Research Paper

Self-Assembled Hydrophobic Honokiol Loaded MPEG-PCL Diblock Copolymer Micelles

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Purpose. Honokiol showed potential application in cancer treatment, but its poor water solubility restricts its clinical application greatly. So, we designed a self-assembled monomethoxy poly(ethylene glycol)-poly(ε-caprolactone) (MPEG-PCL) micelle to load honokiol to overcome its poor water solubility.

Methods. We synthesized MPEG-PCL diblock copolymer that could self-assemble into monodisperse micelles at the particle size of ca.18 nm in water. Honokiol was loaded into MPEG-PCL micelle by direct dissolution method assisted by ultrasound, without any surfactants, organic solvents, and vigorous stirring.

Results. The blank MPEG-PCL micelles (100 mg/mL) did not induce any hemolysis *in vitro* and showed very low toxicity *ex vivo* and *in vivo*. Honokiol could be molecularly incorporated into MPEG-PCL micelles at the drug loading of about 20% by direct dissolution method assisted by ultrasound. After loaded into MPEG-PCL micelles, honokiol maintained its molecular structure and anticancer activity *in vitro*. Honokiol could be sustained released from MPEG-PCL micelles *in vitro*. The honokiol loaded MPEG-PCL micelles could be lyophilized without any adjuvant.

Conclusion. The prepared honokiol formulation based on self-assembled MPEG-PCL micelle was stable, safe, effective, easy to produce and scale up, and showed potential clinical application.

KEY WORDS: honokiol; Micelle; MPEG-PCL; nanomedicine; self-assembly.

INTRODUCTION

Due to their poor water solubility, many hydrophobic drugs are greatly restricted in clinical development. Honokiol, 3′,5-di-2-propenyl-1,1′-biphenyl-2,4′-diol, is a constituent of the Chinese medicinal herb *Magnolia officinalis/grandiflora*, and its molecular structure is presented in Fig. 1. Honokiol has been demonstrated to possess the effects of anti-thromboxane, anti-inflammation, anti-oxidation, anti-arrhythmia, central depression, muscle relaxing, and anti-anxiety (1–5). Previous reports demonstrated that honokiol could treat cancers by causing cell cycle arrest (6,7), inhibiting angiogenesis (8), and inducing apoptosis (9–12). Meanwhile, honokiol could overcome conventional drug resistance (13–15) and also showed synergistic antitumor effect with other anticancer

agents such as cisplatin or adriamycin (16,17). When many studies around the pharmacodynamics and pharmacology of honokiol attract great attention, questions around the formulation of honokiol should also be attended due to the fact that honokiol is water insoluble. Therefore, it is interesting to develop a good formulation for the lipophilic honokiol.

The nanotechnology shows promising application in drug delivery that accounts for the main part of nanomedicine (18). Biodegradable polymeric nanoparticles are highlighted as advanced drug delivery systems for cancer therapy (19–22). Now, some novel anticancer drugs based on biodegradable polymer nanoparticles have been marketed (23). The nanotechnology provided a novel method to overcome the poor water solubility of lipophilic drugs (24–26). We try to make honokiol intravenously injectable by nanotechnology. In this article, we designed a novel self-assembled MPEG-PCL micelle and developed an excellent honokiol formulation based on the self-assembled MPEG-PCL micelle without using any organic solvents, surfactants, and vigorous stirring. The MPEG/PCL copolymer is biodegradable and biocompatible (27,28). The prepared MPEG-PCL micelle was non-toxic, easy to produce, mono-dispersed, and small enough (ca.18 nm). The honokiol loaded MPEG-PCL micelle (ca.21 nm) was stable, easy to produce and scale up, and had high drug loading (20%). Previously, many protocols have been performed in our laboratory to make honokiol injectable, including liposomal honokiol (15–17), honokiol

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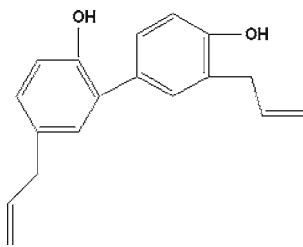
Fig. 1. Molecular structure of honokiol.

loaded F127 micelle (29), honokiol loaded PCL-PEG-PCL nanoparticles (30,31), and honokiol loaded self-assembled PCL-PEG-PCL micelles (32). This work was precisely designed on the basis of our previous studies. This honokiol formulation based on MPEG-PCL micelle was better than previously reported honokiol formulations. The honokiol loaded MPEG-PCL micelle might be an excellent honokiol formulation that could meet the requirement of clinical application.

MATERIALS AND METHODS

Materials

Materials included monomethoxy poly(ethylene glycol) (MPEG, *Mn* = 2000, Aldrich, USA), ε-caprolactone (ε-*CL*, Alfa Aesar, USA), stannous octoate (Sn(Oct)$_2$, Sigma, USA), Dulbecco's modified Eagle's medium (DMEM, Sigma, USA), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, Sigma, USA), acetonitrile (AN, Fisher Scientific, UK), dimethyl sulfoxide (DMSO, KeLong Chemicals, China), and dichloromethane (DCM, KeLong Chemicals, China), petroleum ether (KeLong Chemicals, China). Honokiol was purified in our laboratory by high-speed counter-current chromatography (HSCCC) method (33).

Sprague-Dawley (SD) rats, at weight of 200±20 g, were used for acute toxic study. The animals were purchased from the Laboratory Animal Center of Sichuan University (Chengdu, China). The animals were housed at temperature of 20–22°C, relative humidity of 50–60% and 12 h light-dark cycles. Free access to food and water was allowed. All the animals would be in quarantine for a week before treatment. All animal care and experimental procedures were conducted according to Institutional Animal Care and Use guidelines.

