

Improving aqueous solubility and antitumor effects by nanosized gambogic acid-mPEG₂₀₀₀ micelles

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Abstract: The clinical application of gambogic acid, a natural component with promising antitumor activity, is limited due to its extremely poor aqueous solubility, short half-life in blood, and severe systemic toxicity. To solve these problems, an amphiphilic polymer-drug conjugate was prepared by attachment of low molecular weight (ie, 2 kDa) methoxy poly(ethylene glycol) methyl ether (mPEG) to gambogic acid (GA-mPEG₂₀₀₀) through an ester linkage and characterized by ¹H nuclear magnetic resonance. The GA-mPEG₂₀₀₀ conjugates self-assembled to form nanosized micelles, with mean diameters of less than 50 nm, and a very narrow particle size distribution. The properties of the GA-mPEG₂₀₀₀ micelles, including morphology, stability, molecular modeling, and drug release profile, were evaluated. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide) tests demonstrated that the GA-mPEG₂₀₀₀ micelle formulation had obvious cytotoxicity to tumor cells and human umbilical vein endothelial cells. Further, GA-mPEG₂₀₀₀ micelles were effective in inhibiting tumor growth and prolonged survival in subcutaneous B16-F10 and C26 tumor models. Our findings suggest that GA-mPEG₂₀₀₀ micelles may have promising applications in tumor therapy.

Keywords: gambogic acid, poly(ethylene glycol)-drug conjugate, micelle, antitumor, toxicity

Introduction

Gambogic acid, the major active ingredient of gamboge, a brownish to orange resin exuded from the *Garcinia hanburyi* tree in Southeast Asia, possesses significant anticancer activity both in vitro and in vivo.¹⁻⁴ Multiple mechanisms may be involved in its potent antitumor effect, including induction of apoptosis, cell cycle arrest, telomerase inhibition, antiangiogenesis activity, and an antimetastasis effect.⁵⁻¹⁰ Unfortunately, gambogic acid has poor solubility (less than 0.5 µg/mL), a short half-life (less than 1 hour in dogs and less than 20 minutes in rats), and low bioavailability, which limits its application.^{11,12} Solubilizers such as Cremophor® EL (polyethoxylated castor oil, Sigma-Aldrich, St Louis, MO, USA) and L-arginine have been introduced to solve these problems.¹¹⁻¹⁴ However, these agents may cause a series of side effects, such as vascular stimulation, hemolytic toxicity, hypersensitivity reactions, nephrotoxicity, neurotoxicity, and cardiotoxicity.¹⁵ Further, rapid plasma elimination of gambogic acid cannot be avoided by these formulations.^{12,16} These defects greatly limit the therapeutic effect of gambogic acid. Therefore, it is necessary to develop structural modifications or a new dosage form of gambogic acid.

Conjugation of hydrophilic polymers with small molecule drugs to produce polymer-drug conjugate systems has been demonstrated to be a viable formulation strategy for utilizing hydrophobic drugs in a water-soluble manner, which may offer advantages over the corresponding parent drugs, including fewer side effects, improved solubility, passive tumor targeting, an improved pharmacokinetic profile, and

lower plasma concentrations. This passive tumor targeting is achieved primarily through the enhanced permeability and retention effect. In past decades, conjugation of hydrophilic polymers with hydrophobic anticancer drugs (such as paclitaxel, doxorubicin, camptothecin, and curcumin) to produce polymer-drug conjugate systems has been proven to be a viable formulation strategy.^{17–25} A large number of hydrophilic polymers could be chosen as water-soluble macromolecule carriers; among them, poly(ethylene glycol) (PEG) is considered to be one of the best candidates. Several chemotherapeutic agents have been conjugated to PEG with covalent bonds, including a PEG-curcumin conjugate, a PEG-paclitaxel conjugate, and a PEG-camptothecin conjugate, resulting in homogeneous water-soluble prodrugs with an extended circulatory life and altered biodistribution.^{26–29} Tang et al³⁰ reported that using PEG as a water-solubilizing unit was a useful strategy for increasing the water solubility of gambogic acid, which is hydrophobic. Ding et al³¹ synthesized a series of gambogic acid-PEG conjugates with different amino acid and dipeptide spacers, which showed satisfactory water solubility compared with gambogic acid, and the circulatory retention time, biodistribution, and bioavailability of the conjugates were remarkably improved. However, the bioactivity and toxicity of gambogic acid-PEG conjugates in vivo have not been reported thus far.

On the basis of these studies, we developed a nanosized drug delivery system with the aims of increasing the aqueous solubility, improving the therapeutic efficacy, and reducing the toxicity of gambogic acid to normal tissues. In this study, an amphiphilic polymer-drug conjugate was prepared by condensation of low molecular weight monomethoxy-poly(ethylene glycol) (mPEG)-2000 with gambogic acid (GA-mPEG₂₀₀₀) through an ester linkage, which then formed micelles. The physicochemical properties of the formulations were evaluated, and their in vitro cytotoxicity, in vivo antitumor effect, and toxicity were also investigated.

Materials and methods

Materials

Gamboge resin was obtained from Chengdu Herb Market (Sichuan, People's Republic of China). mPEG (average molecular weight 2,000) and Dulbecco's Modified Eagle's Medium were purchased from Sigma-Aldrich. Gambogic acid was prepared and purified in our laboratory. Acetonitrile of high-performance liquid chromatography (HPLC) grade was purchased from Fisher Scientific (Loughborough, UK). Chloroform, methanol (analytical grade), 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC), and N-hydroxysuccinimide were

purchased from Chengdu Kelong Chemical Co, Ltd (Chengdu, People's Republic of China). Other reagents were of analytical grade. Deionized water (>18 MU, Purelab®; Classic Components Corporation, Torrance, CA, USA) was used in all experiments.

Cells and animals

Murine colon adenocarcinoma cells (C26) and murine melanoma cells (B16-F10) were purchased from the American Type Culture Collection (Rockville, MD, USA). The cells were grown in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum (Sigma-Aldrich). Primary human umbilical vein endothelial cells (HUVECs) were isolated from human umbilical cord veins and grown in EBM-2 (endothelial basal medium-2) with Single Quots (Lonza, Walkersville, MD, USA) containing vascular endothelial growth factor and other growth factors. HUVECs at passages 2–4 were used for all experiments. The above cells were maintained at 37°C in a humidified incubator containing 5% CO₂.

Balb/c and C57BL/6J mice (age 6–8 weeks, weight 18–22 g) were used for the in vivo antitumor tests and toxicity studies. The animals were purchased from the Laboratory Animal Center of Sichuan University, and housed in sex-segregated cages at a controlled temperature of 20°C–22°C, a relative humidity of 50%–60%, and 12-hour light-dark cycles. The animals were provided with standard laboratory chow and tap water ad libitum. After a 1-week acclimation period, the animals were used for the experiments. All animal procedures were performed following the protocol approved by the Institutional Animal Care and Treatment Committee of Sichuan University (Chengdu, People's Republic of China). All mice were treated humanely throughout the experimental period.

Measurements

¹H nuclear magnetic resonance (NMR) spectra were determined on an Avance 400 (Bruker Corporation, Ettlingen, Germany) spectrometer (400 MHz) using deuterated chloroform as the solvent. Ultraviolet-visible spectra were recorded on a UV-2401 PC spectrophotometer (Shimadzu, Tokyo, Japan). HPLC detection was done on a 2996 detector (Waters Corporation, Milford, MA, USA). Chromatographic separations were performed on a reversed phase C₁₈ column (4.6 mm × 150 mm, 5 μm, SunFire; Waters Corporation). The column temperature was kept at 28°C, and the detection wavelength was 292 nm. Acetonitrile/0.1% formic acid (80/20, v/v) solution was used as the mobile phase, and the flow rate was 1 mL per minute.

