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Synthesis, properties and application of a novel series of one-ended monooleate-modified poly(ethylene glycol) with active carboxylic terminal

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Abstract

Novel series of one-ended monooleate-modified poly(ethylene glycol) with active carboxylic terminal were synthesized by two steps of chemical modification of poly(ethylene glycol) by esterification, one with succinic anhydride to obtain active carboxylic terminal, and the other with oleic acid to obtain sufficiently hydrophobic terminal. The associative polymers were characterized by IR and ^{1}H NMR, their molecular weights were measured by gel permeation chromatography (GPC). The results showed that the synthesis procedure allows us to reach total grafting. The electron microscopy images, associative behaviour of the polymers in aqueous solution and their abilities to prolong the circulation time of emulsion in mice were also investigated.

Keywords: Hydrophobically associating PEG; Surface modifier; Long circulation

1. Introduction

After a long period of inspiration by biological membranes, polymeric membrane technologies have now been established in impressively large scale. For example, surface active amphiphilic block or comb copolymers – with blocks from, e.g. poly(ethylene glycol) (PEG) or a fluorinated polymer – had been added during membrane formation, and the 'grafting-to' reactions had been used to functionalize membranes with hydrophilic macromolecules (e.g. PEG or poly(vinyl pyrrolidone)) [1]. PEG is biocompatible, non-toxic and hydrophilic, widely used for the preparation of a variety of biomaterials [2,3]. PEG is non-biodegradable, however, the lower molecular weight of PEG (less than 10,000) is easily removed from human body through kidney membrane [4]. In recent years, a wide variety of amphiphilic associated polymers containing PEG segment have been reported, such as distearoyl phosphatidylethanolamine/methoxypoly(ethylene glycol) (DSPE-PEG) [5–7], dipalmitoyl phosphatidylethanolamine/methoxypoly-(ethylene glycol) (DPPE-PEG) [8], 1-palmityl-2-oleyl phosphatidylethanolamine/methoxypoly(ethylene glycol) (POPE-PEG) and hydrogenated soy phosphatidylethanolamine/methoxypoly(ethylene glycol) (HSPE-PEG) [9]. These associated polymers were widely used in the biomedical field as surface modifiers of some particulate drug-carriers like conventional liposomes, emulsion and nanoparticles. As surface modifiers of these particulate drug-carriers, for example, conventional emulsion (oil in water), amphiphilic associated polymer with core-shell structure are formed through the segregation of insoluble hydrophobic group (phosphatidylethanolamine derivatives) into the oil core of emulsion, which is surrounded by a dense PEG shell composed of hydrophilic PEG chains, which endows these particulate drug-carriers with a stealth character in the blood compartment, achieving a long circulation in the body. However, the expense of phosphatidylethanolamine derivatives, and the difficulties in obtaining large quantities of this lipid,

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make therapeutic applications of PEG-containing drug-carriers impractical. These considerations have prompted us to search for inexpensive alternatives to phosphatidylethanolamine derivatives-modified PEG which are equally or more capable of prolonging circulation half-lives of these particulate drug-carriers.

The lipid moiety of the associated polymer must be sufficiently hydrophobic to firmly anchor the hydrophilic coat to the surface of oil core. On the other hand, presence of the pendant carboxyl groups on polymer is expected to enhance the biodegradability of the polymer and to facilitate further modifications of the polymer, such as attaching drug molecules, short peptides and oligosaccharides onto the carboxyl groups [10]. Furthermore, the blood levels of particulate drug-carriers containing PEG-derivatives which have functional groups at their terminals, such as carboxylic terminal, were higher than that of the carriers containing PEG-derivatives which have no functional groups [11]. In this study, a novel series of one-ended monooleate-modified PEG with active carboxylic terminal were synthesized, their properties and abilities to prolong the circulation time of emulsion in mice were also investigated.