Synthesis of MPEG-PCL Diblock Copolymer

MPEG-PCL diblock copolymer with the designed molecular weight of 4,000 was synthesized by ring-opening polymerization method (34). Briefly, calculated amount of ε-caprolactone and MPEG (*Mn* = 2000) (weight ratio = 1:1) were introduced into a dry glass ampoule under nitrogen atmosphere, and Sn(Oct)$_2$ (0.5% *w/w* of total feed stock) was added. The ampoule was kept at 130°C. During polymerization, the system was stirred slowly. Six hours later, the system was rapidly heated to 180°C under vacuum for another 20 min. After cooling to room temperature under nitrogen atmosphere, the MPEG-PCL copolymer was first dissolved in dichloromethane and reprecipitated from the filtrate using AR grade excess cold petroleum ether. Then the mixture was

filtered and vacuum dried to constant weight. The obtained purified MPEG-PCL copolymer was kept in air-tight bags in desiccator before use.

Preparation of Blank or Honokiol Loaded MPEG-PCL Micelles

Blank MPEG-PCL micelles were prepared by self-assembly method (32). Briefly, MPEG-PCL copolymer was directly added in water followed by increasing temperature to 50°C. Due to the amphiphilic properties, the MPEG-PCL copolymer self-assembled into micelles triggered by temperature increasing. Five minutes later, the micelles solution was cooled to room temperature for further application. After blank MPEG-PCL micelles were obtained, the honokiol was loaded into MPEG-PCL micelles by direct dissolution method assisted by ultrasound. Briefly, different amount of honokiol was added into the micelles solution in ultrasound water bath (JY92-2D, Ningbo Scientz Biotechnology Co., China) presented with ice. Thirty minutes later, the suspension was filtered by filter paper and a syringe filter (pore size: 220 nm) (Millex-LG, Millipore Co., 109 USA) to remove the insoluble drugs. Finally, the honokiol loaded MPEG-PCL micelles were lyophilized for further application.

Determination of the Physicochemical Properties of Prepared Polymer and Micelles

Gel permeation chromatography (GPC, Agilent 110 HPLC, America) was used to determine macromolecular weight of MPEG-PCL copolymers. MPEG-PCL copolymer was dissolved in freshly distilled tetrahydrofuran (THF) at a concentration of 1–2 mg/mL. The THF was eluted at a rate of 1.0 mL/min through two Waters Styragel HT columns and a linear column. The internal and column temperatures were kept at 35°C. The macromolecular weight was calculated from elution volume of polystyrenes with narrow molecular weight distribution.

Particle size distribution spectra of micelles were determined by laser diffraction particle size detector (Nano-ZS, Malvern Instrument, UK). The zeta potential of honokiol loaded nanoparticles in water was measured by Malvern Zeta analyzer (Nano-ZS, Malvern Instrument, UK). The temperature was kept at 25°C during measuring process. And all results were the mean of 3 test runs.

The morphology of prepared micelles was observed under a transmission electron microscope (TEM) (H-6009IV, Hitachi, Japan): micelles were diluted with distilled water and placed on a copper grid covered with nitrocellulose. The samples were negatively stained with phosphotungstic acid and dried at room temperature.

Crystallographic assays were performed on honokiol powder, blank MPEG-PCL micelles, and honokiol loaded MPEG-PCL micelles by X-ray diffractometer (XRD) (X'Pert Pro, Philips, Netherlands) using Cu Kα radiation.

The stability of honokiol loaded MPEG-PCL micelles was evaluated as follows: honokiol loaded MPEG-PCL micelles in water were kept at 4°C, and the particle size of micelles was detected at predetermined time point in the following month.
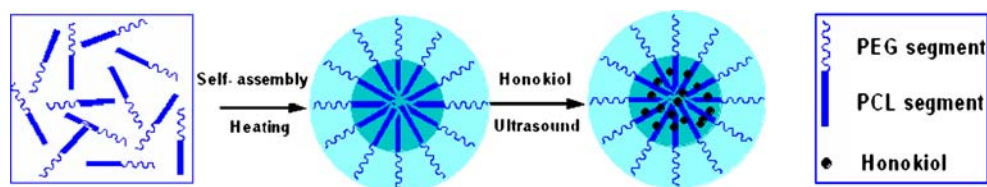
Fig. 2. Preparation scheme of honokiol loaded MPEG-PCL micelles.

The concentration of honokiol was determined by High Performance Liquid Chromatography (HPLC, Waters Alliance 2695). Solvent delivery system was equipped with a column heater and a plus autosampler. Detection was taken on a Waters 2996 detector. Chromatographic separations were performed on a reversed phase C18 column (4.6×150 mm-5 μm, Sunfire Analysis column). And the column temperature was kept at 28°C. Acetonitrile/water (60/40, v/v) was used as eluent at a flow rate of 1 mL/min.

