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Preparation of MPEG–PLA nanoparticle for honokiol delivery *in vitro*

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ABSTRACT

Honokiol (HK) shows potential application in cancer treatment, but its poor water solubility restricts clinical application greatly. In this paper, monomethoxy poly(ethylene glycol)–poly(lactic acid) (MPEG–PLA) was synthesized by ring-opening polymerization and processed into nanoparticle for honokiol delivery. Chemical structure of the synthesized polymer was confirmed by ^{1}H NMR, and its molecular weight was determined by gel permeation chromatography (GPC). Honokiol loaded MPEG–PLA nanoparticles were prepared by solvent extract method. And particle size distribution, morphology, drug loading, drug release profile and anticancer activity *in vitro* were studied in detail. The described honokiol loaded MPEG–PLA nanoparticles in this paper might be a novel formulation for honokiol delivery.

1. Introduction

Honokiol (HK), 3′,5-di-2-propenyl-1,1′-biphenyl-2,4′-diol, is a constituent of Chinese medicinal herb *Magnolia officinalis*/*grandiflora*. It has a variety of pharmacological effects, such as anti-inflammatory, antithrombotic, anti-arrhythmic, anti-oxidative, central depressant, muscle relaxant and anxiolytic effects (Lo et al., 1994; Maruyama and Kuribara, 2000; Liou et al., 2003). In the past decades, many researches suggested that honokiol had anticancer activity and showed potential application in cancer treatment (Bai et al., 2003; Ahn et al., 2006; Lee et al., 2006; Battle et al., 2005; Xu et al., 2006; Hahm and Singh, 2007; Sheu et al., 2007). But honokiol is hydrophobic, vascular administration of honokiol is very difficult. Therefore, it is interesting to develop novel formulations for honokiol delivery.

Nanotechnology shows promising application in drug delivery system that accounts for the main part of nanomedicine (Wagner et al., 2004). Recently, biodegradable polymeric nanoparticles are highlighted as advanced drug delivery system for cancer therapy (Service, 2005; Ganta et al., 2008). Nowdays, many studies of anticancer drugs based on biodegradable polymer nanoparticles have been performed in the preclinical and clinical research (Wang et al., 2008). Nanotechnology provided a novel method to overcome the poor water solubility of hydrophobic drugs (Saffie-Sieverb et al., 2005). After hydrophobic drug entrapped into amphiphilic polymeric nanoparticles, drug loaded nanoparticles could be well and stably dispersed in water solution to meet the requirement of intravenous injection. Previously, we have successfully prepared the liposomes (Luo et al., 2008; Jiang et al., 2008; Hou et al., 2008) and nanoparticles/micelle (Gou et al., 2008a,b; Gong et al., 2008; Wei et al., 2009; Gou et al., 2009a,b) suitable for the hydrophobic drug delivery. However, these polymers based on MPEG and PCL have not been approved by Food and Drug Administration (FDA) for the application of clinical intravenous injection. Therefore, there is a long way in clinical application for nano-drug based on these polymers. And works around seeking an excellent material for nano-drugs should be continued. MPEG–PLA diblock copolymer with great biodegradability and compatibility has been widely applied in drug delivery system. MPEG–PLA nanoparticle was one widely studied intravenously injectable drug vectors (Gref et al., 1994; Matsumoto et al., 1999; Zhang et al., 2006). Meanwhile, nano-drug based on MPEG–PLA has been paid clinical study (Rapoport, 2007).

In this paper, we intend to develop a novel honokiol formulation based on MPEG–PLA nanoparticle suitable for vascular administration. The prepared honokiol loaded MPEG–PLA nanoparticle was characterized, and drug release profile has been studied. Meanwhile, the hemolytic test and MTT had been done to evaluate the safety of MPEG–PLA nanoparticle as intravenous drug vector. And the therapeutic evaluation of honokiol loaded MPEG–PLA nanomedicine will be studied later in our research group.