General synthesis method for GA-mPEG₂₀₀₀ conjugates

Isolation and purification of gambogic acid

Fifty grams of dry gamboge resin was suspended in 150 mL of pyridine and stirred at 80°C–90°C for 30 minutes to form a pyridine salt of gambogic acid. After filtering through Kieselguhr (Sigma-Aldrich), 20 mL of water was added to the filtrate. The mixture was cooled to 4°C overnight, and the precipitate was collected and washed with pyridine solution (70%, v/v) and water several times. After drying under vacuum, the resulting yellow powder was dissolved in 250 mL of ethyl ether and heated under reflux for 30 minutes, then filtered, concentrated, and precipitated using petroleum ether; the yellow precipitate of the pyridine salt of gambogic acid was then collected and dried. The solid obtained was dissolved in 120 mL of ethyl ether and washed with aqueous HCl (1 M) and water. The ether solution was then dried over sodium sulfate and evaporated to yield an orange powder of 3.8 g gambogic acid (purity 98.6%, w/w, analyzed by HPLC).

Synthesis of GA-mPEG₂₀₀₀

Gambogic acid (345 mg, 0.55 mmol), EDC (191.7 mg, 1 mmol), and 4-dimethylaminopyridine (61 mg, 0.5 mmol) were dissolved in 10 mL of CH₂Cl₂ in an ice water bath, and mPEG₂₀₀₀ (1 g, 0.5 mmol) was then added. The resulting mixture was stirred overnight at room temperature. The solution was washed in 1 M HCl (3 × 10 mL), water (2 × 10 mL), and brine (2 × 10 mL), dried over Mg₂SO₄, and concentrated in vacuo. The precipitate was suspended in 10 mL of ethyl ether under stirring for 30 minutes to remove the free gambogic acid. The solution was filtered and the yellow precipitate was obtained. The crude product was further purified by silica gel column chromatography using a step gradient of methanol (2%–5%) in CH₂Cl₂ to remove the free mPEG₂₀₀₀, giving rise to GA-mPEG₂₀₀₀ (747 mg, 57%) as a buff-colored solid. A single spot was visualized with iodine vapor by thin liquid chromatography analysis (dichloromethane to methanol, 10:1). The ¹H NMR spectrum of GA-mPEG₂₀₀₀ was studied using an Avance 400 spectrometer (400 MHz), and CH₃Cl was used as the solvent.

Preparation and characterization of GA-mPEG₂₀₀₀ micelles

GA-mPEG₂₀₀₀ micelles were prepared using a direct dissolution method assisted by ultrasonication. GA-mPEG₂₀₀₀ (84 mg) was dissolved in 10 mL of distilled water and sonicated at 25°C for 1 minute. The control sample was prepared by dissolving 22 mg of L-arginine solubilizer and

20 mg of gambogic acid (GA-L) in 10 mL of distilled water and then sonicating the sample at 25°C for 3 minutes. The final concentrations of GA-mPEG₂₀₀₀ in the micelles and gambogic acid in the GA-L solution were determined by HPLC assay. A direct observation method was used to evaluate the solubility of the GA-mPEG₂₀₀₀ conjugates, and the equivalent solubility value was calculated depending on the measured solubility.

The particle size and zeta potential of the micelles were determined by dynamic light scattering using a Zetasizer Nano ZS-90 instrument (Malvern Instruments, Malvern, UK). The sample suspension (gambogic acid concentration 2 mg/mL) was diluted ten times with deionized water before measurement. The refractive index was 1.330. The temperature was kept at 25°C during the measuring process. All tests were run in triplicate, and mean values are reported.

The morphology of the prepared micelles was observed by transmission electron microscopy (TEM) and atomic force microscopy (AFM). For TEM, the samples were diluted with distilled water and placed on a copper grid covered with nitrocellulose. The samples were negatively stained with phosphotungstic acid and dried at room temperature, after which images were taken using a TEM device (H-6009IV; Hitachi, Japan). For AFM, the prepared micelle suspension was diluted with deionized water and deposited onto freshly cleaved mica lamella. The sample was dried for 3 hours at room temperature. AFM images were taken by tapping mode in air on an AFM device (SPA400; Seiko, Tokyo, Japan).

After preparation, the micelles were kept at 4°C and 25°C, respectively, and the stability of the formulations was evaluated qualitatively by observation of aggregates and particle size measurement. A homogeneous solution implies stability of the drug system, whereas the presence of precipitation or marked changes of particle size indicates instability.

Molecular modeling study

Construction of three-dimensional

GA-mPEG₂₀₀₀ structures

First, gambogic acid was built with MarvinSketch (<http://www.chemaxon.com>) and optimized at the molecular mechanical level using the MMFF94 method.³² Meanwhile, mPEG₂₀₀₀ was constructed using Hyperchem³³ software (Hypercube Inc., Gainesville, FL, USA). The gambogic acid and mPEG₂₀₀₀ structures were then merged together in the Hyperchem workspace (Figure 1A). GA-mPEG₂₀₀₀ was then optimized at the molecular mechanical level using the orthogonal partial least squares³⁴ method with the steepest descent algorithm. It was further optimized at the same

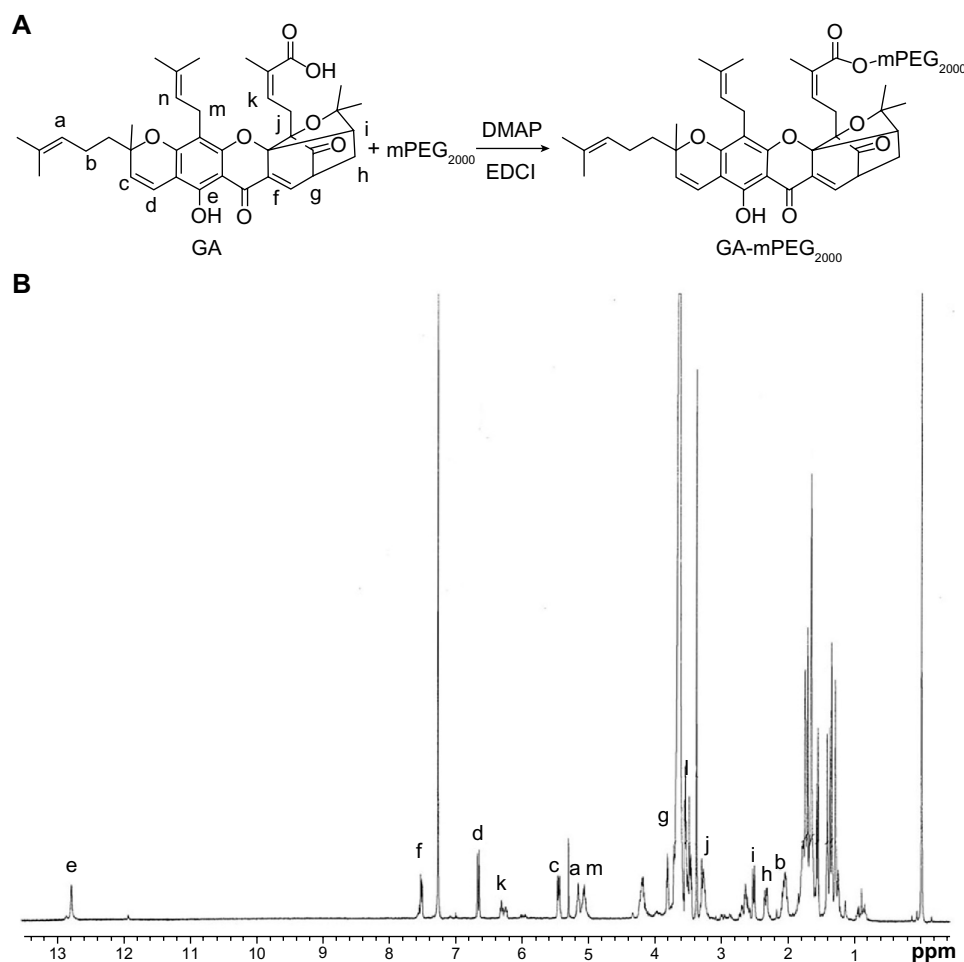


Figure 1 Synthesis and identification of GA-mPEG₂₀₀₀ copolymers. **(A)** Chemical reaction scheme for preparing the GA-mPEG₂₀₀₀ conjugate. **(B)** ¹H nuclear magnetic resonance spectrum of GA-mPEG₂₀₀₀ in CD₃Cl.