Drug loading (DL) and encapsulation efficiency (EE) were determined as follows. Briefly, 0.2 mL of drug loaded MPEG-PCL micelles slurry was induced into pre-weighed Eppendorf tube and was lyophilized to constant weigh. Afterward, the dried deposit was dissolved in 0.1 mL dichloromethane (DCM) and was diluted by acetonitrile. The amount of honokiol in the solution was determined by HPLC. At last, drug loading (DL) and entrapment efficiency (EE) of drug loaded nanoparticles were calculated according to Eqs. (1) and (2):

$$DL = \frac{\text{Amount of drug}}{\text{Amount of polymer + drug}} \times 100\% \quad (1)$$

$$EE = \frac{\text{Exptl. drug loading}}{\text{Theor. drug loading}} \times 100\% \quad (2)$$

Safe Study of the Blank MPEG-PCL Micelles

Hemolytic Test In Vitro

The hemolytic study was performed on MPEG-PCL micelles *in vitro*. Briefly, 0.5 mL of MPEG-PCL micelles

(100 mg/mL) in normal saline was diluted into 2.5 mL by normal saline and added into 2.5 mL of rabbit erythrocyte suspension (2%) in normal saline under 37°C. Normal saline and distilled water were employed as negative and positive control, respectively. Three hours later, the erythrocyte suspension was centrifuged and the color of the supernatant was compared with controls. If the supernatant solution was absolute achromatic, it was implied that MPEG-PCL micelles did not induce any hemolysis. In contrast, MPEG-PCL micelles would induce hemolysis if the supernatant solution was red.

Cytotoxicity of MPEG-PCL Micelles

The cytotoxicity of MPEG-PCL micelle was evaluated by cell viability assay on L929 cell line and HEK293 cell line. Briefly, L929 and HEK293 cells were plated at a density of 5×10^3 cells per well in 100 μL DMEM medium in 96-well plates and grown for 24 h. The cells were then exposed to a series of MPEG-PCL micelles at different concentrations for 48 h, and the viability of cells was measured using the MTT method.

Acute Toxicity of MPEG-PCL Micelles In Vivo

The acute toxicity of MPEG-PCL micelles was evaluated on SD rats. Briefly, MPEG-PCL micelles (100 mg/mL) in normal saline were intravenously injected into SD rats at the dosage of 2 g/kg, and normal saline was used as the control (12 rats per group: six female and six male). In the following week, the adverse effects were observed. On day 7, the rats were sacrificed. The blood was withdrawn from a caudal vena cava. The heart, liver, spleen, lung, and kidney were subjected to the histological evaluation.
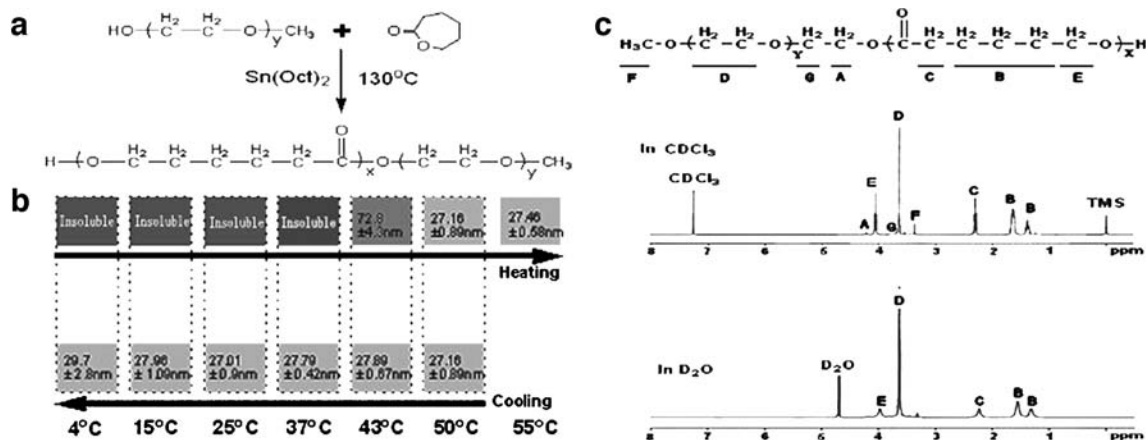

Fig. 3. Preparation and characterization of MPEG-PCL copolymer. **a** synthesis scheme of MPEG-PCL copolymer; **b** the effect of temperature on the micellization of MPEG-PCL diblock copolymer in water; **c** ^{1}H-NMR spectra of MPEG-PCL copolymer in CDCl$_3$ and D$_2$O respectively.

Release Study *In Vitro*

To determine the release kinetics of honokiol from micelles, 0.5 mL of honokiol loaded MPEG-PCL micelles or honokiol in DMSO solution (as control) was placed in a dialysis bag (molecular mass cutoff 8~14 kDa). The dialysis bags were incubated in 30 mL of phosphate buffer (pH 7.4) containing Tween80 (0.5%) at 37°C with gentle shaking, and incubation medium was replaced by fresh incubation medium at predetermined time points. The released drug was quantified, and the cumulative release profile with time was demonstrated. This study was repeated 3 times, and the result was expressed as Mean value ±Sd.

Anticancer Activity of Honokiol Loaded MPEG-PCL Micelles *In Vitro*

The anticancer activity of prepared honokiol praeparatum was evaluated by MTT method *in vitro*. The cytotoxicity of empty MPEG-PCL micelles, honokiol and honokiol-loaded MPEG-PCL micelles on cisplatin-sensitive human ovarian cancer (A2780s) cells was evaluation by cell proliferation assays. Briefly, A2780s cells were plated at a density of 5×10^3 cells per well in 100 μL of RPMI 1640 medium in 96-well plates and grown for 24 h. The cells were then exposed to a series of empty MPEG-PCL micelles, free honokiol or micelle-encapsulated honokiol at different concentrations for 48 h, and the viability of cells was measured by the MTT method. These assays were repeated 6 times, and results were expressed as Mean value ±Sd.