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2. Experimental

2.1. Materials

Methyl poly(ethylene glycol) (MPEG, M_n = 5000) and D,L-lactide were synthesized in our lab. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and stannous octoate ($Sn(Oct)_2$), RPMI 1640, DMEM, were purchased from Sigma (USA). Dimethyl sulfoxide (DMSO) and dichloromethane (DCM) were purchased from KeLong Chemicals (Chengdu, China). Acetonitrile (AN) was purchased from Fisher Scientific (UK). Honokiol was purified in our laboratory by HSCCC method (Chen et al., 2007).

All the chemicals used in this work were all analytical pure grade, and used as received except PEG.

2.2. Synthesis of MPEG–PLA copolymer

MPEG–PLA diblock copolymer was synthesized by ring-opening polymerization of lactide in the presence of mono-methyl poly(ethylene glycol) (MPEG). Briefly, an calculated amount of D,L-lactide, MPEG and $Sn(Oct)_2$ were first added into a flamed three-necked glass flask under the protection of nitrogen atmosphere. Then, the mixture was heated to 130 °C under mechanical stirring. Ten hours later, the reaction system was degassed under vacuum for another 45 min followed by being cooled to room temperature under the protection of nitrogen atmosphere. Finally, the crude product of MPEG–PLA copolymer was reprecipitated from dichloromethane with excess pre-cold petroleum ether. The mixture was filtered and vacuum dried to constant weight. The obtained purified MPEG–PLA copolymer was kept in air-tight bags in desiccator before the further use.

2.3. Preparation of blank or honokiol loaded MPEG–PLA nanoparticles

Honokiol loaded MPEG–PLA nanoparticles were prepared by solvent extract method. Briefly, 400 mg of MPEG–PLA and 50 mg of HK were co-dissolved in 10 ml acetone kept at 50 °C for 5 min to form organic phase. Then, the organic phase was quickly added into water under moderate mechanical stirring. After 10 min, with the diffusion of acetone into water, amphiphilic MPEG–PLA block copolymer could self-assemble into nanoparticles and its hydrophobic core encapsulated HK in aqueous solution. Finally, the obtained nanoparticles were placed in a dialysis bag (molecular weight cutoff 8000–14,000 Da) to dialyze against distilled water for 3 days to remove the remained acetone. Blank MPEG–PLA nanoparticles were prepared by the same method except that honokiol powder was not added.

2.4. Physicochemical properties

^{1}H NMR spectra of MPEG–PLA copolymer (in $CDCl_3$) were recorded on Varian 400 spectrometer (Varian, USA) at 400 MHz using tetramethylsilane as an internal reference standard. The gel permeation chromatography (GPC) measurements were conducted at 25 °C with a instrument of HPLC (Agilent 110, USA). The samples were dissolved in freshly distilled tetrahydrofuran (THF) at a concentration of 1 mg/ml. THF was eluted at a rate of 1.0 ml/min.

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

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

Crystallographic assay was performed on HK powder, blank MPEG–PLA nanoparticle, and HK loaded MPEG–PLA nanoparticles by X-ray diffractometer XRD) (X'Pert Pro, Philips, Netherlands) using Mo Kα radiation.

The concentration of HK was determined by High Performance Liquid Chromatography (HPLC) Instrument (Waters Alliance 2695). Solvent delivery system 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 mm × 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. Detection wavelength was 254 nm.

Drug loading and entrapment efficiency were determined as follows. Briefly, 0.2 ml of drug loaded MPEG–PLA nanoparticle was introduced into pre-weighed EP tube and was lyophilized to constant weigh. Afterwards, the dried deposit was dissolved in 0.1 ml dichloromethane and was diluted by acetonitrile. Meanwhile, the amount of honokiol in the solution was determined by HPLC. Drug loading (DL) and encapsulation efficiency (EE) of drug loaded nanoparticles were calculated according to Eqs. (1) and (2):

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

$$\mathrm{EE}(\%) = \frac{\text{experimental drug loading}}{\text{theoretical drug loading}} \times 100 \quad (2)$$

2.5. Hemolytic test in vitro

The hemolytic study was performed on MPEG–PLA nanoparticle *in vitro* (Gou et al., 2009c). Briefly, 0.5 ml sample at different concentrations 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 used 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 implied that there was no hemolysis. In contrast, hemolysis occurred when the supernatant solution was red.

