Thermosensitive Micelles–Hydrogel Hybrid System Based on Poloxamer 407 for Localized Delivery of Paclitaxel

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ABSTRACT: A thermosensitive micelles–hydrogel hybrid system based on Poloxamer 407 (P407) was prepared to resolve the fast erosion and low loading capability of lipophilic drug of P407 gels for local chemotherapy. Different amounts of glutaraldehyde (GA) were applied to generate cross-linked networks with carboxymethyl chitosan (CMCS) interpenetrated in P407 gels, in which paclitaxel (PTX)-loaded *N*-octyl-*O*-sulfate chitosan micelles (PTX-M) were dispersed uniformly. The *in vitro* characteristics of CMCS-modified P407 gels (PTX-M-MG) were performed by examining the viscosity, swelling ratio, mechanical property, and drug release, while the *in vivo* evaluation included tissue distribution and anticancer efficacy through intratumoral administration in hepatoma solidity cell (Heps) tumor-bearing mice. The results showed that PTX-M-MG containing 0.05% (w/v) GA possessed lower viscosity, higher swelling ratio, stronger mechanical property, and longer term drug release, in which the loading efficiency of PTX was enlarged by the introduction of PTX-M. Moreover, PTX-M-MG revealed a prolonged retention at tumor sites, lasting for 20 days, and a superior tumor inhibition rate (64.27%) with reduced toxicity compared with Taxol®, PTX-M, and PTX-M loaded unmodified P407 gels (PTX-M-P407). It can be concluded that PTX-M-MG is a promising local delivery system for hydrophobic drug in cancer therapy, providing both improved efficacy and relieved side effects. © 2013 Wiley Periodicals, Inc. and the American Pharmacists Association J Pharm Sci 102:2707–2717, 2013

Keywords: cancer chemotherapy; controlled release; hydrogels; micelles; biomaterials; poloxamer 407; localized delivery

INTRODUCTION

Hydrogel has been widely used for tissue scaffolds, and drug and cell delivery systems in regenerative medicine because of its unique characteristics.[1–3] Thereinto, *in situ* gel has attracted more attention for the reason of its transition of sol to a gel state by the variation of environment, which assembles such advantages of solution and hydrogel as accurate dosage, ready to use, good compliance for patients in sol state; prolonged drug action, reduced side effects, and low frequency with which drugs need to be administered when turned into gel state.[4–7] In particular, thermosensitive hydrogel is one of the most frequently applied carriers because of the conveniently controllable adjustment of temperature.[8,9] In addition, *in situ* gel via intratumoral (i.t.) injection has been explored in cancer therapy because of the distinguished advantages of longer exposure time in tumor mass and less systemic exposure, which might enhance antitumor activity and reduce side effects.[10–12]

P407 is a hydrophilic linear triblock polymer which can be transformed from sol to a gel through micellization and gelation at a certain concentration and temperature.[13] P407 gels have been widely used in drug delivery systems, especially the local delivery, because P407 can be administered in liquid form and serve on sustained release depot at body temperature.[14,15] However, the major defect of P407 gels as a sustained release system is the rapid erosion in the physiological environment, which is induced by the macrodilution of body fluid, making the concentration of P407 to drop below the critical gelation concentration level.[16,17] In addition, the low loading capacity for poor soluble drugs of P407 gels narrows the application of drug carriers as well.[18,19] Therefore, it would be desirable to develop a P407-modified

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hydrogel possessing moderative disaggregation and high load of poor soluble drugs.

Carboxymethyl chitosan (CMCS) is a water soluble derivative of chitosan (CS) with excellent biocompatibility, which has been under investigation in a wide range of biomedical applications, such as tissue-engineering scaffolds and drug-delivery carriers.[20–22] Furthurmore, CMCS has abundant 2-NH_2 groups that can be cross-linked by glutaraldehyde (GA) to generate a network, which promotes the mechanical intensities and prevents it from the fast erosion of P407 gels.

Paclitaxel (PTX) is an effective chemotherapeutic agent by stabilized microtubules and mitotic arrest. It has shown broad-spectrum activity in several solid tumors.[23,24] Despite the clinical advances represented by PTX, it has a low therapeutic index because of the extremely hydrophobic property, which can be associated with serious side effects. PTX-loaded *N*-octyl-*O*-sulfate chitosan (NOSC) micelles (PTX-M), firstly prepared by our group, could significantly increase the water-solubility of PTX to 1000-fold with the drug-loading content about 40%, meanwhile reducing the toxicity of PTX, which is suitable for the P407 gels to improve the loading capability of PTX.[25]

Herein, we prepared an injectable micelles–hydrogel hybrid system for intratumoral drug delivery in this investigation. PTX-M was dispersed in P407 gels, in which a CMCS network was cross-linked with various concentrations of GA that interpenetrated P407 gels. The CMCS-modified P407 gels (PTX-M-MG) were developed with a view to overcome the fast dissolution and improve the mechanical strength of P407 gels, and enlarge the loading capability and extend the release period of PTX. Besides, tissue distribution and antitumor activity of PTX-M-MG after intratumoral administration were studied to further image the potential for pharmaceutical use.

