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α-Tocopherol succinate-modified chitosan as a micellar delivery system for paclitaxel: Preparation, characterization and in vitro/in vivo evaluations

Na Liang[a], Shaoping Sun[b], Xuefeng Li[a], Hongze Piao[a], Hongyu Piao[a], Fude Cui[a,*], Liang Fang[a]

[a] School of Pharmacy, Shenyang Pharmaceutical University, No. 103, Wenhua Road, Shenyang 110016, China
[b] School of Chemistry and Material Science, Heilongjiang University, No. 74, Xuefu Street, Harbin 150080, China

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ABSTRACT

α-Tocopherol succinate hydrophobically modified chitosan (CS-TOS) containing 17 α-tocopherol groups per 100 anhydroglucose units was synthesized by coupling reaction. The formation of CS-TOS was confirmed by ^{1}H NMR and FT-IR analysis. In aqueous medium, the polymer could self-aggregate to form micelles, and the critical micelle concentration (CMC) was determined to be 5.8×10^{-3} mg/ml. Transmission electron microscopy (TEM) observation revealed that both bare and paclitaxel-loaded micelles were near spherical in shape. The mean particle size and zeta potential of drug-loaded micelles were about 78 nm and +25.7 mV, respectively. The results of DSC and XRD analysis indicated that paclitaxel was entrapped in the micelles in molecular or amorphous state. In vitro cytotoxicity and hemolysis study revealed the effectiveness and safety of this delivery system, which was further confirmed by the in vivo antitumor evaluations. It can be concluded that the CS-TOS was a potential micellar carrier for paclitaxel.

1. Introduction

Paclitaxel (PTX), as one of the most exciting chemotherapeutic drugs, which exerts its antitumor effect primarily by stabilizing the microtubules during mitosis, has been successfully used in the clinical treatment of several cancer types, especially breast and ovarian cancer (Singla et al., 2002). Due to its poor water solubility (approximately <2 μg/ml) (Liggins et al., 1997), PTX is currently solubilized in a 50:50 mixture of Cremophor EL and dehydrated ethanol as Taxol®. Unfortunately, Cremophor EL was shown to induce severe side effects like hypersensitivity, neurotoxicity, and nephrotoxicity (Gelderblom et al., 2001). Therefore, many attempts have been made to find less toxic and better tolerated carriers for PTX delivery.

Among these new delivery systems, polymeric micelles have attracted increasing interest (Alani et al., 2010; Qu et al., 2009; Sawant et al., 2008; Zhang et al., 2010) because of their special characteristics, such as good solubilization efficiency (Montazeri Aliabadi et al., 2005), high stability upon dilution (Torchilin, 2007), and reducing non-selective reticuloendothelial system (RES) scavenge (Kataoka et al., 2000). The nanoscale dimensions of polymeric micelles also permit the efficient accumulation in tumor tissues via the enhanced permeability and retention (EPR) effect (Maeda et al., 2000). Polymeric micelles have unique core–shell architecture, which is composed of hydrophobic segments as the inner core and hydrophilic segments as the outer shell in aqueous medium. Poorly water-soluble drugs can be solubilized within the core by hydrophobic interactions (Kwon and Okano, 1996).

To date, numerous amphiphilic block or graft copolymers have been synthesized for micellar drug delivery applications. Among them, chitosan has been extensively studied due to its excellent biocompatibility, biodegradability, nontoxicity, and low immunogenicity (Chen et al., 2006; Francis Suh and Matthew, 2000). However, natural chitosan has its intrinsic limitation for it can be dissolved only in acidic water, and after hydrophobically modification, ideal micelles were hard to be prepared. In view of that, water-soluble chitosan with low molecular weight and high degree of deacetylation was chosen as the hydrophilic part of the polymer in our study, whose amphiphilic derivatives have been demonstrated as potential carriers for micelles of hydrophobic drugs by many researchers (Chen et al., 2003; Hu et al., 2008; Huo et al., 2010; Liu et al., 2004; Ngawhirunpat et al., 2009). On the other hand, α-tocopherol is an excellent solvent for many poorly soluble drugs because of its good lipophilic nature (Nielsen et al., 2001). It may therefore provide sufficient capacity for hydrophobic drugs.

Abbreviations: CS, chitosan; α-TOS, α-tocopherol succinate; CS-TOS, α-tocopherol succinate-modified chitosan; PTX, paclitaxel; EDC, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride; NHS, N-hydroxysuccinimide; TNBS, 2,4,6-trinitrobenzene sulfonic acid; DS, degree of the substitution; EE, drug encapsulation efficiency; DL, drug loading.

* Corresponding author at: 32# Box, School of Pharmacy, Shenyang Pharmaceutical University, No. 103, Wenhua Road, Shenyang 110016, China.
Tel.: +86 24 23986355; fax: +86 24 23986355.
E-mail addresses: cuifude@163.com, syphucuifude@163.com (F. Cui).

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In consideration of that, α-tocopherol succinate was introduced to the polymer.

In this study, we developed the polymeric micelle system of α-tocopherol succinate-modified chitosan (CS-TOS) for paclitaxel delivery. The amphiphilic chitosan derivative was synthesized by coupling reaction. In aqueous medium, the conjugate can self-assemble to form micelles. Based on this property, paclitaxel was incorporated into the micellar core for intravenous delivery. The preparation, characterization, properties of the micelles, and in vitro/in vivo evaluations were studied in detail.

2. Materials and methods

2.1. Materials

Water-soluble chitosan (CS) with molecular weight of 30 kDa and degree of deacetylation > 90% was purchased from Kittolife Co., Ltd., Seoul, Korea. α-Tocopherol succinate (α-TOS) was a kind gift from Xinchang Pharmaceutical Co., Ltd., Zhejiang, China. 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS) were purchased from Shanghai Medpep Co., Ltd., Shanghai, China. 2,4,6-Trinitrobenzene sulfonic acid (TNBS), pyrene (purity > 99%), and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were obtained from Sigma Chemical Co., St. Louis, USA. Paclitaxel (PTX, purity of 99.9%) was purchased from Tianfeng Bioengineering Technology Co., Ltd., Liaoning, China. Cremophor EL was kindly supplied by BASF Corp., Ludwigshafen, Germany. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), and penicillin–streptomycin mixture were purchased from Gibco BRL, Carlsbad, USA. All other chemicals and solvents were of analytical or chromatographic grade and used without further purification. Distilled water or Milli-Q water was used in all experiments.

2.2. Animals and cell line

The New Zealand rabbit (male, weighing 2 kg) and specific pathogen-free female Kunming mice (5–6 weeks old, weighing 20–25 g) were purchased from Laboratory Animal Center of Shenyang Pharmaceutical University, Liaoning, China. MCF-7 cells (human breast cancer cells) were acquired from American Type Culture Collection, Manassas, USA.

Procedures involving animals complied with ethical guidelines and were approved by the Shenyang Pharmaceutical University Animal Ethics Committee.

2.3. Synthesis of α-tocopherol succinate-modified chitosan (CS-TOS)

The CS-TOS was synthesized by the coupling reaction of carboxyl group of α-tocopherol succinate with amino group of chitosan in the presence of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and N-hydroxysuccinimide (NHS), referring to the procedure described by Lee et al. (1998) with minor modification.