2.6. In vitro release study

To determine the *in vitro* release kinetics of honokiol from nanoparticles, 0.5 ml of HK loaded MPEG–PLA nanoparticles slurry was placed in a dialysis bag (molecular mass cutoff 8000–14,000 Da), and 0.5 ml of HK solution in DMSO (1 mg/ml) was used as control. The dialysis bags were incubated in 30 ml of phosphate buffer (pH = 7.0) containing Tween 80 (0.5%, w/v) at 37 °C with gentle shaking, then incubation medium was replaced by fresh medium at predetermined time points. The released drug was quantified, and the cumulative release profile with time was demonstrated. This study was repeated three times, and result was expressed as mean value ± SD.

2.7. Analysis of cytotoxicity

The cytotoxicity of blank MPEG–PLA nanoparticle, free HK and nanoparticle encapsulated HK on cisplatin-sensitive (A2780s) human ovarian cancer cells was evaluation by cell proliferation assay. 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 empty MPEG–PLA nanoparticle, free HK or nanoparticle encapsulated HK at different concentrations for 48 h. And free HK dissolved in DMSO at the concentration of HK 1 mg/ml and diluted with RPMI 1640 medium to obtain various concentrations of honokiol solution. The cells viability was measured by the MTT method. Mainly, each cell was added to 20 μl 5 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) solution and treated for 4 h at 37 °C. Then, the supernatant was fully removed and 150 μl DMSO was added to per cell, oscillating it for 30 min. After solubilization, the amount of blue formazan produced by viable cell was measured at 570 nm. The absorbance of the formed formazan was proportional to the number of cells plated. The additional manual counting of the cells confirmed the linearity between the number of viable cells and the absorbance values. Mean percentage of cell survival relative to that of untreated cells was estimated from data from six individual experiments, and results were expressed as mean value ± SD.

3. Results and discussion

Traditionally, almost half of new molecular entities identified by pharmaceutical industry screening programs have failed to be developed, because their poor water solubility made their formulation difficult or even impossible. It was reported that honokiol, as a multi-functional drug, has great potential in disease therapy especially in cancer therapy. Its lipophilicity made administration difficult. So, an excellent honokiol formulation was needed. Nanotechnology also provides a novel method to overcome the poor water solubility of hydrophobic drugs. MPEG–PLA nanoparticle was regarded as safe drug vectors and widely used in drug delivery (Riley et al., 2001; Dong and Feng, 2004; Lu et al., 2005). Meanwhile, there is still nano-drug based on MPEG–PLA nanoparticles that was proved by FDA to be applied in clinic (Kim et al., 2004). In this paper, MPEG–PLA was synthesized and processed into nanoparticles to load HK to overcome the water solubility of honokiol. A novel honokiol formulation based on MPEG–PLA nanoparticles was developed.

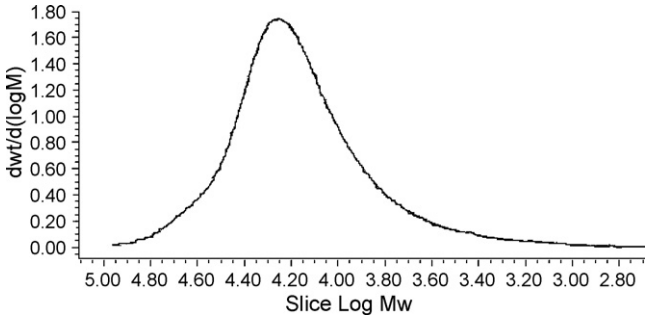

Fig. 2. GPC curve of prepared MPEG–PLA.

3.1. The ^{1}H NMR and the GPC of MPEG–PLA copolymers

The molecular structure and molecular weight of prepared MPEG–PLA copolymers were characterized by ^{1}H NMR and GPC. As shown in Fig. 1, peaks "a" and "c" were assigned to methyl group and methylene protons of $-CH_3$, and –CH– in PLA units, respectively. The peak "b" was attributed to methylene protons of PEG oxyethylene units. The very weak peak "e" was, respectively attributed to methylene protons of $-O-CH_2-CH_2-$ in PEG end block that linked with PLA blocks. The ^{1}H NMR spectrum suggested that the MPEG–PLA diblock copolymer was successfully synthesized.