MATERIALS AND METHODS

Materials

Poloxamer 407 (P407, M_w = 12, 600, PEO_{99}-PPO_{67}-PEO_{99}) was provided by Badische Anilin- and Soda-Fabrik (BASF). CMCS (carboxylation degree of 45%) was obtained from Nantong Lushen Bioengineering (Nantong, People's Republic of China). NOSC was synthesized by Zhang and group.[25,26] PTX was from Yew Pharmaceutical Company Ltd. (Jiangsu, People's Republic of China). Human hepatocyte cell line L02, and mice hepatoma solidity cell (Heps) were presented from Nanjing University, People's Republic of China. RPMI-1640 medium (Hyclone®), fetal bovine serum, penicillin–streptomycin solution (Hyclone®), phosphate buffered saline (PBS, Hyclone®), 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazoliumbromide (MTT) was provided by Sunshine Biotechnology Company Ltd. (Nanjing, People's Republic of China). Trypsin (Gibco®) was purchased from Pufei Bio-Technology (Shanghai, People's Republic of China). All other chemicals and reagents were of analytical grade. Male ICR mice (18–20 g), purchased from Nantong University (People's Republic of China), were maintained under controlled temperature and humidity conditions with free access to food and water. The experiments were carried out in compliance with the National Institute of Health Guide for the Care and Use of Laboratory Animals.

Preparation of Unloaded CMCS/GA-Modified P407 Hydrogels (P407-CMCS/GA)

The "cold method" was adopted for preparing P407 gels as described.[27] To prepare the P407-CMCS/GA with different concentrations of GA (0.025%, 0.05%, and 0.1% final concentrations, w/v), at first P407 was dispersed in the CMCS solution (P407 19%, CMCS 1.5%, w/v), and then the mixed solution (P407-CMCS) was kept at 4°C overnight to ensure the complete dissolution of P407. After that, 0.1 mL of GA solutions with different concentrations was added to 4.9 mL of P407-CMCS solutions, and eddied thoroughly to initiate the cross-linking reaction.

Measurement of Lower Critical Solution Temperature (LCST) and Gelation Time

The tube-inverting method was used to determine the sol–gel transition temperature and time.[28] The samples (1 mL) were added into 5 mL tubes (10 mm inner diameter) at 4°C and heated in a temperature-controllable water bath from 18 to 40°C at a heating rate of 1°C/min, the temperature when the liquid did not flow for 30 s was recorded as LCST. The gelation time was determined by incubating samples in a water bath at a constant temperature and inverting the tubes every 0.5 min, the time at which the liquid did not flow for 30 s was recorded as gelation time.

Rheological Studies

Rheological experiments were carried out by a rheometer (Physica MCR 301, Anton Paar, Ostfildern, Germany) with plate geometry in oscillation mode.[29] All the measurements were performed at a fixed frequency of 1 Hz and a strain of 0.1% with a gap size of 0.5 mm. To prevent the evaporation of samples, a solvent trap was used in conjunction with liquid paraffin. The viscosities of P407 and P407-CMCS solutions along with storage modulus (G′) and the loss modulus (G″) were acquired with the change of temperature at a rate of 1°C/min from the beginning temperature, 18°C. The gelation temperature was gotten when G′

and G″ were equivalent in value. Differently, viscosities of P407-CMCS/GA solutions were continuously measured at 20°C, and data were recorded automatically for 2 min.

Swelling Behavior

The swelling ratio (SR) of hydrogels was measured in a water bath at 37°C as previously described.[30] The P407-CMCS solutions were firstly mixed with different amounts of GA sufficiently for 2 min at room temperature, and then placed in a 37°C water bath to form the gel. The fresh-made samples were weighted and immersed in 20 mM phosphate buffer solutions (PB, pH 6.8). At predetermined time intervals, these samples were weighted after removing the surface solution with a filter paper; next they were returned to the same container with fresh medium. The SR is calculated as follows: $SR\,(\%) = (W_t - W_o)/W_o \times 100$, where W_o is the weight of the original hydrogel and W_t is the weight of hydrogel at various swelling times.

Mechanical Property

Mechanical property of hydrogels was performed with texture analyzer TA-XT2 (SMS, Stable Micro Systems Ltd., Surrey, UK).[31] The prepared hydrogels were transferred into glass bottles and placed in a 37°C water bath to allow complete gelation, meanwhile avoiding the introduction of bubbles. The analytical probe (5 mm diameter) was then pushed into each sample with a defined rate (1 mm/s) and to a defined depth (3 mm). At least five replicate analyses of each sample were performed.

In Vitro Cytotoxicity

In vitro cytotoxicity of unloaded hydrogels was performed on human hepatocyte cell line L02 as normal cells by MTT assay. About 1×10^5 cells per well were seeded in 96-well plates (Costar, Cambridge, MA). After 24 h culturing, the cells were treated with diluted P407-CMCS/GA solution for 24 h, 48 h, and 72 h, respectively. Subsequently, 20 μL of MTT PBS solution (5 mg/mL) was added into each well. After incubating for 4 h at 37°C, the medium was removed, and 150 μL of dimethyl sulfoxide was added and shaken trice. The readings were taken at 570 nm using Eliasa (Thermo Scientific).

Preparation of PTX-M-MG

PTX-M was prepared by dialysis as described previously.[25,26] In brief, PTX (16 mg) and NOSC (16 mg) were dissolved in 0.355 mL of dehydrated ethanol and 4 mL of distilled water, respectively. Subsequently, PTX solution was added into the NOSC solution drop by drop under constant stirring. The mixed solution was subjected to dialysis against deionized water overnight. Then the micelle solution was filtrated through a 0.45 μm pore-sized membrane and lyophilized by a freeze dryer system to obtain the dried powers of PTX-M. The drug-loading content (LC%) was calculated using the following equations: $LC\% = (W_{PTX\ in\ PTX\text{-}M}/W_{PTX\text{-}M}) \times 100\%$, where $W_{PTX\ in\ PTX\text{-}M}$ is the weight of PTX loaded in the micelles, and $W_{PTX\text{-}M}$ is the weight of micelles. The particle size and polydispersity index (PDI) of the PTX-M were assayed by dynamic light scattering method (Brookhaven Instruments Corporation, Georgia). PTX-M was dispersed in deionized water with 0.05% (w/v) GA or 1.5% (w/v) CMCS for different times, respectively.

