A Novel Strategy Based on Ligand-switchable Nanoparticle Delivery System for Deep Tumor Penetration

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Methods

Materials.

All chemicals were purchased from J&K chemical Ltd., USA unless otherwise stated. Mal-PEG$_{5k}$-OH was purchased from Shanghai YareBio Ltd., China. TAT and R8 were purchased from Shanghai GL Biochem Ltd., China. Cy5 NHS ester was purchased from Lumiprobe Co., USA. Pexidartinib (PLX-3397) was purchased from Shanghai Selleck Chemicals, China. Doxorubincin-HCl was purchased from Jingyan chemical Ltd., China.

Synthesis of Block Copolymers.

PEG-*b*-PCL, PAE-*b*-PCL, Cy5-PEG-*b*-PCL. The synthesis and characterization of PEG-*b*-PCL, PAE-*b*-PCL, and Cy5-PEG-*b*-PCL were referred to our previous description.[1]

TAT-PEG-*b*-PCL and R8- PEG-*b*-PCL. Mal-PEG-*b*-PCL was synthesized as shown in Fig. S1. In brief, Mal-PEG-OH (0.5 g, 0.1 mmol) and CL(0.57 g, 5 mmol) were dissolved in 5 mL of toluene, followed by addition of one drop of Sn(Oct)2 into the solution. After freeze-degas-thaw cycles for three times, the reaction mixture was stirred at 110 °C for 12 h. Then the solvent was precipitated into excess diethyl ether to obtain the crude product. The precipitate was dried under vacuum. TAT-PEG-*b*-PCL and R8-PEG-*b*-PCL were synthesized by click reaction of maleimide and sulfydryl. Briefly, Mal-PEG-*b*-PCL (100 mg, 0.01 mmol) was dissolved in 5 mL of DMF, followed by

dispersing in 10 mL of pH 7.4 PBS buffer solution. TAT (25 mg, 0.015 mmol) was added into the solution. The mixture was stirred in the dark at 30°C for 24 h. The mixture was transferred into a dialysis bag (MWCO 3500) and dialyzed against water for 2 days to remove the excess free peptides. The TAT-PEG-*b*-PCL was obtained after lyophilization.[2] ^{1}H-NMR spectra showed that the double bonds of maleimide group (δ: 6.7 ppm) disappeared (Fig. 1B). Similarly, R8-PEG-*b*-PCL were obtained. (Fig. S4B)

TAT-PAE-*b*-PCL and R8- PAE-*b*-PCL. Mal-PAE-*b*-PCL was synthesized as shown in Fig. S2. Briefly, Maleimidopropionic acid (42.3 mg, 0.25 mmol) was dissolved in 1mL of CH_2Cl_2 followed by addition of Oxalyl chloride (63.5 μL, 0.75 mmol). Under catalysis of DMF, the mixture was stirred at room temperature for 12 h. The mixture was evaporated to remove the solvent. 5mL of CH_2Cl_2 was added and evaporated again, followed by repeating three times to remove excess Oxalyl chloride. 2 mL of CH_2Cl_2 and PAE-*b*-PCL (0.5 g, 0.05 mmol) were added, followed by stirring at room temperature for 12 h. The mixture was transferred into a dialysis bag (MWCO 3500) and dialyzed against pH 6.5 PB solution for 2 days to remove the small molecules. The double bonds of maleimide group (δ: 6.7 ppm) appeared, indicated the Maleimidopropionic acid had successfully conjugated with PAE-*b*-PCL (Fig. S5A and S5B). In a similar way, detailed protocols for the synthesis of TAT-PAE-*b*-PCL and R8- PAE-*b*-PCL were referred to those of TAT-PEG-*b*-PCL and R8-PEG-*b*-PCL, and reacted at pH 6.5 PBS buffer solution in the dark at 30°C for 24 h. After dialysis and freeze-drying, the products were characterized by^{1}H-NMR spectra. The disappearance of double bonds of maleimide group also demonstrated that TAT or R8 had successfully introduced into the end terminal of PAE-PCL (Fig. S5C and S5D).

Preparation of sulfydryl-switchable micelles (MSPM^{SSH}). 5mg PCL-*b*-PEG and 5mg PCL-*b*-PAE-Mal dissolved in THF/DMSO (95/5) with the concentration of 5 mg ml^{-1}, and dropwise added into phosphate buffer (pH ~ 6.5, 20 mM) solution under ultrasonic shaking, following by sonication for 20 min. 1mg dithioglycol was added into the mixture and allowed to reacting at 30°C for 12 h. Subsequently, these solutions were transferred into a dialysis bag (MWCO 3500) and dialyzed against PB solution (pH 7.4, 1mM) for 2 days to remove excess dithioglycol, followed by

ultrafiltration to anticipant concentration.

