Two-pronged anti-tumor therapy by a new polymer-paclitaxel conjugate micelle with an anti-multidrug resistance effect

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Abstract

Cancerous tumors are still a major disease that threatens human life, with tumor multidrug resistance (MDR) being one of the main reasons for the failure of chemotherapy. Here, a reduction-sensitive polymer prodrug micelle, mPEG-DCA-SS-PTX (PDSP), was manufactured with a new polymer inhibitor of drug-resistance as a carrier to overcome MDR and improve the anti-tumor effect of paclitaxel (PTX). The PDSP micelles display good stability, double-responsive drug release, and excellent biocompatibility. The PDSP micelles reduced the cytotoxicity of PTX to normal HL-7702 cells, and enhanced that to SMMC-7721 and MCF-7 cells in vitro. Improved sensitivity of A549/ADR to PDSP was also observed in vitro. Furthermore, in vivo experiments show reduced systemic toxicity and enhanced therapeutic efficacy of DOX to H22 subcutaneous tumor-bearing mice. This work proves the reduction sensitive polymer prodrug micelles carried by the new polymer inhibitor can be used as an alternative delivery system to target tumors and reverse MDR for paclitaxel and other tumor-resistant drugs.

1 Introduction

So far, cancer is still a leading cause of human death in the world[1-3], with multidrug resistance (MDR) being one of the main reasons for the failure of chemotherapy[4]. Thus, reversing tumor MDR has become a research focus of pharmaceutical scientists. At present, there are usually two ways to reverse MDR: one is to increase drug penetration into tumor cells, and the other is to inhibit the efflux of drugs. Nano-drugs can promote the drug delivery to tumor tissue through the EPR effect and can increase drug penetration into cells by endocytosis. However, traditional nano-drugs cannot inhibit the drug efflux proteins on the cell membrane. Therefore, the study of nano-drugs combined with efflux protein inhibitors is gaining more and more attention[5, 6]. It has been reported that a reduction sensitive polycurcumin encapsulated paclitaxel micelles, pH-sensitive micelles co-loading with paclitaxel and dithiram, redox-responsive micelles co-loading with doxorubicin and indomethacin, paclitaxel-loaded d- α -tocopherol succinate modified chitosan micelles, and so on[6-9]. These nano-drugs combined with efflux protein inhibitors significantly reversed tumor MDR. However, small molecular efflux protein inhibitors often have problems with leakage and serious side effects. At present, the approved polymers for overcoming the MDR effect includes TPGS, Pluronic, Cremophor EL, and so on[10]. But their nanomicelles have weak stability due to their large critical micelle concentration (CMC). Therefore, a new polymer, methoxy polyethylene glycol-deoxycholic acid (mPEG-DCA, as seen in Scheme 1), was synthesized to overcome MDR in our lab. It was confirmed that mPEG-DCA has a better anti-MDR effect than TPGS in drug-resistant cells, and a subsequently better anti-tumor effect (data not shown). Therefore, a new nano-drug delivery system was manufactured using mPEG-DCA as a carrier for antineoplastic drugs to treat tumors in a two-pronged manner in this study.

Paclitaxel (PTX) is an active anti-tumor drug isolated from the bark and wood of the Pacific *Taxus mairei*. As an anti-cancer superstar, PTX has a therapeutic effect on a variety of cancers, such as breast, ovarian, head and neck neoplasm, and prostate[11, 12] cancers. PTX is a well-known BCS class IV drug with poor solubility and permeability due to a large number of hydrophobic groups in its structure[13]. To overcome

its poor aqueous solubility, a 50:50 mixture of Cremophor EL and ethanol has been used as a solvent for PTX in a commercial product[14]. Cremophor EL, however, has shown many side effects (including hypersensitivity, nephrotoxicity, and neurotoxicity [15, 16]) which seriously limit its clinical application. Also, many studies have proven that PTX is a substrate of the membrane-bound drug efflux pump P-gp, often resulting in limited efficacy against drug resistant tumors[10, 17, 18]. In order to overcome these deficiencies, pharmaceutical scientists have developed a variety of PTX nanoparticles, such as nanosuspensions[19], nanoparticles[20], liposomes [21], nanoemulsions[22], micelles[23, 24], nanoassemblies[25], and so on. These nanoparticles can partially avoid the side effect of commercial PTX injection (Taxel), but are still not effective in overcoming tumor drug resistance.

In this study, a reduction sensitive polymer-paclitaxel prodrug was constructed by using mPEG-DCA as carrier for PTX, which used disulfide bonds as a linker and self-assembled into nano-micelles to exhibit the properties of anti-MDR, tumor targeting, and high stability (Scheme 1)[26-28]. Firstly, mPEG-DCA-PTX polymer prodrug was synthesized (Shown in Scheme 2), then nano-micelles were prepared by thin-film dispersion-sonication method. Pharmaceutical properties of the micelles, in vitro cytotoxicity, in vivo anti-tumor activity, and biosafety were then investigated. In order to explore its targeting in vivo, the tissue distribution test was also carried out in tumor-bearing mice.

2 Materials And Methods

2.1 Materials

PTX was purchased from Nanjing Jingzhu Bio-technology Co., Ltd. (Nanjing, China). PTX injection (XINERJIN, 30 mg/5 mL) was bought from the ShuangLu Pharm Co. Ltd. (Beijing, China). The reference substances of PTX (purity, 99.8%) and Diazepam (purity, 99.8%) were bought from Shanghai YuanYe biotechnology co., LTD. Dimethylaminopyridin (DMAP, purity, >98%), 3, 3'-Dithiodipropionic acid (DTDP, purity > 98%), succinic anhydride (SA, purity, >98%), CaH₂ (purity > 98.5%), N, N'-dicyclohexyl-carbodiimide (DCC, purity, >98%), and Glutathione (GSH) were purchased from Aladdin Reagent Co., Ltd. (Shanghai, China). Succinic anhydride (purity, >99% GC), DMSO-d₆, and CDCl₃ were purchased from J&K Scientific Ltd. (Beijing, China). Pyrene (purity, 97%) was purchased from Shanghai Jingchun Bio-technology Co., Ltd. (Shanghai, China). Acetyl chloride was obtained from Tianjin Fuyu Fine Chemical Co., Ltd. All chemical reagents were analytical grade and used without further treatment. The mPEG-DCA was synthesized by our Lab. Dialysis tubing (MWCO 2000/350) was obtained from Shanghai Lvniao Technologies, Inc. (Shanghai, China). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl Tetrazolium bromide (MTT), RPMI 1640 medium, Dulbecco's modified Eagle medium (DMEM), penicillin-streptomycin (Amresco, USA), 0.25% (w/v) trypsin-EDTA solution and fetal bovine serum (FBS, Gibco, USA) were purchased from Sigma-Aldrich. H22 cells were gifted by Professor Du Gangjun at Henan University.

2.2 Synthesis of polymer prodrugs

2.2.1 Synthesis of dithiodipropionic anhydride (DTDPA)

Firstly, dithiodipropionic anhydride (DTDPA) was synthesized by the published methods [29]. Briefly, DTDP (5 g) was dissolved in acetyl chloride (50 mL), stirred at 600 r/min, and then refluxed at 75 °C for 7 h. After the reaction was completed, the solvent was removed by vacuum rotary evaporator and the product precipitated by ether. The product was vacuum dried at 40 °C to obtain DTDPA.

