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Preparation, Identification, and Evaluation of PEGylated Puerarin

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ABSTRACT: Cardiovascular disease is one of the most serious diseases threatening human health. Puerarin has been widely used in china as a cardiovascular agent. However, a major clinical limitation is its adverse effects of hemolysis. We have addressed this issue by PEGylation and synthesized a puerarin prodrug by covalently linking puerarin to the drug carrier monomethoxy-poly(ethylene glycol) (mPEG). The covalent linkage was validated by UV, FTIR, ^{1}H-NMR, and matrix-assisted laser desorption ionization-time-of-flight mass spectrometry. The hemolysis assay showed that the prodrug produced minimal hematotoxicity. The hydrolysis profile showed that the prodrug was stable in a low pH environment and can continuously release the active drug in physiological condition *in vitro*. A pharmacokinetic study showed a prolongation of the elimination half-life and a favorable distribution profile in organs, suggesting that the prodrug preparation produced fewer side effects on liver than the injectable form of puerarin normally used. Additionally, the protective effect of the prodrug on acute myocardial ischemia model implied better therapeutic effect than injectable puerarin. We show that the puerarin PEG conjugation is a promising prodrug that produces fewer side effects, exhibits better aqueous solubility, and is more effective than puerarin. © 2012 Wiley Periodicals, Inc. J. Appl. Polym. Sci. 000: 000–000, 2012

KEYWORDS: PEGylation; puerarin; prodrugs; pharmacokinetics; synthesis; hemotoxicity; pharmacodynamics

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INTRODUCTION

Puerarin, 4′,7-dihydroxy-8-C-glucosylisoflavone, is an active monomer isolated from *Radix Puerariae lobata* (Willd.) Ohwi. It has been shown to be effective in angiocardiopathy and clinically such as hypertension, coronary heart disease, and angina pectoris.[1,2] Unfortunately, oral absorption of puerarin is poor and erratic, due to its low solubility in water, with ~ 70% of the orally administered dose excreted in the feces after 72 h.[3] Because of poor oral bioavailability, puerarin is often prepared as injectable (i.e., intravenous) for its used in the clinical studies. Adverse reactions accompanied with iv puerarin had been reported including skin eruption, shock, and hemolysis.[4,5] These adverse effects have limited clinical applications of puerarin. Attempts to reduce adverse reactions by iv puerarin were made in many persectives. Liu et al. reported that by adding phospholipid and making an emulsion the bioavailability of puerarin in rats was improved by 2–4 times.[6] Attaching alkylamino radical or alkyl group to the 4′-OH position could enhance the water solubility of puerarin by two times.[7] However, adverse effects of hemolysis persisted. Because of adverse reactions resulting from injectable, oral administrations using improved formulations such as sustained-release tablets, or solid lipid nanoparticle were employed in many studies.[8–10] Although these formulations reduced the risk of hemolysis, the advantage of and need for an injectable in viewing of its high bioavailability and rapid onset of drug effects should be recognized. Thus, it is desirable that an injectable with a favorable aqueous solubility and minimal side effects can be developed and implemented for clinical uses.

In this article, a polymer drug delivery system for puerarin is presented. The polymer drug delivery system for small molecular drug has been used to overcome pharmaceutical issues such as unwanted side effects, limited aqueous solubility, poor biocompatibility, and immunogenicity.[11] Poly(ethylene glycol) (PEG) was often used as the polymeric carrier due to its biocompatibility, amphiphilic, and dissolvability in both water and organic solvent.[12] PEG, as approved by FDA systemic human used, can easily covalently bond to the intended molecule and achieved the PEGylation process.[13] Through PEGylation, toxicity

of molecules could be altered or even reduced. Additionally, PEGylation decreases the hydrophobicity of the intended small molecule. PEGylation has been successfully used in protein and oligonucleotides drugs for these purposes.[14,15]

In this study, we selected Gly as a linker in the PEG and made it a polymer drug carrier for the puerarin. The resulting product is a PEG prodrug of puerarin. Analytical characterization was carried out by TLC, UV–vis, FTIR, ^{1}H-NMR, and matrix-assisted laser desorption ionization-time-of-flight mass spectrometry (MALDI-TOF-MS). The key adverse effect of injectable puerarin, hemolytic property, was evaluated using our prodrug evaluated *in vitro*. The stability of prodrug was investigated in buffer solutions at different pH. Furthermore, the pharmacokinetic parameters were studied in mice. Last, the protective effect of prodrug on cardiocerebrovasular disease was compared with puerarin injectable.

EXPERIMENTAL

Materials

Chemical. Puerarin was purchased from Ankang Heye Maidisen Plant Pharmaceutical (Shan-xi, China). The following materials, with the suppliers indicated, were used as provided below: monomethoxy-poly(ethylene glycol) (M_W = 5000, $mPEG_{5000}$) Sigma Aldrich Chemie GmbH (Riedstr, Germany); Glysine (Gly) Sangon (shanghai, China); 4-dimethylaminopyridine (DMAP), NJ; 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDCI) Wanxiang Chemical (Henan, China); 4-nitrophenyl chloroformate (*p*NPC) Johnson Matthey Company (London, England). The AST, CK, and LDH kits were provided by Nanjing Jiancheng Bioengineering Institute (Nanjing, China). All organic solvents were from Hangzhou Chemical Reagent (Zhejiang, China). The solvent were dried by following methods: pyridine (heated with CaH_2 to reflux for 2 h), CH_2Cl_2 (heated with P_2O_5 to reflux for 2 h), and isopropanol (adjusted pH to 5.5–7.5 with Na_2CO_3 and heated with CaH_2 to reflux for 2 h).