2. Experimental section

2.1. Materials

Succinic anhydride and oleic acid were purchased from Shanghai Chemical Reagent Co. (Shanghai, China) and used as received. Poly(ethylene glycol) with number average molecular weights of 400 (PEG400), 1000 (PEG1000), 2000 (PEG2000), 4000 (PEG4000), 6000 (PEG6000) and 10,000 (PEG10000) with dihydroxyl end groups were precipitated from THF solution into hexane. $SOCl_2$ was freshly distilled before use. DMF and pyridine were refluxed over KOH and then distilled. Dioxane and toluene were freshly distilled over sodium metal before use. Dichloromethane was refluxed over P_2O_5 and then distilled. Scutellarin standard (purity >98%) was purchased from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Soybean oil (Beiya Industrial Co., Ltd, Liaoning, China), Lipoid E80 (Lipoid, Germany), Pluronic F68 (BASF, Germany) were used as received. All other reagents were of AR 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. Characterization

IR spectra were recorded on a Nicolet Impact 410 FT-IR instrument. NMR spectra were obtained on a Bruker AV 300 Avance spectrometer. Deuterated chloroform ($CDCl_3$) was used as solvent and tetramethylsilane (TMS) was used as internal standard. Number average molecular weight (M_n) and polydispersity index (I_p) of the polymers were determined by gel permeation chromatography (GPC) using a Waters 501 coupled with a Waters 401 refractive index detector. Four Waters Styragel columns were used: HR0.5 (molar weight from 0 to 1000 g/mol), HR1 (molar weight from 100 to 5000 g/mol), HR3 (molar weight from 500 to 30,000 g/mol) and HR4 (molar weight from 5000 to 500,000 g/mol) conditioned in THF. The eluant used was THF (HPLC Grade from SDS) at 1.0 ml/min. All chromatograms were recorded at 25° C using narrow distribution PEG calibration standards (between 200 and 12,000 g/mol). All data were processed on a PC using Baseline 810 software (Waters). Differential scanning calorimetry (DSC) was carried out for the powder samples by using Linkam DSC 600 (Linkam Scientific Instrument Ltd, UK), the temperature range was 25–120 °C and heating rate was 10 °C/min.

2.3. Synthesis of one-ended monooleate-modified poly(ethylene glycol) with active carboxylic terminal

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 terminal (Scheme 1).

2.3.1. Poly(ethylene glycol) with active carboxylic terminal (PEG-COOH)

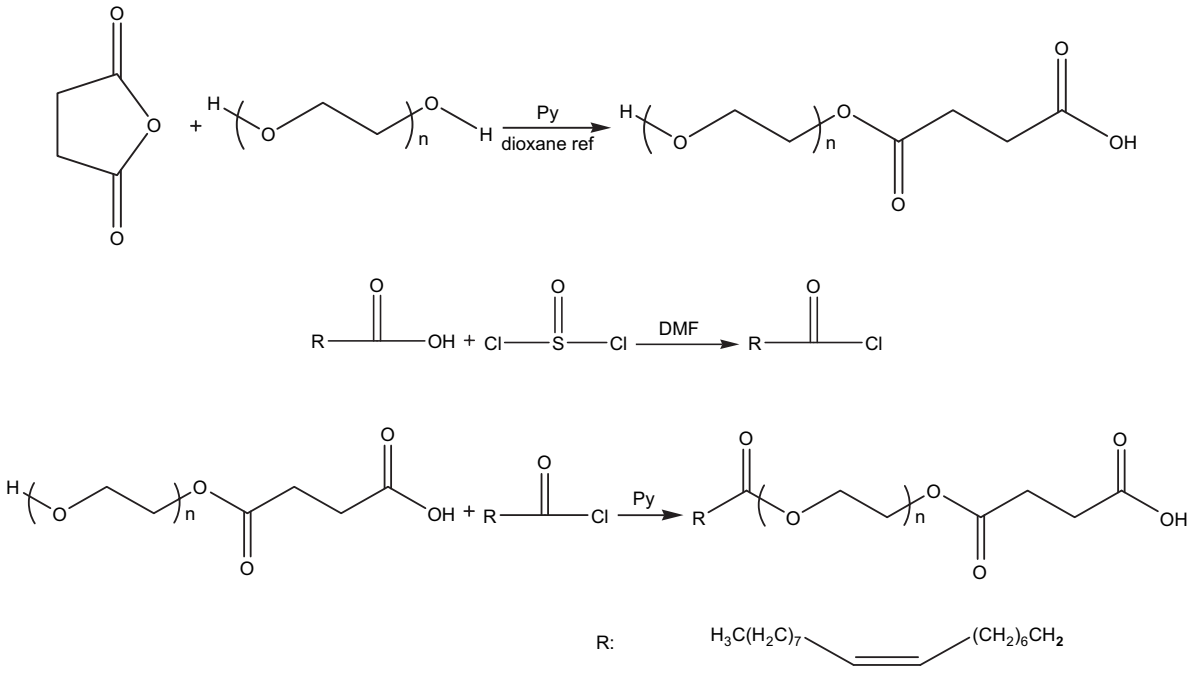
PEG (0.01 mol) and succinic anhydride (0.01 mol) were dissolved in dioxane (200 ml), pyridine added as catalyst. And the above solution was refluxed until the reaction equation was attained. The reaction mixture was cooled to the room temperature and ethyl ether was added dropwise to the mixture until no more precipitate appeared, the mixture was stirred in an ice bath for 30 min and filtrated. The precipitate was redissolved in CH_2Cl_2, and ethyl ether was added until no more precipitate appeared, then the mixture was stirred in an ice bath for 15 min, and the precipitate was filtered off and dried.

