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Synthesis and characterization of a novel polydepsipeptide contained tri-block copolymer (mPEG–PLLA–PMMD) as self-assembly micelle delivery system for paclitaxel

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ABSTRACT

A series of biodegradable polydepsipeptides based new triblock copolymers, poly (ethylene glycol)-poly(L-lactide)–poly(3(S)-methyl-morpholine-2,5-dione) (mPEG–PLLA–PMMD) have been synthesized and characterized as self-assembly micelle delivery system for paclitaxel (PTX). Compared to the $mPEG_{2000}$–$PLLA_{2000}$ diblock copolymers, the triblock copolymers present more benefits such as lower CMC value, positive-shifted zeta potential, better drug loading efficiency and stability. Among the triblock polymers, $mPEG_{2000}$–$PLLA_{2000}$–$PMMD_{1400}$ micelles present low cytotoxicity and promote the anti-cancer activity of PTX on A-549 and HCT-116 cells. In addition, $mPEG_{2000}$–$PLLA_{2000}$–$PMMD_{1400}$ micelles prolongs the circulation time of PTX in rat after i.v. injection (5 mg/kg) than that of $mPEG_{2000}$–$PLLA_{2000}$ micelles and Taxol®. The half life ($t_{1/2\beta}$), mean residence time (MRT), $AUC_{0-\infty}$ and clearance (CL) for PTX-loaded $mPEG_{2000}$–$PLLA_{2000}$–$PMMD_{1400}$ micelles are determined to be 1.941 h, 2.683 h, 5.220 μg/mL h (1.8-fold to $mPEG_{2000}$–$PLLA_{2000}$ group), 0.967 L/h kg^{-1}, respectively. In conclusion, $mPEG_{2000}$–$PLLA_{2000}$–$PMMD_{1400}$ copolymer could be developed as one of the promising vectors to anti-cancer agents for chemotherapeutics.

1. Introduction

Paclitaxel (PTX) is a natural diterpenoid which is isolated from the extract of the bark of *Taxus brevifolia*. It is one of the most effective chemotherapeutic drugs and has been widely used for a variety of cancers (Mekhail and Markman, 2002). Since PTX is very slightly soluble in aqueous solutions (0.6 μg/mL) (Goldspiel, 1997), its commercial pharmaceutical product (Taxol®) is formulated in a mixture of Cremophor® EL/absolute ethanol (1:1 in volume). However, large amount application of Cremophor EL has been reported to cause many side effects such as hypersensitivity reactions, myelosuppression and neurotoxicity (Weiss et al., 1990). To overcome these problems, great efforts have been made to develop new delivery systems for PTX, e.g. liposomes (Klibanov et al., 1991), nanoparticles (Danhier et al., 2009a; Xiao et al., 2009; Yu et al., 2010) and polymeric micelles (Huh et al., 2005; Zhang et al., 2009). Among them, biodegradable block copolymeric micelles (BCMs) are expected to be one of the effective vectors to improve the bioavailability of PTX with specific delivery and minimal side-effects (Maeda et al., 2001, 2000). Currently, an mPEG–PLA BCM encapsulated PTX system (Genexol®-PM) is under phase II clinical trial in Korea and US.

Although Genexol®-PM exhibits less toxicity than Taxol®, it is also reported to accumulate PTX in liver and spleen. To achieve a higher PTX uptake in tumors, 3-fold dose of Genexol®-PM (compared to Taxol®) is needed. In addition, Genexol®-PM is also reported to decrease the circulation time and AUC of PTX in vivo (Kim et al., 2001). The structural instability resulted micelle aggregation and drug leakage accelerate its elimination by mononuclear phagocytic systems (MPS) (Kedar et al., 2010; Mikhail and Allen, 2009). All these mentioned limitations restrict the further applications of polymeric vectors in enhancing permeability and retention effects (EPR effect) for PTX at the site of solid tumors (Ruenraroengsak et al., 2010). Therefore, developing of a novel polymer by structural modifications as drug vector is necessary (Watanabe et al., 2006; Yang et al., 2007; Zhang et al., 2009).

Recently, several biocompatible poly amino acid based polypeptide derivatives have been developed by some investigators (Chen

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et al., 2006; Leopold and Friend, 1995; Zhang et al., 2009) and successfully applied for preparation of stabilized and/or functionized micelles (Bae and Kataoka, 2009; Cha et al., 2009; Prompruk et al., 2005; Yokoyama et al., 2004). Inspired by these investigations, introducing poly amino acid derivatives into the structure of copolymers has been considered to be another strategy for stabilized micellar drug systems (Gaucher et al., 2005). The stabilized micelles might enhance the circulation time of drugs and reduce the uptake by MPS thus facilitating their accumulation in tumor, so such structural modifications are not only expected to significantly improve the physicochemical properties but also in vivo performance of polymeric materials and become one of efficient approaches for delivery of chemotherapeutic drug.

In the present study, a novel type of biodegradable poly (ethylene glycol)-poly(L-lactide)–poly(3(S)-methyl-morpholine-2,5-dione) (mPEG–PLLA–PMMD) triblock copolymers has been designed and synthesized. Their structural characteristics are identified by using DSC and ^{1}H NMR analysis. Simultaneously, the morphology, size distribution, surface potential, stability, drug encapsulation and cytotoxicity of the triblock polymeric micelles are also characterized. Furthermore, pharmacokinetic profiles of mPEG–PLLA/mPEG–PLLA–PMMD encapsulated PTX are evaluated by comparing the in vivo pharmacokinetic properties to those of Taxol®.

2. Materials and methods

2.1. Materials

Paclitaxel (PTX) was purchased from Xi'an Sanjiang Bio-Engineering Co. Ltd. (Xi'an, China). Monomethoxy poly(ethylene glycol) (mPEG, Mn 2000) and stannous octoate were purchased from Sigma–Aldrich Co. (St. Louis, MO, USA). L-Lactide (Pharm. grade) was purchased from PURAC Far East Pte Ltd. (Singapore) and recrystallized by ethyl acetate twice before use. L-Alanine (analytical grade) was purchased from Shanghai Biochemical Reagent Company (Shanghai, China). All other reagents were of analytical grade or HPLC grade.

2.2. Synthesis of 3(S)-methyl-2,5-morpholinedione, mPEG–PLLA and mPEG–PLLA–PMMD

2.2.1. 3(S)-Methyl-2,5-morpholinedione

3(*S*)-Methyl-2,5-morpholinedione (MMD) was synthesized by a two-step reaction as described previously (Fung and Glowaky, 2003; Ouchi et al., 1998). Firstly, L-alanine (35.0 g) was dissolved in 150 mL water:diethyl ether/1:1 (v/v) after addition of 100 mL NaOH solution (4 M). A freshly prepared chloroacetyl chloride solution (1 g/mL in diethyl ether, 50 mL) and NaOH solution (4 M, 50 mL) were continuously injected into the mixture under stirring (400 rpm/min) and the pH value of the mixture was kept at 11 throughout the reaction. After the reaction, the aqueous layer was acidified to pH 1 and extracted by ethyl acetate. The combined organic layer was washed with saturated NaCl solution and dried by $MgSO_4$. The crystal of *N*-(chloroacetyl)-L-alanine was obtained under vacuum concentrating. Secondly, *N*-(chloroacetyl)-L-alanine (20.2 g) and triethylamine (13.1 g) were dissolved in 400 mL of *N*,*N*-dimethylformamide (DMF) and reacted at 90 °C for 6 h under N_2 protection. The mixture was allowed to stand for overnight at 4 °C. After removing the crystallized salt and DMF/TEA by filtration and vacuum desiccation, the crude MMD was obtained by precipitating in 50 mL of chloroform. Further purification was performed by recrystallizing in cold diethyl ether.

2.2.2. Diblock copolymers of mPEG–PLLA

$mPEG_{2000}$–$PLLA_{2000}$ was prepared as our previously reported method (Lu et al., 2008). Briefly, $mPEG_{2000}$ and L-lactide were dissolved in 20 mL 0.5% (w/w) $Sn(Oct)_2$ toluene solution in a flame-dried and nitrogen-purged flask. The flask was sealed and maintained at 150 °C for 12 h to complete the reaction. The synthesized polymer was recovered by dissolving in chloroform followed by precipitation in ice-cold diethyl ether. The resultant precipitate was filtered and dried at room temperature in vacuum.

2.2.3. Triblock copolymers of mPEG–PLLA–PMMD

$mPEG_{2000}$–$PLLA_{2000}$ and MMD were put into a pre-heated silanized flask. The flask was evacuated and refilled with dry N_2 several times then placed in a preheated oil bath (150 °C). When MMD was melted, 50 mg of stannous octoate (in 10 mL toluene) was added under vigorous stirring. After toluene evaporated, the polymerization was performed at 130 °C for 12 h and stopped by putting the tube into freezer. The synthesized triblock copolymer was recovered by dissolving in chloroform followed by precipitation in petroleum ether. The resultant precipitate was filtered, washed with isopropanol and dried in vacuum at 35 °C for 12 h. Triblock copolymers with different PMMD blocks were synthesized as the same method by changing the monomer/prepolymer ratio.

