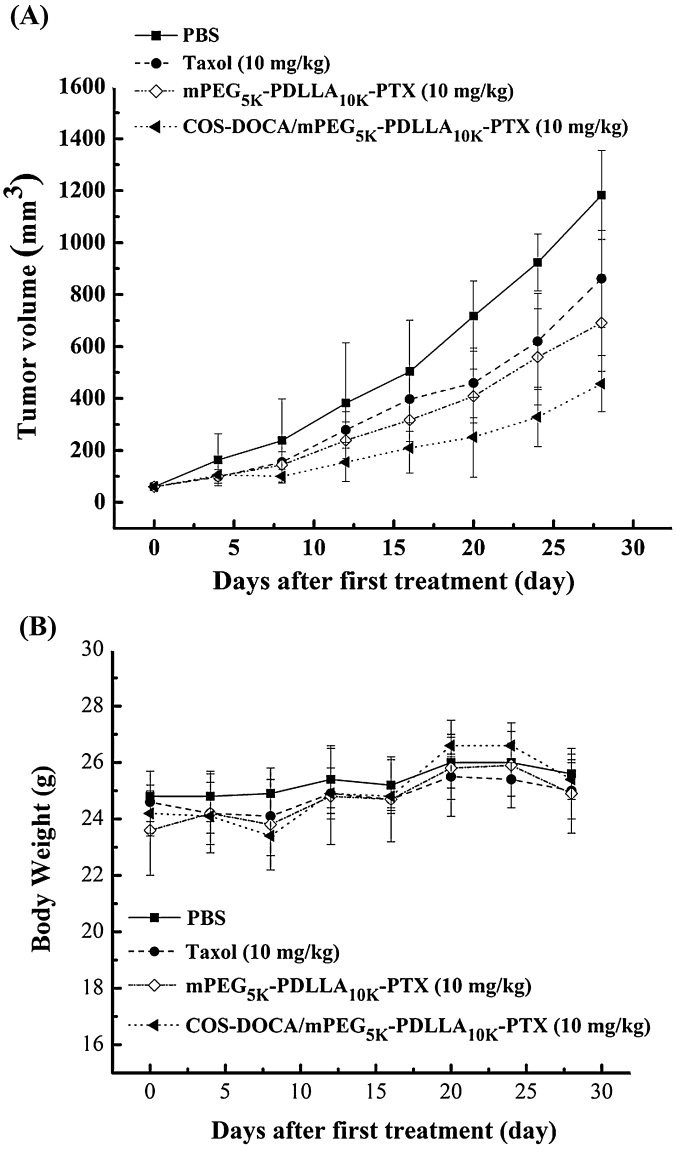


Fig. 9. (A) Antitumor effect of control and PTX-loaded COS-DOCA/mPEG-PDLLA on BALB/c tumor-bearing nude mice. (B) Body weight change of BALB/c tumor-bearing nude mice after intravenous injection according to a dose schedule regimen of three injections at 3 days intervals, ($n = 7$)

4. Conclusion

In this study, we designed and synthesized the mixed micelles composed of COS-DOCA and mPEG-PDLLA entrapping the poorly soluble anticancer drug PTX. The COS-DOCA/mPEG-PDLLA mixed micelles with ideal drug-loading content and encapsulation efficiency had a particle size around 40 nm. In vitro, PTX release was sustained as a result of encapsulation into the inner DOCA and PDLLA cores of the micelles. For MGC-803, HeLa and A549 cells, PTX-loaded COS-DOCA/mPEG -PDLLA mixed micelles mixed micelles displayed noticeable antitumor efficacy. Cellular uptake experiment indicated the addition of COS-DOCA can avoid the phagocytolysis of macrophages and facilitate the uptake of tumor cells. Zebrafish as a toxicological model revealed the toxicity of PTX-loaded mixed micelles is much lower than Taxol. Our pharmacokinetic studies demonstrated that PTX-loaded COS-DOCA/mPEG-PDLLA mixed micelles could increase the blood circulation time of PTX. In addition, the mixed micelles exhibited remarkable antitumor activity on A549 tumors in vivo and reduced

toxicity. All the evidences suggest the PTX-loaded COS-DOCA/mPEG-PDLLA mixed micelles developed in this study may be a potential delivery system to load hydrophobic anticancer drugs for the enhanced cancer treatment.

Acknowledgements

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