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Preparation, characterization and evaluation of breviscapine lipid emulsions coated with monooleate–PEG–COOH

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ABSTRACT

Series of monooleate-modified PEG with active carboxylic terminus on the other end (MO–PEG–COOH) were used to modify the lipid emulsions surface to prepare a sterically stabilized lipid emulsions for carrying Traditional Chinese Medicine – breviscapine. Based on the research of relationship between polymer structure and prolonged circulation activity, we developed an optimized formulation and a technological method to prepare the sterile and stable MO–$PEG_{10,000}$–COOH (Bre–LE–$PEG_{10,000}$) coated breviscapine lipid emulsions (Bre–LE) for intravenous administration. Follow the optimum preparation, the average particle size, polydispersity index, zeta potential, Ke value and content of final product were determined to be (207.1 ± 8.5) nm, 0.197 ± 0.005, (−33.6 ± 2.0) mV, (21.1 ± 2.3)% and (95.0 ± 1.8)% respectively (n = 3). The characteristics, stability and safety of Bre–LE–$PEG_{10,000}$ were also studied with Bre–LE as a control. Increased plasma concentration by surface modification of the lipid emulsions may enhance the pharmacological activity of breviscapine to promote blood circulation.

1. Introduction

Particulate drug-carriers, after intravenous administration, are removed from blood circulation by cells of the reticuloendothelial system (RES). Poly(ethylene glycol) (PEG) is a biocompatible, non-toxic and hydrophilic polymer, which is widely used in developing a surface modifying material (Trubetskoy and Torchilin, 1995; Xiong et al., 2006a; Chen et al., 2010; Kutscher et al., 2010; Zhao et al., 2011). Long-circulation (sterically stabilized) particulate drug-carriers, like liposomes, solid lipid nanoparticles, gold nanoparticles (Prencipe et al., 2009) and single-walled carbon nanotubes (SWNTs) (Liu et al., 2008), have been prepared by modifying their surface with hydrophilic PEG derivatives to reduce their RES uptake and increase their circulation half-time. At present, although there is no consensus about the mechanism how PEG modified particulate drug-carriers are removed from the circulation, it is hypothesized that PEG mediates a prolonged circulation time by opposing opsonization of particulate drug-carriers (Liu et al., 1995).

Breviscapine is one of cardiovascular drugs extracted from a Chinese herb *Erigeron breviscapinus* (Vant.) Hand.-Mazz (Xiong et al., 2009). According to records of Chinese ancient book "Dian Nan Ben Cao", it can promote blood circulation, remove blood stasis and dredge the meridian passage. It contains mainly scutellarin (primary active ingredient, 4′,5,6-tetrahydroxyflavone-7-*O*-glucuronide) and little apigenin-7-*O*-glucuronide. Scutellarin has poor solubility in water, and can soluble in ether, chloroform, ethanol, acetic acid and acetone. It is only stable in acidic conditions and rather unstable in alkaline solutions. The lipid emulsions (LE, oil-in-water emulsions stabilized by lipid surfactants), used as carrier for breviscapine, might improve the chemical stability of drug, increase drug loading efficiency, decrease irritation on the surrounding tissue as well as control and modify its pharmacokinetics and tissue distribution. Lipid emulsions as particulate drug-carriers can be produced on large industrial scale and sterilized by autoclaving but avoid drug leakage from carriers like liposomes (Allen and Cleland, 1980; Thomas and Tirrell, 2000). However, the adsorption of plasma proteins onto intravenously injected lipid emulsions is considered to be the same crucial factor determining the organ distribution (Göppert and Müller, 2003) as that of liposomes. Increasing plasma concentration of breviscapine by surface modification of the lipid emulsions may be sorely needed to enhance the pharmacological activity of promoting blood circulation, removing blood stasis and dredging the meridian passage.

A novel series of monooleate-modified PEG with active carboxylic terminus (MO–PEG–COOH) has been synthesized and

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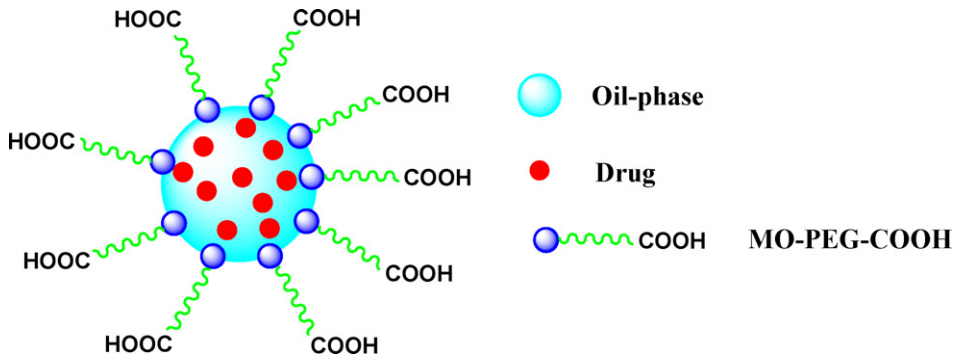

Scheme 1. The scheme of breviscapine lipid emulsions coated with MO–PEG–COOH.

characterized previously (Xiong et al., 2006a). This associated polymer is inexpensive, easily synthesized to obtain large quantities of product with high purity, multifunctional and especially fits for surface modification of emulsions. The lipophilic moiety of the associated polymer is sufficiently hydrophobic to firmly anchor the hydrophilic coat to the surface of oil core because the lipophilic moiety of polymer (monooleate-end) and the component of oil phase (oleic acid) are isogenous. On the other hand, the presence of free pendant carboxyl groups on the polymer is expected to enhance the biodegradability of the polymer and to facilitate further modifications of the polymer, such as conjugation with drug molecules, short peptides and oligosaccharides (Guan et al., 2005).