RESULTS

In this paper, we prepared a novel honokiol formulation: honokiol loaded MPEG-PCL micelles. The preparation scheme was presented in Fig. 2. First, blank MPEG-PCL micelles were prepared by heat-induced self-assembly from MPEG-PCL diblock copolymer that was synthesized by ring-opening polymerization. Then, honokiol was directly incorporated into MPEG-PCL micelles by direct dissolution method assisted by ultrasound. After honokiol loaded MPEG-PCL micelles were prepared, they was lyophilized to powder that was well re-soluble. In this procedure, an excellent honokiol formulation was obtained without using any organic solvents, vigorous stirring, and surfactants.

Preparation and Characterization of MPEG-PCL Micelles

The MPEG-PCL copolymer with the designed molecular weight of 4,000 was synthesized by ring-opening polymerization method using Sn(Oct)$_2$ as catalyst at the temperature of 130°C, and the synthesis scheme was shown in Fig. 3a. The effect of temperature on the micellization of prepared MPEG-PCL copolymer in water was demonstrated (Fig. 3b). The MPEG-PCL copolymer was insoluble in water at room temperature. With an increase in temperature from 37 to 50°C, micellization of MPEG-PCL occurred and particle size of MPEG-PCL micelles decreased. When the temperature was higher than 50°C, the temperature had little effect on the particle size of MPEG-PCL micelles. Meanwhile, when the temperature decreased from 50 to 4°C, the size of micelles did not change immediately. The micellization of MPEG-PCL
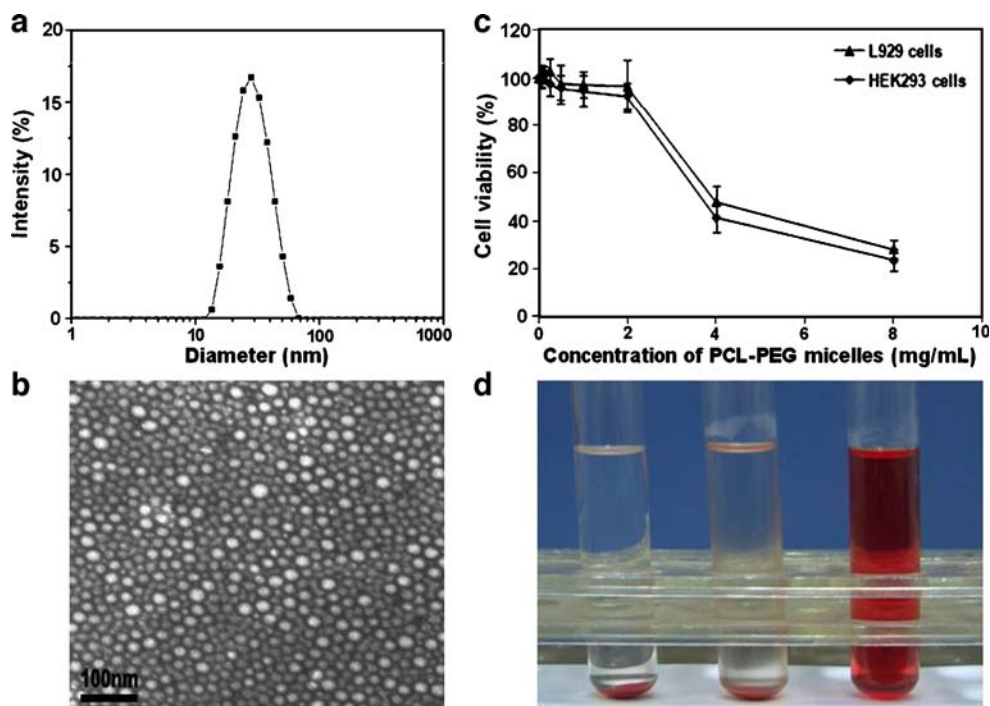

Fig. 4. Characterization of blank MPEG-PCL micelles. **a** size distribution spectrum determined by laser diffraction size detector; **b** morphology of MPEG-PCL micelles determined by TEM; **c** cytotoxicity of MPEG-PCL micelles on L929 cells and HEK293 cells; and **d** observation of the result of hemolytic study on MPEG-PCL micelles (1, MPEG-PCL micelles at the concentration of 100 mg/mL; 2, normal saline used as the negative control; and 3, distilled water used as positive control).

Table 1. Properties of Honokiol Loaded MPEG-PCL Micelles

Code	HK/MPEG-PCL (w/w)	Drug loading (%)	Encapsulation efficiency (%)	Dynamic Particle size (nm)	PDI
H1	1/20	4.57±0.097	95.81±1.94	29.19±0.52	0.112±0.01
H2	3/20	11.69±0.69	88.20±4.63	30.54±1.56	0.086±0.021
H3	5/20	17.27±0.66	83.49±2.66	31.67±0.77	0.062±0.027
H4	8/20	20.74±1.30	65.40±3.29	31.16±0.93	0.069±0.027
H5	12/20	27.41±2.91	62.93±4.99	165±4.33	0.150±0.022
[a]H6	8/20	18.53±1.91	56.87±4.87	41.43±3.1	0.158±0.031

[a] These samples were prepared by direct dissolution method in the absence of ultrasound.