Briefly, the water-soluble chitosan was dissolved in distilled water and α-TOS was dissolved in dimethylformamide, both by sonication treatment in water bath at room temperature (Sonic Purger Model KH7200DB, Kunshan Ultrasonic Instruments Co., Ltd., Shanghai, China). To activate the carboxyl group of α-TOS, equal amount (1.5 equivalents of α-TOS) of EDC and NHS were added into the chitosan solution, which allowed formation of the amide linkage by reacting with the primary amino groups of chitosan. Afterwards, the α-TOS solution was added to the chitosan solution in dropwise manner. After completely dripped, the reaction mixture was kept in agitation at room temperature in dark condition. Twenty-four hours later, the mixture was poured into methanol/ammonia solution (7/3, v/v). The resulting precipitate was collected by centrifugation, and washed thoroughly with methanol to remove the unreacted α-TOS.

After above processes, the yellow gel precipitate was dissolved in 20 ml acidic distilled water, and then dialyzed against distilled water by using dialysis membranes (MWCO: 3.5 kDa, Viskase Companies Inc., USA) for 48 h in order to remove other water-soluble by-products. Finally, the dialyzed solution was lyophilized to get the CS-TOS powder (Freeze Dryer Model FD-1C-50, Boyikang Experimental Instrument Co., Ltd., Beijing, China).

2.4. Characterization of CS-TOS

2.4.1. Proton nuclear magnetic resonance (^{1}H NMR) analysis

To confirm the formation of CS-TOS, high-resolution ^{1}H NMR spectra were collected using a Bruker Avance spectrometer (AV-600, Bruker, Karlsruhe, Germany) operated at 600 MHz. Native and modified chitosan were dissolved in the mixture of deuterated water (D_2O) and tetradeuteroacetic acid (CD_3COOD) at a concentration of 1% (w/v).

2.4.2. Fourier-transform infrared (FT-IR) analysis

In order to determine the chemical interaction between CS and α-TOS, FT-IR spectra of CS, α-TOS, their physical mixture and CS-TOS were recorded in KBr discs on Fourier-transform infrared spectrometer (Bruker IFS-55, Bruker, Switzerland) in the range from 4000 to 400 cm^{-1}.

2.4.3. Measurement of the degree of substitution (DS)

The degree of the substitution is defined as the number of α-tocopherol groups per 100 anhydroglucose units (amino groups) of CS-TOS, and it was determined by TNBS method (Bernkop-Schnürch and Krajicek, 1998), which measures the amount of remaining primary amino residues on the polymer using 2,4,6-trinitrobenzene sulfonic acid (TNBS reagent).

2.4.4. Determination of critical micelle concentration (CMC)

To prove the potential of hydrophobic microdomain formation, the CMC of CS-TOS in aqueous medium was determined using a spectrofluorophotometer (F-2500 FL Spectrophotometer, Hitachi Ltd., Japan) with pyrene as the fluorescence probe (Kalyanasundaram and Thomas, 1977; Zhao et al., 1990).

2.5. Preparation of PTX-loaded CS-TOS micelles

The incorporation of PTX into polymeric micelles was carried out by a probe-type ultrasonic method. The concrete steps were as follows: firstly, 10 mg of CS-TOS was dissolved in 10 ml of distilled water, then desired volume of PTX-acetone solution at the concentration of 1 mg/ml was added quickly into the aqueous phase under probe-sonication, and the resultant mixture was further ultrasonicated at 400 W for 30 min (JY92-II, Ningbo Scientz Biotechnology Co., Ltd., China). To keep the sample solution from being heated, the sonication was carried out in an ice bath, with the pulse function on for 3 s and off for 2 s. In order to remove unloaded PTX from the mixture, the product was centrifuged at 4000 rpm for 10 min. Subsequently, the resultant supernatant was lyophilized at a condenser temperature of −52 °C and pressure of less than 20 Pa to obtain PTX-loaded CS-TOS micelles.

2.6. Characterization of PTX-loaded CS-TOS micelles

2.6.1. Differential scanning calorimetry (DSC) analysis

Four samples including PTX, blank micelles, the physical mixture of PTX and blank micelles, and PTX-loaded micelles were

weighted and sealed in the aluminum pans, then scanned from 25 to 320 °C at a heating rate of 4 °C/min on the Mettler Toledo DSC Star System (Mettler Toledo, Switzerland). The micellar solutions were lyophilized before the test. Analysis was performed under a nitrogen gas atmosphere.

2.6.2. X-ray diffraction (XRD) analysis

XRD analysis was performed using an X-ray diffractometer (Geigerflex, Rigaku Co., Japan) with Cu Kα radiation in the range of 3–50° (2θ) at 30 kV and 30 mA. Samples were scanned at a scanning speed of 2°/min and step size of 0.02°.

2.6.3. Transmission electron microscopy (TEM) observation

The morphology of blank and PTX-loaded micelles was observed with transmission electron microscope (Jeol JEM1200EX, Tokyo, Japan) operated at an accelerating voltage of 60 kV. Negative staining of samples was performed as follows: one drop of sample solution was placed onto a copper grid, which was coated with carbon. After air-drying for 5 min, samples were negatively stained by using a 2% (w/v) phosphotungstic acid solution, and air-dried before observation.

2.6.4. Measurement of particle size and zeta potential

The size of the polymeric micelles as number-weighted hydrodynamic diameter, their distribution, and zeta potential were determined by dynamic light scattering (DLS) method using Nicomp 380/ZLS (Nicomp Instruments, Particle Sizing Systems, USA).

2.7. Reverse-phase HPLC analysis of PTX

2.7.1. Measurement of PTX concentration in the micellar solution

The concentrations of PTX were determined using a reverse-phase HPLC system, which consisted of a mobile phase delivery pump (Jasco PU-980 Intelligent HPLC pump, Jasco, Japan) and a UV detector (Jasco UV-975 Intelligent UV/Vis detector, Jasco, Japan). For separation, a Diamonsil™ C18 reverse-phase column (200 mm × 4.6 mm, 5 μm, Dikma Technologies Inc., China) was used. The mobile phase was composed of acetonitrile and water at the ratio of 60:40 (v/v). The flow rate and column temperature were set at 1.0 ml/min and 30 °C, respectively. The UV absorbance was determined at 227 nm with injection volume of 20 μl.

2.7.2. Determination of drug encapsulation efficiency (EE%) and drug loading (DL%)

The drug encapsulation efficiency and drug loading of the PTX-loaded micelles were measured as follows: before lyophilization, 100 μl of PTX-loaded micelles solution with CS-TOS concentration of 1.0 mg/ml was added into the ultrafilter (Vivaspin 500, MWCO 10k, Sartorius Co., Germany), and centrifuged at 10,000 rpm for 10 min. The PTX amount in the ultrafiltrate was regarded as unentrapped drug amount (W_1), which was measured by HPLC method. For determination of the total PTX amount (W_0) in the solution, another 100 μl of identical PTX-loaded micelles solution was diluted 100-fold by methanol under ultrasonic treatment to extract PTX from the micelles. The drug encapsulation efficiency (EE%) and drug loading (DL%) of PTX-loaded micelles could be calculated by the following equations:

$$\text{EE\%} = \frac{(W_0 - W_1)}{W_0} \times 100\% \quad (1)$$

$$\text{DL\%} = \frac{(W_0 - W_1)}{(W_0 - W_1 + 100)} \times 100\% \quad (2)$$

where W_0 is the total PTX content in 100 μl of the solution, and W_1 is the PTX content in the ultrafiltrate. The unit was microgram.