2.3.2. Oleoyl chloride

Oleic acid (0.01 mol) was dissolved in $SOCl_2$ (3 ml), and DMF was added as catalyst, refluxed for 5 h, and the crude product was obtained after evaporation of the excess $SOCl_2$.

2.3.3. Monooleate-modified poly(ethylene glycol) with active carboxylic terminal (MO-PEG-COOH)

PEG-COOH (0.01 mol) and oleoyl chloride (0.01 mol) were dissolved in CH_2Cl_2 (200 ml), and pyridine was added as acid binding agent, refluxed for 24 h, the precipitated pyridine hydrochloride was removed by filtration, and the filtrate was concentrated under reduced pressure. The crude product was recrystallized from ethyl acetate and discolored by active carbon. There was no oleoyl chloride in the product when the reaction ended. It was confirmed by thin layer chromatography (TLC) (*n*-butanol:water:acetic acid = 4:1:1). The spots were detected by placing the TLC plate in an iodine vapor chamber. After evaporation of ethyl acetate, the product was dissolved in normal saline and filtrated. For isolating MO-PEG-COOH from the mixture of MO-PEG-COOH and PEG-COOH, the filtrate was passed through a Sepharose CL-6B column using normal saline as eluant, every 5 ml a tube, lyophilized. A little methanol was added to each tube and analyzed by



Scheme 1. Reaction scheme of MO-PEG-COOH.

TLC, visualized by bromine. The tubes that contained MO-PEG-COOH were collected, centrifuged (3000 rpm, 30 min), and the final product was obtained after lyophilizing the supernatant.

2.4. Scanning electron microscopy (SEM) and transmission electron microscopy (TEM)

SEM images were obtained at 20 kV on a JSM-5310-LV microscope (Jeol, Japan). The sample was sputter coated with a thin layer of gold before testing. TEM observation of MO-PEG-COOH in double distilled water was carried out at 75 kV with H-7000 (Hitachi, Japan), which was negatively stained with 2% phosphotungstic acid and placed on a copper grid coated with film.

2.5. Viscosity measurements

Measurements were carried out on Ubbelhode No. 1c capillary (ROTH) immersed in a thermostated bath at 25 °C. Flow time was measured and converted into viscosity from the conversion charts provided by the manufacturer. These kinematic viscosities allow the reduced viscosity of the samples to be calculated.

2.6. Preparation of conventional emulsion and different molecular MO-PEG-COOH-modified emulsions

Anti-thrombus drug scutellarin was selected as model drug to investigate the prolonged-circulation property of MO-PEG-COOH associated polymers *in vivo*. The conventional scutellarin emulsion was prepared as follows. The oil phase was prepared by dissolving scutellarin (0.04%) in the mixture of Lipoid E80 (1.2%) and soybean oil (10%) using a sonicator (B5200S-DT Sonicator, Branson Ultrasonics Co. Ltd, Shanghai, China). Nitrogen gas was bubbled through this mixture for 15 min. To make the aqueous phase, Pluronic F68 (2%) was dissolved in a mixture of glycerol (2.25%) and double distilled water that had been pretreated with nitrogen gas for 15 min. A preemulsion was 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 3 min. Final emulsification was completed by passing the preemulsion through a homogenizer (EmulsiFlex-05 High Pressure Homogenizer, Avestin Inc., Canada) at 15,000 psi for five times. The same procedures were applied for preparing the different molecular MO-PEG-COOH-modified emulsions as it for the conventional emulsion except for different molecular MO-PEG-COOH (2 mmol/l) added to the aqueous phase. The particle diameter of these preparations was 200 to 300 nm.