in water might be due to its special amphiphilicity related to the special molecular structure and molecular weight. To our knowledge, PCL/PEG copolymer is a semi-crystalline polymer having a low glass transition temperature. Due to the semi-crystalline nature, micellization of MPEG-PCL did not occur in water at room temperature. When temperature increased from room temperature to ca.50°C, the MPEG-PCL changed from semi-crystalline to amorphous phase that allowed amphiphilicity triggered micellization of MPEG-PCL to occur. Moreover, the ^{1}H-NMR study of MPEG-PCL in CDCl₃ and D₂O were performed and results were shown in Fig. 3c. The sharp peaks at 3.60 and 3.32 ppm of the ^{1}H-NMR spectrum of MPEG-PCL in CDCl₃ were attributed to methylene protons of –CH₂CH₂O– and –OCH₃ end groups in PEG blocks, respectively. Peaks at 1.32, 1.56, 2.30, and 4.06 ppm were assigned to methylene protons of –(CH₂)₃–, –OCCH₂–, and –CH₂OOC– in PCL blocks, respectively. The very weak peaks at 4.20 and 3.82 ppm were attributed to methylene protons of –O–CH₂–CH₂– in PEG end blocks that linked with PCL blocks, respectively. The ^{1}H-NMR spectrum of prepared MPEG-PCL in CD₃Cl indicated that MPEG-PCL copolymer was successfully synthesized. At the same time, the number average molecular weight (Mn) of the prepared MPEG-PCL block copolymers was calculated from ^{1}H-NMR spectrum according to Eqs. (3)–(5) (35):

$$I_D/I_A = 4 \times Y/2 \tag{3}$$

$$I_E/I_A = 2 \times X/2 \tag{4}$$

$$M_{n(MPEG-PCL)} = M_{n(PEG)} + M_{n(PCL)}$$
$$= 44 \times (Y+1) + 31 + 114 \times X \tag{5}$$

where I_A, I_D, and I_E are integral intensities of peaks at about 4.20, 3.60, and 4.06 ppm, respectively, in the ^{1}H-NMR spectrum of MPEG-PCL in CD₃Cl. "X" and "Y+1" were the block numbers of PCL and PEG respectively in MPEG-PCL copolymers. Results indicated that the Mn of prepared MPEG-PCL copolymer was ca.4050, which was very consistent with the theoretical value of 4000. Meanwhile, the molecular weight of MPEG-PCL was also determined by gel permeation chromatography (GPC). Results was presented as follows: Mn: 3010, Mw: 4750, and PDI: 1.58. Moreover, [1]H-

NMR spectrum of MPEG-PCL micelles in D₂O was also recorded and shown in Fig. 3c. Compared with the ^{1}H-NMR spectrum of MPEG-PCL copolymer in CDCl₃, the PCL resonances broadened, whereas the peaks associated with PEG did not change; this occurrence has been attributed to MPEG-PCL micelle formation. This result also suggested that the PCL segments of MPEG-PCL became the core of MPEG-PCL micelle and PEG segments became the shell (36).

The particle size distribution spectrum of MPEG-PCL micelles shown in Fig. 4a suggested that these mono-disperse micelles had the dynamic particle size of 27 nm and did not form any aggregates in water. The zeta potential of MPEG-PCL micelles was −5.4 mV. Meanwhile, according to the TEM image of MPEG-PCL micelles as presented in Fig. 4b, it could be observed that these micelles had the mean particle size of ca.18 nm and were spherical and uniform. To evaluate the cytotoxicity of the MPEG-PCL micelles *in vitro*, MTT assays of MPEG-PCL micelles were performed on L929 cells and HEK293 cells (Fig. 4c). It could be found that MPEG-PCL micelles at a concentration lower than 2 mg/mL did not greatly affect the cell viability of both L929 cells and HEK293 cells. MPEG-PCL micelles (> 2 mg/mL) showed some concentration-dependent cytotoxicity on cells. Otherwise, the hemolytic study of MPEG-PCL micelles was performed, and the result implied that MPEG-PCL micelles (100 mg/mL) did not induce hemolysis *in vitro* (Fig. 4d). Acute toxic study of MPEG-PCL micelles indicated that intravenous administration of MPEG-PCL micelles at the dosage of 2 g/kg did not cause mortality in rat model. Meanwhile, there were no signs of abnormal behavioral reactions. The condition of teeth, oral cavity, eyes, and eye slits were normal. There was no difference in food consumption between the groups throughout the study. In Fig. 5, it could be found that intravenous administration of MPEG-PCL micelles at the dosage of 2 g/kg did not induce any histological damage on heart, liver, spleen, lung, and kidney of mice.