2.2.2 Synthesis of mPEG-DCA-DTPA and mPEG-DCA-SA

mPEG-DCA-DTPA: 3.5 mmol of DTDPA was placed into 30 mL of DMF in a three-neck flask, and stirred to dissolve completely in a nitrogen and ice bath. 0.7 mmol of mPEG-DCA and 0.7 mmol DMAP were dissolved in 10 mL of DMF and added dropwise into the above DTDPA solution. The reaction was carried out for 24 h at 30 °C. DMF was removed by vacuum rotary evaporation. The residue was dissolved in a moderate amount of dichloromethane (DCM). DCM solution was extracted with 0.1 mol/L hydrochloric acid aqueous solution and a saturated NaCl solution twice, respectively. Dehydration of the above DCM solution was executed with the anhydrous magnesium sulfate. The solution was concentrated and mixed with anhydrous diethyl ether to obtain the precipitate, which was collected by filtration and dried under vacuum at 37 °C.

mPEG-DCA-SA: 3.5 mmol of succinic anhydride (SA), 0.7 mmol of mPEG-DCA, and 0.7 mmol DMAP were dissolved in 40 mL of DMF, and stirred in nitrogen for 2 h at room temperature. Then the reaction was continued for 24 h at 30 °C. Purification and collection of the products were the same as the above method for mPEG-DCA-DTPA.

2.2.3 Synthesis of mPEG-DCA-PTX prodrugs

0.35 mmol mPEG-DCA-DTPA and 0.7 mmol DMAP were dissolved in anhydrous DCM and stirred at 30 °C for 60 min. 0.35 mmol PTX and 0.8 mmol DCC were added and reacted at 30 °C for 48 h. The reaction solution was filtered to remove before the concentration was carried out. The concentrated solution was mixed with diethyl ether to gain a precipitate, which was collected by filtration and dried at 35 °C in a vacuum. The mPEG-DCA-SS-PTX (PDSP) was purified using flash chromatography with DCM:methanol (80:1) as the eluent on a silica column. Further purification and collection of mPEG-DCA-CC-PTX (PDCP) was obtained by the same process as described for mPEG-DCA-SA.

2.2.4 Characterization of PDSP and PDCP

¹H NMR spectra was recorded on an AVANCE 300 MHz spectrometer (Switzerland). Fourier transform infrared spectroscopy (FTIR) analyses were carried out with an Avatar 360 Fourier transform infrared spectrophotometer (America) using KBr pellets. The CMCs of PDSP and PDCP were also investigated by fluorescence spectroscopy with pyrene as a fluorescence probe. Briefly, PDSP and PDCP micelles were diluted to a concentration range of 5.0×10^{-5} mg/mL to 5.0×10^{-1} mg/mL. Five milliliters of diluted PDSP and PDCP micelles solutions were mixed with 0.5 mL of pyrene acetone (6.0×10^{-6} mol/L), respectively. The mixtures were then sonicated for 10 min and stored overnight in a dark place at room temperature to remove the acetone completely. The fluorescence intensity of the samples at an emission wavelength of

372 nm was recorded by a fluorescence spectrophotometer (Hitachi F-7000, Japan). The CMC was then estimated by using the inflection point on the fit curve of the fluorescence intensity ratios (I_{333}/I_{339}) (excitation wavelengths of 333 nm and 339 nm) versus the log concentration of polymer prodrug.

2.3 Preparation of polymer prodrug micelles

20 mg of PDCP was dissolved in 10 mL of CH_2Cl_2 through stirring. After complete dissolution, CH_2Cl_2 was removed to form films of the polymer prodrug by rotary evaporation, which were then dried overnight under vacuum. The films were hydrated using ultrapure water at a room temperature to form a suspension. These were sonicated at 100 W using probe sonography to obtain micelles. According to the results of single-factor tests, it was found that the hydration temperature, ultrasonic power, and the counts of ultrasound were critical factors that influence the dimension and size distribution of micelles. Therefore, the central composite design (CCD)-response surface method was used to optimize the fabricated process with particle size and polydispersity index (PDI) as the responsive indexes within a minimum number of experiments. Three independent variables (factors) and their levels in the central composite design experiments are listed in Tab. 1. A total of 27 experiments and their determined values of dependent variables (responses) are listed in Tab. 2. Design Expert 8.0.6.1 software was used to fit the experimental results to obtain an optimal polynomial mathematical model. The optimal process parameters were selected from response surface diagrams.

The PDSP micelles and blank micelles (BM) were fabricated using these optimal process parameters, except that PDCP was replaced by PDSP or mPEG-DCA at the equivalent amount. To obtain the PTX injection, PTX was dissolved in a 1:1 ratio of ethanol and Cremopher EL.

2.4 Particle size, zeta potential and Particle morphology

Particle size, size distribution (PDI), and zeta potential of polymer prodrug micelles were measured using a Nano-ZS90 ZetaSizer (Malvern Instruments, Malvern, UK). The freshly prepared micelles were placed onto a carbon-coated copper grid and dried at room temperature overnight in a vacuum oven. The dimensions and morphologies of micelles were imaged on a JEOL 2010 transmission electron microscopy (TEM, JEOL Co., Japan).

2.5 Stability

To study the stability of PDSP and PDCP micelles, storage, dilution, and plasma stability experiments were carried out. In the storage stability test, micelles were stored at 4 °C and room temperature for 15 days and the sizes were determined. In the dilution stability test, micelles were diluted respectively to 10, 20, 50, 100 times with distilled water and particle size analysis was executed half an hour later. In the plasma stability test, the micelles were incubated with rat plasma at 37 °C for 48 h in a shaker (ZWY-103D, Shanghai Zhicheng, China) at a shaking speed of 100 rpm. After that, 2 mL of the samples were withdrawn at a pre-determined time of 0, 4, 8, 16, 24, 48 h, and the sizes were analyzed. All measurements were carried out in triplicate.

2.6 *In vitro* release

The GSH reduction-sensitive release test of PTX from the PDSP micelles was carried out using dialysis. One milliliter of micelles was transferred into dialysis tubes (MWCO 2000 Da) and dialyzed against 20 mL of either pH 7.4 PBS containing 0.5% Tween-80 and different concentrations of GSH (0 μ M, 10 μ M, 20 mM and 40 mM) or pH 5.8 PBS containing 0.5% Tween-80 and 20 mM GSH. The drug release from the PDSP micelles without a disulfide bond was also investigated as a control. The dialysis tubes were shaken in an air thermostatic oscillator at 150 rpm and 37 °C. At predefined time intervals, 1 mL of released medium was withdrawn for testing and replaced with 1 mL of fresh medium at the same temperature. The samples were filtered through 0.22 μ m filters, where the concentration of PTX was determined at 227 nm with UV detection on a Waters 2695-2489 HPLC system (Waters Co., Ltd., Milford, MA, USA) and Waters Symmetry C18 column (250 mm $\times$ 4.6 mm, 5 μ m) whose temperature was set at 30 °C. The mobile phase consisted of acetonitrile/water (50:50, v/v) and set at a flow rate of 1.0 mL/min. All experiments were performed in triplicate. Finally, time-cumulative release curves under different conditions were drawn.