In the present study, series of MO–PEG–COOH were used to modify the lipid emulsions surface to prepare a sterically stabilized LE for carrying traditional Chinese medicine (Scheme 1). Based on the research of relationship between polymer structure and prolonged circulation activity, we developed an optimized formulation for the preparation of sterile and stable breviscapine lipid emulsions (Bre–LE) coated with MO–PEG–COOH (Bre–LE–PEG) for intravenous administration. The physicochemical properties, stability, the ability to evade capture by the reticuloendothelial system and to prolong the circulation time in mice were studied. The safety of Bre–LE–PEG was also investigated in contrast to Bre–LE.

2. Experimental

2.1. Materials

Breviscapine was provided by Jiangsu Chia-tai Tianqing Pharmaceutical Co. Ltd. (Jiangsu, China). The percentage of scutellarin and apigenin-7-*O*-glucuronide was 96.4% and 3.6%, respectively. Scutellarin standard (purity >98%) was purchased from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Lipoid E80 (egg yolk lecithin with 80–85% of phosphatidylcholine) was purchased from Lipoid GmbH (D-Ludwigshafen, Germany). Poloxamer 188 was purchased from BASF (China) Co., Ltd. (Shanghai, China). Tween 80, Brij 35 and Mrij 59 were purchased from Nanjing Will Chemical Co., Ltd. (Nanjing, China). Monooleate-modified poly(ethylene glycol) with oxymethyl terminal (MO–PEG–OCH_3, with PEG number average molecular weights of 400, 2000) was purchased from Liaoning Kelong Fine Chemical Co., Ltd. (Liaoning, China). Triton X-100 was purchased from Sigma. Fetal calf serum was purchased from Hangzhou Sijiqing Bioengineering Material Co., Ltd. Other chemicals used were of analytical grade. Kunming mice (male, 8 weeks old, 18–22 g) were purchased from the Experimental Animal Center of China Pharmaceutical University (Jiangsu, China).

2.2. Optimized preparation of Bre–LE–PEG

Series of MO–PEG–COOH with PEG number average molecular weights of 400–10,000 Da have been prepared in previous studies. The synthesis consists of two steps of chemical modification of PEG by esterification, one with succinic anhydride to obtain active carboxylic terminal, and the other with oleic acid to obtain sufficiently hydrophobic terminus (Xiong et al., 2006a).

The type, amount and method of MO–PEG–COOH added to Bre–LE–PEG were optimized as reported previously (Xiong et al., 2010). In detail, method I (external added method): the oil phase was prepared by dissolving scutellarin (0.04 g) in the mixture of Lipoid E80 (1.2 g), vitamin E (V_E) (0.6 g) and soybean oil (10 g, containing 1.2 g oleic acid). To make the aqueous phase, Poloxamer 188 (2.0 g) and acquired amount of varying molecular weight MO–PEG–COOH or MO–PEG–OCH_3 dissolved in a mixture of glycerol (2.25 g) and 100 ml double distilled water. The emulsions were prepared by mixing the oil phase and the aqueous phase with a constant speed stirrer (XHF-1 Stirrer, Shanghai Xinda BioChem Instrument Co. Ltd., Shanghai, China) at 8000 rpm for 60 s, then passing the mixture through a homogenizer (EmulsiFlex-05, Avestin, Canada) at 20,000 psi for twenty times. The final products were filter sterilized through a 0.22 μm filter into a sterile container. Brij 35, Mrij 59, Tween 80, MO–PEG_{400}–OCH_3 and MO–PEG_{2000}–OCH_3 were also used as control surface modifiers in prolonged circulation property. These polymers were added in the same manner as MO–PEG–COOH. Emulsions without surface modifier were also prepared.

Another two methods of modifying the surface of particulate drug-carriers were as follows. Method II (internal added method): Bre–LE–PEG was prepared in the same manner as mentioned above except acquired amount of polymer was added to oil phase (Wheeler et al., 1994; Liu and Liu, 1995). Method III (incubation method): the same procedures were applied for preparing Bre–LE–PEG as described above except 50 ml of double distilled water was added and no polymer was added in aqueous phase, after homogenization the emulsions were mixed with an equal volume of polymer solution followed by incubation at room temperature for 1 h (Takeuchi et al., 1998, 2001).

2.3. Measurement of size distribution and zeta potential

Particle size and width of the distribution (polydispersity index, PI) were determined by photon correlation spectroscopy (Msater-Sizer 3000, Malvern Instruments Co., Worcestershire, UK). Data were analyzed in terms of intensity, volume, or number weighted distribution and reported as *z*-average diameter. The zeta potential (ζ) was measured by Malvern Zetasizer 3000 (Malvern Instruments Co., Worcestershire, UK). All samples were 1:100 diluted with 2.25% glycerin.