2.8. In vitro hemolysis test

To investigate the hemolytic potential of PTX-loaded micelles, in vitro hemolysis test was performed referring to the method described by Burt et al. (1999). At first, rabbit blood was collected from the marginal ear vein, and the fibrinogen was removed by stirring with a glass rod. Afterwards, the blood was washed with 0.9% NaCl, and the supernatant was discarded after centrifugation at 3000 rpm for 10 min. The above process was repeated until the supernatant solution was achromatic. Finally, the red blood cells at the bottom of the centrifuge tube were diluted with normal saline to obtain a 2% suspension (v/v). For determination, different amounts of lyophilized PTX-loaded micelles were dispersed in 0.9% NaCl, and 2.5 ml of the mixture was added into the tubes with 2.5 ml of 2% erythrocyte suspension in each. The final concentrations of PTX ranged from 10 to 200 μg/ml. After gently mixing and 4 h of incubation at 37 °C, samples were centrifuged at 3000 rpm for 10 min, then the supernatant was collected and analyzed for released hemoglobin by spectrophotometric determination at 540 nm (UV-Vis Spectrophotometer Model 752, Shanghai Spectrum Instruments Co., Ltd., China). Normal saline and distilled water were employed to obtain the negative and positive control, respectively. The degree of hemolysis was calculated by the following equation. For comparison, the Cremophor EL-based formulation was also tested.

$$\text{Hemolysis}\ (\%) = \frac{(A_{sample} - A_{0\%})}{(A_{100\%} - A_{0\%})} \times 100\% \quad (3)$$

where A_{sample}, $A_{0\%}$, and $A_{100\%}$ are the absorbance of the samples, negative control, and positive control, respectively.

2.9. In vitro cytotoxicity against MCF-7 cells

The cytotoxicity of free drug in dimethyl sulfoxide (DMSO), Cremophor EL-based PTX formulation, PTX-loaded CS-TOS micelles, and CS-TOS itself against MCF-7 cells was assessed by MTT method. The experiment was carried out as follows: 50 μl of MCF-7 cells which were in the logarithmic growth phase were seeded in a 96-well microtiter plate at the density of 1×10^4 cells per well. The Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin solution was utilized as the cell culture medium. Cells were cultivated at 37 °C in a humidified atmosphere containing 5% CO_2. When the cell confluence reached to 75%, the cells were incubated with samples for 24 h at the equivalent drug concentrations of 0.0625, 0.125, 0.25, 0.5, 1, and 2 μM. After incubation, 10 μl of MTT solution (5 mg/ml in 0.02 M phosphate buffer) was added to each well, and the plate was incubated for a further 4 h. At the determined time, unreacted MTT was removed by aspiration, and 100 μl of DMSO was added to dissolve the purple formazan crystals. The absorbance was measured at 570 nm using a Bio-Tek Synergy HT plate reader (Bio-Tek Instruments Inc., USA). Untreated cells were taken as the control with 100% viability. Cell viability was calculated as follows:

$$\text{Cell viability}\ (\%) = \frac{\text{absorbance of cells exposed to samples}}{\text{absorbance of untreated cells}} \times 100\% \quad (4)$$

2.10. In vivo antitumor efficacy study

Specific pathogen-free female Kunming mice, 5–6 weeks old, weighing 20–25 g were used for this study. The mice were housed five per cage at 21 ± 2 °C and 50–60% relative humidity with a 12 h light/dark cycle. Sterilized food and water were accessible ad libitum, and all the animals were acclimatized to laboratory conditions for a week before the experiments.

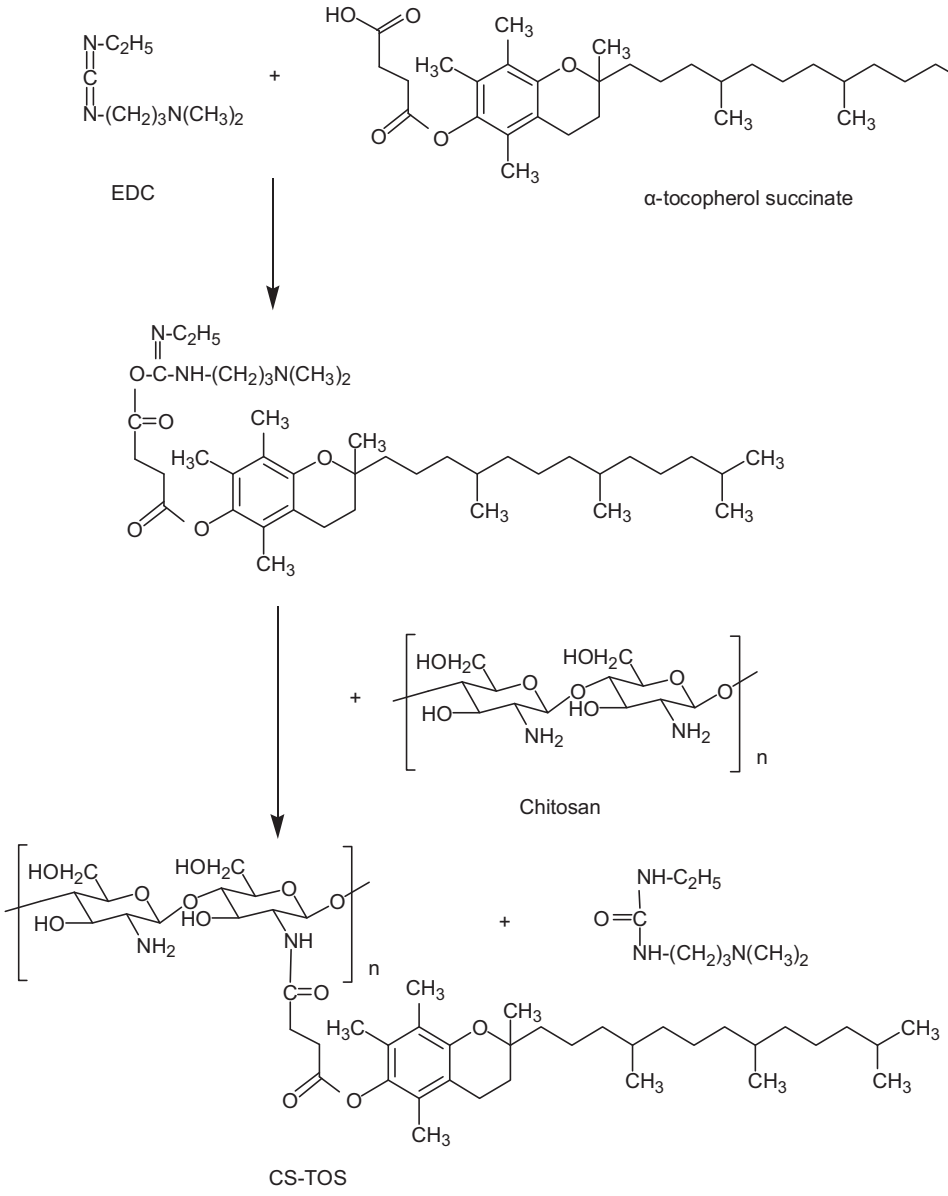

Fig. 1. Scheme of the coupling reaction between CS and α-TOS through amide linkage formation.