2.7 Reduction-responsibility of micelles

To investigate reduction-response of polymer prodrug micelles, 20 mL of samples were mixed with glutathione to obtain a final GSH concentration of 10 μ M, 20 mM and 40 mM. The samples were then placed in an air thermostatic oscillator with a shaking speed of 100 rpm at 37 °C. After incubation for 24 h, the dimension of the micelles was measured by Nano-ZS90 ZetaSizer. All samples were tested three times.

2.8 Hemolysis test

Hemolytic assays of two polymer prodrug micelles were performed using fresh rat blood. First, the micellar solution was diluted with distilled water for a PTX concentration of 0.1, 0.3, 0.5 mg/mL. According to literature[30], the rat blood sample was prepared by the following method: 8 mL of fresh rat blood with anticoagulant was diluted with 10 mL of 0.9 % sodium chloride solution to obtain the diluted blood samples. 10 mL of either micellar solution at different concentrations, distilled water (positive control), or 0.9% sodium chloride (negative control) were placed in EP tubes, respectively. Then, 0.2 mL of diluted blood samples were added into each EP tube and gently blended. All sample tubes were placed in a water thermostatic shaker with a speed of 100 rpm at 37 °C and incubated for 2 h. Finally, the samples were centrifuged at 159 g for 10 min, and the supernatant was transferred into a colorimetric plate. The absorbance of each sample was measured at 545 nm on a UV-2600 ultraviolet-visible spectrophotometer (Shimadzu Co., Ltd., Tokyo, Japan), and the relative hemolytic rates (RHR%) were calculated by the following equation.

$$\text{RHR}\% = \frac{D_s - D_{nc}}{D_{pc} - D_{nc}} \times 100\%$$

D_s is the absorbance of the micelle samples, D_{nc} is the absorbance of negative control, and D_{pc} is the absorbance of positive control. The experiments were conducted in triplicate, and the data is shown as the mean values plus standard deviation ($\pm$ SD).

2.9 In Vitro Cytotoxicity Assays

2.9.1 Cell Culture

HL-7702 cells, SMMC-7721 cells, and MCF-7 cells were provided by Kaiji Biotech Co., Ltd. (Nanjing, China). A549/PTX cells were gifted by Professor Guang Han in Henan University. HL-7702 cells were cultured in DMEM medium while the other three cell lines were cultured in RPMI 1640 medium; all cell lines were then placed in a cell culture incubator with a 5% CO₂ atmosphere at 37 °C. The cells were subcultured with 0.25% trypsin-EDTA (EDTA=ethylenediaminetetraacetate) upon reaching 80-90% confluence. For further experiments, cells were seeded in a 96-well plate with 1×10^4 cells/well. After incubation for 24 h to allow cell attachment, the cells were treated with various samples at different PTX/micelle concentrations for the following tests.

2.9.2 Toxicity to normal cells

To study the biocompatibility of the micelles and polymer, the toxicity of BM, PDCP, and PDSP micelles toward HL-7702 cells were evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay[31]. Briefly, the BM, PDCP, and PDSP micelles were added and co-incubated with HL-7702 cells for 24 h or 48 h at concentrations of 2, 5, 10, 20, 50, and 100 μ g/mL in a 96-well plate. Untreated cells were used as control. After 24 h or 48 h of treatment the cells were washed, and 10 μ L of MTT (5 mg/mL) was added to each well. The cells were incubated for an additional 4 h at 37 °C. The medium was then discarded, and DMSO (100 μ L) was added to dissolve the formazan crystals formed. The absorbance in each well was determined at a wavelength of 570 nm with a multidetector microplate reader (Hidex Chameleon V, Turku, Finland). Each drug concentration was tested in triplicate.

2.9.3 In Vitro Cytotoxicity Assays

In this study, the efficiency of the several formulations against three cancer cells (SMMC-7721, MCF-7, and A549/PTX) was studied using a free PTX solution as a reference. Briefly, three cell lines were co-incubated with PTX injection, PTX injection+BM, PDCP, PDSP, PDCP+GSH, and PDSP+GSH for 24 h or 48 h at different concentrations of PTX. For SMMC-7721 and MCF-7 cells, the concentrations of PTX were set at 0.04, 0.2, 1, 5, 25 μ g/mL. PTX at concentrations of 0.4, 2, 10, 50, and 250 μ g/mL was used to inhibit the growth of A549/PTX cells. After 24 h or 48 h of co-incubation, the cell viability assays were carried out by the MTT method described in the above section. Each experiment was executed in triplicate.

2.10 In vivo antitumor activity

All animal experiments were evaluated and approved by the Ethics Committee for Animal Experimentation of Henan University (License No. HUSOM-2017-236). Five to six weeks old SPF Kunming

mice (20±2 g, Code No. 41003100004675) bearing an H22 tumor with a volume of 100 mm³ were randomly divided into four groups (n=10): (1) saline, (2) PTX injection, (3) PDCP micelle, and (4) PDSP micelle. No significant difference in body weight was observed between these groups. The formulations were administrated via tail vein every other day for seven injections with an equivalent PTX dose of 5 mg/kg. During the period of administration, body weight and tumor size was monitored daily, and tumor volume was calculated according to the formula $\text{Volume}=0.5\times(\text{width})^2\times(\text{length})$. Mice were sacrificed at 24 h post-final injection, and the liver, kidney, heart, lungs, spleen, and tumor were collected. All tissues were washed with cold normal saline (0.9% NaCl) to remove the blood on the surface, followed by quick-drying with tissue paper. After that, the tumor was weighed, and all tissues were buried in wax and sliced to perform hematoxylin and eosin (H&E) staining for pathology. The tumor weight and volume inhibition ratio (IR_w and IR_v) were calculated using the following formula[32]:

$$\text{IR}(\%) = \left(\frac{D_{\text{control}} - D_{\text{test}}}{D_{\text{control}}} \right) \times 100\%$$

where D_{control} is the tumor weight or volume for the saline group and D_{test} is the tumor weight or volume for the groups treated by drugs. The images of tissue sections were carried out under a microscope after H&E staining.

2.11 In vivo Tissue distribution

To evaluate the biodistribution of two polymer prodrug micelles in this study, H22 tumor-bearing mice with a tumor volume of 500 mm³ were randomly divided into four groups (n=24) (saline, PDCP micelles, PDSP micelles, and PTX injection). With saline as control, drug formulations were administrated intravenously at a dose of 10 mg/kg PTX via the caudal vein. All animal experiments were evaluated and approved by the Ethics Committee for Animal Experimentation of Henan University (License No. HUSOM2021-078). Six mice in each group were sacrificed at 2, 6, 12, and 24 h after administration. The liver, kidney, heart, lung, spleen, and tumor were collected, respectively. All tissue samples were stored at -20 °C until analysis. The concentration of PTX in tissues was determined by HPLC as described above, using acetonitrile/water (50:50, v/v) as the mobile phase. The area under the concentration-time curve (AUC) in all the tissues was calculated using trapezoid method. We calculated the tumor targeting rate (TTR) of three formulations according to the following formula:

$$\text{TTR} = \frac{(AUC_{0-t})_{\text{tumor}}}{\sum_{i=1}^n (AUC_{0-t})_{\text{normal}}}$$

3 Results And Discussion

3.1 Synthesis and characterization of MDCP and MDSP

The $^1\text{H-NMR}$ and IR spectra confirmed the formation of the PDSP and PDCP prodrugs (Fig. S1-3). In the PDSP spectrum (Fig. S2), the methylene proton peak of the hydroxyl group of paclitaxel shifted from δ 4.42 ppm to δ 6.3 ppm, and the carboxyl peak in mPEG-DCA-DTPA disappeared. In the PDSP spectrum, there were similar results. These results indicate that PDSP and PDCP have been synthesized successfully.