To prepare the PTX-M-MG, first a certain concentration of CMCS (1.5%, w/v) and PTX-M solutions (4 mg/mL, 5 mL) were mixed as a solvent of P407, and then GA was added in (0.05% GA in final, w/v) to have an intense agitation.

Dispersibility of PTX-M in PTX-M-MG

To investigate the dispersibility of micelles in PTX-M-MG, the cross-sectional images of hydrogels were examined with field emission scanning electron microscope (FE-SEM, Sirion 200, Holland) as reported previously.[32] Samples of FE-SEM were obtained by freeze drying, and the cross-sectional area was sputter coated with platinum before examination. P407-modified hydrogels loaded with same amount of PTX (PTX-MG) were used as a control.

In Vitro Drug Release

A membraneless model was used to assay the release behavior.[33] 0.5 mL of PTX-M-MG was placed in a tube and incubated at 37°C for 30 min. Then 40 mL of PB (pH 6.8), containing 1% Tween80 preequilibrated at experimental temperature, was added as release medium, satisfying the sink condition. The release study was conducted in a SHY-2A thermostatic shaker under 37°C at 50 ± 10 rpm. At predetermined time, 2 mL of the release medium was collected and replaced with fresh medium. The concentration of PTX was determined by high-performance liquid chromatography (HPLC). The HPLC was equipped with a C18 column (250 × 4.6 mm, Diamonsil, People's Republic of China) at 35°C and a ultraviolet spectrophotometer at 227 nm. The mobile phase was a mixture of methanol and water (75:25, v/v). The samples were delivered at a flow rate of 1.0 mL/min. To explore the possible drug release routes from hydrogel, 1 mL purified water was used as a release medium, which was collected at predetermined time and examined by transmission electron microscope (TEM, Hitachi H-7650, Japan).

Development of Heps Tumor-Bearing Mice Model

For tumor formation, Heps cells from ascites of tumor-bearing mice were harvested and suspended in PBS. Then 0.2 mL of cell suspension was inoculated subcutaneously in the same flank of each mouse. The tumor volume (V) was calculated with the following formula: $V = [\text{length} \times (\text{width})^2]/2$.[34]

Tissue Distribution

Once the solid tumor reached about 100 mm^3, the animals were divided into four groups randomly. Taxol®, PTX-M, PTX-M-P407, and PTX-M-MG were intratumorally administrated at a dose of 20 mg/kg. At predetermined time points, blood samples were collected by retro-orbital plexus puncture into heparinized tubes and plasma were obtained by centrifugation. Immediately after blood sampling, animals were sacrificed by cervical dislocation, and organs (tumor and liver) were harvested. Tissue samples were rinsed in saline, wiped with filter paper to remove the surface fluid, and weighted.

The concentrations of PTX were determined using HPLC. In brief, 100 μL of plasma or 200 μL of tissue homogenate was mixed with 200 μL of acetone for 5 min. Then, the mixtures were centrifuged at 12,000 rpm for 10 min. 20 μL of the supernatant was introduced into the HPLC system.

Antitumor Activity

When the solid tumor reached about 100 mm^3, the animals were randomly divided into six groups ($n = 15$): (1) negative control group (sterile normal saline, i.t.); (2) positive control group one (Taxol® intravenous (i.v.), every other day for four times); (3) positive control group two (Taxol® i.t.); (4) PTX-M (i.t.); (5) PTX-M-P407 (i.t.); (6) PTX-M-MG (i.t.). PTX formulations were given at a dose of 20 mg/kg and all the intratumorally treated groups were subjected to a single administration.

To evaluate the antitumor efficacy, the change of tumor volume, *in vivo* toxicity, survival rate, and body weight were investigated. Tumor size and body weight in each group were determined every other day for eight days after administration, then the animals were sacrificed by cervical dislocation and tumors were harvested followed by photographing. The survival time of six groups treated as above were also monitored and the median survival time of each group was calculated by GraphPad Prism 5.[35]

Statistical Analysis

Results are given as mean ± standard deviation (SD). Statistical significance was analyzed by one-way analysis of variance and the Bonferroni test. $^{*}p < 0.05$ was considered as a significant difference, and $^{**}p < 0.01$ was considered as extremely significant difference.

RESULTS AND DISCUSSION

Sol–Gel Transition Behavior

Lower critical solution temperature values of P407 and P407-CMCS solutions acquired by tube-inverting method were 29.96 ± 0.25°C and 28.03 ± 0.15°C, respectively, making clear that they were reversibly thermosensitive. In contrast, adding GA (0.025%–0.10%, w/v) to P407-CMCS solutions would finally transform the P407-CMCS/GA solutions to a nonthermosensitive hydrogels after a period of time because of the cross-linking reactions between CMCS and GA (Fig. 1), as reported elsewhere.[10] The cross-linked CMCS network interpenetrated the P407-CMCS/GA gels might become more serried as the cross-linking time increased, avoiding the occurrence of disaggregation of collective P407 micelles or P407 gels when the temperature was below LCST, eventually losing the thermosensitivity of the hydrogels. In addition, higher concentrations of GA shortened the period when the sol state was maintained below LCST (data not shown) because of the speedier formation of cross-linked CMCS network, caused by higher concentrations of GA.