Fig. 5. Histological staining of the organs of rats treated with normal saline or MPEG-PCL micelles at the dosage of 2 g/kg. (a), (b), (c), (d), and (e) were heart, liver, spleen, lung, and kidney in the control group, respectively. (f), (g), (h), (i), and (j) were heart, liver, spleen, lung, and kidney in the treat group (MPEG-PCL, 2 g/kg), respectively.
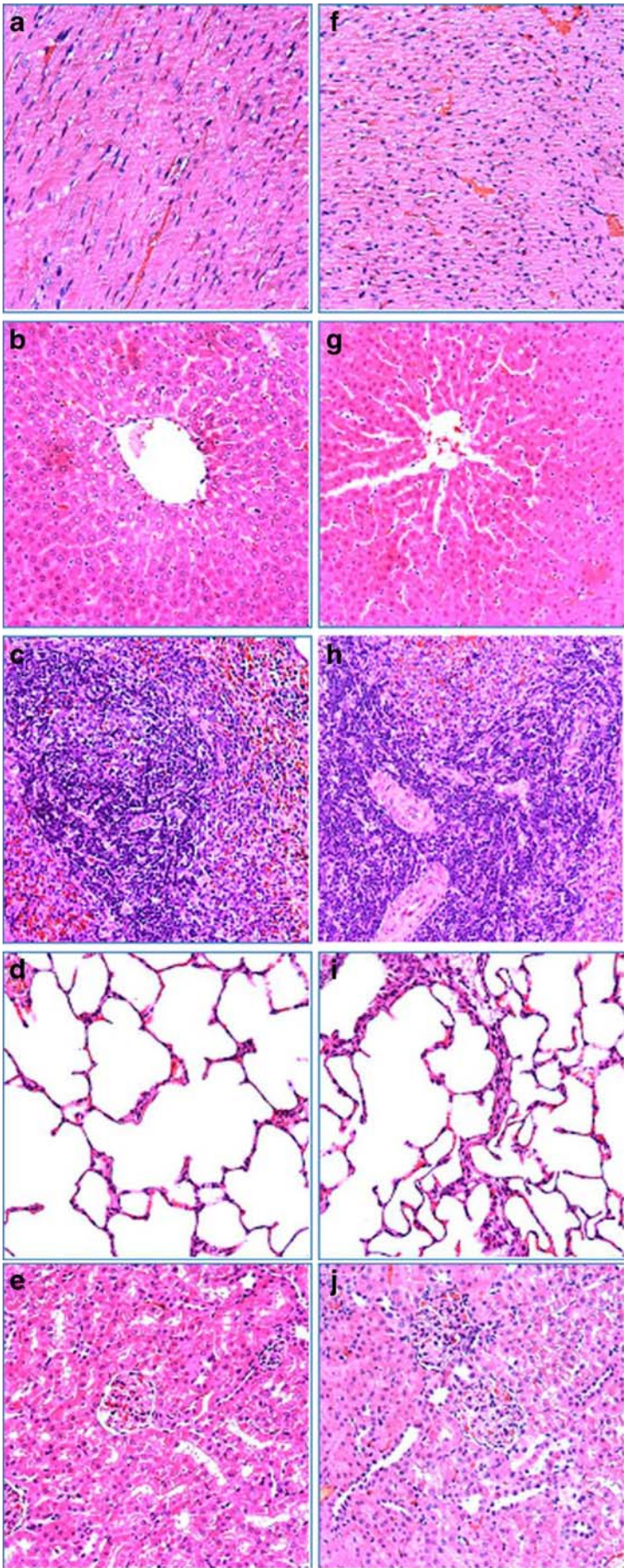
Honokiol Loaded MPEG-PCL Micelles

The effect of drug/micelle mass ratio in feed on the properties of honokiol loaded MPEG-PCL micelles was presented in Table 1. With an increase in honokiol/micelle mass ratio, drug loading increased and encapsulation efficiency decreased. When honokiol/micelles mass ratio increased form 1/20 to 8/20, the particle size of obtained drug loaded micelles did not increase significantly. But the particle size increased form ca.30 nm to 165 nm when the honokiol/micelles increased form 8/20 to 12/20. So, honokiol/micelles mass ratio in feed at 8/20 was suggested in this study, and honokiol loaded MPEG-PCL micelles with drug loading of ca.20% were characterized in detail. To discuss the effect of ultrasound on the properties of obtained micelles, honokiol loaded MPEG-PCL micelles was prepared by direct dissolution method in the absence of ultrasound. In the absence of ultrasound, honokiol also could be dissolved into MPEG-PCL micelles, but longer incubation time (5 h) should be applied to sufficiently dissolve honokiol into MPEG-PCL micelles. Meanwhile, the honokiol loaded MPEG-PCL micelles prepared in the absence of ultrasound had lower drug loading and encapsulation efficiency, and larger particle size than those prepared assisted by ultrasound. This phenomenon indicated that the ultrasound could make honokiol faster and more sufficiently dissolved into MPEG-PCL micelles. As shown in Fig. 6a, the white lyophilized powder of honokiol loaded MPEG-PCL micelles and the re-dissolved transparent micelles solution could be observed. When the

honokiol loaded MPEG-PCL micelles were lyophilized, adjuvant was not used. To our knowledge, the good re-solubility of lyophilized nano-drug might be an important precondition of clinical application of that nano-drug. This honokiol loaded MPEG-PCL micelle powder has good re-solubility. The TEM image of the honokiol loaded MPEG-PCL micelles was shown in Fig. 6b. These drug loaded micelles had the mean particle size of 21 nm and were spherical and uniform. Meanwhile, the particle size distribution of MPEG-PCL micelles indicated that these honokiol loaded MPEG-PCL micelles are monodisperse and did not form any aggregates in water (Fig. 6c). In Fig. 6d, the XRD spectra of honokiol, MPEG-PCL micelles, and honokiol loaded MPEG-PCL micelles were shown. It could be found that the characteristic XRD peaks of honokiol disappeared in the XRD spectrum of honokiol loaded MPEG-PCL micelles that might indicate that honokiol was molecularly incorporated in MPEG-PCL micelles. Meanwhile, the HPLC spectra of honokiol, MPEG-PCL micelles, and honokiol loaded MPEG-PCL micelles were presented in Fig. 6e. The elution time of honokiol did not change after it was incorporated into MPEG-PCL micelles, which might indicate that honokiol was physically incorporated into MPEG-PCL micelles and maintained its molecular structure. Finally, the stability of the honokiol loaded MPEG-PCL micelles was determined as shown in Fig. 6f. The mean particle size of honokiol loaded MPEG-PCL micelles did not change markedly in four weeks at 4°C. According to Fig. 6, it was indicated that honokiol was molecularly incorporated into MPEG-PCL micelles and main-
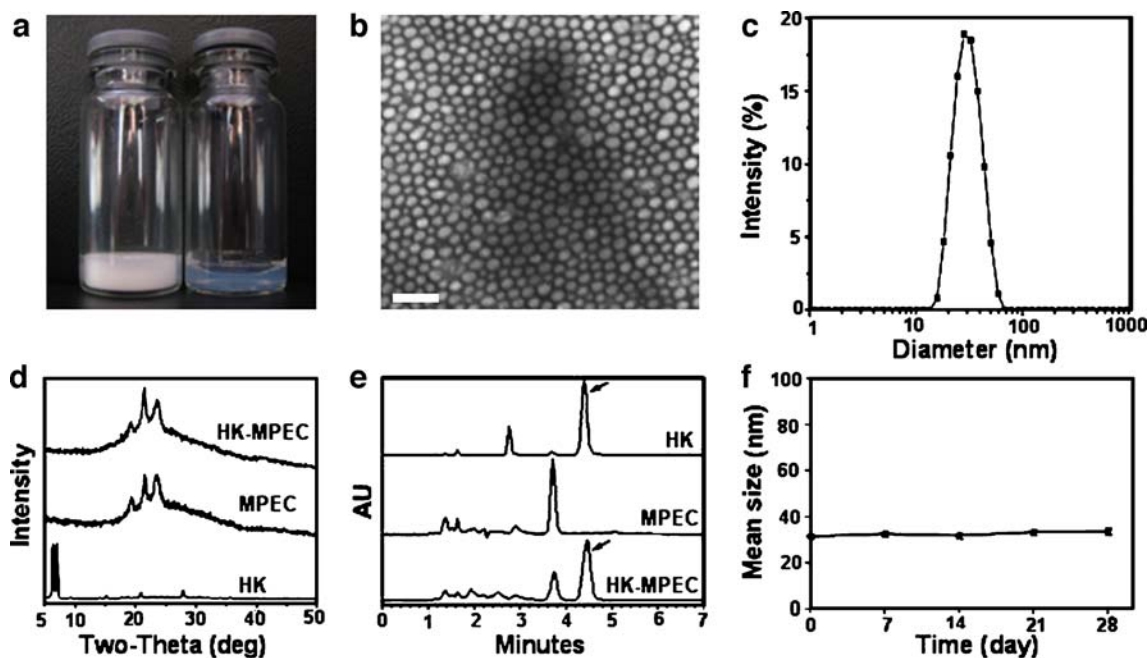