The FT-IR diagrams of mPEG-DCA, PTX, mPEG-DCA-SA, mPEG-DCA-DTPA, PDCP, and PDSP are shown in Fig. S3. In the spectra of PDSP and PDCP, the asymmetric and symmetric stretching vibration peaks of $\text{V}_{\text{C-H}}$ in DCA's structure were shown at 2945.3 cm^{-1} and 2887.4 cm^{-1} , while the stretching vibration peaks of $\text{V}_{\text{C=O}}$ in the carboxyl and ester were shown at 1730.2 cm^{-1} . These peaks were similar to those in the spectra of mPEG-DCA-SA and mPEG-DCA-DTPA. The characteristic absorption peaks of PTX also appeared in the spectra of the two polymer prodrugs, particularly the ketone carbonyl $\text{V}_{\text{C=O}}$ at 1737.9 cm^{-1} ; the carbonyl peak of the amide group at 1651.1 cm^{-1} , and the monosubstituted benzene ring at 707.9 cm^{-1} . Therefore, the results suggest that PTX was successfully bonded with mPEG-DCA-SA and mPEG-DCA-DTPA. The results also showed that the CMCs of PDSP and PDCP were $19.1\text{ }\mu\text{g/mL}$ and $9.3\text{ }\mu\text{g/mL}$, which was smaller than the CMCs of polymers CSA-ss-DOCA, TPGS and Pluronic reported in the kinds of literature[33-35]. Small CMC will be beneficial to the formation of micelles[24, 29].

3.2 The results of process optimization

To prepare uniform and stable polymer prodrug micelles, the preparation process was optimized with the central composite design, which is a statistical tool to present the quantitative relationship between independent variables and responses values[36]. In this study, the different levels of three experimental factors (seen in Tab. 1) were employed in the central composite design experiments. The particle size (Y_1) and PDI (Y_2) were used as the responses (dependent variables). The experimental results were fitted to multiple linear equations, quadratic polynomials, and cubic polynomials (seen in Eq. S1~ S6) of the effects of hydration temperature (X_1), ultrasonic power (X_2), and the counts of ultrasound (X_3) on the responses (Supporting Information) using the Design Expert 8.0.6.1 software. The fitting degree of the equation is good if its correlation coefficient R value is close to 1. The cubic polynomial model was found to be optimal to describe the correlation between the change of variables and the subsequent responses (seen in Eq. S5 and S6).

The three-dimensional response surface was drawn in line with the fitted cubic polynomial equation (Fig. S4). As the predicted values come closer to the actual ones, the points on the scatterplot fall closer to the line. If they are not, another model must be checked to improve the fit. If the points are all very close to the line, the model is expected to offer good predictability[37]. Fig. S4 shows good prediction capability of the selected models for both Y_1 and Y_2 . According to the optimal region on the surface, the best process was obtained as follows: hydration temperature at $43\text{ }^\circ\text{C}$, ultrasonic power at 90 W , and ultrasonic frequency at 14 times. Under the optimized conditions, the experimental validation of the proposed model was

conducted using three batches of sample. The polymer prodrug micelles used in the rest of the study were manufactured using this optimization process.

3.3 Characterization of particle size, zeta potential, and morphology

To confirm the form of the polymer prodrug micelles, the mean particle sizes, PDIs, zeta potentials, and morphology of PDSP and PDCP micelles were studied. Fig. 1 shows that the average particle size of PDSP and PDCP were both about 200 nm with a generally uniform size distribution, as indicated by their low PDIs in Tab. S1. Zeta potential of the micelles was approximately -9 to -15 mV as shown in Tab. S1, indicating that these nanoparticles might have good stability because of the strong homogeneous charge repulsion between particles.

TEM images (Fig. 1A and 1B) demonstrate that the PDSP and PDCP micelles possess spherical morphology at smaller measured sizes compared to the size data obtained by dynamic light scattering, which was attributed to the fact that these micelles were dried and their polyethylene glycol outer layer shrunk after losing water in the TEM experiments.

3.3 Stability of Micelles

Fig. 2A shows the dilution stability of the PDSP and PDCP micelles. The particle size of PDSP and PDCP micelles did not change significantly when diluted up to 100 times, which suggested that PDSP and PDCP micelles could have good stability when diluted by the blood in body[24]. Fig. 2B shows the storage stability of PDSP and PDCP micelles at room temperature and 4 °C. The particle size of all micelles hardly changed at room temperature and 4 °C within 12 days. These results clearly show that both micelles had good storage stability over the measured period of time. Fig. 2C shows the plasma stability of the polymer prodrug micelles. The particle sizes of PDSP and PDCP micelle did not change significantly for 48 h after being mixed with rat plasma, which suggests that they could remain stable when administered intravenously[32].

3.4 In vitro release

In some studies, it has been confirmed that the NP prodrugs developed with disulfide-bonds could be sensitive to reductants, thereby releasing drug on-demand[38, 39]. In this study, we determined the reduction-responsive drug release in GSH-containing medium at different concentrations and pH values, to predict the stability in blood circulation and the efficiency of disulfide-bond-bridged prodrug micelles response to the microenvironment of tumor cells. As shown in Fig. 2D, the disulfide-bond-bridged prodrug micelles exhibited GSH-triggered drug release. It can be seen that about 75% and 95% of PTX was released from PDSP micelles within 48h when incubated in PBS (pH 7.4) and 20 mM and 40 mM of GSH. However, only about 30% of PTX was generated within 48 h in 10 μ M and 0 μ M GSH solution (pH 7.4), which are equivalent concentrations of GSH in blood and extracellular matrix (about 2~20 μ M)[40]. These results imply that the PDSP prodrug had good stability in blood and keen response to a high

concentration of GSH in tumor cells. Thus, the prodrug could specifically deliver a drug into tumor cells [24]. In contrast, less than 30% of PTX was released from PDCP micelles after incubation in all medium with different concentrations of GSH and pH value, which indicated that PDCP micelles were not sensitive even when co-incubated with large concentrations of GSH. Additionally, Fig. 2D shows that over 95% of PTX was released from PDSP prodrugs when incubated in PBS (pH 5.8) with 20 mM of GSH, which implies that PDSP micelles should be sensitive to an acidic environment in tumor cells. Consequently, reduction-sensitive prodrugs can selectively release drugs based on the amount of external GSH and the weak acid-environment.

3.5 Reduction-response of micelles

The size changes of PDSP and PDCP prodrug micelles after incubation with different concentrations of GSH could further reflect their sensibility to a reductive environment. In this section, the micelle size's response to the reductive environment was investigated and the results are shown in Fig. 2E and 2F. As shown in Fig. 2E, the average size of PDCP micelles had not changed after incubation for 24 h at many different concentrations of GSH, and hardly changed even at high GSH concentrations (40 mM GSH for 24 h) or for longer periods of time (20 mM GSH for 48 h). This indicates that PDCP was not sensitive to the reductive environment in tumor cells. However, the particle size of PDSP micelles increased significantly with time when incubated in 20 mM GSH for 48 h or 40 mM GSH for 24 h, as shown in Fig. 2F. These results suggest that PDSP micelle had good reduction sensitivity, which was consistent with the results of the in vitro release test.