Preparation and characterization of ligand-switchable nanocarriers (LSNs). The synthesis and characterization of all the block copolymers were described in the Supporting Information (Fig. S1, S2, S4, and S5). Referred to previous work,[1] all micelles were prepared through nanoprecipitation technique. Briefly, the block polymers were respectively dissolved in THF/DMSO (95/5) with the concentration of 5 mg mL^{-1}. According to predetermined formulation (Table S1), the certain amount of polymer solutions was mixed together and dropwise added into phosphate buffer (pH ~ 5.5, 20 mM) solution under ultrasonic shaking, following by sonication for 20 min. Subsequently, these solutions were transferred into a dialysis bag (MWCO 3500) and dialyzed against PB solution (pH 7.4, 1 mM) for 2 days, followed by ultrafiltration to anticipant concentration. DOX-loaded, PLX-3397-loaded and Cy5-labeled micelles were prepared in similar method. The formulations were shown in Table S2 and S3. The characterizations of all micelles were determined using dynamic light scattering (DLS), zeta potential and transmission electron microscopy (TEM).

Evaluation the ligand-switchable capability of LSNs *via* QCM. To assess the ligand-switchable potential, we firstly confirmed the location of PAE's terminal at different pH condition *via* quartz crystal microbalance (QCM). Briefly, 0.01 mL $MSPM^{SSH}$ solution (1 mg mL^{-1}) was respectively added in 0.99 mL phosphate buffer (PB, pH 7.4 and 6.5, 10 mM). After stabilizing with PB solution for 0.5 h, the interactions between micelles with gold chips at different pH condition were determined using QCM.

***In Vitro* Drug Release.** 1 ml of DOX-loaded micelles solution with initial DOX

concentration of 0.05 mg ml^{-1} was added to dialysis bag, and dialyzed against 14 ml of 10 mM buffer solution (pH 5.0, 6.5 and 7.4) under vigorous stirring. At set time points, 1 ml of dialysis fluid was taken out for fluorescence measurement (excitation at 490 nm) and replaced with an equal volume of fresh buffer. The amount of released DOX was determined by measuring the intensity of the emission (590 nm) using free DOX as standard. The same experiments were repeated triplicate and taken the average.

The cytotoxicity assessment. HepG2 cells were seeded into a 96-well plate at an initial density of 5000 cells per well in 100 μL DMEM medium. After an incubation of 24 h, the culture medium of each well was removed and replaced with 80 μL of fresh medium, then 20 μL micelles (MSPM, $MSPM^{TAT}$, $MSPM^{STAT}$, $MSPM^{R8}$, and $MSPM^{SR8}$) solution with different concentration (0, 25, 50, 500, 2500, and 5000 μg/mL) was added to each well. After 24-hour incubation, the culture medium was removed and replaced with 10% of CCK8 solution. Two hours later, the absorbance (at 570 nm) of each well was determined using microplate reader. The cytotoxicity was expressed in a percentage of the control group. With similar method, the cytotoxicity of DOX-loaded micelles at different pH (pH 7.4 and 6.5) were performed.

Cellular Uptake. The cellular uptakes of all micelles were demonstrated by confocal laser scanning microscopy (CLSM) and flow cytometry (FCM). HepG2 cells were seeded into confocal dish at a density of 5000 cells per well. After an incubation of 24 h, the culture medium of each dish was removed and replaced with 1 mL fresh medium with different pH (pH 7.4 and 6.5) and 250 μL DOX-loaded micelles solution. After 2-hours incubation, the solution was removed. These cells were washed three times with

1 mL PBS, fixed with 1 mL 4% paraformaldehyde solution, followed by observation with CLSM after staining by 4',6-diamidino-2-phenylindole (DAPI). Similarly, HepG2 cells were seeded into 12-well plates at an initial density of 10000 cells per well and treated with DOX-loaded micelles after an incubation of 24 h. 2 hours later, the medium was removed and each well was washed three times. 0.5 mL trypsin solution was added into each well for 1-min incubation, then 0.5 mL fresh medium was added, followed by 5-min centrifugation. The supernatant was removed and the cells were washed three times. Finally, 1 mL 4% paraformaldehyde solution was added to fix and disperse the cells for flow cytometric measurement.

Penetration in multicellular tumor spheroids. HepG2 multicellular tumor spheroids were cultivated in agarose-coated 48-well plates. When the tumor spheroids grew to 150nm, the medium was removed and replaced with 400 μL fresh medium with different pH (pH 6.5 and 7.4) and 100 μL DOX-loaded micelles solution (0.05 mg DOX/mL). After 10 min of incubation, the mixture was removed and replaced with fresh medium, followed by incubation of 4 h. Next, the micelles-treated tumor spheroids were transferred to confocal dish and washed with PBS three times. Finally, the penetrations of all micelles at different pH were observed using CLSM.

***In vivo* pharmacokinetic study.** All animal studies were performed in accordance with the Regulations for the Administration of Affairs Concerning Experimental Animals (Tianjin, revised in June 2004) and adhered to the Guiding Principles in the Care and Use of Animals of the American Physiological Society, and were approved by the Animal Ethics Committee of Nankai University (Tianjin, China). All protocols within

the study involving animals were approved by the Institutional Animal Care and Use Committee, Nankai University. In this part, the pharmacokinetic study mainly referred to the Cheng's work of our group. Standard curves of the Cy5-labeled micelle solution (Cy5-MSPM, Cy5-MSPM^{R8}, and Cy5-MSPM^{SR8}) were established before administration (Fig. S9). The rats (n = 3) were injected intravenously via the tail vein with Cy5-labeled micelles at a dose of 5 mg kg-1, respectively. At predetermined time points (5 min, 15 min, 30 min, 1 h, 1.5 h, 2 h, 3 h, 4 h, 6 h, 8 h, 12 h, and 24 h), blood samples were collected into heparinized tubes. The blood samples were centrifuged at 4000 rpm for 10 min to remove the blood cells, the plasma fractions were collected and measured by a fluorescence spectrometer (excitation at 625 nm and emission at 670 nm). The amount of the micelle remaining in blood was determined according to the standard curves. The percentage of the injected dose (%ID) was calculated by comparing the amount of micelle remaining in blood with the total injected dose. Blank samples from mice administrated with physiological saline were analyzed as the background fluorescence of the plasma.