Laboratory Animals. ICR mice weighing 20 ± 2 g were obtained from Laboratory Animal Center, Zhejiang Chinese Medicine University, Hangzhou, China. The animal handling procedures complied with the China National Institutes of Healthy Guidelines for the Care and Use of Laboratory Animals and were approved by local committee review prior to initiation of the studies.

Analytical Methods. The progress of each reaction product was detected with TLC on general silicone rubber sheets, spots were observed under 254 nm ultraviolet light followed by visible light after developed with I_2/KI. The UV absorption was performed by using UV8500 UV–vis Spectrophotometer (Techcomp) and infrared by FTIR spectrophotometer (FTIR200 Thermo Nicolet), in which the samples were pressed into a potassium bromide pellet prior to IR absorption spectra. The ^{1}H-NMR spectra were recorded on AVANCE III 500 MHz spectrometer (Bruker) at room temperature using solvent DMSO-d^6.

Reversed-phase HPLC was carried out with UltiMate 3000 system (DIONEX) equipped with a quaternary pump, an autosampler, and photodiode array detector set at 250 nm. Separation was achieved using a Acclaim 120 C_{18} column (250 × 4.6 mm^2, 5 μm), and a C_{18} precolumn of the same packing with a mobile phase: acetonitrile (eluant A) and ddH_2O (eluant B). The elution was performed by the following gradient: 10% of A, from 10% A–100% A over the first 5 min, followed by 6 min at 100% A. The column was injected with 20 μL of the prepared sample, eluted with constant flow rate at 1 mL/min under conditions described above at 30°C.

Molecular weight was obtained from MALDI-TOF MS using a 4800 Plus MALDI TOF/TOF^{TM} Analyzer (AB SCIEX). The analysis was carried out in a liner mode and the data for the 2-ns pulses of the 337 nm nitrogen laser were averaged for each spectrum. The liner and positive-ion TOF detection were performed using an acceleration voltage 25 kV and a laser intensity of ~ 10% higher than the threshold. Prior to data analysis the mass spectra were smoothed with Savitsky–Golay algorithm. A saturated solution of α-CHCA in 50% acetonitrile containing 0.1% TFA, as the final concentration, was used as the matrix solution. $mPEG_{5000}$, $mPEG_{5000}$—OCO—Gly and $mPEG_{5000}$—OCO—Gly—Puerarin were dissolved in water resulting in a concentration of 1 mg/mL. The sample-matrix solutions were prepared a ratio of 1 : 2. Each mixture was thoroughly vortex and 1 μL of the sample-matrix solution was deposited onto the sample plate and dried by rapid vacuum evaporation.

Synthesis of $mPEG_{5000}$—OCO—Gly—Pur Conjugates

Synthesis of $mPEG_{5000}$—pNP. The mPEG was activated using the *p*NPC method.[16] $mPEG_{5000}$ (20 g; 10 mmol) was dissolved in 150 mL toluene and the solvent was evaporated to dryness to remove water under vacuum. The residue was dissolved in 300 mL anhydrous CH_2Cl_2, *p*NPC (6.465 g; 30.0 mmol) prior to addition of 6 mL pyridine. The reaction completed in 20 h under room temperature with continuous stirring and TLC monitoring. The fluid was condensed to 5 mL under vacuum before adding diethyl ether (250 mL) at 4°C. The reaction lasted for 2 h and the product was filtrated and dried under vacuum. Yield: 19.899 g (96.32 %, w/w).

- TLC: $CHCl_3$: CH_3OH = 7 : 0.5 (v/v)
- ^{1}H-NMR (DMSO-d_6), δ(ppm): 3.244 (3H, s, —OCH_3, and mPEG), 3.517(br s,CH_2OCH_2), 7.584, 7.566 (2H,d, CHPh), 8.337, 8.318 (2H,d, CHPh).

Synthesis of $mPEG_{5000}$-OCO-Gly. Glycine (3.028 g; 40 mmol) was weighed and dissolved in 160 mL ddH_2O/ acetonitrile (3/2, v/v) prior to adding $mPEG_{5000}$—*p*NP (10.285 g; 5.0 mmol).[17] The pH was adjusted to 8 by 3 mL Et_3N and the reaction was completed in 5 h at room temperature with constant stirring, followed by TLC monitoring. The solution was extracted with 500 mL diethyl ether after the pH was adjusted to 2 using HCl. The remaining water layer was separated and extracted with 500 mL chloroform for three times. The organic phase was dried over Mg_2SO_4 and filtered to small volume accompanied by drops of diethyl ether (totaled 500 mL). The intermediate was recovered by filtration and dried under vacuum. Yield: 9.696 g (92.30%, w/w).

- TLC: $CHCl_3$: CH_3OH : HCOOH = 7 : 1.5 : 0.1 (v/v)
- IR (KBr): v = 1718.78 (C=O), 2889.00 (CH_2, OCH_3), 114.92, 1060.70 (C—O—C), 1467.43(CH_2CH_2) cm^{-1}.

- ^{1}H-NMR (DMSO-d_6), δ (ppm): 3.246 (3H, s, —OCH_3, and mPEG), 3.515(br s, CH_2CH_2O), 5.811(1H, s, and NH), 4.218 (2H, s, and CH_2OCO), 7.309 (1H, s, and COOH).

Synthesis of $mPEG_{5000}$—OCO—Gly—Puerarin. Puerarin (1.23 g; 2.955 mmol), DMAP (0.241 g; 1.97 mmol), and EDCI (0.755 g; 3.94 mmol) were dissolved in 50 mL anhydrous pyridine. The mixture was stirred on ice, while the $mPEG_{5000}$—OCO—Gly (5 g; 0.985 mmol) was dissolved in 10 mL of anhydrous pyridine, and was slowly added into the mixture (drop by drop). The reaction finished in $\sim$ 5 h and subsequently tested by TLC. The solution was further reduced condensed and crystallized with isopropanol at -20°C for 2 h, followed by filtering and washing by diethyl ether. The final residue was recrystallized twice and dried under vacuum. Yield: 4.856 g (89.93%, w/w).