3.6 Biocompatibility in vitro

The hemolysis test could be used to estimate the toxicity of nanoparticles to red blood cells. When the hemolysis ratios are lower than the maximum hemolysis rate (5%) for the medical device requested by FDA[30, 41], the nanoparticles are considered to have good hemocompatibility. In this study, hemolysis was not observed after the two polymer prodrug micelles were incubated with rat blood for 2 h (as shown in Fig. 3A). The hemolysis rates calculated by the absorbance were less than 5% even for the highest concentration of PDSP and PDCP micelles (0.5 mg/mL as shown in Fig. 3B), which indicates that the two polymer prodrug micelles did not contribute to RBC membrane related toxicity. Based on these results, it seems that all of the PTX micelles had good blood compatibility.

To study the biosafety of polymer prodrug micelles, the in vitro cytotoxicity tests of BM, PTX, PTX+BM, PDCP, and PDSP micelles to normal HL-7702 cells was carried out by an MTT assay. Fig. 3C shows the effect of BM on the proliferation of HL-7702 cells, where its inhibition rate was less than 10%. This implies that mPEG-DCA had very good biosafety. As shown in Fig. 3D, the viability of cells treated by the two drug-loaded micelles was more than 75% at a concentration of 25 µg/mL after incubation for 24 h, and the cell viability was still over 65% even incubated for 48 h. This indicates that the PDCP and PDSP micelles were safe. The cytotoxicity of PDCP and PDSP micelles to HL-7702 cells was not significantly different. However, the PTX injection and PTX injection+BM showed strong cytotoxicity for HL-7702 cells, where the cell viability was lower than 40% when incubated at a concentration of 25 µg/mL for 48 h (seen

in Fig. 3E). This suggests that the PTX injection had high cytotoxicity to normal cells, primarily attributed to excipients such as Cremophor EL[19, 24]. However, the cytotoxicity of the PTX injection and PTX injection+BM to HL-7702 cells was almost equivalent, which further confirmed that mPEG-DCA was biocompatible. Therefore, PDGP and PDSP micelles could eliminate toxicities induced by the solubilizer in the PTX injection, and are highly biocompatible.

3.7 In vitro cytotoxicity and anti-drug resistance of tumor cells

The results of cytotoxicity for the PTX injection, PTX injection+BM, PDGP, PDGP+GSH, PDSP, and PDSP+GSH to SMMC-7721 cells are shown in Fig. 4. As shown in Fig. 4A and 4B, the survival rate of SMMC-7721 cells decreased with an increase of PTX concentration after 24 h or 48 h incubation. Moreover, the IC_{50} of six formulations against SMMC-7721 cells after incubation for 48 h was significantly lower than that for 24 h (Tab. S2), suggesting that the toxicity of each formulation to SMMC-7721 cells increased with an increase in incubation time. These results also suggest that the anti-tumor activity of each formulation was not only time-dependent but also concentration-dependent. More importantly, it was found that the cell survival rate (51% and 42%) in the PTX injection+BM group was lower than that of PTX injection (59% and 53%) at 24 h and 48 h, and that the corresponding IC_{50} was 1.88 and 0.83 times lower than that of the PTX injection, respectively. This suggests that BM may have activity in killing tumor cells. At 24 h and 48 h, the IC_{50} values of the PDGP+GSH group were slightly lower but not significantly different than that of the PDGP group, which indicated that even high concentrations of GSH could not influence the anti-tumor activity of PDGP because of its non-responsiveness to GSH. However, the IC_{50} of the PDSP group was 1.94, 1.79 times greater than that of the PDSP+GSH group, and significantly lower than those of other four groups, indicating that high concentration of GSH in cells could enhance the anti-tumor activity of PTX in PDSP due to its reduction-sensitivity. It can be seen from Fig. 4C and 4D that the cytotoxicity of all formulations to MCF-7 cells was similar to those of SMMC-7721 cells, which also confirms the fact that the reduction-sensitivity of PDSP could enhance the anti-tumor activity of PTX. These results suggest that PDSP could deliver PTX to tumor cells and release PTX in response to the strong reductive environment of the tumor, thus improving the anti-tumor activity of PTX[25, 42].

In order to study the effect of various formulations on anti-tumor drug resistance, their cytotoxicity to the drug-resistant cell line A549/PTX cells was investigated. From Fig. 4E and 4F, it can be seen that the inhibitory efficiency of each formulation on the growth of A549/PTX cells increased as PTX concentration and incubation time also increased, indicating that all formulations inhibited the proliferation of A549/PTX cells in a concentration-dependent and time-dependent manner. At 24 h and 48 h, the inhibitory effect of PTX injection+BM on the growth of A549/PTX cells was 7.34 and 2.42 times lower than those of the PTX injection, which this difference was larger than those of sensitive cell lines SMMC-7721 and MCF-7. These results suggest that BM can not only kill tumor cells but also reverse tumor drug resistance. This anti-tumor activity of BM would cooperate with PTX to kill the tumor in a two-pronged way for PDSP micelles. For PDGP+GSH and PDGP, the difference of their IC_{50} against A549/PTX

cells was similar to those for drug sensitive cell lines SMMC-7721 and MCF-7, which indicates that high concentrations of GSH could not improve the activity of PDCP to drug-resistant cells. This result was attributed to the fact that PDCP did not respond to GSH to release drugs and mPEG-DCA in tumor cells, so mPEG-DCA did not reverse the drug resistance of tumor cells. On the other hand, the IC_{50} of PDSP+GSH against A549/PTX was 1.08 and 1.16 times lower than those of PDSP, indicating that the high level of GSH increased the anti-tumor activity of PDSP due to its reduction sensitivity. In addition, we can also find that the IC_{50} ratios of PDSP to PDCP against A549/PTX cells were larger than those of drug sensitive cell lines SMMC-7721 and MCF-7, suggesting that PDSP increases the inhibitory effect of PTX on A549/PTX cell more dramatically. The above result indicates that PDSP can significantly reverse drug resistance of the tumor.

3.8 In vivo anti-tumor activity and toxicity evaluation

Fig. 5A lists the dimension, shape of the tumor, and the trends of change in tumor volume in various groups after intravenous administration over 14 days. Tumors treated with saline grew rapidly and reached 2465 mm³ on the 14th day. However, the tumors in mice administered with PTX injection, PDCP micelles, and PDSP micelles only grew to approximately 1427, 896, and 352 mm³, respectively. This indicates that the tumor growth was inhibited by these drug-loaded formulations at different levels. Compared with the PTX injection, the PDSP micelles could significantly inhibit tumor growth ($P < 0.01$), which may be attributed to its reduction sensitive drug release. Moreover, the reduction insensitive PDCP micelles displayed much weaker inhibitory effects on tumor growth compared with the PDSP micelles ($P < 0.01$). The tumor inhibition rates were calculated by comparing the average tumor volume and mass in the mice treated by the three PTX formulations compared to those treated by saline (Fig. 5B and Fig. S5). Over all formulations, PDSP micelles displayed the highest tumor inhibition rates, which suggests that the PDSP micelles have better antitumor activity compared with the other two formulations.