Abbreviations: GA, gambogic acid; mPEG₂₀₀₀, methoxy poly(ethylene glycol) methyl ether (molecular weight 2,000); DMAP, 4-dimethylaminopyridine; EDC, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride.

level with the molecular mechanical plus method³⁵ using the Fletcher-Reeves³⁶ algorithm.

Next, the optimized GA-mPEG₂₀₀₀ was subjected to a series of molecular dynamics simulations so as to obtain a lower energy minimum. The solvent effect was implicitly considered using the CHARMM27^{37,38} force field in the process of molecular dynamics simulation. The molecular dynamics simulations included three stages: heating from 0 K to 400 K, simulating at 400 K, cooling from 400 K to 300 K, and running at 300 K. At each stage, the simulated time is set to 100 picoseconds.

Simulation on self-assembly of GA-mPEG₂₀₀₀

Two GA-mPEG₂₀₀₀ molecules, the structures of which had been annealed using molecular dynamics, were first merged together. Two kinds of conformations were purposefully arranged. In the first conformation, the gambogic acid moieties of the two molecules were arranged

face-to-face. In the second conformation, they were arranged back-to-back. They were then simulated using Langevin dynamics. The friction coefficient and random seed were set as 0.05 per picosecond and -1,111, respectively. The solvation effect was treated as mentioned above. Simulation times for the first conformation and the second conformation were set to 100 picoseconds and 300 picoseconds, respectively.

In vitro drug release

Release of gambogic acid from the GA-L formulation and GA-mPEG₂₀₀₀ micelles in vitro was monitored by a dialysis method. Dialysis was carried out at 37°C using Spectra/Por dialysis membranes (Spectrum Laboratories, Inc, Rancho Dominguez, CA, USA) with a molecular weight cutoff of 1 kDa and phosphate-buffered saline (pH 7.4) as the sink solution. The molecular weight cutoff of the dialysis membrane only allows for diffusion of the free drug. The initial concentration of gambogic acid in the GA-L formulations

was 20 mg/mL and the initial concentration of GA-mPEG₂₀₀₀ in the micelles was 84 mg/mL (gambogic acid equivalent concentration, 20 mg/mL). At scheduled intervals, 1 mL of the dialysis medium was collected and the same volume of fresh medium was added immediately. The concentration of gambogic acid in the dialysis medium was monitored by HPLC assay. The percentage release was calculated according to the following equation:

$$\text{Drug release percentage (\%)} = (W_{\text{release}}/W_{\text{total}}) \times 100\%$$

where W_{total} is the total amount of gambogic acid in the GA-L formulation or micelles and W_{release} is the amount of gambogic acid released from the GA-L formulation or GA-mPEG₂₀₀₀ micelles into the dialysis medium.

Cytotoxicity assay

The in vitro cytotoxicity of the conjugates was quantified by measuring the IC₅₀ (drug concentration inhibiting 50% of cells) in a murine melanoma cell line (B16-F10), a colon carcinoma cell line (C26), and HUVECs. B16-F10 and C26 cells were cultured in Dulbecco's Modified Eagle's Medium containing 10% heat-inactivated fetal calf serum at a density of 1×10^5 cells per 180 μL per well in 96-well microtiter plates and allowed to proliferate at 37°C in a humidified incubator with 5% CO₂ for 24 hours. HUVEC cells were cultured in Dulbecco's Modified Eagle's Medium containing low serum growth supplement, 100 IU/mL penicillin, and 100 $\mu\text{g}/\text{mL}$ streptomycin at a density of 1×10^5 cells per 180 μL per well in 96-well microtiter plates and allowed to proliferate at 37°C in a humidified incubator with 5% CO₂ for 24 hours. Next, 20 μL serial dilutions of gambogic acid and GA-mPEG₂₀₀₀ (final equivalent gambogic acid concentration, 0.1–3.2 mg/mL) were added to the respective wells, and all samples were prepared and measured in quintuplicate for each concentration. After 48 hours of incubation, 20 μL of 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide (MTT, 5 mg/mL per well) was added and the plates were incubated for a further 4 hours. The supernatant was then removed, followed by addition of 150 μL of dimethylsulfoxide to each well. Fifteen minutes later, the absorbance at 570 nm was detected using a microplate reader and the IC₅₀ values for each compound were calculated from absorbance versus dilution factor plots.

In vivo tumor model and treatment plan

The antitumor activity of GA-mPEG₂₀₀₀ micelles was investigated in the B16-F10 and C26 models. In the B16-F10 model, C57BL/6J mice were injected subcutaneously with 50 μL of

cell suspension containing 5×10^5 B16-F10 cells on day 0. In the C26 model, Balb/c mice were injected subcutaneously into the right flank with 50 μL of C26 cell suspension containing 2×10^6 cells at day 0. Once tumors reached 150–250 mm³ in volume, the mice were randomized into three groups (ten mice per group). Tumor-bearing mice were injected intravenously every 2 days for 2 weeks with 100 mL of normal saline (control), GA-L (4 mg/kg bodyweight), or GA-mPEG₂₀₀₀ micelles (16.8 mg/kg body weight, gambogic acid 4 mg/kg equivalent), respectively. Tumor measurements were performed (according to the formula $0.52 \times \text{length} \times \text{width}^2$) and the mice were weighed and examined for antitumor activity every two (B16-F10 model) or three (C26 model) days. Mouse survival times were observed to document further the antitumor activity and toxicity of GA-L and GA-mPEG₂₀₀₀ micelles.

Statistical analysis

The data are expressed as the mean and standard deviation. Statistical differences were determined using the Student's *t*-test. Differences were considered to be statistically significant at $P < 0.05$.

Results

Synthesis and identification of GA-mPEG₂₀₀₀

The synthesis of GA-mPEG₂₀₀₀ was according to the references in the literature with some modifications.^{30,39} Hydroxy-terminated mPEG₂₀₀₀ was directly attached to gambogic acid at the 30-carboxy group. The main advantage of coupling one gambogic acid molecule at one end of mPEG₂₀₀₀ is the high selectivity of the only one hydroxyl in mPEG₂₀₀₀, which results in high yield and purity of GA-mPEG₂₀₀₀. The mPEG₂₀₀₀ was reacted with excess gambogic acid to guarantee that mPEG₂₀₀₀ was totally reacted. However, there was still some free mPEG₂₀₀₀ observed with iodine vapor by thin layer chromatography (dichloromethane to methanol, 10:1). The crude product was firstly purified in ethyl ether to remove free gambogic acid and then purified by gradient elution column chromatography.

