

Protein–Polymer Hybrid Nanoparticles for Drug Delivery

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Polymeric nanoparticles have much medical promise with a large number of therapeutic nanoparticles presently in clinical trials or approved for clinical use.^[1] Using nanoparticles, it may be possible to achieve improved delivery of hydrophobic drugs, targeted delivery of drugs to specific cells by ligand attachment,^[2] and sustained and triggered release.^[3] Proteins of albumin were considered to be efficiently taken up by cells and specifically accumulated in tumor cells based on their interactions with some specific protein receptors in caveolae and caveolae-mediated endothelial transcytosis as well as on tumor cells.^[4] Here, we report a new drug delivery platform based on a type of nanoparticle which is self-assembled from a bovine serum albumin (BSA)-poly(methyl methacrylate) (PMMA) conjugate. BSA, a nutrient to cells, is biodegradable and biocompatible and could be used as a model protein for albumins; PMMA, which is a thermoplastic, is also approved by FDA for medical applications. As shown in **Scheme 1**, uniform core-shell spherical nanoparticles of BSA-PMMA could be prepared with hydrophobic anti-cancer drugs being encapsulated in the hydrophobic core (made of PMMA) of the nanoparticles by a simple nanoprecipitation method. The camptothecin-encapsulated BSA-PMMA nanoparticles show enhanced anti-tumor activity both in vitro and in animals. In an animal study, a ~79% prohibition of tumor growth was observed when using camptothecin-encapsulated nanoparticles compared with free drug.

Protein-hydrophilic polymer conjugates have been extensively investigated and are relatively easy to synthesize in aqueous solution by a “grafting onto” or “grafting from” approach.^[5] Although protein-hydrophobic polymer

conjugates are expected to have interesting additional functionalities due to their amphiphilic molecular structures, however, to our knowledge, the synthesis of protein-hydrophobic polymer conjugates has been recognized to be difficult mainly because of the insolubility of hydrophobic polymer in aqueous solution. In this study, the conjugate made of BSA and hydrophobic PMMA was successfully synthesized according to our previous report.^[6] BSA was dissolved in pure dimethyl sulfoxide (DMSO) at 50 °C at a concentration of 2 mg/mL, followed by addition of acrylic acid *N*-hydroxysuccinimide ester (NAS) to introduce double bonds on the protein surface. Methyl methacrylate (MMA) and 2,2'-azobis(2-methylpropionitrile) (AIBN) were added to conduct the polymerization at 60 °C to form the BSA-PMMA bioconjugates. Each BSA was confirmed to be modified with ~51 acryloyl groups as determined by MALDI-TOF mass spectrometry (see Supporting Information). ¹H-NMR was used to confirm the chemical composition of the conjugate (see Supporting Information). The content of BSA in the conjugate was varied from ~4 wt% to ~82 wt% as determined by ¹H-NMR, depending on the feed ratio of MMA to BSA. The molecular sizes of the denatured BSA, BSA-PMMA (weight ratio 4.6:1) conjugate, and BSA-PMMA (weight ratio 1.6:1) conjugate in DMSO were determined, by dynamic light scattering (DLS) (**Figure 1A**), to be ~9 nm, ~16 nm, and ~20 nm, respectively. These values indicate that the attachment of PMMA chains to the BSA protein increases the size of the macromolecule. No free BSA was detected in the product, as determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (**Figure 1B**).

By a simple nanoprecipitation method, self-assembled spherical nanoparticles with diameters around 100 nm were obtained (**Figure 1C**). The BSA:PMMA weight ratio and the concentration of the conjugate in solution may be changed to control the nanoparticle size and surface charge (**Table 1**), allowing us to achieve optimal pharmacokinetic properties of the nanoparticles. The size of the nanoparticles decreases and the number of negative charges on the surface increases with an increasing ratio of BSA to PMMA (**Table 1**). One possible explanation for this behavior is that at a higher BSA:PMMA ratios, more hydrophilic BSA molecules are located at the surface of the nanoparticles with hydrophobic PMMA in the core. The BSA:PMMA weight ratio of 1.6:1 and the conjugate concentration of 0.1 mg/mL result in nanoparticles with a favorable size of 100 nm and a zeta potential of –50 mV for drug delivery applications.^[7] It is well-known that cationic nanoparticles could be effectively taken up by cells. However, cationic nanoparticles may also cause non-specific binding and toxicity to cells in some cases, due to their strong