2.4. Measurement of stability constant (Ke)

Stability constant was determined by centrifugation–spectrophotometry method (Xi et al., 2000; Xiong et al., 2010). In brief, the supernatant of emulsions before and after centrifugation (4000 rpm for 15 min) was diluted by water to an appropriate concentration. Then the turbidity of diluted sample was determined by spectrophotometry (500 nm) and water blank was used as a reference. The stability constant Ke (%) = $(A_0 - A)/A_0 \times 100\%$. A_0 and A are the absorption of the diluted supernatant before and after centrifugation, respectively.

2.5. Determination of scutellarin in emulsions and biosamples by RP-HPLC assay

The concentration of scutellarin in emulsions was measured by RP-HPLC. The emulsions were dissolved by using 10% Triton X-100 in ethanol as emulsion breaking agent. A 20 μl aliquot of the supernatant fluid of broken emulsions was injected into HPLC column for assay. Biosamples (including plasma, liver and spleen) were collected as previously described (Xiong et al., 2011). In brief, 200 μl methanol was added to 100 μl aliquot of plasma or tissue homogenate (10%, w/v) with 1% sodium bisulfate. Then, the mixed samples were shaken and centrifuged at 12,400 rpm for 10 min at 4 °C. A 20 μl aliquot of the supernatant fluid was injected into HPLC for assay. The chromatographic system (Xiong et al., 2006b) consisted of a Waters 510 HPLC pump and a Waters 486 Absorbance UV detector (Waters Corp., Milford, MA, USA). The wavelength of this detector was set to 335 nm. The HPLC system was controlled by a computer employing the Millennium 2010 ChemStation software. The analytical column was a reverse phase Hypersil C_{18} column (250 mm × 4.6 mm, 5 μm particle size; Dalian Elite Analytical Instrument Co., Ltd., Dalian, China) maintained in a column oven (Timberline Instruments, Boulder, CO, USA). The mobile phase was composed of methanol–water–glacial acetic acid (40:60:1). Elution was performed isocratically at 40 °C at a flow-rate of 1.0 ml/min.

2.6. Stability

Physical and chemical stability of Bre–LE–$PEG_{10,000}$ after dilution were studied. Because the distribution of nanoparticles *in vivo* depends on the particle size, it is important for emulsion to maintain the drug loaded emulsion droplet to reach targeting organ without changes of particle size in blood. The physical stability evaluated in this paper was to mimic the physical stability of emulsion droplet in blood. For study of physical stability, Bre–LE–$PEG_{10,000}$ was diluted by PBS (pH 7.4) containing 10% fetal calf serum at 37 °C. At 6, 12 and 24 h after dilution, the particle size and polydispersity index were measured. To investigate ability of carriers to protect drug from chemical degradation after dilution by PBS or PBS containing 10% fetal calf serum, Bre–LE–$PEG_{10,000}$ without V_E (Bre–LE–$PEG_{10,000}$-1) was used to avoid the interference from chemical protection of V_E. The same procedures were applied for preparing Bre–LE–$PEG_{10,000}$-1 as the one for Bre–LE–$PEG_{10,000}$ except that no V_E was added to the oil phase. Dilution stability experiment was carried out as follows (Xiong et al., 2010): Bre–LE–$PEG_{10,000}$-1 was diluted 10-fold with PBS or 10% fetal calf serum at 37 °C. The samples were withdrawn at appropriate intervals and prepared as biosamples disposing procedure mentioned above. The scutellarin remained in the diluted samples was determined by HPLC.

Long-term stability of Bre–LE–$PEG_{10,000}$ was evaluated after storage at room temperature for up to 6 months. The particle size, polydispersity index, zeta potential, stability constant Ke and content of scutellarin in emulsions were determined as a function of the storage time. The content of scutellarin was determined by HPLC. The particle size, polydispersity index, zeta potential and stability constant Ke were measured as described previously.

2.7. Biodistribution of scutellarin in mice

Kunming mice with an average weight of 20 g were used in this study. Different PEG molecule attached Bre–LE–PEG were injected intravenously into the tail vein of the mice (25 mg/kg). Animals were sacrificed under ether anesthesia at 30 min after administration. Blood was collected in heparin-coated tubes and centrifuged at 2500 rpm for 5 min to separate the plasma. Liver and spleen were removed, weighed and homogenized (10%, w/v) in a solution of 1% sodium bisulfate in normal saline. All samples were immediately frozen at −20 °C until analysis.

2.8. Elementary safety assay

Hemolysis test was as follows (Xiong et al., 2004). Blood was withdrawn from New Zealand white rabbit (2.5 kg) ear vein and defibrinated by collection in vacutainer tubes containing glass beads and then rocking the tubes gently for 30 min. A 10 ml aliquot of defibrinated blood and 10 ml of normal saline were mixed and centrifuged. The supernatant was discarded and repeated this step 3 times till no red color was observed in the supernatant. Erythrocytes were diluted by normal saline to 2% (v/v). For testing the hemolysis property of emulsions, 0.1, 0.2, 0.3, 0.4, 0.5 ml of Bre–LE or Bre–LE–$PEG_{10,000}$ were added to the mixture of 2% erythrocyte solution and 2.4, 2.3, 2.2, 2.1 2.0 ml of normal saline, respectively. The mixture of 2.5 ml of 2% erythrocyte solution and 2.5 ml of normal saline was used as control.