2.3. Structural identification of copolymers

After dissolving in $CDCl_3$ and/or D_2O, the ^{1}H NMR spectra of MMD, mPEG–PLLA and mPEG–PLLA–PMMD were recorded with a Bruker Avance spectrometer (AV-500; Bruker, Karlsruche, Germany) operating at 500 MHz. In addition, FT-IR spectra were obtained with KBr tablets from 64 scans at 2 cm^{-1} resolution using a Bruker FT-IR spectrometer (Tensor 27, Bruker, Germany).

2.4. Characterization of copolymers

2.4.1. Physicochemical properties of copolymers

The physicochemical properties of various copolymers including melting point (T_m), glass-transition temperature (T_g), intrinsic viscosity (η) and mean molecular weight (Mn) were determined and compared. Thermal characteristic data were collected with a Linkham DSC600 thermo plate at the heating rate of 5 °C/min. Molecular weights were calculated from peak integration of ^{1}H NMR spectra. Intrinsic viscosities of copolymers in water were determined by ubbelohde viscometer.

2.4.2. Critical micelle concentrations (CMCs)

The CMCs of mPEG–PLLA–PMMD copolymers were determined using pyrene as an extrinsic probe. An aliquot of 100 μL pyrene solution in acetone was transferred into a test tube and evaporated to dryness. 10 mL of polymeric micelles (0.05–2.0 mg/mL) was added to the tube and incubated at room temperature over night under stirring. The final concentration of pyrene was 5×10^{-7} M. Steady-state fluorescence spectra were obtained with a Shimadzu RF-5301PC luminescence spectrometer at room temperature. The measured emission wavelength was set at 392 nm and band widths of excitation and emission were set to 5 nm and 3 nm, respectively. The ratios of the excitation spectra's fluorescent intensities at 338 nm and 333 nm (I_{338}/I_{333}) were calculated and plotted against the logarithm of polymer mass concentration.

2.5. Preparation and characterization of PTX-micelles

2.5.1. Preparation of PTX-micelles

PTX loaded micelles were prepared using a glass-filter method which has been reported for liposome preparation (Katayama

et al., 2002). Briefly, 20 mg of PTX was dissolved in 20 mL acetonitrile/methanol (8:2, v/v) with 240 mg copolymers, the mixture was infiltrated into a G5 glass-filter (pore size: 1.5–2.5 μm) and the solvent was evaporated with a gentle stream of N_2 at room temperature. The drug–polymer layer formed on the glass-filter was hydrated with 20 mL of normal saline (25 °C) for 10 min and sonicated for 3 min at 25 °C (Ultrasonic Homogenizer Model KH2200DB, Hechuang, Inc., China). The micelles were passed through the filter repeatedly by alternately pressing syringes connected to both sides of the filter. Finally the mixture was filtrated through a 0.22 μm filter (Millipore, USA). Thereafter, 2.5 mL of the filtrate was transferred into a glass vial and lyophilized with a LGJ-10 lyophilizer (Gongyi Yuhua Instrument Co., Ltd., Gongyi, China) after addition of mannitol ($W_{polymer}$:$W_{manitol}$/1:1). The obtained PTX-micelles were kept at 4 °C for further experiments.

2.5.2. Quantification of PTX

Quantification of PTX in PTX-micelles was conducted using an HPLC system (SHIMADZU LC-10AD pump liquid chromatograph) coupled with a Diamonsil C-18 analytical column (250 mm × 4.6 mm I.D., 5 μm, Dikma Technology Company, China) maintained at 30 °C. The isocratic elution with a mobile phase of methanol and water (72:28, v/v) was used at a flow rate of 1.0 mL/min for separation of analytes. The analytes were monitored at the UV wavelength of 228 nm. The drug loading and entrapment efficiencies of PTX in micelles were evaluated by Eqs. (1) and (2):

$$\text{Loading efficiency}\,(\%) = \left[\frac{M_{PTX}}{M_{PTX} + M_{Polymer}}\right] \times 100 \quad (1)$$

$$\text{Entrapment efficiency}\,(\%) = \left(\frac{[PTX]_{solubilized}}{[PTX]_{added}}\right) \times 100 \quad (2)$$

in which M_{PTX}, $M_{Polymer}$, $[PTX]_{solubilized}$ and $[PTX]_{added}$ were the amount of PTX loaded in the micelles, the amount of copolymers in formulations, the concentration of PTX in micelles (after filtration) and the concentration of PTX in formulations (before filtration), respectively.

2.5.3. Size distribution and zeta potential of micelles

Droplet size distribution of micelles was determined using a Malvern Zetasizer 3000 system (Malvern instruments Ltd., Malvern, UK) based on dynamic light scattering. Measurements were performed at a fixed angle of 90° to the incident light and data were collected over a period of 3 min. Zeta potential of micelles were also recorded. The samples were diluted properly before tests.

2.5.4. Stability of PTX loaded micelles

To assess the stability of PTX-loaded micelles in aqueous solutions, PTX-loaded micelles (PTX:Copolymers/1:12, w/w) were dispersed into normal saline to yield the concentrations of PTX were about 1.0 mg/mL. All samples were kept at 25 °C until 96 h. The particle size and drug content of each micelles in solutions at 6 h, 12 h, 24 h, 48 h and 96 h were determined and compared.

2.5.5. In vitro release of PTX loaded micelles

In vitro release of PTX from micelles was conducted with a dialyzed method as reported previously (Danhier et al., 2009b). PTX-micelles were dispersed in phosphate buffered saline (PBS) with different pH and the final PTX concentrations were about 0.5 mg/mL. 1 mL of the micelles were transferred into a dialysis pocket (MWCO 8000–10,000, Spectra/Por, Houston, TX, USA) and immersed into 100 mL release medium (0.5% Tween 80 in PBS, pH 7.4, pH 6.8, pH 5.0, pH 4.0, 37 °C) under magnetic stirring at 300 rpm. 2 mL of the samples was collected and the equal volume of fresh medium was refilled at time intervals for 48 h. Concentrations of PTX in the release medium were determined by HPLC analysis.

2.5.6. Morphology study of PTX-loaded micelles

The morphology study of PTX-micelles were operated using a transmission electron microscope (Hitachi H-7650 transmission electron microscope, JOEL Ltd., Japan) at an accelerating voltage of 200 kV. A drop of PTX-loaded micelles was deposited onto a carbon-coated copper grid (200 meshes). The films on the grid were negatively stained by a drop of uranyl acetate solution (0.5%, w/v) for 90 s. The excess solution was removed with a filter paper, and followed by a thorough air-drying. The stained films were then photographed.

2.6. In vitro anti tumor activity

Mitochondrial activity of the cell was determined by MTT assay. Human lung adenocarcinoma cell A-549 and colorectal carcinoma cell HCT-116 (Cell Resource Center of China Science Academy) were seeded in 96-well plates at the density of 5×10^3 cells/well and cultured with 200 μL of Mc5A medium supplemented with 10% heat-inactivated FBS and 1% penicillin/streptomycin (100 IU/mL) in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C. For experiments, the cells were incubated with 180 μL Mc5A medium containing Taxol®, PTX-loaded mPEG–PLLA–PMMD or mPEG–PLLA micelles at the PTX concentration ranged from 0.001 to 20 μg/mL for 48 h. After another 4 h incubation with addition of 20 μL freshly prepared MTT solution (5 mg/mL), the culture medium was replaced with 150 μL of DMSO. The absorbance of the dissolved formazan dye was read at 570 nm using a microplate reader (Powerwave X, Bio-TEK Instruments, Inc., USA). Drug-free treated cells were taken as negative control (100% viability) and cells without addition of MTT were used as blank to calibrate the spectrophotometer. Cytotoxicities of blank micelles and Cremophor® EL/ethanol (50/50, v/v) solubilizer to A-549 and HCT-116 cells were assessed using the above method after diluting to the same fold as their corresponding PTX-loaded formulations.

2.7. Pharmacokinetics

Male Wistar rats (230–250 g) supplied by the New Drug Screening Center of China Pharmaceutical University were fed on a standard laboratory diet with free access to water under the controlled temperature at 20–22 °C and relative humidity of 50% with 12 h light/dark cycle. Prior to drug administration, the animals were fasted but allowed to have free access to water overnight. The experiment was conducted after approval by the Animal Ethics Committee of the China Pharmaceutical University.