In vivo antitumor efficacy of the micelles was evaluated with the animal tumor models. The mice were injected subcutaneously in the armpit of right anterior limb with 0.2 ml of cell suspension containing 2.02×10^6 U14 cervical cancer cells. Day of tumor inoculated was assigned as day 0. On day 3, the animals were weighed and randomly divided into following groups ($n = 10$): a control group (normal saline); two Taxol groups (10 mg/kg and 20 mg/kg); and two PTX-loaded micelles groups (10 mg/kg and 20 mg/kg); and the treatment was initiated. The control saline or preparations were intravenously administered via the tail vein every 3 days for four times. At day 13, all the animals were sacrificed by cervical dislocation, and the tumor mass was removed, photographed and weighted. The antitumor efficacy of each formulation was evaluated by tumor inhibition rate (TIR), which was calculated by the following formula. In addition, for safety evaluation, the body weight of each mouse was recorded every day.

$$\mathrm{TIR} = \frac{(\text{tumor weight of normal saline group} - \text{tumor weight of treated group})}{\text{tumor weight of normal saline group}} \times 100\% \quad (5)$$

2.11. Statistical analysis

Each experiment was performed in triplicate. Values were expressed as mean ± standard deviation (SD). Statistical data analysis was performed using the Student's *t*-test with $P < 0.05$ as the level of significance.

3. Results and discussion

3.1. Preparation of CS-TOS

CS-TOS was synthesized by attaching the carboxyl groups of α-TOS to the primary amino groups of CS through the formation of amido bonds. Firstly, the water soluble EDC reacted with the carboxyl group of α-TOS to form an active ester intermediate. Then the intermediate can react with the primary amino of CS to form an amido bond. The function of NHS was to stabilize the intermediate, so that the yield could be higher. The remained α-TOS was washed by methanol, and the remained EDC, NHS, and by-product (isourea)

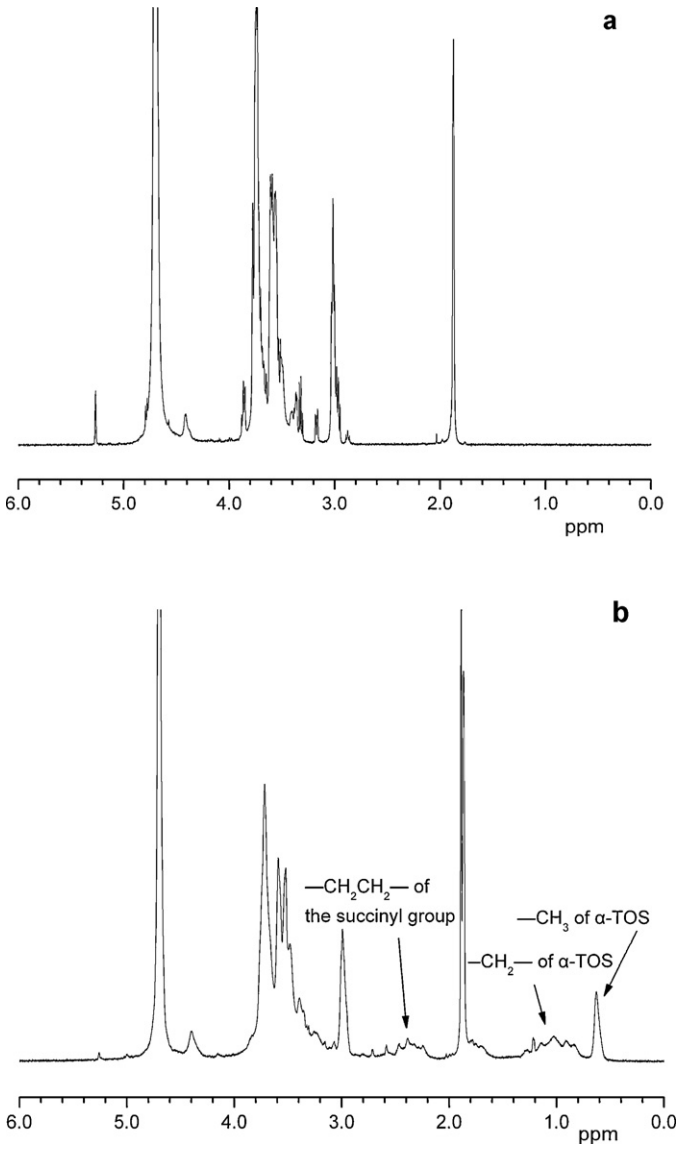

Fig. 2. ^{1}H NMR spectra of (a) CS and (b) CS-TOS.

could be easily removed by dialysis with water. The scheme of the reaction between CS and α-TOS is shown in Fig. 1.

3.2. Characterization of CS-TOS

3.2.1. ^{1}H NMR analysis

^{1}H NMR spectra were used to confirm the binding between CS and α-TOS. As shown in Fig. 2, the proton peaks at 0.63 and 0.83–1.31 ppm, which were attributed to the methyl and methylene protons of the long-chain alkyl group of α-TOS, were observed in the spectrum of CS-TOS, while no such peaks existed at the same chemical shifts in the spectrum of CS. Furthermore, the newly appeared signals at 2.26–2.72 ppm belonged to the methene hydrogen (—$COCH_2CH_2$—) of the succinyl group, which was agree with the report by Xu et al. (2007). These results certified the successful synthesis of CS-TOS.

3.2.2. FT-IR analysis

The combination between CS and α-TOS was further confirmed by FT-IR analysis. The FT-IR spectra of CS, α-TOS, their physical mixture and the final product are shown in Fig. 3.

The spectrum of CS indicated that peaks at 1633 and 1522 cm^{-1} were assigned to C–O stretching vibration of carbonyl group (amide I band) and N—H bending vibration of a secondary amine (amide II band), respectively. Compared with CS, the characteristic peak at 1598 cm^{-1} corresponding to benzene skeleton vibration was observed in the spectrum of CS-TOS, and it indicated that the α-TOS was combined to CS. Furthermore, the new signals at 1652 and 1560 cm^{-1} assigned to the amide bands I and II suggested the formation of a new amido link. All these differences implied the success of grafting α-TOS onto the amino groups of CS.

3.2.3. Degree of the substitution (DS) of CS-TOS

The degree of amino substitution of CS-TOS was determined by TNBS method. The method is based on the reaction of the terminal amino group with 2,4,6-trinitrobenzene sulfonic acid to form a yellow colored product, which had maximum UV absorbance at the wavelength of 344 nm and the absorbance is proportional to the number of primary amino groups in CS-TOS. From the standard curve, which was obtained by a series of solutions with increasing amount of CS, the DS of CS-TOS in this experiment was calculated as 17%.

3.2.4. CMC of CS-TOS

CMC value is a parameter indicative of the micelle's stability upon dilution. Micelles can be formed only when the concentration of the polymer is higher than its CMC. Polymeric micelles with remarkable lower CMC are generally more stable. In order to determine the CMC of CS-TOS, fluorescence measurements were carried out using pyrene as the fluorescent probe.