Vascular irritation test was as follows (Xiong et al., 2004). New Zealand white rabbits (2 ± 0.058 kg) were provided by the Central Animal Laboratory of China Pharmaceutical University. Bre–LE or Bre–LE–$PEG_{10,000}$ was injected intravenously into ear-edge of one of rabbit's ear (7 mg/day) once-a-day for 3 days. As a control, normal saline was injected into the other ear. The rabbits were killed by aortic bleeding under pentobarbital anesthesia in the 24 h after last administration. The ear was removed. The cut place was the injection site 1.3 (near-injection terminal) and 4.0 cm (far-injection terminal) towards the heart. Then the tissue was 10% formalin-fixed, paraffin embedded and HE stained for evaluation of vascular irritation.

To investigate elementary toxicity of Bre–LE–$PEG_{10,000}$, the mice were divided into three groups of ten animals. The mice were injected intravenously into the tail vein with normal saline, Bre–LE (prepared as reported previously (Xiong et al., 2010)) or Bre–LE–$PEG_{10,000}$ (13 mg/kg) once-a-day for 7 days. After the last administration, animals were euthanized and their liver and kidneys were removed, 10% formalin-fixed, paraffin embedded and HE stained for evaluation of liver and kidneys toxicity.

3. Results and discussion

3.1. Optimized preparation of Bre–LE–PEG

The results of optimized adding method of MO–PEG–COOH are as follows. Method II (internal added method) is not suitable for MO–PEG–COOH because these series polymers do not dissolve in oil phase. The process of method I (incubation method), in which the constant speed stirring and homogenization were involved, is not feasible before adding half of the polymer solution because of the excessive viscosity of the emulsion. Additionally, the phenomena of creaming and emulsion-broken were observed within 12 and 24 h, respectively in static condition. Method I (external added method) is an easy method for the preparation

Table 1
Effects of varying compositions of breviscapine emulsions on biodistribution in mice (mean ± S.D., n = 3–5).

Composition of emulsions[a]	% Injected dose (ID) per tissue[b]				Blood/RES
	Blood	Liver	Spleen	RES[c]	
None	3.35 ± 0.57[d]	1.48 ± 0.62	0.25 ± 0.10	1.73	1.94
Brij 35	3.39 ± 0.95	1.29 ± 0.55	0.23 ± 0.04	1.52	2.23
Mrij 59	3.56 ± 0.80	1.39 ± 0.54	0.22 ± 0.07	1.61	2.21
Tween 80	3.89 ± 0.81	1.18 ± 0.37	0.19 ± 0.04	1.37	2.84
MO–PEG_{400}–OCH_3	3.41 ± 0.93	1.39 ± 0.60	0.29 ± 0.14	1.68	2.03
MO–PEG_{400}–COOH	3.57 ± 0.76[d]	1.43 ± 0.38	0.23 ± 0.06	1.66	2.15
MO–PEG_{2000}–OCH_3	3.48 ± 1.02	1.31 ± 0.42	0.32 ± 0.08	1.63	2.13
MO–PEG_{2000}–COOH	3.73 ± 0.74[d]	1.36 ± 0.33	0.19 ± 0.07	1.55	2.41
MO–PEG_{6000}–COOH	4.02 ± 0.87[d]	1.14 ± 0.59	0.17 ± 0.10	1.31	3.07
MO–$PEG_{10,000}$–COOH	4.94 ± 1.27[d]	1.00 ± 0.32	0.10 ± 0.05	1.10	4.59

[a] 160–220 nm particle size distribution; 2 mmol/l of polymer added in emulsions.
[b] 30 min after injection.
[c] RES = liver + spleen.
[d] Citing from Ref. Xiong et al. (2006a).

of MO–PEG–COOH-coated breviscapine lipid emulsions. Furthermore, the coated emulsions were stable for six months without any creaming or emulsion-broken phenomenon. Therefore, method I – external added method was chosen as the optimal preparation method.

The results of optimized type of MO–PEG–COOH added to Bre–LE–PEG are as follows. Emulsions with varying compositions were prepared by method I for *in vivo* long circulating studies. From these results we decided which molecular weight of PEG should be picked for optimal preparation. The results are showed in Table 1. The injected dose of drug was calculated based on the theory that blood volume was 7.3% of body weight. RES organs include liver, spleen and bone marrow. The drug captured by bone marrow was negligible (Woodle et al., 1992). Therefore the amount of drug captured by RES could be calculated approximately as the amount of drug captured by liver plus that by spleen. In this study, we used the ratio of drug in blood to the drug in RES (blood/RES) as prolonged-circulation index to evaluate the long-circulating ability of polymer. As the present results show, almost every surface modifier can prolong the circulation time of emulsions in blood and to avoid uptake by the liver and spleen. The common characteristic of these surface modifiers is all these polymers have flexible long hydrophilic chain. Synthesized polymers with large molecular weight PEG (PEG_{6000} and $PEG_{10,000}$) attached have higher capability to enhance circulation longevity of emulsions and reduce their uptake by RES than commercial polymers such as Brij 35, Mrij 59 and Tween 80. Normal emulsions without PEG and other hydrophilic surface modifiers showed low blood levels, suggesting that emulsions of this size range (160–220 nm) are readily taken up by the RES.

For the same molecular weight of PEG, as can be seen in Table 1, the group of the synthesized polymers with free –COOH terminus showed slightly increased blood level of emulsions (3.73 ± 0.74% ID), correspondingly slightly decreased RES uptake (1.55% ID) and increased blood/RES ratio (2.41) in contrast to that with $–OCH_3$ terminus (blood = 3.48 ± 1.02% ID, RES = 1.63% ID and blood/RES = 2.13).