***In vivo* tumor accumulation and penetration.** HepG2 cells were inoculated into the left abdomen of BALB/c nude mice, followed by 3 weeks of culture. When the tumors reached about 200 mm^3, these mice were randomly divided into three groups and intravenously injected with Cy5-MSPM, Cy5-MSPM^{R8} and Cy5-MSPM^{SR8} with the same level of Cy5. At 1 h, 6 h, 24 h post-administration, one mouse of each group was sacrificed for harvesting the major organs (heart, liver, spleen, lung, and kidney) and tumor. *Ex vivo* imaging was conducted by the Kodak IS *in vivo* FX imaging system.

Finally, the tumors were collected, embedded by paraffin, and cut into 8-μm-thick sections. These sections were stained by DAPI and anti-CD31-FITC, and observed using CLSM.

Hematology and Biochemistry Analysis. Hematological and biochemical examination were performed by automatic blood analyzer (Celltace, Japan) and automatic biochemical analyzer (Vitalab, Holland). The ICR mice was treated with three type of micelles at the dose of 20 mg kg^{-1} every other day for two times. Hematological and biochemical analysis were performed at 1 day after the last administration (Day 1) and 1 day after seven successive administrations (Day 7). The whole blood samples were collected from eyes at the designated time points. Briefly, 100 μL of blood sample was collected into heparinized tube for hematological analysis. 500 μL of blood sample was collected into a 1.5 mL centrifuge tube and serum was harvested by centrifugation at 3500 rpm for 10 min for biochemical analysis.[3]

***In vivo* antitumor efficacy and combination therapy.** HepG2 tumor-bearing mice (BALB/c) were cultivated in advance. When the tumor volume reached about 100 mm^3, the tumor bearing mice were randomly divided into six groups. From Day 1, the mice were weighed and administrated with PBS (control), free DOX, free PLX-3397, $MSPM^{SR8}$/DOX, $MSPM^{SR8}$/PLX-3397 and $MSPM^{SR8}$/DOX + $MSPM^{SR8}$/PLX-3397 (at day 1, 3, 5, 7 and 9). The dose of DOX and PLX-3397 were fixed at 5 mg kg^{-1} and 5 mg kg^{-1} body weight. Weight of mice and tumor volume (Tumor volume (mm^3) = Length × $Width^2/2$) were measured at determined time points for three weeks.

Hematoxylin/eosin (H&E) and immunohistochemical staining. At day 21, all

tumor-bearing mice were anesthetized with 8% chloral hydrate and sacrificed for collecting the tumors. Tumor samples were fixed for 48 h in 4% paraformaldehyde, embedded in paraffin and cut into 8-μm-thick sections, followed by hematoxylin/eosin (H&E) and terminal deoxynucleotidyl transferasemediated deoxyuridine triphosphate nick end (TUNEL) staining. The resulting sections were observed using optical microscope (Leica DMI6000 B) and CLSM. The analysis of apoptotic index in tumor tissue was performed using ImageJ.

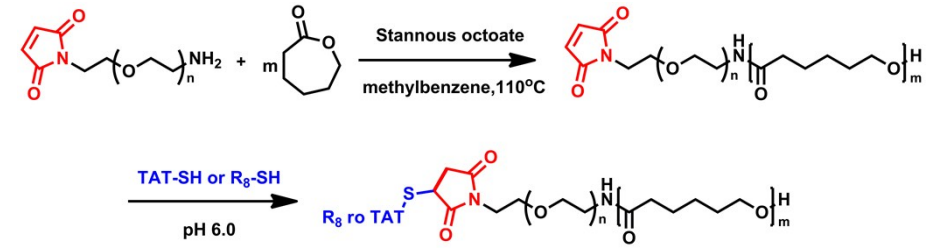


Fig. S1 Synthesis of TAT-PEG-*b*-PCL and R8- PEG-*b*-PCL.