The number-average molecular weight of MPEG–PLA block copolymers and PEG/PLA block ratios were calculated from ^{1}H NMR spectra according to Eqs. (3)–(7):

$$\frac{I_b}{I_d} = \frac{4(x-2)}{2} \tag{3}$$

$$\frac{I_c}{I_d} = \frac{3(y-2)}{2} \tag{4}$$

$$M_{n(\mathrm{PEG})} = 44x + 31 \tag{5}$$

$$M_{n(\mathrm{PLA})} = 72y \tag{6}$$

$$M_{n(\mathrm{MPEG-PLA})} = M_{n(\mathrm{PEG})} + M_{n(\mathrm{PLA})} \tag{7}$$

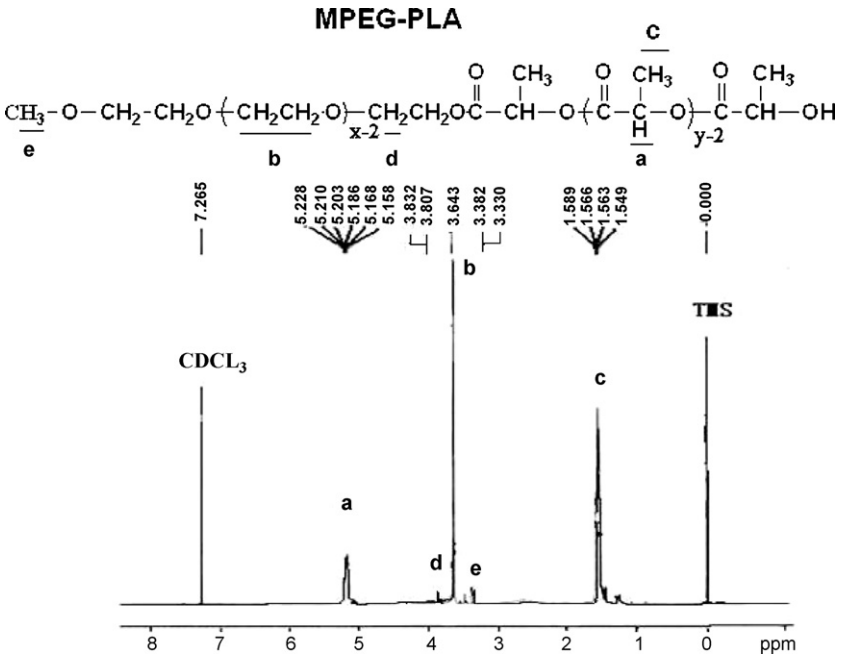

Fig. 1. ^{1}H NMR spectrum of MPEG–PLA in $CDCL_3$.

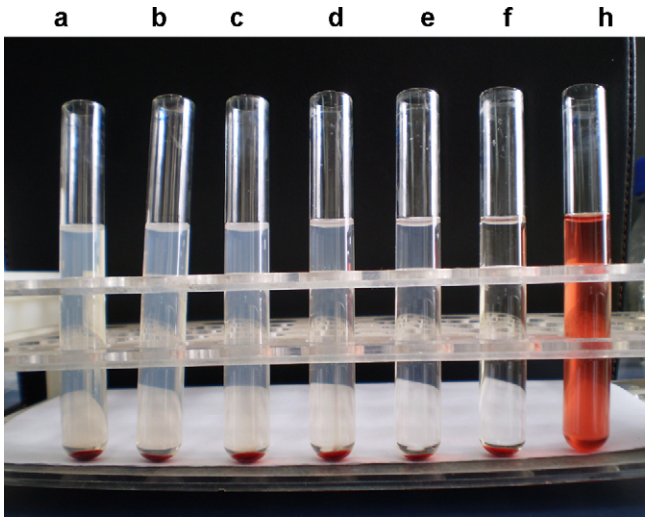

Fig. 3. Hemolytic test on the MPEG–PLA nanoparticles. The images were taken on 3 h after reaction. (a) The concentration of MPEG–PLA nanoparticles is 40 mg/ml; (b) 32 mg/ml; (c) 24 mg/ml; (d) 16 mg/ml; (e) 8 mg/ml. (f) Sample is normal saline used as negative control and (h) sample is distilled water used as positive control.