For the PEG attached polymers with –COOH terminus, as the present results show, the order of their activities of prolonged circulation is MO–$PEG_{10,000}$–COOH (blood/RES = 4.59) > MO–PEG_{6000}–COOH (blood/RES = 3.07) > MO–PEG_{2000}–COOH (blood/RES = 2.41) > MO–PEG_{400}–COOH (blood/RES = 2.15). It can be concluded that these potential activities are PEG's molecular weight dependent. MO–$PEG_{10,000}$–COOH, which showed the best blood residence level at 30 min after injection in Table 1, was used to prepare MO–PEG–COOH coated breviscapine lipid emulsions in the next experiments. Therefore, MO–$PEG_{10,000}$–COOH was chosen as the optimal type of MO–PEG–COOH.

Table 2
Characteristics of breviscapine lipid emulsions before and after coated with MO–$PEG_{10,000}$–COOH (mean ± S.D., n = 3).

Conc. of polymer (mmol/l)	Particle size (D, nm)		D(coated)/D(original)[a]
	Original	Coated	
1	209.1 ± 6.4	196.3 ± 8.7	0.939
2	↓	181.1 ± 4.7	0.866
3	↓	179.9 ± 8.5	0.860

[a] D(original) = particle size of non-coated breviscapine lipid emulsions; D(coated) = particle size of breviscapine lipid emulsions coated with MO–$PEG_{10,000}$–COOH.

The results of the optimization of the amount of MO–PEG–COOH added to Bre–LE–PEG were as follows. As shown in Table 2, the particle size of MO–$PEG_{10,000}$–COOH coated emulsions was decreased with the increasing concentration of polymer in the aqueous phase of emulsions, suggesting the surface activity of the polymer with one hydrophilic terminus and another lipophilic terminus. The ratio of particle size of breviscapine lipid emulsions coated with MO–$PEG_{10,000}$–COOH to that of non-coated breviscapine lipid emulsions decreased significantly when the concentration of polymer increased from 1 mmol/l (D(coated)/D(original) = 0.939) to 2 mmol/l (D(coated)/D(original) = 0.866). But the increase of polymer concentration from 2 mmol/l to 3 mmol/l did not result in significant change in D(coated)/D(original) (from 0.866 to 0.860, respectively). Fig. 1 showed the change in the zeta potential with different concentration of polymer in aqueous phase. The increased zeta potential of breviscapine lipid emulsions after coated with MO–$PEG_{10,000}$–COOH originated from negatively charged group of carboxyl at the terminal of the polymer. The increased absolute value of zeta potential was of benefit to the stability of emulsions *in vitro* and *in vivo*. There was no significant increase between zeta

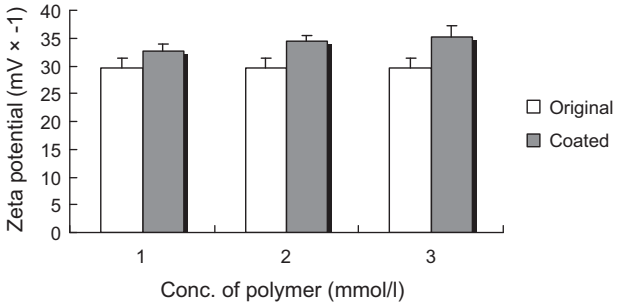

Fig. 1. Zeta potential of breviscapine lipid emulsions before and after coated with MO–$PEG_{10,000}$–COOH (mean ± S.D., n = 3).

Table 3
Effects of concentration of MO–$PEG_{10,000}$–COOH in breviscapine emulsions on biodistribution in mice (mean ± S.D., n = 3–5).

Conc. of polymer (mmol/l)[a]	% Injected dose (ID) per tissue[b]				Blood/RES
	Blood	Liver	Spleen	RES	
1	4.12 ± 0.60	1.21 ± 0.48	0.11 ± 0.05	1.32	3.49
2	4.94 ± 1.27[c]	1.00 ± 0.32	0.10 ± 0.05	1.10	4.59
3	4.76 ± 0.46	0.94 ± 0.21	0.11 ± 0.03	1.05	4.62

[a] 160–220 nm particle size distribution.
[b] 30 min after injection.
[c] Citing from Ref. Xiong et al. (2006a).

potential of emulsions coated with 2 mmol/l polymer and that of 3 mmol/l polymer.

The change of biodistribution and prolonged circulation index as a function of concentration of polymer also confirmed this trend (Table 3). The drug amount of breviscapine lipid emulsions coated with MO–$PEG_{10,000}$–COOH in blood increased significantly when the concentration of polymer increased from 1 mmol/l (4.12 ± 0.60% ID) to 2 mmol/l (4.94 ± 1.27% ID), and drug amount in liver decreased from 1.21 ± 0.48% ID to 1.00 ± 0.32% ID. But the change of drug amount in spleen was insignificant. Being coated with 2 mmol/l polymer and that of 3 mmol/l polymer resulted in no significant increase between blood/RES value of emulsions. Because the particle size was reported by z-average diameter, the value of particle size was including other particles in the suspension besides emulsion droplets coated with MO–$PEG_{10,000}$–COOH when the polymer concentration increased over the necessary concentration for coating emulsion droplets, for example, micelle of MO–$PEG_{10,000}$–COOH. And the micelle formed by MO–$PEG_{10,000}$–COOH was smaller than these emulsion droplets. Therefore the z-average diameter of suspension decreased with polymer concentration increasing over the necessary concentration for coating emulsion droplets. Based on the results above, it can be concluded that there was no more spare space for coating on the surface of the emulsion particles and no more increase in thickness of the coating layer with an increase in the concentration of the polymer from 2 mmol/l to 3 mmol/l. Therefore, 2 mmol/l was chosen as the optimal amount of MO–$PEG_{10,000}$–COOH added to the aqueous phase of emulsions.