Rheology Studies

The rheological behaviors likewise demonstrated that P407 and P407-CMCS solutions were temperature dependent. Figure 2a shows that the viscosity of the P407-CMCS solution increased with the temperature in a manner that was similar to the rheological behavior of the P407 solution. However, the presence of CMCS slightly decreased the gelation temperature approximately 2°C, as a result of the addition of hydrophilic CMCS hindering the interaction of P407 micelles and water molecules, which was beneficial for the entanglement of P407 micelles associated with the formation of P407-CMCS hydrogels. Besides, the study on the influence of different amounts of GA on the viscosity of P407-CMCS/GA solutions was also conducted (Figure 2b). To prevent any possible difference between the thermosensitive and nonthermosensitive P407-CMCS/GA solutions, thermosensitivity was maintained while the viscosity was measured. The increase of the viscosity of the P407-CMCS/GA solution, as the increase in the amount of GA and the reaction time, may have been due to the formation of the CS network within it, which obstructed the flow of the solution. Moreover, the reaction time was fixed at 2 min, which was enough for the *in vivo* injection, followed by turning into a semisolid drug reservoir quickly.

Swelling Behavior

The P407 hydrogels are well known to dissolve promptly in aqueous environment and with low SRs,[10,36] which resembles the results in this report,

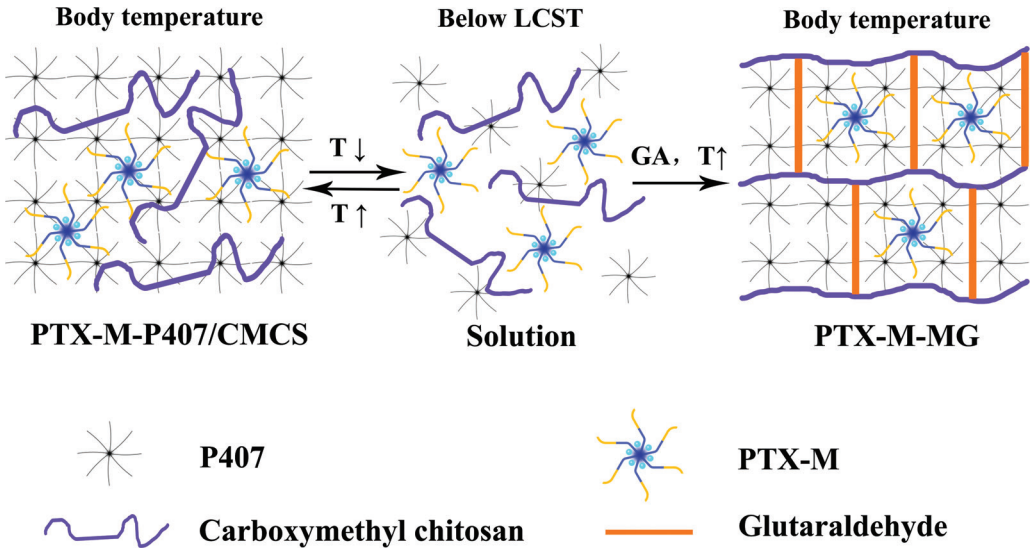

Figure 1. A schematic representation of the thermosensitive micelles–hydrogel hybrid system based on P407.

that 19% (w/v) of P407 hydrogels were completely dissolved in PB (pH 6.8) *in vitro* for 6 h. To reduce the rate of dissolution, the cross-linked CMCS network was incorporated in the P407-CMCS/GA hydrogels, which had higher SR and lower erosion rate than P407 and P407-CMCS hydrogels (data not shown) did. Notably, the maximum SRs of P407-CMCS/GA hydrogels were found to vary from the amount of GA