Figure 1A shows the synthesis procedure for GA-mPEG₂₀₀₀. The ¹H NMR spectrum of GA-mPEG₂₀₀₀ demonstrates successful synthesis of GA-mPEG₂₀₀₀. Figure 1B shows a multiplet at $\delta 3.58$ – 3.72 (ppm) that was attributed to the repeating units in mPEG₂₀₀₀, and the peaks at 3.38 ppm were assigned to the three methyl protons in mPEG₂₀₀₀. Protons of gambogic acid can be found at 12.78 ppm (6-H), 7.50 ppm (10-H), 6.65 ppm (4-H), 6.33–6.23 ppm (27-H), 5.48–5.42 ppm (3-H), 5.16–5.07 ppm (32-H, 37-H), 3.82 ppm

(11-H), 3.58–3.54 ppm (31a-H), 3.50–3.46 ppm (31b-H), 3.30–3.26 ppm (26-H), 2.52 ppm (22-H), 2.32 ppm (21a-H), and 2.09–2.02 ppm (36-H), and all of the other multiple signals at 1.78–1.30 came from the protons in gambogic acid. These results demonstrate further that the conjugate was successfully prepared.

Characterization of GA-mPEG₂₀₀₀ micelles

Solubility of GA-mPEG₂₀₀₀ conjugates

The appearance of the prepared GA-mPEG₂₀₀₀ micelles is shown in Figure 2. Gambogic acid formed a turbid yellow suspension in water, indicating that gambogic acid could not be dissolved in aqueous solution (Figure 2A), whereas a clear and transparent solution of GA-mPEG₂₀₀₀ micelles could be

observed (Figure 2B), indicating its good solubility in water. Compared with the extremely poor solubility of gambogic acid (0.5 µg/mL), GA-mPEG₂₀₀₀ exhibited satisfactory aqueous solubility, being 2.7×10^5 -fold (135.8 mg/mL) that of gambogic acid.

Particle size and morphology of GA-mPEG₂₀₀₀ micelles

The GA-mPEG₂₀₀₀ micelles were suitable for an injectable formulation with a small particle size. The mean (\pm standard deviation) vesicle diameter of the micelles was 9.41 ± 2.91 nm ($n=3$) as determined by dynamic light scattering, and the polydispersity index (PDI) was 0.193 ± 0.072 (Figure 2B). Further, AFM and TEM analyses confirmed that the micelles were spheroids with a regular shape and a size

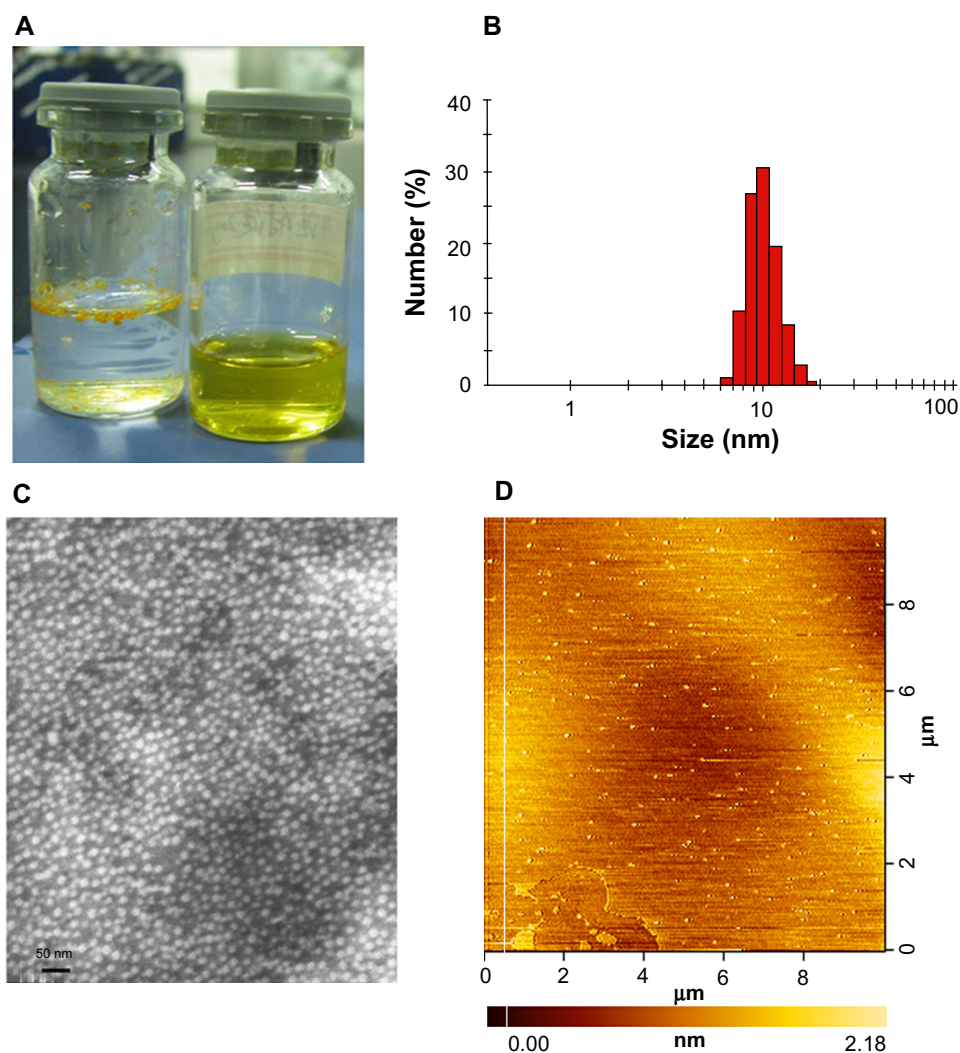


Figure 2 Preparation and characterization of GA-mPEG₂₀₀₀ micelles. **(A)** Appearance of gambogic acid in various dosage forms (**left**) mixture of gambogic acid and water, (**right**) GA-mPEG₂₀₀₀ micelles. **(B)** Size distribution of GA-mPEG₂₀₀₀ micelles. **(C)** Transmission electron microscopic images of typical GA-mPEG₂₀₀₀ micelles. **(D)** Atomic force microscopic images of typical GA-mPEG₂₀₀₀ micelles.

Abbreviations: GA, gambogic acid; mPEG₂₀₀₀, methoxy poly(ethylene glycol) methyl ether (molecular weight 2,000).

distribution of <50 nm (Figure 2C–D). The diameter of the micelles observed by AFM and TEM agreed with the results of the particle size analysis. These results demonstrate that the GA-mPEG₂₀₀₀ micelles were uniformly dispersed and had a very narrow particle size distribution.

Stability of GA-L and GA-mPEG₂₀₀₀ micelles

From observation of the aggregates, we found that the GA-L solution became turbid after 3 days and yellow precipitation could be observed after 5 days, indicating that the GA-L solution was not a stable formulation, whereas the GA-mPEG₂₀₀₀ micelle solution remained clear and transparent for 30 days. From particle size measurement of the GA-mPEG₂₀₀₀ micelles, we found that the average size and PDI of the micelles did not change significantly over 30 days (Figure 3). These results indicate that the GA-mPEG₂₀₀₀ micelle formulation was a homogeneous and stable drug system.