- TLC: $CHCl_3$:CH_3OH =7:1.5 (v/v)
- IR (KBr): ν=893.07 (OH), 1633.8,1515.68 (Ph), 3390.56 (OH, NH) cm^{-1}
- ^{1}H-NMR (DMSO-d_6), δ (ppm): 3.249 (3H, s, —OCH_3, mPEG), 3.518 (br s, CH_2CH_2O), 6.816,6.799 (2H, d, H—Ar, and H-2′,6′),6.969 (1H, br s, H—Ar, and 6-H),7.377 (1H, s, and NH), 7.430,7.413 (2H, d, H—Ar, and H-3′,5′), 7.928,7.911(1H, d, H—Ar, and H-5), and 8.342 (1H, s, and HO—Ar).

Hemolysis Assay

Hemolysis assay was performed by using fresh whole blood from healthy human volunteers (with agreements). Erythrocytes were isolated by centrifugation at 2000 rpm for 10 min, washed three times with 0.1*M* phosphate buffered saline (PBS, pH 7.4) solution, and suspended in PBS at a 50% of hematocrit. The prodrug and the puerarin injectable were prepared in PBS buffer to give final puerarin concentration in a range from 2 to 5 mg/mL and an aliquot of 490 μL solution in sterile tubes, into which erythrocytes (10 μL) was added. The PBS was used as the negative control and ddH_2O was used as the positive control. The samples were mixed and incubated for 36 h at 37°C. All the tubes were shaked at intervals of 2 h and recorded by photographs.

Evaluation of Drug Release

Characteristics of hydrolysis prodrug were evaluated by dissolving the prodrug (1 mg/mL) at pH 2.2 (0.1*M* Na_2HPO_4 2.0 mL–0.1*M* citric acid 98.0 mL), pH4.6 (0.1*M* Na_2HPO_4 46.75 mL–0.1*M* citric acid 53.25 mL), pH 6.2 (0.1*M* Na_2HPO_4 18.5 mL–0.1 mol/L NaH_2PO_4 81.5 mL), pH 7.4 (0.1*M* Na_2HPO_4 81.0 mL–0.1 mol/L NaH_2PO_4 19.0 mL), pH 8.0 (0.1*M* Na_2HPO_4 94.7 mL–0.1 mol/L NaH_2PO_4 5.3 mL), pH 10.0 (0.2*M* glycine 50.0 mL–0.2*M* NaOH 32.0 mL added H_2O to 200 mL). The entire sample solutions were kept at 37 $\pm$ 0.5°C, a sample of 20 μL was collected at predetermined time points, and analyzed by HPLC free puerarin and the prodrug remained.

Pharmacokinetic Investigation

Pharmacokinetic Studies. Pharmacokinetic studies were carried out using three groups of ICR mice. Animal cares and procedures were followed institutional guidelines that comply with national and international laws and policies. Puerarin and prodrug dissolved in physiological saline respectively were administered to the mouse via tail vein at a volume of 0.2 mL/mouse and dosage of 10 mg Puerarin/kg body weight based on previous studies. Blood was collected through the eyes into heparin-treated tubes (10 μL; 500 IU/mL) in triplicate at the following time points: 2, 5, 7, 10, 15, 30, 45, 60, 90, and 120 min. The blood samples were centrifuged at 4000 rpm for 10 min, and plasma was removed and stored at -20°C until analysis. After blood collection, all mice were euthanized by cervical dislocation, and spleen, kidneys, lungs, liver, and heart were collected and then stored at -20°C until analysis.

Plasma and Tissue Sample Analysis. Plasma (0.2 mL) was treated with 2 mL methanol under vortex for 1 min, kept in -20°C for 30 min to allow precipitation of any protein, followed by centrifugation at 12,000 rpm for 10 min. The supernatant was evaporated to dryness under vacuum. In case of puerarin, the residues were reconstituted with 0.2 mL methanol, centrifuged at 12,000 rpm for 10 min, and the supernatant (20 μL) analyzed by HPLC under condition reported above. For the prodrug, the residue was first hydrolyzed with 50 μL of NaOH 1*M* at 40°C for 1 h, neutralized with 25 μL of H_3PO_4 and then analyzed by HPLC.

Each organ was weighed, treated with methanol (4 mL) and homogenized. The tissue was sonicated for 30 min in a water bath and centrifuged for 10 min at 12,000 rpm. The supernatant was dried under vacuum and the residue was processed and analyzed as above.

Protective Effect of Puerarin and Prodrug on Cardiocerebrovasular Disease. A pilot study was conducted previously with doses of puerarin(10, 20, and 30 mg/kg) to determine the dose dependent effects on pituitrin (Pit)-induced acute myocardial ischemia (AMI) model in mice,[18] we found that all of 10, 20, and 30 mg/kg of puerarin have effective in reducing the levels of CK, LDH, and AST, and puerarin at dose 20 and 30 mg/kg was more significantly ($P < 0.05$ and $P < 0.01$, respectively, date not show) in decreased the levels of CK, LDH, and AST. Hence, we chose the dose 20 mg/kg of puerarin for further studies.

For this study, the mice were random divided into three groups as follow: model, puerarin injectable (20 mg/kg) and prodrug (266.67 mg/kg; equivalent to 20 mg puerarin/kg). Animals were fasted but allowed water overnight prior to the experiments. Test drugs or their vehicle (normal saline) was intravenously administered 10 min before administered Pit (i.p, 30 U/kg). After 2 h, blood samples (1 mL) were collected and incubation in 37°C for 20 min, and then followed by centrifugation at 4000 rpm for 10 min to separate the serum. The levels of CK, LDH, and AST in the serum were evaluated by kits.