2.7. Abilities to prolong the circulation time of emulsion in vivo

In order to study the prolonged-circulation property of MO-PEG-COOH associated polymers after the administration of conventional scutellarin emulsion and different molecular MO-PEG-COOH-modified scutellarin emulsions, 25 mg/kg scutellarin in each preparation was injected intravenously into the tail vein of Kunming mice. At 30 min after administration, five animals were sacrificed under ether anesthesia. Blood was processed and used for high-performance liquid chromatography (HPLC) analysis [12]. In brief, 10 μl of 10% sodium bisulfate solution was added to each 100 μl aliquot of mouse plasma sample to prevent oxidative degradation

of scutellarin. A 200 μl aliquot of methanol was added to precipitate protein. Then, the samples were mixed with shaking on an SW-80A vortex shaker (Shanghai Medical University Instrument Plant, Shanghai, China) for 5 min. After centrifugation for 10 min and 10,000*g* at 4 °C (Refrigerated Centrifuge 3K30, Sigma, Germany), a 20 μl aliquot of the supernatant fluid was injected into HPLC for assay. The chromatographic system 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 × 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) and protected by a guard column (10 × 4.6 mm) packed with the same material. 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.

3. Results and discussion

3.1. Synthesis and characterization of MO-PEG-COOH

The critical molar ratio depends first of all on the functionality of the components and also on the reactivity of functional groups involved in the formation of bonds [13]. It was difficult for synthesis reaction producing PEG-COOH to come to equilibrium. In general, the higher molecular weight was contained in PEG, the longer time was required. It may be due to that the reaction system had less hydroxyl groups at ends of PEG and initiation sites when the molecular weight of PEG became higher. After the reaction, the products can be easily purified by simply precipitating the associative polymer by addition of a poorly polar solvent (typically ethyl ether) and removing the unreacted materials and by-products by filtration.

To improve the reactivity of oleic acid, and also to reduce side reaction, to enhance selectivity of the reaction, oleic acid was converted to acyl chloride. The acyl chloride is rather easy to obtain and more reactive to the corresponding oleic acid. In the final esterification reaction, total conversion was obtained by pyridine, which catalyzed pyridine-hydrochloride salt formed. There are many methods for the separation of polydisperse polymers, such as adsorption chromatography, size-exclusion chromatography and liquid chromatography at the critical condition which has proved to be especially effective in the analysis of end-functionalized polymers [14]. In this paper, MO-PEG-COOH and PEG-COOH were separated through a Sepharose CL-6B column. The principle is, in water, the hydrophobic effect of oleate in MO-PEG-COOH is the driving force for micelles formation (cf. Section 3.2, Fig. 5). Therefore, MO-PEG-COOH and PEG-COOH (soluble in water and no micelles formation) could be isolated through a Sepharose CL-6B column.

Fig. 1 shows the FT-IR spectra of PEG (a), PEG-COOH (b) and the MO-PEG-COOH (c). The strong absorption band at

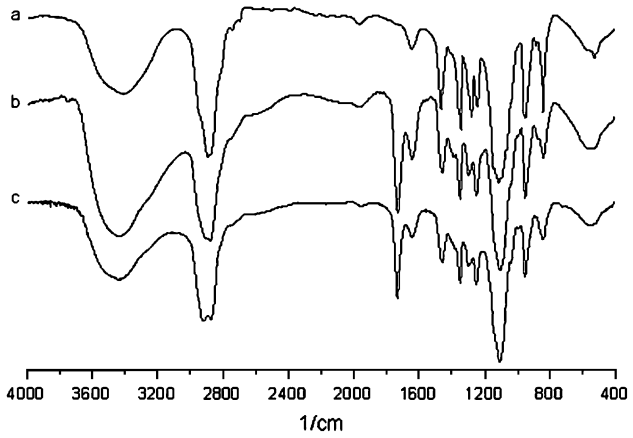


Fig. 1. FT-IR spectra of (a) PEG4000, (b) PEG4000-COOH, and (c) MO-PEG4000-COOH.