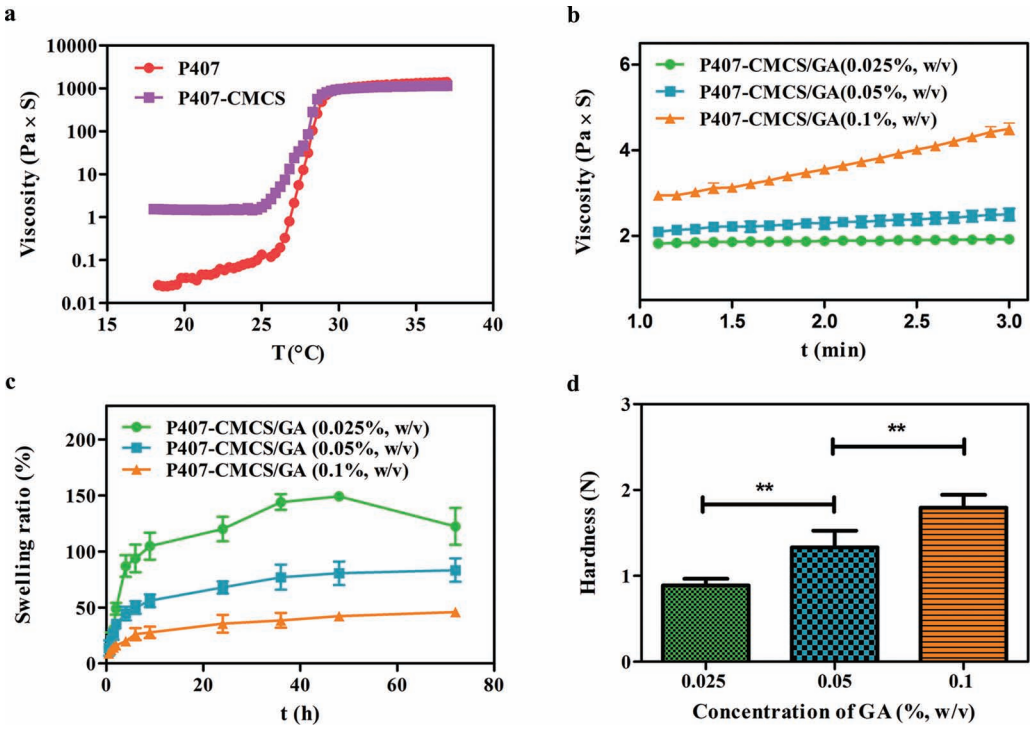

Figure 2. Characterization of P407-modified hydrogels (P407-CMCS/GA). (a) Viscosities of P407 and P407-CMCS solutions according to the increase of temperature from 18 to 37°C. (b) Viscosities of P407-CMCS/GA solutions containing GA at concentrations of 0.025%, 0.05%, and 0.1% (w/v) according to the increase of reaction time at 20°C. (c) Swelling behaviors of P407-CMCS/GA hydrogels with GA at concentrations of 0.025%, 0.05%, and 0.1% (w/v). (d) The mechanical properties of P407-CMCS/GA hydrogels with different amounts of GA. Each data is represented as means ± SD ($n = 3$, $^{**}p < 0.01$).

in the hydrogels, meaning that less amount of GA produced higher maximum SR (Figure 2c). Interestingly, although P407-CMCS/GA hydrogels with 0.05% and 0.1% (w/v) GA had the capability of maintaining the integrity until 72 h, P407-CMCS/GA hydrogel containing 0.025% (w/v) GA started to lose its weight implying the proceeding of dissolution of hydrogels after 48 h *in vitro*, which indicated that a higher concentration of GA might create a denser CMCS network, bind more aggregated P407 micelles or hydrogels in compartments during hydration, and consequently prevent the prompt dissolution of the hydrogels.

Mechanical Property

Mechanical property is also a key parameter of hydrogels for pharmaceutical applications.[37] As the weak mechanical property of P407 hydrogels, P407-modified hydrogels were explored to improve the mechanical property and achieve a relatively strong and yet elastic hydrogel.[17,37] The hardness of P407 hydrogel was under detectability because of its loose mechanical property, whereas the hardness of P407-CMCS/GA hydrogels was greatly enlarged along with the increasing amount of GA due to the increasing degree of cross-linking (Figure 2d). However, a higher degree of cross-linking might create a more brittle structure.[37] Hence, P407-CMCS/GA hydrogels with 0.05% (w/v) GA was chosen to acquire both stronger mechanical property and higher SR for the following researches.

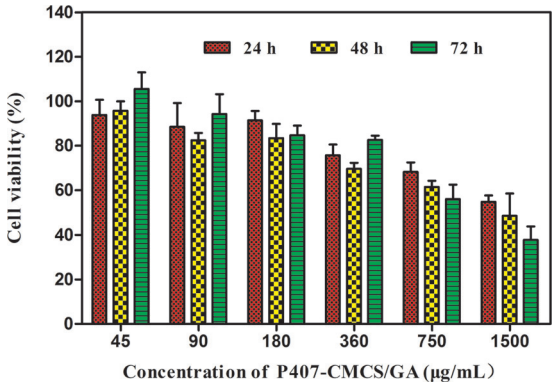

Figure 3. Cell viabilities of L02 cells treated with blank P407-CMCS/GA as a function of incubating time and the concentration of materials. Each data point is represented as mean ± SD ($n = 5$).

In Vitro Cytotoxicity

As shown in Figure 3, cell survival ratios exhibited the concentration relevance rather than the time dependence after treatment with diluted P407-CMCS/GA solution for 24, 48, or 72 h on L02 cells. Even though cell toxicities increased gradually with the increase of concentration of P407-CMCS/GA solution, cell survival ratios were all above 80% when the concentration of P407-CMCS/GA was lower than 360 μg/mL. It indicated that P407-CMCS/GA solution hardly had any toxicity to normal cells and was appropriate for drug delivery as a carrier.

Table 1. The influence of GA or CMCS on size of PTX-M ($n = 3$)

	PTX-M		PTX-M-GA		PTX-M-CMCS	
Time (Days)	Size (nm)	PDI	Size (nm)	PDI	Size (nm)	PDI
0	125.5 ± 0.4	0.29 ± 0.01	124.6 ± 0.1	0.27 ± 0.02	153.5 ± 2.3	0.24 ± 0.02
1	132.9 ± 0.9	0.24 ± 0.02	121.9 ± 0.6	0.28 ± 0.01	180.9 ± 2.9	0.26 ± 0.02
3	146.9 ± 0.1	0.24 ± 0.02	132.8 ± 1.7	0.29 ± 0.01	187.1 ± 2.3	0.24 ± 0.01
5	144.8 ± 3.5	0.29 ± 0.02	145.6 ± 1.0	0.29 ± 0.01	147.9 ± 5.0	0.21 ± 0.03
10	131.6 ± 0.4	0.29 ± 0.01	131.0 ± 1.0	0.29 ± 0.02	161.8 ± 1.6	0.17 ± 0.03

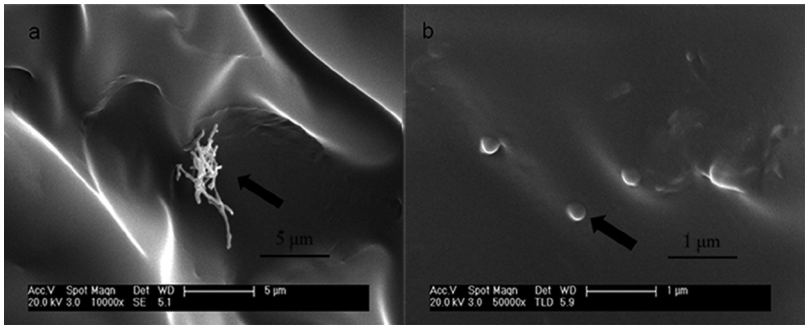

Figure 4. Cross-sectional images of (a) PTX and (b) PTX-micelles in modified P407 gels (the arrow indicates the aggregates of PTX in (a), and PTX-M in (b)).