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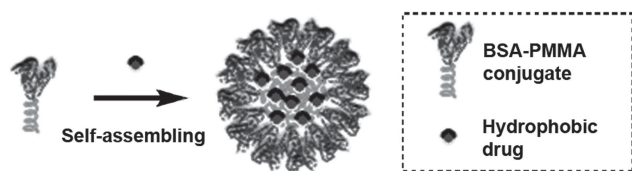
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Scheme 1. Formation of macromolecular micellar aggregates of BSA-PMMA conjugates in solution.

interactions with negatively charged cell membranes. Anionic nanoparticles have lower non-specific binding and lower toxicity to cells compared with cationic nanoparticles and could also be efficiently taken up by various types of cell lines.^[7] In the following in vitro and in vivo studies, we formulated BSA-PMMA nanoparticles using the conjugate with the BSA:PMMA weight ratio of 1.6:1.

To visualize the cell uptake of the nanoparticles using fluorescence microscopy, FITC was covalently attached to BSA-PMMA conjugates by reacting with the amine groups of the BSA in DMSO, followed by nanoprecipitation to produce the fluorescent nanoparticles. To demonstrate cell uptake of the nanoparticles, we use two types of cells, human colon cancer cells (HCT116 cells) and adipose stem cells (ASCs). After incubating the nanoparticles (0.05 mg/mL) with cells for 4 h at 37 °C, cells were washed three times with PBS (pH 7.4) and observed by fluorescence microscopy. Figure 1D and E show that the fluorescent nanoparticles are captured by both types of the cells.

We have encapsulated camptothecin (CPT) as a model chemotherapy drug in the BSA-PMMA nanoparticles.

Table 1. The size and zeta potential of the nanoparticles prepared at different BSA:PMMA ratios and different concentrations.

BSA:PMMA weight ratio	Polymer concentration [mg/mL]	Size [nm]	Zeta potential [mV]
0.04:1	0.1	452 ± 25	-37.4 ± 2.0
0.8:1	0.1	313 ± 23	-41.2 ± 1.5
1.6:1	0.1	119 ± 19	-49.5 ± 2.2
1.6:1	0.08	88 ± 8	-46.3 ± 2.0
1.6:1	0.02	63 ± 5	-42.8 ± 2.4

Camptothecin is used for treating a wide range of tumors, including advanced human colon, ovarian, and esophageal carcinomas.^[8] However, it is poorly soluble in water^[9] and easily converted at physiological conditions to a less active form.^[10] By encapsulation of CPT, nanoparticles could help disperse the drug in aqueous solution, prevent it from degradation, and deliver this drug into cells. During the preparation of CPT-encapsulated BSA-PMMA nanoparticles, the pH of the BSA water solution was adjusted to 5 to avoid the deactivation of CPT in basic solution. The high concentration (120 mg/mL) of BSA-PMMA conjugate in chloroform solution helped to dissolve the CPT in chloroform in making nanoparticles by nanoprecipitation. CPT-encapsulated nanoparticles were dispersed in a release medium (PBS, pH 7.4 containing 2% (w/v) Tween 80).^[11] As shown in **Figure 2A**, at the loading ratio of 25 wt% to prepare the CPT-encapsulated nanoparticles, the release of a high initial burst of drug occurs. The encapsulation ratio was determined to be ~11 wt% (weight percentage of drug