Three groups of rats with at least six in each group were dosed with PTX solution (in Chremophor EL:Ethanol/50:50, v/v), PTX loaded mPEG–PLLA micelles and PTX loaded mPEG–PLLA–PMMD micelles intravenously at the dose of 5 mg/kg, respectively. Serial venous blood samples (0.25 mL) were collected into heparinized tubes at 0.08, 0.17, 0.25, 0.50, 0.75, 1, 2, 3, 4, 6, 8 h after drug administration and centrifuged immediately. A 100 μL aliquot volume of plasma was transferred into a 10 mL centrifuge tube and extracted with 5 mL of ethyl ether after addition of 20 μL internal standard (Diazepam, 500 ng/mL). The mixture was vortex-mixed for 3 min and centrifuged at 12,000 rpm for 10 min. The organic layer was separated and evaporated. The residues were re-constituted with 100 μL methanol and 20 μL of which were injected into the HPLC for analysis. The chromatography condition was similar to the in vitro quantification method described in Section 2.5.2 but with the mobile phase of methanol:water/68:32 (v/v). Pharmacokinetic parameters were calculated by DAS 2.0 Pharmacokinetics Software (Chinese Society of Mathematical Pharmacology) with

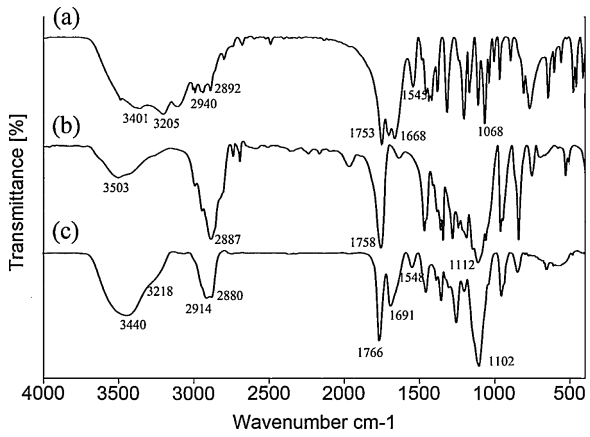


Fig. 1. FT-IR spectra recorded from KCl tablet of MMD (a), mPEG–PLLA (b) and mPEG–PLLA–PMMD (c).

compartmental or non-compartmental approaches. Results were presented as mean ± S.D.

2.8. Data analysis

Differences between the three formulations in parameters were statistically evaluated by a one-way analysis of variance test using SPSS Version 11.5. A value of $p < 0.05$ was considered significantly for all tests.

3. Results and discussion

3.1. Synthesis of mPEG–PLLA–PMMD copolymers

mPEG–PLLA–PMMD was finally synthesized by ring-opening copolymerization of 3(*S*)-methyl-2,5-morpholinedione and mPEG–PLLA. 2,5-Morpholinedione derivative is a effective cyclopeptide to introduce polypeptide structure in polymers (Ouchi et al., 2002; Wang and Feng, 1998), the synthesis route of 3(*S*)-methyl-2,5-morpholinedione and the polymerization process are shown in Scheme 1. As shown in Fig. 1, mPEG–PLLA–PMMD triblock copolymers presented a strong signal at 1766 cm^{-1} which came from the carbonyl group (C═O) in both PLLA and PMMD segments. The signal at 1691 cm^{-1} could be assigned to the amide group in MMD unit. The stretching frequency at 1102 cm^{-1} indicated the ester group (–O–C═O) existed in PLLA and PMMD chain. Compared to mPEG–PLLA, the wider and stronger absorption band from 3200 cm^{-1} to 3440 cm^{-1} assigned to the stretch of N–H group in PMMD suggested the successful introduction of MMD unit into the copolymer.

The ^{1}H NMR spectra of the MMD, mPEG–PLLA and mPEG–PLLA–PMMD are illustrated in Fig. 2(a–c). The peaks corresponding to mPEG, PLLA and PMMD segment were clearly observed in mPEG–PLLA–PMMD spectrum (Fig. 2c) and could be assigned as follows: 1.56 (NH–CH–C*H*$_3$, 3H), 1.63 (O–CH–C*H*$_3$, 3H), 3.38 (O–C*H*$_3$, 3H), 3.6–3.8 (O–C*H*$_2$–C*H*$_2$, 4H, PEG segment), 4.42 (NH–C*H*–CH$_3$, 1H), 4.8–4.9 (O═C–C*H*$_2$–O, 2H, PMMD segment), 5.15 (O═C–C*H*–O, 1H, PLLA segment), 7.92 (CH–N*H*, 1H). Compared to the spectrum of the MMD monomer, the chemical shifts of triblock copolymer do not change greatly except for the down-field shift of –NH– signal from 7.15 to 7.92 ppm. In addition, the solubility test was adopted to identify whether the polymers were triblock copolymers or a mixture of homopolymer of PMMD and mPEG–PLLA, the triblock copolymer showed good solublity in $CHCl_3$ because of the introduction of hydrophobic PLLA while the homopolymer of PMMD was insoluble in $CHCl_3$.

Table 1
Characteristics of block copolymers with varying composition.

Polymers	T_g (°C)	T_m (°C)	η (dL/g)
$mPEG_{2000}$	–	59.3	–
$mPEG_{2000}$–$PLLA_{2000}$	−22.4	52.3	0.787
$mPEG_{2000}$–$PLLA_{2000}$–$PMMD_{700}$	−26.7	47.7	0.713
$mPEG_{2000}$–$PLLA_{2000}$–$PMMD_{1400}$	−31.1	44.5	0.641
$mPEG_{2000}$–$PLLA_{2000}$–$PMMD_{2800}$	−37.4	38.9	0.581

3.2. Characterization of copolymers

3.2.1. Physicochemical properties of mPEG–PLLA–PMMD triblock copolymers

The T_m, T_g and η values of mPEG–PLLA–PMMDs with different block ratios are listed in Table 1. Different from the results reported for 6-methyl-2,5-morppholindione and caprolactone copolymers (Ye et al., 1994), decreased T_g and T_m for mPEG–PLLA–PMMD triblock copolymers were observed while compared to $mPEG_{2000}$–PLA_{2000} diblock copolymers. For $mPEG_{2000}$–PLA_{2000}–$PMMD_{2800}$, the T_g and T_m are −37.4 °C and 38.9 °C, respectively, which are much lower than $mPEG_{2000}$–PLA_{2000} (T_g −22.4 °C, T_m 52.3 °C). It might be attributed to the flexibility of PMMD segment and inhibition of the interaction among mPEG–PLLA chains. Lower intrinsic viscosities were also observed in all three triblock copolymers. The intrinsic viscosity of mPEG–PLLA dropped from 0.787 dL/g to 0.581 dL/g when PMMD block was introduced with a molecular weight of 2800. The decreased viscosity suggested lower extent of chain stretching of copolymer molecular in solvent. We propose that more spherical shape particles existed in mPEG–PLLA–PMMD micelles than those in mPEG–PLLA micelles.

Fig. 2(d) shows the ^{1}H NMR spectrum of mPEG–PLLA–PMMD in D_2O. The specific signals of –CH_3 (δ = 1.61) and –CH– (δ = 5.18) in PLLA backbone were both weakened compared to its ^{1}H NMR spectrum in $CDCl_3$ (Fig. 2c), where the peaks of –CH_2 (δ = 4.85–4.95) and –CH– (δ = 4.45) in MMD unit were not significantly changed owing to its hydrophilic property. Therefore, mPEG–PLLA–PMMD triblock polymers might be able to form a micelle with a PLLA core and a lateral shell constituted by mPEG and PMMD segments.

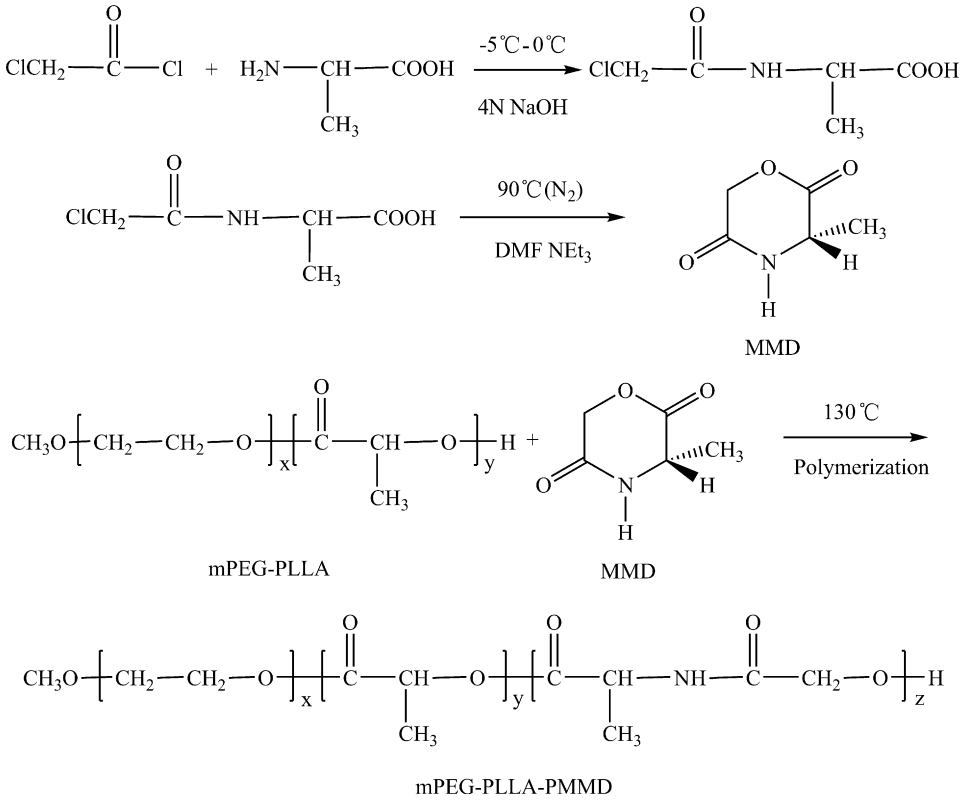
3.2.2. Measurement of critical micelle concentration

The critical micelle concentration was obtained by fluorescent determination. As listed in Table 2, lower CMC value could be determined when higher PMMD segment ratio was introduced in. As illustrated in Fig. 3, the intensity ratios of I_{338}/I_{333} increased more rapidly for $mPEG_{2000}$–$PLLA_{2000}$–$PMMD_{1400}$ triblock copolymers, the CMC value (0.0025 g/L) was significantly lower compared to $mPEG_{2000}$–$PLLA_{2000}$ (0.0171 g/L). Generally, the lower CMC values for those amphiphilic copolymers might be attributed to the higher hydrophobicity produced by the higher hydrotropic block ratio (Kang and Leroux, 2004). In this study, we obtained a copolymer with less CMC value by introducing a hydrophilic PMMD segment. It might reveal that strong polydepsipeptide interactions were existing among polymer molecules in aqueous medium, this interaction force may reduce core fluidity and enhance the formation of stable micelle structure (Burt et al., 1999; Yamamoto et al., 2002).