The changing trend in body weight of mice is shown in Fig. 5C, from which it can be seen that there was no significant change in body weight of all animals within 14 days of administration. For the groups treated with PDSP and PDCP prodrug micelles, the low fluctuation of body weight might be attributed to the fact that the two polymer prodrug micelles did not induce observed systemic toxicity. The bodyweight of mice in the PTX injection group had no significant change, which was consistent with the results mentioned in previous literature[38, 43]. Therefore, the systemic toxicity of each formulation could not be accurately reflected by the change of body weight, such that further studies on biosafety by other methods is necessary. Fig. 5D and Tab. S3 showed the survival rates of tumor-bearing mice in various test groups. The survival rates of mice injected with the PDSP micelles groups reached 100%, but that of the PTX injection group was only 60%. Therefore, the PDSP micelles may have better biosafety compared with the PTX injection.

The images of primary organ slices stained by hematoxylin-eosin (H&E) are shown in Fig. 6. No obvious abnormalities were observed in the slices of the heart, liver, and kidney for the PDSP and PDCP groups. However, the PTX injection induced severe pathological changes in the heart, liver, and kidney with

distinct cell degeneration, inflammatory cell infiltration, and hemorrhage. These results suggest that PDSP and PDCP had good biocompatibility, and that the PTX injection had obvious side effects[21, 31]. In histological images of tumor slices, all the groups except for the saline group showed different levels of tumor cell necrosis, which was characterized by distinct debris. Among these treatment groups, tumor tissues treated by PDSP micelles exhibited the highest level of necrosis and the fewest number of proliferating cells, indicating strong inhibition of malignant proliferation. In summary, our results suggest that the PDSP micelles exhibit enhanced antitumor activity and less systemic toxicity.

3.9 Biodistribution test

As shown in Fig. 7, the biodistribution of PTX was significantly influenced by the PTX micelle formulations. Firstly, the PTX injection displayed high concentrations in various tissues at 2 h after administration and was quickly eliminated from all the tissues thereafter, which indicated that the PTX injection is rapidly distributed and eliminated in vivo. The concentration of free PTX from all formulations was very high at 2 h in the kidney, which may be attributed to the fact that the kidney is one of the main organs for the excretion of drugs. PTX concentration determined from the two micelle groups at 2 h showed the similar to that from PTX injection group in various normal tissues besides the liver and kidney, and was high in the kidney within 6 h, which was similar to that reported by Zhang et al[21]. These results may be attributed to the abrupt release and leakage of a small amount of PTX from micelles in the physiological environment, as indicated by the results of in vitro release test in pH7.2 medium. We do not know what the long-term effects of this may be, but our results warrant close examination of potential side effects in future preclinical studies. From the PDSP micelles group, most PTX can be observed in the liver within 24 h, possibly due to the liver-targeting property of deoxycholic acid in the polymer prodrug, which could mean that PDSP would have a better effect on an in-situ tumor of liver[44-47]. The accumulation of PTX from PDSP and PDCP micelles in tumor tissues at 24 h was significantly higher than that of PTX injection (5.66 and 2.72 times, $P<0.001$ and $P<0.05$). And, the TTR of PDSP and PDCP micelles in tumor tissues within 24 h was higher than that of PTX injection (seen from Fig. S6, 1.62 and 1.33 times). This phenomenon may be attributed to the EPR effect[42, 48-50], which was favorable for the suppression of tumor cells. As shown in Fig. 5, the level of free PTX detected in tumors treated with PDSP micelles was higher than that of PDCP micelles within 6 h~24 h, which may be due to the specific and rapid release of PTX from PDSP micelles induced by a high level of GSH in the tumor cells, and the inhibitory effect of mPEG-DCA on drug efflux. These results explain why the dimension of tumors in mice treated by PDSP micelles was significantly smaller than those treated with PDCP micelles.

Declarations

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References

1. Smith RA, Andrews KS, Brooks D, Fedewa SA, Manassaram-Baptiste D, Saslow D, Wender RC: **Cancer screening in the United States, 2019: A review of current American Cancer Society guidelines and current issues in cancer screening.** *CA Cancer J Clin* 2019, **69**:184-210.
2. Zheng P, Liu Y, Chen J, Xu W, Li G, Ding J: **Targeted pH-responsive polyion complex micelle for controlled intracellular drug delivery.** *Chinese Chemical Letters* 2020, **31**:1178-1182.
3. Wang Y, Wang X, Zhang J, Wang L, Ou C, Shu Y, Wu Q, Ma G, Gong C: **Gambogic acid-encapsulated polymeric micelles improved therapeutic effects on pancreatic cancer.** *Chinese Chemical Letters* 2019, **30**:885-888.
4. Paul S, Moye-Rowley WS: **Multidrug resistance in fungi: regulation of transporter-encoding gene expression.** *Front Physiol* 2014, **5**:143.
5. Wu J, Lu Y, Lee A, Pan X, Yang X, Zhao X, Lee RJ: **Reversal of multidrug resistance by transferrin-conjugated liposomes co-encapsulating doxorubicin and verapamil.** *J Pharm Pharm Sci* 2007, **10**:350-357.
6. Huo Q, Zhu J, Niu Y, Shi H, Gong Y, Li Y, Song H, Liu Y: **pH-triggered surface charge-switchable polymer micelles for the co-delivery of paclitaxel/disulfiram and overcoming multidrug resistance in cancer.** *Int J Nanomedicine* 2017, **12**:8631-8647.
7. Wang J, Wang F, Li F, Zhang W, Shen Y, Zhou D, Guo S: **A multifunctional poly(curcumin) nanomedicine for dual-modal targeted delivery, intracellular responsive release, dual-drug treatment and imaging of multidrug resistant cancer cells.** *J Mater Chem B* 2016, **4**:2954-2962.
8. Zhou Y, Wang S, Ying X, Wang Y, Geng P, Deng A, Yu Z: **Doxorubicin-loaded redox-responsive micelles based on dextran and indomethacin for resistant breast cancer.** *Int J Nanomedicine* 2017, **12**:6153-6168.
9. Zhang X, He F, Xiang K, Zhang J, Xu M, Long P, Su H, Gan Z, Yu Q: **CD44-Targeted Facile Enzymatic Activatable Chitosan Nanoparticles for Efficient Antitumor Therapy and Reversal of Multidrug Resistance.** *Biomacromolecules* 2018, **19**:883-895.