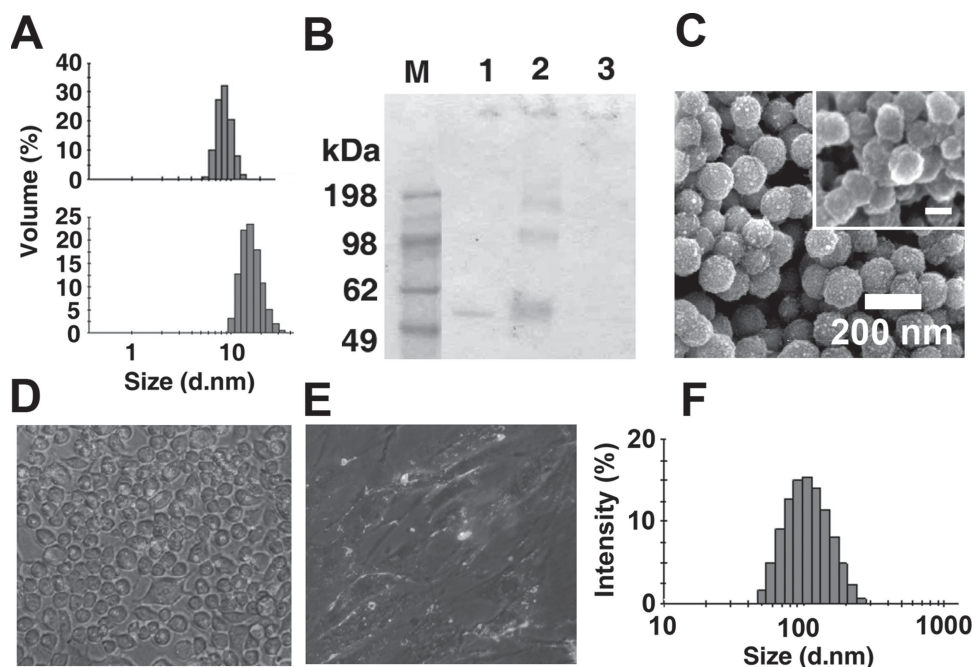


Figure 1. A) DLS of denatured BSA and BSA-PMMA conjugate (BSA:PMMA weight ratio of 4.6:1) in DMSO. B) SDS-PAGE electropherogram of native BSA (lane 1), denatured BSA (lane 2), and BSA-PMMA conjugate (BSA:PMMA weight ratio of 4.6:1) (lane 3). C) SEM image of BSA-PMMA nanoparticles (BSA:PMMA weight ratio of 4.6:1) (Insets: CPT-encapsulated BSA-PMMA nanoparticles, Scale bar: 100 nm). D) and E) Fluorescence microscopy images of HTC116 cells and ASCs cells treated with FITC-labeled BSA-PMMA nanoparticles. F) DLS of the CPT-encapsulated BSA-PMMA nanoparticles in PBS (pH 7.4) at the concentration of 7 mg/mL.

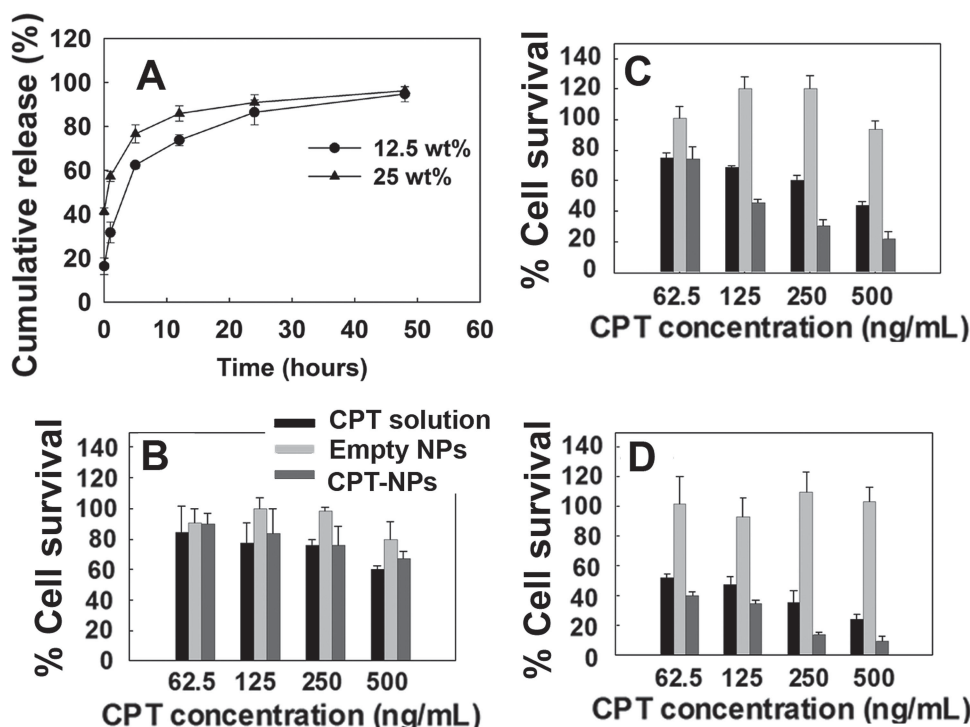


Figure 2. A) Cumulative release of CPT from BSA-PMMA nanoparticles in PBS (pH 7.4) containing 2% (w/v) Tween 80 for different loading percentages of the drug camptothecin. B,C,D) Percentage of HCT116 cell survival after treatment with different formulations of CPT solution and CPT-encapsulated nanoparticles for 24, 48, 72 h.