This fluorescence method is based on pyrene's sensitivity to the hydrophobicity of its microenvironment. Pyrene molecules have a very low solubility in water and preferentially solubilize into the hydrophobic domain. At low concentrations, the polymer molecules only existed as single chains, and the fluorescence intensity kept constant. When the concentration was above the CMC value, micelles were formed. The pyrene molecules entered into the hydrophobic cores of micelles and strongly emitted, leading to the increase of fluorescence intensity. Furthermore, the third peak in the emission spectra of pyrene increased more significantly than the first peak. Thus the ratio between the fluorescence intensity of peak III (384 nm) and peak I (375 nm) (I_3/I_1) increased markedly.

As shown in Fig. 4, CMC can be obtained from the intersection of two straight lines, one of which was the fitted line at low concentrations and the other was the fitted line on the rapid rising part of the curve (Fischer et al., 1998). The CMC was calculated to be 5.8×10^{-3} mg/ml, which was much lower than that of the low molecular weight surfactants. This result suggested that the CS-TOS micelles may remain stable in the dilute condition and preserve their structure without dissociation.

3.3. Preparation of PTX-loaded CS-TOS micelles

Blank micelles were prepared without difficulty by dispersing CS-TOS in distilled water under probe-sonication due to its inherent self-assembly ability in aqueous environment. It was reported that drugs could not be incorporated into the micelles easily once the micelle structure was formed completely (Zhang et al., 2004), thus the PTX-acetone solution was added simultaneously with the probe-sonication treatment. In general, the α-TOS segments of CS-TOS molecule self-assembled to form a hydrophobic core, while the hydrophilic CS segments were pushed into water phase as the outer shell, which suggested that the formation of the micelles may be due to either hydrophobic–hydrophobic interactions or van der Waals interactions between PTX molecules and the hydrophobic groups of the polymer. By the method described in Section 2.5, the PTX-loaded CS-TOS micelles were prepared successfully. The

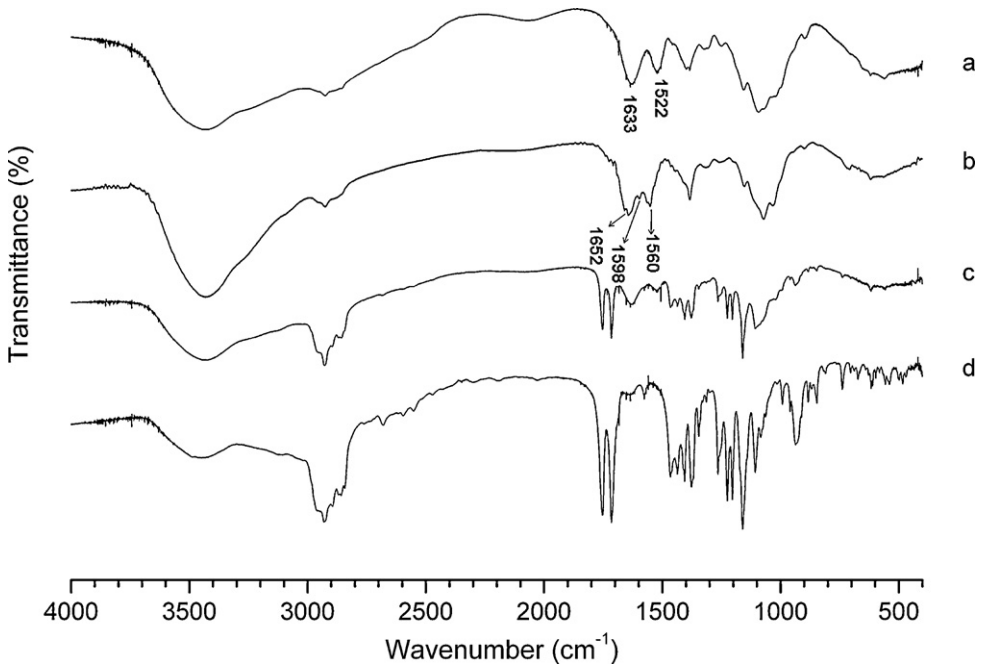

Fig. 3. FT-IR spectra of (a) CS, (b) CS-TOS, (c) physical mixture of CS and α-TOS, and (d) α-TOS.

drug encapsulation efficiency reached to 65.0 ± 4.2%, and the drug loading capacity was as high as 7.7 ± 0.7%.

3.4. Characterization of PTX-loaded CS-TOS micelles

3.4.1. DSC analysis

To confirm the existent form of PTX in the polymeric micelles, DSC analysis was carried out for PTX, blank micelles, their physical mixture, and PTX-loaded micelles. As shown in Fig. 5, the DSC thermogram of PTX revealed one endothermic peak at 222.7 °C and one exothermic peak at 233.9 °C, which were attributed to the melting and decomposition of PTX, respectively. Blank micelles presented no sharp peaks in the curve. For their physical mixture, all characteristic peaks of each component were shown only with a little shift. The PTX-loaded micelles showed a similar curve to blank micelles, which suggested that PTX existed in an amorphous state in the micelles.

3.4.2. XRD analysis

To further evaluate the crystallization behavior of PTX-loaded micelles, the XRD patterns of PTX, blank micelles, their physical mixture and PTX-loaded micelles are shown in Fig. 6. For PTX, there were four intense peaks at 2θ of 5.08°, 8.41°, 10.67° and 11.95°, and numerous small peaks between 15° and 30°. The lyophilized blank micelles gave two broad peaks around 2θ of 15° and 22°. When PTX and the blank micelles were physically mixed, typical crystal peaks of PTX were still observed with weakened intensity, which suggested that PTX did not achieve dispersion in the micelles at molecular level. In the pattern of lyophilized PTX-loaded micelles, there were no diffraction peaks of PTX, but two broad peaks similar to those of blank micelles. Therefore, it can be concluded that PTX was entrapped in the hydrophobic core of the CS-TOS micelles in molecular or amorphous state.

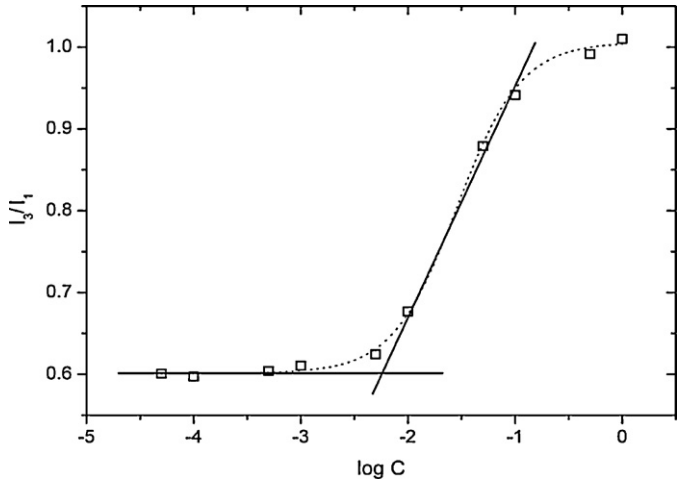

Fig. 4. Plot of the fluorescence intensity ratio (I_3/I_1) as a function of the logarithm concentration of CS-TOS.