10. Cao Y, Wei Z, Li M, Wang H, Yin L, Chen D, Wang Y, Chen Y, Yuan Q, Pu X, et al: **Formulation, Pharmacokinetic Evaluation and Cytotoxicity of an Enhanced-penetration Paclitaxel Nanosuspension.** *Curr Cancer Drug Targets* 2019, **19**:338-347.
11. Kou L, Sun R, Xiao S, Cui X, Sun J, Ganapathy V, Yao Q, Chen R: **OCTN2-targeted nanoparticles for oral delivery of paclitaxel: differential impact of the polyethylene glycol linker size on drug delivery in vitro, in situ, and in vivo.** *Drug Deliv* 2020, **27**:170-179.
12. Li H, Li J, He X, Zhang B, Liu C, Li Q, Zhu Y, Huang W, Zhang W, Qian H, Ge L: **Histology and antitumor activity study of PTX-loaded micelle, a fluorescent drug delivery system prepared by PEG-TPP.** *Chinese Chemical Letters* 2019, **30**:1083-1088.
13. Zhang B, Xue A, Zhang C, Yu J, Chen W, Sun D: **Bile salt liposomes for enhanced lymphatic transport and oral bioavailability of paclitaxel.** *Pharmazie* 2016, **71**:320-326.
14. Fjallskog ML, Frii L, Bergh J: **Is Cremophor EL, solvent for paclitaxel, cytotoxic?** *Lancet* 1993, **342**:873.
15. Rowinsky EK, Donehower RC: **Paclitaxel (taxol).** *N Engl J Med* 1995, **332**:1004-1014.
16. Malingr[©] MM, Terwogt JM, Beijnen JH, Rosing H, Koopman FJ, van Tellingen O, Duchin K, Huinink WW, Swart M, Lieverst J, Schellens JH: **Phase I and pharmacokinetic study of oral paclitaxel.** *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* 2000, **18**:2468.
17. Jang SH, Wientjes MG, Au JLS: **Kinetics of P-glycoprotein-mediated efflux of paclitaxel.** *Journal of Pharmacology and Experimental Therapeutics* 2001, **298**:1236-1242.
18. Gallo JM, Li S, Guo P, Reed K, Ma J: **The effect of P-glycoprotein on paclitaxel brain and brain tumor distribution in mice.** *Cancer Res* 2003, **63**:5114-5117.
19. Li M, Chen Y, Yin L, Wang H, Cao Y, Wei Z, Wang Y, Yuan Q, Pu X, Zong L: **Formulation and Stability Evaluation of Structure-Altered Paclitaxel Nanosuspensions Stabilized by a Biocompatible Amino Acid Copolymer.** *Sci Adv Mater* 2017, **9**:1713-1723.
20. Yu M, Su D, Yang Y, Qin L, Hu C, Liu R, Zhou Y, Yang C, Yang X, Wang G, Gao H: **D-T7 Peptide-Modified PEGylated Bilirubin Nanoparticles Loaded with Cediranib and Paclitaxel for Antiangiogenesis and Chemotherapy of Glioma.** *ACS Appl Mater Interfaces* 2019, **11**:176-186.
21. Zhang J, Du Q, Song X, Gao S, Pang X, Li Y, Zhang R, Abliz Z, He J: **Evaluation of the tumor-targeting efficiency and intratumor heterogeneity of anticancer drugs using quantitative mass spectrometry imaging.** *Theranostics* 2020, **10**:2621-2630.
22. Ding D, Sun B, Cui W, Chen Q, Zhang X, Zhang H, He Z, Sun J, Luo C: **Integration of phospholipid-drug complex into self-nanoemulsifying drug delivery system to facilitate oral delivery of paclitaxel.** *Asian*

23. Liu Y, Sun J, Cao W, Yang J, Lian H, Li X, Sun Y, Wang Y, Wang S, He Z: **Dual targeting folate-conjugated hyaluronic acid polymeric micelles for paclitaxel delivery.** *Int J Pharm* 2011, **421**:160-169.
24. Chang S, Wang Y, Zhang T, Pu X, Zong L, Zhu H, Zhao L, Feng B: **Redox-Responsive Disulfide Bond-Bridged mPEG-PBLA Prodrug Micelles for Enhanced Paclitaxel Biosafety and Antitumor Efficacy.** *Front Oncol* 2019, **9**:823.
25. Luo C, Sun B, Wang C, Zhang X, Chen Y, Chen Q, Yu H, Zhao H, Sun M, Li Z, et al: **Self-facilitated ROS-responsive nanoassembly of heterotypic dimer for synergistic chemo-photodynamic therapy.** *Journal of Controlled Release* 2019, **302**:79-89.
26. Luo C, Sun J, Liu D, Sun B, Miao L, Musetti S, Li J, Han X, Du Y, Li L, et al: **Self-Assembled Redox Dual-Responsive Prodrug-Nanosystem Formed by Single Thioether-Bridged Paclitaxel-Fatty Acid Conjugate for Cancer Chemotherapy.** *Nano Lett* 2016, **16**:5401-5408.
27. Zong L, Li X, Wang H, Cao Y, Yin L, Li M, Wei Z, Chen D, Pu X, Han J: **Formulation and characterization of biocompatible and stable I.V. itraconazole nanosuspensions stabilized by a new stabilizer polyethylene glycol-poly(beta-Benzyl-L-aspartate) (PEG-PBLA).** *Int J Pharm* 2017, **531**:108-117.
28. He M, Yu L, Yang Y, Zou B, Ma W, Yu M, Lu J, Xiong G, Yu Z, Li A: **Delivery of triptolide with reduction-sensitive polymer nanoparticles for liver cancer therapy on patient-derived xenografts models.** *Chinese Chemical Letters* 2020.
29. Zong L, Wang Y, Qiao P, Yu K, Hou X, Wang P, Zhang Z, Pang X, Pu X, Yuan Q: **Reduction-sensitive poly(ethylene glycol)-polypeptide conjugate micelles for highly efficient intracellular delivery and enhanced antitumor efficacy of hydroxycamptothecin.** *Nanotechnology* 2020, **31**:165102.
30. Liu MC, Liu B, Sun XY, Lin HC, Lu JZ, Jin SF, Yan SQ, Li YY, Zhao P: **Core/Shell Structured Fe(3)O(4)@TiO(2)-DNM Nanospheres as Multifunctional Anticancer Platform: Chemotherapy and Photodynamic Therapy Research.** *J Nanosci Nanotechnol* 2018, **18**:4445-4456.
31. Luo C, Sun J, Sun B, Liu D, Miao L, Goodwin TJ, Huang L, He Z: **Facile Fabrication of Tumor Redox-Sensitive Nanoassemblies of Small-Molecule Oleate Prodrug as Potent Chemotherapeutic Nanomedicine.** *Small* 2016, **12**:6353-6362.
32. Pu X, Zhao L, Li J, Song R, Wang Y, Yu K, Hou X, Qiao P, Zong L, Chang S: **A polymeric micelle with an endosomal pH-sensitivity for intracellular delivery and enhanced antitumor efficacy of hydroxycamptothecin.** *Acta Biomater* 2019, **88**:357-369.
33. Liu H, Wu S, Yu J, Fan D, Ren J, Zhang L, Zhao J: **Reduction-sensitive micelles self-assembled from amphiphilic chondroitin sulfate A-deoxycholic acid conjugate for triggered release of doxorubicin.** *Mater Sci Eng C Mater Biol Appl* 2017, **75**:55-63.