inside the nanoparticle). As the CPT loading ratio decreased from 25 wt% to 12.5 wt%, a lower initial burst was achieved and the encapsulation ratio was determined to be ~10 wt%. In both formulations, the encapsulated CPT was released from the nanoparticles over a period of 48 h. The drug release from the BSA-PMMA nanoparticles when incubated with cells, is possibly from the diffusion of drug molecules from the nanoparticles and the degradation of nanoparticles, which is the main mechanism of drug release observed for polymer nanoparticles.^[12] The CPT-encapsulated BSA-PMMA nanoparticles with a 12.5 wt% loading ratio were chosen for the following in vitro and in vivo anti-tumor activity assay.

The CPT-encapsulated BSA-PMMA nanoparticles were analyzed for their ability to retard the proliferation of tumor cells. HCT116 cells were exposed to CPT in a 10% v/v DMSO solution containing CPT or CPT-encapsulated nanoparticles with the same drug concentration. The final concentration of DMSO in the cell culture medium is 0.5% (v/v), which has no measurable effect on cell viability. Empty nanoparticles were used as a control. The viability of cell populations was then assessed by the MTT method^[13] at 24 h, 48 h, and 72 h. After 24 h, no significant variance in cell viability was measured with different CPT concentrations. As shown in Figure 2B-D, the dose-dependent cytotoxic effect of the CPT solution was evident cumulating in approximately 50% HCT116 survival at 500 ng/mL after 48 h and at 62.5 ng/mL after 72 h. The encapsulation of CPT into the nanoparticles resulted in marked improvements of the anti-tumor activities. After 48 h, 50% survival was observed at 125 ng/mL. After 72 h, less than 20% survival was observed at the CPT concentration

above 250 ng/mL. The empty nanoparticles showed excellent biocompatibility. Around 100% cell survival was observed for cells treated with different concentrations of empty nanoparticles. After 48 h (Figure 2C), a slight promotion of cell growth (~120% cell survival) was observed, which might be caused by the empty nanoparticles that contain BSA, which serves as a nutrient for cells. The promotion effect disappeared after 72 hours, possibly because that the BSA-PMMA nanoparticles have been depleted by cell growth.

We tested the in vivo anti-tumor efficiency of CPT-encapsulated BSA-PMMA nanoparticles by i.v. injection into mice with subcutaneous colon cancer tumors. The size of CPT-loaded BSA-PMMA nanoparticles in PBS (pH 7.4) at a concentration of 7 mg/mL was measured to be 100 nm (PDI = 0.121) by DLS (Figure 1F). The small size and narrow size distribution of the nanoparticles in solution made this formulation very suitable for i.v. injection. The dose of CPT (9 mg/kg) was based on the literature.^[14] CPT is very insoluble in aqueous solution and is acutely lethal when given to mice by i.v. injection at 9 mg/kg (0.18 mg/20 g mouse) due to the particulate matter in the drug suspension. Thus, we followed the protocol introduced by Davis and his colleagues^[14] for the administration of free CPT. In the experiment, the CPT was suspended in a vehicle of 0.5% methylcellulose and 0.1% Tween 80 and administered by i.p. injection as an attempt to maximize its efficiency. As shown in **Figure 3**, the tumor volume in CPT nanoparticle-treated mice was significantly smaller than that of mice treated with free CPT. For example, injection of CPT nanoparticles at 9 mg/kg suppressed tumor size by 67% at day 7, com-

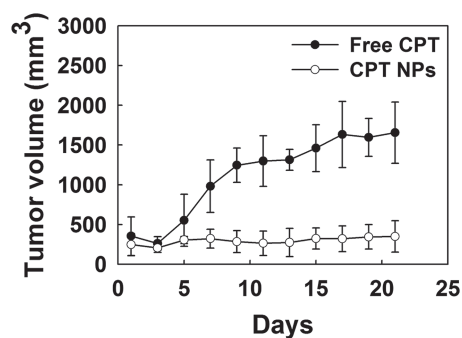


Figure 3. Anti-tumor efficiency using free CPT and CPT-encapsulated BSA-PMMA nanoparticles in mice bearing subcutaneously implanted tumors.