1116 cm^{-1} in Fig. 1(a) was attributed to the stretching vibration of the ether group of PEG. The characteristic absorption bands at 1730 cm^{-1} in Fig. 1(b) was due to the C=O stretching vibration. It can also be observed at 1731 cm^{-1} in Fig. 1(c).

Fig. 2 is the ^{1}H NMR spectrum of MO-PEG-COOH. The proton signals at 4.24 ppm belonged to the vinyl methine group (H–C=C–H) of oleic acid. The signals at 3.66 ppm were attributed to the methylene protons in PEG. The proton signals at 2.65 ppm were assigned as the two methylene groups ($-CH_2C=O$) of succinic anhydride. And the signals at 2.31 ppm belonged to the methylene group ($-CH_2C=O$) of oleic acid.

Characteristics of MO-PEG-COOH with different molecular weights are presented in Table 1. The functionalisation ratios were determined from the ^{1}H NMR signal integration (4.24 ppm) of the methine group of the hydrophobic group.

The states of MO-PEG-COOH with PEG number average molecular weights of 400 and 1000–10,000 were liquid and solid, respectively. The DSC traces of MO-PEG-COOH (with PEG number average molecular weights of 1000–10,000)

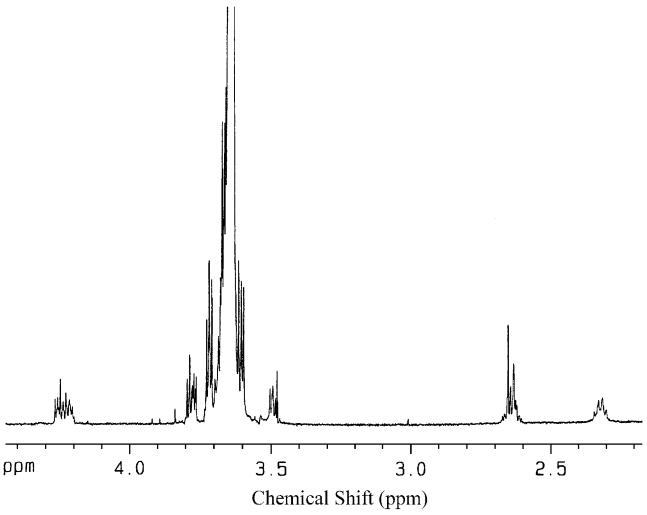


Fig. 2. ^{1}H NMR spectrum of MO-PEG4000-COOH (in $CDCl_3$).

Table 1
Characteristics of different molecular MO-PEG-COOH

Polymer	M_n GPC H_2O (g/mol)	I_p	Grafting ratio (^{1}H NMR) (%)
MO-PEG400-COOH	760	1.08	96
MO-PEG1000-COOH	1350	1.11	95
MO-PEG2000-COOH	2350	1.10	94
MO-PEG4000-COOH	4340	1.13	95
MO-PEG6000-COOH	6340	1.10	96
MO-PEG10,000-COOH	10,340	1.14	95

were all single endothermal peaks (melting peaks). The melting points of MO-PEG-COOH with PEG number average molecular weights of 1000, 2000, 4000, 6000 and 10,000 were 38.7, 59.1, 66.4, 68.2 and 71.8 °C, respectively. Fig. 3 shows the DSC trace of MO-PEG4000-COOH.

3.2. SEM and TEM of MO-PEG-COOH

The morphology of MO-PEG-COOH associated polymers (with PEG number average molecular weights of 1000–10,000) was amorphous. It demonstrated there was no crystal structure formed. Fig. 4 shows the SEM images of PEG4000 and MO-PEG4000-COOH. The TEM images of MO-PEG-COOH associated polymers (with PEG number average molecular weights of 400–10,000) in double distilled water were similar to each other, except that the particle size of lower molecular weight of MO-PEG-COOH was smaller. After dissolving in double distilled water, MO-PEG-COOH formed micelles suspended in the water. The particles were spherical and relatively uniform in size. It was also the proof that MO-PEG-COOH (form micelles in water) and PEG-COOH could be isolated through a Sepharose CL-6B column. Fig. 5 shows the TEM image of MO-PEG4000-COOH in double distilled water.