3.3. Preparation and characteristics of PTX-loaded micelles

3.3.1. Preparation of PTX-loaded-micelles

Before the preparation of drug loaded micelles, the particle sizes and zeta potentials of blank copolymers micelles were determined and listed in Table 2. The zeta potential values shift to positive with the increased molecular weights of PMMD block, larger



Scheme 1. The synthesis route of 3(*S*)-methyl-2,5-morpholinedione and the polymerization process of mPEG–PLLA–PMMD.

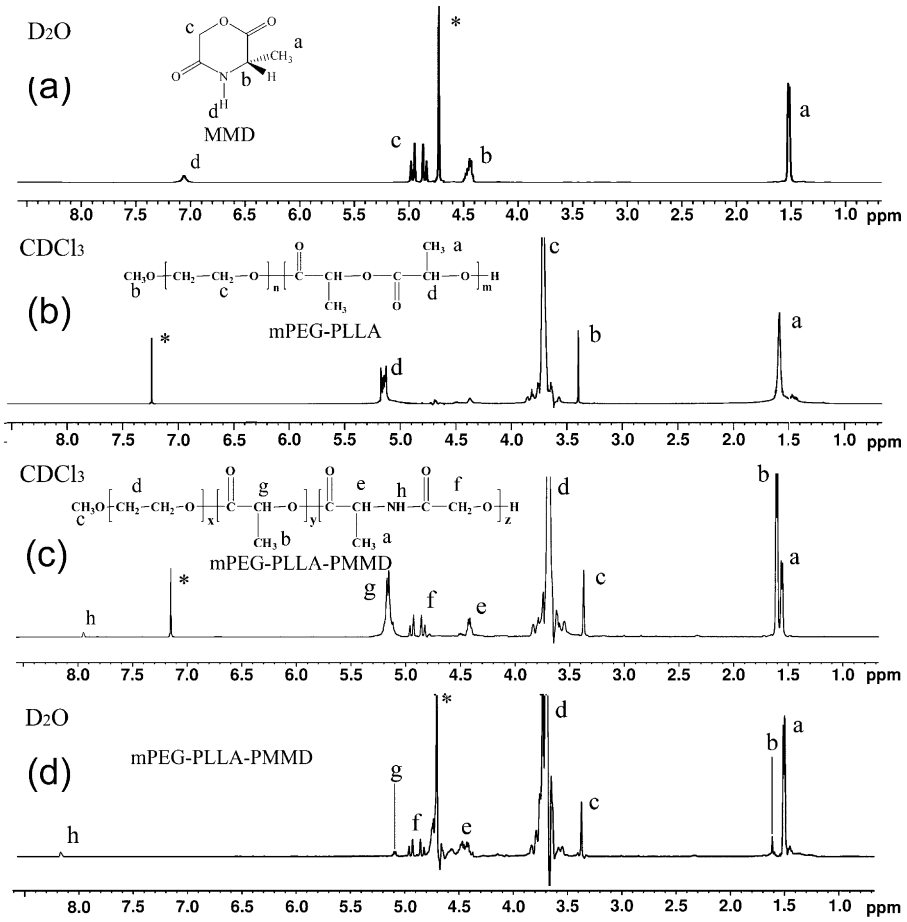


Fig. 2. ^{1}H NMR spectra of (a) MMD in D_2O, (b) mPEG–PLLA in $CDCl_3$, (c) mPEG–PLLA–PMMD in $CDCl_3$ and (d) mPEG–PLLA–PMMD in D_2O. *Solvent peak.

Table 2
Characteristics of self-assembly blank micelles and PTX loaded micelles. Data of size, zeta and poly index are shown as mean ± S.D. (n = 3).

Polymers[a]/PTX–polymer micelles[b]	Size (nm)[c]	Zeta (mV)[c]	Poly index[c]	CMC (g/L)[d]
$mPEG_{2000}$–$PLLA_{2000}$	48.9 ± 6.9	−9.1 ± 3.8	0.216	0.0175
$mPEG_{2000}$–$PLLA_{2000}$–$PMMD_{700}$	38.1 ± 5.4	2.8 ± 4.2	0.179	0.0064
$mPEG_{2000}$–$PLLA_{2000}$–$PMMD_{1400}$	42.5 ± 7.1	7.6 ± 3.3	0.225	0.0025
$mPEG_{2000}$–$PLLA_{2000}$–$PMMD_{2800}$	94.5 ± 6.0	9.4 ± 1.9	0.267	0.0014
PTX–$mPEG_{2000}$–$PLLA_{2000}$	86.4 ± 7.4	−8.5 ± 2.4	0.227	–
PTX–$mPEG_{2000}$–$PLLA_{2000}$–$PMMD_{1400}$	67.4 ± 6.5	7.9 ± 3.1	0.214	–

[a] Molecule weight was calculated from peak integration of ^{1}H NMR spectra.
[b] PTX-loaded copolymer micelles were prepared using $mPEG_{2000}$–$PLLA_{2000}$ or $mPEG_{2000}$–$PLLA_{2000}$–$PMMD_{1400}$ with a PTX concentration about 1 mg/mL.
[c] Results obtained in normal saline after proper dilution.
[d] Critical micelle concentration determined in deionized water.

size particles was formed for $mPEG_{2000}$–$PLLA_{2000}$–$PMMD_{2800}$ micelles. After reservation for 2 weeks at 25 °C, precipitation and flocculation were noticed for $mPEG_{2000}$–$PLLA_{2000}$ and $mPEG_{2000}$–$PLLA_{2000}$–$PMMD_{2800}$ micelles while opalescence was observed for $mPEG_{2000}$–$PLLA_{2000}$–$PMMD_{700}$ and $mPEG_{2000}$–$PLLA_{2000}$–$PMMD_{1400}$ micelles. It suggested that aggregation of micelles could be prevented by introducing PMMD segment with proper monomer ratios. The strong hydrogen bonding effect in $mPEG_{2000}$–$PLLA_{2000}$–$PMMD_{2800}$ micelles might result in formation of larger particles. Therefore, $mPEG_{2000}$–$PLLA_{2000}$–$PMMD_{1400}$ was selected to prepare PTX-loaded micelles in further study.

As listed in Table 2, both PTX-loaded $mPEG_{2000}$–$PLLA_{2000}$–$PMMD_{1400}$ and $mPEG_{2000}$–$PLLA_{2000}$ micelles were prepared with narrow size distributions and mean diameters of 70–90 nm. To evaluate the solubilization of copolymers to PTX, PTX-loaded micelles were prepared with different amount of PTX and 240 mg of copolymers. The solubilization and entrapment efficiency of PTX in micelles against amount of PTX added were illustrated in Fig. 4. Similar to the previous report for PTX loaded mPEG–PCL micelles (Letchford et al., 2009), precipitation was observed obviously after the maximum solubilized concentration (to what extent the drug could be solubilized by copolymers using a proper preparation method) was achieved, it might be due to the aggregation caused by the complex drug–drug and drug–polymer interactions, for PTX–mPEG–PLLA–PMMD micelles, solubilized PTX concentration increased with increased amount of PTX in formulation (from 8 to 48 mg), for PTX–mPEG–PLLA micelles, solubilized PTX concentration decreased when PTX was added more than 32 mg in formulation, the maximum solubilization of PTX by $mPEG_{2000}$–$PLLA_{2000}$–$PMMD_{1400}$ micelles at concentration of 2.0 mg/mL with drug–polymer ratio of 1:6 is significantly higher than that by $mPEG_{2000}$–$PLLA_{2000}$ micelles at 1.0 mg/mL with a drug–polymer ratio of 1:12, so the drug loading efficiency was increased from 7.7% to 14.3%. The enhanced solubilization of PTX by $mPEG_{2000}$–$PLLA_{2000}$–$PMMD_{1400}$ triblock copolymers could be explained by the stabilized inner PLLA core by PMMD block (as illustrated in Scheme 2), the more rigid structure may decrease thermal motion of polymer molecules and prevent drug aggregation, or even create higher non-covalence bonding effect with the incorporated drugs (Lee et al., 2004).