The critical parameters of optimum formulation and fabrication method of making Bre–LM–PEG were: 2 mmol/l of MO–$PEG_{10,000}$–COOH was added to the aqueous phase of emulsions by external added method.

3.2. Characterization of Bre–LM–$PEG_{10,000}$

Follow the optimum preparation found above, the average particle size, polydispersity index, zeta potential, Ke value and content of final product were (207.1 ± 8.5) nm, 0.197 ± 0.005, (−33.6 ± 2.0) mV, (21.1 ± 2.3)% and (95.0 ± 1.8)%, respectively (n = 3). Bre–LM–$PEG_{10,000}$ is safe for intravenous administration because the size distribution was narrow and the particle size was smaller than 1 μm (Lv et al., 2006). The results of zeta potential and Ke value prefigure the Bre–LM–$PEG_{10,000}$ maybe stable for a long period.

3.3. Stability

The effects of polymer coating on the physical dilution stability of emulsions in fetal calf serum were evaluated by measuring the change in particle size and polydispersity index (Fig. 2). The particle size and polydispersity index of noncoated emulsions after incubation in fetal calf serum for 24 h increased approximately 1.15-fold and 1.8-fold, respectively, which suggested that aggregation and/or fusion of emulsions occurred in the serum. The aggregation and/or

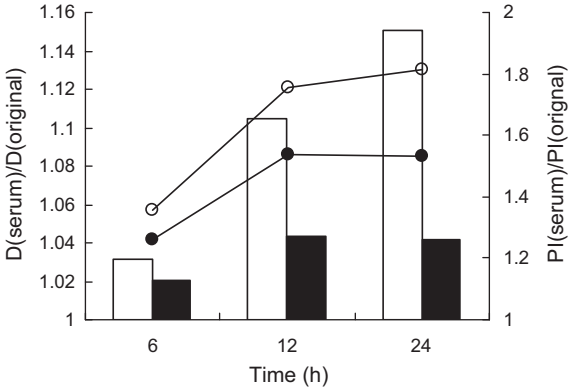

Fig. 2. Effect of polymer coating with MO–$PEG_{10,000}$–COOH on the change in particle size (D) (bar) and polydispersity index (PI) (circle) of breviscapine lipid emulsions during incubation in 10% fetal calf serum (n = 3). Breviscapine emulsions (empty symbol); breviscapine emulsions coated with MO–$PEG_{10,000}$–COOH (dark symbol).

fusion were impeded by the MO–$PEG_{10,000}$–COOH coating as shown in Fig. 3. The polymer layer on the surface of emulsions may effectively prevent the adsorption of aggregation promoting components such as protein in serum.

Drug was diluted before it was injected. The chemical dilution stability experiment was designed to simulate the chemical stability *in vivo*. The dilution stability is very important to drugs with poor

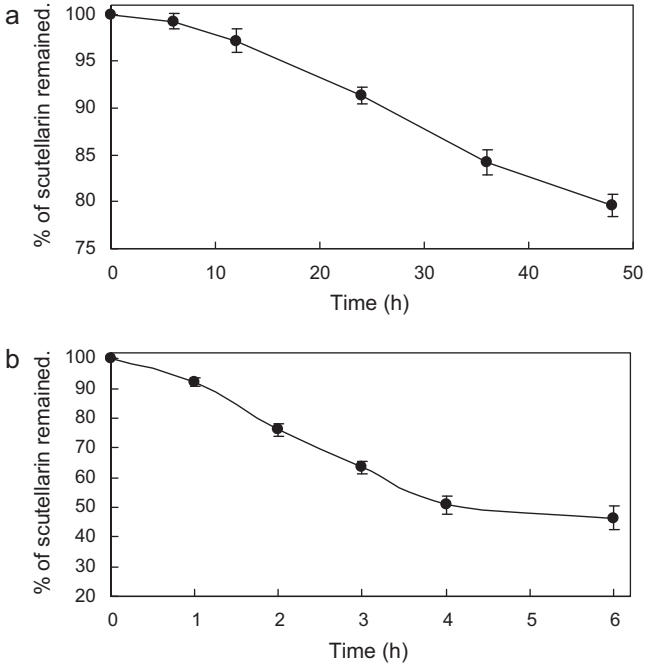

Fig. 3. Degradation of scutellarin in Bre–LM–$PEG_{10,000}$ diluted with pH 7.4 PBS (a) and fetal calf serum (b) at 37 °C (mean ± S.D., n = 3).

Table 4
Long-term stability of Bre–LM–$PEG_{10,000}$ under storage at room temperature (mean ± S.D., $n = 3$).