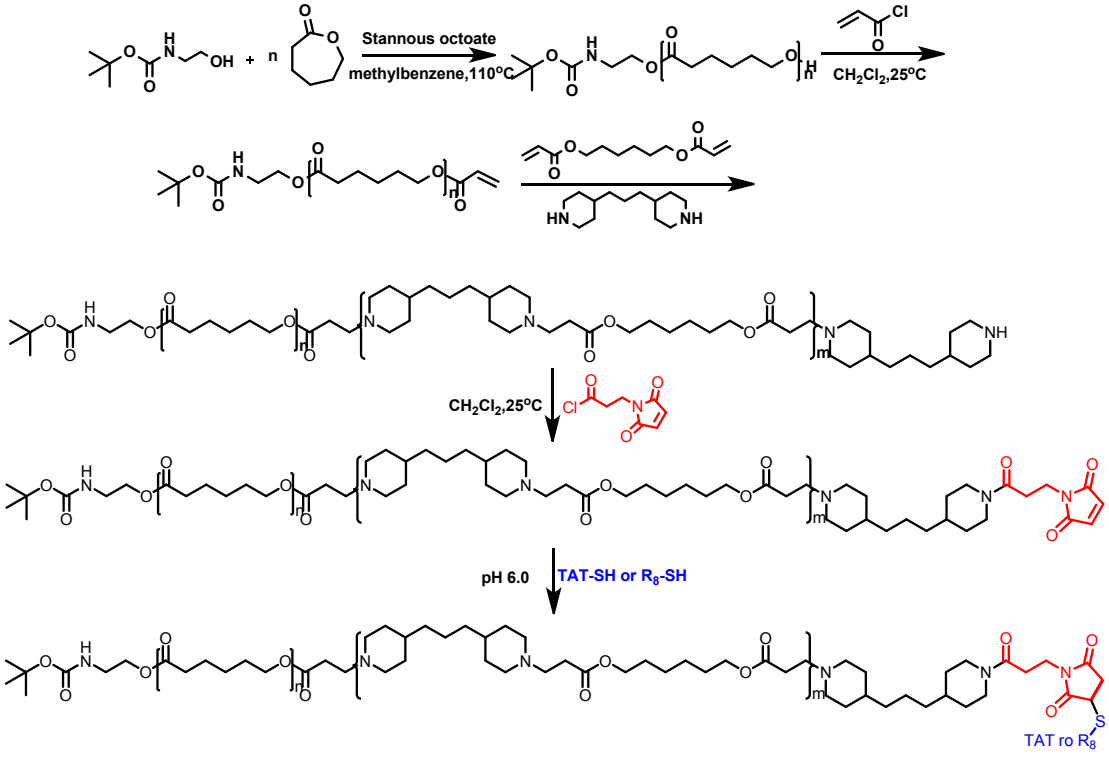
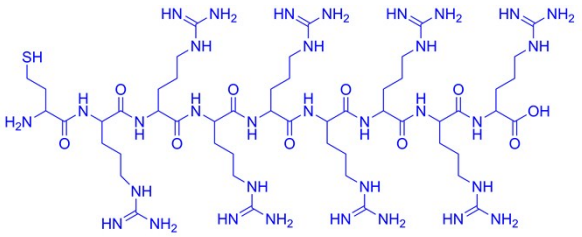
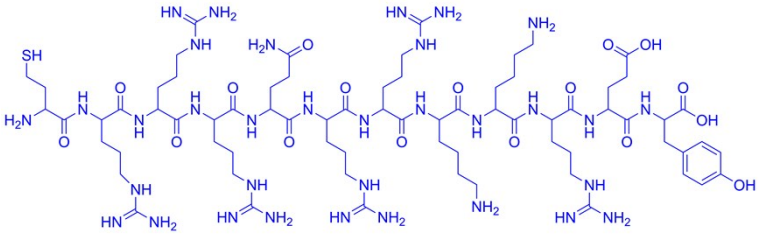


Fig. S2 Synthesis of TAT-PAE-*b*-PCL and R8- PAE-*b*-PCL.



R8



TAT

Fig S3. The structure of R8 and TAT.

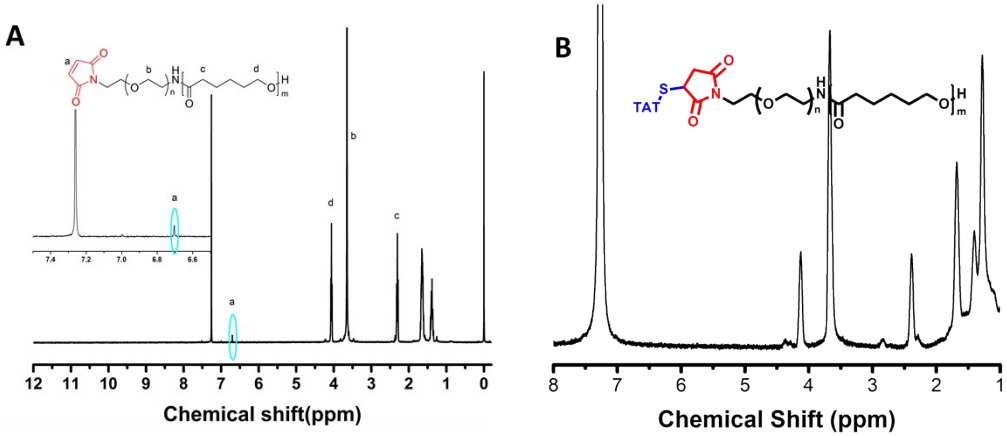


Fig. S4 The ^{1}H NMR spectra of polymers. (A). Mal-PEG-*b*-PCL in $CDCl_3$. (B). TAT-PEG-*b*-PCL in $CDCl_3$.