3.3.2. Stability of PTX loaded micelles

As shown in Fig. 5, PTX-loaded $mPEG_{2000}$–$PLLA_{2000}$ micelles present a faster drug leakage rate than that of PTX-loaded $mPEG_{2000}$–$PLLA_{2000}$–$PMMD_{1400}$ micelles. The relative drug content for PTX-loaded $mPEG_{2000}$–$PLLA_{2000}$ micelles dropped to 80% in 6 h and the particle size increased to 180 nm in 12 h, oversaturation drug loading (Richter et al., 2010) may exist with PTX–$mPEG_{2000}$–$PLLA_{2000}$ micelles when drug–polymer ratio was fixed at 1:12, an equilibrium could be quickly achieved after micelles were prepared. Therefore, drug precipitation occurred in several hours at 25 °C. The instable PTX loading of mPEG–PLA micelles was also mentioned before (Shin et al., 2009). In addition, no oversaturation was observed for mPEG–PLLA–PMMD micelles

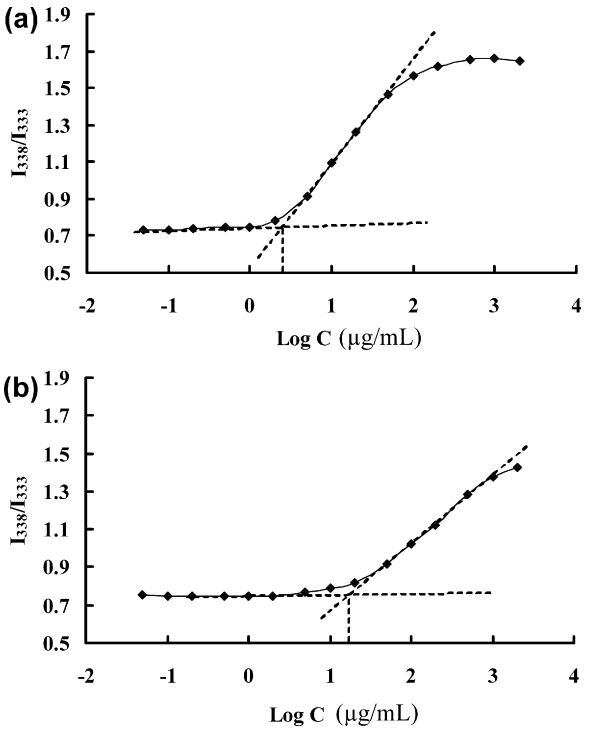


Fig. 3. Fluorescence intensity ratio variation of I_{338}/I_{333} for pyrene emission against the concentration of copolymers: (a) $mPEG_{2000}$–$PLLA_{2000}$–$PMMD_{1400}$ and (b) $mPEG_{2000}$–$PLLA_{2000}$.

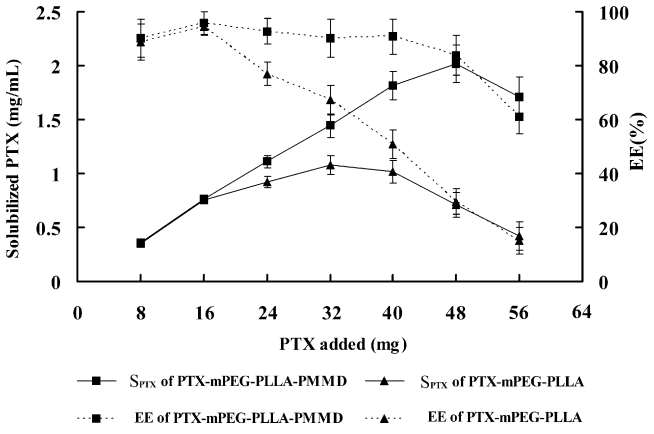
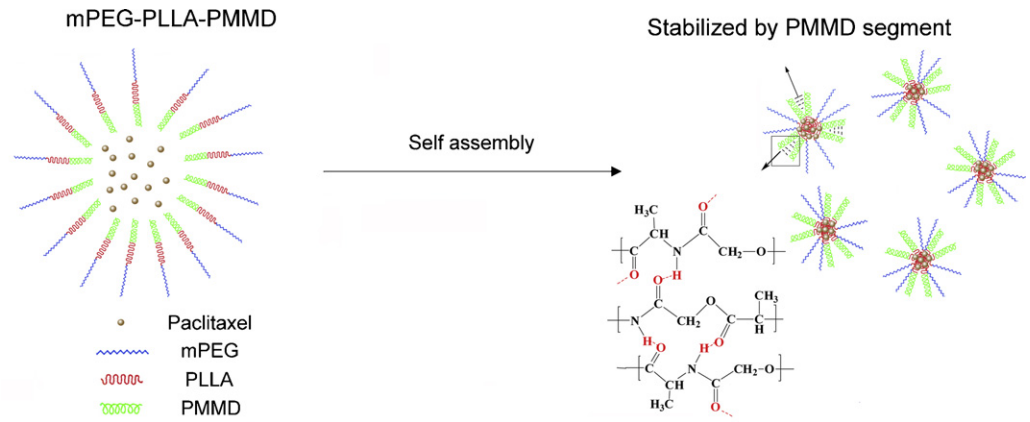


Fig. 4. Curves of PTX added in formulation against the concentration of PTX encapsuled in micelles and the entrapment efficiency (EE). Data points and error bars represent the mean ± S.D. (n = 3).



Scheme 2. Self-assembly of PTX loaded mPEG–PLLA–PMMD micelles.

under the same conditions. The lower drug leakage and particle size increasing rate (<150 nm after 48 h) suggested the increased stability of PTX encapsulated in the core of micelles.

3.3.3. *In vitro release of PTX loaded micelles*

A typical two-phase release behavior for PTX–mPEG$_{2000}$–PLLA$_{2000}$–PMMD$_{1400}$ micelles has been determined, the release profiles are illustrated in Fig. 6. A significant pH-sensitive release of PTX from mPEG$_{2000}$–PLLA$_{2000}$–PMMD$_{1400}$ micelles was observed, the inner core of triblock micelles may shrink at acidic surrounding and more stable structures were constituted to prevent the drug release. Except for a burst release found in PTX–mPEG$_{2000}$–PLLA$_{2000}$ micelles, the release rate appeared to be faster with triblock copolymers at pH 7.4. More than 60% of PTX was released in 48 h. Generally, micelles with higher drug–polymer affinity provide a lower drug release rate (Cha et al., 2009; Yoo and Park, 2001), however, the slow drug release rate with mPEG–PLLA micelles might be attributed to drug–polymer co-aggregation due to the turbid appearance of the micelles after test. The co-aggregation formed during the dialysis process led to an increasing micelle particle size and these particles could be seen as a matrix which can prevent drug diffusion. Another reason might be the dissociation of micelles. More dissociation occurred for mPEG$_{2000}$–PLLA$_{2000}$ micelles then large drug precipitation particles were formed and a fake "sustained-release" profile was presented while this is not expected since the aggregation could increase the elimination of the micelles in vivo by the mononuclear phagocytic system (MPS) and reduce drug retention in circulation.

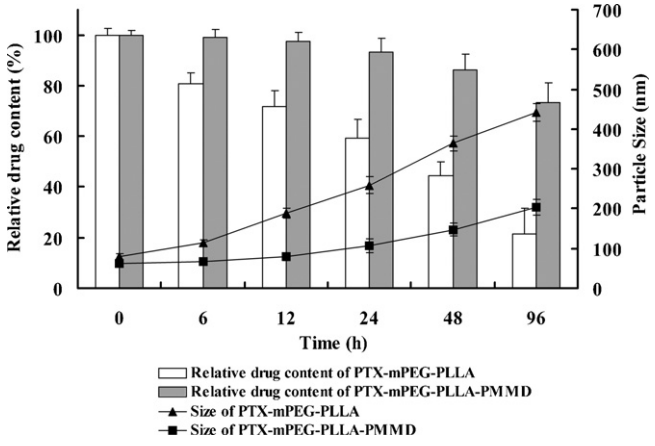


Fig. 5. The change of entrapment efficiency and particle size of PTX loaded mPEG$_{2000}$–PLLA$_{2000}$ and mPEG$_{2000}$–PLLA$_{2000}$–PMMD$_{1400}$ micelles at 25 °C in 96 h.

Moreover, the drug leakage caused by unstable micellar structure also resulted in unchanged pharmacokinetics due to plasma protein binding. The release study further demonstrated the different drug loading property for two kinds of copolymers.

3.3.4. *Morphology study of PTX-loaded micelles*

The lyophilized samples were properly diluted using normal saline before imaging work. The morphology of PTX-loaded micelles detected by TEM was shown in Fig. 7. mPEG$_{2000}$–PLLA$_{2000}$–PMMD$_{1400}$ micelles exhibited a spherical shape with nano sizes about 80 nm (Fig. 7a). In contrast, a fusion state was observed for mPEG$_{2000}$–PLLA$_{2000}$ micelles (Fig. 7b). mPEG$_{2000}$–PLLA$_{2000}$ diblock copolymer with higher CMC value perhaps have instable thermodynamic property, the molecule could be easily detached from core-shell structure and reconstituted with other detached polymeric molecule to form larger particles. The improved morphology stability of mPEG–PLLA–PMMD micelles is consistent with the results obtained from the viscosity, CMC and stability tests.