Storage time (month)	Particle size (nm)	Polydispersity index	Zeta potential (mV)	Ke (%)	Content of scutellarin (%)
0	207.1 ± 8.5	0.197 ± 0.005	−33.6 ± 2.0	21.1 ± 2.3	95.0 ± 1.8
1	219.4 ± 4.5	0.203 ± 0.011	−25.8 ± 1.5	22.0 ± 0.7	94.0 ± 1.1
3	216.5 ± 7.1	0.215 ± 0.008	−24.2 ± 2.6	24.5 ± 3.3	93.8 ± 1.3
6	228.5 ± 5.4	0.220 ± 0.006	−18.7 ± 4.2	24.1 ± 1.5	92.6 ± 1.0

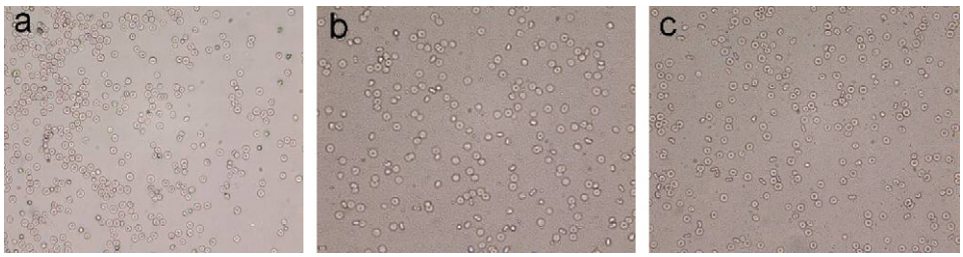

Fig. 4. Representative photomicrographs of red blood cell in haemolyticus test. (a) Control; (b) Bre–LE (0.0067%); (c) Bre–LE–$PEG_{10,000}$ (0.0067%).

chemical stability, since degradation after being injected is one of the key factors making the plasma half-life shorter. The results of dilution stability in PBS and 10% fetal calf serum are shown in Fig. 3. The results show there is a nearly linear degradation of scutellarin in both PBS and 10% fetal calf serum. The first-order rate constants of Bre–LE–$PEG_{10,000}$-1 were 0.0051 in PBS and 0.1411 in fetal calf serum, respectively. It was reported the first-order rate constants of Bre–LE were 0.0050 in PBS and 0.1484 in fetal calf serum, respectively (Xiong et al., 2010). The results show the polymer coating could not further protect drug from chemical degradation after dilution by PBS and fetal calf serum in contrast to Bre–LE. The *in vivo* chemical stability is closely related to pharmacokinetices property. The half-life ($t_{1/2}$) of breviscapine was short (18.38 min) (Xiong et al., 2011). The longer half-life of breviscapine loaded in Bre–LE–$PEG_{10,000}$ would be expected in the further study.

The long-term stability data for Bre–LE–$PEG_{10,000}$ under storage at room temperature is summarized in Table 4. The particle size, polydispersity index and Ke increased 1.10-, 1.12- and 1.14-fold from 0 to 6 months, respectively. Absolute value of zeta potential and content of scutellarin decreased gently. Based on the data, Bre–LE–$PEG_{10,000}$ was physically and chemically stable for 6 months at room temperature. The physical stability of particulate

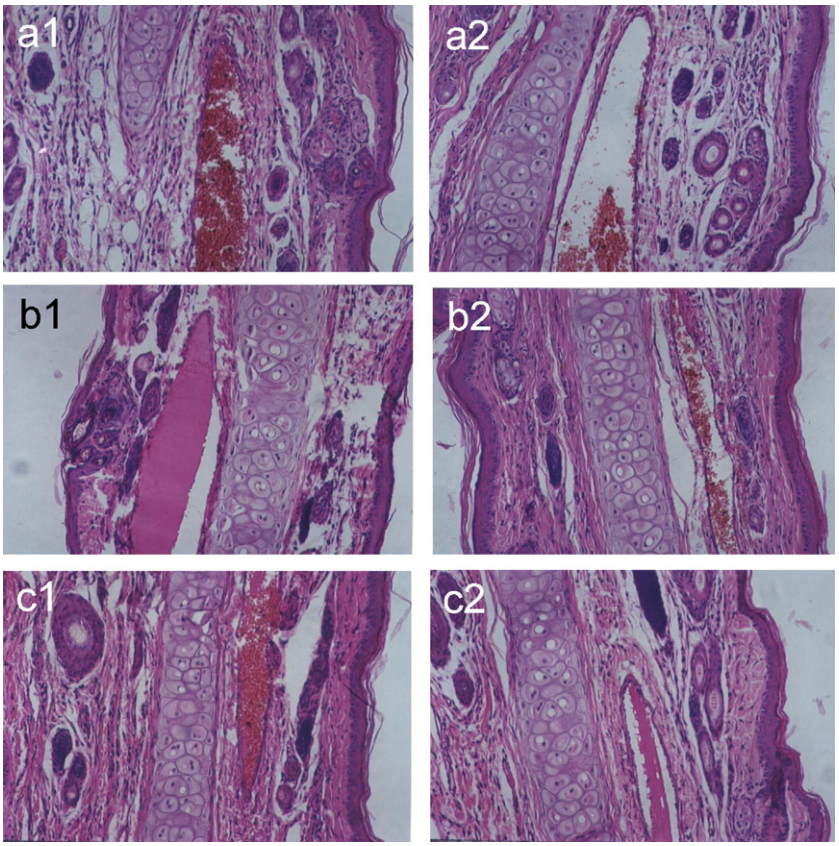

Fig. 5. Representative pathology slide photomicrographs of rabbit ear vein slices in vascular irritation test. (a) Physiological saline (control); (b) Bre–LE; (c) Bre–LE–$PEG_{10,000}$. 1: near to injection site (1.3 cm); 2: far from injection site (4.0 cm).