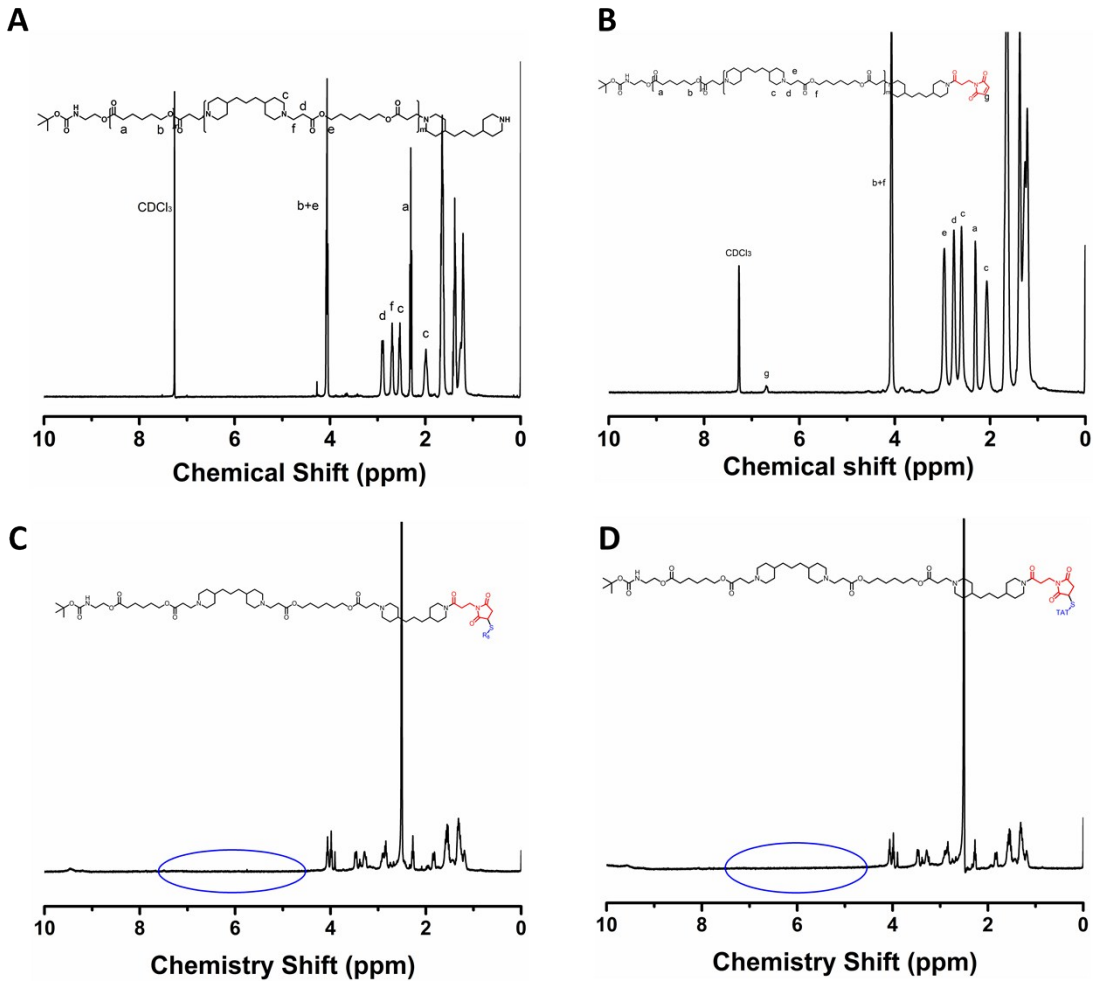


Fig. S5 The ^{1}H NMR spectra of polymers. (A). PAE-*b*-PCL in $CDCl_3$. (B). Mal-PAE-*b*-PCL in $CDCl_3$. (C). R8-PAE-*b*-PCL in DMSO-d_6. (D). TAT-PAE-*b*-PCL in DMSO-d_6.