Statistical Analysis

The data was presented in mean $\pm$ SD. Statistical analysis was performed using the Student's *t*-test, $P < 0.05$ and $P < 0.01$ were considered statistically significant and highly significant, respectively.

RESULTS AND DISCUSSION

Synthesis and Characterization of PEG Conjugate

PEG prodrug for puerarin was synthesized according to Figure 1. The extremity of $mPEG_{5000}$ was first activated by pNPC to attach to Glycine (Gly), the hydroxyl group of puerarin was esterified with the carboxyl of Gly in the catalyzed of EDCI and

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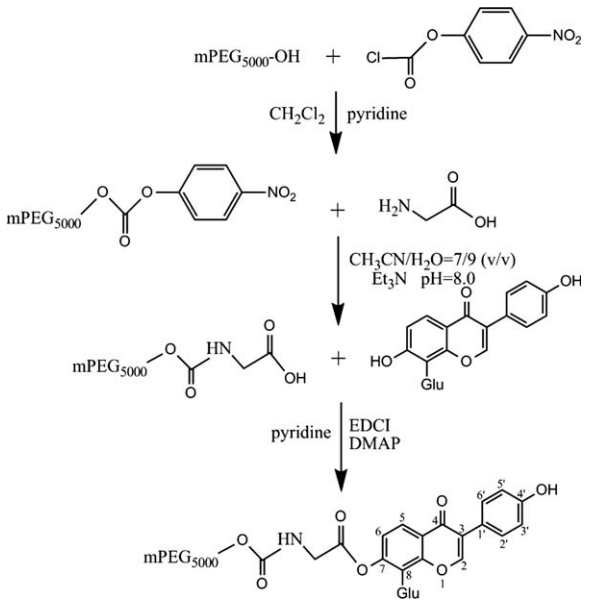

Figure 1. Synthesis scheme of the puerarin-PEG conjugate.

DMAP with pyridine as solution. The characterization of the PEG conjugate was evaluated by TLC, UV, FTIR, and ^{1}H-NMR. Samples from each synthetic step were first analyzed by TLC, which manifested that almost all were single points on the plate (Figure 2), and unavoidable, there are still a bit unreacted $mPEG_{5000}$ in the product, which cannot be separated by solution precipitation. Because of the instability, the intermediate $mPEG_{5000}$—*p*NP was reacted immediately to $mPEG_{5000}$—OCO—Gly. Both the carbonyl peak of $mPEG_{5000}$—OCO—Gly appearing at 1718.78 cm^{-1} in the IR spectrum and the hydrogen chemical shift of 5.811(1H, s, and NH), 4.218 (2H, s, and CH_2OCO), and 7.309(1H, s, and COOH) in the ^{1}H-NMR spectrum confirmed its structure. After repeated recrystallization, only one blove fluorescent spot and no free puerarin under the 254 nm UV light was

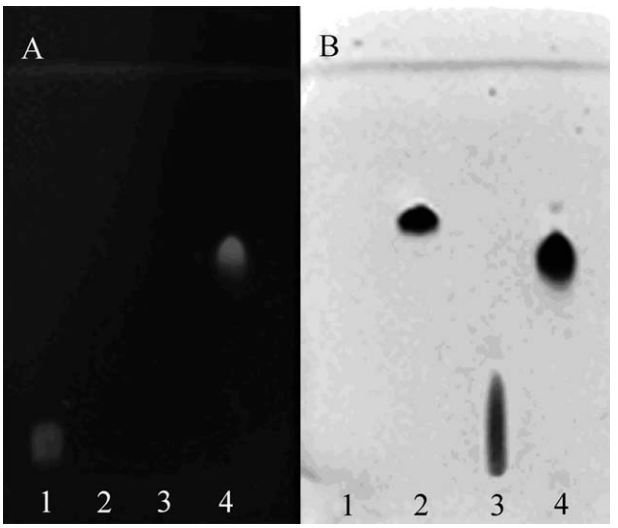

Figure 2. The identification results of TLC (A: Samples detected under the 254 nm UV light. B: Samples developed with I_2/KI. 1–4: Puerarin, $mPEG_{5000}$, $mPEG_{5000}$-OCO-Gly, and $mPEG_{5000}$-OCO-Gly-puerarin, respectively).

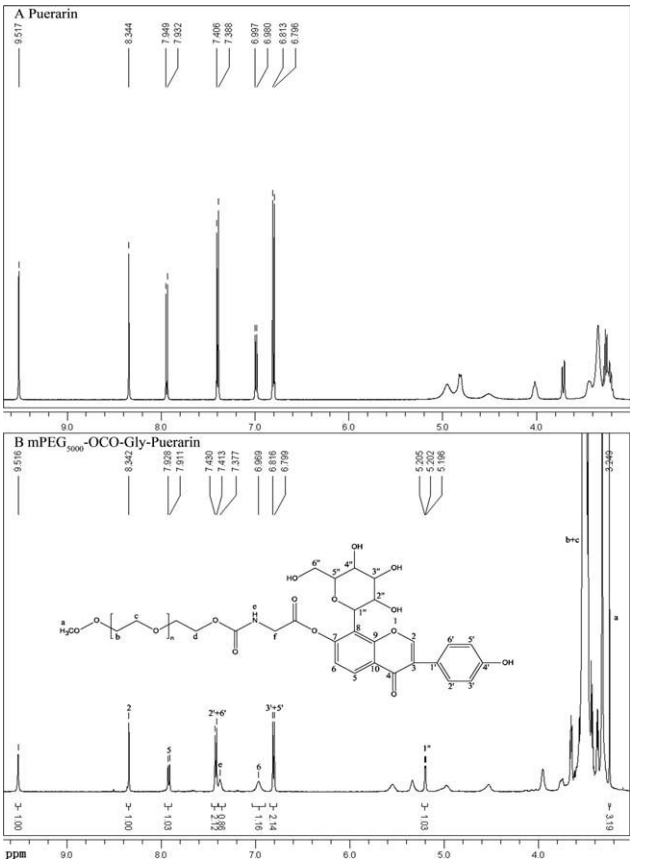

Figure 3. The ^{1}H-NMR spectrum of the final product (DMSO-d^6).