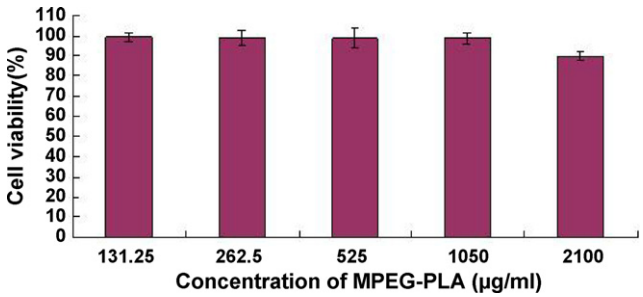

Fig. 4. Cytotoxicity of blank MPEG–PLA nanoparticles on human ovarian cancer cells: A2780s (cisplatin-sensitive).

where I_b, I_c, and I_d were integral intensities of peaks at about 3.6, 1.5 and 3.8 ppm, respectively in ^{1}H NMR spectrum of MPEG–PLA copolymers (Fig. 1). x and y were the respective block number of PEG and PLA, respectively in macromolecular structure of MPEG–PLA copolymers. And the molecular weight of MPEG–PLA was found 10,708 Da.

As shown in Fig. 2, the molecular weight distribution datum of MPEG–PLA was normal distribution. And the macromolecular weight distribution (polydispersity, PDI, M_w/M_n) was 1.33. Only a single peak existed in Fig. 2, which indicated the mono-distribution