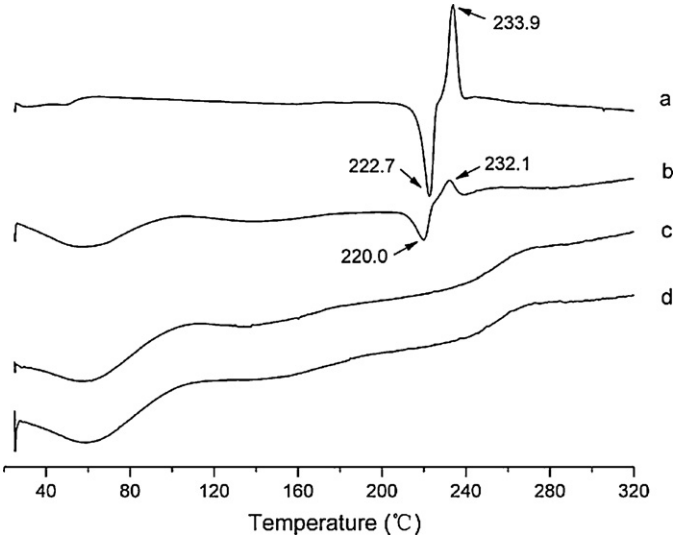

Fig. 5. DSC spectra of (a) PTX, (b) physical mixture of PTX and blank micelles (1:12, w/w), (c) blank micelles, and (d) PTX-loaded micelles (drug loading = 7.7%).

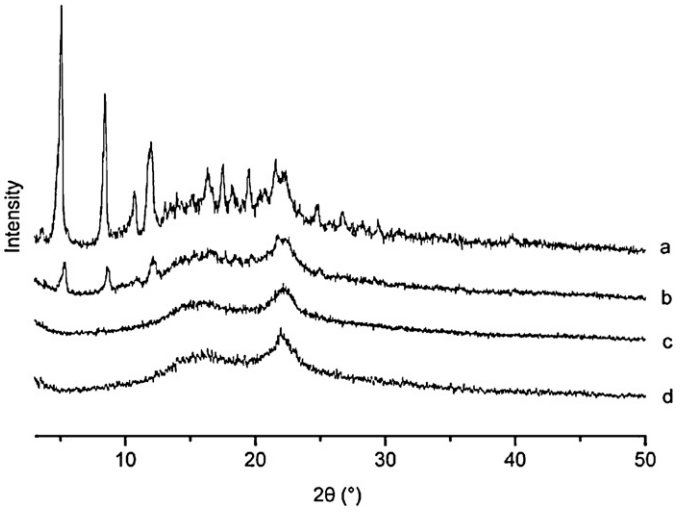

Fig. 6. XRD spectra of (a) PTX, (b) physical mixture of PTX and blank micelles (1:12, w/w), (c) blank micelles, and (d) PTX-loaded micelles (drug loading = 7.7%).

3.4.3. TEM observation

TEM micrographs of the bare and PTX-loaded micelles presented in Fig. 7 indicated that CS-TOS was capable to form micelles and entrap PTX in their hydrophobic cores. Both bare and drug-loaded micelles had near spherical shape. It was obvious that micelles loaded with PTX were larger than the bare ones, which indicated the incorporation of PTX into the micelles.

3.4.4. Particle size and zeta potential

The micelles were studied by DLS method in order to measure their effective diameters and population distribution. The results showed that both bare and PTX-loaded micelles had narrow size distribution. Compared with the corresponding blank micelles (10.8 ± 0.5 nm), PTX-loaded micelles had a much larger size of 77.6 ± 11.4 nm. It also indicated that the drug was solubilized inside the micelles, which was consistent with the report by Hu et al. (2006).

Furthermore, it was noted that the particle size measured by DLS was bigger than that observed by TEM. This discrepancy may result from the difference between the dried state and the hydrated state, in which the respective measurement performed (Kim et al., 2000), but it was consistent that after drug loading, the micelles became bigger than the blank ones.

Zeta potential is an important parameter indicating the stability of micellar systems. It was reported that under a relatively high surface charge, particles can repel each other with a strong electrostatic repulsion force, thus increase the stability of the system. In this study, the bare micelles were positively charged with zeta potential of $+27.9 \pm 2.7$ mV due to the ionized amino groups of CS. After PTX loading, the positive potential decreased very slightly and insignificantly to $+25.7 \pm 2.1$ mV, which may be explained by the change of particle size and surface charge density. From the result, the stability of the micellar system could be confirmed.

3.5. In vitro hemolytic test

Surfactants are known to cause hemolysis of red blood cells. For amphiphilic polymers are analogs of low molecular weight surfactants, they may have the potential to cause hemolysis. In order to determine whether the PTX-loaded micelles formulation is safe for intravenous injection, the level of hemolysis was measured and compared with the Cremophor EL-based formulation. It was clear that although the concentration of PTX increased from 10 μg/ml to 200 μg/ml, the hemolytic activity of PTX-loaded micelles was almost negligible, with the hemolysis percentage no more than 5.4%, while the Cremophor EL-based formulation induced hemolysis up to 10.4%. The results demonstrated that PTX-loaded CS-TOS micelles were less likely to cause hemolysis after intravenous administration.

3.6. In vitro cytotoxicity study

To estimate the effectiveness of PTX-loaded CS-TOS micelles, in vitro cytotoxicity study was performed by MTT method. From Fig. 8, it can be observed that in the concentration range used for PTX formulations, the bare micelles did not show significant cytotoxicity with more than 90% of MCF-7 cells survived. In the drug loading groups, it was encouraging to observe that PTX-loaded micelles and Cremophor EL-based formulation with equivalent doses showed similar cytotoxicity against MCF-7 cells after 24 h incubation, and the cell viability was dependent on the drug concentration. At low concentrations (0.0625–0.5 μM), only free PTX in DMSO was effective to suppress the cell growth, while the other two formulations of PTX displayed no cytotoxicity, which may be attributed to the relatively low amount of drug released from the carriers. At high concentrations of 1 and 2 μM, both PTX-loaded micelles and Cremophor EL-based formulation showed decreased cell growth, which was comparable to free PTX. Moreover, an interesting phenomenon was found that at low concentrations (0.0625–0.25 μM), the cell viabilities of drug loading groups were a little higher than those of the bare micelles groups. The mechanism will be further studied. All in all, these findings demonstrated that PTX-loaded micelles had the potency similar to that of Cremophor EL-based formulation, and CS-TOS should be a safe drug carrier.

3.7. In vivo antitumor efficacy

Based on the effective cytotoxicity, the antitumor effect of PTX-loaded micelles in U14-tumor-bearing mice was compared with that of Taxol by measuring the tumor growth inhibition. As shown in Fig. 9, PTX-loaded micelles markedly inhibited the growth of U14 tumor at doses of 10 and 20 mg/kg, compared with that observed in control group ($P < 0.05$). Furthermore, at the dose of 10 mg/kg, the micelles displayed slightly better tumor inhibition efficacy than Taxol, with the TIR of 68.0% compared to 66.6%, and the difference was not statistically significant ($P > 0.05$). Moreover, at the dose of 20 mg/kg, PTX-loaded micelles showed even enhanced antitumor efficacy with the TIR of 77.5%.

On the other hand, for Taxol 20 mg/kg group, only 5 animals survived till day 13, while none of the animals treated with micelles at equivalent dose died during the experimental period, which was consistent with the maximum tolerated doses of Taxol at 13.5 mg/kg after intravenous administration (Danhier et al., 2009). Moreover, the intravenous administration of Taxol resulted in development of immediate ataxia, decreased activity and enhanced respiration, while the PTX-loaded micelles were well tolerated.