Fig. 6. Physicochemical characterization of the honokiol loaded MPEG-PCL micelles. **a** the appearances of the lyophilized honokiol loaded MPEG-PCL micelles powder (left) and re-dissolved micelles in normal saline (right); **b** the morphology determined by TEM (the bar is 100 nm); **c** the particle size distribution spectrum determined by laser diffraction size detector; **d** the XRD spectra of honokiol loaded MPEG-PCL micelles (HK-MPEC), MPEG-PCL micelles (MPCE) and honokiol (HK); **e** the HPLC spectra of free honokiol (HK), MPEG-PCL micelles (MPCE), and honokiol loaded MPEG-PCL micelles (HK-MPEC); **f** stability study of honokiol loaded MPEG-PCL micelles at 4°C in 4 weeks.
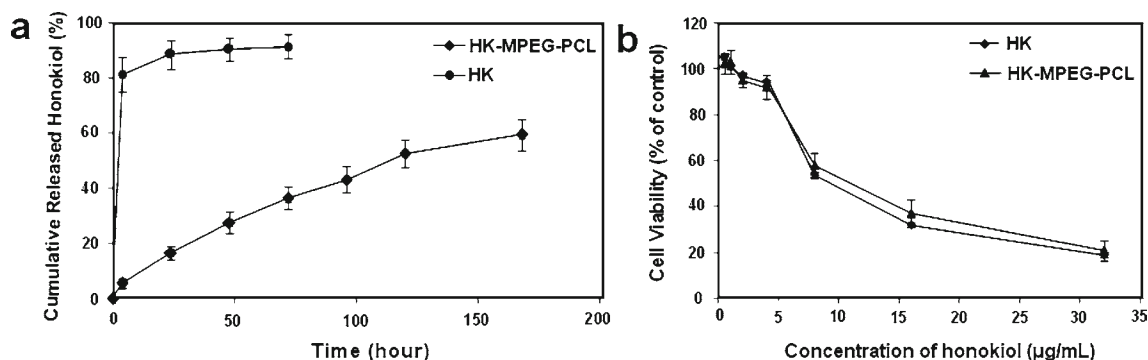
Fig. 7. The release profile and anticancer activity of honokiol loaded MPEG-PCL micelle *in vitro*. **a** the release profile of honokiol from MPEG-PCL micelles determined by a dialysis method; and **b** the cytotoxicity of honokiol (HK) and MPEG-PCL micelle encapsulated honokiol (HK-MPEG-PCL) on A2780s cells *in vitro*.

tained its molecular structure; the obtained honokiol loaded MPEG-PCL micelles had the mean particle size of ca.21 nm and were mono-disperse, stable, and re-soluble after lyophilized.

Release profile of honokiol loaded MPEG-PCL micelles indicated that honokiol could be released from micelles in a sustained manner *in vitro* as shown in Fig. 7a. The sustained release of honokiol from MPEG-PCL micelles might be due to 1), the diffusion of honokiol from the micelle to the release medium and 2), degradation and collapse of micelles induced release of honokiol to the medium. Finally, the anticancer activity of honokiol loaded MPEG-PCL micelles, compared to free honokiol, was evaluated on cisplatin-sensitive human ovarian cancer (A2780s) cells. Empty MPEG-PCL micelle at the concentration of 1 mg/mL did not suppress the proliferation of A2780s cells at all (data not shown). According to Fig. 7b, it was shown that the honokiol loaded MPEG-PCL micelles maintained the anticancer activity of honokiol. So, the prepared honokiol loaded MPEG-PCL micelle could release honokiol in an extended profile and efficiently suppress the proliferation of A2780s cells *in vitro*.