Molecular modeling study

Three-dimensional structure of GA-mPEG₂₀₀₀

As demonstrated in Figure 4A, the initial conformation of GA-mPEG₂₀₀₀ was a curve-like mPEG₂₀₀₀ covalently linking with gambogic acid. Kinetic simulations were done to find

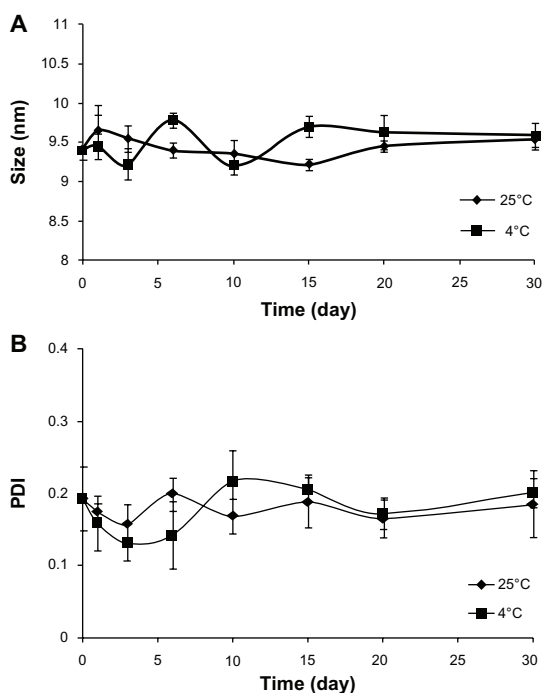


Figure 3 Stability of GA-mPEG₂₀₀₀ micelles. (A) Average size of GA-mPEG₂₀₀₀ micelles stored at 25°C and 4°C. (B) PDI of GA-mPEG₂₀₀₀ micelles stored at 25°C and 4°C.

Notes: n=3 samples per group per time point; bars, mean ± standard deviation.

Abbreviations: GA, gambogic acid; mPEG₂₀₀₀, methoxy poly(ethylene glycol) methyl ether (molecular weight 2,000); PDI, polydispersity index.

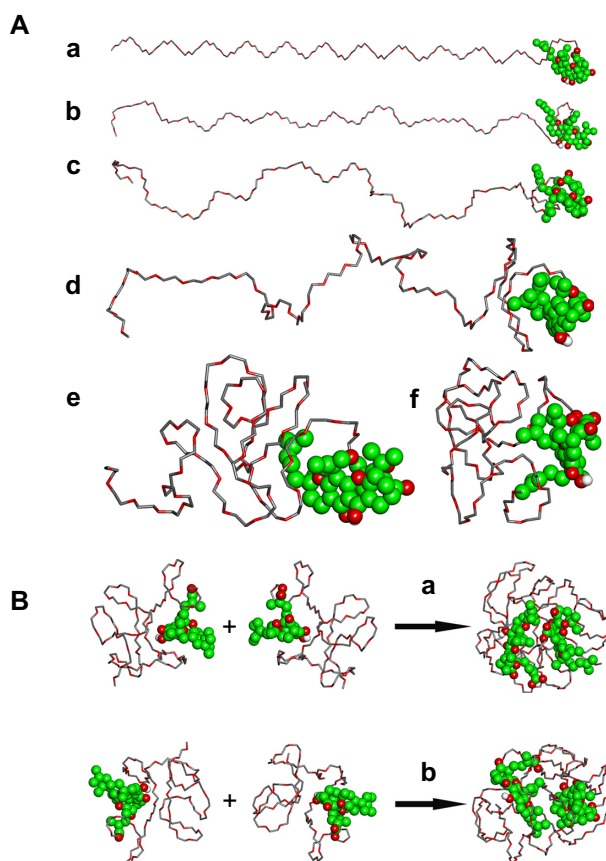


Figure 4 Molecular modeling studies of GA-mPEG₂₀₀₀ micelles. (A) Conformations of GA-mPEG after being heated for 0 (a), 9.875 (b), 20.010 (c), 30.082 (d), 39.925 (e), and 100 (f) picoseconds, respectively. (B) Interaction between GA-mPEG₂₀₀₀ molecules resulting from face-to-face GA-GA initial conformation (a) and back-to-back GA-GA conformation (b). GA was depicted with Corey-Pauling-Koltun space filling style, its carbon atoms were colored with green, and its oxygen atoms were colored with red.

Abbreviations: GA, gambogic acid; mPEG₂₀₀₀, methoxy poly(ethylene glycol) methyl ether (molecular weight 2,000).

an energy minimum for GA-mPEG₂₀₀₀. At the heating stage, GA-mPEG₂₀₀₀ was heated from 0 K to 400 K with a temperature step of 30 K. It can be seen from Figure 4A that the mPEG₂₀₀₀ moiety of GA-mPEG₂₀₀₀ changes its shape first and then gradually entwines around gambogic acid.

It must be a reasonable phenomenon due to the fact that the GA-mPEG₂₀₀₀ is in a polar solvent environment. As mentioned above, the solvation (water) effect was considered implicitly in the process of molecular dynamics simulation, because we know that gambogic acid is hydrophobic. The GA-mPEG₂₀₀₀ tries to increase its interaction with the polar solvent environment by reducing the hydrophobic moiety on its exposing surface. When mPEG₂₀₀₀ changes its shape and entwines around gambogic acid, the exposed hydrophobic area is reduced effectively. The equilibrated structure for GA-mPEG₂₀₀₀ is shown in Figure 4A(f).

Simulation on the self-assembly of GA-mPEG₂₀₀₀

In order to understand in detail the mechanism of interaction between GA-mPEG₂₀₀₀ molecules, Langevin dynamics simulations were conducted on clusters composed of two GA-mPEG₂₀₀₀ molecules. When two GA-mPEG₂₀₀₀ molecules were arranged so that the gambogic acid parts of the molecules were face-to-face (as shown in Figure 4B[a]), the gambogic acid moieties approached each other after 100 picoseconds of Langevin dynamics simulation. When two GA-mPEG₂₀₀₀ molecules were arranged so that the gambogic acid moieties were back-to-back (as shown in Figure 4B[b]), the gambogic acid parts of the two molecules were finally close to each other after 300 picoseconds of Langevin dynamics simulation. These results suggest that, in a polar solvent environment, the gambogic acid moieties of GA-mPEG₂₀₀₀ tend to move closer and form the core of the micelle, whereas the mPEG₂₀₀₀ moieties tend to stay in the external area and form the shell of the micelle, agreeing with the data in Figure 2.

In vitro release profile

A modified dialysis method was used to investigate the in vitro drug release behavior of the GA-L and GA-mPEG₂₀₀₀ micelles. The molecular weight cutoff of the dialysis membrane only allows for diffusion of free gambogic acid. As shown in Figure 5, the GA-mPEG₂₀₀₀ micelles showed a

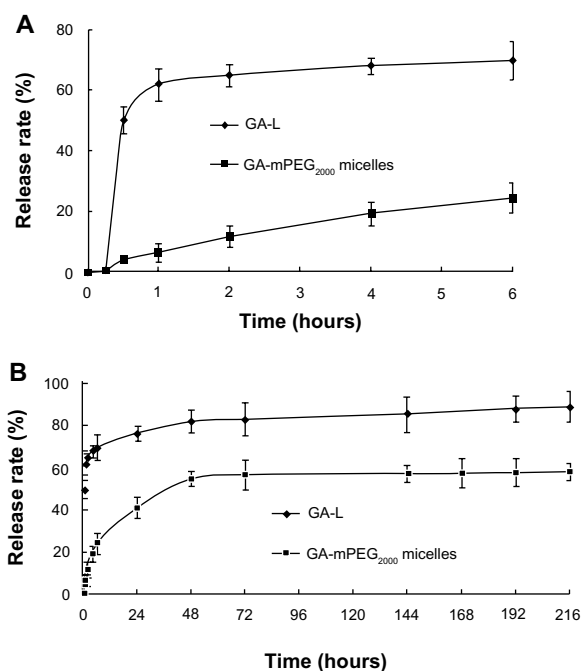


Figure 5 Time course of GA release from the GA-L formulation (◆) and GA-mPEG₂₀₀₀ micelles (■) at 37°C and pH 7.4 in 6 hours (A) and 216 hours (B).