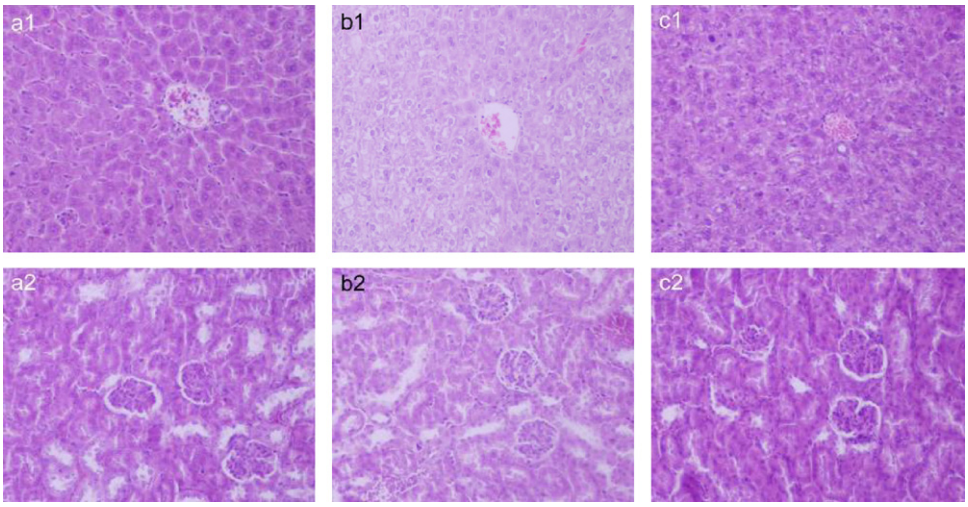

Fig. 6. Representative photomicrographs of liver and kidney slices of mice. (a) Physiological saline (control); (b) Bre–LE; (c) Bre–LE–$PEG_{10,000}$. 1: liver; 2: kidney.

drug carrier is an important factor in prolonged circulation and delivering drug to the target other than the RES.

3.4. Safety assay

The safety assay about long-circulation particulate drug-carriers coated with PEG derivatives has been less reported. Hemolysis and vascular irritation are very important tests for parenteral preparations before animal experiments and clinical trial because vessels and erythrocytes are the initial parts of the body meeting and interacting with intravenously injected preparations. For safety assay of Bre–LE–$PEG_{10,000}$, hemolysis test *in vitro* and vascular irritation test in rabbits *in vivo* were carried out. The red color of solution in the tube of hemolysis test did not become darker with increasing concentration of Bre–LE and Bre–LE–$PEG_{10,000}$. In addition, there was neither broken nor agglutinated erythrocytes observed under microscope (Fig. 4) after 0.0013–0.0067% Bre–LE or Bre–LE–$PEG_{10,000}$ was added in 2% erythrocyte solution for 0.5–3 h.

The results of vascular irritation in rabbits are shown in Fig. 5. The results showed there was no swelling, thrombus, degeneration or inflammatory cell infiltration observed after intravenous administration of Bre–LE and Bre–LE–$PEG_{10,000}$ at the dosage of 2.5 mg/kg in the pathology slide photomicrographs of normal, Bre–LE and Bre–LE–$PEG_{10,000}$ groups.

The results of elementary toxicity in mice are shown in Fig. 6. The liver slice of normal saline group showed normal liver cells arranged in cords. No degeneration or necrosis was observed. The liver slices of both Bre–LE group and Bre–LE–$PEG_{10,000}$ group showed liver cells also arranged in cords. But the cytoplasma was looser, and symptoms of steatosis were observed in a few of liver cells. It has been reported that hepatic steatosis characterized by increased saturated fatty acids leads to liver injury (Pachikian et al., 2008). It might be caused by the saturated fatty acids of soybean oil in the formulation of both Bre–LE and Bre–LE–$PEG_{10,000}$. However, the slight injury of liver cell in Bre–LE–$PEG_{10,000}$ group was no more serious than that of Bre–LE group. The results of kidney slice showed no significant difference among physiological saline group, Bre–LE group and Bre–LE–$PEG_{10,000}$ group, no degeneration and necrosis was observed. The results suggested there was no kidney toxicity observed in Bre–LE group and Bre–LE–$PEG_{10,000}$ group. All in all, it can be concluded that the MO–$PEG_{10,000}$–COOH coating could not cause further damage of the liver and kidneys after intravenously administration at the dosage of 13 mg/kg for 7 days compared to Bre–LE.

4. Conclusions

We developed optimized formulation and fabrication method for a novel polymer coated lipid emulsion to carry a traditional Chinese medicine – breviscapine based on the properties of varying formulation and preparation *in vitro* and *in vivo*. The Bre–LE–$PEG_{10,000}$ optimizely prepared in this paper was more stable in fetal calf serum and in the storage condition at room temperature for up to 6 months than Bre–LE. Incorporation of PEG-derivatives into Bre–LE decreased uptake efficiency by RES, resulting in enhanced stability and a prolonged circulation time *in vivo*. There was no more toxicity found in the safety tests after coating Bre–LE with MO–$PEG_{10,000}$–COOH. It lays down the groundwork for clinical application of the novel polymer of MO–$PEG_{10,000}$–COOH. Based on the biodistribution results of breviscapine in blood and RES, higher pharmacological activity of Bre–LE–$PEG_{10,000}$ in circulation system than that of Bre–LE in further pharmacodynamics study may be expected.

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