detected [Figure 2(A)], when developed with I_2/KI, the color of blove fluorescent spot changed to chocolate brown without the spot of $mPEG_{5000}$—OCO—Gly [Figure 2(B)]. The absence of free drug was further verified by HPLC with single peak [Figure 4(A)]. Additionally, the structure of final product was verified by UV (data not shown) and IR spectrum (see data above). There may be concerns that whether all hydroxyl groups of the puerarin were successfully linked. The ^{1}H-NMR spectra in Figure 3 showed that proton signal of H-3′, 5′, H-2′, 6′were unchanged, however, the signals of H-5 and H-6 were shifted remarkably, and the peak shape of H-6 was changed clearly as well. All these evidences indicated that PEGylation had taken place at the 7-OH instead of 4′-OH. Additionally, the integration ratio of —OCH_3/2-H was 1/3.19, which was very close to the theory ratio (1/3) suggesting that puerarin molecule was reacted with mPEG derivative in a mole ratio of 1/1.

HPLC results manifested both puerarin and its PEG conjugate with specific single elution peaks (Figure 4). However, comparing the retention time of puerarin (13.6 min) to that of the PEG conjugate (19.8 min), the retention time of puerarin was significantly delayed by PEGylation, which provided additional evidence for success obtained the target derivative described above. Furthermore, the compounds from each synthetic step were verified by MALDI-TOF MS. As demonstrated in Figure 5, $mPEG_{5000}$—OCO—Gly showed a single but widely dispersed peak at *m*/*z* 5080 [Figure 5(B)], which would be equivalent to CO—Gly (*m*/*z* 103) conjugated with 1 unit of $mPEG_{5000}$ [*m*/*z*

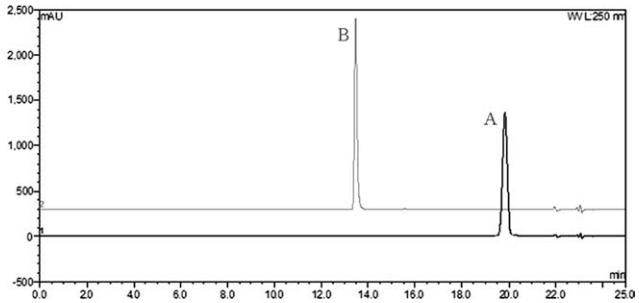

Figure 4. HPLC chromatograph of (A) prodrug and (B) puerarin.

4968, Figure 5(A)]; meanwhile, the prodrug showed a dominant average M_W of 5476 [Figure 5(C)], equivalent to $mPEG_{5000}$—OCO—Gly (m/z 5080) conjugated with 1 unit of puerarin (m/z 416). The MALDI-TOF MS further confirmed the polydispersity of the PEG molecules and more importantly, it clarified the reaction mole ratio of each step, which was consistent with the results of ^{1}H-NMR.

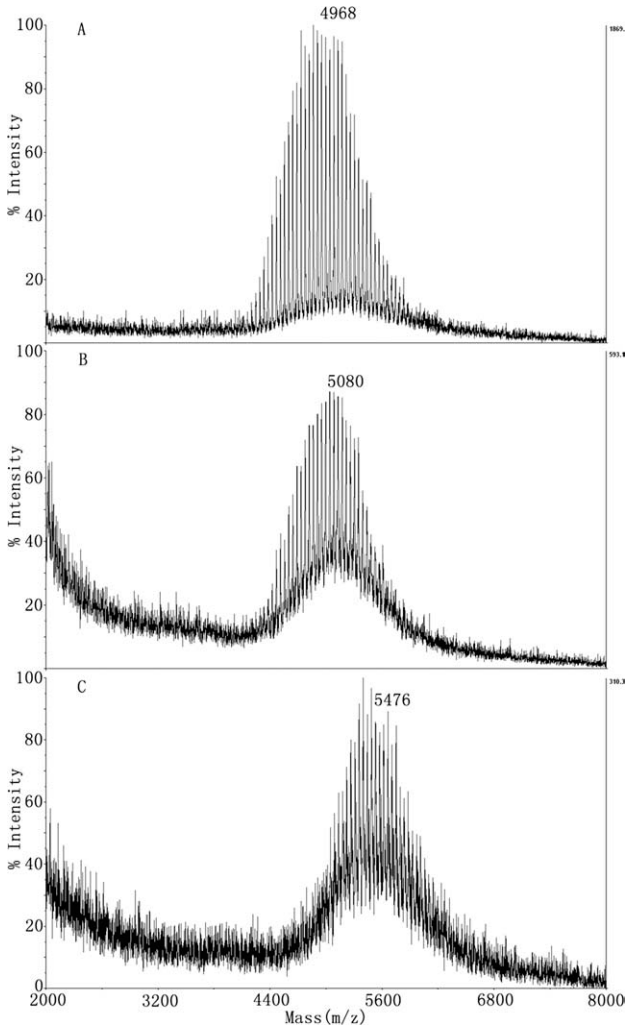

Figure 5. MALDI-TOF MS of $mPEG_{5000}$ (A), $mPEG_{5000}$-OCO-Gly (B), and $mPEG_{5000}$-OCO-Gly-Pur (C).