3.4. *In vitro anti-tumor activity*

Significant cytotoxicity was observed for Taxol® vehicle when the concentration was higher than 0.1 μg/mL (corresponding to PTX concentration in formulation) and the cell viability was no

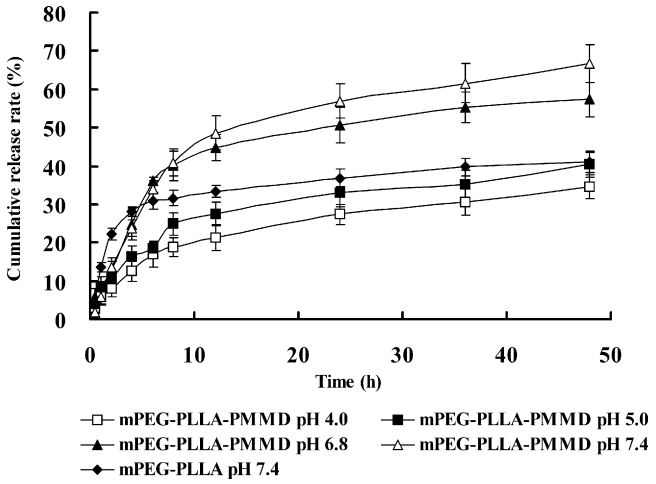


Fig. 6. PTX release profile from mPEG$_{2000}$–PLLA$_{2000}$–PMMD$_{1400}$ micelles in PBS solution at different pH. PTX–mPEG$_{2000}$–PLLA$_{2000}$ release curve at pH 7.4 was also made. All tests were performed at 37 °C with 0.5 wt% Tween 80 as a sink condition. Data represent mean ± S.D. (n = 3).

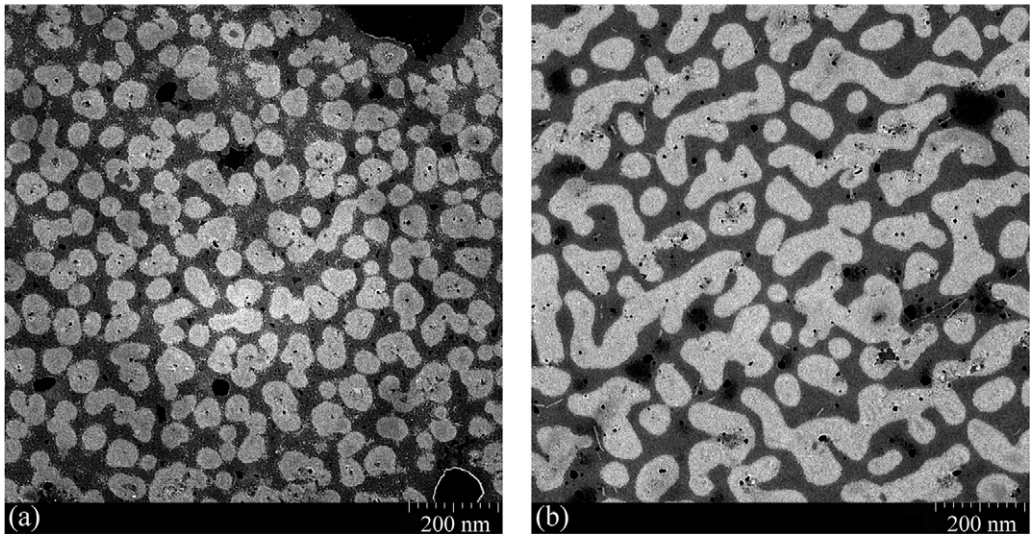


Fig. 7. Transmission electron microscopic (TEM) images of the PTX loaded micelles. (a) PTX–mPEG$_{2000}$–PLLA$_{2000}$–PMMD$_{1400}$ micelles and (b) PTX–mPEG$_{2000}$–PLLA$_{2000}$ micelles. Bars represent 200 nm.

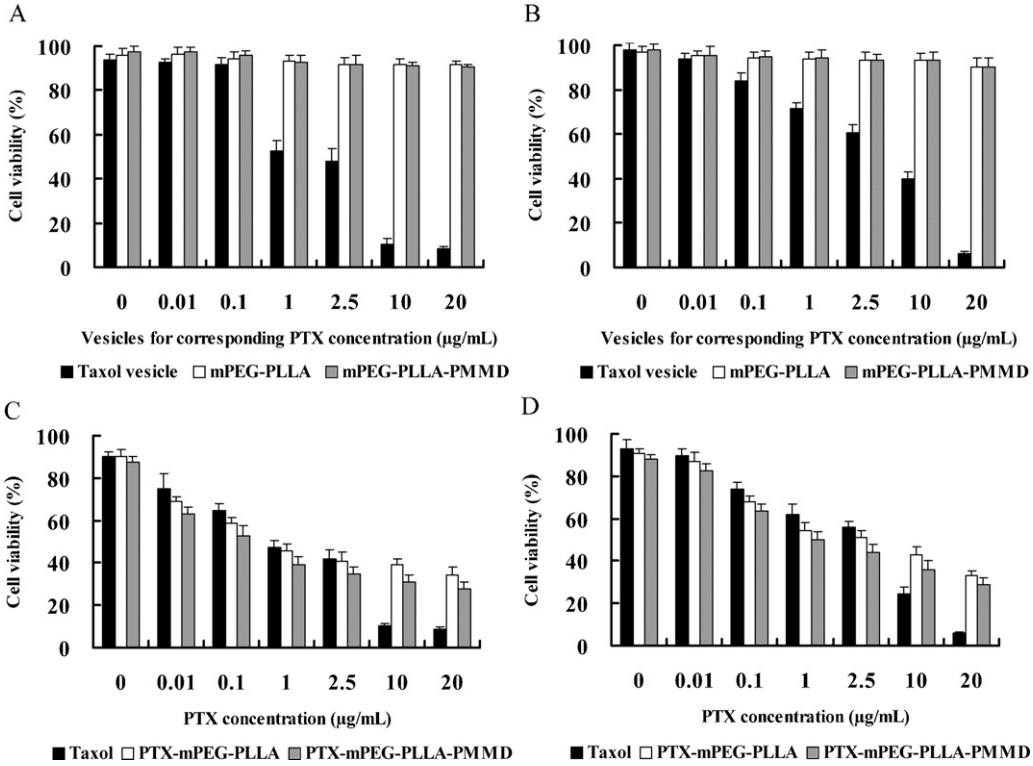


Fig. 8. Viability of the A-549 (A) and HCT-116 (B) cells as a function of varying concentrations of blank carriers (mPEG$_{2000}$–PLLA$_{2000}$–PMMD$_{1400}$, mPEG$_{2000}$–PLLA$_{2000}$ and Cremophor EL/Ethanol, the concentrations are represented as corresponding PTX concentration). In vitro cytotoxicity of various formulations of PTX against A-549 (C) and HCT-116 (D) cells. Each bar represents average ± S.D. (n = 5).

Table 3
Compartmental pharmacokinetic parameters of paclitaxel following a single intravenous (5 mg/kg) administration of Taxol®, PTX–mPEG–PLLA–PMMD and PTX–mPEG–PLLA micelles. Data are shown as mean ± S.D. (n = 6).

Parameters	Taxol®	mPEG–PLLA–PMMD micelles	mPEG–PLLA micelles
$t_{1/2\alpha}$ (h)	0.134 ± 0.036	0.039 ± 0.022*	0.063 ± 0.033*
$t_{1/2\beta}$ (h)	1.194 ± 0.581	1.941 ± 0.163*,†	1.547 ± 0.161*
V_d (L/kg)	0.818 ± 0.060	0.844 ± 0.333†	1.318 ± 0.270*
CL (L/h kg^{-1})	1.775 ± 0.393	0.967 ± 0.144*,†	1.791 ± 0.160
$AUC_{(0-t)}$ (μg/mL h)	2.848 ± 0.413	5.078 ± 0.691*	2.661 ± 0.247
$AUC_{(0-\infty)}$ (μg/mL h)	2.934 ± 0.649	5.220 ± 0.753*,†	2.800 ± 0.258

* $p < 0.05$ compared to Taxol®.
† $p < 0.05$ compared to PTX–mPEG–PLLA micelles.

Table 4
Non-compartmental pharmacokinetic parameters of paclitaxel following a single intravenous (5 mg/kg) administration of Taxol®, PTX–mPEG–PLLA–PMMD and PTX–mPEG–PLLA micelles. Data are shown as mean ± S.D. ($n = 6$).