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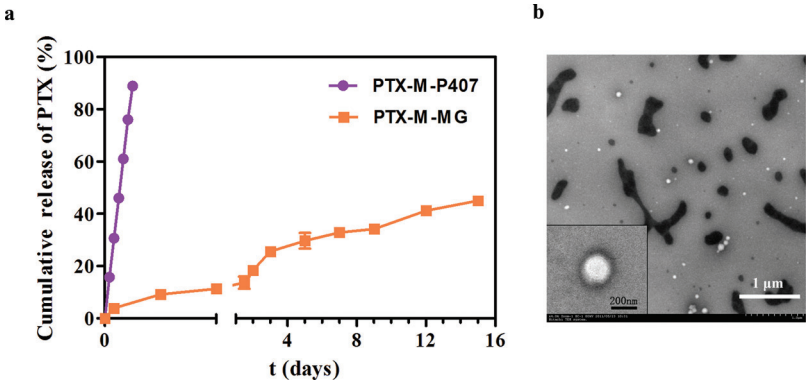

Figure 5. (a) The cumulative release of PTX from P407 and modified P407 hydrogels in PB (pH 6.8) containing 1% Tween 80 *in vitro* at 37°C for 15 days. Each data point is represented as mean ± SD ($n = 3$). (b) TEM images of the released micelles from PTX-M-MG in distilled water medium.

Dispersibility of Micelles in P407-M-MG Hydrogels

PTX, a poor water-soluble drug, can be partly solubilized in P407 solution, however, the solubility is too low to achieve the treatment threshold of PTX as before.[15] As a result, PTX-M was preferred to enhance the loading capacity of PTX in P407 hydrogels. The introduction of PTX-M had no influence on the LCST and gelation time of P407 hydrogels (data not shown). Whether the addition of CMCS or GA affected the stability of PTX-M, a test of particle size was operated as shown in Table 1. The results displayed that GA could hardly impact the size of PTX-M, while CMCS slightly increased the size of PTX-M by virtue of the entanglement between hydrophilic CMCS and the hydrophilic shell of micelles. It demonstrated that CMCS or GA did nothing to the stability of PTX-M.

To examine how PTX dispersed in P407-M-MG hydrogels, the cross-sectional images obtained by FE-SEM are exhibited in Figure 4. PTX-MG revealed the dendritic crystallization of PTX, indicating the insolubilization of loading cargo, whereas PTX-M were uniformly dispersed in PTX-M-MG hydrogels without seeing the crystallization of PTX, implying that the presence of PTX in P407-M-MG hydrogels was in the form of micelles and with no leakage from PTX-M.

In Vitro Drug Release

The *in vitro* cumulative releases of PTX from P407 and P407-M-MG hydrogels are exhibited in Figure 5a. The burst releases of PTX trapped in P407 hydrogels were significantly faster ($p < 0.01$, $n = 3$) than those from P407-M-MG hydrogels (30.7 ± 1.7% vs. 4.0 ± 1.1% at 2.0 h), and the releases were sustained only for 6 h caused by the fast erosion of P407 hydrogels, inducing the complete release of PTX-M into the release medium. Interestingly, the duration of

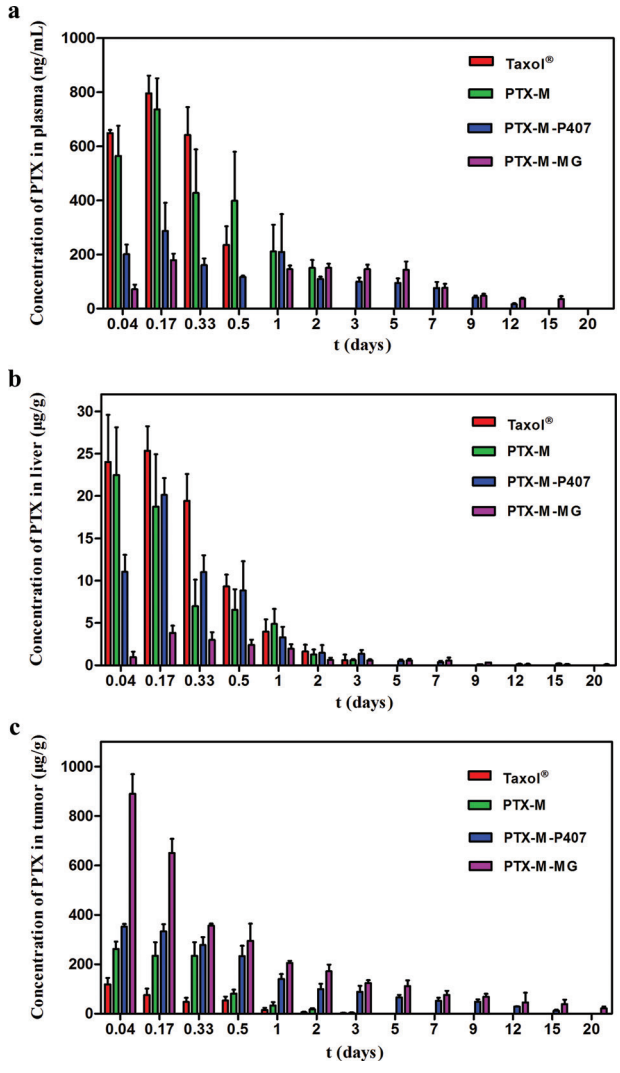

Figure 6. Tissue distribution of PTX after intratumoral administration of Taxol®, PTX-M, PTX-M-P407, and PTX-M-MG in Heps-bearing mice in blood, liver, and tumor. Each data point is represented as mean ± SD ($n = 3$).