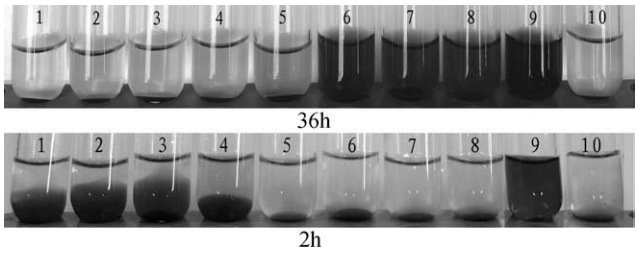

Figure 6. The hemolytic properties of puerarin and prodrug at different concentrations. 1–4: The samples were treated with prodrug (contained puerarin 2–5 mg/mL, respectively); 5–8: The samples were treated with puerarin injectable (puerarin = 2–5 mg/mL, respectively); 9: The sample was treated with ddH_2O; 10: The sample was treated with PBS. The Erythrocytes were obtained from the same healthy volunteer.

In the first two steps, we are able to achieve high yields with some modifications of reaction conditions. About the last step, it is difficult to maintain the yield with a single product because that puerarin structure is complex and exhibits two hydroxyl groups. Thus, the molar ratio of puerarin versus mPEG must be >1, according to the chemical reaction principle. It is possible to reach a molar ratio of 3 or greater and that the molar ratio increases as the products converge to become only one main substance. Our exploration uncovered that the reaction solvent plays an important role in achieving our goal. The facts that hydrophobic solvent could not effectively and completely dissolved puerarin and that hydrophilic solvents (e.g., DMSO, DMF, $CHCN_3$, and THF) tend to give multiple byproducts were evident. We found that only pyridine can give one main product after optimization (data not show) and recrystallization. Han et al. described that the 7-OH was in priority to be deprotonated over 4′-OH in alkalescent conditions.[19] Thus, in our work, the pyridine is not only the resolution but also the catalyst. Moreover, our data indicated that 7-OH of puerarin is more active than 4′-OH, which is consistent with the study of Yuan et al.[20]

Hemolysis Properties

The main purpose of this study is to solve the hematotoxicity of puerarin by PEGylation, so we evaluated the hemolytic properties of the PEG conjugate by treating erythrocytes obtained from health humans. In the preliminary study, hemolysis was apparent when concentrations of puerarin reached 3 mg/mL at 37°C for 36 h (Figure 6). The erythrocytes treated with prodrug were deposited slower than that treated with puerarin injectable after 2 h, likely due to the flocculation effect of PEG. To alleviate concerns on any hemolytic activity of the prodrug, 4 mg/mL of puerarin was used in a separate study. Irrespective of PEGlyation, $mPEG_{5000}$ did not show any hemolytic activity [Figure 7(b)], instead it stabilized the cell membrane when physically mixed with puerarin, as shown by the injectable [Figure 7(e)]. The membrane stabilizing effect of PEGylation was much more pronounced than that of the physically mixing group (following a long duration of incubation) [Figure 7(f)]. It is worthy to point out that no hemolysis in any sample from the PEGylation group [Figure 7(c)], whereas the puerarin injectable [Figure 7(d)] showed nearly 100% of hemolysis after 36 h incubation. These results showed that PEG could stabilize the

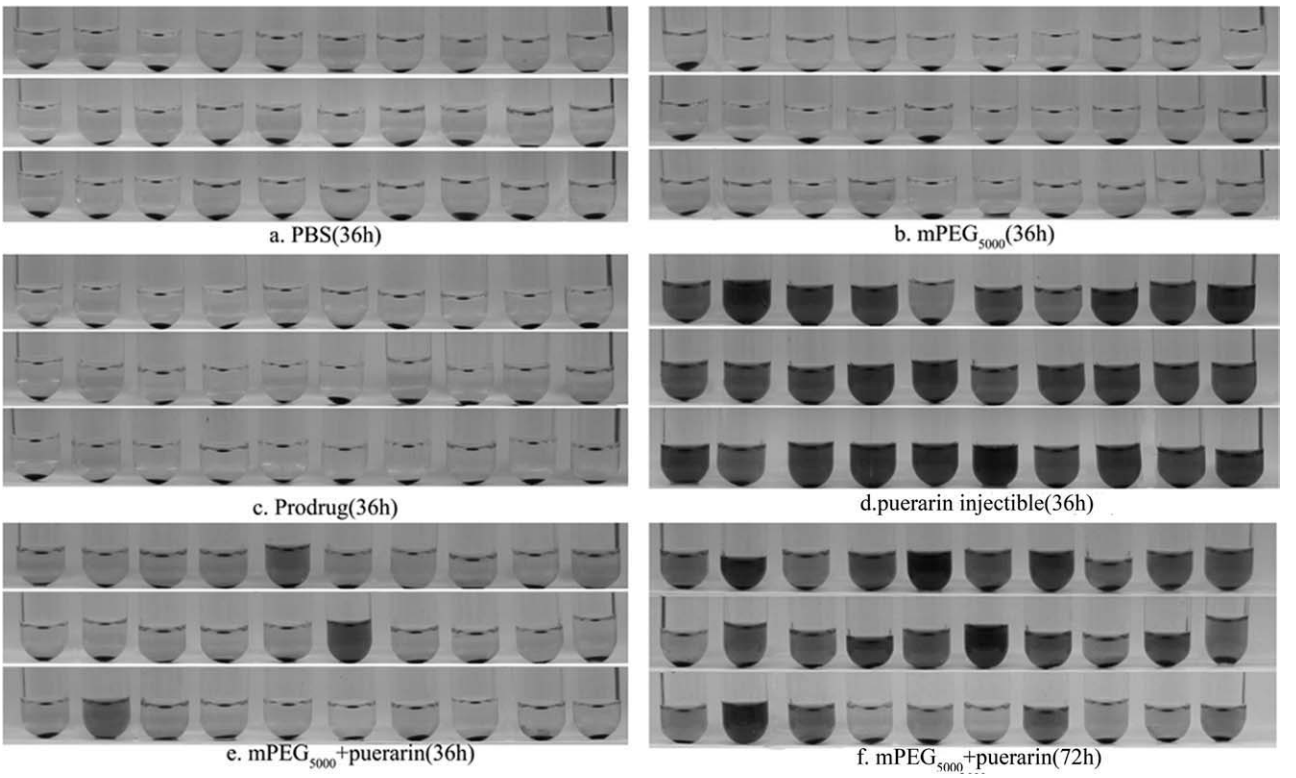

Figure 7. The hemolytic properties of the prodrug. Each tube represented one erythrocyte collection from an individual healthy volunteer.