Notes: n=3 samples per group per time point; bars, mean ± standard deviation. **Abbreviations:** GA, gambogic acid; mPEG₂₀₀₀, methoxy poly(ethylene glycol) methyl ether (molecular weight 2,000); L, L-arginine.

much slower cumulative release rate compared with the rapid release profile for GA-L. Approximately 69% of the gambogic acid was released into the medium in the GA-L group within 6 hours, whereas only 24% of gambogic acid was released from the micelles. Over a 2-week period, the mean cumulative release rate in the GA-mPEG₂₀₀₀ micelle group was much lower than that in the GA-L group (58.30% ± 4.28% versus 89.37% ± 7.43%, respectively). The in vitro drug release data show that gambogic acid could be released from the micelles in a sustained manner for an extended period.

Cell cytotoxicity assay

The in vitro biological efficacy of the GA-mPEG₂₀₀₀ micelles was evaluated against B16-F10, C26, and HUVEC cells using the MTT method. The data in Table 1 show that both GA-L and the micelles were cytotoxic to tumor cells and HUVEC cells. Compared with GA-L, the IC₅₀ values for the GA-mPEG₂₀₀₀ micelles were 2.7–3.5 times greater than those for gambogic acid.

Antitumor effects and toxicity of GA-L and GA-mPEG₂₀₀₀ in vivo

Mice subcutaneously injected with B16-F10 melanoma cells or C26 colon carcinoma cells were used to compare the antitumor activity of GA-L with that of GA-mPEG₂₀₀₀ micelles. In both models (Figures 6 and 7), tumor growth rates in mice treated with GA-L or GA-mPEG₂₀₀₀ micelles were markedly delayed, and GA-mPEG₂₀₀₀ micelles were more efficient than GA-L in suppressing growth of tumors. On day 20 after treatment, the T/C, defined as the ratio of the mean tumor volume in the treated group to that in the control group treated with normal saline, was 0.52 ($P < 0.05$) in mice treated with GA-L and 0.39 ($P < 0.05$) in mice treated with GA-mPEG₂₀₀₀ micelles (Figure 6B). Similar results were observed in C26 tumor-bearing mice (Figure 7B); on day 22 after treatment, the T/C value was 0.35 ($P < 0.05$) in mice treated with GA-L and 0.20 ($P < 0.01$) in mice treated with GA-mPEG₂₀₀₀ micelles. In addition, in both tumor models,

Table 1 Half maximal inhibitory concentration of different types of cells after incubation with the different drugs for 48 hours

	IC ₅₀ ± SD (GA equivalent, μM)	
	GA	GA-mPEG ₂₀₀₀ micelles
B16-F10	0.33 ± 0.05	1.16 ± 0.27
C26	0.80 ± 0.16	2.67 ± 0.38
HUVEC	0.81 ± 0.09	2.15 ± 0.27

Abbreviations: GA, gambogic acid; IC₅₀, half maximal inhibitory concentration; SD, standard deviation; HUVECs, human umbilical vein endothelial cells; mPEG₂₀₀₀, methoxy poly(ethylene glycol) methyl ether (molecular weight 2,000).

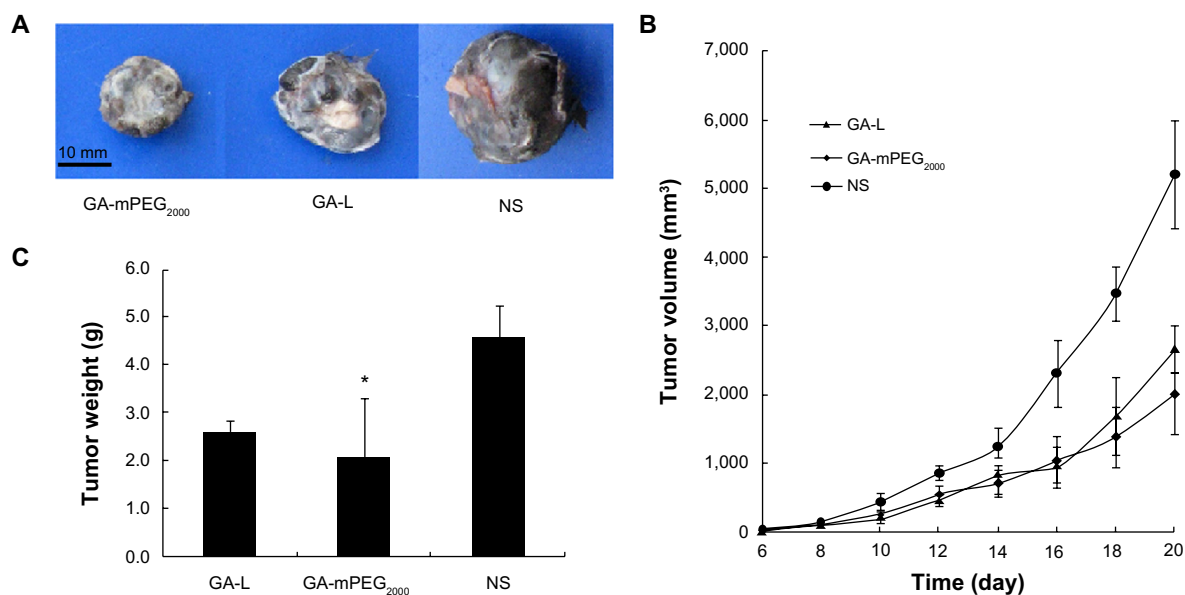


Figure 6 Antitumor study of GA and GA-mPEG₂₀₀₀ micelles in B16-F10 model. **(A)** Representative photographs of subcutaneous tumors in the GA-L group, GA-mPEG₂₀₀₀ micelle group, or NS group. **(B)** Comparison of tumor volume after treatment with GA-L (▲), GA-mPEG₂₀₀₀ micelles (◆), or NS (●). **(C)** Weight of subcutaneous tumors in each group.

Notes: n=5 mice per group per time point; bars, mean ± standard deviation. *P<0.05.

Abbreviations: GA, gambogic acid; mPEG₂₀₀₀, methoxy poly(ethylene glycol) methyl ether (molecular weight 2,000); L, L-arginine; NS, normal saline.

tumor weights in the GA-mPEG₂₀₀₀ micelle group after the final treatment were markedly lower than in the free drug and normal saline control group (Figures 6C and 7C). In the B16-F10 tumor-bearing mouse model (Figure 6C), mean tumor weights in the GA-mPEG₂₀₀₀ micelle group (2.07 ± 1.24 g)

decreased by 20% and 55% ($P<0.05$) compared with the GA-L group (2.6 ± 0.24 g) and normal saline control group (4.51 ± 0.65 g), respectively. In the C26 tumor-bearing mouse model (Figure 7C), mean tumor weights in the GA-mPEG₂₀₀₀ micelle group (0.504 ± 0.42 g) decreased by 58% and 83%