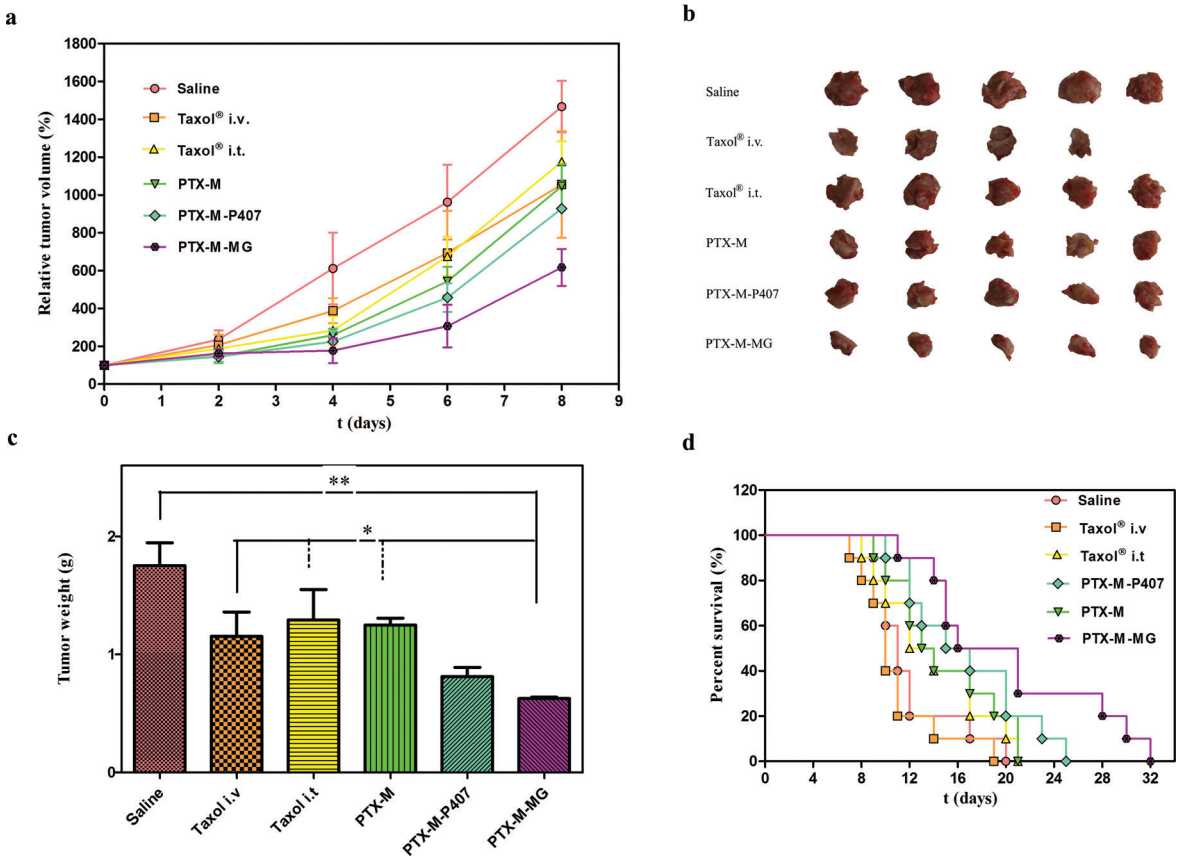

Figure 7. Antitumor activity induced by PTX in Heps tumor-bearing mice after a single dose of i.t. injections of Taxol®, PTX-M, PTX-M-P407, PTX-M-MG, and multiple doses of i.v. administration of Taxol®. (a) Tumor growth (PTX-M-MG vs. saline, Taxol® i.v., and PTX-M-P407, $^{*}p < 0.05$; PTX-M-MG vs. PTX-M, $^{**}p < 0.01$) (error bars are mean ± SD, $n = 10$). (b) Images of excised tumor. (c) Tumor weight ($^{*}p < 0.05$, $^{**}p < 0.01$, error bars are mean ± SD, $n = 5$); (d) Survival rate.

releases of PTX from P407-M-MG hydrogels was significantly extended to more than 15 days by reducing the diffusion rates of PTX or PTX-M from the compartments of CMCS networks, and exceeding the diffusion rates of those in chitosan-based or other thermosensitive hydrogels under similar release conditions *in vitro*.[10,38] In other words, the incorporation of CMCS/GA network into P407-M-MG hydrogels prolonging the *in vitro* drug release to a large extent was excellent for P407-M-MG hydrogels as a drug depot in tumor mass.

There were two routes of drug releasing from micelles–hydrogel systems in principle. One was that the drug molecules released from micelles in the compartment of hydrogel network followed by diffusing into the dissolution medium, the other was that the drug-loaded micelles diffused into the medium through aqueous channel directly and subsequently released the encapsulated drug molecules.[32] To make certain the releasing route of PTX, we detected the concentration of PTX in the distilled water medium and took photographs by TEM. The concentration of PTX surpassed its saturated solubility in distilled water medium, and micelles were found in TEM images (Figure 5b) with particle size about 200 nm, nearly the size of PTX-M in the absence of GA (about 187 nm), suggesting that a portion of PTX released from PTX-M-MG hydrogels were in the form of micelles, that is, the second release route did exist in this system. However, details of the mechanisms, by which the releases of PTX from PTX-M-MG hydrogels were continuing, must be investigated in the future.