3.3. Solution property

The different molecular MO-PEG-COOH associated polymers have similar solution properties. Fig. 6 shows the variation of the reduced viscosity according to the polymer concentration for MO-PEG4000-COOH. The study reveals a marked associative behaviour: the reduced viscosity is systematically higher than that of the non-modified PEG-COOH and the evolution with the concentration is not linear. The property distinguishes associated polymers from other polymers concerning the viscosity enhancement. This particular rheological property is explained by the incompatibility

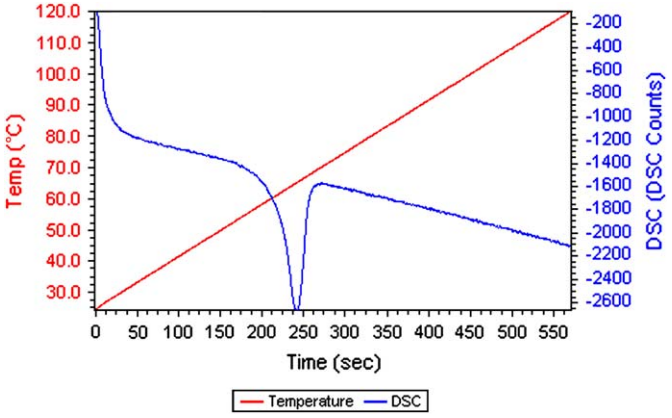


Fig. 3. DSC trace of MO-PEG4000-COOH.

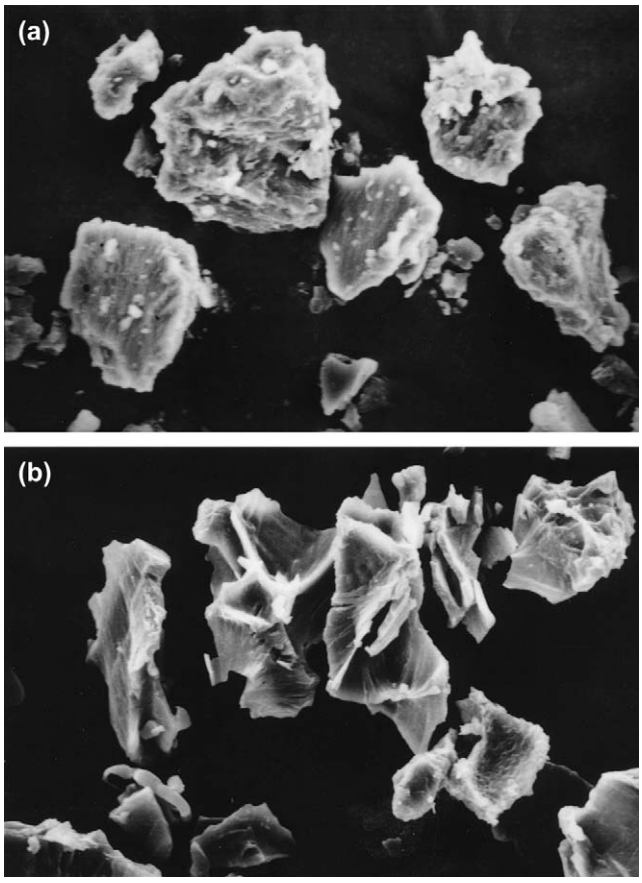


Fig. 4. SEM images of (a) PEG4000 and (b) MO-PEG4000-COOH at ×500 magnification.