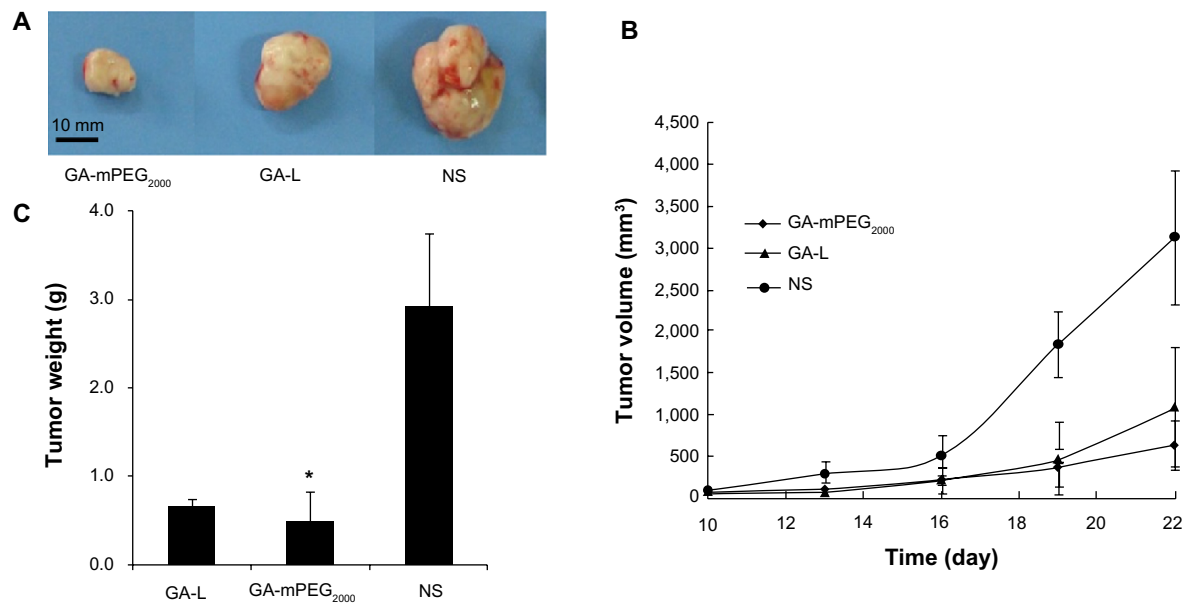


Figure 7 Antitumor study of GA and GA-mPEG₂₀₀₀ micelles in C26 model. **(A)** Representative photographs of subcutaneous tumors in the GA-L group, GA-mPEG₂₀₀₀ micelle group, or NS group. **(B)** Comparison of tumor volume after treatment with GA-L (▲), GA-mPEG₂₀₀₀ micelles (◆), or NS (●). **(C)** Weight of subcutaneous tumors in each group.

Notes: n=5 mice per group per time point; bars, mean ± standard deviation. *P<0.05.

Abbreviations: GA, gambogic acid; mPEG₂₀₀₀, methoxy poly(ethylene glycol) methyl ether (molecular weight 2,000); L, L-arginine; NS, normal saline.

($P < 0.05$) compared with the GA-L group (1.2 ± 0.72 g) and normal saline control group (2.922 ± 0.83 g), respectively. These results suggest that the GA-mPEG₂₀₀₀ micelles enhanced the antitumor effects of gambogic acid alone, and markedly delayed tumor growth rates in mice.

Throughout the experiment, the mice tolerated the scheduled doses of GA-mPEG₂₀₀₀ micelles and did not show obvious changes in fur, behavior, or eating. However, when mice were treated with GA-L intravenously, they struggled and demonstrated piloerection every time. As shown in Figure 8A and B, weight gain was normal in mice from the normal saline group, but decreased slightly in the GA-mPEG₂₀₀₀ micelle group, while that in the gambogic acid group was significantly lower than that of the other two groups. During our study, we did not observe any

treatment-related mortality in the GA-mPEG₂₀₀₀ micelle group; however, mice in the GA-L group started to die after the third (B16-F10 model) and second (C26 model) drug treatments. Moreover, for both models, we observed a substantial increase in the life span of mice in the GA-mPEG₂₀₀₀ micelle group. In the B16-F10 model, 50% of mice treated with GA-mPEG₂₀₀₀ micelles survived for more than 24 days ($P < 0.01$, Figure 8C). In contrast, all mice treated with GA-L died within 18 days. In the C26 model, 70% of mice treated with GA-mPEG₂₀₀₀ micelles survived for more than 31 days ($P < 0.01$, Figure 8D). In contrast, all mice treated with GA-L died within 28 days. These results suggest that GA-L is lethal in mice, whereas GA-mPEG₂₀₀₀ micelles decrease the toxicity of gambogic acid and prolong the lifespan of the animals.

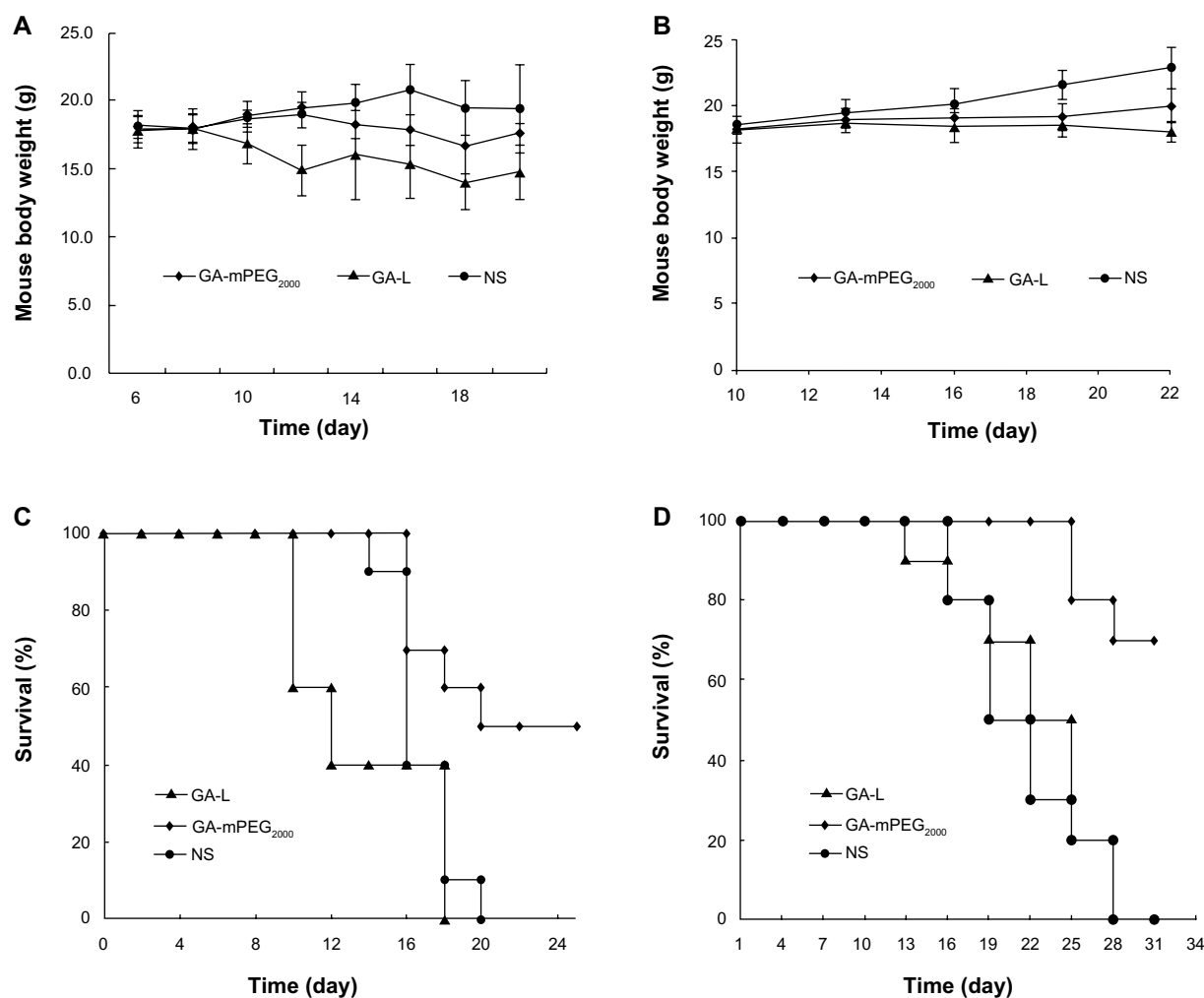


Figure 8 Toxicity study of GA and GA-mPEG₂₀₀₀ micelles in B16-F10 and C26 models. **(A)** Curves for body weight in B16-F10 tumor-bearing mice after treatment with GA-L (▲), GA-mPEG₂₀₀₀ micelles (♦), or NS (●). **(B)** Curves for body weight in C26 tumor-bearing mice after treatment with GA-L (▲), GA-mPEG₂₀₀₀ micelles (♦), or NS (●). **(C)** Survival curves for B16-F10 tumor-bearing mice after treatment with GA-L (▲), GA-mPEG₂₀₀₀ micelles (♦), or NS (●). **(D)** Survival curves for C26 tumor-bearing mice after treatment with GA-L (▲), GA-mPEG₂₀₀₀ micelles (♦), or NS (●).