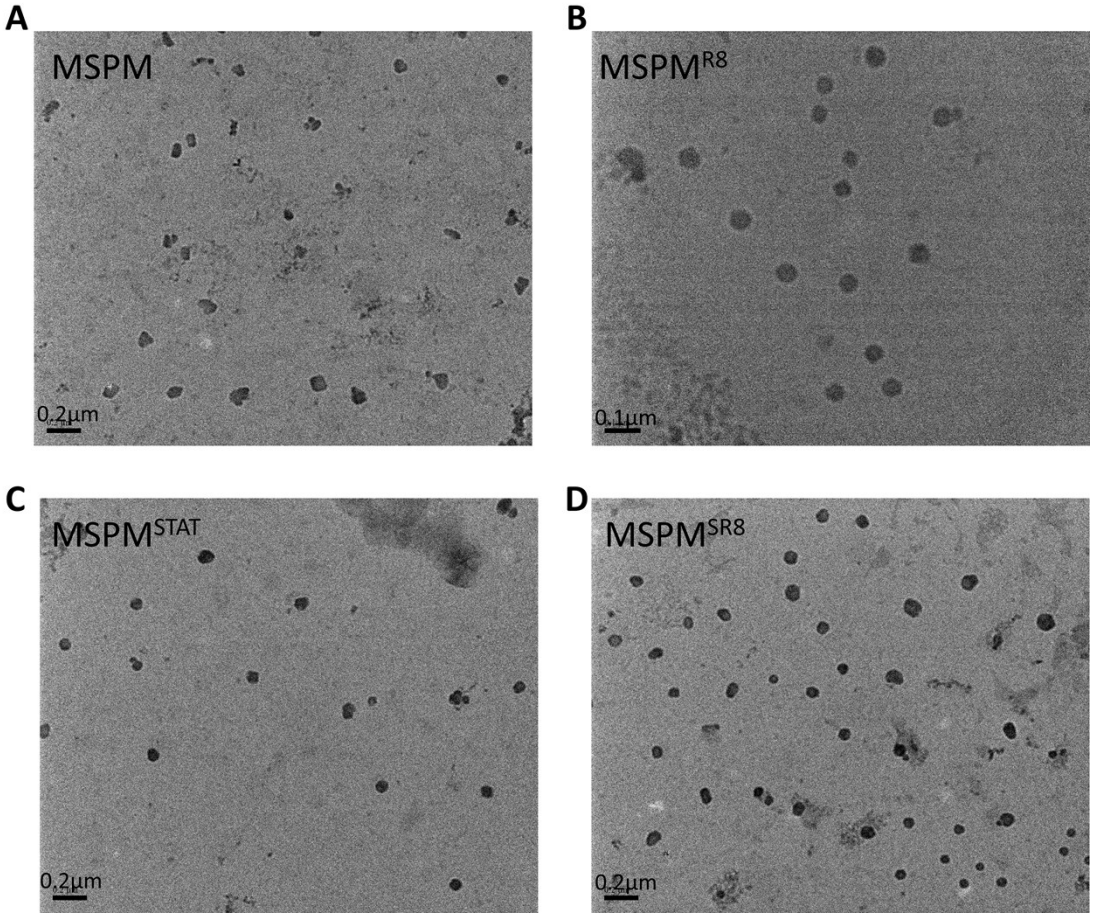


Fig. S6 TEM images of micelles. (A). MSPM. (B). MSPM^{R8}. (C). MSPM^{STAT}. (D). MSPM^{SR8}.

Table S1. Formulations of micelles.

micelle	PEG-*b*-PCL (mg)	CPP-PEG-*b*-PCL (mg)	PAE-*b*-PCL (mg)	CPP-PAE-*b*-PCL (mg)
MSPM	2.5	0	2.5	0
MSPM^{TAT}	2.0	0.5	2.5	0
MSPM^{SR8}	2.5	0	2.0	0.5
MSPM^{R8}	2.0	0.5	2.5	0
MSPM^{SR8}	2.5	0	2.0	0.5

Table S2. Formulations of Cy5−labeled micelles.

micelle	PEG-*b*-PCL (mg)	CPP-PEG-*b*-PCL (mg)	PAE-*b*-PCL (mg)	CPP-PAE-*b*-PCL (mg)	Cy5-PEG_{2k}-*b*-PCL
MSPM	2.0	0	2.5	0	0.5 mg
$MSPM^{TAT}$	1.5	0.5	2.5	0	0.5 mg
$MSPM^{SR8}$	2.0	0	2.0	0.5	0.5 mg
$MSPM^{R8}$	1.5	0.5	2.5	0	0.5 mg
$MSPM^{SR8}$	2.0	0	2.0	0.5	0.5 mg

Table S2. Formulations of DOX-loaded or PLX-3397-loaded micelles.

micelle	PEG-PCL (mg)	CPP-PEG-PCL (mg)	PAE-PCL (mg)	CPP-PAE-PCL (mg)	DOX or PLX-3397
MSPM	2.0	0	2.5	0	1 mg
$MSPM^{TAT}$	1.5	0.5	2.5	0	1 mg
$MSPM^{SR8}$	2.0	0	2.0	0.5	1 mg
$MSPM^{R8}$	1.5	0.5	2.5	0	1 mg
$MSPM^{SR8}$	2.0	0	2.0	0.5	1 mg