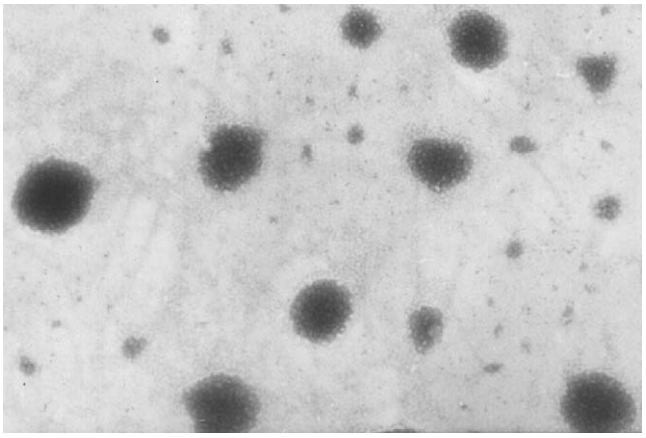


Fig. 5. TEM image of MO-PEG4000-COOH in double distilled water at ×20,000 magnification.

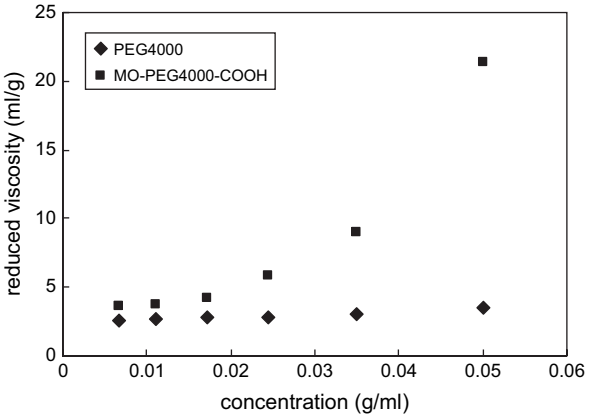


Fig. 6. Concentration study of MO-PEG4000-COOH at 25 °C.

between various groups, for example, hydrophilic and hydrophobic groups, within the same macromolecule. Depending on the importance of the repulsion and attraction forces (steric, electrostatic, etc.), these compounds may have a more or less marked antagonistic character [15–19]. A suited combination of grafted layer and membrane barrier structure with steric effect will be essential to particulate drug-carriers.

3.4. Abilities to prolong the circulation time of emulsion in vivo

The percent of the injected dose in blood is summarized in Table 2. Incorporation of the examined PEG-derivatives into scutellarin emulsion appreciably increased the blood level of emulsion at 30 min after injection. Conventional emulsion without PEG showed low blood levels. When MO-PEG-COOH was incorporated in emulsion, increasing the PEG molecular weight from 400 to 10,000 caused an increase in the blood level from 3.57 to 4.94%.

Incorporating a polymeric surface modifier during coating, forming, or a similar process is an important method for controlling surface properties [20]. The finding that coating of the particles with a hydrophilic polymer gave a slow clearance rate resulted in a major improvement of the utility of such particles. The hydrophilic coating is thought to mask the surface from opsonins marking the particle for uptake by mononuclear phagocyte system (MPS), primarily by liver Kupffer cells and spleen fixed macrophages [21,22]. We believe that the reduced MPS uptake and prolonged blood circulation are achieved by steric stabilization of the associated polymer in emulsion [7,23], consistent with the steric force of the associated polymers in solution.

Table 2
Effects of MO-PEG-COOH on blood *in vivo* for scutellarin emulsion in mice

Preparation	Percentage of dose (%)
Conventional emulsion	3.35 ± 0.57
MO-PEG400-COOH-modified emulsion	3.57 ± 0.76
MO-PEG1000-COOH-modified emulsion	3.60 ± 0.72
MO-PEG2000-COOH-modified emulsion	3.73 ± 0.74
MO-PEG4000-COOH-modified emulsion	3.89 ± 0.80
MO-PEG6000-COOH-modified emulsion	4.02 ± 0.87
MO-PEG10,000-COOH-modified emulsion	4.94 ± 1.27

Conventional emulsion and those containing various derivatives of PEG were injected *via* the tail vein in mice and their percent of the injected dose in blood was estimated at 30 min after injection. Results are expressed as mean ± SD ($n = 5$).

4. Conclusion

The synthesis of a novel one-ended monooleate-modified poly(ethylene glycol) with active carboxylic terminal allows us to obtain complete grafting of the hydrophobic end-group oleate. GPC and ^{1}H NMR were used to determine molar mass, polymolecularity and grafting ratio. The activity of MO-PEG-COOH in prolonging the circulation time of scutellarin emulsion was proportional to the average molecular weight of PEG.

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