DISCUSSION

Cancer is one of the major causes of mortality, and the worldwide incidence of cancer continues to increase. Although there are already some classic anticancer drugs in clinic, they are severely insufficient, and novel anticancer agents are very desirable. Honokiol was demonstrated as a novel anticancer agent or chemosensitizer by many scientists (6–17). But honokiol is lipophilic, and problems around its formulations should be addressed prior to its clinical application (15–17,29–32). In this paper, we successfully developed a novel honokiol formulation based on MPEG-PCL micelles. Commonly, drug loaded polymeric micelles are prepared by emulsion solvent extract method, dialysis method, etc. (37). When emulsion solvent extract method is used to prepare polymer nanoparticles, organic solvents, surfactants, and violent stirring are always necessary (38,39). The problems around the residual of organic solvents and surfactants in products and the harm of organic solvents to operant and environment are undesirable. Meanwhile, the violent stirring depends greatly on the instruments which would restrict the industrial manufacture. Otherwise, those drug loaded nanoparticles prepared by emulsion solvent evaporation method are always larger than 100 nm. When dialysis method was used to prepare drug loaded polymer

nanoparticles, obtaining grams or even kilograms of products seems to be very difficult (40). So, it is very interesting to develop an advanced method to prepare drug loaded polymer nanoparticles (41). Previously, PEG/PCL nanoparticle/micelle has been prepared to deliver drugs by above mentioned methods (31,36,42–44). In this paper, we prepared honokiol loaded MPEG-PCL micelles by direct dissolution method that did not need any organic solvents, surfactants, and vigorous stirring. Meanwhile, this direct dissolution method is easy to scale up. Kilograms of honokiol loaded MPEG-PCL micelles might be easily prepared by this method. So, the direct dissolution method might be an attractive method to prepare honokiol loaded polymeric nanoparticles.

Previously, many protocols have been performed to make honokiol injectable. Chen *et al.* prepared liposomal honokiol [15–17]. When that liposomal honokiol was prepared, toxic dichloromethane and methanol were used. Gou *et al.* prepared honokiol loaded F127 micelle by emulsion solvent evaporation method. But Pluronic F127 is not biodegradable and has some toxicity *in vivo*. Later, Gou *et al.* prepared honokiol loaded PCL-PEG-PCL nanoparticles by emulsion solvent evaporation method. In that protocol, Pluronic F127 and ethyl acetate were used as surfactant and organic solvent, respectively. Those honokiol loaded PCL-PEG-PCL nanoparticles had the particle size of 161 nm (30). Wei *et al.* prepared a novel honokiol loaded PCL-PEG-PCL micelle by self-assembly method without using any organic solvents and surfactants. But those micelles had low drug loading of 6.4% and encapsulation efficiency of 44%. Meanwhile, the particle size of those micelles was not stable, although those micelles had the mean particle size of 61 nm (32). According to our previous studies, PCL/PEG copolymers showed thermo-sensitive property in water, and we successfully invented novel thermo-sensitive hydrogels based on PCL/PEG copolymers (34,35,45). PCL/PEG copolymers with different molecular structure and molecular weight would result in different thermo-sensitive properties. In this paper, on the basis of previous work, we re-designed a novel MPEG-PCL diblock copolymer with molecular weight of ca.4,000 to load honokiol by direct dissolution method. The synthesized MPEG-PCL copolymer could self-assemble into mono-dispersed micelles in water at the temperature of ca.50°C (Fig. 3b). According to the cytotoxicity study *in vitro* (Fig. 4c), hemolytic study *in vitro* (Fig. 4d), and acute toxic study *in vivo* (Fig. 5), the prepared MPEG-PCL micelle might

be a safe candidate as intravenous drug vector. It was also exciting that honokiol, rather than paclitaxel and camptothecin, could be directly dissolved into the micelles assisted by ultrasound. Moreover, the obtained honokiol loaded MPEG-PCL micelles had many excellent properties, they were 1), mono-disperse (PDI = 0.069±0.027, Table 1); 2), small size (21 nm, Fig. 6b); 3), high drug loading (ca.20%, Table 1); 4), stable (Fig. 6f); 5), able to sustain release of honokiol *in vitro* (Fig. 7a); and 6), maintained the anticancer activity of honokiol *in vitro* (Fig. 7b). Otherwise, Tang *et al.* demonstrated that 10- to 20-nm micelles containing doxorubicin improve the drug's penetration, accumulation, and antitumor activity (46). The honokiol loaded MPEG-PCL micelles with the particle size of 21 nm might be more efficient in cancer treatment than previously reported honokiol loaded nanoparticles with larger size *in vivo*. So, the obtained honokiol loaded MPEG-PCL micelle has many advantages over previously reported honokiol formulations and might be a better formulation than many previously reported honokiol formulations.

In summary, we prepared mono-disperse honokiol loaded MPEG-PCL micelles without using any organic solvents, surfactants, and gravis stirring. The honokiol loaded MPEG-PCL micelles overcame the poor water solubility of honokiol, maintained the anticancer activity of honokiol *in vitro*, and could sustain release of honokiol *in vitro*. Meanwhile, the employed MPEG-PCL micelle was a safe candidate as intravenous drug delivery system. The prepared honokiol loaded MPEG-PCL micelle is an excellent honokiol formulation and has potential clinical application.

CONCLUSION

We successfully prepared an excellent honokiol formulation based self-assembled MPEG-PCL micelle. This honokiol formulation was original, stable, safe, effective, and easy to produce and scale up. This honokiol formulation has potential clinical application.

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