red cell but could not antagonize the hemolytic activities of puerarin. Nevertheless, PEGylation had provided an effective means to reduce the risk of hemolysis.

As one of the major therapeutic agents for angiocardiopathy in China, puerarin injectable was widely used in the last decade. Because of the hemolysis side effect, production, and sale of puerarin injectable by manufacturers has been stagnant. The key focus of the current project is to decrease or eliminate the hemolytic effect of puerarin injectable. Our data suggested that the PEGylation appeared to be able to overcome the adverse hemolytic effects resulting from puerarin injectable. We also demonstrated that PEGylation provides an effective pharmaceutical means to reduce the risk of hemolysis. Researches hold different views on the hemolytic mechanism of puerarin, some suggested that it interfered with the integrity of cell membrane,[21] and others showed evidences immune complex-mediated hypersensitivity that occurred clinically.[22,23] Neverthless, prodrug we designed and presented in this report appeared to be able to stabilize the cell membrane of the erythrocyte. The data are consistent with works performed by others that showed PEG was capable of ameliorating or eliminating the immunogenicity of parent drug.[24,25] However, the final efficacy and safety in regard to the immunotoxicity of the prodrug need to be substantiated clinically, and the machine of the prodrug avoiding hemolytic could be further studied.

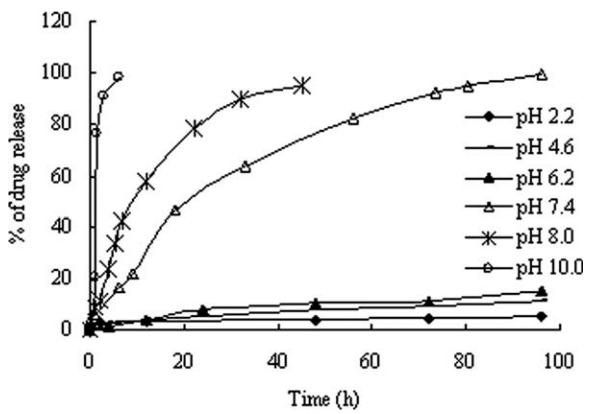

Figure 8. Rate-pH profile for the hydrolysis of the prodrug in different pH buffer solution.

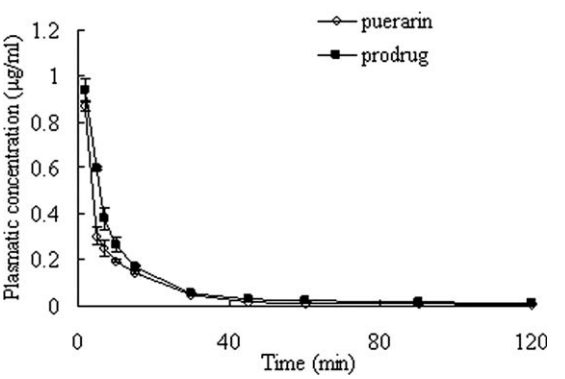

Figure 9. Plasma concentration-time profile following the intravenous administration of puerarin injectable and prodrug in mice ($n = 3$).

Table I. The Main Pharmacokinetic Parameters of Puerarin Injectable and Prodrug After IV Administration in Mice

Compound	$t_{1/2}\alpha$ (min)	$t_{1/2}\beta$ (min)	$AUC_{0-\infty}$ (μg/min/mL)	CL (mL/min/kg)	V_c (mL/kg)
Puerarin injection	0.86 ± 0.20	11.21 ± 0.71	9.12 ± 0.25	1.09 ± 0.04	3.29 ± 0.75
Prodrug	3.24 ± 0.20[a]	26.53 ± 1.64[a]	11.78 ± 0.17[a]	0.85 ± 0.01[a]	6.26 ± 0.51[a]

[a]Statistical significance at $P < 0.05$.
Data represent mean ± SD ($n = 3$).

In Vitro Release Studies

Release kinetic of the prodrug has significant impacts on safety and efficacy studies *in vivo*, and on the information can be derived from the release profile of the prodrug *in vitro*. Hydrolysis process for the prodrug was carried out in a series of buffers with different pH, as shown in Figure 8. The PEG conjugate is subjected to hydrolyzation to 3.20% at pH 2.2, 4.06% at pH 4.6, and 4.24% at pH 6.2 after 12 h. However, hydrolyzation percentage could reach up to 42.17% at pH 8.0, and nearly 100% at pH 10.0 after 6 h. At pH 7.4, the hydrolyzation percentage of the prodrug reached 46.51% after 18 h treatment. These data indicated that PEG conjugates were more stable under acidic environment and tended to undergo hydrolyzation under alkaline conditions, the results were correspond to the study of Liu et al.,[26] in that study, a PEG conjugation of puerarin on glycosyl (PEG-PUE) was researched, and degradation behavior result of PEG-PUE showed that the PEG-PUE degraded relatively slow in pH 5.0 and 2.0 when compared with pH 9.0. The result may provide some useful information that the water pH value of the prodrug should be controlled at acidic pH in favor of maintaining stability of prodrug. To some extent, the release kinetic profiles explained the no hemolytic effect of the prodrug, but it is paradoxical when incubation time comes to 72 h (all prodrug should be hydrolyzed), almost all physical mixture treated samples were hemolyzed [Figure 7(f)], but no hemolytic samples were observed in prodrug treatment group (data not show here). Further study should be carried out.