Parameter	Taxol®	mPEG–PLLA–PMMD micelles	mPEG–PLLA micelles
$AUC_{(0-t)}$ (μg/mL h)	2.477 ± 0.437	4.773 ± 0.823*,†	2.481 ± 0.166
$AUC_{(0-\infty)}$ (μg/mL h)	2.701 ± 0.602	5.091 ± 0.825*,†	2.710 ± 0.200
$AUMC_{(0-t)}$ (μg/mL h^2)	2.275 ± 0.815	10.780 ± 2.706*,†	4.131 ± 0.659*
$AUMC_{(0-\infty)}$ (μg/mL h^2)	3.541 ± 1.950	13.747 ± 2.885*,†	5.810 ± 1.282*
MRT (h)	1.233 ± 0.433	2.683 ± 0.193*,†	1.956 ± 0.210*
$t_{1/2}$ (h)	1.037 ± 0.415	1.687 ± 0.167*	1.758 ± 0.197*
CL (L/h kg^{-1})	1.987 ± 0.471	1.005 ± 0.170*,†	1.850 ± 0.141
V_d (L/kg)	2.634 ± 0.570	2.448 ± 0.437†	4.242 ± 0.581*

* $p < 0.05$ compared to Taxol®.
† $p < 0.05$ compared to PTX–mPEG–PLLA micelles.

more than 10% at 10, 20 μg/mL. However, mPEG–PLLA–PMMD and mPEG–PLLA copolymers exhibited lower cytotoxicity at high concentration of 20 μg/mL (90% cell viability) (Fig. 8A and B). Regardless of the cytotoxicity induced by Taxol® vesicle (Huo et al., 2010), incorporation of PTX into micelles significantly enhanced the anti-cancer activity of PTX both on A-549 and HCT-116 cells at the concentration ranged from 0.001 to 1.0 μg/mL (Fig. 8C and D). The enhanced anti-cancer effect of PTX micelles might be attributed to the hypersensitizing effect of copolymers (Wei et al., 2009) and higher drug uptake through endocytosis pathway (Xiao et al., 2011). In addition, after the 2-day exposure to PTX–mPEG–PLLA–PMMD micelles, the viability of cells was significantly lower than that of PTX–mPEG–PLLA within a wide concentration range (0.01–10 μg/mL, $p < 0.05$). The IC_{50} of triblock, diblock copolymers and Taxol® (calculated based on the concentration from 0.001 to 2.5 μg/mL) for A-549 and HCT-116 cells were 0.208 ± 0.054, 0.470 ± 0.078, 0.693 ± 0.091 μg/mL; 1.03 ± 0.126, 2.130 ± 0.177, 4.820 ± 0.596 μg/mL, respectively. The lower CMC value and positive zeta potential of mPEG–PLLA–PMMD triblock copolymers might help to increase the cell binding and endocytosis of micelles thus enhancing the PTX uptake. The novel structure of copolymer might also affect the microenvironment of cell membrane and alter its protein structure and function thus decreasing P-gp mediated drug efflux from the cells (Xiao et al., 2011).

3.5. Pharmacokinetic analysis

The pharmacokinetic profiles of PTX after i.v. bolus administration of Taxol® and PTX-loaded $mPEG_{2000}$–$PLLA_{2000}$/$mPEG_{2000}$–$PLLA_{2000}$–$PMMD_{1400}$ micelles (5 mg/kg, equal to PTX) are illustrated in Fig. 9 and the calculated pharmacokinetic parameters are summarized in Table 3.

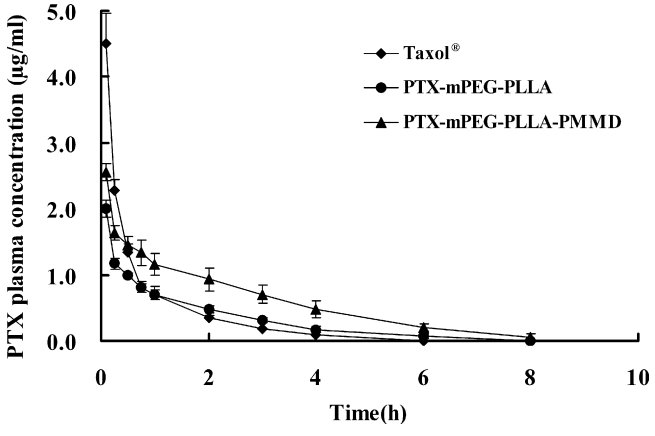


Fig. 9. Paclitaxel plasma concentration–time profile following i.v. bolus administration of 5 mg/kg paclitaxel to a rat. Data are shown as mean ± S.D. ($n = 6$).

As shown in Fig. 9, the plasma level of PTX exhibited a biphasically decline in all groups. Although the plasma concentrations of PTX for PTX–$mPEG_{2000}$–$PLLA_{2000}$ and PTX–$mPEG_{2000}$–$PLLA_{2000}$–$PMMD_{1400}$ micelles were lower than that of Taxol® at 0.08 and 0.17 h after administration, both formulations significantly prolonged the retention of PTX in circulation system. The drug circulation time extended to 8 h for PTX–mPEG–PLLA–PMMD group, which was substantially longer than that of PTX–mPEG–PLLA group. The elimination half life ($t_{1/2\beta}$), area under the plasma concentration–time curve ($AUC_{0-\infty}$) and clearance (CL) for Taxol®, PTX-loaded mPEG–PLLA and mPEG–PLLA–PMMD micelles were 1.194 h, 2.934 μg/mL h, 1.775 L/h kg^{-1}, 1.547 h, 2.800 μg/mL h, 1.791 L/h kg^{-1} and 1.941 h, 5.220 μg/mL h, 0.967 L/h kg^{-1}, respectively. Similar results could be obtained using non-compartmental model fitting (Table 4). The mean residence time (MRT) of PTX–mPEG–PLLA–PMMD group increased statistically compared to other two preparations. Higher distribution volume (V_d) was observed in mPEG–PLLA group which was similar to the results reported in mice (Kim et al., 2001). The study demonstrated that the elimination of mPEG–PLLA micelles delivery system was fast or drug leakage was fairly rapid in vivo (Chen et al., 2008), thus no significant difference was observed on AUC value while compared to Taxol®. In contrast, the relative higher initial plasma concentration and longer drug retention of PTX encapsulated in mPEG–PLLA–PMMD micelles suggested polydepsipeptides stabilized micelles may provide more stable structure in vivo. Therefore, mPEG–PLLA–PMMD micelles is considered to be a potential vector to reduce the passive uptake and elimination of PTX by MPS and increase the EPR effect.

4. Conclusion

In this study, a serial of mPEG–PLLA–PMMD triblock copolymers have been synthesized by ring-opening polymerization of 3(*S*)-methyl-2,5-morpholinedione and mPEG–PLLA. mPEG–PLLA–PMMD had lower CMC value than mPEG–PLLA, and could well self-assemble in aqueous medium to form micelles with particle size lower than 100 nm. The positive-shift zeta potential has been determined with the increased molecular weights of PMMD block. $mPEG_{2000}$–$PLLA_{2000}$–$PMMD_{1400}$ showed a better solubilizing effect for PTX (2.0 mg/mL) than $mPEG_{2000}$–$PLLA_{2000}$ (1.0 mg/mL), the drug loading efficiency thus increased to 14.3% compared to 7.7% using $mPEG_{2000}$–$PLLA_{2000}$. $mPEG_{2000}$–$PLLA_{2000}$–$PMMD_{1400}$ micelles exhibit spherical shape within nano-size scale under TEM observation and presents lower drug leakage and particle size increasing rate than that of $mPEG_{2000}$–$PLLA_{2000}$ micelles. In contrast, an fusion state has been noticed for $mPEG_{2000}$–$PLLA_{2000}$ micelles. In addition, the faster in vitro release of PTX from $mPEG_{2000}$–$PLLA_{2000}$–$PMMD_{1400}$

micelles than $mPEG_{2000}$–$PLLA_{2000}$ has been determined in PBS at pH 7.4 at 37 °C. The reason might be attributed to the aggregation or dissociation of the $mPEG_{2000}$–$PLLA_{2000}$ copolymers. It is considered that introducing of PMMD segment can enhance the stability of PTX-loaded micellar system. In summary, the novel self-assembly micelle system cannot only increase the solubility of PTX in aqueous solutions, but also enhances its anti-cancer activity on A-549 and HCT-116 cells, prolong its retention in circulation system thus improve its targeted disposition in vivo. The presented results also indicate that polydepsipeptides modification may be an effective approach to improve the properties of existing copolymer micelles.

Acknowledgements

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References

Bae, Y., Kataoka, K., 2009. Intelligent polymeric micelles from functional poly(ethylene glycol)–poly(amino acid) block copolymers. Adv. Drug Deliv. Rev. 61, 768–784.

Burt, H.M., Zhang, X., Toleikis, P., Embree, L., Hunter, W.L., 1999. Development of copolymers of poly(D,L-lactide) and methoxypolyethylene glycol as micellar carriers of paclitaxel. Colloids Surfaces B: Biointerfaces 16, 161–171.

Cha, E.J., Kim, J.E., Ahn, C.H., 2009. Stabilized polymeric micelles by electrostatic interactions for drug delivery system. Eur. J. Pharm. Sci. 38, 341–346.

Chen, H., Kim, S., He, W., Wang, H., Low, P.S., Park, K., Cheng, J.X., 2008. Fast release of lipophilic agent from PEG–PDLLA micelles revealed by in vivo Forster resonance energy transfer imaging. Langmuir 24, 5213–5217.

Chen, W., Chen, H., Hu, J., Yang, W., Wang, C., 2006. Synthesis and characterization of polyion complex micelles between poly(ethylene glycol)-grafted poly(aspartic acid) and cetyltrimethyl ammonium bromide. Colloids Surfaces A: Physicochem. Eng. Aspects 278, 60–66.

Danhier, F., Lecouturier, N., Vroman, B., Jérôme, C., Marchand-Brynaert, J., Feron, O., Préat, V., 2009a. Paclitaxel-loaded PEGylated PLGA-based nanoparticles: in vitro and in vivo evaluation. J. Control. Release 133, 11–17.