pared with the free CPT-treated group. Further reduction in tumor growth was observed with repeat nanoparticle injections at day 9. For CPT nanoparticle-treated mice, a 80% tumor volume reduction was achieved at day 17 compared with free CPT-treated group. At day 21, the median tumor volume of the CPT nanoparticle-treated mice is 348 mm³, which is slightly (40%) increased compared with the tumor volume at day 1 (247 mm³). However, for free CPT-treated group, at day 21, the median tumor volume 1655 mm³, which is 368% increased compared with the tumor volume at day 1. Thus, a ~79% prohibition of tumor growth was observed when using CPT encapsulated nanoparticles compared with free CPT. When using the CPT-encapsulated BSA-PMMA nanoparticles for *in vivo* anti-tumor therapy, two effects are anticipated to increase the uptake of the drug-loaded nanoparticles by cancerous cells: (1) the enhanced permeability and retention (EPR) effect of solid tumors would allow more long-circulating drug-loaded nanoparticles to be accumulated in the tumor tissue;^[15] (2) drug-loaded albumin nanoparticles, one example of which is the FDA-approved Nab-paclitaxel,^[4] were considered to be probably accumulated more in cancerous cells than in non-cancerous cells based on some specific protein receptors in caveolae and caveolae-mediated endothelial transcytosis as well as on tumor cells.^[4] In our study, the BSA protein was denatured in DMSO for conjugation. A recent paper about utilizing denatured BSA-coated liposomes as drug delivery carriers has proven that the coating of denatured BSA could also increase the stability of the nano-carriers in plasma and enhanced the cellular uptake efficiency of the vehicle.^[16]

It is known that PMMA is biocompatible but not biodegradable *in vivo*. A number of studies have tried to develop PMMA-based nanoparticles for drug delivery.^[17] No obvious toxicity was observed by *i.v.* injection of PMMA nanoparticles *in vivo*.^[18] In our study we utilized the BSA, which is biodegradable, to copolymerize with PMMA. As described in the supporting information, one BSA molecule was covalently bound with about 51 PMMA chains. Each attached PMMA chain only has 6–8 of repeating units (MMA) in the BSA-PMMA conjugate we used for *in vivo* studies. After the degradation of BSA *in vivo*, only oligomers of MMA (6–8 of repeating units with 600–800 Da in molecular weight) would exist. In the *in vitro* and *in vivo* study, we observed that empty

BSA-PMMA nanoparticles have good biocompatibility and no toxicity to cells or mice.

In summary, we have developed a new protein–polymer hybrid nanoparticle platform consisting of a hydrophobic polymeric core of PMMA and a hydrophilic protein shell of BSA. We have demonstrated that the hybrid nanoparticles have tunable size and surface charge, ease of modification, excellent biocompatibility, and efficient cell uptake. The preparation process is simple and easy to scale up, which suggests its use for drug delivery to cells. As an example of this type of drug delivery vehicles, compared with free drug formulation, the camptothecin-encapsulated BSA-PMMA nanoparticles shows enhanced anti-tumor activity both *in vitro* and *in animals*.

Experimental Section

Materials: Albumin from bovine serum (lyophilized powder, A3294), acrylic acid *N*-hydroxysuccinimide ester (NAS), methyl methacrylate (MMA), 2,2'-azobis(2-methylpropionitrile) (AIBN), dimethyl sulfoxide are purchased from Sigma-Aldrich and used without further treatment. NOD/LtSz-scid IL2R γ null (NOG) mice were purchased from The Jackson Laboratory (Bar Harbor, ME).