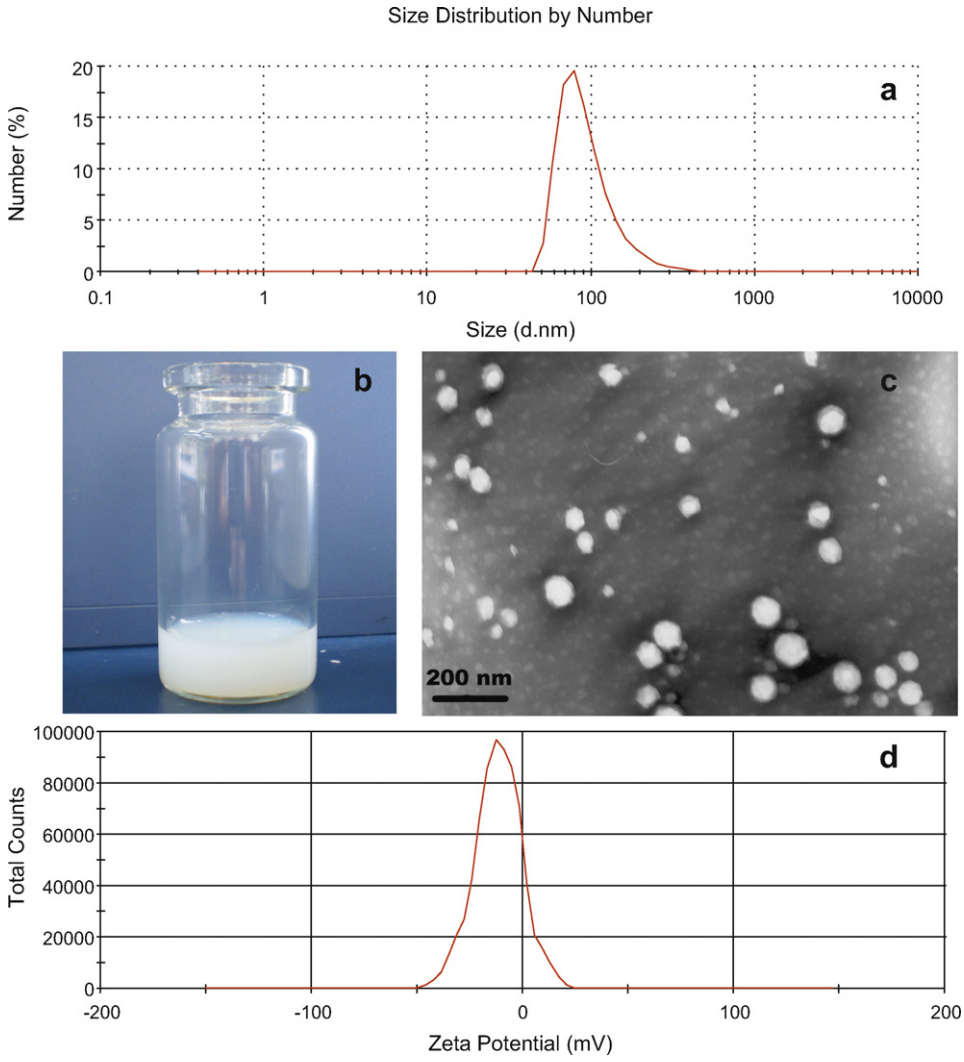

Fig. 5. Characterization of MPEG–PLA nanoparticle. (a) Size distribution spectrum determined by laser diffraction size detector, mean size 96 ± 2 nm; (b) optic image; (c) morphology of MPEG–PLA nanoparticles determined by TEM; (d) zeta potential determined by laser diffraction zeta detector, mean zeta potential −11.3 ± 1.7 V.

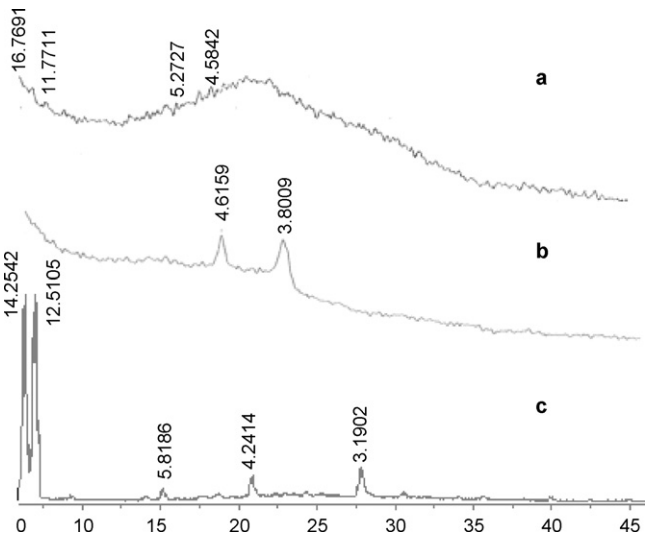

Fig. 6. X-ray diffraction spectra. (a) Blank MPEG–PLA nanoparticle crystal; (b) honokiol-loaded MPEG–PLA nanoparticles; and (c) free honokiol.

of molecular weight. Through GPC, the macromolecular weight of MPEG–PLA was found to be about 10,060 Da. ^{1}H NMR and GPC results indicated that the MPEG–PLA copolymer was prepared successfully.

3.2. Safety evaluation of MPEG–PLA nanoparticles in vitro

Hemolytic test was performed on MPEG–PLA nanoparticles. As shown in Fig. 3, MPEG–PLA nanoparticles at the concentration of 40 mg/ml did not cause any hemolysis on rabbit erythrocyte comparing with the negative control (normal saline). Meanwhile, the cytotoxicity of blank MPEG–PLA was evaluated by cell viability assay on A2780 cells which was shown in Fig. 4. According to Fig. 4, cell proliferation was not suppressed by MPEG–PLA nanoparticle (2 mg/ml) *in vitro*, and non-toxicity of prepared MPEG–PLA nanoparticles was implied.

3.3. Physical characterization of honokiol loaded MPEG–PLA nanoparticle

HK loaded MPEG–PLA nanoparticle with drug loading of ca. 10% was chosen for characterization. According to Fig. 5(a), the prepared nanoparticle had the mean particle size of 96 ± 2 nm with the polydisperse index (PDI) of $0.1.85 \pm 0.014$. It suggested that the obtained nanoparticle was mono-dispersed and stable in the water solution. The appearance of prepared HK nanoparticle suspension was shown in Fig. 5(b). TEM photo as shown in Fig. 5(c) indicated that the prepared nanoparticle had sphere appearance with the mean particle size of ca. 80 nm. Meanwhile, the zeta potential of the nanoparticle was -11.3 ± 1.7 mV as shown in Fig. 5(d).