34. Collnot EM, Baldes C, Wempe MF, Kappl R, Huttermann J, Hyatt JA, Edgar KJ, Schaefer UF, Lehr CM: **Mechanism of inhibition of P-glycoprotein mediated efflux by vitamin E TPGS: influence on ATPase activity and membrane fluidity.** *Mol Pharm* 2007, **4**:465-474.
35. Gao Y, Li LB, Zhai G: **Preparation and characterization of Pluronic/TPGS mixed micelles for solubilization of camptothecin.** *Colloids Surf B Biointerfaces* 2008, **64**:194-199.
36. Tung NT, Tran CS, Pham TM, Nguyen HA, Nguyen TL, Chi SC, Nguyen DD, Bui TB: **Development of solidified self-microemulsifying drug delivery systems containing l-tetrahydropalmatine: Design of experiment approach and bioavailability comparison.** *Int J Pharm* 2018, **537**:9-21.
37. Taheri M, Bagheri M, Moazeni-Pourasil RS, Ghassempour A: **Response surface methodology based on central composite design accompanied by multivariate curve resolution to model gradient hydrophilic interaction liquid chromatography: Prediction of separation for five major opium alkaloids.** *J Sep Sci* 2017, **40**:3602-3611.
38. Sun B, Luo C, Yu H, Zhang X, Chen Q, Yang W, Wang M, Kan Q, Zhang H, Wang Y, et al: **Disulfide Bond-Driven Oxidation- and Reduction-Responsive Prodrug Nanoassemblies for Cancer Therapy.** *Nano Lett* 2018, **18**:3643-3650.
39. Yu W, Shevtsov M, Chen X, Gao H: **Advances in aggregatable nanoparticles for tumor-targeted drug delivery.** *Chinese Chemical Letters* 2020, **31**:1366-1374.
40. Chi Y, Yin X, Sun K, Feng S, Liu J, Chen D, Guo C, Wu Z: **Redox-sensitive and hyaluronic acid functionalized liposomes for cytoplasmic drug delivery to osteosarcoma in animal models.** *J Control Release* 2017, **261**:113-125.
41. Yang X, Cai X, Yu A, Xi Y, Zhai G: **Redox-sensitive self-assembled nanoparticles based on alpha-tocopherol succinate-modified heparin for intracellular delivery of paclitaxel.** *J Colloid Interface Sci* 2017, **496**:311-326.
42. Yang B, Wang K, Zhang D, Ji B, Zhao D, Wang X, Zhang H, Kan Q, He Z, Sun J: **Polydopamine-modified ROS-responsive prodrug nanoplatfrom with enhanced stability for precise treatment of breast cancer.** *RSC Advances* 2019, **9**:9260-9269.
43. Sun B, Luo C, Zhang X, Guo M, Sun M, Yu H, Chen Q, Yang W, Wang M, Zuo S, et al: **Probing the impact of sulfur/selenium/carbon linkages on prodrug nanoassemblies for cancer therapy.** *Nat Commun* 2019, **10**:3211.
44. Chen ZP, Zhu JB, Chen HX, Xiao YY, Feng MS, Cai H, Chen J, Cai BC: **Synthesis of a novel polymer bile salts-(polyethylene glycol)2000-bile salts and its application to the liver-selective targeting of liposomal DDB.** *Drug Dev Ind Pharm* 2010, **36**:657-665.

45. Chen ZP, Xiao L, Liu W, Liu D, Xiao YY, Chen J, Liu X, Li WD, Li W, Cai BC: **Novel materials which possess the ability to target liver cells.** *Expert Opinion on Drug Delivery* 2012, **9**:649-656.

46. Putz G, Schmider W, Nitschke R, Kurz G, Blum HE: **Synthesis of phospholipid-conjugated bile salts and interaction of bile salt-coated liposomes with cultured hepatocytes.** *J Lipid Res* 2005, **46**:2325-2338.

47. Parks DJ, Blanchard SG, Bledsoe RK, Chandra G, Consler TG, Kliewer SA, Stimmel JB, Willson TM, Zavacki AM, Moore DD, Lehmann JM: **Bile acids: natural ligands for an orphan nuclear receptor.** *Science* 1999, **284**:1365-1368.

48. Li Z, Zhu J, Wang Y, Zhou M, Li D, Zheng S, Yin L, Luo C, Zhang H, Zhong L, et al: **In situ apolipoprotein E-enriched corona guides dihydroartemisinin-decorating nanoparticles towards LDLr-mediated tumor-homing chemotherapy.** *Asian J Pharm Sci* 2020, **15**:482-491.

49. Wang Y, Song J, Chow SF, Chow AH, Zheng Y: **Particle size tailoring of ursolic acid nanosuspensions for improved anticancer activity by controlled antisolvent precipitation.** *Int J Pharm* 2015, **494**:479-489.

50. Hashizume H, Baluk P, Morikawa S, McLean JW, Thurston G, Roberge S, Jain RK, McDonald DM: **Openings between Defective Endothelial Cells Explain Tumor Vessel Leakiness.** *The American Journal of Pathology* 2000, **156**:1363-1380.

Tables

Tab. 1 The factors and levels of the central composite design test.

Factors	Levels		
	-1	0	1
Hydration temperature X_1 (°C)	35	45	55
Ultrasonic power X_2 (W)	60	100	140
The counts of ultrasound X_3 (Times)	10	20	30

Tab. 2 The results of the central composite design test.

No.	Factors			Responsive values	
	X_1	X_2	X_3	Size (nm)	PDI
1	-1	-1	-1	163.4	0.417
2	-1	-1	0	181.4	0.38
3	-1	-1	1	268.8	0.522
4	-1	0	-1	174.9	0.444
5	-1	0	0	207.6	0.426
6	-1	0	1	197.1	0.493
7	-1	1	-1	221.6	0.536
8	-1	1	0	216.8	0.527
9	-1	1	1	279.8	0.581
10	0	-1	-1	179.6	0.408
11	0	-1	0	174.7	0.347
12	0	-1	1	256	0.553
13	0	0	-1	228.7	0.35
14	0	0	0	185	0.404
15	0	0	1	187.6	0.422
16	0	1	-1	202.7	0.359
17	0	1	0	183.3	0.321
18	0	1	1	207.1	0.482
19	1	-1	-1	170.8	0.325
20	1	-1	0	169.9	0.507
21	1	-1	1	223.1	0.521
22	1	0	-1	152.5	0.434
23	1	0	0	142.8	0.437
24	1	0	1	195.4	0.448
25	1	1	-1	232.4	0.51
26	1	1	0	220.8	0.599
27	1	1	1	232	0.532

Figures

Figure 1

The particle size and TEM photos of PDSP (A) and PDSP (B) micelles.

Figure 2

The results of in vitro stability and reduction-sensitivity test. A: Dilution stability; B: Storage stability; C: Plasm stability; D: Reduction-sensitive release; E: Reduction-sensitivity of PDCP micelles; F: Reduction-sensitivity of PDSP micelles.

Figure 3

In vitro biosafety tests. A: Hemolysis (a: Distilled water, b: Saline, c-d: PDSP at 0.1, 0.3, 0.5 mg/mL, f-h: PDSP at 0.1, 0.3, 0.5 mg/mL); B: Hemolysis rate; C: The cytotoxicity of BM to HL-7702 cells; D: The cytotoxicity of PDSP, PDSP, PTX injection and PTX injection+BM to HL-7702 cells at 24 h; E: The cytotoxicity of PDSP, PDSP, PTX injection and PTX injection+BM to HL-7702 cells at 48 h.

Figure 4

The cytotoxicity of six PTX formulations to SMCC-7702 cells (A, B), MCF-7 cells (C, D), and A549/PTX cells (E, F) at 24 h and 48 h.

Figure 5

The results of in vivo anti-tumor activity to H22 tumor in mice. A: The change in tumor volume and the tumor shape (n=10), **P<0.01, ***P<0.001; B: The inhibition rate of tumor volume, *P<0.05, ***P<0.001; C: The change in the bodyweight of mice for 14 days; D: The curve of mice survival for 14 days.

Figure 6

The images of the heart, liver, kidney, and tumor slices stained with H&E dyes after treatment by different PTX formulations.

Figure 7

Body distribution of PTX after i.v. administration of PDSP, PDCP and PTX to mice at 2 h, 6 h, 12 h, 24 h (n=6), *P<0.05, ***P<0.001.

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