Additionally, the change in body weight in tumor-bearing animals was used as a toxicity marker. In untreated group, the body weight significantly increased by 20% during the 13 days after inoculation. Meanwhile, in PTX-loaded micelles groups with doses of 10 mg/kg and 20 mg/kg, there was a slight increase in body mass as a result of natural animal growth. However, the toxic effect was observed in 20 mg/kg Taxol group, for significant decrease in body weight was found, but the weights of tumor lumps were no lower than that of the corresponding micelles-treated group.

Taken together, these studies demonstrated that the PTX-loaded micelles had similar or even better therapeutic effects on U14 tumors, but significantly reduced the toxic effects compared to

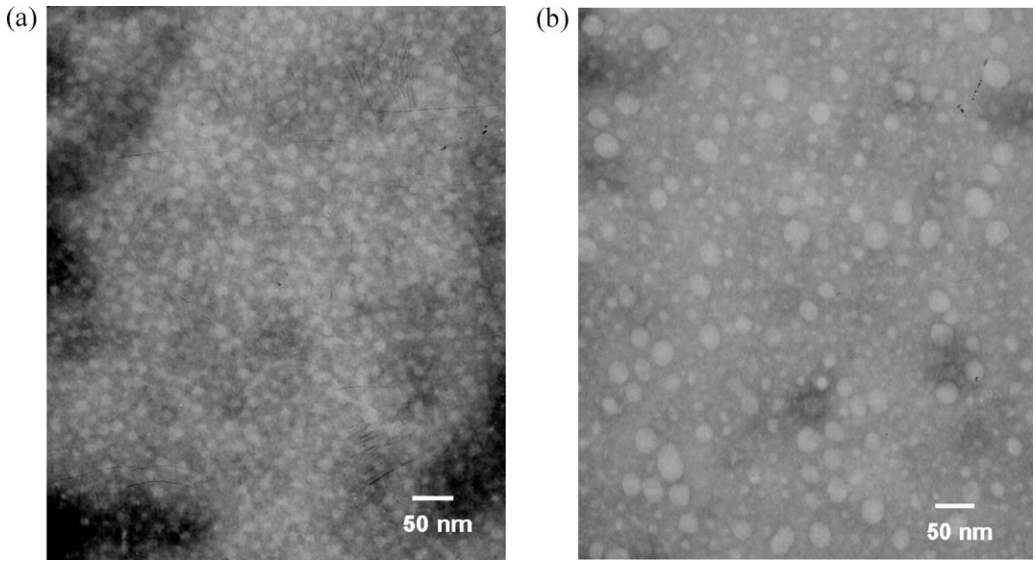

Fig. 7. TEM images of (a) bare micelles and (b) PTX-loaded micelles (scale bar indicates 50 nm).

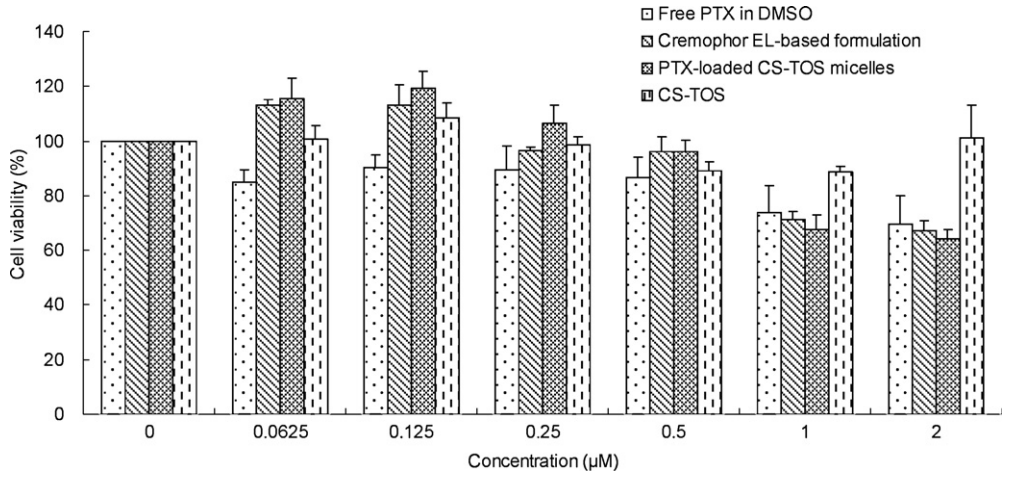

Fig. 8. In vitro cytotoxicity of various PTX formulations and CS-TOS against MCF-7 cell line after 24 h incubation. Data were plotted as the mean ± SD of three measurements.

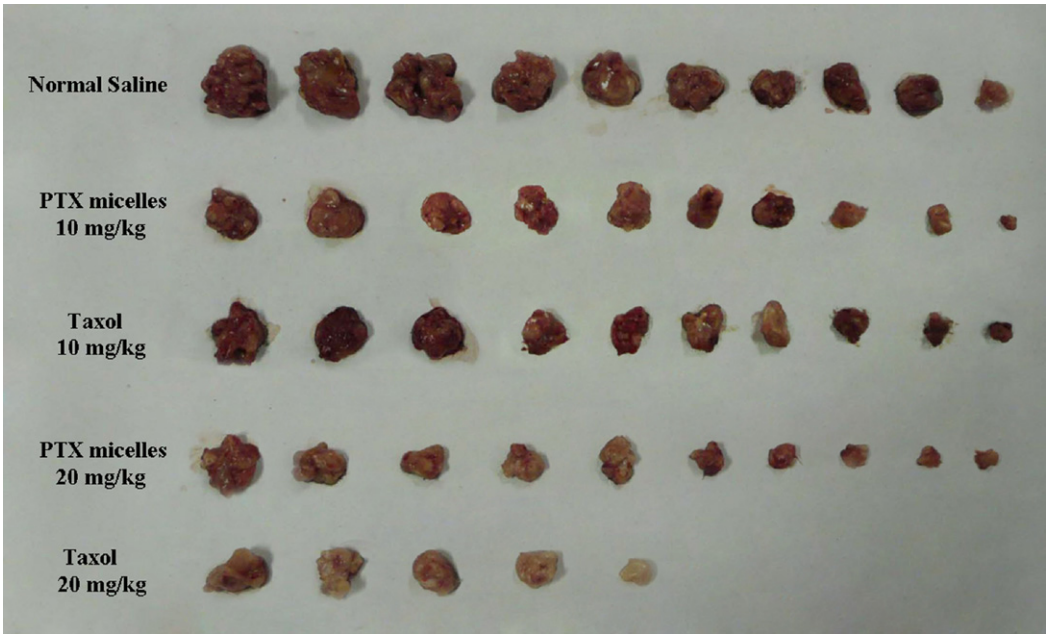

Fig. 9. Photographs of tumors from each treatment group excised on day 13.

Taxol. It can be speculated that PTX-loaded micelles may allow higher doses of PTX than Taxol to increase its efficacy.