Synthesis of BSA-PMMA Conjugates: BSA was dissolved in pure DMSO stirring at 50 °C, for 24 h, followed by centrifugation at 7000 rpm for 30 min at 20 °C to remove the undissolved residues. Concentration of BSA in pure DMSO was determined to be 2.36 mg/mL by the Bradford protein assay. NAS (dissolved in DMSO at a concentration of 21.5 mg/mL) was added to the above DMSO solution containing BSA, with a molar ratio of NAS to BSA being 142:1, followed by stirring at 25 °C for 5 h. The number of acryloyl groups on each BSA was determined by MALDI-TOF mass spectrometry. Then, MMA was added at concentrations of 1 M, 0.2 M, or 0.1 M, followed by addition of AIBN (10 mM) to initiate the polymerization at 60 °C. After reaction at 60 °C for 20 hours, the solution was poured into methanol/ethyl ether (1:8, v/v) mixture to precipitate the products, and washed with methanol/ethyl ether mixture for three times. After drying at vacuum, the yields were 75%, 63%, and 57%, respectively. ¹H-NMR was recorded on a Varian Inova 600 MHz spectrometer in DMSO-*d*₆. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was used to compare the molecular weight of the BSA and BSA-PMMA conjugate.

Nanoprecipitation Procedure: BSA-PMMA conjugate was dissolved in chloroform (0.2 mL) at a concentration of 120 mg/mL, followed by injection into the water solution (1.8 mL) containing 13 mg/mL of BSA (the pH of the BSA water solution was adjusted to 5.0 by adding HCl solution) under magnetic stirring. After probe sonication of the above suspension at 4 °C for 1 min, the dry powder of the nanoparticles was obtained after freeze-drying. For preparation of drug-encapsulated nanoparticles, CPT was dissolved in the chloroform solution containing the BSA-PMMA conjugate, followed by the same nanoprecipitation and freeze-drying procedure to obtain the powder of drug-encapsulated nanoparticles.

***In vitro* and *in vivo* Experiment:** Human colorectal cancer cells HCT116 were plated in 96-well microplates at 5.0 × 10³ cells per well in 190.0 μL of complete medium and allowed to adhere under incubation at 37 °C and 5% CO₂. Twenty-four hours later, 10.0 μL of medium containing different CPT solutions or CPT-encapsulated

nanoparticulate suspensions were added to wells and incubated for 24, 48, and 72 h. Wells containing control cells for all preparations were treated with 10 μ L of medium. Percentage cell survival after each time point was determined by the MTT colorimetric assay. The medium was replaced by 200 μ L of MTT solution prepared in media to give a final concentration of 0.5 mg/mL. This was incubated for 2 h at 37 °C and 5% CO₂ to allow for the formation of purple water insoluble formazan. MTT solution was replaced with 100 μ L DMSO and mixed through a pipette and incubated at 37 °C and 5% CO₂ for 2 h to dissolve formazan. Optical density was measured at 570 nm using a microwell plate reading spectrophotometer and compared to control cells. Cell survival was then expressed as a percentage of the control cells, which were taken to represent the 100% cell survival for each respective time point. NOG mice (15–16 weeks old) were injected s.c. with 2×10^7 HCT116 colon cancer cells approximately 10–18 days before dosing. The tumor volume was determined by measuring the tumor in two dimensions with calipers and calculated using the formula: tumor size = (length \times width²)/2. Treatment was initialized when the mean tumor size reached approximately 100–500 mm³ (day 1). The animals were sorted into 2 groups. Each group consisted of 4 mice with tumor sizes ranging from 130 to 610 mm³ with group mean tumor sizes of 240–350 mm³. For group one, as the positive control, free CPT was administered by intraperitoneal injection (i.p. injection). CPT is very insoluble in aqueous solution and is acutely lethal when given to mice i.v. at 9 mg/kg (0.18 mg/20 g mouse) due to the particulate matter in the drug suspension. Thus, CPT was suspended in a vehicle of 0.5% methylcellulose and 0.1% Tween 80 and administered i.p. in an attempt to maximize its efficacy. Mice were given CPT at 9 mg/kg once daily on days 1 and 7 with a dosing volume of 200 μ L. For group two, mice were treated with CPT nanoparticles. The dry powder of CPT nanoparticles was suspended in PBS (pH 7.4) at a concentration of 7 mg/mL with the assistance of probe sonication. All treatments were administered intravenously by tail vein injection (i.v. injection) once daily on days 1 and 7 at 9 mg of CPT/kg. Tumor sizes were measured for the duration of the experiment. Each animal was euthanized when the tumor weight reached the predetermined end point size (2000 mm³), body weight loss exceeded 20%, or other signals of sickness, such as breathing problems, failure to eat and drink lethargy or abnormal posture were observed.

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