Tissue Distribution

The tissue distribution profiles of Taxol®, PTX-M, PTX-M-P407, and PTX-M-MG were compared after a single intratumoral administration in Heps tumor-bearing mice. As anticipated in Figures 6a and 6b, Taxol® and PTX-M exhibited higher concentrations of PTX in plasma or liver than PTX-M-P407 and PTX-M-MG in the first 24 h after administration; however, PTX in Taxol® and PTX-M were hardly detected after 3 days whereas PTX in PTX-M-P407 and PTX-M-MG hydrogels were above the detection limit till 7 and 15 days, respectively. The reason that PTX in PTX-M-P407 could continually release for a longer

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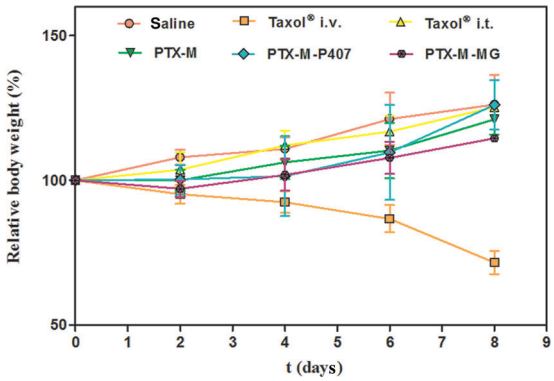

Figure 8. Alteration in the body weight of Heps tumor-bearing mice as a function of time after treating with saline, Taxol® i.v., Taxol® i.t., PTX-M, PTX-M-P07, and PTX-M-MG. Results are given as mean ± SD. ($n = 5$).

time *in vivo* than *in vitro* was PTX-M-P407 in tumor compartment with a lower dissolution ration, which slowed down the release of PTX. Notably, after administration, concentrations of PTX loaded in PTX-M-MG hydrogels in liver were all low than 5 μg/g, far below other formulations. The hepatic metabolism and side effects of PTX were reduced by the reason that PTX-M-MG hydrogels restricted the release of PTX. Figure 6c plots the concentrations of PTX in tumor sites. PTX-M-MG hydrogels possessed the highest tumor concentration of PTX during the test period and still had about 26.44 μg/g of PTX in tumor site until 20 days when PTX in other groups had been under detectability, which meant that PTX-M-MG hydrogels could be detained in tumor pass for a long time, as well as continuingly released the PTX to obtain the excellent antitumor activity.

In Vivo Antitumor Efficacy Assay

The average tumor volume is shown in Figures 7a and 7b. An enhanced tumor inhibition effect in the drug-treatment groups was observed, especially in the PTX-M-MG hydrogels, which significantly reduced tumors volume by an average 2.38-, 1.71-, 1.91-, 1.70-, and 1.50-fold compared with saline, Taxol® (i.v.), Taxol® (i.t.), PTX-M, and PTX-M-P407 groups. Likewise, the final tumor weight was measured and is displayed in Figure 7c. The results revealed that PTX-M-MG hydrogels inhibited tumor growth most efficiently, and the inhibition rate calculated by tumor weight was 64.27%, followed by PTX-M-P407 (56.80%), PTX-M (28.76%), Taxol® (i.v.) (26.17%), and Taxol® (i.t.) (21.06%) in that order. It could be found that the results in Figure 7a–c are in well agreement with each other. Besides, the survival times in response to treatment groups were a considerable index for comparing the antitumor effectiveness (Figure 7d). Mice treated with PTX-M-MG groups significantly prolonged the median survival time of tumor-bearing mice to 18.5 days compared with Taxol® (i.v.) (10 days). All these results suggested that PTX-M-MG hydrogels were more effective to inhibit tumor growth and extend animal survival. This prominent performance was probably acquired by the longer retention of PTX-M-MG hydrogels at tumor site, in respect that tumor cells would be more affected by PTX when prolonging the contact times between PTX and tumor cells, because of the specific cell-cycle affection of PTX;[24,39] on the other hand, the inhibition of P-glycoprotein (P-gp) efflux by P407 or NOSC could increase the cytotoxicity of chemotherapeutics.[25,40] Furthermore, the released drug in micellar form could improve the cellular uptake through endocytosis pathway, ultimately resulting in a superior anticancer activity.

Moreover, mice injected at tumor sites gained similar growth curves of body weights. In contrast, about 30% weight loss existed in intravenous injection group (Figure 8), indicating the severe side effects and toxicity of Taxol® (i.v.). Thereby, intratumoral administration might be a safer approach for drug delivery compared to systemic administration.

CONCLUSIONS

New intratumoral injectable PTX-M-MG hydrogels were constructed and characterized in this paper. The incorporation of CMCS-GA cross-linked networks finally transformed the thermosensitive P407 gels into nonthermosensitive hydrogels with higher swell ratios, stronger mechanical property, and improved drug-release profile. The parameters suggested that P407-CMCS/GA hydrogels containing 0.05% (w/v) GA could be a remarkable drug carrier for *in situ* injection. Afterward, the introduction of PTX-M greatly enhanced the loading capability of hydrophobic drug in P407 gels. Eventually, the obtained PTX-M-MG hydrogels were examined in *in vivo* research. All dates showed that PTX-M-MG hydrogels had excellent performance as a drug depot, including extending retention time at tumor sites, reducing hepatic metabolism, improving antitumor efficiency, and weakening side effects of PTX when compared with Taxol®, PTX-M, and PTX-M-P407. In a word, the injectable micelles–hydrogel system may provide a platform for the insoluble drug to achieve enhanced localized delivery and treatment efficacy with minimal side effects.

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