4. Conclusions

In this study, we have successfully synthesized a novel hydrophobically modified chitosan with hydrophobic α-tocopherol succinate and water soluble chitosan. Due to the amphiphilic property, the conjugate could self-assemble to form micelles in aqueous medium with a lower CMC and had good solubilization ability for paclitaxel. The drug loading content could reach up to 7.7%. The PTX-loaded micelles have a small size of less than 100 nm with narrow size distribution. In vitro cytotoxicity study and hemolysis test indicated the effectiveness of PTX-loaded CS-TOS micelles and the safety of the carrier, which was further confirmed by the in vivo antitumor test. Taking the advantages above, it can be concluded that CS-TOS is a promising micellar carrier for paclitaxel delivery.

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References

Alani, A.W.G., Bae, Y., Rao, D.A., Kwon, G.S., 2010. Polymeric micelles for the pH-dependent controlled, continuous low dose release of paclitaxel. Biomaterials 31, 1765–1772.

Bernkop-Schnürch, A., Krajicek, M.E., 1998. Mucoadhesive polymers as platforms for peroral peptide delivery and absorption: synthesis and evaluation of different chitosan–EDTA conjugates. J. Control. Release 50, 215–223.

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 Surf. B: Biointerfaces 16, 161–171.

Chen, X.-G., Lee, C.M., Park, H.-J., 2003. O/W emulsification for the self-aggregation and nanoparticle formation of linoleic acid-modified chitosan in the aqueous system. J. Agric. Food Chem. 51, 3135–3139.

Chen, X.-G., Liu, C.-S., Liu, C.-G., Meng, X.-H., Lee, C.M., Park, H.-J., 2006. Preparation and biocompatibility of chitosan microcarriers as biomaterial. Biochem. Eng. J. 27, 269–274.

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

Fischer, A., Houzelle, M.C., Hubert, P., Axelos, M.A.V., Geoffroy-Chapotot, C., Carré, M.C., Viriot, M.L., Dellacherie, E., 1998. Detection of intramolecular associations in hydrophobically modified pectin derivatives using fluorescent probes. Langmuir 14, 4482–4488.

Francis Suh, J.K., Matthew, H.W.T., 2000. Application of chitosan-based polysaccharide biomaterials in cartilage tissue engineering: a review. Biomaterials 21, 2589–2598.

Gelderblom, H., Verweij, J., Nooter, K., Sparreboom, A., 2001. Cremophor EL: the drawbacks and advantages of vehicle selection for drug formulation. Eur. J. Cancer 37, 1590–1598.

Hu, F.-Q., Ren, G.-F., Yuan, H., Du, Y.-Z., Zeng, S., 2006. Shell cross-linked stearic acid grafted chitosan oligosaccharide self-aggregated micelles for controlled release of paclitaxel. Colloids Surf. B: Biointerfaces 50, 97–103.

Hu, F.-Q., Wu, X.-l., Du, Y.-Z., You, J., Yuan, H., 2008. Cellular uptake and cytotoxicity of shell crosslinked stearic acid-grafted chitosan oligosaccharide micelles encapsulating doxorubicin. Eur. J. Pharm. Biopharm. 69, 117–125.

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.

Kalyanasundaram, K., Thomas, J.K., 1977. Environmental effects on vibronic band intensities in pyrene monomer fluorescence and their application in studies of micellar systems. J. Am. Chem. Soc. 99, 2039–2044.

Kataoka, K., Matsumoto, T., Yokoyama, M., Okano, T., Sakurai, Y., Fukushima, S., Okamoto, K., Kwon, G.S., 2000. Doxorubicin-loaded poly(ethylene glycol)-poly(β-benzyl-L-aspartate) copolymer micelles: their pharmaceutical characteristics and biological significance. J. Control. Release 64, 143–153.

Kim, C., Lee, S.C., Kang, S.W., Kwon, I.C., Kim, Y.-H., Jeong, S.Y., 2000. Synthesis and the micellar characteristics of poly(ethylene oxide)–deoxycholic acid conjugates. Langmuir 16, 4792–4797.

Kwon, G.S., Okano, T., 1996. Polymeric micelles as new drug carriers. Adv. Drug Deliv. Rev. 21, 107–116.

Lee, K.Y., Kwon, I.C., Kim, Y.H., Jo, W.H., Jeong, S.Y., 1998. Preparation of chitosan self-aggregates as a gene delivery system. J. Control. Release 51, 213–220.

Liggins, R.T., Hunter, W.L., Burt, H.M., 1997. Solid-state characterization of paclitaxel. J. Pharm. Sci. 86, 1458–1463.

Liu, C.-G., Desai, K.G.H., Chen, X.-G., Park, H.-J., 2004. Linolenic acid-modified chitosan for formation of self-assembled nanoparticles. J. Agric. Food Chem. 53, 437–441.

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.

Montazeri Aliabadi, H., Brocks, D.R., Lavasanifar, A., 2005. Polymeric micelles for the solubilization and delivery of cyclosporine A: pharmacokinetics and biodistribution. Biomaterials 26, 7251–7259.

Ngawhirunpat, T., Wonglertnirant, N., Opanasopit, P., Ruktanonchai, U., Yoksan, R., Wasanasuk, K., Chirachanchai, S., 2009. Incorporation methods for cholic acid chitosan-g-mPEG self-assembly micellar system containing camptothecin. Colloids Surf. B: Biointerfaces 74, 253–259.

Nielsen, P.B., Müllertz, A., Norling, T., Kristensen, H.G., 2001. The effect of α-tocopherol on the in vitro solubilisation of lipophilic drugs. Int. J. Pharm. 222, 217–224.

Qu, G., Yao, Z., Zhang, C., Wu, X., Ping, Q., 2009. PEG conjugated N-octyl-O-sulfate chitosan micelles for delivery of paclitaxel: in vitro characterization and in vivo evaluation. Eur. J. Pharm. Sci. 37, 98–105.

Sawant, R.R., Sawant, R.M., Torchilin, V.P., 2008. Mixed PEG-PE/vitamin E tumor-targeted immunomicelles as carriers for poorly soluble anti-cancer drugs: improved drug solubilization and enhanced in vitro cytotoxicity. Eur. J. Pharm. Biopharm. 70, 51–57.

Singla, A.K., Garg, A., Aggarwal, D., 2002. Paclitaxel and its formulations. Int. J. Pharm. 235, 179–192.

Torchilin, V., 2007. Micellar nanocarriers: pharmaceutical perspectives. Pharm. Res. 24, 1–16.

Xu, X., Li, L., Zhou, J., Lu, S., Jie, Y., Yin, X., Ren, J., 2007. Preparation and characterization of N-succinyl-N′-octyl chitosan micelles as doxorubicin carriers for effective anti-tumor activity. Colloids Surf. B: Biointerfaces 55, 222–228.

Zhang, C., Ping, Q., Zhang, H., 2004. Self-assembly and characterization of paclitaxel-loaded N-octyl-O-sulfate chitosan micellar system. Colloids Surf. B: Biointerfaces 39, 69–75.

Zhang, W., Shi, Y., Chen, Y., Yu, S., Hao, J., Luo, J., Sha, X., Fang, X., 2010. Enhanced antitumor efficacy by paclitaxel-loaded pluronic P123/F127 mixed micelles against non-small cell lung cancer based on passive tumor targeting and modulation of drug resistance. Eur. J. Pharm. Biopharm. 75, 341–353.

Zhao, C.L., Winnik, M.A., Riess, G., Croucher, M.D., 1990. Fluorescence probe techniques used to study micelle formation in water-soluble block copolymers. Langmuir 6, 514–516.