Otherwise, the XRD spectra of blank MPEG–PLA nanoparticle and HK loaded MPEG–PLA nanoparticle were presented in Fig. 6. In Fig. 6(c), the characteristic peaks of honokiol are the first two which were at 14.2542 and 12.5105. These two peaks disappeared in HK loaded nanoparticle shown in Fig. 6(b), and it implied that honokiol was molecularly incorporated in MPEG–PLA nanoparticle. With the introduction of hydrophobic HK, the crystallinity of MPEG–PLA was slightly changed. So the peaks at 4.6 and 3.8 appeared in Fig. 6(b).

The release profile *in vitro* was evaluated. A sustained release manner could be visibly observed when HK released from MPEG–PLA nanoparticle as shown in Fig. 7. Only 53% HK released from the nanoparticles within 24 h, while free HK released about 100% into the outside media. These physical properties indicated

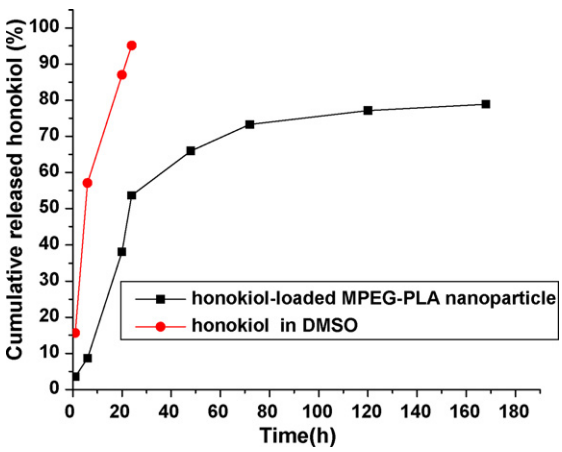

Fig. 7. *In vitro* release profile of honokiol from MPEG–PLA nanoparticles in PBS (pH = 7.0) and DMSO at 37 °C.

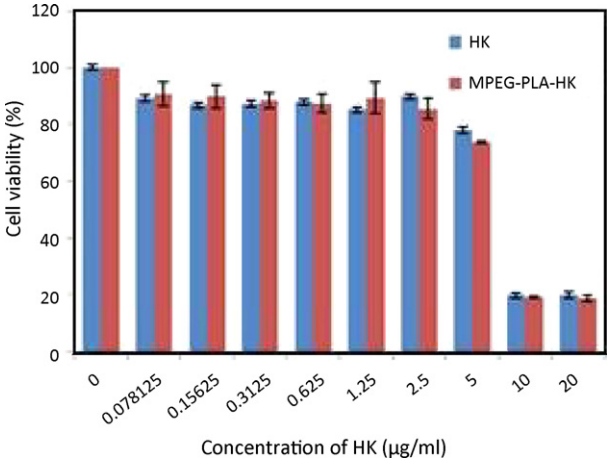

Fig. 8. Cytotoxicity of honokiol loaded MPEG–PLA nanoparticles on human ovarian cancer cells A2780s.

that the prepared HK loaded MPEG–PLA nanoparticle was a novel honokiol formulation which could meet the requirement of intravenous injection.

3.4. Anticancer activity of honokiol loaded MPEG–PLA nanoparticle

The cell viability assays were performed to evaluate the anticancer activity of HK loaded MPEG–PLA nanoparticles and free HK. Free honokiol and honokiol loaded nanoparticle significantly decreased the viability of A2780s cells with increase in honokiol concentration. Fig. 8 showed the influence of drug concentration and nanoparticle on cell viability. The results indicated that the cytotoxicity of HK loaded MPEG–PLA nanoparticle was comparable to that of free honokiol and IC50 was 8.45 μg/ml. It implies that HK loaded MPEG–PLA nanoparticle might had great potential application of anticancer effect on cisplatin-sensitive (A2780s) human ovarian cancer cells *in vitro*.

4. Conclusion

MPEG–PLA copolymer was successfully synthesized and processed into nanoparticle to load honokiol. Encapsulating honokiol in MPEG–PLA nanoparticle made hydrophobic honokiol to be injectable. The honokiol loaded MPEG–PLA nanoparticle might be a novel honokiol formulation.

Acknowledgements

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