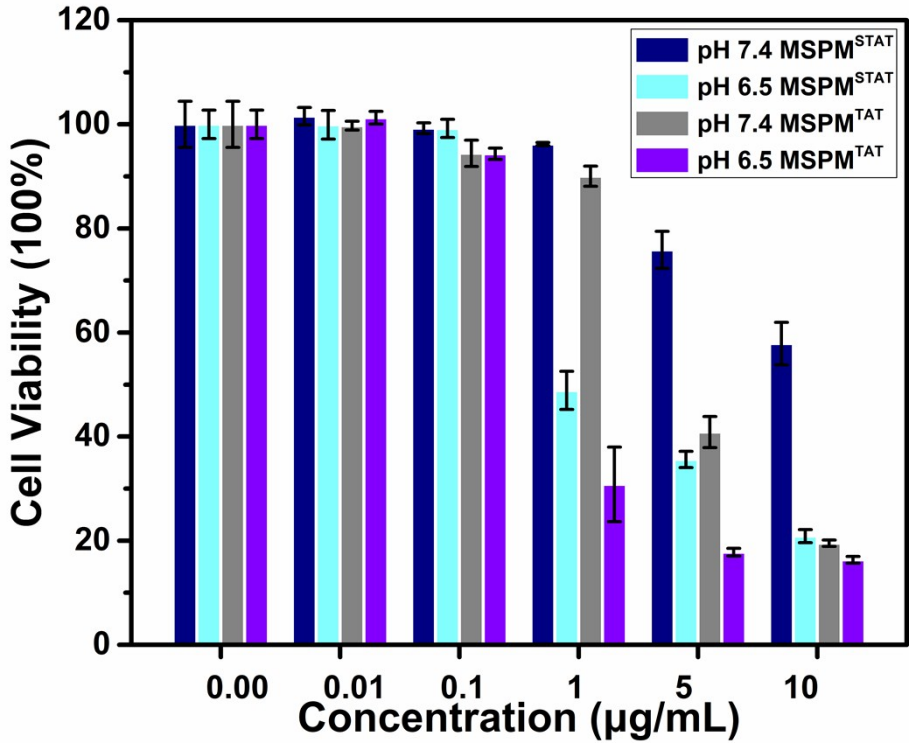


Fig. S7 Cytotoxicity of DOX-loaded micelles ($MSPM^{TAT}$/DOX and $MSPM^{STAT}$/DOX) at different condition.

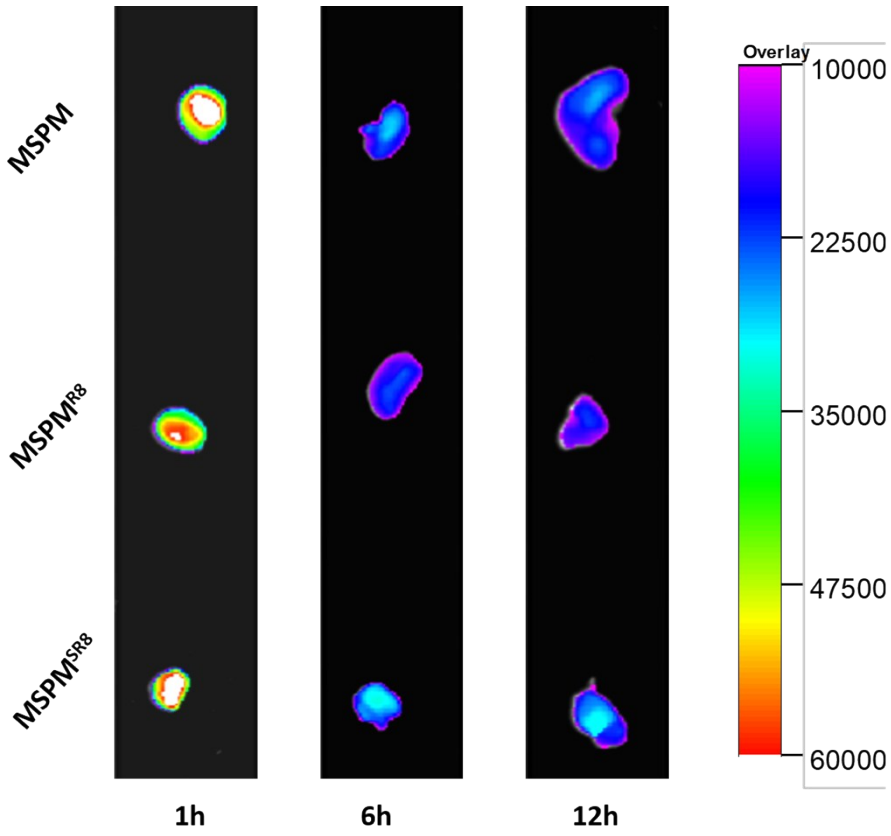


Fig. S8 The *ex vivo* fluorescence imaging of the tumor at 1h, 6h, and 24h.

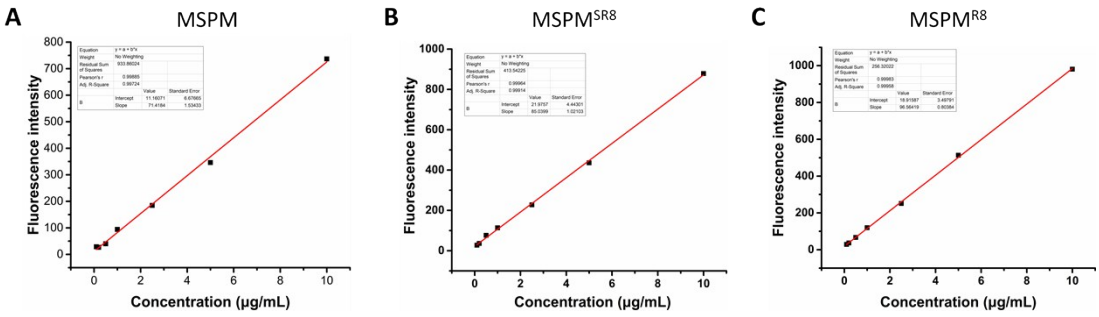


Fig. S9 The standard curves of Cy5-labeled micelles. (A). Cy5-MSPM. (B). Cy5-MSPM^{SR8}. (C). Cy5-MSPM^{R8}.