Notes: n=10 mice per group per time point; bars, mean \pm standard deviation.

Abbreviations: GA, gambogic acid; mPEG₂₀₀₀, methoxy poly(ethylene glycol) methyl ether (molecular weight 2,000); L, L-arginine; NS, normal saline.

Discussion

In this study, low molecular weight PEG (ie, 2 kDa) was conjugated to the hydrophobic drug, gambogic acid (molecular weight 628.75 Da), and this amphiphilic conjugate was used to form micelles as a colloidal carrier. As shown in Figure 2, the GA-mPEG₂₀₀₀ conjugate formed nanosized micelles with a mean particle size of about 10 nm. These particles could be evenly dispersed in water, enabling the micelles to penetrate leaky vasculature and spontaneously accumulate in solid tumors via the enhanced permeability and retention effect.⁴⁰ The maximum drug loading achieved in this formulation was 135.8 mg/mL of physically entrapped gambogic acid, indicating that the polymer-drug conjugate markedly improved the solubilization capacity of gambogic acid. Achievement of this high drug loading level highlights the influence of drug-excipient compatibility as demonstrated in previous studies.⁴¹

Over 30 days, the GA-mPEG₂₀₀₀ micelle solution remained clear and transparent, and the average size and PDI did not change significantly (Figure 3). Therefore, conjugating gambogic acid with biocompatible water-soluble mPEG₂₀₀₀ resulted in a homogeneous and stable dosage form in aqueous solution with high drug loading and a small particle size, which makes gambogic acid more suitable for intravenous administration.

From the *in vitro* drug release profile (Figure 5), we found that GA-mPEG₂₀₀₀ micelles had much slower release behavior compared with GA-L, and the gambogic acid in the GA-mPEG₂₀₀₀ micelles had greatly increased stability compared with GA-L. Drug release from micelles might be due to hydrolysis of the conjugate.⁴² This attenuated drug release suggests potential applicability of polymeric micelles as a controlled drug delivery system that could result in a more favorable pharmacokinetic profile *in vivo* and minimize exposure of healthy tissues, while increasing accumulation of drug at the tumor site.^{43–45}

An *in vitro* cytotoxicity study showed that GA-mPEG₂₀₀₀ micelles retain the cytotoxicity of gambogic acid to B16-F10, C26, and HUVECs, indicating that polymeric prodrugs do not lose the anticancer and antiangiogenic activity of gambogic acid. However, compared with free gambogic acid, the IC₅₀ values for the GA-mPEG₂₀₀₀ micelles were increased by 2.7–3.0-fold. This suggests that the cytotoxicity is mainly due to the native gambogic acid released from the GA-mPEG₂₀₀₀ micelles, and the relatively lower cytotoxicity seen might be induced by the slower release behavior of gambogic acid from micelles over 48 hours. These results are consistent with the drug release profile mentioned earlier.

Conjugation of drug molecules with biocompatible water-soluble polymers, eg, PEG, has been shown to increase their apparent aqueous solubility, provide protection from degradation, and/or improve their pharmacokinetics and biodistribution.^{46–49} Conjugation to PEG as a carrier could enable passive targeting to solid tumors via the enhanced permeability and retention effect.^{50–52} Our *in vivo* antitumor study showed that GA-mPEG₂₀₀₀ micelles were more efficient in suppressing growth of tumors and prolonging the life span of mice compared with GA-L (Figures 6–8). The underlying mechanisms might involve three phases. First, aggregation of the GA-mPEG₂₀₀₀ conjugate to form micelles could protect the ester linkage from hydrolysis and protect the encapsulated drug from enzymatic degradation. Second, micelles with a small size could penetrate the leaky vasculature and accumulate in tumor tissue via the enhanced permeability and retention effect. Third, the micelles may release the drug slowly at tumor sites, resulting in higher drug concentrations in the tumor and lower drug concentrations in other tissues. Multiple mechanisms may be involved in the antitumor effect of gambogic acid, including induction of apoptosis, cell cycle arrest, telomerase inhibition, antiangiogenesis activity, and an antimetastasis effect.^{5–10} Our *in vitro* cytotoxicity study showed that gambogic acid kills tumor cells, and this also occurs *in vivo*. Gambogic acid can also inhibit angiogenesis, resulting in suppression of tumor growth *in vivo*. Therefore, the anticancer effect of GA-mPEG₂₀₀₀ micelles may be induced by inhibiting angiogenesis and directly killing tumor cells.

In this study, all mice showed abnormal changes in their fur and behavior during and after administration of GA-L, indicating that this formulation is irritating to mice. Interestingly, administration of the GA-mPEG₂₀₀₀ micelles did not induce such changes in these animals, suggesting that the nanosized assemblies of GA-mPEG₂₀₀₀ caused less toxicity and irritation compared with GA-L. Further, the body weight of mice in the GA-L group was significantly decreased after treatment, but not in the GA-mPEG₂₀₀₀ micelle group. Given that body weight loss is used as an indicator of the adverse effects of drugs and chemicals,⁵³ our results demonstrate the decreased toxicity of GA-mPEG₂₀₀₀ micelles compared with GA-L. Meanwhile, we observed high mortality in the GA-L group when tumor volume was <300 mm³, suggesting that these deaths were not caused by the tumor but by drug toxicity. Encouragingly, we did not observe these phenomena in the GA-mPEG₂₀₀₀ micelle group, suggesting that the GA-mPEG₂₀₀₀ micelles did not have lethal toxicity in mice. Our results further confirm the decreased toxicity of GA-

mPEG₂₀₀₀ micelles compared with GA-L. Two underlying mechanisms might be involved. First, conjugation with mPEG₂₀₀₀ may decrease the toxicity of gambogic acid.³¹ Second, PEGylated micelles could prolong blood residence time and reduce the risk of nonspecific accumulation of the drug in the body.⁵⁴ As a result, the drug might be more distributed at the tumor site, but less accumulated in normal tissue, thus reducing drug side effects.

To our knowledge, this work is the first concerning use of GA-mPEG₂₀₀₀ in melanoma and colon cancer therapy in vivo. Our results suggest that intravenous administration of GA-mPEG₂₀₀₀ micelles may have potential application in treating melanoma and colon cancer.

Conclusion

Biodegradable mPEG₂₀₀₀ conjugates of the natural antitumor agent gambogic acid were prepared. These GA-mPEG₂₀₀₀ conjugates could self-assemble into micelles with better solubility in water as compared with gambogic acid. Moreover, the GA-mPEG₂₀₀₀ micelles enhanced drug stability and antitumor effects, prolonged survival time, and were less toxic to mice than gambogic acid. Our results indicate that such polymer-drug conjugate systems could solve many of the problems associated with insoluble chemicals and natural anticancer agents, and at the same time achieve excellent properties in drug delivery systems.

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Disclosure

The authors report no conflicts of interest in this work.

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