Danhier, F., Magotteaux, N., Ucakar, B., Lecouturier, N., Brewster, M., Preat, V., 2009b. Novel self-assembling PEG-p(CL-co-TMC) polymeric micelles as safe and effective delivery system for Paclitaxel. Eur. J. Pharm. Sci. 73, 230–238.

Fung, F.N., Glowaky, R.C., 2003. Bioabsorbable polydepsipeptides, their preparation and use. EP0322154A2.

Gaucher, G., Dufresne, M.H., Sant, V.P., Kang, N., Maysinger, D., Leroux, J.C., 2005. Block copolymer micelles: preparation, characterization and application in drug delivery. J. Control. Release 109, 169–188.

Goldspiel, B.R., 1997. Clinical overview of the taxanes. Pharmacotherapy 17, 110–125.

Huh, K.M., Lee, S.C., Cho, Y.W., Lee, J., Jeong, J.H., Park, K., 2005. Hydrotropic polymer micelle system for delivery of paclitaxel. J. Control. Release 101, 59–68.

Huo, M., Zhang, Y., Zhou, J., Zou, A., Yu, D., Wu, Y., Li, J., Li, H., 2010. Synthesis and characterization of low-toxic amphiphilic chitosan derivatives and their application as micelle carrier for antitumor drug. Int. J. Pharm. 394, 162–173.

Kang, N., Leroux, J.C., 2004. Triblock and star-block copolymers of N-(2-hydroxypropyl)methacrylamide or N-vinyl-2-pyrrolidone and D,L-lactide: synthesis and self-assembling properties in water. Polymer 45, 8967–8980.

Katayama, K., Kato, Y., Onishi, H., Nagai, T., Machida, Y., 2002. Preparation of novel double liposomes using the glass-filter method. Int. J. Pharm. 248, 93–99.

Kedar, U., Phutane, P., Shidhaye, S., Kadam, V., 2010. Advances in polymeric micelles for drug delivery and tumor targeting. Nanomed.: Nanotechnol. Biol. Med. 6, 714–729.

Kim, S.C., Kim, D.W., Shim, Y.H., Bang, J.S., Oh, H.S., Kim, S.W., Seo, M.H., 2001. In vivo evaluation of polymeric micellar paclitaxel formulation: toxicity and efficacy. J. Control. Release 72, 191–202.

Klibanov, A.L., Maruyama, K., Beckerleg, A.M., Torchilin, V.P., Huang, L., 1991. Activity of amphipathic poly(ethylene glycol) 5000 to prolong the circulation time of liposomes depends on the liposome size and is unfavorable for immunoliposome binding to target. Biochim. Biophys. Acta: Biomembr. 1062, 142–148.

Lee, J., Cho, E.C., Cho, K., 2004. Incorporation and release behavior of hydrophobic drug in functionalized poly(D,L-lactide)-block-poly(ethylene oxide) micelles. J. Control. Release 94, 323–335.

Leopold, C.S., Friend, D.R., 1995. In vitro study drug for the assessment of poly(L-aspartic acid) as a drug carrier for colon-specific drug delivery. Int. J. Pharm. 126, 139–145.

Letchford, K., Liggins, R., Wasan, K.M., Burt, H., 2009. In vitro human plasma distribution of nanoparticulate paclitaxel is dependent on the physicochemical properties of poly(ethylene glycol)-block-poly(caprolactone) nanoparticles. Eur. J. Pharm. Biopharm. 71, 196–206.

Lu, Y., Li, J., Wang, G., 2008. In vitro and in vivo evaluation of mPEG–PLA modified liposomes loaded glycyrrhetinic acid. Int. J. Pharm. 356, 274–281.

Maeda, H., Sawa, T., Konno, T., 2001. Mechanism of tumor-targeted delivery of macromolecular drugs, including the EPR effect in solid tumor and clinical overview of the prototype polymeric drug SMANCS. J. Control. Release 74, 47–61.

Maeda, H., Wu, J., Sawa, T., Matsumura, Y., Hori, K., 2000. Tumor vascular permeability and the EPR effect in macromolecular therapeutics: a review. J. Control. Release 65, 271–284.

Mekhail, T.M., Markman, M., 2002. Paclitaxel in cancer therapy. Expert Opin. Pharmacother. 3, 755–766.

Mikhail, A.S., Allen, C., 2009. Block copolymer micelles for delivery of cancer therapy: transport at the whole body, tissue and cellular levels. J. Control. Release 138, 214–223.

Ouchi, T., Miyazaki, H., Arimura, H., Tasaka, F., Hamada, A., Ohya, Y., 2002. Synthesis of biodegradable amphiphilic AB-type diblock copolymers of lactide and depsipeptide with pendant reactive groups. J. Polym. Sci.: Part A: Polym. Chem. 40, 1218–1225.

Ouchi, T., Seike, H., Nozaki, T., Ohya, Y., 1998. Synthesis and characteristics of polydepsipeptide with pendant thiol groups. J. Polym. Sci. A: Polym. Chem., 36.

Prompruk, K., Govender, T., Zhang, S., Xiong, C.D., Stolnik, S., 2005. Synthesis of a novel PEG-block-poly(aspartic acid-stat-phenylalanine) copolymer shows potential for formation of a micellar drug carrier. Int. J. Pharm. 297, 242–253.

Richter, A., Olbrich, C., Krause, M., Hoffmann, J., Kissel, T., 2010. Polymeric Micelles for parenteral delivery of Sagopilone: physicochemical characterization, novel formulation approaches and their toxicity assessment in vitro as well as in vivo. Eur. J. Pharm. Biopharm. 75, 80–89.

Ruenraroengsak, P., Cook, J.M., Florence, A.T., 2010. Nanosystem drug targeting: facing up to complex realities. J. Control. Release 141, 265–276.

Shin, H.C., Alani, A.W.G., Rao, D.A., Rockich, N.C., Kwon, G.S., 2009. Multi-drug loaded polymeric micelles for simultaneous delivery of poorly soluble anticancer drugs. J. Control. Release 140, 294–300.

Wang, D., Feng, X.D., 1998. Copolymerization of caprolactone with (3S)-3-[(benzyloxycarbonyl)methyl]morpholine-2,5-dione and the ^{13}C NMR sequence analysis of the copolymer. Macromolecules 31, 3824–3831.

Watanabe, M., Kawano, K., Yokoyama, M., Opanasopit, P., Okano, T., Maitani, Y., 2006. Preparation of camptothecin-loaded polymeric micelles and evaluation of their incorporation and circulation stability. Int. J. Pharm. 308, 183–189.

Wei, Z., Hao, J., Yuan, S., Li, Y., Juan, W., Sha, X., Fang, X., 2009. Paclitaxel-loaded Pluronic P123/F127 mixed polymeric micelles: formulation, optimization and in vitro characterization. Int. J. Pharm. 376, 176–185.

Weiss, R.B., Donehower, R.C., Wiernik, P.H., 1990. Hypersensitivity reactions from Taxol. J. Clin. Oncol. 8, 1263–1268.

Xiao, K., Luo, J., Fowler, W.L., Li, Y., Lee, J.S., Xing, L., Cheng, R.H., Wang, L., Lam, K.S., 2009. A self-assembling nanoparticle for paclitaxel delivery in ovarian cancer. Biomaterials 30, 6006–6016.

Xiao, L., Xiong, X., Sun, X., Zhu, Y., Yang, H., Chen, H., Gan, L., Xu, H., Yang, X., 2011. Role of cellular uptake in the reversal of multidrug resistance by PEG-b-PLA polymeric micelles. Biomaterials 32, 5148–5157.

Yamamoto, Y., Yasugi, K., Harada, A., Nagasaki, Y., Kataoka, K., 2002. Temperature-related change in the properties relevant to drug delivery of poly(ethylene glycol)-poly(L-lactide) block copolymer micelles in aqueous milieu. J. Control. Release 82, 359–371.

Yang, L., Zhao, Z., Wei, J., El Ghzaoui, A., Li, S., 2007. Micelles formed by self-assembling of polylactide/poly(ethylene glycol) block copolymers in aqueous solutions. J. Colloid Interface Sci. 314, 470–477.

Ye, W.P., Gu, Z.W., Yang, J.Y., Xu, L., Chen, X.L., Hou, M.S., 1994. Synthesis and characterization of novel biodegradable block copolymers of 6-methylmorpholindione and caprolactone. Acta Polym. Sin. 5, 609–614.

Yokoyama, M., Opanasopit, P., Okano, T., Kawano, K., Maitani, Y., 2004. Polymer design and incorporation methods for polymeric micelle carrier system containing water-insoluble anti-cancer agent camptothecin. J. Drug Target. 12, 373–384.

Yoo, H.S., Park, T.G., 2001. Biodegradable polymeric micelles composed of doxorubicin conjugated PLGA-PEG block copolymer. J. Control. Release 70, 63–70.

Yu, D.H., Lu, Q., Xie, J., Fang, C., Chen, H.Z., 2010. Peptide-conjugated biodegradable nanoparticles as a carrier to target paclitaxel to tumor neovasculature. Biomaterials 31, 2278–2292.

Zhang, L., Rakotondradany, F., Myles, A.J., Fenniri, H., Webster, T.J., 2009. Arginine–glycine–aspartic acid modified rosette nanotube–hydrogel composites for bone tissue engineering. Biomaterials 30, 1309–1320.