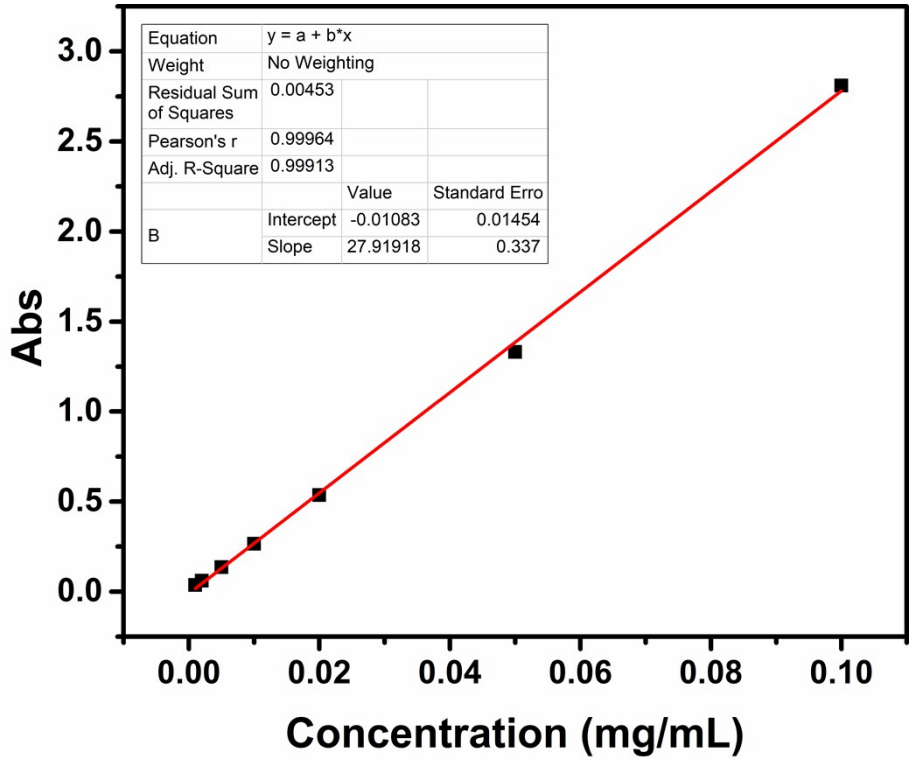


Fig. S10 The standard curves of PLX-3397 in DMSO at 307 nm.

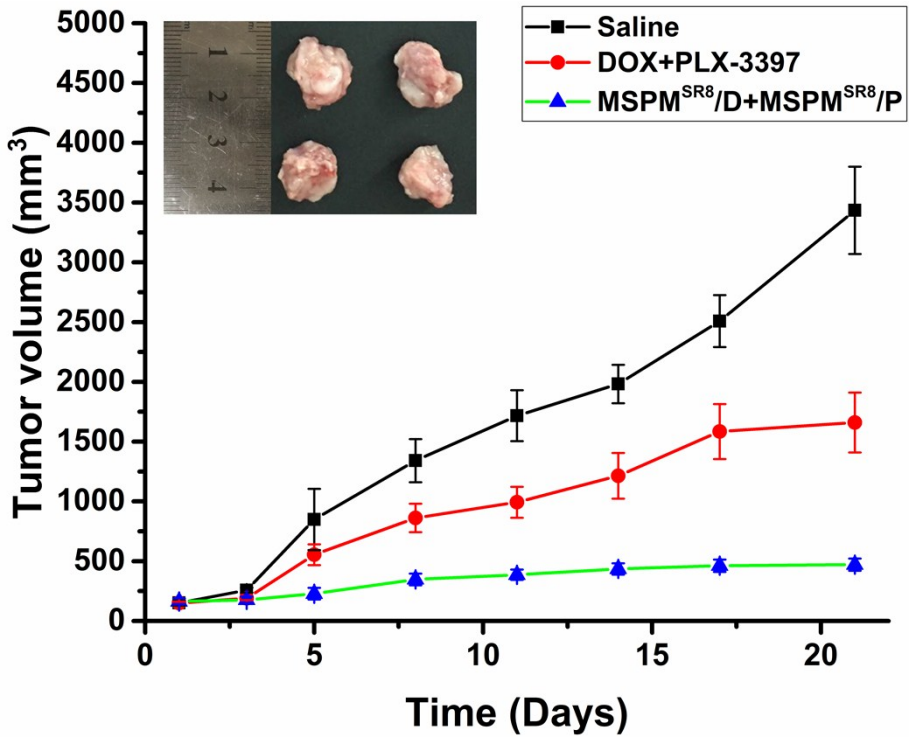


Fig. S11 HepG2 tumor growth curves after injection with saline, free DOX + PLX-3397 and MSPMS^{R8}/DOX + MSPM^{SR8}/PLX-3397 at a dose of 5 mg (DOX) kg^{-1} and 5 mg (PLX-3397) kg^{-1} body weight, respectively.

1. T. Cheng, Y. Zhang, J. Liu, Y. Ding, H. Ou, F. Huang, Y. An, Y. Liu, J. Liu and L. Shi, *ACS Appl Mater Interfaces*, 2018, **10**, 5296-5304.
2. E. Jin, B. Zhang, X. Sun, Z. Zhou, X. Ma, Q. Sun, J. Tang, Y. Shen, E. Van Kirk, W. J. Murdoch and M. Radosz, *J. Am. Chem. Soc.*, 2013, **135**, 933-940.
3. C. Yang, L. Chu, Y. Zhang, Y. Shi, J. Liu, Q. Liu, S. Fan, Z. Yang, D. Ding, D. Kong and J. Liu, *ACS Applied Materials & Interfaces*, 2015, **7**, 2735-2744.