Pharmacokinetic Investigation

The prodrug and puerarin were injected to mice to evaluate the pharmacokinetic character *in vivo*. The calibration curves used for the determination of puerarin in plasma and tissue was $C = 0.0011\ A + 0.0004$ ($r = 0.9994$), with a linear range of 0.8–32 μg/mL. The limit of detection was 0.1 μg/mL, and the recovery rate >94%. Puerarin levels from prodrug administration (133 mg/kg, iv; equivalent to 10 mg puerain/kg) in the plasma at different times were compared with those from puerarin injectable (10 mg/kg, iv) and displayed in Figure 9. These levels were used to estimate pharmacokinetic parameters using statistical moment analysis, as shown in Table I. Plasma total puerarin concentrations (include free puerarin and PEG conjugated puerarin) were higher at all time points in the prodrug group than the puerarin injectable group. Plasma $AUC_{0-\infty}$ for the prodrug (11.78 ± 0.17 μg/min/mL) was 1.29-fold higher than those of puerain injectable (9.12 ± 0.25 μg/min/mL). Additionally, a significantly longer ($P < 0.05$) elimination half-life was noted with prodrug (26.53 ± 1.64 min) as compared with puerarin injectable (11.21 ± 0.71 min). Table II showed the $AUC_{0-\infty}$ of puerarin injectable and prodrug distribution in tissues. The puerarin concentration of the prodrug in liver was significantly ($P < 0.01$) reduced as compared with puerain injectable, suggesting that PEGylation played a role in ameliorating hepatotoxicity of puerarin. Furthermore, the $AUC_{0-\infty}$ of prodrug in the heart was 1.48-fold higher than that of puerarin injectable, implying a better therapeutic effect provided by prodrug than by puerarin injectable on angiocardiopathy. PEG is primarily excreted by kidney ($M_W < 30$ KDa) or liver ($M_W > 20$ KDa), depending the molecule size,[13,27] as supported by our data that $AUC_{0-\infty}$ values in kidney of the prodrug were significantly higher. The pharmacokinetic study on rat about puerarin and PEG-PUE showed that the PEG-PUE had several times increased in half-time and mean residence time,[28] which also suggest that the PEGyated puerarin could provide prolonged plasma level of puerarin.

Effect of Puerarin and Prodrug on Pit-induced AMI Mice

In most cases, safety of the drug is a primarily concern, and then the therapeutic efficacy should be addressed as well. The prodrug we designed has avoided the hemolytic effectively, but the biological efficacy of the prodrug as an anticardiovascular drug still has to be verified. As shown in Table III, both of puerarin injectable and prodrug can significantly ($P < 0.05$) decreased the levels of CK, LDH, and AST in serum when compared with model group. Furthermore, when compared with puerarin injectable, the mice treated with prodrug showed significantly ($P < 0.05$) decreased in the case of CK and AST, which implied the prodrug has better therapeutic effect on cardio-cerebral-vascular disease than puerarin injectable. This likely to be attributed by extended elimination half time and improved biodistribution effects of the prodrug, especially in the heart (the $AUC_{0-\infty}$ was 1.48-fold higher than puerarin).

CONCLUSIONS

In this study, we presented a new anhemolytic, water-soluble PEGylated prodrug form of puerarin. The hemolysis properties

Table II. $AUC_{0-\infty}$ of Puerarin Injectable and Prodrug in Tissues

	$AUC_{(0-\infty)}$ (μg/min/g)	
Tissues	Puerarin injection	Prodrug
Kidney	64.47 ± 1.51	96.57 ± 1.42[b]
Liver	35.33 ± 1.16	19.83 ± 0.61[b]
Lung	66.51 ± 3.48	76.67 ± 0.45[a]
Heart	43.91 ± 1.22	64.77 ± 2.56[b]
Spleen	93.95 ± 4.75	94.69 ± 2.32

[a]Statistical significance at $P < 0.05$, [b]Statistical significance at $P < 0.01$.
Data represent mean ± SD ($n = 3$).

Table III. Levels of CK, LDH, and AST in AMI Mice

Groups	CK (IU/L)	LDH (IU/L)	AST (IU/L)
Model	2210.56 ± 160.14	6135.14 ± 334.72	112.62 ± 4.16
Puerain injectable	1905.38 ± 145.12[a]	5525.52 ± 221.23[a]	98.23 ± 7.86[a]
Prodrug	1702.89 ± 90.24[a,b]	5355.12 ± 260.13[a]	90.95 ± 3.65[a,b]

[a]Statistical significance at $P < 0.05$ when prodrug compared to puerarin, [b]Statistical significance at $P < 0.05$ when prodrug compared to model.
Data represent mean ± SD ($n = 10$).

on human blood cell, hydrolysis kinetics *in vitro*, pharmacokinetic behavior *in vivo*, and biological efficacy of the prodrug were investigated. Our data showed that the PEG conjugation could reduce the risk of hemolysis that occurred during puerarin administration. At the same time, the prodrug could release puerarin rather constantly in physiological buffer, and exhibited favorable pharmacokinetics, with a longer elimination half-life and advantageous biodistribution pattern in organs. Furthermore, the therapeutic effect of the prodrug on AMI model was very encouraging which implied better application than puerarin injectable. Our approach by using PEGylation technique has provided a useful pharmaceutical model for prodrug design and its manufacturing, especially for circumventing adverse drug reactions.

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